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Identification of Dantrolene Binding Sites in Porcine Skeletal Muscle Sarcoplasmic Reticulum*

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Dantrolene, an intracellularly acting skeletal muscle relaxant, inhibits Ca^{2+} release from the sarcoplasmic reticulum during excitation-contraction coupling by an unknown mechanism. The drug is used to treat malignant hyperthermia, a genetic sensitivity to volatile anesthetics which results in the massive release of intracellular Ca^{2+} from affected skeletal muscle. We hypothesize that determination of the site of action of dantrolene will lead to further understanding of the regulation of sarcoplasmic reticulum calcium release. We report the identification of specific dantrolene binding sites in porcine skeletal muscle sarcoplasmic reticulum using a rapid filtration binding assay for [^{3}H]dantrolene. The binding isotherm in the heavy sarcoplasmic reticulum fraction indicates a single binding site with a K_d of 277 ± 25 nM and a B_{max} of 13.1 ± 1.5 pmol/mg of protein. Pharmacological specificity is characterized by inhibition of [^{3}H]dantrolene binding with unlabeled dantrolene, or azumolene, a physiologically active congener, but not with aminodantrolene, which is physiologically inactive. Drug binding is maximal at pH 6.5–7.5, requires no Ca^{2+} or Mg^{2+}, and is inhibited by salt concentrations above 100 mM. [^{3}H]Dantrolene binding is greatest in the sarcoplasmic reticulum, which contains the ryanodine receptor, the primary calcium release channel. No binding is detected in the fractions enriched for sarcolemma or transverse tubules. We suggest that dantrolene inhibits calcium release from the sarcoplasmic reticulum by either direct or indirect interaction with the ryanodine receptor.

Dantrolene (1-[[5-(*p*-nitrophenyl)furfurylidine]amino]hydantoin sodium) is a hydantoin derivative that acts as a postsynaptic muscle relaxant (1) and is the only known effective treatment for malignant hyperthermia (MH),[1] a genetic disorder of excitation-contraction coupling (ECC) in skeletal muscle (2). MH is triggered in susceptible individuals by volatile anesthetics and depolarizing muscle relaxants (3) and is characterized by a pattern of muscle physiology which resembles aberrant ECC. MH is exemplified by a massive release of Ca^{2+} from the sarcoplasmic reticulum (SR), which results in hypercontracture, hypermetabolism, elevated temperatures, and death, if not treated with dantrolene (3). Porcine stress syndrome, a porcine model for MH triggered by volatile anesthetics or stress, is also characterized by an exaggerated increase in intracellular Ca^{2+} and is linked to a genetic defect in the ryanodine receptor (RyR), the SR calcium release channel (4, 5). Dantrolene inhibits the development or progression of MH by decreasing the levels of myoplasmic Ca^{2+} in both human and porcine skeletal muscle (2, 6, 7). The evidence suggests that dantrolene does so by inhibiting the release of SR Ca^{2+} (7) rather than its reuptake into the SR (8).

The mechanism of ECC-induced Ca^{2+} release in skeletal muscle has been reviewed extensively (9–13). Briefly, ECC is initiated by a wave of depolarization across the sarcolemma which is sensed by the transmembrane dihydropyridine receptor (DHPR), an L-type calcium channel present in the transverse tubules, specialized invaginations of the sarcolemma. The DHPR apposes the RyR in the SR membrane forming the triad junction (14) and is poised to sense depolarization and transmit a signal that results in Ca^{2+} release via the RyR (9). This signal transduction involves intramembrane charge movement across the DHPR rather than the calcium current seen in cardiac muscle (9). The exact relationship of this signaling event to the opening of the RyR is not clear but may involve a direct interaction of the cytoplasmic loop of the DHPR with the RyR (15). The net result of this interaction is the opening of the RyR, Ca^{2+} release from the SR into the myoplasm, and the initiation of skeletal muscle contraction.

RyR opening can be modulated by a number of ligands and cellular processes, including caffeine, ATP, ruthenium red, the immunophilin FK506-binding protein (FKBP12), Ca^{2+}, and phosphorylation (12). Furthermore, the cellular architecture of the triad junction suggests that at least one of a number of proteins known to span the junction, triadin, may modulate signal transduction and/or RyR opening (16). Most studies suggest that dantrolene acts to inhibit Ca^{2+} release from the SR at a site distal to the DHPR (7, 17). Only one study, in amphibian skeletal muscle, suggests that dantrolene may affect charge movement across the DHPR (18). Theoretically, therefore, the pharmacological action of dantrolene to reduce myoplasmic Ca^{2+} may occur as a result of the drug's interaction with the DHPR, the RyR, or any of the proteins, known or uncharacterized, of the triadic junction. Identifying the molecular target of dantrolene will likely delineate the component(s) that regu-

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[1] The abbreviations used are: MH, malignant hyperthermia; ECC, excitation-contraction coupling; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; DHPR, dihydropyridine receptor; HPLC, high performance liquid chromatography; DMF, dimethyl formamide; HSR, heavy sarcoplasmic reticulum; LSR, light sarcoplasmic reticulum; Mes, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

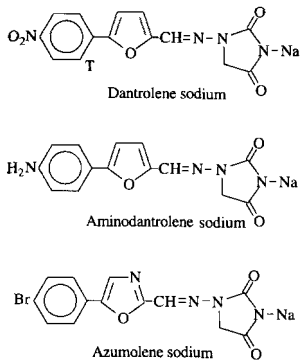

FIG. 1. **Dantrolene and its congeners.** Dantrolene and azumolene are physiologically active skeletal muscle relaxants, whereas aminodantrolene is inactive. *T* denotes the position of tritium in [^{3}H]dantrolene.

lates SR Ca^{2+} release during ECC in both normal and MH-susceptible skeletal muscle.

We have therefore begun a program to identify the molecular target of dantrolene. In this paper we report the development of a pharmacological assay demonstrating the existence of discrete dantrolene binding sites in skeletal muscle SR membranes. Although previous attempts have been made to identify a dantrolene receptor by radioactive ligand binding assays (19) or by fluorescence measurements (20), the former assay suffered from poor reproducibility (21) and both from the inability to distinguish specific from nonspecific binding. Here we characterize a specific, reproducible assay for [^{3}H]dantrolene binding to pig skeletal muscle membranes. In addition, as dantrolene is such a hydrophobic compound (22), we also describe many of the experimental obstacles overcome during development of the assay.

EXPERIMENTAL PROCEDURES

Dantrolene sodium·3½H_2O, azumolene sodium·2H_2O, and aminodantrolene sodium were gracious gifts of Proctor & Gamble, Norwich, NY. [^{3}H]Dantrolene was custom synthesized by ChemSyn Science Laboratories, Lenexa, KS. [^{3}H]Ryanodine (61.5 Ci/mmol) and [^{3}H]PN200-110 (78 Ci/mmol) were purchased from DuPont NEN and Amersham Corp., respectively. Phenylmethylsulfonyl fluoride, benzamidine, leupeptin, and pepstatin A were purchased from Boehringer Mannheim. All HPLC solvents were purchased from J. T. Baker and were HPLC grade. Cytoscint ES and hyamine hydroxide were purchased from ICN, and glass fiber filters (GF/C) were from Whatman. Other filters were gifts from Gelman and Stratagene, as noted. Common laboratory chemicals were purchased from Sigma.

