ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol. Exp. 1997, 57: 329-332

Interaction of sulfhydryl reagents with K^+ transport in adrenal chromaffin granules

Adam Szewczyk[1], **Nikolai A. Lobanov**[2], **Marcin Nowotny**[1] and **Maciej J. Nałęcz**[1]

[1]Department of Molecular and Cell Neurobiology, Nencki Institute of Experimental Biology, 3 Pasteur St., 02-093, Warsaw, Poland, Email: adam@nencki.gov.pl; [2]Institute of Bioorganic Chemistry, Belorussian Academy of Sciences, 5/2 Zhodinskaya St., Minsk 220141, Belarus

Short communication

Abstract. In the present study the functional role of SH groups in the Ca^{2+} -independent K^+ -selective channel activity in the membrane of bovine adrenal gland chromaffin granules has been studied. Ionic channel activity has been estimated using $^{86}Rb^+$, a K^+ analogue, flux measurements. The inhibition of chromaffin granules K^+ channel by SH modifying agents, such as N-ethylmaleimide, mersalyl and phenylarsenoxide, is described.

Key words: potassium transport, SH groups, chromaffin granules, adrenal glands

Potassium channels in various intracellular compartments, including synaptic vesicles (Woodbury 1995) and mitochondria (Szewczyk et al. 1996), have been described. Recently, Ca^{2+}-independent K^+ selective channel (K^+_{CG} channel) of large conductance in the membrane of adrenal gland chromaffin granules has been identified (Arispe et al. 1992, Ashley et al. 1994). The K^+_{CG} selective channel was found to be inhibited by TEA and insensitive to the electrical potential and to Ca^{2+} or charybdotoxin - a blocker of a Ca^{2+}-activated K^+ channel of large conductance (Arispe et al. 1992). Recently, the K^+_{CG} channel was reported to be controlled by both inhibitory and stimulatory heterotrimeric GTP--binding proteins (Arispe et al. 1995). It has been suggested that the K^+_{CG} channel plays a role in regulating the vesicle membrane potential and thus the process of exocytosis of catecholamines from adrenal chromaffin cells (Arispe et al. 1992).

The sulfhydryl groups (SH groups) are known to be important for the function of many membrane transport systems (for review see Van Inwaarde et al. 1992). These include also various potassium channels (Alekseev 1992, Lee et al. 1994, Han et al. 1996). In the present study the flux measurements (Garty et al. 1983, Garty and Karlish 1989) of $^{86}Rb^+$, a K^+ analogue, were used in order to study properties of the chromaffin granule K^+ channel since this method allows specific measurements in an heterogeneous population of vesicles. Experiments have been performed under conditions where only electrogenic transport of $^{86}Rb^+$ was measured. This simple and convenient flux assay, combined with the marker enzyme estimations, allowed a valuable measuring of the K^+_{CG} channel activity. In the present report it has been used to study the effect of sulfhydryl reagents on the electrogenic K^+ transport in chromaffin granules vesicles.

Bovine adrenal medullae were fractionated as described by Brocklehurst and Pollard (1990) leading to crude chromaffin granule preparations. The crude chromaffin granule pellet was resuspended in the homogenization buffer, overlaid on 1.7 M sucrose, 1 mM EGTA, 1 mM $MgSO_4$, 5 mM Hepes, pH 7.3, and centrifuged at 100,000 x g for 60 min at 4°C. The granular pellet was then resuspended in 25 volumes of solution containing 1mM $MgSO_4$, 20 mM Hepes, pH 7.3, and lysed by freezing and thawing. Subsequently, the suspension of chromaffin granule membranes was centrifuged at 48,000 x g for 30 min. The obtained pellet was resuspended in the lysis buffer, and the centrifugation was repeated. Further purification has been carried out by repeating resuspension of the pellet, its placing over a 1 M sucrose cushion and centrifugation. In detail, membrane suspension (2.5 ml) was overlaid on top of 12 ml of solution containing 1 M sucrose, 10 mM Hepes/KOH, pH 7.3 in Beckman SW28.1 rotor tubes and centrifuged at 100,000 x g for 90 min. The chromaffin granule membranes remaining on the top of the sucrose cushion were collected and resuspended in 25 volumes of 10 mM Hepes/KOH buffer, pH 7.4. The membranes were then centrifuged at 48,000 x g for 30 min and, subsequently, the pellet was resuspended in the buffer containing 100 mM KCl, 10 mM Hepes/Tris, pH 7.4 at a protein concentration of 1 mg/ml. Aliquots of this final suspension were frozen. The presence of a chromaffin granule membrane marker, cytochrome b_{561}, was measured by difference spectrum between dithionite-reduced and oxidized states at 561 nm (Zinder et al. 1978).

