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Modulation of the chemotactic peptide- and immunoglobulin G-triggered respiratory burst in human neutrophils by exogenous and endogenous adenosine

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Abstract

The effects of exogenous and endogenous adenosine on the production of oxygen metabolites in neutrophils triggered by the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or immunoglobulin G (IgG)-opsonized yeast particles, were investigated. By using luminol-enhanced chemiluminescence, we found that adenosine A_1 receptor activation did not affect, whereas adenosine A_2 receptor activation, through a mechanism involving the cyclic AMP (cAMP)–protein kinase A signalling pathway, both inhibited the fMLP- and IgG-triggered respiratory burst. The adenosine-induced inhibition was however more pronounced after exposure to fMLP than to IgG-yeast. Stimulation with fMLP caused an extracellular accumulation of endogenous adenosine, which indicates that this event is a negative-feedback mechanism preventing an uncontrolled activation of chemoattractant-stimulated neutrophils. On the contrary, exposure of neutrophils to IgG-yeast did not appear to accumulate extracellular adenosine, probably due to increased adenosine deaminase activity during phagocytosis. In conclusion, this work accentuates the importance of adenosine, both exogenously applied and endogenously formed, as an inflammatory agent modulating the respiratory burst during the different phases in neutrophil activation.

Keywords: Adenosine; Neutrophil; Respiratory burst; Chemiluminescence; cAMP

1. Introduction

Stimulation of neutrophils with different types of stimuli results in an increased oxygen consumption, termed as the respiratory burst. By the action of a membrane bound NADPH-oxidase and via other enzymatic reactions, molecular oxygen is converted to various toxic oxygen compounds, e.g., superoxide anions, hydrogen peroxide and chloramines, which are used in the killing of foreign pathogens (Rosen et al., 1995). A normally functioning NADPH-oxidase is vital in host-defence, since defects in this enzyme-complex, as shown in chronic granulomatous disease, ultimately lead to recurrent life-threatening infections (Roos, 1994).

In addition to the beneficial role in host defence, the release of toxic oxygen radicals and proteolytic enzymes from activated neutrophils can cause injury to normal tissues during inflammatory processes and ischemic syndromes (Ernst et al., 1987; Weiss, 1989). Among the biochemical processes to limit severe tissue injury during these conditions, the release of endogenous adenosine is considered to be one of the most important. For instance, Cronstein et al. (1986) have shown that adenosine released from cultured endothelial cells inhibits neutrophil-mediated injury of these cells. Furthermore, anti-inflammatory drugs such as methotrexate and sulfasalazine are suggested to act by increasing the extracellular level of adenosine (Cronstein et al., 1993; Gadangi et al., 1996). Adenosine has been shown to exert both pro- and anti-inflammatory effects on neutrophil function by interacting with high affinity adenosine A_1 and low affinity adenosine A_2 receptors, respectively. Occupancy of adenosine A_1 receptors

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promotes adhesion, chemotaxis and phagocytosis, whereas adenosine A_2 receptor occupancy inhibits most neutrophil functions including the respiratory burst (for reviews, see Cronstein, 1994; Fredholm, 1997). Adenosine is predominantly formed (both intra- and extracellularly) through breakdown of AMP by the action of 5′-nucleotidases and can be metabolized by adenosine kinase-mediated phosphorylation to AMP, or through deamination by adenosine deaminase, giving inosine as a product (Fox and Kelley, 1978). Adenosine also readily traverses cell membranes via specific nucleoside transport proteins (Thorn and Jarvis, 1996). The level of extracellular adenosine can be modulated by the use of specific inhibitors interfering with the metabolism or transport of this nucleoside (Cronstein, 1995). It was recently shown that the novel adenosine kinase inhibitor GP515 (4-amino-1-(5-amino-5-deoxy-1-β-D-ribofuranosyl)-3-bromo-pyrazolo-[3,4-D]pyrimidine), by increasing the extracellular level of adenosine, not only decreased neutrophil accumulation into inflamed tissue, but also reduced histamine- or bradykinin-induced vascular leakage (Rosengren et al., 1995).

Cyclic AMP (cAMP) is considered to be a physiological modulator of oxygen radical production in stimulated neutrophils, and it is well known that an elevation of intracellular cAMP by physiological cAMP agonists (e.g., prostaglandin E_2, histamine or β-adrenergic drugs) inhibits the respiratory burst elicited by the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Coffey, 1992). The role of cAMP in mediating the adenosine A_2 receptor-induced reduction of the respiratory burst in neutrophils is controversial. Previous findings show that occupancy of adenosine A_2 receptors via G_s proteins activates adenylate cyclase (Stiles, 1992), and several investigators have thus suggested that adenosine reduces fMLP-stimulated production of oxygen radicals by elevating cAMP (Iannone et al., 1987; Nielson and Vestal, 1989; Mueller et al., 1992; Fredholm et al., 1996). However, other researchers have reached the opposite conclusion, i.e., that cAMP is not involved in the inhibitory effects of adenosine (Cronstein et al., 1988, 1992a; Cronstein and Haines, 1992; Revan et al., 1996; Spisani et al., 1996).

The effects and mechanisms of adenosine on neutrophil oxygen radical production in response to phagocytic stimuli have been poorly investigated. Kubersky et al. (1989) found that adenosine attenuated complement-opsonized zymosan-stimulated, but not immune complex-stimulated oxygen radical production. We have previously shown that adenosine or an adenosine deaminase inhibitor, inhibits the stimulatory effects of platelets on the respiratory burst triggered by immunoglobulin G (IgG)-opsonized yeast particles (Zalavary et al., 1996).

In the present study, we evaluated the role of exogenous and endogenous adenosine in the regulation of neutrophil oxygen radical production, triggered by two different types of stimuli, fMLP and IgG-opsonized yeast particles, by using luminol-amplified chemiluminescence.

