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INHIBITION OF NEUTROPHIL FUNCTION BY ASPIRIN-LIKE DRUGS (NSAIDS): REQUIREMENT FOR ASSEMBLY OF HETEROTRIMERIC G PROTEINS IN BILAYER PHOSPHOLIPID

STEVEN B. ABRAMSON,* JOANNA LESZCZYNSKA-PIZIAK, ROBERT M. CLANCY, MARK PHILIPS and GERALD WEISSMANN

Department of Medicine, Division of Rheumatology, New York University Medical Center and the Hospital for Joint Diseases, New York, NY, U.S.A.

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Abstract—Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit neutrophil functions via mechanisms that are independent of their effects on prostaglandin biosynthesis. We examined the effects of sodium salicylate and piroxicam on GTP/GDP exchange by a regulatory G protein (Gαi). Plasma membrane and cytosol of human neutrophils were prepared by nitrogen cavitation and discontinuous sucrose density centrifugation. Salicylate (3 mM) and piroxicam (50 μM) reduced [^{35}S]GTPγS binding to purified plasma membranes [65 ± 3.7 and 75 ± 5.3% ($P < 0.003$) of control, respectively]. Membrane-associated G$\alpha/\beta\gamma$ was solubilized by treatment of plasma membranes with sodium cholate. NSAIDs did not inhibit binding of GTP to solubilized G$\alpha/\beta\gamma$ derived from detergent-treated plasma membranes. Lipid reconstitution was achieved by detergent dialysis followed by the addition of bilayer liposomes (phosphatidycholine). Salicylate and piroxicam inhibited GTPγS binding to G$\alpha/\beta\gamma$ derived from solubilized plasma membranes reconstituted in phosphatidylcholine vesicles (bilayer structures) but had no effect when phosphatidylethanolamine (hexagonal phase II structure) was used for reconstitution. Salicylate and piroxicam had no effect on GTP binding to cytosolic fractions in which soluble Gαi exists as a free subunit, suggesting that the effect required either assembly of Gαi/$\beta\gamma$ heterotrimer or the presence of a lipid bilayer. Although the addition of purified bovine $\beta\gamma$ subunits to dialyzed cytosol increased both the total GIP binding capacity and the pertussis toxin-dependent ADP-ribosylation of Gαi, consistent with assembly of a G protein heterotrimer, NSAIDs had no effect on GTP binding. In contrast, NSAIDs *inhibited* GTP binding to heterotrimeric $G\alpha_{cytosol}/\beta\gamma_{bovine}$ when the complex was inserted into bilayer liposomes. The data indicate that salicylate and piroxicam disrupt neutrophil function via their capacity to interfere with GTP/GDP exchange at an α subunit of a regulatory G protein, an effect which requires assembly of the active heterotrimer Gαi/$\beta\gamma$ in a phospholipid bilayer.

Key words: nonsteroidal antiinflammatory drugs, neutrophils, G proteins

Non-steroidal anti-inflammatory drugs (NSAIDs)† exert some of their anti-inflammatory effects by inhibiting the prostaglandin H (PGH) synthase of a variety of cell types [1]. However, NSAIDs also inhibit the activation of neutrophils by chemoattractants such as *N*-formyl-methione-leucine-phenylalanine (FMLP), leukotriene B_4 (LTB_4) and C5-derived peptides [2–4]. This pharmacological property of NSAIDs is not due to the inhibition of prostaglandin biosynthesis and is shared by sodium salicylate, an ineffective inhibitor of PGH synthase [2].

The intimate mechanism(s) by which NSAIDs inhibit neutrophil function remains unclear. However, there is evidence to suggest that these agents interfere with processes regulated by a membrane-associated, pertussis toxin-sensitive GTP binding protein (G protein) [5–7]. For example, NSAIDs inhibit the binding of the chemoattractant FMLP to the neutrophil plasma membrane, inhibit ligand-induced calcium movements and phospholipid remodeling, and block the pertussis toxin-dependent ADP-ribosylation of Gα [2, 5, 6, 8]. They have no effect on signal transduction after calcium ionophores or phorbol myristate acetate [6].

Recently, we reported that NSAIDs inhibit the binding of [^{35}S]GTPγS to a G protein within purified neutrophil membrane preparations [5]. Since activation of a G protein follows the displacement of GDP by GTP on the α subunit, interference by NSAIDs with guanine nucleotide exchange would be expected to disrupt post-receptor transduction events [5, 7, 9–11]. In the current studies we examined the lipid and subcellular site requirements for the NSAID effect on [^{35}S]GTPγS binding. The data indicate that the capacity of NSAIDs to inhibit GTP/GDP exchange requires the assembly of the heterotrimeric G$\alpha/\beta\gamma$ complex within the supporting architecture of a phospholipid bilayer. These observations may represent a common mechanism

* Corresponding author: Dr. Steven B. Abramson, Department of Rheumatology and Molecular Medicine, Hospital for Joint Diseases, 301 East 17th Street, New York, NY 10003. Tel. (212) 598-6119; FAX (212) 598-6582.

† Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; FMLP, *N*-formyl-methionine-leucine-phenylalanine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; and Hex_{II}, hexagonal II.

by which NSAIDs exert diverse effects on membrane-dependent processes in a variety of cell types.

