ELSEVIER

Biochemical Pharmacology 61 (2001) 1551–1559

Biochemical Pharmacology

Involvement of A_3 receptors in the potentiation by adenosine of the inhibitory effect of theophylline on human eosinophil degranulation: possible novel mechanism of the anti-inflammatory action of theophylline

Charles I. Ezeamuzie*

Department of Pharmacology and Toxicology, Faculty of Medicine, Kuwait University, Safat 13110, Kuwait

Received 7 August 2000; accepted 28 September 2000

Abstract

The current use of theophylline in asthma is based on both the bronchodilatory and the anti-inflammatory effects. The exact mechanism of these actions is still controversial and may include the inhibition of adenosine 3′,5′-monophosphate phosphodiesterase enzyme (PDE) and antagonism of adenosine receptors. In this study, the mechanism of the anti-inflammatory action was investigated by studying the inhibition by theophylline of complement C5a (C5a)-induced degranulation of human eosinophils and its interaction with adenosine. Theophylline (10–1000 μM) inhibited C5a-induced release of eosinophil peroxidase (EPO) in a concentration-dependent manner with an IC_{50} of 233.5 μM and a maximal inhibition of 90.3 $\pm$ 3.0%. In contrast, the PDE4 inhibitor rolipram (up to 50 μM) had no effect. The adenosine A_3 receptor agonist N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine (IB-MECA) also inhibited release (IC_{50} = 7.5 μM), but neither adenosine itself nor the selective A_1 and A_2 agonists and antagonists had any significant effect, even at 100 μM. The inhibition produced by clinically relevant concentration of theophylline (50 μM) was potentiated by ineffective concentrations of exogenous adenosine and additive to that produced by IB-MECA. The potent and selective A_3 antagonist MRS 1220, but not the A_1 or A_2 antagonists, significantly reversed the inhibitory effect of theophylline. These results suggest that therapeutic concentrations of theophylline inhibit human eosinophil partly by acting as an A_3 agonist. Together with the potentiation of theophylline action by adenosine, perhaps via the A_3 receptors, these novel actions may, at least in part, contribute to the mechanism of the anti-inflammatory action of this drug *in vivo*.

Keywords: Theophylline; Adenosine; A_3 receptors; Eosinophil; Degranulation; Asthma

1. Introduction

Theophylline is widely used in the treatment of bronchial asthma. Its clinical benefit is believed to be derived not only from its bronchodilatory effect but also, and perhaps more importantly, from its anti-inflammatory activities [1–3]. Experimental and clinical data have demonstrated that at therapeutic concentrations, theophylline inhibits the late response to inhaled allergens by inhibiting the airway inflammation and the associated bronchial hyperreactivity [1,4,5].

In the bronchial inflammation of asthma, eosinophils are known to play a key role [6,7]. Besides the release of inflammatory mediators such as leukotrienes and oxygen radicals, eosinophils contain several granule-derived cationic proteins such as EPO, ECP, EDN, and MBP. When released by the infiltrating bronchial eosinophils these toxic proteins cause airways epithelial damage, resulting in the development of bronchial hyperreactivity [8,9]. There is now ample evidence that eosinophils are important targets of the anti-inflammatory actions of theophylline. Apart from

* Tel.: +965-531.2300x6329; fax: +965-531.8454.

E-mail address: ezeamuzie@hsc.kuniv.edu.kw (C.I. Ezeamuzie).

Abbreviations: C5a, complement fragment 5a; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; OPD, O-phenylenediamine; CGS 21680, 2-[[2-[4-(2-carboxyethyl)phenyl]-ethyl]amino]-*N*-ethylcarboxamidoadenosine; CPA, N-cyclopentyladenosine; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 1,3-dipropyly-8-cyclopentylxanthine; IB-MECA, N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine; MBP, major basic protein; MRS1220, 9-chloro-2-(2-furyl)-5-phenylactylamino[1,2,4]triazolo[1,5-c]quinazoline; PDE, phosphodiesterase; cAMP, adenosine 3′,5′-cyclic monophosphate.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00613-X

the inhibition of eosinophil influx into the bronchial tissues of allergen challenged animals and man, theophylline has been shown to directly suppress several functions of eosinophils such as the release of superoxide ions and granule proteins [10,11], lipid mediators and chemotaxis [12].

For a long time, the molecular mechanism of action of theophylline has been a subject of intense debate. Among the prevalent hypotheses are the inhibition of cAMP and cGMP phosphodiesterases [13] and the antagonism of adenosine receptors [14]. Phosphodiesterase inhibition results in the increase in the intracellular levels of cAMP. The latter is an effective inhibitor of the various responses of inflammatory cells [10,15]. In human eosinophils, the predominant PDE isoenzyme is PDE4 [16,17]. Although at high concentrations (IC_{50} = 300–661 μM) theophylline can inhibit this enzyme [11,18], at therapeutic concentrations (27–80 μM) [19], there is little or no inhibition. Furthermore, selective inhibitors of PDE4 enzyme such as rolipram failed to inhibit eosinophil degranulation [11] thus suggesting that PDE inhibition alone is insufficient for the manifestation of this effect. These findings suggest that the exact mechanism of the *in vivo* anti-inflammatory action of this drug remains to be elucidated.

In addition to the A_1 and A_2 receptors, adenosine is now known to mediate some of its effects through the novel A_3 receptors [20,21], and these receptors have been found to be richly expressed on human eosinophils [22]. Recently, we and others have shown that the A_3 receptors on human eosinophils mediate anti-inflammatory effects, specifically the inhibition of degranulation [23] and chemotaxis [22,24]. On the basis of this new information, it becomes necessary to reassess the role of adenosine, especially the A_3 receptors *vis-a-vis* the mechanism of action of theophylline.

The purpose of this study was therefore to investigate the mechanism of inhibition of eosinophil degranulation by theophylline with particular respect to its interaction with adenosine receptors.

