Subcellular-membrane characterization of [^{3}H]ryanodine-binding sites in smooth muscle

Zhen-Du ZHANG, Chiu-Yin KWAN* and Edwin E. DANIEL

Smooth Muscle Research Program, Department of Biomedical Sciences, McMaster University Health Sciences Centre, Hamilton, Ontario, Canada L8N 3Z5

The plant alkaloid ryanodine, known to interact selectively with the intracellular Ca^{2+}-release channel in skeletal and cardiac muscles, has been repeatedly reported to affect smooth-muscle contractile functions that are consistent with its intracellular action at the Ca^{2+}-release channel sites. Direct evidence for the binding of [^{3}H]ryanodine to smooth-muscle membranes is sparse. Following our recent detailed characterization of functional effects of ryanodine and a preliminary report on the presence of [^{3}H]ryanodine binding sites in rat vas deferens smooth muscle, we now report in this study a detailed characterization of binding of [^{3}H]ryanodine to smooth muscle at the subcellular-membrane level. The ryanodine receptor in rat vas deferens muscle layer is primarily of smooth-muscle origin and is localized at the subcellular membrane site that is consistent with its role as a Ca^{2+}-release channel in the sarcoplasmic reticulum (SR). Ryanodine binding to its receptor is Ca^{2+}-dependent, with half-maximal binding occurring within the physiologically relevant cytosolic Ca^{2+} concentration. It is also sensitive to many factors, including change in Mg^{2+} concentration, ionic strength and osmolarity across the membrane vesicles. Agents known to inhibit (Ruthenium Red, Mg^{2+}) or enhance (caffeine, Na^+, K^+) the Ca^{2+}-induced Ca^{2+} release also inhibit or enhance the binding of ryanodine. Quantitative differences in ryanodine receptors exist among smooth muscles and do not seem to parallel their SR contents. Results from the present study indicate both the need and the basis for future investigations of the functional role of the ryanodine receptor in different smooth muscles.

INTRODUCTION

Ryanodine, a neutral alkaloid extracted from *Ryania speciosa*, has long been used as a pharmacological tool to study intracellular Ca^{2+}-release mechanisms in muscle [1]. Labelling of the Ca^{2+}-release channels on intracellular membranes using [^{3}H]ryanodine binding to the sarcoplasmic reticulum (SR) membrane has been well studied in skeletal muscle and cardiac muscle [2]. Morphologically, this ryanodine receptor is localized to the cisternae of SR and identified as part of the feet structures [3]. Ryanodine receptors from these tissues have been purified, reconstituted into lipid bilayers [3] and cloned [4].

Functional studies in smooth-muscle preparations revealed that ryanodine also modified mechanical responses [5–7] as well as Ca^{2+} mobilization [8–10]. In spite of the general sparseness of SR in smooth muscle, these functional studies suggest that ryanodine indeed interacts with the intracellular Ca^{2+}-release channels. However, feet structures like those found in skeletal and cardiac muscles have not been identified in smooth muscle. Also, direct radioligand-binding studies of [^{3}H]ryanodine to better-defined smooth-muscle membranes have been surprisingly few. Chadwick et al. [11] failed to identify any [^{3}H]ryanodine-binding sites in the crude membrane fraction of bovine aortic smooth muscle, although they were able to purify the receptor for Ins(1,4,5)P_3 from the same tissue. On the other hand, ryanodine receptors have recently been isolated from pig aortic smooth muscle and reconstituted into lipid bilayers to demonstrate its Ca^{2+}-release-channel activity [12], but the number of binding sites for [^{3}H]ryanodine was too low to allow further characterization of its subcellular localization and binding properties. Several functional studies have consistently suggested that the action of ryanodine is consistent with its intracellular action at the Ca^{2+}-release site of the agonist-sensitive Ca^{2+} store [13–15]. In a preliminary study using rat vas deferens (RVD) smooth muscle [13], we have observed specific binding of [^{3}H]ryanodine to the membrane fractions at a reasonably high level, so that further detailed characterization of [^{3}H]ryanodine binding could be made technically possible, as demonstrated in the present study.

The specific objectives of the present study include (*a*) detailed characterization of the subcellular localization of [^{3}H]ryanodine binding sites in RVD smooth muscle, (*b*) determination of the factors that modify or optimize the [^{3}H]ryanodine binding, and (*c*) investigation of the prevalence of ryanodine receptors in different smooth-muscle preparations under optimized binding conditions.

MATERIALS AND METHODS

Tissue handling and membrane preparation

Male Wistar rats, weighing 250–450 g, were killed by cervical dislocation. The whole length of both RVDs was excised and immersed immediately in ice-cold buffer containing 0.25 M sucrose and 10 mM imidazole, 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, small blood vessels and epithelium were meticulously removed from the smooth-muscle layers as previously reported [16]. Subcellular membranes were isolated by differential centrifugation and sucrose-density-gradient centrifugation in accordance with the procedures developed in this laboratory [16]. The flow chart of the membrane isolation procedure is shown in Figure 1. Membranes from other smooth-muscle tissues were studied only

Abbreviations used: SR, sarcoplasmic reticulum; RVD, rat vas deferens; MIC2, microsome 2; PNS, postnuclear supernatant; B_{max}, maximum binding.

* To whom correspondence and reprint requests should be addressed.

