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NON-STEROIDAL ANTI-INFLAMMATORY DRUGS: EFFECTS ON A GTP BINDING PROTEIN WITHIN THE NEUTROPHIL PLASMA MEMBRANE

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Abstract—Sodium salicylate and other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit neutrophil functions via unknown mechanisms. To examine their site of action in the neutrophil we have studied discrete events within the plasma membrane which depend upon the normal function of a GTP binding protein (G protein). We demonstrated that sodium salicylate and piroxicam inhibit neutrophil activation in response to stimuli which require signal transduction via a G protein (e.g. formyl-methionine-leucine-phenylalanine) but have no effect on stimuli which do not (e.g. phorbol myristate acetate, ionomycin). NSAIDs blocked the ADP-ribosylation of the pertussis toxin substrate in human neutrophils. This effect was associated with the capacity of NSAIDs to block pertussis toxin-dependent inhibition of neutrophil functions. Finally, NSAIDs inhibited the binding of GTPγS, a stable analog of GTP, to purified neutrophil membrane preparations. The data indicate that salicylate and other NSAIDs interact with a G protein in the neutrophil plasmalemma and thereby uncouple post-receptor signalling events.

Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs†) exert some of their anti-inflammatory effects by inhibiting the 11-cyclo-oxygenases of platelets and blood vessels [1]. However, NSAIDs also inhibit the activation of neutrophils by a variety of agents such as the chemoattractants formyl-methionine-leucine-phenylalanine (FMLP), leukotriene B_4 (LTB_4), and C5-derived peptides [2–11]. This pharmacological action of NSAIDs is independent of their inhibition of prostaglandin synthesis and is a property shared by sodium salicylate, a poor inhibitor of cyclooxygenase [2]. It has been proposed that these actions of NSAIDs provide an additional explanation for their anti-inflammatory effects [2, 10].

We and others have shown previously that NSAIDs disrupt normal signalling events in the neutrophil: the binding of the chemoattractant FMLP is reduced, calcium movements and membrane phospholipid remodelling are inhibited and, paradoxically, the production of cyclic AMP is increased [2, 6]. These events are regulated by one or more signal-transducing GTP binding proteins (G proteins) within the plasmalemma [12–15]. Therefore, we have examined the effects of NSAIDs on specific G protein dependent functions. The data indicate that NSAIDs alter the properties of the G protein and may so disrupt post-receptor transduction events within the plasmalemma.

MATERIALS AND METHODS

Chemicals. FMLP was obtained from Vega Biotechnologies Inc. (Tucson, AZ). Phorbol myristate acetate (PMA) and ionomycin were obtained from Calbiochem (San Diego, CA). Dimethyl sulfoxide (DMSO), sodium salicylate (NaS), indomethacin, and piroxicam were obtained from the Sigma Chemical Co. (St. Louis, MO). Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA). GTPγS and GTPγ^{35}S were obtained from the Du Pont Co. NEN Research Products (Boston, MA).

Neutrophil function. Superoxide anion generation was measured as the superoxide dismutase inhibitable reduction of cytochrome *c* by the batch method [16]. Neutrophil aggregation was monitored by changes in light transmission in a dual-channel aggregometer (Payton Associates, Buffalo, NY) as described [2].

Preparation of neutrophil membranes. Suspensions containing 98 ± 1% human neutrophils were prepared from heparinized blood as described [2]. Neutrophils were suspended in ice-cold Tris–HCl buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.5) with EDTA (0.5 mM) at 100×10^6 cells/mL and treated with the protease inhibitor aprotonin (100 kUnits/mL). Neutrophil suspensions were then disrupted with a tissue homogenizer (Polytron; Brinkmann Instruments, Westbury, NY). Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. The suspension was centrifuged in a Sorvall RC-3 at 2500 rpm for 20 min at 4°. The supernatant was removed

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† Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; FMLP, formyl-methionine-leucine-phenylalanine; DMSO, dimethyl sulfoxide; NaS, sodium salicylate; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; and Pt_x, pertussis toxin.

