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PDE isoenzymes as targets for anti-asthma drugs

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ABSTRACT: Phosphodiesterase (PDE) isoenzyme profiles of human cell preparations and tissues have been analysed by a semiquantitative method using selective PDE inhibitors and activators. Neutrophils, eosinophils and monocytes contain PDE IV exclusively. Lymphocytes, alveolar macrophages and endothelial cells contain PDE IV and PDE III, and in addition, PDE I is measured in macrophages and PDE II in endothelial cells. These basal cell-specific PDE isoenzyme profiles appear to be modified by: 1) substrate concentration; 2) kinase-dependent phosphorylation; and 3) regulated rate of synthesis. Therefore, PDE isoenzyme profiles represent dynamic patterns, which apparently adapt to pathological and environmental conditions.

In parallel functional studies, the influence of mono-selective (rolipram, PDE IV; motapizone, PDE III), dual-selective (zardaverine) and non-selective (theophylline) PDE inhibitors were compared. Corresponding to isoenzyme analysis, it was demonstrated that both PDE III and PDE IV have to be inhibited for complete suppression of either tumour necrosis factor- α (TNF- α) release from macrophages, or lymphocyte proliferation (PDE III/IV cells). In eosinophils (PDE IV cells) platelet-activating factor (PAF)-induced chemotaxis or C5a-stimulated degranulation are only weakly inhibited by rolipram alone. After addition of a β_2 -agonist, however, the efficacy of rolipram is enhanced due to concomitant influence of synthesis and breakdown of cyclic adenosine monophosphate (cAMP). Theophylline inhibits PDE isoenzyme activities and functions of inflammatory cells with similar potency, and exhibits higher functional efficacy as compared to rolipram.

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Tissue-specific isoenzyme profiles

Phosphodiesterase (PDE) inhibitors possess the potential to interfere with functions of nearly every cell type present in airway tissue, demonstrating that cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) are involved in the regulation of cellular activation. Studies with selective inhibitors indicate that inflammatory cells, immune cells or smooth muscle cells react differently to selective PDE inhibitors and should, therefore, be differently equipped with PDE isoenzymes I–V. In order to understand the action of selective inhibitors and, furthermore, to optimize and target new anti-inflammatory or immunomodulatory PDE inhibitors, a method based on measurement of PDE activity was developed which makes it possible to quantitate PDE isoenzymes in tissue homogenates. An overview of the tissue-specific PDE isoenzyme equipment of cells which participate in airway inflammation will be presented and compared to functional inhibition by selective and non-selective PDE inhibitors of the respective cells.

The semiquantitative evaluation of the isoenzyme patterns uses defined concentrations of selective inhibitors

for PDE III (motapizone, 1 μ M), PDE IV (rolipram, 10 μ M) or PDE V (zaprinast, 10 μ M), which at these concentrations completely inhibit the corresponding isoenzyme without grossly affecting others [1]. In the presence of the combination of motapizone and rolipram, the activities of PDE I or PDE II are quantified by addition of either Ca/calmodulin or cyclic guanosine monophosphate (cGMP). This method (for details see [2]) evaluates absolute activities of the various isoenzymes at the standard substrate concentration of 0.5 μ M cAMP or cGMP, and describes the activity ratios between individual isoenzymes under these conditions.

The isoenzyme profiles of macrophages [3] and lymphocytes [4] isolated from bronchoalveolar lavage (BAL) and peripheral blood, respectively, are summarized in figure 1. Macrophages contain large amounts of PDE I, equivalent activities of PDE III and IV, and smaller amounts of PDE V. In T-lymphocytes, which were carefully depleted of platelets, the prominent isoenzyme is PDE IV. PDE III is also present and minor activities of PDE I and PDE V were found. The ratio PDE IV/PDE III was about 1 in macrophages and 2.5 in lymphocytes. In neither cell type PDE II was detected in considerable

Fig. 1. – PDE isoenzyme profiles of: a) human alveolar macrophages; and b) peripheral blood CD4⁺ T-lymphocytes. Soluble (□) and particulate (▨) fractions were prepared from the isolated cells by sonication and centrifugation, as described in [2]. The fractional inhibition or activation of the hydrolysis of 0.5 μ M cAMP or 0.5 μ M cGMP in both fractions by selective inhibitors or activators (10 μ M rolipram (PDE IV); 1 μ M motapizone (PDE III); 10 μ M zaprinast (PDE V); 5 μ M cGMP (PDE II); 250 μ M Ca²⁺ + 150 nM calmodulin (PDE I)) was determined, and PDE isoenzyme activity was calculated as the difference between PDE activity in the presence and absence of these additives. Data are given as mean $\pm$ SEM from six experiments. PDE: phosphodiesterase; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate.

amounts. With regard to the subcellular distribution, it is apparent that PDE III is predominantly membrane-bound, whereas PDE IV is found in the cytosolic compartment.

