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THE HETEROGENEITY OF ION CHANNELS IN CHROMAFFIN GRANULE MEMBRANES

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Abstract: Chromaffin granules are involved in catecholamine synthesis and traffic in the adrenal glands. The transporting membrane proteins of chromaffin granules play an important role in the ion homeostasis of these organelles. In this study, we characterized components of the electrogenic $^{86}Rb^{+}$ flux observed in isolated chromaffin granules. In order to study single channel activity, chromaffin granules from the bovine adrenal medulla were incorporated into planar lipid bilayers. Four types of cationic channel were found, each with a different conductance. The unitary conductances of the potassium channels are 360 ± 10 pS, 220 ± 8 pS, 152 ± 8 pS and 13 ± 3 pS in a gradient of 450/150 mM KCl, pH 7.0. A multiconductance potassium channel with a conductivity of 110 ± 8 pS and 31 ± 4 pS was also found. With the exception of the 13 pS conductance channel, all are activated by depolarizing voltages. One type of chloride channel was also found. It has a unitary conductance of about 250 pS in a gradient of 500/150 mM KCl, pH 7.0.

Key words: Chromaffin granule, Intracellular channel, Potassium channel, Chloride channel, Black lipid membrane

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Abbreviations used: BLM – black lipid membrane technique, I – single-channel current amplitude, K_{CG} – large conductance potassium channel, P_o – open-probability; U – potential, U_{rev} – reversal potential, γ – ion conductance, τ_o – mean open lifetime, τ_c – mean closed lifetime

INTRODUCTION

Ion channels selective for potassium and chloride ions are present in all intracellular membranes [1]. They have been found in the endo/sarcoplasmic reticulum, Golgi apparatus, lysosomes, endosomes and mitochondria [2]. Recently, mitochondrial potassium channels have become a focus of researchers' attention due to the observation that the pharmacological modulation [3, 4] of their activity yields a cytoprotective effect in different cell types [5, 6]. These channels are also present in the membranes of secretory vesicles such as chromaffin and zymogen granules, and synaptic vesicles [7]. Intracellular pathways for K^+ and Cl^- transport via ion channels are involved in a variety of processes, such as organelle volume regulation, charge compensation and pH homeostasis in specific cellular compartments [8-10].

Ion channels are also present in the membranes of chromaffin granules from the adrenal medulla. Chromaffin granules are involved in catecholamine synthesis and traffic. By the process of exocytosis, the chromaffin cell secretes a complex mixture of catecholamines, ATP, ions, proteins and peptides. The uptake of catecholamine into chromaffin granules is driven by the pH gradient [11].

The chromaffin granule ion channels were investigated via electrophysiological and macroscopic flux measurements. Single channel recordings were obtained both with the patch-clamp technique and after fusion of purified granule membranes with a bilayer membrane (BLM). Several different cation-selective channels were described after the incorporation of intact chromaffin granules, but only two types of highly selective K^+ channel could be reconstituted from a preparation of chromaffin granule "ghosts" [12, 13]. A K^+-selective, large conductance (~160 pS in symmetrical 400 mM KCl) channel was described in [12]. It was insensitive to charybdotoxin, a blocker of the Ca^{2+}-activated K^+ channel of large conductance. The channel's activity was also unaffected by Ca^{2+} and potential across the bilayer [12]. It was also reported that the chromaffin granule K^+ channel was controlled by both inhibitory and stimulatory heterotrimeric GTP-binding proteins [14]. A similar channel, highly selective for potassium, but with a different conductance (~400 pS in symmetric 450 mM KCl), was described in [13]. This channel was insensitive to both Ca^{2+} and charybdotoxin, and was blocked by TEA^+. There is also evidence of anion channels in chromaffin granule membranes [15-17].

