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Evidence for a K^+ channel in bovine chromaffin granule membranes: single-channel properties and possible bioenergetic significance

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Abstract. A K^+ channel was incorporated into voltage-clamped planar lipid bilayers from bovine chromaffin granules and resealed granule membranes ("ghosts"). It was not incorporated from plasma membrane-rich fractions from the adrenal medulla. The channel had a conductance of ~400 pS in symmetric 450 mM KCl, with the permeability sequence $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$, and was insensitive to both Ca^{2+} and charybdotoxin. It exhibited complex gating kinetics, consistent with the presence of multiple open and closed states, and its gating was voltage-dependent. The channels appeared to incorporate into bilayers with the same orientation, and were blocked from one side (the side of vesicle addition) by 0.2-1 mM TEA^+. The block was slightly voltage-dependent. Acidification of resealed granule membranes in response to external ATP (which activated the vacuolar-type ATPase) was significantly reduced in the presence of 1 mM intralumenal TEACl (with 9 mM KCl), and parallel measurements with the potential-sensitive dye Oxonol V showed that such vesicles tended to develop higher internal-positive membrane potentials than control vesicles containing only 10 mM KCl. 1 mM TEA^+ had no effect on proton-pumping activity when applied externally, and did not directly affect either the proton-pumping or ATP hydrolytic activity of the partially-purified ATPase. These results suggest that chromaffin granule membranes contain a TEA^+-sensitive K^+ channel which may have a role in regulating the vesicle membrane potential.

Key words: K^+ channel – Chromaffin granule – Membrane reconstitution – Planar bilayer

Introduction

Adrenal chromaffin secretory vesicles (chromaffin granules), and resealed granule membranes ("ghosts"), can be prepared by straightforward subcellular fractionation protocol which yield large amounts of material of high purity (e.g. Apps et al. 1980). These preparations have provided an important model system in which to study certain aspects of biogenic amine accumulation (Njus et al. 1986; Johnson 1988). In particular, although recent important advances in our understanding of the molecular mechanisms of synaptic transmission have largely come from studies of more directly-relevant neuronal cells, where the machinery for transmitter release is likely to be both more abundant and more efficient, our overall knowledge of the bioenergetics of secretory vesicles is still based on studies of the chromaffin granule. Even so, considerable disagreement remains over the presence of specific ion channels in the membranes of such vesicles, and their biological role. This is a significant problem, since the activation of such channels could drastically modify both membrane potentials and ion distributions, with important consequences for the accumulation of biogenic amines (as described below). In addition, it has been suggested that channels may be involved in vesicle-plasma membrane fusion, and it is now generally accepted that the appearance of a channel-like "fusion pore", derived at least partly from vesicular proteins, is a central event in exocytosis (Monck and Fernandez 1992).

A recent in vitro study described the incorporation into planar lipid bilayers of several different cation channels from intact chromaffin granules, but only one type of channel, a Ca^{2+}-independent K^+ channel, could be reconstituted from preparations of chromaffin granule "ghosts" (Arispe et al. 1992). The argument that increasing the purity of the preparation had removed channels located in contaminating membranes, leaving a chromaffin granule-specific channel in the ghosts, does not of course *prove* the channel's origin – for example, a minor channel-containing fraction might simply have been co-purified with the ghost membranes. Nevertheless, such studies are highly suggestive, and there is firmer evidence for the presence of ion channels in chromaffin granule membranes from patch-clamp studies (Picaud et al. 1984). Also, there are several reports detailing the reconstitution

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of ion channels from synaptic vesicle preparations (Rahamimoff et al. 1988; Thomas et al. 1988; Sato et al. 1992), and from neurohypophysial secretory vesicles (Stanley et al. 1988), supporting the idea that ion channels may be a basic component of vesicles involved in regulated exocytosis. Chromaffin granules are an attractive system for further study because of the high degree of purity that can be achieved.

In this study, we have attempted to identify chromaffin granule-specific channels by adopting a combined approach involving single ion channel reconstitution and recording, together with macroscopic assays of both vesicle acidification and membrane potential. Chromaffin granule membranes contain a vacuolar (V-type) proton-pumping ATPase (Percy et al. 1985; Moriyama and Nelson 1987; Forgac 1989) which hydrolyses cytoplasmic ATP and pumps protons into the granule lumen. The pH within the lumen of intact granules in the presence of MgATP at pH 7.0 is about 5.5, and there is a membrane potential of about 50 mV, inside positive (Holz 1979; Johnson and Scarpa 1979). Similar values are found in resealed "ghosts" (Phillips and Apps 1980). As in chemiosmotic coupling in mitochondria, the proton electrochemical potential drives secondary transport systems, and in fact both components of the protonmotive force are utilised to provide the striking amine accumulation (to an intragranular concentration of ~0.6 M) observed in chromaffin granules (Njus et al. 1986). Functional ion channels would clearly interact with such a system, and we therefore decided to measure both the electrical potential and ΔpH in chromaffin granules in the presence of substances shown to modify the behaviour of putative ion channels. This paper describes the properties of a high-conductance, TEA^+-sensitive K^+ channel reconstituted from chromaffin granule and "ghost" membranes. The channel could not be reconstituted from plasma membrane vesicles, and we provide further evidence to suggest that it did indeed originate from chromaffin vesicles, where it may have a significant role in vesicle bioenergetics.

Methods

Membrane preparations

Fresh bovine adrenal glands were collected on ice from a local abattoir and chromaffin granule membranes and resealed membrane "ghosts" were prepared from the adrenal medullae as previously described (Apps et al. 1980). The "ghosts" were resealed in 0.3 M sucrose, 10 mM HEPES, pH 7.4 (room temperature). The counterion to HEPES was Na^+, K^+ or TEA^+, or combinations of these, the total cation concentration being 5.9 mM. Plasma membrane fractions were prepared in parallel by a modification of the method of Meyer and Burger (1979). "Ghosts" destined for bilayer reconstitution were suspended in 0.3 M sucrose containing 10 mM HEPES/KOH (pH 7.4). These vesicles were either used immediately, or were snap-frozen in small aliquots in liquid N_2 and stored at −70 °C for up to 3 months. Membrane vesicles ("ghosts") destined for assay of proton translocation and ATPase activity, and for ATPase purification and reconstitution, were stored for up to 3 months at −20 °C in 10 mM "Na-HEPES", i.e. 10 mM HEPES neutralized with NaOH (pH 7.4), or "K-HEPES" or "TEA-HEPES", or mixtures as described in due course, with 0.1 mM EDTA and 1 mM dithiothreitol. There was only minor loss of activity on storage. Protein concentrations were measured by the Folin-Lowry method following TCA precipitation (Peterson 1977; Markwell et al. 1981). These were 10–20 mg/ml for granule membranes and ~5 mg/ml for "ghosts" and plasma membrane vesicles.

Single-channel reconstitution

These experiments were carried out at room temperature. Chromaffin granule membranes, resealed granule membrane "ghosts", or plasma membrane vesicles were incorporated into voltage-clamped planar lipid bilayers formed from a decane dispersion of 15 mg/ml palmitoyloleoyl phosphatidylethanolamine (POPE) with 15 mg/ml palmitoyloleoyl phosphatidylserine (POPS). The lipid suspension was drawn across a 0.3 mm hole in a styrene co-polymer cup (Polypenco Q200.1, Amari Plastics, Glasgow, UK) separating two solution-filled chambers designated *cis* (the side of subsequent vesicle addition) and *trans*. The chambers were connected to a Biologic RK 300 patch-clamp amplifier (Intracel, Shepreth, UK) via Ag/AgCl electrodes and agar salt bridges containing 150 mM KCl. The *cis* chamber was voltage-clamped relative to the *trans* chamber, which was grounded, and all potentials are quoted as *cis-trans*, with positive ("outward", *cis* to *trans*) currents at potentials > the cation channel reversal potential. The closed state (marked in each figure) is the baseline current in the absence of open channels. Positive currents are shown as upwards deflexions, unless specifically indicated (in Fig. 8, all openings are displayed as downwards deflexions to facilitate direct comparisons). Membrane vesicles (5–10 μg protein/ml) were added to the *cis* chamber in the presence of a salt gradient (usually 450:150 mM KCl, *cis* > *trans*), and fusion (at 0 μV) was accelerated by adding 1 mM $CaCl_2$ to the *cis* chamber, and by stirring. After one or more channels appeared, further fusion was usually prevented by perfusing the *cis* chamber with fresh, vesicle-free solution. Transmembrane currents were routinely low-pass filtered (8-pole Bessel, −3 dB at 0.3 kHz, or other frequencies as indicated), and sampled by a LabMaster DMA A/D converter at 5–10 times the −3 dB frequency for storage on disc using Axotape software (Axon Instruments, Foster City, CA, USA). The recordings for illustrations were post-filtered using a digital Gaussian filter, and analysis was in the main carried out by using pClamp software (Axon Instruments) and PS-Plot (Polysoft, Salt Lake City, UT, USA). The relative permeability of the channel for different cations with respect to K^+ was assessed by measuring reversal potentials with 450 mM KCl *cis* and 450 mM chloride salts of the test monovalent cation *trans*, with appropriate activity corrections:

