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Solubilization and reconstitution of voltage-dependent calcium channel from bovine cardiac muscle. Ca^{2+} influx assay using the fluorescent dye Quin2

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Highly purified sarcolemmal membranes, prepared from fresh bovine heart left ventricle, were solubilized by *n*-octyl β-D-glucopyranoside and reconstituted into proteoliposomes with soybean phospholipids by the detergent-dialysis method. Ca^{2+} flux into the proteoliposomes was determined using the fluorescent probe Quin2. A membrane potential (negative in the proteoliposome interior) that was created by K^+ diffusion mediated by valinomycin accelerated the Ca^{2+} influx. The voltage-dependent Ca^{2+} influx was dependent on pretreatment of the sarcolemmal membranes with Bay K 8644 and was inhibited by various calcium antagonists including nicardipine ($K_{0.5} = 4.5 \cdot 10^{-7}$ M), verapamil ($K_{0.5} = 9.2 \cdot 10^{-9}$ M), diltiazem ($K_{0.5} = 26 \cdot 10^{-8}$ M) and ω-conotoxin ($K_{0.5} = 9.5 \cdot 10^{-9}$ M).

Introduction

Calcium influx across sarcolemmal membranes through voltage-dependent calcium channels initiates and modulates cardiac muscle contraction [1–3]. Slow inward calcium currents through the calcium channels are blocked by clinically important cardioactive drugs, especially calcium antagonists, such as dihydropyridine derivatives, verapamil and diltiazem, which have been applied for the treatment of cardiovascular disorders. The binding proteins for these drugs from skeletal muscle have been identified, purified and characterized by several groups [4,5]: recently, the primary structure of dihydropyridine-binding protein has been determined [6]. However, the functional aspects of these binding proteins and how they are related to the calcium channel are still not clearly understood. Furthermore, it is not known whether these calcium antagonist binding proteins from skeletal muscle are identical to those in sarcolemmal membranes. Therefore, it is very important to isolate the physiologically active calcium channel proteins from the membranes of cardiac muscle. In order to accomplish this, the solubilization of the calcium channel proteins from sarcolemmal membranes and their reconstitution into an appropriate artificial membrane system are obviously required.

Furthermore, the Ca^{2+} flux through the channel protein should be determined by the most convenient and reliable method. The function of various channels, such as acetylcholine receptors [7], sodium channels [8] and skeletal muscle transverse tubules calcium channels [9], in reconstituted liposomes have been successfully assessed by measuring the movement of radioactive ions.

In the present study, a non-ionic dialyzable detergent, *n*-octyl β-D-glucopyranoside, was used

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to solubilize the sarcolemmal membranes, and then the solubilized proteins were reconstituted into proteoliposomes. Using the fluorescent probe Quin2, we demonstrated that the Ca^{2+} infux is dependent on membrane potential, enhanced by the calcium agonist, Bay K 8644, and inhibited by various calcium antagonists. Furthermore, the inhibitory effect of ω-conotoxin, a neuronal calcium channel inhibitor [10,11], was also demonstrated.

The single channel activities observed with the solubilized protein incorporated into a planar lipid bilayer have been reported elsewhere [12].

Experimental procedures

Materials. Quin2 was purchased from Dojin (Kumamoto). Soybean phospholipids were purchased from Associated Concentrates (Woodside, NY) and partially purified as described [13]. Valinomycin was purchased from Boehringer Mannheim (Mannheim), and *n*-octyl β-D-glucopyranoside (octyl glucoside) was from Sigma (St. Louis). Bay K8644, nicardipine, verapamil, diltiazem and ω-conotoxin were generous gifts from Bayer (Wuppertal), Yamanouchi Pharmaceuticals (Tokyo), Eizai (Tokyo), Tanabe Seiyaku (Osaka), and Peptide Research Institute (Osaka), respectively.

All other reagents were of the highest purity available from commercial sources.

Preparation of highly purified cardiac sarcolemmal membranes. Bovine hearts were obtained from a local abattoir immediately after killing. Sarcolemmal membranes were prepared from bovine ventricular muscles according to the method of Kuwayama and Kanazawa [14], which was modified slightly. All procedures were carried out at 4°C. In order to avoid any proteolytic degradation, 0.01 mg/ml of leupeptin and 0.1 mM phenylmethylsulfonyl fluoride were added to all buffers used.

