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EFFECTS OF FIVE ANION CHANNEL BLOCKERS ON THROMBIN- AND IONOMYCIN-ACTIVATED PLATELET FUNCTIONS

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Abstract—The inhibitory effects of anion channel blockers were evaluated on aggregation, intracellular Ca^{2+} rises, and the production of arachidonic acid metabolites in human platelets. Inhibitors included five anion channel blockers: phloretin, probenecid, pyridoxal phosphate, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS) and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS). The degree of inhibition by each of these agents was dose-dependent on thrombin-activated platelet function. These agents generally had no significant inhibitory effects on ionomycin-activated platelet functions. It is suggested that anion mobilization plays a major role in the receptor-mediated activation of platelet functions, but only a minor role in Ca^{2+} ionophore-induced platelet activation. It is also suggested that several agents may have properties unrelated to anion channel blockers. Phloretin may be a selective cyclooxygenase inhibitor, and probenecid may inhibit phospholipase A_2. DIDS and SITS may interfere with certain aggregation-inducing mechanisms.

Upon activation by stimulators, platelets respond with various functional changes, including a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), production of arachidonic acid metabolites, release of intracellular granule constituents and aggregation. The role of cation mobilization, especially that of Na^+, has been well established by the works of Limbird *et al.* [1–3]. They demonstrated that elimination of Na^+ from the extracellular fluid suppresses platelet activation induced by several stimulators. They reported the same result for the case where amiloride and its analogues that inhibit Na^+ mobilization were present. However, little is known about the effects of permeant anions (Cl^-, OH^-) on the expression of platelet functions. The presence of anion channels in human platelets has been suggested by the finding that platelet membranes react with a monospecific anti-band 3 antibody that recognizes the anion carriers present on red blood cells [4]. In 1977, Pollard *et al.* demonstrated that several anion channel blockers inhibit the secretion of serotonin by thrombin-activated platelets [5]. Their findings suggested that anion mobilization plays an important role in platelet activation. However, the effects of anion channel blockers on other platelet functions have not thus far been evaluated.

In the present study, an attempt was made to define the effects of five anion channel blockers on platelet functions. These five anion channel blockers included phloretin, probenecid, pyridoxal phosphate, 4, 4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS), and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS). The effects of these agents have previously been studied most intensively in human red blood cell membranes where they were found to bind to the anion transport protein (band III) and block anion exchange [6–9]. The drugs' structures are diverse. However, they are generally anionic aromatic compounds impermeant to biological membranes. We evaluated the effects of each of these agents on four parameters of platelet function. The platelet functions evaluated included (1) $[Ca^{2+}]_i$ rises, (2) aggregation, (3) production of 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and (4) production of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). (3) and (4) are arachidonic acid metabolites produced by the pathways of cyclooxygenase and 12-lipoxygenase, respectively.

MATERIALS AND METHODS

Agents. Aequorin and ionomycin were obtained from Baxster-Travenol (Tokyo, Japan), and Calbiochem (CA, U.S.A.), respectively. Phloretin, probenecid, pyridoxal phosphate, DIDS and SITS were obtained from Sigma Chemical Co. (MO, U.S.A.). All other laboratory reagents and solvents were of analytical grade or better.

Preparation of platelets. Citrate anti-coagulated blood was obtained by venepuncture from healthy human donors. No donors had been on any medication for at least two weeks preceding the experiment. The blood was centrifuged at 60 *g* for 15 min to obtain platelet-rich plasma.

Aequorin-loading and measurement of $[Ca^{2+}]_i$. Aequorin loading was performed essentially as described by Johnson *et al.* [10]. Platelets in platelet-rich plasma were washed once with a modified

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Hepes–Tyrode's buffer. This buffer contains 129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 10 mM Hepes pH 7.2 and 5.5 mM glucose. Cells were resuspended and incubated for 1 hr at 0° in each of the following solutions:

Solution A: NaCl 150 mM, Hepes 5 mM, ATP 5 mM, $MgCl_2$ 2 mM, EGTA 10 mM, Aequorin 0.2 mg/ml, prostaglandin E_1 1 μM (PGE_1);

Solution B: NaCl 150 mM, Hepes 5 mM, ATP 5 mM, $MgCl_2$ 10 mM, EGTA 0.1 mM, PGE_1 1 μM.

