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ATP-Sensitive K^+ Conductance in Pancreatic Zymogen Granules: Block by Glyburide and Activation by Diazoxide

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Summary. The properties of transporters (or channels) for monovalent cations in the membrane of isolated pancreatic zymogen granules were characterized with an assay measuring bulk cation influx driven by a proton diffusion potential. The proton diffusion potential was generated by suspending granules in an isotonic monovalent cation/acetate solution and increasing the proton conductance of the membrane with a protonophore. Monovalent cation conductance had the sequence $Rb^+ > K^+ > Na^+ > Cs^+ > Li^+ >$ N-methyl glucamine$^+$. The conductance could be inhibited by Ca^{2+}, Mg^{2+}, Ba^{2+}, and pharmacological agents such as quinine, quinidine, glyburide and tolbutamide, but not by 5 mM tetra-ethyl ammonium or 5mM 4-aminopyridine, when applied to the cytosolic surface of the granule membrane. Over 50% of K^+ conductance could be inhibited by millimolar concentrations of ATP or MgATP. The inhibition by MgATP, but not by ATP itself, was reversed by the K^+ channel opener diazoxide. The inhibitory effect is probably by a noncovalent interaction since it could be mimicked by nonhydrolyzable analogs of ATP and by ADP. The reversal of MgATP inhibition by diazoxide may be mediated by phosphorylation since it was not affected by dilution, and was blocked by the protein kinase inhibitor H7. The properties of the K^+ conductance of pancreatic zymogen granule membranes are similar to those of ATP-sensitive K^+ channels found in the plasma membrane of insulin-secreting islet cells, neurons, muscle, and renal cells.

Key Words secretory granules · sulfonylurea · potassium channel · quinine · barium · ATP

Introduction

The exocrine pancreas is capable of regulated secretion of NaCl as well as of digestive enzymes. Both processes have been intensely investigated and may be coupled to some extent (DeLisle & Hopfer, 1986). NaCl secretion is thought to be the product of "active" Cl^- transport across the acinar cell and electrophoretic movement of Na^+ through the paracellular space (Petersen & Gallacher, 1988). Secretion of digestive enzymes takes place through incompletely understood processes that are morphologically characterized by fusion of discrete storage organelles, the zymogen granules, with the apical plasma membrane and movement of their contents into the acinar lumen and down the ducts (Palade, 1975). Both processes are subject to physiological regulation by hormones and second messengers (Kimura et al., 1986). For secretion of digestive enzymes to occur in permeabilized acinar preparations, both Cl^- and K^+ appear to be required as the major ionic constituents (Fuller, Eckhardt & Schulz, 1989*a*). To get insight into the role of electrolyte movement across the granule membrane, several investigators have studied regulation and properties of Cl^- conductance and electrically silent Cl/bicarbonate exchange in the granular membrane (Gasser, DiDomenico & Hopfer, 1988*a*; Fuller et al., 1989*b*; Thévenod, Gasser & Hopfer, 1990) since methods became available to isolate pancreatic zymogen granules that are stable in physiological saline solutions (DeLisle et al., 1984). In addition, a K^+ conductance has been described in pancreatic and parotid zymogen granule membranes, based on a lysis assay that depends on permeability increases to Cl^- with the ionophore tripropyl-tin and to protons with a protonophore, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Gasser, DiDomenico & Hopfer, 1988*b*).

The granule membrane becomes part of the luminal plasma membrane with fusion and thus should contain the granular K^+ conductance; therefore, one would expect K^+ secretion into the lumen of exocrine glands. However, the primary secreted fluid in the pancreas is low in K^+ (Ishikawa & Kanno, 1991), suggesting that the luminal K^+ conductance is regulated in vivo. In contrast to an abundance of information about basolateral K^+ conductance in epithelial cells in general, very little is known about luminal K^+ channels. Regulated K^+ secretion is known to occur through luminal K^+ channels in several epi-

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thelial tissues, such as the colon (Dawson, 1991) and renal tubular cells (Stokes, 1990). Two types K^+ channels have been characterized in the luminal membrane of renal cortical collecting duct cells: (i) a "maxi" K^+ channel (Hunter et al., 1984) that has a low open probability under physiological conditions and is therefore probably not involved in K^+ secretion; (ii) a low-conductance K^+ channel that displays a high open probability (Frindt & Palmer, 1989) and is blocked by millimolar concentrations of ATP as well as activated by PK A plus MgATP (Wang & Giebisch, 1991).

Since the isolated pancreatic zymogen granule preparation provides a convenient experimental system to study regulation of transporters that act from the cytosolic side, we investigated the effects of physiological and pharmacological agents that are known to affect K^+ channels in other systems, such as ATP and its analogs, the inhibitors quinine and antidiabetic sulfonylureas, and as well as the K^+ channel opener diazoxide. To better characterize the native K^+ transport pathway, we employed a simplified lysis assay that avoided the ionophore tripropyl-tin. The results indicate that the granular K^+ conductance has characteristics of ATP-sensitive channels found in the plasma membrane of cardiac and skeletal muscle, neurons, pancreatic β-cells, smooth muscle and renal cells (Noma, 1983; Cook & Hales, 1984; Spruce et al., 1985; Standen et al., 1989; Ashford et al., 1990; Wang & Giebisch, 1991). To this date, it is unique as to its location in membranes bounding intracellular organelles.

Materials and Methods

Materials

ATP, adenosine 5'[$\beta\gamma$-methylene]triphosphate (App[CH2]p), adenosine 5'-[$\beta\gamma$-imido]-triphosphate (App[NH]p), adenosine tetraphosphate (Attp), adenosine 5'-[γ-thio]triphosphate (ATP[S]) (all Na^+ or Li^+ salts), phenylmethanesulphonyl fluoride (PMSF), EGTA, EDTA, bovine serum albumin (98–99% BSA, essentially fatty-acid free), imidazole, Percoll, cesium acetate, CCCP, quinine, quinidine, staurosporine, 1-(5-isoquinolylsulfonyl)-2-methylpiperazine (H7), diazoxide, tetra-ethyl ammonium chloride (TEA Cl), 4-aminopyridine, N-methyl-glucamine$^+$ (NMG^+), $BaCl_2$, and sucrose were obtained from Sigma (St. Louis, MO). Rubidium acetate was from Aldrich Chemical (Milwaukee, WI). MES, MOPS, HEPES and Tris were purchased from Research Organics (Cleveland, OH). 3,3',4',5-tetrachlorosalicylanilide (TCS) was from Eastman Kodak (Rochester, NY) and tri-*n*-propyl-tin (TPT) from Alfa Products (Danvers, MA). Tolbutamide and glyburide were generous gifts of Hoechst-Roussel Pharmaceuticals (Somerville, NJ). All other chemicals were of the highest analytical grade available. Stock solutions of water-insoluble compounds, such as glyburide (also called glibenclamide), diazoxide, or H7, were prepared daily in dimethylformamide (DMF) or dimethylsulfoxide (DMSO) (usually 1% vol/vol). Ionophores were dissolved in 100% ethanol.

Methods

Isolation of Granules

Zymogen granules (ZG) were isolated from the pancreatic gland of male Sprague-Dawley rats exactly as described earlier (Thévenod et al., 1990).

