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Effects of MK-447 on thrombin-induced aggregation, secretion of ATP, and $[Ca^{2+}]_i$ mobilization in rabbit platelets

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AIM: To study the effects of MK-447 on aggregation release reaction and intracellular calcium mobilizartion by thrombin. **METHODS:** Aggregation and release reaction were assessed by light transmission and ATP content in rabbit citrate platelet-rich plasma (PRP), and cytosolic-free calcium was measured by fluorescence and imaging. **RESULTS:** MK-447 (2-aminomethyl-4-*t*-butyl-6-iodophenol hydrochloride) induced a decrease in light transmission (DLT), so called platelet shape change, without detectable aggregation and secretion of ATP, and increased intracellular calcium concentration ($[Ca^{2+}]_i$) slightly in washed single platelet loaded with Fura 2, the peak value being about 160 nmol $\cdot L^{-1}$. These effects were not inhibited by egtazic acid 3 mmol $\cdot L^{-1}$ or indometacin 3 μmol $\cdot L^{-1}$. The pretreatment of PRP with MK-447 700 μmol $\cdot L^{-1}$ reduced the DLT by thrombin, potentiated and enhanced thrombin-induced aggregation and secretion of ATP in a concentration-dependent manner. Thrombin-induced $[Ca^{2+}]_i$ mobilization (peak value: 369 ± 45 nmol $\cdot L^{-1}$) was further enhanced by the administration of MK-447 at 2 min before the addition of thrombin, and the peak value reached 623 ± 121 nmol $\cdot L^{-1}$ ($P < 0.01$). **CONCLUSION:** MK-447-induced platelet shape change was involved in intracellular calcium release in this preparation. MK-447 enhanced thrombin-induced aggregation and release reaction and these effects of MK-447 on aggregation and release reaction by thrombin might result from the synergistic effect of intracellular calcium mobilization.

KEY WORDS platelet aggretation; adenosine diphosphate; calcium; thrombin; MK-447.

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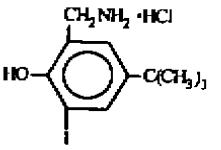
MK-447 (2-aminomethyl-4-*t*-butyl-6-iodophenol hydrochloride) is an anti-inflammatory agent by scavenging oxygen-derived free radicals released during the conversion of prostaglandin PGG_2 to PGH_2[1], and shows dual effects on PG endoperoxide biosynthesis by acting as a tryptophan-like cofactor of PG hydroperoxide synthase[2-4]. MK-447 stimulated epoprostenol (PGI_2) generation in isolated rat aorta from endogenous arachidonic acid (AA) whereas thromboxane (TX) generation from washed rabbit platelets was not stimulated[5]. Collagen (10 $\mu g \cdot mL^{-1}$)-induced platelet aggregation was not affected by MK-447 300 mmol $\cdot L^{-1}$, but inhibited by its analog[6]. The platelet aggregation includes platelet shape change with centralization of granules, platelet aggregation, and release reaction which were due to different mechanisms and related one another, and automatically happened when the aggregating agents were added to the platelets. It is very hard to know the mechanism of each part, especially the

platelet shape change and centralization of granules. In the present paper, the effects of MK-447 on shape change, thrombin-induced aggregation and secretion of ATP, and intracellular calcium mobilization in single platelet were investigated.



MK-447

MATERIALS AND METHODS

Reagents Lucifferin-lucifferase (Chrono-Log Cotp, Havertown PA, USA) for measuring the secretion of ATP was dissolved in saline (40 mg·L^{-1}) before use and stored in cold and dark area. ATP (Sigma Co) was dissolved in distilled water (1 mmol·L^{-1}) and stored at −20 ℃ and diluted with saline to 1 μmol·L^{-1} before use. MK-447 (Merck, Sharp & Dohome) was dissolved in saline 10 mg·mL^{-1}, maximum) and stored at 4 ℃ and diluted with Tris-buffered saline before use. Thrombin (Topical, Bovine Origin, Parke-Davis Division of Warner-Lambert Co, USA) was kept as −20 ℃ and dissolved with Tris- or HEPES-buffer just before use. Egtazic acid (Sigma Co) was dissolved in distilled water, adjusted to pH 7.4 and stored at room temperature (22 ℃). Fura 2 and Fura 2-AM (Wako Pure Chemicals, Osaka, Japan) were used for Ca^{2+} calibration curve and platelet loading.

