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Role of cyclic AMP in the modulation of IgE production by the β_2-adrenoceptor agonist, fenoterol

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Role of cyclic AMP in the modulation of IgE production by the β_2-adrenoceptor agonist, fenoterol. O. Coqueret, D. Demarquay, V. Lagente. ©ERS Journals Ltd 1996.

ABSTRACT: We have previously demonstrated that the β_2-adrenoceptor agonist, salbutamol, potentiates the effect of interleukin-4 (IL-4) on immunoglobulin E (IgE) production by human peripheral blood mononuclear cells (PBMC).

This study was undertaken to further define the activities of these drugs and the role of cyclic-adenosine monophosphate (cAMP) in the modulation of IgE production.

Our results indicate that fenoterol (1 µM) potentiated IL-4-induced IgE production and IgE messenger ribonucleic acid (mRNA) expression. Moreover, this effect was associated with an increase in intracellular cAMP levels. The activities of this drug on IgE production were mimicked by cell permeable cAMP analogues, such as dibutyryl-cAMP (db-cAMP) (100 µM) or Sp-AMP (10 µM). The potentiating effect of fenoterol on IgE production was markedly inhibited in the presence of protein kinase A (PKA) inhibitors: H8 (10 µM) and Rp-AMP (10 µM), suggesting that its effects are likely to depend on the activation of the cAMP pathway. Additionally, the potentiating effect of fenoterol was also blocked in the presence of indomethacin. Fenoterol potentiated IL-4-induced IgE production from purified B-cells activated through their CD40 antigen. This effect was associated with an increase in cellular viability. Therefore, the activities of this drug on PBMC are likely to be mediated by the activation of another cellular type.

Taken together, these results show that fenoterol potentiates the IL-4-induced IgE production *via* the cAMP pathway, but that this enhancement could not be explained by a direct effect on B-lymphocytes.

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Inhaled β_2-adrenoceptor agonists are the most effective of the available bronchodilator drugs used to treat asthma. They induce bronchial smooth muscle relaxation and are potent inhibitors of mediator release by mast cells [1, 2]. However, despite this beneficial effect, recent studies have suggested that the regular use of β_2-adrenoceptor agonists in asthma has, controversially, been claimed to lead to a further progression of the disease [3, 4], and may be associated with increased morbidity and even mortality [5]. Indirect evidence also suggests that β_2-adrenergic agonists play a role in the modulation of immunity. For example, β_2-adrenergic stimulation of lymphocytes produces changes in such parameters as T-cell proliferation [6], antibody synthesis [7] and eicosanoid production [8]. Following the activation of the β_2-adrenoceptor, these drugs induce an enhancement of adenosine 3',5'-cyclic-monophosphate (cyclic-AMP, cAMP) levels [9]. This increase is followed by the activation of cytoplasmic cAMP-dependent protein kinase A (PKA), the translocation of the kinase to the nucleus and ultimately the phosphorylation of nuclear transcription factors, such as the cAMP response element-binding protein (CREB) [10]. Thus, cAMP plays a key role in the regulation of lymphocyte activation, but its effect depends on the cell type, signals and stages of cell-cycle activation.

Despite the clinical importance of immunoglobulin E (IgE) in allergic reactions, little attention has been paid to the effect of β_2-adrenoceptor agonists on the regulation of the production of this antibody. We have previously demonstrated that salbutamol potentiates the effect of interleukin-4 (IL-4) on IgE production from human peripheral blood mononuclear cells (PBMC), which might be explained by an inhibition of interferon-γ (IFN-γ) production [11]. The same effect was observed *in vitro* and *in vivo* in the mouse, where it could be associated with an increased release of Th2-type lymphokines from murine splenocytes [12]. The present study was undertaken to further characterize the effect of the β_2-adrenoceptor agonist, fenoterol, on human IgE production. In addition, the role of the intracellular cAMP level and PKA activation was analysed.

