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Evidence for Involvement of a Zymogen Granule Na^+/H^+ Exchanger in Enzyme Secretion from Rat Pancreatic Acinar Cells

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Abstract. We have characterized a Na^+/H^+ exchanger in the membrane of isolated zymogen granules (ZG) from rat exocrine pancreas and investigated its role in secretagogue-induced enzyme secretion. ZG Na^+/H^+ exchanger activity was estimated by measuring Na^+ or Li^+ influx and consequent osmotic swelling and lysis of ZG incubated in Na- or Li-acetate. Alternatively, intragranule pH was investigated by measuring absorbance changes in ZG which had been preloaded with the weak base acridine orange. Na^+- or Li^+-dependent ZG lysis was enhanced by increasing inward to outward directed H^+ gradients. Na^+-dependent ZG lysis was not prevented by an inside-positive K^+ diffusion potential generated by valinomycin which argues against parallel operation of separate electrogenic Na^+ and H^+ permeabilities and for coupled Na^+/H^+ exchange through an electroneutral carrier. Na^+- and Li^+-dependent ZG lysis was inhibited by EIPA (EC_{50} ~25 μM) and benzamil (EC_{50} ~100 μM), but only weakly by amiloride. Similarly, absorbance changes due to release of acridine orange from acidic granules into the medium were obtained with Na^+ and Li^+ salts only, and were inhibited by EIPA, suggesting the presence of a Na^+/H^+ exchanger in the membrane. Na^+ dependent lysis of ZG was inhibited by 0.5 mM MgATP and MgATP-γ-S by about 60% and 35%, respectively. Inhibition by MgATP was prevented by incubation of ZG with alkaline phosphatase (100 U/ml), or by the calmodulin antagonists calmidazolium (0.75 μM), trifluoperazine (100 μM) and W-7 (500 μM), suggesting that the ZG Na^+/H^+ exchanger is regulated by a ZG membrane-bound calmodulin-dependent protein kinase. Na^+ dependence of secretagogue (CCK-OP)-stimulated amylase secretion was investigated in digitonin permeabilized rat pancreatic acini and was higher in acini incubated in Na^+ containing buffer (30 mM NaCl/105 mM KCl buffer; 6.4 ± 0.4% of total amylase above basal) compared to buffer without Na^+ (0 mM NaCl/135 mM KCl buffer; 4.7 ± 0.4% of total amylase above basal, $P < 0.03$). EIPA (50 μM) reduced CCK-OP-induced amylase secretion in Na^+ containing buffer from 7.5 ± 0.6% to 4.1 ± 0.8% ($P < 0.02$). In the absence of Na^+ in the buffer, CCK-OP-stimulated amylase release was not inhibited by 50 μM EIPA. The data suggest that an amiloride insensitive, EIPA inhibitable Na^+/H^+ exchanger is present in ZG membranes, which is stimulated by calmodulin antagonists and could be involved in secretagogue-induced enzyme secretion from rat pancreatic acini.

Key words: Sodium/hydrogen exchange — Amiloride — Ethylisopropylamiloride — Calmodulin dependent protein kinase — Calmodulin antagonists — Calmidazolium

Introduction

Na^+/H^+ exchangers are involved in various cellular functions, such as intracellular pH, cell volume regulation, cell proliferation and transcellular Na^+ absorption [27]. Four isoforms of the Na^+/H^+ exchanger, NHE 1–4 have been cloned [19, 32]. NHE 1 is present in the basolateral plasma membrane of nearly all mammalian epithelial cells, NHE 2–3 are predominantly expressed in the apical membrane of epithelial cells, such as intestine and kidney, where they mediate transcellular Na^+ transport [27]. NHE 1–3 are inhibited by the drug amiloride and its analogues, but their sensitivity to these inhibitors differs between isoforms.

Much less is known about Na^+/H^+ exchange in the membrane of intracellular organelles. Na^+/H^+ exchangers have been described in mitochondria, chromaffin granules and renal endocytotic membranes. They seem

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to differ from the plasma membrane Na^+/H^+ exchangers with regard to their sensitivity to inhibitors, substrate specificity and size [2, 9, 11, 12, 13, 21, 28].

In the exocrine pancreas, studies on isolated permeabilized acinar cells indicate that secretagogue-induced secretion of digestive enzymes is enhanced by electrolyte movements across the zymogen granule membrane [8]. A Cl^- conductance, a Cl^-/anion exchanger and a K^+ conductance have been described in the membrane of isolated zymogen granules [10, 24–26] and implicated in the mechanism of stimulated enzyme secretion from isolated pancreatic acini [7]. In the present work, we provide evidence for the presence of a Na^+/H^+ exchanger in the membrane of isolated zymogen granules from rat pancreas, which is regulated by a zymogen granule membrane-bound protein kinase. In addition, we demonstrate that CCK-OP-induced amylase secretion from permeabilized pancreatic acinar cells is Na^+-dependent and blocked by EIPA, suggesting that the Na^+/H^+ exchanger is involved in enzyme secretion.

Materials and Methods

Adenine nucleotides, collagenase (Type III from *Clostridium histolyticum*, 790 U/mg), cholecystokinin-octapeptide (CCK-OP), bovine serum albumin (98–99% BSA, essentially fatty-acid free), carbonylcyanide m-chlorophenylhydrazone (CCCP), valinomycin, amiloride, alkaline phosphatase (type VII-NL from bovine intestinal mucosa), calmodulin and calmidazolium were obtained from Sigma (Deisenhofen, Germany). Trifluoperazine hydrochloride was from LC Laboratories/Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Benzamil was acquired from RBI-Bio Trend (Köln, Germany) and acridine orange was from Atlanta (Heidelberg, Germany). Pefabloc® SC [4-2-aminoethyl)-benzolsulfonylfluoride hydrochloride], and trypsin-inhibitor (from hen egg white) were purchased from Boehringer (Mannheim, Germany). Percoll and Phadebas® Amylase kit were from Kabi Pharmacia (Freiburg, Germany). Ethylisopropylamiloride (EIPA) was a gift from Dr. H.J. Lang (Hoechst, Frankfurt, Germany). All other reagents were of the highest analytical grade available.

Methods

Isolation of Zymogen Granules

Zymogen granules (ZG) were isolated from the pancreatic glands of male Wistar rats (180–300 g, Charles River Wiga GmbH, Sulzfeld, Germany) as described earlier [25, 26].

Measurement of Cation Conductance

Cation conductance of pancreatic ZG was assayed according to a previously reported protocol for the quantitative evaluation of macroscopic ion fluxes through endogenous conductance pathways of ZG membranes [5, 25]. This assay relies on the measurement of osmotic lysis of ZG, which have been resuspended in buffered isotonic salt solutions, after addition of electrogenic ionophores allowing for membrane permeation of counterions. Granule lysis causes a decrease in absorbance of the suspension measured at a wavelength of 540 nm in a Beckman DU-64 spectrophotometer at 37°C.

