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Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production 133–140

Coupling of Antagonistic Signalling Pathways in Modulation of Neutrophil Function

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Modulation of neutrophil activation by catecholamines reflects a fine-tuning by coupling inhibitory and stimulatory receptor pathways. The catecholamine isoproterenol (ISO) binds to beta-adrenergic cell surface receptors and thereby inhibits cell responses such as O_2^- production stimulated by formyl peptides. However, ISO did not inhibit O_2^- generation activated by 1 μM ionophore A23187, the protein kinase C activators phorbol ester (PMA, 100 ng/ml) and oleoylacetylglycerol (OAG, 50 μM), and the G-protein activator NaF (40 mM). Furthermore, the overall kinetics of oxidant production in the presence of ISO were unchanged when cells were stimulated with PMA, OAG, A23187, and NaF. These results would imply that neither intracellular calcium, the activation of protein kinase C, nor the activation of G-protein are the primary target of the inhibitory pathway. Accordingly, pertussis toxin did not block PMA or NaF-stimulated superoxide generation. In contrast, formyl peptide-dependent GTPase activity is inhibited by ISO in sonicated cell preparations. Since ISO increases the cAMP concentration in the cell, the possibility is raised that a cAMP-dependent kinase inhibits signal transduction in part by blocking the interaction of this receptor with its G-protein.

Key words: superoxide, neutrophils, cell response modulation

Local recruitment of neutrophils, the most numerous white blood cells in circulation, to the sites of inflammation, is the first line of defense by the host against invading microorganisms. Migration of neutrophils into the tissue depends on activation of a number of cell responses, including release of granule enzymes and superoxide anions [1–3]. Host tissue damage by these highly toxic substances may be limited by a delicate balance between stimulatory and inhibitory events in the activated neutrophil.

Abbreviations used: BSA, bovine serum albumin; cAMP, adenosine 3':5'-cyclic monophosphate; DTT, dithiotreithol; FLPEP, N-formyl-norleu-leu-phe-norleu-tyr-lys-fluorescein; ISO, L-(−)-isoproterenol; PMA, phorbol 12-myristate 13-acetate; OAG, oleoylacetylglycerol; W-7, (N-6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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Stimuli such as bacteria-derived formyl peptides induce inositol phospholipid breakdown and activate cellular functions. On the other hand, stimuli that elevate intracellular cAMP levels antagonize such activation and inhibit a number of cell responses. Beta-adrenergic agonists, prostaglandins of the E series, histamine, and adenosine bind to inhibitory receptors which are coupled to G_s, the guanine nucleotide binding protein stimulatory for adenylate cyclase [4–9].

In a previous study we showed that among the adrenergic agonists, ISO is the most effective and can be inhibited by the beta-blocker propranolol [10]. The rank order of potency (isoproterenol > epinephrine > norepinephrine) is consistent with $beta_2$-adrenergic receptor mediated inhibition. The speed and potency of, for example, catecholamine-mediated inhibition is remarkable. Five hundred beta-receptors are able to inhibit stimulation of superoxide generation by up to 10^5 formyl peptide receptors [11]. Thus massive amplification of the inhibitory signal is needed. However, little information is available as to the mechanism by which catecholamines exert their inhibitory action. Inhibition of intracellular signalling processes like Ca^{2+}-elevation at low peptide concentrations and phosphoinositide metabolism were reported [10]. On the other hand, we demonstrated that ISO does not compete for the formyl peptide receptor nor does it alter the receptor number on the cell surface or change the receptor-ligand interaction [10]. Despite the massive inhibition of superoxide generation, ISO has no or little effect on cell functions involving the cytoskeleton. Inhibition of phagocytosis and chemotaxis was minimal. This indicates that the modulation of cell activation through beta-receptors may play an important physiological role. The inhibitory pathway may be important in limiting the extent or location of tissue injury caused by neutrophil activation without interfering with the chemotactic properties of a migrating cell.