Methods

Reagents

The following reagents were obtained as noted. Recombinant human interleukin-4 (IL-4, 1×10^7 U·mg^{-1}) was purchased from Biotrans (Los Angeles, CA, USA). Foetal calf serum (FCS), phosphate-buffered saline (PBS),

glutamine, penicillin and RPMI 1640 were from Flow (Irvine, Scotland). Fenoterol, N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (db-cAMP), indomethacin, N-5-isoquinolinesulphonamide (H8), ortho-phenylenediamine (OPD), bovine serum albumin (BSA) and saline sodium phosphate ethylenediamine tetra-acetic acid (EDTA) (SSPE) buffer were purchased from Sigma (St Louis, MO, USA). Cholera toxin (CT) was from Calbiochem (La Jolla, CA, USA). Sp and Rp-cyclic 3',5'-hydrogen phosphorothioate adenosine triethylammonium salt (Sp- and Rp-AMP) were from RBI (Natick, MA, USA). Purified IgE antibodies used as standards were obtained from Stallergenes (Fresnes, France). Purified mouse anti-human IgE monoclonal antibodies (moAbs) and anti-CD40 moAbs were from Pharmingen (San Diego, CA, USA). Tween 20 was obtained from Merck (Darmstadt, GFR). Anti-CD20 moAbs were from Becton Dickinson (San Jose, CA, USA).

Isolation of peripheral blood mononuclear cells

Human PBMC were isolated from healthy nonatopic donors by density gradient centrifugation on Ficoll (Eurobio, Les Ulis, France). The cells recovered at the interface were washed twice by centrifugation at 1,800 rpm in RPMI 1640, and were resuspended in a culture medium consisting of RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 50 U·mL^{-1} penicillin and 50 mg·mL^{-1} streptomycin (RMPI-FCS) at a final concentration of 2×10^6 cells·mL^{-1}.

B-lymphocyte preparation

Normal B-lymphocytes were purified from PBMC according to the experimental procedure described by MILTENYI *et al*. [13]. PBMC resuspended in PBS solution containing 1% BSA plus 0.01% sodium azide plus 5 mM EDTA, were incubated with paramagnetic beads (200 mL of the solution provided by the manufacturers for 10^8 cells) conjugated to anti-CD19 moAbs. Cells were next deposited on specially designed columns and separated into positive and negative populations using a high magnetic field (Miltenyi Biotec, Sunnyvale, CA, USA). Unlabelled CD19- cells were eluted from the column, whilst CD19+ B-cells were recovered by vigorous washing of the column matrix after its removal from the magnet. After staining purified B-cell preparations with fluorescein isothiocyanate (FITC)-conjugated specific anti-CD20 moAbs (Becton Dickinson, Moutain View, CA, USA), fluorescence analysis was performed with a FACSstar+ (Becton Dickinson, Sunnyvale, CA, USA) and showed that they contained >98% B-lymphocytes. B-lymphocytes were then resuspended in RPMI-FCS at a final concentration of 1×10^6 cells·mL^{-1} and were assessed for viability by trypan blue exclusion after 13 days of culture.

Measurement of IgE production

PBMC or purified B-lymphocytes were cultured for 13 days, after which supernatants were harvested and tested for their IgE concentrations. Briefly, 96-well enzyme-linked immunosorbent assay (ELISA) plates (Maxisorp, Nunc) were coated overnight at 4°C with 100 µL·well^{-1} (10 µg·mL^{-1}) of anti-IgE moAb in 0.1 M $NaHCO_3$ buffer, pH 9.6. After five washes with PBS added with 0.05% Tween 20 (PBS-Tween), the plates were incubated for 1 h at 37°C with RPMI-FCS to saturate nonspecific protein-binding sites. One hundred microlitres per well of culture supernatants or defined solutions of IgE standards was then added and the plates were incubated for 2 h at 37°C. After washing the plates five times with PBS-Tween, 100 µL of 1/10,000 diluted peroxidase-labelled anti-IgE moAb was added to each well, and incubation was continued for 2 h at 37°C. After five washes with PBS-Tween, 100 µL of OPD (0.5 mg·mL^{-1}) in 0.1 M citric acid and 0.1 M Na_2HPO_4 buffer was added. The reaction was stopped with 100 µL of H_2SO_4, and absorbance was assessed at 492 nm with an ELISA reader (Dynatech, Alexandria, VA, USA). The sensitivity of the assay was 300 pg·mL^{-1}. No cross-reactivity has been observed with others subclasses of antibodies.