Using a Polytron homogenizer (Kinematica, Switzerland), minced ventricles (700 g of tissue, wet weight) were homogenized four times, each time for 20 s at medium speed, with a 20 s interval between each, in mannitol buffer containing 0.25 M mannitol, 70 mM Tris-HCl (pH 7.4) and the proteinase inhibitors. The homogenate was centrifuged at $1900 \times g$ for 20 min, and then the precipitate was resuspended with the mannitol buffer, homogenized and centrifuged as above. The procedure was repeated three times, and the resulting supernatants were combined and filtered through a layer of nylon gauze (50 mesh). The filtered supernatant was centrifuged at $70100 \times g$ for 25 min, and the resulting precipitate was suspended in the mannitol buffer and homogenized by four strokes in a Potter-Elvehjem homogenizer with a tightly fitting Teflon pestle. The homogenate was then layered onto 40 ml of 0.64 M sucrose containing 20 mM imidazole-HCl (pH 7.4) and centrifuged at $70100 \times g$ for 90 min. After centrifugation, the turbid layer at the mannitol/sucrose interface was carefully withdrawn with a Pasteur pipette, diluted 3-fold with the mannitol buffer and centrifuged at $70100 \times g$ for 25 min. The resulting precipitate was resuspended in the mannitol buffer and stored in liquid nitrogen in small aliquots until use. The yield of sarcolemmal membranes was about 85 μg of protein from 1 g wet weight of ventricular muscles. The ouabain-sensitive Na^+/K^+-ATPase activity was measured in each preparation as a marker enzyme of plasma membranes. Routinely, the activity was in the range of 25–30 μmol/mg protein per h, comparable with the previous study [15,16], indicating that the preparations obtained were highly purified.

Solubilization of sarcolemmal membranes and reconstitution into proteoliposomes. The phospholipid suspension was prepared as follows: asolectin (10 mg) was suspended in 1 ml of a solution containing 25 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$ and 2% octyl glucoside, and this suspension was sonicated three times with a probe-type sonicator (Branson, model 200) for 3 min at 0°C under an argon atmosphere.

Sarcolemmal membranes (450 μg of proteins) were solubilized with 2% octyl glucoside in the mannitol buffer (total volume of 180 μl) with or without Bay K 8644 (1 μM). The supernatant obtained after centrifuging the solubilized membranes at $150000 \times g$ for 60 min was mixed with the phospholipid suspension described above and dialyzed against 1 l of solution containing 25 mM Tris-HCl (pH 7.4)/2 mM $MgCl_2$/0.1 mM dithiothreitol at 4°C for 48 h [17]. The proteoliposomes thus formed were collected by centrifuga-

tion at 150000 × *g* for 60 min and washed with buffer containing 25 mM Tris-HCl (pH 7.4)/2 mM $MgCl_2$. The pellet was resuspended in 1 ml of a buffer containing 0.4 M KCl/25 mM Tris-HCl (pH 7.4)/1 mM $MgCl_2$ (K^+-loading buffer) and then quickly frozen at −80°C. Quin2 (10 mM) was also added to the K^+-loading buffer when necessary. After 5 min, the suspension was thawed at room temperature and sonicated by the probe-type sonicator using 20 × 0.5 s pulses. The freeze-thaw-sonication procedure was repeated two more times [18]. Then the suspension was centrifuged at 150000 × *g* for 60 min, and the resulting pellet was resuspended in 2 ml of the K^+-loading buffer.

The internal volume fraction of the proteoliposomes was estimated to be approx. 2.5% of the total volume of the suspension according to the method of Oku et al. [19].

Measurement of Ca^{2+} influx into proteoliposomes. Ca^{2+} influx into the proteoliposomes was estimated by measuring the internal Ca^{2+} concentration through monitoring the fluorescence intensity change of Quin2 preloaded as described above. The fluorometry was carried out with a fluorospectrophotometer (Hitachi 650-60) at an excitation of 339 nm and an emission of 490 nm. Proteoliposomes suspended in the K^+-loading buffer (20 μl) were added into 1.98 ml of a reaction mixture consisting of 0.4 M NaCl/25 mM Tris-HCl (pH 7.4) at 35°C. After 2 min of preincubation, $CaCl_2$ was added to a final concentration of 1 mM, followed by the addition of 1 μl of valinomycin (0.1 mg/ml of methanol). The fluorescence intensity before the addition of $CaCl_2$ (F_0) and the initial rate of increase of fluorescence intensity after the addition of valinomycin (ΔF per min) were used for the calculation of the normalized initial rate of fluorescence intensity increase ($\Delta F/F_0$ per min).

