

Effects of Extracellular ATP on Ion Transport Systems and $[Ca^{2+}]_i$ in Rat Parotid Acinar Cells

Comparison with the Muscarinic Agonist Carbachol

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ABSTRACT The effects of extracellular ATP on ion fluxes and the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were examined using a suspension of rat parotid acinar cells and were contrasted with the effects of the muscarinic agonist carbachol. Although ATP and carbachol both rapidly increased $[Ca^{2+}]_i$ about threefold above the resting level (200–250 nM), the effect of ATP was due primarily to an influx of Ca^{2+} across the plasma membrane, while the initial response to carbachol was due to a release of Ca^{2+} from intracellular stores. Within 10 s, ATP (1 mM) and carbachol (20 μ M) reduced the cellular Cl^- content by 39–50% and cell volume by 15–25%. Both stimuli reduced the cytosolic K^+ content by 57–65%, but there were marked differences in the rate and pattern of net K^+ movement as well as the effects of K^+ channel inhibitors on the effluxes initiated by the two stimuli. The maximum rate of the ATP-stimulated K^+ efflux ($\sim 2,200$ nmol K^+ /mg protein per min) was about two-thirds that of the carbachol-initiated efflux rate, and was reduced by $\sim 30\%$ (vs. 60% for the carbachol-stimulated K^+ efflux) by TEA (tetraethylammonium), an inhibitor of the large conductance (BK) K^+ channel. Charybdotoxin, another K^+ channel blocker, was markedly more effective than TEA on the effects of both agonists, and reduced the rate of K^+ efflux initiated by both ATP and carbachol by $\sim 80\%$. The removal of extracellular Ca^{2+} reduced the ATP- and the carbachol-stimulated rates of K^+ efflux by 55 and 17%, respectively. The rate of K^+ efflux initiated by either agonist was reduced by 78–95% in cells that were loaded with BAPTA to slow the elevation of $[Ca^{2+}]_i$. These results indicated that ATP and carbachol stimulated the efflux of K^+ through multiple types of K^+ -permeable channels, and demonstrated that the relative proportion of efflux

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through the different pathways was different for the two stimuli. ATP and carbachol also stimulated the rapid entry of Na^+ into the parotid cell, and elevated the intracellular Na^+ content to 4.4 and 2.6 times the normal level, respectively. The rate of Na^+ entry through $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport and $\text{Na}^+\text{-H}^+$ exchange was similar whether stimulated by ATP, carbachol, or ionomycin, and uptake through these two carrier-mediated transporters accounted for 50% of the ATP-promoted Na^+ influx. The remainder may be due to a nonselective cation channel and an ATP-gated cation channel that is also permeable to Ca^{2+} . The consumption of extracellular ATP by an ecto-ATPase (apparent $K_{0.5}$ for ATP is 0.93 mM) on the plasma membrane limited the duration of the response to ATP when large concentrations of cells were used. The effects of ATP on $[\text{Ca}^{2+}]_i$ and ion fluxes were blocked specifically by DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), and were more potent in the absence of Mg^{2+} , suggesting that the active nucleotide moiety was ATP^{4-} . These studies suggest that ATP may function as a neurotransmitter and modulate fluid secretion by stimulating Ca^{2+} -sensitive Cl^- and K^+ channels and multiple Na^+ uptake pathways in the rat parotid acinar cell. Although some of these pathways were similar to those activated by carbachol, others were unique to ATP, which suggests that purinergic receptors may play a regulatory role in salivary secretion.

INTRODUCTION

Studies performed using a variety of cells have demonstrated that extracellular ATP can cause various changes, including the alteration of ion fluxes, cell growth, and the contraction and relaxation of smooth muscle (Stone, 1981; Chahwala and Cantley, 1984; Gordon, 1986; Reilly et al., 1987). In at least some systems, the effects of ATP may be mediated by specific purinergic receptors. Such receptors have been classified into multiple subtypes: those that are sensitive to adenosine (P_1 type) and those which are sensitive to ATP (P_2 type) (Burnstock, 1978). Moreover, subdivisions of the P_2 -type purinergic receptor have also been suggested (Burnstock and Kennedy, 1985; Gordon, 1986). Extracellular ATP has been reported to activate Ca^{2+} -sensitive ion channels by elevating the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Burgess et al., 1981; Gallacher, 1982; Sauve et al., 1988), and recently it was reported that extracellular ATP directly gated a channel in smooth muscle cells that was permeable to Na^+ and Ca^{2+} ions (Benham and Tsien, 1987). The activity of ecto-ATPases, which hydrolyze ATP (Knowles, 1988; Lin and Russell, 1988), and ectokinases, which use ATP to phosphorylate the outer plasma membrane (Ehrlich et al., 1986), may also play a role in some of these diverse effects of ATP. In addition to these specific effects, extracellular ATP can permeabilize some types of cells, including mast cells (Tatham et al., 1988) and transformed cells in culture (Heppel et al., 1985). ATP is stored and cosecreted with classical neurotransmitters, and may itself be a neurotransmitter (Fredholm and Hedqvist, 1980; Stone, 1981; Gordon, 1986), providing a physiological rationale for the effects of ATP.

Recent evidence suggests that ATP may be an important neurotransmitter for the parotid gland. The parotid acinar cell has been used as a model system to study the activation of ion channels by neurotransmitters as well as a model to study the production of biochemical second messengers that couple receptor activation to the

stimulation of fluid and electrolyte secretion. The earliest events involved in fluid secretion involve the elevation of $[Ca^{2+}]_i$, and the activation of Cl^- channels in the apical membrane and K^+ channels in the basolateral membrane (for review, see Petersen and Gallacher, 1988). In parotid acinar cells, muscarinic, alpha-adrenergic, and substance P receptors are linked to phospholipase C, and receptor agonists cause the rapid production of inositol polyphosphate compounds, including inositol 1,4,5-trisphosphate ($InsP_3$), which mobilizes Ca^{2+} from intracellular stores and elevates $[Ca^{2+}]_i$. Gallacher (1982) reported that extracellular ATP altered membrane conductances and stimulated ^{86}Rb efflux in the mouse parotid acinar cell. In previous studies we observed that extracellular ATP caused large increases in $[Ca^{2+}]_i$ in rat parotid acinar cells in suspension, and that this response appeared to be due to the activation of a P_2 purinergic receptor on the plasma membrane (McMillian et al., 1987a, 1988). Unlike the phospholipase C-linked agonists, the effects of ATP were not mediated by a GTP-dependent protein, and ATP elevated $InsP_3$ by only a minor degree, which appeared to be secondary to its effects in elevating $[Ca^{2+}]_i$ (McMillian et al., 1988). In contrast, some effects of extracellular ATP on other cell types were coupled by GTP-dependent proteins involved in the activation of phospholipase C, and ATP produced significant increases in $InsP_3$ levels (Okajima et al., 1987; Dubyak et al., 1988).

In the present study, we characterized more fully the responses of rat parotid cells to extracellular ATP and compared its effects with those of the muscarinic agonist carbachol. Previously we found that both ATP and carbachol activated a net efflux of K^+ from rat parotid cells (McMillian et al., 1987a), and electrophysiological studies indicated that both agonists stimulated a Ca^{2+} -activated large conductance K^+ channel and a nonselective cation channel (McMillian et al., 1988). The effluxes of K^+ and Cl^- initiated by carbachol and other phospholipase C-linked agonists were coupled to increases in $[Ca^{2+}]_i$, and most of the carbachol-stimulated Na^+ entry was mediated by $Na^+-K^+-2Cl^-$ cotransport and Na^+-H^+ exchange (Soltoff et al., 1989). Therefore, additional experiments were performed to measure the activation of ion fluxes by extracellular ATP, to determine the dependence of these fluxes on $[Ca^{2+}]_i$, and to compare these responses to the effects elicited by carbachol. In addition, we also extend our previous observations of carbachol-initiated K^+ fluxes. The results demonstrated that ATP activates some responses in common with the phospholipase C-linked agonists, but that it also has unique effects. ATP may function as a neurotransmitter and play a role in controlling fluid secretion by the parotid cell.

Preliminary reports have been published in abstract form (Soltoff et al., 1986, 1988a-c).

METHODS

Cell Preparation

A suspension containing single cells and small clusters of multiple cells was prepared from freshly dissected rat parotid glands as described previously (McMillian et al., 1987a). At the completion of the procedure, the cells were suspended in a HEPES/Ringers solution of the following composition, in millimolar: 120 NaCl, 5 KCl, 2.2 $MgCl_2$, 1 $CaCl_2$, 20 HEPES, 5

betahydroxybutyrate, 10 glucose, and 0.1% bovine serum albumin; pH 7.4. In most experiments the cells were resuspended in the solutions described below.

Intracellular Free Ca^{2+} ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured using fura 2-AM (Molecular Probes, Eugene, OR) as described elsewhere (McMillian et al., 1987b). In experiments performed under Ca^{2+} -free conditions, cells were maintained in Ca^{2+} -containing solution and EGTA (20 mM) was added ~2 min before the addition of agonist.

$^{45}Ca^{2+}$ Uptake

Cells were suspended in solution A (see below) containing 1 mM $CaCl_2$ and were added to a stirred thermostated chamber at 37°C. After an equilibration period of 20 min, $^{45}Ca^{2+}$ was added (5 μ Ci/ml) to the cells either in the absence of stimuli (to measure the basal uptake of Ca^{2+}) or simultaneously with carbachol (20 μ M) or ATP (1 mM). Samples (200 μ l) of the suspension were removed and pelleted through a wash solution and oil layer as described previously (Soltoff et al., 1989). Radioactivity in the pellet was added to scintillation fluid (Liquiscint) and counted in a liquid scintillation counter. A sample of the extracellular medium was also counted to determine the specific activity. The rate of Ca^{2+} uptake was calculated from the initial linear increase in $^{45}Ca^{2+}$ uptake and was normalized to cell protein. In the presence of ATP, the linear period was about 30 s, but this period lasted for several minutes during the basal or carbachol-stimulated influx.

K^+ Fluxes

Alterations in cellular K^+ content were monitored at 37°C using an extracellular K^+ -sensitive electrode (Microelectrodes, Inc., Londonderry, NH) as reported previously (Soltoff et al., 1989). In some studies the rate of K^+ efflux was measured within the initial linear period of K^+ release, which varied for ATP and carbachol (see Fig. 4). Generally, the rates were calculated over a 0.01–0.015 and 0.025–0.05 min period for carbachol and ATP, respectively. To measure the total releasable K^+ content, 25–50 μ M digitonin was added to the suspension to permeabilize the cells at the end of each measurement. A sample of the suspension was collected for protein determination.

