Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 282-288

NOT CA^{2+} BUT CAMP IS THE SECOND MESSENGER FOR MORPHOLOGICAL CHANGES IN RAT MEGAKARYOCYTE

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Received April 28, 1995

ATP and thrombin both induced Ca^{2+} mobilization from intracellular Ca^{2+} store site of megakaryocyte, the progenitor cell of platelet (Uneyama C., Uneyama H. and Akaike N. (1993) J. Physiol.(Lond.), 470,73-749). Since in platelet, thrombin is known as a strong agonist and ADP is known as a weak agonist, we further investigated the effect of these agonists on megakaryocyte. Thrombin induced Ca^{2+} mobilization, 5-hydroxy tryptamine (5-HT) release and aggregatory morphological changes in megakaryocyte, but ATP induced only Ca^{2+} mobilization. Thrombin-induced 5-HT release was inhibited by adenylate cyclase-activating drugs, and the morphological changes could be induced by H-8, an inhibitor of cAMP-dependent protein kinase. These results suggest that the Ca^{2+} mobilization is not sufficient to induce morphological changes, and the signal to cause morphological changes in megakaryocyte may be cAMP.

Megakaryocyte is the progenitor cell of platelet and it is believed that platelet is produced by fragmentation of the cytoplasm of megakaryocyte [1]. And megakaryocyte shares many functional properties with platelet including uptake of 5-HT [2], phagocytic activity [3], responsiveness to bioactive substances [4,5] and proteoglycan and lipid composition [6,7]. However, some differences of membrane between platelet and megakaryocyte that indicated simple physical fragmentation could not explain the production of platelet from megakaryocyte have been also reported [8]. At present, it is still unclear how the number of platelet in blood is regulated and by what mechanism platelet is produced from megakaryocyte in vivo.

As it is difficult to obtain pure and much amount of megakaryocytes from living animals, the study about megakaryocytes has been limited. Recent advances in the technique to monitor physiological responses of single cell allowed us to study the property of living cell without purification or cultivation. The perforated whole-cell patch-clamp method reported by Horn and Marty [9] is the best technique for megakaryocyte [10,11]. We have previously reported that rat megakaryocyte responded to both ATP and ADP showing periodic activation of Ca^{2+}-dependent K^+ current (Ca^{2+} oscillation).

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0006-291X/95 $5.00

Thrombin was another agonist of megakaryocyte to induce Ca^{2+} mobilization [12]. In platelet, thrombin and ADP are known to be classified into different class of agonist, thrombin as a strong agonist that induced both platelet aggregation and release reaction by itself, and ADP as a weak agonist that induced platelet aggregation only in the presence of another stimuli [13]. Thus we compared the effect of ATP with that of thrombin on megakaryocyte. The reactions studied were, Ca^{2+} mobilization estimated as I_{KCa} by perforated whole cell patch-clamp method, 5-HT release by spectrofluorometry and morphological changes by scanning electron microscopy (SEM). These studies revealed the different roles of second messengers in cellular physiological responses.

MATERIALS AND METHODS

Preparation of the cell

Adult male and female Wistar rats weighing 250-500 g were anaesthetized by the inhalation of an overdose of diethylether and exanguination. The femoral bones were isolated from each animal and the bone marrow was washed out with standard external solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM N-2-hydroxtethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4) by the use of a disposable syringe and needle. The solution was filtered through a 75 μm nylon mesh to eliminate a large mass of cells and bone pieces.

Electrical measurements

The bone marrow cell suspension was transferred to a recording chamber (Falcon, Primaria tissue culture dish, diameter 35 mm) and left for a few hours at room temperature until the cells stably settled on the base of the chamber. The whole-cell current of megakaryocytes were measured with the modified nystatin perforated patch technique as previously described [10,11].

