Acta Neurobiol. Exp. 2001, 61: 1-12

ATP-sensitive K^+ transport in adrenal chromaffin granules

Adam Szewczyk[1], **Nikolai A. Lobanov**[2], **Anna Kicińska**[1], **Grażyna Wójcik**[3] and **Maciej J. Nałęcz**[3]

[1]Laboratory of Intracellular Ion Channels and [3]Laboratory for Mechanism of Transport Through Biomembranes, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St., 02-093 Warsaw, Poland;
[2]Laboratory of Protein Chemistry, Institute of Bioorganic Chemistry, Belarus National Academy of Sciences, 5/2 Kuprevicha St., Minsk 220141, Belarus

Abstract. In the present study the influx of $^{86}Rb^+$, a K^+ analogue, was studied in mitochondria, microsomes and chromaffin granules prepared from adrenal gland medulla. The most active electrogenic $^{86}Rb^+$ transport was found in the membrane fraction identified as chromaffin granules by marker enzyme estimation. The transport was found to be sensitive to ATP, ATPγS, ADP and to the triazine dyes, but not to AMP and cAMP. The inhibition induced by ATP was observed in the absence of externally added Mg^{2+}, suggesting that a free nucleotide, rather than the ATP-Mg complex, was required for inhibition. Furthermore, the $^{86}Rb^+$ influx was found to be inhibited by Mg^{2+} alone, but not by Ca^{2+} and antidiabetic sulfonylureas. The $^{86}Rb^+$ influx was not stimulated by potassium channel openers. In conclusion, our results indicate that an electrogenic, ATP-sensitive potassium transport system operates in the chromaffin granule membrane.

Correspondence should be addressed to A. Szewczyk, Email: adam@nencki.gov.pl

Key words: potassium transport, adrenal chromaffin granules, ATP, potassium channel openers

INTRODUCTION

Potassium and chloride selective channels exist in the membrane of intracellular organelles such as mitochondria, sarco/endoplasmic reticulum, nucleus, synaptic vesicles and secretory granules (for review see Szewczyk 1998). Ion channels have been also reported in membrane of chromaffin granules from adrenal medulla (Pazoles and Polard 1978, Pollard et al. 1979, Picaud et al. 1984, Arispe et al. 1992, Ashley et al. 1994). The chromaffin granules of the adrenal medulla contain the endogenous catecholamines. They are also involved in catecholamines synthesis and traffic, both within and out side the cell (for review see Kirshner et al. 1987). The uptake of hormones, driven by pH gradient (ΔpH), from the cytosol into chromaffin granules is catalyzed by a catecholamine carrier (Angeletti et al. 1985). Secretion of hormones occurs as a result of fusion of chromaffin granule vesicles with the plasma membrane of the adrenal chromaffin cell.

The chromaffin granule ion channels have been investigated after fusion of a granule membranes with planar bilayer membranes, followed by single channel recordings. Several different cation channels were described after incorporation of intact chromaffin granuls "ghosts" (Arispe et al. 1992, Ashley et al. 1994). A K^+ selective, large conductance (~160 pS in symmetrical 400 mM KCl) channel was described by Arispe et al. (1992). It was insensitive to charybdotoxin, a blocker of a Ca^{2+}-activated K^+ channel of large conductance (Arispe et al. 1992). Channel activity was also unaffected by Ca^{2+} and potential across the bilayer (Arispe et al. 1992). It was also reported that the chromaffin granule K^+ channel was controlled by both inhibitory and stimulatory heterotrimeric GTP-binding proteins (Arispe et al. 1995). Similar, highly selective for potassium, but with a different conductance (~400 pS in symmetric 450 mM KCl) channel has been described by Ashley (1994). The channel was insensitive to both Ca^{2+} and charybdotoxin, and was blocked by TEA^+. The inhibition by TEA^+ was slightly voltage-dependent (Ashley et al. 1994).

A key problem concerning single channel recordings in planar bilayer membranes is the purity of the applied membrane preparation. Therefore, we have applied the flux measurements using $^{86}Rb^+$, a K^+ analog, as described by others (Garty et al. 1983, Garty and Karlish 1989), to study the properties of a chromaffin granule K^+ transport. Recently, we have used successfully this approach to show that electrogenic K^+ transport in chromaffin granules is blocked by sulfhydryl reagents (Szewczyk et al. 1997) and some potassium channel blockers (Lobanov et al. 1997). Transport measurements were performed under such conditions that only an electrogenic influx of $^{86}Rb^+$ into chromaffin granules was measured. This simple and convenient flux assay, combined with marker enzyme estimations, forms a valuable method for measuring K^+ channels activity in a heterogeneous population of the chromaffin membrane vesicles. This permitted us to confirm, *via* different experimental design, the presence of K^+ transport system in chromaffin granule membranes. In addition, we were able to demonstrate some new properties of the electrogenic K^+ transport operating in chromaffin granules.

In this paper we report that the chromaffin granule membranes possess an electrogenic transport system, selective for potassium ions. We have also shown that this transporting system is sensitive to ATP and Mg^{2+}, but not to the antidiabetic sulfonylureas or potassium channel openers.

METHODS

Materials

$^{86}RbCl$, with a specific radioactivity of 20 Ci/mmol, was purchased from Polatom (Poland). [^{3}H]glibenclamide, 48.5 Ci/mmol, was supplied by DuPont NEN (Germany). A23187 and bafilomycin A_1 was from Sigma (USA). Glibenclamide was purchased from Research Biochemicals Incorporated (USA) and glipizide from Hoechst (Germany). U-37883A was generously offered by Upjohn Company (USA). All other chemicals were of the highest purity commercially available.