Ribonucleic acid (RNA) isolation and Northern Blot assays

Total RNA was isolated from PBMC using the method described by CHOMCZYNSKI and SACCHI [14]. Aliquots of RNA (20 µg) were electrophoresed in 1% agarose, 6% formaldehyde gels, electrotransferred to Hybond N^+ membranes (Pharmacia, St-Quentin en Yvelines, France), and fixed by UV irradiation. Prehybridization and hybridization were performed at 68°C in a solution containing 0.5% sodium dodecyl sulphate (SDS), Denhardt 5×, SSPE 5×, dextran sulphate 5×, salmon sperm 20 µg·mL^{-1} and formamide 50%. Hybridization was performed overnight in a fresh solution containing a ^{32}P-labelled RNA probe specific for the constant regions of the ε chain of IgE (Cε) (1×10^6 cpm·mL^{-1}). The membranes were washed for 30 min at room temperature in 1× standard sodium citrate (SSC)/0.1% SDS, and twice in 0.1× SSC/0.1% SDS at 68°C. They were then autoradiographed for 5 days at -80°C on RPN Hyperfilm (Amersham) using two intensifying screens.

RNA probe

In order to detect the IgE mRNA, a probe complementary to its sequence was constructed. A complementary deoxyribonucleic acid (cDNA) corresponding to a part of the CH2-CH4 domain of the Cε molecule was obtained by reverse transcription-polymerase chain reaction (RT-PCR) experiments using oligonucleotides corresponding to nucleotides 228–244 and 830–846 of the published sequence [15]. These oligonucleotides were flanked by EcoR1 and BamH1 sites to facilitate further cloning. One milligram of total RNA from the U266B1 cell line (kindly provided by G. Delespesse, University of Montreal, Montreal, Canada) was reverse transcribed

using a commercial kit (Promega, Madison, WI, USA) for 15 min at 42°C. cDNA (5 mL) was then amplified by 35 cycles of PCR at an annealing temperature of 65°C. The PCR products were electrophoresed in Tris-borate/EDTA buffer and an expected band of 490 base pairs (bp) was excised from the gel, purified out of agarose (Plasmid Midi kit, Qiagen, Chatsworth, CA, USA) and digested with EcoR1 and BamH1 (Stratagene, La Jolla, CA, USA). This DNA was ligated overnight at 15°C with pBluescript II KS (-) (Stratagene) treated with calf intestine alkaline phosphatase. The XL1 Blue bacteria (Stratagene) were then transformed with the ligation mixture. The white colonies obtained after blue/white selection were analysed for the presence of a 490 bp insert. One "positive" clone was selected and partially sequenced to confirm the presence of the insert using a commercial sequencing kit (Applied Biosystem, Foster, CA, USA). The plasmid containing the insert was then used for *in vitro* transcription of a ^{32}P-labelled riboprobe using a commercial kit (Stratagene).

Determination of intracellular cAMP

A commercial radioimmunoassay (RIA) cAMP-test was used (NEN, Dreieich, Germany). PBMC (2×10^6 cells·mL^{-1}) were incubated in RPMI-FCS for 5 min with the indicated stimuli, then harvested, washed in cold PBS and incubated for 20 min in 0.1 M HCl at 4°C [16].

