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Extracellular ATP increases free cytosolic calcium in rat parotid acinar cells

Differences from phospholipase C-linked receptor agonists

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The effects of extracellular ATP on intracellular free calcium concentration ($[Ca^{2+}]_i$), phosphatidylinositol (PtdIns) turnover, amylase release and Ca^{2+}-activated membrane currents were examined in isolated rat parotid acinar cells and contrasted with the effects of receptor agonists known to activate phospholipase C. ATP was more effective than muscarinic and α-adrenergic agonists and substance P as a stimulus for elevating $[Ca^{2+}]_i$ (as measured with quin2). The ATP effect was selectively antagonized by pretreating parotid cells with the impermeant anion-exchange blocker 4,4′-di-isothiocyano-2,2′-stilbenedisulphonate (DIDS), which also inhibited binding of [α-^{32}P]ATP to parotid cells. By elevating $[Ca^{2+}]_i$, ATP and the muscarinic agonist carbachol both activated Ca^{2+}-sensitive membrane currents, which were measured by whole-cell and cell-attached patch-clamp recordings. However, there were marked contrasts between the effects of ATP and the receptor agonists linked to phospholipase C, as follows. (1) Although the combination of maximally effective concentrations of carbachol, substance P and phenylephrine had no greater effect on $[Ca^{2+}]_i$ than did carbachol alone, there was some additivity between maximal ATP and carbachol effects. (2) Intracellular dialysis with guanosine 5′-[β-thio]diphosphate did not block activation of ion channels by ATP, but did block channel activation by the muscarinic agonist carbachol. This suggests that a G-protein is involved in the muscarinic response, but not in the response to ATP. (3) Despite its pronounced effect on $[Ca^{2+}]_i$, ATP had little effect on PtdIns turnover in these cells, in contrast with the effects of carbachol and other Ca^{2+}-mobilizing agents. (4) Although ATP was able to stimulate amylase release from parotid acinar cells, the stimulation was only $33 \pm 9\%$ of that obtained with phospholipase C-linked receptor agonists. These differences suggest that ATP increases $[Ca^{2+}]_i$ through specific activation of a pathway which is distinct from that shared by the classical phospholipase C-linked receptor agonists.

INTRODUCTION

ATP is stored and co-secreted with many neurotransmitters and, similarly to its metabolite adenosine, may act as a neuromodulator [1–3]. The effects of extracellular ATP on cells appear to be mediated through specific P_2-type purinergic receptors and ectokinases, and are pharmacologically distinct from effects mediated by adenosine (via P_1-type receptors) [3,4]. Although a large number of cell types respond to extracellular ATP, the potency and effectiveness of ATP and its analogues vary widely among the systems studied [4,5]. This complexity of responses, coupled with a poorly developed P_2-purinergic pharmacology, has slowed understanding and acceptance of possible physiological roles for ATP. Different subtypes of P_2 purinergic receptors undoubtedly contribute to the differences in observed responses to ATP [4,5]. For example, ATP acts directly to contract smooth muscle and indirectly, through release of endothelia-derived relaxation factor, to relax smooth muscle in dog blood vessels; contraction and relaxation responses are selectively evoked with different ATP analogues [6].

Even in hepatocyte preparations, a well-studied model system offering a large, relatively homogeneous, population of cells, there appear to be multiple types of purinergic receptors. Extracellular ATP has been shown to increase the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in hepatocytes and to activate Ca^{2+}-dependent ion fluxes and metabolic processes [7–11]. These effects of ATP in liver cells are complicated by actions of its metabolite adenosine, which activates hepatic adenylate cyclase through P_1 purinergic receptors [9,11,12]. P_2-type hepatic responses to ATP have been extensively characterized, however. ATP potently (half-maximal effects at about 1 μM) elevates $[Ca^{2+}]_i$ and stimulates glucose production [10,11]. Among other analogues, ADP and adenosine 5′-[γ-thio]triphosphate are typically almost as potent and effective as ATP, but methylene derivatives of ATP are nearly ineffective at comparable concentrations [9–12]. In common with other Ca^{2+}-mobilizing agonists in this system, such as phenylephrine, vasopressin and angiotensin II, ATP stimulates phosphatidylinositol (PtdIns) breakdown [11,12]. There also is evidence that hepatic P_2-type purinergic receptors are coupled through guanine-nucleotide-binding proteins to other second-

Abbreviations used: $[Ca^{2+}]_i$, intracellular free calcium concentration; PtdIns, phosphatidylinositol; DIDS, 4,4′-di-isothiocyano-2,2′-stilbenedisulphonate; GDP[S], guanosine 5′-[β-thio]diphosphate; PBS, Dulbecco's phosphate-buffered saline containing glucose; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; InsP_2, InsP_3, InsP_4, inositol bis-, tris- and tetrakis-phosphate respectively.

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messenger pathways, although the contributions of these pathways to effects of ATP on $[Ca^{2+}]_i$ or metabolism are unclear [12,13]. At high concentrations (0.1–1 mM), ATP has been reported to be much more effective than ADP in stimulating Ca^{2+} uptake by hepatocytes [8,14], and there are characteristic differences in the effects of ATP and ADP on $[Ca^{2+}]_i$ transients in these cells [15]. It appears likely that there are at least two P_2-type purinergic receptors linked to Ca^{2+} mobilization in liver cells.

Elevation of $[Ca^{2+}]_i$ appears to be a common effect of ATP in many tissues. Since the mechanism of action of other Ca^{2+}-mobilizing agonists (muscarinic and α-adrenergic agonists and the undecapeptide substance P) is relatively well characterized in rat parotid acinar cells, this tissue is a useful system for studying the effects of extracellular ATP. Gallacher [16] originally reported that the electrophysiological response of parotid glands from mice (but not rats) to ATP was indistinguishable from that to other Ca^{2+}-mobilizing agonists. Recently we re-examined the effects of extracellular ATP in dispersed rat parotid acinar cells. We found that ATP elevates $[Ca^{2+}]_i$ in these cells in a highly specific and reversible manner consistent with the activation of a P_2-type purinergic receptor [17]. This effect on $[Ca^{2+}]_i$ was similar to that observed with carbachol, which acts via muscarinic receptors in parotid cells. Like carbachol, extracellular ATP stimulated K^+ release, which was rapidly followed by re-uptake of K^+ into the cells [17]. $[Ca^{2+}]_i$ responses to both agonists could be observed in the absence of extracellular Ca^{2+} [17]. Among ATP analogues examined, ADP had minimal effects (in contrast with responses in hepatocytes) and adenosine 5′-[γ-thio]triphosphate was the only effective agonist; this nucleotide-selectivity resembles that for ATP effects reported in certain other cell types [18–21]. In the absence of Mg^{2+}, ATP was much more potent in elevating $[Ca^{2+}]_i$, arguing against ectokinase mediation of ATP effects in the parotid [17]. An inhibitory effect of Mg^{2+} on ATP responses has been noted in other systems, and ATP^{4-} has been proposed as the actual effector molecule in these systems [18,19]. ATP is complexed with neurotransmitters rather than with Mg^{2+} in synaptic vesicles, and ATP^{4-} transiently may reach high concentrations in synapses, allowing for physiological stimulation of this P_2-type purinergic effector system and elevation of $[Ca^{2+}]_i$.

