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Ca^{2+}-dependent production of reactive oxygen metabolites by human neutrophils in response to fluorinated propranolol analogues

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Abstract

Fluorinated analogues of propranolol, namely trifluoroethyl propranolol (F3), pentafluoropropyl propranolol (F5), and heptafluorobutyl propranolol (F7), were found to induce reactive oxygen metabolite (ROM) production in human neutrophils in a dose-dependent manner. Preincubation of neutrophils with the calcium chelator BAPTA-AM or the tyrosine kinase inhibitor genistein inhibited this ROM production. Direct measurements of intracellular calcium revealed that these analogues caused a transient increase in intracellular calcium. In addition, these fluorinated analogues of propranolol caused a transient increase in actin polymerization. The effects of these compounds were found to be dependent upon the degree of fluorination of the parent compound. Propranolol, on the other hand, had no direct effect on ROM, calcium, or actin polymerization when added alone to neutrophils, although it did modify responses of cells to various stimuli. Whereas ROM production induced by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine was enhanced in a dose-dependent manner, the response to the particulate stimulus, latex beads, was abolished.

Keywords: Neutrophils; Calcium; Fluorinated analogues; ROM; Propranolol

1. Introduction

The antioxidant properties of propranolol have been advocated for a number of years [1–4]. The suggestion of antioxidant activity for propranolol suffered a serious setback when reports that propranolol inhibited xanthine oxidase [5], one of many enzymes that produce reactive oxygen species, could not be confirmed. The antioxidant role for propranolol is thought to be mediated by its ability to partition within plasma membranes and scavenge ROM, especially products of lipid peroxidation [6,7]. Since partitioning into the plasma membrane is an attribute of hydrophobicity, the possibility existed that increasing hydrophobicity of propranolol by fluorination [8–10] would increase its putative antioxidant effects. In the present work, we tested the effect(s) of propranolol and its fluorinated analogues on human neutrophil.

The neutrophils constitute the first line of defense against invading microorganisms. The cells are capable of chemotaxis towards the invaders, phagocytosis, and intracellular killing of ingested microbes in a well-coordinated manner that ensures stimulus–response coupling. Intracellular killing occurs via several mechanisms of which transient production of ROM is a major player [11,12]. The neutrophils' non-mitochondrial oxidase system reduces molecular oxygen to ROM in response to many soluble and particulate stimuli. Thus, activation of this oxidase system provides a good model for studying the antioxidant properties of propranolol and its fluorinated analogues. Here, we demonstrate that whereas propranolol per se had no direct effect on ROM production, its fluorinated analogues activated ROM production directly and in a dose-dependent manner. The extent of activation was dependent upon the degree of fluorination. This activation was accompanied by transient actin polymerization and transient calcium mobilization. Inhibition of calcium mobilization or tyrosine phosphorylation inhibited ROM production by these analogues. Pretreatment of cell with propranolol abolished the ability of these cells to produce ROM. Our results suggest a prooxidant rather than antioxidant role for fluorinated analogues of propranolol.

Abbreviations: PA, phosphatidic acid; ROM, reactive oxygen metabolites; fMLP, formyl-methionyl-leucyl-phenylalanine; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid, AM, tetra-acetoxymethylester; and 2, 7 DECFDA, 6-carboxy-2,7-dichlorodihydrofluorescein diacetate.

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2. Materials and methods

2.1. Materials

Racemic propranolol was supplied by Berk Pharmaceuticals and fluorinated analogues, namely heptafluorobutyl propranolol (F7), pentafluoropropyl propranolol (F5), and trifluoroethyl propranolol (F3), were synthesized as described previously [13]. Fura-2-AM (1-(2,5-carboxyoxazol-2-yl)-6-aminobenzofuran-5oxyl)-2-(2-amino-5-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetoxymethyl ester), BAPTA-AM, genistein, 2,7 DCFDA, and rhodamine-phallacidin were all purchased from Molecular Probes. All other reagents were Analar grade and were purchased from Sigma and BDH Chemicals.

2.2. Preparation of human neutrophils

Human peripheral blood neutrophils were prepared by dextran sedimentation of heparinized whole blood obtained from healthy donors and centrifuged through Ficoll–Paque as described previously [14]. Contaminating red blood cells were removed by hypotonic lysis with isotonic NH_4Cl. The remaining cells were suspended in Krebs–HEPES medium (pH 7.4) containing 120 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25 mM HEPES, and 0.1% BSA and were further purified through neutrophil isolation medium (Cardinal Associates). Final purity and viability were both between 98–99% as indicated by flow cytometry and trypan blue dye exclusion tests.