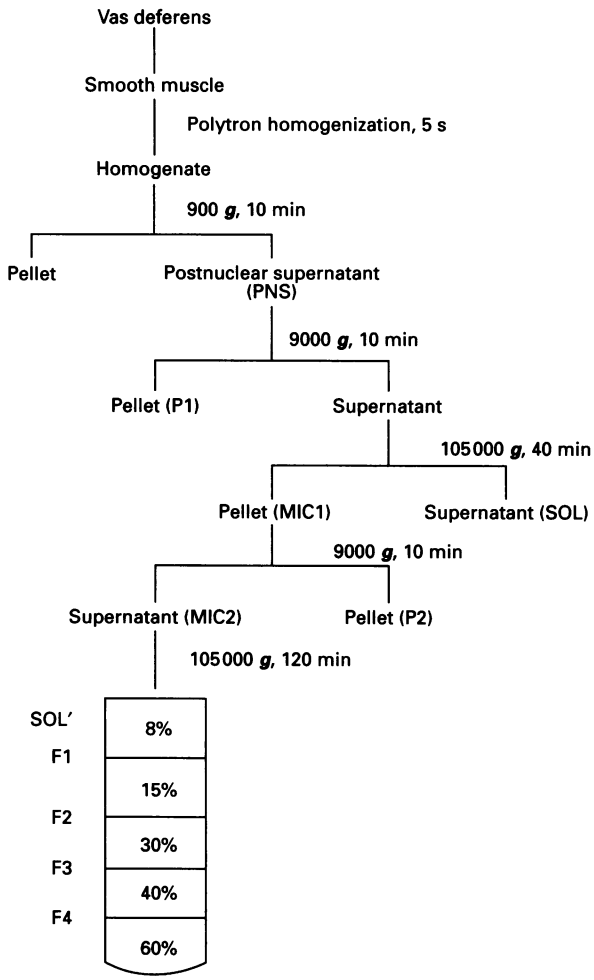

Figure 1 Flow chart for the isolation of subcellular membranes from RVD smooth muscle

at the microsomal level (MIC2) and were prepared under the same conditions as employed for RVD for comparative purposes.

Membrane markers

Several marker enzyme activities were monitored in each subcellular fraction in order to define the subcellular nature of isolated membrane fractions. Methods and the use of these membrane marker enzymes have been reviewed previously in detail [17]. NADPH–cytochrome *c* reductase activity, a putative SR marker, was measured by monitoring the reduction of cytochrome *c* by NADPH at 550 nm in a potassium phosphate buffer, pH 7.5, containing 1 mM KCN, 50 mM cytochrome *c* and 0.2 mM β-NADPH. Cytochrome *c* oxidase activity, indicative of the presence of mitochondrial inner membranes, was measured in 50 mM potassium phosphate buffer, pH 7.5, with 68 μM cytochrome *c* reduced by sodium hydrosulphite as the substrate.

5′-Nucleotidase (5′-AMPase) and Mg^{2+}-ATPase activities, the plasma-membrane markers, were determined as the amount of P_i released from 5 mM Na-AMP or 5 mM Na_2-ATP over a reaction time of 30 min or 15 min respectively at 37 °C in 50 mM imidazole, pH 7.5, containing 10 mM $MgCl_2$ [18]. [^{3}H]Prazosin binding [19] and ^{3}H-PN200-110 binding [20] were employed as additional plasma-membrane markers. Specific [^{3}H]saxitoxin binding (tetrodotoxin being used as displacing agent to define specific binding) was applied as a neuronal membrane marker in accordance with the method developed in this laboratory [21,22].

Binding of [^{3}H]ryanodine

Unless otherwise specified, binding of [^{3}H]ryanodine was routinely performed in a medium consisting of 0.3 M KCl, 0.5 mM $CaCl_2$, 25 mM Tris, 1 mM dithiothreitol and 5–7 nM [^{3}H]ryanodine at pH 7.4 in a total volume of 250 μl with an incubation period of 2 h. Binding was routinely carried out at 25 °C, since pilot experiments showed that increasing temperature to 37 °C did not give further improvement, whereas binding at 4 °C was drastically decreased. The non-specific binding was determined simultaneously in the presence of excess (10 μM) of unlabelled ryanodine. The specific binding is defined as the difference between the total and the non-specific binding. The reaction was started by adding 100 μl membrane fractions (20–50 μg of protein), and was terminated immediately after 120 min of incubation by filtration through Whatman GF/F filters with a subsequent addition of 4 ml of ice-cold buffer followed by washes with 4 × 4 ml of buffer. Any deviation from the above standard binding conditions is specified in the corresponding Figure and Table legends. The radioactivity of each sample retained by the filter was counted on a Beckman liquid-scintillation counter with 45% counting efficiency.

Data analysis

The saturation curves were analysed by the method of Scatchard (B/F versus B plot; B and F are the amounts of specifically bound and free ligand respectively). K_d and $B_{max.}$ represent the dissociation constant for the receptor–drug complex and the total number of binding sites respectively. Saturation-binding data were analysed by the EBDA (equilibrium binding data analysis) computer program, which was designed to analyse saturation studies and drug-displacement studies [23], and the CDATA computer program.

In kinetics studies, the association rate constant (K_1) and dissociation rate constant (K_2) were calculated from the following equations:

$$\ln[B_{eq}/(B_{eq}-B)] = K_{obs}t \quad (1)$$

$$\log(B/B_0) = -K_2t \quad (2)$$

$$K_1 = [K_{obs}-K_2]/F \quad (3)$$

in which B is the binding measured at various time intervals, B_{eq} is the binding at the plateau level, B_0 is the binding at zero time, and K_{obs} is the slope of the linear eqn. (1).