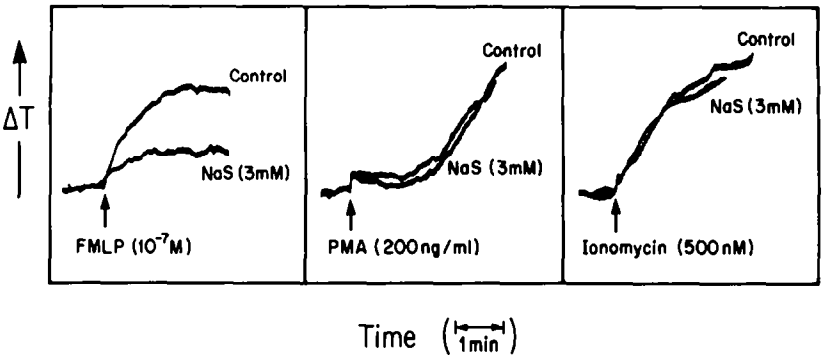

Fig. 1. Effect of sodium salicylate on neutrophil aggregation in response to FMLP (10^{-7} M), PMA (200 ng/mL) and ionomycin (500 nM). Neutrophils were preincubated with buffer or drug (5 min, 37°) before exposure to stimulus. Change in light transmission was recorded over time. Aggregation curves are representative of three experiments performed in duplicate.

(polymorphonuclear leukocyte membrane layer) and was centrifuged in a Beckman SW 55 Ti rotor at 37,000 rpm for 40 min at 4°. The pellet was washed once with Tris–HCl buffer, pH 7.5, 0.5 mM EDTA, aprotonin (100 kUnits/mL). Membranes were resuspended in the same buffer at approximately 1 mg protein/mL and stored at −70°. The protein content of membrane preparation was determined by the method of Lowry *et al.* [17].

Ligand binding assays. All experiments were performed at 30° and each determination was performed in duplicate. Membrane preparations (1–5 μg protein) were treated with sodium salicylate (1 mM) or piroxicam (50 μM) for 5 min before incubation with 1 μM GTPγ^{35}S (5,000–10,000 cpm/pmol) in 40 μL total volume of 25 mM Hepes, pH 8, 20 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM diothiothreitol (DTT), 100 mM NaCl. After 60 min, the samples were filtered through 25-mm Whatman GF/F glass fiber filters and washed with four successive 4-mL volumes of ice-cold 10 mM Hepes, pH 8, 100 mM NaCl, 5 mM $MgCl_2$. The radioactivity retained on the filter was measured by liquid scintillation counting as described [18]. Non-specific binding was determined by incubating samples in the presence of 100-fold excess unlabeled GTPγS and was less than 20% of the total binding. To examine the effect of pertussis toxin on the binding of GTPγS to neutrophil membranes, some membranes were incubated in the presence of pertussis toxin as described below (0.1 μg/mL) (30 min, 37°) before addition of GTPγ^{35}S.

Treatment of neutrophils with pertussis toxin. To examine the effects of NSAIDs on the inhibition of neutrophil function by pertussis toxin, neutrophils were preincubated (90 min, 37°) in the presence or absence of various concentrations of pertussis toxin alone or with pertussis toxin plus an NSAID, and then washed, before stimulation with FMLP (10^{-7} M). Preliminary experiments indicated that lower concentrations of pertussis toxin were required to inhibit superoxide anion generation rather than to inhibit aggregation. Therefore, the concentration of pertussis toxin utilized in the superoxide experiments was 50 ng/mL compared to 200 ng/mL in studies of aggregation.

