**ABSTRACT**

Ryanodine, a neutral alkaloid, is a widely used pharmacological tool in the studies of muscle excitation-contraction coupling. The specific binding sites for ryanodine have been identified to exist on sarcoplasmic reticulum (SR) in both skeletal muscle and cardiac muscle. The ryanodine receptor has been purified from different tissue types. The purified ryanodine receptor from skeletal muscle was found to be identical with foot structure, a protein spanning the gap between t-tubule and SR membrane. The ryanodine receptor was also found to have Ca²⁺ channel activity, a Ca²⁺-induced Ca²⁺ release channel. In functional studies with isolated SR vesicle or single channel, ryanodine was found to have dual effects on Ca²⁺ channel. At lower concentrations, ryanodine locked Ca²⁺ channel in the open state, but fully closed the channel at higher concentrations. In smooth muscle, ryanodine was also found to affect intracellular Ca²⁺ movement in the functional studies using intact tissue. However, direct evidence was not available for the presence of ryanodine receptor in smooth muscle before this research was carried out.

In the present study, a high affinity binding site was found located on SR membranes of rat vas deferens (RVD) smooth muscle (K_d=5.6 nM, B_max=435 fmol/mg). The ryanodine receptor in smooth muscle shared many similarities with that of skeletal muscle, but was not identical. The time required for [³H]ryanodine binding to microsomal fraction was about two hours to reach a steady state, and [³H]ryanodine could be
dissociated from its binding site by 20 fold dilution. However, the dissociation was very slow and incomplete when initiated by excess ryanodine. The [3H]ryanodine binding in smooth muscle was Ca²⁺ dependent. The affinity was increased with increased Ca²⁺ concentrations, but the B.max was unchanged. The [3H]ryanodine binding also increased with higher ionic strength and higher osmolarity, but later has less effect. Many factors that affect Ca²⁺-induced Ca²⁺ release (CICR) channel activity were also found to affect [3H]ryanodine binding in my study; e.g., both Mg²⁺ and ruthenium red inhibited binding and caffeine potentiated it, especially in the presence of a low Ca²⁺ concentration. In the present study, I also showed that varied levels of [3H]ryanodine binding site existed among different smooth muscles. This variation was not correlated with the density of innervation or the SR content of different smooth muscles.

In dog mesentery artery smooth muscle, a low affinity binding site (K.≈269 nM) was also identified, in addition to the high affinity binding site.

In the present study, I also tried to show functional effect of ryanodine on the CICR channel using subcellular membrane vesicles from vas deferens. Ryanodine, at higher concentrations, inhibited oxalate-stimulated Ca²⁺ uptake, an effect which was observed as early as 5 minutes after uptake was initiated. This inhibitory effect was partially additive to that of cyclopiazonic acid (CPA), a potent SR Ca²⁺ pump inhibitor, when these agents were used at submaximal concentrations. However, when CPA was used at a maximal concentration, ryanodine had no additional effect. Ryanodine at concentrations of 10⁻⁹ to 5x10⁻⁸ M, did not significantly change the Ca²⁺ release rate. In

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To my parents
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LIST OF ABBREVIATIONS

BSA bovine serum albumin
PNS post nuclear supernatant
MIC microsomal
Sol soluble fraction
P Pellet
RVD rat vas deferens
DMA dog mesenteric artery
MOPS 3-(N-morpholino)propanesulfonic acid
Tris tris(hydroxymethyl)aminomethane hydrochloride
STX saxitoxin
TTX tetrodotoxin
5'-ND 5'-nucleotidase
DTT 1,4 dithiothreitol
Bmax maximum binding
SR sarcoplasmic reticulum
ER endoplasmic reticulum
PNS post nuclear supernatant
IP3 inositoll,4,s-trisphosphate
ER endoplasmic reticulum
IP3 inositoll,4,s-trisphosphate
TRC voltage operated Ca2+ channel
ROC receptor opened Ca2+ channel
ICR Ca2+ induced Ca2+ release
CICR Ca2+ induced Ca2+ release
DHP dihydropyridine

RATIONALE

It is well known that cytoplasmic Ca2+ is the key ion in triggering muscle contraction. Under resting conditions, the cytoplasm Ca2+ level remains in the range of 10^-7 - 10^-6 M, while the extracellular Ca2+ concentration is 10,000 - 100,000 fold higher. It is well recognized that sarcoplasmic reticulum (SR) serves as an intracellular Ca2+ pool. The elevation of cytoplasm Ca2+ which triggers muscle contraction may be due to intracellular Ca2+ release and/or extracellular Ca2+ influx depending on the muscle type. In striated muscle, especially skeletal muscle, the amount of SR is abundant. Although a type of L-type Ca2+ channel protein has been identified in plasma membrane, especially T-tubules, the connection is triggered by Ca2+ released from SR following membrane excitation in skeletal muscle. In cardiac muscle, Ca2+ entry through L-type Ca2+ channels induces Ca2+ release from SR. However, the exact role of the L-type Ca2+-channel proteins in excitation-contraction coupling is still unclear in skeletal muscle; most evidence suggest they function as voltage sensors. During the past 10 years, a neutral alkaloid, ryanodine, was found to bind to the foot structure, a protein spanning between T-tubule plasma membrane and SR in striated muscle. The purified ryanodine receptor has been demonstrated to be identical to the foot structure and contains a Ca2+-channel. Therefore, ryanodine has been widely used as a selective tool to study the mechanisms of excitation-contraction coupling in striated muscle, where it serves as a marker for a voltage-operated (skeletal muscle) or...
a Ca\(^{2+}\)-induced (cardiac muscle) Ca\(^{2+}\) release site in SR.

In smooth muscle, at least two types of Ca\(^{2+}\) channels have been identified in SR: the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) channel (the ryanodine receptor in striated muscle) and isolated 1,4,5-trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) release (IRCR) channel. In morphological studies, the distribution of SR in smooth muscle is different from that observed in skeletal muscle. No direct evidence demonstrates the existence of a foot structure between SR and plasma membrae in smooth muscle.

Smooth muscle contains less SR than striated muscle. The dependence of intracellular Ca\(^{2+}\) for muscle contraction varies among different smooth muscles, and thus the relative importance and the roles of SR contributing to the contraction of different smooth muscles are unclear. One difficulty in studying the roles of SR in smooth muscle contraction is the lack of a specific intracellular Ca\(^{2+}\) channel blocker or opener. Having been employed as a marker for Ca\(^{2+}\) releasing channel on SR in striated muscle, ryanodine may also mark intracellular Ca\(^{2+}\) release sites in smooth muscle. In functional experiments, ryanodine has been extensively shown to block Ca\(^{2+}\) release from SR and/or deplete the intracellular Ca\(^{2+}\) pool. The locus or mechanism of the action of ryanodine in smooth muscle, however, remains unclear.

In this study, I first demonstrated the location of ryanodine binding sites (ryanodine receptors) in membrane fractions from smooth muscles using radioligand binding technique and then further characterized this receptor at the subcellular membrane level. I also studied the effect of ryanodine on SR Ca\(^{2+}\) transport using isolated smooth muscle microsomal fractions enriched in SR. These studies provide direct evidence showing the location of ryanodine binding sites in smooth muscle and suggest possible action of ryanodine on smooth muscle SR.

1. INTRODUCTION

1.1 Muscle Contraction

1.1.1 Roles of Ca\(^{2+}\)

Ca\(^{2+}\) is an important ion playing a key role in many physiological events. The contractions of skeletal, cardiac and smooth muscle, which contribute to the body movement, blood circulation and activities of internal organs, are controlled by hormonal and/or neuromodulatory factors. However, contractile activities of muscles are controlled ultimately by regulation of the intracellular Ca\(^{2+}\) level which triggers a cascade of events leading to muscle contraction.

In smooth muscle, it is generally believed that the major mechanism of regulation of contraction is through binding of Ca\(^{2+}\) to calmodulin and of the Ca\(^{2+}\)-calmodulin complex to the catalytic subunit of myosin light chain kinase (Hill and Murphy, 1989). This is followed by myosin light chain phosphorylation that permits activation of myosin ATPase by actin. The subsequent hydrolysis of ATP provides the energy for the sliding cycle of the contractile filaments resulting in the muscle shortening.

In skeletal muscle, the binding site for Ca\(^{2+}\) is troponin which in turn forms a complex with tropomyosin. The formation of this complex due to Ca\(^{2+}\) binding causes conformation changes of the tropomyosin and "uncover" the active sites of the actin. This allows contraction to proceed according to the sliding filament model theory of muscle contraction (Ashley et al., 1991). In some non-muscle tissues, Ca\(^{2+}\) sometimes acts as a second messenger by activating various proteins and ion channels which in turn mediates many activities within the cell, such as neurotransmitter release and cell secretion.

1.1.2 Excitation-contraction coupling

In the muscle cell, the cellular mechanisms that link surface membrane excitation with the elevation of cytoplasm Ca\(^{2+}\), and thus, the initiation of contraction are collectively termed excitation-contraction coupling. The contraction of striated muscle is initiated by membrane depolarization. In smooth muscle, the contraction can be initiated by both depolarization and by binding of agonists to the surface membrane receptors. Under physiological conditions, the cytoplasm Ca\(^{2+}\) level in the resting muscle is less than 10\(^{-7}\) M which is more than 10,000 fold lower than that in the extracellular space (greater than 10\(^{-5}\) M) (Rega, 1980). Inside the cell, SR is found to contain a high concentration of Ca\(^{2+}\) (greater than millimolar) (Boudet et al., 1984; Tiro, 1990) which is comparable to the Ca\(^{2+}\) level in the extracellular space. It has been well demonstrated that both Ca\(^{2+}\) channels and Ca\(^{2+}\) pumps are present on plasma membrane (PM) and SR membrane (Tsien and Tsien, 1990; Grover, 1985). Thus, both extracellular space and SR could serve as the source and the sink of Ca\(^{2+}\) for muscle contraction and relaxation. These pumps and channels then
regulate the level of Ca\(^{2+}\) in the cytoplasm during muscle contraction/relaxation. When striated muscle is stimulated, the major source of Ca\(^{2+}\) is that released from SR through its Ca\(^{2+}\) channel during muscle contraction.

However, the exact mechanism by which membrane excitation triggers Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) pool is not clear, but may involve an L-type-Ca\(^{2+}\) channel acting as a voltage sensor. A number of hypotheses have been proposed to account for the coupling of membrane excitation to the elevation of intracellular Ca\(^{2+}\) in skeletal muscle. These proposed mechanisms can be generally divided into two classes. One type of mechanism involves the release of a second messenger following excitation of the plasma membrane, e.g., IP\(_3\) and Ca\(^{2+}\) (Abdel-Latif, 1986; Bentidge 1993). The second type of mechanism relies on a direct structural linkage between a voltage sensor on the surface membrane and a Ca\(^{2+}\)-channel in the SR membrane, by way of a foot protein in skeletal muscle (Ashley et al., 1991). Over several decades, study on excitation-contraction coupling has attracted many investigators. Since skeletal muscle has well-developed SR and skeletal muscle contraction depends mainly on Ca\(^{2+}\) release from SR, skeletal muscle has been commonly used in those studies. During the past 10 years, ryanodine, a plant alkaloid, has been widely used as a tool both to label internal Ca\(^{2+}\)-releasing channels and also to control channel activity (Lai and Meissner, 1989). Since most previous studies of ryanodine receptor were carried out on skeletal muscle, the skeletal muscle structure is first briefly reviewed.

1.2.1 The structure of SR in skeletal muscle

The endoplasmic reticulum (ER) is a tubular and flat vesicular structure present in the cytoplasm. The walls of ER are constructed of lipid bilayer membranes containing large amounts of proteins, similar to the cell membrane (Guyton, 1991). In general, ER has a variety of functions in terms of synthesis of proteins and lipid substances, transportation of some substances and Ca\(^{2+}\) storage. In the muscle cells, the ER network is specifically named sarcoplasmic reticulum (SR). In skeletal muscle, an extensive SR is present in the cytoplasm and has a special anatomical and functional organization that is extremely important in the control of muscle contraction by regulating intracellular Ca\(^{2+}\) level (Figure 1).

In skeletal muscle, SR has been divided into four types according to their structural locations: terminal cisternae, intermediate cisternae, longitudinal cisternae and fenestrated cisternae (Lai and Meissner, 1989). The endfolding of PM into the cells forms transverse (T)-tubules which are closely associated with terminal cisternae. At these T-SR junctions, there is a 12 nm gap separating the two membranes. However, under the electron microscope, this gap can be seen to be crossed at intervals by a large protein structure which has been identified and named the foot structure (Figure 2) (Frazzini-Armstrong, 1970). From the electron-micrographs so viewed by negative staining, the foot structure was identified as four-leaved, square shaped complex which has a dense central core region with an apparent 2-nm hole in the centre (Wagenknetz et al., 1989).

Using differential-speed centrifugation, two distinct types of membrane vesicles,
Figure 2. An illustration of terminal cisternae, T-tubule and foot structure relationships in skeletal muscle (adapted from Ashley et al., 1991).

1.1.2.2 Ca\textsuperscript{2+}-channels in skeletal muscle

Ca\textsuperscript{2+}-channels have been identified in both the plasma membrane (PM) and the SR membrane in skeletal muscle (Tien and Tien, 1990). Using electrophysiological methods, it has been confirmed that Ca\textsuperscript{2+} moves across the surface membrane in depolarized muscle fibers (Ashley et al., 1991). The plasma membrane Ca\textsuperscript{2+}-channel was found predominantly in the T-tubule. It has been shown that glycerol treatment could uncouple the T-tubules electrically from the surface membrane of the muscle fibres (Fujino et al., 1961). The Ca\textsuperscript{2+} current was either reduced greatly or abolished in these detubulated fibres (Nicolás et al., 1980). The depletion of Ca\textsuperscript{2+} in the T-tubules could also decrease Ca\textsuperscript{2+} current (Almers et al., 1981). The presence of Ca\textsuperscript{2+} channel in PM was also demonstrated using a radiolabelled Ca\textsuperscript{2+} channel antagonist, e.g. [\textsuperscript{3}H]PN200-110 (Wilson et al., 1991). However, the Ca\textsuperscript{2+} entry through the surface membrane of skeletal muscle is, in fact, not necessary for twitch activation of contraction. Skeletal muscle fibres are capable of sustained contractile activity in the absence of external Ca\textsuperscript{2+}.

The major source of Ca\textsuperscript{2+} for skeletal muscle contraction is from the SR through Ca\textsuperscript{2+}-release channels. These Ca\textsuperscript{2+}-channels are concentrated in isolated SR in heavy vesicles that are thought to be derived from the terminal cisternae. Ca\textsuperscript{2+} influx from SR vesicles is activated by caffeine, Ca\textsuperscript{2+} and adenosine nucleotides, and is inhibited by Mg\textsuperscript{2+} (Kim et al., 1983; Meissner et al., 1986). Using cyanide as a marker, Ca\textsuperscript{2+}-channels have been isolated from skeletal SR (Lai et al., 1988b) and incorporated into a planar lipid bilayer. Reconstituted channels showed the characteristics similar to those of native...
Ca\(^{2+}\) channels in SR (Details will be described in section 1.3.2). However, certain Ca\(^{2+}\)-
channel blockers, e.g. D600 and dihydropyridines (DHP), blocked contraction resulting
from prolonged membrane depolarization (Ashley et al., 1991). It has been proposed
that normal coupling between the T-tubule membrane and SR Ca\(^{2+}\) release is disrupted by Ca\(^{2+}\-
channel blockers. T-tubule membranes proteins that bind DHP may function as voltage
sensors that link surface membrane activation to release of Ca\(^{2+}\) from the SR (Ashley et
al., 1991). No evidence of a chemical messenger to mediate skeletal muscle contraction
exists.

1.1.3 General structure of smooth muscle cell

Smooth muscle cells are small compared to striated muscle fibres. For their size,
the cell surface areas of smooth muscle cells are extensive. A high surface area-to-volume
ratio is characteristic of smooth muscle cells. On the cell surface, caveolae which are
flask-shaped invaginations of the cell membrane can be observed (Garfield and Somlyo,
1985). The basal lamina does not penetrate into the caveolae but passes over their necks
(the width of the narrowest part of the neck is about 35 nm) without change in appearance.
The cavity of a caveolae is accessible to extracellular space tracers, and the
caveolae are stable structures that are not rapidly internalized into cytoplasmic vesicles.

Between the rows of caveolae, the cell membrane is occupied by dense bodies that
are attachment sites of the contractile apparatus. Between the smooth muscle cells, two
types of junctions are well recognized and are of well-established functional significance.

three types of the SR were proposed: peripheral, intermediate, and central. The peripheral
sarcoplasmic reticulum lies beneath the plasma membrane, both across and along the
longitudinal axis of the cell, forming a network among the caveolae. This peripheral SR
is tubular and occasionally in close contact with the plasmalemma. The intermediate SR
is radially arranged, connecting the peripheral SR with the central SR. The central SR is
located deeply in the central sarcoplasmic axis among the myofilaments. SR identified in
smooth muscle is indeed an intracellular structure different from the caveolae, since it is
not penetrated by the extracellular markers (such as ferritin and colloidal lectinum)
(Devine et al., 1972).

1.2 Intracellular Ca\(^{2+}\) pools in smooth muscle

To study the distribution and properties of Ca\(^{2+}\) handling in smooth muscles, a
large variety of methods has been used in either intact muscle strips or isolated cells.
These methodologies include: techniques of morphology, pharmacological techniques and
subcellular membrane isolation techniques (Daniel et al., 1983). It has been
demonstrated that SR is present in sufficient abundance to serve as an intracellular source
and sink of calcium (van Breemen and Sijtsa, 1989). That smooth muscle contraction can
result from Ca\(^{2+}\) release from intracellular pool has been demonstrated using either skinned
smooth muscle or intact muscle preparation. Ca\(^{2+}\) influx and Ca\(^{2+}\) release were dissociated
by the use of either a Ca\(^{2+}\)-free medium or an inhibitor of Ca\(^{2+}\) influx (e.g. La\(^{3+}\), Ca\(^{2+}\)
channel antagonists). Under the conditions mentioned above, norepinephrine induced a
transient contraction in rabbit aorta which was accompanied by a rapid release of cellular
Ca\(^{2+}\) and an increase in "Ca efflux (Kawasaki and Weiss, 1988; Petersen and Tsien, 1998). 
Caffeine which is known to mobilise internal Ca\(^{2+}\) could also cause a transient contraction
in vascular smooth muscle in the presence or absence of external Ca\(^{2+}\). 