Statistical analysis

Results are expressed as mean±SEM of at least three experiments and were compared using the nonparametric Mann-Whitney statistical test.

Results

Effect of IL-4 and fenoterol on IgE production and on IgE mRNA expression

In earlier studies, we demonstrated that the β_2-adrenoceptor agonist, salbutamol potentiates the effect of IL-4 on IgE production by human PBMC. This effect was confirmed in this study by the experiments using fenoterol and presented in figure 1. Fenoterol, which is ineffective in inducing IgE production when added alone to the cell cultures (<0.3 ng·mL^{-1}), enhanced the IgE production induced by IL-4 in a dose-dependent manner, with a peak effect observed at 1 µM.

To further investigate the mechanism underlying this effect, Northern Blot experiments were conducted to assess the effects of these drugs on IgE mRNA expression. Results showed that IL-4 (30 U·mL^{-1}) induced the appearance of a 2.2 kb productive IgE mRNA after 10 days of culture (fig. 2). When the cells were stimulated with IL-4 in the presence of fenoterol (1 µM), results showed that this drug potentiated the effect of IL-4 at the IgE mRNA level. Densitometric analysis of three different experiments indicated that fenoterol induced a marked increase in the mRNA expression compared to the effect of IL-4 added alone to the cell cultures (data not shown).

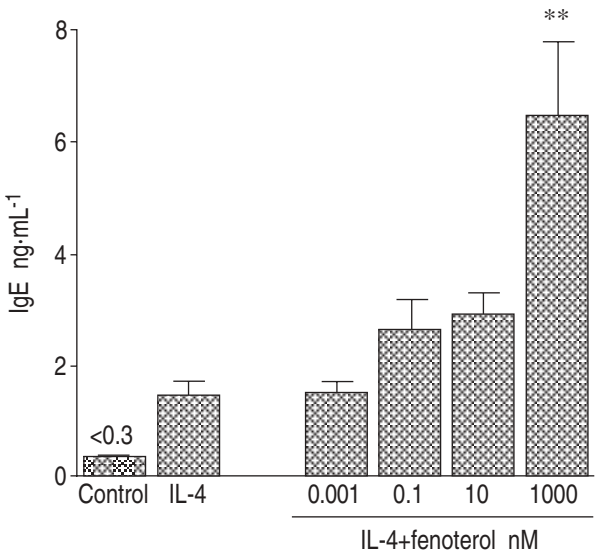

Fig. 1. – Effect of fenoterol on the IL-4-induced IgE production by human PBMC. PBMC (2×10^6 cells·mL^{-1}) obtained from healthy donors were added with IL-4 (30 U·mL^{-1}) and cultured for 13 days in the presence or absence of fenoterol (0.001–1000 nM). Cell-free supernatants were harvested and stored frozen prior to IgE determinations by ELISA (mean±SEM of three experiments). **: $p<0.01$, compared to IL-4-stimulated cells. IL-4: interleukin-4; IgE: immunoglobulin E; PBMC: peripheral blood mononuclear cells; ELISA: enzyme-linked immunosorbent assay.