2. Materials and methods

2.1. Chemicals

The materials and their sources were as follows: dextran (Kabi-Pharmacia, Uppsala, Sweden); sodium metrizoate (Nycomed Pharma, Oslo, Norway); Ficoll–Paque (Pharmacia Biotech, Uppsala, Sweden); rabbit polyclonal anti-yeast IgG (Dakopatts, Glostrup, Denmark); N^6-cyclopentyladenosine (CPA), 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), erythro-9-(-2-hydroxy-3-nonyl)adenine (EHNA), iodotubercidin and 4-(3-butoxy-4-metoxybenzyl)-2-imidazolidinone (Ro 20-1724; Research Biochemicals, Natick, MA, USA); catalase and *S*-(4-nitrobenzyl)-6-thioguanosine (NBTG; Boehringer Mannheim, Mannheim, Germany); *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89; Calbiochem, San Diego, CA, USA); 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol (ZM 241385; Tocris Cookson, Bristol, UK); adenosine, 2′-deoxyadenosine, 5′-*N*-ethylcarboxamidoadenosine (NECA), adenosine deaminase, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), horse radish peroxidase, inosine, isoproterenol, 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), phorbol myristate acetate (PMA), dibutyryl cAMP, superoxide dismutase and theophylline (Sigma, St. Louis, MO, USA). All reagents were of analytical grade. Some chemicals were dissolved in dimethyl sulfoxide (DMSO) and further diluted in buffer (final DMSO concentration was at maximum 0.2%). Equivalent concentrations of DMSO were used in control experiments. The substances tested in this study had no significant effect on cell viability, as assessed by Trypan blue exclusion.

2.2. Isolation of human neutrophils

Neutrophils were isolated from heparinized human peripheral blood, essentially according to the method described by Böyum (1968). In short, erythrocytes were removed by dextran-sodium metrizoate sedimentation and hypotonic lysis. The preparation was then centrifuged on a Ficoll–Paque gradient to exclude lymphocytes, monocytes and platelets. A Ca^{2+}-free Krebs–Ringer phosphate buffer, supplemented with 10 mM glucose and 1.5 mM $MgSO_4$ (KRG, pH 7.3), was used for washing. After isolation, the neutrophils were suspended in KRG supplemented with 1 mM $CaCl_2$ (KRG with Ca^{2+}) and the cell density was determined with a Coulter Counter ZM Channelyser 256 (Coulter-Electronics, Luton, UK). The isolated neutrophils were stored on ice before use.

2.3. Preparation of phagocytic prey

Heat-killed *Saccharomyces cerevisiae* (10^8 particles/ml) were IgG-opsonized by incubation (37°C, 30

min) with purified rabbit anti-yeast IgG (20 μg/ml) in the presence of 20% heat-inactivated (56°C, 30 min) human serum (Zalavary et al., 1996). After incubation the yeast particles were washed in KRG with Ca^{2+} and resuspended to the appropriate concentration. In some experiments IgG-yeast, opsonized in the absence of serum, were used as prey.

2.4. Chemiluminescence

Neutrophils (1×10^6/ml) suspended in KRG with Ca^{2+} in polycarbonate tubes were preincubated for 5 min at 37°C in the absence or presence of the chemicals to be tested (when testing H89, preincubation was performed for 10 min), supplemented with luminol (final concentration 50 μM), stimulated with fMLP (10^{-7} M) or IgG-opsonized yeast particles (5×10^6/ml) and monitored for chemiluminescence (CL) in a 6-channel Biolumat LB 9505 (Berthold, Wildbaden, Germany). Since peroxidase is often a limiting factor in luminol-enhanced CL, extra peroxidase (horse radish peroxidase; 4 U) was added to the tubes in some experiments. Measurements in the horse radish peroxidase-system reflected the total CL-activity. The intracellular part of the CL-response was defined as the activity obtained in the presence of superoxide dismutase (200 U) and catalase (2000 U), large molecular O_2^- and H_2O_2 scavengers, respectively (Dahlgren et al., 1991; Bengtsson et al., 1996).

2.5. Statistics

Data are expressed as the mean ± S.E.M. Statistical differences between means were assessed by the unpaired, two-tailed Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Exogenous adenosine inhibits both fMLP- and IgG-stimulated production of oxygen metabolites through adenosine A_2 receptor occupancy

Exposure of human neutrophils (1×10^6/ml) to fMLP (10^{-7} M) or IgG-opsonized yeast particles (5×10^6/ml) resulted in different kinetics of the CL-responses. The chemotactic peptide caused an immediate response that peaked seconds after stimulation and thereafter decreased rapidly within 10 min to basal levels. In contrast, exposure of neutrophils to IgG-yeast induced a slower CL-response, that peaked after 20 min and lasted for more than 60 min. Low concentrations of adenosine (10^{-13}–10^{-9} M), which promote binding to the adenosine A_1 receptor (Cronstein, 1994), did not significantly influence the generation of oxygen metabolites, whereas higher concentrations (10^{-9}–10^{-5} M), promoting binding to the adenosine A_2 receptor (Cronstein, 1994), dose-dependently inhibited the production in response to both types of stimuli (Fig. 1A). Adenosine more effectively inhibited the fMLP-triggered than the IgG-triggered respiratory burst, with a maximum inhibition of 78 ± 1% at 10^{-6} M adenosine ($n = 6$) and 36 ± 4% at 10^{-5} M ($n = 4$), respectively. To investigate whether the effects of adenosine on NADPH-oxidase activity are mediated by occupation of different classes of adenosine surface receptors, we used N^6-cyclopentyladenosine (CPA) and 5′-*N*-ethylcarboxamidoadenosine (NECA), agonists for the adenosine A_1 and the adenosine A_2 receptor, respectively. The addition of CPA (10^{-12}–10^{-8} M) did not affect the fMLP-induced CL-response, whereas it slightly reduced the CL elicited by IgG-yeast (Fig. 1B). Similar effects of CPA were obtained when using submaximal

