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EFFECT OF A STIMULANT OF GUANYLATE CYCLASE, SIN 1, ON CALCIUM MOVEMENTS AND PHOSPHOLIPASE C ACTIVATION IN THROMBIN-STIMULATED HUMAN PLATELETS

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Abstract—The effects of sin 1, a metabolite of an antianginal agent, molsidomine, were investigated on human platelet activation induced by thrombin. This drug promoted a slight inhibition of serotonin release in a medium containing 1 mM Ca^{2+} or 1 mM EGTA (from 63% to 46% and from 57% to 41% of total serotonin secretion, respectively, with the highest dose used). Under these conditions, Ca^{2+} movements, monitored by quin 2 fluorescence, were markedly impaired. The most pronounced effect was towards Ca^{2+} influx, which presented a rapid inhibition with low doses. In the presence of external calcium, thrombin raised cytoplasmic free Ca^{2+} concentration from 100 nM to 1277 nM. This was reduced to 466 nM and 175 nM with 10^{-7} M and 10^{-4} M sin 1, respectively. Ca^{2+} mobilization from internal stores was less inhibited, since cytoplasmic free Ca^{2+} concentration was reduced from 148 nM with thrombin alone to 134 nM with 10^{-4} M sin 1. In addition to Ca^{2+} movements, sin 1 was tested on [^{32}P] phosphatidic acid synthesis resulting from phospholipase C activation induced by thrombin. Phosphatidic acid labelling displayed a maximal inhibition of 43–50% with the highest doses of sin 1 (10^{-4} M–10^{-3} M) with or without Ca^{2+} in the incubation medium. However, this effect appeared much more sensitive to sin 1 in the presence of external Ca^{2+} (25% at 10^{-7} M sin 1 with external Ca^{2+} against 12% at the same sin 1 concentration with EGTA). This discrepancy might be explained by the difference of cGMP level obtained when platelets were treated by sin 1 in the presence or in the absence of Ca^{2+} in the medium. This study shows that the major target of sin 1 via cGMP is not platelet phospholipase C as previously described, but inhibition of Ca^{2+} influx through plasma membrane.

Platelet activation induced by various physiological agents such as thrombin is followed by a rapid hydrolysis of inositol phospholipids by a specific phosphodiesterase (phospholipase C). This generates two second messengers, inositol-1,4,5-*tris*-phosphate (IP_3) and diacylglycerol (DAG) derived from phosphatidylinositol-4,5-bisphosphate (PIP_2) [1–4]. In platelets, DAG stimulates phosphorylation of a 40 kDa protein through activation of protein kinase C [5, 6], whereas IP_3 can mobilize Ca^{2+} from intracellular stores such as dense tubular system [7, 8]. The rise in cytosolic free Ca^{2+} concentration occurring during platelet activation is mainly due to an extracellular influx through the plasma membrane, involving yet unidentified putative Ca^{2+} channels, possibly linked to receptors [9–12]. However, a small part of this rise in cytosolic free Ca^{2+} can be attributed to some release from internal stores promoted by IP_3 [12]. Actually, such a concept is based on studies performed using quin 2 as a fluorescent probe. The low values of cytoplasmic free calcium concentration detected in platelets stimulated in the absence of extracellular Ca^{2+} might well be due to the high Ca^{2+} buffering capacity of quin 2, which is introduced in mM amounts into cell cytoplasm [9–13]. Indeed, the use of other fluorescent Ca^{2+} indicators such as fura 2 revealed that mobilization of intracellular Ca^{2+} could be sufficient to raise cytoplasmic free Ca^{2+} concentration to μM values [14]. This might indicate that Ca^{2+} influx is not so critical for platelet responses, which might still remain maximal under conditions where only Ca^{2+} from dense tubular system is mobilized.

Ca^{2+}-dependent pathways such as myosin light chain phosphorylation or phospholipase A_2 activation act in synergism with Ca^{2+}-independent pathway such as phospholipase C and protein kinase C activation [15, 16].