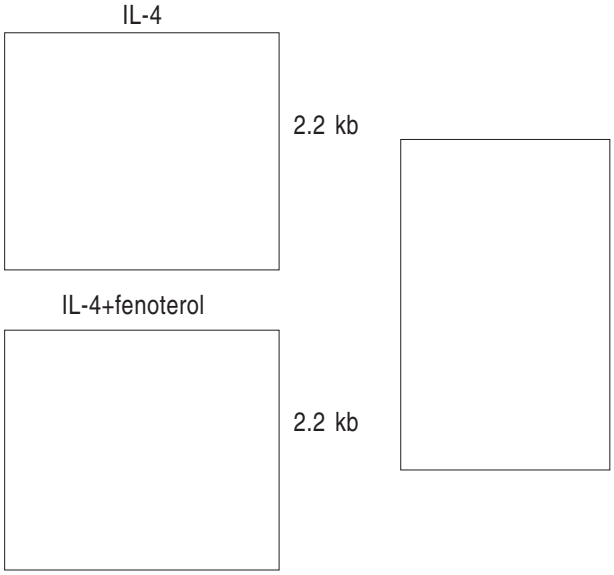

Fig. 2. – Fenoterol potentiated the expression of the IL4-induced IgE mRNA. PBMC were stimulated for 10 days with IL-4 (30 U·mL^{-1}) alone or with IL-4 and fenoterol (1 µM) together (IF). Total cellular RNA was isolated, size fractionated (20 µg·lane^{-1}) and blotted onto a nylon membrane. The membranes were hybridized with a ^{32}P-labelled RNA probe specific for IgE. The ethidium bromide staining pattern of the membrane from which the Northern Blot was made shows equivalence of RNA loading because IL-4 was found to significantly inhibit the expression of β-actin mRNA IF means RNA obtained from cells stimulated with IL-4 and fenoterol (1 µM). 28S and 18S are constant of sedimentation of ribosomal RNA. RNA: ribonucleic acid; mRNA: messenger RNA. For further abbreviations see legend to figure 1.

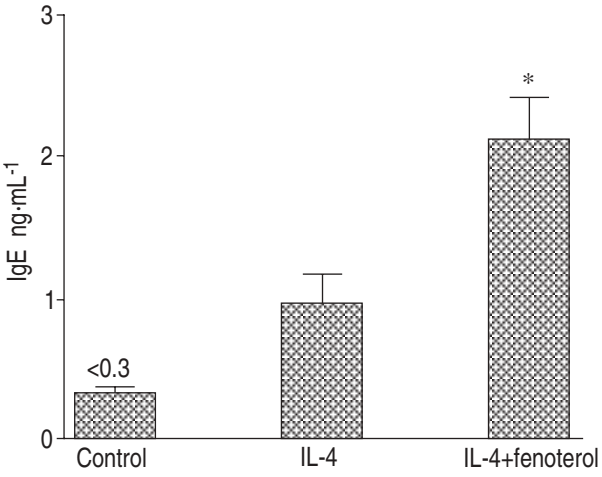

Fig. 3. – Fenoterol enhanced the IL-4-induced IgE production by purified B-cells. Purified B-lymphocytes (1×10^6 cells·mL^{-1}) were cultured with anti-CD40 MoAbs (1 mg·mL^{-1}) and IL-4 (30 U·mL^{-1}) alone or with IL-4 and fenoterol (1 μM) together. Supernatants were harvested after 13 days of culture and IgE concentrations were measured by specific ELISA. Results are expressed as mean±SEM of three different experiments. *: $p<0.05$ compared to IL-4-stimulated cells. For abbreviations see legend to figure 1.

Effect of fenoterol on purified B-lymphocytes

We also studied the effect of fenoterol on purified B-lymphocytes activated through their CD40 antigen. Fenoterol potentiated IgE production by purified B-cells stimulated with a combination of IL-4 and anti-CD40 MoAbs, but to a lesser extent as compared to the effect observed on PBMC. Compared to the effect of IL-4 added alone to the cell cultures, a two- to threefold significant increase ($p<0.05$) was observed (fig. 3). Additionally, results presented in table 1 indicate that fenoterol (1 μM) increased the number of viable B-lymphocytes recovered when B-cells were simulated by anti-CD40 in the presence of IL-4.

Effect of fenoterol on cAMP levels

We investigated whether the effect of fenoterol on PBMC was associated with increases in intracellular cAMP levels. No effect on cAMP production was observed when the cells were stimulated with IL-4 for 5 min (30 U·mL^{-1}). Fenoterol induced a nonsignificant decrease of cAMP production as compared to nonstimulated control cells (table 2). By contrast, when the cells were stimulated with IL-4 and fenoterol together (1 μM), a significant increase ($p<0.01$) in cAMP production was noted (table 2) as compared to IL-4-stimulated cells.

