INVESTIGATIONS OF CHEMOSENSORY MECHANISMS IN CULTURED GLOMUS CELLS OF THE RAT CAROTID BODY USING PATCH CLAMP/ WHOLE-CELL RECORDING

By

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ABSTRACT

The mammalian carotid body is a small chemosensory organ, located at the carotid bifurcation which senses blood levels of oxygen, carbon dioxide, and acidity and controls breathing. In response to these chemosensory stimuli nerve impulses emanating from the carotid body are conveyed along the carotid sinus nerve and forward information to the respiratory control centre in the brainstem. The chemosensors in the carotid body were presumed to be the glomus or type I cells, which contain neurotransmitters and receive a direct sensory innervation, although relatively little is known about their physiology. The goals of this thesis were to characterize the electrophysiological properties of glomus cells from the rat carotid body and determine if they are modified by exposure to the chemosensory stimuli. The use of cell cultures of dissociated carotid bodies allowed virtual isolation of glomus cells making them accessible for study by the modern electrophysiological patch clamp/whole-cell technique. This high resolution method is ideally suited for electrophysiological recording from small cells (such as the glomus cells) and allows the measurement of current flow through single ion-channel proteins.

Whole-cell recording from glomus cells under voltage clamp revealed the presence of voltage-activated Na^+, K^+, and Ca^{2+} currents as well as leakage anion (Cl^-/HCO_3^-) channels which were unmasked in physiological bicarbonate/CO_2-containing media. Acute exposure to hypoxia ($Po_2 \approx$ 20-30 Torr) caused a selective suppression of K^+ currents in glomus cells while chronic exposure (up to 2 weeks) to hypoxia ($Po_2 \approx$ 50 Torr) promoted the selective induction of Na^+ channels and caused hypertrophy of these cells. Both these effects appear to be mediated via the second messenger cAMP and may

account for the increased sensitivity and enlargement of the carotid body following chronic hypoxia. The other chemosensory stimuli, extracellular acidification and hypercapnia, decreased both Na^+ and K^+ currents in glomus cells. The effects of both these stimuli were likely mediated via cytoplasmic acidification as directly lowering pH_i had similar effects on the membrane currents and carbonic anhydrase activity, which is present in these cells cytoplasm, is known to cause acidification in the presence of CO_2.

A discussion of the intrinsic physiological mechanisms of chemotransduction by glomus cells revolves around modulations of the voltage-activated K^+, and Na^+ currents during chemosensory stimulation causing alterations in intracellular Ca^{2+} and pH. These changes in Ca^{2+}_i and pH_i cause neurotransmitter release onto the sensory nerve endings finally leading to increased breathing by a reflex pathway through the respiratory control centre in the brainstem.

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GENERAL INTRODUCTION

The carotid body is a small peripheral chemosensory organ which helps control ventilation in mammals and birds during changes in arterial Po_2, Pco_2, and pH. The first anatomical description of the carotid body was in the early 1700's but it was not until 1926 when De Castro proposed that the carotid body might be a sensory organ for substances transported in the blood (see Eyzaguirre and Zapata 1984). Analogous chemosensory structures have been found in lower vertebrates and have been identified as the carotid labyrinth in reptiles (Ishii et al 1985) and amphibians (Kusakabe et al 1991), and in addition, chemoreceptors have been localized to the first gill arch in teleosts (Burleson and Milsom 1990). The mammalian organ is highly organized and discrete unlike the structures in lower vertebrates. The carotid body is reported to have the largest blood flow per unit weight of any body tissue (McDonald 1981), has a sizable O_2 consumption indicating a high metabolic rate (Eyzaguirre and Zapata 1984), and is innervated by sensory nerve fibers which project to the respiratory centre in the brainstem. The main cell types of the carotid body are the glomus (or type I) cells, many of which are innervated, and the supporting sustentacular (or type II) cells. The actual mechanisms by which this organ responds to changes in O_2, CO_2, and pH in the blood are poorly understood despite

many years of research, in part because of the difficulty in obtaining reliable physiological data from the small ($\approx$ 10 μm) glomus cells using conventional techniques. Indeed, up until a few years ago, the actual sensors of the chemosensory stimuli were in doubt, and both the glomus cells and apposed sensory nerve terminals were leading candidates (McDonald 1981; Eyzaguirre and Zapata 1984).

Cell types in the carotid body

The carotid body is a small organ (1-2 mm. in diameter) which lies in the bifurcation of the carotid artery in mammals. During development, the primordia of the carotid body can be distinguished as early as 14 days of gestation in the rat embryo (Kondo 1975) and 6 weeks of gestation in the human embryo (McDonald 1981). Grafting experiments using the chick-quail chimera method (Le Lievre and Le Douarin 1975) showed that the majority of cells in the avian carotid body are of neural crest origin (Pearse et al 1973).

The adult carotid body is comprised of two main cell types, the glomus or type I cells and the sustentacular or type II cells. Glomus cells constitute from 57% of the total cells in human carotid bodies to 79% in rat carotid bodies (McDonald 1981). Some researchers have further separated glomus cells into subtypes based on: i) the size of their cytoplasmic dense-cored vesicles (DCV), e.g. cells with large DCV (110-120 nM) = type A, cells with small DCV ($\approx$ 90 nM) = type B (McDonald and Mitchell 1975; McDonald 1981); ii) staining properties, e.g. dark or light types of glomus cells based on electron density of cytoplasm under transmission electron

microscopy (McDonald 1981); iii) type of neurotransmitter present, e.g. type A = dopamine, type B = norepinephrine (McDonald 1981); and iv) type of acetylcholine receptors (AChR) present, e.g. one type = muscarinic AChR, another type = nicotinic AChR (Chen and Yates 1984). Glomus cells are arranged in small groups (≈ 30 cells) or clusters called glomoids which are in close proximity to blood vessels.

Sustentacular cells range from 21% to 43% of the total cell population (McDonald 1981) and these cells envelop the glomus clusters in an intimate association. Sustentacular cells are generally considered to be "glial-like" elements of the carotid body and have many similarities to Schwann and satellite cells which are found in close relation to nerve cells and fibers elsewhere in the peripheral nervous system (Eyzaguirre and Zapata 1984; McDonald 1981). These cells may play a role in response to injury and may be contractile (McDonald 1981), but are not thought to play a direct role in the chemotransduction process. The most striking difference between glomus and sustentacular cells, at the ultrastructural level, is the presence of many dense-cored vesicles in glomus cells but not in sustentacular cells (McDonald and Mitchell 1975; McDonald 1981). These vesicles contain putative neurotransmitters, mainly catecholamines, which will be discussed later.

A third cell type found in small numbers in carotid bodies are ganglion cells. These relatively large neuronal cells (20-40 μm) comprise less than 1% of total carotid body cells. In fact, data from studies on the rat carotid body indicate that only 25 of 11,500 cells are ganglion cells (McDonald 1981). These cells are of two types;

i) parasympathetic, migrating along the glossopharyngeal nerve, and ii) sympathetic, originating from the superior cervical ganglion (McDonald and Mitchell 1975). It has been suggested that the ganglion cells play a role in the vasomotor pathways which constrict or dilate the capillaries in the carotid body in response to various stimuli (McDonald 1981).

Innervation and synaptic interactions in the carotid body

The carotid body is innervated mainly by sensory fibers of the carotid sinus nerve, a branch of the glossopharyngeal nerve and sympathetic (motor) fibers which travel along the postganglionic trunk of the superior cervical ganglion. Axonal degeneration studies in the rat carotid body have shown that 95% of the nerve endings are from the petrosal (sensory) ganglion of the glossopharyngeal nerve (McDonald and Mitchell 1975). Retrograde-labelling studies have confirmed that neuronal cell bodies which lie in the distal one-third to one-half of the petrosal ganglion have axons terminating in the carotid body (Katz and Black 1986). However 5% of the axons which end in the carotid body are sympathetic (motor) in origin (McDonald and Mitchell 1975).

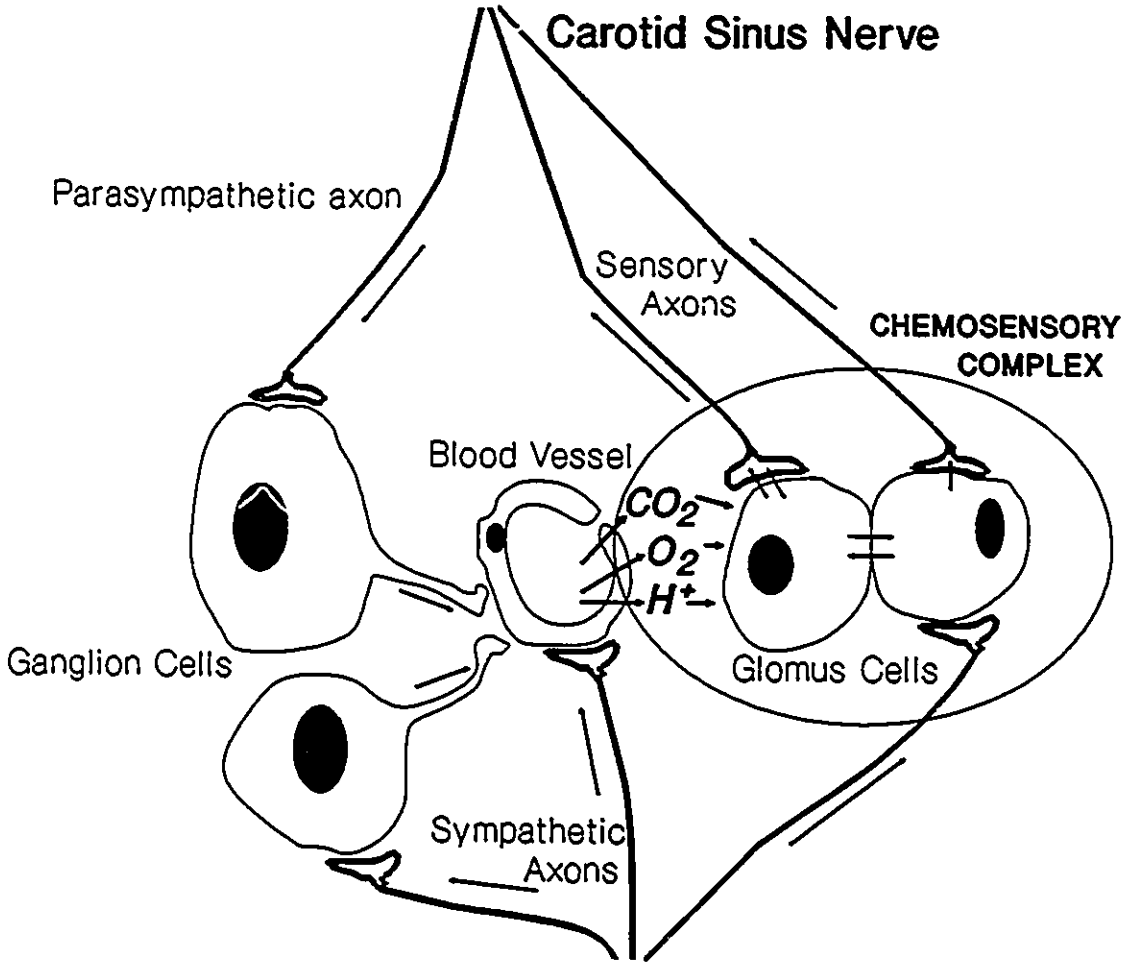
An important characteristic of the intact carotid body is the highly organized pattern of synaptic connections found: i) between nerve endings and glomus cells, ii) between adjacent glomus cells, and iii) between nerve endings and blood vessels. In 1975, McDonald and Mitchell published a comprehensive ultrastructural study on the innervation of the rat carotid body describing these interconnections. Afferent nerve

endings (95% of total) could be either presynaptic or postsynaptic to glomus cells and indeed many of these contacts formed reciprocal synapses. Two types of vesicles, large dense-cored vesicles, and smaller clear-cored vesicles occurred at these synapses. These vesicles contain putative neurotransmitters which may be important in chemosensory processing in the carotid body (see below). Sensory endings on glomus cell processes had a larger ratio of clear-cored vesicles in relation to dense-cored vesicles, while endings on glomus cell bodies contained an equal ratio. McDonald and Mitchell (1975) found that 5% of the contacts between sensory nerve endings and glomus cells showed reciprocal synapses. These are closely-paired synapses between two adjacent cells in which one cell is postsynaptic at one synapse and presynaptic at the other. Reciprocal synapses, as well as gap junctions, were also seen between neighbouring glomus cells which suggest that intercellular communication is important. The contact between the sensory nerve ending and the glomus cell has been the focus of much attention as it is the presumed site where chemotransduction and the relay of chemosensory information occurs and is often referred to as the "chemosensory complex" (Eyzaguirre and Zapata 1984).

In addition to the synaptic interactions between glomus cells and afferent nerve endings the blood vessels entering the carotid body are innervated by both sympathetic nerves and parasympathetic nerves (McDonald 1981). The carotid body is a highly vascularized organ receiving a large blood flow from a small branch of the external carotid artery. The organization and innervation of the capillary networks through the carotid body allow both the total blood flow to the organ and the internal blood flow

to be precisely controlled (Eyzaguirre and Zapata 1984). The dual innervation pattern, sympathetic and parasympathetic, allows blood flow to be precisely controlled through the carotid body by constriction or dilation of the blood vessels (Eyzaguirre and Zapata 1984).

A summary of the neuronal and vascular circuitry in the carotid body is shown below and is adapted from several sources (Biscoe and Duchen 1990b; Eyzaguirre and Zapata 1984; McDonald and Mitchell 1975).



The representation above indicates why attempts to understand the mechanisms of chemotransduction from studies on intact carotid bodies *in vivo* have been difficult. The complex synaptic interactions and the frequent inability to separate effects due secondarily to circulatory changes have added to problems in the interpretation of physiological data from *in vivo* and *in vitro* carotid body recordings (see below).

Neurotransmitters in the carotid body

There are several types of potential neurotransmitters in the carotid body including catecholamines, acetylcholine, 5-hydroxytryptamine (serotonin), and peptides such as substance P, met-enkephalin, and atrial natriuretic peptide (ANP) (McDonald 1981; Wang,Z.-Z et al 1991b). Studies in the rat, rabbit, cat, and pig have shown the predominant catecholamine to be dopamine (58-86%), whereas norepinephrine (14-37%) and epinephrine (0-6%) are found in smaller amounts (McDonald 1981). Carotid bodies release dopamine on exposure to hypoxia in a calcium-dependent manner since it can be prevented by the use of a calcium-free, high magnesium perfusate (Fidone et al 1982). This effect can be mimicked in dissociated carotid body cultures of glomus cells exposed to hypoxia in the absence of any nervous (sensory or motor) inputs (Fishman et al 1985). Dopamine (DA) is also released from the isolated carotid body *in vitro* in response to low pH and elevated CO_2 (Rigual et al 1991). In general, injections of DA through *in vitro* carotid body preparations appeared to have an inhibitory effect on the chemosensory discharge from the carotid body but this result was highly variable and seemed to be species

dependent (Eyzaguirre and Zapata 1984). Intracellular recordings from glomus cells also produced variable results with the majority of cells ($\approx$ 70%) depolarizing in response to the application of DA (Goldman and Eyzaguirre 1984; Monti-Bloch and Eyzaguirre 1990). A recent patch-clamp study has shown that DA, in nanomolar concentrations, can attenuate the voltage-activated calcium current in rabbit glomus cells (Benot and Lopez-Barneo 1990) suggesting that glomus cells may have an autoinhibitory feedback pathway dependent on DA.

There is evidence for the presence of the neurotransmitter acetylcholine (ACh) in the carotid body (McDonald 1981). Since both acetylcholinesterase and a high-affinity uptake mechanism for choline has been localized to glomus cells it is likely that ACh is synthesized in these cells (Fidone et al 1977; Nurse 1987). The role of ACh in chemotransduction is still unclear though some workers propose it is the sensory neurotransmitter that relays chemosensory information from the glomus cells to the apposed sensory nerve endings (Eyzaguirre and Zapata 1984; Fidone et al 1977). In some early studies perfusion of ACh through *in vitro* carotid body preparations showed a pronounced increase in the afferent discharge (Eyzaguirre and Koyano 1965b). Other studies determined that different subpopulations of glomus cells contain either nicotinic or muscarinic ACh receptors (Chen and Yates 1984) and intracellular recordings from glomus cells has shown that the majority of cells depolarize when exposed to either ACh, nicotine, or bethanecol (muscarinic agonist) but these results were quite variable (Eyzaguirre et al 1990; Monti-Bloch and Eyzaguirre 1990). Therefore, like in the case for dopamine, the studies with ACh

were variable which may, in part, be due to species differences (Eyzaguirre and Zapata 1984).

There have been fewer studies on the roles of the remaining putative neurotransmitters in the carotid body, eg. serotonin, substance P, met-enkephalin, and atrial natriuretic peptide (ANP). Both substance P or met-enkephalin cause depolarization of the majority of glomus cells (Eyzaguirre et al 1990; Monti-Bloch and Eyzaguirre 1990). A recent study has localized ANP-like immunoreactivity in glomus cells of the cat carotid body and moreover ANP is a potent inhibitor of chemosensory discharge from the carotid body in response to hypoxia (Wang, Z.-Z. et al 1991b).

The physiological roles of these neurotransmitters during the chemosensory responses of the carotid body are still unclear and are beyond the goals of this thesis.

Theories of chemotransduction

There are two major theories for chemotransduction in the carotid body which both involve the 'chemosensory complex'. In the first theory the sensory nerve endings in the carotid body are the transducing element of the chemosensory stimuli, low Po_2, high Pco_2, and low pH, while the glomus cells act as dopaminergic interneurons which modulate the response (McDonald and Mitchell 1975; McDonald 1981). In the second theory the glomus cells are the primary receptor element which sense changes in Po_2, Pco_2, and pH and release neurotransmitter onto the apposed

sensory nerve ending (Eyzaguirre and Zapata 1984). In over 60 years of physiological studies researchers have attempted to elucidate the mechanisms of chemotransduction in the carotid body and determine the primary sensor for the chemosensory stimuli.

Physiological studies of the carotid body

Physiological recordings of the electrical activity from the carotid body were first performed in the early 1930's by Heymans who received the Nobel prize for Physiology and Medicine in 1938 for his contributions (see Biscoe 1971; Eyzaguirre and Zapata 1984). These extracellular recordings were taken from the carotid sinus nerve (sensory) and showed changes in activity in response to asphyxia. Other early studies showed a hyperbolic relationship between arterial Po_2 and carotid sinus nerve activity (Eyzaguirre and Koyano 1965a) while a linear relationship was obtained for the other chemosensory stimuli, Pco_2 and pH (Eyzaguirre and Koyano 1965a; Eyzaguirre and Zapata 1984). Several groups still use *in vivo* preparations to study chemotransduction mechanisms in the carotid body (Fitzgerald et al 1990; Di Giulio et al 1990; Shirahata and Fitzgerald 1991a,b) while others use *in vitro* preparations, in which the carotid body and sinus nerve are isolated from the animal (Baron and Eyzaguirre 1977; Eyzaguirre et al 1989; Iturriaga et al 1991; Rigual et al 1991). A major problem in these studies is that the site of action of the chemosensory stimulus cannot be easily determined since recordings are usually from the afferent nerve, at a point far removed from the transduction site. Hence effects occurring at the glomus cell could not be unambiguously separated from those at the nerve terminal. To this

end, direct physiological studies have been attempted to determine the effects of chemosensory stimuli on glomus cells.

Glomus cell physiology

Glomus cells have proven to be difficult to characterize physiologically because of their small size (8-10 μm diameter) and inaccessibility *in situ*. Attempts to make the cells more accessible include the use of carotid body slices (Eyzaguirre et al 1989) though by far the most successful approach to date has been the use of freshly-isolated glomus cells (Duchen et al 1988; Lopez-Barneo et al 1988; etc.), or cell cultures of the dissociated carotid body (Fishman and Schaffner 1984; Fishman et al 1985; Nurse 1987,1990; Stea and Nurse 1989,1991a,b). These cultures in particular provide an attractive alternative to studies *in situ* for the investigations of chemosensory mechanisms. First, glomus cells can be isolated and observed under direct visual control thereby facilitating electrophysiological recording. Second, the fluid and gaseous environment can be precisely controlled allowing studies of the direct effects of chemosensory stimuli on these cells following short or long-term exposure. As will be seen later all of these advantages were exploited in this thesis.

The first electrophysiological studies on glomus cells used conventional intracellular recording techniques, where a glass microelectrode is used to impale a cell and measure its membrane potential. Many electrophysiological studies on glomus cells based on intracellular recording have been reported (Eyzaguirre et al 1983,1990; He et al 1991a,b; Monti-Bloch and Eyzaguirre 1990) but unfortunately this technique

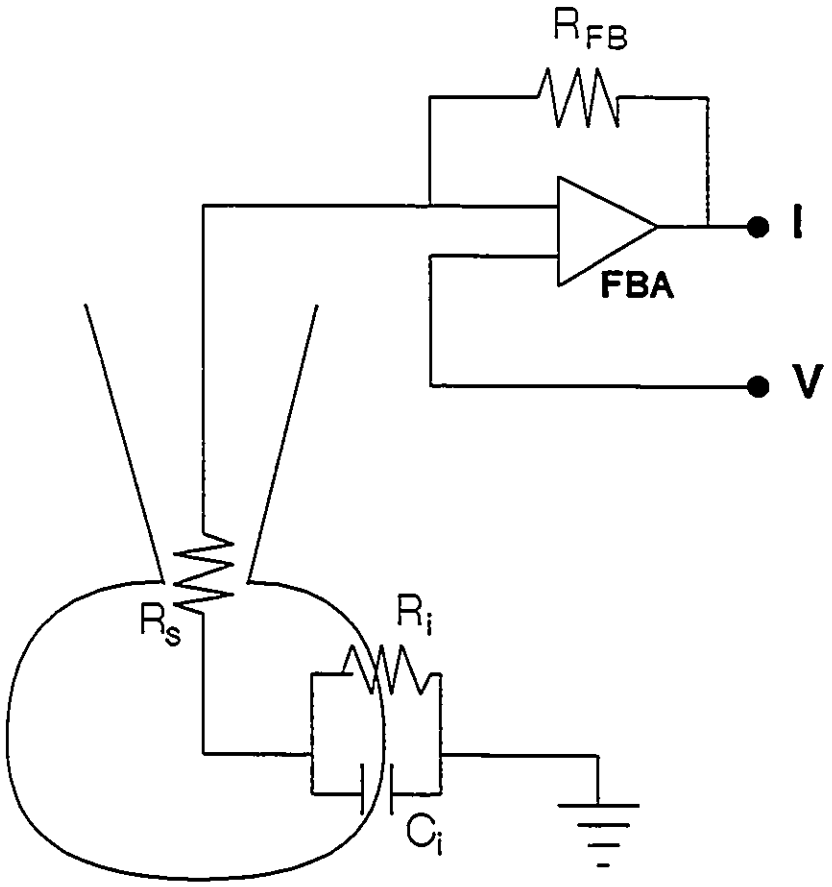
is not ideal for small cells (e.g. glomus cells) due to the problem of damage introduced by the microelectrode. This damage creates a shunt around the electrode and often leads to an artefactual depolarization of the cell. For example, by this method reported resting potentials for glomus cells ranged from -20 to -36 mV (Baron and Eyzaguirre 1977; Eyzaguirre et al 1983; He et al 1991) whereas recent estimates based on the more-reliable patch clamp technique (see below) suggest the true value is at least -50 mV (Duchen et al 1988).

Electrophysiological studies using the patch clamp technique

Since the pioneering studies of Hodgkin and Huxley on the squid axon in the 1950's, electrophysiologists have applied voltage clamp analyses to dissect the ionic currents in both excitable and non-excitable cells. The voltage clamp circuit uses a feedback amplifier to maintain a constant potential across the cell membrane by injecting an equal and opposite current to match the natural current fluctuations in the membrane. Ideally this injected current can then be read as a mirror image of the cell's intrinsic currents at the potential tested (Jones 1990). In this way Hodgkin and Huxley (1952) determined that sodium and potassium ions carried the currents responsible for the action potential in the giant axon of the squid, *Loligo*. They used a particular voltage clamp method consisting of two wires inserted into the axoplasm of the giant axon, one to measure potential and the other to inject current (Hille 1984; Hodgkin and Huxley 1952). Since this technique could not be performed on intact cells a variety of other voltage clamp methods, employing basically the same

technology, was introduced. These included the double gap method (sucrose gap), suction pipette method, and the most commonly used two-microelectrode voltage clamp (Hille 1984; Jones 1990). These methods allowed the characterization of the electrophysiological properties of a number of cell types. However, small cells such as certain types of neurons and a variety of neuroendocrine cells could not be studied with these conventional voltage clamp methods due to the damage caused by impaling these cells with one, let alone two, electrodes. Fortunately, in 1976, Erwin Neher and Bert Sakmann introduced a novel method called the patch clamp technique, which allowed voltage clamp recordings from not only small cells, but also small membrane patches in which single ion channel proteins could be studied. This discovery led to the award of the Nobel Prize for Physiology or Medicine to Neher and Sakmann in 1991 (see Neher and Sakmann 1992). Basically this technique utilizes the voltage clamp principles discussed above except that only one electrode is needed and the manipulations are less invasive. In brief, a fire-polished glass pipette with a tip $\approx$ 1 μm in diameter is brought in contact with the cell surface and a small amount of suction is applied. Frequently this causes a high-resistance seal to be established between the glass and membrane (Hamill et al 1981). This is the cell-attached configuration which allows one to measure single channel currents in the membrane patch. The patch of membrane can then be pulled away while remaining attached to the pipette, because of the mechanical strength of the membrane-glass seal, resulting in an inside-out patch. This configuration allows precise control of the fluid environment on both sides of the patch of membrane permitting accurate studies of

ion-selectivity of ion channels. A third manipulation achieved by applying more suction while in the cell-attached mode results in the whole-cell configuration. The added suction causes the patch of membrane to rupture underneath the pipette permitting access to the cell interior allowing currents across the whole cell to be measured. A fourth configuration can be established by pulling away the pipette from the cell, while in the whole-cell configuration, leaving an outside-out patch of membrane (Hamill et al 1981). In this thesis the whole-cell configuration was extensively used in Chapters 1 through 4 (in Chapter 5 the inside-out configuration was used) and a schematic diagram illustrating the circuit is shown below (adapted from Jones 1990, Hille 1984):



In the diagram above the feedback amplifier (FBA) holds the membrane potential at the level set by the control (V). The current is then measured as a voltage drop across the high-resistance (1-10 GΩ) feedback resistor (R_{FB}). If the series resistance (R_s) is much less than the cell's input resistance (R_i) then a fairly accurate reading of the whole-cell currents can be achieved but in larger cells where R_i is low the series resistance must be compensated for. The membrane capacitance (C_i) can be measured by recording the capacity current during a voltage step (Jones 1990). This whole-cell recording technique is ideal for small cells as no microelectrode impalement is needed and often these cells have higher input resistances. However, although this technique has several advantages over conventional intracellular recording there are some disadvantages, the major of which is the dialysis of the intracellular contents after rupture of the membrane patch (Hamill et al 1981).

Perforated-patch recording

An interesting solution to the problem of dialysis during conventional whole-cell recording was devised by Horn and Marty (1988) in which an antibiotic, nystatin, was added to the pipette solution before a patch clamp experiment. The cell attached patch was formed as usual (Hamill et al 1981) but instead of rupturing the membrane by applying suction, nystatin inserts into the membrane patch forming perforations which allow recording of whole-cell currents. Due to the action of nystatin on the membrane this method was dubbed 'perforated-patch' recording (Horn and Marty 1988). Nystatin forms pores in membranes which are permeable to both monovalent

cations and anions but not divalent ions (Kleinberg and Finklestein 1984) or any larger molecules such as cAMP or cGMP. This allows the experimenter to control the concentrations of monovalent ions inside the cell without washing out the important second messengers, metabolites or buffers in the cytoplasm (Horn and Marty 1988). The advantages of the perforated patch technique over conventional whole-cell recording were capitalised upon in many of the experiments described in this thesis.

Patch clamp studies on glomus cells

The first studies using the patch clamp technique on freshly-isolated rabbit glomus cells were reported in 1988 (Duchen et al 1988; Lopez-Barneo et al 1988). Since then several other studies have used this technique to study the electrophysiology of freshly-isolated glomus cells in response to a variety of stimuli (Biscoe and Duchen 1989,1990a,b; Hescheler et al 1989; Lopez-Lopez et al 1989; Peers 1990a,b; Urena et al 1989; etc.). These studies along with the work described in this thesis have led to new ideas of the mechanisms underlying chemotransduction in the carotid body and have provided evidence for the glomus cell being the primary sensor of the chemosensory stimuli.

Goals of the thesis

The goals of this thesis were to: i) use a cell culture system of dissociated rat carotid body cells to allow direct access to the putative chemoreceptor glomus cells; ii) use the high-resolution electrophysiological method known as the patch-clamp

technique (Hamill et al 1981) and the novel perforated-patch method, which are especially suited for recording from small cells, to characterize the physiology of glomus cells; iii) determine the effects of the various natural stimuli, e.g. hypoxia, acidity, and hypercapnia, on the electrophysiological characteristics of these cells; and iv) propose mechanisms by which the carotid body responds to natural chemosensory stimuli.

In Chapter 1 I will describe the electrophysiological characteristics of glomus cells using both conventional and perforated-patch recording techniques. In Chapter 2 the effects of hypoxia, both acute and chronic, on the membrane properties of these cells will be addressed. Chapter 3 three deals with the effects of acidity on the membrane currents in glomus cells. The effects of hypercapnic stimuli will be discussed in Chapter 4. Chapter 5 is a single channel study of anion channels found in membrane patches of glomus cells. Finally, Chapter 6 is a discussion of the physiological relevance of the above findings in regards to the mechanisms of chemoreception by glomus cells.

A number of the experiments described in the following chapters have been recently reported (Stea and Nurse 1989, 1991a, 1991b, 1992; Stea et al 1991).

CHAPTER 1

Electrophysiological Characteristics

of

Rat Glomus Cells

INTRODUCTION

Glomus cells have been difficult to characterize electrophysiologically because of their small size (8-10 μm diameter) and inaccessibility *in situ*. There have been several attempts to make the cells more accessible, e.g. carotid body tissue slices (Eyzaguirre and Monti-Bloch 1982), but recent studies utilizing short and long term cultures of dissociated carotid bodies have allowed glomus cells to be studied in virtual isolation (Duchen et al 1988; Fishman and Schaffner 1984; Fishman et al 1985; Hescheler et al 1989; Nurse 1987, 1990; Peers 1990a,b; Stea and Nurse 1991a,b; Urena et al 1989; etc.). Early electrophysiological studies on glomus cells were based on intracellular recordings (see review Eyzaguirre and Zapata 1984) but were unreliable due to the problem of damage introduced by the microelectrode in these small cells. For example, typical resting potentials reported for glomus cells with intracellular recording ranged from -20 to -26 mV (Eyzaguirre et al 1983; Oyama et al 1986a) whereas recent estimates based on the patch clamp technique

(Hamill et al 1981) suggest the true value is likely to be > -50 mV (Duchen et al 1988). Studies by the latter technique have also shown that glomus cells have excitable membranes and are capable of generating action potentials (Duchen et al 1988; Lopez-Barneo et al 1988; see however Stea and Nurse 1991a); these properties were not previously detected with intracellular recordings presumably because of the low resting potentials which resulted in inactivation of the voltage-dependent sodium channels.

The work described in this chapter will deal with the electrophysiological characterization of cultured rat glomus cells using the patch-clamp technique as well as the novel perforated-patch method (Horn and Marty 1988) which allows more stable long-term recordings by preserving cytoplasmic integrity. This involved measurements of the passive membrane properties, i.e. input resistance and capacitance, as well as a dissection of the various voltage-dependent ionic currents carried by the major cations, Na^+, K^+, and Ca^{2+}. This knowledge will be used in subsequent chapters to test whether any of the active membrane properties are modulated by exposure to chemosensory stimuli and therefore provide evidence for or against the putative chemoreceptor functions of glomus cells.

METHODS

Glomus cell culture

Carotid body cells from 1 day-old to adult rats (Wistar; Charles River,Quebec) were grown in dissociated primary cell cultures and used in the experiments described below. Rats were stunned by a blow to the head and the carotid bifurcation was excised bilaterally and placed in ice-cold L-15 plating medium (Gibco, Grand Island, NY). Carotid bodies were isolated and incubated at 37°C for 30 min in medium containing Ca-Mg-free Hanks' Balanced Salt Solution (HBSS), 0.1% collagenase (Gibco), 0.1% trypsin (Gibco), 0.01% deoxyribonuclease (Millipore Corp., Freehold, NJ), and 1% penicillin-streptomycin. The enzyme solution was carefully removed and replaced with growth medium (see below) to inactivate any remaining enzyme. The carotid bodies were mechanically teased apart and slowly triturated before plating the resulting cell suspension into collagen-coated central wells (diameter ca. 8 mm) of modified 35 mm petri dishes. Typically, 0.1 ml of the cell suspension from 24 carotid bodies was placed into each of 6 wells. The cells were grown at 37°C in a humidified atmosphere of 95% air: 5% CO_2 for 1 - 68 days before they were used in the patch-clamp experiments.

The growth medium consisted of F-12 nutrient medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 80 U/l insulin (Sigma Chemical Co., St.Louis, MO), 0.6% glucose, 2 mM glutamine, and 1% penicillin-streptomycin (Gibco).

Conventional whole-cell recording

Just prior to recording, cultures were rinsed and bathed with a physiological salt solution (see Solutions), and mounted on the stage of an inverted phase contrast microscope (Leitz Diavert). The reference electrode consisted of a polyethylene tube filled with 0.9% saline:2% agar connecting the bathing solution to a pool of 150 mM KCl that was grounded via an Ag:AgCl electrode. The tube contacted the bathing solution via an insertion in a stainless steel block that was placed in the culture dish. Patch pipettes were fabricated from Corning 7052 glass (1.5 mm O.D.) pulled in a Brown-Flaming horizontal puller (Model P-80; Sutter Instruments Co., San Francisco, CA). The pipettes were fire-polished and their resistance varied between 2-10 MΩ. In a few experiments stray glass capacitance was reduced by the application of sylgard (Dow Corning Corp., Midland, MI) to the pipette tips. Giga-ohm (GΩ) seals, typically between 2-10 GΩ, were formed between the pipette and identified glomus cells following application of gentle pipette suction (Hamill et al 1981); additional suction was applied to obtain the whole-cell configuration. Most experiments were performed at room temperature.

