Inhibitory Action of 4-Aminopyridine on Ca$^{2+}$-ATPase of the Mammalian Sarcoplasmic Reticulum*

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In the isolated guinea pig diaphragm muscle, 4-aminopyridine (4-AP) elicited a marked potentiation of twitch contraction evoked by direct electrical stimuli. Although tetraethylammonium (TEA) and charybdotoxin only slightly potentiated twitch contraction, 4-AP, but not TEA, also augmented a contractile response to caffeine. These effects of 4-AP on muscle contraction could not be interpreted by a simple inhibition of potassium channels on the plasma membrane. In the fragmented sarcoplasmic reticulum (SR) prepared from the guinea pig psoas muscle, 4-AP inhibited the ATP-driven Ca$^{2+}$ uptake from the extravesicular medium. Furthermore, 4-AP at concentrations less than 10 mM elicited a selective inhibition of Ca$^{2+}$-activated SR ATPase in a competitive manner against the Ca$^{2+}$ concentration of the medium and 10 mM 4-AP showed the unsurmountable inhibition. 4-AP at 30 mM apparently inhibited activities of other ATPases such as Na$^{+}$,K$^{+}$- and myosin ATPases. In contrast, other potassium channel blockers such as TEA, apamin, charybdotoxin, and glibenclamide did not inhibit the SR function. These results suggest that, although the specific concentration range is rather small, 4-AP elicited an inhibition of SR Ca$^{2+}$-pumping activity, leading to the marked potentiation of muscle contractile responses to electrical stimuli and caffeine.

The inhibitory action of 4-aminopyridine (4-AP)$^1$ on potassium channels of plasma membrane has been revealed after the finding of its stimulatory effects on neuromuscular transmission through experimental and clinical studies in Bulgaria (1). Although the 4-AP-induced increase in the quantity of acetylcholine liberated from nerve endings has been established (1, 2), 4-AP, especially at higher concentrations around the millimolar range, could increase contractility of rat and frog skeletal muscle preparations through the direct action on muscle cells (3-5). Since, in the presence of 4-AP, changes in membrane potential were not always associated with contractile response (6), and the relaxation time was prolonged (3), it has been suggested that 4-AP may have a direct action on the resequestration of Ca$^{2+}$ in the muscle cell. Also, in cardiac muscle of the dog ventricle, the prolongation of the action potential was much smaller than the prolongation of the relaxation time, suggesting that the primary action of 4-AP may be to alter the intracellular Ca$^{2+}$ metabolism (7). Our preliminary experiments using diaphragm muscle of the guinea pig revealed that, in contrast to tetraethylammonium (TEA), another potassium-channel blocker (8), 4-AP elicited a marked augmentation of contractile responses to caffeine which is known to release Ca$^{2+}$ from the SR (9). Thus, we made SR preparations and investigated the effects of 4-AP on the SR Ca$^{2+}$-pumping activity and Ca$^{2+}$-activated ATPase activity. We report here that 4-AP has a direct action on the mammalian sarcoplasmic reticulum (SR) which may associate with increase in contractility.

MATERIALS AND METHODS

Contractile Response—Male guinea pigs (about 400 g) were stunned and exsanguinated. Diaphragm muscle was excised and cut into four strips. The strip was suspended in a medium bubbled with 95% O$_2$, 5% CO$_2$ with the following composition (in mM): NaCl, 136.8; KCl, 5.4; CaCl$_2$, 2.5; MgCl$_2$, 1.0; NaHCO$_3$, 24; and glucose, 5.5 (37°C, pH 7.4). Twitch contractions were evoked by strong electrical stimuli (5-msec pulses, supramaximal voltage, 0.1 Hz), which directly stimulated muscle cells, and were recorded isometrically, as reported elsewhere (10).

