Direct binding of verapamil to the ryanodine receptor channel of sarcoplasmic reticulum

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ABSTRACT Radioligand binding experiments and single channel recordings demonstrate that verapamil interacts with the ryanodine receptor Ca^{2+} release channel of the sarcoplasmic reticulum of rabbit skeletal muscle. In isolated triads, verapamil decreased binding of [^{3}H]Ryanodine with an IC_{50} of ~8 μM at an optimal pH 8.5 and pCa 4.3. Nitrendipine and *d-cis*-diltiazem did not interfere with binding of [^{3}H]Ryanodine to triads, suggesting that the action of verapamil does not involve the dihydropyridine receptor. Single channel recordings showed that verapamil blocked Ca^{2+} release channels by decreasing open probability, duration of open events, and number of events per unit time. A direct interaction of verapamil with the ryanodine receptor peptide was demonstrated after purification of the ~400 kDa receptor protein from Chaps-solubilized triads. The purified receptor displayed high affinity for [^{3}H]Ryanodine with a K_d of ~5 nM and a B_{max} of ~400 pmol/mg. Verapamil and D600 decreased [^{3}H]Ryanodine binding noncompetitively by reducing the B_{max}. Thus the presence of binding sites for phenylalkylamines in the Ca^{2+} release channel was confirmed. Verapamil blockade of Ca^{2+} release channels may explain some of the paralyzing effects of phenylalkylamines observed during excitation-contraction coupling of skeletal muscle.

INTRODUCTION

The 175 kDa α_1 peptide of the dihydropyridine (DHP) receptor serves as a binding site for three classes of Ca^{2+}-entry blockers such as dihydropyridines, phenylalkylamines, and benzothioazepines (Catterall et al., 1988). Prototype compounds for the three groups are nitrendipine, verapamil, and diltiazem, respectively. In the transverse tubular membrane of skeletal muscle, the radioligand binding affinities of Ca^{2+} blockers to the α_1 receptor fall within the submicromolar range, ~1 nM for nitrendipine (Fosset et al., 1983), 30 nM for verapamil (Galizzi et al., 1984), and 200 nM for diltiazem (Galizzi et al., 1986). Although L-type Ca^{2+} channels of skeletal muscle are blocked by DHPs at submicromolar concentrations (Valdivia and Coronado, 1990), only a small percent of DHP binding to the tubular membrane of skeletal muscle is involved in blockade of Ca^{2+} currents (Schwartz et al., 1985). The specific enrichment of DHP receptors in the tubular membrane (Fosset et al., 1983) and the fact that the α_1 peptide of the DHP receptor is homologous to the voltage-gated Na^+ channel (Tanabe et al., 1987; Tanabe et al., 1988; Ellis et al., 1988), suggested that α_1 may have a voltage-sensing function unrelated to Ca^{2+} entry into the cell (Rios and Brum, 1987; Tanabe et al., 1988).

Eisenberg et al. (1983) showed that one such process in which Ca^{2+}-entry blockers are implicated, other than Ca^{2+} entry into the cell, is excitation-contraction coupling. In skeletal muscle, Ca^{2+} entry through Ca^{2+} channels does not contribute to muscle contraction (Gonzales-Serratos et al., 1982) and removal of external Ca^{2+} does not immediately affect contraction (Armstrong et al., 1972). Nevertheless, D600 (methoxyverapamil) was reported to produce paralysis of skeletal muscle fibers (Eisenberg et al., 1983). In the presence of phenylalkylamines, skeletal muscle turned into a mechanically refractory state in which fibers did not contract in response to electrical stimulation or to depolarization by elevated potassium. Intramembrane charge movement is also absent in paralyzed fibers, and this has been held as evidence that D600 uncouples voltage-sensors in the transverse tubular membrane from Ca^{2+} release in the sarcoplasmic reticulum membrane (Hui et al., 1984; Caputo and Bolanos, 1987; Brewe et al., 1987; Erdmann and Luttgau, 1989; Fill and Best, 1989). In a recent study, the phenylalkylamine binding site responsible for muscle paralysis has been presumed to be on the α_1 DHP receptor because K_d values estimated following recovery from paralysis after exposure of fibers to D888 (desmethoxyverapamil) agreed well with the radioligand binding K_d of [^{3}H]D888 to the DHP receptor (Erdmann and Luttgau, 1989).

We describe a new class of pharmacologically active binding sites for phenylalkylamines, specifically verapamil, in skeletal muscle. The site turned out to be directly on the ryanodine receptor channel of the sarcoplas-

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mic reticulum membrane (Smith et al., 1985, 1986, 1988). This binding site differs from that on DHP receptors in that neither dihydropyridines nor diltiazem can replace, or compete with, phenylalkylamines. The interaction of verapamil with the ryanodine receptor resulted in an inhibition of [^{3}H]Ryanodine binding. The alkaloid ryanodine is known to produce paralysis in skeletal and cardiac muscle (Jenden and Fairhurst, 1969) and has been found to bind with nanomolar affinity to the "foot" protein that bridges the gap between the transverse tubule and the junctional sarcoplasmic reticulum (SR) in triads of skeletal muscle (Inui et al., 1987; Block et al., 1988; Lai et al., 1988). A tetramer of this protein is sufficient to reconstitute ryanodine-sensitive Ca^{2+} release channels in planar bilayers (Imagawa et al., 1987; Smith et al., 1988; Ma et al., 1988; Lai et al., 1988). We find that occupancy of these low affinity sites on the ryanodine receptor by verapamil leads to inhibition of Ca^{2+} release channels. This observation is relevant to the in vivo actions of phenylalkylamines and to studies that use micromolar levels of verapamil and related derivatives. A preliminary report of this work appeared in abstract form (Valdivia and Coronado, 1989; Valdivia et al., 1990).

MATERIALS AND METHODS

Purification of triads from rabbit skeletal muscle

Triads were purified from adult rabbit white skeletal muscle using the pyrophosphate variant of Mitchell et al. (1983). The following protease inhibitors were added during homogenization: Pepstatin A (1 μM), Iodoacetamide (1 mM), phenylmethylsulfonyl fluoride (PMSF, 0.1 mM), Leupeptin (1 μM), and Benzamidine (1 mM). Purified triads were immediately used or stored for no more than 2 wk at −80°C. Thawed samples were discarded after use. Routinely, the [^{3}H]PN200-110 binding capacity was 12 pmol/mg, the [^{3}H]Ryanodine binding capacity was 9 pmol/mg, and the ATP-dependent Ca^{2+} uptake capacity was 15 nmol/mg triad protein.