Other analytical methods. Protein concentration was determined by the method of Lowry et al. [20]. Na^+/K^+-ATPase activity was measured as ouabain-sensitive ATPase with a reaction mixture containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 3 mM ATP, 1 mM EDTA, 50 mM Tris-HCl (pH 7.5) with or without 1 mM ouabain at 37°C [21]. Inorganic phosphate liberated from ATP during the assay was determined by the method of Fiske and SubbaRow [22].

Results

Effect of calcium agonist Bay K 8644 on Ca^{2+} influx

Highly purified sarcolemmal membranes were solubilized with octyl glucoside and reconstituted into proteoliposomes as described in Experimental Procedures. In order to study the effect of the calcium agonist Bay K 8644, sarcolemmal membranes were treated with the drug at the concentration of 1 μM before the solubilization, and the drug was also present during the reconstitution. In Fig. 1, typical traces of the fluorescence of Quin2 entrapped in the proteoliposomes are shown. When $CaCl_2$ was added to the reaction mixture, a slight increase of the fluorescence intensity was observed with simple liposomes (L), which may be due to residual Quin2 existing on the exterior of the liposomes. On the other hand, significant increases of the fluorescence intensity were observed with proteoliposomes reconstituted with sarcolemmal membrane proteins. Particularly, proteoliposomes containing Bay K 8644-treated proteins (B(+) proteoliposomes) responded to the $CaCl_2$ addition much more than those containing untreated proteins (B(−) proteoliposomes). These results indicate that pretreatment of the sarcolemmal membranes with Bay K 8644 keeps the calcium channel protein in the open state. The addition of valinomycin resulted in further increases of fluorescence intensity: here again, the response was more prominent with B(+) proteoliposomes, and virtually no change was observed with simple liposomes. Since valinomycin permeates K^+ down its concentration gradient from the proteoliposome interior to the exterior portion, generating a membrane potential (more negative in the proteoliposome interior), the valinomycin-elicited increase of the fluorescence intensity reflects the voltage-driven Ca^{2+} influx, which is also enhanced by the pretreatment with Bay K 8644. Thus, the calcium channel activity can easily be assayed by the present method, whose validity has been confirmed, as shown in Fig. 2. The initial rate of fluorescence intensity increase ($\Delta F/F_0$ per min) was almost proportional to the amount of protein, over the range of 60–620 μg, added to a fixed amount of phospholipids (10 mg), and thus the amount of pro-

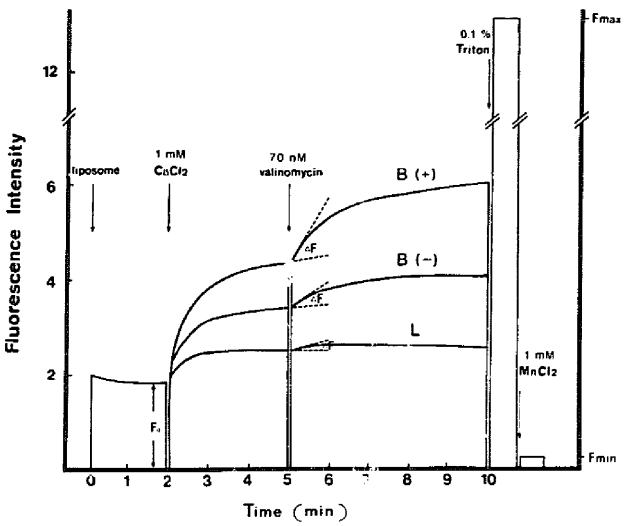

Fig. 1. Typical traces of the fluorescence change of Quin2 entrapped in proteoliposomes. 20 μl of reconstituted proteoliposomes or simple liposomes were added to 1.98 ml of reaction mixture consisting of 0.4 M NaCl/25 mM Tris-HCl (pH 7.4). After 2 min incubation, 1 mM $CaCl_2$ was added, followed by the addition of valinomycin (1 μl of 0.1 mg/ml of methanol) at 5 min. At the end of each assay, 0.1% Triton X-100 and 5 mM $MnCl_2$ (final concentration) were added to estimate F_{max} and F_{min}, respectively. Traces B(+), B(−) and L are with Bay K 8644-treated proteoliposomes, Bay K 8644-untreated proteoliposomes and simple liposomes, respectively.

teins (300–400 μg) used for routine assays was in the most reliable range. The sarcolemmal membranes must be treated with Bay K 8644 before their solubilization, as demonstrated in Fig. 3. In non-treated membranes, addition of Bay K 8644 into the reaction mixture failed to activate the Ca^{2+} influx into proteoliposomes containing either Bay K 8644 pretreated or untreated proteins.

