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ACCELERATED COMMUNICATION

14,15-*cis*-Episulfide-eicosatrienoic Acid, an 'Epoxygenase' Eicosanoid Analog, Inhibits Ionophore- But Not Thrombin-Induced Platelet Aggregation

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SUMMARY

An 'epoxygenase' eicosanoid analog, 14,15-*cis*-episulfide-eicosatrienoic acid, has several unique pharmacological effects on platelets. These include (i) inhibition of ionophore A23187- but not thrombin-induced activation, (ii) inhibition of thromboxane B_2 biosynthesis derived from endogenous but not exogenous arachidonic acid, and (iii) attenuation of ionophore-mediated increases in cytosolic Ca^{2+} when extracellular or membrane Ca^{2+} is available but not when these pools are excluded. Neither elevation of cyclic AMP levels, a potent inhibitory process, nor direct antagonism of the prostaglandin H_2/thromboxane A_2 receptor is responsible for the actions of 14,15-*cis*-episulfide-eicosatrienoic acid. These properties distinguish 14,15-*cis*-episulfide-eicosatrienoic acid from other antiaggregatory substances.

Lipids derived from cyclooxygenase- or lipoxygenase-catalyzed oxidation of arachidonic acid have prominent effects on platelets and the cell biology of thrombosis (1, 2). Recently, a new group of eicosanoids, the EETs, termed "epoxygenase" metabolites, have been described (3–5). The EETs influence stimulus-response coupling in several cell types (6); however, little is known about their effects on platelets. Previously, we reported that certain 14,15-EET isomers inhibited cyclooxygenase activity and, using platelets as a cellular model, we found that they also inhibited arachidonic acid-induced aggregation. Contrary to expectations, this antiaggregatory effect was not due to inhibition of cyclooxygenase (7). Paradigms for lipid mediator action based on precedents with other eicosanoids may be inappropriate for the EETs. For instance, they do not act via receptor-mediated processes (8, 9) and they occur as endogenous constituents of cells, including platelets and endothelial cells (10–12). Our initial results and these unusual traits prompted further pharmacological investigations using a stable heteroatom-containing analog, 14,15-*cis*-episulfide-ET. We report that it has a unique effect on calcium ionophore- and thrombin-induced aggregation, inhibiting the former much more than the latter. This distinguishes it from other antiaggregatory substances.

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Experimental Procedures

Materials. Arachidonic acid (NuChek Prep); PGE_1, TxB_2, U46619 (9,11-dideoxy-9,11-methanoepoxy-prostaglandin $F_{2\alpha}$, and BM 13,177 [4-[2-(benzenesulfonamido-*o*-ethyl)-phenoxyacetic acid]] (The Upjohn Co.); calcium ionophore A23187 or its nonfluorescent analog 4-bromo-A23187 (Calbiochem); indo-1 acetoxymethyl ester (Molecular Bioprobes); IBMX, human thrombin, collagen, and LDH assay kits (Sigma Chemical); and 2′,5′-ddA (PL Biochemicals) and cyclic AMP assay kits (New England Nuclear) were used. L655,240 [3-[1-(4-chlorobenzyl-5-fluoro-3-methyl-indol-2-yl]-2,2-dimethylpropanoic acid], a TxA_2 antagonist, was supplied by Dr. Cecil Pickett (Merck-Frosst, Canada). EETs, including 14,15-*cis*-EET, 14,15-*trans*-EET, 14,15-*cis*-episulfide-ET, and 14,15-*cis*-aza-ET, were synthesized as described (13, 14).

Isolation of human platelets. Human blood (9 volumes) collected in 3.8% (w/v) trisodium citrate (1 volume) was centrifuged at 200 × *g* for 20 min, and the PRP was isolated. Platelets (3–5 × 10^8 platelets/ml) were washed in citrate buffer, pH 6.5, and resuspended in Hanks' balanced salt solution, 0.02 M HEPES buffer, pH 7.2, containing 1.5 mM Ca^{2+}, 0.8 mM Mg^{2+}, and 1 mg/ml albumin (15). In certain cases, PRP was incubated with 0.5 mM aspirin for 10 min to inhibit the

ABBREVIATIONS: EET, epoxyeicosatrienoic acid; ET eicosatrienoic acid; PGE_1, prostaglandin E_1; TxB_2, thromboxane B_2; IBMX, isobutylmethylxanthine; 2′,5′-ddA, 2′,5′-dideoxyadenosine; PRP, platelet-rich plasma; LDH, lactate dehydrogenase; TxA_2, thromboxane A_2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PGH_2, prostaglandin H_2.

platelet cyclooxygenase enzyme. To label platelet dense granules for quantitation of the release reaction, PRP was incubated with [^{14}C] serotonin (16).

