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Effects of solubilization and vanadate/glutathione complex on inhibitor potencies against eosinophil cyclic AMP-specific phosphodiesterase

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Treatment of membranes from guinea-pig peritoneal eosinophils with deoxycholate and NaCl solubilized >95% of the particulate cyclic AMP-specific phosphodiesterase (PDE IV). Solubilized PDE IV was at least 10 times more potently inhibited by selective PDE IV inhibitors (e.g. rolipram, denbufylline) than bound enzyme. Vanadate/glutathione complex (V/GSH) activated membrane-bound PDE IV and also increased potencies of these same inhibitors by at least 10-fold. Neither solubilization nor V/GSH markedly influenced the inhibitory activities of non-selective inhibitors (e.g. trequinsin, dipyridamole). Inhibitor effects on solubilized PDE IV and cyclic AMP accumulation in intact cells were strongly correlated. These results suggest a biologically important site on eosinophil PDE IV which is concealed or partially concealed in freshly prepared membranes and is exposed by solubilization or V/GSH.

Cyclic AMP; Phosphodiesterase; Eosinophil; Inhibitor

1. INTRODUCTION

Cyclic AMP-specific phosphodiesterase (PDE IV) is present in many inflammatory cells [1]. In guinea-pig eosinophils, PDE IV is the predominant and, perhaps, only PDE isozyme present with greater than 92% of the activity being tightly membrane-bound [2]. Inhibitors of PDE IV, alone, potently inhibit eosinophil superoxide ($O^{\cdot 2-}$) generation [2,3]. This effect occurs with a minimal elevation of intracellular cyclic AMP levels, although the order of potency of several PDE inhibitors in reducing $O^{\cdot 2-}$ generation correlates well with their effectiveness in increasing cyclic AMP accumulation when adenylate cyclase is stimulated with a β-agonist [2]. Surprisingly, inhibitor effects on membrane-bound cyclic AMP PDE and whole cell function (cyclic AMP/ $O^{\cdot 2-}$) are poorly correlated [2]. For example, trequinsin is equipotent with rolipram and denbufylline in inhibiting the particulate PDE IV but is at least 100-fold less effective than the latter two compounds in elevating cyclic AMP and reducing $O^{\cdot 2-}$ generation. It was proposed that the entry of trequinsin into eosinophils is, somehow, hindered [2]; however, this explanation is difficult to reconcile with the fact that this compound is a very potent effector of other cell types [4].

We have undertaken further studies on eosinophil PDE IV and demonstrated dissimilar inhibitory actions of rolipram and trequinsin indicating different sites of action which may explain the aforementioned anomolies.

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2. MATERIALS AND METHODS

2.1. *Materials*

Cyclic [2,8-^{3}H]AMP (41 Ci/mmol) and [8-^{3}H]GMP (13.8 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., UK). Rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrolidone] and AH-21-132 [(±)-*cis*-6-(*p*-acetamidophenyl)-1,2,3,4,4a,10b-hexahydro-8,9-dimethoxy-2-methylbenzo-{c}{1,6}naphthyridine] were synthesized by the Department of Discovery Chemistry, Rhône-Poulenc Rorer Ltd. (Dagenham, Essex, UK). Denbufylline (BRL 30892; 1,3-di-*n*-butyl-7-[2'-oxopropyl]-xanthine) was a gift from Beecham Pharmaceuticals (Epsom, Surrey, UK). Trequinsin (HL-725; 9,10-dimethoxy-2-mesitylimino-3-methyl-3,4,6,7-tetrahydro-2*H*-pyrimido(6,1-a]isoquinolin-4-one) was supplied by Hoechst Pharmaceuticals (Hounslow, Middx., UK). Ro-20-1724 [1-4-(3-butoxy-4-methoxybenzyl)-2-imidozolidinone) was obtained from Roche Products Ltd. (Welwyn Garden City, UK). The cyclic AMP radioimmunoassay kit was purchased from NEN Chemicals GmbH. All other chemicals were obtained from Sigma Chemical Co., BDH Chemicals (both of Poole, Dorset, UK) and Rhône-Poulenc Ltd. (Eccles, Manchester, UK). Male Dunkin Hartley guinea-pigs were purchased from a local supplier.

2.2. *Preparation of guinea-pig eosinophils and preparation of subcellular fractions*

Guinea-pig peritoneal eosinophils were purified and subcellular fractions prepared according to [2].

2.3. *Solubilization and partial purification of cyclic AMP PDE*

The membrane-bound cyclic AMP PDE was solubilized by homogenizing freshly prepared membranes with a Dounce homogenizer (10 strokes) in 4 ml of homogenization buffer containing deoxycholate (DOC) (0.5%) and NaCl (100 mM). The homogenate was centrifuged at 100,000 × *g* for 30 min and the supernatant containing the solubilized activity removed and the pellet resuspended in an equal volume of homogenization buffer.