Although the function of SR in smooth muscle is still not fully defined, increasing
evidence suggests that Ca\(^{2+}\) released from SR is directly or indirectly involved in smooth
muscle contraction. Functional conduction between PM and SR has been suggested
recently. Evidence has shown that the emptying of intracellular Ca\(^{2+}\) store and refilling
of the store can facilitate Ca\(^{2+}\) entry into the cells. In rabbit ear artery, the rate of filling
and the maximum degree of filling depend on the external Ca\(^{2+}\) concentrations (Carnesi
and Dougan, 1981; Carnesi et al., 1990). In this tissue, blocking the SR Ca\(^{2+}\) pump
failed to prevent the refilling of an empty norepinephrine-sensitive Ca\(^{2+}\) store (Misia
now, 1989). This suggests a refilling pathway in which no Ca\(^{2+}\) pump is involved.
Evidence was also obtained from the studies in this laboratory. In dog mesenteric artery
(DMA), a store exists which can be refilled in the absence of Ca\(^{2+}\) pumping (Loe et
al., 1992). The refilling of this store could be blocked by the PM Ca\(^{2+}\) channel blocker
nifedipine and enhanced by the PM Ca\(^{2+}\) channel opener Bay KB644 in DMA as well as
dog trachea (Bourgeaud et al., 1991b). However, another Ca\(^{2+}\) store was also identified
that is refilled via a Ca\(^{2+}\) pump in DMA (Low et al., 1992). These studies indicate that
different Ca\(^{2+}\) stores and different refilling mechanisms in smooth muscle cell exist.
1.2.1 Ca\textsuperscript{2+} Channels

Smooth muscle contraction is triggered by the elevation of cytosolic Ca\textsuperscript{2+}. It is generally believed that the increase of Ca\textsuperscript{2+} level involves both Ca\textsuperscript{2+} influx from the extracellular space and Ca\textsuperscript{2+} release from an intracellular pool through different Ca\textsuperscript{2+} channels (Musienska et al., 1992). Ca\textsuperscript{2+} entry via PM Ca\textsuperscript{2+} channels (e.g., after KCl depolarization) can effectively trigger a full contraction in most smooth muscle preparations in the presence of extracellular Ca\textsuperscript{2+}. Three major types of Ca\textsuperscript{2+} channels have been proposed to exist on the PM: voltage operated Ca\textsuperscript{2+} channel (VOCC), receptor operated Ca\textsuperscript{2+} channel (ROCC) and nonselective leak channels (van Breemen and Saida, 1989; Spedding and Pauletta, 1992; Tsien and Tsien, 1990). Among them, the VOCCs have been well characterized for its functional activity with specific pharmacological tools; e.g., Ca\textsuperscript{2+} channel antagonists, such as the dihydropyridine derivatives which block L-type Ca\textsuperscript{2+} channels. The availability of radiolabeled Ca\textsuperscript{2+} antagonists has allowed direct demonstration and detailed characterization of VOCC in some smooth muscles (Wibo et al., 1987; Higo et al., 1988).

The existence of ROCC has been controversial. It has been noted that some agonists could cause the entry of Ca\textsuperscript{2+} into smooth muscle cells followed by contraction without a change of the membrane potential (van Breemen, 1989). However, the lack of a specific tool, e.g., selective antagonist for ROCC, has limited the study of ROCC in smooth muscle, and direct evidence for such a channel is not available. The question is still open whether these channels may belong to the class of nonspecific calcium channels.

Two major Ca\textsuperscript{2+} release mechanisms have been demonstrated in smooth muscle cells: IP\textsubscript{3} induced Ca\textsuperscript{2+} release (IUCR) and Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR) (van Breemen and Saida, 1989). These two Ca\textsuperscript{2+} release mechanisms are believed to be mediated by two separate Ca\textsuperscript{2+} release channels located on SR. The existence of these two types of Ca\textsuperscript{2+} release channels has been demonstrated by a large body of functional experimental evidence. These two Ca\textsuperscript{2+} release channels have been fully characterized in smooth muscle by functional studies. One of them, the IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} channel has been successfully purified (Mourey et al., 1990; Chaddwick et al., 1990).

1.2.1.1 ICR Channel

Insoluble 1,4,5-trisphosphate (IP\textsubscript{3}) is one of the hydrolytic products of membrane phosphoinositide. IP\textsubscript{3} is well known as a principal second messenger linking cell-surface receptors that activate phosphoinositide C and the increase in intracellular free Ca\textsuperscript{2+} that occurs when such receptors are activated (Berridge and Irvine, 1989). The action of IP\textsubscript{3} is terminated by dephosphorylation by IP\textsubscript{3} phosphatase which has been shown to remove the 3-phosphate from IP\textsubscript{3} to produce IP\textsubscript{2} (Connolly et al., 1986). That IP\textsubscript{3} can release Ca\textsuperscript{2+} from the SR and promote smooth muscle contraction has been generally accepted (van Breemen and Saida, 1989; Musienska et al., 1992). IP\textsubscript{3} can activate the Ca\textsuperscript{2+} release via ICR channel on the SR of smooth muscle, leading to the initial, transient phase of Ca\textsuperscript{2+} mobilization.

In the muscle preparation, the use of detergents (e.g., saponin, digitonin) to permeabilize the PM while preserving SR function (skinned smooth muscle cell) (Saida and Naramura, 1978) allowed the demonstration of direct effects of IP\textsubscript{3} on a Ca\textsuperscript{2+} release channel on SR. The addition of IP\textsubscript{3} to skinned vascular smooth muscle preparation resulted in Ca\textsuperscript{2+} release and muscle contraction (Sasazuki et al., 1985). IP\textsubscript{3} has also been shown to stimulate Ca\textsuperscript{2+} release from arteric SR vesicles (Watanabe and Benovolensky, 1987). Light activation of cyclic IP\textsubscript{3} predisposed into muscle cells, showed that it rapidly released SR Ca\textsuperscript{2+} at submicromolar concentrations (Waller et al., 1987).

The existence of a IP\textsubscript{3} sensitive Ca\textsuperscript{2+} channel has been demonstrated in smooth muscle. High densities of [H]IP\textsubscript{3} receptor binding sites in the vas deferens and other smooth muscle have been reported as shown in table 1 (Hashimoto et al., 1986; Erlich and Watanas, 1988; Iino et al., 1988; Vesperinas et al., 1989). Recently, this IP\textsubscript{3} receptor protein has been purified from bovine sartorius smooth muscle microsomes (Chaddwick et al., 1990) and from rat vas deferens (RVD) smooth muscle membranes (Mourey et al., 1990). In a crude microsomal fraction of RVD, a high affinity IP\textsubscript{3} binding site was identified with $K_d = 10.4 \text{ nM}$ and $B_{max}$ of 3.7 pmol/mg (Mourey et al., 1990). This binding protein was then purified 65 fold without changing its affinity for IP\textsubscript{3}, binding. The purified IP\textsubscript{3} receptor is a protein with M, 260K. Da. IP\textsubscript{3} binding to RVD receptor was optimal in alkaline conditions and could be completely inhibited by 20 µg/ml heparin. Although Ca\textsuperscript{2+} inhibited [H]IP\textsubscript{3} binding in brain, it had no effect on [H]IP\textsubscript{3} binding to RVD smooth muscle preparations. The receptor purified from bovine sartorius has similar properties to the RVD IP\textsubscript{3} receptor, similar abundance ($B_{max}$) and $K_d$, and similar inhibition by heparin.

The sartorius IP\textsubscript{3} receptor has a lower molecular mass (224K Da) than that of RVD IP\textsubscript{3} receptor. When reconstituted into a planar bilayer, the purified sartorius IP\textsubscript{3} receptor constitutes an IP\textsubscript{3} gated ion channel (Erlich and Watanas, 1988; Meyrleiner et al., 1991). This channel could be activated specifically by phorbol ester relevant concentrations of IP\textsubscript{3}. Low concentrations of heparin, shown by Kobayashi et al., (1988) to inhibit IP\textsubscript{3} actions, blocked the IP\textsubscript{3}-gated channel current.

A cDNA encoding the cerebellar IP\textsubscript{3} receptor has been successfully cloned and sequenced (Mignery et al., 1990). The amino acid sequence of the IP\textsubscript{3} receptor has significant homology with that of the skeletal muscle ryanodine receptor. The regions near the COOH terminus and in four of the transmembrane regions in the COOH-terminal part of the two receptor proteins share the most similarity. The amino acid sequences of the cardiac and skeletal muscle ryanodine receptor also share more than 80% homology in the same regions where similarities are observed between the primary sequences of the skeletal muscle ryanodine receptor and the IP\textsubscript{3} receptor. These conserved regions might play an important role in the function of all three Ca\textsuperscript{2+} release channels.
1.2.1.2 CICR channel

The CICR mechanism was initially demonstrated in skeletal muscle (Ford and Podolsky, 1970). In smooth muscle, the CICR mechanism has also been demonstrated using skinned smooth muscle preparations (Boeh et al., 1981; Sales, 1982; Strat and Dixon, 1983). The threshold for Ca\(^2\) to initiate Ca\(^2\) release through this channel was estimated to be in the low micromolar range of Ca\(^2\) (Boeh et al., 1981; Sales, 1982).

Propranolol and ruthenium red block CICR while eAMP potentiates this release (Sales and van Breemen, 1984). Caffeine releases SR Ca\(^2\) by facilitating CICR and lowering the concentration of Ca\(^2\) (10\(^{-8}\) M) required to initiate Ca\(^2\) release as shown in guinea pig mesenteric artery (Boeh et al., 1981). Caffeine can increase the affinity of Ca\(^2\) binding to the CICR Ca\(^2\) channel. Recent studies demonstrate that ryanodine can selectively act on CICR channels in both striated muscle and smooth muscle. A detailed review will be provided later (See: 1.3.2, 1.3.3, and 1.3.5).

1.2.2 Ca\(^2\) pumps

The contraction-relaxation cycle of smooth muscle, the removal of Ca\(^2\) from the cytosol is another important step. Two distinct forms of Ca\(^2\)-transport ATPase (Ca\(^2\) pump) have been demonstrated, one localized in the plasma membranes and another in the SR membrane (Groves, 1985; Eggemont et al., 1988). They are responsible for the Ca\(^2\) extrusion to the extracellular space and Ca\(^2\) recycling into the SR respectively.

The evidence of the presence of Ca\(^2\)-pumps in both PM and SR comes from studies of ATP-dependent, azide insensitive Ca\(^2\) uptake by subcellular membrane vesicles.

Two modes of Ca\(^2\) uptake were distinguished. One, which was azide-insensitive, correlated well with PM markers. Another, which was azide-activated, did not correlate with plasma membrane markers (Daniel et al., 1983). It has been noted that in skeletal muscle SR the Ca\(^2\) uptake was azide-stimulated, whereas in the erythrocytes, lacking SR, Ca\(^2\) uptake was azide-insensitive (Hassall, 1978; Sarkadi, 1980).

The PM Ca\(^2\)-pump in smooth muscle is similar to the erythrocyte type Ca\(^2\)-transport ATPase (Eggemont et al., 1988). It is a 140K Da calmodulin binding protein. The Ca\(^2\)-pump associated with SR is a 105K Da protein (Eggemont et al., 1988; Rasmussen et al., 1983). Both polyclonal and monoclonal antibodies raised against the 140K Da Ca\(^2\)-transport ATPase inhibited (Ca\(^2\)-Mg\(^2\))-ATPase activity and reduced the Ca\(^2\) uptake in the purified PM fraction. However, these antibodies hardly affected the Ca\(^2\) uptake and the (Ca\(^2\)-Mg\(^2\))-ATPase activity in the SR fraction (Verlot et al., 1985; Verlot et al., 1986).

The ratio of the number of PM Ca\(^2\)-pump to the SR Ca\(^2\)-pumps is different among different types of smooth muscle. For example, a relatively higher amount of SR Ca\(^2\)-ATPase than PM Ca\(^2\)-ATPase was found in bovine aorta and main pulmonary artery smooth muscle. In contrast, porcine stomach smooth muscle contains a higher content of PM Ca\(^2\)-ATPase (Eggemont et al., 1988). This indicates that the contribution of each Ca\(^2\) pump to the regulation of the intracellular Ca\(^2\) level may vary in different species and types of smooth muscle.

1.3 Ryanodine

1.3.1 General introduction

Ryanodine, a neutral alkaloid, was originally extracted from the stem and root of the plant ryania species (Jenden and Fairhurst, 1969). It was characterized as early as 1948 following its isolation and crystallization. Ryanodine, C\(_8\)H\(_{16}\)NO\(_4\) is freely soluble in ethanol, chloroform, acetone, ether and water. It is stable in neutral aqueous solution and will withstand prolonged boiling (Jenden and Fairhurst, 1969). There are two major active components of this alkaloid: ryanodine and 9,21-dihydroxyryanodine. Their structures are shown in figure 3. In a functional study using guinea pig aorta (Ito et al., 1989), these two compounds were shown to have similar effects on caffeine-induced Ca\(^2\) release. They also had similar characteristics, such as dose dependence and the effects on intracellular Ca\(^2\). Ryanodine was originally used as an insecticidal agent. After it was shown to have profound effects on muscles from a variety of vertebrates and invertebrates, many investigators were attracted to study its pharmacological properties (Jenden and Fairhurst, 1969). During the first 20 years after ryanodine was isolated, the pharmacological effects of ryanodine were most clearly shown in muscle. These effects of ryanodine varied with species and types of muscle. In vertebrate skeletal muscle, ryanodine induced a slow irreversible contraction (Jenden and Fairhurst, 1969). The maximal effect was reached within 30-60 min with 10\(^{-6}\)-10\(^{-9}\) M ryanodine in most vertebrate skeletal muscles. In
Contrast to its effects on skeletal muscle, ryanodine has a negative inotropic effects on mammalian cardiac muscle, i.e. ryanodine caused a progressive decline in contractile force (Jarden and Fairstar, 1969).

Ca²⁺ transport was thought to be the key step on which ryanodine acted to affect muscle contraction. However, the exact site of action was not clear. During the 1980s, major clarification occurred from studies of the sites of interactions in various muscle types using Radiolabelled ryanodine and studies on the purified ryanodine receptor (Lai and Melzner, 1989). The study of the ryanodine receptor has been most complete in skeletal and cardiac muscle. In striated muscle, it is now well accepted that ryanodine binds to the SR Ca²⁺-channel which forms a complex with the foot structure (Ashley et al., 1991). The ryanodine receptor has also been identified and purified from non-muscle cells, e.g. brain (McPherson et al., 1991) and even from non-excitable cells, e.g. liver (Shoshan-Barmatz et al., 1991).

1.3.2 The ryanodine receptor in striated muscle

1.3.2.1 Radioligand binding studies

The existence of specific intracellular ryanodine receptors was initially inferred from the structure-activity relationships for ryanodine-stimulated Ca²⁺ mobilisation. Confirmation came from purification and functional reconstitution of these receptors. Radioligand binding studies formed an essential part of this substantial progress and they will continue to contribute to our understanding of the relationship between receptor occupancy and response.

The first radiolabelling of ryanodine was reported by Fairstar using the bromination method to label the pyrrole ring shown in figure 3 (Fairstar, 1971; Fleischer et al., 1985). Now, the tritiated ryanodine has become commercially available with a usable specific activity. This enabled people to identify and purify ryanodine receptors from striated and some non-muscle tissue. The first report of ryanodine binding site in SR appeared in 1985 (Pessah et al., 1985). [³H]ryanodine was shown to bind specifically to heavy SR membranes from skeletal and cardiac muscle with high affinity in a Ca²⁺ dependent manner. An additional lower affinity [³H]ryanodine binding site was also found to be present in cardiac muscle preparations (table 2). Subsequently, such a low affinity binding site was also found in skeletal muscle (table 2). Some discrepancies summarized in this table may reflect merely the very different conditions under which the membrane preparations and radioligand binding assays were performed.
This possibility is supported by the fact that many factors may affect ryanodine binding to its receptors. The binding affinity and/or receptor site density of [3H]ryanodine in striated muscle SR were increased by iontrophic gramicidin, caffeine and adenosine nucleotides and decreased by Mg++ and ruthenium red (Pennah et al., 1987; Inagawa et al., 1987; Michalk et al., 1988).

The poor reversibility of ryanodine binding has been demonstrated by many studies (Lai et al., 1988). In kinetic studies, ryanodine binds to the receptor very slowly. The maximum binding required at least 2 hours. The association of [3H]ryanodine from the high-affinity binding site initiated by excess amounts of unlabelled ryanodine was very slow and incomplete. However, ryanodine could be dissociated from the high-affinity binding site faster when dissociation was initiated by a 20-fold dilution in the absence of unlabelled ryanodine. These phenomena were interpreted as the presence of a low-affinity binding site in addition to the high-affinity binding site in skeletal muscle (Lai et al., 1989). The occupation of the low-affinity binding site by unlabelled ryanodine appeared to cause a conformational change of ryanodine receptor and lock in the [3H]ryanodine bound to the high-affinity sites, thus preventing its dissociation from the receptors. Similar low-affinity binding sites were also identified in both skeletal and cardiac muscle by the method of saturation experiments (table 2). It has also been proposed that there are multiple conformational states of the ryanodine receptor or multiple binding sites for ryanodine binding (Book et al., 1992).

The ryanodine receptors from skeletal and cardiac muscle SR share many similarities. The SR from both muscle types contains high and low affinity binding sites. They both have a slow association profile and poor reversibility. However, some differences have also been noted between the skeletal muscle ryanodine receptor and that of cardiac muscle. The cardiac ryanodine receptor is apparently more sensitive to activation by Ca++ than the skeletal ryanodine receptor (Zimanyi and Pennah, 1991) and less sensitive to inhibition by Mg++ than the skeletal muscle form (Michalk et al., 1988; Zimanyi and Pennah, 1991).

1.3.2.2 Purification of the ryanodine receptor

The radioligand binding experiments have provided direct evidence of the existence and the location of ryanodine receptor in striated muscle. The purification was facilitated by the finding that ryanodine receptor could be solubilized from skeletal and cardiac SR membranes in a buffer containing the detergent Chaps and high salt concentrations (Pennah et al., 1986). Ryanodine receptors were then successfully purified by different methodologies, including: sequential column chromatography (Inui et al., 1987), immunofinity chromatography (Smith et al., 1988), and density-gradient centrifugation (Lai et al., 1988b).

The purified ryanodine receptor in skeletal muscle was identified as a high-molecular weight protein of M, 350-450K Da in the form of an oligomeric complex of apparent sedimentation coefficient 30S. The cardiac ryanodine receptor has also been purified which comprised a single high-molecular-weight protein of M, 400K Da which sedimented as a large complex of 30S (Lai et al., 1988a).

The purified ryanodine receptor from skeletal muscle bound [3H]ryanodine with high-affinity, similar to that found using crude membrane preparations (table 2). The ryanodine binding capacity to the purified receptor was dramatically increased with maximum binding site density (Bmax) of up to 500 pmol/mg protein. High-affinity ryanodine binding to the purified receptor depended on Ca++ in micromolar range as observed for the membrane-bound receptor (Lai and Meissner, 1989). [3H]ryanodine bound to purified receptor from cardiac muscle with both high and low affinities (table 2) (Inui et al., 1987).

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mg)</th>
<th>Kd2 (pmol/mg)</th>
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<tr>
<td>Cardiac</td>
<td>36</td>
<td>0.49</td>
<td></td>
<td></td>
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<tr>
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<tr>
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<td>Cardiac</td>
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<td>47400</td>
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<tr>
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<td>81.5</td>
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<tr>
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<tr>
<td>Cardiac</td>
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<tr>
<td>Skeletal</td>
<td>11.8</td>
<td>13</td>
<td>118</td>
<td>16.5</td>
</tr>
</tbody>
</table>

1: micromoles, 2: purified protein, 3: (Ca++/100M), 4: (Ca++/100M), ND: not detected.
1.3.2.3 Structure of ryanodine receptors

Following its purification, the detailed structure of the ryanodine receptor has been observed using freeze-fracture electron-micrographs. The shape of the purified ryanodine receptor was shown to be similar to that of the foot structures that bridges the gap between the SR and T-tubule membranes in skeletal muscle (Lai et al., 1988b). The receptor is a square particle made up of four globular subunits. The sides of the square are 26 nm and there appears to be a 2 nm hole in the centre (Lai et al., 1988b). Another four holes were also seen around the centre hole. The receptor is inserted into the SR membrane such that the square can be seen on the face of the membrane (Figure 2).

Molecular biological techniques in receptor studies have greatly facilitated the understanding of structural properties of ryanodine receptor and its significance for the receptor function. Cloning and sequencing analyses of cDNAs have revealed the primary structure of the ryanodine receptor from skeletal muscle (Takahama et al., 1989; Zorzato et al., 1990) as well as from heart (Ono et al., 1990) and from brain (Hakimzadeh et al., 1992). Studies of the expression of this receptor suggest the involvement of various regions of receptor molecules in receptor functions such as ligand binding, and receptor regulation.

As suggested by the deduced sequence from cDNA cloned from rabbit skeletal muscle, the ryanodine receptor protein consists of a sequence of 5,032 amino acids (Takahama et al., 1989). The calculated Mr of the ryanodine receptor is 565,223 Da. However, the human ryanodine receptor cDNA sequence, which has also been cloned, encoded a protein of 5,032 amino acids with a molecular weight of 565,584 Da (Zorzato et al., 1990). In the later study, major differences were found between the sequences of rabbit skeletal muscle and human skeletal muscle in the region between residues 1872 and 1923.

