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DIFFERENTIAL REGULATION OF EARLY PHASE AND LATE PHASE RESPONSES IN HUMAN NEUTROPHILS BY cAMP

R. HUGH DANIELS,* IAN N. BIRD, MAXINE E. HILL and MICHAEL J. FINNEN
Yamanouchi Research Institute (U.K.), Littlemore Hospital, Littlemore, Oxford OX4 4XN, U.K.

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Abstract—The elevation of intracellular levels of cyclic AMP by forskolin stimulation of adenylate cyclase regulates early and late phase neutrophil responses differentially. Early phase neutrophil responses as measured by shape change in response to chemotactic factors, transmigration across a polycarbonate membrane and priming were unaffected by forskolin-induced elevation of intracellular cAMP. Late phase neutrophil responses such as release of superoxide anions, activation of phospholipase A_2 and platelet activating factor (PAF) synthesis were inhibited by increasing intracellular cAMP through the addition of 10 μM forskolin for 10 min prior to stimulation. *N*-Formyl-methionyl-leucyl-phenylalanine-stimulated arachidonic acid release fell from 9.3% (untreated cells) to 4.6% in forskolin-treated cells. PAF generation was also inhibited from 430 pg/10^6 cells in untreated cells to background levels in forskolin-treated cells (110 pg/10^6 cells). Also, the reduction of cytochrome *c* by superoxide anions fell from 4.2 nmol/10^6 cells in the absence of forskolin to 2.0 nmol/10^6 cells following forskolin treatment. These results indicate that in neutrophils the elevation of cAMP acts differentially on cellular responses, not affecting early activation events, but markedly inhibiting late events such as the release of inflammatory mediators.

Neutrophils are important effector cells during an acute inflammatory response. The signal transduction pathways, regulating the many processes neutrophils use to move from the circulation to a site of inflammation, have been extensively investigated but still remain unclear. Neutrophils must carry out their complex activities in the right sequence and in the right place. The behaviour of neutrophils when stimulated by a variety of chemotactic agonists can be simplistically divided into "early" and "late" events dependent on the signal molecule and its concentration [1]. "Early phase" neutrophil activation generates cellular responses which would occur upon initial stimulation of neutrophils *in vivo*, such as changes in adhesive properties, locomotory activity and priming of the neutrophil to subsequent stimulation of metabolic processes [2, 3]. The "late phase" of neutrophil activation represents those events which occur once a neutrophil has migrated across the endothelium, through the tissue and arrived at the site of damage or infection, and is marked by generation of the respiratory burst, activation of phospholipases (leading to pro-inflammatory lipid mediator release) and phagocytosis.

The most extensively used chemotactic agonist in studies of neutrophil responses is *N*-formyl-methionyl-leucyl-phenylalanine (fMLP†). At nanomolar concentrations, fMLP elicits changes in adhesion and movement of neutrophils (early events) [2]. Higher concentrations (micromolar) of fMLP induce neutrophil superoxide anion production, hydrolytic enzyme release and phagocytosis (late events) [4]. Other chemotactic agonists [e.g. interleukin-8 (IL-8)] that fully activate early responses are poor activators of late responses. However, this is often accompanied by an augmentation of late responses to a subsequent agonist such as micromolar concentrations of fMLP [5]. Several chemotactic agonists have been shown to have similar "priming" effects on neutrophils [6–8]. The mechanism by which priming occurs or how neutrophils differentiate early and late phase responses remains unclear despite intense investigation. Evidence suggests that more than one signal transduction pathway is involved in neutrophil activation but the signals involved in particular pathways and the role of effectors generated by different agonists still remain an enigma [9–11]. A greater understanding of the pathways which lead to differentiation of early and late events would provide useful information on mechanisms which regulate neutrophil signal transduction.

