mation of poly(dA-dT)·poly(dA-dT) and of poly(dG-dC)·poly(dG-dC) Z-DNA.

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Reconstitution of the Voltage-Sensitive Calcium Channel Purified from Skeletal Muscle Transverse Tubules†

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ABSTRACT: The purified calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubule membrane consists of three subunits: α with M_r 135 000, β with M_r 50 000, and γ with M_r 33 000. Purified receptor preparations were incorporated into phosphatidylcholine (PC) vesicles by addition of PC in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and removal of detergent by molecular seive chromatography. Forty-five percent of the α, β, and γ polypeptides and the [^{3}H]dihydropyridine/receptor complex were recovered in association with PC vesicles. The rate of dissociation of the purified and reconstituted dihydropyridine/receptor complex was identical with that in T-tubule membranes, and allosteric modulation by verapamil and diltiazem was retained. The reconstituted calcium antagonist receptor, when occupied by the calcium channel activator BAY K 8644, mediated specific $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ transport into the reconstituted vesicles. $^{45}Ca^{2+}$ influx was blocked by the organic calcium antagonists PN200-110 ($K_{0.5}$ = 0.2 μM), D600 ($K_{0.5}$ = 1.0 μM), and verapamil ($K_{0.5}$ = 1.5 μM) and by inorganic calcium channel antagonists ($La^{3+} > Cd^{2+} > Ni^{2+} > Mg^{2+}$) as in intact T-tubules. A close quantitative correlation was observed between the presence of the α, β, and γ subunits of the calcium antagonist receptor and the ability to mediate $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$ flux into reconstituted vesicles. Comparison of the number of reconstituted calcium antagonist receptors and functional channels supports the conclusion that only a few percent of the purified calcium antagonist receptor polypeptides are capable of mediating calcium transport as previously demonstrated for calcium antagonist receptors in intact T-tubules.

Voltage-sensitive calcium channels mediate an increase in cytosolic calcium in response to depolarization and play an important role in excitation–contraction coupling in cardiac and smooth muscle (Hagiwara & Byerly, 1981; Tsien, 1983). The major class of calcium channel in muscle is modulated by dihydropyridine calcium agonists and antagonists (Janis & Triggle, 1983; Schramm & Towart, 1985). These compounds increase and decrease cellular calcium currents by stabilizing long open and closed states of an ensemble of individual calcium channels (Kokubun & Reuter, 1984; Hess et al., 1984). Their binding is subject to allosteric modulation by structurally diverse calcium antagonists that bind to two additional lower affinity sites and either enhance (e.g., diltiazem and other benzothiazepines) or inhibit (e.g., verapamil and other phenylalkylamines) dihydropyridine binding (Schramm & Towart, 1985; Janis & Triggle, 1984).

Skeletal muscle fibers have large voltage-sensitive calcium currents that are restricted to the T-tubule system and are modulated by dihydropyridine calcium channel agonists and

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antagonists (Affolter & Coronado, 1985; Almers et al., 1981; Chiarandini & Stefani, 1983). With T-tubule membranes as starting material, the calcium antagonist receptor has been solubilized with digitonin or CHAPS[1] (Curtis & Catterall, 1983; Glossmann & Ferry, 1983) and purified (Borsotto et al., 1984b, 1985; Curtis & Catterall, 1984). After solubilization and purification in digitonin, the isolated calcium antagonist receptor is a large glycoprotein consisting of three noncovalently associated subunits: α with M_r 135 000, β with M_r 50 000, and γ with M_r 33 000 (Curtis & Catterall, 1984). Although this complex contains the dihydropyridine recognition site of the calcium channel, other aspects of calcium channel function have not been assessed in detergent solution. In this paper, we describe the successful reconstitution of the calcium antagonist receptor into phospholipid vesicles and present an initial analysis of the dihydropyridine-sensitive calcium flux mediated by the purified and reconstituted receptor polypeptides. A preliminary account of some of this work has been presented to the Society for General Physiology (Curtis & Catterall, 1985).

Experimental Procedures

Preparation of the Purified Dihydropyridine/Receptor Complex. The calcium antagonist receptor was purified from procedure B membranes by chromatography on WGA–Sepharose, DEAE-Sephadex, and WGA–Sepharose as previously described (Curtis & Catterall, 1984) with three modifications: (i) Diltiazem was not present during receptor labeling and purification. (ii) After occupation of 1% of the calcium antagonist receptors in T-tubule membranes with [^{3}H]PN200-110 (New England Nuclear, Boston, MA), the remaining receptors were saturated with BAY K 8644 (Miles Laboratories, New Haven, CT) by incubation with 2 μM BAY K 8644 except where indicated. (iii) The receptor was eluted from the second WGA–Sepharose column with 200 mM *N*-acetyl-D-glucosamine in calcium or barium uptake medium (10 mM calcium or barium methyl sulfate, 0.3 M sucrose, 10 mM MOPS/tetramethylammonium, pH 7) adjusted to 340 mosM by using a vapor pressure osmometer (Wescor). The soluble [^{3}H]dihydropyridine/receptor complex was assayed by poly(ethylene glycol) (PEG) precipitation in the presence of IgG as carrier protein as described by Curtis and Catterall (1983), and the total amount of receptor was estimated as described by Curtis and Catterall (1984).

