Modulation of extracellular ATP-induced Ca^{2+} responses: role of protein kinases

Lalitha TENNETI and Barbara R. TALAMO*
Neuroscience Program, Tufts University Medical School, 136 Harrison Ave, Boston MA 02111, U.S.A.

Evidence for the modulation of the P_{2z}-purinoceptor for extracellular ATP in dissociated rat parotid cells is presented in studies using compounds that inhibit protein kinases. Preincubation of acinar cells with the protein kinase catalytic-site inhibitors K-252a and staurosporine, as well as with the regulatory-domain inhibitor sphingosine, specifically potentiates the elevation in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) mediated by extracellular ATP, but has no effect on the $[Ca^{2+}]_i$ elevation mediated by muscarinic receptors through phospholipase C activation. Phorbol dibutyrate (PDBu), which activates protein kinase C (PKC), has no modulatory effect on ATP-mediated $[Ca^{2+}]_i$ elevation. Further, pretreatment with PDBu does not reverse or block the effects of K-252a or sphinogosine, arguing against the involvement of PKC. Other pharmacological manipulations indicate that neither calmodulin-dependent nor cyclic-AMP-dependent kinases are involved. Neither the peak intracellular Ca^{2+} mobilization nor the sustained Ca^{2+} entry in response to carbachol or to a Ca^{2+} ionophore (4-bromo-A23187) is altered by the kinase inhibitors that potentiate the $[Ca^{2+}]_i$ response to ATP, indicating that effects on the ATP response are not due to non-specific permeability changes, nor to decreased Ca^{2+} removal from the cytosol. ATP-mediated influx of Mn^{2+} as well as ATP-induced membrane depolarization are potentiated in cells preincubated with K-252a, directly demonstrating that cation influx is enhanced through a P_{2z}-specific route. These results show that P_{2z} responses (or purinoceptors) can be modulated and suggest that phosphorylation events are involved.

INTRODUCTION

The biological actions of extracellular ATP and the intracellular transduction systems through which these actions are mediated have attracted increasing interest as multiple classes of ATP receptors and numerous responsive tissues have been described (Gordon, 1986). For example, ATP enhances superoxide formation in stimulated neutrophils (Ward et al., 1988), activates surfactant phosphatidycholine secretion from alveolar type II cells (Rice et al., 1990) and stimulates histamine secretion from mast cells (Dahlquist and Diamant, 1974). In cultured human airway epithelia, extracellular ATP increases transepithelial Cl^- secretion and apical-membrane of Cl^- permeability, a mechanism that is preserved in cystic-fibrosis airway epithelia which show abnormalities in cyclic-AMP-mediated Cl^- secretion (Stutts et al., 1992). In many cell types ATP increases intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) through heterotrimeric G-proteins and activation of phospholipase C, as in neutrophils (Kuroki et al., 1989; Kuroki and Minakani, 1989) and hepatocytes (Charest et al., 1985; Okajima et al., 1987). In others, it increases $[Ca^{2+}]_i$ by promoting influx of extracellular Ca^{2+}, gating cation channels in lacrimal acinar cells (Sasaki and Gallacher, 1990), neurons (Bean, 1990; Evans et al., 1992) and vascular smooth muscle (Benham and Tsien, 1987). P_{2z} purinergic receptors similar to those found in parotid acinar cells are activated exclusively by tetrabasic ATP^{4-} and may mediate specific increases in membrane permeability, as in macrophages (Greenberg et al., 1988), transformed mouse cell lines (Rozengurt and Heppel, 1979) and mast cells (Cockcroft and Gomperts, 1980). Very little is known about the modulation of these purinergic responses.

Previous studies from our laboratory have shown that ATP specifically and reversibly elevates $[Ca^{2+}]_i$ in fura-2-loaded rat parotid acinar cells by a mechanism different from that utilized by muscarinic receptors on the same cells. Muscarinic agonists promote elevation of $[Ca^{2+}]_i$ through activation of phospholipase C and the polyphosphoinoside cascade. In parotid cells, ATP has little effect on inositol phosphate accumulation and is most potent in the absence of Mg^{2+} (McMillian et al., 1987b, 1988). Examination of the agonist potency series and evidence for the inhibition of the Ca^{2+} response by elevated concentrations of bivalent cations led to the classification of the purinoceptors in parotid acinar cells as P_{2z} (McMillian et al., 1987b, 1993), although general increases in permeability were not observed. More recently, we have observed a biphasic response to ATP under conditions which optimize the detection of a small but potent effect of ATP which can be distinguished pharmacologically from the larger response to ATP^{4-} (McMillian et al., 1993). The high-affinity response is not activated by 3′-*O*-(4-benzoyl)benzoyl-ATP, which potently stimulates the ATP^{4-} response, and it is insensitive to Mg^{2+} and the inhibitors Brilliant Blue G, Reactive Blue 2 and 4,4′-di-isothiocyano-2,2′-stilbenedisulphonate (DIDS), all of which selectively block the effect of ATP^{4-} (McMillian et al., 1988; Soltoff et al., 1989). DIDS and Reactive Blue 2 also inhibit [α-^{32}P]ATP binding, consistent with the idea that they act at the ATP-receptor complex (McMillian et al., 1988, 1993).

