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β-Adrenoceptor-mediated modulation of calcium ionophore activated polymorphonuclear leucocytes

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1 β-Adrenoceptor-mediated modulation of calcium-mediated stimulus-response coupling was studied using calcium ionophore (A23187) activation of polymorphonuclear leucocytes (PMNL). Oxygen metabolite generation was measured with luminol- and lucigenin-dependent chemiluminescence in both whole blood and isolated PMNL.

2 Isoprenaline reduced PMNL response by 53% in a dose-dependent fashion. The effect was saturable, stereoselective, antagonized by propranolol and significant at isoprenaline concentrations as low as 0.01 nM. Fifty % maximal response was induced by 0.26 nM, 3 nM, and 125 nM isoprenaline, adrenaline and noradrenaline respectively.

3 Because the effects of β-adrenoceptor agonists in PMNL have not consistently correlated with measurements of cyclic AMP, alternative means of increasing cyclic AMP were studied. Forskolin and dibutyryl cyclic AMP inhibited PMNL with significant effects at 1.0 μM and 10 μM respectively.

4 The effects of β-adrenoceptor agonists were much greater when PMNL were activated by calcium ionophore compared with opsonized zymosan. Isoprenaline had no effect upon 1-oleoyl-2-acetyl-glycerol activated PMNL.

5 Because catecholamine modulation of oxygen metabolite generation can be characterized pharmacologically, PMNL activation by calcium ionophore is an excellent model for study of β-adrenoceptor function in viable human cells.

6 In contrast to previously described β-adrenoceptor agonist modulation of PMNL function, inhibition of calcium-mediated activation is significant at physiological concentrations. The clinical consequences of such catecholamine effects are dependent upon the mechanism of PMNL activation in a specific circumstance.

Introduction

The polymorphonuclear leucocyte (PMNL) is a primary mediator of inflammation and hypersensitivity. Because β-adrenoceptor agonists are commonly used in the treatment of hypersensitivity response, the effect of these agents upon PMNL function is clinically important. β-Adrenoceptor-mediated inhibition of lysosomal enzyme release (Ignarro & Colombo, 1973; Marone *et al.*, 1980) and superoxide anion generation (Busse & Sosman, 1984) has been demonstrated. However, because studies have described variable degrees of PMNL inhibition and catecholamine effects have only been apparent at high concentrations or in the presence of theophylline (Weismann *et al.*, 1971; Bourne *et al.*, 1971), the physiological significance of these observations is unclear. The variable results may be partly attributed to use of different stimuli for PMNL activation. Because adenosine 3':5'-cyclic monophosphate (cyclic AMP) regulation of calcium mediated response has been demonstrated in many cell types (Rasmussen & Waisman, 1983), β-adrenoceptor agonist modulation of calcium-induced PMNL oxygen metabolite generation was studied in PMNL.

The calcium ionophore A23187 induces calcium influx across the plasma membrane with resultant PMNL aggregation, degranulation and increased oxidative metabolism (DeChatelet *et al.*, 1982). This mechanism of cell activation is distinct from commonly used *in vitro* stimuli such as zymosan or phorbol esters which activate membrane receptors or protein

kinase C respectively (Nishizuka, 1984). Cell activation may be dependent upon synergistic effects of calcium mobilization and protein kinase C activation. The relative importance of the effects of β-adrenoceptor agonists upon each pathway of cell activation is not established.

The PMNL respiratory burst is associated with generation of superoxide anion, hydrogen peroxide, oxygen radical, hydroxyl radical and hypochlorous acid. These oxygen metabolites are not only potent microbicidal agents but are also extremely toxic to human tissues. Because luminol or lucigenin degradation by reactive oxygen molecules is associated with luminescence, inclusion of these agents in the reaction medium provides a sensitive means of detecting PMNL activation. Luminol-dependent chemiluminescenee is relatively non-specific for oxygen metabolites while lucigenin primarily detects superoxide anion (Stevens & Hong, 1984).

This paper describes the inhibition of PMNL oxidative metabolism by β-adrenoceptor agonists, forskolin, and dibutyryl cyclic AMP (db cyclic AMP). The relationship between the β-adrenoceptor-mediated effect and the means of cell activation is examined. The sensitivity of calcium mediated cell activation to cyclic AMP inhibition and the utility of chemiluminescence, as a means for biochemical and pharmacological investigations of stimulus-response coupling in an intact human cell type, is demonstrated.