Ca$^{2+}$-pumping Activity of the Fragmented SR—The Ca$^{2+}$-pumping activity of the fragmented SR was measured using a Ca$^{2+}$ electrode as described elsewhere (11, 12). The heavy fractions of the fragmented SR were prepared from the guinea pig psoas muscle by the modified method of Kim et al. (13). The heavy fraction of the fragmented SR was stored at 0°C and used within 3 days. The fragmented SR (1.5 mg of protein/ml) was incubated in the medium composed of (in mM) KCl, 100; MgCl$_2$, 5; and MOPS, 50 at pH 7.0 (30°C). Then, CaCl$_2$, 50 μM; fragmented SR, 0.5-1 mg of protein/ml; phosphocreatine, 5 mM; and ATP, 1 mM plus creatine kinase, 0.1 mg/ml, were successively added to the medium. After the medium Ca$^{2+}$ was taken up by SR, various drugs were administered. A Ca$^{2+}$ electrode was made using a Ca$^{2+}$ mixture (Fluka, Switzerland).

ATPase Activities—Myosin and deoxycholic acid-treated SR were prepared from the guinea pig psoas muscle according to the methods of Perry (14) and Yamamoto and Tomonura (15), respectively. Pig brain Na$^{+}$,K$^{+}$-ATPase preparation was purchased from Sigma. ATPase activities were determined by measuring the liberated Pi according to the method of Perry (17). Compositions of the reaction medium were as follows: SR preparation, 2 μg protein/ml; CaCl$_2$, at desired concentration, MgCl$_2$, 5 mM; ATP, 2 mM; KCl, 90 mM; EGTA, 1 mM; and HEPES, 30 mM (pH 7.1, 30°C) for SR ATPase; myosin, 0.1 mg/ml; actin, 0.1 mg/ml; KCl, 50 mM; MgCl$_2$, 2 mM; ATP, 2 mM; EGTA, 1 mM; and Tris, 20 mM (pH 6.8, 30°C) for myosin ATPase; and enzyme preparation, 0.01 unit/ml; NaCl, 100 mM; KCl, 20 mM; MgCl$_2$, 5 mM; ATP, 2 mM; and Tris, 50 mM (pH 7.4, 37°C) for Na$^{+}$,K$^{+}$-ATPase. The SR Ca$^{2+}$-ATPase activity was estimated by the difference between ATPase activities in the presence and absence of Ca$^{2+}$. Mg$^{2+}$-ATPase activity of the SR was approximately 2% of the total activity. Free Ca$^{2+}$ concentration was calculated on a computer as described by Fabiato and Fabiato (17).

Statistics and Drugs—Values are expressed as mean ± S.E., and the number of experiments was at least three unless otherwise stated. The statistical analysis was performed by Student’s t test.

The drugs used were: 4-aminopyridine (Tokyo Kasei, Tokyo), tetraethylammonium chloride (Tokyo Kasei), caffeine (Wako Pure.

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$^1$ The abbreviations used are: 4-AP, 4-aminopyridine; TEA, tetraethylammonium; SR, sarcoplasmic reticulum; MOPS, 3-(N-morpholino)propanesulfonic acid.

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RESULTS

Isolated diaphragm muscle of the guinea pig caused twitch contractions in response to strong electrical stimuli which directly activated muscle cells. When 4-AP (0.01–10 mM) was administered, it potentiated twitch contractions dose-dependently (Fig. 1). Low concentrations of 4-AP (e.g., 1 mM or less) elicited a sustained potentiation of twitch contraction. The high concentration such as 10 mM elicited a transient potentiation and an increase in basal tension. In the presence of 3 and 10 mM 4-AP, the twitch amplitude was more than twice of control. Such a strong potentiation of contraction with a similar 4-AP concentration range was also reported in the rat diaphragm (19). The presence of d-tubocurarine (0.1 mM) did not inhibit the potentiation induced by 1 mM 4-AP, suggesting that the 4-AP-induced potentiation is not mediated through the acetylcholine liberation from nerves, as already reported (20). On the other hand, administration of TEA (1–30 mM) also potentiated twitch contraction, but the potentiation was much smaller than that induced by 4-AP. Charybdotoxin (1 µM) but not apamin (1 µM), peptide potassium channel blockers (20, 21), also potentiated twitch contraction by 30% of control, a similar magnitude to that induced by 30 mM TEA (data not shown).

