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Calcium-Independent K^+-Selective Channel from Chromaffin Granule Membranes

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Summary. Intact adrenal chromaffin granules and purified granule membrane ghosts were allowed to fuse with acidic phospholipid planar bilayer membranes in the presence of Ca^{2+} (1 mM). From both preparations, we were able to detect a large conductance potassium channel (*ca.* 160 pS in symmetrical 400 mM K^+), which was highly selective for K^+ over Na^+ (P_K/P_{Na} = 11) as estimated from the reversal potential of the channel current. Channel activity was unaffected by charybdotoxin, a blocker of the [Ca^{2+}]-activated K^+ channel of large conductance. Furthermore, this channel proved quite different from the previously described channels from other types of secretory vesicle preparations, not only in its selectivity and conductance, but also in its insensitivity to both calcium and potential across the bilayer. We conclude that the chromaffin granule membrane contains a K^+-selective channel with large conductance. We suggest that the role of this channel may include ion movement during granule assembly or recycling, and do not rule out events leading to exocytosis.

Key Words chromaffin granule · K^+ channel · planar bilayers channel reconstitution

Introduction

Ion channels occur in the membrane of secretory granules from adrenal chromaffin cells (Picaud et al., 1984), neurohypophyseal granules (Stanley, Ehrenstein & Russell, 1988; Lemos, Ocorr & Nordmann, 1989*a,b*), and synaptic vesicles from *Torpedo* electric organ (Rahamimoff et al., 1988, 1989). Since transmitter release and hormone secretion are known to occur as a consequence of fusion of secretory vesicle membranes with plasma membranes, it has been attractive from the earliest instance to hypothesize that these channels could be involved in the fusion event (Picaud et al., 1984; Stanley & Ehrenstein, 1985; Stanley, Ehrenstein & Russell, 1988; Lemos et al., 1989*a,b*; Rahamimoff et al., 1989, 1990). The contributions of the channels have been envisioned to be the regulation of osmotic gradients to support the work of membrane fusion and volume mixing (Pollard et al., 1979*a,b* 1984; Creutz & Pollard, 1980; Pollard, Pazoles & Creutz, 1980; Stanley & Ehrenstein, 1985; Finkelstein, Zimmerberg & Cohen, 1986; Stanley et al., 1988) and, to support direct contact between fusion membrane partners (Breckenridge & Almers, 1987). Indeed, a possible relationship between channel and fusion properties has been directly demonstrated for the case of synexin (annexin VII), which is a cytosolic, calcium-binding protein with both Ca^{2+}-channel and membrane fusion properties (Pollard & Rojas, 1987; Pollard, Burns & Rojas, 1988). In addition, formation of the "fusion pore" (Ornberg & Reese, 1981) has been associated with conductance changes (Breckenridge & Almers, 1987). Nonetheless, no firm evidence exists to allow correlation of these conductance changes with synexin, or indeed with channels which are otherwise *resident* in vesicle membranes.

A key problem facing the investigation has been the difficulty of identifying and studying the properties of the genuine vesicle membrane channel proteins. Indeed, the characteristics have proven varied, and sometimes unique to different vesicle types. Anion channels were first detected biochemically in bovine chromaffin granules (Pazoles & Pollard, 1978; Pollard et al., 1979*a*; Pollard et al., 1980), and later found to occur in rat neurohypophysis (Stanley et al., 1988), and fused *Torpedo* secretory vesicles (De Riemer et al., 1987; Rahamimoff et al., 1988). Furthermore, [Ca^{2+}]-dependent K^+ channels have been reported in rat hypophyseal granules (Stanley et al., 1988; Lemos et al., 1989*a,b*). In addition, Rahamimoff et al. (1990) reported two types of cation selective channels in *Torpedo* vesicles which were characterized as being of large conductance, low selectivity, calcium activated and voltage regulated. However, of the increasing number of proteins found to be localized in small synaptic vesicles (for references *see* Knaus et al., 1990) and, to some extent, in adrenal chromaffin granules (Picaud et al., 1984), only synaptophysin has been shown to exhibit cation-selective channel activity (Thomas et al., 1988). Thus, a compelling interpretation of these channels in terms of regulation of exocytosis has

proven elusive, and may prove increasingly difficult as the details of the complex biochemistry of these organelles become fully appreciated (Lee et al., 1992).

In this regard, recent elemental imaging studies of secretory granules both inside adrenal chromaffin cells and following isolation, clearly indicate that concentrations of K^+ and Na^+ can change substantially during subcellular fractionation (Ornberg, Kuijpers & Leapman, 1988). Furthermore, the ion content of the isolation buffer was found to directly affect the ionic composition within granules. For example, the concentrations of K^+ and Na^+ within the granules are 83 and 0 mM, respectively, in intact adrenal chromaffin cells. However, following isolation in a Na-containing buffer, the K^+ and Na^+ concentrations changed to 40 and 19 mM, respectively. Yet, other constituents such as nitrogen (ATP and catecholamines), phosphorous (ATP), calcium and magnesium remained constant (Ornberg et al., 1988). The simplest interpretation of these data could be that cation channels occur within the vesicle membranes, and that their function is compatible with sustained stability of the granules. However, chromaffin granule membranes have never been studied from this perspective before, and it is not even known whether they contain such channels. Indeed, adrenal chromaffin granules are notorious for their impermeability to ions other than chloride and protons (Brocklehurst & Pollard, 1988*a*,*b*).

We have therefore searched for possible cation channels in granule membranes, using a method suitable to fuse the vesicles (as either intact granules or granule ghosts) with planar lipid bilayers and to detect channel activity. In this paper we report that intact chromaffin granules possess, at least, two types of K^+ channels, one of which is frequently observed in highly purified chromaffin granule membrane ghosts. This channel is of large conductance (*ca.* 160 pS), is high selective for K^+ over Na^+, and is *insensitive* to Ca^{2+} or charybdotoxin, a blocker of the $[Ca^{2+}]$-activated K^+ channel of large conductance (Anderson et al., 1988; MacKinnon & Miller, 1988). We concluded that the properties of this channel may best be interpreted in terms of a system important in granule assembly or recycling, but do not exclude a role in catecholamine equilibrium distribution or exocytosis.