For partial purification, 1 ml of solubilized cyclic AMP PDE activity containing 2–4 mg protein was applied to a PD-10 desalting column (Pharmacia). The eluate was applied to a DEAE-trisacryl column (0.7 × 1 cm) pre-equilibrated with column buffer (20 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM *p*-tosyl-L-lycine-chloromethyl ketone, pH 7.5). The column was washed with 10 ml of column buffer and PDE activities eluted with a linear gradient of NaCl (0–0.7 M, 14 ml) in column buffer. The flow rate was 1 ml·min^{-1} and 1 ml fractions were collected.

2.4. *Measurement of PDE activity*

PDE activity was determined according to [5]. The IC_{50} values (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds examined were determined from concentration–response curves in which concentrations ranged from 0.6 nM to 1 mM. At least three concentration–response curves were generated for each agent. For the determination of V_{max} and K_m values, the concentration of cyclic AMP was varied while the amount of ^{3}H-labelled cyclic AMP remained constant. The data were evaluated by an iterative procedure (computer program written by Dr. M. Vlitos, Computer Department, Rhône-Poulenc Rorer Ltd.) for analysis of complex kinetics [6].

Protein was determined [7] with bovine serum albumin as the standard. Vanadate/glutathione complex (V/GSH) was prepared and added to enzyme assays according to [8]. The nomenclature for cyclic nucleotide PDE adopted in this paper is based on [9].

2.5. *Measurement of eosinophil cyclic AMP*

Cell incubations and measurement of cyclic AMP was as described [2]. The EC_{50} values (concentration which produced 50% of maximal cyclic AMP response) for the compounds examined were determined from concentration–response curves in which concentrations ranged from 0.032 μM to 500 μM.

3. RESULTS

3.1. *Solubilization of eosinophil particulate PDE IV*

Gentle homogenization (Dounce) of eosinophil membranes in buffer containing DOC (0.5%) (critical micelle concentration of DOC = 0.21%) and NaCl (100 mM) solubilized almost all (>95%) the cyclic AMP PDE activity. This treatment released less than 50% of the particulate proteins. Solubilized PDE IV eluted as a single peak from DEAE-trisacryl at a NaCl concentration of 300 mM (Fig. 1.).

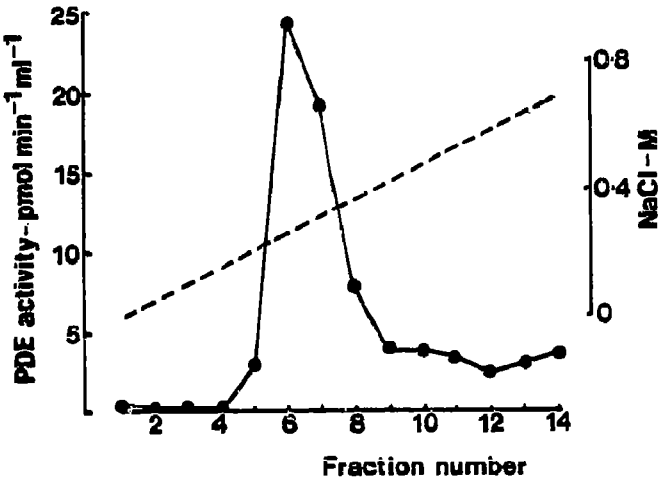

Fig. 1. DEAE-trisacryl chromatography of solubilized cyclic AMP PDE activity. Fractions were assayed for cyclic AMP PDE activity (1 μM substrate) in the presence of 200 μM EGTA.

