Monoclonal Antibodies against MDR1 P-glycoprotein Inhibit Chloride Conductance and Label a 65-kDa Protein in Pancreatic Zymogen Granule Membranes*

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The regulation of Cl⁻ and cation conductances by the nonhydrolyzable ATP analog adenosine 5'-(β,γ-methylene)triphosphate (AMP-PCP) was characterized in isolated zymogen granules (ZG) from pancreatic acinar cells. ZG were purified from rat pancreas homogenate by Percoll gradient centrifugation. Cl⁻ conductance was assayed by suspending ZG in isotonic KCl buffer and measuring osmotic lysis induced by maximal permeabilization of ZG membranes (ZGM) for K⁺ with the K⁺ ionophore valinomycin (Val). This resulted in influx of K⁺ through the artificial pathway and of Cl⁻ through endogenous channels. To measure cation conductances ZG (pH, 6) were suspended in pH 7 buffered isotonic monovalent cation acetate salts. The pH gradient was converted into an outside-directed H⁺ diffusion potential by maximally increasing H⁺ conductance of ZGM with the protonophore carbonyl cyanide m-chlorophenyl hydrazone. Osmotic lysis of ZG was induced by H⁺ diffusion potential driven influx of monovalent cations through endogenous channels and non-ionic diffusion of the counterion acetate. In the absence of Val, ZG were stable in KCl buffer up to 2 h. AMP-PCP enhanced osmotic lysis 4-fold compared to control, due to activation of Cl⁻ conductance by AMP-PCP and K⁺ influx through an AMP-PCP-insensitive nonselective cation pathway, which could be blocked by 0.1 mM Ba²⁺, 0.5 mM quinine, or 0.2 mM flufenamate. In addition, a K⁺ and Rb⁺ selective cation conductance was found which was completely blocked by 0.5 mM AMP-PCP or 0.5 mM quinine. AMP-PCP induced Cl⁻ conductance was strongly inhibited by two monoclonal antibodies against MDR1 P-glycoprotein (JSB-1 and C219; 5-10 μg/ml), but not by a monoclonal antibody against the cystic fibrosis transmembrane conductance regulator (M3A7; 5 μg/ml) or by mouse IgG. The AMP-PCP-insensitive nonselective cation conductance was not blocked by monoclonal antibodies against MDR1 P-glycoprotein (MDR1). Immunoblot studies of ZG membranes revealed the presence of a major immunoreactive protein band of ≈65 kDa with both monoclonal antibodies against MDR1, but no protein of the approximate size of MDR1 (≈170 kDa) was detected. We propose that the Cl⁻ channel or a regulator of the channel, that is activated by the non-hydrolyzable ATP analog AMP-PCP in ZG membranes, is a member of the ATP-binding cassette superfamily of transporters and may have homology to MDR1 P-glycoprotein.

Upon stimulation by secretagogues, such as acetylcholine or cholecystokinin, or their second messengers diacylglycerol and inositol triphosphate/CA²⁺, pancreatic acinar cells secrete NaCl, fluid, and digestive enzymes. The mechanisms of regulated NaCl secretion in exocrine gland cells have been intensively investigated (1). NaCl secretion is thought to involve "active," cytoplasmic accumulation of Cl⁻ above its electrochemical equilibrium through three interlinked transporters at the basolateral plasma membrane acting together as a Cl⁻ pump: the (Na⁺ + K⁺)-ATPase, the Na⁺-K⁺-2Cl⁻ cotransporter and, depending on the exocrine gland under study, a K⁺-selective or nonselective cation channel (2, 3). NaCl and fluid secretion is controlled by cytosolic Ca²⁺ signals which regulate the opening of the cation channels required for recirculation of K⁺ through the cotransporter. Cl⁻ secretion occurs through activation of luminal Cl⁻ channels, which are regulated by internal Ca²⁺ as well (4, 5). Na⁺ flow may occur through paracellular pathways and the leaky tight-junctions into the electrically negative lumen.

Regulated secretion of digestive enzymes takes place through poorly understood processes that are morphologically characterized by fusion of zymogen granules with the apical plasma membrane and release of granular contents into the acinar lumen (6). Using permeabilized pancreatic acinar cells we have demonstrated that the Ca²⁺ and cAMP signaling pathways stimulate secretion of digestive enzymes (7). This type of studies also revealed an absolute dependence of regulated exocrine enzyme secretion on the ionic environment of the secretory granules: isosmotic replacement of Ca²⁺ or K⁺ by impermeant ions, or application of Cl⁻ and K⁺ channel blockers abolished hormone- or second messenger-induced enzyme secretion (8).

Based on these observations, the presence of hormonally regulated Cl⁻ and K⁺-selective channels in the membranes of ZG has been postulated: upon fusion of ZG with the luminal plasma membrane, the increased influx of salt and water through the granule Cl⁻ and K⁺ channels would promote enzyme secretion, possibly by enhanced decondensation and "flushing-out" of macromolecular enzymes (8, 9). Cl⁻ and K⁺ permeabilities have been characterized in isolated ZG (9-12). In addition to regulation by protein kinase-mediated protein phosphorylation (11, 12), the Cl⁻ and K⁺ permeabilities in ZG are modulated by ATP binding. The K⁺ conductance is blocked by ATP and its non-hydrolyzable analogs (12). These properties together with its pharmacological characteristics (inhibition by

1 The abbreviations used are: ZG, zymogen granules; AMP-PCP, adenosine 5'-(β,γ-methylene)triphosphate; ATP-PNP, adenosine 5'-(β,γ-imidodiphosphate; ATPyS, adenosine 5'-(γ-thio)triphosphate; CCP, carbonic anhydrase; CFTR, cystic fibrosis transmembrane conductance regulator; CHRC5, colchicine-resistant Chinese hamster ovary cells; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; mAb, monoclonal antibody; MDR1, multidrug resistance P-glycoprotein; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid.

