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The Potency and Kinetics of the β-Adrenergic Receptors on Human Neutrophils

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SUMMARY

The binding ot catecholamines to the β-adrenergic receptors on human polymorphonuclear leukocytes rapidly inhibits cell responses stimulated by chemoattractant ligands. As a first step in understanding the mechanism of the inhibition, we investigated the number of β-receptors required to optimally block superoxide anion production, a response that is measured kinetically by a convenient spectrophotometric assay for the reduction of cytochrome *c*. We found that after blockade of 50–60% of the β-adrenergic receptors with an irreversible antagonist, maximal inhibition of the response was still elicited by isoproterenol (ISO). Next we investigated the kinetics with which superoxide generation is inhibited. We found that half-maximal inhibition was observed at 3×10^{-8} M ISO, which approximates the K_d because many of the receptors are involved. Cell responsiveness recovered when propranolol was added between the time of ISO and chemoattractant addition. From the recovery we estimated that the half-time for ISO dissociation is less than 10 sec. Finally, we examined the rate at which cell responses decay following ISO administration after chemoattractant. Optimal rates of inhibition, turning off oxidant production in seconds, occur at ISO concentrations $\geq 3 \times 10^{-7}$ M. Taken together, these observations are consistent with an association rate constant for ISO estimated to be $\geq 10^8$ $M^{-1}min^{-1}$ and a dissociation rate constant ≥ 4 min^{-1}. These results are discussed in terms of the available data concerning the binding of agonists to β-adrenergic receptors.

Many cell responses are regulated by an interplay between stimulatory and inhibitory receptors. The best studied example is regulation of adenylate cyclase activity, which is stimulated by several receptors linked to G_s (for example β-adrenergic receptors, prostaglandin receptors, and glucagon receptors) and is inhibited by receptors linked to G_i (for example α_2-adrenergic receptors and some types of muscarinic and opiate receptors) (1, 2).

We, as well as others, have begun to investigate neutrophil activation as another system in which cellular response is stimulated by some receptors and inhibited by others (3–7). Stimulatory ligands for the neutrophil (for example N-formyl peptides, leukotriene B_4, and C5a) activate this cell by evoking both chemotaxis and the release of potent inflammatory mediators such as oxidants and proteases. An inhibitory pathway is activated by catecholamines (β_2-receptors), prostaglandins of the E series, adenosine (A2 receptors), and histamine (H2 receptors) (6, 8–10). These agents all stimulate adenylate cyclase but inhibit neutrophil function.

Catecholamines inhibit the release of lysosomal enzymes and superoxide anions in response to stimuli such as formyl peptides. Furthermore, inhibition of intracellular signalling events that link activation of the formyl peptide receptor to cell response was observed. Phosphoinositide metabolism and intracellular Ca^{2+} elevation were affected by 10^{-6} M ISO (3). Cell functions such as phagocytosis and chemotaxis, which involve the cytoskeleton, are less or variably inhibited. Accordingly, minimal inhibition of actin polymerization and right angle scatter were observed (3). This could indicate that the cell activation process through formyl peptide receptors may not be entirely obliterated by the ISO pathway.

The mechanism by which the occupancy of 10^3 β-adrenergic receptors is capable of inhibiting the oxidant production stimulated by up to 10^5 formyl peptide receptors is incompletely understood (11). The inhibitory pathway is presumably important in limiting the extent or location of tissue injury caused by neutrophil activation.

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ABBREVIATIONS: G_s and G_i, stimulatory and inhibitory GTP-binding proteins regulating adenylate cyclase; BIM, N^6-(Bromoacetyl)-N^1-[3-(4-indolyoxy)-2-hydroxypropyl]-(*Z*)-1,8-diamino-*p*-menthane; FLPEP, *N*-formyl-norleu-leu-phe-norleu-tyr-lys-fluorescein; ISO, L-(−)-isoproterenol; PROP, propranolol; *t*-boc, *t*-butoxy-carbonyl-phe-leu-phe-leu-phe; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

Aside from its physiological roles in host defense and tissue injury, the oxidant-generating system in the neutrophil provides a unique opportunity to evaluate the inhibitory aspects of the β-adrenergic/G_s pathway. The production of superoxide anion is conveniently measured in neutrophils as a spectrophotometric reduction of cytochrome *c* (12). In this system, the interplay between stimulatory and inhibitory pathways can be evaluated kinetically and in real-time. Using this approach, we are able to evaluate both quantitative and kinetic aspects of this novel pathway. An analysis of the speed of this pathway and the number of β-receptors required to activate it and a prediction of the binding rate constants of the β-adrenergic agonist are presented in this report. A separate study details the biochemistry of this pathway.

Experimental Procedures

Materials

Fluorescein-labeled FLPEP was prepared and characterized as previously described (13). FLPEP was used as a stimulus rather than nonfluorescent peptide in order to compare results in this report with earlier observations (14, 15). The N-formyl peptide receptor blocker *t*-boc was obtained from Vega Biotechnologies, Inc. (Tucson, AZ). ISO, cytochrome *c* (type IV; Sigma Chemical Co., St. Louis, MO) and *dl*-PROP hydrochloride (Aldrich Chemical Co., Inc., Milwaukee, WI) were obtained commercially. BIM was a gift from Dr. Josef Pitha (National Institutes of Health, Bethesda, MD).