2. Materials and methods

2.1. Isolation of human peripheral blood eosinophils

Fresh blood was obtained from consenting healthy or mildly atopic adults who have taken no medications in the last 72 hr. Eosinophils were purified by a slight modification of the immunomagnetic method [25]. Briefly, three parts of sodium citrate-anticoagulated (13 mM final) blood was mixed with one part of 1% (w/v of 0.9% saline) hydrated methylcellulose solution to sediment the erythrocytes over 30 min at room temperature. The leucocyte-rich supernatant was collected and centrifuged at 200 *g* for 10 min at room temperature. After aspirating off the platelet-rich supernatant, the pelleted leucocytes were washed in "wash buffer" (Ca^{2+}- and Mg^{2+}-free, 10 mM HEPES-buffered Hanks balanced salt solution containing 0.25% bovine serum albumin) and resuspended in the same buffer at approximately 10% of the original blood volume. A 2-mL aliquot was then layered on a 2-step percoll gradient (1.080 and 1.090 g/mL) and centrifuged at 900 *g* on Beckman (GS-6R) centrifuge for 20 min at room temperature. The upper layers (mononuclear cells and percoll) were discarded and the pellet (granulocytes) were recovered and washed twice in the same buffer by centrifugation at 250 *g* for 10 min at 4°. After a hypotonic lysis of contaminating erythrocytes with ice-cold distilled water, and readjusting the tonicity with the same volume of double strength saline, the cells were washed, counted and resuspended at a concentration of 2×10^7 cells/mL in the wash buffer. For the eosinophil purification, 1.25 mL of the granulocyte suspension was then mixed with 5 μL of mouse anti-human CD16 monoclonal antibody in a siliconized test tube and incubated on ice for 1 hr with frequent gentle rotation. Cells were then washed twice resuspended in 500 μL of prewashed immunomagnetic beads pre-coated with affinity purified sheep anti-mouse IgG (2×10^8 coated beads) and incubated on ice for 1 hr with frequent tube rotation. The immunomagnetically-labeled neutrophils were removed by magnetic extraction. The purified eosinophils were then recovered by centrifugation and resuspended in reaction buffer (wash buffer containing 2 mM Ca^{2+} and 1 mM Mg^{2+}) for experiments. The eosinophil purity was assessed by differential count of a Wright-Giemsa stained cytosmear. The final cell preparation routinely consisted of over 98% pure eosinophils. Viability was determined by trypan blue exclusion and always exceeded 98%.

2.2. EPO release

Purified eosinophils were used at a concentration of 5×10^5 cells/mL. Fifty microlitres of pre-warmed cell suspension containing 2.5×10^4 cells was dispensed into each well of a microplate. Then, 100 μL of the reaction buffer containing 10 μg/mL cytochalasin B (CB) was added and after 10 min pre-incubation, the cells were stimulated with 50 μL of human recombinant C5a. The mixture was further incubated for 30 min at 37°. It had been determined in pilot experiments that this time was sufficient for the virtual completion of the degranulation process. When the effect of drugs on degranulation was to be studied, the cells were pre-incubated with the drugs for 10 min (unless otherwise stated) before the addition of CB. At the end of the incubation period, reaction was stopped by cooling on ice and after centrifugation at 600 *g*, for 10 min, 50-μL aliquots of the supernatant as well as triton X-100-lysed cells (for total content determination) were taken for the determination of the released enzymes. EPO activity was measured by the OPD method as previously reported [26]. Briefly, OPD substrate solution containing 0.4 mg/mL OPD and 0.4 mg/mL urea hydrogen peroxide in PBS-citrate buffer (pH 4.5) was prepared from SIGMA FAST® OPD tablets. One hundred microlitres of this substrate was added to 50 μL of

the samples in a microplate and incubated for 30 min at 37°. After incubation, the reaction was then stopped with 50 μL of 4M H_2SO_4 and the plate read at 490 nm. EPO release, as index of degranulation, was expressed as percentage of total content, using the amount obtained in half the same number of cells, after lysis, as 50%. The recovery of released EPO activity was usually above 80% at the end of 30 min incubation, but usually lower with more prolonged incubation.

2.3. Chemicals and biochemical reagents

The following reagents and materials were purchased from Sigma Chemical Co.: recombinant human C5a, adenosine, rolipram, theophylline (as aminophylline), percoll, HEPES buffer, bovine serum albumin, OPD, DMSO, cytochalasin B, and all inorganic salts (Sigmaultra). Items obtained from Research Biochemicals Corp., were: IB-MECA, CPA, CGS 21680, DPCPX, DMPX, and MRS 1220. Mouse monoclonal anti-human CD16 antibody (clone FcR gran1) was obtained from CLB, while the magnetic beads (coated with sheep anti-mouse IgG) were supplied by Dynal AS.

Stock solutions of water-insoluble drugs were made in DMSO to concentrations in the range ($1-4 \times 10^{-1}$ M) and then diluted directly in buffer. The final concentration of DMSO present at the highest drug concentrations did not exceed 0.05%—a concentration that has no effect on eosinophil degranulation.

2.4. Statistical analysis

Experimental data are presented as mean ± standard deviation from the number (n) of independent experiments. The drug concentrations producing 50% inhibition of response (IC_{50} values) were calculated using the concentration-effect curves by non-linear regression analysis using GraphPad InPlot (Graph*h*Pad Software Inc.). Statistical significance (p) between treatment groups was determined by the unpaired t-test, while synergism was tested by comparing the effect of two drugs used together with the sum of their individual effects and applying the one-sample t test (InStat, Graph*h*Pad, Software Inc.).

3. Results

3.1. Effect on EPO release

As shown in Fig. 1, in the presence of 5 μg/mL of CB, C5a induced substantial release of EPO from purified eosinophils in a concentration-dependent manner. Release generally began at around 1 nM, and at the highest concentration tested (100 nM), a net release of 44.8 ± 5.3% of the cell content was obtained. No significant EPO release occurred in the absence of CB. The concentration of 10 nM which gave a submaximal release of 24.1 ± 6.0% was chosen for subsequent experiments.