Materials and Methods

Preparation of Chromaffin Granules

Chromaffin granules were prepared from bovine adrenal medulla tissue by homogenization in 0.3 M sucrose and purified on a metrizamide step gradient, between densities 1.120 and 1.104 g/cm^3 as previously described (Pollard et al., 1979*a*; Pazoles et al., 1980). The chromaffin granules were found to be substantially purified from contaminating mitochondria, plasma membranes, and lysosomes. Chromaffin granules, prepared in this way, constitute a virtual model for organelle purification. Briefly, the method included measurement of mitochondrial marker monoamine oxidase by oxidation of radiolabeled tyramine, as described elsewhere (McCaman et al., 1965). Lysosomal marker acid phosphatase was measured by hydrolysis of *p*-nitrophenyl phosphate, as described in Bergmeyer (1974). Plasma membrane marker 5′-nucleotidase activity was evaluated by release of radiolabeled adenosine from adenosine monophosphate (Zinder et al., 1978). Granule content markers, epinephrine and norepinephrine, were measured by the trihydroxyindole method (Anton & Sayre, 1962) as modified in our laboratory (Kelner et al., 1985). Protein was measured by Coomassie blue (Bradford, 1976), as modified for application to chromaffin granule preparations (Pollard et al., 1978). Granule marker dopamine-beta-hydroxylase was measured, as described elsewhere (Nagatsu & Udenfriend, 1972). Finally, granule membrane marker cytochrome *b*-561 activity was measured by the difference between dithionite-reduced and oxidized states at 429 nm (Zinder et al., 1978). However, the activities of marker enzymes for contaminating subcellular organelles are not zero. For this reason, having identified the channel in intact granules, we proceeded to further purify the granule membrane fraction, as described below. Following equilibrium centrifugation, the metrizamide was diluted by 3 volumes of 0.3 M sucrose and centrifuged at 49,000 × *g*. The granules were then resuspended in 0.3 M sucrose and stored at 4°C.

Preparation of Chromaffin Granule Ghosts

Chromaffin granule ghosts were prepared from bovine adrenal medulla tissue by homogenization in 0.3 M sucrose and purification over a 1.6 M sucrose step, as previously described (Brocklehurst & Pollard, 1990). Thereafter, the pellet from the 1.6 M sucrose step was resuspended by homogenization in 50 volumes of ice-cold 5 mM Na-HEPES, pH 7.2, and allowed to remain on ice for 30 min. The solution was centrifuged at 49,000 × *g*, and the resulting pellet then resuspended by homogenization in 5 ml of 0.3 M sucrose to effect a second osmotic shock. Final purification was carried out by centrifugation over a 1 M sucrose step (density = 1.10 gm/cm^3). The ghosts remaining at the top of the gradient after centrifugation at 36,000 rpm were subjected to a final osmotic shock by a 10-fold dilution in buffer. These membranes were then centrifuged at 49,000 × *g*, and resuspended in a minimal volume of buffer before being stored at −70°C.

Our ghost preparation lacks detectable monoamine oxidase and acid phosphatase activity, although it does possess measurable adenylate cyclase activity, which is specific to the chromaffin granule membrane (Zinder et al., 1976, 1977, 1978), as well as low but specific levels of 5′ nucleotidase (Zinder et al., 1978). However, the purified granule membranes lack tubulin and actin, which can be identified on the basis of ^{131}I-labeled tryptic digests of parallel bands or vacant band regions from gels of purified chromaffin cell plasma membranes and chromaffin granule membranes (Zinder et al., 1978). In addition, electron micrographs of purified chromaffin granule membranes are devoid of non-vesicular formed elements (Zinder et al., 1978).

Bilayer Setup

The experimental chamber (made of plexiglass) consisted of two compartments separated by a thin Teflon film. During experiments, the solutions were simultaneously stirred by two Teflon-coated magnets placed in a restricted space at the bottom of each compartment. Ag/AgCl pellet electrodes were immersed in a small pool containing 0.5 M KCl and were electrically connected to the solutions in each compartment via agar bridges (2% agar in 0.5 M KCl). Single channel currents were recorded using a patch-clamp amplifier (EPC-7, List Medical Electronics or Axopatch-1D, equipped with a CV-4B bilayer headstage, Axon Instruments) and were stored on magnetic tape using a PCM/VCR digital system (Digital-4, Toshiba) with a frequency response in the range from DC to 25,000 Hz. Records were made from playbacks through a lowpass filter (8-pole Bessel 902 LPF, frequency devices) set in the range from 200 to 500 Hz.

Bilayer Formation

Planar bilayers were formed by applying a suspension of synthetic palmitoyl-oleolyl-phosphatidyl-ethanolamine (POPE) and phosphatidylserine (PS, 50 mg/ml) in decane. A small glass rod was used to deliver the lipids to a hole of *ca.* 100–150 μm in diameter in a Teflon film separating two compartments that contained the required salt solutions.

Channel Incorporation

To fuse intact granules with a lipid bilayer, we prepared a suspension of the intact vesicles in a solution mimicking the ion composition of the cytosol (in mM): 115 K-glutamate, 36 K-PIPES, 8 $MgCl_2$, 5 NaCl, 5 KCl, and 5 glucose, pH 6.8. Ion channels, presumably present in secretory granules from adrenal chromaffin cells, were incorporated into a bilayer by adding a small volume (5–10 μl) of this suspension of intact vesicles to a different K-HEPES (either 200 or 400 mM) solution containing 1 mM $CaCl_2$ to facilitate membrane fusion. The highly purified ghosts were also resuspended in a K-HEPES solution of the following composition (in mM): 400 K-HEPES, 10 Tris-HEPES, 1 $CaCl_2$, pH 7.4. This high K-HEPES solution was used inside both the *cis* and the *trans* side compartment of the bilayer chamber in all the experiments with ghosts. Granules (or membrane ghosts) were added on only the *cis* side of the chamber, and incorporation occurred directly from the experimental solutions.