5-HT release

Bone marrow cells were suspended in Ca^{2+}-free assay buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.6 mM glucose, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 5 mM HEPES and 0.1% bovine serum albumin, pH 7.4) at 1 to 5 x 10^7 cells/ml. The drugs were dissolved in assay buffer 2 (Ca^{2+}-free assay buffer plus 2 mM $MgCl_2$ and 3.6 mM $CaCl_2$, pH 7.4) and 200 μl of the drug solution was added to 200 μl of the cell suspension. After incubation at 37 °C, 600 μl of ice-cold Ca^{2+}-free assay buffer was added and after centrifugation at 5 °C, 1000xg, 5 min, the supernatant was used for 5-HT assay. Before the assay, 70 % perchloric acid was added to remove protein by centrifugation. 5-HT content in the supernatant was determined by spectrofluorometry according to Nathenas et al. [14], with Hitachi F-4010 spectrofluorometer. The values were compensated with control samples. Data in the figures were statistically analyzed using Student's t test .

SEM

Bone marrow cell suspensions were placed on polylysine-coated slide glass (Matsunami) and settled at least for 30 min at 37 °C under humidified condition. Then the cells were prefixed in 1% glutaraldehyde (0.1 M phosphate buffer, pH 7.4) for 2 hr, postfixed in 1% osmic acid (0.1 M phosphate buffer, pH 7.4) for 1 hr. After the fixation, cells were dehydrated in a graded series of ethanol. Specimens were dried with the critical point drying method, coated with gold and observed by a JSM-840A JEOL scanning electron microscope.

RESULTS

Ca^{2+}-mobilization

Both ATP and thrombin induced oscillatory outward current when monitored with nystatin-perforated whole-cell recording under voltage-clamped condition at a holding potential

(V_H) of -43 mV. This outward current was identified as a K^+-current passed through Ca^{2+}-activating K^+ channel (I_{KCa}) reflecting cytoplasmic Ca^{2+} elevation, thus could be used as an indicator of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) [10]. We showed some of the properties of ATP- and thrombin-induced I_{KCa} in Table 1. As the reaction intensity of the oscillatory I_{KCa} can be quantified by maximum current amplitude (I_{max}) and frequency of the I_{KCa} spikes per sec, we compared these parameters among ATP, ADP and thrombin. The concentration of each agonist used in the table was the concentration that induce the most strong reaction. It is clear that there is no difference between the maximum reaction intensity of purinoceptor- and thrombin-induced oscillatory I_{KCa}. In addition, the mode of regulation also seemed to be the same between ATP and thrombin. Both ATP- and thrombin-induced oscillatory I_{KCa} were completely inhibited by the application of cAMP elevating drugs such as forskolin (1μM) and isobutylmethylxanthin (IBMX)(10 μM) (data not shown).

5-HT release

Then the effects of ATP and thrombin on 5-HT release were investigated. As megakaryocyte is relatively rare in bone marrow cell suspension (about 0.2 % of the total bone marrow cells), we first tried to purify the megakaryocyte according to the method described by Levine and Fedorko [15]. But the purified megakaryocyte showed lower reactivity to ATP and thrombin when monitored by whole-cell patch-clamp method because of their treatment of Ca^{2+}-free buffer and cAMP-elevating drugs for relatively long period (more than 30 min). The purification of megakaryocyte required these condition to inhibit aggregation during the process, but also cause deleterious changes in megakaryocyte function without observable morphological changes. Then we used bone marrow cell suspension without any processing. 1 x 10^7 cell of bone marrow cell contained about 500 pmole of 5-HT, and 1 x 10^7 platelet contained about 200 pmole of 5-HT. When we assume one megakaryocyte produce 4 to 8 x 10^3 platelet [16] and considered that bone marrow cells also contained megakaryocytes of small size, these value indicate that the 5-HT from bone marrow cell suspension are almost derived from megakaryocyte. Therefore, we used unpurified bone marrow suspensions instead of purified megakaryocytes.

Table 1 Characteristics of osillatory K^+ current

Agonists	Imax	Frequency
ATP (30μM)	1*	1*
ADP (1μM)	1.05 ± 0.10	1.04 ± 0.06
Thrombin (30U/ml)	1.02 ± 0.12	0.96 ± 0.16

The values indicated are normalized to those of 30 μM ATP-induced one (*) (I_{max} ; 638.2 ± 71.1 pA (n = 6), frequency ; 0.20 ± 0.02 s^{-1} (n = 6)). Each value represents the mean ± S.E.M. from four to six cells.