Studies carried out on the cyclic nucleotide cyclic AMP (cAMP) have suggested a regulatory role for this molecule in neutrophil activation. Elevation of intracellular levels of cAMP causes the down regulation of a variety of neutrophil responses. These include inhibition of the respiratory burst, phospholipase D activation and phosphatidyl inositol turnover [12–14]. Although most of the evidence implicates cAMP as a down regulator of late events, its role in the regulation of other responses such as chemotaxis, is not clear. Thus, conflicting evidence

* Corresponding author. Tel. (0865) 747100; FAX (0865) 748974.

† Abbreviations: BSA, bovine serum albumin; BSS, balanced salt solution; CMF, calcium- and magnesium-free BSS; DMSO, dimethyl sulphoxide; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; IL-8, interleukin-8; PAF, platelet activating factor; PLA_2, phospholipase A_2; PMNL, polymorphonuclear leucocyte; rhMIL-8, recombinant human monocyte IL-8.

has been generated from systems using different cAMP-elevating agents with various chemoattractants used as agonists [15–18]. For example, certain cAMP-elevating agents such as epinephrine and isoproterenol have been shown to inhibit neutrophil chemotaxis [18], whereas other cAMP-elevating agents such as histamine actually enhance neutrophil movement [15, 19]. In a recent study, Harvath *et al.* [16] demonstrated that elevation of cAMP in neutrophils had quite different effects on chemotaxis, dependent on the chemotactic agonist and the cAMP-elevating agent. No study has yet simultaneously investigated the effects of cAMP elevation on early and late events in rigorously defined systems. We have therefore examined the effects of artificially elevating intracellular cAMP levels (through direct stimulation of adenylate cyclase) on a range of early and late neutrophil responses, in an attempt to clarify the regulatory role of this cyclic nucleotide.

We have investigated the effects of intracellular cAMP on a number of early phase neutrophil responses such as chemotaxis, shape change and priming. Using the same system to elevate intracellular cAMP we also examined fMLP-induced late phase responses including phospholipase A_2 (PLA_2) and NADPH-oxidase activation. Analysis of chemotactic agonist-induced activation of early events, including shape change and priming which have not been investigated previously in this context, demonstrates no effect of elevating intracellular cAMP. However, we find that elevation of intracellular cAMP profoundly inhibits the activation of PLA_2 and the generation of superoxide anions by fMLP. These data suggest a role for cAMP in regulatory mechanisms which differentially control the magnitude of early and late neutrophil responses.

MATERIALS AND METHODS

Materials. Reagents were obtained from the following sources: [^{3}H]arachidonic acid (sp. act. 219 Ci/mmol, Amersham International, Amersham, U.K.); recombinant human monocyte IL-8 (rhMIL-8) (Bachem Inc., Essex, U.K.); cytochalasin B, cytochrome *c* (Type IV, horse heart), dimethyl sulphoxide (DMSO), forskolin, fMLP, heparin (Grade I), bovine serum albumin (BSA, essentially fatty acid free), 0.4% Trypan blue solution (Sigma Chemical Co., Poole, U.K.); BSS (balanced salt solution: NaCl 8 g/L; KCl 0.4 g/L; $CaCl_2$ 140 mg/mL; $MgCl_2 \cdot 6H_2O$ 200 mg/L; glucose 1 g/L; HEPES 2.388 g/L; pH 7.4); CMF (calcium- and magnesium-free BSS). Forskolin was dissolved at 10 mM in DMSO prior to use.

Isolation of peripheral blood neutrophils. Human neutrophils were separated from whole blood essentially as described elsewhere [20]. Briefly, fresh human blood (80 mL) was obtained by venepuncture from healthy adult volunteers and collected into heparin (10 U/mL blood). Neutrophils were separated from red cells by sedimentation with Dextraven 110 (Fisons, Loughborough, U.K.) (one part Dextraven 110: three parts blood) for 30 min at 37°. The leukocyte-rich supernatant was washed in CMF (300 g for 10 min) and the pellet resuspended in 16 mL CMF. The cell suspension was layered on 4 × 6 mL Ficoll-Paque (Pharmacia, Milton Keynes, U.K.) cushions and centrifuged at 300 *g* for 40 min at room temperature. The pellet containing neutrophils was collected and washed in BSS at 300 *g* for 10 min before finally resuspending the pellet in 10 mL of BSS/0.1% BSA. All buffers and reagents used during the preparation of neutrophils and in subsequent experiments were endotoxin free. Neutrophil number and purity were assessed by counting the cells diluted 1:10 in Turk's white cell counting fluid on a modified Neubauer haemocytometer. Neutrophil purity was between 95 and 98% as assessed by cytospin and Giemsa–May Grunwald differentiating stain and the average yield was 1×10^8 neutrophils/80 mL blood. Cell viability as assessed by Trypan blue exclusion was >99%.

