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Cyclic GMP inhibits cytoplasmic Ca^{2+} oscillation by increasing Ca^{2+}-ATPase activity in rat megakaryocytes

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Abstract

The regulatory effects of cyclic GMP on purinoceptor-operated cytoplasmic Ca^{2+} oscillation of rat megakaryocytes were investigated by using whole-cell patch-clamp technique. ATP-induced oscillatory K^+ currents though Ca^{2+}-activated K^+ channels (I_{KCa}s) were depressed by pretreatment with the guanylate cyclase activator, sodium nitroprusside, and a stable membrane-permeable cGMP analogue, 8-bromo-cGMP. The inhibition by sodium nitroprusside was blocked by treatment with a cyclic nucleotide-dependent protein kinase inhibitor, *N*-[2-(methylamino)]-5-isoquinolinesulfonamide · HCl (H-8) (10 μM), but not by a selective cAMP-dependent-protein kinase inhibitor, Rp-cAMPS (100 μM). The oscillatory I_{KCa} directly evoked by intracellular D-myo-inositol-trisphosphate (IP_3) perfusion was also inhibited by the application of sodium nitroprusside. The inhibitory effect of sodium nitroprusside disappeared when the ATP-induced oscillatory I_{KCa} was changed to a monophasic sustained I_{KCa} current by inhibition of Ca^{2+}-ATPase. These results suggested that cGMP depressed Ca^{2+} mobilization by improving Ca^{2+}-ATPase activity by phosphorylation. © 1998 Elsevier Science B.V.

Keywords: cGMP; Ca^{2+} oscillation; Megakaryocyte, rat; Patch-clamp

1. Introduction

Elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers a wide variety of physiological functions in cells. Cells possess, in fact, mechanisms to regulate $[Ca^{2+}]_i$. cGMP is one such regulator of $[Ca^{2+}]_i$. The cyclic nucleotide is synthesized by guanylate cyclase, which is known to be activated by nitrocompounds and their derivatives (Waldman and Murad, 1987). Recent advances in research have revealed that nitrocompounds such as sodium nitroprusside work as nitric oxide (NO) donors to activate guanylate cyclase (Moncada et al., 1997; Schmidt et al., 1993). The signal transduction pathway of NO has been reviewed by other authors (McDonald and Murad, 1995; Lincoln et al., 1995). In blood platelets and vascular smooth muscle, increases in both cAMP and cGMP cause negative regulation of their functions (Bohme et al., 1978; Doni et al., 1991; Collins et al., 1986; Geiger et al., 1992; Maurice and Hasla, 1990). The intracellular mechanisms of these inhibitory actions are not yet understood.

By applying the perforated patch-clamp technique (Uneyama et al., 1993a), we have successfully monitored the real-time change of subcellular Ca^{2+} concentration as the change in the Ca^{2+}-activated K^+ (I_{KCa}) current after agonist stimulation. With this technique, we reported previously that the megakaryocyte, a progenitor cell of platelets, shows oscillatory I_{KCa} after purinergic stimulation. Because of these properties, the megakaryocyte is a good model for investigating the mechanism of intracellular Ca^{2+} oscillations at the single cell level (Uneyama et al., 1993b). The present study was performed with this Ca^{2+} oscillation model to determine the physiological role of cGMP in cytoplasmic Ca^{2+} homeostasis in the rat megakaryocyte.

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2. Materials and methods

2.1. Isolation of rat megakaryocyte

Megakaryocytes were isolated from femoral bones of adult male and female Wistar rats by previously described techniques (Uneyama et al., 1993a). Under a phase-contrast inverted microscope, megakaryocytes could be clearly distinguished from other bone marrow cells by their large size (40–50 μm).

2.2. Electrical measurement of membrane current

Electrical measurements were performed with the nystatin perforated patch (Horn and Marty, 1988; Uneyama et al., 1993a) and the conventional patch recording (Hamill et al., 1981). The resistance between the recording electrode filled with internal solution and the reference electrode in external solution was 4–6 MΩ. The current and voltage were measured with a patch-clamp amplifier (List Medical, EPC-7) and were monitored on a storage oscilloscope (Tektronix, R5113) and a pen recorder (San-ei, RECTI-HORIZ-8K), and were stored on video tapes after digitization with a PCM processor (Nihon Kohden, PCM501ESN). The liquid junction potential between internal and external solutions was approximately −3 mV. The data were adjusted for this potential. All experiments were performed at room temperature (20–22°C).