In the present study, we provide new information regarding the intracellular interaction between stimulatory and inhibitory receptor pathways. We have examined the effect of ISO on the stimulation of superoxide anion generation by various intracellular activators. Our observations raise the possibility that a cAMP-dependent kinase is involved in the inhibition of the signal transduction at the stimulatory receptor or in blocking the interaction of this receptor with its G-proteins.

MATERIALS AND METHODS

Reagents

The fluoresceinated hexapeptide N-formyl-norleu-leu-phe-norleu-tyr-lys (FLPEP) was prepared and stored as previously described [12]. Reagents obtained commercially were L(−)ISO, PMA, OAG, ionophore A23187, ferricytochrome C, superoxide dismutase, nonhydrolyzable ATP, GTP, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (Sigma Chemical Co., St. Louis, MO), and GTP-gamma^{32}P (New England Nuclear Corp., Boston, MA).

Neutrophils

Neutrophils were isolated by the elutriation method from fresh human blood supplemented with 1/7 volume acid citrate dextrose [13]. Cells were then resuspended in ice-cold buffer containing 5 mM KCl, 147 mM NaCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.5 mM glucose, 0.3 mM $MgSO_4$, 1 mM $MgCl_2$, and 10 mM Hepes adjusted to pH 7.4 with 10 N NaOH. Cells were kept on ice at 10^7/ml and warmed up

at 37°C for 5–10 min before the assays. Membranes were prepared by nitrogen cavitation as previously described [14].

Determination of Superoxide Anion Production

The superoxide dismutase inhibitable reduction of cytochrome C was measured by monitoring the change in transmittance of a stimulated cell suspension at 550 nm [15]. Measurements were carried out in a SLM 8000 C spectrofluorometer (SLM Instruments, Inc., Urbana, IL). Cells were suspended in the above buffer containing 1.5 mM Ca^{2+}. Absorbance was calculated from the recorded transmittance by using the computer program provided by SLM. The number of superoxide anions released was calculated by using the mM extinction of cytochrome C of 21.1. The rate of superoxide production of cells measured in the system used was maximal after 60 s and release was largely completed within 3 min. The total number of superoxide anions generated by the cells was therefore measured after 180 s. FLPEP was used as a stimulus in these studies rather than nonfluorescent peptide in order to compare results in this report with earlier observations [16,17].

Determination of GTPase Activity

GTPase activity in sonicated cells and isolated membranes was determined using a modified method outlined elsewhere [18]. Briefly, cells or membranes were resuspended at 10^8/ml in the buffer used above and supplemented with 12.1% sucrose, 1 mM DTT. Cells were then treated for 5 min on ice with 1 mM DFP, sonicated and stored on ice. Twenty microliters of membranes or sonicates were added to 80 μl buffer containing 150 mM NaCl, 20 mM TrisHCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, 0.25 mM ouabain, 1 mM creatine phosphate, 5 U/ml creatine phosphokinase, 0.375 μM GTP, and 0.125 μM GTP [gamma-^{32}P] at a pH of 7.4. All reactants were kept on ice before initiating the assay. The reaction was quenched after 10 min at RT by adding 200 μl of a buffer containing 5% charcoal, 0.5% BSA, and 0.1% dextrose. This mixture was immediately centrifuged in a Beckman microfuge for 1 min. Twice one hundred microliters of the supernatant were removed and counted in a beta-counter by using Ecoscint liquid scintillation cocktail (National Diagnostics, Manville, NJ).

Pertussis Toxin Treatment

Pertussis toxin treatment of cells was carried out for 2 hours at 37°C in the presence of 10 μg/ml toxin in a buffer containing glucose and cytochrome C.