Cell Volume

Intracellular volume was measured at 37°C using the differences in the distributions of $[^3H]H_2O$ and $[^{14}C]sucrose$, as described elsewhere (Soltoff et al., 1989).

$^{36}Cl^-$ Efflux and $^{22}Na^+$ Uptake

Cl^- efflux and Na^+ uptake studies were measured at 37°C using cells equilibrated with $^{36}Cl^-$ and $^{22}Na^+$, as previously described (Soltoff et al., 1989). The rate of stimulated Na^+ uptake was evaluated by measuring the increase in the $^{22}Na^+$ content after the addition of ouabain (3 mM) followed by the agonist about 20 s later. In some experiments, DIDS (200 μ M) was added to suspensions of parotid cells at least 10 min before ATP.

Oxygen Consumption

The rate of oxygen consumption (QO_2) of the parotid cell suspension was monitored using a Clark-type oxygen electrode (model 5331; Yellow Springs Instrument Co., Yellow Springs, OH; or model 125/05; Instech Laboratories, Horsham, PA) and an oxymeter (model 53 oxygen monitor; Yellow Springs Instrument Co.; or model 102B; Instech Laboratories). Cells

were washed two times, resuspended in the appropriate solution, and incubated at 37°C for 15–20 min before monitoring the QO_2 . A sample of the suspension was collected after each measurement to normalize the QO_2 to the cell protein content. Unless otherwise stated, in all experiments ouabain (2.5 mM) was added after the appropriate stimulus, and the data are presented as the ouabain-sensitive portion of the QO_2 .

Ecto-ATPase Activity

ATPase activity was measured in a coupled assay system that contained enzymes to regenerate ATP from ADP. The rate of ATP hydrolysis was measured at 37°C by monitoring the disappearance of NADH in the presence of lactate dehydrogenase (LDH) plus pyruvate generated from the rephosphorylation of ADP by pyruvate kinase and phospho(enol)pyruvate. The constituents of this system were as follows: 140 mM NaCl, 9 mM KCl, 4.5 mM $MgCl_2$, 20 mM HEPES, 1.4 mM phospho(enol)pyruvate (K^+ salt), 20 U/ml LDH, 5 mg/ml NADH, and 3.5 U/ml pyruvate kinase. The assay was conducted at 340 nm using a Hewlett Packard 8451A Diode Array Spectrophotometer (Palo Alto, CA). To measure the ecto-ATPase activity, intact parotid cells were added to the above solution containing 2.5 mM ouabain and different concentrations of Tris ATP (0–3 mM, added in a 1:1 ratio with $MgCl_2$). Ouabain was present as a precaution to block potential Na,K-ATPase activity (from nonintact cells) which would otherwise contribute to the measured ATPase activity. When indicated, cells were pretreated with 200 μ M DIDS for ~60 min at room temperature before monitoring the ATPase activity.

Buffering the $[Ca^{2+}]_i$ with BAPTA

In some experiments, the cells were loaded with BAPTA, a Ca^{2+} chelator (Tsien, 1980), to buffer elevations in $[Ca^{2+}]_i$. In these studies the cells were exposed to 25 μ M BAPTA-AM (Molecular Probes, Eugene, OR), the permeable form of the chelator, for at least 20 min before commencing any measurements.

Solutions

The composition of the normal solution (solution A) in which the cells were suspended was as follows, in millimolar: 116.4 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1 NaH_2PO_4 , 25 Na HEPES, 1.8 $CaCl_2$, 1 butyrate, 5.6 glucose; pH 7.4. In Cl^- replacement experiments, Na isethionate, KNO_3 , and $Ca(NO_3)_2$ were substituted for NaCl, KCl, and $CaCl_2$, respectively. In studies performed in the absence of Ca^{2+} , either $CaCl_2$ was replaced with equimolar NaCl and 500 μ M EGTA was added to the solution, or else cells were initially suspended in normal medium and 2 mM or more EGTA was added to chelate the Ca^{2+} .

Materials

Male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) were used in all experiments. $^{36}Cl^-$, $[^{14}C]sucrose$, and $[^3H]H_2O$ were purchased from Dupont Co. (Wilmington, DE). $^{22}Na^+$, $^{36}Cl^-$, and $^{45}Ca^{2+}$ were purchased from Amersham Corp. (Arlington Heights, IL) or Dupont Co. (Wilmington, DE). Other chemicals and their sources were as follows: nystatin (mycostatin) (Calbiochem-Behring Corp., San Diego, CA); dinonylphthalate (ICN Biochemicals, Inc., Plainview, NY); silicone oil (Aldrich Chemical Co., Milwaukee, WI). Tetraethylammonium Cl^- (TEA) was obtained from Sigma Chemical Co. (St. Louis, MO) or Eastman Kodak Co. (Rochester, NY). Furosemide, phospho(enol)pyruvate, LDH, hexokinase, and NADH were obtained from Sigma. All other chemicals were reagent grade or better. Dimethylamiloride was synthesized as previously described (Cragoe et al., 1967). Purified charybdo-

toxin (Anderson et al., 1988) was a generous gift from Dr. Christopher Miller (Brandeis University, Waltham, MA).

ATP

Unless otherwise specified, ATP (vanadate free, Na⁺ salt) (Sigma) or ATP-gamma-S (Sigma) was added in a 1:1 ratio with MgCl₂ (or MgSO₄ in Cl⁻-free experiments). Tris ATP (Sigma) was used in the ecto-ATPase studies.

Protein Determination

Tissue samples were dissolved in 0.2 N NaOH/0.1% sodiumdodecyl sulfate, and the protein content was determined using the procedure of Lowry et al. (1951) using bovine serum albumin as the protein standard.

Statistics

The results are presented as the mean ± the standard error. The number of experiments (*n*) refers to the number of individual parotid preparations examined.

RESULTS

Effects on [Ca²⁺]_i and Ca²⁺ Influx

The effect of ATP on [Ca²⁺]_i (fura 2 fluorescence) was compared with that of several other Ca²⁺-mobilizing agonists (Fig. 1 A). Unless otherwise noted, all stimuli were used at concentrations that had maximal effects on [Ca²⁺]_i. ATP was generally more effective at elevating [Ca²⁺]_i than phospholipase C-linked agonists. In paired studies (*n* = 12), the basal [Ca²⁺]_i was 263 ± 22 nM, and carbachol (20 μM) and ATP (1 mM) elevated [Ca²⁺]_i to 647 ± 83 and 1,104 ± 214, respectively. In a previous report we found that substance P, carbachol, and alpha-adrenergic agonists elevated [Ca²⁺]_i (measured using fura 2) to ~275, 175, and 35% above basal levels (Soltoff et al., 1989). The rise in [Ca²⁺]_i by ATP was reversed by hexokinase (McMillian et al., 1987a), which consumed the added ATP, or by Mg²⁺ (Fig. 1 A), which complexes with ATP (see below). In the absence of other additions, [Ca²⁺]_i remained elevated at near maximal levels for at least 20 min after the addition of ATP. The present findings were similar to our previous observations of the effects of ATP on [Ca²⁺]_i, which were made using quin 2 (McMillian et al., 1987a), except that [Ca²⁺]_i was observed to rise more rapidly due to the relatively lower Ca²⁺ buffering provided by fura 2.

ATP and carbachol also rapidly elevated [Ca²⁺]_i in the absence of extracellular Ca²⁺. Under these conditions, the increase in [Ca²⁺]_i by ATP was transient (Fig. 1 B) and was much less than that produced in the presence of extracellular Ca²⁺ (Fig. 1 A). However, the elevation above basal levels persisted for at least 20 min. After the transient elevation and return of [Ca²⁺]_i, the subsequent addition of carbachol did not further alter [Ca²⁺]_i (Fig. 1 B). In contrast, when carbachol was added first, it produced a rapid transient elevation of [Ca²⁺]_i comparable to that seen in the presence of Ca²⁺, and the subsequent addition of ATP produced a small increase in [Ca²⁺]_i (Fig. 1 C).

Consistent with the greater dependence of ATP on extracellular Ca²⁺ to elevate

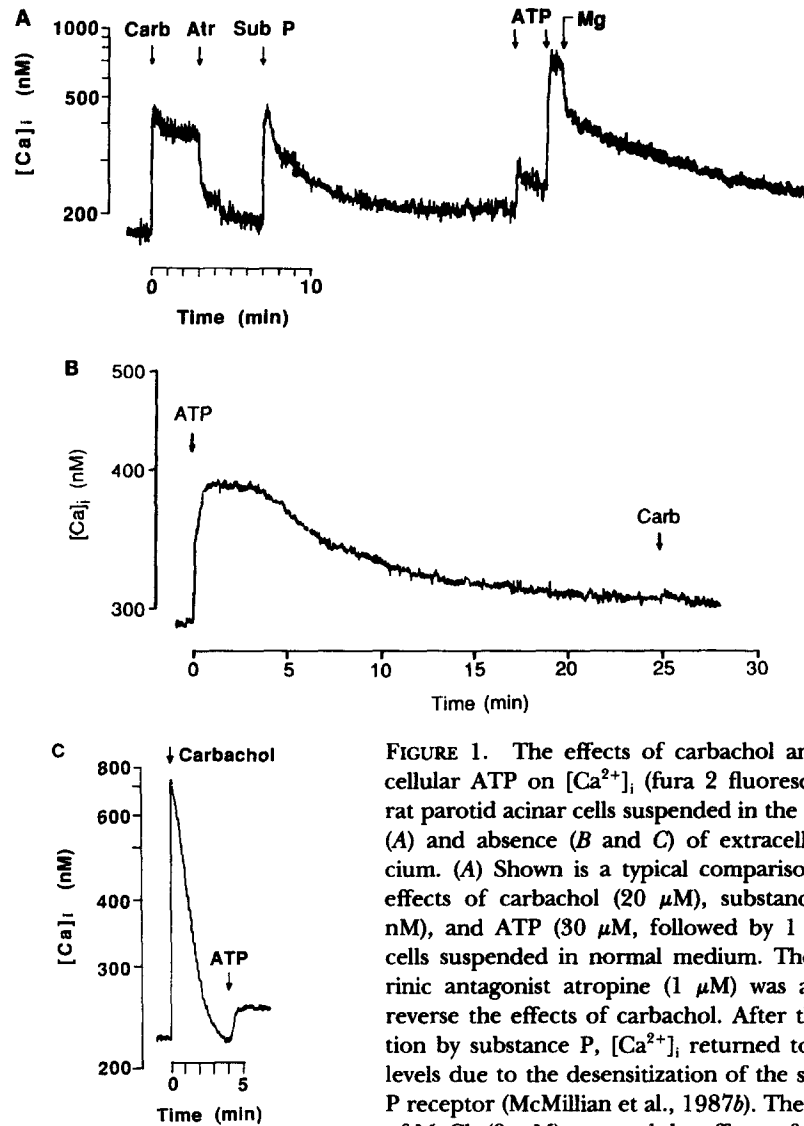


FIGURE 1. The effects of carbachol and extracellular ATP on $[Ca^{2+}]_i$ (fura 2 fluorescence) in rat parotid acinar cells suspended in the presence (A) and absence (B and C) of extracellular calcium. (A) Shown is a typical comparison of the effects of carbachol ($20 \mu M$), substance P (10 nM), and ATP ($30 \mu M$, followed by 1 mM) on cells suspended in normal medium. The muscarinic antagonist atropine ($1 \mu M$) was added to reverse the effects of carbachol. After the elevation by substance P, $[Ca^{2+}]_i$ returned to control levels due to the desensitization of the substance P receptor (McMillian et al., 1987b). The addition of $MgCl_2$ (9 mM) reversed the effects of ATP (see