$^{86}Rb^+$ flux measurements through ion-conducting pathways were performed essentially as described before (Garty et al. 1983, Garty and Karlish 1989). The chromaffin vesicles were prepared to contain 100 mM KCl inside. Integrity of the vesicles was tested by estimation of membrane potential (with safranine O), after application of valinomycin. Shortly before starting the assay, the external 100 mM K^+ was replaced with $Tris^+$ ion on DOWEX ion-exchange resin that resulted in the formation of a K^+ gradient and, subsequently, in the efflux of K^+ from chromaffin granule vesicles. An electrical diffusion potential (negative inside) was thus established in the vesicles containing active K^+ channels. The addition of $^{86}Rb^+$ (a K^+ analogue) to the external solution under such conditions should lead to the uptake of $^{86}Rb^+$, against the K^+ gradient exclusively in the vesicles containing active channels. The process may occur due to the $^{86}Rb^+$ equilibration according to the diffusion potential previously built up across the granular membrane, what also amplifies the sensitivity of transport measurements. In addition, the $^{86}Rb^+$ uptake does not affect the potential itself.

An application of this method offers essential advantages to studies on intracellular ion channels. Procedures of tissue fractionation often lead to heterogeneous membrane vesicles, creating difficulties in interpretation of the single channel activity measurements in black lipid membranes. Instead, flux studies with membrane vesicles, plus estimation of suitable marker enzymes, allow to correlate the measured transport activity with a specific intracellular compartment. Also, flux measurements enable studies of numerous ionic channel effectors within a short time.

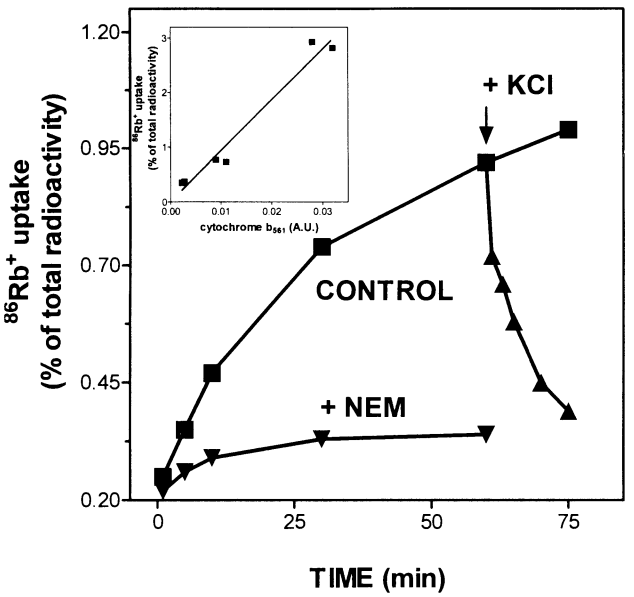

Fig. 1. Time course of $^{86}Rb^+$ uptake into the chromaffin granules. Accumulation of radioactivity (■) into the chromaffin granules was measured as described below. At the time point indicated by an arrow, 30 mM KCl was added to the reaction mixture (▲). Accumulation of radioactivity in presence of 3 mM N-ethylmaleimide (NEM) is also shown (▼). Measurements were performed as follows: aliquots of 100 µl of the chromaffin granule vesicles suspension were applied to small Dowex (50-X8 Tris form) columns and eluted with 1.5 ml of 175 mM sucrose. This step was performed to exchange the external potassium cations into $Tris^+$. The assay was initiated 30 s later by the addition of 2 - 4 µCi of $^{86}RbCl$ (specific radioactivity of 20 Ci/mmol, Polatom, Poland). Subsequently, in order to separate the vesicles from the medium, 100 µl aliquots of the reaction mixture were applied to Dowex columns (50-X8 Tris form), and vesicles were eluted directly into the counting vials by addition of sucrose solution, as previously. The amount of $^{86}Rb^+$ trapped within the vesicles (eluted from the Dowex columns) was estimated by scintillation counting. Measurements were performed at 20°C. The $^{86}Rb^+$ content is expressed as a percentage of the initial total radioactivity in the vesicle reaction medium, or as a percentage of the control (sample without added reagents). Figure shows a representative time course of ^{86}Rb uptake for 4 different experiments. Inset: Linear correlation ($r^2 = 0.965$) between the $^{86}Rb^+$ uptake found in various adrenal medulla membrane vesicle preparations (submitochondrial particles, microsomes, chromaffin granules) and the level of cytochrome b_{561}. The accumulation of radioactivity was measured 45 min following addition of $^{86}RbCl$ to the vesicles (performed as described above).

Figure 1 presents the time course of $^{86}Rb^+$ uptake into chromaffin granule vesicles, expressed as a percentage of the total radioactivity in the sample. Addition of 30 mM KCl, causing depolarization of the diffusion potential, promoted a rapid efflux of $^{86}Rb^+$ from the vesicles. In the presence of 3 mM N-ethylmaleimide accumulation of $^{86}Rb^+$ was found to be very low.