Four positively transmembrane segments (named M1, M2, M3 and M4) in the C-terminal of ryanodine receptor were suggested (Takahama et al., 1989). It is also suggested that the C-terminus encompasses the channel-forming region and is responsible for the Ca\(^{2+}\) channel activity. The regions which are assigned to the cytoplasmic side of the SR membrane and are close to segment M1 are proposed to be the binding sites of modulators, e.g. Ca\(^{2+}\), adenine nucleotides, caffeine, calmodulin etc. (Figure 4). The rest of the molecule is cytoplasmic and lies between the T-tubule and SR membranes and thus is the foot region seen in electron micrographs. Computer survey revealed that the sequence of the ryanodine receptors was different from that of other channels or proteins of known sequence (Takahama et al., 1989; Zorzato et al., 1990) although they shared some similar sequences with the IP\(_3\) receptor (Miyagawa et al., 1989). The expression of the cDNA encoding the ryanodine receptor in Chinese hamster ovary cells yielded a protein having identical properties to the ryanodine receptor (Penzner et al., 1989). The purified ryanodine receptor protein has molecular mass of 400-450kDa (a monomer). It has been suggested that ryanodine receptor form a complex of four monomers (one tetramer) in the membranes.

As suggested by biochemical and physiological studies, the cardiac and skeletal muscle ryanodine receptors are similar but not identical. In an immunological study, some antibodies that recognized the cardiac receptor on immunoblot analysis did not recognize the protein in the microsomes isolated from skeletal muscle (Imagawa et al., 1989). This is confirmed by comparison of the amino acid sequence of the skeletal and cardiac ryanodine receptors. The rabbit cardiac muscle ryanodine receptor is composed of 4,969 amino acids with M\(_r\) 564,711 Da (Ono et al., 1990). This is 63 amino acids shorter than the skeletal muscle isoform. The amino acid sequence of the rabbit cardiac ryanodine receptor shows 60% overall identity with that of the skeletal form (Ono et al., 1990; Nishizumi et al., 1990). These two forms of the ryanodine receptor are derived from two different genes. The cardiac receptor gene is located on human chromosome 1, and the skeletal muscle receptor gene is on chromosome 19 (Mochimaru et al., 1990).

1.3.3 Ryanodine receptor in other tissues

1.3.3.1 Ryanodine receptor in nerve tissue

As mentioned before, Ca\(^{2+}\) plays several important roles in regulating various cellular functions and Ca\(^{2+}\) release from intracellular stores is a major source of cytoplasmic Ca\(^{2+}\) in some tissues. In nerve tissues, the release of transmitter at the motor nerve terminal is mediated by Ca\(^{2+}\) elevation in the nerve ending cytoplasm. A growing body of evidence indicates that the nerve terminal has some sites to store Ca\(^{2+}\) (Blatstein et al., 1980). At mouse neuromuscular junctions, ryanodine has been demonstrated to facilitate Ca\(^{2+}\)-dependent release of transmitter (Nishizumi et al., 1990).

Using caffeine, studies in central and peripheral neurons have demonstrated the presence of intracellular Ca\(^{2+}\) store sites similar to the SR. Possible roles of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in raising intracellular free Ca\(^{2+}\) concentrations were also suggested (Neuring and McBurney, 1984; Thayer et al., 1985). As in the muscle cells, several lines of evidence suggest that the ryanodine receptor is involved in Ca\(^{2+}\) signalling within neurons. For example, ryanodine has been found to inhibit the Ca\(^{2+}\) release from the caffeine-sensitive Ca\(^{2+}\) store sites in sympathetic ganglia (Kawai and Watanabe, 1989; Thayer et al., 1988) and sensory neurons (Thayer et al., 1988). Ryanodine binding sites have also been extensively identified and studied in neuronal tissues and the receptor protein has been recently purified and cloned (McPherson et al., 1991; Hakimzadeh et al., 1992).

In redisoligand binding studies, high affinity \(^{3}H\)ryanodine binding sites were identified in rat brain microsomes (Ashby, 1989). The affinity of this binding site is within the range of that of striated muscle. The maximum binding of ryanodine to the brain microsomes is much less than that of striated muscle, possibly due to the smaller content of ER in brain. \(^{3}H\)Ryanodine binding was further characterized in the rabbit cerebral microsomes (Kawai et al., 1991) and both high and low affinity binding sites were identified. \(^{3}H\)Ryanodine binding to the rabbit cerebral microsomes was Ca\(^{2+}\)-dependent and significantly increased by AMP-PNP (5'-adenylylimidodiphosphate) and caffeine. Mg\(^{2+}\) and ruthenium red could inhibit \(^{3}H\)ryanodine binding. The specific ryanodine binding in cerebral microsomes was not affected by 10 \muM IP\(_{3}\) in the absence or presence.
of 1 mM AMP-PPNP at 50 μM Ca\(^{2+}\) (Kawai et al., 1991). The brain ryanodine receptor has been purified 6000-fold with no change in \(^{3}H\)ryanodine binding affinity (McPherson et al., 1991). This receptor contains a 50k Da protein subunit, which is identified by anti-peptide antibodies against the skeletal and cardiac muscle ryanodine receptor. When incorporated into a lipid bilayer, this ryanodine receptor showed Ca\(^{2+}\) channel activity which is ryanodine and caffeine sensitive and IP\(_{3}\) insensitive.

The brain ryanodine receptor is smaller than that from both skeletal and cardiac muscle. It is composed of 4872 amino acids with M, 551,901 Da (Nakamura et al., 1992). Between brain-skeletal muscle and brain-cardiac muscle, ryanodine receptors share 70% and 67% identities in amino acid sequence respectively. As in skeletal muscle, both cardiac and brain ryanodine receptor consist of two main parts. One consists of the highly hydrophobic segments (M1, M2, M3 and M4) in the carboxyl-terminal half of the molecule. The large remaining cytoplasmic region corresponds to the foot structure. The potential ligand binding sites are also located on the putative cytoplasmic side.

1.3.3.2 Ryanodine receptor in liver

In rat liver, \(^{3}H\)ryanodine bound to high affinity binding sites in microsomal subfractions with a \(K_a\) of 10 nM and \(B_{max}\) of 200 fmol/mg of protein (1200 fmol/mg by centrifugation method) (Sheshou-Banetz et al., 1991). The specific binding reached equilibrium in 1-2 minutes. \(^{3}H\)Ryanodine was also rapidly dissociated from its binding sites by addition of excess unlabelled ryanodine at 37 °C (approximately 30 seconds).

\(^{3}H\)Ryanodine binding in liver was Ca\(^{2+}\) independent. The binding could be slightly stimulated by NaCl, Mg\(^{2+}\), ATP and IP\(_{3}\), but strongly inhibited by caffeine.

Other than the above-mentioned tissues, ryanodine was also found to be involved in the Ca\(^{2+}\) mediated activity in some other tissues which include endothelial cell (Leah et al., 1992) and sea urchin egg (Gallone et al., 1991). Using molecular biological techniques, it was demonstrated that two different types of ryanodine receptors were present in different tissues: RY1 and RY2 (Sorrentino and Volpe, 1993; McPherson and Campbell, 1993). Now, increasing evidence indicates that a third form ryanodine receptor, RY3, is present in many different types of tissue including smooth muscle and some non-muscle type tissues (Sorrentino and Volpe, 1993; McPherson and Campbell, 1993), (table 3).
Table 3. Distribution Of Expression Of Three Ryanodine Receptor Genes

<table>
<thead>
<tr>
<th>Tissues</th>
<th>RYA1</th>
<th>RYA2</th>
<th>RYA3</th>
</tr>
</thead>
<tbody>
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<td>Skeletal muscle</td>
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<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>-</td>
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<td>Kidney</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Brain</td>
<td>+</td>
<td>+++</td>
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<td>Heart</td>
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(Adapted from Sorrentino and Volpe, 1993.)

3.3.4 The functional effects of ryanodine on striated muscle

Ryanodine can cause irreversible skeletal muscle contraction or cardiac muscle relaxation (Jarden and Fairhurst, 1969). This could be due to either blocking of the Ca²⁺ release channel on the SR or depleting Ca²⁺ from the SR Ca²⁺ pool by opening this channel. Both radioligand binding studies and functional studies suggested that ryanodine bound preferentially to the Ca²⁺ channel which was in the open state (Michalk et al., 1988; Ito et al., 1988). This preference of ryanodine for the open channels is supported by the fact that many factors which activate the Ca²⁺ channel can also increase ryanodine binding to the receptor; e.g. Ca²⁺, caffeine and ATP (Panah et al., 1987; Imanaga et al., 1987; Michalk et al., 1988). It has been proposed that at low concentrations, ryanodine locks the Ca²⁺ channel in the open state after binding. High concentrations of ryanodine appear to completely close the channel (Lai and Melaner, 1989). These hypotheses are supported by studies in skeletal and cardiac muscle using different approaches. In studies of Ca²⁺ loading and Ca²⁺ release in isolated heart SR preparations, the apparently paradoxical observed effects of ryanodine have been interpreted to be due either to activation or inhibition of a Ca²⁺ permeable pathway depending on the ryanodine concentration used (table 4).

An exciting development was the incorporation of the isolated receptor protein into planar bilayer to give channels which were identical to the native Ca²⁺ channels on SR, heavy vesicles (Imanaga et al., 1987; Lai et al., 1988b; Smith et al., 1988). The receptor protein acts as a Ca²⁺ channel with properties of conductance and gating behavior similar

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Method</th>
<th>Effects of ryanodine</th>
<th>references</th>
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<tbody>
<tr>
<td>Skeletal</td>
<td>SR vesicle</td>
<td>0.01-1μM increased Ca²⁺ release</td>
<td>Failure and Hasselhöf, 1970</td>
</tr>
<tr>
<td>Cardiac</td>
<td>SR vesicle</td>
<td>40μM inhibited CICR</td>
<td>Chamberlin et al., 1984</td>
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<tr>
<td>Cardiac</td>
<td>SR vesicle</td>
<td>20-50μM Ca²⁺ uptake</td>
<td>Peter and Lipford, 1985</td>
</tr>
<tr>
<td>Skeletal</td>
<td>SR vesicle</td>
<td>0.01-1μM increased Ca²⁺ release</td>
<td>Melaner, 1986</td>
</tr>
<tr>
<td>Cardiac</td>
<td>SR vesicle</td>
<td>0.01-1μM increased Ca²⁺ release</td>
<td>Lattanzo et al., 1987</td>
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<td>Cardiac</td>
<td>SR vesicle</td>
<td>20-30μM increased Ca²⁺ release</td>
<td>Peter et al., 1988</td>
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<td>Skeletal</td>
<td>Single channel</td>
<td>30μM block sub.</td>
<td>2mM block</td>
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<tr>
<td>Cardiac</td>
<td>Single channel</td>
<td>1-5μM block sub.</td>
<td>50μM increased Ca²⁺ uptake</td>
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<tr>
<td>Skeletal</td>
<td>Single channel</td>
<td>10μM block sub.</td>
<td>1mM block</td>
</tr>
<tr>
<td>Aorta</td>
<td>Single channel</td>
<td>1mM block</td>
<td>1mM block</td>
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back sub.: locked in the subconductance state.
block: fully closed the channel.
single channel: recombinating purified receptor into lipid bilayer and recorded ion current.
1.3.5 The functional effects of ryanodine on smooth muscle

Although ryanodine receptors have been purified and fully characterized in some tissues, the study of this receptor in smooth muscle has been mostly limited to functional studies. Although results derived from different studies have been inconsistent and controversial, ryanodine has also been widely shown to affect the mechanical activity and the Ca²⁺ mobilization by smooth muscles. Io et al. (1986) reported that ryanodine inhibited SR Ca²⁺ release through a CICR Ca²⁺-channel. They found that 30 μM ryanodine inhibited both norepinephrine (NE) and caffeine-induced rise contractions of guinea pig aortic smooth muscle and prevented Ca²⁺ efflux from this tissue into Ca²⁺ free medium induced by NE and caffeine. However, some subsequent reports showed that ryanodine may depolarize the Ca²⁺ store rather than inhibit Ca²⁺ release. In rabbit aortic smooth muscle, ryanodine also reduced the NE-induced contraction and NE-stimulated Ca²⁺ efflux rate. However, ryanodine alone stimulated Ca²⁺ efflux and decreased net cellular Ca²⁺ content in smooth muscle (Hwang et al., 1987; Kanzawa et al., 1988). In rat aorta, ryanodine alone with 1.25 mM Ca²⁺ induced a slowly developing rise in tension (Jalsu-Schaeffer and Freiston, 1988). The increased Ca²⁺ release from SR was also demonstrated using Fura-2 fluorescence technique in aorto vein smooth muscle (Eirne et al., 1988). In isolated bovine and porcine coronary artery smooth muscle cells, ryanodine also caused [Ca²⁺]i increase measured by fura-2 (Wagner-Mann et al., 1992). Ryanodine did not change [Ca²⁺]i when muscle cell was pretreated with caffeine. These results suggested that ryanodine caused Ca²⁺ release from intracellular stores which is caffeine-sensitive. Other tissue which have been reported to be affected by ryanodine include: guinea pig portal vein, pulmonary artery and tunica media (Liao et al., 1988), tracheal smooth muscle (Gethoff and Lipton, 1983; vas deferens (Kobay et al., 1988; Vesperina, 1989) as shown in table 5.

However, unlike in striated muscle, the dual effects of ryanodine on Ca²⁺ channel in smooth muscle have not been directly observed; e.g. ryanodine inhibiting Ca²⁺-channel at higher concentrations and locking Ca²⁺-channel in the subconductance state at lower concentrations. Based on the finding from striated muscle, the effect of ryanodine on smooth muscle has been mostly interpreted to involve locking the Ca²⁺ channels in the open state between the concentration of 10-30 μM. In support of this, ryanodine has been shown to act on the CICR channels only when it is open in skinned fibre of tunica and vascular smooth muscle (Liao et al., 1988). In this study, after its first application, ryanodine did not affect either Ca²⁺ movement measured by fura-2 or smooth muscle contraction. However, if the muscle fibre was treated with caffeine or Ca²⁺, both of which open CICR channels, ryanodine abolished Ca²⁺ release or muscle contraction induced by caffeine but contraction still occurred to a high-K solution. That ryanodine depleted Ca²⁺ store could be further supported by the fact that after treatment with ryanodine in the presence of Ca²⁺, A23187 could not release any Ca²⁺ from the stores (Kanzawa et al., 1988). This indicates that no Ca²⁺ was left in the store after ryanodine treatment, since A23187 releases the stored Ca²⁺ by making membranes permeable to Ca²⁺. However, the ability of ryanodine to deplete intracellular Ca²⁺ stores might be exploited by inhibition of the SR Ca²⁺ pump as well as by opening of CICR channels (see 5.3).
1.3.6 The relation of IP3 and ryanodine sensitive Ca2+ release channels in smooth muscle

As mentioned before, there are two major Ca2+ release channels in SR in smooth muscle: the CICR Ca2+ channel and the IP3-sensitive Ca2+ channel. The locus of ryanodine's effect on smooth muscle has been demonstrated to be the CICR Ca2+ channel on SR. Evidence has been provided in guinea pig taenia smooth muscle that there are two types of Ca2+ pools in smooth muscle, one with both CICR and ICR channels and one with only ICR channels (Lino et al., 1988). In this instance, the amount of Ca2+ released by the application of IP3 was about twice as large as that by caffeine alone before the ryanodine treatment. The pretreatment of ryanodine could partially decrease IP3 induced Ca2+ release but abolished caffeine induced Ca2+ release. The fraction of intracellular Ca2+ pool containing both CICR and ICR channels was proposed to be: about 40%, 5% and 60% in taenia caeci, portal vein and pulmonary artery respectively. The caffeine-sensitive store has also been proposed to be part of a larger IP3-sensitive store in rat aorta preparation (Nagana and D'Ocón, 1992). However, a controversial result was also obtained in vascular smooth muscle fibres that the IP3-sensitive store may be part of the caffeine-sensitive store (Hwang and van Beurman, 1987; Kaumsma and al., 1998). The degree of overlap between Ca2+ pools containing ICR and/or CICR is not clear. It may vary between different types of smooth muscle or different species. The procedures for evaluating overlap all assume that ryanodine only affects CICR channels and do not consider the possibility that it may inhibit the SR-Ca2+ pump (see 5.3).

In smooth muscle, the functional effects of ryanodine on SR CICR Ca2+ channel were studied mostly in intact muscle strips or in skinned muscle preparations. Unlike the findings in striated muscle, reports showing the dual effects of ryanodine in smooth muscle are sparse. Among the functional experiments, the ryanodine concentrations used were in the range of 1 to 30 μM. Most experiments showed that ryanodine depleted Ca2+ from smooth muscle SR possibly by locking CICR Ca2+ channel in the open state, except for the report from Ho et al. (1986) showing that ryanodine blocked this channel in the guinea pig aortic smooth muscle. However, the only direct measurement of the effect of ryanodine on CICR Ca2+ channel showed that this channel was fully blocked by 1 mM ryanodine, while ryanodine at lower concentration had no effect on the Ca2+ channel (Herrmann-Frank et al., 1991).

Due to these controversial findings, the mechanism of action of ryanodine on smooth muscle is still not fully understood. More investigations are required to solve this issue. In this laboratory, the method for Ca2+ transport study using subcellular membrane vesicles has been well established. The microsomal fraction obtained from RVD smooth muscle by differential speed centrifugation has been shown to have strong ability to accumulate Ca2+ in the presence of ATP. However, due to the small content of SR in smooth muscle, the microsomal fraction may contain the vesicles mainly derived from plasmalemma. In order to increase the Ca2+ uptake signal by SR vesicle, potassium oxalate can be present in the Ca2+ uptake experiments. Potassium oxalate has been shown to selectively penetrate into the vesicle derived from SR (Greger and Kwan, 1984), complex.
2. TECHNICAL CONSIDERATIONS AND EXPERIMENTAL DESIGN

2.1 Subcellular membrane fractionation

Ligand binding is one of the most common approaches to the identification and characterization of receptors. Binding studies on broken cell preparations from tissues of smooth muscle may provide direct evidence for the presence and location of the receptor in the cell. In the present study, the method of subcellular fractionation was used to separate and purify various cellular organelles or their components, e.g., plasma membranes, SR, mitochondria.

The techniques for subcellular membrane isolation have been well developed in this laboratory (Kwan et al., 1983a, Kwan et al., 1983b). The broken cellular membranes of varied size and density can be separated by different speeds of centrifugation. Further centrifugation of the membrane on a discontinuous sucrose gradient can also yield more enriched single membrane fractions because there are characteristic differences of the densities of SR and PM membranes. To define the components obtained by the procedures mentioned above, a number of marker enzymes were employed. The methods of the assay and the distribution of these enzymes have been previously reported.

from this laboratory also showed a very large catecholamine-stimulated Ca²⁺ uptake by SR derived from RVD smooth muscle. RVD may have a well-developed SR system for intracellular Ca²⁺ accumulation (Grove and Kwan, 1984). Therefore, RVD was chosen as the primary tissue for the initial characterization of ryanodine receptors and study of the effect of ryanodines on Ca²⁺ transport.

Most smooth muscle tissues are innervated by autonomic nerves. However, the density of nerve fibers and varicosities varies considerably between tissues. RVD is one of the highly innervated smooth muscles. In RVD smooth muscle, each cell is thought to be associated with one or more nerve terminals (Richardson, 1962). Some other smooth muscle tissues, e.g., saccic smooth muscle, are more sparsely innervated and varicosities contact only a small percentage of the smooth muscle cells in the muscle bundle. A specific ryanodine binding site has been demonstrated in neurons and the receptor protein has been purified from this tissue (Ashby, 1989; McPherson et al., 1991). Therefore one of the goals of this study was to clarify whether ryanodines in rat vas deferens binds to smooth muscle or neuronal tissue, and whether the density of the ryanodine binding site is related to the innervation of different smooth muscles. In this laboratory, [³H]ryanodine binding assay has been used to identify nerve varicosities amongst smooth muscle membranes (Ahmad et al., 1988; Kurota et al., 1987), so this technique was used in the present study.

