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ATP-regulated chloride conductance in endoplasmic reticulum (ER)-enriched pig pancreas microsomes

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The Cl^- conductance of endoplasmic reticulum-enriched pancreatic microsomes was identified. Its regulation by nucleotides was investigated by measuring the rate of cation ionophore-induced microsome swelling in the presence of an inward Cl^- gradient. The conductance was solubilized and reconstituted into liposomes. The Cl^- conductance in intact microsomes was inhibited by stilbene (10^{-4} M) and indanyloxyacetic acid (10^{-5} M) derivatives. ATP increased Cl^- conductance with half-maximal stimulation at $8 \cdot 10^{-6}$ M. Other trinucleotides (GTP, CTP and UTP) were without effect at 10^{-4} M. The non-hydrolysable analogue of ATP, adenosine 5'-[$\beta\gamma$-methylene]triphosphate ($AppCH_2p$) increased Cl^- conductance with a potency similar to that of ATP. The same concentration of adenosine 5'-[γ-thio]triphosphate ($ATP\gamma S$) which is a substrate for kinases, had no effect. ATP stimulation of Cl^- conductance was inhibited by stilbene derivatives. The data suggest the presence of at least one ATP-binding site, and show that the ATP does not need to be hydrolyzed and that its spatial conformation is important for activating the Cl^- conductance. Solubilized microsomal proteins reconstituted into liposomes retained their stilbene-inhibited, ATP-stimulated Cl^- conductance. A 167 kDa protein was detected by anti-CFTR antibodies in the intact microsomes, but not in the solubilized proteins. The 64 kDa protein (a component of a ubiquitous Cl^- channel) was detected in the both intact and solubilized microsomes. These results suggest that this Cl^- conductance is not a CFTR protein.

Introduction

A growing body of experimental evidence indicates that chloride conductance is important in a number of biological preparations. One of the key functions of chloride conductance is in ion transport across epithelia [1]. Chloride (Cl^-) conductive pathways are believed to influence the regulation of volume and/or fluid secretion coupled to the exocytosis of macromolecules in such organs as the pancreas [2,3]. Within the cell, chloride conductance controls intracellular vesicle acidification [4–6] and regulates the volume of the Golgi aparatus [7–9]. Epithelia contain several chloride channels [10–16] that differ in their electrophysiological properties and their regulation. The Cl^- channels in the rough endoplasmic reticulum of the exocrine pancreas may also control Ca^{2+} uptake [17].

Many studies have examined the regulation of Cl^- conductance, particularly its activators. Calcium, protein kinase A, and protein kinase C all activate Cl^- channels in epithelia [14,18,19], yet no drugs have been found to activate such channels. It has recently been reported that adenosine triphosphate, ATP, directly modulates chloride conductance in the zymogen granules of the rat pancreas, and regulates Cl^- channels in airway epithelial cells [20,21]. ATP has also been found to stimulate chloride secretion by cystic fibrosis, CF, airway cells, and to contribute to the activation of a channel protein encoded by the gene responsible for abnormal Cl^- transport in the secretory epithelia of CF patients, the CFTR protein [22,23].

The aim of the present study was 3-fold. First, to identify the chloride conductance moiety in endoplasmic reticulum-enriched microsomes from pig pancreas. Second, because of the importance of ATP which also stimulates the Cl^-/OH^- anion exchanger in the pancreatic ER [24], to characterize Cl^- conductance regulation by nucleotides. Lastly, to solubilize and reconstitute this conductance into liposomes. The chloride conductance in intact endoplasmic reticulum-enriched microsomes and reconstituted liposomes containing solubilized microsomal proteins were unmasked and its properties were investigated by measuring the rate of cation ionophore-induced volume changes in isoosmotic salt solutions [20,24]. The Cl^- conductance in

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endoplasmic reticulum-enriched pancreatic microsomes was inhibited by stilbene derivatives and an indanyloxyacetic acid derivative. The CFTR protein was present in the intact microsomes, but not in the solubilized proteins, the 64 kDa protein – a component of the ubiquitous Cl^- channel – was detected in both intact and solubilized proteins. The Cl^- conductance was activated by ATP, which did not need to be hydrolyzed to exert its action. The data suggest that there are ATP-binding sites on the channel and/or the regulatory protein. The Cl^- conductance was solubilized and reconstituted in liposomes. The reconstituted conductance retained its ATP regulation site and its sensitivity to inhibitors.

