Pergamon

Biochemical Pharmacology, Vol. 50, No. 12, pp. 2033–2038, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02104-3

ISOPRENALINE INDUCTION OF cAMP-PHOSPHODIESTERASE IN GUINEA-PIG MACROPHAGES OCCURS IN THE PRESENCE, BUT NOT IN THE ABSENCE, OF THE PHOSPHODIESTERASE TYPE IV INHIBITOR ROLIPRAM

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(*Received* 13 *April* 1995; *accepted* 5 *September* 1995)

Abstract—The long-term effects of incubating freshly isolated, elicited guinea-pig peritoneal macrophages with the β-adrenoceptor agonist isoprenaline and the selective inhibitor of phosphodiesterase (PDE) IV rolipram, on adenosine-3′,5′-cyclic phosphate (cAMP)-specific PDE IV activity have been investigated. The level of cAMP PDE activity in macrophages was unaffected by long-term exposure of cells to rolipram alone. In contrast, in the presence of isoprenaline (10 μM), a concentration-related (0.05–50 μM) increase in cAMP PDE activity was observed in the cytosolic and particulate fractions. Incubation with isoprenaline alone did not affect macrophage cAMP PDE. cAMP PDE activities in homogenates of control cells and macrophages treated with isoprenaline (10 μM) and rolipram (5 μM) (3-fold activation) were inhibited by the selective PDE IV inhibitor, rolipram, with similar potencies (IC_{50}: 2–3 μM). The increase in cAMP PDE activity in response to rolipram and isoprenaline was completely blocked by cyclohexamide (10 μg/ml). Incubating macrophages for 10 min with rolipram increased cAMP accumulation in the presence, but not in the absence, of isoprenaline (10 μM) over the same concentration range that induction of cAMP PDE activity was observed. cAMP levels remained elevated for at least 1 hr. Isoprenaline (10 μM) alone induced a transient elevation in cAMP levels that peaked at 2 min and had returned to basal levels by 10 min. Protein kinase A activity (PKA) was increased almost 10-fold (at 10 min) by exposing cells to rolipram plus isoprenaline and remained elevated for at least 4 hr. Isoprenaline alone induced a small (2-fold) increase in PKA activity and rolipram alone was without effect.

Key words: cAMP phosphodiesterase; macrophages; rolipram; isoprenaline

cAMP§-specific PDE IV is a family of isoenzymes that specifically hydrolyzes cAMP with high affinity (Km – 2 μM) and is selectively inhibited by compounds such as rolipram and Ro-20-1724 [1]. Agents that elevate cAMP accumulation exert two types of control on cAMP PDE—short-term and long-term upregulation [2–4]. Short-term activation of cAMP PDE is one of several mechanisms to rapidly turn off the cAMP signal, whereas enzyme induction involving *de novo* protein synthesis, which has been observed in several cell types, occurs as cells attempt to adapt to prolonged elevation of the second messenger.

The well-documented induction of cAMP PDE occurs after prolonged (2 or more hr) exposure of cells to cAMP analogues or agents that elevate intracellular levels of the second messenger and is blocked by inhibitors of mRNA or protein synthesis [2]. Upon removal of the stimulus, cAMP PDE activity decays slowly to basal levels over several hours [2]. Short-term activation of one PDE IV subtype (PDE IV_D) by agents that stimulate adenylate cyclase through a PKA-dependent phosphorylation has recently been reported [3, 4]. Only a 'long-form', splice variant of PDE IV_D ($RNPDEIV_{D3B}$) containing a consensus sequence for phosphorylation by PKA has been shown to be activated in this manner [3].