The mechanism by which ATP elevates $[Ca^{2+}]_i$ in parotid cells is unclear. Although ATP has been demonstrated to activate phospholipase C through at least one type of P_2-receptor [11,12,22], and thus elevate $[Ca^{2+}]_i$ through InsP_3 generation, there is some controversy as to the contribution of this pathway to P_2-purinergic effects on $[Ca^{2+}]_i$ in other systems [18,21,23,24]. Cockcroft & Gomperts [18] long ago noted that ATP-induced PtdIns breakdown in mast cells appeared to be the result rather than the cause of elevation of $[Ca^{2+}]_i$. Since the effects of ATP in parotid and mast cells share a similar agonist potency series and are inhibited by Mg^{2+}, the possibility was examined that ATP might elevate $[Ca^{2+}]_i$ in parotid cells independently of phospholipase C. In the present study, we have compared a number of ATP effects with those of known phospholipase C-linked receptor agonists, and the following results show that extracellular ATP acts through a pathway distinct from that shared by parotid muscarinic, α-adrenergic and substance P receptors.

EXPERIMENTAL

Dissociated acinar-cell preparation

One or two virus-free male Sprague–Dawley rats (Charles River Laboratories, Kingston, NY, U.S.A.) weighing 200–350 g were killed by cardiac puncture under chloral hydrate anaesthesia (400 mg/kg) and the parotid glands were removed to warmed oxygenated Dulbecco's phosphate-buffered saline containing 10 mM-glucose (PBS). The glands were carefully and extensively cleaned free of fat, lymph nodes, blood vessels, connective tissue and large ducts under a dissecting microscope. Dissociated cells were prepared by a modification of the method of Kanagasuntheram & Randle [25]. Glands were minced and washed with Ca^{2+}- and Mg^{2+}-free PBS containing 1 mM-EDTA, and incubated in this buffer with trypsin (3.75 mg/5 ml) for 7 min at 37 °C. The trypsin solution was then removed, and Ca^{2+}- and Mg^{2+}-free PBS containing 3.75 mg/5 ml of soya-bean trypsin inhibitor was added. The parotid fragments were centrifuged at about 100 *g* for 5 min and the pellet was resuspended in 2.5 ml of Hepes/Ringer solution (NaCl 120 mM, KCl 5 mM, $MgCl_2$ 2.2 mM, $CaCl_2$ 1.0 mM, Hepes 20 mM, β-hydroxybutyrate 5 mM, glucose 10 mM and bovine serum albumin 1.0%, pH 7.4). PBS containing 3.75 mg of collagenase (Type II; Cooper) in 2.5 ml was then added, and the parotid fragments were incubated with constant shaking (100 cycles/min) under O_2 for 40–60 min at 37 °C, and intermittently triturated with a 10 ml plastic pipette to disperse clumps. Parotid cells and small clumps were filtered through 210 μm-pore-size polyethylene mesh (Spectrum) and collected by layering over 4% bovine serum albumin in PBS (pH 7.4) and centrifuging at approx. 100 *g* for 5 min. The resulting cell pellet was washed by resuspension in Hepes/Ringer solution and centrifuged again. The yield was typically 5×10^7 cells/rat. Viability was determined (after washing in PBS to remove albumin) by Trypan Blue exclusion and was generally > 95%. Parotid cells were resuspended in oxygenated Hepes/Ringer and kept in a 25 ml flask at about 20 °C. After incubation for 20 min at 37 °C, the ATP content of these cells was 10.3 ± 0.8 nmol/mg of protein ($n = 5$ preparations), indicating that the cells are healthy [25]. Although cells were used only for 8–10 h after preparation for the present studies, amylase responses to agonists persisted for at least 24 h and quin2 responses for 48 h.

Experiments with quin2

Parotid cells were loaded with quin2 acetoxymethyl ester and $[Ca^{2+}]_i$ was measured as described previously [17]. Ester hydrolysis (loading) of quin2 was monitored by following the spectral shift of the emission wavelength from 440 nm to 492 nm. Qualitatively similar results to those presented were obtained in preliminary experiments with fura 2 acetoxymethyl ester (Molecular Probes). Maximally effective concentrations of drugs were determined in preliminary experiments by determination of cumulative concentration–response curves.

ATP binding

Parotid cells were washed twice and resuspended in PBS containing 1 mM-EDTA. These conditions minimize ecto-ATPase activity and select for ATP^{4-}, the presumed true agonist. DIDS (100 μM) or an equal volume of

dimethyl sulphoxide (final concn. 0.1%, v/v) was added, and cells were preincubated at 37 °C for 10 min. [α-^{32}P]-ATP (diluted to a final concn. of 0.1 μM- or 0.3 μM-ATP) was added to cells to start the incubation. ATP or $MgSO_4$ was added as indicated in Fig. 4. Samples (100 μl; about 10^6 cells and 10^6 c.p.m.) were removed at indicated times and added to Microfuge tubes containing 900 μl of ice-cold PBS layered over 400 μl of a dinonyl phthalate/silicone oil (2:3, v/v) mixture. After micro-centrifuging and pelleting cells with bound [α-^{32}P]ATP, the aqueous layer and most of the oil layer were removed by aspiration and Microfuge tips containing pellets were cut off and counted for radioactivity in a scintillation counter.

Patch-clamp recordings

All recordings were made in a Basal Medium Eagle solution (NaCl 116.4 mM, KCl 5.4 mM, $MgSO_4$ 0.8 mM, NaHepes 25 mM, glucose 5.6 mM, NaH_2PO_4 1 mM, $CaCl_2$ 1.8 mM, pH 7.4) at 21–23 °C. The patch electrodes, for both whole-cell and single-channel recordings, contained 140 mM-KCl, 1 mM-NaCl, 1 mM-EGTA and 10 mM-Hepes adjusted to pH 7.2 with KOH. Patch electrodes were pulled from borosilicate glass (W-P Instruments, New Haven, CT, U.S.A.) to an outer diameter of 1–3 μm and lightly fire-polished just before use.

Whole-cell and single-channel currents were recorded with the use of an EPC-7 patch clamp (Medical Systems Corp., Greenvale, NY, U.S.A.). Current signals were filtered at 1 kHz (8-pole Bessel filter; Frequency Devices, Haverhill, MA, U.S.A.). The current signals were digitized at 100 μs for higher-resolution traces (see Fig. 5) and at 10 ms for all other current traces.