Materials and Methods

Materials

All chemicals were obtained from commercial sources and were of the highest grade available. EDTA, valinomycin, Hepes, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), leupeptin, ATP, CTP, UTP, adenosine 5′-[γ-thio]triphosphate (ATPγS), adenosine 5′-[βγ-methylene]triphosphate ($AppCH_2p$), n-octyl β-D-glucopyranoside (NOG), and bovine serum albumin fraction V (BSA), CHAPS, Triton X-100, gelatin, horseradish peroxidase-Protein A were from Sigma. 4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and carbonylcyanide 3-chlorophenylhydrazone (CCCP) were from Fluka. ECL was from Amersham. Molecular mass standards were from Bio-Rad. Cholesterin was from Boehringer. Indanyloxyacetic acid (IAA 94-95) was a gift from Dr. D. Landry (Columbia University, New York). Cleaned asolectin was a gift from Dr. M. Akabas (Columbia University, New York). Other chemicals were from Merck. Polyclonal antibodies against the 64 kDa protein (anti-64 kDa), were a generous gift from Dr. Q. Al-Awqati (Columbia University, New York), and polyclonal antibodies against the peptide 181 (CFTR amino acid residues 415–427, exon 9, pre-NBF) from Dr. W.B. Guggino (John's Hopkins University, Baltimore).

Methods

Tissue preparation

Pancreas from adult pigs were transported on ice from the slaughter house and all subsequent manipulations were performed at 4°C. The preparation of endoplasmic reticulum (ER)-enriched microsomes is described elsewhere [24]. The resulting vesicles were stored at −80°C when needed they were rapidly thawed and the enzymes assayed.

Enzyme assays, protein determination

Protein concentration, RNA (marker of ER), Na^+/K^+-ATPase (marker of plasma membrane) and cytochrome-*c* oxidase (marker of mitochondria) were measured as described in Refs. 24–27. The microsomal fraction obtained after centrifugation at $109\,000 \times g$ was enriched in RNA (4.3-fold) and depleted of Na^+/K^+-ATPase (0.09-fold) and cytochrome-*c* oxidase (0.5 fold) compared to the original homogenate [24,28].

Measurement of microsomal Cl^- permeability

Chloride transport was examined by measuring the osmotic swelling of microsomes using a cation probe, and light scattering [2,3,29].

Light scattering. For transport assays, the vesicles were suspended in a buffer containing 150 mM potassium gluconate, 20 mM Hepes-Tris (pH 7), at a protein concentration of 10 mg/ml. The microsomes (≈ 5 μl vesicles ≈ 50 μg protein) were then added to 1 ml of iso-osmotic assay buffer (150 mM KCl, 20 mM Hepes-Tris (pH 7)) with or without an appropriate concentration of K^+ ionophore, valinomycin (25 μg/ml) or H^+ ionophore, CCCP (3.3 μg/ml). The kinetics of swelling were followed by measuring the time-dependent change in the scattering of microsomes at 540 nm. The Cl^- conductive pathway was identified by permeabilizing the vesicles for cations using valinomycin or CCCP. Using such conditions for light-scattering experiments implies that the endogenous Cl^- conductive pathway becomes rate-limiting for the influx of salt and water, resulting in microsomal swelling. Thus, when artificial cation conductance is high, the endogenous Cl^- conductive pathway, if present, becomes rate limiting for salt entry, causing the swelling. The cation ionophores were prepared as stock solutions in 95% ethanol and were added to the KCl buffer to obtain a given concentration of ionophore (see above) and 0.01% ethanol. This concentration of ethanol did not influence the swelling of the vesicles. The absorbance was continuously measured for 1 min at room temperature, using a Hitachi U-2000 spectrophotometer (Braun Sci, Paris, France).

Analysis of light scattering data. The increase in microsomal volume corresponding to the Cl^- conductive pathway was determined by measuring absorbance for 1 min in the presence of a cation ionophore. The results were quantified by measuring the initial relative rate constant (IRC) which corresponds to the decrease in optical density ($10^{-3} \cdot OD$) measured within one second after adding vesicles to the assay buffer ($\Delta OD/s$). It was estimated from the data (absorbance vs. time) recorded by a spectrophotometer. The changes in IRC measured under different experimental conditions are expressed as a percent of their respective controls. Because the values of IRC varied from one day to another, each set of appropriate controls was

taken as 100%. The IRC test values for a given day were compared to the controls for the same day.

All experiments were repeated at least three times; each included 4–10 determinations of $\Delta OD/s$ for control and test conditions. The results are expressed as means ± S.E., and analysed using Student's unpaired *t*-test.

ATP was used as the Mg^{2+} salt, other nucleotides were Na^+ salts dissolved in 5 mM $Mg(CH_2COOH)_2$. The solutions containing nucleotides were buffered to pH 7. All experiments were performed at room temperature.

Solubilization

Two series of solubilization experiments were performed, the first with NOG, and the second with CHAPS or Triton X-100. Pancreatic microsomes were solubilized with NOG by incubating them at 2 mg/ml (or 5 mg/ml for reconstitution and electrophoresis) in 0.25 M sucrose, 1.4% NOG, 10 mM imidazole, 10% glycerol (pH 7) plus proteinase inhibitors for 30 min at 4°C. For solubilization with CHAPS (10 mM) or Triton X-100 (0.8%), the buffer was 100 mM KCl, 5 mM Hepes (pH 7.4), 5 mM Tris, 0.5 mM $MgCl_2$. The solubilized and non-solubilized proteins were spun at 109000 × *g* for 40 min at 4°C (Beckman Optima XL-70, rotor 70 Ti) and the insoluble proteins in the pellet were discarded. The solubilized protein were quantified by the BCA method [30].