$$P_{K^+}/P_{y^+} = a_{y^+}/a_{K^+} \cdot \exp(-FE_r/RT)$$

where P and a represent the cation permeabilities and activities respectively of K^+ and the test cation, E_r is the reversal (equilibrium) potential (mV), and F/RT is 0.039 per mV.

ATPase reconstitution and activity

The vacuolar ATPase from adrenal chromaffin membranes was solubilized, partially purified and functionally reconstituted into proteoliposomes by the method of Pérez-Castiñeira and Apps (1990). Purifications typically gave a 5–6 fold enrichment of ATPase activity with ~15% recovery. ATPase activity was assayed at 30 °C by monitoring the conversion of NADH to NAD^+ in the presence of pyruvate kinase and lactate dehydrogenase (Percy et al. 1985). ATPase activities were directly proportional to protein concentration, and were reduced by >90% on adding 5 nmol/mg of the specific V-type ATPase inhibitor, Bafilomycin A_1 (Bowman et al. 1988).

Assay of H^+-translocation

H^+-translocation was assayed as previously described (Pérez-Castiñeira and Apps 1990). Briefly, the fluorescence quenching of 9-amino 6-chloro 2-methoxyacridine (ACMA, gift of Dr R. Kraayenhof, Free University, Amsterdam) was monitored in a Perkin-Elmer SP3000 fluorimeter set to excitation and emission wavelengths of 420 nm and 480 nm respectively. The assay buffer (0.5 ml), which was constantly stirred, contained 0.3 M sucrose, 10 mM HEPES/NaOH (pH 7.4), 3 mM K_2SO_4, 2 mM $MgSO_4$, 1.8 μM ACMA, and either chromaffin granule "ghosts" (60 μg protein), or ATPase-containing proteoliposomes (2 μg protein), and was thermostated at 30 °C. Proton-pumping was initiated by the addition of 1 mM ATP. The fluorescence response of ACMA was calibrated by "back-titration": after maximal ATP-dependent quenching had been achieved, small aliquots of 0.2 M HCl were added to decrease instantaneously the pH of the medium, thereby reducing ΔpH and decreasing the fractional quench (Q). ACMA was found to obey the relationship derived by Schuldiner et al. (1972):

$$\Delta\text{pH} = \log [Q/(1 - Q)] - \log(V_{in}/V_{out})$$

although the apparent value of V_{in}/V_{out} (the ratio of the intravesicular volume to the volume of the medium) was much larger than expected (Haigh et al. 1989; Casadio 1991). Proton pumping was also reduced by Bafilomycin A_1, in parallel with ATPase activity.

Membrane vesicle potentials

"Ghosts" (approximately 100 μg protein/ml) were incubated under control conditions in 0.5 ml of buffer containing 4 μM Oxonol V, 0.3 M sucrose, 10 mM HEPES/NaOH (pH 7.4), 2 mM $MgSO_4$ and 1 mM ATP, monitoring fluorescence emission at 645 nm on excitation at 585 nm. In other respects the experimental conditions were similar to those used to assay proton pumping, and the results of all fluorescence assays were recorded using an X-Y pen recorder or, in some cases, the analogue data were digitized (at 100 Hz) and stored on a computer disc.

Materials

The lipids for bilayer reconstitution were obtained from Avanti Polar Lipids (Birmingham, AL, USA), and stored in $CHCl_3$ for up to 6 months at −70 °C. The lipids for ATPase reconstitution were obtained from Lipid Products (Nutfield, UK). All reagents were of the best available grades.

Results

Reconstitution of single-channel activity

Control bilayers (capacitance ~250 pF, conductance <5 pS) showed no channel-like activity, but we routinely observed positive unit current transitions of ~10 pA within 30–40 min of adding chromaffin granule membranes or "ghosts" to the *cis* bilayer chamber. These currents mostly appeared to be single-channel events, but occasionally 2 or even 3 channels appeared together in a previously quiescent bilayer (consistent with their incorporation by vesicle fusion). The channels were usually similar in amplitude and gating behaviour from experiment to experiment, and under the fusion conditions we adopted the positive currents we regularly observed at zero mV could only have been due to K^+ ions flowing *cis-to-trans*. It was also clear from differences in channel gating kinetics at positive and negative holding potentials (described in more detail below) that the channels almost always incorporated with the same orientation. We occasionally observed other cation channels with lower or higher conductances from both chromaffin granule membrane and resealed "ghost" preparations, but collected examples of an anion channel in only 1–2% of all experiments. These other channels occurred too infrequently for detailed analysis, but it remains possible that further manipulation of the fusion conditions might encourage their appearance (cf. Hayman et al. 1993). Examples of single-channel recordings with a particularly high open probability are illustrated in Fig. 1 (trace a), which also shows examples of less frequently-observed anion and cation channels (traces b and c respectively). These could be unequivocably identified as cation or anion channels by the direction of current flow in the presence of the ionic gradient (i.e. positive currents at 0 mV represent a net flow of K^+ *cis to trans*, while negative currents at 0 mV represent a net flow of Cl^- *cis to trans*, e.g. Fig. 1 b). Although channels were also incorporated from plasma membrane fractions, they did not resemble the channels seen with chromaffin granule or "ghost" membranes. As channels appeared to incorporate more readily from resealed "ghost" membranes, these were used in preference to fragmented granule membranes for most of the subsequent experiments.