Assay for Cation Conductance of ZG

Cation conductance of pancreatic ZG was assayed by a modification of a previously reported method for evaluation of ionic permeabilities of ZG membranes to electrolytes (DeLisle & Hopfer, 1986). In brief, ZG were resuspended in buffered isotonic salt solution and the granule membrane was made highly permeable to the major counter-ion with ionophores. Under these conditions, bulk salt influx into the intragranular space and the resulting granular lysis is limited by membrane permeability to the ion of interest. The kinetics of lysis was measured in a spectrophotometer as time-dependent change in absorbance at 540 nm (A_{540}) and quantified as times for lysis of half the granules. It had previously been shown that granular lysis is linearly related to absorbance (DeLisle & Hopfer, 1986). The inverse half-time was considered proportional to the rate constant of lysis and thus a relative rate constant. Typically, 50–70 μg of granules was suspended in a cuvette containing 3 ml buffered salt solution; this amount corresponds to an initial absorbance of 0.3–0.4. Absorbance was usually recorded continuously for 15 min at 37°C with a Beckman model DU-50 spectrophotometer equipped with a Peltier constant-temperature chamber, an automatic six-unit sampler and a kinetics Soft-Pak module. Data were stored and analyzed using a Symphony spreadsheet program (Lotus Development, Cambridge, MA).

Unless otherwise indicated, granules were suspended in buffered, 150 mM monovalent cation/acetate solutions and electrogenic cation influx into the granules was initiated by the addition of 16 μM CCCP. To control the magnitude of the pH gradient, a permeant buffer was employed, which for most experiments consisted of 50 mM imidazole titrated to the desired pH with acetic acid. All transport experiments were carried out at 37°C.

Calculation of Free Ca Concentrations

The free Ca^{2+} concentrations of Ca/EGTA buffers were calculated by the program of Fabiato (1988).

Modelling

The use of acetate salts for the lysis assay presents some interesting complications for transport experiments because the protonated, uncharged form of acetic acid would rapidly equilibrate across the granule membrane and thereby represents an acid and osmotic load on the intragranular space. Similar considerations hold for imidazole. To get some estimate of the quantitative changes for experimental design as well as for analysis of data, the intragranular pH and the granular volume changes were modeled.

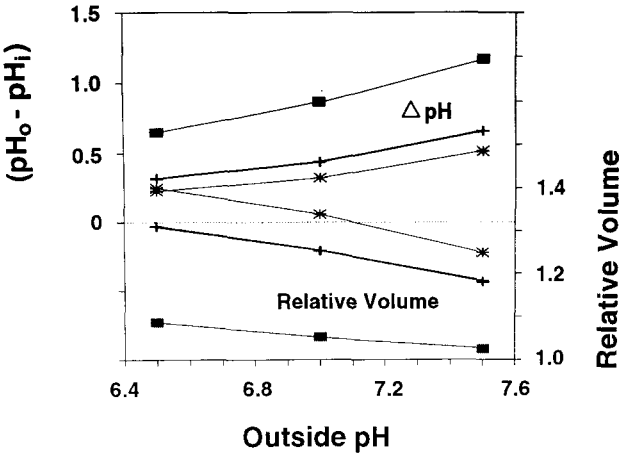

Fig. 1. Modelling of transmembrane pH gradient (ΔpH, left) and change in granular volume (*Relative Volume*, right) generated by 150 mM K^+ acetate and imidazole buffer adjusted with acetate to different pH values (*see* Appendix). Imidazole concentrations: (■) 0, (+) 50, (*) 100 mM, adjusted to the desired pH with acetic acid. The three upper traces demonstrate increases in ΔpH with increasing outside pH at different imidazole concentrations. The three lower traces demonstrate that an osmotic load due to increasing imidazole concentrations results in volume changes up to ~40% (difference in vertical direction).

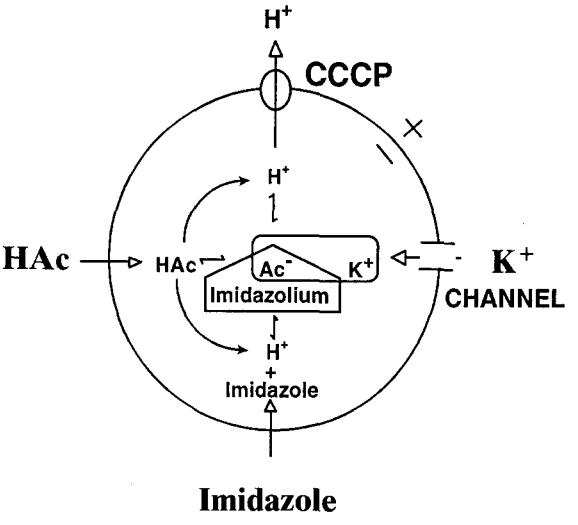

Fig. 2. Model of assay for cation conductance by protonophore-induced osmotic lysis of pancreatic zymogen granules, when suspended in an isotonic solution of a mono-valent cation/acetate solution. The arrows indicate the net flux of solutes into and out of granules. The driving force is the acetic acid concentration gradient. Acetic acid permeates through the lipid membrane and dissociates within the grandules to provide protons for protonation of imidazole as well as efflux from the acid interior. The proton efflux depends on exogenous protonophores, such as CCCP. It generates a diffusion potential that, in turn, energizes K^+ influx through K^+ channels in the membrane.

Assumptions and data incorporated into the model, and a brief description of the program, are provided in the Appendix. Figure 1 shows predicted pH differences between the intragranular space and the medium (more acidic inside) as well as increases in volume with increasing concentrations of the imidazole/acetate buffer in the pH range of 6.5 to 7.5. The modelling indicates that, in the pH range 6.5 to 7.5, imidazole/acetate buffer can be used to alter intragranular pH with only a moderate osmotic load resulting in volume changes of up to 40%. This conclusion is supported by the stability of granules suspended in Rb^+ or K^+ acetate plus imidazole/acetate buffers without protonophores up to pH 7.5 (half-times > 2 hr), even though the membrane has relatively high conductances for Rb^+ and K^+ (*see* Results).

Analysis of Transport Data and Statistics

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 of 15 min if the half-time was not reached. Experiments with K^+ acetate, CCCP, and valinomycin to ensure high K^+ permeability served as controls for complete granule lysis and to measure the residual absorbance due to membrane fragments. This residual absorbance amounts to about 4% of the initial value; it was taken into account for calculating half-times of lysis. The slope of the absorbance change with time was estimated by linear regression of the digitized data.

The reproducibility of transport measurements on samples from the same granule preparation was within 5%. Unless otherwise indicated, all experiments were repeated at least three times with different granule preparations. Variability is provided as means ± SD of different preparations. If figures show single experiments, they are representative of at least three experiments with qualitatively similar results. When drugs were added in DMF, DMSO, or ethanol, the control experiments received the same amount of carrier solvent.Statistical analysis of data was carried out with the Statgraphics program using paired Student's *t* test. Results with levels of $P < 0.05$ were considered significant.

Results

Validation of Assay for Cation Conductance

Granule lysis measures the end point of bulk osmolyte influx and swelling. It provides a convenient and robust method for initial characterization of the membrane permeability and transporters in the granule membrane. In the case of a salt as the major osmolyte in the medium, it is possible to selectively choose highly permeant anions or cations so that the lysis rate becomes limited by the permeability to the counter-ion. To evaluate cation conductance, i.e., to make electrogenic cation influx rate limiting for lysis, acetate salts were used as major osmolytes. This experimental set-up is based on the rationale illustrated in Fig. 2: acetic acid, but not acetate,

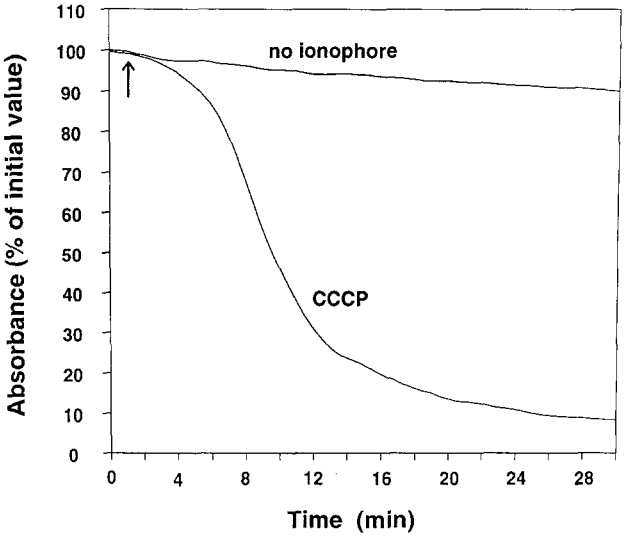

Fig. 3. Protonophore-induced osmotic lysis of pancreatic zymogen granules. CCCP-induced lysis measured in terms of decrease of A_{540} of a granule suspension. Zymogen granules were suspended in 150 mM K^+ acetate buffered with 50 mM imidazole, which had been adjusted with acetic acid to pH 7.0, 0.1 mM EGTA, and 1 mM EDTA. Without addition of a protonophore, the granules remain intact for over 2 hr in this medium at 37°C (*no ionophore*). Addition of 16 μM CCCP (CCCP trace) at the arrow accelerated granular lysis, reflected by the decrease in absorbance.

