Characterization of the Solubilized Charybdotoxin Receptor from Bovine Aortic Smooth Muscle

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ABSTRACT: Monoiodotyrosine ([^{125}I]ChTX) binds with high affinity to a single class of receptors present in bovine aortic smooth muscle sarcolemmal membranes that are functionally associated with the high-conductance Ca^{2+}-activated K^+ channel [maxi-K channel; Vázquez, J., et al. (1989) *J. Biol. Chem. 265*, 20902–20909]. Cross-linking experiments carried out with this preparation in the presence of [^{125}I]ChTX and disuccinimidyl suberate indicate specific incorporation of radioactivity into a protein of M_r 35 000. The smooth muscle ChTX receptor can be solubilized in active form in the presence of selected detergents. Treatment of membranes with digitonin releases about 50% of the ChTX binding sites. The solubilized receptor retains the same biochemical and pharmacological properties that are characteristic of toxin interaction with membrane-bound receptors. The solubilized receptor binds specifically to wheat germ agglutinin–Sepharose resin, suggesting that it is a glycoprotein. Functional ChTX binding sites can also be solubilized in 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS). Sucrose density gradient centrifugation of either digitonin or CHAPS extracts indicates that the ChTX receptor has a high apparent sedimentation coefficient ($s_{20,w}$ = 23 and 18 S, respectively). Cross-linking experiments indicate that the appearance of the 35-kDa membrane protein correlates with ChTX binding activity after both wheat germ agglutinin–Sepharose and sucrose density gradient centrifugation steps. Given the high apparent sedimentation coefficient of the ChTX receptor, the 35-kDa membrane protein may be a subunit of a higher molecular weight complex which forms the maxi-K channel in smooth muscle sarcolemma.

High-conductance (ca. 250 pS) Ca^{2+}-activated K^+ channels (maxi-K channels)[1] are present in both electrically excitable and electrically nonexcitable cells and are thought to play a role in modulating cellular stimulus–response coupling (Peterson & Maruyama, 1984; Latorre et al., 1989). These channels, whose gating is regulated by the level of cytoplasmic Ca^{2+}, as well as by the membrane potential, are characteristically blocked by low nanomolar concentrations of charybdotoxin (ChTX; Miller et al., 1985), a 37 amino acid basic peptide isolated from venom of the scorpion *Leiurus quinquestriatus* var. *hebraeus* (Gimenez-Gallego et al., 1988; Sugg et al., 1990). ChTX has been radioiodinated, and the monoiodotyrosine adduct of ChTX ([^{125}I]ChTX) was shown to block the vascular smooth muscle maxi-K channel (Vázquez et al., 1989) with similar properties as those of native toxin. Binding studies indicate that [^{125}I]ChTX receptor sites in bovine aortic smooth muscle sarcolemma are functionally associated with maxi-K channels, making [^{125}I]ChTX the only biochemical tool so far identified for investigating the structural components of the channel protein.

The structure of the maxi-K channel has not yet been characterized. In order to approach this problem, [^{125}I]ChTX was used as a probe to determine various properties of the channel protein. The results presented in this paper indicate that the ChTX receptor can be solubilized in good yield and in functional form from bovine aortic sarcolemma using the detergent digitonin. The channel protein was then analyzed by wheat germ agglutinin–Sepharose chromatography or sucrose density gradient centrifugation. In addition, cross-linking studies identified a 35-kDa membrane protein as being part of the ChTX receptor. These data provide the first biochemical insight into the structure of the maxi-K channel. A preliminary report of these findings has been made in abstract form (Vázquez et al., 1988).

Experimental Procedures

Materials. Leiurus quinquestriatus var. *hebraeus* venom was obtained from Alomone Labs, Jerusalem, Israel, while *Buthus tamulus* venom was purchased from Sigma. Na^{125}I was obtained from Amersham Corp., while the reagents IODO-GEN, CHAPS, and disuccinimidyl suberate were supplied by Pierce Chemical Co. Digitonin (lot 1115) was purchased from Gallard–Schlesinger Chemical Manufacturing Corp. WGA–Sepharose was supplied by Pharmacia–LKB. The protein-gold reagent was from Integrated Separation Systems. All other reagents were purchased from commercial sources and were of the highest purity commercially available. Glass fiber filters (GF/C) were obtained from Whatman.

Purification and Iodination of Toxins. ChTX and iberiotoxin (IbTX) were purified to homogeneity from *Leiurus quinquestriatus* and *Buthus tamulus* venoms, respectively, as outlined previously (Gimenez-Gallego et al., 1988; Galvez et al., 1990). ChTX was iodinated using the IODO-GEN method as described (Vázquez et al., 1989). Stock solutions of

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[1] Abbreviations: maxi-K channel, high-conductance Ca^{2+}-activated K^+ channel; ChTX, charybdotoxin; [^{125}I]ChTX, monoiodotyrosine charybdotoxin; WGA, wheat germ agglutinin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IbTX, iberiotoxin; PMSF, phenylmethanesulfonyl fluoride; PEG, poly(ethylene glycol); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TEA, tetraethylammonium ion; HPLC, high-performance liquid chromatography; DTT, dithiothreitol.

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toxins were prepared for use in 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin.

Preparation of Bovine Aortic Smooth Muscle Sarcolemmal Membrane Vesicles. Highly purified sarcolemmal membrane vesicles derived from bovine aortic smooth muscle were prepared by discontinuous sucrose density gradient centrifugation of a microsomal fraction as previously outlined (Slaughter et al., 1989). Membranes were resuspended in 160 mM NaCl/20 mM Tris-HCl, pH 7.4, frozen in liquid N_2, and stored at −70 °C.

