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ELEVATION OF CYTOPLASMIC FREE CALCIUM CONCENTRATION BY STABLE THROMBOXANE A_2 ANALOGUE IN HUMAN PLATELETS*

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SUMMARY: 9, 11-Epithio-11, 12-methano-thromboxane A_2 (STA_2), a stable analogue of thromboxane A_2, caused a rapid rise in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]i$) in human platelets as measured with the fluorescent Ca^{2+} indicator quin2. Concomitantly, this compound induced phosphorylation of myosin light chain which is catalyzed by Ca^{2+}, calmodulin-dependent protein kinase. These reactions were fast enough to trigger serotonin release. 13-Azaprostanoic acid, a receptor level antagonist of thromboxane A_2 inhibited STA_2-induced elevation of $[Ca^{2+}]i$, phosphorylation of myosin light chain and serotonin release. These results provide evidence that STA_2 interacts with a thromboxane A_2 receptor which leads to elevation of $[Ca^{2+}]i$.

When platelets are stimulated by various agents such as thrombin and collagen, TXA_2[1/] is generated from arachidonic acid which is liberated from the membrane phospholipid (1). Although this substance is among the most potent platelet-activating agents thus far identified, the mechanism of its action has not been fully understood because of its inherent instability. To overcome this problem, several compounds have been synthesized as TXA_2 mimic substances (2-4). U-46619, like TXA_2 causes platelet activation and vasoconstriction but it is more structually related to endoperoxides than TXA_2. Although CTA_2 is a structural analogue of TXA_2 and induces

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1/ The abbreviations used are: TXA_2, thromboxane A_2; U-46619, 9, 11-methanoepoxy prostaglandin H_2; CTA_2, carbocyclic thromboxane A_2; $[Ca^{2+}]i$, cytoplasmic free Ca^{2+} concentration; 13-APA, 13-azaprostanoic acid; SDS, sodium dodecyl sulfate; PGE_1, prostaglandin E_1.

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vasoconstriction, unexpectedly it does not activate platelets (3). Recently, a new stable TXA_2 analogue, STA_2, has been synthesized and characterized (4). In contrast to CTA_2, this compound shows both biological activities of TXA_2 in platelets and vessels.

There are several lines of evidence suggesting that TXA_2 activates platelets by mobilizing Ca^{2+} into cytoplasm (5). However, direct evidence in support of this action of TXA_2 is lacking, since actual measurements of $[Ca^{2+}]i$ in platelets have not been previously achieved. Recently, Rink et al. (6) have measured successfully $[Ca^{2+}]i$ in intact platelets by means of the fluorescent Ca^{2+} indicator quin2 and they have shown for the first time that thrombin actually raises $[Ca^{2+}]i$ in intact platelets. Therefore, using this method, we examined whether STA_2 increased $[Ca^{2+}]i$ through a TXA_2 receptor. The phosphorylation of myosin light chain was also measured during the action of STA_2, since this reaction has been described to be stimulated by a rise of $[Ca^{2+}]i$ (7-9).

EXPERIMENTAL PROCEDURES

Washed human platelets were prepared as described by Baenziger and Majerus (10). STA_2 and 13-APA were donated by Ono Pharmaceutical Co.. Quin2 acetoxymethyl ester was purchased from Dojindo Laboratories. [^{14}C]Serotonin (58 mCi/mmol) and ^{32}Pi (carrier free) were obtained from New England Nuclear and the Radiochemical Centre, respectively. $[Ca^{2+}]i$ was measured by means of the fluorescent Ca^{2+} indicator quin2. Washed platelets were incubated for 25 min at 37°C with Hepes buffered saline (145mM NaCl, 5mM KCl, 1mM $MgSO_4$, 0.5mM Na_2HPO_4, 10mM Hepes, 5mM glucose, pH7.4 at 37°C) containing 5μM quin2 acetoxymethyl ester. The suspension was washed 2 times with Hepes buffered saline to remove any extraneous dye. Fluorescence was recorded at 37°C in Hitachi fluorescence spectrophotometer 650-60. Excitation was at 339 nm and emission at 500 nm. $[Ca^{2+}]i$ was calculated from fluorescence signals as described by Rink et al. (6). Labeling of platelets with ^{32}Pi was carried out as described by Lyons et al. (11). The platelet proteins labeled with ^{32}Pi were subjected to SDS-polyacrylamide slab gel electrophoresis, stained, dried on a filter paper and then exposed to an X-ray film to prepare an autoradiogram. Electrophoresis was carried out as described by Laemmli (12). The relative radioactivity of each band was quantified by densitometric tracing of the autoradiogram as described previously (13). Serotonin release was quantified by measuring radioactive material released from the platelets which were preloaded with [^{14}C]serotonin as described by Haslam et al. (14).