Perforated-patch recording

Perforated-patch recordings were obtained from glomus cells by using essentially the same technique as described by Horn and Marty (1988). The pore-forming antibiotic nystatin (Sigma) was dissolved in methanol (5 mg/ml) or dimethyl sulfoxide (DMSO; 50 mg/ml) and ultrasonicated for 5 min. This stock was added to

the pipette solution used in the whole-cell experiments (see below) at a concentration of 150-500 μg/ml. The use of DMSO instead of methanol resulted in improved solubility of the nystatin, a more rapid decrease in the series resistance, and an earlier commencement of whole-cell recording. Typically, after 5-25 min exposure to the nystatin solution in the cell-attached mode, series resistance values decreased to $\approx$ 10 MΩ and experimental protocols were initiated. Control experiments with DMSO (without nystatin) in the pipette had no obvious effects on these cells.

Series resistance was typically 10 - 15 MΩ for perforated-patch and $<$ 10 MΩ for conventional whole-cell recordings and was compensated in most experiments. Junction potentials, which varied typically from 2-10 mV, were cancelled at the beginning of the experiment.

Data analysis

Whole-cell currents were recorded with a World Precision Instruments (S7050A) or a Dagan 3900 patch clamp module and probes equipped with 1 GΩ headstage feedback resistors. Records were simultaneously displayed on an oscilloscope (Tektronix, 5111A), digitized with an AXOLAB 1100 computer interface (Axon Instruments, Burlingame, CA) and stored on disk in a IBM compatible computer using PCLAMP software (Axon Instruments). Data were analyzed using PCLAMP software and traces shown in the text were plotted on a Hewlett Packard Laserjet III printer after subtraction of the capacitance and leakage currents and filtering at 1000 to 10000 Hz. Steady state inactivation curves were generated from

the peak inward Na^+ and Ca^{2+} currents elicited by steps from various holding potentials for several seconds. These data were fitted with smooth curves according to a modified version of the Boltzmann equation (e.g. Clark et al 1990):

$$h = \frac{I_{Na}(V_m)}{I_{Na_{max}}} = \frac{1}{\exp\frac{(V_m + V_{1/2})}{S_{1/2}} + 1}$$

where h is the steady state inactivation determined by dividing the peak I_{Na} at each holding potential (V_m) by the max I_{Na} recorded. The variables $V_{1/2}$ (holding potential where I_{Na} was half maximal) and $S_{1/2}$ (slope factor at $V_{1/2}$) were adjusted for best fit of the curve to the observed data by a curve fitting program. Most of the results are represented in the text by the mean value of the data plus or minus the standard error (S.E.). In most cases, data were compared using a Student's t-test with the level of significance set at $p < 0.01$.

Solutions

Most experiments were performed using extracellular fluid of the following composition (mM): NaCl,140 (or NaCl,135 + KCl,5); $CaCl_2$,1; $MgCl_2$,2; glucose,10; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES),10 at pH 7.4 with a measured osmolarity of ≈ 290 mOsmoles/l. Other sets of experiments were performed using a bicarbonate-buffered external medium containing the following (mM): NaCl,110; KCl,5; $NaHCO_3$,24; $CaCl_2$,2; $MgCl_2$,2; glucose,10;

sucrose,12; bubbled with 5% CO_2 to maintain the external pH $\approx$ 7.4 and a measured osmolarity of $\approx$ 290 mOsmoles/l. The stock pipette solution for whole-cell and perforated-patch experiments either contained (mM): i) KCl,140 (or KCl,135 + NaCl,5); $CaCl_2$,1; ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA),11 ($[Ca^{2+}] \approx 10^{-8}$); HEPES,10 at pH 7.2 or ii) potassium glutamate or gluconate,105; KCl,35; $CaCl_2$,1; EGTA,11; HEPES,10 at pH 7.2. or iii) KCl,135; NaCl,5; $CaCl_2$,0.1; HEPES,10 at pH 7.2. All solutions were filtered through a 0.45 μm millipore filter before use.

Tetrodotoxin (TTX; Sigma) was added to the bathing solution at a final concentration of 0.2 to 2 μM to block voltage-dependent Na^+ current. To block voltage-dependent K^+ currents tetraethylammonium (TEA; 5-25 mM) was added to the bath. In addition K^+ currents were eliminated using a K^+-free pipette solution containing the following (mM): CsCl,105; TEA chloride,25; NaCl,10; $CaCl_2$,1; EGTA,11; HEPES,10 at pH 7.2. External Ca^{2+} was replaced by 2 mM cobalt (Co^{2+}) in several experiments designed to determine the contributions of Ca^{2+} to the inward current and to the outward Ca^{2+}-activated K^+ currents. All solutions were added to the recording chamber under gravity and withdrawn by vacuum suction.

RESULTS

Appearance of glomus cells in culture

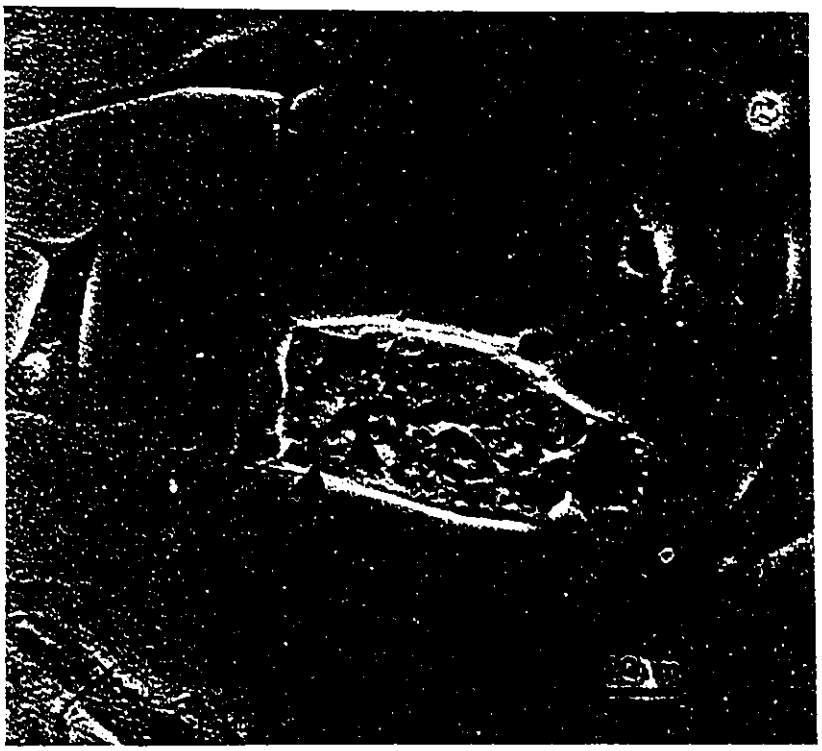
Glomus cells grow in discrete clusters which are readily identifiable in culture as shown in Fig.1. These cells survive for many weeks *in vitro* with the oldest cell recorded from in this study being over 9 weeks in culture. Generally glomus cells were rounder during the first week in culture which facilitated whole-cell recording. In older cultures the cells tended to flatten out resulting in a lower probability of obtaining stable whole-cell recordings. The data presented below were obtained mainly from glomus cells which were members of an identifiable cluster but a few isolated single glomus cells were also studied. Since there were no obvious differences in the properties of single cells, data from both groups were pooled.

The occasional cell within glomus cell clusters produced virtually no active currents and displayed large capacitative transients under voltage clamp. These cells were not studied in detail but may be sustentacular (type II) cells (see however, Duchen et al 1988).

Passive membrane properties of glomus cells

The results presented below are based on electrophysiological experiments on over 400 glomus cells. More than half of these were studied with the perforated-patch method (Horn and Marty 1988) and the remaining with conventional whole-cell recording (Hamill et al 1981). The results were generally comparable though with the

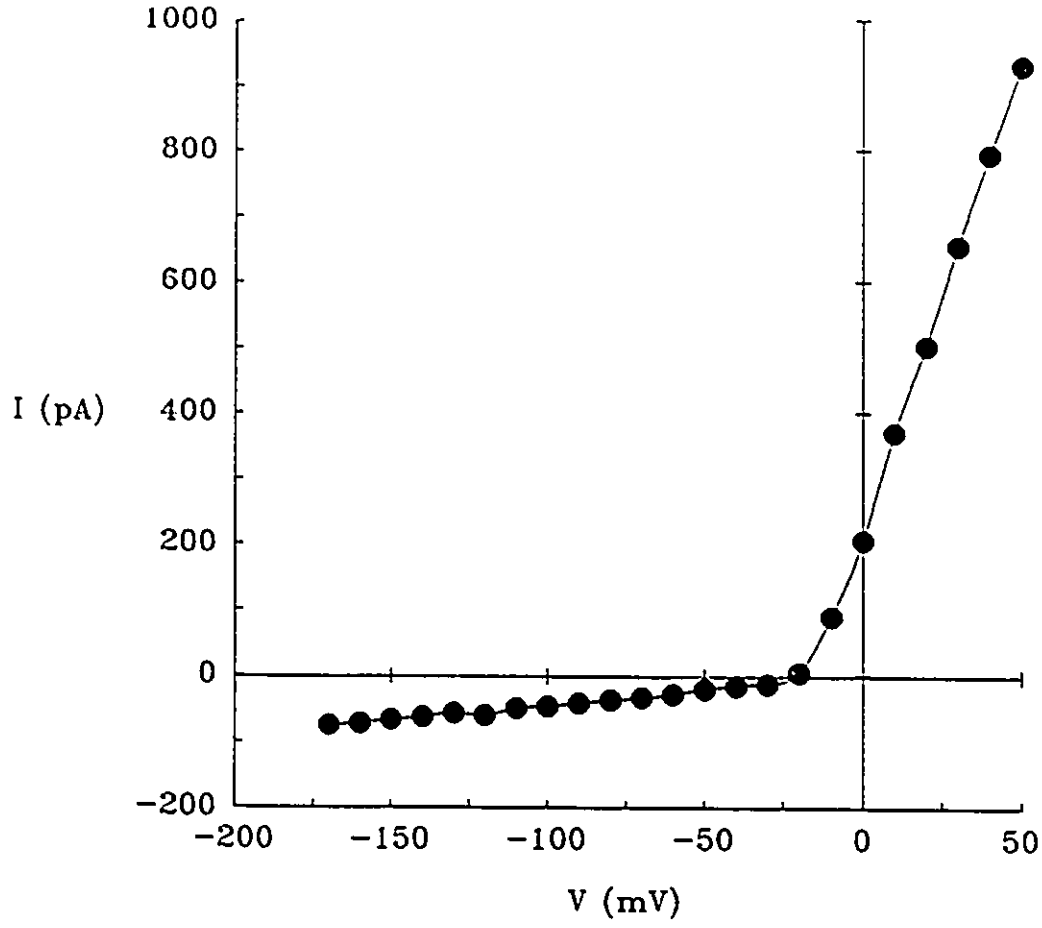
FIG.1. Phase contrast micrograph of a glomus cell cluster from a culture of dissociated rat carotid body. Arrow indicates typical glomus cell used in patch clamp experiments. Note clearly visible nuclei and nucleoli in glomus cells.



perforated-patch technique the preparation was more stable and recordings lasted typically between 15 and 30 min, although several cells persisted for more than 1 hour. Data from both methods were pooled and described below.

In voltage-clamp experiments in HEPES-buffered external media cultured glomus cells had input resistances (inverse of leakage conductance) of 2.64 +/- 0.15 GΩ (mean +/- S.E.; n=376) as determined from the slope of the I/V relation generated by voltage steps from holding potentials of -70 or -60 mV to potentials from -170 to -40 mV. In this voltage range the I/V curve was linear indicating a passive leakage conductance; steps more depolarized than -40 mV elicited voltage-activated conductances (Fig.2). The mean input resistance calculated from glomus cells in culture for more than 3 weeks was slightly lower (1.7 GΩ; n=28) than that from cells less than 1 week in culture (2.7 GΩ; n=181) but these values were not significantly different. Many cells, including electrically excitable ones, have resting potentials which are governed by leakage conductances dependent on K^+ ions (Hille 1984). However, based on intracellular studies, it has been suggested that the resting potential of glomus cells is governed largely by the movements of Cl^- ions (Eyzaguirre et al 1983; Oyama et al 1986a) and the intracellular Cl^- concentration was estimated to be ≈ 20 mM using ion-sensitive microelectrodes (Oyama et al 1986a). In experiments designed to simulate this level of intracellular Cl^- (decreasing the intracellular Cl^- from 140 mM to 30 mM; see Methods) there was a small but significant increase in the input resistance from ≈ 2.2 GΩ (n=270) to ≈ 2.9 GΩ (n=100). In other experiments where K^+ ions were eliminated from the pipette (see

FIG.2. I-V relation for a cultured glomus cell. Voltage clamp currents (I) were measured during steps from a holding potential of -60 mV to various test potentials (V). The linear portion of the I-V relation at hyperpolarizing voltage steps below -40 mV was used to estimated the leakage conductance (and input resistance). Depolarizing steps to -30 mV and above resulted in the opening of voltage-activated currents (measured 30 -35 ms after the voltage step).


1000
800
600
400
200
0
−200
I (pA)
−200
−150
−100
−50
0
50
V (mV)

Methods) there also was a significant ($p < 0.01$) rise in the input resistance from ≈ 2.6 GΩ (n=376) to ≈ 4.4 GΩ (n=51). These results suggest that both Cl^- and K^+ ions contributed to the leakage conductance of glomus cells during recording in control HEPES-buffered external media (HBM).

Since carotid body chemoreceptors are sensitive to elevated CO_2 (hypercapnia) it was of interest to compare the passive properties of glomus cells in a more physiological HCO_3^-/CO_2 buffered extracellular fluid. Further, glomus cells are known to contain carbonic anhydrase (Nurse 1990) which could influence pH_i in HCO_3^-/CO_2 media. Indeed the input resistance of glomus cells was found to be markedly different when exposed to bicarbonate-buffered media (BBM)(Stea and Nurse 1991b). On switching from HBM to BBM equilibrated with 5% CO_2 to maintain a constant external pH of 7.4 (see methods) the input resistance of glomus cells significantly decreased by a factor of three ($p < 0.0001$) to 850 +/- 92.2 MΩ (mean +/- S.E.; n=116). This effect was completely reversible in many cases (Fig.3A,B) and was observed soon after switching from HBM to BBM. In a few cases, however, the decrease in resistance was seen after switching back from BBM to HBM. Associated with the decrease in input resistance in BBM were large fluctuations in the whole-cell leakage current possibly due to increased channel activity. In a few cases this noise appeared to result from the opening of large-conductance channels (Fig.4) and the single channel conductance was estimated at ≈ 300 pS from records where discrete current steps allowed the generation of an I-V curve (Fig.4). The opening of only 3 or 4 of these large conductance channels could

FIG.3. Contrasting effect of bicarbonate-buffered media (BBM) vs HEPES-buffered media (HBM) on leakage currents in cultured glomus cells. A: Switching the perfusate from HBM to BBM (HCO_3^-/5% CO_2) caused a pronounced and reversible increase in the leakage current and a noticeable increase in channel noise. B: The I-V relation for this same cell indicated the leakage conductance was higher in BBM than in HBM and this change was completely reversible; holding potential = -60 mV.

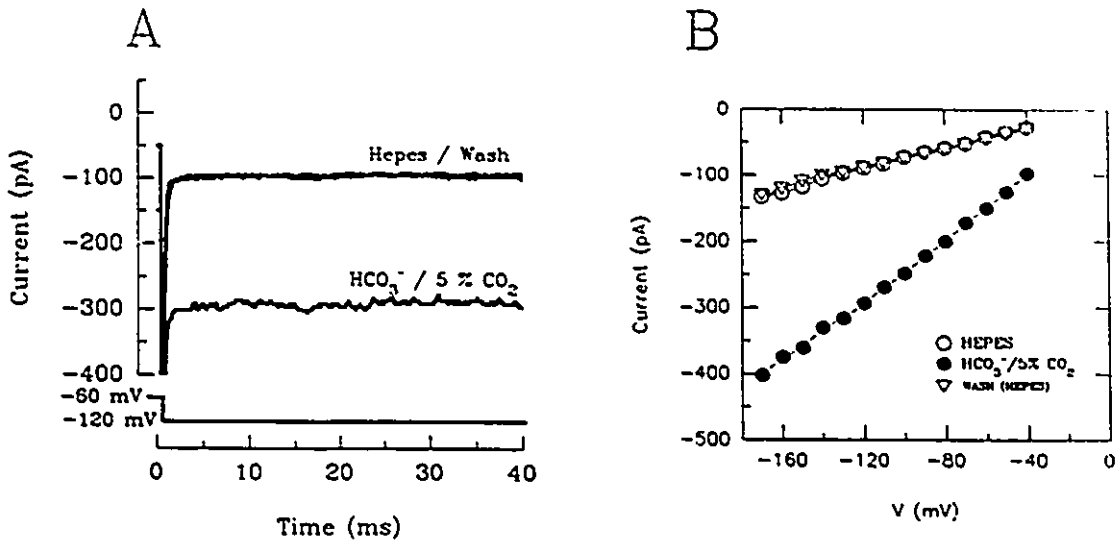
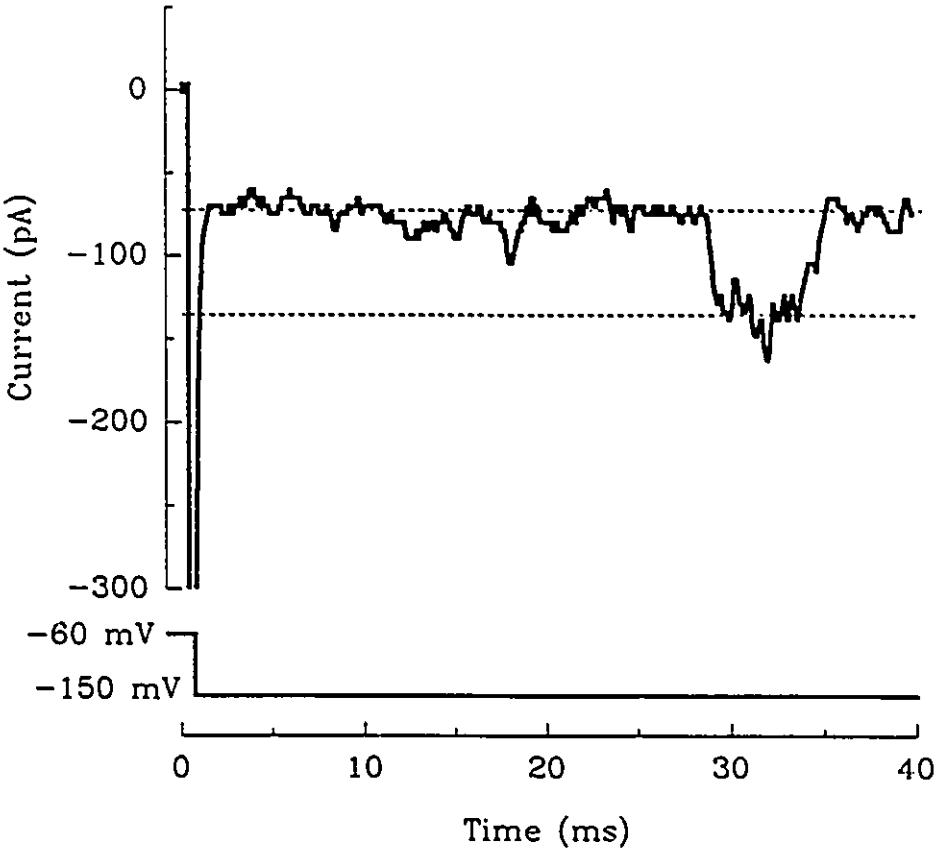

A
B
Current (pA)
0
-100
-200
-300
-400
-60 mV
-120 mV
Hepes / Wash
HCO3- / 5 % CO2
0
10
20
30
40
Time (ms)
Current (pA)
0
-100
-200
-300
-400
-500
-160
-120
-80
-40
0
V (mV)
HEPES
HCO3-/5% CO2

FIG.4. Putative single channel opening superimposed on whole-cell leakage currents in BBM. In this cell a discrete current step was observed (dotted lines) corresponding to the opening of a large conductance channel.


0
−100
−200
−300
Current (pA)
−60 mV
−150 mV
0
10
20
30
40
Time (ms)

account for the decrease in input resistance seen after switching to BBM from HBM. To probe for the ion selectivity of the increased conductance in BBM ion-substitution experiments were carried out. In K^+-free solutions (n=15) a significant decrease in the input resistance occurred (mean $\approx$ 350 MΩ) as illustrated in Fig.5 suggesting that K^+ ions were not the major current carrier of the increased leakage currents in BBM. The possibility that Cl^- ions might contribute significantly to this leak was tested using 9-anthracene carboxylic acid (9-AC), a known blocker of chloride channels in membrane patches of glomus cells (Stea and Nurse 1989; Chapter 5). In 11 of 14 experiments the increased leakage conductance in BBM was abolished by 5 mM 9-AC (Fig.6); in the remaining cases addition of 9-AC had no effect. Though these results suggest that Cl^- ions are likely the major carriers of the leakage current in BBM, the known non-specific effects of 9-AC renders this conclusion tentative.

The increase in leakage conductance in BBM could arise from the acidification of pH_i following hydration of CO_2 , catalysed by carbonic anhydrase known to be present in glomus cells (Nurse 1990). Alternatively a change in cell volume or osmotic stress, on switching from HBM to BBM, could lead to opening of the leakage channels (Ubl et al 1988). To probe for the latter possibility cells were exposed to a hypoosmotic solution consisting of HBM diluted with H_2O to $\approx$ 145 - 190 mOsmoles/l. In 28 glomus cells exposed to hypoosmotic HBM, 20 showed a significant decrease ($p < 0.01$) in input resistance from 2.4 +/- 0.5 GΩ to 809 +/- 110 MΩ (mean +/- S.E.). Fifteen of these cells showed complete reversibility of this

FIG.5. Leakage conductance in bicarbonate-buffered media in the absence of K^+ ions. A pronounced increase in leakage current was still observed upon switching from HEPES-buffered media (HBM) to BBM (HCO_3^-/5% CO_2) when K^+ ions were eliminated from the recording solutions. This suggests that the leakage current observed during recording in BBM is not carried by K^+ ions.

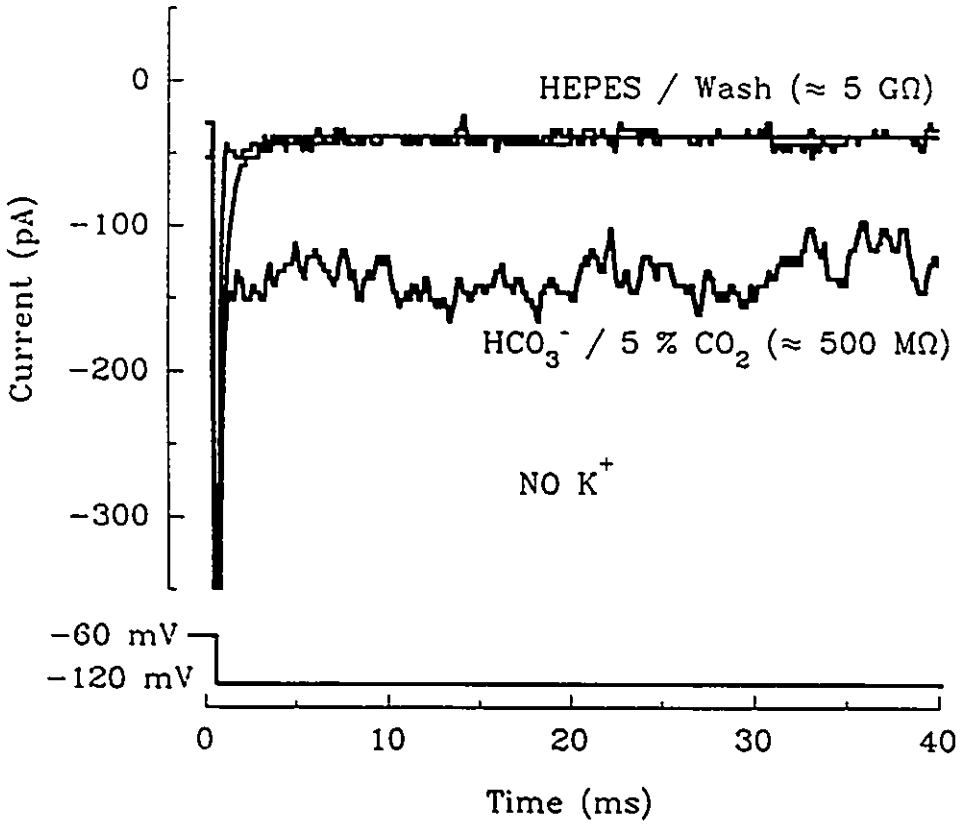
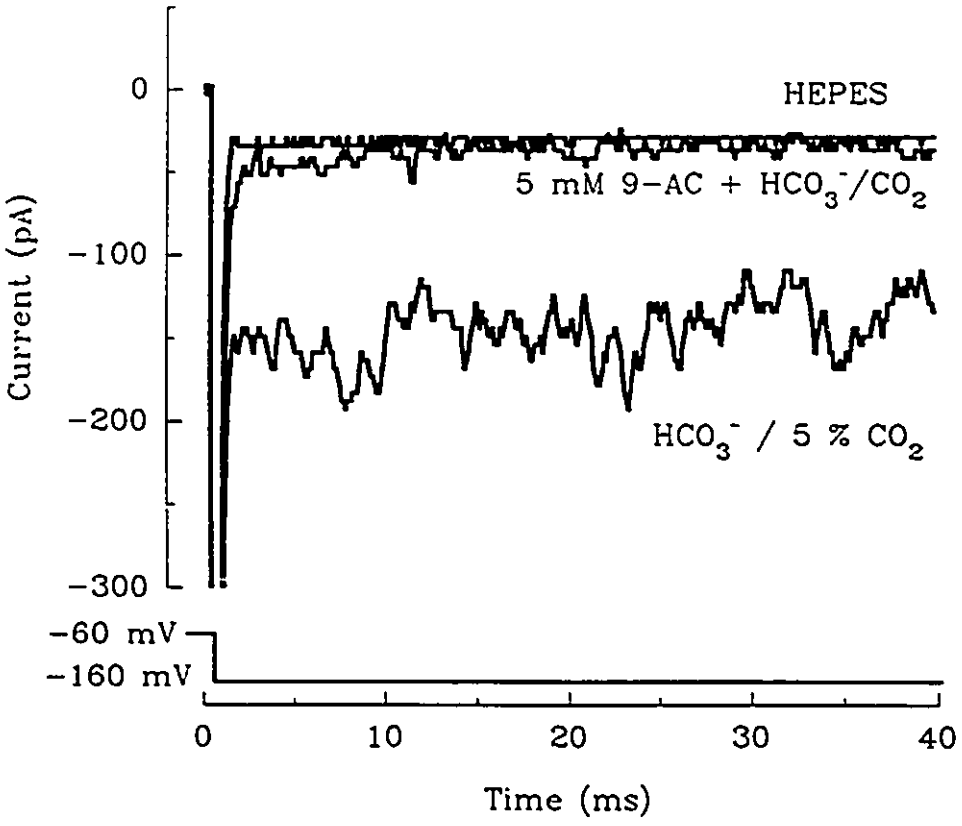

0
−100
−200
−300
Current (pA)
HEPES / Wash (≈ 5 GΩ)
HCO3- / 5 % CO2 (≈ 500 MΩ)
NO K+
−60 mV
−120 mV
0
10
20
30
40
Time (ms)

FIG.6. Effect of 9-anthracene carboxylic acid (9-AC) on the leakage current in a glomus cell recorded in BBM. The addition of 5 mM 9-AC, a chloride channel blocker, to the BBM caused a reduction in the leakage current in this cell to the level recorded in HBM; this is consistent with Cl^- ions being the major carriers of the leakage current in BBM.


0
−100
−200
−300
Current (pA)
HEPES
5 mM 9−AC + HCO_3^-/CO_2
HCO_3^- / 5 % CO_2
−60 mV
−160 mV
0
10
20
30
40
Time (ms)

effect when returned to isoosmotic HBM (Fig.7). In eight cells the input resistance did not change on exposure to hypoosmotic HBM. These experiments raise the possibility that changes in cell volume may trigger opening of the leakage channels in BBM.

Another passive membrane property that can be directly measured by voltage clamp is the input capacitance which is directly proportional to membrane surface area. The input capacitance (C_i) of glomus cells was determined by integration of the capacity current transient recorded under voltage clamp and then dividing the resulting charge (q) by the voltage step (V):

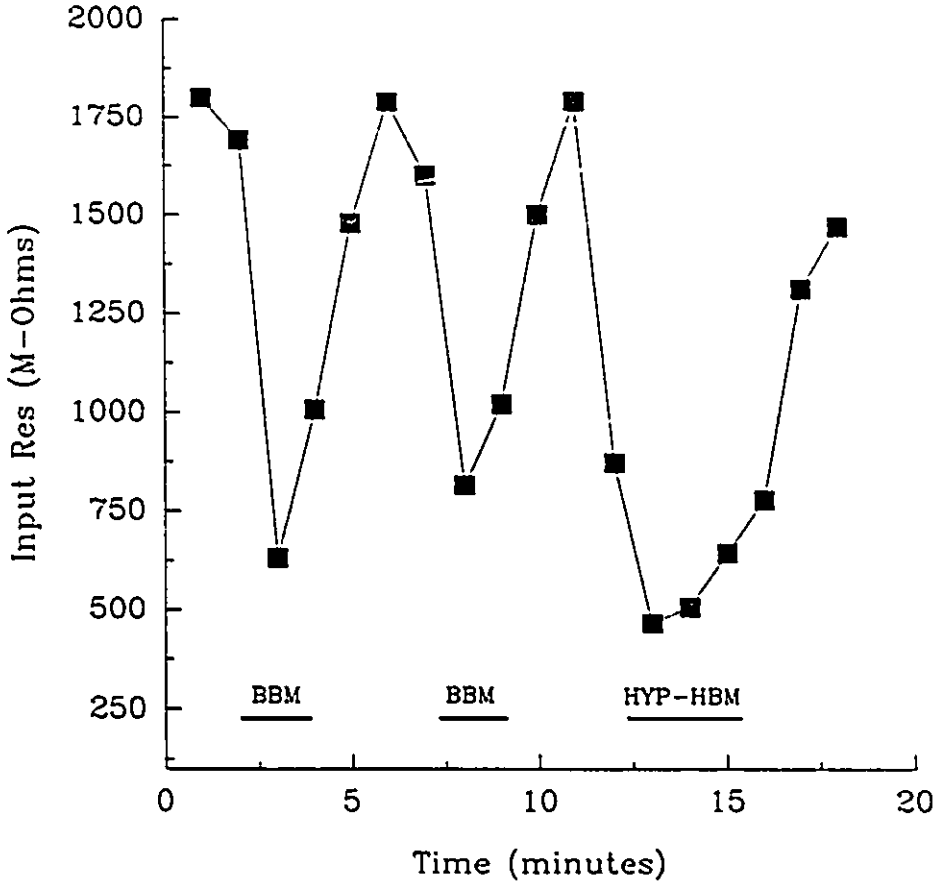
$$C_i = \frac{q}{V}$$

Rat glomus cells in this study had an input capacitance (C_i) of 6.4 +/- 0.2 pF (n=201) which is very close to the values reported for adult rabbit glomus cells (6.2 pF, Duchen et al 1988; 7.3 pF, Urena et al 1989). These capacitance values correlate to a cell of approximately 10 μm in diameter using the formula below assuming a spherical cell with a specific membrane capacitance (SMC) of 1 $\mu F/cm^2$:

$$Surface\ Area = 4\pi r^2 = \frac{C_i}{SMC}$$

This estimate is very close to the actual size of 8 -15 μm reported for glomus cells *in vivo* (McDonald 1981) and *in vitro* (Duchen et al 1988).

FIG.7. Time course of changes in input resistance recorded from a glomus cell exposed to BBM and hypoosmotic HBM. Switching from HBM to BBM caused reversible decreases in the input resistance. In addition perfusion of a hypoosmotic HBM (osmolarity $\approx$ 190 mOsmoles/L) caused a similar reversible decrease in the input resistance. The cells were bathed with HBM except during the intervals indicated by the lower horizontal lines when BBM (HCO_3^-/5% CO_2) or hypoosmotic HBM (HYP-HBM) was perfused. These results suggest that changes in cell volume, or osmotic stress, may trigger the increased leakage conductance in BBM.


2000
1750
1500
1250
1000
750
500
250
Input Res (M-Ohms)
BBM
BBM
HYP-HBM
0
5
10
15
20
Time (minutes)

Active membrane properties of rat glomus cells

In normal external and internal solutions (see methods) large depolarizing steps from a holding potential of -70 or -60 mV to -30 mV or more depolarized resulted in voltage-dependent currents becoming activated which consisted of a small transient inward current followed by a prolonged outward current (Fig.8A,B) recorded under voltage clamp. Determinations of the ionic basis of these currents is described below.

Na^+ currents

The transient inward current was activated upon stepping to -30 mV (Fig.8B) which is similar to the threshold for activation of freshly isolated rabbit glomus cells ($\approx$ -40 mV, Urena et al 1989), and other closely-related neural crest derivatives (small intensely-fluorescent or SIF cells $\approx$ -40 mV, Alexander 1991; chromaffin cells $\approx$ -40 mV, Fenwick et al 1982; sympathetic neurons $\approx$ -30 mV, Belluzzi and Sacchi 1986). This current rapidly inactivated over 5 ms and the steady state inactivation curve for the transient inward current in a cultured rat glomus cell recorded in normal solutions (see Methods) is shown in Fig.9. The $V_{1/2}$ (holding potential where I_{Na} was half-maximal) was -59.4 +/- 4.9 mV (n=5) which is similar to the $V_{1/2}$ for the voltage-activated Na^+ current in a variety of electrically-excitable cells including rabbit glomus cells (-50 mV, Urena et al 1989), small intensely-fluorescent or SIF cells of sympathetic ganglia (-55 mV, Alexander 1991), and principal sympathetic neurons (-56 mV, Galvan and Sedlmeir 1984) but is more negative than the $V_{1/2}$ in chromaffin cells (-35 mV, Fenwick et al 1982). The peak inward current was quite

FIG.8. Whole-cell voltage-activated currents recorded from cultured glomus cells under voltage clamp. A: Small transient inward followed by a prolonged outward current (upper trace) in response to a voltage step from -60 to 0 mV (lower trace). B: Leak subtracted peak inward currents (filled circles) and peak outward current (open triangles) in the same cell following a voltage step from a holding potential of -60 mV to various test potentials (V).