Platelet aggregation. Platelet suspensions (1.0 ml) were incubated for 2 min at 37° with 0–100 nmol of 14,15-*cis*-episulfide-ET or related compounds. Platelets were stimulated with arachidonic acid (3 μM final concentration), the TxA_2 mimetic U46619 (1 μM), thrombin (0.02–0.1 units/ml), collagen (5 μg/ml), or the ionophore A23187 (0.5–2.5 μM). The agonists were added in 10 μl of aqueous buffer containing 1 mg of albumin/ml and negligible amounts (<0.02%) of organic solvents. Aggregation was monitored photometrically. The aggregation rate refers to the change in light transmission versus time for the linearly increasing portion of the aggregation curve. The maximal response refers to the percentage of transmittance after platelets were fully aggregated, typically at 2 min after addition of agonist. TxB_2 was determined by immunoassay (7). Cyclic AMP levels were determined by incubating platelet suspensions (1.5 ml) for 0.5 or 2 min at 37° with 14,15-*cis*-episulfide-ET and then quenching at 4° with EGTA at a final concentration of 5 mM. After centrifugation for 1 min at 14,000 × *g*, platelets were suspended in 1 ml of ethanol and lysed by sonication. The supernatant fluid was isolated and evaporated, and the residue was reconstituted in 0.1 ml of water. Cyclic AMP was determined by immunoassay. In certain experiments, 0.2 mM IBMX, a phosphodiesterase inhibitor, was used to prevent metabolism of cyclic AMP.

Determination of platelet cytosolic Ca^{2+} levels. Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) were determined by fluorometry (17), using an Aminco-Bowman instrument with a thermostatted cuvet chamber. Platelets (2×10^8 cells/ml) in Ca^{2+}/Mg^{2+}-free Tyrode's buffer containing 2 μM PGE_1 and 0.5% bovine albumin were incubated at 25° for 45 min to incorporate 2 μM indo-1 acetoxymethyl ester. Portions (0.50 ml) of these platelets were centrifuged for 10 sec at 10,000 × *g*, and the supernatant fluid was discarded. The residual fluid was removed by washing with Tyrode's buffer, without disruption of the platelet pellet. Finally, the platelets were resuspended in Tyrode's buffer. Suspensions (0.5 ml) of platelets plus 14,15-*cis*-episulfide-ET (0–48 μM) were transferred to a quartz cuvet and incubated for 1 min at 37° in a fluorometer. Fluorescence was monitored during this incubation and after the addition of agonist. The emission wavelength was 410 nm; the excitation wavelength was 331 nm, minimizing any contribution from unhydrolyzed indo-1 (18). Maximum fluorescence was measured by addition of 0.1% Triton X-100 detergent and 0.01 M $CaCl_2$. Minimum fluorescence was determined by addition of 6 mM $MnCl_2$. Experiments were performed with buffers containing various amounts of Ca^{2+}, Mg^{2+}, and EGTA, as specified in Results. $[Ca^{2+}]_i$ values, estimated as described (18, 19), represent the mean ± standard error of three or more experiments. The K_d for indo-1 was 250 nM.

Reversibility of effects of 14,15-*cis*-episulfide ET. Five milliliters of control platelets or platelet suspensions with 15 μM 14,15-*cis*-episulfide-ET were incubated for 2 min at 37°. An aliquot (1.0 ml) from each was stimulated with U46619 (1 μM) to establish the initial aggregation responses. The remaining portions were mixed with 20 ml of citrate washing buffer, pH 6.5, containing 5 mg/ml of bovine albumin, to sequester lipids (20). After centrifugation at 400 × *g* for 5 min, the platelets were resuspended in Hanks' balanced salt solution and an aliquot (1.0 ml) was stimulated again with U46619 (1 μM), to determine whether the effect of the episulfide analog could be reversed by washing.

Effect of 2′,5′-ddA on inhibition of platelet aggregation. 2′,5′-ddA inhibits adenylate cyclase and reverses the antiaggregatory effects of cyclic AMP on platelets (21). Platelets with or without 80 μM 2′,5′-ddA were incubated at 37° for 2 min; then, 3 μM PGE_1 or 12–30 μM 14,15-*cis*-episulfide-ET was added for 1 min before stimulation with 0.5 μM A23187. Aggregation and cyclic AMP levels were monitored for reversal by 2′,5′-ddA.

Statistics. Statistical comparisons were based on paired *t* tests or, for multiple comparisons, analysis of variance with Bertoli's or Dunnet's *post hoc* test.

