J Anesth (2004) 18:107–112
DOI 10.1007/s00540-004-0228-6

Journal of
Anesthesia

Toborinone and olprinone, phosphodiesterase III inhibitors, inhibit human platelet aggregation due to the inhibition of both calcium release from intracellular stores and calcium entry

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Abstract
Purpose. We investigated the inhibitory effects of toborinone and olprinone on human platelet aggregation and calcium mobilization.
Methods. Washed human platelets were preincubated with toborinone or olprinone, then exposed to 0.015 U·ml^{-1} of thrombin. Aggregation curves were measured using an aggregometer. Effects of toborinone or olprinone on changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were measured fluorometrically using fura-2 acetoxymethyl ester (fura-2). Levels of intracellular cyclic 3′,5′-adenosine monophosphate concentration ($[cAMP]_i$) were also measured, using enzyme-linked immunosorbent assay (ELISA) techniques.
Results. The concentrations required to cause 50% inhibition of aggregation (IC_{50}) induced by thrombin were 9.7 ± 0.9 μM for toborinone and 3.6 ± 0.2 μM for olprinone. Both drugs at IC_{50} significantly elevated $[cAMP]_i$ levels and significantly inhibited Ca^{2+} release from intracellular stores. Release of $[Ca^{2+}]_i$ induced by thrombin was 272.9 ± 87.1 nM, 153.3 ± 28.7 nM, and 138.9 ± 58.2 nM in the control, toborinone, and olprinone groups, respectively ($P < 0.02$). Calcium influx through calcium channels in the plasma membrane was also suppressed by toborinone and olprinone.
Conclusion. Toborinone (9.7 μM) and olprinone (3.6 μM) inhibit human platelet aggregation, though these concentrations are higher than their therapeutic plasma concentrations. The inhibitory effects of both drugs are related to the inhibition of both Ca^{2+} release and Ca^{2+} entry through $[cAMP]_i$ elevation.

Key words Toborinone · Olprinone · Phosphodiesterase III inhibitor · Platelet

Introduction

Toborinone and olprinone are phosphodiesterase III (PDE III) inhibitors that are used to treat heart failure [1–3]. As noncatecholamine, nonglycosidic inotropic agents, they increase myocardial intracellular cyclic 3′,5′-adenosine monophosphate concentration ($[cAMP]_i$) by selective inhibition of cardiac PDE III enzymes, and they increase intracellular calcium delivery, thereby increasing myocardial contractility. These agents also increase cAMP in vascular smooth muscle, resulting in direct vasodilator activity. These combined positive inotropic and vasodilatory effects are ideal for treating severe congestive heart failure. Conversely, however, by increasing cAMP, these agents may affect intracellular calcium concentration ($[Ca^{2+}]_i$) and inhibit platelet aggregation. These agents evidently have the potential to provide beneficial antithrombotic properties [4], particularly in unstable angina and myocardial infarction. We have recently reported that milrinone, at clinical concentrations (0.9 μM), inhibits thrombin-induced platelet aggregation, an effect that is predominantly mediated by the suppression of calcium release from the dense tubular system [5]. Other studies have reported the inhibition of platelet aggregation and intracellular calcium mobilization by the PDE III inhibitors, milrinone and amrinone [6–8]. However, information regarding the inhibition of platelet aggregation and calcium mobilization in human platelet-rich plasma (PRP) is currently unavailable for toborinone and olprinone. Calcium signaling has been considered to be important in numerous platelet processes. The present study examined the effect of olprinone and toborinone on platelet aggregation and on intracellular calcium mobilization.

Subjects, materials, and methods

The Ethics Committee on Human Research of Kyoto Prefectural University of Medicine approved this study. Written, fully informed consent was obtained from all participants.

