Action of rolipram on specific PDE4 cAMP phosphodiesterase isoforms and on the phosphorylation of cAMP-response-element-binding protein (CREB) and p38 mitogen-activated protein (MAP) kinase in U937 monocytic cells

Simon J. MACKENZIE and Miles D. HOUSLAY[1]

Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Davidson and Wolfson Buildings, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

U937 monocytic cells are shown here to express a range of PDE4, cAMP-specific phosphodiesterase (PDE) isoenzymes: the long isoenzymes, PDE4A4, PDE4D5 and PDE4D3, plus the short isoenzyme, PDE4B2. These isoenzymes provide around 76 % of the total cAMP PDE activity of U937 cells. The specific activities of the total PDE4A, PDE4B and PDE4D activities were 0.63 ± 0.09, 8.8 ± 0.2 and 34.4 ± 2.9 pmol/min per mg of protein respectively. The PDE4 selective inhibitor, rolipram, inhibited immunopurified PDE4B and PDE4D activities similarly, with IC_{50} values of approx. 130 nM and 240 nM respectively. In contrast, rolipram inhibited immunopurified PDE4A activity with a dramatically lower IC_{50} value of around 3 nM. Rolipram increased phosphorylation of cAMP-response-element-binding protein (CREB) in U937 cells in a dose-dependent fashion, which implied the presence of both high affinity (IC_{50} value approx. 1 nM) and low affinity (IC_{50} value approx. 120 nM) components. Rolipram dose-dependently inhibited the interferon-γ (IFN-γ)-stimulated phosphorylation of p38 mitogen-activated protein (MAP) kinase in a simple monotonic fashion with an IC_{50} value of approx. 290 nM. On this basis, it is suggested that rolipram inhibition of PDE4A4 is involved in regulating CREB phosphorylation but not IFN-γ-stimulated p38 MAP kinase phosphorylation. PDE4A4 was also selectively activated by challenge of U937 cells with either bacterial lipopolysaccharide (LPS) or IFN-γ through a process which was attenuated by both wortmannin and rapamycin. It is proposed that the PDE4A4 isoform is involved in compartmentalized cAMP signalling responses in U937 monocytes.

Key words: interferon-γ, lipopolysaccharide, phosphoinositide 3-kinase, protein kinase A, rapamycin.

INTRODUCTION

It has long been appreciated that compartmentalization plays an important role in generating specificity in the cAMP-signalling processes [1–4]. However, the molecular machinery responsible for this is now only just becoming apparent. This appears to depend upon a bewildering array of enzymes involved in generating, controlling, and then responding to, changes in the intracellular levels of cAMP. Thus up to nine forms of adenylyl cyclase responsible for cAMP production, and over 30 forms of phosphodiesterases (PDEs), are able to hydrolyse cAMP [5]. The localization of these enzymes, either to specific intracellular sites or to complexes within the cytosol, is believed to be responsible for generating gradients of cAMP. These are seemingly sampled by particular pools of protein kinase A (PKA) that are either anchored at specific intracellular sites or found in complexes with other signalling proteins within the cytosol. Such multiplicity of components and their ability to interact with other proteins is believed to underpin compartmentalized cAMP responses [1–4].

The PDE4 family of cAMP-specific phosphodiesterases represent an important pharmacological target, as selective inhibitors can exert both anti-inflammatory and anti-depressant effects [6–10]. Four distinct genes, named PDE4A, PDE4B, PDE4C and PDE4D, encode a family of 15 or more PDE4 isoforms [10–13]. These isoforms each have a common core, consisting of a central catalytic unit and C-terminal tail. However, alternative mRNA splicing and the use of distinct promoters generates the so-called 'long isoforms' that are characterized by the presence of two regions of homology called upstream conserved region (UCR)1 and UCR2, as well as 'short' isoforms that lack UCR1. Individual isoforms are distinguished by unique N-terminal regions that are encoded by distinct exons found at the extreme 5′ end of these genes [10]. The generation of such a large family of enzymes suggests that particular PDE4 isoforms may have specific roles. To date, however, no systematic analysis has been done to try and determine the relative activities of different PDE4 enzyme families in a particular cell type and to see if inhibition of either particular subclasses or isoforms is associated with any change in the regulation of cAMP signalling.

We show here that resting U937 monocytic cells express the PDE4A4 (GenBank accession number U18087), PDE4B2 (GenBank accession number L20971), PDE4D3 (GenBank accession number L20970) and PDE4D5 (GenBank accession number AFO12073) isoenzymes, where the activity of PDE4D > PDE4B ≫ PDE4A. Exploiting the acute sensitivity of PDE4A4 activity to be inhibited by rolipram, leads us to suggest that cAMP signalling processes are compartmentalized in these cells. Thus rolipram inhibition of the PDE4A4 isoform regulates phosphorylation of the cAMP-response-element-binding protein (CREB) transcription factor, but is seemingly not involved in the

Abbreviations used: CREB, cAMP-response-element-binding protein; LPS, lipopolysaccharide; IFN-γ, interferon-γ; cAMP PDE, cAMP phosphodiesterase; PKA, protein kinase A; MAP, mitogen-activated protein; mAb, monoclonal antibody; PI-3K, phosphoinositide 3-kinase; UCR, upstream conserved region.

[1] To whom correspondence should be addressed (e-mail M.Houslay@bio.gla.ac.uk).

© 2000 Biochemical Society

inhibition of interferon-γ (IFN-γ)-stimulated phosphorylation of p38 mitogen-activated protein (MAP) kinase, a key enzyme involved in regulating cellular inflammatory and stress responses [14–16]. We suggest that the PDE4A4 isoform may have a distinct functional role in these cells. In this regard it is shown that PDE4A4 is selectively activated by challenge of U937 cells with the pro-inflammatory mediators lipopolysaccharide (LPS) and IFN-γ through a process blocked by both the phosphoinositide 3-kinase (PI-3K) inhibitor, wortmannin, and by the immunosuppressant, rapamycin.

MATERIALS AND METHODS

RPMI, fetal-calf serum, glutamine, Dowex 1X8-400, LPS (*Salmonella minnesota*), and anti-rabbit IgG–horseradish peroxidase antibody were all obtained from Sigma. Kinase inhibitors and IFN-γ were from Calbiochem, Nottingham, U.K. Bradford reagent was from Bio-Rad. Protease inhibitors were from Boehringer Mannheim. Monoclonal antibodies (mAb) specific for human PDE4A, PDE4B and PDE4D classes were generously given by Dr Sharron Wolda (ICOS Corp, Seattle, WA, U.S.A). Anti-mouse IgG–horseradish peroxidase antibody, [^{3}H]cAMP, and ECL® reagents were from Amersham International.