text). (B) In the absence of calcium, the addition of ATP (1 mM) rapidly elevated $[Ca^{2+}]_i$, although the level was below that observed in the presence of calcium (compare B with A). Carbachol ($20 \mu M$) was ineffective when added after ATP. (C) Carbachol ($20 \mu M$) produced a rapid elevation of $[Ca^{2+}]_i$ to a level similar to that seen in the presence of calcium (A), followed by a rapid return to normal levels. The subsequent addition of ATP ($250 \mu M$) produced a small but significant elevation of $[Ca^{2+}]_i$. These results are typical of observations made using at least five preparations.

$[Ca^{2+}]_i$, ATP was more effective than carbachol in stimulating $^{45}Ca^{2+}$ entry into parotid cells. The basal rate of $^{45}Ca^{2+}$ uptake into cells suspended in 1 mM Ca^{2+} was 0.38 ± 0.08 nmol/mg per min ($n = 4$). Carbachol (20 μ M) increased this by 50%, but the uptake rate in the presence of ATP (1 mM) was about 10-fold as large, 3.83 ± 0.47 (4) nmol/mg per min. Thus, for ATP but not for carbachol, the influx of Ca^{2+} across the plasma membrane contributed greatly to the initial rise in $[Ca^{2+}]_i$ seen in cells suspended in normal (Ca^{2+} -containing) medium.

The rate of the elevation of $[Ca^{2+}]_i$ by ATP was markedly slower in cells loaded with the Ca^{2+} chelator BAPTA (not shown). BAPTA had similar effects on the responses to carbachol and other phospholipase C-linked agonists (Soltoff et al., 1989). Alterations in the effects of ATP and carbachol in the absence of extracellular Ca^{2+} or when the cells were loaded with BAPTA were used to assess the contributions of $[Ca^{2+}]_i$ to the activation of various ion channels and ion fluxes.

ATP Stimulates the Release of Cl^-

Within 10 s after the addition of ATP, the cellular Cl^- content (165.2 ± 8.3 (16) nmol Cl^- /mg protein) was reduced by $39.2 \pm 2.1\%$ (7) (Fig. 2). However, in cells loaded with BAPTA, the $^{36}Cl^-$ efflux was greatly delayed, and the reduction in Cl^- content ($2.4 \pm 3.3\%$, $n = 3$) within the first 10 s was not significant. These results are consistent with the rapid activation by ATP of a Ca^{2+} -sensitive Cl^- channel. Carbachol (50.2% reduction), other phospholipase C-linked agonists, and ionomycin (47.4%) also promoted substantial reductions in the cellular Cl^- content within 10 s and their effects were also greatly delayed in BAPTA-treated cells (Soltoff et al., 1989).

Extracellular ATP and Carbachol Induce the Net Efflux of K^+

Both ATP and carbachol stimulated the rapid efflux of K^+ (Fig. 3). The initial rates of K^+ efflux stimulated by ATP and carbachol were $2,162 \pm 172$ (25) and $3,293 \pm 180$ (28) nmol K^+ /mg per min, respectively. In paired studies, the ATP-stimulated efflux rate was $34.6 \pm 4.5\%$ (25) less than the maximum carbachol-stimulated release rate. The rate of the ATP-initiated efflux was similar to that initiated by the Ca^{2+} ionophore ionomycin (1 μ M), $1,955 \pm 364$ (7) nmol/mg per min. In contrast, the ouabain-induced "leak" of K^+ (Fig. 3) was only 22 ± 3 (3) nmol/mg per min, which was $\sim 1\%$ of the agonist-stimulated rates. The markedly higher rates of agonist-stimulated K^+ effluxes indicate the large increases in K^+ permeability and the activation of K^+ -permeable ion channels (McMillian et al., 1988) by ATP and carbachol. The K^+ content of the unstimulated cells was 468.8 ± 15.6 (38) nmol/mg. Using the value for cell volume (3.56 μ l/mg) previously reported (Soltoff et al., 1989), the intracellular K^+ concentration was 132 mM. A similar value (135 mM) was determined using ^{42}K by Nauntofte and Dissing (1988). ATP and carbachol promoted the transient loss of $65.3 \pm 1.8\%$ (31) and $57.3 \pm 1.7\%$ (38), respectively, of the intracellular K^+ content, a reduction of more than 270 nmol K^+ /mg protein, and more than half of the efflux occurred within 10–15 s (Fig. 3).

The characteristic patterns of the net movement of K^+ in response to carbachol (i.e., the rapid loss of K^+ followed by the partial reuptake to a sustained altered level (Fig. 3) and other phospholipase C-linked agonists were similar to the characteristic

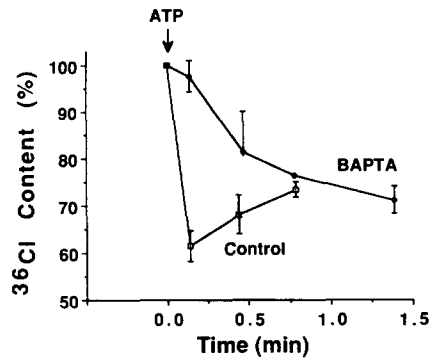


FIGURE 2. The effect of ATP (1 mM) on the chloride content of rat parotid acinar cells equilibrated with $^{36}Cl^-$. Shown are the results for control cells (\square) and cells loaded with BAPTA (\diamond). Samples were collected at various times after the addition of ATP (time 0), and the results were normalized to the chloride content of unstimulated cells (see text for absolute values). $n = 3$ (except $n = 2$ where no bars are shown).

agonist-induced alterations of $[Ca^{2+}]_i$: after reaching a peak level, the carbachol-induced increase in $[Ca^{2+}]_i$ declined to a level that was maintained above the basal level (Fig. 1 A; and Soltoff et al., 1989). However, although the ATP-promoted elevation of $[Ca^{2+}]_i$ was well maintained (McMillian et al., 1987a), the ATP-promoted loss of K^+ was not sustained. After a relatively short period of time, the cellular K^+ content returned to normal levels (Fig. 3) due to the activity of the plasma membrane ecto-ATPase, which reduced the concentration of ATP in the external medium (see below) and effectively removed the stimulus. If a second bolus of ATP was added after the K^+ reuptake commenced, a K^+ loss similar to that induced by the initial exposure to ATP occurred (not shown). In contrast, the effects of ATP on $[Ca^{2+}]_i$ were sustained because dilute cell suspensions were used in the fura 2 studies

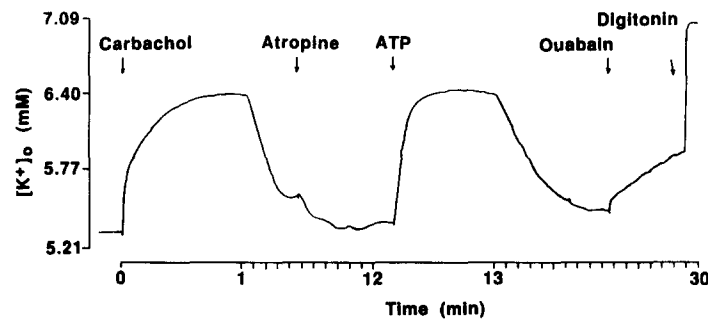


FIGURE 3. The effects of carbachol (20 μM), ATP (1 mM), and ouabain (2.5 mM) on net potassium efflux and reuptake by rat parotid cells in suspension. Potassium flux was monitored using an extracellular potassium-sensitive electrode. An increase or decrease in the extracellular potassium concentration indicates a net efflux or influx, respectively. The carbachol-induced K^+ loss was followed by a recovery phase, which resulted in the maintenance of cell K^+ at a new altered level that was fully returned to normal by the addition of 2 μM atropine (see Soltoff et al., 1989). The ATP-induced K^+ loss was also followed by a recovery phase. Note that the time scale is expanded 10-fold during the carbachol- and ATP-promoted effluxes in order to record accurately the rapid efflux kinetics, and that the recovery phases occur more slowly. The relatively slow efflux observed when the Na pump was inhibited by ouabain is in marked contrast to the rapid effluxes produced by the agonist-induced activation of ion channels. At the end of the experiment, digitonin was added to measure the potassium content. See text for further details.

(<5% as concentrated as those used in the K^+ efflux experiments), and the ecto-ATPase activity was insufficient to substantially diminish the extracellular ATP concentration over the normal time course of the $[Ca^{2+}]_i$ measurements.

The reuptake of K^+ also involved factors in addition to $[Ca^{2+}]_i$. The net K^+ flux in stimulated cells, as well as in unstimulated cells, was determined by the relative balance between channel-induced K^+ efflux pathways and the various K^+ influx pathways. The agonist-induced rapid release of K^+ indicated the initial activation of the former; subsequently, the $Na^+K^+2Cl^-$ cotransport system and the Na,K -ATPase (Na^+ pump) were activated (see below) and participated in the reuptake of K^+ .