Formation of planar bilayers and single channel recording

Planar bilayers were composed of brain phosphatidylethanolamine and brain phosphatidylserine at a 1:1 weight ratio (Avanti Polar Lipids, Birmingham, AL) dissolved in decane (Aldrich Chemical Co., Milwaukee, WI). Bilayers were formed on a 0.3-mm diam hole in a Lexan polycarbonate partition. Triad preparations (0.1–0.2 mg) were added to the *cis* solution composed of 0.25 M CsCl, 10 μM $CaCl_2$, and 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) titrated with TrisHCl (Tris[hydroxymethyl] aminomethane) to pH 7.4. Thus, Cs^+ instead of Ca^{2+} was the major carrier of current (Smith et al., 1988). SR Cl^- channels were separated from release channels on the basis of reversal potential (E_{Cl} = +37 mV, E_{Cs} = −37 mV). In ~30% of vesicle fusions, release channels could be recorded in the absence of Cl^- channels and these cases were used for analysis. The total recording time was ~120 min from three preparations of triads. The *trans* solution was the same except the CsCl which was 0.05 M. *Cis* solution was connected via an Ag/AgCl electrode and an agar/KCl bridge to the head-stage input of a List L/M EPC 7 amplifier (List Electronic, DA-Eberstadt, FRG). Trans solution was held at ground potential using the same electrode arrangement. Recordings were filtered through a low-pass Bessel (Frequency Devices, Haverhill, MA) at 1.5 kHz and digitized at 4 kHz. Acquisition, storage, and analysis were done on a PC/AT computer using Keithley Instruments Inc.-DAS 570 software (Cleveland, OH).

Ryanodine binding assay

[^{3}H]Ryanodine binding assays were carried out for 90 min at 36°C in 400 μl of 0.15 M NaCl, 50 μM $CaCl_2$, 20 mM Tris-HCl, pH 8.5 (other buffers, free Ca^{2+}, and pH, are indicated in figure legends). [^{3}H]Ryanodine (60 mCi/mmol; Du Pont-New England Nuclear, Wilmington, DE) was diluted directly into the incubation medium to achieve concentrations in the saturable range of 1–30 nM. Ca^{2+}-EGTA buffers containing 1 mM Na_2EGTA (ethylenediamine tetraacetic acid) were used when desired free Ca^{2+} was ≤100 μM. Free Ca^{2+} concentrations were calculated using a computer program and were verified with a Ca^{2+} electrode. The following buffers purchased from Sigma Chemical Co. (St. Louis, MO) were used for the indicated range of pH, Mes (2[*N*-Morpholino] ethanesulfonic acid, pH 6–6.5); Mops (3-[*N*-Morpholino] propanesulfonic acid, pH 7–7.5); Tris (Tris[hydroxymethyl] aminomethane, pH 8–9; and Caps (3-[Cyclohexyl amino]-1-propanesulfonic acid, pH 10–11). Verapamil or D600 were delivered from stock solutions in methanol. Final concentration of methanol in the incubation media was ≤1% vol/vol. Same volume of methanol was always added to control samples and had no effect on [^{3}H]Ryanodine binding. During incubation, triads (0.1–0.2 mg/ml), solubilized membranes (0.1–0.2 mg/ml) or purified receptor (6–10 μg/ml) were the last reagent added to the media. Samples (0.1–0.4 ml) were filtered on Whatman Inc. GF/B glass fiber filters (Clifton, NJ) and washed twice with 5 ml ice-cold water. A Brandel M-24R Cell Harvester (Gaithersburg, MD) was used for filtration. In assays of solubilized receptor, filters were soaked for 15 min in 5% polyethylenimine. Nonspecific binding corresponds to the total binding measured in the presence of 10 μM unlabeled ryanodine and was subtracted from each sample. Ryanodine was purchased from Calbiochem (La Jolla, CA).

Verapamil binding assay

[^{3}H]Verapamil (80 mCi/mmol, Du Pont-New England Nuclear) was directly diluted into 400 μl of incubation medium (40 mM Tris-HCl, pH 8.5) to achieve concentrations in the range of 2–100 nM. Triads (0.05–0.1 mg/ml) were the last reagent added. Samples (0.4 ml) were filtered on Whatman GF/C glass fiber filters and washed twice with 5 ml incubation medium containing 200 mM choline chloride. Binding to filters (~8% of total binding) was determined separately (always in parallel experiments) for each concentration of [^{3}H]Verapamil. Nonspecific binding corresponds to the total binding measured in the presence of 10 μM cold verapamil, when [^{3}H]Verapamil concentration was 1–30 nM; or in the presence of 20 μM cold verapamil when [^{3}H]Verapamil concentration was 40–100 nM. In displacement experiments of bound [^{3}H]Verapamil, the cold displacer (verapamil, nitrendipine, ryanodine) was added from a stock solution in methanol. Same volumes of methanol added to controls had no effect. Stock solutions prepared by serial dilution in water gave erroneous results, presumably due to a high nonspecific binding of drugs to glass test tubes. Dissociation constant were determined according to Cheng and Prusoff (1973) using the

relation $IC_{50} = K_d\ (1+[L^*]_{0.5}/K_d^*)$, where IC_{50} is the concentration of inhibitor that induces half-displacement of bound [^{3}H]ligand, $[L^*]_{0.5}$ is the concentration of free [^{3}H]ligand at half-displacement, K_d^* and K_d are dissociation constants for receptor and [^{3}H]ligand, and receptor and unlabeled ligand, respectively. Verapamil and D600 were gifts from Knoll AG (Ludwigshafen, FRG).