RESULTS

Effects of ISO on the Stimulatory Signalling Pathway

Stimulation of oxidant production with high doses of the phorbol ester PMA, the diacylglycerol analogue OAG, the calcium ionophore A23187, and the G-protein activator NaF was not affected by optimal ISO concentrations (Table I). Only the formyl peptide receptor-mediated activation of cell response was blocked by adding 10^{-6}M ISO 30 s before stimulation. In contrast, the calmodulin antagonist W-7 blocked all five stimulators tested. W-7 is reported specifically to antagonize calmodulin without affecting protein kinase C [19]. Furthermore, the overall kinetics of oxidant production in the presence of ISO were unchanged when cells were stimulated with PMA,

TABLE I. Effects of ISO on the Activation of Cells by Different Stimuli*

Additions	Superoxide anions produced (nmol $O_2^-/10^6$ cells)
FLPEP	13.4 ± 0.2
FLPEP + ISO	2.2 ± 0.9
PMA	21.0 ± 0.2
PMA + ISO	20.4 ± 1.0
OAG	12.7 ± 0.3
OAG + ISO	13.1 ± 0.4
A23187	5.2 ± 0.6
A23187 + ISO	5.4 ± 0.6
NaF	6.6 ± 1.3
NaF + ISO	6.1 ± 0.4
FLPEP + W-7	1.0 ± 0.3
PMA + W-7	2.6 ± 0.1
OAG + W-7	n.d.
A23187 + W-7	1.0 ± 0.2
NaF + W-7	0.9 ± 0.3

*Cells were stimulated with the reagents listed and superoxide anion generation was monitored over a time period of 8 min. ISO was added 30 s before the stimuli. Cells were preincubated with the calmodulin blocker W-7 5 min at 37°C. The concentrations of the reactants were FLPEP, 1 nM; PMA, 100 ng/ml; OAG, 50 μM; A23187, 50 μM; NaF, 40 mM; ISO, 1 μM; W-7 100 μM. Values are the mean ± S.E. of three determinations of a representative experiment. n.d.: not determined.

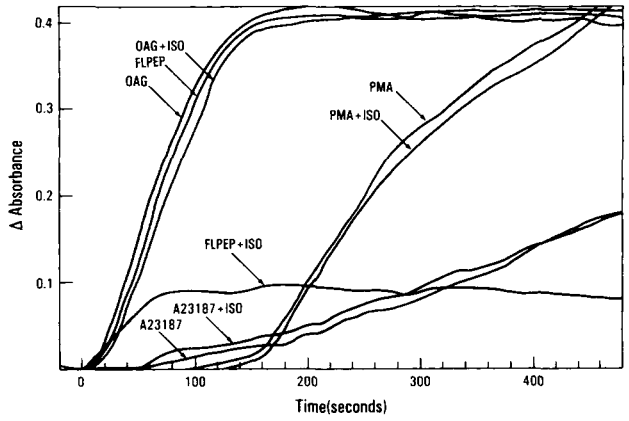

Fig. 1. Time course of superoxide generation upon stimulation of cells at time 0 by FLPEP (1 nM), PMA (100 ng/ml), OAG (50 μM), and A23187 (50 μM) in the presence of 1.5 mM Ca^{2+}. ISO (10^{-6}M) was added to the cells 30 s before stimulation. Data are representative of three independent experiments.

OAG, and A23187 (Fig. 1). Similar observations were made in the presence of 40 mM NaF (Fig. 2). Neither the maximal rate nor the magnitude of oxidant production was changed in the presence of ISO.