When external Ca²⁺ was removed, twitch contraction gradually decreased (Fig. 1A, c and d). During the falling phase of twitch contraction, an administration of 10 mM 4-AP elicited a similar increase in twitch contraction to that in the normal medium (Fig. 1A, c), being consistent to the reported results in frog muscle (4). 10 mM 4-AP also raised the basal tension in the absence of external Ca²⁺; although 30 mM TEA elicited a very small potentiation in the absence of external Ca²⁺ (Fig. 1A, d).

Next, we examined the effects of 4-AP on the contractile response of the diaphragm muscle to caffeine (Fig. 2). An administration of 3 mM caffeine elicited a small contraction with the magnitude of 12.9 ± 1.61% (n = 26) compared with the twitch amplitude evoked by supramaximal electrical stimuli. The presence of 4-AP (1–10 mM) also elicited a small contraction in a dose-dependent manner (Fig. 2). In the presence of 4-AP at 1 mM, but not 0.3 mM, the caffeine-induced contraction was augmented approximately five times as much as control. Raising 4-AP concentrations to 3 and 10 mM attenuated the degree of augmentation gradually, but at 10 mM 4-AP, the contractile response to caffeine was still twice greater than control, whereas the presence of 30 mM TEA did not elicit contraction or augment the caffeine-induced contraction (Fig. 2).

To understand the biochemical mode of action, we made SR preparations from the guinea pig psoas muscle (a white muscle). Using a Ca²⁺ electrode, Ca²⁺-pumping activity of fragmented SR and caffeine-induced Ca²⁺ release from SR were measured (Fig. 3). An addition of ATP to the SR-incubated medium gradually lowered the medium Ca²⁺ concentration, indicating the active Ca²⁺ uptake by SR. After the Ca²⁺ level was lowered, administration of caffeine (0.5 mM) elicited a transient increase in the medium Ca²⁺ concentration, as reported previously (11, 12). The presence of 4-AP (10 mM) delayed the active Ca²⁺ uptake and prolonged the duration of Ca²⁺ increase induced by caffeine. In the presence of 10 mM 4-AP, time to reach the nominally Ca²⁺-free level after the ATP addition prolonged, 1.37 ± 0.05 (n = 4), the times control significantly (p < 0.05), suggesting that the presence of 4-AP inhibits the SR Ca²⁺-pumping activity, whereas 3 mM 4-AP did not show significant effects on the Ca²⁺ uptake process and caffeine-induced response. On the other hand, 30 mM TEA did not affect the active Ca²⁺ uptake or the caffeine-induced Ca²⁺ release. Potassium channel blockers of 1 µM charybdotoxin, 1 µM apamin, and 10 µM glibenclamide (22) or potassium channel activators (23) of 10 µM cromakalim and 10 µM nicorandil did not affect the active Ca²⁺ uptake or the caffeine-induced Ca²⁺ release (data not shown).

Effects of 4-AP on the various ATPase activities were investigated (Fig. 4). When the Ca²⁺-ATPase of the deoxycholate-treated SR prepared from the guinea pig psoas was
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Fig. 3. Effects of 10 mM 4-AP (upper panel) and 30 mM TEA (lower panel) on Ca\textsuperscript{2+}-pumping activity and caffeine-induced response of the fragmented sarcoplasmic reticulum (SR) prepared from the guinea pig psoas muscle. Calibration for the medium Ca\textsuperscript{2+} concentration is shown on the left of the upper panel; arrows indicate the addition of each concentration of Ca\textsuperscript{2+}. The level before addition of Ca\textsuperscript{2+} represents the nominally Ca\textsuperscript{2+}-free concentration, about 1 \textmu M. Lowercase letters represent the following: a, addition of CaCl\textsubscript{2}; b, addition of the fragmented SR; c, addition of phosphocreatine; and d, addition of ATP and creatine kinase to the medium. A prompt reduction and the following gradual decrease in Ca\textsuperscript{2+} concentration after addition of ATP are due to the formation of the ATP-Ca\textsuperscript{2+} complex and the active Ca\textsuperscript{2+} uptake by SR, respectively.