The relative effectiveness of phospholipase C-linked agonists and ionomycin on both $[Ca^{2+}]_i$ elevation and the amount of K^+ released was as follows: ionomycin > carbachol \geq substance P > phenylephrine (Soltoff et al., 1989). The efficacy of ATP ranked between ionomycin and carbachol for both measurements, suggesting that the ATP-initiated loss of intracellular K^+ was closely related to the $[Ca^{2+}]_i$ elevation. To evaluate this relationship, additional experiments were performed by altering Ca^{2+} mobilization, and the effects of K^+ channel blockers were examined to identify the pathways responsible for the agonist-initiated efflux of K^+ .

Effects of Inhibitors on K^+ Efflux

The carbachol-initiated efflux displayed a transient rapid component, which lasted 1–2 s, followed by a much slower phase (Fig. 4 A). In contrast, the ATP-initiated efflux proceeded at or close to its initial rate for a much longer period of time (Fig. 4 B), as did the ionomycin-stimulated efflux. The agonist-initiated effluxes were affected differently by inhibitors of K^+ channels (Table I). The maximum rate of the ATP-promoted K^+ efflux was reduced by ~30% by TEA (15 mM), which blocks the BK (or maxi K) channel (Iwatsuki and Petersen, 1985; Suzuki et al., 1985). TEA was almost twice as effective in reducing the carbachol-stimulated efflux (Soltoff et al., 1989; and Table I). In paired studies ($n = 9$), TEA (15 mM) reduced the initial ATP- and carbachol-stimulated K^+ efflux rates to similar values, $1,720 \pm 260$ and $1,543 \pm 208$ nmol/mg per min, respectively. A lower concentration (2 mM) of TEA (Fig. 4) was about half as effective as 15 mM TEA (Table I). In contrast, TEA had little effect on the transient amount of K^+ released by ATP, although it greatly reduced the carbachol-induced K^+ loss (Table I). These results suggested the following: (a) the activation of the TEA-sensitive BK channels contributed to a substantial portion of the efflux initiated by ATP and carbachol; (b) other K^+ -permeable ion channels were also activated by both stimuli; and (c) the TEA-insensitive channels played a greater role in the ATP-stimulated efflux, and permitted a nearly normal amount of intracellular K^+ to be released.

Barium, another inhibitor of K^+ channels (Latorre and Miller, 1983), reduced the carbachol-stimulated K^+ release rate by 40% (Soltoff et al., 1989), and also reduced the rate of the ATP-promoted K^+ efflux (not shown). However, this effect was difficult to interpret because it was complicated by the ability of divalent cations to complex with ATP, thereby reducing its effectiveness (see below).

Charybdotoxin (CTX), a protein isolated from scorpion venom, has been shown to block a variety of Ca^{2+} -activated K^+ channels (for review, see Moczydlowski et al., 1988). CTX was much more effective than TEA in reducing the agonist-stimulated K^+ efflux (Table I, Fig. 4). 100 nM CTX was ~40% more effective than 15 mM TEA

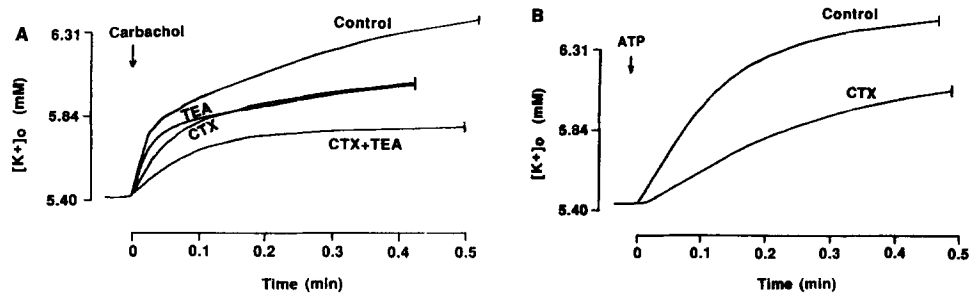


FIGURE 4. The effects of TEA and CTX on the agonist-stimulated K^+ effluxes of rat parotid cells in suspension. TEA (2 mM) and/or CTX (10 nM) were added ~ 3 min before 20 μM carbachol (A) or 1 mM ATP (B). Shown are representative results obtained using several aliquots of cells taken from the same preparation, and each aliquot was used at a similar protein density (3.5–3.9 mg/ml). For illustrative purposes the effluxes in response to either agonist are shown as commencing at the same extracellular K^+ concentration, although the actual initial concentrations varied slightly ($<4\%$) in the experiments shown. The cumulative results of similar experiments are presented in Table I.

TABLE I
Inhibitory Effects of Various Conditions on the Release of K^+ from Parotid Acinar Cells Stimulated by ATP and Carbachol

	% Inhibition of K^+ release rate	
	ATP	Carbachol
TEA (15 mM)	32.1 \pm 4.2 (9)	58.7* \pm 5.0 (9)
TEA (2 mM)	13.9 \pm 4.7 (8)	27.3* \pm 6.1 (11)
CTX (100 nM)	81.1 \pm 2.4 (3)	83.6 \pm 1.2 (3)
CTX (10 nM)	64.1 \pm 2.0 (5)	54.9 \pm 4.9 (6)
CTX (10 nM) + TEA (2 mM)	68.7 \pm 1.2 (3)	71.2 \pm 5.5 (4)
0 Ca_o	54.5 \pm 4.1 (8)	16.9* \pm 12.0 (6)
BAPTA	78.2 \pm 3.8 (5)	94.6* \pm 2.6 (4)

ATP (1 mM) and carbachol (20 μM) were added to rat parotid acinar cells suspended in solution A, and K^+ release was measured as shown in Fig. 4. When present, TEA and CTX were added 2–3 min before the addition of carbachol or ATP. BAPTA-treated cells were exposed to BAPTA-AM (25 μM) for at least 20 min before the addition of agonist. In 0 Ca_o experiments, cells were incubated for 20 min in solution A, resuspended in calcium-free solution A, and 500 μM EGTA was added 2 min before the addition of agonist. The effects of the inhibitors on the initial rates of carbachol- and ATP-stimulated K^+ release and on the amount of K^+ release were normalized to the results obtained using cells from the same preparation in the absence of any inhibitors. For the rates of K^+ efflux promoted by ATP and carbachol, respectively, the inhibitory effect of 2 mM TEA (added alone) plus 10 nM CTX (added alone) was 104.3 \pm 10.3% (3) and 103.9 \pm 16.0% (4) of the inhibitory effect produced by 2 mM TEA and 10 nM CTX added simultaneously. The absolute rates of K^+ release are presented in the text. ATP and carbachol promoted the transient loss of 312.5 \pm 13.5 ($n = 31$) and 274.8 \pm 11.8 ($n = 37$) nmol K^+ /mg protein, respectively. Compared with these control values, the inhibitors reduced these losses by the following percentages, for ATP and carbachol, respectively: 15 mM TEA: 9.2 \pm 3.8% (12), 56.2 \pm 3.5% (11); 2mM TEA: 7.7 \pm 3.7% (8), 22.3 \pm 4.0% (10); 100 nM CTX: 33.1 \pm 9.0% (3), 68.2 \pm 2.3% (3); 10 nM CTX: 26.4 \pm 3.8% (5), 39.6 \pm 2.9% (6); 0 Ca_o : 16.4 \pm 3.9% (10), 30.3 \pm 7.5% (6); BAPTA: 31.1 \pm 8.0% (9), 54.9 \pm 7.8% (5). The number of different preparations is shown in parentheses.

*Includes data previously presented in Soltoff et al., 1989.

in reducing the carbachol-stimulated rate of efflux, and was more than twice as effective as 15 mM TEA in reducing the ATP-stimulated rate of efflux. A much lower concentration of CTX (10 nM) was 66–79% as effective as 100 nM CTX in reducing the agonist-stimulated rates of efflux (Table I). TEA (2 mM) and CTX (10 nM) displayed an additive inhibitory effect on the carbachol-initiated K^+ efflux rate (Fig. 4 A; Table I). The marked differences between the inhibitory effects of TEA and CTX were further indications that ATP and carbachol both activated multiple K^+ -permeable ion channels (see Discussion).

Dependence of K^+ Efflux on $[Ca^{2+}]_i$

To evaluate the Ca^{2+} dependence of K^+ efflux, the activation of the efflux pathways was examined under conditions that affected Ca^{2+} mobilization and the ability of cells to maintain elevated levels of $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+} , the rate of ATP-stimulated K^+ efflux was reduced by ~55%, but the amount of K^+ lost was reduced by only 16% (Table I). This indicated that extracellular Ca^{2+} supported a large portion of the initial release of K^+ , but that in the absence of Ca^{2+} the efflux continued for a time sufficient to promote the nearly normal loss of K^+ . In contrast to these effects, there was a lesser reduction in the initial rate of K^+ efflux but a significant reduction in the K^+ loss produced by carbachol in the absence of Ca^{2+} (Table I; and Soltoff et al., 1989). Along with the results shown in Fig. 1 C, these results suggested that the initial release of K^+ promoted by carbachol was due to Ca^{2+} , released from intracellular stores, and that the maintenance of this K^+ efflux required extracellular Ca^{2+} . In cells loaded with BAPTA, the rates of both ATP- and carbachol-initiated effluxes were greatly reduced, and the amounts of K^+ lost also were reduced significantly (Table I). Thus, although there were marked differences between the effluxes promoted by these two stimuli, their effects depended on the rapid elevation of $[Ca^{2+}]_i$ to activate the K^+ -permeable channels and promote the efflux of K^+ , which is consistent with the activation of Ca^{2+} -sensitive ion channels.

Effect of ATP on Cell Volume

ATP produced a significant reduction in the cellular volume of parotid cells, as might be expected since it promoted a substantial loss of intracellular K^+ and Cl^- . Within ~10 s, the earliest time at which the response was measured, the cell volume was decreased by $14.8 \pm 3.2\%$ (4) (Fig. 5). The reduction was maintained for at least 5 min, but by 10 min it had begun a return toward the initial values. The return appeared to be due to the reestablishment of the normal ionic content of the cell, in part due to the reuptake of K^+ initiated upon the consumption of the added ATP (see below). The magnitude of the ATP-induced decrease in volume was similar to that produced by ionomycin (16%) but less than that produced by carbachol (25%) within 10 s (Solttoff et al., 1989).