Table 1. – Effect of fenoterol on the recovery of viable B-lymphocytes activated by anti-CD40 antibodies and IL-4

	Viable cells $\times10^5$
Control	2.5±0.5
IL-4	3.5±1.2
IL-4+fenoterol	4.8±2.3

B-lymphocytes were seeded at 1×10^6 cells·well^{-1} in the presence of anti-CD40 MoAbs and with or without IL-4 (100 U·mL^{-1}). Cultures were performed in the presence or absence of 1 μM fenoterol. After 13 days of culture, viable B-lymphocytes were enumerated using the trypan blue dye exclusion method (mean±SEM of three experiments). MoAb: monoclonal antibody; IL-4: interleukin-4.

Table 2. – Effect of IL-4 and fenoterol on cAMP production (pmoles·10^{-6} cells) by PBMC

	None	IL-4 (30 U·mL^{-1})
Control	103±10	103±24
Fenoterol (1 μM)	52±12	413±48**

Cells (2×10^6 cells·mL^{-1}) were stimulated for 5 min with IL-4 (30 U·mL^{-1}) or fenoterol (1 μM) alone or with a combination of IL-4 and fenoterol. cAMP concentrations were then measured as described in the Materials and Methods section. Results are expressed as mean±SEM of four different experiment. **: $p<0.01$ compared to IL-4-stimulated cells. cAMP: cyclic adenosine monophosphate; PBMC: peripheral blood mononuclear cells; IL-4: interleukin-4.

Effect of cell permeable cAMP analogues on IgE production by PBMC

Because many of the effects of β_2-adrenoceptor agonists are thought to be mediated through increases in intracellular cAMP levels, the effect of db-cAMP (100 μM) and Sp-AMP (10 μM) were then examined in separate experiments. As shown in figure 4, both drugs (100 μM) significantly potentiated the effect of IL-4 on IgE production ($p<0.05$). The increases were 3–4 times higher with these compounds than with IL-4 alone.

Role of PKA in the modulation of IgE production by fenoterol

To determine whether the increase in IgE production is mediated through cAMP-dependent phosphorylation,

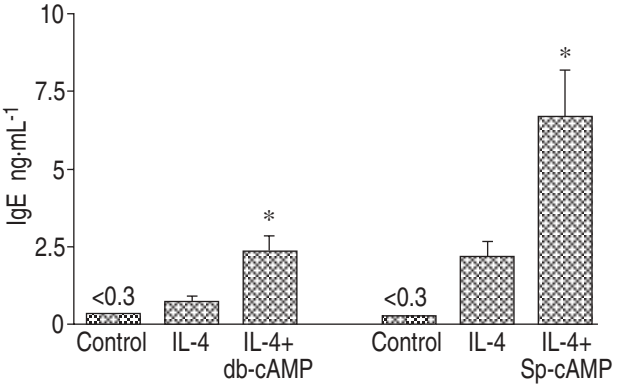

Fig. 4. – Effect of cell permeable cAMP analogues on IgE production by PBMC. PBMC were cultured with IL-4 (30 U·mL^{-1}) alone or IL-4 plus db-cAMP (100 μM) or IL-4 plus Sp-AMP (10 μM). Supernatants were harvested after 13 days of culture and IgE concentrations were measured by specific ELISA. Results are expressed as mean±SEM of three different experiments. *: $p<0.05$ compared to IL-4-stimulated cells. cAMP: cyclic adenosine monophosphate; db-cAMP: dibutyryl-cAMP. For further abbreviations see legend to figure 1.

