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Purification and reconstitution of potassium channel proteins from squid axon membranes

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Voltage-dependent K^+ channels are responsible for repolarization of the cell membrane during the late phase of the action potential. Here we report the purification of proteins from squid axon membranes which bind the K^+-channel blocker noxiustoxin (NTX), and their subsequent functional reconstitution in planar bilayers. The NXT-affinity purified proteins had M_r values of 60000 ± 6000, 160000 ± 15000 and 220000 ± 20000. Their incorporation into bilayers resulted in single-channel currents with three conductances, the most frequent one of 11 pS in 300/100 mM KCl (*cis/trans*). The voltage dependence, reversal potential and bursting behavior suggest that these are the K^+ channels involved in the squid axon action potential.

Ion channel reconstitution; Squid axon; Delayed rectifier; Planar bilayer

1. INTRODUCTION

The squid axon delayed rectifier responsible for the repolarization of the cell membrane during the late phase of the action potential was the first K^+ channel described [1,2]. However, its purification, as well as the purification of other types of K^+ channels, which are the most diversified [18], has not been achieved due to the lack of high affinity compounds. Previously, it was shown that noxiustoxin (NTX), a 4.3 kDa peptide toxin obtained from the venom of the Mexican scorpion *Centruroides noxius*, is a potent blocker of the delayed rectifier in the squid axon [4]. In the present work NTX was used to purify NTX-binding proteins from a detergent extract of squid axon membranes. The purified proteins were reconstituted in a functional form into planar lipid bilayers.

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2. MATERIALS AND METHODS

2.1. *Solubilization of membranes and affinity column*

Batches of approximately 10 g of squid axons were routinely used to prepare the rich membrane fraction I, as described [6]. The membranes from fraction I were solubilized in a buffer containing the following composition: 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in 10 mM Hepes-Na buffer, pH 7.4, 150 mM NaCl, 2.5 $CaCl_2$, 0.02% phosphatidylcholine, plus the following protease inhibitors: phenylmethylsulfonyl fluoride (0.1 mM), iodoacetamide (1 mM), 1:10 phenanthroline (1 mM) and pepstatin A (1 μM). The preparation was incubated for 1 h at 4°C and spinned down (60000 × *g*) for 20 min. The soluble material (3.2 mg in 6 ml) was incubated with 5 ml of NTX-affinity beads (capacity: 1 mg of NTX/5 ml resin) for 1 h at 25°C and loaded into the column. The column was washed with 30 ml of buffer A (same as solubilization buffer, but decreasing CHAPS concentration to 0.1%) and the bound protein was eluted with buffer A containing 1 mM 4-aminopyridine (4-AP). Approximately 30 μg of protein was recovered.

2.2. *Binding of [^{125}I]NTX to crude axonal membranes*

Membranes of squid axons obtained by centrifugation in 0.33 M sucrose, after disruption (Polytron) and homogenization (teflon pestle), display a B_{max} of 0.81 pmol/mg protein when assayed with 60 nM iodinated NTX. Incubation was performed in 5 mM Tris-HCl buffer, pH 8.0, in artificial sea

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water. Membranes were incubated 50 min at 25°C and free [^{125}I]NTX separated by centrifugation.

2.3. *Cross-linking of [^{125}I]NTX to purified proteins*

Protein eluted from the affinity column with 4-AP was dialyzed and incubated for 20 min with 300 nM [^{125}I]NTX, either in the absence or the presence of 20 μM cold NTX. After incubation, the protein content of each tube was cross-linked by the addition of disuccinimidyl suberimidate (final concentration 1 mM) in dimethylsulfoxide. Samples were run in the SDS-PAGE system as described above, stained, dried and placed in the Kodak holder with appropriate films for autoradiography.