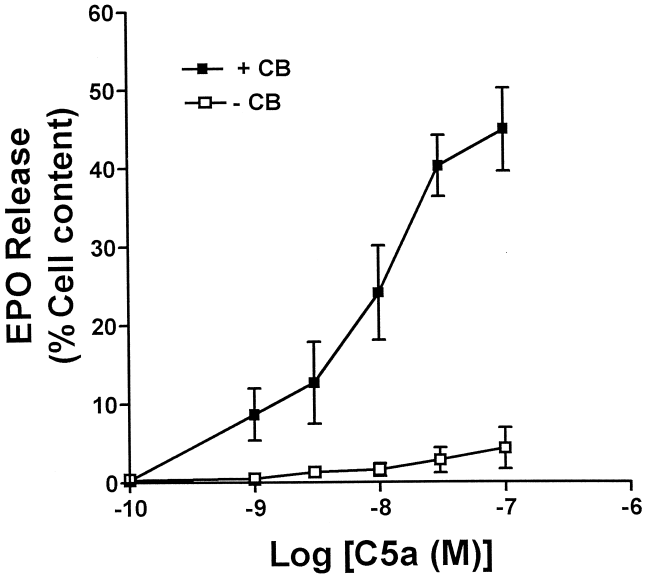

Fig. 1. Release of EPO from human eosinophils induced by human recombinant C5a in the presence and absence of 5 μg/mL cytochalasin B. Spontaneous releases of (0–3%) have been subtracted from all values. Values are mean ± SD for 8 experiments.

In the concentration range 10–1000 μM, theophylline inhibited EPO release in a concentration-dependent manner (Fig. 2). The IC_{50} (95% CI) was 233.5 μM (208.7–276.2 μM), $N = 7$, and almost complete inhibition (90.3 ± 3.0%) at 1000 μM. At the concentration of 50 μM which is well within the therapeutic range of 27–80 μM, the drug produced a significant inhibition of 28.8 ± 4.7%, $P < 0.05$. In contrast, neither the potent PDE IV inhibitor rolipram (up to

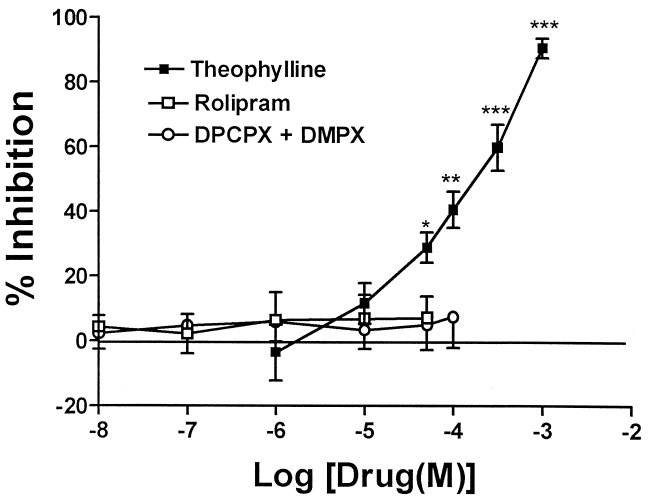

Fig. 2. The effect of theophylline, rolipram and the combination of the selective A_1 and A_2 receptor antagonists—DPCPX and DMPX on EPO release induced by C5a (10 nM). The uninhibited release was in the range 15–32% of cell content. Values are means ± SD for 6 experiments. *$P <$ 0.05, **$P < 0.01$; ***$P < 0.001$.

50 μM) nor the combination of the selective adenosine A_1 receptor antagonist DPCPX with the A_2 receptor antagonist DMPX, had any significant effect. From these results it seemed unlikely that the inhibition of PDE4 or/and the antagonism of A_1 and A_2 receptors were important in the mechanism of inhibition of eosinophil degranulation by theophylline.

Since agents that activate the adenylyl cyclase enzyme are known to synergize with inhibitors of PDE4, the effect of theophylline and rolipram on degranulation were compared in the presence of salbutamol (1 μM). This concentration of salbutamol had no effect on degranulation. As shown in Fig. 3a, in the presence of salbutamol, there was only a modest potentiation resulting in a small, non-significant, shift in the theophylline concentration-response curve to the left. Much more dramatically, the presence of salbutamol converted the inactive rolipram to a highly effective inhibitor of degranulation with an $IC_{50} \approx 1$ μM and maximal inhibition of 65.3 ± 6.8% at 30 μM (Fig. 3b). These results confirm that the inhibition of PDE4 alone is insufficient to inhibit eosinophil degranulation and that an additional signal (perhaps cAMP generation) was required.

3.2. Effect of adenosine and its analogues on EPO release

In view of above, as well as the recent studies showing that the newly discovered adenosine A_3 receptors mediated inhibitory actions on human eosinophils [22–24], a possible role of this receptor subtype in the mechanism of action of theophylline was explored. As shown in Fig. 4, the selective adenosine A_3 receptor agonist IB-MECA produced a concentration-dependent inhibition of EPO release. The IC_{50} (95% CI) was 7.5 μM (4.0–13.6 μM), and at the highest concentration tested (100 μM), the mean percentage inhibition was 75.3 ± 8.1%. Both adenosine itself and the selective A_2 agonist CGS 21680 produced only small, statistically non-significant, effects at high concentrations (10–100 μM), whereas the selective A_1 agonist CPA was completely without effect. The interaction of theophylline with these adenosine agonists was subsequently studied.

3.3. Interaction between theophylline and adenosine receptor agonists

As the results in Fig. 5 show, pre-incubation of eosinophils with theophylline in the presence of in-effective concentrations of adenosine resulted in a surprising potentiation of the effect of theophylline (10–100 μM). The interactions appeared to be synergistic since the effects obtained with the combination were generally higher that the sum of the effects of the individual drugs. For example, the inhibitions produced by theophylline alone (10, 50, and 100 μM) were 6.0 ± 3.7%, 25.3 ± 6.7%, and 37.5 ± 8.2%, respectively, whereas the inhibition produced by adenosine alone (100 μM) was only 11.6 ± 5.6%. In the presence of this concentration of adenosine, the inhibitions by theophylline were 35.7 ± 5.3%, 58.4 ± 6.4% and 68.0 ± 5.2%, respectively (Fig. 5a). These latter values are all significantly higher than the sum of the corresponding individual drug effects, $P < 0.05$, ($N = 4$–7), thus suggesting synergistic interaction. The interaction with the lower concentration of adenosine (10 μM) was, however, essentially additive. Theophylline also interacted with the A_3 receptor agonist IB-MECA in essentially additive manner (Fig. 5b). For example, the inhibitions were 23.3 ± 6.7% for theophylline (50