ADP-Ribosylation of the pertussis toxin substrate in the neutrophil plasma membrane. Neutrophil membranes were prepared as above. Membrane preparations containing approximately 20 μg of protein were incubated for 30 min at 30° in 0.1 mL of ADP-ribosylation reaction mixture consisting of 0.15 mM Hepes (pH 8), 10 mM thymidine, 5 mM $MgCl_2$, 1 mM ATP, 0.3 mM GTP, 10 μM [^{32}P]NAD (2 μCi), and pertussis toxin (0.1 μg/mL). Pertussis toxin was activated with 30 mM DTT. The reaction was terminated by adding Laemmli sample buffer, followed by boiling for 3 min. Then the samples were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography as described [15, 19]. To determine the effect of NSAIDs on the capacity of pertussis toxin to ADP-ribosylate the G protein of intact neutrophils, a "two-stage" experiment was performed. Membranes were prepared from intact neutrophils that had been exposed previously to pertussis toxin (90 min, 37°). These membranes were then exposed again to pertussis toxin (30 min, 30°) in the presence of [^{32}P]NAD in the reaction mixture as described above. In some experiments, intact neutrophils were incubated in the presence of both pertussis toxin and an NSAID before membrane preparation and the "second stage" ADP-ribosylation.

RESULTS

Effects of sodium salicylate and piroxicam on the activation of neutrophils in response to diverse stimuli. As shown in Fig. 1 sodium salicylate effectively inhibited neutrophil aggregation in response to FMLP (10^{-7} M). Salicylate significantly inhibited aggregation at concentrations as low as 1 mM; the IC_{50} was between 2 and 3 mM. In contrast, sodium salicylate had no effect on aggregation in response to PMA or ionomycin, stimuli which bypass receptor–G protein interaction. The effect of piroxicam was comparable to that of salicylate (data not shown). These results are consistent with

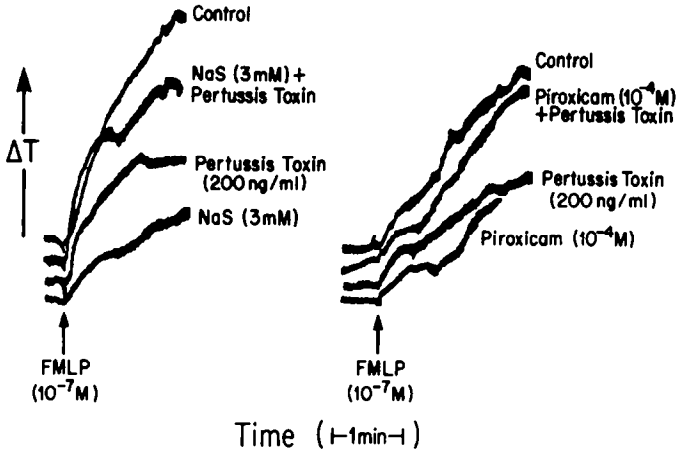

Fig. 2. Effects of sodium salicylate and piroxicam on the inhibition of neutrophil aggregation by pertussis toxin. Neutrophils were incubated (90 min, 37°) in buffer (control) or in the presence of pertussis toxin alone, NSAID alone, or pertussis toxin plus NSAID and then washed thrice before exposure to FMLP. The aggregation curves which illustrate the effect of NSAID alone were performed on cells not washed following the preincubation period since washing prevented the inhibition of aggregation by NSAID, although it did not affect their reversal of the pertussis toxin effect. Aggregation curves are representative of three experiments.

previous reports that NSAIDs inhibit ligand-induced stimulation (e.g. leukotriene B_4, FMLP, C5a) but have little effect on stimuli which do not engage specific cell surface receptors [2, 9, 10].

Effects of NSAIDs on the inhibition of neutrophil function by pertussis toxin. The above observations indicated that salicylate and piroxicam, like pertussis toxin, interfere with neutrophil responses regulated by a signal-transducing G protein. In the neutrophil, pertussis toxin catalyzes the ADP-ribosylation of an ~40 kDa α-subunit (G_α) of an immunologically distinct GTP binding protein [12, 20, 21]. To determine whether NSAIDs exerted effects at this G protein, neutrophils were preincubated with pertussis toxin in the presence or absence of drug and then stimulated with FMLP (10^{-7} M). Results were compared to control responses elicited from neutrophils incubated with buffer alone before stimulation. It should be noted that following the 90-min incubation period, except where indicated, neutrophils were washed prior to exposure to FMLP.

