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Short Communication

# Cyclopiazonic acid and thapsigargin induce platelet aggregation resulting from $Ca^{2+}$ influx through $Ca^{2+}$ store-activated $Ca^{2+}$- channels

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**Abstract**

The effects of cyclopiazonic acid and thapsigargin, selective inhibitors of the endoplasmic reticulum $Ca^{2+}$-ATPase pump, on the platelet aggregation were investigated using washed rat platelets prepared by chromatography on Sepharose 2B columns. In $Ca^{2+}$-free medium, cyclopiazonic acid and thapsigargin did not induce aggregation, but in the presence of 1 mM $Ca^{2+}$, platelet aggregation was induced in a concentration-dependent manner. Cyclopiazonic acid- and thapsigargin-induced platelet aggregation was blocked by 1 mM $Ni^{2+}$ but not by 100 $\mu$M indomethacin or 1 $\mu$M nifedipine. In aequorin-loaded platelets, cyclopiazonic acid and thapsigargin caused sustained elevation of the cytosolic $Ca^{2+}$ concentration, an effect which was blocked by $Ni^{2+}$, a non-selective $Ca^{2+}$ channel blocker and SK&F 96365 (1-{$\beta$-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenyl}-1 H-imidazole hydrochloride), a putative receptor-operated $Ca^{2+}$ channel antagonist. The above results indicated that both cyclopiazonic acid and thapsigargin induced platelet aggregation and elevation of cytosolic $Ca^{2+}$ concentration, that extracellular $Ca^{2+}$ was essential for cyclopiazonic acid- and thapsigargin-induced platelet aggregation, and that platelet aggregation may be associated with $Ca^{2+}$ influx through $Ca^{2+}$ store-activated $Ca^{2+}$ channels. © 1998 Elsevier Science B.V.

*Keywords:* Cyclopiazonic acid; Thapsigargin; Platelet, rat; $Ca^{2+}$; Aggregation; Aequorin

## 1. Introduction

Cyclopiazonic acid, thapsigargin and 2,5-di-*tert*-butyl-1,4-benzohydroquinone (tBuBHQ) have been shown to be potent and selective inhibitors of the sarcoplasmic reticulum/endoplasmic reticulum ($Ca^{2+}$-ATPase pump in skeletal, cardiac and vascular smooth muscles (Seidler et al., 1989; Deng and Kwan, 1991; Shimamoto et al., 1992; Janczewski and Lakatta, 1993). These $Ca^{2+}$-pump inhibitors cause an elevation of cytosolic $Ca^{2+}$ concentration in intact cells via the spontaneous release of $Ca^{2+}$ from intracellular stores as a result of prevention of $Ca^{2+}$ sequestration by endoplasmic reticulum (Brune and Ullrich, 1991; Low et al., 1992; Janczewski and Lakatta, 1993). Therefore, they have been widely used in the study of the role of intracellular $Ca^{2+}$ in various cellular events, including platelet activation, aggregation and secretion (Brune and Ullrich, 1991; Malcolm and Fitzpatrick, 1992; Tao and Haynes, 1992; Authi et al., 1993; Kimura et al., 1993; Dachary-Prigent et al., 1995). However, different $Ca^{2+}$-pump inhibitors seem to exert different effects on platelet activity. For example, coagulant activity and microparticle formation during platelet activation depend on an increase in cytosolic $Ca^{2+}$ levels and are inhibited by thapsigargin and cyclopiazonic acid, but not by tBuBHQ (Dachary-Prigent et al., 1995). Furthermore, thapsigargin induces aggregation and secretion responses, but tBuBHQ only induces shape change (Authi et al., 1993). The effect of cyclopiazonic acid on platelet aggregation has not been investigated. Recently, the aggregation activity of human platelets elicited by thapsigargin has been attributed in part to the response secondary to endogenous thromboxane $A_2$/prostaglandin $H_2$ formation which is inhibited by aspirin and indomethacin (Malcolm and Fitzpatrick, 1992; Authi et al., 1993). However, in our preliminary experiments, we did not observe the inhibitory effect of indomethacin on thapsigargin-induced aggregation of chromatographically isolated rat platelets. In view of the above

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findings, we now used washed rat platelets isolated by filtration chromatography on Sepharose 2B column to study the action of cyclopiazonic acid as well as the effect of $Ca^{2+}$ on platelet aggregation, compared to the effect of thapsigargin.