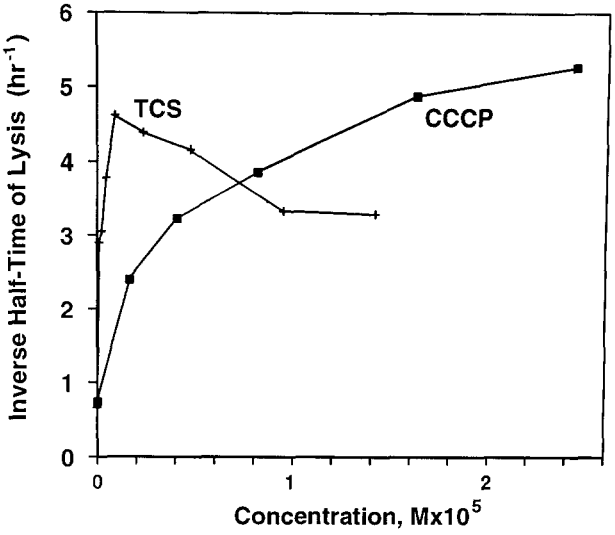

Fig. 4. Dependence of K^+ acetate influx and granule lysis on protonophore (CCCP or TCS) concentration. Zymogen granules were suspended in K^+ acetate buffer as described for Fig. 3 and half-times of lysis measured after addition of the protonophores CCCP or TCS (*see* Methods). CCCP and TCS were added from stock solutions in 100% ethanol. The final ethanol concentration was kept constant at 1% (vol/vol).

easily moves across biological membranes so that acetic acid would flow into granules when the outside acetate concentration is high; acetic acid dissociates inside the granules, increasing the proton concentration. In the presence of a protonophore, the proton concentration gradient across the membrane is converted to an inside negative diffusion potential that serves as driving force for cation influx. Continuous acetic acid influx maintains the proton gradient and proton efflux, the membrane potential and cation flux until the granules lyse due to osmotic swelling.

The use of a protonophore to initiate the generation of a diffusion potential and cation influx has the advantage that granule stability can be evaluated before protonophore addition, and thus helps to determine unspecific effects of any experimental manipulation. As shown in Fig. 3 for a solution of 150 mM K^+ acetate, 1 mM EDTA, and 50 mM imidazole, titrated to pH 7.0 with acetic acid, granules are stable with half-times >2 hr in the absence of protonophores, indicating low permeabilities to K^+, acetate (in contrast to the high permeability to the protonated acetic acid), or H^+ (DeLisle et al., 1984). Addition of 16μM CCCP considerably enhanced the rate of lysis, revealing the presence of a K^+ conductance in the membrane. 90% of all preparations had these long half-times of >2 hr in the absence of protonophores and could be used for measurements of the K^+ conductance. For one series of 21 preparations, the inverse half-time related to the K^+ conductance was $3.6 \pm 2.3\ hr^{-1}$ (lysis rate with CCCP), in contrast to $0.50 \pm 0.16\ hr^{-1}$ in the absence of CCCP ($P < 0.001$). All preparations exhibited a K^+ conductance, although the magnitude differed. Further experiments were conducted similarly as shown in Fig. 3 with an initial period in which baseline granule lysis rate was measured in the absence of a protonophore to exclude major changes in unspecific "leakiness" of the membrane by experimental manipulations.

To ensure that the proton conductance of the membrane was not rate limiting for lysis, titrations with protonophores were carried out. As shown in Fig. 4 maximal rates of lysis were reached with 15–25 μM CCCP or 1μM for TCS at an external pH of 7.0. At pH 7.5, maximal CCCP effects were already seen with about 1.5 μM (*data not shown*). With maximal proton conductance provided by added protonophores, the cation conductance then becomes the rate limiting step for overall cation/acetate influx and lysis.

To evaluate the dependence of cation influx on the magnitude of the diffusion potential, the magnitude of the pH-gradient across the granule membrane was manipulated. Changes in the pH gradient were brought about in two ways: (i) by varying me-

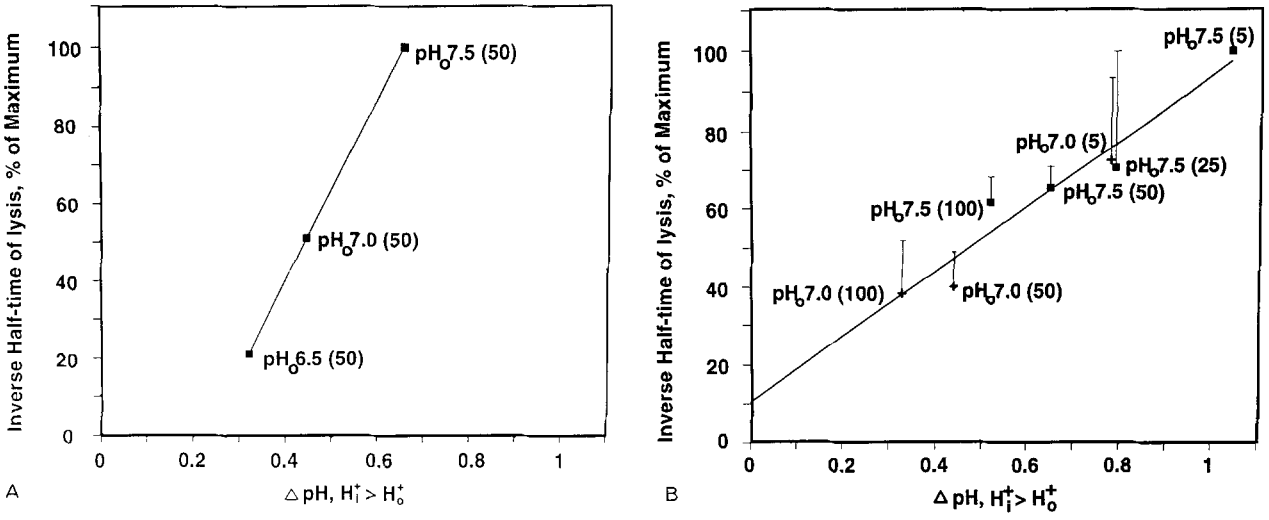

Fig. 5. pH gradient as driving force for K^+ influx into zymogen granules. (*A*) Granules were incubated in 150 mM K^+ acetate/50 mM imidazole buffer and the outside pH (pH_o) adjusted to 6.5, 7.0, or 7.5 with acetic acid. The transmembrane pH gradient ($pH_{inside} < pH_{outside}$, $H_i^+ > H_o^+$) was calculated as the difference between outside pH and intragranular pH estimated from the "SCoP" model in the Appendix. Half-times of lysis were measured after addition of 16 μM CCCP (*see* Methods) to generate H^+ diffusion potentials and inverse half-times expressed relative to the maximum at pH_o of 7.5. Numbers in parentheses refer to imidazole concentration. (*B*) Granules were incubated in 150 mM K^+ acetate and varying concentrations of imidazole (5, 25, 50, or 100 mM) at pH 7.0 or pH 7.5. Other experimental conditions and calculations were as described for *A*. The graph provides mean values ± SD of three different granule preparations.

dium pH at constant imidazole concentration, or (ii) by varying imidazole concentration at constant medium pH. The magnitude of the pH-gradient in each case was estimated from modeling experiments detailed in the Appendix.

