A STUDY OF PROTEIN PHOSPHORYLATION IN CENTRAL DOPAMINERGIC NEURONS

by

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Abstract

The addition of dopamine to homogenates or slice preparations from rat caudate nucleus results in a 100-150% increase in tissue cAMP levels. Since protein kinases are the only known physiological receptors for cAMP, it has been hypothesized that the response of the postsynaptic neurons to dopamine may be mediated in part through the phosphorylation of specific membrane proteins. This study examines the effects of dopamine on cAMP and its associated protein kinase in caudate nucleus.

Caudate tissue contains a Type II protein kinase that is activated by cAMP, and that catalyzes the phosphorylation of several synaptic membrane proteins. Dopamine, however, did not appear to enhance the phosphorylation of these proteins either in broken cell or intact preparations, although increases in cAMP could be demonstrated under similar experimental conditions.

Destruction of postsynaptic neurons using the neurotoxin kainic acid significantly reduced cAMP-dependent protein kinase activity. Destruction of presynaptic nerve terminals, on the other hand, had no effect on the activity of this enzyme system. However, this procedure was associated with an increase in dopamine receptor sensitivity as measured by an increase in dopamine-mediated turning behaviour. This behavioural response was not abolished by intrastriatal administration of kainic acid, although this technique reduced dopamine-sensitive adenylate cyclase and cAMP-dependent protein phosphorylation by 80-90%.

The administration of chronic haloperidol also produced behavioural supersensitivity, as well as increases in the number of postsynaptic dopamine

receptors, but this was not associated with comparable increases in the activity of dopamine-sensitive adenylate cyclase, or in cAMP-dependent protein phosphorylation. These studies therefore do not support a role for cAMP or cAMP-dependent protein kinases in the mediation of postsynaptic dopamine receptor supersensitivity.

Rat caudate nucleus also contains a number of proteins that are phosphorylated by Ca^{++}-dependent protein kinases. Substrates for calmodulin-dependent and independent protein kinases were identified and characterized on the basis of their solubilization properties and response to neuroleptic drugs. Dopamine enhanced the phosphorylation of two of these proteins in rat striatal slices. The effects of dopamine on protein phosphorylation could be distinguished from those of depolarizing agents such as KCl or veratridine, and were not mimicked by 8-bromo-cAMP.

The present studies demonstrate that increases in cAMP produced by dopamine have no measurable effect on cAMP-dependent protein phosphorylation. Although these data do not definitely preclude a role for cAMP-dependent protein kinases in the regulation of postsynaptic function, they indicate that the physiological relevance of dopamine-mediated increases in cAMP should be re-examined.

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Chapter I

Introduction

1.1 General Introduction and Rationale

During the past decade a vast body of literature has accumulated regarding the role of dopamine as a neurotransmitter in the central nervous system. The impetus for this extensive research effort has derived in part from the well-known association of dopamine with specific clinical disorders. For example, degeneration of dopaminergic neurons in the substantia nigra and the subsequent impairment of dopaminergic transmission in the nigro-striatal tract is thought to be a primary feature of the pathophysiology of Parkinson's disease (Hornykiewicz, 1966).

Another clinical disorder that has been attributed to altered dopaminergic function is schizophrenia (Hornykiewicz, 1977; Snyder et al., 1974; Matthysse and Lipinski, 1975; Davis, 1976; Snyder, 1976; Snyder, 1981). The evidence for the involvement of dopamine in schizophrenia stems from the observation that antipsychotic drugs, that are used in the treatment of schizophrenia, exert their effects by binding dopamine receptors (Snyder et al., 1974; Iversen, 1975; Seeman, 1980; Snyder, 1976) thereby blocking synaptic transmission in central dopaminergic neurons.

The success of the neuroleptics as therapeutic agents in the treatment of schizophrenia gave rise to the "dopamine hypothesis of schizophrenia" (Snyder, 1976) which in its simplest form states that schizophrenia is the result of overactivity of dopaminergic pathways in the brain. Although the brains of schizophren-

ic patients contain normal levels of dopamine (Crow et al., 1979), they do show an increase in the number of dopamine receptors in the corpus striatum (caudate nucleus and putamen) as well as in the mesolimbic area (Owen et al., 1978; Lee et al., 1978c; Lee and Seeman, 1980; Crow et al., 1979). These studies indicate that the primary defect in schizophrenia may be an increase in the sensitivity of postsynaptic receptors to dopamine. Other investigators, however, have questioned this interpretation since the reported increases in the number of dopamine receptors in the brains of schizophrenics appear to be confined to patients previously maintained on neuroleptic medication (Reynolds et al., 1981; MacKay et al., 1980). As will be discussed in more detail later, prolonged disruption of dopaminergic transmission leads to an adaptive increase in the number of postsynaptic dopamine receptors, and this is thought to be a biochemical correlate of increased receptor sensitivity.

In recent years biochemical studies have attempted to characterize the properties of the "dopamine receptor." Research in this area has been complicated by the fact that mammalian brain contains several classes of dopamine receptor with different biochemical properties (Seeman, 1980). Although the pharmacological characteristics of these receptors have been well-defined (Seeman, 1980), their functions in vivo, and the intracellular mechanisms by which they achieve their effects have not been clearly elucidated. In view of the inherent heterogeneity of dopamine receptors within the brain (Cools and Van Rossum, 1980; Kebabian and Calne, 1979), it is unlikely that the physiological response of these receptors to dopamine is mediated through a single biochemical process.

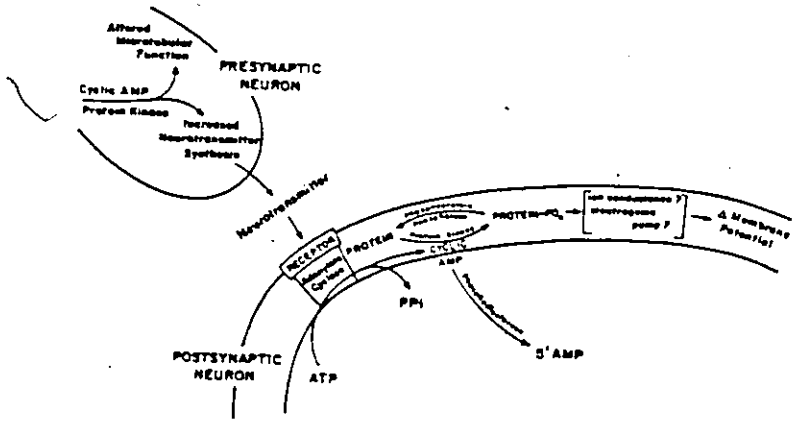
Kebabian and associates (1972) have isolated an adenylate cyclase from areas of brain rich in dopamine nerve terminals that is selectively stimulated by dopamine and its agonists, and is inhibited by antipsychotic agents such as

haloperidol and chlorpromazine. The enzyme is localized postsynaptically (Kebabian et al., 1972; Minneman et al., 1978; Schwartz et al., 1978; Garau et al., 1978; Creese et al., 1977a), and mediates the intracellular formation of cAMP which is believed to function as a "second messenger" for the effects of the transmitter (Greengard, 1976). Although not all dopamine receptors in the brain are linked to adenylate cyclase (Seeman, 1980), this enzyme displays many of the pharmacological properties of the indirectly characterized dopamine receptor (Clement-Cormier et al., 1974; Kebabian et al., 1972; Iversen, 1975) and has provided a useful in vitro model for investigating some of the intracellular events that accompany dopaminergic transmission.

Synaptosomal membranes contain specific proteins that are phosphorylated by a cAMP-regulated process. The enzymes that catalyze this reaction are known as cAMP-dependent protein kinases. Greengard and his colleagues have proposed that the phosphorylation of these membrane proteins may play an important role in synaptic transmission, a hypothesis that has obtained much experimental support (Greengard, 1979; Williams, 1979; Reddington and Mehl, 1979; Kelly et al., 1979). A schematic representation of this process is shown in Figure 1.

Although there is reason to believe that dopamine agonists or antagonists might preferentially alter cAMP-dependent protein kinase activity, as yet the effect of dopamine on the phosphorylation of synaptic membrane proteins is unclear. In addition, since synaptic membranes also contain a number of proteins the phosphorylation of which is not cAMP-regulated, the possibility that dopamine might influence cAMP-independent phosphorylation must also be considered.

In light of the above, the aim of this work was: (1) to investigate the effects of dopamine agonists and antagonists on protein phosphorylation in rat



Proposed roles for cyclic AMP and protein phosphorylation in neuronal function include regulation of neurotransmitter synthesis in presynaptic terminals (*a*), regulation of microtubular function (*b*) (indicated, purely for convenience, as occurring in the presynaptic axon; microtubules occur in dendrites, soma and axon of neurones), and generation of postsynaptic potentials (*c*). The sequence of events by which neurotransmitter, released from presynaptic terminals, produces an electrophysiological response in the postsynaptic cell is conceived as follows. The released neurotransmitter activates a neurotransmitter-sensitive adenylate cyclase present in the membrane of the postsynaptic cell, leading to the production of cyclic AMP in the immediate vicinity of the postsynaptic membrane. The newly formed cyclic AMP activates a cyclic AMP-dependent protein kinase present in the postsynaptic membrane. This activated protein kinase catalyses the phosphorylation of a substrate protein also present in the postsynaptic membrane, converting it from the non-phosphorylated to the phosphorylated state. A key element of this model is that this substrate protein controls the permeability of the postsynaptic membrane. Phosphorylation of the substrate protein leads, through a change either in ion conductance or in the rate of an electrogenic pump, to a change in membrane potential, the "postsynaptic potential". Since the postsynaptic potential is transient in nature, enzymatic machinery must exist which terminates this sequence of events. This enzymatic machinery includes a phosphodiesterase that hydrolyses the cyclic AMP to 5'-AMP and a phosphoprotein phosphatase that converts the substrate protein back to the non-phosphorylated form, leading to the termination of the postsynaptic potential.

Figure 1: A schematic representation of the role of protein kinases in synaptic function

caudate nucleus, and (2) to determine whether changes in the sensitivity of dopamine neurons are accompanied by changes in the phosphorylation of specific synaptic membrane proteins. Investigation of the biochemical mechanisms that mediate dopamine receptor function is important both from the point of view of understanding the process of dopaminergic transmission, as well as from the point of view of obtaining a better appreciation of the response of the postsynaptic neuron to neuroleptics. This information may provide an approach to the manipulation of disorders involving enhanced or suppressed dopaminergic function. A summary of the relevant literature is presented below.

1.2 Anatomy and Physiology of the Nigro-Striatal Dopamine System

Through the use of the Falck-Hillarp histofluorescence technique Anden et al. (1964) demonstrated the nigro-striatal pathway, that originates in the pars compacta of the substantia nigra, and terminates in the corpus striatum (see Figure 2). The clinical finding that Parkinson's disease is the result of a decrease in the dopamine content of the caudate nucleus secondary to degeneration of the substantia nigra led to the proposal that this pathway was the principal source of striatal dopamine in man (Hornykiewicz, 1966).

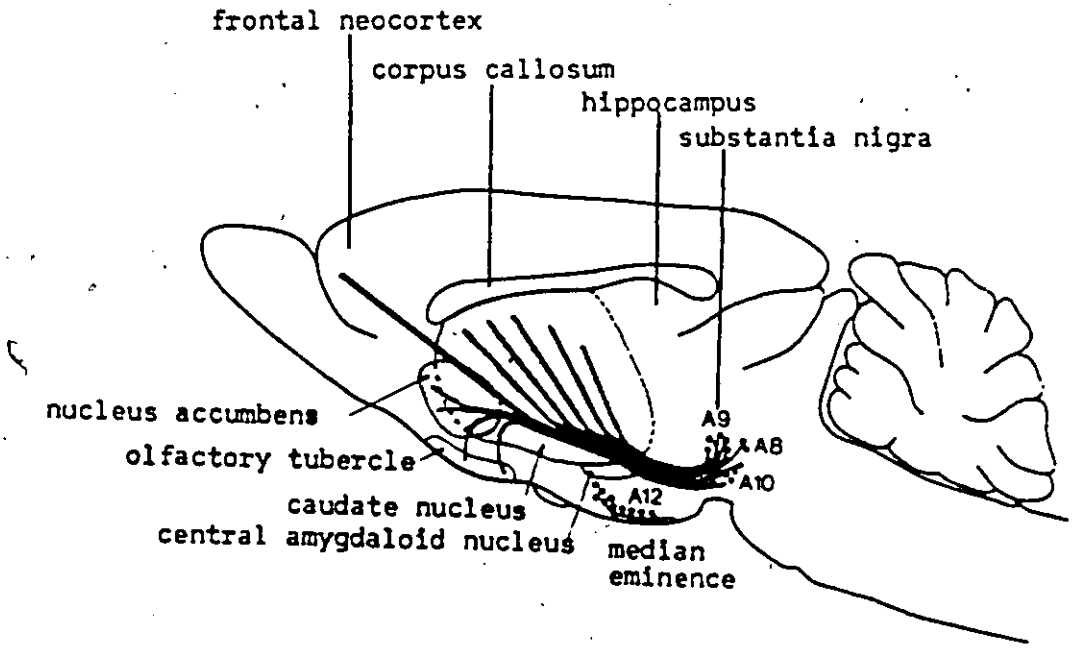


Figure 2: A schematic representation of the dopamine pathways in rat brain

The nigro-striatal pathway additionally includes descending gabanergic (Hattori et al., 1975; McGeer et al., 1977) and Substance P (Hong et al., 1977)

fiber tracts that project to the substantia nigra. The cell bodies of the gabanergic and Substance P neurons are localized in the striatum and can be selectively destroyed by injections of kainic acid, a neurotoxin that causes degeneration of cell soma and dendrites while sparing axons and nerve terminals (Coyle and Schwartz, 1976; Hattori and McGeer, 1977; Coyle et al., 1978; Hong et al., 1977). Gabanergic fibers synapse with dendrites of dopaminergic neurons in the substantia nigra (Hattori and McGeer, 1977; Hattori et al., 1975) and exert an inhibitory influence on dopamine activity (Bartholini et al., 1975). Dopamine released from these dendrites (Korf et al., 1976; Geffen et al., 1976) stimulates the release of GABA from nigral nerve endings (Reubi et al., 1977).

The activity of dopaminergic neurons is additionally regulated through synaptic pathways within the striatum. The dopaminergic afferents to the striatum synapse with excitatory cholinergic neurons (Hattori et al., 1976) that are intrinsic to the basal ganglia (Lynch et al., 1972; Hattori et al., 1976; McGeer et al., 1975). Dopamine and its agonists inhibit acetylcholine release, whereas dopamine antagonists have the opposite effect (Bartholini et al., 1977). Acetylcholine, in turn, enhances the presynaptic release of dopamine (Giorguieff et al., 1976; De Belleroche and Bradford, 1978) an effect that appears to be mediated in part by nicotinic receptors localized on dopamine nerve endings (McGeer et al., 1979).

Cholinergic neurons also stimulate gabanergic neurons whose axons leave the striatum to form inhibitory synapses with dopamine neurons in the substantia nigra (McGeer et al., 1976a). This neuronal feedback loop between the substantia nigra and the striatum provides one mechanism for the regulation of dopaminergic transmission in the nigro-striatal pathway (Figure 3).

Systemic administration of pharmacological agents that facilitate dopaminergic transmission produces an inhibition of neuronal activity in the stri-

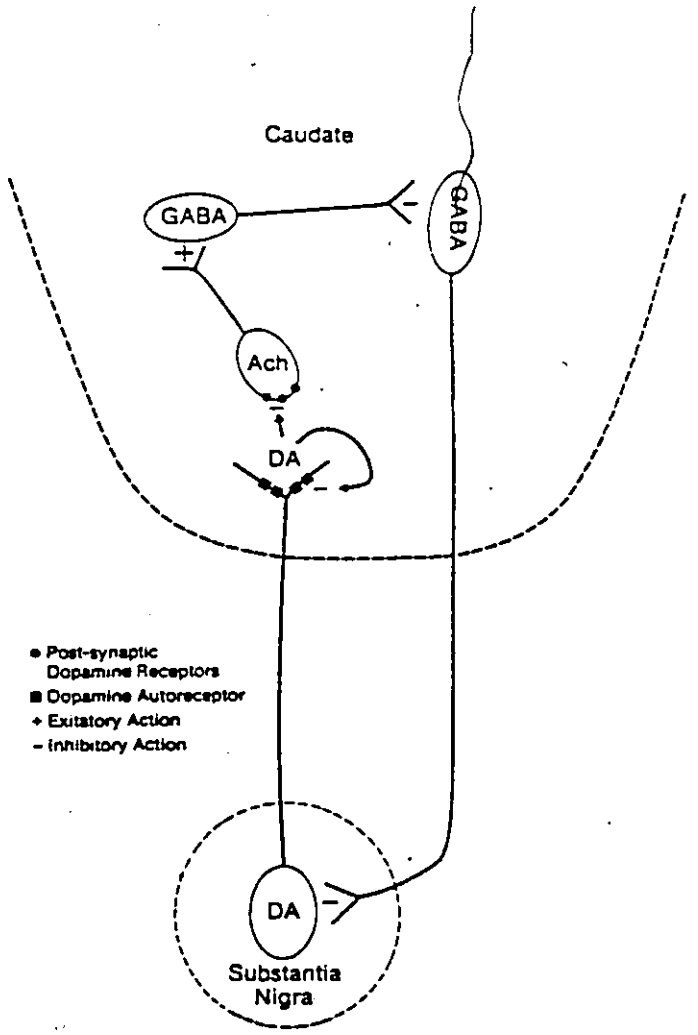


Figure 3: Negative-feedback mechanisms for nigro-striatal dopamine neurons: Local (autoreceptor) and neuronal feedback loop.

atum (Groves et al., 1975) and a corresponding decrease in the firing rate of dopamine neurons in the substantia nigra (Rebec and Groves, 1975; Bunney et al., 1973). In contrast, administration of drugs that block dopamine receptors, produces an increase in dopaminergic nerve impulses (Groves et al., 1975; Aghajanian and Bunney, 1977; Bunney and Grace, 1978) and in the activity of caudate nucleus neurons (Anden et al., 1970). These results are consistent with a variety of studies demonstrating that dopamine has an inhibitory effect on postsynaptic neurons in the caudate nucleus (Connor, 1970; Feltz, 1969; McLennan and York, 1967).

1.3 Dopamine Receptors in the Mammalian Brain

The mammalian brain contains different classes of dopamine receptors that can be distinguished on the basis of physiological, pharmacological and biochemical criteria (Cools and Van Rossum, 1980; Kebabian and Calne, 1979; Titeler et al., 1978; Creese, 1980; Iversen et al., 1980; Spano et al., 1980). Using a modification of the nomenclature originally proposed by Kebabian and Calne (1979), Seeman (1980) has identified four sites of dopamine action that can be classified according to their binding affinities for dopamine and neuroleptics. The D-1 site is linked to dopamine-sensitive adenylate cyclase, and is stimulated by micromolar concentrations of dopamine, and inhibited by micromolar concentrations of neuroleptics. The D-2 site is stimulated by micromolar concentrations of dopamine, and inhibited by nanomolar concentrations of neuroleptics. The D-3 site is sensitive to nanomolar concentrations of dopamine and micromolar concentrations of neuroleptics. The D-4 site has a high affinity for both dopamine and neuroleptics (Figure 4).

1.3.1 Postsynaptic Regulation of Dopamine Function

A. Properties of D-1 Receptors

As discussed previously, one of the physiological effects of dopamine is to increase intracellular levels of cAMP through activation of a specific adenylate cyclase. Dopamine stimulation of adenylate cyclase has been demonstrated in homogenates (Kebabian et al., 1972; Clement-Cormier et al., 1974), and in slices (Forn et al., 1974; Wilkening and Makman, 1975) of rat caudate nucleus. A number of analogues that possess dopamine-like activity in vivo have also been shown to increase the activity of dopamine-sensitive adenylate cyclase (Makman et al., 1975; Miller et al., 1974; Kebabian et al., 1972; Iversen, 1975).

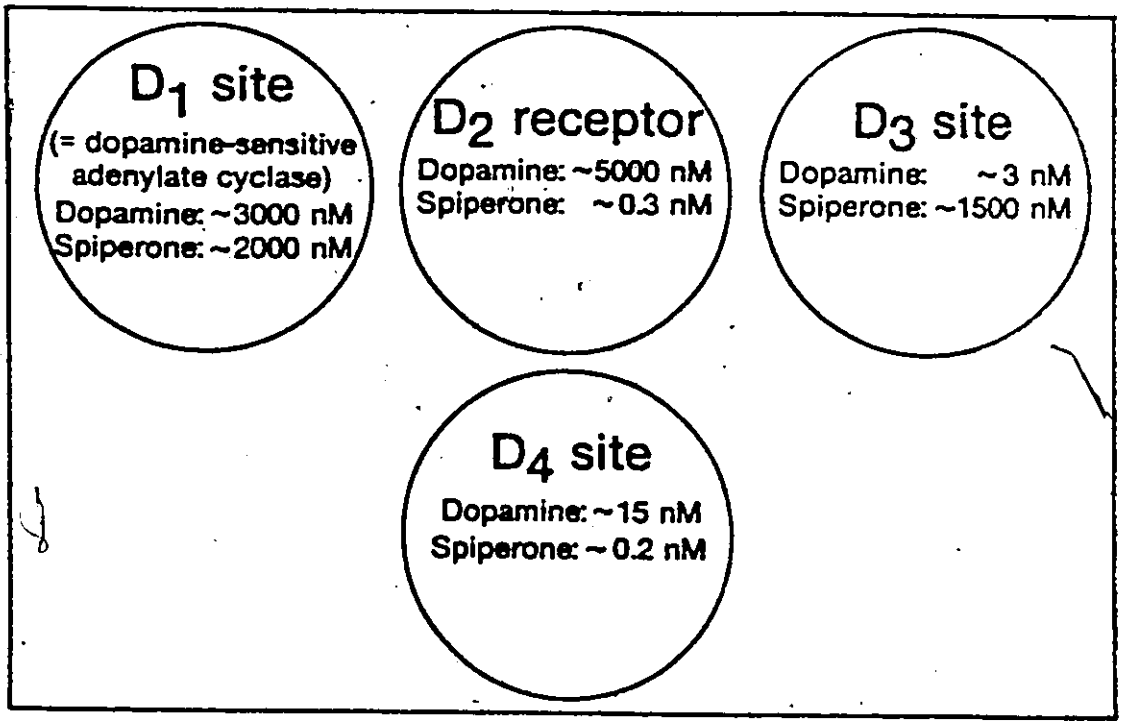


Figure 4: A classifification of dopamine receptors in mammalian brain

As cAMP is a well-established intracellular messenger for a number of hormonally-mediated events (Robison et al., 1971), and is capable of amplifying hormonal responses by binding and activating specific cAMP-dependent protein kinases (Rubin and Rosen, 1975; Glass and Krebs, 1980; Krebs and Beavo, 1979), this provides a possible mechanism for the physiological changes that accompany dopaminergic transmission.

It has been suggested, based on work in other neuronal systems including cerebellar Purkinje cells (Siggins et al., 1974; Hoffer et al., 1972), and sympathetic ganglia (Kebabian et al., 1975) that the electrophysiological effects of dopamine in caudate nucleus might be mediated through the postsynaptic genera-

tion of cAMP. Although the neurophysiological consequences of dopamine stimulation in the caudate remain somewhat controversial (Herrling and Hall, 1980), most of the experimental evidence indicates that dopamine functions as an inhibitory neurotransmitter in this brain region (Siggins, 1978).

Consistent with the hypothesized role of cAMP as an intracellular mediator of dopaminergic transmission, iontophoretic administration of cAMP to caudate nucleus neurons depresses their firing rate, an effect that is potentiated by phosphodiesterase inhibitors (Siggins et al., 1974). These results are observed following presynaptic destruction of dopamine nerve terminals with 6-Hydroxydopamine indicating that the electrophysiological changes produced by dopamine and cAMP occur in the postsynaptic membrane (Siggins et al., 1974).

Biochemical studies have also confirmed a postsynaptic localization of adenylate cyclase. Lesions of dopamine cell bodies in the substantia nigra do not alter the activity of dopamine-sensitive adenylate cyclase within either the nigra (Kebabian and Saavedra, 1976) or the striatum (Krueger et al., 1976), indicating that the cyclase is located on cellular elements other than dopamine neurons. Conversely, destruction of postsynaptic neurons in the striatum with kainic acid spares the dopaminergic nerve terminals but abolishes adenylate cyclase activity (McGeer and McGeer, 1976; Schwartz and Coyle, 1977; Di Chiara et al., 1977a). Measurement of dopamine receptors using labelled antagonists further indicates that about 40-50% of dopamine receptors are unaffected by kainic acid treatment (Murrin et al., 1979; Schwartz et al., 1978; Creese et al., 1977a), and are therefore not linked to adenylate cyclase.

Antipsychotic drugs, that inhibit dopaminergic activity, are potent competitive antagonists of the dopamine-sensitive adenylate cyclase (Kebabian et al., 1972; Clement-Cormier et al., 1974; Miller et al., 1974). There is a good cor-

relation between the ability of the phenothiazines to inhibit the dopamine-sensitive adenylate cyclase and their clinical potency in vivo (Iversen, 1975). This relationship, however, does not apply to the butyrophenones, that exhibit strong antipsychotic activity in vivo (Janssen, 1965; Iversen, 1975) but are relatively weak antagonists of the cyclase in vitro (Clement-Cormier et al., 1974 Krueger et al., 1975). The physiological significance of the observed inhibition of the dopamine-sensitive adenylate cyclase by neuroleptics has been disputed (Seeman, 1980) since this effect is only observed at concentrations of neuroleptic that are far in excess of circulating drug levels reported in vivo (Seeman, 1977; Nakahara et al., 1978).

The enzyme adenylate cyclase consists of at least three components: a receptor component located at the outer surface of the membrane containing a recognition site for binding of hormones or transmitters, and a catalytic unit and nucleotide regulatory component on the cytosolic side of the membrane (Rodbell, 1980). The regulatory component contains sites for the binding of guanine nucleotides which have been shown to modulate the hormonal sensitivity of the enzyme (Tell et al., 1975; Lefkowitz, 1974; Rodbell et al., 1975; Chen et al., 1980). These agents stimulate basal as well as dopamine-sensitive adenylate cyclase activity in homogenates (McSwigan et al., 1980) as well as membrane fractions from rat caudate nucleus (Clement-Cormier et al., 1975; Roufogalis et al., 1976a; Sulakhe et al., 1977).

Brain adenylate cyclase activity exhibits a biphasic response to Ca^{++} with stimulation occurring at low Ca^{++} concentrations and inhibition at higher concentrations (Brostrom et al., 1975; Lynch et al., 1977). The Ca^{++} sensitivity of the enzyme is conferred by calmodulin (Brostrom et al., 1978; Cheung et al., 1975; Lynch et al., 1976;) which combines with the enzyme to form an active complex (Liu and Cheung, 1976). Two forms of adenylate cyclase have been iso-

lated, one dependent on calmodulin and the other not (Westcott et al., 1979). Sensitivity to calmodulin can be restored by the addition of a dissociated protein factor tentatively identified as the GTP-binding subunit of the cyclase (Toscano et al., 1979). More recent studies, however, have shown that calmodulin exerts its effects directly on the catalytic subunit of the enzyme and does not require the presence of the guanyl nucleotide binding subunit (Salter et al., 1981).

Tissue levels of cAMP are additionally regulated by cyclic nucleotide phosphodiesterase the enzyme that catalyzes its hydrolysis to 5'-AMP. Rat or bovine brain phosphodiesterase is partly soluble and partly particulate (Cheung and Salganicoff, 1967; De Robertis et al., 1967). Calmodulin increases the activity of the soluble enzyme, while having no effect on the particulate enzyme (Cheung, 1971; Cheung et al., 1975; Lynch et al., 1977). The soluble enzyme that is activated by calmodulin has been identified as the high K_m form of the enzyme that hydrolyzes cAMP with a high K_m and cGMP with a low K_m (Cheung et al., 1978) and is the only one of several forms of phosphodiesterase that is affected by calmodulin (Kakiuchi et al., 1973).

Binding of calmodulin to the enzyme increases its catalytic activity but has little effect on its substrate affinity (Klee et al., 1979; Morrill et al., 1979). The calmodulin-activated form of phosphodiesterase is located postsynaptically (Ariano and Appleman, 1979) as are calmodulin (Wood et al., 1980) and adenylate cyclase (Rechardt and Harkonen, 1977; Minneman et al., 1978; Garau et al., 1978). This adds further support to the hypothesis that cAMP may mediate some aspects of synaptic transmission, and indicates, moreover, that the postsynaptic regulation of cyclic nucleotide levels intimately involves calmodulin.

B. Properties of D-2 Receptors

The D-2 receptors are distinguished from the D-1 and D-3 classes of receptors by their high affinity for neuroleptic drugs (Seeman, 1980). A number of lines of evidence indicate that this population of receptors mediates many of the clinical effects attributed to neuroleptic drugs in vivo. Thus the binding affinities for neuroleptic drugs at this site correlate with their antipsychotic potency (Seeman et al., 1975; Meltzer, 1976; Creese et al., 1976a,b; Snyder, 1981; Creese and Snyder, 1977), as well as with the concentration of drug detected in the serum of medicated patients (Seeman 1977).

These receptors have been clearly distinguished from those receptors linked to adenylate cyclase on the basis of a number of biochemical and pharmacological criteria including different anatomical (Garau et al., 1978) and subcellular distributions (Clement-Cormier and George, 1978; Laduron et al., 1978) antagonism by different classes of neuroleptic drugs (reviewed by Seeman, 1980), susceptibility to GTP inhibition of agonist binding (Creese et al., 1979) and method of adaptation to surgical or pharmacological denervation (Muller and Seeman, 1978; Seeman, 1980).

1.3.2 Presynaptic Regulation of Dopamine Function

As described above, the mechanism by which dopamine exerts its postsynaptic effects is predominantly through interaction with D-1 and D-2 receptors. Dopaminergic function can be regulated additionally by presynaptic "autoreceptors" that provide a local mechanism for the feedback control of dopamine synthesis and release in the vicinity of the nerve terminal (Carlsson, 1977; Bloom, 1977). These autoreceptors or D-3 receptors, using the nomenclature of Seeman (1980), are distributed on dopamine cell processes within the substantia nigra (Bunney et al.,

1973a; Groves et al., 1975; Wilson et al., 1977; Bjorkland and Lindvall, 1975; Korf et al., 1976; Walters and Roth, 1976) or on the preterminal endings of dopamine neurons within the striatum (Bunney and Aghajanian, 1978; Iversen et al., 1976; Westfall et al.,1976).

Activation of these autoreceptors by dopamine or apomorphine results in a decrease in the synthesis and release of dopamine (Roth et al., 1974), an effect that is antagonized by the neuroleptic drug, haloperidol (Carlsson and Lindqvist, 1963; Zivkovic, 1977). These effects persist after transection of the nigro-striatal tract (Kehr et al., 1972) indicating that presynaptic receptor mechanisms may be involved in the regulation of transmitter synthesis in central dopaminergic neurons (Kehr et al., 1972; Roth et al., 1974; Walters and Roth, 1976; Westfall et al., 1976; Argiolas et al., 1982).

In addition, there is electrophysiological evidence for the presence of autoreceptors on dopamine neurons in the substantia nigra (Aghajanian and Bunney, 1977). Agonists and antagonists of dopamine can bind these receptors and directly modulate dopaminergic activity independently of postsynaptic feedback pathways between the striatum and substantia nigra (Bunney and Aghajanian, 1978; Di Chiara et al., 1977b; Garcia-Munoz et al., 1977). Binding studies with tritated ligands have demonstrated a 50% reduction in the number of D-3 sites in postmortem brains of patients with Parkinson's disease (Lee et al., 1978 a,b), or in rats whose nigro-striatal tracts have been lesioned with 6-Hydroxydopamine (Nagy et al., 1978; Fujita et al., 1980). These results indicate that approximately half of the autoreceptor sites in the striatum are located on presynaptic dopamine-containing neurons, an observation that is compatible with the hypothesized role of these receptors in dopamine metabolism and release (Argiolas et al., 1982).

1.3.3 Chemical Lesion and Drug-induced Supersensitivity of Caudate Dopamine Receptors

A. Introduction

Surgical or pharmacological disruption of dopaminergic transmission can result in a state of denervation supersensitivity that is characterized by an increase in the responsiveness of postsynaptic receptors to dopamine (Ungerstedt, 1971a; Klawans et al., 1980; Muller and Seeman, 1978; Reisine, 1981). The development of supersensitivity is thought to be a compensatory response of postsynaptic receptors to the reduced stimulation of these sites by dopamine, although evidence of presynaptic receptor supersensitivity following chronic administration of neuroleptics has also been reported (Bannon et al., 1980; Nowycky and Roth, 1978).

The latter effect is thought to represent an increase in the responsiveness of presynaptic autoreceptors to dopamine, leading to enhanced suppression of dopamine synthesis (Nowycky and Roth, 1978). This would tend to reduce further the availability of synaptic dopamine, and potentiate the development of postsynaptic receptor supersensitivity. Thus dopaminergic transmission involves the complementary regulation of pre as well as postsynaptic receptors both of which are capable of adapting to long-term alterations in dopaminergic function.

These observations have several implications for the treatment of diseases involving disorders of dopaminergic transmission. Thus, the symptoms of schizophrenia can be alleviated by administration of neuroleptics that block postsynaptic dopamine receptors (Matthysse, 1973; Seeman, 1977; Snyder, 1981), or alternatively by treatment with low doses of dopamine mimetics that bind presynaptic autoreceptors and thereby inhibit dopamine synthesis (Inanaga et al., 1975; Smith et al., 1977a; Carlsson, 1978; Gerlach and Luhdorf, 1975; Corsini et al.,

1977). At higher concentrations, dopamine agonists will also bind postsynaptic receptors leading to facilitation of dopaminergic transmission and an exacerbation of psychotic symptoms (Yaryura-Tobias et al., 1970; Angrist et al., 1978; Goodwin 1972; Snyder, 1973). The therapeutic benefits of neuroleptics presumably derive from their ability to bind these receptors and thereby antagonize those aspects of the psychotic process that depend on postsynaptic dopaminergic transmission. Although neuroleptic drugs block dopamine receptors throughout the brain, it has been hypothesized that their antipsychotic properties are more directly related to inhibition of dopamine receptors in the limbic system (Snyder, 1981).

One of the clinical sequelae associated with long-term neuroleptic therapy is tardive dyskinesia, a movement disorder thought to be a pathological manifestation of dopamine receptor supersensitivity secondary to prolonged blockade of dopamine receptors in the corpus striatum (Klawans et al., 1980; Baldessarini and Tarsy, 1980). Chronic administration of dopamine agonists, a procedure that leads to a decrease in the sensitivity of these receptors to dopamine (Di Chiara et al., 1978; Carroll et al., 1977; Tolosa, 1978; Smith et al., 1977b), may be of benefit in the treatment of this condition. Similarly, a potentiation of receptor sensitivity may be clinically desirable in patients with Parkinson's disease in whom deterioration of presynaptic dopaminergic neurons is a central feature (Birkmayer et al., 1975; Lee et al., 1978a; Hornykiewicz, 1975).

B. Behavioural Evidence of Dopamine Supersensitivity

Numerous studies indicate that pharmacological denervation by long-term administration of dopamine-blocking or dopamine-depleting agents enhances dopamine-mediated stereotypic behaviour. This supersensitive behavioural response is seen

following chronic treatment with neuroleptics such as haloperidol (Tarsy and Baldessarini, 1974; Gianutsos et al., 1974; Gnegy et al., 1977a,b; Smith and Davis, 1976; Tye et al., 1977; Voith, 1977), chemical lesions of the nigro-striatal pathway with 6-Hydroxydopamine (Zigmond and Stricker, 1977; Ungerstedt, 1971a; Mishra et al., 1974, 1980) and depletion of catecholamine stores with reserpine (Tarsy and Baldessarini, 1974; Friedman et al., 1975).

C. Biochemical Evidence of Dopamine Supersensitivity

(i) Dopamine Receptor Binding Studies:

Chronic administration of neuroleptic drugs, or denervation of the dopamine-containing neurons in the substantia nigra results in an increase in the density of D-2 receptors in the corpus striatum (Burt et al., 1976; Friedhoff et al., 1977; Kobayashi et al., 1977; Muller and Seeman 1977, 1978; Ebstein et al., 1979; Thal et al., 1979; Creese et al., 1977b; Waddington et al., 1979; Murrin et al., 1979; Nagy et al., 1978; Creese and Snyder, 1979; Mishra et al., 1980). The D-3 sites increase as well, following long-term neuroleptic treatment (Leysen, 1980; Muller and Seeman, 1977; Hitri et al., 1978).

(ii) Dopamine-sensitive Adenylate Cyclase:

The above studies demonstrate that one of the biochemical features of dopamine supersensitivity is an increase in the number of specific dopamine receptors. However, the intracellular changes that accompany altered receptor sensitivity are poorly understood.

The observation that dopamine causes an elevation in intracellular cAMP has provided one biochemical strategy to the approach of this problem. Unfortunately the effects of drug or surgically-induced denervation on the activi-

ty of dopamine-sensitive adenylate cyclase have been difficult to interpret due to conflicting observations. Some investigators have reported increases in the activity of the enzyme following chronic neuroleptic administration (Gnegy et al., 1977a,b; Iwatsubo and Clouet, 1975; Kaneno et al., 1978; Marshall and Mishra, 1980) or lesions of the nigro-striatal tract (Mishra et al., 1974, 1978, 1980), and this has been interpreted as a biochemical correlate of receptor supersensitivity. Additional support for this concept has come from studies demonstrating that the striatal membranes of animals chronically treated with antipsychotic drugs have an increased content of calmodulin (Gnegy and Lau, 1980; Lucchelli, 1980; Gnegy, 1982). As calmodulin has been shown to modulate the activity of brain adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975) as well as 3':5'-nucleotide phosphodiesterase (Cheung, 1971; Teo et al., 1973; Kakiuchi et al., 1973) regulation of the amount of calmodulin in synaptic membranes may be a primary factor in the development of drug-induced supersensitivity.

A key feature in this process appears to be the translocation of calmodulin from the membrane where it activates adenylate cyclase, into the cytosol where it converts the high K_m phosphodiesterase into a low K_m form thereby accelerating the metabolism of cAMP (Gnegy et al., 1977a,c). Thus, long-term stimulation of dopamine receptors is associated with an increase in the soluble calmodulin content and concomitant activation of phosphodiesterase (Hanbauer et al., 1980), whereas chronic blockade of these receptors enhances the membrane content of calmodulin but has no effect on the soluble pool (reviewed in Gnegy, 1982).

The release of calmodulin from the membrane is stimulated by cAMP-dependent protein kinase (Gnegy et al., 1977c), a process that is impaired in rats chronically treated with neuroleptics (Gnegy and Lau, 1980). Moreover,

following the phosphorylation-dependent release of calmodulin from the membrane, dopamine-stimulated adenylate cyclase activity is substantially reduced (Gnegy et al., 1977a). The substrate for the kinase(s) has not been identified although there is some suggestion that it may be a calmodulin-binding protein (Gnegy et al., 1977b).

The above results notwithstanding, a number of investigators have been unable to detect an increase in the sensitivity of adenylate cyclase to dopamine following chronic neuroleptic treatment (Von Voigtlander et al., 1975; Heal et al., 1976; Rotrosen et al., 1975; Roufogalis et al., 1976b; Hyttel, 1978) or denervation of the nigro-striatal tract (Freedman et al., 1981; Krueger et al., 1976). This may be due to methodological differences between different studies, but in general the observed effects of long-term neuroleptic therapy on adenylate cyclase activity have been small (Muller and Seeman, 1978) as have the increases in membrane calmodulin content (Gnegy et al., 1977 a,b).

Moreover, since inhibition of the dopamine-sensitive adenylate cyclase requires concentrations of neuroleptic that are considerably higher than circulating drug levels reported in vivo (Seeman, 1977; Nakahara et al., 1978) one wonders whether changes in the activity of this enzyme system are a reliable measure of drug-induced supersensitivity. Although calmodulin has been implicated in dopamine receptor supersensitivity, the specificity of the neuroleptic-induced effect on calmodulin must be questioned since the interaction of these drugs with calmodulin is dependent on their hydrophobicity not clinical potency (Roufogalis, 1981).

Consistent with this, a number of pharmacological agents that do not block dopamine receptors are capable of binding calmodulin and inhibiting its activity (Roufogalis, 1981). Neuroleptics also inhibit other cellular enzymes

including phospholipid-sensitive kinase (Mori et al., 1980; Schatzman et al., 1981) and this might indirectly affect dopaminergic function particularly since phospholipids appear to be involved in the coupling of dopamine receptors to striatal adenylate cyclase (Anand-Srivastava and Johnston, 1981).

In summary, the evidence that calmodulin and adenylate cyclase are involved in the mediation of dopamine receptor supersensitivity is still far from conclusive. In view of the fact that many of the effects of neuroleptics appear to be mediated by D-2 receptors, which are not related to adenylate cyclase, and that this population of receptors is reliably increased in supersensitive animals (Seeman, 1980) it will be important to elucidate the biochemical mechanism by which changes in the sensitivity of these receptors are induced.

1.4 cAMP-Dependent Phosphorylation in Brain

1.4.1 Introduction

There is substantial evidence that hormonally-induced increases in intracellular cAMP are accompanied by activation of a specific class of enzymes known as cAMP-dependent protein kinases (reviewed in Rubin and Rosen, 1975, Nimmo and Cohen, 1977, Glass and Krebs, 1980; Krebs and Beavo, 1979). These enzymes catalyze the transfer of the terminal phosphate of ATP onto serine and threonine residues of a variety of substrate proteins. Cyclic nucleotide dependent protein kinase activity was initially demonstrated in skeletal muscle by Walsh et al. (1968) and subsequently shown by Kuo and Greengard (1969) to be distributed ubiquitously in the tissues of phylogenetically diverse species. This latter observation led to the proposal that cAMP exerts most if not all of its cellular effects through activation of cAMP-dependent protein kinases.

The discovery that nervous tissue contains a cAMP-dependent protein kinase that is capable of catalyzing the phosphorylation of exogenous (Maeno and Greengard, 1972; Miyamoto et al., 1971) and endogenous protein substrates (Johnson et al., 1972; Ueda et al., 1973), coupled with the observation that the actions of a number of neurotransmitters are mediated through cAMP (Bloom et al., 1980; Nathanson, 1977; Daly, 1975), led to the proposal that this enzyme system might regulate some aspects of synaptic function (Greengard, 1978, 1979). In support of this, the enzyme, its protein substrates, and a phosphoprotein phosphatase are highly concentrated in synaptic membranes (Ueda et al., 1975; Weller and Morgan, 1976; Uno et al., 1977a,b) as are the enzymes adenylate cyclase (Clement-Cormier et al., 1975; Laduron et al., 1976; Weiss and Costa, 1968; De Robertis et al., 1967) and phosphodiesterase (De Robertis et al., 1967; Weiss and Costa, 1968).

1.4.2 Biochemical Properties of cAMP-dependent Protein Kinases

Two isozymes of the cAMP-dependent protein kinase have been identified by ion-exchange chromatography on DEAE cellulose (Corbin et al., 1975), and by their biochemical (Corbin et al., 1975; Hofmann et al., 1975) and immunological (Fleisher et al., 1976) properties. The kinase from rabbit skeletal muscle and bovine heart have been used as prototypes for the Type I and Type II isozymes respectively, the relative distribution and concentration of the two enzymes varying depending on the tissue source (Sugden and Corbin, 1976, Bechtel et al., 1977). Both enzymes have a symmetric tetrameric structure consisting of two regulatory (R) subunits and two catalytic (C) subunits. In the inactive state the R and C subunits are associated, the R subunit impeding the activity of C by shielding the catalytic site (Hoppe et al., 1978a; Witt and Roskowski, 1975; Todhunter and Purich, 1977; Corbin et al., 1978; Flockhart et al., 1980).

Binding of cAMP to the R subunit causes dissociation of the enzyme into an R_2 $[cAMP]_4$ complex and a catalytic component that exhibits phosphotransferase activity (Brostrom et al., 1970; Tao et al., 1970; Gill and Garren, 1970). The stoichiometry of cAMP binding to R is 4 mol of cAMP per regulatory subunit dimer (Corbin et al., 1978; Weber and Hilz, 1979; Builder et al., 1980). Activation of the kinase by cAMP can be depicted as follows:

$$R_2C_2 + 4\,[cAMP] = R_2[cAMP]_4 + 2C$$

The catalytic subunits from the two isozymes have been purified and shown to be virtually identical (Hofmann et al., 1975; Bechtel et al., 1977). Each subunit has a molecular weight of 40,000 and is readily inactivated by a heat stable protein inhibitor found in many tissues (Appleman et al., 1966; Ashby and Walsh, 1972).

The specific biochemical and functional properties associated with the two types of cAMP-dependent protein kinases appear to be attributed to differences in their regulatory subunits (Hofmann et al., 1975). For this reason, elucidation of the molecular mechanisms involved in dissociation and reassociation of the two enzymes has been an important area of research.

The type I protein kinase is readily dissociated and activated by salt, histone and cAMP (Corbin et al., 1975), contains high affinity binding sites for Mg ATP (Hofmann et al., 1975), reassociates rapidly in the presence of Mg ATP (Corbin et al., 1975; Hofmann et al., 1975), and has a regulatory subunit of molecular weight 48,000. The Type II kinase is not readily dissociated by high ionic strength, and has a cAMP binding protein of molecular weight 55,000 (Rubin et al., 1972) that is phosphorylated by the catalytic subunit.

Termination of the effects of cAMP involves rapid dissociation of cAMP from the R_2 $[cAMP]_4$ complex as a result of C binding (Smith et al., 1981).

Phosphodiesterase, the enzyme that degrades cAMP into 5'-AMP, is incapable of metabolizing cAMP bound to R (Brostrom et al., 1971). Therefore, termination of the effects of cAMP must occur through a sequential process in which cAMP is first released from the R_2 $[cAMP]_4$ complex, followed by its inactivation by phosphodiesterase (Chau et al., 1980).

In the case of the Type 1 kinase, release of cAMP from R and recombination of R and C to form inactive holoenzyme is greatly facilitated by Mg ATP (Wilchek et al., 1971; Brostrom et al., 1971; Beavo et al., 1975). This nucleotide modulates the activity of the Type I kinase by binding to a high affinity site on the holoenzyme (Beavo, et al., 1975; Schwechheimer and Hofmann, 1977). The ATP binding site appears to be the same as the catalytic site on C or a component of it, the affinity of which is greatly enhanced by association with R (Hoppe et al., 1978 a,b). Binding of ATP results in stabilization of the holoenzyme such that the affinity of the enzyme for cAMP is reduced (Haddox et al., 1972; Beavo et al., 1975; Hofmann et al., 1975), as is its capacity to dissociate in the presence of salt and histone (Corbin et al., 1975).

Since levels of ATP in the cell are considerably higher than the K_m for binding of ATP to either the holoenzyme or free C (Schwechheimer and Hofmann 1977; Beavo et al., 1975), it is likely that one of the functions of the high affinity site is to insure that ATP binds preferentially to the inactive holoenzyme, rather than to free C, thereby preventing excessive activation of C under physiological conditions (Beavo et al., 1975).

As described above, ATP is an important modulator of Type I protein kinase activity. Dissociation and activation of the Type II enzyme on the other hand, appears to be regulated through phosphorylation of the regulatory subunit by C (Erlichman et al., 1974). Autophosphorylation of the Type II kinase regula-

tory subunit (R_{II}) occurs rapidly through an intramolecular mechanism that is not dependent on cAMP, and may represent 20-30% of the activity expressed in the presence of cAMP (Rubin et al., 1972; Erlichman et al., 1974; Todhunter and Purich, 1977; Corbin et al., 1978; Potter and Taylor, 1979).

Phosphorylation of R_{II} can also occur through an intermolecular process catalyzed by free C in response to dissociation by cAMP (Erlichman et al., 1974). Both the "phospho" and "dephospho" forms of the enzyme are rapidly and completely dissociated by cAMP (Rangel-Aldao and Rosen, 1976; Hofmann et al., 1975). Both reassociate in the absence of cAMP (Erlichman et al., 1974) however, dephosphorylated R_{II} recombines with C several times faster than phosphorylated R_{II} (Rangel-Aldao and Rosen, 1976). This effect is magnified in the presence of physiological concentrations of ATP, as ATP tends to inhibit reassociation of the Type II subunits (Rangel-Aldao and Rosen, 1977).

Dephosphorylation of R_{II} can occur by reversal of the kinase reaction (Rosen and Erlichman, 1975) or by activation of phosphoprotein phosphatase (Chou et al., 1977; Erlichman et al., 1975). Since the concentration of ATP in the cell far exceeds the amount required for autophosphorylation (Erlichman et al., 1974), and the phosphorylated holoenzyme is not a substrate for phosphoprotein phosphatase (Chou et al., 1977; Erlichman et al., 1975), it has been proposed that most of the enzyme in vivo is in a phosphorylated state (Rangel-Aldao and Rosen, 1976; Rangel-Aldao et al., 1979). Studies of phosphorylation in intact animals support this contention (Uno, 1980).

1.4.3 Characteristics of cAMP-dependent Protein Kinases in Brain

The cAMP-dependent protein kinase from brain has been extensively purified and characterized (Uno et al., 1977a; Rubin et al., 1979). In contrast to other tissues, a high proportion of the enzyme is membrane-bound (Hofmann et al., 1977; Corbin et al., 1975; Maeno et al., 1971). Soluble and particulate fractions of brain contain both the Type I and Type II protein kinases (Rubin et al., 1979; Corbin et al., 1975; Walter et al., 1978b; Lohmann et al., 1980).

The Type II kinase is highly concentrated in membrane and cytosol fractions (Walter et al., 1978b) and appears to mediate the phosphorylation of a number of neuronal membrane proteins (Walter et al., 1979a). It shares a number of biochemical properties in common with other Type II kinases including a 55,000 dalton regulatory subunit (Rubin et al., 1979), autophosphorylation (Ul'masov et al., 1980; Walter et al., 1977, 1978; Walter and Greengard, 1978; Maeno et al., 1974; Rubin et al., 1979), and response to salt and histone (Corbin et al., 1975).

Despite these biochemical similarities, the brain enzyme has immunological characteristics that distinguish it from other Type II enzymes (Erlichman et al., 1980; Rubin et al., 1979). The functional implications of this have not been determined, although the fact that the brains of different mammalian species consistently contain high levels of the Type II kinase, while the relative distribution of the two enzymes in other tissues is markedly heterogeneous (Sugden and Corbin, 1976; Bechtel et al., 1977) may indicate that this enzyme is uniquely adapted to nervous system function.

The results of lesion, biochemical and histochemical studies indicate that the majority of brain adenylate cyclase resides postsynaptically. Therefore one might also expect cAMP-dependent protein kinases to be localized postsynaptically. Consistent with this, destruction of postsynaptic neurons with kainic acid

leads to a substantial reduction in both the amount of Type II cAMP-dependent protein kinase, as well as one of its principal substrates, Protein I, in synaptic membrane fractions of rat caudate nucleus (Sieghart et al., 1978).

The role of the Type I enzyme in neuronal function has not been elucidated. Based on the fact that it does not catalyze the phosphorylation of synaptic membrane proteins, and that its activity is not altered by kainic acid treatment (Walter et al., 1979a) both of which distinguish it from the Type II enzyme, it has been concluded that the Type I enzyme is probably not involved in synaptic function (Walter et al., 1979a). However, since kainic acid destroys cell bodies and dendrites while leaving axons intact, a possible presynaptic role for the Type I kinase can not be excluded.

1.4.4 Substrates of cAMP-dependent Kinases in Brain

Synaptic membrane fractions from mammalian brain contain several proteins whose phosphorylation is stimulated by cAMP (Ueda et al., 1973; Ueda and Greengard, 1977). One of these proteins has been identified as the regulatory subunit of the Type II protein kinase (Rubin et al., 1979; Lohmann et al., 1980).

Another prominent substrate for the enzyme, Protein I, consists of two related polypeptide chains with molecular weights of 86,000 (Protein Ia) and 80,000 (Protein Ib) respectively (Ueda and Greengard, 1977). This protein appears to be specifically localized in neuronal tissue (De Camilli et al., 1979; Bloom et al., 1979; Sieghart et al., 1978; Goelz et al., 1981), where it is primarily associated with synaptic vesicles and postsynaptic membranes (De Camilli et al., 1979; Bloom et al., 1979). These latter pieces of evidence, coupled with the fact that the appearance of Protein I in development parallels synaptic ontogenesis (Lohmann et al., 1978a,b) has led to the proposal that this protein is intimately involved in the modulation of synaptic function (Dolphin et al., 1980).

Consistent with this, studies by Greengard's group have shown that the phosphorylation of Protein I can be altered by a number of substances that are known to affect neuronal activity including cyclic nucleotides (Forn and Greengard, 1978), depolarizing agents (Forn and Greengard, 1978; Nestler and Greengard, 1980), and anesthetic and convulsant drugs (Strombom et al., 1979). In addition, the putative neurotransmitters serotonin and dopamine have been shown to increase the phosphorylation of Protein I in rat facial motor nucleus and bovine superior cervical ganglion respectively (Dolphin and Greengard, 1981; Nestler and Greengard, 1980).

Interpretation of the functional significance of the modifications in Protein I phosphorylation is complicated by the fact that increases in its state of phosphorylation were evaluated using a retrospective technique in which subsequent labelling of the extracted protein using an exogenous protein kinase demonstrated a decrease in the incorporation of [^{32}P]-phosphate in stimulated relative to control slices, an observation compatible with increased incorporation of cold phosphate into the protein during prior exposure to test substances.

The fact that some labelled phosphate was still incorporated into Protein I using the "back phosphorylation" technique indicates that the protein is probably not maximally phosphorylated as a result of stimulation in vivo. Subtraction of the difference between the amount of phosphorylation that occurs in the extracted protein under optimal assay conditions, and that assumed to have occurred in vivo may lead to an overestimate of the degree of stimulated phosphorylation in the latter case.

As yet there has been no demonstration that neurotransmitters affect the state of phosphorylation of Protein I in slices of cerebral cortex (Forn and Greengard, 1978) or in synaptosomes (Krueger et al., 1977). This may reflect the

complexity of synaptic interconnections and the multiplicity of neurotransmitter receptors in cortical tissue. Although the effect of dopamine on the phosphorylation of synaptic membrane proteins remains uncertain, one group of investigators has shown that dopamine enhances the incorporation of ^{32}P into rat striatal slices. Preincubation of these tissues with dopamine antagonists inhibited this effect (Williams, 1976). These studies did not determine which striatal proteins were affected by treatment with dopamine. Subsequent investigations by the same group revealed that dopamine produced a small and generalized increase in the phosphorylation of caudate nucleus proteins. These effects were not mimicked by cAMP, and were only seen in the presence of high levels of ATP (Hullihan et al., 1979). Moreover, as these latter studies were conducted in homogenates, one cannot conclude that the observed changes in protein phosphorylation were synaptic in origin.

Although there is an increasing amount of circumstantial evidence implicating the role of cAMP-mediated phosphorylation in neuronal function, few studies have convincingly demonstrated that changes in the electrical activity of neurons are associated with altered phosphorylation of neuronal membrane proteins. Some support for this hypothesis has come from studies in invertebrates. Injection of the catalytic subunit of cAMP-dependent protein kinase into the abdominal ganglion of Aplysia causes a decrease in the K^+ current, and a prolongation of the action potential leading to increased Ca^{++} influx (Kaczmarek et al., 1980; Castellucci et al., 1980). These effects are normally produced by the neurotransmitter serotonin (Klein and Kandel, 1980). The additional Ca^{++} influx into the membrane increases the amount of transmitter released from the presynaptic nerve terminal (Castellucci et al., 1980). The endogenous substrate for the kinase is not known, although it is thought to be a component of the K^+ channel or a protein associated with it.

1.5 Calcium-Dependent Phosphorylation in Brain

1.5.1 Calmodulin-dependent Protein Phosphorylation

A. Introduction

Ca^{++} is involved in the regulation of a number of neuronal processes (Ruben, 1970; Baker, 1971), yet little is known about the molecular mechanisms by which Ca^{++} exerts its effects. The elucidation of the biochemical mechanisms underlying Ca^{++}-regulated cellular processes has been greatly facilitated by the demonstration that many of the effects of Ca^{++} are mediated through specific binding proteins (Kretsinger, 1979).