Results

Identification of 14,15-*cis*-episulfide-ET as an inhibitor of platelet activation. We examined EET analogs to identify one that inhibited platelet aggregation maximally with no corresponding inhibition of cyclooxygenase activity. An analog with sulfur replacing the oxygen of the oxirane ring, 14,15-*cis*-episulfide-ET, was optimal (Table 1). The concentration required for half-maximal inhibition of aggregation induced by arachidonic acid (IC_{50}, mean ± standard error, more than three experiments), 6.0 ± 1.8 μM, was comparable to 3.9 ± 1.0 μM, the IC_{50} for 14,15-*cis*-EET, the most potent of the naturally occurring EETs, under these conditions. Unlike 14,15-*cis*-EET, its episulfide analog did not inhibit cyclooxygenase activity. Incubation of arachidonic acid with platelets produced 167 ± 20, 162 ± 13, 163 ± 16, and 135 ± 5 ng of TxB_2/ml in the presence of 0, 10, 30, and 100 μM 14,15-*cis*-episulfide-ET, respectively. These values (mean ± standard error, three or more experiments) were indistinguishable by analysis of variance ($p > 0.8$). 14,15-*cis*-Episulfide-ET also inhibited U46619-induced aggregation (IC_{50} = 3.4 ± 1.1); thus, it blocks aggregation induced by natural or synthetic agonists for the PGH_2/TxA_2 receptor, independent of an effect on cyclooxygenase.

Effects on collagen-stimulated platelets. 14,15-*cis*-Episulfide-ET inhibited collagen-induced aggregation with an IC_{50} of 13.0 ± 3.0 μM, and it inhibited TxB_2 formation derived from collagen-mediated release of endogenous arachidonic acid (Fig. 1). This contrasts with its effect on TxB_2 formation derived from exogenous arachidonic acid. Collagen-dependent aggregation was fully inhibited in the presence of appreciable TxB_2. The effects of 14,15-*cis*-episulfide-ET were distinct from those of cyclooxygenase inhibitors or PGH_2/TxA_2 receptor antagonists. With 0.5 mM aspirin, a cyclooxygenase inhibitor, the two responses (aggregation and TxB_2 formation) correlated closely. With BM 13,177 and L655,240, two structurally distinct PGH_2/TxA_2 receptor antagonists, the two responses were completely dissociated. For instance, L655,240 inhibited the rate and magnitude of aggregation by 90%; however, it did not inhibit TxB_2 formation. The TxB_2 concentrations were 79 ± 9, 93 ± 10, and 109 ± 4 ng/ml (mean ± standard error, three experiments) in the presence of 0.3, 1, and 5 μM L655,240, respectively. These values were indistinguishable by analysis of variance ($p > 0.8$) from the control value of 89 ± 9 ng/ml.

TABLE 1

Effect of 14,15-EET analogs on aggregation and TxB_2 formation by platelets stimulated with arachidonic acid or U46619, a TxA_2 mimetic

Platelets incubated for 2 min at 37° with 0–100 μM levels of 14,15-EET analogs were transferred to a cuvet containing arachidonic acid (3 μM) to initiate aggregation. The initial rate and maximal response were measured to determine the extent of inhibition. TxB_2 formation, 3 min after addition of arachidonic acid, was determined by immunoassay. Similar experiments were performed using 1 μM U 46619, an agonist for the PGH_2/TxA_2 receptor. Values represent the mean ± standard error of three or more experiments.

Compound	Arachidonic acid		U 46619 Aggregation, IC_{50}
	Aggregation, IC_{50}	TxB_2 formation, IC_{50}	
	μM	μM	μM
14,15-*cis*-EET	3.9 ± 1.0	12 ± 3	6.7 ± 2.7
14,15-*cis*-episulfide ET	6.0 ± 1.8	>100	3.4 ± 1.1
14,15-*trans*-EET	8.5 ± 1.3	>100	19.8 ± 10.5
14,15-*cis*-aza-ET	55.0 ± 5.0	>100	15

