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Membrane Stabilizing, Anti-Oxidative Interactions of Propranolol and Dexpropranolol with Neutrophils

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ABSTRACT. We have investigated the effects of the β-adrenoreceptor-blocking agent, propranolol (9–300 μM) on several pro-inflammatory activities of human neutrophils *in vitro*. Superoxide production by calcium ionophore (A23187)-activated neutrophils was particularly sensitive to inhibition by low concentrations (9–18.7 μM) of this drug. However, inhibition of superoxide generation by neutrophils activated with phorbol myristate acetate (PMA), opsonized zymosan (OZ), and arachidonate (AA) only occurred with higher concentrations of propranolol, and coincided with decreased intracellular calcium fluxes, phospholipase A_2 (PLA_2) activity and synthesis of platelet-activating factor (PAF). Propranolol possessed neither cytotoxic nor superoxide-scavenging properties but, using a haemolytic assay of membrane-stabilizing activity, this agent neutralized the membrane-disruptive effects of the bioactive phospholipids, lysophosphatidylcholine (LPC), PAF, and lysoPAF (LPAF). A mechanistic relationship between the anti-oxidative and membrane-stabilizing properties of propranolol was suggested by the observation that pretreatment of neutrophils with LPC or PAF eliminated the inhibitory effects of the drug on superoxide generation by PMA-activated neutrophils. Dexpropranolol, a stereoisomer with minimal β-blocking activity, and propranolol were equally effective with respect to their membrane-stabilizing and anti-oxidative interactions with neutrophils, but several other β-blocking agents (atenolol, metoprolol, sotalol, and timolol) did not possess these activities. Inhibition of oxidant generation is, therefore, not a common property of β-blocking agents and, in the case of propranolol, appears to occur as a consequence of membrane-stabilization rather than by β-receptor-directed effects. BIOCHEM PHARMACOL 52;2:341–349, 1996.

KEY WORDS. neutrophils; propranolol; dexpropranolol; oxidants; membrane stabilization

In addition to its beneficial cardiovascular effects, the non-selective β-adrenoreceptor-blocking agent, propranolol, has also been reported to modulate the functions of lymphocytes and neutrophils. Short-term administration of propranolol to patients with lepromatous leprosy [1], chronic acne [2], rheumatoid arthritis [3], essential hypertension [4], or hyperthyroidism [5], as well as to healthy control subjects [5], is accompanied by enhanced proliferative responses of circulating lymphocytes *in vitro*. These enhancing effects of propranolol on the proliferative responses of mitogen-activated lymphocytes apparently do not involve β-adrenoreceptors because similar effects were observed following administration of dexpropranolol, a stereoisomer with minimal β-blocking activity [5].

Propranolol has also been reported to potentiate the adhesive [6, 7] and migratory [1–3, 6–9] activities of neutrophils *in vitro* and *in vivo*, and to modulate the pro-oxidative activities of these cells [9–11]. Several biochemical mechanisms, including β-blocking effects [7], oxidant-scavenging properties [9], and membrane-stabilizing activity [10], as well as inhibition of phosphatidate hydrolase [12] and protein kinase C [11], have been proposed to explain these potentially important, albeit secondary, interactions of propranolol with neutrophils. However, the relative contributions of these various mechanisms to propranolol-mediated modulation of neutrophil function remain to be established.

The primary objective of the present study was to identify the sites and mechanisms of the anti-oxidative interactions of propranolol and dexpropranolol with human neutrophils *in vitro*.

MATERIALS AND METHODS

Chemicals and Reagents

Propranolol, its dextroisomer, D(+)-propranolol, and atenolol were provided by ICI (South Africa) Ltd., and metoprolol, sotalol, and timolol were obtained from the South

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† *Abbreviations:* AA, arachidonic acid; C/E, cellular:extracellular ratio; CaI, calcium inonphore; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; LECL, lucigenin-enhanced chemiluminescence; LPC, lysophosphatidylcholine; LPAF, lyso-platelet-activating factor; OZ, opsonized zymosan; PAF, platelet-activating factor; PKC, protein kinase C; PLA_2, phospholipase A_2; PMA, phorbol 12-myristate 13-acetate.

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African affiliates of Ciba-Geigy Ltd., Bristol-Myers Squibb Ltd., and Merck, Sharp & Dohme, Ltd., respectively. These various cardioselective and noncardioselective, β-adrenoreceptor-blocking agents were dissolved in indicator-free Hanks' balanced salt solution (HBSS), pH 7.4, and used, in the case of propranolol and dexpropranolol, at a final concentration range of 9–300 μM in the various assays described below. The other β-blocking agents were used at a fixed, final concentration of 300 μM in assays of neutrophil superoxide generation and membrane stabilization.

Unless indicated, all other chemicals and reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and radiochemicals from Du Pont NEN Research Products (Boston, MA, U.S.A.).

Neutrophils

Human neutrophils were obtained from heparinized (5 units of preservative-free heparin/mL) venous blood of healthy adult volunteers, and separated from mononuclear leucocytes by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden)-metrizoate (Nyegaard, Oslo, Norway) cushions at 400 *g* for 25 min at room temperature. The resultant pellet was suspended in PBS (0.15 M, pH 7.4) and sedimented with 3% gelatin for 15 min at 37°C to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%) were resuspended to 1×10^7/mL in PBS and held on ice until used.