Authentication of [^{3}H]Dantrolene and Determination of Specific Activity—[^{3}H]Dantrolene (Fig. 1) was custom synthesized by ChemSyn using the four-step reaction scheme of Snyder *et al.* (1), with the first step involving tritiation of *o*-bromoaniline by catalytic halogen replacement. [^{3}H]Dantrolene was received as the crystalline salt (·3½ H_2O) and dissolved in dimethylformamide (DMF). DMF was chosen as the solvent for two reasons: 1) dantrolene is soluble in DMF to a concentration of 10 mM (data not shown), and 2) it can be stored at −20 °C as a solution, essential for radioactive compounds, since freezing tends to concentrate solute locally, thus increasing the likelihood of radiolysis (23). Stock solutions of unlabeled dantrolene were dissolved in DMF, ethanol, or water. Both labeled and unlabeled dantrolene were chromatographed by HPLC on a Lichrosorb 100 RP-18 column (5 μm, Merck), 12.5 × 0.4 cm, using 25% acetonitrile in 20 mM potassium phosphate buffer, pH 7.4, as solvent phase, and peaks identified by absorbance at 385 nm (24). In the case of [^{3}H]dantrolene, 0.2-ml fractions were collected, and 50-μl aliquots were counted in 4 ml of Cytoscint ES in an LKB 1209 RackBeta liquid scintillation counter. Both unlabeled and [^{3}H]dantrolene chromatographed as single peaks with a retention time of 3.2 min (data not shown).

Specific activity was determined as follows. Unlabeled dantrolene was weighed in triplicate and dissolved in DMF or ethanol to a 10 mM final concentration. The samples were then diluted serially into ethyl acetate, a solvent in which dantrolene acquires fluorescent properties (20), and excitation and emission maxima were determined to be 383 and 540–580 nm, respectively, in an MPF-66 Perkin-Elmer fluorescence spectrophotometer (data not shown). DMF and ethanol concentrations up to 10% do not quench dantrolene fluorescence in ethyl acetate (data not shown). Fluorescence emission at 540 nm (λ_{ex}, 383 nm) was measured as a function of increasing dantrolene concentration and was found to be linear over the range of 1–500 nM (INPLOT 4.0, GraphPad, $r = 0.995$, data not shown). A standard graph of fluorescence intensity *versus* dantrolene concentration was then constructed, and the concentration of [^{3}H]dantrolene was determined from the above graph by measuring the fluorescence emission of the radioactive samples. To determine the specific activity of [^{3}H]dantrolene, triplicate aliquots of known concentration were counted in Cytoscint ES using an LKB RackBeta 1209 liquid scintillation counter (counting efficiency 59%). The resultant specific activity of [^{3}H]dantrolene was found to be 8.92 Ci/mmol. During the course of the experiments described below, specific activity was routinely assessed and corrected for tritium exchange.

Determination of Dantrolene and Congener Solubility—Solubility of dantrolene and congeners was determined by filtration assay based on the finding that dantrolene and congeners coprecipitate (see data below). Samples containing 1 ml of assay buffer (see below) were mixed with 60,000 cpm [^{3}H]dantrolene and increasing concentrations of unlabeled dantrolene or congener in borosilicate glass tubes, vortexed, and allowed to stand at 20 °C for 1 h. The final concentration of DMF was 0.25%. Samples were filtered rapidly through Whatman GF/C filters (average pore size 1.2 μm), as described for [^{3}H]dantrolene binding below, and washed with 1 × 5 ml of binding buffer at room temperature, and the amount of radioactivity bound to the filters was determined. Increases in filter-bound radioactivity in the presence of unlabeled drug are presumably due to coprecipitation (25).

Preparation of Pig Skeletal Muscle Membrane Fractions—Pig (Yorkshire × Landrace or homozygous normal Yorkshire) fast twitch skeletal muscle were gifts of Dr. Donald Wilkerson, Department of Surgery, UMDNJ-Robert Wood Johnson Medical School, and Dr. Charles F. Louis, Department of Veterinary Biology, University of Minnesota. Freshly dissected muscle was frozen immediately in liquid nitrogen and maintained at −72 °C until use. Membrane fractions corresponding to the sarcolemma, transverse tubules, light (LSR) and heavy sarcoplasmic reticulum (HSR) were prepared, with slight modification (26), according to the method of Meissner (27). Briefly, 150–160 g of tissue was homogenized in a Waring blender (2 × 30 s, high speed) with 750 ml of ice-cold buffer containing 5 mM Tris maleate, pH 6.8, and 0.1 M NaCl. The homogenate was centrifuged at 2,600 × *g* for 30 min at 4 °C. The supernatant was saved and the pellet rehomogenized in the Waring blender with 150 ml of buffer, 1 × 30 s at high speed, and centrifuged as above. The supernatants were combined, filtered through six layers of cheesecloth, and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was discarded and the pellet resuspended in 30–40 ml of 0.6 M KCl, 5 mM Tris-Mes, pH 6.8, with two or three strokes of a motor-driven, Teflon-glass homogenizer. These salt-treated microsomes were sedimented at 120,000 × *g* for 60 min. The supernatant was discarded and the pellets resuspended in a total of 30 ml of 5 mM Tris-Mes buffer, pH 6.8, containing 0.4 M KCl, 10% sucrose (w/v), and 20 μM $CaCl_2$. Ten ml of the resuspended pellet was layered onto a discontinuous sucrose gradient (5 ml of 40%, 8 ml of 35%, 8 ml of 30%, and 5 ml of 20% sucrose containing 0.4 M KCl, 20 μM $CaCl_2$, in 5 mM Tris-Mes, pH 6.8) and centrifuged overnight at 130,000 × *g*, with slow acceleration and no braking at the end of the run. Membrane fractions at the density interfaces were aspirated carefully, diluted slowly to approximately 10% sucrose in buffer (0.1 M KCl, 5 mM Tris-Mes, pH 6.8), and repelleted at 95,000 × *g* for 40 min at 4 °C. The pellets were resuspended slowly in 1–2 ml of 5 mM Na-PIPES buffer, pH 6.8, containing 10% sucrose and 0.1 M KCl, aliquoted, frozen in liquid N_2, and stored at −70 °C until use. The following protease inhibitors were present at all stages of membrane fractionation: 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin A, and 0.5 mg/ml benzamidine. Protein was determined by the method of Bradford (28). Membrane fractions were characterized for their ability to bind both [^{3}H]ryanodine (29) and the dihydropyridine antagonist [^{3}H]PN200-110 (30).