Solubilization of the ChTX Receptor. Sarcolemmal membrane vesicles, at a protein concentration of 5 mg/mL, were incubated with 1% digitonin at 37 °C for 10 min and then subjected to centrifugation at 180000*g* for 60 min. The supernatant (S_1) was removed and the pellet homogenized in the original volume with 20 mM NaCl/20 mM Tris-HCl, pH 7.4 Digitonin was added to a final concentration of 2%, and following incubation at 37 °C for 10 min, the material was clarified by centrifugation as described above. This process was repeated 4 more times. The resulting supernatants (S_{2-6}) were pooled and stored at 4 °C. All buffers used for solubilization contained 1 mM PMSF and 1 mM iodoacetamide.

For solubilization with CHAPS, membranes were incubated with 2% detergent at 37 °C for 10 min, and soluble material was separated by centrifugation as indicated above. Insoluble material was subjected to one more extraction under identical conditions.

Binding Assays. The association of [^{125}I]ChTX with bovine aortic sarcolemmal membrane vesicles was determined in the presence of 0.1% digitonin as outlined previously (Vázquez et al., 1989). Binding of radiolabeled toxin to the solubilized receptor was measured by incubating aliquots of solubilized material in 0.05% digitonin with [^{125}I]ChTX, in the absence or presence of various other test agents, at room temperature in a total volume of 200 μL. At the end of the incubation period, 10 μL of a solution of 50 mg/mL bovine γ-globulin was added, and the protein was precipitated by addition of 4 mL of ice-cold 10% (w/v) PEG (MW ~8000) in 200 mM NaCl/20 mM Tris-HCl, pH 7.4. The precipitate was immediately collected onto GF/C glass fiber filters, which were then washed twice with 4 mL of ice-cold PEG buffer. The filters had been presoaked in 0.3% poly(ethylenimine). Nonspecific binding was determined in the presence of 10 nM ChTX. In each experiment, triplicate assays were routinely performed, and the data were averaged. The standard error of the mean of these replicates was typically less than 3%.

Analysis of Data. Data from saturation binding experiments were subjected to Scatchard analysis, and linear regression was performed to obtain the equilibrium dissociation constant (K_d), and the maximum receptor concentration (B_{max}). The correlation coefficients were typically greater than 0.97. Data from competition experiments were analyzed by the method of Cheng and Prusoff (1973) to determine K_i values.

WGA–Sepharose Chromatography. Digitonin-solubilized receptor was adjusted to 200 mM in NaCl and incubated with WGA–Sepharose, batchwise, overnight at 4 °C, in the presence of protease inhibitors. The suspension was then placed in an empty column, and the fluid phase was collected until the WGA–Sepharose resin was packed. The column was washed with 5 bed volumes of 200 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.2% digitonin. Specifically bound glycoproteins were eluted with 200 mM *N*-acetyl-D-glucosamine dissolved in the above buffer, and 4-mL fractions were collected.

Sucrose Density Gradient Centrifugation. Digitonin- or CHAPS-solubilized material was layered onto a 7–25% (w/v) sucrose gradient prepared in 20 mM Tris-HCl, pH 7.4, containing either 0.1% digitonin or 0.1% CHAPS, respectively, and protease inhibitors. The gradient was subjected to centrifugation for 13 h at 34000 rpm in a Beckman SW 40 Ti rotor. The gradient was fractionated into 0.6-mL fractions, and the distribution of binding sites and protein was determined. The $s_{20,w}$ values were determined by extrapolation from the migration of the marker proteins thyroglobulin ($s_{20,w}$ = 19.2 S) and catalase ($s_{20,w}$ = 11.3 S) run in parallel.

Cross-Linking Experiments. For experiments with native membranes, sarcolemmal vesicles (0.15 mg of protein/0.6 mL) were incubated with 120 pM [^{125}I]ChTX in the absence or presence of other test agents, in 20 mM NaCl, 20 mM Hepes–NaOH, pH 7.4, 0.1% digitonin, and 0.1% bovine serum albumin, for 30 min at room temperature. Membranes were collected by centrifugation at 70000 rpm for 20 min in a Beckman TLA-100.2 rotor. Pellets were resuspended in 0.5 mL of 200 mM NaCl/10 mM TAPS–NaOH, pH 9.0, and disuccinimidyl suberate was then added to a final concentration of 0.18 mM. Samples were incubated at room temperature for 1 min, and the reaction was stoppped by addition of 500 mM Tris-HCl, pH 7.4. Membranes were collected by centrifugation, washed twice, and resuspended in SDS–PAGE sample buffer, in the absence or presence of 50 mM dithiothreitol. For cross-linking of the soluble ChTX receptor, samples were dialyzed against 20 mM TAPS–NaOH, pH 9.0, and 0.1% digitonin and were then incubated with 120 pM [^{125}I]ChTX for 30 min at room temperature. NaCl was next added to a final concentration of 100 mM, followed by the addition of 0.54 mM disuccinimidyl suberate. The cross-linking reaction was terminated by addition of SDS–PAGE sample buffer. All samples were subjected to SDS–PAGE using either 5–20% or 11% acrylamide gels. Gels were dried and exposed at −70 °C to Kodak XAR-5 film using Lightning Plus intensifying screens.

Protein Determination. Protein concentration was determined using either the amidoblack dye method (Newman et al., 1982) or the Gold method (Stoscheck, 1987), with bovine serum albumin as a standard.

Results

Identification of the ChTX Receptor by Cross-Linking. To identify the protein(s) involved in binding ChTX, cross-linking experiments were carried out with [^{125}I]ChTX and bovine aortic sarcolemmal membrane vesicles employing a variety of different reactive reagents. Neither derivatization of the toxin amino groups with *N*-succinimidyl 6-[(4-azido-2-nitrophenyl)amino]hexanoate nor coupling of toxin carboxyl groups to either 4-(glycinamido)benzophenone or 4-(cysteylamido)-benzophenone resulted in ChTX adducts which were useful as photoprobes for identifying the receptor. In the former case, the adducts were biologically inactive, while in the latter case, no coupling to the C-terminus of the toxin occurred. Therefore, the use of bifunctional cross-linking reagents was considered. For these experiments, sarcolemmal membrane vesicles were incubated with [^{125}I]ChTX in low ionic strength buffer, in the absence or presence of various test agents until equilibrium was achieved. The incubation mixture was next subjected to centrifugation, and membranes were resuspended in high ionic strength media. Disuccinimidyl suberate was added, and after 1 min, the reaction was quenched, and the samples were processed for SDS–PAGE and autoradiography.