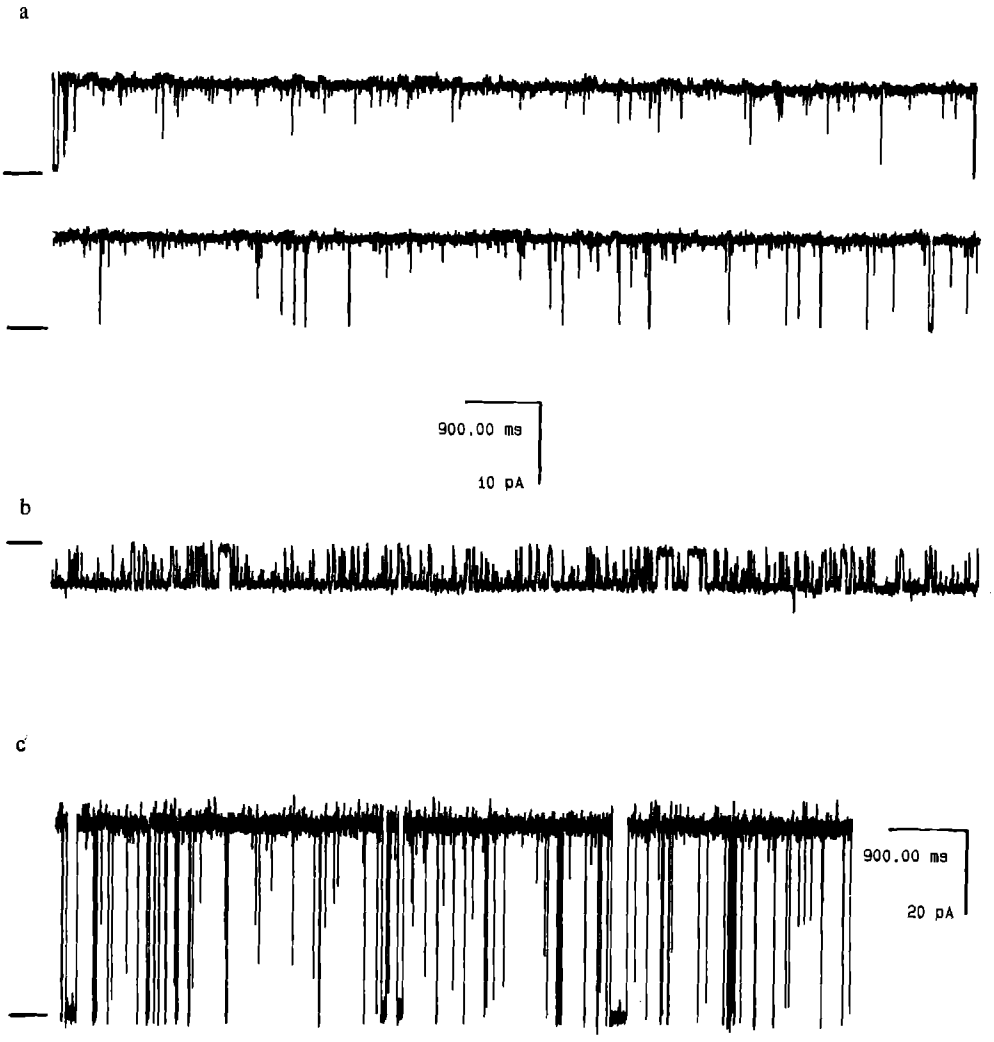


Fig. 1a–c. Single-channel recordings at a holding potential of zero mV from "ghost" membranes incorporated into bilayers exposed to 450 mM KCl *cis*, 150 mM *trans*. The closed levels, corresponding to currents through the lipid bilayer (always < single-channel currents), are indicated (–). **a** "Positive" currents from openings of a cation channel (i.e. net flux of K^+ flowing *cis* to *trans* at 0 mV. **b** "Negative" currents from an anion channel (a net flux of Cl^- flowing *cis* to *trans* at 0 mV). **c** incorporation of a high-conductance K^+ channel. Recordings were low-pass filtered at 200 Hz, 100 Hz and 500 Hz respectively

Conductance and selectivity studies

The conductance and selectivity of the channel were examined in detail. In symmetric 450 mM KCl the current/voltage (I/V) relation (e.g. Fig. 2) appeared to be linear when examined between membrane potentials of ±80 mV, with a single-channel slope conductance of 390±30 pS (mean±SD, $n=12$). In these, as in all other experiments, care was taken to minimise or offset any junction potential (see Hayman et al. 1993), and as a result reversal potentials in symmetric solutions were usually within ±2 mV of zero. I/Vs were also linear in 450 mM *cis* vs 150 mM *trans* KCl gradients (e.g. Fig. 3), with a reversal potential of −23.0±5.0 mV (mean ±SD, $n=10$; conductance 350±21 pS, $n=12$). This may be compared with the value of −23.5 mV for the reversal potential predicted by the Nernst equation in an ideally K^+-selective channel under these conditions, after appropriate corrections from standard tables to give ionic activities. The relative cation permeability sequence (Table 1) $P_K > P_{Rb} > P_{Cs} > P_{Na} > P_{Li}$) is typical for a K^+ channel (Hille 1984), but the permeability ratios should only be regarded as approximate values because although rectification was not apparent in the I/Vs, in most cases E_r was obtained by extrapolation (it was rare to observe actual current reversal under these conditions). Having established the fact that the channel was highly selective for K^+ over Cl^-, with a typical K^+ channel permeability sequence, we examined its conductance behaviour in more detail (Fig. 4). In KCl solutions, single-channel conductance were analysed by assuming Michaelis-Menten type kinetics, to give an apparant affinity (K_m) of 42 mM, saturating at a conductance of 490 pS (all concentrations were corrected by appropriate activity coefficients). Taken together, these

Table 1. Relative cation selectivities

trans cation	E_r, mV (mean±SD)	P_{cation}/P_K (mean±SD)
K^+	(0)	(1)
Rb^+	−7.7±1.5	0.72±0.14
Cs^+	−14.5±0.60	0.53±0.02
Na^+	−29.3±1.5	0.33±0.02
Li^+	−39.0±2.1	0.24±0.04

Data from single-channel experiments ($n=3$ or 4 independent channels for each condition) in 450 mM KCl *cis*, vs 450 mM test cation Cl *trans*. All calculations used calculated activities

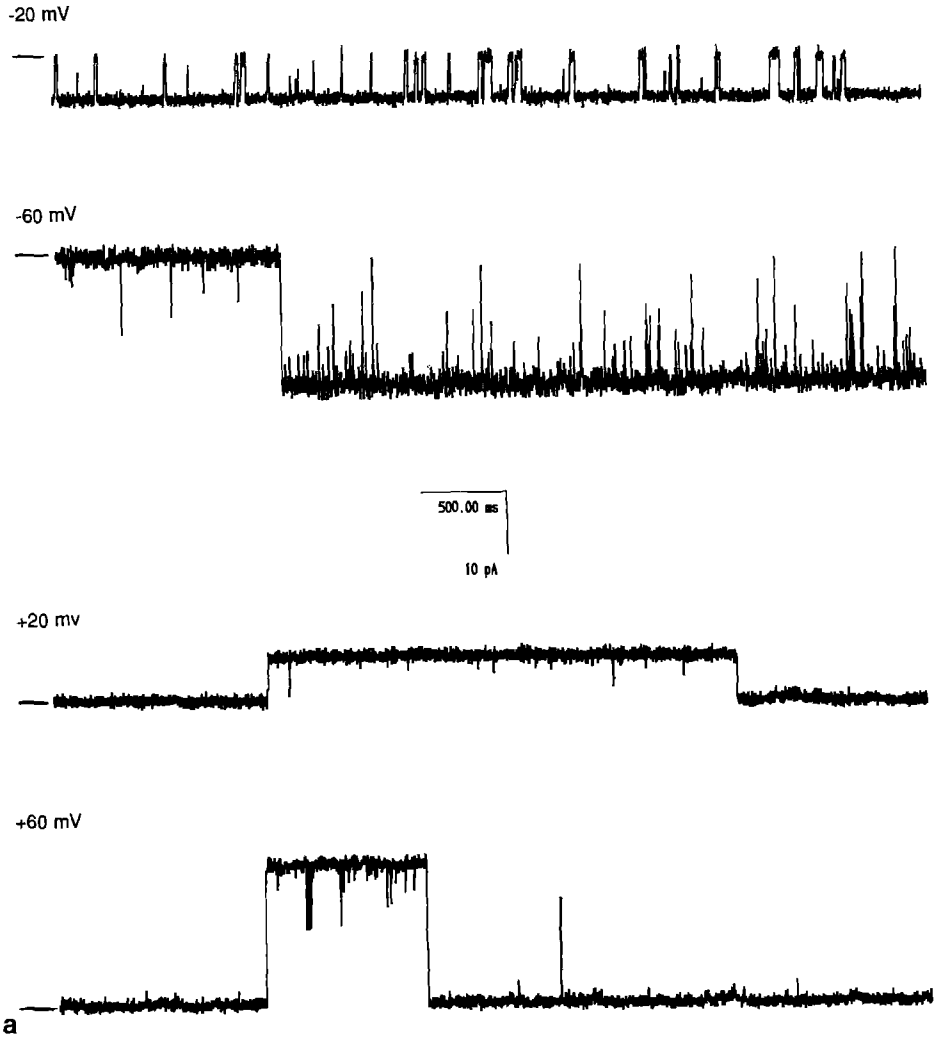


Fig. 2. Selected single-channel recordings **a**, and current/voltage relationship **b**, under symmetric ionic conditions (450 mM KCl). Channel conductance 396 pS. In part **a**, the closed levels are marked (−). Filtered at 200 Hz

results firmly identified the channel as a high-conductance K^+ channel, and raised the question: was it a maxi-K channel?

