

copy (Figs. 1 and 2). The greatest strength of these assay systems lies in the ability to monitor two parameters simultaneously. In comparing responses of Ca^{2+} release patterns with the $I_{\text{Ca,C}}$ within the same cell, subtle differences between the pathways can be identified and studied. Much information has been published on the muscarinic receptor subtypes and the final Ca^{2+} response.³⁻⁶ Currently we are focusing our efforts on the different steps between receptor activation and Ca^{2+} increases, including identification of G proteins and their effectors. Molecular biology techniques, such as the use of antisense oligonucleotides, will be useful in identifying individual steps in the pathways. The greatest promise lies in further development of new fluorescent probes sensitive to levels of second messengers. For example, development of a ratioing InsP_3 -sensitive dye would be most useful in separating the roles of Ca^{2+} and InsP_3 . With improvement in microscope speed or sensitivity, responses could be viewed at a much smaller scale, perhaps allowing better correlation between intracellular structure and pathway responses.

[28] Purification and Reconstitution of N-Type Calcium Channel Complex from Rabbit Brain

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Introduction

Voltage-sensitive Ca^{2+} channels play important roles in the regulation of intracellular calcium concentrations in cell types as diverse as muscle cells, neuroendocrine cells, and neurons. Calcium that enters the cell through voltage-sensitive channels acts as a second messenger in cellular processes including the initiation of cardiac and smooth muscle excitation-contraction coupling and synaptic vesicle fusion with the plasma membrane leading to neurotransmitter release in neurons. Calcium channels have been classified into four different types (L, N, T, and P), each of which can be identified by pharmacological and biophysical properties.¹ The best characterized voltage-sensitive Ca^{2+} channel is the skeletal muscle dihydropyridine (DHP)-sensitive Ca^{2+} channel. This channel is essential in the process of excitation-contraction coupling, and it is thought to function both as a calcium channel and as a voltage sensor in excita-

¹ B. P. Bean, *Annu. Rev. Physiol.* **51**, 367 (1989).

tion-contraction coupling.² The skeletal muscle DHP receptor has been purified and is composed of four subunits, α_1 (170K), $\alpha_2\delta$ (175K), β (52K), and γ (32K),³ all of which have been cloned.⁴ However, a number of α_1 subunits, the pore-forming component of voltage-sensitive Ca^{2+} channels, have been cloned and shown to be products of five genes (A, B, C, D, and E type).⁵⁻⁷ These α_1 subunits share homology with the skeletal muscle α_1 subunit. Thus, molecular biology has provided another means of classifying the voltage-sensitive Ca^{2+} channels. The L-type Ca^{2+} channels (C and D type) are also present in neurons, and their channel activity is modulated by dihydropyridines. The P-type Ca^{2+} channel (A type), first identified in Purkinje neurons, is blocked by ω -Aga-IVA, a funnel web spider toxin. The snail toxin ω -conotoxin GVIA binds with high affinity and exerts an inhibitory effect on the N-type Ca^{2+} channel (B type). These channels are also neuro-specific.

There is a substantial amount of evidence indicating that N-type Ca^{2+} channels are responsible for the voltage-activated release of neurotransmitters in a variety of neurons. The specific N-type Ca^{2+} channel blocker ω -conotoxin GVIA directly inhibits presynaptic Ca^{2+} currents.⁸ ω -Conotoxin GVIA binding sites have also been localized precisely at active zones in the presynaptic membrane where neurotransmitter release occurs.^{9,10} The N-type Ca^{2+} channels have been shown to be inhibited by norepinephrine by acting through α_2 -adrenergic receptors. This modulation of the ω -conotoxin-sensitive Ca^{2+} current is thought to involve G proteins.¹¹⁻¹³ Because the role G proteins play in the modulation of N-type Ca^{2+} channels may provide a significant mechanism in regulating neurotransmitter release, this chapter focuses on the purification and reconstitution of the N-type Ca^{2+} channel from rabbit brain.

² T. Tanabe, K. G. Beam, J. A. Powell, and S. Numa, *Nature (London)* **336**, 134 (1988).

³ K. P. Campbell, A. T. Leung, and A. H. Sharp, *Trends Neurosci.* **11**, 425 (1988).

⁴ R. J. Miller, *J. Biol. Chem.* **267**, 1403 (1992).

⁵ T. P. Snutch, J. P. Leonard, M. M. Gilbert, H. A. Lester, and N. Davidson, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3391 (1990).

⁶ T. P. Snutch, J. Tomlinson, J. P. Leonard, and M. Gilbert, *Neuron* **7**, 45 (1991).

⁷ Y. Mori, T. Friedrich, M. Kim, A. Mikami, J. Nakai, P. Ruth, E. Bosse, F. Hofmann, V. Flockerzi, T. Furuichi, K. Mikoshiba, K. Imoto, T. Tanabe, and S. Numa, *Nature (London)* **350**, 398 (1991).