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CL– Conductance in Zymogen Granules and MDR1 P-glycoprotein

Introduction

Glibenclamide and activation by diazoxide (12) suggest that the K+ conductance is mediated by channels similar or identical to ATP-sensitive K+ channels found in the plasma membrane of islet cells, neurons, muscle, and renal cells (13). The CL– conductance is activated by ATP and non-hydrolyzable analogs of ATP, such as AMP-PCP or AMP-PNP (14).

Recently, two members of the ATP binding cassette superfamily of transporters with properties of CL– channels have been cloned: the cystic fibrosis conductance transmembrane regulator (CFTR) (15) and the multidrug resistance P-glycoprotein (MDR1) (16). They are activated by ATP, the mechanisms of activation, however, are only partially understood. The CFTR CL– channel is activated by protein kinase A- and C-mediated phosphorylation (17), but activation may also require ATP binding and hydrolysis (18–20). The MDR1 associated CL– current is induced by swelling and requires allolytic interaction with ATP for activation (21, 22).

A long-term goal of our studies is the molecular identification of the major transporters present in the ZG membrane and to understand their role in the membrane transport of counterions. Monoclonal antibodies (mAb) are potentially useful tools in the identification strategy. They can detect proteins of interest in cell membranes and localize them in intact cells. Occasionally, they also alter the function of the protein studied, providing information linking structure and function. In the present study, we have examined the effect of mAb against MDR1 and CFTR on the ion conductance pathways of pancreatic ZG and performed immunoblot analysis of purified ZG membranes. The data indicate that the two mAb directed against cystolic epitopes of MDR1, JSB-1 and C219, are functional blockers of the CL– conductance of pancreatic ZG membranes that is activated by the non-hydrolyzable ATP analog AMP-PCP. A ZG membrane protein of ~65 kDa was labeled by both antibodies against MDR1 and could represent the CL– channel or a regulator of the channel.

Experimental Procedures

Materials

AMP-PCP sodium salt, valinomycin, carbonyl cyanide n-chlorophenylhydrazone (CCCP), quinine hydrochloride, fluoride acid, and mouse immunoglobulin (mouse IgG) were obtained from Sigma (Deisenhofen, Germany). Horseradish-peroxidase conjugated sheep anti-mouse immunoglobulin (mouse IgG) were obtained from Sigma (Deisenhofen, Germany). Harsheradish-peroxidase conjugated sheep anti-mouse IgG was from Amersham (Braunschweig, Germany). Anti-P-glycoprotein 170–180 (JSB-1–25) was purchased from Boehringer (Mannheim, Germany). P-glycoCHEK C 219 MAB (for the detection of P-glycoprotein) (24) was from Isotopen Diagnostik CIS GmbH (Dreieich, Germany), and anti-CFTR mouse monoclonal antibody M3A7 (25) was kindly provided by Drs J. R. Riordan and N. Kartner, University of Toronto, Canada.

Methods

Isolation of ZG and Purification of ZG Membranes—Zymogen granules were isolated from the pancreatic glands of male Wistar rats (180–300 g, Charles River Wiga GmbH, Sulzfeld, Germany) as described earlier (12). Granule membranes were purified as described previously (26), with slight modifications: ZG were diluted about 10-fold in an ice-cold hypotonic lysis buffer containing 0.1 mM MgSCN, 5 mM EDTA, 10 mM HEPES, 50 mM KSCN, adjusted to pH 7.9 with Tris, plus a protease inhibitor mixture (10 µg/ml leupeptin, 1 mM benzamidine, 0.2 mM PefablocSC, and 50 µg/ml trypsin inhibitor), and lysis was allowed to proceed on ice for 1 h. The clear suspension was centrifuged at 200,000 x g for 90 min. The pellet containing purified ZG membranes was stored in liquid nitrogen. ZG membranes were suspended to provide 10 µg of protein/ml.

Assays for Ion Conductances of ZG—CL– and cation conductances of pancreatic ZG were assayed according to a previously reported method for quantitative evaluation of macroscopic ion fluxes through endogenous conductance pathways of ZG membranes (10, 12). This assay relies on the measurement of osmotic lysis of ZG resuspended in buffered isotonic salt solution following addition of anionic ionophore for counterions. If the membrane permeability for counterions is low, ZG will remain stable in isotonic solutions for several hours in the absence of ionophore, because no net salt accumulation can occur into the intragranular space. Addition of an electrogenic ionophore maximally permeabilizes the ZG membranes for the counterion, and net salt influx can take place through the endogenous ion conductance and the artificial pathway. The osmotic load of the intragranular space attracts water, the granules swell and lyse. The end point of salt influx through endogenous conductance, lysis, can take place through the endogenous conductance pathway, but not by the flux of counterions through the shunt pathway. Consequently, the slope of the decrease in absorbance with time will represent an estimate of the rate of ions transported through the endogenous conductance pathway. Anion conductance was measured by resuspending ZG in a solution buffered with 20 mM HEPES to pH 7.0 (iso-osmotic 150 mM KCl) in a cuvette, adding 5 µM valinomycin, which selectively and maximally permeabilizes ZG membranes to the major cation K+.

