Epoxyeicosatrienoic Acids Inhibit Ca\(^{2+}\) Entry into Platelets Stimulated by Thapsigargin and Thrombin*

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The epoxyeicosatrienoic acids derived from the cytochrome P-450 pathway of arachidonic acid metabolism have a unique platelet antiaggregatory profile. This prompted us to examine their influence on cellular Ca\(^{2+}\) mobilization. 14,15-cis-Epoxyeicosatrienoic acid and related compounds inhibited the rise in cytosolic Ca\(^{2+}\) following agonist stimulation of platelets by thapsigargin, a receptor-independent agonist, and thrombin, a receptor-dependent agonist. The epoxyeicosatrienoic acids selectively inhibited the entry of Ca\(^{2+}\) from the exterior of the platelets but did not alter Ca\(^{2+}\) discharge from intracellular pools. The magnitude of inhibition by 14,15-cis-epoxyeicosatrienoic acid was proportional to the rate of Ca\(^{2+}\) entry. 14,15-cis-Epoxyeicosatrienoic acid also inhibited the rate of influx of Mn\(^{2+}\), a cation which enters platelets via pathways similar to Ca\(^{2+}\). The magnitude of inhibition was proportional to the rate of Mn\(^{2+}\) entry, suggesting that epoxyeicosatrienoic acids act on divalent cation channels in a fashion which depends on the state of opening of the channel. Selective inhibition of Ca\(^{2+}\) entry into platelets may account for the antiaggregatory effects of the epoxyeicosatrienoic acids. We are unaware of other endogenous compounds exhibiting this property, suggesting that epoxyeicosatrienoic acids may be useful to probe agonist-stimulated cellular Ca\(^{2+}\) mobilization in nonexcitable cells.

Epoxyeicosatrienoic acids (EETs)


The abbreviations used are: EET, epoxyeicosatrienoic acid; ET, eicosatrienoyl; HEL, human erythroleukemia; HEPES, 4-(2-hydroxyethyl) piperazine-N,N'-bis(2-ethanesulfonic acid); PRP, platelet-rich plasma; PG, prostaglandin; TxA₂, thromboxane; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate; TMB-8, a receptor-dependent agonist. The epoxyeicosatrienoic acids selectively inhibited the entry of Ca\(^{2+}\) from the exterior of the platelets but did not alter Ca\(^{2+}\) discharge from intracellular pools. The magnitude of inhibition by 14,15-cis-epoxyeicosatrienoic acid was proportional to the rate of Ca\(^{2+}\) entry. 14,15-cis-Epoxyeicosatrienoic acid also inhibited the rate of influx of Mn\(^{2+}\), a cation which enters platelets via pathways similar to Ca\(^{2+}\). The magnitude of inhibition was proportional to the rate of Mn\(^{2+}\) entry, suggesting that epoxyeicosatrienoic acids act on divalent cation channels in a fashion which depends on the state of opening of the channel. Selective inhibition of Ca\(^{2+}\) entry into platelets may account for the antiaggregatory effects of the epoxyeicosatrienoic acids. We are unaware of other endogenous compounds exhibiting this property, suggesting that epoxyeicosatrienoic acids may be useful to probe agonist-stimulated cellular Ca\(^{2+}\) mobilization in nonexcitable cells.

EXPERIMENTAL PROCEDURES

Materials—Acetylsalicylic acid, apyrase, and thrombin (Sigma); PGE\(_1\), 8,9-cis-EET, 11,12-cis-EET, 14,15-cis-EET (Cayman Chemical or Oxford Biomedical) and its heteroatom analog, 14,15-cis-episulfide-deeicosatrienoic acid (10); thapsigargin (LC Services); ploneric acid and fura-2-acetoxyethyl ester fluorescent Ca\(^{2+}\) indicator (fura-2/AM; Molecular Probes); the voltage-dependent Ca\(^{2+}\)-channel antagonist verapamil (Biomol); the intracellular Ca\(^{2+}\)-channel blocker TMB-8 (8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride; Aldrich) (16), and human erythroleukemia cells (ATCC) were used.

Preparation of Human Platelets—Human blood (9 volumes) collected in 3.8% trisodium citrate (1 volume) was centrifuged at 200 × g for 20 min, and the platelet-rich plasma (PRP) was isolated. PRP was incubated with 0.5 mM aspirin for 15 min to inhibit the cyclooxygenase enzyme, eliminating indirect effects from PGH\(_2\)/TxA\(_2\) formation (17, 18). Washed platelets were prepared and resuspended in Tyrode’s buffer, pH 7.2, 1.5 mM Ca\(^{2+}\), 0.8 mM Mg\(^{2+}\), and 0.1 unit/ml apyrase (19). Albumin was left out to prevent it from binding EETs and thapsigargin. For several experiments platelets were suspended in a Ca\(^{2+}\)- and Mg\(^{2+}\)-free buffer.