The application of $^{86}Rb^+$ flux measurements to chromaffin granules proved the existence of a potent electrogenic, K^+-selective transport system in the granular membrane. Using this technique, it was shown that K^+ electrogenic transport is blocked by ATP and nucleotide analogues [18], by Mg^{2+}, Ba^{2+} and Zn^{2+} [18, 19], and by SH-modifying agents such as N-ethylmaleimide or mersalyl [20]. We recently reported that $^{86}Rb^+$ flux is blocked by low pH [21].

The aim of this study was to analyze the single channel components of the K^+ electrogenic flux observed in chromaffin granule membranes. We characterized ion channels which may contribute to monovalent ion transport

through chromaffin granule membranes. Channel activity was registered using the planar lipid bilayer system. This allowed us to measure single channel activity after the reconstitution of purified chromaffin granule membranes. Both the conductance and gating parameters of the observed channel activity were analyzed.

MATERIALS AND METHODS

Chemicals

The asolectin from soybean, potassium chloride, calcium chloride, *n*-decane and chloroform were from Sigma (USA). All the other chemicals were of the highest purity commercially available.

Subcellular fractionation of adrenal glands

Bovine adrenal medullas were fractionated essentially as previously described in [22]. The purification of the chromaffin granules and the purity of the membrane preparations were confirmed via a marker enzyme estimation, as previously described [18]; we observed a linear correlation between $^{86}Rb^+$ uptake and the level of cytochrome b_{561} (the marker enzyme for chromaffin granule membranes). In different chromaffin cell membrane preparations, such as those from submitochondrial particles, microsomes and chromaffin granules (the correlation coefficient was $r^2 = 0.98$), and no correlation was found between rubidium uptake and the levels of other enzymes like cytochrome c oxidase, glucoso-6-phosphatase and 5'-nucleosidase, which are respectively markers for the inner mitochondrial membrane, endoplasmic reticulum and plasma membrane.

$^{86}Rb^+$ uptake into chromaffin granules

The principle of the applied flux assay was originally described by others [23, 24]. Briefly, chromaffin granule vesicles containing an inner concentration of 100 mM KCl were prepared. Shortly before the assay, external K^+ was replaced with $Tris^+$. As a result of the K^+ concentration gradient, an electrical diffusion potential was established in those vesicles containing active K^+ channels. The addition of the $^{86}Rb^+$ isotope, a K^+ analogue, to the external solution led to the uptake of $^{86}Rb^+$ due to its equilibration with the membrane potential, but did not affect the level of the potential itself. It is important to note that $^{86}Rb^+$ accumulation occurs selectively into those vesicles containing open K^+ channels, thus enhancing the sensitivity of the transport measurements.

Asolectin purification

The asolectin used for the planar lipid bilayer technique was purified according to the following procedure: 10 g asolectin was dissolved in 50 ml of chloroform, followed by the addition of ice cold acetone. This mixture was kept at 0ºC for 1 h. The lipid precipitate was centrifuged at 2500 rpm for 15 min, and the supernatant was removed. The asolectin was stored at -80ºC.

Black lipid membrane technique

The planar lipid membrane was formed by spreading phospholipid solution (painted bilayer). Planar phospholipid bilayers were formed in a 250 μm diameter hole which separated two chambers (internal volumes: *cis* 2 ml and *trans* 3 ml). The chambers contained 450/150, 450/450 mM or 500/150 mM KCl, 5 mM Hepes, pH 7.0 (adjusted with KOH). To improve membrane stability, the outline of the aperture was coated with a lipid suspension, and dried with N_2 prior to bilayer formation. The planar phospholipid bilayers were painted using asolectin in *n*-decane at a final concentration of 25 mg of lipid/ml. The formation and thinning of the bilayer were monitored by capacitance measurements. The final capacitance values ranged from 110 to 200 pF. Electrical connections were made using Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. A voltage was applied to the *cis* compartment of the chamber, and the *trans* compartment was grounded. Suspensions of chromaffin granules in 300 mM sucrose, 10 mM Hepes, pH 7.2 (adjusted with KOH) were added to the *trans* compartment. pH changes in the *cis* and *trans* chambers were obtained by the addition of a fixed amount of HCl or KOH. All measurements were carried out at room temperature.