Reconstitution

Reconstitution was begun immediately after centrifugation at 109000 × *g*. Dry asolectin and cholesterol were each dissolved in chloroform (100 mg/ml), and the solutions were mixed to give 30% (w/w) cholesterol and a total of 10 mg phospholipids. The solvent was removed under vacuum for 5 h and the lipids were suspended in the reconstitution buffer (assay buffer: 150 mM potassium gluconate, 20 mM Hepes-Tris (pH 7), supplemented with 0.8% NOG). The lipid dispersion was sonicated for 25 min in a water-bath type sonicator (Branson 2200, OSI, Paris, France). The solubilized proteins (1 mg) were mixed with 10 mg sonicated phospholipids and dialyzed against one liter of assay buffer for 24 h; the assay buffer was changed twice during that period. The proteoliposomes formed were tested for Cl^- conductance activity by measuring the rate of iso-osmotic swelling of the vesicles in the presence of an inward Cl^- gradient.

Gel electrophoresis and Western blotting

ER-enriched intact and solubilized microsomal proteins (30 μg/lane) were separated by SDS-PAGE (Laemmli [31]). The proteins were transferred from the polyacrylamide gel to a PVDF membrane by electroblotting for immunodetection experiments.

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 10% or 6% polyacrylamide gel (Mini-protean gel apparatus, Bio-Rad).

Intact and solubilized microsomal proteins were prepared for electrophoresis in 75 mM Tris (pH 8.8), 2% SDS (w/v), 10% glycerol, 0.01% Bromophenol blue, plus 10% β-mercaptoethanol. The samples were heated to 37°C for 12 min. The gels (10% or 6%) were run for 60 min at 25 mA/gel. The running buffer contained 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 0.1% SDS (w/v). After electrophoresis, the gel was stained for protein with Coomassie brilliant blue R250 for 15 min and destained with 7% acetic acid/5% methanol.

Immunodetection. The proteins separated on either 10% or 6% gels were electroblotted onto PVDF membranes (0.2 μm, Bio-Rad) in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% methanol for 1 h at 100 V and 4°C (Bio-Rad Transblot cells).

Anti-64 kDa antibodies. The proteins were separated on 10% SDS-PAGE and transferred to PVDF as indicated above.

The blots were incubated with 5% non-fat milk in the wash buffer (200 mM NaCl, 50 mM Tris-HCl (pH 7.4)) for 1 h to block the non-specific binding sites.

Polyclonal antibodies against the 64 kDa protein were used diluted 1 : 10000 in 5% non-fat milk, in wash buffer overnight, at 4°C.

All subsequent steps were performed at room temperature.

The strips were thoroughly rinsed and exposed to the horseradish peroxidase-Protein A (Sigma) diluted 1 : 10000 in wash buffer (containig 5% non-fat milk) for 2 h. The blots were rinsed and exposed to enhanced chemiluminescence detection solution (ECL, Amersham).

Anti-CFTR antibodies. The proteins were separated on 6% SDS-PAGE and transferred to the PVDF membrane as above. Polyclonal antibodies against the CFTR-peptide were characterized and immunodetection procedure was as described by Zeitlin et al. [32]. Antigen-antibody complexes were detected by exposing the blots to enhanced chemiluminescence detection solution (ECL, Amersham).

Results

A Cl^- conductive pathway in ER-enriched pancreatic microsomes

Fig. 1 shows the light scattering measurements for pancreatic microsomes in 150 mM KCl, 20 mM Hepes (pH 7). Absorbance decreased only slightly, indicating moderate vesicle swelling due to the influx of KCl and water. We infer that the permeability of microsomes to

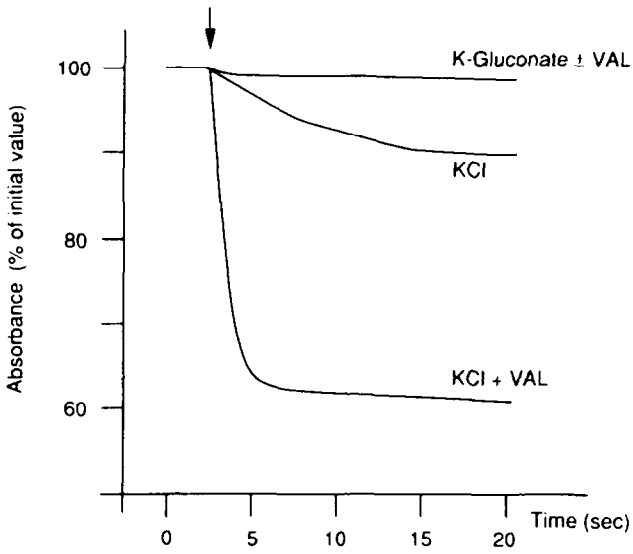

Fig. 1. Swelling of ER-enriched pancreatic microsomes in the presence and absence of valinomycin. Microsomes were suspended in 150 mM potassium gluconate solution. The arrow indicates the introduction of microsomes into a KCl or potassium gluconate solution. There was a significant decrease in absorbance in KCl-buffered solutions containing 25 μg/ml valinomycin. The initial IRC for this experiment was 40 ΔOD/s.