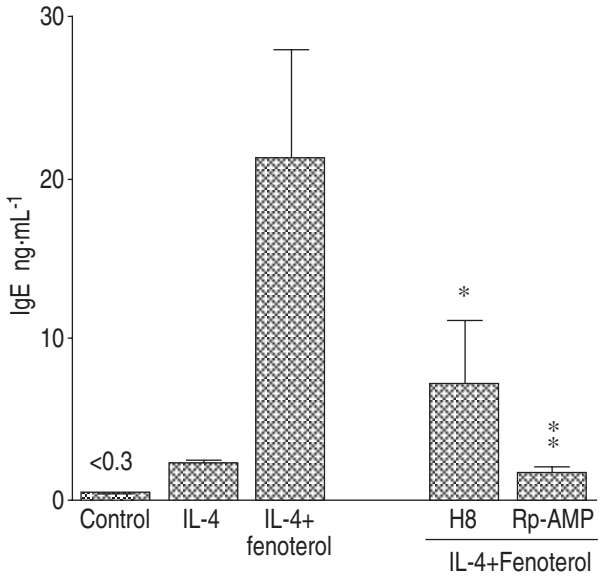

Fig. 5. – The potentiating effect of fenoterol on IgE production was partially reversed in the presence of PKA inhibitors. PBMC were preincubated for 30 min with H8 (10 μM) or Rp-AMP (10 μM). IL-4 (30 U·mL^{-1}) and fenoterol (1 μM) were then added alone or in combination and the cells were cultured for 13 days. Results are expressed as mean±SEM of three different experiments. *: $p<0.05$; **: $p<0.01$, compared to the results obtained in the presence of IL-4 and fenoterol. H8: N-5-isoquinolinesulphonamide. For further abbreviations see legend to figure 1.

inhibitors of PKA were used. H8 and Rp-AMP both compete for the adenosine triphosphate (ATP)-binding site of the catalytic subunit but do not alter the binding of cAMP to the regulatory subunits of PKA. Cells were pretreated for 30 min with H8 (10 μM) or Rp-AMP (10 μM) and were then stimulated with either IL-4 alone or with IL-4 and fenoterol together. No effect of the inhibitors was observed on the IL-4-induced IgE production (data not shown). By contrast, the potentiating effect of fenoterol on IgE production was significantly inhibited by H8 ($p<0.05$) and Rp-AMP ($p<0.01$) (fig. 5).

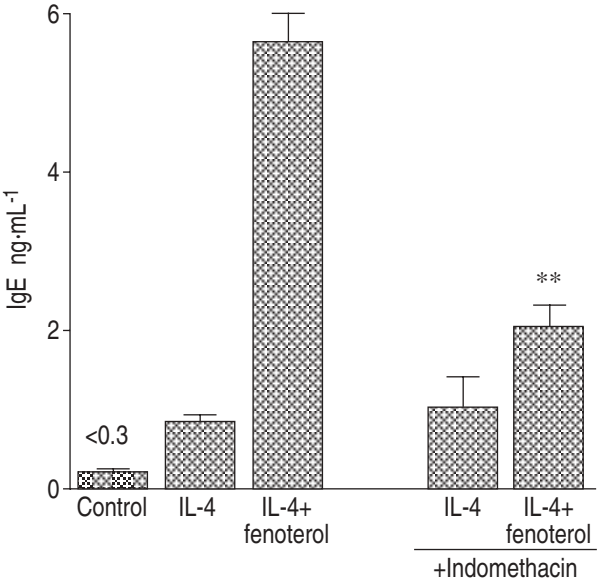

Fig. 6. – The potentiating effect of fenoterol on IgE production was partially reversed in the presence of indomethacin. PBMC were preincubated for 30 min with indomethacin (10 μM) alone or a combination of IL-4 (30 U·mL^{-1}) and fenoterol (1 μM). The cells were cultured for 13 days (results are expressed as mean±SEM of three different experiments. **: $p<0.01$, compared to the results obtained in the presence of IL-4 and fenoterol. For abbreviations see legend to figure 1.