Since bulk salt influx into the intragranule space and the resulting granule lysis are limited by the flux of ions through the endogenous conductance pathway, but not by the flux of counterions through the shunt pathway, the slope of the decrease in absorbance with time will represent an estimate of the rate of ions transported through the endogenous conductance pathway. Half-time of granule lysis was estimated from the slope of the decrease in absorbance with time between addition of ionophore 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 lysis, which were considered proportional to the rate constant of lysis. Unless otherwise indicated, data were expressed as means ± SEM of different preparations. Statistical analysis was carried out with the Statgraphics program using Student's *t*-test. Results with levels of $P < 0.05$ were considered significant.

To measure cation conductance, ZG were suspended in 150 mM monovalent cation/acetate solutions containing 1 mM EDTA and buffered with 50 mM imidazole (pH 7.0, adjusted with acetic acid). Since the intragranule pH is about 6.5, and the membrane permeability for protons is low [25], an inside-to-outside directed H^+ concentration gradient of ~0.5 pH units between intragranule space and incubation solution was generated. Cation influx through endogenous cation pathways was initiated by addition of 16 μM of the electrogenic protonophore CCCP, which maximally permeabilizes the granule membrane to H^+ and converts the H^+ concentration gradient into an inside negative H^+ diffusion potential. The inside negative granule membrane potential, in turn, energizes cation influx through endogenous cation permeabilities. Anion influx occurs through the uncharged molecule acetic acid, which permeates through the lipid membrane by nonionic diffusion and dissociates to provide the intragranule space continuously with protons for protonation of imidazole as well as for proton efflux from the acidic interior [25]. Under these conditions, cation influx through endogenous cation permeabilities is rate-limiting.

Measurement of Na^+/H^+ Exchange

The Na^+/H^+ exchange activity was measured by two different methods: ZG were either resuspended in isotonic cation acetate salts buffered with imidazole as described above for cation conductance but in the absence of the electrogenic protonophore CCCP. Osmotic lysis under these conditions will be the consequence of electroneutral, proton gradient-dependent influx of monovalent cations through an endogenous transporter.

Alternatively, Na^+/H^+ exchange was monitored by measuring absorbance changes of the H^+-dependent accumulation of the weak base acridine orange into acidic compartments [22]. An increase of intragranule pH causes deprotonation of acridine orange and efflux of the uncharged form of acridine orange from the intragranule space until a new steady state of distribution is reached. This change can be monitored by measuring the change in absorbance of acridine orange at 493–540 nm [20]. For that purpose, 70–100 μg of the purified ZG were acidified by preincubation for 1 hr in 40 μl homogenization buffer (pH 5.5) containing 300 μM acridine orange. The ZG suspension was then diluted in a cuvette in 3 ml of a buffer containing (in mM): 300 mannitol, 5 HEPES, 5 $MgSO_4$ (pH titrated to 7.5 with Tris/HCl), preincubated for 5 min with solvent or drugs, and 150 mM monovalent cations were added as sulfate salts to generate an inside-directed cation gradient as driving force. The buffered salt solutions were hypertonic (545 mOsm), therefore no lysis occurred during the measurements [8]. The time-dependent increases in absorbance measured as the absor-

bance difference between 493 nm and 540 nm, were determined in an Aminco DW-2000 UV-VIS spectrophotometer (Colora, Lorch, Germany) at 25°C. Data were collected for 1 min, stored using an Aminco DW-2000 software package and analyzed with a Symphony spreadsheet program.

Isolation of Pancreatic Acini and Assay for Amylase Release

The preparation of dispersed acini by collagenase digestion has been previously described [7, 14]. The pancreas from one male Wistar rat (130–160 g) was trimmed free of fat and connective tissue, minced to a fine paste and suspended in 10 ml digestion buffer containing (in mM): 130 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 KCl, 1.2 KH_2PO_4, 0.01% trypsin inhibitor, 0.2% bovine serum albumin (w/v), 10 glucose and 20 HEPES (pH 7.4). Collagenase digestion was carried out in two steps. Tissue was first treated with 600 U collagenase for 15 min, then with 900 U for 30 min at 37°C under a continuous supply of O_2. Intact acini were incubated with O_2 in buffers of the following composition (in mM): 20 HEPES, 1.2 KH_2PO_4, 2 $MgCl_2$, 0.1 $CaCl_2$, 0.01% trypsin inhibitor, 0.2% bovine serum albumin (w/v), 10 glucose, pH titrated to 7.4 with KOH or NaOH and addition of either 135 KCl, 135 NaCl, or different isotonic combinations of both salts. Aliquots of the isolated acini were permeabilized for 10 min 2 ml incubation buffer containing 5 μg/ml digitonin. Following permeabilization, test substances were preincubated for 5 min and the secretagogue CCK-OP (1 nM) was added. Samples of 200 μl acinar suspension were taken shortly before and 30 min after addition of CCK-OP and centrifuged for 30 sec at 14,000 rpm in an Eppendorf microfuge. The supernatant was removed, amylase release from acini was determined using the Phadebas® amylase test kit (Kabi Pharmacia, Freiburg, Germany). To determine total amylase activity, acini were lysed in ''diluent solution'' containing (in mM): 10 Na_2HPO_4/10 NaH_2PO_4 buffer (pH 7.8), 0.1% sodium dodecyl sulfate and 0.1% bovine serum albumin for 1 hr and further sonicated for 10 min. Amylase release was expressed as the percentage of total amylase activity present in the pancreatic acinar suspension. The initial amylase content before addition of CCK-OP was subtracted for each value.

Statistics

Means were calculated ± SEM. Statistical analysis of data was carried out with the Statgraphics program using paired and unpaired Student's *t*-test, results with levels of $P < 0.05$ were considered significant.

Abbreviations

Zymogen granules (ZG), lysis rate of zymogen granules per hour (hr^{-1}), carbonyl-cyanide m-chlorophenylhydrazone (CCCP), ethylisopropylamiloride (EIPA), adenosine 5′[βγ-methylene] triphosphate (AMP-PCP), adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S), cholecystokinin octapeptide (CCK-OP), protein kinase A (PKA), protein kinase C (PKC).

Results

Cation Selectivity of ZG Lysis with and without the Protonophore CCCP

In the presence of CCCP, lysis of ZG incubated in acetate salts of monovalent cations was high with Rb^+, whereas only slight permeation was detected with Li^+ and an intermediate lysis was found with K^+, Na^+ and Cs^+ (Fig. 1*A*). Lysis was complete within 20 min. The sequence observed is similar to that found in a previous report on a K^+-selective conductance from ZG [25]. In the absence of CCCP, when no electrogenic cation flux should take place, substantial ZG lysis was also observed, but only for ZG incubated in Na- and Li-acetate (Fig. 1*B*), indicating the presence of a transporter selective for Na^+ or Li^+ but not for other cations. However, the kinetics of lysis were much slower in the absence of CCCP (compare time scales in *A* and *B*). An estimate of the selectivity of ZG cation conductance was obtained by subtracting the rates of ZG lysis in the absence of CCCP from those in the presence of CCCP. The permeability sequence for electrogenic movement of monovalent cations was $Rb^+ \gg K^+ > Cs^+ \geqslant Na^+ > Li^+$, and $Na^+ > Li^+$ for the electroneutral pathway. Calculated mean inverse