⁸ E. F. Stanley and G. Goping, *J. Neurosci.* **11**, 985 (1991).

⁹ S. J. Smith and G. J. Augustine, *Trends Neurosci.* **11**, 458 (1988).

¹⁰ R. Robitaille, E. M. Adler, and M. P. Charlton, *Neuron* **5**, 773 (1990).

¹¹ B. P. Bean, *Nature (London)* **340**, 153 (1989).

¹² M. R. Plummer, A. Rittenhouse, M. Kanevshy, and P. Hess, *J. Neurosci.* **11**, 2339 (1991).

¹³ A. H. Delcour and R. W. Tsien, *Science* **259**, 980 (1993).

The availability of a high-affinity radiolabeled compound, ^{125}I -labeled ω -conotoxin GVIA, for the receptor site on the N-type Ca^{2+} channel has made this receptor ideal for investigating the structure and function of neuronal Ca^{2+} channel complexes. Also it has been demonstrated that the purified DHP receptor from skeletal muscle, reconstituted in artificial lipid bilayers, is directly stimulated by G_s , the G protein of adenylyl cyclase (adenylate cyclase).¹⁴ Therefore, the reconstitution of the N-type Ca^{2+} channel complex should provide a direct method for testing the regulatory role G proteins exert on neuronal Ca^{2+} channel activity.

Procedures

Isolation and Purification of Crude Brain Membranes from Rabbit Brain

Rationale. ^{125}I -Labeled ω -conotoxin binding experiments with crude brain membranes suggest that the receptor is present at the range of 200 to 300 fmol/mg of protein, a level 1000-fold less than the DHP receptor in skeletal T-tubule preparations, thus making the purification of the N-type Ca^{2+} channel more difficult. Because the DHP-sensitive Ca^{2+} channels from skeletal muscle are localized in the T-tubules, dihydropyridine binding can be enriched from homogenates about 5-fold in KCl-extracted skeletal muscle microsomes. Washing of skeletal muscle microsomes with KCl enriches for the DHP receptor by removing extrinsic proteins. Because the ω -conotoxin-sensitive Ca^{2+} channels are widely distributed throughout the brain, no method is available to enrich substantially these channels in purified membrane preparations without drastically reducing the total yield of the receptor. However, KCl washing of crude brain membranes yields a membrane preparation with twice the B_{max} of ^{125}I -labeled ω -conotoxin compared to crude brain membranes (400–600 fmol/mg protein). Thus, KCl-extracted crude brain membranes are used as the starting material for the purification of the N-type Ca^{2+} channel.

Buffers

Medium I: 50 mM Tris–maleate, pH 7.4 0.5 mM EDTA

Medium II: 20 mM Tris–maleate, pH 7.4, 0.303 M sucrose

All buffers contain a cocktail of protease inhibitors including aprotinin (0.5 $\mu\text{g}/\text{ml}$), benzamidine (100 $\mu\text{g}/\text{ml}$), leupeptin (92.5 $\mu\text{g}/\text{ml}$), pepstatin A (0.5 $\mu\text{g}/\text{ml}$), and phenylmethylsulfonyl fluoride (PMSF, 40 $\mu\text{g}/\text{ml}$).

¹⁴ A. Yatani, Y. Imoto, J. Codina, S. L. Hamilton, A. M. Brown, and L. Birnbaumer. *J. Biol. Chem.* **263**, 9887 (1988).

Procedure. All steps are performed at 4°. Four fresh whole brains from New Zealand White rabbits (3–4 kg) without the meninges are immediately placed in 200 ml of medium I to remove excess blood prior to homogenization. Approximately 1600 g of brain tissue is homogenized three times in 400 ml of medium I for 30 sec at speed 5 with a Brinkmann PTA 20S Polytron (Westbury, NY). The homogenate is then centrifuged at 17,300 rpm (35,000 g_{\max}) for 20 min in a Beckman type 45 Ti rotor (Fullerton, CA). The supernatants are discarded. The pellets are resuspended in 400 ml of medium II with 0.6 M KCl and gently stirred for 20 min. The sample is then filtered through six layers of cheesecloth and centrifuged at 40,000 rpm (186,000 g_{\max}) for 60 min in a Beckman type 45 Ti rotor. Again, the supernatants are discarded and the pellets extracted by gently stirring for 20 min with medium II containing 0.6 M KCl. The sample is filtered through six layers of cheesecloth and centrifuged at 25,000 rpm (72,660 g_{\max}). The pellets are resuspended in medium II to a protein concentration of approximately 20–25 mg/ml. Protein concentrations are determined by the method of Lowry *et al.*¹⁵ as modified by Peterson using bovine serum albumin (BSA) as a standard.¹⁶