Superoxide generation

This was measured using an LECL† method [13]. Neutrophils were preincubated for 15 min at 37°C in 900 μl HBSS containing 0.2 mM lucigenin in the presence and absence of propranolol and dexpropranolol (9–300 μM). Spontaneous and stimulus-activated LECL responses were, then, recorded in an LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of 100 μL of the following stimuli of neutrophil membrane-associated oxidative metabolism: the calcium ionophore A23187, (1 μM), PMA (25 ng/mL), OZ (0.5 mg/mL) and AA (10 μM). LECL readings were integrated for 5-sec intervals and recorded as millivolts × seconds^{-1} (mV.s^{-1}). Additional experiments were performed to investigate the following: (1) the duration of preincubation of neutrophils at 37°C with propranolol and dexpropranolol (75–300 μM) on the PMA-activated LECL responses of these cells; (2) the durability of the effects of propranolol and dexpropranolol (150 and 300 μM) on the PMA-activated LECL responses of neutrophils following washing (twice) of cells that had been exposed to the drugs for 15 min at 37°C; (3) the effects of atenolol, metoprolol, sotalol, and timolol (300 μM) on the LECL responses of PMA-activated neutrophils; (4) the effects of the membrane-destabilizing phospholipids, LPC and PAF on propranolol and dexpropranolol (18.75, 37.5, and 75 μM) interactions with neutrophils; to avoid cytotoxicity the phospholipids were added as 3 incremental doses of 2 μM at 5-min intervals (6 μM total dose of LPC and PAF), and (5) superoxide-scavenging activity of propranolol and dexpropranolol (9–300 μM) using a cell-free xanthine (1 mM)-xanthine oxidase (17 milliunits/mL) superoxide-generating system.

Oxygen Consumption

A 3-channel, Clark-type oxygen electrode (model DW1, Hansatech Ltd, King's Lynn, Norfolk, U.K.) was used to measure the effects of propranolol and dexpropranolol (150 and 300 μM) on O_2 consumption by PMA-activated neutrophils (2×10^6/mL). The reduction in PO_2 was monitored for 15 min after the addition of PMA.

NADPH-Oxidase Activity

Neutrophils (5×10^6/mL) were preincubated for 30 min at 37°C in the presence and absence of propranolol and dexpropranolol (300 μM), followed by addition of PMA. After a 10-min incubation, the cells were centrifuged at 4°C and the pellets resuspended in 0.34 M sucrose supplemented with 0.5 mM phenylmethyl-sulphonyl fluoride (Calbiochem Corp., La Jolla, CA, U.S.A.) and disrupted by sonication. Cellular debris was removed by centrifugation and the membrane fractions in the supernatants harvested following centrifugation at 70,000 *g* for 30 min. The membrane pellets were dispersed in 1 mL sucrose and assayed for NADPH-oxidase activity using LECL. Reaction mixtures (1 mL) contained lucigenin, membrane fractions (200 μL), and NADPH (2 mM), which was added last to initiate superoxide generation. In an additional series of experiments designed to investigate the effects of propranolol and dexpropranolol on fully-assembled NADPH-oxidase, the drugs (150 and 300 μM) were preincubated for 10 min at 37°C with purified membrane fractions prepared from control PMA-activated neutrophils, followed by addition of NADPH and measurement of LECL.

Intracellular Calcium Fluxes

These were measured spectrofluorometrically using fura 2 (Calbiochem) as the calcium-sensitive indicator [14]. Neutrophils (1×10^7/mL) were pre-loaded with fura 2 (2 μM) for 30 min at room temperature in HBSS, washed twice, and resuspended in HBSS. The fura 2-loaded cells (1×10^6/mL) were then preincubated with propranolol or dexpropranolol (9–300 μM) for 5–10 min at 37°C, then activated with 1 μM of the synthetic chemotactic tripeptide, FMLP [15] or 30 μM AA in the thermoregulated cuvette of a Hitachi 650-10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm, respectively. Intracellular calcium concentrations

($[Ca^{2+}]_i$) were calculated by procedures that have been described previously [14].

PAF Synthesis and Phospholipase A_2 Activity

For these experiments, cell numbers, incubation times, and stimulant concentration were established in a series of preliminary experiments. In both assays, the cells were preincubated with propranolol or dexpropranolol for 15 min at 37°C. PAF was assayed in the supernatants of control and drug-treated, Cal (2.5 μM)-activated neutrophils, using a commercially-available radioimmunoassay (Du Pont NEN) after a 10-min incubation at 37°C. In additional experiments designed to control for possible interactions of propranolol and dexpropranolol with PAF, the drugs were added retrospectively to PAF-containing supernatants from control, Cal-activated neutrophils. The mixtures were incubated for 15 min and the PAF quantitated by radioimmunoassay. In a similar series of experiments, the immunoreactivity of reagent PAF (6.25–100 ng/mL) was measured following a 15-min exposure to propranolol and dexpropranolol (300 μM).