Fig. 5. Inhibitory effects of 4-AP on the deoxycholate-treated SR Ca\textsuperscript{2+}-ATPase activity in the presence of various concentrations of Ca\textsuperscript{2+}. A shows the concentration-ATPase activity curves for Ca\textsuperscript{2+} in the absence and presence of 4-AP (2, 4, 6, and 10 mM). pCa represents the negative logarithm of free Ca\textsuperscript{2+} concentration. B shows the Dixon-plot (26) drawn from A by taking the mean values of ATPase activity in the presence of various tested concentrations of 4-AP at pCAs 6, 6.2, and 6.5. The value in the presence of 10 mM was omitted, since 10 mM 4-AP apparently elicited an unsurmountable inhibition. The ordinate represents the reciprocal of the ATPase activity. Each symbol represents the mean value of duplicated measurements. The mean value of crosses of three linear regression lines gives the apparent dissociation constant of 2.4 mM for 4-AP.

nearly maximally activated in the presence of 1 \textmu M Ca\textsuperscript{2+}, 4-AP (0.3–30 mM) dose-dependently inhibited the ATPase activity. Against the basal ATPase (Mg\textsuperscript{2+}-activated ATPase) of the deoxycholate-treated SR and the Na\textsuperscript{+},K\textsuperscript{+}-ATPase of the hog brain, 0.1–3 mM 4-AP did not elicit inhibition and 10 mM 4-AP inhibited these ATPase activities by 7–10% of control (Fig. 4). The actin-activated myosin ATPase prepared from the guinea pig psoas was not affected by 3 and 10 mM 4-AP (Fig. 4). 4-AP at 30 mM abolished the SR Ca\textsuperscript{2+}-ATPase activity and inhibited other tested ATPase activities by 30–55% of control (Fig. 4). In addition, 10 mM 4-AP did not inhibit the myosin ATPase activities in the presence of Ca\textsuperscript{2+} or in the presence of EDTA and K\textsuperscript{+} (data not shown). Other potassium channel blockers or activators of 1 \textmu M charybdotoxin, 1 \textmu M apamin, 1 \textmu M glibenclamide, 10 \textmu M cromakalim, and 10 \textmu M nicotine did not inhibit any kind of ATPase activities tested (data not shown). The effects of TEA on ATPase activities could not be tested, since it interfered with the formation of the P\textsubscript{i}-molybdate complex for the determination of liberated P\textsubscript{i} through ATPase reaction.

Fig. 4. Inhibitory effects of 4-AP (0.3–30 mM) on the Ca\textsuperscript{2+}-activated ATPase of the deoxycholate-treated SR prepared from the guinea pig psoas muscle, compared with the effects on various tested ATPases. The SR Ca\textsuperscript{2+}-ATPase activity (Ca\textsuperscript{2+}-SR, ○) was determined at 1 \textmu M free Ca\textsuperscript{2+} plus 5 mM Mg\textsuperscript{2+}. Mg\textsuperscript{2+}-SR (■) represents the SR Mg\textsuperscript{2+}-ATPase activity at 5 mM Mg\textsuperscript{2+} without Ca\textsuperscript{2+}. Myosin (■) represents actin-activated myosin ATPase from the guinea pig psoas in the presence of 0.1 mg/ml actin, 0.1 mg/ml myosin, and Na\textsuperscript{+},K\textsuperscript{+} (●) represents Na\textsuperscript{+},K\textsuperscript{+}-ATPase from the hog brain in the presence of 100 mM Na\textsuperscript{+} and 20 mM K\textsuperscript{+}. 100% of the ATPase activity means the activity determined in the absence of 4-AP. Each symbol indicates the mean value of duplicated measurements. 4-AP inhibited the SR Ca\textsuperscript{2+}-ATPase activity with a 50% inhibition concentration of 5.5 mM.
tion-ATPase activity relationship to the right in a parallel manner (Fig. 5A). 4-AP at 10 mM apparently showed an unsurmountable inhibition of the SR Ca\textsuperscript{2+}-ATPase activity. Using values of ATPase activity in the presence of various tested concentrations of 4-AP at pCa 6, 6.2, and 6.5, the Dixon plot (28) for the determination of enzyme inhibitor constants (Fig. 5B) reveals that 4-AP inhibits the SR Ca\textsuperscript{2+}-ATPase in a competitive manner against Ca\textsuperscript{2+} activation with the apparent dissociation constant of 2.4 mM, although the 4-AP concentration for the competitive inhibition is limited less than 10 mM.