The results of these experiments are shown in Figure 1. It is apparent that only a major protein is labeled in the presence

Table I: Solubilization of [^{125}I]ChTX Binding Sites from Bovine Aortic Sarcolemmal Membrane Vesicles[a]

	[^{125}I]ChTX binding		protein			
	pmol	%	mg	%	sp act. (pmol/mg)	x-fold purification
M	6.7	100	48	100	0.14	1.0
S_1	0.48	7	16	33	0.03	0.2
S_2	1.16	17	6.8	14	0.17	1.2
S_3	0.70	10	3.2	6.6	0.22	1.6
S_4	0.45	6.7	1.6	3.3	0.28	2.0
S_5	0.36	5.4	1.0	2.1	0.36	2.6
S_6	0.24	3.6	0.62	1.3	0.38	2.7
S_{2-6}	3.1	46	13.6	28	0.23	1.6
WGA	2.44	36	0.96	2.0	2.54	18
SG•	0.22	33[b]	0.088	1.8[b]	2.49	18

[a] Bovine aortic sarcolemmal membrane vesicles were solubilized with digitonin and fractionated by either WGA–Sepharose chromatography or sucrose density gradient centrifugation, as indicated under Experimetnal Procedures. Aliquots of each fraction were incubated with 30 pM [^{125}I]-ChTX in a medium consisting of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% digitonin, and 0.1% bovine serum albumin, at 22 °C until equilibrium was achieved. M represents the starting membrane material and S_n represents various supernatant fractions obtained after centrifugation while SG• represents fractions 3–6 of the sucrose density centrifugation gradient shown in Figure 5. [b] Values have been corrected to take into account that only 1:10 of the solubilized sample was applied to the gradient.

of [^{125}I]ChTX (lane A). This protein has an apparent molecular weight of 35K. Importantly, when increasing concentrations of native ChTX (100 pM–10 nM) are included during the binding reaction, labeling of this protein is decreased (lanes B–D). Labeling of this moiety also displays other pharmacological properties which are expected if this protein were associated with the ChTX receptor. Thus, IbTX (100 pM–10 nM; lanes E–G), 1 mM TEA (lane H), and 1 mM KCl (lane I), other known inhibitors of the ChTX binding reaction (Vázquez et al., 1989; Galvez et al., 1990), specifically prevent covalent incorporation of [^{125}I]ChTX into the 35-kDa membrane protein. Taken together, these results suggest that the 35-kDa protein identified in these studies is part of the ChTX receptor in aortic smooth muscle.

In some experiments, another membrane protein (MW ca. 95K) appears to be labeled, but at low intensity. However, it is not associated with the ChTX receptor, since such labeling is not affected by pharmacological agents that inhibit interaction of ChTX with its receptor (lanes A–I). As the electrophoresis protocol was carried out under reducing conditions (i.e., in the presence of 50 mM DTT), samples were also subjected to SDS–PAGE in the absence of reduction to determine if the 35-kDa protein is associated with distinct molecular weight component(s) by disulfide bridges. The mobility of the 35-kDa protein is not altered under these conditions (not shown), suggesting either that this protein alone forms the ChTX receptor or that other components are not linked to this portion of the ChTX receptor through disulfide bonds.

Solubilization of the ChTX Receptor from Vascular Smooth Muscle. In order to optimize conditions for solubilization of the ChTX receptor in active form from bovine aortic sarcolemma, preliminary experiments were carried out in which sarcolemmal membrane vesicles were treated at 4 °C with different detergents. The detergents selected for these experiments included digitonin, Triton X-100, CHAPS, octyl glucoside, and dodecyl β-D-maltoside. These agents were all employed at final concentrations of 1%. After separation of insoluble from soluble material by centrifugation, [^{125}I]ChTX binding was assessed in both soluble and particulate fractions. Although some, if not all, toxin binding activity disappeared from the particulate material after exposure to each of the detergents listed above, only small amounts of binding activity were observed in the soluble fraction, and only if either digitonin or CHAPS was used for solubilization.

Given these results, further experiments were carried out to improve extraction of the ChTX receptor from membranes. Increasing the detergent concentration did not increase the yield of soluble receptor at 4 °C with either digitonin or CHAPS. However, raising the temperature from 4 to 37 °C during the detergent incubation step led to a significant increase in the amount of receptor solubilized in active form. Nevertheless, under these conditions, only 5 or 7% of total receptor sites were detected in the binding assay after extraction with 1% digitonin or 1% CHAPS, respectively. Therefore, particulate material obtained after this treatment was reextracted with each of these detergents and soluble material again separated by centrifugation. Results from these experiments indicate that an additional amount of receptor could now be extracted with digitonin. In addition, increasing

