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^-) 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^- 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^- 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^- 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^- production in human neutrophils by isoprenaline. However, in our experiments, Rp-cAMPS by itself *inhibited* the fMLP-stimulated O_2^- production. We show here that this inhibition is due to adenosine pres-

ent 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.

2. MATERIALS AND METHODS

Human neutrophils from young healthy male and female volunteers were isolated as previously described [6] and O_2^- 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 × 10⁶ 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^- 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.

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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^-, superoxide anion; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; XAC, xanthine amine congener.

3. RESULTS AND DISCUSSION

fMLP (1 μM) stimulated the formation of 6.01±0.33 nmol $O_2^-/10^6$ cells. Rp-cAMPS, at a concentration used in other intact cell experiments (100 μM) [2–5] unexpectedly produced a profound inhibition of fMLP-stimulated O_2^- 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^- 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^- production, with XAC (0.1 μM; 13.1 ± 0.5 nmol $O_2^-/10^6$ cells) being more potent than DPCPX (0.1 μM; 9.9 ± 0.8 nmol $O_2^-/10^6$ cells). The inhibitory effect of Rp-cAMPS (100 μM) was blocked by XAC (0.1 μM) and was unaffected by DPCPX at the same concentration. The inhibitory effect of adenosine (0.1 μM) 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 sub-
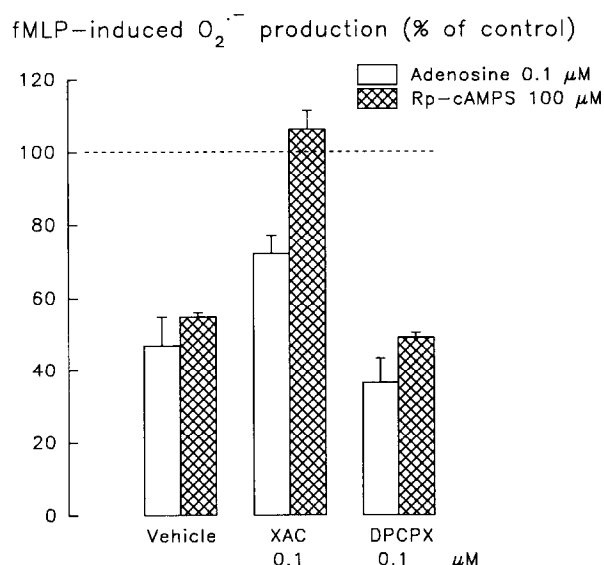
Fig. 2. The effect of adenosine desaminase on the inhibition by isoprenaline (ISO), Rp-cAMPS, cAMP and adenosine (ADO) of the fMLP (1 μM) stimulated production of O_2^- from human neutrophils. Test substances and adenosine desaminase (ADA, 0.02 mg/ml) or vehicle were added to the cells 3 min before addition 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.