[^{3}H]Dantrolene Binding Assay—[^{3}H]Dantrolene binding to pig skeletal muscle membrane fractions was determined by a rapid filtration assay. Membrane protein, at the appropriate concentration, was incubated in buffer (10 mM Na-HEPES or Tris-HCl, pH 7.4), with various concentrations of [^{3}H]dantrolene, in the presence or absence of either 30 μM unlabeled dantrolene or 150 μM azumolene for 60 min at 37 °C in the dark, in a final reaction volume of 250 or 500 μl, in triplicate. [^{3}H]Dantrolene binding to HSR at 37 °C reached equilibrium by 60 min (data

not shown). The final concentration of DMF or ethanol in the assay mixture was less than 1%. A 50-μl unfiltered aliquot/sample was placed in a vial containing 4 ml of Cytoscint ES, and radioactivity was determined by liquid scintillation counting to calculate directly the total ligand concentration/sample. Samples were then filtered rapidly through Whatman GF/C filters using a Hoefer model FH225V filtration apparatus (suction at 10 mm Hg for best separation). Filters were washed rapidly (<2 s) with 1 × 5 ml of ice-cold binding buffer and treated overnight with 200 μl of 1 M hyamine hydroxide/filter to hydrolyze the membranes and proteins. Increasing the number of washes served only to decrease the total radioactivity measured and did not increase the signal-to-noise ratio (data not shown). Radioactivity was then determined by liquid scintillation counting in 4 ml of Cytoscint ES. Total binding was that achieved in the absence of unlabeled drug, and nonspecific binding, that measured in the presence of 150 μM azumolene (Fig. 1), a more water-soluble, physiologically active congener of dantrolene (31). Since the maximum solubility of dantrolene (20 °C, pH 7.4) was determined to be 30 μM (see below), we used azumolene to determine the nonspecific binding of [^{3}H]dantrolene. Specific binding was determined by subtracting nonspecific binding from total binding. The data for this and the other binding assays described below were analyzed by nonlinear regression analysis using INPLOT. Control assays were run in the absence of skeletal muscle membranes to determine the degree of [^{3}H]dantrolene binding to the filters and to ensure that there was no specific binding to filters, as has been observed in other systems (32, 33).

[^{3}H]Ryanodine Binding Assay—[^{3}H]Ryanodine binding to skeletal muscle membrane fractions was determined according to the method of Valdivia *et al.* (29). Briefly, increasing concentrations of [^{3}H]ryanodine (1–50 nM) were incubated with membrane fractions in a buffer containing 20 mM Tris-HCl, pH 8.5, 0.15 M NaCl, and 50 μM $CaCl_2$, at 37 °C for 90 min. Samples were filtered through Whatman GF/C filters using a Brandel cell harvester and washed with ice-cold buffer (2 × 5 ml). The filters were counted for radioactivity in Cytoscint ES. Nonspecific binding corresponded to the binding measured in the presence of 10 μM ryanodine. Data were analyzed as above. The K_d and B_{max} for [^{3}H]ryanodine binding to HSR were 7.0 ± 0.05 nM and 9.0 ± 1.6 pmol/mg of protein, respectively.

[^{3}H]PN200-110 Binding Assay—[^{3}H]PN200-110 binding to skeletal muscle membrane fractions was measured as described previously (30). Membranes were incubated at room temperature (25 °C) in a solution containing 50 mM Tris-HCl, pH 7.4, with different concentrations of [^{3}H]PN200-110 (0.1–5 nM), in the dark. Membrane-bound ligand was separated from the free ligand by using the Brandel cell harvester as described for the [^{3}H]ryanodine binding assay, and the radioactivity on the filter was counted in Cytoscint ES. One μM nitrendipine was used to determine the nonspecific binding. Data were analyzed as above. The K_d and B_{max} values for [^{3}H]PN200-110 binding to the transverse tubules were 0.8 ± 0.1 nM and 16 ± 2.4 pmol/mg of protein, respectively.

RESULTS

Solubility Limits of Dantrolene and Congeners—Dantrolene is an extremely hydrophobic drug, and therefore it precipitates easily out of aqueous solution. Hence, prior to development of the binding assay, the solubility limits of dantrolene and its congeners, azumolene and aminodantrolene, were determined by coprecipitation, as described under "Experimental Procedures." The approximate solubility maxima at pH 7.4, 20 °C, were determined to be 30, 50, and 300 μM for dantrolene, aminodantrolene, and azumolene, respectively. The above solubility limit for dantrolene is in clear agreement with the value of 35.1 μM determined by Salata and Jalife (34). Solubility limits for these compounds at 37 °C are not significantly different than those determined at 20 °C (data not shown).

Nonspecific Binding of [^{3}H]Dantrolene—In developing the rapid filtration assay for [^{3}H]dantrolene binding to pig skeletal muscle membranes, we immediately became aware of the propensity of dantrolene to bind nonspecifically to many laboratory materials. During attempts at developing a centrifugation assay, we found that [^{3}H]dantrolene binds nonspecifically to the sides of polypropylene tubes (data not shown). Moreover, polypropylene induces the precipitation of 30 μM azumolene in the presence of nanomolar amounts of [^{3}H]dantrolene (data not shown). Silanization of polypropylene significantly reduced nonspecific binding (about 50%), but the same process enhanced nonspecific binding to glass (data not shown). Despite silanization, however, use of polypropylene tubes in centrifugation assays for [^{3}H]dantrolene binding to skeletal muscle membranes resulted in unacceptable intersample variation. These tubes, therefore, are unsuitable for use with dantrolene or its congeners. Rapid filtration, rather than centrifugation, was chosen as an assay method. Various filter media were tested for their ability to bind [^{3}H]dantrolene nonspecifically, using the rapid filtration method described above. Glass fiber filters (GF/C) were the only ones not to bind significant amounts of [^{3}H]dantrolene (Table I). Preincubating these filters with unlabeled dantrolene, various detergents, or chemicals commonly used to inhibit nonspecific binding in other systems did not reduce nonspecific binding (Table II). Hence, untreated Whatman GF/C filters were used in the assay described below.

TABLE I
Adsorption of [^{3}H]dantrolene to various filters

The adsorption of [^{3}H]dantrolene to various filters was determined using a filtration manifold (Hoefer). [^{3}H]Dantrolene (189,600 cpm) in 250 μl of 10 mM Na-HEPES, pH 7.4, was filtered rapidly and filters washed with 5 ml of cold buffer. Radioactivity retained on the filters was determined by liquid scintillation counting (n = 3).

Filter media	Adsorbed
	cpm
Glass fiber (GF/C)	4,450 ± 90
Nitrocellulose (Stratagene)	103,100 ± 10,200
Nitrocellulose (Amersham)	133,400 ± 9,800
TF-450 (PTFE, Gelman)	—[a]
Duralon (Nylon, Stratagene)	85,800 ± 6,800
Nyalaflo 45 (Nylon, Gelman)	143,500 ± 10,300
Supor 800 (polysulfone, Gelman)	76,800 ± 4,200
Versapore 800 (Acrylic copolymer, nylon, Gelman)	15,600 ± 3,100
GN-4 (cellulose acetate/nitrate, Gelman)	78,800 ± 4,400

[a] Filtration was not possible.