PLA_2 activity was measured according to the extent of release of radiolabelled AA (^{3}H-AA) from Cal-activated neutrophils [16]. Briefly, neutrophils (1×10^7/mL) were coincubated with 5 μCi/mL ^{3}H-AA [5,6,8,9,11,12,14,15-^{3}H(N), 160 Ci/mmol, Du Pont NEN] for 30 min at 37°C in Ca^{2+}-free HBSS, washed twice, and resuspended to 1×10^7/mL in HBSS. Following preincubation with propranolol and dexpropranolol for 15 min at 37°C, the cells (5×10^6 in 2 mL) were activated with 2.5 μM calcium ionophore. After 5 min of incubation at 37°C, the reactions were terminated and the lipids extracted by addition of 5 mL of *n*-hexane/isopropranolol/concentrated HCl (300:200:4, v/v/v). The upper organic phase was separated, dried under a stream of nitrogen, reconstituted in 100 μL of hexane/isopropranolol (3:2, v/v) and ^{3}H-AA quantitated by radiometric TLC [16].

Protein Kinase C Activity

The effects of propranolol and dexpropranolol (150 μM) on the activity of purified PKC from rat brain (Boehringer Mannheim, Germany) were measured using a commercial, colourimetric PKC assay kit (Pierce, Rockford, IL, U.S.A.). Briefly, the drugs were co-incubated with PKC (1.6 mU/mL) for 15 min at 30°C, after which an aliquot of the mixture (10 μL) was transferred to vials containing 8 μg of dye-labeled PKC substrate (glucose synthase peptide), 10 mM Tris assay buffer containing 2 mM ATP, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, and phosphatidyl-L-serine (5 μg) as activator, to give a final reaction volume of 25 μL. Vials were incubated for 30 min at 30°C, after which phosphorylated and nonphosphorylated peptide was separated by centrifugation through a SpinZyme™ affinity separation unit (Pierce). Following elution, the amount of phosphorylated peptide was determined spectrophotometrically at 570 nm.

Cellular ATP Levels

After 30 min incubation at 37°C in HBSS, neutrophil ATP levels were measured in the lysates of control and propranolol- or dexpropranolol-treated cells (10^6) using a luciferin/luciferase chemiluminescence method [17].

Cellular Uptake of Propranolol

Binding of ^{3}H-propranolol to neutrophils was measured as previously described [18]. Briefly, 70 μL of silicone oil was introduced into microcentrifuge tubes containing 25 μL of 2 M NaOH. Neutrophil suspension (2×10^6 cells in 200 μL) containing 1 μCi of ^{3}H-propranolol (specific activity 21.2 Ci/mmol, Du Pont NEN; 3.75 nanomoles carrier drug added) was incubated over the silicone oil for 15 min at 37°C, after which the unbound and neutrophil-associated drug were separated by centrifuging the cells through the oil at 12,000 *g* for 3 min. After freezing the tubes at −70°C, the neutrophil pellets were sliced from the bottom of each tube and solubilized in Protosol® (Du Pont NEN). Solubilized pellets and cell-free supernatants were then assayed for ^{3}H-propranolol, using a liquid scintillation spectrometer. Results are expressed as the C/E ratio of the drug following calculation of the intracellular volume by addition of [^{3}H]-H_2O (Du Pont NEN) to neutrophils.

Membrane Stabilization

The membrane-stabilizing potential of propranolol and dexpropranolol (75 and 300 μM) was measured using a haemolytic assay. Sheep erythrocytes were washed 3 times and resuspended to 5% in HBSS. The erythrocytes (final concentration of 0.5%) were then co-incubated with the drugs for 30 min at 37°C, followed by the addition of LPC, PAF, and lysoPAF (LPAF) at concentrations (5–8 μM) that caused partial haemolysis. After 5 min, intact erythrocytes were removed by centrifugation and the supernatants assayed spectrophotometrically at 415 nm for haemoglobin content. The potential of atenolol, metoprolol, sotalol, and timolol (500 μM) to protect against LPC-induced haemolysis was also investigated.

The protective effects of propranolol and dexpropranolol (75 and 300 μM) against hypotonic lysis of sheep erythrocytes were also investigated in a limited series of experiments.

Interactions of Propranolol and Dexpropranolol with LPC

The ultraviolet absorption spectra of mixtures of propranolol and dexpropranolol (100 and 200 μM) and LPC (70–2000 μM), relative to those of identical concentrations of

the individual agents, were measured using a Pye Unicam SP 1700 double-beam UV spectrophotometer.

Statistical Analysis

The results of each series of experiments are expressed as the mean values ± the standard error of the mean (SEM). Where appropriate, levels of statistical significance were calculated by Student's *t*-test.