There is much interest in the therapeutic potential of selective PDE IV inhibitors, because they dampen the functions of a wide number of inflammatory cells and exhibit anti-inflammatory activities *in vivo* [5, 6]. The long-term effects of PDE IV inhibitors on components of the cAMP cascade in inflammatory cells is an important issue that only few studies have addressed. Recently, it was demonstrated that the β-adrenoceptor agonist salbutamol induces PDE IV activity in the established monocytic cell line, U937, and that this effect is enhanced by rolipram [7] which, alone, has no effect. The increase in cAMP hydrolytic activity in response to the β-agonist correlates, in a concentration-dependent manner, with salbutamol-induced cAMP accumulation and PKA activation. It was proposed that the increased cAMP PDE activity impairs the ability of PGE_2 to elevate cAMP and antagonizes the activation of U937 cells by inflammatory mediators [8].

We have investigated the effects on cAMP PDE activity of exposing freshly isolated guinea-pig peritoneal macrophages to rolipram in the absence and presence of the β-adrenoceptor agonist isoprenaline. Rolipram, alone, had no effect on macrophage cAMP PDE activity; however, cells exposed to a combination of rolipram

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§ Abbreviations: cAMP, adenosine-3′,5′-cyclic phosphate; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; HBSS, Hanks balanced salt solution; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase (protein kinase A).

plus isoprenaline had 2–3-fold greater cAMP hydrolytic activity compared to controls. Unlike U937 cells, incubation of macrophages with a β-adrenoceptor agonist (isoprenaline) alone did not affect cAMP PDE activity.

MATERIALS AND METHODS

Materials

Cyclic [2,8-^{3}H] AMP (50 Ci/mmole, 1 mCi) was purchased from Amersham International (Amersham, Bucks, U.K.). Rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrolidone] was synthesized by the Department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd (Dagenham, Essex, U.K.). The cAMP radioimmunoassay kit and [γ-^{33}P] ATP (3000 Ci/mmol) were purchased from NEN Chemicals GmbH. Donor horse serum was purchased from Flow Laboratories Ltd. (Irvine, Scotland). Other cell culture reagents and the PKA assay kit were from Gibco BRL (Paisley, Scotland). All other chemicals were obtained from Sigma Chemical Co., BDH Chemicals (both of Poole, Dorset, U.K.) and Prolabo (Eccles, Manchester, U.K.). Male Dunkin Hartley guinea pigs were purchased from a local supplier.

Preparation of guinea-pig macrophages

Male Dunkin Hartley guinea pigs (250–400 g) were injected (i.p.) with 0.5 mL of donor horse serum three times over the course of 1 week. At least 5 days after the third injection, the guinea pigs were killed by CO_2 asphyxiation. A ventral incision was made and 50 mL of HBSS without Ca^{2+} poured into the abdominal cavity. The abdomen was gently massaged for approximately 1 min and the peritoneal exudate was aspirated and centrifuged at 250 × g for 10 min at 20°C. The supernatant was discarded and the pellet washed once (10 mL HBSS) and resuspended in 5% heat-inactivated FCS in HBSS. Aliquots of the cell suspension (1 mL) were layered onto a discontinuous (55% and 70% v/v) Percoll gradient in HBSS. The gradients were centrifuged (250 × g, 30 min, 20°C) and the macrophages (>85% pure), recovered from the surface of the 55% gradient layer (1.067 g mL^{-1}), were resuspended in 10 mL HBSS. Total cell counts were determined using a Coulter counter and differential cell counts were obtained from cytospin slides fixed in methanol and stained with Wright-Giemsa.

Cell culture

Macrophages, obtained by peritoneal lavage, were washed twice in sterile HBSS and centrifuged at 250 × g for 10 min at 20°C. After resuspending them in HBSS, the cells were plated at a density of 3×10^5 cells/cm^2 and incubated for 30 min at 37°C to remove nonadherent cells. The macrophages (>95% pure) were then cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 12.5 units/mL penicillin, 6.25 units/mL streptomycin, and 2% FCS for the indicated times at 37°C in 5% CO_2.