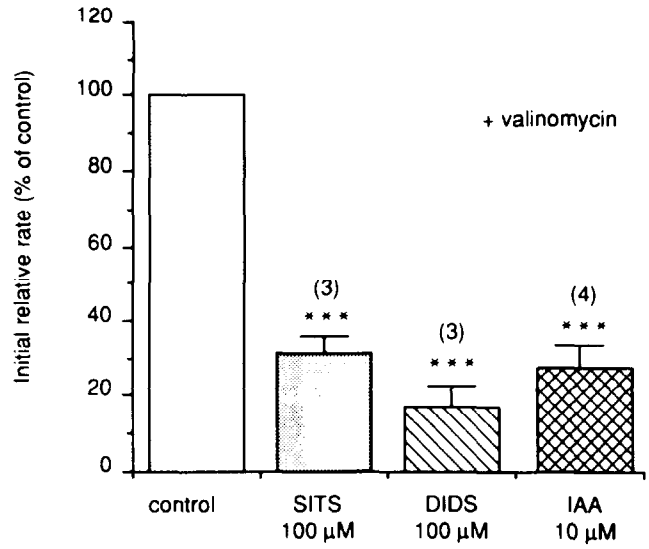

Fig. 2. Effects of anion transport inhibitors on valinomycin-induced changes in the Cl^- conductance of ER-enriched pancreatic microsomes. The control IRC in the presence of valinomycin was 10–16.5 ΔOD/s. Numbers in brackets indicate the number of independent experiments. *** $P < 0.005$ using Student's t-test for unpaired values.

KCl is rather low under these conditions. The electrogenic cation ionophores, valinomycin or CCCP, caused a significant drop in absorbance, indicating greater microsomal swelling. The swelling was quite rapid: 95% of the response occurred within 20 s. The initial relative rate constant, IRC was 7–45 ΔOD/s, regardless of the ionophore used (Table I). This variability in relative rates of decrease in absorbance has been reported previously [3,20]. It has been attributed to variations in the relative permeabilities of microsomes in steady state, from one experiment to another. The increased IRC in the presence of a cation ionophore indicates that the cation permeability of intact microsomes is rate limiting for salt entry and swelling, while the endogenous chloride permeability is quite high. Vesicles were placed in potassium gluconate buffer plus valinomycin to check that the ionophore-induced swelling of microsomes was Cl^--dependent. The decrease in absorbance under these conditions was smaller then in KCl buffer (Fig.1). This small decrease in absorbance was probably due to non-specific swelling. The microsomes isolated from porcine exocrine pancreas swelled in the iso-osmotic solutions in the presence of an inward Cl^- gradient in a voltage-dependent manner, suggesting the existence of Cl^- conductance.

TABLE I

Effects of cationic ionophores on iso-osmotic ER-enriched microsomal swelling in the presence of an inward Cl^- gradient

	ΔOD/s	n
Control	11.0± 5.3	9
Valinomycin	20.0±10.4 *	22
CCCP	21.2± 9.2 *	9

Results are means ± S.E. n, number of independent experiments. * $P < 0.025$ using Student's t-test for unpaired values.

The regulation of endogenous microsomal Cl^- conductance was further studied by performing all experiments in the presence of 25 μg/ml valinomycin using light scattering. The results are expressed as the percentage change in IRC values as compared to control IRC measured in the presence of valinomycin (100%).

Effects of anion transport inhibitors

Three anion transport inhibitors were tested: SITS, DIDS and IAA 94-95. SITS and DIDS are well known inhibitors of Cl^- transport [2,33]; IAA 94-95 inhibits the Cl^- conductance of kidney microsomes and is a derivative of IAA-94 [34–36]. Incubation of vesicles with inhibitor (0.1 mM SITS, 0.1 mM DIDS for 10 min, or 10 μM IAA 94-95 for 1 h) decreased IRC values by 70% (Fig. 2), indicating inhibition of Cl^- conductance.