At the end of the second incubation, platelets were separated from unloaded aequorin by Sepharose CL-2B gel filtration using Hepes–Tyrode's buffer as elutant. Aequorin-loaded platelets were suspended at a concentration of 2×10^5 cells/μl in Hepes–Tyrode's buffer with 100 μM of Ca^{2+}. One milliliter of this suspension was used to measure aggregation and aequorin luminescence in a Platelet Ionized Calcium Aggregometer (Chrono-Log, PA, U.S.A.). An appropriate concentration of each inhibitor was added to the platelet suspension and the mixture was incubated for 5 min at 37°. After 0.1 U/ml thrombin or 0.5 μM ionomycin had been added to the platelet suspension, aequorin luminescence was serially measured. Aequorin light signals were calibrated as described by Johnson *et al.* [10].

Aggregation. Platelet aggregation and aequorin luminescence were measured simultaneously using a Platelet Ionized Calcium Aggregometer. The maximum aggregation slope was determined as described by Ware *et al.* [11].

Analysis of arachidonic acid metabolism by high performance liquid chromatography (HPLC). The production of arachidonic acid metabolites and aequorin-detected $[Ca^{2+}]_i$ were measured simultaneously. Ten minutes after the addition of stimuli, the cell suspension was added to 4 times the volume of ethyl acetate. The mixture was acidified to pH 3.0 by 0.2 M HCl. Also, 450 ng prostaglandin B_2 was added as an internal standard. Extraction and evaporation were performed as described by Kanaji *et al* [12]. The evaporated residue was dissolved in 100 μl methanol and subjected to reversed-phase HPLC using a TSK-Gel ODS-80T_M (4.6 × 150 mm, Toyo Soda, Tokyo, Japan). The mobile phase consisted of methanol/water/acetic acid (75:25:0.01, v/v) at a flow rate of 1 ml/min. The column effluent was monitored at 270 nm for PGB_2. It was monitored at 235 nm for 12-hydroxy-heptadecatrienoic acid (HHT) and 12-hydroxy-eicosatetraenoic acid (12-HETE). HHT and 12-HETE were identified using chromatography on pure samples. The amounts of HHT or 12-HETE were determined by comparing their peak areas with that of the internal standard.

RESULTS

Platelet activation induced by thrombin and ionomycin

Thrombin, at a dose of 0.1 U/ml, elevated platelet $[Ca^{2+}]_i$ to 4.8 ± 1.5 μM, and induced aggregation with a maximal aggregation rate of 31.2 ± 4.3 (% optical density reduction/min). HHT and 12-HETE production rates induced by 0.1 U/ml thrombin were 42.1 ± 13.8 and 33.6 ± 10.3 (ng/2 × 10^8 cells), respectively.

Ionomycin, at a dose of 0.5 μM, elevated platelet $[Ca^{2+}]_i$ to 14.2 ± 2.3 μM, and induced aggregation with a maximal aggregation rate of 35.8 ± 5.4 (% optical density reduction/min). HHT and 12-HETE production rates induced by 0.5 μM ionomycin were 295 ± 57, and 418 ± 52 (ng/2 × 10^8 cells), respectively.

Arachidonic acid released by phospholipase A_2 is metabolized by platelets through either cyclooxygenase or 12-lipoxygenase pathway. The amount of HHT, a non-enzymatic product of prostaglandin G_2, is known to correlate well with the amount of thromboxane A_2. Thus, HHT has been shown to be a good marker of the cyclooxygenase activity [13]. 12-HETE, a final product of 12-lipoxygenase, serves as a marker of 12-lipoxygenase. Both HHT and 12-HETE were measured in the present study because they are ultraviolet-detectable and can be measured simultaneously using high performance liquid chromatography.

Effects of DIDS

DIDS, in a dose-dependent manner, inhibited four platelet functions induced by 0.1 U/ml thrombin (Fig. 1A). The ID_{50} values were approximately 50 μM for aggregation and $[Ca^{2+}]_i$ rises, and approximately 25 μM for HHT and 12-HETE production. DIDS had no inhibitory effect on HHT and 12-HETE production induced by 0.5 μM ionomycin (Fig. 1B). DIDS inhibited ionomycin-induced aggregation in a dose-dependent manner, but even at a concentration of 200 μM the inhibitory effect was not complete; 46 ± 6% inhibition at 200 μM of DIDS, while it completely inhibited thrombin-induced aggregation at a concentration of 200 μM. $[Ca^{2+}]_i$ rises induced by ionomycin were reduced slightly by DIDS at high concentrations.