Preparation of subcellular fractions

The cells were collected, washed twice with ice-cold HBSS, and homogenized on ice in 3–3.5 mL homogenization buffer (20 mM Tris-HCl, pH 7.5, 2mM $MgCl_2$, 1 mM dithiotheitol, 5mM EDTA, 0.25 M Sucrose, 20 μM p-tosyl-l-lycine-chloromethyl-ketone, 10 μM leupeptin, and 2000 units/mL aprotinin) with a Dounce homogenizer (25 strokes). The homogenate was centrifuged at 105,000 × g for 30 min at 4°C, the supernatant was collected, and the pellet resuspended in an equal volume of homogenization buffer. The supernatant and particulate fraction were then assayed for cyclic nucleotide PDE activity.

Measurement of PDE activity

PDE activity was determined by the two-step radioisotope method of Thompson *et al.* [9]. The reaction mixture contained 20 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 0.2 mM ethyleneglycol-bis-(β-aminoethyl ester) N, N, N′, N′-tetraacetic acid (EGTA) and 0.05 mg/mL bovine serum albumin. Unless otherwise stated, the substrate concentration was 1 μM.

The IC_{50} values (concentration that produced 50% inhibition of substrate hydrolysis) for the compounds tested were determined from concentration-response curves in which concentrations ranged from 0.1 nM to 100 μM. At least three concentration-response curves were generated for each agent.

Protein was determined as described by the method of Lowry *et al.* [10], using bovine serum albumin as standard.

Categorization of PDE isoenzymes

The nomenclature for the different cyclic nucleotide PDEs adopted in this paper is based on that of Beavo and Reifsnyder [11].

Measurement of macrophage cAMP accumulation

Macrophages, in HBSS, were plated into 6-well plates (Nunc) (2 million per well) and incubated for 30–45 min at 37°C. Nonadherent cells were removed and RPMI 1640 medium, containing 2% FCS, with or without effectors, was added. Incubations were terminated by removal of medium, rapid washing of the cell monolayer (ice-cold HBSS), and addition of 1 mL of ice-cold trichloroacetic acid (TCA) (5%). The TCA extracts were briefly sonicated (10 sec), centrifuged (3000 × g) for 15 min and the supernatants removed to a clean tube. TCA was removed with 3 washes of water-saturated ether (5 vols). The last traces of ether were removed with 3 washes of water-saturated ether (5 vols). The last traces of ether were removed by gassing with nitrogen. Samples were acetylated and cAMP quantified by radioimmunoassay (RIA, NEN Chemicals GmbH). In some experiments, cAMP was measured in the untreated medium from the macrophage incubations.

Measurements of cAMP-dependent protein kinase (PKA) activity

Macrophages were plated into 6-well cell culture plates (2 million per well) and incubated in HBSS for 30–45 min, as above. The medium, containing nonadherent cells, was removed and RPMI-1640 medium without or with rolipram (5 μM) and/or isoprenaline (10 μM) was added. The cells were incubated for the specified periods at 37°C, after which the medium was aspirated and the cells washed rapidly with ice-cold HBSS. Extraction buffer (50 mM KH_2PO_4, pH 6.8, 10 mM EDTA, 0.2 mM dithiothreitol, 125 mM NaCl, 0.5 mM 3-isobuty-1-methylxanthine [IBMX], 0.2% Triton X-100) (0.5 mL) was then added to wells, the cells scraped from the surface, and then homogenized with a Dounce homogenizer (20 strokes).