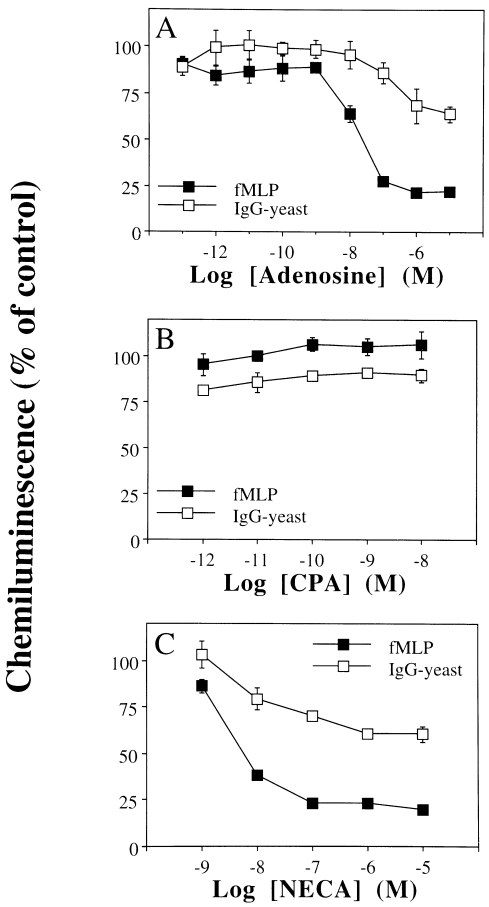

Fig. 1. Effects of adenosine and adenosine analogues on the production of oxygen metabolites. Neutrophils (1×10^6/ml) were incubated for 5 min at 37°C in the absence (control) or presence of adenosine (10^{-13}–10^{-5} M; A), the adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA; 10^{-12}–10^{-8} M; B) or the adenosine A_2 receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA; 10^{-9}–10^{-5} M; C). The cells were then stimulated with fMLP (10^{-7} M; filled squares) or IgG-opsonized yeast particles (5×10^6/ml; open squares) and monitored for luminol-amplified chemiluminescence. The data are based on integral values of chemiluminescence recorded over a period of 10 min (fMLP) or 30 min (IgG-yeast). Results are expressed as percent of control chemiluminescence and represent the mean ± S.E.M. of four to nine separate experiments (the error bars sometimes lie within the size of the symbols).

doses of stimuli (fMLP: 1, 2.5 and 5×10^{-8} M; IgG-yeast: 0.5, 1 and 2×10^{6} particles/ml; data not shown). Both the fMLP- and IgG-stimulated production of oxygen metabolites were more sensitive to an inhibition by the adenosine A_2 agonist NECA (10^{-9}–10^{-5} M) than to adenosine (Fig. 1A and C). However, the maximal inhibition by NECA did not differ from that obtained with adenosine ($80 \pm 2\%$ reduction, $n = 9$, and $40 \pm 4\%$ reduction, $n = 5$; at 10^{-5} M NECA after exposure to fMLP and IgG-yeast, respectively). The selective adenosine A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680; 10^{-9}–10^{-5} M) did not significantly differ from NECA (10^{-9}–10^{-5} M) in the potency of inhibiting the production of oxygen radicals (data not shown).

To further demonstrate that the adenosine-induced inhibition of CL is mediated by binding of adenosine to its specific receptors, we tested the effects of the naturally occurring ribose-modified adenosine analogue 2′-deoxyadenosine, which has poor affinity to adenosine receptors (Gilbertsen, 1987). As compared to adenosine, 2′-deoxyadenosine (10^{-5} M) did not affect the fMLP-induced CL-response ($2 \pm 2\%$ reduction, $n = 5$), whereas it slightly reduced the IgG-stimulated production of oxygen radicals ($18 \pm 2\%$ inhibition, $n = 5$). Adenosine is rapidly deaminated to inosine by adenosine deaminase in vivo. Exogenous inosine (10^{-5} M) affected the CL-responses in the same way as 2′-deoxyadenosine, i.e., no dramatical effects were registered ($1 \pm 3\%$ and $21 \pm 5\%$ reduction of the fMLP- and IgG-triggered respiratory burst, respectively; $n = 4$). Further evidence for an extracellular target site of adenosine was obtained by the finding that the specific adenosine transport inhibitor *S*-(4-nitrobenzyl)-6-thioguanosine (NBTG; 10^{-5} M) did not reverse the inhibitory effects of adenosine (data not shown). The possibility that adenosine decreases CL by acting as an oxygen metabolite scavenger and/or by interfering with the light emitting reaction, e.g., by reacting with luminol, was excluded by the finding that adenosine did not affect the CL-response elicited by the potent NADPH-oxidase activator phorbol myristate acetate (PMA, 10^{-7} M; data not shown).

3.2. The effects of adenosine on the intra- and extracellular production of oxygen radicals

The CL-technique enables a separate measurement of extra- and intracellularly produced oxygen radicals, respectively (Dahlgren et al., 1991). Exposure of neutrophils to fMLP caused an initial potent extracellular CL-response, followed by a weaker and more delayed intracellular response. Of the total production of oxygen radicals induced by fMLP, $75 \pm 4\%$ ($n = 5$) was released extracellularly and $25 \pm 4\%$ ($n = 5$) intracellularly (Fig. 2A). On the contrary, IgG-opsonized yeast particles predominantly induced an intracellular CL-response ($75 \pm 6\%$ of the total

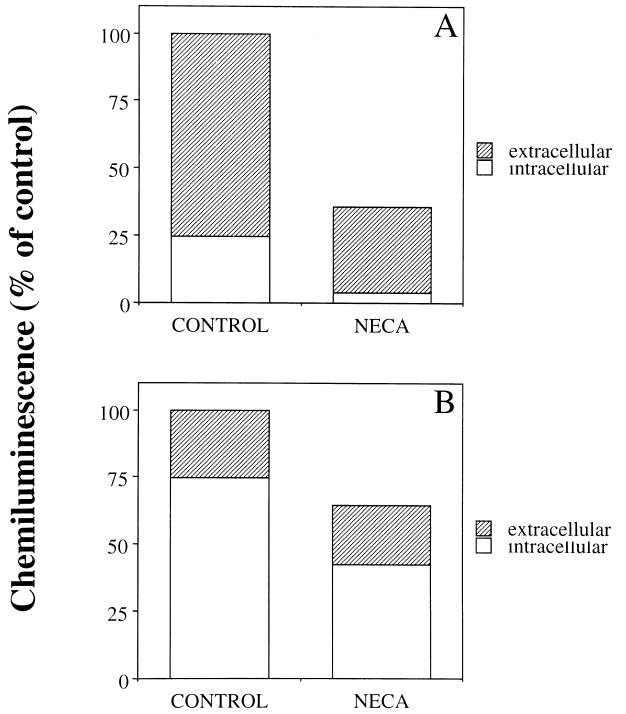

Fig. 2. Effects of adenosine A_2 receptor activation on the extra- and intracellular generation of oxygen metabolites. Neutrophils (1×10^{6}/ml) were incubated for 5 min at 37°C in the absence (control) or presence of NECA (10^{-6} M). The cells were then stimulated with fMLP (10^{-7} M; A) or IgG-opsonized yeast particles (5×10^{6}/ml; B) and monitored for luminol-amplified chemiluminescence in the presence of extra peroxidase (horse radish peroxidase, 4U; total activity is shown by the open + hatched area) or a combination of the scavengers superoxide dismutase (200 U) and catalase (2000 U; intracellular activity is shown by the open area). The data are based on integral values of chemiluminescence recorded over a period of 10 min (fMLP) or 30 min (IgG-yeast) and are expressed as percent of the neutrophil control stimulated in the presence of horse radish peroxidase. The values represent the mean of five separate experiments.