RESULTS

Neutrophil LECL Responses

The effects of propranolol and dexpropranolol on superoxide production by activated neutrophils are shown in Fig. 1. At concentrations of 9.4 μM, 9.4 μM, 75 μM, and 9.4 μM and upwards, propranolol caused statistically significant inhibition of the LECL responses of neutrophils activated with calcium ionophore ($P < 0.03$–$P < 0.02$), AA ($P < 0.04$–$P < 0.01$), opsonized zymosan ($P < 0.0001$–$P < 0.0003$), and PMA ($P < 0.07$–$P < 0.0002$), respectively, with respective IC_{50} values of 15 μM, 78 μM, and 81 μM. The corresponding concentrations of dexpropranolol were

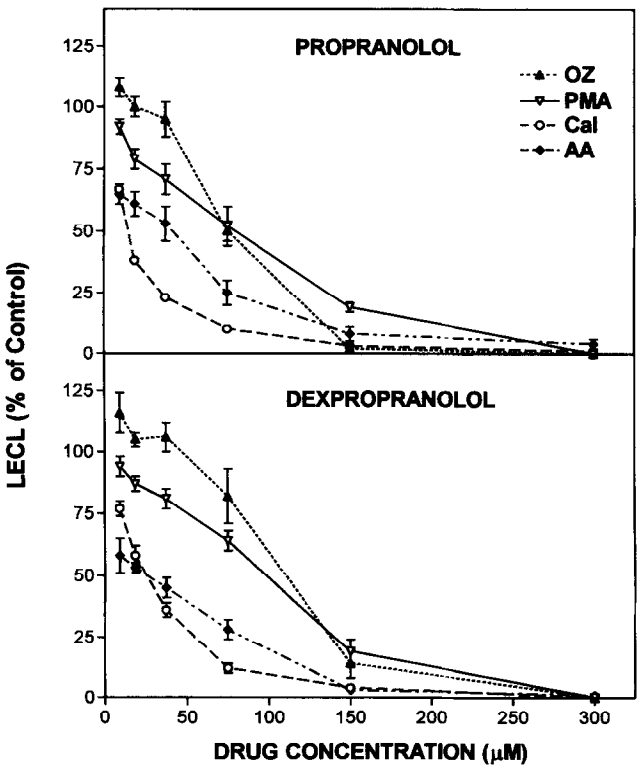

FIG. 1. The effects of propranolol and dexpropranolol on the peak LECL responses of neutrophils activated with OZ, PMA, Cal and AA. The results of 3–14 experiments are expressed as the mean percentage ± SEM of the corresponding drug-free control systems. The absolute peak values for the drug-free control systems activated with OZ, PMA, Cal, and AA were 9087 ± 814, 4358 ± 601, 3384 ± 442, and 2809 ± 465 mV.s^{-1}, respectively, and were reached after 7, 11, 14, and 4 min for each stimulus, respectively.

9.4 μM ($P < 0.02$–$P < 0.02$), 9.4 μM ($P < 0.02$–$P < 0.04$), 150 μM ($P < 0.002$–$P < 0.0003$) and 18.75 μM ($P < 0.002$–$P < 0.001$).

Extending the duration of preincubation of neutrophils with propranolol or dexpropranolol did not increase the sensitivity of the cells to the inhibitory effects of these agents. Following 15 min of preincubation at 37°C, the mean percentages of PMA-activated LECL of the drug-free control system were 33 ± 1 and 45 ± 0 for neutrophils exposed to 150 μM propranolol and dexpropranolol, respectively. After 120 min of preincubation, the corresponding values were 35 ± 2 and 37 ± 2.

Washing of neutrophils that had been co-incubated with propranolol and dexpropranolol was associated with slight loss of inhibition of PMA-activated LECL at 150 μM, but not with 300 μM of these agents. For unwashed neutrophils continuously exposed to propranolol at concentrations of 150 μM and 300 μM for 30 min prior to activation, the mean percentages of PMA-stimulated LECL of the drug-free control system were 20 ± 0 and 2 ± 0, respectively. The corresponding responses of washed, matched neutrophils were 31 ± 1 and 1 ± 0, respectively. Similar results were obtained with dexpropranolol (not shown).

At a fixed concentration of 300 μM, neither atenolol, metoprolol, sotalol, nor timolol affected the LECL responses of PMA-activated neutrophils. The peak LECL values of control neutrophils and for cells treated with 300 μM propranolol, dexpropranolol, atenolol, metoprolol, sotalol, and timolol were 3920 ± 249, 41 ± 5, 38 ± 4, 4097 ± 294, 3486 ± 231, 3528 ± 178, and 3631 ± 282 mV.s^{-1}, respectively.

The effects of pretreatment of neutrophils with LPC or PAF on propranolol-mediated inhibition of PMA-activated LECL responses are shown in Fig. 2. At all concentrations tested, propranolol caused significant ($P < 0.003$–$P < 0.0001$) inhibition of the LECL responses of PMA-activated neutrophils, which was significantly ($P < 0.002$–$P < 0.0002$) antagonized by inclusion of both LPC and PAF. These bioactive phospholipids also antagonized the inhibitory effects of dexpropranolol on superoxide generation by activated neutrophils.

Using the cell-free xanthine/xanthine oxidase superoxide-generating system, neither propranolol nor dexpropranolol at concentrations of up to 300 μM possessed superoxide-scavenging properties. The LECL responses of the drug-free control system, and for systems containing either propranolol or dexpropranolol (300 μM), were 872 ± 49, 854 ± 91, and 1372 ± 538 mV.s^{-1}, respectively.