## 2. Materials and methods

### 2.1. Preparation of washed platelets

Blood (3–5 ml/rat) drawn from the carotid artery or heart of male Wistar rats weighing 250–275 g was dissolved into 1/10 volume of 3.8% trisodium citrate and centrifuged at 1,000 rpm for 10 min. The supernatant, platelet-rich plasma (PRP), was applied to a sepharose 2B column ($2 \times 15$ cm) and platelet fractions were eluted with 0.05 M Tris–HCl buffer (pH 7.2), containing 140 mM NaCl, 1 mM $MgCl_2$, 0.1% glucose and 0.4% bovine serum albumin. A platelet suspension of $2 \times 10^5$/ml was prepared using the above elution buffer (Kawai et al., 1994).

### 2.2. Platelet aggregation

Aggregation was quantified photometrically as the maximal rate of change in light transmission through a 200 $\mu$l sample of washed platelet preparation at 37°C under constant stirring in the test solution. The reference contained only the above Tris buffer. Platelet aggregation was measured using the Chrono-Log aggregometer (USA) controlled by an IBM computer through a Agg-Link interface connecting to two recorders, allowing simultaneous measurement of two samples (Huang and Kwan, 1996). $Ca^{2+}$ was added before the addition of other reagents to induce aggregation unless otherwise indicated. Nifedipine and indomethacin were incubated for 10 min with the platelet preparation before the addition of other reagents.

### 2.3. Measurement of cytosolic $Ca^{2+}$ in aequorin-loaded platelets

Cytosolic $Ca^{2+}$ was monitored as chemiluminescence in aequorin-loaded platelets (Yamaguchi et al., 1986) according to procedures described earlier (Johnson et al., 1985). Briefly, rat PRP acidified to pH 6.1 with 1 M citric acid was washed with HEPES-buffer saline (0.14 M NaCl, 2.7 mM KCl, 0.1% bovine serum albumin, 0.1% glucose and 3.8 mM HEPES pH 7.6) containing 5 mM EGTA and 1 $\mu$M prostaglandin $E_1$. The platelets were then suspended in 90 $\mu$l of the same buffer and 10 $\mu$l of 3 mg/ml aequorin solution. Dimethylsulfoxide (DMSO) was added stepwise to obtain a final concentration of 6% in the suspension and the mixture was incubated for 2 min. The suspension was subsequently diluted with 1 ml of HEPES-buffer saline, centrifuged, and washed 3 times, each time with 1 ml of the same HEPES. Finally, the platelets were suspended, $3–5 \times 10^5$ platelets/ml, in the HEPES buffer saline containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for the determination of platelet cytosolic $Ca^{2+}$ in the Chrono-Log aggregometer.

