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Dual modulation of chloride conductance by nucleotides in pancreatic and parotid zymogen granules

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The regulation of Cl^- conductance by cytoplasmic nucleotides was investigated in pancreatic and parotid zymogen granules. Cl^- conductance was assayed by measuring the rate of cation-ionophore-induced osmotic lysis of granules suspended in iso-osmotic salt solutions. Both inhibition and stimulation were observed, depending on the type and concentration of nucleotide. Under optimal conditions, the average inhibition measured in different preparations was 1.6-fold, whereas the average stimulation was 4.4-fold. ATP was inhibitory at 1–10 μM but stimulated Cl^- conductance above 50 μM. Stimulation by ATP was more pronounced in granules with low endogenous Cl^- conductance. The potency of nucleotides in terms of inhibition was ATP > adenosine 5′-[γ-thio]triphosphate (ATP[S]) > UTP ⩾ CTP ⩾ GTP ⩾ guanosine 5′-[γ-thio]triphosphate (GTP[S]) ⩾ ITP. The potency with respect to stimulation had the following order: adenosine 5′-[βγ-methylene]triphosphate (App[CH_2]p) > ATP > guanosine 5′-[β-thio]diphosphate (GDP[S]). Adenosine 5′-[βγ-imido]triphosphate (App[NH]p) was also stimulatory, and was more potent than ATP in the parotid granules, but less potent in the pancreatic granules. Aluminium fluoride stimulated Cl^- conductance maximally at 15–30 μM-Al^{3+} and 10–15 mM-F^-. F^- was less effective at higher concentrations. Protein phosphorylation by kinases was apparently not involved, since the nucleotide effects (1) could be mimicked by non-hydrolysable analogues of ATP and GTP, (2) showed reversibility, and (3) were not abolished by the protein kinase inhibitors 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) or staurosporine. The data suggest the presence of at least two binding sites for nucleotides, whereby occupancy of one induces inhibition and occupancy of the other induces stimulation.

INTRODUCTION

Secretion of fluid and electrolytes by epithelia primarily involves transcellular transport of Cl^- which is electrogenic and energy-dependent. The current cellular model for Cl^- secretion in epithelia is based on electrophysiological and transport studies [1]. It postulates the following sequence of events: Cl^- entry into the cell against an electrochemical gradient mediated by an electroneutral, loop diuretic-sensitive $Na^+/K^+/2Cl^-$ cotransporter at the basolateral plasma membrane; Cl^- exit down an electrochemical gradient via a Cl^- channel at the luminal plasma membrane; and electroneutrality through the basolateral exit of K^+ and Na^+ and the paracellular diffusion of Na^+. Cl^- secretion is a regulated process [2]. It is stimulated by secretagogues and intracellular messengers which activate both K^+ conductance at the basolateral plasma membrane and luminal Cl^- conductance. The presence of a regulated apical Cl^- channel has been established in several secretory epithelia [3]. However, the transport processes involved in Cl^- secretion and its regulation in exocrine pancreas and parotid gland are less well understood.

Another aspect of secretion concerns exocytosis of macromolecular products. Exocytosis occurs as the result of a 'fusion–fission' reaction between secretory granules and the apical membrane of secretory cells [4]. It is also a regulated process, stimulated by hormones and intracellular messengers [5].

Recently, we have isolated osmotically stable secretory granules from different epithelia [6] and studied ion transporters present in the membranes of these granules. We have observed Cl^- conductance, a Cl^-/anion exchanger and K^+ conductance [7,8]. Moreover, we showed that Cl^- transport across the granular membrane is activated by pretreatment *in vivo* with secretagogues [9]. We postulated that the Cl^- conductance present in the granular membrane is inserted into the luminal membrane during exocytosis and contributes to the increase in overall cellular Cl^- conductance, measured using electrophysiological techniques, upon hormonal stimulation [10], as well as 'flushing out' of macromolecular exocrine products during exocytosis.

The isolated granules provide a convenient experimental system with which to study the regulation of ion transporters. In the present study, we show that the Cl^- conductance present in the membrane of pancreatic and parotid zymogen granules is modulated by different cytoplasmic nucleotides and their non-hydrolysable analogues.

MATERIALS AND METHODS

Materials

ATP, CTP, UTP, ITP, GTP, adenosine 5′[βγ-methylene]triphosphate (App[CH_2]p), adenosine 5′-[βγ-imido]-triphosphate (App[NH]p) (all Na^+ salts), phenylmethanesulphonyl fluoride (PMSF), EGTA, BSA (98–99% albumin, essentially fatty-acid free), imidazole, valinomycin, nonactin, gramicidin (Dubos), Percoll and sucrose were from Sigma (St. Louis, MO, U.S.A.). Mes, Mops, Hepes and Tris were purchased from Research Organics, Inc. (Cleveland, OH, U.S.A.). Adenosine 5′-[γ-thio]triphosphate (ATP[S]) was from Calbiochem (San Diego, CA, U.S.A.). Guanosine 5′-[γ-thio]-triphosphate (GTP[S]) and guanosine 5′-[β-thio]diphosphate (GDP[S]) were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). All other chemicals were from common commercial sources and were of the highest analytical grade available.

Abbreviations used: App[CH_2]p, adenosine 5′-[βγ-methylene]triphosphate; App[NH]p, adenosine 5′-[βγ-imido]triphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; G-protein, guanine-nucleotide-binding regulatory protein; GTP[S], guanosine 5′-[γ-thio]triphosphate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; NBF, nucleotide-binding fold; PMSF, phenylmethanesulphonyl fluoride.

