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CHARACTERIZATION OF THE Ca^{2+} COORDINATION SITE REGULATING BINDING OF Ca^{2+} CHANNEL INHIBITORS d-cis-DILTIAZEM, (±)BEPRIDIL AND (-)DESMETHOXYVERAPAMIL TO THEIR RECEPTOR SITE IN SKELETAL MUSCLE TRANSVERSE TUBULE MEMBRANES

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SUMMARY. Ca^{2+} inhibits (-)[^{3}H]desmethoxyverapamil, d-cis-[^{3}H]diltiazem and (±)[^{3}H]bepridil binding to skeletal muscle transverse-tubule membranes with a half-maximum inhibition constant, $K_{0.5}$ = 5 ± 1 μM. This value is close to that of the high affinity Ca^{2+} binding site which controls the ionic selectivity of the Ca^{2+} channel found in electrophysiological experiments suggesting that the Ca^{2+} coordination site which regulates the ionic selectivity is also the one which alters binding of the Ca^{2+} channel inhibitors investigated here. Ca^{2+} and (-)D888 bind to distinct sites. Occupation of the Ca^{2+} coordination site decreases the affinity of (-)D888 for its receptor by a factor of 5. Other divalent cations have the same type of inhibition behavior with the rank order of potency Ca^{2+} ($K_{0.5}$ = 5 μM) > Sr^{2+} ($K_{0.5}$ = 25 μM) > Ba^{2+} ($K_{0.5}$ = 50 μM) > Mg^{2+} ($K_{0.5}$ = 170 μM).

INTRODUCTION. Inhibitors of voltage-dependent Ca^{2+} channels have important therapeutic actions in angina pectoris, cardiac arrhythmias and hypertension (1-3). One class of inhibitors includes molecules of the 1,4-dihydropyridine series such as nitrendipine, nifedipine or PN 200-110 (4), another class of inhibitors includes a chemically heterogenous group of compounds comprising verapamil and its analogs D600 and desmethoxyverapamil (D888), bepridil and diltiazem (3-8). [^{3}H]verapamil, (-)[^{3}H]D888, d-cis-[^{3}H]-diltiazem and (±)[^{3}H]bepridil binding sites have now been identified in skeletal muscle membranes (9-15). The number of binding sites for 1,4-dihydropyridine inhibitors, for verapamil-like drugs, for diltiazem and for bepridil, in transverse-tubule (T-tubule) membranes seems to be identical (B_{max} = 50-75 pmol/mg protein) (15). Binding studies have shown that there is on T-tubule membranes a single and common binding site for bepridil, diltiazem, verapamil and D888 (10, 15-16) distinct from the dihydropyridine binding site.

Electrophysiological experiments have shown that blockade of Ca^{2+} currents by verapamil, D600 (17-18) and diltiazem (17, 19) is antagonized by raising external Ca^{2+}.

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These observations suggested to us that biochemical experiments using the corresponding tritiated Ca^{2+} channel inhibitors or their analogs might provide informations on the Ca^{2+} binding sites modulating the binding of verapamil, D600 or diltiazem. This paper analyzes the inhibitory effect of Ca^{2+} and other divalent cations on the interaction between (-)[^{3}H]D888, d-cis-[^{3}H]diltiazem and (±)[^{3}H]bepridil and their receptors.

MATERIALS AND METHODS. T-tubule membranes were prepared from rabbit white skeletal muscle according to (20-21) as modified in (11) in the presence of 0.1 mM phenylmethylsulfonylfluoride to minimize proteolytic degradation. T-tubule membranes were stored in liquid nitrogen at a concentration of 5-7 mg/ml of protein in 20 mM Hepes/NaOH buffer at pH 7.6. Protein concentration was determined according to Hartree (22). In standard equilibrium binding assays, membranes were incubated at 25°C in 1 ml of a solution containing 10 mM Hepes/NaOH buffer at pH 7.6 with the required concentrations of various ^{3}H-ligands. Incubation lasted 60 min for (-)[^{3}H]D888, 90 min for d-cis-[^{3}H]diltiazem and 20 min for (±)[^{3}H]bepridil. Incubations were stopped by rapid filtration of 400 µl samples of the incubation mixture through Whatman GF/C filters under reduced pressure. The filters were washed three times with 8 ml of a cold solution made of 100 mM Tris/Cl buffer, pH 7.6. Non-specific binding was measured using 1 µM (-)D888, 10 µM d-cis-diltiazem and 1 µM (+)bepridil for the respective ^{3}H-ligands. Experiments in duplicate were systematically carried out. Free calcium concentrations between 0.1 and 10 µM were buffered using hydroxyEDTA (N-hydroxyethylenediaminetriacetic acid) and calculated according to (23-24). Around pH 7.6, the equilibria concerned are :