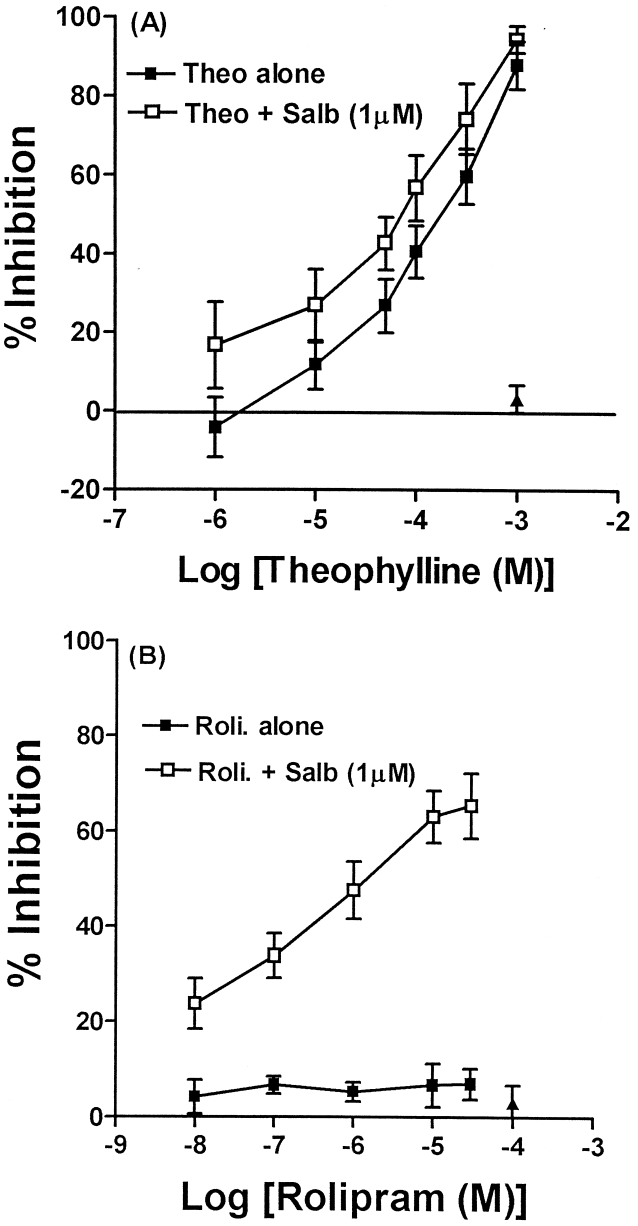

Fig. 3. The effect of salbutamol on the ability of theophylline (a) and rolipram (b) to inhibit the release of EPO induced by C5a (10 nM). The uninhibited releases were in the range 15–32% of cell content. Cells were first pre-incubated with theophylline or rolipram for 10 min, then for a further 5 min with salbutamol before being stimulated for release. The un-joined symbol (closed triangle) represents the effect of salbutamol alone. Values are means ± SD for 7 experiments.

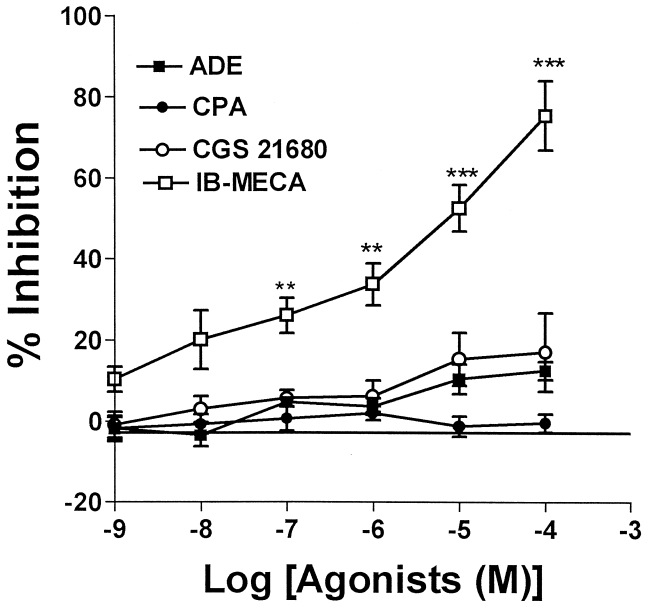

Fig. 4. The effect of adenosine and the various adenosine receptor agonists on EPO release induced by C5a (10 nM). The uninhibited releases were in the range 15–32% of total cell content. Values are means ± SD for 5 experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

μM) and 20.3 ± 5.0% for IB-MECA (1 μM) separately, but 46.1 ± 5.1% for the combination. In the presence of 2.5 μM concentrations of the selective A_1 and A_2 receptor agonists, CPA and CGS 21680, respectively, the effect of theophylline either was not changed or was slightly reduced (Figs. 5c and d).

3.4. Reversal of the effect of theophylline by adenosine A_3 receptor antagonist

Because theophylline is a known antagonist at the A_1 and A_2 receptors and IB-MECA is a selective A_3 agonist, there arose the question whether theophylline could be acting as an A_3 agonist in the same way as IB-MECA. To investigate this possibility, the effect of the selective A_3 antagonist MRS 1220 [27] on theophylline-induced inhibition of degranulation was carried out. As shown in Table 1, preincubation of eosinophils with MRS 1220 (1.0 and 2.5 μM) resulted in a significant and concentration-dependent reversal of the inhibition produced by low concentrations of theophylline. For example, at 2.5 μM MRS 1220 reduced the inhibition produced by theophylline (50 μM) from 27.8 ± 5.5% to 13.7 ± 4.5%, $P < 0.01$, $N = 6–7$, and that of 100 μM theophylline from 37.4 ± 7.8% to 21.3 ± 6.9%, $P < 0.05$, $N = 6–7$. There was no reversal of inhibition at the highest concentration of theophylline (1000 μM). The drug also produced a significant reversal of the effect of IB-MECA (1 μM), as well as that of the combination of IB-MECA and theophylline (50 μM). Furthermore, the antagonist completely abolished the small inhibition produced by adenosine (10 and 100 μM), and in fact at 2.5 μM it produced small enhancement of release. The enhanced inhibitory effects produced by the combination of the two concentrations of adenosine with theophylline were also reversed significantly by the A_3 receptor antagonist, $P < 0.05$. In contrast, neither the inhibitory effects of theophylline nor those of its combination with adenosine or IB-MECA were significantly affected by the presence of a mixture of the selective A_1 receptor antagonist DPCPX and the A_2 receptor antagonists DMPX (2.5 μM each). None of the three antagonists had any EPO-releasing activity of their own or affected EPO estimation.