To measure cation conductance, zymogen granules were suspended in 150 mM monovalent cation/acetate solutions containing 1 mM EDTA, buffered with 50 mM imidazole, and titrated to pH 7.0 with acetic acid. Since the intragranular pH is more acidic than the incubation medium, an inside-to-outside directed proton concentration gradient between the intragranular space and incubation solution was generated. To control the magnitude of this pH gradient, the permeant buffer imidazole was employed. Under these conditions, the transmembrane pH gradient was about 0.5 pH units (12). Cation influx through endogenous cation conductances was initiated by addition of 16 µM CCCP, an electrogenic protonophore. CCCP maximally permeabilizes the granular membrane to protons and converts the proton concentration gradient to an inside-negative proton diffusion potential. The inside-negative granular membrane potential, in turn, energizes cation influx through endogenous cation permeabilities of the ZG membrane. Acetate does not permeate the ZG membrane. When the buffer acetate concentration is high, anion influx occurs through the uncharged molecule acetic acid, which permeates through the lipid membrane by non-ionic diffusion and associates to provide the intragranular space continuously with protons for protonation of imidazole as well as for proton efflux from the acid interior (12). In this assay H+ permeation and non-ionic diffusion of imidazole acetate are not rate-limiting. Intragranular accumulation of cation acetate salts, water influx, and osmotic lysis of the granules are determined by cation influx through endogenous cation permeabilities. Cation conductances were estimated by adding 3 ml of buffered salt solution, which corresponds to an initial absorbance of 0.3–0.4. Absorbance was usually recorded continuously for 20–50 min at 37 °C with a Beckman DU-64 spectrophotometer equipped with a Peltier constant-temperature chamber, an automatic six-unit sampler, and a kinetics Soft-Pac Module. Data were stored using a Beckman Light Scatter program and analyzed using a Symphony spreadsheet program (Lotus Development GmbH, Munich, Germany).

Analysis and Validation of Transport Data—The half-time of granular lysis was estimated from the slope of the decrease in absorbance with time between ionophore addition and either experimental half-time, or the entire observation period if the half-time was not reached. The slope of the absorbance change with time was estimated by linear regression of the digitized data. Lysis rates were expressed as half-times of granular lysis or its reciprocal value, i.e. the inverse half-time of lysis which was considered proportional to the rate constant of lysis. Linear regression analysis of the data actually represents an oversimplification of the actual kinetics of lysis. For instance, in experiments with AMP-PCP carried out in the absence of ionophore, lysis occurred after some latency (see Fig. 2). For these curves linear regression analysis was carried out either from the latency period or from the onset of lysis. Lysis rates calculated from the onset of lysis were ~2-fold higher than those calculated from the entire observation period. However, the dose response curves for AMP-PCP (Fig. 3) or for the effect of antibodies on lysis (Fig. 5) were not different. In these experiments, lysis values for ionophores and antibodies were calculated from the data of the entire observation period.

Unless otherwise indicated, experiments were repeated at least with three different granule preparations and data expressed as means ± SEM.
RESULTS

The Non-hydrolyzable ATP Analog AMP-PCP Blocks K+ Conductance—When ZG were isolated from exocrine pancreas as described under “Methods,” they were osmotically stable in isotonic K-acetate/imidazole solution up to 2 h, unless the nonhydrolyzable ATP analog AMP-PCP was added to the cuvette, which resulted in enhanced lytic lysis of the granules (Fig. 1A). This behavior is explained as follows. In the presence of the nonhydrolyzable ATP analog AMP-PCP, the proton concentration gradient across the membrane is converted to an inside-negative diffusion potential that serves as driving force for cation influx through the endogenous K+ permeability. Continuous acetic acid influx maintains the gradient and membrane potential. The endogenous K+ conductance present in the membrane becomes rate-limiting for K+ influx and the osmotic load (net uptake of K-acetate) induces first granular swelling and finally granular lysis.

Incubation of granules in the presence of the non-hydrolyzable ATP analog AMP-PCP inhibited K+ conductance in a dose-dependent fashion (Fig. 1, A and B). A maximal reduction of inverse half-time of lysis to 52 ± 14% of the controls without AMP-PCP was observed at a concentration of 0.25 mM AMP-PCP; higher concentrations (up to 1 mM) did not further inhibit K+ conductance (Fig. 1B). The experiments were carried out in the presence of 5 mM Mg2+. Qualitatively similar results were found in the absence of Mg2+, although inhibition by AMP-PCP was less pronounced (not shown). This confirms a previous report, where concentrations of AMP-PCP up to 5 mM in the absence of Mg2+ reduced K+ conductance by about 40% (12).

