High-Level Expression and Functional Reconstitution of *Shaker* K^+ Channels†

Tsoyue Sun, Amir Aghazadeh Naini, and Christopher Miller*

Howard Hughes Medical Institute, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254-9110

Received April 21, 1994; Revised Manuscript Received June 8, 1994®

ABSTRACT: Voltage-gated K^+ channels were expressed in COS cells transiently transfected with a plasmid carrying a cDNA for an inactivation-removed *Shaker* K^+ channel driven by an adenovirus promoter. Channel expression was followed by immunological detection, binding of radioactive charybdotoxin (CTX), and functional reconstitution into planar lipid bilayers. About 10^7 channels per transfected cell are expressed on the plasma membrane. The expressed channels are glycosylated and competent to bind CTX with the expected characteristics. Channels observed after insertion into planar lipid bilayers displayed the voltage-dependent gating, conduction, and ion selectivity behavior expected for this channel. Channels were solubilized in several detergents without loss of CTX binding activity. The results make plausible a systematic attack on the purification of milligram-level amounts of functional K^+ channels from a heterologous expression system.

The voltage-gated K^+ channels of electrically excitable membranes have been under intense molecular scrutiny in recent years for two reasons: their central importance in the operation of electrically active cells, and the ease of their molecular manipulation and high-resolution electrophysiological analysis. As a result, the K^+ channels provide a satisfying example of the relation between membrane protein function and underlying molecular architecture (Miller, 1991; Sigworth, 1993). However, since K^+ channels are sparsely and heterogeneously expressed in natural tissues, progress in their protein-level biochemistry has been slow. Indeed, heroic efforts have been required to purify even miniscule quantities of these proteins from natural sources such as mammalian brain or muscle (Parcej & Dolly, 1989; Scott et al., 1994; Garcia-Calvo et al., 1994).

Since many K^+ channel genes are now well characterized, several attempts have been made to produce biochemically usable amounts of these proteins by high-level heterologous expression as a first step toward large-scale purification and, ultimately, direct structure determination by electron crystallography (Kühlbrandt et al., 1994). The widely-used baculovirus-Sf9 expression system produces K^+ channel protein in vast abundance (Klaiber et al., 1990; Li et al., 1994; Santacruz-Toloza et al., 1994a), but in our hands, most of this is found in nonfunctional insoluble aggregates (Klaiber, 1991); accordingly, final yields of purified product have been low (Santacruz-Toloza et al., 1994a; Li et al., 1994) and reconstitution into planar lipid bilayers unsatisfactory (P. Stampe and C. Miller, unpublishable). Moreover, once intact cells have been fragmented or membranes solubilized into detergent micelles, the functional competence of K^+ channel protein has been thus far impossible to quantify; until this problem is solved, all attempts to purify K^+ channels from heterologous expression systems will remain under a cloud of uncertainty that properly inhibits progression to two-dimensional crystallization studies.

We now report three improvements directed at ameliorating these difficulties in high-level heterologous expression of K^+ channels. First, we use a mammalian transient transfection system that produces approximately 10^7 channels per cell. Second, we exploit an engineered high-affinity ligand, *N*-ethylmaleimide-labeled charybdotoxin (NEM-CTX), to quantify and characterize assembled, tetrameric *Shaker* K^+ channels in both isolated membrane fragments and detergent extracts. Third, we show that the channels produced can be functionally reconstituted into model membranes accessible to direct electrical recording.

MATERIALS AND METHODS

Biochemicals and Reagents. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) or from Promega (Madison, WI). Recombinant *N*-glycanase was from Genzyme, alkaline phosphatase-conjugated anti-IgG antibodies were from Promega, and prestained SDS–PAGE molecular weight markers were from Bio-Rad (Richmond, CA). *N*-[1,2-^{3}H]Ethylmaleimide was obtained from New England Nuclear (Boston, MA). CHAPS was from Pierce (Rockford, IL), and PMSF, sulfopropyl-Sephadex C25, cholic acid, *n*-dodecyl D-maltoside, and endoglycosidase H were from Sigma. The detergents $C_{12}E_8$ and LDAO were from CalBiochem–Novabiochem (La Jolla, CA). Mixed soybean lipids (asolectin) were from Associated Concentrates (Woodside, NY).

All experiments employed a *Drosophila Shaker* B K^+ channel (Schwarz et al., 1988) cDNA modified in two ways. First, we exploited a 41-residue N-terminal deletion, Δ6–46, that eliminates fast N-type inactivation (Hoshi et al., 1990). Second, the channel's affinity for charybdotoxin (CTX) was increased 2000-fold by a point mutation, F425G (Goldstein & Miller, 1992, 1993). For some experiments, we also added an eight-residue epitope from human rhodopsin onto the C-terminus of the *Shaker* construct for the purpose of immunological detection. These engineered *Shaker* channels show normal, delayed rectifier-like electrophysiological behavior when expressed in *Xenopus* oocytes.

We used a mammalian expression plasmid, pMT3, a derivative of pMT2 (Sambrook et al., 1989), which carries the adenovirus major late promoter. The two cloning sites of pMT2, 5′ *Pst*I and 3′ *Eco*RI, were replaced by 5′ *Eco*RI and 3′ *Not*I. This plasmid was a generous gift from Dr. D. Oprian.

† This work was supported in part by NIH Grant GM-31768.
® Abstract published in *Advance ACS Abstracts,* August 1, 1994.

0006-2960/94/0433-9992$04.50/0

The above *Shaker* construct was inserted into pMT3 using the *Eco*RI and *Not*I sites. The *Eco*RI recognition site is followed by the 4 base pair sequence CACC immediately preceding the initiation codon.

Charybdotoxin variants were produced as fusion proteins in *Escherichia coli* and purified as described in detail (Park et al., 1991; Stampe et al., 1994). For radioactive labeling of CTX, we used the "spinster cysteine" strategy (Shimony et al., 1994) in which an unpaired cysteine is substituted at position 19, a location on the CTX molecular surface that is physically far removed from the toxin's zone of close contact with its receptor (Stampe et al., 1994; Goldstein et al., 1994). The mutant toxin (CTX-R19C, prepared and stored as a mixed disulfide with glutathione) was exposed to 0.5 mM DTT for 30 min at pH 8.3, a procedure that selectively reduces the unpaired cysteine at position 19 while maintaining the toxin's three structurally essential disulfides intact. This reduced toxin was immediately isolated on reversed-phase HPLC (Shimony et al., 1994) and reacted with [^{3}H]NEM (44 Ci/mmol) for 1 h in 0.75 M NaCl/0.4 M MOPS, pH 6.9. The NEM-labeled CTX was purified on HPLC and stored in 0.75 M NaCl, 0.5 mg/mL BSA, and 0.4 M MOPS, pH 7.0, at −20 °C. The concentration of NEM-CTX determined by scintillation counting using ^3H_2O as standard agreed within 20% with that measured by the 280-nm extinction coefficient of CTX (Park et al., 1991).

