Cytoplasmic pH regulates ATP-induced Ca^{2+} -dependent K^{+} -current oscillation in rat megakaryocytes

Chikako UNEYAMA,*† Hisayuki UNEYAMA,† Michihito TAKAHASHI* and Norio AKAIKE†

*Division of Pathology, National Institute of Health Sciences, Tokyo 158, and †Department of Neurophysiology, Tohoku University School of Medicine, Sendai 980, Japan

The effect of cytoplasmic pH (pH_i) on ATP-induced Ca^{2+} oscillation of rat megakaryocytes was investigated by the whole-cell patch-clamp technique. Megakaryocytes responded to extracellular ATP and showed the periodic activation of K^{+} channels, which reflects the oscillation in intracellular free Ca^{2+} concentration. Intracellular alkalinization by 20 mM NH_4Cl resulted in inhibition of the oscillatory response, and intracellular acid-

ification by 20 mM sodium acetate enhanced the response. NH_4Cl also inhibited the $\text{Ins}(1,4,5)\text{P}_3$ -induced oscillation. Sodium acetate had no effect on the InsP_3 -induced oscillation, but enhanced the guanosine 5'-[γ -thio]triphosphate-induced response. These results indicate that pH_i modulates the ATP-induced cellular response at least at two points.

INTRODUCTION

Some biologically active substances are known to affect intracellular pH (pH_i). For instance, thrombin was reported to change platelet pH_i , at first in an acidic and later an alkaline direction (Sage et al., 1990; Zavoico et al., 1986). But the role of pH_i in the cellular response remains unclear, and controversial results have been reported on the role of Ca^{2+} mobilization in platelets (Siffert and Akkerman, 1987; Zavoico and Cragoe, 1988). One of the reasons for such confusion seems to be the small size of the platelet. As it is difficult to investigate platelets at the single-cell level, most studies were performed on platelet suspensions. In these experimental conditions, the results are the sum of each response and include adverse side reactions such as aggregation. Recently we have reported that the progenitor cell of platelets, the megakaryocyte, could be a useful model for investigation of agonist-induced intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) oscillations (Uneyama et al., 1993). Megakaryocytes resemble platelets in many points, including their phagocytic property and glycoprotein composition (Fedorco, 1977), and the large size (30–70 μm) enabled us to detect the response in single-cell level easily. Thus it may be informative to know the single-cell response of megakaryocytes for further understanding of platelets. Here we report the effect of pH_i on ATP-induced K^{+} -current oscillation of megakaryocytes.

MATERIALS AND METHODS

Preparation

Adult male and female rats weighing 250–350 g were anaesthetized 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 with a disposable syringe with a needle. After filtration through 75 μm -pore nylon mesh to eliminate large masses of cells, the solution containing bone-marrow cells was transferred to a recording chamber. The chamber was left for a few hours at room temperature until the cells settled stably on the base of the recording chamber.

Megakaryocytes could be clearly distinguished from other

bone-marrow cells under a phase-contrast inverted microscope by their large size (30–50 μm).

Solutions

The ionic composition of the standard external solution was (in mM): 150 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl, 10 glucose and 10 Hepes. The pH was adjusted to 7.4 with Tris base. The internal solution for the nystatin perforated patch was (in mM) 150 KCl and 2 Hepes, and the pH was adjusted to 7.2 with Tris base. Nystatin was added at a final concentration of 150 $\mu\text{g}/\text{ml}$ to the internal solution just before use. The internal solution for conventional whole-cell patch was (in mM): 150 KCl, 5 ATP, 2 MgCl_2 and 2 Hepes.

Electrical measurements

Whole-cell currents of the megakaryocytes were recorded with nystatin-perforated and conventional patch-recording configurations (Horn and Marty, 1988; Uneyama et al., 1992) under voltage-clamped conditions at room temperature (21–24 °C). The resistance between the recording electrode and the Ag–AgCl reference electrode was 4–6 M Ω . The current was measured with a patch-clamp amplifier (List, EPC7), and monitored simultaneously on a storage oscilloscope (Tektronix, 511A) and a thermal-head pen recorder (Sanei, Rectihoriz-8K21) after filtration at 1 kHz.

Measurement of pH_i

The pH_i was measured with the fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Isolated rat bone-marrow cells were incubated with 5 μM BCECF acetoxymethyl ester (AM) for 40 min on glass coverslips inside a culture dish. Subsequently, the coverslips were washed three times with the standard external solution and mounted in a chamber on the stage of an inverted fluorescence microscope. pH_i was measured by ratio imaging of BCECF fluorescence excited at 440 and 490 nm and imaged through a 510 nm dichroic reflector and a 520 nm long-pass filter using an Argus 50 image

Abbreviations used: pH_i , intracellular pH; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; GTP[S], guanosine 5'-[γ -thio]triphosphate.

