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# Preparations of *Rp*-cyclic adenosine 3′,5′-phosphorothioate (*Rp*-cAMPS) can contain biologically active amounts of adenosine

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Superoxide anion ($O_2^{\cdot-}$) production from human neutrophils stimulated by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 1 μM) was inhibited by preparations of the inhibitor of cAMP-dependent protein kinase, *Rp*-cyclic adenosine 3′,5′-phosphorothioate (*Rp*-cAMPS, 100 μM). This effect of *Rp*-cAMPS was reversed by xanthine amine congener (0.1 μM), an adenosine receptor antagonist, and by low concentrations of adenosine desaminase (0.02 mg/ml). HPLC analysis shows that these preparations of *Rp*-cAMPS contained concentrations of adenosine which could produce significant inhibition of fMLP-induced $O_2^{\cdot-}$ production. These results suggest that *Rp*-cAMPS should be used with caution in cells or tissues containing adenosine receptors, and that preparations of *Rp*-cAMPS should be treated with adenosine desaminase before use to avoid activation of adenosine receptors.

Signal transduction; *Rp*-cyclic adenosine 3′,5′-phosphorothioate; Adenosine; Superoxide anion formation; Human neutrophil

## 1. INTRODUCTION

Stimulation of receptors which activate adenylate cyclase potently inhibit the fMLP-stimulated $O_2^{\cdot-}$ production from neutrophils, presumably through production of cAMP and activation of cAMP-dependent protein kinase (for review see [1]). An inhibitor of cAMP-dependent protein kinase would be expected to attenuate the effects of activators of adenylate cyclase, and to enhance the effect of fMLP on $O_2^{\cdot-}$ production.

*Rp*-cAMPS, a cell-permeable inhibitor of cAMP-dependent protein kinase, which reversibly binds to the cAMP binding site on the regulatory subunit of cAMP-dependent protein kinase (for review see [2]), has been widely used in experiments to investigate the role of the adenylate cyclase/cAMP pathway in receptor activity in intact cells (for example see [3–5], see also [2] and references therein). We wished to use *Rp*-cAMPS to investigate the role of cAMP in the inhibition of fMLP-induced $O_2^{\cdot-}$ production in human neutrophils by isoprenaline. However, in our experiments, *Rp*-cAMPS by itself *inhibited* the fMLP-stimulated $O_2^{\cdot-}$ production. We show here that this inhibition is due to adenosine present in preparations of *Rp*-cAMPS, rather than *Rp*-cAMPS itself, activating adenosine receptors. These data indicates that *Rp*-cAMPS should be used with caution in cell preparations containing adenosine receptors.

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*Abbreviations:* cAMP, cyclic adenosine 3′,5′-monophosphate; *Rp*-cAMPS, *Rp*-cyclic adenosine 3′,5′-phosphorothioate; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; $O_2^{\cdot-}$, superoxide anion; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; XAC, xanthine amine congener.

## 2. MATERIALS AND METHODS

Human neutrophils from young healthy male and female volunteers were isolated as previously described [6] and $O_2^{\cdot-}$ production was monitored at 550 nm by continuous measurement of ferricytochrome reduction, inhibitable by superoxide dismutase, as described in [7]. Test substances were added to cuvettes containing $1 \times 10^6$ neutrophils in a total volume of 0.5 ml and the cuvettes were pre-incubated for 3 min at 37°C before the addition of fMLP to initiate $O_2^{\cdot-}$ production. Analysis of *Rp*-cAMPS was performed with a L-6200 HPLC pump (Merck, Darmstadt, Germany) on a LiChrosphere RP-8 select B (Merck) reverse-phase silica column (124 × 4 mm) using a fixed wavelength UV-detector (LDC, Riveiera Beach, CA) at 254 nm. In some experiments the peaks were analyzed with a diode array detector (Perkin-Elmer, Überlingen, Germany). The eluent consisted of 2.5% acetonitrile and 20 mM trimethyl ammonium formate buffer adjusted to pH 4.0; the elution rate was 1.5 ml/min. Injections of 20 μl were made from a 10 mM stock solution of *Rp*-cAMPS.