Transfection of COS Cells and Membrane Preparation. Growth and transfection of COS cells were as described for expression of visual pigments in the same system (Oprian et al., 1987), except that cells were transfected at 50% confluency. Cells were harvested 60 h after transfection by scraping into phosphate-buffered saline (PBS), 150 mM NaCl, and 10 mM NaP_i, pH 7.0. These were used immediately for intact-cell binding or were further processed for preparation of membrane fragments at 4 °C. For membrane preparation, cells were resuspended in 1 mL per 10 cm diameter plate of 150 mM KCl, 2 mM $MgCl_2$, and 5 mM EGTA, adjusted to pH 10.6 with NH_4OH. (Control membranes in which the high-pH exposure was omitted displayed similar levels of CTX receptor sites, but were much more resistant to fusion into planar lipid bilayers.) The cells were lysed by forcing the resuspension through a 25-gauge needle 4 times with vigor. The homogenate was layered on each prechilled step sucrose gradient (10 mL sample, 14 mL of 20% w/v sucrose and 14 mL of 38% w/v sucrose in 20 mM MOPS–KOH, pH 7.1) and was centrifuged in an SW28.1 rotor for 45 min at 25 000 rpm. The turbid band at the 20–38% sucrose interface was collected, diluted 4 times with cold water, and pelleted at 40 000 rpm in a Ti 50.2 rotor for 45 min. Membranes were resuspended in 250 mM sucrose/10 mM Hepes–KOH, pH 7.3. Aliquots of the membrane resuspension were quickly frozen with dry ice and stored at −70 °C.

Immunoblotting of Shaker Protein Expressed in COS Cells. COS cells were harvested as above and incubated for 1 h in PBS containing 17 mM CHAPS and 10 μg/mL PMSF. The nuclei were pelleted in a microcentrifuge for 3 min at 14 000 rpm. The sample was run on 8% SDS–PAGE gels (Laemmli, 1970) and was transferred onto nitrocellulose filter paper. Filters were preincubated 1 h at 37 °C in 5% nonfat dry milk in 0.15 M NaCl/10 mM Tris-HCl, pH 7.4, and were shaken overnight in monoclonal anti-1D4 antibody (0.05 μg/mL). After several washes in 0.15 M NaCl, 10 mM Tris-HCl, and 0.05% NP-40, pH 7.4, an alkaline phosphatase-conjugated anti-mouse antibody was added to a concentration of 0.1 μg/mL dilution for 2 h. Filters were washed as before, reacted with NBT and BCIP in 100 mM NaCl/5 mM $MgCl_2$, and rinsed with water.

[^{3}H]NEM-CTX Binding Assays. (A) Intact Cells. For a single binding assay, 1/20 plate of harvested COS cells was suspended in 100 μL of PBS containing 1 mg/mL BSA and the desired concentration of [^{3}H]NEM-CTX (0.02–30 nM). Control samples for determination of nonspecific binding also contained 1000 nM unlabeled CTX. After 30 min on ice, cells were microcentrifuged for 30 s, and the supernatant was removed for estimation of free [^{3}H]NEM-CTX. To the cell pellet was added 100 μL of 17 mM CHAPS at room temperature for 30 min, and insoluble material was pelleted as above. The supernatant was counted in 15 mL of scintillation fluid to determine the bound [^{3}H]NEM-CTX.

(B) COS Membrane Fragments. Membranes from 1/10 plate of COS cells were resuspended in 50 μL of binding buffer (50–150 mM NaCl, 10 mM Hepes–NaOH, pH 7.1, and 1 mg/mL BSA) containing [^{3}H]NEM-CTX. After 30 min incubation on ice, membranes were centrifuged in an airfuge at 100000*g* for 10 min. Supernatants were removed as above, and pellets were gently but quickly washed with 100 μL of high-salt buffer (300 mM NaCl/10 mM NaP_i, pH 7.0). Membrane pellets were then solubilized and counted as above.

(C) Detergent-Solubilized K^+ Channels. Intact COS cells or crude membranes were extracted for 1 h on ice in PBS containing the desired concentration of detergent and 0.5 mg/mL asolectin. The extract was pelleted in the airfuge as above, and [^{3}H]NEM-CTX was added to the supernatant, which contained solubilized *Shaker* channels. After a 30-min equilibration, each 50–100 μL sample was applied to a 1-mL cation-exchange column (SP-Sephadex C25) that had been equilibrated in extraction buffer and centrifuged immediately before use for ~20 s in a clinical centrifuge. After application of the sample, 300 μL of extraction buffer was added, and the column was centrifuged for 1 min in the clinical centrifuge to remove free toxin. The solution passing through the column, which contains the receptor-bound toxin, was counted for radioactivity.

(D) Displacement of [^{3}H]NEM-CTX by Unlabeled CTX. For each sample, membrane fragments containing ~100 fmol of CTX receptor sites were suspended in 100 μL of 150 mM NaCl binding buffer with [^{3}H]NEM-CTX at a total concentration 50–100-fold over its K_D. In addition, the solution contained varying concentrations of unlabeled CTX. Samples were equilibrated at room temperature 45 min, and then membranes were pelleted in the airfuge as above, except that pellets were counted without washing. Data were analyzed as relative NEM-CTX binding normalized to B_0, the value in the absence of unlabeled CTX, according to

$$\text{relative binding} = \{B_o - K_{app} - [\text{CTX}]_T + [(B_0 - K_{app} - [\text{CTX}]_T)^2 + 4K_{app}B_o]^{1/2}\}/2B_o \quad (1)$$

Here, $[\text{CTX}]_T$ is the *total* concentration of CTX present; K_{app} is the apparent dissociation constant of CTX, and is given in terms of the true dissociation constants of the two ligands: $K_{app} = K_{CTX}(1 + [\text{NEM-CTX}]/K_{\text{NEM-CTX}})$. For this analysis to be valid, NEM-CTX must be in large excess over receptor site concentration, as in these conditions.

Planar Lipid Bilayers. The planar lipid bilayer system and the method for incorporation of channel-bearing membrane vesicles have been described in detail (Miller, 1986; Miller et al., 1987). In these experiments, K^+ currents were measured with an Axopatch 200-A patch-recording amplifier, and membranes of capacitance 50–100 pF were cast from decane solutions of 14 mg/mL phosphatidylethanolamine +

6 mg/mL phosphatidylcholine (1-palmitoyl-2-oleoyl) on polystyrene partitions with holes of 100–150 μm diameter (Wonderlin et al., 1990). Recording solutions contained 10 mM Hepes–KOH (pH 7.3), 0.1 mM EDTA, and 350 KCl (*cis* chamber) or 70 mM KCl (*trans* chamber). In some cases, 200 mM NaCl was also added to the *trans* chamber after incorporation of channels. Approximately 5 μl of membrane preparation was added to the *cis* chamber with stirring until channels appeared, with the voltage held at −50 mV and periodically pulsed from −80 to −30 mV (*trans* side negative). Channels most often inserted with the cytoplasmic side exposed to the *trans* chamber, as could easily be determined by the polarity of voltage activation and the sidedness of CTX block. The physiological voltage convention is used throughout, with the external side of the channel defined as zero voltage.

