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Two distinct cytosolic calcium responses to extracellular ATP in rat parotid acinar cells

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1 Increasing concentrations of ATP (0.5 μM–300 μM) produced a biphasic increase in intracellular calcium concentration $[Ca]_i$ in rat parotid acinar cells, reflecting two distinct Ca_i responses to extracellular ATP.

2 In the absence of Mg^{2+} (with 3 mM $CaCl_2$ in the buffer solution), the more sensitive response was maximal at 3–5 μM and was not further increased by 30 μM ATP. This response to ATP was not well maintained and was blocked by ADP (0.5 mM). A second, much larger increase in Ca_i was observed on addition of 300 μM ATP. This larger effect, which we have described previously, appears to be mediated by ATP^{4-}, and was selectively reversed by 4,4'-di-isothiocyanato-dihydrostilbene-2,2'-disulphonate as well as by high concentrations of α,β-methylene ATP.

3 Among ATP analogues, only the putative P_{2Z} agonist, 3'-0-(4-benzoyl)benzoyl-ATP distinguished between the two responses. This analogue was at least 10 fold more potent than ATP in stimulating the ATP^{4-}-response, but did not evoke the more sensitive response. The agonist potency series for both responses to ATP was identical for other analogues examined (ATP > ATPγS = 2-methylthio ATP (a P_{2Y}-selective agonist) >> ADP, ITP and α,β-methylene ATP (a P_{2X}-selective agonist)).

4 Although the effect of ATP^{4-} could best be characterized as a P_{2Z}-type purinoceptor response, this effect was strongly and selectively blocked by reactive blue 2, a putative P_{2Y}-purinoceptor antagonist. Reactive blue 2 may bind to and block P_{2Z} purinoceptors since [γ^{32}P]-ATP binding to parotid cells was inhibited by this compound.

5 In contrast to the response to ATP^{4-}, the more sensitive response to ATP was potentiated by reactive blue 2 and was less affected by increases in external Mg^{2+} and Ca^{2+}.

6 Parasympathetic denervation selectively increased the more sensitive response, suggesting that it may be physiologically regulated.

Keywords: Purinergic; P_{2Z}-receptor; rat parotid cells; intracellular calcium; nonspecific cation channel; benzoylbenozyl ATP; ATP responses; 4,4'-diisothiocyanato stilbene-2,2'-disulphonate (DIDS); reactive blue 2; parasympathetic denervation

Introduction

ATP is stored and released with biogenic amine neurotransmitters and may itself act as a neurotransmitter or neuromodulator (Burnstock, 1986; Evans *et al.*, 1992). Extracellular ATP elevates the intracellular free calcium concentration $[Ca]_i$ in many tissues, in some cases by activating phospholipase C (Boyer *et al.*, 1989; Boyer & Harden, 1989), and in others by directly gating calcium-permeable ion channels (Benham & Tsien, 1987; Friel & Bean, 1988; Diverse-Pierluissi *et al.*, 1991). ATP-stimulated increases in Ca_i may modulate physiological responses and provide a simple index for characterizing ATP receptors and their mechanisms of action.

Previously we reported that ATP specifically and reversibly elevates Ca_i in rat parotid acinar cells in a manner consistent with activation of P_2-purinoceptors (McMillian *et al.*, 1987a; 1988; Soltoff *et al.*, 1989; 1990a). The effect of ATP on Ca_i is inhibited by increasing the concentration of divalent cations, suggesting that ATP^{4-} is the active species (McMillian *et al.*, 1987a). This purinoceptor effect is reversed on breakdown of ATP to ADP (McMillian *et al.*, 1987a; 1988). We have shown previously that the calcium response in rat parotid acinar cells is not activated by most ATP analogues (McMillian *et al.*, 1987a) and that non-hydrolyzable analogues and ADP are inactive or only weakly active on ATP-stimulated ion fluxes as well (Soltoff *et al.*, 1990b). Pretreatment of cells with 4,4'-diisothiocyanato stilbene-2,2'-disulphonate (DIDS) specifically inhibits the ATP response in parotid cells and also inhibits [α^{32}P]-ATP binding, consistent with an action of DIDS at the parotid ATP receptor complex (McMillian *et al.*, 1988; Soltoff *et al.*, 1990b). ATP elevates Ca_i in parotid cells by a mechanism which differs from that shared by muscarinic, substance P receptors and α-adrenoceptors, all of which activate phospholipase C. ATP has little effect on [^{3}H]-inositol phosphate accumulation, and electrophysiological effects of ATP do not require guanine nucleotide binding proteins, in contrast to muscarinic effects (McMillian *et al.*, 1988; Soltoff *et al.*, 1990a). Effects of ATP on Ca_i in parotid cells are additive with the effects of other calcium-mobilizing agonists, indicating that ATP mobilizes a calcium pool distinct from that mobilized by phospholipase C-linked receptor agonists (McMillian *et al.*, 1988; Soltoff *et al.*, 1990a). A similar phospholipase C-independent increase in Ca_i in response to ATP has been reported in a number of other cell types (Richards *et al.*, 1987; Buisman *et al.*, 1988; El-Moatassim *et al.*, 1989; Sasaki & Gallacher, 1990; Li *et al.*, 1991). ATP has a much weaker effect on amylase release from parotid cells than do muscarinic and α-adrenoceptor agonists and substance P, probably due to its failure to activate protein kinase C, which may play an important role in exocytosis in these cells (McMillian *et al.*, 1988). The mechanism of action of ATP in parotid cells is unknown, but present data are consistent with an ATP-gated non-selective cation channel (Soltoff *et al.*, 1992).

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ATP receptors (P_2-purinoceptors) differ from adenosine receptors (P_1-type), being much less sensitive to blockade by methylxanthines (Burnstock, 1986). There appear to be several subtypes of ATP receptors (Gordon, 1986). Smooth muscle P_2-purinoceptors have been subdivided into P_{2x} and P_{2y} types on the basis of differential sensitivity to the agonists, α,β-methylene ATP and 2-methylthio ATP, and to the antagonists arylazidoaminoproprionyl ATP ($ANAPP_3$) (Fedan et al., 1985) and reactive blue 2 (Burnstock & Warland, 1987; Houston et al., 1987), respectively. P_{2x}-receptors respond with a potency order α,β-methylene ATP > ATP > 2-methylthio ATP; these receptors rapidly desensitize in response to α,β-methylene ATP (and to a lesser extent with ATP) and also appear more sensitive to inactivation by $ANAPP_3$ (Burnstock & Kennedy, 1985). In contrast, P_{2y}-receptors display the reverse potency order and are more sensitive to blockade by reactive blue 2 (Houston et al., 1987). A third type of purinoceptor, designated P_{2z}, requires uncomplexed ATP (ATP^{4-}) for activation; in physiological buffer solutions most of the ATP is complexed with divalent cations and consequently higher concentrations of ATP are required to stimulate this receptor (Cockroft & Gomperts, 1980; Gordon, 1986). The P_{2z}-receptor appears to be coupled to cellular permeabilization to solutes of a restricted size in mast cells (Tatham et al., 1988; Greenberg et al., 1988), and in some cell lines (Weisman et al., 1984; Gonzalez et al., 1989), but this does not appear to be the case in rat parotid acinar cells (McMillian et al., 1988). It has been suggested that benzoylbenzoyl ATP [3'-0-(4-benzoyl)benzoyl-ATP] is a selective P_{2z} agonist (Erb et al., 1990), although recent evidence suggests that this compound also activates P_{2y}-receptors (Boyer & Harden, 1989).

