[^{125}I]Margatoxin, an Extraordinarily High Affinity Ligand for Voltage-Gated Potassium Channels in Mammalian Brain†

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Received February 13, 1995; Revised Manuscript Received June 29, 1995⊗

ABSTRACT: Monoiodotyrosine margatoxin ([^{125}I]MgTX) specifically and reversibly labels a maximum of 0.8 pmol of sites/mg of protein in purified rat brain synaptic plasma membrane vesicles with a dissociation constant of 0.1 pM under equilibrium binding conditions. This K_d value was confirmed by kinetic experiments (K_d of 0.07 pM), competition assays employing native margatoxin (MgTX) (K_i of 0.15 pM), and receptor saturation studies (K_d of 0.18 pM). Thus, this toxin represents the highest affinity, reversible radioligand for any membrane-bound receptor or ion channel described to date. [^{125}I]MgTX binding in this system is modulated by charybdotoxin (K_i of 5 pM), kaliotoxin (K_i of 1.5 pM), and the agitoxins I and II (K_i's of 0.1 and 0.3 pM, respectively), in a noncompetitive manner. Moreover, α-dendrotoxin displayed a K_i value of 0.5 pM. Iberiotoxin was without any effect, suggesting that the receptor site is likely to be associated with a voltage-gated K^+ channel complex. [^{125}I]MgTX binding is inhibited by cations that are established blockers of voltage-dependent K^+ channels (Ba^{2+}, Ca^{2+}, Cs^+). The monovalent cations Na^+ and K^+ stimulate binding at low concentrations before producing complete inhibition as their concentrations are increased. Stimulation of binding results from an allosteric interaction that decreases K_d, whereas inhibition is due to an ionic strength effect. Affinity labeling of the binding site in rat brain synaptic plasma membranes employing [^{125}I]MgTX and the bifunctional cross-linking reagent, disuccinimidyl suberate, causes specific and covalent incorporation of toxin into a glycoprotein of an apparent molecular weight (M_r) of 74 000. Deglycosylation studies reveal an M_r for the core polypeptide of the MgTX receptor of 63 000. Immunoprecipitation studies, employing sequence-directed antibodies indicate that at least K_v1.2 and K_v1.3 are integral constituents of the rat brain MgTX receptor.

Margatoxin (MgTX),[1] a 39 amino acid peptidyl toxin, has recently been purified from venom of the new world scorpion *Centruroides margaritatus*, and its primary structure has been determined (Garcia-Calvo et al., 1993). Out of all the voltage-gated K^+ channels tested so far, K_v1.3, K_v1.5, K_v1.6, and K_v3.1, by heterologous expression of the channels in *Xenopus* oocytes, MgTX has been characterized as a potent inhibitor of the voltage-gated K^+ channel, K_v1.3 (Garcia-Calvo et al., 1993). This channel is also present in human T lymphocytes (Leonard et al., 1992). The molecular identity of MgTX was verified by chemical synthesis (Bednarek et al., 1994) and by expressing the peptide in *Escherichia coli* as part of a fusion protein, followed by cleavage and purification of the resulting product (Garcia-Calvo et al., 1993). Both synthetic and recombinant MgTX display identical properties as the native peptide (Garcia-Calvo et al., 1993; Bednarek et al., 1994).

Several peptidyl toxins purified from various scorpion venoms have been shown to interact with specific types of K^+ channels. Charybdotoxin (ChTX) is the best-studied of all these agents. Originally described as an inhibitor of the high-conductance, Ca^{2+}-activated K^+ (maxi-K) channel present in muscle and neuroendocrine cells (Miller et al., 1985), ChTX was later found to inhibit a member of the *Shaker* family of voltage-dependent K^+ channels, K_v1.3, that is expressed in neurons, and monoiodotyrosine ChTX ([^{125}I]-ChTX) has been used to characterize high-affinity binding sites in rat brain synaptic plasma membranes which, on the basis of the resulting pharmacological profile, are believed to be associated with K_v1.3. (Vázquez et al., 1990). Similar results have been obtained by monitoring [^{125}I]ChTX binding to K_v1.3 channels in human T lymphocytes (Deutsch et al., 1991). MgTX displays significant sequence homology with several previously identified peptidyl K^+ channel inhibitors [e.g., ChTX, iberiotoxin (IbTX), kaliotoxin (KTX), and the agitoxin family (AgTX); see, e.g., Gimenez-Gallego et al. (1988), Galvez et al. (1990), Romi et al. (1993), and Garcia et al. (1994)], and it was characterized as a potent inhibitor of [^{125}I]ChTX binding to rat brain synaptic membranes, although it appeared that the toxin did not behave as a strictly competitive inhibitor (Garcia-Calvo et al., 1993).

† Supported by the multidisciplinary research Grant S6611-MED and Grant P11187-MED, both from the Austrian Research Foundation, and by an APART fellowship (Austrian Program for Advanced Research and Technology) from the Austrian Academy of Sciences.

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⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1995.

[1] Abbreviations: AgTX, agitoxin; BSA, bovine serum albumin; BTP, bistrispropane; α-DTX, α-dendrotoxin; [^{125}I]ChTX, monoiodotyrosine charybdotoxin; ChTX, charybdotoxin; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; [^{125}I]MgTX, monoiodotyrosine margatoxin; IbTX, iberiotoxin; IC_{50}, concentration causing half-maximal inhibition; k_1, association rate constant; k_{-1}, dissociation rate constant; K_d, dissociation constant; K_i inhibition constant; KTX, kaliotoxin; MgTX, margatoxin; n_H, pseudo-Hill slope; NxTX, noxiustoxin; TEA, tetraethylammonium.

0006-2960/95/0434-13627$09.00/0

This study reports the reversible binding properties of monoiodotyrosine MgTX ([^{125}I]MgTX), a novel high-affinity ligand for voltage-dependent K^+ channels. Several representatives of these structurally related toxins (e.g., ChTX, KTX) have been radiolabeled (Vázquez et al., 1990; Romi et al., 1993), but a subpicomolar dissociation constant has not been reported for any of these probes. Our results indicate that [^{125}I]MgTX represents the highest affinity reversible ligand for an ion channel to be characterized to date. Due to its high affinity and specificity, [^{125}I]MgTX is expected to be a valuable tool for further investigation of the pharmacology, physiology, subunit composition, and tissue distribution of voltage-gated K^+ channels.

