Korean J Physiol Pharmacol
Vol 7: 25–28, February, 2003

4-Aminopyridine Inhibits the Large-conductance Ca^{2+}-activated K^+ Channel (BK_{Ca}) Currents in Rabbit Pulmonary Arterial Smooth Muscle Cells

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Ion channel inhibitors are widely used for pharmacological discrimination between the different channel types as well as for determination of their functional role. In the present study, we tested the hypothesis that 4-aminopyridine (4-AP) could affect the large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) currents using perforated-patch or cell-attached configuration of patch-clamp technique in the rabbit pulmonary arterial smooth muscle. Application of 4-AP reversibly inhibited the spontaneous transient outward currents (STOCs). The reversal potential and the sensitivity to charybdotoxin indicated that the STOCs were due to the activation of BK_{Ca}. The BK_{Ca} currents were recorded in single channel resolution under the cell-attached mode of patch-clamp technique for minimal perturbation of intracellular environment. Application of 4-AP also inhibited the single BK_{Ca} currents reversibly and dose-dependently. The membrane potential of rabbit pulmonary arterial smooth muscle cells showed spontaneous transient hyperpolarizations (STHPs), presumably due to the STOC activities, which was also inhibited by 4-AP. These results suggest that 4-AP can inhibit BK_{Ca} currents in the intact rabbit vascular smooth muscle. The use of 4-AP as a selective voltage-dependent K^+ (K_V) channel blocker in vascular smooth muscle, therefore, must be reevaluated.

Key Words: BK_{Ca}, Pulmonary artery, Smooth muscle, 4-Aminopyridine

INTRODUCTION

Activity of potassium channels controls membrane potential (V_m), which in turn regulates cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in pulmonary arterial smooth muscle (Nelson & Quayle, 1995; Yuan, 1995). To date, three types of potassium channels have been described in pulmonary arterial smooth muscle cells (PASMCs): voltage-dependent K^+ (K_V) channels, large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, and ATP-sensitive K^+ channels (Clapp 1995; Park et al, 1997; Yuan et al, 1998; Bae et al, 1999). Ion channel inhibitors are widely used for pharmacological discrimination between the different channel types as well as for determination of their functional role. 4-Aminopyridine (4-AP) is a potent inhibitor of K_V currents with a half-block at 0.2~1 mM (Noack et al, 1990; Robertson & Nelson, 1994). It has been thought that, in the vascular smooth muscle, action of millimolar concentrations of 4-AP is selective for K_V channels (Nelson & Quayle, 1995; Post et al, 1995; Yuan, 1995)

Recently, Petkova-Kirova et al. (2000) reported that 4-AP could inhibit the rat vascular smooth muscle BK_{Ca} currents by making intracellular pH (pH_i) alkaline, where the intracellular Ca^{2+} was buffered by a pH-sensitive Ca^{2+}-chelator EGTA. In addition, Hayabuchi et al. (1998) reported that acidosis increased the BK_{Ca} activities not by directly changing $[H^+]_i$ but by enhancing $[Ca^{2+}]_i$ under the experimental condition, where $[Ca^{2+}]_i$ of cytoplasmic side is buffered by EGTA. However, when the $[Ca^{2+}]_i$ in the solution of intracellular side were strictly maintained constant, for example, by using a pH-insensitive Ca^{2+} buffer BAPTA, the opposite phenomenon i.e. inhibition of BK_{Ca} by intracellular acidification was observed (Hayabuchi et al, 1998; Petkova-Kirova et al, 2000), suggesting that both $[Ca^{2+}]_i$ and BK_{Ca} are simultaneously influenced by pH_i.