2.4. *Planar lipid bilayers*

Bilayers were made according to the Mueller-Rudin [13] technique using diphytanoylphosphatidylcholine (20 mg of lipid per ml of *n*-decane, Avanti). They were formed over a 250 μm diameter aperture in a 50 μm thick plastic septum that separated two 400 μl teflon chambers. The hole was pretreated with the same lipid solution used for bilayer formation and allowed to dry under a N_2 stream. A patch-clamp amplifier (Biologic RK-300) was used to record the current and to control the voltage across the bilayer. Ag/AgCl electrodes in series with 1 M KCl agar bridges were used. The voltage was applied to the *cis* side of the bilayer and the *trans* side was held at virtual ground. In all the records, channel opening is shown as an upward deflection if the applied potential is positive. The membrane currents were recorded on a modified digital audio processor connected to a VCR [17], and the data analyzed off line. Ion channel reconstitution was performed using the fusion strategy [14], as follows: either liposomes containing the purified receptor, or the purified soluble receptor (< 1 μg/ml), were added to the *cis* side compartment. Liposomes were formed by dialysing the purified protein at 4°C for 3 days against 50 mM KCl, Hepes-K 10 mM, pH 7.4. Thereafter, they were sonicated for 2 min in the presence of 0.6 M sucrose. Experiments were performed at 20 ± 2°C.

3. RESULTS AND DISCUSSION

Axon membranes from the squid *Loligo vulgaris* were used in this study since NTX, a toxin purified from the Mexican scorpion *Centruroides noxius*, selectively affects their delayed rectifier [4,5]. After isolating axonal membranes as described in section 2, they were solubilized in a buffer containing 2% CHAPS. The solubilized material was incubated with NTX-Affigel P-10 beads and the protein bound to the NTX-Affigel P-10 (~30 μg) was eluted from the column with 1 mM of 4-AP and 0.1% of CHAPS. Assuming that all the recovered protein corresponds to channel proteins, the maximum purification in this step would be 100-fold (see fig.1). This purification procedure was repeated three times with comparable results.

Gel electrophoresis of purified NTX-receptor showed mainly four peptides (lane 2, fig.1). Two strong bands with apparent M_r values of 53 ± 3 and 60 ± 6, and two additional faint bands corresponding to higher M_r forms. The protein pattern obtained with the purified preparation is in good agreement with data obtained by cross-linking the purified material with [^{125}I]NTX (lane 3, fig.1). The NTX binding sites are found in proteins with M_r of 66 ± 6 (broad doublet), 160 ± 15 and 220 ± 20. The 66 kDa band protein is within the same range as that proposed for the K^+ channel of the *Shaker* locus of *Drosophila* [8] and those of the mouse and rat brain K^+ channels [9–11]. As shown in lane 4, fig.1, the lower molecular mass