Reconstitution of the Purified Calcium Antagonist Receptor into Phospholipid Vesicles. In the standard procedure to reconstitute the calcium antagonist receptor, 40–80 pmol of receptor was eluted from the second WGA–Sepharose column in a total volume of 0.8 mL of calcium or barium uptake medium and mixed with 2.2 mL of 1.5% (w/v) CHAPS containing 1% egg phosphatidylcholine (PC; Sigma Chemical Co.) in the corresponding medium. The 3-mL sample was immediately applied to a 1-cm × 30-cm column of Sephadex G-50 presaturated with PC equilibrated in the corresponding medium. The column was eluted with a flow rate of 0.25 mL/min with uptake medium, and 1-mL fractions were collected. All operations were carried out at 4 °C. Two 1-mL fractions were recovered that contained 35–45% of the applied [^{3}H]PN200-110/receptor complex associated with large phospholipid vesicles clearly seen due to their ability to scatter visible light. The amount of the [^{3}H]PN200-110/receptor complex incorporated into these vesicles was assayed by the collection of vesicles on the GF/F filters. Recovery by using this procedure was greater than 90% when determined with [^{14}C]PC (Amersham) labeled vesicles.

Measurement of $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$ Uptake into Vesicles. To measure $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$ uptake into protein-free vesicles or vesicles containing the reconstituted calcium antagonist receptor, external divalent cations were removed by application of 200 μL of vesicle suspension to a 2-mL Sephadex G-50 column equilibrated wiith sucrose medium (0.34 M sucrose, 10 mM MOPS/tetramethylammonium, pH 7.0, 2 μM BAY K 8644, 340 mosM) and centrifugation at 1000*g* for 1 min. Uptake into 50–100 μL of vesicles in sucrose medium was initiated by addition of 50 μL of sucrose medium containing 2 μCi of $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$ (New England Nuclear). After incubation at 37 °C for the indicated time, uptake was terminated by adding the sample to a 0.5-cm × 10-cm column of Chelex 100 (50–100 mesh, Bio-Rad) equilibrated with sucrose medium containing 1 mg/mL bovine serum albumin. Vesicles were immediately eluted from the column with 1 mL of sucrose medium containing 1 mg/mL bovine serum albumin. Background after application of 2 μCi of isotope to a Chelex column was 130 cpm detected in the eluate (99% removal of free divalent cation). Recovery of [^{14}C]PC-labeled vesicles applied to Chelex columns was >95%.

To correct for the background leak of $^{45}Ca^{2+}$ into the vesicles, the same lipid solution was reconstituted without the calcium antagonist receptor to produce protein-free vesicles. These were assayed for $^{45}Ca^{2+}$ uptake at the same time as the receptor-containing vesicles. After correction for any internal volume difference between the receptor-containing and the protein-free vesicles, the specific $^{45}Ca^{2+}$ uptake attributable to the reconstituted receptor was determined by subtracting the background $^{45}Ca^{2+}$ uptake into the protein-free vesicles prepared at the same time.

Determination of Internal Volume. The internal volume of vesicles was determined directly by reconstituting in the presence of uptake medium containing $^{45}Ca^{2+}$ or [^{14}C]sucrose followed by removing external $^{45}Ca^{2+}$ on Chelex columns and [^{14}C]sucrose by filtration on GF/F filters as described above. For comparison of samples used first for uptake experiments, the internal volume was determined by adding vesicles in sucrose medium to medium containing $^{45}Ca^{2+}$ and 10 μM A23187. After 20 min at 37 °C, external $^{45}Ca^{2+}$ was removed on Chelex columns. Internal barium did not significantly alter ionophore-mediated calcium uptake.

SDS Gel Electrophoresis. Lyophilized samples of sucrose gradient fractions were dissolved in sample buffer (3% SDS, 12 mM EDTA, 30 mM Tris-HCl, pH 7.6) at 100 °C in the presence of *N*-ethylmaleimide and placed in boiling water for 3 min. Samples were then analyzed on a discontinuous gel system according to Laemmli (1970) consisting of a stacking gel of 3% acrylamide and a running gel containing a 5–15% (w/v) linear acrylamide gradient. The gels were silver stained according to the method described by Oakley et al. (1980), and the amount of subunits in individual fractions was quantitated by densitometry scans.

Results and Discussion

Reconstitution of the Purified Calcium Antagonist Receptor in Phospholipid Vesicles. Since digitonin solubilizes membranes by interaction with cholesterol and does not efficiently solubilize phospholipid, another detergent must be used to add solubilized phospholipid prior to reconstitution. Previous

[1] Abbreviations: PC, phosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; WGA, wheat germ agglutinin; DEAE, diethylaminoethyl; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PEG, poly(ethylene glycol).