As expected, preincubation with sodium salicylate alone inhibited neutrophil aggregation (Fig. 2). Interestingly, salicylate did not inhibit aggregation effectively if cells were washed immediately before exposure to FMLP. Treatment of neutrophils with pertussis toxin inhibited neutrophil aggregation to 47 ± 1.6% of control (Table 1), an inhibition which was not reversed by washing. Salicylate interfered with the pertussis toxin effect: the incubation of neutrophils with both pertussis toxin and salicylate (3 mM) before exposure to FMLP resulted in less inhibition of aggregation than that observed in the presence of pertussis toxin alone (67.6 ± 8% of control ($P < 0.01$), compared to pertussis toxin alone) (Table 1). This reversal by salicylate of the effect of the toxin could be demonstrated only if the neutrophils were washed before exposure to FMLP,

Table 1. Effect of sodium salicylate on the inhibition of neutrophil aggregation by pertussis toxin

Treatment	Neutrophil aggregation (% of control)
Pertussis toxin	47.4 ± 1.6*
Pertussis toxin	
+NaS (1 mM)	60.3 ± 3.8†
+NaS (2 mM)	65.6 ± 12.4
+NaS (3 mM)	67.6 ± 8.0‡

Neutrophils were exposed to buffer, pertussis toxin or pertussis toxin plus sodium salicylate (90 min, 37°) and then washed before exposure to FMLP (10^{-7} M). Results are expressed as the per cent of control ± SEM (N = 3) of cells incubated in buffer alone before exposure to FMLP. Control value for aggregation was 11.4 ± 1.3 cm^2/min.

* Significantly different compared to the buffer control ($P < 0.01$).

†‡ Significant differences between cells treated with pertussis toxin alone versus pertussis toxin plus salicylate: † $P < 0.05$, and ‡ $P < 0.01$.

presumably due to the removal of the direct inhibitory effects of the drug by washing.

Piroxicam (50 and 100 μM) also interfered with pertussis toxin dependent inhibition: pertussis toxin alone reduced FMLP-induced aggregation to 56.5 ± 7.3% of control; this compared to a reduction to 67.9 ± 9.7% of control in the presence of both piroxicam and pertussis toxin ($P < 0.05$) (Fig. 2).

These data are consistent with our previous observation regarding the effect of sodium salicylate on the capacity of pertussis toxin to inhibit superoxide anion [22]. In those studies, while salicylate alone did not reduce effectively FMLP-dependent

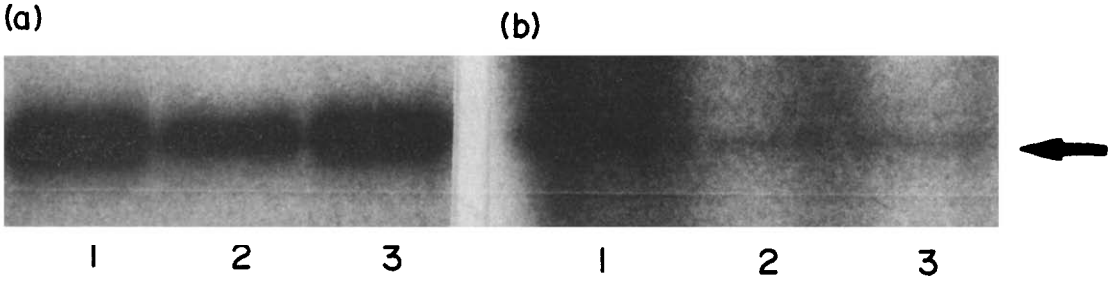

Fig. 3. Effects of sodium salicylate and piroxicam on ADP-ribosylation of the 41 kDa pertussis toxin substrate (arrow) of neutrophil membrane preparations. Membranes were incubated with NSAIDs for 5 min and then exposed to pertussis toxin (PT) and [^{32}P]NAD for 30 min as described in Materials and Methods. Panel (a): 0.1 μg/mL PT; Panel (b): 0.04 μg/mL PT. Lane 1: control; lane 2: 3 mM sodium salicylate; lane 3: 50 μM piroxicam.

superoxide anion production, it (at concentrations of 1–3 mM) reversed the inhibitory capacity of pertussis toxin.