2.3. Measurement of reactive oxygen species production

The production of ROM by neutrophils was measured using luminol-dependent chemiluminescence (LDCL) as described previously [15–17]. To confirm ROM production, we used 2,7 DCFDA essentially as described previously [18]. Briefly, 10^6 neutrophils were incubated with 1 μM 2,7 DCFDA (room temperature for 15 min). Cells were washed and allowed to adhere to glass coverslips (10 min at room temperature). Coverslips were washed and secured between two plates of a custom-designed coverslip holder, placed onto a microscope stage, and viewed using the Leica TCS confocal microscope (Leica-Kaki). Images were obtained at a temporal resolution of 400 msec/image. Changes in fluorescence were color-coded and displayed at the specified time intervals.

2.4. Actin polymerization measurements

The extent of actin polymerization was measured by the increase in binding sites for rhodamine-phallacidin as described previously [19,20]. Briefly, neutrophils (10^7/mL) were incubated at 37° for 10 min in a stirred temperature-controlled chamber. Samples (100 μL) were drawn and the cells fixed in 3.7% formaldehyde in PBS for 15 min at room temperature. Propranolol or fluorinated propranolol (1.5–200 μM) was added to the cells and 100-μL samples were drawn at different time intervals and fixed as above. Fixed samples were washed and permeabilized in 0.5% Triton X-100 in PBS for 5 min, washed three times with PBS, and then stained with rhodamine-phallacidin (0.33 μM) for 1 hr at room temperature. Fluorescence intensity of washed cells was measured using flow cytometry (FACScan, Becton Dickinson).

2.5. Measurement of calcium

Neutrophils were loaded with 1-(2,5-carboxyoxazol-2-yl)-6-aminobenzofuran-5oxyl)-2-(2-amino-5-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetoxymethyl ester (fura-2-AM) as described previously [21]. The cells were washed, placed on glass coverslips, and allowed to adhere for 15 min at room temperature. Coverslips with adherent cells were rinsed with Krebs–HEPES. Coverslips were secured between two plates of a custom-designed coverslip holder, placed onto a heated microscope stage (33°), and intracellular free calcium ($[Ca^{2+}]_i$) measurements were performed on individual cells using the IonVision dual-excitation system (ImproVision) as described previously [14].

3. Results

3.1. Effect of fluorinated propranolol on reactive oxygen species production in human neutrophils

Luminol-dependent chemiluminescence was used to investigate the effect of propranolol and its fluorinated analogues on reactive oxygen species production by human neutrophils. Treatment of cells with propranolol (50–300 μM) had no apparent effect on ROM production in resting human neutrophils. However, the response to fMLP (1 μM) was enhanced by propranolol in a dose-dependent manner with maximum potentiation occurring at 200 μM (Fig. 1a). Both the *S*-(+) and *R*-(−) propranolol enantiomers were equally active. In contrast, the response to activation by particulate stimuli (unopsonized latex beads) was inhibited by pretreatment of neutrophils with propranolol (Fig. 1b).

Heptafluorobutyl propranolol (F7), induced a rapid and dose-dependent increase in ROM that peaked within minutes (Fig. 2a). The time for peak ROM production varied between preparations of neutrophils from different donors (range 1–8 min). Other fluorinated analogues, namely pentafluoropropyl propranolol and trifluoroethyl propranolol (F5 and F3, respectively), were also effective with the degree of activation being dependent upon the number of fluorine atoms in the substituted side chain. Unless otherwise stated, data presented in the figures refer to results obtained with the most potent analogue F7 as an example of the effects of these compounds on human neutrophils.

To confirm the effect of F7 on ROM production, we

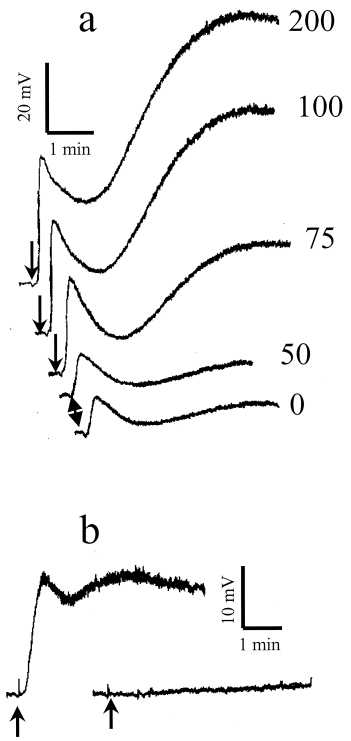


Fig. 1. Effects of propranolol on ROM production by human neutrophils as measured by luminol-dependent chemiluminescence. (a) fMLP-induced ROM production by cells pretreated with propranolol for 2 min at the indicated doses (0–200 μM). Arrowheads indicate the time of addition of the stimulus fMLP (1 μM). (b) Latex bead (10^8/mL)-induced ROM production (left trace) is inhibited by pretreatment of cells with 100 μM of propranolol. Arrowheads indicate the time of addition of the beads. The traces are representative of at least 5 experiments with neutrophils isolated from three different donors.