In order to verify whether the measured $^{86}Rb^+$ uptake activity is localized in the chromaffin granules membranes, both the transport activity and the cytochrome b_{561} level (maker enzyme of chromaffin granule membranes) were estimated in different populations of vesicles obtained during adrenal gland fractionation. Figure 1 (inset) shows the comparison of $^{86}Rb^+$ uptake with the level of cytochrome b_{561} in different subcellular fractions: submitochondrial particles, microsomes and chromaffin granule ghosts. The high $^{86}Rb^+$ uptake was found to correlate very well with the level of cytochrome b_{561} (Fig. 1). Additionally, no correlation was found of the 5-nucleotidase, glucoso-6-phosphatase or cytochrome c oxidase activities with $^{86}Rb^+$ uptake in microsomes and submitochondrial particles, respectively (data not shown). Hence, it is concluded that the measured channel activity is located in chromaffin granules mem-

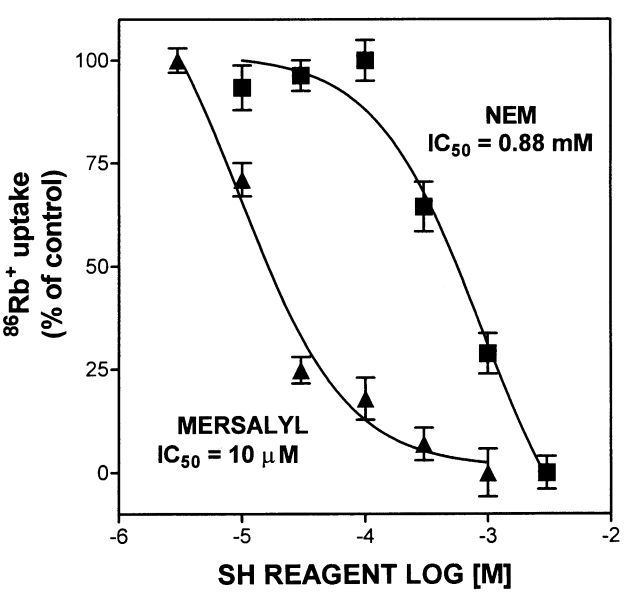

Fig. 2. Concentration dependence of the effect of mersalyl and N-ethylmaleimide on $^{86}Rb^+$ uptake into chromaffin granules. Measurements of the $^{86}Rb^+$ uptake into chromaffin granules were performed as described in the legend to Fig. 1. Points represent mean ± SD. Figure shows results of one representative experiment out of three independent experiments.

brane. In addition, with no diffusion potential created (no K^+ gradient) the accumulation of $^{86}Rb^+$ was found to be very low, confirming that the applied assay measures an electrogenic transport of K^+ (data not shown).

Figure 2 shows the effect of SH-reagents (NEM, mersalyl) on $^{86}Rb^+$ uptake into the chromaffin granules vesicles. Both NEM and mersalyl were found to inhibit the measured transport. Figure 2 shows the concentration dependence of the inhibitory effects exerted by NEM ($IC_{50} = 0.88 \pm 0.08$ mM) and mersalyl ($IC_{50} = 10 \pm 2$ μM). In addition, $^{86}Rb^+$ uptake was found to be inhibited by phenylarsenoxide ($IC_{50} = 34 \pm 4$ μM) (data not shown), what supplies strong evidence for the presence of vicinal SH-groups in K^+_{CG} channel.

In conclusion, results of the present investigation point to the sensibility of electrogenic K^+ transport system in chromaffin granules to SH modifying agents. The described inhibitory effects could prove useful in further studies dedicated to the identification of a specific inhibitor of the granular potassium channel.

This study was supported in part by grant No. 6 P203 003 04 to Adam Szewczyk. In addition, the fellowship No. 325 from UNESCO/MCBN (to Nikolai A. Lobanov) is gratefully acknowledged.

Alekseev S.I. (1992) Interaction of sulfhydryl reagents with A-type channels of Lymnaea neurons. Biochim. Biophys. Acta 1110: 178-184.

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 from chromaffin granule membranes. J. Membr. Biol. 130: 191-202.

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

Brocklehurst K.W., Pollard H.B. (1990) Cell biology of secretion. In: Peptide hormone secretion. A practical approach (Eds. .C. Hutton and K. Siddle). IRL/Oxford, Oxford, p. 233-255.

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

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

Han J., Kim E., Won-Kyung H., Earm Y.E. (1996) Sulfhydryl redox modulates ATP-sensitive K^+ channels in rabbit ventricular myocytes. Biochim. Biophys. Res. Commun. 219: 900-903.

Lee K., Ozanne S.E., Hales C.N., Ashford M.L.J. (1994) Effects of chemical modification of amino and sulfhydryl groups on K_{ATP} channel function and sulfonylurea binding in CRI-G1 insulin-secreting cells. J. Membr. Biol. 139: 167-181.

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.

Van Inwaarden P.R., Driessen A.J.M., Konings W.N. (1992) What we can learn from the effects of thiol reagents on transport proteins. Biochim. Biophys. Acta 1113: 161-170.

Woodbury D.J. (1995) Evaluation of the evidence for ion channels in synaptic vesicles. Mol. Membr. Biol. 12: 165-171.

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

Received 5 May 1997, accepted 30 September 1997