Effects of ATP

As ATP has been found to regulate Cl^- conductance in pancreatic zymogen granules [20], the direct effect of ATP (0.1 μM–2 mM) on ER-enriched pancreatic microsomes was investigated. ATP stimulated the valinomycin-induced swelling of microsomes (Fig. 3). The IC_{50} (the concentration of ATP that increased IRC by 50% of its maximal ATP-induced stimulation)

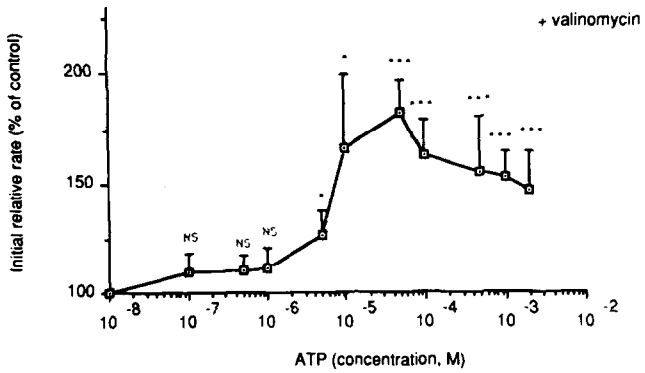

Fig. 3. Effect of ATP on the Cl^- conductance of ER-enriched pancreatic microsomes. Initial relative rates (IRC) of valinomycin-induced microsomal swelling are expressed as percentages of control values without adenine nucleotides. The control IRC in the presence of valinomycin was 8–22 ΔOD/s. The ATP concentrations were 10^{-7} to $5 \cdot 10^{-5}$ M ($n = 3$), 10^{-4} M ($n = 6$) and $5 \cdot 10^{-4}$ to $2 \cdot 10^{-3}$ M ($n = 5$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ using Student's t-test for unpaired values.

was 8 μM. Thus, the voltage-dependent Cl^- conductance was increased by ATP. The stimulation decreased slightly at high concentrations (≥ 0.1 mM) of ATP, suggesting the presence of two ATP binding sites.

The effects of ATP on Cl^- conductance were blocked by 0.1 mM DIDS (Fig. 4).

Effects of non-hydrolysable ATP analogues

The effects of non-hydrolysable ATP analogues were measured in the presence of 5 mM $Mg(CH_2COOH)_2$, to test whether the increase in Cl^- conductance by ATP involved the membrane-bound endogenous protein kinases which might use ATP as a substrate. Preliminary experiments showed that these molecules had their maximal effect at 100 μM (data not shown). The non-hydrolysable analogue of ATP, $AppCH_2p$, increased IRC, but ATPγS, which is a kinase substrate, was without effect (Table II). These results

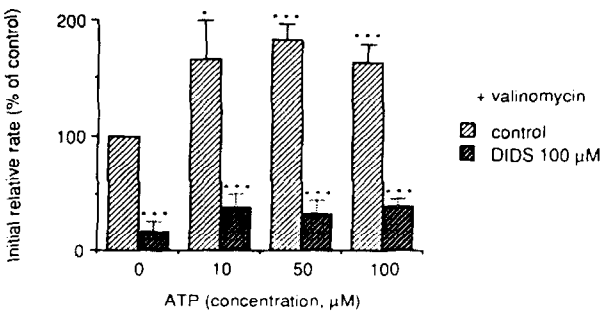

Fig. 4. Inhibition of the ATP-stimulated Cl^- conductance of ER-enriched pancreatic microsomes. The microsomes were incubated for 10 min with 100 μM DIDS prior to measurements. The control IRC in the presence of valinomycin was 11.2–14.1 ΔOD/s. All experiments were performed three times ($n = 3$). * $P < 0.05$ and *** $P < 0.005$ using Student's t-test for unpaired values.

TABLE II

Effects of ATP derivatives on the Cl^- conductance of ER-enriched pancreatic microsomes

	Initial relative rate in the presence of valinomycin (% of control)	n
Control	100	12
ATP	162.9 ± 16.0 ***	6
ATPγS	81.4 ± 12.1 n.s.	3
$AppCH_2p$	177.8 ± 44.4 **	3

The initial relative rate constant in the presence of valinomycin was 5–24 ΔOD/s. n, number of independent experiments. ** $P < 0.01$, *** $P < 0.005$ and n.s. = non-significant, using Student's t-test for unpaired values.

indicate that unhydrolyzed ATP is active. It also suggests that the spatial conformation of the ATP molecule is very important for its binding.

Effect of trinucleotides

The presence of purinergic/pyrimidinergic receptors was assessed by testing the effect of other trinucleotides on Cl^- conductance. The three nucleotides tested, GTP, CTP and UTP at 100 μM in the presence 5 mM $Mg(CH_2COOH)_2$, did not modify valinomycin-induced microsomal swelling (Fig. 5). Thus the Cl^- conductance was not modulated by these nucleotides, suggesting the absence of purinergic/pyrimidinergic receptors.