investigated its effect fluorimetrically using the ROM-sensitive dye 2,7 DCFDA [18]. We found that F7 induced a transient increase in 2,7 DCFDA fluorescence consistent with a rise in ROM production (Fig. 2b). The maximum increase was noted at around 5 min, but again the time to reach peak ROM production was donor-dependent. Unlike propranolol, these compounds exhibited an additive effect on latex bead-induced ROM production (Fig. 2c). Whether these analogues acted on the same site that propranolol binds to was tested by treating these cells with propranolol (200 μM) prior to challenge with the analogues. Under such conditions, propranolol totally abolished the response to F7 (Fig. 2d).

To probe the mechanism through which F7 evokes ROM production, we tested the involvement of calcium, tyrosine phosphorylation, and actin polymerization. We found that pretreatment of neutrophils with the calcium chelator BAPTA-AM (1 μM) or the tyrosine kinase inhibitors genistein (20 ng/mL) and herbimycin-A (1 μg/mL) abolished ROM production by fluorinated analogues (Fig. 3a–c).

Since the neutrophil oxidase system is under the control of filamentous (F)-actin [22–24], the possibility existed that agents which modify the state of intracellular actin would also modify ROM responses. This possibility was tested using the actin-depolymerizing agent cytochalasin B (CB) [23,24] and the actin-polymerizing agent jasplakinolide (Jas) [25,26]. Whereas CB (10 μM) potentiated the effect of these analogues on ROM production, as exemplified in Fig. 4a where the ROM response to F7 was significantly enhanced, Jas (10 μM) significantly reduced ROM production by F7 (Fig. 4b), suggesting that the response to these analogues was dependent upon the status of polymerized actin within the neutrophils.

3.2. Effect of fluorinated propranolol on actin polymerization in human neutrophils

One of the earliest events following neutrophil activation is a rapid and transient increase in the cellular content of polymerized actin [19,20,27]. Using flow cytometry to measure the binding of rhodamine-phallacidin to F-actin, we found that fluorinated propranolol caused a rapid and transient increase in the number of binding sites for rhodamine-phallacidin (Fig. 4c). This is consistent with a transient rise in actin polymerization, with an initial rapid phase reaching a maximum in 10–20 sec, followed by a decay phase to prestimulatory levels by 60–80 sec. Interestingly, this ability was also dependent upon the number of fluorine atoms in the substituted side chain. The maximum effect on actin polymerization occurred with the pentafluoropropyl propranolol (F5, 200 μM). Propranolol, on the other hand, had no apparent effect on the state of polymerized actin in resting cells within the time scale of these experiments. However, responses to fluorinated analogues were altered by pretreatment of cells with propranolol. The degree of alteration depended upon the analogue used, such that the response to F7 was only marginally inhibited whereas responses to F5 and F3 were totally abolished by pretreatment of cells with propranolol (200 μM).

3.3. Effect of fluorinated propranolol on calcium homeostasis

The involvement of calcium in the molecular mechanism of F7-induced ROM production was suggested by our finding that pretreatment of neutrophils with BAPTA-AM abolished ROM production. We therefore tested the involvement of calcium directly using the calcium indicator fura-2. Resting neutrophils exhibited a calcium level of 62 $\pm$ 3 nM. Upon treatment with F7 (15 μM), intracellular free calcium transiently rose to 606 $\pm$ 22 nM (average peak calcium concentration per cell) before decaying back to the prestimulatory levels (Fig. 5a and b). The response was both asynchronous and heterogeneous with the number of cells responding and the extent of the calcium transient being dependent upon the concentration of F7. The latter is demonstrated in Fig. 5c. This effect on intracellular calcium was dependent upon the degree of fluorination of the propranolol analogues, with the potency being F7 $>$ F5 $>$ F3. The