The Ca_i response to ATP in parotid cells is characterized by a broad cumulative concentration-response curve. A small response is apparent at less than 5 μM ATP (regardless of Mg^{2+} concentration) and a larger effect is obtained only at much higher concentrations of ATP (50–300 μM, depending on Mg^{2+} concentrations) (McMillian et al., 1987a; 1988). Recently, with a change in collagenase and trypsin lots, our parotid cell preparations show higher sensitivity to ATP. In the present study we examined the characteristics and pharmacology of the more sensitive Ca_i response to ATP and further characterized the response to ATP^{4-}, enabling us to assign these responses to subtypes of the P_{2z}-purinoceptor class. These results have recently been published as an abstract (McMillian et al., 1990).

The physiological responses to neurotransmitter stimulation of rat parotid acinar cells are altered by denervation of the gland. After parasympathetic denervation of the parotid gland, a heterologous supersensitivity develops to carbachol, noradrenaline and substance P (Ekstrom, 1980; Ekstrom & Wahlestedt, 1982). The effect of parasympathetic denervation on the response to ATP also was investigated to see whether the purinoceptor response is similarly regulated.

Methods

Cell preparations

Parotid glands were removed from male Sprague/Dawley rats (Taconic Farms, Germantown, NY; 150–250 g) and acinar cells were prepared as previously described (McMillian et al., 1988). The small, more sensitive response to ATP became much larger when different dispersing enzyme lots were used (Worthington collagenase CLS1 No. 4177, lot No. 49A095) and trypsin (Sigma, Cat No. T-8253, lot No. 18F-0828). Previous studies were on cells prepared with collagenase (Worthington, CLS Type 2, No. 14174, lot No. 48E295) and trypsin (Sigma); some data obtained with earlier preparations are included since a relatively pure response to ATP^{4-} was obtained. For supersensitivity studies, unilateral parasympathetic postganglionic denervations were performed as previously described (Talamo et al., 1979), and the cells were prepared from denervated and contralateral control parotid gland three to five weeks later.

Ca_i measurements

Cells were loaded with fura-2 acetoxymethyl ester as previously described (McMillian et al., 1987b), and suspended in appropriate medium in a cuvette, with continuous stirring. Ca_i was estimated by determining the ratio of fluorescence emitted at 505 nm when excited at 340 and 380 nm, assuming a K_d of 224 nM for fura-2. Ratios were determined on a Perkin-Elmer LS5 spectrofluorimeter equipped with a computer and a fura-2 programme which allowed ratios to be obtained every 7–8 s. Drug additions were made to the cuvette on-line, with continuous recording. In each run, a correction was made for dye leakage by addition of 250 μM $MnCl_2$ which was then chelated with diethylenetriamine penta acetic acid (DTPA, 1 mM); maximum fluorescence was defined by the addition of digitonin (50 μM) and minimum fluorescence was determined with 100 mM EGTA (McMillian et al., 1987a,b). In some experiments, fluorescence was directly recorded during excitation at 340 nm. In all experiments where Mg^{2+} was added to reverse the effects of ATP, the concentration of Mg^{2+} was 10 mM. All experiments were repeated at least two times with similar results, or more times as indicated.

[$\gamma^{32}P$]-ATP binding

Binding of [$\gamma^{32}P$]-ATP to parotid cells was determined as previously described for [$\alpha^{32}P$]-ATP (McMillian et al., 1988). When present, reactive blue 2 was preincubated with cells for 10 min prior to initiation of the binding reaction with radiolabelled ATP. Binding was carried out for 10 min and cells were then sedimented and washed. Triplicate assays were carried out for each condition in each experiment.

Materials

$ANAPP_3$ was a kind gift from Dr J.S. Fedan; ryanodine, octanol and hexanol were gifts from Dr K. Dunlap. MK801, ketamine and [$\gamma^{32}P$]-ATP (New England Nuclear) were gifts from Drs C. Harrington, J. Kauer & D. Chikaraishi, respectively. 2-Methylthio ATP was purchased from Research Biochemicals Incorporatated; DIDS and 4,4'-diisothiocyanato-dihydrostilbene-2,2'-disulphonate (dihydroDIDS) were from Molecular Probes; ATP and adenosine-5'-O-(3-thiotriphosphate) (ATPγS) were from Boehringer Mannheim. DTPA was obtained from Fluka Chemie AG. Reactive blue 2 (Cibacron blue), benzoylbenzoyl ATP, ATP dialdehyde (adenosine 5'-triphosphate-2',3'-dialdehyde) and α,β-methylene ATP and other drugs were obtained from Sigma, unless otherwise indicated.

Results

Biphasic Ca_i response to ATP

The Ca_i response to ATP in rat parotid cells was characterized by a broad concentration-response curve, even under conditions optimal for eliciting the effect of ATP^{4-} (1 mM $CaCl_2$, 0 $MgCl_2$). At a higher calcium concentration (3 mM $CaCl_2$, 0 $MgCl_2$), a biphasic cumulative concentration-response curve for ATP was clearly evident (Figure 1a). A small, potent ATP-effect (half-maximal at about 1 μM, maximal at about 3–5 μM) could be distinguished from the effect of ATP^{4-} (which became apparent when the concentration of ATP was increased from 60 to 300 μM) (Figure 1a). The rate of increase in Ca_i in cells exposed to concentrations of ATP higher than 300 μM was slower than the response to low concentrations of ATP. While the Ca_i response to low con-