Effects of SITS

SITS, an analogue of DIDS, also inhibited thrombin-induced production of HHT and 12-HETE in a dose-dependent manner, with an ID_{50} of approximately 50 μM. It was generally less potent than DIDS in inhibiting thrombin-induced platelet activation. SITS, at all concentrations tested, had no suppressive effect on ionomycin-induced platelet activation, except for aggregation which SITS, at a concentration of 400 μM, inhibited by 32 ± 3%.

Effects of phloretin

Phloretin effectively diminished all the thrombin-induced platelet functions (Fig. 2A). There was a large difference between the ID_{50} value for HHT production (approximately 3 μM) and that for 12-HETE production (25 μM). Note that the ID_{50} values for HHT production and 12-HETE production were essentially the same for DIDS and SITS.

In ionomycin-induced activation, phloretin inhibited HHT production with an ID_{50} value of approximately 9 μM, while 12-HETE production was enhanced (Fig. 2B). Phloretin had no effect on either ionomycin-induced aggregation or $[Ca^{2+}]_i$ rises.

Effects of probenecid

Probenecid inhibited thrombin-induced production of HHT and 12-HETE almost equally, with an

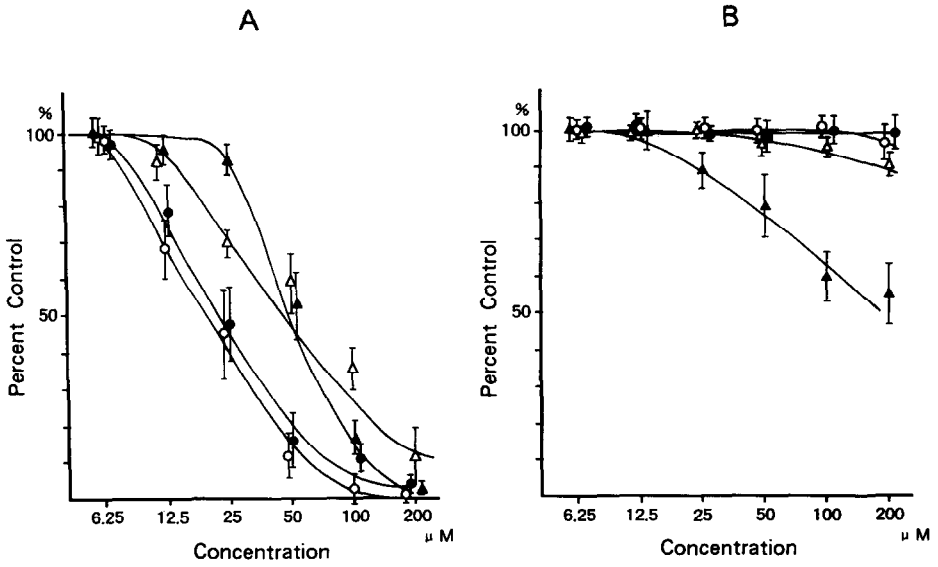

Fig. 1. Effect of DIDS on thrombin- and ionomycin-activated platelet functions. The indicated concentration of DIDS was added to the aequorin-loaded platelet suspension, and the mixture was incubated for 5 min at 37°. Then, 0.1 U/ml thrombin or 0.5 μM ionomycin was added to initiate the reaction. Maximum aggregation rate, $[Ca^{2+}]_i$ rises, HHT and 12-HETE production were determined, as described in Materials and Methods. (A) Thrombin activation; (B) ionomycin activation. Open circle, HHT; closed circle, 12-HETE; open triangle, $[Ca^{2+}]_i$; closed triangle, aggregation. The data presented are the means ± SE of four experiments.

