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Kinetic characteristics of thrombin receptor-mediated responses in rat megakaryocytes

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Abstract

Kinetic characteristics of thrombin receptor-mediated responses on rat megakaryocytes were examined by the use of the perforated patch clamp combined with the rapid drug exchange system termed the 'Y-tube method'. The application of thrombin evoked repetitive Ca^{2+}-activated K^+ current (I_{KCa}) in a concentration-dependent manner. The characteristic features for thrombin-induced response compared with purinoceptor-induced response were the long latency, long washout time and fast desensitization. The similar I_{KCa} as thrombin was induced by trypsin. Thrombin- and trypsin-induced I_{KCa} were both inhibited by the protease inhibitor, SBTI, and the washout time for thrombin was markedly shortened (7.4 ± 2.2 s) when thrombin was washed out by a solution containing soybean trypsin inhibitor. A synthetic thrombin receptor agonist peptide induced I_{KCa} oscillation with shorter latency than thrombin.

Keywords: Thrombin receptor; Megakaryocyte, rat; Ca^{2+}-activated K^+ current; Patch clamp, perforated

1. Introduction

Thrombin is a multifunctional protease fulfilling an important role in both hemostasis and thrombosis. In addition to its role in blood coagulation, thrombin acts on a wide variety of cells via a unique proteolytic cleavage of cell surface receptor. Vascular smooth muscle cells and fibroblasts, for instance, migrate and proliferate in response to thrombin (Tani et al., 1991; Berk et al., 1991), and endothelial cells are also activated by the protease (Herbert et al., 1994). In addition, Greco et al. (1996) reported that two types of thrombin receptors are required for optimal activation of platelets. Thus we tried to analyze the features of thrombin receptors in megakaryocytes, the progenitor cells of platelets.

In previous reports, we showed that pharmacological properties of receptors on the megakaryocyte could be easily examined by applying the perforated patch-clamp technique (Akaike et al., 1993; Uneyama et al., 1993a,b,c, 1994). With this technique, we could detect the thrombin receptor-operated actions on megakaryocytes by monitoring the Ca^{2+}-activated K^+ current (I_{KCa}). In addition to the patch-clamp technique, we also employed a rapid drug exchange system called the 'Y-tube method' which completely exchanges the external solution surrounding a cell within 20 ms (Nakagawa et al., 1991). This system enables us to analyze the kinetics of the thrombin response within seconds. Here, we show some properties of the thrombin receptor on rat megakaryocytes.

2. Materials and methods

2.1. Preparation of megakaryocytes

Adult male and female rats weighing 250–350 g were anesthetized by inhalation of diethyl ether and killed by cutting the carotid artery. Femoral bones were isolated and the bone marrow was washed out with standard external solution by using a disposable syringe and needle. After filtration by a 75 μm nylon mesh to eliminate large masses of cells, the solution containing bone marrow cells was transferred to a recording chamber. The chamber was left

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for a few hours at room temperature until the cells settled on the base of the chamber. Megakaryocytes could be clearly distinguished from other bone marrow cells under phase-contrast inverted microscope by their large size (30–50 μm).

2.2. Solutions

The ionic composition of the standard external solution was (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES. The pH was adjusted to 7.4 with Tris base. For the Ca^{2+}-free solution, $CaCl_2$ in the standard solution was replaced with equimolar $MgCl_2$, and 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added. The internal solution for the perforated patch was (in mM) 150 KCl and 10 HEPES, and the pH was adjusted to 7.2. Nystatin was added to the internal solution to a final concentration of 150 μg/ml. The internal solution for conventional whole-cell patch was (in mM): 150 KCl, 2 Mg-ATP, 10 HEPES, and the pH was adjusted to 7.2.

2.3. Electrical measurements

Whole-cell currents of the megakaryocytes were recorded with the modified perforated patch-clamp technique of Horn and Marty (1988) as reported before (Uneyama et al., 1992, 1993d) at room temperature (21–24°C). Some experiments were performed using a whole-cell patch-clamp technique (Hamill et al., 1981). The liquid junction potential between internal and external solutions was approximately −3 mV. The data were not compensated for this potential. The resistance of the recording electrode was 5–10 MΩ. The current was measured using a patch-clamp amplifier (List Medical, EPC-7) and simultaneously recorded on a pen recorder (Sanei, RECTI-HORIZ-8K) after changing the signal to the digital mode using a digital audio processor (Nihon Kohden, PCM501ESN).