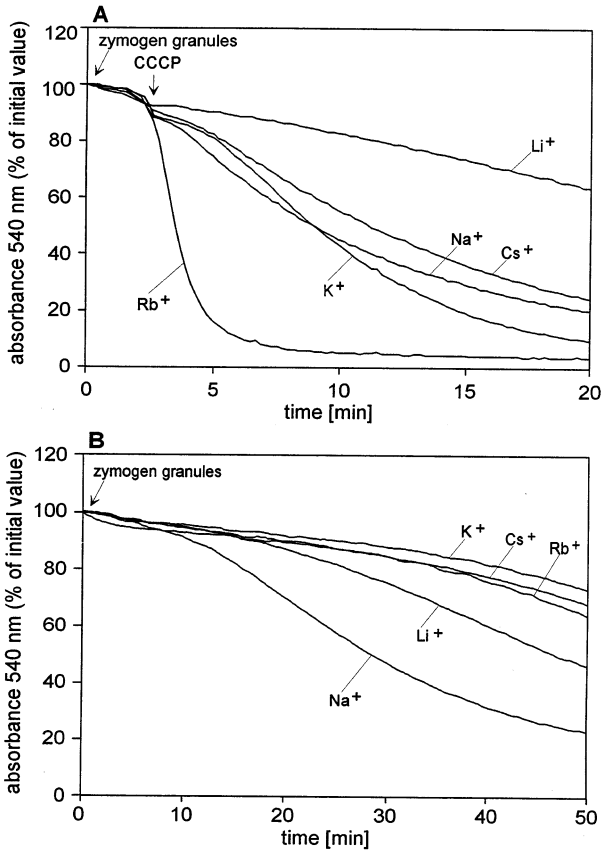

Fig. 1. Dependence of ZG-lysis on monovalent cations in the presence (*A*) or absence (*B*) of the protonophore CCCP. Zymogen granules were suspended in isotonic medium containing 150 mM of the monovalent cation acetate salts as indicated in the figure, and incubated at 37°C for the indicated time period with (*A*) or without (*B*) the electrogenic protonophore CCCP (16 μM). The kinetics of lysis were monitored by measuring the changes in optical density at 540 nm. The curves (*A*) and (*B*) are representative of 25, respective 39 different experiments.

Table 1. Comparison of the rates of ZG lysis in the presence and absence of the protonophore CCCP (16 μM) for various monovalent cation acetate salts

Acetate salts	Lysis rates of ZG suspended in 150 mM monovalent cation acetate salts		
	Lysis rates (+ CCCP)	Electroneutral (− CCCP)	Electrogenic (Lysis rates − electroneutral)
Rb^+	44.4 ± 3.5	0.7 ± 0.05	43.7 ± 3.5
K^+	6.3 ± 0.4	0.5 ± 0.04	5.7 ± 0.4
Na^+	5.1 ± 0.4	2.2 ± 0.17	2.7 ± 0.4
Cs^+	4.0 ± 0.3	0.6 ± 0.07	3.4 ± 0.3
Li^+	2.2 ± 0.3	1.2 ± 0.15	1.0 ± 0.4

(n = 10, period of measurement 30 min, lysis rates hr^{-1} ± SEM).

half-times of lysis for ten different experiments are summarized in Table 1. Na- and Li-acetate-dependent lysis in the absence of CCCP could be the consequence of Na^+/H^+ exchange or parallel operation of Na^+ and H^+ permeabilities. We have therefore tested this hypothesis further.

Effect of ΔpH on ZG Lysis in the Absence of CCCP

To measure the dependence of cation influx into ZG on the magnitude of the outward-directed H^+ gradient, the pH-gradient across the granule membrane was varied. ZG were suspended in imidazole buffer containing 150 mM Na-, Li- or K-acetate at pH 6.5, 7.0 or 7.5. The pH gradient across the zymogen granule membrane was estimated, similarly as described in [25]. As shown in Fig. 2, rates of ZG lysis in Na-acetate were 1.8 ± 0.3 hr^{-1} at pH 7.0 with an estimated ΔpH of 0.44, decreased to 1.3 ± 0.3 hr^{-1} in pH 6.5 buffer (ΔpH of 0.32) and increased to 2.0 ± 0.2 hr^{-1} at pH 7.5 (ΔpH of 0.65). With Li-acetate lysis rates were calculated to 1.0 ± 0.1 hr^{-1} at a transmembrane ΔpH of 0.44, were reduced to 0.6 ± 0.2 hr^{-1} at a ΔpH of 0.32 and increased to 1.3 ± 0.1 hr^{-1} at a ΔpH of 0.65. The values for Na- and Li-acetate at a ΔpH of 0.65 and 0.32 were significantly different from those found at a ΔpH of 0.44 ($P < 0.01$). However, with K-acetate lysis rates were identical (0.6 ± 0.1 hr^{-1}) at all transmembrane pH gradients tested. Qualitatively similar results were obtained when acetate was substituted by propionate (*not shown*).

Effect of Amiloride and of the Amiloride Analogues Ethylisopropylamiloride (EIPA) and Benzamil on ZG Lysis

Different concentrations of the known Na^+/H^+ exchange inhibitors amiloride (50 μM–1 mM), EIPA (5–200 μM) and benzamil (10–500 μM) were tested on the lysis of ZG in Na- or Li-acetate buffers. Dose-response curves for inhibition of ZG lysis in 150 mM Na-acetate (*A*) and Li-acetate (*B*) by amiloride, EIPA and benzamil are shown in Fig. 3. Dose-response curves and EC_{50} were calculated using sigmoidal dose-response curve fitting. EIPA, a specific inhibitor of Na^+/H^+ exchangers blocked osmotic lysis with EC_{50} values of ~25 μM in Na-acetate and ~20 μM in Li-acetate. EIPA was more potent than the specific Na^+ channel blocker benzamil, which showed half-maximal inhibition of ZG lysis at ~100 μM in Na- or Li-acetate buffer. High concentrations of amiloride were needed to inhibit ZG lysis, half-maximal inhibition in Li-acetate occurred at ~200 μM, whereas in Na-acetate amiloride only weakly inhibited ZG lysis (Fig. 3).

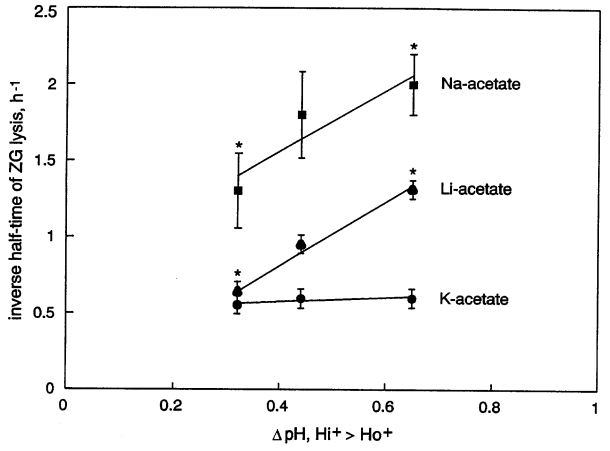

Fig. 2. Na- or Li-acetate dependent lysis rates are enhanced by an inside-to-outside directed H^+ gradient. ZG were suspended in standard incubation buffer containing 150 mM of Na-, Li- or K-acetate, and the pH (pH_o) was adjusted to 6.5, 7.0 or 7.5 with sulfuric acid. The transmembrane pH gradient (Δ pH; $H_i^+ > H_o^+$) was defined as the difference between outside pH and intragranule pH and calculated as described in [25]. Means ± SEM are calculated from 5–6 different preparations. *$P \leq 0.01$, Student's *t*-test for paired comparison with ZG lysis at pH 7.0 in the same cation salt.