Methods

Subjects

Studies were performed in subsets of 25 healthy, drug-free volunteers between the ages of 20 and 45 years. Blood samples were drawn from an antecubital vein after subjects had been supine for 20 min. All subjects were asked to abstain from methylxanthine-containing foods for one day prior to study.

PMNL isolation

Human PMNL were isolated from blood anticoagulated with EDTA (2 mg ml^{-1}) using a modification of the method of Boyum (1968). Leukocyte-rich plasma was obtained after dextran sedimentation, layered on a Ficoll-Hypaque gradient (Sp. gr.1.077), and centrifuged at 150 *g* for 25 min. PMNL were washed and contaminating red blood cells removed with hypotonic lysis for 20 s in distilled water. Cell counts were performed with a haemocytometer and a suspension of 2×10^5 cells ml^{-1} prepared in PBS. PMNL were kept at 0°C during isolation. The procedure yielded preparations of 95% PMNL which were over 95% viable, as evaluated by trypan blue exclusion. Total time for PMNL isolation was approximately 1 h.

Whole blood suspension

Blood anticoagulated with heparin (100 u ml^{-1}) was drawn from volunteers immediately before use. After haematocrit and white blood cell counts were obtained, whole blood was diluted 1:100 in PBS for measurement of chemiluminescence responses.

Measurement of chemiluminescence

Chemiluminescence was measured at room temperature using a Beckman LS-7500 (Beckman Instruments, Fullerton, CA) scintillation counter set in the out-of-coincidence mode. Buffer and all reagents were placed in polyethylene scintillation vials in a volume of 1 ml. A 1 ml aliquot of cell suspension (whole blood or purified PMNL) was then added sequentially to each vial. The final cell suspension contained 5×10^4 PMNL ml^{-1} in purified PMNL preparations and from 2×10^4 to 7×10^4 PMNL ml^{-1} in whole blood preparations. Zymosan activated preparations contained 2×10^5 PMNL ml^{-1}. Scintillation counting was performed immediately after addition of cells and then at 3 to 6 min intervals depending upon number of samples in an experiment. Data were analysed using a computer and integration (total luminescence or area under the curve) was performed over desired time intervals. Integration provided more reproducible data than peak values, response at a given time, or slope (rate of activation). Experiments were repeated in duplicate or triplicate on each blood sample. Variation between identical samples within an experiment was approximately 5%.

Data analysis

Because absolute PMNL response following activation demonstrated interindividual variability, measurements were standardized as % of control response using the same cell preparation, simultaneous activation and parallel detection of luminescence. Dose-response analysis was performed using integrated luminescence over 10 min. Computer assisted simultaneous curve fitting as described by De Lean *et al.* (1978) was used to compare statistically IC_{50}, slope and maximal response. The antagonistic effects of propranolol were analysed using Schild regression analysis (Arunlakshana & Schild, 1959). Isoprenaline dose-response (10 pM to 1 μM) and the effects of propranolol (100 pM to 3 μM) in the presence of 1 μM isoprenaline were evaluated in parallel experiments using the same blood specimen. Agonist dose-ratios (ratio of agonist concentration with propranolol to agonist without propranolol that cause same degree of

inhibition) were calculated for each concentration of propranolol.

Reagents

Dulbecco's phosphate buffered saline (composition in $g\,l^{-1}$: NaCl 8, KCl 0.2, Na_2HPO_4 1.25, KH_2PO_4 1) was prepared with 1 mg ml^{-1} glucose, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS). A23187 was dissolved in dimethylsulphoxide (DMSO) at a concentration of 10 mM and stored at −20°C. Immediately before each experiment, A23187 was added to the buffer. Zymosan was heated at 100°C for 20 min in distilled water, cooled, opsonized in serum for 20 min at 37°C, and stored at −20°C. 1-Oleoyl-2-acetyl glycerol (OAG) (1 μM), zymosan (0.5 mg ml^{-1}) or A23187 (0.2 μM) were utilized for PMNL activation. Luminol was dissolved in DMSO at a concentration of 10 mg ml^{-1}, diluted in distilled water to a concentration of 2 mM and stored at room temperature. Lucigenin was dissolved in distilled water. The final luminol and lucigenin concentrations were 0.4 μM and 1 μM respectively. Isoprenaline, adrenaline or noradrenaline dilutions were prepared each morning in distilled water with sodium metabisulphite 1 mg ml^{-1} as preservative. Forskolin was dissolved in ethanol and then diluted to appropriate concentrations in distilled water. Propranolol, db cyclic AMP and sodium butyrate were prepared in appropriate dilutions in distilled water the day of the experiments. All controls contained appropriate concentrations of metabisulphite, DMSO or ethanol.

