Extracellular nucleotides stimulate receptor-mediated calcium mobilization and inositol phosphate production in human fibroblasts

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In human skin fibroblasts, low concentrations of extracellular ATP stimulated $^{45}\text{Ca}^{2+}$ efflux from a slow-turnover intracellular pool, accompanied by inositol phosphate generation. These effects of ATP were not due to a generalized increase in plasma-membrane permeability. The EC_{50} (concn. giving 50 % stimulation) for ATP was dependent on Ca^{2+} and Mg^{2+} concentrations in a manner which indicates that a form of ATP uncomplexed with bivalent cations is the active species. The rank order of potency of nucleotides was: $\text{ATP} = \text{UTP} > \text{adenosine } 5'-[\gamma\text{-thio}]\text{triphosphate} > \text{ITP} > \text{ADP} > \text{UDP} > \text{other nucleoside triphosphates}$. Adenosine $5'-[\alpha\beta\text{-methylene}]\text{triphosphate}$, adenosine $5'-[\beta\gamma\text{-methylene}]\text{triphosphate}$ and 2-methylthio-ATP were inactive. Thus the nucleotide specificity of this receptor is different from that of previously characterized P_2 purinoceptors. Nucleotide-stimulated $^{45}\text{Ca}^{2+}$ mobilization and inositol phosphate production were markedly inhibited by phorbol ester, and partially inhibited by pertussis-toxin pretreatment. These findings suggest that the coupling of nucleotide receptor to phospholipase C is mediated both by a pertussis-toxin-sensitive G-protein and by a pertussis-toxin-insensitive mechanism.

INTRODUCTION

Extracellular ATP is known to induce a variety of responses in several cell types (reviewed by Gordon, 1986). These responses include contraction of visceral smooth muscle (Maguire & Satchell, 1981), the regulation of vascular tone (Pearson, 1988), and the control of various secretory and metabolic processes, including histamine release by mast cells (Dahlquist & Diamant, 1974; Cockcroft & Gomperts, 1979b), glycogenolysis (Buxton *et al.*, 1986), insulin release (Loubatieres-Mariani *et al.*, 1979), surfactant secretion (Rice & Singleton, 1987) and prostacyclin release by endothelial cells (Needleman *et al.*, 1974; Pearson *et al.*, 1983; Boeynaems & Galand, 1983). Both mitogenic and anti-mitogenic effects of ATP have been reported to occur in lymphocytes (Gregory & Kern, 1978; Ikehara *et al.*, 1981). The receptors mediating these diverse effects of ATP have been grouped under the term P_2 purinoceptors, of which several subtypes have been delineated pharmacologically (Burnstock & Kennedy, 1985; Gordon, 1986).

In several cell types P_2 receptors have been shown to be coupled to the activation of inositol phospholipid breakdown, thereby controlling the generation of inositol phosphates and diacylglycerol, second messengers which mediate Ca^{2+} mobilization and protein kinase C activation (Berridge & Irvine, 1984; Nishizuka, 1986). This type of response to ATP has been reported in endothelial cells (Hallam & Pearson, 1986; Piroton *et al.*, 1987), Ehrlich ascites cells (Dubyak & De Young, 1985), hepatocytes (Charest *et al.*, 1985), aortic myocytes (Phaneuf *et al.*, 1987), alveolar cells (Rice & Singleton,

1987), the HL60 cell line (Dubyak *et al.*, 1988) and primary pituitary cells (Van der Merwe *et al.*, 1989).

An entirely different phenomenon caused by extracellular ATP is a non-selective cell-membrane permeabilization, which occurs in mast cells (Cockcroft & Gomperts, 1979a) and in transformed mouse cell lines (Rozengurt & Heppel, 1979). This effect is strictly specific for ATP (not other nucleotides) and requires relatively high ATP concentrations ($> 100 \mu\text{M}$ total ATP at physiological bivalent-cation concentrations). Both types of response (phospholipase C activation and permeabilization) appear to occur in J774 cells (Greenberg *et al.*, 1988).