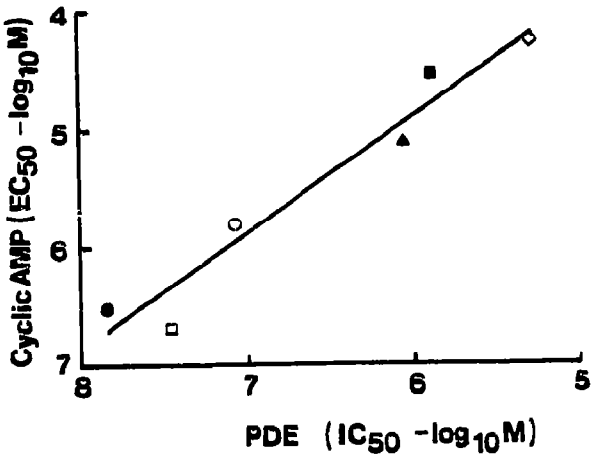

Fig. 2. Stimulation of cyclic AMP accumulation in intact eosinophils as a function of inhibition of solubilized PDE IV. Eosinophil cyclic AMP data are expressed as EC_{50} values ($-\log_{10}$M) and cyclic AMP PDE data (see Table I) as IC_{50} values ($-\log_{10}$M). Cyclic AMP and PDE studies were performed on different preparations of cells. Each point is the mean of at least 3 determinations. Regression analysis (Fig P computer program BIOSOFT) demonstrated that stimulation of cyclic AMP accumulation as a function of PDE IV inhibitory activity is highly significant (r=0.97, P<0.001, n=6). The symbols represent rolipram (●), denbufylline (□), Ro-20-1724 (○), trequinsin (▲), AH-21-132 (■) and IBMX (◇).

3.2. *Inhibitor potency against solubilized PDE IV*

Rolipram, denbufylline and Ro-20-1724 potently inhibited solubilized PDE IV (Table I). The inhibitory potencies of these compounds were at least 10-fold greater than against the bound, particulate enzyme. In contrast, a number of other, non-selective PDE inhibitors (trequinsin, AH-21-132, dipyridamole, 3-isobutyl-1-methyl xanthine (IBMX)) exhibited similar inhibitory potencies against the bound and solubilized eosinophil PDE IV (Table I). Regression analysis of the relationship between the inhibitory potencies (IC_{50}'s μM) of

Table I

Inhibitor potencies against bound and solubilized particulate PDE IV from eosinophils. Eosinophil bound and solubilized PDE IVs were measured with 1 μM substrate

Inhibitor	IC_{50} (μM) Eosinophil PDE IV	
	Bound	Solubilized
Rolipram	0.23 ± 0.02 (n=10)	0.014 ± 0.003 (n=9)
Dipyridamole	8.1 ± 1.4 (n= 8)	5.0 ± 0.58 (n=3)
Trequinsin	0.36 ± 0.08 (n= 9)	0.89 ± 0.20 (n=8)
AH-21-132	3.4 ± 1.2 (n= 4)	1.3 ± 0.31 (n=3)
Denbufylline	0.36 ± 0.08 (n= 6)	0.035 ± 0.009 (n=4)
Ro-20-1724	0.92 ± 0.35 (n= 3)	0.083 ± 0.023 (n=3)
IBMX	4.8 ± 1.6 (n= 3)	5.3 ± 2.3 (n=3)

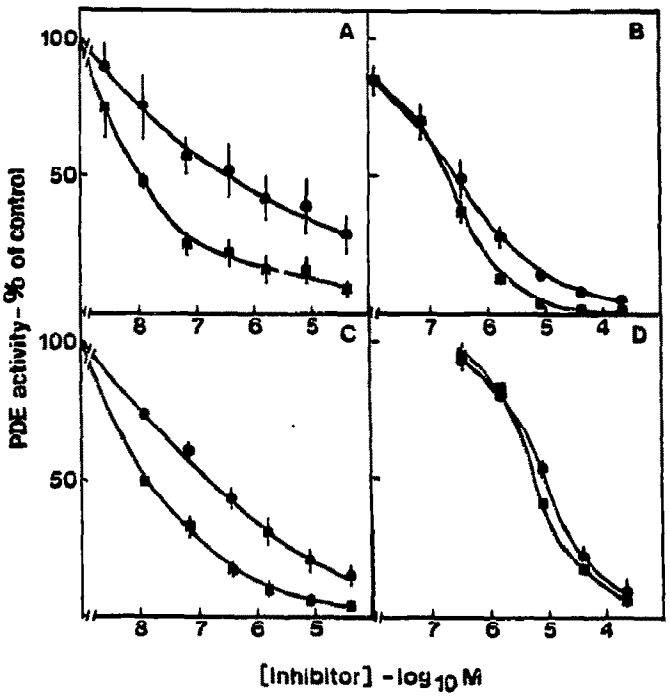

Fig. 3. Inhibition of bound, particulate cyclic AMP PDE by rolipram, denbufylline, trequinsin and dipyridamole in the presence and absence of V/GSH. PDE activity assayed in the presence of the indicated concentrations of rolipram (panel A), trequinsin (panel B), denbufylline (panel C) and dipyridamole (panel D) with (■) and without (●) V/GSH. The results represent the means ± S.E.M. of between 3 and 5 experiments.

inhibitors against solubilized PDE IV and their efficacies in elevating cyclic AMP levels in intact cells (EC_{50}'s μM) indicated a very strong (r=0.97, P<0.001, n=6) correlation (Fig. 2.). Inhibitor effects on bound, particulate PDE IV and cyclic AMP accumulation were relatively weakly correlated (r=0.63, P=0.18, n=6).