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Isolation of zymogen granules

Zymogen granules were isolated from the pancreas or parotid gland of male Sprague–Dawley rats (150–250 g; Zivic Miller Laboratories Inc., Allison Park, PA, U.S.A.), as described earlier [8,9,11]. Briefly, rats (fed *ad libitum*) were ether-anaesthetized and killed by cervical dislocation. The pancreas and/or both parotid glands were quickly removed into ice-cold homogenization buffer containing 250 mM-sucrose, 50 mM-Mops, 0.1 mM-$MgSO_4$, 0.1 mM-EGTA (about 100 nM free Ca^{2+}), 1 mg of defatted BSA/ml and 0.2 mM-PMSF (added from a 100 mM stock solution in anhydrous dimethyl sulphoxide immediately before homogenization), and the pH was adjusted to 7.0 by titration with NaOH. Exocrine glands were freed from fat and connective tissues, crudely minced with scissors and ground to small pieces by 10 strokes at 500 rev./min using a motor-driven Potter–Elvehjem glass/Teflon homogenizer. Tissue was disrupted by rapid decompression of a pressure bomb (45 ml internal volume; Parr Instrument Co., Moline, IL, U.S.A.) filled with an inert gas, such as argon or nitrogen, at 3450 kPa (500 p.s.i.) for 1 min. This procedure disrupts all cells, but releases zymogen granules intact, as judged by phase-contrast microscopy. The resulting homogenate was supplemented with Percoll-containing buffer to a final concentration of 40% Percoll (v/v), 250 mM-sucrose, 50 mM-Mes, 25 mM Mops (carry-over from homogenization buffer), 2 mM-EGTA, 0.2 mM-$MgSO_4$, 1 mg of BSA/ml, and 0.2 mM-PMSF, and the pH was adjusted to pH 6.5 with NaOH. Zymogen granules were isolated on a continuous Percoll gradient which was formed by centrifugation at 20000 *g* for 20 min in a Sorvall SS 90 vertical rotor in a Sorvall RC-5B centrifuge (both from du Pont Instruments, Newtown, CT, U.S.A.). The pancreatic or parotid zymogen granules formed a distinct dense band at the bottom of the gradient (density 1.13–1.15 g/ml) which was collected for further studies.

Measurement of granular Cl^- permeability

The Cl^- permeability characteristics of zymogen granules isolated from pancreatic and parotid glands were assayed as described in detail previously [11]. Zymogen granules were suspended in buffered iso-osmotic KCl, NaCl or LiCl solutions and osmotic lysis was started by addition of the electrogenic cation ionophores valinomycin (with KCl), nonactin (with NaCl) or gramicidin (with LiCl). The kinetics of lysis were monitored by measuring the time-dependent changes in light scatter (absorbance) of the granule suspension at 540 nm. It has previously been shown that the granular concentration is linearly related to the absorbance [7]. Typically, 50–70 μg of granules, corresponding to an initial absorbance of 0.3–0.4, was used for experiments. The Cl^- conductance was identified by selectively and maximally permeabilizing the granular membrane for the major cation in the buffer (K^+, Na^+ or Li^+) with an appropriate concentration of ionophore. Under these conditions the endogenous Cl^- permeability becomes rate-limiting for gradient-driven influx of salt and water, resulting in granular swelling and lysis. Purified granules (30–60 μl) were added to 3 ml of a standard buffer used for measurements of Cl^- conductance in light-scatter experiments in a cuvette containing 1 mM-EGTA, 0.1 mM-$MgSO_4$ and 50 mM-imidazole (titrated to pH 7.0 with acetic acid) or 20 mM-Hepes (titrated to pH 7.0 with KOH), and 150 mM of the K^+, Na^+ or Li^+ chloride salt. After 2–3 min of incubation, cation ionophores were added from a stock solution of 95% ethanol to a final concentration of 1 μM-valinomycin, -nonactin or -gramicidin and 0.5% (v/v) ethanol. Absorbance was measured continuously for 15–30 min at 37 °C using a Beckman model DU-50 spectrophotometer equipped with a Peltier constant-temperature chamber, an automatic 6-unit sampler and a kinetics Soft-Pak module. Data were stored and analysed using a Symphony spreadsheet program (Lotus Development Corp., Cambridge, MA, U.S.A.).

Analysis of transport data and statistics

Cl^- conductance was quantified by measuring the decrease in absorbance over time for at least 15 min (parotid) or 25 min (pancreas), starting with addition of an electrogenic ionophore. Experiments with permeant SCN^- salts served as controls to give complete lysis and the maximal decrease in absorbance, usually to 5–10% of the initial value. The half-time of lysis (time of half-maximal decrease in absorbance) was estimated from the slope of the absorbance change with time between ionophore addition and either the experimental half-time or the entire observation period if the half-time was not reached. The slope was calculated by linear regression of the digitized data.

The reproducibility of transport measurements on samples from the same granule preparation was within 5%. All experiments were repeated at least three times with different granule preparations and means ± S.D. were calculated. If Figures show single experiments, they are representative of at least three experiments with qualitatively similar results. Data were analysed using the Statsgraphics program (STSC, Inc., Rockville, MD, U.S.A.), using paired Student's *t* test, or correlation analysis. Significance was considered at levels of $P < 0.05$.

RESULTS

Characteristics of granular Cl^- conductance

Zymogen granules isolated from exocrine pancreas and parotid cells according to the procedure given in the Materials and methods section were osmotically stable in KCl buffer, with a half-life of over 4 h [e.g. for parotid see Fig. 1 (control trace); pancreatic granules behaved identically (results not shown)]. Addition of the electrogenic K^+ ionophore valinomycin resulted in granule lysis (Fig. 1). This behaviour is explained as follows. The artificial K^+ conductance induced by valinomycin and the endogenous Cl^- conductance permit influx of KCl down its chemical gradient; water follows, resulting in swelling and osmotic lysis of granules. As the artificial valinomycin-induced K^+ conductance is high, the endogenous Cl^- conductance present in the membrane becomes rate-limiting for KCl influx and subsequent lysis of granules.