In view of the coupling of ATP receptors to phospholipase C in other cell types, we investigated the effects of extracellular nucleotides in human skin fibroblasts. We report that in these cells phospholipase C is activated by a nucleotide receptor different from the hitherto characterized subclasses of P_2 purinoceptors.

MATERIALS AND METHODS

Materials

myo-[2- ^3H]inositol, *myo*-[2- ^3H]inositol 1-phosphate, 2-deoxy-D-[2,6- ^3H]glucose and [^3H]uridine were purchased from Amersham International. $^{45}\text{CaCl}_2$ was from New England Nuclear. Guanosine $5'-[\gamma\text{-thio}]\text{triphosphate}$ was from Boehringer-Mannheim Biochemicals (Mannheim, Germany), and 2-methylthio-ATP was from Research Biochemicals (Natick, MA, U.S.A.). Staphylococcal α -toxin was obtained from Dr. S. Bhakdi, Institute of Medical Microbiology, University of Giessen, Giessen,

Abbreviations used: EC_{50} , concn. giving 50 % stimulation; PMA, phorbol myristate acetate.

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Germany. Pertussis toxin was from List Biological Laboratories (Campbell, CA, U.S.A.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Methods

Cell culture. Human fibroblasts grown from forearm skin biopsies were maintained in Dulbecco's Modified Eagle's Medium (GIBCO, Grand Island, NY, U.S.A.), with 10% (v/v) foetal-calf serum (GIBCO) in humidified CO₂/air (1:9) at 37 °C, and were used between passages 8 and 30. Experiments were performed with confluent quiescent monolayers of cells in 6- or 12-well culture plates (Nunc, Copenhagen, Denmark). ATP-stimulated ⁴⁵Ca²⁺ efflux was observed in five fibroblast lines derived from different individuals (results not shown). All data presented were obtained with a single fibroblast line designated F199.

⁴⁵Ca²⁺ efflux. Cells were loaded for 1–1.5 h in buffer A containing 5 μCi of ⁴⁵Ca²⁺/ml and 0.2 mM-CaCl₂. Buffer A comprised (mM): NaCl 140; KCl 4; MgCl₂ 1; Na₂HPO₄ 1.4; glucose 8.3; Hepes 20 (pH 7.4); Phenol Red 6 mg/l; and bovine serum albumin (fatty acid free, Miles Laboratories) 0.5 g/l. The cells were then washed six times with buffer A containing 1 mM-Ca²⁺ at 37 °C, and were placed on a slowly rotating shaker for 10 min during each of the last four washes. This washing procedure allows exchange of ⁴⁵Ca²⁺ bound to extracellular sites and located in rapidly exchanging intracellular compartments. After washing, the basal fractional efflux rate of the remaining ⁴⁵Ca²⁺ was 4–5% per 6 min in unstimulated cells, corresponding to a kinetic compartment of average half-life (*t*_{1/2}) = 80–100 min. ⁴⁵Ca²⁺ fractional efflux was derived by expressing ⁴⁵Ca²⁺ released during the efflux period (usually 6 min) as a percentage of total cellular ⁴⁵Ca²⁺ present at the start of the efflux period. Initial total cellular ⁴⁵Ca²⁺ was calculated by adding effluxed radioactivity to that remaining in the cells after solubilization with 0.5% SDS. Except where otherwise stated, stimulations were carried out in the presence of 1 mM-Ca²⁺ and -Mg²⁺. To facilitate comparisons between experiments using different nucleotides, fractional-efflux data were normalized by expressing the fractional efflux as a percentage of that in response to 100 μM-ATP (maximal response), which was included in each experiment.

