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A COMPARISON OF THE ABILITIES OF ACETYLSALICYLIC ACID, FLURBIPROFEN AND INDOMETHACIN TO INHIBIT THE RELEASE REACTION AND PROSTAGLANDIN SYNTHESIS IN HUMAN BLOOD PLATELETS

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1 A quantitative comparison has been made of the abilities of acetylsalicylic acid, flurbiprofen and indomethacin to inhibit the adenosine diphosphate (ADP)-induced platelet release reaction and to inhibit the synthesis of prostaglandins from arachidonic acid.

2 Experiments were carried out on human platelets that had been incubated with the agents *in vitro* and on platelets obtained from volunteers who had ingested standard doses of the drugs.

3 The results obtained for acetylsalicylic acid show that there is a close relation between the release reaction and the synthesis of prostaglandins in platelets.

4 Flurbiprofen and indomethacin appear to inhibit the release reaction rather more effectively than they inhibit the synthesis of prostaglandins. It is possible that these agents inhibit the release reaction by another mechanism.

Introduction

Blood platelets undergo a secretory process known as the platelet release reaction. They also have biochemical pathways for the synthesis of prostaglandins. These properties may be related in that agents that induce the release reaction also stimulate prostaglandin synthesis (Smith & Willis, 1970; Smith, Ingerman, Kocsis & Silver, 1973), and some of the agents that inhibit the release reaction also inhibit prostaglandin synthesis, (Smith & Willis, 1971; Vargaftig & Zirinis, 1973; McIntyre & Philp, 1977). Experiments carried out on platelets *in vitro* and using models of the thrombotic process indicate that the platelet release reaction may be important in thrombogenesis (White & Heptinstall, 1978).

To gain further information on the relation between the release reaction and prostaglandin synthesis in platelets we have compared quantitatively the abilities of three anti-inflammatory agents, acetylsalicylic acid, flurbiprofen and indomethacin, to inhibit these platelet properties.

Methods

Preparation of platelet rich plasma

(a) *For release reaction.* Blood was collected by clean venepuncture from healthy human volunteers and aliquots (9.0 ml) were dispensed into siliconized glass centrifuge tubes that contained 3.8% (w/v) trisodium citrate dihydrate (1.0 ml) as anticoagulant. The platelets in the citrated whole blood were then labelled by adding a 25 μM solution of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT (specific activity 0.8 Ci/mmol) in 150 mM NaCl, 20 μl per ml of blood. Platelets rapidly accumulate 5-HT in freshly drawn blood; a control experiment showed that uptake was complete within 15 min at 20°C. Citrated platelet-rich plasma (PRCP) was prepared from this blood by centrifugation at 300 *g* for 10 min. It was then transferred to other siliconized-glass tubes and the platelet count was determined with a Coulter counter (Model F_N). Platelet-poor plasma (PPCP) was prepared by centrifuging the residual blood at 1200 *g* for 20 min and was used to dilute the PRCP to a platelet count of 300,000/μl. The PRCP was then used in experiments to determine the extent of the platelet release reaction, the [^{3}H]-5-HT serving as a marker for the release of intragranular material from the platelets.

(*b*) *For prostaglandin synthetase activity (PGSA).* Aliquots (9.6 ml) of blood were also collected into 100 mM disodium edetate (EDTA, 0.4 ml) as anticoagulant. In this case the platelets were not labelled; the blood was centrifuged immediately to obtain EDTA platelet-rich plasma (PREP), and the platelet count

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was adjusted to 300,000/μl, using autologous PPEP. The PREP was used to assess PGSA in the platelets.

During the above procedures and when samples of PRCP and PREP were subsequently incubated with the anti-inflammatory agents, the samples were kept under an atmosphere of 5% CO_2 in air. This stops the rise in pH that is sometimes observed when blood and plasma samples are handled.

Determination of the effect of anti-inflammatory agents on platelets in vitro

When the experiments were carried out wholly *in vitro*, appropriate aqueous solutions (40 μl/ml) of the agents were added to samples of PRCP or PREP so that final concentrations of 0, 10^{-7}, 10^{-6}, 3×10^{-6}, 10^{-5} and 10^{-4} M were achieved. The solutions of indomethacin were prepared with the aid of sodium hydroxide; the final pH of the solutions (up to pH 8) did not alter the pH of the PRCP or PREP. All the samples were incubated at 37°C for 30 min and the effect of the agents on the extent of the release reaction or on PGSA was then determined.

