Cyclic AMP inhibition of fMet-Leu-Phe-dependent metabolic responses in human neutrophils is not due to its effects on cytosolic Ca^{2+}

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Cyclic AMP powerfully inhibits the fMet-Leu-Phe-dependent respiratory burst and exocytosis of azurophilic and specific granules without affecting Ca^{2+} release from intracellular stores. The elevation of $[\text{Ca}^{2+}]_i$ induced by fMet-Leu-Phe is short-lived in cyclic AMP-treated cells and similar to that of untreated cells stimulated in the absence of external Ca^{2+} . Nevertheless, in these latter cells fMet-Leu-Phe induces metabolic activation. We therefore suggest that the inhibitory action of cyclic AMP on neutrophil responses is not due to its effects on $[\text{Ca}^{2+}]_i$ homeostasis.

In many different cell systems the interaction of agonists with specific membrane receptors is coupled to rapid changes in the level of $[\text{Ca}^{2+}]_i$ and cyclic AMP (Tsien *et al.*, 1984; Berridge, 1975). In general the activation of Ca^{2+} -mobilizing receptors also induces a turnover of phosphatidylinositol (Michell, 1979; Cockcroft, 1981).

In leucocytes, receptors that induce $[\text{Ca}^{2+}]_i$ elevation activate cell responses, whereas procedures which raise intracellular cyclic AMP antagonize such stimulation (Smolen *et al.*, 1980; Simchowicz *et al.*, 1980; Weissman *et al.*, 1971; Ignarro *et al.*, 1974). A similar inhibitory role of cyclic AMP has been shown in platelets (Salzman, 1972; Chiang *et al.*, 1975). The mechanism by which cyclic AMP inhibits leucocyte and platelet responses is, however, unclear.

Recently, on the basis of measurements of $[\text{Ca}^{2+}]_i$ performed with the fluorescent indicator quin2, it has been suggested that in human platelets cyclic AMP-dependent reactions can regulate $[\text{Ca}^{2+}]_i$, possibly by inhibiting Ca^{2+} mobilization from intracellular stores or by stimulating Ca^{2+} extrusion from the cytosol (Feinstein *et al.*, 1983; Zavoico & Feinstein, 1984). In the present study we have investigated the relationship between intracellular increase of cyclic AMP, modifications of $[\text{Ca}^{2+}]_i$ and metabolic responses

in human neutrophils activated with the chemotactic peptide fMet-Leu-Phe.

Our results show that the mechanism by which cyclic AMP inhibits fMet-Leu-Phe-induced respiratory and secretory responses is independent of modifications of $[\text{Ca}^{2+}]_i$.

Materials and methods

Preparation of neutrophils

Neutrophils, more than 98% pure, were prepared by dextran sedimentation and centrifugation on Ficoll Hypaque gradients (Boyum, 1968) using citrate-anticoagulated venous blood obtained from healthy adult donors. After hypo-osmotic lysis of contaminating erythrocytes, the cells were suspended in medium containing 138 mM-NaCl, 6 mM-KCl, 1.2 mM-MgCl₂, 1 mM-P_i, 0.5 mM-CaCl₂, 5.6 mM-glucose and 20 mM-Hepes (pH 7.4). In some experiments 4 mM-NaHCO₃ was also present.

Metabolic studies

The respiratory response was measured as O₂ consumption at 37°C with a Clark oxygen electrode as previously described (Rossi *et al.*, 1981) using 1.5×10^7 neutrophils, unless otherwise indicated, suspended in the above medium supplemented with 5 µg of cytochalasin B/ml and 2 mM-NaN₃ in order to avoid the inactivation of the chemotactic peptide used as stimulus.

Secretion assay

Human neutrophils (10^7 /ml) suspended in the above medium containing cytochalasin and NaN₃

Abbreviations used: $[\text{Ca}^{2+}]_i$, ionized cytosolic Ca^{2+} ; PMA, phorbol 12-myristate, 13-acetate; PGE₁, prostaglandin E₁; Hepes, 4-(2-hydroxymethyl)-1-piperazine-ethane sulphonic acid.