Specific Binding of [^{3}H]Dantrolene to HSR—To quantitate the interaction between [^{3}H]dantrolene and its binding sites in HSR at equilibrium, increasing concentrations of [^{3}H]dantrolene were incubated with HSR membranes, in the presence or absence of azumolene, and radioactivity was determined as described under "Experimental Procedures." Nonspecific binding, that measured in the presence of 150 μM unlabeled azumolene, was subtracted from total binding, that measured in the presence of [^{3}H]dantrolene alone (Fig. 2*A*), to give a specific binding curve (Fig. 2, *A* and *B*). Using nonlinear regression analysis (INPLOT) to analyze the calculated specific binding data, we determined that the resultant curve is best described as a rectangular hyperbola, indicating a single class of binding sites. The calculated K_d and B_{max} for [^{3}H]dantrolene binding to HSR under these experimental conditions are 277 ± 25 nM and 13.1 ± 1.5 pmol/mg of protein, respectively. These results were obtained despite a signal-to-noise ratio between 8 and 15%. Recent binding experiments carried out with [^{3}H]dantrolene dissolved in ethanol instead of DMF yielded a signal-to-noise ratio of 20–30% without any significant changes in the binding parameters (K_d, 273 ± 25 nM; B_{max}, 12.9 ± 1.2 pmol/mg of protein; Fig. 3, *A* and *B*). Indeed, the nonspecific, and, hence, the total [^{3}H]dantrolene bound, decreased by approximately 50% when the drug was dissolved in ethanol, as compared with that seen when the drug was dissolved in DMF (see Figs. 2*A* and 3*A*). When control assays were carried out as described under "Experimental Procedures" but without membrane protein, "specific" binding of [^{3}H]dantrolene to glass fiber filters was not seen.

Inhibition of [^{3}H]Dantrolene Binding to HSR by Dantrolene Congeners—We determined the ability of dantrolene congeners

TABLE II
Nonspecific binding of [^{3}H]dantrolene to GF/C filters: effect of various treatments

Adsorption of [^{3}H]dantrolene to glass fiber filters treated with various solutions was determined as described in Table I.

Treatment	Bound[a]
	cpm
None	4,450 ± 90
Polyethyleneimine (1%)	12,500 ± 2,400
Bovine serum albumin (1%)	8,700 ± 870
Ficoll (1%)	9,700 ± 650
Gelatin (1%)	11,300 ± 770
Dichlorodimethyl silane	10,100 ± 940
Poly-L-lysine (0.5%)	14,520 ± 800
Tween 20 (1%)	7,200 ± 450
CHAPS (1%)	6,500 ± 340
Glycerol (1%)	5,300 ± 210
Dantrolene (1 μM)	6,500 ± 250

[a] Total counts filtered, 189,600 cpm.

to inhibit specific [^{3}H]dantrolene binding to HSR to assess pharmacological specificity. [^{3}H]Dantrolene (200 nM) was incubated with 50 μg of HSR membrane protein in the presence of increasing concentrations of dantrolene, azumolene, or aminodantrolene (a physiologically inactive congener (35)), in binding buffer for 60 min at 37 °C, and radioactivity was determined. The results, presented in Fig. 4, show that dantrolene and azumolene have nearly identical inhibition characteristics, whereas aminodantrolene, at concentrations up to its solubility maximum, has no activity in this system. The apparent K_i values for dantrolene and azumolene are approximately 528 nM. This value was calculated using the Cheng-Prusoff correction, in which K_i is a function of both the radiolabeled ligand concentration and the concentration of inhibitor at which 50% of ligand binding to receptor is inhibited (36).

Comparison of Specific [^{3}H]Dantrolene, [^{3}H]PN200-110, and [^{3}H]Ryanodine Binding Sites in Subcellular Skeletal Muscle Membrane Fractions—To compare the subcellular distribution of [^{3}H]dantrolene and [^{3}H]ryanodine binding sites, we measured their specific binding to skeletal muscle membrane fractions corresponding to sarcolemma, transverse tubules, LSR, and HSR, prepared as described under "Experimental Procedures." Fifty μg of membrane protein from each fraction was incubated, in triplicate, with [^{3}H]dantrolene (200 nM), [^{3}H]PN200-110 (0.8 nM), or [^{3}H]ryanodine (5 nM), in the presence or absence of appropriate concentrations of the unlabeled counterpart of each; specific binding was determined by the assay system described for each drug under "Experimental Procedures." The concentration of each labeled ligand in the experiment approximated the calculated K_d for that ligand. The results, shown in Table III, indicate a parallel distribution of [^{3}H]ryanodine and [^{3}H]dantrolene binding sites in pig skeletal muscle membrane fractions. The LSR fraction showed approximately half the [^{3}H]dantrolene and [^{3}H]ryanodine binding sites as compared with HSR. No specific binding of either drug was seen in the sarcolemma or the transverse tubule fraction, with the latter possessing the highest concentration of [^{3}H]PN200-110 binding sites.

Effects of pH, and Mono- and Divalent Cations, on Specific [^{3}H]Dantrolene Binding to HSR—The binding of [^{3}H]ryanodine to its receptor, the primary calcium release channel of the SR, is affected by pH, divalent cations, and ionic strength (11, 12, 37). We therefore investigated the effects of pH and increasing concentrations of NaCl, KCl, Ca^{2+}, or Mg^{2+} on the specific binding of [^{3}H]dantrolene (200 nM) to HSR, as described under "Experimental Procedures." As shown in Fig. 5, the optimum pH for [^{3}H]dantrolene binding is between 6.5 and 7.5, with a rapid falloff in specific binding above pH 7.5. At pH 6.0, dantrolene precipitates out of solution, and by pH 9.0 specific binding is completely abolished. Both NaCl and KCl inhibit the binding of [^{3}H]dantrolene to HSR (Fig. 6), with 20–40% control binding evident at 150 mM. The specific binding of [^{3}H]dantrolene is not greatly inhibited by Ca^{2+} or Mg^{2+}, as is shown in Fig. 7, although the patterns of the inhibition curves are somewhat different. Calcium, at concentrations above 10 μM, inhibits its specific binding to a maximum of approximately 60% control values. On the other hand, the maximal inhibitory effects of Mg^{2+} on [^{3}H]dantrolene binding also reach 60% control, but at a concentration of 20 mM.