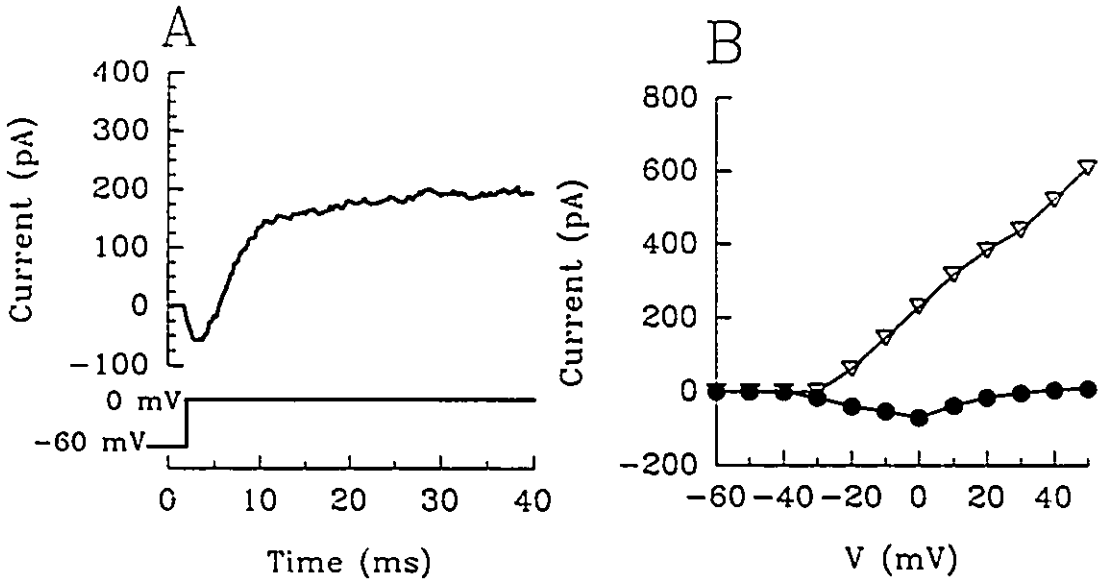
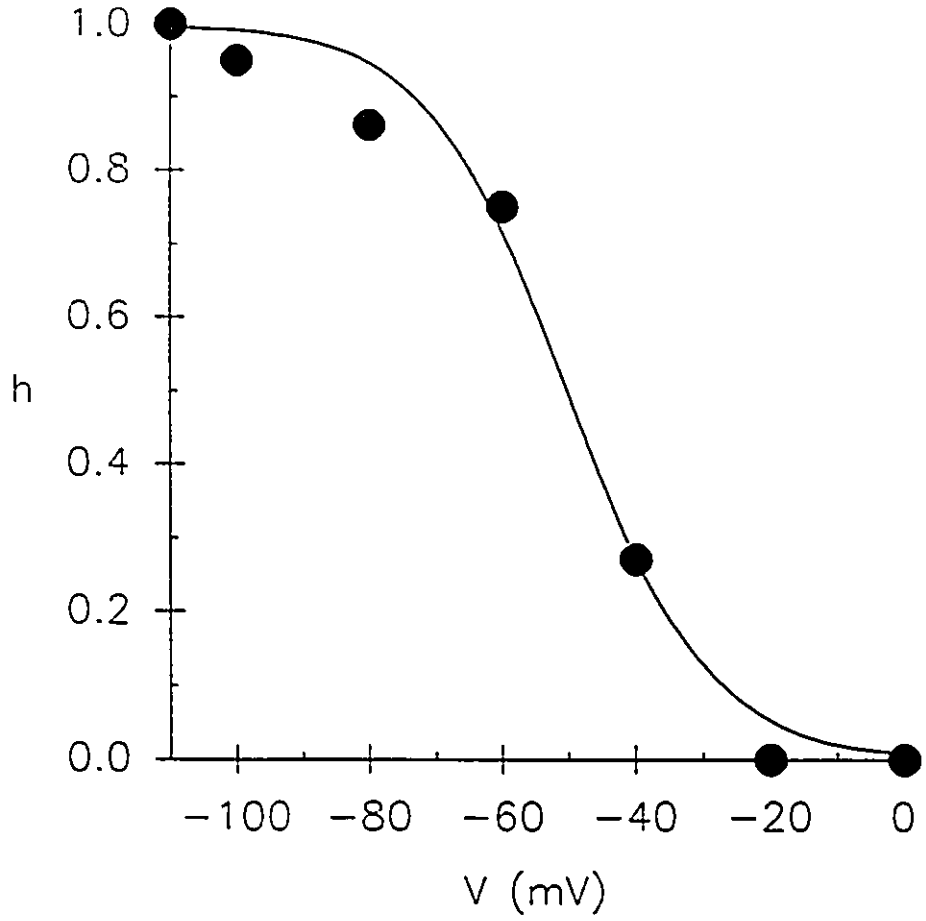

A
400
300
200
100
0
−100
Current (pA)
0 mV
−60 mV
0
10
20
30
40
Time (ms)
B
800
600
400
200
0
−200
Current (pA)
−60 −40 −20 0 20 40
V (mV)

FIG.9. Steady state Na^+ inactivation curve from a cultured glomus cell in normal recording solutions. Peak transient Na^+ currents were measured at each holding potential and divided by the maximum Na^+ current measured from a holding potential of -110 mV to give the steady-state inactivation (h; filled circles). These data were fitted with a smooth curve generated from a modified version of the Boltzmann equation (see methods).


1.0
0.8
0.6
h
0.4
0.2
0.0
−100
−80
−60
−40
−20
0
V (mV)

variable from cell to cell ranging from almost negligible to 500 pA with a mean (+/- S.E.) of 40.0 +/- 3.1 pA (n=376). The fast transient inward current was largely abolished by 0.2 - 2 μM tetrodotoxin (TTX) in 12 experiments (Fig.10) consistent with the presence of a conventional Na^+-dependent mechanism; also in two experiments where external Na^+ was replaced, with the large impermeant cation choline, the transient inward current was suppressed. Cells with larger peak Na^+ currents displayed action potentials upon depolarization in current clamp mode (e.g. Fig.11A), while the majority of cells (which had small Na^+ currents) produced only small active responses (Fig.11B). These transient Na^+ currents were smaller than those reported in freshly-dissociated adult rabbit glomus cells (460-2000 pA: Duchen et al 1988; Urena et al 1989) and reflects a lower density of voltage-dependent Na^+ channels in cultured rat glomus cells which appear to be of a comparable size. The density of Na^+ channels in cultured glomus cells was calculated from the formula:

$$Density = \frac{Number\ of\ channels}{surface\ area} = \frac{gNa_{max}/\gamma_{Na}}{C_i/SMC}$$

where gNa_{max} is the maximum Na^+ conductance; γ_{Na} is an estimate of the unitary Na^+ channel conductance (17 pS; see Fenwick et al 1982); C_i is the average input capacitance (6.4 pF; see passive properties above); and SMC is the specific membrane capacitance (1μF/cm^2). The maximum Na^+ conductance, gNa_{max}, was obtained from the relation:

FIG.10. Effect of tetrodotoxin (TTX) on the transient inward current in a cultured glomus cell. The transient inward current in this cell was almost completely blocked by the addition of 1 μM TTX to the bath while the outward current was unaffected. This suggests that a large portion of the transient inward current was carried via typical TTX-sensitive Na^+ channels.

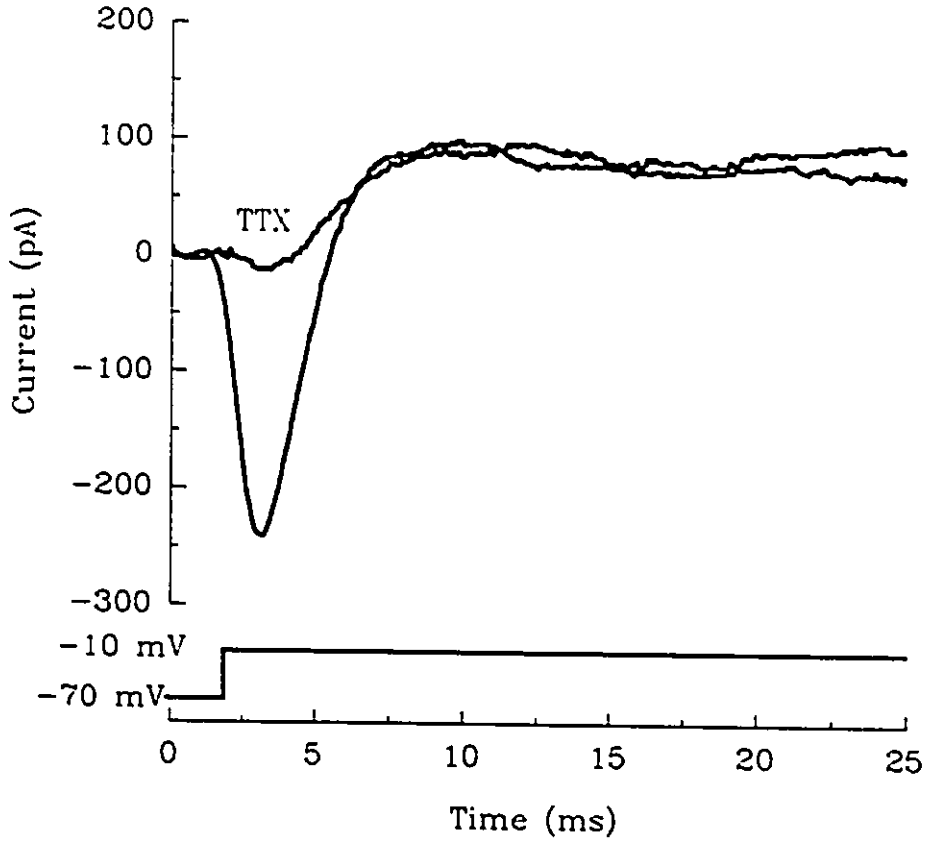
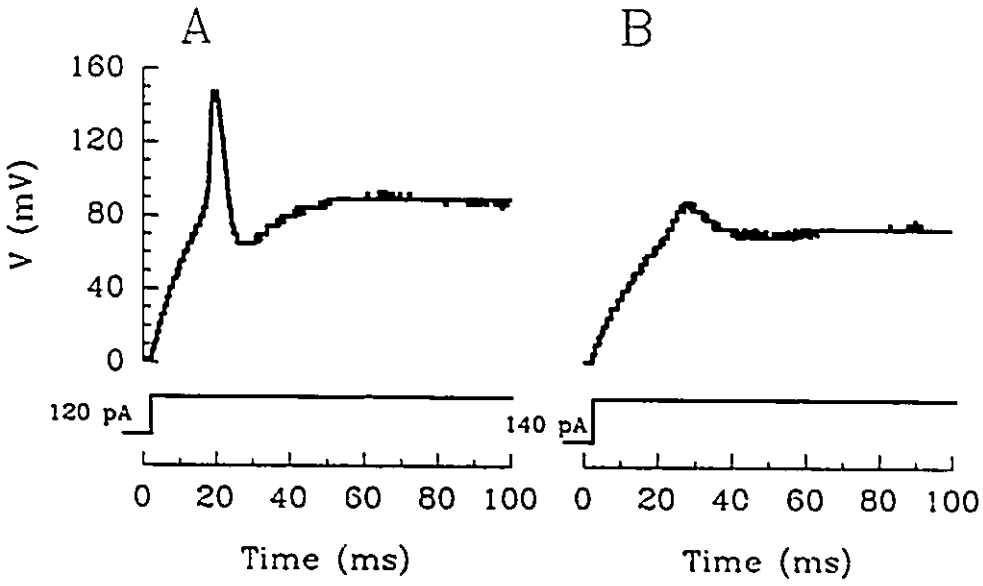

200
100
0
−100
−200
−300
Current (pA)
TTX
−10 mV
−70 mV
0
5
10
15
20
25
Time (ms)

FIG.11. Current clamp recordings from glomus cells. A: An action potential ($\approx$ 80 mV amplitude) was elicited when depolarizing current (120 pA; lower trace) was applied to this cell. B: When depolarizing current (140 pA; lower trace) was injected into another glomus cell only a small active response ($\approx$ 20 mV amplitude) was observed. This is likely due to a lower density of Na^+ channels in this cell which is more typical of the majority of glomus cells tested.


A
B
160
120
80
40
0
V (mV)
120 pA
140 pA
0 20 40 60 80 100
0 20 40 60 80 100
Time (ms)
Time (ms)

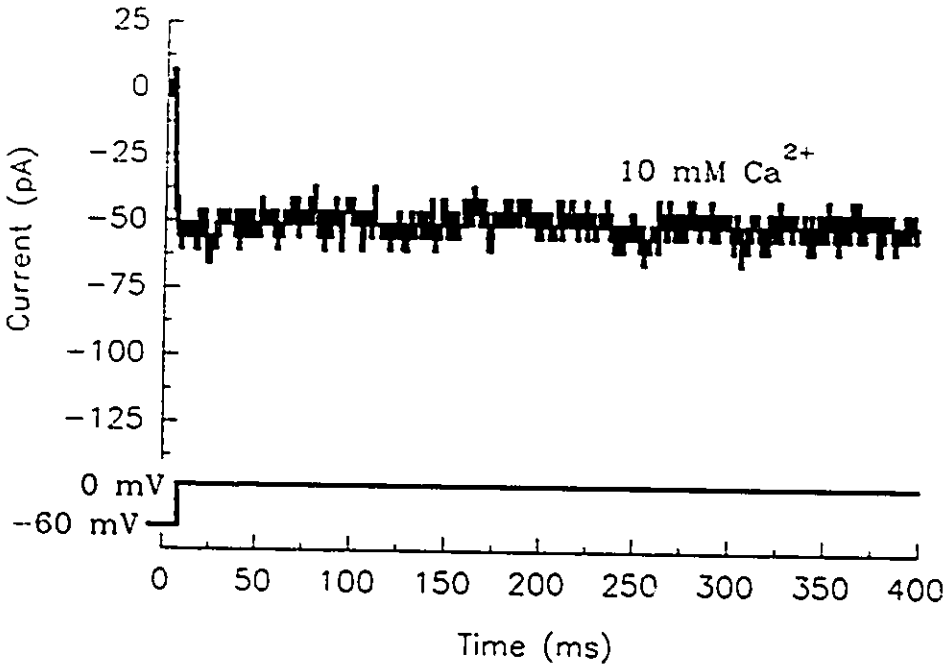
$$gNa_{max} = \frac{I_{max}}{E_m - E_{Na}}$$

where I_{max} = the maximum inward current estimated from the peak current recorded during a step from -60 to 0 mV, assuming that at -60 mV half the Na^+ channels are inactivated (see Fig.9); E_m = test potential (0 mV); E_{Na} = sodium equilibrium potential. The calculated Na^+ channel density in rat glomus cells was 0.09 channels/μm^2 which is considerably less than that for bovine chromaffin cells (1.5 to 10 Na^+ channels/μm^2; Fenwick et al 1982) and for freshly-isolated adult rabbit glomus cells (0.8 to 3.7 channels/μm^2; my calculations using data of Duchen et al 1988 and Urena et al 1989). This difference in Na^+ channel density between rat and rabbit glomus cells may well be due to a species difference rather than due to a difference in age since recordings from a few glomus cells isolated from adult rats did not produce significantly larger peak inward currents (mean=22.8 +/- 8.4 pA; n=9).

Ca^{2+} currents

Another component to the inward current was only observed when K^+ currents were blocked by Cs^+ ions and TEA in the pipette. This current was activated at $\approx$ -30 mV but unlike the transient Na^+ current was prolonged and did not inactivate, at least over 400 ms in a few experiments (e.g. Fig.12). In K^+-free media this slow inward current had a peak value of 34.8 +/- 3.8 pA (n=51) while the peak transient inward Na^+ current was 51.3 +/- 10.3 pA (n=51) in these same cells. This current

FIG.12. Prolonged inward Ca^{2+} current observed during recording in K^+-free media. With 10 mM Ca^{2+} in the bath and K^+ ions replaced by Cs^+ and TEA in the pipette a prolonged inward current was seen in this glomus cell which did not seem to inactivate at least over 400 ms. The small Na^+ current in this cell is hardly visible over the augmented Ca^{2+} current.


25
0
−25
−50
−75
−100
−125
Current (pA)
10 mM Ca2+
0 mV
−60 mV
0
50
100
150
200
250
300
350
400
Time (ms)

was carried by Ca^{2+} ions since it was augmented when the extracellular Ca^{2+} concentration was increased from 2 mM to 10 mM (n=15) and was completely abolished when extracellular Ca^{2+} was replaced by 2 mM Co^{2+} (n=7; see Fig.13). Application of 5 μM methoxyverapamil (D-600), an organic calcium channel blocker (Hille 1984) known to block the sustained L-type Ca^{2+} current in bovine chromaffin cells (Bossu et al 1991a), caused approximately a 50% reduction (n=2) in this prolonged inward current. In two experiments the $V_{1/2}$ estimated from the steady state inactivation curves was $\approx$ -40 mV (Fig.14) which is shifted in the depolarized direction from the $V_{1/2}$ for the Na^+ current (see also Kostyuk et al 1981; Stea and Nurse 1992). The density of calcium channels in glomus cells was estimated using the same procedures discussed above for the Na^+ channels. Assuming a single channel conductance (γ_{Ca}) of 5 - 10 pS (Hille 1984) the density was 0.03 channels/μm^2, which is three-fold lower than the density of Na^+ channels. Thus the slow component of the inward current recorded from cultured rat glomus cells under voltage clamp appeared to be due to the movement of Ca^{2+} ions through L-type calcium channels as reported for rabbit glomus cells (Duchen et al 1988; see also Peers and Green 1991).

K^+ currents

The prolonged outward current recorded under voltage clamp (Fig.8A) reached a plateau approximately 20 ms after the voltage step. The peak outward current had a mean value of 752.9 +/- 28.0 pA (n=376) when the cell was held at -70 or -60 mV and stepped to +50 mV and generally did not inactivate at least over the first 50 ms.

FIG.13. Ca^{2+} currents from a cultured glomus cell recorded in K^+-free solutions. When Ca^{2+} was replaced by 2 mM cobalt the prolonged inward current was abolished but a small transient Na^+ current remained. Elevating the external Ca^{2+} from 2 mM to 10 mM caused a pronounced increase in the prolonged current in this cell.

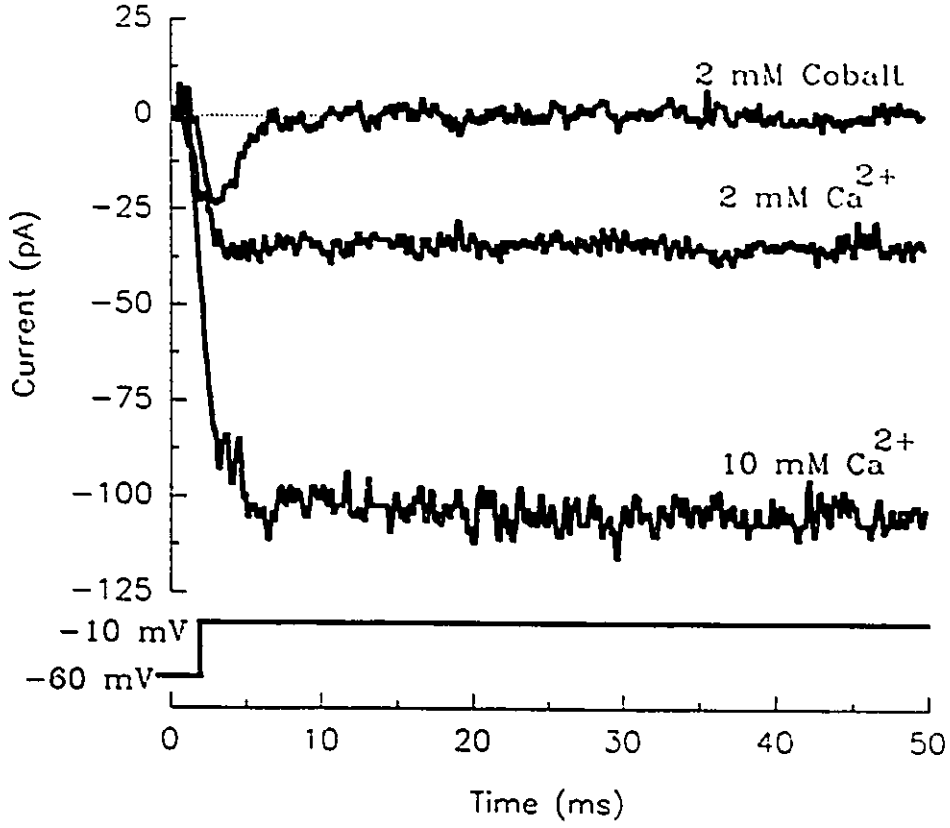
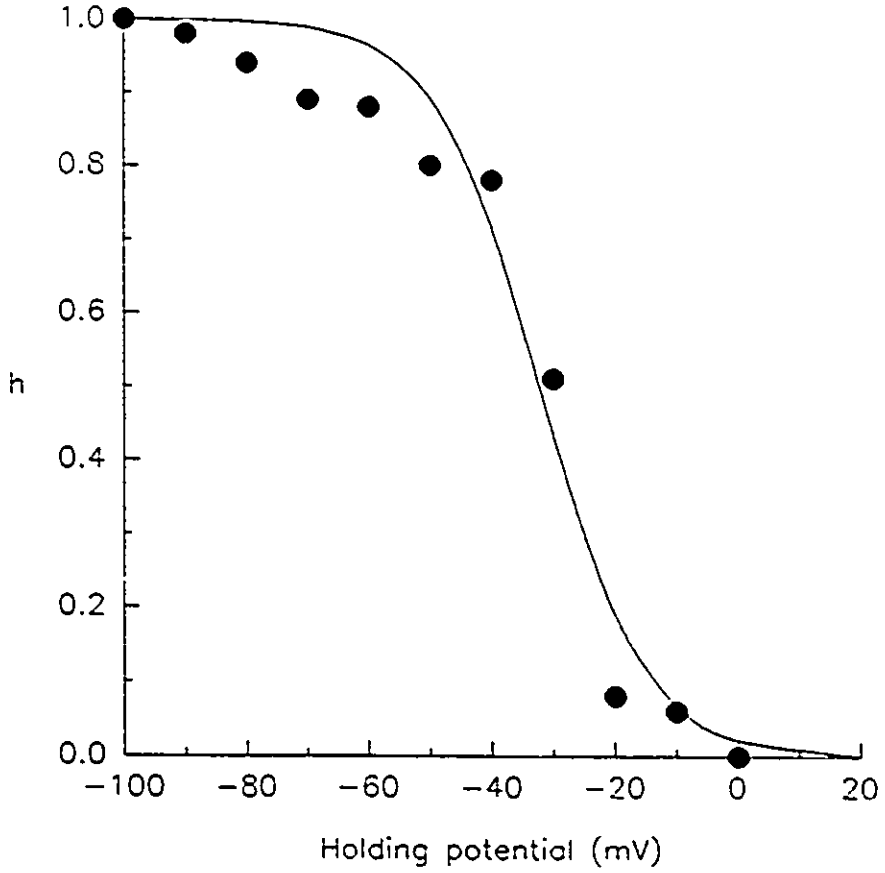
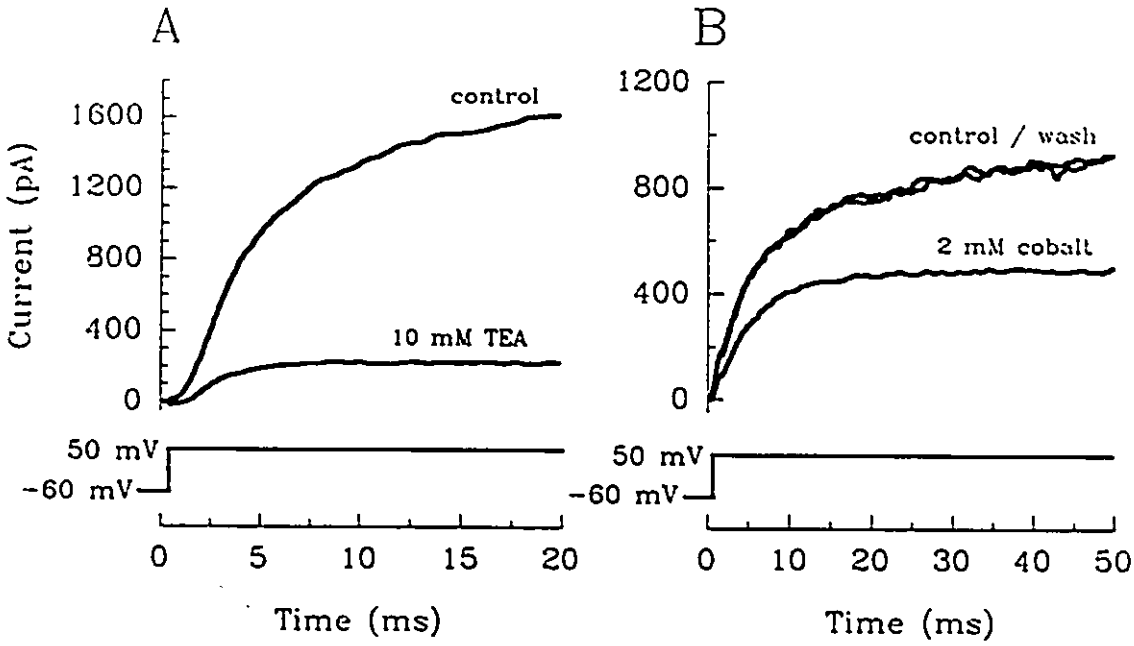

25
0
−25
−50
−75
−100
−125
Current (pA)
2 mM Cobalt
2 mM Ca2+
10 mM Ca2+
−10 mV
−60 mV
0
10
20
30
40
50
Time (ms)

FIG.14. Steady state Ca^{2+} inactivation curve from cultured glomus cell during recording in K^+-free media. Data (filled circles) were fitted with a smooth curve generated from a modified version of the Boltzmann equation (see methods); h represents peak Ca^{2+} current measured after the transient Na^+ current was inactivated ($\approx$ 10 ms) from each holding potential divided by the maximum Ca^{2+} current (measured at -100 mV). Note the slight shift to the right ($\approx$ 20 mV for $V_{1/2}$) compared with the Na^+ inactivation curve in Fig.9.


1.0
0.8
0.6
0.4
0.2
0.0
h
−100
−80
−60
−40
−20
0
20
Holding potential (mV)

The voltage dependent outward current had a threshold for activation between -30 and -20 mV (Fig.8B; triangles) and was carried by K^+ ions since it was eliminated when K^+ ions were substituted by Cs^+ and TEA ions in the pipette (n=51). Further, external application of 5 mM TEA, a known blocker of a variety of voltage-activated K^+ channels (Hille 1984), reduced the outward current by 75 +/- 4.5% (Fig.15A; n=7). The presence of a significant Ca^{2+}-activated component to the outward current was indicated following blockade of Ca^{2+} entry by substituting external Ca^{2+} with cobalt (2 mM). In these experiments there was a marked and reversible decrease in the outward current by 52.7 +/- 7.8% (Fig.15B; n=6) along with a reduction in current noise possibly due to the closing of large conductance Ca^{2+}-activated K^+ channels (Ganfornina and Lopez-Barneo 1991). The presence of a substantial Ca^{2+}-activated component to the outward K^+ current has also been reported in other studies on isolated glomus cells (Hescheler et al 1989; Peers 1990a,b; Stea et al 1991). The density of Ca^{2+}-activated K^+ channels in cultured rat glomus cells was 0.022 K^+_{Ca} channels/μm^2 calculated as described above assuming the Ca^{2+}-activated component of the K^+ current was $\approx$ 50% of the total current (see above) and was carried by large conductance K^+_{Ca} channels ($\gamma \approx$ 200 pS, Marty 1989).

FIG.15. Effect of tetraethylammonium (TEA) and cobalt on the outward K^+ current in glomus cells. A: The addition of 10 mM TEA, a K^+ channel blocker, to the bath caused a large reduction in the K^+ current in this cell. B: Reduction in whole-cell K^+ current in glomus cell during equimolar replacement of external Ca^{2+} with Co^{2+}. The decrease in current noise in the presence of cobalt may reflect the blockade of the larger conductance Ca^{2+}-activated K^+ channels in these cells.


A
B
control
10 mM TEA
control / wash
2 mM cobalt
Current (pA)
1600
1200
800
400
0
1200
800
400
0
50 mV
-60 mV
50 mV
-60 mV
0
5
10
15
20
0
10
20
30
40
50
Time (ms)
Time (ms)

DISCUSSION

This chapter describes the electrophysiological properties of cultured glomus cells from a chemosensory organ, the rat carotid body, using both conventional whole-cell recording (Hamill et al 1981) and the new perforated patch method (Horn and Marty 1988). This is the first study in which glomus cells were studied by the latter recording technique. In this method the presence of the pore-forming antibiotic, nystatin, in the pipette limits diffusive exchange between cell interior and patch pipette to small monovalent ions (Horn and Marty 1988), thus preserving cytoplasmic integrity. Though the preparation was more stable and responses were more readily reversible with the perforated-patch technique the ionic currents recorded with the two methods were similar. The results reported here are qualitatively similar to those reported in other studies using the patch clamp technique on glomus cells (Duchen et al 1988; Hescheler et al 1989; Peers 1990a; Urena et al 1989). However, as discussed below, some important aspects of glomus cell physiology, not hitherto recognized, were uncovered during the present study.

Leakage currents in glomus cells

Several early studies using intracellular recording suggested that Cl^- ions are important in governing the resting potential of glomus cells though the problem of cell damage by electrode impalement made this conclusion tentative (Eyzaguirre et al 1983; Oyama et al 1986a). Our findings suggest that both Cl^- and K^+ movements

contribute to the leakage conductance or input resistance of glomus cells in conventional HEPES-buffered external media. The high input resistances ($\approx$ 2.6 GΩ) agreed well with the values from other patch clamp studies on glomus cells (Duchen et al 1988; Urena et al 1989) but were much higher than the values reported from intracellular recordings ($\approx$ 40 MΩ; Eyzaguirre et al 1983). It has been suggested that the presence or absence of electrotonic coupling between glomus cells during recording may account for these discrepancies (Monti-Bloch and Eyzaguirre 1990; He et al 1991a). Alternatively, damage caused by intracellular impalement of these small cells may be the cause of the low input resistances by the introduction of a shunt around the electrode (see Duchen et al 1988). Interestingly, using the patch clamp technique, the input resistance of glomus cells was found to be markedly depressed ($\approx$ three-fold) when recording in a more-physiological bicarbonate-buffered media (BBM) instead of the conventional HEPES-buffered media (HBM). These findings indicated that leakage channels were opened during recording in BBM. The presence of large current steps superimposed on the whole-cell currents, the insensitivity of these currents to removal of K^+ ions, and the sensitivity of the leakage currents to the chloride channel blocker 9-AC suggested that large conductance anion channels were opened in BBM. If so, the opening of $\approx$ 3-4 such channels could account for the increase in leakage conductance. The opening of these leakage channels may be due to removal of HEPES or the consequence of addition of HCO_3^- and/or CO_2 to the external solution. HEPES has been shown to block large conductance anion channels reconstituted from human platelets (Manning and Williams 1989) and inhibit

outwardly rectifying anion channels in an epithelial cell line (Hanrahan and Tabcharani 1990). As discussed in Chapter 5, a large conductance, HCO_3^--permeable, chloride channel (blocked by 9-AC) was recorded in inside-out patches from glomus cells (see also Stea and Nurse 1989) raising the possibility that HEPES may block these channels in the whole cell, and that this block is removed in BBM. However this may not provide a complete explanation since the channels were frequently open in membrane patches in HBM where the mean open probability was 0.6 (Stea and Nurse 1989; see Chapter 5). Alternatively, changes in cell volume, for example produced by alteration of intracellular osmolytes, may be the stimulus for opening of these channels. For example, in cell-attached patches from cultured kidney cells exposure to hypotonic media ($\approx$ 190 mOsmoles/L) causes opening of ion channels (Ubl et al 1988). In support of this idea, artificially induced cell swelling by the use of a hypoosmotic HBM mimicked the effect of BBM in several cases. Indeed the carotid body is sensitive to changes in osmolarity since during perfusion of hyperosmotic and hypoosmotic media the chemosensory discharge from this organ increased and decreased, respectively, in an *in vitro* preparation (Gallego et al 1979). However, in contrast to my findings, the above study found glomus cell input resistance, measured by microelectrode impalement, increased on exposure to hypoosmotic media (Gallego et al 1979). A possible modulator for the above membrane responses in glomus cells may be atrial natriuretic peptide or factor (ANP) which has recently been localized to cat glomus cells (Wang, Z.-Z. et al 1991b). Atrial natriuretic peptides are produced by the heart in response to a variety of stimuli

(including hypoosmolarity) and evoke a number of vasodilatory, natriuretic, and diuretic responses in various tissues, e.g. decreasing cell volume in heart cells (Clemo et al 1991). ANP is a potent inhibitor of carotid sinus nerve discharge in the cat carotid body evoked by hypoxia suggesting that it may modulate the membrane properties of glomus cells (Wang, Z.-Z. et al 1991b).

Therefore it is plausible that *in vivo*, when glomus cells are in a physiological bicarbonate-buffered milieu which facilitates opening of anion channels, Cl^- may contribute significantly to the resting potential of these cells.