3.3. *Stimulation of PDE IV by vanadate/glutathione complex*

Exposure of eosinophil membranes to Na_3VO_4 (1.4 mM) and GSH (2.8 mM) (V/GSH), which has previously been shown to activate membrane-bound, hormone-sensitive PDE III in adipocytes and hepatocytes [8,10], caused a large, reversible stimulation of cyclic AMP PDE activity. Neither Na_3VO_4 nor GSH, when added alone, influenced activity. As reported previously [2], the bound particulate eosinophil PDE exhibited complex kinetics. Analysis of the data [6] revealed K_m values of 1 μM and 40 μM for the high- and low-affinity components, respectively. V/GSH did not greatly influence the K_m values (3 μM and 48 μM) but the V_{max} values were greatly increased (0.05 and 0.4 nmol·min^{-1}·ml^{-1}, without V/GSH; 0.4 and 2 nmol·min^{-1}·ml^{-1}, with V/GSH). The V/GSH-induced stimulation of PDE IV activity was only observed in membranes prepared from freshly isolated eosinophils; the bound, particulate PDE in membranes from cells stored frozen (−70°C) for 1 day or longer was inhibited by V/GSH.

Solubilized PDE IV also exhibited non-linear kinetics, but the affinity of the enzyme for substrate (K_ms=22 μM and 101 μM) was reduced in comparison to the bound, particulate enzyme while the V_{max} values (0.3 and 3 nmol·min^{-1}·ml^{-1}) were greatly increased. V/GSH exerted a much smaller effect on the solubilized PDE compared to the bound, particulate activity. Substrate affinities (K_ms=13 μM and 84 μM) and V_{max} values (0.4 and 4 nmol·min^{-1}·ml^{-1}) were marginally increased by V/GSH. V/GSH had no effect on partially purified solubilized PDE IV.

3.4. *Effect of V/GSH on inhibitor potency*

Exposure of eosinophil membranes to V/GSH increased the inhibitory potencies of rolipram (IC_{50}'s=0.24 ± 0.12 μM, n=5, without V/GSH; 0.02 ± 0.01 μM, n=5 with V/GSH) and denbufylline (IC_{50}'s=0.29 ± 0.16 μM, n=3, without V/GSH; 0.02 ± 0.01 μM, n=3, with V/GSH) by approximately 10-fold. In contrast V/GSH only slightly increased the potency of trequinsin (IC_{50}'s=0.32 ± 0.09 μM, n=5, without V/GSH; 0.2 ± 0.05 μM, n=5, with V/GSH) and the IC_{50} value of dipyridamole (8 ± 1.2 μM, n=3) was unaffected (Fig. 3). In contrast to the results obtained on the bound enzyme, V/GSH had no effect on the inhibitory potency of rolipram on solubilized PDE IV. Trequinsin also exhibited similar inhibitory potencies against solubilized PDE IV in the absence and presence of V/GSH (data not shown).

4. DISCUSSION

In view of the evidence suggesting only one cyclic AMP PDE (ref [2], Fig. 1), a possible explanation for differences in the inhibitory actions of selective PDE IV inhibitors and non-selective inhibitors requires invoking the existence of two sites on the enzyme. The rolipram group of compounds, at high concentrations, and the trequinsin group both interact at a site (perhaps the catalytic site) which is designated Sc. Rolipram and other PDE IV inhibitors, as well as acting at Sc, can also potently interact at a second site which is designated Sr. In freshly prepared membranes, rolipram and trequinsin would act predominantly at Sc with Sr being inaccessible or only partially accessible. V/GSH, acting indirectly, by inducing a conformational change in the bound, particulate enzyme, would expose Sr so increasing the potencies of selective PDE IV inhibitors. Solubilization would also expose Sr explaining why V/GSH did not influence rolipram potency on liberated activity.

The poor correlation between intracellular cyclic AMP accumulation and inhibitor actions on untreated membrane-bound PDE IV indicates, perhaps, that the enzyme is not maintained in its native form following

cell disruption. In contrast, a very strong correlation was demonstrated between IC_{50} values of inhibitors against solubilized PDE IV and their potencies in elevating intracellular cyclic AMP implying that, in intact eosinophils, cyclic AMP PDE exists in a form similar to the solubilized or V/GSH-treated enzyme. Furthermore, it suggests the potential importance of the putative Sr in regulating eosinophil function.

Finally, several studies [11–13] indicate that the biological actions of rolipram result from a relatively weak, competitive inhibition of PDE IV. Our results suggest that a reappraisal of this view is warranted.

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