Biological actions of purines on rat megakaryocytes: potentiation by adenine of the purinoceptor-operated cytoplasmic Ca^{2+} oscillation

¹Chikako Uneyama, *Hisayuki Uneyama, Michihito Takahashi & *Norio Akaike

Division of Pathology, National Institute of Health Sciences, Setagaya-ku, Tokyo 158, and *Department of Bio-Plasticity, Faculty of Medicine, Kyushu University, Fukuoka, 812 Japan

We have found that adenine enhanced the purinoceptor-operated cytoplasmic Ca^{2+} oscillation in rat megakaryocytes at submillimolar concentrations. Guanine and other nucleic acid bases had no effect on this system. Adenine enhanced the reaction intensity but had no effect on the threshold concentration of ATP to evoke the oscillation.

Keywords: Adenine; purinoceptor; cytoplasmic Ca^{2+} oscillation; rat megakaryocyte; Ca^{2+} -activated K^+ current

Introduction We have previously reported that rat megakaryocytes responded to extracellularly applied ATP and ADP by showing periodic activation of a Ca^{2+} -dependent K^+ current (I_{KCa}) reflecting oscillatory changes in cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) induced by novel subtype of purinoceptor (Uneyama *et al.*, 1993a). As the feature of I_{KCa} oscillation in megakaryocytes is an advantage in investigating the physiological properties of single cells, we can obtain many interesting data with this experimental system (Uneyama *et al.*, 1993b,c; Akaike *et al.*, 1993; Uneyama *et al.*, 1994). Thus we studied the effect of purines and pyrimidines, and found that adenine had an enhancing effect on the purinoceptor-induced I_{KCa} oscillation. This is the first account of the modulatory effect of adenine on receptor function.

Methods The isolation technique for megakaryocytes was described previously (Uneyama *et al.*, 1993a). Megakaryocytes were used for electrical experiments within 8 h after isolation. Electrical measurements were performed with the modified perforated patch recording of Horn & Marty (1988) as previously described (Uneyama *et al.*, 1993a,b). The resistance between the recording electrode filled with internal solution and the reference electrode in external solution was 4–6 M Ω . The liquid junctional potential between the pipette solution and the standard external solution was approximately –3 mV. The data were not compensated for this potential. All experiments were performed at room temperature (20–22°C). The ionic composition of standard external solution was (in mM): NaCl 150, KCl 5, MgCl_2 1, CaCl_2 2, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris [hydroxymethyl]aminomethane (Tris)-OH. The composition of the patch-pipette solution was (in mM): KCl 150 and HEPES 10. The pH was adjusted to 7.2 with Tris-OH.

H-8 (N-[2-methylamino]ethyl]-5-isoquinoline sulphonamide dihydrochloride) was obtained from Seikagaku Kogyo (Tokyo, Japan) and all other drugs were obtained from Sigma (St. Louis, U.S.A.). All drugs were dissolved in the standard external solution just before use. Drugs were applied by a rapid application method termed the 'Y-tube' method, as described by Murase *et al.* (1990). With the use of this technique, the drugs could be applied rapidly to megakaryocytes within 20 ms.

Results At first, we examined the Ca^{2+} mobilizing effects of purines (adenine and guanine) and pyrimidines (cytosine and

uracil) on rat megakaryocytes. None of these drugs induced any detectable outward current at concentrations up to 1 mM (data not shown). However, in the presence of ATP (1 μM) or ADP (0.01 μM), adenine markedly enhanced I_{KCa} spikes induced by the purinoceptor stimuli. A typical current trace is shown in Figure 1a. Adenine (1 mM) evoked I_{KCa} spikes only in the presence of ATP in a good reproducible manner, but another purine, guanine, had no effect. Pyrimidines also had no enhancing effect on the I_{KCa} at concentrations up to 1 mM (data not shown). The action of adenine occurred 14 ± 3 s ($n = 7$) after the drug application and diminished 31 ± 11 s ($n = 7$) after the drug wash out.

As previously reported (Uneyama *et al.*, 1993a), the EC_{50} value for ATP was about 3 μM , and the concentration (1 μM) of ATP used in Figure 1a was the threshold one to evoke I_{KCa} . The synergistic action of adenine and sub-threshold (0.3 μM) and over-maximal (100 μM) concentrations of ATP was, therefore, examined (Figure 1b). Adenine (1 mM) had no effect in the presence of these sub-threshold or supra-maximal concentrations of ATP. Thus, the enhancing effect by adenine was dependent on the ATP concentration, but the receptor sensitivity for ATP was not so dramatically changed as in the case of Ca^{2+} and Mg^{2+} (Uneyama *et al.*, 1993a).