[^{3}H]Inositol phosphate accumulation

Parotid cells were loaded with [^{3}H]inositol, washed, exposed to agonists for the times indicated, and processed as previously reported [26]. [^{3}H]Inositol phosphates were separated on Dowex 2 columns as published [27]. There was considerable variability in [^{3}H]inositol incorporation among different cell preparations ($n = 11$) when expressed per mg of protein (175000 ± 40000 c.p.m./mg of protein). In contrast, the percentage of [^{3}H]inositol (total or water-soluble) that accumulated in the phosphate fractions in response to each agonist was much more reproducible across different cell preparations, as noted by others [28]. Since water-soluble [^{3}H]inositol and its phosphorylated metabolites are co-processed in the assay (and thus subjected to the same losses), the ratios of the phosphorylated metabolites relative to total water-soluble [^{3}H]inositol were determined for each sample. Stimulation by agonists relative to basal values was then determined. For [^{3}H]inositol-release experiments, cells were pelleted after exposure to agonists for 60 min, and the percentage of [^{3}H]inositol in the supernatant relative to total water-soluble [^{3}H]inositol in the cells was determined. Basal release averaged 11 ± 5%.

Amylase and lactate dehydrogenase release

Amylase and LDH experiments were carried out 6–8 h after preparation of cells. The cells were washed three times in warmed oxygenated glucose-free Hepes/Ringer solution and then distributed to warmed Microfuge tubes containing the drugs or neurotransmitters to be examined, and incubated in a shaking water bath at 37 °C. After incubation for 60 min, the cells were pelleted in a MISCO micro-centrifuge, and a sample of supernatant was removed. For amylase experiments, pellets and supernatants were frozen and thawed, and diluted appropriately to give activity in the linear response range. Amylase activity was assayed at room temperature by the method of Bernfeld [29]. Amylase release was calculated as a fraction of the total (in supernatant and pellet) and then expressed as percentage of maximal isoprenaline (10 μM)-stimulated release. Normalizing to isoprenaline stimulation corrected for variability in basal and maximal responses among experiments. LDH release into the supernatant was assayed by the method of Bergmeyer *et al.* [30], which measures the conversion of NADH into NAD^+ at 339 nm in a spectrophotometer, with pyruvate as a substrate.

Statistics

Values are given as means ± S.E.M. Unless otherwise indicated, experimental groups were compared with control groups by Student's unpaired two-tailed t test.

Materials

Stock solutions of Na_2ATP (Sigma, St. Louis, MO, U.S.A.; product no. 2383) were made up with equimolar $MgCl_2$ at pH 7.0–7.4 unless otherwise noted. Although ATP was much more potent in the absence of Mg^{2+} [17], possible permeabilization artifacts [31] were avoided by using the MgATP complex. [^{3}H]Inositol and [α-^{32}P]ATP were purchased from New England Nuclear (Boston, MA, U.S.A.). GDP[S] was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Phentolamine was a gift from Ciba–Geigy (Summit, NJ, U.S.A.). Unless otherwise indicated, materials were obtained from Sigma.

RESULTS

Effects on cytoplasmic Ca^{2+}

The effects of ATP on $[Ca^{2+}]_i$ in rat parotid acinar cells, as estimated from changes in quin2 fluorescence, were compared with the effects of muscarinic and α-adrenergic agonists and substance P (Fig. 1 and Table 1). Although each agonist rapidly elevated $[Ca^{2+}]_i$ from the basal value of about 150 nM, there were characteristic differences among the responses to maximally effective concentrations of these agonists (Fig. 1). After exposure of parotid cells to the muscarinic agonist carbachol, the peak increase in $[Ca^{2+}]_i$ rapidly declined to a somewhat lower value, which was maintained for as long as examined (up to 60 min). Substance P frequently produced a similar maximal increase in $[Ca^{2+}]_i$ but, in contrast with carbachol, the subsequent decline in $[Ca^{2+}]_i$ was much faster and more complete (Fig. 1). The α-adrenergic agonists phenylephrine and noradrenaline produced smaller increases in $[Ca^{2+}]_i$ (Table 1) which were maintained at the elevated value for up to 60 min. Atropine and phentolamine, blockers respectively of muscarinic and α-adrenergic receptors, rapidly reversed the effects on $[Ca^{2+}]_i$ of carbachol and phenylephrine or noradrenaline (Fig. 1, Table 1) and prevented the increase in $[Ca^{2+}]_i$ if added before the agonists. ATP was significantly more effective at increasing $[Ca^{2+}]_i$ in parotid cells than were the other agonists (Table 1), and the elevated $[Ca^{2+}]_i$ was maintained at the peak value throughout the experiment. ATP was effective in the presence of substance P and muscarinic and α-adrenergic

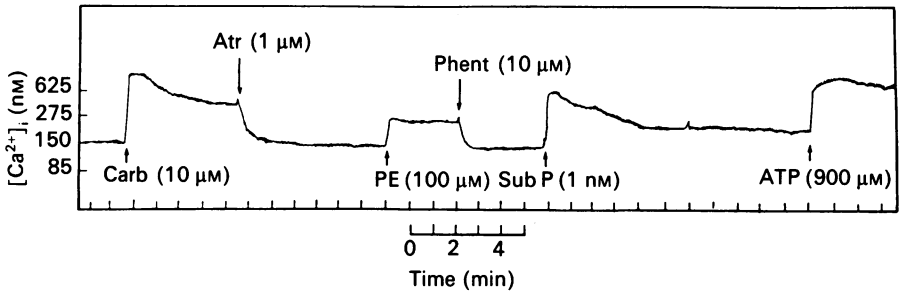

Fig. 1. Effect of different agonists on $[Ca^{2+}]_i$ in dissociated rat parotid acinar cells

Cells were prepared and $[Ca^{2+}]_i$ was measured as described previously [17]. The order of agonist and antagonist addition [carbachol (Carb), atropine (Atr), phenylephrine (PE), phentolamine (Phent), substance P (Sub P), then ATP] is typical of the traces summarized in Table 1.

Table 1. Comparison of the effects of ATP and other agonists on $[Ca^{2+}]_i$ in rat parotid acinar cells

$[Ca^{2+}]_i$ was determined as described in the Experimental section. Typically, all agonists were tested in the same trace, in the order shown in Fig. 1. Sensitivity and maximal responses to ATP were not consistently affected whether ATP was added before or after other agonists. The concentrations of agonists were maximal for $[Ca^{2+}]_i$ effects, as determined from concentration–response studies. These results were determined from ten cell preparations; $*P < 0.05$ relative to basal, $\dagger P < 0.05$ relative to highest phospholipase C-linked receptor agonist response (paired t test).