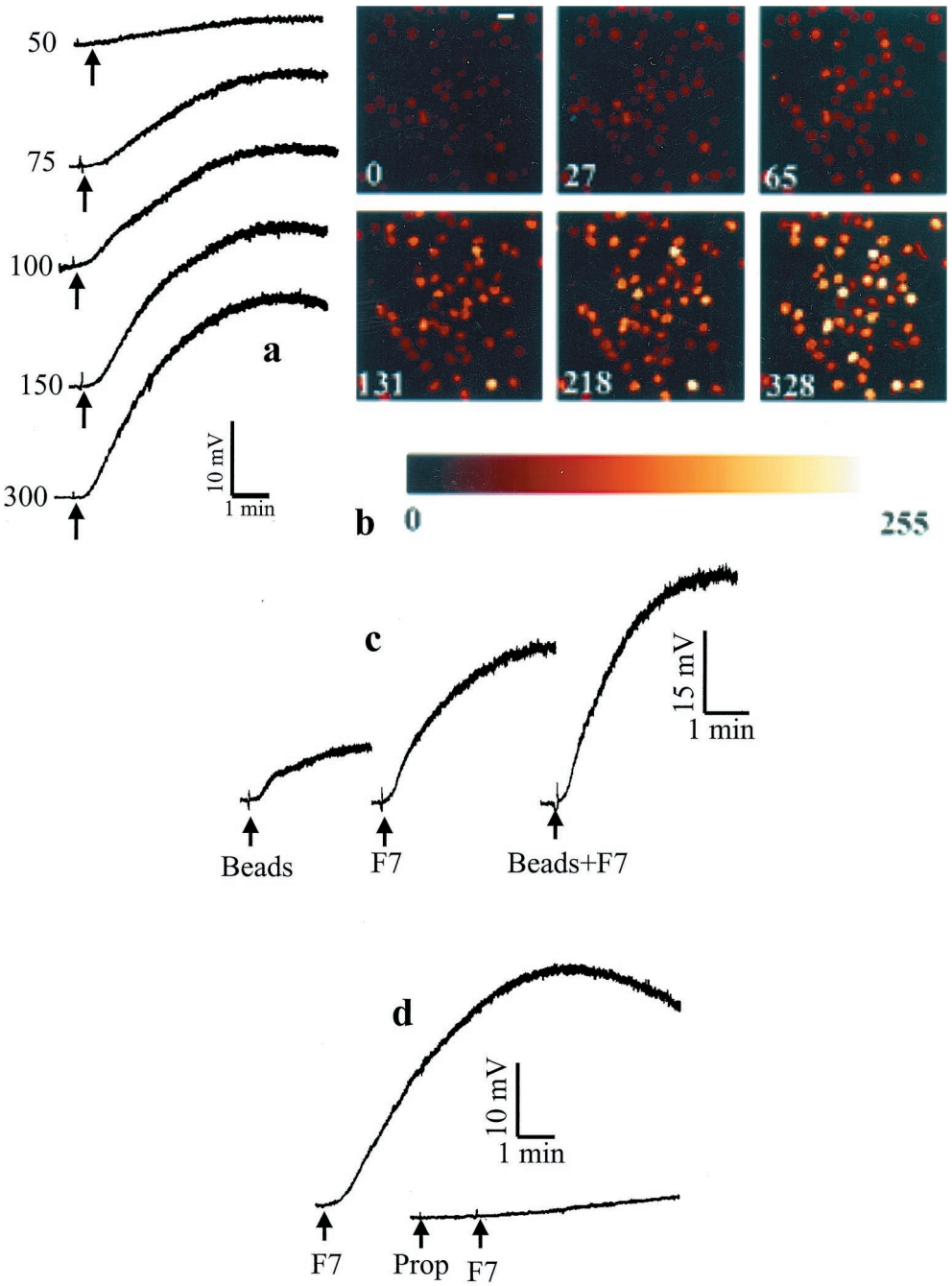


Fig. 2. Effect of the fluorinated analogue of propranolol (F7) on ROM production as measured by luminol-dependent chemiluminescence. (a) Dose–response traces of human neutrophils treated with F7 as indicated by the numbers on the left. (b) Confocal micrographs showing increased fluorescence intensity of 2,7 DCFDA-labeled human neutrophils at the indicated time intervals (in seconds) in the bottom left corner of each image. The fluorescence intensity is coded according to the color bar. Scale bar is 20 μm. (c) Additive effect of F7 (100 μM) + beads (10^7) on ROM production by neutrophils. (d) Inhibition of F7-induced ROM production by treatment of cells with propranolol (Prop, 200 μM) prior to F7 (100 μM).