Inositol phosphate production. Cells were labelled for 24 h in serum-free Medium 199 (GIBCO) containing 5 μCi of [³H]inositol/ml. After preincubation in buffer A containing 1 mM-Ca²⁺ and 10 mM-LiCl for 10 min, cells were stimulated with nucleotides in the same buffer for 20 min. Incubations were terminated by aspirating the medium, adding 0.5 ml of ice-cold 0.5 M-HClO₄ containing 5 mM-EDTA and 1 mM-DTPA (diethylenetriaminepenta-acetic acid) (Delahunty *et al.*, 1988), and 90 μg of phytic acid hydrolysate, prepared as described by Wreggett & Irvine (1987). After neutralization with 0.5 M-KOH and sedimentation of the precipitate at 4 °C for 30 min, the supernatants were chromatographed on Dowex anion-exchange resin as described by Martin (1986), except that total inositol phosphates were eluted in a single step with 1 M-ammonium formate/0.1 M-formic acid. The putative inositol phosphates were co-eluted with authentic *myo*-[2-³H]inositol 1-phosphate.

Incorporation of *myo*-[³H]inositol into phospholipids was determined by washing the HClO₄-insoluble cell residue three times with HClO₄, and solubilizing it with 0.1 M-NaOH.

Monitoring plasma-membrane permeability. Cells were loaded for 1 h in glucose-free buffer A containing 1 mM-Ca²⁺ and 0.4 μCi of 2-deoxy[³H]glucose/ml at 37 °C, then washed six times with buffer A. Then 1 ml of buffer A was added, and 0.1 ml samples were removed at the indicated times for determination of radioactivity. Efflux was expressed as a percentage of initial cellular radioactivity determined after lysis of the cells with 0.5% SDS. A similar procedure was used to monitor efflux of [³H]uridine metabolites.

Monitoring of nucleotide purity. Nucleotides were analysed for purity on a Spectra Physics h.p.l.c. system with a Hi Chrom APSHYP2537 anion-exchange column, eluted with a linear gradient of potassium phosphate from 50 mM (pH 2.5) to 500 mM (pH 3.8).

Data analysis. Concentrations of ATP⁴⁻, MgATP, CaATP, NaATP, KATP and HATP were calculated by using a computer program employing absolute stability constants as listed by Fabiato (1981) and adjusted for use at 37 °C as described by Martell & Smith (1976). The apparent stability constants calculated for our experimental conditions (pH 7.4) were (M⁻¹): MgATP 19215; CaATP 5916; NaATP 5.82; KATP 5.30. EC₅₀ values for agonists were determined by fitting dose-response curves for nucleotide-stimulated ⁴⁵Ca²⁺ efflux by least-squares non-linear regression to the equation

$$E = E_{\max} \cdot [N] / (EC_{50} + [N])$$

where E represents ⁴⁵Ca²⁺ fractional efflux (%/6 min), [N] is nucleotide concentration, and E_{max} is the ⁴⁵Ca²⁺ fractional efflux in response to 100 μM-ATP. Each Figure shows results from a single experiment which was representative of 2–4 similar experiments. Points represent the mean of duplicate wells, with error bars equal to the difference between duplicates. Error bars not drawn lie within the size of the symbol.

RESULTS

Extracellular ATP mobilizes Ca²⁺ and stimulates inositol phosphate production

In human fibroblasts, extracellular ATP stimulated the efflux of ⁴⁵Ca²⁺ from a slow-turnover (*t*_{1/2} = 80 min) intracellular pool (Fig. 1). The rate of ⁴⁵Ca²⁺ efflux was increased 16-fold over basal during the first 3 min of exposure to 100 μM-ATP, and the pool of ⁴⁵Ca²⁺ mobilized by ATP was essentially depleted of radioactivity after 6 min of stimulation. ATP-stimulated ⁴⁵Ca²⁺ efflux was largely independent of extracellular Ca²⁺, indicating that it is not a consequence of increased Ca²⁺ entry, but represents release of Ca²⁺ from intracellular stores (Fig. 1).