Subcellular fractionation of the adrenal glands

Fractionation of adrenal medulla was performed in order to obtain mitochondria, chromaffin granules and microsomes (mixture of plasma membrane and endoplasmic reticulum). Bovine adrenal medulla were essentially fractionated as previously described by Brocklehurst and Pollard (1990). Medulla from fresh bovine adrenal glands were removed and homogenized in 0.3 M sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.3. The

homogenate was filtered through surgical gauze, and the filtrate was centrifuged at 800 x g for 15 min. The supernatant was further centrifuged at 20,000 x g for 30 min at 4°C and the subsequent supernatant was centrifuged at 100,000 x g for 60 min at 4°C to separate the cytosol and microsomal (100,000 x g pellet) fraction. The pellet from the 20,000 x g centrifugation contained mitochondria, which were gently swirled off the top of the pinky chromaffin granule pellet. The crude chromaffin granule pellet was resuspended in the homogenization buffer, overlaid on 1.6 M sucrose, 1 mM EGTA, 1 mM $MgSO_4$, 10 mM Hepes, pH 7.3, and centrifuged at 135,000 x g for 60 min at 4°C. The granule pellet was resuspended in 25 volumes of solution containing 1 mM EGTA, 5 mM Hepes, pH 7.5 and lysed by freeze-thawing. Subsequently, the suspension of chromaffin granule membranes was centrifuged at 48,000 x g for 30 min. The obtained pellet was resuspended with the same volume of the lysis buffer and the centrifugation was repeated. Final purification was accomplished by centrifugation of resuspended pellet in a suitable volume of lysis buffer (approx. 1 mg/ml) over a 1 M sucrose cushion. The membrane suspension (2.5 ml) was overlaid above 12 ml of solution containing 1 M sucrose, 1 mM EGTA, 10 mM Hepes/KOH, pH 7.5 in Beckman SW28.1 rotor tubes and centrifuged at 100,000 x g for 90 min. The chromaffin granule membranes remaining at the top of the sucrose cushion were collected and resuspended in 25 volumes of 10 mM Hepes/KOH buffer, pH 7,4. These membranes were then centrifuged at 48,000 x g for 30 min, and the membrane pellet was resuspended (at a final protein concentration of about 1 mg/ml) in a buffer containing 100 mM KCl, 10 mM Hepes/Tris, pH 7.4. The aliquots of this final suspension were frozen. For the experiments where other monovalent cations were closed into chromaffin vesicles, the final pellet was suspended in the media containing either 100 mM LiCl, NaCl, RbCl or CsCl.

Preparation of mitochondria and submitochondrial particles

Adrenal medulla mitochondria were prepared according to Johnson and Lardy (1967) using 75 mM sucrose, 225 mM mannitol, 3 mM Hepes-KOH (pH 7.4) and 1 mM EGTA as the isolation medium.

Submitochondrial particles (SMP) were prepared from mitochondria by means of sonication and ultracentrifugation. In brief, freshly prepared mitochondria were suspended in a medium containing 150 mM KCl, 10 mM Tris-HCl, pH 7.4, at a protein concentration of 10 mg of protein per ml. After use of a Branson sonicator (Switzerland) (20 x 45 s with 10 s intervals, at 50% duty), the sample was centrifuged for 10 min. at 100,00 x g to remove unbroken mitochondria. Then, the obtained supernatant was centrifuged for 35 min. at 100,000 x g, and the final pellet containing SMP was suspended in 100 mM KCl, 20 mM Hepes, pH 7.4, and frozen.

$^{86}Rb^+$ flux measurements

The isotope flux through ion-conducting pathways was performed essentially as described previously (Garty et al. 1983, Garty and Karlish 1989). In brief, for transport experiments with membrane preparations the external K^+ was removed by passing the vesicles through a cation-exchange column (Dowex 50-X8 Tris form). Aliquots of 200 µl of the vesicle suspension (1 mg protein/ml; chromaffin granules ghosts, SMP or microsomes) were applied to small Dowex columns and eluted with 1.5 ml of 175 mM sucrose. This step exchanged the external potassium cations into $Tris^+$ and diluted the suspension by about 5 fold. Various reagents (ATP, ADP, $MgCl_2$, bafilomycine A_1, atractyloside, oligomycine, and others) were added to the vesicle suspension, as indicated. The assay was initiated 30 s later by adding 25-50 µl of $^{86}RbCl$ (2-4 µCi). Vesicles were incubated with the isotope under different conditions and for the times given in the legends to the figures. Subsequently, in order to separate the vesicles from the medium, 100 µl aliquots of the reaction mixture were applied to 2-3 cm columns of Dowex 50-X8 (Tris form) placed in Pasteur pipettes, and vesicles were eluted directly into counting vials by addition of 1.5 ml of ice-cold 175 mM sucrose solution. Prior to use, the columns were washed with 2 ml of 175 mM sucrose followed by 2 ml of 175 mM sucrose containing 25 mg/ml of bovine serum albumin and stored at 4°C. The amount of $^{86}Rb^+$ trapped within the vesicles was estimated by scintillation counting. The $^{86}Rb^+$ content was expressed as a percentage of the initial total radioactivity in the vesicle reaction medium or as a percentage of control (sample without added reagents).

Marker enzyme activity measurements

5'- Nucleotidase and glucose-6-phosphatase were assayed by measuring the release of inorganic phosphate

from AMP and from glucose-6-phosphate, respectively (Aronson et al. 1974). The assay for inorganic phosphate was based on the method of Fiske and SubbaRow (1925).

ATPase activity was determined spectrophotometrically following oxidation of NADH (Cintrón and Pedersen 1979) in the ATP-regenerating system (Pullman et al. 1960) using a dual wavelength spectrophotometer (Shimadzu, Model UV-3000) at 340 nm versus 400 nm (Bogucka et al. 1990).

Cytochrome c oxidase activity, a mitochondrial inner membrane marker enzyme, was measured as previously described by Storrie and Madden (1990).

The presence of the chromaffin granule membrane marker, cytochrome b_{561} was measured by the difference spectrum between dithionite-reduced and oxidized states at 561 nm (Zinder et al. 1978).

Binding of [^{3}H]-glibenclamide to membrane vesicles

Binding of [^{3}H]glibenclamide to chromaffin membranes was performed as described previously (Szewczyk et al. 1996a).

Chromaffin granules transmembrane potential ($\Delta\Psi$) measurements

Chromaffin granules transmembrane potential was measured with safranine O as previously described (Åkerman et al. 1976). Changes of absorption, reflecting changes of granular $\Delta\Psi$ after addition of 40 ng/ml valinomycin, were measured with Shimadzu dual-wavelength spectrophotometer (UV-3000).

Protein concentration assay

Protein concentration was measured using the Bio-Rad Protein Assay kit according to the instructions of the manufacturer.