Determination of the effect of ingestion of anti-inflammatory agents on platelets

Volunteers ingested standard doses of the agents. The doses were either 300 mg acetylsalicylic acid (Aspirin), 50 mg flurbiprofen (Froben) or 25 mg indomethacin (Indocid). PRCP or PREP were prepared at various times after the drug had been taken and immediately tested for the extent of the platelet release reaction or PGSA. The effects of acetylsalicylic acid were determined for up to 14 days after the drug had been taken; the effects of flurbiprofen or indomethacin were determined 2 h, 24 h and 48 h after the drug had been taken.

Platelet release reaction

The ability of platelets in PRCP to undergo the release reaction was determined by stirring samples of the PRCP with adenosine diphosphate (ADP) and measuring the amount of [^{3}H]-5-HT that was released. The procedure was as follows. Six aliquots (480 μl) of each preparation were dispensed into glass test-tubes. Four of these samples were mechanically stirred at 37°C for 4 min, two controls being left on the bench. A solution of ADP in 150 mM NaCl (20 μl) was then added to the four stirred samples such that the final concentration of ADP in each was 10 μM, and these samples were then stirred for a further 6 min. Saline (20 μl) was added to the controls. To stop the release reaction and to avoid any further release during the subsequent handling of the samples a solution of 14 mM acetylsalicylic acid in 150 mM NaCl (50 μl) was added to all the samples and the tubes were then placed in ice. As the acetylsalicylic acid was added at the end of the experiment it had no effect on any release that had occurred while the samples were being stirred with ADP and did not interfere with any effect of the anti-inflammatory agent under test. When the tubes were cold the platelets were separated from their supernatant plasma by centrifugation at 2500 *g* for 7 min. Samples (50 μl) of the supernatant plasmas from test and control tubes, as well as samples of untreated PRCP, were then analysed for [^{3}H]-5-HT. They were transferred to vials that contained a mixture (10 ml) of 0.5% (w/v) PPO and 10% (w/v) BBS3 solubilizer in toluene and counted in a Beckman scintillation counter. After determining the mean amount of [^{3}H]-5-HT that had been released from the platelets by ADP (by subtracting the level of [^{3}H]-5-HT in the supernatants of the control tubes from the level in the supernatants of the tubes to which ADP had been added), and after determining the amount of [^{3}H]-5-HT that had been accumulated by the platelets (by subtracting the level of [^{3}H]-5-HT in the supernatants of the control tubes from the level in untreated PRCP), the amount of [^{3}H]-5-HT that had been released was expressed as a percentage of that which had been accumulated.

Prostaglandin synthetase activity (PGSA)

PGSA was determined by the technique described by Cockbill & Heptinstall (1978) in which 0.5 mM arachidonic acid is used as substrate and the amount of malondialdehyde (MDA) that is produced by platelets is measured. Briefly, two samples (1 ml) of PREP were transferred to tubes containing phosphate-buffered saline (pH 7.4, 4 ml) in which the arachidonic acid had just been dispersed. Prostaglandin synthesis was then allowed to proceed for 1 h at 37°C. The reaction was terminated by adding perchloric acid. Autologous platelet-poor plasma (PPEP) was always treated in the same way to allow for any auto-oxidation of arachidonic acid. After the precipitated protein had been removed from the samples by centrifugation, the MDA was complexed to thiobarbituric acid, extracted into acid butanol, and quantitated spectrophotometrically at 534 nm. The PGSA was expressed as total MDA produced by 10^9 platelets.

Agents and materials

Acetylsalicylic acid and indomethacin were purchased from the Sigma Chemical Co; flurbiprofen was a gift from the Boots Co. Ltd. These pure materials were used for the *in vitro* studies. When the agents were to be ingested the proprietary preparations were Aspirin, Froben and Indocid. Some of the other