Measurement of intracellular cAMP. Neutrophils (10^6) were suspended in 200 μL BSS/0.1% BSA and then exposed to 10 μM forskolin (prepared at 10 mM in DMSO) or 0.1% DMSO as control for various times. The reaction was terminated by the addition of 400 μL 100% ethanol. The suspension was then vortexed and allowed to settle. The supernatant was removed and the pellet was resuspended in 100 μL 70% ethanol and vortexed again. The two supernatants were mixed and spun at high speed for 20 sec in an Eppendorf microfuge. The remaining supernatant was removed and dried down in a Christ CMC-1 speed vac at 50°. The extract was resuspended in the appropriate assay buffer and assessed using the Amersham ^{125}I-cAMP scintillation proximity assay, sensitive to 0.2 pmol cAMP/tube.

Measurement of superoxide release. The release of superoxide anions was measured using a superoxide dismutase-inhibitable cytochrome *c* reduction assay as described previously [5]. Briefly, 25 μL of cells (2×10^7/mL in BSS) were added to 175 μL of assay mixture (50 μM cytochrome *c*, 0.1 μM cytochalasin B and 1 μM fMLP) in BSS just before reading the plate. The plate was read at 10 sec intervals over 3 min at 550 nm in a microplate reading spectrophotometer (Molecular Devices, Menlo Pk, CA, U.S.A.). The mean V_{max}mO.D./min was converted into nmol cytochrome *c* reduced/min/10^6 cells using the extinction coefficient 29.5 for the relevant cytochrome *c*. To assess the effect of elevated cAMP the cells were preincubated for 10 min at 37° with 10 μM forskolin (or 0.1% DMSO as a control) before addition to the assay mixture.

Measurement of arachidonic acid release. Measurement of arachidonic acid release was carried out as described previously [21]. Briefly, human neutrophils were resuspended at 10^7 cells/mL in BSS/0.1% BSA and labelled with [^{3}H]arachidonic acid (1 μCi/10^7 cells) for 45 min at 37°. The cells were then washed three times in BSS/0.1% BSA (300 *g* for 10 min) and finally resuspended at a cell dilution of 2×10^6 cells/mL in BSS/BSA. To assess arachidonic acid release by neutrophils, 0.5 mL of cell suspension in the presence of 0.5 μg/mL cytochalasin B was incubated for 10 min at 37° with 2×10^{-7} M fMLP. When investigating arachidonic acid release from primed cells the tubes were pre-incubated with rhMIL-8 (10^{-8} M) for 10 min prior to the addition of fMLP. To elevate intracellular levels of cAMP

the cells were incubated for 10 min at 37° with 10 μM forskolin (control cells contained 0.1% DMSO) before the addition of any stimuli. To terminate the experiment, labelled cells were centrifuged at 4000 *g* for 20 sec and 400 μL of the supernatant were added to 6 mL of Ultima Gold Scintillant (Canberra Packard, Berks, U.K.). The amount of [^{3}H]-arachidonic acid released was determined by scintillation counting and expressed as a percentage of total counts incorporated. Control cells were incubated over a similar time course with cytochalasin B alone.