2.3. Solutions

The ionic composition of standard external solution was (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 10 glucose. The pH was adjusted to 7.4 with tris[hydroxymethyl]aminomethane (Tris)–OH. The composition of the perforated patch-pipette solution was (in mM): 150 KCl and 10 HEPES. The pH was adjusted to 7.2 with Tris–OH. The conventional patch-pipette solution contained (in mM): 150 KCl, 2 ATP-Mg and 10 HEPES. The pH was adjusted to 7.2 with Tris–OH.

2.4. Drugs

Sodium nitroprusside, ATP, ADP, 8-bromo-cGMP, cyclopiazoic acid (CPA), thapsigargin, nystatin and D-myo-inositol-trisphosphate (IP_3) were obtained from Sigma (St. Louis, MO, USA), and H-8 (*N*-[2-(methylamino)]-5-isoquinolinesulfonamide · HCl) was from Seikagaku Kogyo (Tokyo, Japan). W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide · HCl) and IBMX (3-isobutyl-1-methylxanthine) were from Funakoshi (Tokyo, Japan). All drugs were dissolved in the pipette or standard external solution just before use. Drugs were applied by using a rapid application method termed the 'Y-tube' method, as described elsewhere (Murase et al., 1989). With this technique, the drug could be rapidly applied to a megakaryocyte within 20 ms.

3. Results

In all of the following experiments, megakaryocytes were voltage-clamped at a holding potential (V_H) of −43 mV. Under voltage-clamp conditions and with the nystatin-perforated technique, megakaryocytes showed oscillatory K^+ currents in response to external application of ATP. The current was identified as the I_{KCa} characterized in previous papers (Uneyama et al., 1993a). In the present study, we regarded the I_{KCa} as an indicator of changes in subcellular Ca^{2+} concentration.

In the first experiments, we examined the direct effect of the drugs used here on the Ca^{2+}-activated K^+ channel, by using A23187-permeabilized megakaryocytes (Akaike et al., 1993). Sodium nitroprusside (100 μM), 8-bromo-cGMP (3 mM), cyclopiazonic acid (1 μM), Rp-cAMPS (100 μM) and H-8 (30 μM) had no observable effects on the macroscopic I_{KCa} (data not shown). Thus, the effects of the drugs used in this experiment were not the result of direct interactions with the K^+ channel.

3.1. Effects of cGMP-elevating agents on agonist-induced oscillatory I_{KCa}

Fig. 1Aa shows the effect of a guanylate cyclase activator, sodium nitroprusside, on the ATP-induced oscillatory I_{KCa}. External application of sodium nitroprusside reversibly depressed the ATP-induced oscillatory I_{KCa} in a concentration-dependent manner. The application of sodium nitroprusside lowered the frequency of the ATP-induced I_{KCa} and caused a gradual decrease in its amplitude. The inhibitory activity of sodium nitroprusside was seen as a change in either the frequency or maximum amplitude of the current, or total integrated value, as previously observed in the case of cAMP-elevating agents (Uneyama et al., 1993b; Akaike et al., 1993). The quantitative amplitude changes in the inhibitory potency of sodium nitroprusside are shown in Fig. 1Ab. Sodium nitroprusside had little influence on the first transient peak of I_{KCa} but inhibited more potently the subsequent oscillatory I_{KCa}. Exogenous application of a membrane-permeable cGMP analogue, 8-bromo-cGMP (3 mM), also depressed the ATP-induced currents (Fig. 1B) in similar manner. Similar results were obtained when ADP and thrombin were used as intracellular Ca^{2+} mobilizer instead of ATP (data not shown). Thus, in rat megakaryocytes, cGMP inhibited the Ca^{2+} mobilization elicited by various agonist stimuli.