Effects of NSAIDs on the ADP-ribosylation of G_α. The above observation that salicylate and piroxicam interfered with the inhibitory effects of pertussis toxin suggested that the drugs might prevent the ADP-ribosylation of G_α. Figure 3 demonstrates that this is indeed the case: as shown by autoradiography, the 41 kDa pertussis toxin substrate of membranes preincubated with either sodium salicylate or piroxicam incorporated significantly less ^{32}P than did the substrate of control membranes. Quantitation of radiolabel incorporated by densitometry revealed that salicylate and piroxicam inhibited pertussis toxin dependent ADP-ribosylation to 69.1 ± 1.7 and 83.4 ± 1.8% of control values respectively ($P < 0.006$).

A "two-stage" exposure was also performed which mimicked the conditions under which NSAIDs blocked the effects of pertussis toxin on neutrophil functions. In the first stage, intact neutrophils were incubated with buffer alone, pertussis toxin alone, or pertussis toxin plus an NSAID under conditions sufficient to ADP-ribosylate the G protein. No source of radiolabeled NAD was available during this first stage. Membranes prepared from these cells were then exposed again to pertussis toxin plus radiolabeled NAD in a standard ADP-ribosylation protocol [12, 15, 19], as shown in Fig. 4. The exposure of control membranes (prepared from neutrophils incubated in buffer alone) to pertussis toxin in the presence of [^{32}P]NAD ("stage 2") resulted in the ADP-ribosylation of an approximate 40–41 kDa substrate, as expected. A significant decrease in "stage 2" radiolabel incorporation was observed when membranes were derived from neutrophils previously incubated with pertussis toxin, indicating a preemptive "stage 1" ADP-ribosylation of the G protein in the intact cell. However, if intact neutrophils were incubated with both pertussis toxin plus an NSAID (salicylate, piroxicam or indomethacin), "stage 2" ADP-ribosylation was not impeded (Fig. 4). Thus, NSAIDs prevented the pertussis toxin-catalyzed "stage 1" ADP-ribosylation of G_α in intact neutrophils. The data were concordant with the observation that NSAIDs reduced the inhibitory effects of pertussis toxin on neutrophil function (Table 1, Fig. 2).