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Received: May 2, 2003 / Accepted: January 14, 2004

Platelet preparation

Blood samples from the antecubital veins of eight healthy volunteers who had not taken any drugs for at least 2 weeks were collected into plastic tubes containing acid-citrate-dextrose solution, comprising 85 mM sodium citrate, 70 mM citric acid, and 110 mM glucose. The ratio of blood to acid-citrate-dextrose solution was 4:1. Samples were centrifuged at 110 *g* for 15 min and the upper layer of PRP was removed. PRP samples were incubated with 3 mM fura-2 acetoxymethyl ester (fura-2) for 15 min at 37°C, then centrifuged at 250 *g* for 15 min in the presence of 0.3 U·ml^{-1} apyrase. A washed platelet suspension (WPS) loaded with fura-2 was obtained by discarding the supernatant and resuspending the pellet in Tyrode-Hepes buffer at pH 7.4 [9]. To examine aggregation curves and intracellular calcium mobilization, and to measure cAMP concentrations, numbers of platelets in the WPS were adjusted to 10^5 cells·μl^{-1}, using a Coulter Counter Model II (Coulter Electronics, Hialeah, FL, USA).

Determination of concentrations of toborinone and olprinone required to cause 50% inhibition of aggregation (IC_{50}) induced by thrombin

Platelet aggregation curves were investigated, using a turbidimetric method, in samples. Aliquots of WPS (245 μl) were placed into a silicon-coated glass tube, maintained at 37°C, and stirred at 1000 rpm throughout the experiments. Extracellular free calcium concentrations were adjusted to 1 mM using $CaCl_2$, and mixtures were incubated for 1 min with 5 μl of toborinone (seven concentrations, 0.2–200 μM) or olprinone (seven concentrations, 0.0656–65.6 μM). Platelet aggregation induced by thrombin (final concentration, 0.015 U·ml^{-1}) was measured for 5 min, using an aggregometer (Hema Tracer 601; Nikoh Bioscience, Tokyo, Japan). The baseline optical density point was defined as 0% for WPS and 100% for distilled water. To samples in the control group, 5 μl of distilled water was added. The IC_{50} values of toborinone and olprinone were calculated using an equation of the first degree.

Measurement of intracellular cyclic 3′,5′-adenosine monophosphate concentration ($[cAMP]_i$)

After the incubation of PRP with distilled water, or with toborinone (final concentration, 9.7 μM) or olprinone (final concentration, 3.6 μM) for 1 min at 37°C in the presence of 1 mM $CaCl_2$, samples were stimulated for 5 min with 0.015 U·ml^{-1} of thrombin. Reactions were stopped by the addition of ice-cold 99.8% ethanol. Samples were centrifuged at 1000 *g* for 20 min at 4°C, then $[cAMP]_i$ was measured in the supernatant, using an enzyme immunoassay kit, according to the non-acetylation protocol described by the manufacturer (Amersham International, Amersham, Buckinghamshire, UK). Values for Results were expressed as pmol·10^{-8} platelets.

Measurement of intracellular free Ca^{2+} mobilization

Aliquots of WPS (490 μl) loaded with fura-2 were added to a fluorometric cuvette and stirred at 1000 rpm in a fluorometer (CAF-110; JASCO, Tokyo Japan). After the incubation of samples with 10 μl of distilled water (control) or with toborinone (final concentration, 9.7 μM) or olprinone (final concentration, 3.6 μM) for 1 min at 37°C in the presence of 1 mM $CaCl_2$ to record basal fluorescence intensity, stimulation was performed for 5 min, using 0.015 U·ml^{-1} thrombin. Excitation wavelengths were 340 nm and 380 nm, and the emission wavelength was 505 nm [10]. Changes in $[Ca^{2+}]_i$ were monitored using the fura-2 340/380-fluorescence ratio, calculated according to the equation described by Tsien et al. [11], using a dissociation constant for fura-2 and $[Ca^{2+}]_i$ of 224 nM.

As nickel ions (Ni^{2+}) block the influx of extracellular calcium, $NiCl_2$ (final concentration, 1 mM) was added to fura-2-loaded human platelets instead of $CaCl_2$. Under these conditions, the effects of toborinone or olprinone on thrombin-induced calcium release from intracellular stores such as the dense tubular system were examined [12,13].