Chemicals

Unlabelled ryanodine (lot SA-889A) was purchased from Research Biochemical Inc. (Wayland, MA, U.S.A.). [^{3}H]Ryanodine (sp. radioactivity 60 or 95 Ci/mmol), [^{3}H]prazosin (sp. radioactivity 26 Ci/mmol), [^{3}H]PN200-110 (sp. radioactivity 78 Ci/mmol) and [^{3}H]saxitoxin (sp. radioactivity 20 Ci/mmol) were obtained from NEN Research Products (Boston, MA, U.S.A.). DL-Dithiothreitol, β-NADPH, cytochrome *c* and sodium salts of ATP and AMP were all purchased from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Subcellular distribution of [^{3}H]ryanodine-binding sites

In distribution experiments, [^{3}H]ryanodine was used at a concentration near the K_d value (4–8 nM). Figure 2 shows that the

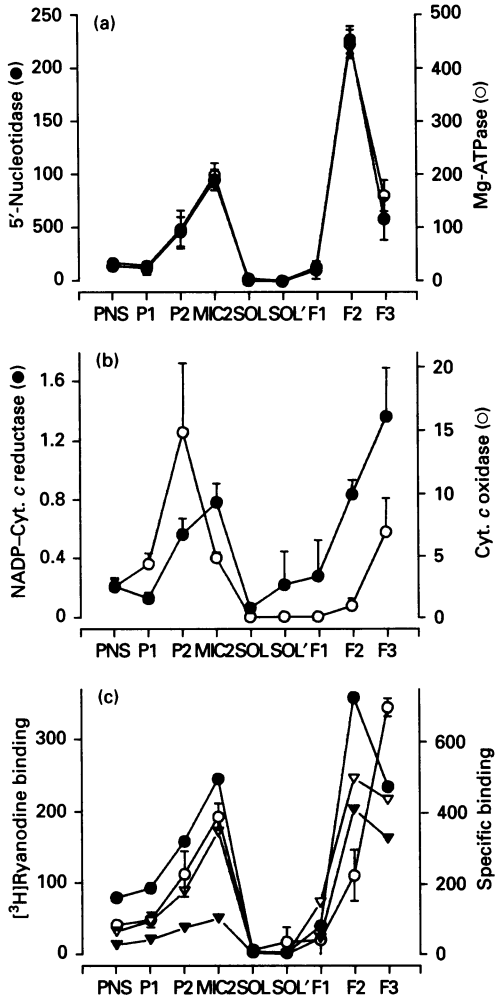

Figure 2 Subcellular-membrane characterization

(**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 (Cyt.) *c* reductase (Δ*A*/min per mg), and mitochondrial-inner-membrane marker, cytochrome *c* oxidase (Δ*A*/min per mg). Measurements of these membrane markers enzyme and of [^{3}H]ryanodine binding (**c**; ○) were all carried out on the same membrane preparation. Each data point represents the mean ± S.D. of 3 experiments. Radioligand binding (fmol/mg) to surface membrane receptors ([^{3}H]prazosin; ▼) and ion channels, such as [^{3}H]saxitoxin (●) or [^{3}H]PN200-110 (▽), was also studied (**c**) with the same membrane preparation; measurements were performed in triplicate.

MIC2 fraction obtained by differential centrifugation was enriched in the plasma membranes and SR membranes, as indicated by high levels of 5′-nucleotidase, Mg^{2+}-ATPase, [^{3}H]prazosin binding (α_1-adrenoceptor) and [^{3}H]PN200-110 binding (L-type Ca^{2+} channels) as plasma-membrane markers and NADPH–cytochrome *c* reductase activity as a SR membrane marker. Further purification was introduced by subfractionating the MIC2 fraction on a discontinuous sucrose gradient. It is also clearly shown in Figure 2 that [^{3}H]ryanodine binds to SR-enriched membrane fraction (F3) with the highest number of binding sites.

Since RVD is a highly innervated tissue, the possible presence of ryanodine receptors in the synaptosomes or the synaptosomal membranes of the nerve varicosities was examined by comparing the subcellular distribution of [^{3}H]ryanodine binding and [^{3}H]saxitoxin binding in these fractions. These distribution profiles are consistent with a locus of ryanodine binding to SR

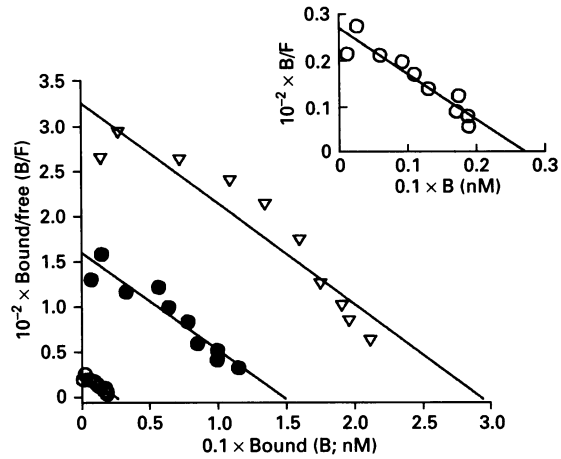

Figure 3 Representative Scatchard plot for [^{3}H]ryanodine binding to PNS (○), MIC2 (●) and F3 (▽)