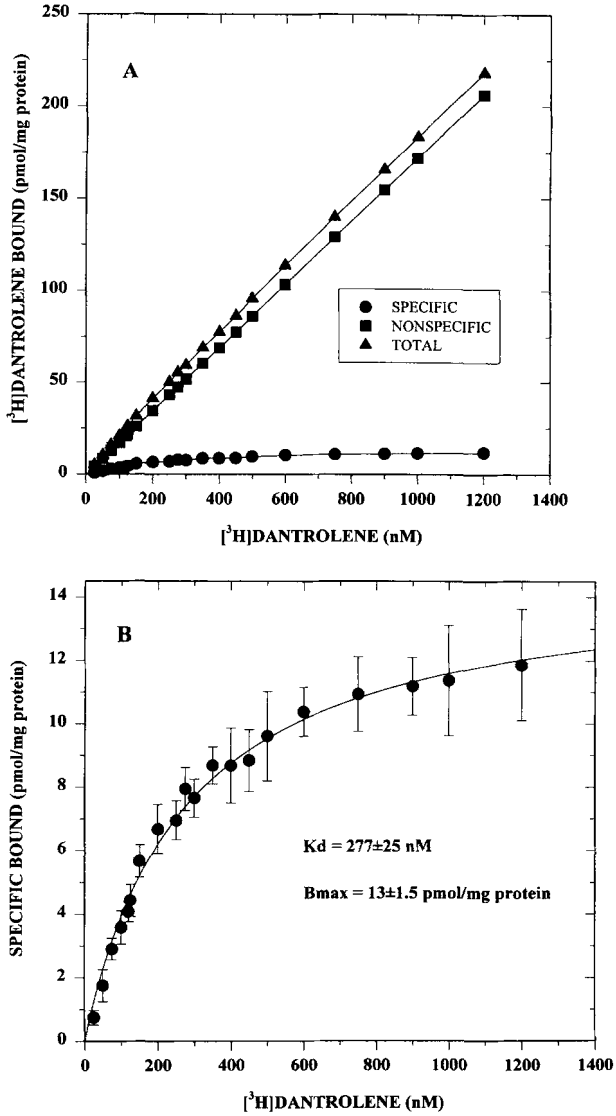

FIG. 2. **Binding of [^{3}H]dantrolene to HSR membranes.** *Panel A*, total, nonspecific, and specific binding of [^{3}H]dantrolene to porcine HSR membranes as determined by rapid filtration assay described under "Experimental Procedures." Each point is the mean of triplicate determinations ± S.E. from four different experiments on membranes derived from three separate animals. Stock [^{3}H]dantrolene solutions were made in DMF. *Panel B*, specific binding, the difference between total and nonspecific binding expanded from *panel A*.

DISCUSSION

Dantrolene, originally synthesized by Snyder *et al.* (1), is one of a series of hydantoin derivatives found to be active as muscle relaxants. Ellis and co-workers (39, 40) presented evidence that dantrolene inhibited ECC in mammalian skeletal muscle, acting distal to the neuromuscular junction, and Van Winkle (7)

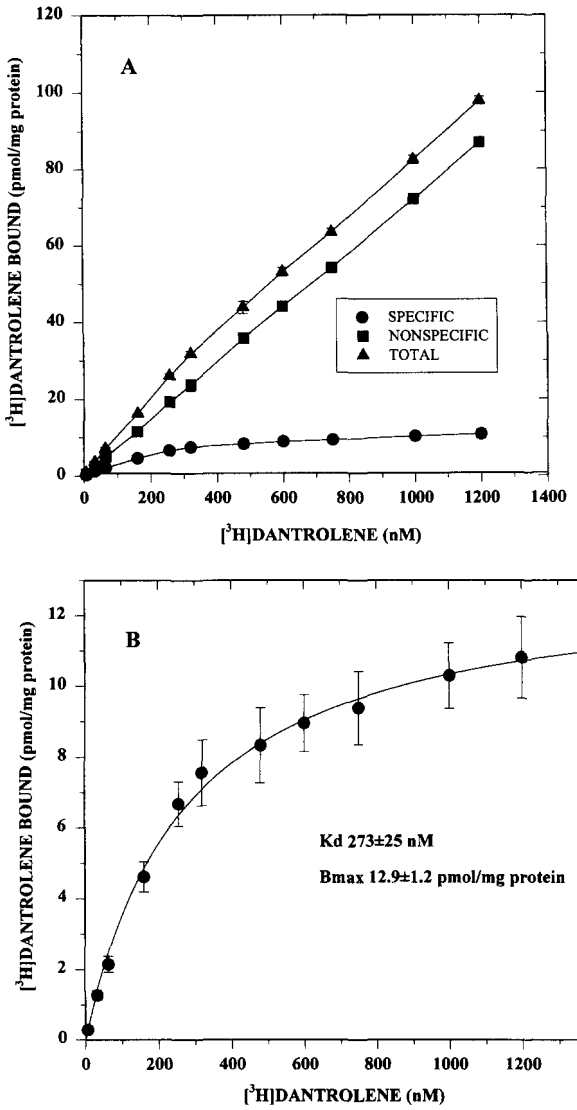

FIG. 3. **Binding of [³H]dantrolene to HSR membranes.** *Panel A*, conditions were identical to those in Fig. 2 except that stock [³H]dantrolene solutions were made in ethanol ($n = 3$). *Panel B*, specific binding curve generated using data expanded from *panel A*.

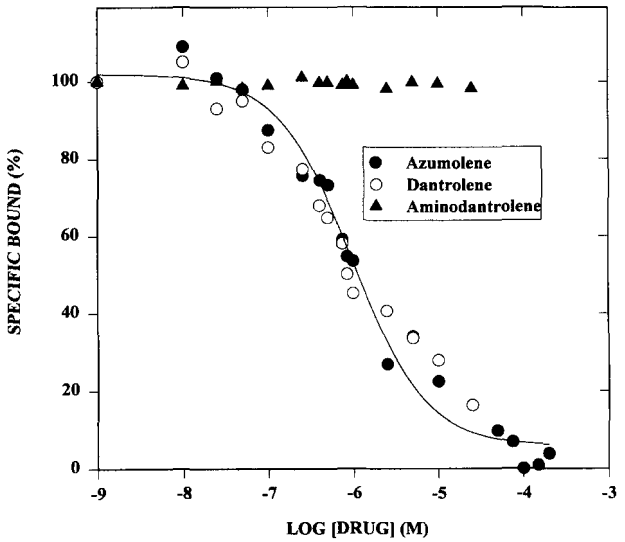

FIG. 4. **Inhibition of [³H]dantrolene binding to HSR membranes.** HSR membranes were incubated with [³H]dantrolene (200 nM) and increasing concentrations of dantrolene, azumolene, or aminodantrolene as described under "Results." Binding was determined using rapid filtration, as described under "Experimental Procedures."

TABLE III

Subcellular distribution of [³H]dantrolene, [³H]ryanodine, and [³H]PN200–110 binding sites in pig skeletal muscle membrane microsomes

Porcine skeletal muscle was fractionated as described under "Experimental Procedures." Fifty μg of membrane protein from each fraction was incubated, in triplicate, with [³H]dantrolene (200 nM), [³H]ryanodine (5 nM), or [³H]PN200–110 (0.8 nM), in the presence or absence of appropriate concentrations of respective unlabeled drugs, and specific binding was determined by the assay system described under "Experimental Procedures."

Membrane fraction	[³H]Dantrolene binding	[³H]Ryanodine binding	[³H]PN200–110 binding
	%	%	%
1 (sarcolemma)	0	0	0
2 (T-tubules)	0	0	100
3 (LSR)	52	55	17
4 (HSR)	100	100	30

demonstrated *in vitro* evidence that the drug exerted its effects in skeletal muscle by suppressing Ca^{2+} release from the SR. Gronert *et al.* (41) and Harrison (42) were the first to report on the successful use of dantrolene in the treatment of porcine MH. Soon after, dantrolene was shown to be effective in treating human MH (43), and the drug has been the mainstay of therapy since that time. The effectiveness of dantrolene in treating MH has been associated with the drug's ability to suppress the rise in intramyoplasmic Ca^{2+} resulting from the triggering of the syndrome (6, 44). Although the molecular mechanism of action of the drug is not yet known, its elucidation will help define the regulation of Ca^{2+} release from skeletal muscle SR. One experimental approach is to develop a pharmacological binding assay that will ultimately lead to the identification of a putative dantrolene receptor, via direct ligand-receptor interaction. As a first step toward realization of this goal, we have developed an assay demonstrating the binding of [³H]dantrolene to a specific receptor(s) in skeletal muscle.