One of the best characterized of the Ca^{++}-binding proteins is a heat-stable acidic protein of molecular weight 17,000 known as calmodulin (Means and Dedman, 1980; Klee et al., 1980; Cheung, 1980). Calmodulin is found in most mammalian tissues (Smoake et al., 1974), and is particularly enriched in brain (Watterson et al., 1976) where it is thought to regulate a variety of cellular functions associated with synaptic transmission (Roufogalis, 1980).[1] These include activation of the enzymes cyclic nucleotide phosphodiesterase (Cheung, 1971; Kakiuchi et al., 1973), adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975), myosin light chain kinase (Dabrowska et al., 1977) and the synaptic plasma membrane [Ca^{++} + Mg^{++}] ATPase (Kuo et al., 1979; Sobue et al., 1979).

As described previously, one of the important roles of calmodulin is the regulation of cAMP metabolism, a function which emphasizes the close interrelationship between Ca^{++} and cAMP in the control of cellular processes. The regulation of cAMP metabolism is accomplished by a dual mechanism that involves first the stimulation of adenylate cyclase by calmodulin in the membrane

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[1] Unless otherwise indicated, all processes attributed to calmodulin in the text refer to the Ca^{++}-calmodulin complex.

and second, regulation of the rate of cAMP degradation through calmodulin activation of the high K_m phosphodiesterase in the cytosol (Cheung, 1980).

The role of calmodulin in synaptic function is additionally supported by histochemical studies indicating that it is localized predominantly in postsynaptic neurons and that its distribution parallels that of phosphodiesterase confirming its involvement in cAMP metabolism (Wood et al., 1980). Biochemical evidence that calmodulin regulates the activity of the Ca^{++}-activated ATPase in synaptosomes (Sobue et al., 1979; Kuo et al., 1979), as well as the release of catecholamines from synaptic vesicles (De Lorenzo et al., 1979; De Lorenzo, 1982) indicate that it is involved in presynaptic function as well.

B. Calmodulin-dependent Protein Kinases

Recent studies have shown that the Ca^{++}-dependent phosphorylation of a number of proteins in synaptic membranes (Schulman and Greengard, 1978a,b; O'Callaghan et al., 1980a, 1980c) and synaptic vesicles (De Lorenzo et al., 1979) is mediated through calmodulin. Calmodulin also regulates the phosphorylation of a number of proteins in brain cytosol (O'Callaghan et al., 1980 a,b; Yamauchi and Fujisawa, 1979) including the enzyme tryptophan hydroxylase (Kuhn et al., 1980; Yamauchi and Fujisawa, 1979) that regulates the biosynthesis of serotonin. Brain tissue contains at least three distinct calmodulin-dependent protein kinases: myosin light chain kinase (Dabrowska et al., 1978; Yagi et al., 1978;), phosphorylase b kinase (Cohen et al., 1978; Ozawa, 1973) and one or two additional enzymes that regulate the phosphorylation of neuronal specific Protein I (Kennedy and Greengard, 1981) as will be discussed later.

1.5.2 Phospholipid-sensitive Protein Kinase

Another species of Ca^{++}-dependent protein kinase, requiring phospholipid as a cofactor has been identified in a wide range of mammalian tissues (Kuo et al., 1980; Takai et al., 1977a,b; Minakuchi et al., 1981). The enzyme is highly concentrated in brain (Kuo et al., 1980) where it is distributed in soluble and particulate fractions (Kuo et al., 1980) along with its endogenous protein substrates (Wrenn et al., 1980). The enzyme is highly stimulated by phosphatidylserine as well as phosphatidylinositol (Takai et al., 1979; Wrenn et al., 1980; Kaibuchi et al., 1981; Kishimoto et al., 1980; Takai et al., 1981). Synaptic membranes are also capable of activating the purified enzyme in the presence of Ca^{++} (Takai et al., 1979). In the presence of phospholipid, the affinity of the enzyme for Ca^{++} is increased several fold (Kuo et al., 1980). This suggests that this enzyme system may be highly responsive to increases in Ca^{++} influx following neuronal depolarization.

1.5.3 Inhibition of Ca^{++}-dependent Protein Kinases by Neuroleptic Drugs

Levin and Weiss (1976, 1979) have demonstrated that certain antipsychotic drugs can bind to the Ca^{++}-calmodulin complex thereby preventing calmodulin from activating its target enzymes. The phenothiazine trifluoperazine has been shown to be particularly effective at inhibiting calmodulin-mediated processes (Levin and Weiss, 1977; 1978; 1980), although this appears to be a structurally non-specific interaction (Roufogalis, 1981). Ca^{++} has been shown to expose hydrophobic regions of calmodulin (Tanaka and Hidaka, 1981) and the ability of antipsychotics to bind and inhibit calmodulin appears to be a function of their hydrophobicity not their specific clinical properties (Roufogalis, 1981).

Consistent with these observations, antipsychotic drugs, including trifluoperazine have also been shown to inhibit phospholipid-sensitive kinase (Mori et

al., 1980; Wrenn et al., 1981; Schatzman et al., 1981), although the latter enzyme appears much less sensitive to the effects of these drugs and the inhibition can be overcome by phosphatidylserine (Schatzman et al., 1981). High levels of calmodulin, however, do not reverse the drug-induced inactivation of the calmodulin kinase (Schatzman et al.,; 1981) indicating that antipsychotics bind calmodulin with a higher affinity than they do the phospholipid-sensitive kinase.

1.5.4 Substrates for Ca^{++}-dependent Protein Kinases in Brain

As discussed earlier, synaptic membranes contain two neuronal specific proteins, Proteins Ia and Ib that are phosphorylated by a cAMP-dependent protein kinase (Ueda et al., 1973; Ueda and Greengard, 1977; Lohmann et al., 1980). Treatment of intact synaptosomes (Sieghart et al., 1979; Krueger et al., 1977) or brain slices (Forn and Greengard, 1978) with depolarizing agents such as K^+ or veratridine, increases the phosphorylation of these same proteins through a Ca^{++}-dependent process.

Dibutyryl cAMP and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) also increase the phosphorylation of these proteins in brain slices, but these effects do not require extracellular Ca^{++} (Forn and Greengard, 1978).

Subsequent investigations revealed that Protein I is phosphorylated at three sites (Huttner et al., 1981). One site is phosphorylated by cAMP-dependent protein kinase as well as by a Ca^{++}-dependent protein kinase (Kennedy and Greengard, 1981; Huttner et al., 1981; Huttner and Greengard, 1979). The kinase that phosphorylates this region of the Protein I molecule has been tentatively identified as calmodulin-sensitive, based on the fact that its activity is inhibited by trifluoperazine (Kennedy and Greengard, 1981). The kinase however, does not require calmodulin for activation, and is localized exclusively in the cytosol

(Kennedy and Greengard, 1981). Since phospholipid kinase is also activated by Ca^{++}, inhibited by trifluoperazine and found in brain cytosol (Kuo et al., 1980; Schatzman et al., 1981; Wrenn et al., 1980) this enzyme may also mediate the phosphorylation of this fragment of Protein I. In line with this, Wu et al. (1982) have recently shown that an 87,000 dalton acidic protein that co-migrates with Protein Ia on sodium-dodecyl sulphate polyacrylamide gel electrophoresis is localized in synaptosomal cytosol and phosphorylated by the phospholipid-sensitive protein kinase. This polypeptide was initially identified as a proteolytic fragment of Protein Ia.

The two other sites in Protein I are phosphorylated by calmodulin-dependent protein kinase (Kennedy and Greengard, 1981; Huttner and Greengard, 1979). The kinase is found in cytosolic as well as particulate fractions and is very sensitive to calmodulin (Kennedy and Greengard, 1981). The Ca^{++}-dependent phosphorylation of Protein I is decreased by intrastriatal injections of kainic acid (Sieghart et al., 1980) indicating that this protein kinase activity is neuronal in origin.

Although purified Protein I is a substrate for calmodulin-dependent protein kinase (Kennedy and Greengard, 1981; Huttner and Greengard, 1979), the phosphorylation of Protein I in synaptic membranes appears to be only marginally enhanced by calmodulin (Sieghart et al., 1980). Ca^{++}-dependent phosphorylation of Protein I, on the other hand, is readily demonstrated in intact synaptosomes incubated with $^{32}P_i$ (Sieghart et al., 1979, 1980; Krueger et al., 1977). These discrepancies may be accounted for by differences in the experimental system used to measure protein phosphorylation. Alternatively, the Ca^{++}-dependent phosphorylation of Protein I in intact synaptosomes may be mediated independently of calmodulin.

The cAMP-dependent phosphorylation of Protein I occurs in a collagenase-resistant portion of the molecule, whereas the sites that are regulated by Ca^{++} and calmodulin are collagenase-sensitive (Huttner et al., 1981). It has recently been proposed that the collagenase-sensitive region of Protein I may be involved in binding of the protein to synaptic membranes (Ueda, 1981). Since the cAMP-dependent phosphorylation of Protein I is probably a postsynaptic event (Sieghart et al., 1978), whereas the Ca^{++}-dependent phosphorylation of Protein I appears to be enriched in presynaptic elements such as synaptosomes (Krueger et al., 1977; Sieghart et al., 1979, 1980), the differential phosphorylation of Protein I by Ca^{++} and cAMP-dependent protein kinases may provide a molecular mechanism for pre and postsynaptic regulation of the activity of this protein respectively.

Ca^{++} has been shown to mediate the phosphorylation of other proteins in synaptic membranes. Two of these proteins, designated DPH-L and and DPH-M, have molecular weights of 51,000 and 62,000 respectively, and are phosphorylated by a calmodulin-dependent protein kinase (Schulman and Greengard, 1978a,b; De Lorenzo et al., 1979). Recent studies have demonstrated that these proteins correspond to the alpha and beta subunits of neurotubulin (Burke and De Lorenzo, 1981, 1982; De Lorenzo, 1982).

Phosphorylation of these proteins occurs in intact synaptosomes and synaptic vesicles in response to depolarizing agents, and is temporally correlated with an increase in Ca^{++} uptake and neurotransmitter release (De Lorenzo et al., 1979). None of these effects are observed if Ca^{++} is omitted from the incubation medium. These results indicate that phosphorylation of specific vesicle proteins by calmodulin-dependent protein kinase may mediate some of the effects of Ca^{++} on vesicular function and transmitter release.

An additional role of Ca^{++}-dependent phosphorylation in transmitter release is suggested by the observation that synaptosomes contain an "actin-like" protein whose phosphorylation is enhanced by Ca^{++} (Hershkowitz, 1978; Hofstein et al., 1980), although other investigators have provided strong evidence that this protein is in fact the alpha subunit of pyruvate dehydrogenase (Morgan and Routtenberg, 1980; Browning et al., 1981a; Sieghart, 1981, Magilen et al., 1981). An interaction between synaptosomal actin and vesicular myosin, analogous to the process of excitation-contraction coupling in muscle, has been proposed as the basis for vesicular fusion and neurotransmitter release in nerves (Berl et al., 1973).

Substrates for Ca^{++}-dependent protein kinases have also been identified in cerebral cortex cytosol. Four proteins of molecular weights 95,000, 73,000, 50,000 and 15,000 are phosphorylated by phospholipid-sensitive kinase. The enzyme also phosphorylates a membrane protein of 21,000 daltons, (Wrenn et al., 1980). Substrates for the calmodulin-dependent protein kinase in brain cytosol include a 55,000 dalton protein as well as a 50,000 and 60,000 dalton protein believed to be the same as proteins DPH-L and DPH-M described earlier (O'Callaghan et al., 1980a,b).

1.6 Research Objectives

The original goal of these studies was to investigate the effect of dopamine on the phosphorylation of synaptic membrane proteins and to determine whether this effect was mediated by Ca^{++} or cAMP-dependent protein kinases. The nigrostriatal system was selected as a model in which to test this hypothesis for the following reasons:

1. The corpus striatum contains a high density of dopamine terminals and the dopamine receptors in this region have been well characterized.
2. The effects of dopamine can be selectively stimulated or inhibited by well-characterized pharmacological agonists and antagonists
3. Well-defined behavioural models (open-field behaviour, ipsilateral and contralateral turning) and biochemical models (dopamine-sensitive adenylate cyclase, numbers of dopamine receptors) exist for verifying the effects of pharmacological and surgical denervation of striatal tissue.
4. The synaptic interconnections in this system have been well-investigated, and methods are available for selectively disrupting these interconnections. The activity of protein kinases can be measured under these conditions thus yielding information regarding pre and post-synaptic localization of these enzymes.
5. Both Ca^{++} and cAMP have been shown to be important in the regulation of dopamine function in this system. The corpus striatum also contains Ca^{++} and cAMP-dependent protein kinases. Therefore, the effects of dopamine on both of these enzyme systems can be studied using established biochemical techniques.
6. Chronic blockade of dopamine receptors in the corpus striatum results in behavioural and biochemical alterations that reflect an increase in dopamine receptor sensitivity. Changes in cAMP and calmodulin have been shown to accompany this process. Therefore, the effects of altered dopaminergic function on the activities of cAMP and calmodulin-dependent protein kinase can be investigated.

7. Clinical relevance: altered dopamine function has been demonstrated in a variety of clinical conditions including Parkinson's disease, Huntington's Chorea, Gilles de la Tourette's Syndrome, drug induced dyskinesias, and schizophrenia. It is hoped that an improved understanding of the biochemistry of dopamine transmission might lead to more selective methods of therapeutic intervention in these disorders.

The hypothesis that dopamine alters the phosphorylation of synaptic membrane proteins was tested using a variety of research strategies. As a prelude to studies of cAMP-dependent phosphorylation it was first important to demonstrate that dopamine stimulated adenylate cyclase activity in preparations of rat caudate nucleus. Although the effects of dopamine on adenylate cyclase have been investigated by other workers, aspects of these studies were reduplicated to establish that the results obtained in the present study were compatible with published observations. This approach was taken to ensure that subsequent studies of cAMP-dependent phosphorylation could be correlated with measures of dopamine-sensitive adenylate cyclase activity.

The second research objective was to characterize cAMP-dependent protein kinase activity in rat caudate nucleus. The aim of these studies was to determine the subcellular and anatomical localization of the enzyme and its protein substrates as well as some of its biochemical properties.

The third research objective was to examine Ca^{++} and calmodulin-dependent protein kinase activity in rat caudate nucleus, as substrates for these enzymes have been implicated in the regulation of neuronal function (Sieghart et al., 1979; De Lorenzo, 1982; O'Callaghan et al., 1980a, 1980b; Sieghart, 1981; Greengard, 1979).

Having characterized some of the properties of Ca^{++} and cAMP-dependent protein kinases and their protein substrates the fourth objective was to determine the effect of dopamine on Ca^{++} and/or cAMP-dependent protein phosphorylation in striatal slices, and whether this effect could be antagonized by neuroleptic drugs. Consistent with the hypothesized role of protein kinases in synaptic function, pharmacological agents that facilitate or inhibit depolarization in brain slices were simultaneously investigated for their effects on protein phosphorylation.

Finally, if protein phosphorylation is involved in dopaminergic transmission, surgical or pharmacological disruption of dopamine pathways may lead to an adaptive increase in protein kinase activity or an alteration in the phosphorylation of substrate proteins. Therefore, the effects of chronic neuroleptic administration and surgical denervation on protein kinase activity were also investigated. Since both cAMP and calmodulin have been implicated in dopamine receptor supersensitivity the kinases that they activate may participate in the development of this process.

Chapter II

Materials and Methods

2.1 Animals

Male Wistar rats ranging in weight from 250-300 g were obtained from Woodlyn Farms, Guelph, Ontario. They were housed in an isolated room with controlled temperature and a 12 hour light-dark cycle. Food and water were available ad libitum.

2.2 Materials

The following chemicals obtained from commercial sources were used in this study: Histone (Type II-A), cAMP, GTP, ATP, dopamine, apomorphine-HCl, 6-Hydroxydopamine, kainic acid, 2-Chloro-adenosine, veratridine, dithiothreitol (DTT), norepinephrine, and calmodulin (Sigma Chemical Co., St.Louis, Mo.); 3-isobutyl-1-methylxanthine (Aldrich, Milwaukee Wisconsin); 4-(2-hydroxyethyl) 1-piperazin ethanesulfonic acid (HEPES, Calbiochem, California). Electrophoresis reagents were obtained from Bio-Rad (Mississippi), with the exception of acrylamide (BDH Chemicals, Toronto). Haloperidol (Haldol) was from McNeil Laboratories (Stouffville, Ont.), trifluoperazine (Stelazine-TFP) was from Smith, Kline and French (Mississauga, Ont.). [^{32}P] ATP (>3000 Ci/mmol) was purchased from Amersham (Mississauga, Ont.) as the triethylammonium salt. [^{32}P] orthophosphate was purchased from Amersham. [^{3}H] cAMP (32 Ci/mmol) was purchased from New England Nuclear (Boston, MA) as the ammonium salt. 8-N_3-[^{32}P] cAMP was purchased from ICN. (Irvine, Cal.). All other chemicals were standard

reagent grade. Double distilled, deionized water was used throughout these studies.

2.3 Experimental Procedures

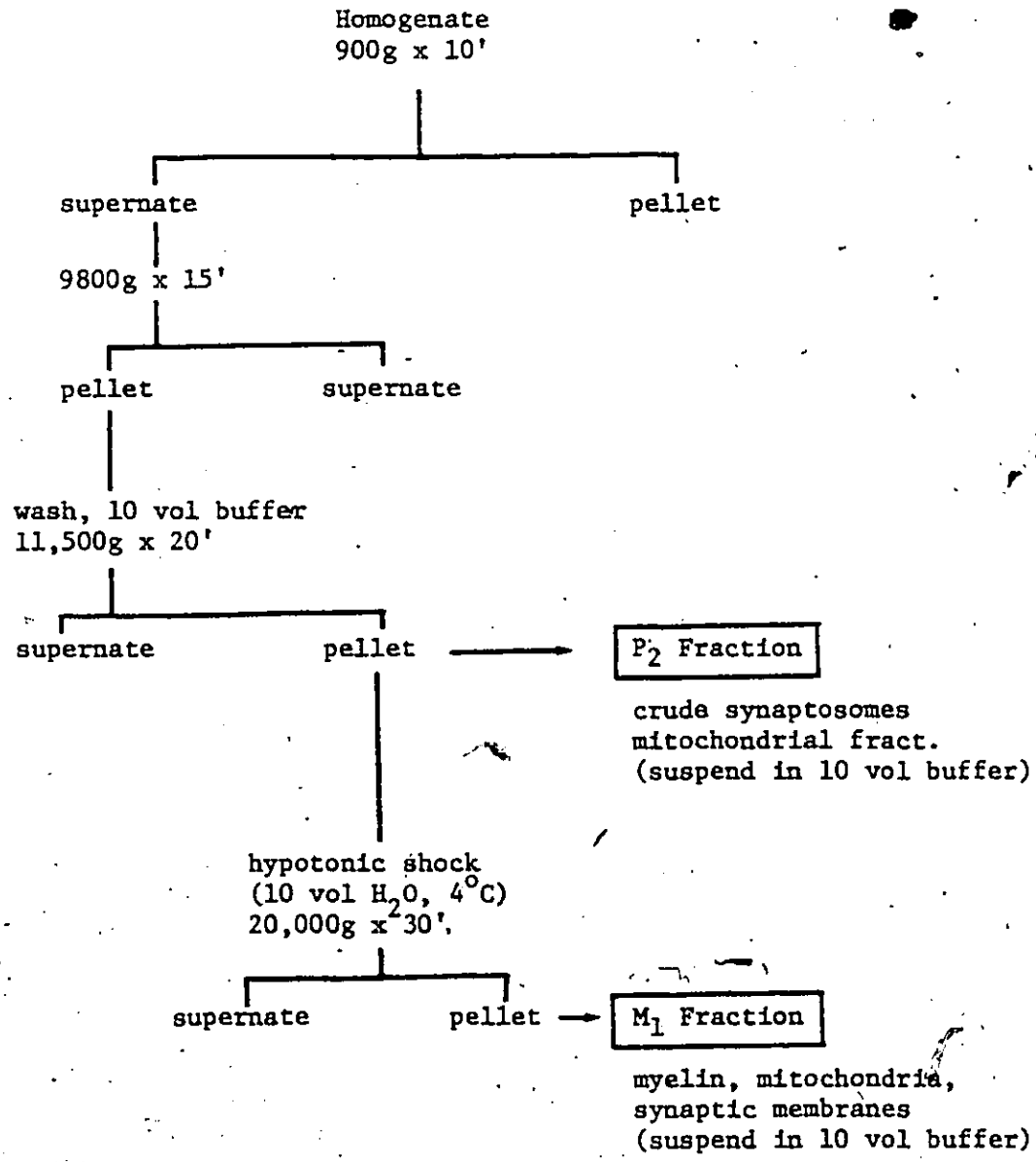
2.3.1 Preparation of Synaptic Membrane Fractions from Rat Caudate Nucleus

Synaptic membrane fractions were prepared by the following modification of the procedure of De Robertis et al. (1967). All operations were carried out at 4°C. Male Wistar rats were decapitated, and the caudate nuclei were quickly dissected and homogenized by hand in a Teflon-glass homogenizer in 10 volumes of ice-cold 0.32 M sucrose, 10 mM potassium phosphate buffer (pH 7.4). In some experiments the homogenization buffer included 2 mM EGTA and 0.3 mM PMSF (phenyl-methyl sulfonyl fluoride, Sigma) to inhibit proteolysis. Synaptosomes (P fraction) and synaptic membranes (M_1 fraction) were prepared by differential centrifugation as shown in Scheme I.

2.3.2 Determination of Adenylate Cyclase Activity in Homogenates and in Synaptic Membrane Preparations from Rat Caudate Nucleus

Adenylate cyclase activity was assayed by the method of Mishra et al. (1974) with slight modifications. Crude homogenates of striatal tissue were prepared by homogenizing tissues at 4°C in medium containing 2 mM Tris-maleate buffer (pH 7.4), 0.8 mM EGTA at a 1:50 dilution (wet weight:volume). Synaptic membrane fractions (M_1) were prepared as previously described (Scheme I) using a homogenizing buffer containing 10 mM potassium phosphate (pH 7.4), 0.32 M sucrose, 2 mM EGTA and 0.3 mM PMSF. M_1 fraction (.04 ml) was added to a 200 µl reaction mixture containing 80 mM Tris-maleate (pH 7.4), 2 mM $MgSO_4$, 1 mM

Scheme I


Homogenate
900g x 10'
supernate
pellet
9800g x 15'
pellet
supernate
wash, 10 vol buffer
11,500g x 20'
supernate
pellet
P2 Fraction
crude synaptosomes
mitochondrial fract.
(suspend in 10 vol buffer)
hypotonic shock
(10 vol H2O, 4°C)
20,000g x 30'
supernate
pellet
M1 Fraction
myelin, mitochondria,
synaptic membranes
(suspend in 10 vol buffer)

IBMX, and dopamine (or other test substances) as described in Results. The reaction was initiated by the addition of 1 mM ATP and was carried out for 2.5 min at 37°C in a shaking water bath.

Assays of adenylate cyclase activity in the M_1 fraction were identical except that the ATP concentration was 0.3 mM, and GTP (10 µM) was included in the reaction mixture as described by Clement-Cormier et al. (1975). Particulate matter was removed by low speed centrifugation, and 25 µl portions of the supernatant fluid were assayed for cAMP as described by Brown and Makman (1972). All samples were assayed in duplicate. Variation between repeated samples was less than 10%.

Binding reactions were carried out in a 200 µl reaction mixture containing 50 mM sodium acetate (pH 3.5), 0.25 mg/ml BSA, 10 nmol cAMP (32 µCi/nmol), 50 µl of binding protein from rat brain, and 25 µl of caudate supernate. After incubation for 90 min, samples were diluted with 1 ml ice cold 0.2 M potassium phosphate (pH 6.0) and filtered (Millipore Type HA). Filters were washed three times with 2 ml buffer, dried under a heat lamp, and counted in a toluene-based scintillator. Tissue levels of cAMP were determined by reading sample values from a binding curve produced by titrating increasing quantities of cold cAMP into the assay mixture. Levels of cAMP from 0.5-25 pmol were reliably detected using this approach. Adenylate cyclase activity was measured as pmol cAMP formed per mg protein during a 2.5 min incubation in the presence of 1 mM ATP (basal activity) and various concentrations of dopamine. Background activity (cAMP produced in the absence of previous compounds) was subtracted from values obtained for basal and dopamine-stimulated activities.

2.3.3 Endogenous cAMP-dependent Protein Phosphorylation

The reaction mixture (final volume, 100 µl) contained 60 µl of tissue preparation (2-3 mg protein/ml), 10 mM potassium phosphate (pH 7.4), 2 mM $MgSO_4$, and 0.2 mM EGTA. Samples were equilibrated at 37°C for 1 min in a shaking water bath prior to initiation of the reaction with 0.5 nmol ATP (specific activity 4 µCi [^{32}P] ATP) with or without 10 µM cAMP. Incubations were carried out for 30 s and terminated by the addition of 0.05 ml SDS stop solution as described in Table I. Samples were heated in a boiling water bath for 10 min, and 75 µl portions (50-70 µg protein) were electrophoresed (see Section 7).

2.3.4 Solubilization and Dissociation of Membrane-associated Protein Kinases and their Protein Substrates from Rat Caudate Nucleus

Membrane-bound protein kinases and their endogenous protein substrates were solubilized from the M_1 fraction using 0.5 M NaCl, or 1% Triton X-100 plus 0.1 mM DTT in the absence or presence of 10 µM cAMP. The M_1 fraction was divided into 0.5 ml portions to which was added 100 µl of one of the following solutions: (a) water; (b) 60 µM cAMP; (c) 3 M NaCl; (d) 3 M NaCl, 60 µM cAMP; (e) 6% Triton X-100, 0.6 mM DTT; (f) 6% Triton X-100, 0.6 mM DTT, 60 µM cAMP. Samples were incubated on ice for 30 min then centrifuged at 150,000 x g for 30 min. The resulting supernatant and membrane fractions were assayed for protein kinase activity.

2.3.5 Photoaffinity-labelling Procedure

The covalent labelling of cAMP-binding subunits of protein kinases was carried out using the photoaffinity analogue 8-N_3-[^{32}P] cAMP. The standard reaction mixture (100 µl volume) contained 60 µl protein, 1 mM 2-mercaptoethanol, 0.5 mM IBMX, and 0.5 µM 8-N_3-cAMP (specific activity 4-8 Ci/mol). Specific binding was

TABLE 1

Composition of Gel Mixtures Used for Electrophoresis

Additions: (ml)	Separating Gel 18%	Separating Gel 6.4%	Stacking Gel 5%
Water	35	33.0	5.4
Buffer	16.0	16.0	2.5
32% Acrylamide/ 0.8% bis-acrylamide	36.0	13.0	1.6
40% Sucrose	8.0	1.3	
10% Glycerol			0.1
10% SDS	0.65	0.65	0.1
TEMED	0.016	0.048	0.005
10% AMP	0.2	0.2	0.15

Gels were prepared from a stock solution of 32% by weight of acrylamide and 0.8% by weight of N,N'-bis-methylene acrylamide. The final concentrations in the separating gel were as follows: 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The gels were polymerized chemically by the addition of tetramethylethylenediamine (TEMED) and ammonium persulphate (AMP). The stacking gels contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS. The electrode buffer (pH 8.3) contained 0.025 M Tris and 0.192 M glycine and 0.1% SDS. The samples were solubilized in 0.065 M Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue as tracking dye. (After Laemmli, 1970).

assessed by incubating some samples in the presence of a molar excess of cAMP (20 µM). In other experiments photoaffinity labelling was carried out with samples previously exposed to cAMP during extraction of synaptic membranes with either detergent or NaCl as described earlier. Preincubations were carried out for 30 min in the dark at 4°C and samples then irradiated for 10 min with a Mineralite hand lamp at a distance of 8-10 cm. Samples were agitated periodically to ensure efficient labelling. These samples were then mixed with 50 µl of SDS-stop solution, boiled for 5 min, and electrophoresed.

2.3.6 Histone Kinase Assay

The reaction mixture used for studying protein phosphorylation (final volume, 100 µl) contained 40 µl M_1 protein, 10 mM potassium phosphate (pH 7.4), 2 mM $MgSO_4$, 0.2 mM EGTA, and 50 µg of histone (5 mg per ml). The assay mixture was equilibrated at 37°C for 1 min in a shaking water bath prior to the initiation of the reaction by 0.25 nmol ATP (specific activity, 3 µCi/nmol) in the absence or presence of 10 µM cAMP. The reaction was carried out for 5 min and was terminated by the addition of 1 ml of 0.3 N perchloric acid, 5 mM phosphoric acid. Samples were placed on ice for 20 min, and the precipitate was collected on glass fibre filters. Filters were then dried and counted in a liquid scintillation counter with 5 ml of aqueous flour. One unit of histone kinase activity was defined as that amount of enzyme that transferred 1 pmol of ^{32}P from [^{32}P] ATP to recovered protein in 1 min at 37°C.

2.3.7 Electrophoretic Methods

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinuous buffer system of Laemmli (1970). A 6.4-18% acrylamide gradient was set up using 200 ml flasks connected by Tygon tubing which remained clamped until gels were poured. The composition of gel mixtures is shown in Table I. The acrylamide gradient was reinforced with a sucrose density gradient to reduce unwanted diffusion, and the TEMED concentration in the 6.4% gel was increased to ensure that gelation spread from the top of the gel downwards, thereby minimizing distortion due to convection currents during polymerization as described by Margolis and Kenrich (1968) and Jeppessen (1974). These modifications greatly enhanced the resolving power of the gels. All components of the gel mixture except ammonium persulfate were added to the gradient flasks which were then placed on two magnetic stirrers which were elevated 10 cm above the top of the gel apparatus. The solutions in the two flasks were gently stirred, and the ammonium persulfate was added to initiate polymerization. Degassing was not necessary if solutions were not overly agitated during the mixing process.

The acrylamide solution was conducted by gravity into a gel mold (22 cm x 14 cm x 1.5 mm) via polyethylene tubing which led from the 18% flask. The gel was poured to a height of 20 cm, and was overlayered with 2 ml of n-butanol. The gel was allowed to polymerize overnight, the gel surface was rinsed several times with deionized water, and then a Teflon well former was placed over the surface of the separating gel. A 5% acrylamide stacking gel (see Table I) was dispensed over the top of the running gel. After 15 min of polymerization the well formers were removed, wells were rinsed with water, and samples to be electrophoresed (75 µl) were overlayed with reservoir buffer. Molecular weight standards

obtained from Bio-Rad were diluted 1:20 in SDS buffer and electrophoresed in parallel.

Electrophoresis was carried out at 4°C at 40 mA using a Bio-Rad power supply until the tracking dye was within 1 cm of the bottom of the gel. The gel was removed from the apparatus and stained overnight in 2-propanol, 10% acetic acid and 0.1% Coomassie Brilliant Blue R-250. Gels were destained in 10% acetic acid, 30% methanol for at least 24 hours with several changes of medium. Gels were then placed on Whatman 1 mm filter paper, covered with Saran Wrap, and dried under vacuum using a Bio-Rad Model 224 slab gel drier.

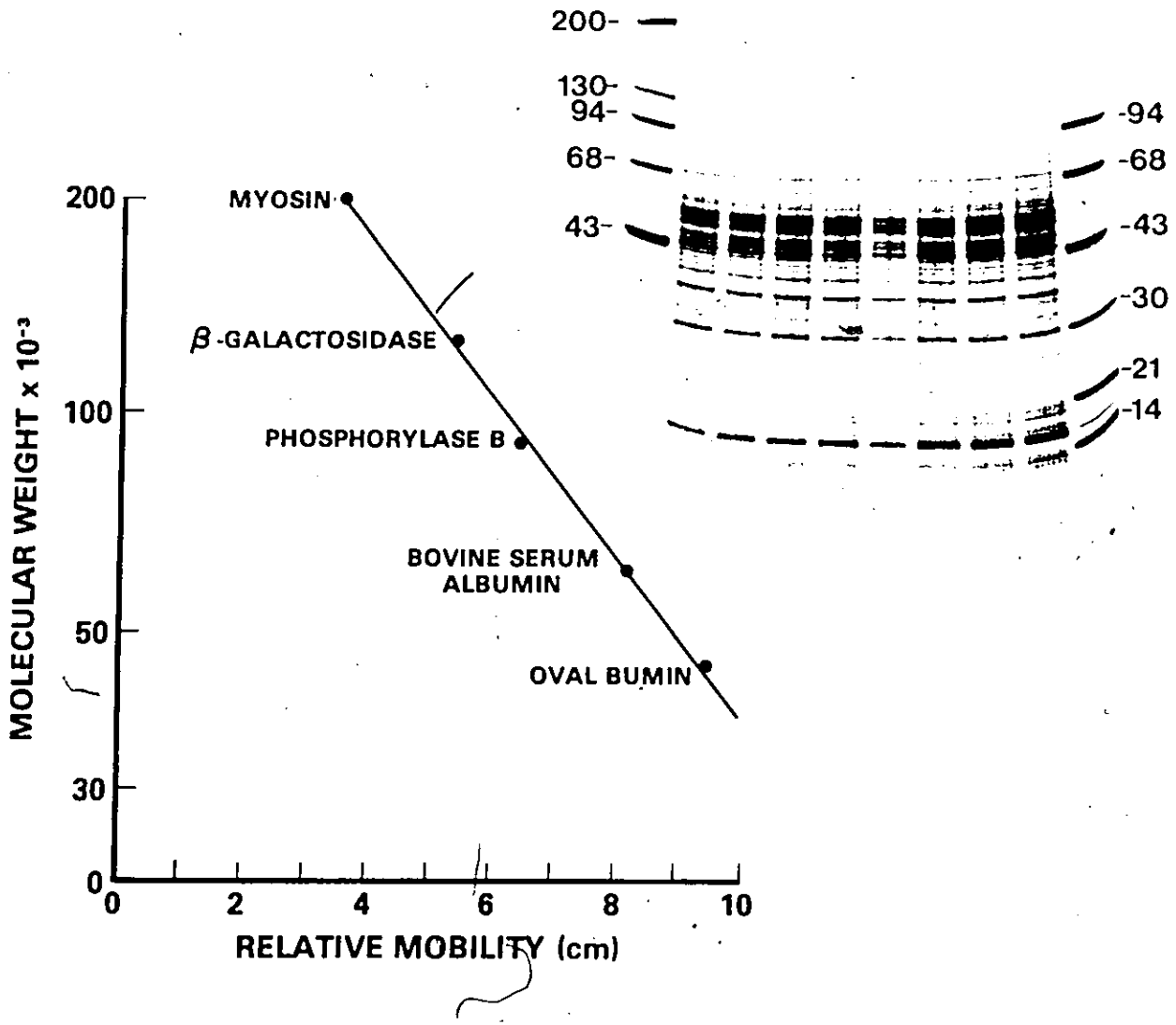
2.3.8 Autoradiography

Dried gels were placed on a sheet of Kodak X-Omat film (XRP-50) in a dark room and sealed in a double envelope of aluminum foil to prevent light penetration. The package was sandwiched between two sheets of plywood which were clamped to maximize contact between gel and film. Films were developed in a Kodak X-Ray processing machine following a 5 day exposure. Apparent molecular weights of visualized phosphoproteins were estimated from a standard curve of log molecular weight versus mobililty that was obtained by using polypeptides of known molecular weight (Figure 5).

2.3.9 Densitometry

Intensity of bands on the film was quantified by scanning autoradiograms with a Joyce-Loebl densitometer. To correct for the background density of the films, base lines for the optical density of different proteins were constructed by connecting the troughs on either side of the base of a protein peak and then measuring the height of the peak. This method has been used by a number of investigators and has been shown to correlate well with measurements of phosphate

Figure 5. Standard Curve for Molecular Weight Determinations in SDS-PAGE. Molecular weight standards obtained from Bio-Rad (see **Materials**) were electrophoresed at 4°C at 40 mA for 8 h in a 6.4 - 18% acrylamide gradient gel as described in **Methods.** The relative electrophoretic mobilities of these proteins were plotted against their known molecular weights. Inset: Coomassie staining of gel showing molecular weight-markers (lanes 1, 10) and synaptic membrane proteins from rat caudate nucleus (lanes 2-9).


200-
130-
94-
68-
43-
-94
-68
-43
-30
-21
-14
MYOSIN
β-GALACTOSIDASE
PHOSPHORYLASE B
BOVINE SERUM ALBUMIN
OVAL BUMIN
MOLECULAR WEIGHT x 10⁻³
200
100
50
30
0
0
2
4
6
8
10
RELATIVE MOBILITY (cm)

incorporation using liquid scintillation counting (Ueda et al.,1973). Due to the large number of phosphorylated proteins in the caudate nucleus, and the difficulty involved in reproducibly isolating proteins from the gels and counting them, autoradiography followed by densitometer scanning was selected as a more suitable method of quantifying endogenous phosphorylation.

2.3.10 Lesion Procedures

Male Wistar rats were anesthetized with Nembutal (50 mg/kg) and placed in a stereotaxic apparatus.

A. 6-Hydroxydopamine (6-OHDA) Lesions

A stainless steel cannula was implanted into the right substantia nigra using the following co-ordinates from the Atlas of Pellegrino and Cushman: anterior: 2.8 mm; lateral: - 2.2 mm; ventral: - 3.0 mm. 6-OHDA was dissolved in saline solution containing 0.4 mg/ml of ascorbic acid, and was dispensed through a cannula in a 4 µl volume at a rate of 1 µl/min. Control rats were infused with saline ascorbate. Seven to ten days after surgery the effectiveness of the lesion was confirmed by observations of rotational behaviour induced by systemic injections of apomorphine (2 mg/kg). Circling behavior was measured by counting the number of turns made during a 15 min period following drug injection. Animals that showed a consistent turning response (> 12 turns per 10 min) were considered to have reliable lesions of the substantia nigra (Ungerstedt and Arbuthnott, 1970), and were used for assays of cAMP-dependent protein kinase activity. The average yield of synaptic membrane material from lesioned striata was about 20-25% less than in control striata. Therefore samples were diluted with homogenizing buffer to the same protein concentrations prior to assay.

B. Kainic Acid Lesions

Kainic acid (5 nmol) in 1 µl of 0.2 M potassium phosphate (pH 7.4), was dispensed via a cannula into the right caudate nucleus at a rate of 0.25 µl/min. Sham-operated rats received buffer alone. Stereotaxic co-ordinates for the caudate were: anterior: 7.9 mm; lateral: 2.7 mm; ventral: 1.5 mm.

2.3.11 Chronic Haloperidol Studies

A. Treatment Regimen

This was based on the procedure of Friend et al. (1978). Rats ware maintained on a graduated dosage of haloperidol beginning with 0.64 mg/kg daily for 4 days. This was followed by 1.28 mg/kg daily for 4 days, and 2.5 mg/kg for 4 days. Finally, rats received 5.0 mg/kg daily for 8 days. Control rats were maintained on the vehicle of 1% tartaric acid. All injections were intraperitoneal. Biochemical and behavioural tests were done on the sixth day after the last injection.

B. Biochemical Measures.

(i) Dopamine-sensitive Adenylate Cyclase Activity:

The striata from haloperidol and vehicle-injected rats were quickly dissected, and dopamine-sensitive adenylate cyclase activity in homogenate and synaptic membrane fractions was assayed as described previously (Section 3.2).

(ii) cAMP-dependent Protein Phosphorylation:

Neuronal membrane fractions were prepared and equal amounts (100-160 µg) of protein from drug-treated and control striata were assayed for endogenous cAMP-dependent protein phosphorylation as described earlier (section 3). Phosphorylated proteins were separated by electrophoresis and visualized by autoradiography.

(iii) Dopamine Receptor Assay:

Dopamine receptor binding was measured according to the method of Seeman et al., (1975) using [^{3}H] spiroperidol as ligand. These studies were carried out in collaboration with G. Jawahir of the Department of Neurosciences (McMaster University). Changes in the number and/or affinity of dopamine receptors in the striata of haloperidol and control rats were assessed via Scatchard analysis.

2.3.12 Behavioural Measures

Six days following the termination of drug treatment, rats were injected with apomorphine HCl (1 mg/kg intraperitoneally) and rated for stereotyped behaviour according to the scoring system shown in Table II. To minimize experimental bias, rats were coded and injected by one person and assessed by a second observer who was unaware of the order of presentation of the various animals. Rats were tested 15 min after the apomorphine injection for a period of 2 min. Differences in mean stereotypy scores between haloperidol and vehicle groups were assessed using the Wilcox Rank Score Test.

2.3.13 In Vitro Drug Studies

Concentrated stocks of haloperidol (13 mM) and trifluoperazine (2.5 mM) were diluted in incubation buffer to the appropriate concentrations. Control samples received equivalent amounts of the drug vehicle. Veratridine was dissolved in DMSO and then diluted 100-fold in the assay buffer to give the required concentration (100 µM). Control samples received DMSO alone.

Table II: Scoring system used for the estimation of the intensity of stereotypy (from Costall and Naylor, 1973).

Score	Description of stereotyped behaviour
0	Normal inactive, or sleepy
1	Normal active
2	Discontinuous sniffing, constant exploratory activity
3	Continuous sniffing, periodic exploratory activity
4	Continuous sniffing, discontinuous gnawing, biting or licking; brief periods of locomotor activity
5	No exploratory activity; animals are restricted to one spot, continuous gnawing, biting or licking

2.3.14 Preparation and Incubation of Caudate Nucleus Slices

Male Wistar rats were killed by decapitation, caudate nuclei were dissected out on ice, and tissue slices 0.275-0.3mm thick were prepared using a McIlwain tissue chopper. Slices were placed in a 13 x 100 mm test tube containing 4 ml of Krebs-Ringer buffer (KRB). The composition of the KRB was as follows (in mM): NaCl, 132; KCl, 4.8; $MgSO_4$, 2.4; HEPES, 20; EGTA, 0.1; $CaCl_2$, 1.1 (1 mM free Ca^{++}). The KRB was oxygenated by bubbling with 95% O_2, 5% CO_2 for 30 min in a water bath at 37°C and adjusted to pH 7.4 with NaOH prior to use. Slices were separated from each other by gently vortexing, and individual slices were placed in 10 x 75 mm test tubes containing 1 ml of KRB. The slices were incubated for 90 min in a shaking water bath at 37°C with replacement of the KRB every

20 min. Preincubation of striatal slices has been shown to significantly reduce endogenous levels of cAMP associated with decapitation stress. This is important to the detection of dopamine-stimulated cAMP accumulation in this preparation (Wilkening and Makman, 1975).

2.3.15 Determination of Intracellular cAMP Levels in Slices of Rat Caudate Nucleus

Slices were prepared and incubated as described above. Following the 90 min preincubation, the KRB was removed from the slices, and replaced with 0.25 ml of KRB containing various test agents in the presence of 0.75 mM IBMX. As reported by others, (Kayaalp et al.,1981) it was found that reliable dopamine stimulation could not be observed in the presence of anti-oxidants such as ascorbic acid; therefore, test substances were dissolved in KRB immediately prior to use. Dopamine inhibitors were made up in KRB with or without dopamine before being added to the slices.

At the end of the incubation period, test tubes were placed in a boiling water bath for 20 min to extract cAMP. Duplicate 50 µl portions of the incubation medium were assayed for cAMP using the method of Brown and Makman (1971) as described earlier. The remaining slice was solubilized in 250 µl of 0.2 N NaOH, heated at 80°C for 20 min, and then assayed for protein concentration.

2.3.16 Protein Phosphorylation in Slices

Slices were preincubated for 90 min in KRB either individually or in groups of 8-10 slices as described above, then incubated for 30-60 min in 1 ml of KRB containing 0.25 mCi of [^{32}P]-orthophosphate. The medium was removed, slices were rinsed twice in 4 ml of KRB, and test substances (250 µl) were added to initiate the reaction. Following the incubation, slices were removed from the test medi-

um and rapidly homogenized in 150 µl of KRB plus 75 µl of SDS stop solution (Laemmli, 1970). Samples were boiled for 10 min, and portions were removed for electrophoresis and protein determinations.

2.3.17 Protein Determinations

Two methods of determining protein concentration were used. The procedure of Lowry et al., (1951) was used for all samples except those which had been solubilized in SDS stop solution, since both SDS and mercaptoethanol are known to interfere with this assay. These samples were assayed by a modification of the Coomassie Brilliant Blue G-250 method (Zaman and Verwilghen, 1979), in which excess SDS is removed prior to protein determination. Solubilized samples (20 µl) were diluted to 250 µl with water and then 0.45 ml of 100 mM potassium phosphate (pH 7.4) was added to precipitate the SDS. After mixing, samples were centrifuged (1500 x g) for 10 min at room temperature to sediment the precipitate. Supernate (250 µl) was transferred into a 13 x 100 mm test tube containing 2.75 ml of a 0.05% solution of Coomassie Brilliant Blue G-250 in 0.3 M perchloric acid. After mixing, absorbance at 600 nm was measured using a Zeiss spectrophotometer. Standard curves were constructed using BSA solubilized in SDS buffer. Protein values were in the same range as those obtained using the Lowry method. Samples were then diluted to the lowest common protein value with additional SDS buffer. Bromophenol blue tracking dye (2 µl) was added to each sample, and 75 µl portions were subjected to electrophoresis.

2.3.18 Statistical Methods

Statistical analysis was conducted in appropriate cases using the Analysis of Variance and Student's t-test. In all cases where results were found to be statistically significant ($p<0.05$), specific F and t-values and their associated levels of significance are presented in either the figure legends or text. Unless otherwise specified, where no level of significance is given, results should be interpreted as being non-significant.

In some instances, differences between experimental groups were assesed using the Analysis of Variance (ANOVA) followed by contrast studies or t-tests to provide a more meaningful breakdown of the data. Use of these tests is indicated in the appropriate figure legends.

Chapter III

Results

3.1 Properties of Dopamine-Sensitive Adenylate Cyclase in Rat Caudate Nucleus

Four parameters of enzyme activity were selected based on studies of dopamine-sensitive adenylate cyclase activity reported in the literature. They were: (1) subcellular localization of the enzyme in synaptic membranes, (2) response of the enzyme to neuroleptic drugs, (3) pre vs. postsynaptic localization of the enzyme, and (4) evidence of supersensitivity in response to chronic administration of neuroleptic drugs.

3.1.1 Dopamine Stimulation in Homogenates and Synaptic Membrane Fractions of Rat Caudate Nucleus

As has been reported by others (Kebabian et al., 1972; Clement-Cormier et al., 1974; Mishra et al., 1974), dopamine caused a marked stimulation of adenylate cyclase activity in homogenates of rat caudate nucleus (Figure 6A). Combined data for different experiments indicated that dopamine produced a 2 to 2.5-fold increase in adenylate cyclase activity ($p<.05$). The stimulation of adenylate cyclase was concentration-dependent with highest levels observed at 100 µM dopamine (data not shown).

Subcellular fractionation of homogenates of caudate nucleus was carried out as described in **Materials and Methods.** Synaptic membranes were isolated and analyzed for dopamine-sensitive adenylate cyclase activity. In confirmation of previous findings, (Clement-Cormier et al., 1975), the specific activity

Figure 6. Adenylate Cyclase Activity in Rat Caudate Nucleus. Crude homogenates (A) and synaptic membrane fractions (B) were prepared from rat caudate nuclei and assayed for adenylate cyclase activity at 37 °C for 2.5 min as described in Methods. Activity is expressed as pmol cAMP formed per mg protein per min assayed in the absence (open bars) or presence (closed bars) of 100 μM dopamine, and results represent the mean ± SEM of three independent experiments assayed in duplicate. Protein was determined by the method of Lowry et al., (1951). Control: no drug added TFP: assayed in the presence of 100 μM trifluoperazine; Hal: 100 μM haloperidol. Statistical significance was assessed using a 2-way ANOVA. Comparisons between drug and control groups were further assessed using simple t-tests.

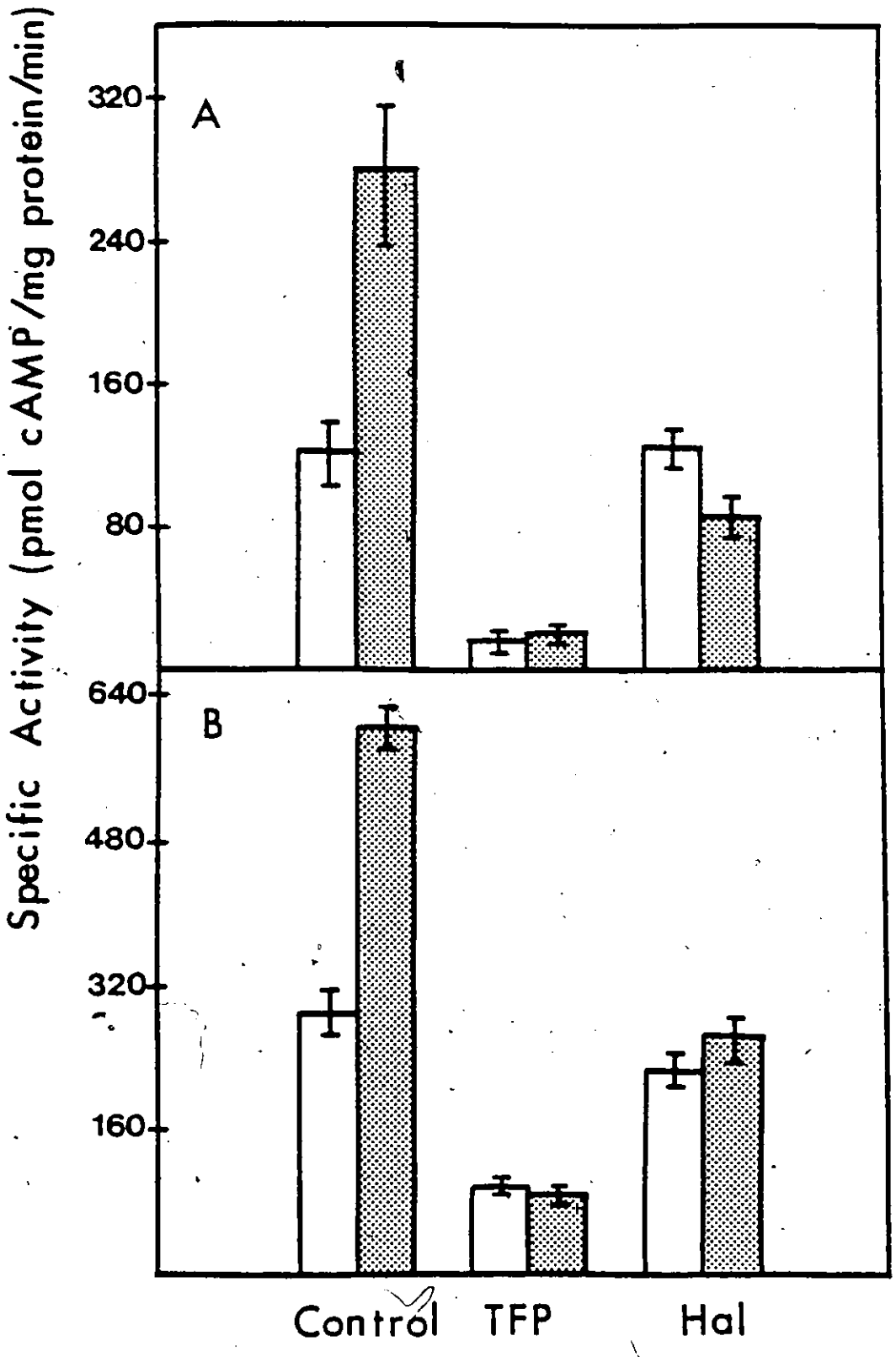
ANOVA

HAL	$F_{1,19}$	P value
drug	26.7	<.001
DA	18.0	<.001
drug x DA	9.4	<.001

TFP	$F_{2,15}$	P value
drug	89.5	<.001
DA	17.3	<.001
drug x DA	17.7	<.001

T-tests

	t (5 df)	
open bars: control vs TFP	6.11	p<002
closed bars: control vs TFP	4.26	p<.01
open bars: control vs HAL	1.87	ns
closed bars: control vs HAL	6.78	p<.001


Specific Activity (pmol cAMP/mg protein/min)
320
240
160
80
A
640
480
320
160
B
Control
TFP
Hal

of the dopamine-sensitive adenylate cyclase was higher in synaptic membranes (Figure 6B) than in crude homogenates of caudate tissue (Figure 6A). As has been reported by others (Clement-Cormier et al., 1975) dopamine stimulation of the enzyme in synaptic membranes required GTP (Figure 7). Basal enzyme activity was also slightly elevated by GTP but this was not statistically significant. Therefore, assays of dopamine-sensitive adenylate cyclase activity in synaptic membranes were routinely carried out in the presence of 10 μM GTP as described by Clement-Cormier et al., (1975).

3.1.2 Inhibition of Dopamine-sensitive Adenylate Cyclase Activity by Neuroleptic Drugs

Neuroleptic drugs, that block dopamine receptors, have been shown to inhibit dopamine-sensitive adenylate cyclase activity (Kebabian et al., 1972; Clement-Cormier et al., 1974; Miller et al., 1974). In the present study, haloperidol (100 μM) caused a 60-70% reduction in dopamine-stimulated adenylate cyclase activity ($p<.001$), but only slightly depressed basal enzyme activity (Figure 6). This effect was observed in crude homogenates (Figure 6A) as well as in synaptic membrane fractions (Figure 6B). Trifluoperazine (100 μM) inhibited basal activity 70-80% ($p<.05$), and dopamine-stimulated activity by 90% ($p<.05$) in both tissue preparations (Figures 6A and 6B). Similar results have been reported by Gnegy and Treisman (1981).

3.1.3 Neuronal Localization of Dopamine-sensitive Adenylate Cyclase

Destruction of striatal neurons with kainic acid substantially decreases striatal adenylate cyclase activity (Garau et al., 1978; Minneman et al., 1978; Schwartz et al., 1978). Since kainic acid destroys cell bodies and dendrites but spares presynaptic fibers, these results indicate that the dopamine-sensitive adenylate cyc-

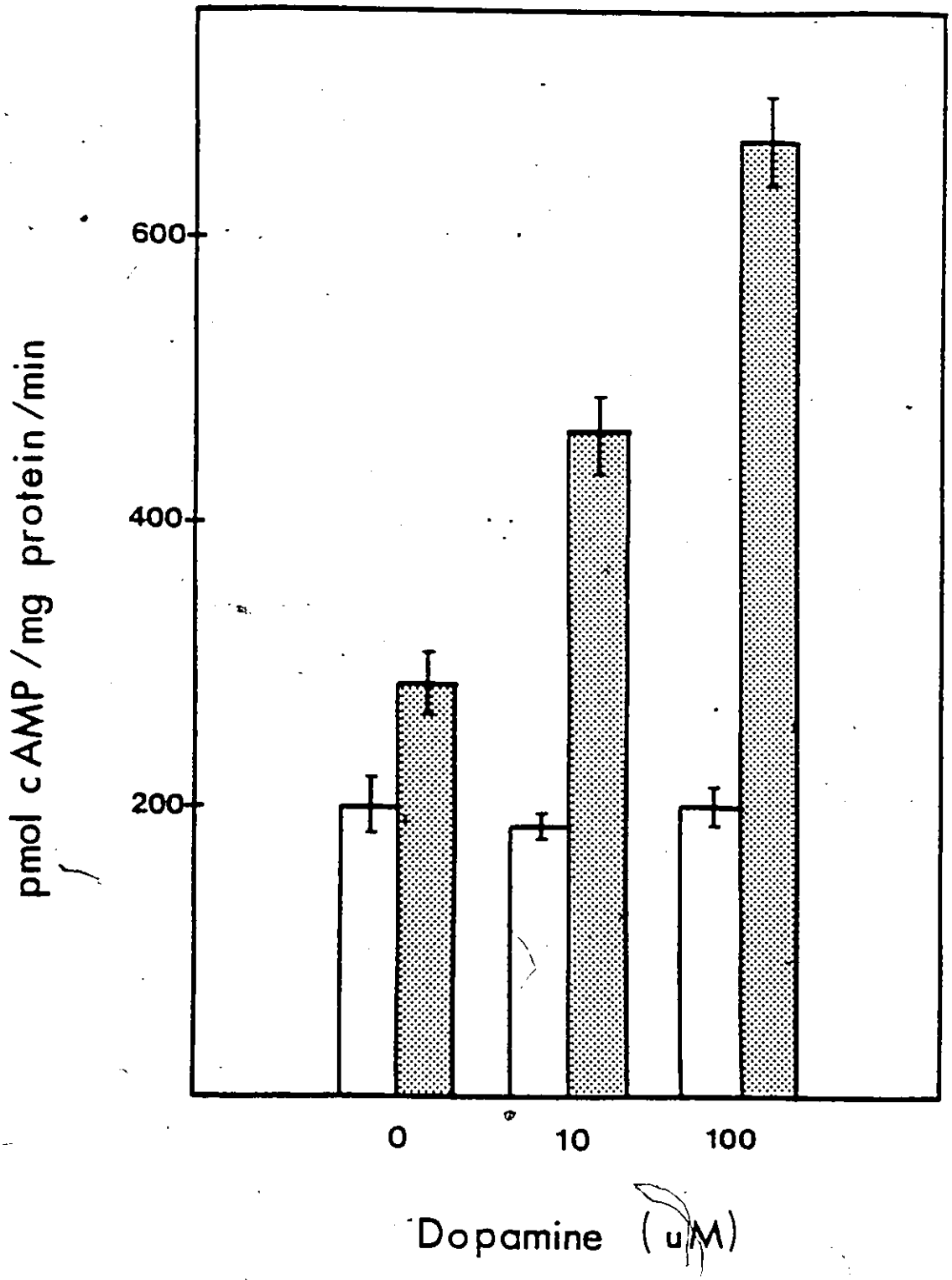
Figure 7. Effect of GTP on Dopamine-Stimulated Adenylate Cyclase Activity. Synaptic membrane fractions from rat caudate nucleus were prepared as described in Methods, and dopamine-sensitive adenylate cyclase activity was assayed in the absence (open bars) or presence (closed bars) of 10 μM GTP. Results are of four independent experiments assayed in duplicate (mean ± SEM). Data were analyzed using a 2-way ANOVA followed by paired t-tests to compare adenylate cyclase activity in the absence or presence of GTP.