MATERIALS AND METHODS

Materials. MgTX was expressed in *E. coli* as part of a fusion protein and purified to homogeneity as previously described (Garcia-Calvo et al., 1993). MgTX was iodinated using procedures similar to those described for radiolabeling of ChTX (Vázquez et al., 1989). Synthetic ChTX and IbTX were purchased from Peninsula Laboratories. Recombinant AgTX I and II were a gift from Dr. Rod MacKinnon, Harvard Medical School, Boston, MA. Na^{125}I was bought from New England Nuclear Corp. Glass fiber filters (GF/C) were from Whatman. Polyethyleneimine and bovine serum albumin (BSA) were purchased from Sigma. All other reagents were obtained from commercial sources and were of the highest purity grade commercially available.

Preparation of Purified Synaptic Plasma Membrane Vesicles from Rat Brain. Rats (Wistar, 150−250 g of either sex) were killed by guillotine, and their brains were rapidly dissected. This material was placed in ice-cold homogenization buffer (320 mM sucrose, 1 mM K_2EDTA, and 10 mM TRIS/HCl, pH 7.4). Synaptosomes were prepared by a previously published method (Feigenbaum et al., 1988). Synaptic plasma membrane vesicles were purified in a sucrose gradient after osmotic lysis of the synaptosomes. These vesicles were resuspended in 50 mM TRIS/HCl, pH 7.4, flash-frozen in liquid nitrogen and stored at −80 °C. Binding activities were stable for at least 6 months.

Binding Assays. All binding assays were carried out in 12 × 75 mm or 13 × 100 mm polystyrene tubes (Sarstedt Nos. 55.467 and 55.478). The incubation medium (4−8 mL) consisted of 20 mM TRIS/HCl, pH 7.4, and 0.1% BSA (buffer A). Nonspecific binding was defined in the presence of 100−300 pM recombinant MgTX, and incubation was carried out at 22−25 °C typically for 120 min. All serial toxin dilutions were performed in a BSA-containing buffer and directly added to the incubation mixture to avoid adsorbtion phenomena. Drug dilutions were made similarly from a DMSO stock solution. A final DMSO concentration of 0.1% was never exceeded, and this concentration was without effect on binding (data not shown). At the end of incubation, the reaction mixture was rapidly filtered through Whatman GF/C glass fiber filters that had been presoaked for at least 60 min in 0.3% (w/v) polyethyleneimine, followed by 2 washes with ice-cold filtration buffer (20 mM TRIS/HCl, pH 7.4, 150 mM NaCl; 4 mL per wash). A Brandell cell harvester was used to perform the filtration procedure. Employing this protocol and at a K_d concentration of radioligand and receptor, nonspecific binding represented less than 3% of the total amount of radioactivity added, and specific binding is >95% of total binding. No displaceable binding of [^{125}I]MgTX was detected from GF/C filters under these conditions. In each experiment, duplicate or triplicate assays were routinely performed, and the data were averaged.

Cross-Linking of [^{125}I]MgTX to Rat Brain Synaptic Membranes. Rat brain synaptic plasma membrane vesicles (0.1 mg/mL) were incubated with 0.1 nM [^{125}I]MgTX in 20 mM TRIS/HCl, pH 7.4, 0.1% BSA until equilibrium was achieved (45 min at room temperature). The membranes were washed twice with ice-cold $NaPO_4^{3-}$ buffer. Disuccinimidyl suberate (DSS) dissolved in DMSO (100 mM) was added with vigorous agitation up to a final concentration of 1 mM and was reacted for 2 min on ice. Thereafter, the incubation mixture was adjusted to a final concentration of 100 mM TRIS/HCl, pH 7.4, to quench the unreacted cross-linking reagent. The membranes were collected by centrifugation (45 000*g* for 15 min), washed twice in buffer as above, resuspended in SDS−PAGE sample buffer (4%−6% SDS, 1% β-mercaptoethanol), and separated by 10% SDS−PAGE. The gels were dried and subjected to autoradiography at −80 °C for 1−2 days using Kodak XAR film.

Deglycosylation of [^{125}I]MgTX-Labeled Rat Brain Synaptic Plasma Membrane Vesicles. [^{125}I]MgTX-labeled rat brain membranes were boiled for 10 min in 0.5% SDS, 50 mM β-mercaptoethanol to denature the preparation, and then the samples were adjusted to 10 mM TRIS/HCl, pH 7.4, 1.5% Triton X-100, 5 mM β-mercaptoethanol. Recombinant *N*-glycosidase F (0.2 units) was added, and incubation proceeded for 12 h at 37 °C. The reaction was stopped by addition of boiling SDS−PAGE sample buffer. The deglycosylated samples was analyzed on a 10% SDS−PAGE gel in parallel with a control which had been incubated under identical conditions without *N*-glycosidase F present.

Solubilization of the MgTX Receptor, Antibody Production, and Immunoprecipitation Studies. Rat brain MgTX receptors were solubilized for 30 min on ice with digitonin in the presence of 500 mM KCl. All solubilization details have been published previously (Garcia-Calvo et al., 1994). Polyclonal sera were raised against unique regions of the *Shaker*-type K^+ channels $K_v1.2$ and $K_v1.3$. The sequences of the synthetic peptides employed and their locations within the primary amino acid sequence are QEG VNN SNE DFR EEN LKT AN (anti-$K_v1.2_{(461-480)}$) and DEK DYP ASP SQD VFE AAN (anti-$K_v1.3_{(211-228)}$). The amino acid numbering refers to (Stühmer et al., 1989). The sequence-directed antibodies were produced against peptides synthesized on a lysine core linked to a solid-phase peptide synthesis support. For immunization, the conjugates (≈1 μmol of peptide/immunization), emulsified in Freund's adjuvant, were injected into each of two rabbits. The procedure was repeated 1 month later, and serum collection was begun 2 weeks thereafter. Antibody production was monitored by ELISA analysis following standard protocols. The respective immunoprecipitation protocols have been published previously (Knaus et al., 1994a) except that all precipitation studies were exclusively performed in 20 mM TRIS/HCl, pH 7.4, 60 mM NaCl, 0.1% digitonin, 0.1% BSA.