Methods

Neutrophils. Neutrophils were obtained from fresh venous blood of normal human female volunteers and drawn into 1/7 volume of acid citrate dextrose. The cells were isolated by the elutriation method of Tolley *et al.* (16), washed in a buffer containing 147 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.3 mM $MgSO_4$, 0.2 mM KH_2PO_4, 1.4 mM Na_2HPO_4, 1 g/liter glucose, and 1.5 mM $CaCl_2$ (pH 7.4), and resuspended in the same buffer system without calcium at 10^7 cells/ml. Cells were diluted to 2.7×10^6 cells/ml into the above buffer containing $CaCl_2$ and supplemented with 10 mM HEPES, adjusted to pH 7.4 with NaOH and equilibrated at 37° for several minutes before assay of oxidant production (3).

Measurement of superoxide anion production. The superoxide dismutase-inhibitable reduction of cytochrome *c* was monitored by recording the change in the transmittance at 550 nm of a stimulated cell suspension, corrected for the contribution of cell light-scattering changes in the presence of superoxide dismutase (12). The concentration of cytochrome *c* in the sample was 120 μM and the superoxide dismutase concentration in the controls was 60 μg/ml. Spectroscopic measurements were made in a SLM 8000 C spectrofluorometer (SLM Instruments Inc., Urbana, IL). The transmittance data were converted to absorbance values by computer (17). The rates of superoxide anion production were derived from the slopes of the absorbance data using the computer program divided by SLM Instruments (Fig. 1). In order to prevent high doses of ISO from interfering with the oxidant assay (3) we used 10^{-6} M as the optimal inhibitory concentration of superoxide anion production.

Blocking of β-receptors with BIM. β-Adrenergic receptors were blocked with BIM by incubating cells (2×10^7/ml) for 2 hr. The buffer contained 147 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.3 mM $MgSO_4$, 0.2 mM KH_2PO_4, 1.4 mM Na_2HPO_4, 1 g/liter glucose, 2 mg/ml bovine serum albumin (pH 7.4), and increasing concentrations of BIM. The incubation was stopped on ice and cells were washed three times at 4° in the same buffer system without bovine serum albumin. Subsequently cells were either used for ^{125}I-pindolol binding studies or incubated for an additional hour at 37°. This further incubation was to maintain conditions equivalent to those used for the determination of free β-receptors (see below). Superoxide anion production in response to formyl peptide stimulation, in the presence and absence of 10^{-6} M ISO, was then monitored.

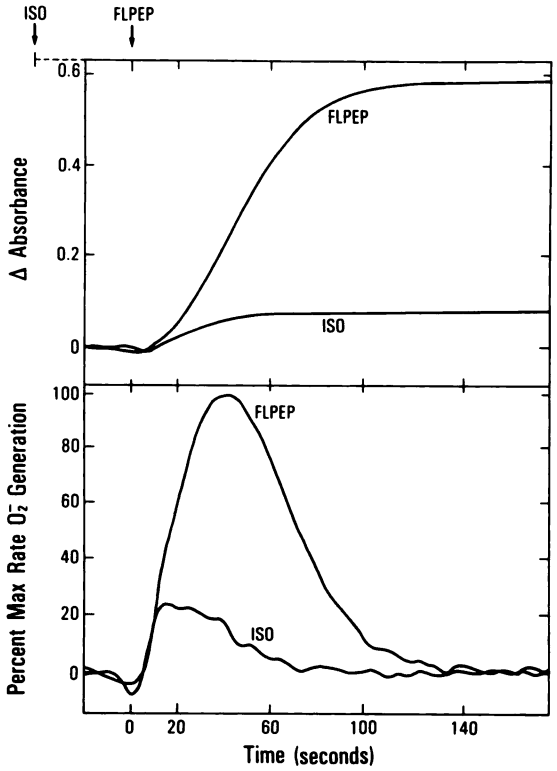

Fig. 1. Rate of superoxide production from absorbance data. Cells (4×10^6/1.5 ml) were stimulated with 1 nM FLPEP in the presence or absence of 10^{-6} M ISO, which was added 40 sec before FLPEP. The superoxide dismutase-inhibitable reduction of cytochrome *c* was monitored by measuring A_{550}. An increase in absorbance of 0.6 (*upper panel*) corresponds to 25.4 μM reduced cytochrome *c*. The *lower panel* shows the same data but plots the rate of change of absorbance over time. The rate is plotted as percentage of maximal superoxide production in the absence of ISO. This is the form in which we display superoxide production in the rest of the figures, with the exception of Fig. 5A.