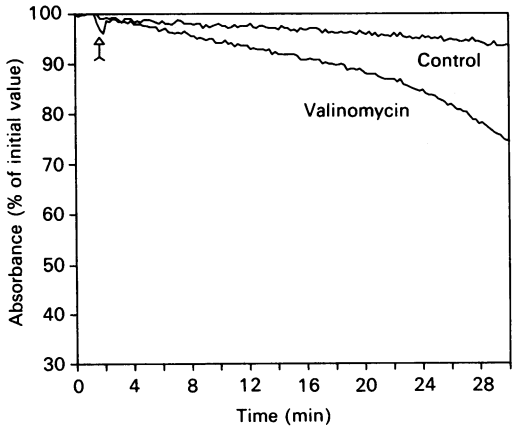

Fig. 1. Osmotic lysis of parotid zymogen granules induced by valinomycin.

Granules were suspended in a buffered KCl solution at 37 °C in a cuvette. In the control experiment no valinomycin was added and the granules remained osmotically stable. No significant absorbance changes (λ = 540 nm) were observed. Addition of 1 μM-valinomycin to the granules (arrow) resulted in significant granular lysis.

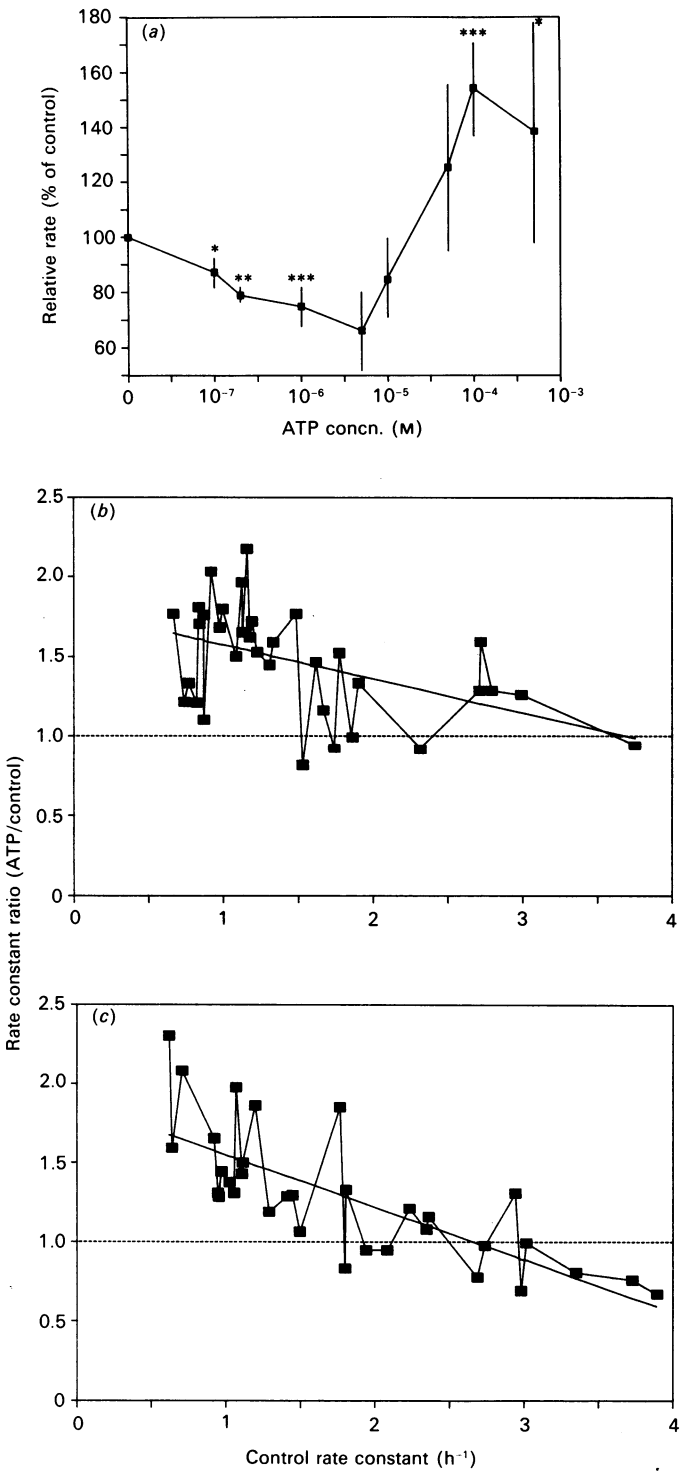

Fig. 2. Effect of ATP of Cl⁻ conductance of pancreatic (*a*,*b*) and parotid (*c*) zymogen granules

(*a*) Relative rates (inverse half-times) of valinomycin-induced lysis (see Fig. 1) are expressed as percentages of control values without adenine nucleotides. A value of 100% equals 1.54 ± 0.67 h^{-1} (mean ± S.D. of 12 experiments and preparations). Granules were suspended in iso-osmotic KCl buffer plus 5 mM-$MgCl_2$. $^*P < 0.025$; $^{**}P < 0.01$; $^{***}P < 0.001$, using Student's t test for one-sample analysis of paired values. (*b*,*c*) The ratio of relative rate constants with and without 100 μM-ATP was plotted as a function of rate constants without ATP. The correlation coefficient r was determined by correlation analysis, and its significance level against the null hypothesis based on the Student's t distribution was calculated. (*b*) $y = -0.214x + 1.787$; $r = 0.476$; $n = 34$; $P < 0.01$. (*c*) $y = -0.329x + 1.875$; $r = 0.751$; $n = 33$; $P < 0.01$.