Ecto-ATPase Activity

An ATP-consuming activity on the outer membrane of the cells was measured using intact parotid cells in suspension. The ATP dependence (apparent $K_{0.5} = 0.93$ mM) is shown in Fig. 6. The activity was diminished only slightly by pretreating the cells with DIDS, which, along with its reduced form dihydroDIDS, has been reported to

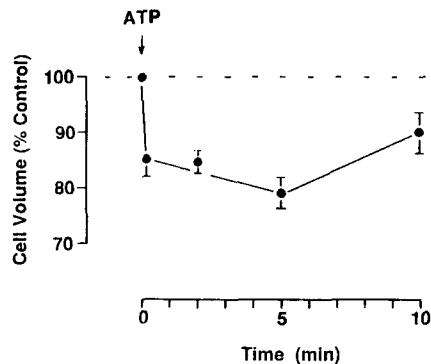


FIGURE 5. The effect of ATP (1 mM) on the intracellular volume of rat parotid acinar cells suspended in solution A. Cell volume was measured using $[^3H]H_2O$ and $[^{14}C]sucrose$ (see Methods), and the results were normalized to the volume measured before the addition of ATP (time 0), represented by the broken line. $n = 4$.

be a nonspecific inhibitor of other ATPases, including the renal Na,K-ATPase (Pedemonte and Kaplan, 1988) and an ecto-ATPase in hepatocytes (Knowles, 1988). The relative lack of effect of DIDS (Fig. 6), which greatly inhibits the ion transport effects of extracellular ATP, suggests that the ecto-ATPase does not play a role in the origination of the effects on the ion transport systems. The precise role of the ecto-ATPase in the parotid acinar cell is not known, but it may serve to limit the time of effectiveness of any ATP released in a neurotransmitter-like fashion (see Discussion).

Extracellular ATP and Carbachol Increase Intracellular Na^+

The effects of ATP on stimulating Na^+ influx and elevating the intracellular Na^+ content were measured using $^{22}Na^+$. The basal Na^+ content was 73.8 ± 4.9 (12) nmol/mg, similar to that determined previously (Soltoff et al., 1989). Using the intracellular volume of $3.56 \mu l/mg$, this represents an intracellular Na^+ concentration of ~ 20 mM. ATP was much more effective than carbachol in elevating Na^+ . The maximum levels to which ATP and carbachol elevated the Na^+ content were 4.4 and 2.6 times the basal content, increases of ~ 225 and 125 nmol/mg, respectively (Fig. 7). Since the cells shrank after the addition of these stimuli, the intracellular Na^+ concentration was increased to >85 and 55 mM, respectively. Maximal elevations were achieved nearly 1 min after the additions of the stimuli, and after 10 min the Na^+ content remained elevated at more than twice the basal level.

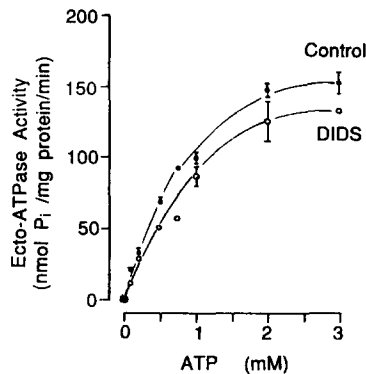


FIGURE 6. The effect of different concentrations of ATP on the ecto-ATPase activity of rat parotid cells in suspension. The ATPase activity of control and DIDS (200 μM)-treated cells was measured in the presence of 100 μM to 3 mM ATP as described in Methods. $n = 3-4$.

Unidirectional rates of Na^+ uptake were measured in a manner similar to the alterations of the intracellular Na^+ content, except that ouabain was added (to block Na^+ pump-mediated $^{22}\text{Na}^+$ efflux) immediately before the addition of ATP. Ouabain (added alone) promoted the accumulation of Na^+ at a very low rate, 8 ± 1 (16) nmol/mg per min, which is a reflection of the low basal rate of the ion transport systems involved in Na^+ entry into the parotid cell. The addition of extracellular ATP increased this rate by more than 45 times, to 395 ± 13 (22) nmol/mg protein per min. The carbachol- and ionomycin-stimulated unidirectional rates of Na^+ uptake were 265 and 590 nmol/mg per min, respectively (Soltoff et al., 1989). Thus, there was a qualitative correlation between the effect of these three agents (carbachol, ATP, and ionomycin) on $[\text{Ca}^{2+}]_i$ and Na^+ entry.

DIDS (200 μM), which blocked the ATP-promoted rise in $[\text{Ca}^{2+}]_i$ (McMillian et al., 1988), was also effective in blocking the effects of ATP on Na^+ uptake. The initial rate of ATP-stimulated $^{22}\text{Na}^+$ uptake in DIDS-treated cells was reduced to 22.3 ± 3.3 (3) nmol Na^+ /mg per min, which was only 5% of the normal ATP-stimu-

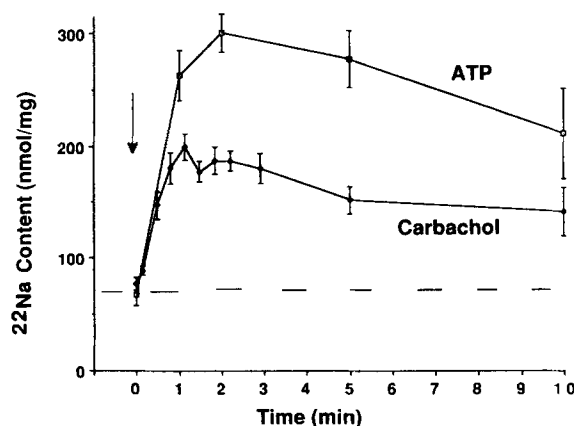


FIGURE 7. The effects of ATP (1 mM) and carbachol (20 μM) on the sodium content of rat parotid cells in suspension. Cells suspended in solution A were equilibrated with $^{22}\text{Na}^+$ for at least 20 min before the addition of stimuli (time 0). The combined results from 3 to 4 (for ATP) or 4 to 8 (for carbachol) preparations are shown. The unstimulated value measured before the addition of agonist is represented as a broken line.

lated influx rate and was close to the basal uptake rate measured in the presence of ouabain alone.

ATP Stimulates the Rate of Oxygen Consumption

As might be expected from their effects on Na^+ accumulation (Fig. 7) and K^+ efflux (Figs. 3 and 4), ATP and carbachol caused a rapid increase in the QO_2 of rat parotid cells, and this increase was reversed by the subsequent addition of ouabain (2.5 mM) (Fig. 8 A). Stimulation of the QO_2 by ATP or phospholipase C-linked agonists was also prevented by prior addition of ouabain to the cells (not shown), demonstrating that ATP was not acting as a mitochondrial uncoupler. The stimulatory effect of ATP on the QO_2 was reversed by adding hexokinase to the suspension to consume the added ATP (Fig. 8 B). When added before ATP, DIDS prevented ATP from stimulating the QO_2 (not shown); when added after ATP (Fig. 8 C), it reversed the effect of ATP.

Due to the tight coupling between mitochondrial oxidative phosphorylation and

the Na,K-ATPase activity, the ouabain-sensitive QO_2 is a quantitative measurement of Na^+ pump activity in intact epithelial cells (Mandel and Balaban, 1981). The ouabain-sensitive ATP-stimulated QO_2 was 18.4 ± 1.0 (14) nmol O_2 /mg protein per min, similar to the carbachol-stimulated value (18.0) previously determined (Soltoff et al., 1989). Assuming an ATP: O_2 ratio of 6:1 for ATP produced by oxidative phosphorylation, and a Na^+ :ATP ratio of 3:1 for the energetic cost of the Na^+ pump activity, the ATP-stimulated Na^+ pump activity was ~ 330 nmol Na^+ /mg protein per min. This stimulation was promoted by the combination of the large decrease in intracellular K^+ (Fig. 3) and increase in Na^+ (Fig. 7), which stimulated the Na^+ pump activity by greatly increasing the intracellular Na^+/K^+ ratio (Soltoff and Mandel, 1984; Soltoff et al., 1989). The effect of ATP on QO_2 was similar to that of nystatin, a cationophore that increases Na^+ entry and K^+ efflux, and which can be

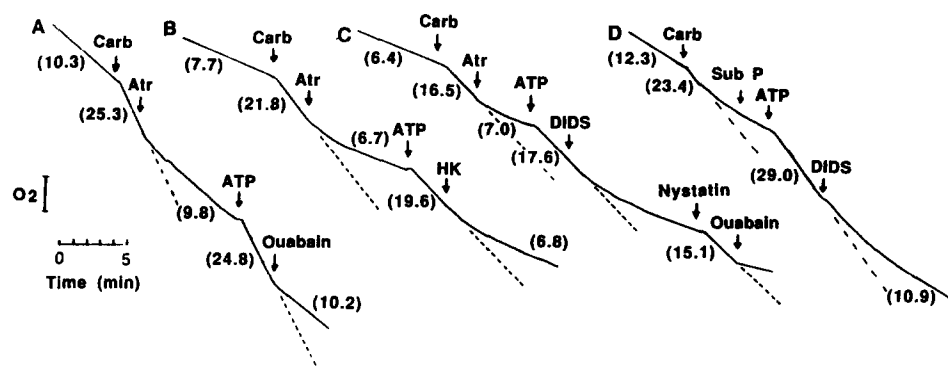


FIGURE 8. The effect of various stimuli on the oxygen consumption of rat parotid cells in suspension. The reduction of the O_2 in a closed chamber is shown as a function of time. The cells were suspended in solution A (A–C) or in calcium-free solution A to which $500 \mu M$ EGTA was added (D). The following concentrations of agents were added: $20 \mu M$ carbachol, $1 \mu M$ atropine, $1 mM$ ATP, $2.5 mM$ ouabain, $1 U/ml$ hexokinase, $200 \mu M$ DIDS, $0.4 mM$ nystatin, $10 nM$ substance P. The numbers in parentheses are the rates of oxygen consumption (in nmol O_2 /mg protein per min) calculated from the linear portion of O_2 consumption, represented by a dotted line in some traces. Each trace is representative of those obtained in at least four separate preparations.

used to evaluate the maximum Na^+ pump activity in intact cells (Soltoff and Mandel, 1984; Soltoff, 1986). The ouabain-sensitive nystatin-stimulated QO_2 was 18.9 nmol O_2 /mg protein per min (Soltoff et al., 1989), which represents a Na^+ pump activity of 340 nmol Na^+ /mg protein per min. Thus, ATP stimulated the Na^+ pump to a near-maximal rate.