Radioligand binding to β-adrenergic receptors. Binding studies were conducted either with intact cells or with washed membranes. Intact neutrophils (10^6) were incubated with 200 pM of ^{125}I-pindolol (New England Nuclear, Boston, MA) in a total volume of 0.5 ml of the above-described buffer containing 2 mg/ml bovine serum albumin. The incubation was carried out at 37° for 60 min and stopped by adding 10 ml of ice-cold phosphate-buffered saline diluted 1:10 with distilled water. Samples were incubated 10 min on ice to reduce nonspecific binding. Samples were then filtered over Whatman GF/C fiberglass filters and the filters were washed with 20 ml of cold phosphate-buffered saline. Radioactivity retained on the filters was determined in a γ-counter. Nonspecific binding was determined by binding of ^{125}I-pindolol in the presence of 10^{-6} M PROP. In some experiments we also measured ^{125}I-pindolol binding that could be blocked by unlabeled CGP-12177, a β-adrenergic antagonist that binds to surface, but not sequestered, receptors (18). These experiments demonstrated that virtually all the receptors were on the cell surface.

Membranes were prepared by nitrogen cavitation as previously described (19). Binding to about 50 μg of membranes was conducted with 6 concentrations of ^{125}I-pindolol (10–400 pM) in a buffer containing 50 mM Tris-HCl, 8 mM $MgCl_2$, 0.5 mM EGTA at pH 7.5 Incubations were for 90 min at 25° and were terminated by filtering the membranes over Whatman GF/C filters that were washed with 20 ml of buffer. Nonspecific binding, defined as binding in the presence of 10^{-6} M PROP, was subtracted from the total binding. The number of available receptors was determined by nonlinear regression analysis of specific binding data to an equation describing binding to a single class of sites.

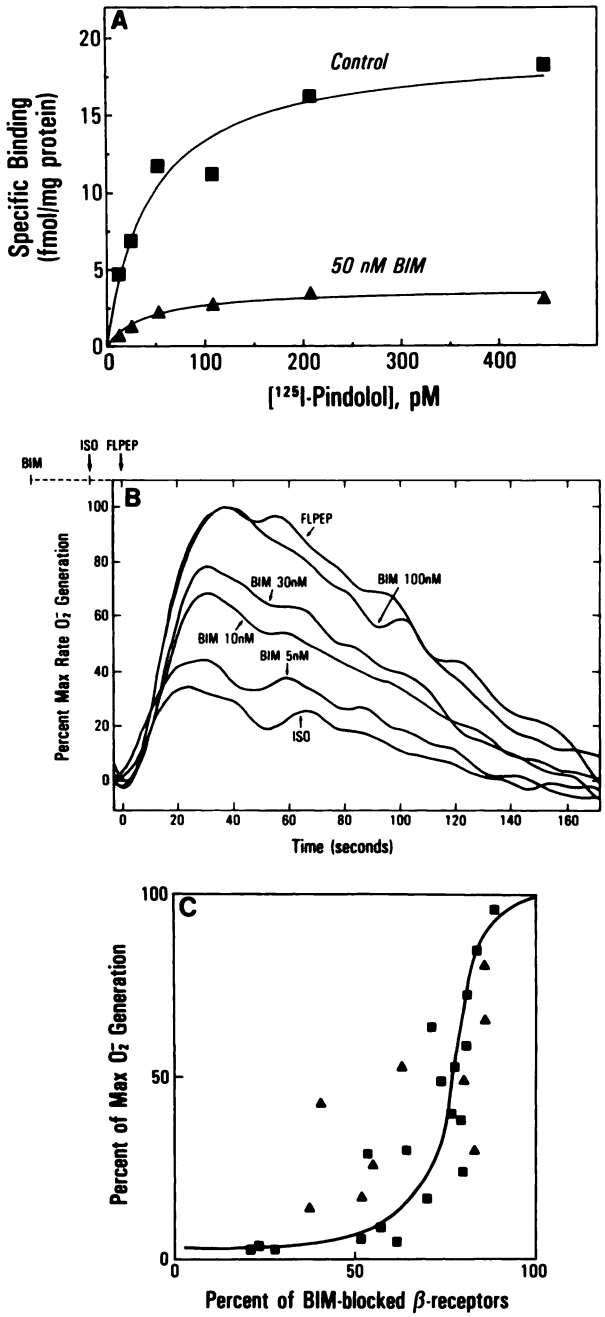

Fig. 2. Relationship of β-receptor occupancy to cell function. A, Specific binding of ^{125}I-pindolol to membranes prepared from untreated cells (■) and cells pretreated with 50 μM BIM (▲). Specific binding was calculated by subtracting binding of ^{125}I-pindolol to membranes in the presence of 1 μM PROP (nonspecific binding) from the total binding. Nonspecific binding relative to total binding was typically about 10% at low concentrations of ^{125}I-pindolol and did not exceed 25% at the highest concentration of radiolabeled antagonist used. B, Modulation of the rate of superoxide anion production by 10^{-6} M ISO as a function of preincubation of cells with BIM. Cells were preincubated with various concentrations of BIM and were washed. They were then stimulated at time 0 by addition of 1 nM FLPEP. 10^{-6} M ISO was added 40 sec before stimulation. The rate of change of oxidant production is plotted as in the *lower panel* of Fig. 1. The data are normalized so that the rate of oxidant production in the absence of ISO is defined to be 100%. Data are representative of observations in more than 10 separate experiments. C, Oxidant production as a function of receptor availability. Cells were preincubated with BIM and washed. Inhibition of superoxide production was quantitated as the percentage of maximal inhibition observed in cells not preincubated with BIM. Superoxide anion production was complete after 200 sec.