3.2. Involvement of cyclic nucleotide-dependent kinase in sodium nitroprusside-mediated I_{KCa} inhibition

To confirm that the inhibition of I_{KCa} by cGMP was mediated by activation of cGMP-dependent protein kinase,

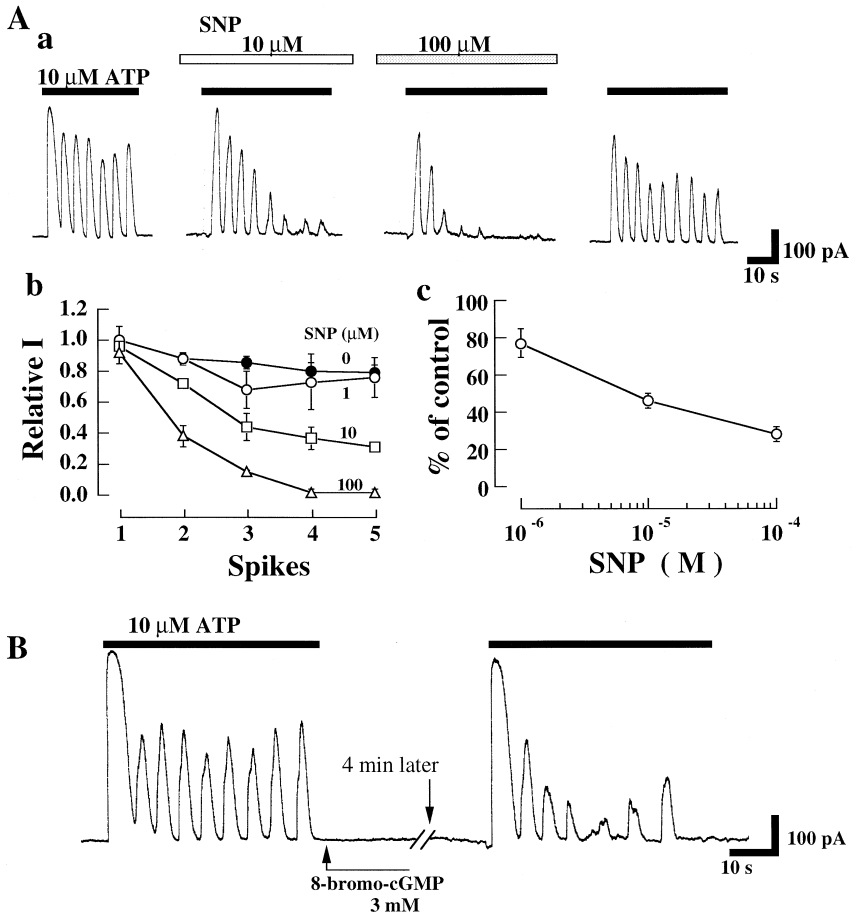

Fig. 1. Inhibition by sodium nitroprusside of agonist-induced oscillatory I_{KCa}. Records were obtained by the nystatin-perforated patch technique. A megakaryocyte was voltage-clamped at a V_H of −43 mV. (A) Concentration-dependent inhibition by sodium nitroprusside (SNP). (a) Current traces from the same cell. Drugs were continuously applied for the period shown by the bars above each response. Sodium nitroprusside was added for 30 s. Quantitative results are summarized in (b) and (c). Each transient current was normalized to the amplitude of the first spike (b). Current traces were subsequently integrated with respect to the baseline for 40 s while the K^+ current oscillation was being evoked and shown as a percentage change of the integrated current in the presence or absence of drugs tested (c). Concentration-inhibition curve for sodium nitroprusside. Each point and vertical bar represent mean and ±S.E.M. from three experiments. (B) Effect of the membrane-permeable cGMP analogue, 8-bromo-cGMP. 8-Bromo-cGMP (3 mM) was added for 4 min before ATP application. The current trace is typical of three reproducible observations.