Voltage-activated properties of glomus cells

Rat glomus cells contained conventional voltage-gated Na^+ channels, sensitive to TTX, Ca^{2+} channels which were similar to the L-type (Nowycky et al 1985), and K^+ channels which had a large Ca^{2+}-activated component. These properties are qualitatively similar to those reported for adult rabbit glomus cells (Duchen et al 1988; Urena et al 1989). However the inward Na^+ currents in rat glomus cells (including those from the adult) were much smaller on average than those reported for adult rabbit and indeed the calculated density of Na^+ channels in rat glomus cells was much less than in adult rabbit glomus cells. Spiking appears to be common in adult (Duchen et al 1988; Lopez-Barneo et al 1988) but not embryonic (Delpiano and Hescheler 1989) rabbit glomus cells. Although a minority of rat glomus cells with larger inward currents did elicit spiking under current clamp in this study, it appears not to be a common feature of these cells in either short or long-term culture. The

possible role of Na^+ channels and cell spiking in hypoxic chemotransduction will be discussed in Chapter 6. A prolonged L-type Ca^{2+} current (Nowycky et al 1985) was also present in rat glomus cells and similar currents have been described in both adult (Duchen et al 1988; Urena et al 1989) and embryonic (Hescheler et al 1989) rabbit glomus cells. It is probable that these channels play a major role in the chemotransduction process as calcium influx is directly linked to transmitter release in the carotid body (Rocher et al 1991). Finally the outward voltage-activated K^+ current found in rat glomus cells had a substantial Ca^{2+}-activated component. In other systems Ca^{2+}-activated K^+ channels play a variety of physiological roles such as, modulating repetitive firing in neurons (Hille 1984) or regulating secretion in endocrine cells (Blatz and Magleby 1987). As intracellular Ca^{2+} is likely an important mediator of transduction processes in glomus cells (discussed in Chapter 6) these Ca^{2+}-activated K^+ channels may also be linked to the chemotransduction process.

Consequently the ionic currents described above (Cl^-, Na^+, Ca^{2+}, and K^+) may be modulated in glomus cells in response to various sensory stimuli, e.g. hypoxia (Chapter 2), acidity (Chapter 3) and hypercapnia (Chapter 4).

CHAPTER 2

Effects of Hypoxia

on the

Electrophysiological Properties

of Glomus Cells

INTRODUCTION

One of the major roles for the mammalian carotid body is to sense changes in arterial Po_2 and reflexly control ventilation (Biscoe 1971; De Castro 1926; Eyzaguirre and Zapata 1984). During hypoxia the afferent signal increases along the carotid sinus nerve, which innervates the carotid body, and this stimulates the respiratory control centre resulting in increased breathing. At the commencement of these studies the leading candidates for the O_2 sensors were the glomus or type I cells, which form discrete clusters in the carotid body, and the sensory nerve endings which form morphological synapses with the glomus cells (McDonald 1981). In recent years attention has focused on glomus cells as chemotransducers since the initial finding by Lopez-Barneo et al (1988) in the rabbit, that glomus cells contained an O_2-sensitive K^+ current. Several other studies using patch clamp whole-cell recording have confirmed this result (Hescheler et al 1989; Lopez-Lopez et al 1989; Peers 1990a,b;

Stea and Nurse 1991a; see however Biscoe and Duchen 1990a) and in addition there are direct single-channel recordings of Po_2-sensitive K^+ channels in membrane patches of rabbit glomus cells (Delpiano and Hescheler 1989; Ganfornina and Lopez-Barneo 1991). These findings suggest a mechanism where glomus cells would become depolarized in response to hypoxia due to closing of the O_2-sensitive K^+ channels leading to increased transmitter release and a subsequent enhancement of the afferent signal from the carotid body (Lopez-Barneo et al 1988).

Some of the experiments reported in this chapter were designed to test whether rat glomus cells also contain an O_2-sensitive K^+ current, and whether this property persists in culture. In these acute experiments exposure to low Po_2, or hypoxia, lasted only a few minutes. However, prolonged hypoxia (for days or weeks) is known to cause a number of morphological and physiological changes in the carotid body *in vivo*. For example, conditions of chronic hypoxia, as occurs in humans and animals living at high altitude (Edwards et al 1971a) or in patients with hypoxic lung disease (Edwards et al 1971b), cause enlargement of the carotid body as well as a time-dependent sensitization of this chemosensory pathway (Barnard et al 1987; Nielsen et al 1988). These adaptations are thought to be important during acclimatization to hypoxia though the underlying mechanisms are unknown. The above structural and functional changes may be mediated via a direct action of low arterial Po_2 on the carotid-body oxygen sensors, i.e. glomus cells, or indirectly, by way of blood-borne factors arising elsewhere in the circulation. The ability to study rat glomus cells in culture (Nurse 1987,1990) allowed a direct examination of the effects of hypoxia,

acute or chronic, on the electrical properties of these cells without the external circulatory or synaptic influences present *in vivo*.

The results presented below are based on experiments dealing with the effects of acute and chronic hypoxic exposure on the electrophysiological properties of cultured rat glomus cells. In the first group of experiments the original finding of Lopez-Barneo et al (1988) that acute hypoxia caused a decrease in the outward K^+ current in rabbit glomus cells was confirmed in this system. However, two further questions arose. First, did intracellular dialysis during conventional whole-cell recording modify the normal response to hypoxia? Indeed a major involvement of intracellular metabolites is implicit in the 'metabolic' hypothesis of chemoreception, one of the popular theories proposed to account for the carotid body's response to hypoxia (reviewed by Eyzaguirre and Zapata 1984). Second, is this effect of hypoxia specific to glomus cells or is it a general feature shared by other cell types? To probe for possible contributions of dialysed components to changes in membrane properties during hypoxia results based on conventional whole-cell recording (Hamill et al 1981) were compared with the new perforated-patch technique (Horn and Marty 1988), which is known to minimize 'wash-out' of cytoplasmic constituents. To address the question of specificity of the O_2-sensitive K^+ current the effect of hypoxia was tested on membrane currents of; i) sensory petrosal neurons, which innervate glomus cells (McDonald 1981) and are part of the chemosensory complex of the carotid body (Eyzaguirre and Zapata 1984), and ii) cultured small intensely-fluorescent (SIF) cells which are dopaminergic interneurons from the superior cervical ganglion and are

thought to be developmentally related to glomus cells of the carotid body (Doupe et al 1985; Kobayashi 1971; McDonald and Blewett 1981). In the second group of experiments the effects of chronic hypoxia on the membrane properties of glomus cells were investigated by exposing carotid body cultures to normoxic (20% O_2) and hypoxic (6% O_2) atmospheres for up to 2 weeks. The effects of prolonged elevation of intracellular cAMP (using the membrane permeant analog dibutyryl cAMP) were also investigated since hypoxia is known to increase $cAMP_i$ in the carotid body *in vivo* (Delpiano and Acker 1991; Perez-Garcia et al 1990; Wang, Z.-Z. et al 1991a).

METHODS

The procedures for the glomus cell culture, whole-cell and perforated-patch recording, and data analysis were identical to those previously described in Chapter 1.

Cultured small intensely fluorescent (SIF) cells

The procedures used for growing SIF cells from the superior cervical ganglia (SCG) of the rat were similar to those described by Doupe et al (1985). Briefly, 1 to 3-day-old rat pups were killed by a blow to the head and the SCG removed and placed in L-15 plating medium. Dispersed cells of the SCG were obtained by combined enzymatic and mechanical dissociation as described above and grown in culture dishes on a thin layer of Matrigel (Collaborative Research, Bedford, MA). Typically, the cell suspension from 8 ganglia was plated into 5 dishes. Selection for SIF cell survival was obtained by the addition of 0.5-5 μM dexamethasone (Sigma Chemical Co., St.Louis, MO) to the growth medium (Doupe et al 1985) which consisted of F-12 nutrient medium supplemented with 10% fetal bovine serum (FBS), 0.6% glucose, 2 mM L-glutamine, and 1% penicillin-streptomycin (Gibco). SIF cells were grown under the same incubation conditions as the glomus cells for 7-14 days before they were used in whole-cell experiments.

Cultured petrosal neurons

Sensory petrosal ganglia were dissected from 0 - 12 day old rat pups (Wistar, Charles River, Quebec) and dissociated using both enzymatic and mechanical procedures. Pups were first stunned by a blow to the head and decapitated before removing the carotid bifurcation and surrounding ganglia of the IXth and Xth cranial nerves. In some experiments both the petrosal and juxtaposed jugular ganglia were used without attempting to separate them. In other experiments petrosal 'enriched' cultures were obtained by first pulling on the glossopharyngeal (IXth) nerve and separating the attached petrosal ganglion with the aid of two pairs of forceps. This procedure at least provided an enrichment for the more distal region of the petrosal ganglion where the chemosensory afferents are located (Katz and Black 1986). The excised ganglia were incubated for 1 hr at 37°C in an enzyme solution containing 0.1% collagenase/ 0.1% trypsin (Gibco, Grand Island, NY). The enzyme was then replaced by growth medium (see below), and the tissues were mechanically dissociated and triturated to yield a cell suspension. The cells were grown on a thin layer of Matrigel (Collaborative Research, Bedford, MA) applied to the central wells of modified 35 mm tissue-culture dishes. The growth medium consisted of F-12 nutrient medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 80 U/l insulin (Sigma Chemical Co., St.Louis, MO), 0.6% glucose, 2 mM glutamine, and 1% penicillin-streptomycin (Gibco). Cultures were grown at 37°C in a humidified atmosphere of 95% air: 5% CO_2 for 18 h to 43 days before they were used in the patch-clamp/whole-cell experiments.

Solutions

Most of the hypoxia experiments were performed using extracellular fluid and pipette solutions identical to those described in Chapter 1. To simulate acute hypoxic conditions the perfusion fluid was bubbled with 100% N_2 continuously for 20-30 min until the Po_2 fell below 20 Torr before it was applied to the cells. Whole-cell currents were recorded within 2 min of perfusing the low Po_2 solutions; in this period it was found during control experiments that the actual Po_2 in the culture dish did not exceed 30 Torr.

In chronic experiments glomus cells were cultured as described above, and two days after plating the cells were exposed to one of the following treatments for up to 14 days: i) A hypoxic environment (6% O_2, 88% N_2, 5% CO_2); ii) A normoxic environment (20% O_2, 75% N_2, 5% CO_2) with 1 mM dibutyryl cAMP (Sigma) added to the nutrient media; iii) A normoxic environment with 1 μg/ml of NGF (a gift from Dr. E. Cooper) in the media. In addition control cultures were grown in the usual normoxic environment as described in Chapter 1, Methods.

Data analysis

All treatments were compared to controls with a Student's t-test and the level of significance was set at $p < 0.01$. Peak Na^+ currents were measured corresponding to a voltage step from -60 to 0 mV and then divided by the input capacitance of the cell to give the Na^+ current density. Steady-state K^+ currents were measured for a step from -60 to +50 mV (after $\approx$ 30 ms) and then divided by the capacitance to give the K^+ current density.

RESULTS

Effects of acute hypoxia on whole-cell currents in cultured glomus cells

Exposure of 53 cultured glomus cells to hypoxia (Po_2 ≈ 20-30 Torr) during conventional whole-cell recording resulted in each case in an immediate decrease in the peak outward current (Fig.16A,B); the small inward current was unaffected. Complete reversibility following perfusion of normoxic solutions (Po_2 ≈ 160 Torr) was demonstrated in 17 cases, where the mean outward current (+/- S.E.) decreased by 17 +/- 2.4% in response to hypoxia during voltage steps from -70 or -60 to +50 mV. In the remaining 36 cases recovery did not occur before deterioration of the preparation terminated the experiment. Conceivably intracellular dialysis was the main contributing factor to cell deterioration since as discussed below the preparation was more stable and the responses were more readily reversed with the perforated-patch configuration. In twenty perforated-patch experiments where glomus cells were exposed to acute hypoxia, 15 cells showed a reversible decrease in outward current by 14 +/- 1.6%. In general this property, i.e. the reversible decrease in outward current due to hypoxia, persisted in rat glomus cells even after several weeks in culture (Fig.17) although the magnitude of the decrease declined after 1 week in culture.

The observed decrease in outward current due to hypoxia is most easily explained by a decrease in K^+ conductance. First, substitution of glutamate for most of the chloride in the intracellular (pipette) solution did not alter the immediate response to hypoxia (n=11) suggesting a negligible contribution due to chloride

FIG.16. Effect of acute hypoxia on whole-cell currents in cultured glomus cells. A: outward K^+ current is reversibly decreased ($\approx$ 30%) on exposure (within 5 min) to a hypoxic solution (P_{o2} $\approx$ 20 Torr) with conventional whole-cell recording; note small decrease in inward current during wash was likely due to washout of the Ca^{2+} current. B: peak outward currents in the same cell following voltage steps from a holding potential of -70 mV before (open triangles; control), during (filled circles; hypoxia), and after (open circles; wash) exposure to hypoxia.

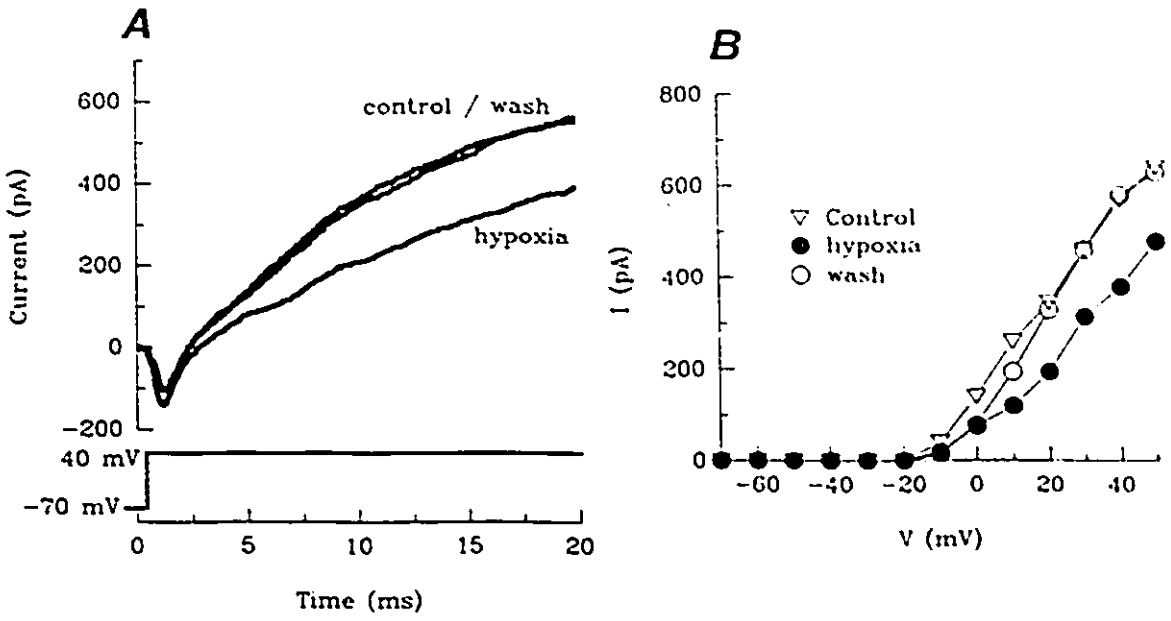
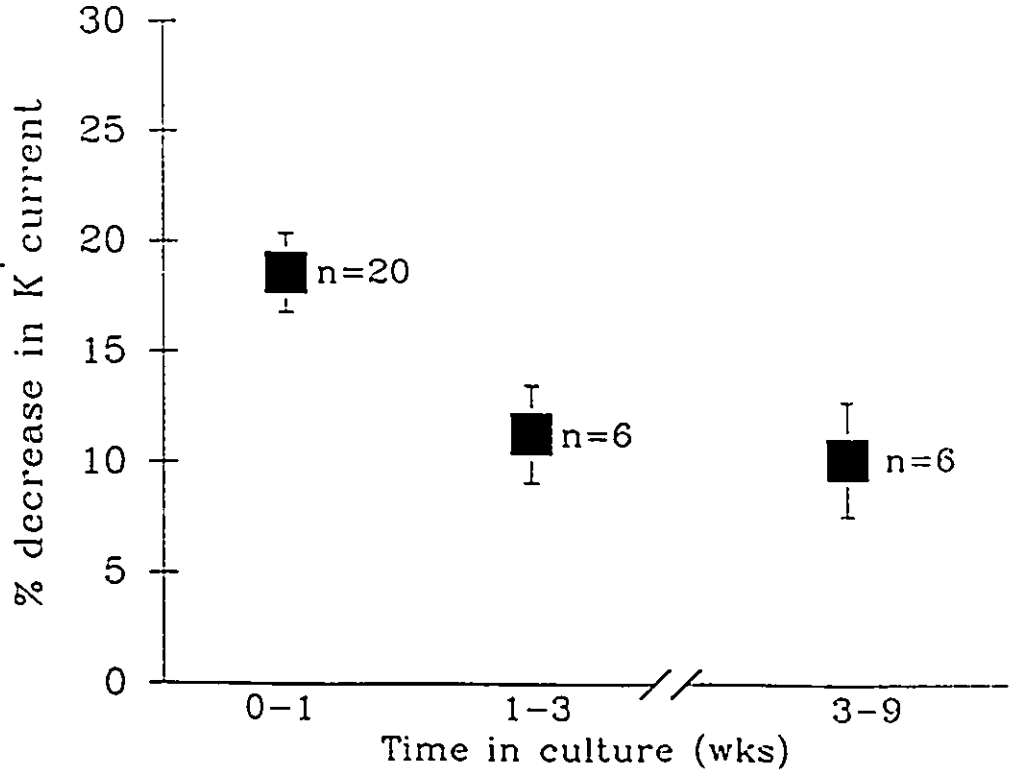

A
600
400
200
0
−200
Current (pA)
control / wash
hypoxia
40 mV
−70 mV
0
5
10
15
20
Time (ms)
B
800
600
400
200
0
I (pA)
Control
hypoxia
wash
−60
−40
−20
0
20
40
V (mV)

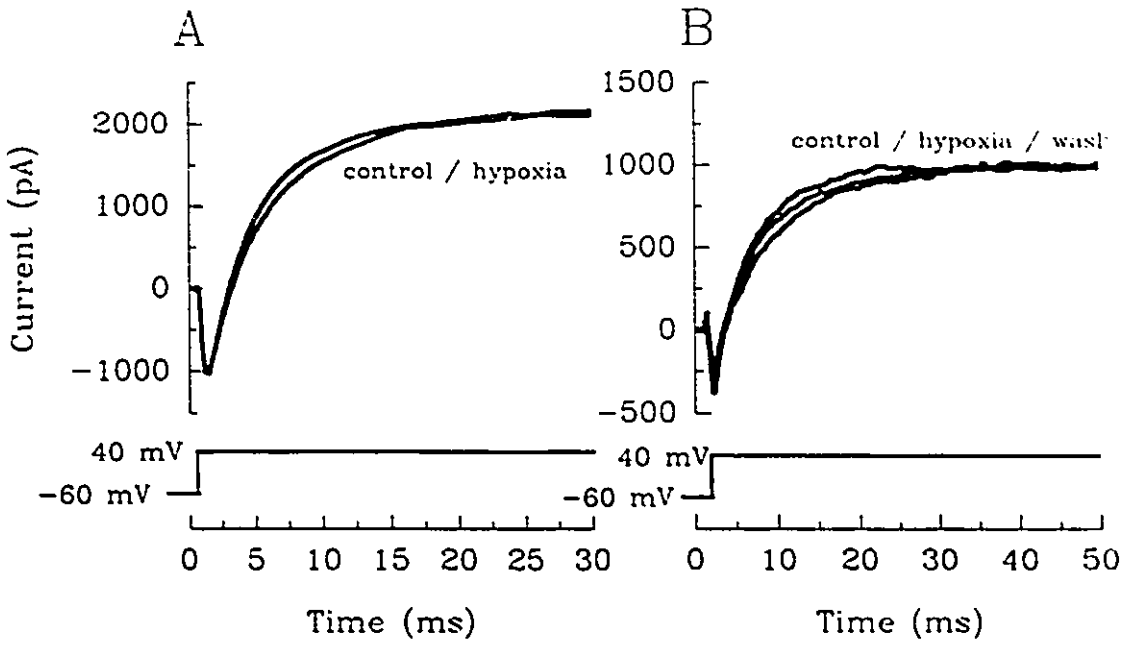
FIG.17. Effect of acute hypoxia on short and long-term cultures of glomus cells. The reversible decrease in outward K^+ current caused by hypoxia ($Po_2 \approx 20$ Torr) is apparent even in cells grown for up to 9 weeks in culture but is lower than the 20% decrease recorded from cells less than 1 week in culture.


30
25
20
15
10
5
0
% decrease in K+ current
n=20
n=6
n=6
0–1
1–3
3–9
Time in culture (wks)

movements although extracellular chloride was not changed in these experiments. Second, in eight experiments when K^+ currents were abolished by the substitution of Cs^+ for K^+ ions in the pipette, hypoxia had no effect. These results on cultured rat glomus cells therefore support the recent findings of an O_2-sensitive K^+ conductance in isolated rabbit glomus cells (Lopez-Barneo et al 1988; Lopez-Lopez et al 1989; Hescheler et al 1989; see also Peers 1990a,b).

The question arose whether this O_2-sensitive K^+ current was specific to glomus cells. To address this, the effects of hypoxia were compared on a closely-related cell type, the small intensely fluorescent or SIF cells from sympathetic ganglia and on sensory petrosal neurons, which innervate the carotid body and form synapses with glomus cells (McDonald and Mitchell 1975). In contrast to its effects on glomus cells, hypoxia had no detectable effect on the macroscopic currents recorded from SIF cells ($n=10$) or petrosal neurons ($n=15$) under voltage clamp (Fig.18A,B). These experiments suggest that the O_2-sensitive outward K^+ current described above is a specific feature of glomus cells and is undoubtedly related to their chemosensory function.

FIG.18. Lack of effect of acute hypoxia on whole-cell currents in cultured small, intensely-fluorescent (SIF) cells or petrosal neurons. A: Unlike glomus cells, the outward K^+ current recorded from SIF cells was unaffected by exposure to a hypoxic solution ($P_{O2} \approx 20$ Torr); voltage step (lower trace) from -60 mV to +40 mV. B: Perfusion of a hypoxic ($Po_2 \approx 20$ Torr) external solution elicited no change in the active currents in this petrosal neuron.

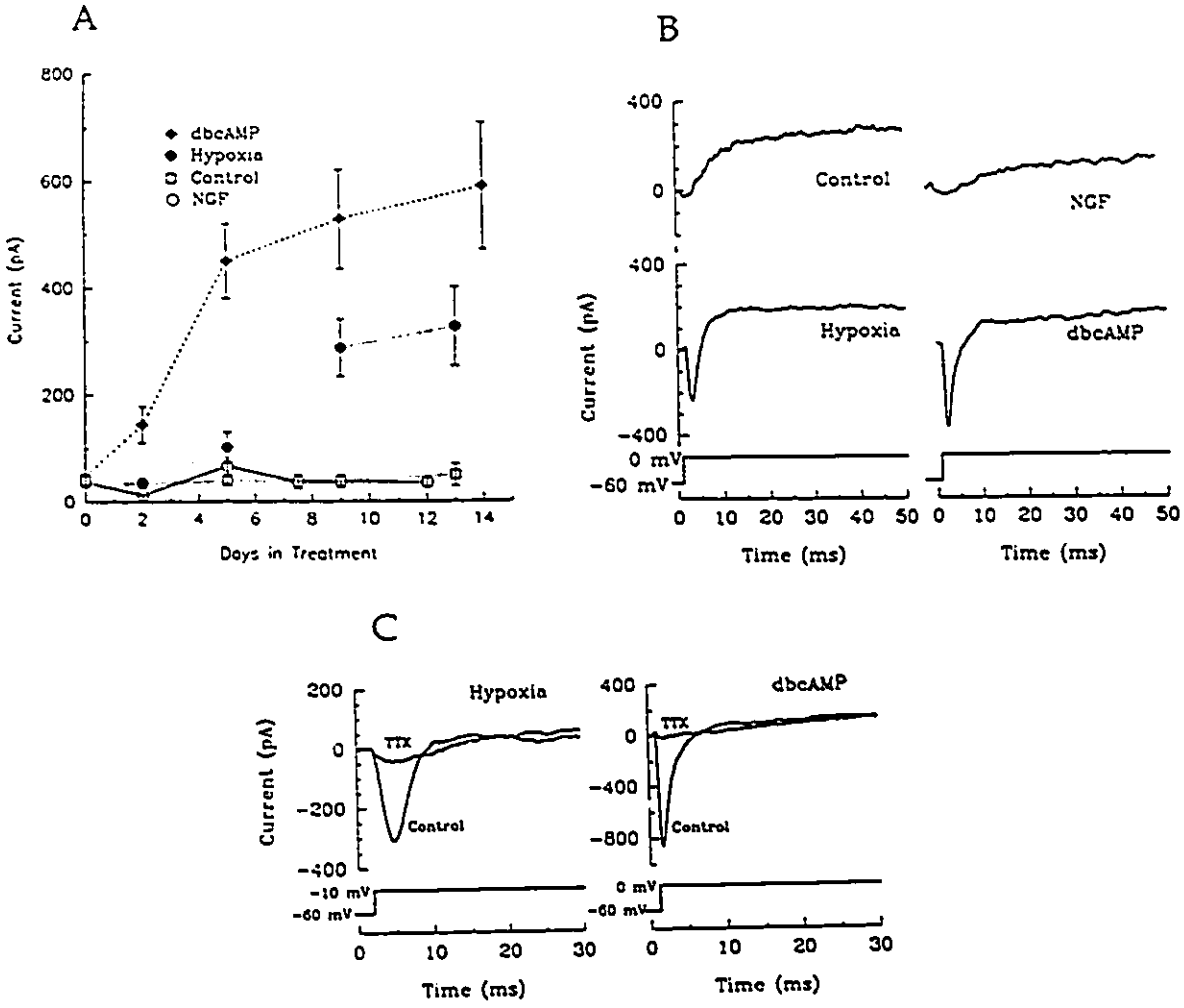

A
B
2000
1000
0
−1000
1500
1000
500
0
−500
Current (pA)
control / hypoxia
control / hypoxia / wash
40 mV
−60 mV
40 mV
−60 mV
0 5 10 15 20 25 30
0 10 20 30 40 50
Time (ms)
Time (ms)

Effects of chronic hypoxia on membrane properties of glomus cells

Following exposure of carotid body cultures to chronic hypoxia both the active and passive properties of glomus cells were significantly modified. Alterations in the active properties will be considered first. After 5 days of exposure to chronic hypoxia ($Po_2 \approx 50$ Torr) there was a significant ($p < 0.01$) rise in the peak inward current compared to controls (Fig.19A,B). The effect increased with exposure time and seemed to plateau at $\approx$ 320 pA by day 13 in hypoxia (Fig.19A,B). Control glomus cells grown in normoxic conditions for similar durations showed little deviation from a peak inward current of $\approx$ 40 pA (Fig.19A,B; see also Chapter 1). The large rise in peak inward current after hypoxic exposure was attributed to an induction of voltage-activated Na^+ channels since application of 0.5 to 1 μM tetrodotoxin (TTX) to the bath substantially decreased or abolished this increase in all cells tested (Fig.19C, left trace; n=13).

To probe for possible underlying mechanisms of Na^+ channel expression during hypoxia a membrane permeant analog of cAMP, dibutyryl cAMP (dbcAMP; 1 mM), was added to the culture media (see Methods) since it has been shown that hypoxia causes a rise in intracellular cAMP in glomus cells (Wang, Z.-Z. et al 1991a), and elevated $cAMP_i$ stimulates Na^+ channel expression in PC 12 cells (Kalman et al 1990) and in skeletal muscle cells (Offord and Catterall 1989). In the presence of 1 mM dbcAMP under normoxic conditions glomus cells showed an even greater increase in Na^+ current than hypoxia (Fig.19A; filled diamonds).This increase was significant ($p < 0.01$) compared to controls after only 2 days of treatment but seemed to plateau

FIG.19. Comparison of the effects of chronic hypoxia, dibutyryl cAMP (dbcAMP), and NGF on the development of inward Na^+ currents in cultured chemosensory glomus cells. A: Time course of the effect of each treatment on the peak inward Na^+ current elicited by a voltage step from -60 to 0 mV; control (normoxia, $Po_2 \approx 160$ Torr;□), hypoxia ($Po_2 \approx 50$ Torr;●), 1 mM dbcAMP (◆), and 1 μg/ml NGF (○). The progressive increase in peak Na^+ current is already significant ($p < 0.01$) at 5 days in hypoxia ($Po_2 \approx 50$ Torr) and at 2 days in dbcAMP; at no time was there a significant change during NGF treatment. As in previous studies (Stea and Nurse 1991a), control (normoxic) glomus cells had a mean peak Na^+ current of ≈ 50 pA which remained relatively constant over time in culture (□). Each point represents mean (+/- S.E.) of a minimum of 9 cells from at least two separate platings; graph is based on recordings from over 250 glomus cells. B: Whole-cell current traces recorded from glomus cells grown under various conditions. The control glomus cell, grown in a normoxic environment, had a typical small transient inward current followed by prolonged outward K^+ current; voltage step (lower traces) from -60 mV to 0 mV. The NGF-treated cell (upper right) showed no obvious difference from the control. In contrast, glomus cells exposed to hypoxia ($Po_2 \approx 50$ Torr) and to dbcAMP (1 mM) for 9 days showed a pronounced increase in the inward current but no apparent change in the outward K^+ current. C: Effect of tetrodotoxin (TTX) on the inward current in glomus cells exposed to hypoxia or dbcAMP. The inward current recorded from glomus cells exposed to hypoxia ($Po_2 \approx 50$ Torr) or 1 mM dbcAMP for 9-10 days was almost completely blocked by 1 μM TTX. The residual inward current is likely due to the small Ca^{2+} current known to be present in these cells.

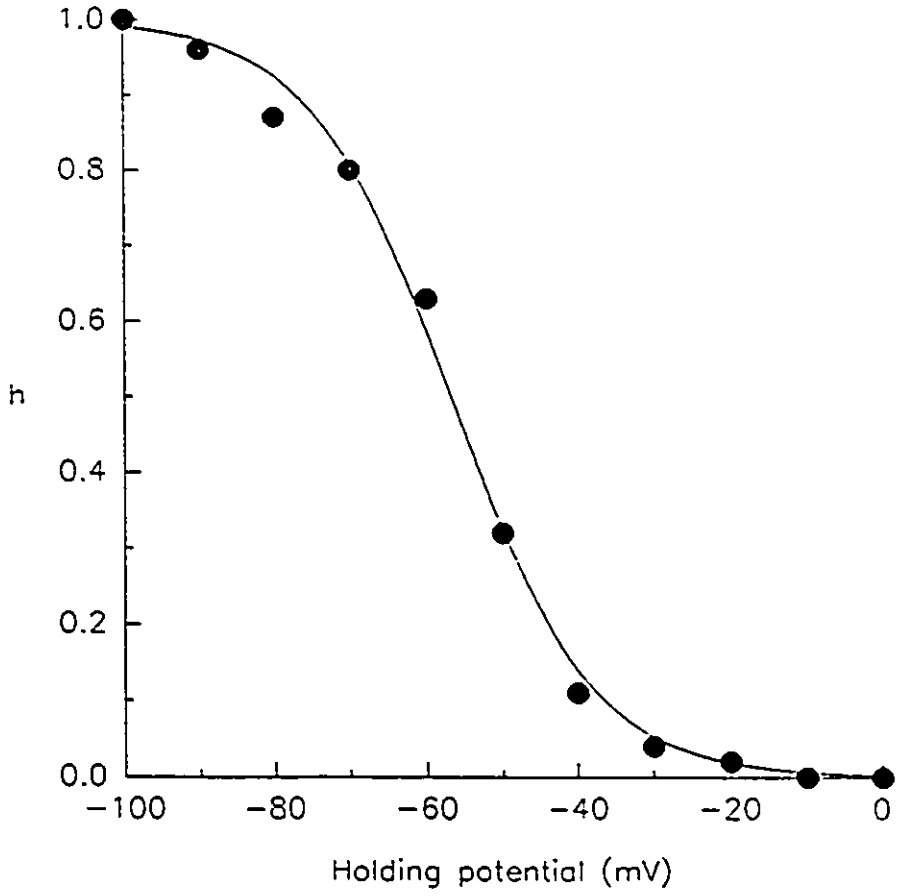

A
B
C
dbcAMP
Hypoxia
Control
NGF
Current (pA)
Days in Treatment
Control
NGF
Hypoxia
dbcAMP
0 mV
-60 mV
Time (ms)
TTX
-10 mV

after 9 days of treatment at ≈ 550 pA (Fig19A,B). As with hypoxia, the dbcAMP-treated cells had peak inward currents that were largely blocked by TTX (Fig.19C, right trace; n=11) indicating the presence of typical voltage-gated Na^+ channels. The steady-state inactivation curve for the Na^+ current in dbcAMP-treated glomus cells (e.g. Fig.20) appeared similar to that for control glomus cells (see Chapter 1) indicating the effects of dbcAMP on the magnitude of the Na^+ current was not simply due to a shift in the steady-state inactivation. In a few experiments the effects of another cyclic nucleotide, cGMP, were also tested since hypoxia is also known to affect levels of cGMP in glomus cells (Wang, Z.-Z. et al 1991a). Five glomus cells exposed to the membrane permeant analog dibutyryl cGMP (200 μM) in the culture media for 8 days showed no obvious changes in the inward Na^+ current.

The question arose whether Nerve Growth Factor (NGF) might act as an intermediary signal for the hypoxic response since NGF induces Na^+ channel expression in PC 12 cells (Kalman et al 1990; Pollock et al 1990) and during embryonic development at least some glomus cells appear sensitive to NGF (Aloe and Levi-Montalcini 1980). To investigate this glomus cells were cultured in normoxic conditions with the addition of 1 μg/ml NGF to the culture media (see Methods). As shown in Fig.19A,B, treatment of carotid body cultures with 1 μg/ml NGF had no significant effect on the inward Na^+ current. Thus, it is possible that hypoxia increases the number of Na^+ channels in glomus cells by a cAMP-mediated pathway but not by locally elevating NGF.