MATERIALS AND METHODS

Subcellular fractionation

Neutrophils (PMN) were isolated from peripheral blood of normal volunteers as described [12]. Neutrophils were disrupted by N_2 cavitation (350 psi × 20 min at 4°) in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 10 mM HEPES, pH 7.3) plus protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, chymostatin and aprotinin). Post-nuclear supernatants were subjected to discontinuous (40, 50, and 60%; w/v) sucrose density centrifugation (160,000 *g* for 2 hr). The resulting subcellular fractions, cytosol (CS), plasma membrane (PM), specific granules (SG) and azurophilic granules (AG), were collected manually. Enzyme assays for lactate dehydrogenase, alkaline phosphatase, vitamin B_{12} binding and β-glucuronidase demonstrated that this method produces highly purified neutrophil fractions, as we have reported [13].

[^{35}S]GTPγS binding

Equilibrium [^{35}S]GTPγS binding to neutrophil subcellular fractions was quantitated by the method of Northup *et al.* [14]. Subcellular fractions (CS 10–30 μg, PM 1–5 μg, SG 1–5 μg) were preincubated with NSAIDs for 5 min at 30°, and then were incubated for 40 min at 30° in 40 μL of binding buffer [20 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM diothiothreitol, 100 mM NaCl, and 1 μM GTP (3000–5000 cpm/pmol), 50 mM Tris–HCl, pH 8.0]. Reactions were terminated by the addition of ice-cold buffer (5 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris, pH 8.0) followed immediately by rapid filtration. Nitrocellulose (NC) filters were used for cytosolic and solubilized fractions, and Whatman GF/F filters were used for intact plasma and specific granule membranes. Bound [^{35}S]GTPγS was quantitated by scintillography.

Characterization of liposomes

Phosphatidylcholine (PC) liposomes and non-bilayer hexagonal (II) (Hex_{II}) phase phosphatidylethanolamine (PE) were prepared as described [15–17]. PC and PE were greater than 98% pure as assessed by iodine visualization of the phospholipids after separation by two-dimensional thin-layer chromatography on silica gel G (250 mesh), using $CHCl_3$:CH_3OH:H_2O (65:25:5) as the solvent system for the first dimension and $CHCl_3$:CH_3COCH_3:CH_3OH:CH_3:CH_3COOH:H_2O (3:4:1:1:0.5) as the solvent system for the second dimension. A dry film of PC or PE was suspended by agitation on a Vortex mixer in 20 μM Fura-2, 0.145 M KCl, 0.5 mM EGTA, 25 mM Tris, pH 8.0. The resulting lipid preparations were refrigerated overnight, then passed, at room temperature, through a Sephadex G-50 column, and eluted with the buffer to remove unsequestered Fura-2. Fura-2 leakage from liposomes was monitored by measuring the excitation fluorescence intensity at 340 nm in the presence of a calcium-containing buffer with a Perkin–Elmer 650-10S fluorescence spectrophotometer. Liposome integrity was determined by measuring the Triton X-100 latency of Fura-2 fluorescence as described [17, 18].

Reconstitution of solubilized plasma membranes

Two hundred micrograms of purified plasma membranes in 1000 μL of 20 mM Tris (pH 7.4) 100 mM NaCl was solubilized by adding 10% sodium cholate solution up to a final concentration of 0.2%. This concentration of cholate in the above buffer was just below the critical micelle concentration [16, 19]. The sample was left on ice for 30 min. The solution was centrifuged at 100,000 *g* for 30 min. The supernatant was dialyzed using the PIERCE microdialyzer system 100. Solubilized plasma membranes, free of detergent, were reconstituted with 100 μM PC or PE vesicles prepared as described.

Reconstitution of cytosolic Gα with purified βγ subunits

Purified bovine brain βγ was supplied by Paul Sternweis (Vanderbilt University, Nashville, TN). Cytosol protein was diluted to 1 mg/mL with either 20 mM Tris (pH 8.0), 100 μM PC, 20 mM $MgCl_2$ or 20 mM Tris (pH 8.0), 0.2% sodium cholate, 20 mM $MgCl_2$. βγ Subunits were added to a final concentration of 4 μg/mL, and the samples were left on ice for 2 hr. Reconstituted cytosol was preincubated with NSAIDs for 5 min and then was incubated with [^{35}S]GTPγS binding buffer for 30 min at 30°. Reactions were terminated by diluting samples with ice-cold 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 100 mM NaCl followed by rapid filtration through nitrocellulose filters. Bound [^{35}S]GTPγS was quantitated by scintillography.

ADP-ribosylation

Twenty micrograms of cytosol in the presence or absence of purified βγ subunits (4 μg/mL) was incubated for 60 min at 30° in 40 μL of buffer containing activated pertussis toxin (5 μg/mL), 1 mM ATP, 10 mM thymidine, 5 μM [^{32}P]NAD (1 μCi/sample), 100 mM Tris (pH 8.0). Pertussis toxin was activated by a 10-min incubation at 30° with 30 mM dithiothreitol. The reaction was terminated by adding Laemmli buffer and heating samples at 100° for 5 min. Radiolabeled proteins were resolved by 10% SDS–PAGE and detected by autoradiography.

Immunoblot

Immunoblot analysis was performed according to the technique of Towbin *et al.* [20]. Subcellular fractions (5–10 × 10^7 PMN equivalents) were subjected to 10% SDS–PAGE and transferred to NC. Nitrocellulose filters were blocked with 5% non-fat dry milk (Carnation) in phosphate-buffered saline and probed with G protein subunit-specific rabbit polyclonal antisera 8730 (anti-Gα) or 5356 (anti-Gβ), provided by David Manning (University of Pennsylvania). Immunolocalized proteins were visualized by ^{125}I-protein A binding (3 × 10^5 cpm/mL) followed by autoradiography.

