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NUCLEOTIDE-ACTIVATED CHLORIDE CHANNELS IN LYSOSOMAL MEMBRANES*

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Lysosomal membrane vesicles purified from rat liver contain a basal chloride conductance that was enhanced in the presence of ATP, non-hydrolysable ATP-analogs and, to a lesser extent, GTP. Other nucleotides, including AMP, ADP and cAMP, as well as CTP and UTP were not effective. Following fusion of the vesicles with an artificial phosphatidylethanolamine/phosphatidylserine bilayer, we found that ATPγS dramatically increased the incidence of 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS)- sensitive chloride channels with a unitary slope conductance of approx. 40 pS in 300 mM/50 mM KCl buffers and 120 pS in symmetrical 300 mM KCl buffers. Since similar results were obtained with AMP-PNP, the results indicate that lysosomes contain a chloride permeable ion channel that is activated by ATP through allosteric interaction.

Lysosomes, eukaryotic organelles involved in the degradation of both intra- and extracellular materials, are characterized by an acidic internal pH maintained through an ATP-driven electrogenic H^+-pump [1]. In addition, lysosomes contain specific carriers for low molecular weight metabolites, such as neutral and anionic sugars and amino acids [2-4]. Recently, an anion conductance has been proposed to counter balance the membrane potential generated by the flow of the positively charged protons [5-7], the identity of the anion conductance however has not yet been established. In our study, rat liver lysosomal membranes were used to characterize Cl^- conductances both at the macroscopic level (*i.e.* by means of quenching of 6-methoxy-N-(3-sulfopropyl)-quinolium (SPQ) fluorescence in

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Abbreviations used: ATPγS, adenosine-5'-O-(2-thiodiphosphate); AMP-PNP, adenylylimidodiphosphate; DIDS, 4,4'-diisothio-cyano-stilbene-2,2'disulphonic acid.

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vesiculated membranes) and at the single channel level (*i.e.* upon reconstitution in planar lipid bilayers). The role of the lysosomal chloride conductance could be classified as an ATP-activated Cl^- channel whose biophysical and regulatory characteristics were clearly different from CFTR, the cystic fibrosis gene encoded Cl^- channel.

MATERIALS AND METHODS

Materials: All nucleotides were purchased from Boehringer Mannheim (FRG). Phospholipids were obtained from Avanti Polar Lipids, Birmingham AL, U.S.A., SPQ from Calbiochem., La Jolla CA, U.S.A. and catalytic subunit of protein kinase A from Promega, Madison, WI, U.S.A. All other chemicals were from Sigma Chem. Co., St.Louis, MO, U.S.A..

Preparation of lysosomal membrane vesicles: Rat liver lysosomal membranes were prepared as previously described [2]. The purity of the membranes was assessed by determining the specific activities of β-glucocerebrosidase and tartrate-inhibitable acid phosphatase, both lysosomal marker enzymes, and alkaline phosphatase and ouabain-sensitive Na^+/K^+ ATPase, marker enzymes for both plasma membranes and (early) endosomal membranes. Lysosomal membrane markers were 90 - fold enriched in the final preparation (recovery: 8%) [2]. Electronmicroscopical examination showed a homogeneous population of vesicles totally free of recognizable mitochondrial or RER debris (data not shown). The membrane orientation upon resealing is considered to be random [8]

SPQ-quenching: Lysosomal membrane vesicles were resuspended in a buffer containing 20 mM HEPES (pH=7.0), 300 mM mannitol, 6 mM $MgSO_4$, 3 mM Ca/EGTA (pCa=6), 1 mM $NaVO_3$ and 20 mM SPQ. Nucleotides were trapped into the vesicles through a single cycle of freeze-thawing in liquid nitrogen. The KCl-induced rate of SPQ quenching was quantitated using a Perkin Elmer LS-3B fluorescence spectrometer interfaced to a micro-computer and rate constants (k_1) were determined as previously described [9].