RESULTS

Validation of the assay for K^+ transport into chromaffin granules

The principle of the applied flux assay was originally described by others (Garty et al. 1983, Garty and Karlish 1989). In brief, we prepared chromaffin granules vesicles containing an inner concentration of 100 mM KCl (or other monovalent cation chloride salts, as indicated) (Fig.1). Shortly before the assay the external K^+ was replaced with Tris ion (for details see Methods). As a result of a K^+ gradient, an electrical diffusion potential was established in vesicles containing active K^+ channels (Fig. 1). The addition of $^{86}Rb^+$ isotope, a K^+ analog, to the external solution, led to the uptake of $^{86}Rb^+$ due to its equilibration with the membrane potential, but not affecting the level of the potential itself. It is important to notice, that ^{86}Rb accumulation occurs selectively into the vesicles containing active K^+ channels thus amplifying sensitivity of the transport measurements (Fig. 1). Figure 2 presents the time course of $^{86}Rb^+$ uptake into chromaffin granule vesicles (expressed as a percentage of total radioactivity in the sample). Addition of 30 mM

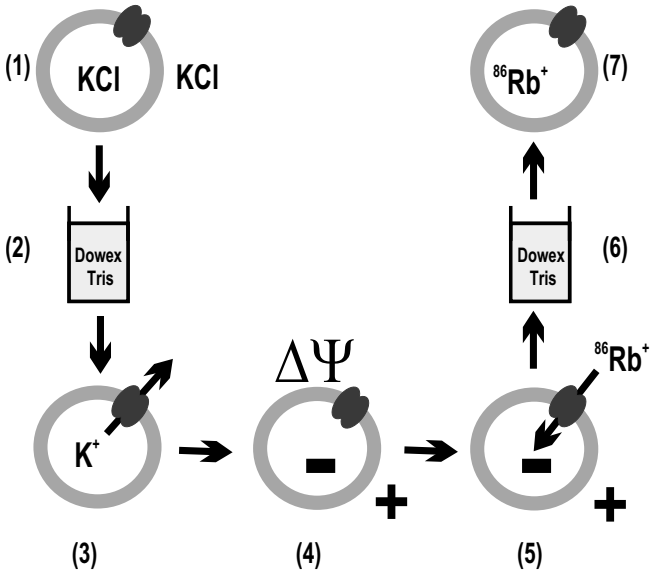

Fig. 1. Schematic representation of the assay applied in this report. (1) Chromaffin granules were prepared in the presence of 100 mM KCl. (2) Chromaffin granules suspension was applied to Dowex-Tris column and external potassium cations were exchanged into $Tris^+$. (3) In chromaffin granules containing active channels potassium cations start to leak out of vesicles. (4) This leads to formation of diffusion potential – negative inside of vesicles. (5) The transport assay was initiated by adding radioactive $^{86}RbCl$. Vesicles were incubated with the isotope under different conditions and for the times given in legends to the figures. (6) Subsequently, in order to measure $^{86}Rb^+$ accumulation in chromaffin granules reaction mixture was applied on Dowex column to remove external $^{86}Rb^+$. (7) The amount of $^{86}Rb^+$ trapped within the vesicles was measured by scintillation counting. For details of the experimental protocol see Methods.

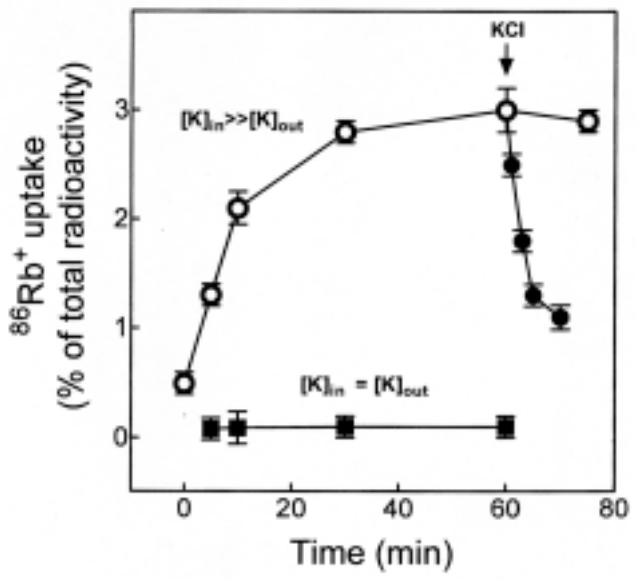

Fig. 2. Time course of $^{86}Rb^+$ uptake into the chromaffin granules. After addition of $^{86}RbCl$, accumulation of radioactivity was measured as described in the Methods (❍). At the time indicated by an arrow, 30 mM KCl was added to the reaction mixture (●). Accumulation of radioactivity without removal of external potassium is also shown (■).Values are means ± SD for triplicate determination. Measurements were performed at 20°C.

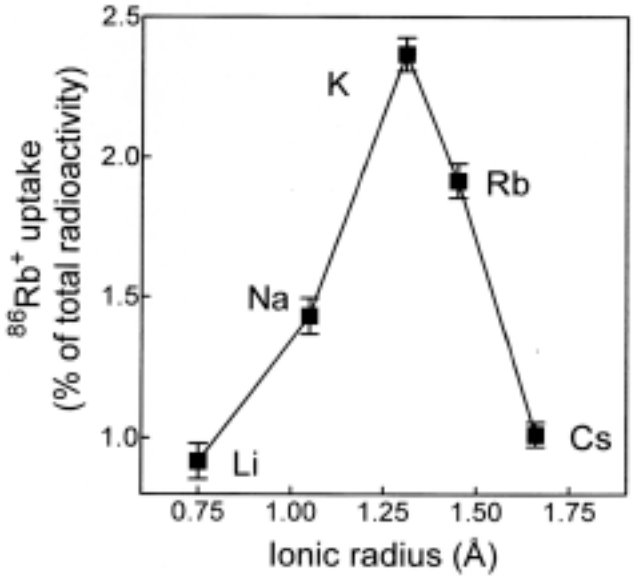

Fig. 3. Specificity of $^{86}Rb^+$ uptake into chromaffin granules. Chromaffin granules were preloaded with the media containing 100 mM LiCl, KCl, RbCl or CsCl. After addition of $^{86}RbCl$, accumulation of radioactivity was measured as described in the "Materials and Methods" over 45 min. Values are means ± SD for triplicate determination. Measurements were performed at 20°C.

KCl, which caused depolarization of the diffusion potential, promoted a rapid efflux of $^{86}Rb^+$ from the vesicles. In the absence of K^+ gradient (no diffusion potential was created) accumulation of $^{86}Rb^+$ was found to be very low (Fig. 2). This result suggests that the K^+ transport pathway operates by electrogenic rather than electroneutral mechanism.

To certify the cation specificity of the observed transport process, experiments were carried out using different monovalent cations entrapped into the chromaffin granule vesicles followed by $^{86}Rb^+$ accumulation measurements. Figure 3 shows the effects of different ionic compositions within vesicles on $^{86}Rb^+$ uptake into chromaffin granule vesicles. As presented, the highest accumulation of radioactivity was observed with the presence of K^+ and Rb^+ within vesicles. This is because only these cations were able to efflux effectively from vesicles leading to the formation of a high diffusion potential as a driving force for $^{86}Rb^+$ accumulation. This observation indicated the specificity of the measured transport phenomenon for K^+ ions.