2.2 Structure of vas deferens

The vas deferens are direct continuation of the ducts of the epididymis in the male genital system. The whole length of vas deferens can be divided into two portions: the prostatic and the epididyral parts. The wall of the vas deferens consists of several layers: (from inside to outer layer) epithelium; inner longitudinal, circular and outer longitudinal muscle layers; and mesenteric or connective tissue layer. Smooth muscle makes up about 70-75% of the vas deferens cross-sectional area (Cohen et al., 1979). The outer longitudinal muscle layer comprises 32-60% of the total vas deferens cross-sectional area, whereas the inner longitudinal plus and circular muscle layers make up 39-59% of the total vas deferens cross-sectional area. Previous studies showed that a relatively higher smooth muscle component or mesentsral component containing SR could be obtained from this tissue during subcellular membrane fractionation (Kwan et al., 1983a). Studies

2.3 Theory for radioligand binding data analysis

The study of receptor involves several major experimental approaches: pharmacological or functional studies, and radioligand binding studies. The availability of radioactively labelled ligands that retain their biological activity has allowed the direct identification of binding sites for these radiolabelled agents. In target tissues they elicit appropriate biological responses. In the present study, the radioligand-receptor binding is the major technique employed. In the radioligand binding study, the calculations about receptor density, affinity, kinetics, heterogeneity and cooperative interactions between receptors are inferred from the application of a variety of theoretical assumptions and mathematical relationships. Many factors, such as the selection of radioligand, and the definition of specific binding, may affect the conclusions drawn from radioligand binding studies.

2.3.1 Scatchard analysis

The Scatchard plot (or Rosenthal plot) is based on a mathematical model which assumes that radioligand-receptor binding involves a bimolecular interaction following the law of mass action (Scatchard, 1949; Rosenthal, 1977). Therefore, radioligand binds reversibly to the receptor in a concentration-dependent fashion and is able to reach saturation. From the saturation isotherm, by measuring the specific ligand binding (B) at equilibrium and knowing the concentration of the free ligand (F) in the incubation...
medium, the equilibrium dissociation constant (\(K_d\)) and the maximum number of binding sites (\(B_{max}\)) can be estimated from the equation:

\[
\frac{B}{F} = \frac{B_{max}}{K_d} - \frac{B}{K_d}
\]

This equation can also be plotted in the form of \(B/F\) against \(B\) which fits a best line by linear regression. This plot is named the Scatchard-Rasenthal plot. The \(K_d\) can be estimated from this plot as the negative of the slope (-1/\(K_d\)) and \(B_{max}\) is obtained from the intercept of the line on the x intercept.

From the Scatchard plot, a straight line often suggests one single binding site without any interaction between these binding sites. If there are multiple binding sites or a cooperative interaction between the binding sites exist, a curvilinear line will provide the best fit in a Scatchard plot. Another plot, the Hill plot which is also derived from the saturation isotherm is usually used simultaneously with Scatchard plot to suggest the existence of multiple binding sites or cooperativity.

\[
\log \left( \frac{B}{B_{max}} \right) = \log[L] - \log[K_d]
\]

The Hill plot is obtained by plotting the log of radioligand concentration (log[L]) against log\(B/(B_{max}-B)\), \(n\) is the Hill coefficient which is determined by the slope of the plot. If the Hill coefficient is not significantly different from unity, it suggests a single class of association experiment. The association and dissociation rate constants are expressed as \(K_a\) and \(K_d\), respectively. \(K_a\) can be calculated by estimating the \(t_{1/2}\) which is the half time to reach equilibrium binding. In this case, the \(B = 1/2B_{max}\) and the equation (3) can be transformed to:

\[
K_a = 0.693t_{1/2}
\]

When log \(B/(B_{max}-B)\) is plotted against time where \(B\) is the amount of bound ligand at time \(t\), \(K_{on}\) can be estimated from the slope of the plot. The association rate constant then can be calculated from equation (5).

The equilibrium binding constant \(K_d\) can also be determined from the kinetic experiment as:

\[
K_d = K_a/K_{on}
\]

Usually, the \(K_d\) estimated from kinetic experiment should be very close to that determined from the Scatchard plot in the saturation experiment, if the underlying assumptions have been fulfilled. A discrepancy suggests the need for careful evaluation of possible complexity in the binding reactions.

Binding sites. If negative cooperativity or multiple sites with different affinities exist, \(n\) will be less than one.

2.3.3 Kinetics of Binding

This includes two types of experiments to study association and dissociation. The association experiment is designed to examine the time required to reach steady state for radioligand binding to the receptor. The dissociation experiment is designed to determine the time course for the radioligand to dissociate from the receptor. After reaching the steady state in the presence of a certain amount of radioligand with tissue, the dissociation can be initiated by two different procedures: either diluting the ligand with an excess of buffer, or adding an excess amount of non-radioactive ligand. The association and dissociation rate constants then can be estimated from the following equations:

\[
K_{on}t = 2.303 \log \left( \frac{B_{max}}{B_{eq}} \right)\]

\[
B_{eq}/B_{max} = e^{-K_{on}t}
\]

\[
K_{on} = \frac{K_{off} \cdot K_{on}}{[L]}
\]

In these equations, \(B_{eq}\) is the bound radioligand in the steady state, \(B\) is the bound radioligand at different times (\(t\), \([L]\) is the free radioligand concentration used in the

2.3.3 Competition experiment

This is another important experiment in a radioligand study which can yield two major parameters, \(K_{IC}^c\) and \(K_c\). After \(K_{IC}^c\) is estimated from the competition curve, \(K_c\) can be determined using the equation below (Cheng and Pravoff, 1973):

\[
K_c = IC_{50}/(1+([L]/K_c))
\]

\(IC_{50}\) is the concentration of unlabelled ligand which could displace 50% of specifically bound radioligand. \(K_c\) is the equilibrium dissociation constant for unlabelled ligand in the presence of radioligand (\([L]\)). The equation is only applied when receptor concentration ([R]) is much less than \(K_c\). If \([R]\) is greater or equal to \(K_c\), the value of \(K_c\) from this equation may be an overestimation of the true value.

The competition experiment can be carried out by incubating a fixed concentration of radioligand with an increasing concentration of unlabelled ligand. If the total bound radioligand is 100 and 1 without or with presence of unlabelled ligand ([L]), respectively, log \(B/(B_{max}-B)\) can be plotted against log([L]). After \(IC_{50}\) is read from competition curve, \(K_c\) then can be calculated subsequently. The Hill coefficient \(n\) can be estimated as slope from this plot. As with the Hill coefficient in saturation experiment, if it is close to unity, it often indicates the existence of one single binding site. When \(n\) is less than one, it often suggests the existence of multiple binding sites. When labelled and unlabelled ligands are
the same or chemically similar, the $K_i$ calculated from competition experiment is often very close to $K_i$ determined from Scatchard plot.

2.4 The objectives

The conclusions about the location of ryanodine binding sites and the effect of ryanodine in smooth muscle are mainly based on the functional studies using either intact tissue or skinned muscle cells. Studies at the subcellular and molecular level are surprisingly few. Although ryanodine receptors have been well studied in striated muscles, considering the difference in the structure of skeletal muscle and smooth muscle and different dependence on intracellular and extracellular Ca$^{2+}$ for contraction, it is necessary to characterize ryanodine receptors and their functions in different types of smooth muscles. Due to the presence of another Ca$^{2+}$ channel, ICR channel on SR, and homology between these two channels in structure as discussed above, further characterization of the relation between these two Ca$^{2+}$ release channels is also important. My research project had the following objectives:

1. To investigate the location of ryanodine receptor in rat vas deferens using $[^{3}H]$ryanodine. This included the demonstration that the ryanodine receptor is of smooth muscle origin and located on SR of smooth muscle.

2. To characterize the ryanodine receptor in RVD smooth muscle in detail and to optimize the ryanodine binding conditions. $[^{3}H]$ryanodine binding to different smooth

3. MATERIALS AND METHODS

3.1 Materials

$[^{3}H]$ryanodine (specific radioactivity 60 or 95 Ci/mmol), $[^{3}H]$pyrazoxin (specific radioactivity 26 Ci/mmol), $[^{3}H]$IPN200-110 (specific radioactivity 78 Ci/mmol), and $^{45}$CaCl$_2$ (2.5 mCi/mL) were obtained from NEN Research Products (Boston, MA, U.S.A.). (Weyland, MA, U.S.A.), Nifedipine, phenolthrin, tetradotalin (TTX), cyclopiazonic acid (CPA), dithiothreitol (DTT), ruthenium red, B-NADPH, cytochrome c and sodium salts of ATP and AMP, Ca$^{2+}$ isophosphate A23187 were all purchased from Sigma (St. Louis, MO, U.S.A.). Trichloroacetic acid (TCA) was obtained from BDH.

3.2 Tissue handling of RVD

Male Wistar rats, weighting 250-450 g, were killed by ether cervical dislocation or CO$_2$ gas. The whole length RVD on both sides was excised and immersed immediately in ice-cold sucrose/imidazole buffer (0.25 M/10 mM, pH 7.4). Trimming of RVD was performed on the top of a glass plate kept cold on ice. The surrounding fat, connective tissues, nerve fibres and small blood vessels were meticulously removed from the smooth muscle layers. The whole RVD duct was cut open longitudinally. The epithelium was then peeled off with a pair of forceps. The cleaned tissue was then peeled and stored in a -70 or -20°C freezer until used for experiments.

3.3 Membrane preparation of RVD smooth muscle

The subcellular membranes were isolated by differential centrifugation and sucrose-density-gradient centrifugation in accordance with the procedures developed in this laboratory (Kwan et al., 1983b). The whole procedure is summarized as a flow chart shown in figure 6. Briefly, the cleaned whole length RVD was finely minced in sucrose/imidazole buffer (0.25 M/10 mM, pH 7.4) with a pair of scissors. The minced RVD smooth muscle was then homogenized using a polycarbonate PT20 (Brinkman Instruments Co., Switzerland) at a tissue/volume ratio (RVD and sucrose/imidazole) of 1g/11ml for 5 seconds. The crude homogenate was first centrifuged at 900 g for 10 minutes. The pellet was discarded. The supernatant, named post-nuclear supematant (PNS) was further centrifuged at 9,000 g for 10 minutes. The pellet than obtained was resuspended and named the P1 fraction. The supernatant was centrifuged at 105,000 g for 40 minutes (60 Ti rotor, Beckman LS50, L565 or L550-B ultracentrifuge). The supernatant after this spin contains soluble protein and was named SOL. The pellet is the crude microsomal fraction (MIC1). MIC1 was resuspended and was then centrifuged again at 9,000 g for 10 minutes. The supernatant obtained is MIC2 and the pellet is P2. The
plasma membrane and SR membrane fractions in MOC2 could be further separated by 
loading MOC2 on the discontinuous sucrose density gradients and centrifuging at 105,000 
g for 120 minutes (SW40 rotor, Beckman L59, L565 or L300-B ultracentrifuge). The 
gradients consisted of 2 ml 60%, 2 ml 40%, 3 ml 30% and 2 ml 15% sucrose (weight/weight) from the bottom to the top of the centrifuge tube. The various fractions 
(F1, F2, F3, F4) were removed at the sucrose interfaces. The whole membrane preparation 
procedure was performed at 4 °C. Each pellet was resuspended in sucrose/imidazole buffer 
using a Teflon-coated hand-held homogenizer. All fractions obtained during membrane 
isoaltion were then assayed for the marker enzymes or binding activities.

Figure 5. Flow chart for the isolation of subcellular membranes from RVD smooth 
muscle.

3.4 Tissue handling and membrane preparation of DMA

Mongrel dogs of either sex were used in this study, following euthanasia by a 
procedure approved by McMaster University Animal Care Committee. Dogs were killed 
by an overdose (100 mg/Kg) of pentobarbital. The mesenteric vasculature was quickly 
removed and placed in ice-cold sucrose/MOPS (250mM/10mM) buffer. The tissue was 
subsequently trimmed according to the procedure previously developed in this laboratory 
(Kwon et al., 1983b). The entire network of mesenteric arteries was separated from 
mesenteric vein and nerve. The surrounding fat and connective tissue were removed. The 
cleaned mesenteric arteries were then stored in a -70 or -20 °C freezer until used for 
experiments.

The detailed membrane isolation procedure for DMA smooth muscle has also been 
previously described (Kwon et al., 1983b). In my experiments, the membrane preparation 
method was the same as that described above for RVD smooth muscle, except that the 
initial homogenization by the polytron PT20 was performed in a tissue/buffer ratio of 
1g/10ml for 15 seconds. The flow-chart of this membrane preparation is illustrated in 
figure 6.

3.5 Membrane preparation for other tissues

In this study, some other tissues were also used for comparative purposes. These
include: rat skeletal muscle and cardiac muscle, dog aorta and vas deferens, rat aorta, mesenteric and tail arteries, rabbit and guinea pig vas deferens. The skeletal muscle was taken from the hind leg of Winter rat. The membrane preparation procedure for rabbit and guinea pig vas deferens was the same as that described for RVD. In the studies of [3H]thyronine binding to other tissues, the method of the membrane preparation was the same as that used for DMA. For these tissues, only MGD2 was used in the experiments.

Figure 6. Flow chart for the isolation of subcellular membranes from DMA smooth muscle. The preparation of subcellular membranes from other smooth muscles (as listed in Table 7, except for vas deferens) also followed this flow chart as mentioned in the text.

3.6 Enzyme assays

3.6.1 5'-Nucleotidase

Used as a plasma membrane marker, the activity of this enzyme was determined as the amount of inorganic phosphate (P) released from Na-AMP. The reaction was carried out at 37°C for 30 minutes in the presence of 5 mM Na-AMP, 50 mM imidazole, 10 mM MgCl₂, and pH 7.5 (Kwan and Ramal, 1985). The reaction was stopped by adding trichloroacetic acid (TCA) to the reaction buffer. The tubes were then centrifuged for 10 minutes and the supernatant was taken for the measurement of P. Spectrophotometric measurement was performed on a Beckman Model DU-88 spectrophotometer at a wavelength of 700 nm.

3.6.2 Mg²⁺-ATPase

This enzyme was measured under similar conditions to those used for the assay of 5'-Nucleotidase except that the reaction time was 15 minutes. The enzyme activity was determined as the amount of inorganic phosphate (P) released from Na₂-ATP.

3.6.3 NADPH cytochrome c reductase

This enzyme was measured by monitoring the reduction of cytochrome c by NADPH in a potassium phosphate buffer (50 mM). The buffer contained 1 mM KCN, 50 mM cytochrome c and 0.2 mM β-NADPH at pH 7.5. A Beckman Model 25 double beam...
spectrophotometer (at a wavelength of 550 nm) was used for this enzyme activity measurement.

3.6.4 Cytochrome c oxidase

The measurement of this enzyme activity was performed using the same spectrophotometer as that for NADPH cytochrome c (at a wavelength of 550 nm). The activity was measured in 50 mM potassium phosphate buffer, pH 7.5, with 68 μM cytochrome c reduced by sodium hydrosulfite as the substrate.

3.7 Protein assay

The protein concentration in each membrane fraction was determined according to the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used to make a standard.

3.8 [3H]ryanodine binding to RVD

3.8.1 Binding condition

The assay for [3H]ryanodine binding in RVD was routinely performed in a medium consisting of 0.3 M KCl, 0.5 mM CaCl₂, 25 mM Tris and 1 mM dithiothreitol (DTT) at pH 7.4. Unless otherwise specified, 5-7 nM [3H]ryanodine was used in the experiment in a total volume of 250 μl with an incubation period of 2 hours. Binding was routinely indicated times.

3.8.3 Equilibrium binding experiments

The reaction was started by addition of MIC2 (or PBS, P3 as indicated in the Results section) (20-50 μg protein). The membrane was then incubated for 2 hours in the presence of increasing concentrations of [3H]ryanodine (0.5 - 35 nM). The reaction was terminated by filtration as described above.

3.8.4 Competition experiments

In these experiments, MIC2 fraction was incubated with varied concentrations of unlabelled ryanodine. The [3H]ryanodine concentration was fixed near the K₅₀ value. The incubation period was 2 hours.

3.9 [3H]ryanodine binding to DMA and other tissues

A medium consisting of 0.6 M KCl, 0.5 mM CaCl₂, 25 mM Tris and 1 mM DTT at pH 7.4 was defined as providing optimized condition. [3H]ryanodine binding to DMA smooth muscle preparations and all other tissues was carried out under this condition. Other procedures of the binding assay were the same as that for RVD.

3.10 [3H]ryanodine binding

The [3H]ryanodine binding was performed according to the method previously reported (Ahmad et al., 1988; Kruta et al., 1987). Briefly, the reaction medium contained 50 mM Tris-HCl, 0.2% BSA at pH 7.4. The membranes were incubated with this medium at 25 °C in the presence of [3H]ryanodine (1 nM) with total volume 250 μl for 30 minutes. Non-specific binding was determined simultaneously in the presence of 1 μM ryanodine (RTX). The specific binding was the difference between total and the non-specific binding. The reaction was started by the addition of membranes to the incubation tubes and terminated by the addition of 3 ml ice-cold buffer and filtration through Whatman GF/F filters followed by two washes with 3 ml buffer. The filters were kept in the scintillation fluid overnight in the dark before being counted for the radioactivity.

3.11 [3H]ryanodine binding

The incubation medium for this binding assay contained 50 mM MOPS and 10 mM MgCl₂ at pH 7.0 (Shi et al., 1989). The reaction was started by adding membranes into the medium to a total volume 250 μl and incubated for 30 minutes at 25 °C. The binding was terminated by adding 5 ml ice-cold buffer into the incubation tubes and rapid filtration through Whatman GF/F filters followed by 3x5 ml wash with buffer. The specific binding was defined as the binding displaceable by 10 μM phenelzamine. The filters were placed into the counting vials with 5 ml of scintillation fluid and kept overnight in the dark before being counted for the radioactivity.
3.12 \(^{3}H\)PN200-110 binding

This binding assay was performed according to Wibo and Godfraind (1988). The binding was carried out in Tris buffer (50 mM) at pH 7.4. The reaction was started by adding membrane into the medium to a total volume of 1 ml in the presence of 0.1 nM \(^{3}H\)PN200-110 and then incubated for 30 minutes at 37 °C. The binding was stopped by rapid filtration of reaction medium through GF/F filters followed by 3x3 ml wash with buffer. The filters were transferred into the scintillation fluid and left overnight in the dark and then counted for the radioactivity. The free-specific binding was determined in the presence of 10\(^{6}\) M nifedipine. Since nifedipine is light sensitive, the reaction was carried out in the water bath which was covered to avoid the light.

3.13 Data analysis

The data from saturation and competition binding experiments were analyzed by the EBDA (equilibrium binding data analysis) computer programme, which was designed to analyze saturation studies and drug displacement studies (M. Persson, 1983). The data from association and dissociation experiments were analyzed by the Kinetic computer programme. Binding parameters, including \(K_d\), \(B_{max}\), \(t_0\), IC\(_{50}\), \(K_{app}\) and \(K_i\), were calculated by computer analysis. The calculated results of competition experiments were further analyzed by CDATA87 computer programme for one or two binding site model.