Reconstitution of chromaffin granule membranes into the planar lipid bilayer

The quality of the lipid bilayers was checked at 0 mV and ± 50 mV before the addition of the chromaffin granule membrane suspension, and they showed no channel-like activity. The electrically silent membranes had a capacitance of 110-200 pF and a conductance of 10 pS. 10-50 minutes after the addition of chromaffin granule "ghosts" to the *trans*-bilayer chamber, channel-like activity could be observed.

Data recording and analysis

The current was measured using a Bi-layer Membrane Admittance Meter (model ID 562, IDB, Gwynadd, UK). The signal was filtered at 0.2 kHz (Low Pass Bessel Filter 4 Pole, Warner Instrument Corp.), digitized (A/D converter 1401, Cambridge Electronic Design, UK) and transferred to a PC for off-line analysis using CED Electrophysiology Package V6.41 and pClamp6 software (Axon Instruments), and plotted in Microcal Origin.

Channel conductances were obtained as follows. In the experiments, all the observable current steps were measured for each voltage, and their amplitudes were plotted versus voltage. Channel conductances were then derived from the slopes of the linear regression lines to the data points. The conductances obtained for each individual experiment were then averaged over the number of experiments performed under the same conditions. The results are expressed as ± SDs.

Ion selectivity was measured under 450/150 mM KCl (*cis*/*trans*) asymmetrical conditions. The reversal potential U_{rev} was obtained as a voltage for which the corresponding linear regression intersected the horizontal axis of the I-V curves, and was compared to the value calculated for K^+ according to the Nernst equation.

RESULTS

Rubidium flux into chromaffin granules

Fig. 1 shows the rate of $^{86}Rb^+$ uptake into chromaffin granule vesicles (expressed as the percentage of total radioactivity present in the sample). In the absence of a K^+ gradient (no diffusion potential was created), the accumulation of $^{86}Rb^+$ was low. The rubidium accumulation was high in the presence of a diffusion potential. Adding 30 mM KCl (at 60 mins), which caused a depolarization of the diffusion potential, promoted a rapid efflux of $^{86}Rb^+$ from the vesicles. This result suggests that the K^+ transport pathway operates by an electrogenic rather than electroneutral mechanism.

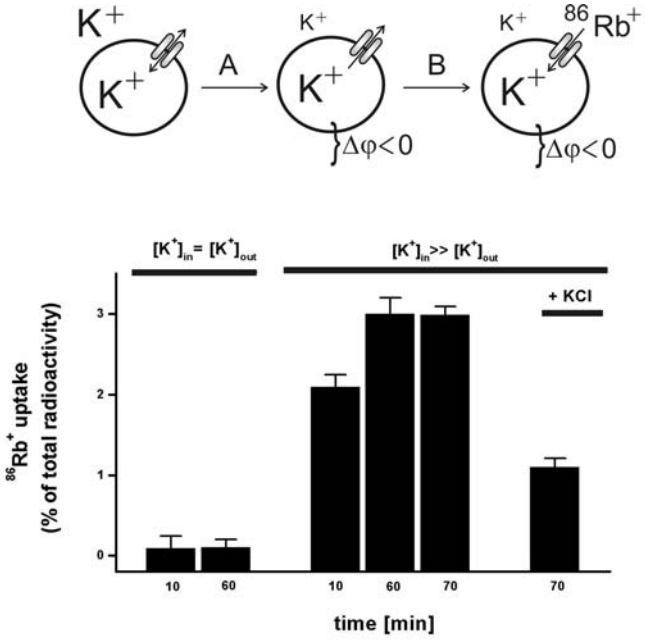

Fig. 1. $^{86}Rb^+$ uptake into the chromaffin granules. Depending on the ratio of the potassium concentration inside the vesicle ($[K^+]_{in}$) to the concentration outside the vesicle ($[K^+]_{out}$), a diffusion potential is created (top A). The addition of $^{86}Rb^+$ causes radioisotope influx into the granules (top B). The time course is shown for $^{86}Rb^+$ uptake into the chromaffin granules in the absence of a potassium diffusion gradient, in the presence of the gradient, and 10 minutes after the addition of KCl, which diminishes the potassium gradient.