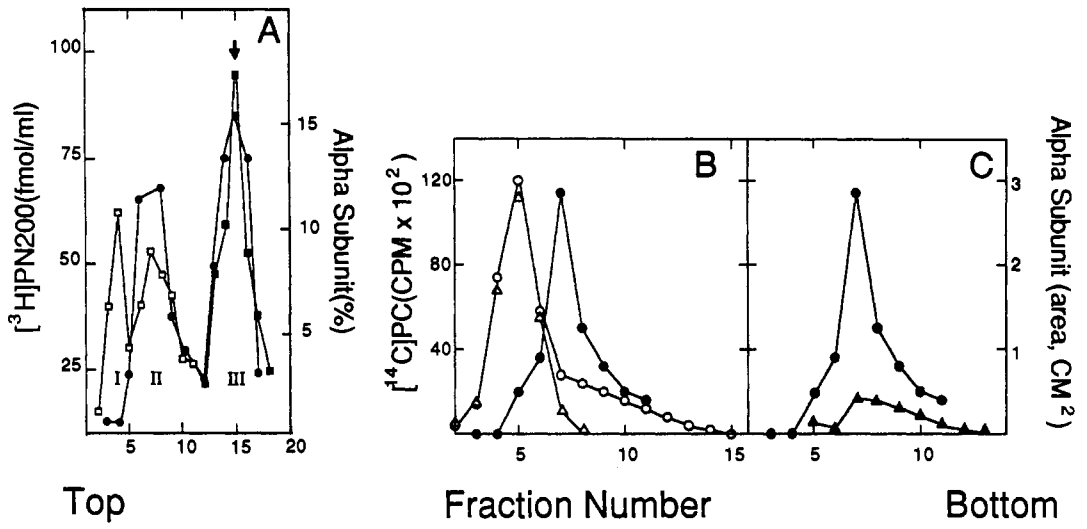

FIGURE 1: Sucrose gradient analysis of the calcium antagonist receptor reconstituted into phospholipid vesicles. (A) The reconstituted [^{3}H]PN200-110/receptor complex was sedimented on a 5–20% sucrose gradient containing 0.1% digitonin for 1.5 h at 210000g in a VTi50 rotor. [^{3}H]PN200-110 associated with vesicles was determined by filtration on GF/F filters (□), and [^{3}H]PN200 associated with soluble receptor (fractions 13–19) was quantitated by PEG precipitation (■). The distribution of calcium antagonist receptor polypeptides was determined by SDS gel electrophoresis of sucrose gradient fractions and densitometry scans of silver stained gels. The percent of the total α subunit found throughout the gradient is shown as the area of the integrated densitometric scanning peaks in cm^2 (●). The normal 20S position of soluble receptor on a parallel gradient is marked by an arrow. (B) The purified [^{3}H]PN200-110/receptor complex was reconstituted with tracer amounts of [^{14}C]PC and sedimented on a 3–12% sucrose gradient without digitonin for 2.5 h at 210000g. The distribution of [^{14}C]PC associated with receptor-containing vesicles (○) or protein-free vesicles sedimented on a parallel gradient (△) is shown. The amount of the α subunit in each sucrose gradient fraction is shown (●) and the β and γ polypeptides had a similar distribution. (C) The distribution of the α subunit in (B) (●) is compared to the [^{14}C]PC associated with receptor-containing vesicles (▲) derived by subtracting the receptor-containing and protein-free [^{14}C]PC profiles in (B).

studies of the stability of the calcium antagonist receptor in different detergents (Glossmann & Ferry, 1983; Borsotto et al., 1984a) indicated that CHAPS was the best candidate. Good recovery of receptor complex and vesicles with a large internal volume (1–2 μL/mg PC) was obtained with removal of detergent by gel filtration on Sephadex G-50 columns. In order to determine the efficiency of incorporation of the purified calcium antagonist receptor into phospholipid vesicles prepared by these methods, the reconstituted vesicle fraction containing the [^{3}H]PN200-110/receptor complex was analyzed by sedimentation through linear 5–20% sucrose gradients containing 0.1% digitonin. Since this concentration of digitonin will maintain the calcium antagonist receptor in solution but will not solubilize PC vesicles, calcium antagonist receptors in reconstituted vesicles should band at an equilibrium density near the top of the gradient while the remaining digitonin-solubilized receptor continues to migrate through the gradient as a 20S complex. Three peaks of [^{3}H]PN200-110 were observed (Figure 1A, □ and ■). Peak I is located at the position of protein-free vesicles (not shown) and contains dissociated [^{3}H]PN200-110 but no calcium antagonist receptor detectable by SDS–polyacrylamide gel electrophoresis (●). Peak II contains PC vesicles, bound [^{3}H]PN200-110 (□), and calcium antagonist receptors (●). Peak III represents the remaining solubilized [^{3}H]PN200-110/calcium antagonist receptor complexes (■) migrating through the gradient with a sedimentation coefficient of 20 S. In three experiments, a mean of 45% of the [^{3}H]PN200-110/receptor complex and the calcium antagonist receptor α subunit was recovered in peak II, the reconstituted vesicle peak. This efficiency of incorporation is similar to that given in previous reports of digitonin-solubilized proteins reconstituted by a similar procedure (Kelleher et al., 1983; Hekman et al., 1984).