2.4. Drugs

Thrombin (from bovine plasma), Na-ATP, trypsin, nystatin and soybean trypsin inhibitor (SBTI) were purchased from Sigma (St. Louis, MO, USA). The activity of thrombin is expressed in NIH units. Hirudin and aprotinin were obtained from Seikagaku Kogyo (Tokyo, Japan). The activity of hirudin is expressed in anti-thrombin units. One anti-thrombin unit is the amount which neutralizes one NIH unit of thrombin (fibrinogen clotting assay) at 37°C. Thrombin receptor agonist peptide (Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe) was from Peninsula Laboratories. D-*myo*-Inositol trisphosphate was from Dojindo Lab. (Kumamoto, Japan). Drugs were applied by the use of a rapid application method termed the 'Y-tube' method, as described elsewhere (Nakagawa et al., 1991). With this technique, the solution surrounding an isolated megakaryocyte could be completely exchanged within 20 ms.

3. Results

3.1. Characterization of thrombin-induced current in rat megakaryocyte

Rapid application of thrombin induced periodic outward current activation of megakaryocytes at a holding potential (V_H) of −40 mV. This current reversed its direction around V_H of -81 ± 3 mV ($n = 3$), and was diminished by pretreatment of the cell for 1 h with a membrane-permeant Ca^{2+}-chelating agent, BAPTA-acetoxymethyl ester at 10 μM (data not shown, $n = 2$). Furthermore, internal perfusion with Cs^+ instead of K^+-containing internal solution also abolished the thrombin-induced outward current (data not shown, $n = 7$). These results in addition to previous reports (Uneyama et al., 1993a, 1995) indicated that the thrombin-activated current was Ca^{2+}-dependent K^+-current (I_{KCa}) similar to that previously seen in ATP stimulation.

3.2. Concentration-response relationships

Then, the concentration-response relationships were investigated. Fig. 1A shows the typical current traces obtained from one cell, and shows the definition of the terms. 'Latency' is the period required to evoke the first current spike after application of the drug. 'Frequency' is the number of spikes per second. 'Washout time' is the time required to abolish the current completely after washout of the drug. Fig. 1B shows the concentration dependency of the thrombin-induced I_{KCa}. The minimum concentration of thrombin to evoke the I_{KCa} was 3 U/ml, and the maximum frequency could be induced at 30 U/ml. In Fig. 1C, the concentration-dependent changes in I_{max}, frequency, latency and washout time are summarized. I_{max}, frequency and washout time increased and latency decreased as the concentration of applied thrombin was increased in each cell. These concentration-dependent changes of those parameters had almost the same trends as previously reported in case of ATP and ADP (Uneyama et al., 1993a).

3.3. Pharmacological properties

The pharmacological features of the receptor involved in the thrombin-induced I_{KCa} were examined by using some types of proteases and their inhibitors. A serine protease inhibitor, SBTI (0.2 mg/ml) abolished the thrombin-induced I_{KCa} (Fig. 2A). On the other hand, the same concentration of SBTI had no effect on the ATP-induced I_{KCa} (Fig. 2B). Hirudin (20 U/ml), a strong inhibitor of thrombin (Tam et al., 1979), abolished the thrombin-in-

duced I_{KCa} (Fig. 2C). Pretreatment of the cell with hirudin (20 U/ml) also failed to inhibit the ATP response (data not shown, $n = 2$).

The I_{KCa} oscillation was also induced by the application of a serine protease. As shown in Fig. 3A, the serine protease, trypsin (0.1 mg/ml), induced I_{KCa} oscillations that were indistinguishable from those induced by thrombin. The trypsin-induced I_{KCa} was inhibited by SBTI (0.2 mg/ml) but not by hirudin (20 U/ml). Trypsin always induced I_{KCa} oscillation in thrombin-sensitive cells (data not shown, $n = 21$). Aprotinin at 0.1 mg/ml acted as an inhibitor of trypsin. However, other types of proteases were inactive on rat megakaryocyte. Chymotrypsin had no effect up to 1 mg/ml (Fig. 3B). Thermolysin, collagenase and elastase (1 mg/ml each) also had no effect (data not shown, $n = 3$). These results confirmed that the thrombin-induced I_{KCa} is caused by the protease activity of thrombin. In addition, the features of thrombin-induced response such as latency, washout time, oscillatory pattern, etc., were almost the same as the features of trypsin-induced response.