Solubilization and detection of microsomal proteins

A series of experiments were performed to find the conditions for solubilization of ER-enriched microsomes. The total protein concentration of intact microsomes (before solubilization) was 2 mg/ml. Treatment with 10 mM CHAPS solubilized 50%, 0.8% Triton

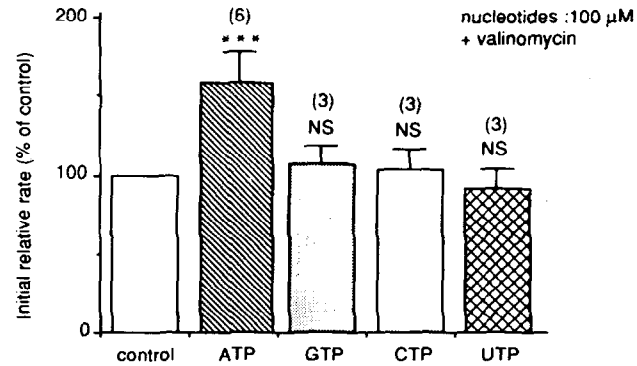

Fig. 5. Effects of trinucleotides on the Cl^- conductance of ER-enriched pancreatic microsomes. The control IRC in the presence of valinomycin was 8–15 ΔOD/s. The nucleotides were tested at 100 μM in the presence of 5 mM $Mg(CH_2COOH)_2$. Numbers in brackets indicate the number of independent experiments. *** $P < 0.005$ and n.s. = non-significant using Student's t-test for unpaired values.

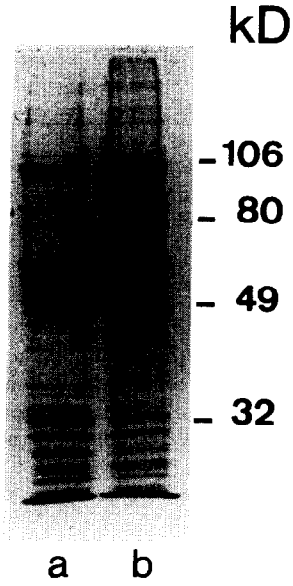

Fig. 6. Coomassie blue staining of intact and solubilized microsomal proteins separated by SDS-PAGE/10%, in presence of 10% β-mercaptoethanol. Lane a: 30 μg intact microsomal protein. Lane b: 30 μg solubilized microsomal proteins.

X-100 solubilized 38%, and 1.4% NOG solubilized 53% of the total proteins. Reconstitution experiments were performed using microsomal proteins solubilized with NOG, since NOG was more readily removed by dialysis than was CHAPS. CMC (critical micellar concentration) for NOG is 22 mM and for CHAPS is 6 mM.

Fig. 6 shows the SDS-PAGE protein profiles before and after protein solubilization under reduced conditions. Proteins with molecular masses close to 80 kDa and 45 kDa were lost from the solubilized proteins. The presence of CFTR protein and/or of a component of the ubiquitous Cl^- channel, 64 kDa protein [37], was determined by running Western blots of intact microsomes and solubilized proteins (Fig. 7). The anti-CFTR peptide (pre-NBF) antibodies recognized a single 167 kDa protein band of a size consistent with CFTR, in intact microsomes, but not in the solubilized material (Fig. 7,e). The 64 kDa protein was detected in both intact microsomes and solubilized proteins (Fig. 7,a,b).

Reconstitution of an ATP-regulated Cl^- conductance

Fig. 8 shows the light scattering of solubilized ER-enriched microsomal proteins reconstituted into asolectin/cholesterol liposomes. Control experiments (liposomes alone) showed little change in absorbance (1.9 ± 0.7 ΔOD/s, $n = 6$). The control initial rate constant in the absence of valinomycin was 2.7 ± 1.0 ΔOD/s ($n = 7$), while the initial relative rate constant of proteoliposomes in the presence of valinomycin was 4.7 ± 0.7 ΔOD/s ($n = 7$), indicating the reconstitution of Cl^- conductance. These changes were inhibited by