the effect of an inhibitor of cyclic nucleotide-dependent protein kinases, H-8, was examined (Fig. 2A). ATP-induced I_{KCa} was continuously inhibited during sodium nitroprusside (30 μM) application. Under these conditions, addition of H-8 (30 μM) abolished the inhibitory effect of sodium nitroprusside. The action of H-8 appeared 40 ± 12 s after its application. As H-8 inhibits non-selectively both cGMP- and cAMP-dependent processes, at the concentration used in this experiment, we tested the involvement of cAMP in the sodium nitroprusside-mediated blockade by using a selective cAMP-dependent protein kinase inhibitor, Rp-cAMPS. As shown in Fig. 2B, the ATP-induced I_{KCa} spikes were abolished by application of an adenylate cyclase activator, forskolin (1 μM), and the inhibitory action of forskolin was blocked by applying 200 μM Rp-cAMPS to the cell. However, in order to block the I_{KCa} spikes, 100 μM sodium nitroprusside had to be applied in the presence of the inhibitor. This indicates that cAMP-dependent protein kinase was not involved in the action of sodium nitroprusside.

3.3. Effect of sodium nitroprusside on IP_3-induced oscillatory I_{KCa}

In our previous report we showed that the ATP-induced I_{KCa} current is mediated by intracellular release of IP_3 (Uneyama et al., 1993b). To investigate the effect of sodium nitroprusside on I_{KCa} currents directly evoked by IP_3, we applied IP_3 to the cell and induced an oscillatory I_{KCa} (Fig. 3). We used a pipette solution containing 10 μM IP_3 and, under conventional whole-cell conditions, introduced IP_3 into the cell. The intracellular application of IP_3 immediately induced an oscillatory I_{KCa}, and the amplitude of I_{KCa} was reversibly depressed by the application of either sodium nitroprusside or a phosphodiesterase in-