In medium containing physiological (1 mM) concentrations of Ca²⁺ and Mg²⁺, the EC₅₀ of ATP for stimulating ⁴⁵Ca²⁺ efflux was 4 μM total ATP (Table 1). This corresponds to a calculated ATP⁴⁻ concentration of 0.1 μM. A maximally effective concentration of ATP released 35–50% of the total cellular ⁴⁵Ca²⁺ during 6 min of stimulation, a somewhat smaller response than

Fig. 1. Time course of ATP-stimulated $^{45}\text{Ca}^{2+}$ efflux and dependence on extracellular Ca^{2+}

Cells were loaded with $^{45}\text{Ca}^{2+}$ and washed before addition of ATP ($100\ \mu\text{M}$) at zero time to all cells, except for controls ($\circ$). The following concentrations of Ca^{2+} were present from 9 min before ATP addition: 1 mM ($\bullet$); 0.1 mM ($\triangle$); nominally Ca^{2+} -free ($\blacktriangle$); Ca^{2+} -free plus 0.2 mM-EGTA ($\square$).

Fig. 2. ATP stimulation of inositol phosphate production and effect of pertussis toxin

Cells were prelabelled for 20 h with $\text{myo-}[^3\text{H}]\text{inositol}$ in the absence ($\circ$) or presence ($\bullet$) of 100 ng of pertussis toxin/ml, before stimulating for 30 min with ATP in the presence of Li^+ . Total inositol phosphates are expressed as a percentage of radioactivity in total cellular phospholipids.

Table 1. Potencies of nucleotides for $^{45}\text{Ca}^{2+}$ mobilization in human fibroblasts

EC_{50} values were determined from dose-response curves for $^{45}\text{Ca}^{2+}$ fractional efflux measured during a 6 min stimulation with nucleotides. Values shown are means of two to six experiments. The stimulation medium contained 1 mM- Mg^{2+} and $-\text{Ca}^{2+}$.

Group	Nucleotide	EC_{50} (μM)
1 (ATP contamination excluded)	ATP	4.2
	UTP	5.2
	ATP (γ -thio analogue)	16
	ITP	20
	ADP	45
	UDP	75
2 (ATP contamination not excluded)	GTP	150
	XTP	240
	ATP ($\beta\gamma$ -imido analogue)	380
	CTP	520
	TTP	690
	dATP	> 100
	GTP (γ -thio analogue)	> 100
3 (inactive)	AMP, adenosine, 2-methylthio-ATP, $\alpha\beta$ - and $\beta\gamma$ -methylene analogues of ATP, GDP (β -thio analogue)	> 1000

that achieved by $1\ \mu\text{M}$ -bradykinin (60–75 %) or 10 % foetal-calf serum (65–80 %; results not shown).

In cells prelabelled with $[^3\text{H}]\text{inositol}$, ATP stimulated inositol phosphate production with $\text{EC}_{50} = 8\ \mu\text{M}$ in the presence of 1 mM- Mg^{2+} and $-\text{Ca}^{2+}$ (Fig. 2). ATP-stimulated inositol phosphate accumulation in the presence of Li^+ was linear with time up to at least 20 min, indicating that ecto-nucleotidase activity probably does not significantly alter the action of nucleotides under our experimental conditions, and that the nucleotide receptors do not rapidly desensitize.

Since it has been reported that ATP can cause non-selective plasma-membrane permeabilization in some cell types (Cockcroft & Gomperts, 1979a; Rozengurt & Heppel, 1979; Steinberg *et al.*, 1987), we examined the effect of ATP on the efflux of 2-deoxy $[^3\text{H}]\text{glucose}$ metabolites from pre-loaded cells, as an index of plasma-membrane integrity. ATP (1 mM) did not cause any increase in 2-deoxy $[^3\text{H}]\text{glucose}$ efflux, in contrast with a 5-fold increase caused by the pore-forming staphylococcal α -toxin (results not shown). Similarly, ATP did not increase the rate of loss of radioactivity from cells pre-loaded with $[^3\text{H}]\text{uridine}$ (results not shown).