RESULTS AND DISCUSSION

Fig. 1 shows time courses of serotonin release and fluorescence response in platelets which were stimulated by STA_2. 0.1 μM STA_2 evoked a rapid rise in $[Ca^{2+}]i$ from the basal level of about 100 nM to at least 1-2 μM. Elevated $[Ca^{2+}]i$ then declined rapidly. This rise was fast enough to trigger serotonin release. Using quin2, Feinstein et al. (15) and our group (16) have reported recently that PGE_1, which increases platelet cyclic AMP, inhibits thrombin-induced elevation of $[Ca^{2+}]i$ in platelets. STA_2-induced elevation of $[Ca^{2+}]i$ was also inhibited by this prostaglandin (data not shown). It is well known that phosphorylation of myosin light chain is increased in platelets which are stimulated by various agents such as thrombin, collagen and Ca^{2+} ionophore A23187 (11, 17, 18). This protein phosphorylation results from Ca^{2+} stimulation of a specific protein kinase in the presence of calmodulin (7, 8). Then, the next experiment was designed to determine whether STA_2 also induced phosphorylation of myosin light chain. When platelets were stimulated by STA_2, phosphorylation of myosin light chain was increased rapidly as shown in Fig. 2[2/]. As expected from $[Ca^{2+}]i$ response to STA_2, maximum extent of this protein phosphorylation was obtained within 30 s of stimulation followed by dephosphorylation.

Recently, Le Breton et al. (20) have reported that 13-APA acts as a specific antagonist of TXA_2 at a receptor level. In the last set of experiments, we examined the effect of 13-APA on STA_2-induced elevation of $[Ca^{2+}]i$, phosphorylation of myosin light chain and serotonin release. 13-APA inhibited STA_2-induced elevation of $[Ca^{2+}]i$ markedly as shown in Fig. 3. In the presence of 100 μM

2/ Under these conditions, a protein with a molecular weight of 40,000 was also heavily phosphorylated. This protein is phosphorylated by a distinct protein kinase (protein kinase C) (13, 19) and the activation of protein kinase C by the action of STA_2 will be described elsewhere.

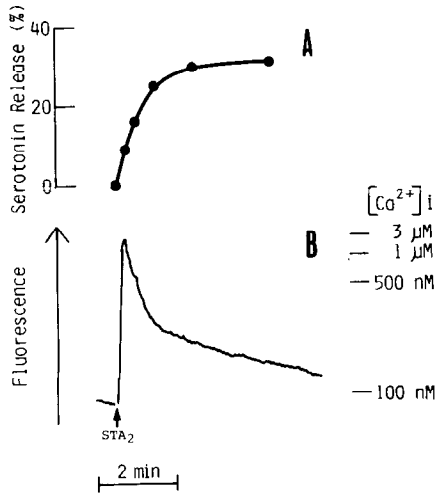

Fig. 1. Time courses of serotonin release and quin2 fluorescence response in platelets stimulated by STA_2. Samples of platelets which were loaded with both [^{14}C]serotonin and quin2 were stimulated by STA_2 (0.1 μM) in Hepes buffered saline containing 1 mM $CaCl_2$. Serotonin release was quantified and quin2 fluorescence was recorded as described under "EXPERIMENTAL PROCEDURES". Serotonin release is expressed as % release of [^{14}C]serotonin in the cells just prior to stimulation. A, serotonin release; B, quin2 fluorescence response.

13-APA, 0.1 μM STA_2 could raise [Ca^{2+}]i only to 300-400 nM. Phosphorylation of myosin light chain and serotonin release were also inhibited by 13-APA (Fig. 4). Inhibition by this agent of these