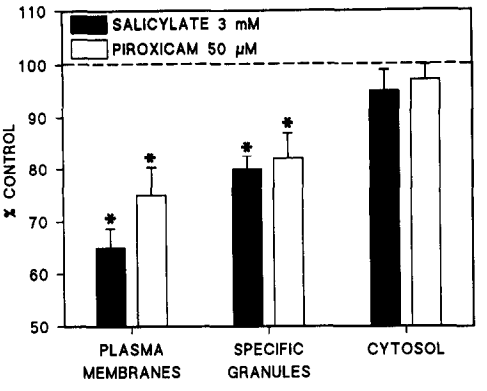

Fig. 1. Effect of NSAIDs on [^{35}S]GTPγS binding to neutrophil subcellular fractions. Plasma membranes (1–5 μg), specific granules (1–5 μg) and cytosol (10–30 μg) were preincubated with either sodium salicylate or piroxicam for 5 min before the addition of [^{35}S]GTPγS (see Materials and Methods). Reactions were terminated after 30 min. Results are the means ± SEM of four experiments. Each batch of subcellular fractions was prepared from neutrophils harvested from at least three donors. Key: (*)$P < 0.01$ versus buffer control. Total binding activity (pmol/10^8 PMN) in subcellular fractions was distributed as follows: plasma membrane, 119 ± 24; specific granule membrane, 11 ± 2; and cytosol, 114 ± 20.

RESULTS

Effects of NSAIDs on [^{35}S]GTPγS binding to purified plasma membranes

Neutrophil cavitates were separated into plasma membrane, specific granule membrane and cytosol fractions as we have described [13]. Figure 1 demonstrates the effects of sodium salicylate and piroxicam on the binding of the nonhydrolyzable analog of GTP, [^{35}S]GTPγS, to the purified plasma membrane fraction. Plasma membranes were incubated in the presence or absence of drug for 5 min before the addition of 1 μM [^{35}S]GTPγS. Total binding reached equilibrium at 30 min; nonspecific binding was less than 20% of the total. The nucleotide specificity of the GTP binding assay has been established by this and other laboratories [7, 13, 14]. Salicylate (1, 2 and 3 mM; 160–480 μg/mL) reduced the specific binding of [^{35}S]GTPγS to purified plasma membranes to 88 ± 3 ($P < 0.05$), 84 ± 4 ($P < 0.01$) and 65 ± 3.7% (< 0.0001) of control, respectively. Piroxicam (10 and 50 μM; 3–15 μg/mL) reduced GTP binding to 89 ± 3 ($P < 0.05$) and 75 ± 5.3% ($P < 0.003$) of control values, respectively. It should be noted that NSAID concentrations which effectively inhibited GTP binding are achievable following the oral administration of drug, and are comparable to those which inhibit neutrophil functions such as homotypic aggregation and superoxide anion production [2].

Determination of the effects of NSAIDs on [^{35}S]-GTPγS binding by the subcellular location of Gα

We next examined the effects of salicylate and piroxicam on the specific binding of [^{35}S]GTPγS in distinct subcellular locations: the plasma membrane, specific granule membrane and cytosol. Over 95% of the [^{35}S]GTPγS binding activity was recovered in these three fractions. Total binding activity (pmol/10^8 PMN) was distributed as follows: plasma membrane 119 ± 24 (48%), specific granule membrane 11 ± 2 (4%) and cytosol 114 ± 20 (46%). Figure 1 illustrates the effects of salicylate and piroxicam on [^{35}S]GTPγS binding at equilibrium to each of the subcellular fractions. Both NSAIDs inhibited GTP binding to plasma and specific granule membranes. Neither drug, however, affected GTPγS binding to the cytosol fraction.

The guanine nucleotide binding domain of the G protein resides on the α subunit, whnich is present in the plasma membrane, specific granule and cytosolic components of the neutrophil. Within the plasmalemma, the α subunit is complexed with the hydrophobic βγ subunit [21–24]; only uncomplexed α subunits have been demonstrated in cytosol [24, 25]. To determine the distribution of the α and βγ subunits in our three subcellular fractions, immunoblotting with antisera specific for either Gα or Gβ was performed. Immunodetectable Gα was present in all three fractions, as expected (Fig. 2). In contrast, immunodetectable Gβ was found only in plasma and specific granule membrane fractions. These observations differ from those of Bokoch *et al.* [26] who did not detect G protein subunits in specific granule fractions, but are consistent with the findings of others [24, 27, 28].

Effect of ADP-ribosylation on GTPγS binding to plasma membrane and cytosol

Plasma membranes and cytosol contain multiple high and low molecular weight GTP binding proteins. To determine whether the NSAID effect was exerted at Gαi of the signal transducing heterotrimer, we examined the effects of pertussis toxin treatment on GTP binding. As shown in Fig. 3, the ADP-ribosylation of Gαi reduced the total GTPγS binding capacity of plasma membranes to 66 ± 7% ($P < 0.001$) of control values. NSAIDs did not inhibit GTP binding to pertussis toxin-treated plasma membranes. Pertussis toxin treatment inhibited GTP binding to monomeric Gα in cytosol to 87 ± 3% ($P < 0.01$) of control. Since the efficiency of the ADP-ribosylation of Gαi is reduced in the absence of βγ subunits, these studies likely underestimate the quantity of Gαi present in cytosol relative to that in plasma membranes. Thus, the data indicate that a minimum of 30–40% of plasmalemmal and 15–20% of cytosolic GTP binding capacity can be attributed to Gαi. The disruption of GTP/GDP exchange by NSAIDs appears to be targeted to this chemoattractant receptor-coupled G protein.