Then, to investigate whether the effects of trypsin and thrombin were induced by the same receptor, we performed the cross-desensitization study of each of the responses. Thrombin was applied repeatedly to cause desensitization of the receptor, and then trypsin was applied (Fig. 3C). Trypsin failed to induce any response after desensitization of the thrombin response, but even under this condition, subsequent application of 10 μM ATP still induced oscillatory I_{KCa}. In addition, repeated applications

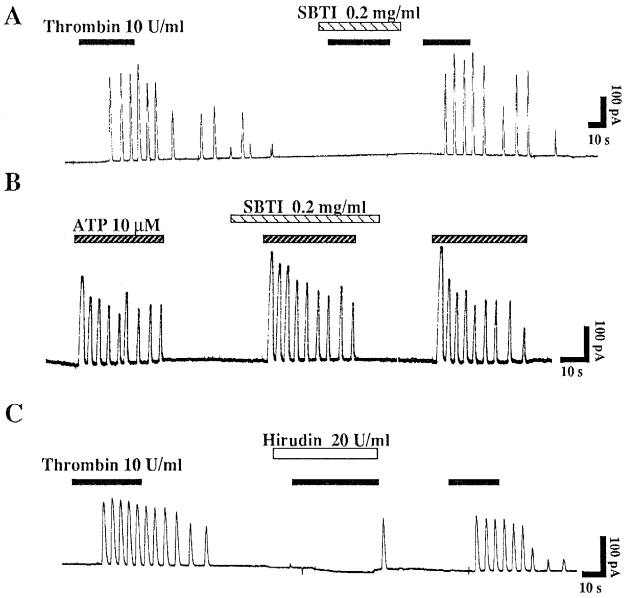

Fig. 2. Effects of hirudin and protease inhibitor on the thrombin-induced oscillatory I_{KCa}. Each drug was applied during the period indicated by the horizontal bar. The current traces indicated in the figure were typical of three to five experiments.

of trypsin also caused desensitization, and such cells did not respond to thrombin (data not shown, $n = 3$).

3.4. Kinetic properties of thrombin stimulation

In the following experiments, the temporal kinetics of thrombin-induced I_{KCa} were compared to that of another