Pretreatment of cells with BIM changed the K_d of ^{125}I-pindolol binding less than 2-fold. In control membranes the number of receptors ranged from 14 to 41 fmol/mg of protein, and K_d ranged from 16 to 48 pM.

Modeling. Predictions of binding of the ligand (L) to the receptor (R) were calculated by numerical integration using the computer program PROTEUS (to be discussed elsewhere) with the equations describing reversible one-step binding,

$$L + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} L{\cdot}R$$

with the rate constants for association, k_{on}, and dissociation, k_{off}. The rate of formation of the ligand-receptor complex ($L{\cdot}R$) is described by the following equation:

$$d(LR)/dt = (k_{on})\ (R_f)(L_f) - (k_{off})\ (LR)$$

In this equation L_f and R_f are the free concentrations of ligand and receptor, and LR is the concentration of ligand-receptor complex.

Results

β-Receptor occupancy requirement for inhibition of superoxide production. The fractional occupancy of β-receptors necessary to turn off stimulated cells was determined by partially blocking β-receptors with the irreversible binding antagonist BIM (20). Cells were pretreated with increasing concentrations of BIM for 2 hr at 37°. Subsequently, the cells were washed and the relative number of free β-receptors was determined by measuring the binding of a radioactive antagonist, ^{125}I-pindolol. Alternatively, membranes were prepared from BIM-treated cells and free β-receptors on membranes were determined by ^{125}I-pindolol binding. In saturation binding isotherms to these membranes, the K_d of ^{125}I-pindolol binding was relatively unaffected (less than 2-fold change) when membranes were prepared from cells preincubated with BIM (Fig. 2A). The number of binding sites, however, was reduced. In a parallel experiment, the ability of ISO to inhibit oxidant production of intact cells preincubated with BIM was determined (Fig. 2B). An ISO concentration of 10^{-6} M saturated β-receptors not blocked with BIM and maximally inhibited oxidant production (3). The effect of ISO on oxidant production was blocked 50% by preincubation with 10 nM BIM and was completely blocked by preincubation with 100 nM BIM. Fig. 2C compares blocking of β-receptor binding with blocking of β-receptor function. ISO retained maximal activity and inhibited oxidant production even when half the β-receptors were blocked by BIM. Blockade of more receptors resulted in decreased β-adrenergic receptor function. Thus, maximal inhibition of oxidant production could occur even when some receptors were inactivated. However, full inhibition could only occur when a substantial fraction of the β-receptors (40–50%) were available.

The effect of ISO on superoxide production was blocked when the β-adrenergic antagonist PROP was added to the cells before stimulation (Fig. 3). Half-maximal blocking of the turn-off rate induced by 10^{-6} M ISO was observed with 30–50 nM PROP.

Kinetics of the inhibitory pathway. The IC_{50} for the superoxide production was estimated from the kinetic dose-response curves in Fig. 4. Cells were preincubated with ISO for

Available receptor number was determined by the binding of 200 pM ^{125}I-pindolol to intact cells (■) or from full binding isotherms (using six concentrations of ^{125}I-pindolol) to membranes (▲). The receptor number was normalized to the number of receptors on cells incubated without BIM.

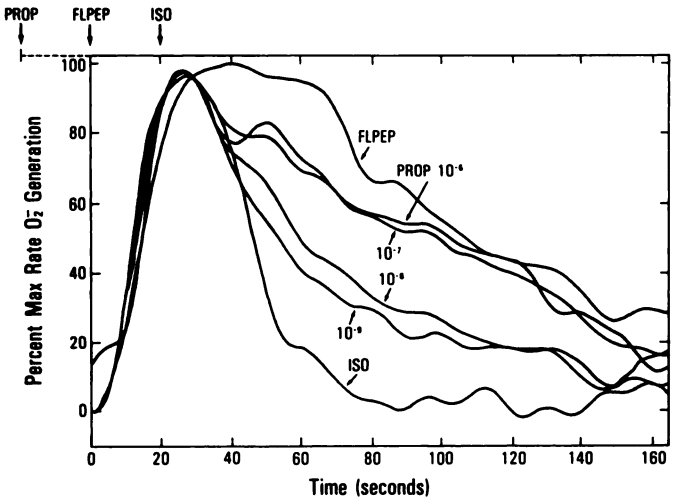

Fig. 3. Dose-response curve for PROP blocking the effects of ISO on oxidant production. The rate of oxidant production is plotted as a function of time after stimulation of cells. PROP at the concentrations indicated was added 30 sec before stimulation of the cells with 1 nM FLPEP at time 0. ISO (10^{-6} M) was added at time 20 sec. One of three experiments is shown.