Reconstitution into planar lipid bilayers: Planar lipid bilayers of phosphatidyl-ethanolamine and phosphatidylserine (7:3; wt/wt) were painted onto a 0.3 mm hole drilled in a polystyrene cup [10]. Lysosomal vesicles (5 - 25 μg of protein) were added to the cis compartment which contained 300 mM KCl, 10 mM HEPES (pH=7.2) and 1 mM of $CaCl_2$, $MgCl_2$ and EGTA, and was continuously stirred. The buffer in the trans compartment was identical except for [KCl], which was 50 mM. In some experiments, [KCl] in the trans compartment was raised to 300 mM, to obtain a symmetrical salt solution. Chloride channels were identified by a negative current at a -40 mV holding potential, the K^+ equilibrium potential, and the absence of a significant current at +40 mV (Cl^- equilibrium potential). The cis chamber was connected to a pulse generator, leaving the trans compartment at virtual ground. Currents were measured by feeding the trans compartment into the current-to-voltage converter of a voltage clamp system. Signals were filtered at 300 Hz prior to data analysis.

RESULTS AND DISCUSSION

Highly purified lysosomal membrane vesicles isolated from rat liver were loaded with SPQ, a halogen-sensitive fluorochrome demonstrated to be suitable for quantitating

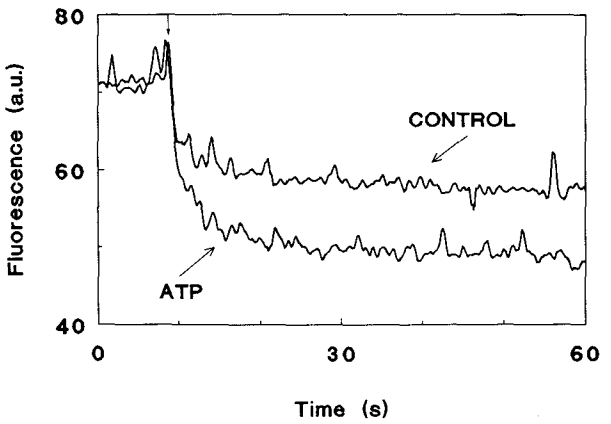

Figure 1. Rate of SPQ quenching. Cl^--influx in control (- ATP) and ATP-containing rat liver lysosomal vesicles determined in presence of valinomycin (10 μM) using the Cl^--sensitive probe SPQ. Quenching of SPQ was started by the addition of KCl (arrow; final concentration: 25 mM) and terminated by addition of Triton X-100 (final concentration: 0.05 %). The experiment shown is representative of 6 independent experiments.

transmembrane chloride fluxes [11]. In the presence of the potassium ionophore valinomycin, *i.e.* when generation of a counteracting membrane potential is inhibited, trapped ATP (1 mM) markedly increased the rate of KCl-induced (25 mM) SPQ quenching (Fig.1), indicative for an increased intravesicular Cl^- concentration. ATP not only enhanced the rate of SPQ quenching, but also increased the amplitude of the rapid phase of the response dramatically (Fig. 1), suggesting that in addition to an increased membrane permeability to Cl^-, cryptic Cl^- channels became activated (c.f ref.[12]). The rapid quenching of SPQ observed is not secondary to activation of the proton pump since, in inside-out vesicles, the presence of an ATP stimulated efflux of protons would result in a reduced rather than an increased rate of SPQ quenching. In further experiments however, ATPγS was used instead of ATP to preclude any secondary effects of proton-pump induced pH gradients on the lysosomal Cl^- conductance. As demonstrated in Table 1, ATPγS, in the

Table 1. Effects of ATPγS on lysosomal Cl^- fluxes. SPQ-quenching experiments were performed as described in Figure 1. Rate constants (k_I) were calculated and expressed as mean or mean ± SEM. Number of independent experiments are indicated in parentheses. Asterisks indicate a significant difference from the control ($p < 0.05$; Student *t* test).

	k_I of SPQ quenching (s^{-1})		
	Control	ATP	ATPγS
None	0.18 ± 0.01 (3)	0.26 (2)	0.25 ± 0.02* (4)
Valinomycin	0.23 ± 0.01 (6)	0.37 ± 0.10* (6)	0.37 ± 0.03* (6)