Effect of NSAIDs on [^{35}S]GTPγS binding to solubilized plasma membrane fractions reconstituted with phospholipids

Solubilized plasma membranes. The above experiments indicated that while GTPγS binding capacity was distributed throughout all subcellular fractions examined, the inhibitory effect of NSAIDs on GTP binding was limited to plasma membrane and specific

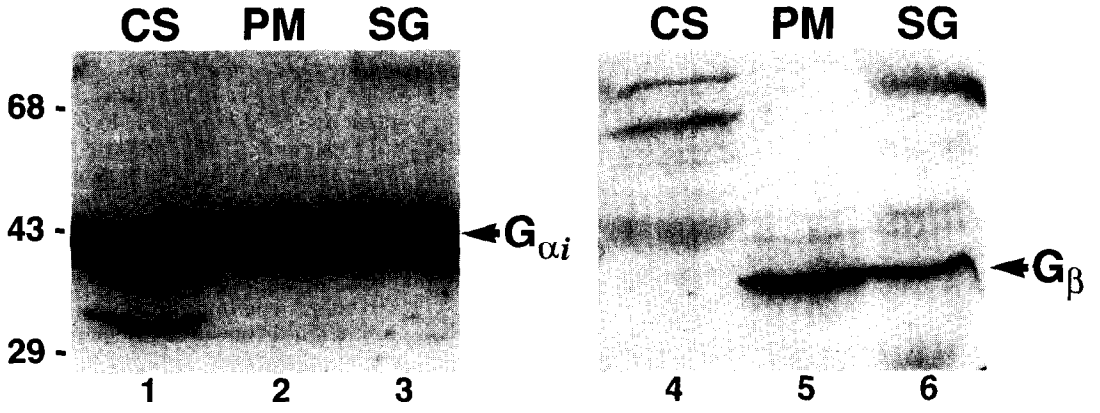

Fig. 2. Immunodetection of Gαi and Gβ in neutrophil subcellular fractions. Cytosol (CS, lanes 1 and 4), plasma membrane (PM, lanes 2 and 5) and specific granules (SG, lanes 3 and 6) derived from 1–40 × 10^7 neutrophils were analyzed by 12% SDS–PAGE (molecular weight of standard protein in kDa, left), transferred to nitrocellulose, and probed with antiserum 8730 (anti-Gαi, lanes 1–3) or 5356 (anti-Gβ, lanes 4–6) followed by ^{125}I-protein A. Immunodetected proteins were visualized by autoradiography. Deformation of the band corresponding to cytosolic Gαi is due to the large amount of actin that migrates slightly slower than Gαi in this gel system.

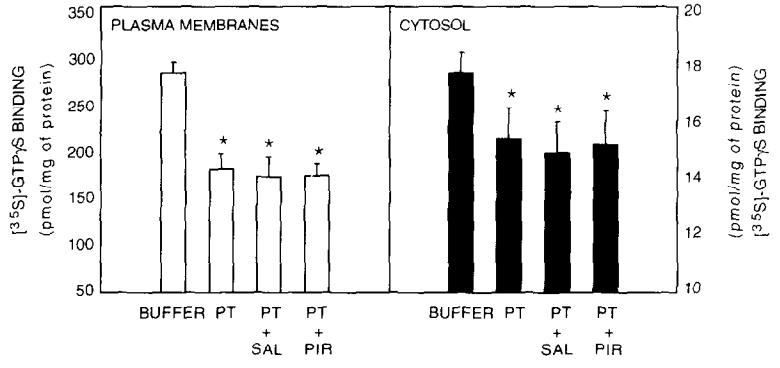

Fig. 3. Effect of ADP-ribosylation on GTPγS binding to plasma membrane and cytosol. Cytosol (20 μg) and plasma membranes (5 μg) were incubated for 30 min at 30° in 40 μL of buffer containing activated pertussis toxin (5 μg/mL), 1 mM ATP, 10 mM thymidine, 5 μM NAD, 100 mM Tris (pH 8.0). Twenty microliters of each sample was then mixed with 20 μL of [^{35}S]GTPγS binding buffer and incubated for 40 min at 30°. Reactions were terminated by the addition of ice-cold buffer (4 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris, pH 8.0) followed immediately by rapid filtration. Bound [^{35}S]GTPγS was quantitated by scintillography. Results are the means ± SEM of three experiments. Total binding activity (pmol/mg protein) in cytosol and plasma membranes was 31 ± 7 and 195 ± 10, respectively. Key: (*)$P < 0.01$ versus buffer control. Abbreviations: PT, pertussis toxin; PIR, piroxicam; and SAL, salicylate.

granule membrane components. This suggested either a requirement for the intact heterotrimeric Gα/βγ complex (absent from cytosol) or the restriction of NSAID action to a lipid environment. To test whether the heterotrimeric complex was sufficient for NSAID action, membrane proteins were extracted and solubilized by the addition of 0.2% sodium cholate followed by dialysis. Detergent extraction does not disrupt the oligomeric complex [24]. Solubilized plasma membrane fractions containing Gα/βγ were incubated in the presence or absence of NSAID for 5 min before the addition of 1 μM [^{35}S]GTPγS. Total and specific binding of GTPγS to solubilized membrane fractions (pmol/mg protein) did not differ significantly from intact membrane preparations (data not shown). As shown in Fig. 4, neither salicylate nor piroxicam inhibited [^{35}S]GTPγS binding to solubilized Gα/βγ present in detergent-treated plasma membranes.