Agonist specificity of the nucleotide receptor

The EC_{50} for ATP-induced $^{45}\text{Ca}^{2+}$ efflux was dependent on the Mg^{2+} concentration in the extracellular medium. Increasing $[\text{Mg}^{2+}]$ caused the dose-response curve for ATP to shift to the right, without altering the maximal effect (Fig. 3). This indicates that, although MgATP is the form present in highest concentration, it is not the principal agonist at the receptor, and suggests that one of the other forms of ATP (ATP^{4-} , NaATP , KATP or HATP) is mainly responsible for the ATP effects.

Fig. 3. Effect of Mg^{2+} concentration on ATP-stimulated $^{45}\text{Ca}^{2+}$ efflux

Cells were loaded with $^{45}\text{Ca}^{2+}$, washed, and finally rinsed with Mg^{2+} -free buffer before stimulating for 6 min with ATP in efflux buffer containing the following Mg^{2+} concentrations: 0 ($\circ$); 0.3 mM ($\bullet$); 2 mM ($\square$); 8 mM ($\blacksquare$). $[\text{Ca}^{2+}]$ was 0.3 mM throughout. The curves were fitted by least-squares non-linear regression of the data to a single-site binding equation as described under 'Methods'.

To characterize the specificity of the nucleotide receptor, EC_{50} values for various nucleotides were determined from dose-response curves of $^{45}\text{Ca}^{2+}$ fractional efflux (Table 1). All the nucleotides which had Ca^{2+} -mobilizing activity showed the same maximal effect, despite widely differing potencies, suggesting that they all activate the same receptor. This maximal effect was clearly different from (and less than) the maximal responses to bradykinin and foetal-calf serum (results not shown). In addition, maximally effective concentrations of ATP and UTP were non-additive in their capacity to mobilize $^{45}\text{Ca}^{2+}$ (results not shown),

constituting further evidence that these nucleotides activate the same receptor.

To exclude the possibility that the activity of these compounds was due to ATP contamination, UTP, ITP, ADP, UDP and adenosine 5'-[γ -thio]triphosphate (ATP[S]) were analysed by anion-exchange h.p.l.c., under conditions where a 2% contamination by ATP would have been detectable. UTP, ITP and ATP[S] were found to contain significant amounts of their respective nucleoside diphosphates, but no detectable ATP. ADP and UDP were not significantly contaminated with nucleoside triphosphates. Therefore the activity of these nucleotides cannot be ascribed to ATP contamination. It was not possible to exclude that the activity of the weak agonists (those with $\text{EC}_{50} > 150 \mu\text{M}$) may have been due to contamination with ATP or UTP, since even a small (< 2%) contamination with these agonists could explain their apparent activity.

Inhibition of nucleotide-stimulated inositol phosphate production and Ca^{2+} mobilization by pertussis toxin and phorbol ester

ATP stimulation of both inositol phosphate production (Fig. 2) and $^{45}\text{Ca}^{2+}$ mobilization (Fig. 4) was partially inhibited by pretreatment of cells with pertussis toxin. Pertussis toxin was maximally effective at a concentration of 30 ng/ml, which inhibited the response to a maximally effective concentration of ATP (30 μM) by 55–70%. Pertussis toxin (100 ng/ml) inhibited inositol phosphate production by 40–50%, and, as with $^{45}\text{Ca}^{2+}$ mobilization, the maximal extent of ATP stimulation was diminished without a major shift in EC_{50} (Figs. 2 and 4). In parallel experiments, bradykinin-stimulated $^{45}\text{Ca}^{2+}$ mobilization was not inhibited by pertussis toxin (results not shown).