Forskolin was obtained from Calbiochem-Behring, La Jolla, CA. All other reagents were obtained from Sigma Chemicals, St Louis, Mo.

Results

Effects of β-adrenoceptor agonists

PMNL oxygen metabolite generation (detected with either luminol or lucigenin luminescence) induced by A23187 was inhibited by low concentrations of isoprenaline (Figure 1). Significant effects were detectable using 0.01 nM isoprenaline (88% control in whole blood, 81% control in PMNL, both $P < 0.05$) in most subjects. PMNL activation was inhibited by an average of 53% at isoprenaline concentrations over 0.1 μM. The IC_{50} (50% maximal response) ranged from 0.01 nM to 0.8 nM with a mean of 0.26 ± 0.07 nM in 15 young individuals. The IC_{50} was consistent in a single blood specimen but variable between individuals and in the same individual on different days. The effects of isoprenaline were markedly diminished when the drug was introduced more than 5 min after PMNL activation. The decreased effect of isoprenaline was also observed at high concentrations (1 mM) that would minimize changes in drug response due to decreased receptor affinity (Figure 2). Although the frequency of chemiluminescence measurements did not allow detection of minor changes in activation kinetics, no significant effects of the β-adrenoceptor agonists on time to peak response were identified. Because β-adrenoceptor affinity changes rapidly and may be altered by procedures required for PMNL isolation, studies were also performed within 5 min of phlebotomy using a whole blood preparation. Isoprenaline potency and maximal effect were not significantly different whether evaluated in whole blood or isolated PMNL. The degree of inhibition by isoprenaline was not dependent upon magnitude of response or cell concentration. Isoprenaline (100 nM) inhibited PMNL activation to 45 ± 6% of control using 2×10^4 cells ml^{-1}, 51 ± 4% of control using 8×10^4 cells ml^{-1} and 47 ± 8% of control using 4×10^5 cells ml^{-1} (samples from 3 subjects).

Isoprenaline inhibition was much greater when PMNL were activated by A23187 rather than opsonized zymosan or OAG (Figure 3). Isoprenaline had decreased effect with zymosan or OAG activation whether total PMNL chemiluminescence, peak chemiluminescence, equivalent time intervals, or

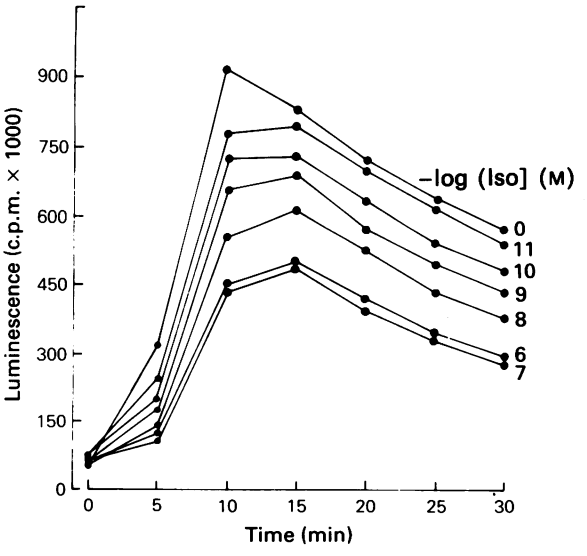

Figure 1 The effects of isoprenaline (Iso) upon the polymorphonuclear leucocyte (PMNL, 5×10^4 ml^{-1}) respiratory burst using luminol-dependent chemiluminescence. Isolated PMNL were activated by 0.2 μM A23187 at time zero. Values indicate the results of a single experiment and are representative of experiments in 15 subjects.