Insensitivity to Ca^{2+} and ChTX

We therefore studied the channel's inhibitor profile, and the role of Ca^{2+} as a potential channel activator. Experiments with potential activators and inhibitors were repeated at least 3 times on different channels, and additions were always made to both *cis* and *trans* chambers. Neither the removal of Ca^{2+}, or the addition of charybdotoxin (ChTX, Miller et al. 1985), appeared to inhibit the channel (Fig. 5). This behaviour was quite distinct from that of Ca^{2+}-activated K^+ channels present in the chromaffin cell plasma membrane (Marty 1981; Yellen 1984; Marty and Neher 1985), whose activation is essentially regulated by (cytoplasmic) [Ca^{2+}] between $\sim$10 nM to $\sim$10 μM, with loss of voltage-dependence in 1 mM Ca^{2+} (because the activation curve is then grossly shifted to negative potentials). In addition, such channels should be blocked by 10–40 nM ChTX (Robitaille et al. 1993). Thus it appears unlikely that the channel described here originated from contaminating chromaffin cell plasma

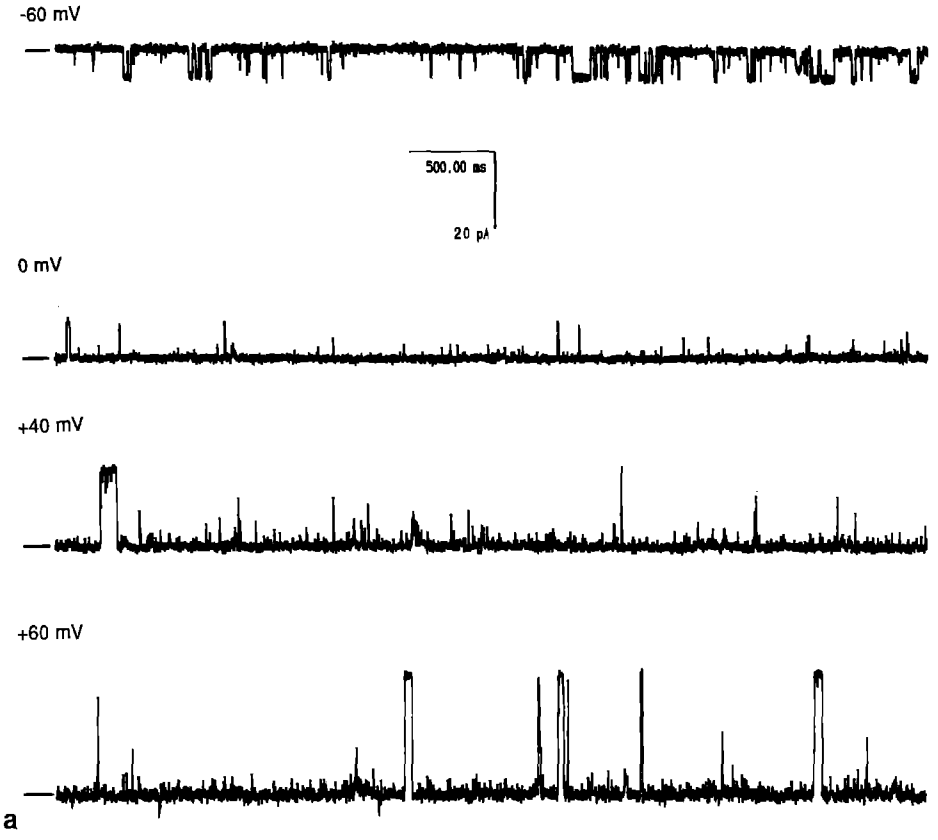


Fig. 3. Selected single-channel recordings **a** and current/voltage relationship **b**, under asymmetric ionic conditions (450 mM KCl *cis* vs 150 mM KCl *trans*). Channel conductance 318 pS, E_r −32 mV. In part **a**, the closed levels are marked (−). Filtered at 200 Hz

membranes, unless it is a novel channel which has remained undetected in patch-clamp studies.

Preliminary gating analysis

The channels showed clear differences in gating kinetics between negative and positive holding potentials (Fig. 6). At negative potentials, channel activity switched between two patterns of gating: long bursts of openings interrupted by frequent brief closures, and long mainly-closed episodes interrupted by frequent brief openings. Depending on the individual channel open probability, this characteristically gave rise to rather "symmetric" recordings (Fig. 6a), although in general the open state was noisier (as expected). In comparison, at positive holding potentials, there were many fewer short closures during (relatively-long) bursts of openings, and also fewer short openings during the main interburst intervals. This gave much "cleaner" recordings (Fig. 6c), and the differences between positive and negative potentials were readily apparent in virtually every experiment. As mentioned previously, the pattern of voltage-dependent behaviour (where apparent) was the same in every channel examined, strongly suggesting that they always became incorporated into the bilayer with the same orientation. A more detailed analysis of the typical gating behaviour of a single channel is presented in Fig. 7. These data (5.4 min) were filtered at 1 kHz (−3dB, 8-pole Bessel) and digitized at 10 kHz before analysis (excluding events lasting less than 0.4 ms, whose lifetimes cannot be adequately resolved under these conditions). There were 2 clearly separable distributions of open lifetimes at both +40 mV and −40 mV, and while there may be only 2 closed lifetime distributions at −40 mV, the distribution at +40 mV clearly had multiple (at least 3) components. However, a large proportion of the short-lived events, especially the shortest closures, were too brief to be adequately resolved (note especially the flickery closures from the open state in Fig. 6b).

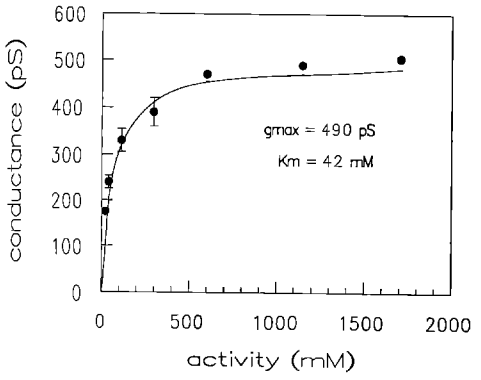


Fig. 4. Conductance vs activity relationship. Standard deviations are shown are for 4 independent experiments, and other measurements are the average of at least 2 independent experiments. The data were fitted directly to a single-site (Michaelis-Menten) model

Block by TEA^+

Although Ca^{2+} and ChTX showed no effects on the reconstituted channel, many K^+ channels are blocked at one or more sites by tetraethylammonium (TEA^+, Jan and Jan 1992). It is well-known that, depending on recording noise and filtering, individual blocking events may be too brief to be individually resolved (leading to an apparent reduction in single-channel current amplitude, a "smooth" block), and the binding sites may be located with the voltage-gradient (making the block voltage-dependent). TEA^+ blocked the channel described in this study, with little reduction in current amplitude with up to 5 mM TEA^+ *cis*, but a clear reduction in amplitude (which was slightly more marked at negative holding potentials) on adding lower concentrations to the *trans* side. The effects of adding low (up to 0.5 mM) concentrations of