Three fractions were obtained from same membrane preparation. The experiments were performed at the same time under the same conditions. Inset is the expanded Scatchard plot for PNS. The calculated K_d and $B_{max.}$ values for this experiment are 8.5 nM and 163 fmol/mg for PNS, 8 nM and 910 fmol/mg for MIC2, and 7.8 nM and 1775 fmol/mg for F3, respectively. The Hill coefficients for PNS, MIC2 and F3 are 0.97, 0.98 and 0.95.

membranes and not to the contaminating synaptosomal membranes. Consistent with this finding is also the lack of [^{3}H]ryanodine binding to any of the membrane fractions derived from paravascular nerves of mesenteric vasculature (results not shown).

Equilibrium binding and the effect of Ca^{2+}

Having established the primary subcellular membrane sites for [^{3}H]ryanodine, we carried out the equilibrium binding experiments on three subcellular fractions of various degrees of purity, i.e. postnuclear supernatant (PNS; the starting material), MIC2 (containing plasma membranes and SR) and F3 (SR-enriched fraction), in order to examine whether the membrane isolation and purification procedure affects [^{3}H]ryanodine binding. The K_d value for MIC2 calculated from 10 separate experiments is 5.6 ± 1.4 nM. The non-specific [^{3}H]ryanodine binding to the MIC2 fraction is about 30% of the total [^{3}H]ryanodine bound in the presence of a concentration of [^{3}H]ryanodine near the K_d value. Figure 3 shows the Scatchard plot of the saturation profiles for [^{3}H]ryanodine binding to three subcellular membrane fractions over a wide concentration range of [^{3}H]ryanodine, 0.5–35 nM. The parallel linear Scatchard plots suggest that only one homogeneous class of high-affinity binding site exists for [^{3}H]ryanodine in RVD within the concentration range used. Its density was determined by the amount of SR membrane present in each fraction. 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) and the Hill-coefficient values were all near unity. These results strongly suggest that membrane purification procedures had little effect on the binding properties of [^{3}H]ryanodine. Therefore, all subsequent characterization of [^{3}H]ryanodine binding was performed using MIC2 fraction unless otherwise specified.

Figure 4 shows that binding of [^{3}H]ryanodine to MIC2 increases with increasing free Ca^{2+} concentrations in the medium up to 2 μM Ca^{2+}, reaching half-maximal binding at 0.14 μM Ca^{2+} and maintaining the plateau level at as high as 500 μM

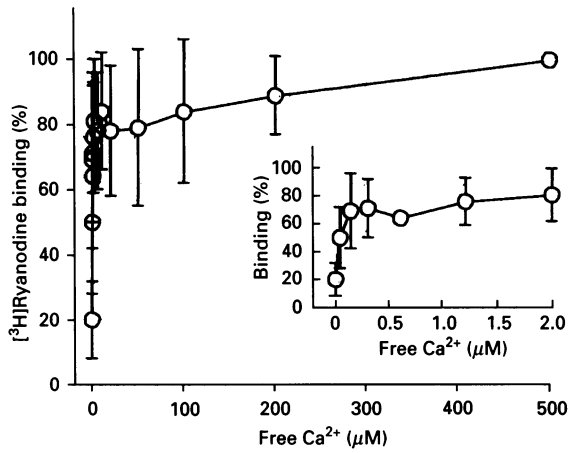

Figure 4 Ca^{2+}-dependence of specific binding of [^{3}H]ryanodine to MIC2

Free Ca^{2+} was buffered with 100 μM EGTA in the presence of 0.3 M KCl in accordance with the calculations of Grover et al. [44]. The binding at 500 μM Ca^{2+} is taken as 100%. Symbols and error bars represent means ± S.D. of three experiments. Inset is the expanded plot which shows binding from 0 to 2 μM free Ca^{2+}.

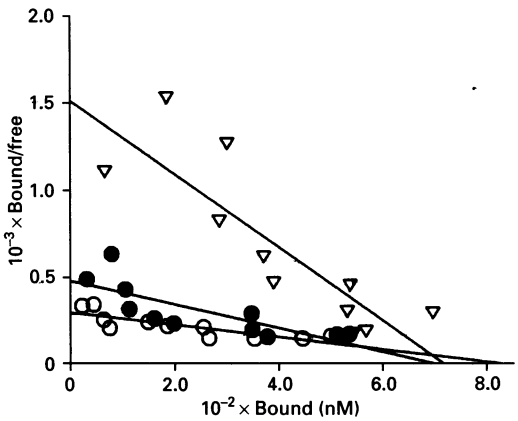

Figure 5 Scatchard plot of [^{3}H]ryanodine binding to MIC2 under different free Ca^{2+} concentrations: 0.14 μM (○), 2 μM (●) and 500 μM (▽)

These results were obtained in triplicate by using the same membrane preparation. Free Ca^{2+} at desired concentrations was buffered by 100 μM EGTA in the presence of 0.3 M KCl. The calculated values for K_d, $B_{max.}$ and h are listed in Table 1.

Table 1 Binding parameters for [^{3}H]ryanodine binding to RVD smooth-muscle microsomes at different Ca^{2+} concentrations

Results were obtained from three different experiments (means ± S.D.)