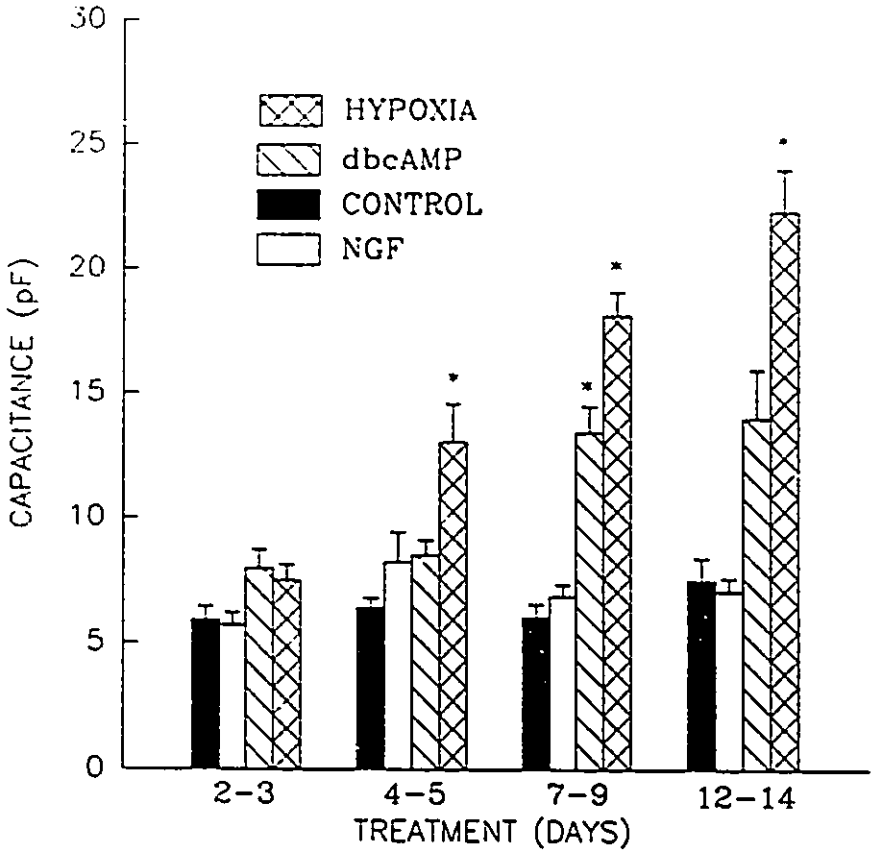
The second major effect of chronic hypoxia was on a passive membrane

FIG.20. Steady state Na^+ inactivation curve from glomus cell grown in the presence of 1 mM dbcAMP for 5 days. Data (filled circles) were fitted with a smooth curve generated from a modified version of the Boltzmann equation (see methods); h represents the peak Na^+ current from each holding potential divided by the maximum Na^+ current (from a holding potential of -100 mV). Note that this curve is very similar to the steady state inactivation curve for an untreated glomus cell (Fig.9) suggesting that dbcAMP-treatment did not alter Na^+ currents by shifting the steady-state inactivation.


1.0
0.8
0.6
h
0.4
0.2
0.0
−100
−80
−60
−40
−20
0
Holding potential (mV)

property of glomus cells, i.e. input capacitance, which is proportional to surface area. As described in Chapter 1 this value is typically ≈ 6 - 7 pF for control cells grown in normoxic conditions. After 5 days in hypoxia (Po_2 ≈ 50 Torr) cultured glomus cells showed a significant two-fold increase (≈ 13 pF; n=13) in their input capacitance which reached more than 3 times (≈ 23 pF; n=12) the control value after 13 days in culture (Fig.21). An estimate of the cell size calculated from input capacitance values and assuming a spherical shape (see Chapter 1) indicated that cells exposed to hypoxia for 13 days were ≈ 27 μm in diameter while control cells were ≈ 15 μm. As discussed later these novel findings provide at least a partial explanation for carotid body hypertrophy, that is known to occur *in vivo* when animals and humans are exposed to chronic hypoxia (Edwards et al 1971a,b; Dhillon et al 1984; McGregor et al 1984). As with the increase in number of Na^+ channels (see above), the hypertrophic effect of hypoxia on glomus cells could be mimicked qualitatively by growing the cells under normoxia in the continuous presence of 1 mM dbcAMP, but not NGF (Fig.21). However, hypoxia (Po_2 ≈ 50 Torr) was more effective than 1 mM dbcAMP in promoting hypertrophy in glomus cells, whereas the converse was true for the increase in number of Na^+ channels (cf. Fig.19A & Fig.21). Direct measurements of cultured rat glomus cells after fixation and staining for tyrosine hydroxylase-immunoreactivity confirmed that in both hypoxia and dbcAMP-treated cultures the average diameter of glomus cells (estimated from the mean of the short and long axes) was significantly larger ($p < 0.01$) than in untreated normoxic controls after 9 - 14 days (A.Jackson, personal communication).

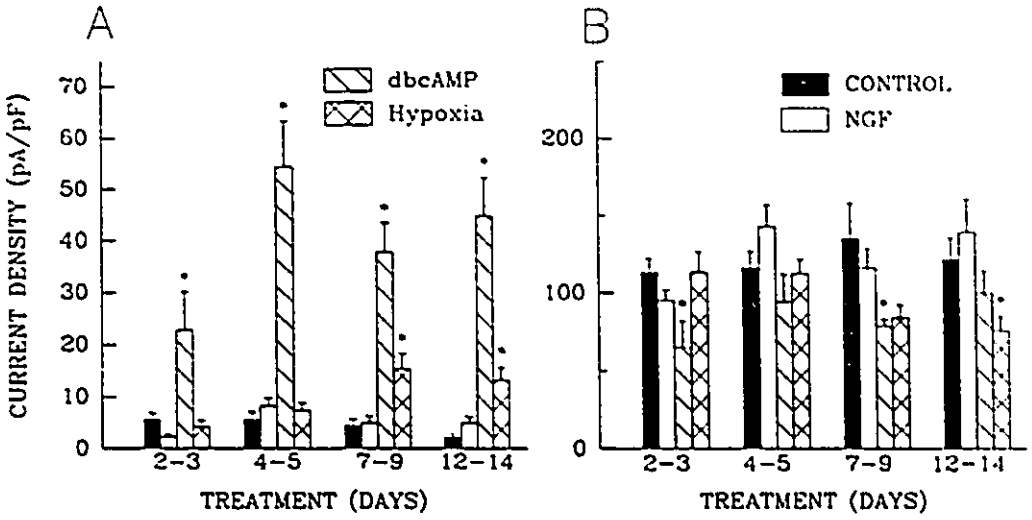
FIG.21. Hypertrophy of chemosensory glomus cells induced by hypoxia and dbcAMP but not NGF. After 5 days in a hypoxic ($Po_2 \approx 50$ Torr) environment the input capacitance (which is proportional to cell surface area) is significantly (*; $p < 0.01$) increased relative to age-matched controls; a progressive increase was observed with longer exposure times up to 14 days. The continuous presence of 1 mM dbcAMP in the media for 9-14 days also caused a significant, though less pronounced, increase in input capacitance in glomus cells grown under normoxic conditions. Control and NGF-treated (1 μg/ml) cells showed no significant changes in capacitance over 14 days in culture. Input capacitance was determined by integration of the capacity current transient in response to a voltage step and dividing the resulting charge by the voltage step.


30
25
20
15
10
5
0
CAPACITANCE (pF)
HYPOXIA
dbcAMP
CONTROL
NGF
*
*
*
*
2-3
4-5
7-9
12-14
TREATMENT (DAYS)

Specificity of the effects of chronic hypoxia on the membrane currents in glomus cells

Did the progressive increase in inward Na^+ current produced by chronic hypoxia and dbcAMP (Fig.19A) result simply from the accompanying increase in growth or hypertrophy of glomus cells (Fig.21) or from a specific inductive effect? To answer this question a comparison of the current densities for both voltage-activated Na^+ and K^+ currents was performed. Current densities were calculated by dividing the peak currents by the membrane capacitance (pA/pF). As shown in Fig.22A, chronic hypoxia resulted in a significant increase in inward Na^+ current density by 7-9 days, though the even more pronounced effect of dbcAMP was already quite substantial by 2-3 days of treatment. Once induced, the increased Na^+ current density persisted at least 2 weeks for both treatments. For example the Na^+ channel density in glomus cells exposed to chronic hypoxia (13 days) or dbcAMP (14 days) was 0.21 and 0.60 channels/μm^2 respectively compared to 0.09 channels/μm^2 calculated for normoxic controls (see Chapter 1). In contrast, the outward K^+ current density remained relatively constant and indeed the trend was towards a decrease rather than an increase after 1 week of treatment with hypoxia or dbcAMP (Fig.22B). The density of K^+ channels, after hypoxia (13 days) or dbcAMP (14 days) treatment, was calculated at 0.37 and 0.44 channels/μm^2 respectively. These are slightly lower than the value 0.59 channels/μm^2 calculated for the K^+ channel density in normoxic glomus cells in Chapter 1. In addition, the normalized density of leakage channels (obtained on dividing the leakage conductance by membrane capacitance) was not

FIG.22. Comparison of the effects of chronic hypoxia, dbcAMP, and NGF on the density of Na^+ and K^+ channels in glomus cells. A: Changes in Na^+ current density in glomus cells exposed to various treatments. Significant (*) increases in the peak Na^+ current density were recorded after 9 days in hypoxia ($Po_2 \approx 50$ Torr). Dibutyryl cAMP (1 mM) caused significant increases as early as 2 days after exposure. B: K^+ current density in glomus cells exposed to various treatments. K^+ current density in hypoxia and dbcAMP-treated cells was equal to or less than that in control cells at all time points. In fact, significant (*) reductions in the K^+ current were seen after 7 - 14 days in both hypoxia and dbcAMP-treated glomus cells. Notes: NGF-treated cells did not differ significantly from controls at any time point. Significance levels ; student's t-test; * = $p < 0.01$. To calculate Na^+ current density, peak Na^+ currents elicited by voltage steps from -60 to 0 mV were divided by the input capacitance of the same cell. In the case of the K^+ current density, steady-state K^+ currents elicited from a step of -60 to +50 mV (after $\approx$ 30 ms) were divided by the input capacitance.


A
B
dbcAMP
Hypoxia
CONTROL
NGF
CURRENT DENSITY (pA/pF)
70
60
50
40
30
20
10
0
200
100
0
2-3
4-5
7-9
12-14
TREATMENT (DAYS)
TREATMENT (DAYS)

significantly altered by either treatment. These data suggest that both hypoxia and dbcAMP promote a specific induction of Na^+ channels in glomus cells. Whether the density of calcium channels, also known to be present in these cells (see Chapter 1) is similarly augmented requires further study.

DISCUSSION

In this chapter I have described the electrophysiological changes in cultured rat glomus cells exposed to acute hypoxia ($Po_2 \approx$ 20-30 Torr; 2 to 5 minutes) and to prolonged hypoxia ($Po_2 \approx$ 50 Torr; up to 14 days). These results have elucidated some of the possible mechanisms by which changes in O_2 can directly affect the electrophysiological properties of chemoreceptive glomus cells of the mammalian carotid body.

O_2-sensitive K^+ current in glomus cells

The finding of an O_2-sensitive K^+ current in cultured rat glomus cells confirms recent reports based on studies of freshly-isolated rabbit glomus cells (Lopez-Barneo et al 1988; Lopez-Lopez et al 1989; Hescheler et al 1989; see also Peers 1990a,b). In addition, the O_2-sensitive K^+ current was found to persist in long-term cultures (> 8 weeks) and is likely to mediate a physiological (membrane) response in the intact cell during exposure to hypoxia. These cultures of glomus cells were derived mainly from perinatal rats, suggesting that the channels underlying this current appear early in development and remain effective in the foreign culture environment even in the absence of the cells' normal sensory inputs. The reason for the apparent decrease in the magnitude of the O_2-sensitive K^+ current over several weeks in culture is presently unclear though this may be related to the abnormal culture environment. A significant aspect of these experiments was that the specific effect of hypoxia on

membrane K^+ conductance of the glomus cell was similar with both conventional whole-cell and perforated-patch recording. This study is the first report in which glomus cells were studied by the latter recording technique. In this method the presence of the pore-forming antibiotic, nystatin, in the pipette limits diffusive exchange between cell interior and patch pipette to small monovalent ions (Horn and Marty 1988), thus preserving intracellular small molecules e.g. second messengers, metabolites. Though the preparation was more stable and responses were more readily reversible with perforated-patch recording, no additional changes in membrane current above that recorded with the conventional whole-cell method were detected on exposure to hypoxia. Thus the closing of a special class of O_2-sensitive K^+ channels appears to be the main membrane event associated with acute hypoxia in both 'intact' and dialysed glomus cells.

Is the O_2-sensitive K^+ conductance described above a unique property of glomus cells or is it shared with other cell types? A positive answer would emphasize the notion that glomus cells evolved to play a key role in chemoreception in the cardio-pulmonary system. I therefore asked whether this property was present in sensory petrosal neurons or in small intensely fluorescent (SIF) cells of sympathetic ganglia. Petrosal neurons were chosen since they form reciprocal synapses with glomus cells *in vivo* (McDonald and Mitchell 1975) and their terminals form part of the chemosensory complex in the carotid body (Eyzaguirre and Zapata 1984). On the other hand, SIF cells were chosen since; i) they are considered similar or identical to glomus cells by ultrastructural and biochemical criteria and are also derived from the

embryonic neural crest (Doupe et al 1985; Kobayashi 1971; McDonald and Blewett 1981), ii) like glomus cells they are often found clustered around fenestrated capillaries (McDonald and Blewett 1981), and iii) they can be grown in dissociated cell culture where they express several of the phenotypes also found in glomus cells (Doupe et al 1985). Interestingly, none of the cultured petrosal or SIF cells appeared to contain the O_2-sensitive K^+ conductance found in glomus cells. Thus it may well be that the properties responsible for O_2-sensing are distributed uniquely in the glomus or type I cells of the carotid body.

Chronic hypoxia induces physiological changes in glomus cells

In this study chronic hypoxia (over a two week period) was found to induce a selective increase in Na^+ channels, as well as hypertrophy in glomus cells. These novel findings suggest plausible mechanisms by which chronic hypoxia can produce the known altered physiological and morphological responses in the mammalian carotid body. First the sensitization of the carotid chemoreceptor response, thought to be involved in the ventilatory acclimatization to hypoxia (Barnard et al 1987, Nielsen et al 1988), may well be due to an increased Na^+ channel density in glomus cells since Na^+ channels play a major role in generating excitability (spiking) in a number of neurons and neuroendocrine cells (Hille 1984). In addition, it was found that the induction of Na^+ channels in glomus cells could be mimicked qualitatively by artificially raising $cAMP_i$ (with the membrane-permeant dibutyryl cAMP) under normoxic conditions. Since chronic hypoxia is known to elevate $cAMP_i$ levels in the

carotid body (Delpiano and Acker 1991; Perez-Garcia et al 1990; Wang W.-J. et al 1991) and specifically in glomus cells (Wang, Z.-Z. et al 1991a), the above results suggest that hypoxia may induce Na^+ channels in glomus cells via a cAMP-mediated pathway. It is noteworthy that elevated $cAMP_i$ can cause Na^+ channel induction in an adrenal chromaffin cell line, PC 12 cells (Kalman et al 1990) and skeletal muscle cells (Offord and Catterall 1989). In contrast, although nerve growth factor (NGF) can cause Na^+ channel induction in PC 12 cells (Kalman et al 1990; Pollock et al 1990), and appears to be important in the embryonic development of glomus cells (Aloe and Levi-Montalcini 1980), it appears not to mediate induction of Na^+ channels in glomus cells during chronic hypoxia.

Second, enlargement of the carotid body *in vivo* after exposure to chronic hypoxia (Edwards et al 1971a,b; Dhillon et al 1984; McGregor et al 1984) may be partly due to a direct effect in promoting glomus cell hypertrophy as cultured glomus cells almost doubled in size after 13 days in hypoxia ($Po_2 \approx 50$ Torr). This finding suggests that external blood-borne factors are not necessary for hypoxia-induced glomus cell hypertrophy (McGregor et al 1984) as the role of circulation is eliminated from these dissociated carotid body cultures (Nurse 1987,1990). This study also suggests that an increase in $cAMP_i$ is a likely mediator of the hypertrophic effect. Similarly, elevating $cAMP_i$ in related PC 12 cells causes an observable increase in cell size (Kalman et al 1990) but this response is initiated by NGF unlike the situation in glomus cells where NGF is not involved. This hypertrophy observed in glomus cells may also play a role in the sensitization of the carotid chemoreceptor response

mentioned above. An increase in the dopamine content of the carotid body has been measured after chronic hypoxia (Pallot and Barer 1982) and since glomus cells are the major store of this neurotransmitter in the carotid body (McDonald 1981) then it is conceivable that hypertrophic glomus cells contain more neurotransmitter than their smaller normoxic counterparts. This may lead to increased release onto the sensory nerve terminals in response to chemosensory stimuli and subsequently amplify afferent discharge. In addition it has been suggested that there might be increased contact between the larger glomus cells and the apposed nerve endings which could also intensify sensory signals from the carotid body (McGregor et al 1984).

Although both the induction of Na^+ channels and hypertrophy of glomus cells are induced by artificially raising intracellular cAMP under normoxic conditions, these changes are not exactly parallel to those seen after chronic hypoxia. Since, in addition to cAMP elevation (Wang, Z.-Z. et al 1991a) hypoxia elicits other responses in glomus cells including the elevation of intracellular calcium (Biscoe and Duchen 1990) and a decrease in cGMP levels (Wang, Z.-Z. et al 1991a) it is not surprising that elevating $cAMP_i$ did not precisely duplicate the effects of chronic hypoxia.

The implications of the above effects on the mechanisms of chemotransduction in the carotid body will be discussed in Chapter 6.

CHAPTER 3

Effects of Acidity on Membrane Currents of Glomus Cells

INTRODUCTION

Another chemosensory stimulus which excites the mammalian carotid body and increases ventilation is acidity, irrespective of the changes in arterial Po_2 or Pco_2 (see Eyzaguirre and Zapata 1984). In earlier studies it was thought that a likely location for the sensing of H^+ ions, or pH, was the sensory nerve ending which is part of the chemosensory complex of the carotid body (see Biscoe 1971). However, more recent studies using high resolution electrophysiological and spectrofluorimetric techniques have implicated the glomus, or type I cell, as the primary sensor for changes in arterial pH (Biscoe and Duchen 1990; Buckler et al 1991a,b; He et al 1991a; Lopez-Lopez et al 1989; Peers and Green 1991; Stea et al 1991). Many researchers have implicated H^+ ions as a mediator in chemosensory responses by the carotid body. Indeed a popular hypothesis regarding chemotransduction by the carotid body is dubbed the 'acidic' hypothesis. Initially it was thought that changes in extracellular H^+ were key to the chemosensory responses (see Eyzaguirre and Zapata 1984)

however, later studies suggested that intracellular pH may play a fundamental role in the chemotransduction process (Hanson et al 1981). Interestingly, both Buckler et al (1991a,b) using the pH-sensitive fluoroprobe SNARF-1, and He et al (1991a) using pH-sensitive microelectrodes found that changes in external pH were directly linked to changes of intracellular pH in glomus cells. Therefore as suggested in the 'acidic' hypothesis (Hanson et al 1981) it may be changes in intracellular pH which are physiologically important in the carotid body's responses to extracellular acidity.

The purpose of the investigations presented in this chapter was to determine the effects of intracellular (pH_i) and extracellular pH (pH_o) on the membrane currents in cultured rat glomus cells described in Chapter 1. In general glomus cells contain a variety of voltage-activated K^+ currents of which several types are known to be modulated by pH_i in other systems including pancreatic cells (Cook et al 1984), lymphocytes (Deutsch and Lee 1989), and some excitable cells (Moody 1984). Therefore, it was plausible that K^+ currents in glomus cells could be similarly affected. Indeed as was the case for hypoxia (Chapter 2), a suppression of K^+ current by acidity could in theory lead to excitation of the chemoreceptors. The approach used to study effects of pH on membrane currents in glomus cells was different from that used in other systems (Peers and Green 1991), where the normal cytoplasmic milieu was disrupted due to intracellular dialysis with artificial perfusates (e.g. during conventional whole-cell recording; see Hamill et al 1981); this in turn could disturb the cell's intrinsic mechanisms for regulating pH_i and also directly affect any whole-cell currents dependent on cytoplasmic integrity. To circumvent these limitations I

used the perforated-patch technique for whole-cell recording (Horn and Marty 1988), in which the pore-forming antibiotic, nystatin, in the patch pipette limits diffusive exchange to small monovalent ions. In this way recovery of membrane currents following perturbation in pH_i should not be influenced by pipette buffers and should occur coincident with the restoration of pH_i by the cell's normal regulatory mechanisms. To manipulate pH_i conventional methods were used, including bath application of the K^+/H^+ ionophore nigericin (Thomas et al 1979), and of a weak acid (Roos and Boron 1981). In many experiments the effects of external pH on membrane currents were also tested.

METHODS

The procedures for the glomus cell culture, whole-cell and perforated-patch recording, and data analysis were identical to those previously described in Chapter 1.

Solutions

Most experiments were performed using extracellular fluid of the following composition (mM): NaCl,135; KCl,5; $CaCl_2$,2; $MgCl_2$,2; glucose,10; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES),10 at pH 7.4. The stock pipette solution for conventional whole-cell and all perforated-patch experiments contained (mM): KCl,135; NaCl,5; $CaCl_2$,1; ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA),11 ($[Ca^{2+}] \approx 10^{-8}$); HEPES,10 at pH 7.2. External pH was altered using normal extracellular fluid of pH 7.0 and 6.5. Cytoplasmic acidification was induced by adding: i) 2.5 - 5.0 μg/ml (3.3 - 6.6 μM) of the K^+/H^+ ionophore nigericin to the extracellular solution, and ii) 20 mM Na acetate to the external solution. A third method of cytoplasmic acidification, exposure to CO_2, will be described in Chapter 4. In some experiments, the possible role of the Na^+/H^+ antiporter in the recovery from acidification was tested by adding 0.1 mM amiloride, a known blocker of this antiporter, to the external solution. All solutions were added to the recording chamber under gravity and withdrawn by vacuum suction.

RESULTS

The experiments described below deal with the effect of both intracellular and extracellular pH changes on the membrane properties of cultured glomus cells of the rat. The passive properties of these cells (input resistance and input capacitance) were not obviously affected by any of the treatments described below which perturb pH_i or pH_e and will not be described further in this chapter. The active currents were significantly affected by alterations of pH_i and pH_e as described below.

Effect of cytoplasmic acidification

Two methods were used to acidify the cytoplasm of the cultured glomus cells. First, as depicted in Fig.23 the ionophore nigericin was used to promote cytoplasmic acidification by inserting into the cell membrane as electroneutral K^+/H^+ exchangers which rapidly stimulate H^+ influx and K^+ efflux (Thomas et al 1979). Second, a weak acid (e.g. acetate) was added to the external solution to produce a fall in pH_i following entry of the undissociated form into the cell (Roos and Boron 1981). By combining these procedures with the perforated-patch method it was possible to record changes in whole-cell currents during an acid load and following recovery in the presence of the cell's natural mechanisms for regulating pH_i. The addition of 3 - 6 μM nigericin to the external solution (pH = 7.4) caused a rapid (2 - 5 min) decrease in the outward K^+ current (Fig.24A,B,C). This effect was reversible following washout of the drug and generally, recovery occurred rapidly and was

FIG.23. Schematic representation of a perforated-patch recording from a glomus cell indicating the action of the ionophore nigericin in inducing cytoplasmic acidification. The antibiotic, nystatin forms pores permeable to monovalent ions (Horn and Marty 1988). The ionophore nigericin forms electroneutral K^+/H^+ exchangers in the cell membrane when added to the external perfusate (Thomas et al 1979). An outwardly directed K^+ gradient will stimulate H^+ influx through these exchangers acidifying the intracellular milieu.

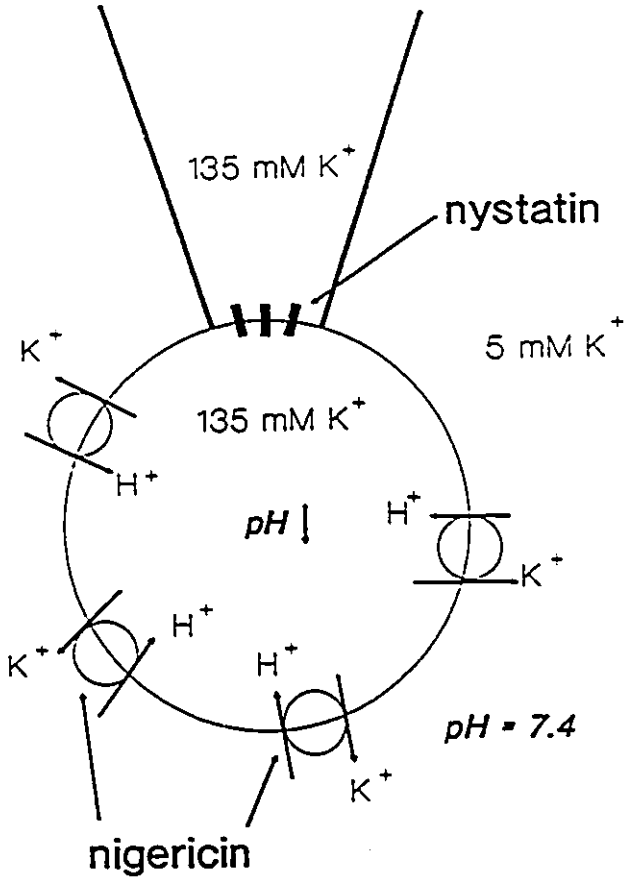
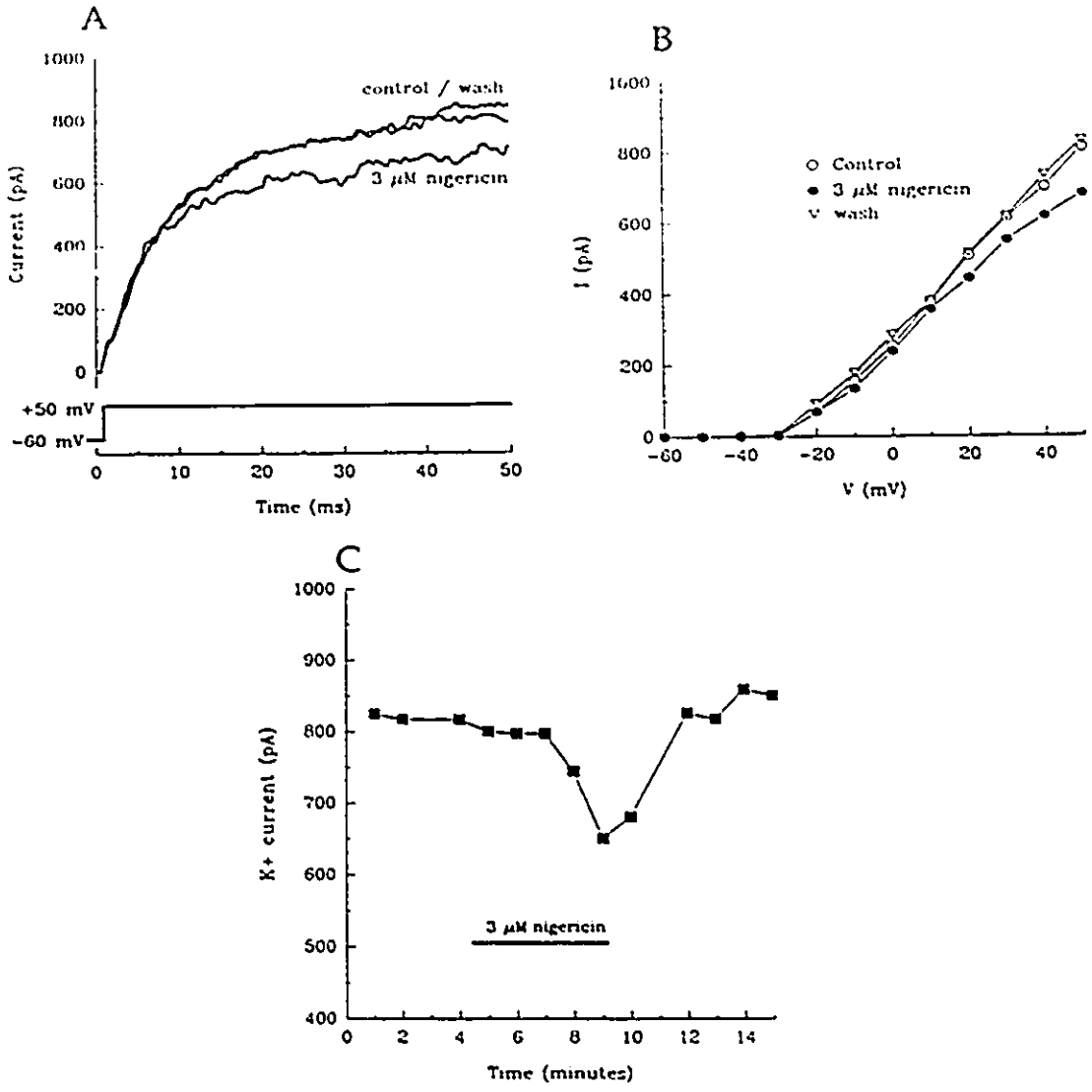

135 mM K+
nystatin
5 mM K+
K+
135 mM K+
H+
pH
H+
K+
H+
K+
H+
pH = 7.4
K+
nigericin

FIG.24. Effect of cytoplasmic acidification on whole-cell currents from cultured rat glomus cells using the perforated-patch recording method. A: Reduction of K^+ current in response to cytoplasmic acidification induced by addition of 3 μM nigericin; lower trace, voltage step. B: I-V curve from the same cell of steady state outward K^+ current before, during and after addition of nigericin. C: Time course of reduction and recovery of outward K^+ current in response to a brief exposure to nigericin (3 μM); lower horizontal bar indicates period during which nigericin was present in the perfusion fluid.


A
1000
800
600
400
200
0
Current (pA)
control / wash
3 μM nigericin
+50 mV
-60 mV
0
10
20
30
40
50
Time (ms)
B
1600
800
600
400
200
0
I (pA)
Control
3 μM nigericin
wash
-60
-40
-20
0
20
40
V (mV)
C
1000
900
800
700
600
500
400
K+ current (pA)
3 μM nigericin
0
2
4
6
8
10
12
14
Time (minutes)

complete (Fig.24C). In 17 experiments, nigericin caused the K^+ current to decrease reversibly by 20.5 +/- 2.1% (mean +/- S.E.). The addition of a weak acid (20 mM acetate) to the external solution also caused a reversible decrease in the K^+ current by 21.3 +/- 3.1% (n=4). Due to the small size of the inward Na^+ current in most glomus cells it was difficult to quantify the effect of an acid load on this current. However, in a few cells nigericin caused a noticeable decrease in the Na^+ current.

To test for possible mechanisms of recovery from cytoplasmic acidification the Na^+/H^+ antiport blocker, amiloride, was added to the external medium soon after removal of nigericin. The addition of 0.1 mM amiloride (n=5) inhibited recovery of the outward K^+ current from an acid load, induced by 3 μM nigericin (Fig.25). This result contrasts with the rapid recovery of the K^+ current usually seen (Fig.24C) after removal of nigericin (in the absence of amiloride).

Effect of external pH

By lowering the pH of the external solution from 7.4 to 7.0 no appreciable change in the outward K^+ current (< 5%) was observed in 20 of 24 cases. This contrasts with other studies on glomus cells where a decrease in K^+ current occurred in response to changes in pH_e from 7.4 to 7.0 (Lopez-Lopez et al 1989; Peers 1990a). However, in this study a change in pH_e from 7.4 to 6.5 caused a reversible decrease in the K^+ current by 22.6 +/- 5.0 % (mean +/- S.E.; n=14: Fig.26A). Na^+ currents in glomus cells were also decreased when pH_e was reduced from 7.4 to 6.5 (Fig.26B) but quantification was difficult due to the small size of the current.

FIG.25. Time course of the effect of cytoplasmic acidification and of cobalt application on steady-state K^+ current in a single glomus cell. A rapid (within 2 min) reduction in K^+ current was observed during addition of 3 μM nigericin. Recovery of the K^+ current from this acid load was prevented by addition of 0.1 mM amiloride, an inhibitor of the Na^+/H^+ antiport. A reversible decrease in K^+ current was also observed during perfusion of a weak acid, acetate (20 mM). Finally, replacement of external Ca^{2+} with Co^{2+} caused over a 50% decrease in K^+ current and this is likely due to elimination of the Ca^{2+}-activated K^+ component. The lower horizontal bars indicate the period during which the indicated drug was present in the perfusion fluid. All values represent steady state K^+ currents recorded during voltage steps from -60 to +50 mV.