Results

Cationic Channels from Intact Chromaffin Granules

To minimize the lysis of adrenal chromaffin granules we suspended them in a solution known to represent the ion content of chromaffin cell cytosol (Ornberg et al., 1988). To facilitate the fusion of the vesicles with the bilayer, we added $CaCl_2$ (1 mM) to both sides. Exposure of the *cis* side of the bilayer to intact granules caused the otherwise electrically silent bilayer to acquire ion channel activity. In the specific example shown in Fig. 1*A*, three channels were found to be expressed, of which two were of identical conductance. Regions of the traces in Fig. 1*A* (*a–d*) are also shown in Fig. 1*B* using expanded time scale. In region "*a*", to the far right of the record, there is evidence of two channels which open simultaneously. The record of the activity of the channel with a conductance equal to 96 pS has superimposed on it two openings of a channel with a conductance of 76 pS. By contrast, in record "*d*" two 96 pS channels open simultaneously. As anticipated from the data in Fig. 1*A* and *B*, we were able to distinguish at least two separate, high conductance channels, for which the current-voltage (*I-V*) curves had nonlinear characteristics as shown in Fig. 1*C*. We also noted in all our experiments that the kinetics of these channels seemed insensitive to the potential gradient.

Although the method used to prepare the adrenal chromaffin granules employed in the preceding experiments yielded highly purified intact vesicles (Pollard et al., 1979*a,b*), we now included additional purification steps to exclude possible contaminating membranes. Furthermore, to study any possible modulation of the K^+-channel activity by intracellular molecules, we decided to minimize the concentration of non-membrane granule constituents. We therefore lysed adrenal chromaffin cell granules and prepared highly purified granule ghosts by a procedure employing two separate gradients and three osmotic lysis steps (*see* Materials and Methods). The channel activity acquired when intact chromaffin granules were allowed to fuse with the bilayer was remarkably similar to that observed if these purified chromaffin granule ghosts were used instead.

Potassium Channels from Purified Chromaffin Granule Membrane Ghosts

Assuming that the main anions used in our solutions (glutamate, HEPES and PIPES) are not permeant, K^+ ions might seem to be the main ions carrying the current. To increase the amplitude of the single channel current, we augmented the concentration of K^+ from physiological 156 (Fig. 1) to either 400 (*see* Figs. 2, 4–7) or 200 mM (Fig. 3).

As shown in Fig. 2, we were able to detect only one type of K^+ channel with chromaffin granule ghosts as the donor membrane. This conclusion is based on 27 incorporations out of 29 attempts using three different preparations of granule ghosts. Indeed, from one preparation to the next, the only variability was a difference in the frequency of open-

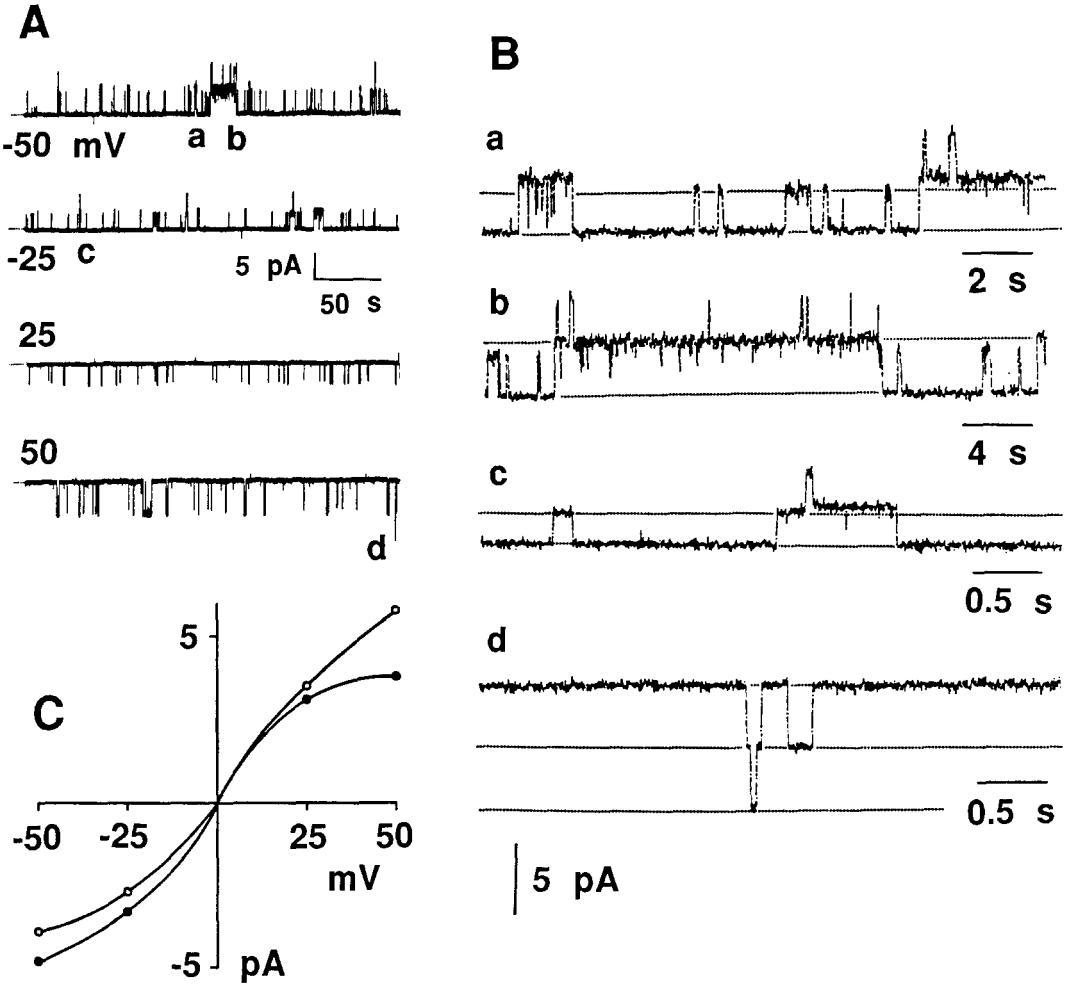

Fig. 1. Incorporation of cationic channel activity resulting from the fusion of intact chromaffin granules with the bilayer. Symmetrical K^+ solution (in mM: 115 K-glutamate, 36 K-PIPES, 8 $MgCl_2$, 5 NaCl, 5 KCl, 5 $CaCl_2$, pH 7.4) in both compartments of the bilayer chamber. (*A*) Four consecutive segments of a continuous record at different transmembrane potentials as indicated in mV above each record. Upward and downward deflections represent open channel currents. (*B*) Segments *a-d* were taken from the records on part *A* and are shown on expanded time scale. For *a-b* upward deflections represent open channel currents (at −50 mV). For *c-d* downward deflections represent open channel currents. (*C*) *I-V* curves for the two channel events present in the records.

ings. In Fig. 2, records from two different experiments are shown in *A* and *B*, respectively. Indeed, the frequency of channel openings in Fig. 2*B* is higher than that shown in Fig. 2*A*. The records in Fig. 2*B* also exhibit long lasting silent periods (for further kinetic properties *see* next section). Based on the *I-V* curve depicted in Fig. 2*C*, we found the channel to be relatively linear between −40 and 40 mV, with a slope conductance of *ca.* 160 pS.