The Non-hydrolyzable ATP Analog AMP-PCP Activates Cl- Conductance and Induces Osmotic Lysis in the Absence of Valinomycin—When ZG were incubated in isotonic HEPES-buffered KCl solution, they were osmotically stable up to 4 h. Addition of the electrogenic potassium ionophore valinomycin (5 μM) enhanced lysis of the granules (Fig. 2) allowing influxes of K+ and Cl- down their respective electrochemical gradients (through the artificial K+ conductance and the endogenous Cl- conductance), followed by water influx. As the valinomycin-induced K+ conductance was high, the endogenous Cl- conductance present in the membrane became rate-limiting for KCl flux and subsequent lysis of granules. We had previously demonstrated that adenine nucleotides, such as ATP (above 50 μM), or the non-hydrolyzable analogs AMP-PCP and AMP-PNP increased Cl- conductance in pancreatic and parotid ZG; the most pronounced effects were observed with AMP-PCP which in-

![Fig. 1](https://via.placeholder.com/150)

**Fig. 1.** A, protonophore-induced osmotic lysis of pancreatic ZG and inhibition of granular lysis by the non-hydrolyzable ATP analog AMP-PCP. ZG were incubated as described under “Methods” plus or minus the indicated concentration of AMP-PCP and incubated in the cuvette at 37 °C for 5 min. Measurements were started at the arrow, and the protonophore CCCP (16 μM) was added, where indicated. B, dose response curve for block of K+ conductance by AMP-PCP in pancreatic ZG. Control inverse half-times of lysis (100%) measured in the absence of AMP-PCP were 13.3 ± 4.6 h⁻¹. *p < 0.05; **p < 0.02; ***p < 0.001, by Student’s t test for paired comparison of conditions plus or minus AMP-PCP. Results are means ± S.D. of three to four different experiments.

![Fig. 2](https://via.placeholder.com/150)

**Fig. 2.** Effect of AMP-PCP on osmotic lysis of pancreatic ZG incubated in HEPES-buffered medium (150 mM KCl) with or without valinomycin. ZG were incubated in the presence or absence of 0.5 mM of the nonhydrolyzable ATP analog AMP-PCP. At the arrow, 5 μM valinomycin was added, where indicated, and recording of the data was started. These data are typical of 50 different experiments.
creased Cl⁻ conductance up to 4-fold at a final concentration of 0.1 mM (Fig. 2). This ionophore independent osmotic lysis apparently required activation of specific ion transporters, since no lysis occurred, when KCl was replaced by sucrose or mannitol mole by mole (not shown). The increase in osmotic lysis observed with AMP-PCP in the absence of valinomycin showed a half-maximal effect at ~50 μM and was maximal above 0.25 mM (Fig. 3). Inverse half-times of lysis in controls without valinomycin were calculated to 0.40 ± 0.05 h⁻¹ and increased to 1.46 ± 0.06 h⁻¹ by addition of 0.25 mM AMP-PCP (p < 0.001). In other words, 0.25 mM AMP-PCP stimulated osmotic lysis in the absence of valinomycin by ~4-fold.

Valinomycin Independent ZG Lysis Induced by AMP-PCP Involves a Nonelective, AMP-PCP Insensitive, Cation Conductance Pathway—Following replacement of K-acetate by Na-acetate, CCCP also induced granule lysis, suggesting Na⁺ flux through a Na⁺ permeable electrogenic ion pathway (Table I). AMP-PCP at concentrations ranging between 0.1 and 1 mM did not significantly inhibit the Na⁺ permeable conductive pathway (5.6 ± 3.5 h⁻¹ in controls versus 4.8 ± 3.0 h⁻¹ in the presence of 0.5 mM AMP-PCP). We tested in a similar manner the inhibitory effect of AMP-PCP on monovalent cation conductance pathways, we tested various inorganic and organic pharmacological agents which block other K⁺ channels and/or nonselective cation channels. We compared the inhibitory effect of these agents on the cation conductance of granules incubated in K-acetate, which predominantly permeates the K⁺-selective pathway, and of granules incubated in Na-acetate, which almost exclusively permeates the nonselective cation pathway (see Table I).

In this set of experiments AMP-PCP at the maximal inhibitory concentration of 0.5 mM significantly decreased K⁺ conductance to 68 ± 15% of the controls without nucleotide (p < 0.001), i.e., it mainly blocked the K⁺-selective conductive pathway (Fig. 4). Na⁺ conductance was not inhibited by AMP-PCP (91 ± 11% of control; not significant). Quinine, a lipophilic inhibitor of certain types of K⁺ channels, inhibited K⁺ conductance in a dosedependent way. Half-maximal inhibition was obtained at ~0.1 mM quinine and maximal inhibition at 0.5 mM (not shown). This concentration significantly reduced K⁺ conductance to 9 ± 4% of controls (p < 0.001). A maximal inhibitory concentration of quinine of 0.5 mM was also significantly reduced the nonselective cation conductance measured with Na⁺ as major cation to 21 ± 11% of controls (p < 0.001) (Fig. 4). This indicates that quinine blocks both the K⁺-selective pathway and the nonselective cation conductance. Ba²⁺, depending on the type of K⁺ channel, blocks from the outside or from the cytosolic side of the channel (31). In the experimental conditions of this study, putative channel proteins of zymogen granule membranes have their cytosolic domains exposed to the incubation medium. Surprisingly, micromolar concentrations of Ba²⁺ (ranging between 10 and 100 μM) selectively inhibited nonselective cation conductance. 100 μM Ba²⁺ inhibited Na⁺ conductance to 14 ± 2% of controls (p < 0.001), whereas the same concentration of Ba²⁺ reduced K⁺ conductance to 56 ± 15% of controls (p < 0.001) (Fig. 4). Since the AMP-PCP-sensitive (K⁺-selective) and -insensitive components represent about 50% of total K⁺ conductance each (see Table I and Fig. 4), the almost complete inhibition of Na⁺ conductance (~90% inhibition) and the inhibition of K⁺ conductance by ~45% with 100 μM Ba²⁺ indicate