Cell culture

The U937 monocytic cell line was maintained in RPMI, with 10% fetal-calf serum and 2 mM glutamine, at a cell density between 1×10^6 and 8×10^6 cells per ml in an incubator at 37 °C with 5% CO_2. For experimental work, the cells were diluted to 2×10^6 cells per ml in serum-free medium the previous day. Cells treated with inhibitors were incubated with these for 20 min prior to the addition of inflammatory agents. The reaction was stopped by centrifugation of the cells at 800 ***g*** for 3 min at 4 °C. The medium was removed and the cells were washed with ice-cold PBS and then re-centrifuged. The resulting cell pellet was resuspended in lysis buffer [25 mM Hepes (pH 7.5), 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% glycerol, and 1% Triton X-100] with added protease inhibitors (see [17,18]), and centrifuged at 12000 ***g*** for 10 min at 4 °C. The supernatant was retained for further analysis.

Immunoblotting

Immunoblotting was performed using PDE4-subclass-specific antisera/antibodies, previously described in [17–20]. Briefly, cell lysates were boiled for 2 min in sample buffer, subjected to SDS/PAGE, then transferred to nitrocellulose for immunoblotting. Each of the four PDE4 subclasses differs at their C-terminal regions [10]. Both peptides and glutathione S-transferase fusion proteins, reflecting unique sequences within these regions, have been used previously to generate specific PDE4A, PDE4B and PDE4D mAbs and polyclonal antisera to all four PDE4 classes [17,18,20].

Separation of proteins for Western blot analysis of p70S6 kinase, extracellular-signal-regulated protein kinase (or ERK) and p38 MAP kinase was performed using a low-bisacrylamide gel solution, as described in [19,21]. The p70S6 kinase polyclonal antiserum and anti-phosphotyrosine antibody were obtained from Santa Cruz Biotechnology, Wiltshire, U.K. The CREB, serine-133-phospho-CREB, phospho-p38 MAP kinase (Thr-180/Tyr-182) and the control p38 MAP kinase antibodies were from New England Biolabs (Hitchin, Herts., U.K.). Quantitative analyses of phospho-CREB and phospho-p38 MAP kinase were done by densitometric scanning over a linear range as detailed before [17].

Immunoprecipitation and PDE assays

Selective immunoprecipitation of the enzymes of the four PDE4 classes was performed as described previously [19,22]. As discussed before [19,22], sufficient antiserum was used to ensure that all isoenzymes of the target PDE4 subclass were selectively immunoprecipitated; these were then subjected to the PDE assay. PDE assays were done by a modification [23] of the two-step Thompson and Appleman method [24]. Determinations of total cellular PDE activity and the amounts of PDE3 and PDE4 components were performed with fresh cell lysates. As described before [22,25], determination of the total PDE3 and PDE4 activities was done using 1 μM cAMP as substrate and 10 μM of either the PDE3 selective inhibitor, cilostimide [26], or the PDE4 selective inhibitor, rolipram [10].

RESULTS

Detection of PDE4 isoenzymes by immunoblotting

The C-terminal regions of each of the four PDE4 families are distinct. This feature has been exploited to generate and describe antisera and mAbs that are specific for each of the four PDE4 families (reviewed in [10]). As all isoenzymes within any particular PDE4 subfamily differ only by virtue of their distinct N-terminal region, such C-terminal antisera are able to detect all members from a particular family. The isoenzymes within a particular PDE4 subfamily can then be discriminated on the basis of size differences on SDS/PAGE gels [10]. Utilizing such an approach to probe U937 cell homogenates, we identified, using a PDE4A-specific mAb (Figure 1), a single immunoreactive species of 125 ± 2kDa which co-migrated with the recombinant long PDE4A4 (pde46) isoenzyme [17].

Using a mAb specific for enzymes of the PDE4B family [18] we detected a single immunoreactive species of 82 ± 3 kDa (Figure 1). This co-migrated with recombinant human PDE4B2, the only known 'short' PDE4B isoenzyme [10,18].

Using an antiserum specific for enzymes of the PDE4C family, we failed to detect any immunoreactive species expressed in U937 cells. Using this antiserum, we were able to identify recombinant human PDE4C2 [27], which served as a positive control (results not shown).

Using a mAb specific for enzymes of the PDE4D family [20], we detected two immunoreactive species (Figure 1), of 95 ± 2 kDa and 105 ± 3 kDa by SDS/PAGE. These reflect the reported sizes [20] of the long human isoenzymes PDE4D3 and PDE4D5 respectively. Indeed, the two PDE4D immunoreactive species found in U937 cells co-migrated with the corresponding recombinant forms of PDE4D3 and PDE4D5 (Figure 1). In addition, using an antiserum specific for the N-terminal region of PDE4D5 [21], we identified only the more slowly migrating 105 ± 2 kDa species found in U937 cells, confirming the identity of this more slowly migrating PDE4D species (results not shown).

Various investigators [28–31] have employed reverse transcriptase-PCR to identify PDE4 transcripts in monocytes and U937 cells. However, such analyses were done using primers only able to identify whether transcripts were present for a particular PDE4 subfamily rather than being able to identify specific isoenzymes. Such studies have demonstrated that transcripts for unidentified isoenzyme members of PDE4A, PDE4B and PDE4D, but not PDE4C, were present both in U937 cells and in human monocytic cells. In this study, however, we have explicitly identified the particular PDE4 isoenzymes that are expressed in