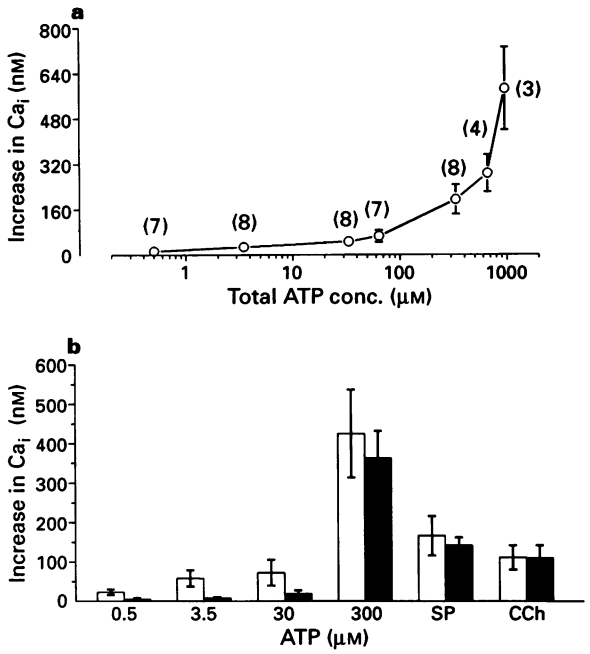

Figure 1 (a) ATP concentration-response curve for Ca_i elevation: The increase in Ca_i over baseline (mean ± s.e.mean) was determined for cumulative ATP concentrations ranging from 0.5–963 μM. At each data point, the number of independent experiments is shown in parentheses. For all experiments, the maximum reached within the first 3 data points (about 22 s) after addition of agonist is taken, although the Ca_i continues to rise slowly for ATP concentrations greater than 300 μM. The calculated concentration of ATP^{4-} (3 mM Ca^{2+}, 0 mM Mg^{2+}) (Fabiato, 1988) is given in parentheses following each value of total added ATP: 0.5 μM ATP (0.023 μM ATP^{4-}), 3.5 μM (0.14 μM), 33.5 μM (1.6 μM), 63 μM (3 μM), 363 μM (19 μM), 663 μM (38 μM), 963 μM (61 μM). Higher concentrations of ATP were not tested because substantial amounts of extracellular calcium are chelated. (b) Effect of ADP on Ca_i responses to ATP, carbachol (CCh) and substance P (SP): The Ca_i response to various agonists was determined in the presence and absence of ADP (0.5 mM). Peak responses are shown for each agonist, $n = 3$ experiments. Ca^{2+} and Mg^{2+} concentrations were 3 and 0 mM respectively. Open columns are controls. Solid columns show responses in the presence of ADP. Note that 30 μM ATP is not significantly more effective than 3.5 μM ATP and that ADP blocks effects of low concentrations of ATP but not effects of 300 μM ATP or of SP (0.1 μM) or CCh (100 μM).

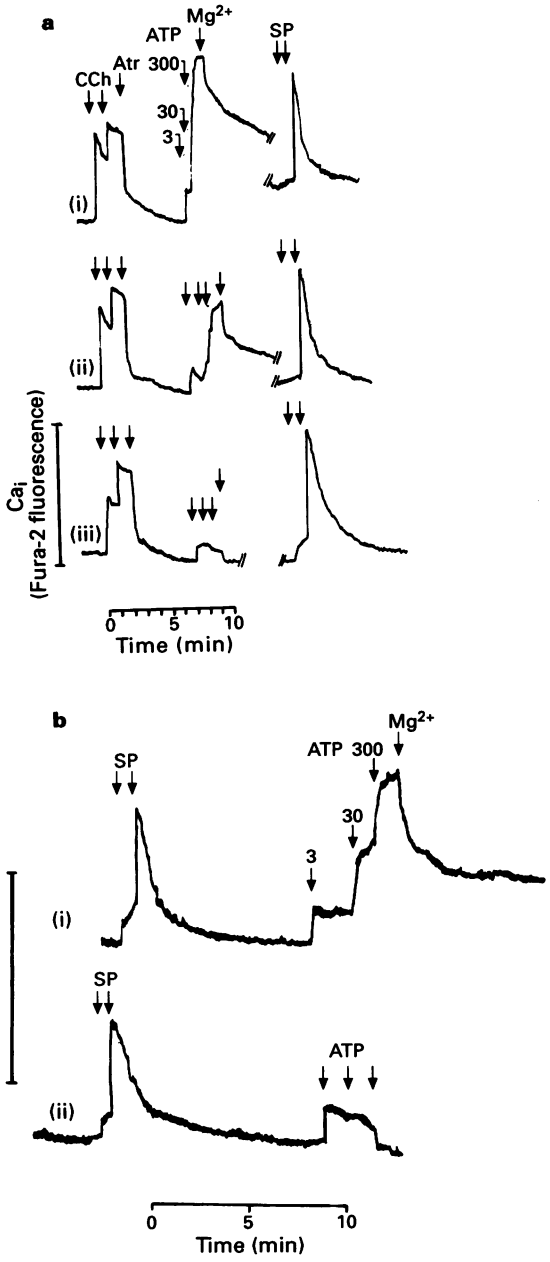

Figure 2 Separation of the two ATP-induced Ca_i responses with (a) Mg^{2+} and (b) 4,4'-diisothiocyanato stilbene-2,2'-disulphonate (DIDS). Ca_i responses to different agonists were tested in the presence of various concentrations of Mg^{2+} or DIDS. Ca^{2+} concentration was 1 mM in each experiment. Where additions are indicated by arrows, the concentrations are: carbachol (CCh) = 3 μM; 100 μM. Atropine (Atr) = 10 μM. Substance P (SP) = 30 pM; 100 nM. Mg^{2+} = 10 mM. ATP concentrations (in μM) are indicated in the figure. Agonists are added in the same sequence in each experiment. These traces are representative of ten or more experiments. (a)i is the control. Mg^{2+} concentrations in the cell suspension medium are: (i) 0; (ii) 1 mM; (iii) 10 mM. Breaks in traces represent time for the ATP response to reverse after high Mg^{2+} addition. This break represents 20 min for (i), 5 min for (ii), and 0 min for (iii). The presumed effect of ATP^{4-} is blocked by increasing concentrations of Mg^{2+}, while the responses to a low concentration of ATP, and CCh and SP responses are unaffected. (b) DIDS selectively blocks the effect of a high concentration of ATP while a low concentration of ATP, and SP, are unaffected. (b)(i) is the control; (b)(ii) Cells were pretreated with 150 μM DIDS for 40 min and washed before assay.

centrations of ATP reached a peak value within 15–23 s, the response to high concentrations continued to increase. We usually added Mg^{2+} within 45–60 s after the addition of concentrations of ATP higher than 300 μM, because reversal was slowed at these high concentrations and because cell damage ensued after prolonged exposure to high Ca_i . The responses in Figure 1a were calculated as the maximal increases in Ca_i within the first 22 s (3 data points) of addition of ATP rather than the maximal elevation in Ca_i after prolonged exposure to the agonist. The maximum peak Ca_i value of the more sensitive response to ATP was typically smaller than the Ca_i responses to maximal concentrations of substance P or the muscarinic agonist, carbachol. These phospholipase C-linked receptor agonists served as useful controls in these studies. Additional manipulations with various agents also demonstrated that the two responses could be distinguished. ADP (0.5 mM), which produced a much smaller Ca_i increase (6 ± 6 nM, $n = 4$) than did 3.5 μM ATP (75 ± 27 nM, $n = 4$) in the same cell preparation, blocked the potent effect of ATP without obviously affecting the Ca_i response to ATP^{4-} (or carbachol or substance P) (Figure 1b). Even when the responses to 3 μM carbachol and 3 μM ATP were of the same magnitude (Figure 4a), ADP blocked only the response to ATP (Figure 4f). The more sensitive response to ATP was not blocked by high concentrations of Mg^{2+} (Figure 2a) (nor Ca^{2+} (Figure 4e)), which lowers the free ATP^{4-} concentration. The concentration of ATP^{4-} available at 3, 30 and 300 μM total ATP in the presence of 1 mM Ca^{2+} and 0, 1 or 10 mM Mg^{2+} was calculated to be (0.36, 3.6,