Thrombin induced 5-HT release at higher concentrations (over 3 U/ml) and ATP and ADP did not release 5-HT at all (Fig. 1A). A23187, which induce irreversible and continuous $[Ca^{2+}]_i$ increase in megakaryocyte induced 5-HT release. This may be caused by cell lysis as A23187 treated cells almost died and disappeared in 30 min. Thus the value of 5-HT release induced by high-concentration of A23187 could be regarded as maximum release. No additional effect was observed with combined treatment of thrombin and ATP or ADP (data not shown). As thrombin is known to activate G_i to decrease cellular cAMP level as well as mobilizing Ca^{2+} [17], we then examined the effect of cAMP on 5-HT release from bone marrow cells. Fig.1B shows the effect of forskolin and IBMX on thrombin-induced 5-HT release. Forskolin inhibited both control and thrombin-induced 5-HT release from bone marrow cells, and IBMX also seemed to inhibit the thrombin-induced 5-HT release. These results suggest that the 5-HT release from megakaryocyte is negatively regulated by cAMP.

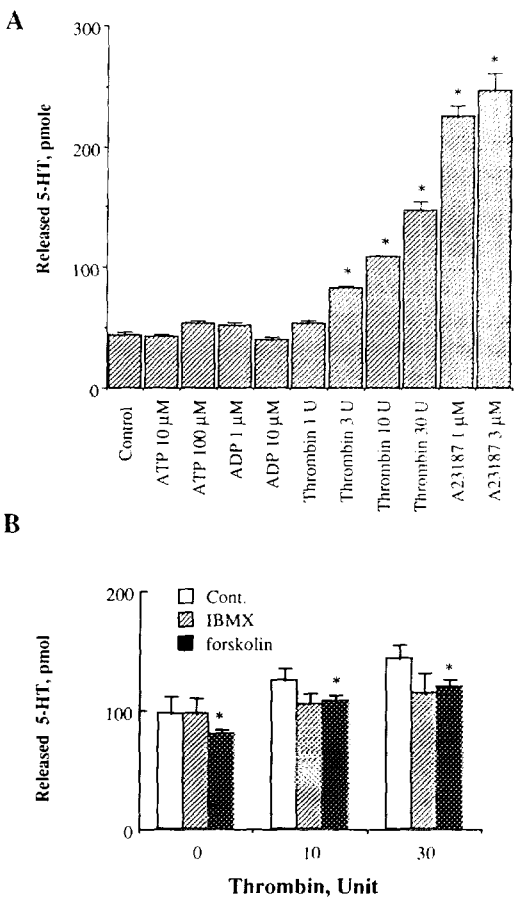


Figure 1. 5-HT release from bone marrow cells(A). The released 5-HT from bone marrow cells, 1.0 x 10^7 per tube. The incubation was for 60 min at 37 °C. B; Bone marrow cell suspension (1.5 x 10^7 cells/tube) was incubated at 37 °C for 30 min under mild shaking with 100 μM IBMX (shaded column) or 10 μM forskolin (closed column). Each value represents mean ± S.E.M. from three samples. * represent the significant level of $p<0.05$ as tested for the difference between the experimental and corresponding control.