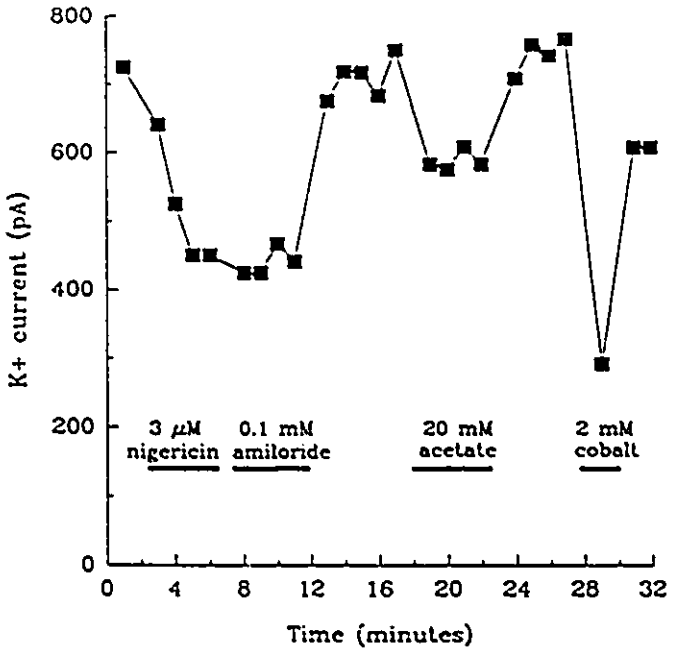
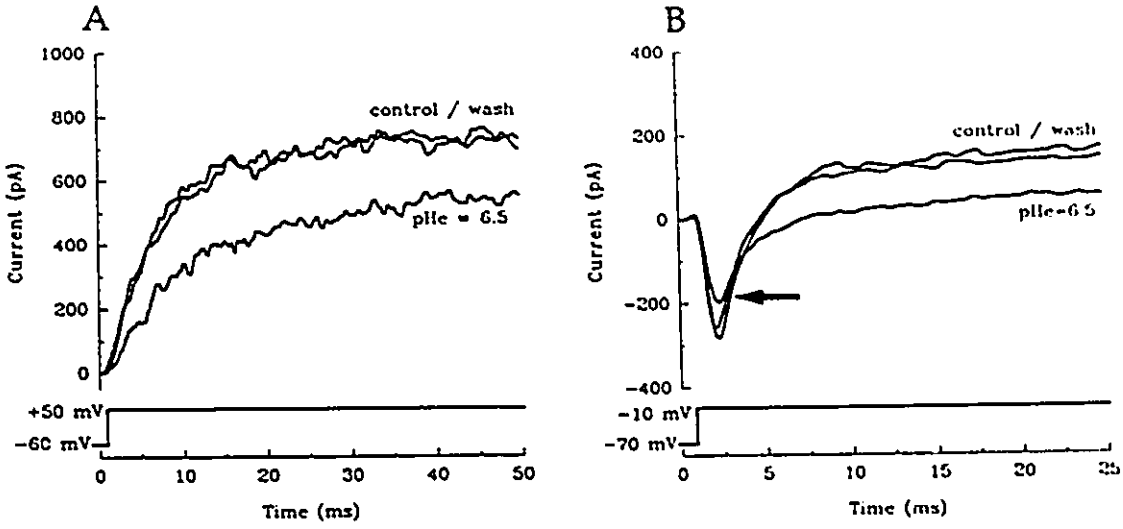

800
600
400
200
0
K+ current (pA)
3 μM
nigericin
0.1 mM
amiloride
20 mM
acetate
2 mM
cobalt
0
4
8
12
16
20
24
28
32
Time (minutes)

FIG.26. Effect of extracellular acidification on whole-cell currents in glomus cells. A: Reducing pH_e from 7.4 to 6.5 caused a reversible decrease in the outward K^+ current. B: A decrease in the inward Na^+ current (arrow) was also observed during the same treatment; lower traces, voltage steps.


A
1000
800
600
400
200
0
Current (pA)
control / wash
pHe = 6.5
+50 mV
-60 mV
0
10
20
30
40
50
Time (ms)
B
400
200
0
-200
-400
Current (pA)
control / wash
pHe=6.5
-10 mV
-70 mV
0
5
10
15
20
Time (ms)

Thus both cytoplasmic acidification, induced by application of nigericin or acetate, and extracellular acidification (from 7.4 to 6.5) caused pronounced decreases ($\approx$ 20%) in the voltage-activated currents in glomus cells.

DISCUSSION

In this study I have primarily used the nystatin perforated-patch recording method (Horn and Marty 1988) to study the effects of pH_i and pH_e on membrane currents in cultured glomus cells of the rat carotid body. The attractive feature of this approach was that cytoplasmic washout was prevented thereby maintaining the natural pH buffering mechanisms in the cells. This technique allowed the study of membrane currents both during and after cytoplasmic acidification induced by bath application of the K^+/H^+ ionophore nigericin (Thomas et al 1979) or of a weak acid, acetate (Roos and Boron 1981), at constant pH_e. To my knowledge this approach has not been used previously to study the effects of pH_i and pH_e on whole-cell currents in 'intact' cells, though it appears to be advantageous for small cells in which a two electrode voltage clamp is not practical. There are several reports however, on the effects of pH_i on membrane currents in dialysed preparations (Moody 1984; Peers and Green 1991). For example, Deutsch and Lee (1989) studied whole-cell membrane currents in lymphocytes by using pipette solutions of varying pH or by adding the proton ionophore FCCP to equilibrate external and internal pH.

Effect of pH on active currents

Cytoplasmic acidification induced by the addition of 3 μM nigericin to the external perfusate had a marked effect on the K^+ currents in glomus cells. The outward K^+ current decreased by $\approx$ 20% and recovered completely upon removal of

the ionophore. These results appeared not to be specific for glomus cells as other work in this laboratory (Alexander 1991) has demonstrated that a closely-related cell type, the small intensely-fluorescent (SIF) cells also showed a marked depression in the voltage-activated K^+ (and Na^+) currents during perfusion with nigericin (see also; Stea et al 1991). Since cytoplasmic acidification induced by perfusion of a weak acid (acetate) produced similar decreases in the K^+ current in glomus cells compared to the results seen with nigericin it is unlikely that the action of this ionophore on K^+ currents was due to a non-specific effect. Although various K^+ currents are decreased by lowered pH_i (inward rectifying I_K, the delayed rectifier I_K; Moody 1984), one current likely to be affected in these glomus cells is the Ca^{2+}-activated K^+ current (Peers 1990a,b; Urena et al 1989) which is known to be inhibited by a decrease in pH_i in freshly-isolated glomus cells (Peers and Green 1991) and pancreatic β-cells (Cook et al 1984). This current appears to represent a major component of the overall K^+ current in cultured rat glomus cells since over 50% of the outward current was blocked by equimolar substitution of Ca^{2+} with Co^{2+} (see Chapter 1).

In addition, the inward Na^+ current appeared to be reduced in glomus cells following cytoplasmic acidification but this was difficult to quantify due to the small size of the inward current (see Chapter 1). Also the Na^+ current in the squid axon is suppressed by lowering pH_i (Moody 1984). A large reduction (39%) in the voltage-activated Na^+ current was observed in closely-related SIF cells during nigericin perfusion (Alexander 1991; see also Stea et al 1991).

Application of amiloride to cultured glomus cells after an acid load inhibited

recovery of the K^+ current. The drug amiloride is known to inhibit the Na^+/H^+ antiporter, an electroneutral control system that aids in recovery from cytoplasmic acidification in many different cell types (Roos and Boron 1981). Thus our results are consistent with the involvement of the Na^+/H^+ antiporter in pH regulation in glomus cells (Buckler et al 1991a; Fitzgerald et al 1990), though the possibility of a direct effect of amiloride on Ca^{2+} and/or K^+ currents is not excluded (Tang et al 1988).

Extracellular acidification produced a substantial decrease in K^+ currents in glomus cells in other studies (Lopez-Lopez et al 1989; Peers 1990) when pH_e was reduced to pH 7.0 from pH 7.4. In contrast, in my study this level of extracellular acidification had no detectable effect though a lowering of pH_e to 6.5 produced a comparable reduction ($\approx$20%) in K^+ current. Further, the results were similar with both the conventional whole-cell and perforated-patch recording methods. Reasons for this discrepancy are unclear but may be related to species differences. Also the glomus cells in this study were kept longer in culture (> 3 days) before recording. Since recent studies indicate that changes in pH_e causes corresponding changes in pH_i in glomus cells (Buckler et al 1991a,b; He et al 1991a), the possibility is raised that the effects of external acidification are mediated through an internal mechanism. In the present study both a pH_e of 6.5 and cytoplasmic acidification with 3 μM nigericin caused an almost identical decrease in the K^+ current which could be explained if a similar cytoplasmic acidification was caused by both treatments. Indeed the ionophore nigericin should affect the pH_i in the ratio $[K^+]_i/[K^+]_e = [H^+]_i/[H^+]_e$ (Thomas et al 1979) which would theoretically drop the pH_i in these glomus cells to $\approx$ 6.0 in the

steady state and in the studies by He et al (1991a) and Buckler et al (1991b) a pH_e of 6.5 decreased pH_i of glomus cells to $\approx$ 6.0 and $\approx$ 6.6 respectively. Thus effects of pH_e may generally be mediated via alterations in pH_i in glomus cells.

Since a recent study on pancreatic acinar cells has shown a direct relationship between pH_i and intracellular Ca^{2+} levels (Tsunoda 1990) it is likely that interactions between these two variables are important in the mechanisms controlling the release of neurotransmitters from glomus cells during chemosensory stimulation (discussed in detail in Chapter 6).

CHAPTER 4

Effects of Hypercapnic Stimuli on Membrane Properties of Glomus Cells

INTRODUCTION

The third major stimulus which elicits a chemosensory response from the mammalian carotid body is an increase in arterial Pco_2, or hypercapnia. However, the stimulus/response relationship for carotid body chemoreceptors is different for a hypercapnic versus a hypoxic stimulus. The afferent discharge in the carotid sinus nerve increases linearly with increasing Pco_2 both during recording from *in vivo* (Fitzgerald et al 1990) and *in vitro* (Eyzaguirre et al 1989) carotid body preparations. In contrast, lowering Po_2 elicits an exponential rise in the afferent discharge from the carotid body (Eyzaguirre and Koyano 1965a, Eyzaguirre and Zapata 1984). Nevertheless, even though its effects are less pronounced than hypoxia, hypercapnia does have a direct effect on the carotid body causing increased ventilation. The mechanism and site of action of a hypercapnic stimulus in the carotid body had remained elusive for many years. However, the idea that CO_2 effects are mediated through local pH changes has been considered for some time and was formally known

as the 'acidic' hypothesis of chemoreception (Hanson et al 1981). Experiments that led to this hypothesis indicated that the ventilatory response to a hypercapnic stimulus was slowed by the presence of inhibitors of carbonic anhydrase which catalyses the reaction:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

According to this scheme, the H^+ ions produced by the above reaction would act as the intermediary stimulus for increased ventilation when CO_2 levels are increased. Though the actual localization of carbonic anhydrase (CAH) in the carotid body remained speculative for many years (see Eyzaguirre and Zapata 1984) it now appears that CAH, and consequently where H^+ ions would be produced during hypercapnia, is specifically found in the cytoplasm of glomus cells (Nurse 1990; Ridderstrale and Hanson 1984; Rigual et al 1985). This cytoplasmic localization of CAH would account for the rapid decrease in pH_i seen in glomus cells when exposed to media containing CO_2 (Buckler et al 1991a,b; He et al 1991b), and indeed in one of these studies the application of the membrane-permeant CAH inhibitor, acetazolamide, caused the rate of intracellular acidification to be considerably slowed (Buckler et al 1991a). Also of interest are the recent findings that perfusion of acetazolamide or methazolamide (membrane-permeant CAH inhibitors) through *in vitro* carotid body preparations suppressed the chemosensory discharge during transient increases in CO_2 (Iturriaga et al 1991; Rigual et al 1991). These studies suggest that CAH is essential for the chemosensory response to hypercapnia which is probably mediated through a

fall in pH_i in glomus cells.

In this chapter I will describe the influence of increasing CO_2 levels on the membrane properties of cultured rat glomus cells. The effects of the membrane-permeant CAH inhibitor, acetazolamide, will also be evaluated. This study is the first and only report in which whole-cell currents in glomus cells were recorded in physiological bicarbonate/CO_2-buffered media instead of the commonly-used HEPES-buffered media (see Stea and Nurse 1991b). Recent studies have shown that the use of HEPES buffer may complicate physiological interpretation in many experiments. For example, HEPES has been shown to block anion channels in several systems (Hanrahan and Tabcharani 1990; Manning and Williams 1989). Further, in HEPES-buffered media the measured intracellular pH is significantly higher than in physiological bicarbonate-buffered media (Buckler et al 1991a; Gaillard and Dupont 1990), and this may indirectly affect the gating of ion channels, e.g. ATP-sensitive K^+ channels in pancreatic β-cells (Carroll et al 1988). Since glomus cells contain the enzyme carbonic anhydrase (Nurse 1990), which is likely to affect pH_i homeostasis in the presence of CO_2, as well as voltage-activated channels that are sensitive to pH_i (Stea et al 1991; Chapter 3), it is possible that recordings in HCO_3^--free media may reflect an abnormal physiology. Indeed, Thomas (1989) has recently cautioned "... he who works in bicarbonate-free media risks studying cellular and molecular pathology rather than physiology".

METHODS

The procedures for the glomus cell culture, perforated-patch recording, and data analysis were identical to those previously described in Chapter 1.

Solutions

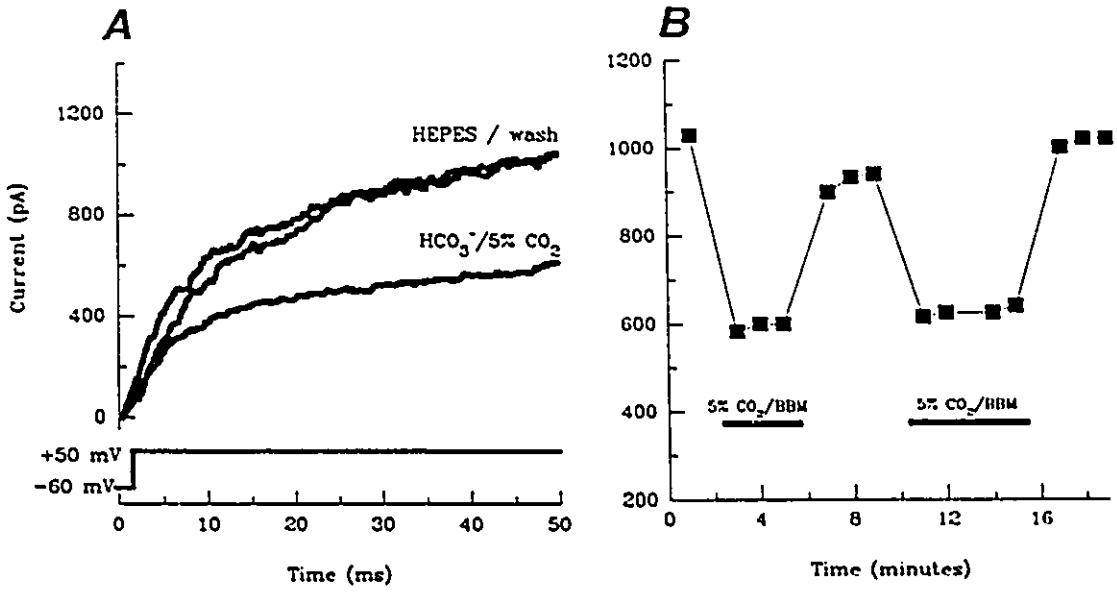
Most experiments were initiated using as the perfusate HEPES-buffered media (HBM) of the following composition (mM): NaCl,135; KCl,5; $CaCl_2$,2; $MgCl_2$,2; glucose,10; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES),10 at pH 7.4 with a measured osmolarity of $\approx$ 290 mOsmoles/l. In the experiments described in this chapter the perfusate was frequently switched to a bicarbonate-buffered media (BBM) containing the following (mM): NaCl,110; KCl,5; $NaHCO_3$,24; $CaCl_2$,2; $MgCl_2$,2; glucose,10; sucrose,12; bubbled with 5% CO_2 (external pH $\approx$ 7.4) or with 10% CO_2 (external pH $\approx$ 7.2) and a measured osmolarity of $\approx$ 290 mOsmoles/l. The BBM was continuously and rapidly ($\approx$ 5 ml/min) perfused so as to maintain the CO_2 levels in the recording chamber close to the equilibrium levels since some loss of CO_2 was likely from the recording chamber but unlikely through the silicon tubing carrying the perfusate. The CO_2 solutions were equilibrated using a CO_2-mixing pump (generously provided by Gord McDonald) which could be rapidly switched to any desired level of CO_2. The stock pipette solution for all experiments contained (mM): KCl,135; NaCl,5; $CaCl_2$,0.1; HEPES,10 at pH 7.2. All solutions were filtered through a 0.45 μm millipore filter before use.

RESULTS

Effect of BBM on voltage-activated membrane currents

In Chapter 1 of this thesis I described a marked decrease in input resistance in glomus cells upon switching from HEPES-buffered media (HBM) to bicarbonate/ 5%CO_2-buffered media (BBM). This was attributable mainly to the opening of anion channels in BBM, resulting in an increase in leakage currents (see Chapter 1). In addition to this effect the voltage-activated K^+ current in glomus cells was also affected but in an opposite manner. Changing the perfusion fluid from HBM to BBM (+ 5% CO_2) caused a rapid decrease in the K^+ current by 23.9 +/- 1.8% (n=56) which was reversible in most cases when HBM was reintroduced (Fig.27A,B). The decrease in K^+ current was seen within 2-3 min of switching solutions (Fig.27B) and persisted during BBM perfusion. The effect of BBM perfusion on the voltage-activated Na^+ current in glomus cells was more difficult to quantify since this current was relatively small in these cells (see Chapter 1; Stea and Nurse 1991a), although in a few cases where the current was larger a reversible decrease was also seen. A likely explanation for the decrease in K^+ current in BBM is an acidification of pH_i which, as described in Chapter 3 (Stea et al 1991), suppressed both the voltage-activated K^+ and Na^+ currents in glomus cells. Previous work in this laboratory showed that cultured glomus cells contained intracellular carbonic anhydrase activity (Nurse 1990; see also Rigual et al 1985). Since CO_2 is freely membrane permeable, BBM containing CO_2 should cause rapid intracellular

FIG.27. Effect of bicarbonate-buffered media (BBM) on K^+ currents in glomus cells. A: Switching from HEPES-buffered media (HBM) to BBM (HCO_3^-/5% CO_2) caused a reversible decrease in the voltage-activated outward K^+ current; all traces have the leakage currents subtracted. B: Time course of the effect of BBM on outward K^+ currents in a glomus cell. A rapid (within 2-3 min) and completely reversible decrease in the K^+ current was observed upon switching from HBM to BBM. Note: between treatment intervals with BBM (lower bars) this cell was perfused in HBM.

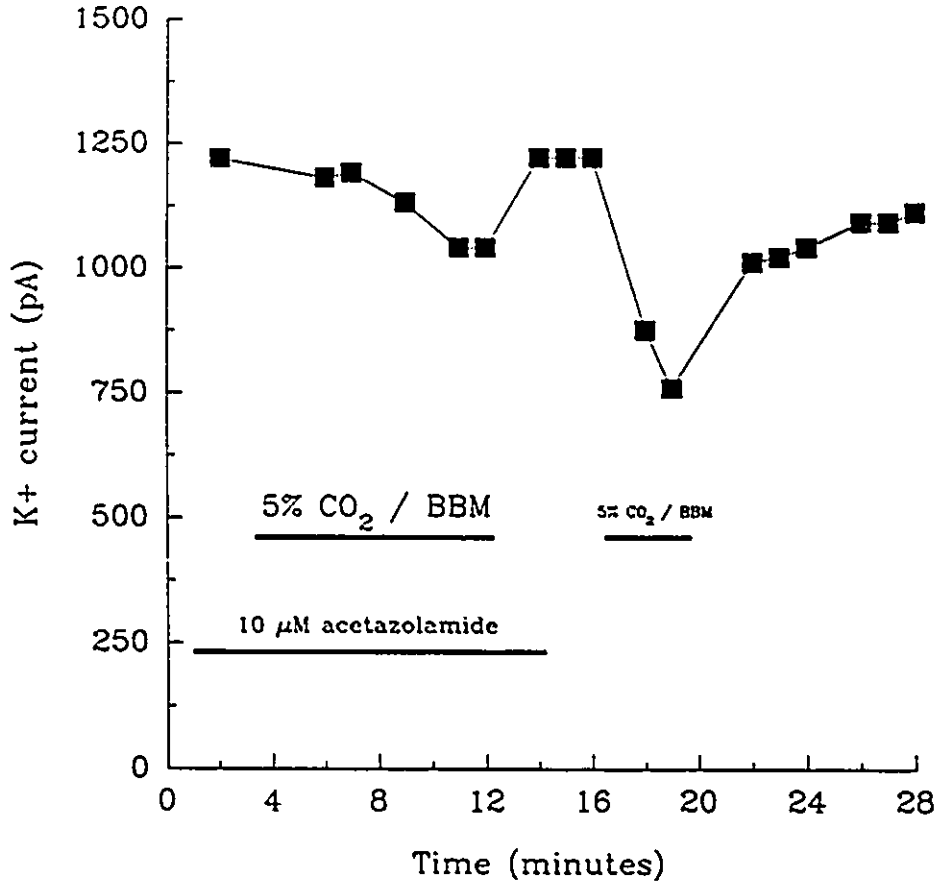

A
1200
800
400
0
Current (pA)
HEPES / wash
HCO3-/5% CO2
+50 mV
-60 mV
0
10
20
30
40
50
Time (ms)
B
1200
1000
800
600
400
200
5% CO2/BBM
5% CO2/BBM
0
4
8
12
16
Time (minutes)

acidification due to the hydration of CO_2 to H^+ (and HCO_3^-) catalyzed by carbonic anhydrase. Hence, inhibition of carbonic anhydrase should slow the rate of intracellular acidification and consequently, the time course of the decrease in K^+ current. To test this a membrane-permeant sulfonamide, acetazolamide, which inhibits carbonic anhydrase activity in glomus cells (Nurse 1990) was added to the BBM. In four experiments with 10 μM acetazolamide present in the perfusate, a marked delay (5-7 min) in the decrease in K^+ current was observed upon switching from HBM to BBM (Fig.28). When the same cells were exposed to BBM without acetazolamide there was the usual rapid (2-3 min) fall in K^+ current (Fig.28). In addition to this delay, the decrease in K^+ current recorded in BBM with acetazolamide (9.8 +/- 2.1%; n=4) was much less than in the same glomus cells perfused with BBM minus acetazolamide (31.8 +/- 6.5%; n=4). Presumably, the effects seen during recording with acetazolamide in the bath were due to an inhibition of carbonic anhydrase (CAH) activity inside the glomus cells. This decrease in activity would be reflected in a lower rate of hydration of CO_2 to H^+ ions, thereby slowing cytoplasmic acidification as shown directly with the pH-sensitive fluoroprobe SNARF-1 (Buckler et al 1991a).

Effect of hypercapnic stimulus on voltage-activated currents in glomus cells

Modification of the passive (see Chapter 1) and active properties of glomus cells (see above) on switching from HEPES-buffered media (HBM) to physiological 5% CO_2/HCO_3^--buffered media did not answer the question whether the membrane currents are altered during a hypercapnic stimulus. To simulate hypercapnia, the BBM

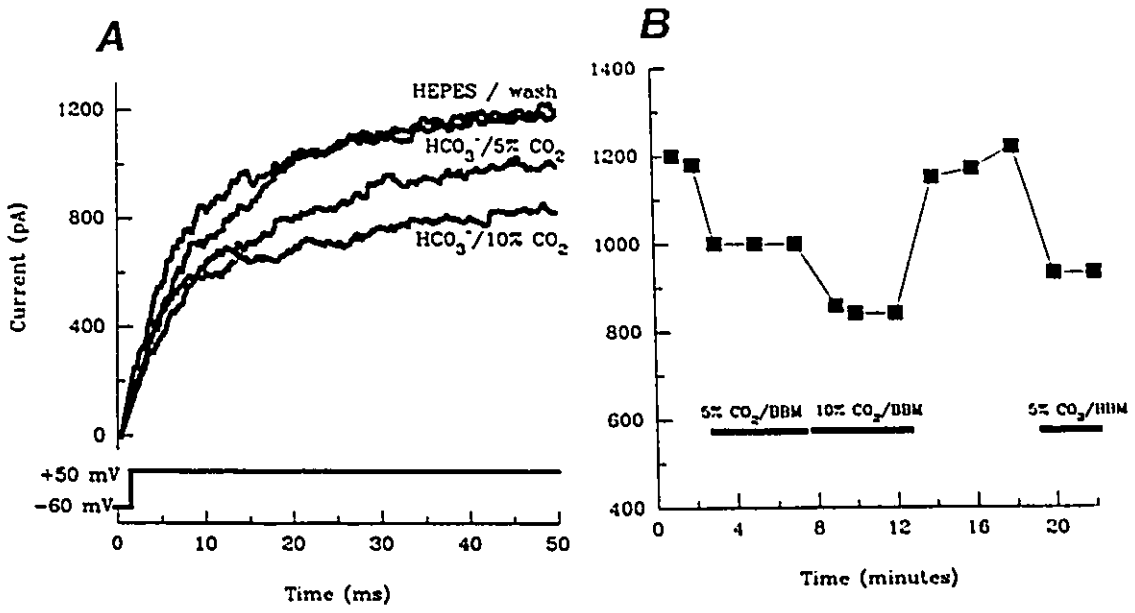
FIG.28. Acetazolamide suppresses the effect of BBM on K^+ currents in glomus cells. In the presence of a carbonic anhydrase inhibitor, acetazolamide (10 μM; lower horizontal bar), the reduction in K^+ current seen upon switching from HBM to BBM (+ 5% CO_2; upper horizontal bars) was markedly slowed and reduced as compared to the effect seen in the same cell without acetazolamide. All current values were measured corresponding to voltage steps from -60 to 50 mV. Note: between treatment intervals with BBM the culture was perfused with HBM +/- acetazolamide.


1500
1250
1000
750
500
250
0
K+ current (pA)
5% CO2 / BBM
10 μM acetazolamide
0
4
8
12
16
20
24
28
Time (minutes)

was equilibrated with 10% CO_2 instead of 5% CO_2 (5%BBM) during continuous perfusion of the cultures. Although the external pH decreased from ≈ 7.4 to ≈ 7.2 in these experiments, from earlier studies (see Chapter 3) this change in pH_e was not expected to cause any significant effects on the electrophysiological properties of glomus cells. In 12 experiments where glomus cells were first exposed to HBM, then 5% CO_2/BBM, and next switched to 10% CO_2/BBM there was a marked, reversible decrease in the voltage-activated K^+ current in 10% CO_2/BBM in addition to that already seen after switching from HBM to 5% CO_2/BBM (Fig.29A,B). The K^+ current recorded in 10% CO_2/BBM was on average 14.3 +/- 2.2% (n=12) smaller than in 5% CO_2/BBM and the suppression was usually complete within 3-4 min (Fig.29B), a time course similar to that seen on switching from HBM to 5% CO_2/BBM. As regards to the leakage currents, however, switching from 5% CO_2/BBM to 10% CO_2/BBM had no additional effect superimposed on the large increase seen on switching initially from HBM to 5% CO_2/BBM (see Chapter 1).

These experiments indicated that simulated hypercapnia, i.e. increasing CO_2 levels from 5% to 10%, caused a significant reduction in the voltage-activated K^+ current in glomus cells which was likely due to the concomitant decrease in pH_i caused by the CAH-catalysed hydration of CO_2.

FIG.29. Effects of varying CO_2 levels on whole-cell K^+ currents in glomus cells. A: Switching from HBM to bicarbonate-buffered media (BBM) equilibrated with 5% CO_2 ($pH_e \approx 7.4$) caused a rapid decrease in the K^+ current in this glomus cell. BBM equilibrated with 10% CO_2 ($pH_e \approx 7.2$), which simulates hypercapnia, caused a further decrease in K^+ current. The effect was completely reversible upon perfusion with control HBM. B: Time course of decrease in K^+ current in a glomus cell exposed to BBM equilibrated with 5% and 10% CO_2. K^+ currents (voltage step = -60 to 50 mV) decreased rapidly ($\approx$ 2 min) on switching from HBM to BBM equilibrated with 5% CO_2. A further decrease was observed when a hypercapnic BBM (10% CO_2) was perfused onto this cell. These effects were readily reversible. Note: between treatment intervals with BBM (lower horizontal bars) culture was perfused with HBM.


A
HEPES / wash
HCO_3^-/5% CO_2
HCO_3^-/10% CO_2
Current (pA)
1200
800
400
0
+50 mV
-60 mV
0
10
20
30
40
50
Time (ms)
B
1400
1200
1000
800
600
400
5% CO_2/DBM
10% CO_2/DBM
5% CO_2/HBM
0
4
8
12
16
20
Time (minutes)

DISCUSSION

Increased levels of CO_2 in the circulation is the main stimulus to increase breathing in mammals. The main site of action of this stimulus in the central chemoreceptive neurons in the medulla oblongata (Loeschcke 1982). These neurons are synaptically linked to inspiratory neurons in close proximity which can directly increase ventilation. Collectively these groups of neurons are sometimes referred to as the respiratory control centre. However, another set of chemosensory cells responsive to changes in CO_2 are found in the peripherally-located carotid body (Eyzaguirre and Koyano 1965a; Eyzaguirre and Zapata 1984; Fitzgerald et al 1990); these cells are also linked to changes in ventilation by a reflex pathway through the respiratory centre in the brainstem. Many recent studies have implicated the glomus or type I cell as the actual sensor for changes in CO_2 due mainly to the demonstrations of the direct link between CO_2 levels and pH_i in glomus cells (Buckler et al 1991b; He et al 1991b), the ability of pH_i to modify the active membrane currents (Chapter 3; Peers and Green 1991; Stea et al 1991), and the localization of carbonic anhydrase activity inside these cells (Nurse 1990).

Increasing CO_2 levels modifies membrane currents in glomus cells

Increasing the level of CO_2 in the bathing media from $< 0.1\%$ (atmospheric) to 5% in this study at a constant extracellular pH ($\approx$ 7.4) caused two major changes in the membrane currents recorded from cultured rat glomus cells. First, a large

increase in the leakage conductance (which governs resting potential) possibly due to an opening of anion channels was observed (discussed in Chapter 1). Second, there was a pronounced decrease in the voltage-activated currents in glomus cells. This decrease was further augmented when the level of CO_2 was increased from 5% to 10% even though the resulting change in extracellular pH or pH_e (7.4 to 7.2; constant $[HCO_3^-]$) was previously shown to be insufficient to have a direct effect on the membrane currents (see Chapter 3; Stea et al 1991). The decrease in outward current recorded in CO_2-containing solutions was attributable to the resulting acidification of pH_i which directly suppressed voltage-activated currents in glomus cells as demonstrated in Chapter 3. Indeed increasing CO_2 levels have been shown to decrease pH_i, at least transiently, in glomus cells using the pH-sensitive fluoroprobe SNARF-1 (Buckler et al 1991b), or by the use of pH-sensitive microelectrodes (He et al 1991b). Presumably this decrease in pH_i accompanying CO_2 introduction is due to the hydration of the CO_2 to HCO_3^- and H^+ ions, catalyzed by intracellular carbonic anhydrase.

Carbonic anhydrase activity in glomus cells

Carbonic anhydrase (CAH) is thought to play a physiological role during the hypercapnic response of the carotid body (Hanson et al 1981). Several studies have indicated that CAH is present in the carotid body (Ridderstrale and Hanson 1984) and specifically to glomus cells (Nurse 1990; Rigual et al 1985). Other investigations where permeant CAH inhibitors, acetazolamide or methazolamide, were perfused

through *in vitro* carotid body preparations indicated marked decreases in chemosensory discharge in response to a hypercapnic stimulus (Iturriaga et al 1991; Rigual et al 1991). In the present study acetazolamide (10 μM) caused an obvious delay in the decrease of voltage-activated K^+ current in glomus cells recorded in CO_2-containing, bicarbonate-buffered media. In addition, the change in the steady-state level of the K^+ current was less in the presence of acetazolamide than in its absence. Since it is likely the decrease in K^+ current is due to a fall in pH_i (see Chapter 3; Stea et al 1991) these results indicate that inhibiting CAH activity in glomus cells prevents the rapid changes in pH_i that normally occurs during a hypercapnic stimulus. In fact this has been shown directly in isolated glomus cells, in a study by Buckler et al (1991a), where perfusion of acetazolamide slowed the fall in pH_i on exposure to CO_2, although the steady state level was not greatly affected. Thus it appears that hypercapnia directly affects glomus cells by decreasing pH_i which in turn may act as an intermediary signal in the chemosensory pathway in glomus cells. The possible physiological roles of CO_2 and pH_i in chemoreception will be discussed in Chapter 6.

Summary of contrasting effects of HEPES vs bicarbonate/CO_2-buffered media on whole-cell currents in glomus cells

The present findings indicate two important differences in physiological properties of glomus cells during recordings in bicarbonate-free and bicarbonate/CO_2-containing media. First, previous electrophysiological studies indicated that these cells have small leakage currents and consequently high input resistances (2 - 4 GΩ) in

bicarbonate-free, HEPES-buffered media (Duchen et al 1988; Hescheler et al 1989; Stea and Nurse 1991a). In contrast, during perfusion with HCO_3^-/CO_2-buffered media a three-fold increase in input conductance was observed in most cells indicating that leakage channels, possibly permeable to anions, were opened during these recordings (see Chapter 1). Second, in the present study switching from HEPES-buffered media to HCO_3^-/CO_2-buffered media caused a significant decrease in K^+ currents which is attributed to acidification of pH_i catalysed by CAH in glomus cells (Nurse 1990; Rigual et al 1985).

In conclusion, the significance of studies on glomus cells in HEPES-buffered media before and after chemosensory stimulation must be reevaluated since these cells behave much differently in physiological HCO_3^-/CO_2-buffered solutions.

CHAPTER 5

Bicarbonate-Permeable Anion Channels in Membrane Patches from Cultured Glomus Cells

INTRODUCTION

Recent studies using patch clamp / whole-cell recording techniques indicated that rabbit glomus cells contain voltage-gated Na^+, K^+, and Ca^{++} channels (Duchen et al 1988; Hescheler et al 1989; Peers 1990; Urena et al 1989) and as described in Chapter 1 of this thesis, cultured rat glomus cells were shown to have similar types of ion channels (see also Peers 1990a,b; Stea and Nurse 1991a). However, only the voltage-activated channels, especially the O_2-sensitive K^+ channel (Lopez-Barneo et al 1988; Hescheler et al 1989; Stea and Nurse 1991a), have been the focus of these studies and little attention has been directed to the channels governing the leakage conductance which controls the resting potential. Only a few studies have identified individual ion channels in isolated patches of glomus cell membrane (Delpiano and Hescheler 1989; Duchen et al 1988; Ganfornina and Lopez-Barneo 1991; Stea and Nurse 1989). Two of these studies described the voltage-activated, O_2-sensitive K^+ channel in membrane patches from rabbit glomus cells (Delpiano and Hescheler 1989;

Ganfornina and Lopez-Barneo 1991). A few cell-attached recordings in another study suggested that leakage K^+ channels were present in rabbit glomus cells (Duchen et al 1988).