A retrocontrol of these mechanisms may be exerted by cyclic nucleotides. Cyclic AMP acts as a second messenger that mediates inhibition of platelet function, mainly by suppressing agonist induced cytosolic Ca^{2+} rise and regulating platelet Ca^{2+} homeostasis [17]. Cyclic GMP may act as a positive messenger for various hormones (neurotransmittors, growth factors etc.) [18]. In human platelets, this cyclic nucleotide seems to be a negative rather than a positive messenger. It was reported that thrombin or collagen stimulation induces an increase of cGMP level [19], which can also be promoted by different drugs such as nitrosocompounds (sodium nitroprusside) or cGMP analogs (8-bromo cGMP). In the latter case, this results in inhibition of platelet functions [20–23]. It was proposed that cGMP prevents Ca^{2+} mobilization and influx induced by throm-

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† Abbreviations used: IP_3, inositol-1,4,5-*tris*phosphate; DAG, diacylglycerol; PAF-acether, platelet activating factor (1-*O*-alkyl)-2-acetyl-*sn*-glycero-3-phosphocholine); IP_4, inositol-1,3,4,5-tetrakisphosphate.

bin, acting on receptor-linked generation of DAG [21]. Takai *et al.* [21] showed a blockade of thrombin-induced breakdown of inositol phospholipids, which parallels an inhibition of 40 kDa protein phosphorylation and of serotonin secretion.

In the present study, we used a guanylate cyclase activator, sin 1, an active metabolite of molsidomine. This compound inhibits platelet aggregation [24], probably owing to its ability to increase cGMP level [24, 25]. In order to study the mechanism of action of this drug, we tried to determine the effects of sin 1 on the different steps described above, such as Ca^{2+} fluxes and phospholipase C activation in thrombin stimulated human platelets.

MATERIALS AND METHODS

Materials. Quin 2 AM (quin 2 acetoxymethylester) and human thrombin (3000 NIH units/mg protein) were obtained from Sigma (Saint-Louis, MO). 5-[^{3}H] hydroxytryptamine (serotonin, 18 Ci/mmol), 8 [^{3}H] guanosine 3',5'-cyclic phosphate kit and [^{32}P] orthophosphate were from the Radiochemical Centre (Amersham, U.K.). Sin 1 was supplied by Hoechst (Paris, France).

Preparation of platelet suspensions. Fresh blood, obtained from human volunteer donors free of any medication for at least one week, was drawn onto acid citrate dextrose as an anticoagulant [26]. Platelet-rich plasma, obtained by centrifugation at 120 *g* for 20 min (20°), was incubated at 37° in the presence of [^{32}P]*o*-phosphate (100 μCi/ml) for 30 min. Quin 2 AM (15 μM) was then added for experiments where fluorescence was determined and incubation was continued for 30 min. Platelets were then acidified to pH 6.5 with citric acid, sedimented at 1000 *g* for 15 min (20°) and suspended in the same volume, compared to plasma, of Tyrode-Hepes buffer (pH 7.35) according to Lagarde *et al.* [26], resulting in a platelet concentration of about 3×10^8 cells/ml.

Determination of serotonin secretion. Serotonin release was assessed essentially as in [27]. Details are given in the legends of the figures.

Measurement of cytoplasmic free calcium. This was performed at 37° using a Jobin-Yvon spectrofluorimeter (model YJ 3C) essentially as described by Rink *et al.* [9]. Owing to some interference towards fluorescence occurring at high doses of sin 1, this was used for these experiments only up to 10^{-4} M sin 1, at which concentration no interference could be observed.

Determination of cyclic GMP content of platelets. Platelets were incubated as described in the incubation procedure, the reaction was stopped by adding EDTA (5 mM, final concentration), followed by boiling for 3 min. Samples were centrifuged at 1500 *g* for 10 min. Cyclic GMP in the supernatant was measured by radioimmunoassay.