The PKA activity of macrophage Triton X-100 extracts was determined with an assay kit (GIBCO) in which incorporation of ^{33}P from [γ-^{33}P]ATP into the synthetic, specific substrate, kemptide, was measured. Briefly, 10 µL of the macrophage extract were added to an assay mixture (total volume 40 µL) containing 50 µM kemptide, 0.1 mM [γ-^{33}P] ATP (20 µCi/mL), 10 mM $MgCl_2$, 0.25 mg/mL bovine serum albumin, 50 mM Tris/HCl (pH 7.5) in the absence and presence of 10 µM cAMP and/or 1 µM synthetic cAMP-dependent protein kinase inhibitor (GIBCO). Incubations were allowed to proceed for 15 min before the reaction was stopped by pipetting 20 µL of the assay mixture onto phosphocellulose ion-exchange paper discs, which were then washed twice, extensively, in phosphoric acid (1%, v/v) and then twice with water. Individual discs were placed in scintillation vials and ^{33}P measured by liquid scintillation spectrometry after addition of scintillation cocktail (Lumagel, Lumac-LSC B.V., Olen, Belgium). The extent of PKA activation was expressed as the activity ratio, which is the ratio of specific activity in the absence of added cAMP to that in the presence of sufficient cAMP to fully activate the enzyme (–cAMP/+cAMP activity ratio).

Statistical analysis

Results are expressed as means ± SEM. Data were compared by unpaired two-tailed Students *t*-test and were considered to be statistically significant when P was less than 0.05.

RESULTS

Effects of rolipram and isoprenaline on cAMP phosphodiesterase activity

Rolipram alone had little effect on cAMP phosphodiesterase activity; however, in the presence of 10 µM isoprenaline, it induced a concentration-dependent increase in cAMP PDE activity, with a maximal response being observed at 8 µM (Fig. 1). Following exposure to rolipram and isoprenaline, cAMP PDE activity was increased in both the cytosolic (A) and particulate (B) fractions (Fig. 1). The percentage of total cAMP PDE activity associated with the two subcellular fractions did not change. In control cells, particulate cAMP PDE accounted for 81 ± 9.2% of total activity, whereas in treated cells it accounted for 83 ± 6.3%.

cAMP PDE activity increased within 2 hr following exposure of cells to rolipram (5 µM) plus isoprenaline (10 µM) and remained elevated for at least 20 hr (Fig. 2). The increase in cAMP PDE induced by rolipram and isoprenaline was completely blocked by cyclohexamide (Fig. 3). Neither the PDE III inhibitor, SK&F 94120 (5 µM), nor the PDE V inhibitor, zapranast (5 µM), influenced cAMP PDE activity following incubation with adherent macrophages.

cAMP hydrolytic activity in homogenates of guinea-pig peritoneal macrophages was almost totally inhibited by rolipram (IC_{50}: 2.8 µM) (Fig. 4). A similar potency (IC_{50}: 2.6 µM) for rolipram was observed in homogenates from cells treated with isoprenaline (10 µM) plus rolipram (5 µM), in which a 3-fold increase in cAMP PDE activity was observed (Fig. 4). The inhibitory potency of rolipram was increased approximately 20-fold when macrophage membranes were exposed to vanadate/glutathione complex (data not shown). Neither