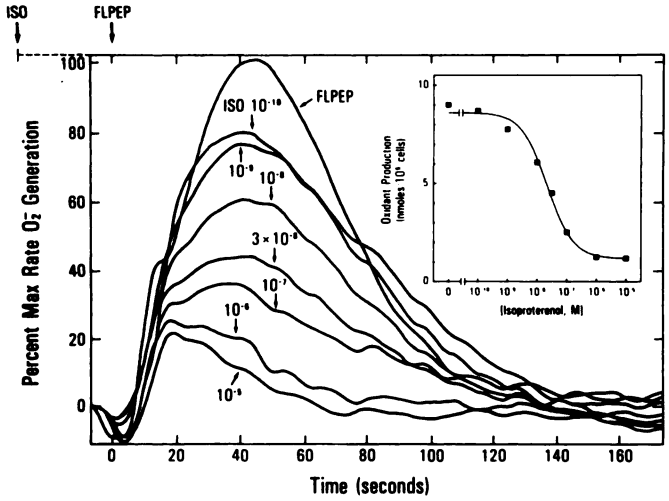

Fig. 4. Dose-response curve for ISO inhibition of oxidant production. Cells were equilibrated with the ISO concentrations indicated for 40 sec before stimulation with 1 nM FLPEP at time 0. The rate of oxidant production is compared with FLPEP-stimulated control cells. *Inset*, Total superoxide anion production from the same experiment is plotted as a function of ISO concentration on a logarithmic scale. The data are fitted by computer to a sigmoid curve with a Hill slope of −1 ($r^2 = 0.992$). The data are representative of four independent experiments.

40 sec and then were stimulated with *N*-formyl chemoattractant peptide. Desensitization of β-adrenergic receptors is not significant during this short preincubation period with ISO (3). Half-maximal inhibition of the rate of oxidant production was observed at 3×10^{-8} M ISO (Fig. 4). ISO decreased the amount of superoxide production without significantly altering the duration of superoxide production. Furthermore, the dose-response curve of total superoxide anion production measured over a time period of 180 sec displays the typical sigmoid characteristics of a reversible bimolecular reaction for the binding of ISO to the β-adrenergic receptors (Fig. 4, *inset*). The measurement of total oxidant production as a function of ISO concentration also demonstrates that maximal inhibition of cell response was achieved with 10^{-6} M ISO. Higher ISO concentrations (10^{-5} M) did not significantly increase the inhibitory effect on total oxidant production.

We estimated the dissociation rate constant for ISO binding to the β-receptor by measuring the rate at which ISO inhibition of FLPEP-stimulated oxidant production could be reversed by the β-adrenergic antagonist PROP (Fig. 5). The cells were preincubated with ISO for 40 sec before stimulation with FLPEP. At various times before stimulation PROP was added to block the effects of ISO (Fig. 5A). Oxidant production of cells was recorded as a measure of cell function. When PROP was added together with peptide (time 0) the inhibition by ISO was substantial and lasted at least 1 min. When PROP was added 30 sec before the peptide, ISO had no effect and superoxide generation was complete. Thus, virtually all the ISO must have dissociated from the β-receptors and been replaced by PROP during the 30-sec incubation. This suggests that ISO