© 2000 Biochemical Society

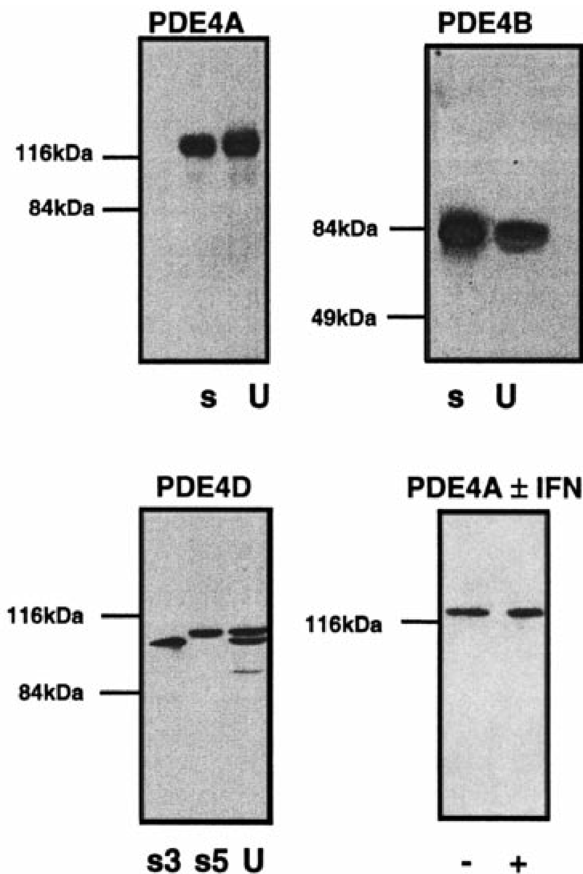

Figure 1 Immunoblotting of PDE4 isoenzymes in U937 cells

Immunoblotting was performed using the specific PDE4 antibodies shown. PDE4A: U, U937 lysate; s, recombinant PDE4A4 standard; PDE4B: U, U937 lysate; s, recombinant PDE4B2 standard; PDE4D: U, U937 lysate; s3, PDE4D3 standard; s5, recombinant PDE4D5 standard; PDE4A ± IFN: U937 cell lysates challenged with (+) or without (−) IFN-γ (10 μg/ml) for 20 min and blotted for the PDE4A specific antibody. Recombinant enzymes were expressed in transiently transfected COS7 cells. These show typical immunoblots of U937 cell lysates from experiments performed three times. For analysis of PDE4A, 100 μg of cell protein was used per lane, with 50 μg of protein used per lane for studies of PDE4B and PDE4D.

U937 cells at the protein level. These species are the long isoenzymes PDE4A4, PDE4D5 and PDE4D3, and the short isoenzyme, PDE4B2.

Determination of relative PDE4 activities by immunoprecipitation

We have previously demonstrated [17,19] that these various antibodies and antisera can be used to immunoprecipitate selectively members of the various PDE4 subclasses without altering either their activity or sensitivity to inhibition by rolipram. Here, we generated lysates of U937 cells which released more than 98 % of the endogenous PDE4 activity and immunoreactivity in a soluble form. This has allowed us to determine the relative activities of each of the three PDE4 subclasses represented in U937 cells. Taking equal amounts of U937 cell extract on a protein basis, we first determined the amount of antibody needed to immunoprecipitate all of a particular PDE4 subclass. Blotting the post-immunoprecipitation supernatants to check for complete depletion ensured this. Additionally, we immunoblotted the immunoprecipitated material with antibodies specific for each of the various PDE4 subfamilies to ensure that selective immunoprecipitation had occurred in each instance (results not shown). We then expressed the activity of individual immunoprecipitated PDE4 subclasses in terms of a specific activity value that was related to the total cell protein extract probed by immunoprecipitation. This allowed for direct comparisons to be made. Such analyses yielded comparative PDE activities in U937 cells of 0.63 ± 0.09, 8.8 ± 0.2 and 34.4 ± 2.9 pmol/min per mg of protein extract for the total PDE4A, PDE4B and PDE4D activities respectively (means ± S.D. shown for three independent experiments; 1 μM cAMP used as substrate). We also performed immunoprecipitation experiments with the PDE4C specific antisera but, consistent with the immunoblotting results, failed to identify any immunoprecipitated PDE activity (< 0.01 pmol/min per mg of protein). Additionally, we noted that > 98 % of the PDE activity associated with these various immunoprecipitates could be inhibited by rolipram, indicating this activity was entirely that due to PDE4. Assessment was done at 10 μM rolipram with 1 μM cAMP as a substrate; conditions where inhibition of PDE4 can be expected to be near complete, yet inhibition of other PDE species will be negligible [10]. Note that in the absence of U937 extract, none of the adsorbed antibody complexes exhibited any cAMP phosphodiesterase activity (< 0.01 pmol/min per mg of protein).

PDE activity of total U937 cell lysates, when 1 μM cAMP was used as substrate, had activities of 62.2 ± 2.5 pmol/min per mg of lysate protein in the absence of rolipram, and 14.7 ± 3.1 pmol/min per mg of lysate protein in the presence of 10 μM rolipram. Thus altogether, PDE4 isoenzymes provide the major PDE activity in U937 cells (47.5 pmol/min per mg of lysate protein). This is of the order of 76 % of the total PDE activity found in U937 cell lysates. In addition, we can determine that the combined PDE4 activity found in the PDE4A, PDE4B and PDE4D immunoprecipitates accounted for > 94 % of the rolipram-inhibited activity of the lysate, confirming that the immunoprecipitation procedure employed here isolated essentially all of the expressed PDE4 activity.

In addition, we used the PDE3 selective inhibitor, cilostimide (10 μM) [26] to gauge PDE3 activity in extracts. This showed that only approx. 9 ± 2 % of the cAMP PDE activity in U937 cells was due to PDE3.

Inhibition of PDE4 isoenzymes by rolipram

Rolipram serves as the paradigm of a PDE4 selective inhibitor [7,9,10], with purified recombinant forms of the four different PDE4 enzyme classes appearing to be inhibited similarly with IC_{50} values (1 μM used as cAMP substrate) of around 100–1000 nM [10]. It has, however, been proposed that certain PDE4 isoenzymes can adopt distinct conformational states that can be discriminated by different sensitivities to inhibition by rolipram [7,9,10]. We undertook to examine whether the rolipram sensitivity of the various PDE4 families expressed in U937 cells was similar or not and whether it was altered in cells that had been treated with the pro-inflammatory mediator, IFN-γ (Figure 2). We observed that the PDE4B2 activity was similarly inhibited with IC_{50} values of 132 ± 25 nM in untreated cells and 138 ± 50 nM in IFN-γ-treated cells. The combined PDE4D3 and PDE4D5 activities were also similarly inhibited, with IC_{50} values of 245 ± 83 nM observed in untreated cells and 238 ± 90 nM in IFN-γ-treated cells. To our surprise, however, the PDE4A4 activity in U937 cell extracts was dramatically more sensitive to inhibition by rolipram than these other PDE4 isoenzymes, exhibiting an IC_{50} value of 2.4 ± 0.7 nM when immunopurified from untreated U937 cells. A similar IC_{50} value