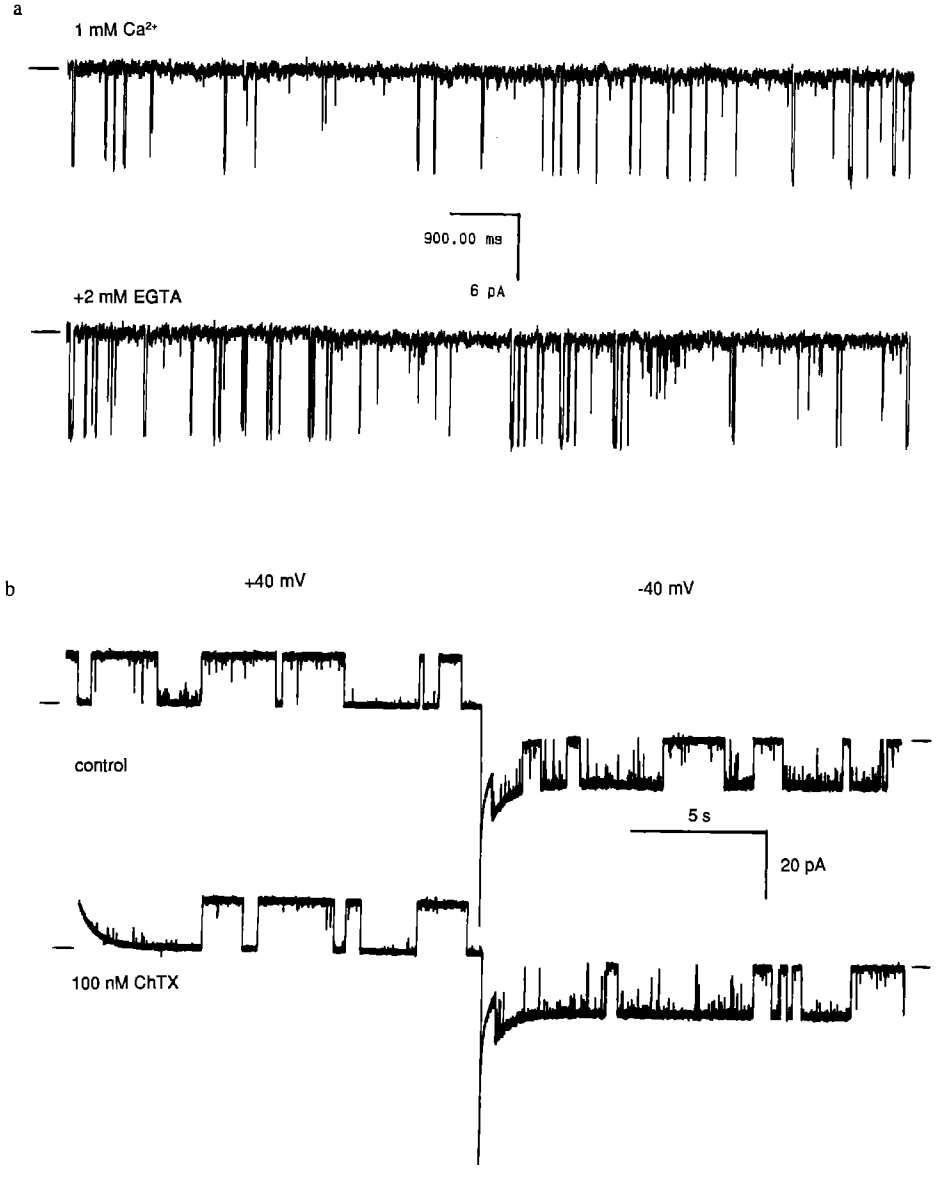


Fig. 5. Lack of effect of Ca^{2+} **a** and ChTX **b** on single-channel activity. Both experiments were carried out in symmetric 450 mM KCl. In part **a**, calculated free $[Ca^{2+}] < 20$ nM after EGTA addition, holding potential -25 mV, closed levels marked $(-)$. In part **b**, another channel was pulsed between ± 40 mV after adding ChTX-free buffer (*upper trace*), or 100 nM ChTX, to both chambers. All recordings filtered at 200 Hz

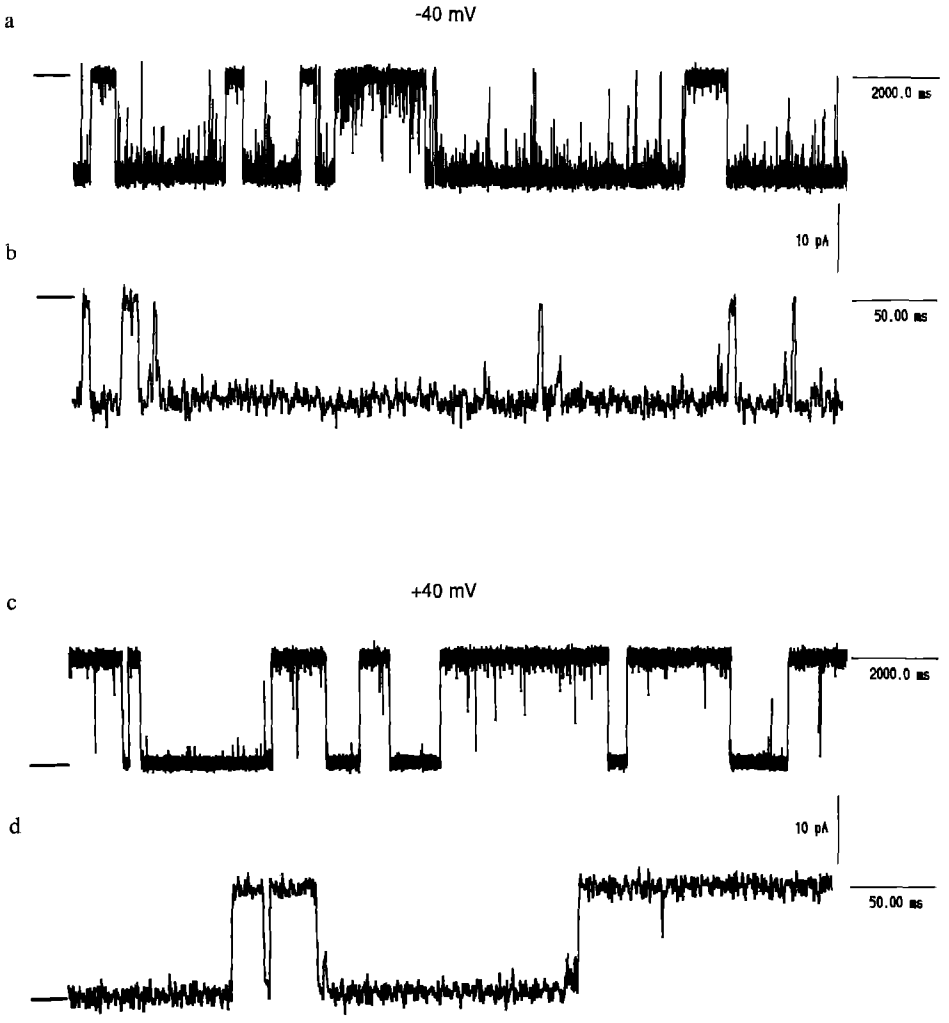


Fig. 6. Channel recordings in symmetric 450 mM KCl at holding potentials of −40 mV **a, b** and +40 mV **c, d**, illustrating typical kinetics on both long (seconds) and short (tens of ms) time scales. Filtered at 200 Hz (**a** and **c**: time scale bar 2 s) or 500 Hz (**b** and **d**: *time scale bar* 50 ms), closed levels marked (−)