Platelet-rich plasma (PRP) Blood was collected from carotid artery of rabbits (New Zealand White, 2.93±0.3 kg) under ether anesthesia into the plastic tubes containing 1/10 volume of 3.8 % trisodium citrate and centrifuged at 200×g for 10 min for getting PRP. The platelet counts in PRP was adjusted to 6×10^{11}·L^{-1} by Coulter Counter™ (Thrombo Counter-C, Counter Electronics, Hialeah FL, USA) and kept at 22 ℃ before use. Platelet-poor plasma (PPP) was obtained by further centrifugation of aliquot of PRP at 2000×g for 10 min at 4 ℃. All experiments with PRP were finished within 6 h after getting PRP.

Grouping To check the effects of MK-447 itself on the light transmission in PRP, the experiments were divided into 2 groups: (1) PRP + Tris/HCl buffered saline or egtazic acid + MK-447 to record the light transmission and ATP release simultaneously by MK-447; (2) washed platelet + HEPES buffered saline or egtazic acid + MK-447 to evaluate the effect of MK-447 on $[Ca^{2+}]_i$ mobilization.

For the effects of MK-447 on thrombin-induced shape change, aggregation, relesae reaction, and $[Ca^{2+}]_i$ mobilization, the experiments were: (1) PRP + Tris/HCl buffered saline or MK-447 + thrombin; (2) washed platelet + HEPES buffered saline or MK-447 + thrombin. In the experiments with PRP and washed single platelets in each group, 12—15 samples were made and over 30 cells were measured at least from 6 rabbits.

Platelet aggregation The platelet aggregation was quantified by the change of light transmission[7] with 4-channel aggregometer (PAT-4A, Nippon Denshi Kagaku Co, Tokyo, Japan) and 4-channel recorder (T-626DS, Nippon Denshi Kagaku Co, Tokyo, Japan). The light transmission change for PRP was set at 0 % and that for PPP was set at 100 %. After 2 min of preincubation at 37 ℃, PRP (250 μL) in the cuvette was further incubated for 2 min with Tris-buffered saline 20 μL (Tris/HCl buffer 150 mmol·L^{-1}; 0.9 % saline =1:4, pH 7.4) prior to the addition of aggregating agent (10 μL) with stirring by a silliconized magnetic bar.

Platelet release reaction The release reaction of platelets was assessed by luminescence of ATP released in the medium from the dense granules during platelet aggregation[8] with 2-channel lumi-aggregometer (Chrono-Log Corp, Havertown PA, USA) and 2-channel recorder (B-281L, Rikadenki Kogyo Co, Tokyo, Japan). The amount of ATP released was expressed as μmol per 4×10^5 platelets.

Washed platelet and Fura 2 loading For measuring the cytosolic-free calcium concentration ($[Ca^{2+}]_i$) in single platelet, the washed rabbit platelet was prepared. Blood collected from a carotid artery of rabbits with 1/7 acid citrate-dextrose solution (ACD, sodium citrate 85 mmol·L^{-1}, citrate acid 71 mmol·L^{-1}, and glucose 110 mmol·L^{-1}) was centrifuged at 200×g for 15 min for getting PRP, and its remnant precipitate was centrifuged again at 800×g at the 25 ℃ for 20 min for getting platelet pellets which were suspended with a Ca^{2+}-free HEPES buffer (NaCl 145, KCl 5,

$MgSO_4$ 1, glucose 5 and HEPES 10 mmol·L^{-1}, pH 7.4 at 22 ℃). The washed platelet counts were adjusted to 6×10^{11}·L^{-1} and then the washed platelet was loaded with Fura 2-AM 2 μmol·L^{-1} (final concentration) at 37 ℃ for 15 min. The Fura 2-AM-loaded platelet pellet was obtained by centrifugation at 800× *g* at 25 ℃ for 20 min and resuspended in Ca^{2+}-free HEPES buffer to obtain the final concentration of 2×10^{11} platelets·L^{-1}. Extracellular Ca^{2+} concentration was adjusted to a final concentration of 1 mmol·L^{-1} at 5—10 min before measuring $[Ca^{2+}]_i$ mobilization using digital image fluorescence microscope and computer assistant imaging and analysing system.