Phospholipid reconstitution. We next performed studies to determine whether the reconstitution of solubilized G proteins derived from plasma membranes with exogenous phospholipids could restore the NSAID effect. We compared the effects of PC and PE, under conditions in which PC adopts the lamellar phase (liposome) and PE assumes the hexagonal (II) phase configuration, consisting of hexagonally packed cylinders of lipid surrounding

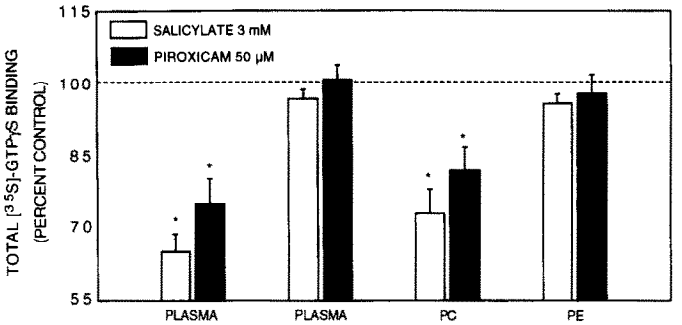

Fig. 4. Effect of NSAIDs on [^{35}S]GTPγS binding to solubilized and phospholipid reconstituted plasma membranes. Plasma membranes (1–5 μg) were solubilized with 0.2% sodium cholate. The detergent was removed by dialysis against 25 mM Tris, pH 7.4 (4 hr with four changes of buffer). Plasma membrane proteins were reconstituted into lipid bilayer by the addition of 20 μM phosphatidylcholine (PC) liposomes of 20 μM phosphatidylethanolamine (PE). Intact, solubilized and reconstituted plasma membranes were preincubated for 5 min with either sodium salicylate or piroxicam before the addition of [^{35}S]GTPγS binding buffer (see Materials and Methods). Results are the means ± SEM of three experiments. Key: (*)$P < 0.01$ versus buffer control.

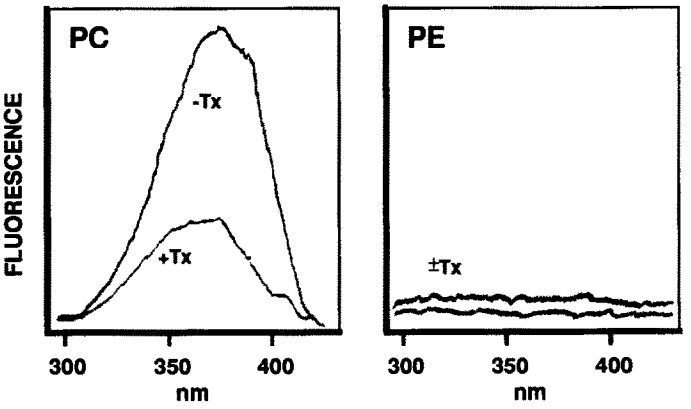

Fig. 5. Effect of Triton X-100 on excitation spetra of PC or PE containing Fura-2. A dry film of PC or PE was suspended by agitation on a Vortex mixer in 20 μM Fura-2, 0.145 M KCl, 0.5 mM EGTA, 25 mM Tris (pH 8.0). The samples were passed through a Sephadex G-50 column and eluted with the buffer to replace the external Fura-2. Excitation fluorescence spectra at 340 nm were taken for each preparation in the presence of external Ca^{2+}, without and with Triton X-100, which disrupts liposomal vesicles to free Fura-2.

central aqueous channels toward which the polar head groups are oriented [15, 18, 29]. To confirm the formation of the predicted phospholipid configuration, we examined the capacity of PC and PE to sequester the fluorescent probe Fura-2. PC and PE suspensions were prepared in the presence of Fura-2 and passed through a Sephadex G-50 column. The presence of lipid-entrapped Fura-2 was established by measuring excitation fluorescence intensity in the presence and absence of Triton X-100. As shown in Fig. 5, bilayer PC, but not hexagonal (II) phase PE, formed closed vesicles as determined by the Triton X-100 lability of Fura-2 fluorescence.