Determination of Cytosolic Ca\(^{2+}\) Concentration in Platelets—PRP was incubated with 2 μM fura-2/AM and 0.02% ploneric acid for 15 min at 37 °C. Platelets containing fura-2/AM were washed as described above, incubated for an additional 25 min at 37 °C, and then centrifuged and resuspended in Tyrode’s buffer (128 mM NaCl, 25.8 mM NaHCO\(_3\), 1.25 mM sucrose, 10 mM glucose, 8.8 mM HEPES, 5 mM KCl, 1.3 mM NaH\(_2\)PO\(_4\)) to a final concentration of 2–3 × 10\(^{10}\) platelets/ml. 14,15-cis-EET (0–60 μM), 14,15-cis-episulfide ET (0–60 μM), 8,9-cis-EET (60 μM), 11,12-cis-EET (60 μM), or verapamil (25 μM), dissolved in methanol, were transferred to an acrylic cuvette, and the solvent was evaporated; TMB-8 was dissolved in water and added directly to the cuvette 2 min before stimulation. Platelets in Tyrode’s buffer (2.4 ml) were transferred to the cuvette containing EET and equilibrated for 1 min at 37 °C. Platelets were stimulated with 0.1–1 μM Tg or 2 units/ml thrombin; high dose thrombin was used to assure rapid and complete discharge of Ca\(^{2+}\) from intracellular stores. Experiments were performed with Ca\(^{2+}\)- and Mg\(^{2+}\)-free buffer or after adjustment of the Ca\(^{2+}\) and Mg\(^{2+}\) concentrations to 1.5 mM with CaCl\(_2\) and 0.8 mM with MgCl\(_2\). In some experiments 1.5 mM CaCl\(_2\) and 0.8 mM MgCl\(_2\) were added to Ca\(^{2+}\)- and Mg\(^{2+}\)-free buffer 1–7 min after stimulation of platelets. This protocol separates discharge from influx and facilitates precise determination of Ca\(^{2+}\) entry rates. Fluorescence was determined with an H & L Instruments spectrofluorometer (Series 300), at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. Fluorescence was monitored for 1 min prior to addition of thapsigargin (0.1–1.0 μM), or other lipid mediators of thrombosis and hemostasis.

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thrombin (2 units/ml). Cytosolic Ca\(^{2+}\) levels, [Ca\(^{2+}\)]
were determined by fluorometry (20, 21), according to the formula [Ca\(^{2+}\)]
= \(K_a \times f \times (R - \frac{R_{max}}{R_{max} - R})\), where \(R\) is the ratio of the measured fluorescence at 500 nm after excitation at 340 and 380 nm, respectively; \(R_{max}\) was determined by lysing the cells with 0.1% Triton X-100, and \(R_{min}\) was obtained in the presence of 10 mM EGTA and 125 mM Tris (pH=9.0); \(f\) is the ratio from excitation of fura-2 in 380 nm in the free (\(R_{max}\)) and fully bound (\(R_{max}\)) state; and \(K_a = 224\) nm at 37 °C (21).

**Di &non(6) Cation Influx in Platelets**—Platelets were loaded with fura-2 as described above. 80 \(\mu\)M MnCl\(_2\) was added to Ca\(^{2+}\)-free Tyrode’s in some cases to use the fluorescence-quenching properties of Mn\(^{2+}\) as an alternative measure of cation influx (22). The initial rates of stimulated Mn\(^{2+}\) influx for control and EET-treated platelets were estimated by linear regression of the quenching of fura-2 fluorescence (22, 29). Thus, replacement of Ca\(^{2+}\) influx into cells. In Ca\(^{2+}\)-free buffer containing 80 \(\mu\)M Mn\(^{2+}\), fluorescence measured at 360 nm decreased slowly due to the basal permeability of resting platelets to divalent cations; EETs had no detectable effect on Mn\(^{2+}\) influx in unstimulated platelets. Stimulation by 1 \(\mu\)M thapsigargin caused fluorescence at 360 nm to decrease rapidly; treatment with 14,15-cis-EET or 14,15-cis-episulfide-ET prior to thapsigargin stimulation decreased the rate of quenching by 12 ± 4% (p < 0.05, n = 4) and 35 ± 12% (p < 0.05, n = 6), respectively. When platelets were stimulated with 1 \(\mu\)M thapsigargin for 3 min, followed by addition of Mn\(^{2+}\), fluorescence quenching due to agonist-stimulated influx of Mn\(^{2+}\) increased rapidly in control cells. 14,15-cis-EET inhibited the rate of quenching by 73 ± 3% (p < 0.05, n = 3) (Fig. 2). This is consistent with inhibition of divalent cation influx by EETs. The rate of quenching of fura-2 fluorescence was consistently greater when Mn\(^{2+}\) was added after stimulation than when Mn\(^{2+}\) was present in the medium before stimulation.