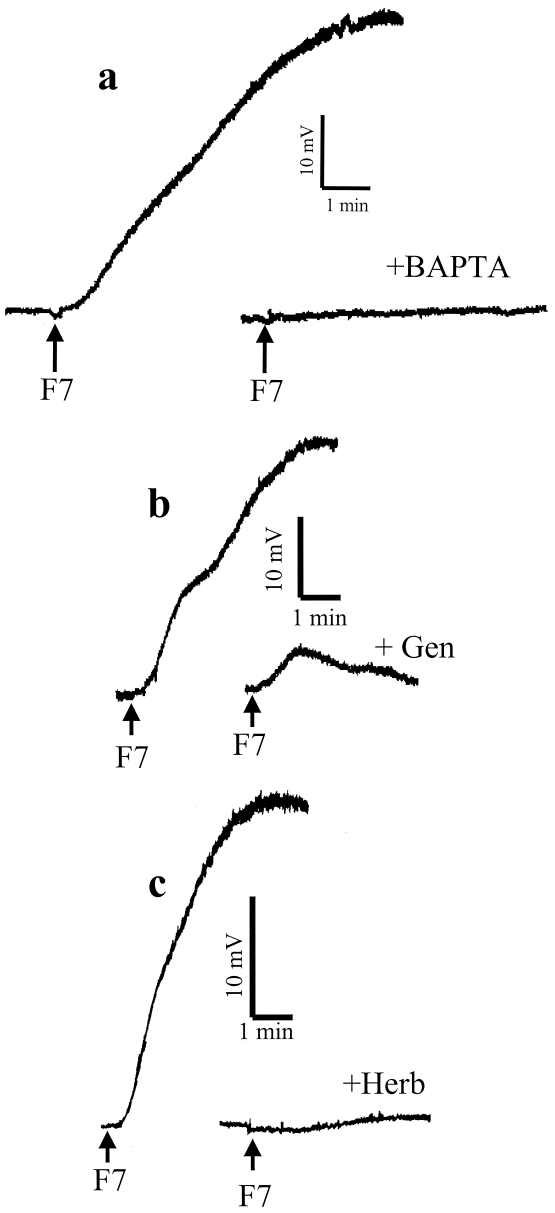


Fig. 3. Effect of fluorinated analogues of propranolol on ROM production. (a) Pretreatment of human neutrophils with BAPTA-AM (1 μM, 30 min at room temperature, right-hand trace) abolishes ROM production due to F7 (100 μM). (b) Inhibition of F7-induced ROM production by pretreatment of cells with genistein (20 μng/mL). (c) Inhibition of F7-induced ROM production by pretreatment of cells with herbimycin (10 μM). The data are representative of at least 5 experiments with neutrophils obtained from different donors.