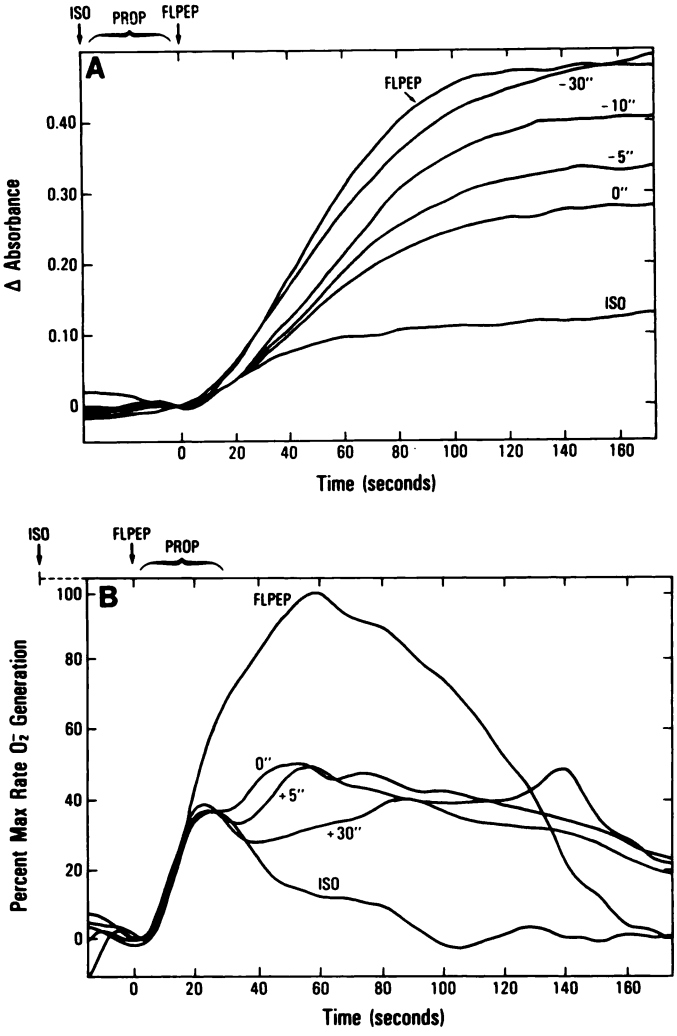

Fig. 5. Dependence of superoxide anion generation on the displacement of ISO by PROP. A, Cells were incubated with 10^{-6} M ISO 40 sec before stimulation of cells by 1 nM FLPEP at time 0. Stimulated and ISO-inhibited cells not preincubated with PROP served as controls. Data are representative of observations from five independent experiments with cells from different donors. The superoxide dismutase-inhibitable change in absorbance is plotted as a function of time. PROP was added 30, 10, 5, or 0 sec before the peptide. B, The rate of superoxide anion generation is plotted as a function of time after stimulation of cells with 1 nM FLPEP. PROP was added 0, 5, or 30 sec after stimulation of cells.

dissociated from its receptors very rapidly. When PROP was added 10 sec before the peptide, reversion of ISO inhibition was about 75%, or about half of the reversion that occurs when PROP and FLPEP were added simultaneously. This suggests that the $t_{1/2}$ for the dissociation of ISO from β-receptors is less than 10 sec. Adding the β-blocker after the peptide resulted in a delay of partial recovery of cell response (Fig. 5B). The delay was measured to be about 15–20 sec, as was best demonstrated when PROP and peptide were added simultaneously at time 0.

Fig. 6 shows the dose-response curves for turning off the oxidant production when ISO is added after cells had been maximally stimulated by peptide. The kinetics of the decay of cell responses after ISO addition should be consistent with the ISO association rate. The rate of oxidant production is plotted as a function of time. As a control, an antagonist of the chemotactic peptide receptor (*t*-boc, 10^{-5} M) was used to displace the peptide from the stimulatory receptors. This antagonist saturates available stimulatory receptors within 1 sec and permits the decay of the production of superoxide anions (14). ISO at 3×10^{-7} M rapidly inhibited cell responses with initial rates indistinguishable from those of *t*-boc. As a rough estimate it may therefore be concluded that adequate receptor occupancy to inhibit cell responses is achieved within a few seconds. Computer simulation of ISO binding shows calculated receptor occupancy for ISO as a function of concentration (Fig. 7). The rate constants used in the calculation are $k_{off} = 4\ min^{-1}$ and $k_{on} = 10^8\ M^{-1}min^{-1}$ (see Discussion). Equilibration of ISO with more than 50% of the receptors occurs within 10 sec for concentrations above 10^{-7} M. This is consistent with the rapid turn-off of stimulated cells by high ISO concentrations shown in Fig. 6.

Discussion

The number of receptors required to saturate the inhibitory pathway. Neutrophils exhibit a total of ~10^6 receptors for stimulatory ligands (C5a, immune complexes, leukotriene B_4, platelet activating factor, formyl peptide, etc.) and perhaps 2×10^4 receptors for inhibitory ligands, (prostaglandin E_1, adenosine, histamine, and β-agonists) (21). It is remarkable that the output of no more than 10^3 β-adrenergic receptors is able to blunt the output of at least 5×10^4–10^5 formyl peptide receptors. In order to further quantitate this relationship, we investigated the output of the inhibitory pathway as a function of the availability of its receptors. We used BIM, an irreversible antagonist, to partially block the β-receptors and determined that only about 50% of the residual receptors were required to evoke the maximal effect of this pathway (Fig. 2). These excess receptors are not "spare" in the sense that they are uncoupled from cell response. Rather, it appears that only 50% of the β-adrenergic receptors are required to saturate the inhibitory pathway when a large concentration of ISO is used. Proof of this hypothesis will require knowledge of the pathway (presumably involving G_i, cAMP, and protein kinase A) and a demonstration that the biochemical events are saturated by less than all of the receptors. Evidence for the latter arises from the observation that preincubation of cells with up to 5 nM BIM did not alter the kinetics of the turn-off of cell response by 10^{-6} M ISO added during maximal stimulation of cells (not shown). Because 5 nM BIM blocks 50–60% of the β-adrenergic receptors, the rapid turn-off was achieved with only 50% of the β-adrenergic receptors.