**RESULTS**

**Modulation of Thapsigargin-induced Ca\(^{2+}\) Mobilization in Platelets**—Thapsigargin elevates cytosolic Ca\(^{2+}\) levels in several cell types, including platelets (17, 28, 29). This reflects a receptor-independent discharge of Ca\(^{2+}\) from an IP\(_3\)-sensitive, intracellular pool when endogenous PGI\(_2\)/TXA\(_2\) production is eliminated by aspirin (17). "Epoxygenase" eicosanoids had no significant effect on [Ca\(^{2+}\)]
when platelets suspended in Ca\(^{2+}\)-free buffer were incubated with thapsigargin (Fig. 1, upper panels). 14,15-cis-EET lowered [Ca\(^{2+}\)]
from a control value of 183 ± 6 to 165 ± 12 nm (p < 0.05, n = 7), whereas 14,15-cis-episulfide ET enhanced [Ca\(^{2+}\)]
from 165 ± 15 to 196 ± 17 nm (p < 0.05, n = 5). However, EETs inhibited the rise in [Ca\(^{2+}\)]
when thapsigargin (Fig. 1, lower panels).

**[Ca\(^{2+}\)]\(i\) only in a Ca\(^{2+}\)-containing medium. a and b, representative tracings of [Ca\(^{2+}\)]
of thapsigargin-stimulated platelets in Ca\(^{2+}\)-free medium; c and d, representative tracings of [Ca\(^{2+}\)]
of Tg-stimulated platelets in Ca\(^{2+}\)-containing medium. Solid lines, control responses; dotted lines, responses in the presence of 60 \(\mu\)M 14,15-cis-EET (a and c) or 14,15-cis-episulfide ET (b, 60 \(\mu\)M; d, 15 \(\mu\)M).**

![Fig. 1. 14,15-cis-EET and 14,15-cis-episulfide ET inhibit [Ca\(^{2+}\)] only in a Ca\(^{2+}\)-containing medium. a and b, representative tracings of [Ca\(^{2+}\)] of Tg-stimulated platelets in Ca\(^{2+}\)-free medium; c and d, representative tracings of [Ca\(^{2+}\)] of Tg-stimulated platelets in Ca\(^{2+}\)-containing medium. Solid lines, control responses; dotted lines, responses in the presence of 60 \(\mu\)M 14,15-cis-EET (a and c) or 14,15-cis-episulfide ET (b, 60 \(\mu\)M; d, 15 \(\mu\)M).](image1)