© 2000 Biochemical Society

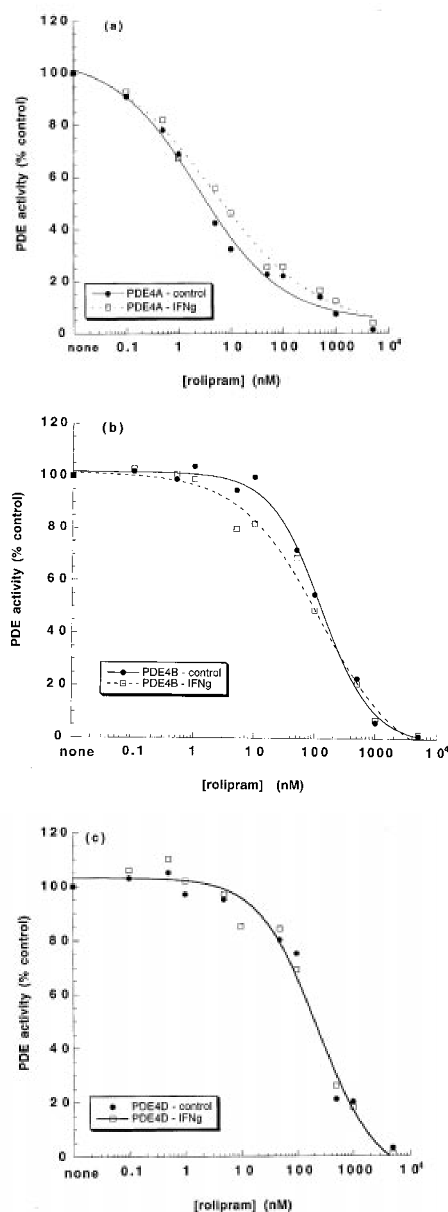

Figure 2 Rolipram inhibition of PDE4 isoenzyme classes in U937 cells

Inhibition by rolipram of the PDE4A (**a**), PDE4B (**b**) and PDE4D (**c**) activity from resting U937 cells. Specific polyclonal antibodies were used to selectively immunoprecipitate these activities for analysis. The activities analysed represent the total isoenzyme fraction of each of these subfamilies. There is no PDE4C activity in U937 cells. These are a single set of data, representative of three independent experiments done on three separate occasions.

of 4.6 ± 1.4 nM was observed, however, for PDE4A4 from IFN-γ-treated U937 cells (means $\pm$ S.D. shown for three independent experiments).

Rolipram increases CREB phosphorylation in U937 cells

Having established that PDE4A4 is exquisitely sensitive to inhibition by rolipram, we set out to determine if rolipram at such low levels could affect phosphorylation of the transcription factor CREB, which provides a major route through which cAMP exerts effects on gene expression [32]. This protein is phosphorylated by PKA at serine-133, leading to its activation.

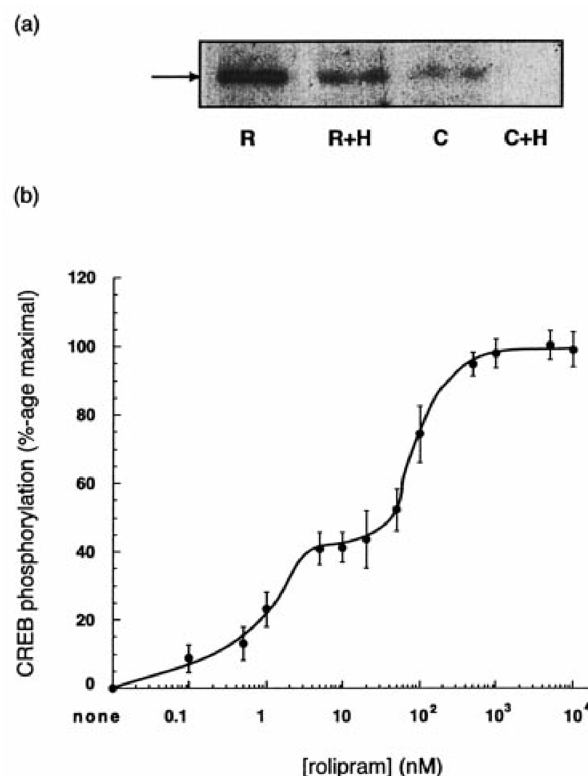

Figure 3 Rolipram stimulates CREB phosphorylation in U937 cells

U937 cells were incubated with or without rolipram prior to harvesting. Extracts (50 μg of protein per lane) were subjected to SDS/PAGE and then blotted with a serine-133 P-CREB-specific antibody that detected this 38 kDa species. Incubations were for 20 min. (**a**) Effect of 10 μM rolipram (R) alone; rolipram and 0.5 μM H89 (R + H); basal/no additions (C); and basal plus 0.5 μM H89 (C + H). (**b**) U937 cells were incubated for 20 min with the indicated concentrations of rolipram. They were then harvested, subject to SDS/PAGE and immunoblotted for serine-133 P-CREB whose level was then determined by densitometric scanning of gels over linear range (see the Materials and methods section). Means $\pm$ S.D. are shown for 3 independent experiments, taking the maximal level as 100%.

Such a phosphorylated form of CREB can be detected specifically by immunological means [32]. We demonstrate here that the treatment of U937 cells with rolipram alone served to promote CREB phosphorylation (Figure 3a). That such an effect was mediated by PKA can be inferred by the fact that incubating U937 cells with the PKA inhibitor, H89 (Figure 3a) ablated this effect. Interestingly, rolipram promoted CREB phosphorylation in a biphasic, dose-dependent fashion (Figure 3b). One component was of apparent high affinity, exhibiting an EC_{50} value of 1.2 ± 0.4 nM that was similar to the IC_{50} value observed for inhibition of PDE4A4 by rolipram (2.4 nM). The second component was of apparent lower affinity, exhibiting an EC_{50} value of 125 ± 32 nM (means $\pm$ S.D. for three independent experiments) which was of a similar magnitude to the IC_{50} values observed for inhibition of the PDE4B (132 nM) and PDE4D (245 nM) activities by rolipram. Under all such conditions, we also immunoblotted samples with an antibody to CREB itself in order to assess total CREB levels. These did not alter in any of the experimental conditions described above (results not shown).

Rolipram inhibits IFN-γ-stimulated p38 MAP kinase phosphorylation in U937 cells

Activation of p38 MAP kinase plays a critical role in determining cellular responses to stress and, in particular, the response of cells