Solubilization and Purification of N-Type Calcium Channel

Rationale. The procedure for purifying the N-type Ca^{2+} channel from brain was developed based on our knowledge of the purification and subunit composition of the skeletal muscle DHP receptor. The skeletal muscle DHP receptor was purified with the use of ion-exchange chromatography, wheat germ agglutinin (WGA)-agarose chromatography, and sucrose density gradient centrifugation. Because of the low density of ω -conotoxin receptors in brain, an immunoaffinity purification step is needed to obtain useful amounts of the receptor. A number of monoclonal and polyclonal antibodies to the individual subunits of the DHP receptor were tested for the ability to immunoprecipitate the solubilized ω -conotoxin receptor. Monoclonal antibody VD2₁ against the β subunit of the L-type Ca^{2+} channel immunoprecipitates greater than 85% of the solubilized ω -conotoxin receptor, demonstrating that the N-type Ca^{2+} channel complex contains a β subunit with a similar epitope as the DHP receptor. However, additional steps are necessary to provide a homogeneous purification of the N-type Ca^{2+} channel. The α_2 subunit of the DHP receptor is highly glycosylated and binds to immobilized WGA. Therefore,

¹⁵ O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

¹⁶ G. L. Peterson, *Anal. Biochem.* **83**, 346 (1977).

the use of WGA-agarose provides a powerful method for the purification of the N-type Ca^{2+} channel.

The WGA-agarose resin along with others, such as heparin agarose, were tested for the ability to bind solubilized ^{125}I -tagged ω -conotoxin-labeled receptor from brain membranes. Although WGA-agarose is able to bind approximately 50% of the ^{125}I -tagged ω -conotoxin-labeled receptor, the elution conditions of this procedure have been determined to be inefficient for a potential purification step. However, heparin-agarose binds 85 to 90% of the solubilized ω -conotoxin receptor, which could be eluted using increasing ionic strength. For large-scale preparations, heparin agarose column chromatography provides a 4-fold purification of the receptor. Finally, the large size of the N-type Ca^{2+} channel complex (21 S) also makes it possible to use sucrose density gradients to increase the purity of the N-type Ca^{2+} channel. Thus, the N-type Ca^{2+} channel is purified using ion-exchange chromatography, immunoaffinity chromatography, and sucrose density gradient centrifugation.

Reagents. ^{125}I -Labeled ω -conotoxin GVIA (1600 Ci/mmol) is obtained from Amersham (Arlington Heights, IL). Digitonin is obtained from ICN (Costa Mesa, CA) and purified as previously described,¹⁷ heparin-agarose is from Sigma (St. Louis, MO), and hydrazide avidin gel is from Unisyn Technologies Inc. Monoclonal antibody VD2₁-agarose¹⁸ and anti-46K-agarose are prepared according to the instructions provided with the hydrazide avidin gel with 3 mg of antibody cross-linked per milliliter of swollen gel.

Buffers

Buffer I: 10 mM HEPES, pH 7.4, 100 mM NaCl, 0.2 mg/ml BSA

Buffer II: 10 mM HEPES, pH 7.4

Buffer III: 10 mM HEPES, pH 7.4, 1.0 M NaCl, 1.0% digitonin
% w/v

Buffer IV: 10 mM HEPES, pH 7.4, 0.3 M NaCl, 0.1% digitonin

Buffer V: 10 mM HEPES, pH 7.4, 0.4 M NaCl, 0.1% digitonin

Buffer VI: 10 mM HEPES, pH 7.4, 0.7 M NaCl, 0.1% digitonin

Buffer VII: 50 mM CAPS, pH 10.0, 0.6 M NaCl, 0.1% digitonin

Protease inhibitors are added from stock solutions to the concentrations employed for the purification of the receptor. Calpain inhibitors I and II (2 $\mu\text{g}/\text{ml}$) are also added to the solubilization and elution buffers. These protease inhibitors are included in the preparation to decrease proteolysis of the $\alpha_{1\text{B}}$ subunit (230K) of the receptor.

¹⁷ A. T. Leung, T. Imagawa, and K. P. Campbell, *J. Biol. Chem.* **262**, 7943 (1987).

¹⁸ J. Sakamoto and K. P. Campbell, *J. Biol. Chem.* **266**, 18914 (1991).

Prelabeling with ^{125}I -Labeled ω -Conotoxin GVIA. To follow the purification of the N-type calcium channel throughout each experimental step, a small portion of brain membranes is labeled with the specific channel blocker ^{125}I -labeled ω -conotoxin GVIA before the solubilization. Two milligrams of KCl-extracted brain membranes is suspended in 2 ml of buffer I, and ^{125}I -labeled ω -conotoxin GVIA is added to a final concentration of 0.5 nM as previously described.¹⁸ The sample is incubated at room temperature for 60 min and vortexed periodically. The membranes are centrifuged for 10 min at 100,000 rpm in a TLA Beckman 100.3 rotor, and the supernatant is removed and discarded. The resuspended pellet, which contains the labeled N-type Ca^{2+} channels, is added to the solubilization mixture as described below. The ^{125}I -labeled ω -conotoxin GVIA–N-type Ca^{2+} channel complex is very stable at 4° (half-life >12 hr). Under these conditions, the amount of radiolabeled Ca^{2+} channels in 2 mg of brain membranes is sufficient to trace the receptor throughout the purification.