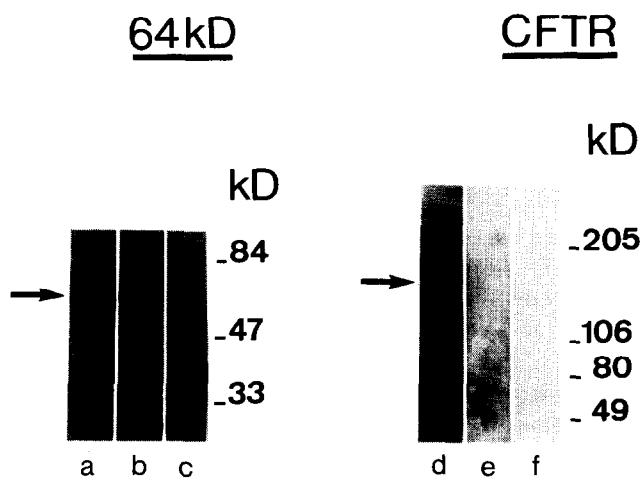

Fig. 7. Western blot analysis of intact and solubilized microsomal proteins. 64 kDa: Intact and solubilized microsomal proteins were subjected to SDS-PAGE on 10% gel, transferred to PVDF membrane and incubated with anti-64 kDa antibodies as described in Materials and Methods. Lanes a and c: Intact microsomal proteins. Lane b: Solubilized microsomal proteins. Lane c: The blots incubated only with horseradish peroxidase-protein A. CFTR: Intact and solubilized microsomal proteins were subjected to SDS-PAGE on 6% gel, transferred to PVDF membrane and incubated with anti-CFTR antibodies as described in Materials and Methods. Lanes d and f: Intact microsomal proteins. Lane e: Solubilized microsomal proteins. Lane f: the blots incubated only with horseradish peroxidase-anti-rabbit IgG.

DIDS and IAA 94-95, confirming the presence of the Cl^- conductance in the liposomes. ATP (100 μM) also increased the initial rate of swelling of proteoliposomes in the presence of valinomycin ($115.5 \pm 5.6\%$, $n = 3$,

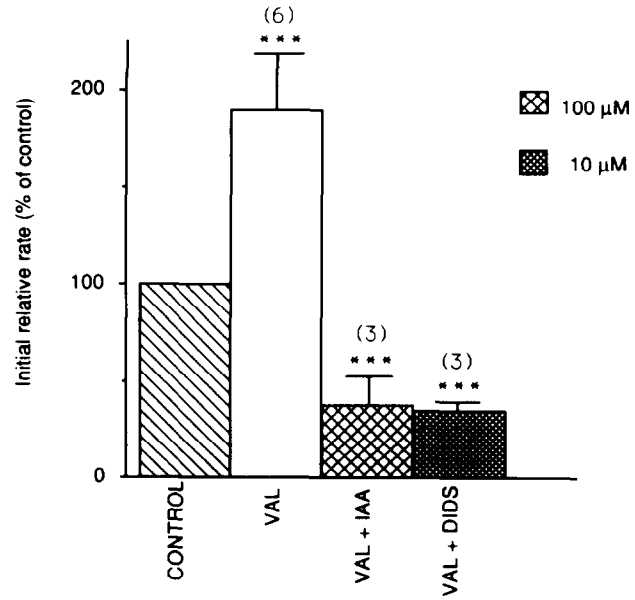

Fig. 8. Effects of valinomycin and anion transport inhibitors on ER-enriched solubilized proteins reconstituted into liposomes. Histogram summarizing the reconstitution results. Numbers in brackets indicate the number of independent experiments. *** $P < 0.005$ using Student's t-test for unpaired values.

*$P < 0.01$), whereas GTP was without effect ($103.9 \pm 9.2\%$, $n = 3$, n.s.).

Discussion

A macroscopic assay, the iso-osmotic swelling of microsomes measured by light scattering, suggests the presence of Cl^- conductance in the ER-enriched pancreatic microsomes. ER-enriched pancreatic microsomes swell via Cl^- conductive pathways, because the endogenous cation permeability is quite low. The results suggest that microsomes contain a voltage-dependent Cl^- conductance that is regulated by ATP and inhibited by stilbene derivatives and IAA 94-95. The Cl^- conductance can be solubilized and reconstituted into liposomes. The reconstituted system is stimulated by ATP and is sensitive to inhibitors.

The enzymatic markers determinated indicate that the microsomes were enriched in endoplasmic reticulum, but may have been slightly contaminated by plasma membranes. The experimental protocol for microsome preparation used was chosen to minimize contamination with plasma membranes (for details, see Refs. 24, 28).

Activation of Cl^- conductance by ATP in ER-enriched microsomes may be an important factor in the regulation of endoplasmic reticulum volume, leading to the secretion of macromolecules. ATP-regulated Cl^- conductances are present in both the ER and zymogen granules [20]. However, the Cl^- conductance in the ER is not inhibited by low concentrations of ATP, as is that of zymogen granules [20]. This difference may reflect incomplete processing of the Cl^- channel protein in the ER. If ER and zymogen granules contain the Cl^- conductance composed of the same protein(s), then the final target of the proteins responsible for the Cl^- conductance described here is the plasma membrane. The proteins are transported from the ER, where they are synthesized, to the Golgi aparatus, where they are fully processed, and then to the zymogen granules, from where they are delivered to the plasma membrane. Zymogen granules play a direct role in the secretion of macromolecules by the exocrine pancreas and are believed to contribute to the increase in overall cellular Cl^- conductance by delivering chloride channels to the luminal membrane during exocytosis [20]. The mechanism described above could be an additional mechanism involved in the secretion of fluid and electrolytes in exocrine acinar cells, which is mainly regulated by Ca^{2+}-dependent processes [38].