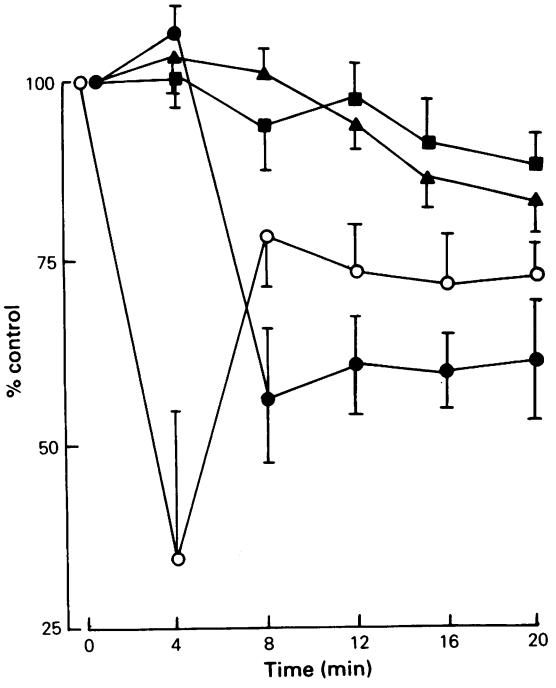

Figure 2 The effects of a high concentration of isoprenaline (1 mM) when introduced at 0 (○), 3.5 (●), 7.5 (▲), or 11.5 (■) min. Values represent the means, with vertical lines indicating s.e.mean, of experiments in three subjects (each repeated in triplicate) using a whole blood preparation with luminol detection and A23187 activation. Effects of isoprenaline introduced at 0 or 3.5 min were statistically significant ($P<0.05$) at any subsequent measurement.

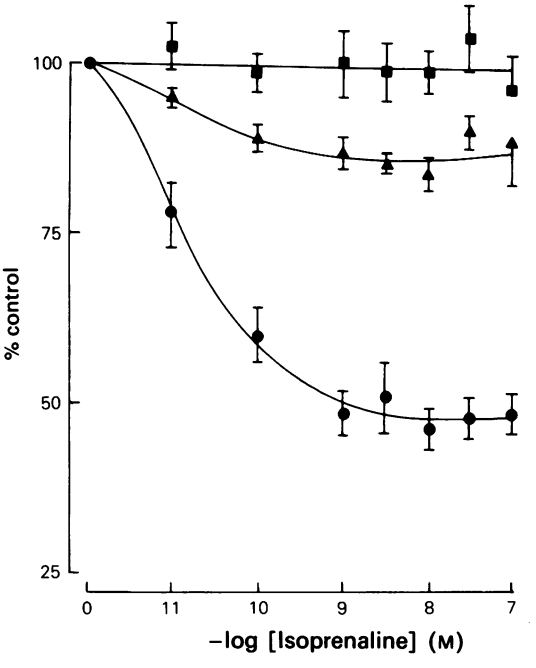

Figure 3 The effects of isoprenaline introduced at time of polymorphonuclear leucocyte (PMNL) activation with 0.2 μM A23187 (●), 0.5 mg ml^{-1} opsonized zymosan (▲) or 1 μM 1-oleoyl-2-acetyl glycerol (■). Values represent the means, with vertical lines indicating s.e.mean, of specimens from five subjects. Luminol-dependent chemiluminescence was integrated over 0–20 min using isolated PMNL.

periods of equal magnitude of response were analysed. Isoprenaline had a minimal or no effect when introduced at 5 or 10 min after zymosan activation.

Pharmacological characterization of the effects of the β-adrenoceptor agonists

Isoprenaline inhibition of chemiluminescence was stereoselective. The IC_{50} (50% maximum inhibition) was 0.26 nM using (−)-isoprenaline and 84 nM using (+)-isoprenaline ($P<0.05$). Slope factors and maximum inhibition were not significantly different (maximum inhibition measured using 1 mM concentrations, $P>0.05$). Comparison of agonists demonstrated that isoprenaline was more potent than adrenaline which was more potent than noradrenaline. The IC_{50} values for (−)-isoprenaline, (−)-adrenaline and (−)-noradrenaline were respectively 0.26 ± .07 nM, 3 ± 0.8 nM, and 125 ± 105 nM. The IC_{50} for adrenaline or noradrenaline was significantly different from the IC_{50} for isoprenaline ($P<0.05$). Maximum inhibition and slope factors were not significantly different.

(±)-Propranolol caused a concentration-dependent antagonism of the inhibition due to isoprenaline. Propranolol in the absence of isoprenaline had no effect on PMNL chemiluminescence at concentrations less than 10 μM. Schild regression analysis demonstrated that the effects of propranolol were competitive (slope of unity) and similar in whole blood or purified PMNL preparations (Figure 4). The pA_2 of propranolol was 10.29 in whole blood and 10.24 using purified PMNL.