[Ca^{2+}] (μM)	K_d (nM)	$B_{max.}$ (fmol/mg)	h
0.14	18 ± 5	426 ± 7	0.98 ± 0.01
2	17 ± 5	462 ± 27	0.99 ± 0.01
500	5 ± 0	435 ± 11	1.00 ± 0.02

Ca^{2+}. The effects of Ca^{2+} on the equilibrium binding of [^{3}H]ryanodine to MIC2 fraction are shown in Figure 5, with the binding parameters summarized in Table 1. Clearly, within the physiologically relevant range of cytosolic Ca^{2+} concentration

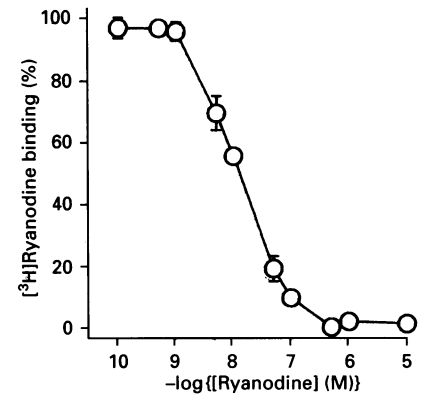

Figure 6 Competition curve obtained by increasing concentrations of unlabelled ryanodine with [^{3}H]ryanodine at 5 nM (near K_d value)

Data points represent means ± S.D. of four separate experiments. The data were analysed by EBDA computer program.

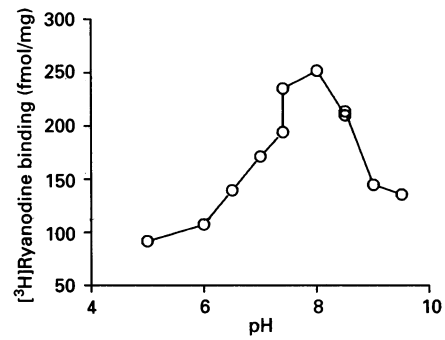

Figure 7 Dependence of specific [^{3}H]ryanodine binding on pH

The experiments were carried out in the membrane of the MIC2 fraction in the presence of 500 μM Ca^{2+} and 0.3 M KCl. The pH was buffered by imidazole (pH 5–7.4), Tris (pH 7.4–8.5) or glycine (pH 8.5–9). Each data point represents the average of two separate experiments performed in triplicate.

(0.14 and 2.0 μM), the affinity for ryanodine, as reflected from the K_d values determined from the slope of the Scatchard plot, remained practically unchanged. At 500 μM Ca^{2+}, however, the K_d value for ryanodine decreased by 3–4-fold. $B_{max.}$ and Hill-coefficient values, however, were quite comparable under these conditions.

Kinetics and displacement of [^{3}H]ryanodine binding

For the kinetic studies, some of the results have been previously published [13]. [^{3}H]Ryanodine at concentrations near the K_d value was incubated with the MIC2 fraction. Binding was started by addition of membrane fraction to the incubation medium under standard binding conditions. The plateau level of [^{3}H]ryanodine binding occurred after 2 h with $K_{obs.} = 0.028 \pm 0.004$ min^{-1} ($n = 4$). Dissociation was initiated, after incubation for 3 h, by 20-fold dilution with wash buffer. [^{3}H]Ryanodine dissociated from the binding site with a K_2 value of 0.016 nM·min^{-1} (average of two separate experiments). The calculated K_d from K_2/K_1 is 6.0 ± 1.9 nM, is excellent agreement with K_d values determined in equilibrium binding experiments.

Figure 6 shows the displacement of [^{3}H]ryanodine binding by

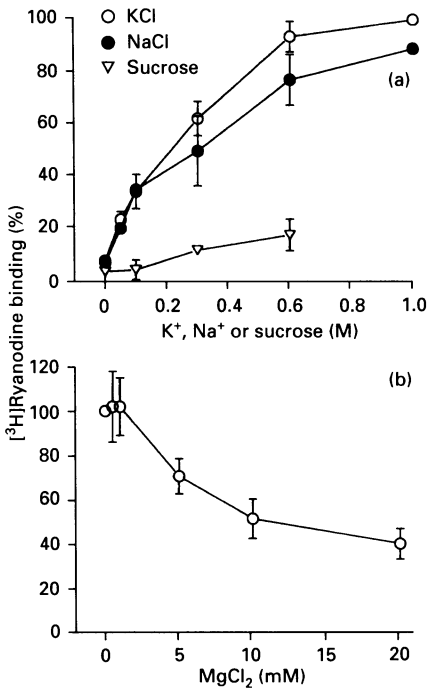

Figure 8 Comparison of the effects of different ions on the specific binding of [³H]ryanodine to MIC2

The experiments were performed in the presence of 500 μM Ca^{2+}. (**a**) The specific binding of [³H]ryanodine at 1 M KCl is taken as 100%. The experiment on the effect of sucrose was performed in the absence of KCl. (**b**) The inhibitory effect of $MgCl_2$ was studied in the presence of 500 μM Ca^{2+} and 0.3 M KCl. The specific binding in the absence of $MgCl_2$ is taken as 100%. Each point and error bar represents mean ± S.D. of three separate experiments.