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were incubated at 37°C for 5 min in polystyrene tubes in the presence or absence of the inhibitors. Thereafter fMet-Leu-Phe (100 nM) or PMA (33 nM) were added and the incubation was continued for a further 5 or 10 min respectively. After this time the cell suspension was rapidly pelleted by centrifugation at 8000g for 30 s in a microcentrifuge (Eppendorf). The supernatants were assayed for released β -glucuronidase (Gianetto & De Duve, 1955) as a marker of secretion of azurophilic granules, vitamin B₁₂-binding protein (Kane & Peters, 1975) as marker of secretion of specific granules and lactate dehydrogenase (Wroblewski & La Due, 1955) as a marker of cytosolic components. The values of stimulated secretion were corrected for spontaneous release and then calculated as a percentage of the total content measured on sonicated untreated neutrophils. Lactate dehydrogenase release was always less than 5% in all determinations.

fMet-Leu-[³H]Phe binding

The binding of fMet-Leu-[³H]Phe to neutrophils was measured by using a rapid filtration technique as previously described (De Togni *et al.*, 1983). Non-specific binding was defined as the amount of binding not inhibited by a 500-fold excess of unlabelled fMet-Leu-Phe and specific binding as the total amount of fMet-Leu-[³H]Phe bound minus the non-specific binding.

Transmembrane potential

Membrane potential was monitored with the lipophilic fluorescent dye bis-(1,3-diethylthio-barbiturate)-trimethineoxonal (bis-oxonol) (Rink *et al.*, 1980). Briefly, when this dye moves from aqueous solution into a non-polar environment, as when it binds to membranes or proteins, its fluorescence increases. Depolarization of the membranes increases the transfer of the dye anions from the external solution onto binding sites inside the cell, thus increasing the net fluorescence. Excitation and emission wavelengths were 540 and 580 nm respectively. Bis-oxonol was added at a concentration of 100 nM to a stock cell suspension kept at room temperature, from which samples were taken and equilibrated at 37°C for 5 min before starting the experiments.

[Ca²⁺]_i measurements

Cytosolic Ca²⁺ was monitored with quin2 (Tsien *et al.*, 1982; Di Virgilio & Gomperts, 1983). Neutrophils were suspended in the standard medium described above at a concentration of 5 × 10⁷/ml and equilibrated at 37°C for 5 min. Then quin2 acetoxymethyl ester (quin2-AM) was added at a final concentration of 15 μM. After 15 min, the cells were diluted to 1 × 10⁷/ml with warm medium

and left at 37°C for a further 40 min. After loading the neutrophils were washed and kept at room temperature until used. The intracellular quin2 concentration was between 0.24 and 0.35 nmol/10⁶ cells. Fluorescence measurements were performed in a Perkin-Elmer 650-40 fluorescence spectrophotometer equipped with a thermostat-controlled cuvette holder and magnetic stirring. Excitation and emission wavelengths were 339 and 492 nm respectively; the excitation slit width was 3 nm and emission slit width 10 nm. The fluorimeter was equipped with two cut-off filters, UV D 25 and UV 35, for excitation and emission respectively in order to minimize light-scattering artifacts. Fluorescence calibration as a function of [Ca²⁺]_i was performed by lysing the cells with Triton X-100 (0.1%) at pH 8.67 in the presence of EGTA and Tris (6.6 mM and 40 mM respectively) in order to give the zero-Ca²⁺ fluorescence ($F_{\min.}$) and then by adding back excess Ca²⁺ to give $F_{\max.}$. [Ca²⁺]_i was calculated according to:

$$[\text{Ca}^{2+}]_i = K_d(F - F_{\min.}) / (F_{\max.} - F)$$

where K_d is 115 nM and F is the fluorescence of the intracellular indicator before lysis (Tsien *et al.*, 1982).

Reagents

fMet-Leu-Phe, PMA, cytochalasin B, PGE₁ and dibutyryl cyclic AMP were purchased from Sigma; fMet-Leu-[³H]Phe was from New England Nuclear; [⁵⁷Co] cyanocobalamin from Amersham International and quin2 from Calbiochem. All the reagents employed were of the highest available purity.

Results

Fig. 1 shows that in human neutrophils the elevation of intracellular cyclic AMP by means of externally added dibutyryl cyclic AMP and theophylline completely inhibits the respiratory burst caused by fMet-Leu-Phe. Fig. 2 shows that this effect is not due to an inhibition of the binding of the peptide, since in the presence of dibutyryl cyclic AMP the binding of fMet-Leu-[³H]Phe is only slightly decreased at short incubation times.