![Fig. 2. EETs inhibit the quenching of fura-2 fluorescence by Mn\(^{2+}\) influx. fura-2-loaded washed platelets were incubated in a Ca\(^{2+}\)-free medium and stimulated with 1 \(\mu\)M Tg. MnCl\(_2\) (80 \(\mu\)M) was added 3 min later to monitor divalent cation entry by fura-2 quenching. The fluorescence was measured at an excitation wavelength of 360 nm. Solid line, control responses; dotted line, Mn\(^{2+}\) quenching with 60 \(\mu\)M 14,15-cis-EET.](image2)
EETs Inhibit Ca\(^{2+}\) Entry following Depletion of Intracellular Ca\(^{2+}\) Stores by Thapsigargin or Thrombin—Receptor-dependent activation of phospholipase C with thrombin (30) or inhibition of dense tubular Ca\(^{2+}\)-ATPase by thapsigargin (28) depletes the intracellular storage pools of their Ca\(^{2+}\) content. This depletion is accompanied by influx of Ca\(^{2+}\) from the extracellular space to the cytosol according to the “capacitative” model of Ca\(^{2+}\) entry (31). Addition of Ca\(^{2+}\) to the buffer, after stimulation, temporally separates the discharge and influx phases. This facilitates an unambiguous distinction between the two processes. In a Ca\(^{2+}\)-free medium, thapsigargin (1 \(\mu\)M) caused a slow, sustained rise in [Ca\(^{2+}\)], which was maximal by 1 min (Fig. 3a). 14,15-cis-EET did not alter this rise due to Ca\(^{2+}\) discharge. In treated cells [Ca\(^{2+}\)], was 165 ± 12 nM (n = 7), a value indistinguishable from the control level 183 ± 6 nM (p > 0.05, n = 7). Addition of Ca\(^{2+}\) to the buffer 3 min after stimulation increased [Ca\(^{2+}\)], to 701 ± 92 nM in control platelets (Fig. 3a); 14,15-cis-EET reduced this to 559 ± 21 nM (p < 0.05). 11,12-cis-EET (60 \(\mu\)M) also inhibited Ca\(^{2+}\) influx, whereas 8,9-cis-EET (60 \(\mu\)M) had a consistent, but statistically insignificant inhibitory effect (Table I). 14,15-cis-EET also inhibited the influx of Sr\(^{2+}\) in a similar protocol (data not shown). The hydration product of 14,15-cis-EET, 14,15-dihydroxyoctadecatrienoic acid, and 15(S)-hydroxyeicosatetraenoic acid were both inactive. Results were similar with thrombin. In Ca\(^{2+}\)-free buffer thrombin (2 units/ml) caused a rapid rise in [Ca\(^{2+}\)], followed by its gradual decline toward the resting level within 5 min (Fig. 3b). 14,15-cis-EET did not inhibit this initial, IP\(_3\)-mediated discharge of intracellular Ca\(^{2+}\). In treated cells [Ca\(^{2+}\)], was 377 ± 73 nM (n = 3), a value indistinguishable from the control level of 488 ± 54 nM (n = 3) (Fig. 3b). Addition of Ca\(^{2+}\) 3 min after stimulation with thrombin reversibly increased [Ca\(^{2+}\)], to 455 ± 10 nM; 14,15-cis-EET reduced this to 293 ± 22 nM (p < 0.05, n = 3). Treatment of platelets with 14,15-cis-episulfide ET yielded similar results (not shown). Control experiments with TMB-8 and verapamil verified the selective effect exhibited by EETs. TMB-8, an intracellular Ca\(^{2+}\) antagonist, decreased both intracellular Ca\(^{2+}\) discharge and subsequent Ca\(^{2+}\) influx induced by thapsigargin (Fig. 3c). Verapamil, a voltage-sensitive Ca\(^{2+}\)-channel blocker, did not inhibit Ca\(^{2+}\) discharge or entry induced by thapsigargin or thrombin (data not shown), consistent with the lack of voltage-gated Ca\(^{2+}\) channels on platelets (32, 33).

The capacitative model of cellular Ca\(^{2+}\) regulation predicts that the rate of Ca\(^{2+}\) entry into cells is inversely proportional to its concentration within cellular storage pools (31). We examined whether this model applies to platelets, and if so, whether the inhibitory effect of EETs varied with the rate of Ca\(^{2+}\) entry. Brief stimulation by thapsigargin should discharge little Ca\(^{2+}\) from intracellular pools; thus, the rate of Ca\(^{2+}\) entry into the cytosol for “refilling” should be slow. Prolonged stimulation by thapsigargin should discharge substantial Ca\(^{2+}\) from intracellular pools; thus, the rate of Ca\(^{2+}\) entry should be rapid. Platelets in Ca\(^{2+}\)-free buffer were stimulated with 0.1 \(\mu\)M thapsigargin for 1, 3, 5, or 7 min, then Ca\(^{2+}\) was added. The rate and magnitude of increase in [Ca\(^{2+}\)], after addition of Ca\(^{2+}\) to the buffer, was proportional to the duration of stimulation (Fig. 4a), consistent with predictions based on Putney’s capacitative model (31). 14,15-cis-EET (60 \(\mu\)M) inhibited the rate of rise in [Ca\(^{2+}\)], in all cases, but its effect correlated with the rate of Ca\(^{2+}\) influx (Fig. 4b).

### Table I

**Effects of 8,9- and 11,12-cis-EET on intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) entry in Tg-stimulated platelets**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount of [Ca(^{2+})] condition</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ce(^{2+})-free (\pm) Ce(^{2+})</td>
</tr>
<tr>
<td>Control (1 (\mu)M Tg)</td>
<td>231 ± 4</td>
</tr>
<tr>
<td>8,9-cis-EET (60 (\mu)M)</td>
<td>235 ± 9</td>
</tr>
<tr>
<td>11,12-cis-EET (60 (\mu)M)</td>
<td>225 ± 21</td>
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</table>

*Maximal elevation in [Ca\(^{2+}\)], in Ca\(^{2+}\)-free medium.