response, $n = 5$; Fig. 2B). NECA (10^{-6} M) effectively reduced the large extracellular production of oxygen radicals induced by fMLP, by about 60%, and also markedly inhibited the smaller intracellular production by more than 80% (Fig. 2A). The predominant intracellular CL-activity obtained during phagocytosis was reduced by over 40% by NECA, whereas the small extracellular CL-activity was almost unaffected (14% inhibition; Fig. 2B).

3.3. Neutrophils release endogenous adenosine when exposed to fMLP but not to IgG-yeast

Release of endogenous adenosine has been considered to function as a negative feedback mechanism in neutrophil activation (Cronstein, 1994). We studied this autocrine mechanism by preincubating neutrophils with adenosine deaminase (0.25 U/ml), which deaminates extracellularly located adenosine to inosine, or the adenosine receptor antagonist theophylline (10^{-5} M). We found that

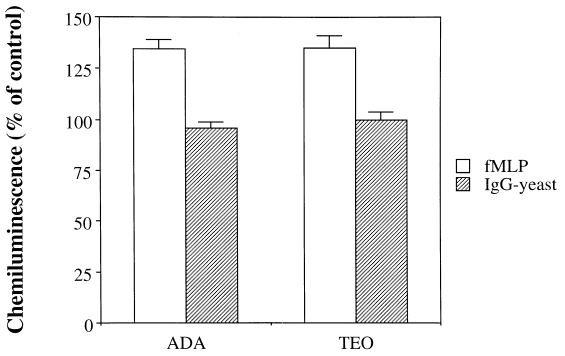

Fig. 3. Effects of adenosine deaminase and theophylline on the generation of oxygen metabolites. Neutrophils (1×10^6/ml) were incubated for 5 min at 37°C in the absence (control) or presence of adenosine deaminase (ADA; 0.25 U/ml) or the adenosine receptor antagonist theophylline (TEO; 10^{-5} M). The cells were then stimulated with fMLP (10^{-7} M; open bars) or IgG-opsonized yeast particles (5×10^6/ml; hatched bars) and monitored for luminol-amplified chemiluminescence. The data are based on integral values of chemiluminescence recorded over a period of 10 min (fMLP) or 30 min (IgG-yeast). Results are expressed as percent of control chemiluminescence and represent the mean ± S.E.M. of nine separate experiments.

these agents enhanced the fMLP-induced CL-response by about 35%, whereas they did not affect the IgG-stimulated response (Fig. 3). Similar effects, i.e., stimulatory effects on the fMLP-triggered production of oxygen radicals, were obtained by the selective adenosine A_{2A} receptor antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385; 10^{-5} M), but not with the specific adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 10^{-5} M; data not shown). This suggests that adenosine is released into the neutrophil suspension after stimulation with fMLP and reduces the respiratory burst by occupying adenosine A_2 receptors, whereas exposure of neutrophils to IgG-yeast does not accumulate adenosine extracellularly. The presence of adenosine deaminase, theophylline or ZM 241385, but not DPCPX, reversed the inhibitory effects of exogenous adenosine on oxygen radical production, further implicating that the suppressive effects are not mediated by metabolization of adenosine to inosine, but via binding of adenosine to surface adenosine A_2 receptors on the neutrophil (data not shown).

3.4. Inactivation of adenosine kinase or adenosine deaminase differently affects fMLP- and IgG-stimulated production of oxygen radicals

The adenosine kinase inhibitor iodotubercidin and the adenosine deaminase inhibitor erythro-9-(-2-hydroxy-3-nonyl)adenine (EHNA) have been extensively used to elucidate the role of endogenous adenosine in various types of cells (Newby, 1981; Van Waeg and Van den Berghe, 1991; Barankiewicz et al., 1995). We found that EHNA and iodotubercidin, respectively, dose-dependently (10^{-7}–10^{-5} M) reduced the CL-responses induced by both types of stimuli (Fig. 4A and B). Iodotubercidin more effectively reduced the fMLP-triggered than the IgG-triggered respiratory burst, with a maximum inhibition of $53\pm2\%$ ($n=8$) and $18\pm3\%$ ($n=8$), respectively, at 10^{-5} M iodotubercidin (Fig. 4A). The situation was opposite considering EHNA, which more potently reduced IgG-stimulated production of oxygen radicals (maximum inhibition: $25\pm2\%$ and $43\pm2\%$ at 10^{-5} M EHNA after exposure to fMLP and IgG-yeast, respectively, $n=8$; Fig. 4B). The inhibition of the neutrophil respiratory burst by iodotubercidin or EHNA was reversed ($P<0.05$) by theophylline (10^{-5} M), indicating that the inhibitory effects are mediated by an increased accumulation of extracellular