Monocytes, eosinophils and neutrophils isolated from human blood contain PDE IV almost exclusively, which is located predominantly in the cytosolic compartment (fig. 2). These data are derived from pure cells which were prepared by an immunomagnetic method [5], and by elutriation in the case of monocytes [3].

PDE isoenzyme profiles of several cell types isolated from human tissues are summarized in table 1. Three characteristic groups are obvious: 1) "PDE IV cells" represented by neutrophils, eosinophils, monocytes and epithelial cells [2, 5, 6]; 2) "PDE III/IV cells" represented by endothelial cells, macrophages and T-lymphocytes [1, 3, 4]. Endothelial cells in addition contain PDE II [1] and macrophages PDE I and PDE V [3]; and 3) "PDE III/V cells" are typically represented by platelets [7], and vascular smooth muscle cells [8].

Regulation of PDE isoenzyme activity

These PDE isoenzyme profiles make it possible to compare the different cyclic nucleotide hydrolysing

Fig. 2. – Phosphodiesterase (PDE) isoenzymes of white blood cells. a) Polymorphonuclear cells (PMNs) from human peripheral venous blood were separated by centrifugation on Ficoll. b) Eosinophils were obtained by staining PMNs with anti-CD16 MACS microbeads and separated from neutrophils in a magnetic field using the MACS procedure (purity 99.9%) [5]. c) Monocytes were isolated by Percoll density separation and further purified by countercurrent centrifugal elutriation [3]. Cells were homogenized (Branson sonifier) and PDE isoenzyme patterns were determined at 0.5 μ M cyclic nucleotide substrate concentration in the soluble (□) and particulate (▨) fractions. Data presented are the mean $\pm$ SEM from three (PMNs), and six (eosinophils) and three (monocytes) experiments.

capacities of various cells at basal conditions. Under the influence of mediators, such as hormones or cytokines, these activities may change to a certain degree and with typical time courses:

1). PDE III activity may be enhanced due to phosphorylation by protein kinase A (PKA), protein kinase C (PKC) or insulin-dependent serine kinase (ISK) [9–11]. These changes in activity represent short-term regulation within minutes.

2). PDE IV may be upregulated by *de novo* synthesis, as shown in a monocyte and a keratinocyte cell line [12, 13]. This increased activity is triggered by enhanced cAMP and PKA levels and occurs within hours.

Table 1. — PDE isoenzyme profiles of various human cell types

	I	II	PDE III	IV	V
Neutrophils	-	-	-	+++	-
Eosinophils	-	-	-	+++	-
Monocytes	(+)	-	(+)	++	-
Macrophages (BAL)	++++	-	++++	+++	++
T-lymphocytes	-	-	+	++	-
Endothelial cell (HUVEC)	+	++++	+++	+++	-
Epithelial cell (primary culture)	++	(+)	(+)	++	(+)
Platelets	(+)	+	++	(+)	+++
Bronchi	+	++	++	++	++
Arteria pulmonalis	++	-	+++	++	+++

PDE activity (pmol·min⁻¹·mg protein⁻¹): (+): 1–3; ++: 4–10; +++: 10–30; ++++: 40–70; +++++: >80. PDE: phosphodiesterase; BAL: bronchoalveolar lavage; HUVEC: human umbilical vein endothelial cells.

3). Due to individual K_m values of PDE III and PDE IV (0.3 and 2 μ M cAMP, respectively) their activities may change in the presence of different substrate concentrations *in vitro* as well as *in situ*.

4). In the allergic state, an increased PDE IV activity was found in monocytes [14], which may be related to the modified activation threshold of these cells in the atopic organism.

Functional inhibition of inflammatory cells

Lipopolysaccharide (LPS)-induced release of tumour necrosis factor- α (TNF- α) from BAL macrophages was partially reduced by mono-selective inhibitors, such as rolipram or motapizone (fig. 3) [15]. However, when both drugs were added in combination, complete suppression of TNF- α release was observed. Analogous

Fig. 3. — Inhibition of LPS-induced TNF- α release from human alveolar macrophages. Macrophages adhered to microtitre plates (2×10^5 cells·well⁻¹) during 90 min and were then incubated for 18 h in the presence of LPS ($1 \mu\text{g} \cdot \text{mL}^{-1}$) at the given substance concentrations. TNF- α was determined in the supernatant by an immunoradiometric assay, and the percentage of inhibition was calculated ($n=8$; mean $\pm$ SEM). LPS: lipopolysaccharide; TNF- α : tumour necrosis factor- α . ■: motapizone + rolipram (1:1); □: rolipram; ▼: motapizone.