Table 1 shows that the secretion of azurophilic and specific granules caused by fMet-Leu-Phe is also strongly inhibited by the rise in intracellular cyclic AMP. In the presence of both dibutyryl cyclic AMP and theophylline the inhibition was 97% and 83% for the release of azurophilic and specific granules respectively. When PGE₁, which is known to activate the adenylate cyclase, was used instead of dibutyryl cyclic AMP, secretion was inhibited to a smaller extent, although still significantly (50%). Interestingly, the release of

Table 1. *Effect of dibutyryl cyclic AMP, theophylline and prostaglandin E₁ on fMet-Leu-Phe-induced β-glucuronidase and vitamin B₁₂-binding protein release*

fMet-Leu-Phe was at 100nM, dibutyryl cyclic AMP 1mM, theophylline 2mM, PGE₁ 20μM. Values represent means ± S.E.M. for the number of experiments given in parentheses. For other experimental details see the Materials and methods section.

Additions	β-Glucuronidase		Vitamin B ₁₂ -binding protein	
	Release (%)	Inhibition of release (%)	Release (%)	Inhibition of release (%)
fMet-Leu-Phe	47.3 ± 3.3 (4)	—	45.6 ± 3.1 (4)	—
fMet-Leu-Phe + dibutyryl cyclic AMP	23.4 ± 6.1 (4)	50.5	30.3 ± 5.0 (4)	28.7
fMet-Leu-Phe + theophylline	17.9 ± 7.6 (4)	62.2	17.3 ± 5.0 (4)	56.9
fMet-Leu-Phe + theophylline + dibutyryl cyclic AMP	1.4 ± 1.2 (4)	97.0	6.7 ± 2.5 (4)	81.7
fMet-Leu-Phe + PGE ₁	23.9 ± 2.9 (3)	49.5	29.4 ± 3.5	35.5
fMet-Leu-Phe + PGE ₁ + theophylline	12.2 (2)	74.3	20.6 (2)	54.8

Fig. 1. *Inhibition by dibutyryl cyclic AMP of fMet-Leu-Phe-stimulated O₂ consumption*
fMet-Leu-Phe, 100nM, was added (arrow) to 1.5 × 10⁷ neutrophils suspended in standard buffer in the absence (a) or presence (b) of 1mM-dibutyryl cyclic AMP and 2mM-theophylline.

Table 2. *PMA-induced vitamin B₁₂-binding protein release is insensitive to cyclic AMP inhibition*

PMA was at 32nM; for other experimental details see Table 1 and the Materials and methods section.

Additions	Release of vitamin B ₁₂ -binding protein (%)
PMA	41.5 ± 3.5 (4)
PMA + dibutyryl cyclic AMP	41.4 ± 3.5 (4)
PMA + theophylline	39.7 ± 2.7 (4)
PMA + theophylline + dibutyryl cyclic AMP	40.9 ± 4.4 (4)
PMA + PGE ₁	43.3 (2)
PMA + PGE ₁ + theophylline	43.9 (2)

Fig. 2. *Kinetics of specific binding of fMet-Leu-[³H]Phe to cyclic AMP-inhibited neutrophils*
fMet-Leu-[³H]Phe was 50nM; cell concentration was 1.5 × 10⁷/ml. ●, Cells suspended in the standard buffer; ○, cells suspended in the standard buffer plus 1mM-dibutyryl cyclic AMP and 2mM-theophylline.

azurophilic granules was always more affected than that of specific granules. The reason for this differential sensitivity to cyclic AMP is not clear, but it is known that the two types of granules are differently released in response to various stimuli (Smith & Iden, 1979).

Table 2 shows that the secretion of specific granules induced by PMA is unaffected by dibutyryl cyclic AMP, theophylline and PGE₁. PMA is believed to act through a Ca²⁺-independ-

dent mechanism, possibly by direct stimulation of protein kinase C (Di Virgilio *et al.*, 1984). Data for β -glucuronidase release are not shown, since PMA is a very weak secretagogue for azurophilic granules.

Fig. 3 shows that the respiratory burst induced by PMA is also not modified by dibutyl cyclic AMP and theophylline.