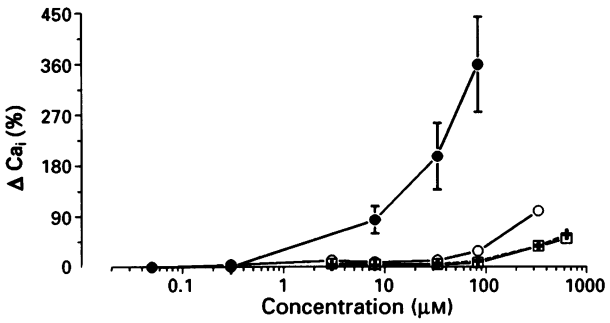

Figure 3 Benzoylbenzoyl ATP is a selective agonist for the ATP^{4-} response. ATP and various analogues were tested at different concentrations for their ability to elevate Ca_i. Each point represents the mean and s.e.mean (vertical bars) of three or more independent experiments in the presence of 1 mM Ca^{2+} and no added Mg^{2+}. Where error bars are not shown, they fall within the borders of the symbol. The increment in Ca_i (Δ Ca_i (%)) is given relative to the response to 300 μM ATP, which is set at 100%. Symbols are: benzoylbenzoyl ATP (●); ATP (○); ATPγS (+); 2-methylthio ATP (□). Benzoylbenzoyl ATP was more potent and more effective at increasing Ca_i than ATP or other ATP analogues (2-methylthio ATP and ATPγS). ATP and other analogues produced a small increase in Ca_i at low concentration, evidenced by the long, shallow concentration-response curve. Benzoylbenzoyl ATP was ineffective in stimulating the more sensitive response and the concentration-response curve was thus much steeper than that of other analogues examined.

43 μM), (0.15, 1.3, 15 μM) or (0.02, 0.19, 2.0 μM) respectively (Fabiato, 1988). Additionally, the more sensitive ATP response was not blocked by pretreatment of cells with DIDS (Figure 2b).

Effects of ATP analogues

Analogues of ATP were examined for effects on Ca_i. Among those analogues tested, only benzoylbenzoyl ATP was more potent than ATP as a stimulus for the response to ATP^{4-}. The maximum effective concentration of benzoylbenzoyl ATP could not be determined in these experiments since concentrations much higher than 30 μM quenched the fura-2 signal. Under conditions that favoured the effect of ATP^{4-} (0 Mg^{2+}, 0.5–1 mM Ca^{2+}), this putative P_{2z}-purinoceptor agonist was at least 30 fold more potent than ATP, which in turn was approximately 10 fold more potent than the P_{2y} agonist, 2-methylthio ATP (Figure 3). Benzoylbenzoyl GTP (0.3 μM–300 μM) was ineffective ($n = 2$). ATPγS was approximately equipotent to 2-methylthio ATP. It was not possible to examine a full range of concentrations for some of the analogues on the ATP^{4-}-response because extracellular calcium was complexed at high concentrations of analogues, but ADP, ITP, GTP and 8-Br ATP had little effect even at 1 mM (<5% of the response to 300 μM ATP; $n = 3$ or more for each compound). α,β-Methylene ATP had no effect on Ca_i in parotid cell suspensions ($n = 7$).

Under conditions selective for the more sensitive response (10 mM Ca^{2+} and 0 Mg^{2+}, or 300 μM DIDS pretreatment), ATP was more effective than any of the analogues. Benzoylbenzoyl ATP (0.3–30 μM) had no stimulatory effect ($n = 4$ cell preparations). Although it was difficult to quantify due to the small magnitude of the more sensitive effect, the potency order for other analogues appeared similar to that found for ATP^{4-}. Concentrations of 30–100 μM ATPγS and 2-methylthio ATP were required to produce effects similar to that obtained with a maximally effective concentration of ATP (3 μM). At 1 mM, ITP and ADP were 10–20% as effective but 8-Br ATP appeared inactive ($n = 3$ preparations for each compound). The non-hydrolyzable analogue adenosine 5'-(β,γ-imido)triphosphate (AppNHp) was approximately 10% as effective as ATP on both Ca_i responses at concentrations 10 fold higher than that required for maximal ATP effects ($n = 3$). Compounds known to block purinoceptor responses in other systems, $ANAPP_3$ (1 mM), 8-azido ATP (1 mM) and 5'-fluorosulphonyl-benzoyladenosine (5 mM), had no effect on either response to ATP in parotid cells even after activation with u.v. light ($n = 3$ or more experiments with each compound).

Reactive blue 2 selectively blocks the ATP^{4-} effect

Reactive blue 2, a putative P_{2y}-type purinoceptor inhibitor, was found to block specifically the response to ATP^{4-} under conditions which allowed the two responses to ATP to be easily separated (3 mM Ca^{2+}, 0 Mg^{2+}) (Table 1). This blocking effect is readily apparent in Figure 4. Reactive blue 2 (Figure 4b,c,d) or high Ca^{2+} (10 mM) (Figure 4e) selectively decreased the effect of high concentrations of ATP while ADP (Figure 4f) selectively blocked the effect of low concentrations of ATP. At the high concentrations of reactive blue 2 (10 and 30 μM) the response to carbachol also was inhibited in this experiment, although the effect is not significant for the pooled data of Table 1. Reactive blue 2 did reduce the

Table 1 Effect of reactive blue 2 on Ca_i response to ATP and other calcium-mobilizing agonists

		Reactive blue 2 concentration [μM]							
		0	*(n)*	3	*(n)*	10	*(n)*	30	*(n)*
ATP	0.5 μM	23 ± 7	(5)	51 ± 15	(4)	50 ± 19	(4)	57 ± 22	(5)
	3.5 μM	58 ± 21	(6)	93 ± 20	(5)	100 ± 25	(5)	97 ± 26	(6)
	30 μM	73 ± 33	(4)	135 ± 17	(3)	131 ± 34	(3)	103 ± 37	(4)
	300 μM	461 ± 110	(6)	306 ± 45	(5)	211 ± 33*	(5)	166 ± 39*	(6)
Δ (300 μM)–(3.5 μM)		403 ± 92		213 ± 45*		111 ± 27*		68 ± 20*	
(% inhibition)		(0)		(17 ± 22)		(48 ± 15)		(75 ± 6)	
Substance P (10^{-7} M)		167 ± 50	(6)	155 ± 39	(5)	207 ± 43	(5)	211 ± 40	(6)
Carbachol (10^{-4} M)		112 ± 31	(6)	107 ± 22	(5)	104 ± 19	(5)	95 ± 14	(6)

Numbers represent mean ± s.emean increase in Ca_i (nM) over basal levels which averaged 252 ± 70 nM, 269 ± 51 nM, 288 ± 44 nM, and 300 ± 48 nM at 0, 3, 10 and 30 μM reactive blue respectively. Δ (300 μM)–(3.5 μM) indicates the increment in Ca_i at 300 μM ATP above the value at 3.5 μM ATP, an indication of the response to ATP^{4-}. (*n*) = number of cell preparations. Extracellular calcium was 3 mM; no Mg^{2+} was added. Ca_i changes were estimated by the ratio method.
*$P < 0.05$ when compared to response in the absence of reactive blue 2 by Newman-Keul's multiple range test. Although reactive blue 2 tended to increase Ca_i responses to low concentrations of ATP (responses to 30 μM ATP or less were increased to 241 ± 44% of the responses observed in the absence of reactive blue 2), no significant potentiation was observed for any one concentration of ATP or reactive blue 2 (Newman-Keul's multiple range test).