At concentrations up to 600 ng/ml, pertussis toxin had no obvious effects on cell morphology and did not alter the amount of $^{45}\text{Ca}^{2+}$ taken up by cells during the loading period (results not shown), indicating that it did

Fig. 4. Effect of pertussis toxin on ATP-stimulated $^{45}\text{Ca}^{2+}$ efflux

Cells were incubated for 20 h with or without pertussis toxin, then loaded with $^{45}\text{Ca}^{2+}$, washed and stimulated with ATP for 6 min. For the toxin-treated cells, pertussis toxin was also present throughout the loading and washing procedure. Symbols: (a) 200 ng of pertussis toxin/ml ($\bullet$), control ($\circ$); (b) 30 mM-ATP ($\bullet$), control ($\circ$).

Fig. 5. Inhibition of ATP-stimulated $^{45}\text{Ca}^{2+}$ efflux by phorbol ester

After loading with $^{45}\text{Ca}^{2+}$ and washing, cells were preincubated for 5 min with 10 nM-PMA (□), 100 nM-PMA (△), or buffer only (○) before stimulating for 6 min with efflux buffer containing PMA as in the preincubation and ATP at the indicated concentrations.

not cause a major perturbation of cellular Ca^{2+} metabolism, nor did it have a general toxic effect on the cells. In addition, pertussis toxin (100 ng/ml) had no effect on total incorporation of *myo*-[^3H]inositol into phospholipids over the 20 h prelabelling period (results not shown).

Pretreatment of cells with phorbol myristate acetate (PMA) markedly inhibited $^{45}\text{Ca}^{2+}$ mobilization and inositol phosphate generation stimulated by nucleotides; 100 nM-PMA virtually abolished the response to a submaximal ATP concentration (10 μM), whereas the effect of a supramaximal ATP concentration (1000 μM) was inhibited by 75% (Fig. 5). UTP-stimulated $^{45}\text{Ca}^{2+}$ mobilization was inhibited to a similar degree by PMA (results not shown). In cells stimulated with 100 μM -ATP for 20 min in the presence of Li^+ , total inositol phosphate accumulation was increased to $475 \pm 24\%$ of control; in parallel cells pretreated with 100 nM-PMA for 5 min, the stimulation was decreased to $204 \pm 25\%$ of that in control cells.

DISCUSSION

We report here that in normal human skin fibroblasts low concentrations of extracellular nucleotides evoke $^{45}\text{Ca}^{2+}$ release from a slow-turnover pool, and stimulate inositol phosphate generation. The following lines of evidence indicate that these responses to nucleotides are due to receptor-mediated phospholipase C activation: (a) ATP did not cause non-selective plasma-membrane permeabilization, and the ATP concentration range over which the effects occurred was much lower than that reported for permeabilization; (b) nucleotide-stimulated $^{45}\text{Ca}^{2+}$ mobilization and inositol phosphate production were markedly inhibited by activation of protein kinase C, a feature typical of receptor-phospholipase C coupled systems; and (c) $^{45}\text{Ca}^{2+}$ mobilization and inositol

phosphate production were sensitive in part to inhibition by pertussis toxin.

In physiological extracellular medium (140 mM- Na^+ , 4 mM- K^+ , 1 mM- Ca^{2+} and - Mg^{2+} , pH 7.4), ATP exists in a number of forms, with the following distribution: MgATP (71%), CaATP (22%), NaATP (3.0%), KATP (0.8%), ATP^{4-} (2.4%) and HATP (1.2%). Our results indicate that CaATP is not the predominantly active form at the human fibroblast nucleotide receptor, since ATP-stimulated $^{45}\text{Ca}^{2+}$ mobilization was not greatly decreased in Ca^{2+} -free medium containing EGTA. The EC_{50} for ATP increased with increasing extracellular $[\text{Mg}^{2+}]$, as would be expected if ATP^{4-} , NaATP, KATP or HATP were the active form at the receptor, and MgATP was inactive. However, the observed degree of rightward shift in the ATP dose-response curve with increasing $[\text{Mg}^{2+}]$ (Fig. 3) was slightly less than the theoretical calculated shift that would be expected on the assumption that MgATP is inactive. This result therefore suggests that MgATP is not completely inactive, but behaves as a weak agonist at the receptor.