In pilot studies, drugs reported to act at ATP-binding sites of protein kinases were tested to see whether they acted as ATP analogues at the binding site of the parotid-cell purinoceptor. The results of these experiments suggested rather that the drugs might be acting on protein kinases, which in turn modulate the cellular responses to extracellular ATP. Modulation of ATP

Abbreviations used: 4-BrA23187, 4-bromo-A23187; 8-BrcAMP, 8-bromo cyclic AMP; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CDTA, 1,2-diaminocyclohexane-*NNN′N′*-tetra-acetic acid; DIDS, 4,4′-di-isothiocyano-2,2′-stilbenedisulphonate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; Iso H-7, 1-(5-isoquinolinesulphonyl)-3-methylpiperazine; IBMX, isobutylmethylxanthine; PKA, protein kinase A (cyclic AMP-dependent protein kinase); PKC, protein kinase C; PDBu, 4β-phorbol 12,13-dibutyrate; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide.

* To whom correspondence should be addressed.

responsivity has not been reported previously. To explore these effects further, the $[Ca^{2+}]_i$ response to ATP was monitored during treatment with compounds reported to inhibit protein kinase through either the catalytic or the regulatory domain. Comparison of the effects of the drugs on responses activated by ATP and on responses activated via other receptor- or ionophore-mediated pathways on the same cells allowed us to explore the specificity of the modulation and to gain insight into the influx or efflux pathways for Ca^{2+} that are activated by ATP.

EXPERIMENTAL

Dissociated acinar-cell preparation

Dissociated parotid acinar cells were prepared by a modification of the method of Kanagasuntheram and Randle (1976) as described previously (McMillian et al., 1987a). The cell pellet was suspended in Hepes/Ringer buffer of the following composition (in mM): NaCl, 120; KCl, 5; $MgCl_2$, 2.2; $CaCl_2$, 1; Hepes, 20; β-hydroxybutyrate, 5; glucose, 10; BSA, 0.1%; pH 7.4. For all experiments the stock solutions of various inhibitors were prepared in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide in the assay was never more than 0.2% of the volume.

$[Ca^{2+}]_i$ was assayed in cells loaded with 0.5 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.) (McMillian et al., 1987a). Washed cells were suspended in Mg^{2+}-free Hepes/Ringer buffer, and 0.5 ml of the cell suspension was added to a cuvette containing 1.5 ml of Mg^{2+}-free Hepes/Ringer buffer that was otherwise identical with the isolation buffer except that the concentration of Ca^{2+} was elevated to 3 mM to enhance detection of the response to low concentrations of ATP. Fluorescence was measured in a Perkin–Elmer LS5 spectrofluorimeter by using wavelengths of 340 and 380 nm for excitation and 505 nm for emission. $[Ca^{2+}]_i$ was estimated by the ratio method, by using a K_d of 224 nM for Ca^{2+} binding to fura-2 (Grynkiewicz et al., 1985). Maximum and minimum fluorescence values were obtained by using digitonin (50 μM) and EGTA (100 mM) respectively. $[Ca^{2+}]_i$ values were corrected for the contribution of extracellular fura-2 fluorescence, determined by adding 100 μM $MnCl_2$ to quench the extracellular fura-2, followed by addition of 200 μM 1,2-diaminocyclohexane-*NNN'N'*-tetra-acetic acid (CDTA) to chelate the added Mn^{2+}.

For Mn^{2+}-influx studies, the quenching of the fura-2 fluorescence emission signal by Mn^{2+} was monitored at an excitation wavelength of 360 nm, where changes in $[Ca^{2+}]_i$ do not contribute to the signal (Merritt et al., 1989).

Plasma-membrane permeability was assayed by monitoring the uptake of ethidium bromide, which shows enhanced fluorescence on binding to nuclear DNA (Tatham et al., 1988). Ethidium bromide (20 μM) was added to the cell suspension 1 min before ATP, and the fluorescence was recorded for 15–20 min, with 360 nm and 580 nm as excitation and emission wavelengths respectively. Maximum fluorescence was determined by adding 50 μM digitonin.

Membrane potential was measured with the potential-sensitive dye bis-oxonol (100 nM). Depolarization of the membranes slowly increases the transfer of the dye from the external solution to binding sites inside the cell, thus enhancing its fluorescence (De Togni et al., 1984; Greenberg et al., 1988). Cells suspended in appropriate solutions (for Na^+-free solutions, NaCl was replaced by *N*-methyl-D-glucamine) were equilibrated for 5 min with bis-oxonol; fluorescence then was measured in the spectrofluorometer at 6 s intervals, by using excitation and emission wavelengths of 540 and 580 nm respectively. The value observed before stimulation was subtracted from the fluorescence signal measured after addition of ATP (300 μM).

Amylase release was determined in cells that were washed three times in warmed oxygenated glucose-free Hepes/Ringer solution and distributed (200 μl portions) to tubes containing 1 μM 4β-phorbol 12,13-dibutyrate (PDBu) or buffer. After incubation at 37 °C for 60 min, the cells were pelleted and the pellets and supernatants were frozen and thawed and diluted appropriately to give enzyme activity in the linear-response range. Amylase activity was assayed by the method of Bernfeld (1955). The fraction of total amylase released into the supernatant was calculated, and the effect of stimulation was determined by subtracting basal release (no drugs added).