$$\text{hydroxyEDTA}^{3-} + H^+ \underset{}{\overset{K_1}{\rightleftharpoons}} \text{hydroxyHEDTA}^{2-} + H^+ \overset{K_2}{\rightleftharpoons} \text{hydroxyH}_2\text{EDTA}^-$$

with $pK_1 = 9.89$ and $pK_2 = 5.41$. The apparent equilibrium dissociation constant for Ca^{2+} binding to hydroxyEDTA ($K_{hydroxyEDTA}$) was calculated according to (23) using $pK_{hydroxyEDTA^{3-}} = 8.3$; $pK_{hydroxyEDTA} = 5.9$ at pH 7.6 and 25°C. hydroxyEDTA by itself was without effect on (-)[^{3}H]D888, d-cis-[^{3}H)diltiazem and (±)[^{3}H]bepridil binding assays.

Chemicals. d-cis-[^{3}H]diltiazem (160 Ci/mmol) was obtained from Amersham, (-)[^{3}H]D888 (83 Ci/mmol), (+) and (-)D888 were from Knoll AG, (±)[^{3}H]bepridil (35-40 Ci/mmol) was from CEA, and CERM, France ; (+)bepridil was from CERM, France ; d-cis-diltiazem was from LERS-Synthelabo, France. Chloride salts were used for divalent cations. All other compounds are from standard sources.

RESULTS AND DISCUSSION

Ca^{2+} is essential for defining the ionic selectivity of voltage-dependent Ca^{2+} channels in skeletal muscle (25) in cardiac muscle (26) and in neuronal cells (27). When external Ca^{2+} is removed, the ionic channel becomes permeable to monovalent cations such as Na^+, K^+, Li^+, Rb^+ and Cs^+. Ca^{2+} ions at very low concentrations ($K_{0.5}$ = 0.7 - 2 µM) and several other divalent cations such as Sr^{2+}, Ba^{2+}, Co^{2+}, Mn^{2+} and Mg^{2+} reversibly block this inward current of monovalent cations. In the presence of Ca^{2+} in the external medium, the channel is permeable to Ca^{2+} and to Ca^{2+} analogs such as Ba^{2+},and no more to monovalent cations. The inset of Fig. 1 illustrates equilibrium binding of (-)[^{3}H]D888 to T-tubule membranes. Specific binding was obtained by the

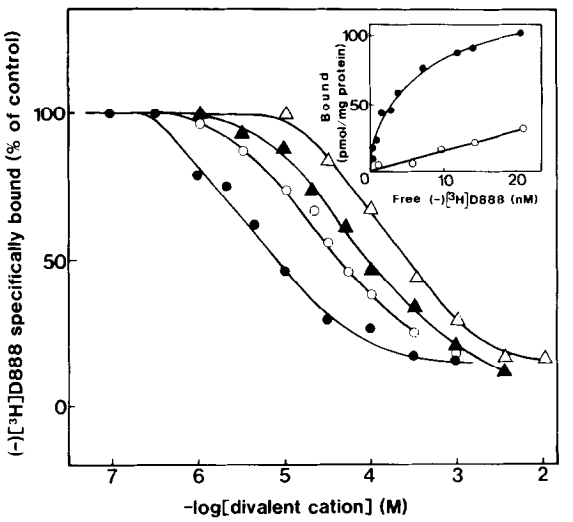

Fig. 1. Inhibition of (-)[^{3}H]D888 (0.4 nM) binding to T-tubule membranes (0.01 mg/ml) by inorganic divalent cations. Inset, equilibrium binding of (-)[^{3}H]D888 to T-tubule membranes in the absence (●) and in the presence (○) of 1 μM (-)D888. Main panel, inhibition of specific (-)[^{3}H]D888 binding by increasing concentrations of Ca^{2+} (●), Sr^{2+} (○), Ba^{2+} (▲), Mg^{2+} (△).

difference between total and non-specific binding. The Scatchard plot of the specific binding (not shown) is consistent with a single class of site with an equilibrium dissociation constant (K_D) of 1.5 nM and a maximum binding capacity (B_{max}) of 70 pmol/mg of protein. The main panel of Fig. 1 shows that divalent cations such as Ca^{2+}, Sr^{2+}, Ba^{2+} and Mg^{2+} inhibit specific (-)[^{3}H]D888 binding to T-tubule membranes. Half-maximum inhibition occurs with cation concentrations ($K_{0.5}$) in the range of 5 to 170 μM with the following rank order of potency, Ca^{2+} ($K_{0.5}$ = 5 μM) > Sr^{2+} ($K_{0.5}$ = 25 μM) > Ba^{2+} ($K_{0.5}$ = 50 μM) > Mg^{2+} ($K_{0.5}$ = 170 μM). These experiments were carried out in 10 mM Hepes/NaOH buffer. In experiments carried out in 50 mM Tris-Cl buffer, $K_{0.5}$ values for Ca^{2+}, Sr^{2+} and Ba^{2+} were shifted towards lower affinity constants (100 times for Ca^{2+}) (11-12).