The endogenous Cl⁻ conductance was quantified by the rate of change in absorbance of a granular suspension in the presence of valinomycin. The relative rate constants (measured as inverse half-life) of pancreatic granules in the presence of valinomycin ranged between 0.62 and 4.58 h^{-1}, with a mean value (± S.D.) of 1.72 ± 0.87 h^{-1} ($n = 65$). This variability in Cl⁻ conductance had also been observed in previous studies and is attributed to variations in the secretory state of animals at the time of death ('semi-activated state') [9]. The range of Cl⁻ conductance of parotid granules yielded relative lysis rate constants between 0.61 and 4.41 h^{-1} (mean ± S.D. 1.74 ± 1.11 h^{-1}; $n = 42$).

It is noteworthy that most of the experiments with pancreatic zymogen granules were performed in imidazole/acetate buffer, whereas the buffer used for parotid granules was Hepes. Imidazole/acetate permeates membranes better than Hepes and rapidly equilibrates intragranular with extragranular pH. Though a pH gradient seems to affect the activity of zymogen granule ion transporters [8], the effects of nucleotides on Cl⁻ conductance described in this study were independent of the presence or absence of a pH gradient.

Effect of ATP on Cl⁻ conductance

Based on earlier evidence indicating that secretagogues and thus intracellular signalling pathways stimulate the Cl⁻ conductance of isolated pancreatic granules, we directly investigated the effect of cyclic AMP-dependent protein kinase on pancreatic and parotid Cl⁻ conductance. In these experiments, we observed that 2 mM-ATP also seemed to stimulate Cl⁻ conductance independently from added cyclicAMP-dependent protein kinase (see Fig. 3*a*). A more detailed study of the ATP effects over a wide range of concentrations (0.1–500 μM) revealed that low ATP concentrations decreased and high concentrations increased Cl⁻ conductance. The results with pancreatic granules are summarized in Fig. 2(*a*) in terms of changes in relative lysis rate constants. Maximal inhibitory effects were observed between 1 and 5 μM-ATP; in contrast, maximal stimulation was at 100 μM-ATP. On average, Cl⁻ conductance was decreased by about 25% at 1 μM-ATP and was increased by 55% at 100 μM-ATP. This dual modulation of Cl⁻ conductance was consistently observed in 12 different preparations, although the magnitude varied. Qualitatively similar observations were obtained with parotid gland granules (Fig. 3*b* and Table 1). This dual effect of ATP on Cl⁻ conductance indicates the presence of stimulatory and inhibitory adenine nucleotide binding sites on proteins associated with Cl⁻ conductance.

To explain the quantitative variability of the stimulatory ATP effect, which may result from differences in the activation state of granules [9], the effect of 100 μM-ATP on Cl⁻ conductance was correlated with the baseline Cl⁻ conductance (in the absence of ATP). Figs. 2(*b*) and 2(*c*) show that for both pancreatic and parotid granules the change in Cl⁻ conductance induced by 100 μM-ATP depended on the activation state of the granules; less-activated Cl⁻ transporters were stimulated to a larger extent by ATP, whereas when granular Cl⁻ conductance was high, 100 μM-ATP had no effect or was even inhibitory (see Fig. 2*c*).

Effects of non-hydrolysable ATP analogues on Cl⁻ conductance

Since membrane-bound endogenous protein kinases might use ATP as a substrate to phosphorylate the Cl⁻ transporter, we tested the effects of different non-hydrolysable ATP analogues on Cl⁻ conductance in both granule preparations [12]. Figs. 3(*a*) and 3(*b*) show actual lysis curves with the different ATP analogues used at maximal effective concentrations, and Table 1 provides a statistical overview of these types of experiments. ATP[S] lowered Cl⁻ conductance, similar to the effects of low (micromolar) concentrations of ATP. In contrast, App[NH]p and App[CH_2]p

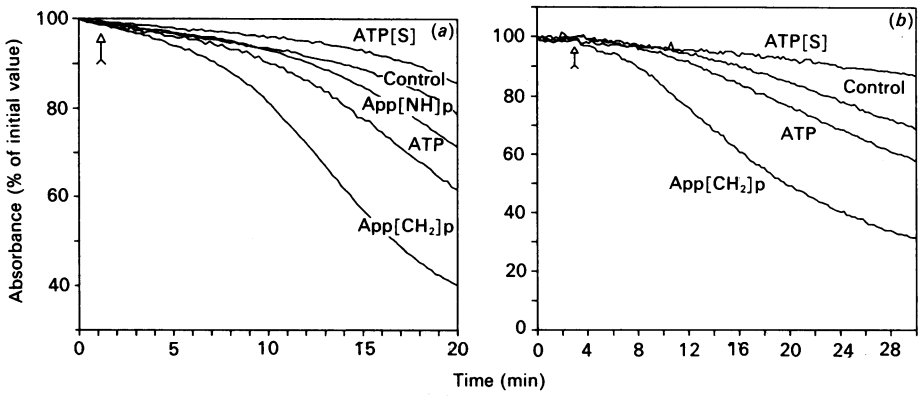

Fig. 3. Effect of adenine nucleotides on Cl^- conductance of zymogen granules from pancreas (*a*) or parotid gland (*b*)

ATP (100 μM) or its non-hydrolysable analogues ATP[S] (10 μM), App[CH_2]p or App[NH]p (both 100 μM) were added to a buffered KCl solution which also contained 5 mM-$MgCl_2$. Addition of 1 μM-valinomycin (arrow) initiated KCl influx and osmotic lysis. These results are typical for three different preparations.