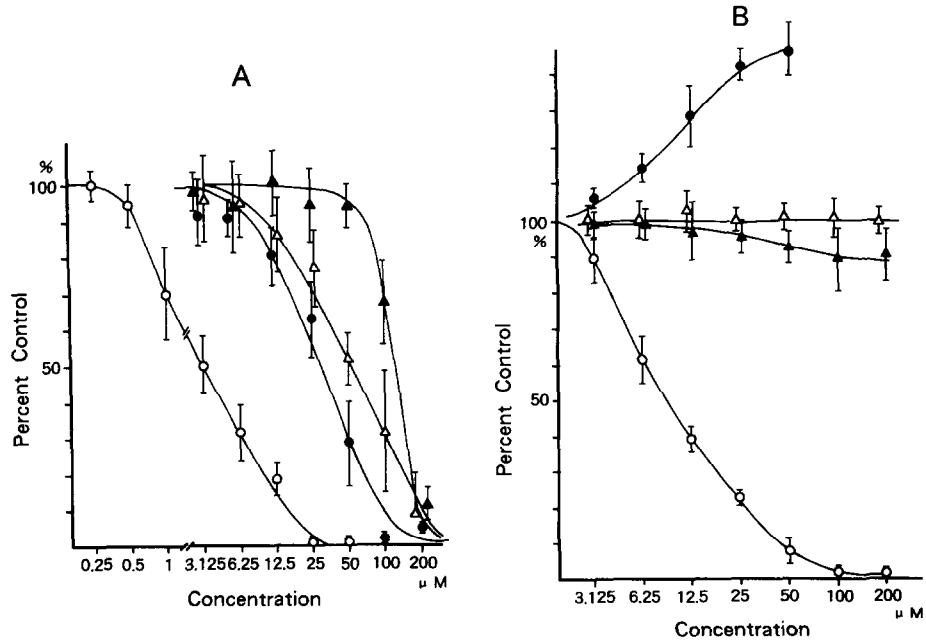

Fig. 2. Effect of phloretin on thrombin- and ionomycin-activated platelet functions. The indicated concentration of phloretin was added to the aequorin-loaded platelet suspension, and the mixture was incubated for 5 min at 37°. Then, 0.1 U/ml thrombin or 0.5 μM ionomycin was added to initiate the reaction. Maximum aggregation rate, $[Ca^{2+}]_i$ rises, HHT and 12-HETE production were determined, as described in Materials and Methods. (A) Thrombin activation; (B) ionomycin activation. Open circle, HHT; closed circle, 12-HETE; open triangle, $[Ca^{2+}]_i$; closed triangle, aggregation. The data presented are the means ± SE of four experiments.

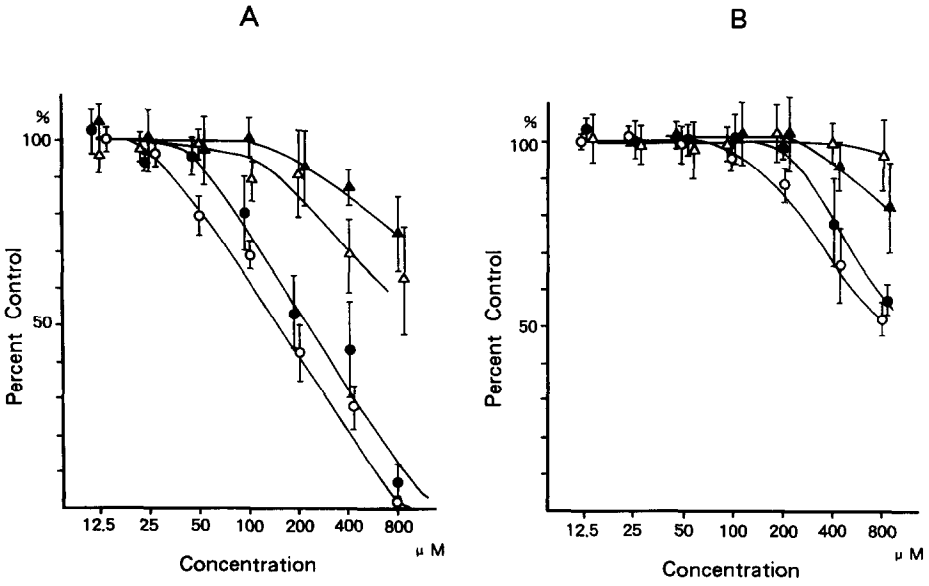

Fig. 3. Effect of probenecid on thrombin- and ionomycin-activated platelet functions. The indicated concentration of probenecid was added to the aequorin-loaded platelet suspension, and the mixture was incubated for 5 min at 37°. Then, 0.1 U/ml thrombin or 0.5 μM ionomycin was added to initiate the reaction. Maximum aggregation rate, $[Ca^{2+}]_i$ rises, HHT and 12-HETE production were determined, as described in Materials and Methods. (A) Thrombin activation; (B) ionomycin activation. Open circle, HHT; closed circle, 12-HETE; open triangle, $[Ca^{2+}]_i$; closed triangle, aggregation. The data presented are the means ± SE of four experiments.