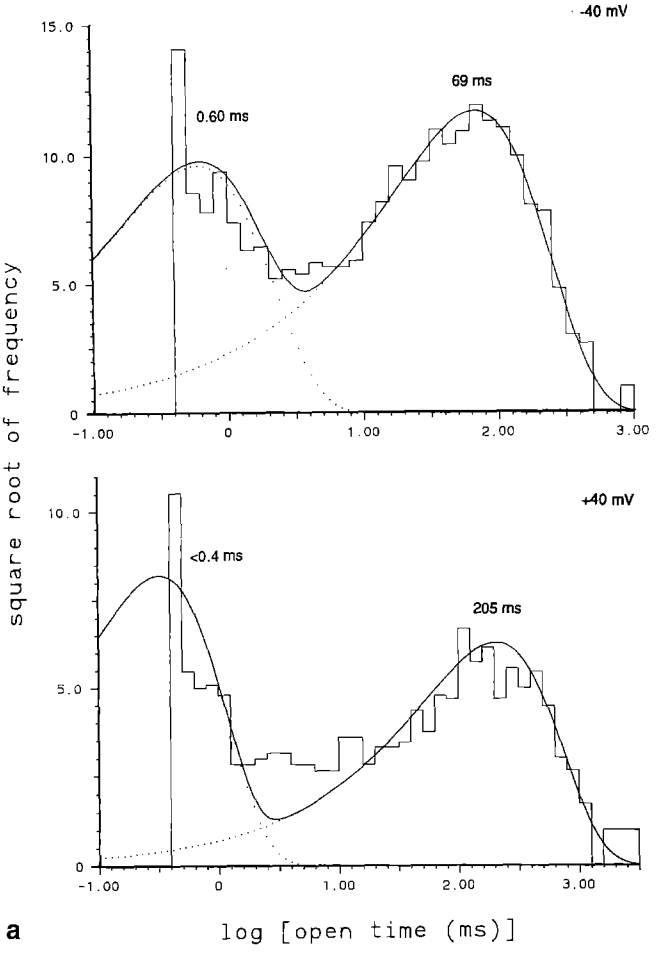
the blocker to both the *cis* and *trans* sides of reconstituted channel are illustrated in Fig. 8. In this diagram, the usual current convention is ignored in order to facilitate direct comparisons between single-channel current amplitudes. These findings were consistent with the presence of at least one blocker binding site located near the *trans* entrance, and the block was modelled by using the equations of Woodhull (1973), Hille and Schwarz (1978) and Coronado and Miller (1979; but also see Cukierman et al. 1985), where the blocker binding and unbinding rates are expicitly voltage-dependent. Similar treatments have subsequently been applied very widely (e.g. Yellen 1984) to give the apparent binding affinity, usually reported as the zero-voltage dissociation constant, $K_b(0)$, as well as to measure the fractional distance of the site down the voltage drop (δ, measured as part of $z\delta$, the "effective valence" of the blocking reaction):

$$I = I_0/[1 + [TEA^+]/K_b(0) \cdot \exp(z\delta FV/RT)]$$

Data were fitted directly by a non-linear least squares method to analyse block induced by up to 3 mM TEA^+ added to the *trans* side of reconstituted channels, giving $K_b(0) = 0.47 \pm 0.13$ mM and $\delta = -0.21 \pm 0.06$ (both means $\pm$SD, $n = 6$). Figure 9 shows one example of this analysis. Having identified a channel blocker, binding with a dissociation constant of approximately 1 mM at a site some 20% across the voltage drop from the *trans* side, we proceeded to use TEA^+ as a tool to investigate whether a TEA^+-sensitive K^+ channel might indeed be located in resealed "ghost" membranes.

Proton-transport and vesicular membrane potential

ATP-dependent H^+-translocation into "ghosts" and proteoliposomes was monitored by following the fluorescence quenching of the permeant weak base, ACMA (Schuldiner et al. 1972; Pérez-Castiñeira and Apps 1990; Casadio 1991). In resealed "ghosts", both the rate and extent of acidification depended on the nature of the counterion present with the HEPES buffer within the vesicles (Fig. 10 and Table 2). In the traces shown in Fig. 10, initial fluorescence was 90% (10 on the scale) in each case. The small initial increase in fluorescence produced by the addition of ATP has been noted before (Percy et al. 1985) but is only apparent when the subsequent rate of quenching is low. Partial or complete replacement of internal but not external K^+ with TEA^+ reduced both the rate of quenching and the ΔpH finally achieved. In K^+-containing "ghosts", quenching could be



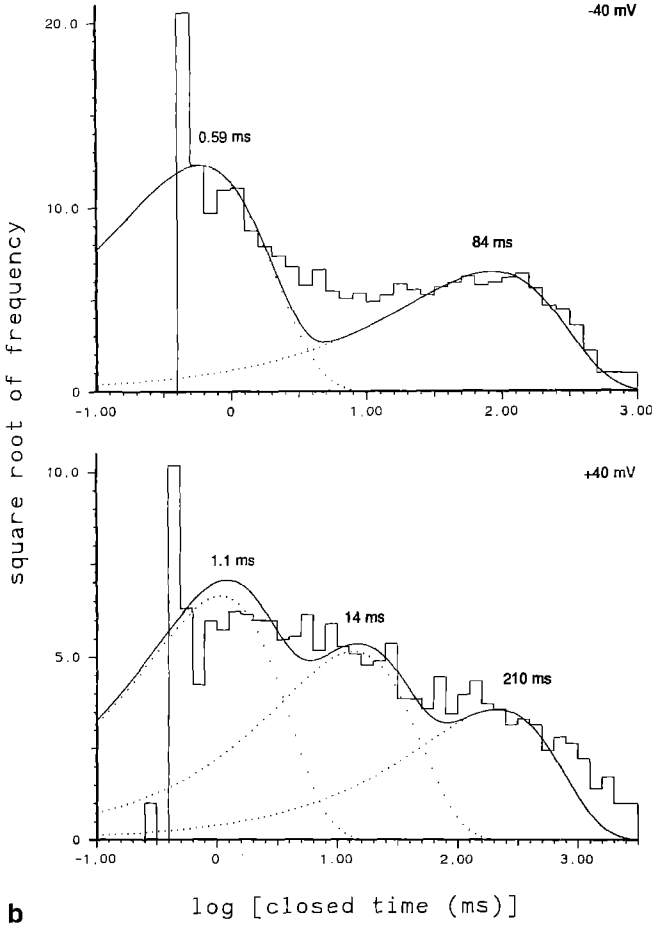


Table 2. Effect of internal cation on ATP-dependent acidification of chromaffin granule membrane "ghosts"

Internal ion	K^+	K^+/TEA^+	TEA^+	Na^+
Relative quench rate (min^{-1}, ±SD)	0.57±0.12	0.21±0.02	0.045±0.006	0.164±0.019
ΔpH (±SD)	1.50±0.05	1.14±0.07	0.83 ±0.05	1.19 ±0.04
number of expts	8	6	6	6
P (comparing – ΔpH)		<0.01	<0.01	<0.01

The experiments were carried out as described in the text, and the results were analysed by computing the standard errors of the differences of the means of control and test conditions. The ratio of $[TEA^+]$ to $[K^+]$ was the same as that in Fig. 10, trace b), i.e. 1:9. P is the probability of the null hypothesis being true

augmented by including either valinomycin (1 μM) or NaSCN (10 mM) in the external medium (Fig. 10). In the presence of valinomycin the quenching trace appeared biphasic, but this has not been investigated further (Fig. 10, upper trace d). In the presence of thiocyanate, the ACMA-quenching traces for "ghosts" containing K^+, Na^+, TEA^+ or K^+/TEA^+ as counterions to HEPES were identical (not shown). In contrast, the inclusion of 10 mM HEPES/TEA^+ mixtures (i.e. 5.9 mM TEA^+) inside reconstituted proteoliposomes containing the partially-purified ATPase had no inhibitory effect on ATP-driven H^+-translocation (not shown). Although ACMA quenching obeys the theoretical relationship derived by Schuldiner et al. (1972), it does not allow a direct estimate of ΔpH, since the value of V_{in} does not correspond to the directly-measured intravesicular volume. However, ΔpH has been directly measured in "ghosts" by analysis of methylamine distribution (Phillips and Apps 1980), and in the presence of a permeant ion (I^-) to minimise $\Delta\psi$, ΔpH = 1.5 under the conditions used in the present experiments. This permits calculation of its value under other conditions from the maximal quench achieved (Table 2). Initial dQ/dt has been used to provide an estimate of the rate of acidification, which should be directly proportional to the rate of proton translocation (Bennett and Spanswick 1983). TEA^+ (as the HEPES or Cl salt, up to 10 mM), did not affect the rate of ATP hydrolysis by "ghosts" or by proteoliposomes (not shown). Membrane potentials in "ghost" vesicles were measured quantitatively by using the potential-sensitive dye Oxonol V. An internal (positive) membrane potential was generated rapidly on adding ATP to the suspensions (Fig. 11): this was least marked in K^+-containing "ghosts". However, the