Effect of Na^+ and Li^+ Concentration Gradients on ZG Lysis

Na^+ or Li^+ concentration gradients were also varied to study their role as driving force for lysis of ZG in the absence of CCCP. Na- or Li-acetate concentrations in the medium were varied between 150 mM and 100 mM, while the tonicity was held constant at 350 mOsm by addition of K-, Rb- or Cs-acetate. With decreasing concentrations of Na-acetate ZG lysis rates decreased maximally by ~20% when Na^+ was isosmotically replaced by 50 mOsm Cs^+ (Fig. 4). However, with K^+ or Rb^+ a reduction of Na^+ concentration by 25 mM almost completely abolished ZG lysis (Fig. 4). Similar results were

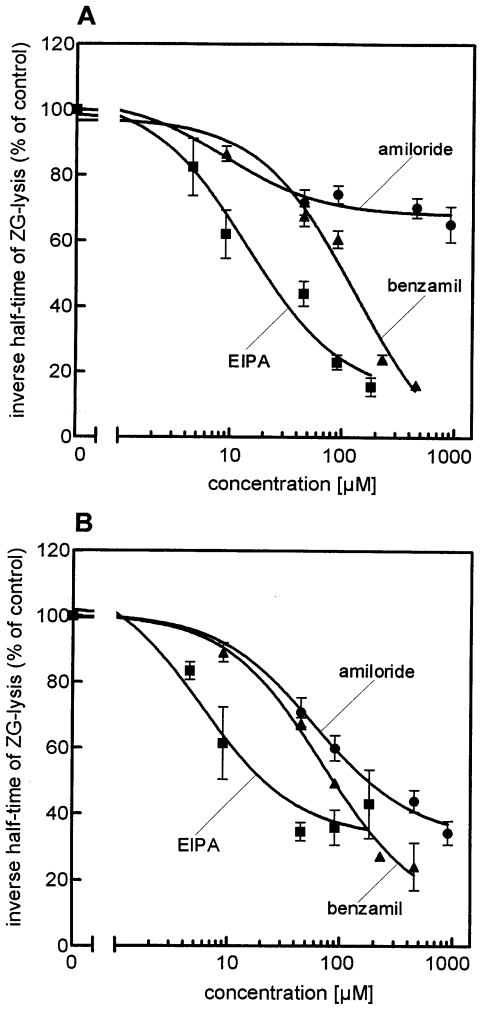

Fig. 3. Concentration-response curves for inhibition of ZG-lysis by amiloride and its analogues in Na- or Li-acetate buffers. Osmotic lysis of ZG in 150 mM Na-acetate (*A*) or 150 mM Li-acetate (*B*) were measured in the presence of increasing concentrations of amiloride, EIPA and benzamil. Inverse half-times of lysis were plotted as percent of controls in Na-acetate (100% = 2.27 ± 0.08 hr^{-1}, $n = 36$) or Li-acetate (100% = 1.19 ± 0.04 hr^{-1}, $n = 29$). Values represent means ± SEM of 4–36 experiments. When drugs were added in DMSO the control experiments received the same amount of carrier solvent.

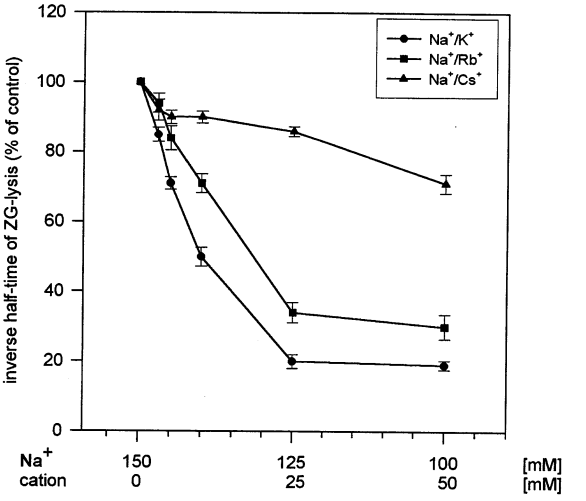

Fig. 4. Effect of isosmotic replacement of buffer Na^+ by K^+, Rb^+ or Cs^+ on ZG lysis. ZG were suspended in incubation buffer containing 50 mM imidazole at pH 7.0. Na-acetate concentration in the medium was varied between 150 mM and 100 mM, while the tonicity was held constant at 150 mM by addition of K-, Rb- or Cs-acetate. Values are expressed as percent of inverse half-time of lysis in 150 mM Na-acetate (100% = 2.34 ± 0.07 hr^{-1}, $n = 30$). Means ± SEM of 4–12 different preparations.

obtained for Li^+-dependent osmotic lysis of ZG (*not shown*). This suggested that K^+ and Rb^+ either directly inhibit the Na^+/H^+ exchanger in the ZG membrane or that electrogenic influx of small amounts of K^+ or Rb^+ through endogenous K^+ and Rb^+ selective channels prevent Na^+ influx through Na^+ selective channels.

To determine whether an influx of K^+ or Rb^+ into the ZG through K^+- and Rb^+-selective channels is responsible for the reduction of ZG lysis, we added 0.5 mM AMP-PCP which completely blocks the K^+- and Rb^+-selective conductance of ZG [24, 25] and should abolish the effect of K^+ on ZG lysis. However, AMP-PCP did not prevent the inhibitory effect of 25 mM K^+ on ZG lysis in Na-acetate buffer (Table 2). We also used the K^+ ionophore valinomycin, which should generate an inside-positive K^+ diffusion potential and prevent ZG lysis in Na-acetate buffer. Valinomycin did not prevent, but rather enhanced ZG lysis in the presence of 5 or 25 mM K^+ in the medium (Table 2).