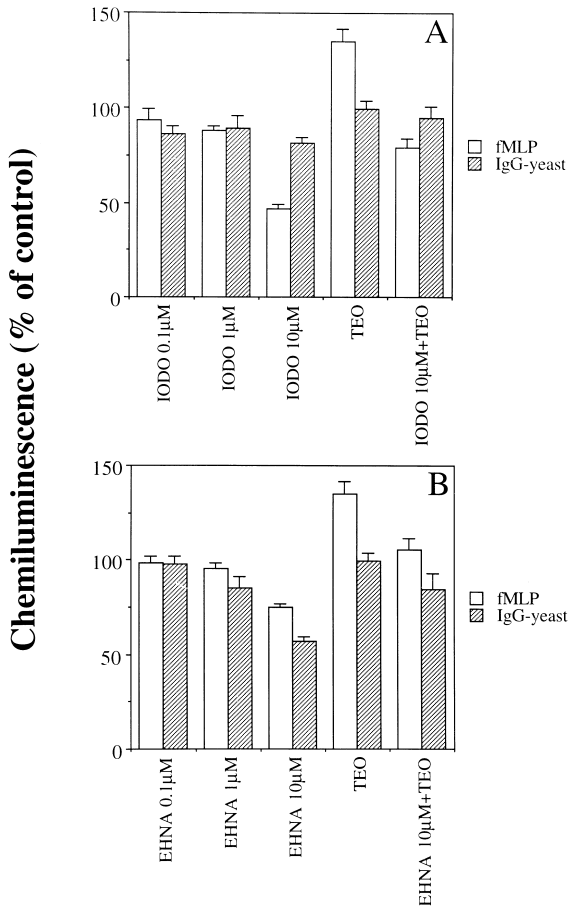

Fig. 4. Inactivation of adenosine kinase or adenosine deaminase differently affects the fMLP- and IgG-triggered respiratory burst. Neutrophils (1×10^6/ml) were incubated for 5 min at 37°C in the absence (control) or presence of the adenosine kinase inhibitor iodotubercidin (IODO, 10^{-7}–10^{-5} M; A), the adenosine deaminase inhibitor erythro-9-(-2-hydroxy-3-nonyl)adenine (EHNA, 10^{-7}–10^{-5} M; B), theophylline (TEO; 10^{-5} M; A and B) or the combinations iodotubercidin (10^{-5} M) and theophylline (10^{-5} M; A), or EHNA (10^{-5} M) and theophylline (10^{-5} M; B). The cells were then stimulated with fMLP (10^{-7} M; open bars) or IgG-opsonized yeast particles (5×10^6/ml; hatched bars) and monitored for luminol-amplified chemiluminescence. The data are based on integral values of chemiluminescence recorded over a period of 10 min (fMLP) or 30 min (IgG-yeast). Results are expressed as percent of control chemiluminescence and represent the mean ± S.E.M. of five to eight separate experiments.

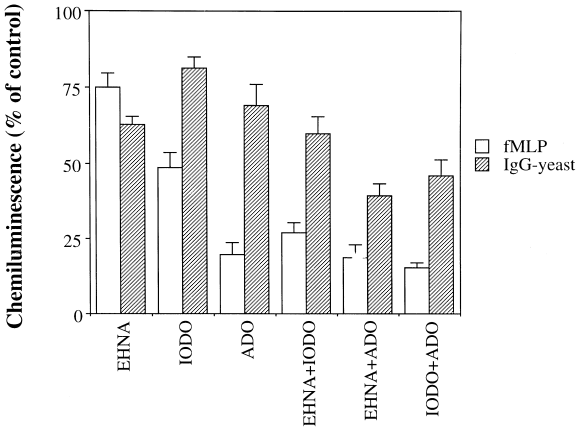

Fig. 5. Combining adenosine with EHNA or iodotubercidin further reduces IgG-stimulated but not fMLP-stimulated oxygen radical production. Neutrophils (1×10^6/ml) were incubated for 5 min at 37°C in the absence (control) or presence of EHNA (10^{-5} M), iodotubercidin (IODO; 10^{-5} M), adenosine (ADO; 10^{-5} M) or various combinations of these agents. The cells were then stimulated with fMLP (10^{-7} M; open bars) or IgG-opsonized yeast particles (5×10^6/ml; hatched bars) and monitored for luminol-amplified chemiluminescence. The data are based on integral values of chemiluminescence recorded over a period of 10 min (fMLP) or 30 min (IgG-yeast). Results are expressed as percent of control chemiluminescence and represent the mean ± S.E.M. of four to five separate experiments.

adenosine, activating adenosine surface receptors (Fig. 4A and B). The specificity of EHNA (10^{-5} M) was confirmed, since it blunted ($P < 0.05$) the stimulatory effect of adenosine deaminase (0.25 U/ml) on the fMLP-induced CL-response from 135 ± 4% ($n = 13$) to 97 ± 5% ($n = 5$).

A combination of EHNA (10^{-5} M) and iodotubercidin (10^{-5} M) more strongly inhibited ($P < 0.05$) the production of oxygen radicals in response to fMLP but not to IgG-yeast, as compared to the inhibition obtained by these agents alone (Fig. 5). Combining adenosine (10^{-5} M) with EHNA (10^{-5} M) or iodotubercidin (10^{-5} M) did not further reduce fMLP-stimulated CL, whereas these combinations further inhibited ($P < 0.05$) IgG-stimulated CL (Fig. 5).

Cronstein et al. (1987) have suggested that adenosine deaminase from serum can be adsorbed to the yeast cell walls during the opsonization procedure, which thereby might explain the inhibitory effect of EHNA (Figs. 4 and 5), and the lack of effect of exogenous adenosine deaminase or theophylline (Fig. 3), on the yeast-stimulated production of oxygen radicals. However, we found that EHNA (10^{-5} M) induced similar relative inhibition when exposing neutrophils to IgG-yeast, which had been opsonized in the absence of serum (43 ± 7% and 43 ± 8% inhibition after stimulation with IgG-yeast, opsonized in the absence and presence of serum, respectively; $n = 6$). Furthermore, treatment of neutrophils with theophylline or exogenous adenosine deaminase did not affect the production of oxygen radicals, elicited by IgG-yeast opsonized in the absence of serum (data not shown).