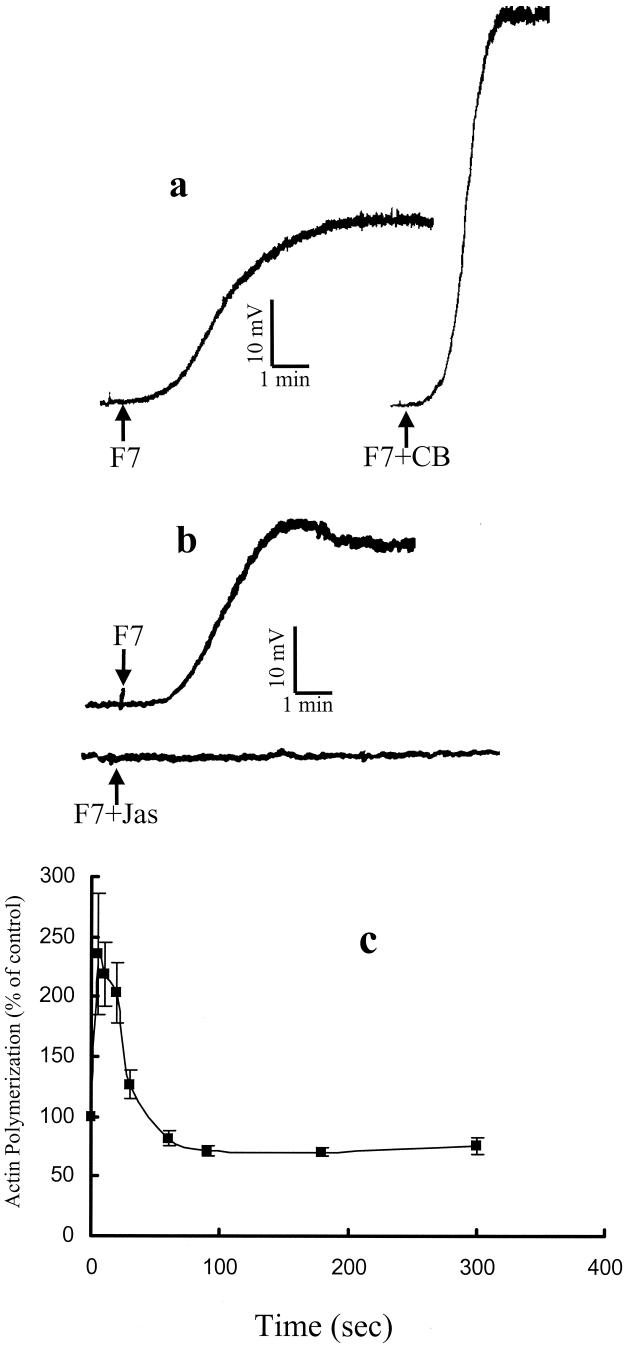


Fig. 4. Effect of fluorinated propranolol in the presence of F-actin-modifying agents. (a) The effect of cytochalasin B (CB, 10 μM) on F7-induced ROM production. (b) Effect of jasplakinolide (Jas, 10 μM) on F7-induced ROM production. (c) Direct effect of fluorinated propranolol on actin polymerization as measured by changes in the number of binding sites for rhodamine-phallacidin. The effect on actin polymerization is most evident with pentafluoropropyl propranolol (F5, 200 μM). Each data point is the mean ± SEM, N = 3.

parent compound propranolol, on the other hand, had no apparent effect on calcium homeostasis (Table 1).

4. Discussion

The antioxidant role of beta-blockers has been advocated by a number of investigators [28–30]. This role was suggested to be mediated by the ability of these compounds to partition within biological membranes and scavenge reactive oxygen metabolites, especially lipid peroxides [6,7]. Since fluorination increases hydrophobicity [8,9], thus enhancing partitioning of these compounds to the plasma membrane, we investigated the antioxidant/pro-oxidant