Measurement of PAF release. Neutrophils (2×10^6) were incubated for 10 min at 37° with either 0.1% DMSO or 10 μM forskolin in the presence of cytochalasin B. The cells were then stimulated for a further 10 min at 37° with 10^{-6} M fMLP in a final volume of 0.5 mL BSS/0.1% BSA. To extract the lipids 0.5 mL of 1:2 (v/v) of chloroform:methanol was added, and the mixture vortexed and then spun at 4000 *g* in a microfuge for 30 sec. The aqueous phase and the protein layer were removed and the chloroform was evaporated off in a Christ CMC-1 speed vac at 50° for 1 hr. The lipids were reconstituted in BSS/BSA and the amount of PAF was assessed using the highly specific Amersham [^{3}H]PAF scintillation proximity assay, sensitive to 20 pg PAF/tube (cross-reactivity with lyso-PAF < 0.01%) (Amersham International).

Shape change assay. Purified human neutrophils in BSS/BSA were incubated for 10 min at 37° either in the presence of 10 μM forskolin or 0.1% DMSO. The cells were then left untreated or stimulated with 10^{-9} M fMLP or 10^{-8} M rhMIL-8 for a further 10 min at 37°. After the incubation period the neutrophils were fixed by the addition of 0.25% glutaraldehyde (BDH, Poole, U.K.) for 10 min at room temperature. Cells were centrifuged at 500 *g* for 5 min, the pellet was resuspended in phosphate-buffered saline and neutrophils were examined under the microscope (Zeiss Axioskop) and scored for shape-changed cells as described [3].

Chemotaxis assay. The assay performed was modified from a previously published method [22] and used Transwell 6.5 mm diameter tissue culture well inserts with a 3 μm pore polycarbonate membrane base suspended in 24 well plates (Costar, Cambridge, MA, U.S.A.). The medium used for the assay and prewetting the inserts was RPMI 1640 with 0.1% BSA. Polymorphonuclear leucocytes (PMNLs) (10^5) that had been incubated with 10 μM forskolin or 0.1% DMSO for 10 min at 37° were placed in prewetted well inserts. Medium containing 5×10^{-8} M fMLP was present in the well below. Inserts containing PMNLs were incubated for periods of up to 1 hr at 37° in a humidified incubator. At the end of the incubation period each insert was removed from its well and the base washed gently in fluid from the well. The well medium was then removed, centrifuged and any non-adherent cells pelleted. Each cell pellet was resuspended in 100 μL of phosphate-buffered saline and added back to the relevant well. The myeloperoxidase assay was used to determine how many cells had passed through the insert filter. Assay buffer (300 μL) (0.05 M sodium citrate, 0.2% Triton X-100, pH 4.0, containing 2 μL of 60% hydrogen peroxide and 2, 2 azino bis (3 ethylbenzthiazoline)-6-sulphonic acid) was added to each well. Samples from each well were read at 405 nm in a plate-reading spectrophotometer (Molecular Devices, Menlo Pk, CA, U.S.A.). In most cases, optical densities obtained were compared against a standard curve of known dilutions of PMNLs (set up and assayed with the samples) and the percentage of PMNLs migrated calculated for each insert. The chemotactic assay was performed on five occasions with control and forskolin-treated PMNL samples run as six replicates in each assay.

Statistical analysis. Student's *t*-test was used to carry out statistical analysis of the data.

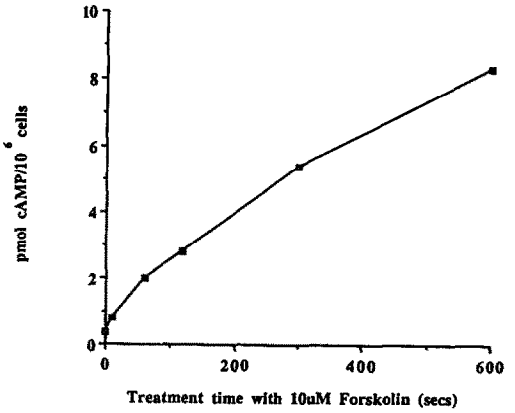

Fig. 1. Measurement of intracellular cAMP levels in human neutrophils incubated at 37° and treated with 10 μM forskolin at various time-points. The levels of cAMP were measured in a commercially available radioimmunoassay (see Materials and Methods). Each point represents the mean for triplicate determinations and the above graph is representative of three separate experiments.