As indicated above, low selectivity for cations has been a hallmark of the secretory vesicle membrane cationic channels hitherto reported (De Riemer et al., 1987; Rahamimoff et al., 1988; Lemos et al., 1989*a,b*; Lee et al., 1992). We therefore investigated the selectivity of the present chromaffin granule ghosts channel by recourse to a measurement of the reversal potential of the single channel current (V_{rev}) in the presence of K-HEPES on the *cis* side and K-HEPES plus Na-HEPES on the *trans* side.

We calculated the *I-V* curve from the mean amplitude of the channel current at different potentials (Fig. 3). For this experiment we used intact granules and the channel incorporated was exposed to 200 mM K-HEPES on the *cis* side and 100 mM K-HEPES plus 100 mM Na-HEPES on the *trans* side. Samples of the recorded channel activity at different potentials are shown on the right (Fig. 3). From these records (K^+ as the charge carrier) and the reversal potential (−15 mV), it is clear that the cationic channels incorporated together with the membranes of

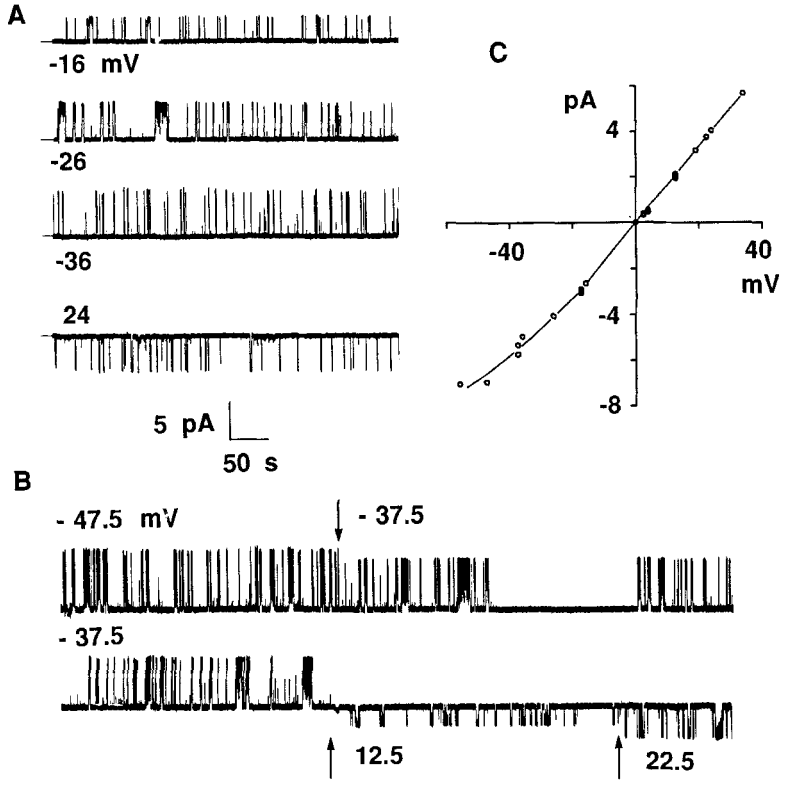

Fig. 2. Incorporation of potassium-channel activity resulting from the fusion of highly purified chromaffin granule membrane ghosts with the bilayer. (*A,B*) Single K^+-channel activity from two different preparations of granule ghosts. At −16, −26 and −36 mV for panel *A* (or −47.5 and −37.5 mV for panel *B*) upward deflections represent open channel currents. Symmetrical K-HEPES solution (400 mM) in both compartments. (*C*) Values for the *I-V* curve were obtained from amplitude histograms constructed from databases of at least 2000 events.

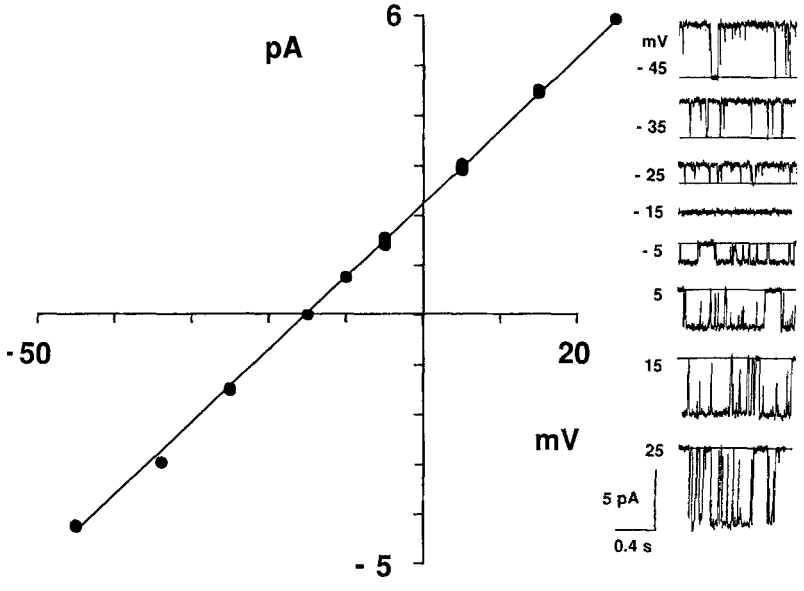

Fig. 3. Potassium selectivity of the cation channel from granule ghosts. Vertical axis represents the mean value from the amplitude histogram of single channel events at the indicated transmembrane potential (abscissa). Composition of the solution (in mM): *Cis* side, 200 mM K-HEPES, pH 7; *Trans* side, 100 K-HEPES, 100 Na-HEPES, pH 7. Segments of the channel activity with K^+ as charge carrier at each transmembrane potential are shown on the right side of the *I-V* curve. The closed state of the channel is indicated by the line. Slope for the straight line, 148.6 pS.