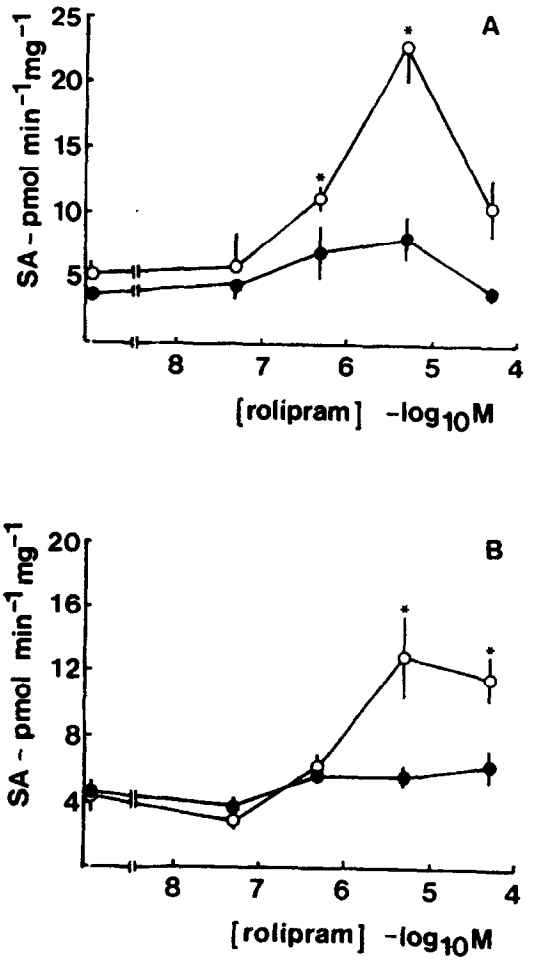

Fig. 1. Concentration/response relationship for stimulation of cAMP PDE by rolipram in macrophages treated with isoprenaline. Cells (20×10^6) were incubated with (○) or without (●) 10 µM isoprenaline in the presence of increasing concentrations of rolipram (0–50 µM) for 18 hr. Cytosolic (A) and particulate (B) fractions were prepared as described in the Methods section and cAMP PDE activity was measured with 1 µM substrate. Specific activity values (SA) represent the means ± SEM (vertical bars) for 3 experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$ for significant difference from control.

cGMP (10 µM) nor calcium (2 mM) plus calmodulin (10 U/mL) affected cAMP PDE in either control or treated cells. The cGMP-inhibited PDE (PDE III) inhibitor, siguazodan, only weakly inhibited cAMP PDE activity in homogenates from control and treated cells (IC_{50} > 100 µM).

Effect of rolipram and isoprenaline on cAMP accumulation

Rolipram alone exerted little effect on cAMP accumulation (Fig. 5); however, in the presence of isoprenaline (10 µM), a dose-related increase in cAMP accumulation was observed during a 10-min incubation (Fig. 5). Isoprenaline alone induced a transient increase in cAMP that had returned to background levels by 20 min. Following exposure of cells to rolipram (5 µM) plus isoprenaline (10 µM), intracellular cAMP remained elevated for at least 1 hr (Fig. 6A) in spite of a large

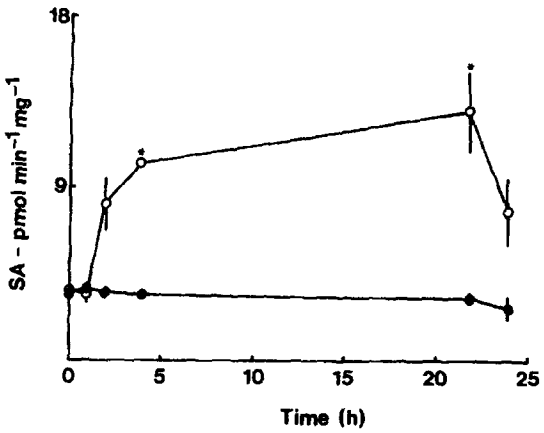

Fig. 2. Time/response relationship for stimulation of cAMP PDE activity in macrophages by isoprenaline and rolipram. Cells (20×10^6) were incubated for the indicated time periods with 5 μM rolipram in the presence (○) or absence (●) of isoprenaline (10 μM). At the end of the incubations, cells were homogenized and homogenate cAMP PDE activity was assayed immediately. Specific activity values (SA) represent the means ± SEM (vertical bars) for 3 experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$ for significant difference from control.

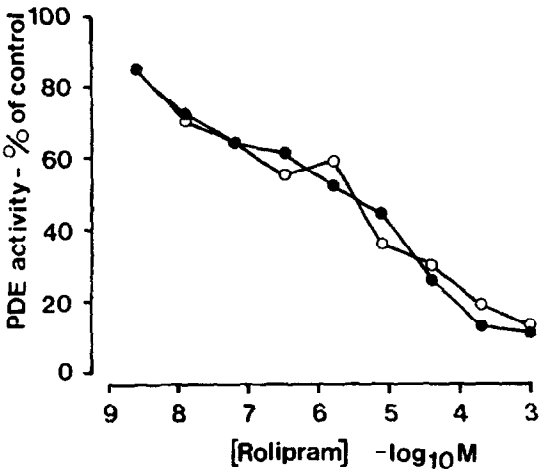

Fig. 4. Effect of rolipram on cAMP PDE activity in homogenates from control and treated macrophages. Macrophages (20×10^6 cells) were incubated in the absence (○) and presence (●) of isoprenaline (10 μM) and rolipram (5 μM), as described in the Methods section. The cAMP PDE activity in homogenates was measured in the presence of increasing concentrations (0.003–1000 μM) of rolipram. The data represent the results from a typical experiment performed in triplicate.