Single channel recording from chromaffin granules

Potassium channels

The black lipid membrane technique was adopted to study the properties of the single channel in chromaffin granules. Many single channel activities were found. The most characteristic is that of the large conductance channel K_{CG}.

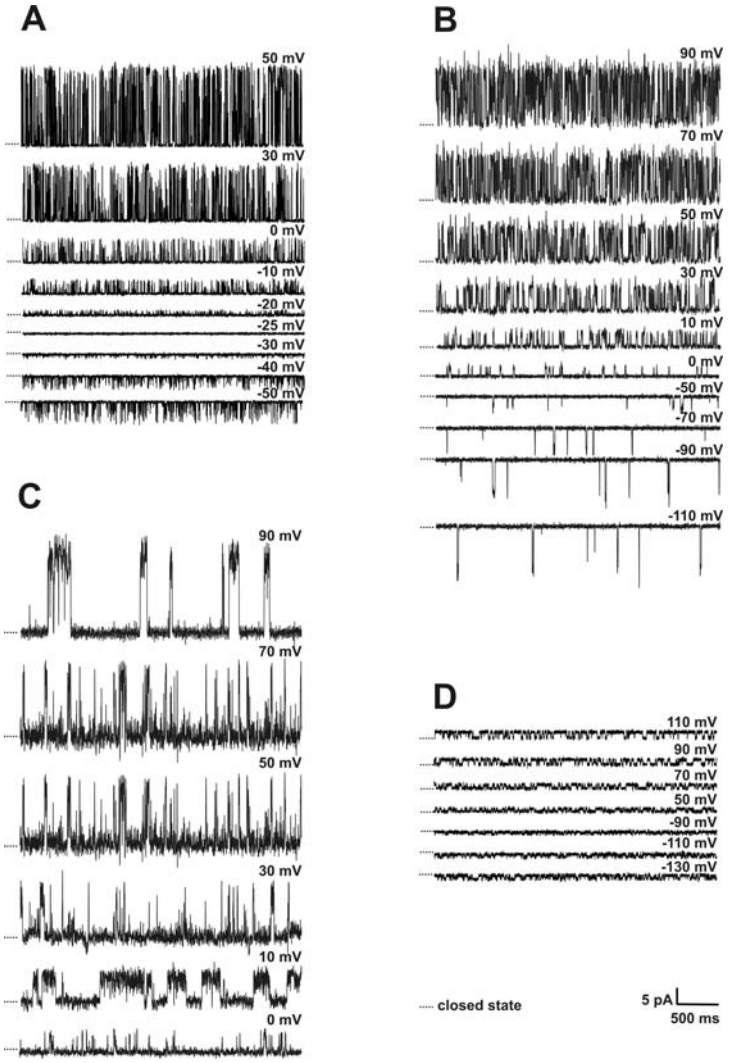

Fig. 2. Single channel recordings of four different potassium channels of chromaffin granules. Different holding potentials. 450/150 mM KCl, 5 mM Hepes/KOH pH 7.0 *cis*/*trans* buffers. The recordings were low-pass filtered at 0.2 kHz. Common scale adopted. Closed levels marked as ···. A – K_{CG} potassium channel with a large conductance of 360 ± 10 pS; B – K_{152} potassium channel with a conductance of 152 ± 8 pS; C – K_{220} potassium channel with a conductance of 220 ± 8 pS; K_{13} – potassium channel with a conductance of ~13 ± 3 pS.