monophasic inhibition curves resulted from treatment with dual-selective PDE inhibitors, such as zardaverine and tolafentrine or non-selective theophylline and pentoxifylline. The median inhibitory concentration (IC₅₀) values for inhibition of TNF- α release by various PDE inhibitors are summarized in table 2. These observations are in accordance with the isoenzyme profile of macrophages, suggesting that inhibition of either PDE III or PDE IV results in a partial attenuation of total cAMP-hydrolysing capacity. Only if both isoenzymes are simultaneously inhibited will intracellular cAMP concentrations and PKA activity be sufficiently elevated for a complete inhibition of TNF- α release.

Similar effects of PDE inhibitors on T-lymphocyte proliferation were observed [15]. Preparations of peripheral blood monocytes were stimulated with anti-CD3 antibody BMA031 and proliferation was quantitated after 72 h by ³H-thymidine incorporation. Partial inhibition was obtained by rolipram and motapizone, whereas in the presence of the combination motapizone/rolipram (1:1) monophasic inhibition curves and complete suppression of T-lymphocyte proliferation were observed. Correspondingly, 100% suppression was also obtained with dual-selective compounds, zardaverine and tolafentrine, and the respective IC₅₀ values are summarized in table 2. These data suggest that in "PDE III/IV cells" both isoenzyme activities have to be inhibited for complete suppression of cellular functions - at least under the stimulating conditions given.

Theophylline appears to inhibit both TNF- α release and lymphocyte proliferation with high efficacy but low potency, mirrored by IC₅₀ values in the range 100–600 μ M (table 2). These high IC₅₀ values seem to be typical for *in vitro* experiments, where cell preparations are studied in the absence of their tissue environment. If those mediators which are present under *in vivo*

Table 2. — Influence of PDE inhibitors on human alveolar macrophages and T-lymphocytes

	IC ₅₀ μ M	
	TNF- α release	Lymphocyte proliferation
Rolipram + motapizone	0.46 $\pm$ 0.12	0.08 $\pm$ 0.03
Zardaverine	1.0 $\pm$ 0.3	1.3 $\pm$ 0.3
Tolafentrine	4.7 $\pm$ 1.3	1.5 $\pm$ 0.2
Pentoxifylline	403 $\pm$ 70	ND
Theophylline	553 $\pm$ 97	112 $\pm$ 21

TNF- α release from LPS-stimulated human alveolar macrophages was determined after 18 h incubation in the presence of various concentrations of the given drugs. Human peripheral blood mononuclear cells were isolated and purified according to standard procedures [16], and incubated (4×10^5 cells·well⁻¹) for 44 h in the presence of anti-TCR/CD3 monoclonal antibody (BMA031, $10 \mu\text{g} \cdot \text{mL}^{-1}$). ³H-thymidine ($0.5 \mu\text{Ci} \cdot \text{mL}^{-1}$) incorporation was measured for 4 h and inhibition of proliferation by PDE inhibitors was determined. IC₅₀ values were calculated from the concentration-inhibition curves by nonlinear regression analysis. Data are presented as mean $\pm$ SEM, ($n=5-12$). PDE: phosphodiesterase; IC₅₀: median inhibitory concentration; TNF- α : tumour necrosis factor- α ; LPS: lipopolysaccharide; TCR: T-cell receptor; ND: not determined.

conditions, such as adenylate cyclase-activating adrenaline, prostaglandin E₂ (PGE₂) or adenosine, are also added *in vitro*, lower IC₅₀ values should be expected. The simultaneous acceleration of cAMP generation by these mediators and inhibition of cAMP breakdown by PDE inhibitors evoke a synergistic effect, which in the case of theophylline results in an enhanced sensitivity (and lower IC₅₀) values towards this drug. This synergism will be demonstrated and discussed here as an example of stimulated eosinophil cationic protein (ECP) release from human eosinophils.

Eosinophils contain PDE IV exclusively and it might be expected that their activation should be efficiently reduced by PDE IV inhibitors. Therefore, the effect of various PDE inhibitors on eosinophil chemotaxis stimulated either by platelet-activating factor (PAF) or interleukin-5 (IL-5) was studied [16]. Interaction of rolipram with PAF-induced chemotaxis surprisingly demonstrated flat inhibition curves, resulting in IC₅₀ values in the range of 1 μ M, and only 60% inhibition at 10 μ M rolipram (fig. 4). In contrast, theophylline exhibited total inhibition at 1 mM, and an IC₅₀ value of approximately 30 μ M (corresponding to 5.4 μ g·mL⁻¹) was calculated from these data. No difference between cells from normal and atopic donors was obtained for PAF-induced chemotaxis.