© 2000 Biochemical Society

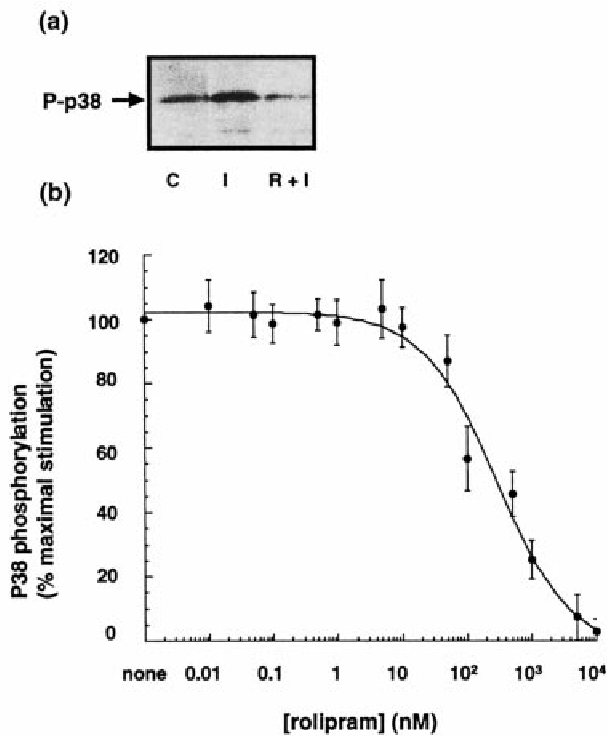

Figure 4 Rolipram inhibits the phosphorylation of p38 MAP kinase in U937 cells

U937 cells were incubated as indicated prior to being harvested, whereupon 75 μg samples of protein from the resultant lysate were run upon an SDS/PAGE gel. The activated, phosphorylated form of p38 MAP kinase was detected using the phospho-p38 MAP kinase (Threonine-180/Tyrosine-182) antibody. (**a**) Cells were untreated (C), treated with 10 μg/ml IFN-γ for 10 min (I) or treated with 10 μM rolipram for 20 min and then challenged with 10 μg/ml IFN-γ for 10 min (R + I). Shown here is a Western blot representative of an experiment carried out at least four times. (**b**) Cells either were not pretreated or were pretreated with increasing concentrations of rolipram in DMSO for 20 min prior to stimulation with 10 μg/ml IFN-γ for 10 min. Cells were then harvested and samples taken for quantitative Western blotting. The data show means $\pm$ S.D. for 4 separate experiments taking the level of phosphorylation of p38 MAP kinase observed on stimulating the cells with IFN-γ alone as 100%.

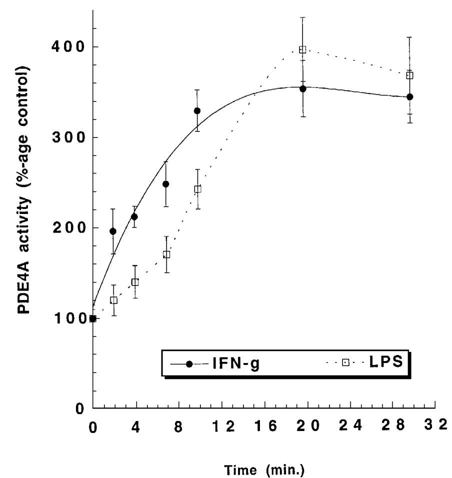

Figure 5 LPS and IFN-γ cause the time-dependent activation of PDE4A activity in U937 cells

U937 cells were incubated with either IFN-γ (10 μg/ml) or LPS (1 μg/ml) and harvested at the indicated times prior to lysis and selective immunoprecipitation of PDE4A using a specific pAb. The immunoprecipitated material was then subjected to determination of PDE activity (1 μM cAMP substrate). Activity is shown relative to that for untreated cells (100%). Means $\pm$ S.D. are shown for 3 independent experiments.

to pro-inflammatory cytokines [14–16]. p38 MAP kinase was originally identified as a protein kinase that was activated by LPS stimulation of a macrophage cell line [16]. Since then, both LPS and IFN-γ have been shown to activate p38 MAP kinase in B- and T-cells [14,15], macrophages [33] and neutrophils [34]. Activation has also been deduced to occur in monocytes because the compound SB202190, a specific inhibitor of p38 MAP kinase, served to block LPS- and IFN-γ-induced gene activation [35]. Stimulatory phosphorylation of p38 MAP kinase, on Thr-180/Tyr-182, can readily be detected using a phospho-p38 MAP kinase antibody. Here we show that IFN-γ elicited the phosphorylation of p38 MAP kinase in U937 monocytic cells (Figure 4a). Intriguingly, we show here, to our knowledge for the first time, that rolipram could inhibit this process (Figure 4a). However, in marked contrast with its effect on CREB phosphorylation, rolipram elicited its inhibitory effect on p38 MAP kinase phosphorylation in a simple, monotonic, dose-dependent fashion (Figure 4b), for which a single IC_{50} value of 294 ± 99 nM (means $\pm$ S.D. for three independent experiments) could be derived. This value is of a similar magnitude to the IC_{50} values observed for inhibition of the PDE4B (132 nM) and PDE4D (245 nM) isoenzymes by rolipram.

Increased PDE4A activity elicited by IFN-γ and LPS challenge

The activation of human monocytic cells by the pro-inflammatory mediators LPS and IFN-γ can be attenuated by PDE4 selective inhibitors [7,8,36–38]. Our demonstration that rolipram can regulate the phosphorylation of both CREB and p38 MAP kinase in U937 cells adds to the notion that the functioning of PDE4 enzymes is of importance in regulating signalling responses in monocytic cells. Indeed, one might surmise that an increase in PDE4 activity serves to facilitate pro-inflammatory signalling responses. In this study we have defined the PDE4 isoforms present in U937 cells and established selective immunopurification as a means of evaluating the separate activity of the three PDE4 sub-classes represented in U937 cells. We thus set out to determine if LPS and IFN-γ caused acute changes in PDE4 activity in these cells.

We demonstrated that IFN-γ caused a rapid, time-dependent activation of PDE4A activity in U937 cells (Figure 5). This was a selective effect as no increase in the activity of either PDE4B or PDE4D enzymes occurred upon challenge of U937 cells with IFN-γ (Table 1). Additionally, we did not detect any immunoprecipitated PDE4C activity in extracts from either treated or untreated cells. The increase in PDE4A activity was quite profound, being approx. 3.5-fold after 20 min incubation with IFN-γ (Figure 5 and Table 1). This effect, however, was not accompanied by any IFN-γ-mediated change in the expression level of endogenous PDE4A4 or any induction of a new PDE4A isoenzyme, as evident from immunoblotting extracts of treated cells with a pan-PDE4A antibody (Figure 1). Neither did we observe any change in the expression or induction of new immunoreactive PDE4B and PDE4D species (results not shown), nor was there any induction of any PDE4C species (results not shown). We noted that treatment of cells with the transcriptional inhibitor actinomycin D did not alter the ability of IFN-γ to increase PDE4A activity in U937 cells (Table 1). This is consistent with the notion that IFN-γ serves to activate the pre-existing

© 2000 Biochemical Society

PDE4A4 species expressed in U937 cells rather than causing any induction of new enzyme protein.

As found with IFN-γ treatment, treating U937 cells with bacterial LPS had no effect on the activity of the PDE4B, PDE4C and PDE4D enzymes (Table 1). However, LPS challenge caused a time-dependent increase of approx. 3-fold in PDE4A (Table 1 and Figure 5). As noted for IFN-γ, LPS challenge for a period of up to 30 min did not cause any change in the expression of the existing immunoreactive PDE4 species found in these cells or the appearance of any new PDE4 species (results not shown). Neither did IFN-γ challenge alter the sensitivity of these enzymes to inhibition by rolipram (see above).