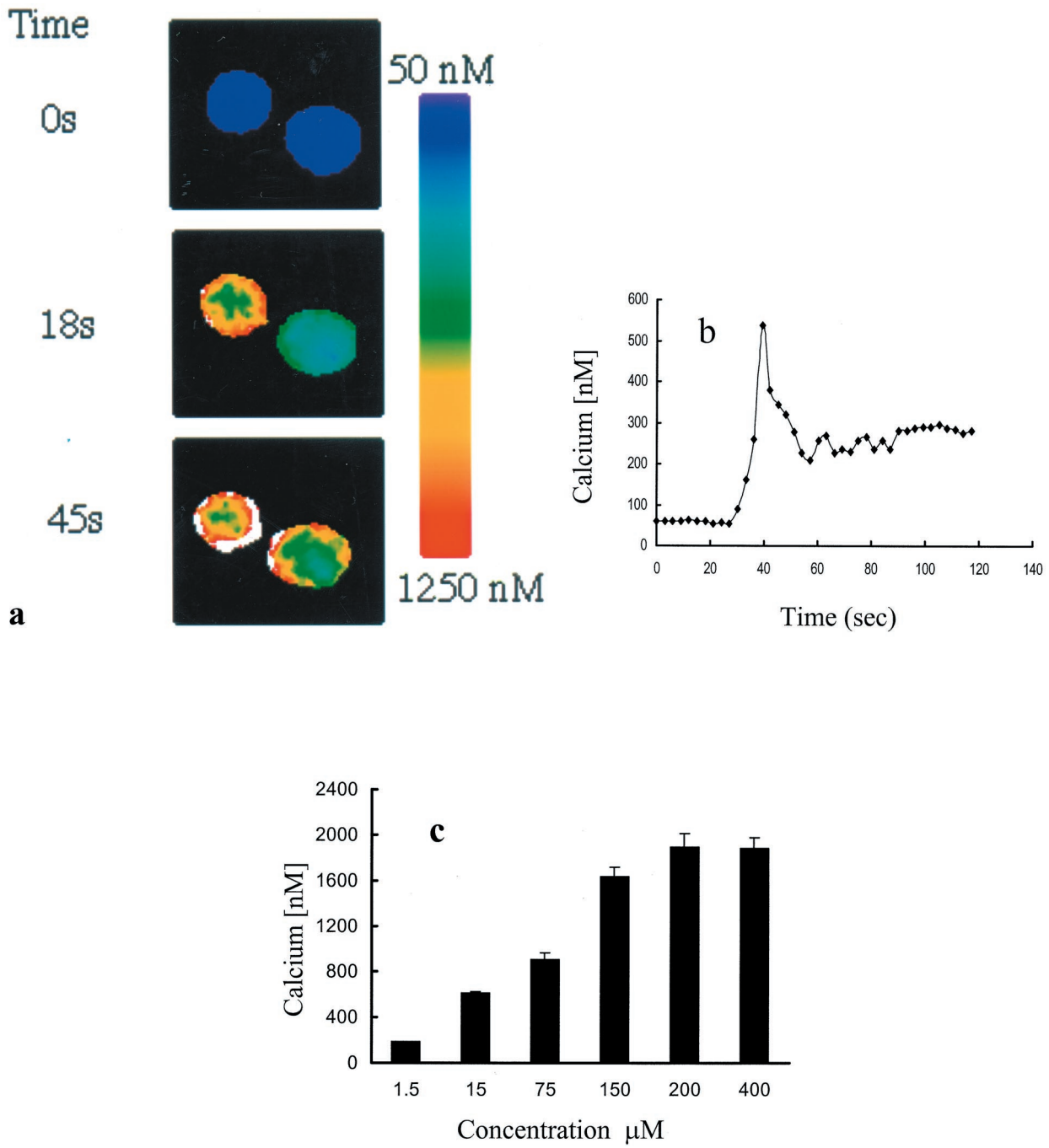
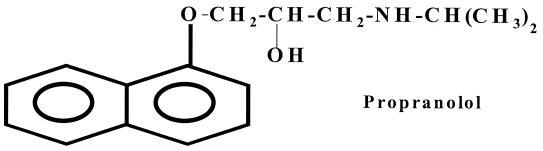
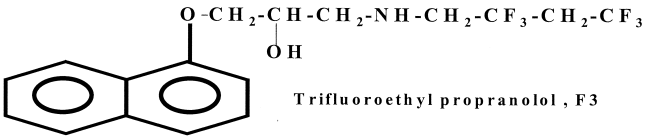
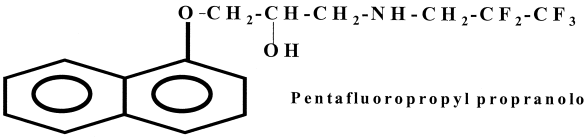
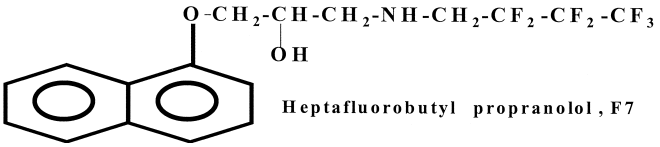


Fig. 5. Changes in calcium homeostasis following stimulation with fluorinated propranolol. (a) Intracellular calcium maps of resting (time 0 msec) and F7-stimulated neutrophils at the indicated time intervals (in seconds). The calcium maps are color-coded such that high calcium is indicated by warm colors. (b) Calcium changes in a single neutrophil following treatment with F7. This is a representative experiment performed with cells obtained from at least three different donors. (c) Dose–response relationship between the extent of the calcium transient (peak calcium) and the concentration of F7 used. This is a representative experiment of at least 4 experiments performed on neutrophils obtained from three different donors.

properties of propranolol and its fluorinated analogues using human neutrophils as a biological source of ROM.

We found that pretreatment of neutrophils with propranolol had no apparent effect on ROM in resting neutrophils, although the response to ROM-inducing stimuli was modified. Formylated peptide fMLP-induced ROM production was enhanced by pretreatment of the cells with propranolol, suggesting a pro-oxidant role. In contrast, the neutrophil response to the particulate stimulus, latex beads, was totally abolished by pretreatment with propranolol, suggesting an antioxidant role. This apparent anomaly may underlie the conflicting reports in the literature regarding the antioxidant role of beta-blockers. Since ROM can be generated via at least two molecular mechanisms (Ca^{2+}-dependent and -in-

Table 1
A summary of the potency of propranolol and its analogues on the various parameters examined

Structure	ROM	ACTIN	Calcium
 Propranolol	0	0	0
 Trifluoroethyl propranolol , F3	+	+	+
 Pentafluoropropyl propranolol , F5	++	+++	++
 Heptafluorobutyl propranolol , F7	+++	++	+++

Potency is graded from no effect (0) to maximum effect (+++).

dependent [31]), we propose that the observed differences in response to propranolol may be dependent upon the mechanism of ROM generation.