Analysis of Data. The results from saturation-binding experiments were subjected to a Scatchard analysis, and linear regression was performed to obtain the equilibrium dissociation constant (K_d) and maximal receptor concentration (B_{max}). The correlation coefficient for these plots was typically >0.98. Data from competition experiments were

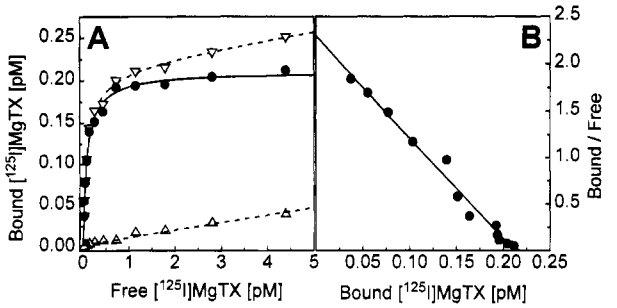

FIGURE 1: Binding of [^{125}I]MgTX to rat brain synaptic plasma membrane vesicles. (A) Saturation binding analysis. Rat brain synaptic plasma membrane vesicles (0.2 μg/mL) were incubated with increasing concentrations (0.025−4.6 pM) of [^{125}I]MgTX. The incubation medium consisted of 20 mM TRIS/HCl, pH 7.4, and 0.1% BSA. The binding reaction was carried out at 22 °C until equilibrium was achieved. Total binding (▽, dashed line) and nonspecific binding determined in the presence of 100 pM MgTX (△, dashed line) are represented. Specific binding (●, solid line) was assessed from the difference between total and nonspecific ligand binding. For this experiment, a K_d of 0.091 pM and a B_{max} value of 0.83 pmol/mg of protein were measured. (B) Analysis of [^{125}I]MgTX binding at equilibrium. Specific binding data from A are presented in the form of a Scatchard representation.

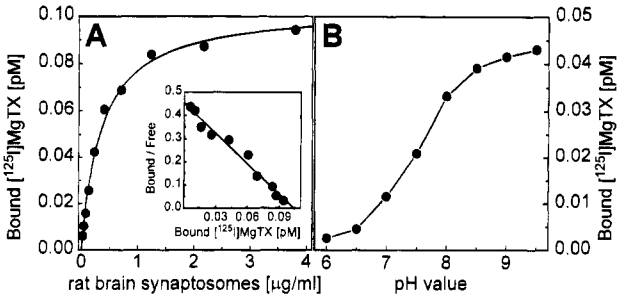

FIGURE 2: Receptor−saturation study and pH dependency of [^{125}I]-MgTX binding to rat brain synaptic plasma membrane vesicles. (A) Saturation study. Membranes (0.05−3.8 μg/mL of membrane protein) were incubation with 0.14 pM [^{125}I]MgTX until equilibrium was achieved. Specific binding data (total binding−nonspecific binding) are presented. Under these experimental conditions, a binding ability for [^{125}I]MgTX of 68% was determined. Inset, data from A are presented in the form of a Scatchard transformation, in which bound receptor (which equals bound radioligand) is plotted against the ratio of bound *versus* free receptor, a binding ability (x-intercept) of 68% and a K_d (−1/slope) of 0.18 pM were determined. (B) pH dependence. Membranes (0.08 μg/mL of membrane protein) were incubated with 0.14 pM [^{125}I]MgTX at different pH values in BTP buffer until equilibrium was achieved. Specific binding data are shown.

computer-fitted to the general dose−response equation (DeLeau et al., 1978) and then analyzed by the method published by Linden (1982) to determine K_i values. The association rate constant (k_1) was determined by employing the second-order rate equation $k_1 = [1/(L_0 - R_0)][(L_o - RL_t)/(R_0 - RL_t)]$ where L_0 is the total concentration of ligand, R_0 the total receptor concentration, and RL is the receptor−ligand complex at one given time point t. The dissociation rate constant (k_{-1}) for [^{125}I]MgTX was determined from the first-order plot of ligand dissociation *versus* time.

Protein Determination. The concentration of membrane protein was determined by the method published by Bradford (1976) using BSA as a standard.

RESULTS

Characterization of MgTX-Binding Sites in Rat Brain Synaptic Membranes. When purified rat brain synaptic plasma membrane vesicles are incubated with increasing concentrations of radiolabeled toxin until equilibrium is achieved, [^{125}I]MgTX associates with membranes in a concentration-dependent manner (Figure 1A). Repetition of this experiment in the presence of 100 pM nonradiolabeled toxin results in a pattern of [^{125}I]MgTX association which is linearly dependent on radioligand concentration. The specific binding of MgTX, defined as the difference between total radiolabeled toxin binding and binding in the presence of excess native peptide, is a saturable function of [^{125}I]-MgTX concentration. A Scatchard analysis of these data (Figure 1B) indicates the presence of a single class of binding sites with a K_d of 0.09 pM and a B_{max} of 0.8 pmol/mg of protein. These are typical parameters for the MgTX interaction, since in different membrane vesicle preparations, K_d values of 0.08−0.15 pM and corresponding B_{max} values of 0.7−1.1 pmol/mg of protein have been measured. Inclusion of 50 mM NaCl in this assay increased the K_d to 0.9 pM without exhibiting any significant effect on B_{max} values (data not shown). To confirm the extremely high binding affinity of this toxin in rat brain synaptosomal membranes, receptor saturation experiments were performed (Figure 2A). Saturating the radioligand with increasing concentrations of receptor yielded a dissociation constant for [^{125}I]MgTX of 0.18 pM. This value is in good agreement with data obtained from radioligand saturation studies. The binding ability of the radiolabeled toxin as estimated from these experiments was >70% of the total radioactivity added. At a K_d concentration (0.1 pM), [^{125}I]MgTX binding to synaptic membrane protein was linear up to a concentration of 0.3 μg of membrane protein per mL. Toxin binding was increased by increasing the pH value of the incubation medium from pH 6.0 to 9.5 (Figure 2B), thereby reaching a plateau of specific binding between pH 8.5 and 9.5. This finding presumably reflects the titration of charged residues in the external vestibule of the MgTX receptor, since it is widely accepted that this class of peptidyl toxins interacts with K^+ channels via a strong electrostatic interaction of positively charged toxin residues with negatively charged residues in the external channel mouth.

The kinetics of MgTX binding have been measured to ascertain whether the toxin interacts with its receptor site through a simple bimolecular reaction. The data shown in Figure 3A indicate that when vesicles are incubated with [^{125}I]MgTX, the toxin associates with synaptic plasma membranes in a time-dependent fashion, and equilibrium is achieved in approximately 30 min. The nonspecific binding component for MgTX, determined in the presence of excess unlabeled toxin (100 pM), is time-independent and has been subtracted from the experimental data shown in Figure 3A. A semilogarithmic transformation of these data (Figure 3A, inset) using the second-order rate equation yields a linear plot, and the slope of this representation gives k_1. The mean of all individual experiments determining the association rate constant for MgTX (n = 4), calculated as described under Materials and Methods is 1.3×10^9 M^{-1} s^{-1}. This value is at least 20-fold higher than the diffusion control rate expected for a small peptide and indicates a strong electrostatic interaction between the positively charged toxin (net charge of +6) and negative charges located on the MgTX receptor.