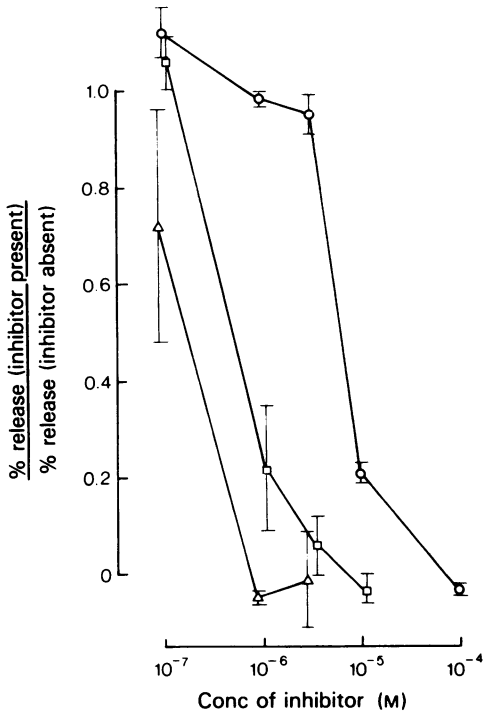

Figure 1 Inhibition of the release reaction by acetylsalicylic acid (○), flurbiprofen, (□) and indomethacin (△) *in vitro*. The results for acetylsalicylic acid and flurbiprofen are the mean of 2 determinations, vertical lines show range of results; those for indomethacin are the mean of 3 determinations; vertical lines show s.e. mean. The percentages of [^{3}H]-5-hydroxytryptamine released in the control experiments were 38% and 53% (for acetylsalicylic acid), 19% and 34% (for flurbiprofen) and 30%, 48% and 50% (for indomethacin).

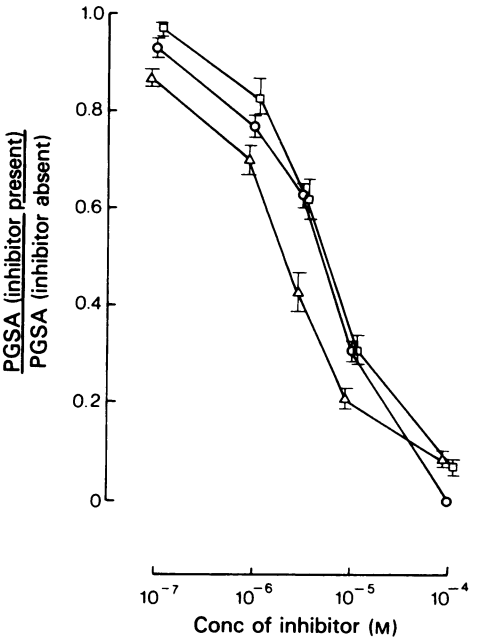

Figure 2 Inhibition of prostaglandin synthetase activity (PGSA) by acetylsalicylic acid (○), flurbiprofen (□) and indomethacin (△) *in vitro*. The results for all three agents are the mean of four determinations; vertical lines show s.e. mean.

materials used were: [^{3}H]-5-hydroxytryptamine binoxalate (28 Ci/mol) from New England Nuclear; 5-hydroxytryptamine (creatinine sulphate complex), arachidonic acid (grade 1) and adenosine diphosphate (sodium salt, grade 1) from Sigma; BBS3 solubilizer from Beckman.

Results

The effects of the anti-inflammatory agents on the platelet release reaction in vitro

The effects on the platelet release reaction of incubating platelets with each of the three agents are shown in Figure 1, where the ratio of the percentage of [^{3}H]-5-HT released in the presence of inhibitor to the percentage of [^{3}H]-5-HT released in the absence of inhibitor is plotted against inhibitor concentration. The results show that the release reaction was inhibited by 50% when either 6×10^{-6} M acetylsalicylic acid, 4×10^{-7} M flurbiprofen or 2×10^{-7} M indomethacin was present.

The effects of anti-inflammatory agents on prostaglandin synthetase activity in vitro

The effects on PGSA of incubating platelets with each of the three agents are shown in Figure 2, where the ratio of PGSA in the presence of inhibitor to PGSA in the absence of inhibitor is plotted against inhibitor concentration. The results show that PGSA was inhibited by 50% when the platelets had been incubated with either 5×10^{-6} M acetylsalicylic acid, 5×10^{-6} M flurbiprofen or 2×10^{-6} M indomethacin.