3.14 Ca\(^{2+}\) transport

3.14.1 Ca\(^{2+}\) uptake

The azide-insensitive Ca\(^{2+}\) uptake was carried out routinely in media containing 250 mM sucrose, 50 mM imidazole, 5 mM ATP, 100 \(\mu\)M CaCl\(_2\), 1 mM MgCl\(_2\) and 5 mM sodium azide, pH 7.4 (27 °C). A trace amount of \(^{45}\)Ca was added. For caffeine-stimulated Ca\(^{2+}\) uptake, 5 mM potassium oxalate was also added in the medium. The uptake was started by adding the membrane fraction into the medium (containing 20 \(\mu\)g protein). The experiments were performed at 37 °C with a total volume of 250 ml. After incubation for an indicated time, the reaction was terminated by immediate filtration of the reaction buffer through millipore filters (type HA, 0.45 \(\mu\)m pore size), followed by 2x3 ml washes with ice cold wash buffer containing sucrose/imidazole (250 mM/50 mM) at pH 7.4. The filters were routinely predawn in 0.1 M KCl for 1-2 hours before filtration. The filters were then transferred into 4-5 ml of scintillation fluid and left overnight in the dark before being counted. The radioactivity of each sample was counted for 2 minutes in a Beckman Scintillation counter.

Ca\(^{2+}\) binding to the membrane fraction was determined simultaneously in the presence of 10\(^{-8}\)M Ca\(^{2+}\) ionophore, A23187, in the medium. ATP-dependent Ca\(^{2+}\) accumulation is referred to as the \(^{45}\)Ca retained in the absence of A23187 minus that in the presence of A23187.

3.14.2 Ca\(^{2+}\) release

In these experiments, the membrane vesicles were preloaded with Ca\(^{2+}\) under the condition described above for the indicated time. Ca\(^{2+}\) release was initiated by adding releasing buffer A or B to the preloaded vesicles. The releasing buffer A contained 30 mM sucrose, 150 mM imidazole, 0.3 M KCl, with or without 100 \(\mu\)M Ca\(^{2+}\). The releasing buffer B contained 50 mM sucrose, 150 mM imidazole, 0.3 M KCl and 100 \(\mu\)M EGTA, with or without 100\(\mu\)M Ca\(^{2+}\).

The paired t-test was used for statistical analysis in this study.

4. RESULTS

4.1 \(^{3}H\)Hydroxylamine binding in RVD

4.1.1 Optimization of binding conditions

Under the conditions described in method section for \(^{3}H\)Hydroxylamine binding, preliminary experiments showed that similar levels of \(^{3}H\)Hydroxylamine binding were obtained at 25 °C and at 37 °C as measured in microsomal fractions (figure 7). However, the binding level was very low when the experiments were performed at 0 °C. These experiments were performed under identical conditions except for the temperature, (i.e. the same membrane preparation was simultaneously assayed at the indicated temperatures). Therefore, the subsequent experiments were all carried out at 25 °C.

The effect of protein concentrations on \(^{3}H\)Hydroxylamine binding was also tested. Changing the protein concentration from 20 to 100 \(\mu\)g/ml did not significantly affect \(^{3}H\)Hydroxylamine binding per mg protein to the microsome fraction. The preliminary experiments also showed that \(^{3}H\)Hydroxylamine binding capacity could be preserved by storing cleaned RVD smooth muscle or isolated subcellular membranes fractions in a -20 °C freezer. Therefore, either fresh or frozen membrane fractions were used for ligand binding experiments in the subsequent experiments of this study.
4.1.2 Subcellular membrane characterization

The method for subcellular membrane isolation has been well established in this laboratory (Kwan et al., 1983a; 1983b) and the isolated membrane fractions have been characterized using biochemical markers, morphological methods and 45Ca transport. In the present study, several marker enzyme activities were monitored in each subcellular fraction in order to define the subcellular nature of isolated membrane fractions under the conditions used in my study. As shown in figure 8, the M2 fraction obtained by differential centrifugation was enriched in plasma membranes and SR membranes, as indicated by the presence of high levels of 5'-nucleotidase and Mg2+-ATPase, as plasma membrane markers and of NADPH-cytochrome c reductase activity as a SR membrane marker. [3H]dexamethasone binding (α2-adrenoceptor) and [3H]PN200-110 binding (L-type Ca2+ channels) were also used as additional plasma membrane markers (figure 9).

The SR- and P2-enriched fractions were further purified by subfractionating the M2 fraction on a discontinuous sucrose gradient. As shown in figure 8, P3 is the SR enriched membrane fraction. Since RVD is a highly innervated tissue, [3H]tachykinin binding was employed as a marker to assess the possible contamination of fractions by sympathetic membranes of the nerve variocotytes. As shown in figure 9, although [3H]tachykinin binding was also enriched in the M2 fraction, the highest level of binding was found in the P2 fraction paralleling the distribution of plasma membrane markers, but not SR markers. The P2 fraction was enriched in mitochondria membranes as indicated by cytochrome C oxidase, SOL and SOL1 contain mainly soluble protein which had very low enzyme activities. Also P1 and P4 showed very low enzyme activities or ligand binding capacity, suggesting that these fractions may be derived from the connective tissues rather than smooth muscle cells.

The total recovery of protein and enzymes activities was >90%. This indicates that little of the protein and enzymes activities were lost during the entire membrane isolation process.

4.1.3 The distribution of [3H]yosodine binding

The association of [3H]yosodine binding sites and SR membrane swelling was substantiated by evaluating the subcellular distribution of [3H]yosodine binding to different fractions. The highest density of [3H]yosodine binding was to SR-enriched membrane fractions (F3), as defined by NADPH-cytochrome c reductase activity (figure 9). The coefficient constant between the distributions of [3H]yosodine binding and NADPH-cytochrome c reductase activity is very close to one (0.95). The density of [3H]yosodine binding site did not parallel the activity of any plasma membrane marker. The peaks of [3H]tachykinin and [3H]yosodine binding were located in the different fractions. This indicates that this [3H]yosodine binding site is of smooth muscle origin. Consistent with this finding is also the lack of [3H]yosodine binding to any of the membrane fractions derived from paravascular nerves of mesenteric vasculature (results not shown). Although M2 fraction contained a large portion of plasma membrane, M2 was still used as the major fraction for most detailed characterizations in order to conserve tissue.

Figure 7. The effect of temperature on [3H]yosodine binding to M2 fraction of RVD.
The association experiments were carried out at 0, 25 and 37 °C respectively. The data represent the average of two experiments performed in triplicate.
Figure 8. Subcellular membrane characterization by marker enzymes. (a) Plasma membrane marker enzymes: 5'-nucleotidase (μmol/h per mg) and Mg-ATPase (μmol/h per mg). (b) Endoplasmic reticulum marker enzyme, NADPH-cytochrome C reductase (ΔA/min per mg), and mitochondrial-inner-membrane marker, cytochrome C oxidase (ΔA/min per mg). Measurements of these membrane marker enzymes were all carried out on the same membrane preparation. Each data point represents mean±SD of three experiments.

Figure 9. The distribution of radioligand binding (fmol/mg) to surface membrande receptors ([3H]prazosin, PRA) and Ca²⁺ channels ([3H]PN200-110, PN) or synaptosome marker ([3H]jasplakinolide, SXT). The data were obtained from one representative experiment in triplicate using the same membrane preparation. [3H]Rimodine binding (RYA, fmol/mg) is the mean of 3 experiments with SD.
4.1.4 Equilibrium binding

After the binding conditions were established and the primary subcellular membrane site for \(^{3}H\)yosadine binding was deﬁned, equilibrium binding experiments were carried out on the microsomal fraction (MIC2). Due to the limited quantity of radioligand, 0.5 to 35 nM \(^{3}H\)yosadine was used in these experiments. The speciﬁc \(^{3}H\)yosadine binding and corresponding Scatchard plots are shown in Figure 10. The mean \(K_d\) value for MIC2 fraction was 5.6±1.4 nM (\(n=10\)). The nonspeciﬁc \(^{3}H\)yosadine binding to this fraction was about 30% of the total \(^{3}H\)yosadine bound in the presence of a concentration of \(^{3}H\)yosadine near the \(K_d\) value.

In order to examine whether the membrane isolation and puriﬁcation procedure affected \(^{3}H\)yosadine binding, the equilibrium binding was also performed on three subcellular fractions of various degrees of purity of SR, i.e., FNS, MIC2 and F3, and the affinities of \(^{3}H\)yosadine binding to these fractions were compared. Figure 11 shows the Scatchard plots of the saturation proﬁles for \(^{3}H\)yosadine binding to these subcellular membrane fractions over 0.5–35 nM \(^{3}H\)yosadine. The parallel linear Scatchard plots suggest that only one homogeneous class of high afﬁnity binding site exists for \(^{3}H\)yosadine in these preparations within the concentration range of yosadine used. The Hill-coefﬁcient values were all near unity. Figure 12 shows one representative experiment performed in triplicate. Similar results were obtained in two additional experiments, in which \(B_{max}\) values consistently increased with increasing SR content, whereas the \(K_d\) values were comparable (5-7 nM). These results suggest that membrane puriﬁcation procedure had little effect on the binding properties of \(^{3}H\)yosadine. The subsequent characterization of \(^{3}H\)yosadine binding was performed routinely using MIC2 fraction unless otherwise speciﬁed.

4.1.5 Association and dissociation

The association and dissociation experiments were performed in the presence of \(^{3}H\)yosadine near the \(K_d\) concentration (4-7 nM). As shown in Figure 12a, \(^{3}H\)yosadine binding to MIC2 fraction reached a plateau after 2 hours of incubation. The calculated \(K_{on}\) is 0.028±0.004 min\(^{-1}\) (\(n=4\)). The \(^{3}H\)yosadine binding level was not changed by prolonged incubation for 3-6 hours at 25°C.

After 3-hour incubation, the dissociation was initiated by either addition of excess unlabelled yosadine (10\(^{-6}\) M) or 20 fold dilution with reaction buffer. \(^{3}H\)yosadine dissociated from the binding site by 20 fold dilution with a \(K_d\) value of 0.016 nM min\(^{-1}\) (average of two separate experiments). The \(K_d\) value calculated from \(K_{on}/K_{off}\) is 6.0±1.9 nM. This is in excellent agreement with \(K_d\) determined in the equilibrium binding experiment described above. However, \(^{3}H\)yosadine was unable to dissociate from its binding site in the presence of excess unlabelled yosadine (Figure 12b).

4.1.6 Competition experiments

Figure 13 shows the competition of \(^{3}H\)yosadine binding by unlabelled yosadine at indicated concentrations. The concentration of \(^{3}H\)yosadine was chosen near the \(K_d\)

value. The results were analyzed by EBD and CDATA87 computer programme. The calculated \(K_{d}\) is 6.7±2.0 nM, which is similar to the \(K_d\) values obtained in saturation and kinetic studies. The calculated slope of Hill plot is very close to unity (\(n=4\)). Further analysis of the results by CDATA87 computer programme indicated that the data ﬁtted the model for one binding site.
Figure 11. Representative Scatchard plots for [3H]ryanodine specific binding to PNS (○), M2C2 (●) and F3 (△). Three fractions were obtained from the same membrane preparation and the binding experiments were carried out at the same time under the same conditions. Inset is the expanded Scatchard plot for PNS.

Figure 12. Kinetic study of specific [3H]ryanodine binding to M2C2 of RVD. (A) Time course for the association reaction. (B) Time course for the dissociation reaction. After the membrane was incubated for 3 hours, dissociation was initiated by the addition of unlabelled ryanodine (10 μM) (●) or by a 20 fold dilution with wash buffer (○).
4.1.7 Effect of Ca\(^{2+}\) on \(^{3}H\)ryanodine binding

The ryanodine receptor has been demonstrated to be the CRICR channel complex in striated muscle. The association of ryanodine with its binding site has been reported to be related to the conditions of the channel (open or closed) (Iino et al., 1988). The effect of Ca\(^{2+}\), which can open CRICR channel, on \(^{3}H\)ryanodine binding was studied by changing free Ca\(^{2+}\) concentration in the medium. The Ca\(^{2+}\) concentrations were controlled by BOTA in accordance with the calculations of Grover et al. (1982). Figure 14 shows the Ca\(^{2+}\) dependence of \(^{3}H\)ryanodine binding to CRICR. \(^{3}H\)ryanodine binding increased with increasing free Ca\(^{2+}\) concentrations in the medium up to 2 \(\mu\)M Ca\(^{2+}\). The half-maximum binding happened at 0.14 \(\mu\)M free Ca\(^{2+}\) and remained at the plateaus level from 2 \(\mu\)M Ca\(^{2+}\) to as high as 500 \(\mu\)M.

The effect of Ca\(^{2+}\) on the equilibrium binding was then examined. The \(B_{\text{max}}\) and Hill coefficient values, as determined by Scatchard plot, were quite comparable under different Ca\(^{2+}\) concentrations as shown in table 6. The affinity for ryanodine, as reflected from \(K_d\) values remained unchanged within the physiologically relevant range of cytosolic Ca\(^{2+}\) concentrations (0.14 and 2.0 \(\mu\)M) (table 6). However, the affinity was increased 3-4 fold by further elevating Ca\(^{2+}\) concentration (500 \(\mu\)M).

4.1.8 Effects of ionic strength, osmolarity, and pH

The ionic strength was controlled by changing NaCl or KCl concentrations in the reaction medium. Figure 16 shows that an increase in ionic strength caused a progressive
increase in the binding of [H]pyridoxine which reached a plateau at 0.6 M. An imposed increase in the osmolarity across the membrane vesicles with increasing concentrations of sucrose also caused a progressive increase in [H]pyridoxine binding (Figure 14). This augmentation of [H]pyridoxine binding by sucrose is smaller than that by NaCl or KCl. The K_d and the Hill coefficient for [H]pyridoxine binding remained unchanged in 0.3M and 0.6 M KCl.

Figure 17 shows that [H]pyridoxine bound to RVD smooth muscle MCI2 fraction in a pH dependent manner, with optimum binding at pH 7.5-8.0. The pH was controlled by imidazole, tris and glycine buffering reagents in the pH range of 5.0-7.4, 7.4-8.5 and 8.5-9.5, respectively while the other ingredients in the buffer remained the same.

4.1.9 Effects of Caffeine, MgCl_2, and Ruthenium Red

Several factors have been reported to affect CICR channel activity. In my ligand binding studies, caffeine significantly enhanced [H]pyridoxine binding in the presence of low Ca^{2+} concentration (0.14 μM), but had no effect on binding in the presence of high concentration of Ca^{2+} (500 μM) (Figure 18).

MgCl_2 inhibited [H]pyridoxine binding in a concentration dependent manner (Figure 19). Ruthenium red, like Mg^{2+}, has been commonly used as an intracellular Ca^{2+} channel blocker in different muscle types. In the present study as shown in figure 20, ruthenium red, up to 10 μM, potently inhibited [H]pyridoxine binding to MCI2 fraction, reaching its maximum effect at 10 μM.

Table 6. Parameters for [H]pyridoxine binding to RVD smooth muscle microsomes at different Ca^{2+} concentrations. Results were obtained from three different experiments (mean±SD).

<table>
<thead>
<tr>
<th>Ca^{2+} (μM)</th>
<th>K_d (μM)</th>
<th>B_max (mol/mg)</th>
<th>n_H</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.14</td>
<td>18±5</td>
<td>426±7</td>
<td>0.98±0.01</td>
</tr>
<tr>
<td>2</td>
<td>17±5</td>
<td>452±27</td>
<td>0.99±0.01</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>435±11</td>
<td>1.00±0.02</td>
</tr>
</tbody>
</table>

Figure 14. Ca^{2+} dependence of specific [H]pyridoxine binding to MCI2 of RVD. Free Ca^{2+} was buffered with 100 μM EGTA. The [H]pyridoxine binding at 500 μM is taken as 100%. Symbols and error bars represent mean ± SD of three experiments. Inset is an expanded plot for [H]pyridoxine binding from 0 to 2 μM free Ca^{2+}.

Figure 15. Scatchard plots of [H]pyridoxine binding to MCI2 at 0.14 (O), 2 (●) and 500 (+) μM Ca^{2+}. Each result represents one typical experiment performed in triplicate by using the same membrane preparation.
Figure 16. The effect of ionic strength on specific [3H]yanodine binding. The experiments were performed in the presence of 500 μM Ca⁴⁺. The experiment on the effect of sucrose was performed in the absence of KCl. The specific binding of [3H]yanodine at 1 M KCl is taken as 100%. Data represents mean±SD, n=3.

Figure 17. Dependence of specific [3H]yanodine binding on pH. Each data point represents the average of two separate experiments performed in triplicate.

Figure 18. The effect of caffeine on specific [3H]yanodine binding to MChR in the presence of 0.3 M KCl and 300 μM (●) or 0.14 μM (○) Ca⁴⁺. The specific [3H]yanodine binding in the absence of caffeine is taken as 100%. The results represent mean±SD from three experiments.
4.1.10 [3H]pyramidine binding in microsomal fractions from different smooth muscle tissues

The purpose of this experiment was to compare the levels of [3H]pyramidine binding in different smooth muscles. In the initial experiments, the levels of [3H]pyramidine binding in some tissues were very low under the condition used for RVD. Since increasing ionic strength could enhance the binding level, the concentration of KCl was increased to 0.6 M in the binding medium and this medium was used to optimize the binding of [3H]pyramidine. Under this condition, the relative number of [3H]pyramidine binding sites in M2 fractions varied with the smooth muscle preparations as listed in Table 7. The corresponding level of [3H]pyramidine binding in the M2 fractions from skeletal muscle obtained under the same binding condition is also listed for comparison. Among the smooth muscle preparations listed in Table 7, RVD smooth muscle had the highest number of [3H]pyramidine binding sites under the same binding conditions. However, the levels of [3H]pyramidine binding to smooth muscle membranes were substantially lower than that in skeletal muscle.

4.1.11 Correlation between [3H]pyramidine and [3H]oxotremorin binding in different smooth muscles

As described above, the level of [3H]pyramidine binding varied considerably among different muscle preparations. Due to variation in the densities of innervation of these tissues, the varied levels of [3H]pyramidine receptors in different smooth muscle preparations might be accounted for by the contaminating nerve varicosities. [3H]pyramidine binding to equivalent membrane fractions (M2) from tissues with different densities of innervation was compared to [3H]oxotremarin binding, which is a neuronal membrane marker. Densities of [3H]pyramidine and [3H]oxotremarin binding in different tissues were plotted as shown in Figure 21. No correlation was found between these two binding (correlation coefficient=0.02), suggesting that [3H]pyramidine binding was primarily of smooth muscle origin. Among the tissues examined, RVD smooth muscle showed the highest number of [3H]pyramidine binding sites. The [3H]oxotremarin binding level was high in DMA preparation while [3H]pyramidine binding was lower in this tissue than in RVD.
Table 7. Specific $[^3H]$hydramine binding to MIC2 isolated from different smooth muscles under the optimized condition. The binding medium contained 500 nM Ca$^{2+}$, 0.6 M KCl, 25 mM tris and 1 mM DTT at pH 7.4. The data represent mean±SD, except for dog trachea, in which two separate experiments were performed.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Binding (fmol/mg)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Skeletal muscle</td>
<td>1578±384</td>
<td>3</td>
</tr>
<tr>
<td>RVD</td>
<td>391±82</td>
<td>6</td>
</tr>
<tr>
<td>Dog vas deferens</td>
<td>97±28</td>
<td>3</td>
</tr>
<tr>
<td>Guinea-pig vas deferens</td>
<td>200±39</td>
<td>3</td>
</tr>
<tr>
<td>Rat mesenteric artery</td>
<td>87±31</td>
<td>4</td>
</tr>
<tr>
<td>Dog mesenteric artery</td>
<td>87±30</td>
<td>4</td>
</tr>
<tr>
<td>Rat aorta</td>
<td>23±18</td>
<td>4</td>
</tr>
<tr>
<td>Dog aorta</td>
<td>40±26</td>
<td>4</td>
</tr>
<tr>
<td>Dog trachea</td>
<td>0, 29</td>
<td>2</td>
</tr>
<tr>
<td>Dog mesenteric nerve</td>
<td>not detectable</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 21. Correlation between $[^3H]$hydramine binding and $[^3H]$saxitoxin binding (fmol/mg) in different smooth muscles (MIC2) (including: RVD, rat mesenteric artery and vein, rat aorta, rat tail artery, dog vas deferens, dog mesenteric artery and vein, dog aorta, guinea pig vas deferens). The data represent the average of 2-4 separate experiments performed in triplicate. (correlation coefficient=0.02)

4.2 $[^3H]$hydramine binding in DMA

4.2.1 The effect of Ca$^{2+}$ on specific $[^3H]$hydramine binding

Free Ca$^{2+}$ concentrations in the medium were controlled by EGTA similarly to that described for RVD. 0.6 M KCl was used in these experiments. The level of $[^3H]$hydramine binding to MIC2 increased with increasing free Ca$^{2+}$ concentrations up to 2 μM. The half-maximal binding occurred at 0.14 μM free Ca$^{2+}$ and binding reached a plateau from 2 μM Ca$^{2+}$ to as high as 500 μM (Figure 22).