Solubilization. The ^{125}I -tagged ω -conotoxin-labeled membrane pellet is homogenized in 1 ml of buffer II by drawing it up and down in a pipette tip. The sample along with approximately 900 mg of brain membranes is added to buffer III to a final volume of 300 ml. The final protein and digitonin concentrations are 3 mg/ml and 1%, respectively. After gentle stirring for 1 hr at 4°, the membrane suspension is centrifuged at 35,000 rpm (142,000 g_{max}) for 37 min in a Beckman type 45 Ti rotor. The supernatant, which contains the solubilized N-type Ca^{2+} channel complex, is slowly diluted with 700 ml of buffer II while gently stirring to reduce ionic strength. The final volume of 1000 ml has a protein concentration of 0.9 mg/ml, a digitonin concentration of 0.3%, and an NaCl concentration of 0.3 M.

Heparin-Agarose Chromatography. The solubilized ω -conotoxin receptor is passed through a column containing 50 ml of heparin-agarose preequilibrated in buffer IV at a flow rate of 5 ml/min. The heparin-agarose column is extensively washed with 5 column volumes of buffer IV and then 8 column volumes of buffer V. The bound receptor is eluted from the column with buffer VI at a flow rate of 2 ml/min. Aliquots of the eluted fraction are counted in a γ counter to determine ^{125}I -labeled ω -conotoxin radioactivity.

VD2₁-Agarose Affinity Chromatography. The fractions containing the ω -conotoxin binding activity are pooled (90–100 ml) and incubated overnight at 4° with 8 ml of VD2₁-agarose preequilibrated with buffer II containing 0.6 M NaCl and 0.1% digitonin. After extensive washing, the ω -conotoxin receptor is eluted from the VD2₁-agarose column with buffer VII and neutralized immediately. This step provides greater than 1000-fold enrichment of the receptor complex. The VD2₁-agarose-purified receptor

periodically contains a variable amount of a contaminating protein (46K) which is removed by preadsorbing the VD₂-agarose-eluted receptor with an immunoaffinity resin prepared using polyclonal antibodies raised against the 46K protein. Anti-46K polyclonal antibodies do not immunoprecipitate ¹²⁵I-labeled ω -conotoxin labeled receptor.

Sucrose Density Gradient Centrifugation. The ω -conotoxin receptor is concentrated to 0.6 ml in an Amicon (Danvers, MA) ultrafiltration cell using a YM100 membrane and layered onto a linear 5–30% sucrose density gradient (12.5 ml). Gradients are centrifuged at 4° in a Beckman VTi 65.1 rotor for 100 min at 215,000 *g*. Fractions (0.6 ml) are collected from the top of the gradients using an ISCO Model 640 density gradient fractionator (Lincoln, NE) and counted in a Beckman γ counter (Fullerton, CA) counter. The receptor is present in gradient fractions 8–12.

Subunit Composition and Properties. The purified N-type Ca²⁺ channel complex from rabbit brain consists of several subunits. Figure 1 shows a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the various steps in the purification of the N-type Ca²⁺ channel. Samples from each purification step are separated on a 3–12% linear SDS–polyacrylamide gel and stained with Coomassie blue. The purified N-type Ca²⁺ channel is composed of a 230K subunit (α_{1B}) tightly associated with a 140K subunit (α_2 , reduced), a 57K subunit (β), and a novel 95K subunit. The positions of these subunits are indicated by the arrows in Fig. 1 (α_1 , α_2 , 95K, and β). The subunits of the receptor complex comigrate on the sucrose gradient with the peak of ¹²⁵I-labeled ω -conotoxin binding activity. For comparison, Fig. 1 also shows a Coomassie blue-stained 3–12% polyacrylamide gel of 25 μ g of purified skeletal muscle DHP receptor. The subunits of the L-type Ca²⁺ channel complex are indicated by the arrows (α_1 , α_2 , β , and γ). Analysis of the sucrose density gradient fraction with affinity-purified sheep polyclonal antibodies to each subunit demonstrates that all four subunits of the N-type Ca²⁺ channel comigrate on the sucrose density gradient and are immunologically distinct. Furthermore, the subunits of the N-type Ca²⁺ channel are coimmunoprecipitated as a receptor complex by affinity-purified antibodies against each of the individual subunits.^{19,20}

Immunoblot analysis shows that affinity-purified polyclonal antibodies to the ω -conotoxin receptor α_2 and β subunits identify the α_2 and β subunits of the skeletal muscle DHP receptor. Also, similar to the α_2 subunit of the skeletal muscle DHP receptor, the α_2 subunit of the ω -conotoxin

¹⁹ D. R. Witcher, M. De Waard, and K. P. Campbell, *Neuropharmacology* **32**, 1127 (1993).