Normally, PDE4 activity is gauged pharmacologically, using rolipram to assess total PDE4 activity in cells. It is salutary then to note that without using the immunoprecipitation strategy described here it is unlikely that the stimulation of PDE4A4 by IFN-γ and LPS would have been identified. Thus although the PDE4A4 isoenzyme provides a small fraction of the total PDE4 activity in U937 cells it appears to be of some importance as it is seemingly acutely regulated by IFN-γ and LPS challenge.

Table 1 Activation of PDE4A by LPS and IFN-γ

Data are shown for immunoprecipitated PDE4A activity unless indicated otherwise. Cells were treated with the indicated ligands for 20 min, harvested, lysed and the indicated PDE4 subfamily immunoprecipitated for PDE activity assay. The PDE activity in these various extracts is given as a percentage relative to that from untreated (control) cells. Specific activities are given in the text. Experiments were performed on three separate occasions with data shown as means ± S.D.

Treatment	PDE4 activity (% relative to control)
Control	(100)
IFN-γ (10 μg/ml)	331 ± 25
LPS (1 μg/ml)	329 ± 40
IFN-γ plus actinomycin D (4 μg/ml)	306 ± 38
IFN-γ plus PD98059 (20 μM)	332 ± 27
IFN-γ plus wortmannin (20 μM)	172 ± 23
IFN-γ plus rapamycin (10 μM)	176 ± 18
LPS plus wortmannin (20 μM)	121 ± 20
LPS plus rapamycin (10μM)	156 ± 21
Control (PDE4B)	(100)
(PDE4B) IFN-γ (10 μg/ml)	90 ± 8
Control (PDE4D)	(100)
(PDE4D) IFN-γ (10 μg/ml)	93 ± 6

Wortmannin and rapamycin attenuate PDE4A activation

We have shown that growth hormone can activate the murine homologue of human PDE4A4 in the F442A pre-adipocyte cell line [19]. This process was inhibited by wortmannin, a PI-3K inhibitor [39]. It was also inhibited by the immunosuppressant rapamycin, a compound that binds to mTOR, a protein which, among other effects, regulates the activation of p70S6 kinase [40]. Both IFN-γ and LPS can activate PI-3K in U937 cells [41,42]. We show here that both wortmannin and rapamycin inhibited PDE4A activation by LPS and IFN-γ (Table 1). In contrast, the MAP kinase/extracellular-signal-regulated kinase inhibitor, PD98059, failed to exert such an effect (Table 1). In order to demonstrate the functionality of these ligands in U937 cells we demonstrated that both IFN-γ and LPS could activate p70S6 kinase (Figure 6). This was evident by their ability to cause the accruement of hyper-phosphorylated states of p70S6 kinase in U937 cells (Figure 6) that migrate more slowly on SDS/PAGE gels and reflect the activated form(s) of this protein kinase. In addition, the effect of such hyper-phosphorylated states was ablated by treatment with either wortmannin or rapamycin (Figure 6), demonstrating the involvement of PI-3K and mTOR in this process.

It was apparent that whilst IFN-γ caused an increase in the hyper-phosphorylated p70S6 kinase forms within 10 min, a somewhat longer period was required for LPS to achieve a similar effect (Figure 6). Indeed, a similar difference was observed for the activation of PDE4A4 by these two ligands (Figure 5). This signifies a close connection between the two processes.

Challenge of F442A pre-adipocytic cells with growth hormone led to the stimulation of the murine homologue of human PDE4A4 by a process that was similarly inhibited by wortmannin and rapamycin [19]. Such treatment caused the PDE4A isoform to migrate more slowly on SDS/PAGE gels. This was not observed here for PDE4A4 enzyme activated in U937 cells using either IFN-γ or LPS. However, specific regions of these homologues show quite profound differences in sequence [10,11,43]. In particular, not only is the extreme C-terminal region highly dissimilar [11], but alterations in this region have been shown to alter enzyme mobility on SDS/PAGE gels [44]. Furthermore, exon 8 of the *PDE4A* gene is hypervariable among animal species and can lead to the insertion of a polyproline stretch seen in human but not rodent forms [11,45]. Such differences may mean that even if these enzymes are similarly activated this may not necessarily lead to similar changes in mobility on SDS/PAGE gels. Nevertheless, we cannot discount the possibility that additional modifications may occur to the murine enzyme that lead to its altered mobility upon activation.

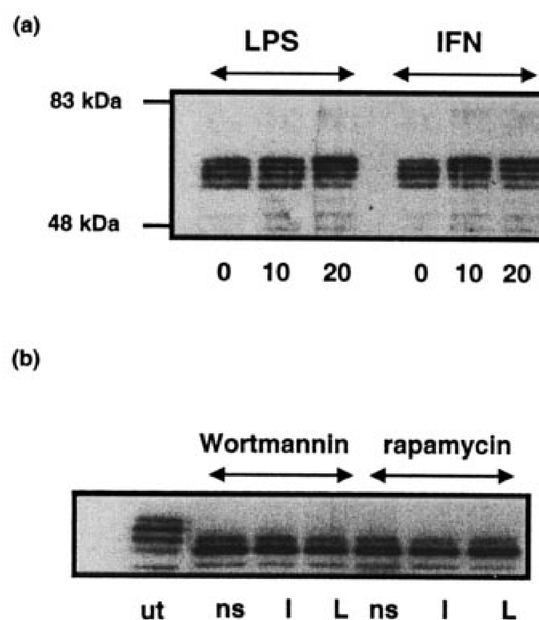

Figure 6 LPS and IFN-γ alter the migration pattern of p70S6 kinase in U937 cells

(**a**) Cells were incubated with either IFN-γ (10 μg/ml) or LPS (1 μg/ml) for the indicated times. They were then harvested, subjected to SDS/PAGE and immunoblotted using a specific polyclonal antibody for p70S6 kinase. This identified the multiple phosphorylated forms of p70S6 kinase which migrate distinctly on SDS/PAGE. (**b**) Either wortmannin (20 nM) or rapamycin (10 nM) was then added to the cells as indicated, together with either no other addition (ns), or 10 μg/ml of IFN-γ (I) or 1 μg/ml of LPS (L). Untreated cells (ut) is shown, in which none of these additions were made. Representative immunoblots are shown from experiments performed three times using 50 μg of cell protein/lane.