Inhibition of the effect of fenoterol on IgE production by indomethacin

The effect of the other cAMP-elevating agents on IL-4-driven IgE production suggests that β_2-adrenoceptor agonists may mediate their effect through endogeneous release of prostaglandin E_2 (PGE_2) by monocytes. To test this hypothesis, PBMC were preincubated with indomethacin (10 μM) for 30 min and then stimulated with IL-4 and fenoterol. No modification was observed on the IL-4-induced IgE production (fig. 6). By contrast, indomethacin significantly inhibited ($p<0.01$) the potentiating effect of fenoterol on IgE production.

Discussion

The results presented in this study indicate that fenoterol and other cAMP analogues potentiate IL-4-induced IgE production by human PBMC. These results were confirmed by the use of another β_2-adrenoceptor agonist, salbutamol (data not shown). To a lesser extent, the same effect was observed on purified B-lymphocytes. The enhanced IgE production by PBMC seems to be mediated through an increase of the productive IgE mRNA expression. The transduction pathway for this potentiating effect depends on PKA activation, since it is blocked by inhibitors of this kinase. Moreover, the same effect was observed when a direct activator of PKA was used. Finally, the potentiating effect of β_2-adrenoceptor agonists is inhibited in the presence of indomethacin, suggesting that their activities might be mediated through a prostaglandin release.

The effect of β_2-adrenoceptor agonists on human IgE production seems to be related to cAMP pathway, since these drugs induce an increase in intracellular cAMP levels. In the present study, this was observed only in the presence of IL-4. Moreover, the cell permeable product db-cAMP, also potentiated the effect of IL-4 on IgE production. Thus, the potentiating effect of β_2-adrenoceptor agonists might be explained by an interaction of cAMP at an unknown stage of IL-4 signalling. This notion finds support in a recent study suggesting a cross-talk between the IL-4 signalling pathway and PKA activation during human B-cell proliferation [17]. Thus, the IL-4 and β_2-adrenergic signalling pathways may overlap one another after the activation of the kinase. This notion is supported in recent studies showing that IL-1 and cAMP induce immunoglobulin (Ig) light chain transcription by activating the nuclear factor-κB (NF-κB) protein [18].

Although previous studies have suggested a direct effect of cAMP on B-cell differentiation, we have no evidence which cells among B-cells, T-cells and monocytes are

implicated in the modulation of IgE production by cAMP. Fenoterol potentiates the IL-4-induced IgE production by purified B-lymphocytes, but this effect seems to be related to enhanced cell viability. Furthermore, the potentiating effect on IgE production is less pronounced on purified B-cells than on PBMC. Thus, the modulation of IgE production by β_2-adrenoceptor agonists is likely to be mediated through another cell type, most likely T-lymphocytes or monocytes. We have previously shown that the enhanced IgE production might be explained by the inhibition of interferon-γ (IFN-γ) production by PBMC [11]. The results presented in this study also suggest that the effect of β_2-adrenoceptor agonists might be explained by an enhanced release of PGE_2. This also suggests that the effect of fenoterol could be explained by the activation of monocytes. Thus, further experiments are required to indicate whether or not the effect of fenoterol could be explained by the release of prostaglandins. Interestingly, it has been reported that PGE_2 potentiates the IL-4-induced IgE production by murine B-lymphocytes [19].

Since the concentrations used in this study are physiologically relevant, the present results suggest that exposure to β_2-adrenoceptor agonists may profoundly influence IgE antibody production. In the presence of IL-4, this effect is associated with an increased intracellular cAMP level followed by an activation of PKA. Moreover, the present results suggest that the increased IgE production might be mediated by the activation of the cyclo-oxygenase pathway. Given the clinical importance of these drugs in the treatment of asthma, further studies are now underway to determine whether β_2-adrenoceptor agonists exhibit the same effects in asthmatic patients.

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