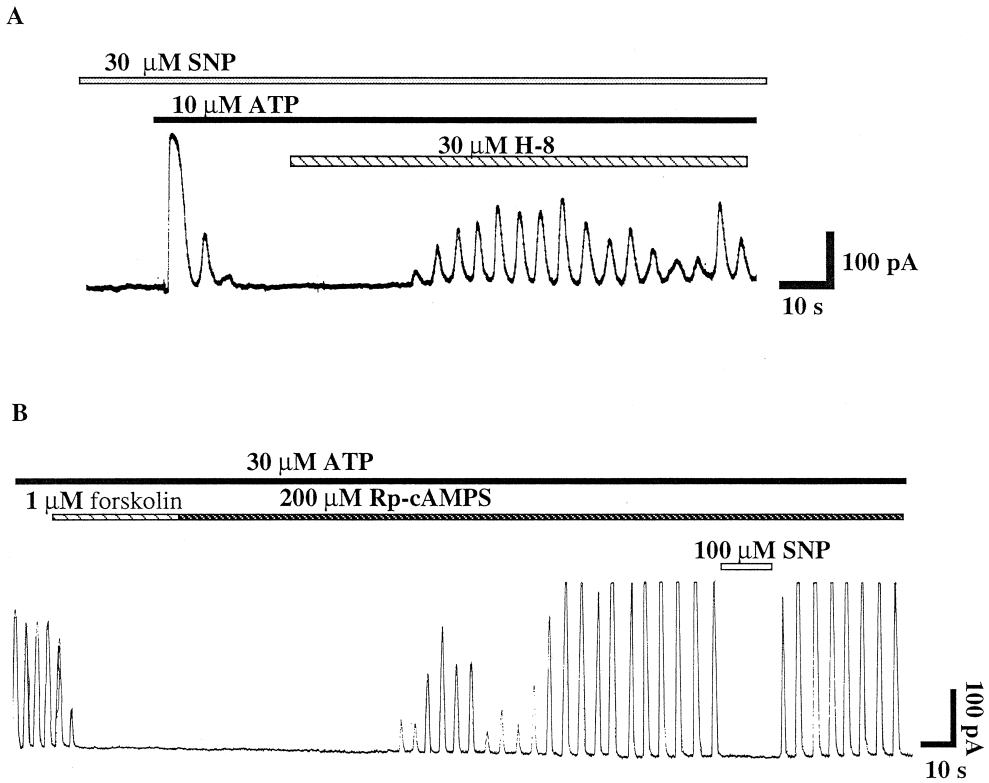

Fig. 2. Involvement of cyclic nucleotide-dependent kinase in sodium nitroprusside-mediated I_{KCa} inhibition. Records were obtained by the nystatin-perforated patch technique. V_H was −43 mV. (A) Effect of H-8 on sodium nitroprusside (SNP)-mediated I_{KCa} inhibition. Drugs were continuously applied for the period shown by the bars above the response. (B) Effect of Rp-cAMPS on 1 μM forskolin (a) and 100 μM sodium nitroprusside (b)-mediated I_{KCa} inhibition. Rp-cAMPS (200 μM) was added for 4 min before ATP application.

hibitor, IBMX. The maximal action of sodium nitroprusside appeared 5 ± 2 s after its application and disappeared 15 ± 2 s after its wash out. The observation that sodium nitroprusside inhibited the response evoked by IP_3 injection suggests that the site of action of cGMP was after the release of IP_3 as well as cAMP.

3.4. Influence of Ca^{2+}-ATPase blockade on the SNP-mediated I_{KCa} inhibition

In a previous report, we showed that the repetitive I_{KCa} resulted in the periodic activation of Ca^{2+}-ATPase (Uneyama et al., 1993b). Therefore, as a next step to identify the target of cGMP, we examined the effect of sodium nitroprusside on the I_{KCa} current after blocking Ca^{2+}-ATPase activity (Fig. 4). In this experiment, we used cyclopiazonic acid (CPA) as a direct inhibitor of the Ca^{2+}-ATPase on the endoplasmic reticulum (Seidler et al., 1989). Pretreatment of a megakaryocyte with CPA (0.1 μM) changed the ATP-induced oscillatory I_{KCa} spikes into a monophasic sustained current. About 4 min after washing the drug out, the sustained current reverted into an oscillatory current (Fig. 4Aa). Similar results were obtained with another Ca^{2+}-ATPase inhibitor, thapsigargin

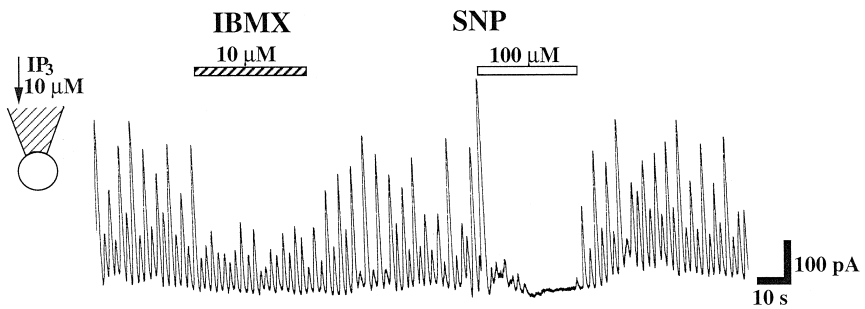

Fig. 3. Effect of sodium nitroprusside on IP_3-induced oscillatory I_{KCa}. Records were obtained by the conventional whole-cell method. The recording pipette was filled with internal solution containing 10 μM-IP_3. Drugs were applied extracellularly during the period indicated by horizontal bars. Each current trace is typical of three to four reproducible observations.