© 2000 Biochemical Society

DISCUSSION

We have identified PDE4A4, PDE4B2, PDE4D3 and PDE4D5 as the sole PDE4 isoenzymes expressed in the U937 monocytic cell line. These enzymes contribute a major fraction of U937-cell cAMP PDE activity and, using selective immunopurification, we have shown that the activity is of PDE4D $>$ PDE4B $\gg$ PDE4A in these cells. Rolipram serves as the paradigm for a PDE4 selective inhibitor, exhibiting IC_{50} values of approx. 100–1000 nM with 1 μM cAMP as substrate [7–10]. Indeed, we observed that the PDE4B and PDE4D activities immunopurified from U937 cells exhibited IC_{50} values of approx. 130–240 nM. However, in marked contrast with this, the IC_{50} value for rolipram inhibition of PDE4A4 activity immunopurified from U937 cells was approx. 3 nM. This value is approx. 600-fold lower than that seen for recombinant PDE4A4 expressed in the cytosol of COS7 cells, *E. coli* and *S. cerevisiae* [10]. It has, however, been suggested that certain PDE4 isoenzymes can be triggered to adopt a distinct conformational state that is identifiable by a heightened sensitivity to inhibition by rolipram [7,9]. The molecular mechanisms, which underpin such changes, have recently begun to be elucidated [10]. In particular, the human PDE4A4 isoenzyme displays a profound increase in its sensitivity to inhibition by rolipram, upon interaction with SH3-domain-containing proteins [45]. This is mediated by SH3-domain interaction with the proline- and arginine-rich LR2 region that links UCR2 to the catalytic unit [45]. Src family tyrosyl kinases, such as Src itself and Lyn, are particularly effective in eliciting this effect and have been shown to be capable of forming complexes with PDE4A4 in either or both particulate and cytosolic fractions from transfected COS1 and COS7 cells [45]. However, we have been unable to co-immunoprecipitate PDE4A4 with either of these tyrosyl kinases from U937 cells (results not shown). We have previously demonstrated that the magnitude of the increase in sensitivity to inhibition by rolipram is dependent upon the particular SH3-domain type with which it is complexed [45]. Here we note a 600-fold shift in rolipram IC_{50} value seen for PDE4A4 in U937 cells, compared with the uncomplexed, recombinant PDE4A4 enzyme analysed in COS7 cells [45]. This is considerably larger than the 20-fold shift elicited upon complexing PDE4A4 with the most effective SH3 domain noted to date, namely that of Lyn [45]. This difference is consistent with the contention that neither Lyn nor Src is complexed with PDE4A4 in U937 cells. We consider it likely, however, that PDE4A4 in U937 cells is in fact complexed with an SH3-domain-containing protein and that this is responsible for eliciting the heightened sensitivity of this enzyme to rolipram. The high specific activity of PDE4 enzymes coupled to low levels of expression will, however, make the identification of any such putative species extremely difficult.

PDE4A4 activity in U937 cells exhibits the highest affinity for inhibition by rolipram that has been reported to date [10]. This is some two orders of magnitude or more lower than the IC_{50} values for rolipram inhibition of the PDE4B and PDE4D activities in U937 cells. Thus low concentrations of rolipram used to inhibit PDE4A4 selectively offer the opportunity to evaluate the role of PDE4A4 in regulating signalling processes in U937 cells. Employing such a strategy, we have been able to show that inhibition of PDE4A4 by rolipram stimulated the phosphorylation of the key transcription factor CREB. This was gauged from our observation that the dose–effect curve comprised two distinct components (Figure 3), where the EC_{50} value for the high-affinity component seemingly reflected the IC_{50} value observed for rolipram inhibition of PDE4A4. In contrast, the EC_{50} value for the low affinity component was similar to the IC_{50} values seen for rolipram inhibition of PDE4B and PDE4D activities. We suggest that this indicates that inhibition of PDE4A4, by low concentrations of rolipram, has a functional effect in stimulating CREB phosphorylation. This is despite the fact that PDE4A provides a relatively minor contribution to the total PDE4 activity in U937 cells.

We also show for the first time, to our knowledge, that rolipram can inhibit the IFN-γ stimulated phosphorylation of p38 MAP kinase. Intriguingly, the dose-dependent nature of this response (Figure 4) was quite different from that seen with rolipram-stimulated CREB phosphorylation (Figure 3), being monophasic with an IC_{50} value similar to that observed for rolipram-mediated inhibition of the PDE4B and PDE4D enzymes rather than that of PDE4A. This suggests that inhibition of PDE4A4 by rolipram may not play a major role in regulating this signalling process.

Our results indicate that cAMP signalling in U937 cells may be functionally compartmentalized, such that PDE4A4 activity serves to regulate CREB phosphorylation but not inhibition of IFN-γ-stimulated p38 MAP kinase phosphorylation. Thus despite the fact that PDE4A4 activity is considerably less than the total PDE4B and PDE4D activities, this isoform may have an important role to play in the regulation of defined aspects of U937 cell functioning. Supportive of the potential importance of this isoform is our observation that both LPS and IFN-γ selectively activated PDE4A4 in U937 cells (Table 1). In both instances this was inhibited by the PI-3K inhibitor, wortmannin, and also by the immunosuppressant, rapamycin. This indicates that PDE4A4 activation may be mediated through a process that involves the PI-3K activation of mTOR, the target for rapamycin action [46]. Whilst mTOR activation leads to the activation of p70S6 kinase, the sequence of events that determines this has yet to be fully elucidated. Indeed, mTOR itself is thought to exhibit protein kinase activity and this can seemingly be inhibited by elevated cAMP levels [47]. It is thus tempting to suggest that either mTOR, or a protein kinase downstream from it, may be responsible for activating PDE4A4. This ability to acutely activate PDE4A4 may be of importance as it could allow pro-inflammatory mediators to try and minimize any possible cAMP-mediated inhibitory action on aspects of the inflammatory response. However, this remains to be explored. Interestingly, it has been shown recently that exposure of human monocytes to LPS caused an increase in the level of transcripts for PDE4B [48]. Whilst neither activity nor protein analyses were done, this might be surmised to lead to the increased expression of PDE4B. Thus pro-inflammatory mediators may exert both acute and chronic effects on cAMP signalling by increasing the activity and expression of specific PDE4 isoforms.

This study contributes to an increasing body of evidence that indicates that cAMP signalling is compartmentalized in many, if not all, cells [1–5]. Whilst PDE4 selective inhibitors have potential therapeutic benefits they also have a variety of associated side effects. The concept developed here, that specific PDE4 isoforms may selectively regulate certain signalling processes, indicates that it is important to understand the role of individual PDE4 isoforms and, perhaps, to develop isoform-selective inhibitors in order to maximize therapeutic benefit.

We thank the Medical Research Council for their financial support.