Purification of ryanodine receptor from Chaps-solubilized triads

Ryanodine receptor was purified on sucrose density gradients. Triads were resuspended in 0.5% Chaps {3-[(3-Cholamidopropyl)dimethylammonio] 1-propanesulfonate, Sigma Chemical Co.}, 1 M NaCl, 40 mM Tris-Maleate pH 7.4, plus protease inhibitors given above. The homogenate was incubated 60 min at 0°C and spun at 40,000 *g* in a Beckman 70.1 Ti rotor (Fullerton, CA). Solubilization of triads under these conditions was always >80%. 1.5-ml aliquotes of supernatant were layered on top of a 32-ml, 5%:20% (wt/vol) linear sucrose gradient containing 0.3 M NaCl, 40 mM Tris-Maleate pH 7.4, 0.1 mM $CaCl_2$, 2.0 mM Dithiothreitol, 50 μM PMSF, 0.15% Chaps, 0.15% Asolecithin (soybean phosphatidylcholine; Avanti Polar Lipids). Gradients were centrifuged at 20,000 *g* in a SW-28 rotor for 15 h at 4°C. Gradient fractions were monitored for protein content, SDS-PAGE (Sodium dodecyl sulfate polyacrylamine gel electrophoresis), and [^{3}H]Ryanodine binding activity. SDS-PAGE was performed according to the Laemmly method. Samples were incubated 15 min at 80°C in 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 10 mM Tris (pH 6.8), and run on a 5–12% linear polyacrylamide gel gradient. A kit from Bio-Rad Laboratories (Richmond, CA) was used for silver staining. Molecular weight standards were myosin, M_r 200,000; β-galactosidase, M_r 116,000; phosphorylase b, M_r 97,400; bovine albumin, M_r 68,000; ovalbumin, M_r 43,000; and carbonic anhydrase, M_r 31,000.

RESULTS

Verapamil inhibition of [^{3}H]Ryanodine binding to triads

Ryanodine binding affinity and binding site density are highly sensitive to key ligands that open or close the Ca^{2+} release channel, such as Ca^{2+}, Mg^{2+}, nucleotides, ruthenium red, and caffeine (Pessah et al., 1986, 1987; Inui et al., 1987; Imagawa et al., 1987). In general, ligands that stimulate [^{3}H]Ryanodine binding also open release channels whereas ligands that inhibit [^{3}H]Ryanodine binding also close or block release channels. Fig. 1, *A* and *B* describe the Ca^{2+} and pH dependence of [^{3}H]Ryanodine binding to triads and the inhibition of specific binding by the Ca^{2+}-entry blocker verapamil. The Ca^{2+} dependence in the absence of verapamil (Fig. 1 *A*, *open circles*) is described by a bell-shaped curve with a peak at ~50 μM [Ca^{2+}] and a maximum binding capacity of 7 pmol/mg protein at 5 nM [^{3}H]Ryanodine (0.15 M KCl, pH 8.5, no added nucleotides). No specific binding was measured at [Ca^{2+}] ≥10 mM or [Ca^{2+}] ≤1 μM. Binding activity at 50 μM [Ca^{2+}] was optimal at pH 9 (Fig. 1 *B*, *open circles*) and decreased at higher or lower pH. No specific binding was measured at pH ≤6 or pH ≥11. For the stimulatory part of the pH curve we estimated a pK_a of ~8.3. However, the inhibitory phase with a midpoint near pH 10 cannot be clearly ascribed to an ionizable group in the receptor structure. In strong basic solutions, ryanodine

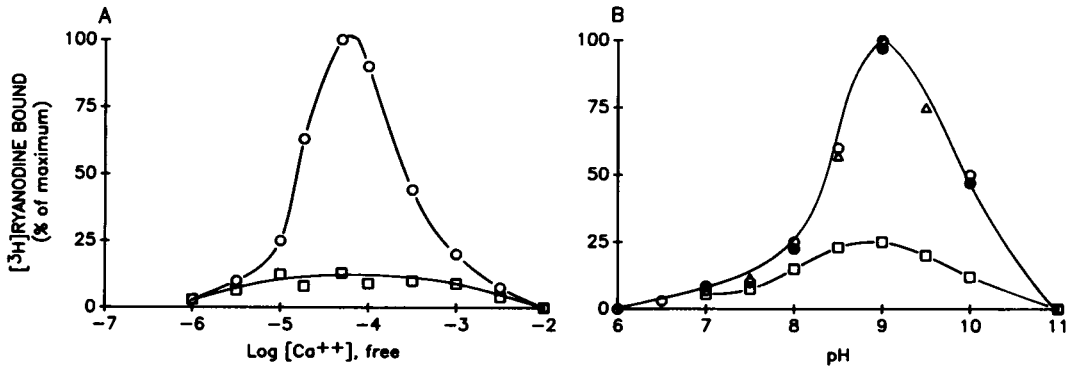

FIGURE 1 Ca^{2+} and pH dependence and verapamil inhibition of [^{3}H]Ryanodine binding to skeletal muscle triads. (*A*) [^{3}H]Ryanodine was incubated 90 min at 36°C with 80 μg purified triads in 0.15 M NaCl, 20 mM Tris-HCl pH 8.5, and the indicated concentration of free Ca^{2+}. [^{3}H]Ryanodine concentration was 5 nM (60 mCi/mmol) and the incubation volume was 400 μl. Open circles correspond to specific binding of [^{3}H]Ryanodine and open squares to specific binding in 20 μM verapamil added at the beginning of the incubation period. 100% corresponds to 3.9 pmol/mg which was the highest specific [^{3}H]Ryanodine binding at 50 μM free Ca^{2+}. (*B*) [^{3}H]Ryanodine was incubated 90 min at 36°C with 80 μg purified triads in 0.15 M NaCl, 150 μM $CaCl_2$, and 20 mM buffer at the indicated pH. Buffers were Mes (pH 6–6.5), Mops (pH 7–7.5), Tris (pH 8–9), and Caps (pH 10–11), titrated with NaOH or HCl. Specific binding of [^{3}H]Ryanodine (*open circles*), plus 20 μM verapamil (*open squares*), or 10 μM nitrendipine (*solid circles*), or 20 μM d-*cis*-diltiazem (*open triangles*). 100% corresponds to 5.4 pmol/mg which was the highest specific [^{3}H]Ryanodine binding at 50 μM free Ca^{2+}, pH 9. (*A*), (*B*) Points are mean of three experiments from separate triad preparations.

undergoes saponification to yield pyrrole-2-carboxylic acid and ryanodol, none of which appear to have pharmacological activity (see Jenden and Fairhurst, 1969).