Values are given as means ± S.E.M. Experimental values were compared with control values by Student's unpaired two-tailed *t* test. In Tables, asterisks indicate level of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fura-2 acetoxymethyl ester, 4-bromo-A23187 (4-BrA23187) and bis-oxonol were obtained from Molecular Probes. ATP (sodium salt), D-sphingosine, 1-(5-isoquinolinesulphonyl)-3-methylpiperazine (Iso H-7), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), PDBu, isobutylmethylxanthine (IBMX) and 8-bromo cyclic AMP (8-BrcAMP) were obtained from Sigma Chemical Co. (St Louis, MO). 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine (H-7) and forskolin were from Calbiochem (La Jolla, CA, U.S.A.). Staurosporine was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). K-252a and K-252b were gifts from Dr. Y. Matsuda of Kyowa Hakko Kogyo Co. (Tokyo, Japan).

RESULTS

Selective effects of K-252a on ATP response

Addition of extracellular ATP rapidly produces a biphasic elevation of $[Ca^{2+}]_i$ in fura-2-loaded rat parotid acinar cells, first generating a small elevation which reaches a plateau value apparent at low concentrations of ATP (3–30 μM), followed by a larger rise which is reached at high concentrations of ATP (300–600 μM) (Figure 1). Evidence for modulation of these

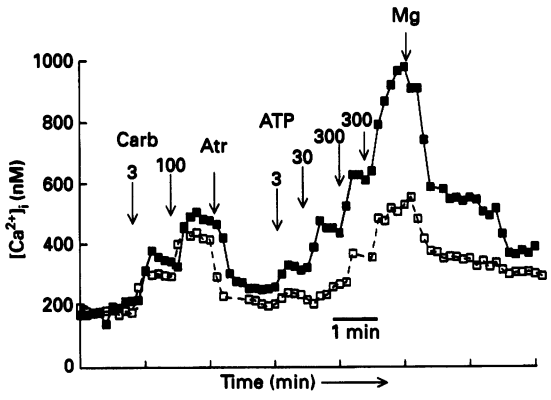

Figure 1 Effect of K-252a on $[Ca^{2+}]_i$ responses to muscarinic agonist and ATP

$[Ca^{2+}]_i$ was measured in cells loaded with fura-2 as described in the Experimental section. Traces show responses to two concentrations of carbachol (Carb; 3 and 100 μM) and four concentrations of ATP (3, 33, 333 and 633 μM), increased cumulatively by successive additions of the nucleotide. Atropine (Atr; 1 μM) reversed the response to carbachol, and Mg^{2+} (10 mM) reversed the response to ATP. □, Control cells (no drugs added); ■, cells pretreated with 1 μM K-252a for 10 min.

Table 1 Effect of protein kinase inhibitors on the $[Ca^{2+}]_i$ responses to carbachol and ATP

Cells were preincubated with the indicated inhibitors for 10 min before stimulation with agonists. Agonists were tested in the same order as shown in Figure 1. Values represent percentage of control (no drugs added) and are means ± S.E.M. of 8–12 cell preparations for K-252a and 3–5 cell preparations for all other drugs (ND, not determined). Asterisks indicate level of significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with response in the absence of inhibitors. Basal $[Ca^{2+}]_i$ levels were not altered by any of the drugs tested.

	Increase in $[Ca^{2+}]_i$ (% of control)					
	K-252a (1 μM)	K-252b (1 μM)	Staurosporine (1 μM)	Sphingosine (30 μM)	H-7 (100 μM)	PDBu (1 μM)
Carbachol (μM)						
3	136±16	94±5	60±4	37±9***	ND	122±29
100	120±13	102±9	84±16	81±10	124±5	116±14
ATP (μM)						
3	228±21***	126±3	119±16	113±19	263±7**	82±10
33	218±18***	114±10	149±23	143±7	180±18*	106±17
333	271±21***	105±2	220±13***	254±32***	113±11	118±17
633	195±26***	113±10	164±2**	174±20**	108±7	105±13

Table 2 Dose-dependent effects of K-252a on ATP response

Cells were preincubated with the indicated concentrations of K-252a for 10 min. The mean ± S.E.M. of the peak $[Ca^{2+}]_i$ response above basal (in nM) is shown for 3–5 cell preparations. Asterisks indicate values significantly different from control (0, no drugs added): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

		$[Ca^{2+}]_i$ (nM)			
ATP (μM)	[K-252a] (μM)... 0	0.1	0.4	1.0	
3	37±3.3	72±1.6*	64±3***	70±6.3***	
33	71±5.6	103±15*	124±6.4***	149±16***	
333	101±8.4	152±20*	218±30**	273±21***	
633	230±20	318±46	364±28*	497±50***	

responses was obtained in studies with protein kinase inhibitors. K-252a, an alkaloid recently isolated from the culture broth of *Nocardiopsis* sp. is a potent inhibitor of protein kinase C (PKC) *in vitro*, and also of cyclic-AMP-dependent protein kinase (PKA) (Kase et al., 1987). In parotid cells, preincubation with 1 μM K-252a for 10 min specifically potentiates the Ca^{2+} response mediated by extracellular ATP. It has no significant effect on the response to the muscarinic agonist carbachol (Figure 1). Both the sensitive but small effect of ATP (3–30 μM) and the larger response to higher concentrations of ATP (ATP^{4-}) are potentiated to nearly the same extent by K-252a pretreatment for 10 min (228±21% and 270±21% of control for 3 μM and 333 μM ATP respectively) (Table 1). A much shorter preincubation for 1 min is ineffective, and extending the preincubation time to 20 min does not further enhance the response beyond the level obtained with a 10 min incubation (results not shown).