O_2 Consumption and NADPH-Oxidase Activity

The effects of propranolol and dexpropranolol (150 and 300 μM) on O_2 consumption and on the activity of NADPH-oxidase in purified membrane fractions prepared from these cells are shown in Table 1. Both activities were significantly decreased by propranolol and dexpropranolol. Addition of both drugs to purified membrane fractions pre-

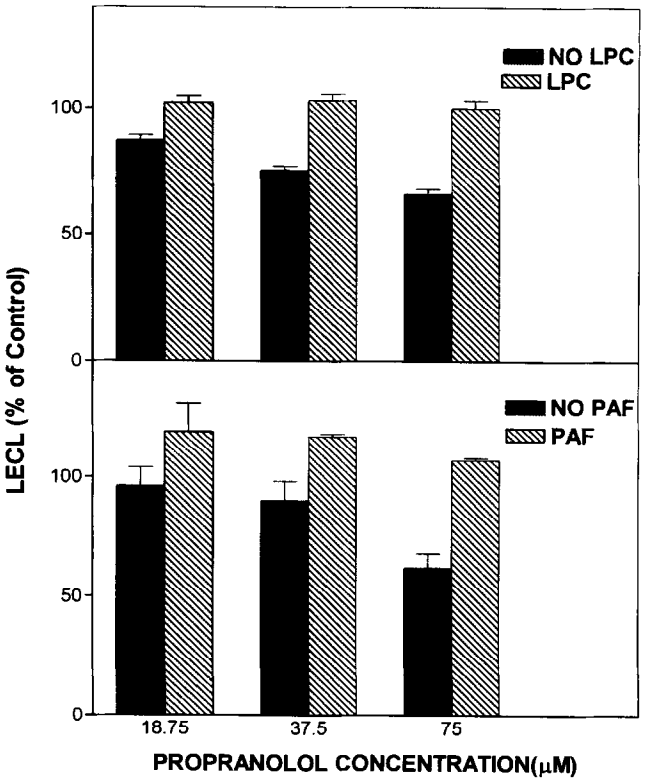

FIG. 2. The effects of LPC and PAF on propranolol-mediated inhibition of the LECL responses of PMA-activated neutrophils. The results of 2–6 experiments are expressed as the mean percentage ± SEM of the corresponding drug-free control systems. The absolute peak LECL values, PMA-activated control neutrophils, and PMA-activated neutrophils exposed to LPC or PAF alone were 3315 ± 871, 4603 ± 908, and 2885 ± 39 mV.s^{-1}, respectively. The corresponding values for PMA-free control systems in the absence of the phospholipids, and with LPC or PAF were 71 ± 18, 91 ± 13, and 62 ± 18 mV.s^{-1}, respectively. The values shown for systems containing propranolol + LPC or PAF were calculated as the mean percentage of the corresponding drug-free phospholipid-treated systems.

pared from PMA-stimulated control cells did not meaningfully affect NADPH-oxidase activity (not shown).

Intracellular Calcium Fluxes, PAF Synthesis, and PLA_2 Activity

The effects of propranolol and dexpropranolol on intracellular calcium fluxes in FMLP-activated neutrophils and on calcium ionophore-stimulated PAF synthesis and PLA_2 activity are shown in Fig. 3. At concentrations of 75 μM and upwards, both agents caused statistically significant ($P < 0.005$) inhibition of FMLP-activated intracellular calcium fluxes that was paralleled by decreased PLA_2 activity and synthesis of PAF, these latter affects achieving statistical significance ($P < 0.025$–$P < 0.001$) at concentrations of 150 and 300 μM with both agents. The inhibitory effects of propranolol and dexpropranolol on the fura-2 fluorescence responses of neutrophils were also evident when AA was used as stimulant (not shown). The inhibitory effects of both drugs on PAF synthesis by neutrophils appeared to be real because (a) the immunoreactivity of reagent PAF was unaffected by coincubation with either propranolol or dexpropranolol, and (b) addition of the drugs to PAF-containing supernatants from activated neutrophils did not affect the reactivity of the phospholipid in the radioimmunoassay.

PKC Activity

Coincubation of PKC with either propranolol or dexpropranolol (150 μM) did not affect enzyme activity. The activity of propranolol- and dexpropranolol-treated PKC was 104 ± 5% and 94 ± 5%, respectively, of the corresponding drug-free control systems (results of 3 experiments).

ATP Levels

Coincubation of neutrophils with propranolol or dexpropranolol at concentrations of up to 300 μM for 30 min at 37°C did not affect cellular ATP levels. The respective values for control neutrophils and those treated with 150 and 300 μM propranolol were 9.2 ± 2.4, 10.9 ± 1.6, and 11.7 ± 2.0 nmoles ATP/10^7 neutrophils, respectively. The corresponding values for cells treated with 150 and 300 μM dexpropranolol were 8.4 ± 1.1 and 12.0 ± 2.3 nmoles ATP/10^7 neutrophils.

Cellular Uptake of Propranolol

In 3 separate experiments, [^{3}H]-propranolol was found to be accumulated by neutrophils to levels that were significantly higher ($P < 0.05$) than extracellular concentrations. The C/E ratios for these experiments (6 replicates in each experiment) were 9.6, 7, and 16.5 (mean ± SEM of 11.3 ± 3).