3.5. The inhibitory effects of adenosine on the respiratory burst involve activation of the cAMP–protein kinase A signalling pathway

It is known that adenosine A_2 receptors are coupled via G_s proteins to adenylate cyclase (Stiles, 1992). Since cAMP elevations and the associated activation of protein kinase A have been found to inhibit neutrophil function, we investigated if adenosine inhibits the respiratory burst by this mechanism. We found that the membrane-permeant cAMP analogue dibutyryl cAMP (3×10^{-4} M), the β-adrenoceptor agonist isoproterenol (10^{-5} M) and the cAMP-specific (class IV) phosphodiesterase inhibitor 4-

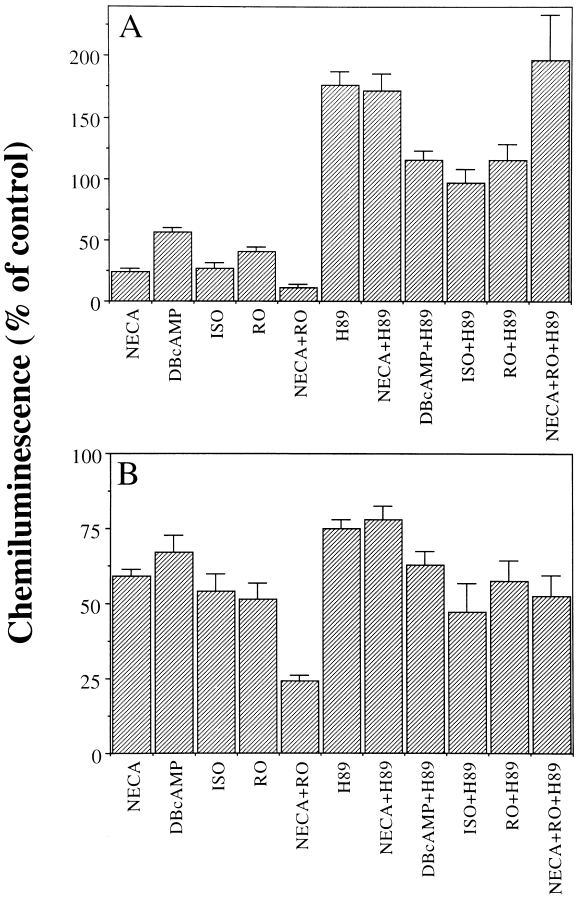

Fig. 6. The adenosine-induced inhibition of the respiratory burst involves activation of the cAMP–protein kinase A signalling cascade. Neutrophils (1×10^6/ml) were incubated for 5 min at 37°C in the absence (control) or presence of NECA (10^{-6} M), dibutyryl cAMP (DBcAMP; 3×10^{-4} M), the β-adrenergic agonist isoproterenol (ISO; 10^{-5} M), the class IV phosphodiesterase inhibitor Ro 20-1724 (RO; 10^{-5} M), the protein kinase A inhibitor H89 (10^{-5} M; 10 min incubation) or various combinations of these agents. The cells were then stimulated with fMLP (10^{-7} M; A) or IgG-opsonized yeast particles (5×10^6/ml; B) and monitored for luminol-amplified chemiluminescence. The data are based on integral values of chemiluminescence recorded over a period of 10 min (fMLP) or 30 min (IgG-yeast). Results are expressed as percent of control chemiluminescence and represent the mean ± S.E.M. of at least five separate experiments.

(3-butoxy-4-metoxybenzyl)-2-imidazolidinone (Ro 20-1724; 10^{-5} M) reduced the fMLP- and IgG-stimulated production of oxygen metabolites in a similar way as NECA (10^{-6} M; Fig. 6). The presence of Ro 20-1724 (10^{-5} M) further enhanced ($P < 0.05$) the inhibitory effects of NECA on both types of CL-responses. The specific protein kinase A inhibitor *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89; 10^{-5} M) (Chijiwa et al., 1990) markedly potentiated the fMLP-stimulated respiratory burst, and completely reversed ($P < 0.05$) the inhibitory effects of NECA, dibutyryl cAMP, isoproterenol, Ro 20-1724 and the combination of NECA and Ro 20-1724. On the other hand, H89 (10^{-5} M) alone slightly reduced the production of oxygen metabolites elicited by IgG-opsonized yeast particles, but still reversed ($P < 0.05$) the inhibitory effects of NECA in the absence or presence of Ro 20-1724. The specificity of H89 as an inhibitor of cAMP-dependent protein kinase was confirmed by the finding that it did not affect the production of oxygen radicals elicited by the protein kinase C activator PMA (data not shown).

4. Discussion

Inflammatory reactions are responsible for tissue injury in several pathological conditions. For example, IgG-mediated phagocytosis and generation of toxic oxygen radicals in response to deposited immune complexes may contribute to the massive organ damage seen in diseases like systemic lupus erythematosus and rheumatoid arthritis (Deo et al., 1997). Adenosine, exogenously applied or endogenously formed, has been shown to exert potent anti-inflammatory effects, e.g., by inhibiting neutrophil-mediated injury in a variety of cellular systems (reviewed by Cronstein, 1995). Consequently, it is relevant to clinically evaluate drugs which affect the adenosine metabolism by locally increase the extracellular level of this nucleoside. Recent studies have indeed shown that methotrexate and sulfasalazine, agents used in the therapy of rheumatoid arthritis, at least partly exert their anti-inflammatory effects due to this mechanism (Cronstein et al., 1993; Cronstein, 1995; Gadangi et al., 1996). Furthermore, the adenosine kinase inhibitor GP515 has shown promise as a potential therapeutic anti-inflammatory agent (reviewed by Firestein, 1996). However, much work remains if the mechanisms behind the immunomodulatory action of adenosine are to be fully elucidated.

Adenosine modulation of neutrophil function has most frequently been studied regarding its effects on the chemoattractant-induced production of oxygen radicals, whereas little is known about how adenosine regulates the phagocytosis-induced production. In the present study, we evaluated and compared how exogenous and endogenous adenosine affect the neutrophil respiratory burst, triggered by fMLP or IgG-opsonized yeast particles.

We have previously shown that adenosine A_1 receptor occupancy stimulates phagocytosis of IgG-opsonized yeast particles by adherent neutrophils (Zalavary et al., 1994). Furthermore, this subtype of adenosine receptor also mediates stimulatory effects on adhesion (Cronstein et al., 1992b) and chemotaxis (Cronstein et al., 1990) in neutrophils. In the present study, however, we found that adenosine A_1 receptor activation did not affect the generation of oxygen metabolites. The absence of stimulatory effects might be explained by the fact that we studied non-adherent neutrophils. The adenosine A_1 receptor-mediated enhancement of neutrophil function might require continuous cell-substratum interactions, i.e., the effects of adenosine are dependent on a crosstalk between adhesion molecules and receptors of fMLP and IgG, respectively. We demonstrated that micromolar concentrations of adenosine reduced the generation of oxygen metabolites in response to both fMLP and IgG-opsonized yeast particles. A marked inhibition by exogenous adenosine of the fMLP-triggered respiratory burst through activation of adenosine A_2 receptors has previously been well characterized (Cronstein et al., 1985; Iannone et al., 1987; Nielson and Vestal, 1989; Fredholm et al., 1996). In the present study, we also found that the IgG-induced production of oxygen radicals was reduced by adenosine through an adenosine A_2 receptor-mediated mechanism, although the effect was not as pronounced as for fMLP-stimulation. The inhibitory effects of adenosine on the IgG-stimulated respiratory burst are not fully correlated to an impaired phagocytic capacity. For instance, we have recently shown that a combination of NECA and Ro 20-1724, or adenosine and EHNA, which in the present study markedly inhibited the production of oxygen radicals, reduced phagocytosis by only 40% (Zalavary and Bengtsson, 1998). Furthermore, unpublished experiments from our laboratory reveal that NECA inhibits the IgG-triggered respiratory burst to the same extent in cytochalasin B-treated neutrophils (i.e., cells unable to phagocytose) as in untreated neutrophils.