Fig. 7. Gating analysis of the channel recording illustrated in Fig. 6. **a**, open time analysis, **b**, closed time analysis. The data were filtered at 1 kHz (−3 db, 8-pole Bessel) and sampled at 10 kHz. Event durations were fitted by maximum likelihood analysis to multiexponential probability density functions containing either 2 or 3 components (shown as *dotted lines*). The mean lifetimes are indicated

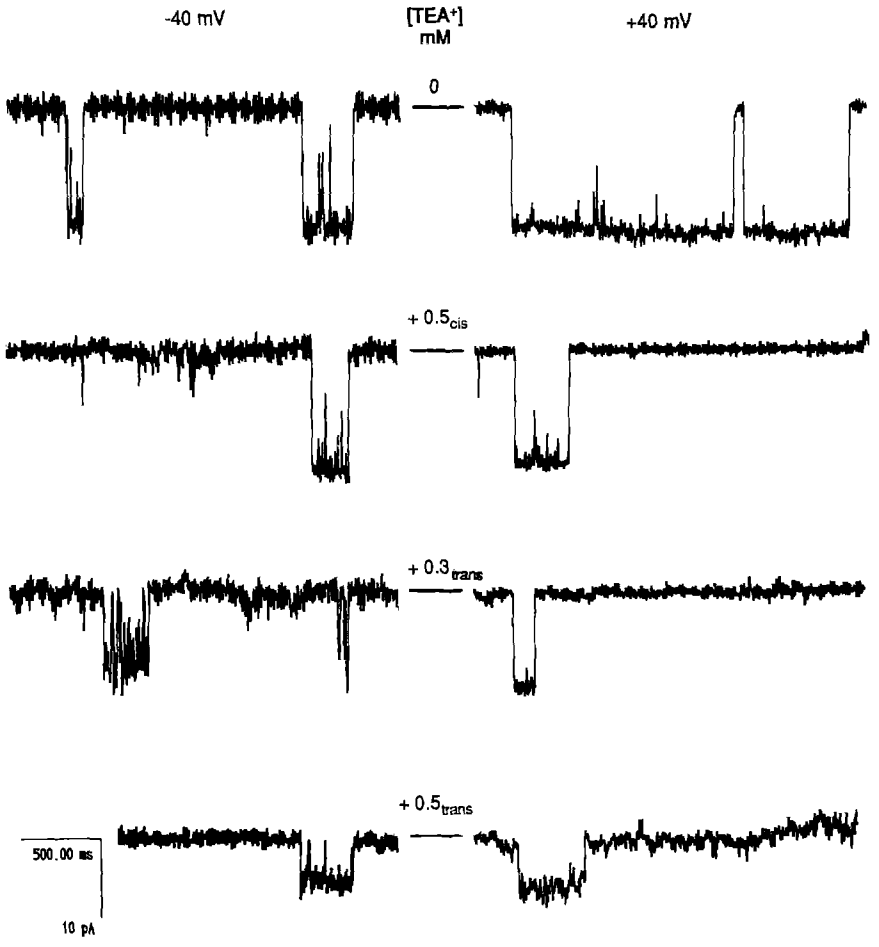


Fig. 8. Channel recordings under control conditions in symmetric 450 mM KCl **a**, and with TEA^+ applied to the channel as indicated **b–d**. Filtered at 200 Hz, closed levels indicated (–). Exceptionally, in this diagram alone, all openings are shown downwards to facilitate direct comparisons at positive and negative holding potentials. In all other diagrams, the normal convention is observed

quenching of the dye was increased by total or partial replacement of K^+ by TEA^+, consistent with the generation, under these conditions, of an increased internal positive membrane potential. These results were reproducible, but because of the relatively low signal-to-noise ratio obtained with Oxonol V, we have not attempted to quantitate them.

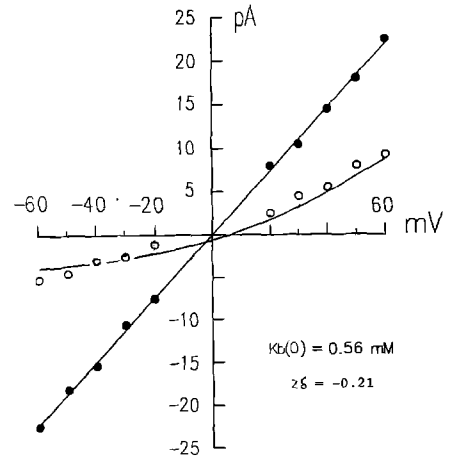


Fig. 9. I/V relations under control conditions (symmetric 450 mM KCl ●) and with 1.7 mM TEA^+ added to the *trans* chamber (○). The latter was fitted directly to the Woodhull equation to give the indicated values for $K_b(0)$ and $z\delta$

Discussion

We have identified a high-conductance cation channel in chromaffin granule membranes which exhibits very high (>100:1) selectivity for K^+ over Cl^-, and displays a typical K^+ channel permeability sequence under biionic conditions. The channel appears to demonstrate "single-site" behaviour in terms of its conductance vs activity relationship, but in fact the fit (to a rectangular hyperbola) is not particularly good, and we have not examined the channel carefully in very low ionic activities, or applied other more decisive tests. It could therefore be a multi-ion channel, typical of other K^+ channels characterized in detail, including maxi-K channels. Its insensitivity to ChTX and to the removal of Ca^{2+} suggests it is *not*, in fact, a Ca^{2+}-dependent K^+ channel [like chromaffin cell plasma membrane K_{Ca} channels, Yellen (1984), which do have a similar conductance.] This, together with the fact that we were unable to incorporate the channel from plasma membrane fractions, militates against a cell-surface origin, and points to an intracellular membrane source. In fact, its properties appear distinct from most previously-described K^+ channels. The discussion about its location will be developed further, in connexion with our parallel experiments at the macroscopic level. The channel has complex gating kinetics, with a minimum of 2 open states (mean lifetimes ~1 ms and ~100 ms) and several closed states (mean lifetimes again ~1 ms and ~100 ms with, at