The effects of ingestion of the anti-inflammatory agents on the platelet release reaction

Volunteers ingested either Aspirin (300 mg), Froben (50 mg) or Indocid (25 mg). The extent of the platelet release reaction was then determined at various times after the drug had been taken (Figure 3). The release reaction was abolished 2 h after taking Froben or Indocid, was reduced 24 h after the agent had been taken and returned to normal after 48 h. In contrast,

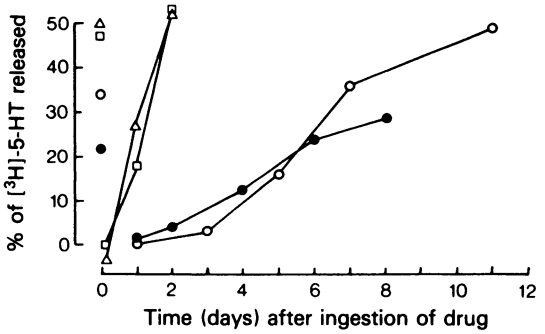

Figure 3 The extent of the release reaction in platelet-rich plasma from 4 individuals before and after they had ingested acetylsalicylic acid (○ and ●), flurbiprofen, (□) or indomethacin (△).

the effect of Aspirin was apparent until about 8 days after the drug had been ingested.

The effects of ingestion of the anti-inflammatory agents on prostaglandin synthetase activity

These experiments were essentially the same as those carried out to determine the effect of ingested agents on the release reaction. The PGSA levels that were obtained for each volunteer are shown in Figure 4. It would appear that PGSA was reduced 2 h after taking Froben or Indocid but that it returned to normal after 24 h. In contrast, 2 h after taking Aspirin, PGSA was abolished and the activity only slowly returned to normal over a period of about 10 days.

Discussion

It has been known for some time that acetylsalicylic acid and indomethacin can inhibit the platelet release reaction (e.g. O'Brien 1968a, b; O'Brien, Finch & Clark, 1970; Weiss, Aledort & Kochwa, 1968; Zucker & Peterson 1968; 1970) and prostaglandin synthesis in platelets (e.g. Smith & Willis, 1971; Vargaftig & Zirinis, 1973), and it is now known that flurbiprofen also inhibits the release reaction (Nishizawa, Wynalda, Suydam & Molony, 1973; Davies, Lederer, Spencer & McNicol, 1974; Sim, McCraw & Sim, 1975; Yasanuga & Ryo 1975). Flurbiprofen is a potent inhibitor of prostaglandin synthesis in other tissues (eg. Crook & Collins, 1975). In this investigation we have compared quantitatively the abilities of these three agents to inhibit the release reaction and platelet PGSA.

To determine the extent of the release reaction before and after platelets had been exposed to anti-inflammatory agents, we chose to stir platelets that had been labelled with [^{3}H]-5-HT, with ADP. The experiments were carried out with suspensions of platelets in plasma that contained citrate as anti-coagulant and labelled platelets were used to facilitate the detection of the released materials. ADP was used as the stimulatory agent because the release reaction induced by this agent appears to be uncomplicated by release of materials via prostaglandin-independent mechanisms (Charo, Feinman & Detwiler 1977). The experiments were carried out in the presence of citrate because this anti-coagulant stimulates the release reaction (Heptinstall & Mulley, 1977; Heptinstall & Taylor, 1978) and thus allows larger differences to be observed between control and inhibited platelet preparations.

To determine prostaglandin synthetase activity (PGSA) we measured the malondialdehyde that is produced when platelets are incubated with arachidonic acid. Malondialdehyde is produced along with prostaglandins as they are synthesized and its measurement has been successfully used to determine

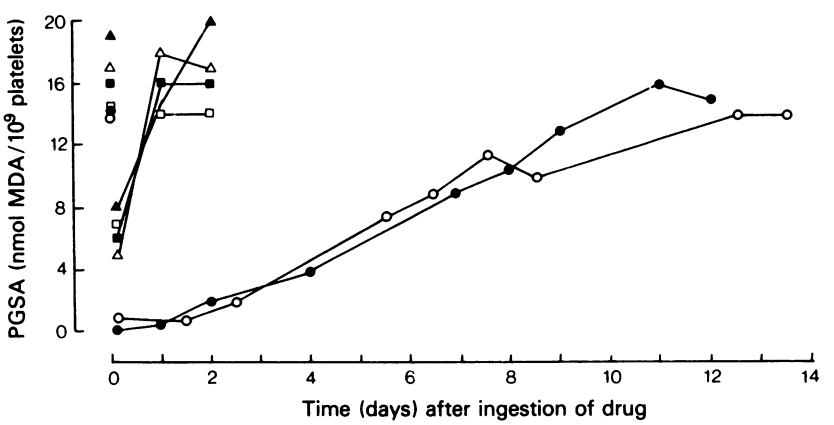

Figure 4 Prostaglandin synthetase activity (PGSA) in platelet-rich plasma from 6 individuals before and after they had ingested acetylsalicylic acid (○ and ●), flurbiprofen (□ and ■) or indomethacin (△ and ▲).