However, at least five different potassium (cationic) channels are present in the membrane of the chromaffin granule. The single channel characteristics of the four potassium channels are shown in Fig. 2. The current versus the potential curves of these four channels are shown in Fig. 3. The open and closed mean time constants, and the open time probabilities are presented in Tab. 1.

Tab. 1. The ion channel of the chromaffin granules – conductances, reversal potential, open and closed time constants and open probabilities (for a given potential). The subscripts represent the membrane potentials for which the time constants were determined: (o) – open state, (s) – substate, n – number of observations.

Channel signature	γ [pS]	U_{rev} [mV]	τ_o [ms]	τ_c [ms]	P_o
K_{CG} (n=22)	360±10	-28±2	τ_{50}=13.0±0.3 τ_{-70}=10.9±0.4	τ_{50}=13.0±0.3 τ_{-70}=10.9±0.4	0.67±0.14 (50) 0.29±0.06 (-70)
K_{220} (n=8)	220±8	-22±2	–	–	–
K_{152} (n=10)	152±8	-28±1	τ_{50}=6.5±0.4 τ_{-70}= 5.1±0.1	τ_{50}=45.7±1.1 τ_{-70}=411±9	0.13±0.04 (50) 0.02±0.01 (-70)
K_{13} (n=12)	13±3	-27±1	τ_{110}=7.4±0.1 τ_{-130}= short	τ_{110}=12.4±0.1 τ_{-130}=15.6±0.2	0.4±0.1 (50) 0.11±0.09 (-70)
K_{110}	110±8	-29±2	τ_{70}=5.1±0.2 (o) τ_{70}=19.0±0.6(o)	τ_{70}=11.8±0.3	0.44±0.06 (50) (o)
K_{31} (n=20)	31±4		τ_{70}=4.4±0.1 (s) τ_{70}=12.4±0.3 (s)		0.34±0.04 (-70) (s)
Cl_{250} (n=5)	250±9	12±2	τ_{20}=4.3±0.2 τ_{20}=50.9±0.8	τ_{20}=7.1±0.4	0.79±0.07 (20)
			τ_{-10}= 3.8±0.5 τ_{-10}= 30.1±0.8	τ_{-10}=20.7±0.9	0.81±0.08 (-10)

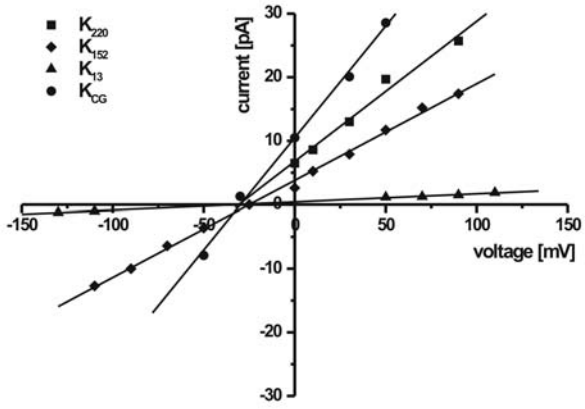

Fig. 3. The current/voltage relationship of the four potassium channels presented in Fig. 2. 450/150 mM KCl, 5 mM Hepes/KOH, pH 7.0 *cis*/*trans* buffers. The recordings were low-pass filtered at 0.2 kHz. The solid lines are drawn to a linear fit of the data.