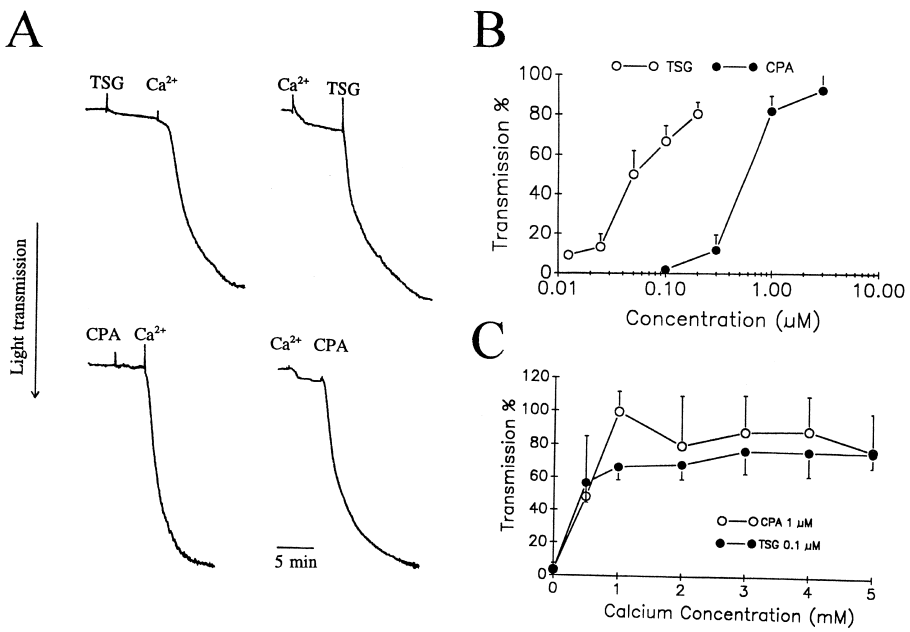


Fig. 1. (A) The effect of 0.1 $\mu$M thapsigargin (TSG) and 1.0 $\mu$M cyclopiazonic acid (CPA) on platelet aggregation in the presence and absence of 2 mM $Ca^{2+}$. (B) Concentration dependence on cyclopiazonic acid and thapsigargin of the platelet aggregation in the presence of 1.0 mM $Ca^{2+}$ (mean ± S.D.; $n = 4–7$). (C) $Ca^{2+}$-concentration dependence of platelet aggregation induced by 1.0 $\mu$M cyclopiazonic acid and 0.1 $\mu$M thapsigargin (mean ± S.D.; $n = 4$). Optimal effect was observed at 1.0 mM $Ca^{2+}$.

### 2.4. Materials

Sepharose 2B was purchased from Pharmacia (Sweden), Arachidonic acid from Chrono-Log (USA). Thapsigargin, cyclopiazonoic acid (from *Penicillium cyclopium*) and aequorin (from jellyfish) and other chemicals were from Sigma (St. Louis).

## 3. Results

Fig. 1A shows that 1.0 $\mu$M cyclopiazonic acid and 0.1 $\mu$M thapsigargin induced prominent aggregation of washed rat platelets in the presence but not in the absence of 1 mM $Ca^{2+}$. The platelet aggregation induced by cyclopiazonic acid and thapsigargin was dependent upon the concentration of thapsigargin and cyclopiazonic acid (Fig. 1B), as well as of $Ca^{2+}$ (Fig. 1C). The maximal effect induced by cyclopiazonic acid was similar to that induced by thapsigargin, but the potency of cyclopiazonic acid was lower than that of thapsigargin by about one log unit (Fig. 1B).

To investigate the route of the entry of $Ca^{2+}$ induced by cyclopiazonic acid or thapsigargin, leading to platelet aggregation, the effect of nifedipine, an L-type $Ca^{2+}$ channel antagonist, and $Ni^{2+}$, a non-selective $Ca^{2+}$ entry blocker, on platelet aggregation was investigated. Nifedipine at 1 $\mu$M did not prevent the platelet aggregation induced by cyclopiazonic acid, thapsigargin, and ADP in the presence of 1 mM $Ca^{2+}$. The rate of platelet aggregation induced by 1 $\mu$M cyclopiazonic acid in the washed platelet preparations with and without preincubation with 1 $\mu$M nifedipine for 10 min was $74.3 \pm 3.9\%$ and $76.5 \pm 9.8\%$, respectively ($n = 6$, $P > 0.05$), while that induced by 0.1 $\mu$M thapsigargin with and without preincubation with nifedipine, was $61.0 \pm 13.6\%$ and $60.8 \pm 11.7\%$, respectively ($n = 6$, $P > 0.05$). In contrast, 1 mM $Ni^{2+}$, a non-specific $Ca^{2+}$ channel blocker, effectively prevented cyclopiazonic acid- and thapsigargin-induced platelet aggregation in the presence of 1 mM $Ca^{2+}$ (Fig. 2A), but did not prevent ADP- and A23187-induced aggregation. The A23187-induced platelet aggregation rate without and with 1 mM $Ni^{2+}$ was $70.8 \pm 4.1\%$ and $61.4 \pm 4.4\%$, respectively ($n = 5$).