ANOVA

Source	$F_{3,20}$	P value
GTP	187.3	<.001
DA	30.4	<.001
GTP x DA	30.0	<.001

Paired t-tests

	t (3 df)	
Control	-2.59	ns
10 DA	11.89	p<.005
100 DA	17.19	p<.001


600
400
200
pmol cAMP/mg protein/min
0
10
100
Dopamine (uM)

lase may participate in the postsynaptic regulation of dopamine function. In confirmation of these findings, it was found that striatal injections of kainic acid caused a 70% reduction in basal ($p<.005$), as well as dopamine-stimulated adenylate cyclase activity ($p<.001$) in homogenates of rat caudate nucleus (Figure 8). However, the magnitude of dopamine stimulation was comparable in lesioned and intact caudates indicating that the decrease in adenylate cyclase activity was due to a loss of enzyme rather than a change in dopamine responsiveness.

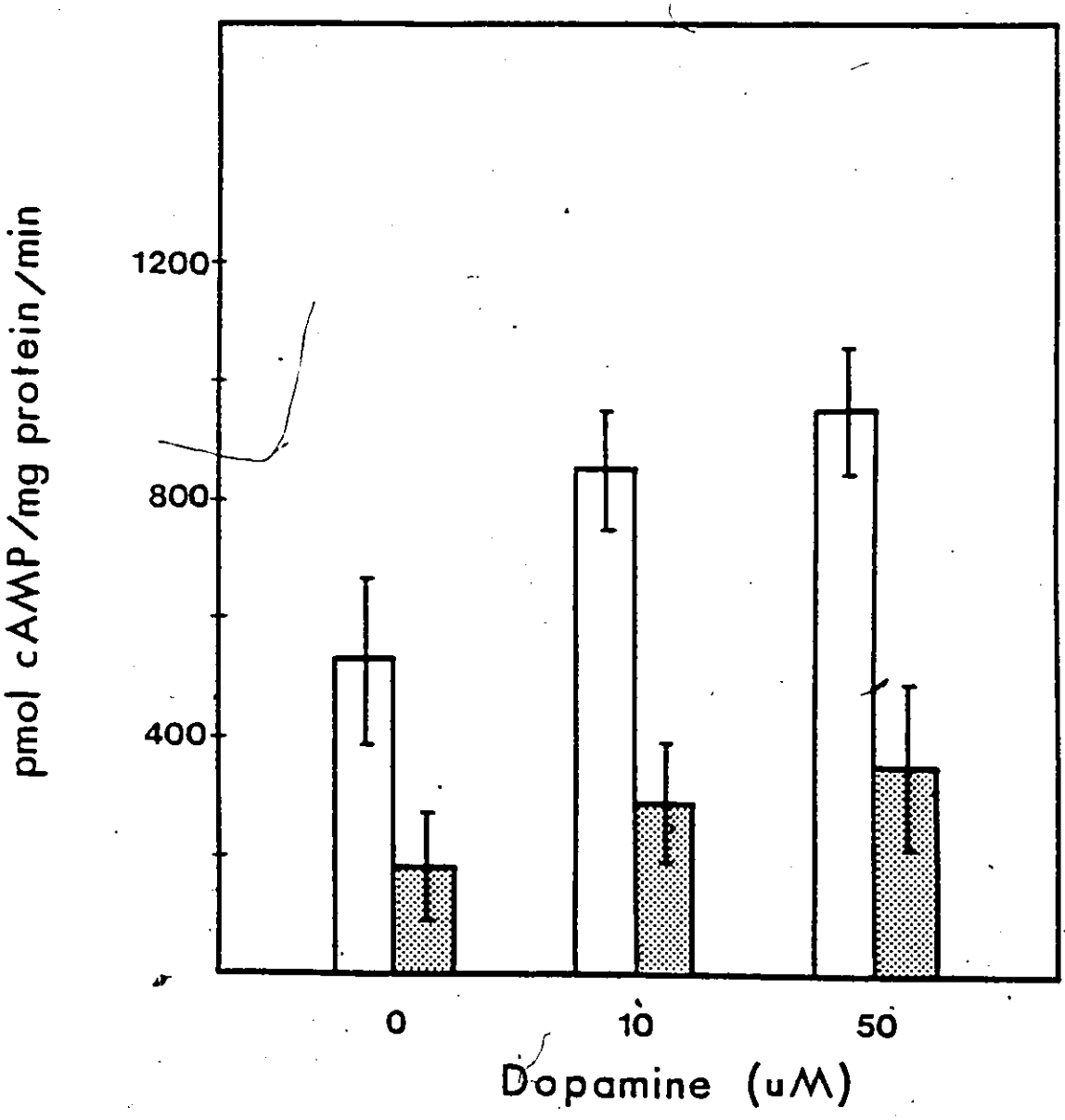
3.1.4 Dopamine Stimulation of Adenylate Cyclase Following Chronic Haloperidol Administration

Biochemical studies have demonstrated that pharmacological blockade of dopamine receptors produces an increase in the activity of the dopamine-sensitive adenylate cyclase, and this has been proposed to be a chemical sign of increased receptor sensitivity (Mishra et al., 1974; Gnegy et al., 1977a, 1977b; Marshall and Mishra, 1980; Iwatsubo and Clouet, 1975). The objective of the present study was to determine whether this experimental strategy could be utilized as a means of investigating cAMP-dependent protein phosphorylation in the caudate since neuroleptic-induced increases in adenylate cyclase activity might be accompanied by similar increases in cAMP-dependent protein kinase activity. In view of the fact that a number of investigators have been unable to demonstrate an effect of chronic neuroleptic administration on dopamine-sensitive adenylate cyclase activity (Von Voigtlander et al., 1975; Heal et al., 1976; Rotrosen et al., 1975; Hyttel, 1978; Roufogalis et al., 1976b), other measures of dopamine receptor supersensitivity were taken to independently assess the effects of haloperidol administration on dopamine function. These included dopamine receptor binding assays and measures of apomorphine-induced stereotyped behaviour, as described

Figure 8. Effect of Kainic Acid on Dopamine-Stimulated Adenylate Cyclase Activity. Kainic acid (5 nmol in 1 µl of 0.2 M potassium phosphate, (pH 7.4) was injected into the right caudate nucleus (closed bars) at a rate of 0.25 µl per min, as described in **Methods.** Left caudates served as sham-operated controls (open bars). Rats were killed 7 days post surgery and adenylate cyclase activity was assayed in individual homogenates of left and right caudate nuclei. Results represent the mean ± SEM of 9 rats assayed in duplicate. Differences in enzyme activity were assessed using ANOVA.

ANOVA

Source	df	F	p
Drug	1	44.13	<.0001
DA	2	7.24	<.002
Between. Rats	8	6.00	<.0001
Error	42		


pmol cAMP/mg protein/min
1200
800
400
0
10
50
Dopamine (uM)

in Methods. Chronic neuroleptic administration has been shown to consistently affect both of these parameters (reviewed by Muller and Seeman, 1978; Seeman, 1980).

A. Adenylate Cyclase.

As shown in Table III, the striata of rats treated chronically with haloperidol showed an increase in dopamine-sensitive adenylate cyclase activity when tissues were assayed in the presence of 100 μM dopamine ($p<.05$). No differences between haloperidol and vehicle groups were detected when enzyme activity was measured at low dopamine concentrations. This contradicts the results of some studies in which chronic neuroleptic administration was associated with a decrease in the amount of dopamine required to stimulate the enzyme (Gnegy and Treisman, 1981; Gnegy et al., 1977a, 1977b). Other investigators, however (Marshall and Mishra, 1980), have reported increased enzyme activity at maximally stimulating concentrations of dopamine, compatible with the results reported here. The findings reported in Table III are therefore representative of results published in the literature, and reaffirm the controversial nature of the role of the dopamine-sensitive adenylate cyclase in drug-induced supersensitivity.

B. Dopamine Receptors

Assays of dopamine receptor binding were performed using the method of Seeman et al. (1975). Figure 9 shows Scatchard plots of [^{3}H] spiroperidol binding in striata of haloperidol and vehicle-treated rats. These experiments were repeated three times, each time comparing Scatchard plots of both haloperidol and control striata within the same experiment. Chronic treatment with haloperidol produced a 60-70% increase in the number of dopamine receptors ($p<.001$). There were no significant changes in receptor affinity. These results are in agreement with pub-

TABLE III

Effect of Chronic Haloperidol on Dopamine-Sensitive Adenylate Cyclase Activity

		EXPERIMENT		
	uM DA	1 (n=6)	2 (n=6)	3 (n=8)
Vehicle				
	0	181.6 ± 11.2	170.1 ± 14.2	350.7 ± 13.5
	0.1		200.8 ± 26.2	
	1.0	220.0 ± 16.6	208.6 ± 28.6	437.4 ± 22.1
	10.0		253.7 ± 30.9	722.2 ± 32.3
	50.0		280.6 ± 27.0	
	100.0			840.1 ± 37.7
Haloperidol				
	0	173.1 ± 9.0	160.3 ± 8.2	393.2 ± 33.3
	0.1		206.7 ± 23.8	
	1.0	215.4 ± 14.8	226.1 ± 25.4	530.7 ± 52.1
	10.0		243.8 ± 19.3	809.1 ± 67.6
	50.0		262.2 ± 13.6	
	100.0			1172.2 ± 137.0 *

Caudate nuclei from rats treated with haloperidol or vehicle were assayed for dopamine-sensitive adenylate cyclase activity as described in Methods. Results are expressed as pmol cAMP formed per mg protein per 2.5 min incubation, and represent the mean ± SEM from 6-8 rats.

* $p < .05$ (Student's t-test)

lished observations (Muller and Seeman, 1978; Clow et al., 1980; Seeman, 1980; Mishra et al., 1980; Ebstein et al., 1979).

C. Behavioural Results

Apomorphine-induced stereotyped behaviour in haloperidol and vehicle-treated rats was assessed using the method of Costall and Naylor (1973) as outlined in **Methods.** The results are summarized in Figure 10. The behaviour of the control rats was characterized predominantly by continuous or discontinuous sniffing, but always with some locomotor activity (mean stereotypy score 2.4). Rats treated chronically with haloperidol exhibited a greatly increased sensitivity to apomorphine ($p<.005$). The significantly higher total stereotypy scores (mean 4.6) were mainly due to an increase in the intensity of the apomorphine response (continuous licking and gnawing) and an abolition of locomotor activity.

These results are in agreement with previous studies demonstrating that animals chronically treated with neuroleptics subsequently show an enhanced behavioural response to apomorphine (Tarsy and Baldessarini, 1974; Voith, 1977; Jackson et al., 1979; Gianutsos and Moore, 1977; Sayers et al., 1975).

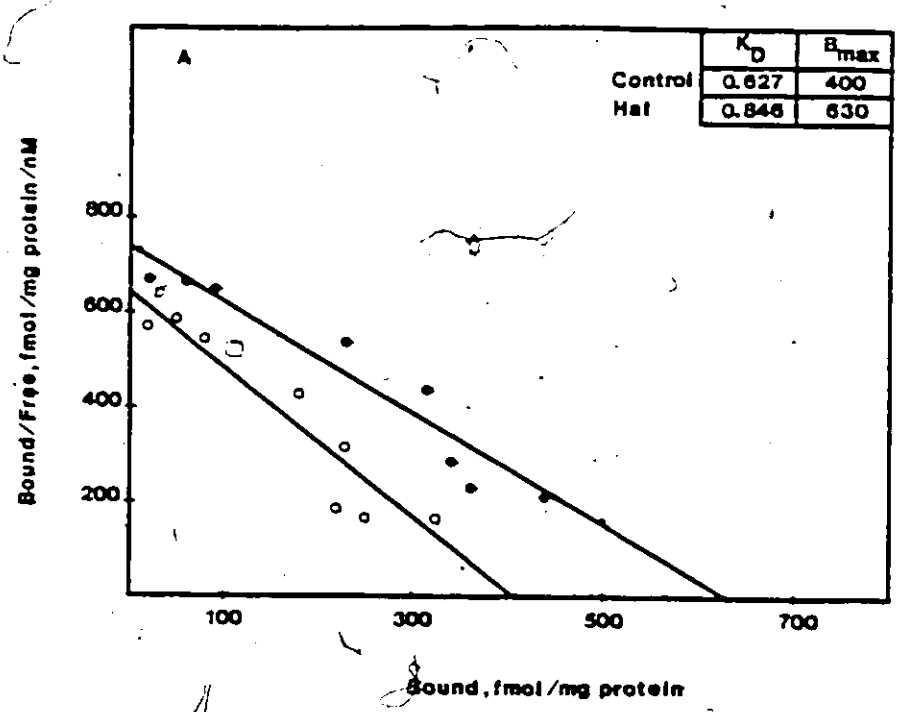
3.2 Identification of Protein Kinase Activity in Rat Caudate Nucleus

The objectives of these studies were: (1) to identify the proteins that were phosphorylated by cAMP and Ca^{++}-dependent protein kinases in rat caudate nucleus, (2) to determine the subcellular and anatomical distribution of these substrates, and (3) to investigate the effects of dopamine on the phosphorylation of these substrates.

Figure 9. [^{3}H] Spiroperidol Binding Following Chronic Haloperidol Administration. [^{3}H] spiroperidol binding was measured using the method of Seeman et al., (1975). Tissue from haloperidol and vehicle-treated rats was pooled separately, and binding of [^{3}H] spiroperidol was plotted by Scatchard analysis. Specific binding was defined as the difference in [^{3}H] spiroperidol binding measured in the presence of (+) and (-) butaclamol. Data were fitted to a straight line using the method of least squares, and differences between the two lines were measured using the Analysis of Variance. Correlation coefficients ranged from -0.87 to -0.96. Data points represent the mean of four individual estimates from control (open circles) and haloperidol-injected (closed circles) rats. (A) and (B): Scatchard plots corresponding to Experiments 1 and 3 respectively shown in Table III.

ANOVA

Source	F value	P value	(df)
A. Diff. in KD	2.39	ns	(1,12)
Diff. in Bmax	26.09	p<.001	(1,13)
B. Diff. in KD	0.28	ns	(1,8)
Diff. in Bmax	37.92	p<.001	(1,9)


A
K_D
B_{max}
Control
0.627
400
Hal
0.846
630
800
600
400
200
100
300
500
700
Bound/Free, fmol/mg protein/nM
Bound, fmol/mg protein

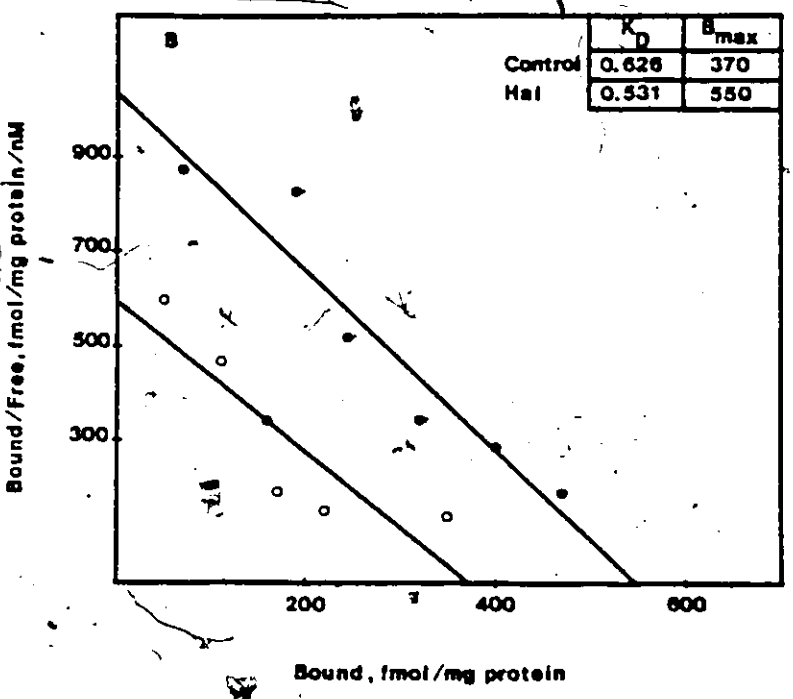
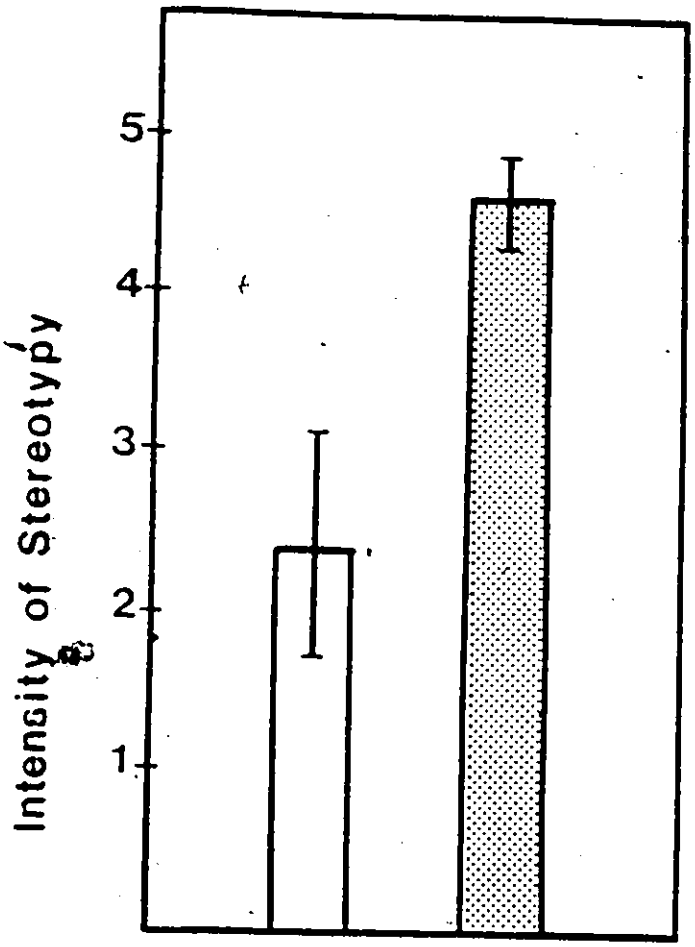

B
K_D
B_{max}
Control
0.626
370
Hal
0.531
550
900
700
500
300
200
400
600
Bound/Free, fmol/mg protein/nM
Bound, fmol/mg protein

Figure 10. Effect of Chronic Haloperidol on Stereotyped Behaviour. Rats were treated for 3 weeks with vehicle (open bar) or haloperidol (closed bar). Six days following the termination of drug treatment rats were injected with apomorphine (1 mg/kg), and tested for stereotyped behaviour using the scoring system of Costall and Naylor, 1973 (see **Methods**). Each column represents the mean ± SEM obtained from 8 rats. Differences between holoperidol and vehicle-treated rats were assesed using the Wilcox Rank Score Test as described in **Methods**.


Intensity of Stereotypy
5
4
3
2
1

3.2.1 Pattern of Protein Phosphorylation in Crude Homogenates of Caudate Tissue

As shown in Figure 11, homogenates of rat caudate nucleus contained several proteins, the phosphorylation of which was enhanced in the presence of cAMP. Time course data revealed that incorporation of labelled phosphate into these proteins was maximal at 5 min of incubation and was markedly decreased by 15 min. The phosphorylation of some proteins, however, (80k, 57k, 55k, 43k and 21k) could still be detected at 30 min.

Similar patterns of cAMP-dependent phosphorylation were observed in the presence of IBMX, a potent phosphodiesterase inhibitor (Figure 12). It was of interest, however, that the phosphorylation of a 55,000 dalton polypeptide was inhibited at 15 and 30 min of incubation in the presence of IBMX. This was the only substrate for the cAMP-dependent protein kinase whose phosphorylation appeared to be altered by IBMX. IBMX alone did not increase cAMP-dependent phosphorylation, indicating that endogenous levels of cAMP were insufficient to activate protein kinases under these assay conditions.

The addition of dopamine to caudate homogenates in the absence or presence of IBMX did not lead to a change in the phosphorylation of any substrates for the cAMP-dependent protein kinase. Surprisingly, dopamine did appear to decrease the phosphorylation of proteins that were substrates for Ca^{++}-dependent protein kinases, and this effect was most pronounced in the absence of IBMX.

Ca^{++} increased the phosphorylation of proteins with molecular weights of 63k, 53k, 20k and 16k, and inhibited the phosphorylation of a 43,000 dalton protein (Figure 11). This latter effect contradicts observations by other workers who have shown stimulation of ^{32}P incorporation into this protein by Ca^{++} (Hof-

Figure 11. Endogenous Protein Kinase Activity of Homogenates of Rat Caudate Nucleus. Caudate nuclei were homogenized in 10 mM potassium phosphate (pH 7.4) containing 0.32 M sucrose, 0.2 mM EGTA, and assayed for endogenous protein phosphorylation as described in **Methods.** Proteins were separated on SDS-PAGE and ^{32}P incorporation was assessed by autoradiography as described in **Methods.** The major phosphoproteins are designated by their polypeptide molecular weights calculated relative to known protein standards as described in the legend to Figure 6. Additions: (a) none (b) 1 mM Ca^{++} (c) 100 µM dopamine (d) 1 mM Ca^{++} plus 100 µM dopamine (e) 5 µM cAMP.

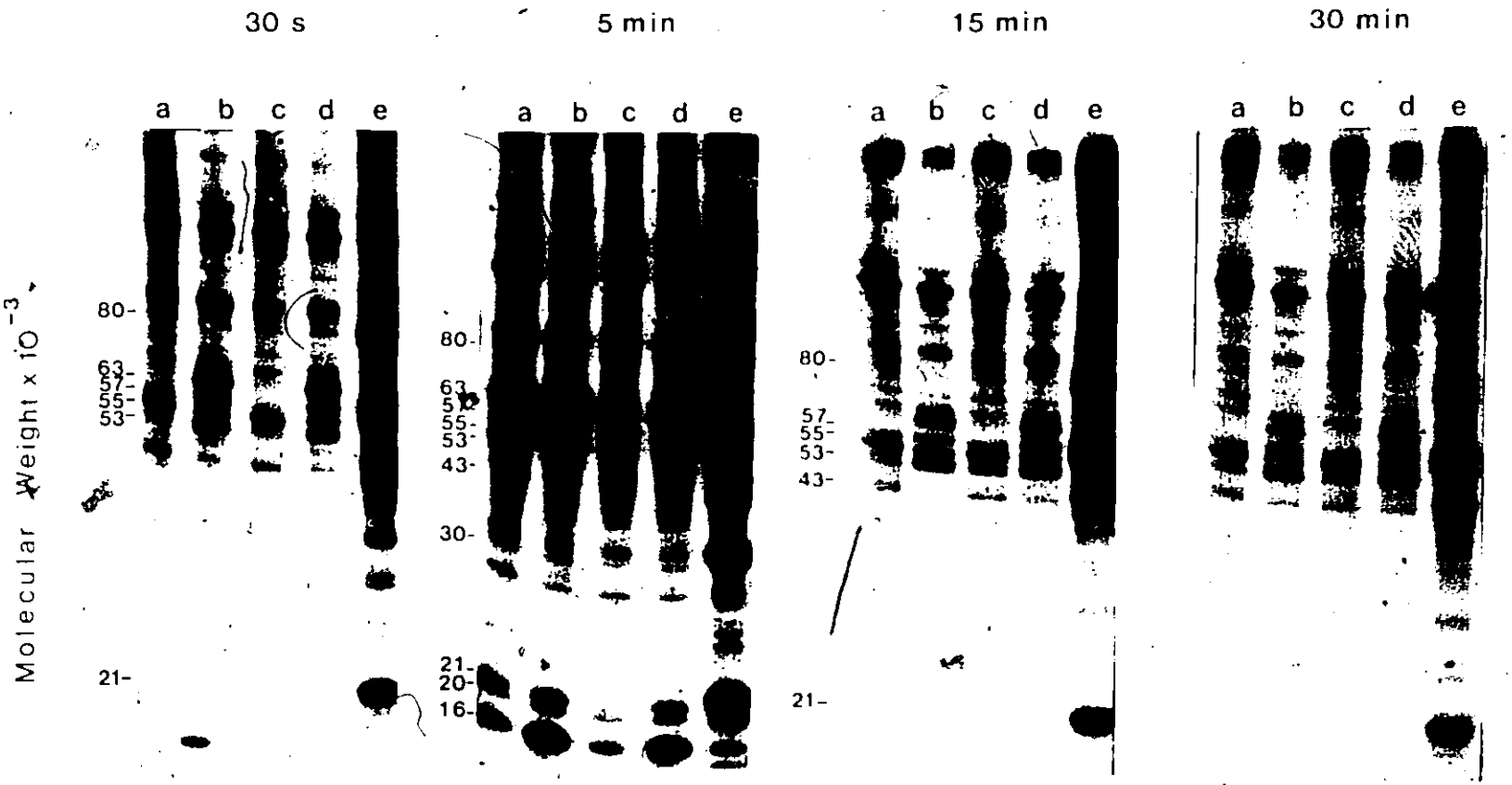
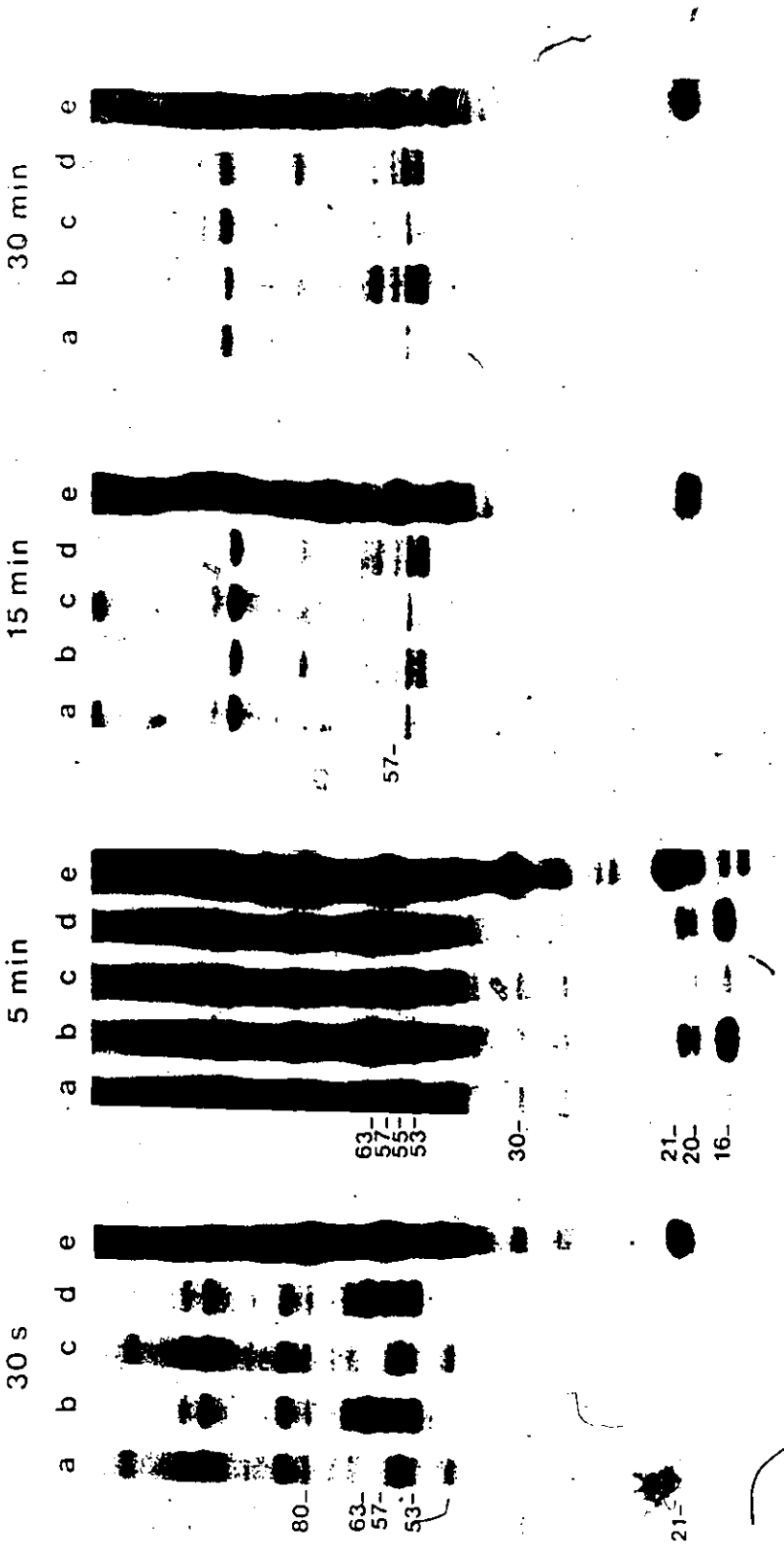

30 s
5 min
15 min
30 min
a b c d e
Molecular Weight x 10^{-3}
80-
63-
57-
55-
53-
43-
30-
21-
20-
16-

Figure 12. Endogenous Protein Kinase Activity of Homogenates of Rat Caudate Nucleus: Effect of IBMX. Homogenates of caudate nucleus were prepared as described in the legend to Figure 12, and assayed for protein kinase activity in the presence of 0.75 mM IBMX. Additions: (a) none (b) 1 mM Ca^{++} (c) 100 µM dopamine (d) 1 mM Ca^{++} plus 100 µM dopamine (e) 5 µM cAMP.


30 min
a b c d e
15 min
a b c d e
57-
5 min
a b c d e
63-
57-
55-
53-
30-
21-
20-
16-
30 s
a b c d e
80-
63-
57-
53-
21-
Molecular Weight x 10^-3

stein et al., 1980). The discrepancy may be due to differences in assay procedures.

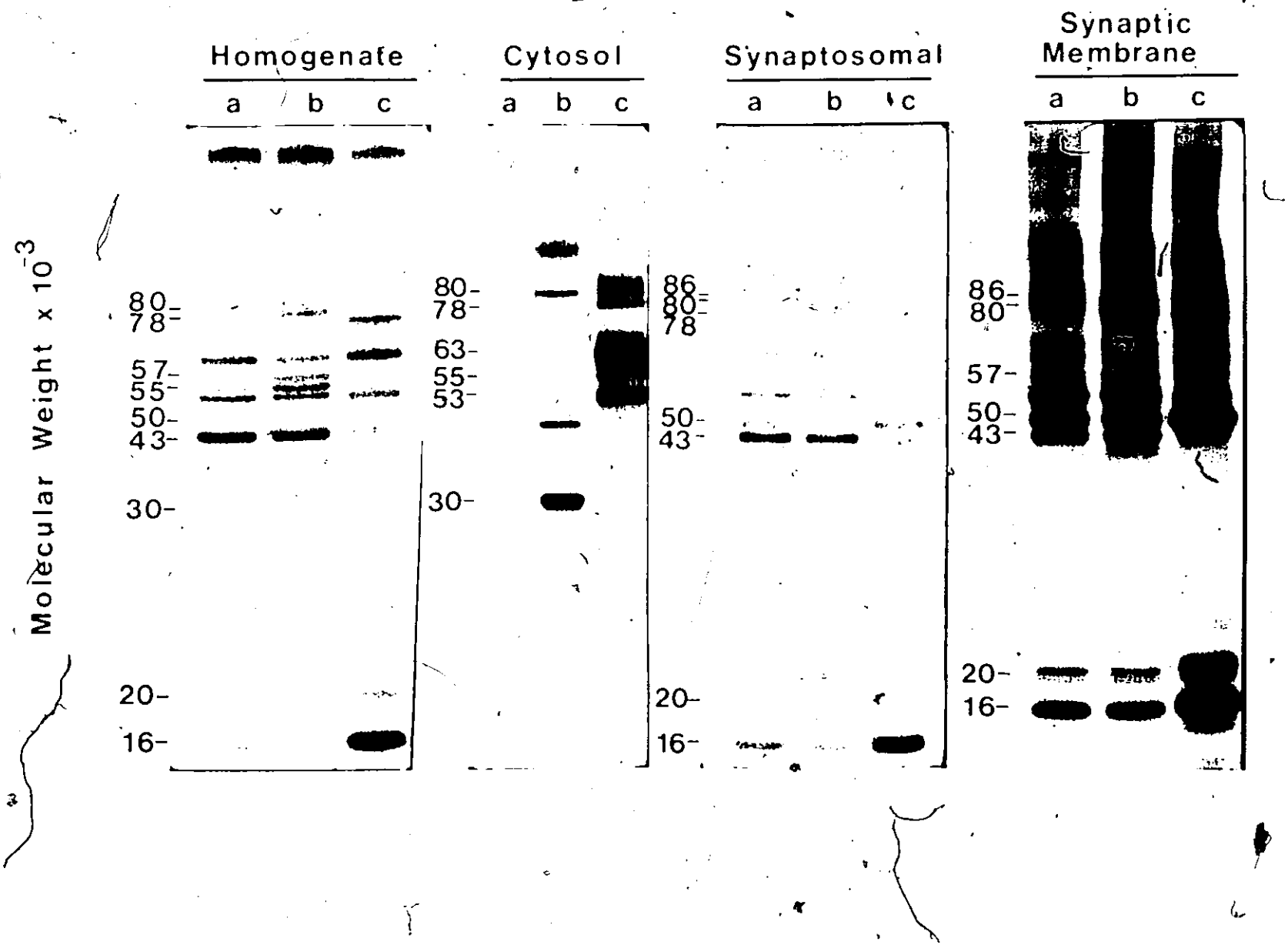
Ca^{++}-dependent protein kinase activity appeared to be maximal at 5 min. Increased phosphorylation of these proteins was also observed at 5 min in the absence of added Ca^{++} and presumably was due to stimulation of the kinase by endogenous Ca^{++} not chelated by EGTA in the homogenizing buffer. By 15 min, the only protein whose phosphorylation could still be detected was 53k.

IBMX apparently reduced the basal as well as the Ca^{++}-stimulated phosphorylation of proteins 63k, 53k, 20k and 16k at 5 min. Methylxanthines have been reported to inhibit protein phosphorylation in brain membranes, homogenates of striatum, and isolated neurons and glia (Weller and Rodnight, 1973; Hullihan et al., 1977; Kinnier and Wilson, 1977), and there is some suggestion that this effect may be due to the ability of these drugs to inhibit adenylate cyclase (Hershkowitz, 1978).

3.2.2 Subcellular Localization of Substrates for cAMP and Ca^{++}-dependent Protein Kinases in Rat Caudate Nucleus

To characterize better the proteins that were phosphorylated by cAMP and Ca^{++}-dependent protein kinases, homogenates of caudate nuclei were subfractionated according to the method of De Robertis et al. (1967) to yield synaptosomal, synaptic membrane and cytosol fractions. The purity of these fractions was not assessed using specific enzyme markers since the protocols for preparing these fractions are well-established and observed patterns of phosphorylation agreed closely with published results. The results, shown in Figure 13, demonstrate that the phosphoproteins previously detected in caudate homogenates were enriched in specific subfractions of rat caudate nucleus.

Figure 13. Subcellular Localization of Substrates for cAMP and Ca^{++}-Dependent Protein Kinase Activity in Rat Caudate Nucleus. Subcellular fractions from rat caudate nucleus were prepared as described in **Methods** and incubated with [^{32}P] ATP for 30 s. Additions: (a) none (b) 5 μM cAMP (c) 1 mM Ca^{++}. Proteins were separated on SDS-PAGE and autoradiographed as described in **Methods.**


Homogenate
Cytosol
Synaptosomal
Synaptic Membrane
a
b
c
Molecular Weight x 10^{-3}
80
78
57
55
50
43
30
20
16
63
53
86

In agreement with published observations, synaptic membranes contained two proteins of M_r=86K and 80k (Proteins Ia and Ib) whose phosphorylation was markedly stimulated by cAMP (Ueda et al., 1973; 1975; Uno et al., 1977b). Two other proteins of M_r=57k and 55k were also identified as substrates for the kinase in agreement with published observations (Lohmann et al., 1980; Walter et al., 1979a; Rubin et al., 1979).

Two substrates for Ca^{++}-dependent protein kinases, 63k and 53k, were phosphorylated in cytosol preparations but were not phosphorylated to any extent in other cell fractions. As will be discussed later, although the phosphorylation of proteins 63k and 53k was not always readily detectable in synaptic membranes, it could be demonstrated in this preparation by the addition of purified calmodulin indicating that these proteins were substrates for calmodulin-dependent protein kinases.

Three other substrates for the enzyme, 50k, 20k and 16k appeared to be membrane proteins as they were not found in the cytosol and they were highly phosphorylated in synaptic membrane fractions. Similar patterns of Ca^{++}-dependent phosphorylation have been reported previously (O'Callaghan et al., 1980a, 1980c; Wrenn et al., 1980). Ca^{++}-mediated inhibition of 43k phosphorylation was also seen most clearly in synaptic membranes.

Since the majority of substrates for cAMP and Ca^{++}-dependent protein kinases appeared to be preferentially localized in synaptic membranes, this tissue preparation was used routinely for the study of dopamine-mediated changes in protein phosphorylation in rat caudate nucleus. A list of the major substrates for cAMP and Ca^{++}-dependent protein kinases in soluble and particulate fractions from rat brain is presented in Table IV.

TABLE IV

Proteins Phosphorylated by Ca++ and cAMP-Dependent Protein Kinases in Soluble and Particulate Fractions from Rat Brain.

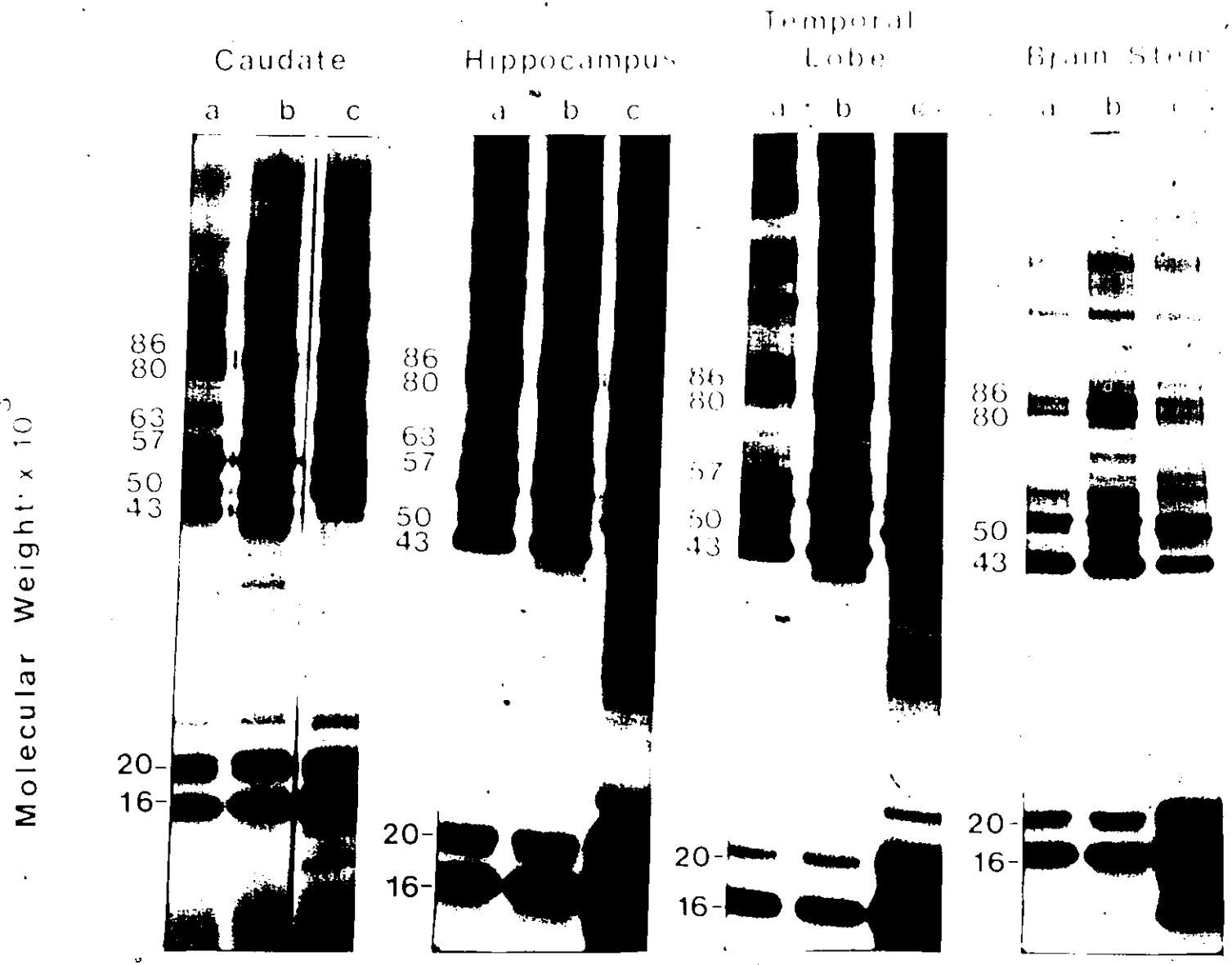
FRACTION	PROTEINS	REFERENCE
Soluble		
cAMP-Dependent:	57k	Lohmann et al. (1980)
Ca++-Dependent:	85k	Wrenn et al. (1980)
	80k	O'Callaghan et al. (1980a)
	55-66k	Yamauchi and Fujisawa (1979)
	45k	Yamauchi and Fujisawa (1979)
	41k	Wrenn et al. (1980)
Particulate		
cAMP-Dependent:	86k (Protein Ia)	Lohmann et al. (1980)
	80k (Protein Ib)	Ueda and Greengard (1977)
	55-57k (R_{II})	Lohmann et al. (1980)
	63k	De Lorenzo et al. (1979)
	53k	Burke and De Lorenzo (1981)
	51k	Schulman and Greengard (1978)
		O'Callaghan et al. (1980c)
	40k	Hofstein et al. (1980c)
	21k	Wrenn et al. (1980)
	20k	Dabrowska et al. (1978)
	16k	Petrali et al. (1980)

3.2.3 Distribution of Substrates for Ca^{++} and cAMP-dependent Membrane Fractions from Different Brain Regions

To determine whether the substrates for the Ca^{++} and cAMP-dependent protein kinases showed particular anatomical distributions the pattern of phosphorylation in hippocampus, temporal lobe and brain stem was also investigated. The objective of this study was to compare the substrates for protein kinases in the caudate nucleus, a dopamine-rich area, with other regions of brain that do not preferentially use dopamine as a neurotransmitter. It was anticipated that different regions of the brain might conceivably show altered substrate specificity for each of the enzymes.

Figure 14 shows the pattern of cAMP-dependent and Ca^{++}-dependent phosphorylation in synaptic membranes from the different brain regions. It is clear from the autoradiographs that the substrates for Ca^{++} and cAMP-dependent protein kinases are distributed throughout the brain and they show virtually identical responses to Ca^{++} and cAMP. These results indicated that the cAMP and Ca^{++}-dependent protein kinases are found ubiquitously in rat brain as are their protein substrates. This would suggest that any change in the phosphorylation of these proteins due to dopamine would probably represent an alteration in enzyme activity rather than a change in the phosphorylation of a specific dopamine-sensitive membrane protein.

Figure 14. Phosphorylation of Synaptic Membrane Proteins from Different Regions of Rat Brain. Synaptic membrane fractions from caudate nucleus, hippocampus, temporal lobe and brain stem were assayed for endogenous protein phosphorylation as described in **Methods.** (a) no addition (b) 5 μM cAMP (c) 1 mM Ca^{++}. Phosphorylated proteins were separated by SDS-PAGE and autoradiographed as described in **Methods.**


Caudate
Hippocampus
Temporal Lobe
Brain Stem
a
b
c
Molecular Weight x 10
86
80
63
57
50
43
20
16

3.3 Biochemical Characterization of cAMP-Dependent Protein Kinase Activity in Rat Caudate Nucleus

3.3.1 Introduction

The above studies demonstrated that rat caudate nucleus contains cAMP and Ca^{++}-dependent protein kinase activity, and that the assay methods used for detecting changes in phosphorylation yielded results compatible with published observations. Preliminary characterization of the substrates of these enzymes demonstrated that they are extensively phosphorylated in synaptic membrane fractions consistent with their hypothesized role in synaptic transmission.

The objective of these studies was to investigate more closely the effects of cAMP on the state of phosphorylation of substrates for the Type II cAMP-dependent protein kinase in synaptic membrane fractions from rat caudate nucleus. The Type II rather than the Type I kinase was selected as a focus of investigation for the following reasons: (1) it has been shown by other investigators that brain contains predominantly Type II cAMP-dependent protein kinase (Sugden and Corbin, 1976; Bechtel et al., 1977) and therefore changes in the activity of this enzyme are likely to be more relevant to neuronal function; (2) since the regulatory subunit of the Type II kinase (R_{II}) is autophosphorylated changes in its level of phosphorylation can be readily assessed using autoradiographic techniques; (3) the Type II kinase appears to mediate the phosphorylation of a number of neuronal membrane proteins including Proteins Ia, Ib and II (Walter et al., 1979a).

Protein II has been identified as the phosphorylated regulatory subunit of the Type II protein kinase (Maeno et al., 1974). More recently this protein has been resolved into two components, Protein IIa and IIb, with molecular weights of

58,000 and 54,000 respectively (Walter et al., 1979a; Lohmann et.al, 1980). Studies of cAMP-dependent phosphorylation in rat brain have identified Protein IIa as the regulatory subunit of the Type II enzyme (Walter et al., 1979a; Lohmann et al., 1980).

Studies in bovine brain, on the other hand, have demonstrated that the Type II regulatory subunit (R_{II}) consists of two proteins of M_r=58,000 and 52,000 (Lohmann et al., 1980). The 58,000 dalton protein has been shown to correspond to the phosphorylated form of the Type II regulatory subunit. Dephosphorylated R_{II} migrates as a separate phosphoprotein on sodium-dodecyl sulfate polyacrylamide gels, and has been shown to have an approximate molecular weight of 55,000 (Rubin et al., 1979; Lohmann et al., 1980; Zoller et al., 1979). The 52,000 dalton phosphoprotein identified by Greengard's group, however, does not represent the dephospho-form of R_{II}, indicating that in bovine brain the Type II regulatory subunit may consist of two proteins (Lohmann et al., 1980).

In both rat and bovine brain, an additional major phosphoprotein in the 50-54,000 dalton range is a substrate for cAMP-dependent protein kinase, and does not co-migrate with any regulatory subunit. This protein has been referred to as Protein IIb (Lohmann et al., 1980; Walter et al., 1979).

As discussed previously, two proteins of M_r=57,000 and 55,000 were shown to be phosphorylated by cAMP-dependent protein kinase in homogenates of rat caudate nucleus (Figure 11). Since preliminary studies had shown that the 57k and 55k proteins showed different patterns of phosphorylation in the presence of cAMP and IBMX (Figures 11 and 12), it was of interest to determine whether these two proteins corresponded to Proteins IIa and IIb described by Greengard's group, or whether they in fact both represented regulatory subunits of the Type II enzyme. Therefore, the solubilization properties of these two proteins, and the effects of cAMP on their phosphorylation were investigated.

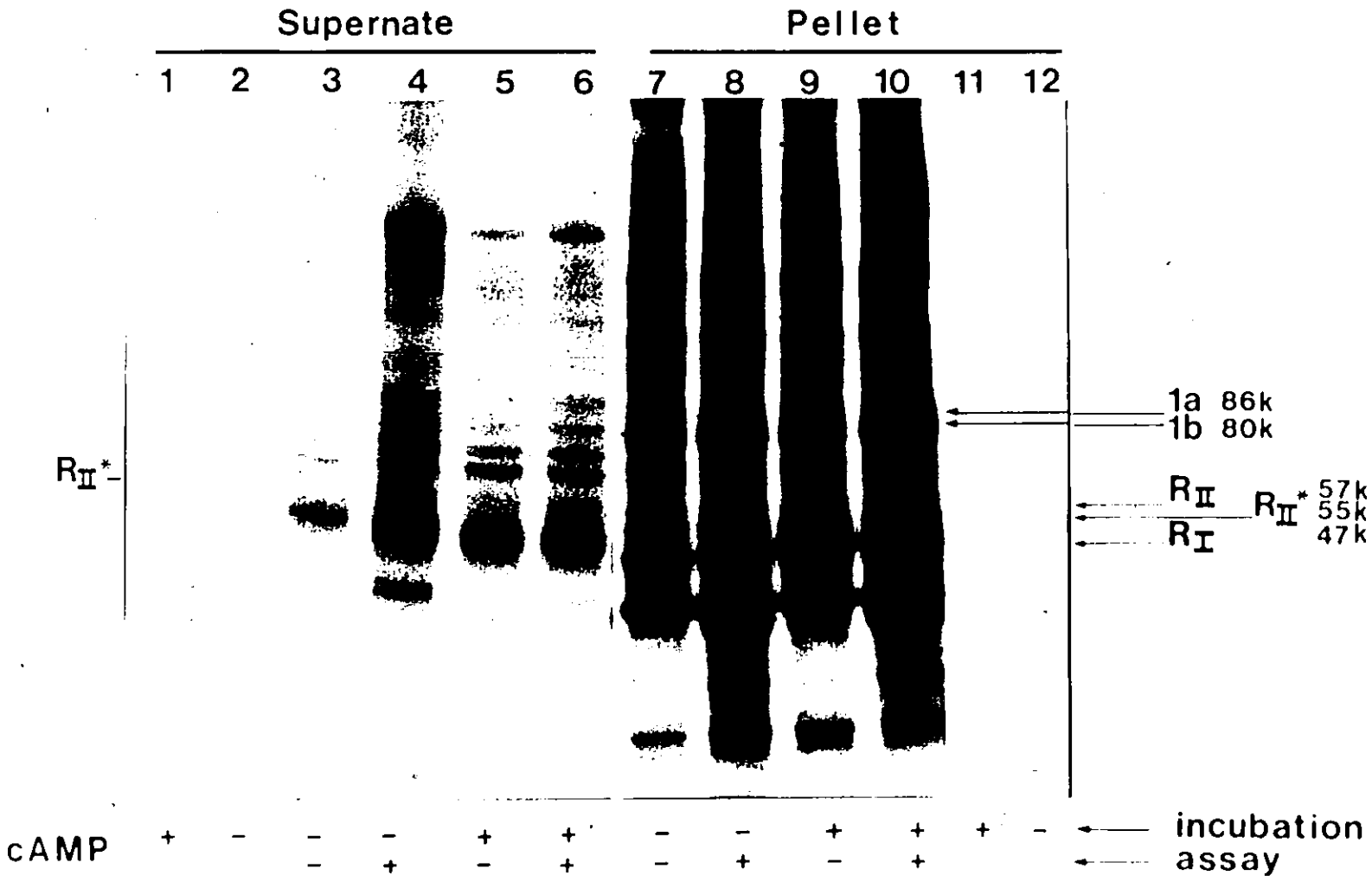
3.3.2 Effects of cAMP on the Solubilization and Dissociation Properties of the Type II Protein Kinase from Synaptic Membrane Fractions of rat Caudate Nucleus

As demonstrated previously, incubation of synaptic membranes from rat caudate nucleus under standard phosphorylating conditions in the presence of 10 μM cAMP led to an increase in the phosphorylation of Proteins Ia, Ib and 57k (compare lanes 7 and 8, Figure 15). Preincubation of membranes in the presence of cAMP led to a large increase in the phosphorylation of the 55k protein when these tissues were assayed in the absence of cAMP (lane 9, Figure 15). The phosphorylation of the 57k protein was also increased but this effect was much smaller than for the 55k protein. One explanation for this effect is preferential dephosphorylation of the 55k band in the presence of cAMP, with enhanced incorporation of labelled phosphate during the subsequent assay. No other membrane proteins appeared to show altered rates of phosphorylation as a result of prior incubation with cAMP.

When cAMP-treated membranes were assayed for protein kinase activity in the presence of cAMP there was no change in the phosphorylation of proteins 57k and 55k, but the phosphorylation of Proteins Ia and Ib was markedly increased (compare lanes 9 and 10, Figure 15).

It was suggested previously that the 57k and 55k proteins might represent the phosphorylated regulatory subunit of Type II cAMP-dependent protein kinase. The regulatory subunits of cAMP-dependent protein kinase can be identified in SDS-PAGE by prior labelling of the regulatory subunits with the photoaffinity analogue 8-N_3-[^{32}P] cAMP. Photoaffinity labelling using 8-N_3-[^{32}P] cAMP revealed that the synaptic membrane fraction from rat caudate nucleus contained three specific cAMP-binding proteins (Figure 15, lane 12). The first of these

Figure 15. Effect of Preincubation with cAMP on cAMP-Dependent Protein Phosphorylation from Rat Caudate Nucleus. Synaptic membranes were preincubated for 30 min at 0°C in the absence (lanes 2,3,4,7,8,12) or presence (lanes 1,5,6,9,10,11) of 10 μM cAMP. Soluble and particulate fractions were prepared by centrifugation at 150,000 x g for 30 min at 4°C, then assayed for endogenous phosphorylation with [^{32}P] ATP in the absence (lanes 3,5,7,9) or presence (lanes 4,6,8,10) of 10 μM cAMP. Photoaffinity labelling of regulatory subunits was carried out on fractions that were pretreated with (lanes 1,11) or without (lanes 2,12) 10 μM cAMP using 8-N_3-[^{32}P] cAMP as described in **Methods.**


Supernate
Pellet
1 2 3 4 5 6 7 8 9 10 11 12
RII*
1a 86k
1b 80k
RII
RII* 57k
55k
RI
47k
cAMP + - - - + + - - + + + -
- + - + - + - +
incubation
assay

bands co-migrated with the 57k protein, the second band was more heavily labelled and co- migrated with the 55k protein, while the third band that had an apparent molecular weight of 47,000 did not co-migrate with any phosphoprotein and is probably the regulatory subunit of the Type I cAMP-dependent protein kinase that does not undergo autophosphorylation.

Photoaffinity labelling of bovine heart and rabbit skeletal muscle that contain high levels of the Type II and Type I enzymes respectively confirmed that the three bands detected in synaptic membranes from rat caudate nucleus corresponded to labelled Type I and Type II regulatory subunits from each of these tissues (data not shown). Photoaffinity labelling of synaptic membranes in the presence of 10 μM cold cAMP completely inhibited the binding of 8-N_3-[^{32}P] cAMP to the Type I regulatory subunit (Figure 15, lane 11). Higher concentrations of cold cAMP (20 μM) were required to inhibit labelling of the Type II subunit (data not shown).

The soluble fraction obtained from these membranes contained a protein of M_r=55,000 that was phosphorylated in the presence of cAMP (Figure 15, lanes 3 and 4). Photoaffinity labelling of this preparation with 8-N_3-[^{32}P] cAMP confirmed that this protein was the autophosphorylated regulatory subunit of the Type II protein kinase (Figure 15, lane 2). The 57k protein was not reliably detected in the soluble fraction by photoaffinity labelling or autophosphorylation with [^{32}P] ATP.