Effects of calcium antagonists on Ca^{2+} influx

The reconstituted proteoliposomes were preincubated with various calcium antagonists for 1 h

TABLE I

EFFECT OF CALCIUM ANTAGONISTS ON Ca^{2+} FLUX

Proteoliposomes were incubated with various amounts of calcium antagonists for 1 h on ice. Ca^{2+} influx ($\Delta F/F_0$ per min $\{\times 10^2\}$) was assayed as described in the legend for Fig. 1. Values are the mean ± S.E. (number of experiments is four) obtained with B(+) proteoliposomes subtracted from those with B(−) proteoliposomes. Numbers in parentheses indicate % of the control.

Concentration (μM)	Nicardipine	Verapamil	Diltiazem	ω-Conotoxin
0	61 ± 13 (100%)	35 ± 4.9 (100%)	44 ± 8.3 (100%)	42 ± 15 (100%)
0.01	47 ± 12 (77%)	12 ± 4.6 (36%) *	23 ± 1.3 (52%)	18 ± 3.8 (43%)
0.1	28 ± 12 (46%)	11 ± 7.0 (33%) *	13 ± 6.0 (30%) *	8.5 ± 5.1 (20%)
1.0	32 ± 16 (53%)	7.3 ± 2.5 (22%) **	12 ± 6.1 (27%) *	0 ± 4.3 *
10	18 ± 7.8 (30%) *	5.5 ± 3.3 (17%) **	0.5 ± 3.4 (1%) **	0 ± 3.6 *
100	10 ± 11 (16%) *	2.5 ± 2.0 (8%) **	0 ± 6.2 **	

* Significantly different from the control, $P < 0.05$

** Significantly different from the control, $P < 0.01$.

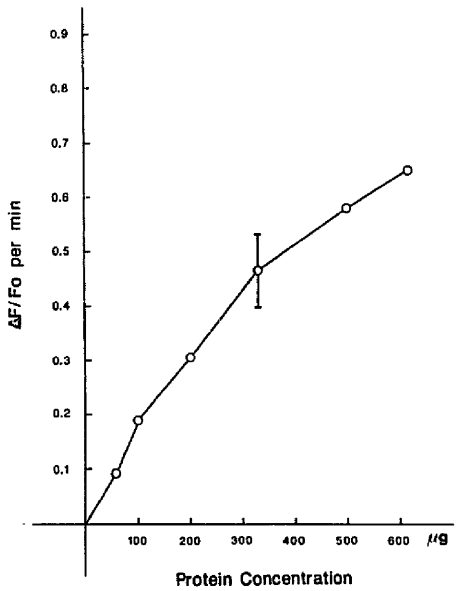

Fig. 2. Initial rate of fluorescence intensity increase ($\Delta F/F_0$ per min) of B(+) proteoliposomes as a function of amount of detergent-solubilized protein added. Reconstituted, K^+ and Quin2-loaded vesicles containing various amounts of detergent-soluble proteins and a fixed amount of soybean phospholipids (10 mg) were prepared as described in the text and Fig. 1. Mean values of two or three determinations and means ±S.E. are shown.