After insertion of channels, a holding potential of −90 or −80 mV was applied. For episodic experiments, depolarizing command pulses were given at 3 s intervals, and currents were collected on computer with a DAP-3200e acquisition board (Microstar Laboratories) and home-written electrophysiological software. Signals were low-pass-filtered at 800 Hz and data sampled at a 700 μs interval. Conductive leakage currents were negligible (<2 pA), but the large, nonlinear capacitance of planar bilayers necessitated subtraction of double-exponential functions fit either to "blank" records containing no channel openings or to repolarizing "tail" currents. The planar bilayer capacitance limits the transient response of the acquisition system; for this reason, single reconstituted *Shaker* channels cannot be observed for the first 3–6 ms after the voltage pulse is applied.

RESULTS

Shaker Channels Expressed in COS Cells. Transient transfection of COS cells has been used for many years for heterologous protein expression. COS cells have also been used to express high levels of an integral membrane protein, rhodopsin (Oprian et al., 1987), and we have adapted this system for expression of *Shaker* K^+ channels. We use an engineered *Shaker* construct in which fast inactivation is removed by deletion of residues near the N-terminus (Hoshi et al., 1990), and which contains a point mutation, F425G, that reveals a high-affinity CTX binding site (Goldstein & Miller, 1992, 1993; Goldstein et al., 1994). In some constructs, we also added to the C-terminus the "1D4" epitope of bovine rhodopsin (Molday & MacKenzie, 1983) for convenient detection of *Shaker* protein on immunoblots. Three days after transfection with a mammalian expression plasmid carrying this K^+ channel construct (Figure 1), we routinely observe a protein band of ~90 kDa, higher than the predicted molecular mass of 72 kDa. Treatment of a detergent extract of these cells with *N*-glycanase shifts this band to ~74 kDa; endoglycosidase-H fails to alter the band mobility, as if most of the protein is glycosylated via the complex carbohydrate, rather than the higher-mannose, pathway (Kornfeld & Kornfeld, 1985). Very little nonglycosylated material is detected, but we always observe low-mobility aggregated *Shaker* protein even in these denaturing gels. The COS cells do not produce enough *Shaker* protein to observe in crude extracts by direct staining of denaturing polyacrylamide gels, in contrast to the very high expression levels seen in the baculovirus-infected Sf9 insect cell system (Klaiber et al., 1990; Li et al., 1994). Therefore, a challenge now is to assess both the amount and the functional state of the COS-expressed *Shaker* protein.

Functional Reconstitution of Shaker Channels in Planar Lipid Bilayers. While we have not been able to subject COS cells to whole-cell or patch-recording methods, we readily observe functional inactivation-removed *Shaker* channels by fusion of COS cell membrane fragments into planar lipid bilayers. *Figure* 2A displays episodic records of these reconstituted channels after fusion of COS membranes in the presence of a KCl gradient. These experiments utilize high

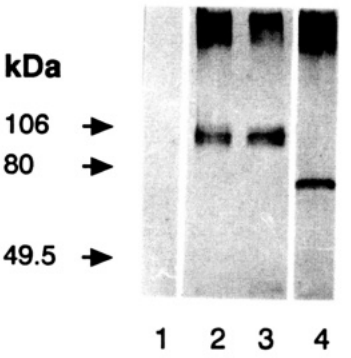

FIGURE 1: Expression of channels in transfected COS cells. Intact COS cells were extracted in CHAPS, and 20 μg of protein was run in each lane for a 1D4 immunoblot, Lane 1, control expressing *Shaker* channel without 1D4 epitope. Lane 2, *Shaker* containing 1D4 epitope. Lane 3, *Shaker–1D4* extract + endoglycosidase H (0.05 unit/mL, 18 h). Lane 4, *Shaker–1D4* extract + *N*-glycanase (100 units/mL, 18 h).

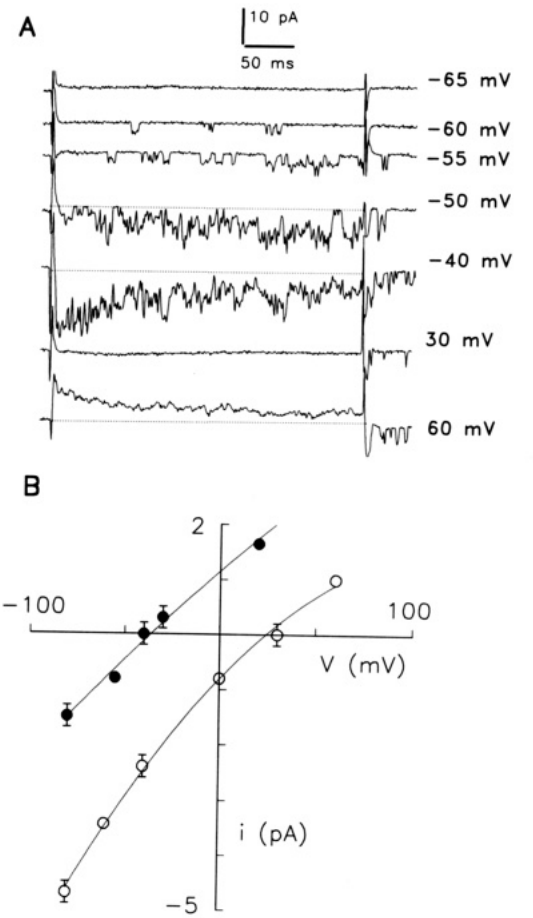

FIGURE 2: Direct observation of reconstituted *Shaker* channels in planar bilayers. COS membranes harvested 3 days after transfection with an inactivation-removed *Shaker* cDNA were inserted into planar bilayers. (A) K^+ currents elicited by 300-ms depolarizing pulses, to the indicated voltages, under "reversed-physiological" conditions (high $[K^+]$ out and low $[K^+]$ in) as described in the text. Approximately 20 channels were present in this membrane. Dashed lines, where present, mark the level of zero current. (B) Open-channel current–voltage relation, under ionic conditions of (A) (○) or with reversed ionic conditions: 350 mM KCl in, 70 mM KCl + 200 mM NaCl out (●).