Assay of this preparation in the presence of cAMP also enhanced the phosphorylation of other proteins in the extract (lane 4) indicating that the phosphorylation of the 55k protein in this case was the result of an intermolecular phosphorylation reaction caused by dissociation of the Type II enzyme by cAMP. In contrast, the increase in phosphorylation of the 55k protein observed following

preincubation of membranes with cAMP was not accompanied by an increase in the phosphorylation of other substrates for the kinase (lanes 5 and 6) indicating that this effect was probably due to intramolecular phosphorylation of 55k by bound catalytic subunit.

Changes in the pattern of distribution of substrates for the Type II cAMP-dependent protein kinase between soluble and particulate fractions were quantified by scanning autoradiographs with a Joyce-Loebl densitometer and obtaining estimates of ^{32}P incorporation into labelled proteins as described in **Methods.** Densitometer tracings of the autoradiographs shown in Figure 15 are depicted in Figure 16. A summary of the observed effects is provided in Table V.

The increase in phosphorylation of the 55k protein in extracts of membranes treated with cAMP was also observed when soluble fractions from synaptic membranes were preincubated with cAMP and then assayed for protein kinase activity (Figure 17, lanes 3 and 4). These results indicated: (1) that the protein kinase activity in the soluble fraction was not dependent on prior exposure of membranes to cAMP; (2) that preincubation of soluble and particulate fractions with cAMP was associated with an increase in the phosphorylation of the 55k protein. Thus it appeared that extracts of synaptic membranes normally contained small amounts of cAMP-dependent protein kinase activity.

Preparation of membranes in buffer containing 2 mM EGTA and 0.3 mM PMSF to inhibit proteolytic degradation gave phosphorylation patterns identical to those obtained when these agents were omitted from the homogenizing medium. It therefore seemed unlikely that the 55k protein was formed from the 57,000 dalton band as a result of proteolysis, although this possibility cannot be completely excluded.

Figure 16. Effect of Preincubation with cAMP on cAMP-Dependent Protein Phosphorylation in Soluble and Particulate Fractions from Synaptic Membranes: Densitometer Tracing of Autoradiogram Shown in Figure 15. Membrane fractions preincubated in the absence or presence of 10 μM cAMP were assayed for endogenous protein kinase activity in the absence (dotted lines) or presence (solid lines) of additional 10 μM cAMP as described in the legend to Figure 9. Autoradiograms were scanned with a Joyce-Loebl Densitometer as described in **Methods.**

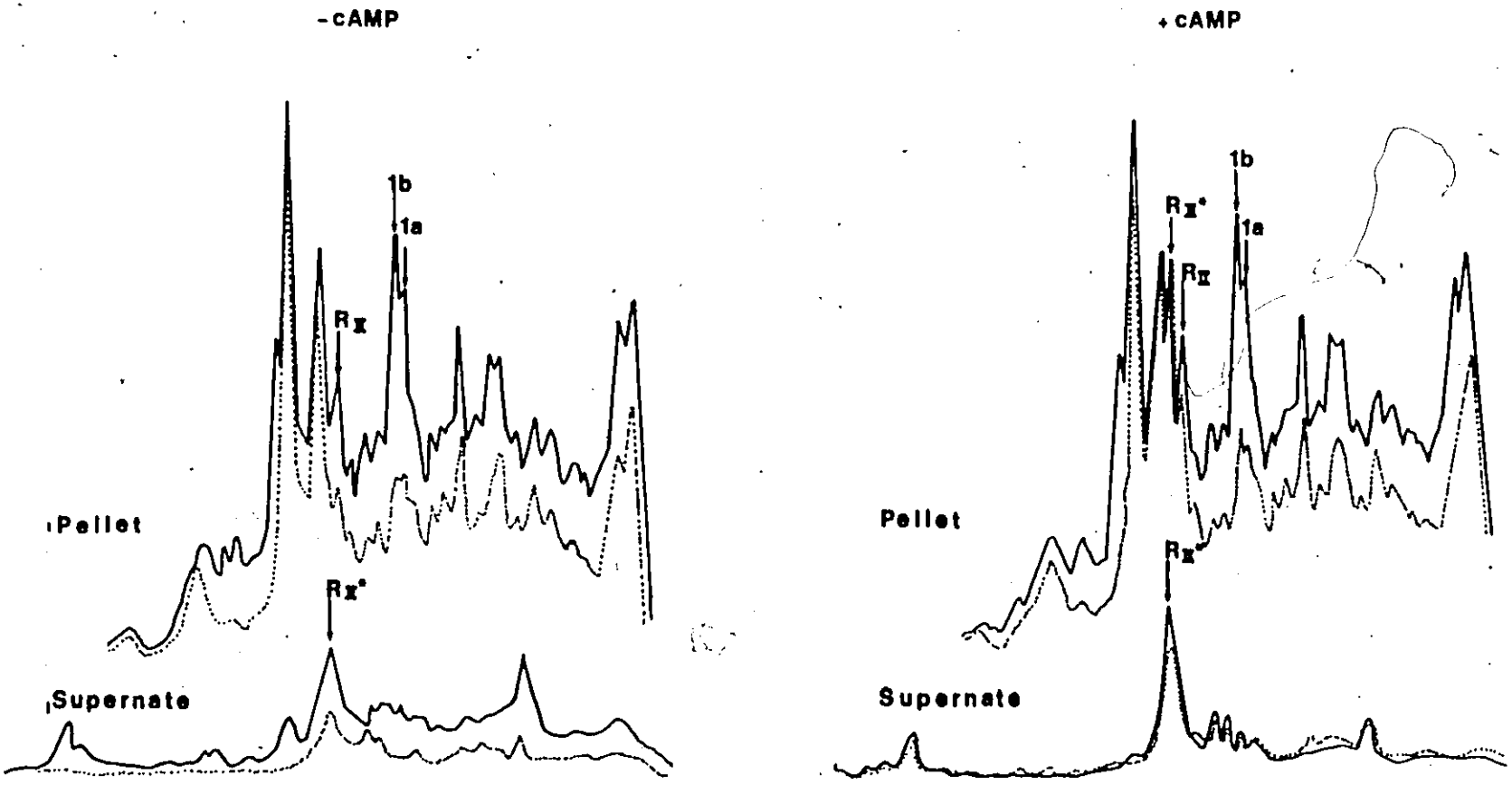

-cAMP
+cAMP
1b
1a
Rx
Rx*
Pellet
Supernate

Figure 17. cAMP-Dependent Phosphorylation in Soluble and Particulate Fractions from Rat Caudate Nucleus. Soluble and particulate fractions were isolated from synaptic membranes by centrifugation as described in the legend to Figure 15, then preincubated for 30 min at 0°C in the absence (lanes 1,2,5,6) or presence (lanes 3,4,7,8) of 10 μM cAMP. Fractions were then assayed for protein kinase activity with [^{32}P] ATP in the absence (lanes 1,3,5,7) or presence (lanes 2,4,6,8) of 10 μM cAMP.

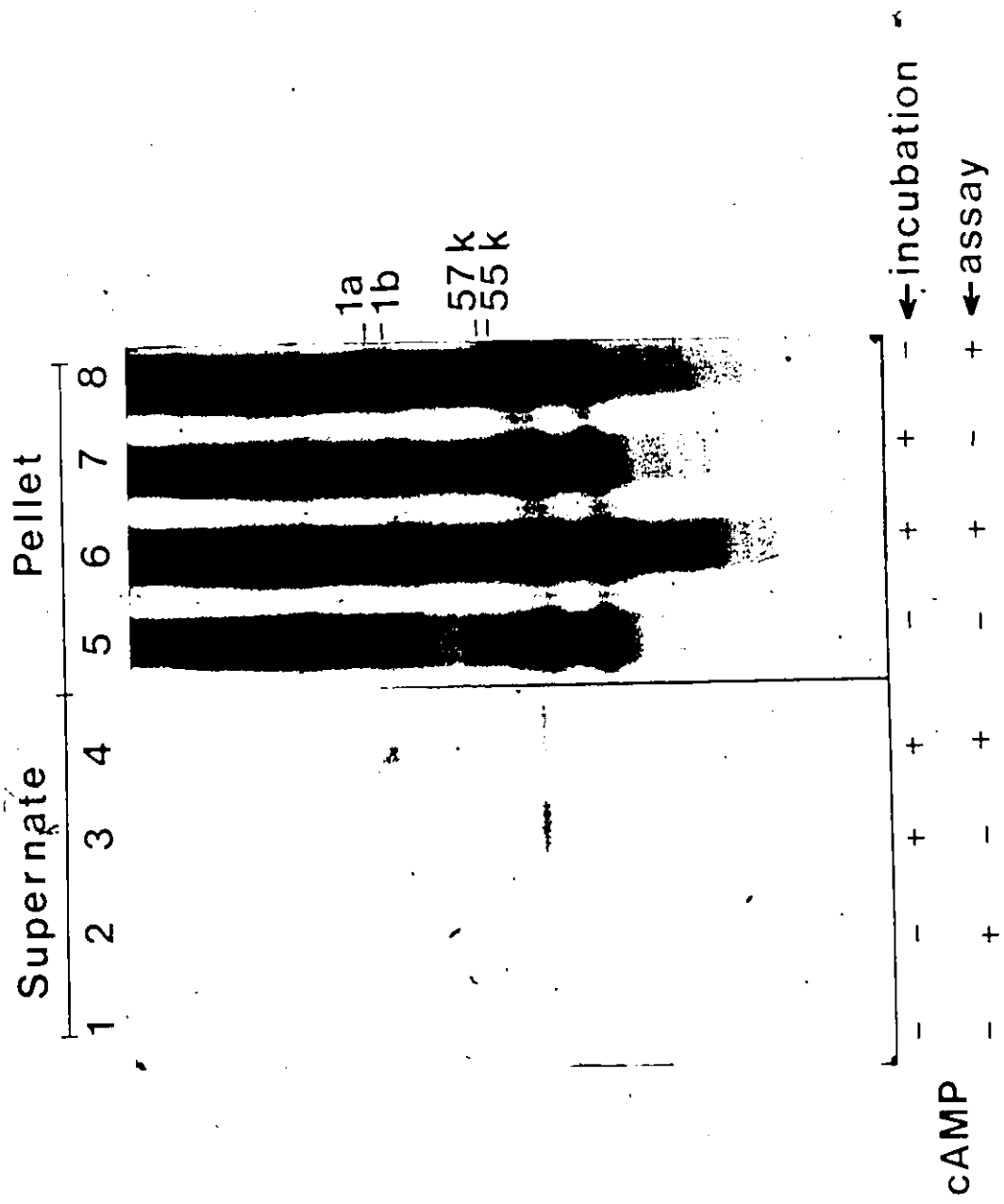

1a
1b
57 k
55 k
Supernate
Pellet
1 2 3 4 5 6 7 8
cAMP
incubation
assay

TABLE V

Effect of cAMP, NaCl and Triton X-100 on Protein Phosphorylation in Soluble and Particulate Fractions from Rat Brain

[32P] Phosphate Incorporation (Arbitrary Units)

		PROTEINS					
Addition to S1 Fraction	Cell Fraction	Ia/Ib -cAMP	Ia/Ib +cAMP	R_{II}(57k) -cAMP	R_{II}(57k) +cAMP	R_{II}*(55k) -cAMP	R_{II}*(55k) +cAMP
Water	particulate	1.97 ±.23	5.63 ±.82	1.0	2.47 ±.14		
	supernate					2.67 ±1.20	3.83 ±1.25
10 uM cAMP	particulate	3.00 ±.58	5.75 ±.91	1.56 ±.23	2.63 ±.43	2.90 ±.53	2.93 ±.82
	supernate					3.43 ±.83	5.43 ±1.13
0.5 M NaCl	particulate	1.47 ±.37	3.87 ±.39	1.57 ±.35	3.23 ±.39		
	supernate	1.1 ±.10	2.60 ±.30			2.00 ±.21	3.67 ±.03
0.5M NaCl + 10 uM cAMP	particulate	1.37 ±.23	1.53 ±.29	0.67 ±.17	0.67 ±.17	2.67 ±.68	2.40 ±.66
	supernate	4.60 ±.60	4.60 ±.83	1.13 ±.40	1.10 ±.49	3.30 ±.62	3.60 ±.66
10% Triton X-100	particulate	3.17 ±.33	7.17 ±.09	3.00 ±.32	5.27 ±.54		
	supernate		3.63 ±.43	5.33 ±1.20	3.33 ±1.20	5.97 ±1.30	6.60 ±1.20
10% Triton X-100 + 10 uM cAMP	particulate	7.33 ±.20	7.67 ±.13	5.93 ±.53	6.13 ±.42		
	supernate		1.77 ±.39	0.53 ±.03	5.70 ±.94	5.50 ±1.5	7.07 ±1.10

Synaptic membrane fractions were preincubated for 30 min at 0°C with water, NaCl or Triton X-100 in the absence or presence of 10uM cAMP. Soluble and particulate fractions were obtained and assayed for cAMP-dependent protein phosphorylation as described in the legend to Figure 15. Autoradiograms from experiments as shown in Figures 15, 18 and 21 were scanned with a Joyce-Loebl Densitometer and ^{32}P incorporation in the absence or presence of cAMP was measured as OD units as described in Methods. Results represent the mean ± SEM from 3 experiments.

The above studies demonstrated (1) that synaptic membranes contain Type I as well as Type II cAMP-dependent protein kinases, (2) that the regulatory subunit of the Type II enzyme can be resolved into two components on SDS gels using photoaffinity labelling and autophosphorylation with [^{32}P] ATP, (3) that preincubation of membranes with cAMP markedly enhances the phosphorylation of the 55k but not the 57k protein.

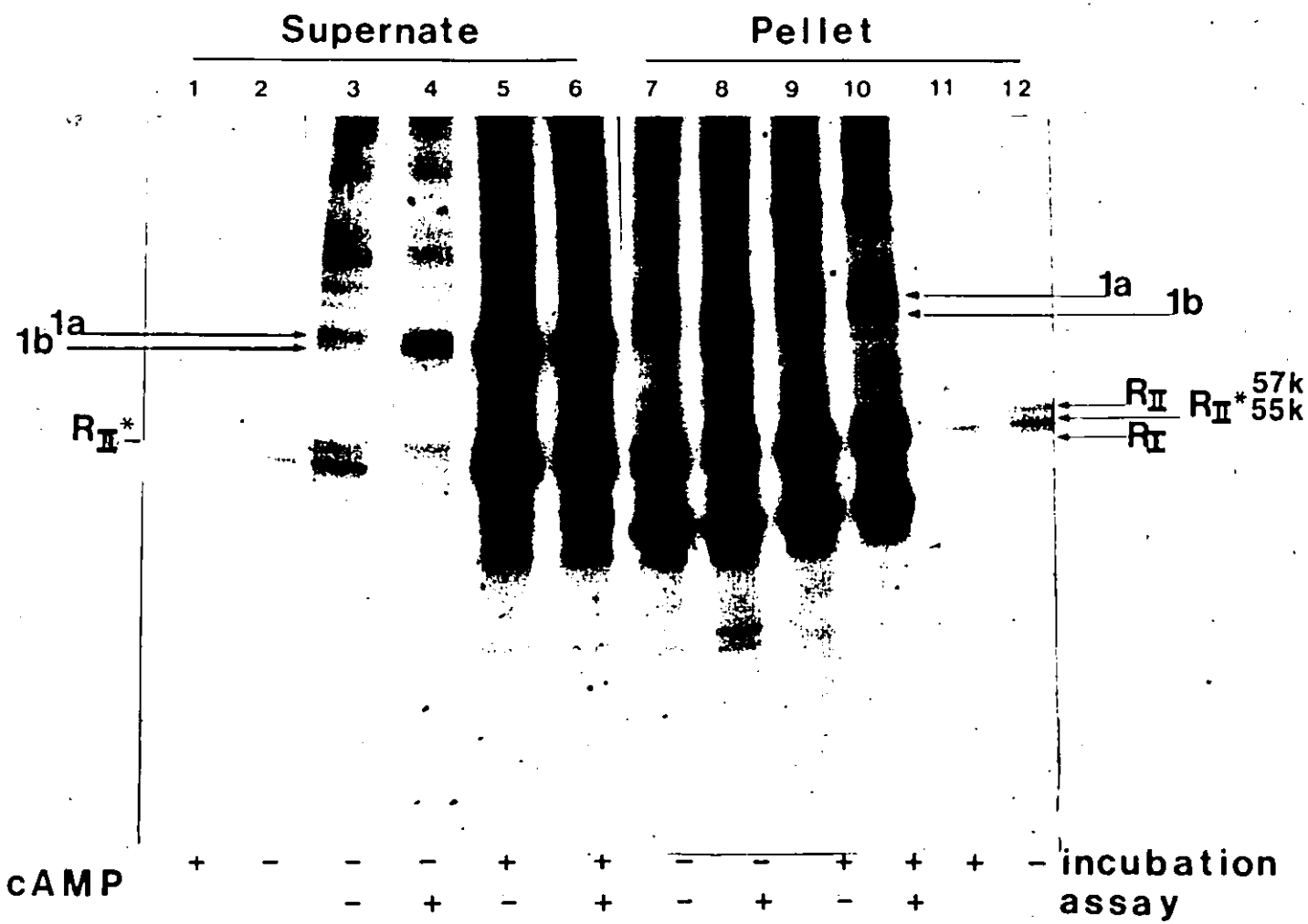
3.3.3 Effects of NaCl on the Solubilization and Dissociation Properties of the Type II Protein Kinase from Rat Caudate Nucleus

Previous studies in non-nervous tissue have shown that activation of membrane-bound protein kinases by cAMP leads to an increase in catalytic activity in the soluble fraction (Corbin et al., 1977). It was of interest to determine whether treatment of synaptic membranes with cAMP would be accompanied by an increase in cAMP-independent phosphorylation in soluble extracts of these membranes. Since non-specific binding of catalytic subunit to plasma membranes occurs in low ionic strength buffers (Corbin et al., 1973; 1977), these experiments were conducted in the presence of 0.5 M NaCl.

As shown in Figure 18, particulate fractions from membranes preincubated with NaCl showed normal patterns of cAMP-dependent protein phosphorylation (compare lanes 7 and 8 in Figures 15 and 18). In contrast, the cAMP-dependent phosphorylation of Proteins Ia and Ib was markedly decreased in particulate fractions obtained from membranes treated with NaCl and cAMP (Figure 18, lanes 8 and 10).

Preincubation of membranes with NaCl and cAMP enhanced the phosphorylation of protein 55k (Figure 18, lanes 9 and 10). Since the cAMP-dependent phosphorylation of endogenous substrates was reduced in membranes pretreated

Figure 18. Effect of NaCl on Protein Phosphorylation in Rat Caudate Nucleus. Synaptic Membrane fractions were incubated at 0°C for 30 min in the absence (lanes 2,3,4,7,8,12) or presence (lanes 1,5,6,9,10,11) of 10 µM cAMP. Soluble and particulate fractions were prepared as described in the legend to Figure 15, and assayed for endogenous protein phosphorylation in the absence or presence of 10 µM cAMP as described in **Methods.** Photoaffinity labelling of NaCl-treated membranes was carried out on soluble and particulate fractions that were pretreated with (lanes 1,11) or without (lanes 2,12) 10 µM cAMP using 8-N_3-[^{32}P] cAMP as described in **Methods.**


Supernate
Pellet
1 2 3 4 5 6 7 8 9 10 11 12
1b
1a
RII*
1a
1b
RII
RII* 57k
55k
RI
cAMP + − − − + + − − + + + − incubation
− + − + − + − + assay

with both NaCl and cAMP, it appeared that NaCl inhibited the reassociation of regulatory and catalytic subunits of the enzyme following their dissociation by cAMP. In support of this interpretation, membranes preincubated with cAMP in the absence of NaCl exhibited normal cAMP-dependent phosphorylation activity, indicating that reassociation of catalytic and regulatory subunits had occurred prior to assay (Figure 15).

Photoaffinity labelling of NaCl-treated membranes identified the regulatory subunits of the Type I and Type II protein kinases as previously shown (Figure 18, lane 12). Labelling of these subunits, however, was less than that observed in non-NaCl treated membranes. This was not due to loss of regulatory subunits from the membrane in the presence of NaCl, since there was no corresponding increase in the number of labelled subunits detected in the supernatant fraction relative to non-NaCl treated controls (see Figure 18, lanes 2 and 12). Instead the reduction in ^{32}P labelling can best be explained by an effect of ionic strength causing inhibition of cAMP binding to regulatory subunit.

Supernatant fractions from NaCl-treated membranes showed low levels of 55k phosphorylation when assayed in the absence of cAMP (Figure 18, lane 3). Phosphorylation of the 57k protein was barely detectable. Photoaffinity labelling of NaCl-treated extracts gave similar results with the majority of labelling occurring in the 55k protein (lane 2). When these extracts were assayed in the presence of cAMP the phosphorylation of the 55k and 57k proteins was decreased relative to controls (Figure 18, lanes 3 and 4), while the phosphorylation of Protein I was slightly increased (Figure 18, lane 4). Previous investigators have shown that Protein I can be extracted from brain membranes using salt (Ueda and Greengard, 1977), so the presence of Protein I in the supernatant was used as an index of protein kinase activity in this fraction.

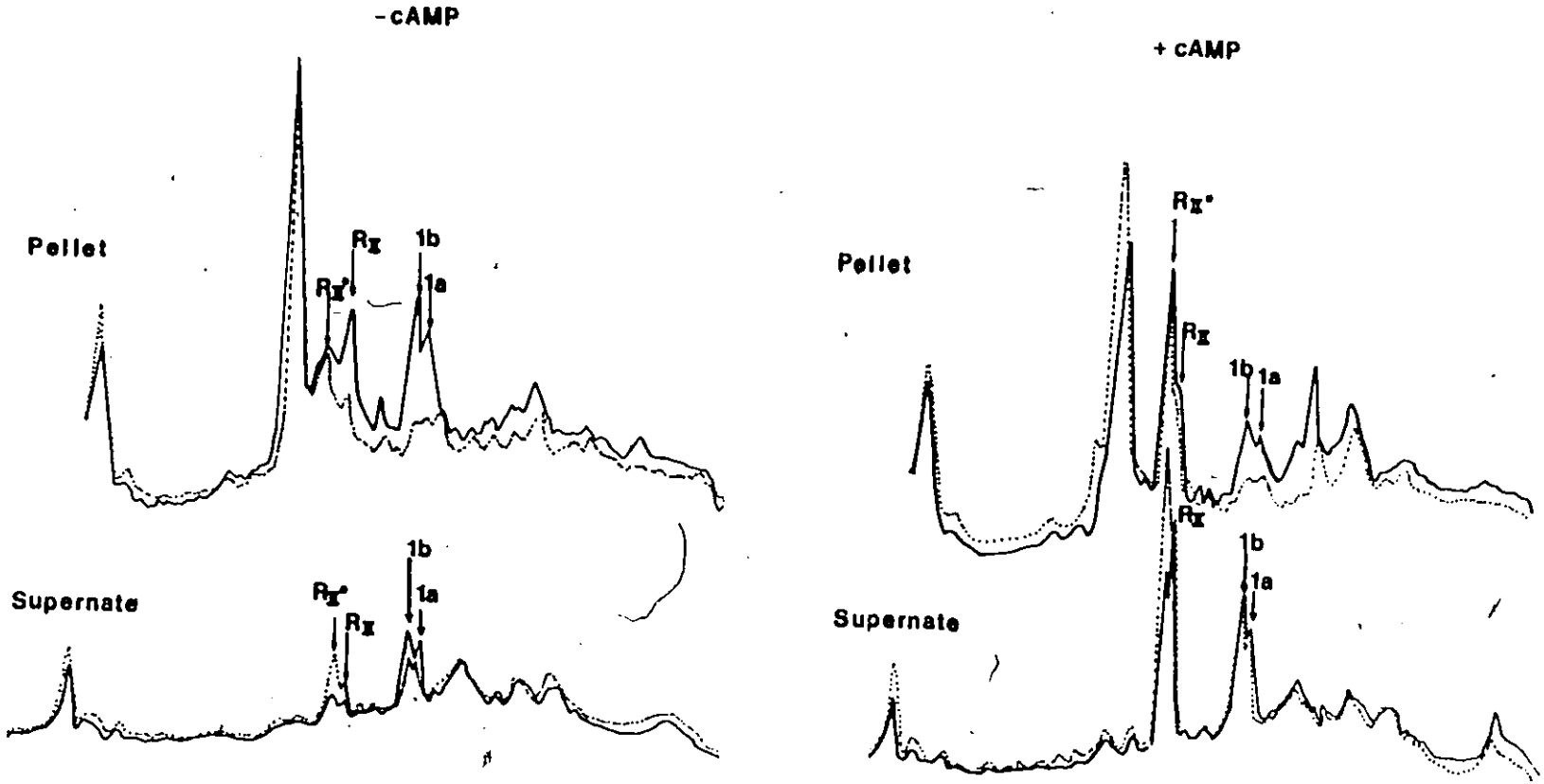
Extracts of membranes prepared in the presence of both NaCl and cAMP, on the other hand, showed a large increase in the phosphorylation of Protein I and 55k. The phosphorylation of 57k was also elevated but this effect was much smaller than for 55k (Figure 18, lane 5). Assay of this preparation in the presence of cAMP produced equivalent results (Figure 18, lane 6). The results of these experiments were quantified using densitometry as shown in Figure 19. Table V provides a summary of the described effects.

These data indicated that treatment of membranes with cAMP and NaCl was associated with a decrease in the cAMP-dependent phosphorylation of proteins in the particulate fraction, and a concomitant increase in cAMP-independent phosphorylation activity in the supernatant fraction. However, the amount of labelled regulatory subunit in either fraction, did not appear to be altered by this procedure, confirming, as others have shown, that membrane-bound R_{II} is not readily solubilized by high ionic strength (Walter et al., 1978).

One interpretation of these results is that incubation of membranes with NaCl and cAMP leads to release of catalytic subunits from the membrane into the cytosol following dissociation of the Type II kinase by cAMP. This view was additionally reinforced by the observation that preincubation of membranes with NaCl and cAMP did not cause an increase in the phosphorylation of Protein I in the particulate fraction as would be expected if the catalytic activity had remained associated with the membrane.

An alternative interpretation of these findings is that solubilization of Protein I was enhanced by the presence of NaCl and cAMP. To distinguish between these two possibilities, the effects of NaCl and cAMP on protein kinase activity were assessed using histone as an exogenous substrate. As shown in Figure 20, particulate fractions from membranes preincubated with water or with cAMP exhibited significant cAMP-dependent protein kinase activity ($p<.02$).

Figure 19. Effect of NaCl on Solubilization of Protein Kinase Activity in Rat Caudate Nucleus. Densitometer Tracing of Autoradiogram Shown in Figure 18. Membrane fractions were incubated with 0.5 M NaCl in the absence or presence of 10 μM cAMP as described in the legend to Figure 18. Soluble and particulate fractions were isolated and assayed for protein kinase activity in the absence (dotted lines) or presence (solid lines) of additional 10 μM cAMP as described in the legend to Figure 15. Autoradiograms were scanned with a Joyce-Loebl Densitometer as described in **Methods.**


-cAMP
Pellet
RII'
RII
1b
1a
Supernate
RII'
RII
1b
1a
+ cAMP
Pellet
RII'
RII
1b
1a
Supernate
RII
1b
1a

Similar results were obtained in the presence of NaCl (compare lanes 1 and 3, and lanes 2 and 4).

Soluble fractions from membranes preincubated with NaCl and cAMP showed a significant increase in cAMP-independent protein kinase activity relative to NaCl-treated controls (t with 7 d.f. = 3.77, $p<.01$). Soluble fractions from membranes treated with water or with cAMP did not show this effect. No cAMP-dependent protein kinase activity was detected in soluble fractions prepared from membranes incubated under any of the above conditions. These results are in agreement with previous data obtained using gel electrophoresis, and support the interpretation that preincubation of membranes with NaCl and cAMP leads to an increase in catalytic activity in the soluble fraction. A criticism of this interpretation is that changes in enzyme activity in the soluble fraction were not accompanied by corresponding decreases in catalytic activity in the membrane fraction. In view of the fact that these studies were conducted using a crude membrane preparation, and changes in soluble protein kinase activity were quite small, alterations in particulate-bound kinase activity might not have been detected under these conditions.

3.3.4 Solubilization of Regulatory Subunits of Type II cAMP-dependent Protein Kinase from Synaptic Membranes of Rat Caudate Nucleus

The previous experiments provided some preliminary evidence that cAMP-independent protein kinase activity can be solubilized from synaptic membranes using NaCl and cAMP. To further examine the distribution of Type II regulatory subunits in supernatant and particulate fractions from rat caudate nucleus, the effects of Triton X-100 on the solubilization of these proteins was examined.

Figure 20. Effect of NaCl and cAMP on Protein Kinase Activity in Soluble and Particulate Fractions from Rat Caudate Nucleus. Synaptic membranes were incubated with 0.5 M NaCl in the absence or presence of 10 µM cAMP as described in the legend to Figure 18. Soluble and particulate fractions, obtained by centrifugation, were assayed for histone kinase activity in the absence (open bars) or presence (closed bars) of cAMP as described in **Methods.** Results represent the mean ± SEM of 8 individual experiments. Statistical analyses were performed using a repeated measures ANOVA with three trial factors, followed by paired t-tests for individual effects.

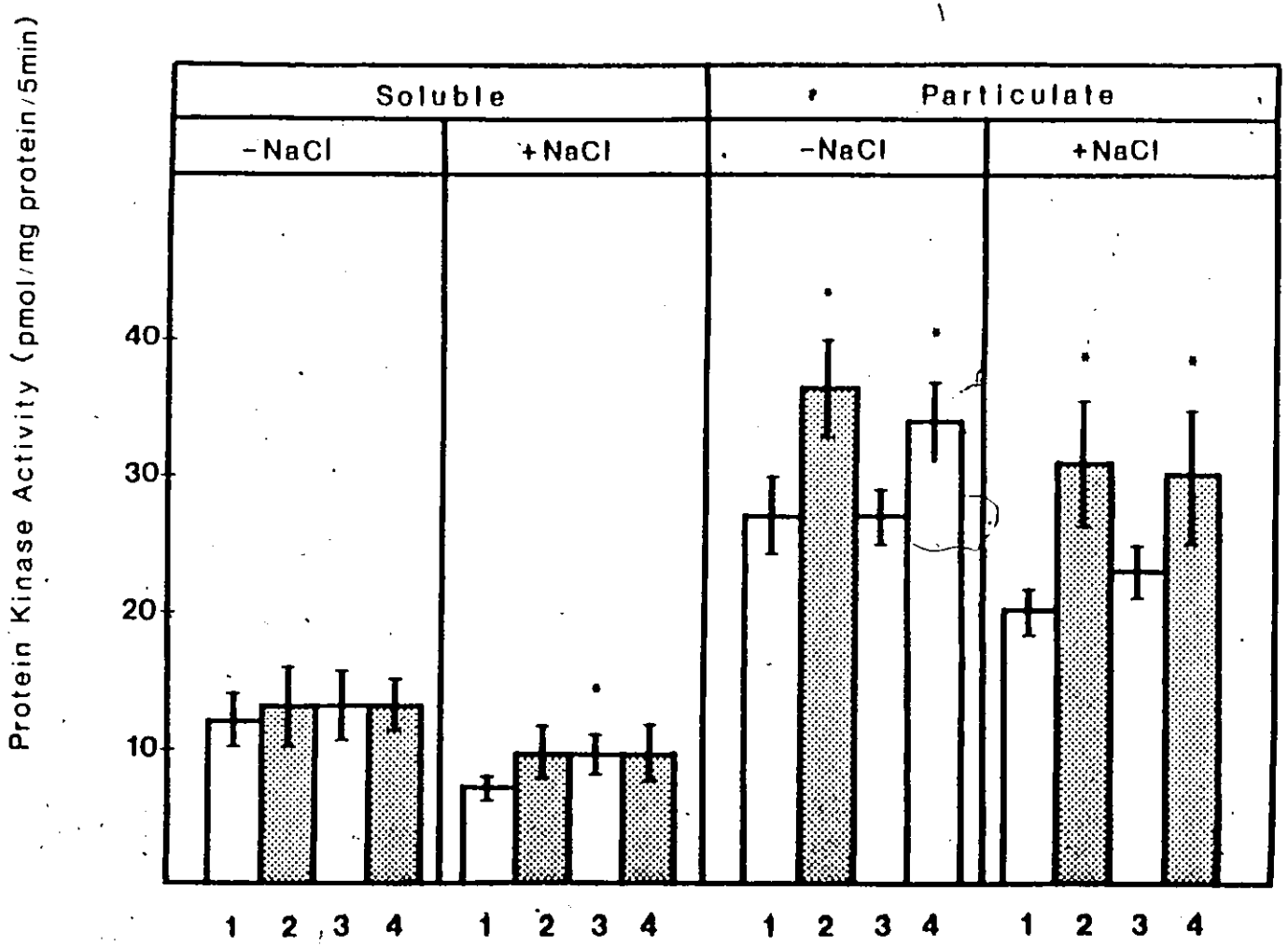
Note: * Significantly different from controls (see text).

Legend

Treatment	cAMP preincubation	cAMP assay
1	-	-
2	-	+
3	+	-
4	+	+

ANOVA

	Soluble		Particulate	
Source	$F_{1,7}$	P	$F_{1,7}$	P
NaCl	3.03	ns	3.67	ns
cAMP preinc.	9.36	<.02	0.11	ns
cAMP assay	1.21	ns	10.71	<.02


Protein Kinase Activity (pmol/mg protein/5min)
Soluble
Particulate
-NaCl
+NaCl
-NaCl
+NaCl
40
30
20
10
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

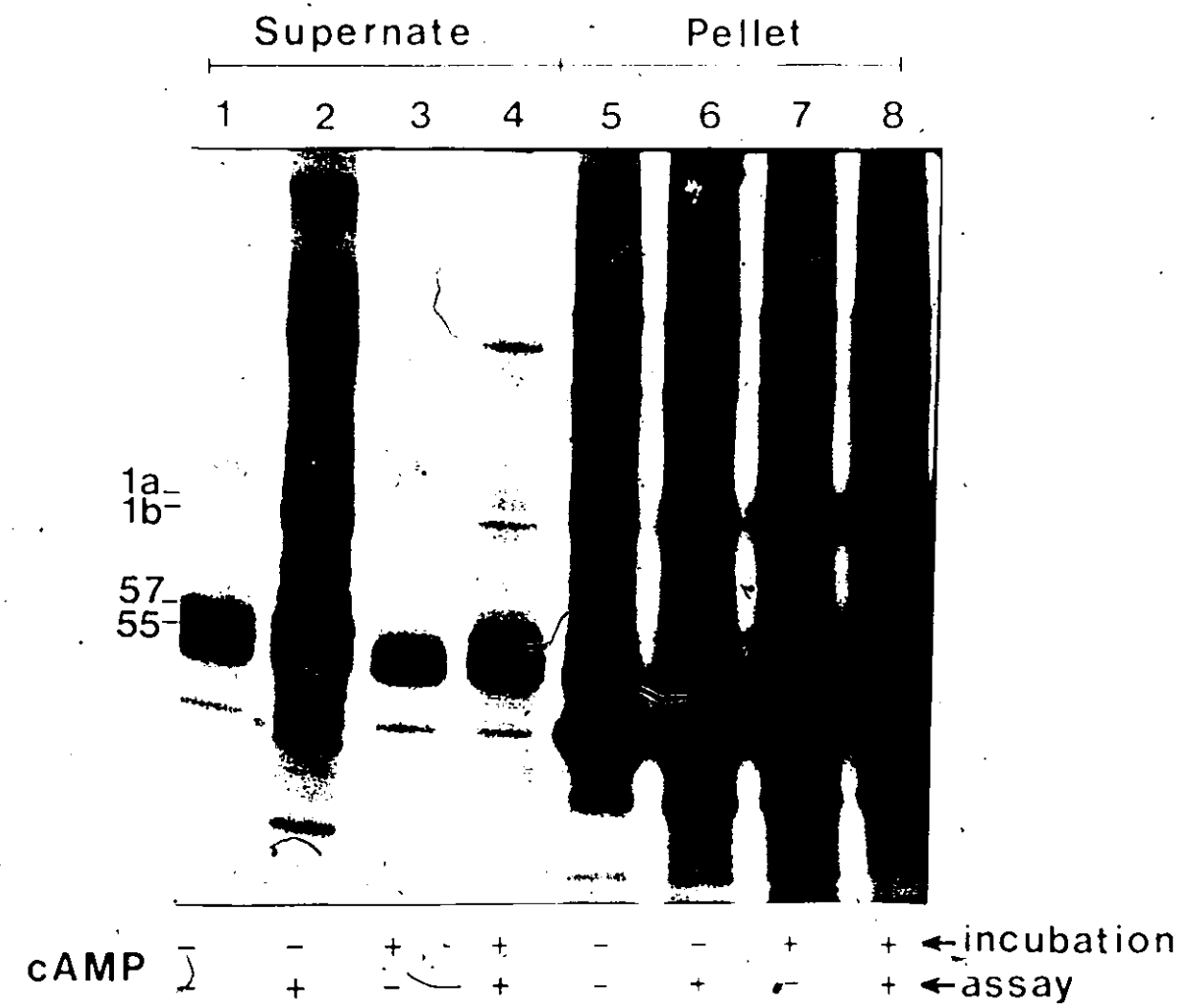
As shown in Figure 21, cAMP increased the phosphorylation of Protein I and 57k in particulate fractions from synaptic membranes preincubated with Triton X-100. The phosphorylation of these substrates was much greater than had been previously observed in membranes prepared without Triton. The increased levels of phosphorylation observed with Triton can be attributed to either a direct effect of Triton on protein kinase activity or to an alteration in the organization of the membrane making protein substrates more available to the enzyme.

Synaptic membranes that had been preincubated with Triton in the presence of cAMP also showed increased phosphorylation of Protein I and 57k (Figure 21, lanes 7 and 8). Phosphorylation of 55k was not seen in the particulate fraction under any of these conditions. These results indicated that dissociation of the membrane-bound protein kinase by cAMP did not result in a loss of catalytic activity from the membrane, since the pattern of phosphorylation seen when membranes were preincubated with cAMP was identical to that observed when untreated membranes were assayed in the presence of cAMP.

Extracts of membranes treated with Triton X-100 and assayed for protein kinase activity in the absence of cAMP showed autophosphorylation of proteins 57k and 55k (Figure 21, lane 1). In the presence of cAMP, the phosphorylation of proteins 57k and 55k was increased as was the phosphorylation of numerous other proteins including Proteins Ia and Ib (Figure 21, lane 2). Since both autophosphorylation and cAMP-dependent phosphorylation could be demonstrated in the extract it was concluded that treatment of synaptic membranes with Triton had led to solubilization of membrane-bound holoenzyme.

Extracts of cAMP-treated membranes, in contrast, did not exhibit autophosphorylation when assayed without cAMP, although increased phosphorylation of 57k and 55k was observed when assays were conducted in the presence

Figure 21. Effect of Triton X-100 on Protein Phosphorylation in Soluble and Particulate Fractions from Rat Caudate Nucleus: Synaptic membranes were incubated at 0°C for 30 min with 1% Triton X-100 in the absence (lanes 1,2,5,6) or presence (lanes 3,4,7,8) of 10 μM cAMP. Soluble and particulate fractions were prepared as described in the legend to Figure 16, and assayed for protein kinase activity with [^{32}P] ATP in the absence (lanes 1,3,5,7) or presence (lanes 2,4,6,8) of 10 μM cAMP.


Supernate
Pellet
1 2 3 4 5 6 7 8
1a
1b
57
55
cAMP
- + + - - + + ←incubation
+ - + - + - + ←assay

of cAMP. A marginal increase in the phosphorylation of Protein I was also detected in the presence of cAMP. Densitometer tracings of the autoradiograph shown in Figure 21 are shown in Figure 22. A quantitative summary of these findings is found in Table V.

To verify that the increase in cAMP-dependent phosphorylation in the supernatant fraction was due to solubilization of the membrane-bound protein kinase, the effects of Triton X-100 on enzyme activity were also measured using histone as an exogenous substrate. As shown in Figure 23, preincubation of membranes with Triton X-100 was associated with a large increase in cAMP-dependent protein kinase activity in the soluble fraction relative to untreated controls ($p<.025$). Similar results were obtained when membranes were preincubated with both Triton X-100 and cAMP. Therefore, the decrease in the cAMP-dependent phosphorylation of endogenous substrates in extracts of membranes treated with Triton plus cAMP, versus Triton alone (Figure 21, lanes 2 and 4), cannot be attributed to differences in the amount of holoenzyme released from the membrane under these two conditions.

3.3.5 Substrate Specificity of the Type II Kinase from Rat Caudate Synaptic Membranes

Previous studies demonstrated that the major substrates for the Type II cAMP-dependent protein kinase in synaptic membranes from rat caudate nucleus were Proteins Ia and Ib, and the regulatory subunit of the enzyme consisting of proteins 57k and 55k. Since the membrane-bound cAMP-dependent protein kinase from synaptic membranes of rat caudate nucleus appeared to share many biochemical properties in common with Type II kinases from other systems including response to cAMP, NaCl, and Triton X-100, it was hypothesized that if the brain enzyme

Figure 22. Effect of Triton X-100 on Protein Phosphorylation in Rat Caudate Nucleus. Densitometer Tracing of Autoradiogram shown in Figure 21. Synaptic membranes were incubated with Triton X-100 in the absence or presence of cAMP as described in the legend to Figure 22. Soluble and particulate fractions were assayed for endogenous protein kinase activity in the absence (dotted lines) or presence (solid lines) of additional 10 μM cAMP as described in the legend to Figure 15. Autoradiograms were scanned with a Joyce-Loebl Densitometer as described in **Methods.**

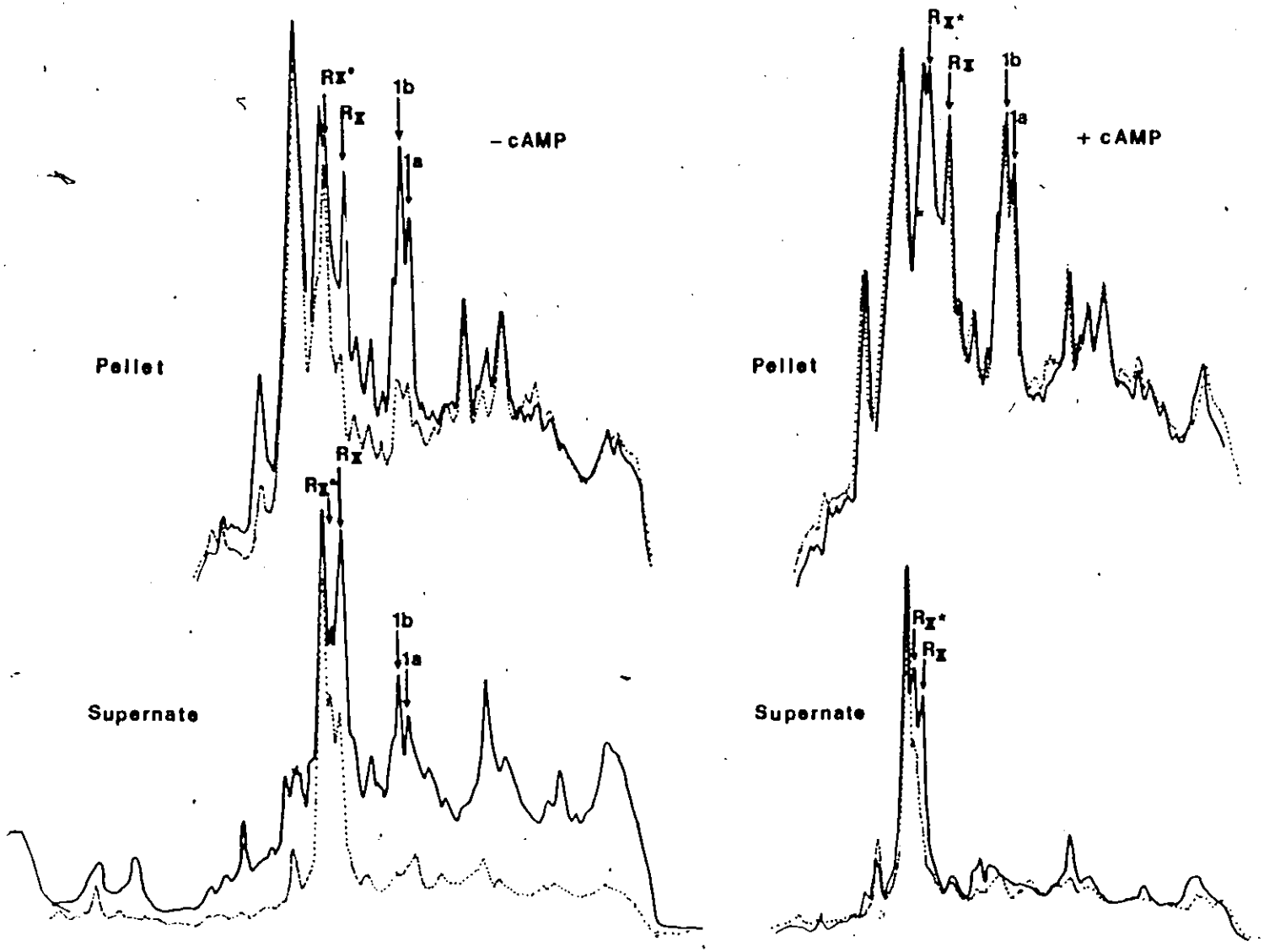

Rx*
Rx
1b
1a
- cAMP
Pellet
Supernate
+ cAMP

Figure 23. Effect of Triton X-100 on Protein Kinase Activity in Soluble and Particulate Fractions from Rat Brain. Synaptic membranes were incubated with Triton X-100 in the absence (open bars) or presence (closed bars) of cAMP as described in the legend to Figure 21. Soluble and particulate fractions were isolated and histone kinase activity was measured as described in **Methods.** Results represent the mean ± SEM of 5 to 7 individual experiments. Data were analyzed using the Analysis of Variance.

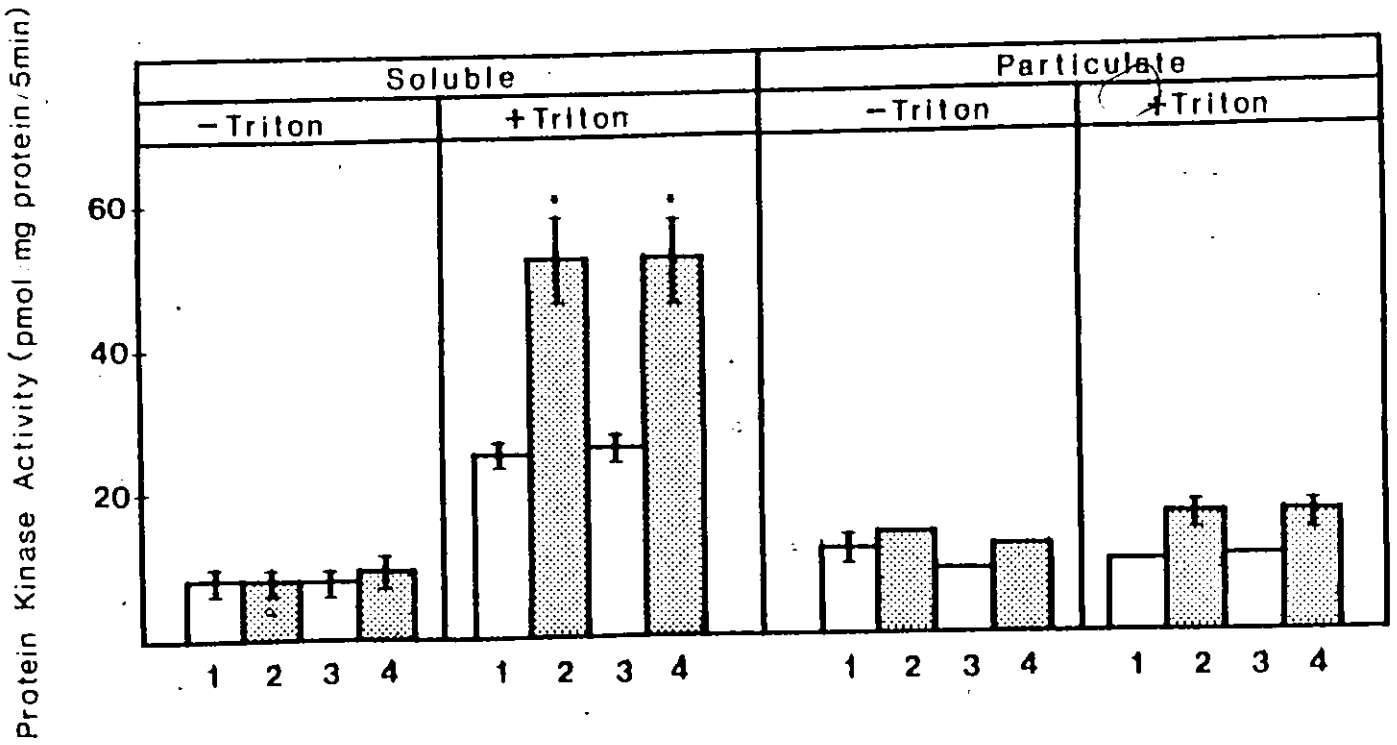
Note: * Significantly different from controls (see text).

LEGEND

Treatment	cAMP preincubation	cAMP assay
1	-	-
2	-	+
3	+	-
4	+	+

ANOVA

	Soluble		Particulate	
Source	$F_{5,42}$	P	$F_{5,42}$	P
Triton	9.76	<.05	0.25	ns
cAMP preinc.	0.66	ns	5.94	ns
cAMP assay	13.24	<.02	7.16	<.05


Protein Kinase Activity (pmol mg protein/5min)
Soluble
Particulate
−Triton
+Triton
−Triton
+Triton
60
40
20
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4

were uniquely involved in the regulation of synaptic function then the specificity of the response would probably reside at the substrate rather than at the enzyme level.

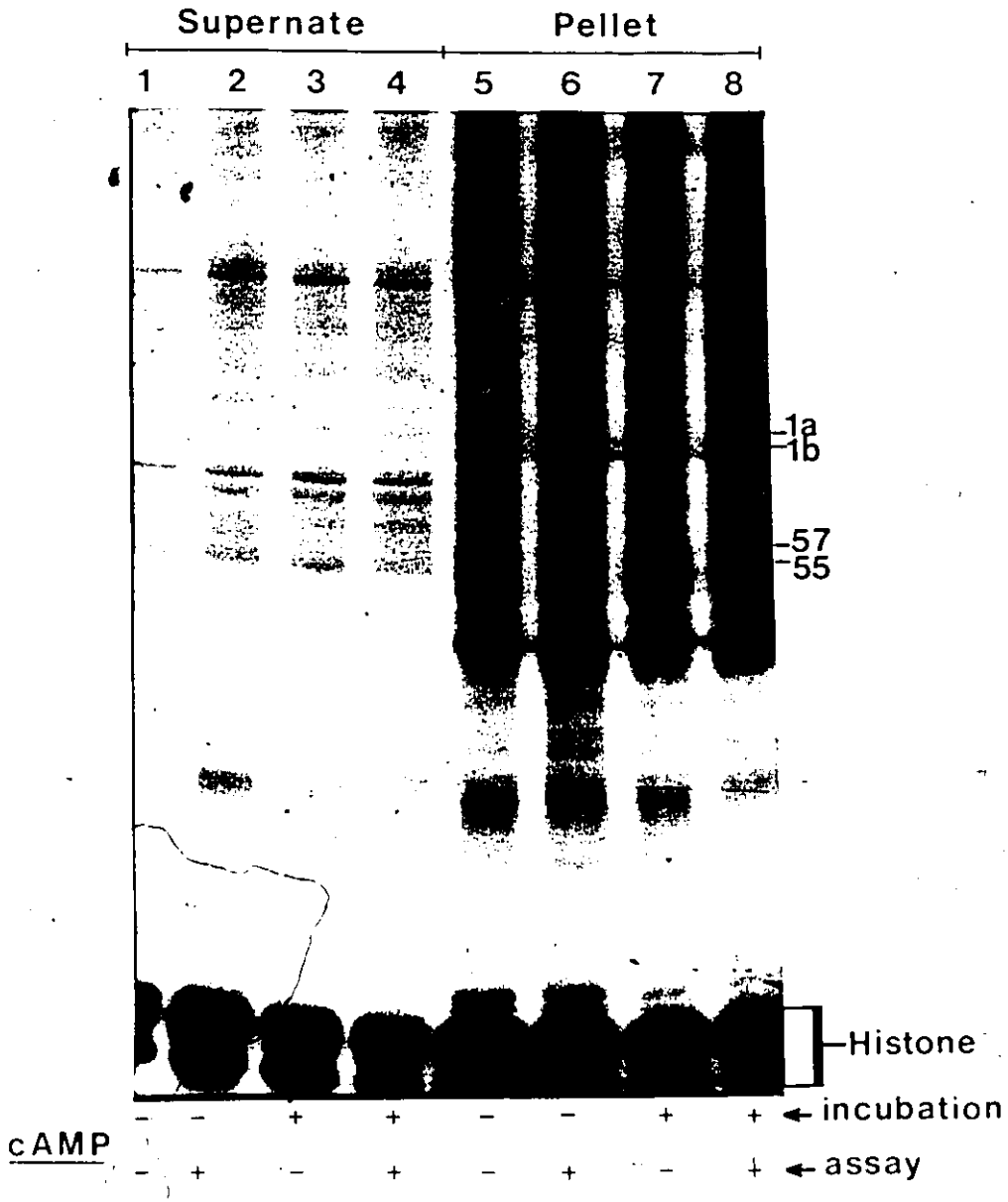
In support of this, one of the principal substrates for the Type II enzyme from brain, Protein I, has been shown to be localized specifically in neuronal tissue (De Camilli et al., 1979; Sieghart et al., 1978; Goelz et al., 1981). It was therefore of interest to determine the effects of competition of histone, an exogenous substrate for the kinase (Uno et al., 1977a), on the phosphorylation of particulate-bound and solubilized Protein I.

As shown in Figure 24, the cAMP-dependent phosphorylation of Protein I was identical in particulate fractions that were assayed in the absence or presence of histone. Protein 57k, on the other hand, that is normally phosphorylated in a cAMP-dependent manner in this preparation, did not show an increase in its phosphorylation when histone was present in the incubation medium (Figure 24, lanes 5 and 6).

Particulate fractions of membranes preincubated with cAMP showed an increase in the phosphorylation of protein 55k when assayed without cAMP (lane 7) as previously described (see Figure 15, lane 9). Assay of this preparation in the presence of cAMP caused an increase in the phosphorylation of Protein I, but led to a decrease in the phosphorylation of 55k (Figure 24, lane 8). Since autophosphorylation of the regulatory subunit could be detected when tissues were assayed in the absence of cAMP (lane 7), these results indicated that histone competitively inhibited the phosphorylation of proteins 57k and 55k by free catalytic subunit.

Undissociated regulatory subunit is also a substrate for the kinase, but this occurs in the absence of cAMP and therefore should be unaffected by

Figure 24. Endogenous cAMP-Dependent Protein Phosphorylation. Effect of Histone. Synaptic membrane fractions were incubated at 0°C for 30 min in the absence (lanes 1,2,5,6) or presence (lanes 3,4,7,8) of 10 μM cAMP. Soluble and particulate fractions, prepared as described in the legend to Figure 15, were assayed for endogenous protein phosphorylation in the presence of 2 μg of calf histone (Type II-A) and [^{32}P] ATP minus (lanes 1,3,5,7) or plus (lanes 2,4,6,8) 10 μM cAMP. The corresponding patterns of phosphorylation obtained in the absence of histone are depicted in Figure 15.