Measurement of $[Ca^{2+}]_i$ in single platelet The Fura 2-loaded washed platelet suspension (50 μL) was layered on a thin cover glass (40 mm×50 mm, thickness: 0.17—0.25 mm, Matsunami Glass Co, Tokyo, Japan) in a 2.5 mm cyclic chamber in diameter at room temperature and observed under the digital image fluorescence microscope (IMT 2-OSP-I, Olympus®, Tokyo, Japan). The fluorescence of Fura 2-loaded washed single platelet, due to excitation at 340 nm and 380 nm, was imaged using U V Apr 100× and inverted to microscope, the video images were obtained using a sillicon-intensified target camera (SIT, Olympus®, Tokyo, Japan) and were stored and digitaly analyzed by inverted microscope system (Argus 100/AC, Hamamatsu Co, Tokyo, Japan). The ratio (R340/380) image was achieved from the division of 340 nm image by 380 nm image and the $[Ca^{2+}]_i$ in single washed platelet was calculated from R340/380 using standard calibration curve of calcium with Fura-2[9].

The response of $[Ca^{2+}]_i$ mobilization to thrombin in rabbit platelets was different among the density subpopulations of platelets[10]. In the present experiments, the densest/largest platelets were selected for measurement of $[Ca^{2+}]_i$.

Statistical analysis All data were expressed as $\bar{x} \pm s$. *P* value was evaluated by *t* test.

RESULTS

Light transmission of PRP and $[Ca^{2+}]_i$ mobilization MK-447 caused the DLT of PRP in a concentration-dependent manner without any platelet aggregation and secretion of ATP recorded simultaneously. In washed platelets, MK-447 induced an increase in $[Ca^{2+}]_i$ and the peak value appeared within 30, and the resullts also showed that MK-447-induced DLT and $[Ca^{2+}]_i$ mobilization were not inhibited by egtazic acid 3 mmol·L^{-1} (Fig 1).

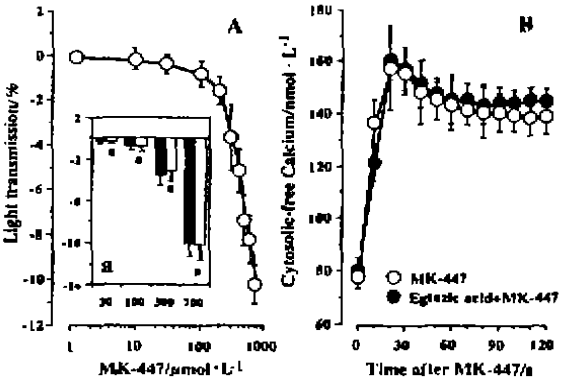


Fig 1. Effect of MK-447 on light transmission in rabbit PRP (A, $n=24$), inhibitory effect of egtazic acid 3 μmol·L^{-1} on DLT (a, $n=14-17$), and $[Ca^{2+}]_i$ mobilization by MK-447 700 μmol·L^{-1} (B, $n=21-23$). Open bar = MK-447, closed bar = MK-447 + egtazic acid. $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$, *vs* MK-447.

DLT by thrombin Thrombin caused transient DLT just before its aggregation, and this DLT was abolished by the pretreatment of MK-447 and the lag phase (LP)[12] just before thrombin-induced aggregation was also markedly shortened by MK-447. (Fig 2).

Aggregation by thrombin Thrombin 0.1 U·mL^{-1} did not induce aggregation in rabbit PRP, but in presence of MK-447 700 μmol·L^{-1}, thrombin 0.1 U·mL^{-1}-induced aggregation was observed, while thrombin 0.3 and 0.5 U·mL^{-1}induced aggregation were significantly enhanced ($P<0.01$) (Fig 2).

ATP released by thrombin Thrombin 0.1 U·mL^{-1} did not induce the ATP release in the absence or presence of MK-447 700 μmol·L^{-1}, whereas the amount of ATP released by thrombin 0.3 and 0.5 U·mL^{-1} was significantly enhanced by the pretreatment of

PRP with MK-447 in 700 μmol·L^{-1} ($P<0.01$) (Fig 2).