Table 1. Effects of adenine nucleotides on pancreatic and parotid Cl^- conductance

Values are means ± S.D. from *n* pancreatic and parotid zymogen granule preparations. A 100% value corresponds to a relative rate constant of 1.74 ± 1.11 h^{-1} for parotid and 1.48 ± 0.64 h^{-1} for pancreatic granules. Unless otherwise indicated, nucleotides were tested at a concentration of 100 μM in a standard KCl incubation buffer containing 5 mM-$MgCl_2$. *$P < 0.02$; **$P < 0.005$; ***$P < 0.001$, using Student's *t* test for one-sample analysis of paired values.

	Relative rate (% of control)		
Nucleotide	Pancreas	Parotid	*n*
Control	100	100	42/47
ATP (1 μM)	75 ± 7***	69 ± 10***	7/5
ATP[S]	76 ± 16**	62 ± 12*	11/4
ATP	142 ± 23***	154 ± 40**	12/11
App[NH]p	120 ± 14	210 ± 60***	5/21
App[CH_2]p	221 ± 62**	435 ± 113**	8/6

increased Cl^- conductance, similar to the effects of high concentrations of ATP (see Fig. 2).

The maximally effective concentrations of different ATP analogues were investigated by varying their concentration over two orders of magnitude. Fig. 4(*a*) shows the results for three different concentrations of ATP[S], App[NH]p and App[CH_2]p (1, 10 and 100 μM) in pancreatic granules, and Fig. 4 (*b*) shows the same protocol for parotid granules. Of all ATP analogues tested, ATP[S] was the most effective in lowering Cl^- conductance, with a maximal effect of about a 1.6-fold decrease in lysis rate at 1 μM-ATP[S]. The ATP[S] dose–response curve resembled that of ATP, in that higher concentrations (above 1 μM) were less inhibitory than the optimal one. Both of the non-hydrolysable ATP analogues App[CH_2]p and App[NH]p [12] increased Cl^- conductance in a concentration-dependent manner, the effects being maximal at 100 μM (Figs. 3*a*, 3*b*, 4*a* and 4*b*). Interestingly, App[CH_2]p was much more effective than App[NH]p in pancreatic zymogen granules (Fig. 4*a*), whereas these compounds were nearly equally effective in the parotid gland (Fig. 4*b*). The reason for this difference remains unclear. The results with non-hydrolysable ATP analogues rule out the possibility that the stimulatory nucleotide effects are mediated by a kinase and phosphate transfer to the transporter.

Another argument for the absence of covalent modification is based on the finding that the stimulatory or inhibitory effects were reversible. When pancreatic or parotid granules were preincubated as a concentrated suspension with 1 μM-ATP[S] or 100 μM-App[CH_2]p and subsequently diluted about 100-fold in incubation buffer, no changes in lysis rate relative to controls (without nucleotides during preincubation) were observed (results not shown). Similar experiments with ATP were consistent with reversibility, but their interpretation was complicated by changes in rates due to the inhibitory effect of residual ATP after dilution.

Furthermore, in three experiments, the protein kinase C inhibitors staurosporine (30–100 nM) [13] and H-7 (50–300 μM) [14] were tested with parotid and pancreatic granules. Their presence did not interfere with the stimulatory effects of 100 μM-ATP.

Effect of other trinucleotides on Cl^- conductance

We have also investigated the effects of other nucleotides, such as GTP, CTP, UTP and ITP, at concentrations between 0.1 and 100 μM. All of these nucleotides affected Cl^- conductance in both granular preparations. Compared with ATP, the pattern of modulation was rather uniform; all nucleotides decreased Cl^- conductance dose-dependently with a maximal decrease of 2-fold. The effects of 100 μM concentrations of these nucleotides on both zymogen granule preparations are illustrated in Fig. 5. Whereas ATP increased Cl^- conductance, GTP, UTP, CTP and ITP (the latter tested only in pancreas) decreased it. The results may indicate the involvement of a G-protein, since GTP can be replaced by other nucleotides, such as ITP and UTP, in activating G-protein coupling of β-adrenergic receptors to adenylate cyclase [15].

Effects of non-hydrolysable GTP analogues on Cl^- conductance

The effects of GTP on GTP-binding proteins can also be mimicked by poorly hydrolysable analogues, such as GTP[S] or by aluminum fluoride, and inhibited by GDP[S] [16]. We therefore tested whether some of the effects observed were mediated by activation of inhibitory or stimulatory G-proteins. Fig. 6 shows the effects of different concentrations of GTP, GTP[S] and GDP[S] in the presence of 5 mM-$MgCl_2$ on Cl^- conductance in exocrine pancreas. GTP[S] decreased Cl^- conductance at least as well as GTP, whereas GDP[S] definitely had no such effect. Significant inhibitory effects were already observed at the low concentration of 1 μM. GTP and GTP[S] at 0.5 mM decreased Cl^-

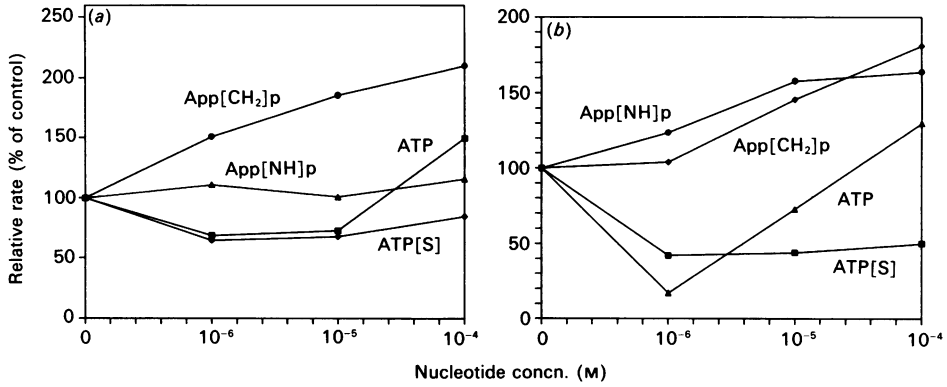

Fig. 4. Effect of adenine nucleotide concentration on Cl⁻ conductance of pancreatic (*a*) and parotid zymogen granules (*b*)

Rates of granular lysis were measured in the absence (control) and presence of adenine nucleotides as shown in Fig. 3. Control rate constants (100%) were 1.48 h^{-1} for pancreatic and 1.15 h^{-1} for parotid zymogen granules. The results are typical for three parotid and five pancreatic granular preparations.