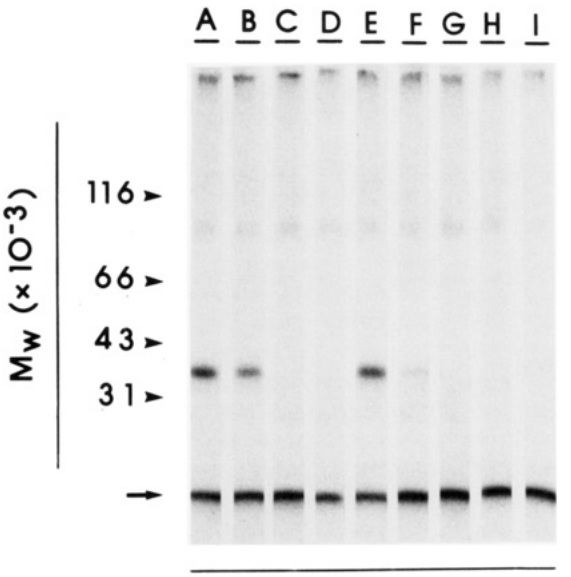

FIGURE 1: Cross-linking of [^{125}I]ChTX to bovine aortic sarcolemma membrane vesicles. Sarcolemma membrane vesicles were incubated with 120 pM [^{125}I]ChTX in a medium consisting of 20 mM NaCl, 20 mM Hepes–NaOH, pH 7.4, 0.1% digitonin, and 0.1% bovine serum albumin for 30 min at room temperature in the absence (A) or presence of either 0.1, 1.0, or 10 nM ChTX (lanes B–D), 0.1, 1.0, or 10 nM IbTX (lanes E–G), 1 mM TEA (H), or 1 mM KCl (I). Membranes were collected by centrifugation, pellets were resuspended in 200 mM NaCl/10 mM TAPS–NaOH, pH 9.0, and disuccinimidyl suberate was added at a final concentration of 0.18 mM. Samples were incubated at room temperature for 1 min, and the reaction was stopped by addition of 500 mM Tris-HCl, pH 7.4. Membranes were collected by centrifugation, washed, and resuspended in SDS–PAGE sample buffer. Samples were subjected to SDS–PAGE using 5–20% acrylamide gels. Gels were dried and exposed to Kodak XAR-5 film. The migration of molecular weight standards is shown. The arrow at the bottom of the gel indicates the dye front.

the digitonin concentration to 2% further increased the yield. On the basis of these findings, four more extractions were carried out in the presence of 2% digitonin, and results from these experiments are shown in Table I. These data are representative findings of over 30 experiments, all of which gave similar results. After these extractions, approximately 50% of the total ChTX receptor and membrane protein were solubilized. However, if the first extraction is discarded, combination of supernatants S_{2-6} yields ca. 45% of total binding sites, but only 28% of membrane protein, resulting in a 1.6-fold purification. The total yield of solubilized ChTX receptor is typical of that found for detergent extraction of other ion channel proteins (Curtis & Catterall, 1983; 1984; Hartshorne & Catterall, 1984; Borsotto et al., 1985; Black et al., 1988). The solubilized material, as demonstrated below, maintains all the binding properties previously observed for interaction of [^{125}I]ChTX with its receptor in intact membranes, and is stable at 4 °C for at least 2 weeks. Moreover, the extract can be frozen quickly in liquid N_2 and stored at −70 °C for several months without loss of activity.

In marked contrast to the results obtained with digitonin, it has not been possible to increase the yield of solubilized receptor using CHAPS. Although greater amounts of receptor appear to be solubilized than with digitonin, given the disappearance of binding activity in the particulate material, recovery of activity, as determined in a binding assay using the PEG precipitation technique, is only approximately 5–7%. One possibility to account for these findings could be loss of affinity for ChTX by receptor in the presence of CHAPS. However, saturation binding experiments with [^{125}I]ChTX, as well as competition experiments in the presence of either native toxin or TEA, indicate that the affinities of the receptor for these agents remain unchanged, with only the maximum number of sites being reduced (not shown). Thus, it is likely that recovery of receptor using the PEG precipitation technique is not quantitative in the presence of CHAPS. Attempts to separate bound from free ligand using Sephadex G-50 columns were unsuccessful due to the lack of significant retention of ChTX in these columns, which gave a poor signal to noise ratio for determining binding activity in soluble extracts. These data, taken together, suggest that a functional ChTX receptor preparation can be obtained in the presence of either digitonin or CHAPS but that quantitative recovery of the receptor on glass fiber filters can only be achieved in the presence of digitonin.

Characterization of the Solubilized ChTX Receptor. To determine whether the digitonin-solubilized ChTX receptor retains the same properties as found for interaction of toxin with binding sites in intact smooth muscle membranes, a variety of different experiments were carried out. Equilibrium binding studies were performed with varying concentrations of [^{125}I]ChTX to ascertain whether ChTX binding remains a saturable high-affinity reaction. Results of these experiments are shown in Figure 2. When soluble receptor (the pool of S_{2-6}) is incubated with increasing concentrations of [^{125}I]-ChTX, there is concentration-dependent association of toxin with PEG-precipitable material (Figure 2A). Nonspecific binding, measured in the presence of excess ChTX, is a linear function of toxin concentration. Specific binding, defined as the difference between total and nonspecific binding, is a saturable function of [^{125}I]ChTX concentration. Representation of these data in a Scatchard analysis indicates the presence of a single class of binding sites, which display a K_d of 39 pM and a B_{max} of 0.54 pmol/mg of protein (Figure 2B). This K_d value is identical to that measured for ChTX association with native membranes under identical ionic strength conditions (not shown), whereas the B_{max} is 1.6-fold higher for the soluble receptor as a result of partial purification during solubilization.

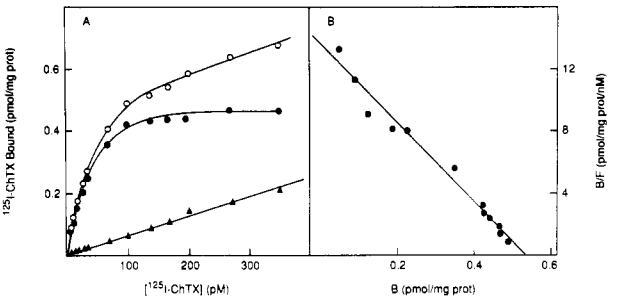

FIGURE 2: Binding of [^{125}I]ChTX to the digitonin-solubilized receptor from bovine aortic smooth muscle. (A) Saturation binding analysis. Aliquots of soluble receptor (2.6 μg) were incubated with increasing concentrations of [^{125}I]ChTX. The incubation medium consisted of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% digitonin, and 0.1% bovine serum albumin. The binding reaction was carried out at 22 °C until equilibrium was achieved. Separation of bound from free ligand was performed as described under Experimental Procedures. Total binding (○) and nonspecific binding determined in the presence of 10 nM ChTX (▲) are represented. Specific binding (●) was assessed from the difference between total and nonspecific binding. (B) Analysis of [^{125}I]ChTX binding at equilibrium. Specific binding data from (A) are presented in the form of a Scatchard representation.