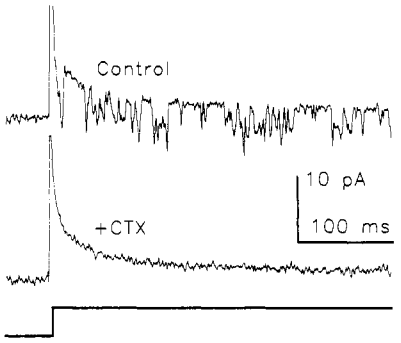

FIGURE 3: CTX inhibition of reconstituted *Shaker* channels. A package of ~10 *Shaker* channels was inserted into a planar bilayer under the conditions of Figure 2A. Voltage was changed from a holding potential of −90 mV to −50 mV where indicated. One-half second of data taken around the voltage step is displayed; with these ionic conditions, channel opening is downward. After several control pulses, CTX (400 nM) was added to the external chamber, and responses were again collected after 20 of stirring. To illustrate the quality of raw data, records are displayed without blank subtraction.

K^+ (350 mM) outside and low K^+ (70 mM) inside, so that pulses from −80 mV to test voltages negative to the equilibrium potential (~38 mV) elicit downward-going, inward current upon channel opening. The records shown were taken after a package of ~20 channels inserted into the membrane. The artifacts arising from imperfect subtraction of the nonlinear capacitance have not been removed, so as to illustrate the settling time of the planar bilayer system.

The channels display the diagnostic hallmarks of *Shaker* K^+ channels. Mild depolarization to −60 mV produces delayed, rare, single-channel openings, while stronger depolarization elicits rapidly activating "oligoscopic" currents due to numerous channels simultaneously open. The steep voltage-dependence of channel activation is apparent from the raw data-traces at low channel activation. In these relatively long (300 ms) voltage pulses, the multichannel currents, e.g., at −40 mV, can be seen to decay slowly after their initial activation, as expected for *Shaker* channels undergoing C-type inactivation (Hoshi et al., 1991). Upon return to the −80 mV holding potential, brief openings of individual channels are often observed; these represent channels returning from C-type inactivation through the open state (Demo & Yellen, 1991). The open-channel *I–V* curve (Figure 2B) yields a chord conductance of 35–40 pS at −60 mV, similar to values measured in oocyte-expressed *Shaker* channels at elevated K^+ concentrations (Heginbotham & MacKinnon, 1993). Currents reverse near 38 mV, the K^+ equilibrium potential under these KCl gradient conditions. Similar results are obtained with channels inserted in the "physiological" orientation—350 mM KCl inside, 70 mM KCl outside, with 200 mM NaCl also present outside—except that the open-channel *I–V* curve reverses with the opposite polarity, as expected for a channel that selects strongly for K^+ over Na^+. External CTX (400 nM) completely eliminates the currents, as shown in the long records of Figure 3. In control experiments using membranes from mock-transfected COS cells, we occasionally observed anion-selective channels, but never the K^+ channels described above.

A typical incorporation event contained 10–20 channels, a gross average that allows an estimate of channel expression. We assume that each incorporation event represents the fusion of a single membrane vesicle with the planar bilayer, and that the typical vesicle is a sphere of 100 nm diameter. Then, if these fusion events are representative of the whole population of membrane vesicles, the channel density in the membranes of transfected cells is 500–1000/μm^2, or about 10^6 channels per cell. While this represents a high density of channels, it is obvious that the estimate has many pitfalls embedded in its assumptions and without corroboration cannot be viewed with any confidence.

Binding of [^{3}H]NEM-CTX to COS-Expressed Shaker Channels. The above calculation highlights the awkwardness and uncertainty of electrophysiological analysis as a quantitative biochemical assay for channel density after disruption of the plasma membrane. A specific ligand would be a useful tool, and we have adapted CTX for this purpose. Many K^+ channels bind scorpion venom peptides of the CTX family with high affinity and specificity at a receptor site located in the channels's externally facing vestibule (MacKinnon & Miller, 1988; Goldstein & Miller, 1993). Detailed studies of the molecular determinants of CTX and of its receptor show that bound peptide makes close contact simultaneously with all four subunits of the tetrameric receptor (Stampe et al., 1994; Goldstein et al., 1994). Mechanistic analysis of CTX–K^+ channel interactions also shows that a critical lysine group on the bound toxin's surface protrudes slightly into the K^+-selective conduction pore of *Shaker* (Park & Miller, 1992; Goldstein & Miller, 1993). For these reasons, we expect CTX binding to provide a quantitative assay for properly assembled tetrameric channels. For many years, it has been difficult to label CTX without compromising its binding activity (Lucchesi et al., 1989), since the close-contact region of the toxin's molecular surface is filled with chemically reactive residues. Recently, however, we showed that an engineered CTX containing a "spinster cysteine" residue located far away from the interaction surface could be conveniently chemically labeled with only slight weakening of binding activity (Shimony et al., 1994). We employ here a CTX derivative labeled with [^{3}H]-*N*-ethylmaleimide to characterize and quantify COS-expressed *Shaker* channels.

Three days after transfection with *Shaker* cDNA, COS cells express a large number of CTX receptors on their surface membranes, as shown in Figure 4A, an equilibrium binding curve measured on intact cells. Toxin binding follows a Langmuir isotherm, with a K_D of 2.9 nM and maximal binding of 14 pmol/plate. Since a plate contains ~10^6 transfected cells, about 8×10^6 channels per transfected cell appear in the plasma membrane. In our experience, expression levels vary from 2 to 20 pmol/plate, but the toxin dissociation constant is always within 20% of the value shown here.

It is of interest to distinguish those channels expressed on the plasma membrane from those hiding, still functional, in the cell's internal membranes. We find that if cells are homogenized vigorously but not fractionated, the amount of toxin binding increases 1.5–2-fold over that measured on intact cells (Figure 4B). If the crude homogenate is then centrifuged onto a 38% sucrose cushion, about half of the binding sites, i.e., the amount observed in the intact cells, are recovered in the membrane fraction, and the rest pellets through the sucrose layer. We suspect that this dense material represents functional muture channels in membranes linked to cytoskeletal proteins, but we have no direct evidence for this guess. In any case, it is clear that most of the *Shaker* channels are surface-expressed 3 days after transfection.