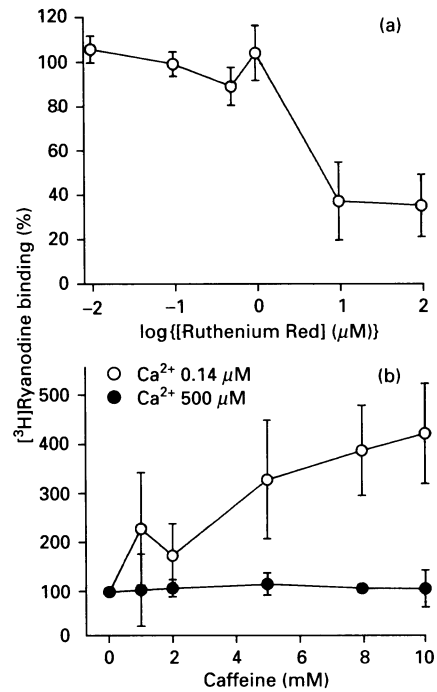

Figure 9 (a) Inhibitory effect of Ruthenium Red on specific [³H]ryanodine binding to MIC2 in the presence of 500 μM Ca^{2+} and 0.3 M KCl; (b) effect of caffeine on specific [³H]ryanodine binding to MIC2 in the presence of 0.3 M KCl and 500 μM or 0.14 μM Ca^{2+}

The specific [³H]ryanodine binding in the absence of caffeine is taken as 100%. The symbols and error bars represent means ± S.D. from three different experiments.

unlabelled ryanodine. The experiments were performed with MIC2 fraction in the presence of [³H]ryanodine at its K_d value. The calculated K_i, 6.7 ± 1.0 nM, was similar to the values obtained in saturation and kinetic studies and had a slope very close to unity ($n = 4$).

Effects of pH, cations and sucrose

Figure 7 shows the effect of pH on [³H]ryanodine binding. Imidazole, Tris and glycine buffering reagents were employed to control the pH of binding medium in the ranges 5.0–7.4, 7.4–8.5 and 8.5–9.5 respectively. [³H]Ryanodine binds to RVD smooth-muscle microsomes in a pH-dependent manner, with optimum binding at pH 7.5–8.0.

Figure 8(a) shows that increasing concentrations of NaCl or KCl each caused a progressive increase in the binding of [³H]ryanodine. An imposed increase in the osmolarity across the membrane vesicles with increasing concentrations of sucrose also caused a progressive increase in [³H]ryanodine binding. This augmentation of [³H]ryanodine binding by sucrose is smaller than that by NaCl or KCl. These results suggest that the non-selective augmentation of binding of [³H]ryanodine by increasing concentrations of univalent cations is primarily due to the high ionic strength of the medium. The K_d and the Hill coefficient for [³H]ryanodine binding remained unchanged in 0.3 M and 0.6 M KCl (results not shown). Unlike KCl, NaCl and $CaCl_2$, $MgCl_2$ inhibits [³H]ryanodine binding in a concentration-dependent manner at either low or high Ca^{2+} concentrations (Figure 8b).

Effects of Ruthenium Red and caffeine

Caffeine significantly enhanced, but Ruthenium Red inhibited, [³H]ryanodine binding to the SR fragments isolated from cardiac and skeletal muscles [24,25]. This is also true for RVD smooth-muscle membranes. Figure 9(a) shows that Ruthenium Red potently inhibited [³H]ryanodine binding with $IC_{50} < 1$ μM. Caffeine enhanced binding of [³H]ryanodine at low Ca^{2+} concentration, but had no effect at high Ca^{2+} concentration (Figure 9b).

[³H]Ryanodine-binding sites in microsomal fractions from different smooth-muscle tissues

Under optimized binding conditions, we have compared the relative number of [³H]ryanodine-binding sites in MIC2 fractions of various smooth-muscle preparations (Table 2). The corresponding levels of [³H]ryanodine binding in the MIC2 fractions from skeletal muscle obtained under the same binding conditions are also listed for comparative purposes. Among the smooth-muscle preparation studied, RVD smooth muscles had the highest number of [³H]ryanodine-binding sites under the same binding conditions, but still substantially lower than that in skeletal and cardiac muscles, as previously reported [2]. Among vascular smooth-muscle preparations, MIC2 of the smaller artery (mesenteric artery) apparently had more [³H]ryanodine-binding sites than that of the larger artery (aorta) in either rat or dog. Scatchard analysis of [³H]ryanodine binding to MIC2 of dog mesenteric arteries indicated a linear profile with a K_d of 6 nM, in excellent agreement with the K_d value obtained from RVD membranes (results not shown).

Table 2 Binding of [^{3}H]ryanodine (6±1 nM) to MIC2 fractions isolated from different tissues

The experiments were performed in medium containing 500 μM Ca^{2+}, 0.6 M KCl, 25 mM Tris and 1 mM dithiothreitol, at pH 7.4, with incubation for 2 h. The data represent means±S.D., except for dog trachea, in which two separate experiments were performed.

Tissue	Binding (fmol/mg)	*n*
Rat skeletal muscle	1587±384	3
RVD	391±82	6
Dog vas deferens	97±28	3
Guinea-pig vas deferens	200±39	3
Rat mesenteric artery	80±31	4
Dog mesenteric artery	87±30	4
Rat aorta	23±18	4
Dog aorta	40±26	4
Dog trachea	0, 29	2
Dog mesentery nerve	Not detectable	3

DISCUSSION

This work represents the first detailed characterization of [^{3}H]ryanodine-binding sites in smooth-muscle membrane preparations. It contains novel information in several aspects: (*a*) the subcellular localization of ryanodine receptors, (*b*) the general binding characteristics of the ryanodine receptors and (*c*) distribution of ryanodine receptors in various smooth-muscle preparations. These aspects are discussed below, along with information derived from other functional studies on the effects of ryanodine on smooth muscles and [^{3}H]ryanodine binding to membranes isolated from other muscle types [26,27].