There are only two previous reports in the literature on the development of assays for dantrolene binding to muscle membranes. Sengupta *et al.* (19) reported the binding of [¹⁴C]dantrolene (specific activity not reported) to pig skeletal and cardiac muscle SR using equilibrium dialysis. However, the receptor concentration (binding sites) used in their assay (6 nM) is higher than the K_d of the high affinity site (5 nM), whereas ideally the concentration should have been $0.1 \times K_d$ to minimize ligand depletion (45). Moreover, being lipophilic, dantrolene binds nonspecifically to both biological membranes as well as the membranes used for equilibrium dialysis experiments (see "Results"), and the authors make no mention of the correction for, or even the extent of, nonspecific binding. Indeed, White *et al.* (21) attempted to reproduce the results of these experiments without success. Dehpour *et al.* (20) demonstrated binding of dantrolene to rabbit skeletal muscle SR membranes using fluorescence techniques. Their methods, however, were unable to distinguish specific from nonspecific binding. Hence, to demonstrate specific binding of dantrolene to a receptor, we have custom synthesized [³H]dantrolene and developed a reproducible binding assay that controls for nonspecific binding.

The major factors we found to be a hindrance during the development of a specific [³H]dantrolene binding assay were the poor solubility of the ligand and its capacity to bind nonspecifically to laboratory materials. Despite these obstacles, we have developed a binding assay that, under the present exper-

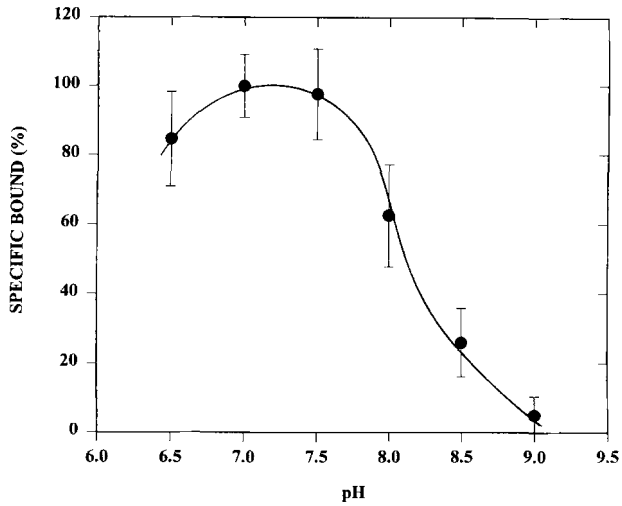

FIG. 5. **Effect of pH on [^{3}H]dantrolene binding.** HSR membranes were incubated with [^{3}H]dantrolene (200 nM) in the presence or absence of 150 μM azumolene at the indicated pH and specific binding determined as described under "Experimental Procedures." Buffers used were 20 mM Na-HEPES (pH 6.0–8.0) or 20 mM Tris-HCl (pH 7.5–9.5).

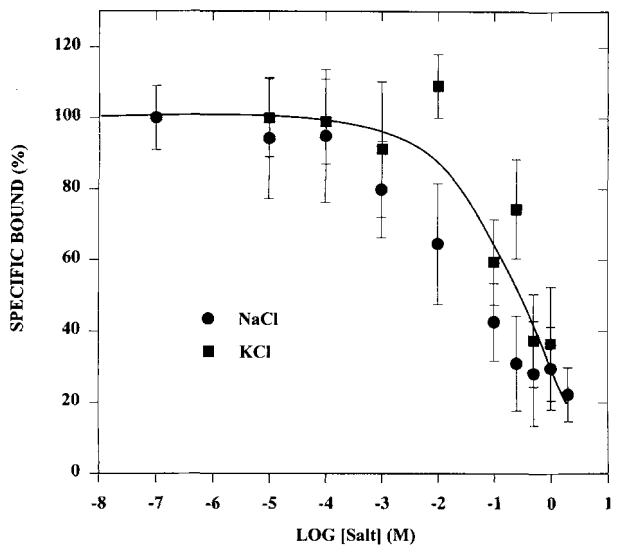

FIG. 6. **Ionic strength dependence of [^{3}H]dantrolene binding to HSR.** Specific [^{3}H]dantrolene binding to HSR was determined as described under "Experimental Procedures," in the presence of increasing concentrations of NaCl or KCl.

imental conditions, indicates the presence of a single class of binding sites with calculated K_d and B_{max} values of approximately 275 nM and 13 pmol/mg of protein, respectively. In ligand binding assays, proper assessment of nonspecific binding should be done at concentrations of unlabeled congener at least 100 times, preferably 1,000 times, the estimated K_d of the ligand (45). Under our assay conditions, dantrolene precipitates at concentrations ≥30 μM. Hence, we could not measure nonspecific binding by maintaining 100-fold excess of unlabeled dantrolene at [^{3}H]dantrolene concentrations above 300 nM without inducing precipitation (25). The specific binding curves shown in Figs. 2 and 3 were generated after determination of nonspecific binding in the presence of 150 μM azumolene rather than dantrolene. However, extrapolation of the nonspecific binding curve generated in the presence of 25 μM dantrolene by linear regression analysis (INPLOT, r = 0.996) to values above 300 nM [^{3}H]dantrolene and subtraction of those calculated values from the directly determined total binding curve yielded a [^{3}H]dantrolene specific binding curve virtually

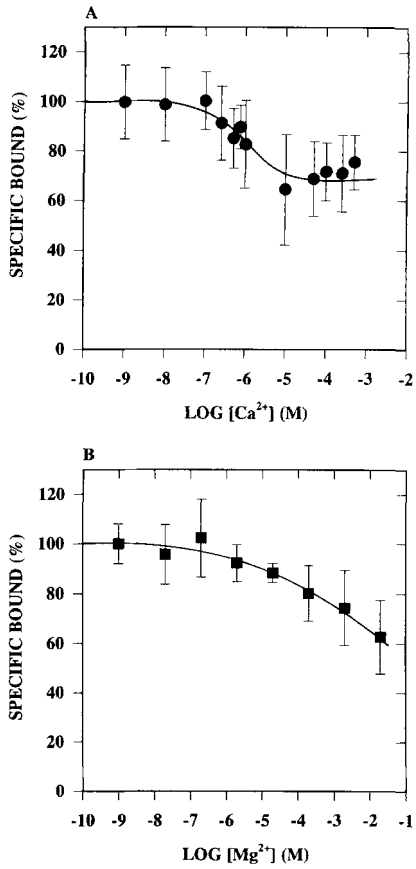

FIG. 7. **Effect of Ca^{2+} and Mg^{2+} on [^{3}H]dantrolene binding to HSR.** Specific [^{3}H]dantrolene binding to HSR was determined in the absence or presence of different concentrations of Ca^{2+} (*panel A*) or Mg^{2+} (*panel B*), as described under "Experimental Procedures."

identical to that generated in the presence of azumolene (data not shown). This demonstrates the validity of our assay using azumolene to measure nonspecific binding.