Changes in medium pH in the range of 6.5–7.5 and of imidazole concentrations up to 100 mM did not significantly affect granular stability in the absence of protonophores. For example, for granules suspended in K^+ acetate and 50 mM imidazole without CCCP, lysis rates over this pH range varied only between 0.8 and 1.2 times the value at pH 7.0. Above pH 7.5 granular stability decreased, while below pH 6.5 it increased disproportionally. These effects at the extremes of pH may be related to intragranular protein disaggregation and aggregation, respectively.

As shown in Fig. 5*A* and *B*, CCCP-dependent K^+ influx, measured as inverse half-time of lysis, is dependent on calculated pH gradient responsible for the membrane potential. Figure 5*A* shows the effect of changes in outside pH from 6.5 to 7.5 at constant imidazole concentration of 50 mM. Figure 5*B* shows the results of another experiment in which the magnitude of the pH gradient was varied by changing the imidazole concentration at constant outside pH of either 7.0 or 7.5. Both experiments together indicate that the magnitude of the diffusion potential generated by the pH gradient has a direct effect on the K^+ influx, as expected for a conductance pathway or channel in the membrane. The slopes are not identical for the two experimental conditions which can be explained by differences in the predicted osmotic load due to intragranular imidazolium and acetate. Osmotic load is another experimental variable that could also influence lysis rates (*see* Appendix and Fig. 1).

Granule lysis rates are also dependent on the concentration of the permeant cation, although in a nonlinear manner. For example, when the dependence on K^+ acetate was measured with sucrose as replacement osmolyte at constant osmolarity and with 50 mM imidazole/acetate at pH 7.0, major increases in lysis rates only occurred between 100 and 150 mM K^+ acetate (Fig. 6). A similar dependence on K^+ acetate was observed when Cs^+ acetate served as replacement osmolyte (i.e., at constant total acetate concentration of 150 mM). Cs^+ acetate can be used as replacement osmolyte for this type of experiment because the membrane is virtually impermeable to Cs^+ (*see below*).

Ionic Selectivity of the Cation Conductance

As shown in Fig. 7, the conductance pathway from pancreatic zymogen granules was highly permeable to Rb^+ and K^+, and less to Na^+. Cs^+, Li^+ and

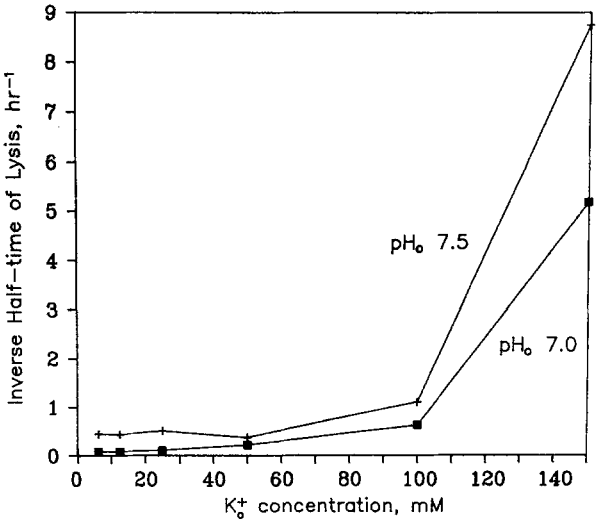

Fig. 6. K^+ influx into zymogen granules is a function of outside K^+ concentration (K^+_o). Granules were suspended in 50 mM imidazole adjusted to either pH_o 7.0 or pH_o 7.5 with acetic acid. K^+ acetate concentrations were varied between 6.25 and 150 mM while the osmolarity was held constant at 300 mOsm by addition of sucrose. Half-times of lysis were determined after addition of 16 μM CCCP (*see* Methods).

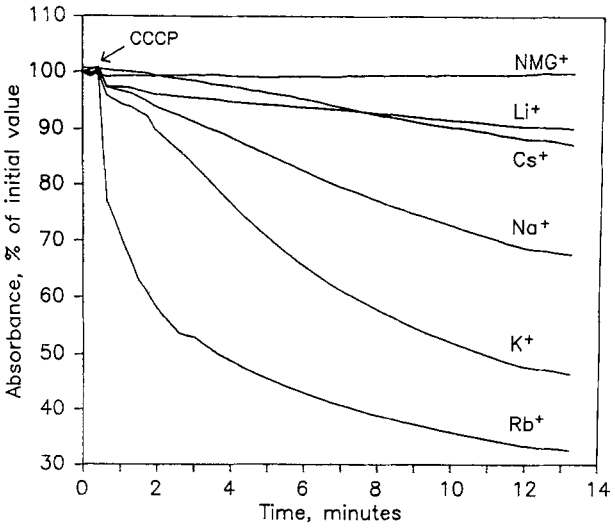

Fig. 7. Selectivity of cation conductance in pancreatic granular membranes. Granules were suspended in a solution consisting of 50 mM imidazole, adjusted to pH 7.0 with acetic acid, 0.1 mM EGTA, 1 mM EDTA, and 150 mM of the acetate salt listed in the figure. 16 μM CCCP was added to the granules indicated by the arrow to generate a proton diffusion potential that can drive cation influx.

NMG^+ did not significantly permeate the channel. The cation selectivity described in Fig. 7 is best represented by Eisenman's selectivity isotherm III (Eisenman, 1962) as applied to biological membranes (Diamond & Wright, 1969), except that Eisenman's sequence III shows a better selectivity of Cs^+ over Na^+. This difference could also result from the known blocking effect of Cs^+ on K_{ATP} channels in other preparations (Quayle, Standen & Stanfield, 1988). The selectivity also follows the cation sequence described by Fuller et al. (1989*a*) in studies on ionic dependence of stimulated protein secretion from pancreatic acinar cells.

Specificity of Blockers

To further characterize the K^+ conductance, pharmacological "fingerprinting" with inorganic and organic agents was employed. It is important to keep in mind that the cytosolic side of zymogen granule membranes is exposed to the medium. This experimental situation is different from that in most cellular investigations with access to the extracytosolic surface of the plasma membrane. This surface corresponds to the inside of granules and, therefore, is not directly accessible in isolated, intact granules. Thus, impermeant, charged or polar K^+ channel blockers that inhibit from the extracellular side may be ineffective when added to granule suspensions even though sensitive K^+ channels may be present in the granule membrane.