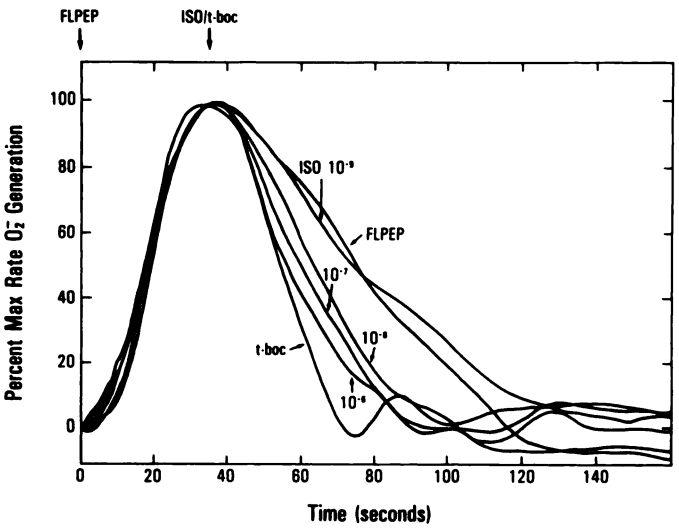

Fig. 6. Kinetics of turn-off of oxidant production by ISO. Cells (4×10^6/1.5 ml) were stimulated by 1 nM FLPEP at time 0. ISO at the concentrations indicated or *t*-boc (10^{-5} M) was added at time 35 sec. The rate of superoxide production is plotted as percentage of control as a function of time. The results shown are typical for ISO effects of superoxide production and were repeated three times with cells from three different donors.

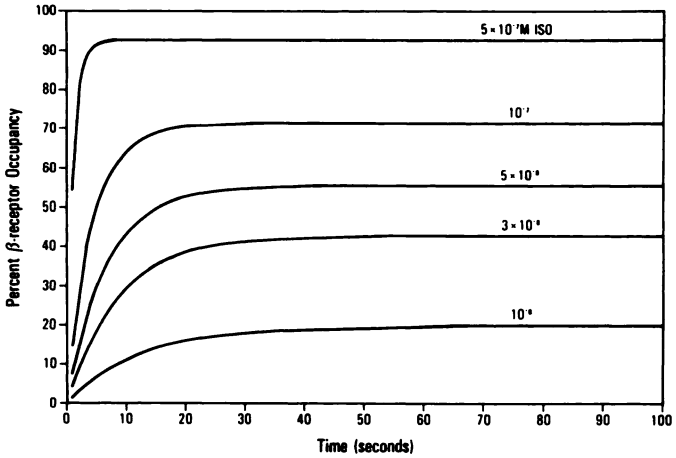

Fig. 7. Computer simulation of ISO binding to β-adrenergic receptors. The percentage of β-adrenergic receptors occupied by ISO is plotted as a function of time. The curves were calculated assuming reversible one-step binding and using the rate constants derived from experimental data ($k_{on} = 10^8\ M^{-1}min^{-1}$, $k_{off} = 4\ min^{-1}$).

The kinetics of the inhibitory pathway. The neutrophil provides an unique opportunity to probe the kinetics of the inhibitory pathway. This may prove useful in understanding the G_i pathway in a system that does not depend on cAMP measurements. We have taken advantage of real-time spectroscopic analysis of neutrophil responses and their modulation by the inhibitory pathway.

The ID_{50} for the inhibition of superoxide production was found to be ~3×10^{-8} M ISO (Fig. 4), a value that was found earlier in steady state measurements to be independent of the stimulus (formyl peptide) concentration. We estimate that 25% of the receptors are required to inhibit 50% of the response (Fig. 2). At 6×10^{-8} M ISO, approximately 50% of the β-receptors are predicted to be occupied, and this is our estimate of the K_d.

A dissociation rate constant for ISO was estimated from Fig.

5. When PROP was added 30 sec before the formyl peptide, the impact of ISO was completely abrogated. Only 5–10 sec of PROP were required to restore 50% of the maximal rate of production. Because the restoration of cell response involves both the dissociation of ISO and the decay of inhibitory pathway associated with ISO binding, we view 10 sec as an upper limit to the half-time of ISO dissociation (k_{off} = 0.69/10 sec = 4 min^{-1}).

The association rate constant calculated from the dissociation rate constant (4 min^{-1}) and the apparent K_d (6×10^{-8} M) is ~10^8 $M^{-1}min^{-1}$. The kinetics of decay of cell responses following ISO is consistent with such a value (Figs. 6 and 7). We observed that 3×10^{-7} M ISO acted as fast as the formyl peptide receptor antagonist.

For the purpose of these calculations, we have made the simplifying assumption that ISO initially binds reversibly to the β-adrenergic receptors with an affinity equal to its EC_{50} in inhibiting cell function. In fact, agonist interaction with receptors is much more complicated than this. In membranes, ISO interacts heterogeneously with the β-receptors. This is usually modeled as two affinity states, a high affinity state corresponding to receptors coupled to G_s and a low affinity state corresponding to uncoupled receptors. It is noteworthy that the EC_{50} value we obtain in functional experiments (60 nM) is quite similar to the K_d of the high affinity site in neutrophil membranes (9–73 nM; Refs. 22 and 23). The K_d for the low affinity sites is reported to be about 300 nM. High concentrations of ISO (10^{-5} M) were not more potent in inhibiting oxidant production when receptors were depleted than was 10^{-6} M ISO in Fig. 2C (not shown). Neither did 10^{-5} M ISO increase the rate of decay of the cell response as in Fig. 6 (not shown). Our data suggest that only the high affinity state of the β-receptor is functionally important. In fact, the observed binding in intact cells is more complicated, with receptor affinity changing over time.