Dissociation at 22 °C of prebound [^{125}I]MgTX from synaptic plasma membranes, initiated by an excess of

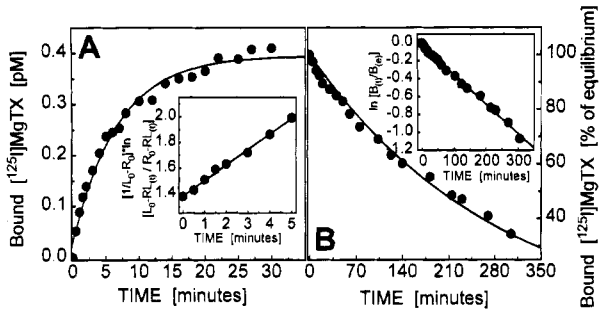

FIGURE 3: Association and dissociation kinetics of [^{125}I]MgTX binding in rat brain synaptic plasma membrane vesicles. (A) Association kinetics. Membrane vesicles were incubated with 0.8 pM [^{125}I]MgTX at 22 °C for different periods of time. Nonspecific binding determined in the presence of 100 pM MgTX is time-invariant and has been subtracted from the experimental points. An association rate constant of 1.92×10^9 M^{-1} s^{-1} was determined. Inset, a semilogarithmic representation of the second-order association reaction, calculated according to the formula described in Materials and Methods. (B) Dissociation kinetics. After incubating vesicles with 0.3 pM [^{125}I]MgTX at 22 °C for 120 min, toxin dissociation was initiated by addition of 100 pM MgTX for different periods of time. A dissociation rate constant of 5.9×10^{-5} s^{-1} was determined. Inset, a semilogarithmic representation of the first-order dissociation reaction.

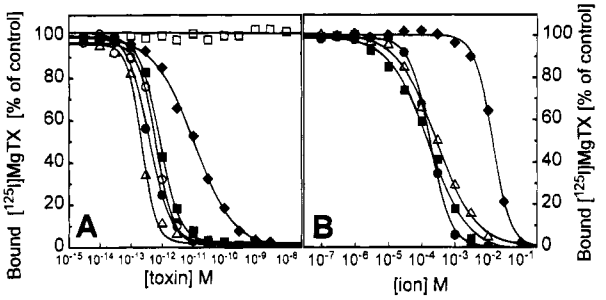

FIGURE 4: Effect of K^+ channel toxins and ions on [^{125}I]MgTX binding to rat brain synaptic plasma membrane vesicles. (A) Toxins. Membrane vesicles (0.2 μg/mL) were incubated with 0.3–0.5 pM [^{125}I]MgTX in the absence or presence of increasing concentrations of either MgTX (●, IC_{50} 0.37 pM, n_H 1.26), ChTX (◆, IC_{50} 10.65 pM, n_H 0.68), α-DTX (■, IC_{50} 0.84 pM, n_H 1.36), AgTX I (△, IC_{50} 0.22 pM, n_H 1.89), AgTX II (○, IC_{50} 0.64 pM, n_H 1.38), or IbTX (□) at 22 °C until equilibrium was achieved. Inhibition by these toxins was assessed relative to an untreated control. (B) Metal ions. The experimental conditions are as described in A. Displacement of [^{125}I]MgTX binding by increasing concentrations of $CaCl_2$ (●, IC_{50} 0.18 mM, n_H 1.41), $BaCl_2$ (■, IC_{50} 0.15 mM, n_H 0.76), CsCl (△, IC_{50} 0.27 mM, n_H 0.74), and TEA (◆, IC_{50} 14 mM, n_H 1.62) were recorded. Displacement curves were computer-fitted as described in Materials and Methods.

unlabeled toxin (100 pM), yields data that can be fit to a single exponential with a $t_{1/2}$ of ca. 190 min (Figure 3B). A semilogarithmic plot of these data generates a linear relationship as expected for a first-order reaction (Figure 3B, inset). The mean value from all individual experiments in which the dissociation rate constant for MgTX was determined is 9.1×10^{-5} s^{-1} ($n = 5$). This value is about 200-fold slower than the rate of [^{125}I]ChTX dissociation from synaptic plasma membranes (Vázquez et al., 1990). Such a finding suggests that the difference in affinities between the two toxins is due to a much higher energy barrier for MgTX that must be overcome in order for it to dissociate from its receptor. The K_d of [^{125}I]MgTX calculated from the mean of these kinetic binding measurements is 0.07 pM, a value in good agreement with that determined by saturation binding to equilibrium. These results suggest that MgTX binding is a completely reversible process that apparently occurs via a single-step reaction.

To determine whether native toxin displays a similar affinity for its receptor in brain as [^{125}I]MgTX, competition experiments were performed. Typical results are illustrated in Figure 4A, and the K_i value for MgTX has been determined from these data by employing the Linden relationship (Linden, 1982). Native MgTX completely displaces [^{125}I]MgTX from its receptor with a K_i value of 0.15 pM. Therefore, monosubstitution of MgTX with iodine does not cause any significant loss in toxin affinity. This is in contrast to results obtained with [^{125}I]ChTX, which suffered a 2- to 3-fold loss in ligand affinity in binding experiments with brain membranes upon iodination.

Pharmacological Profile of the MgTX Receptor in Brain Synaptic Membranes. To determine the pharmacological characteristics of the MgTX site in brain, various compounds that are known to interact with different types of ion channels were tested for their ability to modulate the binding reaction. The first group of agents that were studied are the structurally related toxins ChTX, KTX, and AgTX I and II. As shown in Figure 4A, all of these toxins are able to inhibit completely the binding of MgTX to brain plasma membranes in a concentration-dependent manner. The K_i values ($n = 3-5$) for these toxins are 0.15 pM (MgTX), 5 pM (ChTX), 0.8 pM (KTX), 0.08 pM (AgTX I), and 0.34 (AgTX II). The results with the agitoxins and KTX are noteworthy since, in functional studies, AgTX II is a much higher affinity blocker than AgTX I of all mammalian voltage-gated K^+ channels tested so far (Garcia et al., 1994), and KTX appears to be a selective blocker of $K_v1.3$ (Grissmer et al., 1994). α-Dendrotoxin (α-DTX), a structurally unrelated toxin that interacts with voltage-gated K^+ channels, inhibited with high affinity (K_i of 0.5 pM) binding of [^{125}I]MgTX to its receptor (Figure 4). In marked contrast, IbTX had no effect on MgTX binding in brain at concentrations up to 10 nM (Figure 4). Since all of the toxins mentioned above are known to block specifically voltage-dependent K^+ channels, whereas IbTX is a selective inhibitor of high-conductance Ca^{2+}-activated K^+ channels, the data strongly suggest that the MgTX binding site is functionally associated with a voltage-dependent K^+ channel presumably with the *Shaker*-type K^+ channels, $K_v1.2$ or $K_v1.3$. To ascertain whether ChTX, α-DTX, and the agitoxins inhibit [^{125}]MgTx binding in either a competitive or a noncompetitive fashion, the effects of these toxins were studied under equilibrium-binding conditions. As shown by the Scatchard analysis in Figure 5, ChTX, α-DTX, and AgTX II inhibit MgTX binding by decreasing receptor site density. This pattern suggests that modulation of MgTX binding to membrane-bound K^+ channels by all toxins tested occurs via a noncompetitive interaction.