Platelet aggregation. Platelet aggregation was measured at 37° according to Lagarde *et al.* [26].

Incubation of platelets. Platelet suspensions (0.5 ml aliquots) were equilibrated for 1 min at 37°, after addition of $CaCl_2$ (1 mM) or EGTA (1 mM). Sin 1 was added, the incubation was continued for 1 min and followed by addition of thrombin (0.5 IU/ml). After 1 min, the reaction was blocked by addition of 0.1 ml of 0.2 M EDTA (pH 7.4) and 1 ml of chloroform/methanol (1/1, vol./vol.).

Lipid analysis. Lipids were extracted according to Bligh and Dyer [28] after acidification with 10 N HCl. [^{32}P]-Labelled phospholipids were separated by monodimensional thin-layer chromatography on silicagel plates 0.25 mm thick (Merck, Darmstad, F.R.G.) using the procedure of Jolles *et al.* [29].

The various lipid spots were detected by autoradiography and then scraped directly into scintillation vials containing 10 ml Instagel (Packard). Radioactivity was determined using a Packard Tricarb 4530 spectrometer equipped with automatic quenching correction.

RESULTS

[^{3}H] Serotonin labelled platelets were preincubated for 1 min with various concentrations of sin 1, and then activated by thrombin (0.5 U/ml) for 1 min, in a medium containing Ca^{2+} (1 mM) or EGTA (1 mM). As shown in Fig. 1, sin 1 slightly inhibited thrombin-induced serotonin release which was reduced from 63% to 46% and from 57% to 41%, in the presence or in the absence of Ca^{2+}, respectively, upon addition of 10^{-4}–10^{-3} M sin 1. However, this decrease was never significant. Figure 2 reports aggregation curves obtained at the thrombin concentration (0.5 IU/ml) also used in previous experiments on serotonin release. Under these conditions, sin 1 inhibited platelet aggregation only partially, like serotonin release. A higher degree of

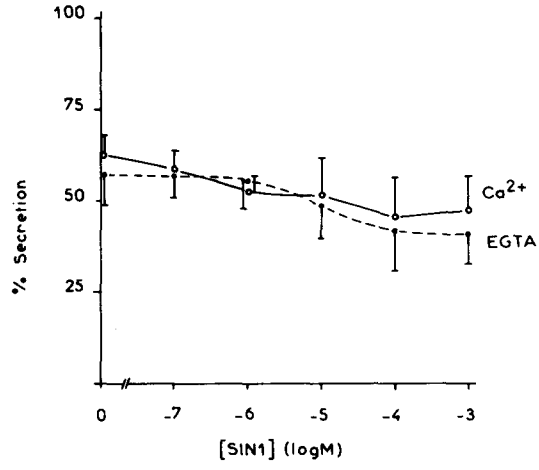

Fig. 1. Inhibitory effect of various concentration of sin 1 on serotonin secretion from platelets stimulated by thrombin. Aliquots of [^{3}H] serotonin labelled platelets were equilibrated for 1 min at 37° after addition of $CaCl_2$ (1 mM) or EGTA (1 mM). Various concentrations of sin 1 were added, incubation was continued for 1 min, followed by addition of thrombin (0.5 IU/ml). After 1 min, the reaction was stopped by adding 0.2 M EDTA (pH 7.4). After centrifugation at 1500 *g* for 5 min [^{3}H] serotonin in the supernatant was measured. Expressed as percentage of total platelet serotonin. Values were deduced for control [^{3}H] serotonin release, which was 4.8 ± 2.3%, independent of sin 1 concentrations. They correspond to the mean ± SEM of 4 experiments.