Further evidence for that adenosine exerts inhibitory effects on the neutrophil respiratory burst through activation of specific surface adenosine A_2 receptors were obtained by the following: (1) the ribose-modified analogue 2′-deoxyadenosine, which has poor affinity to adenosine receptors (Gilbertsen, 1987), failed to mimic the effects of native adenosine; (2) deamination of exogenous adenosine to inosine by adenosine deaminase, or direct addition of inosine, did not affect the production of oxygen radicals in a similar way as adenosine, which indicates that extracellular metabolization of adenosine is not necessary; (3) inactivation of the adenosine transport protein by NBTG did not reverse the suppressive actions of adenosine, showing that cellular uptake of adenosine is not required; and (4) the adenosine receptor antagonists theophylline and ZM 241385, but not DPCPX, reversed the inhibitory effects of adenosine.

The CL-technique makes it possible to determine the relative amount of intracellularly produced oxygen metabolites by adding the membrane impermeable scavengers superoxide dismutase and catalase (Dahlgren et al., 1991). We found that stimulation with fMLP and IgG-opsonized yeast particles induced quite different distributions in the CL-activities. Activation of the oxidase with fMLP predominantly induced an extracellular CL-response, whereas IgG-opsonized yeast particles mainly caused an intracellular CL-response. This reflects that the assembly and activation of the NADPH-oxidase primarily is directed to the plasma membrane and to the internal phagosomal membrane during stimulation with fMLP and IgG-yeast, respectively. NECA markedly decreased the extracellular, but even more the intracellular production of oxygen metabolites induced by fMLP. In contrast, NECA almost exclusively reduced the intracellular phagosomal generation of oxygen radicals during phagocytosis. This finding may explain why Kubersky et al. (1989), who used the cytochrome *C* reduction technique, which measures only extracellularly produced O_2^- (Dahlgren et al., 1991), did not observe any effect of adenosine on the IgG-triggered respiratory burst. The inhibitory action of NECA on the intracellular production of oxygen metabolites coincides with the observation that stimulation of adenosine receptors in rat basophilic leukemia cells enhances the activity of antioxidant enzymes, leading to an increased scavenging of intracellular reactive oxygen species (Maggirwar et al., 1994). However, we found that adenosine neither affected the intracellular nor the extracellular production of oxygen radicals triggered by PMA, thus arguing against such a scavenging mechanism. This finding also suggests that the target for adenosine is upstream to protein kinase C activation in the signalling pathway leading to oxidase activation, and that adenosine does not directly block the assembly of the oxidase components.

In most, if not all tissues, adenosine is produced from AMP by the action of 5′-nucleotidase and is converted into inosine or back into AMP via the reactions catalyzed by adenosine deaminase and adenosine kinase, respectively (Fox and Kelley, 1978). The enzymes 5′-nucleotidase and adenosine deaminase also appear as ecto-enzymes on the cell surface in a variety of cellular systems, although they do not seem to be expressed on neutrophils (Van Waeg and Van den Berghe, 1991; Barankiewicz et al., 1995). Consequently, the extracellular concentration of adenosine is primarily determined by the activity of the above-mentioned enzymes and by the rate of uptake or release of adenosine in cells via the nucleoside transporters. The release of endogenous adenosine has been suggested to act as a negative feedback mechanism in neutrophil activation (Cronstein, 1994). In the present study, we found that removal of extracellular adenosine with adenosine deaminase or blocking of adenosine A_2 receptors, enhanced the production of oxygen metabolites triggered by fMLP, whereas they did not affect the IgG-stimulated production. This suggests either that adenosine is produced in neutrophils after stimulation with fMLP but not after stimulation with IgG-yeast, or that adenosine is produced after stimulation with both types of stimuli, but is rapidly metabolized, e.g., by adenosine deaminase or adenosine kinase during phagocytosis. Our finding that the adenosine deaminase inhibitor EHNA effectively reduced the production of oxygen radicals in response to IgG-yeast, and also potentiated inhibition of the IgG-triggered response by exogenous adenosine, led to the hypothesis that phagocytosis is associated with high adenosine deaminase activity. In correlation, we have recently shown that EHNA inhibits the phagocytic capacity of neutrophils (Zalavary and Bengtsson, 1998). Taken together, our data suggest that depending on which type of stimulus that is presented to the neutrophil, the metabolic pathways and/or transport mechanisms of adenosine are differently regulated to properly control the activity of the neutrophil oxidase during the course of inflammation. Hypothetically, neutrophils exposed to high concentrations of chemoattractants in the blood stream or during their migration towards the site of infection, are sensitive to inhibition by adenosine and also release adenosine by themselves to further prevent an uncontrolled activation. However, during phagocytosis at the inflammatory locus, endogenously released adenosine (from the neutrophil or from other cell types) is rapidly degraded through an increased adenosine deaminase activity, thereby permitting a more efficient killing of the pathogens. Interestingly, Cronstein et al. (1987) have suggested that invading microorganisms adsorb adenosine deaminase present in plasma. The source of adenosine deaminase in the present study is unknown. However, we found that the adenosine deaminase activity does not originate from the phagocytic prey. As mentioned above, neutrophils do not appear to express ecto-adenosine deaminase on the plasma membrane (Van Waeg and Van den Berghe, 1991). It is possible that cytosolic adenosine deaminase is released extracellularly or into the phagosome. Alternatively, adenosine deaminase is present in neutrophil granules, which are mobilized to the plasma membrane or to the early phagosome during contact with prey. Elucidation of this question along with a more careful evaluation of the adenosine metabolism in neutrophils resides in future studies.