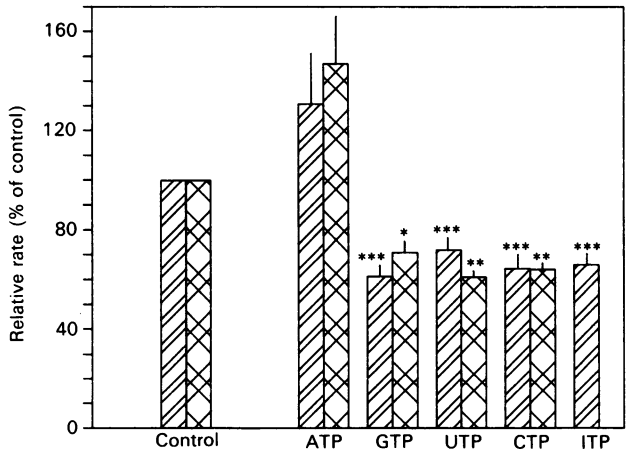

Fig. 5. Effect of trinucleotides on Cl⁻ conductance of pancreatic and parotid zymogen granules

Trinucleotides were tested at 100 μM in pancreatic (▨) or parotid (⊠) zymogen granules and the rates of granular lysis were compared with controls (without nucleotides). Control rate constants (inverse half-times) were 1.74±0.48 h^{-1} for pancreatic and 1.88±0.56 h^{-1} for parotid zymogen granules (means±S.D. of five pancreatic and three parotid preparations). $*P < 0.01$; $**P < 0.005$; $***P < 0.001$, using Student's *t* test for one-sample analysis of paired values.

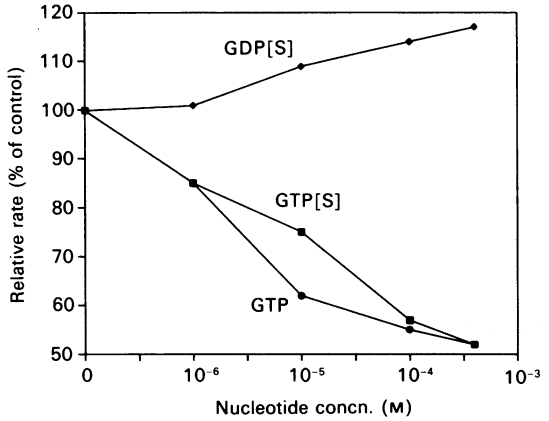

Fig. 6. Effects of GTP and non-hydrolysable analogues on Cl⁻ conductance of pancreatic zymogen granules

For GTP and GTP[S], granules were preincubated for 30 min at 37 °C in the presence of 5 mM-$MgCl_2$ before addition of valinomycin. The experiments with GDP[S] shown in this Figure were performed with 0.1 mM-$MgCl_2$, but without preincubation. A value of 100% is equivalent to a rate constant of 2.47 h^{-1}. The results are typical for four different preparations.

conductance by about 2-fold. A further decrease in Cl⁻ conductance was observed with GTP at millimolar concentrations, whereas the non-hydrolysable analogue showed a plateau at 100–500 μM (results not shown). Optimal conditions for GTP and GTP[S] were a high Mg^{2+} concentration and preincubation for 20–30 min.

The effects of GTP and GTP[S] were reversible upon dilution. When a concentrated granular suspension was first preincubated with the nucleotides and then diluted 1:100 into the transport buffer without nucleotide, the changes in Cl⁻ conductance corresponded to those seen at the final concentration of nucleotide. This result indicates a lack of involvement of covalent reactions in the mechanism of GTP action.

Effect of aluminium fluoride on Cl⁻ conductance

Fig. 7 shows the effects of varying the aluminum fluoride concentration. Aluminium fluoride stimulated Cl⁻ conductance maximally at 15–30 μM-Al^{3+} and 10–15 mM-F^-. Fluoride was less potent at higher concentrations, probably because the active species is replaced by higher aluminum fluoride complexes, e.g. AlF_4^- or AlF_6^{3-}, with increasing fluoride levels [17,17a].

Effects of cations on modulation of Cl⁻ conductance by nucleotides

Inhibition or stimulation of Cl⁻ conductance by nucleotides seemed to be independent of the cation present in the incubation buffer and therefore inherent to the Cl⁻ conductance. When K^+ was replaced by Na^+ and valinomycin by the electrogenic ionophore nonactin, the same pattern of inhibitory and stimulatory effects of nucleotides and non-hydrolysable analogues on Cl⁻ conductance emerged (Fig. 8). ATP and ATP[S] (both at 1 μM) and 0.1 mM-GTP decreased Cl⁻ conductance in the presence of nonactin; ATP and App[CH_2]p (both at 0.1 mM) stimulated Cl⁻ conductance about 2-fold. This pattern matched that observed with valinomycin and K^+ as the major cation, suggesting that the nucleotides effectively modulate the Cl⁻ transporter independently from the accompanying cation. Note that equimolar concentrations of valinomycin in KCl media and nonactin in NaCl media resulted in different rate constants for Cl⁻