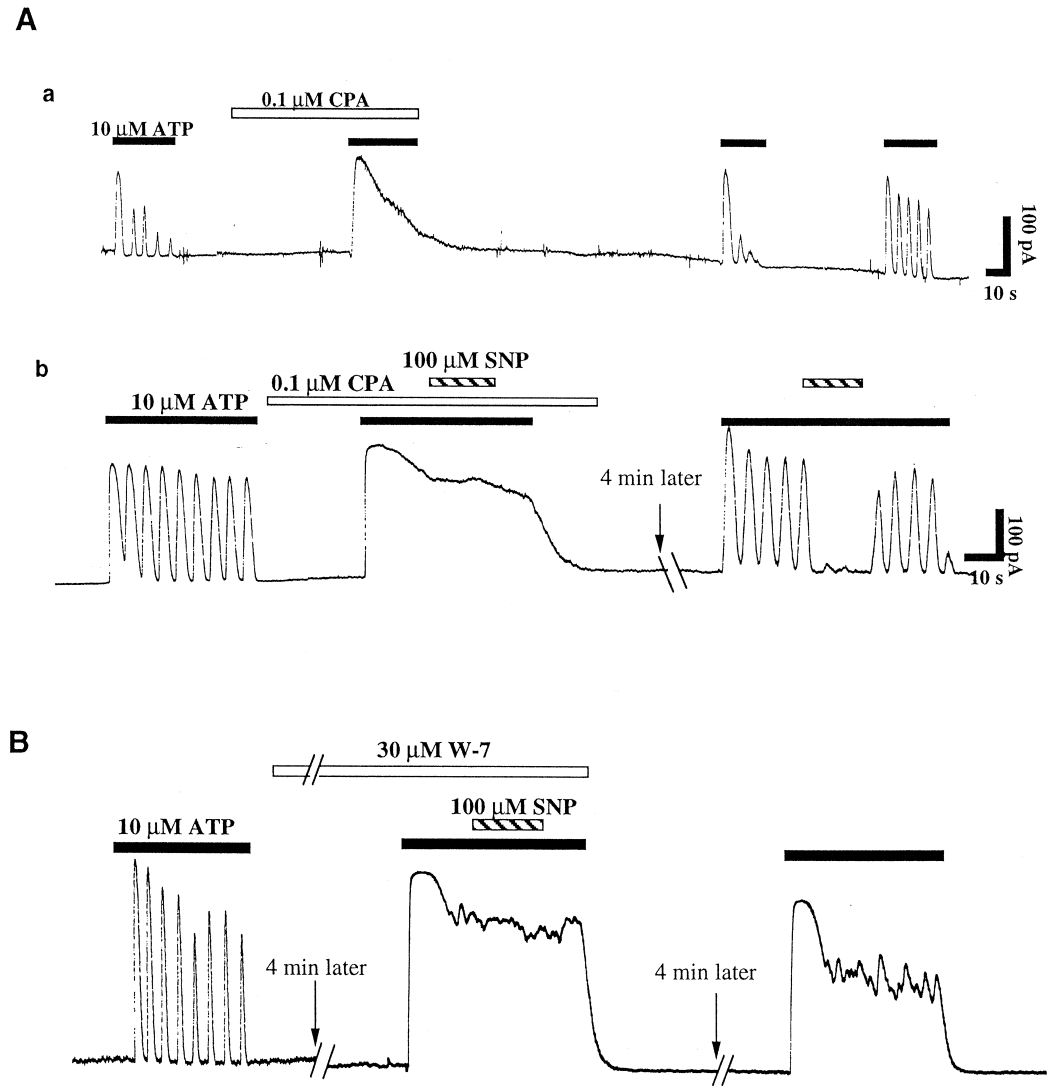

Fig. 4. Effect of cyclopiazoic acid (CPA) (A) and W-7 (B) on the sodium nitroprusside-mediated I_{KCa} inhibition. Records were obtained by the perforated whole-cell method. V_H was −43 mV. Note that effect of 0.1 μM CPA was completely reversible (a) and that CPA blocked the inhibitory effect of sodium nitroprusside (SNP) on the I_{KCa} (b). Drugs were applied extracellularly during a period indicated by horizontal bars. The current trace is typical of three reproducible observations.