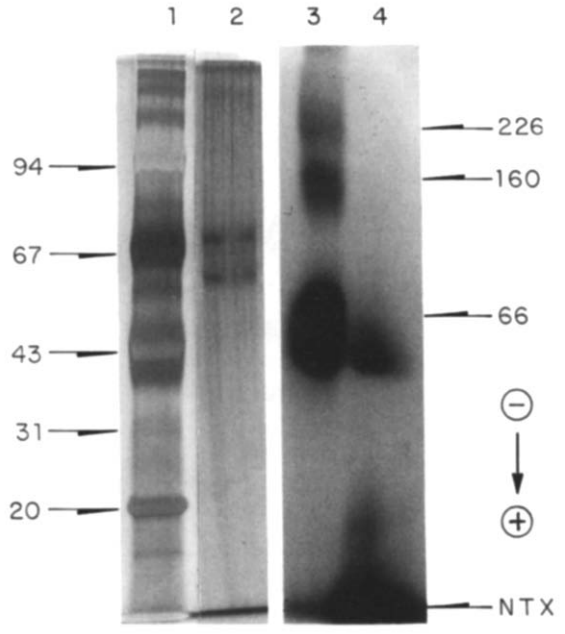

Fig.1. Electrophoresis of purified NTX-binding proteins. Lanes 1 and 2 are silver stained SDS/polyacrylamide (10%) gel electrophoresis (PAGE) [7]. Lane 1 shows molecular weight markers (10 μg each; in kDa): phosphorylase b (94); bovine serum albumin (67); ovalbumin (43); carbonic anhydrase (31); trypsin inhibitor (20). Lane 2 shows the proteins (2 μg) eluted with 4-AP from the NTX-affinity column. Lanes 3 and 4 are autoradiographic pictures of the proteins (0.5 μg) eluted from the affinity column and cross-linked with [^{125}I]NTX ran on a 4–12% gradient SDS-PAGE, thus their electrophoretic mobility is different from lanes 1 and 2. Lane 4 is the same as 3 but with a 75-fold excess cold-NTX added prior to cross-linkage. A parallel lane (not shown) was used to run molecular weight markers (200 kDa myosin, 116 kDa beta-galactosidase, and others similar to lane 1). Arrows on the right indicate approximate M_r of radiolabeled proteins.

peptide (53 kDa) binds NTX non-specifically. The presence of four bands before and after NTX cross-linking, clearly shows that the 160 and 220 kDa polypeptides are not a polymerization artifact of the 66 kDa polypeptide; however, their M_r values (in lane 3) are overestimated due to the covalent attachment of NTX molecule(s). These results show the presence of three distinct peptides in squid axon membranes, which might correspond to three different types of NTX-sensitive K^+ channels. In fact, single-channel currents from patch-clamp of the inside surface of cut-open squid axons, revealed three distinct types of K^+-channel activity [12].

The protein recovered from the affinity column with 4-AP was extensively dialyzed against buffer and used for reconstitution experiments. Fusion with planar lipid bilayers, of either purified soluble receptor or liposomes containing purified receptor gave similar results, indicating that ion channels are present in the purified fractions. Fig.2a shows a long time record of single-channel current fluctuations across a planar membrane following addition of purified receptor protein ($<1\ \mu l\ ml^{-1}$) to the *cis* side. In the presence of a 5-fold KCl concentration gradient, at an applied membrane voltage of 100 mV, the channels display a bursting behavior: grouping in time of opening events separated by relatively long segments of inactivity [2,15]. The mean current of the predominant channel was 1.5 pA (fig.2b), corresponding to a chord conductance of 11 pS (at 100 mV). This value is similar to that of 9–11 pS reported for K^+ channels of squid axon by Conti and Neher [2] and for the smaller K^+ channel reported by Llano et al. [12] in cut-open squid axon. A single-channel conductance of 15 pS was also reported for the delayed rectifier K^+ channels from skeletal muscle [16]. The single-channel conductances have been expressed as chord conductances, $\gamma = i/(E_m - E_k)$, where E_m is the voltage applied to the membrane and E_k is the K^+ reversal potential, because the