the chromaffin granules were highly selective for K^+. The reversal potential (V_{rev}) of −15 mV was determined from the *I-V* curve in Fig. 3 as the potential at which the net flow of current across the open channel is zero. From the equation

$$V_{rev} = RT/F \ln \{(P_{Na}[Na]_{trans} + P_K[K]_{trans}\}/P_K[K]_{cis}\}$$

we calculate the permeability ratio P_K/P_{Na} to be 10. The reversal potential for the bi-ionic condition Na-HEPES in the *trans* side and K-HEPES in the *cis*

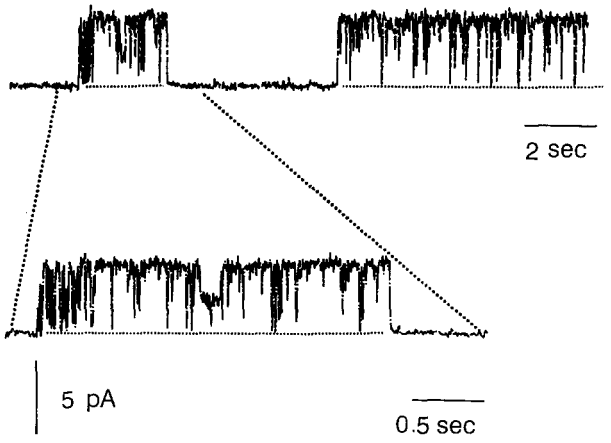

Fig. 4. Multiple open states of the granule ghost channel. Transmembrane potential equal to −50 mV and symmetrical K-HEPES (400 mM). Lower record: expanded time base to show details of the channel activity during a burst (upper record, left side). Horizontal dotted line indicates the closed state of the channel.

side was −62 mV giving a permeability ratio P_K/P_{Na} equal to 11.2. We therefore feel it appropriate to refer to this chromaffin granule ghost channel as a potassium channel (from now on referred to as the CG-type K^+ channel), and thus distinct from the previously described cation selective channels in other secretory vesicle membrane preparations.

Kinetic Behavior of the Chromaffin Granule Ghost K^+ Channel

In all planar bilayer membranes (n = 29) which exhibited the CG-type K^+ channel we observed a very characteristic type of bursting behavior. As illustrated in Fig. 4, we found the channels to go through brief (*ca.* 1 msec) and frequent transitions from the open to the closed state. We also frequently observed single channel current levels of smaller amplitude wihch we interpreted as incomplete closures. One such example is shown in Fig. 4 on an expanded time scale.

From records like those shown in Fig. 5, we also measured the time intervals between channel openings (distribution of closed times) and between channel closings (distribution of open times). The data base used for the open-time and closed-time histograms corresponds to recordings with a signal-to-noise ratio of >10 : 1 (Fig. 5). All events of duration <0.2 msec have been discarded, although omitting such events does introduce some distortion of the observed channel kinetics. The number of events for the data base associated with each histogram was always >2000. The minimum number of open states entered by the CG-type K^+ channel can be estimated determining the number of exponential components necessary to fit the observed open-time distribution (Sakmann & Neher, 1983). As shown in Fig. 6, open-time and closed-time histograms clearly required more than one time constant to fit the points. At a transmembrane potential of −50 mV the time constants were 10.5 and 40.5 msec for the open distribution (Fig. 6, upper right side) and 1.8 and 7.3 msec for the closed distribution (Fig. 6, lower right side). These kinetic parameters were not affected by Ca^{2+} (*see* next section). We concluded that the flickering of the channel current is presumably the consequence of the passage through more than one state during the transition from closed to fully open configuration. The presence of more than one closed configuration for the CG-type K^+ channel might explain the interburst behavior.

The CG-type K^+ Channel is Insensitive to Charybdotoxin and Ca^{2+}

Throughout these experiments we maintained the calcium concentration at 1 mM, both to promote fusion of the ghosts with the target bilayer, and to support what we had anticipated would be the calcium dependence of the ion channel activity. However, early in our studies we noted that removal of calcium or addition of EGTA had no demonstrable effect on the K^+_{CG}-channel activity, once it was incorporated and functional in the bilayer. In addition, to further allay our continuous concerns regarding possible trace contamination of our ghost preparations by non-chromaffin granule membranes, we also tested the CG-type K^+ channels for sensitivity to charybdotoxin. This toxin is a highly selective inhibitor of the [Ca^{2+}]-sensitive K^+ channel of large conductance known to be present in the plasma membrane of chromaffin cells (Fenwick, Marty & Neher, 1982). The record in Fig. 7 illustrates that the activity of the CG-type K^+ channel remained unchanged after the consecutive additions of charybdotoxin (0.1 μM) and EGTA (1.5 mM) to both *cis* and *trans* compartments. We conclude from these experiments that the K^+ channel described here is insensitive to [Ca^{2+}], and very likely derived from the chromaffin granule membrane.

Discussion

A K^+-Selective Channel of High-Conductance is Present in the Adrenal Chromaffin Granule Membrane

The main finding in our study is that the chromaffin granule membrane contains a K^+ selective channel