A number of different metal ions such as Ba^{2+}, Ca^{2+}, and Cs^+, as well as the organic cation TEA, are known to inhibit voltage-gated K^+ channels with different degrees of potency (MacKinnon & Yellen, 1990; Heginbotham & MacKinnon, 1992). When membrane vesicles are incubated with [^{125}I]-MgTX and increasing concentrations of these metal ions, concentration-dependent inhibition of toxin-binding activity occurs with K_i values of 45, 61, and 129 μM, respectively (Figure 4B). In marked contrast, both Na^+ and K^+ produce concentration-dependent stimulation of [^{125}I]MgTX binding at low concentrations, before causing inhibition of toxin binding when concentrations in the high millimolar range

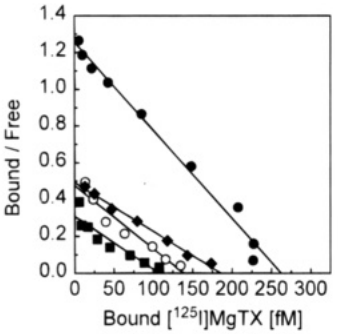

FIGURE 5: Noncompetitive interaction of [^{125}I]MgTX binding with ChTX, α-DTX, and AgTX II. Membrane vesicles were incubated with increasing concentrations of [^{125}I]MgTX (0.011−3.5 pM) in the absence (●, K_d = 0.19 pM, B_{max} = 0.88 pmol/mg of protein) or presence of 20 pM ChTX (◆, K_d = 0.42 pM, B_{max} = 0.58 pmol/mg of protein), 3 pM α-DTX (■, K_d = 0.34 pM, B_{max} = 0.35 pmol/mg of protein), or 1 pM AgTX II (○, K_d = 0.29 pM, B_{max} = 0.44 pmol/mg of protein) at 22 °C until equilibrium was achieved. Specific binding data are presented in each case in the form of a Scatchard representation.

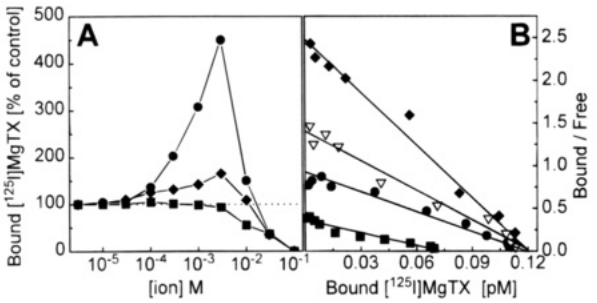

FIGURE 6: Interaction of monovalent cations with [^{125}I]MgTX binding to rat brain synaptic plasma membrane vesicles. (A) Concentration dependency. Membrane vesicles (0.1 μg/mL) were incubated with 0.15 pM [^{125}I]MgTX in the absence or presence of increasing concentrations of either NaCl (◆), KCl (●), or LiCl (■) at 22 °C until equilibrium was achieved. The effects of the monovalent cations were assessed relative to an untreated control. (B) Scatchard analyses. Membrane vesicles were incubated with increasing concentrations of [^{125}I]MgTX (0.01−2.0 pM) in the absence (●, K_d = 0.112 pM) or presence of 3 mM NaCl (▽, K_d = 0.086 pM), 3 mM KCl (◆, K_d = 0.042 pM), or 37 mM KCl (■, K_d = 0.274 pM) at 22 °C until equilibrium was achieved. A B_{max} value of 0.12 pM, corresponding to 0.74 pmol/mg of protein, was determined in all experiments, except for 37 mM KCl present (B_{max} value of 0.07 pM, corresponding to 0.51 pmol/mg of protein). Specific binding data are presented in each case in the form of a Scatchard representation.

are employed (Figure 6A). Stimulation of binding by Na^+ and K^+ is due to an increase in [^{125}I]MgTX affinity, while inhibition of binding by elevated K^+ concentration is due to a decrease in receptor density, as is demonstrated by the data presented in the Scatchard analyses of Figure 6B. Inhibition of binding is likely to be due to an ionic-strength effect since Li^+ also inhibits the toxin interaction at high concentrations but does not produce a stimulation (Figure 6A). Similar results were obtained when the effects of Na^+, K^+, and Li^+ were monitored on [^{125}I]ChTX binding in brain synaptic plasma membranes (Vázquez et al., 1990). TEA exhibits only a moderate inhibitory effect on the binding of MgTX in brain membranes (K_i = 9 mM; Figure 4B). With the exception of K_v1.1 (K_i = 0.1 mM), all other members of the *Shaker* family are much less sensitive to inhibition by TEA (Grissmer et al., 1994). The pattern of modulatory effects produced by these cations on [^{125}I]MgTX binding provides

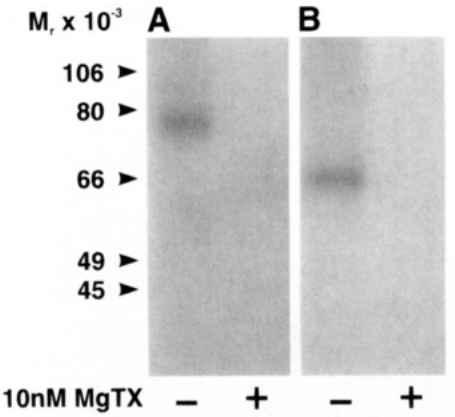

FIGURE 7: Cross-linking of [^{125}I]MgTX and enzymatic deglycosylation of the receptor in rat brain synaptic plasma membrane vesicles. (A) Cross-linking experiments were performed as described under Materials and Methods in the absence or presence of 10 nM MgTX. (B) The MgTX receptor in rat brain synaptic plasma membrane vesicles was visualized by cross-linking the radiolabeled toxin as in A. Deglycosylation employing endoglycosidase F was performed as described under Materials and Methods. Arrows indicate the positions of protein M_r standards.

additional evidence that the peptide is interacting with voltage-dependent K^+ channels.