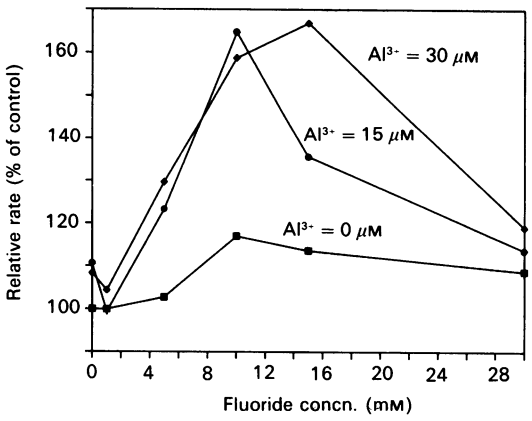

Fig. 7. Effect of aluminium fluoride on Cl^- conductance of pancreatic zymogen granules

Pancreatic zymogen granules were incubated at three different fixed $AlCl_3$ concentrations and various F^- concentrations. For other experimental conditions, see the legend to Fig. 1. Valinomycin-dependent lysis rates were compared with the control rates without aluminium fluoride. Control Cl^- conductance was characterized by a relative rate constant of 0.78 h^{-1}. The results are typical for seven different preparations.

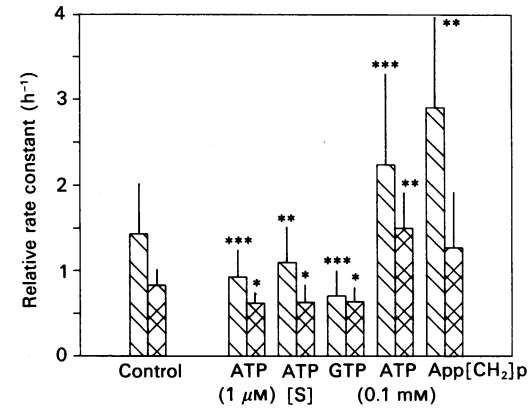

Fig. 8. Comparison of nucleotide effects on Cl^- conductance of pancreatic zymogen granules in the presence of K^+/valinomycin or Na^+/nonactin

The effect of 1 μM-valinomycin (▨) on granular lysis in a 150 mM-KCl buffer was compared with the effects of 1 μM-nonactin (▩) in 150 mM-NaCl buffer. Control rates were measured in the absence of nucleotides. Nucleotide concentrations were, 1 μM- and 100 μMATP, 10 μM-ATP[S], 100 μM-GTP and 100 μM-App[CH_2]p. Results are means ± S.D. of three parotid and six pancreatic preparations. $*P < 0.02$; $**P < 0.005$; $***P < 0.001$ using Student's t test for one-sample analysis of paired values.

conductance in the controls (Fig. 8). This difference could be explained by a lower transport rate of Na^+ via nonactin and hence a lower quasi-steady-state electrical potential driving Cl^- influx [18].

In addition to nonactin and valinomycin, both of which operate as cation carriers, we also tested the channel-forming quasi-ionophore gramicidin (1 μM), with Li^+ as cation. App[CH_2]p and ATP (both at 100 μM) stimulated pancreatic Cl^- conductance (results not shown), as already demonstrated with both valinomycin and nonactin. However, GTP, ATP[S] and low ATP concentrations, which consistently decreased Cl^- conductance in the presence of valinomycin or nonactin (Fig. 8), showed variable effects in both pancreatic and parotid granules (results not shown). The reason for this variability is not understood, but could result from interactions of gramicidin with endogenous membrane transporters.

DISCUSSION

According to current understanding, hormonal stimulation of fluid secretion by secretory epithelia is accompanied by activation of a luminal Cl^- channel [1]. Two major intracellular pathways have been implicated in the regulation of this Cl^- channel: the cyclic nucleotide pathway [19] and the phosphatidylinositol messenger system, which induces elevation of cytosolic [Ca^{2+}] as well as production of diacylglycerol [20,21]. These intracellular signals activate specific protein kinases, i.e. cyclicAMP-dependent protein kinase and Ca^{2+}/phospholipid-dependent protein kinase. Phosphorylation of an associated protein is thought to open the Cl^- channel.

These regulatory mechanisms have been demonstrated in various epithelia and directly at the single channel level by the patch–clamp technique in cell-excised patches of human trachea [22,23]. In other secretory epithelia, such as the lacrimal gland [24], more indirect evidence from whole-cell patch-clamps points to the involvement of the phosphatidylinositol messenger system and the cyclicAMP messenger system. Both regulatory mechanisms are also thought to be operative in the exocrine pancreas and the parotid gland [9,25].

The mechanisms of apical Cl^- channel regulation have recently become a major focus of research in an attempt to elucidate and bypass the cellular defect associated with cystic fibrosis. A mutation of a single gene is probably responsible for the defective regulation of epithelial apical Cl^- channels which underlies most clinical manifestations of the disease, including pancreatic insufficiency [26]. Recent evidence suggests that Cl^- channel phosphorylation by both cyclicAMP-dependent protein kinase and protein kinase C [22,23] are defective in cystic fibrosis.

The results of the present study indicate that normal cytoplasmic nucleotides also exert inhibitory and stimulatory effects on Cl^- conductance. These effects of ATP cannot simply be explained by phosphorylation of transporter-associated proteins even though endogenous protein kinases are present in granule membranes [27,28].

The arguments against phosphorylation underlying the inhibitory effects at micromolar ATP concentrations can be summarized as follows. First, the ATP concentration of 1 μM is well below the K_M of protein kinases for ATP of about 10 μM [29]. Secondly, dilution experiments, performed by preincubating granules with effective inhibitory concentrations of ATP, ATP[S] or other nucleotides and subsequent dilution in a buffer without nucleotide, showed that the effect of nucleotides was reversible. Thirdly, ATP[S] at 10 μM (Fig. 3*a*), as well as the nucleotides GTP, UTP, ITP and CTP at 100 μM (Fig. 5), mimicked the effect of low ATP concentrations on Cl^- conductance, although these substances are generally poor substrates of protein kinases [29,30]. ATP[S] can be used by some kinases; however, in this case one would expect the inhibition to be irreversible, since the thio-phosphoprotein product is not easily hydrolysed by phosphatases.