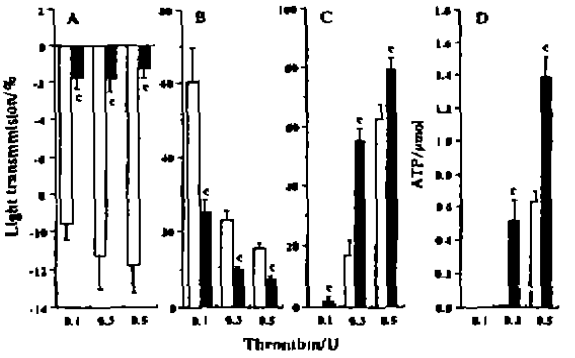


Fig 2. Effects of MK-447 (700 μmol·L^{-1}) on thrombin-induced DLT (A, $n=12-13$), lag phase (B, $n=13$), aggregation (C, $n=14$), and release reaction (D, $n=14-16$) by thrombin. Open bar: MK-447; hatched bar: MK-447 + thrombin. $\bar{x}\pm s$. $^cP<0.01$ *vs* MK-447.

$[Ca^{2+}]_i$ mobilization by thrombin

Thrombin (1.5, 1.5 and 150 U·mL^{-1}) caused an increase in $[Ca^{2+}]_i$ concentration-dependently in washed single platelet. The peak value of thrombin (15 U·mL^{-1})-induced $[Ca^{2+}]_i$ mobilization appeared at 60 s, and was then partially reversed. In MK-447-treated group, the peak of $[Ca^{2+}]_i$ mobilization by thrombin was enhanced markedly ($P<0.01$) without signficant reversion (Fig 3).

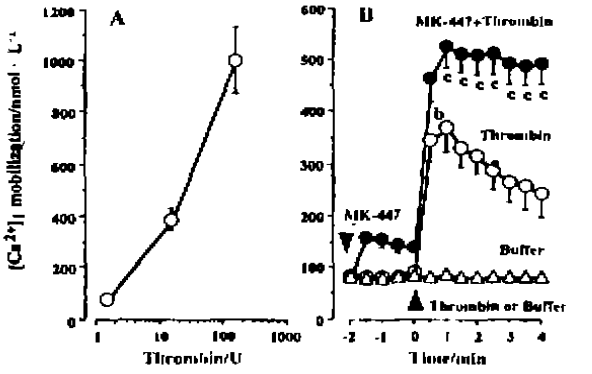


Fig 3. $[Ca^{2+}]_i$ mobilization by thrombin (A, $n=25-27$) and effect of MK-447 700 μmol·L^{-1} on thrombin 15 U·mL^{-1}-induced $[Ca^{2+}]_i$ mobilization (B, $n=27-31$). $\bar{x}\pm s$. $^bP<0.05$, $^cP<0.01$ *vs* thrombin.

DISCUSSION

In the present researches, MK-447 caused a concentration-dependent decrease in light transmission (DLT) through PRP, so called platelet shape change or centralization of granules revealed by electromicroscopy and involved in $[Ca^{2+}]_i$ mobilization[11, 12, 13], without any detectable aggregation and secretion of ATP, in addition, MK-447-induced both DLT and calcium mobilization in single platelets were not related to extracellular calcium influx, this data strongly suggested that the DLT by MK-447 probably mediated by small amount intercellular calcium mobilization through unknown mechanisms. The similar results were recently reported[11] with 5-hydroxytryptamine (5-HT).

MK-447 showed a prompted effects on aggregation, not inhibitions. Thrombin also induced transient DLT before aggregation by its own, but it was reduced and the lag phase (LP) was shortened as well by the pretreatment with MK-447. In other words, thrombin-induced DLT was replaced by the addition of MK-447 prior to thrombin, indicating that MK-447 already caused DLT, ie, the platelets underwent the processes of shape change or centralization of granules and this DLT and $[Ca^{2+}]_i$ mobilization by MK-447 did not recovered before the addition of thrombin, thus, when the administration of thrombin to the PRP, the aggregation was caused directly. Therefore, the platelet shape change or centralization of granules is thought to be an important and necessary procedure in platelet aggregation induced by the almost all of aggregating agents (to be published). The further data indicated that both aggregation and secretion of ATP induced by different doeses of

thrombin were significantly enhanced by MK-447 pretreatment and these effects of MK-447 might be due to the synergistic increase in $[Ca^{2+}]_i$ from intracellular storage sites and calcium influx by both agents.