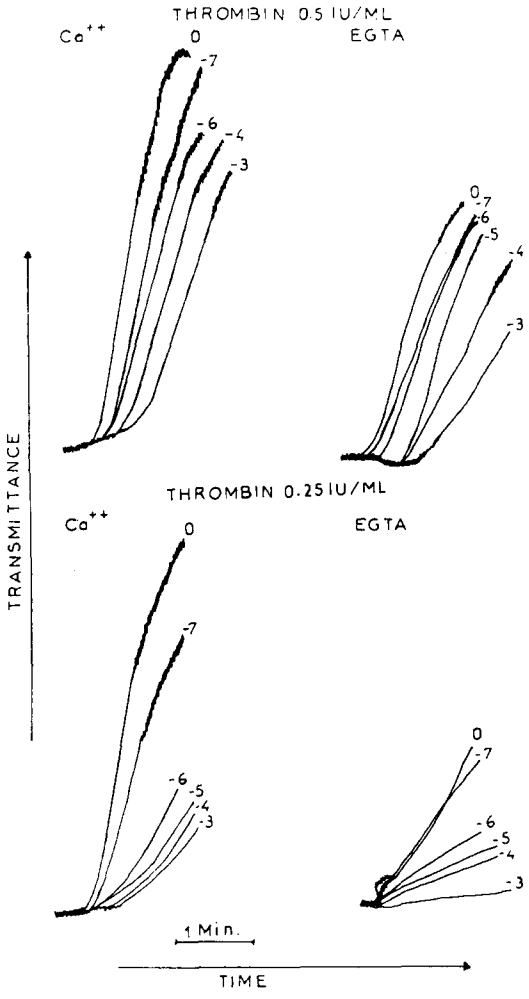

Fig. 2. Human platelet aggregation induced by thrombin 0.25 IU/ml and 0.5 IU/ml in the presence of different doses of sin 1. Typical recording representative of 3 different experiments.

inhibition was observed with a lower thrombin concentration (0.25 IU/ml, Fig. 2). In both cases, platelet aggregation was much lower in the absence of extracellular Ca^{2+}, indicating that Mg^{2+}, which was shown to replace Ca^{2+} in supporting ADP-induced platelet aggregation [30], was less active using thrombin as a stimulant. Under these conditions, aggregation appeared to be somewhat more sensitive to the inhibitory effect of sin 1. Figure 3 represents typical records of calcium movements, monitored by quin 2 fluorescence changes in platelets stimulated by thrombin and pretreated for 1 min by increasing sin 1 concentration. In the presence of Ca^{2+}, where the large fluorescence signal is mainly due to Ca^{2+} influx, sin 1 promoted a dose-dependent inhibition. This was no longer evident when considering the small signal detected in the absence of external Ca^{2+}, which corresponds to Ca^{2+} mobilization from dense tubular system (Fig. 3). More quantitative data about these effects are provided in Figs 4 and 5. In the presence of external Ca^{2+}, the level of cytoplasmic-free Ca^{2+} concentration obtained during thrombin stimulation decreased from 1277 nM with thrombin alone to 466 nM with the lowest dose of sin 1 (10^{-7} M). Although this difference was not significant, owing to large variations in Ca^{2+} signals, a significant decrease was obtained at 10^{-6} M sin 1. This was depressed to 175 nM at 10^{-4} M sin 1 (Fig. 4). The variability observed in basal cytoplasmic Ca^{2+} levels (Fig. 4) was not significant. The remaining increased level of Ca^{2+} observed in Fig. 4 with high doses of sin 1 may be attributed mainly to Ca^{2+} mobilized from internal stores and, to a minimal part, to Ca^{2+} influx. In contrast, Fig. 5 shows a slight effect of sin 1 on Ca^{2+} mobilization, which agrees with this hypothesis.