DISCUSSION

Among tested potassium channel modulators, only 4-AP elicited the inhibition of Ca\textsuperscript{2+}-pumping activity of the fragmented SR and Ca\textsuperscript{2+}-activated SR ATPase. Therefore, this inhibitory action may not be related to the known mode of action, the potassium channel inhibition, but rather may be defined as a novel action for 4-AP. 4-AP at concentrations less than 10 mM elicited a significant inhibition of Ca\textsuperscript{2+}-ATPase activity of the SR but had little or no effect on other tested ATPase activities, whereas 30 mM 4-AP more or less inhibited all tested ATPase activities. These results suggest that 4-AP elicits a specific inhibition of Ca\textsuperscript{2+}-activated SR ATPase activity, although the specific concentration range is narrow.

4-AP at concentrations of 2-6 mM seemed to inhibit the Ca\textsuperscript{2+}-activated SR ATPase activity in a competitive manner against the medium Ca\textsuperscript{2+} concentration. 4-AP at concentrations more than 10 mM elicited an unsurmountable inhibition of the Ca\textsuperscript{2+}-activated SR ATPase activity. The mechanism of action for such a high concentration of 4-AP could not be well defined at present but seems to be nonspecific, since 4-AP at 30 mM apparently elicited the inhibitory effects on activities of Mg\textsuperscript{2+}-activated SR ATPase as well as other tested ATPases. Presumably, these competitive and nonspecific actions of 4-AP at low and high concentrations represent the fact that the Ca\textsuperscript{2+}-pumping activity of the fragmented SR was inhibited only by the high concentration (10 mM) of 4-AP in the presence of a high concentration (50 \muM) of extravesicular Ca\textsuperscript{2+}.

Considering that 4-AP could penetrate the cell across the plasma membrane (1), 4-AP may be capable of interacting with the SR membrane, although the real 4-AP concentration at the cytoplasm could not be determined. Thus, 4-AP elicited an augmentation of contractile response to caffeine, presumably due to the inhibition of resequstration of Ca\textsuperscript{2+} to the SR through the inhibitory effects on Ca\textsuperscript{2+}-pumping activity as described above. The 4-AP-induced potentiation of twitch contraction evoked by electrical stimuli may be explained by the combined mechanism of interference with SR Ca\textsuperscript{2+}-pumping activity and inhibition of potassium channels on the plasma membrane; presumably the former is a major factor for the potentiation than the latter, since other potassium channel blockers of TEA and charybdotoxin elicited much smaller potentiation compared to 4-AP.

Present experiments clearly demonstrate that, although the specific concentration range for 4-AP is small, 4-AP inhibits Ca\textsuperscript{2+}-ATPase activity and active Ca\textsuperscript{2+} uptake of the SR, presumably resulting in the marked potentiation of skeletal and cardiac muscle contractile responses. This mode of action may also account for the smooth muscle response, since, in the absence of external Ca\textsuperscript{2+} plus EGTA, the guinea pig isolated aorta produced a big contractile response to 10 mM 4-AP, but not to 30 mM TEA.2 A similar intervention of 4-AP to intracellular Ca\textsuperscript{2+} metabolism was suggested in nerve cells (27-29). Thus, this newly defined mode of action for 4-AP may be adopted in the cellular responses through excitation-contraction coupling as well as excitation-secretion coupling of nerve cells.

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