Morphological changes

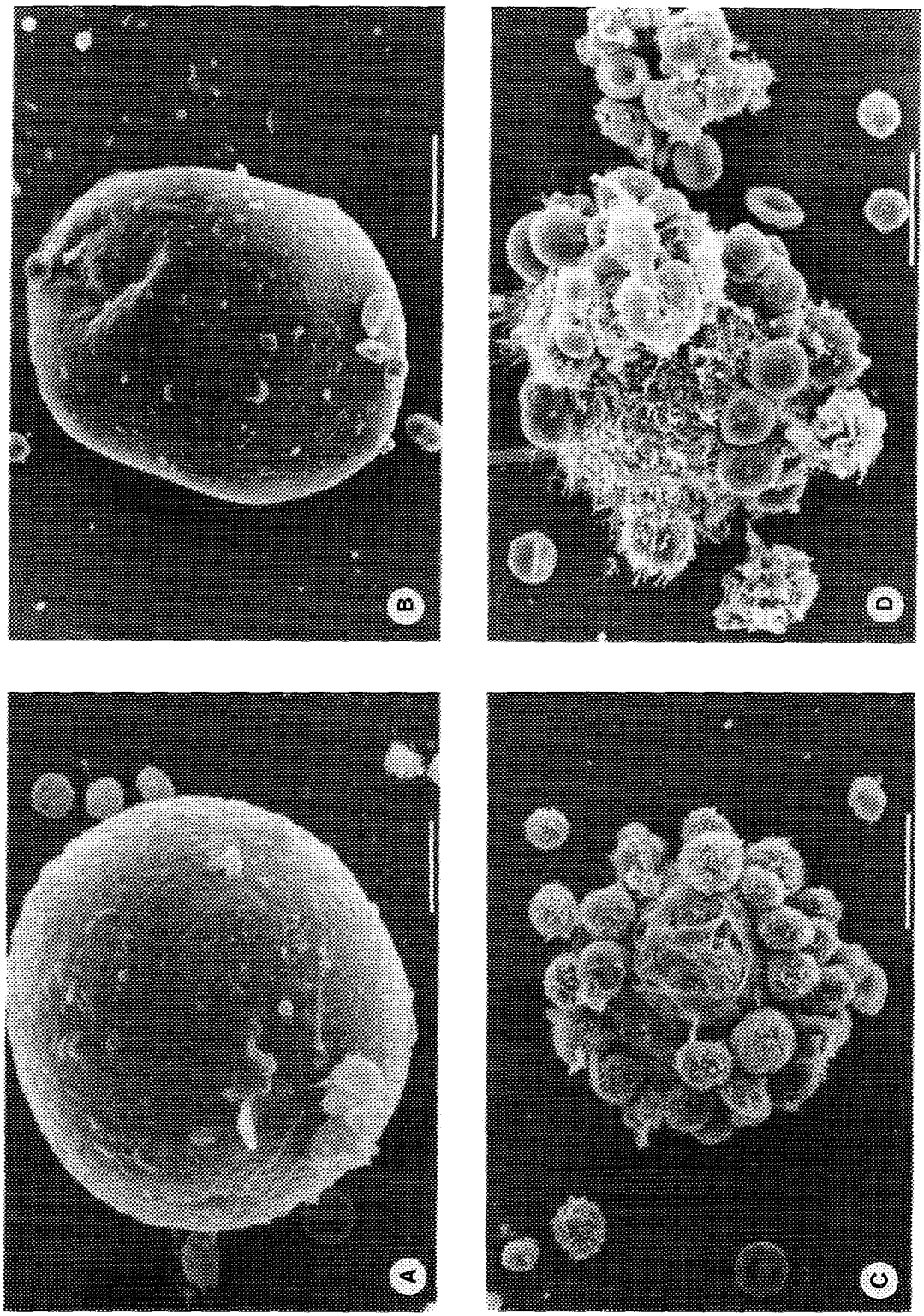
From above experiments, it is suggested that Ca^{2+} mobilization was not sufficient to induce 5-HT release from megakaryocyte. Then we examined the effect at ultra structural level. Megakaryocyte isolated from bone marrow looked like large round ball with relatively smooth surface by SEM (Fig. 2A). The size varied cell to cell, but the surface structures were similar. Such a smooth surface did not altered by ATP treatment (Fig. 2B), but thrombin induced drastic changes in the shape and surface structure (Fig. 2C). Thrombin induced villi-like surface structure of both megakaryocyte and other bone marrow cells which we could not identify, and form a large aggregatory complex. This complex prevent us to see megakaryocyte clearly, but we could recognize the altered morphology of megakaryocyte through the slit of the 'sticky' cells. Megakaryocyte looked like as expanding many pseudopod, surface became irregular and surrounded by filopodium-like structure. In other magakaryocyte, the cytoplasm looked like as divided into many small irregular balls and aggregate leaving large nuclei naked. Similar morphological changes could be induced by treatment of the cell with H-8, an inhibitor of cAMP-dependent protein kinase (Fig. 2C). H-8 induced irregular villi- or filopodium-like structure of megakaryocyte. Though the effect of H-8 seemed to be less strong than thrombin, it was clearly distinguished from the effect of ATP. In addition, 10 μM of Rp-cAMPS, an antagonist of cAMP[18], also induced morphological changes in surface structure of megakaryocyte as in the case of H-8 (data not shown). We can conclude, therefore, not Ca^{2+}-mobilization but decrease in cellular cAMP level induce morphological changes in megakaryocyte.