stances on fMLP-stimulated O_2^- 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^- 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^- production (see Fig. 2), which was substantially reduced by adenosine desaminase. The inhibitory effect of 1 μM adenosine was reduced by this low concentration of adenosine desaminase (see Fig. 2) and the inhibitory effect of 0.1 μM adenosine was also substantially reduced (data not shown). Isoprenaline (10 nM) inhibited fMLP-stimulated O_2^- production to a similar extent as Rp-cAMPS (100 μM). 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^- 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^- production from human neutrophils. Test substances were added to the cells 3 min before addition of fMLP (1 μM). 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.
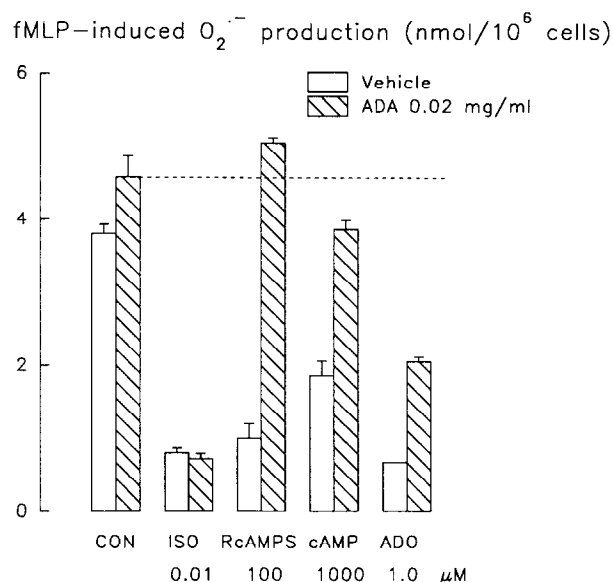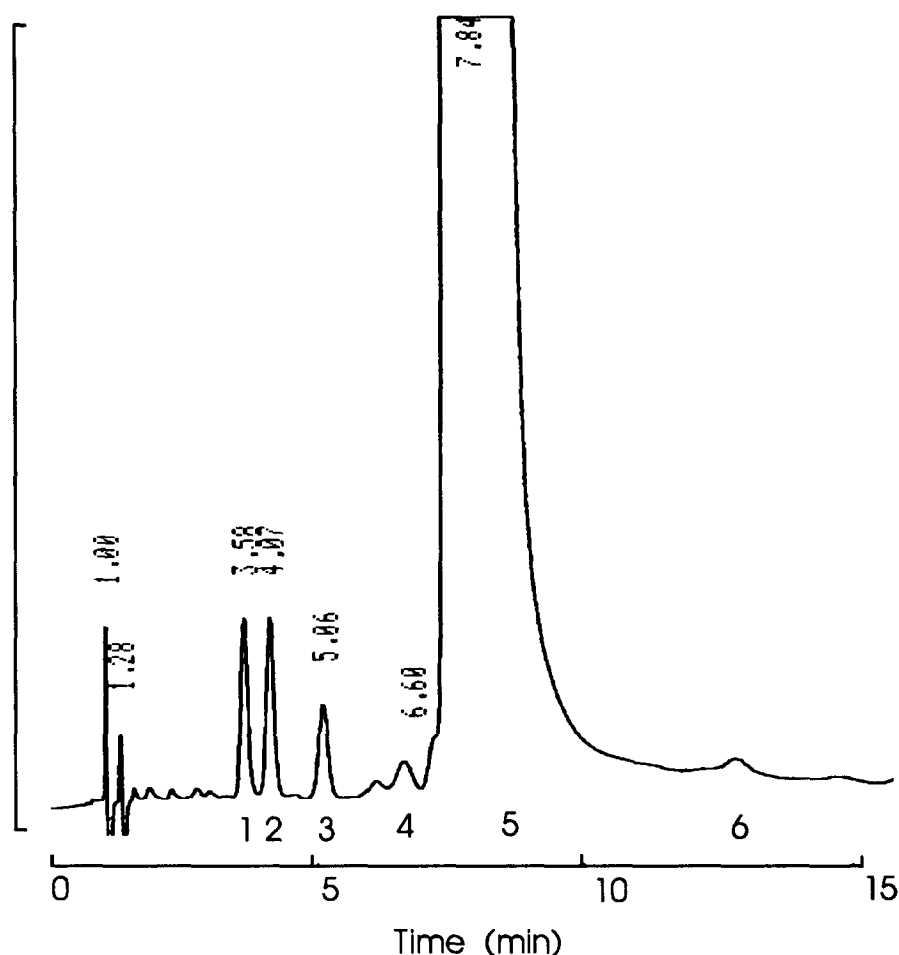
Relative absorbance

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 μM Rp-cAMPS would also contain about 0.05 μM exogenous adenosine. This value is consistent with the data on inhibition of O_2^- production, which suggests that, if adenosine is the sole active agent, its concentration is less than 0.1 μM, as XAC and adenosine desaminase completely blocked the inhibitory effect of Rp-cAMPS preparations but only partially inhibited the effect of 0.1 μM 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^- 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^- 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 deaminase 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 deaminase. Despite the disappearance of the adenosine peak from HPLC traces of both batches, Rp-cAMPS solutions from lot 15 inhibited fMLP-stimulated O_2^- 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^- on cell-permeable cyclic nucleotides.

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

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