ID_{50} value of 200 μM (Fig. 3A). The inhibitory effect of probenecid on thrombin-induced aggregation and $[Ca^{2+}]_i$ rises was not complete even at a concentration of 800 μM.

In ionomycin-induced activation, probenecid at 800 μM reduced HHT and 12-HETE production by 50% (Fig. 3B). It had no significant inhibitory effect on either ionomycin-induced aggregation or $[Ca^{2+}]_i$ rises.

Effects of pyridoxal phosphate

The effects of pyridoxal phosphate on thrombin-induced platelet activation were essentially the same as those of probenecid. Pyridoxal phosphate inhibited thrombin-induced production of HHT and 12-HETE almost equally, with an ID_{50} value of approximately 200 μM (Fig. 4A). Aggregation and $[Ca^{2+}]_i$ rises were also reduced at higher concentrations, but not completely even at 800 μM.

In contrast to probenecid which inhibited ionomycin-induced production of HHT and 12-HETE, pyridoxal phosphate had no significant inhibitory effect on ionomycin-induced platelet activation for all concentrations tested (Fig. 4B).

DISCUSSION

All the agents tested in the present study inhibited thrombin-induced platelet activation. Optimal concentrations for maximal inhibition were, however, diverse. The agents tested had no significant inhibitory effects on ionomycin-induced platelet activation apart from a few cases, suggesting that their common effect is due to inhibiting the receptor-mediated activation of platelets. Although the exact mechanism whereby these agents exert their inhibitory effects remains uncertain, it is most likely that their inhibitory effects on receptor-mediated platelet activation are related to anion mobilization. This would seem to be the case because the common denominator of these structurally dissimilar agents is the blockade of anion mobilization [6–9]. At the same time, our findings suggest that anion mobilization plays only a minor role, if any, in ionomycin-induced activation of platelet functions.

How does anion mobilization contribute to the receptor-mediated activation of platelets? In a wide variety of cells anion channels are known to play an important role in intracellular pH regulation [14, 15]. They participate either in recovery from alkali loading or in acid extrusion with subsequent intracellular alkalinization, depending upon circumstances. In the physiological range of pH, cellular responses are generally potentiated with higher pH values [16, 17]. The influx of hydroxyl ions through anion channels, which results in intracellular alkalinization, has been found to play a pivotal role in serotonin release, shape change, and protein phosphorylation in human platelets [5, 18]. Thus, it is most probable that thrombin-activated anion mobilization contributes to a rise in intracellular pH, at least in microenvironments where initial signal transduction mechanisms reside, triggering the potentiation of platelet activation. In line with this concept, we have demonstrated, with direct measurement of intracellular pH with a pH-sensitive fluorescent dye, that anion mobi-