The entry of manganese ions (Mn^{2+}) was also evaluated, using a quenching technique, to investigate the influx of calcium into the cytosol. $MnCl_2$ (final concentration, 1 mM) was added to fura-2-loaded human platelets, and fura-2 leakage was assessed as a decrease in the fluorescence signal after stimulation by thrombin (calcium-insensitive excitation wavelength of 360 nm). The effects of toborinone or olprinone on thrombin-induced calcium influx through calcium channels in the plasma membrane were also examined [14,15]. Data values were expressed as percentages of the initial fluorescence level.

Materials

We purchased toborinone (Ohtsuka Pharmaceuticals, Tokyo, Japan), olprinone hydrochloride (Eizai, Tokyo, Japan), fura-2 acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan), and apyrase (Sigma Chemical, St. Louis, MO, USA). Thrombin was donated by Mochida Pharmaceuticals (Tokyo, Japan). All other compounds were purchased from commercial sources.

Statistical analyses

Values were expressed as means ± SDs. Aggregation ratios were analyzed using unpaired *t*-tests. Increases in $[Ca^{2+}]_i$ and the release of calcium from intracellular stores were analyzed using general linear regression models for one-way analysis of variance (ANOVA; one between factor), followed by Scheffe multiple-comparison tests. Differences in Ca^{2+} influx measured using Mn^{2+} were analyzed using general linear regression models for two-way ANOVA (one within one between factors). Values of $P < 0.05$ were regarded as significant for all comparisons.

Results

Toborinone and olprinone potently inhibited thrombin-induced platelet aggregation in a concentration-dependent manner (Fig. 1). Both toborinone (20.0 μM) and olprinone (6.5 μM) completely blocked thrombin-induced platelet aggregation. The IC_{50} values were 9.7 ± 0.9 μM for toborinone and 3.6 ± 0.2 μM for olprinone.

The effects of toborinone and olprinone on $[cAMP]_i$ for platelets stimulated with thrombin ($0.015\,U \cdot ml^{-1}$) are shown in Fig. 2. In the resting state without thrombin, $[cAMP]_i$ was 4.5 ± 0.8 $pmol \cdot 10^{-8}$ platelets. After stimulation with $0.015\,U \cdot ml^{-1}$ thrombin, $[cAMP]_i$ did not significantly change (4.5 ± 0.6 $pmol \cdot 10^{-8}$ platelets). Toborinone at 9.7 μM (IC_{50} value) significantly increased $[cAMP]_i$ to 6.4 ± 0.9 $pmol \cdot 10^{-8}$ platelets, compared with the control values ($P < 0.05$). Olprinone at 3.6 μM (IC_{50} value) significantly increased $[cAMP]_i$ to 7.1 ± 1.7 $pmol \cdot 10^{-8}$ platelets, compared with the control ($P < 0.02$). However, no significant difference was noted between the toborinone and olprinone groups.

Resting levels of $[Ca^{2+}]_i$ did not differ significantly between groups (Table 1). Neither of the two agents significantly changed $[Ca^{2+}]_i$ before thrombin stimulation.

Increases in $[Ca^{2+}]_i$ at the peak induced by thrombin in the toborinone (9.7 μM) and olprinone (3.6 μM) groups were significantly reduced compared to the control value ($P < 0.05$ and $P < 0.02$, respectively). When Ni^{2+} was added to samples instead of $CaCl_2$, increases in $[Ca^{2+}]_i$ induced by thrombin in the toborinone (9.7 μM) and olprinone (3.6 μM) groups were significantly lower than the control value ($P < 0.02$ for both; Table 1); Fig. 3 shows traces representing eight independent experiments.