As shown in Table 1, TEA^+ (5 mM) did not block K^+ conductance. In contrast, Ba^{2+} at the same concentration reduced K^+ conductance by 70%. Ca^{2+} (1.5 mM) and Mg^{2+} (5 mM) also inhibited by 38 and 25%, respectively. The inhibitory effect of Ca^{2+} was present already at micromolar free concentration (e.g., 46% inhibition of the lysis rate at 1 μM free Ca^{2+}, relative to controls with 1 mM EGTA), indicating that the K^+ conductance pathway in pancreatic granules is not associated with Ca^{2+}-activated K^+ channels. 4-aminopyridine (5 mM), a lipophilic cation channel blocker was without inhibitory effect. Quinine and its stereoisomer quinidine, other lipophilic inhibitors of certain types of K^+ channels, strongly reduced K^+ conductance; for example, both compounds at 0.5 mM inhibited K^+ conductance by 65% (Table 1 and Fig. 8). The blocker with the highest potency was glyburide, an antidiabetic sulfonylurea derivative. Even at 100 nM, inhibition could be reproducibly demonstrated (~22%). The maximum concentration tested was 100 μM which inhibited about 43% (Fig. 8). In contrast, tolbutamide, another sulfonylurea derivative, inhibited K^+ conductance maximally by 10–15%, even with concentrations up to 1 mM. These results can be compared with the potency of blocking of ATP-sensitive K^+ channels and of binding by various antidiabetic

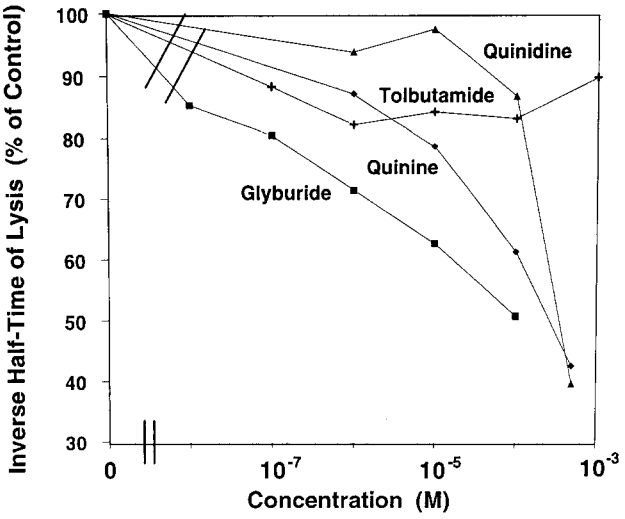

Fig. 8. Dose response curves for blockers of K^+ conductance in pancreatic granules. Granules were suspended in K^+ acetate and incubated with CCCP as in Fig. 3. Control rates (100%) of granular lysis after CCCP addition were measured in the absence of blockers, but presence of the carrier solvent, i.e., 0.1% (vol/vol) DMF. The inverse half-times for controls were 4.3 ± 1.6 hr^{-1}. Blockers were added from a stock solution in DMF with a final DMF concentration of 0.1% (vol/vol).

sulfonylurea analogs in insulin-secreting cells (Schmid-Antomarchi et al., 1987): In the latter system, glyburide was found to be the most potent derivative (K_i = 0.06 nM) and tolbutamide the least potent compound (K_i = 22 μM).

To evaluate whether the Rb^+ conductance was mediated by the same pathway that is responsible for K^+, inhibition was measured for quinine, quinidine, and glyburide with Rb^+ replacing K^+ in the lysis assay. These three agents were as efficacious in the inhibition of Rb^+ influx as for K^+, suggesting the presence of a common transporter or channel for Rb^+ and K^+.

Block by Nucleotides

The block of K^+ conductance by glyburide suggests that the granule membranes contain some type of ATP-sensitive K^+ channel. This class is characterized not only by modulation by specific drugs (e.g., glyburide and diazoxide), but also by inhibition by physiological, i.e., millimolar, concentrations of ATP. Therefore, the effect of ATP up to 5 mM was investigated. As shown in the left part of Fig. 9, 5 mM ATP reduced K^+ conductance to 65 ± 7% of the respective controls without Mg^{2+} (100% = 3.3 ± 1.8 hr^{-1}). 5 mM MgATP was about equally inhibitory as ATP (69 ± 14% of controls), when compared to controls with 5 mM Mg^{2+} (100% = 1.8 ± 0.6 hr^{-1}), but was even more inhibitory (51 ± 25% of controls, calculated from the data but not shown in Figure 9) compared to controls without Mg^{2+} and ATP. Our observations differ from results with pancreatic insulin-secreting cells, where it has been demonstrated that ATP^{4-} is the active component in blocking the ATP-sensitive K^+ channel (Dunne, Illot & Petersen, 1987; Ashcroft & Kakei, 1989). In the islet cells, addition of Mg^{2+} reduces the ability of ATP to inhibit channel activity, and Mg^{2+} removal enhances the potency of ATP. Our data are, however, in agreement with studies in cardiac cells which demonstrate that both MgATP and ATP^{4-} block the ATP-sensitive K^+ channel (Noma, 1983; Kakei, Noma & Shibasaki, 1985; Findlay, 1988).

Table 2 summarizes inhibitory effects of different adenine nucleotides, including poorly hydrolyzable or nonhydrolyzable analogs, in the absence of Mg^{2+}. ATP stood out in this group as the most potent blocker, followed by ATP[S] and App[CH_2]p. The fact that ATP[S] and App[CH_2]p were comparable to ATP suggests that ATP blocks by a noncovalent mechanism, rather than by an enzymatically mediated process associated with ATP hydrolysis. It is noteworthy that even ADP at 5 mM has an effect comparable to that of ATP (Table 2), suggesting that it competes for the same binding site, but with lower affinity than ATP. A[tt]p has the weakest blocking effect of the tested analogs, which can be explained by its larger size and steric restrictions at the nucleotide binding site responsible for channel block.

Activation by Diazoxide

Diazoxide is structurally related to glyburide, but has opposite effects on ATP-sensitive K^+ channels: it selectively activates these channels (Trube, Rorsman & Ohno-Shosaku, 1986) by a mechanism probably involving protein phosphorylation (Dunne, 1989). In pancreatic granules, diazoxide at 0.6 mM reversed the inhibition of K^+ conductance by MgATP, but not by ATP itself (Fig. 9, right). It actually restored the levels of K^+ conductance in about half the preparations to that seen in granules without Mg^{2+} and ATP. If conditions with 5 mM Mg^{2+} are used as controls (5 mM Mg^{2+} itself reduces K^+ conductance from 3.3 ± 1.8 hr^{-1} without to 1.8 ± 0.6 hr^{-1} with Mg^{2+}), then diazoxide actually appears to stimulate (Fig. 9). Diazoxide was without any effect on K^+ conductance in the concentration range of 0.1–1000 μM in the absence of ATP (three experiments, *not shown*) or in the absence of Mg^{2+} (Fig. 10). The requirement of MgATP for the di-

Table 1. Effect of K^+ channel blockers on granular K^+ conductance

	Concentration (mM)	Inverse Half-Time of Lysis[a] (hr^{-1}) mean ± SD	Inhibition (%) mean ± SD	(n)[c]
Control[b]	—	6.1 ± 3.7	—	22
Mg^{2+}	5	4.3 ± 3.0	25 ± 5[d]	13
Ca^{2+}	1.5	3.4 ± 0.2	38 ± 5[d]	3
Ba^{2+}	5	1.6 ± 0.7	70 ± 18[d]	4
TEA^+	5	7.4 ± 0.9	12 ± 4	3
4-Aminopyridine[e]	5	10.0 ± 1.6	−18 ± 10	3
Quinine[e]	0.5	2.4 ± 0.3	64 ± 9[d]	3
Quinidine[e]	0.5	2.1 ± 0.3	66 ± 5[d]	3

[a] Measured after addition of 16 μM CCCP.
[b] Medium: 150 mM K^+ acetate plus 50 mM imidazole adjusted to pH 7.0 with acetic acid.
[c] n = number of preparations tested.
[d] $P < 0.01$, paired Student's t-test.
[e] Added in either DMF or DMSO (final concentration < 0.1%, vol/vol); controls with the same amount of carrier solvent.