Fig. 2B shows that, in the presence of 1 mM $Ca^{2+}$, 1 $\mu$M cyclopiazonic acid induced an elevation of cytosolic $Ca^{2+}$ concentration, manifested as an increase in luminescence signals, all of which could be reversed by 60 $\mu$M SK&F 96365 or 1 mM $Ni^{2+}$, but not by 1 $\mu$M nifedipine (not shown; similar results were also observed with 0.1 $\mu$M thapsigargin). In contrast, the elevation of cytosolic $Ca^{2+}$ concentration induced by 0.1 $\mu$M A23187, a $Ca^{2+}$ ionophore, could not be inhibited by either 60 $\mu$M SK&F 96365 or 1 mM $Ni^{2+}$ since the entry of $Ca^{2+}$ brought about by ionophore does not involve membrane protein channels. However, there seemed to be a slight reduction by SK&F 96365 of the $Ca^{2+}$ level in each case (Fig. 2B). This could have resulted from the overshoot of plasmalemmal $Ca^{2+}$-pump activation in response to sustained elevation of cytosolic $Ca^{2+}$ followed by a very rapid blockade of $Ca^{2+}$ entry. Alternatively, the reduction of cytosolic $Ca^{2+}$ level by SK&F 96365 to slightly below the original resting level may have reflected the presence of non-selective leak channels sensitive to the blockade by SK&F 96365 (Leung et al., 1996).

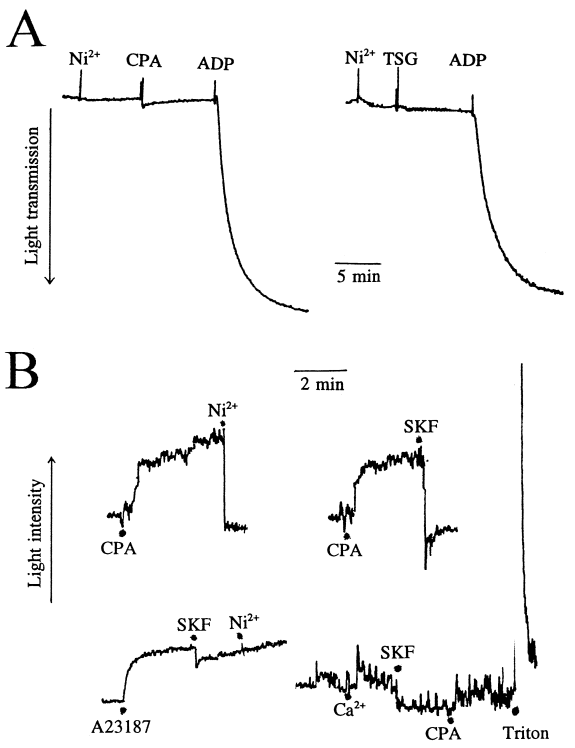


Fig. 2. (A) Prior incubation of 1.0 mM $Ni^{2+}$ with platelets in the presence of 1.0 mM $Ca^{2+}$ inhibited the aggregation induced by 1.0 $\mu$M cyclopiazonic acid (CPA) or 0.1 $\mu$M thapsigargin (TSG), but elicited no effect on aggregation induced by 5.0 $\mu$M ADP. (B) The addition of 1.0 mM $Ni^{2+}$ or 60 $\mu$M SK&F 96365 (SKF) in the presence of 1 mM $Ca^{2+}$ inhibited the elevation of cytosolic $Ca^{2+}$ level induced by cyclopiazonic acid (1.0 $\mu$M), but not that induced by 1.0 $\mu$M A23187. The addition of 1.0 mM $Ca^{2+}$ to platelets in nominally $Ca^{2+}$-free medium elicited a very small rise of cytosolic $Ca^{2+}$ level which was inhibited by 60 $\mu$M SK&F; subsequent application of 1.0 $\mu$M cyclopiazonic acid elicited only a very small transient elevation of aequorin chemiluminescence which was fully expressed upon cell rupture by means of 0.1% Triton X-100 (Triton).