The reconstitution of the calcium antagonist receptor into phospholipid vesicles was also quantitated by analyzing the distribution of protein-free and reconstituted vesicles on sucrose gradients in the absence of detergent. Figure 1B shows the distribution on a sucrose gradient of the [^{14}C]PC of vesicles reconstituted with and without the calcium antagonist receptor. Protein-free vesicles migrated as a sharp symmetrical peak (△), while vesicles reconstituted with purified calcium antagonist receptor exhibited both a main peak and a significant higher density shoulder (○). The peak of calcium antagonist receptor polypeptides (●) in vesicles reconstituted with receptor coincided with the high-density shoulder of [^{14}C]PC vesicles. Thus, this vesicle fraction of higher density contains reconstituted calcium antagonist receptor. Subtracting the distribution of [^{14}C]PC found with protein-free vesicles from that for receptor-containing vesicles in Figure 1B yields a peak of [^{14}C]PC associated with reconstituted receptor (▲) that approximately corresponds to the distribution of the α subunit (Figure 1C). This peak represents a mean of 31% (n = 3) of the total vesicle phospholipid; suggesting that approximately 31% of reconstituted PC vesicles contain reconstituted polypeptides. These preparations therefore provide a suitable system for analysis of the functional properties of the purified receptor complex.

Allosteric Modulation of [^{3}H]PN200-110 Dissociation from Purified and Reconstituted Calcium Antagonist Receptors. In these studies, the calcium antagonist receptors were purified with bound dihydropyridine to stabilize calcium channels in an active state. Dissociation of bound dihydropyridine from both membrane-bound and detergent-solubilized calcium antagonist receptors is accelerated by verapamil and slowed by diltiazem through allosteric interactions (Curtis & Catterall, 1983; Glossmann & Ferry, 1983). These results show that the receptor sites for these different classes of calcium channel modulators are solubilized as a functional complex. The results of Figure 2 show that the purified and reconstituted calcium antagonist receptor retains functional binding sites for diltiazem and verapamil. For both T-tubule membranes and purified calcium antagonist receptors, the [^{3}H]PN200-110/receptor complex has a half-life of 1 min at 37 °C under control conditions in the presence of felodipine, 2.4 min in the

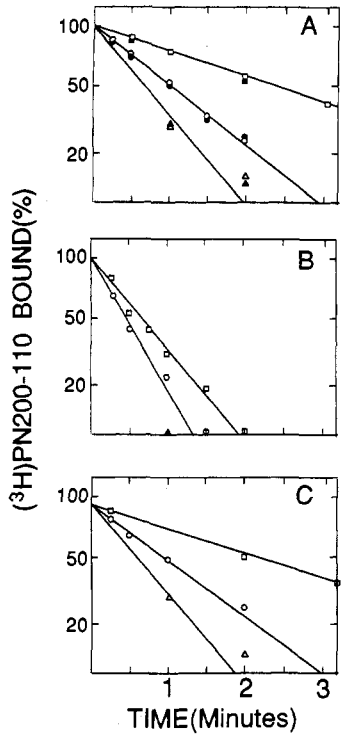

FIGURE 2: Dissociation of the purified and reconstituted [^{3}H]-PN200-110/receptor complex. T-tubule membranes were labeled with 5 nM [^{3}H]PN200-110, and an aliquot was set aside. The remaining calcium antagonist receptor was purified and an aliquot was reconstituted by chromatography on Sephadex G-50 columns as described under Experimental Procedures. At time zero, receptor samples were added to solubilization buffer (185 mM KCl, 1.5 mM $CaCl_2$, 10 mM HEPES/Tris, pH 7.4) containing 1 μM felodipine (○, ●), 1 μM felodipine plus 100 μM diltiazem (□, ■), or 1 μM felodipine plus 100 μM verapamil (△, ▲). After the indicated times at 37 °C, dissociation was terminated by cooling to 4 °C and rapid collection of the [^{3}H]PN200-110/receptor complexes. T-tubules were collected by filtration on GF/C filters, vesicles by filtration on GF/F filters, and solubilized and purified samples by PEG precipitation. Dissociation is expressed as the percent of the starting [^{3}H]PN200-110/receptor complex recovered at time zero. (A) Starting T-tubule membranes (open symbols); the purified calcium antagonist receptor in digitonin (closed symbols). (B) An aliquot (0.8 mL) of the same purified sample as in (A) was mixed with 2.2 mL of 1.5% CHAPS/1% PC, and the rate of dissociation of the complex was determined by PEG precipitation. (C) The purified receptor sample was mixed with CHAPS/PC as in (B) and then reconstituted by chromatography on a Sephadex G-50 column as described under Experimental Procedures. The rate of dissociation of the reconstituted receptor complex was measured by filtration on GF/F filters. Points are the mean of three determinations on separate purifications/reconstitutions.

presence of felodipine plus diltiazem, and 0.7 min in the presence of felodipine plus verapamil (Figure 2A). Addition of 1% PC dispersed in 1.5% CHAPS to the purified calcium antagonist receptor accelerates the rate of dissociation of the PN200-110/receptor complex and reduces the stabilizing effect of diltiazem (Figure 2B), suggesting reduced stability of the solubilized calcium antagonist receptor in CHAPS/PC mixtures. Incorporation of the solubilized calcium antagonist receptor into PC vesicles by separation of detergent on Sephadex G-50 restores the dissociation rates and allosteric interactions characteristic of the [^{3}H]PN200-110 complex in intact T-tubules and digitonin-solubilized calcium antagonist receptor (Figure 2C). Evidently, CHAPS is effectively removed during vesicle formation and the reconstituted PN200-100/calcium antagonist receptor complex regains the stability characteristic of T-tubule membranes.