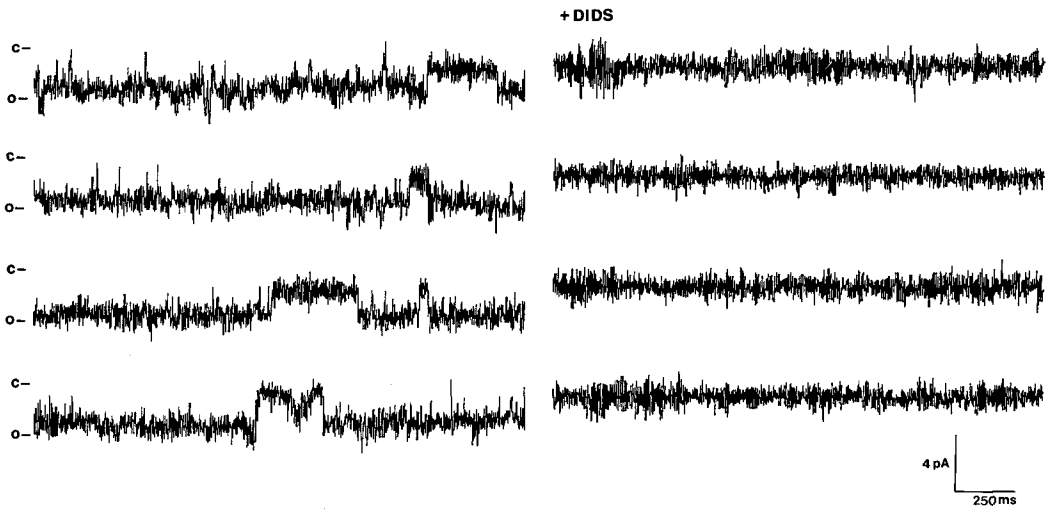

Figure 2. Fusion of rat liver lysosomal membrane vesicles with an artificial PE/PS planar lipid bilayer. Activation of a chloride channel by ATPγS (100 μM) in the absence and presence of DIDS (50 μM). Closed (c) and open (o) states of the channel are indicated. Holding potential: -40 mV.

presence of valinomycin, accelerated SPQ quenching approx. 1.5 - 2 fold, from k_1 = 0.23 ± 0.01 s^{-1} in control to k_1 = 0.37 ± 0.03 s^{-1} in ATPγS-loaded vesicles. The response to ATPγS was comparable to that measured with ATP (k_1 = 0.37 ± 0.10 s^{-1}; n=6), but, since ATPγS is not a substrate for endogenous ATPases, the experimental variation is substantially reduced. Other nucleotides, including cAMP, AMP, ADP as well as CTP and UTP were ineffective in stimulating chloride influx (not shown). A small increase however, was observed by trapping GTP, but not GDP, into the vesicles (k_1 = 0.26 ± 0.01 s^{-1}; n=3; $p < 0.05$).

To characterize the biophysical properties of the anion conductance in more detail, lysosomal membrane vesicles were fused with artificial planar lipid bilayers and the occurrence of anion selective channels was monitored. In the absence of ATPγS, chloride channels were scarcely found (only 2 out of 12 experiments) within the time span of the experiment (30 min). However, in the presence of ATPγS added to both compartments, chloride channels were observed much more frequently, in 7 out of 12 experiments, suggesting that ATP activates cryptic anion channels. Typically, active channels were observed within 15 minutes following vesicle addition. Chloride channels were also observed when ATPγS was added to the trans compartment only (2 out of 3 experiments), indicating that the higher frequency of channel activity is not secondary to ATPγS facilitated fusion of vesicles with the bilayer. Furthermore, in two separate experiments ATPγS was added to a bilayer which previously had been silent for at least 30 minutes, resulting in an almost instantaneous activation of multiple chloride channels. The ATP activated channel was rapidly inhibited upon addition of DIDS (50 μM), an irreversible blocker of anion channels (Fig. 2). The unitary slope conductance of the channel at 0 mV holding potential was