Localization of the channel activity

In order to verify whether the measured $^{86}Rb^+$ uptake was located in chromaffin granule membranes, both transport activity and marker enzyme levels were estimated in different preparations of membrane vesicles obtained during adrenal gland medulla fractionation. The results of these experiments are summarized in Table I. As became apparent from Table I, all membrane preparations used in the present study were to some extent cross-contaminated. However, the highest activity of $^{86}Rb^+$ uptake may be correlated with the presence of cytochrome b_{561}, the marker enzyme for chromaffin granules membranes (Table I). Similarly, the lowest activity was found in the SMP fraction containing the lowest amount of cytochrome b_{561}. We observed a linear correlation between $^{86}Rb^+$ uptake and level of cytochrome b_{561} in different chromaffin cells membrane preparations such as SMP, microsomes and chromaffin granules (correlation coefficient $r^2 = 0.9814$). In opposite, the correlation of $^{86}Rb^+$ uptake and level of other marker enzymes in different membrane preparations was very poor (correlation coefficient $r^2 = 0.1360$ for

Table I

Comparison of the measured $^{86}Rb^+$ uptake activity found in different membrane preparations (submitochondrial particles, microsomes and chromaffin granule membranes) with the activity of marker enzymesmed at 20°C

Enzyme activity[a]	Submitochondrial particles	Microsomes	Chromaffin granule membranes
$^{86}Rb^+$ uptake (% of total radioactivity)	0.36 ± 0.03	0.75 ± 0.06	2.88 ± 0.07
cytochrome b_{561} (A.U./mg protein)	0.002 ± 0.001	0.010 ± 0.001	0.030 ± 0.002
cytochrome c oxidase (A.U./mg protein)	2.8 ± 0.1	1.1 ± 0.1	1.6 ± 0.1
glucoso-6-phosphatase (nmoles/mg protein/min)	8.9 ± 0.9	14.1 ± 0.9	10.9 ± 0.8
5'-nucleotidase (nmoles/mg protein/min)	3 ± 1	35 ± 4	30 ± 5

[a] Values are means ± SD for triplicate determination. Measurements of $^{86}Rb^+$ uptake with the preparation of submitochondrial particles were performed in the presence of oligomycine 1 μg/mg of protein.

cytochrome c oxidase, 0.0001 for glucoso-6-phosphatase and 0.2487 for 5'-nucleotidase). Such an observation leads to the conclusion that the measured transport is located in chromaffin granule membranes but not in the plasma membrane, endoplasmic reticulum or the inner mitochondrial membrane.

Inhibition of $^{86}Rb^+$ uptake by adenine nucleotides and their analogs

Figure 4 shows the effect of adenine nucleotides on $^{86}Rb^+$ uptake into chromaffin granule vesicles. Both 2 mM ATP and ATPγS, a nonhydrolyzable ATP analogue, and 2 mM ADP were able to significantly inhibit the measured transport. In contrast, 2 mM AMP and 100 μM cAMP (data not shown) were found to be much less potent inhibitors of $^{86}Rb^+$ uptake (Fig. 4A). Figure 4B shows the concentration dependence of the inhibition exerted by ATP (EC_{50} = 0.8 ± 0.3 mM) and ADP (EC_{50} = 3 ± 1 mM). The effects of adenine nucleotides shown in Fig. 4 were observed in the presence of bafilomycin A_1 (5 nmoles/mg of protein), a specific V-ATPase inhibitor. This points to the conclusion that ATP inhibition of chromaffin granule K^+ transport was not mediated by some residual transport or hydrolytic activity of granular V-ATPase. The effects of adenine nucleotides were observed also in the presence of 500 μM atractyloside (data not shown). The $^{86}Rb^+$ uptake into chromaffin granules was inhibited by 100 μM nucleotide analogs - triazine dyes: Cibacron Blue F3GA, Reactive red and Reactive yellow (Table II). We also excluded a possibility that adenine nucleotides or triazine dyes disturb granular membrane integrity leading to low accumulation of $^{86}Rb^+$ into vesicles. In the presence of these substances 40 ng/ml valinomycin was still able to generate diffiusion potential measured with safranine O (see Methods) proving integrity of chromaffin granules vesicles during the experiment (data not shown).

Figure 5A shows the effects of adenine nucleotides observed in the presence of magnesium and different divalent cations chelators. In the presence of 2 mM EDTA, ATP was still able to inhibit $^{86}Rb^+$ uptake (Fig. 5A). In order to exclude the possibility that magnesium ions present inside the vesicles affected ATP inhibition in the presence of external EDTA, we also applied 5 μM A23187, a divalent cations ionophore (Fig. 5A). Under these conditions all Mg^{2+} ions, originating from both external and internal compartments, were available for chelation. Similarly to our previous observations, ATP was found to be capable of inhibiting $^{86}Rb^+$ uptake. Interestingly, 2 mM Mg^{2+} was able to slightly potentiate inhibition induced by 2 mM ATP (Fig. 5A).

Inhibitory effects of adenine nucleotides were both observed for the measurements of initial rate of $^{86}Rb^+$ uptake (after 10 min of incubation) and for measurements