*Rp*-cAMPS (lots 12, 13, 15 and 17; BioLog, Bremen, Germany) was dissolved in deionized water to give a stock solution of 10 mM, aliquoted and frozen at −20°C except for one aliquot which was used immediately. *Rp*-cAMPS was kept on ice until addition to the reaction mixture. Enzymatic pretreatment of lots 15 and 17 was performed with adenosine desaminase (Sigma, Deisenhofen, Germany) and 2′,3′-3′,5′-cyclic nucleotide-dependent phosphodiesterases and alkaline phosphatase (Sigma, Deisenhofen, Germany), respectively. XAC (RBI, Natick, MA) was initially dissolved in 0.1 mM acetic acid to give a stock solution of 5 mM and diluted subsequently in reaction buffer (composition given in [7]). DPCPX (RBI, Natick, MA) was initially dissolved in dimethyl sulphoxide to give a stock solution of 10 mM and subsequently diluted in deionized water. Adenosine and cAMP were obtained from Sigma (Deisenhofen, Germany) and were dissolved in deionized water.

## 3. RESULTS AND DISCUSSION

fMLP (1 $\mu$M) stimulated the formation of 6.01±0.33 nmol $O_2^{\cdot-}/10^6$ cells. *Rp*-cAMPS, at a concentration used in other intact cell experiments (100 $\mu$M) [2–5] unexpectedly produced a profound inhibition of fMLP-stimulated $O_2^{\cdot-}$ production (Figs. 1 and 2). This inhibitory effect was seen with solutions made from two separate batches of *Rp*-cAMPS (lots 12 and 13). We suspected that either *Rp*-cAMPS was acting as a weak adenosine receptor agonist, or that the *Rp*-cAMPS preparation contained adenosine. Adenosine has a marked inhibitory effect on fMLP-stimulated $O_2^{\cdot-}$ production from human neutrophils (for review see [1], see also Figs. 1 and 2) and is the starting material for synthesis of *Rp*-cAMPS. XAC, an $A_1/A_2$-adenosine receptor antagonist, and DPCPX, a relatively $A_1$-selective adenosine receptor antagonist (for review see [8]), by themselves potentiated fMLP-stimulated $O_2^{\cdot-}$ production, with XAC (0.1 $\mu$M; 13.1 ± 0.5 nmol $O_2^{\cdot-}/10^6$ cells) being more potent than DPCPX (0.1 $\mu$M; 9.9 ± 0.8 nmol $O_2^{\cdot-}/10^6$ cells). The inhibitory effect of *Rp*-cAMPS (100 $\mu$M) was blocked by XAC (0.1 $\mu$M) and was unaffected by DPCPX at the same concentration. The inhibitory effect of adenosine (0.1 $\mu$M) was also substantially reduced by XAC and not blocked by DPCPX (Fig. 1). These results suggest that some component of the *Rp*-cAMPS preparation was activating $A_2$-adenosine receptors.