**Level of [Ca\(^{2+}\)], 2 min after addition of 1.5 mM Ca\(^{2+}\) to platelet suspension.**

* p < 0.05.
FIG. 4. 14,15-cis-EET effects are dependent on the rate of Ca\(^{2+}\) influx. Platelets in a Ca\(^{2+}\)-free buffer were stimulated with 0.1 μM Tg for 1, 3, 5, or 7 min then Ca\(^{2+}\) was added to the buffer, in each case, to permit influx as in Fig. 3. Under these conditions the rate and extent of extracellular Ca\(^{2+}\) influx into the platelets increased proportional to the duration of stimulation by Tg (upper panel). 14,15-cis-EET inhibited the rate of Ca\(^{2+}\) influx (lower panel) and the extent of Ca\(^{2+}\) elevation (not shown). Rates of Ca\(^{2+}\) entry (nanomolar per s) were calculated from the linear portion of the tracing immediately after addition of Ca\(^{2+}\). Hatched bars, control responses; open bars, response with 60 μM 14,15-cis-EET. *p < 0.05.

FIG. 5. 14,15-cis-EET inhibits Ca\(^{2+}\) entry into Tg-stimulated HEL cells. HEL cells (3 × 10⁶/ml) were incubated in Ca\(^{2+}\)-free medium and stimulated with 10 nM Tg, Ca\(^{2+}\) (1.5 mM) and Mg\(^{2+}\) (0.8 mM) were introduced into the medium 3 min after stimulation, and Ca\(^{2+}\) influx was monitored. Solid line, control; dotted line, 60 μM 14,15-cis-EET.

Elevated [Ca\(^{2+}\)], in control cells to 512 ± 77 nM. Treatment with 14,15-cis-EET reduced this to 386 ± 42 nM (p < 0.05, n = 6) (Fig. 6). Release from intracellular stores by thrombin was also inhibited slightly by 60 μM 14,15-cis-EET (150 ± 16 vs. 134 ± 11; p < 0.05, n = 6; Fig. 6), whereas it had no effect on intracellular release by Tg (Fig. 5). Likewise, 60 μM 11,12-cis-EET had no effect on intracellular release but inhibited Ca\(^{2+}\) entry by 10 nM Tg (data not shown).

**DISCUSSION**

Our data indicate that EETs inhibit the influx of Ca\(^{2+}\) into platelets following receptor-independent discharge of Ca\(^{2+}\) from intracellular pools by thapsigargin or receptor-depend-
observed blockade of uptake and increased loss of Ca\(^{2+}\) by EETs from canine cardiac microsomes, which implies that EETs would elevate [Ca\(^{2+}\)]\(_i\) in those cells. Such an elevation in [Ca\(^{2+}\)]\(_i\) has been observed in anterior pituitary cells (35) and hepatocytes (36). Force et al. (37) observed a modulatory effect on [Ca\(^{2+}\)]\(_i\); vasopressin-induced elevations in [Ca\(^{2+}\)]\(_i\) were attenuated by treatments which prevented EET formation in glomerular mesangial cells. However, our data clearly indicate inhibition, rather than stimulation of Ca\(^{2+}\) levels in platelets.

The effects of EETs were not confined to platelets; they also inhibited Ca\(^{2+}\) influx in HEL cells. The finding that 14,15-cis-EET also inhibited intracellular Ca\(^{2+}\) release by thapsigargin suggests that comparisons of thapsigargin-activated platelets and HEL cells must be made carefully (24). However, Ca\(^{2+}\) mobilization in HEL cells was similar in many respects to that seen in platelets. These cells are useful because: (i) they originate from megakaryocytes, a progenitor for platelets (38), (ii) they do not generate eicosanoids, which might have indirect effects, upon stimulation with thapsigargin or thapsigargin (27), (iii) they are suitable for electrophysiological manipulation, and (iv) they have been maintained in culture and should allow further studies to determine if incorporation of EETs into plasma phospholipids has effects on Ca\(^{2+}\) mobilization (39, 40).

Recent evidence showing generation of EETs by endothelial cells (41), their presence in LDL (42), and increased synthesis in aorta of cholesterol-fed rabbits (43) suggests that these compounds may have a physiological role in hemostasis and thrombosis. If this proves to be the case their mechanism of formation and action differs from and complements other antiaggregatory lipid mediators which regulate vascular events. Furthermore, EETs appear to be useful pharmacological probes to dissect the mechanisms of capacitative and receptor-mediated Ca\(^{2+}\) entry pathways.

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REFERENCES