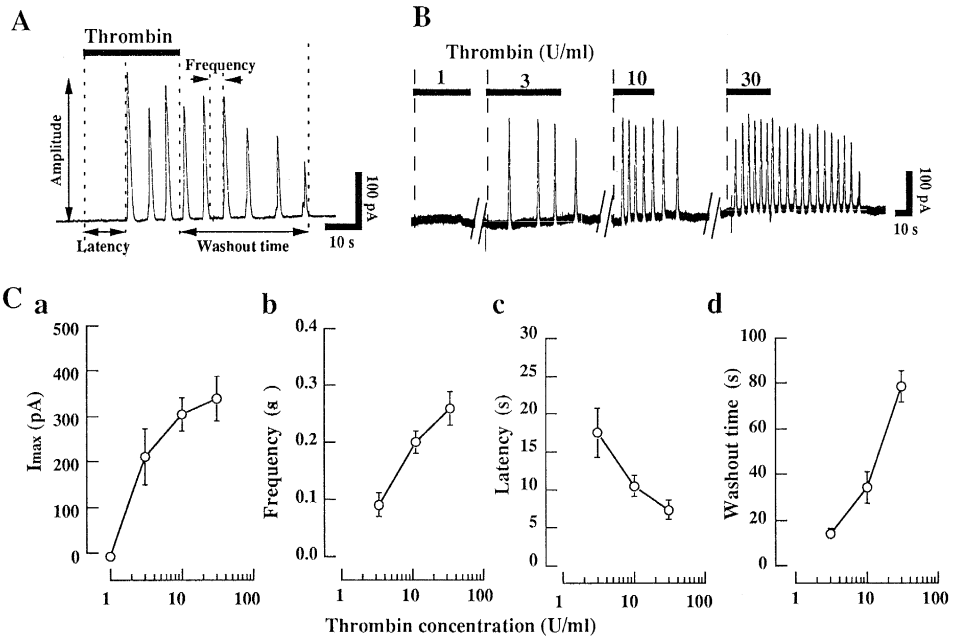

Fig. 1. Concentration dependency of the thrombin-induced response. (A) Typical current traces obtained from a single cell at various concentrations of thrombin. Each concentration of thrombin was applied during the period indicated by closed horizontal bars. The application intervals were more than 2 min. (B) Definition of terms used. Refer to the text for details. C: The concentration dependency of the maximum current amplitude (I_{max}) (a), frequency (b), latency (c) and washout time (d). Quantitative data obtained from four cells. Each line indicates data from a single cell. The same symbol in each figure indicates the same cell.

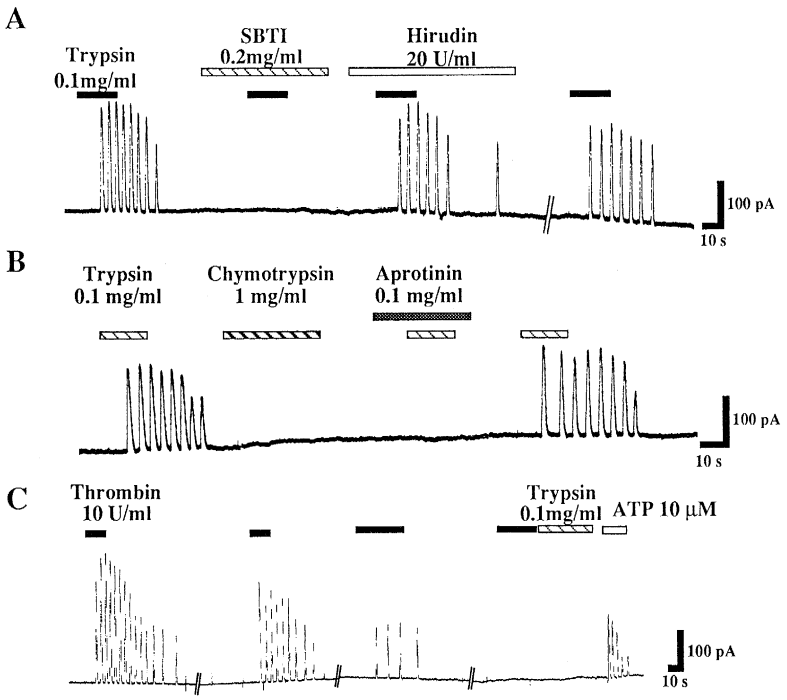

Fig. 3. Trypsin-induced I_{KCa} oscillation. (A and B) Effects of protease and protease inhibitor. (C) Cross-desensitization between the receptors. The holding potential (V_H) was −40 mV. Horizontal bars above each current trace indicate the period of drug application. The current traces were typical from three (A and B) and four (C) cells.

types of stimulation to characterize the thrombin-induced response. Table 1 summarizes each parameter (frequency, latency and washout time) of thrombin-induced I_{KCa} oscillations in comparison with that of purinergic stimulation. The frequency of I_{KCa} evoked by 30 U/ml thrombin was not significantly different from that by 30 μM ATP or 1 μM ADP. Since in a previous report we showed that frequency was estimated as Ca^{2+} mobilizing potency for the applied agonist (Uneyama et al., 1993a), this result indicates that Ca^{2+} mobilizing potency of 30 U/ml throm-