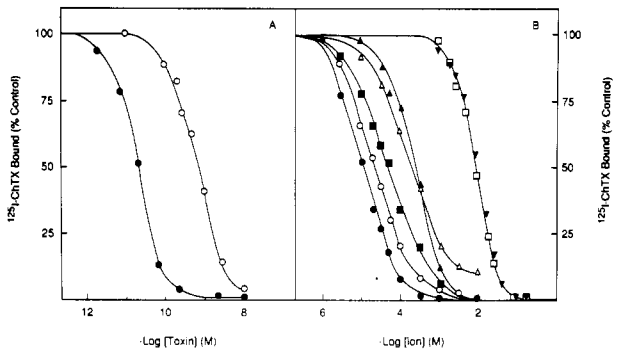

FIGURE 3: Effect of toxins and ions on [^{125}I]ChTX binding to the solubilized ChTX receptor from bovine aortic smooth muscle. Solubilized receptor was incubated with 30 pM [^{125}I]ChTX in a medium consisting of 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% digitonin, and 0.1% bovine serum albumin at 22 °C until equilibrium was achieved: (A) in the absence or presence of increasing concentrations of either ChTX (●) or IbTX (○); (B) in the absence or presence of either $BaCl_2$ (●), KCl (○), CsCl (■), TEA (△), or $CaCl_2$ (▲). In (B), the effects of NaCl (▼) and LiCl (□) were determined by incubating solubilized receptor with 30 pM [^{125}I]ChTX in 20 mM Tris-HCl, pH 7.4, 0.1% digitonin, and 0.1% bovine serum albumin, in the absence or presence of increasing concentrations of either ion. Specific binding data in each case are presented relative to an untreated control.

Competition experiments were also carried out which monitored binding of [^{125}I]ChTX to soluble receptor in the presence of various agents known to affect the binding reaction in membranes. Both native ChTX and IbTX inhibit [^{125}I]-ChTX association to soluble receptor with K_i values of 10 and 458 pM, respectively (Figure 3A). These values are very similar to those previously measured using membrane preparations (Vázquez et al., 1989; Galvez et al., 1990). In addition to toxins, metal ions such as Cs^+, Ba^{2+}, Ca^{2+}, and K^+, as well as the organic cation TEA, are known to bind with high affinity to distinct sites along the ion conduction pathway of maxi-K channels and block ChTX binding to smooth muscle sarcolemma (Vergara & Latorre, 1983; Miller, 1987; Cecchi et al., 1987; Neyton & Miller, 1988a,b; Villarroel et al., 1988; Vázquez et al., 1989). This characteristic behavior is also

manifested with the soluble receptor, where these ions all inhibit toxin binding activity in a concentration-dependent fashion (Figure 3B), displaying equivalent potencies as those previously found with membranes (Vázquez et al., 1989). Another characteristic of toxin interaction with membranes is the dependence of the binding reaction on the ionic strength of the incubation medium. At 10–100 mM concentration, many monovalent cations inhibit ChTX binding in a similar fashion with a very steep concentration dependence (Vázquez et al., 1989). The mechanism by which monovalent cations block toxin binding has been explained based on the way in which ChTX inhibits maxi-K channels, as an effect on the electrostatic interaction between toxin and receptor that is weakened as the ionic strength of the medium is increased. As expected for a fully functional ChTX receptor, the binding reaction in the solubilized preparation displays the same ionic strength dependency as previously observed in membranes (Figure 3B). All these data suggest that the ChTX receptor from vascular smooth muscle has been solubilized in high yield and that the soluble receptor displays the same properties as those characteristic of binding sites in intact membranes.

Wheat Germ Agglutinin–Sepharose Chromatography. In an effort to determine whether the ChTX receptor is a glycoprotein, the digitonin-solubilized preparation was incubated with WGA–Sepharose. Analysis of unbound material contained in the eluent indicates that although 90% of the protein is present, most of the ChTX binding sites are retained on the column (i.e., >80%). Elution of the column with 200 mM *N*-acetyl-D-glucosamine, in the presence of 200 mM NaCl, allows recovery of the receptor (Figure 4A). This procedure results in approximately 18-fold purification of the receptor and suggests that the ChTX receptor is a glycoprotein (Table I). Saturation and competition experiments carried out with material eluted from WGA–Sepharose indicate that the binding properties of this preparation are unchanged from those of the membrane-bound receptor (not shown). Cross-linking experiments carried out with the different fractions obtained from this column, [^{125}I]ChTX, and disuccinimidyl suberate indicate that specific incorporation of radioactivity takes place into a 35-kDa membrane protein that is present in the material eluted from WGA–Sepharose (Figure 4B). No incorporation of radioactivity was observed with unbound material.

Sucrose Density Gradient Sedimentation. The solubilized ChTX receptor in digitonin medium was sedimented through a linear 7–25% sucrose gradient to get some estimate of the molecular size of the protein. After centrifugation, fractions were collected from the gradient, and these were assayed for toxin binding activity and protein concentration. The results are displayed in Figure 5. Specific binding is observed in the region of the gradient which corresponds to an apparent sedimentation coefficient of 23 S. This was determined by extrapolation from the migration of catalase (11.3 S) and thyroglobulin (19.2 S) in parallel gradients, and assumes a linear relationship along the entire gradient. Recovery of activity from the sucrose gradient is essentially quantitative, and a purification factor of ca. 20-fold is achieved (Table I). From a sedimentation coefficient of 23 S, the molecular weight of the receptor and associated digitonin micellar complex can be calculated to be 800K. Since digitonin may contribute as much as 50% to the weight of the solubilized protein–detergent complex, the sucrose density gradient sedimentation step was repeated with receptor solubilized in CHAPS. Results from these experiments indicate that specific toxin binding migrates with an apparent sedimentation coefficient of 18 S (not shown), as determined from the position of both catalase and thyroglobulin in a parallel gradient. Thus, the molecular weight calculated under these conditions (550 K) is smaller than the one calculated with the digitonin-solubilized receptor. In order to determine if ChTX binding activity correlates with migration of the 35-kDa membrane protein, fractions obtained from across the gradient were subjected to cross-linking protocols with [^{125}I]ChTX, followed by SDS–PAGE. Results of these experiments are depicted in Figure 5, and indicate that specific labeling of the 35-kDa membrane protein follows the same profile that is seen for ChTX binding activity. No incorporation of radioactivity was observed in other gradient fractions.