Effect of Furosemide and Removal of Extracellular Cl^- on Na^+ Influx and QO_2

In carbachol-stimulated cells, the majority of Na^+ entry occurs via $Na^+-K^+-2Cl^-$ cotransport (Soltoff et al., 1989). To examine whether this system contributed to the effects of ATP, $^{22}Na^+$ uptake experiments were performed in the presence of furosemide to inhibit $Na^+-K^+-2Cl^-$ cotransport. Furosemide ($1 mM$) reduced the ATP-stimulated Na^+ influx by $30.6 \pm 4.1\%$ (9) (Table II). In absolute terms the

TABLE II
Effect of Furosemide or DMA on the ATP-stimulated Rate of $^{22}\text{Na}^+$ Uptake into Rat Parotid Acinar Cells

Furosemide (1 mM) (n = 9)			DMA (5 μM) (n = 5)		
Control	Furosemide	Δ	Control	DMA	Δ
<i>nmol/mg protein per min</i>					
439 \pm 22	308 \pm 27	131 \pm 17	408 \pm 15	353 \pm 18	55 \pm 15

$^{22}\text{Na}^+$ uptake was measured in paired (\pm inhibitor) experiments. The cells were first equilibrated with $^{22}\text{Na}^+$, and then split into two portions, one of which was exposed to the inhibitor. About 20 s before the addition of 1 mM ATP, 2.5 mM ouabain was added to inhibit efflux through the Na pump. The linear increase in the $^{22}\text{Na}^+$ content upon the addition of ATP was taken as the unidirectional rate of sodium influx.

furosemide-sensitive rate of Na^+ uptake (131 nmol Na^+ /mg per min) was very similar to those measured previously for stimulations by carbachol (142) and ionomycin (127) (Soltoff et al., 1989), although the fraction of the Na^+ uptake rate that was furosemide-sensitive was very different for each stimulus due to their different stimulated rates of Na^+ entry. This indicated that the influx of Na^+ via $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport was similar whether it was initiated by maximal concentrations of carbachol, ionomycin, or ATP. Presumably, the elevation of $[\text{Ca}^{2+}]_i$ to ~ 600 nM by carbachol, the least effective stimuli of these three on $[\text{Ca}^{2+}]_i$, was sufficient to maximally activate $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ transport.

In the presence of 1 mM furosemide or in the absence of extracellular Cl^- (± 1 mM furosemide), the ATP-stimulated ouabain-sensitive QO_2 was reduced by $\sim 10\text{--}15\%$ (Fig. 9). The relatively small effect of these perturbations on the ATP-stimulated QO_2 are in marked contrast to the substantial ($\sim 50\%$) inhibition of the carbachol-stimulated QO_2 produced by furosemide and/or Cl^- removal (Soltoff et al., 1989). This suggests that Na^+ entry through a Cl^- - and furosemide-insensitive pathway, in combination with the loss of intracellular K^+ promoted by ATP, was sufficient to stimulate the Na^+ pump activity of the parotid acinar cell to near normal levels under these conditions.

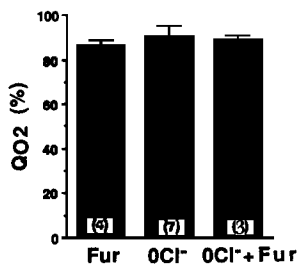


FIGURE 9. The effects of furosemide (1 mM) and/or the absence of extracellular chloride on the stimulation of the ouabain-sensitive QO_2 by ATP (1 mM). Cells were suspended in solution A (control) or in chloride-free medium ± 1 mM furosemide, and the QO_2 was measured as shown in Fig. 8. The cells were exposed to furosemide about 10 min before to ATP. The experimental results were normalized to the effect of ATP on control cells from the same preparation. The number of preparations are shown at the bottom of each bar.

Effect of Inhibitors of Na^+ - H^+ Exchange

A second Na^+ transport system that is present in the parotid cell is the Na^+ - H^+ exchange system, which plays a role in the regulation of pH_i in various epithelial tissues. Dimethylamiloride (DMA), an amiloride analogue that was effective at blocking the activation of Na^+ - H^+ exchange by carbachol (Soltoff et al., 1989), reduced the ATP-stimulated Na^+ uptake rate by 13% (Table II). About 20% of the carbachol-stimulated Na^+ uptake was attributable to influx via Na^+ - H^+ exchange. Notably, the absolute DMA-sensitive rate (55 nmol/mg protein per min) stimulated by ATP was similar to that produced by carbachol (48) and ionomycin (46) (Soltoff et al., 1989). Thus, ATP, carbachol, and ionomycin stimulated a quantitatively similar influx for two Na^+ entry pathways, those of Na^+ - K^+ - $2Cl^-$ cotransport and Na^+ - H^+ exchange.

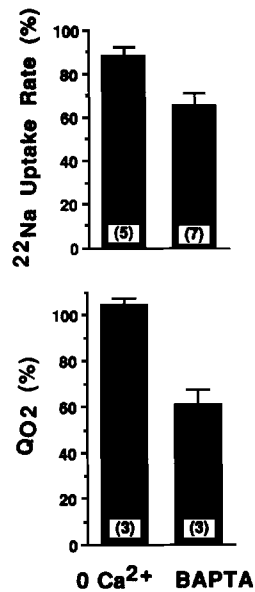


FIGURE 10. The effects of calcium on the stimulation of $^{22}Na^+$ uptake and the ouabain-sensitive QO_2 by ATP (1 mM). Parotid acinar cells were suspended in normal medium (control), in calcium-free medium containing 500 μM EGTA, or in normal medium to which 25 μM BAPTA-AM was added. The experimental results were normalized to the effect of ATP on control cells from the same preparation. The number of preparations is shown at the bottom of each bar.

Dependence of Na^+ Uptake and QO_2 on Ca^{2+}

The rate of ATP-stimulated Na^+ accumulation was diminished by 11% in the absence of extracellular Ca^{2+} , but there was no significant effect on the ouabain-sensitive QO_2 (Fig. 10). The lack of a diminished QO_2 response suggests that ATP increased the intracellular Na^+/K^+ ratio sufficiently such that the Na^+ pump was stimulated to a near-maximal rate, even though there was a reduction in the rate of Na^+ accumulation (Fig. 10) and K^+ efflux (Table I) under these conditions.

The relatively small effect of Ca^{2+} removal on the responses to ATP was in marked contrast to the large reduction in the carbachol-stimulated $^{22}Na^+$ uptake rate and QO_2 , which were inhibited by 40–65% in the absence of Ca^{2+} (Soltoff et al., 1989). The transiency of the QO_2 responses to phospholipase C-linked agonists in the absence of Ca^{2+} also demonstrated that their dependence on extracellular Ca^{2+} differed from that of ATP. As shown in Fig. 8 D, the effect of carbachol on the QO_2

was short-lived. After a brief stimulation, the QO_2 returned to the unstimulated rate, and the subsequent addition of another phospholipase C-linked agonist (here, substance P) did not alter the QO_2 . However, when ATP was added after these agonists, it did cause a large stimulation of the QO_2 . This suggests that ATP stimulated the parotid cells by a mechanism that was, at least in part, distinct from the phospholipase C-linked agonists. The removal of extracellular Ca^{2+} does not alter the ability of phospholipase C-linked agonists to stimulate the production of $InsP_3$ (Aub and Putney, 1985). Since the nystatin-stimulated ouabain-sensitive QO_2 was not significantly altered by the removal of extracellular Ca^{2+} (Soltoff et al., 1989), the activity of the Na^+ pump was not restricted in cells suspended in the Ca^{2+} -free medium. Therefore, the differences between the stimulation of the Na^+ pump activity by ATP and carbachol under these conditions were due to their relative effectiveness in promoting Na^+ entry and/or K^+ efflux.

The dependence of the ATP-stimulated accumulation of Na^+ on $[Ca^{2+}]_i$ also was evaluated using parotid cells loaded with BAPTA. The effect of ATP on the entry of $^{22}Na^+$ was reduced by $34.4 \pm 6.1\%$ (7) in BAPTA-loaded cells (Fig. 10). This decrease was much less than the 95% reduction of either the carbachol- or ionomycin-stimulated Na^+ uptakes (Soltoff et al., 1989). The stimulation of QO_2 by ATP was reduced by $39.0 \pm 6.8\%$ (3) in BAPTA-loaded cells (Fig. 10), and there was a delay in the time that it took ATP to attain the maximum stimulation in QO_2 (not shown).

Nucleotide Specificity of Ion Fluxes

To determine the specificity of the ATP stimulation of ion fluxes, the effects of various nucleotides were examined. ATP- γ -S (1 mM, added 1:1 with $MgCl_2$) stimulated K^+ efflux at a much slower rate (645 nmol/mg per min, $n = 2$), and the loss of K^+ (37.6%, $n = 2$) was much less than that produced by ATP. Other nucleotides (all 1 mM, added with $MgCl_2$), including the nonhydrolyzable analogue AMP-PNP, metabolic products of ATP (adenosine, AMP, and ADP), and GTP, ITP, and dibutyryl cAMP did not measurably alter K^+ efflux, and also did not significantly effect Na^+ influx. These results were consistent with the effects of different nucleotides on $[Ca^{2+}]_i$. Previous studies demonstrated that ATP- γ -S produced a significant elevation (to a maximal level that was 40% as high as that produced by ATP) of $[Ca^{2+}]_i$, and that other nucleotides (including ITP, deoxyATP, and those listed above) were ineffective under these conditions (McMillian et al., 1987a).

ATP Is More Potent in the Absence of Extracellular Mg^{2+}

Since ATP was more potent in elevating $[Ca^{2+}]_i$ in the absence of extracellular Mg^{2+} (McMillian et al., 1987a), we examined whether the effect of ATP on Na^+ uptake was also more potent in the absence of Mg^{2+} . As shown in Fig. 11, in normal medium the apparent $K_{0.5}$ for ATP (added as $MgATP$) was $\sim 500 \mu M$, but in the absence of Mg^{2+} , the $K_{0.5}$ of (Mg -free) ATP was only $30 \mu M$, a similar increase in potency to that seen for the elevation of $[Ca^{2+}]_i$ (McMillian et al., 1987a). These results suggest that ATP^{4-} , not $MgATP$, was the active moiety, and that the effects of ATP were mediated by a purinergic receptor that was highly sensitive to ATP^{4-} .