Unlike in most whole cell current studies where $[Ca^{2+}]_i$ is usually buffered by EGTA or BAPTA, the mechanism by which $[Ca^{2+}]_i$ changes in response to alterations in pH_i must be multi-factorial in intact cells (Marin et al, 1999). Under these intact and physiological conditions, the effect of 4-AP on the BK_{Ca} currents has not yet been investigated. Hence, we investigated the effect of 4-AP on the PASMC BK_{Ca} under the intrinsic pH_i and $[Ca^{2+}]_i$ regulation. In order to ensure the integrity of the cells' intrinsic mechanisms for pH_i and $[Ca^{2+}]_i$ regulation, we used nystatin

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ABBREVIATIONS: 4-AP, 4-aminopyridine; BK_{Ca}, large conductance Ca^{2+}-activated K^+ channel; Kv, voltage-dependent K^+; PASMCs, pulmonary arterial smooth muscle cells; STHPs, spontaneous transient hyperpolarizations; STOCs, spontaneous transient outward currents; Vm, membrane potential.

perforated-patch clamp technique for recording whole-cell current and cell-attached mode for recording single channel current (Hamill et al, 1981; Horn & Marty, 1988), and demonstrated that 4-AP could inhibit the BK_{Ca} currents under these conditions, where cell interior was kept intact.

METHODS

Cell preparation

Rabbits (1.0~2.0 kg) of either sex were anesthetized with sodium pentobarbital (50 mg/kg) and injected with heparin (1000 U/kg) at the same time. The lungs were removed immediately and immersed in normal Tyrode solution. Small pulmonary arteries (outer diameter less than 400 μm), which are the 3rd or 4th branches of the intralobar pulmonary arteries of a lower lobe of either side, were dissected out under the dissecting microscope and incubated at 37°C in Ca^{2+}-free normal Tyrode solution for 15 min. Then, the arteries were transferred to the Ca^{2+}-free normal Tyrode solution containing collagenase and elastase. After an incubation for 30~50 min, the enzymes were washed out by incubating the tissue in enzyme-free, Ca^{2+}-free Tyrode solution for 15 min. Then, cells were isolated by gentle agitation with a fire-polished glass pipette in Kraft-Bruhe (KB) solution. The isolated cells were stored in KB solution at 4°C. A more detailed procedure was described previously (Park et al, 1995).

Solution and drugs

Solution: Cells were transferred to an experimental chamber, mounted on the stage of an inverted microscope (IMT2, Olympus, Japan), and superfused with normal Tyrode solution at the temperature of 34~37°C. Normal Tyrode solution contained (in mM) : NaCl, 143; KCl, 5.4; NaH_2PO_4, 0.33; $CaCl_2$, 1.8; $MgCl_2$, 0.5; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 5; glucose 11; adjusted to pH 7.4 with NaOH. For perforated-patches, nystatin, 200 μg/ml, was added to the pipette solution containing (in mM) KCl, 30; KOH, 118; HEPES, 10; $MgCl_2$, 1, and methane sulfonate, 118; adjusted to pH 7.3 with KOH.

Drugs: All the chemicals were obtained from Sigma (Sigma Chemical Co., USA).

Electrophysiological recordings

Membrane currents and membrane potentials were recorded in nystatin perforated-patch or cell-attached configurations using an Axopatch-1D and Axopatch-200A amplifier (Axon instruments, USA). Data were stored on videotape with a pulse code modulator (Medical System, USA) and digitized with pClamp software 6.01 (Axon Instruments, USA) at a sampling rate of 1~2 kHz and filtered at 0.5~5 kHz. The patch pipettes were pulled from borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a Narishige puller (PP-83, Japan). We used patch pipettes with a resistance of 2~4 MΩ when filled with above pipette solutions. The junction potential between the pipette and standard bath (normal Tyrode) was 6 mV for the perforated-patch clamp recording and 7 mV for the cell-attached patch-clamp recording (pipette negative to the bath, a 3 M KCl agar bridge used as a ground electrode). Membrane potentials presented in this