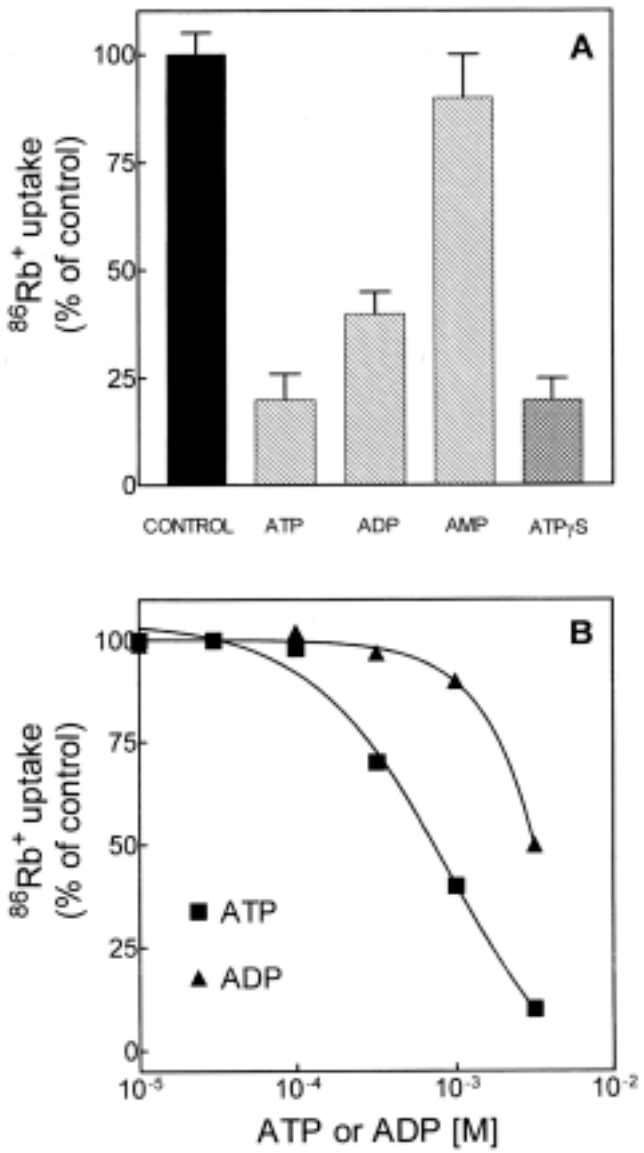

Fig. 4. Effect of adenine nucleotides on $^{86}Rb^+$ uptake into chromaffin granules. A, uptake was measured as described in the Methods. Columns indicate accumulated radioactivity (as a % of control sample, without adenine nucleotides) in the presence of 2 mM ATP, 2 mM ADP, 2 mM AMP, 100 μM cAMP and 2 mM ATPγS. Measurements were performed in triplicate at 20°C, pH = 7.0. B, Concentration dependence of ATP (■) and ADP (•) on $^{86}Rb^+$ uptake into chromaffin granule vesicles. Measurements were performed as described in the Methods. Values are means ± SD for triplicate determination.

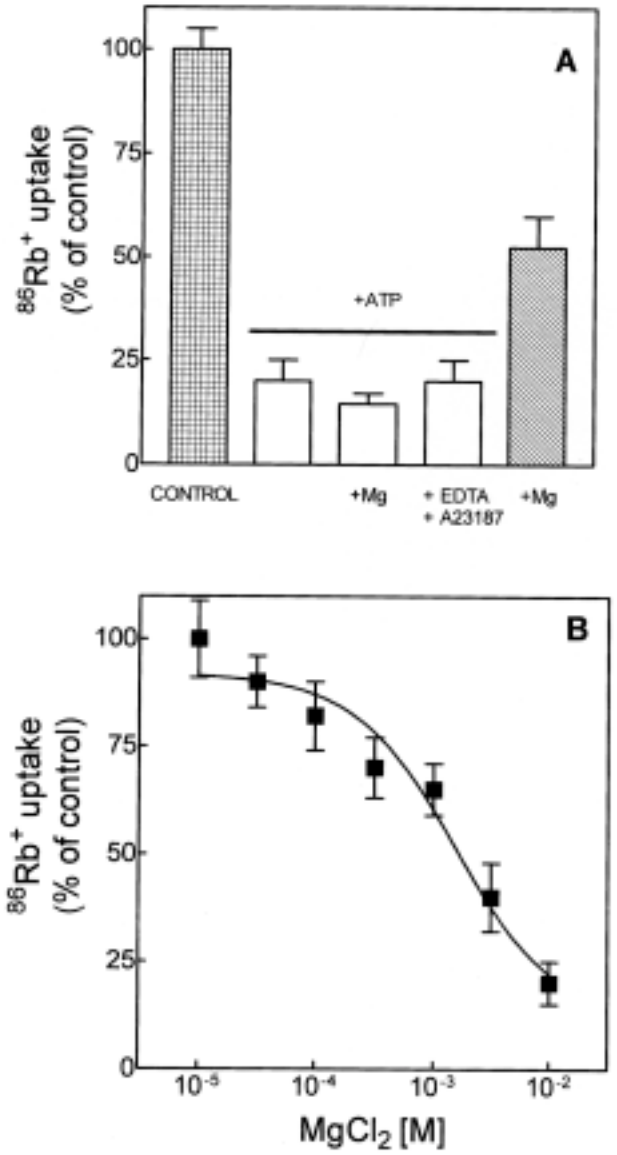

Fig. 5. Effect of ATP and magnesium on $^{86}Rb^+$ uptake into chromaffin granules. A, uptake was measured as described in the legend to Fig. 2. Columns indicate measurements in the presence or absence of 2 mM ATP, 2 mM EDTA + 5 μM A23187 and 2 mM $MgCl_2$. The horizontal bar indicates results obtained in the presence of 2 mM ATP. Values are means ± S.D. for triplicate determination. B, concentration dependence of the effect exerted by $MgCl_2$ on $^{86}Rb^+$ uptake into chromaffin granules vesicles. Values are means ± SD for triplicate determination.

after 30-40 min of incubation. These observations, i.e. inhibitory effect on the initial rate and on the rate measured close to equilibrium, are similar to previous results observed with the use of this kind of flux measurements (Garty et al. 1983, Garty and Asher 1986, Garty et al. 1987).

Inhibition of $^{86}Rb^+$ uptake by magnesium and effects of K^+ channel effectors

Further studies confirmed that free Mg^{2+} was able to inhibit $^{86}Rb^+$ uptake (Fig. 5A). Figure 5B shows the concentration dependence of this effect ($EC_{50} = 1.5 \pm 0.5$ mM).

The activity of the ATP-sensitive K^+ channel (K_{ATP} channel) was described as present in the plasma membrane of different cell types (Lazdunski 1994, Ashcroft 2000) and the inner mitochondrial membrane (mitoK_{ATP} channel) (Inoue et al. 1991, Paucek et al. 1992). One of the common properties of these ionic channels is their sensitivity to antidiabetic sulfonylureas such as glibenclamide or glipizide (Ashcroft and Ashcroft 1992, Szewczyk et al. 1996, 1997). In order to clarify whether the measured K^+ transport in chromaffin granules was catalyzed by a pharmacologically similar protein the effect of antidiabetic sulfonylureas on $^{86}Rb^+$ uptake into chromaffin granules was studied. There was no effect observed for 50 μM glibenclamide and glipizide on the measured transport (Table II). As glibenclamide has a high hydrophobicity, a possibility existed that albumin, which is present in the transport assay (see Materials and Methods), binds to glibenclamide (Aguilar-Bryan et al. 1990) and thus quenched it from the assay medium. In order to exclude this possibility, binding of radioactive glibenclamide to chromaffin granule membranes was studied in the absence of albumin used in transport studies. Using [^{3}H]glibenclamide we were unable to detect specific binding sites for this drug in chromaffin granule membranes (data not shown). This supports the previous observation indicating that the chromaffin granule K^+ transport is not sensitive to antidiabetic sulfonylureas such as glibenclamide and glipizide.