²⁰ D. R. Witcher, M. De Waard, J. Sakamoto, C. Franzini-Armstrong, M. Pragnell, S. D. Kahl, and K. P. Campbell, *Science* **261**, 486 (1993).

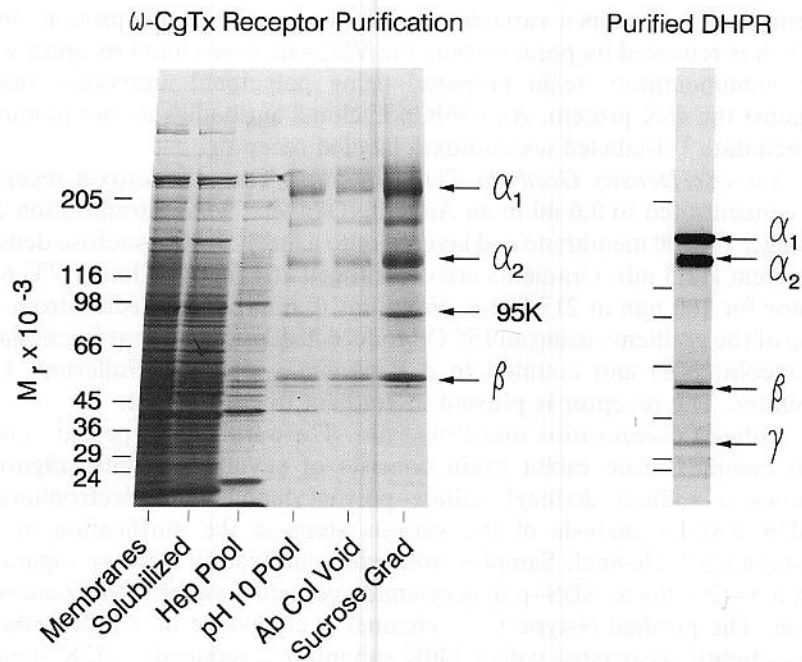


FIG. 1. Gel electrophoresis analysis of N-type Ca^{2+} channel purification and purified skeletal muscle DHP receptor. (*Left*) Samples from each purification step were analyzed by SDS-PAGE and stained with Coomassie blue. The position of the subunits of the N-type Ca^{2+} channel are indicated by the arrows (α_1 , α_2 , 95K, and β). (*Right*) Purified skeletal muscle DHP receptor was analyzed by SDS-PAGE and stained with Coomassie blue. The position of the subunits of the L-type Ca^{2+} channel are indicated by the arrows (α_1 , α_2 , β , and δ). Molecular weight standards are indicated at left. Samples are as follows: membranes, 100 μg of isolated rabbit brain membranes; solubilized, 100 μg of digitonin-solubilized membranes; hep pool, 50 μg of heparin-agarose pooled fractions; pH 10 pool, 100 μg of heparin-agarose pooled fractions; pH 10 eluted pooled fractions; Ab Col Void, 8 μg monoclonal antibody (MAb) VD2₁, pH 10 eluted pooled fractions; Ab Col Void, 8 μg void volume from anti-46K column; and Sucrose Grad, 30 μg of sucrose gradient fractions 10 and 11.

receptor shifts mobility on SDS-PAGE on reduction, yielding δ peptides of 24K and 27K. The WGA, which binds to complex sugars, labels the α_2 subunits of both receptors. *N*-Glycosidase F treatment of the ω -conotoxin receptor shows that the α_2 subunit as well as the 95K subunit contain N-linked sugars. Affinity-purified antibodies to the α_1 and 95K subunits of the N-type Ca^{2+} channel demonstrate that these subunits are present in the ω -conotoxin receptor but not in the skeletal muscle DHP receptor.²⁰

Reconstitution for Single-Channel Analysis

Rationale. The purification of two major Ca^{2+} channels, the skeletal L type and the brain N type, have now been reported.^{20,21} Although both channels are involved in dissimilar cellular functions (excitation–contraction coupling for the skeletal DHP receptor and transmitter release for the ω -conotoxin receptor) each receptor complex is composed of four subunits. However, because the categorization of Ca^{2+} channels into each particular group is based on pharmacological and biophysical properties, reconstitution of the purified receptor provides valuable information on the channel activity of the receptor. It further provides a means to distinguish between biophysical properties specifically due to the purified complex and those that might be due to cellular regulation of the native channel. Specific advantages of reconstitution include easier control over the lipid composition and the solutions on both sides of the membrane. These two factors are either impossible or difficult to control for N-type Ca^{2+} channels in native membranes since (1) channel activity cannot be studied in excised patches, (2) solutions on the inside of a cell cannot be readily controlled, and (3) N-type Ca^{2+} channels are often located in structures which are inaccessible, such as nerve terminals. Therefore, studies on ion-permeation properties and pharmacological regulation of the Ca^{2+} channel are considerably easier for reconstituted channels.