	$[Ca^{2+}]_i$ (nM)	Stimulation (fold)
Basal	185±19	
ATP (1000 μM)	1153±161†	6.33±0.64
ATP (1000 μM)+hexokinase (1 unit/ml)	235±50	
Basal	120±16	
Carbachol (10 μM)	614±195*	3.69±0.50
+Atropine (1 μM)	126±17	
Basal	132±15	
Noradrenaline (10 μM) or phenylephrine (100 μM)	241±45*	1.84±0.23
+Phentolamine (1 μM)	130±14	
Basal	139±18	
Substance P (1 nM)	372±63*	2.97±0.56

agonists as well as their antagonists (Fig. 1), consistent with a direct purinergic effect on acinar cells. As noted previously, the effect of ATP was reversed by removal with hexokinase (Figs. 2 and 3), and was highly specific for ATP; of other purine nucleotides tested, only adenosine 5′-[γ-thio]triphosphate had significant activity [17].

Surprisingly, the combination of maximal concentrations of ATP plus carbachol increased $[Ca^{2+}]_i$ much more than did either agonist alone (Fig. 2). In contrast, carbachol, phenylephrine and substance P appeared to mobilize a common pool of Ca^{2+}, since the combination of maximal concentrations of these agonists had no greater effect than did carbachol alone (Fig. 2*a*). The observation that $[Ca^{2+}]_i$ could be further increased by addition of carbachol to ATP-stimulated cells cannot be explained by separate populations of responding cells, since we observed no additivity of ATP and carbachol effects on $^{22}Na^+$ uptake or K^+ release (S. P. Soltoff, unpublished work). The additivity of maximal ATP and carbachol effects on $[Ca^{2+}]_i$ indicates different Ca^{2+}-mobilizing pathways for these two agonists in parotid cells.

Selective block of the ATP response by DIDS

In a previous study, DIDS was reported to block responses to ATP in Friend erythroleukaemia cells [32]. In the present study, we have examined the effect of this impermeant irreversible antagonist of anion exchange on ATP effects in parotid cells. Pretreatment of cells with DIDS blocked the ATP-induced increase in $[Ca^{2+}]_i$, but did not affect responses to carbachol, phenylephrine or substance P (Fig. 3). All other effects of ATP that we have examined in rat parotid cells were blocked by DIDS pretreatment (see below). It is unlikely that ATP is acting through stimulation of anion exchange, however, since replacement of the major anion, Cl^-, with the impermeant anion isethionate did not alter the $[Ca^{2+}]_i$ response to ATP (M. K. McMillian, unpublished work).

DIDS may inactivate ATP-binding sites on the cell exterior, since a component of [α-^{32}P]ATP binding to parotid cells was blocked by DIDS (Fig. 4). This DIDS-sensitive component was rapidly and selectively displaced by $MgSO_4$, consistent with ATP^{4-} being the moiety which binds [17]. ATP rapidly displaced both DIDS-sensitive and -insensitive [α-^{32}P]ATP binding, arguing

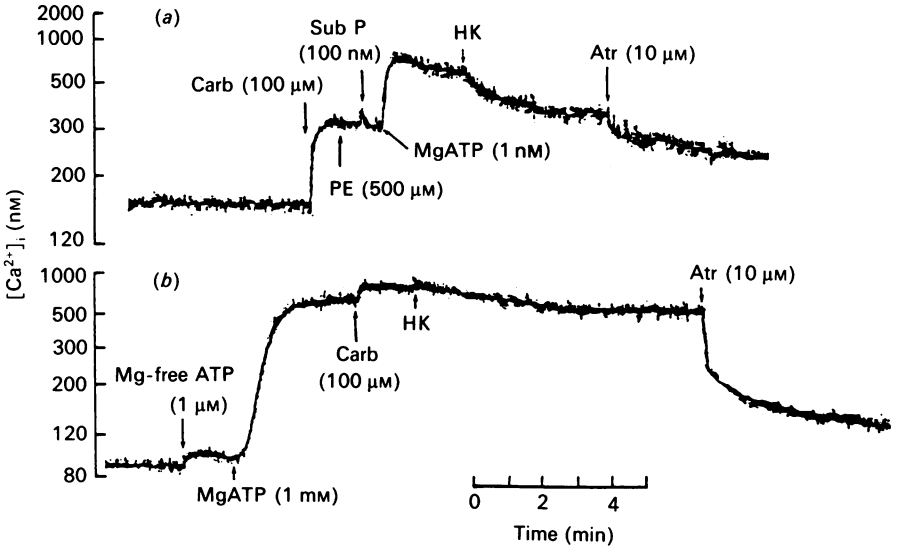

Fig. 2. Partial additivity of carbachol and ATP in elevating $[Ca^{2+}]_i$ in parotid acinar cells

Abbreviations are as indicated in Fig. 1 legend. In trace (*a*), ATP was added after a maximal carbachol (phospholipase C-mediated) effect was obtained and produced a further increase in $[Ca^{2+}]_i$ which was reversed with hexokinase (1 unit/ml). In trace (*b*), after a maximal response to ATP was obtained, carbachol produced a further increase in $[Ca^{2+}]_i$. Owing to the decreased sensitivity of quin2 at higher $[Ca^{2+}]_i$, carbachol effects on top of ATP effects were difficult to quantify. The size of this carbachol response becomes apparent when ATP is first removed with hexokinase (HK) and atropine is added to reverse the carbachol effect. These traces are representative of experiments from three cell preparations.

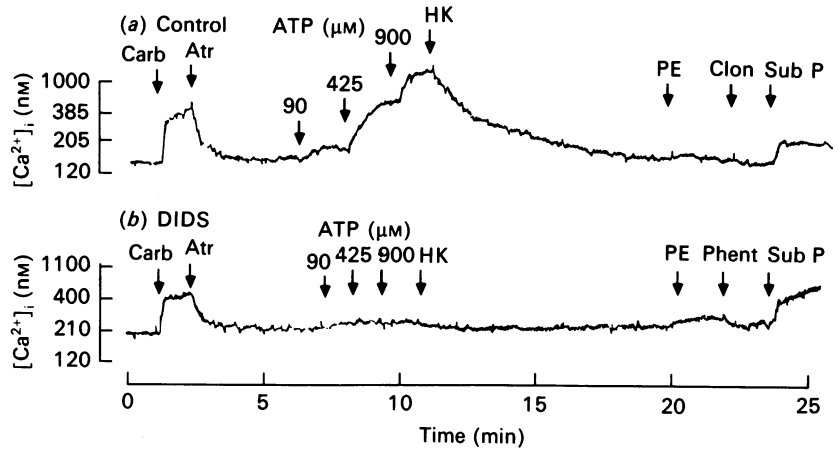

Fig. 3. Selective block of the effect of ATP on $[Ca^{2+}]_i$ in parotid cells by DIDS pretreatment

(*a*) Top trace shows control responses to muscarinic and α-adrenergic agonists and substance P (Sub P; 1 nM), as well as to three (cumulative) concentrations of ATP. Atropine (Atr; 1 μM) reversed carbachol (Carb; 10 μM) effects, and the α-antagonists phentolamine (Phent; 1 μM) or clonidine (Clon; 10 μM) reversed phenylephrine (PE; 100 μM) effects. Note the reversal of the ATP effect on addition of hexokinase (HK; 2.5 units/ml). (*b*) Bottom trace shows cells from the same preparation pretreated with DIDS (150 μM) at room temperature for 40 min. Cells were then washed, and $[Ca^{2+}]_i$ was measured at 37 °C as in Fig. 1. This trace is representative of five experiments with DIDS.

against uptake or trapping of the ligand by the cell pellet (Fig. 4). Further characterization of [α-^{32}P]ATP binding to parotid cells is necessary, but it appears likely that DIDS blocks ATP binding rather than more distal sites in the purinergic effector pathway.