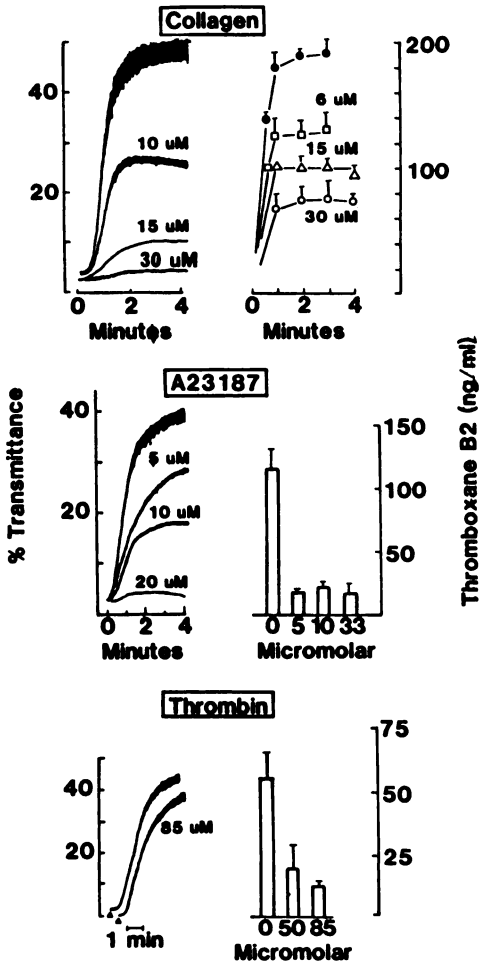

Fig. 1. Effect of 14,15-*cis*-episulfide-ET on aggregation and TxB_2 formation by platelets stimulated with collagen (*upper*), A23187 (*middle*), and thrombin (*lower*). Aggregation *traces* are from a single representative experiment. Collagen (5 μg), A23187 (1 nmol), or thrombin (0.1 units) in 20 μl of Hanks' buffered salt solution with 1 mg of bovine serum albumin/ml was added to 5×10^8 platelets/ml. TxB_2 concentrations (mean ± standard error of more than four experiments) typify the effect of 14,15-*cis*-episulfide-ET as a function of time, for collagen. Data for A23187 and thrombin reflect the results 2 min after stimulation.

Effects on ionophore A23187-stimulated platelets. We examined the effects of 14,15-*cis*-episulfide-ET using agonists that liberate endogenous arachidonic acid but do not require its metabolism for induction of aggregation. Similar to its effect with collagen, the episulfide analog inhibited A23187-induced aggregation, with an IC_{50} of 7.2 ± 2.6 μM. The decline in TxB_2 formation did not parallel the decline in aggregation (Fig. 1). For instance, TxB_2 levels were inhibited, but statistically indistinguishable, in the presence of 5 and 33 μM 14,15-*cis*-episulfide-ET; aggregation was inhibited >95% in the latter case and <25% in the former. With A23187 as an agonist, the effects of 14,15-*cis*-episulfide-ET were readily distinguishable from those of cyclooxygenase inhibitors or PGH_2/TxA_2 receptor antagonists. Aspirin (0.5 mM) reduced TxB_2 formation by >95%; TxB_2 levels declined from 127 ± 9 ng/ml to 6 ± 2 ng/ml. In contrast to the episulfide analog, aspirin did not inhibit the rate or magnitude of A23187-induced aggregation. BM 13,177 and L655,240 did not inhibit either aggregation or TxB_2 formation. Furthermore, 14,15-*cis*-episulfide-ET was effective at inhibiting aspirin-treated platelets stimulated with ionophore. Such platelets do not generate any PGH_2 or TxA_2, excluding an effect on constitutive eicosanoid biosynthesis as a primary mechanism for the antiaggregatory effect (22).

Effects on thrombin-stimulated platelets. Agents rarely exert differential effects on ionophore- and thrombin-induced aggregation. However, even high concentrations of 14,15-*cis*-episulfide-ET did not alter the rate or magnitude of aggregation induced by 0.1 units of thrombin/ml (Fig. 1, *bottom*). In two of 10 experiments, the aggregation responses declined slightly (<10%). Thrombin-mediated TxB_2 formation declined in the presence of 50 and 85 μM 14,15-*cis*-episulfide-ET. TxB_2 concentrations were 24 ± 12 and 9 ± 2 ng/ml, respectively. The latter, only, differed ($p < 0.05$) from the control value of 58 ± 12 ng/ml.

With 0.05 or 0.02 units of thrombin/ml and 85 μM 14,15-*cis*-episulfide-ET, the initial rate of aggregation declined to 68% and 35% of the control value, respectively. At concentrations of <50 μM, 14,15-*cis*-episulfide-ET did not inhibit the response to 0.05 or 0.02 units of thrombin/ml (Table 2). This concentration is supramaximal for inhibition of aggregation by other agonists.

The level of TxB_2 formation stimulated by thrombin was about one half that caused by ionophore. However, the amount of thromboxane produced by an agonist is not necessarily directly related to its contribution to aggregation. Therefore, the fact that ionophore produces more TxB_2 than does 0.1 unit/ml thrombin does not imply that ionophore is more dependent on thromboxane formation (22). We used low doses of thrombin to enhance the detection of inhibition. Table 3 summarizes the IC_{50} values.