† To whom correspondence should be addressed.

analysis system (Hamamatsu Phototonics). The minimal recording interval in our system was 2 s. The fluorescence signal of BCECF was calibrated to pH at the end of the experiment by the method described by Paradiso et al. (1986). The presence of the glass electrode had no specific effect on the resting pH_i and the pH_i changes induced by drugs.

Drug application

Drugs were applied by the use of a rapid application method termed the 'Y-tube' method, as described elsewhere (Nakagawa et al., 1990; Uneyama et al., 1993). By this technique, the solution surrounding an isolated megakaryocyte could be completely exchanged within 20 ms.

Drugs

BCECF-AM was purchased from Molecular Probes. ATP, nystatin and all other chemicals used were purchased from Sigma Chemical Co.

RESULTS

With the nystatin perforated-patch recording, the megakaryocyte responded to the extracellular application of 10 μ M ATP and elicited periodic K^+ currents. The current depends on the $[Ca^{2+}]_i$, and we regarded the activation of K^+ channels as reflecting the rise in $[Ca^{2+}]_i$ in all subsequent experiments (Uneyama et al.,

1993). The effects of drugs on Ca^{2+} -activated K^+ current were examined in 3 μ M ionomycin-treated cells. In this condition, the K^+ channels of the megakaryocyte were fully activated; 20 mM NH_4Cl slightly (less than 5%) depressed the K^+ current, whereas 20 mM sodium acetate had no effect. The ionomycin-treated cells showed similar pH_i changes in response to NH_4Cl and sodium acetate as normal cells.

pH change in megakaryocytes

At first, the change in pH_i during drug treatment was measured. Under our experimental condition, megakaryocytes showed a mean resting pH of 7.29 ± 0.23 ($n = 7$). As shown in Figure 1(a), NH_4Cl caused a rapid alkalosis that was sustained during a continuous drug treatment. The rise of pH_i from control was 0.81 ± 0.15 at the maximum level and decreased gradually. After wash-out of the NH_4Cl , the pH_i decreased to become acidic and then recovered to the original basal level. The pH_i decrease ranged between 0.5 and 0.75 compared with the resting level. The latter acidosis after removal of NH_4Cl depended on the period of exposure to NH_4Cl . On the other hand, 20 mM sodium acetate caused a rapid and transient cytosolic acidosis. The maximum pH_i change was 0.51 ± 0.05 ($n = 4$). The pH_i recovered from the acidic state even in the presence of sodium acetate, and no further change was observed after wash-out of the drug. The acidosis was prolonged by only 2–3 min, and the time course of recovery differed from cell to cell. The pH_i change induced by short-term treatment of NH_4Cl and sodium acetate is shown in Figure 1(b). The pH_i change occurred within 2 s after drug application and recovered rapidly on wash-out. In addition, NH_4Cl did not cause acidosis when the application time was less than 20 s. From the results, we used only short-term application of drugs to modify pH_i . ATP (10 μ M) did not cause a detectable pH_i change under our experimental conditions.

Effect of pH on ATP-induced K^+ -current oscillation

The effects of NH_4Cl and sodium acetate were tested on ATP-induced K^+ -current oscillation. The pH of standard solutions containing drugs was adjusted to 7.4 before application. As shown in Figure 2(a), 20 mM NH_4Cl almost completely inhibited the oscillatory K^+ current. The effect was reversible, the oscillatory response being completely recovered after washing out the drug. On the other hand, 20 mM sodium acetate enhanced the ATP-induced response. ATP (10 μ M) usually causes almost the maximum response, which made it difficult to see the enhancing effect of drugs on the ATP response. But in some cells the responses were not fully activated. One example is shown in Figure 2(b). In this cell, the oscillatory K^+ currents in the control became attenuated rapidly, but addition of 20 mM sodium acetate re-activated the ATP response (enhancement of both current amplitude and frequency). The effect on frequency could be seen even when a low concentration of ATP (1 μ M) was used (results not shown).

Effect of pH on $InsP_3$ -induced K^+ -current oscillation

To clarify further the effect of pH_i , $InsP_3$ was perfused intracellularly by conventional whole-cell patch-recording mode. In this method, the recording electrode was filled with internal solution containing 3 or 10 μ M $InsP_3$, and the pipette solution diffused gradually into the cell after rupture of the patch membrane. Megakaryocytes showed K^+ -current oscillation on intracellular application of $InsP_3$ as well as on external application of ATP. The acidosis induced by sodium acetate had no effect on the $InsP_3$ -induced K^+ -current oscillation (Figure 3a). Intracellular

Figure 1 pH_i changes evoked by 20 mM NH_4Cl and 20 mM sodium acetate on long (a) and short (b) time scales

The ratio images of single megakaryocytes loaded with BCECF were obtained at 20 s (a) or 2 s (b) intervals and calibrated to pH_i . The drugs were applied during the period of each horizontal bar. The line represents a typical one from five (a) or three (b) experiments.