As low concentrations of ATP did not inhibit Cl^- conductance, the channels in zymogen granules and in ER may be different entities. If this is the case, the Cl^- conductance might remain in ER and control intravesicular pH, as it does in Golgi-enriched kidney microsomes [5,36], hence regulating the volume of the ER. It is also possible that the low concentrations of ATP were not inhibitory because of incomplete processing of the protein at the ER.

The way that ATP acts on Cl^- conductance in pancreatic ER is at present unknown. It may stimulate Cl^- conductance by phosphorylation of a Cl^- channel or a channel-regulating protein, or it may bind to a purinergic P_2 receptor, and to the Cl^- channel, or even to a channel-regulating protein. Phosphorylation is unlikely, since ATPγS, which is a protein kinase substrate in the phosphorylation of target proteins [39], has no effect on Cl^- conductance. Purinergic, P_2, and/or pyrimidinergic receptors are probably not coupled to the Cl^- channel of ER-enriched pancreatic microsomes since GTP, CTP and UTP are without effect on Cl^- conductance. However, the stimulation of Cl^- conductance by ATP and AppCH_2p suggests that there are ATP-binding sites on the channel or on the channel-regulating proteins, as in pancreatic zymogen granules [20]. The difference in the effects of AppCH_2p and ATPγS suggests that spatial conformation is important for binding to the channel or to the channel-regulating protein. It is known that the activity of adenine analogues depends greatly on the structure of the molecule. For exemple, an amino group must be present on the purine ring for the activity of adenine nucleotides [40]. This might also hold for the phosphate group at γ position, which binds to the binding sites. Modification of the phosphate group at the γ position by thiol (ATPγS) changes the structure of ATP. This change in the structure is greater than the one caused by introducing CH_2 (AppCH_2p). Therefore, AppCH_2p binds to ATP binding sites, and stimulates the Cl^- conductance, whereas ATPγS is altered so that it does not bind. Similar differences in the actions of AppCH_2p and ATPγS have been observed for the regulation of Cl^- conductance in pancreatic zymogen granules, and for the regulation of an anion exchanger in pancreatic ER [20,24].

It has been recently shown that the CFTR-related Cl^- channel, the protein encoded by the gene responsible for abnormal secretion in cystic fibrosis [39], is regulated by nucleotides [23]. The CFTR protein could be responsible for the Cl^- conductance described here, but the results argue against it. First, the way in which nucleotides regulate the Cl^- conductance in ER-enriched pancreatic microsomes differs from the nucleotide regulation of the CFTR-related Cl^- channel; GTP, UTP and CTP were without effect in the present study, whereas they open CFTR-related Cl^- channel [23]. Second, the CFTR-related Cl^- channnel is insensitive to DIDS and IAA [41], whereas the Cl^- conductance described here is inhibited by these compounds. Third, although the anti-CFTR antibodies detected the 167 kDa protein in the intact microsomes suggesting the presence of CFTR protein in microsomes, this

protein was absent from the solubilized material, whereas both intact and solubilized reconstituted proteins displayed ATP-regulated Cl^- conductance activity. If Cl^- conductance studied here was CFTR protein, protein kinase A (PKA) plus ATP should significantly increase the rate of swelling. Preliminary results show that PKA + Mg^{2+}-ATP did not increase IRC more than Mg^{2+}-ATP alone. It therefore seems unlikely that the CFTR protein is the Cl^- conductance studied here.

The SDS-PAGE protein profiles show that most of the proteins in the microsomes are solubilized by NOG. A component of a Cl^- channel, the 64 kDa protein, was also detected in the solubilized material. Until now there are no reports which indicate whether this chloride channel is modulated by nucleotides. Our working hypothesis is that this protein is also a component of ATP-regulated Cl^- conductance from ER.

The CFTR protein is found in the ductal cells, but not in the acinar cells of the pancreas [42]. Our study shows that 167 kDa protein is present in the ER-enriched microsomes. It seems unlikely that this protein corresponds to microsomes from ducts, since acini account for 99% of the total tissue used for our microsomal preparation. The physiological function of the CFTR protein in the ER of acinar cells, and whether this protein is present in the plasma membranes of these cells both remain to be established.

The ATP-binding sites appear to be resistant to detergent. The rate of valinomycin-induced proteoliposomes swelling was significantly increased by ATP. The relatively small effect of ATP may be due to a partial loss of binding sites during solubilization. Solubilization conditions similar to those used here resulted in an 80% loss of IAA binding sites from kidney microsomes [35]. However, the pancreatic Cl^- conductance remains sensitive to inhibitors even after reconstitution. The alternative explanation is that a few ATP-regulated Cl^- channels were reconstituted into liposomes. This reconstitution of solubilized ATP-regulated Cl^- conductance from ER-enriched pig microsomes will allow purification of the corresponding Cl^- channel and further studies on its biochemical and biophysical properties.

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