K-252a is a very potent modulator of the ATP response: a concentration as low as 100 nM is sufficient to potentiate significantly the effect on $[Ca^{2+}]_i$ (Table 2). K-252b, a cell-membrane-impermeant analogue of K-252a, has no significant effect on the $[Ca^{2+}]_i$ response to ATP or carbachol (Table 1).

Effects of other protein kinase inhibitors

To test further the hypothesis that the ATP response is specifically potentiated by inhibition of protein kinase, we examined the effects of two other inhibitors of protein kinases: staurosporine, which appears to interact directly with the catalytic site of PKC (Tamaoki et al., 1986), and sphingosine, which is a regulatory-domain inhibitor of PKC (Merrill and Stevens, 1989; Hannun and Bell, 1989) as well as an inhibitor of calmodulin-dependent enzymes (Jefferson and Schulman, 1988). Pretreatment of acinar cells for 10 min with 1 μM staurosporine or 30 μM sphingosine (concentrations which do not alter the basal $[Ca^{2+}]_i$ and are not cytotoxic) significantly potentiates the response to ATP (Table 1). Staurosporine and sphingosine appear to enhance selectively the large response to ATP^{4-}, but the more sensitive small response to ATP is not altered. Pretreatment with 100 μM H-7, another potent inhibitor of protein kinases (Hidaka et al., 1984), significantly potentiates the response to low concentrations of ATP, but has no effect on the response to high concentrations (Table 1). However, 100 μM Iso H-7, a structurally related isomer which is a much less effective inhibitor of protein kinases, has no effect at any concentration of ATP (results not shown). With carbachol, pretreatment with staurosporine, sphingosine or H-7 (Table 1) does not change the $[Ca^{2+}]_i$ response to high concentrations of carbachol, although sphingosine does significantly decrease the response to a low concentration of carbachol (Table 1). We also tested the effects of the calmodulin antagonist W-7 (10 μM) on the ATP response. No modulatory effect was observed (results not shown).

Effect of protein kinase activation: PKC and PKA

The above results suggest that potentiation of the ATP response may be due to inhibition of intracellular protein kinases. To test the possibility that activation of known protein kinases would inhibit the ATP response, we added activators of PKA and of PKC. Pretreatment with 1 μM PDBu, which has previously been shown to stimulate exocytosis (protein secretion) in parotid acinar cells (Putney et al., 1984), has no effect on the $[Ca^{2+}]_i$ response to ATP, nor does it alter the Ca^{2+} response to low or high concentrations of carbachol (Table 1), even though it effectively stimulates amylase release in our preparation by 7.5±1% ($n = 3$). Furthermore, if the modulatory effects of the active drugs were mediated by their inhibition of PKC, then the effects should be blocked or reversed by PDBu. This was tested by preincubating cells with 1 μM PDBu for 5 min, followed by 30 μM sphingosine for 5–10 min. Under these conditions sphingosine still potentiates the $[Ca^{2+}]_i$ response to high concentrations

of ATP (227±71% and 174±42% of control for 333 μM and 633 μM ATP respectively). Similarly, PDBu pretreatment does not block the effects of K-252a.

Activation of PKA using 8-BrcAMP (1 mM), a membrane-permeant activator of PKA, or by elevating intracellular cyclic AMP with either forskolin (100 μM), an activator of adenylate cyclase, or IBMX (100 μM), an inhibitor of phosphodiesterase, had no effect on the $[Ca^{2+}]_i$ response to ATP or to carbachol (results not shown).