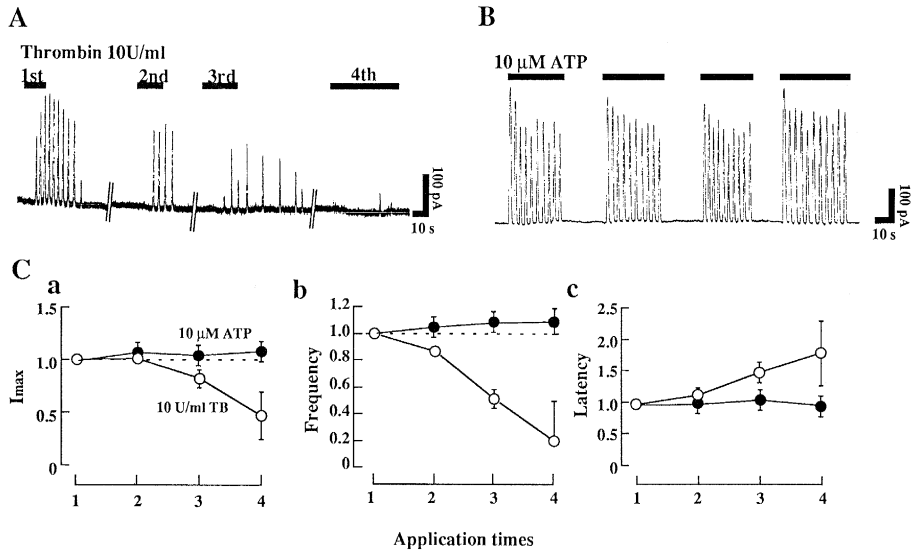

Fig. 4. Desensitization of the thrombin receptor by repetitive stimulations. Typical current traces of the 10 U/ml thrombin (A)- and 10 μM ATP (B)-induced responses. The holding potential (V_H) was −40 mV. Thrombin was applied during the period indicated by the closed horizontal bars. Application was repeated at 2 min intervals. Each trace was obtained from two different cells. (C) Quantitative analysis of desensitization of 10 U/ml thrombin (●) in comparison with that of ATP (○). The effects of repeated application of thrombin up to four times on the maximum current amplitude (I_{max}) (a), frequency (b) and latency (c). In these figures, the values were normalized to the value obtained by the first application (indicated by broken lines).

Table 1
Quantitative parameters of thrombin- and purine-induced I_{KCa} oscillations

Agonists	Concentration	Frequency (s^{-1})	Latency (s)	Washout time (s)
Thrombin	10 U/ml	0.18±0.12	10.40±1.41	34.22±6.99
	30 U/ml	0.26±0.07	7.34±1.31	78.45±6.95
ATP	10 μM	0.21±0.02	1.52±0.31	1.20±0.10
	30 μM	0.25±0.04	1.36±0.21	1.31±0.21
ADP	0.3 μM	0.19±0.05	1.84±0.27	1.01±0.09
	1.0 μM	0.22±0.07	0.94±0.31	1.22±0.31

Each value represents the mean±S.E.M. from four to six cells.

bin might be almost equal to that of 30 μM ATP or 1 μM ADP. In contrast to the frequency, the values of latency and washout time for 30 U/ml thrombin were quite different from those for 30 μM ATP or 1 μM ADP. Thus, long latency and washout time were characteristics for the thrombin response.

Fig. 4 shows the desensitization of the response in comparison with ATP response. Fig. 4C indicates normalized values to the first time reaction as the absolute reaction intensity that differs from cell to cell. When thrombin was applied repeatedly to the same cell at 2 min intervals, the reaction gradually decreased. The quantitative changes are shown in Fig. 4. I_{max} and frequency decreased as the applications were repeated, and the latency increased. However, all parameters of 10 μM ATP-induced I_{KCa} were not changed by the same conditions.

Next, we examined whether the washout time characteristic for thrombin-induced I_{KCa} was caused by strong binding of the enzyme on the receptor or by a specific intracellular signaling mechanism. As thrombin is known to bind to its receptor before cleaving the substrate sequence (activation), the washout time might be caused by the residual thrombin that could not be washed out easily. When thrombin was washed out by an external solution containing 1 mg/ml SBTI, the washout time was markedly

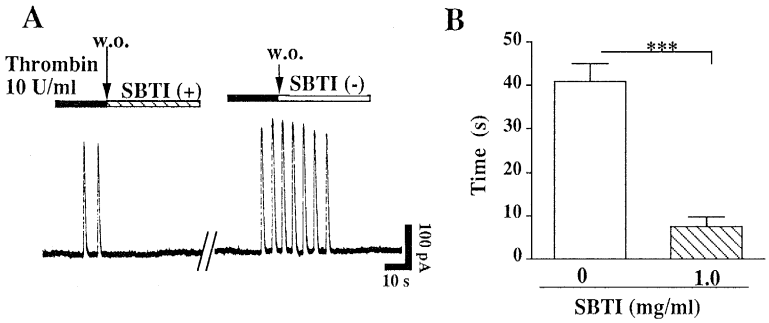

Fig. 5. Effects of SBTI on the washout time of thrombin-induced I_{KCa}. (A) Typical current trace of megakaryocyte stimulated with thrombin and then washed with standard external solution or SBTI-containing external solution. Arrows indicate the time the external solution was changed. (B) Mean washout time when thrombin 10 U/ml was applied for 20 s and then washed by each external solution. The data were obtained from eight cells. * Significantly different from control at $P < 0.01$.