In contrast to propranolol, fluorinated analogues activated the neutrophils directly. The potency of these analogues depended on the degree of fluorination, with F7 being the most potent in many of the responses investigated. This activation caused a transient calcium rise, a transient actin polymerization, and increased ROM production. Furthermore, inhibition of the calcium transient abolished ROM production, suggesting that the analogues affected the calcium-dependent mechanism of ROM production. Although attempts at correlating calcium transient and ROM production in response to the fluorinated analogues have not been carried out, a direct correlation is likely to be the case. This is supported by the following: (i) inhibition of calcium transient by BAPTA-AM inhibited ROM production; (ii) calcium transient always precedes ROM production; and (iii) simultaneous measurement of calcium transient and ROM production has reported a direct correlation between ROM production and the extent of the calcium transient at the time of onset of ROM production [21].

Interestingly, whereas propranolol inhibited ROM production by latex beads, its fluorinated analogues had an additive effect, suggesting a divergent molecular mechanism of action for propranolol and its analogues. This additive effect was not surprising, since the analogues seem to be acting via the calcium-dependent pathway of ROM production, whereas beads induce ROM production in a calcium-independent manner [20]. However, since latex beads are often stabilized with polyanions and these analogues are positively charged at physiological pH, the possibility existed that the effect of analogues on ROM production by beads may be a result of clustering of the beads, thereby altering the ROM response. This possibility seems unlikely for the following reasons: (i) visual examination of the beads failed to show any clusters after treatment with the analogues and (ii) neutrophils displayed an additive response when challenged with beads and fluorinated analogues simultaneously. The finding that agents which modify the cellular actin status also modified ROM production in response to these analogues confirms previous work illustrating that ROM production in neutrophil is inversely proportional to the level of polymerized actin [22–24].

Occupation of β-adrenoceptor by many agonists has been demonstrated to modulate or inhibit calcium-dependent responses, including ROM production by human neutrophils [32–34]. Whether fluorinated propranolol acts through receptor occupation or membrane perturbation has yet to be determined. It is noteworthy, however, that occupation of β-adrenoceptors leads to inhibition of inflammatory responses in these cells [32]. Our data show that fluorinated propranolol stimulates rather than inhibits inflammatory responses.

Propranolol was demonstrated to cause accumulation of PA by inhibition of PA phosphohydrolase, the enzyme responsible for the production of diacylglycerol (DG) from PA [35–37]. Phosphatidic acid has been established as a second messenger for the generation of ROM in human neutrophils [35]. Whereas PA accumulation per se did not trigger ROM production in human neutrophils, fMLP-induced ROM production was potentiated by PA in a dose dependent manner [38]. At least two molecular mechanisms exists for the generation of PA: (i) hydrolysis of (preferentially) phosphatidyl choline by phospholipase D (PLD) and (ii) phosphorylation of DG by DG-kinase [39,40]. Interestingly, inhibition of DG-kinase, which is expected to reduce PA generated from DG, leads to augmentation of ROM production by fMLP [41]. This suggests a minor contribution of PA generated by this pathway and points towards the involvement of PLD in the propranolol signaling pathway. Phosphatidic acid has two possible functions: (i) a Ca^{2+} signaling function and (ii) a source of diacylglycerol [42]. Exogenous PA causes intracellular calcium transient [43, 44]. However, since generated PA exists only in the plasmalemma [42,45], calcium transients invoked by PA would be confined to the immediate environment of the plasma membrane. It therefore follows that if the effects of propranolol on ROM were mediated by PA generation, then calcium transient within the immediate environment of the plasma membrane should ensue. In our experiments, no calcium transients were observed following propranolol encounter, dismissing this possibility. Furthermore, no plasma membrane-localized calcium transients were observed (that is, at the temporal resolution used, seconds) following treatment of neutrophils with fluorinated analogues.

This investigation confirms the previously reported effect of propranolol on human neutrophils [6,32–34]; however, to our knowledge, no previous report describing the pro-oxidant effects of fluorinated analogues has ever been published. Interestingly, propranolol has been on the market for many years, yet no adverse side effect attributed to its pro-oxidant property has ever been reported. The concentrations of propranolol required to enhance ROM production are far higher (approx. 50–100 times) than the plasma concentration observed in patients taking propranolol [46]. Whether such high concentrations exist within microenvironment in vasculature has yet to be established. The question therefore arises as to whether the effects we observed *in vitro* are indeed reflected *in vivo*. Such a question remains to be answered.

Acknowledgments

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