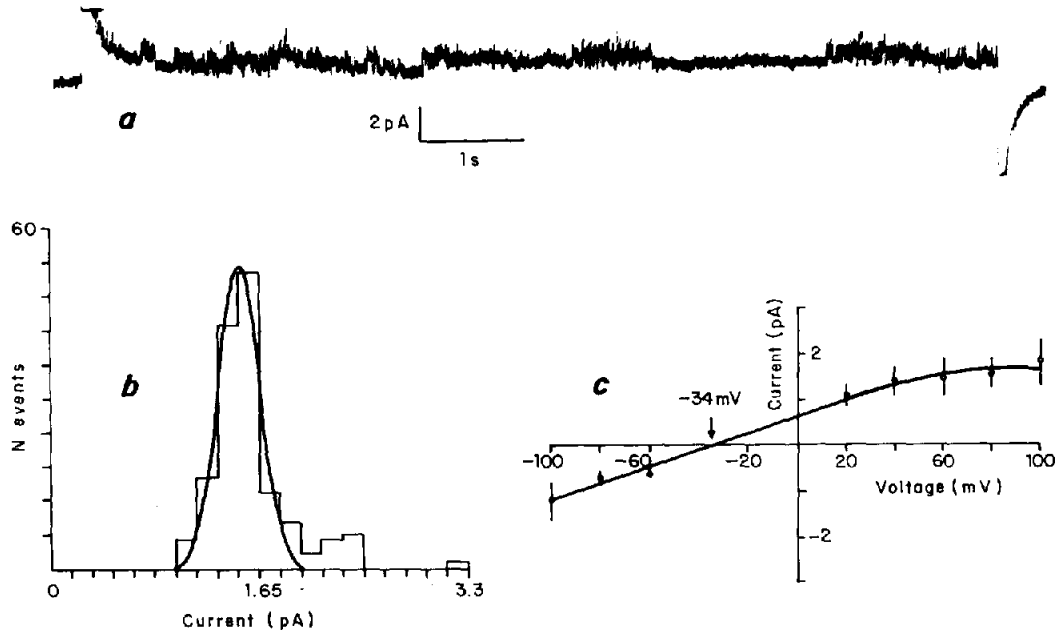

Fig.2. Reconstitution of the purified NTX-binding protein channels into lipid bilayers. (a) Occurrence of single-channel bursts recorded with a membrane potential held at 100 mV in asymmetric solutions. The record was filtered at 100 Hz with an eight-pole Bessel filter and displayed on a Gould chart recorder. The first and last parts of the record show the capacitative transients corresponding to the application and removal of E_m. The solutions bathing the bilayers were asymmetric and contained 0.5 M KCl, 0.1 mM $CaCl_2$, 10 mM Hepes at pH 8.0 in *cis* side, and the same but 0.1 M KCl in *trans*. (b) Amplitude histogram based on single-channel current transitions. The number of analyzed events was 152. The mean amplitude was 1.5 ± 0.2 pA at a membrane potential of 100 mV in asymmetric KCl solutions. (c) Open-channel current voltage relationship. The mean single-channel current at each point was obtained from five different bilayers. The arrow indicates the zero-current voltage. The vertical lines indicate the standard deviation of each point.

single-channel current-voltage relation shows rectification at high positive voltages (fig.2c). This figure also illustrates the selectivity of the single channels. In these experiments we measured the current passing through a single channel in the presence of 5-fold KCl concentration gradient. The reversal potential for the single channel was −34 mV, in agreement with that predicted by the Nernst equation for a cationic selective channel.

Fig.3a shows recordings of single-channel current at different membrane potentials of the more frequently observed unitary current. For large positive voltages, the open probability is higher than for negative ones. In a few records we have encountered two other types of channels (fig.3b). These channels opened infrequently and their single-channel conductances were around 22 and 32 pS (at 100 mV). We have not studied these channels in detail; however, it is possible that they also contribute to the total macroscopic K^+ current elicited by a depolarizing pulse in the squid giant axon membrane. The voltage-dependence of the channel can be seen in the current versus time histograms (fig.3c). The time that the channels spent in the closed current level was less at more positive potentials. In the 100 mV histogram the main current peak (1.5 pA) and two larger ones are evident (c = closed channel current). At 100 mV the open time constants were 0.4 and 2.3 ms (606 events) and the closed ones 17.6 and 89 ms (667