Activation of plasma-membrane currents

Phospholipase C-linked receptor agonists and ATP are both known to stimulate Ca^{2+}-dependent currents in mouse parotid cells [16]. Therefore, electrophysiological responses of single rat parotid cells to ATP, carbachol and the Ca^{2+} ionophore A23187 were compared in patch-clamp experiments. When solutions containing either 1 mM-ATP or 10 μM-carbachol were pressure-ejected on to a cell (see legend to Fig. 5), whole-cell recordings typically exhibited an initial inward current, followed by a larger outward current (Fig. 5*a*). ATP activated membrane currents in 22 of 27 cells tested, and, in another series of experiments, 8 of 11 cells responded to carbachol. Interestingly, the current responses induced

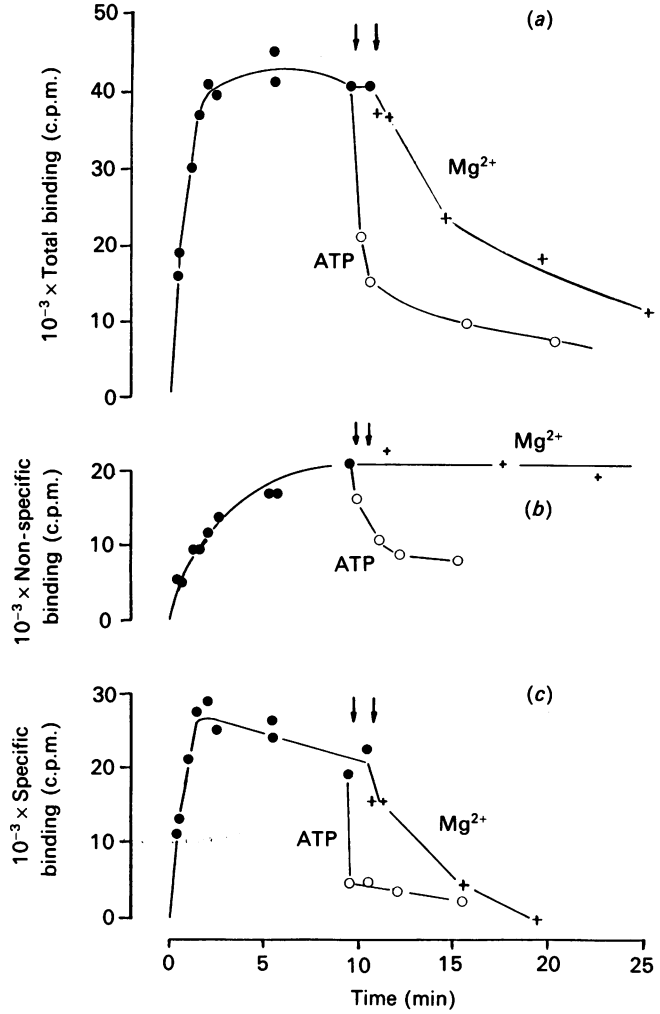

Fig. 4. DIDS blocks [α-^{32}P]ATP binding to parotid cells

Binding experiments were performed as described in the Experimental section. (*a*) Time course of [α-^{32}P]ATP (0.1 μM) binding to parotid cells. Points represent two experiments. In the first, ATP (1 mM) was added at the indicated time, and in the second $MgSO_4$ (5 mM) was added. (*b*) Time course of [α-^{32}P]ATP binding (as above) in the presence of DIDS (100 μM). Note that the remaining binding is not sensitive to Mg^{2+}, though ATP still displaced binding. (*c*) 'Specific' binding of [α-^{32}P]ATP to parotid cells, obtained by subtracting DIDS-insensitive binding (*b*) from total (*a*). The results are typical of results from three cell preparations.

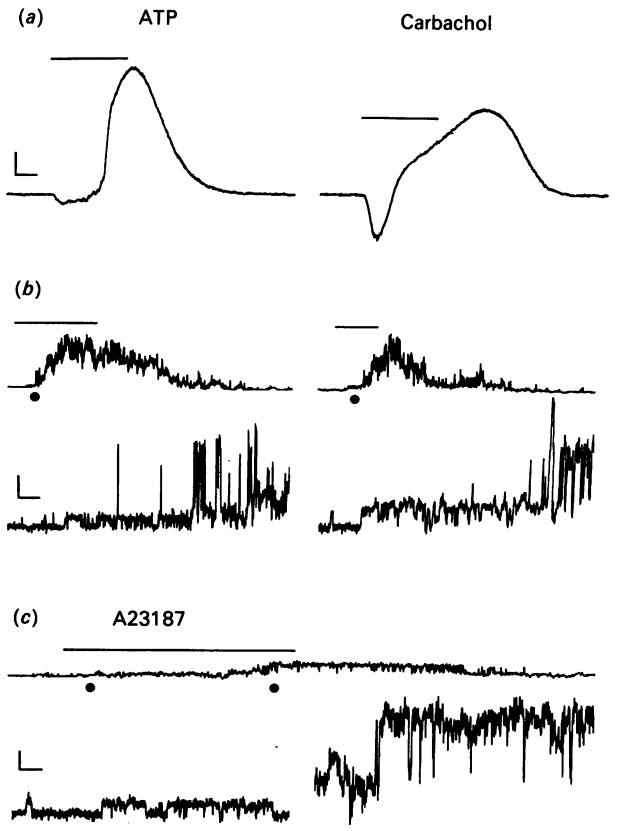

Fig. 5. ATP (1 mM), carbachol (10 μM) and Ca^{2+} ionophore A23187 (1 μM) activate similar membrane currents