In addition to studies on Ca^{2+} movements, sin 1 was tested for its possible effects on phospholipase C activation, which was detected by generation of [^{32}P]-phosphatidic acid in ^{32}P-labelled platelets. As shown in Fig. 6, thrombin promoted an increased

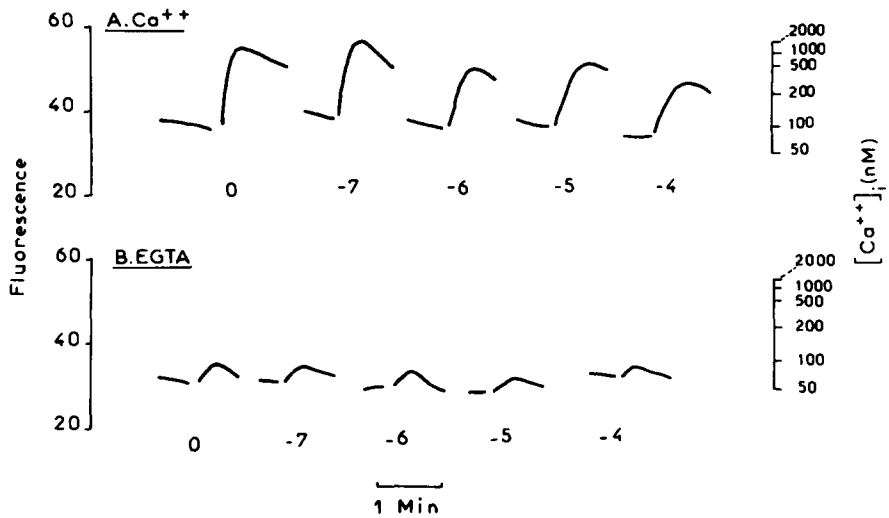

Fig. 3. Fluorescence changes of quin 2-loaded platelets induced by thrombin or thrombin with various concentrations of sin 1 (0, controls without sin 1). Graph recordings are from a typical experiment, representative of 6–7 different experiments.

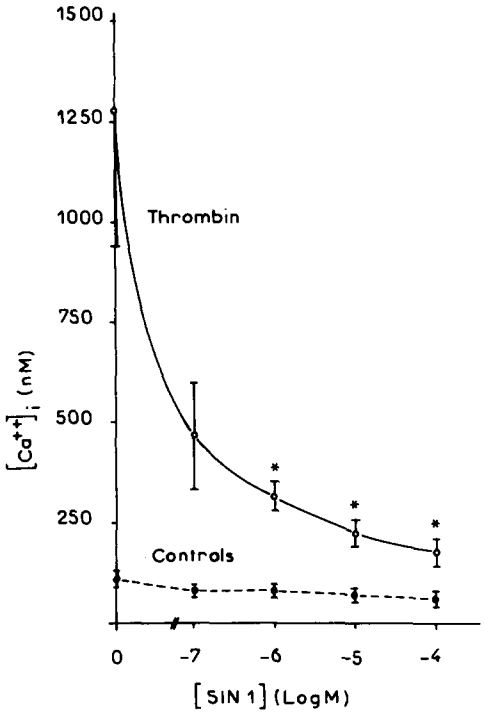

Fig. 4. Dose dependence of the inhibition by sin 1 of calcium movement (mainly influx) induced by thrombin in a Ca^{2+} containing medium. Ca^{2+} concentration is calculated as in [9]. Data (mean ± SEM) are from 6 experiments, described in Materials and Methods. Probability of significance using Student's *t*-test: * $P < 0.05$.

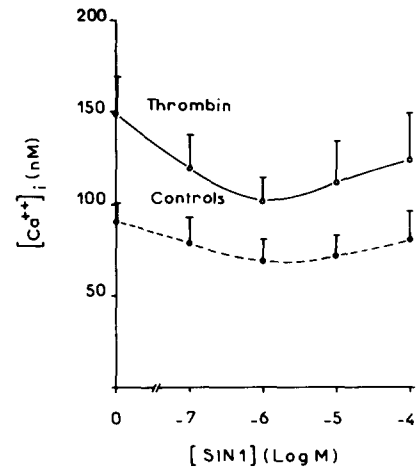

Fig. 5. Dose dependence of the inhibition by sin 1 of calcium mobilization from dense tubular system induced by thrombin in EGTA containing medium. Ca^{2+} is calculated as in [9]. Data (mean ± SEM) are from 7 experiments described in Materials and Methods. Differences were not significant using the Student's *t*-test.