The concentration-dependency of the enhancing action of adenine was investigated. The cells were pretreated with adenine for 1 min before 3 μM ATP application. Adenine increased the frequency of I_{KCa} spikes in a concentration-dependent manner, but did not enhance the current amplitude (Figure 2a). In Figures 2b and c, the quantitative results are shown. Adenine exerted its enhancing action on the current frequency from 1 μM , and the ED_{50} value was about 80 μM , but it failed to increase current amplitude at concentrations up to 1 mM.

Then, we examined whether the enhancing action of adenine was apparent when another agonist was used instead of ATP. We used thrombin as the Ca^{2+} mobilizer, because thrombin also evoked repetitive I_{KCa} spikes in rat megakaryocytes. However, in contrast to purinoceptor stimulation, adenine (1 mM) failed to enhance 5 u ml^{-1} thrombin-induced I_{KCa} oscillation (data not shown). Thrombin-1 5 u ml^{-1} was a submaximal concentration to evoke I_{KCa} oscillation in rat megakaryocytes. In addition, co-administration of adenine (300 μM) and ATP (1 μM) showed the synergistic action in the presence of H-8 (10 μM), an inhibitor of adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase, or forskolin (1 μM), an activator of adenylate cyclase (data not shown). Moreover, even in the absence of Mg^{2+} and Ca^{2+} in the external solution, adenine enhanced the ATP-induced responses (data not shown).

¹ Author for correspondence.

Figure 1 (a) Response to adenine and guanine in the presence and absence of ATP. Note the adenine (1 mM) evoked I_{KCa} spikes only in the presence of 1 μ M ATP. (b) Effect of adenine on the responses induced by low (0.3 μ M) and high (100 μ M) concentrations of ATP. The megakaryocyte was voltage-clamped at a V_H of -40 mV. Drugs were continuously applied for the period shown by the bars above each response. Each current trace was obtained from two different cells.

Figure 2 Concentration-dependent enhancement by adenine of the ATP-induced I_{KCa} oscillation. (a) Typical current traces obtained from the same cell. V_H was -40 mV. Adenine at each concentration was applied 1 min prior to the application of ATP. The current traces were obtained from a single cell, and a typical one of 5 cells examined. (b) Quantitative results obtained from (a). The value of frequency and maximum current amplitude (I_{max}) were normalized to the value obtained with 3 μ M ATP alone. Each value represents the mean $\pm$ s.e. mean of five to six cells.

Discussion Here we demonstrate that adenine enhanced the purinoceptor-induced I_{KCa} oscillation in a concentration-dependent manner. The other nucleic acid bases examined

had no effect. The modulatory action was independent of cyclic AMP and the presence of divalent cations. In previous work, the purinoceptor-operated I_{KCa} was potentiated by reduction of the extracellular divalent cations and inhibited by elevation of intracellular cyclic AMP concentrations (Uneyama *et al.*, 1993a,b; Akaike *et al.*, 1993). Therefore, the modulatory effect of cyclic AMP and the binding of divalent cation to ATP and ADP is not involved in the action of adenine. The thrombin-operated I_{KCa} was, moreover, unaffected by adenine, although that might also be induced by an intracellular signal transduction pathway similar, in part, to the purinoceptor-mediated one. These results suggested that adenine might have a specific receptor site on megakaryocytes. It may be an adenine receptor or a part of a purinoceptor. Though the word 'purinoceptor' is the usual term, the true agonists are not purines, but purine nucleosides or nucleotides (Burnstock & Kennedy, 1985). Thus the discovery of the pharmacological activity of adenine may provide new insight in this area.

Adenine itself is a major intermediate compound of biologically active substances such as ATP and cyclic AMP or nucleic acids consisting of DNA and RNA. When cells are injured or die, these might be released from the intracellular to extracellular spaces and the free base, adenine, might be generated. Furthermore, though adenine has been used as a component of blood preservatives (McShine *et al.*, 1990), adenine has not been regarded as an agonist with any pharmacological function. The findings indicated here will contribute not only to the understanding of the physiology of megakaryocytes but also to the mechanism of blood coagulation.

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