Thrombin-mediated degranulation was unaltered by 14,15-*cis*-episulfide-ET. Control platelets released 33.8 ± 7.5% of their total serotonin; platelets treated with 40 μM 14,15-*cis*-

TABLE 2

Effect of 14,15-*cis*-episulfide-ET on aggregation induced by variable doses of thrombin

Platelet suspensions were incubated with 14,15-*cis*-episulfide-ET, and aggregation was initiated by addition of 0.02–0.1 units/ml thrombin. The aggregation response refers to the change in light transmission versus time for the linearly increasing portion of the aggregation curve. Similar results were obtained by measurement of the magnitude of light transmission at 3 min after addition of agonist. The largest concentration of 14,15-*cis*-episulfide-ET, 85 μM, produced incomplete inhibition with the lowest dose of thrombin. Effects were insignificant for 14,15-*cis*-episulfide-ET concentrations of ≤50 μM.

Thrombin	Aggregation response			
	0[a]	33 μM[a]	50 μM[a]	85 μM[a]
units/ml	*% of control*			
0.1	100		100	100
0.05	100	96	99	68
0.02	100	100	85	35

[a] 14,15-*cis*-Episulfide-ET concentration.

TABLE 3

IC_{50} values for inhibition of platelet aggregation by 14,15-*cis*-episulfide-ET

Agonist	IC_{50}
	μM
Arachidonic acid (3 μM)	6.0 ± 1.8
U46619 (1 μM)	3.4 ± 1.1
Collagen (5 μg/ml)	13.0 ± 3.0
A23187 (2 μM)	7.2 ± 2.6
Thrombin (0.1 units/ml)	>85
Thrombin (0.05 units/ml)	>85
Thrombin (0.02 units/ml)	>50

episulfide-ET released 26.2 ± 6.5% of their serotonin. These values (mean ± standard error, three experiments) were statistically indistinguishable ($p > 0.3$).

Cyclic AMP measurements. The data argue against elevation of cyclic AMP as a mechanism of action, because agents that stimulate adenylate cyclase inhibit aggregation induced by thrombin or ionophore. Platelets incubated for 0.5 min with 30 μM episulfide-ET analog contained 8.1 ± 0.6 pmol of cyclic AMP/10^9 platelets, equivalent to the control level of 6.8 ± 1.5 pmol of cyclic AMP/10^9 platelets. Results were similar with a phosphodiesterase inhibitor included; platelets with 0.2 mM IBMX contained 15.5 ± 3.0 pmol of cyclic AMP/10^9 cells. This was equivalent to 24.3 ± 3.8 and 20.4 ± 4.3 pmol of cyclic AMP/10^9 cells in platelets with 0.2 mM IBMX and 15 or 30 μM 14,15-*cis*-episulfide-ET, respectively. With 0.2 mM IBMX and 3 μM PGE_1 as a positive control, platelets contained 55.9 ± 13.7 pmol/10^9 cells (mean ± standard error, six experiments). Results were similar for 2-min incubations. 2′,5′-ddA, a P site agonist that inhibits adenylate cyclase and reverses the antiaggregatory effect of PGE_1 (21), did not reverse the antiaggregatory effect of 14,15-*cis*-episulfide-ET (Fig. 2). Collectively, these data exclude elevation of cyclic AMP as the primary mechanism accounting for the results with this analog.