Membrane-Stabilizing Activity

The membrane-stabilizing interactions of propranolol and dexpropranolol with sheep erythrocytes are shown in Fig. 4. Both agents, at both concentrations tested (75 and 300 μM), caused significant protection ($P < 0.02$–$P < 0.0001$) against the haemolytic effects of LPC, PAF, and LPAF. However, in comparative experiments performed to investigate the membrane-stabilizing potential of the other β-receptor antagonists, these agents, unlike propranolol and dexpropranolol, were unable to neutralize the membrane disruptive activity of LPC. The mean percentages of LPC 97 μM)-mediated haemolysis of sheep erythrocytes for the control, drug-free system and for systems in which the cells were pretreated with 300 μM propranolol, dexpropranolol, atenolol, metoprolol, sotalol, and timolol were 39 ± 0.7, 8 ± 0.7, 9 ± 0.3, 48 ± 1, 46 ± 0.7, 61 ± 1, and 54 ± 5, respectively.

Pretreatment of sheep erythrocytes with propranolol or dexpropranolol was also accompanied by protection of

TABLE 1. Effects of Propranolol and Dexpropranolol on Neutrophil Oxygen Consumption and NADPH-Oxidase Activity

System	O_2 Consumption ($\mu M/10^6$ cells/15 min)	NADPH-oxidase activity ($mV.s^{-1}$)
Control	76 ± 14*	3833 ± 607†
150 μM Propranolol	48 ± 14	Not done
300 μM Propranolol	18 ± 8	1610 ± 328
150 μM Dexpropranolol	50 ± 12	Not done
300 μM Dexpropranolol	24 ± 4	1655 ± 192

Results are expressed as the mean values ± SEMs of 3* and 5† experiments. Oxygen consumption was monitored for the 15-min period following addition of PMA (25 ng/mL) to neutrophils when uptake was linear. NADPH-oxidase activity was measured in purified membrane fractions prepared from PMA-activated neutrophils, in which intact neutrophils were pretreated with the drugs prior to addition of PMA. The level of NADPH-oxidase activity in membrane fractions prepared from unstimulated neutrophils was 44 ± 11 $mV.^{-1}$. All values shown for the propranolol and dexpropranolol-treated systems are statistically different ($P < 0.05$–$P < 0.002$) from the corresponding control systems.
$P < 0.0551$–$P < 0.0017$

these cells against the haemolytic effects of a hypotonic solution of NH_4Cl. The mean percentages of haemolysis observed in the drug-free control system and in systems treated with 75 and 300 μM propranolol and dexpropranolol were 81 ± 2, 67 ± 2 ($P < 0.0001$), 48 ± 6 ($P < 0.003$), 62 ± 2 ($P < 0.0001$) and 58 ± 1 ($P < 0.0002$), respectively.

Interactions of Propranolol and Dexpropranolol With LPC

Spectrophotometric analysis of mixtures of propranolol and dexpropranolol with LPC did not reveal any interactions between these agents.

DISCUSSION

The effectiveness of dexpropranolol and apparent inactivity of atenolol, metoprolol, sotalol, and timolol suggest that the observed inhibitory effects of propranolol on the generation of superoxide by activated human neutrophils are mediated by β_2-adrenoreceptor-independent mechanisms. At the concentrations used, neither propranolol nor dexpropranolol were cytotoxic for neutrophils, nor did they possess superoxide-scavenging properties, indicating that both agents are true inhibitors of reactive oxidant production by neutrophils. These observations confirm previous studies, in which propranolol was reported to modulate the generation of reactive oxidants by stimulated neutrophils by mechanisms that are unrelated to β-blocking effects [11, 12, 19, 20]. Neutrophils activated with phorbol esters and calcium ionophores are sensitive to the inhibitory effects of propranolol [10–12], and the effects of the drug on neutrophils stimulated with FMLP are divergent, with enhancement or inhibition of superoxide generation observed in the absence or presence of cytochalasin B, respectively [11, 19, 20]. The potentiating effects of propranolol on superoxide generation by FMLP-activated neutrophils have been attributed to inhibition of phosphatidate phosphohydrolase by this agent [11, 12]. Because of this dichotomy of responses, we did not use FMLP as a stimulant of neutrophil membrane-associated oxidative metabolism in the present study, selecting instead, PMA, Cal, OZ, and AA.

In the present study, the inhibitory effects of propranolol on neutrophil superoxide generation were dose-related, and observed with all 4 stimuli of neutrophil superoxide production. There was however, an order of sensitivity, with neutrophil LECL responses activated by PMA and Cal being least and most sensitive to propranolol-mediated inhibition, respectively, with responses activated by AA and OZ having intermediate sensitivity. This type of differential sensitivity to propranolol-mediated inhibition of neutrophils activated with PMA and Cal has been described previously [10]. Interestingly, the inhibitory effects of propranolol on Cal-activated neutrophils were observed with concentrations of this β-adrenoreceptor antagonist that did not appear to influence transductional mechanisms involved in the activation of NADPH-oxidase. However, in the case of the other 3 stimuli of neutrophil membrane-associated oxidative metabolism, it is noteworthy that inhibition of NADPH-oxidase coincided with, and may have been a consequence of, propranolol-mediated interference with neutrophil intracellular calcium fluxes, synthesis of PAF, and activity of PLA_2. These effects on neutrophil calcium and phospholipid metabolism could not be attributed to nonspecific cytotoxicity because cellular ATP levels were unaffected by propranolol at concentrations of up to 150 μM, and were slightly increased at higher concentrations (300 μM) of this agent.