(*a*) Whole-cell recordings from two isolated single cells held at −50 mV. Horizontal bars indicate the length of exposure to the drugs, which were locally applied (within 50 μm) by pressure ejection through a separate glass electrode (tip diameter 5–10 μm). Calibration bar: 50 pA, 1 s. (*b*) Upper traces show cell-attached single-channel recordings from the same cells as in panel (*a*) (ATP left, carbachol right). The patch-electrode potential was held at 0 mV. Bottom traces of panel (*b*) show a small segment of data (indicated by ● in upper traces) expanded 100-fold to illustrate the individual channel openings. Calibration bar: top traces, 15 pA, 1 s; bottom traces, 2.5 pA, 10 ms. (*c*) Cell-attached recordings from a sister cell that was exposed to A23187. Calibration bars are same as in panel (*b*).

by ATP and carbachol could not be distinguished from one another on the basis of either kinetics or amplitude. For both agonists, stimulation of the outward current could be eliminated by dialysing a cell (10 of 10 cells for ATP, 4 of 4 for carbachol) with a zero-K^+/high-Na^+ solution in the patch electrode (140 mM-NaCl, 1 mM-EGTA, 10 m-Hepes, pH 7.2), thus indicating that this current component was normally carried by K^+ ions. Further we found that the inward component was carried by Na^+ ions, since equimolar replacement of Na^+ in the bath and patch electrode with *N*-methyl-D-glucamine prevented either agonist from inducing an inward current response (4 of 4 cells).

To characterize the macroscopic current responses in more detail, single-channel patch recordings were made in the cell-attached configuration. As observed for whole-cell recordings, the examination of ATP- and carbachol-stimulated channel activity revealed no differences in either the classes of channels activated or in the time course of activation (Fig. 5*b*). Two channel types were distinguished on the basis of their single-channel conductances: a small channel corresponding to 31±6 pS (6 cells) and a larger conductance channel corresponding to 130±22 pS (4 cells). Both channel types were Ca^{2+}-sensitive, as demonstrated by increased channel activity in the presence of the Ca^{2+} ionophore A23187 (Fig. 5*c*). In addition, the larger conductance channel could be blocked by 10 mM-tetraethylammonium chloride (applied to an outside-out patch), suggesting that this channel is the BK K^+-selective channel [33]. Notably, the smaller conductance channel was opened

earlier than the Ca^{2+}-activated K^+ channel by each agent (Figs. 5*b* and 5*c*), suggesting a higher sensitivity of this smaller channel to $[Ca^{2+}]_i$. Early activation of a non-specific cation channel would account for the initial inward current observed under whole-cell recording conditions (Fig. 5*a*).

Differential effects of DIDS and GDP[S] on activation of membrane currents

As might be expected on the basis of its selective effect in blocking increases in $[Ca^{2+}]_i$, DIDS blocked ATP-induced, but not carbachol-induced, stimulations of Ca^{2+}-activated ion channels in the plasma membrane. In these experiments, a positive response to either stimulus in a whole-cell recording configuration was first obtained; DIDS (200 μM) was then added to the bath and a second pulse of agonist was applied to the same cell. Carbachol was again effective in stimulating membrane currents (in 4 of 4 cells tested), but the response to ATP was blocked (in all 4 cells tested).

Evidence that different biochemical mechanisms account for the way in which carbachol and ATP activate ion channels was provided by examining the involvement of guanine-nucleotide-binding proteins in the activation of these currents. Patch electrodes were filled with the normal intracellular solution containing 10 mM GDP[S]. Cells were then screened for a positive response to either agonist in the cell-attached patch configuration (Fig. 6*a*). If a cell when tested was positive to either agonist, it was penetrated and whole-cell-clamped and allowed to dialyse for at least 1 min before re-exposure to agonist (Fig. 6*b*). We found that the ATP-induced current responses were unaffected by GDP[S] in 9 of 12 cells tested, whereas carbachol-induced current responses were completely blocked in 7 of 7 cells. In those cells (3 of 12) that failed to respond to a second application of ATP, some critical intracellular component may have been lost, since prolonged dialysis (> 5 min) invariably resulted in a loss of agonist sensitivity, even for control (no GDP[S]) cells. Thus carbachol appears to activate currents through a G-protein blocked by GDP[S] but ATP does not.

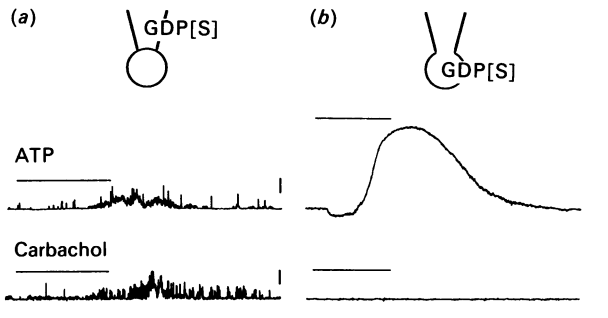

Fig. 6. ATP induction of membrane currents is not blocked by intracellular dialysis with GDP[S]

(*a*) Cell-attached recordings of single-channel currents at a patch potential of 0 mV. The responses to ATP and carbachol are shown for two different cells (upper and lower traces) just before going into the whole-cell recording configuration when GDP[S] (10 mM) is included in the patch pipette. Each cell was exposed to agonist as indicated by the horizontal bars (2 s). (*b*) Whole-cell recordings (at −50 mV) from the same cells shown in panel (*a*). Each cell was exposed to agonist (2 s) after a 1 min dialysis period with the patch-electrode solution. The ATP-induced response is normal, but the carbachol-induced response is blocked. Vertical calibration bars are 15 pA in panel (*a*) and 50 pA in panel (*b*).

Evidence that ATP does not act through phosphoinositide turnover

There was a good correlation between PtdIns turnover and the elevation of $[Ca^{2+}]_i$ for carbachol, substance P and α-adrenergic agonists (Table 2). In contrast, ATP had only a very weak effect on PtdIns turnover in parotid cells (Table 2), and this effect appeared to be secondary to Ca^{2+} mobilization. Even the weakest of the phospholipase C-linked receptor agonists was consistently more effective than ATP in stimulating accumulation of [^{3}H]inositol phosphates (Table 2). Substance P generally produced only short-acting effects on both PtdIns turnover (Table 2) and $[Ca^{2+}]_i$ (Fig. 1), reflecting desensitization [26]. Inositol phosphates in samples rapidly taken after addition of ATP did not reveal any similar short-lived responses. If incubation with ATP was extended for 60 min, measurable accumulation of total [^{3}H]inositol phosphates occurred. This weak effect was not modified by phorbol myristate acetate (PMA) (pretreatment with 10 μM for 30 min gave $102 \pm 6\%$ of control stimulation), in contrast with a general decrease in phospholipase C-linked receptor stimulation after PMA treatment (carbachol, substance P and α-adrenergic stimulation of PtdIns turnover decreased to $84 \pm 3\%$, $73 \pm 7\%$ and $72 \pm 3\%$ of control stimulation; $P < 0.05$ for all). In five experiments in which Ca^{2+} was omitted or complexed with EGTA, the ATP effect was decreased by $74 \pm 10\%$ ($P < 0.05$), but the response to carbachol did not decrease significantly (by $2 \pm 22\%$). Further evidence that alterations in PtdIns turnover are the result rather than the cause of the increase in $[Ca^{2+}]_i$ observed with ATP was provided by experiments with the Ca^{2+} ionophore ionomycin (Table 2*b*), which fully mimicked the ATP effect at 15 s and 2 min (but was toxic at 60 min; see below).