Another modulator of K^+ channels activity, 1mM TEA, was found to have no effect on the measured transport either (Table II). However, 100 μM of the guanidine derivative U-37883A, a non-sulfonylurea K_{ATP} antagonist (Guillemare et al. 1994), was able to inhibit, to some extent, the $^{86}Rb^+$ uptake into chromaffin granules (Table II).

It was shown that both the plasma membrane and the mitochondrial K_{ATP} channels were activated by substances called potassium channel openers (for review see Edwards and Weston 1983, Szewczyk and Marban 1999). However, there was no effect with either 100 μM pinacidil or 100 μM P1060 on the measured transport in chromaffin granules (Table II). The same applied to the potassium channel opener RP66471, known to activate K^+ transport in mitochondria (Szewczyk et al. 1995), which was found without any effect (Table II). Moreover, applied potassium channel openers were also unable to activate ATP- or Mg^{2+}-inhibited $^{86}Rb^+$ influx (data not shown).

Table II

Effect of potassium channel modulators on the $^{86}Rb^+$ influx into chromaffin granules

Substance	Concentration (μM)	$^{86}Rb^+$ Uptake[a] (% of control)
None	-	100 ± 7
sulfonylureas		
glibenclamide	50	102 ± 5
glipizide	50	101 ± 4
triazine dyes		
Reactive red	100	10 ±7
Cibacron blue F3GA	100	25 ± 3
Reactive yellow	100	15 ± 5
potassium channel openers		
pinacidil	100	107 ± 3
P1060	100	97 ± 7
RP 66471	100	110 ± 5
others		
TEA	1000	110 ± 7
U-37883A	100	70 ± 3

[a] Measurements were performed in triplicate as described in Methods. Values are means ± SD. The uptake of $^{86}Rb^+$, measured without additions, after 60 minutes was defined as 100%. Only transport induced by the diffusion potential was considered. Measurements were performed at 20°C.

DISCUSSION

A K^+-selective, electrogenic transporter is present in adrenal chromaffin granule membrane

The value of presented results and interpretation of the data depend on validity of transport assay in terms of reflecting K^+ conductance in chromaffin granule membranes. The flux assay applied in this report was originally described by others (Garty et al. 1983, Garty and Karlish 1989) and was successfully applied to show amiloride-blockable Na^+ channels in toad bladder

microsomes, and veratridine-activated tetrodotoxin--blockable Na^+ channels in rat brain synaptic membranes (Garty et al. 1983). This method, known also as "concentrative uptake", was later applied to measure the activity of Cl^- channels from Torpedo electroplax plasma membrane (Goldberg and Miller 1991). The principle of the assay is as follows. The chromaffin granule vesicles are prepared to contain a high concentration of KCl. Shortly before the assay, the external potassium is replaced by a relatively impermeant Tris cation (Fig. 1). As a consequences of the potassium gradient, an electrical diffusion potential is set up, the magnitude of which is determined by permeabilities of K^+, Cl^- and $Tris^+$ through the membrane. Only in the vesicles containing active potassium channels is the K^+ permeability likely to be much greater than Cl^- and $Tris^+$ permeabilities, and hence in these vesicles a potassium diffusion potential, interior negative, is formed. The isotope that permeates through the channel (in our case $^{86}Rb^+$) when added to the exterior solution, will tend to equilibrate with the membrane potential and thus will accumulate in the vesicles that have $\Delta\Psi$ formed.

Despite of the fact that our preparation of chromaffin granules seems to be cross-contaminated with different types of membranes, there is a clear correlation of observed $^{86}Rb^+$ uptake and the level of cytochrome b_{561} – a marker enzyme for chromaffin granule membranes (see Results section).

Regulation of the chromaffin granule K^+ transport by ATP and Mg^{2+}

The observed transport activity is inhibited by adenine nucleotides (ATP, ADP), but not by antidiabetic sulfonylureas. Free ATP, in the absence of Mg^{2+} ions, appears to be sufficient to inhibit the transport phenomena. ATPMg complex may also inhibit $^{86}Rb^+$ uptake into chromaffin granules vesicles. Transport measurements performed in the presence of 2 mM ATP and 2 mM Mg^{2+} revealed almost 80% inhibition of $^{86}Rb^+$ uptake (Fig. 5A). In this experiment concentration of free ATP and Mg^{2+}, was calculated to be 0.3 mM and 0.45 mM, respectively. Such concentrations of free ATP and Mg^{2+} would be responsible for around 50% inhibition of $^{86}Rb^+$ uptake (see Fig. 4B and Fig. 5B). Actually, higher inhibition was observed what points to the possibility that ATPMg complex may contribute to the overall effect (Fig. 5A).

Inhibitory effects of adenine nucleotides cannot be explained by recently described changes of granular size changes induced by ATP but not by ATPγS (Gualix et al. 1999). In our report both ATP and ATPγS were equally able to inhibit $^{86}Rb^+$ influx into chromaffin granule vesicles.

It is noteworthy that chromaffin granules posses a nucleotide transporter and that the ATP transport into granules is driven by the transmembrane potential positive inside (Bankston and Guidotti 1996, Gualix et al. 1996). We haven't observed any effect of atractyloside, which is able to partially inhibit the nucleotide granular transport, on $^{86}Rb^+$ uptake into chromaffin granules. This excludes a possibility that observed inhibition of transport by ATP is caused by the influx of ATP into chromaffin granules lowering the driving force for $^{86}Rb^+$ uptake.

Possible physiological role of chromaffin granules K^+ transport

Findings of the present study may be important for understanding of physiological role of potassium conductance in chromaffin granules. The chromaffin granules membrane contains a vacuolar- type (V-type) H^+-ATPase, which generates an electrochemical proton gradient, acidifying the granule interior (Forgac 1989, Nelson et al. 2000). Electrogenic potassium transport may play an important physiological role by compensating electric charge transfer produced by the V-ATPase (Ashley et al. 1994). This would enable the formation of a ΔpH, sufficient to drive catecholamine uptake into the chromaffin granules. This hypothesis is also supported by the experiments on the effects of intra-granular cation composition on ATP-dependent acidification of chromaffin granules (Ashley et al. 1994). In fact, a much higher ΔpH was observed with K^+ inside than with TEA^+ (Ashley et al. 1994). Possible physiological role of the adenine nucleotide inhibition of K^+ transport in chromaffin granules is unclear. Adrenal chromaffin granules store catecholamines, together with ATP, at high concentrations (approximately 500 mM and 150 mM, respectively). Hence, cytoplasmic and internal concentration of ATP is high enough to inhibit K^+ transport. It is also likely that granular K^+ transport would be inactive at low pH inside secretory vesicles. On the other hand, extracellular ATP concentration is low, except for short periods of time in the immediate vicinity of release sites of chromaffin cells. Is it, therefore, possible that granular potassium channels become functional during exocytosis? Could they help to locally repolarise the plasma membrane in the immediate vicinity of release

site? Our observation that $^{86}Rb^+$ uptake into chromaffin granules is inhibited by milimolar Mg^{2+}, and considering that magnesium content in chromaffin granules is also in milimolar range (Ornberg et al. 1988) suggest that this cation may play a regulatory role for potassium transport in chromaffin granules.