In conclusion, MK-447 that may be a partial agonist possessed the effects of acceleration, potentiation and enhancement of thrombin-induced aggregation and secretion of ATP due to the synergistic mechanism of cytosolic-free calcium mobilization, another mechanism might be also involved in MK-447 acting as tryptophan-like cofactor of prostaglandin (PG) hydroperoxidase synthase[13,14].

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MK-447对家兔凝血酶诱导的血小板聚集，ATP释放及细胞内游离钙动员的影响

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目的：研究MK-447对家兔凝血酶诱导的血小板聚集释放反应及单细胞内钙水平的影响. **方法**：利用浊度法及测定PRP中ATP的含量评价聚集和释放反应，以荧光图像法分析细胞内钙浓度. **结果**：MK-447仅使兔多血小板血浆(PRP)透光度降低(DLT)，即血小板变形，单血小板$[Ca^{2+}]_i$轻度增加(160 nmol·L^{-1})，并不被依他酸 3 mmol·L^{-1}抑制. MK-447消除凝血酶诱导的DLT，聚集和ATP释放增强，呈剂量依赖性，且凝血酶介导的$[Ca^{2+}]_i$由369

±45 nmol·L^{-1}增加到621±121 nmol·L^{-1}. **结论**: MK-447的血小板变形与其[Ca^{2+}]$_i$释放有关. MK-447增强凝血酶的血小板聚集和ATP释放, MK-447的这一作用可能于[Ca^{2+}]$_i$的协同作用有关.

关键词 血小板聚集; 二磷酸腺苷; 钙; 凝血酶; MK-447

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Chronopharmacokinetics of valproic acid following constant-rate administration in mice and influence of feeding schedule

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AIM: To study the circadian rhythm in pharmacokinetics of valproic acid (VA) and influence of feeding schedule on the rhythm. **METHODS**: Sodium valproate was administered by osmotic minipump technique (1.062 mg·h^{-1}) and iv (50 mg·kg^{-1}) to ICR mice fed under *ad lib* or time-restricted schedules to determine the time-dependent changes of VA kinetics. **RESULTS**: Plasma VA concentration and clearance at steady-state showed circadian rhythms ($P<0.01$). Time-restricted feeding influenced the rhythm of VA kinetics, acrophases of rhythms shifted approximately 12 h. **CONCLUSION**: Timing of dosing is important for VA kinetics and feeding schedule is one of synchronizers in VA kinetics.

KEY WORDS valproic acid, pharmacokinetics, circadian rhythm, drug administration schedule

Valproic acid (VA) is an antiepileptic drug. The toxicity, anticonvulsant actions and kinetics of VA showed circadian rhythm changes in rodents. The circadian rhythm of plasma VA concentrations corresponded well to that of VA anticonvulsant actions and related to the feeding condition[1,2]. The present work was to study the circadian rhythmicity of plasma VA concentration in mice following constant-rate VA administration using osmotic minipumps and to identify the role of feeding schedule on the circadian rhythm of kinetics of VA.

MATERIALS AND METHODS

ICR mice, ♂, 6-wk old (30.2±*s* 2.8 g), were housed 10 per cage from 4-wk old in a standardized light-dark cycle of light on 7:00—19:00, at a room temperature of 24±1 ℃ and a humidity of 60±10 % with food (Oriental Yeast Co, Tokyo, Japan) and water *ad lib* or under a time-restricted feeding schedule (feeding time: 9:00—17:00).

In the study observing the circadian rhythm in plasma VA concentration at the steady-state, 2 groups of 10 mice fed under *ad lib* or time-restricted schedules were anesthetized with ether. A small incision was made in the bake of mice and 2 osmotic minipumps (Model 2001 with 25 mm in length, 7 mm in diameter, Alzet Corp, USA) were implanted subcutaneously into the pockets. The concentration of VA solution used filling one pump was 600 g·L^{-1} of sodium valproate (Valerin, Dainipon Pharmaceutical Co, Japan).

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