Figure 4 shows Mn^{2+} entry, expressed as a percentage decrease in fluorescence at 360 nm. After stimulation by thrombin, fluorescence decreased rapidly within 1 min

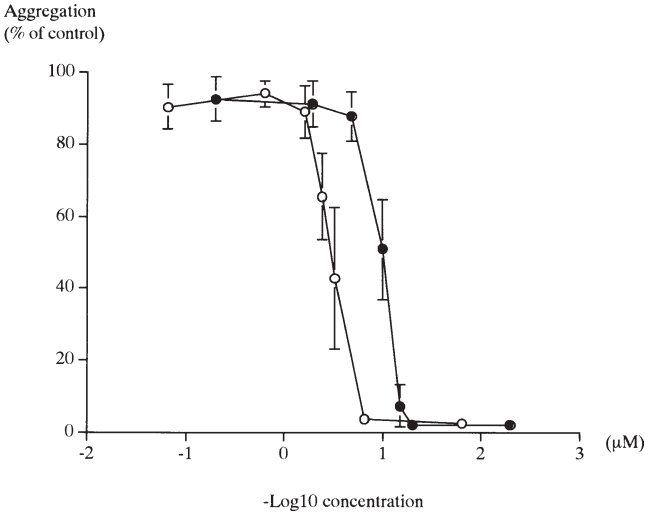

Fig. 1. Concentration-dependent inhibition of thrombin-induced platelet aggregation with toborinone (*filled circles*) and olprinone (*open circles*). Platelets were stimulated with $0.015\,U \cdot ml^{-1}$ of thrombin containing seven concentrations of toborinone or olprinone, and 50% inhibition (IC_{50}) of thrombin-induced aggregation was calculated (average of eight independent experiments)

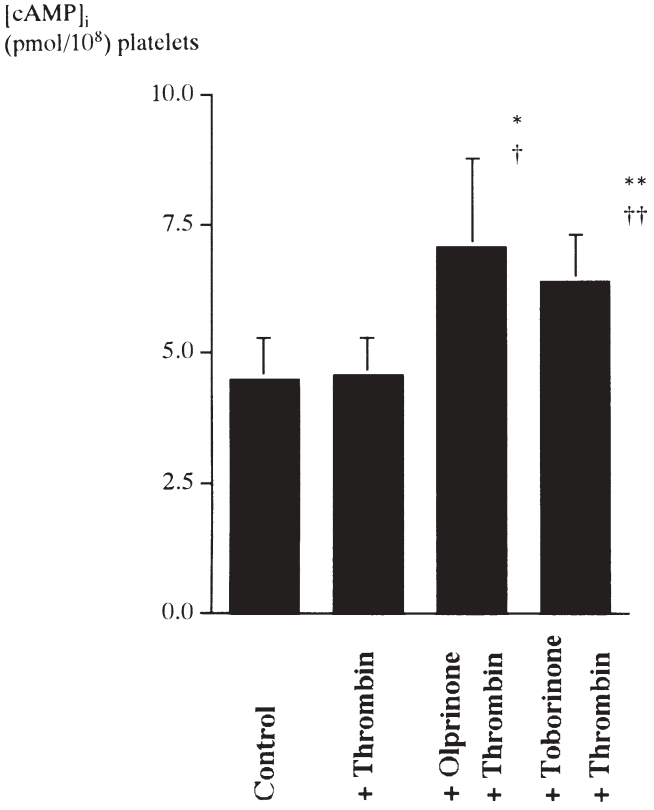

Fig. 2. Effect of toborinone and olprinone on intracellular cyclic 3′,5′-adenosine monophosphate concentrations (*[cAMP]*$_i$) of thrombin-stimulated human platelets. Values for results are expressed as $pmol \cdot 10^{-8}$ platelets. *Symbols* represent means ± SD ($n = 8$ at each point). $^*P < 0.02$; $^{**}P < 0.05$, compared to control value. $^{\dagger}P < 0.02$; $^{\dagger\dagger}P < 0.05$ compared to $0.015\,U \cdot ml^{-1}$ thrombin

Table 1. Resting levels of $[Ca^{2+}]_i$ (nM) and thrombin-stimulated $[Ca^{2+}]_i$ elevation or release