Effects of K-252a on Ca^{2+} removal from the cytosol

Receptor-mediated elevation of $[Ca^{2+}]_i$ also enhances Ca^{2+} efflux from the cell. The enhancement of the $[Ca^{2+}]_i$ response to ATP by K-252a, H-7, staurosporine and sphingosine potentially could result from the effect of these compounds on one or more pathways which regulate $[Ca^{2+}]_i$, e.g. by inhibiting Ca^{2+} removal from the cytosol. To explore the possible role of Ca^{2+} removal in the effect of K-252a, we elevated $[Ca^{2+}]_i$ levels by several mechanisms. Any inhibitory effect of K-252a on the plasma-membrane or intracellular-membrane Ca^{2+} pumps should be revealed under these conditions, resulting in increased levels of $[Ca^{2+}]_i$. (1) Muscarinic stimulation of cells in the presence of extracellular Ca^{2+}. This rapidly elevates $[Ca^{2+}]_i$ to a maximum value (3–4-fold over basal) which then declines to a lower maintained level. (2) Muscarinic stimulation in the absence of extracellular Ca^{2+} (nominally Ca^{2+}-free medium), which increases $[Ca^{2+}]_i$ only transiently. Pretreatment with K-252a has no effect on either the initial release phase or the sustained phase of $[Ca^{2+}]_i$ elevation in response to carbachol under either of these conditions (results not shown). (3) Ionophore-mediated elevation of $[Ca^{2+}]_i$, induced with a non-fluorescent Ca^{2+} ionophore, 4-BrA23187. At ionophore concentrations of 100 nM and 300 nM, $[Ca^{2+}]_i$ is elevated to levels similar to those reached after addition of 30 and 300 μM ATP respectively. 4-BrA23187 elevates $[Ca^{2+}]_i$ gradually, and the levels are maintained for at least 5 min. Cells pretreated with K-252a show no significant alteration in either the peak or the maintained Ca^{2+} response to 4-BrA23187 (results not shown).

K-252a enhances cation entry promoted by ATP

A further test of the hypothesis that the above inhibitors enhance the $[Ca^{2+}]_i$ elevation in response to ATP by acting directly on the non-selective cation-entry pathway was to examine the effect of K-252a on cation influx in response to ATP, by using Mn^{2+} as a surrogate for Ca^{2+}. Measurement of Ca^{2+}-dependent changes in fura-2 fluorescence reflects the net effect on $[Ca^{2+}]_i$ of processes that include both influx and removal of Ca^{2+} from the cytosol by Ca^{2+} pumps and transporters; it therefore does not permit the assessment of Ca^{2+} entry directly. The bivalent cation Mn^{2+} can permeate some Ca^{2+} channels (Lansman et al., 1986) as well as receptor-mediated/non-selective cation channels (Merritt et al., 1989; Hallam et al., 1989; Mertz et al., 1990), but it is not a substrate for plasma-membrane Ca-ATPases (Schatzmann, 1975). When Mn^{2+} binds to fura-2, it displaces Ca^{2+} and causes quenching of the fura-2 signal (Grynkiewicz et al., 1985). Thus the rate of quenching of intracellular fura-2 by Mn^{2+} can be used as an index of Mn^{2+} entry and as a measurement of unidirectional cation influx (Prentki et al., 1987).

Addition of Mn^{2+} to the cell suspension initially causes a small decrease in fluorescence due to its interaction with extracellular fura-2, which is reversed on addition of CDTA, a membrane-impermeant heavy-metal chelator that binds extracellular Mn^{2+} (results not shown). In control cells, the addition of Mn^{2+} leads

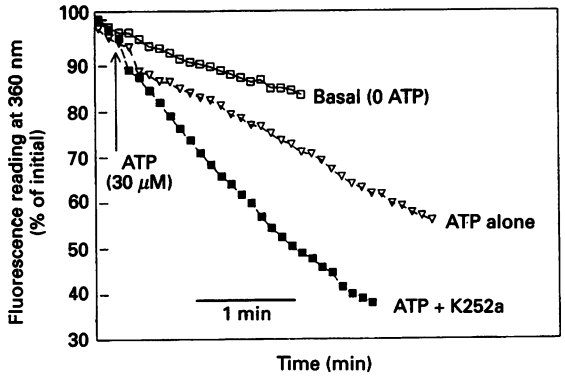

Figure 2 Effect of K-252a on ATP-mediated entry of Mn^{2+} into cells

Fura-2-loaded cells were suspended in nominally Ca^{2+}-free medium. Mn^{2+} (100 μM) was added, and the quenching of fura-2 fluorescence was measured at 360 nm. Addition of Mn^{2+} causes an immediate fall in fluorescence, which is due to the quenching of extracellular fura-2. This value was subtracted from the traces. Values are shown as percentage of initial 360 nm reading. Fluorescence quenching was measured in unstimulated cells (basal; □) and in cells stimulated with ATP (30 μM) either in the absence of K-252a (ATP alone; △) or in cells pretreated with 1 μM K-252a for 10 min (ATP+K-252a, ■). Each trace shown is representative of three others.

to a small, steady, decline in fluorescence (6.4±1.5% of the basal level/min; $n = 3$), due to a slow basal rate of entry of Mn^{2+} into the cells (Figure 2). Addition of 30 μM ATP accelerates the fall in the fluorescence signal (14.1±0.42%/min). K-252a pretreatment further enhances the rates of Mn^{2+} entry in response to ATP (22±0.85%/min) (Figure 2). K-252a alone has no effect on the basal rate of Mn^{2+} entry.