Measurement of Na^+ and Li^+ Flux into ZG with the Dye Acridine Orange

To complement the results obtained with the ZG lysis assay, we determined monovalent cation fluxes into ZG with acridine orange [22] by measuring monovalent cation-dependent alterations of pH gradients across ZG membranes. Granules were equilibrated by preincubation for 1 hr with an homogenization solution buffered to pH 5.5 and containing 300 μM acridine orange and subsequently diluted in 300 mM mannitol buffered to pH 7.5. The granules were incubated for 5 min with solvent or drugs, and 75 mM Na_2-, Li_2-, K_2- or $NMDG_2$-sulfate were then added to the buffer. Na^+ or Li^+ influx through coupled Na^+/H^+ exchange should increase intragranule pH and release acridine orange trapped in the intragranule space. As shown in Fig. 5, the increase in absorbance of acridine orange at 493–540 nm was higher after Na^+ or Li^+ than with K^+ or $NMDG^+$, which are not substrates of Na^+/H^+ exchangers (Fig. 5, Table 3). We also measured the effect of the inhibitors of Na^+/H^+ exchangers

Table 2. Effect of valinomycin (5 μM) and AMP-PCP (0.5 mM) on lysis rates (hr^{-1}) of ZG following isosmotic replacement of Na-acetate by K-acetate

	Lysis rates of ZG		
	150 mM NaAc 0 mM KAc	145 mM NaAc 5 mM KAc	125 mM NaAc 25 mM KAc
Control	2.3 ± 0.2 (16)	1.0 ± 0.2 (3)	0.4 ± 0.1 (16)
Valinomycin	3.0 ± 0.2 (5)	1.9 ± 0.5 (3)	1.3 ± 0.3 (5)
AMP-PCP	2.4 ± 0.1 (3)	Not tested	0.5 ± 0.1 (3)

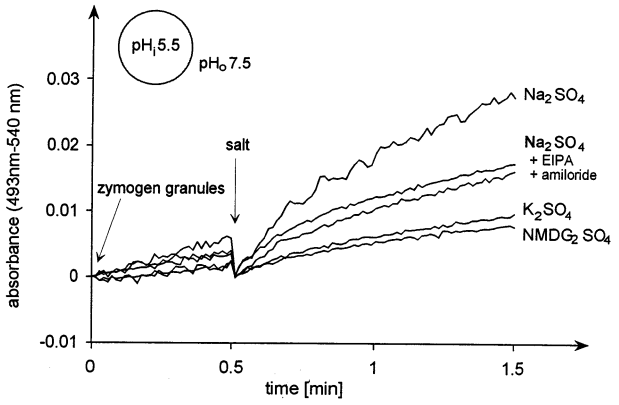

Fig. 5. Measurement of the Na^+/H^+ exchanger of ZG membranes with acridine orange. Absorbance changes of acridine orange (4 μM) at 493–540 nm were measured after addition of 75 mM Na-, K- or NMDG-sulfate salts. Total osmolarity after addition of salts was ~545 mOsm which prevents osmotic lysis of granules. EIPA (100 μM) and amiloride (1 mM) were preincubated with ZG for 5 min before addition of salts. Outside buffer pH (pH_o) was 7.5, the pH inside the ZG (pH_i) was assumed to be 5.5 after a 1 hr preincubation period with pH 5.5 buffer. This experiment is typical for 4–7 different preparations.

Table 3. Effect of 75 mM Na_2^-, Li_2^-, K_2^- or $NMDG_2$-sulfate on acridine orange absorbance of ZG (pH_i = 5.5) incubated in pH 7.5 buffer

	Δ Absorbance of acridine orange (493–540 nm)/min		
Cation	Control	EIPA	Amiloride
Na^+	0.027 ± 0.002	0.019 ± 0.001*	0.019 ± 0.002*
Li^+	0.031	0.022	0.016
K^+	0.016 ± 0.003*	Not tested	Not tested
$NMDG^+$	0.017 ± 0.004*	Not tested	Not tested

Means ± SEM of 1–7 experiments are calculated, where applicable
* $P < 0.05$ by Student's *t*-test for unpaired comparison with Na^+ control

amiloride and EIPA on the absorbance changes of acridine orange induced by Na_2SO_4 or Li_2SO_4. Following a preincubation period of 5 min, amiloride (1 mM) and EIPA (0.1 mM) reduced the Na_2SO_4- or Li_2SO_4-induced absorbance changes of acridine orange.

Effect of Adenine Nucleotides on Na^+ dependent ZG Lysis

Na^+/H^+ exchangers are regulated by kinases and phosphatases [27] and membrane-bound protein kinases and phosphatases have been demonstrated in ZG [3, 23, 31]. We therefore investigated the effect of ATP and ATP-γ-S on the Na^+-dependent lysis of ZG membranes. We also tested the adenine nucleotides ADP and the nonhydrolyzable ATP analogue AMP-PCP which are no substrates of protein kinases [6]. In the absence of Mg^{2+} lysis of ZG incubated in 150 mM Na-acetate/imidazole buffer was similar with 0.5 mM ATP, ATP-γ-S, ADP or AMP-PCP, when compared with controls without nucleotides (Fig. 6*A*, open columns). In the presence of 5 mM $MgSO_4$, lysis rates were significantly reduced with 0.5 mM ATP (1.1 ± 0.1 hr^{-1}, $P < 0.0001$) and with 0.5 mM ATP-γ-S (1.5 ± 0.2 hr^{-1}, $P < 0.0005$) compared to respective controls with Mg^{2+} (2.3 ± 0.1 hr^{-1}), but not with 0.5 mM ADP (2.1 ± 0.2 hr^{-1}) or 0.5 mM AMP-PCP (2.8 ± 1.0 hr^{-1}) (Fig. 6*A*, filled columns). A concentration-response curve of MgATP induced inhibition of Na^+/H^+ exchange is shown in Fig. 6*B*. Maximal inhibition occured at 0.5 mM ATP, half-maximal inhibition of Na^+/H^+ exchanger by MgATP was obtained at ~0.07 mM MgATP. EC_{50} was calculated using one site binding curve fitting. Addition of alkaline phosphatase (100 U/ml) removed the inhibition of Na^+/H^+ exchange induced by MgATP but not that induced by MgATP-γ-S (Fig. 7) whose thiophosphate residue cannot be cleaved from thiophosphorylated proteins by phosphatases [6].

Effect of Calcium/Calmodulin Antagonists on MgATP-induced Inhibition of ZG Na^+-dependent ZG Lysis

To identify the putative ZG membrane-bound protein kinase that may be responsible for inhibition of Na^+-dependent lysis, we tested different protein kinase inhibitors and activators on MgATP effects on ZG incubated in Na-acetate buffer. Reduction of MgATP induced inhibition was only observed after addition of the calmodulin antagonists calmidazolium, trifluoperazine and W-7 (Fig. 8). MgATP induced inhibition of ZG lysis from 2.1 ± 0.1 hr^{-1} to 0.9 ± 0.1 hr^{-1} for controls without antagonists, was prevented by incubation of ZG with 0.75 μM calmidazolium (2.1 ± 0.2 hr^{-1}), 100 μM trifluoperazine (2.1 ± 0.2 hr^{-1}), or 500 μM W-7 (1.5 ± 0.1 hr^{-1}).