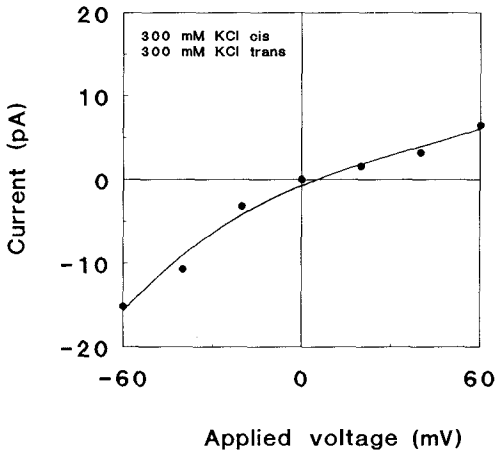

Figure 3. Voltage/current relationship of ATPγS activated Cl⁻-channels. Rat liver lysosomal membranes were fused with a planar lipid bilayer. V/I characteristics were measured in the presence of symmetrical 300 mM KCl buffers.

approx. 40 pS in asymmetrical KCl solutions (300 mM Cis; 50 mM Trans) and approx. 120 pS in symmetrical 300 mM KCl buffers (Fig. 3). The ATPγS-stimulated chloride channel showed a slightly rectifying current to voltage relationship (Fig. 3), whereas the mean open time probability (0.5 ± 0.2) was not significantly affected by changing the applied voltage (not shown).

Different mechanisms could be involved in the nucleotide activation of lysosomal Cl^- channels. First, a chloride conductance activated by protein kinase A mediated phosphorylation is well documented for plasma membranes of epithelial cells (for review see: ref.[13]), and has recently also been established in renal endocytic vesicles [14]. Although we cannot exclude that the chloride channel found in lysosomal membranes could serve as a substrate for protein kinase A, our finding that ATP or GTP by itself and in the absence of cAMP increased the chloride conductance suggests that protein phosphorylation is not involved. This notion is supported by our finding that AMP-PNP (1 mM), an ATP analog that does not serve as a substrate for protein kinases, activates the channel in 3 out of 4 bilayer experiments (data not shown). Furthermore, addition of the catalytic subunit of protein kinase A (150 nM) to ATP activated channels did not increase the open state probability nor did it activate additional channels (n=5; not shown). Secondly, a distinct class of Cl^- selective channels regulated by GTP-binding regulatory proteins was found in plasma membranes of intestinal and renal epithelial cells [9, 15]. Since these channels are activated by GTP but not by ATP, it is unlikely that a similar mechanism is involved in ATP activation of lysosomal anion channels. Taken together our results suggest that the nucleotide-dependent activation of the channel occurs through allosteric interaction rather than protein phosphorylation or activation of G-proteins.

The gene involved in the chloride transport defect observed in cystic fibrosis patients has been identified and cloned [16]. It encodes for a 8-10 pS chloride selective ion channel,

the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), whose activation is triggered by cAMP dependent phosphorylation [17, 18] and requires ATP hydrolysis [19]. Reconstituted in a planar lipid bilayer [20, 21], the properties of CFTR are distinct from the lysosomal Cl^- channel in that the latter channel is activated by ATP, ATPγS and AMP-PNP alone and does not require protein phosphorylation. In addition, the channels differ markedly in single channel conductance and in sensitivity to DIDS. Finally, recent reports indicate that the CFTR Cl^- channel is not involved in lysosomal acidification [22, 23]. Taken together our data suggest that the lysosomal Cl^- channel described is different from both CFTR [22] and protein kinase A activated Cl^- channels in endosomes [14]. However it may well represent another member of the superfamily of A(TP)B(inding)C(assette) transporters, which includes CFTR and the multidrug resistance proteins (P-glycoproteins [23]). The recent finding that MDR-1 functions as an ATP/ATPγS dependent volume activated Cl^- channel [24] supports this notion.

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