When NaF was added to ISO-inhibited cells 75 s after stimulation with FLPEP, cells began to produce O_2^- once again. In the paradigm in which NaF directly activates G protein, activation would appear to bypass the ISO blockade. Furthermore,

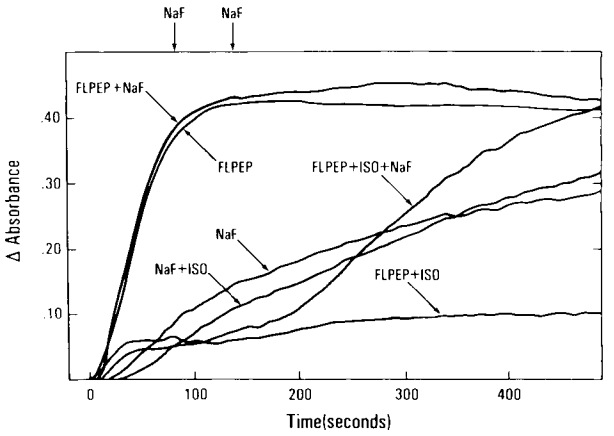

Fig. 2. Time course of superoxide generation measured as change in absorbance of cytochrome C. NaF (40 mM) or FLPEP (1 nM) were added at time 0, and ISO (10^{-6}M) was added to the cells 30 s before stimulation. NaF was added after 75 s to peptide-stimulated cells and after 140 s to peptide-stimulated and ISO-inhibited cells. Similar results were obtained in five independent experiments.

NaF did not activate oxidant production in cells which had stopped making O_2^- in response to FLPEP alone (140 s). This result implies that G proteins had been activated by FLPEP and cannot be further stimulated by NaF (Fig. 2). In analogous experiments, NaF-stimulated cells were not further stimulated by 1 nM FLPEP (not shown). This result would imply that activation of the G protein by NaF uncouples the receptor-mediated response.

Pertussis toxin pretreatment of cells completely blocked receptor-dependent cell response. However, cell response stimulated by PMA or NaF was not affected by pertussis toxin preincubation of cells (Fig. 3).

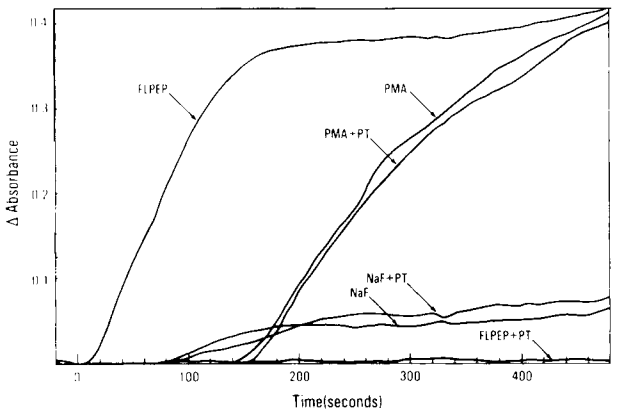

Fig. 3. Time course of superoxide generation of cells stimulated at time 0 with FLPEP, PMA, or NaF. The pertussis toxin treatment (PT) was carried out as outlined in Materials and Methods. The data are representative of three similar experiments.

Effects of ISO on the GTPase Activity

Activation of G-proteins can be measured as a stimulation of their GTPase activity. Figure 4 shows the measurement of GTPase activity in isolated membranes and sonicated cells. In membranes, both 1 nM FLPEP as well as 1 μM ISO stimulated GTPase activity significantly. The stimulation was additive when both ligands were present throughout the incubation time. However, in sonicated cells, the GTPase activity was dramatically inhibited when FLPEP and ISO were present simultaneously. Stimulation by FLPEP or ISO alone was similar to the activation observed in membranes. This line of evidence suggests that the inhibitory mechanism which is lost in membranes but retained in sonicates is not entirely part of the cell membrane.

DISCUSSION

In this report, activation of the stimulatory pathway on different levels of signal transduction provides information regarding the targets for the inhibitory protein kinase. We have demonstrated that stimulation of oxidant production by activation of protein-kinase C with PMA or OAG, and by increasing the intracellular Ca^{2+} concentration with the ionophore A23187 is not affected by ISO (Table I; Fig. 1). These findings suggest that the target of the inhibitory pathway is not the oxidase but must be found in the transduction sequence prior to activation of protein kinase C or the intracellular elevation of Ca^{2+} concentrations. An earlier report showed that the binding, dissociation, and internalization of the stimulatory ligand remains unaffected by ISO [10]. This indicates that ISO does neither directly compete for the FLPEP receptor nor change the processing of the FLPEP-receptor complexes. We have as yet been unable to test whether the interaction of the formyl peptide receptor and G proteins is altered in the presence of ISO.