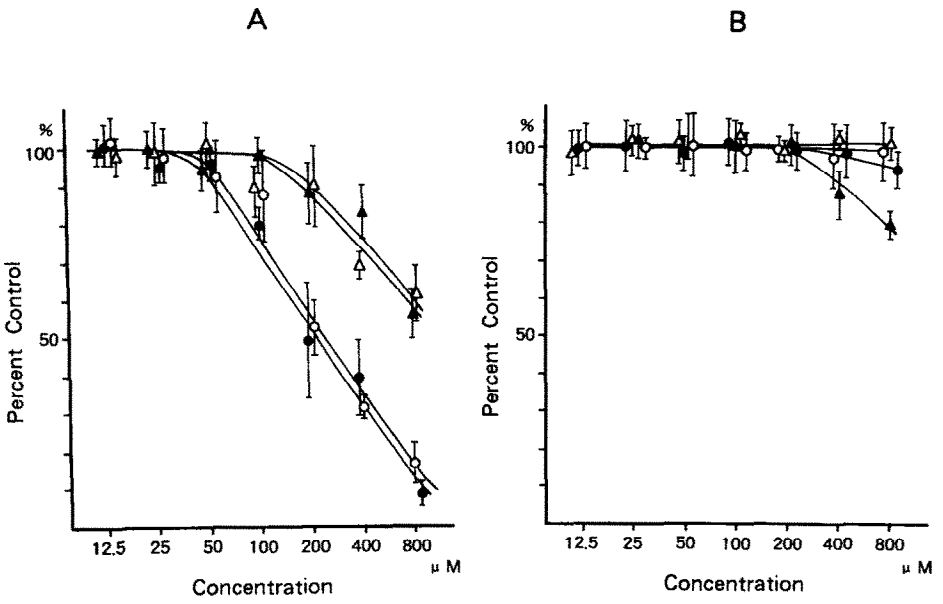

Fig. 4. Effect of pyridoxal phosphate on thrombin- and ionomycin-activated platelet functions. The indicated concentration of pyridoxal phosphate was added to the aequorin-loaded platelet suspension, and the mixture was incubated for 5 min at 37°. Then, 0.1 U/ml thrombin or 0.5 μM ionomycin was added to initiate the reaction. Maximum aggregation rate, $[Ca^{2+}]_i$ rises, HHT and 12-HETE production were determined, as described in Materials and Methods. (A) Thrombin activation; (B) ionomycin activation. Open circle, HHT; closed circle, 12-HETE; open triangle, $[Ca^{2+}]_i$; closed triangle, aggregation. The data presented are the means ± SE of four experiments.

lization does participate in thrombin-induced intracellular alkalinization in certain circumstances and that anion channel blockers inhibit the pH changes [19].

Since thrombin-induced $[Ca^{2+}]_i$ rises were reduced by the presence of these anion channel blockers, it would seem to be the case that one of the sites at which these agents act is proximal to the Ca^{2+}-releasing mechanism. On the other hand, these anion channel blockers inhibit the production of arachidonic acid metabolites with ID_{50} values approximately one fourth of those required for inhibition of $[Ca^{2+}]_i$ rises. Therefore, a mere reduction in $[Ca^{2+}]_i$ rises, which is one of the major factors regulating arachidonic acid release, cannot fully account for the potency of these agents in suppressing the production of arachidonic acid metabolites. With regards to this, it is noteworthy that phospholipase A_2 has a high optimum pH, falling between 8.5 and 10.5, depending upon the reports [20]. Taking all these findings into consideration, it would seem that these anion channel blockers exert additional inhibitory effects on phospholipase A_2 by suppressing intracellular alkalinization.

We have also found several differences between the effects of the various anion channel blockers tested which cannot be accounted for simply on the basis of differences in potency. The platelet functions affected appear to differ from agent to agent. For example, probenecid inhibited ionomycin-induced HHT and 12-HETE production with an ID_{50} of 800 μM, whereas DIDS, SITS and pyridoxal phosphate each had virtually no inhibitory effect on ionomycin-induced production of arachidonic acid metabolites. Since ionomycin-induced $[Ca^{2+}]_i$ rises appear to directly activate phospholipase A_2, and the products of the cyclooxygenase and 12-lipoxygenase pathways were equally reduced in amount, it is most likely that probenecid possesses an antiphospholipase A_2 activity. Also, for thrombin-induced HHT production, phloretin had an ID_{50} (3 μM) far lower than it had for 12-HETE production (approximately 25 μM), whereas other anion channel blockers had almost the same ID_{50} values for both thrombin-induced HHT and 12-HETE production. Moreover, phloretin, while reducing ionomycin-induced production of HHT, inversely increased 12-HETE production. This phenomenon is generally observed with selective cyclooxygenase inhibitors such as aspirin, which, by suppressing the flow of arachidonic acid through the cyclooxygenase pathway, supply more substrate for the 12-lipoxygenase pathway. Thus, it is suggested that phloretin, in addition to being an anion channel blocker, works as a selective cyclooxygenase inhibitor. Finally, DIDS inhibited ionomycin-induced aggregation with an ID_{50} of approximately 200 μM. In contrast, it had no significant effect on $[Ca^{2+}]_i$ rises, HHT production or 12-HETE production. It may be that DIDS interferes with a certain mechanism involved in platelet aggregation, distal to $[Ca^{2+}]_i$ rises and arachidonic acid metabolism.

In the present study, it was found that the common effect of five anion channel blockers on platelet function was the inhibition of thrombin-induced platelet activation. It is suggested that anion mobilization plays an important role in the receptor-mediated platelet activation. It was also found that

probenecid, phloretin and DIDS had certain properties in addition to the blocking of anion channels. All results should be carefully evaluated in the light of these properties.

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