Fig. 4 shows that fMet-Leu-Phe-dependent membrane depolarization is markedly inhibited by dibutyl cyclic AMP and theophylline. However the addition of PMA to cyclic AMP-inhibited cells after the chemotactic peptide causes a normal depolarization. Membrane depolarization induced by the sodium-carrying ionophore gramicidin is shown in Fig. 4 for comparison.

The insensitivity to cyclic AMP inhibition of a

Fig. 3. PMA-induced O_2 consumption is not inhibited by cyclic AMP

PMA (added at the arrow) was 80 nM; cell concentration was 1.5×10^7 /ml. Neutrophils were incubated in the absence (a) or in the presence (b) of 1 mM-dibutyl cyclic AMP and 2 mM-theophylline.

stimulus such as PMA, which acts independently of Ca^{2+} , prompted us to investigate in more detail the effects of the elevation of intracellular cyclic AMP on $[Ca^{2+}]_i$ homeostasis.

Fig. 5 shows the effects of fMet-Leu-Phe on O_2 consumption and $[Ca^{2+}]_i$ in neutrophils incubated with Ca^{2+} (a and b) or without Ca^{2+} (c and d), in the presence (b and d) or absence (a and c) of dibutyl cyclic AMP and theophylline. Autofluorescence traces are shown for each experimental condition at the bottom of the figure. A typical respiratory burst is caused by fMet-Leu-Phe and this is diminished by chelation of extracellular Ca^{2+} by EGTA both in terms of initial rate and in extent. Incubation with dibutyl cyclic AMP and theophylline completely abolishes O_2 consumption in the presence or absence of external Ca^{2+} . In the presence of extracellular Ca^{2+} , fMet-Leu-Phe induces a rapid increase of $[Ca^{2+}]_i$, which slowly falls towards basal levels in about 6–7 min. External Ca^{2+} chelation by excess EGTA slightly diminishes the initial rapid $[Ca^{2+}]_i$ rise and accelerates the decay phase. The rapid initial increase has been interpreted as due to the release of Ca^{2+} from intracellular stores; the larger $[Ca^{2+}]_i$ rise and the slower decay of the $[Ca^{2+}]_i$ transients in the presence of extracellular Ca^{2+} have been attributed to an increase in influx of Ca^{2+} across the plasma membrane (Pozzan *et al.*, 1983; Lew *et al.*, 1984). Our data are consistent with this interpretation. In the presence of dibutyl cyclic AMP and theophylline the rapid initial rise is quantitatively similar to that obtained in the absence of the inhibitors. The kinetics of the $[Ca^{2+}]_i$ transients however are unaffected by external Ca^{2+} chelation and are similar to those of controls in the presence of EGTA.

Discussion

Of the proposed intracellular messengers responsible for the transduction of signals delivered at the plasma membrane level by agonists, Ca^{2+} and cyclic AMP have attracted the most attention. In many cell types different receptors are linked to Ca^{2+} mobilization or cyclic AMP elevation and the two mediators seem to have an antagonist role on cell activation. In particular, in leucocytes and platelets the elevation of intracellular cyclic AMP by different means strongly inhibits cell activation (Smolen *et al.*, 1980; Simchowitz *et al.*, 1980; Salzman, 1972; Chiang *et al.*, 1975). Two main hypotheses have been advanced to explain the mechanism of inhibition by cyclic AMP.

(i) Cyclic AMP affects $[Ca^{2+}]_i$ homeostasis, possibly by blocking Ca^{2+} mobilization from intracellular pools and/or by enhancing Ca^{2+} sequestration (Käser-Glanzman *et al.*, 1977, 1979; Owen &

Fig. 4. Effect of cyclic AMP on membrane depolarization

(a) Cells pretreated for 2 min with 1 mM-dibutyl cyclic AMP and 2 mM-theophylline; (b) control; (c) control depolarized with gramicidin. fMet-Leu-Phe was at 100 nM, PMA was 20 nM, gramicidin was 60 nM. Cells were 3×10^5 /ml.

Le Breton, 1981; Feinstein *et al.*, 1983; Zavoico & Feinstein, 1984).

(ii) Cyclic AMP interferes with phospholipid turnover by inhibiting phospholipase C activity (Tou & Maier, 1976; Takai *et al.*, 1982; Kaibuchi *et al.*, 1982; Imai *et al.*, 1983; Yamanishi *et al.*, 1983; Rittenhouse-Simmons, 1979; Knight & Scrutton, 1984).