Although the permeabilization of mast cells by ATP appears to be an entirely different phenomenon from that described here, it is of interest that ATP uncomplexed with bivalent cations is also the active form mediating permeabilization (Cockcroft & Gomperts, 1979b).

The nucleotide specificity of the human fibroblast receptor is of interest, since nucleotide receptors are heterogeneous and their present classification is rudimentary (Gordon, 1986). The lack of efficacy of AMP and adenosine indicates a P_2 rather than a P_1 purinoceptor (Burnstock & Kennedy, 1985). Four subgroups of P_2 purinoceptors (P_{2X} , P_{2Y} , P_{2T} , P_{2Z}) have been delineated (Gordon, 1986). The human fibroblast receptor does not belong to the P_{2X} group, since the $\alpha\beta$ - and $\beta\gamma$ -methylene-substituted analogues of ATP were inactive. It is also clearly different from the platelet (P_{2T}) receptor, which responds only to ADP and not ATP, and from the mast-cell (P_{2Z}) receptor, which mediates permeabilization, and therefore would appear to belong to the P_{2Y} group. However, since 2-methylthio-ATP was almost inactive, the human fibroblast receptor is different from the classic P_{2Y} receptors described in endothelial cells (Martin *et al.*, 1985), *taenia coli* (Burnstock *et al.*, 1983) and pancreatic β -cells (Bertrand *et al.*, 1987), at which 2-methylthio-ATP is a superactive agonist. The human fibroblast receptor appears to be very similar in specificity to that on Ehrlich ascites-tumour cells (Dubyak & De Young, 1985). Interestingly, the receptor is activated equally well by UTP as by purine nucleotides, and the term 'purinoceptor' therefore seems inappropriate for this class of receptor.

The inhibition of nucleotide-stimulated phospholipase C activation by phorbol ester appears to be an example of a general property of many receptor-mediated phospholipase C signal-transduction systems (Nishizuka, 1986). ATP-induced Ca^{2+} mobilization has previously been shown to be inhibited by phorbol esters in hepatocytes (Charest *et al.*, 1985) and in HL60 cells (Dubyak *et al.*, 1988). It would appear likely that this inhibition results from a C-kinase-mediated phosphorylation; possible sites of this phosphorylation would include the nucleotide receptor, a G-protein coupling the receptor to phospholipase C, or phospholipase C itself.

The coupling of some receptors to phospholipase C is mediated by a pertussis-toxin-sensitive G-protein,

whereas in others the coupling mechanism is insensitive to pertussis toxin (reviewed by Cockcroft & Stutchfield, 1988). In human fibroblasts, pertussis toxin was able to achieve only a partial blockade of coupling, a finding very similar to that reported for HL60 cells by Dubyak *et al.* (1988). These results suggest that the nucleotide receptor coupling to phospholipase C is mediated in parallel by a pertussis-toxin-sensitive G-protein and by a toxin-insensitive mechanism.