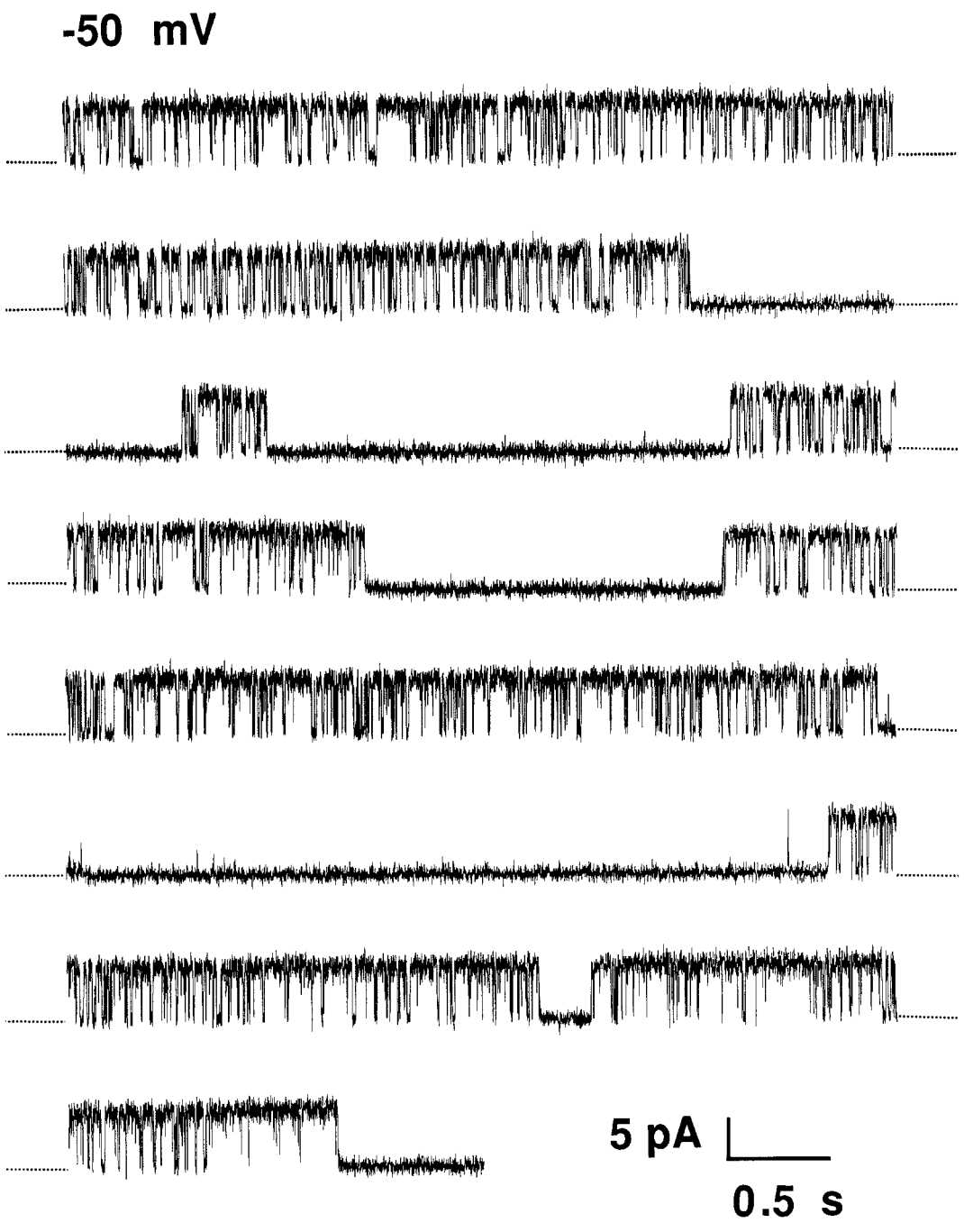

Fig. 5. Bursting behavior of the granule ghost channel activity. Eight segments from a continuous record of the K^+-channel activity. Records made in the presence of symmetrical K-HEPES (400 mM). The closed state of the channel is indicated by the dotted lines on the left and right side of each record.

with large conductance, which lacks sensitivity to either $[Ca^{2+}]$ or charybdotoxin, a selective blocker of the high-conductance $[Ca^{2+}]$-activated K^+ channel (MacKinnon & Miller, 1988). These characteristics clearly distinguish the K^+_{CG}-channel from other K^+ channels previously reported for rat hypophyseal granules (Lemos et al., 1989*a*,*b*; Lee et al., 1992) and secretory cholinergic vesicles from *Torpedo* (Rahamimoff et al., 1989). Furthermore, the high selectivity for potassium ions of the CG-type K^+ channel are clearly distinct from those briefly reported for the non-selective synaptophysin cation

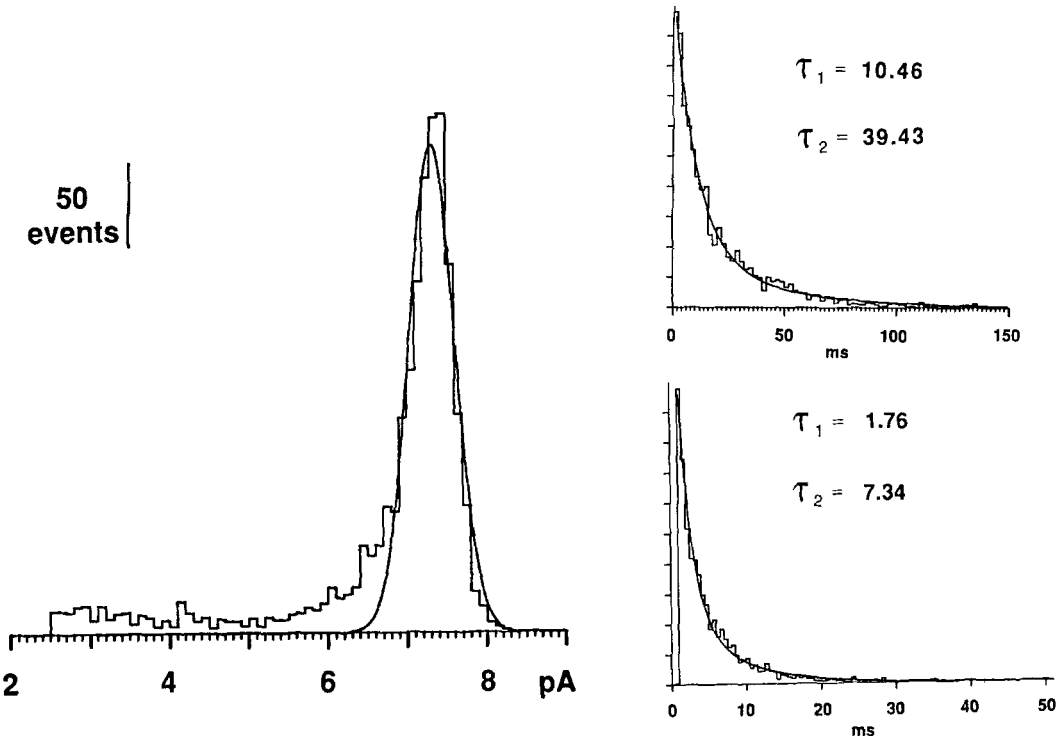

Fig. 6. Amplitude, open- and closed-time histograms of the granule ghost channel. Transmembrane potential equal to −50 mV and symmetrical K-HEPES (400 mM). Left panel: Distribution of single channel current amplitudes. Fitted curve was calculated as

$$dp/di = 1/(2\pi)^{0.5} \exp[-(1 - \langle i \rangle)^2/2\sigma^2]$$

with the following values for the parameters: $\langle i \rangle = 7.3$ pA and $\sigma^2 = 0.3$ pA^2. For the fit, only values smaller than 8.4 pA and greater than 6.5 pA were included. The bars from 2.2 to 6.5 represent different open states of the channel. Right panel: Open- (upper) and closed-time histograms (lower). For each histogram the fitted line corresponds to $NE_o = \Sigma NE_i \exp[-t/\zeta_i]$ where i represents the number of states and NE_o open state event frequency. ζ_i values are given next to the corresponding fitted curve.