Calcium Transport Mediated by the Purified and Reconstituted Calcium Antagonist Receptor. In order to maximize

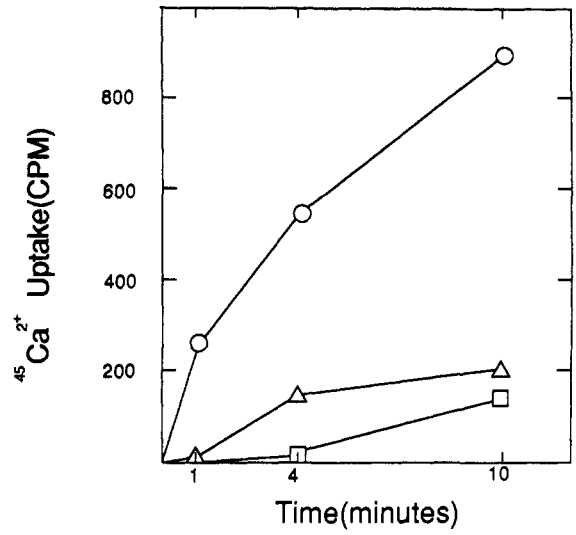

FIGURE 3: $^{45}Ca^{2+}$ uptake into phospholipid vesicles containing the reconstituted calcium antagonist receptor. Calcium antagonist receptor was labeled with tracer amounts of [^{3}H]PN200-110 and saturated and purified in the presence of BAY K 8644. Reconstituted vesicles were incubated at 4 °C for 1 h with 100 μM verapamil (△) or no additions. External calcium was removed by rapid gel filtration. Specific $^{45}Ca^{2+}$ uptake into vesicles was measured for the indicated time as described under Experimental Procedures in the presence of no drugs (□), 2 μM BAY K 8644 (○), or 2 μM BAY K 8644 plus 100 μM verapamil (△). Mean values of three determinations from separate purifications/reconstitutions are presented.

the probability of purification of calcium channels in an active (open) conformation, the calcium antagonist receptors in T-tubule membranes were incubated with sufficient [^{3}H]-PN200-110 to label only approximately 1% of the binding sites. The T-tubule membranes were then incubated in an excess of the specific calcium channel activator BAY K 8644 so that the remaining 99% of the dihydropyridine sites would be occupied by this agent. The calcium antagonist receptors were then solubilized, purified in the continuous presence of BAY K 8644, and reconstituted into PC vesicles as described above.

Initial rates of influx of $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$ into reconstituted PC vesicles were measured under countertransport conditions in which the intravesicular compartment contained a 100-fold higher concentration of unlabeled Ca^{2+} or Ba^{2+} than the incubation solution. These conditions greatly increase the amount of $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$ uptake required to achieve isotopic equilibrium and greatly slow the approach to isotopic equilibrium as described previously for reconstituted sodium channels (Talvenheimo et al., 1982). They therefore maximize the ion flux mediated by reconstituted channels. In 12 preparations of reconstituted calcium channels assayed under these conditions, the initial rate of $^{45}Ca^{2+}$ uptake into vesicles containing the reconstituted calcium antagonist receptor was 2–3-fold greater than influx into protein-free vesicles of the same internal volume. Figure 3 shows the average time course of $^{45}Ca^{2+}$ uptake into vesicles containing the reconstituted calcium antagonist receptor in the presence of BAY K 8644 after correction for the background ion flux into protein-free vesicles prepared and assayed in parallel (○). If the calcium channel activator BAY K 8644 is removed during the reconstitution of the vesicles, $^{45}Ca^{2+}$ influx is markedly reduced, nearly to the level of protein-free vesicles (Figure 3, □). If calcium antagonist receptors were solubilized and purified in the absence of BAY K 8644 and then reconstituted in the presence of BAY K 8644, no increase in $^{45}Ca^{2+}$ influx over the background rate of protein-free vesicles was observed (data not shown). Apparently, the continuous presence of BAY K

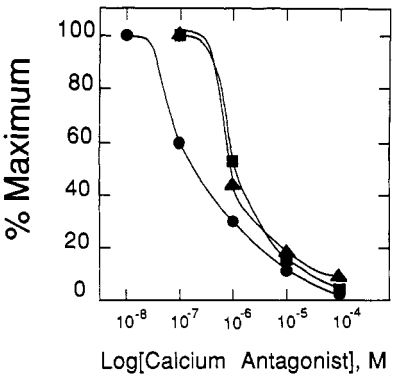

FIGURE 4: Effect of organic calcium antagonists on $^{45}Ca^{2+}$ uptake into reconstituted vesicles. Calcium antagonist receptor was purified and reconstituted in the presence of BAY K 8644. Aliquots of the vesicle preparation were preincubated with the indicated concentration of calcium antagonist for 1 h prior to rapid gel filtration in sucrose medium containing the same concentration of calcium antagonist as described under Experimental Procedures. Uptake was initiated by addition of $^{45}Ca^{2+}$ in sucrose medium containing the appropriate concentration of calcium antagonist, and after 10 min at 37 °C, uptake was terminated by application to Chelex columns. Effects of antagonists on the $^{45}Ca^{2+}$ uptake into protein-free vesicles were determined in an identical fashion. The percent of maximum uptake represents values corrected for the contribution of small antagonist effects on protein-free vesicles: PN200-110 (●); verapamil (■); D600 (▲). All points represent the mean of three determinations on separate purifications/reconstitutions.