In this chapter I will focus on the characteristics of a large conductance anion channel which was frequently observed in cell-free inside-out patches from cultured glomus cells of the rat carotid body (see also Stea and Nurse 1989). Anion channels are widespread and have been described in a variety of other systems, e.g. skeletal muscle (Blatz and Magleby 1985), hippocampal neurons (Francolini and Nonner 1987), olfactory neurons (Maue and Dionne 1987), and certain epithelial cell lines (Nelson et al 1984). Some of these channels play a role in the regulation of the cell's resting membrane potential (Blatz and Magleby 1985). Indeed, Cl^- ions have been shown to be important in governing the resting membrane potential of glomus cells (Eyzaguirre et al 1983; Oyama et al 1986a,b), although these studies were based on intracellular recording and ion-sensitive microelectrodes which may have damaged these small cells. This study represents the only direct description of an anion channel in glomus cells. As discussed later this channel may well play a major role in governing the general physiology of these cells by mediating the fluxes of both chloride and bicarbonate ions.

METHODS

The procedures for the culture of glomus cells were identical to those described in Chapter 1.

Single channel recording

Just prior to recording, cultures were rinsed and bathed with a physiological salt solution (see below) and mounted on the stage of an inverted phase contrast microscope (Leitz Diavert). The reference electrode was a polyethylene tube filled with 0.9% saline:2% agar connecting the bathing solution to a pool of 150 mM KCl that was grounded via an Ag:AgCl electrode. The tube was placed in contact with the bathing solution by inserting it into a stainless steel block which was placed in the culture dish. Patch pipettes were fabricated from Corning 7052 glass (1.5 mm O.D.) pulled in a Brown-Flaming horizontal puller (Model P-80; Sutter Instruments Co., San Francisco, CA). The pipettes were fire-polished and their resistance varied between 5-10 MΩ. Giga-ohm (GΩ) seals, typically between 2-15 GΩ, were formed between the pipette and identified glomus cells following application of gentle pipette suction (Hamill et al 1981). All experiments were performed at room temperature.

Single channel currents were recorded with a World Precision Instruments (WPI) patch clamp module (S7050A) and a probe equipped with a 1 GΩ headstage feedback resistor. Records were simultaneously displayed on an oscilloscope (Tektronix, 5111A), digitized with a digital audio processor (Sony, PCM-501ES)

modified by Unitrade Corp. (Philadelphia, PA) and stored in a video cassette recorder (Sony, SL-HF900). Pipette potentials were displayed on a digital voltmeter mounted on the WPI mainframe (S7100A). Junction potentials, which varied typically from 2-4 mV, were cancelled at the beginning of the experiment and later corrected if the bathing solution was altered during the course of the experiment.

Data analysis

The single channel conductance in media of different ionic compositions was calculated from the slope of the current-voltage (I-V) relation derived from records of single channel currents at different holding potentials. Current amplitudes were measured on a Nicolet 3091 storage oscilloscope after playback from the video tape. Currents of less than 1.5 pA were difficult to distinguish above the baseline noise ($\approx$ 1 pA) and were not included in the I-V relations. Data were filtered at 1000 Hz through a 8-pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA) during playback. The chloride selectivity of the channel was determined from experiments in which sodium and chloride were the only major ions present in the bathing solution. The permeability ratio of sodium to chloride (P_{Na}/P_{Cl}) was calculated using the following form of the Goldman-Hodgkin-Katz equation:

$$E_{rev}=\frac{RT}{F}\ln\frac{[Cl]_b+(P_{Na}/P_{Cl})\,[Na]_p}{[Cl]_p+(P_{Na}/P_{Cl})\,[Na]_b}$$

where E_{rev} is the reversal potential obtained from the single channel I-V relationship;

R is the universal gas constant; T is the absolute temperature; F is Faraday's constant; P is permeability; b refers to the bathing solution; p refers to the pipette solution. The permeability ratio of various anions (A) relative to chloride was determined by substituting the calculated P_{Na}/P_{Cl} into the following modified form of the Goldman-Hodgkin-Katz equation:

$$E_{rev}=\frac{RT}{F}\ln\frac{[Cl]_b+r(P_A/P_{Cl})\,[A]_b+(P_{Na}/P_{Cl})\,[Na]_p}{[Cl]_p+r(P_A/P_{Cl})\,[A]_p+(P_{Na}/P_{Cl})\,[Na]_b}$$

where r is the ratio of the anion:chloride valence.

The kinetics of channel opening and closing were analyzed with the aid of an IBM AT compatible computer and Axess version 1.0 software (Axon Instruments Inc., Burlingame, CA) after playing segments of tape through the Axolab 1100 computer interface (Axon Instruments Inc.). Segments obtained at a constant holding potential from inside-out patches with a single large conductance channel were analyzed for openings and closings over periods lasting 1 to 5 minutes. Histograms of open and closed times were plotted and the mean open and closed times obtained from single exponential curves fitted to the data by a least-squares method. The Kolmogorov-Smirnov non-parametric test was used to determine the goodness of fit of the exponential curves to the observed data. Long openings and closings (> 50 ms) were rare events and were excluded from the histograms. All traces in the text were plotted on a Hewlett-Packard 7035B X-Y recorder.

Solutions

Most experiments were performed on inside-out patches with the patch pipette containing extracellular fluid of the following composition (mM): NaCl,140; $CaCl_2$,0.1; $MgCl_2$,1; glucose,10; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES),10 at pH 7.2, except for a few cases where the calcium concentration was modified as noted below. In several experiments on cell-attached patches the pipette contained (mM): KCl,140; $CaCl_2$,1; $MgCl_2$.2; 11 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA); HEPES,10 at pH 7.2. The bathing solution which typically had a volume of $\approx$ 1 ml, could be rapidly changed by a perfusion system in which the test solution flowed into the culture dish under gravity and was simultaneously withdrawn by manual suction. The bath solution was usually identical to the pipette solution for the initial characterization of the channel. To determine the ion selectivity of the channel the culture was perfused with one or more of 5 different solutions A,B,C,D, and E. Solution A contained (mM): NaCl,70; $CaCl_2$,0.1; $MgCl_2$,1; glucose,10; HEPES,10 at pH 7.2. Solution B contained (mM): NaCl,70; choline chloride,70; $CaCl_2$,0.1; $MgCl_2$,1; glucose,10; HEPES,10 at pH 7.2. Solution C contained (mM): NaCl,70; sodium glutamate,70; $CaCl_2$,0.1; $MgCl_2$,1; glucose,10; HEPES,10 at pH 7.2. Solution D contained (mM): NaCl,70; $NaHCO_3$,70; $CaCl_2$,0.1; $MgCl_2$,1; glucose,10; HEPES,10 at pH 7.5. Solution E contained (mM): NaCl,70; Na_2SO_4,35; $CaCl_2$,0.1; $MgCl_2$,1; glucose,45; HEPES,10 at pH 7.2.

To test the possible effects on channel gating of nucleotides applied to the cytoplasmic face of the cell-free patches, adenosine 3',5'-cyclic monophosphate

(cAMP), guanosine 3',5'-cyclic monophosphate (cGMP), or ATP were dissolved in the bathing solution to give a final concentration of 1 mM. In similar experiments the sensitivity of the channel to pH_i and calcium was tested by appropriate changes in the bathing solution. In the calcium experiments the pipette solution contained (mM): NaCl,140; glucose,10; HEPES,10; EGTA,0.5 at pH 7.2. The bathing solutions were identical except for the addition of the following concentrations of calcium chloride (μM) to obtain the desired free calcium levels: i) 55.7 for 0.01 μM free Ca^{2+}; ii) 279 for 0.1 μM free Ca^{2+}; iii) 467 for 1 μM free Ca^{2+}; and iv) 1000 (without EGTA) for 1 mM free Ca^{2+} (see Magleby and Pallotta 1983).

Drug experiments

The pharmacological properties of the anion channel were tested by perfusing the culture with putative chloride channel blockers or agents known to block chloride transport. These included anthracene-9-carboxylic acid or 9-AC (Aldrich Chemical Co., Milwaukee, WN) at 1-10 mM; 4,4'-diisothiocyanostilbene-2,2'disulfonic acid or DIDS (Sigma) at 0.5 mM; 4-acetamido-4'-isothiocyanostilbene-2,2'disulfonic acid or SITS (Sigma) at 1 mM; and furosemide (Sigma) at 1 mM. In a few experiments 5-Nitro-2(3-phenylpropylamino)-benzoic acid or NPPB, which was kindly donated by Dr.P.Rangachari, was used at a concentration of 10 μM. Both NPPB and 9-AC were first solubilized in dimethyl sulfoxide (DMSO) before dilution in the bathing solution. In control experiments the addition of 1% DMSO to the bathing solution had no detectable effect on channel activity.

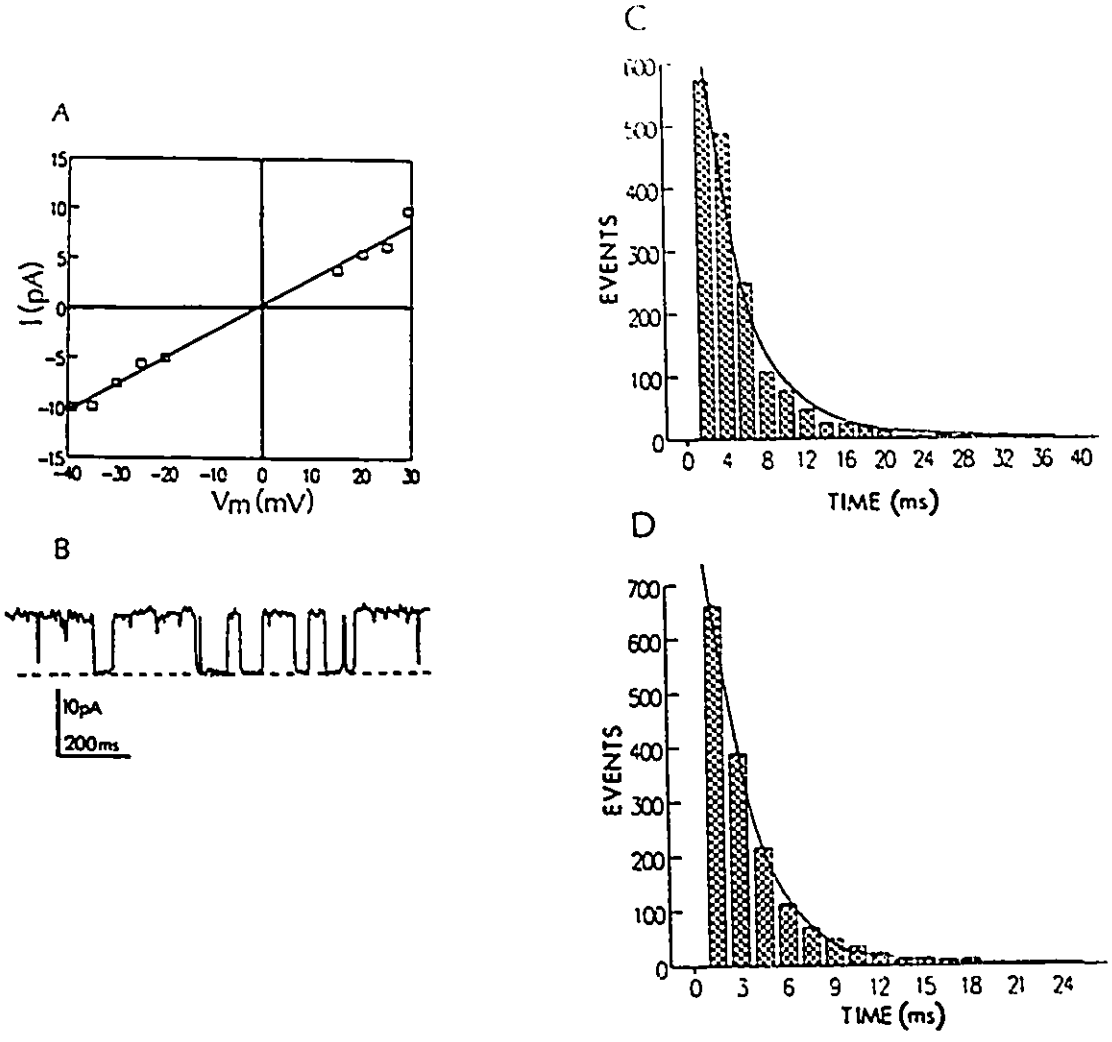
RESULTS

The carotid body cultures used in these experiments were similar to those described in the previous chapters. In these cultures glomus cells grow in confluent islands consisting of a flattened monolayer of usually 10-15 glomus cells, with distinct nuclei and nucleoli. Typically glomus cells free from extracellular matrix were selected for the patch clamp / single channel experiments reported below. Results are based on recordings from over 75 inside-out patches obtained from glomus cell membranes of 1-2 week-old cultures. In the ensuing figures in the text the potential V_m (corrected for junction potentials) represents the conventional membrane potential, i.e. the potential at the cytoplasmic face (exposed to the bath or ground potential in inside-out patches) minus the pipette potential. In addition, according to the usual convention, outward currents are positive and shown as upward deflections, whereas inward currents are negative and shown as downward deflections.

Large conductance channel in glomus cells

In inside-out patches of glomus cells a large conductance channel with linear current-voltage (I-V) characteristics was frequently observed (Fig.30A,B). This channel was present in approximately 75% of the patches and usually 1-3 channels occurred in each patch. The mean single-channel conductance (+/- S.E.), obtained from the slopes of I-V relations (e.g. Fig.30A) was 296 +/- 9.6 pS (n=29) in symmetrical 140 mM NaCl solutions. These single-channel currents reversed direction

FIG.30. Properties of large conductance channel in an inside-out patch of cultured glomus cells. A: I-V relationship of large conductance channel in symmetrical 140 mM NaCl showing a reversal potential (E_{rev}) of 0 mV and a conductance (slope) of 268 pS. B: Single channel currents (filtered at 500 Hz) at a membrane potential (V_m) of +30 mV (dashed line indicates closed state of channel and upward deflections indicate outward current). C: Open duration histogram of channel (V_m = -35 mV) is well-fitted by a single exponential distribution (smooth curve: Kolmogorov-Smirnov; $\alpha > 0.20$) with a calculated mean open time (T_o) of 4.40 +/-0.33 ms (n=1644 events). D: Closed duration histogram (V_m = -35 mV) is well-fitted by a single exponential distribution (smooth curve: Kolmogorov-Smirnov; $\alpha > 0.20$) with a calculated mean closed time (T_c) of 2.64 +/-0.05 ms (n=1622 events). Brief channel openings or closings of less than 500 μs duration were not included in these histograms.


A
I (pA)
Vm (mV)
B
10pA
200ms
C
EVENTS
TIME (ms)
D
EVENTS
TIME (ms)

at V_m = 0 mV as expected in symmetrical solutions.

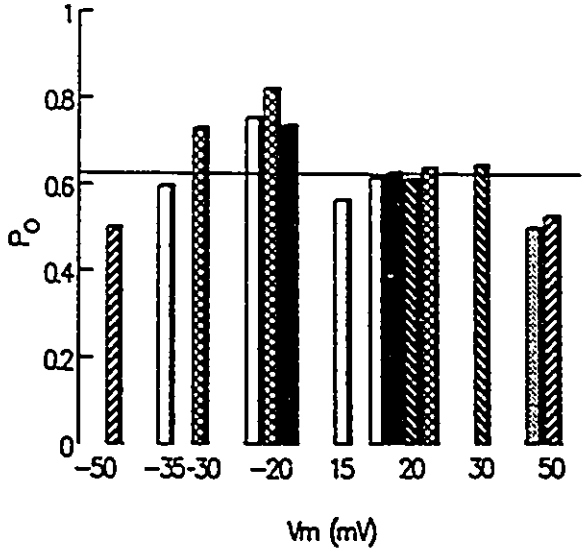
The kinetics of channel opening and closing were analyzed from segments of the single channel records for a constant membrane potential. Histograms of open and closed times are shown in Fig.30C (1644 open events) and Fig.30D (1622 closed events) respectively, for a single channel obtained from one patch, when V_m was held at -35 mV. These histograms are well-fitted by single exponential distributions (Kolmogorov-Smirnov; $\alpha > 0.2$) suggesting that the channel has one open and one closed state. Brief channel openings and closings of less than 500 μs, or flickering, were not included in the analysis. The mean probability of channel opening (P_o) was obtained from the mean open (T_o) and mean closed (T_c) times according to the relation:

$$P_o = \frac{T_o}{(T_o + T_c)}$$

Analysis of approximately 19,000 single-channel events from 6 different patches indicated that P_o (+/- S.E.) was 0.61 +/- 0.03.

To test for possible voltage-sensitivity of the channel P_o was calculated at different positive and negative membrane potentials for 6 different patches. As indicated in Fig.31, P_o remained relatively constant over a wide range of V_m between -50 and +50 mV; this range permitted the most stable recordings and clearest resolution of ion channels in these experiments. These results suggest that the channel was relatively voltage-insensitive over a wide range of membrane potentials.

FIG.31. Effect of membrane potential (V_m) on probability of opening (P_o) of the large conductance channel. Mean P_o value (0.61) indicated by line. Each different bar represents a different inside-out patch (n=6) from which the P_o values were calculated. Data were obtained from approximately 19,000 channel openings. Results show that channel activity was relatively independent of membrane potential (V_m).


1
0.8
0.6
0.4
0.2
0
P_O
-50
-35-30
-20
15
20
30
50
Vm (mV)

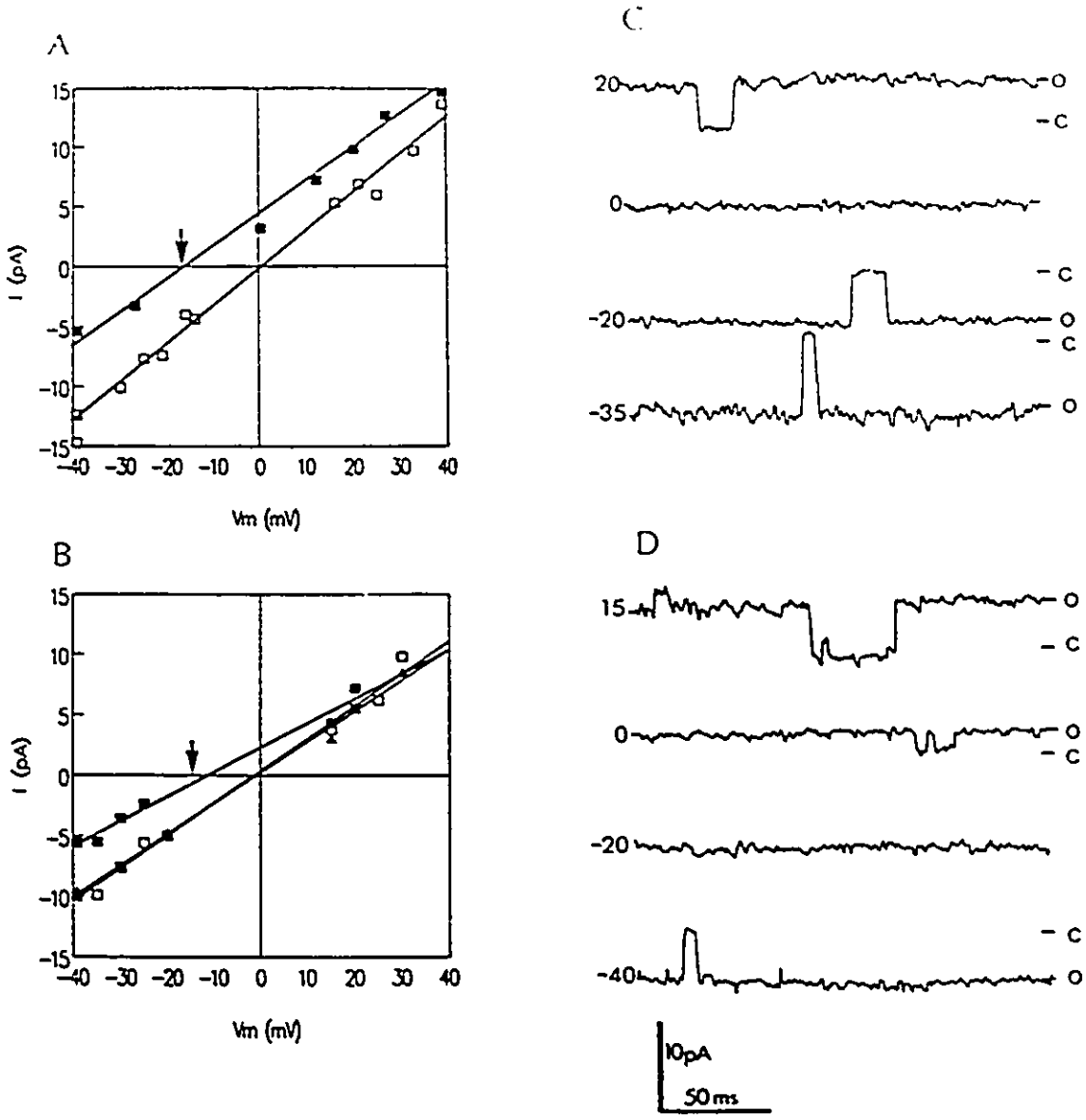
Anion selectivity of channel

Ion-substitution experiments indicated that the channel was more permeable to anions than cations (Fig.32,B,C,D). Decreasing the concentration of NaCl in the bath (solution A, Methods) caused the single channel reversal potential to shift towards the chloride equilibrium potential (Fig.32A). The calculated permeability ratio (P_{Na}/P_{Cl}) of sodium relative to chloride (see above) was 0.11 +/- 0.03 (n=10) indicating that the channel was highly selective for chloride. Equimolar substitution of half of the sodium in the bath by the large cation choline (solution B, Methods) had negligible effect on the single-channel reversal potential, which remained at $\approx$ 0 mV (Fig.32B). Substituting half the chloride in the bath by the anion glutamate (solution C, Methods) resulted in a shift of the reversal potential (Fig.32B,C,D) towards the predicted Nernst (equilibrium) potential for chloride (E_{Cl}= -17.5 mV). The incomplete shift of the reversal potential to E_{Cl} (E_{rev}= -11.5 mV in Fig.32B) can be accounted by a small but finite permeability of the channel to glutamate. Perfusion with 70 mM NaCl:70 mM Na glutamate (solution C) or with 70 mM NaCl (solution A) resulted in a decrease in the mean single-channel conductance (+/- S.E.) to 251 +/- 9.3 pS (n=31) from 296 +/- 9.6 (n=29) in symmetrical 140 mM NaCl solutions.

Anion permeability sequence

The ability of the channel to pass the anions glutamate (glu^-), bicarbonate (HCO_3^-), and sulfate (SO_4^{2-}) was compared by calculating their permeabilities relative to chloride using the modified form of the Goldman-Hodgkin-Katz equation and

FIG.32. Chloride selectivity of large conductance channel. A: Linear single-channel I-V relations in symmetrical 140 mM NaCl (open squares) and with 70 mM NaCl substituted in bath (filled squares) where the reversal potential (E_{rev}) shifts to the chloride equilibrium potential (arrowhead; E_{Cl} = -17.5 mV). B: Linear single-channel I-V relations in symmetrical 140 mM NaCl (open squares), 70 mM NaCl and 70 mM choline chloride (filled triangles), and 70 mM NaCl and 70 mM sodium glutamate in the bath (filled squares). E_{rev} shifts from 0 mV in symmetrical and choline-containing solutions to -11.5 mV in the glutamate-containing solution. C: Single channel currents showing brief channel closures (filtered at 1000 Hz) at various membrane potentials (V_m is indicated at left of trace). Note the reversal potential near 0 mV when the bath contained 140 mM NaCl. D: Single channel currents when the bath contained 70 mM NaCl and 70 mM sodium glutamate. Note the shift in the reversal potential in the negative direction (i.e. towards E_{Cl}). The pipette contained 140 mM NaCl throughout the experiment; o=open, c=closed.


A
B
C
D
I (pA)
Vm (mV)
15
10
5
0
-5
-10
-15
-40 -30 -20 -10 0 10 20 30 40
20
0
-20
-35
O
C
10pA
50 ms

substituting the estimated permeability ratio P_{Na}/P_{Cl} (0.11 +/- 0.03; n=10) obtained from experiments similar to Fig.32A (see Methods). The permeability sequence was: $Cl^- > HCO_3^- > SO_4^{2-} >$ glu$^-$. Notably, bicarbonate passed quite readily through this channel. This was of particular interest since, as discussed in Chapter 4, bicarbonate ions are produced intracellularly in glomus cells during hydration of CO_2, catalysed by carbonic anhydrase (Nurse 1990). The calculated mean permeability ratios (+/- S.E.) of the above anions were: P_{HCO3}/P_{Cl} = 0.71 +/- 0.07 (n=8); P_{SO4}/P_{Cl} = 0.57 +/- 0.08 (n=6); P_{glu}/P_{Cl} = 0.14 +/- 0.05 (n=15). The I-V relation for solutions in which sulfate (solution E, Methods) and bicarbonate (solution D, Methods) were partially substituted for chloride are shown in Fig.33A and B respectively.

Effects of anion transport blockers

Several putative blockers of chloride transport were tested for their ability to block the large conductance channel in inside-out patches. With this configuration the perfused drugs are applied to the cytoplasmic side of the membrane, which is opposite to the usual method of application of these drugs in intact cell preparations (Palade and Barchi 1977). Of those tested in this study only anthracene-9-carboxylic acid (9-AC) was effective. 9-AC reversibly reduced the amplitude of single channel currents at 5 mM and appeared to block the channel reversibly at a concentration of 10 mM (n=9); an example of this blockade is shown in Fig.34. In diaphragm muscle (Palade and Barchi 1977) and hippocampal neurons (Francolini and Nonner 1987) of the rat, as well as in human tracheal epithelium (Welsh 1984), 9-AC is known to be an

FIG.33. Permeability of anion channel to sulfate and bicarbonate ions. A: Single channel I-V relation in symmetrical 140 mM NaCl showing E_{rev} near 0 mV (filled squares). E_{rev} shifts to -5.3 mV when bath solution was switched to 70 mM NaCl and 35 mM Na_2SO_4 (filled triangles), corresponding to a permeability ratio (P_{SO4}/P_{Cl}) of 0.58. B: Single channel I-V relation in symmetrical 140 mM NaCl showing E_{rev} of near 0 mV (filled squares). E_{rev} shifts to -3.6 mV with 70 mM NaCl and 70 mM $NaHCO_3$ in the bath (open squares) corresponding to a permeability ratio (P_{HCO3}/P_{Cl}) of 0.71 indicating HCO_3^- ions are highly permeable through the large conductamce channel.

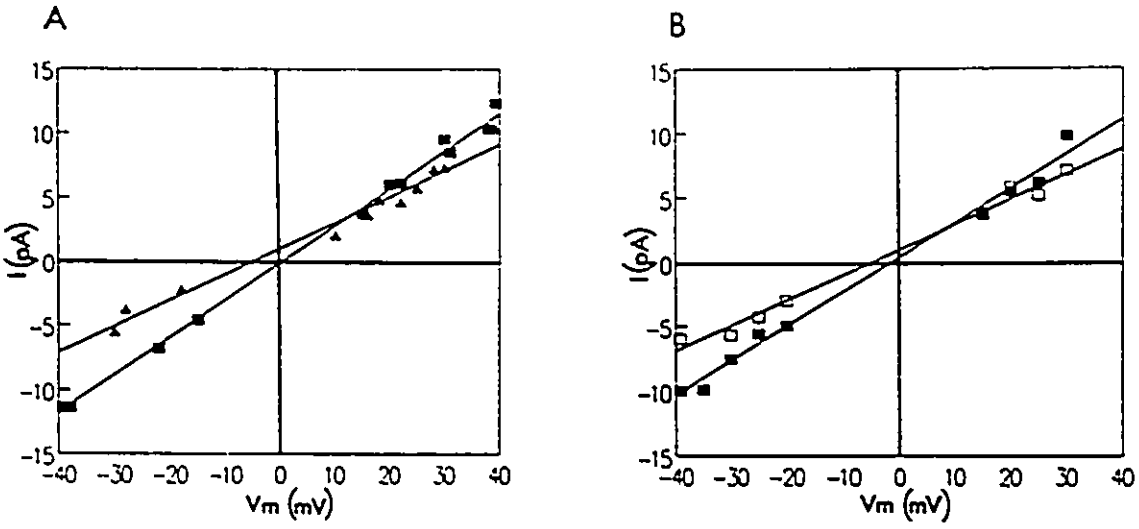
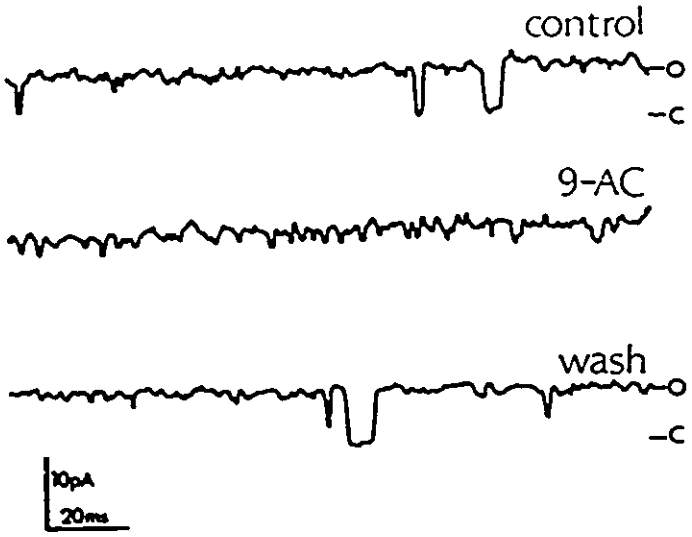

A
B
15
10
5
0
-5
-10
-15
I(pA)
-40 -30 -20 -10 0 10 20 30 40
Vm (mV)

FIG.34. Effect of anthracene-9-carboxylic acid (9-AC) on chloride channel in glomus cells. Single channel currents from an inside-out patch in control symmetrical 140 mM NaCl (top trace), during bath-applied 10 mM 9-AC (middle trace), and following wash out of the drug (lower trace). Blockade by 9-AC is accompanied by an increase in the baseline noise and both effects are reversible. All traces were filtered at 2000 Hz. V_m = +20 mV; o=open, c=closed.


control
o
c
9-AC
wash
o
c
10pA
20ms

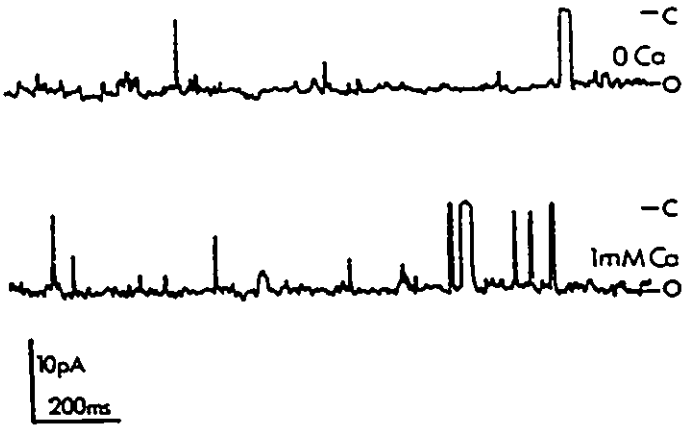
effective blocker of chloride conductance. In the human tracheal epithelium 4 mM 9-AC decreased, in a reversible manner, the single-channel chloride current to about 70% control values in inside-out patches (Welsh 1984); the drug was effective at comparable concentrations in outside-out patches of hippocampal neurons (Francolini and Nonner 1987). In 3 experiments, another less-characterized chloride channel blocker, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) had no noticeable effect on the large conductance channel at a concentration of 10 μM; NPPB is reported to be a potent blocker of chloride conductance in the thick ascending limb of the loop of Henle when applied from the external surface (Wangemann et al 1986).

Finally, the stilbene derivatives, SITS and DIDS at a concentration of 1 mM and 0.5 mM respectively, had no noticeable effect on the channels (n=3); in addition furosemide, at 1 mM, was ineffective (n=3). These compounds block chloride transport in many systems especially those utilizing the Cl^-/HCO_3^- co-transporter (L'Allemain et al 1985).

Effects of "cytoplasmic" Ca^{2+}, pH, or nucleotides

In other systems there is evidence for anion channel gating by certain intracellular ions and small molecules. For example, chloride channels may be regulated by intracellular calcium (Ca_i) in smooth muscle (Byrne and Large 1987), by intracellular pH in skeletal muscle (Blatz and Magleby 1985), and by cAMP and ATP in salt-secreting epithelia (Halm et al 1988; Landry et al 1987). In inside-out patches of glomus cells, the large conductance chloride channel did not appear to be regulated

FIG.35. Lack of effect of internal calcium on anion channel. Single channel currents (filtered at 500 Hz) from inside-out patch with 140 mM NaCl in bath and pipette and no free Ca^{2+} (0.5 mM EGTA present) in bath (top trace). Currents from same patch were unaffected when 1 mM free Ca^{2+} was added to the bath (bottom trace). V_m = -30 mV; o=open, c=closed.