We then examined the effect of adenosine desaminase on the inhibitory effect of *Rp*-cAMPS and other substances on fMLP-stimulated $O_2^{\cdot-}$ production. The inhibitory effect of *Rp*-cAMPS was abolished by adenosine desaminase (0.02 mg/ml), and a small enhancement of fMLP-stimulated $O_2^{\cdot-}$ production was seen (see Fig. 2). These results with this low concentration of adenosine desaminase are particularly significant, as *Rp*-cAMPS is resistant to degradation [2]. cAMP at a 10-times higher concentration than *Rp*-cAMPS (1 mM), produced a smaller inhibition of fMLP-stimulated $O_2^{\cdot-}$ production (see Fig. 2), which was substantially reduced by adenosine desaminase. The inhibitory effect of 1 $\mu$M adenosine was reduced by this low concentration of adenosine desaminase (see Fig. 2) and the inhibitory effect of 0.1 $\mu$M adenosine was also substantially reduced (data not shown). Isoprenaline (10 nM) inhibited fMLP-stimulated $O_2^{\cdot-}$ production to a similar extent as *Rp*-cAMPS (100 $\mu$M). This effect of isoprenaline was unaffected by adenosine desaminase, suggesting that adenosine desaminase was not acting non-specifically. Taken together, these results suggest that the inhibition of fMLP-stimulated $O_2^{\cdot-}$ production by *Rp*-cAMPS, lots 12 and 13, was due to the presence of adenosine or adenosine-like compounds, rather than an effect of *Rp*-cAMPS itself.

HPLC analysis shows that the *Rp*-cAMPS preparations lots 12 and 13 are over 99% pure. Fig. 3 shows the original trace from HPLC analysis of lot 13. In this preparation adenosine, identified by comparison with

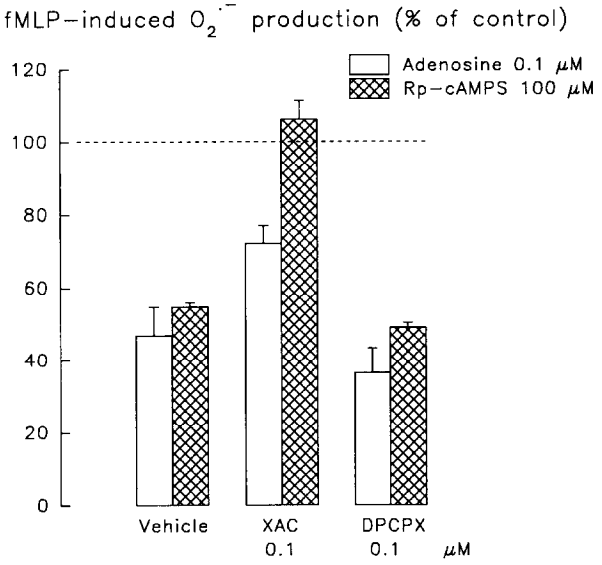


Fig. 1. The effect of adenosine receptor antagonists on the inhibition by *Rp*-cAMPS and adenosine of the fMLP-stimulated $O_2^{\cdot-}$ production from human neutrophils. Test substances were added to the cells 3 min before addition of fMLP (1 $\mu$M). The results shown are the mean ± S.D. of replicate observations ($n$ = 2–3) on neutrophils from a single subject. Similar results were seen in two further experiments on neutrophils from different healthy volunteers. Results are shown as percent of the respective controls, absolute control values are shown in section 3.