Malcolm and Fitzpatrick (1992) reported that human platelets treated with thapsigargin had extensive eicosanoid formation, and the resulting platelet aggregation could be attenuated by ibuprofen and aspirin, thus suggesting an indirect action of thapsigargin on platelet aggregation. Authi et al. (1993), however, reported that thapsigargin still induced aggregation and secretion in indomethacin-treated platelets. In the present study using rat platelets, indomethacin at 100 $\mu$M, causing suppression of the platelet aggregation induced by arachidonic acid from $62.5 \pm 8.6\%$ to $37.3 \pm 14.6\%$ ($n = 6$, $P < 0.05$), failed to prevent platelet aggregation induced by 1 $\mu$M cyclopiazonic acid or 0.1 $\mu$M thapsigargin in the presence of 1 mM $Ca^{2+}$. The maximal platelet aggregation induced by

cyclopiazonic acid with and without preincubation with indomethacin for 10 min was 76.7 ± 9.0% and 77.7 ± 16.8%, respectively ($n = 7$, $P > 0.05$), while that induced by thapsigargin was 74.3 ± 10.2% and 73.8 ± 11.4%, respectively ($n = 6$, $P > 0.05$).

## 4. Discussion

We report here our novel findings that washed rat platelets separated by filtration chromatography on Sepharose 2B column were able to aggregate in the presence of cyclopiazonic acid, a completely reversible inhibitor of the endoplasmic reticulum $Ca^{2+}$ pump. The effects of cyclopiazonic acid were compared to those of the more commonly used irreversible inhibitor of the endoplasmic reticulum $Ca^{2+}$ pump, thapsigargin, and found the following common characteristics: (a) both showed similar concentration dependence, with thapsigargin being more potent than cyclopiazonic acid; (b) the aggregation depended on extracellular $Ca^{2+}$; (c) their effects could not be inhibited by indomethacin or by nifedipine (a specific L-type $Ca^{2+}$ channel blocker), but could be prevented by $Ni^{2+}$ (a non-specific $Ca^{2+}$ channel blocker); and (d) both drugs caused elevation of cytosolic $Ca^{2+}$ at the concentration also effective for platelet aggregation, and the increase in $Ca^{2+}$ could be blocked by SK&F 96365, a receptor-operated $Ca^{2+}$ channel blocker, which has previously been shown to inhibit platelet aggregation (Merritt et al., 1990).

### *4.1. Cyclopiazonic acid-induced platelet aggregation requires extracellular $Ca^{2+}$*

Extracellular $Ca^{2+}$ is essential for both cyclopiazonic acid- and thapsigargin-induced platelet aggregation, because in $Ca^{2+}$-free medium, no platelet aggregation was observed upon addition of cyclopiazonic acid or thapsigargin, despite the fact that these endoplasmic reticulum $Ca^{2+}$-ATPase pump inhibitors can induce small transient elevation of cytosolic $Ca^{2+}$. The requirement of extracellular $Ca^{2+}$ for rat platelet aggregation has also been demonstrated in aggregation induced by Cobra venom peptide, cardiotoxin, which interacts with the platelet cell membrane and enhances membrane permeability to $Ca^{2+}$ (Huang and Kwan, 1996). The optimal effect on platelet aggregation induced by cardiotoxin, as in the present study, was attained at 1–4 mM $Ca^{2+}$. However, in contrast to our present observation, prior addition of 5 mM $Ca^{2+}$ protected the platelets from cardiotoxin-induced aggregation. Since the platelet aggregation induced by cyclopiazonic acid and thapsigargin (present work), which act at an intracellular membrane site, and that induced by ADP (Huang and Kwan, 1996), which acts at the cell membrane receptor site, were not affected by prior addition of 5 mM $Ca^{2+}$, it is unlikely that high concentrations of $Ca^{2+}$ exert any membrane-stabilizing effect, or direct interaction with cyclopiazonic acid.