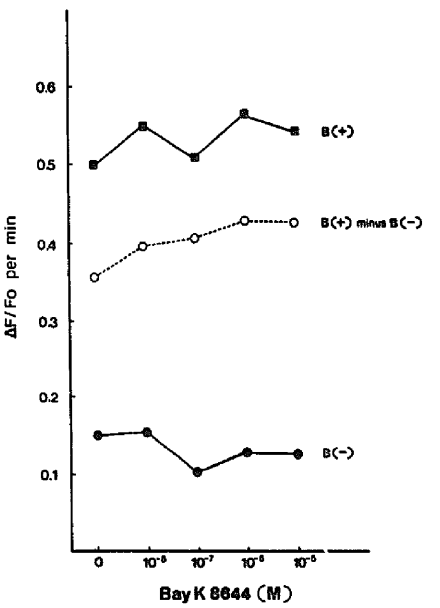

Fig. 3. Effect of post-reconstitutional addition of Bay K 8644 on Ca^{2+} influx. Proteoliposomes containing either Bay K 8644-treated (B(+)) or untreated (B(−)) proteins were incubated with various amounts of Bay K 8644 for 30 min on ice, and then the Ca^{2+} influx was assayed as described in Fig. 1. The initial rate of fluorescence intensity increase ($\Delta F/F_0$ per min) was determined as described under Experimental Procedures and corrected for the background fluorescence change observed with simple liposomes.

on ice. Fig. 4 shows typical fluorescence traces of B(+) proteoliposomes after valinomycin addition in the presence of 1 μM of various calcium antagonists. The increase of fluorescence intensity after valinomycin addition were inhibited in all cases. The effect of various calcium antagonists, including nicardipine, verapamil, diltiazem and ω-conotoxin, at various concentrations on the specific Ca^{2+} flux are shown in Table I. All these antagonists effectively inhibited the Bay K 8644-enhanced fraction of the fluorescence changes. However, the initial rate of fluorescence intensity increase ($\Delta F/F_0$ per min) determined with B(−) proteoliposomes was not influenced by these calcium antagonists, or rather, in the cases of diltiazem and ω-conotoxin, increased slightly at their higher concentrations (data not shown). Thus, it is obvious that calcium antagonists inhibit the specific Ca^{2+} flux, which is dependent on both the membrane potential and Bay K 8644. The antagonist concentrations necessary for half-maximal inhibition ($K_{0.5}$) are $4.5 \cdot 10^{-7}$ M for

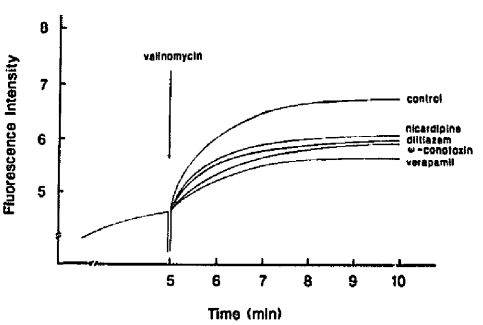

Fig. 4. Typical traces of the fluorescence changes of Quin2 entrapped B(+) proteoliposomes after the addition of valinomycin in the presence of 1 μM nicardipine, verapamil, diltiazem and ω-conotoxin.

nicardipine, $9.2 \cdot 10^{-9}$ M for verapamil, $2.6 \cdot 10^{-8}$ M for diltiazem and $9.5 \cdot 10^{-9}$ M for ω-conotoxin. The inhibitory effects of the antagonists were less prominent and less reproducible when they were added directly to the assay mixture (data not shown).

Discussion

In the present work, sarcolemmal membranes were solubilized with a neutral and mild detergent, octyl glucoside, and reconstituted into proteoliposomes capable of voltage-dependent Ca^{2+} uptake. The Ca^{2+} influx was detected by the fluorescence increase of Quin2 entrapped in the proteoliposomes. It was shown that the Ca^{2+} influx was accelerated by a membrane potential (negative in the proteoliposome interior) generated by K^+ diffusion mediated by the K^+ ionophore valinomycin. Furthermore, pretreatment of sarcolemmal membranes with the calcium agonist Bay K 8644 before solubilization was requisite for the specific Ca^{2+} influx. In addition, the calcium antagonists inhibited only the Bay K 8644-enhanced fraction of Ca^{2+} influx. Thus, we conclude that the difference in the initial rate of Quin2 fluorescence increase of B(+) proteoliposomes and that of B(−) proteoliposomes represents the specific Ca^{2+} influx through the voltage-dependent calcium channels. These results are consistent with the previous study, in which a divalent cation channel current similar to the calcium channel of the intact cardiac muscle was demonstrated [12].

It is not clear whether the applied K^+ diffusion potential increases the open probability of the channel or that it just drives Ca^{2+} ion influx. Since the channel was activated by Bay K 8644, the calcium channel was already open to some extent.