To our knowledge, the pH dependence of [^{3}H]Ryanodine binding has not been reported previously, although Michalak et al. (1988) noticed that [^{3}H]Ryanodine binding at pH 8 was higher than that at pH 6.8. On the other hand, the effect of pH on $^{45}Ca^{2+}$ release from SR vesicles and single Ca^{2+} release channels is well known (Nakamaru and Schwartz, 1972; Shoshan et al., 1981; Sumbilla and Inesi, 1987; Ma et al., 1988). The Ca^{2+} dependence shown in Fig. 1 *A* (measured at pH 8.5) agreed well with that described in other triad preparations by Pessah et al. (1986) at pH 7.1 or Imagawa et al. (1987) at pH 7.4. Our results disagreed with those reported in heavy SR by Lattanzio et al. (1987), however, who observed inhibition of binding but no Ca^{2+}-dependent stimulation. Verapamil at a concentration of 20 μM inhibited binding of [^{3}H]Ryanodine to triads over a wide range of Ca^{2+} and pH (Figs. 1, *A* and *B*, *open squares*). Inhibition was most obvious at micromolar Ca^{2+} and alkaline pH. At 50 μM Ca^{2+} and pH 8.5, 8 μM verapamil displaced 50% of the specific binding of [^{3}H]Ryanodine and 300 μM displaced 96%. At pH 7.0, 20 μM verapamil displaced ~20% of the specific [^{3}H]Ryanodine binding, whereas 1 μM had no effect. Inhibition was specific for verapamil in that prototype drugs from the two other groups of Ca^{2+}-entry blockers, nitrendipine (10 μM, Fig. 1 *B*, *solid circles*) and diltiazem (20 μM, Fig. 1 *B*, *open triangles*) had no effect. The optimal pH for verapamil inhibition was consistent with the pH dependence of [^{3}H]Verapamil binding to transverse tubules (t-tubules) (Galizzi et al., 1984).

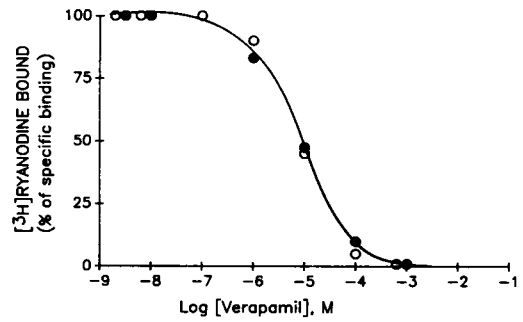

FIGURE 2 Dose-response curve for verapamil inhibition of [^{3}H]Ryanodine binding to triads. [^{3}H]Ryanodine (5 nM) was incubated 90 min at 36°C with 80 μg purified triads in 0.15 M NaCl, 50 μM $CaCl_2$, 20 mM Tris-HCl pH 8.5, and the indicated verapamil concentrations in the absence (*solid circles*) or presence (*open circles*) of 10 μM nitrendipine. 100% corresponds to 3.0 pmol of specific [^{3}H]Ryanodine binding per mg triad protein. Smooth line is a fit with a Hill coefficient $n_H = 0.89$ and $K_d = 10$ μM. Points are mean of two (verapamil plus nitrendipine) or three (verapamil) experiments.

Effect of verapamil is not mediated by DHP receptors

In skeletal muscle fibers (Block et al., 1988), as well as in triads purified by the method used here (Mitchell et al., 1983), there is a physical interaction between the junctional SR and the t-tubule membranes. We therefore considered the possibility that verapamil inhibited binding of [^{3}H]Ryanodine indirectly, by binding to the DHP receptor and causing a conformational change at the [^{3}H]Ryanodine binding site on the ryanodine receptor. Fig. 2 shows dose-response curves for verapamil inhibition of [^{3}H]Ryanodine binding in the presence (*open circles*) and absence (*solid circles*) of 10 μM nitrendipine. Verapamil was only effective at micromolar concentration with an IC_{50} of ~8 μM and furthermore, 10 μM nitrendipine did not alter the dose-response curve. We reasoned that if verapamil inhibited [^{3}H]Ryanodine binding by interacting with the DHP receptor, it should have done so at nanomolar concentrations, that is, at doses known to saturate binding of verapamil to the DHP receptor (Galizzi et al., 1984; see also Fig. 4 *A*). Moreover, the inhibitory effect of verapamil should be abolished by nitrendipine because nitrendipine alone did not inhibit [^{3}H]Ryanodine binding (Fig. 1 *B*, *solid circles*). Nitrendipine, at the concentration tested, is known to displace >90% of verapamil specifically bound to the DHP receptor (Galizzi et al., 1984). Given that the action of verapamil was unaffected by occupancy of the DHP receptor, we concluded that a different class of drug sites was involved in the inhibition of [^{3}H]Ryanodine binding.

Verapamil inhibition of single Ca^{2+} release channels

To identify the site of action of verapamil we investigated its effects on ryanodine-sensitive Ca^{2+} release channels. Fig. 3 *A* (control) shows recordings of Ca^{2+} release channels obtained by the fusion of triads to planar bilayers. The polarity of the inserted channels was the same as in previous studies (Smith et al., 1985), that is, the myoplasmic side faced into the *cis* solution and the intravesicular side faced into the *trans* solution. This was confirmed by the *cis*-activation of channels by ATP and micromolar Ca^{2+} which are myoplasmic activators of SR release channels (Smith et al., 1985, 1986). Instead of Ca^{2+}, we used 250 mM CsCl on the *cis* side and 50 mM CsCl on the *trans* side as current carrier. This eliminated the need for large and unphysiological gradients of trans Ca^{2+} which severely decrease the activity of release channels (Ma et al., 1988). Also Cs^+ blocked SR K^+