8644 is necessary to maintain a conformation of the receptors capable of mediating $^{45}Ca^{2+}$ influx. A recent report of fusion of rat T-tubule membranes with planar phospholipid bilayers stated a similar requirement for the presence of a calcium channel activator to obtain stable recordings from calcium channels (Affolter & Coronado, 1985).

Pharmacological Properties of $^{45}Ca^{2+}$ Influx into Vesicles Containing Reconstituted Calcium Antagonist Receptor. The absolute dependence of influx on the presence of BAY K 8644 provides direct evidence that influx is mediated by reconstituted calcium channels. In this case, $^{45}Ca^{2+}$ influx should also be blocked by inorganic and organic calcium channel antagonists. The organic calcium channel blocker verapamil reduced the initial rate of $^{45}Ca^{2+}$ influx into vesicles containing the reconstituted calcium antagonist receptor to 20% of maximum at a concentration of 100 μM (Figure 3, △). To determine the specificity of this action, the concentration dependence of inhibition of $^{45}Ca^{2+}$ influx in both receptor-containing and protein-free vesicles was examined. Figure 4 shows concentration-effect curves for inhibition by organic calcium antagonists of $^{45}Ca^{2+}$ influx into vesicles containing the reconstituted calcium antagonist receptor. As in previous experiments, the measured $^{45}Ca^{2+}$ influx was corrected for the background rate of $^{45}Ca^{2+}$ influx into protein-free vesicles of the same internal volume assayed under identical conditions. Half-maximal inhibition was observed at approximately 1.5 μM verapamil, 1.0 μM D600, a verapamil analogue, or 0.2 μM PN200-110, a dihydropyridine calcium antagonist. These concentrations are similar to those that give half-maximal inhibition of voltage-sensitive calcium currents in intact skeletal muscle fibers (Chiarandini & Stefani, 1983; Palade & Almers, 1985; Schwartz et al., 1985). This level of inhibition is consistent with the conclusion that the calcium flux in reconstituted vesicles is mediated by functional calcium channels with the pharmacological properties of the dihydropyridine-sensitive calcium channel in T-tubule membranes (Table I).

Although the organic calcium antagonists preferentially blocked the component of $^{45}Ca^{2+}$ influx mediated by reconstituted calcium channels, the inorganic calcium antagonists

Table I: Inhibition of Native and Purified Calcium Channels by Calcium Antagonists

antagonist	$K_{0.5}$ for reconstituted vesicles (μM)	$K_{0.5}$ for protein-free vesicles (μM)[a]	$K_{0.5}$ for intact muscle fibers (μM)[b]
PN200	0.2		0.43
D600	1.0		11.6
verapamil	1.5		15.1
cadmium	10	10	430
nickel	50	ND[c]	680
magnesium	200	200	13,500

[a] Organic calcium channel blockers reduced $^{45}Ca^{2+}$ uptake into protein-free vesicles less than 20% at 100 μM. These small effects were corrected for the data presented. [b] Values for intact muscle from Palade and Almers (1985). [c] ND, not determined.

cadmium, nickel, magnesium, and lanthanum completely blocked all the calcium influx into vesicles containing the reconstituted calcium channel with $K_{0.5}$ values of 10, 50, 200, and 1 μM, respectively (Table I). Moreover, these inorganic antagonists also blocked influx into protein-free vesicles within the same concentration range (Table I). It was therefore not possible to dissociate the specific block of calcium channel mediated $^{45}Ca^{2+}$ flux from the block of nonspecific flux into protein-free vesicles. This is not unexpected since divalent cations should directly compete with external $^{45}Ca^{2+}$ for nonspecific divalent cation binding sites and leakage pathways in phospholipid vesicles. Although we cannot quantitatively separate inhibition of specific and nonspecific $^{45}Ca^{2+}$ flux by inorganic channel blockers, the rank order of potency for their effects ($La^{3+} > Cd^{2+} > Ni^{2+} > Mg^{2+}$) is the same as for inhibition of voltage-sensitive calcium currents in T-tubules (Table I). The block of calcium channels by divalent cations is competitive with permeant divalent cations (Lee & Tsien, 1983). Since the K_D for binding of calcium at its transport sites is approximately 1 μM and the concentration of calcium in our ion flux experiments (100 μM) is 100-fold lower than in the voltage clamp experiments on intact muscle fibers (Palade & Almers, 1985), $K_{0.5}$ values are expected to be 100-fold lower in the reconstituted preparations. Our results approximately agree with this expectation (Table I).