Following characterization studies of lipid structure (bilayers vs Hex_{II}), detergent-solubilized plasma membrane proteins were reconstituted with either PC or PE. Total and specific [^{35}S]GTPγS binding (pmol/mg protein) were again comparable to intact membrane preparations. Figure 4 illustrates the effects of salicylate and piroxicam on specific GTPγS binding to phospholipid-reconstituted plasma membranes. As shown, the insertion of GTP binding proteins into PC liposomes restored the capacity of

Table 1. Effect of NSAIDs on [^{35}S]GTPγS binding to cytosolic Gα reconstituted with purified βγ subunit

Condition	Total GTPγS binding (% of control)	
	Piroxicam (50 μM)	Salicylate (3 mM)
Cytosol	101 ± 2	97 ± 3
Cytosol plus PC liposomes	103 ± 3	97 ± 3
Cytosol plus bovine βγ	104 ± 4	101 ± 3
Cytosol plus bovine βγ and PC liposomes	79 ± 3*	87 ± 0.8†

Results are expressed as percent specific binding compared with corresponding control condition. The total specific binding (pmol/mg protein) for each condition was: cytosol, 18.6 ± 1.0; cytosol plus PC liposomes, 17.8 ± 0.7; cytosol plus bovine βγ, 29.0 ± 2.0; and cytosol plus βγ and PC liposomes, 31.1 ± 1.9. Values are means ± SEM, N = 3.

* $P < 0.001$.

† $P < 0.0001$.

both NSAIDs to inhibit GTP binding: Salicylate (3 mM) and piroxicam (50 μM) reduced GTPγS binding to 74 ± 5 ($P < 0.01$) and 83 ± 4% ($P < 0.01$), respectively. The reconstitution of plasma membrane proteins with PE, which assumed an Hex_{II} phase rather than bilayer configuration, failed to restore the NSAID effect.

To determine whether the addition of multilammelar vesicle to monomeric Gα could restore the NSAID effect, PC was added to cytosolic fractions. Neither salicylate nor piroxicam inhibited [^{35}S]GTPγS binding to cytosol to which PC liposomes had been added (Table 1). Taken together, these experiments indicate that the NSAID effect requires both the presence of a lipid bilayer and the heterotrimeric G protein configuration, which permits association with the bilayer.

Effect of NSAIDs on [^{35}S]GTPγS binding to cytosolic Gα complexed with purified bovine βγ subunits

To further assess the lipid and heterotrimeric requirements of NSAID action, we next examined the effect of NSAIDs on GTP binding to cytosolic Gα complexed with purified bovine βγ subunits in the presence or absence of 100 μM PC vesicles. Successful assembly of a $G\alpha_{cytosol}/\beta\gamma_{bovine}$ heterotrimer in our studies was indicated by two observations. First, as shown in Fig. 6, the addition of bovine βγ to $G\alpha_{cytosol}$ markedly enhanced the incorporation of radiolabeled ADP-ribose into the 41-kDa pertussis toxin-sensitive substrate, as expected [30]. The increase was observed both in the case of solubilized $G\alpha_{cytosol}$ (Fig. 6, panel A) and $G\alpha_{cytosol}$ added to PC liposomes (Fig. 6, panel B). The increase of ADP-ribose incorporation was specific to the pertussis toxin substrate, Gαi: the ADP-ribosylation of a 37-kDa pertussis toxin-independent substrate was unaffected (Fig. 6). Second, the addition of βγ subunits to both cytosol and cytosol plus PC increased total specific GTPγS binding capacity, consistent with assembly of the heterotrimeric complex (Table 1) [30]. Table 1 also demonstrates that NSAIDs did not inhibit GTP binding to cytosolic Gα to which either βγ subunits or PC liposomes had been added. However, both piroxicam (79 ± 3%, $P < 0.001$) and salicylate (87 ± 1%, $P < 0.0001$) significantly inhibited GTPγS binding to the assembled heterotrimer, $G\alpha_{cytosol}/\beta\gamma_{bovine}$, which had been incorporated into PC liposomes.

DISCUSSION

The inhibition of neutrophil activation by sodium salicylate and other NSAIDs is independent of the inhibition of PGH synthase [2]. Previous studies indicate that these agents affect G protein regulated processes, including GTP/GDP exchange by Gα, required for chemoattractant-dependent signalling [5]. In this report we demonstrate that the inhibitory effect of two NSAIDs on [^{35}S]GTPγS binding required the assembly of heterotrimeric G proteins within a lipid bilayer.

The increased binding of GTP to G proteins is associated with an active state in which Gα-GTP subunits stimulate effector enzymes or ion channels. In the neutrophil, the G protein-coupled chemoattractant receptors for both FMLP and C5a are members of the seven-transmembrane-segment class [31]. Signaling in response to these chemoattractants is regulated by a pertussis toxin-sensitive heterotrimeric G protein, $G\alpha i/\beta\gamma$ [10, 11, 32–34]. Following ligand binding, the GTP bound form of the α subunit stimulates phospholipase C dependent phosphoinositol hydrolysis [9, 11, 13, 33]. The capacity of both salicylate and piroxicam to inhibit the binding of a stable analog of GTP, GTPγS, to purified neutrophil plasma membranes therefore suggests a mechanism by which NSAIDs inhibit chemoattractant-dependent signaling. In support of this hypothesis, it is important to note that the concentrations of NSAIDs which effectively inhibit GTP binding, also represent the approximate EC_{50} values for the inhibition of FMLP responses [2, 5]. The importance of the effect on GTP/GDP exchange is further supported by the observation that NSAIDs