Ryanodine receptors are primarily localized in the sarcoplasmic reticulum of RVD smooth muscle

We have compared the subcellular distribution of [^{3}H]ryanodine binding with that of a large variety of membrane markers, which have been very commonly used in the smooth-muscle fractionation technique [17]. Subcellular-distribution studies suggest that the binding sites for [^{3}H]ryanodine are very unlikely to be present in the smooth-muscle plasma membrane, because the distribution of [^{3}H]ryanodine binding sites differs markedly from that of the various plasma-membrane markers employed, such as plasma-membrane-associated enzymes (alkaline phosphodiesterase I, Mg-ATPase and 5′-nucleotidase [16–18]), surface membrane α-adrenoceptors ([^{3}H]prazosin binding [19]) and L-type Ca^{2+} channels ([^{3}H]PN200-110 binding [20]). Our findings indeed suggest that [^{3}H]ryanodine binds to a membrane fraction, consistent with its localization in the SR. For example, the enrichment of [^{3}H]ryanodine-binding sites during membrane fractionation closely paralleled that of NADPH–cytochrome *c* reductase, a widely used putative SR marker enzyme in smooth-muscle fractionation [16]. Upon further subfractionation of RVD microsomes on the sucrose density gradient, NADPH–cytochrome *c* reductase activity is the highest in the F3 fraction; so is the number of binding sites for [^{3}H]ryanodine. Furthermore, we have previously shown that F3 was indeed the fraction that elicited the highest activity of oxalate-facilitated ATP-dependent Ca^{2+} transport [16,28], a property attributed primarily to SR [29,30].

In view of the fact that brain tissues contain [^{3}H]ryanodine-binding sites [31] and that vas deferens is a highly innervated tissue, we also entertained the possibility that the [^{3}H]ryanodine-binding sites in our RVD membrane preparations are of neuronal origin. Several lines of evidence suggest this may not be the case. First of all, we have demonstrated that the binding of [^{3}H]saxitoxin, which is present in nerve varicosities but not in smooth-muscle membranes [21], does not parallel [^{3}H]ryanodine binding. Secondly, in RVD there are no morphologically detectable neuronal cell bodies, and the smooth-muscle cells were innervated by nerve varicosities which do not contain morphologically distinct endoplasmic-reticulum structure, Thirdly, using membranes derived from dog mesenteric paravascular nerve fibres, we could not detect any specific binding of [^{3}H]ryanodine. These results collectively argue against the observed [^{3}H]ryanodine binding sites in RVD being of neuronal origin.

In cardiac muscle, the association between voltage-gated Ca^{2+} channels and ryanodine receptors forming the feet structure as voltage-sensitive Ca^{2+}-release channels varies with the developmental stage [20]. In dog tracheal [32] and mesenteric [33] artery, refilling of ryanodine-sensitive and agonist-sensitive internal membrane Ca^{2+} stores are modulated by BAY K 8644 and nifedipine, suggesting that ryanodine-sensitive Ca^{2+}-release channels in the SR may be closely associated with the voltage-gate Ca^{2+} channels in the plasma membrane. Our findings indicate that [^{3}H]PN200-110- and [^{3}H]ryanodine-binding sites are localized on the plasma membranes and SR, respectively. If these two Ca^{2+} channels were indeed physically associated in the intact cell, such a linkage may not be sufficiently strong to survive the physical disruption during tissue homogenization. Alternatively, physical association between these two types of channels may represent a very small fraction of the total number of Ca^{2+}-channel sites, and thus beyond reliable detection by this technique. After all, the distinct feature of feet structures between the peripheral SR and the plasma membrane in juxtaposition has not been observed in RVD or any smooth-muscle cells.

Many factors can affect ryanodine binding

In RVD smooth-muscle preparation, the K_d for ryanodine binding (6 nM), in the range of 2–500 nM Ca^{2+}, is practically the same as those previously reported in skeletal muscle and cardiac muscle, i.e. 7 nM and 7.9 nM respectively [34,35]. Furthermore, similar K_d values for [^{3}H]ryanodine binding were also obtained from membrane fractions of different purities (e.g. PNS, MIC2 and F3), suggesting that this high-affinity receptor is not susceptible to modification by impurities or the purification processes under our experimental conditions. However, [^{3}H]ryanodine binding to smooth-muscle membrane is highly dependent on Ca^{2+} concentration over a quite narrow range of physiological relevance, with half-maximal binding occurring at about 140 nM Ca^{2+}, which is about the resting cytosolic Ca^{2+} concentration. This is a feature different from that in skeletal muscle and liver. In skeletal muscle, [^{3}H]ryanodine binding is inhibited in a high Ca^{2+} concentration range [2], whereas in the liver [^{3}H]ryanodine binding is Ca^{2+}-independent [36].

Several factors that modify [^{3}H]ryanodine binding to the skeletal-muscle SR were found to have qualitatively similar effects on the binding of [^{3}H]ryanodine to RVD smooth-muscle membranes. These factors include Mg^{2+}, Na^+, K^+, sucrose, Ruthenium Red and caffeine [24–27,37,38].