Activation of exocytosis

The effect of ATP on amylase release from parotid cells was compared with the effects of carbachol and other secretagogues. In contrast with its pronounced effect on $[Ca^{2+}]_i$, ATP was a very poor stimulus for amylase secretion (Table 3). Although much weaker than β-adrenergic agonists, carbachol was significantly more effective than ATP in stimulating amylase release ($P < 0.001$ versus ATP stimulation, $n = 9$ cell preparations; Table 3). The weak effect of ATP on amylase release was, at best, additive with the effects of other secretagogues (Table 3), but, importantly, there was no evidence for an inhibitory effect of ATP on stimulated exocytosis which could have accounted for its weak effect. PMA was a good stimulus for amylase release; this effect was presumably mediated via activation of protein kinase C, since an inactive 4α-phorbol ester had no effect (Table 3). Evidence against a major role for $[Ca^{2+}]_i$ in promoting exocytosis is that Ca^{2+} ionophores promoted the release of the cytoplasmic enzyme LDH as well as amylase (Table 3). Thus toxicity from prolonged exposure to these agents explains the apparently greater stimulation of exocytosis by Ca^{2+} ionophores than by ATP. As previously noted, ATP did not stimulate LDH release in

Table 2. PtdIns turnover in rat parotid acinar cells: comparison of ATP with other agonists

(*a*) PtdIns turnover was indirectly measured by following the accumulation of water-soluble [^{3}H]inositol products as described in the Experimental section. Basal [^{3}H]inositol phosphates amounts were 3.33±0.29% of water-soluble [^{3}H]inositol at 15 s, 2.67±0.39% at 2 min and 4.64±0.63% at 60 min; *$P < 0.05$ relative to basal. (*b*) [^{3}H]Inositol polyphosphates ($InsP_2$, $InsP_3$, $InsP_4$) were batch-eluted after removing [^{3}H]inositol monophosphate with 0.1 M-formic acid/0.2 M-ammonium formate, for 2 min incubation experiments, and [^{3}H]$InsP_2$ with 0.1 M-formic acid/0.4 M-ammonium formate, for 15 s incubations. The basal amounts of [^{3}H]inositol polyphosphates (as percentages of water-soluble [^{3}H]inositol) were 0.29±0.01% at 15 s and 0.41±0.05% at 2 min. Each value represents three or four experiments. For the 15 s experiments (in which the least radioactivity/sample was recovered), basal values averaged 54±8 c.p.m./sample; background was 11±1 c.p.m. Ionomycin was added in dimethyl sulphoxide (final concn. of the latter was 0.1%, v/v).

(*a*)	Accumulation of [^{3}H]inositol phosphates (% of basal)		
	15 s	2 min	60 min
Basal	100±9	100±14	100±14
ATP (1 mM)	107±1	124±12	195±16*
Carbachol (10 μM)	124±4	243±40*	1142±171*
Noradrenaline (10 μM)	117±8	145±11*	429±28*
Substance P (1 nM)	128±8	203±20*	293±102

(*b*)	Accumulation of [^{3}H]inositol polyphosphates (% of basal)	
	15 s ($InsP_3$)	2 min ($InsP_2+InsP_3+InsP_4$)
Basal	100±3	100±12
ATP (1 mM)	112±3	224±20*
Ionomycin (1 μM)	145±40	222±12*
Hexokinase (10 units/ml)	112±19	–
Carbachol (10 μM)	213±13*	863±134*
Noradrenaline (10 μM)	142±2*	361±60*
Substance P (1 nM)	225±29*	622±100*

these experiments [17]. Nor did ATP (1 mM) stimulate [^{3}H]inositol release under identical conditions from cells loaded with this low-molecular-mass permeability marker (experimental/control = 1.06±0.01; for carbachol = 1.05±0.01).

DISCUSSION

Recently we reported that extracellular ATP specifically and reversibly increased $[Ca^{2+}]_i$ and resembled the cholinergic agonist carbachol in stimulating K^+ release in rat parotid acinar cells [17]. In the present study, we have extended this comparison of ATP and carbachol effects and found striking differences. Despite a larger effect on $[Ca^{2+}]_i$, ATP was much weaker than known phospholipase C-linked receptor agonists in stimulating accumulation of [^{3}H]inositol phosphates, and in stimulating amylase exocytosis. In contrast with carbachol, the increase in $[Ca^{2+}]_i$ evoked by ATP appeared to be independent of a guanine-nucleotide-binding protein, and there was partial additivity between maximal ATP and carbachol effects on $[Ca^{2+}]_i$. These differences indicate that ATP elevates $[Ca^{2+}]_i$ through a mechanism distinct from phospholipase C activation.

The partial additivity of maximal ATP and carbachol effects on $[Ca^{2+}]_i$ points to different pools of mobilizable Ca^{2+} as well as different mechanisms of action for the two agonists. The combination of three phospholipase C-linked receptor agonists had no greater effect on $[Ca^{2+}]_i$ than did the most effective alone, confirming that these agonists all mobilize a common pool of Ca^{2+} [34]. It is unclear whether ATP mobilizes Ca^{2+} from an endoplasmic pool which is insensitive to $InsP_3$ [35], or whether ATP, in a manner distinct from phospholipase C-linked receptor agonists, may stimulate intracellular Ca^{2+} release or inhibit Ca^{2+} efflux.

ATP effects on ion channels in parotid cells were indistinguishable from effects of carbachol. We had expected that ATP might activate an additional Ca^{2+}-permeable channel, but obtained no evidence for such a channel in preliminary experiments. Recently, Benham & Tsien [23] have reported that ATP activates a Ca^{2+}-selective channel in smooth-muscle cells. This effect differed from the purinergic effect in parotid cells in a number of ways. The ATP effect in smooth muscle was direct and could not be observed in cell-attached patches, indicating that it was not mediated by a second messenger such as Ca^{2+}. Further, it rapidly desensitized and was unaffected by Mg^{2+}; the non-hydrolysable analogue adenosine 5′-[$\alpha\beta$-methylene]triphosphate was as effective as ATP [23]. These differences argue against a role for this type of ATP-activated Ca^{2+}-channel in mediating purinergic effects in rat parotid cells; however, further study is required to examine a possible contribution of other channels directly activated by ATP [21]. In the present study, ATP (and carbachol) activated two types of channels secondarily to the rise in $[Ca^{2+}]_i$: first, a low-conductance channel, which may correspond to the non-

Table 3. Effect of agonists on amylase secretion and LDH release from rat parotid acinar cells

Amylase and LDH release were determined as described in the Experimental section. Basal amylase release was 7.9±1.4% and isoprenaline (10 μM)-stimulated release was 30.9±4.2% (of the total cellular amylase content). Basal LDH release was 6.9±3.5% of the cellular LDH content. Numbers of experiments for amylase values are given in parentheses; LDH values represent four or five experiments; $*P < 0.05$ relative to basal. Ionophores and phorbol esters were dissolved in dimethyl sulphoxide, at final concentrations of 0.1% and 1% respectively.