	Control (% of control)	Toborinone (% of control)	Olprinone (% of control)
Resting level of Ca^{2+}	101.8 ± 8.4	110.1 ± 17.4	98.1 ± 9.4
Ca^{2+} elevation (Ca^{2+} added)	788.0 ± 150.7 (100.0)	232.8 ± 74.1 (29.5)*	220.6 ± 80.3 (28.0)**
Ca^{2+} release (Ni^{2+} added)	272.9 ± 87.1 (100.0)	153.3 ± 28.7 (56.2)**	138.9 ± 58.2 (50.9)**

*$P < 0.05$; **$P < 0.02$, compared with control
Resting levels of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) did not significantly differ between the groups
Data indicate rise in $[Ca^{2+}]_i$ and ratio (%) of Ca^{2+} elevation or release relative to controls
Values are means ± SD of eight determinations

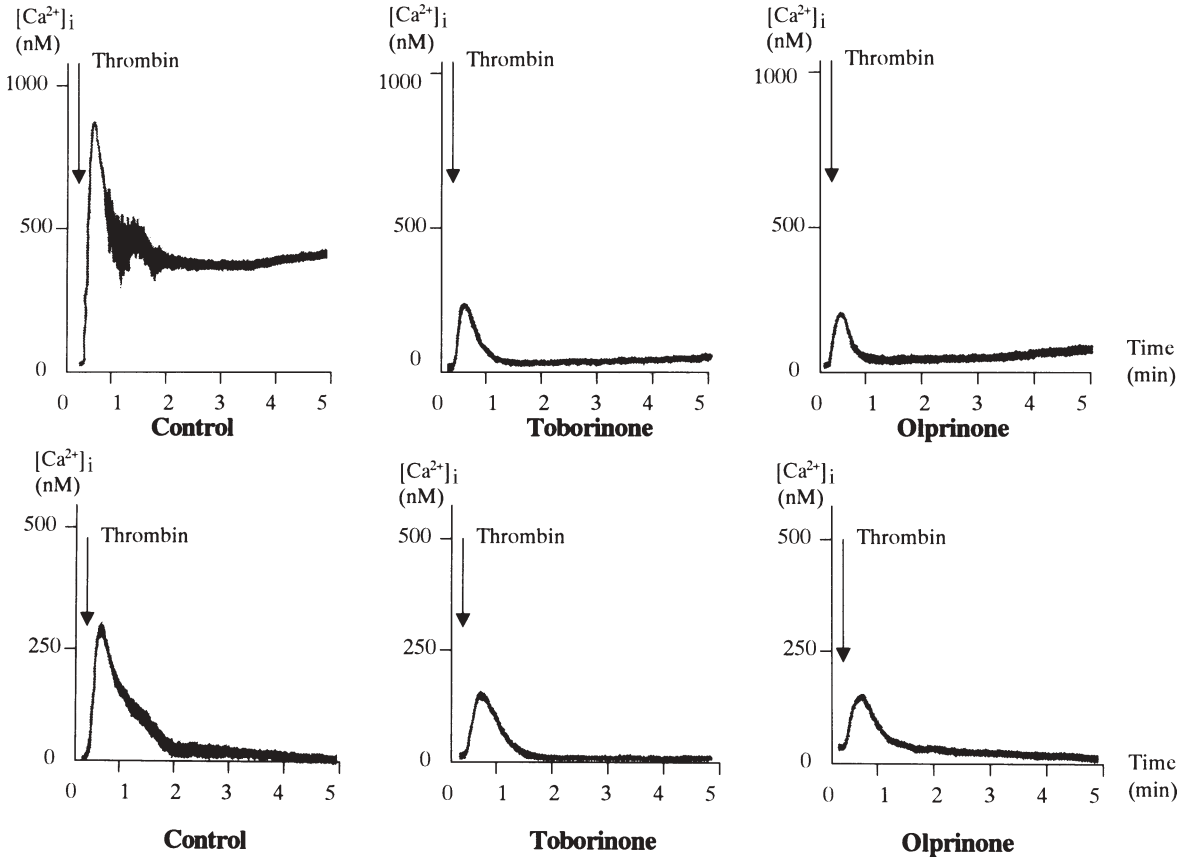

Fig. 3. Effect of toborinone or olprinone on thrombin-induced elevations in intracellular calcium concentration ($[Ca^{2+}]_i$) in platelets. *Upper traces* show thrombin-induced calcium mobilization (both calcium release and influx) and *lower traces* show thrombin-induced calcium release (Ni^{2+} added) from the intracellular stores. Traces are representative of eight independent experiments