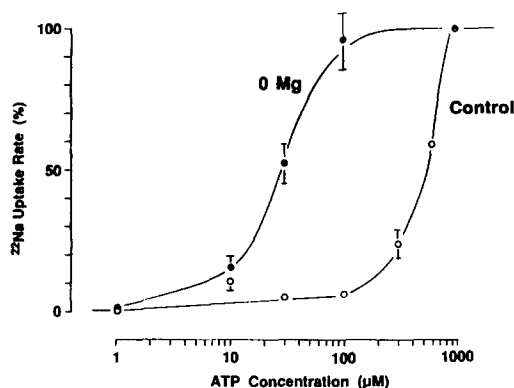


FIGURE 11. The concentration dependence of ATP on $^{22}Na^+$ uptake in the presence and absence of magnesium. Shown are the effects of extracellular ATP added to cells suspended in solution A in the absence (control) or presence (0 Mg) of 2 mM EDTA. ATP was added with (control) or without (0 Mg) an equimolar concentration of $MgCl_2$. The results were normalized to the value of the initial sodium uptake rate stimulated by 1 mM ATP (plus ouabain) minus the uptake rate measured in the presence of ouabain alone. In absolute

terms, this difference was 449.9 ± 12.8 (4) nmol/mg per min and 434.1 ± 18.1 (4) for control and 0 Mg conditions, respectively. Thus, although the potency was increased about 10-fold, the efficacy of ATP was unaltered in the absence of magnesium. Shown are the results from four preparations.

This is also consistent with the ability of Mg^{2+} to reverse the ATP-promoted elevation of $[Ca^{2+}]_i$ (Fig. 1 A).

Since the cells were more sensitive to ATP in the absence of extracellular Mg^{2+} , the effects of various nucleotides on Na^+ uptake were examined under Mg^{2+} -free conditions. Several purine nucleotide analogues stimulated Na^+ entry under these conditions, but none were as effective as ATP (Fig. 12). The order of effectiveness (ATP > ATP- γ -S > AMP-PNP > α,β -methylene ATP ~ β,γ -methylene ATP > ADP) is consistent with a response mediated via a P_2 -type purinergic receptor with a high selectivity for ATP^{4-} , the putative P_{2x} -type receptor (Gordon, 1986).

TEA Reduces the Rate of Na^+ Uptake

Previous results indicated that the stimulation of Na^+ uptake by carbachol appeared to depend, at least in part, on the loss of K^+ and Cl^- subsequent to the elevation of

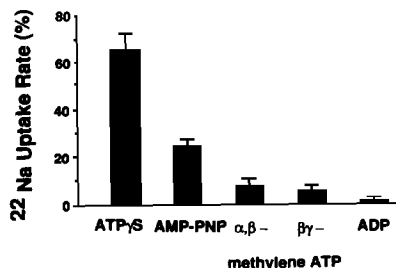


FIGURE 12. The effects of magnesium-free nucleotides (1 mM) on $^{22}Na^+$ uptake into rat parotid acinar cells. Cells were suspended in solution A to which 2 mM EDTA was added ~3 min before 1 mM of the nucleotides was added. $^{22}Na^+$ uptake rates were measured in the presence of ouabain (2.5 mM), and the results were normalized to the difference between the ATP-stimulated rate and the uptake rate measured in the presence of ouabain alone. Shown are the results obtained from three or four preparations.

$[Ca^{2+}]_i$. This suggested that Na^+ uptake or a portion of it might be a regulatory response initiated by the rapid reduction of cell volume produced by the loss of K^+ and Cl^- , which is similar to that seen in other cells (Grinstein et al., 1984). Therefore, the ATP-stimulated Na^+ uptake was measured in the presence of TEA (15 mM) to reduce the efflux of K^+ . The rate of Na^+ uptake was reduced by $20.0 \pm 2.2\%$ (3), an effect much less than the 60% reduction of the response to carbachol (Soltoff et al., 1989). This was not surprising, since the reduction of cell volume by ATP (15%) was less than that produced by carbachol (25%) and since ATP promoted Na^+ uptake through a pathway(s) distinct from those stimulated in response to carbachol (see above).

Effects of BAPTA on Intracellular Ion Content

In the experiments reported in this paper, cells were loaded with BAPTA to buffer $[Ca^{2+}]_i$. Loading the cells with BAPTA also appeared to alter the ion content of the cells. When BAPTA-treated cells were compared with nontreated cells from the same preparation, the Cl^- content was reduced by 32.6 ± 4.0 (4) nmol/mg protein, the K^+ content was increased by 60.2 ± 24.0 (8), and the Na^+ content was reduced by 19.8 ± 3.6 (9). This suggests that when intracellular esterases hydrolyze BAPTA-AM to its free acid form, BAPTA may function as an impermeant anion in the cytosol and substitute for intracellular Cl^- .

DISCUSSION

ATP and carbachol both initiated a rapid increase in $[Ca^{2+}]_i$ and stimulated the net efflux of K^+ and Cl^- and the net influx of Na^+ . Although the overall activation of ion fluxes in response to ATP and carbachol seemed similar, distinct differences in the responses of the rat parotid acinar cell to the two agonists were observed when inhibitors were used to quantitate the activation of specific ion transport systems.

Extracellular Ca^{2+} Contributes to the Elevation of $[Ca^{2+}]_i$ by ATP

While ATP was at least as effective as carbachol in elevating $[Ca^{2+}]_i$ (Fig. 1 A), the initial increase in $[Ca^{2+}]_i$ promoted by ATP was primarily due to the activation of an influx of Ca^{2+} across the plasma membrane, and the initial effects of the "classical" (phospholipase C linked) neurotransmitter agonists were primarily due to the release of Ca^{2+} from intracellular stores. Consistent with these findings, ATP was much less effective in elevating $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Fig. 1 B), but the mechanism for the release of intracellular Ca^{2+} by ATP is unclear. Unlike the phospholipase C-linked agonists (Aub and Putney, 1985; Merritt and Rink, 1987; McMillian et al., 1988), ATP did not substantially increase $InsP_3$ production (McMillian et al., 1988). Extracellular ATP must release Ca^{2+} from intracellular stores via a different intracellular second messenger in the parotid acinar cell, although effects of extracellular ATP on $[Ca^{2+}]_i$ are due to an elevation of $InsP_3$ in other cells (see Introduction). Multiple signal transduction pathways for Ca^{2+} mobilization have been reported in other systems. In Swiss 3T3 fibroblasts, the bombesin- or vasopressin-stimulated elevation of $[Ca^{2+}]_i$ was mediated by an increase in the production of 1,4,5- $InsP_3$, while the platelet-derived growth factor-induced eleva-

tion of $[Ca^{2+}]_i$ was not (Lopez-Rivas et al., 1987). Other reports demonstrated that (intracellular) GTP can release Ca^{2+} from different intracellular pools insensitive to $InsP_3$ (Henne et al., 1987; Nicchitta et al., 1987; Benedetti et al., 1988), and that elevated concentrations of cytosolic Na^+ can exchange for mitochondrial Ca^{2+} . Similar pathways may be activated by ATP, although there is not yet any evidence for a specific mechanism. Of related interest, the elevation of $[Ca^{2+}]_i$ by extracellular ATP in a mouse macrophage cell line was reported to consist of two separate events, the mobilization of Ca^{2+} from intracellular stores, followed by an increase in the influx of Ca^{2+} across the plasma membrane (Greenberg et al., 1988). The early event could be elicited by various purine and pyrimidine nucleotides, which differ from our findings using parotid cells (McMillian et al., 1988).

ATP and Carbachol Activate K^+ Efflux through Multiple Pathways

ATP and carbachol both initiated the rapid efflux of K^+ (Figs. 3 and 4). Since both agonists stimulated an efflux even in the presence of TEA, an inhibitor which blocked the BK channel in patch-clamp studies of parotid acinar cells (Iwatsuki et al., 1985; McMillian et al., 1988), the activation of multiple K^+ -permeable channels must contribute to both of the effluxes. The greater relative effect of TEA in blocking the effects of carbachol suggested that the proportional contribution of K^+ efflux through the BK channel was different for the two stimuli, and that the majority of the ATP-stimulated K^+ efflux was not through the BK channel. As much as 32% of the ATP-promoted K^+ efflux may be via the BK channel, while almost 60% of the carbachol-stimulated K^+ efflux was blocked by 15 mM TEA (Table I).

These conclusions were extended by the results obtained using CTX, which blocks a variety of Ca^{2+} -sensitive K^+ channels (Anderson et al., 1988; Miller, 1988; Moczydlowski et al., 1988). CTX (100 nM) reduced the ATP- and carbachol-stimulated K^+ efflux rates by 81–84%. Similar findings were reported by Nauntofte and Dissing (1988), who found that CTX-containing venom from the scorpion *Leiurus quinquestriatus* blocked ~90% of the carbachol-induced net efflux of ^{42}K in isolated rat parotid acini. Our observation that 100 nM CTX was more effective than TEA in blocking the effects of both carbachol and ATP (Table I) suggests that CTX blocked more than one type of K^+ -permeable channel in the parotid acinar cell, including the BK channel. This is supported by other studies which reported that CTX was an effective inhibitor of a variety of K^+ channels (Moczydlowski et al., 1988; Reinhart et al., 1989). Although CTX was earlier reported to be a specific inhibitor of Ca^{2+} -activated K^+ channels (Smith et al., 1986), it has recently been observed to be effective in blocking other types of K^+ channels (MacKinnon et al., 1988). For example, two Ca^{2+} -insensitive K^+ channels in T lymphocytes were relatively insensitive to TEA but were completely blocked by 5 nM CTX (Lewis and Cahalan, 1988). The data shown in Table I indicate that both ATP and carbachol activated a CTX-sensitive, TEA-insensitive K^+ efflux in parotid cells. This pathway may be the nonselective cation channel, which is also present in the rat parotid acinar cell and was activated by carbachol and ATP (McMillian et al., 1988). Electrophysiological evidence for the presence of both the nonselective cation channel and the BK channel in exocrine secretory cells has been reported (Maruyama and Petersen, 1982, 1984; Marty et al., 1984; Petersen and Maruyama, 1984; Iwatsuki and Petersen, 1985). The data

presented in this paper indicates that not only did the agonist-promoted K^+ efflux occur through at least two K^+ -permeable channels, but that the proportional movement of K^+ through these channels was different for carbachol and extracellular ATP.