Choice of Reconstitution Technique. Three major forms of ion channel reconstitution systems are available: (a) patch clamp of large liposomes²² or painted membranes²³ in which channels have been preinserted, (b) planar bilayers formed from bulk solutions across apertures 50–300 μm in diameter in Teflon septa,²⁴ and (c) lipid monolayers.²⁵ Bilayers formed from monolayers can be used to separate two compartments either of the classic bilayer chamber²⁵ or at the tip of patch electrodes.²⁶ In cases (b) and (c), channels can be formed either by preinsertion into vesicles and subsequent fusion of the vesicles with the bilayer or by direct insertion of channels into the bilayer.

We have limited our choice for the method of reconstitution by the expected properties of the purified receptor. The N-type Ca^{2+} channels

²¹ A. T. Leung, T. Imagawa, B. Block, C. Franzini-Armstrong, and K. P. Campbell. *J. Biol. Chem.* **263**, 994 (1988).

²² D. W. Tank, C. Miller, and W. W. Webb. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7749 (1982).

²³ P. Mueller. *Ann. N.Y. Acad. Sci.* **264**, 247 (1975).

²⁴ P. Mueller, D. Rudin, H. T. Tien, and W. C. Wescott. *Circulation* **26**, 1167 (1962).

²⁵ M. Montal and P. Mueller. *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3561 (1972).

²⁶ R. Coronado and R. Latorre. *Biophys. J.* **43**, 231 (1983).

typically have small current amplitudes in the low picoampere range and have fast open-time durations in the millisecond range.^{20,27} Therefore, reconstitution of the receptor in bilayer chambers is problematic because the capacitance of the large membrane severely limits the recording bandwidth and creates large, complex capacity transients in response to voltage modifications. Although such reconstitutions have been shown for the DHP receptor or the tetrodotoxin (TTX)-sensitive Na⁺ channel, they were mostly done in the presence of drugs (BAY K 8644 and batrachotoxin, respectively) known to affect the open-time constants of both channels. However, compounds with similar functional effects have not been reported for the N-type Ca²⁺ channel.

Because recordings at the tip of patch pipettes allow higher time resolution and better signal-to-noise ratios, we have chosen to study the N-type Ca²⁺ channel by this technique. The tip-dip method presents further distinct advantages: (1) since bilayers at the tip of electrodes cover a smaller surface area, they support stronger voltage gradients (up to 200 mV); (2) contrary to bilayers formed across Teflon holes, tip-dip bilayers do not contain solvents since they are allowed to evaporate from the monolayer; and (3) channels do not need to be preincorporated into vesicles which themselves would form subsequently the basis of the monolayer, and this in turn minimizes considerably the number of protocol steps, which is essential for reducing the amount of proteolysis.

Description of Tip-Dip Technique. In the tip-dip method, the formation of bilayers requires a two-step procedure subsequent to the formation of a monolayer at the surface of the bath solution (Fig. 2). The first step induces the formation of a monolayer by removing the electrode from the bath solution (Fig. 2B), and the second step results in bilayer formation by reimmersion of the pipette, as assessed by the resulting gigaohm seal resistance (Fig. 2C). In some cases, however, these procedures are not needed because of direct formation of the bilayer during the first pipette immersion.

For the formation of monolayers, we use a mixture of bovine brain phosphatidylethanolamine (PE) and phosphatidylserine (PS) in a 1 to 1 weight ratio (PE and PS are from Avanti Polar Lipids, Birmingham, AL). The lipids are stored at -80° and dried under a stream of nitrogen. Dried lipids are then dissolved in *n*-decane at 30 mg/ml. One to three microliters of lipids are then spread on top of the bath solution. Heat-polished electrodes of 7 MΩ resistance are used, which are coated with Sylgard to improve pipette capacitance. Under these conditions, the probability of successful lipid formation exceeds 90%. However, not all the membrane

²⁷ M. De Waard, D. R. Witcher, and K. P. Campbell, *J. Biol. Chem.* **269**, 6716 (1994).