Effects of K-252a on membrane potential

Experimental manipulations that modulate the cation-entry pathway stimulated by ATP would also be expected to modulate the depolarization of the cells, which is secondary to the large movements of cations. Our previous studies suggested that both Na^+ and Ca^{2+} enter through an ATP-activated channel (Soltoff et al., 1990a, 1992), and lead to depolarization of parotid cells. We expected that depolarization would be enhanced by treatments that facilitate the effect of ATP on this channel. Parotid-cell membrane potential was measured with the voltage-sensitive dye bis-oxonal. Depolarization by ATP is marked and is potentiated by preincubation with K-252a (Figure 3a). When Na^+ in the extracellular medium is replaced by *N*-methyl-D-glucamine, ATP is still able to depolarize the cells (Figure 3b). With normal levels of extracellular Na^+, but in the absence of extracellular Ca^{2+} (nominally Ca^{2+}-free), the extent of depolarization is greater than in the presence of Ca^{2+} (Figure 3c). Pretreatment of cells with K-252a potentiates the ATP-mediated depolarization in the presence of both Na^+ and Ca^{2+}, as well as in the presence of either ion alone. Neither K-252b (Figure 3) nor Iso H-7 (results not shown) has any significant effect on the ATP-mediated change in membrane potential.

Effect of ATP and K-252a on membrane permeability

In some cell types, ATP^{4-} increases the permeability to low-molecular-mass solutes. Although this permeabilizing effect has not been detected in our previous studies, we nonetheless examined the effects of ATP and K-252a on membrane permeability. The uptake of ethidium bromide was assessed at

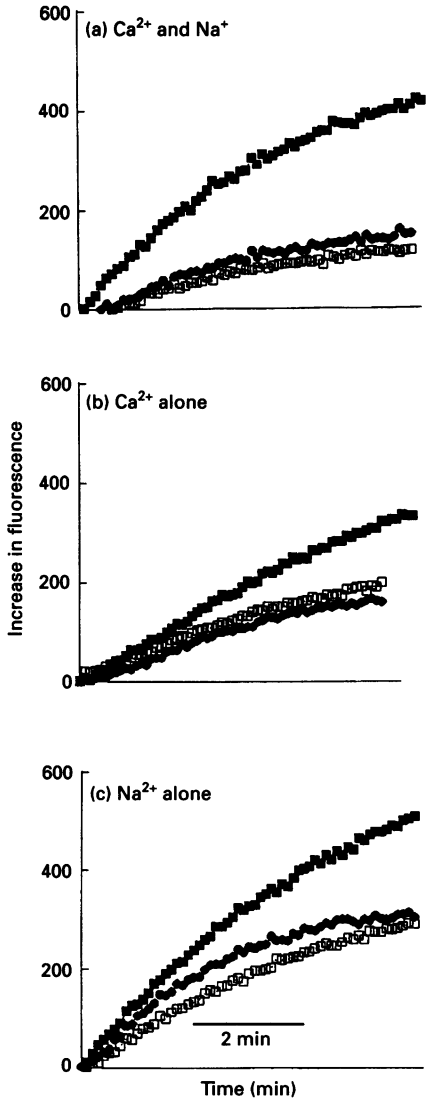

Figure 3 Effect of K-252a on ATP-mediated membrane depolarization

Membrane potential was measured with the potential-sensitive fluorescent dye bis-oxonal. Fluorescence decreases with hyperpolarization and increases with depolarization. Cells were suspended (**a**) in normal medium (with Ca^{2+} and Na^+), (**b**) in the absence of Na^+ (Ca^{2+} alone), or (**c**) in the absence of Ca^{2+} (Na^+ alone). Values represent increase in fluorescence above basal after addition of 300 μM ATP. □, Control cells; ■, cells pretreated with 1 μM K-252a for 10 min before stimulation with ATP; ●, cells pretreated with 1 μM K-252b, as for K-252a. These results were obtained with the same cell preparation. Traces shown are from one experiment and are representative of two others.

concentrations of ATP ranging from 30 to 600 μM (1.4–15.3 μM ATP^{4-}). No increase in permeability to this low-molecular-mass dye is observed on addition of ATP, in either the absence or the presence of K-252a (results not shown).

Effect of K-252a after ATP receptor blockade

Experiments were designed to determine whether drugs thought to act by purinoceptor blockade can prevent the effect of K-252a on the $[Ca^{2+}]_i$ response to ATP. Brilliant Blue G and DIDS block the ATP^{4-} response (Soltoff et al., 1989, 1990b), and DIDS also blocks [^{32}P]ATP binding in parotid acinar cells (McMillian et al., 1988). Pretreatment with Brilliant Blue G (1 μM) and dihydro-DIDS (100 μM), a form of DIDS that does not interfere with fura-2 fluorescence, specifically blocks the ATP^{4-} response, while sparing the small response to low concentrations of ATP. The enhancement of the sensitive ATP/MgATP response by K-252a is preserved, but the ATP^{4-} response is completely blocked (results not shown).

DISCUSSION

In rat parotid acinar cells extracellular ATP elevates $[Ca^{2+}]_i$ in a biphasic manner, with a small increase at low concentrations (high affinity) and a large increase at high concentrations (low affinity), consistent with previous results (McMillian et al., 1993). Based on agonist potency series and other pharmacological evidence, it was previously suggested that two types of P_{2z} purinoceptors are present in parotid acinar cells (McMillian et al., 1993). In the present paper we show that P_2-purinoceptor responses can be modulated by drugs reported to be inhibitors of protein kinases.