The binding isotherms were analyzed by nonlinear regression analysis rather than the conventional Scatchard/Rosenthal plots to reduce the errors in calculation of the binding parameters (45, 46). Scatchard/Rosenthal plots are methods for the linearization of nonlinear data and are valid only when the signal-to-noise ratio is high (46). Such was not the case in our experiments. Indeed, a major impediment to the development of the [^{3}H]dantrolene binding assay was the high degree of nonspecific binding to skeletal muscle membranes. The signal-to-noise ratio in the experiments from which Fig. 2*B* was generated varied between 8 and 15%, whereas, classically, this value should be more than 50% and preferably ≥75% (46). Careful attention to experimental detail, however, has allowed us to obtain highly reproducible results, even when results are compared between different muscle preparations. The binding data shown in Fig. 2 are the individual mean binding values ± S.E. from four different experiments, carried out with HSR membranes obtained from three different animals. The points lie on a single isotherm, demonstrating both the reproducibility and the mathematical defensibility of the results, despite the low signal-to-noise ratio.

We found that the signal-to-noise ratio in the binding assay was dependent on the solvent used to make stock solutions of [^{3}H]dantrolene. This is evident in the higher nonspecific binding of [^{3}H]dantrolene seen when ligand was dissolved in DMF rather than ethanol. Dantrolene, therefore, may form a complex with DMF which allows for greater nonspecific adsorption and/or dissolution into hydrophobic membrane components.

Alternatively, DMF may have a direct effect on the membrane itself, resulting in an increase in the solubility of the drug in the membrane's hydrophobic environment.

The pharmacological specificity of [^{3}H]dantrolene binding was assessed using inhibition assays with unlabeled dantrolene or known physiologically active (azumolene) or inactive (aminodantrolene) congeners (35, 47). Both unlabeled dantrolene and azumolene were equipotent as inhibitors of [^{3}H]dantrolene binding, paralleling their reported equivalence in physiological studies (31, 48). However, the calculated K_i value for dantrolene is different from the calculated K_d value despite the Cheng-Prusoff correction (36), which is likely due to the limitations of the assay: namely, the low specific activity of the radioactive ligand, the poor solubility of the drug, and the relatively low signal-to-noise ratio (22, 23). Indeed, the concentration of the labeled compound in competition binding assays should ideally be $0.1 \times K_d$ (45). However, because of the low specific radioactivity of the synthesized [^{3}H]dantrolene, it is impossible to detect a specific signal at the prescribed ligand concentration. Hence, we chose a concentration of [^{3}H]dantrolene (200 nM) which would give specific counts between 2,000 and 4,000 cpm/assay to obtain reproducible data. Experiments carried out at lower [^{3}H]dantrolene concentrations (25–50 nM) and higher membrane protein concentrations gave inconsistent results because of increases in both nonspecific binding and ligand depletion (data not shown). Despite these limitations, the results described above indicate that the binding of [^{3}H]dantrolene to its putative receptor in skeletal muscle SR is indeed specific and that the binding activity parallels reported physiological activity.

The subcellular distribution of the [^{3}H]dantrolene binding sites was assessed using partially purified membrane fractions corresponding to the four major subcellular membrane regions in skeletal muscle involved in ECC. We found that the [^{3}H]dantrolene binding sites were most abundant in HSR followed by LSR, whereas they were absent from the transverse tubule and sarcolemma fractions. The distribution of [^{3}H]dantrolene binding among these subcellular fractions parallels that of [^{3}H]ryanodine binding. This leads us to suggest that the binding sites for the two ligands either have opposite actions on the same receptor, *i.e.* the RyR, or that the receptors for the two ligands are separate molecules that interact indirectly. The fact that no binding of [^{3}H]dantrolene is detected in the transverse tubule fraction makes it extremely unlikely that dantrolene exerts its effects by inhibiting the action of the DHPR during ECC directly. This diminishes the significance of earlier results showing that azumolene, at very high concentrations, inhibits the binding of the dihydropyridine antagonist [^{3}H]PN200-110 to transverse tubule membranes (49).

The optimum conditions for [^{3}H]dantrolene binding were determined by varying assay solution conditions. These studies allowed us to compare the binding conditions with those reported for [^{3}H]ryanodine binding to the RyR. Maximal [^{3}H]dantrolene binding to its receptor occurs in the pH range 6.5–7.5, whereas maximal [^{3}H]ryanodine binding occurs around pH 8.5 (50), a value at which [^{3}H]dantrolene binding is profoundly inhibited (Fig. 5). Further, [^{3}H]dantrolene binding to the SR requires no Ca^{2+} and is not affected even when the buffer in our binding assay is supplemented with 1 mM EGTA (data not shown). On the other hand, the lack of Ca^{2+} in the binding buffer results in 99% inhibition of [^{3}H]ryanodine binding to the RyR (50, 51). High concentrations of Ca^{2+} in the range that maximally stimulates [^{3}H]ryanodine binding (50, 51) only partially inhibit [^{3}H]dantrolene binding to HSR (Fig. 7*A*). The latter is both physiologically and pharmacologically fortuitous, for were dantrolene binding inhibited by Ca^{2+}, dantrolene could not be effective in reversing MH. Myoplasmic Ca^{2+} concentrations during an episode have been demonstrated to reach sustained concentrations of at least 7–10 μM (44, 52). High ionic strength is essential for [^{3}H]ryanodine binding to the RyR (38, 51), whereas the same inhibits [^{3}H]dantrolene binding (Fig. 6). Yet, the extents of both [^{3}H]dantrolene and [^{3}H]ryanodine binding are similarly inhibited by Mg^{2+} to 60% of control values (see Ref. 51 and Fig. 7*B*). These results indicate that the *in vitro* requirements for dantrolene and ryanodine binding to their receptor sites are distinct. They do not distinguish whether the proposed modulation of the RyR by dantrolene is by a direct or indirect mechanism.

Recent *in vitro* studies using SR membranes or skinned fiber preparations may indicate that dantrolene has a biphasic effect on mammalian skeletal muscle RyR, activation at nanomolar concentrations and inactivation at micromolar concentrations, but do not distinguish whether these are a direct or indirect effect of the drug (53).

In conclusion, we have identified a putative dantrolene receptor in skeletal muscle HSR by developing a specific, reproducible [^{3}H]dantrolene binding assay. Because of the low signal-to-noise ratio evident in our binding assay, we had set the following criteria to convince ourselves of the validity of our conclusions. First, the assay must be reproducible, the results for total and nonspecific binding must be significantly different from each other, and the specific binding curve must fit a mathematically defensible model for receptor-ligand interactions. Second, the assay must demonstrate pharmacological specificity, *i.e.* active congeners of dantrolene should inhibit the binding of [^{3}H]dantrolene, whereas inactive congeners should not. Third, specificity should be evident in the subcellular distribution of binding. The evidence presented herein fulfills these criteria and suggests that the dantrolene binding site modulates the opening of the RyR, directly or indirectly. Future studies will be directed at distinguishing whether the binding site for [^{3}H]dantrolene is on the RyR or on a separate, regulatory molecule.