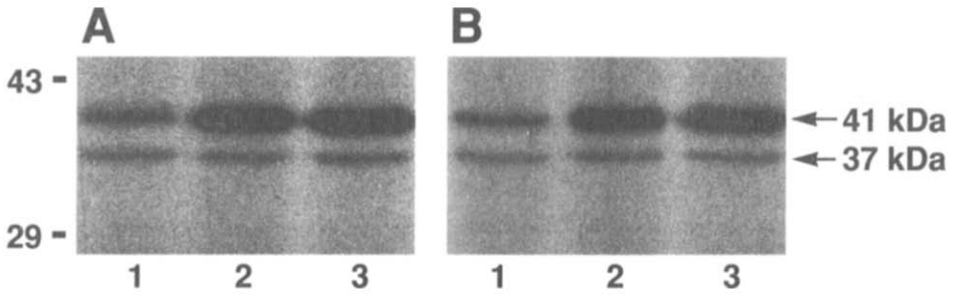

Fig. 6. Effect of $\beta\gamma$ on pertussis toxin-dependent ADP-ribosylation of cytosolic Gα. Twenty micrograms of cytosol was reconstituted with $\beta\gamma$ in the absence (panel A) or presence (panel B) of 100 μM PC liposomes. The samples were ADP-ribosylated in the presence of pertussis toxin (PT) as described in Materials and Methods, resolved on 10% SDS–PAGE, and visualized by autoradiography. Lane(s) 1, cytosol in the absence of bovine $\beta\gamma$ subunits; lane(s) 2, cytosol plus 10 ng of $\beta\gamma$; lane(s) 3, cytosol plus 40 ng of $\beta\gamma$. Note the marked increase of radiolabeled ADP-ribose incorporation by the 41-kDa substrate (Gαi) in the presence of $\beta\gamma$ subunits. $\beta\gamma$ Subunits did not enhance the PT-independent ADP-ribosylation of a 37-kDa protein.

inhibit responses of neutrophils exposed to ligands that engage G protein-coupled receptors (e.g. FMLP, C5a and LTB_4), but have little effect on neutrophil functions in response to phorbol esters or ionomycin, stimuli that bypass G protein-dependent signaling [2, 5, 6].

The [^{35}S]GTPγS binding assay has been used to identify both heterotrimeric G proteins and low molecular weight GTP binding proteins in a variety of cell types [13, 14]. Our findings with regard to the distribution of GTP binding activities of plasma membrane, specific granule and cytosol fractions are in agreement with previous studies [13, 25, 28]. The high concentrations of heterotrimeric GTP binding proteins in neutrophil plasma membrane preparations (1–3% of total membrane protein [26]) make it likely that Gα subunits are a major source of GTP binding activity in this subcellular fraction. We have attempted to determine the contribution of Gαi to total binding by examining the effects of pertussis toxin. There are two major pertussis toxin substrates in plasma membranes that are structurally very similar [35]. Only one major pertussis toxin substrate has been found in neutrophil cytosol but its peptide maps are identical to those of plasma membrane pertussis toxin substrates and the GTP binding regions of these three proteins are highly conserved [35]. In our studies, the pertussis toxin-dependent ADP-ribosylation of Gαi reduced total specific binding of GTPγS to plasma membranes by approximately 35%. Binding to cytosol was reduced by 15–20%. These percentages, therefore, represent the minimum contribution of Gαi to GTP binding in these subcellular fractions. Since the efficiency of ADP-ribosylation of monomeric Gαi is decreased in the absence of $\beta\gamma$ subunits (Fig. 6), it is likely that such an analysis underestimates the relative amount of Gαi present in cytosol. Indeed, autoradiographs indicate that the quantity of immunodetectable Gαi in plasma membranes was equivalent to that of the cytosolic fractions (Fig. 2).

Two lines of evidence indicate that the effects of salicylate and piroxicam on GTPγS binding are exerted at Gαi and not at other GTP-binding proteins. First, as we have reported previously in separate experiments [5], the ADP-ribosylation of plasmalemmal Gαi by pertussis toxin abrogates NSAID inhibition of specific GTPγS binding (Fig. 3). Second, while NSAIDs do not inhibit GTP binding to monomeric Gα in cytosol, binding is inhibited to heterotrimeric $G\alpha_{cytosol}/\beta\gamma_{bovine}$ inserted into PC liposomes. Since the presence of $\beta\gamma$ subunits (which associate specifically with α subunits) is required for inhibition, one must conclude that the NSAID effect is exerted at Gα and not other GTP binding proteins.

The relatively selective effect of NSAIDs on GTP/GDP exchange by Gαi is of interest with regard to the magnitude of inhibition observed. We observed a concentration-dependent inhibition of GTP binding by piroxicam and salicylate which (at pharmacologic concentrations) ranged from 12 to 40%. This magnitude of the observed inhibition is likely to underestimate specific effects of NSAIDs on GTP/GDP exchange by Gαi since the binding assay utilized identifies heterotrimeric and monomeric G protein α subunits (including, but not limited to Gαi), as well as low molecular weight GTP binding proteins [13, 14]. Our studies indicate that the NSAID effect is directed to pertussis toxin-sensitive α subunits that are complexed with $\beta\gamma$ subunits; these heterotrimers should not account for 100% of membrane GTP binding capacity. Therefore, we suggest tht the actual inhibition by NSAIDs of GTP binding specifically to $G\alpha i/\beta\gamma$ heterotrimers exceeds the observed inhibition of total GTP binding (where the denominator, "total GTP binding," reports binding to $G\alpha i\beta\gamma + G\alpha_{other}/\beta\gamma$ + low molecular weight G proteins).