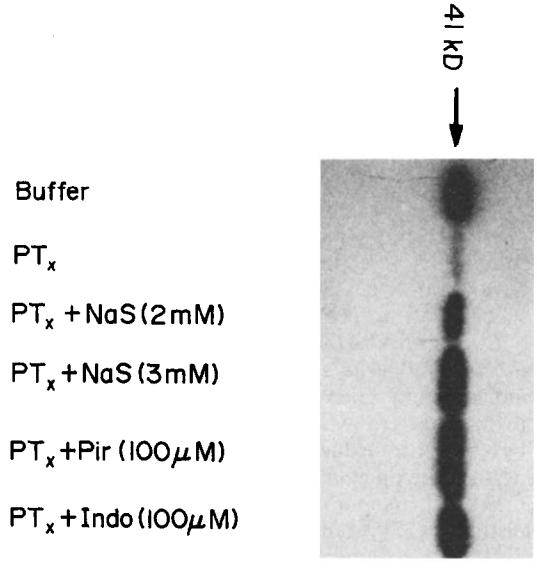

Fig. 4. Effects of NSAIDs on the ADP-ribosylation of the G protein in intact neutrophils. Neutrophils were incubated with buffer, pertussis toxin (PT_x), or pertussis toxin plus an NSAID (90 min, 37°) ("stage 1"). Membranes prepared from these neutrophils were then exposed again to pertussis toxin, in the presence of [^{32}P]NAD (30 min, 37°) ("stage 2"). Protein extracts were subjected to SDS-PAGE followed by autoradiography. The first lane shows the expected "stage 2" ADP-ribosylation of the 40–41 kD pertussis toxin substrate in membranes prepared from cells incubated in buffer alone. The second lane shows that the effect of exposure of intact cells to pertussis toxin during "stage 1" before "stage 2" ADP-ribosylation of the G protein in prepared membranes. The third through sixth lanes demonstrate the effect of adding NSAIDs during the "stage 1" exposure to PT in the intact cell. The autoradiograph is representative of three experiments.

Effects of NSAIDs on the binding of GTPγ^{35}S to neutrophil membranes. Figure 5 shows the effects of sodium salicylate and piroxicam on the binding of the nonhydrolyzable analog of GTP, GTPγ^{35}S, to

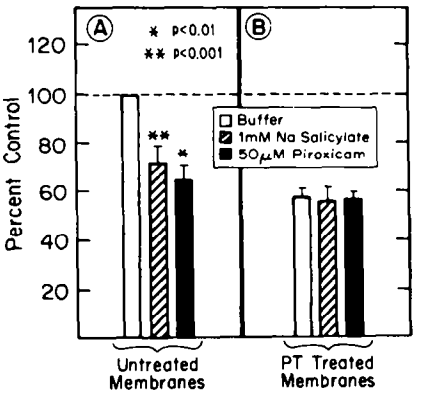

Fig. 5. Effects of pertussis toxin, sodium salicylate and piroxicam on the binding of GTPγ^{35}S to neutrophil membranes. Membranes were prepared in the presence or absence of pertussis toxin (1.0 μg/mL, 30 min, 37°). Binding assays in the presence or absence of NSAID were then performed as described in Materials and Methods. Results are expressed as the per cent of total specific binding by untreated membranes (100% total specific binding = 146 ± 15 pmol/mg protein). Panel A illustrates the effects of salicylate and piroxicam on GTPγ^{35}S binding to membranes prepared in buffer. Panel B illustrates the effect of pretreatment of the membranes with pertussis toxin. Values are means ± SEM, N ≥ 3.

neutrophil membrane preparations. Membrane fractions were incubated in the presence or absence of drug for 5 min before the addition of 1 μM GTPγ^{35}S. Total binding reached equilibrium by 40 min; non-specific binding was less than 20% of total. Sodium salicylate (1 mM) and piroxicam (50 μM) reduced the specific binding of GTPγS at equilibrium to 64 ± 4.2 and 60 ± 3.5% of control respectively ($P < 0.01$). To determine whether these drugs affected binding at G_α, we examined their effects on membranes that had been exposed previously to pertussis toxin. Prior exposure of the membranes to pertussis toxin under conditions sufficient to catalyze ADP-ribosylation reduced the specific binding of GTPγ^{35}S to 56 ± 2.9% of control. Pertussis toxin, in the absence of NAD, and NAD alone, in the absence of pertussis toxin, had no effect on GTP binding. Neither salicylate nor piroxicam affected the binding of GTPγ^{35}S to pertussis toxin treated membranes. These data demonstrate that the ADP-ribosylation of G_α reduces its capacity to bind GTP. In addition, the loss of the NSAID effect in toxin treated membranes suggests a site of action at G_α. In separate experiments, the addition of FMLP (0.1 and 0.01 μM) was shown to have no effect on the binding of GTP to the membranes.

DISCUSSION

We have shown previously that sodium salicylate and other NSAIDs inhibit neutrophil activation by mechanisms that are independent of their capacity to inhibit the prostaglandin H synthase [2, 9, 23]. Several of the earlier observations, such as the effects of NSAIDs on FMLP binding, calcium translocations and intracellular levels of cyclic AMP, suggested that these agents affected G protein regulated processes.