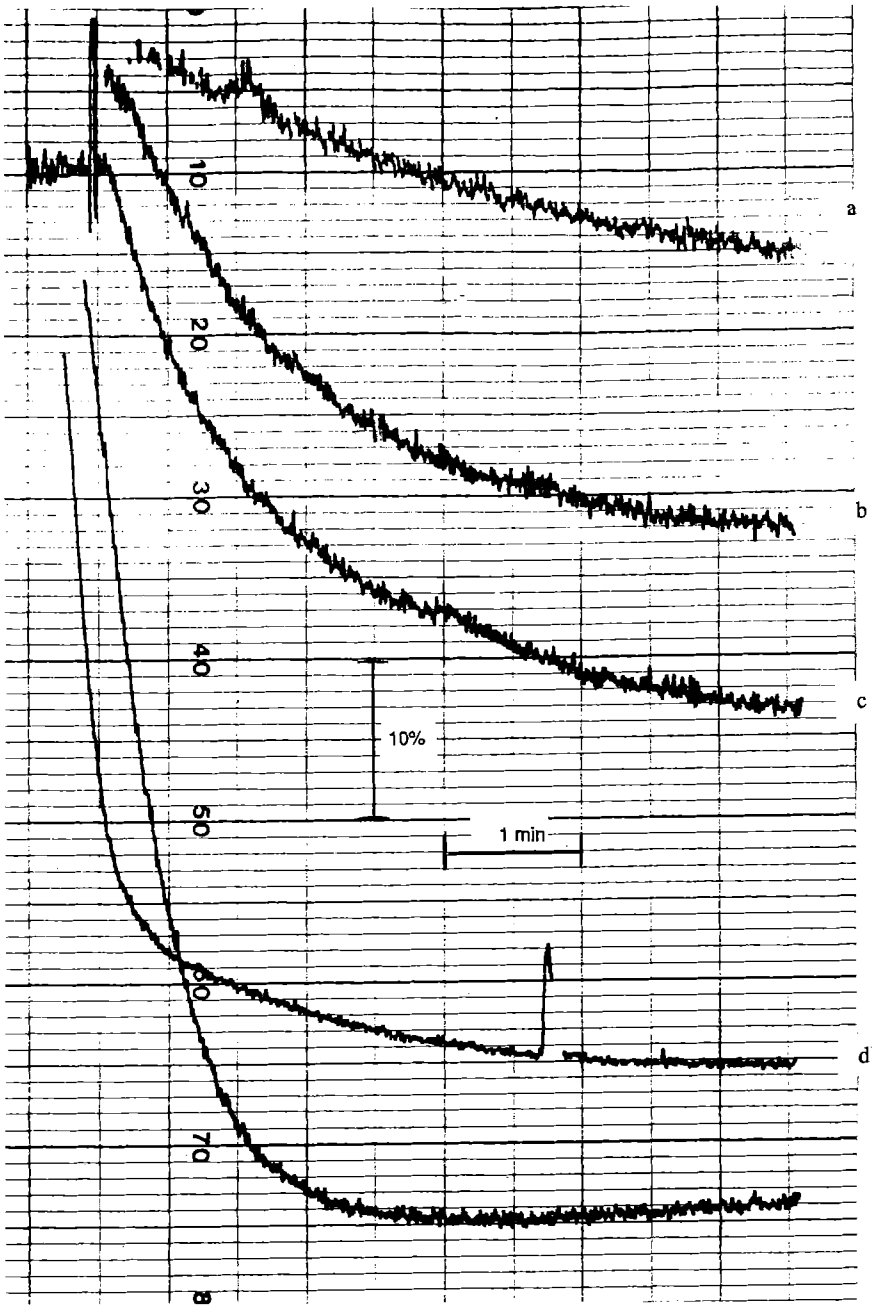


Fig. 10. Time-courses of ATP-dependent quenching of ACMA fluorescence by chromaffin granule "ghosts". "Ghosts" contained 0.3 M sucrose and 10 mM HEPES, pH 7.4. The counterions were: **a** TEA^+; **b** TEA^+/K^+ (1:9); **c** and **d** (two traces) K^+. The external medium (see Methods section) was supplemented with 1 μM valinomycin [**d**, *upper trace*], or 10 mM NaSCN [**d**, *lower trace*]

+40 mV, an additional closed state of intermediate mean duration). The shortest closures, in particular, were very flickery (poorly-resolved) under our recording conditions. The channel certainly appears to be distinct from that characterized by Arispe et al. (1992), which had a much lower conductance (only 160 pS in 400 mM K^+) and very different gating kinetics, although both are insensitive to ChTX and Ca^{2+}.

The demonstration of block by TEA^+ is in many ways a crucial part of this study, although at the single-channel level the analysis we employed has important limitations – for example, the binding site which we examined in some detail will be *physically* located some 20% of the way across the channel from the *trans* side only if the transmembrane voltage gradient is spread across the whole protein, and is linear (both of which are inherently unlikely). However, useful comparisons can be made with other K^+ channels for which TEA^+ has become an important structural probe. These include both Ca^{2+}-activated (including maxi-K) channels and exclusively voltage-dependent channels (see the review by Jan and Jan 1992). In general, TEA^+ block has been observed from both sides of many K^+ channels, and appears to occur at two sites – a "high affinity" site ($K_b(0) \sim 1$ mM), which is accessible externally in the case of plasma membrane channels, and an intracellular site of much lower affinity ($K_b(0) \sim 50$ mM). "Internal" block is always voltage-dependent, but the "external" site may be only barely within the electric field, if at all, although in molluscan neuronal (Hermann and Gorman 1981) and adrenal chromaffin cell (Yellen 1984) plasma membranes, the blockade of K^+ channels (identified as maxi-K chan-

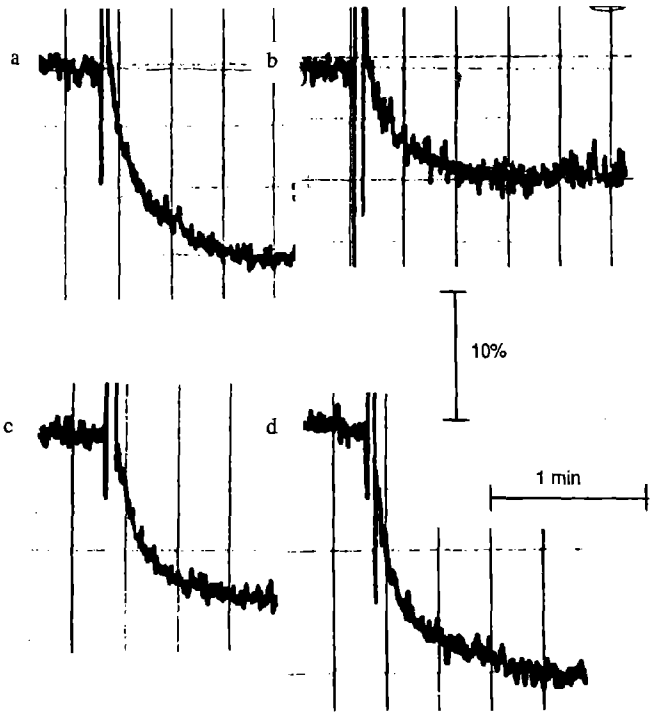


Fig. 11. Time-courses of ATP-dependent quenching of oxonol V. "Ghosts" contained 0.3 M sucrose and 10 mM HEPES, pH 7.4. The counterions were: **a** Na^+; **b** K^+; **c** TEA^+/K^+ (1:9); **d** TEA^+

nels in the latter case) by external TEA^+ clearly does exhibit some voltage-dependence. Maxi-K channels in muscle t-tubules also contain both (relatively) high- and low-affinity TEA^+ binding sites (Latorre et al. 1983). The former site was found to be located near the pore entrance (i.e. not very far down the voltage gradient) on the side opposite to that responsible for Ca^{2+}-sensitivity (identifying this as the lumenal face), and the block was not voltage-dependent.

We have also shown that TEA^+ had a marked effect on the process of V-ATPase mediated acidification of resealed chromaffin granule "ghosts", suggesting that the channel we have identified is important in the regulation of the granule's membrane potential. Addition of 1 mM internal TEA^+ (strictly equivalent to 0.6 mM, accounting for HEPES-buffering of the added TEAOH), but not external TEA^+, grossly limited the extent of acidification (as did substitution of Na^+ for internal K^+). These manipulations did not affect ATP hydrolysis, or proton-pumping by the partially-purified and reconstituted ATPase, suggesting that the pump itself was not directly affected. Instead, these observations support the hypothesis that a TEA^+-sensitive K^+ channel is present in the granule membrane, and allows continuous or intermittent charge-compensation to occur during proton-translocation. This would prevent the build-up of a large, internally positive $\Delta\psi$, which would back-off the pump. In the absence of such a pathway, charge movement by the ATPase would be expected to lead to higher membrane potentials, and this was confirmed in our experiments using the potential-sensitive dye Oxonol V. However, given the rapid rates of ion transport through even partially-activated ion channels (as compared to the turnover of ion pumps), and the relatively high (up to 40 mM) intragranular K^+ concentration (Ornberg et al. 1988; Haigh et al. 1989), it seems likely that we only have a very incomplete picture of this charge-compensation process. Nevertheless, these macroscopic experiments on a well-defined vesicle population lend strong support to the hypothesis that chromaffin granule membranes contain a charge-compensating TEA^+-sensitive K^+ channel. Also, intravesicular access to the site which is sensitive to $\sim$1 mM TEA^+ is topologically consistent with its presence on the *trans* side of reconstituted channels (in this context, see arguments quoted by Ashley 1989).

There are other possible roles for such a channel. For example, it could be more directly involved in amine accumulation, or in the mechanism of vesicle-plasma membrane fusion (either directly or indirectly). It may even be the object of a novel non-constitutive secretory mechanism (e.g. to deliver K^+ channels to the plasma membrane to accelerate membrane repolarization). It would clearly be helpful to pursue this work by carrying out patch-clamp studies on chromaffin granule membranes or "ghosts" (using for example the method of Keller and Hedrich 1992), and it will also be instructive to search for possible cytoplasmic and intralumenal modifiers of the reconstituted channels.

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