To elucidate in greater detail the pharmacological properties of the MgTX receptor in brain, the effects of a wide variety of different ion channel modulators were monitored on the binding reaction. These agents included L-type Ca^{2+} channel blockers (isradipine, desmethoxyverapamil, and diltiazem), N-type calcium channel blockers (ω-conotoxin GVIA), Na^+ channel blockers (tetrodotoxin, saxitoxin), and blockers of other classes of K^+ channels (tolbutamide for ATP-dependent K^+ channels and aflatrem for high-conductance Ca^{2+}-activated K^+ channels; Knaus et al., 1994b). None of these agents, even when tested at high concentrations (1 μM), had any effect whatsoever on [^{125}I]MgTX binding in brain (data not shown). These results suggest that the MgTX receptor in brain synaptic plasma membranes possesses a distinct pharmacology and further supports the hypothesis that this binding site is associated with a voltage-gated K^+ channel since none of these compounds is an accepted modulator of voltage-gated K^+ channels.

Affinity labeling of the [^{125}]MgTX Receptor in Rat Brain Synaptic Membranes. Cross-linking experiments with [^{125}I]-MgTX and rat brain synaptic membranes were performed using the homobifunctional reagent, disuccinimidyl suberate (DSS), in the absence or presence of 10 nM unlabeled MgTX (Figure 7A). Under control conditions (in the absence of competing toxin), one major, although very diffuse staining polypeptide component of M_r 74 000 is identified after cross-linking with a DSS concentration of 0.5−2 mM. This M_r is calculated after subtracting the 4.5 kDa that is contributed to the mass of the cross-linked complex by labeled MgTX. The limited labeling pattern is an initial indication of the specificity of the reaction. The presence of unlabeled MgTX (10 nM) results in complete protection against incorporation of [^{125}I]MgTX into this polypeptide. Several experiments revealed an additional, although very faintly labeled polypeptide of higher M_r (85 000−90 000; data not shown). Treatment of the MgTX-cross-linked brain membrane preparation with recombinant *N*-glycosidase F causes a significant decrease in the apparent molecular weight of the labeled

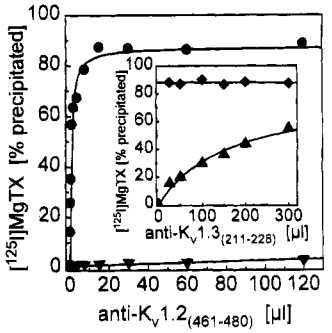

FIGURE 8: Immunoprecipitation of the digitonin-solubilized [^{125}I]-MgTX receptor from rat brain by K_v1.2- and K_v1.3-selective, sequence-directed antibodies. Digitonin-solubilized MgTX-sensitive K^+ channels (≈2.4 fmol/tube) were precipitated with increasing concentrations of anti-$K_v1.2_{(461-480)}$ (●) immobilized on protein A Sepharose. Half-maximal precipitation was achieved at 0.7 µL of crude serum. As a control, anti-$\alpha_{(913-926)}$ (▼), a sequence-directed high-titer antibody against the pore-forming subunit of the Ca^{2+}-activated K^+ channel *Slo*, was used. Inset: Precipitation of MgTX receptors by anti-$K_v1.3_{(211-228)}$ (▲) or anti-$K_v1.3_{(211-228)}$ in the presence of a saturating concentration of anti-$K_v1.2_{(461-480)}$ (20 µL) (◆).

polypeptide (Figure 7B). With the gel system employed (10% SDS−PAGE), the deglycosylated core polypeptide lined up slightly above the bovine serum albumin protein standard (M_w 66.2 kDa), which corresponds (after subtracting a molecular mass of 4.5 kDa contributed by the radiolabeled toxin) to an M_r of 63 kDa. Enzymatic deglycosylation does not alter the diffuse appearance of the MgTX receptor upon SDS−PAGE, which suggests microheterogeneity of the protein core. Considering the M_r of the MgTX receptor, the glycoprotein nature and the diffuse appearance of SDS−PAGE, these data strongly suggest that this polypeptide is a constituent of a voltage-gated K^+ channel in the mammalian brain (see Discussion).

Immunoprecipitation of the [^{125}I]MgTX Receptor in Digitonin-Solubilized Rat Brain Synaptic Plasma Membrane Vesicles. The rat brain MgTX receptor was solubilized, in functionally active form, employing the detergent digitonin. In order to ensure MgTX receptor solubilization with high yield (>70%), 500 mM potassium chloride was present during receptor extraction. Sodium chloride was not able to substitute for potassium. Despite a slight drop in binding affinity for the radiolabeled toxin (K_d = 0.6 pM), the pharmacological profile of the solubilized MgTX receptor is fully retained upon solubilization (data not shown). The soluble MgTX receptor preparation was subjected to immunoprecipitation studies employing sequence-directed antibodies directed against unique sequences of the *Shaker*-type K^+ channel subunits, K_v1.2 and K_v1.3. As a control, samples without antibody were always incubated in parallel, under identical conditions, to determine receptor-bound [^{125}I]MgTX at the end of the incubation period in order to correct for toxin dissociation during precipitation. One antibody, directed against the *Shaker*-type K^+ channel subunit (anti-$K_v1.2_{(461-480)}$) saturably precipitates 86% ± 7% (n = 4) of the brain MgTX receptor (Figure 8). This antibody recognizes the MgTX receptor in a specific manner, since less than 5% of precipitation is observed by preimmune serum, by precipitation in the presence of 3 µM of the respective immunogenic peptide, or by employing a higher-titer and precipitating antibody directed against the α subunit of the high-conductance Ca^{2+}-activated K^+ channel, *Slo*. In parallel experiments, ≈50% (n = 3) of receptor-bound [^{125}I]MgTX is precipitated, in a specific manner, by anti-$K_v1.3_{(461-480)}$). Interestingly, simultaneous precipitation employing saturating concentrations of both anti-$K_v1.2_{(461-480)}$ and anti-$K_v1.3_{(211-228)}$ yields a value not statistically different from anti-$K_v1.2_{(461-480)}$ alone (89% ± 8%). Taken together with the pharmacological profile of the binding reaction, these findings indicate that the gene product of K_v1.2 is a major component of rat brain MgTX receptors and that the K_v1.3 subunit constitutes an additional integral part to the channel complex. As indicated by the coprecipitation studies, MgTX-sensitive K^+ channels appear not to be composed of K_v1.3 without K_v1.2 also forming part of the tetrameric complex. Additional immunoprecipitation studies employing a panel of sequence-directed antibodies directed against most *Shaker*-type K^+ channel subunits, under both native conditions as well as under denaturing conditions (after chemically cross-linking [^{125}I]MgTX to one of the channel subunits), will help to clarify the complete subunit composition of the rat brain MgTX receptor.