DISCUSSION

Platelet is an important component of blood to maintain living organism and the number of platelet is rigidly regulated both in human and in animals. But the mechanism of the regulation remains unclear instead of increasing amount of studies about platelet. As platelet is produced by the fragmentation of its progenitor cell, megakaryocyte, the study about megakaryocyte may be a key point to understand the platelet physiology. Here we demonstrated the responses of magakaryocyte to the two different agonists, ATP and thrombin. We investigated the effect of ATP and thrombin on Ca^{2+}-mobilization by the use of patch-clamp method, 5-HT release as an indicator of release reaction and morphological changes by SEM. These method were applied under careful consideration of the method with the least damage to the cell. For example, image analysis of the fluorescent-dye loaded megakaryocyte [12,19] prevent us to detect oscillatory changes of $[Ca^{2+}]_i$ constantly because Ca^{2+}-chelating fluorescent dye disturbed intracellular Ca^{2+} homeostasis. And popular assay method to detect the release of preloaded radiolabeled 5-HT [20] could not be applied because we had to use bone marrow cell mixture and it seemed to be better to use cells without any delay after bone-isolation.

ATP induced I_{KCa} oscillation, but had no effect on release reaction and cellular morphology. In contrast, thrombin induced I_{KCa} oscillation, release reaction and morphological

Figure 2. Scanning electron micrograph of megakaryocyte treated with agonists. The treatments were as follows: standard external solution for 30 min (A), ATP 30 μM for 30 min (B), thrombin 10 U/ml for 30 min (C) and H-8 3 μM for 2 hours (D). The horizontal bars in each photomicrograph represent 10 μm.


B
D
A
C

change. The mechanism of I_{KCa} oscillation induced by ATP and thrombin seemed to be the same as shown in Table 1. In addition, the effect of cyclopiazonic acid, an inhibitor of Ca^{2+}-ATPase, was also the same and extracellular Ca^{2+} was not required in both ATP and thrombin-induced I_{KCa} oscillation (data not shown). These results indicate that the $[Ca^{2+}]_i$ oscillation evoked by ATP and thrombin shares the same mechanism, involving G-protein activation, IP_3 generation, protein kinase C activation, and negative regulation by cAMP [11]. Thrombin is known to activate both Gq and Gi, and ATP seemed to activate Gq. These results suggest that the signal required to release reaction is the decrease in cAMP, not increase in $[Ca^{2+}]_i$. This hypothesis was confirmed by the fact that morphological change of megakaryocyte could be induced by H-8 (Fig. 2D) and Rp-cAMPS, inhibitors of A-kinase. In addition, no synergistic stimulation of 5-HT release was observed between ATP and thrombin at any concentrations. Though 5-HT release could be induced by Ca^{2+}-ionophore, A23187, it may reflect cellular severe damage as A23187 induce persistent increase in $[Ca^{2+}]_i$ that could not be observed in physiological stimulation.

The idea that $[Ca^{2+}]_i$ is not so important in the morphological change may be peculiar as $[Ca^{2+}]_i$ and protein kinase C is known to play central roles of the release reaction in many cells including platelet [21]. However, it is also the fact in megakaryocyte that ATP mobilize Ca^{2+} and activate protein kinase C [11] without any detectable morphological changes in acute phase. Thus it may be reasonable to think that the roles of these second messengers differ from cell to cell, even between a cell and its direct progenitor cell.

We hope this report contribute the future advances in our understanding of the blood homeostasis and differentiation of the progenitor cell.

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