### Table 1

| Conductance | Rate of granule lysis (h⁻¹) | Mean ± S.D. Mean ± S.D. |
|-------------|-----------------------------|------------------------|
| Total       | Amp-PCP sensitive           | Amp-PCP insensitive     |
| K⁺          | 76.2 ± 42.2                 | 36.3 ± 23.5             | 39.9 ± 13.7             | 8 |
| Na⁺         | 12.4 ± 5.7                  | 7.3 ± 4.9               | 5.1 ± 1.8               | 8 |
| Cs⁺         | 5.6 ± 3.5                   | 0.8 ± 0.7               | 4.8 ± 3.0               | 8 |
| Li⁺         | 5.4 ± 3.2                   | 1.7 ± 1.8               | 3.7 ± 1.8               | 5 |
| Rb⁺         | 2.5 ± 1.0                   | 0.4 ± 0.2               | 2.1 ± 0.8               | 6 |

* p < 0.06 by Student’s t test for unpaired comparison of conditions ±AMP-PCP.
* p < 0.005, by Student’s t test for unpaired comparison of conditions ±AMP-PCP.
* p, not significant.
that Ba$$^{2+}$$ at low micromolar concentrations blocks the nonselective component of K$$^{+}$$ conductance only. Higher, millimolar concentrations of Ba$$^{2+}$$ also block the K$$^{+}$$-selective component of ZG cation conductance (12). The nonsteroidal anti-inflammatory drug flufenamic acid has been used as a blocker of nonselective cation channels in inside-out patches from rat exocrine pancreas (32). As shown in Fig. 5, 200 µM flufenamic acid reduced K$$^{+}$$ conductance to 62 ± 14% of controls. Inhibition of Na$$^{+}$$ conductance was more potent although not complete (inhibition to 41 ± 2% of controls), suggesting that 200 µM flufenamic acid partially inhibits the nonselective cation conductance of ZG.

The results shown in Fig. 4 provide further evidence for the presence of a K$$^{+}$$-selective conductance which is blocked by AMP-PCP and quinine, and of an AMP-PCP-insensitive, nonselective cation conductance which is selectively blocked by micromolar concentrations of Ba$$^{2+}$$, flufenamic acid, and quinine as well.

Activation of ZG Cl$$^{-}$$ Conductance by AMP-PCP Is Abolished by Monoclonal Antibodies against MDR1 P-glycoprotein—Activation of ZG Cl$$^{-}$$ conductance by the non-hydrolyzable ATP analog AMP-PCP (Fig. 2), is reminiscent of a property of a volume-regulated Cl$$^{-}$$ channel associated with MDR1 P-glycoprotein which is activated by ATP, ATP-yS, AMP-PNP, and AMP-PCP (21, 22). We therefore tested two different monoclonal antibodies against MDR1 P-glycoprotein, which are directed against cytosolic domains of the protein, on AMP-PCP induced ZG lysis in the absence of valinomycin (Fig. 5). 0.1 mM AMP-PCP stimulated lysis of ZG incubated in KCl buffer in the absence of valinomycin 3.8–5.2-fold. The commercially available mouse monoclonal antibody against MDR1 P-glycoprotein C219, which binds to the two sequences VQAALD and VQEAALD found 6 residues away from the consensus sequence of the B site of the two ATP-binding domains of MDR1 (24), reduced AMP-PCP induced lysis of ZG in a concentration-dependent manner. In three different experiments the maximally tested concentration of 5 µg/ml C219 reduced ZG lysis induced by 0.1 mM AMP-PCP from 1.7 ± 0.3 h$$^{-1}$$ to 1.1 ± 0.2 h$$^{-1}$$ ($p < 0.025$). Another monoclonal antibody, the JSB-1, which binds to cytosolic domains of MDR1 P-glycoprotein (23), was even more inhibitory. JSB-1 reduced ZG lysis as a function of its test concentration: at the concentration of 5 µg/ml JSB-1 AMP-PCP induced ZG lysis was reduced from 1.5 ± 0.3 h$$^{-1}$$ in controls with AMP-PCP to 0.6 ± 0.1 h$$^{-1}$$ in experiments with AMP-PCP and 5 µg/ml JSB-1 ($p < 0.01; \text{means ± S.D. of three different experiments}$). CFTR is another Cl$$^{-}$$ channel with nucleotide binding domains, which is activated by protein kinase A-mediated phosphorylation and binding of hydrolyzable ATP analogs, which possibly involves ATP hydrolysis (18–20). The monoclonal antibody against the cytosolic, carboxyl-terminal domain of CFTR (M3A7) (24) was tested on AMP-PCP-induced ZG lysis at the same concentrations as the two antibodies against MDR1 P-glycoprotein. Even at the concentration of 5 µg/ml M3A7 had no effect on the enhanced ZG lysis observed with 0.1 mM AMP-PCP (Fig. 5). Also mouse IgG (5 µg/ml) did not reduce the rate of ZG lysis induced by 0.5 mM AMP-PCP (1.5 ± 0.1 h$$^{-1}$$ in controls with AMP-PCP compared to 1.7 ± 0.2 h$$^{-1}$$ for experiments with AMP-PCP and IgG; means ± S.D. of four different experiments).