Na^+ dependence of basal and CCK-OP-induced Amylase Secretion in Permeabilized Pancreatic Acini

To study the possible role of cytosolic Na^+ in secretagogue activation of enzyme secretion, we have varied the cytosolic Na^+ and K^+ concentrations of digitonin perme-

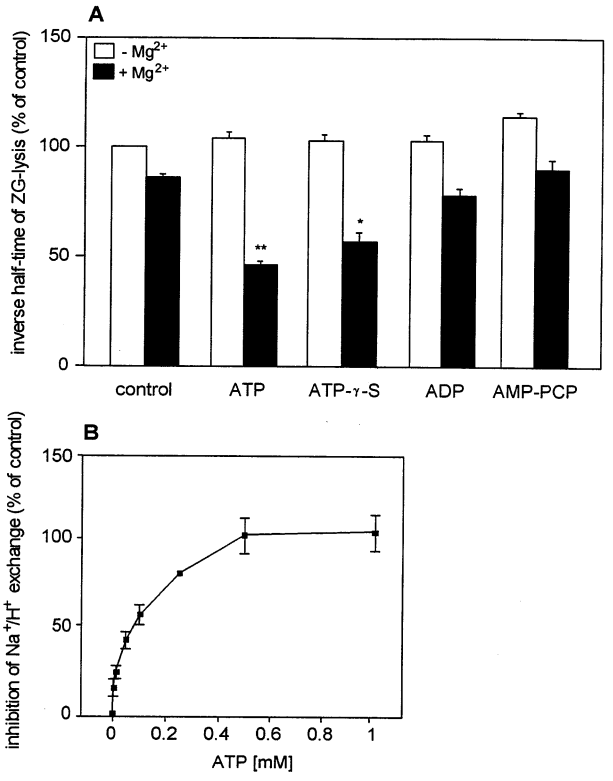

Fig. 6. Mg^{2+}- and adenine nucleotide dependence of Na^+/H^+ exchanger in ZG membranes. ZG were incubated in 150 mM Na-acetate/imidazole buffer with or without 0.5 mM of the adenine nucleotides listed in Fig. 6*A*, and in the absence (open columns) or presence of 5 mM $MgSO_4$ (filled columns). Control rates of lysis (100% = 2.76 ± 0.09 hr^{-1}, $n = 46$) were determined in the absence of Mg^{2+} or nucleotides. Means ± SEM of 3–48 experiments, $*P < 0.0005$, $**P < 0.0001$ paired Student's *t*-test for comparison with the control plus Mg^{2+}. In (*B*) a concentration-response curve for inhibition of Na^+/H^+ exchange of ZG by MgATP is plotted. Relative rates of lysis were calculated as percent of control (100% control = 2.97 ± 0.37 hr^{-1}, $n = 9$) and plotted as percent of the maximal inhibitory effect observed with 1 mM ATP. Values are means ± SEM of 2–9 experiments.

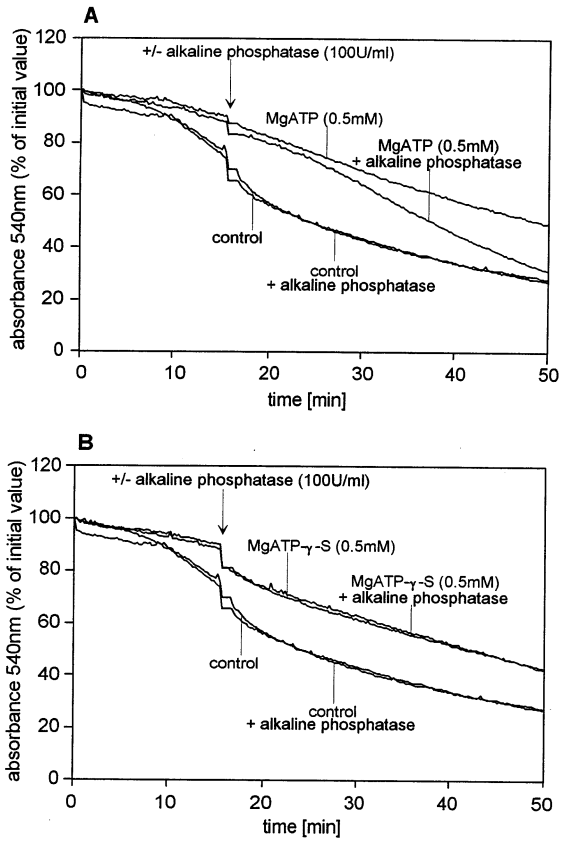

Fig. 7. Effect of alkaline phosphatase on the inhibition of ZG Na^+/H^+ exchanger induced by MgATP and MgATP-γ-S. Absorbance curves show the kinetics of lysis of ZG suspended in standard buffer containing 150 mM Na-acetate and 5 mM $MgSO_4$. At the arrow 100 U/ml alkaline phosphatase were added to ZG preincubated for 10 min with or without 0.5 mM MgATP (*A*) and with or without 0.5 mM MgATP-γ-S (*B*). The experiment is typical of six preparations.

abilized pancreatic acini, and measured CCK-OP-induced amylase secretion. To that end, permeabilized acini were incubated in a buffer containing (in mM): 135 130, 80, 30, 15, 5, or no NaCl. The total concentration of salt was held constant at 135 mM by addition of KCl to the buffer. Amylase released into the medium within 30 min under basal conditions or after addition of a maximal stimulatory concentration of 1 nM CCK-OP [8] was calculated as a percentage of the total amount of amylase in the cell suspension (Fig. 9). Under basal conditions, amylase release was similar in buffers with and without Na^+. However, significantly more amylase was released following CCK-OP stimulation of permeabilized acini incubated in 30 mM NaCl/105 mM KCl containing buffer (6.4 ± 0.4% of total amylase above basal) compared to acini incubated in 0 mM NaCl/135 mM KCl only (4.7 ± 0.4% of total amylase above basal, $P < 0.03$). Further increases in the Na^+ concentration of the incubation buffer resulted in a gradual reduction of CCK-OP-stimulated amylase release (4.9 ± 0.6% at 80 mM NaCl/55 mM KCl, 2.1 ± 0.3% at 130 mM NaCl/5 mM KCl and 3.4 ± 0.6% of total amylase above basal at 135 mM NaCl/0 mM KCl).

Effect of EIPA on Basal and CCK-OP-induced-Amylase Secretion in Permeabilized Pancreatic Acini

To determine whether the Na^+/H^+ exchange of ZG membranes plays a role in Na^+-dependent CCK-OP-induced amylase secretion of permeabilized pancreatic acini, the effect of various concentrations of EIPA was studied in permeabilized acini incubated in buffer containing 30 mM NaCl/105 mM KCl and compared to a buffer containing KCl only. As shown in Fig. 10*A* and *D*, basal amylase release was unaffected by addition of up to 50

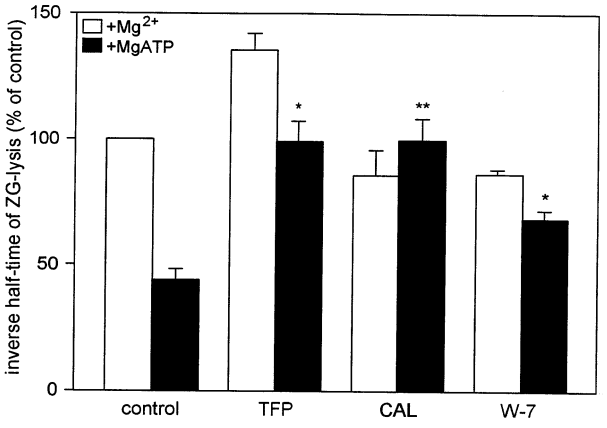

Fig. 8. Effect of calmodulin antagonists on MgATP dependent inhibition of ZG Na^+/H^+ exchanger. ZG were suspended in 150 mM Na-acetate, 50 mM imidazole (adjusted to pH 7.0 with acetic acid), 1 mM EDTA and 5 mM Mg^{2+}. The calmodulin antagonists calmidazolium (CAL, 0.75 μM), trifluoperazine (TFP, 100 μM) and W-7 (500 μM) were incubated for 5–10 min before addition of 0.5 mM MgATP, and ZG lysis was measured as described in the Methods section. Inverse half-times of lysis are plotted as percent of controls with Mg^{2+} (Controls = 2.13 ± 0.13 hr^{-1}, $n = 14$). Means ± SEM of 5–14 experiments, $*P < 0.03$, $**P < 0.0001$ using Student's *t*-test for paired comparison of conditions with MgATP and MgATP + calmodulin antagonist.