Inhibitors of protein kinases may act on either catalytic or regulatory sites of the enzyme, and are for the most part poorly selective for the different types of kinase (Rüegg and Burgess, 1989). Inhibitors which are directed against the ATP-binding site of the catalytic domain include K-252a and K-252b (Kase et al., 1987) and staurosporine (Tamaoki et al., 1986; Nakadate et al., 1988; Wong et al., 1990). Sphinogosine is one of the inhibitors which competes with diacylglycerol for binding to the regulatory domain of PKC (Hannun et al., 1986). Pretreatment of parotid acinar cells with K-252a, a membrane-permeant drug, potentiates the $[Ca^{2+}]_i$ response mediated by both the low- and high-affinity ATP receptors without altering the muscarinic response mediated by $InsP_3$ formation. The membrane-impermeant analogue K-252b is inactive, and preincubation with K-252a is required for the potentiation to develop, indicating that the effects are probably mediated intracellularly. Although the ATP analogues that are most active at the purinoceptor are hydrolysable (McMillian et al., 1993) and therefore are potential phosphodonors for a protein kinase, the requirement for a membrane-permeant kinase inhibitor makes it unlikely that the effects are due to inhibition of an ecto-kinase on the cell surface. Further, $MgATP^{2-}$ rather than ATP^{4-} is the preferred substrate for most kinases, and these experiments were carried out in the absence of Mg^{2+} under conditions optimal for the ATP^{4-} response. Additional evidence for the role of an intracellular kinase comes from the studies with other protein kinase inhibitors, staurosporine, H-7 and sphingosine, which show differential modulatory effects towards either the low-affinity receptor (staurosporine and sphingosine) or the high-affinity receptor (H-7) (Table 1). Although these selective effects cannot easily be explained at present they demonstrate differential sensitivity of these receptors to modulation by various inhibitors.

Other reports suggest a role for PKC in signal transduction by purinoceptors in various cell types. P_{2y}-purinoceptor-mediated elevation of $[Ca^{2+}]_i$ is reported to be completely blocked in neutrophils (Kuroki et al., 1989) and hepatocytes (Charest et al., 1985) by prior incubation with phorbol esters. Recently Rice et al. (1990) reported that sphingosine inhibits surfactant phosphatidylcholine secretion induced by ATP and phorbol esters in alveolar type II cells, implying a role for PKC in regulation of purinoceptor responses. Ca^{2+} mobilization, however, was not altered under the same conditions. Since P_{2y} receptors are linked to the phospholipase C pathway, it is probably that the modulatory effects could be secondary and result from action of these compounds on steps subsequent to phospholipase C activation, rather than to direct effects on the receptors. Our observation

that PDBu, a specific activator of PKC, is not able to reverse or block the effects of sphingosine and K-252a on the ATP response in parotid cells indicates that the action of these two drugs may not be due to their effects on PKC. Although the possibility exists that parotid cells are not sensitive to PDBu or that the PKC may not be sensitive to activation by this phorbol ester, this does not seem likely, since PDBu stimulates amylase secretion from similarly prepared cells. Although sphinogosine inhibits PKC *in vitro* (Hannun et al., 1986), some effects of sphingosine are not mediated through PKC (Winicov and Gershengorn, 1988; Faucher et al., 1988). Calmodulin-sensitive enzymes also are reported to be inhibited by sphingosine, as noted above, but the failure of W-7 to alter the ATP response and the observation that K-252a potentiates ATP-mediated Mn^{2+} influx (in the absence of extracellular Ca^{2+}) in parotid cells suggest that Ca^{2+}/calmodulin is not involved. These observations, as well as the lack of any effect of PKA activators, suggests that another, as yet unidentified, cellular kinase may participate in the modulation of the ATP-receptor response. Other receptors that are modulated by specific protein kinases include the β-adrenergic receptor (Benovic et al., 1986) and rhodopsin (Wilden et al., 1986). In these systems receptor phosphorylation also had a desensitizing effect, consistent with what we report here.

During stimulation, the concentration of $[Ca^{2+}]_i$ is regulated by release of intracellular Ca^{2+} and subsequent entry of $[Ca^{2+}]_i$ from the extracellular medium, as well as by the pathways involved in removal of Ca^{2+} from the cytosol, either by active transport into cellular compartments and/or by efflux into the extracellular space via a Ca^{2+}-ATPase pump. A possible explanation for the enhanced response to ATP that is observed in the presence of kinase inhibitors could therefore be that $[Ca^{2+}]_i$ elevation is secondary to a decrease in Ca^{2+} efflux and/or sequestration, effects that have been reported to be modulated via phosphorylation (Drummond, 1985; Rink and Sage, 1987; Smallwood et al., 1988; Furukawa et al., 1989; Fukuda et al., 1990; Wang et al., 1991). Alternatively, Ca^{2+} entry could be increased, or a combination of modulated entry and efflux could occur.

Several lines of evidence indicate that the effects on the ATP response are due to a specific increase in ATP-mediated cation influx, and not to non-specific membrane permeability changes or a generalized decrease in Ca^{2+} efflux.