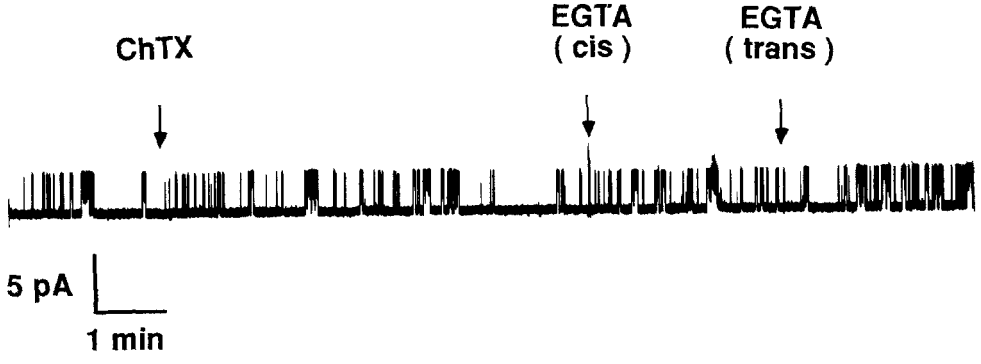

Fig. 7. The granule ghost K^+ channel is insensitive to charybdotoxin and $[Ca^{2+}]$. Transmembrane potential equal to −25 mV and symmetrical K-HEPES (400 mM). Upward deflections represent open channel currents. Charybdotoxin (ChTX) was added to both sides (100 nM ChTX) as indicated (left arrow). EGTA (1.5 mM) was added as indicated to the *cis* side (center arrow) and to the *trans* side (right arrow). During each addition at the time indicated by the arrows, the recording was interrupted and the solutions on both sides were stirred. For this reason the playback does not exhibit the stirring artifacts.

channel (Thomas et al., 1988). Thus, synaptophysin, known to be present in chromaffin secretory vesicles (Obendorf et al., 1988), cannot be responsible for the channel activity described here.

Possible Physiological Role of the CG-Type K^+ Channel

The properties of the chromaffin granule K^+ channel do not necessarily exclude a possible role in secretion, but they do indicate that it might also be reasonable to consider the channel in terms of other granule functions. Some of these other functions, as they impact on establishment of ion gradients, are summarized in Fig. 8, where we delineate, where possible, the previously documented elemental compositions for K^+, Na^+, Ca^{2+} and Cl^- of various chromaffin cell compartments (Ornberg et al., 1988). The granules are assembled in the golgi (Fig. 8, *I*), where they begin to assume the composition of the mature granule. We lack composition data from elemental electron probe microanalysis for this nascent state, and so must leave it blank for the present. The mature granule then moves into the cytosol, where it is poised ready to fuse during exocytosis (Fig. 8, *II*). The concentrations of different ions are known with accuracy and precision for granules in this state (Ornberg et al., 1988), and written within the compartment. Following fusion and exocytosis (Fig. 8, *III*), the loss of granule contents is paralleled by the acquisition of ions and other bulk phase components from the extracellular medium. Substantial experimental data exist to substantiate this claim. For example, the exposure of dopamine β-hydroxylase (DBH) and other glycoproteins on the inner surface of the chromaffin granule during exocytosis allows antibodies to these proteins to be bound and internalized upon retrieval of expended granule membranes (Lingg et al., 1983). The extent of this replacement of granule contents by bulk phase solution can also be appreciated by the fact that retrieval is even accompanied by uptake of macromolecules such as horseradish peroxidase, which are then available for re-secretion (Baker & Knight, 1981; Von Grafenstein, Roberts & Baker, 1986). It is therefore likely that the retrieved ghost is equilibrated with an ionic composition that is similar to that of the medium, and thus entirely different from that of the cytosol or of the mature granule.

The recycling process in the chromaffin cell is also well established on both morphological and biochemical grounds (for review *see* Trifaro & Poisner, 1982) and newer membrane capacitance data is also consistent with this concept (Neher & Marty, 1982; Penner & Neher, 1989; Lindau, 1991). Of course, we need not assume that the chromaffin granule must have a specific ionic composition in order to be competent to accumulate new transmitter. However, it is likely that sometime during the recycling process the intragranular $[Na^+]$ must change from the extracellular concentration, roughly 152 mM, to the concentration of the mature granule (*ca.* 0 mM). Coincidentally, the intragranular $[K^+]$ must increase from 6 mM (extracellular concentration) to 83 mM. This process is represented by Fig. 8, *IV* (retrieval) and *V* (recycling). The time scale for re-secretion of acquired contents from the medium is known to be *ca.* 30 min (Lingg et al., 1983), but no information is available regarding the rate for resetting the intragranular milieu with ions or other granular contents.

We are now in a position to ask in what manner the K^+_{CG}-channel might contribute to any of the above described processes in the chromaffin cell. An obvious possibility is the regulation of chemiosmotic events across the granule membrane (for review *see* Brocklehurst & Pollard, 1988*a*,*b*). It is well-established that the inwardly directed proton pump present in the chromaffin granule membrane creates a proton gradient with the granule interior having a lower pH than the outside. The activity of this proton pump is also expected to make the granule transmembrane electrical potential positive inside. Indeed, while in the absence of MgATP the transmembrane potential of isolated chromaffin granules was estimated as close to -10 mV, in the presence of MgATP the proton pump in the granule membrane generates a potential of 50 to 70 mV inside positive (Pollard et al., 1976; Holz, 1979; Salama, Johnson & Scarpa, 1980). Since transport and accumulation of catecholamines depends on the electrochemical force across granule membrane, one might then ask whether the CG-type K^+-channel might be involved in setting the electrical potential component of the electrochemical gradient across the granule membrane.