Our observations point to a suggestion that the chromaffin granule K^+ transporter belongs to the family of intracellular K_{ATP} channels. Recently, the K^+ channel, sensitive to ATP (mitoK_{ATP} channel) in the inner mitochondrial membrane was characterized (for review see Szewczyk et al. 1996). A similar channel for monovalent cations, highly permeable to Rb^+ and K^+, has been reported in the membrane of pancreatic zymogen granules (Thévenod et al. 1992, Gasser and Holda 1993). The latter channel was also found to be inhibited by a milimolar concentration of ATP (or MgATP), nonhydrolyzable analogs of ATP, ADP, and antidiabetic sulfonylureas.

In conclusion, the results of our investigation support the concept of the existence of an electrogenic K^+ transport system in chromaffin granules. Our results support previous observations that K^+ channels are present in chromaffin granule membranes (Arispe et al. 1992, Ashley et al. 1994). Such a system appears to fulfill an important physiological role in the creation of membrane potential ($\Delta\Psi$) and a ΔpH across the granule membrane necessary for catecholamine uptake. The physiological significance of the adenine nucleotide inhibition of K^+ transport in chromaffin granules remains unclear. Relatively high concentration of those nucleotides in the cytosol and within the granular vesicles suggests that the K^+ transport phenomenon should, in fact, be inhibited under physiological conditions. Probably endogenous activators of K^+ transport must exist to overcome ATP blockade, however, none has been identified yet. Moreover, several potassium channel openers, known to be active in various intracellular systems, were found without any effect in chromaffin granules, as presented herewith. Hence, more studies are required to fully understand the mechanism and the functional role of K^+ transport in chromaffin granules.

Although this study contributes to our understanding of the regulation of potassium transport in chromaffin granule membranes, we have not conclusively demonstrated how adenine nucleotides regulates ion transport in these organelles. It is important to mention that applied flux assay, despite of it usefulness, has some disadvantages. This concerns the possibility of contribution of $^{86}Rb^+/K^+$ exchange or changes of chloride conductance in observed effects. Further studies on single channel activities of chromaffin granule K^+ channels and its interaction with ATP will be needed to resolve these questions.

ACKNOWLEDGEMENTS

This study was supported partially by the grant No. 6 P203 003 04 from the Polish Committee for Scientific Research to Adam Szewczyk and by the fellowship from UNESCO/MCBN to Nikolai A. Lobanov. The authors are highly indebted to Dr. H. Englert from Hoechst (Germany) for providing the glipizide and to Dr. K.D. Meisheri from UpJohn Company (USA) for providing the U-37883A used in this study. Ms. Agnieszka Jabłonowska is acknowledged for her skillful technical assistance at the beginning of these studies.

REFERENCES

Aguilar-Bryan L., Nelson D.A., Vu Q.A., Humphrey M.B., Boyd III A.E. (1990) Photoaffinity labeling and partial purification of the beta cell sulfonylurea receptor using a novel, biologically active glyburide analog. J. Biol. Chem. 265: 8218-8224.

Åkerman K.E.O., Wikström M.K.F. (1976) Safranine as a probe of the mitochondrial membrane potential. FEBS Lett. 68: 191-197.

Angeletti R.H., Nolan J.A., Zaremba S. (1985) Catecholamine storage vesicles: topography and function. Trends Biochem. Sci. 6: 240-243.

Arispe N., De Mazancourt P., Rojas E. (1995) Direct control of a large conductance K^+-selective channel by G-proteins in adrenal chromaffin granule membranes. J. Membr. Biol. 147: 109-119.

Arispe N., Pollard H.B., Rojas E. (1992) Calcium-independent K^+-selective channel from chromaffin granule membranes. J. Membr. Biol. 130: 191-202.

Aronson N.N., Touster Jr., Touster O. (1974) Isolation of rat liver membrane fragments in isotonic sucrose. Methods Enzymol. 31: 190-199.

Ashcroft S.J.H. (2000) The β-cell K_{ATP} channel. J. Membr. Biol. 176: 187-206.

Ashcroft S.J.H., Ashcroft F.M. (1992) The sulfonylurea receptor. Biochim. Biophys. Acta 1175: 45-49.

Ashley R.H., Brown D.M, Apps D.K., Phillips J.H. (1994) Evidence for a K^+ channel in bovine chromaffine granule membranes: single-channel properties and possible bioenergetic significance. Eur. Biophys. J. 23: 263-275.

Bankston L.A., Guidotti G. (1996) Characterization of ATP transport into chromaffin granule ghosts. J. Biol. Chem. 271: 17132-1738.

Bogucka K., Teplova V.V., Wojtczak L., Evtodienko Y.V. (1995) Inhibition by Ca^{2+} of the hydrolysis and the synthesis of ATP in Ehrlich as ascites tumor mitochondria: relation to Crabtree effect. Biochim. Biophys. Acta 1228: 261-266.

Brocklehurst K.W., Pollard H.B. (1990) Cell biology of secretion. In: Peptide Hormone Secretion. A Practical Approach. (Hutton, J.C, and Siddle, K., eds), IRL/Oxford, Oxford, New York, Tokyo, p. 233-255.

Cintrón N.M., Pedersen P.L. (1979) A protein inhibitor of the mitochondrial adenosine triphosphatase complex of rat liver. Purification and Characterization. J. Biol. Chem. 254: 3439-3443.

Edwards G., Weston A.H. (1993) The pharmacology of ATP-sensitive potassium channels. Ann. Rev. Pharmacol. Toxicol. 33: 597-637.

Forgac M. (1989) Structure and function of vacuolar class of ATP-driven proton pumps. Physiol. Rev. 69: 765-796.

Fiske C.H., SubbaRow (1925) The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-400.

Garty H., Asher C. (1986) Ca^{2+}-induced down-regulation of Na^+ channels in toad bladder epithelium. J. Biol. Chem. 261: 7400-7406.