The present studies further explored the effects of sodium salicylate and piroxicam on discrete events which depend upon a GTP binding protein. First, we show that the inhibition by salicylate of neutrophil aggregation required that a signal be transduced via a G protein dependent pathway: aggregation in response to FMLP was inhibited whereas aggregation in response to PMA and ionomycin (stimuli which activate the cell by G protein independent pathways) was not. NSAIDs also inhibit activation in response to C5a [2, 10], leukotriene B_4 [23] and sodium fluoride [23], the last an agent purported to directly activate the G protein [24]. Together, these observations would place the action of NSAIDs distal to receptor–ligand engagement (sodium fluoride inhibited) but proximal to the activation of protein kinase C or the rise of cytosolic calcium (PMA, ionomycin not inhibited).

To examine the hypothesis that NSAIDs interfered with a signal-transducing G protein, two functions were studied: the capacity of the G protein to serve as a substrate for pertussis toxin catalyzed ADP-ribosylation and its capacity to bind a nonhydrolyzable analog of GTP, GTPγ^{35}S. NSAIDs prevented the ADP-ribosylation of the α-subunit of the plasmalemmal G protein by autoradiographic analysis. Concordant with this observation, and most likely as a consequence, both salicylate and piroxicam interfered with the capacity of pertussis toxin to inhibit neutrophil activation. This antagonism between NSAIDs and pertussis toxin, while of unlikely physiological significance, suggests that NSAIDs interfere with a process which can be localized to a known site, G_α.

The second observation which indicated that NSAIDs interfere with the normal function of the G protein was provided by the GTPγ^{35}S binding studies. Increased binding of GTP to the G protein is associated with its activation [15, 21, 23, 25]. In rat cerebral cortex the engagement of adrenergic and muscarinic receptors increases GTP binding to the tissue, an effect which can be blocked by pretreatment of the membranes with cholera toxin and pertussis toxin respectively [26]. In our studies, both salicylate and piroxicam reduced the binding of radiolabeled GTPγS to neutrophil membrane preparations, an effect which would be expected to inhibit G protein dependent functions [26]. It is of interest that pretreatment of the membranes with pertussis toxin also reduced total GTP binding. Pertussis toxin has been shown to induce shifts of electrophoretic mobility of G_α by Western blot analysis [27]. This raises the possibility that the inhibition of cell function by pertussis toxin is due to the induction of structural changes of G_α which, our experiments indicate, reduce its capacity to bind GTP. NSAIDs did not inhibit GTPγS binding to membranes previously treated with pertussis toxin. This would suggest that the NSAID effect on GTP binding is localized to the pertussis toxin substrate.

The mechanism(s) by which NSAIDS interfere with the ADP-ribosylation of the G protein and with GTPγS binding is unclear. NSAIDs as a class are

lipophilic anions which insert into the lipid bilayer and alter membrane viscosity [23, 28]. Plasma membrane viscosity is an important determinant of the capacity of molecules to diffuse within the lipid bilayer and may thereby affect signal transduction. For example, alterations of viscosity have been demonstrated to influence FMLP binding characteristics and cell function [23, 29, 30]. Thus, the intercalation of NSAIDs into the membrane with resultant changes in viscosity may alter the configuration of G_α or prevent access to key catalytic (or binding) sites.

In summary, our data suggest that the inhibition of neutrophil function by NSAIDs, which is independent of the inhibition of prostaglandin synthesis, is due to a disruption of G protein dependent events. Inhibition by NSAIDs was limited to stimuli which required signalling via a G protein. In addition, two processes, ADP-ribosylation and GTPγ^{35}S binding, which can be localized to distinct sites on G_α, were blocked by NSAIDs. These effects may be due to the capacity of NSAIDs to disrupt gel-like regions of the plasmalemma and thereby alter nucleotide–protein and protein–protein interactions within the lipid bilayer.

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