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REFERENCES

- Berridge, M. J. & Irvine, R. F. (1984) *Nature* (London) **312**, 315–321
- Bertrand, G., Chapal, J., Loubatieres-Mariani, M. M. & Roye, M. (1987) *Br. J. Pharmacol.* **91**, 783–787
- Boeynaems, J. M. & Galand, N. (1983) *Biochem. Biophys. Res. Commun.* **112**, 290–296
- Burnstock, G. & Kennedy, C. (1985) *Gen. Pharmacol.* **16**, 433–440
- Burnstock, G., Cusack, N. J., Hills, J. M., Mackenzie, I. & Meghji, P. (1983) *Br. J. Pharmacol.* **79**, 907–913
- Buxton, B., Robertson, S. M. & Olson, M. S. (1986) *Biochem. J.* **237**, 773–780
- Charest, R., Blackmore, P. F. & Exton, J. H. (1985) *J. Biol. Chem.* **260**, 15789–15794
- Cockcroft, S. & Gomperts, B. D. (1979a) *Nature* (London) **279**, 541–542
- Cockcroft, S. & Gomperts, B. D. (1979b) *J. Physiol.* (London) **296**, 229–243
- Cockcroft, S. & Stutchfield, J. (1988) *Philos. Trans. R. Soc. London B* **320**, 247–265
- Dahlquist, R. & Diamant, B. (1974) *Acta Pharmacol. Toxicol.* **34**, 363–384
- Delahunty, T. M., Cronin, M. J. & Linden, J. (1988) *Biochem. J.* **255**, 69–77
- Dubyak, G. R. & De Young, M. B. (1985) *J. Biol. Chem.* **260**, 10653–10661
- Dubyak, G. R., Cowen, D. S. & Mueller, L. M. (1988) *J. Biol. Chem.* **263**, 18108–18117
- Fabiato, A. (1981) *J. Gen. Physiol.* **78**, 457–497
- Gordon, J. L. (1986) *Biochem. J.* **233**, 309–319
- Greenberg, S., Di Virgilio, F., Steinberg, T. & Silverstein, S. C. (1988) *J. Biol. Chem.* **263**, 10337–10343
- Gregory, S. & Kern, M. (1978) *Biochem. Biophys. Res. Commun.* **83**, 1111–1116
- Hallam, T. J. & Pearson, J. D. (1986) *FEBS Lett.* **207**, 95–99
- Ikehara, S., Pahwa, R. N., Lunzer, D. G., Good, R. A. & Modak, M. J. (1981) *J. Immunol.* **127**, 1834–1838
- Loubatieres-Mariani, M. M., Chapal, J., Lignon, F. & Valette, G. (1979) *Eur. J. Pharmacol.* **59**, 277–286
- Maguire, M. H. & Satchell, D. G. (1981) in *Purinergic Receptors* (Burnstock, G., ed.), pp. 49–91, Chapman and Hall, London
- Martell, A. E. & Smith, R. M. (1976) *Critical Stability Constants*, pp. xi–xiii, Plenum, New York
- Martin, T. F. J. (1986) *Methods Enzymol.* **124**, 424–442
- Martin, W., Cusack, N. J., Carleton, J. S. & Gordon, J. L. (1985) *Eur. J. Pharmacol.* **108**, 295–299
- Needleman, P., Minkes, M. S. & Douglas, J. R. (1974) *Circ. Res.* **34**, 455–460
- Nishizuka, Y. (1986) *Science* **223**, 305–312
- Pearson, J. D. (1988) *Biochem. Soc. Trans.* **16**, 480–482
- Pearson, J. D., Slakey, L. L. & Gordon, J. L. (1983) *Biochem. J.* **214**, 273–276
- Phaneuf, S., Berta, P., Casanova, J. & Cavadore, J. C. (1987) *Biochem. Biophys. Res. Commun.* **143**, 454–460
- Pirotton, S., Raspe, E., Demolle, D., Erneux, C. & Boeynaems, J. M. (1987) *J. Biol. Chem.* **262**, 17461–17466
- Rice, W. R. & Singleton, F. M. (1987) *Br. J. Pharmacol.* **93**, 833–838
- Rozengurt, E. & Heppel, L. A. (1979) *J. Biol. Chem.* **254**, 708–714
- Steinberg, T. H., Newman, A. S., Swanson, J. A. & Silverstein, S. C. (1987) *J. Biol. Chem.* **262**, 8884–8888
- Van der Merwe, P. A., Wakefield, I. K., Fine, J., Millar, R. P. & Davidson, J. S. (1989) *FEBS Lett.* **243**, 333–336
- Wreggett, K. A. & Irvine, R. F. (1987) *Biochem. J.* **245**, 655–660

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