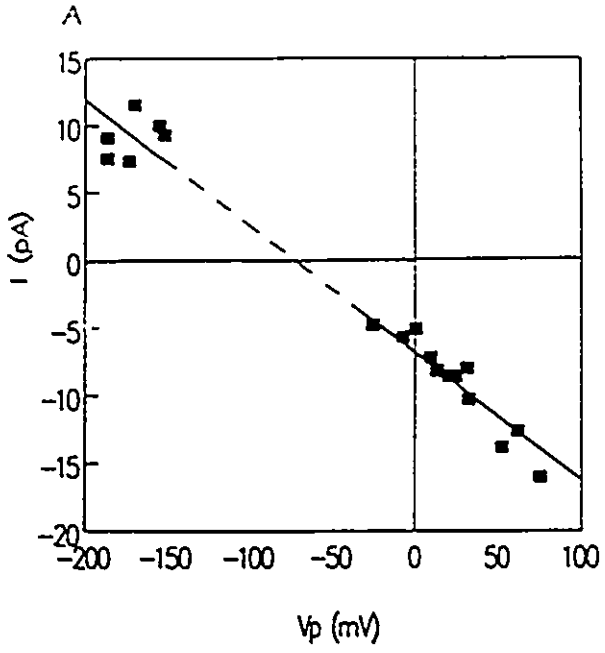

-C
0 Ca
-C
1mM Ca
10pA
200ms

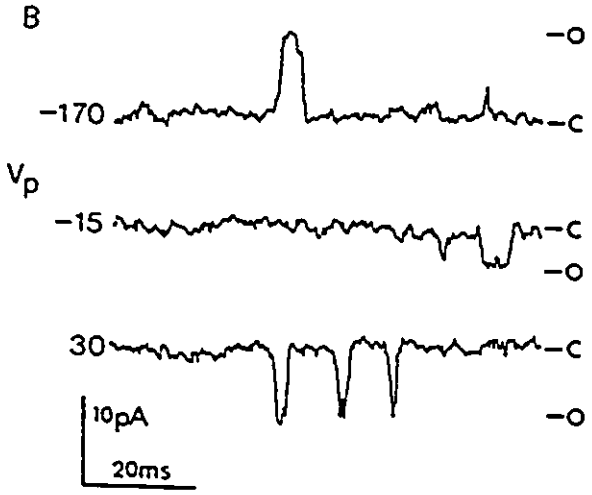
by cytoplasmic calcium (Fig.35). Both the amplitude of the single channel currents and the mean probability of channel opening (P_o) were hardly affected on changing the calcium concentration (from 0 to 0.1 or 1 mM) on the cytoplasmic face of the membrane patch. For example, in one series the calculated P_o in calcium-free solution (0.5 mM EGTA) was 0.72 (n= 1000 events) compared to 0.61 (n= 19,000 events) in 0.1 mM Ca. Similarly, the amplitude of the single-channel currents was not affected by altering the pH_i from 6.5 to 8.0 (n=2) nor by the application of ATP (n=4), cAMP (n=3), or cGMP (n=2) to the bathing solution at a concentration of 1 mM. Though there was no obvious effect on the frequency of channel opening in these experiments, P_o was not computed.

Cell-attached recordings

In cell-attached patches of glomus cells a relatively large conductance channel was observed in about 50 % of the cases with an average of one channel per patch. It had a mean conductance (+/- S.E.) of 109 +/- 6.8 pS (n=7) and had a mean P_o of $\approx$ 0.40 (Fig.36). This channel had approximately 1/3 the conductance of the chloride channel observed in inside-out patches with high (symmetrical) chloride-containing solutions. In experiments on inside-out patches (n=31) there was a decrease in conductance of the chloride channel ($\approx$ 15%) as the chloride concentration at the cytoplasmic face was reduced by one-half. In other studies chloride channels in cell-free patches also showed a marked decrease in conductance as the concentration of the permeant anion is lowered on the cytoplasmic face of the patch (Schwarze and Kolb

FIG.36. Cell-attached recording of large conductance channel in glomus cell. A: Single-channel I-V relation with 140 mM KCl in pipette indicating a conductance (slope) of ≈ 100pS. Note: dotted line indicates uncertainty of reversal potential. B: Single-channel currents at various pipette potentials (V_p) showing reversal of current direction at ≈ -80 mV. All traces were filtered at 2000 Hz; o=open, c=closed.


A
15
10
5
0
−5
−10
−15
−20
I (pA)
−200
−150
−100
−50
0
50
100
Vp (mV)


B
−o
−170
−c
Vp
−15
−c
−o
30
−c
−o
10pA
20ms

1984). Since the chloride concentration in the intact cell is uncertain it is presently unclear whether the large conductance channels seen in cell-attached and cell-free patches represent the same or different channels.

DISCUSSION

The main finding in the present study is the occurrence of a large conductance anion channel in inside-out patches of cultured glomus cells of the rat carotid body. In addition to chloride ions, the most permeable anion tested, bicarbonate ions also passed readily through the channel. These novel findings are of particular interest since these two anions may well play important roles in the physiology of the glomus cell and in the mechanisms of chemoreception in the carotid body (Eyzaguirre et al 1983; see Chapter 6). In view of the apparent importance of chloride ions (Oyama et al 1986a) rather than potassium ions (Eyzaguirre et al 1983; Oyama et al 1986b) in determining the resting membrane potential of the cell, it is plausible that the anion channel described here in cell-free patches may also play a dominant role in the intact cell. In this regard the mean probability of channel opening was relatively independent of membrane potential over the range -50 to +50 mV and therefore the channel would be open at the resting potential which was recently estimated to be $\approx$ -50 mV based on patch clamp / whole-cell recordings from rabbit glomus cells in HEPES-buffered media (Duchen et al 1988). As alluded to in Chapter 4, and as will be discussed in the final Chapter (6), this value of resting potential will need to be revised in view of the difference in resting leakage conductance of glomus cells in physiological HCO_3^-/CO_2-buffered media compared to the conventional HEPES-buffered media. A large conductance channel was also seen in cell-attached patches although it is unclear whether this is the same channel seen in cell-free patches.

Though in the resting cell, the anion channel may still be regulated by as yet unidentified intracellular small ions or molecules, these results suggest that Ca_i (0 - 1 mM), pH_i (6.5 - 8.0), as well as ATP, cAMP, and cGMP (1 mM) have no direct effect on the behaviour of this channel. The large conductance anion channel in glomus cells was quite permeable to bicarbonate (HCO_3^-) ions. This appears to be an important finding and may bear on the mechanisms of the chemosensory response of the carotid body to changes in arterial CO_2 (Eyzaguirre and Zapata 1984; Hanson et al 1981). Conceivably these same anion channels seen in cell-free patches from glomus cells correspond to the leakage (anion) channels that were opened when the glomus cells were switched from HEPES to HCO_3^-/CO_2-buffered media, as described in Chapter 1 and 4. This point is discussed further in the final Chapter.

Comparison with anion channels in other systems

The large conductance anion channel in glomus cells appears unique when compared with other anion channels described in excitable and non-excitable cells. On the basis of size alone, it is clearly different from the smaller conductance channels (with 5-10 fold lower single-channel conductance) described in hippocampal neurons (Francolini and Nonner 1987) and skeletal muscle (Blatz and Magleby 1985) of the rat, and also in a variety of epithelial cells (Halm et al 1988; Welsh 1984). These small conductance channels, however, resemble the large conductance one in glomus cells in that their opening is calcium-independent, and in some cases blockade by 9-AC was demonstrated (Francolini and Nonner 1987; Welsh 1984). Large conductance

anion channels with single channel conductance in the range 210 - 430 pS have also been described in cultured myotubes (Blatz and Magleby 1983, Schwarze and Kolb 1984), olfactory neurons (Maue and Dionne 1987), macrophages (Schwarze and Kolb 1984), and certain epithelial cell lines (Nelson et al 1984). All of these, however, show a moderate-to-strong voltage dependence, unlike the anion channel in the present study. In addition, the anion channel in A6 epithelial cells showed low selectivity for chloride over sulfate and was inhibited by externally-applied SITS (Nelson et al 1984), at a concentration (1 mM) which was ineffective in the present study, when applied to the cytoplasmic face. Whether SITS and DIDS would block the anion channel in glomus cells from the outside requires further studies using the outside-out or whole cell configuration.

CHAPTER 6

Discussion of Mechanisms of Chemotransduction by Glomus Cells of the Carotid Body

Recent evidence, including the results presented in Chapters 2, 3, and 4 of this thesis has suggested a principal role for glomus cells in the transduction of chemosensory stimuli in the carotid body (Biscoe and Duchen 1989,1990a,b; Hescheler et al 1989; Lopez-Barneo et al 1988; Lopez-Lopez et al 1989; Peers 1990a,b; Stea and Nurse 1991a,b; Stea et al 1991). There is general agreement that glomus cells are the sensors which release neurotransmitter onto the apposed sensory nerve endings in response to hypoxia, hypercapnia, and acidity. Among the neurotransmitter candidates in the carotid body are dopamine, norepinephrine, epinephrine, acetylcholine, 5-hydroxytryptamine (serotonin), substance P, met-enkephalin, and atrial natriuretic peptide (Eyzaguirre and Zapata 1984; McDonald 1981; Wang, Z.-Z. et al 1991b). However, it is still unclear which of these putative neurotransmitters convey chemosensory signals from the glomus cell to the sensory nerve endings. The two transmitters which have received the most attention in this regard are dopamine (DA) and acetylcholine (ACh). Dopamine is released from the intact carotid body *in vitro* (Fidone et al 1982; Obeso et al 1992), and also from

cultured carotid body cells in response to hypoxia (Fishman et al 1985). A few studies using *in vitro* carotid body preparations indicated that the sensory discharge recorded along the carotid sinus nerve increases in direct proportion with the amount of dopamine released during stimulation by low pH or high CO_2 (Rigual et al 1984,1991). Acetylcholine has also been implicated as the excitatory neurotransmitter released from glomus cells (see Eyzaguirre and Zapata 1984). Early studies indicated that application of acetylcholine caused an increase in sensory activity in the carotid sinus nerve (Eyzaguirre and Koyano 1965b), but these results were not always consistent (Eyzaguirre and Zapata 1984). There are still many unanswered questions concerning the identity of the excitatory transmitter released from glomus cells in response to chemosensory stimuli, but as these questions were not addressed in this thesis I will not consider them further. In this discussion I will attempt to describe the physiological changes in glomus (or type I) cells in response to each of the chemosensory stimuli, low Po_2, acidity, and elevated Pco_2, and the resulting cellular mechanisms which likely induce neurotransmitter release from these cells.

Resting membrane potential of glomus cells

An important parameter in the physiological interpretation of many recent studies on glomus cells is their resting membrane potential. Resting potentials of $\approx$ -20 to -40 mV have been reported for rat glomus cells (Baron and Eyzaguirre 1977; Eyzaguirre et al 1983; He et al 1991b) but these results are questionable due to

damage caused by impalement of these small cells with intracellular microelectrodes used in these studies. A recent estimate based on patch clamp recording from freshly-isolated rabbit glomus cells suggested the resting potential was closer to -50 mV in HEPES-buffered media (Duchen et al 1988). In view of the fact that the leakage conductance of glomus cells in physiological HCO_3^-/CO_2-buffered media is significantly different from that in HEPES-buffered media, as described in Chapter 1 of this thesis, the true resting potential under physiological conditions needs further re-evaluation (see also Stea and Nurse 1991b). In fact, a recent *in vivo* study has shown that the presence of external CO_2 and HCO_3^- is essential for transduction of hypoxic stimuli in the carotid body (Shirahata and Fitzgerald 1991a). The large increase ($\approx$ 3-fold) in leakage conductance seen in glomus cells exposed to bicarbonate/CO_2 media (Chapter 1) was likely due to the opening of anion channels, since the underlying currents could be blocked by the chloride channel blocker, 9-anthracene carboxylic acid (see Francolini and Nonner 1987); further the increase in leakage conductance was unaffected by elimination of K^+ ions from the recording solutions. These findings qualitatively support earlier proposals, using ion-sensitive microelectrodes, that the resting potential of glomus cells is dependent on Cl^- ions (Oyama et al 1986a) and not K^+ ions (Oyama et al 1986b). If Cl^- movements do indeed govern the resting potential of glomus cells it is quite likely that the large conductance Cl^- channel described in Chapter 5 plays a major role in the leakage conductance (Stea and Nurse 1989). Furthermore, since HCO_3^- ions are quite permeable through these anion channels ($P_{HCO3}/P_{Cl} \approx 0.7$; Chapter 5) it is possible

that HCO_3^- as well as Cl^- ions govern the membrane potential of glomus cells in physiological bicarbonate/CO_2-buffered extracellular solutions (but not in artificial HEPES-buffered solutions).

The following is an attempt to estimate the resting potential (E_m) of glomus cells assuming that the resting conductance is due mainly to large conductance anion channels (Chapter 5; see also Stea and Nurse 1989). This approximation requires knowledge of the intracellular and extracellular concentrations of the permeable anions. Intracellular HCO_3^- concentration ($[HCO_3^-]_i$) can be estimated using the Henderson-Hasselbalch equation:

$$pH_i = pK_a + \log\frac{[HCO_3]_i}{(s)(P_{co_2})}$$

where pH_i is 7.28, measured with the pH sensitive dye SNARF-1 in rat glomus cells bathed in bicarbonate/ 5%CO_2 buffered media (Buckler et al 1991a); the dissociation constant (pK_a) is 6.13; the solubility coefficient (s) for CO_2 is 0.03 (see Buckler et al 1991b). A value of $\approx$ 16 mM was estimated for $[HCO_3^-]_i$ in glomus cells using the above formula. Now using a modified form of the Goldman-Hodgkin-Katz equation one can estimate the membrane potential of glomus cells:

$$E_m = \frac{RT}{F}\ln\frac{[Cl]_i + (P_{HCO_3}/P_{Cl})[HCO_3]_i}{[Cl]_e + (P_{HCO_3}/P_{Cl})[HCO_3]_e}$$

where E_m is the estimated membrane potential, R is the universal gas constant, T is the absolute temperature, F is Faraday's constant, P is permeability, e refers to the extracellular solution, and i refers to the intracellular solution. The value for the permeability ratio of HCO_3^- ions through the Cl^- channels ($P_{HCO_3}/P_{Cl} = 0.71$) was taken from Chapter 5 of this thesis (see also Stea and Nurse 1989). The measured intracellular Cl^- concentration ($[Cl^-]_i$) in glomus cells using ion-selective microelectrodes was $\approx$ 20 mM (Oyama et al 1986a). Extracellular Cl^- and HCO_3^- concentrations were assumed to be $\approx$ 103 and 27 mM respectively (typical plasma levels; Vander et al 1980).

Using the above formula the estimated E_m of rat glomus cells is $\approx$ -35 mV. It is of interest that at this potential the voltage-gated Na^+ channels known to be present in glomus cells would be almost completely inactivated (Chapter 1; Duchen et al 1988; Urena et al 1989). However since rat, and embryonic rabbit, glomus cells generally have very small or negligible inward Na^+ currents and spiking is rare or absent (Hescheler et al 1989; Peers 1990a,b; Stea and Nurse 1991a) inactivation of Na^+ channels at an $E_m \approx$ -35mV should have little influence on the cells' behaviour. In contrast, adult rabbit glomus cells have a higher density of Na^+ channels and spiking

can be routinely recorded, at least in HEPES-buffered media (Duchen et al 1988; Lopez-Barneo et al 1988). If the resting potential in rabbit glomus cells is also around -35 mV under physiological conditions then the spiking mechanism should be inactivated, and theories on chemoreception based on modulation of spike frequency (Lopez-Barneo et al 1988) will need to be re-evaluated. It therefore would be interesting to know the physiological properties of rabbit glomus cells in physiological bicarbonate-buffered media and whether the excitability of these cells differs from that in HEPES-buffered media.

I will use the data obtained from this thesis to suggest a model for chemosensory transduction in the rat carotid body, where glomus cells are the actual chemosensors. Since in the rat, glomus cells have very small Na^+ currents (under normal conditions) it is unlikely that Na^+ channels play a primary role in chemotransduction. However, the other voltage-gated channels, K^+ and Ca^{2+}, are likely to play a major role as described below. As indicated in Chapter 1, both K^+ and Ca^{2+} channels are activated between -40 and -30 mV (see also Duchen et al 1988; Hescheler et al 1989; Stea and Nurse 1991a; Urena et al 1989) and Ca^{2+} channels are only half-inactivated at -40 mV (Chapter 1). Therefore at the estimated resting potential (E_m) of -35 mV small changes in K^+ permeability can significantly influence both the membrane potential as well as inward Ca^{2+} movements across the membrane. However, if the E_m of glomus cells was -50 mV or below as proposed for adult rabbit glomus cells (Duchen et al 1988) the majority of these channels would be closed and consequently would have no effect on the membrane potential. The important role of

the resting potential during chemosensory transduction by glomus cells will be discussed below.

Glomus cells: Adaptable O_2-sensors

As discussed in Chapter 2, rat glomus cells contain a K^+ current which decreases in response to lowered Po_2, in agreement with the original finding of Lopez-Barneo et al (1988) in the rabbit (see also Hescheler et al 1989; Peers 1990a,b; Stea and Nurse 1991a). Single O_2-sensitive K^+ channels have also been recorded from membrane patches of glomus cells (Delpiano and Hescheler 1989; Ganfornina and Lopez-Barneo 1991). Lopez-Lopez et al (1989) suggested that O_2-sensitive K^+ channels may be linked by a G-protein to a hemoglobin-like O_2-sensor located in the plasma membrane of glomus cells. However, in membrane-patches from glomus cells, O_2-sensitive K^+ channels showed no obvious changes when GTPγ-S (a non-hydrolysable analogue of GTP which inhibits G-protein function) was present at the internal face of the membrane (Ganfornina and Lopez-Barneo 1991) suggesting that a G-protein is not involved. It is possible the O_2-sensor is an intrinsic heme-containing membrane protein similar to the one controlling secretion of erythropoietin from liver and kidney cells (Goldberg et al 1988). Interestingly, in a recent study, Acker et al (1989) suggested that the rat carotid body contains a protein that was sensitive to low Po_2 or cyanide and had the spectral properties of the heme-containing enzyme, NADPH oxidase. Although the identity of the actual sensor for changes in O_2 remains

speculative it is clear that a class of K^+ channels in glomus cells close in response to lowered Po_2. Further this property was specific for glomus cells (Chapter 2; see also Stea and Nurse 1991a) since it was not found in the closely-related, neural crest-derived SIF cells or in the sensory petrosal neurons which innervate the carotid body (Chapter 2; Stea and Nurse 1992). The role of these O_2-sensitive K^+ channels will be discussed below.

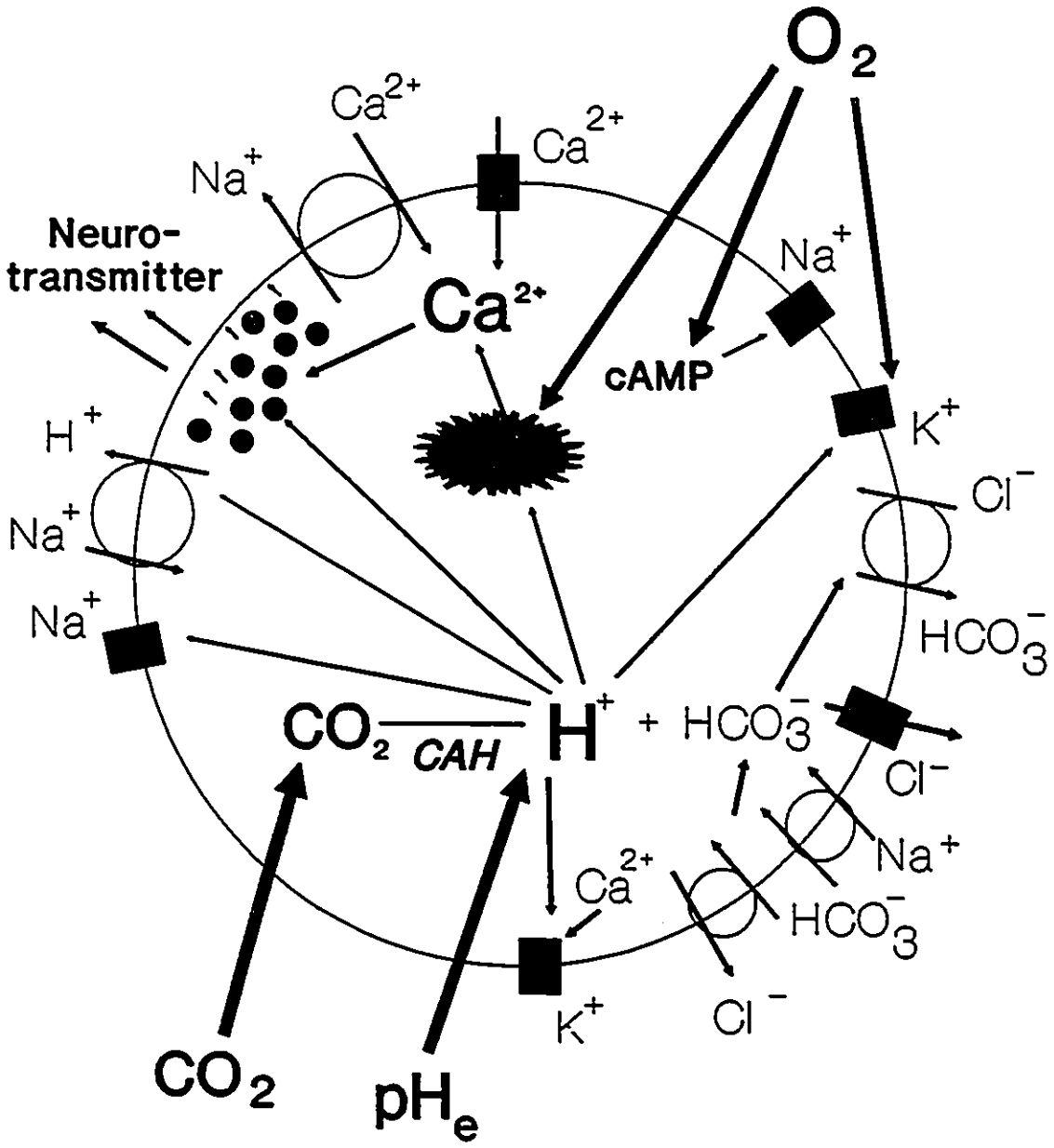
In addition to the immediate chemosensory response of the carotid body to acute hypoxia there is also strong evidence for changes in the physiology and morphology of the carotid body following prolonged exposure to hypoxia, e.g. in animals and humans living at high altitudes (Barnard et al 1987; Edwards et al 1971a; Nielsen et al 1988). As described in Chapter 2 there is an approximate doubling in the size of cultured rat glomus cells exposed to a hypoxic environment for two weeks. This result may account, at least in part, for the observed hypertrophy of the carotid body in humans living at high altitude (Edwards et al 1971a) or rats exposed to chronic hypoxia (McGregor et al 1984). However, an increase in the size of glomus cells cannot adequately explain the increase in chemosensitivity recorded from the carotid bodies of chronically hypoxic animals although an increase in the amount of neurotransmitter in these larger cells is a possibility (Barnard et al 1987; McGregor et al 1984; Nielsen et al 1988). The results obtained in Chapter 2 of this thesis suggest a novel explanation at the cellular level for this altered physiological response. Here it was found that the Na^+ channel density in glomus cells increased progressively during a two week exposure of the cultures to chronic hypoxia. Since the density of voltage-

activated K^+ channels and of leakage channels remained fairly constant, hypoxia seemed to exert a specific effect on the voltage-activated Na^+ channels in glomus cells. Further, it appeared that increases in $cAMP_i$ could be the mediator for this response since artificially elevating $cAMP_i$ in the cultures qualitatively mimicked the increase in Na^+ channel density (Chapter 2), and hypoxia is known to elevate $cAMP_i$ in glomus cells (Wang, Z.-Z. et al 1991a). An increased Na^+ channel density in glomus cells may cause an augmented depolarization initiated by the closing of the O_2-sensitive K^+ channels in response to hypoxia discussed below. This finding, therefore, gives a cellular mechanism underlying the increased sensitivity of the carotid body after exposure to chronic hypoxia. Thus glomus cells appear to be adaptable O_2-sensors capable of responding immediately to hypoxic stimuli or readjusting their responsiveness appropriately depending on environmental conditions.

Physiological role of K^+ channels, pH_i and $Ca^{2+}{}_i$ in Chemoreception

A large body of literature has emerged in the past four years concerning the cell physiology of the putative chemoreceptor in the carotid body, i.e. the glomus or type I cell. Many of these studies have focused on the possible roles of K^+ channels, intracellular Ca^{2+} and intracellular pH in chemotransduction in the carotid body (Biscoe and Duchen 1989,1990a,b; Buckler et al 1991a,b; Iturriaga et al 1991; Nurse

1990; Peers and Green 1991; Rigual et al 1991; Rocher et al 1991; Shirahata and Fitzgerald 1991b; Stea et al 1991; Stea and Nurse 1991b). A schematic representation summarizing the possible physiological roles of K^+ channels, pH_i, and $Ca^{2+}{}_i$ in a glomus cell is shown below. This model is based on results from this thesis and several of the above studies:



Hypoxic Chemotransduction

Hypoxia is a major stimuli which excites the carotid body *in situ* (Eyzaguirre and Koyano 1965a; Eyzaguirre and Zapata 1984) and has been shown to decrease a specific O_2-sensitive K^+ current in glomus cells as seen in Chapter 2 of this thesis (Lopez-Barneo et al 1988; Hescheler et al 1989; Stea and Nurse 1991a). In rat glomus cells with a resting potential of $\approx$ -35 mV this closure of K^+ channels would lead to cell depolarization which would in turn lead to the opening of voltage-activated Ca^{2+} channels known to be present in these cells (Chapter 1; Duchen et al 1988; Hescheler et al 1989; Urena et al 1989). Indeed, it has been shown that the opening of these voltage-activated Ca^{2+} channels, which appear to be L-type Ca^{2+} channels (Chapter 1; Peers and Green 1991), is essential for the increase in chemosensory discharge during hypoxia as shown in an *in vivo* cat carotid body study where the response to a hypoxic stimulus was significantly decreased during perfusion of the L-type Ca^{2+} channel blockers verapamil or nifedipine and was increased when the Ca^{2+} channel agonist BAY K 8644 was perfused (Shirahata and Fitzgerald 1991b). Also, the release of dopamine, known to be stimulated by hypoxia (Fidone et al 1982; Fishman et al 1985), was almost completely blocked in an *in vitro* rabbit carotid body preparation during perfusion of the Ca^{2+} channel blockers, nisoldipine or nitrendipine (Obeso et al 1992). Also this hypoxia-stimulated dopamine release was potentiated by addition of BAY K 8644 (Obeso et al 1992). Alternatively, Biscoe and Duchen (1989, 1990a,b) claim the rise in Ca^{2+}_i during hypoxia is due to release of Ca^{2+} from intracellular stores and Ca^{2+} channels are not involved. In their studies application of Ca^{2+} channel

blockers (cobalt, verapamil, or D-600) to freshly-isolated rabbit glomus cells had little effect on the anoxia-induced rise in Ca^{2+}_i measured with the fluorescent dye Indo-1 (Biscoe and Duchen 1990a). However, in these studies the Po_2 level was often decreased to non-physiological levels (complete anoxia, $Po_2 \approx 0$) by the addition of sodium dithionite and it appeared that eliminating extracellular Ca^{2+} did affect the rise in Ca^{2+}_i seen during anoxia (Biscoe and Duchen 1990a). Therefore, it is likely that the rise in intracellular Ca^{2+} in glomus cells during hypoxia under physiological conditions is due in part to the influx of Ca^{2+} through voltage-activated channels and in part due to release from intracellular stores. A rise in intracellular Ca^{2+} during hypoxia promotes release of the neurotransmitter dopamine from glomus cells (Fishman et al 1985; Obeso et al 1992) which may directly stimulate the sensory nerve endings increasing afferent discharge along the carotid sinus nerve.

Intracellular Ca^{2+} can also have a variety of other physiological effects; e.g. modulation of ion channels (Marty 1989). There is strong evidence that Ca^{2+}-activated K^+ channels are present in glomus cells (Chapter 1; Peers 1990a,b; Stea et al 1991; Urena et al 1989) and there even is a suggestion that this component of K^+ current is also modulated by O_2 during hypoxia (Peers 1990b). However, this proposal has not been supported since in a recent single channel study O_2-sensitive K^+ channels from rabbit glomus cells were not significantly affected by lowering the Ca^{2+} levels to below 1 nM (at the internal surface) which in the same preparation blocked the large conductance (250 pS) Ca^{2+}-activated maxi-K^+ channels (Ganfornina and Lopez-Barneo 1991). In Chapter 1 of this thesis it was found that the Ca^{2+}-activated K^+

component comprised 50% of the outward current in rat glomus cells. The corresponding channels are likely to be opened during increases in Ca^{2+}_i (Marty 1989) which would hyperpolarize the membrane and in turn inhibit calcium influx and the ensuing transmitter release. Therefore Ca^{2+}_i may modulate K^+ channel activity in glomus cells in a negative feedback manner regulating the response to hypoxia.

Hypoxia has also been shown to increase cAMP levels in glomus cells (Wang, Z.-Z. et al 1991a) and a recent study has shown that perfusion of the *in vitro* rabbit carotid body with a specific adenylate cyclase activator, forskolin, for 10 minutes causes a small increase in the carotid sinus nerve discharge and elevated catecholamine release during exposure to relatively low Po_2 (Wang, W.-J. et al 1991). Although the mechanism by which cAMP increases transmitter release in response to acute hypoxia is speculative, it is possible that cAMP can modulate voltage-activated channels in glomus cells (Wang, W.-J. et al 1991).

Chemotransduction of acidic stimuli

Another of the natural stimuli which causes an increase in the afferent impulses in the carotid sinus nerve is a decrease in extracellular pH or pH_e (Eyzaguirre and Zapata 1984). As indicated in several recent studies on glomus cells, changes in pH_e are tightly linked to changes in pH_i (Buckler et al 1991a,b; He et al 1991a,b), which can directly affect gating of ion channels, including Na^+ and K^+ (Chapter 3; see also Peers 1990a; Stea et al 1991) but apparently not Ca^{2+} channels (Peers and Green 1991). The inhibition of K^+ channel activity by acidic pH_i could lead to depolarization

and Ca^{2+} influx (and transmitter release); and this may well be the major pathway mediating chemotransduction of acidity in arterial blood. However, in an *in vitro* rabbit carotid body preparation the release of dopamine during both acidic and hypercapnic (discussed below) stimuli was insensitive to Ca^{2+}-channel blockers but was dependent on extracellular Ca^{2+} and Na^{+} (Rocher et al 1991). The above study suggested that a Na^{+}/Ca^{2+} exchanger in the glomus cell membrane may be responsible for the influx of Ca^{2+} during acidic stimuli. In contrast, it is possible that acidification directly stimulates transmitter release from glomus cells via a calcium-independent mechanism as described in presynaptic nerve terminals in the striatum (Drapeau and Nachshen 1988).

It appears that glomus cells have many pH-regulating mechanisms which may modulate the response of the carotid body to acidity. The transport proteins involved in pH_i homeostasis in glomus cells include; i) an amiloride-sensitive Na^{+}/H^{+} antiporter which functions to extrude H^{+} ions after an acid load (Buckler et al 1991a; Rocher et al 1991; Stea et al 1991), ii) a DIDS-sensitive Cl^{-}/HCO_3^{-} exchanger which is controversially suggested to cause HCO_3^{-} influx (aids recovery from acid load) by Rocher et al (1991) or HCO_3^{-} efflux (acidifying mechanism) by Buckler et al (1991a), iii) a DIDS-sensitive Na^{+}/HCO_3^{-} co-transporter which may aid in pH_i recovery from an acid load (Buckler et al 1991a), and iv) a HCO_3^{-}-permeable anion channel (Chapter 5; see also Stea and Nurse 1989) which would likely aid in efflux of HCO_3^{-} ions from glomus cells (enhancing acidification) since the Nernst potential calculated for HCO_3^{-} ions (E_{HCO3}) was $\approx$ -14 mV (see above) and the estimated resting potential was $\approx$ -35

mV. These transport proteins likely act in concert to rapidly regulate glomus cell intracellular pH after changes elicited by chemosensory stimulation.

Chemotransduction of hypercapnic stimuli

Elevated Pco_2 is also known to increase afferent discharge from the carotid body and this is likely to occur via acidification of pH_i since intracellular carbonic anhydrase activity (CAH) is present in glomus cells (Nurse 1990; Rigual et al 1985). Indeed carbonic anhydrase has been shown to be essential to hypercapnic chemotransduction as CAH inhibitors cause a marked depression in the chemosensory discharge recorded from *in vitro* carotid bodies in response to hypercapnia (Iturriaga et al 1991; Rigual et al 1991). Direct measurements of pH_i using a fluorescent indicator indicate transient decreases in pH_i in glomus cells on exposure to CO_2-containing external media (Buckler et al 1991b). Therefore it is likely that the decreases in the voltage-activated K^+ current in glomus cells during a switch from CO_2-free to bicarbonate/CO_2 (5%) media, and during simulated hypercapnia (increasing CO_2 from 5% to 10%) are due to cytoplasmic acidification (Chapter 4; see also Stea and Nurse 1991b). As suggested above this would allow glomus cells to depolarize transiently on exposure to a hypercapnic stimulus.

Summary of Chemotransduction Mechanisms

In summary, hypoxia causes O_2-sensitive K^+ channels to close, depolarizing the membrane which can cause Ca^{2+} influx through voltage-activated Ca^{2+} channels or

may promote an increase in Ca^{2+}_i from internal stores. Elevated Ca^{2+} can directly affect transmitter release or subsequently open K^+ channels which can in turn regulate this release via a negative feedback mechanism. Both pH_e and hypercapnia cause a decrease in pH_i in glomus cells resulting in closing of voltage-activated K^+ channels and inducing depolarization. This cytoplasmic acidification can affect transmitter release either directly or via changes in Ca^{2+}_i. Glomus cells have a variety of pH regulatory mechanisms, including Na^+/H^+ antiporters, Cl^-/HCO_3^- exchangers, Cl^-/HCO_3^- anion channels, which modulate the above responses and maintain pH homeostasis. This model allows for the effects of hypoxia and the other chemosensory stimuli to be mediated through interactions between K^+ channels, pH_i and Ca^{2+}_i affecting transmitter release from glomus cells onto the sensory nerve-endings.

Future Directions

Since many of the chemotransduction mechanisms proposed in this thesis are based on changes in membrane potential of glomus cells it would be of interest in the future to examine these changes directly. A plausible approach to this study could involve a current clamp analysis of glomus cells during chemosensory stimulation. Ideally, this would be done using perforated-patch recording at 37°C in bicarbonate/ CO_2 media to enhance the physiological significance of the results. Another direction for future studies may involve the reestablishment of the chemosensory complex *in vitro* by culturing sensory petrosal neurons along with glomus cells. This may allow direct study of chemosensory transmission between glomus cell and neuron.

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