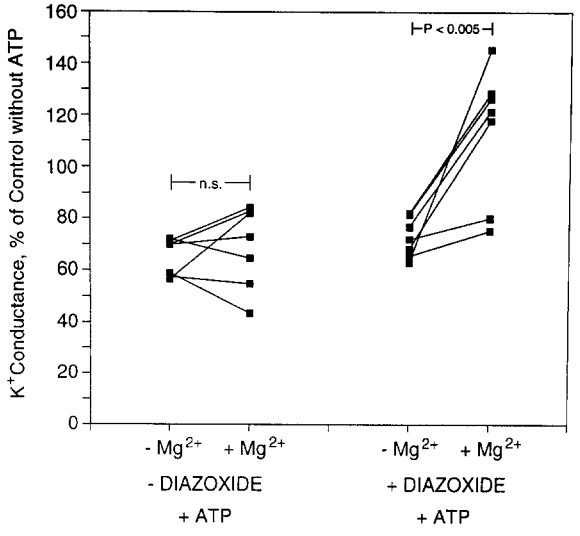

Fig. 9. Mg^{2+} dependence of diazoxide-induced reversal of inhibition of K^+ conductance by ATP. ZG were incubated in 150 mM K^+ acetate, 50 mM imidazole/acetate, pH 7.0, with 5 mM ATP, with or without 5 mM $MgSO_4$, and with or without 0.6 mM diazoxide. Lysis rates were measured after addition of 16 μM CCCP. Separate controls without ATP and diazoxide, but with the carrier solvent DMF (final concentration: 1%, vol/vol), were used for conditions with and without Mg^{2+}. Control rates (inverse half-times) were 3.3 ± 1.8 hr^{-1} without Mg^{2+} and 1.8 ± 0.6 hr^{-1} with Mg^{2+} (means ± SD of seven preparations). These rates were set to 100% for the conditions with and without Mg^{2+}, respectively.

azoxide effect supports the idea of a covalent process, such as phosphorylation, underlying K^+ channel activation.

To test this hypothesis, dilution experiments were carried out. Interactions of ATP with the chan-

Table 2. Inhibition of K^+ conductance by nucleotides

Nucleotide	Rate of granule lysis % of control without nucleotide[a]	
	Mean ± SD 1 μM	Mean ± SD 5 mM
None[b]	100	100
ATP	85 ± 8[d]	55 ± 10[d]
ATP[S]	79 ± 11[c]	58 ± 11[c]
App[CH_2]p	86 ± 8	64 ± 4[d]
App[NH]p	96 ± 6	67 ± 19
ADP	94 ± 2	63 ± 2[c]
A[tt]p	85 ± 8	77 ± 8

[a] Granules were suspended in 150 mM K^+ acetate, 50 mM imidazole (adjusted to pH 7.0 with acetic acid) plus nucleotide at the indicated concentration without Mg^{2+}.
[b] 100% corresponds to an inverse half-time of lysis (after addition of 16 μM CCCP) of 3.2 ± 1.2 hr^{-1}.
[c,d] $P < 0.025$, $P < 0.01$, respectively, by paired Student's t-test to granule lysis in the absence of nucleotides (100%).

nel protein involving noncovalent bonds should reverse upon dilution, whereas covalent interactions, such as phosphorylation should continue. Granules were preincubated as concentrated suspensions for 15 min at 25°C in the absence of K^+ acetate and of a protonophore (no lysis) and subsequently diluted 50- to 100-fold into a K^+ acetate solution plus CCCP to initiate K^+ influx and granular lysis. As summarized in Fig. 10, preincubation with 5 mM ATP and subsequent 50-fold dilution resulted in less inhibition than predicted from previous experiments with 5 mM ATP present during the transport assay (observed with preincubation: 25%; predicted from 13 experi-

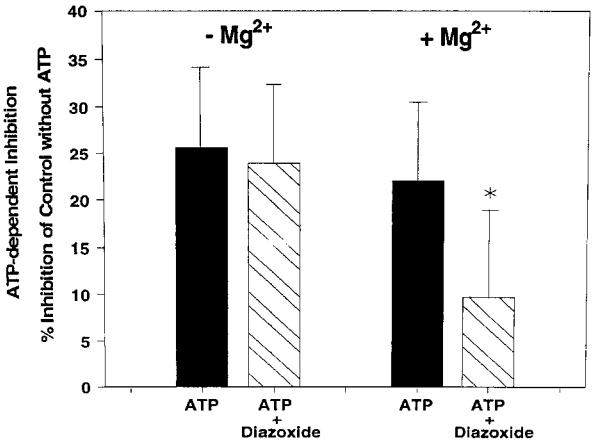

Fig. 10. Reversibility of the ATP block and of the diazoxide-induced activation of K^+ conductance. Granules were preincubated as a concentrated suspension for 15 min at 25°C in homogenization buffer with or without 5 mM $MgSO_4$, with or without 5 mM ATP, or with or without 0.6 mM diazoxide. They were subsequently diluted 50 to 100-fold into 150 mM K^+ acetate, 50 mM imidazole/acetate, pH 7.0, plus 16 μM CCCP, and lysis rates were measured during continued incubation at 37°C. Separate controls without ATP and diazoxide, but with the carrier solvent DMF (final concentration: 1%, vol/vol), were used for conditions with and without Mg^{2+}. Control rates (inverse half-times of lysis) were 10.4 ± 4.2 hr^{-1} without Mg^{2+} and 7.9 ± 4.6 hr^{-1} with Mg^{2+} (means ± SD of five preparations). Inhibition of lysis rates by preincubation with ATP or ATP plus diazoxide was calculated relative to the control rates with or without Mg^{2+}, respectively. *$P < 0.01$, Student's t test for paired comparison of conditions plus/minus diazoxide.

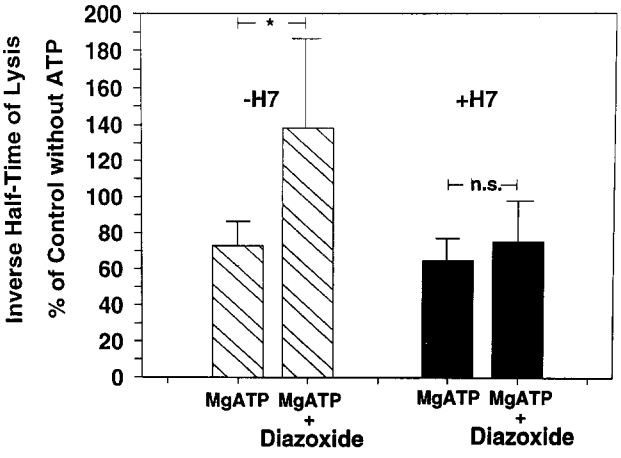

Fig. 11. Protein kinase inhibitor H7 blocks diazoxide-induced activation of K^+ conductance. ZG were incubated in 150 mM K^+ acetate, 50 mM imidazole/acetate, pH 7.0, with or without 5 mM MgATP, with or without 0.6 mM diazoxide, or with or without 3 μM H7. Lysis rates were measured after addition of 16 μM CCCP. Lysis rates without ATP and diazoxide, but with 5 mM $MgSO_4$ and the carrier solvent DMF (final concentration: 1%, vol/vol), served as controls (= 100%). *$P < 0.01$, Student's t-test for paired comparison of conditions plus/minus diazoxide. NS: not significant. Rates are expressed relative to controls without ATP. Results are means ± SD of three ZG preparations.

ments and assuming irreversibility of ATP effect: 41.9 ± 7.8% without and 37.3 ± 18.0% with Mg^{2+}) and close to the one expected for 100 μM ATP (17 ± 11%, $n = 3$). This reversibility of the inhibitory effect is consistent with reversible binding of ATP. As expected from earlier experiments (Fig. 9), the presence of diazoxide had no effect in the absence of Mg^{2+}. In contrast, in the presence of Mg^{2+}, preincubation with 0.6 mM diazoxide resulted in a significant stimulation of K^+ conductance relative to MgATP alone ($P < 0.03$), which shows up in Fig. 10 as less inhibition (only about 10%) relative to the control without ATP. This stimulation cannot be accounted for by the residual 6–12 μM diazoxide present during the transport assay and is consistent with a chemical reaction, i.e., a covalent modification.