Several arguments rule out phosphorylation as the basis for the stimulatory effects of ATP at concentrations above 50 μM, although these concentrations are suitable for protein kinase C [31] and cyclicAMP-dependent protein kinase [29]. First, the ATP analogues App[CH_2]p and App[NH]p, which are not substrates of kinases [12], also increased Cl^- conductance in a concentration-dependent manner (Fig. 4). Secondly, dilution experiments with ATP and these analogues demonstrated the reversibility of the stimulation. Thirdly, staurosporine, a relatively specific inhibitor of protein kinase C [13], and the less

specific compound H-7 [14] had no effect on the increased Cl^- conductance induced by 100 μM-ATP in parotid zymogen granules. However, ATP metabolism may play a role in the dependence of the stimulatory effect of ATP on basal Cl^- conductance. Whereas the stimulation by ATP was much more pronounced when the basal Cl^- conductance was low (Figs. 2*b* and 2*c*), the stimulation by the non-hydrolysable analogue App[CH_2]p was not dependent on basal Cl^- conductance (results not shown).

One way to explain the current data is to postulate the presence of two nucleotide-binding sites, i.e. one inhibitory and one stimulatory binding site, which modulate the activity of Cl^- conductance. The inhibitory site would have a relatively high affinity for ATP[S] and ATP, based on an inhibitory potency (inverse of concentration giving half-maximal inhibition) of different nucleotides, with ATP > ATP[S] > UTP $\geqslant$ CTP = GTP = GTP[S] = ITP. Furthermore, in 'pre-activated' granules with higher Cl^- conductance, occupation of the inhibitory binding site results in a greater effect than in granules with low Cl^- conductance. The stimulatory site requires higher concentrations of ATP than the inhibitory one and also accepts App[CH_2]p and App[NH]p. The stimulatory potency of nucleotides is App[CH_2]p > App[NH]p(parotid) > ATP > App[NH]p(pancreas) > GDP[S]. Reversible binding of nucleotides to either site could induce conformational changes of the ion transporter and thus result in altered Cl^- transport. There is a precedent for this postulated mechanism of action of nucleotides (see below).

At this point, one can only speculate about the physiological importance of the regulatory ATP effects in the cell. They may be useful in coupling secretion to the metabolic state of the cell, promoting secretion at normal concentrations, but having an inhibitory action when ATP levels drop to precariously low values. Alternatively, one can invoke compartmentation, as recently suggested for cyclicAMP in parotid secretion [32] or for adenine nucleotides in inositol 1,4,5-triphosphate-mediated Ca^{2+} flux [33].

The recent identification of the CFTR (cystic fibrosis transmembrane conductance regulator) gene highlights the significance of our observations. A characteristic feature of the predicted protein structure of the gene product is the presence of two sequences resembling consensus nucleotide-binding folds (NBFs) which are distinct from cyclicAMP-dependent protein kinase phosphorylation sites [34]. Omission of a phenylalanine residue at one of these NBFs was observed in cystic fibrosis patients.

This pattern of two NBFs in addition to phosphorylation sites is shared by other membrane-associated proteins involved in the transport of ions or small molecular substances, such as the mammalian multidrug resistance P-glycoprotein [35]. This protein is a target for cyclicAMP-dependent protein kinase [36] and protein kinase C [37], and possesses ATP-, GTP- and App[NH]p-binding sites [38]. The exact role of ATP binding is not understood, although ATP hydrolysis is probably required for transport [39].

The presence of both ATP-binding and phosphorylation sites which modulate ion transporter activity has also been demonstrated for K^+ channels; intracellular ATP inhibits certain types of K^+ channels by decreasing the channel open-probability. The system is best characterized in pancreatic β-cells [40] and cardiac cells [41]. ATP is effective at concentrations varying between 20 and 200 μM and is not hydrolysed, since it can be replaced by equimolar concentrations of App[CH_2]p and App[NH]p [40,42]. However, phosphorylation by protein kinase C activates these K^+ channels [43].

The effects of GTP, GTP[S] and aluminium fluoride (see Figs. 5–7) could also be explained by stimulatory and inhibitory G-proteins being involved in regulating Cl^- channel activity. The nucleotide-gated ion channel model does not exclude involvement of G-proteins. G-proteins could form part of a signalling pathway (sequential model), or could influence transport activity independently (parallel model). Recent studies in permeabilized mast cells [44] and HL-60 cells [45] suggest that (a) G-protein(s), which is termed G_E (for exocytosis), operates at a step distal from the plasma membrane in stimulus–secretion coupling. Both inhibitory and stimulatory effects of guanine nucleotides on secretion have been observed, suggesting the presence of several G-proteins [46]. ATP, XTP and ITP mimicked the effects of GTP and GTP[S] [45]. These G-proteins could be localized in the granular membrane and could modulate gating of the Cl^- transporter.

In summary, our data show that cytosolic nucleotides can substantially increase or decrease Cl^- conductance from isolated zymogen granules, depending on the nature of the nucleotide and its concentration, and also on the activation state of the granules. Maximally, Cl^- conductance can be modulated by up to an order of magnitude (inhibition, 2-fold; stimulation, 5-fold). The similarity of the observations in pancreas and parotid gland indicates that a general regulatory principle is operative. This process can be mimicked by non-hydrolysable analogues of ATP and GTP and apparently does not involve protein phosphorylation by kinases. Other mechanisms for regulation of Cl^- transporters, such as receptors or binding sites for nucleotides and/or G-proteins, must therefore be considered.

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