(1) Membrane permeabilization to small molecules by extracellular ATP^{4-}, such as that reported in mast cells and macrophages (Cockcroft and Gomperts, 1980; Bennett et al., 1981; Steinberg et al., 1987; Tatham and Lindau, 1990), is not observed in parotid cells in either the presence or absence of K-252a. It appears that the enhancement of $[Ca^{2+}]_i$ elevation, depolarization and cation (Mn^{2+}) influx is not secondary to increased permeability for molecules as large as ethidium bromide ($\sim$ 300 Da). This is consistent with previous results, where we noted that ATP did not stimulate [^{3}H]inositol release from parotid cells loaded with this low-molecular-mass permeability marker, nor did it cause leakage of fura-2 or quin 2 (McMillian et al., 1987b, 1988; Soltoff et al., 1990b).

(2) Protein kinase inhibitors do not potentiate the activation of muscarinic receptors which mobilize intracellular stores of Ca^{2+} and subsequently stimulate entry of Ca^{2+} from extracellular sources in parotid cells. The sustained phase of $[Ca^{2+}]_i$ elevation is dependent on extracellular Ca^{2+} and results from the balance between Ca^{2+} entry and Ca^{2+} efflux across the plasma membrane and refilling of intracellular compartments (Poggioli and Putney, 1982). K-252a has no effect on either the peak or the sustained phase of the $[Ca^{2+}]_i$ elevation in response to carbachol, or the recovery to baseline in Ca^{2+}-free medium, indicating the specificity of its effects on the purinergic response.

(3) Increases in cytosolic Ca^{2+} produced by non-receptor-mediated manipulations also are not modified by drugs which facilitate the $[Ca^{2+}]_i$ response to ATP. To test the involvement of various Ca^{2+} transporters with different affinities for $[Ca^{2+}]_i$, the ionophore 4-BrA23187 was used to elevate $[Ca^{2+}]_i$ to two different steady-state levels, comparable with those achieved after stimulation with two different concentrations of ATP. K-252a has no effect on either the peak or the maintained $[Ca^{2+}]_i$ response at either of these $[Ca^{2+}]_i$ levels.

(4) In parotid acinar cells the increase in $[Ca^{2+}]_i$ mediated by ATP is predominantly dependent on the influx of extracellular Ca^{2+}, and ATP markedly stimulates $^{45}Ca^{2+}$ uptake (Soltoff et al., 1992), although a small sustained $[Ca^{2+}]_i$ elevation in response to ATP is sometimes observed in the absence of extracellular Ca^{2+} (McMillian et al., 1987b). Bivalent-cation entry stimulated by ATP was enhanced by K-252a, as evident from the accelerated rate of quenching of fura-2 in the presence of extracellular Mn^{2+}, thus directly demonstrating that potentiation of the ATP response is due to enhanced cation influx.

(5) Cation entry can also be followed by measuring cell depolarization. Cation channels have been implicated in the mechanism of ATP activation in other cells (Krishtal et al., 1983; Nakazawa and Matsuki, 1987; Benham et al., 1987; Friel and Bean, 1988), including lacrimal acinar cells (Sasaki and Gallacher, 1990). Electrophysiological studies in parotid cells also provide evidence for the presence of a non-selective cation channel permeable to Ca^{2+} and Na^+ that is activated by ATP (McMillian et al., 1988; Soltoff et al., 1990a). Activation of such a channel leads to membrane depolarization in the presence of extracellular Ca^{2+} and Na^+ concentrations that exceed the intracellular concentrations, but depolarization itself does not activate Ca^{2+} entry (Soltoff et al., 1992). As shown previously, ATP depolarizes parotid cells, as measured by bis-oxonol fluorescence, in the presence of either Ca^{2+} or Na^+. More importantly, pretreatment with K-252a enhances ATP-induced depolarization, a further demonstration that the effect of the drug is to enhance cation entry through an ATP-activated cation channel.

Collectively, these results agree with the interpretation that the modulatory effects of K-252a are specific to ATP and that the site of action may be at the ATP-mediated cation-influx channel and/or channel/receptor complex. This interpretation is further supported by the effects of Brilliant Blue G and DIDS, which have been shown previously to block the ATP^{4-} response and [^{32}P]ATP binding in parotid acinar cells (Soltoff et al., 1989; McMillian et al., 1993). K-252a is ineffective at the low-affinity site when ATP^{4-} receptors are blocked by Brilliant Blue G or dihydro-DIDS. Thus the receptor site occupied by ATP and competed for by DIDS and Brilliant Blue G seems to be essential for the modulatory effects of K-252a on the ATP^{4-} response, but modulation of the high-affinity site persists under these conditions, reinforcing the evidence for independent activation and regulation of two receptors.

Our results with these inhibitory drugs suggest that P_{2z} responses/purinoceptors are specifically modulated by protein kinase(s). A possible mechanism for regulation is that the receptor (or a protein that regulates it) cycles through phosphorylated and dephosphorylated states, which are differentially sensitive to activation by ATP. Potentiation of the response by inhibitors of protein kinases suggests that the dephosphorylated receptor (or modulator) is more readily activated by ATP. The possibility that the drugs are acting at a different site cannot be ruled out at present. Since all of the effective drugs are hydrophobic, it is possible that they interact with sites on the cytosolic face of the

membrane in the vicinity of the receptor, although a hydrophobic isomer of H-7 is ineffective. Further experiments examining phosphorylation of the purified components of this system will be required to explore this hypothesis.

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