Supernate
Pellet
1
2
3
4
5
6
7
8
1a
1b
57
55
Histone
cAMP
– – + + – – + + ← incubation
– + – + – + – + ← assay

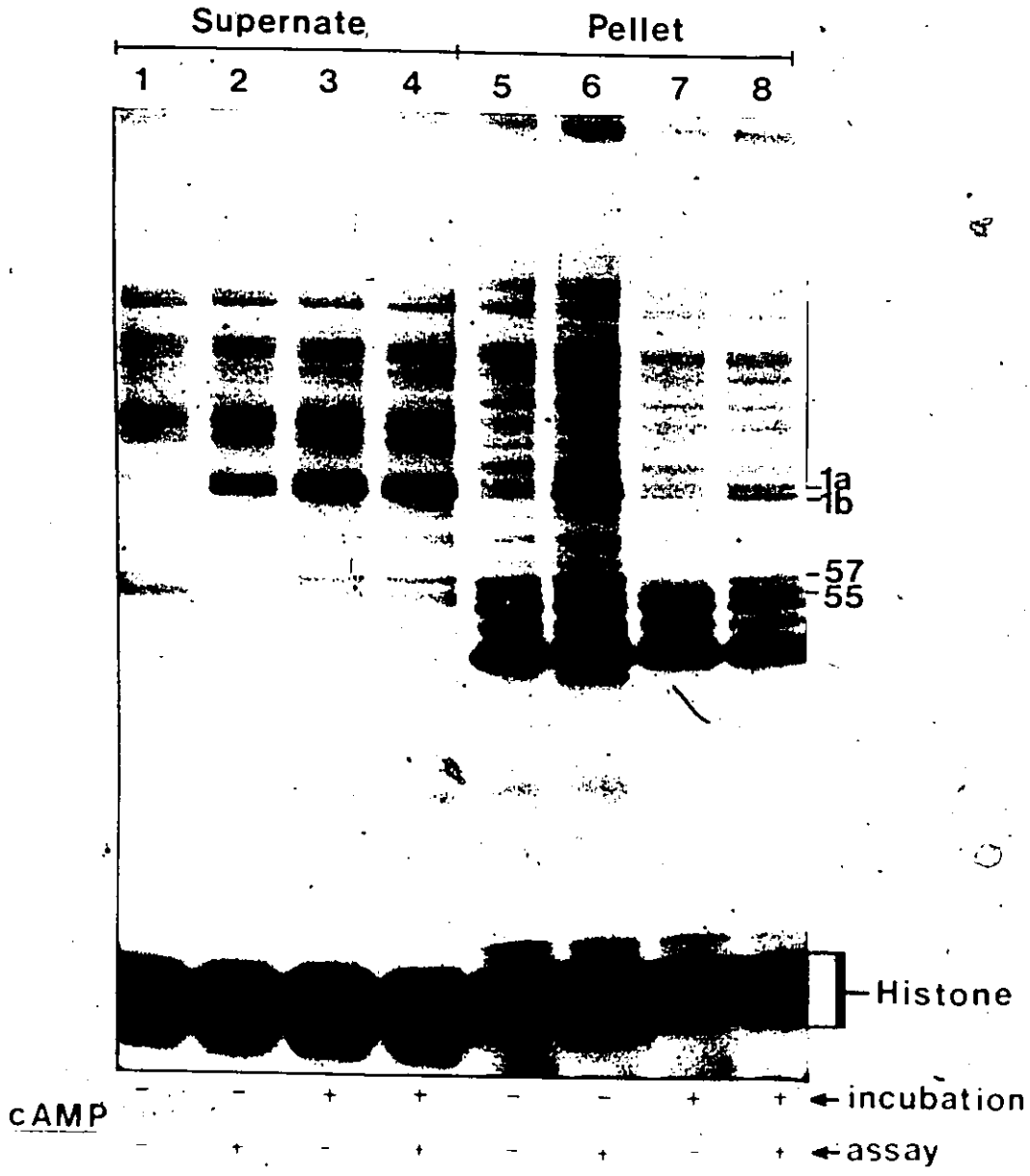
histone. This reinforces the results of the previous studies indicating that the increase in phosphorylation of 55k observed following preincubation of membranes with cAMP represents an intramolecular autophosphorylation reaction.

The phosphorylation of Protein I, however, was maintained in the presence of histone, indicating that the enzyme may exhibit a preference for this substrate since it is known to be phosphorylated by the membrane-bound Type II protein kinase (Walter et al., 1979; Lohmann et al., 1980). Extracts of synaptic membranes prepared either in the presence or absence of cAMP did not show any phosphorylation of endogenous proteins indicating that the phosphorylation of solubilized substrates was also antagonized by histone (Figure 24, lanes 1–4).

When synaptic membranes were extracted with 0.5 M NaCl and the particulate fraction assayed for cAMP-dependent protein kinase activity in the presence of histone, the phosphorylation of Protein I and 57k was increased (Figure 25, lanes 5 and 6).

Particulate fractions from membranes that were preincubated with NaCl and cAMP showed increased phosphorylation of 55k when assayed in the absence of cAMP (Figure 25, lane 7). Assay of protein kinase activity in the presence of cAMP resulted in an inhibition of 55k phosphorylation. Although a small increase in the phosphorylation of Protein I and 57k was observed in the presence of cAMP, the level of phosphorylation of these proteins was equivalent to the basal level of phosphorylation of 55k (lane 8). The reduced phosphorylation of Protein I in particulate fractions of membranes treated with NaCl and cAMP was observed previously, and was attributed to a loss of cAMP-dependent protein kinase activity in the particulate fraction with associated release of catalytic subunits into the supernate.

Figure 25. Effect of NaCl on Endogenous Protein Phosphorylation in Rat Caudate Nucleus. Effect of Histone. Synaptic membrane fractions were incubated at 0°C for 30 min with 0.5 M NaCl in the absence (lanes 1,2,5,6) or presence (lanes 3,4,7,8) of 10 μM cAMP as described in the legend to Figure 18. Soluble and particulate fractions were prepared as described in the legend to Figure 15, and assayed for endogenous protein phosphorylation in the presence of 2 μg of histone (Type II-A) and [^{32}P] ATP minus (lanes 1,3,5,7) or plus (lanes 2,4,6,8) 10 μM cAMP. Corresponding patterns of phosphorylation obtained in the absence of added histone are shown in Figure 18.


Supernate
Pellet
1
2
3
4
5
6
7
8
1a
1b
57
55
Histone
cAMP
incubation
assay

3.3.6 Synaptic Localization of Substrates for cAMP-dependent Protein Kinase in Rat Caudate Nucleus

The studies described in the preceding section investigated some of the biochemical properties of the membrane-bound Type II cAMP-dependent protein kinase and its associated endogenous substrates. Since one of the objectives of this research was to investigate the effects of dopamine on the phosphorylation of synaptic membrane proteins, it was of interest to determine whether procedures that interfere with dopaminergic transmission might alter the phosphorylation of substrates for the Type II protein kinase.

Previous investigators have shown that destruction of postsynaptic membranes with kainic acid leads to a decrease in the amount of Type II kinase and Protein I in preparations of rat caudate nucleus (Walter et al., 1979). These results indicated that the membrane-bound cAMP-dependent protein kinase from rat caudate nucleus was localized predominantly postsynaptically. This is in agreement with the known postsynaptic distribution of adenylate cyclase as discussed earlier.

The effects of presynaptic destruction of dopamine pathways on cAMP-dependent protein phosphorylation are less clear. Hullihan et al.(1979) reported an increase in dopamine-stimulated protein phosphorylation in homogenates of rat caudate nuclei following bilateral injections of 6-hydroxydopamine (6-OHDA) into the nigrostriatal pathway. These results are difficult to interpret, however, since the effects of dopamine on protein phosphorylation were non-specific, they were not mimicked by exogenous cAMP, and were only observed in the presence of high levels of cold ATP. A further limitation to these studies is that they were carried out using crude homogenates, and no attempt was made to identify the phosphorylated substrates or demonstrate that they were synaptic in

origin. Therefore, the aim of the present series of experiments was to compare the effects of kainic acid and 6-OHDA on the phosphorylation of synaptic membrane proteins from rat caudate nucleus. As an adjunct to this work, behavioural effects of these treatments were evaluated.

As discussed in the **Introduction**, unilateral denervation of the caudate nucleus with 6-OHDA is known to produce an increase in turning behaviour when rats are challenged with the dopamine agonist apomorphine, and this has been regarded as a behavioural manifestation of dopamine receptor supersensitivity. It was therefore of interest to determine whether destruction of postsynaptic membranes using kainic acid would abolish 6-OHDA-mediated turning behaviour. For these experiments, rats were randomized to one of the following four experimental groups:

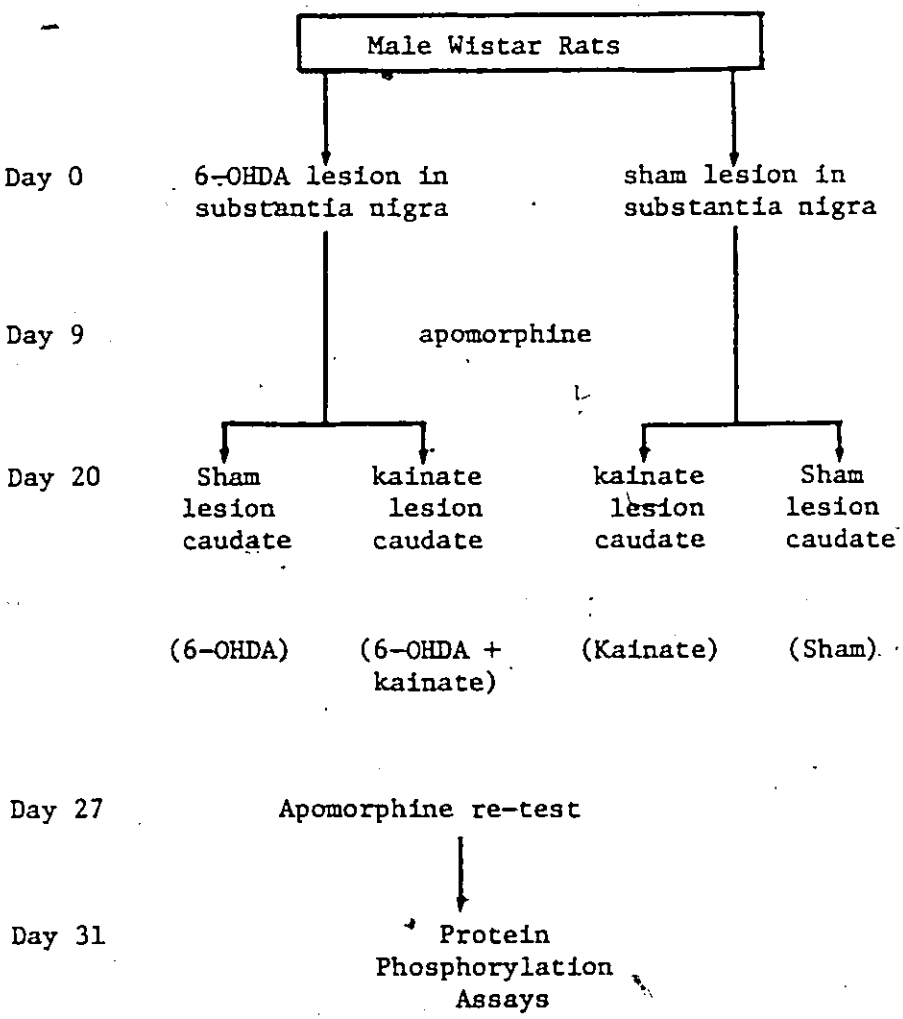
1. Sham: sham lesion in substantia nigra (SN), sham lesion in caudate nucleus (CN)
2. 6-OHDA: 6-OHDA lesion in SN, sham lesion in CN.
3. Kainate: sham lesion in SN,kainic acid lesion in CN.
4. 6-OHDA + Kainate: 6-OHDA lesion in SN, kainic acid lesion in CN.

A summary of the experimental design is shown in Scheme 2.

A. Effects of Lesions on the Phosphorylation of Synaptic Membrane Proteins

Synaptic membranes from intact and lesioned caudate nuclei were prepared and assayed for cAMP-dependent protein kinase activity as described in **Methods.** Representative autoradiographs from control and lesioned caudates from each of the four experimental groups are shown in Figure 26. Rats in Groups 1 and 2 (see above) showed small but significant decreases in the basal and cAMP-stimulated

Scheme 2


Male Wistar Rats
Day 0
6-OHDA lesion in substantia nigra
sham lesion in substantia nigra
Day 9
apomorphine
Day 20
Sham lesion caudate
kainate lesion caudate
kainate lesion caudate
Sham lesion caudate
(6-OHDA)
(6-OHDA + kainate)
(Kainate)
(Sham)
Day 27
Apomorphine re-test
Day 31
Protein Phosphorylation Assays

phosphorylation of proteins Ia and Ib, and 57k on the operated relative to control side. This effect was thought to be the result of tissue damage induced during surgery since it was observed in rats whose caudates had been stereotactically implanted but not lesioned.

Kainic acid lesions of the caudate nucleus, on the other hand, substantially decreased basal and cAMP-dependent phosphorylation activity in confirmation of previous findings. Changes in the level of phosphorylation of Protein I and 57k, the major substrates for the Type II cAMP-dependent protein kinase in synaptic membranes, were quantified using densitometry, and the numerical values obtained were then subjected to statistical analysis. A quantitative summary of changes in the phosphorylation of Protein I and 57k in each of the four experimental groups is presented in Figure 27. As shown in this figure, rats lesioned with kainic acid alone or in combination with 6-OHDA showed significantly less phosphorylation of Protein I and 57k when compared to rats given either 6-OHDA or sham lesions ($p < .001$). The pattern of phosphorylation in kainic acid-treated rats, however, was not significantly different from rats treated with both kainic acid and 6-OHDA, indicating that the major effect on protein kinase activity was due to kainic acid.

These results demonstrated, in agreement with published observations (Walter et al., 1979), that the Type II cAMP-dependent protein kinase and its major phosphorylated substrates, proteins Ia and Ib, are found almost exclusively in postsynaptic membranes. Destruction of presynaptic nerve terminals had no effect on the cAMP-dependent phosphorylation of synaptic membrane proteins, indicating that the Type II protein kinase probably does not play a role in the regulation of presynaptic membrane function.

Figure 26. Phosphorylation of Synaptic Membrane Proteins in Lesioned Rats. The right substantia nigra (SN) and right caudate nucleus (CN) were lesioned with 6-OHDA and kainic acid respectively following the experimental protocol outlined in Scheme I (see text). Synaptic membranes from (a) intact left caudate and (b) lesioned (sham or kainic acid) right caudate nuclei were prepared and assayed for protein kinase activity in the absence (-) or presence (+) of 5 μM cAMP as described in **Methods.** Major phosphorylated substrates for cAMP-dependent protein kinase are labelled as shown.

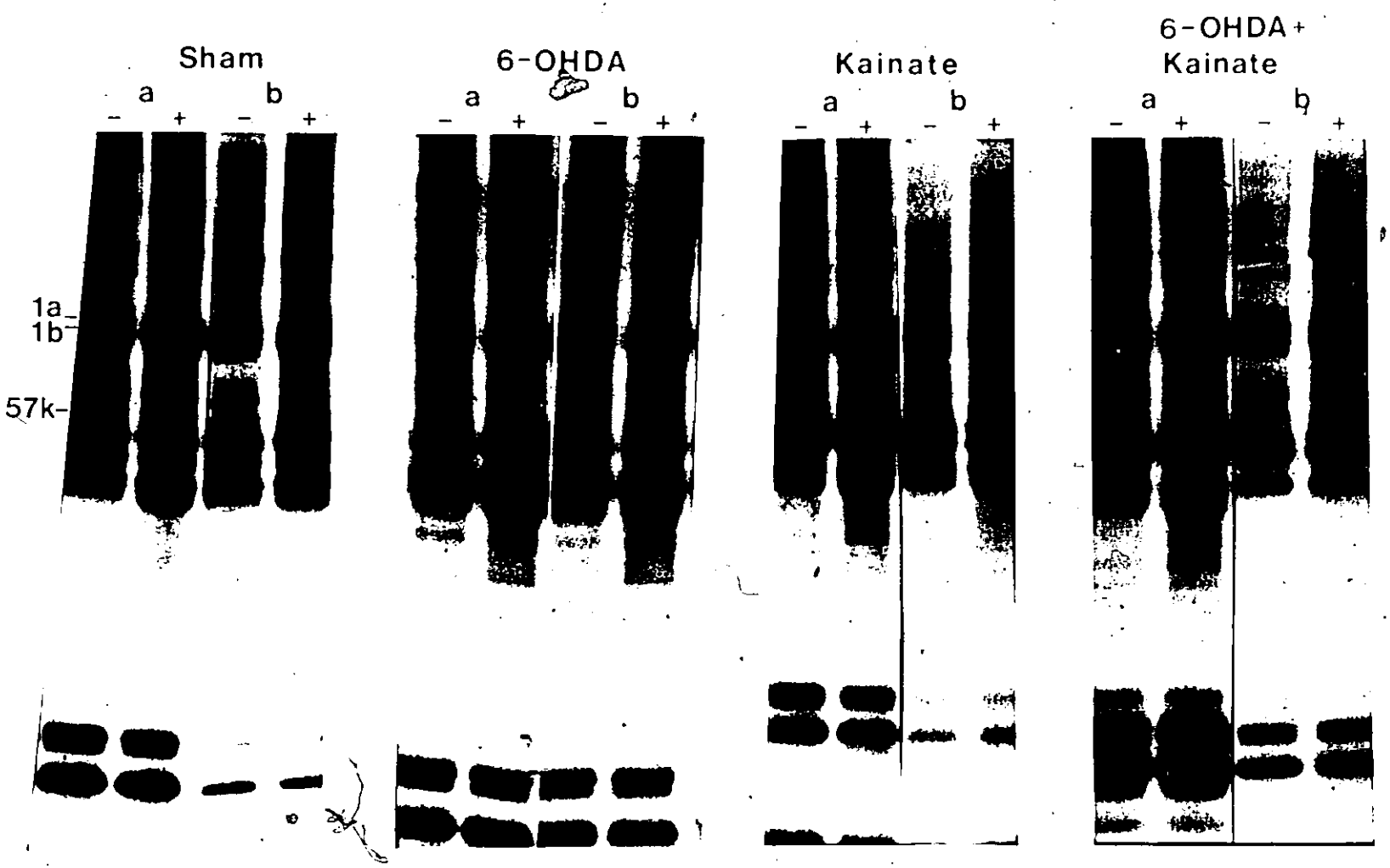

Sham
6-OHDA
Kainate
6-OHDA+
Kainate
a
b
– + – +
1a
1b
57k

Figure 27. Quantification of cAMP-Dependent Phosphorylation in Lesioned Rats.

A. Turning behaviour in response to apomorphine.
B. cAMP-dependent phosphorylation of Proteins Ia,Ib.
C. cAMP-dependent phosphorylation of protein 57k.

Turning behaviour was quantified in rats injected with apomorphine (2 mg/kg/i.p) following the protocol of Scheme I (see text). Rats were monitored for 15 min and results represent the mean ± SEM of cumulative scores for individual groups. Autoradiograms from experiments as shown in Figure 26 were scanned with a Joyce-Loebl Densitometer and ^{32}P incorporation in the absence (open bars) or presence (closed bars) of cAMP was measured as OD units as described in **Methods**. Rats were lesioned as described in the text. Cont: unlesioned left caudates (N=30); Group 1: sham (N=6); Group 2: 6-OHDA (N=7); Group 3: kainic acid (N=8); Group 4: 6-OHDA plus kainic acid (N=9). Results represent the mean ± SEM for individual groups. Differences between groups were measured using a repeated measures ANOVA followed by contrast studies.

ANOVA

	Phosphorylation		Turns	
Source	$F_{3,26}$	P	$F_{3,25}$	P
Between rats	7.2	<.005	19.8	<.001
Within rats	$F_{1,26}$		$F_{1,25}$	
Lesion	417.4	<.001	12.79	<.005
Ia,Ib (B)	371.1	<.001		
57K (C)	101.6	<.001		

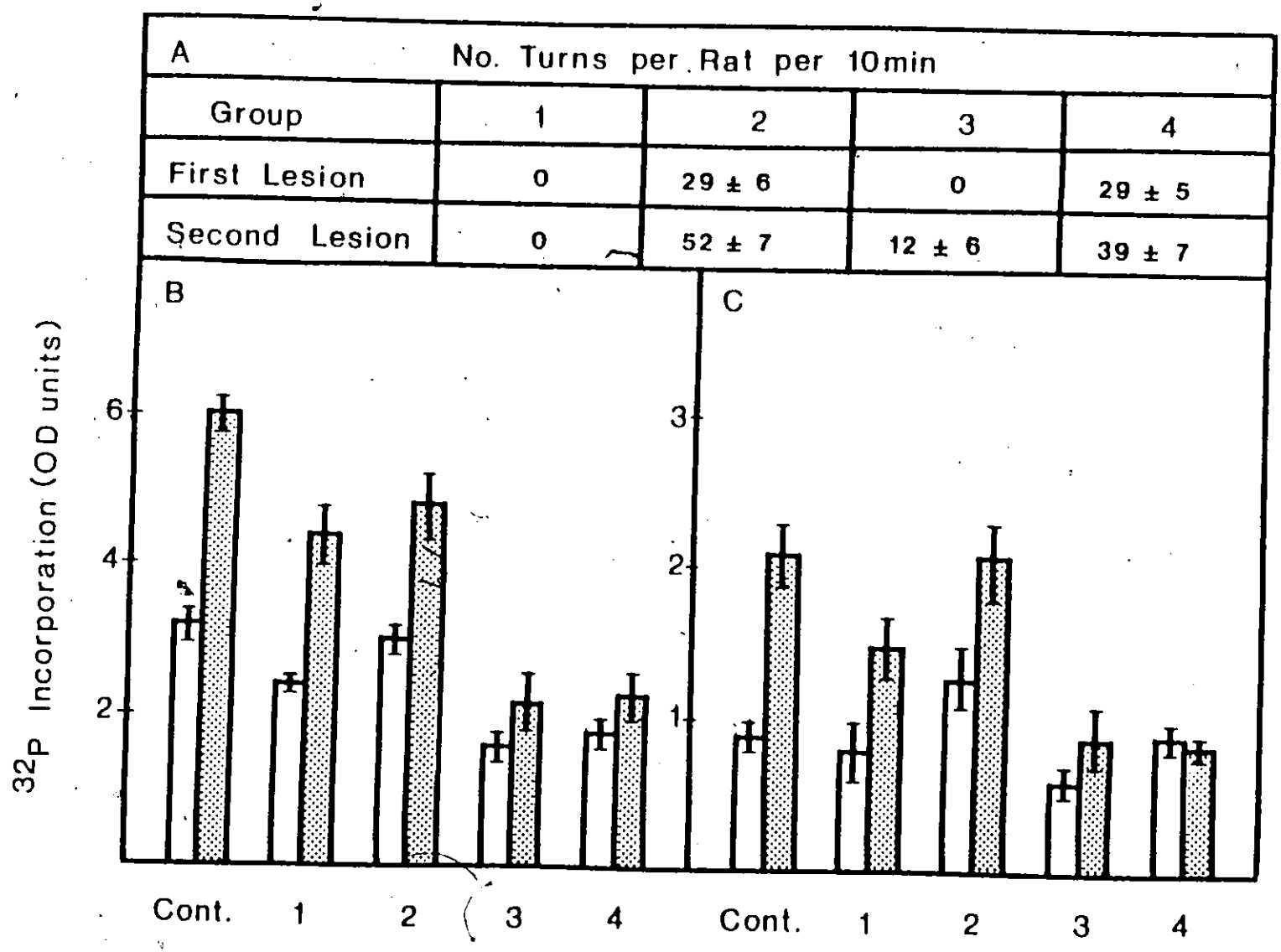
Contrasts

	Phosphorylation		Turns	
Groups	$F_{1,26}$	P	$F_{1,26}$	P
2&4 vs 1&3	2.1	ns	64.2	<.001
1&2 vs 3&4	18.9	<.001	0.02	ns
2&3 vs 1&4	0.7	ns		

A

Group	1	2	3	4
First Lesion	0	29 ± 6	0	29 ± 5
Second Lesion	0	52 ± 7	12 ± 6	39 ± 7

No. Turns per Rat per 10min



Separate measures of cAMP-dependent protein kinase activity using histone as an exogenous substrate confirmed that 6-OHDA lesions had no effect on the activity of this enzyme (data not shown). Since adenylate cyclase is also localized postsynaptically (Minneman et al., 1978; Schwartz et al., 1978; Rechardt and Harkonen, 1977; Garau et al., 1978), these results are consistent with the postsynaptic regulation of protein kinase activity by cAMP.

B. Behavioural Effects of 6-OHDA and Kainic Acid Lesions.

The increase in rotational behaviour seen in 6-OHDA-lesioned animals injected with apomorphine is thought to be due to excessive stimulation of supersensitive dopamine receptors on the denervated side, causing animals to circle in a direction contralateral to the lesion. The direction of rotation, however, appears to have more to do with the site than the side of the lesion. Thus lesions placed more medially in the substantia nigra tend to produce contralateral turning responses, while lesions placed more laterally produce ipsilateral turning responses (Mishra et al., 1980; Thal et al., 1979).

In support of this, stereotactic co-ordinates used in the present study corresponded to the lateral part of the substantia nigra, and 6-OHDA lesions produced consistent ipsilateral turning behaviour in response to apomorphine. In contrast, in previous studies in which 6-OHDA lesions were placed more medially, rats showed contralateral turning in response to apomorphine (data not shown).

The effects of 6-OHDA lesions, 6-OHDA and kainic acid lesions, and kainic acid lesions on apomorphine-induced rotational behaviour are summarized in Figure 27. Rats lesioned initially with 6-OHDA exhibited marked turning behaviour in response to apomorphine, whereas rats given sham lesions did not show this effect. Kainic acid lesions of the caudate nucleus did not modify the

turning behaviour of rats previously lesioned with 6-OHDA. Although kainic acid lesions alone increased turning behaviour in 4 out of 8 rats, this response was not statistically significant.

The results of the present study demonstrate that the increased behavioural responsiveness of 6-OHDA lesioned rats to apomorphine can not be abolished by kainic acid, a neurotoxin that causes destruction of postsynaptic membranes. As discussed previously, an increase in the activity of the dopamine-sensitive adenylate cyclase has been proposed to be one biochemical correlate of dopamine receptor supersensitivity, although the effects of denervation on this enzyme system are controversial.

Since kainic acid produces a 70-90% decrease in the activities of dopamine-sensitive adenylate cyclase and cAMP-dependent protein kinase, the behavioural supersensitivity that accompanies striatal denervation is unlikely to be mediated by these enzyme systems. Since at least 40% of dopamine receptors in the caudate are localized on striatal afferents that are unaffected by kainic acid treatment (Murrin et al., 1979; Minneman et al., 1978; Schwartz et al., 1978; Garau et al., 1978), it is conceivable that this population of receptors may mediate the apomorphine-induced increase in rotational behaviour seen following striatal denervation. This interpretation would also be consistent with the high affinity of apomorphine for presynaptic D-3 receptors (Seeman, 1980).

3.3.7 Effects of Drug-induced Dopamine Receptor Supersensitivity on cAMP-dependent Protein Kinase Activity

The previous investigations revealed that the majority of cAMP-dependent protein kinase activity in rat caudate nucleus was localized postsynaptically. In addition, it was found that denervation of striatal tissue, a procedure that is

known to produce an increase in the number of dopamine receptors and in behavioural sensitivity to apomorphine, was not accompanied by a change in cAMP-dependent protein kinase activity. This indicated that experimental denervation of striatal tissue did not lead to a change in the activity of cAMP-dependent protein kinase or in the number or amount of phosphorylated protein substrates. Since dopamine-sensitive adenylate cyclase activity was not measured in these experiments, the relationship between endogenous cAMP levels and protein kinase activity in denervated striata could not be determined.

As discussed in the **Introduction**, and Section 1 of this chapter, dopamine receptor supersensitivity can also be induced through the chronic administration of neuroleptic drugs. It was therefore of interest to determine whether long-term administration of haloperidol would lead to changes in the phosphorylation of endogenous substrates of the Type II membrane-bound protein kinase. Since dopamine did not affect the activity of cAMP-dependent protein kinases in homogenates of caudate nuclei under the experimental conditions used in the present study (Figure 11) phosphorylation of membrane proteins in haloperidol-treated rats could not be assessed directly.

However, since dopamine did elevate cAMP in homogenates and synaptic membrane preparations of rat caudate nucleus, an effect that was antagonized by haloperidol (Figure 6), it was hypothesized that long-term blockade of dopamine receptors by haloperidol might lead to a significant reduction in endogenous cAMP levels, an event that might be accompanied by compensatory changes in the activity of cAMP-dependent protein kinase. It was anticipated that this change in protein kinase activity might manifest itself as an increase in the level of phosphorylation of endogenous substrates in the presence of cAMP due to either an increase in the amount of protein kinase or its phosphorylated substrates.

Previous studies described in Section 1 demonstrated that the caudate nuclei of rats treated for 3 weeks with increasing doses of haloperidol showed an increase in the number of dopamine receptors (Figure 9), and in apomorphine-induced stereotyped behaviour (Figure 10). The activity of dopamine-sensitive adenylate cyclase was inconsistently affected by chronic haloperidol (Table III). Autoradiograms showing protein phosphorylation in synaptic membranes of vehicle-injected and haloperidol-injected rats are shown in Figure 28. No differences in basal and cAMP-stimulated phosphorylation were detected between the two groups. This result is probably not surprising, since haloperidol-treated rats also failed to show reliable increases in basal and dopamine-stimulated adenylate cyclase activity.

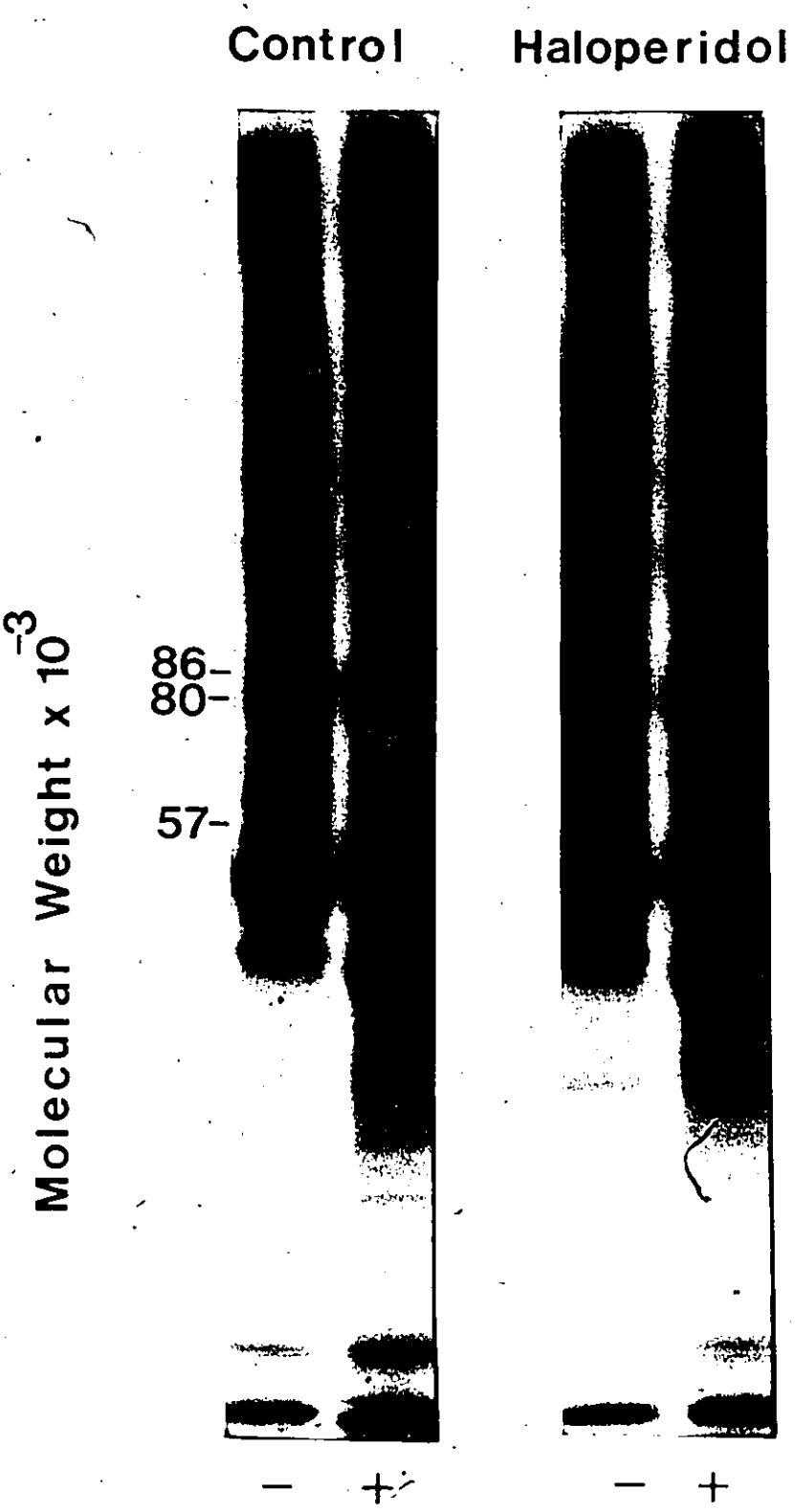
3.4 Biochemical Characterization of Ca^{++}-dependent Protein Kinase Activity in Rat Caudate Nucleus

3.4.1 Introduction

The objective of the previous studies was to characterize cAMP-dependent protein kinase activity in membrane preparations of rat caudate nucleus. This work was based on the hypothesis that dopamine stimulation of postsynaptic receptors might be accompanied by changes in the phosphorylation of specific membrane proteins secondary to dopamine activation of adenylate cyclase. Attempts to demonstrate an effect of dopamine on cAMP-dependent phosphorylation in crude homogenates or synaptic membrane preparations, however, were unsuccessful, in line with the results of previous investigators (Hullihan et al., 1977).

As discussed in the Introduction, and as demonstrated in Section 2, Ca^{++} regulates the endogenous phosphorylation of several neuronal proteins (Fig-

Figure 28. Effect of Chronic Haloperidol on cAMP-Dependent Protein Phosphorylation in Rat Caudate Nucleus. Rats were given i.p. injections of haloperidol for 20 days as described in **Methods.** Control rats received vehicle injections. Synaptic membranes were assayed for endogenous protein phosphorylation in the absence (-) or presence (+) of 5 μM cAMP as described in **Methods.** Phosphorylated substrates are identified by molecular weight.


Control
Haloperidol
Molecular Weight x 10-3
86
80
57
−
+
−
+

ure 13). Although Ca^{++} has not been directly implicated in the regulation of dopamine receptor function, there is increasing evidence that Ca^{++}-dependent protein kinases may mediate many aspects of synaptic function (Greengard, 1978). The objective of the present study was to investigate some of the properties of Ca^{++}-dependent protein kinases and their endogenous substrates in synaptic membrane preparations of rat caudate nucleus as a prelude to studies of the effects of dopamine on phosphorylation in slices.

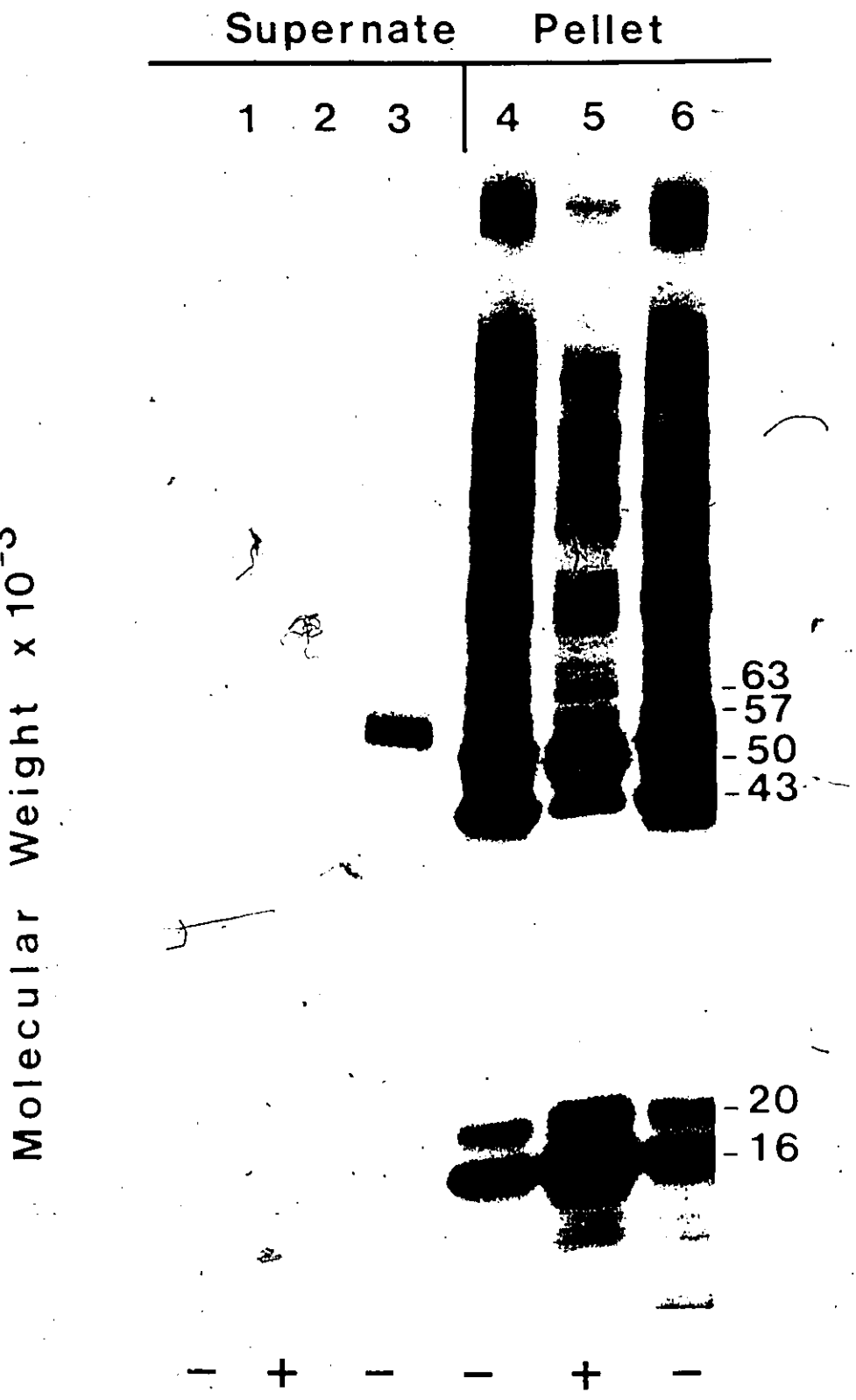
3.4.2 Solubilization of Ca^{++}-dependent Phosphorylation Activity from Synaptic Membranes of Rat Caudate Nucleus

Previous studies had demonstrated that the Type II cAMP-dependent protein kinase and its major phosphorylated substrate proteins could be solubilized from synaptic membranes using NaCl or Triton X-100 indicating that these proteins were integral membrane proteins according to the criteria set forth by Singer (1974). Since Ca^{++}-dependent protein kinases have also been implicated in the regulation of neuronal function, it was of interest to compare the solubilization properties of the Ca^{++} and cAMP-dependent enzymes.

As shown in Figure 29, particulate fractions from membranes preincubated under control conditions contained proteins of M_r=50k, 20k and 16k whose phosphorylation was increased in the presence of Ca^{++}, and a protein of M_r=43k whose phosphorylation was Ca^{++}-inhibited. These results are consistent with previous observations (Figure 13). Supernatant fractions contained a protein of M_r= 63k whose phosphorylation was Ca^{++}-stimulated. In earlier studies, this protein was shown to be highly phosphorylated in cytosol preparations (Figure 13).

When synaptic membranes were incubated either in the presence of 0.5 M NaCl, or 1% Triton X-100, proteins 50k, 43k, 20k and 16k remained bound to

Figure 29. Distribution of Ca^{++}-Dependent Protein Kinase Activity in Soluble and Particulate Fractions from Rat Caudate Nucleus. Synaptic membrane fractions were incubated at 0°C for 30 min in the absence (lanes 1,2,4,5) or presence (lanes 3,6) of 10 μM cAMP. Soluble and particulate fractions were obtained as described in the legend to Figure 15, and assayed for protein kinase activity with [^{32}P] ATP in the absence (lanes 1,3,4,6) or presence (lanes 2,5) of 1 mM Ca^{++}.


Supernate
Pellet
1
2
3
4
5
6
Molecular Weight x 10^{-3}
63
57
50
43
20
16
−
+
−
−
+
−

the particulate fraction, and continued to be phosphorylated in a Ca^{++}-dependent manner (Figure 30). Extraction of membranes in the presence of NaCl and Triton led to an increase in the amount of 20k and 16k measured in the soluble fraction but these proteins were no longer phosphorylated in a Ca^{++}-dependent manner (Figure 30).

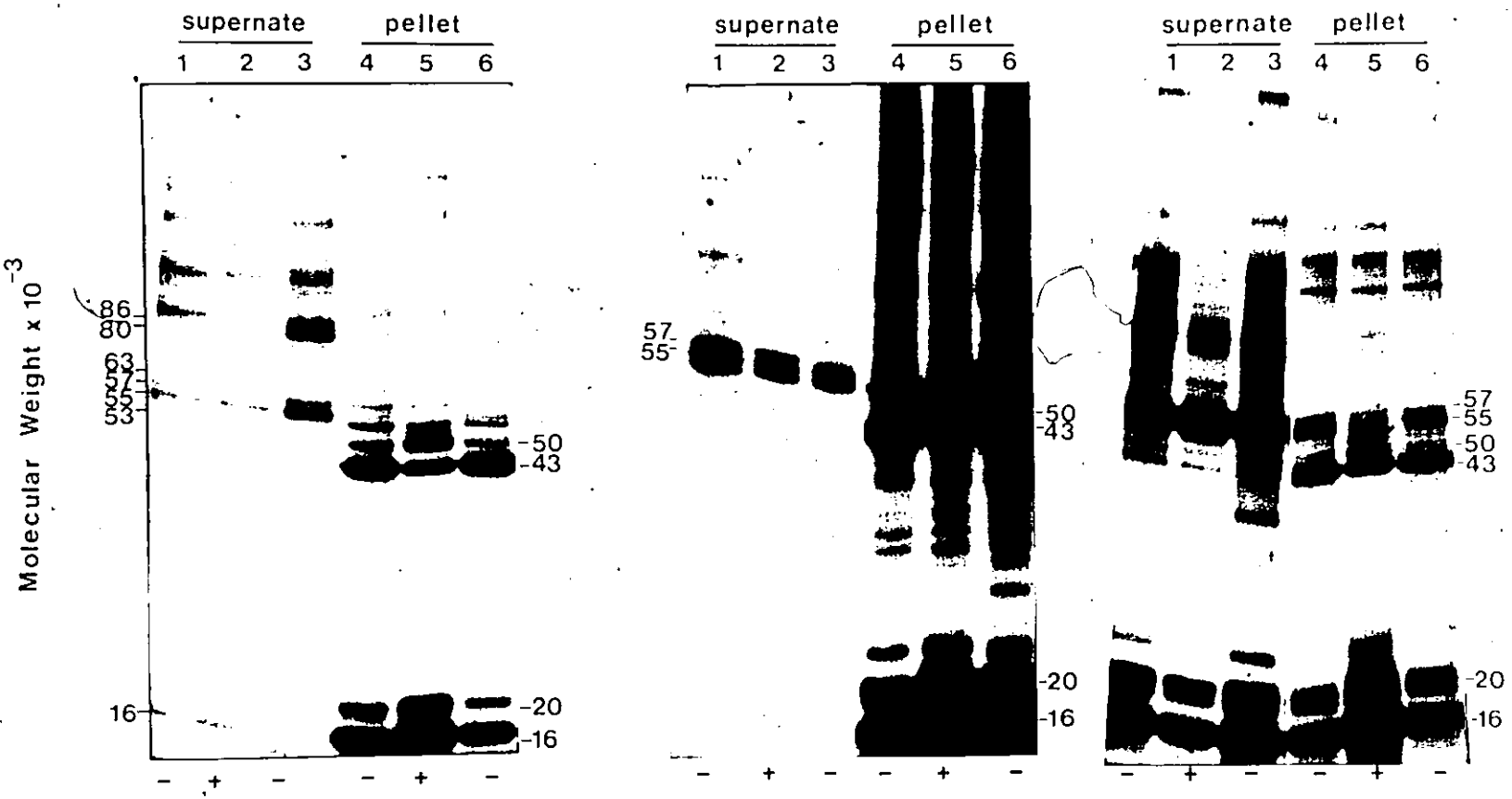
Proteins 50k, 43k and much of 20k and 16k remained associated with the particulate fraction under these conditions, and their phosphorylation continued to be regulated by Ca^{++}. These data indicated that the Ca^{++}-dependent protein kinase and its phosphorylated substrates were integral components of synaptic membranes since they could not be solubilized by the use of salt and/or detergent, procedures that were effective in releasing cAMP-dependent phosphorylation activity from synaptic membranes.

3.4.3 Inhibition of Ca^{++}-dependent Protein Kinase Activity by EGTA and Neuroleptic Drugs

Neuroleptic drugs, by virtue of their capacity to bind calmodulin (Levin and Weiss, 1979) have been shown to inhibit calmodulin-dependent protein kinase (Wrenn et al., 1981; Kuo et al., 1980). These agents also antagonize the phospholipid-sensitive protein kinase (Kuo et al., 1980; Schatzman et al., 1981; Wrenn et al, 1981), presumably as a result of their ability to interact with membrane phospholipids.

As discussed previously (Section 1, and **Introduction**), the neuroleptic trifluoperazine has a high affinity for calmodulin, and it seemed reasonable to use this drug as a probe for calmodulin-dependent protein kinase activity in synaptic membranes from rat caudate nucleus. Haloperidol, on the other hand, has a lower affinity for calmodulin (Levin and Weiss, 1979), but like all neuroleptics has

Figure 30. Effect of NaCl and Triton X-100 on Ca^{++}-Dependent Protein Kinase Activity in Rat Caudate Nucleus. Synaptic membrane Fractions were incubated for 30 min at 0°C with : 0.5 M NaCl (left panel); 1% Triton X-100 (middle panel); 0.5 M NaCl plus 1% Triton X-100 (right panel) in the absence (lanes 1,2,4,5) or presence (lanes 3,6) of 10 μM cAMP. Soluble and particulate fractions were prepared as described in the legend to Figure 16, and assayed for protein kinase activity with [^{32}P] ATP in the absence (lanes 1,3,4,6) or presence (lanes 2,5) of 1 mM Ca^{++}.


supernate
pellet
1 2 3 4 5 6
Molecular Weight x 10^{-3}
86
80
63
57
55
53
16
50
43
20
16
57
55
50
43
20
16
− + − − + −

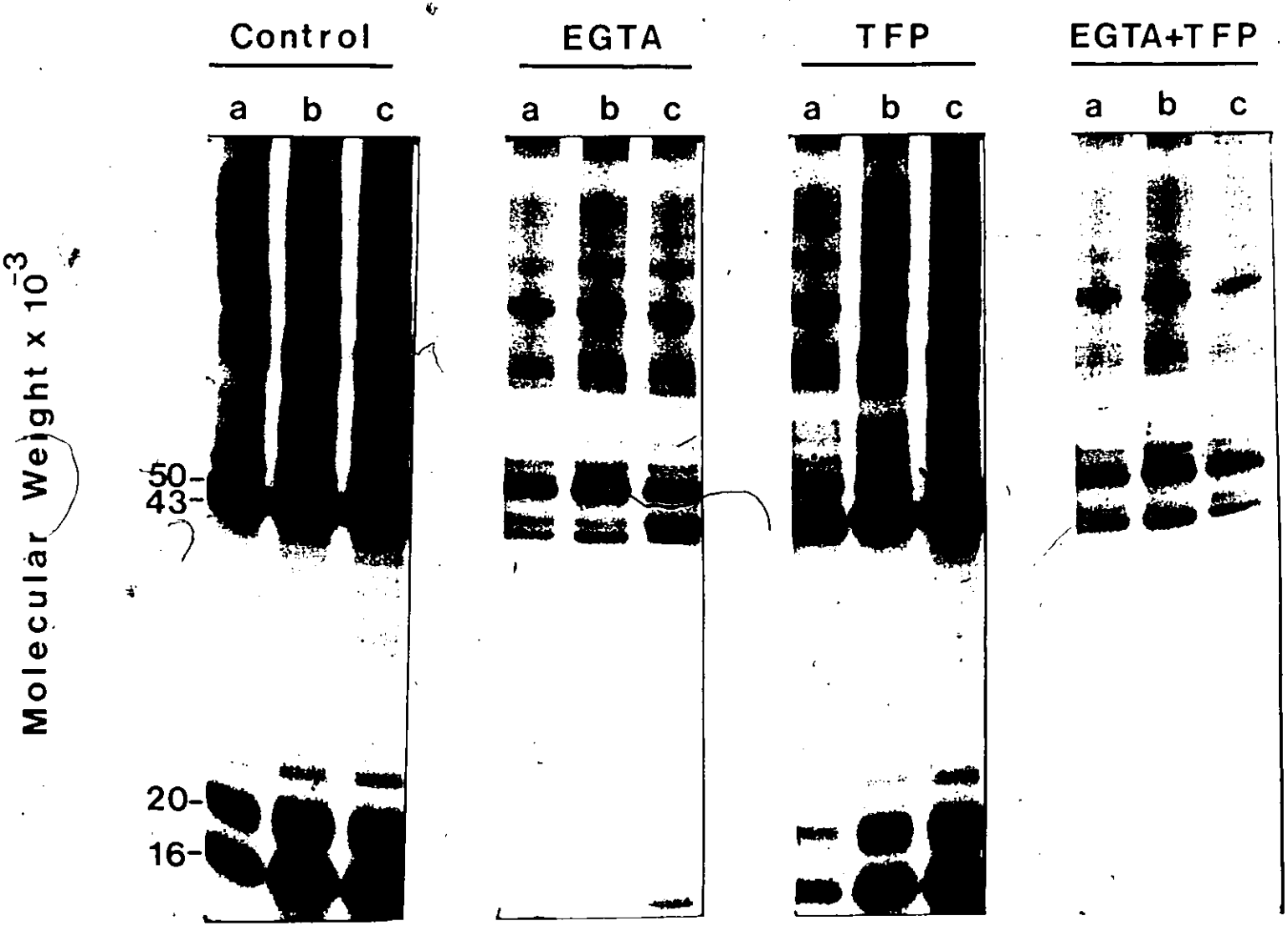
membrane fluidizing effects, particularly at higher drug concentrations (Seeman, 1972).

It therefore seemed important to examine the effects of these drugs on Ca^{++}-dependent protein kinase activity in synaptic membranes. The effects of EGTA, a Ca^{++} chelator, were evaluated for comparison. Membranes used in these studies were prepared both in the presence and absence of the protease inhibitor PMSF, since calmodulin-dependent phosphorylation, in particular, has been shown to be sensitive to proteolysis (Burke and De Lorenzo,1981).

The effects of EGTA and trifluoperazine (TFP) on Ca^{++}-dependent protein phosphorylation are shown in Figure 31. As described previously, Ca^{++} enhanced the phosphorylation of 50k, 20k and 16k, and inhibited the phosphorylation of 43k. These effects were observed at Ca^{++} concentrations of 5 μM and 1 mM, which indicates that the phosphorylation of these proteins occurs at concentrations within the physiological range. When synaptic membranes were prepared in the presence of 2 mM EGTA, the Ca^{++}-dependent phosphorylation of 50k, 20k and 16k was inhibited, this effect being more pronounced when assays were conducted in the presence of 5 μM than in the presence of 1 mM Ca^{++}, indicating that not all the added Ca^{++} was chelated by EGTA. Consistent with this, inhibition of 43k phosphorylation was prevented by EGTA when assays were conducted in the presence of 5 μM but not 1 mM Ca^{++}.

Interestingly, EGTA alone appeared to decrease the basal phosphorylation of 43k which suggested that the effects of Ca^{++} on the phosphorylation of this protein might be biphasic, with stimulation of phosphorylation occurring at low levels of Ca^{++} and inhibition at higher concentrations. In support of this, membranes prepared in the absence of EGTA contain sufficient levels of bound Ca^{++} to stimulate the basal phosphorylation of proteins 50k, 43k, 20k and 16k

Figure 31. Effects of EGTA and Trifluoperazine on Ca^{++}-Dependent Protein Kinase Activity in Rat Caudate Nucleus. Synaptic membranes were prepared as described in **Methods** either in the absence (panels 1,3) or presence (panels 2,4) of 2 mM EGTA. Assays of protein kinase activity were carried out in the absence (panels 1,2) or presence (panels 3,4) of 100 µM trifluoperazine (TFP). Additions: (a) none (b) 5 µM Ca^{++} (c) 1 mM Ca^{++}.


Control
EGTA
TFP
EGTA+TFP
a b c
a b c
a b c
a b c
Molecular Weight x 10^{-3}
50
43
20
16

(Figure 31, compare panels 1 and 2, lane a). Assay of these membranes in the presence of Ca^{++} may therefore lead to inhibition of 43k phosphorylation.

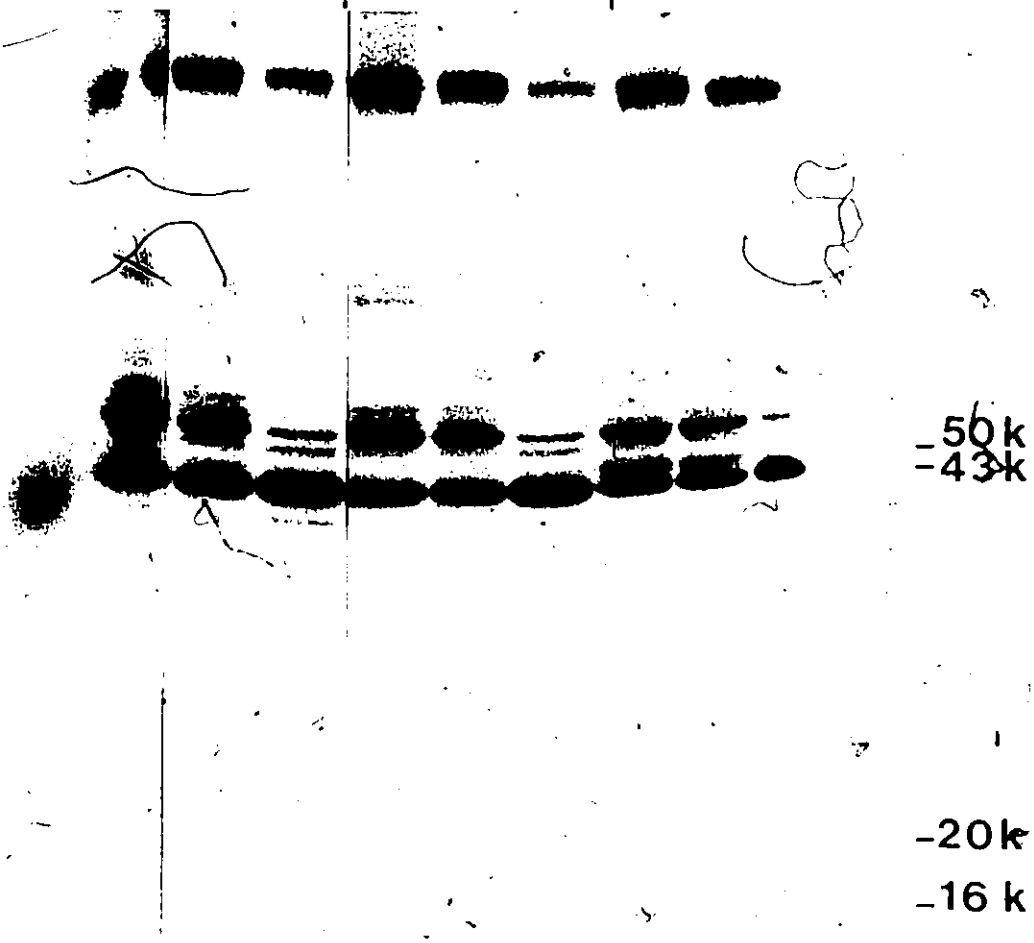
The Ca^{++}-dependent phosphorylation of a protein in this molecular weight range has been shown to be altered by a number of procedures that affect neuronal function. This protein has been identified as the alpha subunit of pyruvate dehydrogenase (Morgan and Routtenberg, 1980; Browning et al.,1981a; Sieghart, 1981; Magilen et al.,1981). Since the 43k protein described in the present study is enriched in mitochondrial subfractions, exhibits Ca^{++}-dependent phosphorylation, and migrates on SDS-PAGE with an apparent molecular weight of 43,000 daltons, it appears that this protein probably corresponds to the alpha subunit of pyruvate dehydrogenase, although biochemical analyses of the two proteins would be required to confirm that they are in fact identical.