Quantitative Features of NEM-CTX Binding. We have characterized the binding of NEM-CTX to these isolated COS membranes for comparison to the CTX–*Shaker* interaction extensively studied by electrophysiological assay in *Xenopus* oocytes (Goldstein & Miller, 1993; Goldstein et al., 1994). Figure 4C displays a binding curve on membrane fragments

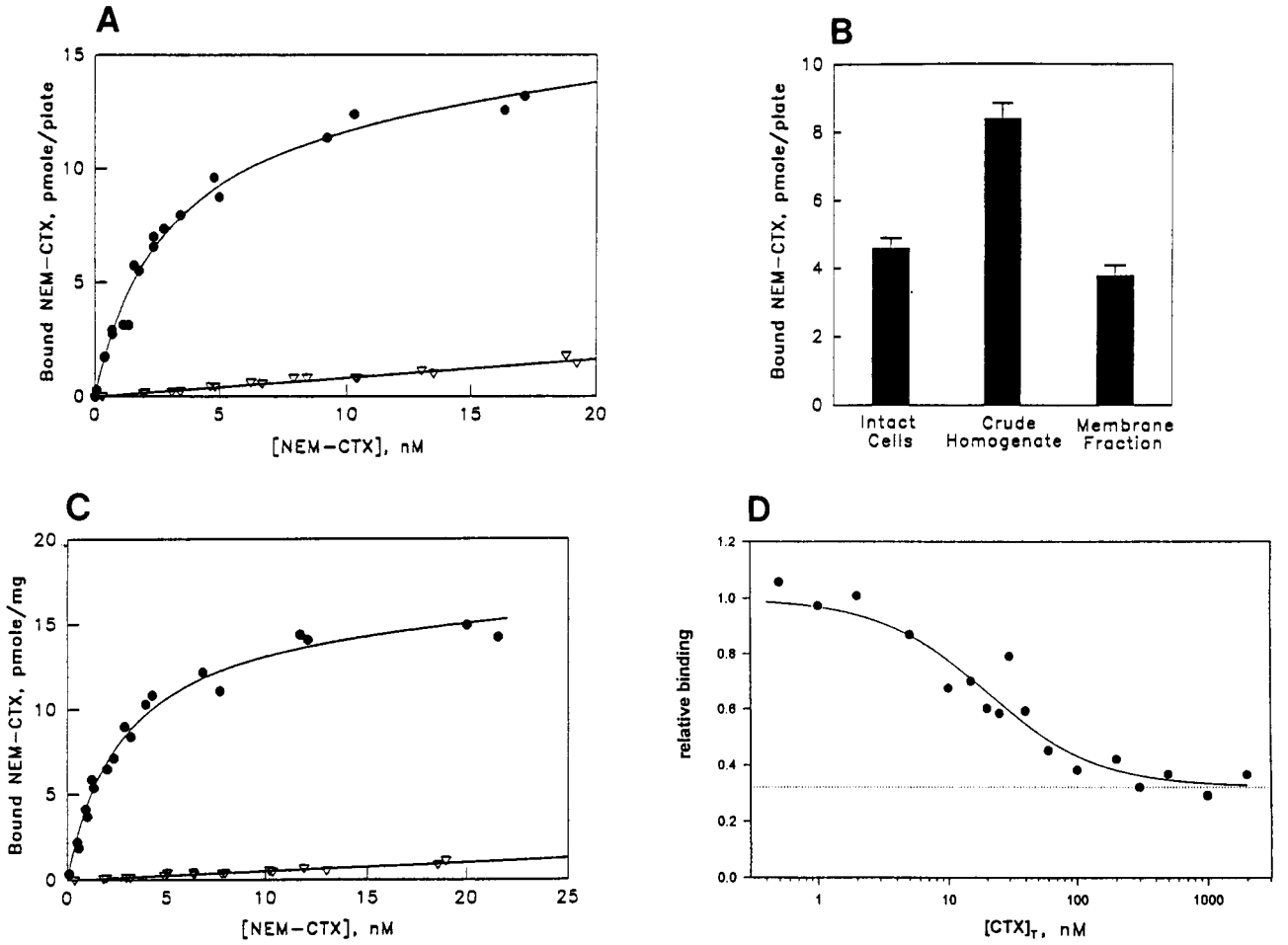

FIGURE 4: Direct binding to COS-expressed CTX receptors. [^{3}H]NEM-CTX equilibrium binding to COS-expressed *Shaker* channels was measured as described under Materials and Methods, in media containing 150 mM NaCl at 21–23 °C. (A) Binding isotherm, intact cells (filled points). The solid curve is drawn, with K_D = 2.9 nM and a maximal binding of 14 pmol/plate, according to a Langmuir isotherm, with the linear term corresponding to nonspecific binding determined directly from the data (open triangles) collected with 1 μM unlabeled CTX present. (B) Comparison of binding by intact cells, crude homogenate, and membrane fragments. Binding assays were carried out at 30 nM NEM-CTX to approximate maximal binding. Bars show means ± SE of triplicate determinations. (C) Binding isotherm, membrane fragments (filled points), as in (A) with K_D = 2.7 nM and a maximal binding of 16 pmol/mg of protein. Open triangles show nonspecific binding. (D) Displacement of [^{3}H]NEM-CTX by unlabeled CTX. The solid curve is drawn according to eq 1, with a 0.24 nM dissociation constant for CTX; the dashed line marks the fraction of nonspecific binding, 0.32 in this experiment.

under conditions similar to those used above on intact cells. The curve remains consistent with a single class of binding sites, with no indications of heterogeneity in affinity. The dissociation constant measured on the membrane fragments is identical to that found for the intact COS cell plasma membrane; the membrane fragments may be stored at −70 °C for several months without alteration of these binding characteristics.

The dissociation constant for NEM-CTX binding to COS membranes, 2.7 nM, is 9-fold higher than that measured electrophysiologically for unmodified CTX in oocytes, adjusted to the same ionic strength as in Figure 4 (Goldstein & Miller, 1993). A more pertinent comparison to make, however, is with a point mutant, CTX-R19Q, since this is isoelectric with CTX-R19C, the precursor of NEM-CTX. The corresponding dissociation constant of CTX-R19Q measured on this K^+ channel in oocytes, 0.9 nM (Goldstein et al., 1994), is within 3-fold of the COS membrane value. Moreover, pilot experiments in oocytes at 150 mM NaCl (Q. Lu and T. Sun, unpublished results) show that nonradioactive NEM-CTX blocks *Shaker* currents with K_D = 3.0 ± 0.2 nM, in excellent agreement with the direct binding measurement here. To compare further the COS and oocyte systems, we performed competition experiments to quantify the binding to COS membranes of authentic CTX (Fgiure 4D). At 150 mM NaCl, unlabeled CTX displaces NEM-CTX competitively, with K_D = 0.24 nM, a value close to that extrapolated to the same ionic strength in oocytes, ~0.3 nM (Goldstein et al., 1994).

The kinetic behavior of the toxin–channel interaction provides an additional comparison in the two expression systems. The labeled toxin dissociates from COS membranes with single-exponential kinetics (Figure 5A). The time constant at 22 °C, 6 min, compares well with the 4-min time constant found in the oocyte system for authentic CTX (Goldstein et al., 1994). Moreover, the affinity of NEM-CTX is lowered 2-fold by replacement of NaCl for KCl (Figure 5B), precisely the same effect observed in the oocyte system for a CTX point mutant (Goldstein & Miller, 1993). On the basis of these detailed characteristics, therefore, the CTX receptors in the two expression systems appear to be fundamentally similar, and by implication the COS system is competent to express with high efficiency properly assembled tetrameric *Shaker* channels.