proportion of the accumulated cAMP being extruded into the extracellular medium (Fig. 6B). By 2 hr, cell-associated cAMP had returned to basal levels (unpublished observations).

Effect of rolipram and isoprenaline on cAMP-dependent protein kinase (PKA)

Exposure of macrophages to rolipram (5 μM) for 10 min did not affect the PKA activity ratio (AR, control cells: 0.075 ± 0.032, $n = 2$; AR, rolipram-treated cells: 0.070 ± 0.027, $n = 3$). Isoprenaline alone stimulated PKA activity slightly (AR: 0.180 ± 0.023, $n = 3$), and a 10-min exposure to a combination of rolipram plus isoprenaline increased PKA activity almost 10-fold (AR: 0.656 ± 0.055, $n = 6$). PKA activity was elevated, but greatly reduced at 4 hr and by 2 hr there was little difference between control and rolipram plus isoprenaline-treated cells (Fig. 7).

DISCUSSION

In view of their therapeutic potential in allergic and chronic inflammatory diseases, the consequences of prolonged exposure of inflammatory cells to PDE IV inhibitors on the regulatory pathways through which they exert their effects warrants consideration. In general, the

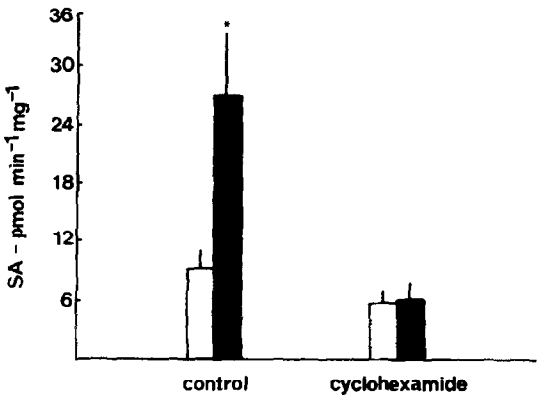

Fig. 3. Effect of protein synthesis inhibitor cyclohexamide on isoprenaline/rolipram-induced stimulation of macrophage cAMP PDE activity. Control (open bars) and isoprenaline (10 μM) plus rolipram (5 μM)-treated (filled bars) cells (20×10^6) were incubated for 18 hr in the absence and presence of 10 μg/mL cyclohexamide. Homogenates were prepared and assayed immediately for cAMP PDE activity. The specific activity values (SA) represent means ± SEM (vertical bars) of 3 experiments performed in triplicate. $*P < 0.05$ for significant difference from control.

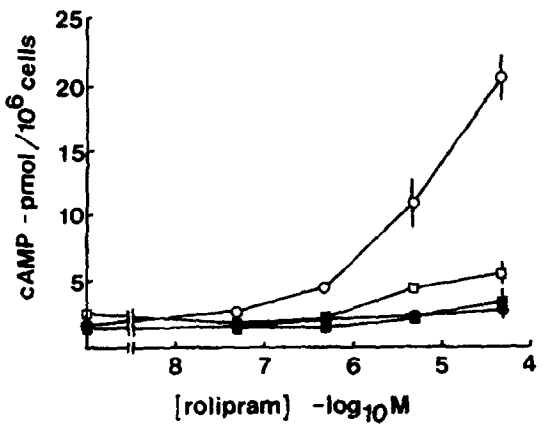

Fig. 5. Concentration relationship for the stimulation of cAMP accumulation in macrophages by rolipram in the absence and presence of isoprenaline. Cells were incubated with the indicated concentration of rolipram in the absence (■, ●) and presence (□, ○) of isoprenaline (10 μM) for 10 min. cAMP was assayed in washed macrophages (●, ○) and in the incubation medium (■, □). These results represent means ± SEM (vertical bars) of 3 separate incubations.