Trifluoperazine (TFP, 100 μM) decreased the basal phosphorylation of 50k, 20k and 16k but had no effect on the phosphorylation of these proteins assayed in the presence of 5 μM or 1 mM Ca^{++} (Figure 31, panel 3, lane a). Lower concentrations of TFP (10 μM) did not affect basal or Ca^{++}-stimulated phosphorylation (data not shown).

TFP and EGTA together produced effects on phosphorylation that were equivalent to those observed with EGTA alone (Figure 31, panel 4). The phosphorylation of 43k, however, was increased to a greater extent in the presence of both agents, indicating as suggested previously, that enhanced phosphorylation of 43k occurs only with very low levels of free Ca^{++}.

The effect of haloperidol on Ca^{++}-dependent phosphorylation is shown in Figure 32. The phosphorylation of proteins 50k, 20k and 16k was partially inhibited by haloperidol concentrations of 100 μM, and completely inhibited by drug concentrations of 1.3 mM. In addition, the basal phosphorylation of 43k was increased by 1.3 mM haloperidol, an effect that was reversed by 1 mM Ca^{++}.

Figure 32. Effect of Haloperidol on Ca^{++}-Dependent Protein Phosphorylation in Rat Caudate Nucleus: Synaptic membranes were 0.3 mM PMSF and 2 mM EGTA. Assays of protein kinase activity were carried out in the absence or presence of Ca^{++} and various concentrations of haloperidol. Additions: (a) drug vehicle (b) 100 µM haloperidol (c) 1.3 mM haloperidol.


Control
5 uM Ca++
1 mM Ca++
50k
43k
20k
16 k
a b c a b c a b c

When these experiments were repeated using membranes prepared in the presence of PMSF and EGTA, the same results were obtained, indicating that the observed patterns of phosphorylation probably were not due to artifactual cleavage of membrane proteins (Figure 33). PMSF, however, did decrease basal but not Ca^{++}-stimulated phosphorylation of 50k, 43k, 20k and 16k.

A reduction in the phosphorylation of these proteins was also observed in independent studies in which the effect of preincubating tissues prior to assay of protein kinase activity was investigated. Other investigators have shown that preincubation of tissues favours dephosphorylation of endogenous substrates leading to an enhancement of cAMP-dependent phosphorylation (Weller and Rodnight, 1973; Ueda et al., 1973; Routtenberg and Ehrlich, 1975). Although preincubation slightly reduced the basal phosphorylation of substrates for the cAMP-dependent protein kinase, it had a much more marked effect on the basal phosphorylation of substrates for the Ca^{++}-dependent protein kinase (Figure 34).

Synaptic membranes that were assayed directly with ^{32}P[ATP] in the absence of added Ca^{++} showed high levels of 50k, 43k, 20k and 16k phosphorylation. Preincubation of these tissues for 1 min at 37°C prior to the addition of ^{32}P[ATP] led to a marked decrease in the phosphorylation of these proteins. Similar results have been reported by others (Dunkley and Robinson, 1981). These data indicate that synaptic membranes contain a phosphatase that rapidly dephosphorylates substrates for Ca^{++}-dependent protein kinase. This view is consistent with earlier experiments in crude homogenates demonstrating a rapid decrease in Ca^{++}-dependent phosphorylation over time (Figure 11).

Figure 33. Effect of EGTA and Trifluoperazine on Ca^{++}-Dependent Protein Phosphorylation in Rat Caudate Nucleus: Addition of PMSF. Synaptic membranes were prepared as described in **Methods** using buffer containing 0.3 mM PMSF with (panels 2,4) or without (lanes panels 1,3) 2 mM EGTA. Assays of protein kinase activity were carried out in the absence (panels 1,2) or presence (panels 3,4) of 100 µM trifluoperazine (TFP). Additions: (a) none (b) 5 µM Ca^{++} (c) 1 mM Ca^{++}. Ca^{++} levels refer to the final concentration of Ca^{++} corrected for the amount of EGTA present in the homogenizing buffer.

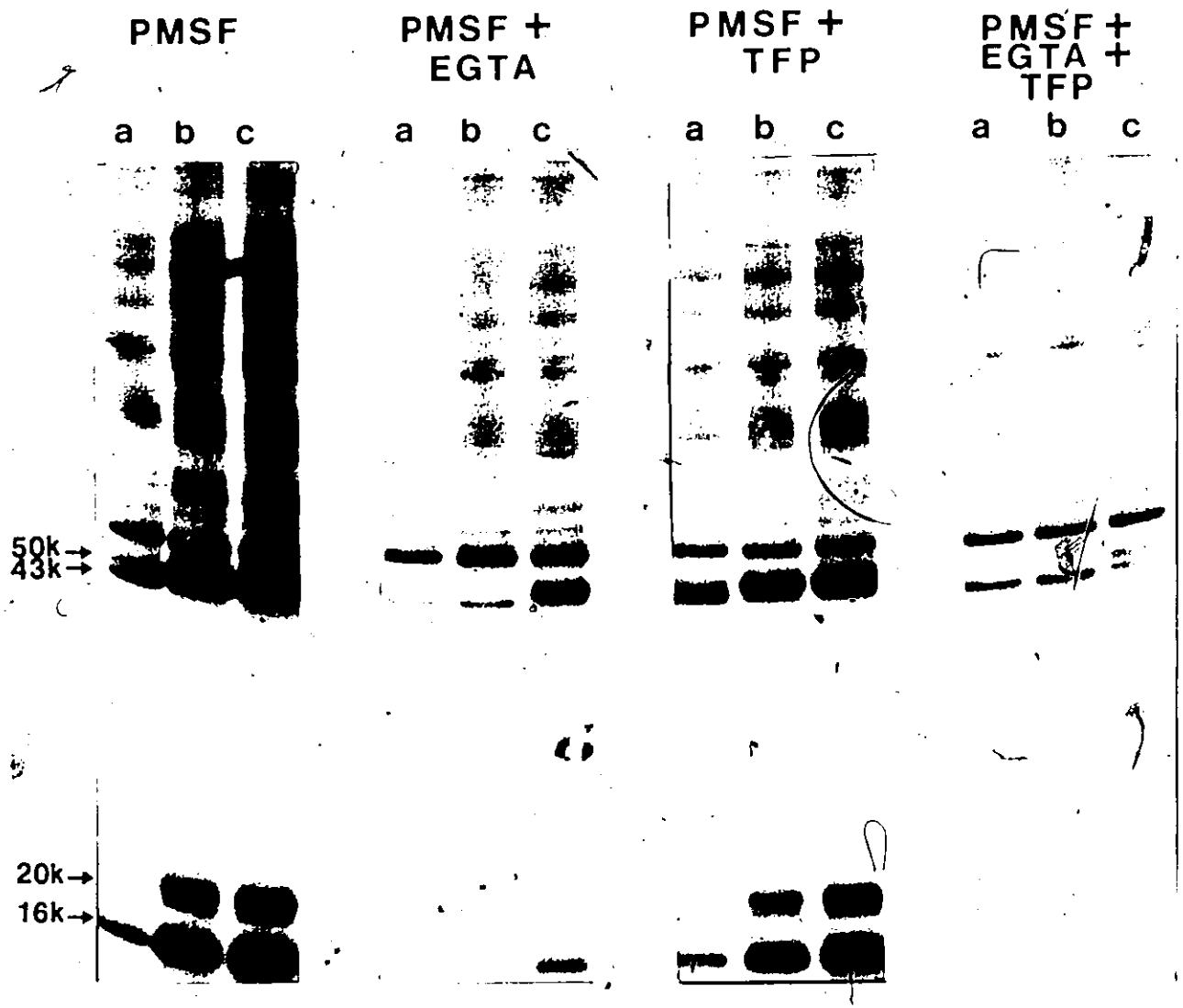
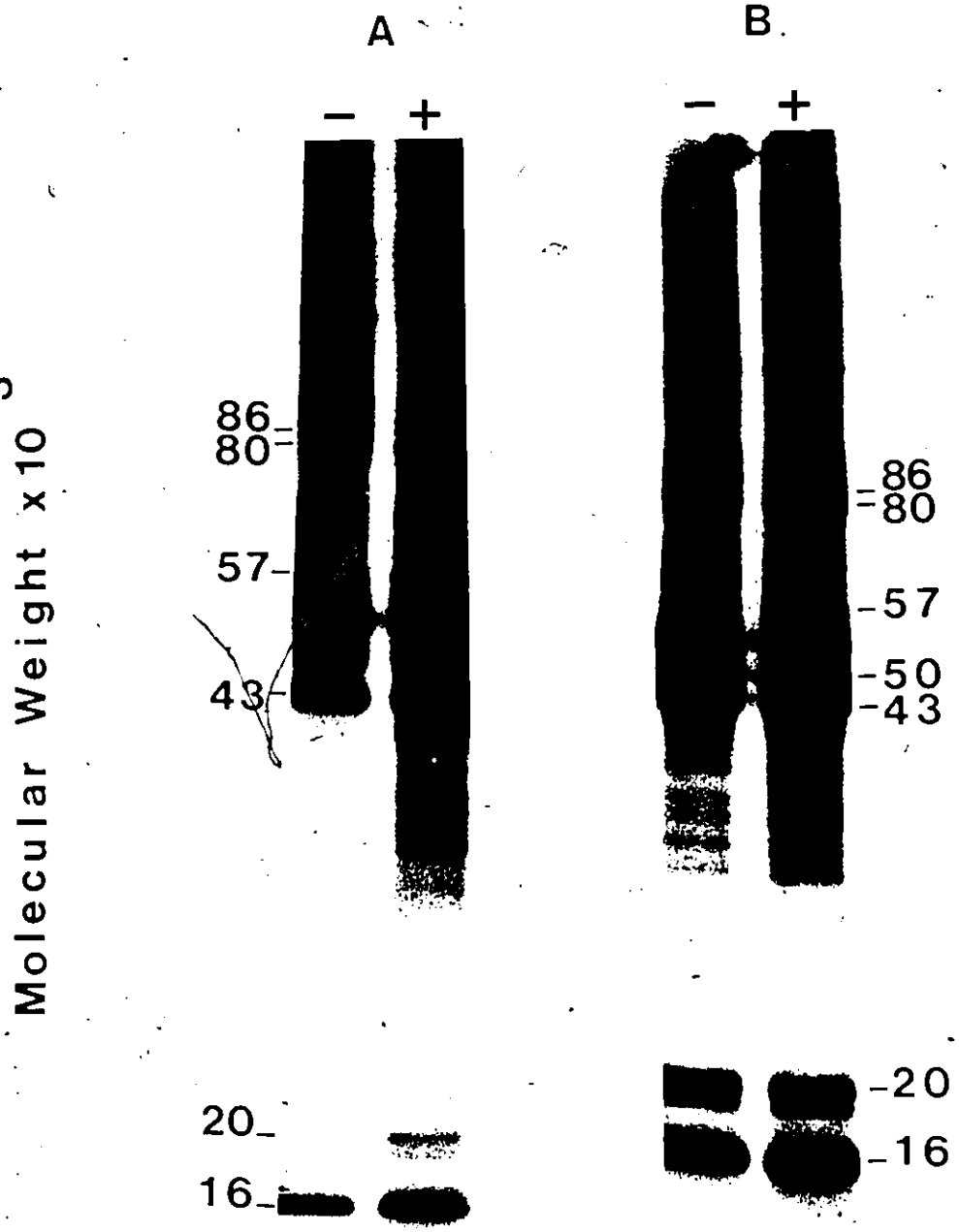

PMSF
PMSF + EGTA
PMSF + TFP
PMSF + EGTA + TFP
a b c
a b c
a b c
a b c
50k
43k
20k
16k

Figure 34. Effect of Preincubation on Protein Kinase Activity in Rat Caudate Nucleus. Synaptic membranes were either preincubated at 37°C for 1 min (A) or not preincubated (B) prior to the addition of [^{32}P] ATP in the absence (-) or presence (+) of 5 μM cAMP.


A
B
−
+
−
+
Molecular Weight x10^{-3}
86
80
57
43
20
16
86
80
57
50
43
20
16

3.4.4 Calmodulin-sensitive Protein Kinase Activity in Synaptic Membranes of Rat Caudate Nucleus

The previous studies demonstrated that the Ca^{++}-dependent phosphorylation of synaptic membrane proteins could be inhibited by EGTA, TFP, and haloperidol. Since haloperidol appeared to be a more potent inhibitor of Ca^{++}-dependent phosphorylation than TFP, it was hypothesized that these drugs might be working through different mechanisms.

One possibility was that TFP, which is known to bind calmodulin with a high affinity (Levin and Weiss, 1979), might preferentially inhibit calmodulin-dependent protein kinase, whereas haloperidol might be a better antagonist of the phospholipid-sensitive kinase. Therefore the effects of haloperidol, TFP and EGTA on calmodulin-dependent protein kinase activity were investigated. Since most of the particulate-bound calmodulin is released into the soluble fraction following the isolation of synaptic membranes from synaptosomes (Schulman and Greengard, 1978a, 1978b), assays were conducted in the presence of purified calmodulin.

As shown in Figure 35, Ca^{++} plus calmodulin stimulated the endogenous phosphorylation of two proteins with apparent molecular weights of 63k and 53k. These proteins have been described in synaptosome preparations (De Lorenzo, 1976), their phosphorylation has been shown to be regulated by calmodulin (Schulman and Greengard, 1978a, 1978b; De Lorenzo et al., 1979), and increases in their phosphorylation have been correlated with the release of neurotransmitters from synaptic vesicles (De Lorenzo and Freedman, 1978; De Lorenzo et al., 1979). More recently, these proteins have been identified as the alpha and beta subunits of neurotubulin (Burke and De Lorenzo, 1981; 1982). Ca^{++} or calmodulin alone did not increase the phosphorylation of these proteins. Ca^{++}, however,

increased the phosphorylation of 50k, 20k and 16k, and inhibited the phosphorylation of 43k as previously described.

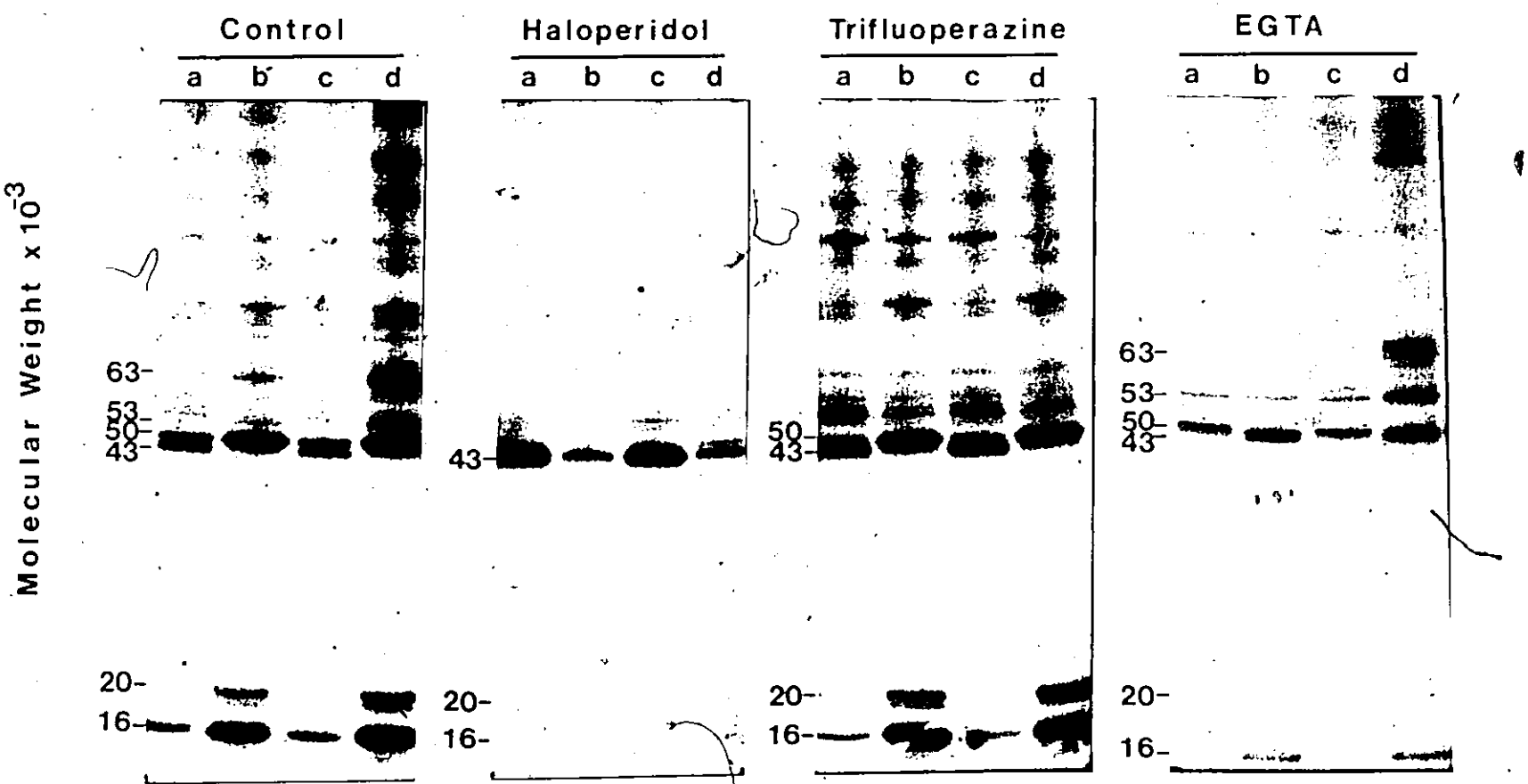
Haloperidol (100 μM) inhibited calmodulin and Ca^{++}-dependent phosphorylation but stimulated the phosphorylation of 43k as shown previously. As expected, this latter effect was much more pronounced in the absence of Ca^{++}. TFP (100 μM) on the other hand did not inhibit the phosphorylation of 50k, 20k or 16k, but completely antagonized the phosphorylation of 63k and 53k by calmodulin.

Since proteins 63k and 53k are also found in soluble fractions of rat brain (Figure 13), it was of interest to examine the effects of haloperidol and TFP on the phosphorylation of these proteins in rat caudate nucleus cytosol. The phosphorylation of 63k and 53k was inhibited by 100 μM TFP and by 1.3 mM HAL (data not shown). Lower concentrations of TFP (10 μM) and HAL (100 μM) did not inhibit the phosphorylation of these proteins. These results are consistent with the observation that haloperidol binds calmodulin with a lower affinity than trifluoperazine (Levin and Weiss, 1979), and agree with earlier observations of inhibition of Ca^{++}-dependent phosphorylation in membrane fractions at these drug doses (Figures 31 and 32).

The inhibition of calmodulin-dependent phosphorylation in soluble and particulate fractions by neuroleptics represents a pharmacological effect of these agents that is independent of their capacity to bind dopamine receptors. In support of this, it has been demonstrated that binding of neuroleptics to calmodulin is a structurally nonspecific interaction that reflects the hydrophobicity of these agents, not their specific antipsychotic properties (Roufogalis, 1981).

EGTA (2 mM) completely inhibited the Ca^{++}-dependent phosphorylation of 50k, and decreased the phosphorylation of 20k and 16k (Figure 35). The

Figure 35. Ca^{++}-Calmodulin-Dependent Protein Kinase Activity in Rat Caudate Nucleus: Effect of EGTA and Neuroleptic Drugs. Synaptic membranes were prepared as described in **Methods** using sucrose–phosphate buffer alone (panels 1,2,3) or in the presence of 2 mM EGTA (panel 4), then assayed for endogenous protein kinase activity as described in **Methods.** Additions: (a) none (b) 5 μM Ca^{++} (c) 2 μg calmodulin (d) 5 μM Ca^{++} plus 2 μg calmodulin. Haloperidol (panel 2) and trifluoperazine (panel 3) were added to assay mixtures to a final concentration of 100 μM.


Control
Haloperidol
Trifluoperazine
EGTA
a b c d
Molecular Weight x10^{-3}
63-
53-
50-
43-
20-
16-

phosphorylation of 43k was inhibited in the absence of Ca^{++}, and slightly increased in the presence of Ca^{++}. These effects of EGTA are in agreement with the results reported earlier. The activity of calmodulin-dependent protein kinase, on the other hand, was not inhibited at this concentration of EGTA, indicating that the phosphorylation of 53k and 63k requires much lower levels of Ca^{++} than the phosphorylation of 50k, 20k and 16k. This is compatible with the hypothesized role of proteins 53k and 63k in mediating the Ca^{++}-dependent release of neurotransmitters. The differential regulation of 43k phosphorylation by neuroleptics and EGTA suggests that this protein may not be a substrate for the same enzyme that phosphorylates 50k, 20k and 16k. Since EGTA inhibits the basal phosphorylation of 43k but enhances the Ca^{++} stimulation of this protein, it appears that 43k is probably phosphorylated in a Ca^{++}-dependent manner in vivo.

3.5 Dopamine-Sensitive Adenylate Cyclase Activity in Slices of Rat Caudate Nucleus

3.5.1 Introduction

As shown previously, rat caudate nucleus contains a number of proteins whose phosphorylation is cAMP-regulated. Dopamine, however, did not increase cAMP-dependent protein phosphorylation in homogenates of caudate nucleus (Figures 11, 12), although it did stimulate adenylate cyclase activity. Since these negative results with dopamine may have been due in part to disruption and dilution of the tissue during the homogenization procedure, the effects of dopamine on cAMP accumulation and protein phosphorylation were re-investigated using striatal slices as it was felt that this preparation might provide a more appropriate model of dopamine function in vivo.

3.5.2 Effect of Incubation Conditions on Dopamine-sensitive Adenylate Cyclase Activity in Rat Striatal Slices

Initial studies of dopamine stimulation of adenylate cyclase in rat striatal slices were conducted according to the procedures of Wilkening and Makman (1975) and Forn et al. (1974). Since adenosine has been shown to stimulate adenylate cyclase in striatal slices through a mechanism that does not involve dopamine receptors (Wilkening and Makman, 1975), the effects of this agent on adenylate cyclase activity were simultaneously investigated to provide an independent measure of cAMP accumulation.

As shown in Figure 36, 2-chloro-adenosine caused a 2.5 fold increase in cAMP levels in striatal slices ($p<.001$), consistent with the results of Wilkening and Makman (1975). Equivalent concentrations of dopamine were without effect. It appeared from these studies that the incubation conditions were compatible with adenosine but not dopamine activation of adenylate cyclase.

Subsequent investigations revealed that the following modifications in the assay procedure were required to detect reliable dopamine stimulation of adenylate cyclase in striatal slices : (1) the removal of ascorbic acid from the incubation medium, since this agent apparently inhibits the binding of dopamine to its receptor (Kayaalp et al., 1981; Heikkila et al., 1981). Instead, dopamine was dissolved in KRB 30 s before it was added to the slices. (2) The inclusion of a phosphodiesterase inhibitor to prevent degradation of accumulated cAMP. (3) Elevation of the dopamine concentration to 1 mM (Table VI). It was of interest in this regard that 100 µM norepinephrine but not 100 µM dopamine caused a 2.5 fold increase in cAMP in rat striatal slices (Table VII). These results are consistent with previous reports of stimulation of adenylate cyclase by norepinephrine in slices of rat caudate nucleus (Forn et al., 1974). For a detailed description of the preparation and incubation of striatal slices, refer to **Methods.**

Figure 36. Effect of 2-Chloro-Adenosine and Dopamine on cAMP Accumulation in Striatal Slices. Slices, prepared as described in **Methods** were preincubated for 90 min in Krebs Ringer Buffer (KRB), and then incubated for 5 min in 0.25 ml of KRB containing 0.6 mM ascorbic acid (Forn et al., 1974), and various test substances. Additions: KRB (control); 100 µM 2-chloro-adenosine (2-Cl-ADO); 100 µM dopamine (DA). Intracellular cAMP levels were determined as described in **Methods.** Protein concentrations were measured using the method of Lowry et al., (1951). Results represent the mean ± SEM of 15 slices assayed in duplicate. Statistical significance was assessed using a one-way ANOVA followed by Dunnett's t-test (Dunnett 1955) to contrast the dopamine and adenosine effects.

ANOVA

Main effects $F_{2,26} = 25.12$ $p<.001$

Contrast Studies

Control vs Ado $t_{26} = 6.64$ $p<.001$

Control vs DA $t_{26} = 1.44$ ns

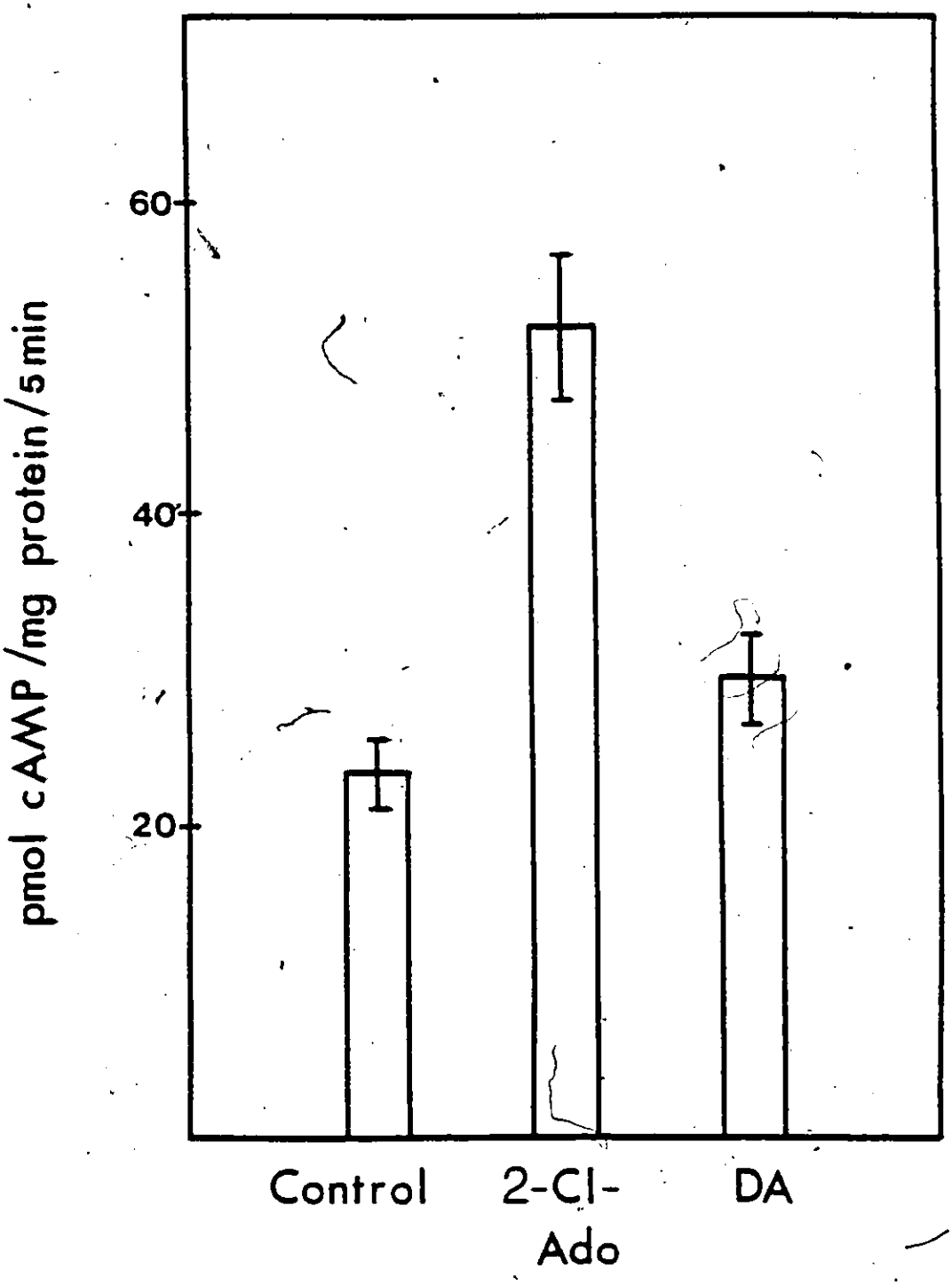

pmol cAMP /mg protein/5 min
60
40
20
Control
2-Cl-Ado
DA

TABLE VI

Effect of Dopamine on cAMP Accumulation in Rat Striatal Slices.

Experiment	pmol cAMP per mg protein per 5 min (−)	(+)
1	21.6	47.0
2	20.2	30.4
3	22.0	45.8
4	18.2	52.2
Mean	20.5	43.8
SEM	±0.9	±4.7

Adenylate cyclase activity in striatal slices was assayed as described in Methods in the absence (−) or presence (+) of 1 mM dopamine plus 3 mM IBMX.

TABLE VII

Increases in cAMP in Rat Striatal Slices: Effects of norepinephrine and dopamine.

pmol cAMP per mg protein per 5 min

Experiment	Control	100uM DA	100 um NE
1	24.2	39.0	54.0
2	21.0	32.4	49.0
3	24.0	28.6	58.4
mean	23.0	33.4	53.8
SEM	±0.1	±1.0	±0.8

Adenylate cyclase activity in striatal slices was assayed as described in Methods in the presence of 3 mM IBMX plus either 100 uM dopamine (DA) or 100 uM norepinephrine (NE).

3.5.3 Effect of IBMX Concentration on Dopamine-sensitive Adenylate Cyclase Activity in Rat Striatal Slices

As mentioned above, preliminary studies had indicated that dopamine stimulation of adenylate cyclase depended on the presence of a phosphodiesterase inhibitor in the incubation medium. Initial experiments had been conducted in the presence of 3 mM IBMX. Since methylxanthines may interfere with other cellular processes independently of their effects on phosphodiesterase, a dose response curve for the effects of IBMX on dopamine stimulation of adenylate cyclase was constructed. As shown in Figure 37, IBMX alone significantly increased cAMP levels in rat striatal slices ($p<.001$). This effect was concentration-dependent, the greatest effect being observed at 3 mM IBMX, the highest level of IBMX tested. IBMX could not be tested at concentrations higher than 3 mM because of solubility limitations.

The magnitude of dopamine stimulation was also dependent on IBMX. The largest increases in dopamine-stimulated adenylate cyclase activity were observed in the presence of 0.75 mM and 3 mM IBMX, these concentrations producing a 75% and 100% increase in cAMP accumulation respectively.

3.5.4 Time Course and Dopamine Concentration Curve

As shown in Figure 38, the effects of dopamine on cAMP accumulation were concentration and time-dependent. Dopamine concentrations of 10 µM and 100 µM did not increase cAMP above basal levels at any of the incubation times tested. This presumably reflected poor tissue permeability to dopamine, since these concentrations of dopamine stimulated adenylate cyclase in broken cell preparations.

Dopamine concentrations of 1 mM, 2 mM, and 4 mM produced 2-2.5 fold increases in cAMP with half-maximal stimulation occurring at about 2.5 min

Figure 37. Effect of IBMX on Dopamine-Stimulated cAMP Accumulation in Rat Striatal Slices. Slices, prepared as described in **Methods**, were preincubated for 90 min in KRB and then incubated for 5 min in 0.25 ml of KRB containing various concentrations of IBMX in the absence (open triangles) or presence (closed triangles) of 1 mM dopamine. IBMX was routinely dissolved in warm KRB (37°C) and dopamine was added to this solution immediately prior to assay. cAMP was assayed as described in **Methods**, and results are expressed as % increase relative to controls representing the mean ± SEM of 9-14 individual determinations assayed in duplicate. Statistical significance was assessed using a 2-way ANOVA followed by contrast studies.

ANOVA

Source	$F_{4,12}$	P value
IBMX	40.42	<.001
DA	41.19	<.01
DA x IBMX	3.84	=.05

Contrasts

Source	$F_{1,12}$	P value
Control vs IBMX	67.8	<.001
75 and 300 μM IBMX vs 750 and 3000 uM	86.43	<.001
750 vs 3000 μM	0.03	ns
75 vs 300 μM	0.32	ns

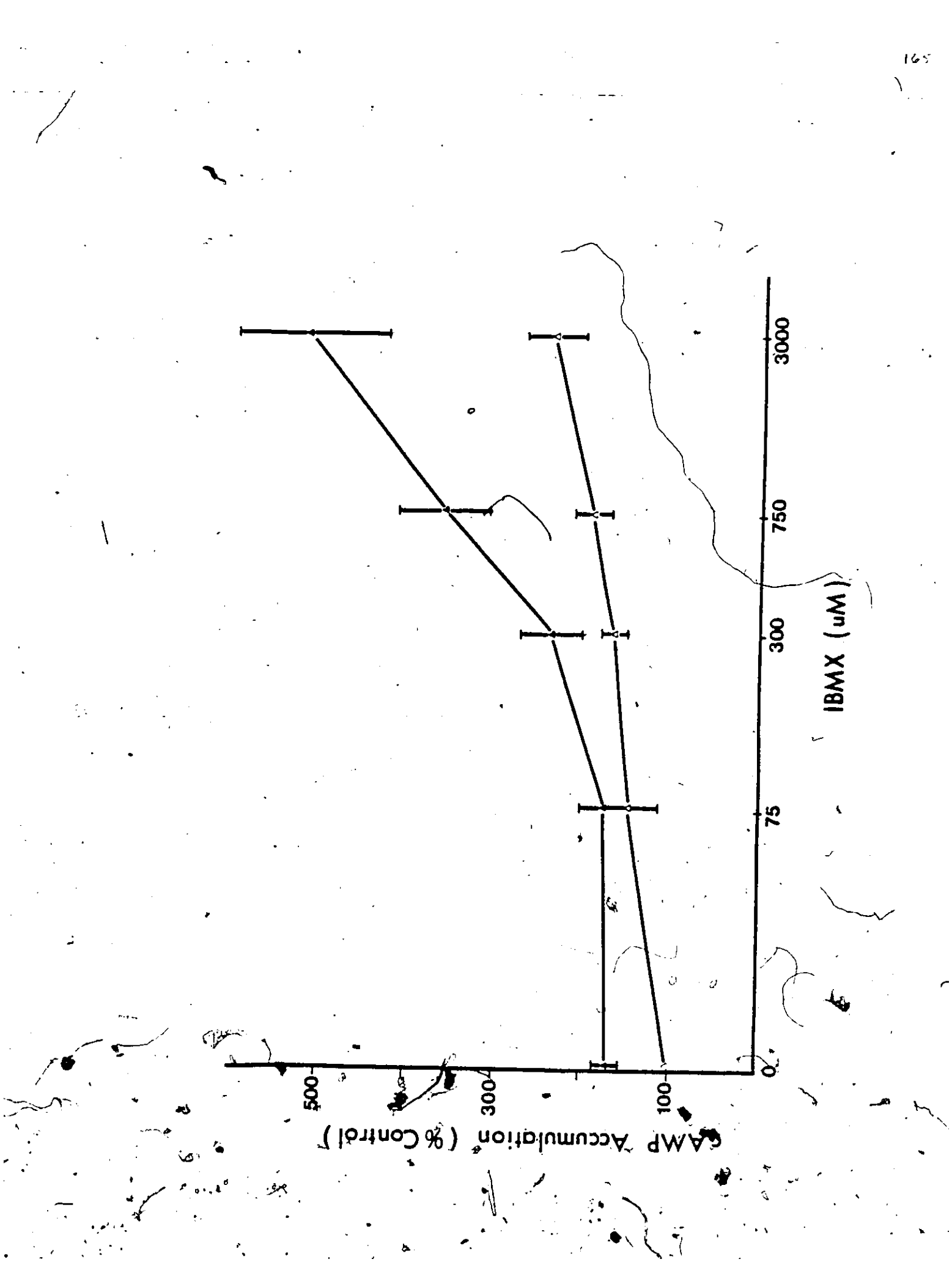
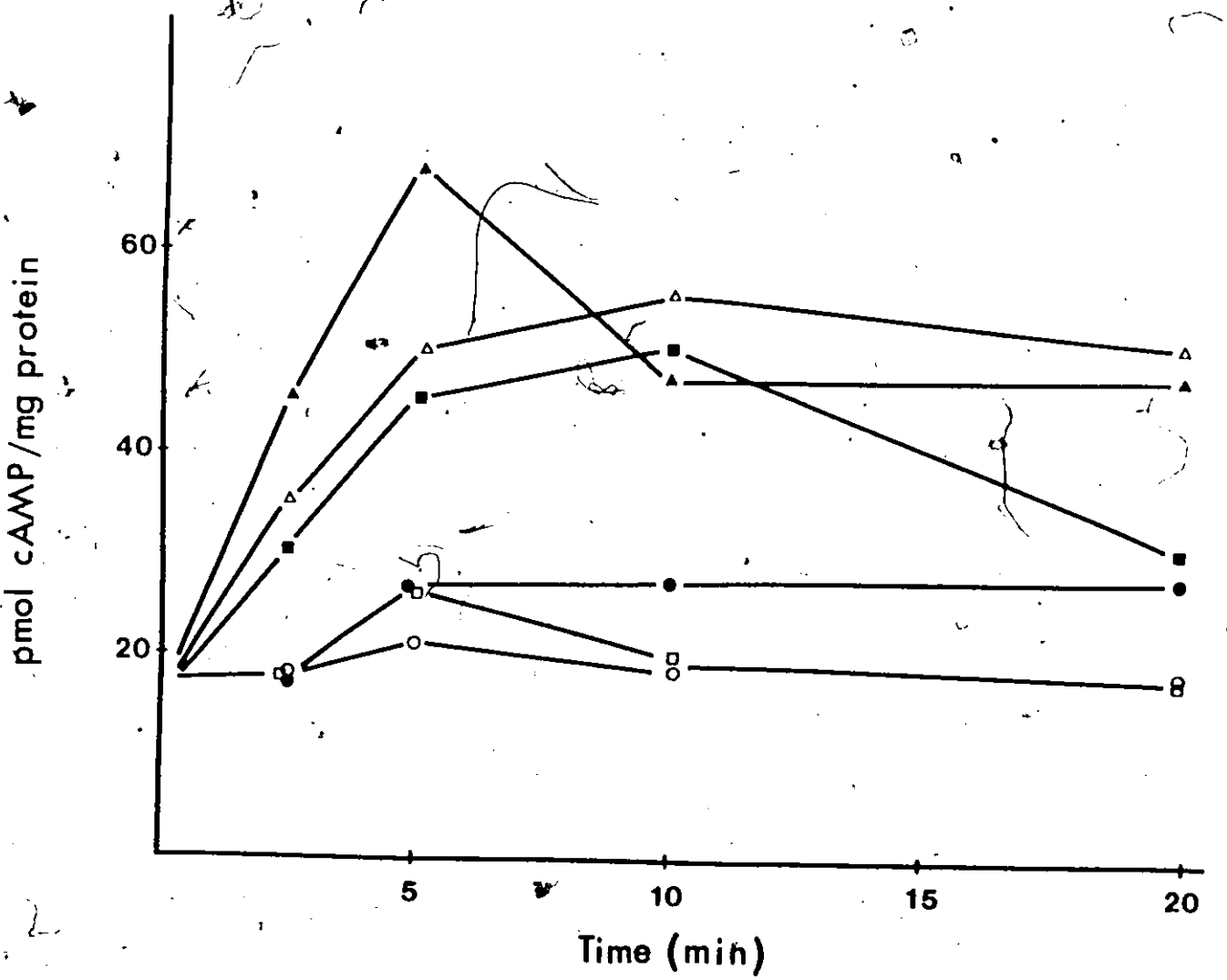

cAMP Accumulation (% Control)
500
300
100
0
75
300
750
3000
IBMX (uM)

Figure 38. Increases in cAMP in Striatal Slices: Effect of Dopamine Concentration and Length of Incubation. Striatal slices, prepared as described in **Methods**, were preincubated for 90 min in KRB, and then incubated in 0.25 ml of KRB containing 0.75 mM IBMX and different concentrations of dopamine. Incubations were carried out for various time periods, and cAMP accumulation was measured as described in **Methods**. Results represent the mean of 4-7 individual slices. Standard error bars have been omitted for clarity. Statistical significance was assessed using a repeated measures ANOVA with 2 trial factors.

o 0 DA □ 10 μM DA ● 100 μM DA
■ 1 mM DA Δ 2 mM DA ▲ 4 mM DA

ANOVA

Source	F value	P value	
time	27.6	< .001	(3,15 df)
DA	3.39	< .01	(5,15 df)
time x DA	1.72	ns	(7,15 df)


pmol cAMP/mg protein
60
40
20
5
10
15
20
Time (min)

of incubation. The effect of dopamine at these concentrations was statistically significant ($p<.01$). Stimulation of adenylate cyclase by dopamine was maximal by 10 min and thereafter began to decline, the most rapid decrease occurring with dopamine concentrations of 1 mM.

3.5.5 Inhibition of Dopamine-stimulated cAMP Accumulation by Neuroleptics

To determine the specificity of dopamine stimulation of adenylate cyclase in striatal slices, the effects of dopamine receptor antagonists on this system were investigated. Previous studies demonstrated that the neuroleptics trifluoperazine (TFP) and haloperidol inhibited dopamine-sensitive adenylate cyclase in homogenates and membrane fractions of rat caudate nucleus (Figure 6).

As shown in Figure 39, haloperidol significantly inhibited dopamine-stimulated cAMP accumulation ($p<.001$), but had no effect on basal cAMP levels. This effect was dose-dependent, with maximal inhibition occurring at haloperidol concentrations of 1.3 mM.

TFP, on the other hand, did not alter cAMP levels either in the absence or presence of dopamine at any of the drug concentrations tested (Figure 39). This was in contrast to results obtained in homogenates where TFP inhibited basal and dopamine-stimulated enzyme activity.

3.5.6 Effects of K^+ on Adenylate Cyclase Activity in Rat Striatal Slices

Depolarization of nerve membranes by electrical stimulation or elevated K^+ has been shown to increase cAMP levels in slice preparations from guinea pig cerebral cortex (Shimuzu and Daly, 1972; Shimuzu et al., 1970a, 1970b), and from rat caudate nucleus (Mishra et al., 1980). It was hypothesized that K^+ might elevate cAMP levels in striatal slices by enhancing the release of dopamine from endogenous catecholamine stores.

Figure 39. Inhibition of Dopamine-Stimulated cAMP Accumulation by Neuroleptics. Slices, prepared as described in **Methods**, were incubated for 10 min in KRB containing 0.75 mM IBMX in the absence (open bars) or presence (closed bars) of 1 mM dopamine, and increasing concentrations of haloperidol or trifluoperazine. cAMP, measured as described in **Methods**, is expressed as % increase relative to controls, and represents the mean ±SEM of 4-12 individual slices. Statistical significance was assessed using a repeated measures ANOVA with 2 trial factors.

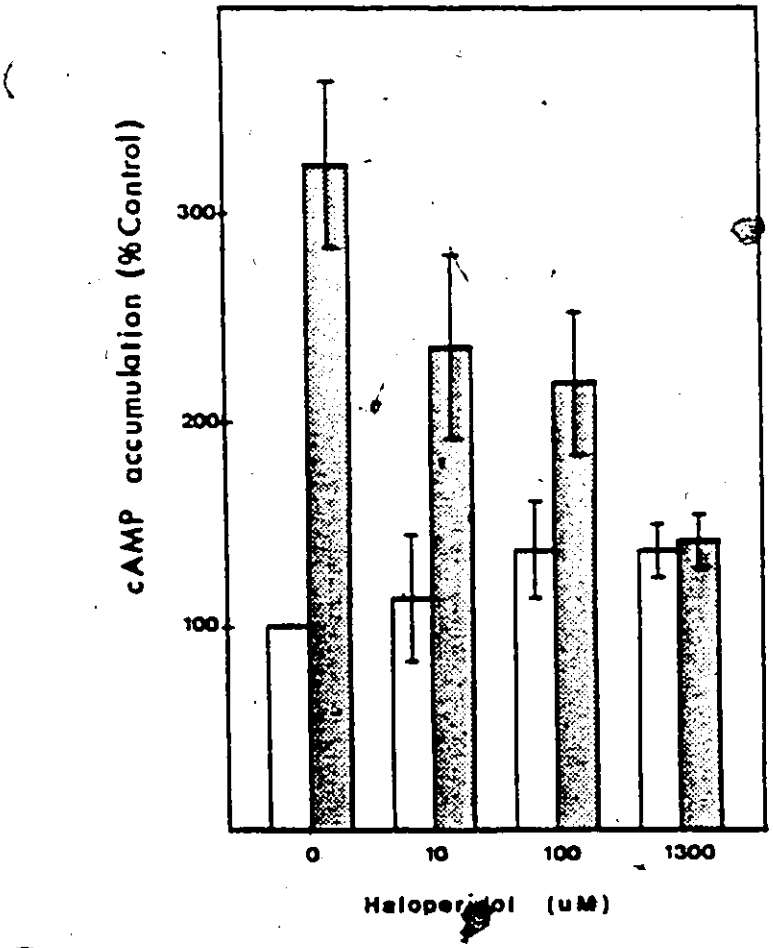
ANOVA

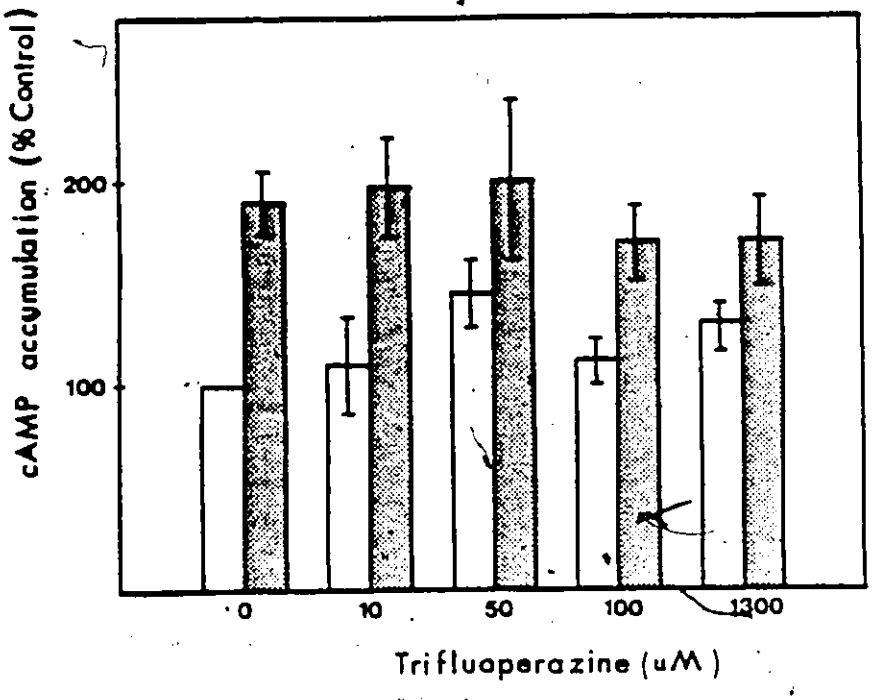
HAL

Source	F	P value
Drug	13.59	<.001 (4,10 df)
DA	206.5	<.001 (1,5 df)
Drug x DA	14.06	<.001 (4,10 df)

TFP

Source	F	P value
Drug	0.28	ns (4,14 df)
DA	49.51	<.001 (1,9 df)
Drug x DA	1.51	ns (4,14 df)


cAMP accumulation (%Control)
300
200
100
0
10
100
1300
Haloperidol (uM)


cAMP accumulation (%Control)
200
100
0
10
50
100
1300
Trifluoperazine (uM)

As shown in Figure 40, K^+ significantly increased cAMP levels in slices of rat caudate nucleus ($p<.005$). Equivalent increases were produced by dopamine alone, or in the presence of 60 mM K^+. These results indicated that endogenous sources of neurotransmitter probably do not contribute significantly to cAMP levels in striatal slices.

3.6 Protein Phosphorylation in Rat Striatal Slices

3.6.1 Introduction

The results of the previous studies demonstrated that dopamine caused a 2-fold increase in cAMP levels in slices of rat caudate nucleus. This effect was dependent on IBMX, and was inhibited by the dopamine antagonist, haloperidol. The next objective was to determine the effects of dopamine on phosphorylation in striatal slices.

3.6.2 Time Course for ^{32}P Uptake

It was anticipated that incubation of rat caudate nucleus slices with [^{32}P] would result in the equilibration of label with intracellular phosphate pools. To determine the time course for ^{32}P incorporation into protein, slices were prepared as previously described and incubated with $^{32}P_i$ for various lengths of time. Phosphorylated proteins were visualized by autoradiography, and the intensity of labelling was quantified using densitometry. The time course for phosphorylation is shown in Figure 41. It is clear from the figure that optimal levels of ^{32}P incorporation were achieved by 30-60 min indicating that exogenous ^{32}P had equilibrated with intracellular pools by this time. Therefore, slices were routinely incubated for 30-60 min with ^{32}P prior to the addition of test agents.

Figure 40. Effect of Dopamine and KCl on cAMP Accumulation in Rat Striatal Slices. Striatal slices were prepared as described in **Methods** and incubated for 5 min under one of the following conditions: Control: (KRB); KCl: (KRB plus 60 mM KCl); DA: (KRB plus 1 mM dopamine); KCl+DA: (KRB plus 60 mM KCl, and 1 mM DA). KRB containing 60 mM KCl contained the same electrolyte composition as normal KRB except that NaCl was replaced by KCl in equimolar concentrations. cAMP was measured as described in **Methods**. Results represent the mean ± SEM of 4–12 individual slices assayed in duplicate. Statistical significance was assessed using a repeated measures ANOVA with 2 trial factors.

ANOVA

Source	$F_{1,5}$	P value
KCl	47.75	<.005
DA	7.67	<.05
KCl x DA	1.39	ns

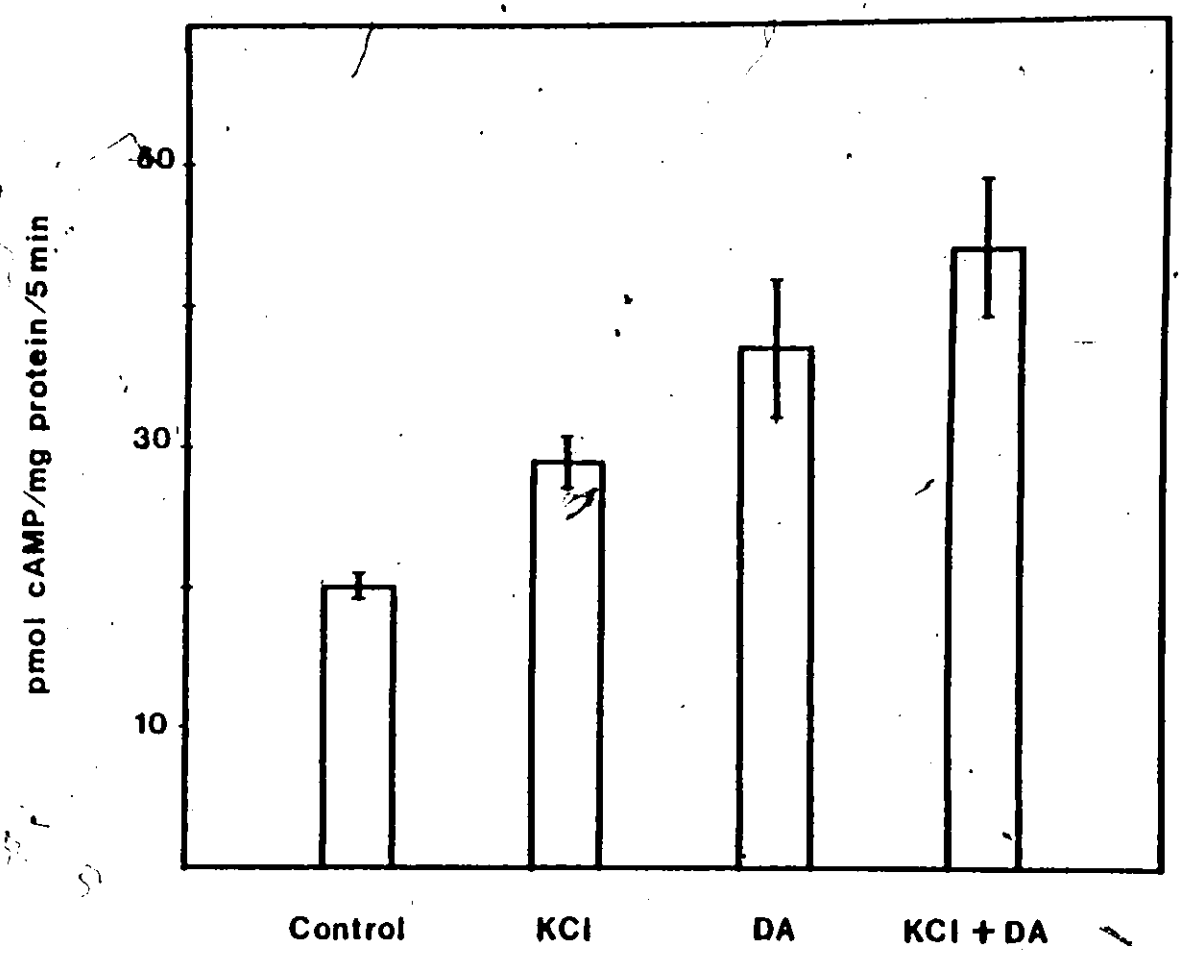
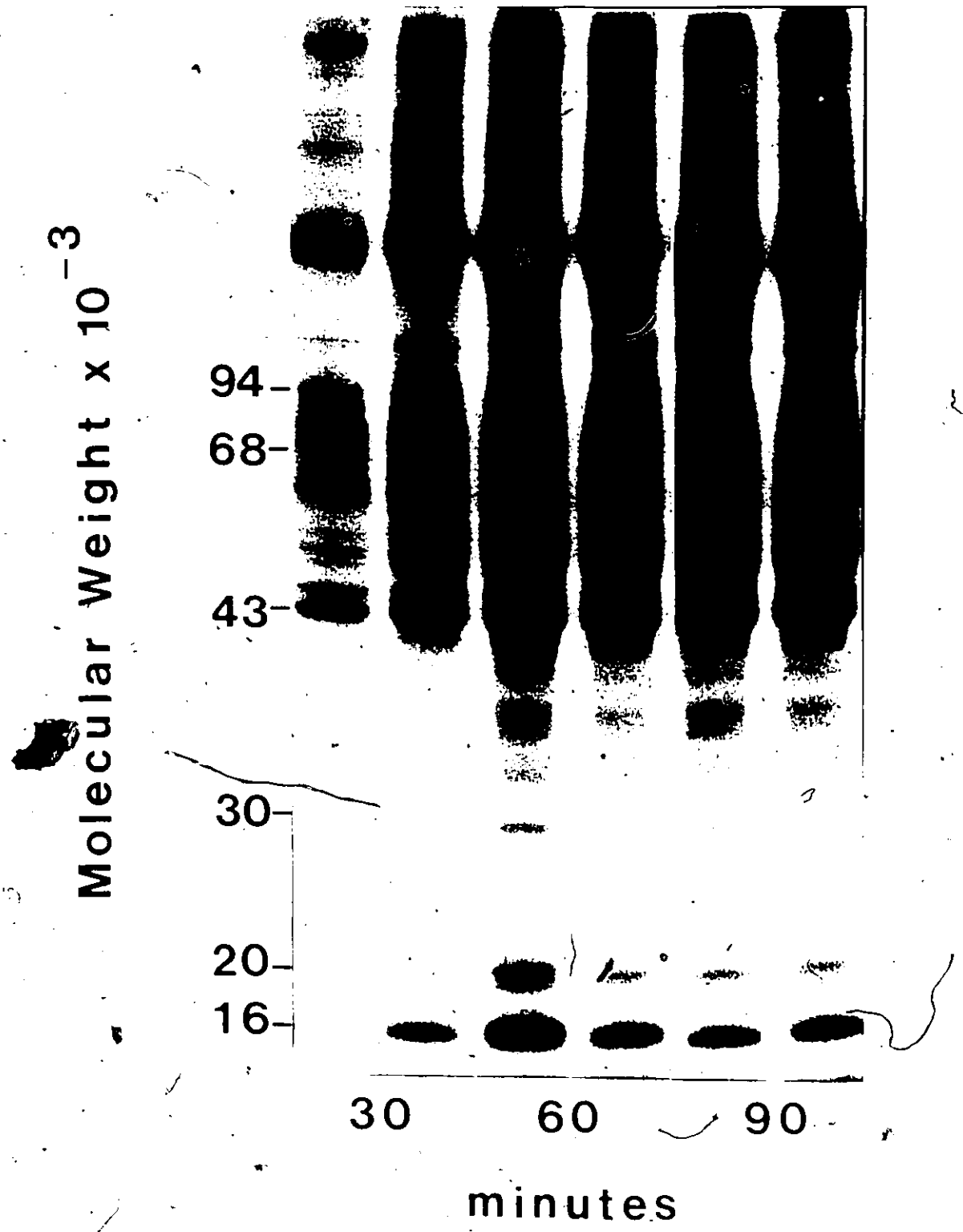

pmol cAMP/mg protein/5 min
60
30
10
Control
KCl
DA
KCl + DA

Figure 41. Incorporation of ^{32}P into Phosphoproteins in Rat Striatal Slices. Slices from rat caudate nucleus were incubated in KRB containing 250 µCi $^{32}P_i$ as described in **Methods.** After 30, 60, or 90 min proteins were solubilized in SDS, separated by electrophoresis, and phosphoproteins were visualized by autoradiography as described in **Methods.** Proteins were identified by molecular weight using known standards as described in the legend to Figure 5.