Effects on intracellular Ca^{2+} levels in activated platelets. Reduced availability of endogenous arachidonic acid may explain the effects of the episulfide analog on TxB_2 formation by platelets stimulated with collagen, A23187, and thrombin. This reduction could originate from a direct effect on phospholipase A_2, or an indirect effect involving cellular Ca^{2+} levels, which influence phospholipase activity. We focused on alteration of Ca^{2+} levels because 14,15-*cis*-episulfide-ET did not inhibit pancreatic phospholipase A_2 and precedents indicate that EETs facilitate Ca^{2+} loss and decrease Ca^{2+} uptake by aortic microsomes (9). In platelets, 14,15-*cis*-episulfide-ET inhibited the rise in $[Ca^{2+}]_i$ following stimulation with 0.5 μM ionophore 4-bromo-A23187. For platelets in buffer with 1.5 mM Ca^{2+}/0.8 mM Mg^{2+}, the $[Ca^{2+}]_i$ levels rose to 220 ± 37 nM. This differed ($p < 0.05$) from the corresponding rise to 106 ± 14 nM in platelet suspensions containing 30 μM 14,15-*cis*-episulfide-ET (Table 4). Results were similar with a Ca^{2+}-free buffer: $[Ca^{2+}]_i$ levels 1 min after addition of 4-bromo-A23187 increased to 168 ± 26 nM in control platelets and to 148 ± 71, 63 ± 8, and 30 ± 13 nM in the presence of 6, 12, and 48 μM 14,15-*cis*-episulfide-ET, respectively. In this case, where the platelet membrane is a source of residual Ca^{2+} for influx into the cytosol, the intracellular Ca^{2+} levels reversed rapidly and returned to levels below the initial basal concentration. This reversal was dose dependent (Fig. 3). There was no cytotoxicity under these conditions. LDH release, as a percentage of total LDH, was 1.9 ± 0.3% in control platelets, 2.3 ± 0.4% in platelets treated with 2.5 μM 4-bromo-A23187, 1.8 ± 0.2% and 2.9 ± 0.9% in platelets treated with 30 and 60 μM 14,15-*cis*-episulfide-ET, respectively, and 2.3 ± 0.2% in platelets treated with 2.5 or 5 μM 4-bromo-A23187 plus 30 μM 14,15-*cis*-episulfide-ET.

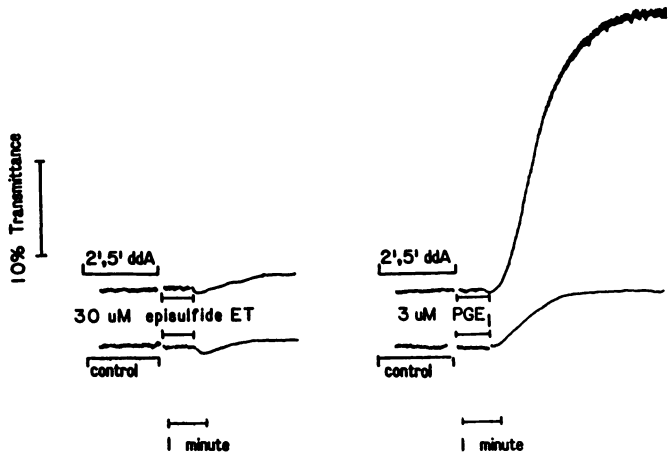

Fig. 2. Effect of 2′,5′-ddA on platelet aggregation inhibited by 14,15-*cis*-episulfide-ET. Platelets were incubated for 2 min at 37° with 80 μM 2′,5′-ddA (21) and then for 1 min with 30 μM 14,15-*cis*-episulfide-ET before stimulation with 0.5 μM A23187. 2′,5′-ddA did not reverse the inhibitory effect of 14,15-*cis*-episulfide-ET (*left*). It did reverse the inhibitory effect of PGE_1, an eicosanoid that stimulates adenylate cyclase (*right*). See text for typical concentrations of intracellular cyclic AMP.

Similarly, 14,15-*cis*-episulfide-ET inhibited the rise of $[Ca^{2+}]_i$ following stimulation with 0.5 μM U46619. $[Ca^{2+}]_i$ levels rose to 214 ± 20 nM in control platelets and to 141 ± 8 nM in platelets treated with 24 μM episulfide-ET.

With 0.5 mM EGTA present to sequester platelet membrane Ca^{2+}, 14,15-*cis*-episulfide-ET had no effect. Following addition of 0.5 μM 4-bromo-A23187, $[Ca^{2+}]_i$ levels increased to 168 ± 25 nM in platelets in Ca^{2+}-free buffer, 126 ± 15 nM in platelets with buffer containing 0.5 mM EGTA, and 118 ± 18 nM in platelets with buffer containing 0.5 mM EGTA and 30 μM 14,15-*cis*-episulfide-ET. These values were equivalent by analysis of variance ($p = 0.3$). Thus, the effect required extracellular or platelet membrane Ca^{2+}.

14,15-*cis*-episulfide-ET did not alter $[Ca^{2+}]_i$ levels in platelets activated by thrombin, consistent with its effect on thrombin-induced aggregation. For platelets in buffer with Ca^{2+}/Mg^{2+}, 0.1 units/ml thrombin increased $[Ca^{2+}]_i$ to 249 ± 14 nM. This was statistically indistinguishable from the corresponding rise to 224 ± 9 nM in platelet suspensions containing 24 μM 14,15-*cis*-episulfide-ET. Results were similar in Ca^{2+}/Mg^{2+}-free buffer.