(data not shown), except that the effect of thapsigargin was irreversible. Thus, the falling phase of I_{KCa} spikes resulted in the rapid removal of Ca^{2+} from the cytoplasm through Ca^{2+}-ATPase activation. Next, the effect of sodium nitroprusside on the sustained I_{KCa} current evoked by CPA treatment was examined (Fig. 4Ab). Sodium nitroprusside (100 μM) had no effect on the sustained I_{KCa}, even when the oscillatory I_{KCa} spikes were suppressed by the treatment with the same concentration of sodium nitroprusside after removal of CPA. In our previous report, we showed that the Ca^{2+}-ATPase of megakaryocyte was also inhibited by treatment with calmodulin inhibitors (Uneyama et al., 1993b). As shown in Fig. 4B, after treatment with a one such drug, W-7 (30 μM), the oscillatory ATP-induced I_{KCa} spikes were changed into sustained currents. Under this condition, the subsequent application of sodium nitroprusside (100 μM) failed to inhibit the sustained I_{KCa}. Furthermore, the IP_3-induced oscillatory I_{KCa} was also changed to a sustained current by the treatment with the Ca^{2+}-ATPase inhibitor. In agreement with the result obtained in Fig. 4, application of 100 μM sodium nitroprusside had no inhibitory effect on the sustained response (data not shown). Thus, sodium nitroprusside lost its inhibitory action on I_{KCa} when Ca^{2+}-ATPase was inhibited.

4. Discussion

In the present study we showed that cGMP inhibited intracellular mobilization of Ca^{2+} in megakaryocytes and investigated its inhibitory mechanism.

As progenitor cells, megakaryocytes share several prop-

erties with platelets (Fedorko, 1977). We clearly showed here that cGMP had an inhibitory action on the agonist-induced oscillatory I_{KCa} in megakaryocytes. Thus, the inhibitory action of cGMP is also a common feature of platelets and megakaryocytes.

Several mechanisms have been proposed to account for the cGMP-dependent $[Ca^{2+}]_i$ reduction, including the activation of Ca^{2+}-ATPase in the plasma membrane and sarcoplasmic reticulum (Yoshida et al., 1991; Johansson and Haynes, 1992), the activation of Ca^{2+}-activated K^+ channels (Duridanova et al., 1995) and the inhibition of inositol 1,4,5-trisphosphate (IP_3) generation (Hirata et al., 1990; Ruth et al., 1993). However, it is difficult to determine which of these possible mechanisms is involved because it is very difficult to investigate platelets at the single cell level with high-time resolution.

At first, we confirmed that sodium nitroprusside, 8-bromo-cGMP and H-8 had no direct effect on Ca^{2+}-activated K^+ channels in megakaryoctes. Therefore, the hypothesis that cGMP acts on the activation process of Ca^{2+}-activated K^+ channels cannot be applied in the case of megakaryocytes. Then we showed that sodium nitroprusside inhibited oscillatory I_{KCa} in a dose-dependent manner. The inhibitory action became greater in the later phase of the reaction (Fig. 1). As this mode of inhibition was not seen with receptor antagonists, we supposed the site of action of cGMP to be the late phase of signal transduction.

In Fig. 2A, we showed that the effect of cGMP required the activation of a cyclic nucleotide-dependent protein kinase. Though several results indicate that there exists 'cross-over' between cAMP and cGMP in the activation of the cyclic nucleotide-dependent protein kinase (Cornwell et al., 1994; Forte et al., 1992), the inhibitory actions of cAMP and cGMP were independent, as shown in Fig. 2B.

In Fig. 3, we showed that sodium nitroprusside inhibited IP_3-induced oscillatory I_{KCa}. This indicates that the site of action of cGMP is after the release of IP_3. Thus, the theory that cGMP inhibits IP_3 generation does not apply in megakaryocytes. This is one of the similar features of cGMP and cAMP in megakaryocytes.

Finally, we indicated that the inhibitory action of cGMP required Ca^{2+}-ATPase activity (Fig. 4). From these results, we suggest that cGMP increases the rate of Ca^{2+} extrusion from the cytoplasm into the endoplasmic reticulum.

In summary, these results are consistent with the conclusion that Ca^{2+}-ATPase is involved in the mechanism of cGMP action in agonist-induced oscillatory I_{KCa} in rat megakaryocytes. By studying agonist-induced oscillatory I_{KCa} in megakaryocytes, we have shown that many modulators affect intracellular Ca^{2+} mobilization (Uneyama et al., 1993a,b,c, 1994a,b,c, 1995a,b, 1997; Akaike et al., 1993; Akaike and Uneyama, 1994). Ca^{2+} homeostasis can be modulated many steps from receptor antagonist to Ca^{2+}-pump. These findings could contribute to the future development of specific drugs.

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