In further experiments, 100 nM IL-5 was used as a chemoattractant, and the effects of rolipram and theophylline were assessed in eosinophils from normal and atopic patients (fig. 5) [17]. Under these conditions, rolipram did not inhibit eosinophil migration. In the presence of 1 mM theophylline, however, chemotaxis was totally suppressed and an IC₅₀ of about 30 μ M was observed with normal cells. With cells from atopic donors, the IC₅₀ value was shifted to 150 μ M, suggesting that the sensitivity of the intracellular signalling pathway for antagonism *via* cAMP-related suppression was downregulated in the atopic state.

Fig. 4. — Inhibition of PAF-induced chemotaxis of human eosinophils. Eosinophils were isolated from blood of normal and atopic donors by dextran sedimentation, separated from mononuclear cells on Percoll and purified by an immunomagnetic method (MACS). Chemotactic responses to PAF (10^{-7} M) were determined using 48-well micro Boyden chambers with 8 μ m pore size PVP-free polycarbonate filters. Drug effects are given as percentage inhibition calculated from six experiments (mean $\pm$ SEM). *: $p < 0.05$. PAF: platelet-activating factor; PVP: polyvinylpyrrolidone. $\circ$: rolipram, normal; $\bullet$: rolipram, atopic; Δ : theophylline, normal; $\blacktriangle$: theophylline, atopic.

Fig. 5. — Inhibition of IL-5-induced chemotaxis of human eosinophils. Chemotactic responses to IL-5 (100 mg·mL⁻¹) were determined as described in legend of figure 4. Drug effects are given as percentage inhibition calculated from six experiments (mean $\pm$ SEM). *: $p < 0.05$. IL-5: interleukin-5. $\circ$: rolipram, normal; $\bullet$: rolipram, atopic; Δ : theophylline, normal; $\blacktriangle$: theophylline, atopic.

In parallel studies with eosinophils, C5a-stimulated release of reactive oxygen species (ROS) and ECP from eosinophils was investigated [5]. In accordance with the chemotaxis studies, rolipram alone did not inhibit ROS or ECP release to a substantial degree (fig. 6). In the presence of salbutamol, however, an inhibitory effect of rolipram was observed. Again, theophylline on its own evoked high efficacy. In the presence of salbutamol, the concentration-response curve for theophylline was shifted to lower concentrations and IC₅₀ values in the range of 100 μ M were calculated. These data demonstrate that for mono-selective drugs, such as rolipram, the simultaneous presence of adenylate cyclase stimulating agents is essential for a sufficient enhancement of cAMP and PKA activity. In contrast, theophylline on its own exhibits higher efficacy. At least in the presence of salbutamol, theophylline inhibits cellular functions in the range of its therapeutic plasma concentrations (30–100 μ M corresponding to 5.4–18 μ g·mL⁻¹).

Fig. 6. — Inhibition of ECP release from C5a-stimulated human eosinophils. Eosinophils from normal donors were isolated by the MACS method and 10^6 cells·mL⁻¹ were stimulated by 100 nM C5a in the presence of the given inhibitor concentrations. After 30 min, cells were sedimented and ECP was determined in the supernatants by RIA. Drug effects are given as percentage inhibition calculated from four experiments (mean $\pm$ SD). ECP: eosinophil cationic protein; RIA: radioimmunoassay. $\circ$: rolipram; $\bullet$: rolipram + salbutamol; Δ : theophylline; $\blacktriangle$: theophylline + salbutamol.

The characteristically higher efficacy of theophylline in comparison to mono-selective rolipram may be due to a mechanistic target additive to PDE inhibition. The previously discussed interaction of xanthines with the inhibitory regulatory protein, Gi [18], which should result in enhanced adenylate cyclase activity, might be one possible explanation. Both inhibition of PDE, which apparently represents the predominant theophylline mechanism, and the speculative Gi interaction would be in agreement with the observation that theophylline effects can be reversed by downstream inhibition, using an inhibitor of PKA [18].

In conclusion: 1) the different cell types in the airways are characterized by cell-specific PDE isoenzyme profiles; 2) PDE isoenzyme activities *in situ* are regulated by several mechanisms; 3) the inhibition of cellular functions by selective PDE inhibitors is in parallel with the specific PDE isoenzyme profile; and 4) theophylline inhibits various functions with higher efficacy as compared to mono-selective PDE inhibitors.

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