labelling of [^{32}P]-phosphatidic acid whose radioactivity was multiplied by 5.0 and 6.0 over controls, in the presence and in the absence of external Ca^{2+}, respectively, but as previously reported [13–31] the difference was not significant. In both cases, sin 1 promoted a significant inhibition of [^{32}P] phosphatidic acid labelling, which attained 43–50% at the highest concentration used (10^{-4}–10^{-3} M) (see Fig. 6). However, [^{32}P] phosphatidic acid synthesis appeared much more sensitive to sin 1 in the presence of external Ca^{2+}, since maximal inhibition was obtained in this case at 10^{-7} M sin 1, whereas the same degree of inhibition required 100–1000-fold higher concentration of sin 1 in the presence of EGTA (Fig. 6).

These various changes were then compared to the effect of sin 1 on platelet cGMP levels. In agreement with previous observations [18], removal of Ca^{2+} from the extracellular medium resulted in a drastic fall of cGMP from 550 fmol to 40 fmol/10^8 platelets. However, in both cases, sin 1 promoted a dose-dependent accumulation of cGMP in platelets, which remained significantly higher in the presence of external Ca^{2+} (Fig. 7). cGMP increase could be observed in both control cells and thrombin-stimulated platelets. In the latter case, thrombin still amplified to some extent the increase in cGMP promoted by sin 1. When comparing these data to those of Figs 4 and 6, a significant inhibition of Ca^{2+} influx and phosphatidic acid synthesis was noted at around 10^{-7} M sin 1, which increased cGMP concentration to about 800 fmol/10^8 cells in the presence of Ca^{2+}. A similar inhibition in the presence of EGTA (Figs 5 and 6) required higher doses of sin 1 (10^{-4}–10^{-3} M) which promoted elevation of cGMP to about the same level (600–800 gmol/10^8 cells).

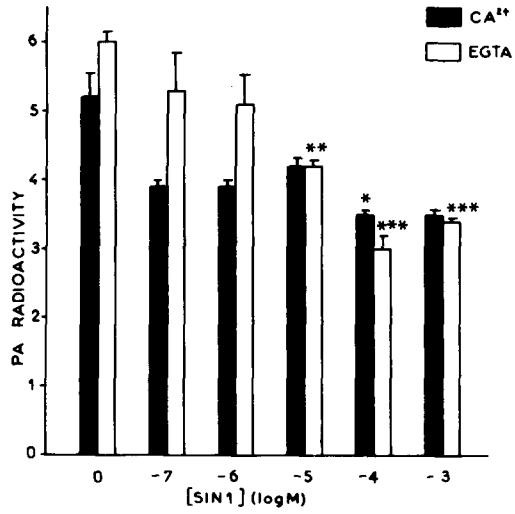

Fig. 6. Effect of various concentrations of sin 1 on [^{32}P] phosphatidic acid synthesis induced by thrombin in a medium containing Ca^{2+} (closed bars) or EGTA (open bars). Experimental conditions are given in Materials and Methods. [^{32}P] Phosphatidic acid (PA) radioactivity represents the ratio of [^{32}P] PA radioactivity to [^{32}P] PA radioactivity in control platelets. Results (mean ± SEM) are from 4 different experiments. Probability of significance using Student's *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