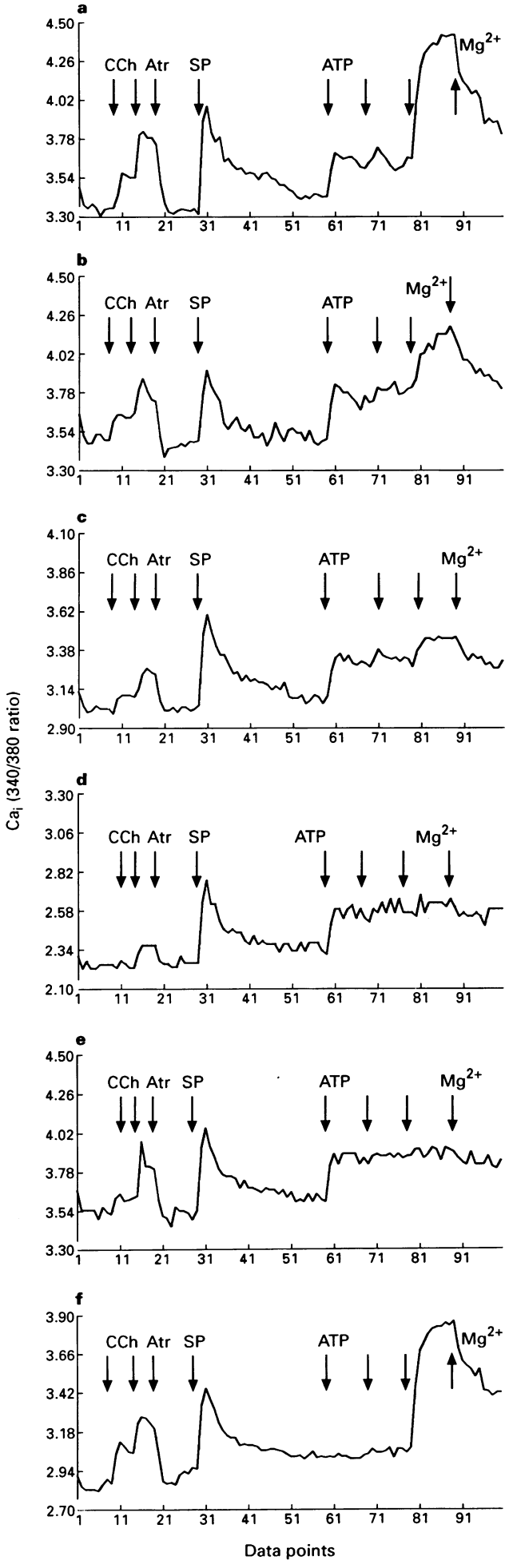

Table 2 Reactive blue 2 displaces [γ^{32}P]-ATP binding to parotid cells.

Reactive blue 2 (μM)	*[γ^{32}P]-ATP bound* (% remaining)
0	100
1	87 ± 10 (4)
3	75 ± 17 (4)
10	78 ± 11 (5)
30	44 ± 11 (7)
100	26 ± 20 (4)

Displacement of [γ^{32}P]-ATP (1–3 μM, usually about 300,000 c.p.m. added) binding by reactive blue 2 was determined after subtracting any binding not displaced by 100 μM ATP (presumably trapped or other non-specific binding: average = 38 ± 5% of total binding, $n = 7$). The mean and s.e.mean are shown for each concentration of reactive blue 2, followed by the number of experiments shown in parentheses. $[Ca^{2+}] = 0$; $[Mg^{2+}] = 0$.

Ca_i response to 10^{-6} M carbachol from 66 ± 19 nM ($n = 3$) to 18 ± 7 nM ($n = 4$) [$P < 0.05$ by Student's 2-tailed unpaired t test]. The more sensitive response to ATP often was potentiated by reactive blue 2 (Table 1).

Reactive blue 2 displaced [γ^{32}P]-ATP binding to parotid cells (Table 2), although this effect varied with different cell preparations. In preparations with pronounced small, potent responses, reactive blue 2 appeared less effective than in preparations which showed a response only to ATP^{4-}. Even so, the antagonism by reactive blue 2 of ATP^{4-} binding (Table 2) showed a similar potency to antagonism of Ca_i responses to ATP^{4-} (Figure 4), and is consistent with blockade of purinoceptors on parotid cells.

A number of compounds known to inhibit effects of ATP and/or calcium mobilization responses in other systems failed to block either of the effects of ATP on Ca_i in parotid cells. All compounds were tested on at least two parotid cell preparations. These included inhibitors of arachidonic acid metabolism, indomethacin (50 μM) and nordihydroguiaretic acid (50 μM), the phospholipase C inhibitor, neomycin (1 mM) and the IP_3 receptor blocker, heparin (1 mg ml^{-1}), three inhibitors of calcium mobilization, ruthenium red (50 μM), caffeine (10 μM) and ryanodine (10 μM), the Ca^{2+}-channel blocker, nifedipine (50 μM), the ATP-sensitive K^+ channel blocker, tolbutamide (1 mM), other K^+-channel blockers [CsCl (15 mM), $BaCl_2$ (2.5 mM), and charybdotoxin (10 nM)], the Cl^--channel blocker picrotoxin (30 μM), glutamate channel blockers [MK801 (50 μM) and ketamine (50 μM)], the Na^+-channel activator veratridine (10 μM), Na_2SO_3 (10 mM) (a shared substituent of DIDS and reactive blue 2), and a putative gap-junction blocker, octanol (150 μM) and an ineffective control alcohol, hexanol (200 μM).

Figure 4 Reactive blue 2 selectively blocks the ATP^{4-} response: (a) control; (b) reactive blue 2, 3 μM; (c) reactive blue 2, 10 μM; (d) reactive blue 2, 30 μM; (e) Ca^{2+}, 10 mM; (f) ADP, 0.5 mM. Data points were recorded every 7–8 s. Drugs were added 10 min prior to addition of agonists, shown by arrows. Additions of the agonists and antagonists were made in the same sequence as designated in the upper panel. The traces from these experiments are representative of those summarized in Table 1. Except as noted, the concentration of Ca^{2+} = 3 mM, Mg^{2+} = 0. Prior addition of reactive blue 2 (3–30 μM) decreased the effect of higher concentrations of ATP without decreasing the response to substance P (SP) or to low concentrations of ATP (b–d). The effect of high concentrations of reactive blue 2 on the response to ATP (d) was similar to that obtained in medium with high Ca^{2+} (e). ADP selectively blocked the more sensitive effect of ATP (f). Additions: carbachol (CCh) = 3 μM, 100 μM; atropine (Atr) = 10 μM; substance P (SP) = 100 nM; ATP = 3 μM, 30 μM, 300 μM.