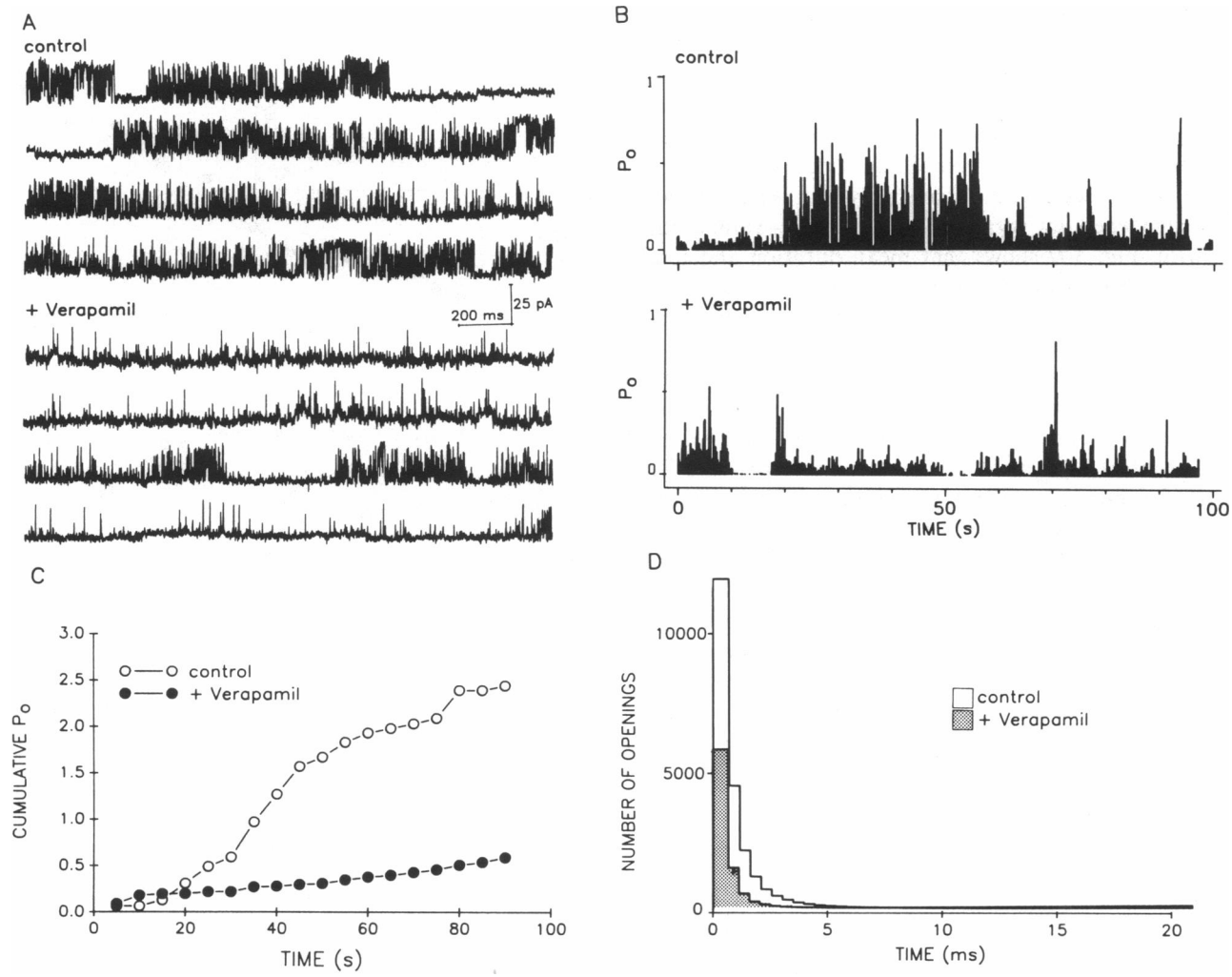

FIGURE 3 Inhibition of Ca^{2+} release channels by verapamil. (*A*) Ca^{2+} release channels at HP +20 mV, 10 μM Ca^{2+} before (control) or after *cis* addition of 50 μM verapamil (+verapamil). (*B*) 100 s of continuous records in control and after addition of verapamil were divided into intervals of 100 ms; P_o in each interval is plotted as a bar of length 0–1. Average P_o was 0.151 in control and 0.038 after addition of verapamil. (*C*) P_o in each interval of 5 s was added to that of the previous interval and the cumulative sum pllted as a function of the recording time. (*D*) Open events of duration time, *t*, or longer are plotted as a function of time, *t*. Number of observations were 11,737 (control) or 3,659 (verapamil).

channels (Coronado and Miller, 1979) and because it has a higher conductance than Ca^{2+} or Na^+ through release channels ($g_{Cs}/g_{Ca} = 2$) (Smith et al., 1988), it improved the signal-to-noise ratio. Channels had a linear current-voltage relationship with a slope conductance of 450 pS and a reversal at potentials more negative than −30 mV. Furthermore, ryanodine at a concentration of 100 nM or less, produced an irreversible decrease in conductance to 250 pS and an increase in mean open time from 0.5 ms in control to 50 ms in the presence of ryanodine (not shown). The ligand gating characteristics of channels in Cs^+ were similar to those measured previously in high *trans* Ca^{2+} (Smith et al., 1985, 1986, 1988). For example, *cis* 5 mM ATP increased channel activity ~five-fold, *cis* 1 mM free Mg^{2+} decreased activity 10-fold, and *cis* 1 μM ruthenium red decreased activity 100-fold (not shown).

Ca^{2+} release channels at +20 mV, 10 μM free Ca^{2+}, and pH 7.4 are shown in Fig. 3 *A*, both before and after addition of 50 μM verapamil. Channel activity monitored over 100 s in each condition had a mean open probability $P_o = 0.15 \pm 0.10$ (SD) in control and decreased to $P_o = 0.038 \pm 0.035$ (SD). The most significant kinetic effects of verapamil were a reduction in the burst time (Fig. 3 *B*) and a reduction in the mean open time (Fig. 3 *D*). Both contributed to an overall decrease in the number of events per unit time, shown in Fig. 3 *C* in a graph of cumulative P_o over time. As indicated by the diary of openings of Fig. 3 *B*, verapamil produced a significant reduction in the

number of long openings, that is in the events with high P_o. The length of each bar in Fig. 3 *B* represents P_o during consecutive sweeps. Each sweep had a duration of 100 ms. Empty spaces, seen only after addition of verapamil, corresponded to null sweeps in which no openings were seen. Successive sweeps containing long openings gave rise to bursts (see Fig. 3 *B*, control) which were substantially shortened by verapamil (Fig. 3 *B*, +verapamil). Likewise, Fig. 3 *D* shows that mean duration and total number of events were reduced from control $\tau_{open} = 1.9$ ms and $n = 11{,}737$ events, to $\tau_{open} \leq 0.6$ and 3,659 events. Duration of closed events (not shown) increased from control $\tau_{closed} = 3$ and 16 ms to $\tau_{closed} = 5$ and 31 ms after addition of verapamil. Similar inhibition was seen when 2 mM Ca^{2+} was added to the trans chamber to establish a Ca^{2+} gradient equivalent to that present across the SR membrane during constant Ca pump activity (Hasselbach and Oetliker, 1983). From these experiments we concluded that verapamil, albeit at relatively high concentrations (>20 μM at pH 7.4), stabilized the release channel in a closed or blocked conformation. This inhibitory effect would explain the reduced affinity of the receptor for [^{3}H]Ryanodine in the presence of verapamil because open channels have a much higher affinity for the alkaloid than closed channels (Pessah et al., 1986, 1987).