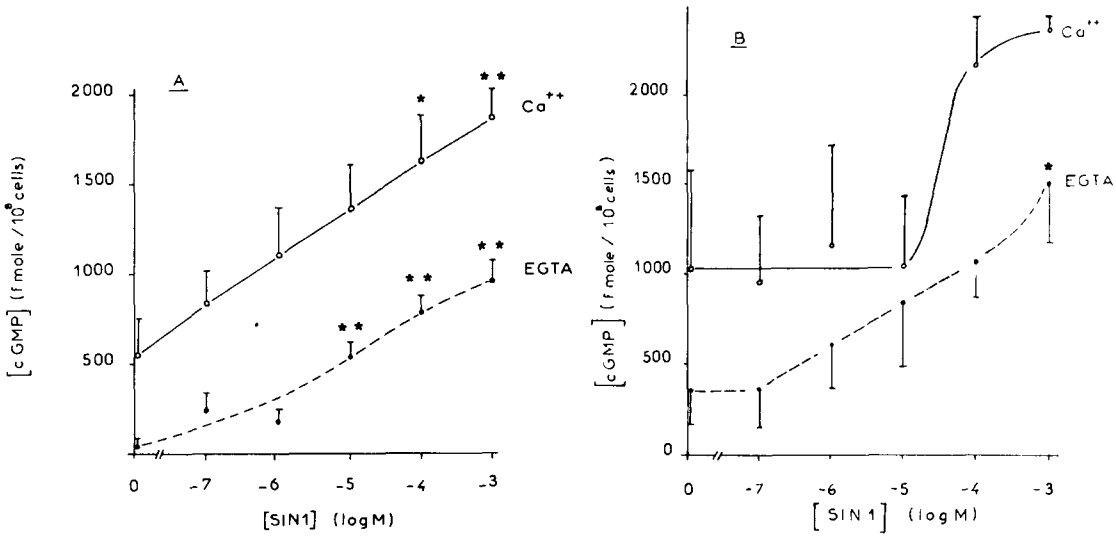

Fig. 7. Effect of increasing concentration of sin 1 on cGMP level of human platelets. (A) sin 1; (B) sin 1 + thrombin. For experimental details see Materials and Methods. Results (means ± SEM) are from 3 different experiments. Probability of significance using Student's *t*-test: * $P < 0.05$; ** $P < 0.01$.

DISCUSSION

This report describes the effects of sin 1 on various platelet responses evoked by thrombin stimulation, such as serotonin release, Ca^{2+} movements and phospholipase C activation. These effects were compared to cGMP increase induced by this drug, since it was previously shown that a major effect of sin 1 on platelets is to stimulate guanylate cyclase activity [24, 25] through a mechanism similar to that exerted by nitro-compounds such as sodium nitroprusside [21–23]. The precise role of this cyclic nucleotide in the regulation of platelet function is not well established. Previous studies have reported a rise in platelet cGMP content elicited by stimulatory agonists [19]. The mechanism of action of cGMP could imply a function of feedback regulator in platelet activation.

Under our experimental conditions, sin 1 triggered an increase in cGMP level, leading to a partial inhibition of serotonin release and phosphatidic acid synthesis induced by thrombin. However, the most important effect was observed on Ca^{2+} influx through the plasma membrane, whereas Ca^{2+} mobilization from internal stores appeared to be much less inhibited. The rise in cytosolic free calcium concentration occurring in EGTA medium depends on phosphodiesteratic cleavage of inositol-phospholipids by phospholipase C, which can occur at basal internal Ca^{2+} concentration [13, 31–32]. This pathway leads to the release of inositol-1,4,5-trisphosphate (IP_3) able to mobilize Ca^{2+} from internal stores (dense tubular system) [7, 8]. So there is a remarkable agreement between the observations that the elevation of cGMP promoted by sin 1 leads to an only partial inhibition of Ca^{2+} mobilization from dense tubular system and of phosphatidic acid synthesis. In other words, a significant phospholipase C activity still remains, thus generating enough IP_3 able to mobilize internal Ca^{2+}. Similar data were previously obtained with BN 52021, a specific antagonist of platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine or PAF-acether) [33].

On the other hand, it is highly improbable that the partial inhibition of phospholipase C activity induced by sin 1 is a consequence of the decrease in Ca^{2+} influx promoted by the drug, since we [13, 31] and others [32] previously reported that phospholipase C stimulation does not require Ca^{2+} influx.