RESULTS

Elevation of intracellular cAMP by forskolin

Neutrophils were exposed to 10 μM forskolin for varying times between 30 sec and 10 min. Intracellular levels of cAMP increased with time to 8.3 pmol/10^6 cells after 10 min (Fig. 1) compared with background levels of 0.37 pmol/10^6 (SD ± 0.1 pmol) which remained constant over the time-course of the assay in DMSO-treated cells. Thus, forskolin increases intracellular levels of cAMP in human neutrophils.

Effect of elevated cAMP on late neutrophil responses

Effect of elevated cAMP on superoxide anion release. Superoxide release from fMLP-stimulated human neutrophils was significantly inhibited by 10 μM forskolin. In DMSO-treated cells 4.2 (SD ± 0.35) nmol of cytochrome *c* were reduced/min/10^6 cells following stimulation with 1 μM fMLP. After 10 min preincubation with 10 μM forskolin this fell to 2.0 (SD ± 0.3) nmol/min/10^6 cells (Fig. 2).

Effect of elevated cAMP on arachidonic acid release. [^{3}H]Arachidonic acid release from labelled neutrophils was used as a measure of PLA_2 activation.

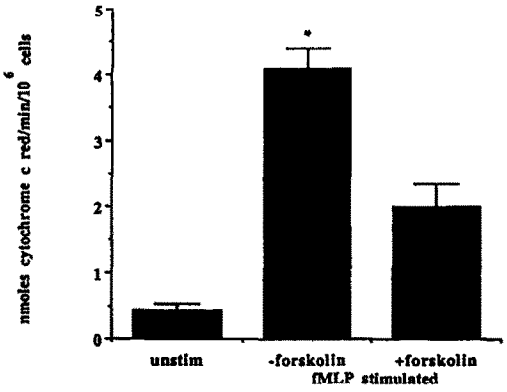

Fig. 2. Superoxide anion release measured by the reduction of cytochrome *c* by human neutrophils. Neutrophils either preincubated for 10 min with 10 μM forskolin or treated with 0.1% DMSO were then stimulated with 1 μM fMLP over a 2 min time-course and the rate of reduction of cytochrome *c* was measured. Each bar represents the mean ($\pm$ SD) of three experiments each carried out in triplicate. The results from forskolin-treated cells were compared to those from DMSO-treated cells using Student's *t*-test: * $P < 0.01$.

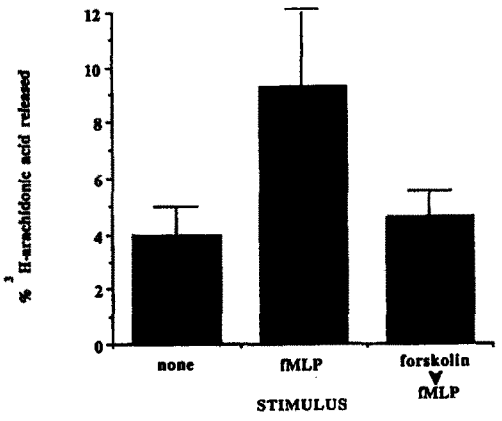

Fig. 3. [^{3}H]Arachidonic acid released from human neutrophils stimulated at 37° with 0.2 μM fMLP for 10 min either with or without a 10 min preincubation with 10 μM forskolin. Each bar represents the mean $\pm$ SD of three experiments each carried out in triplicate. The results from DMSO-treated cells were compared to those from forskolin-treated cells using Student's *t*-test: * $P < 0.01$.

Figure 3 shows that the release of arachidonic acid from neutrophils stimulated with 2×10^{-7} M fMLP was significantly decreased if the cells were pretreated for 10 min with 10 μM forskolin. Release of [^{3}H]-arachidonic acid fell from 9.3% (SD $\pm$ 4.0) to 4.6% (SD $\pm$ 0.9) in the forskolin-treated cells.