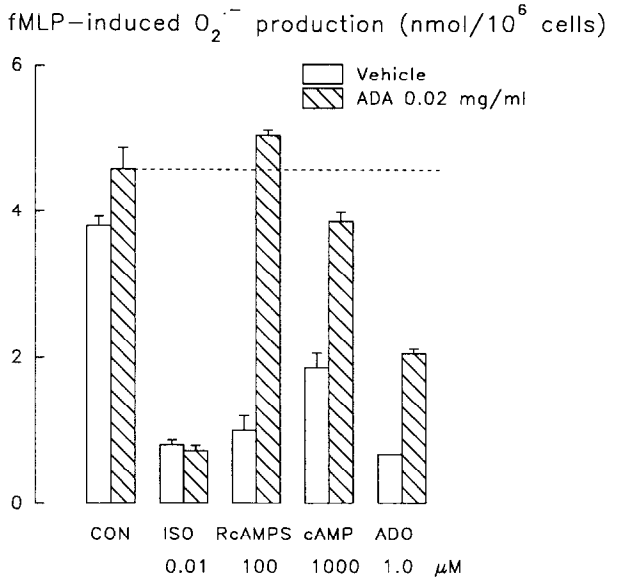


Fig. 2. The effect of adenosine desaminase on the inhibition by isoprenaline (ISO), *Rp*-cAMPS, cAMP and adenosine (ADO) of the fMLP (1 $\mu$M) stimulated production of $O_2^{\cdot-}$ from human neutrophils. Test substances and adenosine desaminase (ADA, 0.02 mg/ml) or vehicle were added to the cells 3 min before addition of fMLP. CON shows the effect of fMLP alone. The results shown are the mean ± S.D. of replicate observations ($n$ = 2–3) on neutrophils from a single experiment representative of at least three separate experiments on neutrophils from different healthy volunteers.

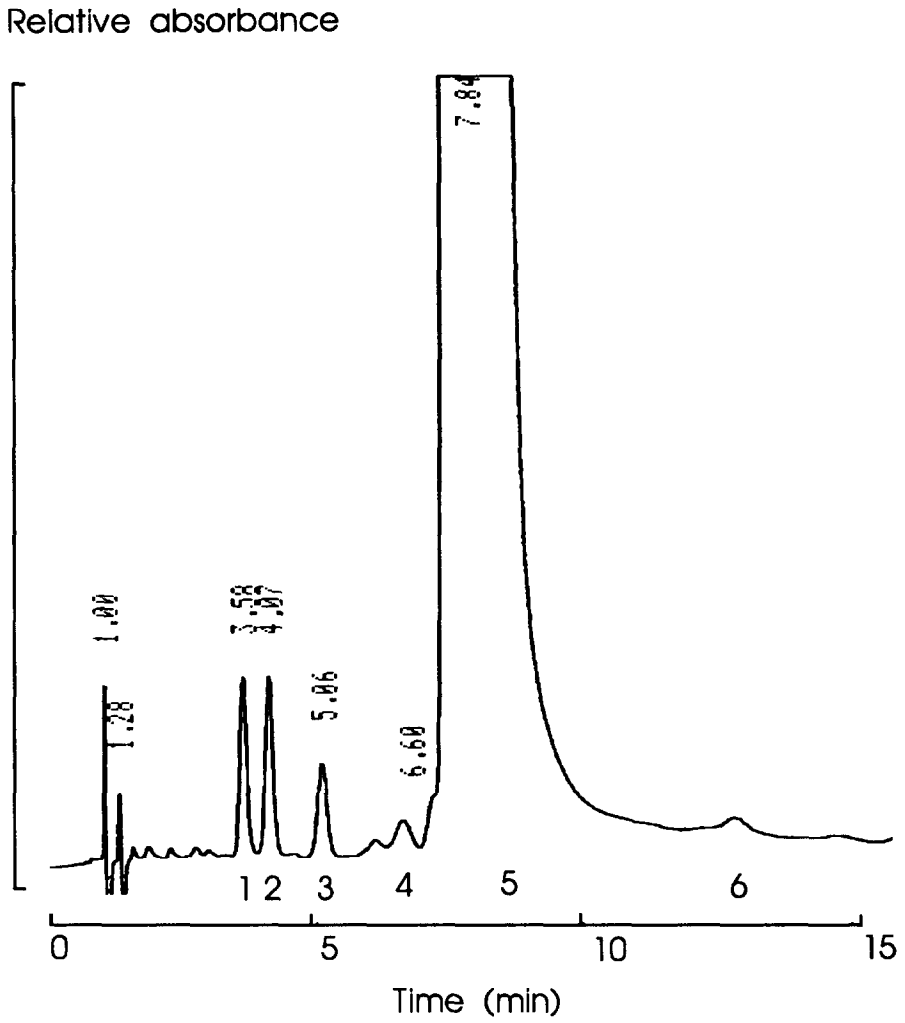


Fig. 3. Original HPLC chromatogram of a sample of *Rp*-cAMPS lot 13, showing relative absorbance at 254 nm. Retention times in min are shown above the peaks. Authentic adenosine is indicated by peak 1 which represents 0.05% of total material. Authentic *Rp*-cAMPS is indicated by peak 5, which represents 99.7% of total material. Other peaks are unknown substances. Similar results were seen for lot 12.