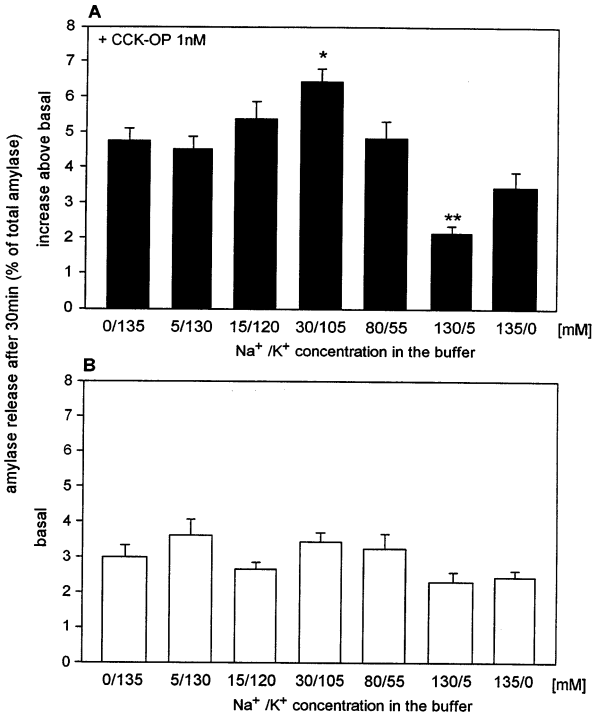

Fig. 9. Effect of varying buffer Na^+ concentrations on CCK-OP-stimulated amylase secretion from permeabilized pancreatic acinar cells. Amylase released in 30 min into the medium under basal conditions (*B*) and after addition of 1 nM CCK-OP (*A*) is plotted as percent of total amylase in the cell suspension. Cells were permeabilized for 10 min with 5 μg/ml digitonin. Incubation buffers contained different Na^+ and K^+ concentrations as described in the figure. The total concentration of Na^+ and K^+ was held at 135 mM for each buffer. CCK-OP-induced increase in amylase release/30 min in various Na^+-containing buffers was calculated as the difference between amylase release after CCK-OP stimulation and basal secretion (*B*). Means ± SEM of 5–18 experiments, $*P < 0.03$, $**P < 0.005$ by unpaired Student's *t*-test for comparison with acini incubated in 135 mM KCl containing buffer.

μM EIPA. In the buffer without Na^+, amylase released in the presence of 1 nM CCK-OP was similar in the absence or presence of 10–50 μM EIPA (Fig. 10*B*). Accordingly, CCK-stimulated amylase release was not significantly different without (6.1 ± 0.7% of total amylase above basal, n = 6) or with 50 μM EIPA (5.0 ± 0.4% of total amylase above basal, n = 6 (Fig. 10*C*). In contrast, 50 μM EIPA significantly reduced amylase secretion from acini incubated in 30 mM NaCl/105 mM KCl buffer in the presence of CCK-OP, when compared with the condition without EIPA (Fig. 10*E*). Consequently, CCK-OP-stimulated amylase release was significantly reduced with 50 μM EIPA, when compared to the condition without EIPA (4.1 ± 0.8% of total amylase above basal *vs.* 7.5 ± 0.6% of total amylase above basal; n = 4–10; $P < 0.02$) (Fig. 10*F*).

Discussion

In the present study, we provide evidence for the presence of a Na^+/H^+ exchanger in isolated zymogen granules of rat pancreatic acini, which may be regulated by a membrane bound calmodulin-dependent protein kinase. Finally, we propose that activation of the putative Na^+/H^+ exchanger of ZG enhances secretagogue activated enzyme secretion.

Evidence for an Electroneutral Na^+/H^+ Exchanger in ZG

ZG lysis in isotonic monovalent cation acetate buffers was H^+ dependent (Fig. 2) and occurred in the absence of ionophore (Fig. 1*B* and Table 1), suggesting electroneutral cation influx coupled to proton efflux. Cation influx occurred only in Na- and Li-acetate buffer, but not in Rb-, K- or Cs-acetate buffer. Similarly, an increase in absorbance of acridine orange caused by H^+ movement from the acidic internal space of ZG ($pH_i = 5.5$) into the incubation buffer (pH 7.5) occurred only after addition of Na^+ or Li^+ salts (Fig. 5 and Table 3). In principle, ZG lysis in Na- or Li-acetate buffer could occur by two different mechanisms: either by activation of a coupled Na^+/H^+ exchanger through an electroneutral carrier or through parallel operation of separate electrogenic Na^+ and H^+ permeabilities, whose net effect would also be electroneutral Na^+/H^+ exchange.

This was tested by measuring ZG lysis in Na-acetate in the presence of K^+ and the K^+ ionophore valinomycin (Table 2). Addition of valinomycin to ZG suspended in buffer with K^+ generates an inside-positive K^+ diffusion