Insets in Fig. 2 illustrate equilibrium binding properties of d-cis-[^{3}H]diltiazem and (±)[^{3}H]bepridil to T-tubule membranes. Scatchard plots for the specific binding component (not shown) indicate the existence of a single class of binding sites. A K_D value of 50 nM and a B_{max} value of 50 pmol/mg of protein were found for d-cis-[^{3}H]diltiazem binding. A K_D value of 20 nM and a B_{max} value of 75 pmol/mg of protein were found for (±)[^{3}H]bepridil binding. The main panel of Fig. 2 shows that both d-cis-[^{3}H]diltiazem

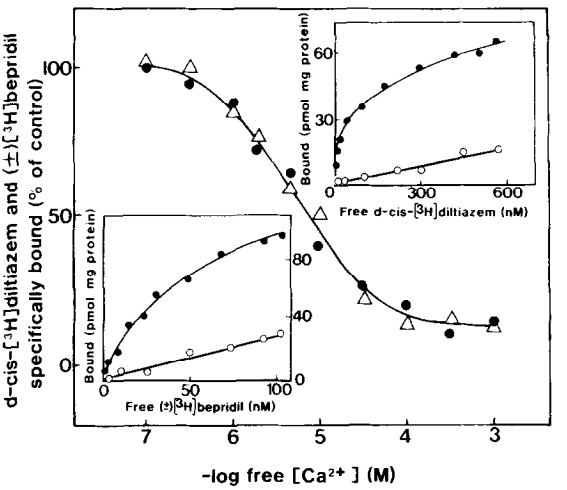

Fig. 2. Inhibition by Ca^{2+} of d-cis-[^{3}H]diltiazem (1.8 nM) and (±)[^{3}H]bepridil (4 nM) binding to T-tubule membranes (0.086 mg/ml for d-cis-[^{3}H]diltiazem and 0.045 mg/ml for (±)[^{3}H]bepridil). Right inset, equilibrium binding is measured by increasing d-cis-[^{3}H]diltiazem (2.5 Ci/mmol) concentrations in the absence (●) and in the presence (○) of 10 μM d-cis-diltiazem. Left inset, equilibrium binding of (±)[^{3}H]bepridil (4 Ci/mmol) in the absence (●) and in the presence (○) of 1 μM (+)bepridil. Main panel, inhibition of specific binding of d-cis-[^{3}H]diltiazem (●) and of (±)[^{3}H]bepridil (△) in the presence of increasing Ca^{2+} concentrations.

and (±)[^{3}H]bepridil binding to T-tubule membranes are also inhibited by Ca^{2+} with a $K_{0.5}$ = 5 ±1 μM.

Fig. 3 shows a Scatchard plot describing (-)[^{3}H]D888 binding to T-tubule membranes in the presence of increasing concentrations of Ca^{2+}. Ca^{2+} induces a decrease of the maximal binding capacity (B_{max}) with a change in the apparent equilibrium dissociation constant K_D for (-)[^{3}H]D888 when Ca^{2+} concentrations are increased from zero (K_D = 1.8 nM, B_{max} = 70 pmol/mg of protein) to 3.5 μM ($K_{D(app)}$ = 2.9 nM, B_{max} = 60 pmol/mg of protein) or 15 μM ($K_{D(app)}$ = 4.3 nM, B_{max} = 57 pmol/mg of protein) and 35 μM ($K_{D(app)}$ = 6.3 nM, B_{max} = 50 pmol/mg of protein).

The plot of $(K_{D(app)}/K_D)$-1 versus Ca^{2+} concentration is hyperbolic (Fig. 3, inset) and indicates that at a saturating Ca^{2+} concentration, $K_{D(app)}$ = 9 nM. This behavior shows that (-)[^{3}H]D888 binds to its receptor both when the Ca^{2+} coordination site is empty and when it is occupied by Ca^{2+}. The K_D value for the Ca^{2+}-free form of the receptor is 1.8 nM, the K_D value for the receptor having its Ca^{2+} site saturated by Ca^{2+} is 9 nM. Ca^{2+} and (-)D888 clearly bind to distinct sites but the occupation by Ca^{2+} of its coordination site decreases the affinity for (-)[^{3}H]D888 by a factor of 5.