Identification of low-affinity sites for [^{3}H]Verapamil in triads

Low-affinity and high-affinity binding sites for [^{3}H]Verapamil in triads are shown in Fig. 4 *A*. Inset in Fig. 4 *A* corresponds to separation of low and high affinity components using Scatchard analysis. A low-density ($B_{max} = 5$ pmol/mg), high-affinity ($K_d = 15$ nM) class of sites saturated at 40 nM [^{3}H]Verapamil whereas a high-density low-affinity component did not exhibit saturation up to a concentration of 100 nM [^{3}H]Verapamil. When triads were prelabeled with 7 nM [^{3}H]Verapamil, displacement with unlabeled verapamil was biphasic (Fig. 4 *B*, *solid circles*). Half-maximum displacement of the high and low affinity components occurred at ~20 nM and 5 μM unlabeled verapamil, respectively. Nitrendipine (Fig. 4 *B*, *open circles*) produced a maximum displacement of 75% of bound [^{3}H]Verapamil with an apparent K_d of ~1.1 nM and a Hill slope of 0.96. This affinity is the same as that of [^{3}H]Nitrendipine binding to DHP receptors (Fosset et al., 1983) and suggested that the high affinity binding of [^{3}H]Verapamil was indeed to the DHP receptor. Also, the total site density of the high affinity component (5 pmol/mg, Fig. 4 *A*) agreed well with the density of nitrendipine receptors in triads (Fairhurst et al., 1983). The fraction of bound [^{3}H]Verapamil sensitive to ryanodine is shown in Fig. 4 *B* (*open triangles*).

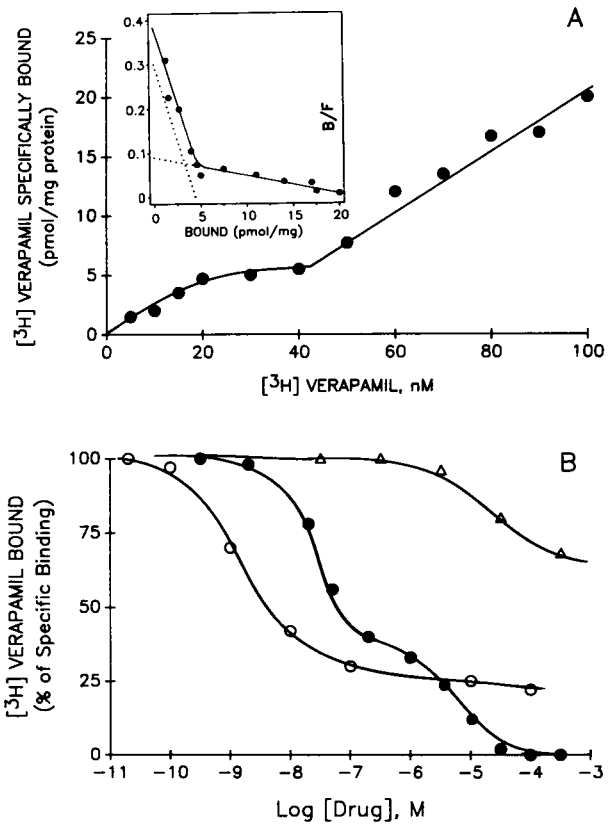

FIGURE 4 Binding of [^{3}H]Verapamil to triads and displacement by unlabeled verapamil, nitrendipine, and ryanodine. (*A*) Purified triads (40 μg) were incubated 60 min at 36°C with [^{3}H]Verapamil (80 mCi/mmol) in 400 μl of 40 mM Tris-HCl pH 8.5. Inset shows Scatchard plot of specifically bound [^{3}H]Verapamil. High affinity site has a $B_{max} = 5$ pmol/mg and $K_d = 15$ nM. Solid line is a least squares regression fit. Dotted lines describe independent binding components. Points are means of two experiments. (*B*) [^{3}H]Verapamil (7 nM) was incubated for 60 min at 36°C with purified triads (120 μg/ml) and the indicated concentration of nitrendipine (*open circles*), verapamil (*solid circles*), or ryanodine (*open triangles*). 100% of specific [^{3}H]Verapamil binding corresponds to 2.5 pmol/mg triad protein. Points are mean of three (verapamil), one (nitrendipine), or three (ryanodine) experiments.

Unlabeled ryanodine inhibited up to 30% of specific binding of [^{3}H]Verapamil at the highest concentration assayed (300 μM ryanodine). Because the ryanodine-sensitive and the nitrendipine-insensitive fractions were roughly the same, we concluded that the low affinity fraction was that responsible for the interaction of verapamil with the ryanodine receptor. Moreover, the apparent K_d for [^{3}H]Verapamil binding to the low affinity nitrendipine-insensitive site (Fig. 4 *B*, *solid circles*) was estimated as 5 μM, which is similar to the IC_{50} for verapamil inhibition of [^{3}H]Ryanodine binding (8 μM, Fig. 2).

Verapamil inhibition of [^{3}H]Ryanodine binding to the purified ryanodine receptor

Micromolar verapamil has a broad spectrum of action which includes inhibition of Na^+ channels (Frelin et al., 1982; Sihra et al., 1984), and inhibition of K^+ channels in neurons (Hume, 1985) and heart (Kass, 1982; Kayana and Katzung, 1984), as well as inhibition of Ca^{2+} transport across the sarcoplasmic reticulum (reviewed by Herbette et al., 1982). We therefore contemplated the possibility that verapamil interacted directly with the ryanodine receptor. Fig. 5 shows [^{3}H]Ryanodine binding experiments performed in the receptor purified to homogeneity from Chaps-solubilized triads. Using the sucrose gradient method of Lai et al. (1988) we identified the purified receptor as a single, ~400 kDa polypeptide sharply distributed between 13.5% and 16.5% wt/vol sucrose (Fig. 5 *A*, lane 2). Arrows indicate molecular weight markers in silver-stained PAGE gels. Polypeptides of lower molecular weight, suggestive of contamination or degradation, did not copurify with the receptor but were abundant in lighter fractions (lane 1). The distribution of the ~400 kDa peptide and the distribution of [^{3}H]Ryanodine binding activity in the sucrose gradient was strictly coincidental (not shown). Fig. 5 *B* (*open circles*) shows that the purified receptor displayed high affinity [^{3}H]Ryanodine binding to essentially a single site with K_d = 5 nM and B_{max} = 400 pmol/mg, in agreement with previous reports using a variety of purification methods (Inui et al., 1987; Imagawa et al., 1987; Lai et al., 1988). The binding of [^{3}H]Ryanodine to the purified receptor in the presence of 30 μM D600 (methoxyverapamil) appears in Fig. 5 *B* (*solid circles*). The phenylalkylamine reduced [^{3}H]Ryanodine binding at all concentrations tested. Inhibition was noncompetitive in nature (Fig. 5 *C*), inasmuch as the K_d did not change but there was a decrease in B_{max} from 400 to 280 pmol/mg, or ~1.4-fold. This result suggested that the verapamil binding site in the purified ryanodine receptor is altogether distinct from the ryanodine binding site.