Effect of elevated cAMP on the generation of PAF. Generation of PAF from human neutrophils stimulated with 10^{-6} M fMLP was completely

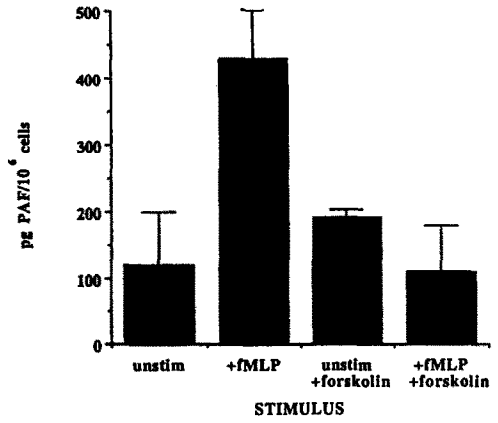

Fig. 4. PAF generation from neutrophils stimulated for 10 min at 37° with 1 μM fMLP either in the presence or absence of 10 μM forskolin (preincubated for 10 min at 37° prior to stimulation). Each bar represents the mean $\pm$ SD of three experiments each carried out in duplicate. Results from forskolin-treated cells were compared to DMSO-treated values using Student's *t*-test; * $P < 0.01$.

inhibited in cells pretreated with 10 μM forskolin. On fMLP activation, levels of PAF in forskolin-treated cells did not rise above unactivated cell background levels (110 pg/10^6 cells), whereas in DMSO-treated control cells PAF levels were significantly increased to 430 pg PAF/10^6 (Fig. 4). Dibutryl cAMP was used to elevate artificially intracellular levels of cAMP and similar results were obtained (data not shown).

Effect of elevated cAMP on early neutrophil responses

Effect of elevated cAMP on chemotaxis. Neutrophil migration across a 3 μm filter in response to fMLP was determined for forskolin-treated and control DMSO-treated cells. Figure 5a shows a typical result; no significant difference ($P > 0.05$) in migration was detected between neutrophils that had elevated levels of cAMP compared with control cells. In addition, no difference could be detected between the two groups after shorter incubation times of 15 and 30 min (Fig. 5b).

Effect of elevated cAMP on neutrophil shape change. Neutrophils were stimulated for 10 min with varying concentrations of fMLP or IL-8 and scored for shape change (Table 1). There was no significant difference in the percentage of neutrophils shape changed with either agonist at any of the concentrations in the presence or absence of 10 μM forskolin ($P > 0.1$).

Effect of elevated cAMP on IL-8 induced priming. [^{3}H]Arachidonic acid-labelled cells were pretreated with either forskolin or DMSO for 10 min. Cells were then primed with 10^{-8} M rhMIL-8 prior to stimulation with 2×10^{-7} M fMLP. Although, as described previously (Fig. 3), the overall response to fMLP was inhibited by pretreatment with forskolin, the level of priming in forskolin-treated cells was comparable to that in DMSO-treated cells