(early phase), then decreased gradually (1–5 min; delayed phase). The percentage decreases in fluorescence in the early phase with toborinone (9.7 μM) and olprinone (3.6 μM) were significantly lower ($P < 0.02$) than that in the control group (74 ± 4%, 85 ± 4%, and 88 ± 3% in control, toborinone, and olprinone groups, respectively). The percentage decrease in fluorescence in the delayed phase (5 min after stimulation by thrombin) with toborinone (9.7 μM) and olprinone (3.6 μM) groups was significantly lower ($P < 0.02$) than that of the control group (63 ± 5%, 78 ± 3%, and 82 ± 4% in the control, toborinone, and olprinone groups, respectively). However, differences in the percentage decrease in fluorescence between the toborinone and olprinone groups were not significant.

Discussion

We investigated the effects of the PDE III inhibitors, toborinone and olprinone, on platelet aggregation and on changes in the intracellular second messenger, $[Ca^{2+}]_i$,

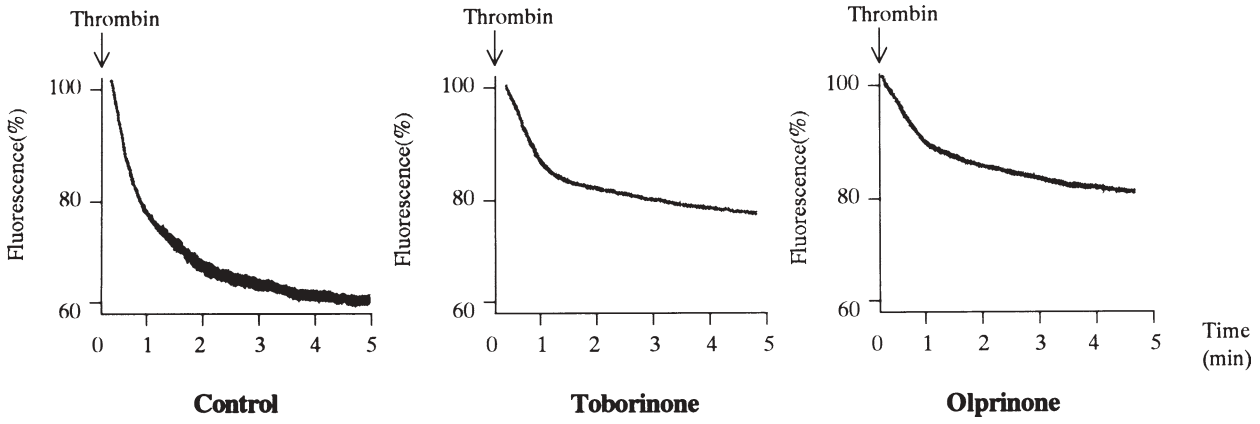

Fig. 4. Effects of toborinone or olprinone on thrombin-induced Mn^{2+} entry. Traces shown are representative of eight independent experiments

using human platelets. Toborinone and olprinone inhibited human platelet aggregation. The inhibitory effects of both drugs were related to the inhibition of both Ca^{2+} release from intracellular stores and Ca^{2+} entry through calcium channels in the plasma membrane.