Dose-response curves for D600 and verapamil, obtained by incubation of the purified receptor with 5 nM [^{3}H]Ryanodine and increasing concentrations of the phenylalkylamine, are given in Fig. 6. D600 (*open circles*) inhibited binding with an IC_{50} of ~80 μM, whereas for verapamil (*solid circles*) the IC_{50} was ~800 μM. This value is 80-fold higher than that measured in intact triads (Fig. 2). In agreement with this shift, we also failed to observe phenylalkylamine effects in purified ryanodine receptor channels up to a concentration of 50 μM verapamil (not shown). This was in spite of the fact that the purified preparation was as active as in previous reports

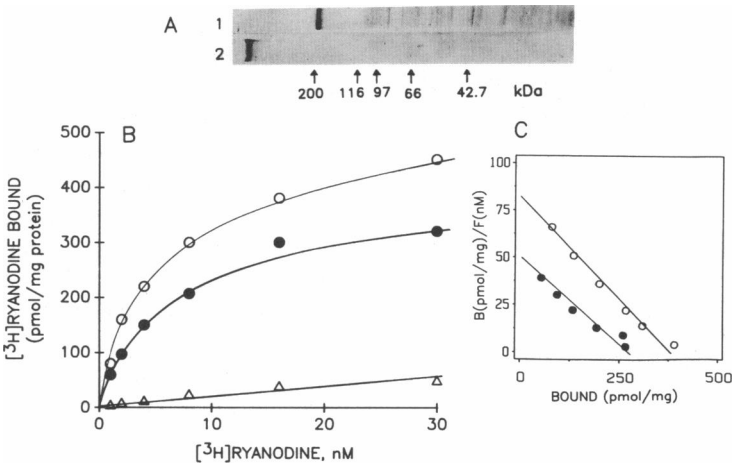

FIGURE 5 [^{3}H]Ryanodine binding to the purified ryanodine receptor and inhibition by D600. (*A*) Silver-stained PAGE gels of 40 μl of 5% (lane 1, 40 μg protein) or 15% (lane 2, 3 μg protein) sucrose gradient fractions of Chaps-solubilized ryanodine receptor. A single band of M.W. ~400 kDa (lane 2) was obtained from 13.5% to 16.5% sucrose. (*B*) Total binding of [^{3}H]Ryanodine to the purified receptor. Fractions pooled from the 13.5% to 16.5% portion of the sucrose gradient were incubated for 90 min at 36°C with the indicated concentrations of [^{3}H]Ryanodine in 300 mM NaCl, 40 mM Tris-Maleate pH 7.4, 100 μM $CaCl_2$, 2 mM Dithiothreitol, 50 μM PMSF, 320 mM Sucrose, 0.15% Chaps, and 0.15% Asolecithin (*open circles*); or in the same buffer plus 30 μM D600 (*solid circles*); or in the same buffer plus 10 μM unlabeled ryanodine (*triangles*). D600 or unlabeled ryanodine were added at the beginning of the incubation period. Receptor concentration was 6 μg/ml. (*C*) Scatchard plot of the specific binding of [^{3}H]Ryanodine. K_d = 5 nM and B_{max} = 400 pmol/mg in the absence of D600 (*open circles*) and K_d = 6 nM and B_{max} = 280 pmol/mg in the presence of D600 (*solid circles*).

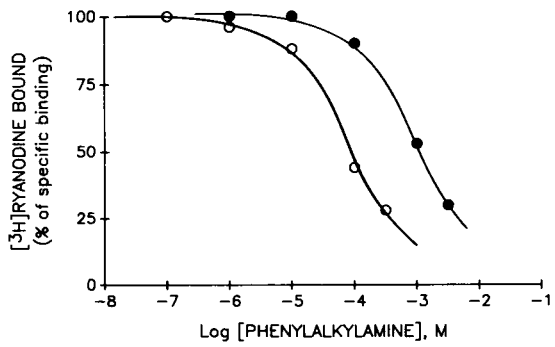

FIGURE 6 Dose-response curve for verapamil and D600 inhibition of [^{3}H]Ryanodine binding to the purified ryanodine receptor. Purified protein (6 μg/ml) was incubated 90 min at 36°C in 300 mM NaCl, 40 mM Tris-Maleate pH 7.4, 100 μM $CaCl_2$, 2 mM Dithiothreitol, 50 μM PMSF, 320 mM Sucrose, 0.15% Chaps, and 0.15% Asolecithin plus 5 nM [^{3}H]Ryanodine and the indicated concentration of D600 (*open circles*) or verapamil (*solid circles*). 100% of specific [^{3}H]Ryanodine binding corresponds to 220 pmol/mg of purified receptor. Points are mean of two experiments for each phenylalkylamine.

(Ma et al., 1988). Evidently there is a drastic decrease in affinity for verapamil in the purified protein. This result is not entirely surprising because the same effect is present for other ligands of the ryanodine receptor. In the case of ruthenium red, there is a shift from IC_{50} = 8 nM in intact triads to 2.5 μM in the purified receptor (Imagawa et al., 1987), or a 300-fold decrease in affinity. For tetraamine palladium and tetraamine platinum, two recently described inhibitors of the Ca^{2+} release channel (Ma et al., 1989), the shift is ~200-fold (unpublished results). Also, the Ca^{2+} required to inhibit [^{3}H]Ryanodine binding is 50-fold higher in the purified than in the native receptor (Imagawa et al., 1987).