4. Discussion

The exact molecular mechanism of the *in vivo* effects of theophylline has been a subject of intense debate for over 50 years. Among the most favored mechanisms are the inhibition of PDEs leading to a rise in the intracellular concentrations of cAMP and the antagonism of adenosine receptors. In this study these two hypotheses were re-assessed with regards to the inhibitory effect of theophylline on C5a-induced human eosinophil degranulation. The results show that theophylline is an effective inhibitor of degranulation with an IC_{50} value of 233.5 μM. At the concentration of 50 μM which is well within the accepted therapeutic plasma concentration of this drug (27–80 μM, corresponding to ≈5–15 μg/mL) [19], the drug achieved a statistically significant inhibition of ≈29%. A similar order of potency has been reported for the release of EDN and cytokines from human eosinophils [11,28]. The current results further showed that in contrast to theophylline, neither rolipram which is a potent PDE4 inhibitor, nor the combination of the selective A_1 receptor antagonist DPCPX and the A_2 antagonist DMPX, had any significant effect. These results would initially appear to suggest that the inhibitory effect of theophylline was both cAMP-independent and unrelated to adenosine A_1 and A_2 receptor blockade. However, since previous reports have shown a synergistic interaction between inhibitors of PDEs and activators of adenylyl cyclase in eosinophils [10,11], the effects of salbutamol on the actions of theophylline and rolipram were examined. In the presence of 1 μM salbutamol which had no effect on degranulation, rolipram became a highly effective inhibitor of degranulation, whereas the effect of theophylline was only marginally enhanced. Together, these results suggest that the inhibition of PDE4, which is the predominant PDE isoenzyme in human eosinophils [16,17], is by itself insufficient for inhibition of degranulation, but that an additional signal—the activation of adenylyl cyclase, was required. With respect to theophylline, its effectiveness in the absence of salbutamol and the small enhancement in the presence of this drug suggests that either that the action of theophylline is largely cAMP-independent or that theophylline is able to generate this additional cAMP signal (or a qualitatively similar signal) for itself, which then interacts with its inhib-