Dibutyryl cyclic AMP and forskolin

db Cyclic AMP inhibited PMNL chemiluminescence at concentrations greater than 10 μM using A23187 activation. Although OAG activated PMNL were also

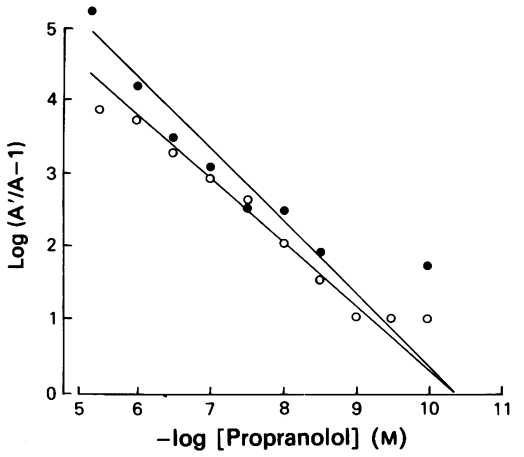

Figure 4 Schild plot of propranolol effect in whole blood (●) or polymorphonuclear leucocyte (PMNL; ○) preparations ($n = 7$). Concentrations in range of 1 nM to 1 μM included in regression analysis. In whole blood: slope = 1.0 ± 0.12; X intercept = 10.29; correlation = 0.965; $P < 0.05$. In PMNL: slope = 0.90 ± 0.04; X intercept = 10.24; correlation = 0.994; $P < 0.05$.

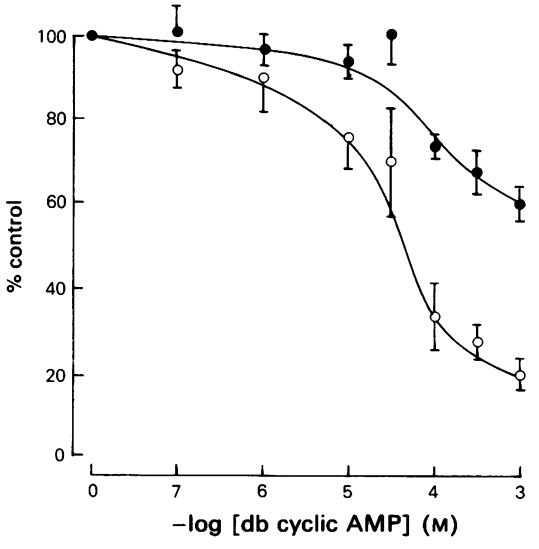

Figure 5 Dibutyryl cyclic AMP incubated with isolated polymorphonuclear leucocytes (PMNL) for 20 min prior to activation by A23187 (○) or 1-oleoyl-2-acetyl glycerol (OAG; ●). Values represent the means with vertical lines indicating s.e.mean, of an integrated response over 0–10 min after A23187 activation of specimens from 5 subjects (ANOVA $F_{1,18} = 25.2$; $P < 0.05$).

inhibited, the effect was much less than that observed with A23187 activation (Figure 5). Butyrate controls had no significant effect. When introduced at the time of PMNL activation, the onset of inhibition by db cyclic AMP was delayed approximately 10 min. When PMNL were preincubated (20 min) with db cyclic AMP prior to addition of A23187 then inhibition occurred immediately after activation and was similar to the effect of isoprenaline.

Forskolin, a diterpene that directly stimulates the adenylate cyclase catalytic subunit, caused a rapid concentration-dependent inhibition of chemiluminescence with 33% decrease at 10 μM ($P < 0.05$) using A23187 activation. Inhibition was significant ($P < 0.05$) for all concentrations greater than 1 μM in purified PMNL and 100 nM in whole blood preparations. Forskolin did not significantly inhibit OAG activated PMNL (Figure 6). Both db cyclic AMP and forskolin caused similar effects with either lucigenin- or luminol-dependent chemiluminescence.

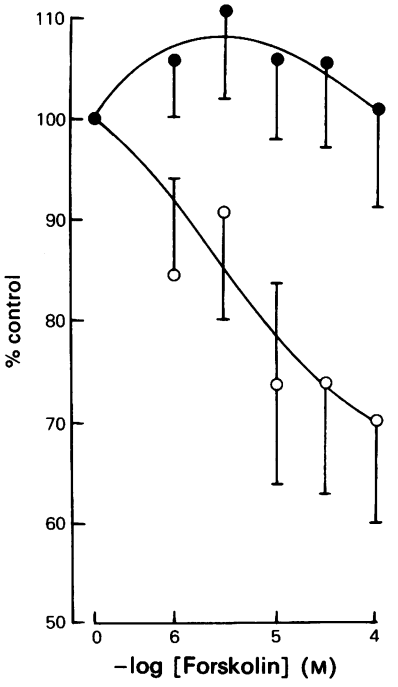

Figure 6 Effects of forskolin on A23187 (○) or 1-oleoyl-2-acetyl glycerol (OAG, ●) activated polymorphonuclear leucocytes (PMNL). Values represent the means, with vertical lines indicating s.e.mean, of specimens from 6 subjects calculated as integrals of response over 10 min following A23187 activation. (ANOVA (paired data) $F_{1,29} = 11.87$; $P < 0.05$).