Caffeine is known to activate Ca^{2+}-induced Ca^{2+} release in smooth muscle. We have also demonstrated that the sensitivity of RVD smooth muscle to the inhibitory effect of ryanodine was enhanced in the presence of caffeine [13]. It has also been shown

that caffeine caused an increase in [^{3}H]ryanodine binding to both skeletal and cardiac muscle SR preparations, but this effect is greater in skeletal-muscle SR [27]. Furthermore, since the relative increase in [^{3}H]ryanodine binding occurred primarily at low Ca^{2+} concentration close to the resting cytosolic Ca^{2+} concentration, caffeine may act by increasing the Ca^{2+}-sensitivity of the [^{3}H]ryanodine binding.

Among various factors that affected [^{3}H]ryanodine binding to the RVD smooth-muscle microsome, the effect of pH was quite different from that observed in skeletal muscle. It is noteworthy that the optimal pH for [^{3}H]ryanodine binding in smooth muscle is somewhat alkaline, but still lies within the physiologically relevant range, whereas the optimal pH for [^{3}H]ryanodine binding to skeletal-muscle membranes, under the same binding conditions, was greater than pH 9 (results not shown) and far from being physiologically relevant. Similarly, it was also reported that ryanodine binding to cardiac-muscle membrane did not reach a plateau up to pH 8.6 [39].

The inhibitory effect of high concentrations of Mg^{2+} (5–20 mM) on the binding of [^{3}H]ryanodine to smooth-muscle membranes is probably of little physiological interest. In skeletal-muscle SR, Mg^{2+} inhibited [^{3}H]ryanodine binding with a IC_{50} of about 0.3 mM [2], and 10 mM Mg^{2+} completely inhibited binding [27,38]. [^{3}H]Ryanodine binding to the hepatic microsome, however, was not affected at all by Mg^{2+} [36]. On the other hand, high concentrations of KCl and NaCl enhance [^{3}H]ryanodine binding in skeletal and cardiac muscle as well as in smooth muscle. This is probably due to the combined effects of ionic strength and osmolarity of the binding medium, since sucrose also produced a qualitatively similar, but smaller, augmentation of [^{3}H]ryanodine binding, as also reported in skeletal-muscle preparations [37]. Scatchard analysis of [^{3}H]ryanodine binding to RVD microsomes at either 0.3 M or 0.6 M KCl yielded a single binding site without a change in K_d (results not shown). Although the physiological relevance of these ionic effects remains obscure, Mg^{2+}-free and high-ionic-strength medium may be used to optimize the conditions for the binding of [^{3}H]ryanodine in preparations in which the binding sites are sparse.

Density of [^{3}H]ryanodine-binding sites in different smooth-muscle tissues is highly variable

In spite of the similar K_d values for [^{3}H]ryanodine binding to SR-enriched membrane fractions in skeletal muscle and smooth muscle, as mentioned above, the number of ryanodine-binding sites varied considerably, even under the optimized binding conditions. Table 2 clearly indicates that in the rat, under similar membrane-separation and [^{3}H]ryanodine-binding conditions, the hind-leg skeletal muscle has the highest number of ryanodine-binding sites. In smooth muscles, in general, the number of ryanodine-binding sites is the least compared with other muscle types, presumably due to their sparse SR content. However, within the smooth-muscle group, microsomal fractions of vas deferens smooth muscle from rat, guinea pig and dog has the highest number of [^{3}H]ryanodine-binding sites, and the [^{3}H]ryanodine binding is very low or practically absent in some smooth-muscle preparations in which functional effects of ryanodine have been demonstrated, e.g. dog trachea [32]. Clearly, there is not a complete correspondence between biochemical and functional analysis of ryanodine as an agent affecting Ca^{2+}-release channels.

Furthermore, it is noteworthy that rat or dog aorta has less ryanodine binding sites than the corresponding mesenteric arteries, although the SR content is believed to be greater in large arteries than in smaller arteries [40]. The above findings and considerations also suggest that the number of ryanodine receptors is probably related to the nature of the mechanisms of excitation–contraction coupling rather than the amount of SR present in the muscle cell. In this connection, another type of Ca^{2+}-release channels which can be labelled with [^{3}H]Ins(1,4,5)P_3 has also been identified and isolated from RVD smooth muscle [41]. Interesting information may be obtained from comparative studies of these two types of SR Ca^{2+}-release channels in these smooth muscles. For example, co-existence of Ca^{2+}-induced Ca^{2+}-release channels and InsP_3-induced Ca^{2+}-release channels in SR, utilizing the same Ca^{2+} pool, has also been functionally demonstrated in skinned smooth-muscle preparations [42]. The very close structural homology of the purified ryanodine and InsP_3 receptors [43] poses an important question: do ryanodine and InsP_3 interact at the same Ca^{2+}-channel site? Studies using RVD smooth-muscle preparation (Z.-D. Zhang, C.-Y. Kwan and E. E. Daniel, unpublished work) indicated that unlabelled ryanodine failed to compete for the [^{3}H]InsP_3 binding site [41]. Also, in hepatocyte membranes, heparin was found to inhibit selectively the [^{3}H]InsP_3 binding without any effect on the [^{3}H]ryanodine binding [36]. Further clarification of the relationship between these two Ca^{2+}-release channels in smooth muscle is needed.

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