4.2.2 Equilibrium binding

$[^3H]$hydramine concentrations 0.5 - 20 nM were used in saturation experiments. The non-specific $[^3H]$hydramine binding was about 30% of the total $[^3H]$hydramine binding in the presence of $[^3H]$hydramine near the $K_{d}$ value. Figure 23 shows specific binding and the corresponding Scatchard plot of the saturation experiments. The calculated $K_{d}$ value was 5.9±0.3 nM and the $B_{max}$ was 171±57 fmol/mg (n=3). The linear Scatchard plot suggested that only one homogeneous class of high affinity binding site existed within the $[^3H]$hydramine concentration used. Evidence for co-operative interactions were not noted under these conditions ($n_{H}=0.93±0.06$).

4.2.3 Kinetic experiments

As with that shown in RVD, $[^3H]$hydramine binding to MIC2 in DMA smooth
muscle was also slow. Binding was initiated by addition of MIC2 to the incubation medium and reached a plateau level after 2 hours as shown in figure 24a. $K_{d}$ was calculated as $0.0029 \pm 0.0006$ min$^{-1}$ from these experiments. After incubation for 3 hours, dissociation was started by either 20 fold dilution with reaction buffer or addition of excess unlabelled ryanodine (20 $\mu$M) (figure 24b). $[{\textit{[H]}}$ryanodine was dissociated from its binding site by dilution with $K_{d}$ value of $0.013 \pm 0.005$ nM$\cdot$min$^{-1}$. The calculated $K_{d}$ from $K_{d}/K_{d}$ was $4.3 \pm 2.8$ nM. However, $[{\textit{[H]}}$ryanodine dissociation was slower and incomplete when dissociation was initiated by adding excess unlabelled ryanodine (figure 24b).

### 4.2.4 Competition experiments

The experiments were performed in the presence of a $[{\textit{[H]}}$ryanodine concentration near the previously determined $K_{d}$. The results were first analyzed by EBDAN programme and plotted as figure 25. The estimated slope for the plot was less than one (0.58 $\pm$ 0.16, $n$=7). The results were then further analyzed by CDATA87 computer programme. Two binding sites with distinct affinities were identified: $K_{d} = 4.0$ nM, $N_{d} = 70\%$; $K_{d} = 269$ nM, $N_{d} = 22\%$.

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**Figure 22.** The effect of free Ca$^{2+}$ on $[{\textit{[H]}}$ryanodine binding to DMA smooth muscle MIC2 fraction. Data represents mean $\pm$ SD of three experiments.

**Figure 23.** Specific $[{\textit{[H]}}$ryanodine binding in saturation experiment. Inset in the corresponding Scatchard plot. This is one representative experiment performed in triplicate. Binding parameters in 3 experiments are described in the text.
figure 24. Kinetics of specific \(^{3}H\)ryanodine binding in MEC2 of DMS. Each point represents mean±SD of 3 separate experiments. (a) Association experiment (b) dissociation was initiated by the addition of 5 ml wash buffer (60) or an excess unlabelled ryanodine (RYA).

Figure 25. Competition curve in the presence of \(^{3}H\)ryanodine near \(K_a\) value. Data points represent mean±SD of 7 separate experiments.
added in the uptake medium. The uptake was initiated by addition of MII2 to the medium. As shown in figure 27, ryanodine inhibited oxalate stimulated Ca\(^{2+}\) uptake in a concentration dependent manner. This inhibitory effect was observed only at higher concentrations of ryanodine.

### 4.3.3 Time dependence of the effect of ryanodine on Ca\(^{2+}\) uptake

Experiments were performed in the presence of 10^{-6} M ryanodine. As shown in figure 26, ryanodine inhibited oxalate stimulated Ca\(^{2+}\) uptake but not in the absence of oxalate. This inhibitory effect could be observed as early as 5 minutes after Ca\(^{2+}\) uptake was started (P<0.05).

### 4.3.4 Comparison of the effect of ryanodine with that of cyclopiazonic acid (CPA)

Figure 28 shows that 10^{-4} M ryanodine had an inhibitory effect similar but smaller than that caused by 30 \(\mu\)M CPA, a known SR Ca\(^{2+}\) pump inhibitor. The amplitude of the inhibition of oxalate stimulated Ca\(^{2+}\) uptake by the addition of both 10^{-4} M ryanodine and 30 \(\mu\)M CPA is the same as caused by 30 \(\mu\)M CPA at both 30 and 60 minutes, but larger than that caused by 10^{-3} M ryanodine alone at 30 minutes. 10^{-6} M ryanodine, 30 \(\mu\)M CPA or both were added into the uptake medium after vesicles were preloaded with Ca\(^{2+}\) (30 min). After addition of ryanodine and CPA or both for another 30 min., the level of Ca\(^{2+}\) uptake was not significantly different from control as shown in figure 28. With submaximal concentrations of ryanodine and CPA, partially additive effect on oxalate stimulated Ca\(^{2+}\) uptake by MII2 was observed as shown in table 8. When both ryanodine and CPA were added, the level of Ca\(^{2+}\) uptake was significantly lower than that when either ryanodine or CPA was present in the medium (P<0.001).

### 4.3.5 The effect of ryanodine on Ca\(^{2+}\) release

The ryanodine concentration which are close to those used in functional studies in other reports, were chosen to test its effect on Ca\(^{2+}\) release from preloaded microsomal vesicles in the presence of oxalate. Figure 29 shows that ryanodine, at both 10^{-4} M and 5x10^{-5} M, has no effect on Ca\(^{2+}\) release induced by 1 mL release buffer containing EGTA (buffer B). In these experiments, ryanodine was added in the release buffer. The preincubation of ryanodine before Ca\(^{2+}\) release was initiated, did not significantly change the Ca\(^{2+}\) release rate.

As mentioned before, Ca\(^{2+}\) can induce Ca\(^{2+}\) release from SR through CICR channel. Mg\(^{2+}\) and ruthenium red can inhibit CICR channel activity. However, the CICR from vesicles of MII2 fraction was not observed under the conditions used in my experiments. In these experiments, Ca\(^{2+}\) release was initiated by the addition of a 20 fold volume of release buffer (buffer A, without EGTA) to the uptake medium after the vesicles were preloaded for 30 min. Ca\(^{2+}\), Mg\(^{2+}\), ruthenium red and ryanodine were all added in the release buffer respectively to observe their effects on Ca\(^{2+}\) release. The addition of Mg\(^{2+}\) and ruthenium red, did not significantly inhibit Ca\(^{2+}\) release. A Mg\(^{2+}\) induced Ca\(^{2+}\) release was not observed under the conditions used in my study.
Figure 26. Time dependence of 10 μM ryanodine effect on Ca²⁺ uptake in the presence (○, ●) or absence (×, ○) of 5 mM oxalate. At 30 minutes, the oxalate stimulated Ca²⁺ uptake in the absence of ryanodine (○) was taken as 100%. Result represents mean±SD of 3 experiments. (×, in the absence of oxalate and ryanodine)

Figure 27. Concentration dependence of the inhibitory effect of ryanodine on oxalate stimulated Ca²⁺ uptake. The MGC fraction was loaded with Ca²⁺ for 30 minutes in the presence of ryanodine at the indicated concentrations. Result represents mean±SD of 3 experiments. The level of Ca²⁺ uptake in the absence of ryanodine was taken as 100%.

Figure 28. Comparison of the effects of ryanodine (1 mM) and CPA (30 μM) in maximally effective concentrations on oxalate-stimulated Ca²⁺ uptake at 30 and 60 minutes. Ryanodine and CPA were also added into the medium after uptake was started 30 minutes and the results are plotted as mean±SD. The level of Ca²⁺ uptake at 60 min was taken as 100%. The results represent mean±SD of 3 experiments. The inhibitory effect of ryanodine was significantly less than that of CPA at 30 minutes (P<0.05). The effect of ryanodine plus CPA is greater than ryanodine alone at 30 minutes (P<0.05).
Figure 29. The effect of ryanodine on Ca\textsuperscript{2+} release. The results are mean±SD from 3 experiments. The microsomal vesicles were preloaded for 30 minutes with Ca\textsuperscript{2+}. Ca\textsuperscript{2+} release was initiated by the addition of 1 ml release buffer B including: EGTA 100 μM, KCl 0.3 M and sucrose/imidazole 250/50 mM, pH 7.4.

Table 8. The effect of ryanodine and CPA on ouabain stimulated Ca\textsuperscript{2+} uptake at submaximal concentrations. Both ryanodine (5x10\textsuperscript{-4} M) and CPA (1 μM) inhibited Ca\textsuperscript{2+} uptake. When both agents were added together at these concentrations, a partially additive inhibition of uptake was observed (P<0.05). The result represents mean±SD from four different experiments. The Ca\textsuperscript{2+} uptake in the absence of both ryanodine and CPA is referred to as 100%.

<table>
<thead>
<tr>
<th></th>
<th>RYA</th>
<th>CPA</th>
<th>RYA + CPA</th>
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<tbody>
<tr>
<td>Ca uptake(%)</td>
<td>70±7</td>
<td>75±8</td>
<td>58±6</td>
</tr>
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</table>

5. DISCUSSION

The present study for the first time provides direct evidence for the presence of specific ryanodine binding sites in smooth muscle and defines their location in the cell. This work also represents the first detailed characterization of [3H]ryanodine binding site in smooth muscle membrane preparations. The followings are the major findings, each of which will be discussed separately:

1. [3H]ryanodine binding in RVD smooth muscle
   a. High affinity [3H]ryanodine binding site was found to be located on SR membranes of RVD smooth muscle.
   b. General characteristics of the ryanodine receptor.
   c. [3H]ryanodine binding to the receptor is regulated by many factors.
   d. The density of ryanodine receptors varies in different smooth muscle preparations

2. [3H]ryanodine binding in DMA smooth muscle
   e. High and low affinity binding sites were identified.
   f. General characterization of ryanodine receptor.

3. The effects of ryanodine on Ca\textsuperscript{2+} transport by microsomal fraction
   g. Effect of ryanodine on Ca\textsuperscript{2+} uptake
5.1 Characterization of [H]ryanodine binding in RVD smooth muscle

5.1.1. Subcellular localization of [H]ryanodine binding site

In smooth muscles, many functional studies suggest that ryanodine acts on internal Ca\(^{2+}\) stores. However, until now, the existence of such a site for ryanodine was not directly demonstrated. Chadwick et al. (1991) failed to detect ryanodine binding site in smooth muscle although they successfully isolated another Ca\(^{2+}\) release channel (IP\(_3\) receptor) from the same tissue. In this laboratory, the techniques for separating smooth muscle cells from connective tissue or nerve varicosities, and its PM and SR from their subcellular organelles have been well established for RVD (Kwan et al., 1983a, 1983b). The purity of each fraction was evaluated by many different markers. Mg-ATPase and 5'-nucleotidase were all shown to be PM associated enzymes (Kwan et al., 1983a, 1983b). NADPH-cytochrome c reductase is a widely used putative SR marker enzyme in smooth muscle membrane fractionation (Kwan et al., 1983a, 1983b). Cytochrome C oxidase is a marker of the inner mitochondrial membrane and has commonly been used as a mitochondrial marker. The enrichment of SR membrane in the F3 fraction prepared from RVD smooth muscle microsomes was also demonstrated by NADPH-cytochrome C reductase and also by using the selective SR Ca\(^{2+}\) pump inhibitor, CPA (Dusty et al., 1993). Oxalate predominantly enhanced Ca\(^{2+}\) uptake by F3 fraction. This increased uptake could be inhibited by CPA.

In the present study, the subcellular distribution of [H]ryanodine binding was first

compared with that of different membrane markers. The results led to the conclusion that the binding site for [H]ryanodine is in the smooth muscle SR membrane. In brief, [H]ryanodine binding site is not on PM membrane, since the distribution of [H]ryanodine binding differed markedly from that of the various PM markers employed.

In figure 8, the M2C fraction showed high activities of enzymes which were used as PM and SR markers. Therefore, M2C contained both PM and SR membrane. The PM and SR membrane in M2C fraction were further separated by centrifugation on a discontinuous sucrose gradient. As shown in figure 8, PM and SR membrane were enriched in F2 and F3 fractions, respectively; i.e. marker enzymes activities were found to be highest in F2 for PM and in F3 for SR. These results are also in agreement with previous findings in the same tissue as characterized with specific marker enzymes activities, morphological approaches and the calcium accumulation technique (Kwan et al., 1983a). The enrichment of PM in F2 was also marked by the presence of surface membrane α-adrenergic receptors, [H]prazosin binding (Sil et al., 1989), and L-type Ca\(^{2+}\) channel shown by [H]FGP200-110 binding (Wibo et al., 1988) (figure 9). Receptors for these ligands were PM specific and F2 contained the highest number of binding sites for these ligands. During membrane fractionation, the enrichment of [H]ryanodine binding sites closely paralleled the level of SR membrane marker, NADPH-cytochrome c reductase, but not the levels of PM markers.

The ryanodine receptor has been identified and purified from brain tissues (McPherson et al., 1991) as well as striated muscle. The brain ryanodine binding site is predominantly on ER membranes and represents a Ca\(^{2+}\) release channel. The rat vas deferens is a highly innervated tissue. However, several lines of evidence suggest that the [H]ryanodine binding site that we detected was not of neuronal origin. First of all, the smooth muscle cells are innervated by nerve varicosities which do not contain morphologically distinct endoplasmic reticulum (ER) structures. In contrast to the varicosities, the neuronal cell bodies that contain ER are not detected morphologically near smooth muscle cell. Secondly, a high affinity [H]jassoxatol binding site has been demonstrated to be present in nerve varicosities but not in smooth muscle membranes (Ahmad et al., 1988). In the present study (figure 5), the distribution of [H]ryanodine binding does not parallel that of [H]jassoxatol binding. Based on these findings, we conclude that [H]ryanodine binding sites are of smooth muscle origin and located on SR membranes. These findings agree with our functional results that ryanodine acts functionally on a Ca\(^{2+}\) channel located on rat vas deferens smooth muscle SR (Bourcier et al., 1991a).

Another concern is the possible physical connection of the ryanodine receptor with PM. In skeletal muscle, ryanodine receptors form the foot structure (Ashley et al., 1991). This foot structure is closely associated with dihydropyridine binding sites (or dihydropyridine receptor, DHP) on PM which have been proposed to serve as a voltage sensor in skeletal muscle. The co-localization of DHP and ryanodine receptors was reported in rat cardiac muscle (Wibo et al., 1991). Direct physical interaction between the DHP receptor and the ryanodine receptor was proposed in striated muscle (Rios and
Brem, 1987). Recently a complex which contained both DEHP receptor and ryanodine receptor was isolated from skeletal muscle (Martí et al., 1994). This complex can be immunoprecipitated with antibodies directed against the ryanodine receptor or against the subunits of the DEHP receptor in a concentration dependent manner. Functional studies from our laboratory suggest that ryanodine-sensitive Ca\(^{2+}\) release channels in the SR may also be associated with the voltage-gated Ca\(^{2+}\) channels in the PM. In dog tracheal (Boureau et al., 1991) and mesenteric artery (Boureau et al., 1991a), refilling of ryanodine sensitive and agonist-sensitive internal Ca\(^{2+}\) stores was modulated by BAY K 8644 and nifedipine. However, in the present study, our findings indicate that (P)Henyodine binding sites are separately located on the PM and SR as shown in figure 9. This strongly suggests that these two ligands binding sites are not associated in smooth muscle. Unlike in skeletal muscle, there is no well-defined foot structure in smooth muscle cell. However, in electron microscopy, some SR structures have been observed to be very close to the PM (Devine et al., 1972). If the ryanodine receptor and the L-type Ca\(^{2+}\) channels were indeed physically associated in the intact cell, such a linkage may be disrupted during the membrane isolation.

Cavocells are structures providing a series of cavities located on the cell surface of smooth muscle. Recently, evidence indicates that cavocell contains many protein structures which have been identified previously in other membrane locations (Pujimoto, 1993; Pujimoto et al., 1992). These cavities may serve as sites for many molecules for transport and possibly for signal transduction. For our purposes, the newly identified protein structures in cavocells may include the Ca\(^{2+}\) binding protein (calmodulin), Ca\(^{2+}\) pump and possibly an IP\(_3\)-like receptor. One of the key steps in these studies is the isolation of cavocell structures from smooth muscle cells. GPI (glycoprophosphatidylinositol) linked proteins were shown to be purified with cavocell (Sergiocomo et al., 1993). 5' nucleotidase is a GPI linked protein and could be cotranschleated in association with cavocell. This enzyme may serve as a useful marker for membrane derived from cavocell during the isolation. In the present study, the enrichment of ryanodine binding and 5' nucleotidase activity was not paralleled during membrane isolation. This finding lessens the possibility that there is a structural connection between ryanodine receptor and cavocell.

5.1.2 The (P)Henyodine binding properties to RVD smooth muscle

Characterization of the ryanodine receptors revealed that a high affinity binding site (K\(_b\) around 6 nM) exists in RVD smooth muscle. This K\(_b\) value was similar to those reported for skeletal and cardiac muscles in some cases (Lai et al., 1989; Inui et al., 1988). However, the maximum (P)Henyodine binding in smooth muscle was much lower than that in skeletal muscle and cardiac muscle. This is probably due to the smaller SR content in smooth muscle cell compared to striated muscle. In saturation experiments, the low-affinity binding site found in striated muscle was not directly identified in our preparations. The Scatchard plot of the saturation isotherms resulted in a straight line (figure 10) suggesting a single class of binding sites. The n\(_b\) from saturation experiment was very close to unity. This suggests a lack of co-operativity between ryanodine receptors. Results from the competition study in RVD smooth muscle membranes also shows one high affinity binding site. The calculated K\(_b\) was very close to the K\(_b\) value estimated from the Scatchard plot. The Hill coefficients obtained from competition experiments were also very close to one. This suggests the presence of one class of binding sites with uniform affinity. The computer analysis of the binding data from the competition experiments was also found to fit best to a one binding site model.

However, the results from the dissociation experiments suggest a possible existence of a low affinity binding site for ryanodine in this tissue. Under our experimental conditions, (P)Henyodine could not be dissociated from its high affinity binding sites by excess amount of unlabelled ryanodine (10 \(\mu\)M). However, (P)Henyodine dissociation could be initiated by a 20 fold dilution with the wash buffer. Similar phenomena have been previously reported in striated muscle by another group (Lai et al., 1989). A possible reason is the presence of a low affinity binding site, which, when occupied, inhibits dissociation of ryanodine from its high affinity binding site as suggested for striated muscle. In striated muscle, two binding sites with different affinities were demonstrated by Scatchard analysis of saturation experiments. In the presence of (P)Henyodine at concentrations near the high affinity K\(_b\) value (low nanomolar value), (P)Henyodine may predominantly be bound to the high affinity binding site. When added in a high concentration (10 \(\mu\)M) following (P)Henyodine binding, the unlabelled ryanodine may also bind to the low affinity binding site, although this was undetectable in saturation or competition studies. The occupation of low affinity binding sites appeared to cause a conformational change of the receptor which locked (P)Henyodine in its high affinity binding site and prevented its dissociation. However, when a dilution method is used to initiate dissociation, few low affinity binding sites are occupied, and (P)Henyodine can dissociate from its high affinity binding sites. So far there is no direct evidence for this explanation in vas deferens SR membrane.