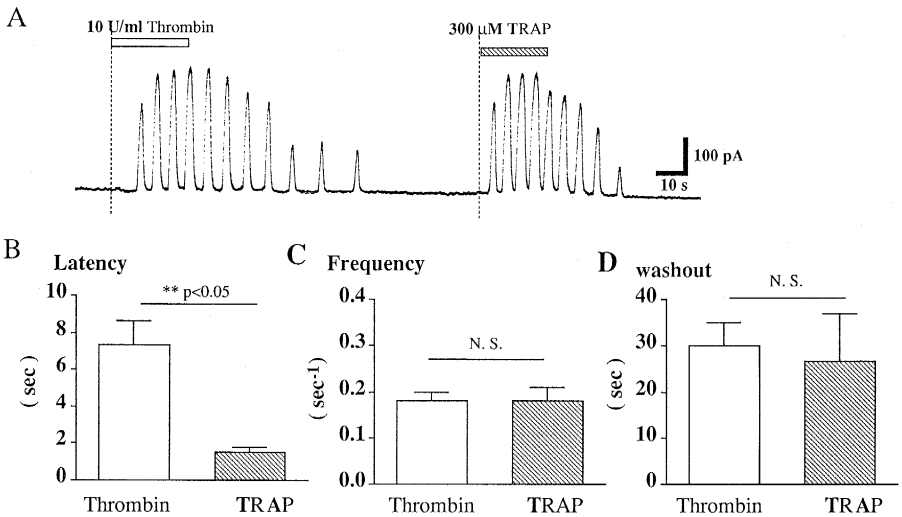

Fig. 6. Comparison of the thrombin- and thrombin receptor agonist peptide (TRAP)-induced I_{KCa} oscillation. (A) Typical current traces obtained from a single cell by thrombin and thrombin agonist. The drugs were applied during the period indicated by the horizontal bars. Mean latency (B), frequency (C) and washout time (D) of each drug were compared. The data were obtained from four cells. ** Significantly different at $P < 0.05$.

shortened (Fig. 5A). The mean washout time when thrombin at 10 U/ml was applied for 20 s and washed by standard external solution was 39.1 ± 4.1 s ($n = 7$), and the mean washout time when washed by external solution containing 1 mg/ml SBTI was 7.4 ± 2.2 s ($n = 7$) (Fig. 5B). Thus, the long-lasting I_{KCa} oscillation after washing out thrombin was produced by proteolytic response of the residual thrombin on the receptor substrate.

Then we examined another characteristic property of the thrombin-induced response, latency. It is known that thrombin cleaves receptor substrate to produce active receptor agonist peptide. Thus we applied the synthetic thrombin receptor agonist peptide (TRAP) to megakaryocytes. As shown in Fig. 6A, TRAP also induced an oscillatory I_{KCa} as in the case of thrombin. However, the latency of TRAP-induced I_{KCa} was significantly shorter than the latency of thrombin-induced I_{KCa} (Fig. 6B). The latency of TRAP-induced I_{KCa} was almost the same as the latency of ATP-induced I_{KCa}. Frequency and washout time were not different between thrombin and TRAP (Fig. 6C and D). Thus the relatively long latency of thrombin-induced response may be caused by the period required to produce a sufficient amount of agonist by the proteolytic action of thrombin.

4. Discussion

In the present study using rat megakaryocytes, we demonstrated that the rapid application of thrombin evoked repetitive Ca^{2+}-activated K^+ current via the serine protease activated receptor. The whole-cell patch-clamp technique combined with the rapid drug application system presented here enabled us to examine the 'on and off' responses after the application and removal of agonists, or the effects of repetitive agonist application on the same cell with high temporal resolution. With this system, we demonstrated the time course of thrombin response and that thrombin and thrombin receptor agonist peptide (TRAP) showed different time dependency to activate intracellular signaling machinery.