Discussion

These studies indicate that luminol- or lucigenin-dependent chemiluminescence induced by A23187 is extremely sensitive to modulation by β-adrenoceptor stimulation. Significant β-adrenoceptor-mediated inhibition of PMNL activation occurred at much lower catecholamine concentrations than previously demonstrated using other techniques. Because chemiluminescence detects very low levels of oxygen metabolite generation, PMNL activation was evaluated at lower concentrations of A23187 and at lower cell concentrations than is possible with other measures of the respiratory burst.

To confirm that observations were not an artifact of the chemiluminescence assay method, pharmacological characteristics of the effects of the β-adrenoceptor agonists were studied. Responses were stereoselective and competitively antagonized by propranolol. Potency ratios of agonists (isoprenaline > adrenaline > noradrenaline) were characteristic of a β-adrenoceptor-mediated response. These results clearly demonstrate β-adrenoceptor-mediated inhibition of PMNL activation and indicate the utility of this technique for studies of receptor stimulation.

The mechanism of the β-adrenoceptor-mediated response is not established in PMNL. Although β-adrenoceptor stimulation is linked to adenylate cyclase activation in most cell types, effects of β-adrenoceptor agonists have not consistently correlated with increased levels of cyclic AMP in PMNL (Marone *et al.*, 1980). Cyclic AMP measurements may not, however, be sensitive to compartmental changes (Pryzwansky *et al.*, 1981). If β-adrenoceptor-mediated responses are mediated by cyclic AMP then alternative means of increasing cyclic AMP should cause similar inhibition of PMNL activation. Because the effects of an exogenous cyclic AMP analogue (db cyclic AMP) and direct adenylate cyclase catalytic subunit activation (forskolin) were similar to the effects of the β-adrenoceptor agonists, the results are consistent with the hypothesis that cyclic AMP is the intracellular mediator of β-adrenoceptor-mediated inhibition in PMNL.

Although isoprenaline caused significant inhibition only when introduced before or soon after PMNL activation (Figure 2), effects of isoprenaline and db cyclic AMP persisted throughout the period of PMNL response. Because the onset of inhibition by db cyclic AMP (without preincubation) was delayed until 10 min after activation, PMNL appear to remain susceptible to the effects of cyclic AMP after activation. It is possible that a change in β-adrenoceptor function following A23187 exposure reduces PMNL sensitivity to isoprenaline. These observations may be a result of increased phospholipase A_2 activity during PMNL activation (Marone *et al.*, 1983) that causes β-adrenoceptor alterations analogous to desensitization (Mallorga *et al.*, 1980).

The marked potency of the β-adrenoceptor agonists in the absence of theophylline distinguish the present results from those of other investigations. Studies of oxygen metabolite generation (superoxide anion measurement or luminol-dependent chemiluminescence) using opsonized zymosan activation (Schopf & Lemmel, 1983; Busse & Sosman, 1984), only demonstrated effects of isoprenaline at concentrations greater than 100 nM. Although there are several methodological variations, the primary distinguishing characteristic of this study was the use of calcium ionophore for cell activation. The PMNL respiratory burst may be induced with either increased cytosolic calcium or protein kinase C activation with OAG which is calcium-independent (Fujita *et al.*, 1984). Because the β-adrenoceptor agonists had a decreased or no effect on OAG activated PMNL and a decreased effect on zymosan activated PMNL (Figure 3) when compared with A23187 activated cells, the β-adrenoceptor-mediated effect is clearly dependent upon the mechanism of PMNL activation.

This study demonstrates that catecholamines may have a significant effect upon PMNL function at clinically relevant concentrations. Both the time of administration of the β-adrenoceptor agonist and mechanism of PMNL activation have been shown to be important in determining catecholamine effects. In addition, it is apparent that determination of oxygen metabolite generation with luminescent probes provides a unique model for *in vitro* study of β-adrenoceptor function in a viable human cell preparation.

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