Identification of the Polypeptides Mediating Specific $^{45}Ca^{2+}$ Uptake. To determine whether the calcium influx stimulated by BAY K 8644 and blocked by PN200-110 and verapamil requires the presence of the α, β, and γ subunits of the calcium antagonist receptor and not other detectable proteins, the calcium antagonist receptor was further purified to near homogeneity by sedimentation through sucrose gradients and each fraction was examined for specifically bound [^{3}H]-PN200-110, polypeptide composition, and the ability to mediate $^{133}Ba^{2+}$ influx when incorporated into PC vesicles. Peak fractions contained only the α, β, and γ polypeptides previously found to be components of the calcium antagonist receptor (Figure 5C). A close quantitative correlation was observed between the presence of the α, β, and γ subunits of the calcium antagonist receptor and the ability to mediate $^{133}Ba^{2+}$ influx (Figure 5A,B). Barium was chosen as the permeant cation in these experiments because it is more specific for voltage-sensitive calcium channels than calcium itself. Barium is not efficiently transported by other calcium transport systems such as the sodium–calcium exchanger and the calcium ATPase. Comparison of the $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ influx mediated by calcium channels reconstituted from the same sucrose gradient preparations revealed a similar initial rate of influx of the two cations consistent with their similar permeability through calcium channels in T-tubules (Almers & McCleskey, 1984).

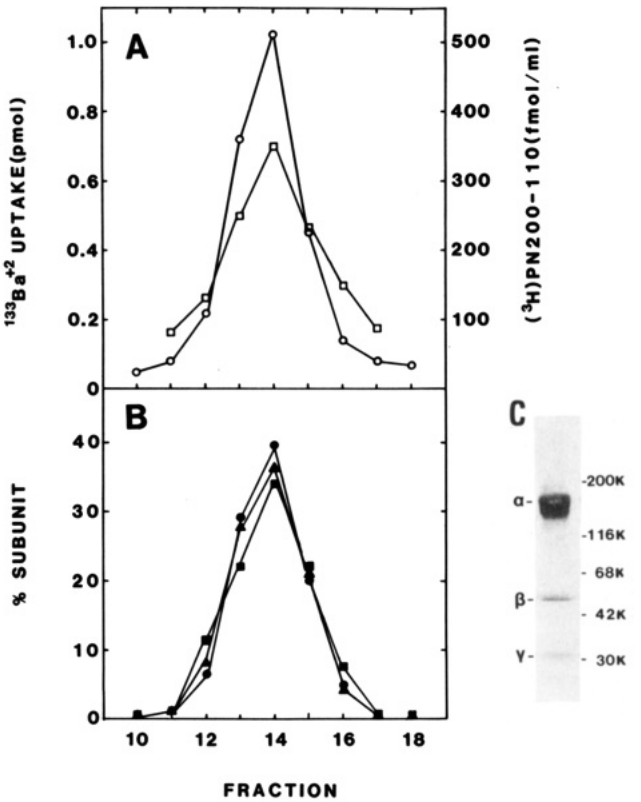

FIGURE 5: Comigration of calcium channels, calcium antagonist receptor subunits, and the [^{3}H]PN200-110/receptor complex on sucrose gradients. Calcium antagonist receptor was saturated with BAY K 8644 and tracer amounts of [^{3}H]PN200-110, purified, and then sedimented on a 5–20% sucrose gradient in barium uptake medium containing 0.1% digitonin and 2 μM BAY K 8644 for 1.5 h at 210000g in a VTi50 rotor. (A) An aliquot of each fraction from the sucrose gradient was taken for determination of the [^{3}H]-PN200-110/receptor complex (○). Aliquots (0.8 mL) of seven fractions from the gradient including the peak of the [^{3}H]PN200-110/receptor complex were individually reconstituted by gel filtration on separate G-50 columns equilibrated with barium uptake medium containing BAY K 8644. After exchange into sucrose medium with BAY K 8644, aliquots of the vesicles reconstituted from the different sucrose gradient fractions were assayed for $^{133}Ba^{2+}$ uptake (□) during a 10-min incubation at 37 °C. All points are the mean of three separate purifications/reconstitutions. (B) The distribution of calcium antagonist receptor polypeptides was determined by lyophilizing the remainder of the sucrose gradient samples, analyzing by SDS gel electrophoresis, and determining the intensity of the silver-stained protein bands by densitometry. Results are presented as the percent subunit present in each fraction, with 100% representing the total integrated area under the peak for each subunit in all nine fractions assayed: α subunit (●); β subunit (■); γ subunit (▲). (C) Silver-stained SDS gel of fraction 14 indicating the purity of the preparation used in panels A and B.

Reconstitution of $^{45}Ca^{2+}$ influx comigrated with the peak of the [^{3}H]PN200-110/receptor complex and the α, β, and γ subunits as illustrated in Figure 5 for $^{133}Ba^{2+}$ influx. These data indicate that the α, β, and γ polypeptides of the purified calcium antagonist receptor are capable of reconstituting a dihydropyridine-sensitive calcium channel and further support the conclusion that calcium antagonist receptors are functional calcium channels.