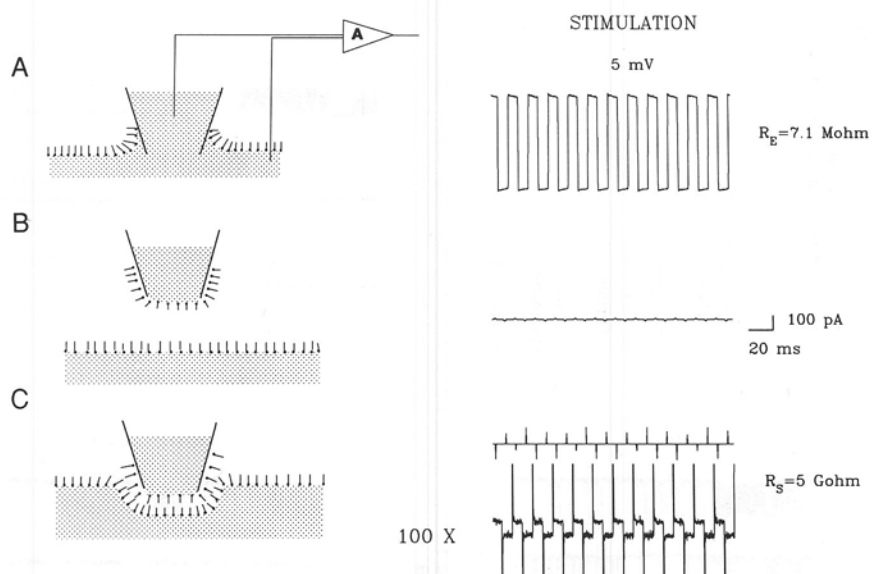


FIG. 2. Tip-dip technique. (A) A patch electrode connected to an amplifier is dipped into the bath covered by a monolayer of lipid mixture. The electrode resistance can be measured in response to a 5-mV pulse. (B) Removal of the electrode results in the formation of a monolayer at the tip of the electrode. (C) Placing the electrode back into the solution results in the formation of a bilayer. This causes an important increase in the electrode resistance. The seal resistance, in response to a 5-mV pulse, is measured after a 100-fold increase in the gain of the amplifier.

formations represent true bilayers, as assessed by the rate of successful recordings. This rate is much lower probably because of the combination of real bilayer formation success and channel presence or incorporation rate. We have frequently performed control experiments in the absence of receptor to test for the stability of the bilayer. Constant voltages applied to the bilayers show that under these conditions no channel-like openings are present, owing to stepwise decrements in seal resistance even in the presence of digitonin at concentrations 10^5 - to 10^7 -fold higher than achieved during the reconstitution of the receptor (Fig. 3A).

Guidelines to Ensure Formation of Good Bilayers. Bilayers formed within the first 10 min result in seal resistances higher than $100 \text{ G}\Omega$ that do not give rise to channel incorporation. This is probably due to an agglomeration of lipid layers favored either by the residual presence of solvent or by an incomplete monolayer formation. We avoid making bilayers 40 min after spreading the lipid because of oxidation of lipids occurring at the air-lipid interface. The presence of PS in combination with

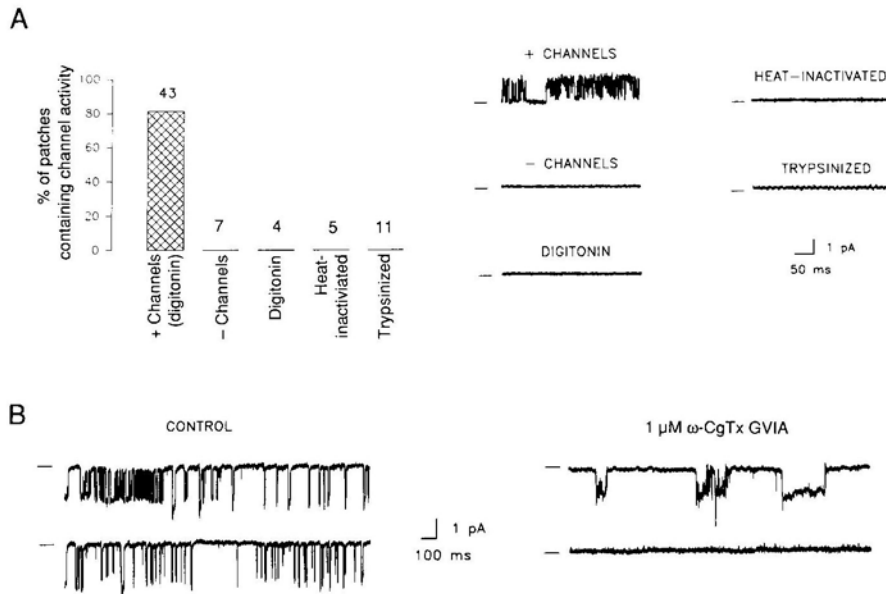


FIG. 3. Reconstitution of purified N-type Ca^{2+} channel complex. (A) The percentage of patches giving rise to channel activity was measured under a variety of conditions: in the presence of 5–20 pmol of receptor, in the absence of channels, in the presence of 0.1% digitonin, and after heat-inactivation or trypsin-digestion of the receptor. Corresponding recordings at right show that channel activity was obtained only in the presence of the receptor. Ionic conditions were as follows: symmetrical 100 mM BaCl_2 , 10 mM HEPES, pH 7.4. The holding potential was +40 mV (+ channels) and +100 mV in other cases. Solid lines represent the closed state of the channel. (B) The N-type Ca^{2+} channel activity is blocked with 1 μM ω -conotoxin GVIA. Symmetrical ionic conditions were used: 100 mM BaCl_2 , 10 mM HEPES, pH 7.4 (electrode), and 1 mM BaCl_2 , 150 mM HEPES, pH 7.4 (bath). The holding potential was -10 mV. Channel activity is blocked 3 min after the addition of 1 μM ω -conotoxin GVIA to the bath.