The suggestion that purinergic receptors activated ion channels in parotid cells was first made by Gallacher (1982), who observed that extracellular ATP altered the membrane potential and stimulated the efflux of ^{86}Rb from mouse parotid glands. ATP has also been observed to cause electrical changes and alterations in ion fluxes in numerous other cells (see Introduction). Extracellular ATP was observed to stimulate two different conductance pathways in bullfrog atrial cells (Friel and Bean, 1988). One conductance was a transient, nonspecific cation-selective channel which was not blocked by the removal of extracellular Ca^{2+} , and which had a reversal potential near -10 mV. The second conductance was better maintained, had a reversal potential near -85 mV, and appeared to be the same Ca^{2+} -sensitive K^+ channel that was stimulated by acetylcholine. These characteristics, as well as the nucleotide specificity ($ATP > ATP\text{-}\gamma\text{-s} > ADP$) are very similar to the results reported here, in which the TEA-sensitive efflux appears to be due to the activation of a Ca^{2+} -sensitive BK channel, and the TEA-insensitive channel may include the activation of the nonspecific cation channel. Extracellular ATP also activated a non-selective cation conductance in a mouse macrophage cell line (Buisman et al., 1988), rat vas deferens (Freil, 1988), and cultured chick myoblasts (Kolb and Wakelam, 1983), and a Ca^{2+} -sensitive K^+ channel in cultured bovine aortic endothelial cells (Sauve et al., 1988).

The removal of extracellular Ca^{2+} affected the amount of K^+ loss promoted by ATP much less than that of carbachol (Table I). This appeared paradoxical since $[Ca^{2+}]_i$ was elevated much less by ATP than by carbachol under these conditions, which severely attenuated the carbachol-stimulated K^+ release. An explanation for this effect of ATP may involve the contrast between the persistence of the small ATP-induced elevation of $[Ca^{2+}]_i$ (Fig. 1 B) and the larger but transient elevation in response to carbachol in the absence of extracellular Ca^{2+} (Fig. 1 C). In patch-clamp studies of these cells, we found a TEA-insensitive ion channel that appeared to be activated by relatively small increases in $[Ca^{2+}]_i$ (McMillian et al., 1988). After application of either ATP or carbachol, this channel opened before the BK channel, suggesting that the former was activated at lower concentrations of $[Ca^{2+}]_i$. The small, long-lasting elevations of $[Ca^{2+}]_i$ induced by ATP in the absence of Ca^{2+} may be sufficient to keep this channel open, allowing for a larger loss of intracellular K^+ . Alternatively, the direct activation of a K^+ -permeable channel by ATP may have accounted for a portion of the efflux. However, as mentioned above, the duration of the efflux is affected by the balance between the efflux and influx pathways, and changes in the influx pathways may also alter the net loss of K^+ .

ATP Activates Ca^{2+} -sensitive Cl^- Channels

One of the initial ion transport events involved in fluid secretion is the activation of Cl^- channels. ATP promoted a rapid efflux of $^{36}Cl^-$ (Fig. 2), as did phospholipase C-linked agonists and ionomycin (Soltoff et al., 1989). The reduced efflux from BAPTA-loaded cells suggests that ATP stimulated a Ca^{2+} -sensitive Cl^- channel.

Other investigators using a similar parotid preparation reported evidence for a cholinergic or Ca^{2+} -activated Cl^- channel by measuring changes in intracellular $^{36}Cl^-$ (Nauntofte and Poulsen, 1986; Melvin et al., 1987) or by whole-cell patch-clamp recordings of rat and mouse parotid glands (Iwatsuki et al., 1985). Due to its effects on $[Ca^{2+}]_i$, ATP appeared to activate the same Ca^{2+} -sensitive Cl^- channel as did phospholipase C-linked agonists. Since specific inhibitors of this channel are not yet available, we cannot rule out an alternative mechanism for the Cl^- efflux, such as loss through a coupled Cl^- -cation cotransport system.

ATP Stimulates Na^+ Accumulation through at Least Three Pathways

ATP and carbachol produced large increases in the Na^+ content of the rat parotid cells (Fig. 7). The effects of various inhibitors on the rate of Na^+ uptake indicated that a portion of the ATP-stimulated Na^+ entry was mediated via the $Na^+-K^+-2Cl^-$ cotransport system and Na^+-H^+ exchange at rates similar to those induced by ionomycin and carbachol. However, the uptake of Na^+ through these two pathways accounted for less than half of the ATP-stimulated Na^+ entry (Table II), while it accounted for 72 and 31% of the carbachol- and ionomycin-stimulated uptakes, respectively (Soltoff et al., 1989). Extracellular ATP also activated Na^+-H^+ exchange and elevated intracellular Na^+ in Ehrlich ascites tumor cells (Weiner et al., 1986). Unlike our studies using parotid cells, the effects of ATP on these and other responses in Ehrlich ascites cells were at least partially mediated via phosphatidylinositol turnover and the activation of protein kinase C.

Two possibilities for additional pathways of Na^+ entry into parotid cells include the nonselective cation channel and an ATP-gated channel. Measurements made in cell-attached patch-clamp studies indicated that an inward current activated by carbachol was quantitatively similar to that activated by ATP and was dependent on extracellular Na^+ (McMillian et al., 1988). The influx of Na^+ through this channel may represent the portion (≤ 75 nmol/mg per min) of the carbachol-stimulated $^{22}Na^+$ uptake that was insensitive to furosemide and DMA. If the ATP-stimulated uptake of Na^+ through this pathway was quantitatively similar to that of carbachol, as was observed for the $Na^+-K^+-2Cl^-$ cotransport and Na^+-H^+ exchange uptake pathways, the sum of these three rates still does not account for the entire ATP-stimulated Na^+ uptake. Recently, Benham and Tsien (1987) reported that extracellular ATP directly activated a cation channel in arterial smooth muscle cells that was permeable to both Na^+ and Ca^{2+} ions. Such a channel may account for a substantial portion of the furosemide- and DMA-insensitive Na^+ uptake as well as for the large influx of Ca^{2+} produced by ATP. The lack of specific channel blockers makes it difficult to state with certainty whether a portion of the ATP-stimulated uptake was through a nonselective cation channel or an ATP-gated channel, and must await additional electrophysiological studies.

Ecto-ATPase Activity

The ATP-consuming activity on the plasma membrane of intact cells (Fig. 6) may serve as a mechanism to limit the effects of extracellular ATP. If ATP is released in neurotransmitter-like fashion from a neuronal source, hydrolysis may terminate its action. Presumably, analogous to other ATPases, the substrate for this enzyme is

MgATP. Although ATP has been reported to be costored without Mg^{2+} with neurotransmitters, Mg^{2+} in the plasma would also limit the potential effects of ATP^{4-} on a purinergic receptor. Under the conditions of our assays, we could not distinguish whether the disappearance of ATP in the presence of intact cells was due to an ectokinase, which could use ATP to phosphorylate a protein on the outer membrane, or to an ATPase which could use the energy of ATP for some other function in addition to removing ATP as a stimulus and limiting the duration of its effectiveness. In the experiments presented here, the consumption of extracellular ATP was especially noticeable in measurements in which a large number of cells were used. In the K^+ flux measurements, 1 mM ATP was added to a suspension of up to 5 mg cellular protein per ml. The ecto-ATPase activity at 1 mM ATP was ~ 100 nmol/mg per min, which would reduce the added ATP by 50% in 1 min. In the cell volume studies, which were conducted at 0.75–1 mg/ml, it would take a longer period of time to reduce ATP to a comparable degree. The restoration of cell volume toward normal levels 10 min after the addition of ATP may be initiated by the diminishment of extracellular ATP.

The Effects of ATP Are Mediated by a Purinergic Receptor Sensitive to ATP^{4-} and Blocked by DIDS

The stimulation of K^+ efflux and Na^+ influx had a high selectivity for ATP over other nucleotides or adenosine compounds. Only ATP- γ -S produced substantial effects, similar to the specificity seen in elevating $[Ca^{2+}]_i$ (McMillian et al., 1987a). ATP^{4-} rather than MgATP appeared to be the active nucleotide moiety, since half-maximal stimulations were obtained with less than one-tenth as much ATP when Mg^{2+} was not present. In addition, other analogues (especially the nonhydrolyzable AMP-PNP) were effective in the absence of Mg^{2+} . This suggests that ATP does not elicit its effects via the activation of ATPases and kinases, which use ATP as a substrate. This hypothesis is further supported by the effectiveness of ATP- γ -S, an unlikely substrate for ecto-ATPases/nucleotidases. Thus, the effects of ATP appear to be mediated through a purinergic receptor that is sensitive to ATP^{4-} .

DIDS appears to block the effects of ATP on all of the physiological responses by preventing the binding of ATP to a site on the plasma membrane (McMillian et al., 1988). The nucleotide specificity suggests that DIDS blocks the binding of ATP^{4-} to a P_2 -type purinergic receptor. DIDS did not block the elevation of $[Ca^{2+}]_i$ by phospholipase C-mediated secretagogues (McMillian et al., 1988), indicating that the intracellular events that couple activation of these receptors to the elevation of $[Ca^{2+}]_i$ were not DIDS sensitive.

CONCLUSIONS

The results presented here have characterized the elevation of $[Ca^{2+}]_i$ and the activation of Cl^- , K^+ , and Na^+ fluxes by the muscarinic agonist carbachol and by extracellular ATP, which appears to activate a purinergic receptor on the rat parotid cell. Extracellular Ca^{2+} was the major source for the large elevation in $[Ca^{2+}]_i$ promoted by ATP, while the release of Ca^{2+} from intracellular stores accounted for the initial elevation of $[Ca^{2+}]_i$ promoted by carbachol. Carbachol and ATP both activated the

efflux of K^+ through multiple K^+ -permeable pathways, but the proportion of K^+ efflux through the different pathways varied for the two agonists. In addition to efflux through the TEA-sensitive BK channel, substantial K^+ loss occurs through a CTX-sensitive pathway, which may include the nonselective cation channel. These studies elucidate the ionic events that initiate fluid secretion in response to neurotransmitters in this tissue, and also suggest that regulation may be exerted by purinergic receptors as well as the classical neurotransmitter receptors. Although the net effects of the different agonists shared some features, the effects of extracellular ATP were not coupled to phospholipase C activation (McMillian et al., 1988). Thus, the neuronal release of ATP may participate in the control of fluid secretion in the parotid acinar cell.

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