Molecular Weight x 10^{-3}
94
68
43
30
20
16
30
60
90
minutes

The pattern of phosphorylation in rat striatal slices was comparable to that previously observed in synaptic membrane fractions. As shown in Figure 42, many of the endogenous substrates for cAMP and Ca^{++}-dependent protein kinases in synaptic membrane fractions were also identified in striatal slices, including proteins of M_r=86k and 80k (Proteins Ia and Ib), 57k, 50k, 43k, 20k and 16k.

3.6.3 Effects of Dopamine and cAMP on Protein Phosphorylation in Rat Striatal Slices

As shown in Figure 43, incubation of striatal slices in the presence of dopamine enhanced the phosphorylation of two proteins with approximate molecular weights of 62-64,000 daltons, and 43k daltons ($p<.05$ and $p<.005$ respectively). The phosphorylation of a 50k, 20k, and 16k protein were also increased by dopamine, but these effects were not consistently observed. A quantitative summary of these data is presented in Figure 44.

The addition of IBMX to the incubation medium did not alter the phosphorylation pattern seen in the absence or presence of dopamine (Figure 43). Although IBMX appeared to enhance the phosphorylation of Proteins Ia, Ib, and 57k in this experiment, this was not a consistent finding. These data suggested that changes in the phosphorylation of striatal proteins observed in the presence of dopamine might be mediated through cAMP-independent protein kinases.

The effect ot 8-bromo-cAMP on phosphorylation in striatal slices was next investigated. Relatively high concentrations of 8-bromo-cAMP were used in these experiments (5 mM), since plasma membranes are relatively impermeable to cyclic nucleotides, and high levels are generally required to observe effects on protein phosphorylation in intact preparations (Forn and Greengard, 1978). As shown in Figure 45, 8-bromo-cAMP increased the phosphorylation of a protein

Figure 42. Patterns of Protein Phosphorylation in Synaptic Membrane and Slice Preparations from Rat Caudate Nucleus. Synaptic membranes were prepared as described in **Methods**, and incubated with [^{32}P] ATP under standard assay conditions (see **Methods**). Additions: none; 5 μM cAMP; 1 mM Ca^{++}. Slices from rat caudate nucleus were preincubated in KRB for 90 min, then incubated for 30 min in KRB containing 250 μCi $^{32}P_i$. Slices were rinsed in KRB to remove extracellular ^{32}P, then incubated for a further 5 min in KRB (0); KRB plus 60 mM KCl (KCl); or KRB plus 1 mM dopamine (DA). Proteins were solubilized in SDS and separated by electrophoresis. Phosphoproteins were visualized by autoradiography, and protein concentration in solubilized slices was estimated using the method of Zaman and Verwilghen, (1979) (see **Methods**). Proteins were identified by molecular weight using known standards as described in the legend to Figure 5.

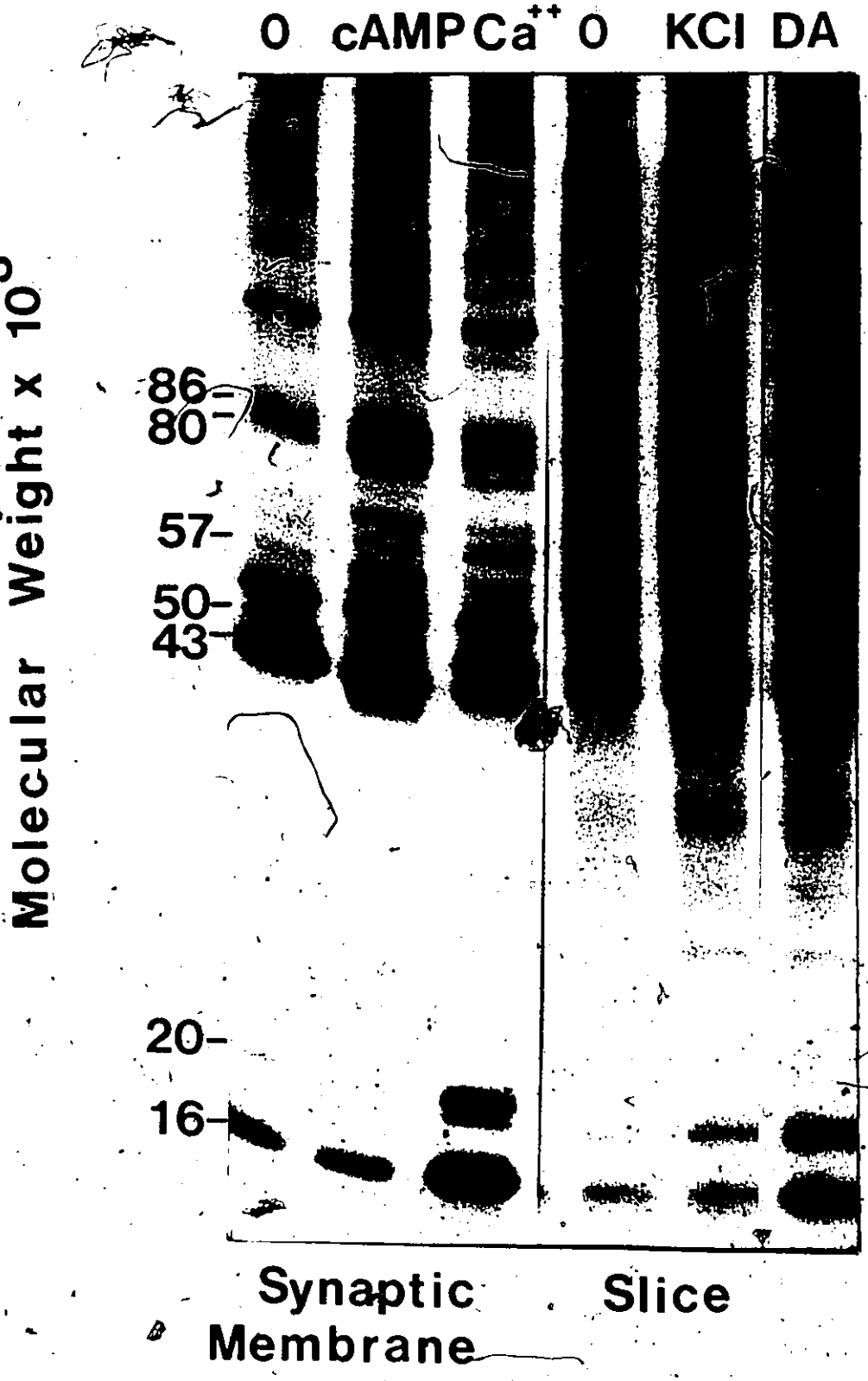

0
cAMP
Ca++
0
KCl
DA
Molecular Weight x 10-3
86
80
57
50
43
20
16
Synaptic Membrane
Slice

Figure 43. Effect of Dopamine on Protein Phosphorylation in Rat Striatal Slices. Rat striatal slices were prepared and incubated with $^{32}P_i$ as described in the legend to Figure 42. Slices were rinsed in KRB to remove extracellular ^{32}P, then incubated for a further 5 min in KRB in the absence (A) or presence (B) of 0.75 mM IBMX with (+) or without (-) 1 mM dopamine. Proteins were solubilized in SDS, separated by electrophoresis, and autoradiographed as described in **Methods.**

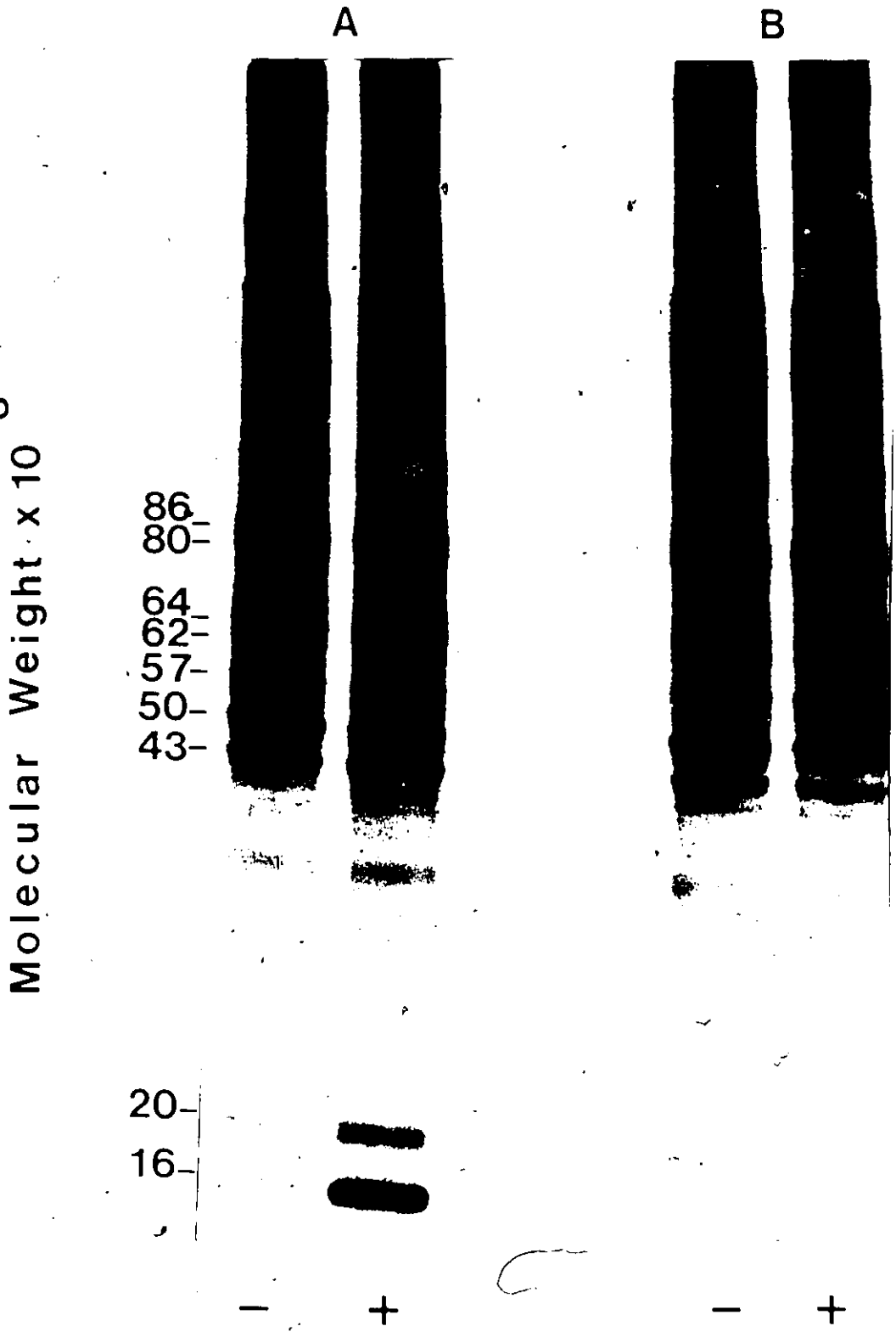
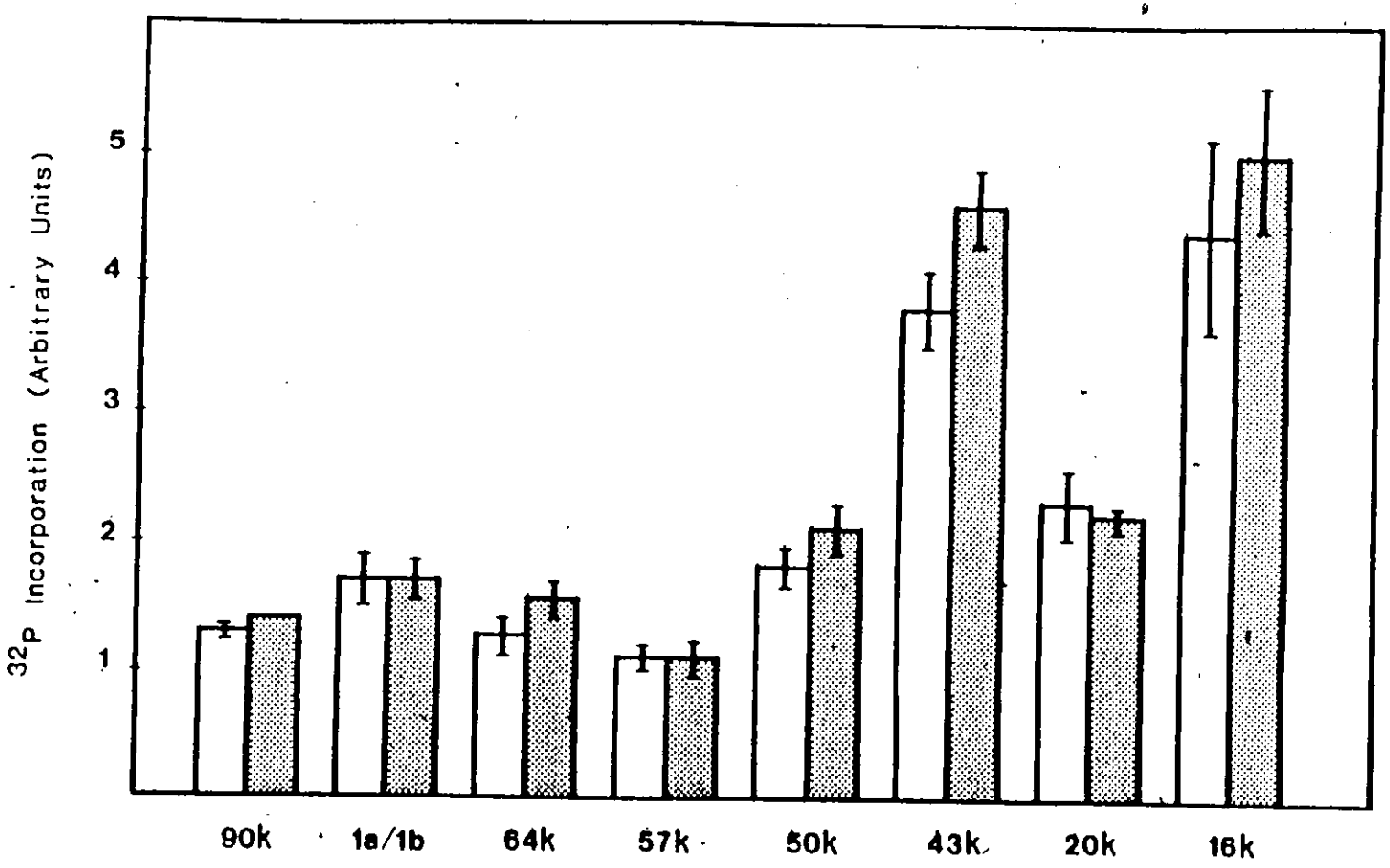

A
B
Molecular Weight x 10^{-3}
86
80
64
62
57
50
43
20
16
−
+
−
+

Figure 44. Dopamine-Stimulated Protein Phosphorylation in Rat Striatal Slices. Slices from rat caudate nucleus were preincubated for 30 min with $^{32}P_i$, rinsed with KRB to remove extracellular $^{32}P_i$, then incubated for a further 5 min in the absence (open bars) or presence (closed bars) of 1 mM dopamine. IBMX was not included in the incubation medium, as it did not appear to influence results obtained with dopamine (see text). Phosphorylated proteins were separated by electrophoresis, and autoradiograms were scanned with a Joyce-Loebl Densitometer as described in **Methods.** Incorporation of ^{32}P into proteins is expressed as OD units. Results represent the mean ± SEM of 12 individual experiments. Major phosphorylated substrates are identified by molecular weight. Data were analyzed using paired t-tests.

T-tests

Proteins	t (11 df)	P value
62-64k	2.64	<.05
43k	3.88	<.005


^{32}P Incorporation (Arbitrary Units)
1
2
3
4
5
90k
1a/1b
64k
57k
50k
43k
20k
16k

with an apparent molecular weight of 57k. As is apparent from this figure, the phosphorylation of Proteins Ia and Ib was also increased in the presence of 8-bromo-cAMP, but this effect was less reproducible, possibly due to the high level of background phosphorylation in these proteins.

In line with this, a number of agents that were observed to increase cAMP levels in striatal slices, including dopamine, norepinephrine, and 2-chloro-adenosine, also failed to produce increases in the phosphorylation of these proteins (data not shown). This may indicate that cAMP-dependent protein kinase exhibits a relative preference for Proteins Ia and Ib in vivo, leading to optimal phosphorylation of these substrates in the presence of low levels of endogenous cAMP. Earlier experiments (Figure 24), demonstrating that the phosphorylation of Proteins Ia and Ib was maintained in the presence of exogenous histone, whereas the phosphorylation of other substrates for the Type II kinase was decreased, support the concept that the phosphorylated form of Proteins Ia and Ib may predominate in vivo.

3.6.4 Effects of Ca^{++} on Phosphorylation in Striatal Slices

Since many of the proteins that were phosphorylated in striatal slices corresponded in molecular weight to previously identified substrates for Ca^{++}-dependent protein kinases in synaptic membrane fractions (Figure 42), it was of interest to examine the effect of Ca^{++} on phosphorylation in striatal slices. Figure 46 demonstrates that incubation of striatal slices in KRB in the presence of Ca^{++} significantly enhanced the phosphorylation of proteins with approximate molecular weights of 62-64k, 50k, 43k, 20k and 16k relative to slices incubated in a Ca^{++}-free medium.

Figure 45. Effect of 8-Bromo-cAMP on Protein Phosphorylation in Rat Striatal Slices. Slices from rat caudate nucleus were preincubated for 30 min with $^{32}P_i$, rinsed with KRB to remove extracellular $^{32}P_i$, then incubated for a further 5 min in the absence or presence of 5 mM 8-bromo-cAMP. Phosphoproteins were separated by electrophoresis and autoradiographed as described in **Methods.**

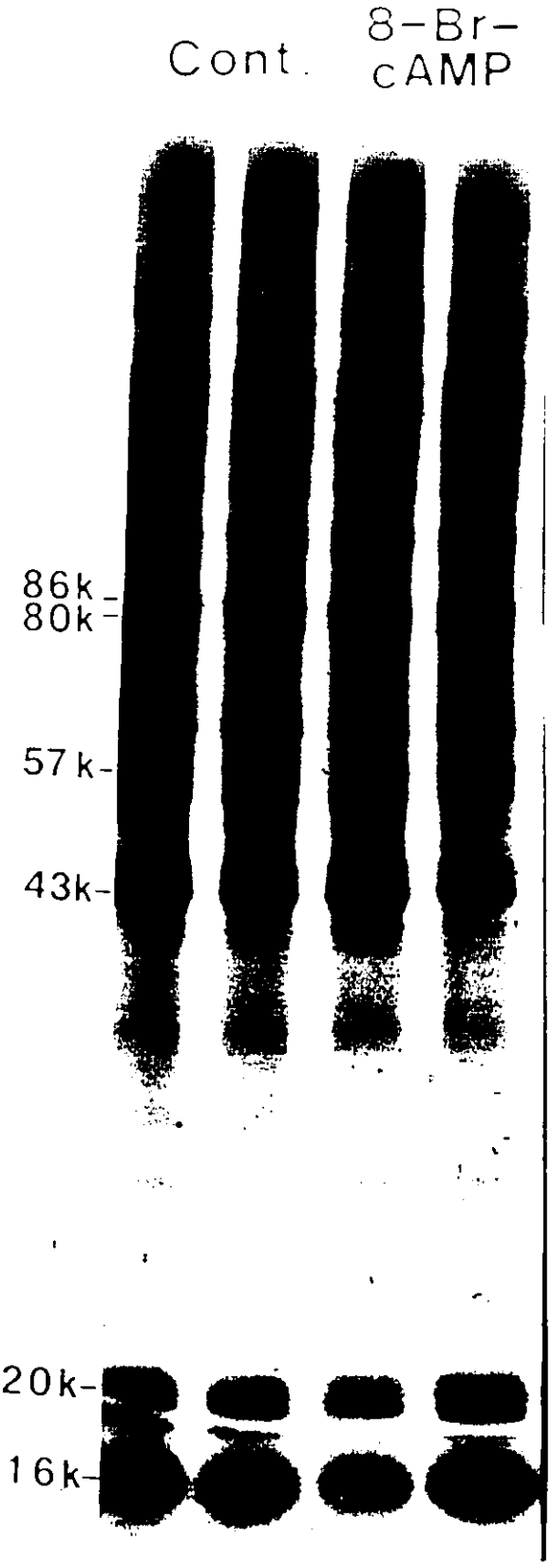
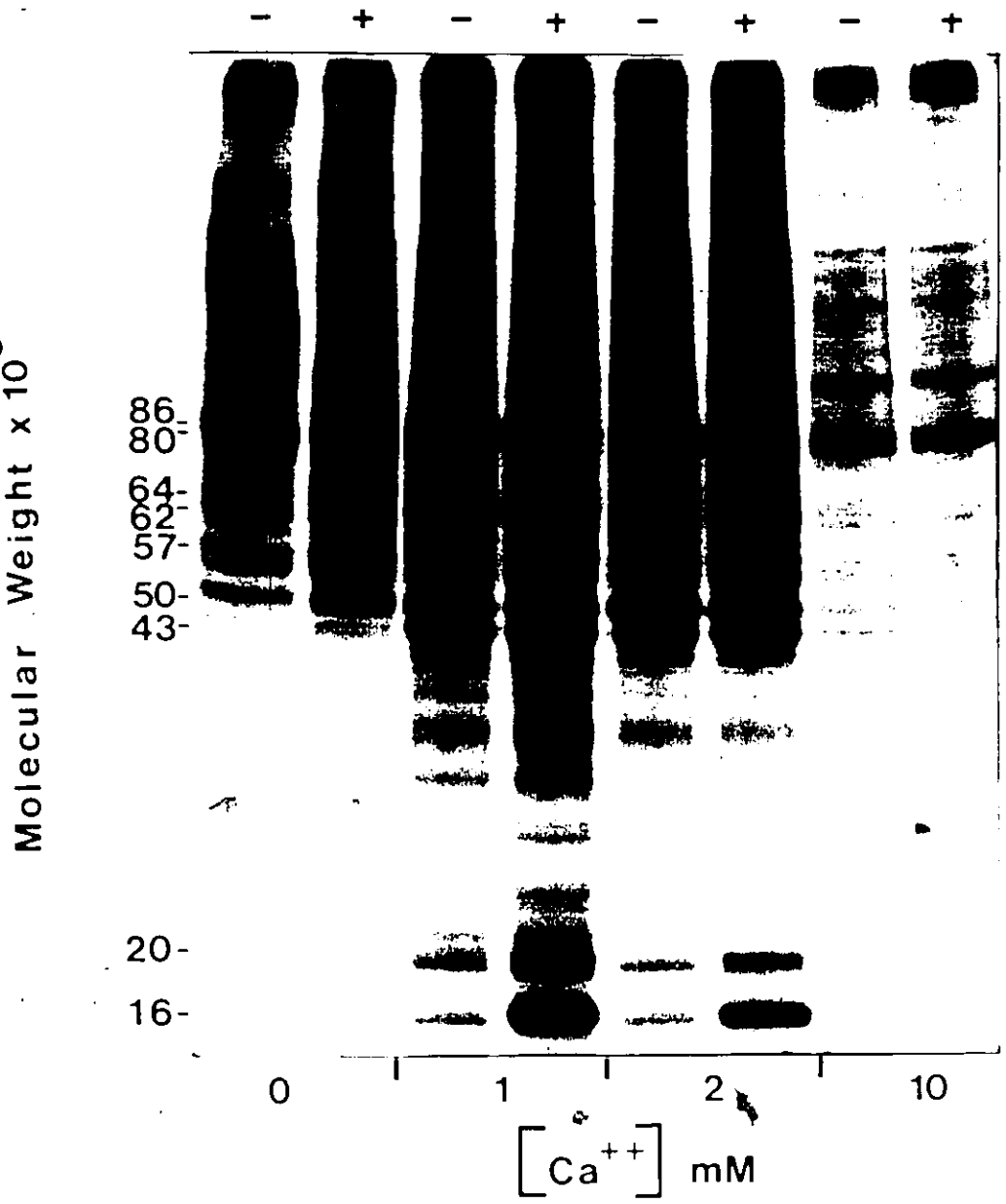

Cont.
8-Br-
cAMP
86k
80k
57k
43k
20k
16k

Figure 46. Effect of Ca^{++} on Protein Phosphorylation in Rat Striatal Slices. Slices from rat caudate nucleus were preincubated in Ca^{++}-free KRB (0.1 mM EGTA), or KRB in the presence of increasing concentrations of Ca^{++}. Levels of Ca^{++} refer to free Ca^{++} not chelated by EGTA. Slices were equilibrated with $^{32}P_i$ for 30 min, rinsed, then incubated for a further 5 min in the absence (-) or presence (+) of 1 mM dopamine using KRB containing the same concentrations of Ca^{++}. Phosphoproteins were identified by autoradiography as described in **Methods.**


- + - + - + - +
Molecular Weight x 10^{-3}
86-
80-
64-
62-
57-
50-
43-
20-
16-
0
1
2
10
[Ca^{++}] mM

The stimulatory effect of Ca^{++} on the phosphorylation of these proteins was concentration-dependent, with optimal levels of phosphorylation occurring with 1 mM Ca^{++}. A slight decrease in phosphorylation was observed with 2 mM Ca^{++}, while in the presence of 10 mM Ca^{++}, phosphorylation was reduced to control levels. Inhibition of phosphorylation by high Ca^{++} concentrations was also observed in synaptic membrane preparations (data not shown). Similar effects of high Ca^{++} have been reported by others (Hofstein et al., 1980). In view of these results, subsequent experiments were conducted in the presence of 1 mM Ca^{++}, the standard concentration of Ca^{++} in KRB.

Figure 46 also shows that dopamine enhanced the Ca^{++}-stimulated phosphorylation of proteins 62-64k and 43k consistent with previous observations (Figure 44). The dopamine effect was inhibited by 10 mM Ca^{++} (Figure 46). The phosphorylation of proteins 20k and 16k was also increased by dopamine in these experiments, although this was not a constant finding.

It should be noted that the degree of phosphate incorporation into protein 16k in the absence or presence of various stimulating agents was highly variable. Although this was not investigated rigorously, staining of gels with Coomassie Blue did not reveal any obvious changes in the amount of phosphorylated substrates following different incubation conditions. It was therefore unlikely that variation in the pattern of 16k phosphorylation was due to differences in the quantity of this protein. An alternative possibility was that labelling of this protein had not reached a steady-state by the time of assay, leading to variable incorporation of ^{32}P during the incubation procedure.

3.6.5 Effects of Depolarizing Agents on Phosphorylation in Striatal Slices

Two depolarizing agents, K^+ and veratridine were tested for their effects on phosphorylation in rat striatal slices. As shown in Figures 47 and 48, when striatal slices were incubated for 5 min in the presence of 60 mM K^+, there was a marked decrease in the phosphorylation of a 90,000 dalton protein ($p<.001$). K^+ also significantly increased the phosphorylation of a protein with an approximate molecular weight of 62-64,000 daltons ($p<.025$), and inhibited the phosphorylation of a 20,000 dalton protein ($p<.05$). Similar effects of K^+ have been observed in intact synaptosomes (Kreuger et al.,1977), although the reduced phosphorylation of a 20,000 dalton protein has not been reported previously.

K^+ also reproducibly altered the pattern of phosphorylation of Proteins Ia and Ib. These two proteins appeared as a closely spaced doublet in autoradiograms from slices incubated with ^{32}P under standard assay conditions (Figure 47, lane 1). However, in the presence of K^+ these proteins became more widely separated, apparently as a result of increased migration of Protein Ib into the gel (Figure 47, lane 3).

Detailed biochemical analyses of Proteins Ia and Ib have demonstrated that they exhibit similar biochemical properties, and an identical primary structure in the region of the phosphorylated serine residues (Ueda and Greengard, 1977). It therefore seemed unlikely that K^+ was differentially altering the phosphorylation of Protein Ib.

An alternative explanation for these results is that K^+ selectively increases the phosphorylation of a protein with a molecular weight almost identical to that of Protein Ib. Consistent with this, Burke and De Lorenzo (1982) have shown that Ca^{++} stimulates the phosphorylation of an 80,000 dalton acidic protein in synaptic vesicles. This protein was clearly distinguished from Greengard's Pro-

Figure 47. Effects of KCl on ^{32}P Incorporation into Rat Striatal Proteins. Slices from rat caudate nucleus were preincubated for 90 min in Ca^{++}-free KRB (0.1 mM EGTA), or in KRB containing 1 mM Ca^{++}. Slices were equilibrated with $^{32}P_i$ for 30 min, then incubated for a further 5 min in KRB with and without Ca^{++} in the absence (-) or presence (+) of 60 mM KCl. Phosphoproteins were separated by electrophoresis and autoradiographed as described in **Methods.**

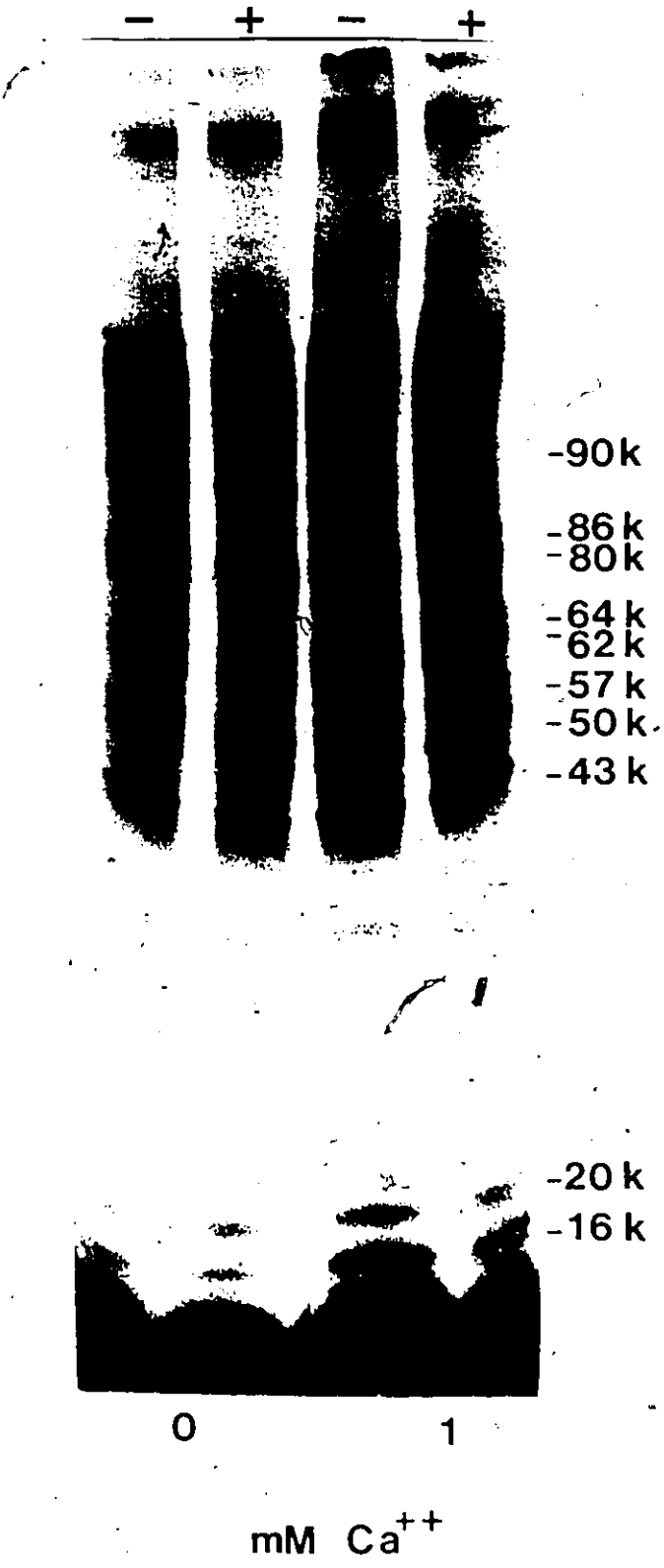
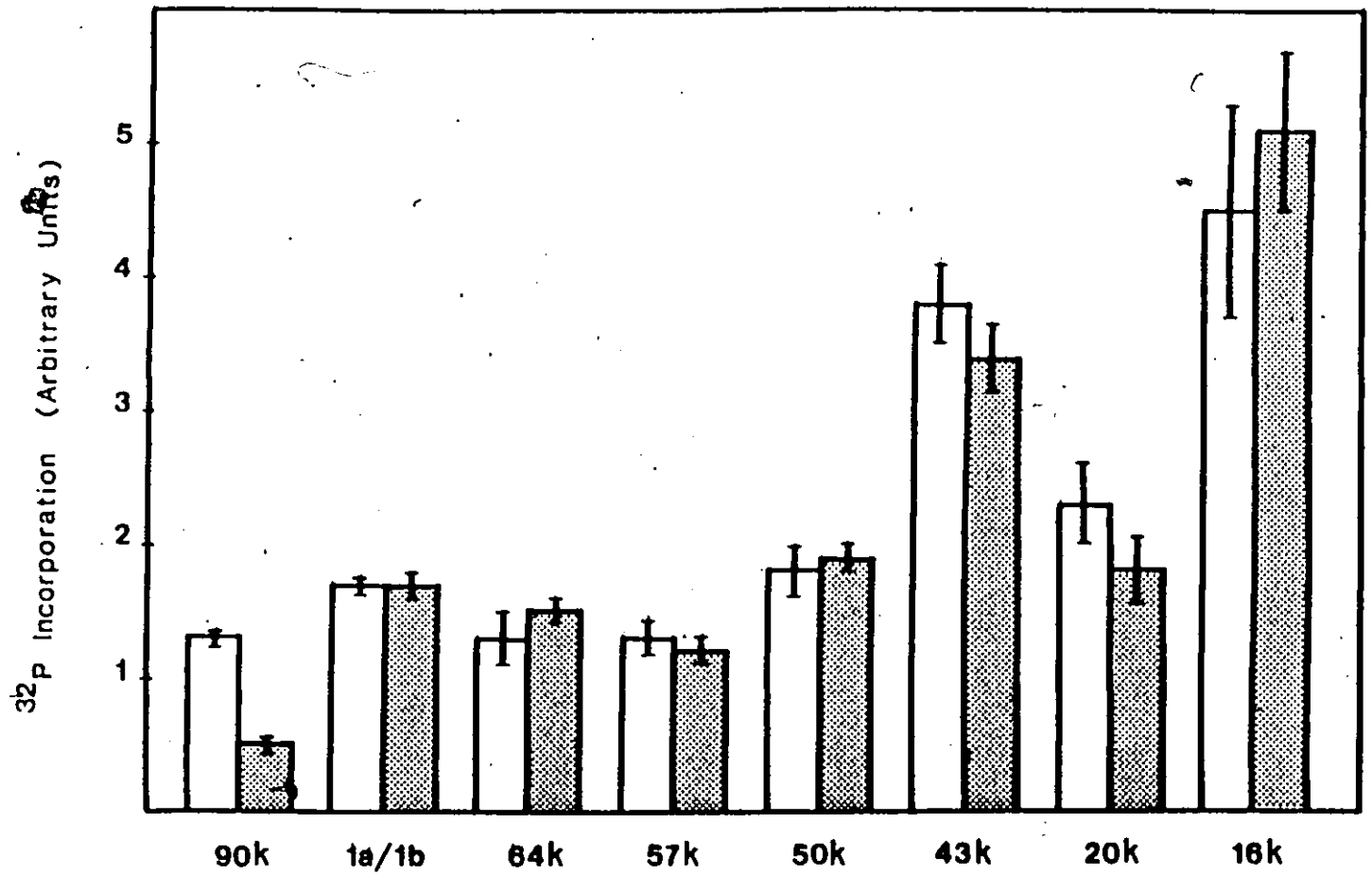

−
+
−
+
-90k
-86k
-80k
-64k
-62k
-57k
-50k
-43k
-20k
-16k
0
1
mM Ca++

Figure 48. Effect of KCl on Protein Phosphorylation in Rat Striatal Slices. Slices from rat caudate nucleus were preincubated in KRB for 90 min, then equilibrated with $^{32}P_i$ for 30 min, rinsed in KRB, and incubated for 5 min in KRB (open bars), or KRB containing 60 mM KCl (closed bars). Phosphoproteins were separated by electrophoresis and autoradiographed as described in **Methods.** ^{32}P incorporation was quantified using densitometry as described in **Methods.** Results are expressed as OD units, and represent the mean ± SEM of 14 individual slices. Major phosphoproteins are identified by their molecular weights. Data were analyzed using paired t-tests.

T-tests

Proteins	t (13 df)	P value
90k	-9.91	<.001
62-64k	2.63	<.05
20k	-2.56	<.05


^{32}P Incorporation (Arbitrary Units)
1
2
3
4
5
90k
1a/1b
64k
57k
50k
43k
20k
16k

tein I on the basis of its isoelectric properties and pattern of phosphorylation following tryptic digestion.

Greengard's group, on the other hand, has recently demonstrated that incubation of brain sysnaptosomes in the presence of K^+ or veratridine increases the Ca^{++}-dependent phosphorylation of an 87k acidic protein (Wu et al., 1982). This protein is phosphorylated by the phospholipid-sensitive protein kinase, and although it co-migrates with Protein Ia on SDS-PAGE, the two proteins can be separated using biochemical techniques. Therefore, it is conceivable that the 78,000 dalton protein described in the present study corresponds to one of the proteins described by these other investigators, and does not represent a shift in the migration of Protein Ib. Tryptic mapping of the phosphoproteins in this molecular weight range will be required to determine which of these interpretations is correct.

As shown in Figure 47, when striatal slices were incubated in Ca^{++}-free KRB, the effect of K^+ on the phosphorylation of 90k and on the migration of Protein Ib was abolished. This is compatible with the results of other workers who have demonstrated that K^+-mediated changes in phosphorylation in synaptosomes and slices require extracellular Ca^{++}_e (Kreuger et al., 1977; Forn and Greengard, 1978).

Although both dopamine and K^+ appeared to alter the phosphorylation of proteins that were previously shown to be substrates for Ca^{++}-dependent protein kinases in both synaptic membrane (Figure 13) and slice preparations (Figure 46), the stimulatory effect of these agents seemed to be confined to specific proteins in this group (compare Figures 44 and 48). Thus the dopamine-mediated increase in the phosphorylation of 43k was not observed in the presence of K^+, and the effects of K^+ on 90k phosphorylation and on the differential migration of

Proteins Ia and Ib were not mimicked by dopamine. This indicated that the effects of dopamine and K^+ on protein phosphorylation probably occurred through separate mechanisms.

Veratridine (100 μM) significantly inhibited the phosphorylation of proteins 90k ($p<.001$), Ia,Ib ($p<.005$), 50k ($p<.01$) and 43k ($p<.001$). The phosphorylation of protein 20k was also decreased, but this did not reach statistical significance. In addition to these effects, veratridine increased the phosphorylation of a 62-64 dalton protein (Figure 49). Greengard's group has reported that veratridine stimulates the phosphorylation of Proteins Ia and Ib in addition to proteins of 50-60,000 daltons (Forn and Greengard, 1978; Kreuger et al., 1977; Nestler and Greengard, 1980). The reason for these discrepancies in veratridine-stimulated protein phosphorylation is not clear at the present time.

As shown in Table VIII, the inhibitory effect of veratridine on the phosphorylation of 90k was antagonized by tetrodotoxin (TTX), an agent that specifically blocks Na^+ channels in nerve membranes. TTX did not alter the phosphorylation of other proteins that were previously shown to be inhibited by veratridine. It is therefore unlikely that the inhibitory effects of veratridine on the phosphorylation of these was mediated through opening of Na^+ channels. As might be expected, TTX did not affect the inhibition of 90k phosphorylation by K^+, since this drug prevents neither K^+ depolarization nor the subsequent entry of Ca^{++} through voltage-dependent channels (Forn and Greengard, 1978). These results, however, must be interpreted with caution in view of the small sample size on which these conclusions were based (n=3).

Figure 49. Effect of Veratridine on Protein Phosphorylation in Rat Striatal Slices. Slices were prepared and incubated with $^{32}P_i$ as described in the legend to Figure 42. After removal of extracellular $^{32}P_i$, slices were incubated for 5 min in the absence (open bars) or presence (closed bars) of 100 µM veratridine. Phosphoproteins were identified using SDS-PAGE and autoradiography, and ^{32}P incorporation was measured by densitometry as described in **Methods**. Results are expressed as OD units and represent the mean ± SEM of 12 individual slices. Major phosphoproteins are identified by their molecular weights. Data were analyzed using paired t-tests.

T-tests

Proteins	t (11 df)	P value
90k	-10.2	<.001
Ia Ib	-3.7	<.005
62-64k	2.4	<.05
50k	-3.2	<.01
43k	-4.3	<.001

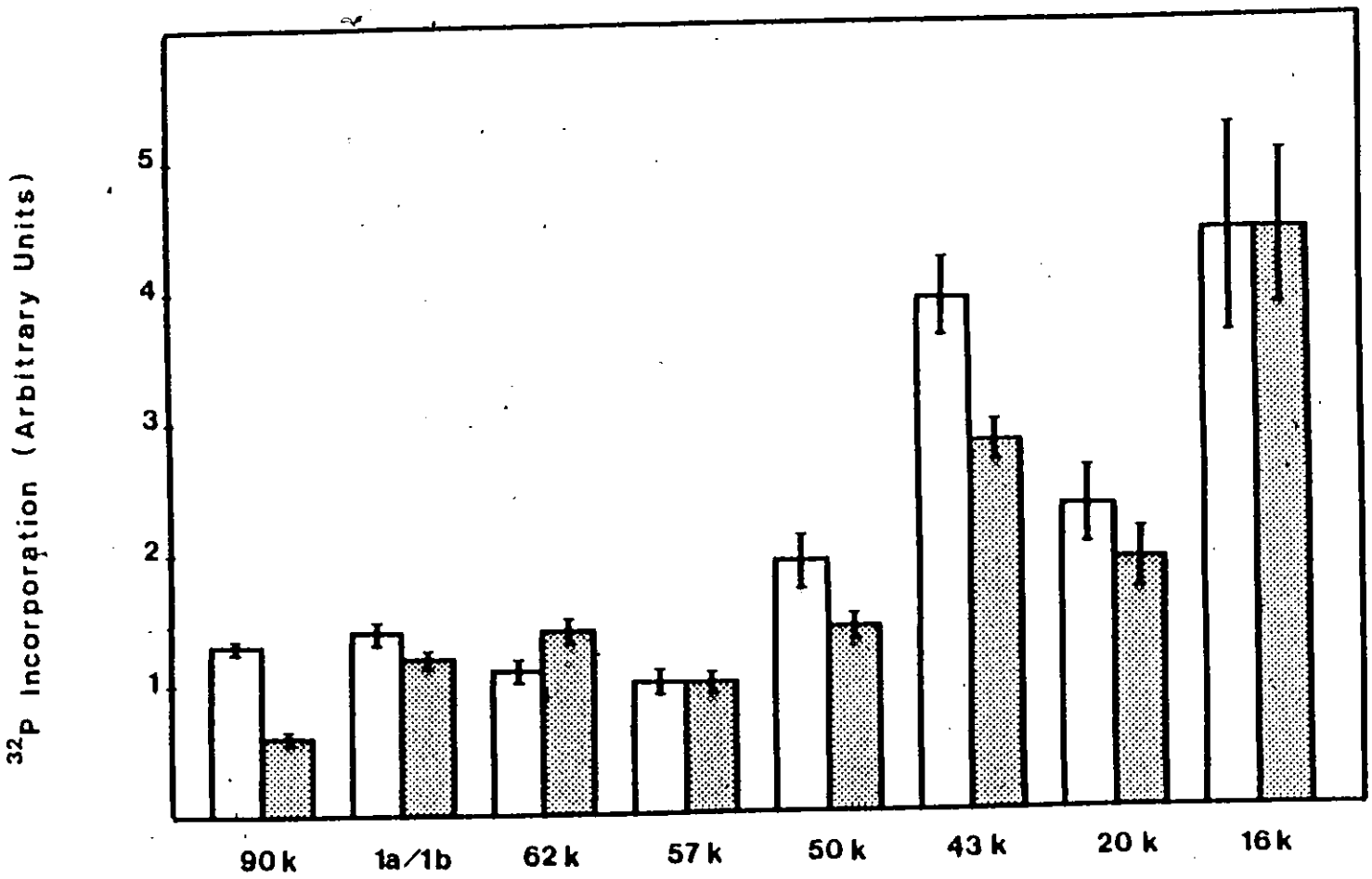

^{32}P Incorporation (Arbitrary Units)
5
4
3
2
1
90 k
1a/1b
62 k
57 k
50 k
43 k
20 k
16 k

TABLE VIII

Effect of TTX on Veratridine and KCl-Mediated Protein Phosphorylation in Rat Striatal Slices.

	Phosphoproteins							
	90k	Ia/Ib	52/64k	57k	50k	43k	20k	16k
Control	1.49	2.01 ±.12	1.13	1.49	1.76 ±.17	3.91 ±.35	3.44 ±.04	7.98
Ver	0.46 ±.02	1.47 ±.03	1.82 ±.13	1.29 ±.01	1.56 ±.02	3.10 ±.10	2.31 ±.13	7.15 ±.35
KCl	0.50	2.03 ±.06	1.84 ±.13	1.58 ±.02	1.87 ±.10	2.83 ±.02	2.94 ±.21	7.89 ±.59
Ver + TTX	1.23 ±.11	1.73 ±.11	1.47 ±.20	1.23 ±.11	1.90 ±.05	3.57 ±.19	2.13 ±.07	5.80 ±.27
KCl + TTX	0.50	2.03 ±.04	1.97 ±.19	1.37 ±.15	1.89 ±.15	3.33 ±.39	2.83 ±.41	7.05 ±.86
TTX	1.28 ±.07	1.45 ±.02	1.33 ±.07	1.49 ±.03	1.75 ±.19	3.43 ±.44	2.65 ±.45	6.30 ±.99

Striatal slices were prepared and incubated with $^{32}P_i$ as described in Methods. Slices were rinsed in KRB, and then incubated a further 5 min in KRB containing 100 uM veratridine (Ver) or 50 mM KCl in the absence or presence of 100 uM TTX. Reactions were terminated with SDS and solubilized proteins were electrophoresed and autoradiographed as described in Methods. ^{32}P incorporation was measured by densitometry. Results are expressed as OD units and represent the mean ± SEM of 3 experiments.

3.6.6 Effects of Haloperidol and Trifluoperazine on Phosphorylation in Striatal Slices

Previous experiments in synaptic membrane fractions had demonstrated an inhibition of Ca^{++}-dependent phosphorylation by neuroleptic drugs (Figure 31). Since striatal slices appeared to contain a number of proteins whose phosphorylation was also Ca^{++}-regulated, it was of interest to determine whether their phosphorylation could be manipulated through pharmacological means. As shown in Figure 50, when striatal slices were incubated in the presence of 1.3 mM haloperidol, there was a large decrease in the phosphorylation of proteins 90k, Ia and Ib, 62-64k, 50k, 43k and 20k.

Incubation of striatal slices with 100 μM trifluoperazine led to a decrease in the phosphorylation of proteins 50k and 16k (Figure 50). Smaller decreases in the phosphorylation of proteins 43k and 20k were also observed, although the limited number of samples used in these studies precluded statistical analysis. The data are consistent, nevertheless, with previous results demonstrating an inhibition of Ca^{++}-dependent phosphorylation by neuroleptics in synaptic membrane fractions (Figures 32 and 35), and are compatible with published observations using broken cell preparations (Schatzman et al., 1981; Wrenn et al., 1980).

In view of these results, the effect of haloperidol on dopamine-dependent phosphorylation was investigated. In confirmation of previous findings, dopamine increased the phosphorylation of protein 43k (Table IX). In the presence of haloperidol, dopamine-stimulated phosphorylation of 43k was markedly inhibited, as was the phosphorylation of other striatal proteins. These results indicated that the inhibitory effects of haloperidol on protein phosphorylation were probably mediated independently of dopamine receptors.

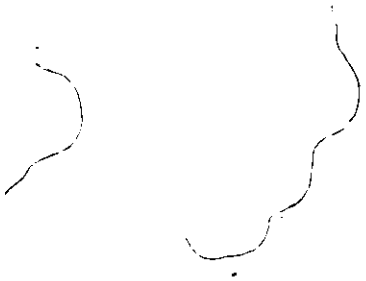


Figure 50. Effect of Neuroleptics on Protein Phosphorylation in Rat Striatal Slices. Slices were prepared and incubated with $^{32}P_i$ as described in the legend to Figure 42. Slices were rinsed in KRB, and incubated for 5 min in KRB containing 1.3 mM haloperidol (open bars) or 1.3 mM trifluoperazine (closed bars). Control slices received the appropriate drug vehicles. Phosphoproteins were identified using SDS-PAGE and autoradiography, and ^{32}P incorporation was measured by densitometry as described in **Methods.** Results are expressed as OD units (% control), and represent the mean ± SEM of 3-5 slices. Major phosphoproteins are identified by their molecular weights. Data were analyzed using paired t-tests.

T-tests

Proteins	t (4 df)	P value
90k	-7.0	<.005
Ia Ib	-9.3	<.001
62-64k	-5.0	<.01
50k	-9.2	<.001
43k	-5.2	<.01
20k	-3.4	<.05

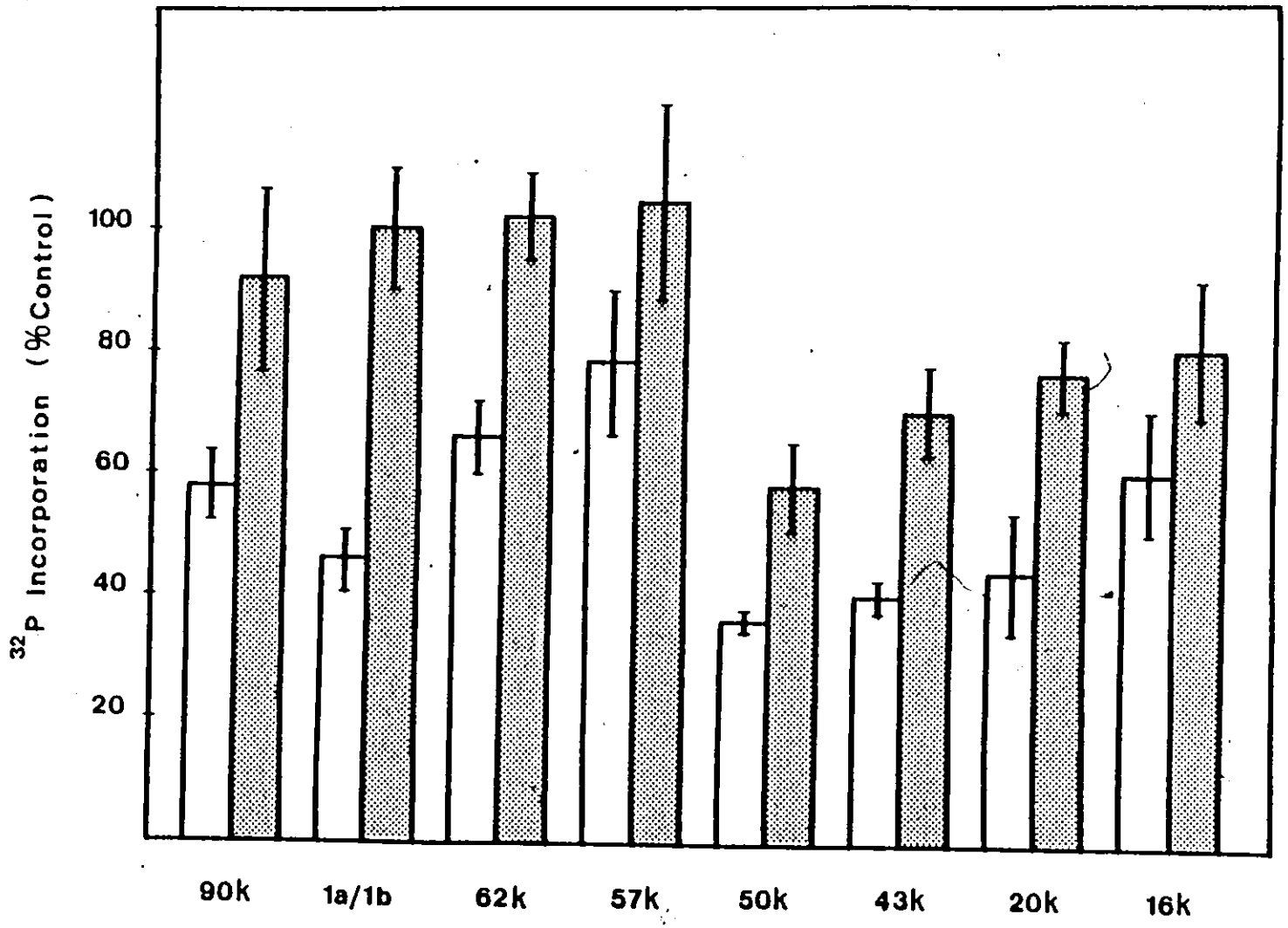

^{32}P Incorporation (%Control)
100
80
60
40
20
90k
1a/1b
62k
57k
50k
43k
20k
16k

TABLE IX

Effect of Haloperidol on Dopamine-Mediated Protein Phosphorylation in Slices

	Control	DA	HAL	DA + HAL	(p)[b]
90k	1.21±.06	1.30±.07 (.64)[a]	0.57±.06 (55.37)*	0.58±.07 (.47)	.001
Ia/Ib	2.17±.26	2.30±.14 (.17)	1.23±.17 (117.1)*	1.18±.17 (.76)	.001
62/64k	1.36±.10	1.69±.10 (2.84)	0.97±.12 (76.49)*	0.93±.08 (5.11)	.001
57k	1.24±.14	1.41±.13 (1.21)	0.77±.22 (23.94)*	0.76±.21 (7.68)	.005
50k	1.63±.24	1.90±.19 (10.66)*	0.50±.13 (90.55)**	0.67±.17 (.24)	.05, .001
43k	4.30±.19	5.30±.21 (16.68)*	2.03±.12 (887.25)**	2.36±.19 (1.51)	.01, .001
20k	2.47±.17	2.56±.09 (.06)	1.20±.19 (53.78)*	1.07±.13 (.67)	.001
16k	3.40±.43	3.80±.34 (.33)	1.84±.21 (130.8)*	1.79±.16 (.37)	.001

Striatal slices were prepared and incubated with ^{32}Pi as described in Methods. Slices were rinsed in KRB, and then incubated a further 10 min in KRB in the absence or presence of 1 mM dopamine (DA), with or without 1.3 mM haloperidol (HAL). Control samples received the appropriate drug vehicle. Reactions were terminated with SDS and solubilized proteins were electrophoresed and autoradiographed as described in Methods. ^{32}P incorporation was measured as described in Methods. Results are expressed as OD units and represent the mean ±SEM of 7 expreiments. Data were analyzed using a repeated measures ANOVA.