REFERENCES

1 Murphy, B. J. and Scott, J. D. (1998) Trends Cardiovasc. Med. **8**, 89–95
2 Colledge, M. and Scott, J. D. (1999) Trends Cell Biol. **9**, 216–221
3 Coghlan, V. M., Bergeson, S.E., Langeberg, L., Nilaver, G. and Scott, J. D. (1993) Mol. Cell. Biochem. **127**, 309–319

© 2000 Biochemical Society

4 Rubin, C. S. (1994) Biochim. Biophys. Acta **1224**, 467–479
5 Houslay, M. D. and Milligan, G. (1997) Trends Biochem. Sci. **22**, 217–224
6 Teixeira, M. M., Gristwood, R. W., Cooper, N. and Hellewell, P. G. (1997) Trends Pharmacol. Sci. **18**, 164–170
7 Torphy, T. J. (1998) Am. J. Respir. Crit. Care Med. **157**, 351–70
8 Dent, G. and Giembycz, M. A. (1995) Clin. Immunother. **3**, 423–437
9 Souness, J. E. and Rao, S. (1997) Cell. Signalling **9**, 227–236
10 Houslay, M. D., Sullivan, M. and Bolger, G. B. (1998) Adv. Pharmacol. **44**, 225–342
11 Sullivan, M., Rena, G., Begg, F., Gordon, L., Olsen, A. S. and Houslay, M. D. (1998) Biochem. J. **333**, 693–703
12 Sullivan, M., Olsen, A. S. and Houslay, M. D. (1999) Cell. Signalling **11**, 735–742
13 Conti, M. and Jin, S. L. C. (1999) Prog. Nucleic Acid Res. **63**, 1–38
14 Schafer, P. H., Wang, L., Wadsworth, S. A., Davis, J. E. and Siekierka, J. J. (1999) J. Immunol. **162**, 659–668
15 Uddin, S., Majchrzak, B., Woodson, J., Arunkumar, P., Alsayed, Y., Pine, R., Young, P. R., Fish, E. N. and Platanias, L. C. (1999) J. Biol. Chem. **274**, 30127–30131
16 Han, J., Lee, J. D., Tobias, P. S. and Ulevitch, R. J. (1993) J. Biol. Chem. **268**, 25009–25014
17 Huston, E., Pooley, L., Julien, J., Scotland, G., McPhee, I., Sullivan, M., Bolger, G. and Houslay, M. D. (1996) J. Biol. Chem. **271**, 31334–31344
18 Huston, E., Lumb, S., Russell, A., Catterall, C., Ross, A. H., Steele, M. R., Bolger, G. B., Perry, M., Owens, R. and Houslay, M. D. (1997) Biochem. J. **328**, 549–558
19 MacKenzie, S. J., Yarwood, S. J., Peden, A. H., Bolger, G. B., Vernon, R. J. and Houslay, M. D. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 3549–3554
20 Bolger, G. B., Erdogan, S., Jones, R. E., Loughney, K., Scotland, G., Hoffmann, R., Wilkinson, I., Farrell, C. and Houslay, M. D. (1997) Biochem. J. **328**, 539–548
21 Hoffmann, R., Baillie, G. S., MacKenzie, S. J., Yarwood, S. J. and Houslay, M. D. (1999) EMBO J. **18**, 893–903
22 Erdogan, S. and Houslay, M. D. (1997) Biochem. J. **321**, 165–175
23 Marchmont, R. J. and Houslay, M. D. (1980) Biochem. J. **187**, 381–392
24 Thompson, W. J. and Appleman, M. M. (1971) Biochemistry **10**, 311–316
25 Spence, S., Rena, G., Sweeney, G. and Houslay, M. D. (1995) Biochem. J. **310**, 975–982
26 Manganiello, V. C., Taira, M., Degerman, E. and Belfrage, P. (1995) Cell. Signalling **7**, 445–455
27 Owens, R. J., Lumb, S., Rees-Milton, K., Russell, A., Baldock, D., Lang, V., Crabbe, T., Ballesteros, M. and Perry, M. J. (1997) Cell. Signalling **9**, 575–585
28 Torphy, T. J., Zhou, H. L., Foley, J. J., Sarau, H. M., Manning, C. D. and Barnette, M. S. (1995) J. Biol. Chem. **270**, 23598–23604
29 Torphy, T. J., Zhou, H. L. and Cieslinski, L. B. (1992) J. Pharmacol. Exp. Ther. **263**, 1195–1205
30 Manning, C. D., McLaughlin, M. M., Livi, G. P., Cieslinski, L. B., Torphy, T. J. and Barnette, M. S. (1996) Mol. Pharmacol. **276**, 810–818
31 Verghese, M. W., McConnell, R. T., Lenhard, J. M., Hamacher, L. and Jin, S.-L. C. (1995) Mol. Pharmacol. **47**, 1164–1171
32 Montminy, M. (1997) Annu. Rev. Biochem. **66**, 807–822
33 Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C. (1995) FEBS Lett. **364**, 229–233
34 Nick, J. A., Avdi, N. J., Young, S. K., Lehman, L. A., McDonald, P. P., Frasch, S. C., Billstrom, M. A., Henson, P. M., Johnson, G. L. and Worthen, G. S. (1999) J. Clin. Invest. **103**, 851–858
35 Manthey, C. L., Wang, S. W., Kinney, S.D. and Yao, Z. (1998) J. Leukocyte Biol. **64**, 409–417
36 Seldon, P. M., Barnes, P. J., Meja, K. and Giembycz, M. A. (1995) Mol. Pharmacol. **48**, 747–757
37 Souness, J. E., Griffin, M., Maslen, C., Ebsworth, K., Scott, L. C., Pollock, K., Palfreyman, M. N. and Karlsson, J. A. (1996) Br. J. Pharmacol. **118**, 649–658
38 Schudt, C., Tenor, H. and Hatzelmann, A. (1995) Eur. Respir. J. **8**, 1179–1183
39 Nakanishi, S., Yano, H. and Matsuda, Y. (1995) Cell. Signalling **7**, 545–557
40 Brown, E. J. and Schreiber, S. L. (1996) Cell **86**, 517–520
41 Herrera-Velit, P. R. N. E. (1996) J. Immunol. **156**, 1157–1165
42 Herrera-Velit, P., Knutson, K. L. and Reiner, N. E. (1997) J. Biol. Chem. **272**, 16445–16452
43 Bolger, G. (1994) Cell. Signalling **6**, 851–859
44 Kovala, T., Sanwal, B. D. and Ball, E. H. (1997) Biochemistry **36**, 2968–2976
45 McPhee, I., Yarwood, S. J., Huston, E., Scotland, G., Beard, M. B., Ross, A. H., Houslay, E. S. and Houslay, M. D. (1999) J. Biol. Chem. **274**, 11796–11810
46 Abraham, R. T. and Wiederrecht, G. J. (1996) Annu. Rev. Immunol. **14**, 483–510
47 Scott, P. H. and Lawrence, Jr., J. C. (1998) J. Biol. Chem. **273**, 34496–34501
48 Ma, D., Wu, P., Egan, R. W., Billah, M. M. and Wang, P. (1999) Mol. Pharmacol. **55**, 50–57

Received 24 November 1999/20 January 2000; accepted 17 February 2000

© 2000 Biochemical Society