The pharmacological significance of the high affinity ryanodine receptor in smooth muscle has not been revealed so far. In functional studies, the commonly used ryanodine concentrations are within the micromolar range. These concentrations are much higher than the K\(_b\) value obtained in this study (about 6 nM), but are close to the low affinity value reported for striated muscle. In striated muscle, dual effects of ryanodine on the Ca\(^{2+}\) release channel have been reported: it locks the channel in the open state at low concentrations and closes the channel at high concentrations. These effects may be mediated by high and low affinity binding sites, respectively. However, in smooth muscle, these dual functional effects have not been observed. Most reports indicate that ryanodine opens the Ca\(^{2+}\) channel on SR of smooth muscle. In recordings from single channels which were isolated from aortic smooth muscle, ryanodine closed the channel at concentration near 1 mM, but an effect of low concentrations of ryanodine on Ca\(^{2+}\) channel activity was not observed (Hermann-Frauck et al., 1991).

Several reasons may contribute to this discrepancy between binding and functional studies. 1. Although high concentrations of ryanodine were added in the buffer in these...
functional experiments, the actual concentration of ryanodine that reached and accumulated near its SR binding site is unknown. Due to the differences between the conditions in the cytosol and those in the ligand binding study, the affinity may vary. Ryanodine binding was found to be regulated by many factors as shown in my study and as previously shown in striated muscle.

In RVD smooth muscle, the existence of a low affinity binding site is still unclear. However, the existence of such a site is suggested by the difference between the $K_d$ value detected in this study (about 5 nM) and the high concentrations used in functional studies to effectively regulate the Ca$^{2+}$ release channel (micromolar range).

Although differences between experimental conditions for binding and functional studies may contribute to the differences between these two values, the possible existence of a low affinity binding site may also partially explain this discrepancy. In functional studies, it is possible that ryanodine affects Ca$^{2+}$ transport by acting on the low affinity binding site. The failure to detect this site in my study may be due to many reasons. One reason is that the concentration range of [3H]ryanodine used in saturation study may have been too narrow (0.5 - 35 nM) to allow the identification of the low affinity binding site in the μM range. In striated muscle, the low affinity binding site has an affinity for ryanodine close to micro molar range. Another reason is that the population of low affinity binding site may be too small to be detected in this smooth muscle. A third reason may be that the binding conditions employed in this study may not favor [3H]ryanodine binding to its low affinity binding site. Many factors can affect [3H]ryanodine binding to its receptor as shown in striated muscle as well as in the present study. The effects of different binding conditions on the binding affinity were reflected in the reports about in striated muscle. For example in table 1, the low affinity binding sites were not always observed in striated muscle depending on the binding and membrane isolation conditions used in these studies.

Another question which must be answered in order to relate binding to functional studies is, what is the explanation of the discrepancy between the association rate of [3H]ryanodine binding and the time required for the functional effect of ryanodine. The association rate determined in binding studies is substantially slower than the rates deduced from functional assays. In the present study, [3H]ryanodine binding reached a maximum after two hours as was reported for striated muscle. However, ryanodine is a lipid soluble compound which can easily enter the cell. In a functional study using intact guinea pig aorta, the addition of ryanodine for only 5 minutes was sufficient to decrease the contraction induced by caffeine (Aoki and Ito, 1988). It took 20 minutes to reach its full effect. It is suggested that maximum binding may not be required for the initiation of ryanodine’s action. Further studies are needed to resolve this discrepancy. Another possible reason for this discrepancy is that the effect of ryanodine observed in functional studies was due to the inhibition of Ca$^{2+}$-pump in SR membranes as suggested in the present study. As shown in figure 26, the inhibition of Ca$^{2+}$ uptake by ryanodine could be observed as early as 5 minutes after the Ca$^{2+}$ uptake was initiated.

5.1.3 Regulation of [3H]ryanodine binding in RVD smooth muscle

The effect of the membrane purification procedure on [3H]ryanodine binding was first of all examined. As shown in figure 11, the $K_d$ values remained the same in PNS, MII2 and P2 with different enrichment of SR membranes. This indicates that the affinity ($K_d$) of the ryanodine receptor was not changed during membrane purification. However, many factors were found to affect [3H]ryanodine binding to its high affinity binding site.

These included: Ca$^{2+}$, Mg$^{2+}$, Na$^+$, K$^+$, rethamine red, caffeine, pH and sucrose. These effects shared some similar features with those caused by the same agents in striated muscle, but they were not identical.

5.1.3.1 Ca$^{2+}$ dependence of [3H]ryanodine binding

As shown in figure 14, [3H]ryanodine binding to RVD smooth muscle SR was highly Ca$^{2+}$ dependent. Ca$^{2+}$, over a very narrow concentration range, stimulated [3H]ryanodine binding. The half maximum binding occurred at about 140 nM of free Ca$^{2+}$, which is very close to the resting cytosolic Ca$^{2+}$ concentration. The affinity of the [3H]ryanodine binding site was not significantly changed in the presence of Ca$^{2+}$ concentrations within the physiologically relevant range (0.14 μM to 2 μM) (table 6). However, further increases in Ca$^{2+}$ concentration could enhance the affinity of the [3H]ryanodine binding site. These features are different from those observed in striated muscle and liver. In the liver, [3H]ryanodine binding was Ca$^{2+}$ independent (Shoham-Barmatz et al., 1993). In both skeletal and cardiac muscle, Ca$^{2+}$ increased [3H]ryanodine binding in a concentration dependent manner at lower concentrations. After reaching a maximum with increasing Ca$^{2+}$ levels, [3H]ryanodine binding was inhibited by further increase of Ca$^{2+}$ concentration (Penuel et al., 1985; Michalak et al., 1988). Compared with findings in skeletal muscle, [3H]ryanodine binding to cardiac muscle SR preparation was less sensitive to Ca$^{2+}$ (Penuel et al., 1985; Michalak et al., 1988). In RVD smooth muscle, the increase of [3H]ryanodine binding reached a maximum at 2 μM of free Ca$^{2+}$. The increase of Ca$^{2+}$ up to 500 μM did not further elevate [3H]ryanodine binding in smooth muscle. Unlike the findings from striated muscle, no inhibition of [3H]ryanodine binding was observed in the present study by higher Ca$^{2+}$ concentrations. It has been proposed that ryanodine bound preferentially to CICR channels which were in the open state (Iino et al., 1988). The presence of Ca$^{2+}$ in the binding medium may affect ryanodine binding by changing the state of the ryanodine receptor (opening it). The unknown state of the Ca$^{2+}$ channel in different preparations under different conditions may contribute to the discrepancies of [3H]ryanodine binding levels reported by different groups.

5.1.3.2 The effects of caffeine, Mg$^{2+}$ and rethamine red on [3H]ryanodine binding

As described before, caffeine is known to activate CICR in smooth muscle. In functional study, the presence of caffeine in the physiological solution enhanced the potency of ryanodine which affected RVD smooth muscle (Bourassa et al., 1991a). In the present study, caffeine also enhanced [3H]ryanodine binding, especially in the
presence of a low concentration of Ca^{2+} which was close to the resting cytosolic
condition. Caffeine may act by increasing the Ca^{2+}-sensitivity of the [dihydropyridine]
binding. In skeletal muscle, in the presence of 1 mM Mg^{2+}, caffeine decreased the
threshold for Ca^{2+} activation of [dihydropyridine] binding and increased the affinity of the
activator site for Ca^{2+} (Pessah et al., 1987). However, caffeine alone caused only small
increases in affinity of the site for [dihydropyridine] in skeletal muscle (Cho et al., 1990).
Caffeine did not significantly affect B_{max} as shown in cardiac muscle, but increased the
affinity for [dihydropyridine] binding in the presence of Mg^{2+} (Pessah et al., 1990).

Mg^{2+} and ruthenium red are two factors known to inhibit CIIR channel in both
striated muscle and smooth muscle. Both Mg^{2+} and ruthenium red inhibited
[dihydropyridine] binding to smooth muscle in my study. In skeletal muscle, Mg^{2+} inhibited
[dihydropyridine] binding with an IC_{50} of about 0.3 mM, and 10 mM Mg^{2+} completely
inhibited binding (Pessah et al., 1986; Cho et al., 90; Zimanyi and Pessah, 1991).
Ruthenium red in the nanomolar range, inhibited [dihydropyridine] to its binding site in
striated muscle. Both compounds caused a decrease in affinity of the site for
[dihydropyridine] in both skeletal and cardiac muscles. However, [dihydropyridine] binding to
RVD smooth muscle SR was less sensitive to the inhibitory effect of these two factors
as shown in figures 19 and 20. My study shows that ruthenium red inhibited [dihydropyridine]
to RVD smooth muscle SR between 1-10 mM and the inhibition is incomplete. Mg^{2+}
inhibited [dihydropyridine] binding to smooth muscle SR only at higher concentrations (5-20
mM). At 20 mM, the highest concentration used in my study, Mg^{2+} caused about 60%

knowing the effects of these regulatory factors on [dihydropyridine] binding, we can
optimize our binding condition, e.g. Mg^{2+}-free and high-ionic-strength medium. This
condition enabled the better characterization of [dihydropyridine] binding site in some
other smooth muscle tissues, which contain a relatively lower level of ryanodine
receptor. In the present study, DMA was chosen for more detailed study as will be
discussed in the next section.

As shown in figure 17, [dihydropyridine] binding to smooth muscle microsomes
reached a maximum in a slightly alkaline, but still physiologically relevant, range. This
result was quite different from that observed in skeletal muscle. In our comparative
experiment with a skeletal muscle preparation, the optimal pH for [dihydropyridine] binding
was greater than 9. A similar result was also reported in cardiac muscle membranes; i.e.,
[dihydropyridine] binding did not reach a plateau at pH values up to 8.6 (Michalski et al.,
1988).

5.1.3.4. Density of [dihydropyridine] binding sites varied among different smooth muscles

Ryanodine has been widely used as a pharmacological tool to study excitation-
contraction coupling in different smooth muscles. We also examined [dihydropyridine]
binding to different smooth muscle preparations and the results are listed in table 7,
which also listed result of skeletal muscle for comparison. In these experiments, the
membrane preparation procedures were similar for the different tissues and [dihydropyridine]
binding to these membrane preparations was carried out under identical conditions.
inhibition of [dihydropyridine] binding to RVD SR. Mg^{2+} may affect the state of ryanodine
receptor (Ca^{2+} channel) by direct competition between Mg^{2+} and Ca^{2+} for the activator
site, therefore it inhibits [dihydropyridine] binding.

5.1.3.3. The effects of ionic strength and pH on [dihydropyridine] binding

Both RCI and NaCl significantly increased [dihydropyridine] binding to RVD smooth
muscle SR in the presence of a maximally effective Ca^{2+} concentration. These effects
could be due to the elevation of ionic strength as has been reported in striated muscle.
However, as shown in figure 16, the presence of sucrose in the binding medium, instead of
RCI or NaCl, also increased the [dihydropyridine] binding level but to a smaller extent.
Therefore, both increased ionic strength and increased osmolality may contribute to the
augmentation of [dihydropyridine] binding in RVD SR, but changes in ionic strength were
more effective. These effects are not fully understood. In different tissues or under
different experimental conditions, the effect of NaCl on [dihydropyridine] binding was
different. For example, in the absence of NaCl, no binding of [dihydropyridine] to skeletal
SR was obtained, while [dihydropyridine] binding to cardiac SR was 80% of that observed
in the presence of NaCl (Michalski et al., 1988). Both affinity and maximum binding site
for [dihydropyridine] were shown to be increased under the high salt condition in cardiac
muscle preparation (Matsudou et al., 1989). The higher ionic strength and higher
osmolality may change the state of the Ca^{2+}-channel to one which favours [dihydropyridine]
binding. The physiological relevance of these ionic effects remains unclear. However,

Among the tissues examined, the skeletal muscle contained the highest density of
ryanodine binding sites. However, within tissues in the smooth muscle group, smooth
muscle from vas deferens of rat, guinea pig and dog contained the highest density of
[dihydropyridine] binding sites. The level of [dihydropyridine] binding was very low or
negligible in some smooth muscle preparations even though functional effects of
ryanodine have been demonstrated in these tissues; e.g. dog trachea (Bourguet et al.,
1991b). The reasons for the lack of correlation between functional and radioligand
binding studies are still not clear. Also, at present, there is no clear structural basis for
these differences.

Among the smooth muscles examined in this study, the varied levels of
[dihydropyridine] binding to the SR seemed not to be due to differences of SR content in
these tissues. According to studies in smooth muscle of rabbit, the large conduit arterioles
contain a higher SR content than do smaller arterioles (Devine et al., 1972). However, as
shown in table 7, the membranes prepared from aorta smooth muscle of rat and dog have
fewer [dihydropyridine] binding sites than do the corresponding mesenteric arterioles. The
densities of nerve varicosities were different among the smooth muscles examined.
However, this varied innervation in different smooth muscles apparently did not
contribute to the density differences of [dihydropyridine] binding sites. The [dihydropyridine]
binding and [dihydropyridine] binding have been compared in the same membrane
preparations obtained from different smooth muscles. As shown in figure 21, no
correlation was found between the density of anaxotxin binding sites and the density

Among the tissues examined, the skeletal muscle contained the highest density of
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binding studies are still not clear. Also, at present, there is no clear structural basis for
these differences.
(\textsuperscript{3}H)ryanodine binding sites. In the present study, the enrichment of SR in various microsomal fractions of different smooth muscle is not clear. The varied enrichment of SR in microsomal fraction may contribute to varied densities of (\textsuperscript{3}H)ryanodine sites among different smooth muscles. To answer this question, the enrichment of SR in microsomes prepared from different smooth muscle should be examined under the same conditions.

It is not clear whether species differences were involved in the varied density of ryanodine binding sites in different smooth muscles. As shown in table 7, the (\textsuperscript{3}H)ryanodine binding level in dog vas deferens smooth muscle is lower than that of RVD smooth muscle. However, the levels of (\textsuperscript{3}H)ryanodine binding to vascular smooth muscles of both dog and rat were very close (table 7). It is possible that smooth muscles from some organs have less ryanodine binding sites than others. Alternatively, special conditions may be required to detect such binding site in certain smooth muscles. Application of an antibody against smooth muscle ryanodine receptor might help to solve this issue. As shown in table 7, DMA contains a maximally high level of (\textsuperscript{3}H)ryanodine binding sites which allowed the further characterization of this receptor.

5.1.3.5. Possible relation between ryanodine and IP\textsubscript{3} binding sites

As mentioned before, ryanodine binding site on SR is considered to represent binding to the CICR channel. Another type of Ca\textsuperscript{2+} release channel (ICR) is also abundant in some smooth muscles. Both channels have been demonstrated to be located on SR and contribute to the release of intracellular Ca\textsuperscript{2+}. However, the distribution of these two channels within smooth muscle cells is still not clear. Evidence from functional studies showed that some intercalated Ca\textsuperscript{2+} pools may contain only one type of Ca\textsuperscript{2+} channel and some may contain both (Zuo, 1988). However, in these functional studies, it was assumed that ryanodine only affected the CICR channel. My study has shown that ryanodine at higher concentrations may inhibit the SR-Ca\textsuperscript{2+} pump. Ryanodine could depoly SR by opening channel to release Ca\textsuperscript{2+} or by inhibiting the SR-Ca\textsuperscript{2+} pump to block Ca\textsuperscript{2+} uptake into SR. If this is true, the interpretation of previous functional studies may be misleading.

Our results may suggest the variation in the relative importance of the two different Ca\textsuperscript{2+} channels in different smooth muscle cells. Those smooth muscle cells which contained more CICR Ca\textsuperscript{2+} channels (ryanodine binding site) may have fewer ICR Ca\textsuperscript{2+} channels (IP\textsubscript{3}, binding site). For example, in intestinal smooth muscle, the level of (\textsuperscript{3}H)ryanodine binding sites is very low, but the level of [\textsuperscript{3}H]IP\textsubscript{3} binding sites is relatively higher (White and Godfraind, 1994). The ratio of [\textsuperscript{3}H]ryanodine and [\textsuperscript{3}H]IP\textsubscript{3} binding sites is 1.9. No test has evaluated the relative contributions of these two channels to excitation-contraction coupling in this tissue. Therefore, the relative importance of the role of CICR and ICR Ca\textsuperscript{2+} channels in excitation-contraction coupling in smooth muscle is still far from being clear. Different excitation-contraction coupling mechanisms may exist among different smooth muscles and may contribute to the variable levels of (\textsuperscript{3}H)ryanodine binding in different smooth muscle preparations. This hypothesis can be further tested by comparing [\textsuperscript{3}H]IP\textsubscript{3} binding and (\textsuperscript{3}H)ryanodine binding levels in different smooth muscles, provided binding studies yield accurate measurements of channel densities. Ideally, those binding studies should be combined with functional studies, providing that the functional effects of ryanodine on smooth muscle SR is clarified (see below).

5.2. Characterization of (\textsuperscript{3}H)ryanodine binding in DMA smooth muscle

The microsomal fraction (M1C2) was used for a detailed characterization of ryanodine receptor in DMA smooth muscle. The isolation of microsomal membranes from this tissue followed the procedure which was developed in this laboratory (Kwan et al., 1983b). A previous study showed that the M1C2 fraction was enriched in PM and SR membranes. Since [\textsuperscript{3}H]ryanodine binding sites have been shown to occur on SR membranes in RVD smooth muscle, detailed characterization was carried out using M1C2 and the binding site was assumed to be on SR.

The specific [\textsuperscript{3}H]ryanodine binding site in DMA smooth muscle shared many characteristics with those from RVD and from skeletal and cardiac muscle. In saturation studies, the Scatchard analysis yielded one high affinity binding site with \(K_d=5\) nM and \(B_{max}=717\) fmol/mg. This \(K_d\) value is comparable to that obtained from RVD smooth muscle. In kinetic studies, the association was similarly slow and reached a plateau only after two hours (figure 24). Like that in RVD smooth muscle, the dissociation of [\textsuperscript{3}H]ryanodine binding from its high affinity binding site by an extensive amount of unlabelled ryanodine was very slow and incomplete (figure 24). However, the specific [\textsuperscript{3}H]ryanodine binding could be rapidly dissociated by 30 fold dilutions with wash buffer. As discussed in the RVD section, this may suggest the presence of a low affinity binding site in this tissue. Additional evidence for the presence of a low affinity binding site comes from competition studies. The \(K_d\) value calculated from [\textsuperscript{3}H]ryanodine competition
data in DMA microsomes was significantly less than one. This indicates that a second binding site may exist in this preparation. In further analyses of these results using the computer programme (COMTAPE), a two binding sites model fitted the data better than a single site model. The $K_d$ value for the high affinity site was very close to that determined from saturation and kinetic studies and the $K_d$ for low affinity binding site was 269 nM, which was close to some of the values reported under some experimental conditions in striated muscle (Pessah et al., 1985).

As shown in RVD, $[\text{H}]$ryanodine binding to SR in DMA was also Ca$^{2+}$ dependent (Figure 22). Ca$^{2+}$ increased specific binding in a very narrow range which was the same as that observed in RVD smooth muscle preparation, but different from other tissues. High concentrations of Ca$^{2+}$ (500 μM) did not inhibit $[\text{H}]$ryanodine binding to DMA SR.

Although different smooth muscles contained varied number of ryanodine receptors, our study suggested that this receptor is probably the same or similar in various smooth muscles based on a comparison of its features. However, the ryanodine receptor of smooth muscles had different ligand binding characteristics from that in striated muscle.