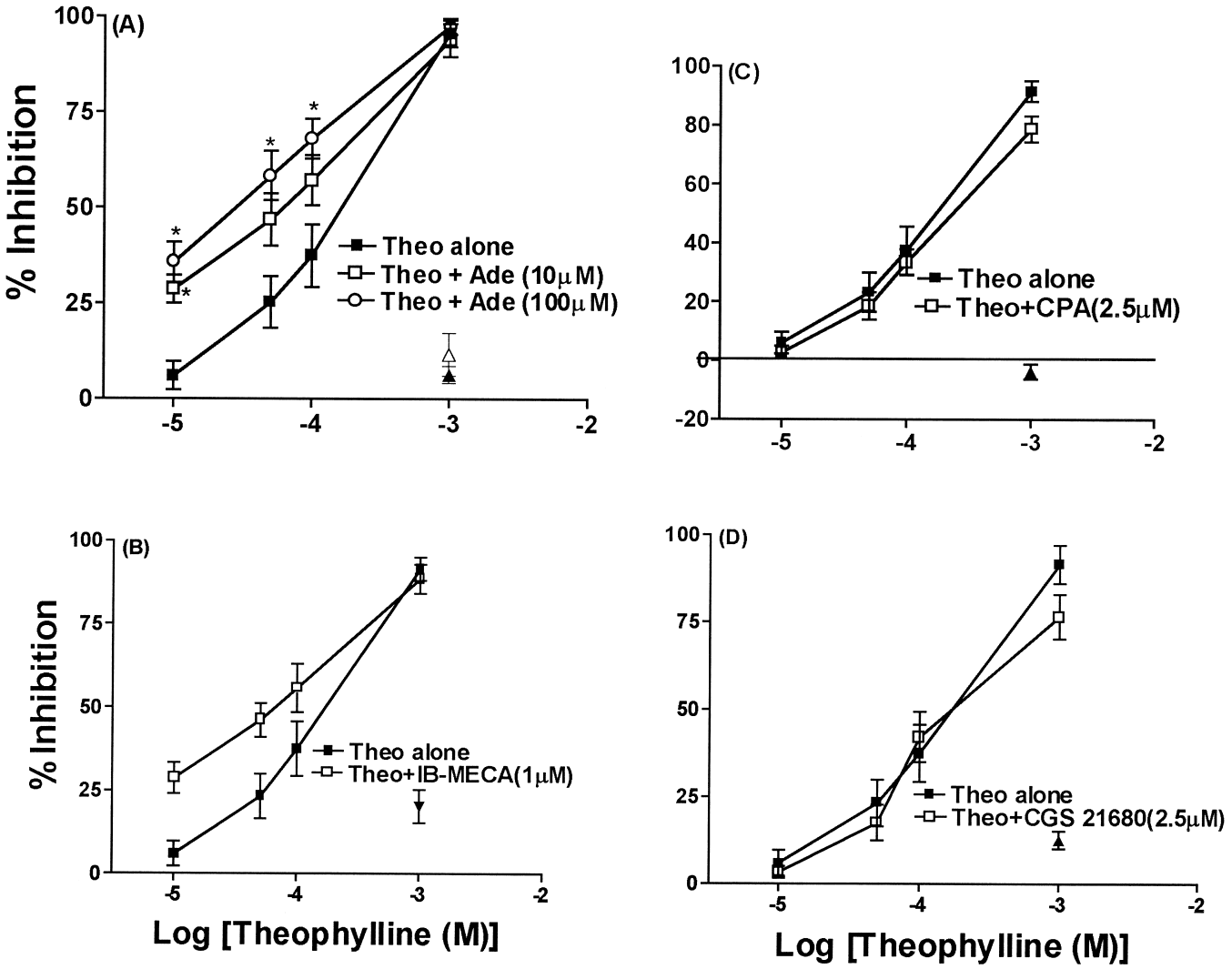

Fig. 5. The interaction of theophylline with adenosine (A) and the various adenosine receptor agonists—IB-MECA (B), CPA (C), and CGS 21680 (D)—on EPO release induced by C5a (10 nM). Cells were first incubated with theophylline for 10 min, then for a further 5 min with the agonists before stimulation for degranulation. The uninhibited releases were in the range 13–35% of total cell content. In (A), the unjoined closed and open triangles represent the effects of 10 μM and 100 μM adenosine alone, respectively. In (B–E), the unjoined closed triangle represents the effect of the given concentrations of the adenosine receptor agonists alone. Values are means ± SD $N = 7$ for A and B, and N = 4 for C and D. *$P < 0.05$ (vs the expected sum of the effects of the individual drugs).

itory action on PDEs. There is no evidence, however, that theophylline can activate the adenylyl cyclase, although its ability to inhibit the inhibitory G_i protein [29] and to directly activate a cAMP-dependent protein kinase [30] have been suggested.

To further investigate, the interaction between theophylline and adenosine on the inhibition of degranulation was studied. The rationale for this was the recent finding that A_3 receptors on human eosinophils mediate inhibition of eosinophil responses such as degranulation [23] and chemotaxis [22,24]. The result indicated that adenosine, which by itself had only a small effect, highly potentiated the inhibitory effect of theophylline. This was a surprising result since theophylline, at therapeutic concentrations, is regarded as an adenosine antagonist (though at A_1 and A_2 receptors) [14]. Thus, adenosine could only possibly take part in this interaction via the A_3 receptors, at least going by the current knowledge about adenosine receptor subtypes. Indeed, a similar interaction was found between theophylline and the selective A_3 agonist IB-MECA. From these results, it is obvious that the inhibitory signals generated by the A_3 receptor activation could add to that generated by theophylline. Thus, one possible explanation for the effectiveness of theophylline in the absence of any interacting signals could be that the drug was acting through the activation of the A_3 receptors, just like IB-MECA. Alternatively, it could be that the drug was simultaneously providing for itself, through A_3 receptors, the extra signal required to interact with any PDE inhibition. This rather unexpected possibility of A_3 agonism was in fact confirmed by the ability of the selective A_3 receptor antagonist MRS 1220 [27] to significantly reverse, in a concentration-dependent

Table 1
Interaction of theophylline with adenosine and IB-MECA on the inhibition of C5a-induced EPO release and the effect of adenosine receptor antagonists

Treatments	% Inhibition of C5a (10 nM)-induced EPO release			
	Buffer ($N = 7$)	MRS 1220 (1 μM) ($N = 6$)	MRS 1220 (2.5 μM) ($N = 6$)	DPCPX + DMPX (2.5 μM each) ($N = 6$)
Buffer	—	2.2 ± 2.6	2.5 ± 1.9	3.2 ± 1.6
Theo (50 μM)	27.8 ± 5.5	18.2 ± 6.0*	13.7 ± 4.5**	25.4 ± 6.6
Theo (100 μM)	37.4 ± 7.8	29.1 ± 7.2	21.3 ± 6.9*	34.7 ± 5.1
Theo (1000 μM)	94.1 ± 4.3	96.6 ± 4.8	89.9 ± 7.2	91.5 ± 5.6
IB-MECA (1 μM)	23.5 ± 5.3	12.5 ± 5.8**	8.3 ± 4.1***	19.9 ± 3.8
Theo (50 μM) + IB-MECA	56.2 ± 4.9	33.9 ± 5.5**	27.9 ± 4.6***	48.6 ± 7.4
Adenosine (10 μM)	4.4 ± 2.4	1.7 ± 2.8	[+ 8.4]	6.2 ± 2.0
Adenosine (100 μM)	8.9 ± 2.6	2.8 ± 4.7	[+ 11.2]	10.8 ± 1.2
Theo (50 μM) + Ade (10 μM)	47.8 ± 7.4	36.3 ± 6.7	29.8 ± 7.1*	39.1 ± 6.6
Theo (50 μM) + Ade (100 μM)	60.6 ± 7.9	40.2 ± 5.6*	37.8 ± 7.2**	55.4 ± 7.3

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (vs buffer).
Values are means ± SD.

manner, the inhibitory effect of theophylline as well as its potentiated effect in the presence of adenosine. However, the fact that MRS 1220—a high affinity antagonist (K_i = 0.7 nM) at the A_3 receptors [31], only partially reversed the effect of low concentrations of theophylline, may suggest that any agonistic effect of theophylline at A_3 receptors is probably small and that additional mechanisms may be involved. As would be expected, the A_1 and A_2 antagonists had no such effect. Thus, the inhibition of human eosinophil degranulation by theophylline cannot be mediated via antagonism of A_1 and A_2 receptors; instead the drug may actually act as an agonist, albeit a weak one, at A_3 receptors in a similar manner as IB-MECA.

The nature of the A_3 receptor-generated inhibitory signal is currently uncertain. Although adenosine can increase intracellular cAMP via A_2 receptors, this is irrelevant here because theophylline itself is an antagonist at this receptor and, if any thing, might even be expected to potentiate degranulation. It is, therefore, difficult not to speculate whether A_3 receptors on eosinophils are positively linked to the adenylyl cyclase system such that theophylline can activate it at the same time as inhibit PDEs. There is currently no evidence to support this view. In fact, it is reported that A_3 receptor activation actually reduces intracellular cAMP [32]. If this is indeed the case in human eosinophils, then other explanations must be sought. Interestingly, however, preliminary data from our on-going studies suggest that A_3 receptor activation may in fact increase (not decrease) agonist-stimulated cAMP accumulation in human eosinophils.