The mechanisms responsible for the adenosine A_2 receptor-mediated inhibition of neutrophil function have been intensely investigated, especially those leading to the marked blockage of the fMLP-triggered respiratory burst (for reviews, see Cronstein, 1994; Fredholm, 1997). Activation of adenosine A_2 receptors neither affects the affinity nor the number of neutrophil receptors for fMLP (Iannone et al., 1987; Cronstein et al., 1989) and IgG (Salmon and Cronstein, 1990). It is well known that adenosine A_2 receptors couple to G_s proteins and thus stimulate adenylate cyclase (Stiles, 1992). Elevations of cAMP inhibit several neutrophil functions, including the

respiratory burst (reviewed by Coffey, 1992), hence adenosine A_2 receptor activation may consistently act via this mechanism. In support, many independent research groups including ours have previously shown that the cAMP–protein kinase A signalling system most likely is important in the adenosine-induced inhibition of neutrophil activities (Iannone et al., 1987; Nielson and Vestal, 1989; Mueller et al., 1992; Zalavary et al., 1994; Fredholm et al., 1996; Zalavary and Bengtsson, 1998). In the present study, the following observations indicate that the adenosine A_2 receptor-mediated inhibition of both fMLP- and IgG-stimulated production of oxygen radicals involves the cAMP–protein kinase A signalling pathway: (1) isoproterenol, dibutyryl cAMP and the class IV phosphodiesterase inhibitor Ro 20-1724 mimicked the effects of NECA; (2) Ro 20-1724 enhanced the inhibitory effects of NECA; and (3) the protein kinase A inhibitor H89 reversed the suppressive actions of NECA. Furthermore, we have recently shown that NECA combined with Ro 20-1724 elevates the cAMP content in both unstimulated and stimulated neutrophils at identical experimental conditions as used in the present study (Zalavary and Bengtsson, 1998). The mechanisms by which the cAMP–protein kinase A signalling system inhibit the respiratory burst in neutrophils have not yet been elucidated, but have been suggested to involve inhibition of receptor/G protein interactions (Mueller and Sklar, 1989), phosphatidylinositol 3-kinase (Ahmed et al., 1995) and phospholipase D (Tyagi et al., 1991). Furthermore, a protein kinase A-mediated phosphorylation of the low-molecular-weight G protein Rap1A (Quilliam et al., 1991) and dephosphorylation of the 47 kDa protein of the phagocytic oxidase (p47 phox) (Bengis-Garber and Gruener, 1996), both important components of the NADPH-oxidase, might more directly inhibit this cellular function. Conversely, other investigators have stated that activation of adenosine A_2 receptors may at least inhibit fMLP-stimulated neutrophil functions via cAMP-independent mechanisms (Cronstein et al., 1988, 1992a; Cronstein and Haines, 1992; Burkey and Webster, 1993; Revan et al., 1996; Spisani et al., 1996). For instance, Revan et al. (1996) have shown that adenosine stimulates the activity of a serine/threonine phosphatase, a mechanism known to attenuate cellular activities. Furthermore, adenosine has been suggested to promote the association of bound fMLP receptors to the cytoskeleton, thereby uncoupling the signal transduction machinery (Cronstein and Haines, 1992). Indeed, adenosine has been found to reduce the G protein activation induced by fMLP (Cronstein et al., 1992a; Burkey and Webster, 1993). However, the fact that adenosine neither affects the fMLP-triggered initial increase in Ca^{2+}, due to mobilization from intracellular stores, nor the rise in inositol(1,4,5)trisphosphate (Walker et al., 1990), do not support that hypothesis.

The discrepancy of whether or not cAMP is involved in adenosine A_2 receptor-mediated signalling in neutrophils, might be related to the use of different classes of protein kinase A inhibitors with various specificity and/or selectivity. In the present study, H89, which is one of the most specific inhibitors of protein kinase A (K_i value of 48 nM; Chijiwa et al., 1990), did not affect the respiratory burst triggered by the direct protein kinase C activator PMA. Furthermore, H89 exerted stimulatory effects of the fMLP-stimulated production of oxygen metabolites. These results strongly support that H89 at least does not interfere with protein kinase C, which is important for the activation of the respiratory burst oxidase (Rider and Niedel, 1987; Curnutte et al., 1994). Unpublished results from our laboratory also show that H89 reverses the adenosine A_2 receptor-induced inhibition of thrombin-stimulated Ca^{2+}-mobilization in platelets, an event that utilizes the cAMP–protein kinase A pathway (Paul et al., 1990).

As described elsewhere (Mitsuyama et al., 1995) and as mentioned above, we found that H89 enhanced the fMLP-triggered production of oxygen radicals. This stimulatory effect correlates with that obtained with adenosine deaminase or adenosine A_2 receptor antagonists, thus supporting that endogenously released adenosine function as a negative feedback modulator of chemoattractant-induced respiratory burst. On the other hand, H89 reduced the phagocytosis-induced respiratory burst. In accordance, we have recently shown that this protein kinase A inhibitor also impairs IgG-mediated actin polymerization, upregulation of CD11b/CD18 and phagocytosis (Zalavary and Bengtsson, 1998), which implies that the cAMP–protein kinase A signalling system is important in regulating motile functions of the neutrophil. This is supported by Ydrenius et al. (1997), who found that neutrophil movement is diminished by blocking protein kinase A. Taken together, these findings strongly argue for that adenosine and the cAMP–protein kinase A signal transduction pathway are important in regulating neutrophil function. However, it must be reminded that other signal transduction events, independent of cAMP, may also be relevant. Further studies are needed to fully elucidate the intracellular mechanisms behind adenosine regulation of neutrophil function.

In summary, we demonstrate that adenosine A_2 receptor occupancy, through a mechanism involving the cAMP–protein kinase A signalling pathway, reduces both fMLP- and IgG-triggered production of oxygen metabolites in neutrophils. Our results also suggest that the metabolism and release of endogenous adenosine constitutes an important autoregulatory system modulating the neutrophil respiratory burst in the inflammatory process.

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