The lipophilic nature of propranolol [21], together with its wide-ranging inhibitory effects on membrane-associated activities of stimulated neutrophils, suggested that the plasma membrane was the probable site of anti-oxidative action of this agent. Lipophilicity is an important physicochemical property that distinguishes propranolol from the other β-blocking drugs used in the present study [21], and probably accounts for the observed high level of intracellular accumulation and, at least in part, the well-recognized membrane-stabilizing properties of this agent [22]. Using a

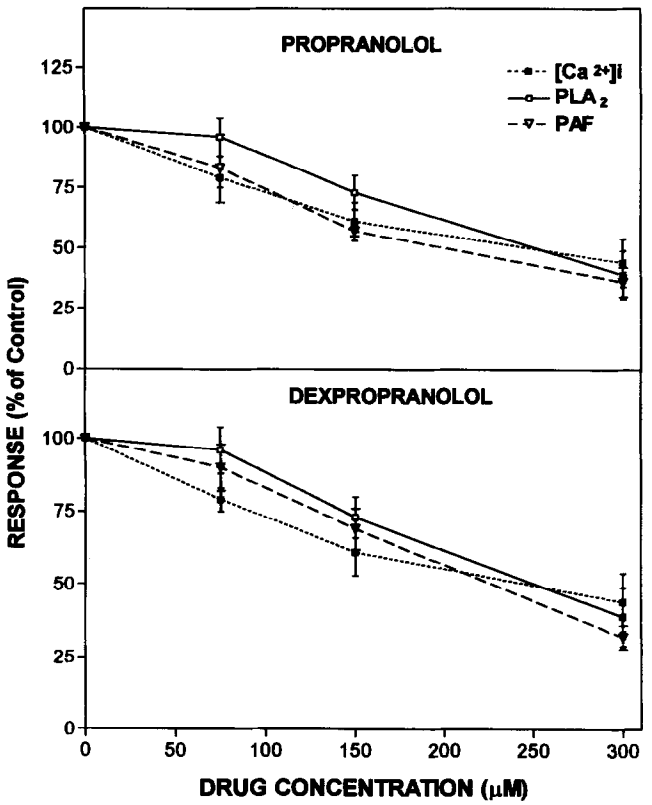

FIG. 3. Effects of propranolol and dexpropranolol on peak intracellular calcium levels $[Ca^{2+}]_i$ in FMLP-activated neutrophils (6 experiments) and on PLA_2 activity (6 experiments) and PAF production (5 experiments) by calcium ionophore-activated neutrophils. The results are expressed as the mean percentage ± SEM of the corresponding drug-free control systems. The mean absolute peak value for $[Ca^{2+}]_i$ in the control systems was 1270 ± 14 nM. The absolute values for release of $[^3H]$-AA by unstimulated and calcium ionophore-activated neutrophils were 5841 ± 207 and 25977 ± 2874 cpm, respectively, and the corresponding value for PAF synthesis by stimulated neutrophils was 21.6 ± 2.3 ng/10^7 cells (unstimulated neutrophils did not generate detectable levels of PAF).

haemolytic assay, we confirmed the membrane-stabilizing properties of propranolol by demonstrating that pretreatment of sheep erythrocytes with either this agent or dexpropranolol, but not with the other β-adrenoreceptor antagonists, increased the resistance of these cells to the haemolytic effects of the bioactive phospholipids LPC, PAF, and LPAF. We were unable to detect complex-forming interactions between propranolol and LPC (spectrophotometric assay) or PAF (radioassay), suggesting that the observed protective effects of this agent are mediated exclusively by membrane-stabilizing mechanisms. This contention is supported by the observation that propranolol also protected sheep erythrocytes against hypotonic lysis.

We also used LPC, LPAF, and PAF to investigate possible relationships between the membrane-stabilizing and anti-oxidative properties of propranolol. Incremental pretreatment of neutrophils with these phospholipids antagonized the inhibitory effects of propranolol (18.75–75 μM) on the generation of superoxide by PMA-activated neutrophils, apparently linking the membrane-stabilizing properties of this agent to its anti-oxidative activity. Antagonism was complete at lower concentrations of propranolol (18.75 and 37.5 μM) and partial at the higher concentration (75 μM), indicating a requirement for higher concentrations of the phospholipids with increasing levels of the drug. The proposed relationship between the membrane-stabilizing and anti-oxidative properties of propranolol is supported by previous studies that have demonstrated that optimum activity of NADPH-oxidase is dependent on the physical state of the neutrophil membrane, which appears to modulate superoxide production by affecting the protein motions, both rotational and lateral, that must take place in the assembly and function of the multicomponent NADPH-oxidase [23, 24]. Agents that increase membrane lateral mobility potentiate NADPH-oxidase, and membrane-stabilizing agents, such as cholesterol and cholesteryl esters, inhibit the production of superoxide [23]. Bioactive phospholipids, including LPC, PAF, and LPAF, are generated during exposure of neutrophils to stimuli of membrane-associated oxidative metabolism and potentiate the activity of NADPH-oxidase [25–29]. Interestingly, the sensitizing effects of lysophosphatides on the pro-oxidative activities of neutrophils are highly correlated with their membrane-disruptive properties [27], suggesting that the membrane-stabilizing interactions of propranolol with these