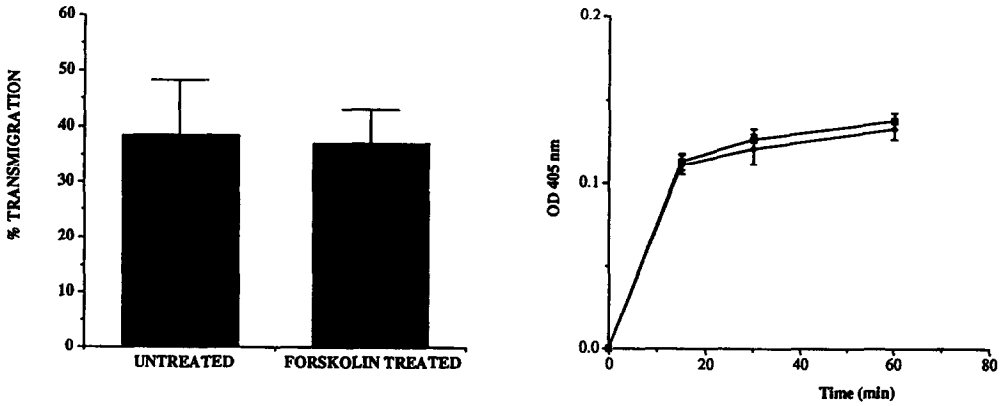

Fig. 5. (a) Neutrophil transmigration through 3 μm filters in response to 0.05 μM fMLP. Cells were pretreated with 10 μM forskolin for 10 min at 37° and then placed on the filters. After 1 hr incubation, treated and untreated cells were collected and transmigration detected using the myeloperoxidase assay. Each bar represents mean ± SD of six inserts per treatment. (b) Neutrophil transmigration through 3 μm filters was measured at various times. Forskolin treated (◆) and control (DMSO-treated) (□) cells were assessed after 15, 30 and 60 min and transmigrated neutrophils expressed as $O.D._{405}$. Each point represents the mean ± SD of six inserts per data-point.

Table 1. Percentage of neutrophils scored as shape changed under phase contrast microscopy (×200) following treatment with the appropriate stimulus and fixing with 0.25% glutaraldehyde

Concn of agonist (M)	Untreated cells % shape changed (±SD)	Forskolin-treated % shape changed (±SD)
fMLP		
10^{-9}	89.6 (2.7)	85.7 (5.7)
10^{-10}	61.5 (2.2)	63.0 (3.0)
10^{-11}	50.5 (30)	49.5 (2.2)
IL-8		
10^{-8}	89.0 (11.0)	88.6 (8.1)
10^{-9}	59.5 (2.0)	57.0 (8.5)
10^{-10}	49.5 (2.2)	49.0 (11.0)
Control (buffer)	12.1 (5.0)	9.9 (4.7)

Forskolin-treated cells were preincubated for 10 min at 37° with 10 μM forskolin before 10 min stimulation with the appropriate dose of agonist; control cells were incubated for the same period with 0.1% DMSO.

Each result represents the mean (±SD) from four separate cell populations with 200 cells counted on each sample.

(Fig. 6). In DMSO-treated cells IL-8-induced priming caused a 48% increase in the response to fMLP and in forskolin-treated cells priming still resulted in a 41% increase in arachidonic acid release (not significantly different: $P > 0.1$).

As with late phase responses we also examined the effect of elevating cAMP with the cell-permeable analogue dibutryl cAMP on early phase neutrophil responses. No inhibition of early events was observed in the presence of this molecule (data not shown).

DISCUSSION

The number and nature of the signal transduction pathways involved in activation and regulation of neutrophil responses has been an area of intense investigation for several decades. One focus of research has been the potential for cAMP as a mediator of neutrophil function, but its precise role still remains uncertain. Most studies on early neutrophil responses such as phosphatidyl inositol turnover, calcium fluxes and chemotaxis have provided conflicting evidence on the consequences of elevating intracellular cAMP [18, 19, 23–25]. For example, the effect of cAMP on neutrophil chemotaxis has received a great deal of attention and yet no clear role for cAMP in regulating chemotaxis has emerged. A possible explanation of this is that these discrepancies result from the wide variety of agents used to elevate cAMP or

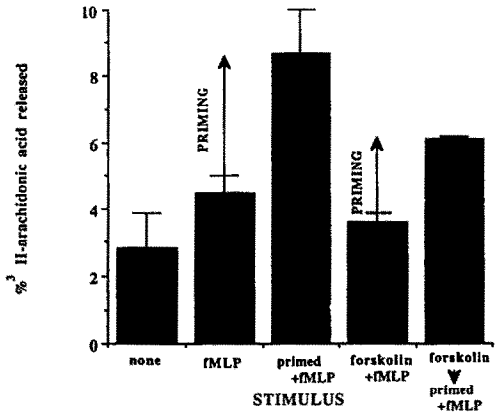

Fig. 6. Effect of elevated intracellular cAMP on primed neutrophil responses. Neutrophils were primed by preincubating cells with 0.01 μM rhMIL-8 for 10 min at 37° before the addition of 0.2 μM fMLP; unprimed cells were treated as in the legend to Fig. 5a. [^{3}H]Arachidonic acid release was then measured from primed and unprimed neutrophils either on cells incubated for 10 min at 37° with 10 μM forskolin or with carrier alone (0.1% DMSO) prior to priming or stimulation. Each bar represents the mean ± SD of three experiments each carried out in triplicate. The arrows represent the percentage increase in arachidonic acid release after priming (see Results).