DISCUSSION

Low-affinity binding sites for phenylalkylamines in skeletal muscle

In the present work, we traced a low-affinity phenylalkylamine binding site to the ryanodine receptor and showed that occupancy of this site by verapamil blocked the release channel. Nitrendipine had no effect on the verapamil-ryanodine receptor interaction which suggested that DHP receptors were not directly involved. A functional interaction of DHP receptors and ryanodine receptors at the triad junction of skeletal muscle has been previously suspected on several grounds. Such coupling may account for (*a*) the influence of DHPs and phenylalkylamines on "voltage sensors" of excitation contraction coupling (Hui et al., 1984; Rios and Brum, 1987; Brewe et al., 1987; Erdmann and Luttgau, 1989); (*b*) the copurification of ryanodine and DHP receptors in membrane preparations of cardiac and skeletal muscle (Williams and Jones, 1983; Knudson et al., 1988); (*c*) the fact that the ryanodine receptor is at the same time the Ca^{2+} release channel and the foot protein that spans the t-tubule SR junction (Block et al., 1989). Thus, the effects of Ca^{2+} antagonists on the ryanodine receptor are important because they may represent the means to determine if a functional coupling exists between the two receptors. Our results do not rule out the existence of such a complex but demonstrate that drug occupancy at t-tubule DHP receptors does not modulate binding of [^{3}H]Ryanodine at the ryanodine receptor.

Although verapamil and D600 block Ca channels in heart (Lee and Tsien, 1983) and skeletal muscle (Valdivia and Coronado, 1989), the pharmacological effects of phenylalkylamines are not restricted to the inhibition of DHP-sensitive Ca^{2+} channels (Herbette et al., 1982; Kass, 1982; Kayana and Katzung, 1984; Hume, 1985; Striessnig et al., 1985; Oeken et al., 1986). Particularly relevant to our findings are previous reports of inhibition of Ca^{2+} transport in the SR by D600 and verapamil (Balzer, 1972; Entman et al., 1972). These effects occur at concentrations higher than those required to saturate drug binding to the DHP receptor (>10 μM; Nayler et al., 1972; Watanabe et al., 1974). Furthermore, the presence in cardiac and skeletal muscle of phenylalkylamine binding proteins other than the DHP receptor, is known. In heart, verapamil receptors are four times more abundant than DHP receptors and only 30% of bound verapamil is displaced by nitrendipine (Garcia et al., 1984). In a solubilized preparation of Ca^{2+} channels of skeletal muscle, Horne et al. (1988) showed that $^{45}Ca^{2+}$ flux activity assayed in liposomes, and the sensitivity of channels to verapamil, resided in fractions with different peptide compositions. The flux activity required the α_1 peptide of the DHP receptor, whereas verapamil sensitivity resided in a fraction which included 165 and 55 kDa proteins distinct from α_1 peptide (Horne et al., 1988). Thus, besides data shown here, there is additional biochemical and electrophysiological evidence to support the existence of pharmacologically active low-affinity phenylalkylamine binding sites in muscle cells.

Implications for excitation-contraction coupling

The presence of a phenylalkylamine-sensitive inhibitory site on the release channel has not been previously considered in the context of muscle paralysis by D600 and related phenylalkylamines. Because caffeine opens release channels (Rousseau et al., 1988) and induces a contracture even in the presence of D600 (Fill and Best,

1989), it was thought that phenylalkylamines could block Ca^{2+} release without affecting release channels. Caffeine does not induce a spontaneous contracture however, in muscle fibers paralyzed with the high affinity phenylalkylamine D888 (Erdmann and Luttgau, 1989). Eisenberg et al. (1983) suggested that D600 blocked contraction at some step after onset of charge movement and before the release of Ca^{2+} from the SR. Brewe et al. (1987) proposed that D600 interacted directly with the "voltage sensor" on the t-tubule membrane to stabilize both the inactive form and the active form of this sensor. Evidence that the α_1 peptide of the DHP receptor forms part of the putative voltage sensor has been provided by direct pharmacological experiments (Hui et al., 1984; Rios and Brum, 1987; Ederman and Luttgau, 1989), and by restoration of contraction in dysgenic mice fibers microinjected with α_1 cDNA (Tanabe et al., 1988). On the other hand, there are numerous observations that suggest that occupancy of the high affinity site in the DHP receptor is not sufficient to induce muscle paralysis. (*a*) In microinjected frog fibers, paralysis may be induced by 30 μM D600 but not 20 μM nifedipine (McCleskey, 1985). (*b*) Cooling of frog fibers to 7°C is a prerequisite for the D600-induced paralysis (Eisenberg et al., 1983) whereas in the same tissue, the binding affinity for [^{3}H]Nitrendipine is about the same at 10° or 37°C (Jaimovich et al., 1986). The B_{max} of [^{3}H]Nitrendipine however, is highly temperature dependent (Jaimovich et al., 1986). (*c*) Doses required to block contraction are two or more orders of magnitude higher than the radioligand binding affinity of the same compound for the DHP receptor. Verapamil, D600, and D888 bind to the α_1 subunit of the DHP receptor with a K_d of ~30 nM, 40 nM, and 1 nM, respectively (Galizzi et al., 1986; Sieber et al., 1987; Galizzi et al., 1984), whereas the concentrations required to block muscle contraction are 30 μM D600 (Eisenberg et al., 1983; McCleskey, 1985); 5–100 μM D600 (Brewe et al., 1987); or 0.05–5 μM D888 (Erdmann and Luttgau, 1989).

In the case of DHPs, the discrepancy between ligand-binding affinities and the electrophysiologically measured affinities, has been attributed to the membrane potential, which at rest holds the DHP receptor in a low affinity state (Bean, 1984; Sanguinetti and Kass, 1984). Whether a voltage-dependence of binding is also present for phenylalkylamines is not entirely known but it is assumed to be similar (Erdmann and Luttgau, 1989). On the other hand, a high phenylalkylamine concentration necessary to paralyze muscle may reflect the fact that the primary target of phenylalkylamines, as far as muscle paralysis is concerned, is not the t-tubule voltage sensor but the ryanodine receptor channel. It must be admitted however, that the conspicuous use dependence and temperature dependence of phenylalkylamine-induced paralysis may be particularly hard to explain within this hypothesis. Even if t-tubule voltage sensors are the main site of action, the block of release channels is practically unavoidable, given that micromolar levels of D600 or D890 are required for paralysis even in skinned muscle fibers (Donaldson et al., 1984; Fill and Best, 1989). Finally, the observation that caffeine stimulates [^{3}H]Ryanodine binding (Pessah et al., 1987) and opens release channels (Rousseau et al., 1988) whereas verapamil inhibits [^{3}H]Ryanodine binding (Fig. 1) and blocks release channels (Fig. 3), suggests that a competitive interaction between the two ligands is likely to occur when both are present. A competition between caffeine and phenylalkylamines, for stimulatory and inhibitory sites, respectively, in the ryanodine receptor, could account for the faster rate of recovery from paralysis observed in muscle fibers treated with D888 plus caffeine (Erdmann and Luttgau, 1989).

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