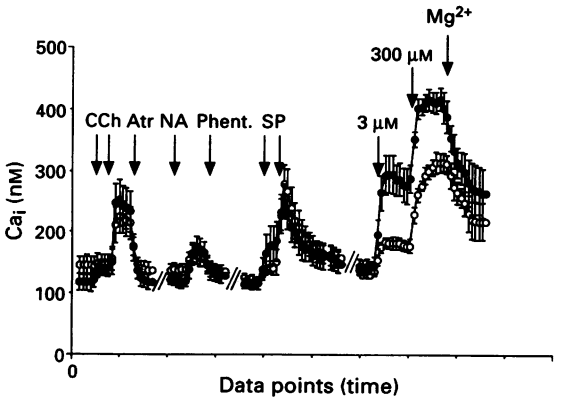

Figure 5 Parasympathetic denervation of rat parotid gland induces selective increases in the more sensitive Ca_i response to ATP: cells were prepared from glands taken from unilaterally denervated rats as described in Methods. The Ca_i responses of preparations from contralateral (unoperated) and denervated glands were compared. Each point represents the average and s.e.mean (vertical bars) of Ca_i values for denervated or contralateral control cell preparations from 4 unilaterally denervated rats; (○) control preparations; (●) denervated preparations. Arrows indicate additions of carbachol (CCh) (3 μM and 100 μM), atropine (Atr) (10 μM), noradrenaline (NA) (30 μM), phentolamine (Phent) (30 μM), substance P (SP) (0.3 and 100 nM), ATP (3 and 300 μM) and $MgCl_2$ (5 mM). Values were determined every 7–8 s; breaks between agonist additions represent 5–10 min pauses (equal for paired denervated and control runs) to allow calcium pools to recover. Note the pronounced increase in the Ca_i response to a low concentration of ATP. This increase also was observed when phospholipase C-coupled receptor agonists were not added prior to stimulation with ATP. The magnitude of the further increase in response to 300 μM ATP was similar in both control and denervated cells (130 and 115 nM respectively). Medium contained Ca^{2+} = 3 mM, Mg^{2+} = 0.

Parasympathetic denervation selectively increases the potent response to ATP

As noted in the introduction, muscarinic receptor, α-adrenoceptor and substance P receptor responses are sensitized following parasympathetic postganglionic denervation of the parotid gland. Sensitivity increases by 3 to 5 fold but there is at best only a small increase in maximal response (Figure 5). The response to 3 μM carbachol was highly variable in cells from control glands of unilaterally denervated animals, but it always was detected in denervated glands. In the course of examining possible supersensitivity to ATP^{4-}, a pronounced increase in the Ca_i response to low concentrations of ATP (3 to 5 μM) was observed (2.8 ± 0.3 fold over contralateral control cell responses, $n = 4$) (Figure 5). Higher concentrations of ATP led to further increases in Ca_i (attributable to ATP^{4-}) which were similar in cells from both denervated and contralateral control glands (Figure 5). The increased response after denervation involved only the more sensitive response, since the increase also was observed in high calcium-containing buffer and in the presence of 30 μM reactive blue 2 ($n = 2$), which eliminated the effect of ATP^{4-}.

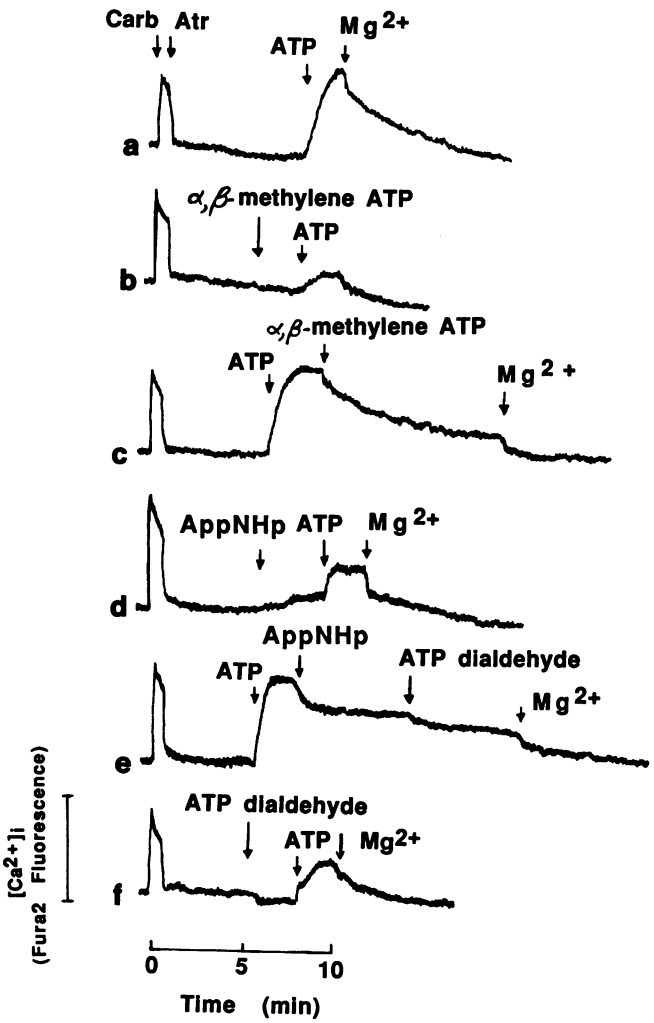

Figure 6 Reversal of the effect of ATP^{4-} by ATP analogues: Ca_i responses to carbachol (CCh) were determined and then various ATP analogues were tested (at 1 mM) for their ability to activate Ca_i responses or to block or reverse the response to ATP (50 μM). Compounds added: (a) ATP; (b) α,β-methylene ATP, then ATP; (c) ATP, then α,β-methylene ATP; (d) 5'-(β,γ-imido)triphosphate (AppNHp), then ATP; (e) ATP, then AppNHp, followed by ATP dialdehyde; (f) ATP dialdehyde, then ATP. Concentrations of other compounds were: $[Ca^{2+}]$ = 1 mM, Mg^{2+} = 0; CCh = 100 μM and atropine (Atr) = 10 μM. Although none of the analogues tested [α,β-methylene ATP ($n = 8$ cell preparations), AppNHp ($n = 6$) and ATP dialdehyde ($n = 3$)] were particularly potent, all partially blocked or reversed the Ca_i response when they were added at 1 mM, a concentration 20 fold higher than that of ATP (50 μM). This particular cell preparation appeared to lack the sensitive response to ATP since Mg^{2+} fully reversed the effect of ATP even in the absence of antagonists. The antagonists did not appear to be acting solely by chelation of extracellular calcium; note that the combination of AppNHp (1 mM) and ATP dialdehyde (1 mM) was no more effective in reversing the response to ATP than pretreatment with AppNHp alone. Note also that AppNHp acted as a weak partial agonist. Antagonism of responses to ATP by analogues, although repeatedly observed, was quite variable, possibly due to inhibition of ATPases by some analogues and potentiation of the more sensitive response to ATP in some preparations.

Reversal of the effect of ATP^{4-}

We hypothesized that inactive compounds or partial agonists that can bind to the ATP^{4-} receptor would reverse the effect of ATP^{4-} on Ca_i. We compared the reversal of the effect of ATP^{4-} by these compounds to reversal by Mg^{2+}, which reduces the concentration of ATP^{4-}.