divalent cations on both sides of the bilayer will ensure membrane stability. The occurrence of artifacts seemed enhanced by reducing either the proportion of PS or the divalent cation concentration to submillimolar levels. Finally, electrode resistance should remain in the range 2 to 10 M Ω since low-resistance electrodes have more unstable bilayers and high-resistance electrodes have a tendency to be obstructed.

Channel Insertion. The rate and number of channels inserted into the bilayer is best controlled by adjusting the final receptor concentration, the level of pipette immersion, and the subsequent perfusion of the bath medium. Higher receptor concentrations are required for channel incorporation occurring after bilayer formation. Furthermore, the receptor con-

centration must be higher when the channels are added to the cup rather than in the electrode owing to the larger bath volume. In contrast, when channels are incorporated into monolayers before patch formation, lower concentrations are required. This is probably because of a better incorporation into monolayers.

Other factors may affect the number of channels in bilayers. We found substantial differences with different lipid amounts added (1 μ l compared to 2 or 4 μ l, for example). The lower the amount of lipids, the higher the channel concentration. This probably arises from (1) a displacement of equilibrium between channels in solution and channels in the lipid phase and (2) an increase in surface areas that really are monolayer in nature, which again suggests a better channel insertion into monolayers. Because the purpose of the experiment is to obtain as consistently as possible a single channel in the bilayer, and because parameters such as the exact concentration of channel in the purified fraction, the amount of lipids added, the surface of the extracellular cup, and the tip surface of the electrode are involved, the experimental conditions need to be defined empirically each time.

It is worth noting that extremely small amounts of purified proteins (5 to 20 pmol) are necessary. Based on the concentrations of N-type Ca^{2+} channels used, experiments performed in a day would require less than 5 μ l of the purified receptor. Because the dilution factor used for recordings of isolated receptors was extremely high, removal of detergent (by polystyrene resins such as Bio-Beads SM-2, Bio-Rad, Richmond, CA) from the purified preparation was not necessary. Figure 3 shows two single-channel recordings that illustrate the specificity of the channel reconstitution. Only bilayers containing intact purified ω -conotoxin receptor are active (symmetrical 100 mM BaCl_2 , voltage electrode +40 mV, Fig. 3A). Like the native N-type Ca^{2+} channel, the reconstituted channel activity is specifically blocked by a bath application of 1 μ M ω -conotoxin GVIA.

Interpretation. In some cases, the number of channels inserted into the bilayer can increase with time. This is true especially when channels are inserted into the electrode where perfusion is not easily feasible. Obviously, there is not a simple bilayer present over the entire area covered by the tip of the electrode. Instead, a lipid bulk at the rim of the electrode may constitute a reservoir where channels can diffuse laterally to decrease or increase the number of functional channels. It is also possible that further lipid or protein diffusion occurs from the surface monolayer into the bilayer along a lipid film present on the glass surface. Increases in lipid amounts at the tip of the electrode would result in the loss of bilayer formation. We have indeed recorded some irreversible increases in seal resistance during the time course of an experiment.

Because these events were more frequent with higher amounts of lipid spreading at the surface of the solution, it does suggest the likelihood of this process. We minimize the occurrence of these events by engaging the electrode deep enough into the solution and by reducing maximally the amount of lipids used (defined as the volume that gives a success rate of at least 50%).

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[29] Infusion of Guanine Nucleotides through Recording Electrodes for Studies on G-Protein Regulation of Ion Currents and Channels

By RODRIGO ANDRADE

Introduction

Intracellular infusion of GTP and guanine nucleotides has been extensively used in electrophysiological experiments to pinpoint the participation of G proteins in the regulation of ion currents and channels. Indeed the use of these compounds has become one of the standard criteria used to implicate heterotrimeric G proteins in membrane phenomena. As a result there is a vast literature regarding the use of GTP and hydrolysis-resistant guanine nucleotides in electrophysiological experiments. Although identical conditions are rarely used to test these compounds, most procedures are simple variants of a few common themes as discussed below.

Use of GTP and Hydrolysis-Resistant Guanine Nucleotides in Whole-Cell Recording

G-protein-coupled receptors are one of the main types of cell surface receptors mediating cell-to-cell communication. Because transmembrane signaling by these receptors is dependent on the hydrolysis of GTP, this nucleotide is an essential cofactor for a variety of transmembrane signaling mechanisms. In intact cells intracellular GTP concentration is maintained by endogenous metabolic pathways. During whole-cell recording, how-