More insight into the possible mechanism underlying diazoxide-induced activation of K^+ conductance in pancreatic granules was derived from paired experiments with protein kinase inhibitors. Figure 11 shows the results on the effect of H7, a blocker of protein kinases A and C (Hidaka et al., 1984), on diazoxide stimulation of the K^+ conductance. H7 (3 μM) did not affect the blocking effect of MgATP (32% inhibition with or without H7 relative to 5 mM Mg controls); in contrast, H7 abolished the activation of K^+ conductance by 0.6 mM diazoxide. Staurosporine, a relatively specific inhibitor of protein kinase C (Tamaoki et al., 1986), when tested at 0.7 nM, had no significant effect (*not shown*).

Discussion

The K^+ Conductance of Zymogen Granules is an ATP-Sensitive K^+ Channel

This study confirms and extends the previous report by Fuller et al. (1987, 1989*a*) who demonstrated K^+ dependence of ^3H-labeled protein secretion in permeabilized rat pancreatic acini. Similarly as in the present study, the authors reported cation dependence of stimulated secretion in the sequence $Rb^+ > K^+ > Na^+ > NMG^+$ (Cs^+ was not tested), and block by cytosolic Ba^{2+} (0.2–2 mM) and quinidine. Subsequently, Gasser et al. (1988*b*) characterized a K^+ conductance pathway in isolated pancreatic and parotid secretory granules with a different cation sequence ($Cs^+ > Rb^+ = K^+ \gg Na^+ > Li^+ > NMG^+$), which was not blocked by Ba^{2+}, TEA^+,

quinine, or 4-aminopyridine. This previous K^+ conductance assay was also based on ionophore-induced osmotic lysis of granules in buffered salt solutions; however, granules were incubated in chloride salts, rather than acetate salts, and proton diffusion potentials were generated with a combination of the artificial Cl^-/OH^- exchanger tri-propyl-tin (TPT) and the protonophore CCCP (Gasser et al., 1988*b*).

The current assay for K^+ conductance has the advantage that it avoids TPT, which could change in unspecific or specific manner the properties of the granule membrane. Indeed, doping the membrane with TPT changes the selectivity of cation conductance measured with the current acetate assay to that seen by Gasser et al. (1988*b*). The altered ion selectivity sequence and the lack of inhibition by many classical K^+ channel blockers argue that the assay with TPT is poorly suited for characterization of granular K^+ channels.

The most interesting observation is probably that the K^+ conductance pathway present in zymogen granule membranes[1] shows the characteristics of the ATP-sensitive, inward-rectifying, K^+-selective channel originally described in heart and pancreatic β-cells (Noma, 1983; Cook & Hales, 1984). This channel is blocked by millimolar concentrations of ATP and its poorly or nonhydrolyzable analogs with or without Mg^{2+}. Although other types of channels are also blocked by cytosolic ATP, such as Ca^{2+}-activated nonselective cation channels (Findlay, Dunne & Petersen, 1985*b*; Cook, Poronnik & Young, 1990), several lines of evidence support the hypothesis that the K^+ transporter from zymogen granules is an ATP-sensitive K^+ channel: (i) ATP-sensitive K^+ channels are highly permeable to Rb^+ and K^+, but less so to Na^+ (Cook & Hales, 1984). Other known K^+ channels in exocrine glands are the Ca^{2+}- and voltage-activated, large conductance K^+ channel in rat and mouse salivary gland acini and pig pancreas (Maruyama, Gallacher & Petersen, 1983; Gallacher, Maruyama & Petersen, 1984) and the low-conductance, Ca^{2+}-activated, and voltage-independent cation channel from mouse and rat pancreatic cells. Both types have ion selectivities that are different from the granular cation conductance: the former shows high selectivity for K^+, but does not allow Rb^+ to pass (Gallacher et al., 1984), and the latter is nonselective (Maruyama & Petersen, 1982). Furthermore, pancreatic granular K^+ conductance was not activated by Ca^{2+} (Table 1). (ii) Glyburide (and to a much lesser extent tolbutamide) blocks K^+ conductance (Fig. 8). The inhibitory concentrations of glyburide are higher than those reported in patch-clamp and $^{86}Rb^+$ efflux studies (Schmid-Antomarchi et al., 1987; Fosset et al., 1988). This difference could be related to an apparent enhancement of tolbutamide sensitivity by cytosolic ADP (Zunkler et al., 1988). Granular membranes also have a different lipid composition (high content of cholesterol and phophatidylserine; LeBel & Beattie, 1984), which results in a low fluidity of the membrane (Gasser, Goldsmith & Hopfer, 1990) and could prevent access to the putative glyburide receptor. Inhibition appears to be specific, since antidiabetic sulfonylureas have no effect on the following K^+ channels: β-cell delayed rectifier (Rorsman & Trube, 1986), Ca^{2+}-activated K^+ channels (Trube et al., 1986), or inwardly rectifying K^+ channels from ventricular muscle cells (Belles, Hescheler & Trube, 1987). (iii) The ATP block is reversed by the K^+ channel opener diazoxide in a Mg^{2+}-dependent manner (Figs. 9 and 10), in agreement with previous reports in insulin secreting cells (Trube et al., 1986; Dunne, 1989). So far, diazoxide-induced activation has been found to be selective for ATP-sensitive K^+ channels and for Ca^{2+}-activated K^+ channels in smooth muscle (Gelband et al., 1990) only. (iv) Quinine and quinidine strongly blocked the granular K^+ conductance. Both compounds have been shown to completely inhibit opening of ATP-sensitive K^+ channels when applied to the internal (Cook & Hales, 1984) or external membrane surface (Findlay, Dunne & Petersen, 1985). In the latter study, it was demonstrated that quinine is a better blocker of ATP-sensitive K^+ channels than of Ca^{2+}-activated K^+ channels.

[1] Rat parotid zymogen granules possess a similar ATP-sensitive K^+ conductance that is inhibited by glyburide and activated by MgATP plus diazoxide. However, its magnitude in terms of granular lysis rates was generally 5–10 times smaller than in pancreatic granules from the same animal.

Dual Mechanism of ATP-Sensitivity

The interaction of ATP with the channel appears to be complex. ATP blocks the channel, while "run-down" of the channel in isolated patches can be prevented by MgATP (Findlay & Dunne, 1986). In addition, recent evidence suggests that phosphorylation may be involved in activation of the channel observed with MgATP and diazoxide (Dunne, 1989). We have used a fourfold approach for probing the mechanisms by which ATP modulates the K^+ conductance in zymogen granules: (i) Mg^{2+} dependence of ATP and diazoxide effects; (ii) replacement of ATP by nonhydrolyzable analogs; (iii) blockers of protein kinases, such as H7 and staurosporine; (iv) dilution following preincubation with high ATP concentrations to test reversibility of the ATP effects.

The results argue that the inhibition by ATP occurs through a Mg^{2+}-independent, reversible, noncovalent interaction with some kind of regulatory site. The inhibition can be mimicked by nonhydrolyzable analogs of ATP, by ADP and by A[tt]p (Table 2) and is not affected by the protein kinase inhibitors H7 and staurosporine. In contrast, the activation by diazoxide plus MgATP was not readily reversible and was prevented by H7, consistent with some kind of covalent modification.

At the present stage one can only speculate about the molecular mechanisms involved in ATP modulation of K^+ conductance. Activation by MgATP could be mediated by channel-independent membrane-associated kinases, such as protein kinase (PK) C (Wrenn, 1984), or putative channel-associated kinase activities (Chung et al., 1991). ATP-dependent K^+ channels in insulin-secreting cells are regulated by PK C (Wollheim et al., 1988; De Weille et al., 1989), though both activation and inhibition have been reported. Activation of ATP-sensitive K^+ channels by PK A has also been described in pancreatic β-cells (Ribalet, Ciani & Eddlestone, 1989) and in cortical collecting duct cells (Wang & Giebisch, 1991).

Inhibition of K^+ channels by ATP in other cells is not caused by simple channel block because changes in membrane potential do not affect it (Sturgess et al., 1986). The simplest explanation for ATP block is binding to a low-affinity, allosteric site of the channel or to associated regulatory proteins, resulting in ATP-dependent activation/inactivation of membrane-bound regulatory proteins.