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REFERENCES

1. Snyder, H. R., Jr., Davis, C. S., Bickerton, R. K., and Halliday, R. P. (1967) *J. Med. Chem.* **10,** 807–810
2. Harrison, G. G. (1988) *Br. J. Anaesth.* **60,** 279–286
3. Rosenberg, H., and Seitman, D. T. (1989) in *Clinical Anesthesia* (Barash, P., Cullen, B. F., and Stoelting, R. K., eds) pp. 459–483, J. P. Lippincott, Philadelphia
4. Fujii, J., Otsu, K., Zorzato, F., De Leon, S., Khanna, V. K., Weiler, J. E., O'Brien, P. J., and MacLennan, D. H. (1991) *Science* **253,** 448–451
5. MacLennan, D. H., and Phillips, M. S. (1992) *Science* **256,** 789–794
6. Lopez, J. R., Gerardi, A., Lopez, M. J., and Allen, P. D. (1992) *Anesthesiology* **76,** 711–719
7. Van Winkle, W. B. (1976) *Science* **193,** 1130–1131
8. Foster, P. S., Hopkinson, K. C., Payne, N., and Denborough, M. A. (1991) *Clin. Exp. Pharmacol. Physiol.* **18,** 489–495
9. Ríos, E., Pizarro, G., and Stefani, E. (1992) *Annu. Rev. Physiol.* **54,** 109–133
10. Ríos, E., and Pizarro, G. (1991) *Physiol. Rev.* **71,** 849–908
11. McPherson, P. S., and Campbell, K. P. (1993) *J. Biol. Chem.* **268,** 13765–13768
12. Meissner, G. (1994) *Annu. Rev. Physiol.* **56,** 485–508
13. Schneider, M. F. (1994) *Annu. Rev. Physiol.* **56,** 463–484
14. Caswell, A. H., and Brandt, N. R. (1989) *J. Bioenerget. Biomembr.* **21,** 149–162
15. Lu, X., Xu, L., and Meissner, G. (1994) *J. Biol. Chem.* **269,** 6511–6516
16. Caswell, A. H., Brandt, N. R., Brunschwig, J.-P., and Purkerson, S. (1991) *Biochemistry* **30,** 7507–7513
17. Roed, A. (1991) *Eur. J. Pharmacol.* **209,** 33–38

18. Hui, C. S. (1983) *J. Physiol.* **337,** 509–529
19. Sengupta, C., Meyer, U. A., and Carafoli, E. (1980) *FEBS Lett.* **117,** 37–38
20. Dehpour, A. R., Mofakham, S., and Mahmoudian, M. (1982) *Biochem. Pharmacol.* **31,** 965–968
21. White, M. D., Collins, J. G., and Denborough, M. A. (1983) *Biochem. J.* **212,** 399–405
22. Livertoux, M. H., Jayyosi, Z., and Batt, A. M. (1988) *Talanta* **35,** 613–619
23. Bylund, D. B., and Yamamura, H. (1990) in *Methods in Neurotransmitter Receptor Analysis* (Yamamura, H. I., Enna, S. J., and Kuhar, M. J., eds) pp. 1–35, Raven Press, New York
24. Wuis, E. W., Grutters, A. C. L. M., Vree, T. B., and Van Der Kleyn, E. (1982) *J. Chromatogr.* **231,** 401–409
25. Pong, S. F., Simmons, M. M., Pong, A. S., Kinney, C. M., Chamberlain, B. K., Cazer, F. D., and Moorehead, T. J. (1989) *Pharmacologist* **31,** 161 (abstr.)
26. Mickelson, J. R., Litterer, L. A., Jacobson, B. A., and Louis, C. F. (1990) *Arch. Biochem. Biophys.* **278,** 251–257
27. Meissner, G. (1984) *J. Biol. Chem.* **259,** 2365–2374
28. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
29. Valdivia, H. H., Hogan, K., and Coronado, R. (1991) *Am. J. Physiol.* **261,** C237–C245
30. Valdivia, H., and Coronado, R. (1988) in *The Calcium Channel: Structure, Function, and Implications* (Morad, M., Nayler, W., Kazda, S., and Schramm, M., eds) pp. 252–271, Springer-Verlag, Berlin
31. Dhillon, D. S., Pong, S. F., and Moorehead, T. J. (1992) *Drug Dev. Res.* **25,** 161–169
32. Cuatrecasas, P., Hollenberg, M. D., Chang, K., and Bennet, V. (1975) in *Recent Progress in Hormone Research* (Greep, R. O., ed) pp. 37–94, Academic Press, New York
33. Cuatrecasas, P., and Hollenberg, M. D. (1976) in *Advances in Protein Chemistry* (Anfinsen, C. B., Edsall, J. T., and Richards, F. M., eds) Vol. 30, pp. 251–451, Academic Press, New York
34. Salata, J. J., and Jalife, J. (1982) *J. Pharmacol. Exp. Ther.* **220,** 157–166
35. Ellis, K. O., and Wessels, F. L. (1978) *Naunyn Schmiedebergs Arch. Pharmacol.* **301,** 237–240
36. Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108
37. Zimanyi, I., and Pessah, I. N. (1991) *J. Pharmacol. Exp. Ther.* **256,** 938–946
38. Pessah, I. N., Francini, A. O., Scales, D. J., Waterhouse, A. L., and Casida, J. E. (1986) *J. Biol. Chem.* **261,** 8643–8648
39. Ellis, K. O., Castellion, A. W., Honkomp, L. J., Wessels, F. L., Carpenter, J. F., and Halliday, R. P. (1973) *J. Pharmaceut. Sci.* **62,** 948–951
40. White, R. L., Wessels, F. L., Schwan, T. J., and Ellis, K. O. (1987) *J. Med. Chem.* **30,** 263–266
41. Gronert, G. A., Milde, J. H., and Theye, R. A. (1976) *Anesthesiology* **44,** 488–495
42. Harrison, G. G. (1975) *Br. J. Anaesth.* **47,** 62–65
43. Kolb, M. E., Horne, M. L., and Martz, R. (1982) *Anesthesiology* **56,** 254–262
44. Lopez, J. R., Allen, P. D., Alamo, L., Jones, D., and Sreter, F. A. (1988) *Muscle Nerve* **11,** 82–88
45. Hulme, E. C., and Birdsall, N. J. M. (1992) in *Receptor-Ligand Interactions: A Practical Approach* (Hulme, E. C., ed) pp. 63–176, IRL Press, New York
46. Bylund, D. B., and Toews, M. L. (1993) *Am. J. Physiol.* **265,** L421–L429
47. Dershwitz, M., and Sreter, F. A. (1990) *Anesth. Analg.* **70,** 253–255
48. Allen, P. D., Lopez, J. R., Sanchez, V., Ryan, J. F., and Sreter, F. A. (1992) *Anesthesiology* **76,** 132–138
49. El-Hayek, R., Parness, J., Valdivia, H. H., Coronado, R., and Hogan, K. (1992) *Biochem. Biophys. Res. Commun.* **187,** 894–900
50. Valdivia, H. H., Valdivia, C., Ma, J., and Coronado, R. (1990) *Biophys. J.* **58,** 471–481
51. Chu, A., Diaz-Munoz, M., Hawkes, M. J., Brush, K., and Hamilton, S. L. (1990) *Mol. Pharmacol.* **37,** 735–741
52. Lopez, J. R., Sanchez, V., Lopez, I., Ryan, J. F., Mendoza, M., Sreter, F. A., and Allen, P. D. (1990) *Anesthesiology* **73,** 109–117
53. Nelson, T., Lin, M., and Suarcz-Kurtz, G. (1995) *Biophys. J.* **68,** 173 (abstr.)