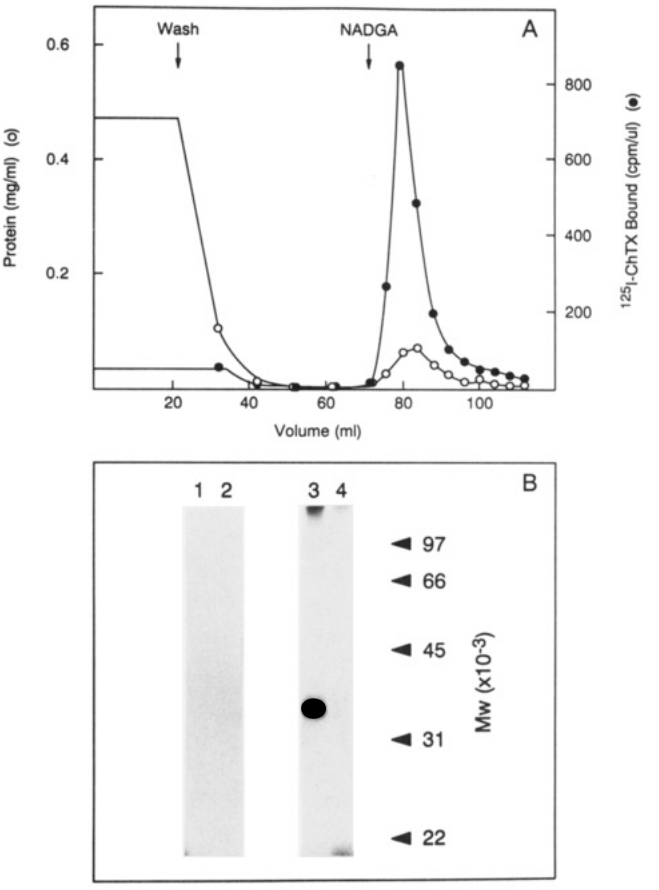

FIGURE 4: Chromatography of the solubilized ChTX receptor on WGA–Sepharose. (A) Elution profile. The S_{2-6} pool of digitonin-solubilized receptor (20 mL) was incubated batchwise with 10 mL of WGA–Sepharose overnight at 4 °C, in the presence of protease inhibitors. The suspension was placed in an empty column, and fractions were collected until the resin was packed. The column was washed with 5 bed volumes of 200 mM NaCl, 20 mM Tris-HCl, pH 7.4 and 0.2% digitonin. Specifically bound glycoproteins were then eluted with 200 mM *N*-acetyl-D-glucosamine (NADGA, arrow) prepared in the above buffer, and 4-mL fractions were collected for determination of [^{125}I]ChTX binding activity (●) and protein concentration (O). (B) Cross-linking pattern with [^{125}I]ChTX. Fractions (ca. 4 μg of protein) corresponding to the pool of unbound material (two left lanes) or NADGA-eluted material (two right lanes) were subjected to cross-linking with [^{125}I]ChTX as described under Experimental Procedures, followed by SDS–PAGE. Lanes 1 and 3 represent cross-linking with radioactive toxin alone, while in lanes 2 and 4, the procedure was repeated in the presence of 10 nM unlabeled ChTX. The migration of molecular weight standards is shown.

Discussion

The results presented in this study demonstrate for the first time that high-affinity binding sites for ChTX can be solubilized in functional form from bovine aortic sarcolemmal