The large conductance potassium channel K_{CG} has a unitary conductance of 360 ± 10 pS in 450/150 mM KCl pH 7.0 buffers and 430 ± 9 pS in symmetrical 450/450 mM KCl pH 7.0 buffers. From the data in Tab. 1, one can conclude that the K_{CG}, K_{152} and K_{13} channels conduct only cations, and that the open time probability is voltage dependent; in negative membrane potentials, the channels are closed. The detailed analysis of the pH and voltage dependency of the most

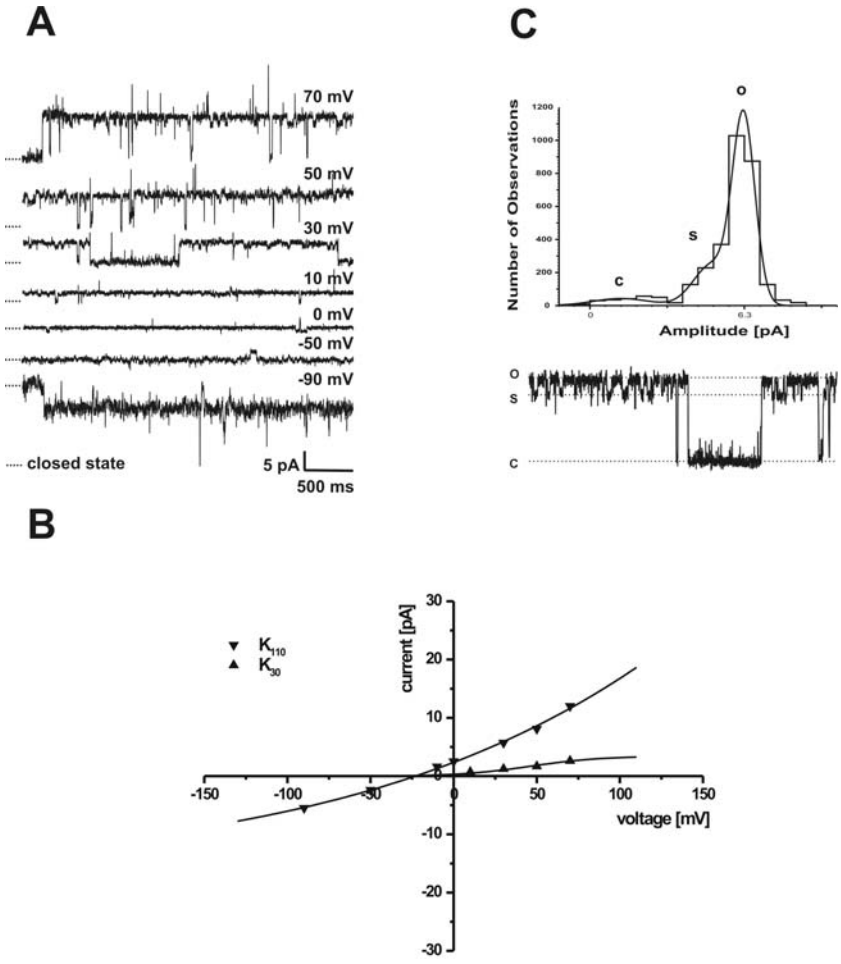

Fig. 4. The multistate potassium channel of a chromaffin granule. 450/150 mM KCl, 5 mM Hepes/KOH, pH 7.0 *cis*/*trans* buffers. A – Single channel recordings. Closed level marked as ···. The recordings were low-pass filtered at 0.2 kHz. B – Current/voltage relationship. C – Amplitude histogram at the holding potential of 50 mV. Levels are marked: c – closed, o – open, s – substate.