PGSA by other investigators (Flower, Cheung & Cushman, 1973; Stuart, Murphy & Oski, 1975; Smith, Ingerman & Silver, 1976).

The results of experiments carried out wholly *in vitro* (Figures 1 and 2) show that all three agents inhibit both the release reaction and PGSA in a concentration-dependent manner. For the release reaction, indomethacin was more effective than flurbiprofen which, in turn, was more effective than acetylsalicylic acid. For PGSA indomethacin was only slightly more effective than flurbiprofen and acetylsalicylic acid. The concentration of acetylsalicylic acid that was required to effect 50% inhibition of PGSA (5×10^{-6} M) was very similar to the concentration required to effect 50% inhibition of the release reaction (6×10^{-6} M). Flurbiprofen and indomethacin were respectively, 12 times and 10 times less potent as inhibitors of PGSA compared with the release reaction.

The results of experiments carried out on platelets withdrawn from volunteers at various times after standard doses of acetylsalicylic acid, flurbiprofen or indomethacin had been ingested are shown in Figures 3 and 4. Again all three drugs inhibited both the release reaction and PGSA, acetylsalicylic acid having an effect on the release reaction that paralleled its effect on PGSA, and flurbiprofen and indomethacin having more effect on the release reaction than on PGSA. A difference between acetylsalicylic acid and the other two agents was also evident in the length of time after the drug had been ingested that platelet activity was affected. The relatively long-lasting effect of acetylsalicylic acid compared with that of indomethacin has been observed before (O'Brien, 1968b, Kocsis, Hernandovich, Silver, Smith & Ingerman, 1973; Gordon, MacIntyre & McMillan 1976; Seuter, 1976; Crook & Collins 1977).

It is known that acetylsalicylic acid inhibits prostaglandin synthesis in platelets by irreversibly acetylating the cyclo-oxygenase responsible for converting arachidonic acid to the prostaglandin endoperoxide intermediates PGG_2 and PGH_2 (Roth, Stanford & Majerus, 1975; Roth & Majerus, 1975); thus the subsequent conversion of these products to thromboxane A_2 (TXA_2), to prostaglandins and to MDA does not occur. The irreversibility of the inhibition accounts for the long-lasting effect of acetylsalicylic acid on PGSA after it has been ingested; PGSA only returns to normal when a completely new generation of platelets has replaced those affected by the drug (Stuart *et al.*, 1975). PGG_2, PGH_2 and TXA_2 are themselves potent inducers of platelet aggregation and of the release reaction (Hamberg, Svensson, Wakabayashi & Samuelsson, 1974; Willis, Vane, Kuhn, Scott & Petrin, 1974; Hamberg, Svensson & Samuelsson, 1975). The parallel effects of acetylsalicylic acid on PGSA and on the release reaction could indicate that the formation of these materials is essential for the ADP-induced release reaction to take place.

Although it is not known how flurbiprofen or indomethacin inhibit the platelet release reaction it has always been assumed that, like acetylsalicylic acid, they act via inhibition of the cyclo-oxygenase involved in prostaglandin synthesis. However, in the present experiments both flurbiprofen and indomethacin appeared to inhibit the release reaction more effectively than they inhibited prostaglandin synthesis. The effect of indomethacin on cyclo-oxygenase, unlike that of acetylsalicylic acid, may be reversible (Stanford, Roth, Shen & Majerus, 1977) and this could account for the different effects of indomethacin on PGSA and on the release reaction in our test systems. However, another possibility is that flurbiprofen and indomethacin both inhibit the release reaction by a mechanism that is independent of the cyclo-oxygenase. Recently Kaplan, Weiss & Elsbach (1978) have shown that low concentrations of indomethacin inhibit phospholipase A_2 of rabbit polymorphonuclear leukocytes. Phospholipase A_2 is the enzyme responsible for liberating arachidonic acid from all membranes before it is converted to prostaglandins. Northover (1977) has shown that indomethacin can act as a calcium antagonist. Movement of calcium between intracellular platelet compartments is needed for the platelet release reaction to occur (Charo, Feinman, & Detwiler, 1976). It is suggested that the effects of flurbiprofen and indomethacin on mechanisms other than the conversion of arachidonic acid to prostaglandins need to be considered before it can finally be concluded that the effect of these drugs on the platelet release reaction is the same as that of acetylsalicylic acid.

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