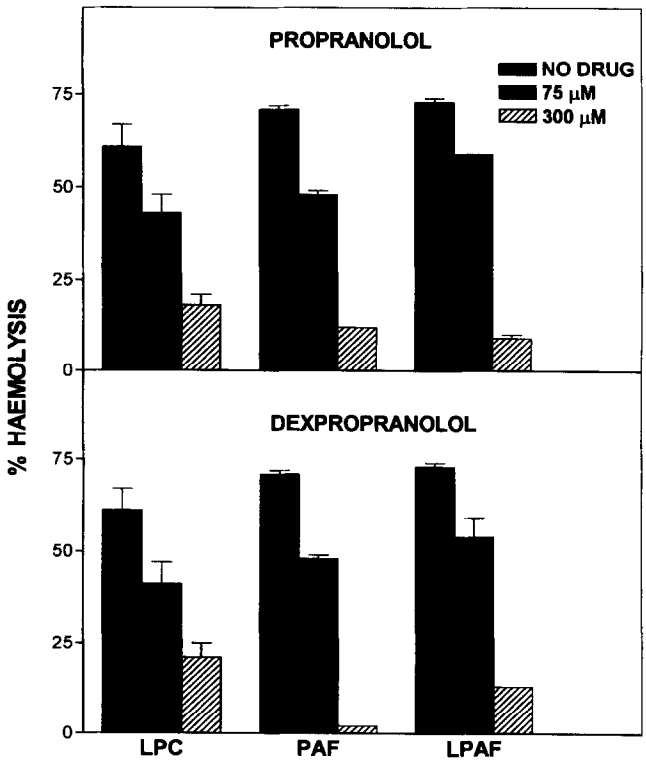

FIG. 4. Effects of propranolol and dexpropranolol (75 and 300 μM) on haemolysis of sheep erythrocytes mediated by LPC, PAF, and LPAF. The results of 4–6 experiments are presented as the mean percentage haemolysis ± SEM.

cells may interfere with the pro-oxidative activities of several endogenously-generated bioactive phospholipids.

Propranolol-mediated inhibition of neutrophil superoxide production apparently occurs by two concentration-dependent mechanisms, both involving membrane stabilization. At low concentrations (<10 μM), the drug selectively inhibits neutrophil superoxide production activated by calcium ionophore. This inhibition occurs in the absence of detectable effects of the drug on transductional mechanisms involved in the activation of NADPH-oxidase, suggesting that the activity of the fully-assembled oxidase is decreased. On the other hand, NADPH-oxidase activity induced by AA, OZ and, especially, PMA is more resistant to low-level membrane stabilization. Intense membrane stabilization with high concentrations of propranolol is required to inhibit superoxide generation by neutrophils activated with these stimuli. However, at these concentrations of propranolol, cellular calcium and phospholipid metabolism is also compromised, suggesting that interference with transductional mechanisms may be the predominant mechanism of decreased activity of NADPH-oxidase at higher concentrations of propranolol. Although PKC has previously been suggested to be a potential target of propranolol-mediated inhibition of NADPH-oxidase [11], our data suggest that membrane stabilization is the probable primary mechanism of anti-oxidative action of this agent, which in all probability would lead to secondary inhibition of PKC. Nevertheless, it is possible that neutrophil isoforms of PKC may be more susceptible to direct inhibition by propranolol. In support of the proposed relationship between membrane stabilization and inhibition of superoxide generation by activated neutrophils, we have recently observed that a series of lipophilic, structurally unrelated compounds, including macrolide antimicrobial agents and the long-acting β_2-agonist salmeterol, also inhibit neutrophil membrane-associated oxidative metabolism by membrane-stabilizing mechanisms (unpublished observations).

It is noteworthy that the concentrations of propranolol that cause membrane stabilization and inhibition of neutrophil membrane-associated oxidative metabolism are remarkably similar to those that potentiate the migratory responses of these cells [9], suggesting a mechanistic relationship between these events. We concede, however, that the concentrations of propranolol used in this study are somewhat higher than serum levels achieved during chemotherapy with this agent [30]. However, several lines of evidence suggest that the membrane-stabilizing, anti-oxidative activities of this β-antagonist may be operative *in vivo*. First, propranolol, as demonstrated in the present study, is concentrated by neutrophils so that serum levels may not be representative of cell and tissue levels. Second, although the anti-oxidative properties of propranolol described here are clearly of secondary clinical importance, it is noteworthy that the circulating leucocyte count, and the neutrophil count in particular, is a well-recognized, independent predictor for future development of several cardiovascular disorders [31, 32]. This is in keeping with a recent report that the decrease in mortality by propranolol in patients with heart disease and complex ventricular arrhythmias is more of an anti-ischaemic than an antiarrhythmic effect [33]. Third, there is some evidence, albeit indirect, that leucocytes from propranolol-treated individuals have increased membrane stability [1–6].

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