DISCUSSION

MgTX is an Extraordinarily High-Affinity Ligand for Voltage-Gated K^+ Channels. The results presented in this manuscript demonstrate that [^{125}I]MgTX binds with very high affinity to a single class of receptor sites in rat brain. Binding of the radiolabeled toxin to these sites occurs through a simple bimolecular reaction with a K_d in the femtomolar range. The same affinity was measured using either equilibrium or kinetic binding conditions. Thus, [^{125}I]MgTX is a radioligand with the highest affinity for any membrane-bound receptor or ion channel introduced so far. The pharmacological profile of the MgTX receptor displays some features that are similar to those previously characterized for the interaction of [^{125}I]ChTX with rat brain synaptic plasma membrane vesicles (Vázquez et al., 1990). Cations such as Ba^{2+}, Ca^{2+}, and Cs^+ displace [^{125}I]MgTX binding with IC_{50} values nearly identical to those found for inhibition of [^{125}I]ChTX binding, and furthermore, Na^+ and K^+ stimulate [^{125}I]MgTX binding at low concentrations as they do [^{125}I]ChTX binding. In addition, several well-characterized toxins that are selective for voltage-dependent K^+ channels modulate MgTX binding in brain with a profile similar to their effects on ChTX binding. The finding that both KTX and α-DTX inhibit with high potency the binding reaction is quite interesting since KTX is only known to block K_v1.3 channels whereas α-DTX displays very low affinity for this channel. There is not a single member of the *Shaker* family of K^+ channels that diplays the pharmacological profile of the brain MgTX receptor. This is the first indication for a heteromultimeric composition of this receptor.

In previous studies employing [^{125}I]ChTX, the affinity of MgTX appears to have been underestimated (Garcia-Calvo et al., 1993). MgTX inhibited [^{125}I]ChTX binding with an IC_{50} value of 10−20 pM, and ChTX displayed a K_i of 8 pM. This inhibition constant for MgTX is (30−100)-fold higher than what has been determined in the present study using direct binding of [^{125}I]MgTX. In contrast, the potency of ChTX in our study was found to be almost the same (K_i = 5 pM) as in the previous study (8 pM). As one possible explanation, the data obtained using [^{125}I]ChTX binding (Garcia-Calvo et al., 1993) could simply reflect the titration

of MgTX by an excess of receptors. Supporting this notion, the apparent IC_{50} value that was determined in the previous study corresponds to the receptor concentration employed in the assay.

Molecular Components of the MgTX Receptor in Rat Brain. In the present study, an affinity-labeling approach and immunoprecipitation experiments were employed to identify molecular components of the high-affinity receptor for MgTX. Labeling of the MgTX receptor in rat brain synaptic plasma membrane vesicles using a bifunctional cross-linking reagent resulted in specific incorporation of the radioligand into a diffuse glycosylated polypeptide with an M_r of 74 000 that after enzymatic deglycosylation yielded a core protein of 63 000. This observation very much parallels findings that have been reported for the bovine and rat α-DTX receptor, where *N*-glycosidase F treatment resulted in a shift in apparent molecular mass from 78 to 65 kDa (Rehm & Lazdunski, 1988; Scott et al., 1990). Even after deglycosylation, the diffuse appearance of the MgTX receptor remained unchanged. Interestingly, diffuse immunoblot bands of comparable size and heterogeneity have also been detected using a monoclonal antibody against the DTX-binding protein (Rehm et al., 1989; Muniz et al., 1992), which consists predominately of the voltage-gated K^+ channel $K_v1.2$ (Scott et al., 1990). Moreover, sequence-directed antibodies against the gene product of $K_v1.2$ in rat brain membranes recognize a diffuse band (consisting of a heterogenous set of polypeptides) with a relative M_r of 75−85 kDa (Sheng et al., 1994). Currently, we have not determined the identity of the pore-forming K^+ channel subunit to which radiolabeled MgTX is cross-linked. But, considering the pharmacological profile of the binding reaction and our data obtained through immunoprecipitation studies under native conditions, $K_v1.2$ as well as $K_v1.3$ could serve as an acceptor for [^{125}I]MgTX in these cross-linking experiments. The apparent molecular mass of the MgTX receptor is within the M_r range as predicted for either $K_v1.2$ (498 amino acids; deduced M_w, 56.8 kDa) or $K_v1.3$ (525 amino acids; deduced M_w, 58.4 kDa) (Stühmer et al., 1989).

A Heterotetrameric K^+ Channel Complex: Basis for High-Affinity Toxin Interactions? Most pharmacological profiles of K^+ channel toxins have been determined using channels that are heterologously expressed in either *Xenopus* oocytes or mammalian cell lines. In this fashion, assays to determine toxin affinity and selectivity are conducted with K^+ channels made up exclusively of four identical subunits. In the mammalian central nervous system, however, the organization of channel subunits appears to be more complex. To date, multiple voltage-dependent K^+ channel gene families have been defined (i.e., *Shaker*, *Shaw*, *Shab*, *Shal*; Covarrubias et al., 1991) and more than 20 K^+ channel genes have been cloned and functionally expressed in heterologous expression systems [for a review see Pongs (1992)]. In mammals, K^+ channel diversity appears to occur, at least in part, from the coassembly of pore-forming subunits representing separate gene products derived from the same K^+ channel family (Christie et al., 1990; Isacoff et al., 1990; McCormack et al., 1990; Ruppersberg et al., 1990; Covarrubias et al., 1991). The application of site-directed antibodies, specific for different K^+ channel subunits (Scott et al., 1994), together with microsequencing of a purified α-DTX receptor preparation (Scott et al., 1990) and immunohistochemical experiments (Wang et al., 1993, 1994; Sheng et al., 1992, 1993; Hwang et al., 1993; Po et al., 1993) provided confirmatory evidence that distinct subunits of voltage-gated K^+ channels indeed do assemble to form heterotetrameric K^+ channels in native neuronal tissues. Our data clearly indicate that the rat brain MgTX receptor is formed, at least, by a heterotetrameric subunit assembly of $K_v1.2$ and $K_v1.3$. Such a heterotetrameric subunit assembly could provide the structural basis to explain the fact that the affinities of all toxins employed in this study are significantly higher than their reported potencies for homotetrameric K^+ channels (Grissmer et al., 1994), even when these experiments are performed under identical ionic strength conditions (data not shown). In a heterotetrameric K^+ channel complex, distinct subunits could contribute unique crucial residues to define a very high affinity receptor which could never be formed in a 4-fold symmetric homotetrameric channel.

In summary, due to its extraordinary high affinity, [^{125}I]-MgTX represents a very valuable tool for future investigations of voltage-gated K^+ channels. This probe may find utility in elucidating the functional role of voltage-gated K^+ channels in native brain tissue and in allowing purification of a distinct subset of K^+ channels through affinity chromatographic approaches. MgTX may also provide a good substrate for site-directed mutagenesis studies which can then be used to reveal those toxin residues that are reponsible for this peptide's high-affinity binding to its receptor site in mammalian brain.