Estimation of the Fraction of Calcium Antagonist Receptors That Are Active Calcium Channels. Although voltage-sensitive sodium and calcium channels have roughly comparable single channel conductances, the initial rate of $^{45}Ca^{2+}$ influx measured in our present reconstitution experiments is much slower than previously observed for sodium channels under similar conditions (Talvenheimo et al., 1982). In order to determine the reason for this apparent discrepancy, we have estimated the fraction of reconstituted vesicles that contain functional calcium channels by comparing the $^{45}Ca^{2+}$ influx mediated by reconstituted calcium channels with that mediated by the calcium ionophore A23187 added to the same vesicles. The initial rate of calcium influx mediated by A23187 is much faster than for reconstituted calcium channels, and the equilibrium level of $^{45}Ca^{2+}$ uptake is 200-fold greater than the maximum $^{45}Ca^{2+}$ uptake mediated by reconstituted calcium channels. Evidently, only 0.5% of the intravesicular volume in the vesicle population is accessible to functional calcium channels. This conclusion was confirmed directly by measuring equilibrium efflux of $^{45}Ca^{2+}$. Less than 2% of reconstituted vesicles lost internal $^{45}Ca^{2+}$ through active calcium channels (data not shown). Since no more than 30% of the PC vesicles contain the reconstituted polypeptides, we conclude that only a small fraction (2–3.3%) of the purified calcium antagonist receptors can form functional calcium channels when reconstituted by our present approach. The apparent low percentage of purified receptors that reconstitute functional channels may result from damage during isolation and reconstitution or may reflect normal physiological regulatory processes. Schwartz et al. (1985) calculated the number of calcium channels and the number of calcium antagonist receptors in the T-tubules in intact muscle and found that, although the receptors were voltage-sensitive, only a few percent of them represent calcium channels that are simultaneously activated during voltage clamp stimuli. Thus, our results with purified channels may accurately reflect the physiological properties of calcium channels in T-tubules.

Conclusion. Our results show that calcium antagonist receptors purified to near homogeneity and consisting primarily of the α, β, and γ polypeptides can be reconstituted to yield functional calcium channels with appropriate pharmacological properties. Evidently, calcium antagonist receptors and voltage-sensitive calcium channels are identical or closely related macromolecules. However, only a small fraction of purified calcium antagonist receptors can be functionally reconstituted. Further work is required to determine whether physiological or biochemical manipulations of T-tubule membranes or purified calcium antagonist receptors can activate a larger fraction of the calcium antagonist receptors to yield functional voltage-sensitive calcium channels.

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Registry No. PN200-110, 88977-22-4; D600, 16662-47-8; Ca, 7440-70-2; Ba, 7440-39-3; La, 7439-91-0; Cd, 7440-43-9; Ni, 7440-02-0; Mg, 7439-95-4; verapamil, 52-53-9.

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Articles

Structure and Properties of the Cellular Receptor for Transforming Growth Factor Type β[†]

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ABSTRACT: Swiss 3T3 cells respond to picomolar concentrations of type β transforming growth factor (TGF-β) with a dose-dependent increase in the formation of colonies in soft agar, a decrease in the growth of cells in monolayer culture, and changes in morphology. This indicates that these cells have functional TGF-β receptors able to mediate a biological response. Binding analysis revealed a single class of TGF-β binding sites (80 000 per cell) with a $K_d \sim 50$ pM. Receptors were affinity-labeled by covalent attachment to ^{125}I-TGF-β with bis(sulfosuccinimidyl) suberate (BS^3). The complexes formed were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of 100 mM dithiothreitol and migrated as $M_r \sim 180\,000$ complexes in 3–10% linear gradient gels. The apparent size of these complexes was larger in gels with a higher percentage of acrylamide. The labeling of the ^{125}I-TGF-β–receptor complexes was inhibited by the presence of excess unlabeled TGF-β but was unaffected by other growth factors. These complexes could be formed by cross-linking whole cells, intact membranes, or solubilized membranes, demonstrating that the TGF-β receptor is located on the plasma membrane and can be solubilized without destruction of its ability to bind TGF-β. A larger $M_r \sim 360\,000$ complex was present in 3–10% linear gradient gels without reduction or after extensive cross-linking, suggesting that the receptor consists of two subunits of similar size attached by disulfide bonds. Since BS^3 is membrane-impermeable, at least a portion of both subunits is located on the outer surface of the plasma membrane. Unlike epidermal growth factor (EGF) receptors, which aggregate and form large complexes at temperatures >4 °C, no temperature-dependent increase in the size of TGF-β–receptor complexes was observed in either Swiss 3T3 or rat 1 cells. Furthermore, no TGF-β-induced phosphorylation was detected under conditions where the EGF receptor kinase was active. These observations indicate that the receptors for TGF-β may differ mechanistically from those of other growth factors, such as EGF.

Transforming growth factors (TGFs)[1] are defined operationally by their ability to cause anchorage-independent growth of NRK cells [for reviews, see Sporn & Todaro (1980), Todaro et al. (1981), and Roberts & Sporn (1986)]. This transforming activity was originally shown by De Larco and Todaro (1978) to be produced by cells in culture after viral transformation and is now known to be due to the cooperative action

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[1] Abbreviations: BS^3, bis(sulfosuccinimidyl) suberate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EGF, epidermal growth factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NRK, normal rat kidney; PBS, Dulbecco's phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TGF, transforming growth factor; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.