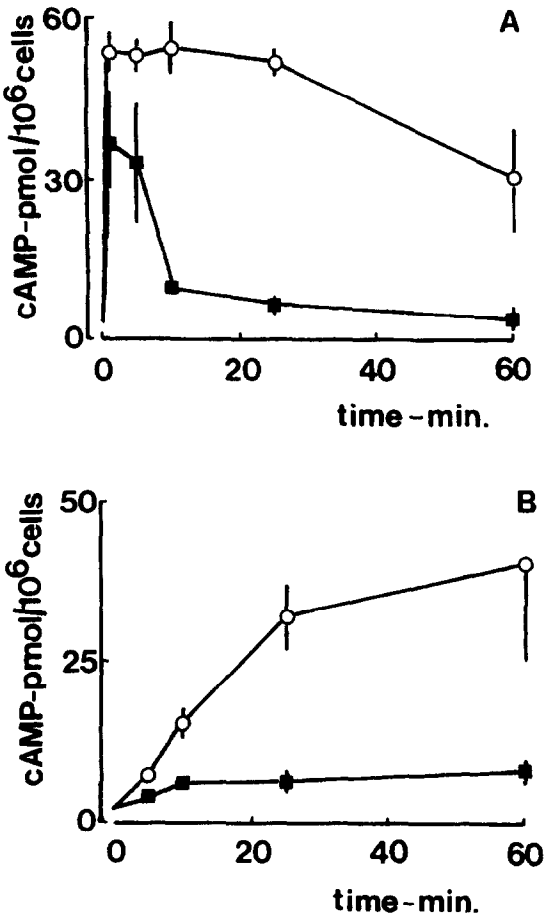

Fig. 6. Time-response relationship for the stimulation of cAMP accumulation by rolipram in the absence and presence of isoprenaline. Cells were incubated with isoprenaline (10 μM) in the absence (■) or presence (○) of 5 μM rolipram for the indicated time periods. cAMP levels in cells (A) and medium (B) were measured as described in the Methods section. The results represent means ± SEM of 3 separate incubations.

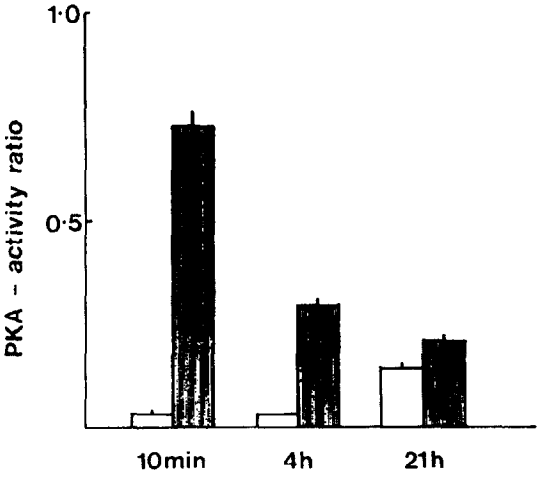

Fig. 7. Time-response relationship for the stimulation of PKA by rolipram and isoprenaline. Cells were incubated in the absence (open bars) or presence (filled bars) of rolipram (5 μM) plus isoprenaline (10 μM) for the indicated time periods as described in the Methods section. The results represent means ± SEM of 4 separate incubations. *$P < 0.01$ for significant difference from control.

effects of isoprenaline and rolipram on macrophage cAMP PDE, cAMP levels, and PKA are similar to those described for salbutamol and rolipram in U937 cells. In both cell types, rolipram alone failed to influence cAMP PDE activity, whereas 2-3 fold increases in hydrolytic activity were observed in combination with β-adrenoceptor agonists (isoprenaline, salbutamol) or prostaglandin E_2 [7]. In contrast to the previous studies on U937 cells [7], however, prolonged exposure of macrophages to a β-adrenoceptor agonist, in the absence of a PDE IV inhibitor, failed to exert a significant effect on cAMP PDE activity.