Although formyl peptide stimulation of neutrophils has been shown to cause a transient 2–3-fold increase in cAMP levels, pretreatment of cells with 10^{-6} M ISO

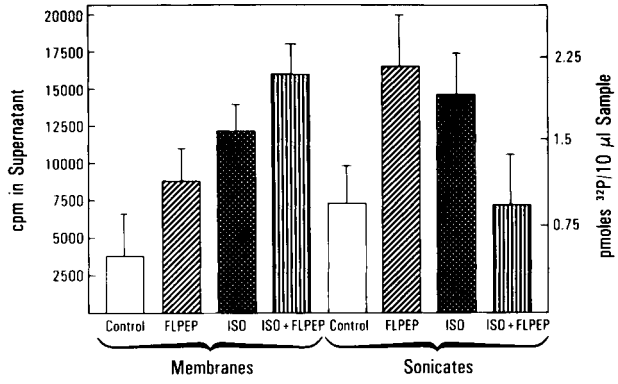

Fig. 4. GTPase activity of membranes or sonicated cells was determined as outlined under Materials and Methods. Picomoles ^{32}P released during a 10-min incubation period at room temperature were calculated from the specific activity of GTP-(gamma-^{32}P) used in the assay. The protein concentration in the samples was typically 1–2 mg/ml.

synergistically increased cAMP levels [10]. Thus the primary regulatory pathways in the neutrophil appear to be dependent upon activation of the adenylate cyclase. This notion was strengthened when similar inhibitory effects were obtained with cells pretreated either with forskolin, an adenylate cyclase agonist, or DBcAMP, a membrane permeable analogue of cAMP [5]. Elevated intracellular cAMP levels may be responsible for the activation of a cAMP-dependent kinase. This activation by anti-inflammatory mediators may lead to a phosphorylation of components of the stimulatory signalling pathway. These phosphorylations may be necessary for blocking the cell responses initiated by chemotactic factors. Phosphorylation of the cytoplasmic domain of receptors was demonstrated to be important in regulation of hormonal responsiveness and transmembrane signalling [20]. Thus uncoupling of the transduction sequence by blocking the interaction between receptor and G-protein would lead to a modulation of cell response. Evidence for this hypothesis came from the observation that ISO reduces the metabolism of phosphoinositides, affecting the stimulation of phosholipase C. Additionally, Figure 4 demonstrates that FLPEP-dependent GTPase activity is significantly inhibited in sonicated cells in the presence of ISO, suggesting that both stimulatory and inhibitory pathways are intact. In contrast, the GTPase activity stimulated by FLPEP and ISO are only additive in membranes, suggesting that the two pathways are no longer coupled, perhaps because soluble elements which interconnect them are lost in the preparation of membranes. However, direct evidence for a phosphorylation of the formyl peptide receptor as a signal terminating event has yet to be shown. Further experiments will have to define the targets of the inhibitory protein kinase activity and proof the involvement of the formyl peptide receptor.

ISO did not inhibit oxidant production activated by NaF (Fig. 2). Activation was also resistant to a pretreatment with pertussis toxin (Fig. 3) [21]. This suggests that ADP-ribosylation of G protein and ISO inhibition do not interfere with fluoride activation of the G proteins. Making the assumption that NaF activates oxidant production primarily by activating G protein [22], our experiments are consistent with the idea that stimulation is blocked in part because the ISO pathway interferes with the receptor-G protein interactions of the stimulatory pathway. However, interpretation of our data may be more complicated since NaF has other effects on cellular components including phosphatases [23,24] and ATPases [25].

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