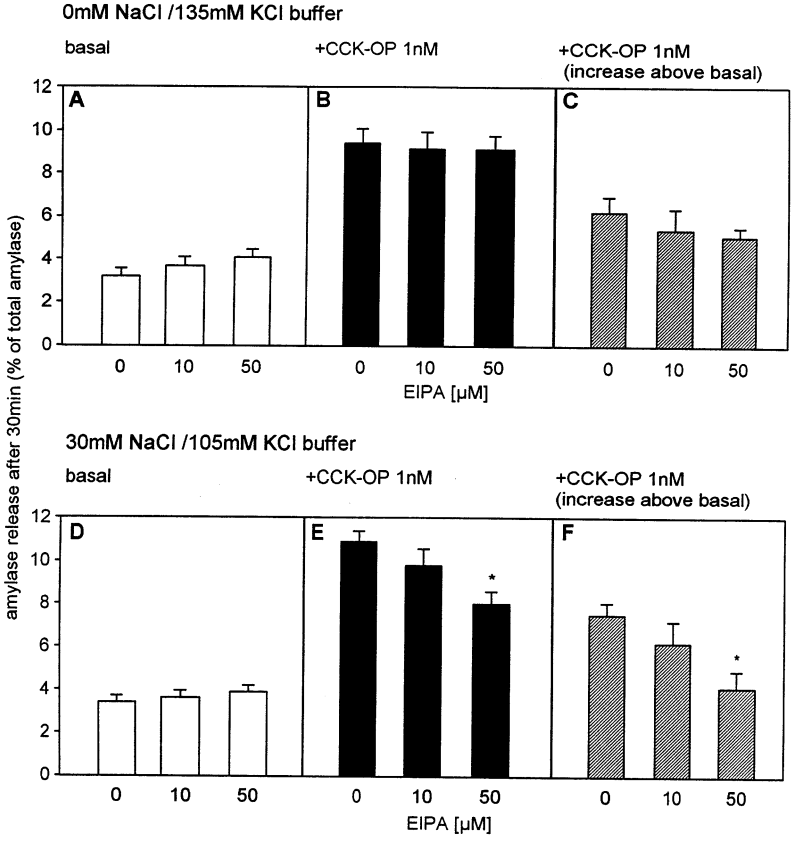

Fig. 10. Effect of EIPA on CCK-OP induced amylase secretion of permeabilized pancreatic acini incubated in buffer with 0 mM NaCl/135 mM KCl (*A,B,C*), or in buffer containing 30 mM NaCl/105 mM KCl (*D,E,F*). Acini were permeabilized with 5 μg/ml digitonin and preincubated for 5 min with EIPA before addition of the secretagogue. The effect of different concentrations of EIPA on amylase released from permeabilized acinar cells/30 min is shown for basal conditions (*A,D*) and following stimulation with 1 nM CCK-OP (*B,E*). The differences between CCK-OP stimulated and basal amylase release are plotted in (*C*) and (*F*). Values are means ± SEM of 3–10 experiments. **P* < 0.02 by unpaired Student's *t*-test for conditions with and without EIPA.

potential which should reduce electrogenic influx of Na^+ through Na^+ permeabilities. However, valinomycin did not prevent Na^+-dependent ZG lysis. Moreover, AMP-PCP, a blocker of the K^+ and Rb^+ selective conductance of ZG [25], did not enhance Na^+-dependent lysis in the presence of 25 mM K^+ (Table 2). These experiments can be taken as evidence for the presence of a Na^+/H^+ exchanger in ZG membranes, which exchanges preferentially external Na^+, or to a lesser extent Li^+ for internal H^+ (Table 1). Indeed, Na^+ and Li^+ are substrates of the Na^+/H^+ exchanger, and transport of Li^+ is kinetically less favorable than the transport of Na^+. Moreover, no appreciable affinity for K^+, Rb^+ or Cs^+ has been identified [1].

The ZG Na^+/H^+ Exchanger is Inhibited by Ethylisopropylamiloride and Benzamil

Amiloride and its analogues exhibit differences in sensitivity to inhibition of the different Na^+/H^+ exchanger isoforms [18]. Orlowski [18] found for NHE 1 and NHE 3 transfected in Chinese hamster ovary cells that amiloride (EC_{50} 100 μM for NHE 3) and EIPA (EC_{50} 2.4 μM for NHE 3) were 100 times less potent inhibitors of NHE 3 than of NHE 1, while benzamil showed the same inhibitory potency for both isoforms (EC_{50} ~100 μM). NHE 2 was equally inhibited by amiloride and EIPA with an EC_{50} of 1–3 μM, whereas nothing is known about the sensitivity of NHE 4 for amiloride and its analogues.

ZG lysis was inhibited by EIPA (EC_{50} ~25 μM) and benzamil (EC_{50} ~100 μM) but was relatively insensitive to inhibition by amiloride (Fig. 3). The relatively high EC_{50} of EIPA suggests that the Na^+/H^+ exchanger of ZG belongs to the ''low-affinity'' NHEs, such as NHE 3 [18]. However, the selectivity of EIPA > benzamil ⩾ amiloride observed for the inhibition of ZG Na^+/H^+ exchanger is unusual and is not in agreement with that described for NHEs, where benzamil was found to be less potent than amiloride [15, 18]. Similar results as in

ZG have been reported for mitochondrial Na^+/H^+ exchangers, which were also inhibited by EIPA and benzamil with EC_{50} values in the range from 50 to 100 μM, but not by amiloride [2, 13, 21]. Therefore, intracellular organelles may express different Na^+/H^+ exchangers which display an inhibitor selectivity distinct from that of plasma membrane Na^+/H^+ exchangers [28].

Regulation of the Na^+/H^+ Exchanger May Involve a ZG Membrane-bound Calmodulin-dependent Protein Kinase

The Na^+/H^+ exchanger of ZG was inhibited by ATP and ATP-γ-S in the presence of Mg^{2+} but not in its absence (Fig. 6). Addition of alkaline phosphatase reduced only the inhibition of Na^+/H^+ exchange induced by MgATP but not that induced by MgATP-γ-S (Fig. 7). This indicates that the Na^+/H^+ exchanger of ZG is inhibited by phosphorylation through an endogenous protein kinase. Previous studies have demonstrated that ZG membranes contain several protein kinases including PKC, PKA and a serine kinase with some characteristics common to casein kinases [3, 4, 23]. Furthermore calmodulin antagonists inhibited a calcium/phospholipid-dependent protein kinase present in ZG membranes [3, 4, 31]. The calmodulin antagonists calmidazolium, trifluoperazine and W-7, which inhibit calmodulin-activated protein kinases [29], were effective in preventing inhibition of Na^+/H^+ exchange by MgATP (Fig. 8). Stimulation of ZG Na^+/H^+ exchange by calmodulin antagonists occurred only in the presence of MgATP, whereas basal ZG Na^+/H^+ exchange remained unaffected. It is therefore likely that the protein kinase which is responsible for inhibition of Na^+/H^+ exchange in ZG membranes belongs to the calmodulin-activated protein kinases.

Physiological Significance

The experiments with permeabilized pancreatic acini strongly suggest that the EIPA inhibitable Na^+/H^+ exchanger from ZG membranes is involved in secretagogue-induced enzyme secretion. CCK-OP-mediated amylase release was enhanced in the presence of 30 mM Na^+ in the incubation buffer (Fig. 9). This process was abolished by addition of 50 μM EIPA (Fig. 10). In contrast, high (130–135 mM) Na^+ concentrations in the medium inhibited CCK-OP-stimulated enzyme secretion. The cytosolic Na^+ concentration could play a role in secretagogue-mediated enzyme secretion under *in vivo* conditions: Following stimulation of intact acinar cells by secretagogues, such as CCK, carbachol or pilocarpin, cytosolic Na^+ concentration increases up to 20–60 mM from resting values around 10 mM [17, 30, 33], possibly due to activation of basolateral Na^+ transporters. The mechanisms by which ZG membrane Na^+/H^+ exchanger could enhance enzyme secretion may be twofold: (i) by an osmotic load due to Na^+ influx, and (ii) by alkalinization of the intragranule space. Such increases in the ionic strength, hydration and deprotonation of the intragranule space are optimal for zymogen deaggregation, decondensation and swelling of the granules to promote fusion and secretion of the stored enzymes [16]. ZG fuse with the luminal membrane of acinar cells during exocytosis. Thus ZG Na^+/H^+ exchange may contribute as well to apical membrane transport processes of pancreatic acinar cells after granule fusion.

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