Reversibility of the effect of 14,15-*cis*-episulfide-ET. The antiaggregatory effect was reversed by washing with citrate buffer containing albumin (5 mg/ml) to sequester available 14,15-*cis*-episulfide-ET. Inhibition was sustained in controls without the washing step, verifying that there was no spontaneous decay of the inhibitor (Fig. 4). A dependence on membrane Ca^{2+} and reversibility are consistent with, but not proof of, a locus of action at the plasma membrane.

Conclusion

Distinctive features of 14,15-*cis*-episulfide-ET include (i) preferential inhibition of A23187- but not thrombin-induced aggregation, (ii) inhibition of TxB_2 biosynthesis derived from endogenous but not exogenous arachidonic acid, (iii) attenuation of ionophore-mediated increases in cytosolic Ca^{2+} when extracellular or membrane pools of Ca^{2+} are available but not when these pools are excluded, and (iv) reversibility. These traits distinguish 14,15-*cis*-episulfide-ET from other antiaggregatory substances. Increases in cellular cyclic AMP or inhibition of cyclooxygenase do not account for the results. Dose-response curves for inhibition of aggregation did not parallel those for inhibition of TxB_2 formation; aggregation induced by arachidonic acid was inhibited fully, whereas TxB_2 formation corresponded to control values. Notably, 14,15-*cis*-episulfide-ET does inhibit aggregation induced by U46619, a synthetic

TABLE 4

Effect of 14,15-*cis*-episulfide-ET on cytosolic Ca^{2+} levels in platelets

Platelets in Tyrode's buffer containing 1.5 mM Ca^{2+} and 0.8 mM Mg^{2+}, buffer without Ca^{2+} and Mg^{2+}, or buffer with 0.5 mM EGTA were incubated with 24 μM episulfide-ET for 1 min at 37° before addition of 4-bromo-A23187 (0.5 μM), thrombin (0.1 units/ml), or U46619 (0.5 μM). Data represent $[Ca^{2+}]_i$ 1 min after the addition of agonist. Values are mean ± standard error of three or more experiments.

Agonist	$[Ca^{2+}]_i$					
	Tyrode's + Ca^{2+}/Mg^{2+}		Tyrode's − Ca^{2+}/Mg^{2+}		Tyrode's + 0.5 mM EGTA	
	−[a]	+[a]	−	+	−	+
	nM					
4-Bromo-A23187	220 ± 37	106 ± 14[b,c]	168 ± 26	27 ± 6[c]	126 ± 15	118 ± 18[b]
Thrombin	249 ± 14	224 ± 9	227 ± 76	264 ± 17		
U46619	214 ± 20	141 ± 8[c]	98 ± 6	80		

[a] −, +, without or with 14,15-*cis*-episulfide-ET.
[b] Concentration of 14,15-*cis*-episulfide-ET = 30 μM.
[c] Significantly different from control ($p < 0.05$).

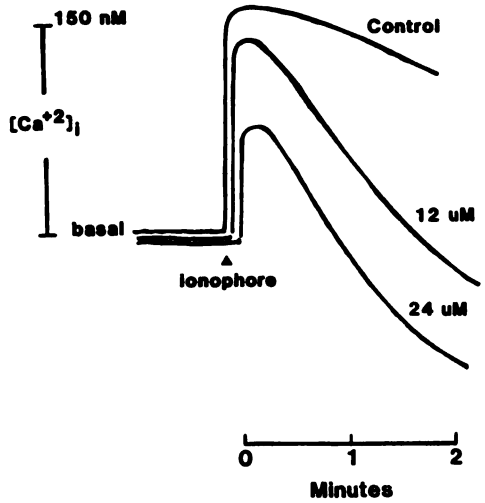

Fig. 3. Effect of 14,15-*cis*-episulfide-ET on cytosolic ionized Ca^{2+} levels in platelets stimulated with 4-bromo-A23187 (0.5 μM). *Traces* depict the rise of $[Ca^{2+}]_i$ following addition of ionophore to platelet suspensions containing 0, 12, or 24 μM 14,15-*cis*-episulfide-ET. The analog had no effect on basal $[Ca^{2+}]_i$ levels. The $[Ca^{2+}]_i$ levels declined in its presence to values below the initial basal level. There was no cytotoxicity due to 4-bromo-A23187 (2.5–5 μM) or to 14,15-*cis*-episulfide-ET (30–60 μM). LDH release was indistinguishable from the control value, 1.9 ± 0.3% of total LDH.