ACKNOWLEDGMENT

We thank Dr. Rod MacKinnon, Harvard Medical School, Boston, MA, for providing the recombinant agitoxins. Drs. Hartmut Glossmann and Jörg Striessnig are very gratefully acknowledged for continuous support and helpful discussions, as are Emanuel Emberger for the ELISA's and Maria Trieb for Western blots in rat brain membranes.

REFERENCES

Bednarek, M. A., Bugianesi, R. M., Leonard, R. J., & Felix, J. P. (1994) *Biochem. Biophys. Res. Commun. 198*, 619.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Christie, M. J., North, R. A., Osborne, B. P., Douglass, J., & Adelman, J. P. (1990) *Neuron 2*, 405.
Covarrubias, M., Wei, A., & Salkoff, L. (1991) *Neuron 7*, 763.
DeLeau, A., Munson, P. J., & Rodbard, D. (1978) *Am. J. Physiol. 4*, E97.
Deutsch, C., Price, M., Lee, S., King, V. F., & Garcia, M. L. (1991) *J. Biol. Chem. 266*, 3668.
Feigenbaum, P., Garcia, M. L., & Kaczorowski, G. J. (1988) *Biochem. Biophys. Res. Commun. 154*, 298.
Galvez, A., Gimenez-Gallego, G., Reuben, J. P., Roy-Contancin, L., Feigenbaum, P., Kaczorowski, G. J., & Garcia, M. L. (1990) *J. Biol. Chem. 265*, 11083.
Garcia, M. L., Garcia-Calvo, M.; Hidalgo, P., Lee, A., & MacKinnon, R. (1994) *Biochemistry 33*, 6834.
Garcia-Calvo, M., Leonard, R. J., Novick, J., Stevens, S. P., Schmalhofer, W. A., Kaczorowski, G. J., & Garcia, M. L. (1993) *J. Biol. Chem. 268*, 18866.
Garcia-Calvo, M., Knaus, H. G., McManus, O. B., Giangiacomo, K. M., Kaczorowski, G. J., & Garcia, M. L. (1994) *J. Biol. Chem. 269*, 676.
Gimenez-Gallego, G., Nvia, M. A., Reuben, J. P., Katz, G. M., Kaczorowski, G. J., & Garcia, M. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3329.
Grissmer, S., Nguyen, A. N., Aiyar, J., Hanson, D. C., Mather, R. J., Gutman, G. A., Karmilowicz, M. J., Auperin, D. D., & Chandy, K. G. (1994) *Mol. Pharmacol. 45*, 1227.
Heginbotham, L., & MacKinnon, R. (1992) *Neuron 8*, 483.

Hwang, P. M., Fotuhi, M., Bredt, D. S., Cunningham, A. M., & Snyder, S. H. (1993) *J. Neurosci. 13*, 1569.
Isacoff, E. Y., Jan, Y. N., & Jan, L. Y. (1990) *Nature 345*, 530.
Knaus, H. G., Eberhart, A., Kaczorowski, G. J., & Garcia, M. L. (1994a) *J. Biol. Chem. 269*, 23336.
Knaus, H. G., McManus, O. B., Lee, S. H., Schmalhofer, W. A., Garcia-Calvo, M., Helms, L. M. H., Sanchez, M., Giangiacomo, K. M., Reuben, J. P., Smith, A. B., III, Kaczorowski, G. J., & Garcia, M. L. (1994b) *Biochemistry 33*, 5819.
Leonard, R. J., Garcia, M. L., Slaughter, R. S., & Reuben, J. P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10094.
Linden, J. (1982) *J. Cyclic Nucleotide Res. 8*, 163.
MacKinnon, R., & Yellen, G. (1990) *Science 250*, 276.
McCormack, J. W., Lin, L. E., Iverson, L. E., & Rudy, B. (1990) *Biochem. Biophys. Res. Commun. 171*, 1361.
Miller, C., Moczydlowski, E., Latorre, R., & Philips, M. (1985) *Nature 313*, 316.
Muniz, Z. M., Parcej, D. N., & Dolly, J. O. (1992) *Biochemistry 31*, 12297.
Po, S. S., Roberds, S. L., Snyders, D. J., Tamkun, M. M., & Bennett, P. B. (1993) *Circ. Res. 72*, 1326.
Pongs, O. (1992) *Physiol. Rev. 72*, S69.
Rehm, H., & Lazdunski, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4919.
Rehm, H., Newitt, R. A., & Tempel, B. L. (1989) *FEBS Lett. 249*, 224.
Romi, R., Crest, M., Gola, M., Sampieri, F., Jacquet, G., Zerrouk, H., Mansuelle, P., Sorokine, O., Van Dorsselaer, A., Rochat, H., Martin-Eauclair, M. F., & Van Rietschoten, J. (1993) *J. Biol. Chem. 268*, 26302.
Ruppersberg, J. P., Schröter, K. H., Sakmann, B., Stocker, M., Sewing, S., & Pongs, O. (1990) *Nature 345*, 535.
Scott, V. E. S., Parcej, D. N., Keen, J. N., Findlay, J. B., & Dolly, J. O. (1990) *J. Biol. Chem. 265*, 20094.
Scott, V. E. S., Muniz, Z. M., Sewing, S., Lichtinghagen, R., Parcej, D. N., Pongs, O., & Dolly, J. O. (1994) *Biochemistry 33*, 1617.
Sheng, M., Tsaur, M. L., Jan, Y. N., & Jan, L. Y. (1992) *Neuron 9*, 271.
Sheng, M., Liao, Y. J., Jan, Y. N., & Jan, L. Y. (1993) *Nature 365*, 71.
Sheng, M., Tsaur, M. L., Jan, Y. N., & Jan, L. Y. (1994) *J. Neurosci. 14*, 2408.
Stühmer, W., Ruppersberg, J. P., Schröter, K. H., Sakmann, B., Stocker, M., Giese, K. P., Perschke, A., Baumann, A., & Pongs, O. (1989) *EMBO J. 8*, 3235.
Vázquez, J., Feigenbaum, P., Katz, G., King, V. F., Reuben, J. P., Roy-Contancin, L., Slaughter, R. S., Kaczorowski, G. J., & Garcia, M. L. (1989) *J. Biol. Chem. 264*, 20902.
Vázquez, J., Feigenbaum, P., King, V. F., Kaczorowski, G. J., & Garcia, M. L. (1990) *J. Biol. Chem. 265*, 15564.
Wang, H., Kunkel, D. D., Martin, T. M., Schwartzkroin, P. A., & Tempel, B. L. (1993) *Nature 365*, 75.
Wang, H., Kunkel, D. D., Schwartzkroin, P. A., & Tempel, B. L. (1994) *J. Neurosci. 14*, 4588.

BI9503209