It has been recently shown that forskolin, PGD₂ and theophylline, agents which elevate intracellular cyclic AMP, strongly reduce the increase of $[Ca^{2+}]_i$ in thrombin-stimulated platelets (Feinstein *et al.*, 1983; Zavoico & Feinstein, 1984). On the other hand, Rink and coworkers (Rink *et al.*, 1982) have demonstrated that thrombin can activate platelets and cause release at or near basal $[Ca^{2+}]_i$, thus suggesting that thrombin might be able to trigger exocytosis by a Ca^{2+} -independent pathway. These latter results suggest that, if the point of inhibition by cyclic AMP is solely at the level of

$[Ca^{2+}]_i$ changes, one would not expect a relevant effect of cyclic AMP on thrombin stimulation.

As far as neutrophils are concerned it has long been known that cyclic AMP inhibits fMet-Leu-Phe responses. Our findings show that it affects $[Ca^{2+}]_i$ homeostasis by shortening the duration of the fMet-Leu-Phe induced rise in $[Ca^{2+}]_i$, whereas it has minor effects on the rate and on the extent of the initial $[Ca^{2+}]_i$ rise. Chelation of extracellular Ca^{2+} does not modify the $[Ca^{2+}]_i$ transients in cyclic AMP-inhibited cells and therefore it can be concluded that, in neutrophils, cyclic AMP at this concentration has no major effect on the release of Ca^{2+} from intracellular stores. Rather our data suggest that cyclic AMP affects the mechanisms responsible either for Ca^{2+} influx into the stores or for Ca^{2+} extrusion from the cytosol. We believe, however, that these effects on $[Ca^{2+}]_i$ are not related to the mechanism of cyclic AMP modulation of neutrophil activation, because, in the

Fig. 5. Effects of cyclic AMP on O_2 consumption and $[Ca^{2+}]_i$.

Upper traces show O_2 consumption, middle traces Ca^{2+} changes and lower traces autofluorescence in cells incubated in 0.5 mM- Ca^{2+} (a), in 0.5 mM Ca^{2+} plus 1 mM-dibutyryl cyclic AMP and 2 mM-theophylline (b), in 2 mM-EGTA (c), and in 2 mM EGTA plus 1 mM dibutyryl cyclic AMP and 2 mM-theophylline (d). All samples were taken from the same batch of quin2-loaded cells, which were incubated for 5 min with or without the inhibitors before starting the experiment. Cell concentration was 2.5×10^6 /ml both in the quin2 experiments and in the O_2 experiments; fMet-Leu-Phe was 100 nM and PMA was 20 nM.

absence of external Ca^{2+} , fMet-Leu-Phe induces $[Ca^{2+}]_i$ transients similar to those observed in cyclic AMP-inhibited cells, and yet neutrophils undergo metabolic activation. Furthermore, in the first 30 s after fMet-Leu-Phe addition, $[Ca^{2+}]_i$ levels are the same both in cyclic AMP-inhibited and in control cells, and by this time the respiratory response is already maximally activated and secretion of granules complete (Cockcroft *et al.*, 1980; Sklar *et al.*, 1982). Our evidence therefore points to a different site for cyclic AMP inhibition, possibly at the level of the generation of another second messenger. Indeed, the formation of an additional intracellular signal besides Ca^{2+} has been postulated in order to explain fMet-Leu-Phe-dependent neutrophil activation (Pozzan *et al.*, 1983). The metabolite which seems to fulfil the requirements for such a role is diacylglycerol, a product of phosphatidylinositol breakdown (Michell, 1983). Diacylglycerol is considered to be the physiological activator of the ubiquitous Ca^{2+} -phospholipid-dependent protein kinase C (Nishizuka, 1984), perhaps acting synergistically with Ca^{2+} (Di Virgilio *et al.*, 1984).

Preliminary results in our laboratory (see also Tou & Maier, 1976) also suggest that the increase

in cellular cyclic AMP inhibits phosphatidylinositol metabolism, thus preventing the formation of diacylglycerol. Under these conditions the $[Ca^{2+}]_i$ rise is an ineffective stimulus, probably because in the absence of diacylglycerol, protein kinase C is insufficiently activated. This interpretation is consistent with the lack of effect of cyclic AMP on neutrophil stimulation by a specific activator of protein kinase C such as PMA.

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