As a main conclusion of this study, the major target of cGMP does not appear to be phospholipase C, as it was concluded from previous reports [21, 23]. These authors have shown a phospholipase C inhibition induced by cGMP, using drugs able to activate guanylate cyclase such as sodium nitroprusside, or a lipophilic analog of cGMP (8-bromo-cyclic GMP), with PAF-acether or thrombin as agonists. In all these studies, we can notice some residual Ca^{2+} mobilization and phosphatidic acid synthesis similar to our results. Thus, it is possible to assume that cGMP-induced inhibition occurs mainly towards Ca^{2+} influx and, to a lesser extent, towards phospholipase C activation. cGMP may interfere with the mechanism regulating Ca^{2+} influx, which is not well established, although Irvine and Moor [24] have shown that inositol-tetrakisphosphate (IP_4) synthesized through phosphorylation of IP_3 by a specific kinase, may be the messenger inducing Ca^{2+} influx. At this point of the study, we could not examine such an effect of cGMP on this particular pathway and IP_4 formation is not yet described in platelets.

cGMP production was less important in a medium containing EGTA than in a Ca^{2+} containing one. This result is consistent with previous reports on other cell systems [18] and would explain a higher inhibition of phospholipase C observed in a medium containing Ca^{2+}. However, as discussed by Goldberg

and Haddox [18], such an effect of Ca^{2+} still remains poorly understood. These authors suggested that Ca^{2+} might activate phospholipase A_2, resulting in the liberation of arachidonic acid with concomitant production of lipoxygenase metabolite, which were shown to stimulate soluble guanylate cyclase [35–41]. Although we previously obtained good evidence for a high sensitivity of platelet phospholipase A_2 to Ca^{2+} [13], our present measurements were performed in non-stimulated cells, where basal activity of phospholipase A_2 should have remained very low.

In conclusion, sin 1, via cGMP, affects platelet activation by inhibiting mainly Ca^{2+} influx through the plasma membrane and to a lesser extent, phospholipase C activation. To the best of our knowledge, this is the first example dealing with platelets where a close dissociation between Ca^{2+} mobilization and Ca^{2+} influx can be observed. On a biochemical point of view, it opens very interesting questions concerning the mechanism of coupling between receptors to various agonists like thrombin and membrane putative Ca^{2+} channels or phospholipase C, respectively. Indeed, these mechanisms might be different, at least in their sensitivity to cGMP.

On a biological and pharmacological point of view, one can observe that the large inhibition exerted by sin 1 towards Ca^{2+} influx actually does not parallel the decrease in serotonin secretion, which seems to depend essentially on phosphatidic acid synthesis, i.e. phospholipase C activation (compare Fig. 1 and Fig. 6). This would indicate that Ca^{2+} influx is not so critical in the regulation of platelet function, at least under the experimental conditions used throughout this study, using relatively large thrombin concentration (0.5 IU/ml). Indeed, aggregation experiments reported in Fig. 2 indicate a higher sensitivity of platelet to sin 1 when thrombin concentration is reduced.

The fact that Ca^{2+} influx apparently does not play a major role in platelet aggregation or secretion would fit with the recent report of Pollock and Rink [14] showing that Ca^{+} mobilization from dense tubular system seems to be sufficient to raise cytosolic free Ca^{2+} concentration to levels as high as 1 μM. Further studies will be necessary to see whether Ca^{2+} influx does not play a more critical role at lower concentrations of agonist evoking a less important phospholipase C activation. Finally, in contrast to platelet, other cell systems, like smooth muscle cells, might be more dependent on Ca^{2+} influx and thus more sensitive to sin 1. In this respect, it is worth recalling that this drug is of a potent vasodilator. So, even if platelets are not the best target of sin 1 and of other activators of guanylate cyclase, they remain a very useful model to study their mechanism of action owing to the possibility to detect in a rather easy way various signalling events such as Ca^{2+} movements and phospholipase C activation.

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