	Stimulation of amylase release (% of that with isoprenaline)	LDH release (fold basal)
Basal	0	1.00
ATP	8±2* (10)	1.19±0.22
Carbachol	20±2* (9)	1.19±0.07
ATP (1 mM)+carbachol (10 μM)	25±5* (3)	–
PMA (1 μM)	30±4* (7)	1.56±0.19
4α-Phorbol 12,13-didecanoate (1 μM)	2±4 (3)	–
PMA (1 μM)+ATP (1 mM)	38±5* (6)	–
A23187 (1 μM)	29±6* (6)	3.4±0.8*
(10 μM)	32±7* (4)	5.0±1.3*
Ionomycin (0.1 μM)	18±4* (4)	2.9±0.6*
(1 μM)	29±5* (5)	4.5±0.9*

selective cation channel described previously in exocrine cells [33], followed by a large, tetraethylammonium-sensitive, K^+ channel which is probably the BK channel [33,36]. Iwatsuki *et al.* [36] have reported that the smaller conductance channel in parotid cells was blocked by a Cl^--channel blocker, but could not rule out a non-selective cation component. Conversely, we have some evidence for the cation channel, but cannot exclude a Ca^{2+}-activated Cl^- channel, which apparently is activated under conditions of fluid secretion in these cells [37]. The similarities of ATP effects to those of carbachol on channel activities, K^+ release [17] and $^{22}Na^+$ uptake and $^{36}Cl^-$ release (S. P. Soltoff, unpublished work) are consistent with a role for neuronally released ATP in parotid salivary-fluid secretion.

Our finding that GDP[S] blocks effects of carbachol, but not of ATP, on Ca^{2+}-activated ion channels argues against a role for a guanine-nucleotide-binding protein in the observed effects of ATP in parotid cells. Since a guanine-nucleotide-binding protein apparently couples muscarinic, α-adrenergic and substance-P receptors to phospholipase C [38], it is not surprising that ATP had little effect on the accumulation of [^{3}H]inositol phosphates. This weak Ca^{2+}-dependent effect of ATP on PtdIns turnover is similar to that described for ATP^{4-} in mast cells [18]. An increase in $[Ca^{2+}]_i$ may explain effects of ADP on PtdIns turnover in platelets as well [24]. The parotid offers the advantage of three relatively well-characterized phospholipase C-linked receptors (muscarinic, α-adrenergic and substance P), which served as controls in characterization of the ATP effect. Without such controls, we would probably erroneously have concluded that ATP acts through phospholipase C in the parotid. Although substance P effects rapidly desensitize in these cells [26,39,40], it is not likely that the ATP effect on PtdIns desensitizes, since the $[Ca^{2+}]_i$ response to ATP is well maintained and the slight purinergic effect on PtdIns turnover is notable only after prolonged $[Ca^{2+}]_i$ elevation.

Consistent with its weak effect on PtdIns breakdown, ATP also had only a weak effect on amylase release, suggesting that diacylglycerol is crucial for effects of phospholipase C-linked receptor agonists on exocytosis. Exocytosis in parotid cells is unusual in that cyclic AMP rather than Ca^{2+} appears to be the major mediator [34,41]. This is readily apparent in the present study, where the strongest (non-toxic) Ca^{2+}-mobilizing agonist, ATP, had minimal effects on amylase release. Clearly, elevated $[Ca^{2+}]_i$ is not a sufficient stimulus for prolonged amylase secretion from parotid cells, although it appears sufficient to activate ion conductances and probably fluid and electrolyte secretion. As noted by a number of other groups, phenylephrine and substance P are as effective as carbachol in stimulating amylase release [42,43] despite their different effects on $[Ca^{2+}]_i$. Presumably activation of protein kinase C by a relatively low concentration of diacylglycerol (which is mimicked by PMA [44]) accounts for the similar effects of the three phospholipase C-linked receptor agonists on amylase secretion, although diacylglycerol has not been directly measured. To some extent, however, the observed importance of the various stimulatory pathways for exocytosis (cyclic AMP > protein kinase C > Ca^{2+}) may reflect our experimental protocol, which requires extended incubation to detect exocytosis. A period of Ca^{2+}-sensitive exocytosis may occur immediately after receptor activation. In contrast with our findings, Gallacher [16] observed a pronounced acute effect of ATP on amylase release from mouse parotid glands. Further experiments are necessary to examine the possibility that ATP is an effective, but transient, activator of exocytosis. However, the finding that ATP is much weaker than carbachol in stimulating amylase release is further evidence for different mechanisms of action for the two agonists.

Together with the specificity and reversibility of ATP effects on parotid cells and the high potency of ATP in the absence of extracellular Mg^{2+} [17], the selective blockade of purinergic effects by DIDS provides evidence

for a discrete purinergic 'receptor' on rat parotid cells. The effect of DIDS on [α-^{32}P]ATP binding suggests that DIDS blocks the purinergic 'receptor' in parotid cells, rather than the mechanism of ATP action. Further characterization of ATP binding to parotid cells will be necessary to clarify the specificity of the DIDS effect on the purinergic response. DIDS reportedly blocks ADP-induced platelet aggregation [45], effects of ATP on Ca^{2+} and Cl^- fluxes in Friend erythroleukaemia cells [32], and elevation of $[Ca^{2+}]_i$ by ATP and ADP in lymphocyte cell lines (P. Rosoff, personal communication). A similar blocking effect of DIDS on ATP and ADP binding would be expected in these systems if ATP and ADP receptors are similar.

In summary, ATP elevates $[Ca^{2+}]_i$ in rat parotid acinar cells by a mechanism which is distinct from the phospholipase C-dependent pathway shared by muscarinic and α-adrenergic agonists and substance P. Although further characterization of the purinergic pathway in these cells is required, the existence of a separate intracellular Ca^{2+}-mobilizing pathway differing from that promoted by PtdIns breakdown is of obvious interest. Such a pathway should allow for synergistic and modulatory actions of ATP when it is released with other neurotransmitters. An understanding of this pathway may help to explain the variety of effects elicited by extracellular ATP in different cell types.

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