There is, however, one striking difference between receptors expressed in the two systems: the ionic strength dependence of toxin affinity. Figure 6 compares the ionic strength dependence of the toxin dissociation constant on the same *Shaker* channel expressed in either COS cells (using NEM-CTX binding) or *Xenopus* oocytes (using a block of macroscopic currents by an isoelectric variant, CTX-R25Q). In

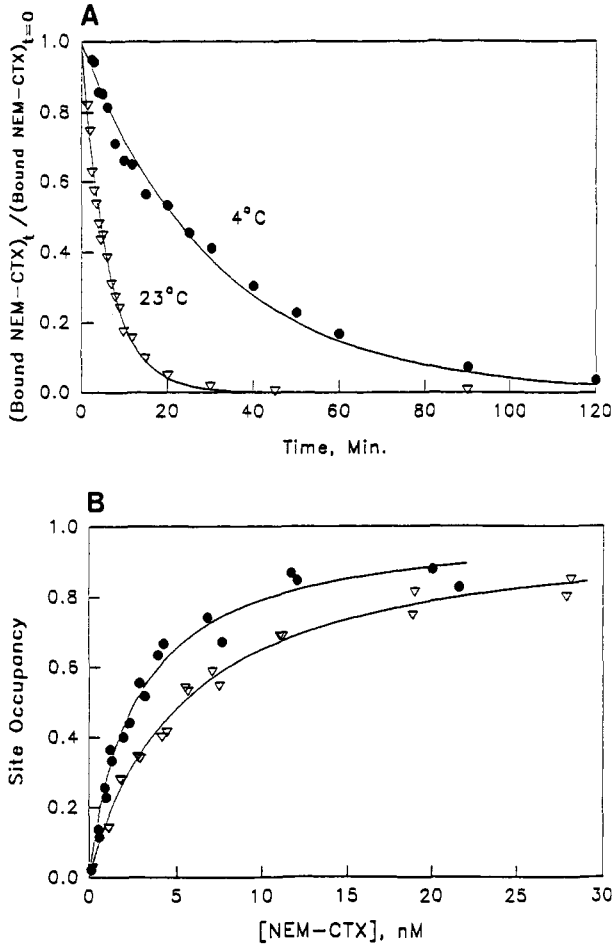

FIGURE 5: NEM-CTX dissociation kinetics and antagonism by K^+. (A) Dissociation kinetics. Membranes were equilibrated at the indicated temperatures with 20 nM NEM-CTX. To initiate NEM-CTX dissociation, 10 μM unlabeled CTX was added at zero time. Aliquots (100 μL) were taken at the indicated times and loaded onto a 1-mL SP-Sephadex column, followed by 300 μL of binding buffer. The solution was quickly blown through the column with a 10-mL syringe and counted for NEM-CTX. The separation of bound from free toxin on the column required about 10 s. (B) Antagonism of toxin binding by K^+. Normalized binding isotherms were measured on membrane fragments, with 150 mM NaCl (solid circles) or KCl (open triangles) present. Solid curves correspond to dissociation constants of 2.6 nM (NaCl) and 5.4 nM (KCl).

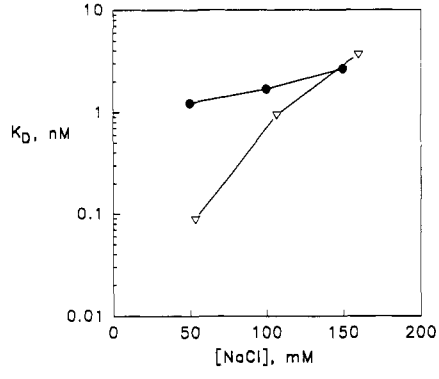

FIGURE 6: Ionic strength dependence of toxin affinity in COS cells and oocytes. NEM-CTX dissociation constants were determined from binding isotherms measured on COS membrane fragments in binding buffer containing 50, 100, or 150 mM NaCl (filled circles). For comparison, open triangles report the K_Ds of CTX-R25Q measured by block of *Shaker* currents in *Xenopus* oocytes (Goldstein & Miller, 1993).

oocytes, toxin binding is strongly dependent on ionic strength, with the affinity weakening ~30-fold as the [NaCl] is raised

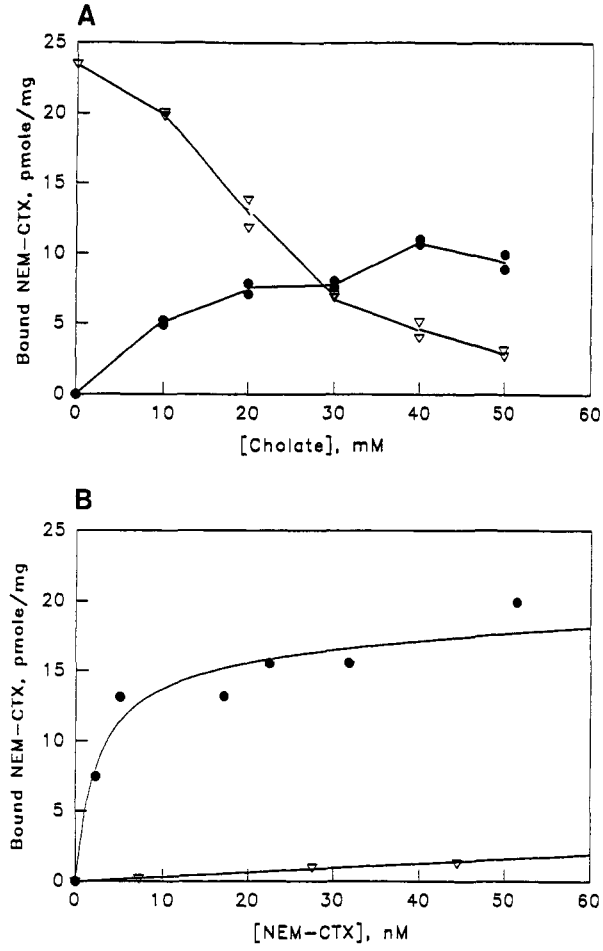

FIGURE 7: Solubilization of CTX receptors in cholate micelles. (A) Cholate extraction curve. Membranes were incubated in the indicated concentrations of cholate, and NEM-CTX binding was measured on both the detergent supernatant (filled circles) and the membrane pellet (open triangles). Bound toxin is reported relative to total protein. (B) NEM-CTX binding isotherm in cholate. Membranes were extracted in 40 mM cholate, and the binding curve was determined as described under Materials and Methods, at 0 °C. Data are reported relative to total protein before extraction.

from 50 to 150 mM. In COS membranes, however, the affinity varies by a mere factor of 2 across the same range. This strkes us as an intriguing dissimilarity suggesting a difference in local surface electrostatics near the receptor sites in the two systems.