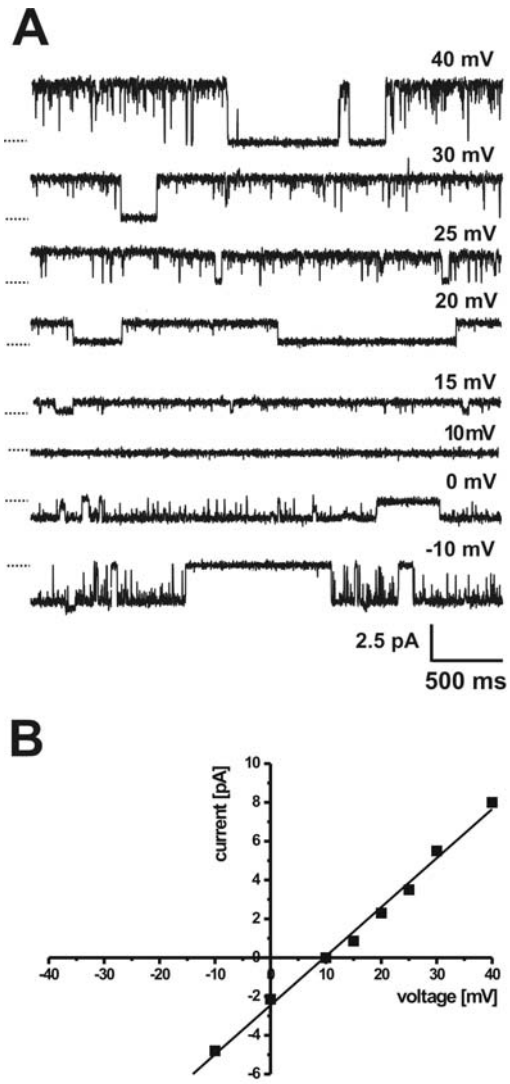

Fig. 5. The anion channel of a chromaffin granule. 500/150 mM KCl, 5 mM Hepes/KOH, pH 7.0 *cis*/*trans* buffers in the presence of 2 mM $CaCl_2$. A – Single channel recordings. The closed level marked as ⋯. The recordings were low-pass filtered at 0.2 kHz. B – Current/voltage relationship.

abundand K_{CG} channel is given elsewhere [21]. The K_{220} channel is slightly less cation selective (U_{rev} ~-22mV), opens only in positive potentials and closes in acidic media. The K_{220} channel was always accompanied by other channels, and thus it was impossible to determine its open and closed time constants. While open-time histograms for the K_{152} channel require only one time constant, the closed-time histograms require two.

The fifth cationic channel is shown in Fig. 4. This channel shows two different amplitudes. It is likely that this activity could be treated as a K_{110} channel with the conductive substate of K_{30}.

The anion selective channel

Anion selective channels were observed in the chromaffin granules, namely Cl_{250}. The presence of anion channels is a rare event, observed in 2% of all our experiments. The anion channel activity could be observed only in the presence of calcium in the medium. The properties of the anion channel are shown in Fig. 5. The unitary conductance of this channel was estimated at 250 ± 9 pS (n = 5)in 500/150 mM KCl, pH 7.0 buffers containing 2 mM of $CaCl_2$ in the *trans* chamber (vesicle addition side), suggesting that the presence of calcium is required on the outer side of the chromaffin granule. The reversal potential was U_{rev} = 12 ± 2 mV, indicating that the channel conducts anions 2.5 times better than cations.

DISCUSSION AND CONCLUSION

Rubidium and potassium ions have a similar hydrated size, and it is known that rubidium can flow through potassium channels. Thus, the radioactive rubidium flux experiment was used to show that in the chromaffin granules, there is a potassium channel-like activity. The rubidium flux into the chromaffin granules was blocked by low pH, ATP, Mg^{2+}, Zn^{2+} and quinacrine [18-20]. In another experiment, it was shown that all the properties observed in the flux experiment could be ascribed to the properties of the large conductance potassium channel K_{CG}, measured using the BLM single channel technique [21]. The large conductance potassium channel showed similar conductivity and properties to the channel described in [13], i.e. it was insensitive to both Ca^{2+} and charybdotoxin, and was blocked by TEA^+ [21]. Since the K_{CG} channel is blocked by low pH but not a positive intravesicular potential, it was concluded that the large conductance channel is responsible for the control of overacidification of chromaffin granules [21].