Physiological Significance

Stanley and Ehrenstein (1985) have proposed that an initial step in the process of exocytosis of neurosecretory granules is activation of Ca^{2+}-dependent K^+ channels present in granular membranes. Electrical coupling to anion pathways would result in salt and H_2O influx, initial osmotic swelling, followed by fusion of the vesicle membrane with the luminal membrane and exocytosis of vesicle contents. Increasing the extragranular tonicity should inhibit secretion (Finkelstein, Zimmerberg & Cohen, 1986), and evidence for this has been provided by Fuller et al. (1989*a*) in protein secretion studies in isolated pancreatic acini. However, studies in beige mouse mast cells (Zimmerberg et al., 1987) and chromaffin granules (Holtz & Senter, 1986) have undoubtedly demonstrated that fusion actually precedes swelling. If swelling occurs in the mast cells of beige mice, it takes place after fusion of granules with the plasma membrane. This observation has been taken to eliminate the swelling hypothesis. However, it is possible that more than one physiological mechanism exists for fusion and that the dominant one in any particular system depends on experimental, physiological, or pathological conditions.

The present study demonstrates that a K^+ conductance pathway with properties similar to ATP-sensitive channels is indeed present in the membrane of zymogen granules. This conductance, however, is not associated with a Ca^{2+}-activated K^+ channel, as suggested by Stanley and Ehrenstein (1985). Under physiological, resting conditions this channel could be closed by the surrounding intracellular, millimolar concentrations of ATP that cause "tonic" inhibition to suppress granular swelling and lysis within the cell. Activation of the channel would require specific regulatory mechanisms to override the ATP block. Unlike in pancreatic β-cells and muscle (Dunne & Petersen, 1991), it is difficult to imagine that the regulation is metabolic in nature, unless ATP is compartmentalized in such a way that the local concentration close to the channel binding site would become very low due to ATP depletion. However, regulatory mechanisms activated by secretagogue hormones could in vivo "override" channel block by ATP, similar to diazoxide plus MgATP in vitro. Phosphorylation induced by PK C and PK A (De Weille et al., 1989; Ribalet et al., 1989), or receptor-mediated activation of GTP-binding proteins (De Weille et al., 1989; Dunne et al., 1989) open ATP-sensitive K^+ channels in the insulin-secreting cell line RINm5F. Similar mechanisms could also activate the K^+ conductance in pancreatic ZG. The K^+ conductance may also have importance for active secretion of K^+ and Cl^- into the intragranular pockets of the acinar lumen formed by fusion of granules with the plasma membrane and "flushing-out" of macromolecular secretory products, as morphologically evidenced by granular swelling after fusion (DeLisle & Hopfer, 1986; Gasser et al., 1988*a*,*b*). To prevent continuous loss of K^+ into the lumen, it must be assumed that the K^+ channel closes subsequent to granular fusion and exocytosis, possibly due to activity of cytosolic phophatases (Burnham, 1985).

It is noteworthy that granular K^+ conductance is inhibited by millimolar concentrations of ATP and its nonhydrolyzable analogs, whereas the Cl^- conductance pathway also present in granular membranes is activated by millimolar concentrations of ATP, App[CH_2]p and App[NH]p (Thévenod et al., 1990); the Cl^- conductance is activated by high concentrations of ATP and by App[CH_2]p and App[NH]p. In addition, Cl^- conductance can also be activated by protein phosphorylation mediated by PK A (Thévenod, Ikebe & Hopfer, 1991). This

could indicate that K^+ and Cl^- transporters are functionally coupled by a complex regulatory process (electrically or protein mediated). Indeed, Cl^- and K^+ conductance pathways appear to be tightly controlled in isolated granules as the granules are stable for hours in isotonic KCl solutions, even though separate Cl^- and K^+ conductance pathways can be demonstrated with added ionophores (Fig. 3, De Lisle & Hopfer, 1986). Extrapolation to resting conditions in vivo would suggest that intracellular ATP activates Cl^- channels, but blocks the K^+ channels, which suffices to prevent massive salt influx. Under stimulated conditions and following fusion events, activation of both K^+ and Cl^- conductance pathways by protein kinases would result in salt influx, granular swelling and exocytosis of secretory materials.

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Appendix

To quantitatively evaluate the effects of acetate and of imidazole buffer on intragranular pH and volume, they were modelled using the simulation package SCoP developed by the National Biomedical Simulation Resource (Duke University, Durham, NC) with support from NIH grant RR01693. The model assumes that the membrane is highly permeable to the uncharged, lipid-soluble species imidazole and acetic acid, but virtually impermeable to the ionic forms imidazolium and acetate. The validity of these assumptions is supported by observations of rapid pH changes in imidazole/acetate buffers (Gasser et al., 1988*b*) and virtual lack of proton conductance in the absence of protonophores (*see* Fig. 3*A*). The latter observation is relevant because the high permeability of both acetate and acetic acid or of imidazole and imidazolium would appear as high proton conductance. Thus, the slow granule lysis rates in imidazole/acetate buffers in the absence of protonophores can only by explained by low acetate as well as imidazolium permeability.

For any given intragranular pH, the concentrations of intragranular acetate and imidazolium can be calculated from acetic acid and imidazole concentrations with the Henderson-Hasselbalch equation. The concentrations of the latter species, in turn, follow from the extragranular pH and total imidazole and acetate concentrations. The difference between intragranular acetate and imidazolium constitutes an acid load which will change the intragranular pH depending on the buffer capacity of intragranular proteins. The intragranular acetate and imidazolium also constitute an osmotic load that results in an increase of the granular volume.

The three equations describing the relationships are listed below, and they are solved simultaneously in SCoP with a built-in block NONLINEAR, which is based on the Newton-Raphson algorithm.

pH_{inside} = function of (pH_{start}, acid load per mg protein, buffering capacity per mg protein). (A1)

Acid load per mg protein = (intragranular volume per mg protein) · (difference between the concentrations of intragranular acetate and imidazolium). (A2)

Intragranular volume = (initial amount of intragranular osmolytes)/((medium osmolarity) − (intragranular osmolarity due to acetate and imidazolium)). (A3)

Due to space limitations, the model is not included, but is available upon request from the corresponding author. It includes the relevant experimental data and their sources, such as pK values for acetic acid and imidazole, granular volume and buffer capacity. Data for intragranular pH and space were taken from earlier publications (DeLisle et al., 1984). The relationship between intragranular pH and acid load (Eq. (A1)) was experimentally determined by titrating lysed granules in lightly buffered water under CO_2-free conditions and subtracting the contribution of the buffered water. The titration curve was nonlinear and therefore fitted to a polynomial with STATGRAF (STSC, Rockville, MD). The highest order polynomial to which the data could be fitted was accepted; it turned out to be a quadratic equation. The numerical values depend on the assumption that the buffering capacity measured in lysed granules is equal to the one in intact granules. Since the intragranular protein concentration is much higher than that of lysed granules and proteins are more aggregated, the measured buffer capacity of lysed granules probably represents an overestimate of the buffer capacity of intact granules. On the other hand, the presence of osmotically inactive space due to aggregated proteins within the granules, would lower the acid (base) load per granule, thus potentially compensating for an overestimate of the buffer capacity. Equation (A3) is based on the assumption that water easily equilibrates, and hence the sum of the concentrations of intragranular osmolytes must equal the sum of those of the extragranular osmolytes. Furthermore, the intragranular osmolytes consist of acetate and imidazolium derived from permeant acetic acid and imidazole in the extragranular medium plus the initially present, impermeant solutes.

Figure 1 is an example of the Δ pH and relative granule size increases at different imidazole concentrations titrated with acetic acid and with the assumption that the osmotically active space is 70% of the total granular volume.