Inhibition of AMP-PCP induced lysis of ZG incubated in KCl buffer by the monoclonal antibodies JSB-1 and C219 (Fig. 5) could be the result of a block of the AMP-PCP activated Cl$$^{-}$$ conductance pathway, of the nonselective cation conductance, or both. We have therefore tested the effect of 5 µg/ml of the monoclonal antibodies JSB-1 and C219 (MDR1 P-glycoprotein) on the lysis of ZG incubated in Na-acetate buffer after addition of CCCP, i.e. on the Na$$^{+}$$ permeant, AMP-PCP-insensitive nonselective cation conductance pathway (see Table I). JSB-1 and C219 (5 µg/ml) had no inhibitory effect on Na$$^{+}$$ conductance (not shown). Similar results were obtained with 5 µg/ml of the monoclonal antibody against CFTR, M3A7. In contrast, when the Cl$$^{-}$$ conductance was investigated by incubating ZG in KCl buffer and adding 5 µM valinomycin, both monoclonal antibodies against MDR1 P-glycoprotein JSB-1 (Fig. 6A) and C219 (Fig. 6B) (5 and 10 µg/ml) inhibited the Cl$$^{-}$$ conductance activated by 0.5 mM AMP-PCP, although the monoclonal antibody C219 was less inhibitory. Mouse IgG (5 and 10 µg/ml) did not affect the Cl$$^{-}$$ conductance activated by AMP-PCP.

MDR1 P-glycoprotein Antibodies Label a Major Immunoreactive band of ~65 kDa in Western Blots of ZG Membranes—Western blots of ZG membrane proteins, which had been separated by SDS-PAGE on 7.5–15% linear gradient gels and transferred to polyvinylidine fluoride membranes, revealed the...
AMP-PCP was added to the cuvette. At the creatic ZG by the monoclonal antibodies against MDR1 P-glycoprotein, JSB-1 (A) and C219 antibodies added, and measurements were started. These data are typical of three experiments. KC1 buffer and preincubated in the cuvette with both monoclonal antibodies against MDR1 P-glycoprotein, membranes with the monoclonal antibody against CFTR, M3A7 (not shown). protein band was found at the location of MDR1 P-glycoprotein. For comparison, when cell lysates of colchicine-resistant CHRC5 cells, which overexpress MDR1 P-glycoprotein (33), blots of pancreas homogenate and ZG membranes with C219 (Fig. 7C). No immunoreactive band was detected in ZG membranes using both JSB-1 and C219 revealed a major immunoreactive protein band of about 65 kDa molecular mass, but no band was detected at the approximate molecular mass of MDR1 P-glycoprotein.

**FIG. 6.** Block of AMP-PCP activated Cl− conductance of pancreatic ZG by the monoclonal antibodies against MDR1 P-glycoprotein, JSB-1 (A) and C219 (B). ZG were suspended in isotonic KCl buffer and preincubated in the cuvette for 10 min with JSB-1 and C219 antibodies or IgG (5 or 10 μg/ml). At the zero time point 0.5 mM AMP-PCP was added to the cuvette. At the arrow, 5 μg valinomycin was added, and measurements were started. These data are typical of three different experiments.

In summary, these results have demonstrated that the two monoclonal antibodies against MDR1 P-glycoprotein, JSB-1 and C219 are functional and specific blockers of a Cl− conductance pathway in ZG membranes which can be activated by the non-hydrolyzable ATP analog AMP-PCP. Western blot analysis of ZG membranes using both JSB-1 and C219 revealed a major immunoreactive band of about 65 kDa molecular mass, but no band was detected at the approximate molecular mass of MDR1 P-glycoprotein.

**DISCUSSION**

The AMP-PCP Insensitive Cation Conductance of ZG Has Characteristics of Non-selective Cation Channels—The enhanced lysis observed with 0.5 mM AMP-PCP in the absence of valinomycin must occur as a consequence of both Cl− and K+ influx through endogenous pathways, resulting in osmotic lysis of ZG. As illustrated in Fig. 1, however, the K+ conductance in ZG membranes is blocked by 0.5 mM AMP-PCP. This K+ conductance pathway therefore cannot account for granular lysis observed in KCl solutions after addition of AMP-PCP and in the absence of valinomycin. An AMP-PCP insensitive K+ permeability is also present in ZG membranes, which accounts for ~50% of total K+ conductance (see Fig. 1B) and may explain K+ influx and osmotic lysis of granules in the absence of valinomycin in conditions where the Cl− conductance has been activated by AMP-PCP (see model of Fig. 8). The major criteria to discriminate between these conductance pathways are their sensitivity to AMP-PCP, their cation selectivity, and their inhibitor sensitivity (Fig. 4 and Table I).

The AMP-PCP-sensitive pathway is selective to K+ and Rb+ only and can be blocked by glibenclamide (12). The AMP-PCP-insensitive, nonselective cation conductance pathway has similar properties to those of nonselective cation channels found in apical and basolateral plasma membranes of secretory tissues and cultured cell lines, but exhibits two major differences. Like other nonselective cation channels it is equally permeable to Na+ and K+ (3, 34, 35). In agreement with previous reports it is inhibited by quinine (34) and selectively blocked by flufenamic acid (32) (see Fig. 4). In contrast to published data (3), adenine nucleotides, e.g. AMP-PCP, did not inhibit the nonselective cation channel of ZG (Fig. 4). Furthermore, Ba2+ was found to specifically block the nonselective component of ZG cation conductance at concentrations ranging between 10 and 100 μM, an effect which has not been described for nonselective cation channels in other cells.