The reason for the failure of adenosine itself, compared with IB-MECA, to significantly inhibit EPO release is currently unclear. Although its uptake and metabolism may contribute, this possibility is inconsistent with the fact that relatively low concentrations of adenosine significantly enhanced superoxide anions release from human eosinophils [23]. Dipyridamole—a nucleoside uptake inhibitor could not be used because it caused a small enhancement of EPO release by itself. One possibility is that this may relate to differences in efficacy of the two agents at the A_3 receptors. A similar low efficacy at the human eosinophil A_2 receptors may explain the lack of significant inhibitory effect seen with CGS 21680. Yukawa and co-workers [33] have reported significant inhibition by A_2 agonists of zymosan-activated superoxide release from eosinophils. This disparity may reflect the different stimuli used in the two studies since it is known that the control of eosinophil responses is generally stimulus-dependent [34].

Extrapolated *in vivo*, a possible scenario may be that theophylline acts firstly by blocking the A_1 and A_2 receptors, thereby allowing endogenous adenosine to activate the A_3 receptors, and secondly by activating the A_3 receptors directly. Such combined A_3 receptor activation, perhaps together with some inhibition of PDEs, may then interact to produce an effective down-regulation of eosinophil functions. If indeed this is so, then the resulting potentiation of the effect of theophylline by endogenous adenosine may help to explain the much-reported disparity between the concentrations of theophylline effective *in vitro* and *in vivo*. Recently, Sullivan *et al*. [4] demonstrated significant *in vivo* anti-inflammatory effect of theophylline in asthmatics at steady state plasma concentrations as low as 36 μM, which is at the lower end of its therapeutic range. Such potency would be unlikely if theophylline was acting solely as a PDE inhibitor for which its IC_{50} is in the range 300–661 μM [11,18].

Adenosine is a ubiquitous mediator released from cells under stress. In asthmatics its concentration in the bronchoalveolar lavage fluid is significantly raised compared with normals [35], and the inhalation of adenosine causes bronchospasm in asthmatics but not in normal subjects [36,37]. The present hypothesis of A_3 receptor-mediated potentiation of theophylline effect by endogenous adenosine is consistent with these observations. Firstly, the higher concentration of adenosine in the asthmatic lung may offer

the explanation why low concentrations of theophylline are effective in asthma. Secondly, the induction of bronchospasm in asthmatics, but not normals, by exogenous adenosine is probably mediated by the pathologically-induced up-regulation of A_1 receptors [38,39], hence the effectiveness of theophylline, acting as A_1 and A_2 receptor antagonist, in inhibiting it [40].

Another *in vivo* situation that can be explained by this hypothesis is the observed higher potency of theophylline in late phase asthmatic response to allergen challenge compared to early phase response [41]. The early response is believed to be mediated mainly by products of degranulating mast cells, especially histamine, whereas the late phase response is a consequence of the subsequent inflammatory processes. Unlike eosinophils and neutrophils, human lung mast cells tend to lack A_3 receptors [22,42].

Recently, theophylline, at therapeutically relevant concentrations, was shown to inhibit IL-5 mediated survival of human eosinophils and to accelerate apoptosis [43]. While this interesting finding can offer explanations for the effectiveness of theophylline in asthma, it is possible that this action was actually A_3 receptor-mediated. In fact, it has been reported that A_3 activation induces apoptosis in a number of cell types, including human eosinophils [44].

It is obvious that the presented work is limited in scope, being based on a single response and employing a single cell type. Much work is required to determine to what extent the present observations apply to other pro-inflammatory cells and under different experimental conditions.

In summary, therapeutic concentrations of theophylline inhibited human eosinophil degranulation and this effect was significantly enhanced by exogenous adenosine, perhaps by acting through the A_3 receptors. Evidence was also found that theophylline may act as a weak agonist at the A_3 receptor. It is proposed that these novel actions may, at least in part, contribute to the mechanism of the anti-inflammatory action of this drug *in vivo*.

Acknowledgments

This study was supported by grant #MR 030 from Research Administration, Kuwait University. I am grateful to Mrs E. Philips for her excellent technical assistance.