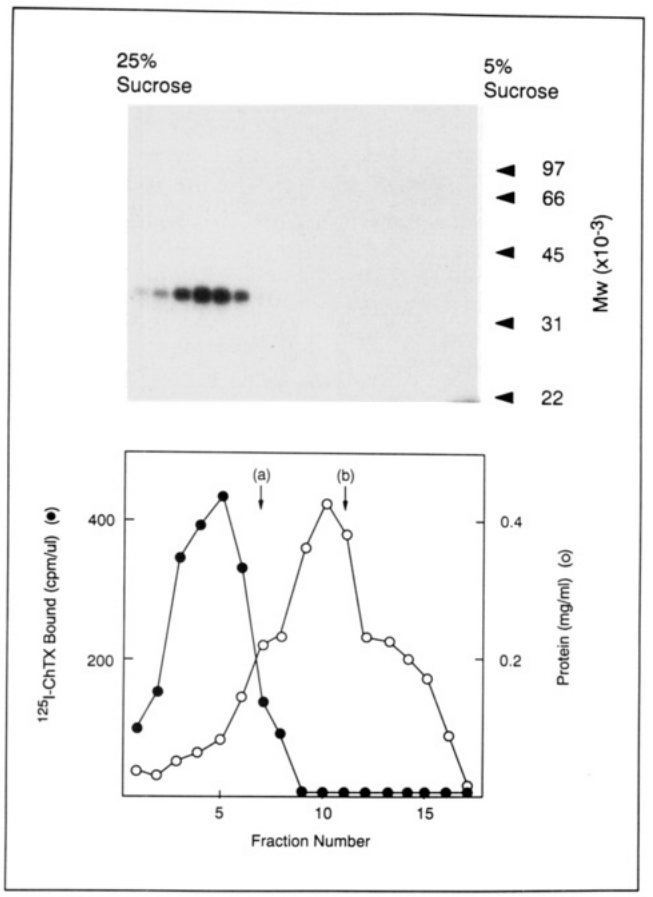

FIGURE 5: Sucrose density gradient centrifugation of the solubilized ChTX receptor. Two milliliters of the S_{2-6} pool of digitonin-solubilized receptor was concentrated to 0.4 mL in a UNISEL Ultracen-30 ultrafilter (Bio-Rad) and layered onto a 10-mL linear sucrose gradient (7–25%) in 20 mM Tris-HCl, pH 7.4, containing 0.1% digitonin and protease inhibitors. The gradient was subjected to centrifugation for 13 h at 34 000 rpm in a Beckman SW 40Ti rotor. Fractions of 0.6 mL were collected for determination of either [^{125}I[ChTX binding activity (●), protein concentration (○), or cross-linking patterns with [^{125}I]ChTX. Migration of the protein standards thyroglobulin (a) and catalase (b) in the sucrose density gradient is indicated by arrows. The cross-linked material was subjected to SDS–PAGE, and the migration of molecular weight standards is shown. Fractions are numbered from the bottom to the top of the gradient.

vesicles in the presence of digitonin. These receptors are believed to be directly associated with the maxi-K channel in smooth muscle. The solubilized ChTX binding sites are very stable, and can be further fractionated either on a WGA–Sepharose column or by sucrose density gradient centrifugation, leading to partial purification of the receptor. In addition, sucrose density gradient centrifugation of soluble receptor gives an $s_{20,w}$ value of 23 S, suggesting that a functional ChTX binding site complex must exist as a very high molecular weight particle. Since cross-linking experiments have identified a protein that is associated with the ChTX receptor which possesses an apparent molecular weight upon SDS–PAGE analysis of 35K, this protein probably represents a subunit of a larger multicomponent complex. Perhaps functional receptor results from association of several of these subunits, as has been proposed with other types of K^+ channels (Christie et al., 1990; Isacoff et al., 1990; McCormack et al., 1990; Ruppersberg et al., 1990).

The solubilization procedure with digitonin involves six consecutive steps in which particulate material is reextracted with detergent at elevated temperature to achieve maximal release of binding sites from the membrane. In this way, approximately 50% of the receptors initially present in membranes can be recovered in completely functional form. This is shown by saturation experiments with the solubilized receptor preparation which demonstrate the presence of a single class of [^{125}I]ChTX binding sites that display a K_d of 39 pM, a value identical to the one measured in native sarcolemmal membranes. Since some preferential extraction of the receptor takes place under the conditions described, a 1.6-fold enrichment in specific activity is observed. The yield of solubilized ChTX receptor is typical of that observed for other membrane-bound proteins, in particular other receptors/ion channels (Curtis & Catterall, 1983, 1984; Hartshorne & Catterall, 1984; Borsotto et al., 1985; Black et al., 1988). Further evidence that digitonin-solubilized receptor possesses the same properties as membrane-bound receptor is provided by the observation that several different modulators of the binding reaction display identical activity in both preparations. These agents include the following: IbTX, an inhibitory peptide 68% homologous with ChTX which has been shown to interact at a distinct site from that of ChTX on maxi-K channels (Galvez et al., 1990); TEA, a relatively high-affinity inhibitor of both ChTX binding and maxi-K channel activity (Villarroel et al., 1988); and several different metallic cations which are known to bind at unique sites located along the ion conduction pathway of the maxi-K channel (Vergara & Latorre, 1983; Miller, 1987; Cecchi et al., 1987; Neyton & Miller, 1988a,b). In addition, monovalent cations, such as Na^+ and Li^+, which produce inhibition of toxin binding in the millimolar concentration range by a mechanism related to relief of the electrostatic interaction between the highly positively charged ChTX and negatively charged residues located in the mouth of the channel (Anderson et al., 1988; MacKinnon & Miller, 1989; MacKinnon et al., 1989), are also active. Since ionic strength effects on toxin binding are preserved in the soluble preparation, these data, together with the other characteristics described above, suggest that not only the ChTX receptor site but also the general protein environment are intact in the digitonin-solubilized preparation.

Perhaps one unique property of the solubilized ChTX receptor is its stability. No apparent loss of activity has been detected after several weeks of storage at 4 °C. In addition, the soluble receptor can be frozen in liquid N_2 and stored at −70 °C for prolonged periods of time. Although proteolytic enzyme inhibitors were routinely present throughout the experiments described herein, identical results are obtained in their absence. The extreme stability of the ChTX binding site is an important consideration in designing a purification scheme using conventional chromatographic techniques, which, given the low specific activity of the starting material, could involve multiple steps and considerable time.

In marked contrast to the results obtained with digitonin, when CHAPS was used as a means of solubilizing the ChTX receptor, low recoveries of activity were routinely noted. Although both disappearance of binding sites from the particulate material and maintenance of toxin affinity in the soluble material were observed, recovery of activity using GF/C filters and the PEG precipitation technique is low. The reason for these findings is unknown. These data, however, indicate that CHAPS is not a useful detergent with which to attempt purification of the ChTX receptor using the currently available assay techniques.

Similar to several other ion channel proteins that have been purified (Curtis & Catterall, 1984; Hartshorne & Catterall, 1984; Cooper et al., 1987; Nakayama et al., 1987; Rehm & Lazdunski, 1988), the ChTX receptor can be specifically

bound to WGA–Sepharose resin and then eluted in functional form in the presence of *N*-acetyl-D-glucosamine. During this procedure, a partial purification of about 15-fold occurs. This result suggests that the receptor is a glycoprotein which could contain either *N*-acetylglucosamine and/or sialic acid residues. As was found with the crude solubilized receptor, WGA–Sepharose-eluted material retains appropriate biochemical properties, and can be stored for prolonged periods of time without any apparent loss of activity.