The Cl− Conductance of Pancreatic Zymogen Granules Has Functional Properties of a Volume-regulated Cl− Channel Associated with MDR1 P-glycoprotein—MDR1 is an ATPase that is responsible for active efflux of various hydrophobic drugs out of cells (36). Valverde and co-workers (21) have demonstrated that hypotonicity causes the appearance of a volume-regulated Cl− current only in cell lines overexpressing MDR1, suggesting that MDR1 is a Cl− channel. This interpretation has been questioned by several reports, which indicate that MDR1 expression may not be correlated to Cl− currents involved in volume regulation (37–39). Despite this controversy the Cl− conductance of ZG has characteristics of the Cl− current described by Valverde and co-workers (21). The MDR1 associated Cl− current is blocked by DIDS, verapamil, and tamoxifen at micromolar con-

![Fig. 7. Western blot analysis of ZG membranes (ZGM), pancreas homogenate (Homo), and colchicine-resistant CHRC5 cell lysates using the monoclonal antibodies against MDR1 P-glycoprotein, JSB-1 and C219. In A and B, 2 μg of protein CHRC5 cell lysate or 40 μg of ZG membranes were loaded; in C the lanes for both pancreas homogenate and ZG membranes contained 100 μg of protein. Proteins were separated by SDS-PAGE, transferred to polyvinylidine difluoride membranes, and probed with 5 μg/ml JSB-1 antibody (A) or 5 μg/ml C219 antibody (B and C). Immunoreaction was assessed by the enhanced chemiluminescence technique (see also "Methods").](image-url)
tein kinase, requires Mg2+ and possibly ATP hydrolysis (18–20).

Finn et al. (44) immunopurified a 200–220-kDa protein from Necturus gallbladder with a channel of pancreatic endoplasmic reticulum. This protein has been identified and purified as the C1- channel protein. Several antibodies raised against C1- channel proteins have been described in intracellular organelles, such as the 70-kDa transport epithelia, such as liver, kidney, pancreas, colon, and jejunum (47). Very little MDR1 was found in intracellular organelles, e.g. Golgi stacks and endoplasmic reticulum (48). Interestingly, C219 reactivity was also found in rat pancreatic acini by peroxidase immunocytochemistry (49). Recently, a 65-kDa MDR1 related protein (“mini-P-glycoprotein”) that is overexpressed in multidrug-resistant cells has been identified in plasma membranes by immunoblot studies with the monoclonal antibody C219 (50). Northern blot analysis with an MDR1 cDNA revealed the presence of a 2.4-kb mRNA, suggesting a truncated or alternatively spliced transcript of MDR1 (50). Its function is not known. Several members of the MDR1-related ATP binding cassette superfamily of transporters have been described in intracellular organelles, such as the 70-kDa peroxisomal protein of mammalian liver (51), or the 170-kDa P-glycoprotein homologue detected in the digestive vacuole of Plasmadium falciparum (52). It is therefore likely that the ~65-kDa protein detected in rat pancreatic ZG membranes also represents a member of the MDR1-related ATP-binding cassette superfamily. It is rather improbable that this 65-kDa protein band is a degradation product of MDR1, since the mAb C219 (which is directed against N- and C-terminal sites of MDR1; Ref. 24) should also detect an equally abundant, complementary fragment of ~100 kDa. Since both monoclonal antibodies against MDR1 (C219 and JSB-1) specifically inhibit AMP-PCP-activated C1- flux, we suggest that this ~65-kDa MDR1-like protein is the C1- channel protein or a regulator of the C1- channel.