To answer this, one might first ascertain whether the channel is ever open, in vivo. One approach to this question is to calculate the Nernst potential for K^+ and compare it with the measured potential. From concentration measurements of rapidly frozen chromaffin cells (Ornberg et al., 1988) we calculate the K^+ potential to be close to 28 mV instead of the measured 50 to 70 mV (positive inside the granule). Thus, for mature chromaffin granules the new K^+ channel may be closed. However, for retrieved ghosts the concentration gradient for K^+ might be capable of sustaining a large negative potential similar to that of the plasma membrane of the chromaffin cell, i.e., -65 mV (Fenwick et al., 1982). At the very least, this potential gradient will favor the catecholamine loading of the granule.

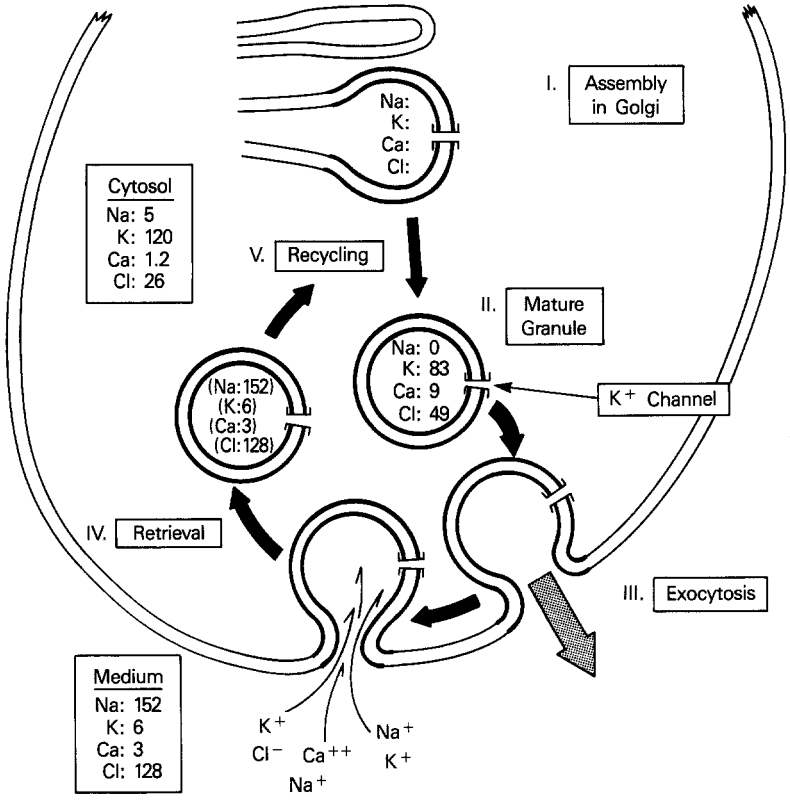

Fig. 8. Possible role of the CG-type K^+ channel in the redistribution of ions in the chromaffin granule. Numbers represent mmol/kg water.

With regard to the granule assembly and recycling processes, it is also certainly possible that the ghost K^+-channel could mediate potassium ion movement to establish the 83 mM K setpoint. During the assembly process, the nascent granule could begin with an intragranular potassium concentration of 123 mM, similar to that of the cytosol. In this case, progress to the mature 83 mM state would have to entail loss of K^+. Previous studies have anticipated that the granule ATPase might be involved in coincident acidification of the nascent granule and, if this occurred, entering H^+ could exchange for exiting K^+. To lose *ca.* 40 mE/l of K^+, the granule would have to acquire 40 mE/l of H^+. Indeed, since the buffering capacity of the granule contents is close to 300 mE/l (Salama et al., 1980), the added protons could be accommodated easily. By contrast, the exchange of endogenous, buffered protons for exogenous potassium with the antibiotic nigericin is known to *destabilize* mature granules (Pazoles & Pollard, 1978). Therefore, we cannot exclude the possibility that the opposite action, of replacing potassium with buffered protons during assembly, could be responsible for the acquired *stability* of the otherwise "hypertonic" granule contents in an isotonic (300 mOsm) cytosolic medium. Clearly, any required potassium movements could be mediated by the new K^+ channel.

In the case of a recycling granule (*step V*), the new CG-type K^+ channel described here could mediate the necessary *addition* of potassium, as described above. Referring to Fig. 8, *step IV*, it is also clear that in addition to potassium movements into the retrieved granule ghost, considerable amounts of sodium will have to be transferred out of the organelle. However, given the selectivity of the K^+_{CG}-channel, it would be unlikely that the K^+ could be involved in Na^+ movement. Perhaps the Na^+/Ca^{2+} exchange activity could be recruited for part of this effort, but the need for sodium movement far exceeds the amount of calcium to be moved in opposite direction. Alternatively, an additional, as yet undetected mechanism may exist for sodium movement. Finally, substantial amounts of chloride must also be moved out of the retrieved granule, but there does exist an anion channel in the granule membrane (Pollard et al., 1979*a*) which could be used for this purpose.

The case for invoking a role for the CG-type K^+ channel in the assembly or recycling process is only compelling in the sense that the chemical data requires a mechanism for moving large amounts of K^+

through the granule membrane. Until the present discovery, there was no basis for understanding how this could happen. We can conclude now that the CG-type K^+ channel is thus a candidate for this transfer mechanism. We do not exclude this or other membrane resident channels from involvement in the exocytosis process, except to emphasize that the properties of the new K^+ channel do not make an obvious case for this possibility. The chemical data also predict that there should also exist a mechanism for moving substantial amounts of sodium across the granule membrane. However, considering the permeability ratio for K^+/Na^+, we must also conclude that it is unlikely that the new K^+ channel could mediate sodium movement. Presumably, another channel, as yet undetected, must also exist in the granule membrane. Indeed, while we initially detected at least two cationic channels in the membranes of *intact* chromaffin granules, we were only able to detect and study one in the purified granule ghost preparation. We conclude that the chromaffin granule membrane remains to be fully investigated in terms of its ensemble of ion conductance mechanisms.

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