[a] F value for each observation (df=1,6)

[b] level of significance for indicated F value (*)

Chapter IV

Discussion

4.1 Identification of Dopamine-sensitive Adenylate Cyclase Activity in Rat Caudate Nucleus

As discussed in the **Introduction**, biochemical studies from several laboratories have shown that dopamine increases cAMP levels in preparations from rat caudate nucleus (Forn et al., 1974; Wilkening and Makman, 1975; Kebabian et al., 1972; Clement-Cormier et al., 1974). These results were confirmed in the present study using homogenates (Figure 6) and slices (Table VI) of rat caudate tissue. The average increase in adenylate cyclase activity or in tissue cAMP produced by optimal concentrations of dopamine was 2.5 fold. This is consistent with published observations. This effect was dependent on the inclusion of phosphodiesterase inhibitors in the assay mixture.

Stimulation of cAMP accumulation in striatal slices required dopamine concentrations of at least 1 mM. This can be attributed to the relative impermeability of intact slices to dopamine since stimulation of adenylate cyclase in homogenates was observed with dopamine concentrations between 10 and 100 uM (Figures 6 and 7). These values correlate reasonably well with the expected concentration of dopamine in the synaptic cleft (1 to 100 uM, Seeman, 1977; Titeler et al., 1978), and are consistent with the low affinity of postsynaptic D-1 receptor sites for dopamine (Seeman, 1980).

The neuroleptic drugs, haloperidol and trifluoperazine (TFP), inhibited dopamine-sensitive adenylate cyclase activity in homogenates and synaptic mem-

brane fractions from rat caudate nucleus (Figure 6), but high concentrations (100 uM) of these drugs were required for this effect to be observed. As explained in the **Introduction**, the concentrations of neuroleptics required to inhibit the dopamine receptor site that is linked to adenylate cyclase (the so-called D-1 site) are over 100 times higher than the therapeutic concentrations detected in the plasma of patients treated clinically with these drugs. Furthermore, there is no correlation between the clinical doses of the neuroleptics and the neuroleptic IC_{50} values at the D-1 site (Seeman, 1980). Although inhibition of dopamine-sensitive adenylate cyclase was obtained with both of these agents, this effect is not likely to be clinically relevant since it is only observed at high drug concentrations.

It was of interest that TFP inhibited basal and dopamine-stimulated adenylate cyclase activity in homogenates but had no effect on enzyme activity in slices. Phenothiazines are known to be more effective antagonists of the dopamine-sensitive adenylate cyclase than the butyrophenones (Creese et al., 1976; Iversen, 1975), and this has been attributed to differences in their lipid solubilities (Seeman, 1972, 1977; Seeman et al., 1974, 1978).

The fact that haloperidol inhibited the effects of dopamine in striatal slices while TFP did not, suggested either that TFP does not produce its main pharmacological effects through blockade of dopamine receptors, or that the population of receptors TFP interacts with is not linked to adenylate cyclase. The fact that TFP displaces dopamine competitively in receptor binding studies and that it is a potent antipsychotic agent (Seeman, 1980), tend to discount the first hypothesis.

The second possibility implies preferential binding of TFP to other dopamine receptors. As discussed in the **Introduction**, neuroleptics bind D-2

receptors with a high affinity (in the nanomolar range) and D-1 receptors (those linked to adenylate cyclase) with a low affinity (micromolar range). Since the amounts of TFP used in the present study were in the micromolar to millimolar range, it was surprising that no inhibition of D-1 receptor activity was observed.

Haloperidol, on the other hand, which has a recognized low affinity for D-1 receptors (Creese and Snyder, 1976a; 1977b; Seeman, 1980; Cross and Owen, 1980; Hyttel, 1980), inhibited dopamine-sensitive adenylate cyclase at millimolar drug concentrations. The capacity of TFP to inhibit adenylate cyclase activity in homogenates may be related in part to its ability to bind calmodulin, a required cofactor for the enzyme in brain (Cheung et al., 1975; Gnegy and Treisman, 1981). TFP, however, may not gain access to calmodulin in intact preparations.

It is important to emphasize at this point that the results described in the present study were obtained at concentrations of TFP in excess of the range associated with specific binding of this drug to calmodulin (Levin and Weiss, 1977, 1979). Therefore these results can probably be attributed to the hydrophobic properties of the drug rather than a specific inhibition of calmodulin-mediated processes. However, since tissue levels of TFP were not measured either in slices or homogenates, this conclusion remains tentative.

In confirmation of previous studies, lesions of striatal neurons with kainic acid caused a marked reduction in striatal dopamine-sensitive adenylate cyclase activity (Figure 8), indicating that the D-1 sites are localized on neurons postsynaptic to the dopamine containing cells.

As detailed in the **Introduction**, denervation of striatal neurons with 6-OHDA, or long-term administration of neuroleptic drugs result in an increase in the dopamine sensitivity of postsynaptic neurons. This enhanced sensitivity can be measured behaviourally as an increase in stereotypy produced in response to

dopamine-mimetic drugs (Tarsy and Baldessarini, 1974; Gianutsos et al., 1974; Gnegy et al., 1977a, 1977b; Tye et al., 1977) or biochemically as an increase in the number of specific dopamine receptors (Burt et al., 1976; Muller and Seeman, 1977, 1978; Creese and Snyder, 1979). Dopamine-sensitive adenylate cyclase activity is inconsistently affected by these manipulations, some authors reporting an increase (Gnegy et al., 1977a, 1977b; Iwatsubo and Clouet, 1975; Marshall and Mishra, 1980), and others no change (Von Voigtlander et al., 1975; Heal et al., 1976; Rotrosen et al., 1975; Roufogalis et al., 1976b; Hyttel, 1978) in the activity of this enzyme system.

In the present study chronic blockade of postsynaptic dopamine receptors using haloperidol produced an increase in the number of [^{3}H] spiroperidol binding sites and in apomorphine-induced stereotyped behaviour (Figures 9 and 10). Although the effect of haloperidol on dopamine-sensitive adenylate cyclase was examined in three separate experiments, a statistically significant difference in enzyme activity between haloperidol and vehicle-injected rats was only demonstrated in one case in which assays were conducted in the presence of 100uM dopamine (Table III). In view of the fact that changes in enzyme activity were not detected when enzyme activity was measured with lower dopamine concentrations (Table III), and that results obtained with 100 uM dopamine were marginally significant ($p<.05$), the bulk of the experimental data indicate that chronic haloperidol administration does not increase the activity of the dopamine-sensitive adenylate cyclase.

The pharmacology of the dopamine-sensitive adenylate cyclase, however, is considerably different from that of the dopaminergic binding sites labelled by [^{3}H] spiroperidol (Marchais and Bockaert, 1980; Hyttel, 1978; Cross and Owen, 1980; Cross and Waddington, 1981). These binding sites are character-

ized by their high affinity for neuroleptics (Seeman, 1980). Moreover, the affinities of these drugs for the D-2 site correlate closely with their ability to antagonize a variety of dopamine-mediated events. These drugs, however, have little or no affinity for receptors linked to dopamine-sensitive adenylate cyclase.

In addition, the affinity of the dopamine-sensitive adenylate cyclase for spiroperidol is very low (Ki=100nM). Thus it is unlikely that [^{3}H] spiroperidol can label the D-1 site in binding experiments where the concentration of labelled ligand is between 0.01 and 5 nM. Moreover, under conditions in which [^{3}H] spiroperidol binding sites are blocked completely with phenoxybenzamine, dopamine-sensitive adenylate cyclase activity is maintained (Marchais and Bockaert, 1980), indicating that the receptor sites associated with [^{3}H] spiroperidol binding and adenylate cyclase activity are different.

Some neuroleptics, particularly of the thioxanthene class, do have a high affinity for the dopamine-sensitive adenylate cyclase. For example, cis-fluphenthixol and piflutixol have K_i values of 1 to 2 nM for inhibition of the dopamine-sensitive adenylate cyclase. The distribution of binding sites for these agents and their affinities for neuroleptic drugs correlate closely with the pharmacology of dopamine-sensitive adenylate cyclase (Hyttel, 1978, 1980; Cross and Owen, 1980; Cross and Waddington, 1981). For example, butyrophenones such as haloperidol are weak inhibitors of dopamine-sensitive adenylate cyclase as well as [^{3}H] fluphenthixol binding (Cross and Owen, 1980). Interestingly, the number of striatal binding sites for [^{3}H] fluphenthixol has been reported to be three times higher than for [^{3}H] haloperidol (Hyttel, 1980). This value may be misleading, however, since the affinity of [^{3}H] haloperidol for CNS dopamine receptors is relatively low compared with other ligands such as [^{3}H] spiroperidol (Quik and Iversen, 1979).

The interpretation of these data is complicated by the fact that there appears to be some cross reactivity between the thioxanthenes and the D-2 class of receptors. At least 20% of receptors labelled by [^{3}H] fluphenthixol are of the D-2 class (Cross and Waddington, 1981). Thus, fluphenthixol is a potent competitive inhibitor of sulpiride, a drug with a recognized high affinity for D-2 receptors (Woodruff and Freedman, 1981). Sulpiride, however, has no effect on the dopamine-sensitive adenylate cyclase (Roufogalis et al., 1976b; Jenner et al., 1980). These data indicate that classes of dopamine receptors labelled by these agents may not be as homogeneous as previously supposed. Given these limitations, it may be inappropriate to draw conclusions about the physiology of dopamine receptors solely on the basis of receptor binding data. Clearly more information is needed about the intracellular mechanisms by which other dopamine receptors exert their effects, before the role of the dopamine-sensitive adenylate cyclase can be placed in its proper context.

Denervation of caudate nucleus neurons using 6-OHDA induced an increase in behavioural sensitivity in response to apomorphine as has been reported by others (Mishra et al., 1974, 1980; Ungerstedt, 1971a; Zigmond and Stricker, 1977; Figure 27). One of the biochemical correlates of this increased sensitivity is thought to be an increase in the activity of dopamine-sensitive adenylate cyclase (Mishra et al., 1974, 1978, 1980), although other investigators have been unable to demonstrate an increase in the activity of this enzyme following denervation (Freedman et al., 1981; Kreuger et al., 1976; Von Voigtlander et al., 1975).

In the present study, the behavioural response to apomorphine in lesioned animals was not abolished by destruction of postsynaptic neurons with kainic acid, a procedure that resulted in a 70-90% decrease in dopamine-sensitive adenylate cyclase activity. It therefore seems unlikely that this population of

receptors is involved in the development of behavioural supersensitivity secondary to 6-OHDA lesions.

Striatal denervation has also been associated with an increase in the number of D-2 receptors (Thal et al., 1979; Creese et al., 1977b; Waddington et al., 1979; Mishra et al., 1980). These have also been shown to be postsynaptically localized (Garau et al., 1978; Murrin et al., 1979), though a substantial number of these neuroleptic binding sites appear to be found on cortical afferents as well (Schwartz et al., 1978; Minneman et al., 1978; Garau et al., 1978). These data indicate that rotational behaviour secondary to unilateral destruction of striatal neurons may be mediated independently of postsynaptic dopamine receptors. Alternatively, a small number of postsynaptic dopamine receptors that were unaffected by the lesion procedure may have been responsible for the increase in behavioural sensitivity.

Studies by Marshall and Ungerstedt (1977) have attempted to determine the neuroanatomical basis of turning behaviour in 6-OHDA lesioned rats. These investigators used electrocoagulation or knife cuts to sever striatal efferent pathways, and they concluded that near total destruction of the neostriatum and parts of the internal capsule was required to abolish apomorphine-induced rotational behaviour in these rats. Since kainic acid destroys neuronal perikarya but leaves axons intact, the results of the present study provide additional evidence that pathways extrinsic to the striatum are involved in the mediation of rotational behaviour. Since the extent of kainic acid lesions was not assessed using histologic procedures, the relationship between the decline in dopamine-sensitive adenylate cyclase activity and the magnitude of striatal damage can not be determined. Other investigators have demonstrated however, that the lesioning procedure used in the present study is associated with large losses of glutamic acid decarboxylase and choline acetyltransferase, and extensive neuronal degen-

eration (McGeer and McGeer, 1976). The large decrease in cAMP-dependent protein kinase activity observed in these rats (Figures 26 and 27) is also consistent with significant destruction of postsynaptic sites. These data tend to exclude cAMP as a potential mediator of apomorphine-induced turning behaviour.

Recent studies have demonstrated that there may be some danger in correlating changes in rotational behaviour with biochemical parameters of dopamine function. For example, ipsilateral rotation and contralateral rotation in response to apomorphine can both be induced following 6-OHDA lesions, And both of these behavioural syndromes are associated with an increase in dopamine-sensitive adenylate cyclase and in the number of [^{3}H] haloperidol binding sites (Thal et al., 1979; Mishra et al., 1980). This contradicts the classical view that rotational behaviour is the product of an imbalance of striatal postsynaptic dopamine receptor function (Anden et al., 1966; Ungerstedt, 1971a) such that animals turn in a direction opposite to the caudate showing the highest dopamine activity.

Although the initiation of circling behaviour is undoubtedly a consequence of disruption of the striato-nigral pathway (Di Chiara et al., 1977c; Tuloch et al., 1978) the coordination and maintenance of this behaviour probably involves other neuronal systems. Studies by other investigators have emphasized the importance of the nucleus accumbens in the mediation of the locomotor component of this behaviour (Kelly and Moore, 1976; Pycock et al., 1978) as well as the contribution of other transmitters such as 5-HT and GABA (Baldessarini et al., 1975; Olpe et al., 1977). Since dopamine receptors were not measured in the present study, no conclusions can be drawn as to the types and number of dopamine receptors remaining after various lesion procedures. Finally, there is some danger in overinterpreting the results of lesion studies, since these procedures can also lead to transynaptic degeneration (Hattori and Fibiger, 1982). Thus the

use of 6-OHDA and kainic acid as selective probes of pre and postsynaptic function must be re-evaluated.

The results presented here strongly indicate that changes in dopamine receptor sensitivity or in dopamine-mediated behaviour are regulated independently of D-1 receptors. One interpretation of the present findings is that the dopamine-sensitive adenylate cyclase may already be functioning at optimal levels under control conditions so that procedures that increase the sensitivity of postsynaptic neurons to dopamine may have little additional effect on enzyme activity. As will be discussed in more detail later, the failure to detect an increase in cAMP-dependent phosphorylation following incubation of striatal slices with dopamine may indicate that basal levels of cAMP are already sufficiently high to fully activate endogenous protein kinases. Since the only mechanism by which cAMP exerts its effects is through activation of cAMP-dependent protein kinases, these data suggest that increases in cAMP produced by dopamine may have little physiological relevance.

Dopamine receptors linked to adenylate cyclase, nevertheless, exhibit a number of properties that are consistent with dopamine function in vivo. Thus, these receptors are localized postsynaptically (Minneman et al., 1978; Garau et al., 1978; Creese et al., 1977a; Schwartz et al., 1978), can be specifically labelled by ligands such as cis-fluphenthixol (Hyttel, 1978, 1980; Cross and Owen, 1980; Cross and Waddington, 1981) are distributed in dopaminergic regions of the brain (McCune et al., 1971; Kebabian et al., 1972; Clement-Cormier et al., 1974; Iversen, 1975) and are antagonized by dopamine receptor blocking agents (Kebabian et al., 1972; Clement-Cormier et al., 1974; Miller et al., 1974).

Although the caudate nucleus has been used extensively as a model for investigating the characteristics of D-1 receptors, evaluation of the role of the

dopamine-sensitive adenylate cyclase is complicated by the heterogeneity of the tissue. This makes it impossible to experimentally isolate D-1 receptors and examine their responses independently of the modulating influences of other dopaminergic and non-dopaminergic neurons. Examination of the literature in which increases in dopamine-sensitive adenylate cyclase have been correlated with measures of postsynaptic dopamine receptor sensitivity reveals that the changes in the activity of this enzyme are comparatively small (see Muller and Seeman, 1978). This effect, however, may be physiologically very significant if it is confined to a discrete population of neurons.

One strategy for assessing the role of D-1 receptors in supersensitivity would be to examine the effects of denervation and chronic haloperidol on [^{3}H] cis-fluphenthixol binding as this ligand has been shown to have a high affinity for D-1 sites, and its pharmacological properties parallel those of the dopamine-sensitive adenylate cyclase (Hyttel, 1978, 1980). If D-1 receptors are involved in the mediation of supersensitivity, one would expect to see an increase in the number and/or affinity of fluphenthixol binding sites, although this has not been demonstrated as yet. This might provide a more sensitive index of D-1 receptor function and assist in resolving the present discrepancies in the literature regarding the role of the dopamine-sensitive adenylate cyclase in vivo.

Some support for the role of dopamine-sensitive adenylate cyclase in the mediation of dopamine receptor supersensitivity has come from studies demonstrating an increase in the calmodulin content of membranes of rats treated chronically with haloperidol (Gnegy, 1982). Since calmodulin is required for the activation of striatal dopamine-sensitive adenylate cyclase (Gnegy and Treisman, 1981), this could provide a mechanism for the increase in the activity of this enzyme in supersensitive animals. This increase in striatal adenylate cyclase

activity has been attributed to a change in the affinity of the enzyme for dopamine rather than an increase in catalytic activity or in the number of enzyme molecules (Gnegy et al., 1977a, 1977b). This is reflected as a decrease in the concentration of dopamine required to stimulate adenylate cyclase in haloperidol-treated rats. Such an effect was not observed in the present study. In contrast to the results of Gnegy's group, differences between haloperidol and vehicle-injected rats were detected when adenylate cyclase activity was assayed in the presence of high (100 uM) but not low (0.1-50 uM) dopamine concentrations (Table III).

4.1.1 cAMP-Dependent Protein Kinase Activity in Rat Caudate Nucleus

As described in the **Introduction**, there is now abundant evidence that most, if not all of the effects of cAMP in various tissues are mediated through protein kinases (Bloom et al., 1975; Bartfai, 1980; Nathanson, 1977; Daly, 1975; Greengard, 1976, 1979, 1980), and since synaptic membranes contain a number of proteins whose phosphorylation is enhanced in the presence of cAMP (Ueda et al., 1973; Ueda and Greengard, 1977; Berman et al., 1980; Weller, 1979; Reddington and Mehl, 1979; Kelly et al., 1979; Ehrlich et al., 1977; Dunkley et al., 1976), it has been hypothesized that some of these proteins may be involved in the regulation of neuronal membrane permeability (Greengard, 1976; see Figure 1).

As discussed in the previous section, dopamine increases cAMP levels in homogenate and slice preparations from rat caudate nucleus. Therefore, the next objective was to investigate the effects of dopamine on the phosphorylation of synaptic membrane proteins from rat caudate nucleus and to determine whether the phosphorylation of these proteins was due to activation of cAMP-dependent protein kinases.

cAMP-dependent protein kinases have been found in both soluble and particulate fractions of mammalian tissues (reviewed by Rubin and Rosen, 1975; Nimmo and Cohen, 1977; Glass and Krebs, 1980; Krebs and Beavo, 1979). Soluble Type II kinases can be distinguished from soluble Type I protein kinases on the basis of a number of biochemical properties (Corbin and Keely, 1977), and it now appears that these properties are shared by the membrane-bound enzymes from brain (Rubin et al., 1979; Walter et al., 1978; Lohmann et al., 1980) as well as from other tissues.

Brain tissue is of particular interest since it contains both Type I and Type II cAMP-dependent protein kinases in soluble and particulate fractions, and in contrast to many other tissues, a substantial portion of the total protein kinase activity is membrane-bound (Hofmann et al., 1977; Walter et al., 1978; Maeno et al., 1971; Uno et al, 1976). The phosphorylation of synaptic membrane proteins from brain has been shown to be mediated by a Type II protein kinase (Walter et al., 1979; Lohmann et al., 1980). Although this enzyme has been extensively purified and characterized (Rubin et al., 1979; Uno et al., 1977), as have some of its protein substrates (Ueda and Greengard, 1977), there have been no detailed studies of the physiological regulation of the particulate enzyme from brain or the nature of its binding to particulate material. Since cAMP-dependent protein kinases are thought to be involved in the regulation of synaptic function, the response of these enzymes to neurotransmitter-mediated increases in cAMP is therefore of some interest.

Corbin and coworkers have demonstrated that epinephrine stimulates cAMP production and activates protein kinase activity in adipose tissue (Corbin et al., 1973b) and heart tissue (Corbin and Keely, 1977; Corbin et al., 1977). They have further shown that in the case of the particulate enzyme from heart, hor-

monally-induced increases in cAMP are associated with translocation of a large percentage of the catalytic subunit activity from the particulate to the supernatant fraction. Since catalytic activity could be readily dissociated from the membrane following activation of the enzyme by cAMP, these investigators have suggested that Type II regulatory subunits may specifically interact with membrane components to strategically position the catalytic subunit near particulate or otherwise compartmentalized substrates. In addition, the release of catalytic subunits from the membrane would facilitate the phosphorylation of soluble as well as membrane-associated substrates.

Although cAMP-dependent protein kinases from cerebral cortex have been shown to possess many of the characteristics of Type II enzymes from other tissues, the brain enzyme appears to be immunologically distinct from the Type II kinase from bovine heart and skeletal muscle (Lohmann et al., 1980; Rubin et al., 1979; Erlichman et al., 1980). It was therefore of interest to determine whether cAMP-dependent protein kinase from brain would exhibit the dissociation and solubilization properties previously established by Corbin and coworkers for the heart enzyme. It was also hoped that these studies might provide some insight into the possible mechanisms by which dopamine mediated increases in cAMP might lead to activation of synaptic membrane protein kinases.

The studies presented here indicate that the phosphorylation of several substrate proteins (Proteins Ia, Ib, and 57K) in synaptic membrane fractions from rat caudate nucleus is catalyzed by a cAMP-dependent protein kinase compatible with the results of others (Walter et al., 1979; Lohmann et al., 1980). Studies with kainic acid showed that the enzyme and its substrates are localized postsynaptically, consistent with published reports (Walter et al., 1979), and the previously established postsynaptic localization of cAMP (Rechardt and Harkonen, 1977;

Minneman et al., 1978; Garau et al., 1978; Figure 8). Destruction of presynaptic dopaminergic neurons, on the other hand, had no effect on cAMP-dependent protein kinase activity (Figures 26 and 27), indicating that this enzyme system may be particularly involved in the regulation of postsynaptic membrane function.

The addition of dopamine to homogenates or synaptic membrane fractions did not increase the phosphorylation of any of the identified substrates for the cAMP-dependent protein kinase. Similar results have been obtained by others (Hullihan et al., 1977), and this has been attributed in part to differences in the concentration of substrate required for the kinase and cyclase reactions (Hullihan et al., 1979).

Another possibility is that the concentration of cAMP required for half maximal stimulation of the brain kinase is in excess of the amount of cAMP generated as a result of dopamine stimulation. Studies by other investigators have shown that the K_a value for cAMP (concentration required for half maximal increase in enzyme activity) is between 0.1 and 0.2 uM (Uno et al., 1977; Beavo et al., 1974), which agrees reasonably well with the concentration of cAMP produced in homogenates of caudate tissue in the presence of dopamine (0.15 to 0.2 uM) under standard assay conditions. However, as pointed out by Beavo et al. (1974) the affinity of the kinase for cAMP in vivo is probably considerably less than the K_a values obtained for the purified enzyme in vitro, since in the latter case enzyme activity is measured at high substrate to enzyme ratios. It is thus apparent that considerable differences may exist between the control of enzyme reaction rates in vivo and in vitro.

Protein kinase activity in the present study was assayed routinely at a concentration of cAMP which was two orders of magnitude higher than the apparent K_a of the enzyme for cAMP. In view of the probable discrepancies

between the substrate requirements of the enzyme in vivo and in vitro, it is not surprising that dopamine failed to increase the activity of cAMP-dependent protein kinase under the present assay conditions. An alternative explanation for these findings is that homogenization of the tissue disrupts the normal arrangement of the dopamine-sensitive adenylate cyclase and cAMP-dependent protein kinase in the membrane leading to uncoupling of the two enzyme systems.

In view of these results, an alternative approach to the investigation of dopamine-mediated effects on cAMP-dependent protein kinase activity was adopted. This entailed modifying dopaminergic transmission through surgical or pharmacological means, and monitoring the effects of these procedures on the phosphorylation of synaptic membrane proteins. Destruction of presynaptic dopamine neurons with 6-OHDA, a procedure that is associated with an increase in the activity of dopamine-sensitive adenylate cyclase and in the number of postsynaptic dopamine receptors (Mishra et al., 1974, 1980; Wilkening and Makman, 1975; Seeman, 1980), had no effect on the pattern of cAMP-dependent protein phosphorylation (Figure 27). Chronic blockade of dopamine receptors with haloperidol also failed to increase cAMP-dependent protein phosphorylation in synaptic membrane fractions from rat caudate nucleus (Figure 28). Since this procedure had no significant effect on dopamine-stimulated adenylate cyclase activity these results were not surprising. These findings, however, are important, since they provide additional evidence that changes in dopamine receptor sensitivity may not involve cAMP.

These results are at variance with recent studies by Lau and Gnegy (1982), demonstrating that rats treated chronically with haloperidol show an increase in cAMP-dependent protein phosphorylation. This was characterized as an increase in the protein kinase activity ratio (Corbin et al., 1973a) using histone

as substrate. The state of protein phosphorylation in haloperidol and control rats, however, was unaffected by exogenous cAMP consistent with the results reported here. The reason for the discrepant observations obtained in the presence of endogenous and exogenous substrate for the kinase is unknown. It is noteworthy that even in control rats, assay of striatal tissue in the presence of cAMP and histone increased cAMP-dependent phosphorylation by only 50%. Since these assays were conducted using crude particulate fractions in which contamination by endogenous protein kinases is likely to be a significant factor, the interpretation of these results is not clear.

These authors also reported an increase in Ca^{++}-dependent protein phosphorylation in rats treated chronically with haloperidol. These results were based on the observation that EGTA inhibited Ca^{++}-dependent phosphorylation to a greater extent in haloperidol-injected rats relative to control rats. This was attributed to an increase in the calmodulin content in the membranes of haloperidol-treated rats.

The effects of haloperidol on Ca^{++}-dependent protein phosphorylation were also examined in the present study. However, no alterations in basal or Ca^{++}-stimulated phosphorylation of endogenous substrates were detected (data not shown). At present there is no explanation for the discrepancies in reported results between these studies.

Since neither dopamine nor procedures that alter dopamine function had any measurable effect on cAMP-dependent phosphorylation in synaptic membrane fractions, the effects of exogenous cAMP on protein kinase activity were investigated. An interesting observation that emerged from this work was the finding that preincubation of synaptic membranes with cAMP subsequently enhanced the phosphorylation of a 55,000 dalton phosphoprotein (Figure 15). In

contrast, when synaptic membranes were assayed directly for protein kinase activity without prior exposure to cAMP, cAMP increased the phosphorylation of a 57,000 dalton protein (Figure 15).

Photoaffinity labelling of these membranes with 8-N_3-[^{32}P] cAMP revealed a major band with a molecular weight of 55,000 and a minor polypeptide with an apparent molecular weight of 57,000 that comigrated with the ^{32}P-labelled proteins. This confirmed that the two proteins probably corresponded to the Type II regulatory subunit (R_{II}). Similar patterns have been observed in bovine brain (Rubin et al., 1979), whereas in rat brain only one protein of molecular weight 52-55,000 daltons has been detected using photoaffinity labelling or autophosphorylation with [^{32}P] ATP (Walter et al., 1978; Lohmann et al., 1980).

The 57k and 55k proteins are thought to correspond to the phospho and dephospho forms of the enzymes respectively (Rubin et al., 1979; Zoller et al., 1979; Walter and Greengard, 1978), the former subunit migrating more slowly on Tris-glycine-SDS gels. Greengard and his colleagues, however, have identified two proteins of 58,000 and 52,000 daltons in bovine brain that are autophosphorylated and photoaffinity labelled with 8-N_3-[^{32}P].cAMP. These proteins apparently do not merely represent the phospho and dephospho forms of the regulatory subunit as their mobility in SDS-PAGE is not altered when photoaffinity labelling is carried out in the presence of catalytic subunit and ATP, a procedure that converts all of the regulatory subunit to the phospho form (Lohmann et al., 1980).

Although the 57k and 55k proteins identified in the present study may represent the phospho and dephospho forms of the regulatory subunit, this possibility seemed unlikely since no evidence of interconversion between the two forms was observed under any of the experimental conditions used. However, photoaffinity labelling in the presence of ATP would be required to exclude this possibility.

The studies presented here provide evidence that the 57k and 55k components of the regulatory subunit show different patterns of phosphorylation depending on the incubation conditions used. Prior exposure of membranes to cAMP results in an increase in the phosphorylation of 55k when assayed in the presence or absence of cAMP. This increase is observed both in supernatant and particulate fractions. Autophosphorylation of the regulatory subunit has been characterized as phosphorylation that occurs as an intramolecular event in the absence of cAMP and as an intermolecular event in the presence of cAMP (Rangel-Aldao and Rosen, 1976). The increase in phosphorylation of 55k following previous exposure to cAMP is therefore consistent with a mechanism of autophosphorylation. Since the holoenzyme is devoid of phosphotransferase activity, and is dependent on cAMP for phosphorylation of free regulatory subunit or exogenous substrates (Rangel-Aldao and Rosen, 1976), the absence of phosphorylation of other membrane proteins under similar assay conditions confirms that the phosphorylation of 55k represents an intramolecular autophosphorylation reaction.

The present results can be explained if, in the presence of cAMP, the 55k protein undergoes dephosphorylation. Since more sites would then be available to incorporate labelled phosphate, the 55k protein would preferentially show an increase in phosphorylation when subsequently assayed with [^{32}P] ATP. It is proposed that preincubation of synaptic membranes with cAMP leads to dissociation of the Type II kinase. Reassociation of catalytic and regulatory subunits is known to occur rapidly following the removal of cAMP (Corbin and Keely, 1977). This is likely to have occurred in the present study since incubations did not include IBMX. Although phosphodiesterase cannot metabolize cAMP bound to the regulatory subunit (Brostrom et al., 1971), considerable hydrolysis of cAMP occurs at 0°C in the absence of phosphodiesterase inhibitors (Corbin et al., 1973). Removal

of free cAMP would tend to favour the formation of inactive holoenzyme according to the following equilibrium equation:

$$R_2C_2 + 4[cAMP] = R_2[cAMP]_4 + 2C$$

Dephosphorylation of the regulatory subunit of the kinase is also stimulated by cAMP (Uno et al., 1977; Ueda et al., 1975), and since the dephospho form of the enzyme reassociates much more rapidly than the phospho form (Rangel-Aldao and Rosen, 1976), this would also promote reassociation of the enzyme subunits under these conditions. Dephosphorylated R_{II} would then be available as a substrate for the catalytic subunit in an intramolecular phosphorylation reaction following the addition of [^{32}P] ATP. This hypothesis assumes that the degradation of cAMP at 0°C is sufficiently slow to enable the activation of endogenous cAMP-dependent phosphatases. NaCl, on the other hand, is known to inhibit reassociation of the Type II enzyme (Corbin et al., 1973) which explains the increase in catalytic activity in the supernate following incubation of synaptic membranes with NaCl and cAMP (Figure 18). The increase in 55k phosphorylation in this case would therefore represent an intermolecular phosphorylation reaction.

Dephosphorylation of 55k probably does not occur substantially under standard incubation conditions (30 s at 37°C) in the presence of cAMP since time course data indicate that dephosphorylation of the regulatory subunit is not apparent until after 2 min of incubation with cAMP (Ueda et al., 1975). Consistent with this, incubation of homogenates for 10-30 min in the presence of cAMP and IBMX leads to preferential dephosphorylation of 55k but not 57k (Figure 12). This effect apparently requires the continued presence of cAMP in the incubation medium, as it is not observed in the absence of IBMX (Figure 11).

The data presented here indicate that the dephosphorylation of proteins 57k and 55k appears to be differentially regulated by cAMP. In view of the

fact that both proteins appear to represent regulatory subunits of the Type II kinase the significance of these results is difficult to interpret. Similar results were obtained when membranes were prepared in the presence of EGTA and PMSF to inhibit protease activity. It is therefore unlikely that the 55k protein arose from proteolytic degradation of the 57k protein.

One of the major difficulties in evaluating the physiological significance of autophosphorylation is that there is comparatively little information available on the role of protein phosphatases in this process. Although the phospho form of the protein kinase appears to predominate in vivo in some tissues (Rangel-Aldao and Rosen, 1976; Rangel-Aldao et al., 1979; Uno, 1980), following dissociation of the enzyme by cAMP, the phosphorylated regulatory subunit is acted upon by a phosphoprotein phosphatase (Chou et al., 1977; Erlichman et al., 1975). Since the dephospho form of the regulatory subunit reassociates with the catalytic subunit much more readily than the phospho form (Rangel-Aldao and Rosen, 1976), the regulation of protein kinase activity in vivo clearly involves the participation of phosphoprotein phosphatases.

Since protein phosphatase activity was not directly measured in these studies, the possible role of this enzyme in mediating some of the observed effects on 55k phosphorylation remains speculative and the interpretation of these results is based solely on differences in the incorporation of labelled phosphate into the 55k protein relative to other membrane substrates for the Type II kinase. As Bar et al. (1981) have pointed out, it is impossible to distinguish between an effect that is mediated by protein kinases or protein phosphatases using endogenous "post hoc" phosphorylation. For example, an increase in the phosphorylation of a specific protein measured in vitro could be due to stimulation of a particular protein kinase or an increased activation in vivo of the appro-

priate phosphatase. The latter effect would increase the amount of dephosphoprotein and allow for more radioactive phosphate to be incorporated in vitro.

One of the limitations of research in the field of protein phosphorylation is the failure to demonstrate that changes in phosphorylation observed in vitro also occur in the intact system. The results of the present study demonstrate that phosphorylation patterns observed in broken cell preparations are not equivalent to those seen under more physiological conditions using brain slices. Although most of the phosphoproteins seen in synaptic membrane fractions were also seen in slices (Figure 42), the relative extent of stimulation by cAMP of ^{32}P incorporation into various proteins differed considerably between the two types of preparations. Thus cAMP markedly enhanced the phosphorylation of Protein I and 57k in synaptic membranes (Figure 13) but had little effect on the phosphorylation of these proteins in striatal slices (Figure 45). As described in Section 3.5.2 of **Results** a variety of agents that increased cAMP levels in striatal slices also failed to increase cAMP-dependent protein phosphorylation activity in this preparation.

These results may be accounted for by differences in the nature of the two experimental phosphorylation systems. Assay of protein kinase activity in homogenates involves a preincubation period in which substrates are dephosphorylated prior to labelling with [^{32}P] ATP, whereas in slices, endogenous substrates for the kinase may already be labelled to a high specific activity prior to the addition of stimulating agents. Therefore, differences in the turnover of labelled phosphate may not be readily apparent. As Sieghart et al. (1980) have pointed out, the relative incorporation of ^{32}P into individual proteins upon incubation of intact tissues with $^{32}P_i$ depends on many factors, including the specific activity of various ATP pools in the tissue, the amount and state of phosphorylation of

various phosphoproteins, and on the accessibility of substrates to various kinases and phosphatases.

The phosphorylation pattern obtained in synaptic membranes, on the other hand, may be the result of artificial juxtaposition of enzymes and substrates in the membrane during the preparation of the tissue. This might enhance the phosphorylation of proteins that would not be accessible to kinases under physiological conditions. An additional source of error is the contamination of synaptic membrane fractions by membrane elements (neuronal and non-neuronal) originating outside synaptic material. For example, protein 43k, that is a prominent substrate for Ca^{++}-dependent protein kinases in synaptic membranes and slices, is thought to be the alpha subunit of pyruvate dehydrogenase, indicating that some of the substrates identified in these preparations evidently derive from mitochondria.

A comparison of the effects of cAMP on the phosphorylation of Protein I in synaptic membranes and slices provides an illustration of the contrasting results obtained using different experimental procedures. As discussed above, Protein I is readily phosphorylated by cAMP-dependent protein kinases in synaptic membranes (Ueda and Greengard, 1977; Figure 13), and changes in its state of phosphorylation have also been demonstrated in brain slices using the "back phosphorylation" technique (Forn and Greengard, 1978). However, none of the effects of cAMP (Forn and Greengard, 1978) or neurotransmitters (Nestler and Greengard, 1980; Dolphin and Greengard, 1981) on the phosphorylation of this protein have been reproduced using direct labelling of intact tissues with $^{32}P_i$. The advantage of the latter approach is that it probably simulates more closely the situation in vivo, but, as alluded to previously, exchange incorporation of label due to endogenous kinases and phosphatases is a significant problem. Although

Greengard's "back phosphorylation" technique avoids this limitation, it is not yet clear whether changes in phosphorylation detected by this method accurately reflect changes in phosphorylation that occur in vivo.

As described above, background labelling of proteins in slices may be sufficiently high to mask increases in phosphorylation due to cAMP. The phosphorylation of Protein I is known to be regulated by cAMP as well as by Ca^{++}-dependent protein kinases (reviewed in Dolphin and Greengard, 1980). In the present study, the phosphorylation of Protein I was maintained when slices were incubated in Ca^{++}-free medium, whereas the phosphorylation of other proteins that were substrates for Ca^{++}-dependent protein kinases was decreased (Figure 46). This may indicate that the phosphorylation of Protein I under these conditions was mediated through cAMP-dependent protein kinases. Small increases in the phosphorylation of this protein were also observed when slices were incubated with 8-bromo-cAMP (Figure 45) or with IBMX (Figure 43), but these were not consistent findings. These results indicate that Protein I may be a substrate for cAMP-dependent protein kinase in striatal slices, although increases in its state of phosphorylation were not readily demonstrated due to high background levels of phosphorylation.

Consistent with the above, dopamine also failed to increase cAMP-dependent phosphorylation in striatal slices. More recently, using the back phosphorylation technique, Greengard's group has demonstrated that dopamine increases the phosphorylation of a 32,000 dalton protein in rat striatal slices. This protein was identified as a substrate for cAMP-dependent protein kinases in striatal homogenates (Walaas et al., 1983). Based on comparison of published autoradiograms with those obtained in the present study, it would appear that the 30k protein shown in Figures 11 and 13 is the same as the 32k protein described by

Walaas et al. (1983). Although the phosphorylation of 30k appears to be cAMP-regulated (Figures 11 and 13), dopamine did not increase its state of phosphorylation either in homogenates (Figures 11 and 12) or in slices incubated with $^{32}P_i$ (Figure 43).

Interestingly, subfractionation of striatal homogenates demonstrated that the 30k protein was preferentially enriched in cytosol fractions (Figure 13), and therefore the observation by Walaas et al. that 32k shows dopamine-dependent phosphorylation is somewhat surprising if dopamine acts through membrane receptors. It is also noteworthy that no effects of dopamine on the phosphorylation of Protein I were reported in the study by Walaas et al.

4.1.2 Ca^{++}-Dependent Protein Kinase Activity in Rat Caudate Nucleus

In contrast to the difficulties associated with demonstrating an effect of cAMP on protein phosphorylation in intact preparations from rat brain, there have been a number of studies documenting an effect of Ca^{++} on the phosphorylation of specific proteins in brain slices and synaptosomes although the identity of these phosphoproteins and their role in neuronal transmission has in most cases remained elusive. In the present study an attempt was made to define the substrates for Ca^{++}-dependent protein kinases in synaptic membrane fractions and slices from rat caudate nucleus. The results of this work demonstrate that Ca^{++} regulates the phosphorylation of specific synaptic membrane proteins from rat caudate nucleus compatible with the results of others (O'Callaghan et al., 1980a, 1980c; Schulman and Greengard, 1978a, 1978b; Kennedy and Greengard, 1981; De Lorenzo et al., 1977; Wrenn et al., 1980; Kuo et al., 1980).

Published studies have shown that the Ca^{++}-dependent phosphorylation of synaptic membrane proteins is dependent on calmodulin (Schulman and Green-

gard, 1978a, 1978b; O'Callaghan et al., 1980a, 1980c). This is indicated by the fact that membranes prepared in the presence of EGTA to remove endogenous calmodulin show a marked reduction in Ca^{++}-dependent phosphorylation. Similar results were obtained in the present study (Figure 31, panel 2). The addition of purified calmodulin to the assay medium was shown to restore Ca^{++}-dependent phosphorylation in studies by Schulman and Greengard (1978a, b) and O'Callaghan et al. (1980a, 1980c).

In contrast to the findings of these investigators, in the present study, the phosphorylation of only two proteins of molecular weights 63,000 and 53,000 daltons appeared to be markedly increased in the presence of Ca^{++}-calmodulin (Figure 35: panel 1, lane d). The regulation of the phosphorylation of proteins 63k and 53k by calmodulin is well-established (De Lorenzo et al., 1979; Schulman and Greengard, 1978a, 1978b). These proteins have been extensively investigated by DeLorenzo and his colleagues, who have demonstrated that their phosphorylation is associated with neurotransmitter release in intact synaptosomes (De Lorenzo, 1976) and synaptic vesicles (De Lorenzo et al., 1979; De Lorenzo and Freedman, 1978). Recent studies have identified these proteins as the alpha and beta subunits of neurotubulin (Burke and De Lorenzo, 1981).

The other substrates for Ca^{++}-dependent protein kinases in synaptic membranes, namely proteins 50k, 20k, and 16k, did not show increases in their phosphorylation in the presence of calmodulin (Figure 35: panel 1, lane 4), although their phosphorylation was substantially decreased by preparing and washing membranes in 2 mM EGTA (Figure 31). The molecular weights of these proteins are similar to those reported by other authors for substrates of Ca^{++}-dependent protein kinases in brain membranes.

The 20,000 dalton protein may be myosin light chain, which has been shown to be phosphorylated by myosin light chain kinase, an enzyme requiring both calmodulin and Ca^{++} for activation (Dabrowska et al., 1978). Wrenn et al. (1980) have also reported the phosphorylation of a 21,000 dalton protein in particulate fractions from guinea pig cerebral cortex that is specifically stimulated by Ca^{++} plus phospholipid. The fact that the phosphorylation of the 20k protein in the present study did not require calmodulin, yet was inhibited by EGTA and haloperidol, suggests that it may in fact correspond to the 21,000 dalton protein described by Wrenn and coworkers. However, as myosin light chain kinase is known to be particularly susceptible to proteolysis, this might also explain the failure to detect calmodulin-dependent phosphorylation of this protein in the present study.

The 16,000 dalton protein described in the present study probably corresponds to myelin basic protein (Petrali et al., 1980; Sulakhe et al., 1980) that has been identified as a substrate for phospholipid-sensitive protein kinase (Minakuchi et al., 1981).

A 50,000 dalton protein has also been identified in studies of Ca^{++}-dependent phosphorylation in brain (Schulman and Greengard, 1978; O'Callaghan et al., 1980), although the properties of this substrate have not been investigated. Protein B-50, a 48,000 dalton phosphoprotein, has been extensively purified and characterized by Gispen's group (Zwiers et al., 1980), and may be equivalent to the 50k protein described by other investigators. The phosphorylation of B-50 is stimulated by Ca^{++} and inhibited by ACTH (Zwiers et al., 1980). The latter process may have behavioural relevance since intramuscular administration of ACTH-like peptides induces excessive grooming and licking behaviour in the rat (Gispen et al., 1975).

The calmodulin and Ca^{++}-dependent protein kinase activities could further be distinguished using pharmacological approaches. The calmodulin-dependent phosphorylation of proteins 63k and 53k was completely inhibited by 100 uM trifluoperazine (TFP), (Figure 35, panel 3), an antipsychotic agent that has been shown to bind calmodulin with high affinity in a Ca^{++}-dependent fashion (Levin and Weiss, 1979). TFP, however, did not inhibit the Ca^{++}-dependent phosphorylation of proteins 50k, 20k, and 16k, although haloperidol at equivalent concentrations, blocked both calmodulin plus Ca^{++} and Ca^{++}-stimulated protein kinase activity (Figure 35, panels 2 and 3)

These results raised the possibility that the Ca^{++}-dependent phosphorylation activity antagonized by haloperidol might be mediated through the phospholipid-sensitive protein kinase (Takai et al., 1979; Kuo et al., 1980; Wrenn et al., 1980). Although published studies using partially purified protein kinase have shown that TFP is a more potent inhibitor of the enzyme than haloperidol (Schatzman et al., 1981), these drugs may interact differently with intact membrane preparations. As phospholipid-sensitive protein kinase activity was not investigated in the present study, this interpretation remains speculative.

The results of the present investigations demonstrate that synaptic membranes from rat caudate nucleus contain calmodulin-dependent as well as calmodulin-independent protein kinase activity. This is indicated by the fact that (1) substrates for Ca^{++}-calmodulin and Ca^{++}-dependent protein kinases do not appear to overlap; (2) the phosphorylation of these substrates can be selectively inhibited by TFP and haloperidol; (3) calmodulin and Ca^{++}-dependent protein kinase activities are distributed in different subcellular fractions: the former is found in soluble and particulate fractions (Figure 13), whereas the latter appears to be confined to membrane fractions (Figures 13, 29, 30).

It would appear from these data that the phosphorylation of at least some membrane proteins is probably regulated independently of calmodulin. Other investigators have maintained, on the other hand, that Ca^{++}-dependent phosphorylation in synaptic membranes is only observed in the presence of added calmodulin (Schulman and Greengard, 1978a, 1978b; O'Callaghan et al., 1980a, 1980c). The reason for the discrepancies between the results of the present study and other published accounts is not clear. The concentration of calmodulin used in the present study does not appear to be a factor, since the amount used was comparable to published reports.

An alternative possibility is that the membrane preparation used in the present study was not fully depleted of endogenous calmodulin. In the paper by Schulman and Greengard (1978b) hypotonic lysis of synaptosomes was carried out for 30 min on ice, in contrast to the present study in which membranes were isolated immediately following the lytic step. This could explain the higher levels of endogenous Ca^{++}-dependent protein kinase in synaptic membranes observed in the present study, but does not account for the fact that the phosphorylation of 63k and 53k was only observed in the presence of exogenous calmodulin, nor does it account for the fact that exogenous calmodulin was unable to restore phosphorylation of any other membrane proteins except 63k and 53k following preparation of membranes with EGTA.

One explanation for the differential phosphorylation of protein substrates in the presence or absence of calmodulin is that these effects may be mediated by different enzymes with different susceptibilities to inhibition by EGTA and neuroleptic drugs. Studies by Yamauchi and Fujisawa (1980), and Kennedy and Greengard (1981), have shown that rat brain contains at least three forms of soluble and one form of particle-associated calmodulin-dependent pro-

tein kinase activity with different specificities for particular substrates. Since proteins 63k and 53k are highly phosphorylated in cytosol fractions of rat brain (Burke and De Lorenzo, 1981; O'Callaghan et al. 1980a, 1980b; Figure 13), whereas the other substrates for Ca^{++}-dependent protein kinases appear to be firmly bound to synaptic membranes (Figures 29 and 30), it is possible that these two groups of proteins may be phosphorylated by different classes of calmodulin-regulated enzymes. Clearly further studies are required to resolve some of these issues.

Another interesting observation that emerged from this work was the finding that the phosphorylation of a 43,000 dalton protein was consistently inhibited by Ca^{++} when synaptic membranes were prepared in the absence of EGTA, and stimulated by Ca^{++} when membranes were prepared in the presence of EGTA (Figure 31, panels 1 and 2). Ca^{++}-mediated inhibition of 43k phosphorylation was also observed in a paper by Sieghart et al. (1980), although this effect was not discussed by these authors.

The neuroleptic haloperidol stimulated the basal phosphorylation of this protein (Figure 35, panel 2). It is probable that this is related to non-specific membrane effects of this drug (Seeman, 1977). Since the basal phosphorylation of this protein can be eliminated by EGTA (Figure 31, panel 2), it appears that its phosphorylation can be supported by endogenous Ca^{++} associated with synaptic membranes. Haloperidol may enhance the phosphorylation of 43k by interacting with hydrophobic regions in the membrane or on the protein thereby increasing the number of phosphorylation sites available to the endogenous protein kinase.

Finn et al. (1980) have shown that the phosphorylation of a 40,000 dalton protein in hippocampus is stimulated by low concentrations of calmodulin and inhibited by TFP. Although the molecular weight of the protein described by Finn

and coworkers is not identical to that of the 43k protein described here, numerous workers have shown changes in the Ca^{++}-dependent phosphorylation of a protein in this molecular weight range (40–43,000 daltons). For example, the in vitro phosphorylation of a 40–43,000 dalton protein has been shown to be affected by injections of pentobarbitol (Conway and Routtenberg, 1979), chronic administration of opiates (Davis and Ehrlich, 1979; O'Callaghan et al., 1979), high frequency electrical stimulation of hippocampal slices (Browning et al., 1979), glutamate stimulation in synaptosomes (Sieghart, 1981), and the acquisition of avoidance learning (Routtenberg, 1980).

Although some researchers have claimed that this protein is equivalent to neuronal actin (Hofstein et al., 1980; Hershkowitz, 1978), the bulk of the recent experimental evidence appears to indicate that this protein corresponds to the alpha subunit of pyruvate dehydrogenase (Kelly et al., 1979; DeBlas et al., 1979; Sieghart, 1981; Magilen et al., 1981; Browning et al., 1981a; Morgan and Routtenberg, 1980). Moreover, there is no good evidence that actin is a substrate for protein kinases.

The enzyme pyruvate dehydrogenase occupies a pivotal role in brain metabolism. It is localized in the mitochondrial matrix, and is regulated by allosteric factors as well as the state of phosphorylaion of the alpha subunit. Dephosphorylation of the enzyme converts the enzyme to its active form and is mediated by a Ca^{++}-stimulated phosphoprotein phosphatase. This enzyme may be involved in the mitochondrial sequestration of Ca^{++}, since mitochondria accumulate Ca^{++} in the presence of inhibitors of pyruvate dehydrogenase kinase (Browning et al., 1981a). Thus the state of phosphorylation of this enzyme may be important in the regulation of intracellular Ca^{++} levels secondary to neuronal stimulation. Alternatively, the phosphorylation of this protein may be associated

with a general change in the metabolic activity of the neuron consistent with altered levels of stimulation.

Many of the effects of Ca^{++} on protein phosphorylation in synaptic membrane fractions were also observed in striatal slices. Thus, Ca^{++} enhanced the phosphorylation of proteins 50k, 43k, 20k and 16k (Figure 46) and these effects were antagonized by neuroleptic drugs (Figure 50). A significant observation that emerged from this work was the finding that dopamine increased the phosphorylation of two proteins with molecular weights of 43k and 62-64k in rat striatal slices. It is significant that protein 43k has been tentatively identified as the alpha subunit of pyruvate dehydrogenase. This may indicate that increases in protein phosphorylation seen in the presence of dopamine are associated with metabolic changes in striatal neurons rather than alterations in membrane permeability.

The phosphorylation of protein 50k was also enhanced by dopamine, but this effect did not reach statistical significance. This represents the first demonstration of an effect of dopamine on protein phosphorylation following incubation of striatal slices with $^{32}P_i$. Recently Hofstein and Segal (1982) using a similar experimental approach have shown that norepinephrine increases the phosphorylation of a number of proteins in rat hippocampal slices, including proteins of molecular weights 47, 43, and 17,000 daltons. These proteins appear to be the same as those identified in the present studies.

Consistent with the results reported here, in the study reported by Hofstein and Segal (1982), incubation of hippocampal slices in the presence of cAMP analogues or the phosphodiesterase inhibitor IBMX failed to increase the phosphorylation of any hippocampal proteins, despite the fact that norepinephrine is known to elevate cAMP in this preparation. As discussed in detail previously,

patterns of protein phosphorylation in brain slices incubated with $^{32}P_i$ are not equivalent to those seen in broken cell preparations, or in slices assayed by the back phosphorylation technique.

The interpretation of the present results is complicated by the fact that the role of Ca^{++} in dopamine receptor function is ill-defined. Although Ca^{++}-dependent protein kinases have been implicated in the control of dopamine synthesis (Yamauchi et al., 1981) and release (De Lorenzo et al., 1979), evidence of postsynaptic regulation of dopamine function through Ca^{++} is more limited. Benardo and Prince (1982) have recently shown that dopamine enhances Ca^{++}-dependent conductance in hippocampal slices. This appears to be a postsynaptic event, and may either be due to increased entry of Ca^{++} into neurons, or enhanced release of Ca^{++} from intracellular stores.

It is difficult to determine in the present study whether the dopamine-mediated effects on protein phosphorylation occur presynaptically or postsynaptically. Studies by other investigators (Sieghart et al., 1980) have shown that Ca^{++}-dependent phosphorylation in synaptosomal and synaptic membrane proteins from rat striatum is substantially decreased following kainic acid lesions. This indicates that many of these proteins are localized postsynaptically. It would be interesting to determine the effects of presynaptic membrane destruction using 6-OHDA on Ca^{++}-dependent phosphorylation in this preparation as well, since many of the neuronal processes attributed to Ca^{++} appear to be presynaptic.

The effects of dopamine on protein phosphorylation in striatal slices could be distinguished from the effects of depolarizing agents such as K^+ or veratridine. Both K^+ and veratridine significantly decreased the phosphorylation of a 90,000 dalton protein in striatal slices (Figures 48 and 49). Similar findings have been reported by others (Kreuger et al., 1977). The veratridine-induced inhibition

of phosphorylation of this protein was reversed by TTX indicating that this effect may at least be partially dependent on Na^+ influx through voltage-sensitive channels (Table VIII).

Although the identity of this protein has not been established, recent studies have shown that the [Ca^{++} + Mg^{++}] ATPase has a subunit of 92,000 daltons that is phosphorylated in association with acetylcholine release (Michaelson and Arissar, 1979; Gordon et al., 1977). The activity of the [Ca^{++} + Mg^{++}] ATPase from brain has been shown to be dependent on calmodulin (Sobue et al., 1979; Kuo et al., 1979). It would be interesting to determine whether this protein bears any similarity to the 90K protein described in the present study.

The results of recent investigations indicate that ion channels are also probable substrates for protein kinases in neuronal membranes. Thus there is evidence that the Na^+ channel from rat brain can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (Costa et al., 1982). Similarly in Aplysia, 5-HT mediated increases in K^+ conductance are mimicked by the catalytic subunit of cAMP-dependent protein kinase (Kaczmarek et al., 1980; Castellucci et al., 1980) and antagonized by the protein kinase inhibitor (Adams and Cervitan, 1982). The substrate for the enzyme is thought to be a specific component of the K^+ channel or a protein related to it .

These studies point to the probable importance of protein kinases in regulating ion fluxes in neuronal membranes and emphasize the necessity of correlating these biochemical changes with meaningful physiological parameters. In the case of dopamine, biochemical studies have preceded an adequate understanding of the physiology of the system. Thus the current controversy as to which population of dopamine receptors is physiologically relevant, those linked to adenylate cyclase or not, is based predominantly on data from receptor binding

studies, the methodology and interpretation of which are themselves subject to dispute.

Although the results of the present series of investigations do not support a role for cAMP-dependent protein kinase in the mediation of dopamine function in rat caudate nucleus, one is still left with the burden of interpreting the significance of the dopamine-mediated increases in cAMP. Given that rat caudate nucleus contains an active membrane protein kinase that is responsive to cAMP, one must assume that increases in intracellular cAMP produced by dopamine must be accompanied by activation of this enzyme. The inability of several investigators to detect such a phenomenon in homogenates or in intact cell preparations indicates that if such changes in phosphorylation are indeed occurring, they cannot be detected by conventional techniques. Indeed the sophisticated biochemical procedures employed by Greengard's group (Nestler and Greengard, 1980) to demonstrate cAMP-dependent and neurotransmitter-dependent alterations in protein phosphorylation in intact brain preparations attests to this point.

Finally, one must address the significance of the dopamine-mediated increase in Ca^{++}-dependent protein phosphorylation. Since there is no information on the mechanism of action of dopamine receptors not linked to adenylate cyclase, it is difficult to determine whether the observed changes in protein phosphorylation are directly mediated through dopamine receptors. Pharmacological approaches would normally be of some value in resolving this issue: However, as previously shown, neuroleptics are potent antagonists of Ca^{++}-dependent protein phosphorylation, and these effects are mediated independently of their capacity to bind dopamine receptors. Also, since the concentration of neuroleptics used in these studies was relatively high, it would be inappropriate to ascribe any of the effects observed with these agents to a specific effect on dopamine receptors.

4.2 Summary

The aim of this work was to examine the effect of dopamine on protein phosphorylation in rat caudate nucleus. Endogenous substrates for cAMP and Ca^{++}-dependent protein kinases were identified as a prelude to studies of dopamine-dependent protein phosphorylation. The results presented here demonstrate that dopamine increases intracellular cAMP and stimulates protein phosphorylation in rat striatal slices, although this latter effect was mediated independently of cAMP. It will be of interest in the future to determine the function of the phosphoproteins identified in this study, and to examine their roles in dopaminergic transmission.

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