The apparent $s_{20,w}$ values of the digitonin- and CHAPS-solubilized ChTX receptor are 23 and 18 S, respectively. These values suggest that the functional ChTX receptor exists as a high molecular weight complex. It is known that digitonin can bind in a 1:1 ratio with protein (Tanford & Reynolds, 1976; Hjelmeland & Chrambach, 1984). Thus, as much as 50% of the weight of the solubilized receptor complex could be due to the presence of this detergent. It is also possible that the receptor could aggregate with other proteins of very high molecular weight, and not separate under the conditions of density gradient centrifugation. Since radiation inactivation measurements have not as yet been performed with the ChTX binding site, this technique could be useful in gaining some insight into the molecular mass of the receptor complex.

Although sucrose density gradient centrifugation studies suggest a large molecular mass for the ChTX receptor, cross-linking protocols with [^{125}I]ChTX, disuccinimidyl suberate, and either aortic sarcolemmal membranes, WGA–Sepharose chromatography fractions, or sucrose density gradient centrifugation fractions indicate that covalent incorporation of radioactivity occurs specifically into a protein that upon SDS–PAGE displays an apparent molecular weight of only 35 K. Labeling of this protein strictly correlates with ChTX binding activity and is prevented by agents which specifically inhibit the ChTX binding reaction, suggesting that it is indeed functionally associated with the receptor complex. Since the mobility of the protein is not altered by use of reducing agents, these results, together with data obtained from sucrose density gradient centrifugation, suggest either that the 35-kDa membrane protein is part of a larger complex or that a functional receptor is made up of multiple copies of the 35-kDa subunit, the members of which are not linked by disulfide bonds. Evidence that different classes of K^+ channels exist as multisubunit complexes has been presented (Christie et al., 1990; Isacoff et al., 1990; McCormack et al., 1990; Ruppersberg et al., 1990). Although the molecular weight of the protein identified in the present study is smaller than other K^+ channel proteins which have been characterized, it is possible that this species could contain all the structural information required to form a channel. Recent studies have shown that significant deletions can be made in one of these K^+ channels (drK1) without significant alteration in the function of the protein (VanDongen et al., 1990). What minimum requirements are necessary for channel function remain to be determined. Recently, a different voltage-dependent K^+ channel with the properties of a delayed rectifier has been cloned by expression from rat kidney, heart, and uterus using a *Xenopus* oocyte system (Takumi et al., 1988; Folander et al., 1990; Pragnell et al., 1990). The resulting cDNA codes for a protein of only 130 amino acids in length, with a single putative transmembrane domain (MW = 14.7 K). It remains to be determined whether this protein forms the channel itself or regulates the activity of an endogenous K^+ channel present in oocytes.

During the last few years, molecular biology approaches have moved rapidly toward the cloning of several different types of voltage-dependent K^+ channels. These K^+ channels have been identified because they share homology with the channel responsible for the "Shaker" phenotype in *Drosophila*, as well as related cloned proteins. Other types of channels, such as the maxi-K and ATP-sensitive K^+ channels, have resisted cloning via these approaches. In addition, expression of maxi-K channels from total mRNA injected into *Xenopus* oocytes has not been straightforward, with only two laboratories reporting some success (Ashcroft et al., 1988; Lu et al., 1990). It has previously been reported that ChTX will block the *Drosophila* Shaker K^+ channel and, therefore, there may be homology in the ChTX binding site of the maxi-K and Shaker related sequences (MacKinnon et al., 1988). However, a homogeneous preparation of ChTX has now been found not to inhibit the Shaker K^+ channel (Oliva et al., 1991). Hence, the idea of using low-stringency hybridization with Shaker probes to identify the maxi-K channel based on similarities in a common ChTX binding site could be misleading. It is possible that the maxi-K channel represents a new family of K^+ channels lacking significant structural homology with those other proteins already cloned. In this respect, the results presented in the present study represent an alternative approach to elucidating the structure of the maxi-K channel, namely, the purification of this protein in order to obtain amino acid sequence information from which to clone the protein.

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Registry No. ChTX, 95751-30-7; IbTX, 129203-60-7.

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New Joint Prediction Algorithm (Q_7-JASEP) Improves the Prediction of Protein Secondary Structure

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ABSTRACT: The classical problem of secondary structure prediction is approached by a new joint algorithm (Q_7-JASEP) that combines the best aspects of six different methods. The algorithm includes the statistical methods of Chou-Fasman, Nagano, and Burgess-Ponnuswamy-Scheraga, the homology method of Nishikawa, the information theory method of Garnier-Osgurthope-Robson, and the artificial neural network approach of Qian-Sejnowski. Steps in the algorithm are (i) optimizing each individual method with respect to its correlation coefficient (Q_7) for assigning a structural type from the predictive score of the method, (ii) weighting each method, (iii) combining the scores from different methods, and (iv) comparing the scores for α-helix, β-strand, and coil conformational states to assign the secondary structure at each residue position. The present application to 45 globular proteins demonstrates good predictive power in cross-validation testing (with average correlation coefficients per test protein of $Q_{7,\alpha} = 0.41$, $Q_{7,\beta} = 0.47$, $Q_{7,c} = 0.41$ for α-helix, β-strand, and coil conformations). By the criterion of correlation coefficient (Q_7) for each type of secondary structure, Q_7-JASEP performs better than any of the component methods. When all protein classes are included for training and testing (by cross-validation), the results here equal the best in the literature, by the Q_7 criterion. More generally, the basic algorithm can be applied to any protein class and to any type of structure/sequence or function/sequence correlation for which multiple predictive methods exist.

Prediction of secondary and tertiary structures of a globular protein from the amino acid sequence remains a major unsolved problem in biology. This is the case despite considerable progress in several key areas, including energy minimization [e.g., Gibson and Scheraga (1986)], molecular dynamics [e.g., Karplus and McCammon (1983), Karplus and Weaver (1976), Levitt and Meirovitch (1983), and Rooman and Wodak (1988)], development of simplified protein potentials [e.g.,