Megakaryocytes responded to thrombin showing oscillatory I_{KCa} as previously reported in the case of ATP (Uneyama et al., 1993a). The maximum current amplitude (I_{max}), frequency and washout time increased as the concentration of thrombin increased (Fig. 1). The thrombin-induced I_{KCa} was blocked by the thrombin antagonists hirudin and protease inhibitor, SBTI (Fig. 2). Thus the enzyme activity of thrombin as a protease was required to activate the receptor. In addition, a protease with wider substrate specificity, trypsin, also evoked I_{KCa} similar to thrombin (Fig. 3) as reported before (Jakobs and Aktories, 1988). The trypsin-induced I_{KCa} was insensitive to hirudin and sensitive to another protease inhibitor, aprotinin. Thrombin and trypsin seemed to activate the same receptor as cross-desensitization between these two agonists was observed (Fig. 3). Chymotrypsin, collagenase, thermolysin and elastase had no effect, showing that the substrate specificity did not match the receptor.

In comparison with ATP response in rat megakaryocyte, characteristic features for the thrombin response were fast desensitization, long washout time and long latency. We investigated these points further. *Desensitization*: As shown in Fig. 4, thrombin-induced response diminished after repetitive application, but application of ATP could induce oscillatory I_{KCa} even after thrombin-induced response disappeared (Fig. 3C). Some lines of evidence suggest that both thrombin and ATP mobilize Ca^{2+} from intracellular Ca^{2+} stores via activation of the phosphatidylinositol cascade (Brass et al., 1991; Hung et al., 1992; Uneyama et al., 1993b). Thus the desensitization of I_{KCa} induced by thrombin might be due to the desensitization at receptor sites, but not at the signal transduction steps to the I_{KCa} opening after the receptor activation. *Washout time*: As shown in Fig. 5, the long washout time of thrombin-induced I_{KCa} was shortened by the presence of SBTI. This result indicates that the long washout time is caused by the residual thrombin and the receptor-bound thrombin is difficult to washout completely by simply exchanging extracellular solution. *Latency*: Fig. 6 indicated that TRAP could induce I_{KCa} oscillation with short latency. Therefore, the long latency of thrombin-induced I_{KCa} may be the time required to produce enough amount of agonist peptide by the proteolytic action. However, an unexpected result was obtained from the TRAP experiment, that was long washout time of TRAP-induced response. Though this washout time was as long as the thrombin-induced response, TRAP-induced washout time was insensitive to SBTI treatment (data not shown). Thus the mechanism may be different from thrombin-induced reaction. As in the case of TRAP, the thrombin receptor must be intact and must have bulk structure. We suppose that such a difference in three-dimensional structure or TRAP-binding-induced changes in receptor structure may inhibit TRAP being washed out easily.

These results agree with previous studies concerning the activation mechanism of the thrombin receptor (Ishii et al., 1993; Vu et al., 1991a,b), which showed the thrombin receptor is cleaved by thrombin at the sequence LDPR/S to generate tethered agonist peptide. According to Vu et al. (1991a), thrombin at first binds to the receptor and then cleaves the receptor. The binding of thrombin to the receptor may be relatively strong and may be difficult to wash out completely by a simple exchange of the external solution. Thus, the main difference between ATP and thrombin-induced responses might be caused by the mode of receptor activation.

In the present experiments, megakaryocytes required relatively high concentrations of thrombin (over 3 U/ml) to evoke the response. In platelets, thrombin is known to activate phospholipase C at lower concentrations (Banga et

al., 1988), and also in our laboratory, platelet responding to thrombin obtained from the same animal showed aggregation and elevation of the intracellular Ca^{2+} concentration at 0.1 U/ml, even when the same lot of thrombin was used (data not shown). Thus the thrombin receptor on megakaryocytes seemed to be the 'moderate affinity' type, which differed from platelet thrombin receptor.

In conclusion, we indicate here the kinetic properties of thrombin receptor on megakaryocytes, and revealed that the thrombin receptor on megakaryocytes differed from platelet thrombin receptor. As we have already suggested in a previous report (Uneyama et al., 1993a), platelet formation from megakaryocyte might not be a simple fragmentation of cytoplasm.

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