The therapeutic plasma concentration of toborinone obtained from a pharmacokinetic study in patients with congestive heart failure was 400–800 ng·ml^{-1} (1.0–2.0 μM) [16]. The therapeutic plasma concentration of olprinone is 20 ng·ml^{-1} (0.066 μM, unpublished data, Eizai Pharmaceuticals, Tokyo, Japan). Given that 90% of toborinone and 81% of olprinone is bound to plasma protein, 9.7 μM of toborinone and 3.6 μM of olprinone would be equivalent to plasma concentrations of 97.0 μM of toborinone and 18.9 μM of olprinone, respectively. Concentrations of either drug required to inhibit platelet aggregation were higher than the clinically effective doses. We recently reported that 0.9 μM of milrinone inhibited platelet aggregation and increased intracellular Ca^{2+} concentration, and was equivalent to a plasma concentration of 3 μM [5]. As 1 μM of milrinone was defined as the concentration close to the therapeutic plasma level, milrinone exerts stronger inhibitory effects on platelet aggregation and calcium mobilization than toborinone or olprinone.

We then studied whether the inhibitory effects of toborinone and olprinone influenced cytosolic Ca^{2+} mobilization. When agonists bind to receptors on the platelet membrane, intracellular second messengers such as inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DG) are activated, resulting in increased free calcium concentrations in the platelet cytosol. Platelet release reactions (inside-out signaling) and tyrosine kinase activation (outside-in signaling) occur and change the conformation of $\alpha_{IIb}\beta_3$, making it competent to bind dimeric fibrinogen [16]. Stirring allows contact between fibrinogen bound on adjacent platelets, leading to platelet aggregation. Toborinone and olprinone significantly inhibited the elevation of cytosolic Ca^{2+}, via the release of Ca^{2+} from intracellular stores and via Ca^{2+} entry across the plasma membrane. Intracellular Ca^{2+} mobilization is antagonized by increasing levels of cAMP and the increasing levels of cAMP block many signal transduction pathways, resulting in the inhibition of platelet aggregation. The process of platelet activation is regulated by levels of the second messenger, cAMP. The $[cAMP]_i$ stimulated by thrombin (0.015 iu·ml^{-1}) was significantly increased by both toborinone and olprinone. Increased cAMP concentrations in the toborinone and olprinone groups in which platelet aggregation was inhibited were only 1.4- to 1.5-fold higher than those of control groups. However, cAMP present in untreated platelets may fully occupy the high-affinity cAMP-binding sites of the regulatory subunits of cyclic nucleotide-dependent protein kinase (PKA) at concentrations of 2.31 ± 0.53 pmol·10^{-8} platelets [18]. In our results, resting levels of platelet cAMP concentration were 4.5 ± 0.8 pmol·10^{-8} platelets, sufficient to occupy the high-affinity cAMP-binding sites of the regulatory subunits of PKA. The inhibitory effects of platelet aggregation occurring with toborinone and olprinone are related to the inhibition of both Ca^{2+} release and Ca^{2+} entry, through small increases in cAMP concentration.

The major target molecules of cAMP in platelets are PKAs, whose effects are mediated through the phosphorylation of specific substrates. This phosphorylation directly affects receptor/G-protein activation and interferes with various signal transduction pathways [19–22], blocking several steps of cytosolic Ca^{2+} elevation and leading to the inhibition of agonist-induced platelet aggregation. Toborinone or olprinone may have had direct or indirect actions in their inhibitory effects on platelet aggregation or Ca^{2+} mobilization.

The present study demonstrated that toborinone and olprinone inhibited human platelet aggregation, though this occurred at concentrations higher than their therapeutic plasma concentrations. These inhibitory effects were related to the inhibition of both Ca^{2+} release and Ca^{2+} entry through $[cAMP]_i$ elevation. Cyclic nucleotides and their regulatory pathways are of particular interest for developing new approaches to treating thrombotic and cardiac disorders. Further study is required to fully understand the effects on platelet function of PDE III inhibitors, such as toborinone and olprinone.

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