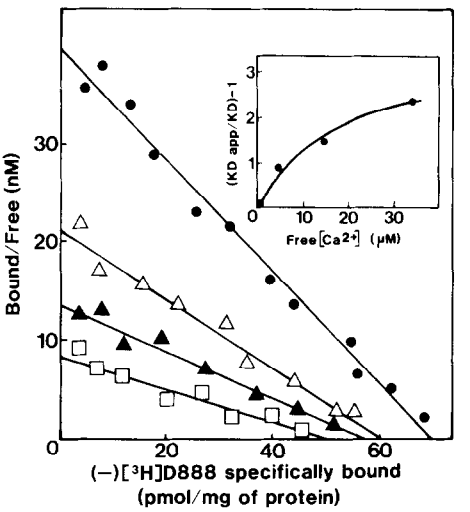

Fig. 3. Specific equilibrium binding of (-)[^{3}H]D888 to T-tubule membranes in the presence of increasing Ca^{2+} concentrations. Equilibrium binding was performed using (-)[^{3}H]D888 prepared at 40 Ci/mmol and 0.01 mg/ml of membranes (pH 7.6 and 25°C). Non-specific binding was measured in the presence of 1 μM (-)D888. Main panel, Scatchard plots of specific (-)[^{3}H]D888 binding in the absence (●) or in the presence of 3.5 μM (△) 15 μM (▲) and 35 μM (□) of Ca^{2+}. Inset, Effect of increasing Ca^{2+} concentrations on the apparent equilibrium dissociation constant ($K_{D(app)}$) for (-)[^{3}H]D888 binding. K_D is the dissociation constant measured in the absence of Ca^{2+}.

At saturating concentrations of Ca^{2+}, B_{max} is decreased by 31 %. The simplest interpretation of this result is that there may be 2 populations or 2 states of (-)D888 receptors in the T-tubule membrane, a major one (69 %) for which Ca^{2+} binding to the coordination site is only modulating the affinity for (-)[^{3}H]D888 by a factor of 5 and a minor one (31 %) for which Ca^{2+} binding has a more drastic effect on the affinity for (-)[^{3}H]D888 (at least a factor of 50) which would result in a decrease of B_{max} instead of a change of $K_{D(app)}$. The 2 different populations or states of receptors would be undistinguishable in their affinity for (-)D888 in the absence of Ca^{2+}.

The affinity found for Ca^{2+} binding from the three ^{3}H-ligand binding experiments, $K_{0.5}$ = 5 ± 1 μM, is similar to the one that has been found in muscle, heart and brain for the Ca^{2+}-calcium channel complex in electrophysiological experiments (K_D = 0.7 - 2 μM) (25-27). Also the order of affinity for Ca^{2+}, Sr^{2+}, Ba^{2+} and Mg^{2+} association at this site found by electrophysiology (25-27), is the same as the one found in (-)[^{3}H]D888 binding experiments. All these results taken together make it likely that the Ca^{2+} coordination site identified by electrophysiology which is in charge of the control of the divalent cation selectivity of the Ca^{2+} channel is the same site that regulates association of Ca^{2+}

channel inhibitors such as D888, diltiazem and bepridil with the voltage-dependent Ca^{2+} channel.

The association of [^{3}H]nitrendipine with the dihydropyridine receptor in skeletal muscle T-tubules was found to be insensitive to Ca^{2+} up to 10 mM Ca^{2+} (16). This result was confirmed with the more active dihydropyridine, (+)[^{3}H]PN 200-110 (not shown). However when EDTA (0.1 mM) was added to the binding assay, it decreased the specific binding of (+)[^{3}H]PN 200-110 by about 50 % and a normal level of binding activity (not shown) could be restored by adding Ca^{2+} ($K_{0.5(Ca^{2+})}$) = 4 μM). These latter results could have suggested the existence of tightly bound Ca^{2+} ions essential for dihydropyridine binding as previously proposed for heart, brain and smooth muscle membranes (28-33) from the same type of experiments. It turned out, however, that the inhibitory effects seen with EDTA were not observed with other Ca^{2+} chelators such as hydroxyEDTA or EGTA (up to 100 μM). Therefore it became clear that the effects seen with EDTA were due to an inhibitory action of EDTA itself and not to the removal of any important Ca^{2+} from the Ca^{2+} channel protein. The Ca^{2+} coordination site which modulates binding of D888, diltiazem and bepridil to the putative Ca^{2+} channel protein does not appear to be involved in the regulation of binding of dihydropyridines to their receptors.

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