The concentration response relationship for rolipram-induced cAMP PDE activation in the presence of isoprenaline closely mirrored that for cAMP accumulation. No significant increase in cAMP accumulation was elicited by rolipram alone, which probably reflects the low rate of cAMP synthesis in unstimulated cells. A transient cAMP response to isoprenaline alone was observed. Whether the rapid turn off of the β-adrenoceptor agonist signal is due the downregulation of the β-adrenoceptor or short-term activation of cAMP PDE activity remains to be determined. A large proportion of the macrophage cAMP produced in response to a combination of isoprenaline and rolipram was extruded. This potentially important regulatory mechanism for controlling intracellular cAMP levels seems to be inadequate, by itself, to control greatly elevated levels of the second messenger because the cells try to adapt by synthesizing *de novo* cAMP PDE.

Previous studies [12] in normal and mutant lymphosarcoma cells, as well as in U937 cells [7], implicate PKA in the induction of cAMP PDE by dibutyryl cAMP and stimulators of cAMP accumulation. Treatment of macrophages with rolipram and isoprenaline caused a 9-fold activation of PKA that was maintained, albeit at a reduced level, after 4 hr. Rolipram, alone, was without effect and only a slight increase in PKA activity was elicited by isoprenaline. In contrast, salbutamol (0.1–1 μM) almost maximally activates PKA in U937 cells [7]. The differences in the PKA responses to β-agonists may, perhaps, explain why cAMP PDE is greatly increased by exposing U937 cells to salbutamol alone, whereas it is not affected by incubating macrophages with isoprenaline. The reasons for the differences in the β-adrenoceptor agonist-induced PKA activation in the two cell types is not immediately apparent because the cAMP responses are similar.

As has been demonstrated previously in other cell types [7], the induction of cAMP PDE is blocked by the inhibitor of protein synthesis, cyclohexamide. Thus, rolipram and isoprenaline induce *de novo* synthesis of cAMP PDE molecules. Because rolipram displays almost identical potencies against cAMP PDE activity in control and treated cells, it seems reasonable to conclude that the induced enzyme is a PDE IV with properties similar to the constitutive enzyme. It should be noted that, unlike murine peritoneal macrophages in which several PDE isoenzymes are present [13], the cAMP hydrolytic activity in guinea-pig macrophages is predominantly PDE IV [14].

It is of interest that increased cAMP PDE activity is detected in both the cytosolic and particulate fractions.

Previous studies have demonstrated that distinct amino acid sequences at the C-terminal end of PDE IV subtypes determine their subcellular location [15]. If this is the case in macrophages, alternative splicing may be responsible for producing the divergent amino acid sequences that permit macrophages to contain both cytosolic and membrane-bound PDE IV. Furthermore, multiple splice variants would be induced by exposure to β-adrenoceptor agonists and PDE IV inhibitors. Alternatively, the native enzyme may be exclusively membrane-bound and homogenization may dissociate a small percentage of the hydrolytic activity. In comparison with the eosinophil [16], guinea-pig macrophage cAMP PDE is relatively easily solubilized [14].

In conclusion, treatment of guinea-pig macrophages with a combination of isoprenaline and rolipram increased cAMP PDE activity, a phenomenon that is associated with greatly increased intracellular cAMP accumulation and activation of PKA. Inhibition of the isoprenaline plus rolipram-elicited increase in cAMP PDE activity by cyclohexamide demonstrates *de novo* protein synthesis. Unlike U937 cells, a β-adrenoceptor agonist failed to increase macrophage cAMP PDE activity in the absence of a PDE IV inhibitor.

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