It was found that with a fixed amount of Quin2 (10 mM), the fluorescence intensity of the Ca^{2+}-Quin2 complex was proportional to the $CaCl_2$ concentration in the range 10^{-4}–10^{-2} M (data not shown). Thus, the intraliposomal Ca^{2+}-Quin2 complex ($[Ca\text{-}Quin2]_i$) at a corresponding time as calculated as follows:

$$[Ca\text{-}Quin2]_i \propto \frac{F - F_{min}}{F_{max} - F_{min}}$$

where F_{max} and F_{min} represent the fluorescence intensity after the addition of Triton X-100 (0.1%) and $MnCl_2$ (5 mM), respectively (see Fig. 1). When Ca^{2+} is not present (namely, when [Ca-Quin2] = 0), F is F_{min}; and when sufficient Ca^{2+} is present, then the concentration of the Ca^{2+}-Quin2 complex is equal to that of Quin2 itself (namely, [Ca-Quin2] = [Quin2]), where $F = F_{max}$. Therefore, the following equation can be established:

$$[Ca\text{-}Quin2]_i = [Quin2] \cdot \frac{F - F_{min}}{F_{max} - F_{min}}$$

From this equation, and assuming that there is a uniform protein distribution in the proteoliposomes, the initial velocity of the specific Ca^{2+} influx into the proteoliposomes was calculated to be approx. 2.13 nmol/mg protein per s, based on the estimation that the internal volume fraction is 2.5% of the total volume of the proteoliposome suspension as described in Experimental procedures. This value is almost comparable with those reported for rat cortical synaptosomes [23] and rat heart cells [24].

Bay K 8644 effectively exerted the Ca^{2+} influx when sarcolemmal membranes were treated with the drug prior to their solubilization: however, the direct addition of the drug into the reaction mixture failed to activate the Ca^{2+} influx (Fig. 3). Since enough Bay K 8644 (1 μM) was already present in the B(+) proteoliposomes, further addition of the drug to the reaction mixture was ineffective. These results indicate that Bay K 8644 may preserve the calcium channel activity during solubilization and reconstitution as described by Curtis and Catterall [9], and that those without Bay K 8644 treatment may be denatured during solubilization, causing them to lose their sensitivity to Bay K 8644.

All the calcium antagonists tested inhibited effectively the Ca^{2+} influx into B(+) proteoliposomes, while they had virtually no effect on B(−) proteoliposomes and simple liposomes. The reasons for the slight increase in B(−) proteoliposomes with ω-conotoxin and diltiazem are unknown. The preincubation of proteoliposomes with the antagonists for 1 h on ice was necessary for the prominent and persistent inhibition of the

Ca^{2+} influx. This has been also described in the reconstitution of voltage-sensitive calcium channels from skeletal muscle transverse tubules [9]. The $K_{0.5}$ values obtained were $4.5 \cdot 10^{-7}$ M for nicardipine, $9.2 \cdot 10^{-9}$ M for verapamil, $2.6 \cdot 10^{-8}$ M for diltiazem, and $9.5 \cdot 10^{-9}$ M for ω-conotoxin. These values, except for that of ω-conotoxin, are in ranges similar to those reported with the voltage-dependent Ca^{2+} current in intact cardiac muscles and heart cells [25–27]. This pharmacological evidence also strongly supports that the calcium channel assayed here is of sarcolemmal origin.

As for ω-conotoxin, it was reported that this peptide inhibited $^{45}Ca^{2+}$ influx into chick brain synaptosomes with a $K_{0.5}$ of 10^{-8} M [28], which was very similar to the value obtained above. Recently, McCleskey et al. [29] reported using electrophysiological techniques, that ω-conotoxin blocked high threshold (or L- and N-type) calcium channels in neurons, which have been defined by Nowycky et al. [30], while other calcium channels, including Aplysia bag cells, guinea pig and frog heart cells, and chick skeletal myotubes, were not persistently affected [29]. This may be contradictory to the results described here. Possible reasons for the discrepancy are: (1) the preincubation of sarcolemmal membranes with the toxin for a longer period caused persistent blocking effects on Ca^{2+} influx. In fact, direct addition of the toxin into the assay mixture during Ca^{2+} flux measurements never caused inhibition; (2) prolonged Bay K 8644 activation-induced ω-conotoxin sensitivity. (3) solubilization caused a conformational change in the channel and/or removal of some membrane factors, which resulted in the exposure of the previously latent ω-conotoxin-sensitive site.

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