Under optimal conditions for a response to ATP^{4-} (in the absence of Mg^{2+} and in the presence of 0.5–1.0 mM Ca^{2+}), 50 μM ATP produced a Ca_i response comparable to maximal muscarinic receptor activation (Figure 6a). This effect of ATP was reversed by the addition of Mg^{2+} (Figure 6a). The effects of benzoylbenzoyl ATP, and of high concentrations of ATPγS and 2-methylthio ATP were reversed similarly by Mg^{2+} ($n = 6$ or more experiments for each compound). We show here that 'inactive' analogues of ATP can block and reverse effects of ATP on Ca_i (Figure 6b–f). Three analo-

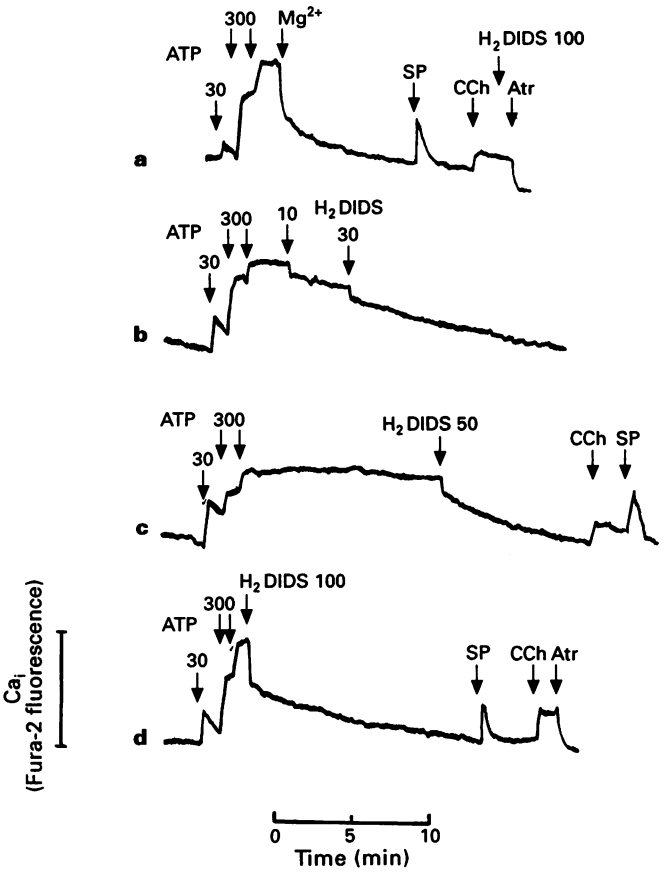

Figure 7 Effect of varying concentrations of 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulphonate (DihydroDIDS) on the Ca_i response to ATP^{4-}: the ability of various concentrations of dihydroDIDS (H_2DIDS) to reverse the response to ATP or to block responses to carbachol (CCh) or substance P (SP) was tested. All concentration values given in the figure are in μM. ATP was added at 30 μM, followed by 300 μM, added twice. SP = 100 nM; CCh = 100 μM; atropine (Atr) = 10 μM. (Note that 100 μM H_2DIDS was as effective as Mg^{2+} in reversing the effect of a high concentration of ATP, without blocking SP or CCh responses). Similar results were obtained on 2 other cell preparations.

gues, α,β-methylene ATP, AppNHp and ATP dialdehyde, which had little or no agonist effects, all partially blocked the response to a subsequent addition of ATP. They also were able to partially reverse the response to a prior addition of ATP. This reversal required a relatively high concentration of the antagonist compound. For example, α,β-methylene ATP was effective only at a concentration of 20:1 relative to ATP and blocked the ATP response by 76 ± 4% ($n = 3$). It seems unlikely that chelation of extracellular calcium accounts for the inhibition by analogues of ATP since benzoylbenzoyl ATP partially overcame the block in the two experiments where it was examined. These experiments illustrate the ability of various ATP analogues to act as antagonists or as partial agonists in these cells.

Another compound which appears to bind to the ATP receptor and which blocks the response to ATP^{4-} is DIDS. Although DIDS pretreatment blocked responses to ATP^{4-} (Figure 2), the fluorescence of this compound limits its utility, and it cannot be used on-line in fura-2 assays. DihydroDIDS, a much less fluorescent derivative of the parent compound, also blocks the response to ATP^{4-} (data not shown). DihydroDIDS specifically reversed the effect of ATP^{4-} on Ca_i (Figure 7b–d), without interfering with the response to carbachol or substance P, consistent with the idea that dihydroDIDS displaces ATP binding to the purinoceptor.

Discussion

The present results provide evidence for two distinct Ca_i responses to extracellular ATP in rat parotid acinar cells. The first response was maximal at low concentrations of ATP (< 5 μM) and was smaller and less well-maintained than the Ca_i response to ATP^{4-} which we have described previously (McMillian *et al.*, 1987a; 1988; Soltoff *et al.*, 1990a). The response to higher concentrations of ATP (ATP^{4-}) does not show a maximum in Figure 1, but in experiments measuring $^{22}Na^+$ influx, maximal rates were reached at 100 μM ATP (with 1.8 mM Ca^{2+} in the absence of Mg^{2+}) (Soltoff *et al.*, 1990a). Rate studies of $^{45}Ca^{2+}$ uptake showed that the response is maximal at 300 μM ATP in the absence of added Mg^{2+}) (Soltoff *et al.*, 1992). ADP selectively blocked the more sensitive response to ATP, while DIDS and reactive blue 2 were selective in inhibiting only the response to ATP^{4-}, as were high concentrations of Mg^{2+} and Ca^{2+} which act by lowering the concentration of ATP^{4-} available on addition of ATP (McMillian *et al.*, 1988; Soltoff *et al.*, 1990a). Benzoylbenzoyl ATP was the only selective agonist and was at least 30 fold more potent than ATP on the ATP^{4-}-specific response, similar to other systems (Gonzalez *et al.*, 1989; Erb *et al.*, 1990). In other experiments which utilized $^{45}Ca^{2+}$ uptake rather than fura-2 fluorescence to assay the response to ATP, dye quenching by benzoylbenzoyl ATP was not a consideration, and higher concentrations of benzoylbenzoyl ATP were tested. Benzoylbenzoyl ATP was equally effective at 30 μM and 100 μM in these studies on rat parotid acinar cells (Soltoff *et al.*, 1992), indicating that maximal stimulation occurs at 30 μM. In fura-2 studies carried out under conditions where the large, low affinity response was blocked, i.e. with 10 mM Ca^{2+} or 300 μM DIDS pretreatment, 30 μM benzoylbenzoyl ATP had no agonist effect on the more sensitive response, which is surprising since this compound binds to ATPases (Williams & Coleman, 1982; Erb *et al.*, 1990) as well as to P_{2y}-purinoceptors (Boyer & Harden, 1989; Boyer *et al.*, 1989). Though generally much weaker, the more sensitive response to ATP may be physiologically important, since this response appears to be neurally regulated, as shown by the increase following parasympathetic denervation.