PROP added to the cells before stimulation blocked the effect of ISO in a dose-dependent fashion (Fig. 3). Because the K_d for PROP is only 10 times lower than for ISO (not shown) a small residual inhibition by ISO was observed even in the presence of high concentrations of PROP.

Two additional features of the inhibitory kinetics are noteworthy. In Fig. 4 we observe that the oxidant production stimulated by FLPEP starts at similar initial rates in the first 20 sec regardless of the ISO concentration. However, although PROP can completely abrogate the ISO effect if added before FLPEP, it cannot restore oxidant production to its uninhibited rate if added after FLPEP (Fig. 5). Perhaps ISO can only elicit inhibitory biochemistry when the peptide receptors are occupied. It has been shown previously that short periods of exposure to ISO do not affect FLPEP binding (3).

Rate constants for the binding of β-agonists. A direct analysis of the kinetics of binding of β-adrenergic agonists is complicated by several factors. First, the available radiolabeled agonists are difficult to work with (24–26). This has meant that most intact cell measurements have required competitive approaches. Second, the affinity of agonists for the β-receptors is time dependent, perhaps reflecting sequestration of the receptors (14, 27, 28). Third, agonist binding to membranes in the absence of guanine nucleotide is heterogeneous, with receptors divided between low and high affinity forms (22, 23). However, in the presence of guanine nucleotide, a situation more analogous to intact cells, the binding is entirely low affinity.

The most successful resolution to date of these limitations has been to use competitive binding approaches in membranes. Here the rate of binding of a labeled antagonist is altered by the presence of an unlabeled agonist (27). Contreras *et al.* (29) found this method to be applicable to β-agonists and -antagonists, as long as all steps had a half-time of >1 min. Therefore, they conducted experiments at 10°. They concluded that the association rate constants for agonists ($3–18 \times 10^6$ $M^{-1}min^{-1}$) are 2 orders of magnitude slower than the association rate constants of antagonists. The dissociation rate constants, in contrast, were similar for agonists and antagonists. They proposed that the slow association rate constants reflect a two-step process in which high affinity binding of agonist can only occur if the receptor has undergone a conformational change. These conclusions are quite intriguing, as they demonstrate a fundamental difference in the manner in which agonists and antagonists bind. It remains to be determined, however, whether the data they obtained at 10° also apply at physiological temperatures.

In a previous study, a functional approach to examine rate constants for ligand binding at the neutrophil formyl peptide receptor was used (14). In neutrophils, the production of oxidants is proportional to the number of formyl peptide receptors occupied (15), and the K_d values obtained from equilibrium binding are comparable to the ED_{50} values of superoxide production. Cell responses decay when antagonists are added to cell suspensions during the course of stimulatory ligand binding. From analysis of this decay, estimates for the rate constants of antagonist interactions with receptors were made (14). The rate constants in this case were consistent with values obtained from competitive binding studies.

Using an analogous approach that, however, involves separate ligands for stimulatory and inhibitory pathways, we estimate an association rate constant for ISO at 37° of 10^8 $M^{-1}min^{-1}$. Although the binding of agonists to the β-receptors may occur faster than functional responses can be measured, the binding cannot occur more slowly. Thus, we view the functional rate constants determined here to be a lower limit for the rate of agonist binding to β-receptors on human neutrophils. The factor that most likely explains the differences between our conclusions and those of Contreras *et al.* (29) is temperature. Our experiments were performed at 37°, theirs at 10°. Temperature may alter both the association rate constant and the putative rate-limiting conformational change.

The results of our study illustrate three aspects regarding the mechanism of β-adrenergic receptors on human neutrophils. First, the rate constants for binding of ISO as parameters for activation of β-adrenergic receptors were estimated from functional data of cell response. These data were obtained at 37° and are likely to represent limits for the kinetics under physiological conditions. The second aspect shows the rapid reversibility of the β-adrenergic receptor activation (Fig. 5). β-Antagonists such as PROP replace ISO at the receptors with a half-time of less than 10 sec and the cell response recovers. This suggests that the β-adrenergic receptors must be occupied by ISO during the course of cell response in order to obtain maximal inhibition. The activation of β-adrenergic receptors is therefore a reversible process and dependent on the continuous occupany by agonists. The third insight into the mechanism

comes from the observation that 500 β-adrenergic receptors are able to inhibit activation by 5×10^4 to 10^5 formyl peptide receptors. Thus, massive amplification of the inhibitory signal is needed, which might be achieved by activation of the adenylate cyclase. Further studies investigating the targets of a cAMP-dependent protein kinase in the stimulatory signaling pathway may further clarify the mechanism of the β-adrenergic receptor system.

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