Figure 2 Effects of 20 mM NH₄Cl and 20 mM sodium acetate on the 10 μ M ATP-induced oscillatory K⁺ currents

The current traces were obtained by nystatin perforated-patch recording configuration at a holding potential of -40 mV. Each drug was applied during a period indicated by horizontal bars. The current traces are typical ones from nine (a) or seven (b) experiments.

Figure 3 Effects of NH₄Cl and sodium acetate on the InsP₃-induced K⁺ currents

The current traces were obtained by conventional whole-cell patch mode with recording pipettes filled with internal solution containing 10 μ M InsP₃. The current traces are typical ones from five (a) or four (b) experiments.

alkalosis induced by NH₄Cl reversibly inhibited the InsP₃-induced oscillation (Figure 3b).

Effect of pH on guanosine 5'-[γ -thio]triphosphate (GTP[S])-induced K⁺-current oscillation

Intracellular application of 10 μ M GTP[S] also evoked an oscillatory K⁺ current with a lag period after the rupture of patch membrane. In this condition, sodium acetate again enhanced the response in a different way from the ATP-induced one (Figure 4). The enhancement by sodium acetate was seen after some delay, and the effect did not diminish quickly after washing out the drug. NH₄Cl inhibited the GTP[S]-induced oscillatory current, as in the cases of ATP- and InsP₃-induced responses.

Figure 4 Effects of NH₄Cl and sodium acetate on the GTP[S]-induced K⁺ currents

The current traces were obtained by conventional whole-cell patch mode with recording pipettes filled with internal solution containing 10 μ M-GTP[S]. The current trace is a typical one from six experiments.

DISCUSSION

Here we report the effect of pH_i on the agonist-induced rise in [Ca²⁺]_i in megakaryocytes by nystatin-perforated whole-cell patch-recording mode. As the megakaryocyte is very sensitive to Ca²⁺-chelating agents such as fura-2, monitoring of the Ca²⁺-activated K⁺ current is the only way to study the intact Ca²⁺ response. Short-term applications of 20 mM NH₄Cl and sodium acetate were used to modify the pH_i, because these were the most reproducible methods to change the pH_i of megakaryocytes. NH₄Cl (20 mM) had weak inhibitory action on K⁺ conductance, but the maximum current amplitude was decreased only by less than 5% in the presence of 20 mM NH₄Cl. Therefore we concluded that the inhibitory action of NH₄Cl was not caused by block of the K⁺ channel itself. Also the open probability of the Ca²⁺-activated K⁺ channel in some types of cells was reported to decrease in acidic pH (Kume et al., 1990). In megakaryocytes at whole-cell level, the effect of acidosis is enhancement of the response, and no such inhibitory action was observed. Thus we could neglect the direct action of acidic pH_i on the K⁺ channel.

The agonist-induced pH_i change was reported in many types of cells, and some hypotheses were proposed concerning the role of pH_i (Ives and Daniel, 1987; Dantluri et al., 1990). But it was not clear at what point the altered pH_i in fact acted. Some investigators mentioned that the pH_i might affect the binding of InsP₃ to the InsP₃ receptor (Worley et al., 1987) and others described that the pH_i might affect the release of Ca²⁺ (Dettbarn and Palade, 1991). From our results, we could define the point of action of acidosis or alkalosis. On the basis of our previous work (Uneyama et al., 1993), the signal-transduction pathway of ATP in megakaryocytes is as follows: extracellular ATP activates a purinoceptor on the megakaryocyte, and the receptor activates phospholipase C through a G-protein, resulting in the production of InsP₃ through activation of phospholipase C. InsP₃ binds to the InsP₃ receptor on the Ca²⁺-storage site, and Ca²⁺ is released from the store. Acidosis enhanced ATP- and GTP[S]-induced K⁺-current oscillation, but had no effect on InsP₃-induced oscillation. The results suggested that the low pH_i acts before the generation of InsP₃ and/or after the activation of G-protein in the signal-transduction pathway. On the other hand, alkalosis inhibited all the ATP-, InsP₃- and GTP[S]-induced K⁺-current oscillation. The high pH_i therefore inhibits some step after InsP₃ production. The candidate steps may be InsP₃ binding to the InsP₃ receptor and/or Ca²⁺ release from the intracellular store site.

These results clearly indicate that there are several pH_i-sensitive steps in the signal-transduction pathway. In addition, we may be able to speculate on the role of pH_i change from these results. According to previous reports, thrombin caused a biphasic change in pH_i in human platelets: a rapid fall, followed by sustained rise (Zavoico et al., 1986; Sage et al., 1990). Thus, if the

situation is the same in both platelet and megakaryocyte, the Ca^{2+} response may be stimulated at the initial phase and inhibited at the later phase by the change of pH_i . This seems to explain the role of pH_i well. Though we need further investigation to confirm this hypothesis, we believe our findings reported here contribute to future experiments.

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