The molecular identity of the observed ion channels remains unclear. They may be voltage-dependent (Kv) [25], inward rectifier (Kir) [26] or large conductance (BK) [27] potassium channels. The chloride channel may belong to the intracellular ion channel family (CLIC), recently shown to be present in intracellular membranes [28], or to the CLCA family, well known to play a role in epithelial secretion [29]. The activation-inactivation process of these channels needs further analysis, especially in the context of its potential role in chromaffin granule exocytosis.

The results of the paper are summarised in Fig. 6, in which six channels of chromaffin granules together with V-ATPase and VMAT catecholamine transporters are shown. Pumps and transporters play a role in loading the granules with catecholamines (VMAT and V-ATPase), channels that provide

electric shunt, controlling catecholamine uptake and protecting the granules from overacidification (K_{CG}). The other potassium channels may play a similar role to the K_{CG} channel, while the calcium-dependent chloride channel may be involved in the secretion of the granules [29].

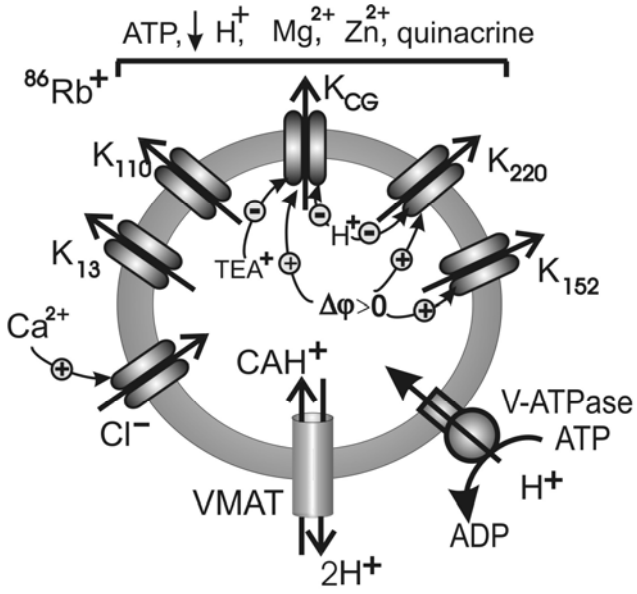

Fig. 6. The channels and transporters of the chromaffin granules. VMAT – catecholamine (CAH^+) transporter, V-ATPase – proton pump; there are five potassium channels and one calcium-dependent chloride channel. Adding TEA^+ and lowering pH closes channels, while increasing the potential difference (Δφ) opens channels. The $^{86}Rb^+$ flux experiments and measurements of a single channel in BLM show that potassium conductivity is affected by the same blockers.

The determination of the channel properties in the case of chromaffin granules is difficult. Our vesicles were 0.2 to 0.3 μm in diameter (data not shown), and the chromaffin granules were 0.16 to 0.33 μm [29]. It is likely that the electrophysiological properties of the chromaffin granule rely on more than a single copy of the channel molecule per granule. Thus, it is not surprising that in a typical experiment (60% of cases), more than one channel is observed simultaneously, i.e. in an incorporated vesicle, there were usually 2-4 channels. In the case of multiple channels, it is difficult to properly determine the electrochemical parameters of each channel. In the remaining 40% of incorporations, when only a single channel activity was observed, five different cationic channels were detected, of which the K_{CG} channel is the most abundant.

Not all the channels detected appeared often enough to enable the determination of all of their electrochemical properties; thus, some are missing from Tab. 1. Apparently, other potassium channels have properties similar to the K_{CG} channel, i.e. they are cation specific, opened by positive potentials and closed by low pH (data not shown). Thus, they may contribute to the macroscopic flux of potassium into chromaffin granules.

Not all the channels are present in a single granule. We were unable to detect any pattern of which channels appear. We are left with two hypotheses: either each chromaffin granule has different ion channels and is in a different physiological state, or the observed heterogeneity represents different states of activity of the same channel molecule. To answer to this question, further study is required.

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