the heterogenous nature of chemotactic agonists [15, 16, 18, 19]. Moreover, it is noteworthy that many studies demonstrating an inhibitory role for cAMP in chemotaxis have needed to employ a phosphodiesterase inhibitor to prevent intracellular degradation of cAMP [16, 23]. There is more consensus, however, over the inhibition effects of cAMP on "late phase" neutrophil responses. Previous work has shown that elevation of cAMP results in the inhibition of superoxide anion generation, degranulation and lipid mediator production [13, 26–29]. However the relationship between the effects of cAMP on early and late events is unclear.

The present work has, for the first time, compared the effects of cAMP on clearly defined early and late phase neutrophil responses under identical conditions. Elevation of intracellular cAMP was achieved by forskolin stimulation of adenylate cyclase prior to measuring fMLP-induced early and late phase responses. Under these conditions, there is a differentiation of the effect of elevated cAMP, with no inhibition of early events but clear inhibition of late events. The effects of elevating cAMP on neutrophil shape change and priming, both defined as early phase responses in the neutrophil repertoire, have not been investigated previously. We find that these two early events as well as chemotaxis remain unaffected by raised cAMP levels (Figs 5 and 6, Table 1). fMLP-induced arachidonic acid release, generation of PAF and activation of NADPH-oxidase (indicators of late phase neutrophil activation) were all significantly inhibited by elevating cAMP prior to stimulation (Figs 2–4). We also found similar results when dibutryl cAMP was used to elevate intracellular cAMP (i.e. inhibition of PLA_2 and NADPH-oxidase but no effect on early responses). However, it could not be ruled out that dibutryl cAMP was having other effects on the cell due to the interference with signal transduction elements by butyrate, as has been reported previously [30].

Our work, therefore, demonstrates that in the absence of a phosphodiesterase inhibitor late phase responses are still inhibited by cAMP whereas early phase responses are unaffected. This suggests that the regulation of neutrophil responses may be dependent on the time and magnitude of elevation of intracellular cAMP concentration and supports the concept that cAMP effects may be differential. If this type of mechanism was reflected *in vivo* it would confer an important negative feedback role for cAMP in the regulation of later neutrophil responses, but not in the regulation of early phase responses.

A further indicator that cAMP is acting as a mediator of neutrophil responses is that most chemotactic stimuli induce an elevation in intracellular levels of cAMP [31–33]. This rise, which has been shown to be very rapid (within 10 sec) and transient (resting levels by 2 min), precedes many activation events suggesting a role for cAMP as an intracellular messenger [32]. However, chemotaxis-related increases in intracellular cAMP can be separated from activation events, such as superoxide generation, again indicating that both temporal and concentration effects may be involved [34].

The ways in which chemotactic agonists cause an elevation in cAMP are poorly understood [for review see 35]. It is not clear what role G-protein-mediated stimulation of adenylate cyclase plays or whether alterations in phosphodiesterase activity are involved. In addition, the way in which cAMP acts within the cell to alter neutrophil behaviour is not well defined. It is known that in many cells, cAMP-dependent protein phosphorylation can play a role in regulating cell function and cAMP-dependent protein kinase activity has been measured in neutrophils [36, 37]. As has been demonstrated in platelets the effects of elevated cAMP would therefore be likely to affect other intracellular signals in some way [24, 38–40]. It may be, therefore, that some cAMP-dependent regulatory process is responsible for differentiating the neutrophil's early and late responses. We are currently investigating at a more detailed level how these early differences in responsiveness to cAMP exert a regulatory effect on neutrophils.

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