Detergent Solubilization of Shaker Channels. In preparing for the purification of a membrane protein, it is necessary to solubilize the protein in detergent and to assess its functionality and stability in micellar solution. We use NEM-CTX binding to do this with COS-expressed *Shaker* channels. Figure 7 illustrates extraction of channels in cholate from membrane fragments, with phospholipid also present in the micellar solution. Figure 7A shows an extraction curve, while the equilibrium binding isotherm in cholate is presented in Figure 7B. Cholate at 40 mM solubilizes about 50% of *Shaker* channels from crude membranes; solubilization in cholate does not alter the NEM-CTX binding affinity. In all detergents tested, the channels deteriorate with time (Figure 8). In cholate or CHAPS, toxin binding activity is maintained satisfactorily for about 10 h at 4 °C; this is also the case for dodecyl maltoside and $C_{12}E_8$; these two detergents, however, weaken the NEM-CTX affinity over 50-fold (data not shown). The detergent LDAO is highly damaging to the channel; no NEM-CTX binding can be detected after only a hour of exposure to this nonionic detergent.

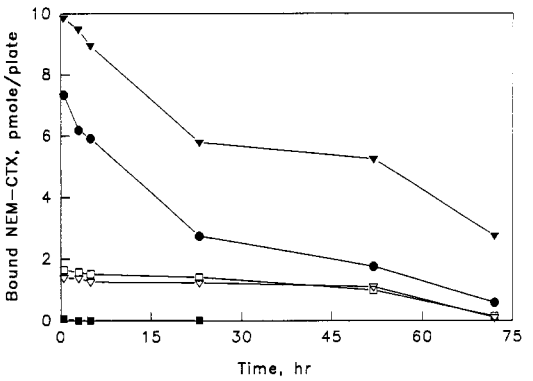

FIGURE 8: Survival of channels in various detergents. Intact COS cells were extracted in PBS containing various detergents at 4 °C, and NEM-CTX binding was assyed, using 30 nM toxin, at the indicated times. (▼) Cholate, 25 mM; (●) CHAPS, 17 mM; (□) $C_{12}E_8$, 1% (▽) dodecyl maltoside, 20 mM; (■) LDAO, 1%.

DISCUSSION

In order to comprehend the relationship between structure and function of K^+ channels, it is necessary to know structure as well as function. This is a compelling motivation for purifying large amounts of channel protein in a functionally active state. For K^+ channels, heterologous expression systems provide the only possible source of protein for this purpose, since these channels are both sparse and heterogeneous in natural tissues. No ideal expression system has been identified to date, and the COS system used here has its own individual mix of advantages and drawbacks. Nevertheless, we consider the system worth pursuing and characterizing. *Shaker* channels are reliably expressed at high levels, up to 10^7 per transfected cell. Most of the channels are found on the plasma membrane, and these appear to be properly processed and functional, as assessed by NEM-CTX binding. The level of expression of *Shaker* channels in COS cells is on the order of 10 pmol, or 3 μg, per 100 cm^2 tissue culture plate, comparable to the levels seen for human rhodopsin in the same system (Oprian et al., 1987). This raises the practical possibility of scaling the expression up to the 1–10 mg level, at which two-dimensional crystallization studies can be plausibly launched.

Before embarking on a large-scale purification effort, however, we should have confidence in the functionality of the expressed channels. We are particularly sensitive to this issue since suffering disappointment upon pursuing an initially hopeful expression of *Shaker* channel protein in the baculovirus-Sf9 system (Klaiber et al., 1990; Klaiber, 1991). In that system, a very large quantity, ~3 mg/L, of *Shaker* protein was expressed by the cells infected with *Shaker* recombinant virus; the plasma membranes of these cells clearly contained functional *Shaker* channels, as shown by whole-cell electrophysiological recording. However, we were unable to solubilize the bulk of this protein in any nondenaturing detergent, and very little of the protein was glycosylated (Klaiber, 1991; Santacruz-Toloza et al., 1994b). Most importantly, in diligent attempts to reconstitute functional channels into planar lipid bilayers, we observed only occasional *Shaker* channels upon fusion of membranes from *Shaker*-expressing Sf9 cells (P. Stampe and C. Miller, unpublished results). In those unhappy experiments, membrane fusion into lipid bilayers proceeded easily, reliably delivering into the bilayers packages of anion-selective channels, endogenous to the Sf9 cells, but the expressed *Shaker* channels appeared rarely and only singly, never in multichannel packages, as would be expected if the native membranes carried a high density of channel protein. We therefore consider the baculovirus-Sf9 system to be unsatisfactory for large-scale production of functional *Shaker* protein. It has, however, been used successfully for purification of small amounts of material of unknown functional competence (Li et al., 1994; Santacruz-Toloza et al., 1994a). In this regard, it is notable that the impressive, symmetric single-particle images recently reported for baculovirus-expressed *Shaker* channels (Li et al., 1994) were obtained with material purified, without a functional assay, either in CHAPS, which in our hands preserves CTX receptor activity, or in LDAO, which destroys it completely (Figure 8).

In our experience with K^+ channel expression, the COS system behaves in a manner strikingly different from the baculovirus-Sf9 system. The *Shaker* channels produced in COS cells are fully glycosylated and appear by available criteria to be functional. The strongest evidence for this conclusion is the reliable behavior of NEM-CTX binding activity. Though binding of this ligand is not a direct assay for electrophysiological activity, it is a strongly suggestive indicator of proper channel architecture, as is also the case of tetrodotoxin binding to Na^+ channels (Tanaka et al., 1983; Hartshorne & Catterall, 1984). A competent CTX receptor requires appropriate assembly of all four subunits of the *Shaker* homotetramer. Binding of the toxin is sensitive to even subtle structural alterations in the channel's outer vestibule (MacKinnon & Miller, 1989; MacKinnon et al., 1990; Goldstein et al., 1994), and so reproducible toxin affinity in the physiologically expected range gives us confidence that the receptor structure, which includes the outer end of the K^+ conduction pore, is intact. Moreover, the electrophysiological properties of the COS-expressed channels reconstituted into planar bilayer membranes—voltage dependnece, K^+ selectivity, and single-channel conductance—are as expected from the behavior of *Shaker* channels extensively described in intact cell membranes. Finally, the number of functional channels per cell estimated crudely from planar bilayer reconstitution is in harmony with the number of CTX receptors measured directly from NEM-CTX binding. This rough agreement argues that the channels observed by bilayer reconstitution mirror the overall population of channels in the membrane fragments, and thus further corroborates the identification of toxin binding with correct function.