The response to ATP^{4-} in parotid cells can best be classified as one mediated via P_{2z}-purinoceptors. The potency order for the agonists (benzoylbenzoyl ATP > ATP > 2-methylthio ATP > α,β-methylene ATP) and the strong inhibition by Mg^{2+} and high concentrations of Ca^{2+} are consistent with activation of a P_{2z}-purinoceptor. A similar potency order (benzoylbenzoyl ATP > ATP > 2-methylthio ATP > AppNHp) for the effect on the ATP^{4-}-response was obtained in $^{45}Ca^{2+}$ uptake experiments (Soltoff *et al.*, 1992). It is unclear whether the 'superagonist' effect of benzoylbenzoyl ATP on Ca_i in parotid cells results from covalent interaction with P_{2z}-receptors and ATPases; however in short-term studies much of the effect is reversed by the addition of Mg^{2+}, suggesting that covalent bonds are not required.

Gallacher (1982) previously noted the low potency of ATP in stimulating ion fluxes in mouse parotid cells and suggested that ATP^{4-} might be the active form, but he suggested that the response was of the P_{2y}-type based on the observation that P_{2y} purinoceptors also activate calcium-dependent K^+ channels in other tissues (Gordon, 1986). Our data clearly show that the response to ATP^{4-} in rat parotid cells is of the P_{2z}-type. While its selectivity for the P_{2z}-receptor must be confirmed, the use of benzoylbenzoyl ATP together with P_{2x}- and P_{2y}-selective agonists should prove useful in subtyping P_2-purinoceptors.

Reactive blue 2 was a potent and selective blocker of the parotid response to ATP^{4-}. This antagonist apparently discriminates P_{2y}-receptors from P_{2x}-receptors (Reilly *et al.*, 1987; Burnstock & Warland, 1987; Houston *et al.*, 1987), but also has a strong effect on parotid P_{2z}-receptors (Soltoff *et al.*, 1989). Reactive blue 2 displaced [γ^{32}P]-ATP bound to

parotid cells as did the inactivating compound DIDS, which forms covalent adducts (McMillian et al., 1988). Blockade of ATP-effects by pretreatment of cells with DIDS, a compound structurally unrelated to ATP, appears to result from covalent inhibition of P_2-type purinoceptors (McMillian et al., 1988). While allosteric effects of these compounds on ATP-binding are possible, it seems likely that reactive blue 2 and DIDS inhibit the response to ATP^{4-} by interfering with the binding of the purine rather than by other means, for example by blocking an ATP-dependent channel. In some experiments (as in Figure 4), reactive blue 2 inhibited the muscarinically-activated increase in Ca_i as well as the response to ATP^{4-}, although the responses to substance P were never inhibited. This finding is in agreement with observations that muscarinic effects are inhibited by reactive blue 2 in other ATP-responsive tissues (Choo, 1981). Coomassie brilliant blue may be a more useful antagonist at P_{2z}-receptors (Soltoff et al., 1989) since this compound does not appear to be a potent blocker of P_{2y}-receptors (Inoue et al., 1991; McMillian, unpublished data).

ATP^{4-} increases the membrane permeability to low molecular weight solutes in many cells, and this permeabilization response shares many features in common with the Ca_i response to ATP^{4-} in parotid cells. However, at the concentrations employed in the present study, which are higher than those used for permeabilization studies in other cells, ATP^{4-} has little if any permeabilizing effect (McMillian et al., 1988). As with the Ca_i response reported here, Mg^{2+} halts permeabilization in response to ATP^{4-}, and this reversal on removal of ATP^{4-} has been interpreted as a resealing of ATP^{4-}-induced lesions in the plasma membrane (Tatham et al., 1988). Reversal of the effects of ATP^{4-} in parotid cells by Mg^{2+} (and to a lesser extent by the more weakly chelated Ca^{2+}), as well as by α,β-methylene ATP and dihydro-DIDS, appears to result from displacement of ATP^{4-} from a specific receptor-like site, rather than through resealing of ATP^{4-}-induced lesions.

The more sensitive Ca_i response to ATP does not fit into any P_2-purinoceptor classification. Although pharmacologically distinguishable from the ATP^{4-}-effect, the more sensitive response resembles this P_{2z}-type response much more than it does the P_{2x}- or P_{2y}-purinoceptor responses. Except for benzoylbenzoyl ATP, the agonist potency series for analogues of ATP was identical for the two Ca_i responses, and the P_2-purinoceptors mediating the two responses may be related. The more sensitive response to ATP was variably present in earlier experiments, and it is possible that the receptor mediating the smaller response to ATP is more sensitive to reagents used in our cell preparation procedure and was lost in previous studies. Another possibility is that the different responses to ATP reflect Ca_i increases in different cell populations or that there is only one receptor for ATP on parotid cells that gives rise to a second as a preparation artifact. However, a similar effect of ATP^{4-} has been reported in cells prepared without enzyme treatment (Richards et al., 1987; Buisman et al., 1988; Tatham et al., 1988; Gonzalez et al., 1989). It is also possible that the variability in responses to ATP accurately reflects the situation *in vivo*. Our finding of two P_2-type purinoceptor-mediated responses on a single cell type is not unique. There are at least two effects of ATP which can be distinguished by the use of analogues of ATP in hepatocytes (Okajima et al., 1987), and there appear to be two or more distinct responses in liver (Cobbold et al., 1988), bullfrog atrial cells (Friel & Bean, 1988), in macrophage and pancreatic cell lines (Greenberg et al., 1988; Li et al., 1991), and in adrenal chromaffin cells (Diverse-Pierluissi et al., 1991).

Breakdown of ATP to ADP may contribute to some of the properties of the more sensitive ATP response. ADP selectivity inhibited this response, and production of ADP by metabolism of ATP may limit the size as well as the duration of the Ca_i elevation. The potentiation of the more sensitive response to ATP by reactive blue 2 may reflect blockade of ecto-ATPases (and of ATPase activity from broken cells).

Denervation increased the Ca_i response to low concentrations of ATP much more than to other calcium-mobilizing agonists, suggesting some specificity in the increased purinoceptor response. Further study is required to determine if metabolism of ATP decreases after denervation (perhaps leading to more ATP and less ADP at the putative receptor). Denervation supersensitivity has been cited as evidence for a physiological role for ATP in the vas deferens (Rohde & Huidobro-Toro, 1988) and is consistent with a physiological effect of ATP in the parotid. Postganglionic parasympathetic denervation of the parotid gland is known to lead to supersensitivity to all agonists which mobilize Ca_i through phospholipase C (Ekstrom, 1980; Ekstrom & Wahlestedt, 1982) (including the agonist noradrenaline which is not depleted). It is unclear if the selective increase in the Ca_i response to low concentrations of ATP reflects changes in denervated cells specific to the P_2-purinoceptor response or if the greater effect reflects differences in the handling of Ca_i in response to ATP relative to phospholipase C-linked receptor agonists.

Our data clearly show that two Ca_i responses to ATP can be distinguished in parotid cells. DIDS, reactive blue 2 and, particularly benzoylbenzoyl ATP are offered as useful pharmacological tools to characterize further the response to ATP^{4-} mediated via P_{2z}-purinoceptors. As yet, we have no selective compounds which affect the more sensitive response to ATP, which may be more important physiologically.

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