**REFERENCES**

1. Petersen, O. H. (1990) J. Physiol. 428, 1–51
2. Maruyama, Y., Galler, D. V., and Petersen, O. H. (1983) Nature 302, 827–829
3. Thorn, P., and Petersen, O. H. (1992) J. Gen. Physiol. 100, 11–25
4. Martin, A., Tan, Y. P., and Trautmann, A. (1984) J. Physiol. 357, 293–325
5. Randriaamapita, C., Chanson, M., and Trautmann, A. (1988) Pflugers Arch. 413, 53–57
6. Palade, G. (1975) Science 189, 347–348
7. Kimura, T., Imamura, K., Eckhardt, L., and Schulz, I. (1985) Am. J. Physiol. 250, G659–G707
8. Fuller, C. M., Eckhardt, L., and Schulz, I. (1989) Pflugers Arch. 415, 385–394
9. Gasser, K. W., DiDomencio, J., and Hopfer, U. (1987) Am. J. Physiol. 253, C293–C299
10. DeLisle, G. R., and Hopfer, U. (1989) Am. J. Physiol. 259, G649–G648
11. Fuller, C. M., Deetjen, H., Piiper, A., and Schulz, I. (1989) Pflugers Arch. 415, 29–36
12. Thibodeau, P., Chathadi, K. V., Jiang, R., and Hopfer, U. (1992) J. Membr. Biol. 129, 253–266
13. Ashcroft, F. M. (1988) Annu. Rev. Neurosci. 11, 97–118
14. Thibodeau, P., Gasser, K. W., and Hopfer, U. (1990) Biochem. J. 272, 119–126
15. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak,
Z. Zielenski, J., Lek, S., Ploivic, N., Choja, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsu, L. C. (1989) Science 245, 1066–1073
16. Chen, C., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1996) Cell 87, 381–399
17. Tabcharani, J. A., Chang, X.-B., Riordan, J. R., and Hanrahan, J. W. (1991) Nature 352, 628–631
18. Andersen, M. P., Berger, H. A., Rich, D. P., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1991) Cell 67, 775–784
19. Quinton, P. M., and Reddy, M. M. (1992) Nature 350, 79–81
20. Baukrowitz, T., Hwang, T.-C., Nairn, A. C., and Gadsby, D. C. (1994) Neuron 12, 473–482
21. Gill, D. R., Hyde, S. C., Higgins, C. F., Valverde, M. A., Muntendig, G. M., and Sepulveda, F. V. (1993) Cell 74, 25–32
22. Valverde, M. A., Diaz, M., Sepulveda, F. V., Gill, D. R., Hyde, S. C., and Higgin, C. P. (1992) Nature 358, 830–833
23. Schepers, J. J., Bulte, J. H. M., Brokke, J. G. P., Bulte, J. H. M., van der Schoot, B. M., and Wessels, J. M., Broxterman, H. J., Kuiper, C. M., Lankelma, J., and Pinedo, H. M. (1995) Int. J. Cancer 62, 389–394
24. Georges, E., Bradley G., Gartipy, J., and Ling, V. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 152–156
25. Kartner, N., Hanrahan, J. W., Jensen, T. J., Naismith, A. L., Sun, S., Ackerley, C. A., Reyes, E. F., Tsai, L.-C., Rommens, J. M., Bear, C. E., and Riordan, J. R. (1991) Cell 64, 681–691
26. Thévenod, F., Haase, W., and Hopper, U. (1992) Anal. Biochem. 202, 54–60
27. Ling, V., and Thompson, I. H. (1974) J. Cell. Physiol. 83, 103–116
28. Laemmli, U. K. (1970) Nature 227, 689–695
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
30. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
31. Hilf, R. (1992 Ionic Channels of Excitable Membranes, 2nd Ed., pp. 130–133, Sinauer Associates Inc., Sunderland, MA
32. Gogolin, H., Dahlem, D., Englert, H. C., and Lang, H. J. (1990) FEBS Lett. 268, 79–82
33. Riordan, J. R., and Ling, V. (1979) J. Biol. Chem. 254, 12701–12705
34. Gogolin, H., and Capek, K. (1990) Biochim. Biophys. Acta 1027, 191–198
35. Gray, M. A., and Argent, B. E. (1990) Biochim. Biophys. Acta 1029, 33–42
36. Gottesman, M. M., and Pastan, I. (1988) Annu. Rev. Biochem. 57, 885–927
37. Rasulo, A., Galietto, I. J. V., Grunen, H. C., and Romeo, G. (1994) J. Biol. Chem. 269, 1452–1456
38. Altenberg, G. A., Deitmer, J. W., Glass, D. C., and Reuss, L. (1994) Cancer Res. 54, 612–622
39. Altenberg, G. A., Vanoye, C. G., Han, E. S., Deitmer, J. W., and Reuss, L. (1994) J. Biol. Chem. 269, 7145–7149
40. Valverde, M. A., Muntendig, G. M., and Sepulveda, F. V. (1993) Pflugers Arch. 435, 552–554
41. Anderson, M. P., Sheppard, D. N., Berger, H. A., and Welsh, M. J. (1992) Am. J. Physiol. 263, L1-L14
42. Begualli, B., Anagnostopoulos, T., and Edelman, A. (1993) Biochim. Biophys. Acta 1152, 319–327
43. Landry, D., Sullivan, S., Nicolaides, M., Rodhead, C., Edelman, A., Field, M., Al-Awqati, Q., and Edwards, J. (1993) J. Biol. Chem. 268, 14948–14955
44. Sinn, A. L., Tsai, L.-M., and Falk, R. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7649–7652
45. Tsai, L.-M., Dillard, M., Rosenberg, R. L., Falk, R. J., Gaido, M. L., and Sinn, A. L. (1991) J. Gen. Physiol. 88, 723–750
46. Chan, H.-C., Kastel, M. A., Nelson, D. J., Hazarika, P., and Dedman, J. R. (1992) J. Biol. Chem. 267, 5411–5416
47. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I., and Willingham, M. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7735–7738
48. Willingham, M. C., Richert, N. D., Cornell, M. W., Tsu, T., Hamada, H., Gottesman, M. M., and Pastan, I. H. (1987) J. Histochem. Cytochem. 35, 1451–1456
49. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I., and Willingham, M. C. (1989) J. Histochem. Cytochem. 37, 159–164
50. Kawai, K., Kusano, I., Ido, M., Sakurai, M., Shiraiishi, T., and Yatani, R. (1994) Biochem. Biophys. Res. Commun. 206, 804–810
51. Kamijo, K., Taketani, S., Yokota, H., Uchi, T., and Hashimoto, T. (1990) J. Biol. Chem. 265, 4534–4540
52. Cowman, A. F., Karch, S., Galatis, D., and Culver, J. G. (1991) J. Cell Biol. 113, 1055–1062