an authentic sample and by a corresponding UV spectrum from a diode array detector, represents 0.05% of the material. However, even though the substance is of high purity, a cuvette with a final concentration of 100 $\mu$M *Rp*-cAMPS would also contain about 0.05 $\mu$M exogenous adenosine. This value is consistent with the data on inhibition of $O_2^{\cdot-}$ production, which suggests that, if adenosine is the sole active agent, its concentration is less than 0.1 $\mu$M, as XAC and adenosine desaminase completely blocked the inhibitory effect of *Rp*-cAMPS preparations but only partially inhibited the effect of 0.1 $\mu$M adenosine.

Our functional results cannot distinguish between adenosine and other by-products of *Rp*-cAMPS synthesis that are adenosine receptor agonists and substrates for adenosine desaminase. However, there appears to be sufficient authentic adenosine present to account for most of the inhibition of fMLP-stimulated $O_2^{\cdot-}$ production.

Regardless of whether adenosine is the sole active agent in these preparations, our results suggest that *Rp*-cAMPS should be used with caution in intact cells or tissues which contain adenosine receptors. Activation of either adenosine $A_1$ receptors (mediating inhibition of adenylate cyclase) or adenosine $A_2$ receptors (mediating activation of adenylate cyclase) could result in misinterpretation of experimental results. Although we have tested only the BioLog *Rp*-cAMPS here, the synthesis and purification method [2] suggests that most commercial preparations must contain some adenosine (or substances with adenosine receptor agonist activity). In addition another potential source for traces of adenosine must be taken into account. In spite of having sufficient stability for most applications in cell culture experiments, *Rp*-cAMPS slowly loses sulfur (0.1%/week at 20°C and 0.2%/year at −20°C) [9] yielding cAMP which is metabolized via adenosine. cAMP release from *Rp*-cAMPS is drastically increased by strong oxidizing

agents. Among others hydrogen peroxide, ozone or oxiranes [10] are able to convert phosphorothioates into corresponding cyclic phosphates. Presently there is no evidence that $O_2^{\cdot-}$ also accelerates degradation of *Rp*-cAMPS to cAMP, but it is reasonable to assume that it would.

So, especially after prolonged storage in solution, there could be a risk of formation of some adenosine, provided that the necessary set of metabolic enzymes is present. For example, this could be the case when using cell culture media containing serum.

In preliminary experiments, batches of *Rp*-cAMPS were enzymatically pretreated during the purification process. One batch (lot 15) was treated with adenosine desaminase and another batch (lot 17) was treated with cyclic nucleotide phosphodiesterases and alkaline phosphatase, to degrade all potential adenosine-releasing nucleotide by-products, and finally with adenosine desaminase. Despite the disappearance of the adenosine peak from HPLC traces of both batches, *Rp*-cAMPS solutions from lot 15 inhibited fMLP-stimulated $O_2^{\cdot-}$ formation to a similar extent as the *Rp*-cAMPS solutions from lots 12 and 13 (data not shown). *Rp*-cAMPS solutions from lot 17 produced a much smaller inhibition, which was reversed by XAC (data not shown). These results suggest that adenosine, or adenosine-like substances, is produced during storage of purified *Rp*-cAMPS. Further experiments are presently underway to determine the best method of removal of contaminants from *Rp*-cAMPS preparations, to reduce degradation of *Rp*-cAMPS during storage and to determine the effects of $O_2^{\cdot-}$ on cell-permeable cyclic nucleotides.

We therefore recommend that at this time all experimental protocols should include either adenosine desaminase or an adenosine receptor blocker to avoid possible interference from activation of adenosine receptors.

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