5.3 The effects of ryanodine on Ca$^{2+}$ transport by RVD smooth muscle microsomes

Although ryanodine has been widely used as a tool to effect the CICR channel in smooth muscle SR, the direct actions of ryanodine on this channel have not been widely studied. This is in contrast to the detailed studies of these channels in striated muscle. Having shown that ryanodine bound to the SR membrane, I tried to study the direct effect of ryanodine on Ca$^{2+}$ transport using isolated subcellular membrane vesicles. One of the difficulties in this study is that the MIIIC fraction was contaminated by vesicles derived from the PM. The inside-out vesicles derived from PM, like those from SR, also have a strong Ca$^{2+}$ accumulating ability. Potassium oxalate has been demonstrated to enter SR but not PM membrane vesicles and to precipitate Ca$^{2+}$. As a result the free Ca$^{2+}$ concentrations in the vesicles are decreased, allowing additional Ca$^{2+}$ uptake. In Ca$^{2+}$ uptake study, oxalate caused significant increase in Ca$^{2+}$ uptake by microsomal fractions isolated from RVD smooth muscle (Grover and Kwan, 1984). Oxalate was used as a tool to enhance the Ca$^{2+}$ uptake signal by vesicles derived from SR in this study. Mitochondria also contains a Ca$^{2+}$ pump and can accumulate large amounts of Ca$^{2+}$.

Aside has been shown to inhibit specifically the Ca$^{2+}$ pump on mitochondrial membrane. In the present study, this compound was also added in the uptake medium to eliminate any Ca$^{2+}$ accumulation by contaminating mitochondria membrane vesicles as shown previously by this laboratory (Kwan et al., 1983b). The present study showed that ryanodine could inhibit oxalate-nonensitive, oxalate-stimulated Ca$^{2+}$ uptake. However, ionophore, A23187, was used to deplete free Ca$^{2+}$ from the vesicles.

5.3.2 The effect of ryanodine on Ca$^{2+}$ uptake

As shown in Figure 27, ryanodine inhibited oxalate-stimulated Ca$^{2+}$ uptake in a concentration-dependent manner. There are two possible explanations for this effect: 1. Ryanodine increased membrane permeability, therefore causing Ca$^{2+}$ leakage from the vesicles. 2. Ryanodine inhibited the Ca$^{2+}$-pump providing uptake. To clarify these questions, experiments were performed to test the time dependence of this inhibitory effect of ryanodine on Ca$^{2+}$ uptake. If ryanodine indeed increased membrane permeability, a progressive decrease in Ca$^{2+}$ uptake would be observed only when the rate of leakage (enhanced by Ca$^{2+}$ accumulation) exceeded the accumulation rate. However, in the present study, ryanodine at 10$^{-7}$ M inhibited 40Ca uptake as early as five minutes after initiation of the Ca$^{2+}$ uptake experiment (Figure 28). This indicates that this effect is most likely due to the inhibition of the Ca$^{2+}$ pump on SR. In support of this, ryanodine was also found to have partial additive effect to that of a submaximal concentration of CPA (Table 8), a known Ca$^{2+}$ pump inhibitor. However, it had no additional effect when added in the presence of a CPA concentration which caused maximal pump inhibition.

CPA was shown to specifically inhibit Ca$^{2+}$ pump located on SR in skeletal muscle (Greger et al., 1988). CPA has later been shown to have a similar inhibitory effect on SR Ca$^{2+}$ pumps in smooth muscle (Ding and Kwan, 1991). In our laboratory,
the effect of CPA on the Ca\(^{2+}\) pump was also studied using mitochondria membrane vesicles derived from RVD smooth muscle SR (Duchy et al., 1993). This compound was shown to be a potent SR Ca\(^{2+}\) pump inhibitor (maximum inhibition about 100% at 10 \(\mu\)M or more). It had little or no effect on the PM Ca\(^{2+}\) pump. Compared with the effect of CPA, ryanodine was less potent in inhibiting the oxalate-stimulated Ca\(^{2+}\) uptake (via the SR Ca\(^{2+}\) pump) as shown in the present study. The inhibition of ryanodine on Ca\(^{2+}\) uptake was incomplete at 1 mM, since addition of 30 \(\mu\)M CPA further decreased the level of Ca\(^{2+}\) accumulation in the SR vesicles (Figure 28). The addition of both compounds together at submaximal concentrations showed a greater inhibition on Ca\(^{2+}\) uptake than that by either agent added separately (Table 3). Therefore ryanodine may act to inhibit uptake into the same Ca\(^{2+}\) pool as does CPA, possibly by the similar mechanism.

In some early works in skeletal muscle and cardiac muscle SR preparations, ryanodine was noted to affect ATPase activity in addition to acting on the CICR channel. However, these observations were controversial. In rabbit skeletal muscle, ryanodine activated the Ca\(^{2+}\) -ATPase of SR with \(K_a=1.49\times10^5 \text{ M}^{-1}\), estimated by measuring the inorganic phosphate released from ATP. However, ryanodine inhibited the Ca\(^{2+}\) uptake level by altering Ca\(^{2+}\) permeability in the same study (Pilheht, 1973). In cardiac muscle SR preparation, ryanodine (100-833 \(\mu\)M) slightly inhibited the Ca\(^{2+}\)-ATPase, but increased oxalate-stimulated Ca\(^{2+}\) uptake (Jones et al., 1979; Feher and Lipford, 1985; Feher et al., 1985). This may have been due to the inhibition of leakage of Ca\(^{2+}\) by way of the CICR channel. In support of this, ryanodine (400 \(\mu\)M) inhibited Ca\(^{2+}\) efflux from purified cardiac SR vesicles (Chamberlain and Fisher, 1984).

5.3.3 The effect of ryanodine on Ca\(^{2+}\) release

In this study, using Ca\(^{2+}\) transport as the index for the channel function, we did not observe functional CICR channels in our RVD smooth muscle SR preparation. Ryanodine (10^{-5} \(\text{M}\)) did not significantly alter Ca\(^{2+}\) release rates (Figure 29). Both Mg\(^{2+}\) and ruthenium red have been reported to inhibit CICR channel activity in striated muscle and smooth muscles (Chamberlain et al., 1984; Endo, 1985). They were also demonstrated to inhibit [H]ryanodine binding to CICR channel in the present study. However, they did not change the Ca\(^{2+}\) release rate under the conditions used in the Ca\(^{2+}\) transport experiments. Thus, functional CICR channel could not be demonstrated in Ca\(^{2+}\) efflux experiments. Since we have demonstrated specific [H]ryanodine binding, the ryanodine binding site on SR is preserved during the membrane isolation process. However, it is possible that the CICR channel following isolation becomes non-functional in this RVD smooth muscle microsomal fraction. Any Ca\(^{2+}\) release could be through a non-specific leakage pathway. An early report has also shown the possible loss of CICR channel activity in isolated SR from dog aortic smooth muscle (Bahrild and Wiener, 1988). In that study, SR, but not caffeine, could induce Ca\(^{2+}\) release from actively loaded SR vesicles of aortic smooth muscle. Under identical condition, caffeine could induce Ca\(^{2+}\) release from SR vesicles prepared from skeletal muscle.

In my radioligand binding study, [H]ryanodine binding to SR reached the plateau in about two hours. However, although ryanodine was allowed to act for less than one hour in my functional study, the short incubation time for ryanodine with SR membrane vesicles in Ca\(^{2+}\) release studies seems not to be the reason why ryanodine did not affect Ca\(^{2+}\) release. In our study, the precipitation of membrane vesicles with ryanodine before initiating Ca\(^{2+}\) release did not change the Ca\(^{2+}\) release rate. In functional study with intact smooth muscle cell preparations from bovine and porcine coronary arteries, ryanodine alone caused a rapid increase in intracellular Ca\(^{2+}\) level. Only 3-6 minutes were required to reach half maximum increase (Wagner-Mann et al., 1992).

The failure to observe effects of ryanodine on the CICR channel in the present study may imply that suitable conditions are required in Ca\(^{2+}\) transport experiment and to restore a channel function during membrane preparation. In striated muscle, that the ryanodine binding site is the CICR channel has been well studied by various experimental methods and the protein has been purified and cloned. However, the reported functional effects of ryanodine on this channel were still varied, depending on the methods used for membrane preparation and on the experimental procedures. As shown in table 3, the dual effects of ryanodine on Ca\(^{2+}\) channel were not always observed in different studies.

In studies of functional effects of ryanodine on Ca\(^{2+}\) transport in striated muscle preparations, the vesicles with passively loaded Ca\(^{2+}\) were used. In our study with smooth muscle, the microsomal fraction contained only a small amount of vesicles derived from SR. Most of the membrane vesicles were derived from PM. Therefore the passive loading of microsomal fraction with Ca\(^{2+}\) will mainly show the accumulation ability of vesicles derived from PM. In order to test Ca\(^{2+}\) transport by vesicles derived from SR, oxalate had to be used. The passive loading of vesicles requires the presence of a high concentration of Ca\(^{2+}\), but the oxalate precipitates the Ca\(^{2+}\) present in the buffer in such experiments precluding this approach.

The exact reason why we did not observe functional CICR channel in isolated microsomes is so far not clear. In striated muscle, some studies show that CICR channel function can be improved under certain conditions, e.g. the phosphorylation of ryanodine receptor. The ryanodine receptors from both skeletal and cardiac muscle, have been shown to be phosphorylated by many different protein kinases: Ca\(^{2+}\)-calmodulin-, cAMP-, and cGMP-dependent protein kinase (Sako et al., 1993). The phosphorylation sites on receptors have also been identified to be at serine-2809 in cardiac muscle and at serine-2843 in skeletal muscle. As increase in maximum [H]ryanodine binding has been observed after phosphorylation of the receptor isolated from cardiac muscles (Takasago et al., 1989). The phosphorylation of ryanodine receptor was also shown to increase Ca\(^{2+}\) channel sensitivity to various ligands in skeletal muscle (Hermann-Frank and Van Assen, 1993); for example: ATP and Ca\(^{2+}\). A suitable condition which can preserve or increase CICR channel activity, needs to be defined for Ca\(^{2+}\) transport experiment using smooth muscle preparations. It is also possible that CICR channel function is modulated by endogenous factor(s). Under my experimental conditions, it is possible that this factor was lost or the association between the this factor and CICR was
The facts that our binding studies gave values for pyranoide binding similar to those in relaxed muscle and that binding was modulated by agents known to affect the CICR channel suggest that receptor for pyranoide in RVD SR membranes is the CICR channel. However the functional studies suggest that RYA at higher concentrations may inhibit the SR Ca\(^{2+}\) pump. However, this effect does not seem to be mediated through the high affinity binding site which was detected in ligand binding experiments in this study. The high affinity RYA binding site probably represents the Ca\(^{2+}\)-release channel. A low affinity binding site in RVD smooth muscle which modulates pyranoide binding to the CICR channel and another site which represents a site of pyranoide’s action on the Ca\(^{2+}\) pump may both exist. Binding to one of these low affinity sites may modulate the inhibitory effect of RYA on the exosite-stimulated, ATP-dependent Ca\(^{2+}\) uptake.

5.4 The pharmacological and physiological significance and possible endogenous ligands for the pyranoide receptors

Unlike in skeletal muscle, the elevation of cytosolic Ca\(^{2+}\) in smooth muscle during excitation-contraction coupling is derived from both the extracellular space and the intracellular Ca\(^{2+}\) store. In some smooth muscles, it comes predominantly from extracellular source. The role of the internal Ca\(^{2+}\) store and release of internal Ca\(^{2+}\) through a CICR and IP\(_3\) mediated channel in smooth muscle is so far not well understood. One of the difficulties is the lack of the specific ligand for the internal Ca\(^{2+}\) channels. The finding of specific pyranoide binding site (pyranoide receptor or CICR channel) in different muscles and non-muscle tissues makes pyranoide a potentially valuable tool to label and control the CICR channel. My studies give support to the use of pyranoide as a valuable tool to evaluate the role of an internal Ca\(^{2+}\) release channel and the internal Ca\(^{2+}\) store in excitation-contraction coupling. Using pyranoide, progress has been made in the study of excitation-contraction coupling in skeletal muscle. Defects in the pyranoide receptor can severely affect the normal process of E-C coupling. For example, malignant hyperthermia is a genetic disease in which patients develop muscle rigidity and fever when exposed to anesthesia. The major cause of the disease was demonstrated to be the abnormal Ca\(^{2+}\) release channel (pyranoide receptor) in skeletal muscle SR (MacLennan and Phillips, 1992).

A large number of chemically diverse substances have been reported to act on the pyranoide-sensitive Ca\(^{2+}\) channel. Caffeine, Ag\(_2\)SO\(_4\), thymol, halothane, dextroseinibide and ATP all release Ca\(^{2+}\) by stimulating the SR pyranoide channel (Lai and Melissor, 1989).

Recently, another compound, chlorethanol was also found to release Ca\(^{2+}\) through pyranoide-sensitive Ca\(^{2+}\) channel on SR (Zoccatto et al., 1993). This compound released Ca\(^{2+}\) from the IP\(_3\)-insensitive store in skeletal muscle, cerebellar microsome and PC12 cells, a caffeine sensitive cell line. To explain the molecular mechanism by which such diverse substances can act on the same molecule, one must postulate the presence of multiple activator binding sites present on the large hydrophilic portion of the receptor.

This region must control a membrane-bound structure encompassing the ion-permeable channel. In the muscle cells, many factors may act on pyranoide receptor and therefore regulate Ca\(^{2+}\) channel activity.

However, the physiological ligand for this pyranoide sensitive Ca\(^{2+}\) channel activation is unknown. A large body of evidence shows that the pyranoide sensitive Ca\(^{2+}\) channel is different from the IP\(_3\)-sensitive Ca\(^{2+}\) channel. Therefore, IP\(_3\) is not likely to be the candidate. Efforts have been made in recent years to identify such an agent and some endogenous compounds have been proposed to serve as second messenger to trigger Ca\(^{2+}\) release through pyranoide sensitive Ca\(^{2+}\) channel under the physiological condition. As described before, Ca\(^{2+}\) itself can activate CICR channel and release Ca\(^{2+}\) from internal Ca\(^{2+}\) store. Ca\(^{2+}\) can serve as the second messenger to operate this channel, but there may also be other modulators of this channel.

Cyclic ADP-ribose (cADP-ribose), a metabolite of NAD\(^+\), is a candidate proposed recently. This compound has been shown to be a potent Ca\(^{2+}\)-stimulating agent and meets some of the criteria of an intracellular messenger. The enzyme responsible for synthesizing cADP-ribose has been found in rabbit liver, brain, heart, spleen and kidney (Rankino et al., 1989) and in pinusary cells (Kobayashi et al., 1991). Two isoforms of this enzyme, termed ADP-ribose cyclase, have been purified. A high affinity cADP-ribose binding site has been identified in sea urchin eggs microsome. The specific cADP-ribose binding site could not be competed for by IP\(_3\) and heparin (Lee, 1991). In a functional study in sea urchin eggs, cADP-ribose was shown to activate the same mechanism as caffeine and pyranoide did, e.g. CICR mechanism, but not the one on which IP\(_3\) acted (Gallione et al., 1991). In this cell, the cADP-ribose-induced Ca\(^{2+}\) release, but not IP\(_3\)-induced Ca\(^{2+}\) release, could be abolished by pretreatment with the pyranoide, caffeine or Ca\(^{2+}\) itself, indicating that cADP-ribose acted on the same Ca\(^{2+}\) pool as pyranoide did.

The role of cADP-ribose as an intracellular messenger is still questioned. Its effect on Ca\(^{2+}\) mobilizing activity has been shown only in sea urchin eggs. Skeletal muscle contains high level of pyranoide receptors (CICR channel). However, cADP-ribose seems not to release Ca\(^{2+}\) from rabbit glycinated skeletal muscle fibres in which the transduction pathway of excitation through T-tubule was disrupted (Gallione et al., 1991). These fibers failed to contract in response to cADP-ribose. No direct evidence is available showing that cADP-ribose binds to the same site as pyranoide does. This can be clarified by doing competition study between these two agents. It is also possible that cADP-ribose as an intracellular agent can cooperate with other agents in some tissue by modulating the Ca\(^{2+}\) channel, for example, by increasing the sensitivity of Ca\(^{2+}\) channel to Ca\(^{2+}\).

Anammum IV is another compound found to exist in the cells which could regulate Ca\(^{2+}\) channel activity. The anamnem form a family of calcium/phospholipid-binding proteins. The sequence analysis indicates that there are eight unique proteins in the anamnem family: annexins I-IV (Duan-Monnot et al., 1990). These proteins are unrelated to calmodulin, protein kinase C, or other calcium binding proteins with regard to
potential structural mechanisms for binding Ca\(^{2+}\) or phospholipid. The cellular roles for
the annexins have not been clearly defined. Evidence supporting annexin VI acts on
ryanodine receptor comes from recent functional study using single channel recording
technique. In an electrophysiological study, annexin VI increased both the probability of
opening and the mean open time of the channel which was isolated from rabbit skeletal
muscle SR and was incorporated into planar lipid bilayer (Diaz-Munoz and Hamilton,
1999). This effect was specific to the trans chamber, which represents the luminal side
of the SR. This channel activity was sensitive to both ruthenium red and ryanodine and
the effect of annexin VI was Ca\(^{2+}\) dependent. However, evidence is insufficient to claim
that annexin VI serves as physiological messenger. Direct evidence is not available that
this agent shares the same binding site with ryanodine.

6. CONCLUDING REMARKS

The present study demonstrates the presence of a specific high affinity ryanodine
binding site in RVD smooth muscle SR using radioligand binding technique. This binding
site is also present in many other types of smooth muscle. This provides direct evidence
of the existence of specific sites for the ryanodine actions which have been shown in
different smooth muscles by functional studies. The ryanodine receptor was characterized
in detail in RVD and DMA smooth muscle preparations. In addition to the high affinity
binding site, a low affinity binding site was also detected in DMA preparation. At high
concentrations, ryanodine has also been shown to inhibit Ca\(^{2+}\) uptake by subcellular
membrane vesicle derived from SR, possibly by acting on the SR Ca\(^{2+}\) pump. This effect
was partially additive to that of CPA, a selective inhibitor of the SR Ca\(^{2+}\) pump, at
submaximal concentrations of each, but not at maximal concentration.

Considerable differences were noted between ryanodine receptor from smooth
muscle and those from other tissues, although they share several similarities. This
suggests that ryanodine receptors in smooth muscle may be different from those of
other tissues in both structures and functions. In the present study, I have also shown that
significant differences in the density of this receptor occur among different smooth
muscle preparations. However, it is not yet possible to correlate such differences with
differences in functional studies. Further studies in this area may provide insight into
differences in excitation-contraction coupling mechanisms in smooth muscles.

The following related areas can be further investigated to better understand
ryanodine receptor functions in smooth muscle:

The relationships between the ryanodine and IP\(_3\) receptors in different smooth
muscles need to be clarified. These receptors represent two different Ca\(^{2+}\) release
channels. Either one may provide for release of intracellular Ca\(^{2+}\) and contraction or both
may be present. The existence of a majority of IP\(_3\) receptors in some smooth muscle cells
can explain why the level of ryanodine receptor is very low in some smooth muscles.
Such a clarification may also reveal the relative importance of these two channels in
different smooth muscles.

The physiological and/or pharmacological significance of ryanodine binding sites
detected in my radioligand binding study are not clear. A functional CICR channel should
be demonstrable using a SR-enriched subcellular membrane vesicle preparation. This
was not accomplished in this study. Suitable conditions for such a study need to be
established. The subcellular membrane isolation procedures may need to be modified to
preserve the CICR channel function. The functional effect of ryanodine on this channel
can then be tested at both low (lower K\(_\text{p}\) value) and high concentrations. The ryanodine
receptor can be purified from smooth muscle and can then be further studied at the
molecular level. These studies can directly reveal the differences between ryanodine
receptors from smooth muscle and those from other tissues.

A search for an endogenous ligand which can modulate a ryanodine sensitive Ca\(^{2+}\)
channel will be valuable. Although some compounds have been suggested to have such
properties, it is still far from clear that any function in smooth muscle.
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