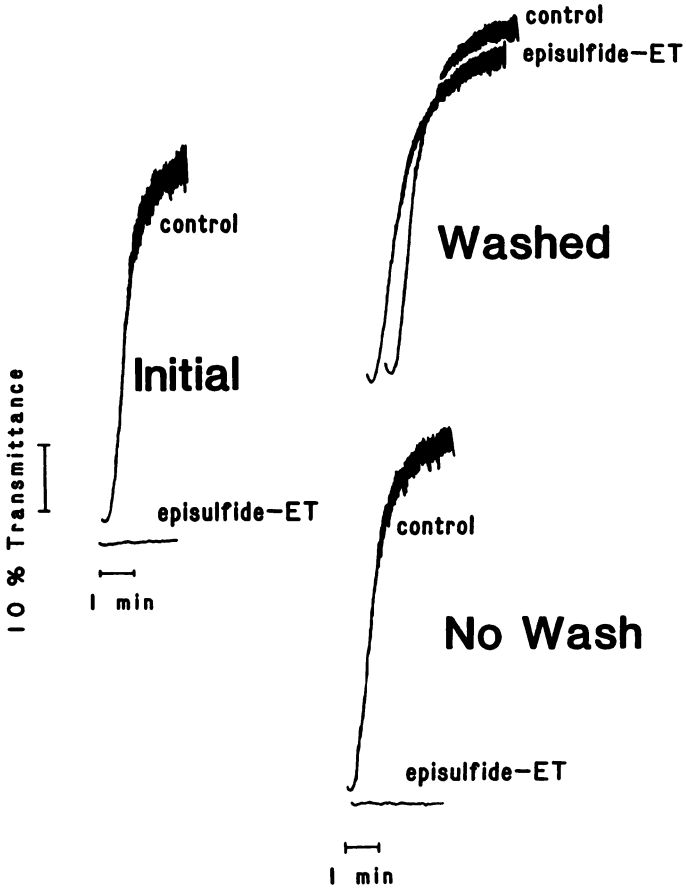

Fig. 4. Reversal of 14,15-*cis*-episulfide-ET antiaggregatory effect. *Left*, initial response to 1 μM U46619 for platelets containing 0 or 15 μM 14,15-*cis*-episulfide-ET. *Upper right*, washing of the platelets with citrate buffer containing 5 mg of bovine albumin to sequester lipids reverses the antiaggregatory effect. *Lower right*, responses with platelets incubated for a corresponding amount of time without a bovine serum albumin wash. There was no decay of inhibitory activity.

PGH_2/TxA_2 agonist, and by A23187, a Ca^{2+} ionophore. Aspirin or other cyclooxygenase inhibitors do not block aggregation induced by these two agonists. In fact, 14,15-*cis*- episulfide-ET inhibited aggregation induced by these agents, using aspirin-treated platelets.

Antagonism of the PGH_2/TxA_2 receptor could account for some data, such as inhibition of arachidonic acid- and U46619-induced aggregation (23). Comparable to 14,15-*cis*-episulfide-ET, receptor antagonists typified by BM 13,177 or L655,240 inhibit aggregation induced by U46619 or arachidonic acid, and they do not inhibit aggregation induced by thrombin. However, other data are inconsistent with this mechanism. Namely, the episulfide analog inhibited both aggregation and TxB_2 biosynthesis initiated by A23187; PGH_2/TxA_2 receptor antagonists did not inhibit either process under similar conditions. Therefore, it is unlikely that 14,15-*cis*-episulfide-ET exerts its effect through PGH_2/TxA_2 receptor antagonism. It is important to stress that the relationship between cytosolic Ca^{2+} and thromboxane is circular; elevations in Ca^{2+} stimulate TxA_2 formation and, conversely, elevations in TxA_2 can stimulate $[Ca^{2+}]_i$ increases. Thus, it seems difficult to dissociate the two processes completely. However, the attenuation of ionophore-mediated increases in cytosolic Ca^{2+} is not likely due to antagonism of TxA_2, because known antagonists do not inhibit ionophore-induced aggregation.

An unusual feature of this antiaggregatory substance is its preferential effect on ionophore- rather than thrombin-induced aggregation. Increases in intracellular Ca^{2+} are associated with

aggregation induced by ionophore and thrombin (24–26); however, there are differences between them. First, thrombin increases Ca^{2+} by ATP-dependent processes and activation of phospholipase C (26, 27); A23187 can increase Ca^{2+} independently of these two processes. Second, compared with thrombin, A23187-facilitated Ca^{2+} mobilization is appreciable and it involves several cellular compartments (28). With thrombin, Ca^{2+} redistribution involves fewer discrete compartments (29); however, these compartments may be directed to the more efficacious or the local release of Ca^{2+}, which is insensitive to the EETs. Therefore, different pools of Ca^{2+} that affect phospholipase A_2-dependent eicosanoid biosynthesis and other activation processes may have differential sensitivity to the EETs.

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