Earlier mechanistic studies have paid close attention to the electrostatic interaction of the positively charged toxin with the negatively charged receptor. Nonspecific through-space electrostatic forces favor CTX binding, as is apparent from the strong dependence of toxin affinity on ionic strength for both muscle Ca^{2+}-activated K^+ channels reconstituted into planar bilayers (Anderson et al., 1988; Park & Miller, 1992) and *Shaker* channels expressed in *Xenopus* oocytes (MacKinnon & Miller, 1989; Goldstein & Miller, 1993; Escobar et al., 1993). The channels expressed in COS cells, however, present a CTX receptor which is far less sensitive to ionic strength variation, as though it is surrounded by less negative surface charge than in the oocyte system. Since the same channel construct was studied in both systems, we infer that a significant amount of negative surface charge is provided by elements other than the channel's acidic amino acid residues. CTX affinity is insensitive to membrane lipid charge (C. Miller, unpublished results), and so that difference between the two systems may lie in different glycosylation patterns; perhaps the channels in oocytes are more heavily sialidated than in the COS cells. This hypothesis is currently being tested.

There are some obvious drawbacks to the COS system as a factory for K^+ channels. First, only 10% of the cells are

transfected, and we have not been able to increase this efficiency. A huge increase in yield might therefore be achieved by rendering all cells active in protein production, as is the case, for example, in vaccinia-based channel expression systems (Leonard et al., 1990). Second, the cells demand growth in monolayer culture; this constraint will make it difficult to scale up to the milligram levels of protein required for structural studies. We are currently attempting to circumvent this problem with microcarrier techniques. Third, we do not yet have a direct assay for *nonfunctional* channels; the binding and reconstitution assays developed here detect only functional protein. The glycosylation characteristics of the channels suggest that nearly all the *Shaker* protein has been processed through the Golgi apparatus and is therefore in an advanced stage of maturity; this is not by itself an argument for functional health, but it provides grounds for optimism. With many points for possible improvements, the COS expression system is not likely to survive unchanged as a source of choice for large-scale purification of *Shaker* K^+ channels. However, the approaches and assays introduced here should be easily transferrable to future expression systems, and they set the stage for a more rational and systematic attack on purification and eventual crystallization of these K^+ channel proteins.

ACKNOWLEDGMENT

We thank Dr. Dan Oprian for the expression plasmid pMT3, and Qiang Lu for measurements of NEM-CTX block of *Shaker* channels expressed in oocytes.

REFERENCES

Anderson, C., MacKinnon, R., Smith, C., & Miller, C. (1988) *J. Gen. Physiol. 91*, 317–333.
Demo, S. D., & Yellen, G. (1991) *Neuron 7*, 743–753.
Escobar, L., Root, M. J., & MacKinnon, R. (1993) *Biochemistry 32*, 6982–6987.
Garcia-Calvo, M., Knauss, H. G., McManus, O. B., Giangiacomo, K. M., Kaczorowski, G. J., & Garcia, M. L. (1994) *J. Biol. Chem. 269*, 676–682.
Goldstein, S. A. N., & Miller, C. (1992) *Biophys. J. 62*, 5–7.
Goldstein, S. A. N., & Miller, C. (1993) *Biophys. J. 65*, 1613–1619.
Goldstein, S. A. N., Pheasant, D. J., & Miller, C. (1994) *Neuron 12*, 1377–1388.
Hartshorne, R. P. & Catterall, W. A. (1984) *J. Biol. Chem. 259*, 1667–1675.
Heginbotham, L., & MacKinnon, R. (1993) *Biophys. J. 65*, 2089–2096.
Hoshi, T., Zagotta, W. N., & Aldrich, R. W. (1990) *Science 250*, 533–538.
Hoshi, T., Zagotta, W. N., & Aldrich, R. W. (1991) *Neuron 7*, 547–556.
Klaiber, K. (1991) Ph.D. Thesis, Brandeis University, Waltham, MA.
Klaiber, K., Williams, N., Roberts, T. M., Papazian, D. M., Jan, L. Y., & Miller, C. (1990) *Neuron 5*, 221–226.
Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–634.
Kühlbrandt, W., Wang, D. N., & Fujiyoshi, Y. (1994) *Nature* (London) *367*, 614–621.
Laemmli, U. K. (1970). *Nature* (London) *227*, 680–685.
Leonard, R. J., Karschin, A., Jayashee-Aiyar, S., Davidson, N., Tanouye, M. A., Thomas, L., Thomas, G., & Lester, H. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7629–7633.
Li, M., Unwin, N., Stauffer, K. A., Jan, Y. N., & Jan, L. Y. (1994) *Curr. Biol. 4*, 110–115.
Lucchesi, K., Ravindran, A., Young, H., & Moczydlowski, E. (1989) *J. Membr. Biol. 109*, 269–281.
MacKinnon, R., & Miller, C. (1988) *J. Gen. Physiol. 91*, 335–349
MacKinnon, R., & Miller, C. (1989) *Science 245*, 1382–1385.
MacKinnon, R., Heginbotham, L., & Abramson, T. (1990) *Neuron 5*, 767–771.
Miller, C. (1986) *Ion Channel Reconstitution*, Plenum Press, New York.
Miller, C. (1991) *Science 252*, 1092–1096.
Miller, C., Latorre, R., & Reisin, I. (1987) *J. Gen. Physiol. 90*, 427–449.
Molday, R. S., & MacKenzie, D. (1983) *Biochemistry 22*, 653–660.
Oprian, D. D., Molday, R. S., Kaufman, R. J., & Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8874–8878.
Parcej, D. N., & Dolly, J. O. (1989) *Biochem. J. 257*, 899–903.
Park, C. S., & Miller, C. (1992) *Biochemistry 31*, 7749–7755.
Park, C. S., Hausdorff, S. F., & Miller, C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2046–2050.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Santacruz-Toloza, L., Huang, Y., John, S. A., & Papazian, D. M. (1994a) *Biochemistry 33*, 5607–5613
Santacruz-Toloza, L., Perozo, E., & Papazian, D. M. (1994b) *Biochemistry 33*, 1295–1299.
Schwarz, T. L., Tempel, B. L., Papazian, D. M., Jan, Y.-N., & Jan, L.-Y. (1988) *Nature* (London) *331*, 137–142.
Scott, V. E. S., Rettig, J., Parcej, D. N., Keen, J. N., Findlay, J. B. C., Pongs, O., & Dolly, J. O. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1637–1641.
Shimony, E., Sun, T., Kolmakova-Partensky, L., & Miller, C. (1994) *Protein Eng. 7*, 503–507.
Sigworth, F. J. (1993) *Q. Rev. Biophys. 27*, 1–40.
Stampe, P., Kolmakova-Partensky, L., & Miller, C. (1994) *Biochemistry 33*, 443–450.
Tanaka, J. C., Eccleston, J. F., & Barchi, R. L. (1983) *J. Biol. Chem. 258*, 7519–7526.
Wonderlin, W. F., Finkel, A., & French, R. J. (1990) *Biophys. J. 58*, 289–297.