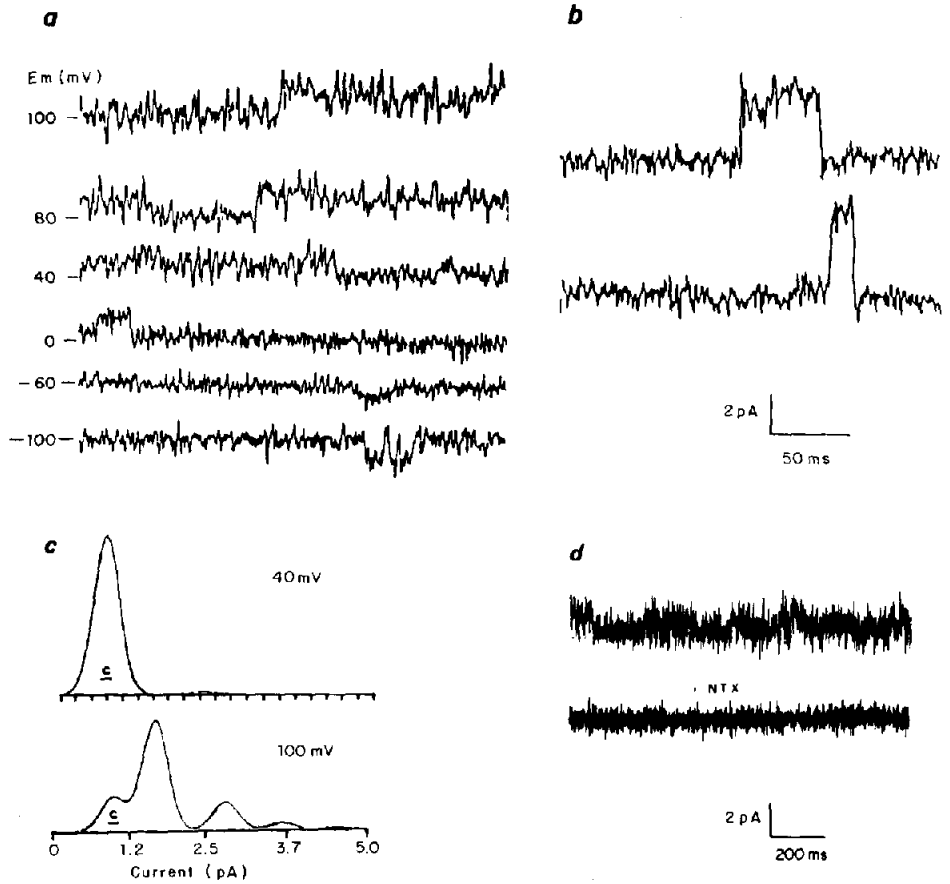

Fig.3. Properties of the main channel. (a) Representative single-channel records at the indicated voltages (E_m), of the predominant channel filtered at 300 Hz, digitized at 2 kHz sampling interval and plotted by computer. (b) Current records of the two less frequent K^+-channels incorporated into bilayers. (c) Current level histograms from a bilayer. Records were filtered at 100 Hz and each histogram was constructed with 28 s of records at the indicated potentials. Each peak represents the percent of time that the current spent in each level. c indicates the basal bilayer current (closed state of the channel). (d) NTX block of the channel. Addition of 2 μM NTX to the *cis* compartment blocked the current fluctuations. Upper record is the control before NTX addition. Membrane potential: 100 mV.

events). The biexponential fit of the open and closed dwell histograms agrees with the complex bursting kinetics seen in fig.2a. Preliminary results indicate that the K^+-channel activity is blocked by NTX (fig.3d) and tetraethylammonium (TEA^+). However, in some cases, after seeing blockage, we have observed increases in the current which could be due to further channel incorporation since the NTX receptor protein loaded liposomes are very fusogenic.

The results demonstrate that the purified NTX binding protein displays single-channel activity when incorporated into planar lipid bilayers. A main 11 pS conductance and two larger ones were observed. The reversal potential, the voltage dependence and the preliminary results with NTX and TEA^+ suggest that the purified fraction contains the delayed K^+ channel from the squid giant axon. To our knowledge, this is the first report in the literature showing both reconstitution and activity of a K^+ ion channel isolated by affinity column from a solubilized membrane preparation, and the used strategy could be useful for the purification of K^+ channels from other membrane preparations.

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