### *4.2. Cyclopiazonic acid-induced aggregation utilizes $Ca^{2+}$ entry via non-selective cation-channels*

These results obtained with nifedipine and $Ni^{2+}$ indicate that the platelet aggregation induced by $Ca^{2+}$-pump inhibitors was not associated with $Ca^{2+}$ entry via nifedipine-sensitive L-type $Ca^{2+}$ channels, and support the contention that aggregation may utilize $Ca^{2+}$ release-activated, non-selective cation channels as observed in endothelial cells (Pasyk et al., 1995) and vascular smooth muscle cells (Deng and Kwan, 1991; Low et al., 1992). Indeed, thapsigargin has been reported to increase cytosolic concentration of $Ca^{2+}$ and $Na^{+}$ in human and rat platelets (Heemskerk et al., 1994). Elevation of the cytosolic $Na^{+}$ concentration was more pronounced in $Ca^{2+}$-free medium, indicating that some of the $Na^{+}$ enters through $Ca^{2+}$ entry pathways (Kimura et al., 1993). Such a contention is also in accordance with the fact that platelet aggregation was also inhibited by SK&F 96365 (Merritt et al., 1990) and tetrandrine (Chen et al., 1996), which were reported to inhibit platelet aggregation via non-L-type $Ca^{2+}$ channels.

### *4.3. Cytosolic $Ca^{2+}$ concentration measurements agree with aggregation studies*

The results (Fig. 2B) obtained with aequorin-loaded rat platelets are well consistent with those of aggregation studies as presented in Fig. 1A and Fig. 2A. In the absence of cyclopiazonic acid, $Ca^{2+}$ alone caused only a small increase of luminescence, this being consistent with the small changes seen in the aggregation experiment (Fig. 1A). Subsequent addition of 60 $\mu$M SK&F 96365 prevented the sustained rise of cytosolic $Ca^{2+}$ concentration induced by 1.0 $\mu$M cyclopiazonic acid, which was similar to that elicited by 1.0 $\mu$M cyclopiazonic acid in $Ca^{2+}$-free medium (not shown). Apparently, the small transient elevation of cytosolic $Ca^{2+}$ by cyclopiazonic acid due to $Ca^{2+}$ release in the absence of $Ca^{2+}$ entry (in $Ca^{2+}$-free medium or in the presence of $Ni^{2+}$) was not capable of causing platelet aggregation (see Fig. 1A and Fig. 2A), thus reinforcing the essential role of $Ca^{2+}$ entry in the process leading to platelet aggregation.

Although the results obtained in aggregation studies are qualitatively consistent with those of cytosolic $Ca^{2+}$ measurements, we would point out that chromatographically washed rat platelets were chemically more sensitive, so that DMSO concentrations higher than 1.0% (v/v) also caused membrane aggregation. This precluded the use of DMSO-facilitated loading of aequorin for chromatographically isolated platelets and we used the EGTA wash/centrifugation method originally proposed by Johnson et al. (1985). It is possible that some endogenous substances which prevent spontaneous platelet aggregation may have been removed chromatographically from the platelet membranes.

### 4.4. Conclusion

Unlike receptor agonists, cyclopiazonic acid and thapsigargin interfere specifically with the internal $Ca^{2+}$ store, causing $Ca^{2+}$ release-activated $Ca^{2+}$ entry, which in turn leads to platelet aggregation. The relatively less potent effect of cyclopiazonic acid (compared to thapsigargin) on platelet aggregation also agrees well with its less potent inhibitory effect on endoplasmic reticulum $Ca^{2+}$-ATPase (Darby et al., 1996). The present findings let us to conclude that cyclopiazonic acid, like thapsigargin, causes aggregation of washed rat platelets via influx of $Ca^{2+}$ as a result of depletion of intracellular $Ca^{2+}$ stores, independent of endogenous lipid mediator formation.

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