A central observation of these studies regarding the site of NSAID action is that the effects of both drugs were restricted to membrane-containing compartments of fractionated neutrophils. Salicylate and piroxicam inhibited the binding of [^{35}S]GTPγS only to plasma and specific granule membranes; binding to monomeric Gα present in cytosol (46%

of total cellular binding capacity) was unaffected. This suggests a requirement for the interaction of NSAIDs either with the intact heterotrimer or the association of Gαi with a phospholipid bilayer. The heterotrimeric configuration alone was by no means sufficient for NSAID action: GTPγS binding to solubilized, Gα/βγ extracted from plasma membranes was not inhibited by either salicylate or piroxicam nor was the absence of an effect due to functional alteration of the capacity of the oligomeric complex to bind GTPγS: total and specific binding activity was unaffected by the solubilization process.

The absence of an NSAID effect on [^{35}S]GTPγS binding to Gα present in cytosol and to the solubilized heterotrimeric complex derived from plasma membrane indicates a lipid requirement for NSAID action. To confirm this we employed PC which adopts a lamellar phase and PE which adopts a nonbilayer hexagonal II phase structure. These structural differences, noted previously by scanning electron microscopy [15, 16, 29], were confirmed in our studies by the capacity of PC but not PE to form intact vesicles which entrap Fura-2. Our experiments demonstrate that the capacity of salicylate and piroxicam to inhibit GTPγS binding can be restored if solubilized Gα/βγ heterotrimers derived from plasma membranes are incorporated into PC liposomes. In contrast, the insertion of Gα/βγ in hexagonal II phase (nonbilayer) lipid structures of PE did not restore the NSAID inhibitory effect. In these latter structures, hexagonally packed cylinders of lipid surround central aqueous channels toward which the polar head groups are oriented [15, 16, 29]. The conclusion that the heterotrimeric configuration and a lipid bilayer were both essential for NSAID action was confirmed by the $G\alpha_{cytosol}/\beta\gamma_{bovine}$ reconstitution experiments. NSAIDs did not inhibit GTP binding to monomeric $G\alpha_{cytosol}$ nor to $G\alpha_{cytosol}$ to which PC liposomes had been added. Sternweis had previously shown that free Gαi and Gαo require βγ subunits in order to insert into PC liposomes [36], consistent with the notion that the association of a hydrophilic α subunit with hydrophobic sites of plasma membrane is due to its coupling with prenylated Gβγ [25, 37]. We therefore reconstituted $G\alpha_{cytosol}$ with $\beta\gamma_{bovine}$ subunits in the presence or absence of PC liposomes. The effectiveness of heterotrimeric assembly was confirmed both by an increase of pertussis toxin-dependent ADP-ribosylation and of total GTP binding capacity. The results of these experiments confirmed those which analyzed the effects of NSAIDs on Gα/βγ derived from detergent-treated plasma membranes. Salicylate and piroxicam inhibited GTP binding to the $G\alpha_{cytosol}/\beta\gamma_{bovine}$ heterotrimer only if the complex was inserted into PC (bilayer) liposomes. The magnitude of inhibition was less than that observed for intact plasma membranes. This is not unexpected since it would be unlikely that the efficiency of heterotrimer reconstitution and lipid insertion would be complete. The restoration of NSAID inhibition of the magnitude observed, approximately half that of intact membranes, suggests that 50% heterotrimeric ($G\alpha_{cytosol}/\beta\gamma_{bovine}$ reconstitution/lipid insertion has been achieved.

Although our studies demonstrate a requirement for both lipid and the heterotrimeric configuration, the data suggest that the selectivity of the NSAID effect resides not in the lipids but in the structure of the G protein. Membrane association alone via an isoprenylated tail (common to both the βγ subunit and low molecular weight G proteins) is not sufficient to impart NSAID sensitivity. This suggests that the NSAIDs may interact with the heterotrimer only after a configurational change which results following association of the α subunit with βγ. Whether NSAIDs act at the GTP binding site or elsewhere on the heterotrimer is unknown. However, the membrane requirement for inhibition suggests that the site of NSAID action may be at the lipid–protein interface.

The recognition that NSAIDs must act within a lipid bilayer is consistent with the known physicochemical properties of these agents: NSAIDs are anionic, planar, lipophilic compounds that intercalate into and alter the viscosity of both plasma membranes and liposomes [6, 38]. Their capacity to interfere with GTP/GDP exchange may provide new insight into a common mechanism by which a variety of mediators and pharmacological agents regulate cell activation. For example, we have reported previously that arachidonic acid, released from cell membrane stores, acts as a second messenger in neutrophils through its capacity to increase the binding of GTPγS to the plasmalemmal G protein [7]. Avissar *et al.* [39] have shown that the engagement of adrenergic and muscarinic receptors increases GTP binding to membranes prepared from rat cerebral cortex, an effect that is blocked by pretreatment of the membranes with cholera toxin and pertussis toxin, respectively. Like these bacterial toxins, the psychotropic agent lithium blocks agonist-induced increases of GTP binding to rat cerebral cortex membranes, a property which has been proposed as a pharmacological mechanism for lithium action [39].

In summary, our data show that the inhibition of GTPγS binding by NSAIDs required the assembly of the heterotrimeric G protein in a phospholipid bilayer. We suggest that the intercalation of lipophilic aspirin-like drugs into the plasmalemma interferes with topological molecular interactions, such as GTP/GDP exchange, which are essential for normal signal transduction. This property may account not only for the inhibition of neutrophil functions by these agents, but also for diverse effects on membrane-dependent processes.

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