Garty H., Asher C., Yeger O. (1987) Direct inhibition of epithelial Na^+ channels by a pH-dependent interaction with calcium, and by other divalent ions. J. Membr. Biol. 95: 151-162.

Garty H., Karlish S.J.D. (1989) Ion channel-mediated fluxes in membrane vesicles: selective amplification of isotope uptake by electrical diffusion potential. Methods Enzymol. 172: 155-164.

Garty H., Rudy B., Karlish S.J.D. (1983) A simple and sensitive procedure for measuring isotope fluxes through ion-specific channels in heterogenous populations of membrane vesicles. J. Biol. Chem. 258: 13094-13099.

Gasser K.W., Holda J.R. (1993) ATP-sensitive potassium transport in pancreatic secretory granule membrane. Am. J. Physiol. 264: G137-142.

Goldberg A.F.X., Miller C. (1991). Solubilization and functional reconstitution of a chloride channel from Torpedo californica electroplax. J. Membr. Biol. 124: 199-206.

Gualix J., Abal M., Pintor J., Garcia-Carmona F., Miras-Portugal M.T. (1996) Nucleotide vesicular transporter of bovine chromaffin granules. Evidence for a mnemonic regulation. J. Biol. Chem. 271: 1957-1965.

Gualix J., Alvarez A.M., Pintor J., Miras-Portugal M.T. (1999). Studies of chromaffin granule functioning by flow cytometry: transport of fluorescent epsilon-ATP and granular size increase induced by ATP. Receptors Channels 6: 449-461.

Guillemare, E., Honore, E., De Weille, J., Fosset, M., Lazdunski, M., and Meisheri, K.D. (1994) Functional receptors in Xenopus oocytes for U-37883A, a novel ATP-sensitive K^+ channel blocker: comparison with rat insulinoma cells. Molec. Pharmacol. 46: 139-145.

Inoue I., Nagase H., Kishi K., Higuti T. (1991) ATP-sensitive K^+ channel in the mitochondrial inner membrane. Nature 352: 244-247.

Johnson D., Lardy H.A. (1967). Isolation of liver or kidney mitochondria. Methods Enzymol. 10: 94-96.

Kirshner N., Corcoran J.J., Caughey B., Korner M. (1987) Chromaffin vesicle function in intact cells. Ann. N. Y. Acad. Scien. 493: 207-219.

Lazdunski M. (1994) ATP-sensitive potassium channels: an overview. J. Cardiovasc. Pharmacol. 24 (suppl. 4): S1-S4.

Lobanov N.A., Szewczyk A., Wójcik G., Nowotny M., Nałęcz M.J. (1997) Effects of K^+ channel inhibitors on potassium transport in bovine adrenal chromaffin granules. Biochem. Molec. Biol. Int. 41: 679-686.

Nelson N., Perzov N., Cohen A., Hagai K., Padler V., Nelson H. (2000) The cellular biology of proton-motive force generation by V-ATPases. J. Exp. Biol. 203: 89-95.

Ornberg R.L., Kuijpers G.A., Leapman, R.D. (1988) Electron probe microanalysis of the subcellular compartments of bovine adrenal chromaffin cells. Comparison of chromaffin granules *in situ* and *in vitro*. J. Biol. Chem. 263: 1488-1493.

Paucek P., Mironova G., Mahdi F., Beavis A.D., Woldegiorgis G., Garlid K.D. (1992) Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K^+ channel from rat liver and beef heart mitochondria. J. Biol. Chem. 267, 26062-26069.

Pazoles C.J., Pollard H.B. (1978) Evidence for stimulation of anion transport in ATP-evoked transmitter release from isolated secretory vesicles. J. Biol. Chem. 253: 3962-3969.

Picaud S., Marty A., Trautmann A., Grynszpan-Winograd O., Henry J.P. (1984) Incorporation of chromaffin granule membranes into large-size vesicles suitable for patch--clamp recording. FEBS Lett. 178: 20-24.

Pollard H.B., Pazoles C.J., Creutz C.E., Zinder O. (1979). The chromaffin granule and possible mechanisms of exocytosis. Int. Rev. Cytol. 58: 159-197.

Pullman M.E., Penefsky H.S., Datta A., Racker E. (1960) Partial resolution of the enzymes catalyzing oxidative phosphorylation. I Purification and properties of soluble, di-nitrophenol-stimulated adenosine triphosphatase. J. Biol. Chem. 235: 3322-3329.

Storrie B., Madden E.A. (1990) Isolation of subcellular organelles. Methods Enzymol. 182: 203-225.

Szewczyk A. (1998). The intracellular potassium and chloride channels: properties, pharmacology and function. Mol. Membr. Biol. 15: 49-58.

Szewczyk A., Czyż A., Wójcik G., Wojtczak L., Nałęcz M.J. (1996) ATP-regulated K^+ channel in mitochondria: pharmacology and function. J. Bioenerg. Biomembr. 28: 147-152.

Szewczyk A., Lobanov N.A., Nowotny M., Nałęcz M.J. (1997) Interaction of sulfhydryl reagents with K^+ transport in adrenal chromaffin granules. Acta Neurobiol. Exp. 57: 329-332.

Szewczyk A., Marban E. (1999). Mitochondria: a new target for K channel openers? Trends Pharmacol. Sci. 20:157-161.

Szewczyk A., Pikuła S., Wójcik G., Nałęcz, M.J. (1996a) Glibenclamide inhibits mitochondrial K^+ and Na^+ uniports induced by magnesium depletion. Int. J. Biochem. Cell. Biol. 28: 863-871.

Szewczyk A., Wójcik G., Lobanov N.A., Nałęcz M.J. (1997) The mitochondrial sulfonylurea receptor: identification and characterization. Biochem. Biophys. Res. Comm. 230: 611-615.

Szewczyk A., Wójcik G., Nałęcz M.J. (1995) Potassium channel opener, RP66471, induces membrane depolarization of rat liver mitochondria. Biochem. Biophys. Res. Comm. 207: 126-132.

Thévenod F., Chathadi K.V., Jiang B., Hopfer, U. (1992) ATP-Sensitive K^+ conductance in pancreatic zymogen granules: Block by glyburide and activation by diazoxide. J. Membr. Biol. 129: 253-266.

Zinder O., Hoffman P.G., Bonner W.M., Pollard H.B. (1978) Comparison of chemical properties of purified plasma membranes and secretory vesicle membranes from bovine adrenal medulla. Cell Tissue Res. 188: 153-170.

Received 15 September 2000, accepted 9 November 2000