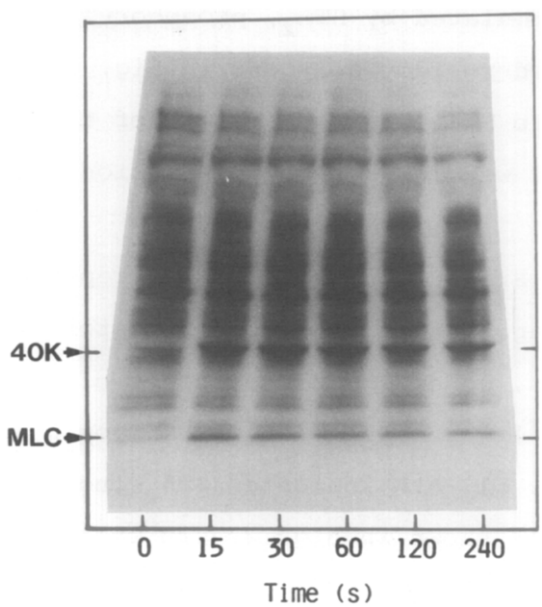

Fig. 2. Time course of phosphorylation of myosin light chain induced by STA_2. Samples of platelets which were loaded with ^{32}Pi were stimulated by STA_2 (0.1 μM) for various periods of time as indicated in Hepes buffered saline containing 1 mM $CaCl_2$. Radioactive proteins were analyzed as described under "EXPERIMENTAL PROCEDURES". MLC indicates myosin light chain.

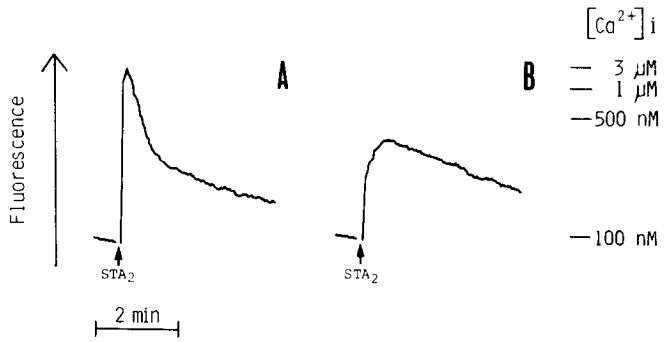

Fig. 3. Effect of 13-APA on STA_2-induced quin2 fluorescence response. Samples of platelets which were loaded with quin2 were preincubated with 13-APA (100 μM) or saline for 1 min at 37°C in Hepes buffered saline containg 1 mM $CaCl_2$ and then incubated with STA_2 (0.1 μM). Quin2 fluorescence was recorded as described under "EXPERIMENTAL PROCEDURES". A, with saline; B, with 13-APA.

reactions was more pronounced at the lower concentrations of STA_2. These results provide evidence that STA_2 induces elevation of $[Ca^{2+}]i$ and platelet activation through a platelet TXA_2 receptor.

There is some debate upon the mechanism by which TXA_2 causes Ca^{2+} mobilization. Previous studies in isolated platelet vesicles

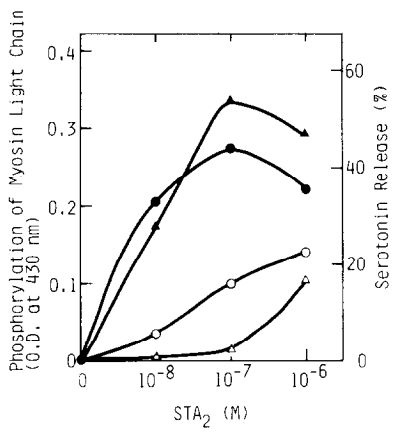

Fig. 4. Effect of 13-APA on STA_2-induced phosphorylation of myosin light chain and serotonin release. Samples of platelets which were loaded with ^{32}Pi or [^{14}C]serotonin were preincubated with 13-APA (100 μM) or saline for 1 min at 37°C in Hepes buffered saline containing 1 mM $CaCl_2$ and then incubated with various concentrations of STA_2 as indicated. The incubation was carried out for 15 s at 37°C to measure phosphorylation of myosin light chain and for 1 min at 37°C to measure serotonin release. Background value for phosphorylation of myosin light chain, which occurred in the absence of STA_2, was about 0.12 O.D.. This value was subtracted from each point. Serotonin release is expressed as % release of [^{14}C]serotonin in the cells just prior to stimulation. (●——●, ○——○), phosphorylation of myosin light chain; (▲——▲, △——△), serotonin release. Closed symbols, with saline; open symbols, with 13-APA.

have suggested that TXA_2 functions as an intracellular Ca^{2+} ionophore to release Ca^{2+} from intracellular stores (21). However, as mentioned above, Le Breton et al. (20) have synthesized a specific receptor level antagonist of TXA_2 and, using this agent, they have suggested that TXA_2 does not act as a Ca^{2+} ionophore but it must interact with a specific receptor to cause Ca^{2+} mobilization (22). Although the mechanism by which native TXA_2 causes platelet activation has not been investigated in this study, the present results support the concept that TXA_2 induces Ca^{2+} mobilization and platelet activation through its specific receptor.

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