References

[1] Pauwels RA. New aspects of the therapeutic potential of theophylline in asthma. J Allergy Clin Immunol 1989;83:548–53.
[2] Barnes PJ, Pauwels RA. Theophylline in the management of asthma: time for reappraisal? Eur Respir J 1994;7:579–91.
[3] Page CP. Recent advances in our standing of the use of theophylline in the treatment of asthma. J Clin Pharmacol 1999;39:237–40.
[4] Sullivan PJ, Bekir S, Jaffer Z, Page C, Jeffery P, Costello J. Anti-inflammatory effects of low-dose oral theophylline in atopic asthma. Lancet 1994;343:1006–8.
[5] Banner KH, Page CP. Anti-inflammatory effects of theophylline and selective phosphodiesterase inhibitors. Clin Exp Allergy 1996;26:(Suppl 2)2–9.
[6] Frigas E, Gleich, GJ. The eosinophil and the pathophysiology of asthma. J Allergy Clin Immunol 1986;77:527–37.
[7] Barnes PJ. New concepts in pathogenesis of bronchial hyperresponsiveness and asthma. J Allergy Clin Immunol 1989;83:1013–26.
[8] Motijima S, Frigas E, Leogering DA, Gleich GJ. Toxicity of eosinophil proteins for guinea pig tracheal epithelium in vitro. Am Rev Respir Dis 1989;139:801–5.
[9] Laitinen LA, Heins M, Laitinen A, Kava T, Haahtela T. Damage of the airway epithelium and bronchial reactivity in patients with asthma. Am Rev Respir Dis 1985;131:599–606.
[10] Kita H, Abu-Ghazaleh RI, Gleich GJ, Abraham RT. Regulation of Ig-induced eosinophil degranulation by adenosine 3,5, cyclic monophosphate. J Immunol 1991;146:2712–8.
[11] Hatzelmann A, Tenor H, Schudt C. Differential effects of non-selective and selective phosphodiesterase inhibitors on human eosinophil functions. Br J Pharmacol 1995;114:821–31.
[12] Tenor H, Hatzelmann A, Church MK, Schudt C, Shute JK. Effects of theophylline and rolipram on leukotriene C4 (LTC4) synthesis and chemotaxis of human eosinophils from normal and atopic subjects. Br J Pharmacol 1996;118:1727–35.
[13] Bergstrand H. Phosphodiesterase inhibition and theophylline. Eur J Respir Dis 1980;61:37–44.
[14] Fredholm BB, Persson CGA. Xanthine derivatives as adenosine antagonists. Eur J Pharmacol 1982;81:673–6.
[15] Dent G, Giembycz MA, Rabe KF, Barnes PJ. Inhibition of eosinophil cyclic nucleotide PDE activity and opsonized zymosan-stimulated respiratory burst by type IV-selective PDE inhibitors. Br J Pharmacol 1991;103:1339–46.
[16] Barnes PJ. Cyclic nucleotides and phosphodiesterases and airways functions. Eur Respir J 1995;8:457–62.
[17] Schudt C, Tenor H, Hatzelmann A. PDE isoenzymes as targets for antiasthma drugs. Eur Respir J 1995;8:1179–83.
[18] Cohan VL, Showell HJ, Fisher DA, Pazoles CJ, Watson JW, Turner CR, Cheng JB. In vitro pharmacology of the novel phosphodiesterase type 4 inhibitor, CP-80633. J Pharmacol Exp Ther 1996;278:1356–61.
[19] Rang HP, Dale MM. Pharmacology. Edinburgh: Churchill Livingstone, 1991.
[20] Sajjadi FG, Firestein GS. cDNA cloning and sequence of the human A_3 adenosine receptor. Biochem Biophys Acta 1993;1179:105–7.
[21] Salvatore CA, Jacobson MA, Taylor BE, Linden J, Johnson RG. Molecular cloning and characterization of the human A_3 adenosine receptor. Proc Natl Acad Sci USA 1993;90:10365–9.
[22] Walker BAM, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D, Bai TR. Adenosine A_3 receptor expression and function in eosinophils. Am J Respir Cell Mol Biol 1997;16:531–7.
[23] Ezeamuzie CI, Philips E. Adenosine A_3 receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release. Br J Pharmacol 1999;127:188–94.
[24] Knight K, Zheng X, Rocchini C, Jacobson M, Bai T, Walker B. Adenosine A_3 receptor stimulation inhibits migration of human eosinophils. J Leukoc Biol 1997;62:465–8.
[25] Hansel TT, De Vries IJM, Iff T, Ris S, Wandzilak M, Betz S, Blaser K, Walker C. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. J Immunol Meth 1991;145:105–10.
[26] Kroegel C, Yukawa T, Dent G, Venge P, Chung KF, Barnes PJ. Stimulation of degranulation from eosinophils by platelet activating factor. J Immunol 1989;142:3518–26.
[27] Jacobson KA, Park K-S, Jiang J-L, Kim Y-C, Olah ME, Stiles GL, Ji X-D. Pharmacological characterization of novel A_3 adenosine receptor-selective antagonists. Neuropharmacology 1997;36:1157–65.

[28] Shute JK, Tenor H, Church MK, Holgate ST. Theophylline inhibits the release of eosinophil survival cytokines—is Raf-1 the protein kinase A target. Clin Expt Allergy 1998;28(Suppl.3):47–52.
[29] Parsons WJ, Ramkumar V, Stiles GL. Isobutylmethylxanthine stimulates adenylate cyclase by blocking the inhibitory regulatory protein G_i. Mol Pharmacol 1988;34:37–41.
[30] Tomes C, Rossi S, Moreno S. Isobutylmethylxanthine and other classical cyclic nucleotide phosphodiesterase inhibitors affect cAMP-dependent protein kinase activity. Cell Signal 1993;5:615–21.
[31] Kim Y-C, Ji X-D, Jacobson KA. Derivatives of the triazoloquinazoline adenosine antagonist (CGS 15943) are selective for the human A_3 receptor subtype. J Med Chem 1996;39:4142–8.
[32] Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O. Molecular cloning and characterization of a novel adenosine receptor: the A_3 adenosine receptor. Proc Natl Acad Sci USA 1992;89:7432–6.
[33] Yukawa T, Kroegel C, Chanez P, Dent G, Ukena D, Chung KF, Barnes PJ. Effect of theophylline and adenosine on eosinophil function. Am Rev Respir Dis 1989;140:327–33.
[34] Ezeamuzie CI, Al-Hage M, Nwankwoala RNP. The effect of salmeterol on human eosinophils is both stimulus- and response-dependent. Int J Immunopharmacol 1997;19:421–30.
[35] Driver AG, Kukoly CA, Ali S, Mustafa SJ. Adenosine in bronchoalveolar lavage fluid in asthma. Am Rev Respir Dis 1993;148:91–7.
[36] Cushley MJ, Tattersfield AE, Holgate ST. Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. Br J Clin Pharmacol 1983;15:161–5.
[37] Church M, Holgate S. Adenosine-induced bronchoconstriction and its inhibition by nedocromil sodium. J Allergy Clin Immunol 1993;93:190–4.
[38] El-Hashim A, D'Agostino B, Matera MG, Page CP. Characterization of adenosine receptors involved in adenosine-induced bronchoconstriction in an allergic rabbit model. Br J Pharmacol 1996;119:1262–8.
[39] Nyce JW, Metzger WJ. An anti-sense oligonucleotide against A_1 receptors inhibiting allergen-induced airway responsiveness in allergec rabbits. Nature 1997;97:721–5.
[40] Mann JS, Holgate ST. Specific antagonism of adenosine-induced bronchoconstriction in asthma by oral theophylline. Br J Clin Pharmacol 1985;19:685–92.
[41] Pauwels R, Van Rebterghem D, Van Der Straeten M, Johanesson N, Persson CGA. The effect of theophylline and enprofylline on allergen-induced bronchoconstriction. J Allergy Clin Immunol 1985;76:583–9.
[42] Bai TR, Weir T, Walker B, Salvatore CA, Johnson RG, Jacobson MA. Localization of adenosine A_3 receptors by in-situ hybridization in human lung. FASEB J 1994;8:A800.
[43] Yasui K, Hu B, Nakazawa T, Agematsu K, Komiyama A. Theophylline accelerates human granulocyte apoptosis not via phosphodiesterase inhibition. J Clin Invest 1997;7:1677–84.
[44] Kohno Y, Ji X-D, Mawhorter SD, Bochner B, Sei Y, Koshiba M, Jacobson KA. Activation of adenosine A_3 receptors on human eosinophils raises $[Ca^{2+}]_i$ and induces apoptosis. Drug Dev Res 1996;37:182.