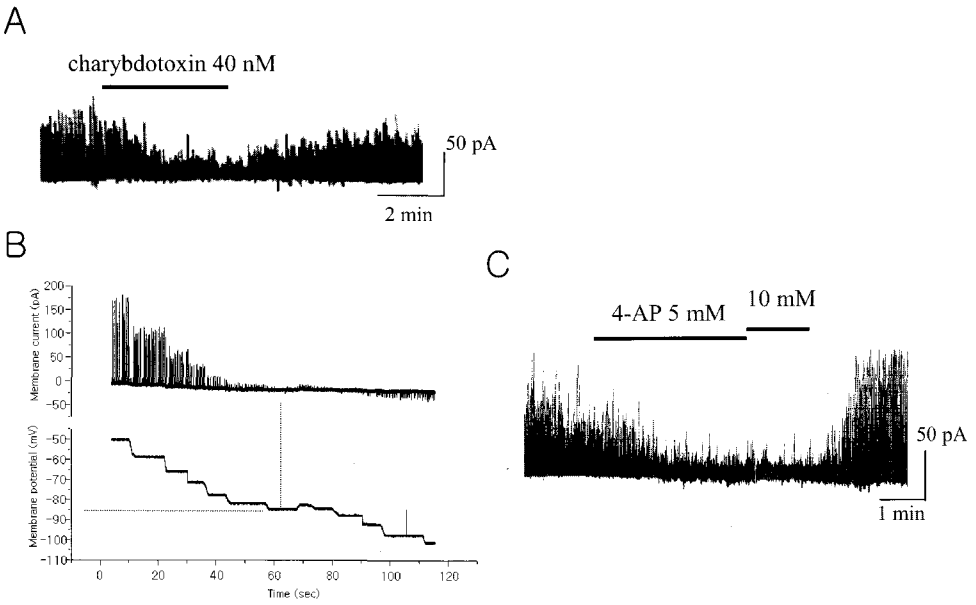

Fig. 1. Effect of 4-AP on the spontaneous transient outward currents (STOCs) of rabbit pulmonary arterial smooth muscle cell. A; Representative chart record of the STOCs and their inhibition by charybdotoxin, a specific BK_{Ca} inhibitor. The STOCs were recorded at the holding potential of −41 mV in the nystatin perforated-patch clamp mode. B; Measurement of the reversal potential of the STOCs by changing the holding potential. C; Representative chart records of STOC inhibition by 4-AP under the same experimental condition as that of A. Each trace from the different cells.

paper were corrected for these junction potentials in each experimental condition.

RESULTS

Effect of 4-AP on STOCs

Under the perforated-patch configuration of patch-clamp technique, spontaneous transient outward currents (STOCs) were usually recorded, when the membrane potential was held above −40 mV. The STOCs were inhibited by 40 nM charybdotoxin (Fig. 1A). In some cells, of which STOC activities were high enough, we could obtain the reversal potential of STOCs (Fig. 1B). They were around −85 mV, which is close to the calculated K^+ equilibrium potential (−87.9 mV). These results indicate that STOCs recorded under the present experimental condition was due to the activation of BK_{Ca} currents. The application of millimolar concentrations of 4-AP reversibly inhibited the STOCs (Fig. 1C, 7 cells out of 11 cells tried). The results shown in Fig. 1 suggest that 4-AP could inhibit the BK_{Ca} currents under a physiological experimental condition, where the intrinsic $[Ca^{2+}]_i$ regulation system was minimally perturbed.

Effect of 4-AP on single BK_{Ca}

To determine whether 4-AP indeed could inhibit the BK_{Ca} currents of intact PASMC, we recorded the single BK_{Ca} currents using the cell-attached configuration of patch-clamp technique. Fig. 2 shows a representative single channel recording at the pipette potential of +7 mV. The patch in Fig. 2 contained at least 2 channels. The activities of the recorded currents markedly increased by a brief application of 10 mM caffeine (n=4, data not shown). Addition of charybdotoxin to the pipette solution prohibited the current recording (n=6, data not shown). Assuming the resting membrane potential of PASMCs to be ~−40 mV (Bae et al, 1999), the actual trans-membrane potential is about −47 mV. Under this experimental condition, the amplitude of single channel currents was 11.1±1.1 pA (n=4), and from this the single channel conductance was calculated to be 235.6±22.9 pS (n=4). These results indicate that the recorded current was through BK_{Ca}. The single BK_{Ca} currents were reversibly and dose-dependently inhibited by 4-AP (Fig. 2). The application of 4-AP decreased not only the channel activity (open probability), but also current amplitude (Fig. 2). The decrease of single channel current amplitude by 4-AP might be interpreted to suggest that 4-AP interfered with the permeation of the BK_{Ca} and decreased the single channel conductance. Alternatively, 4-AP might have depolarized membrane potential by inhibiting other type of K^+ currents, since the trans-membrane potential across the BK_{Ca} is membrane potential minus pipette potential under the present experimental condition. If the latter were correct, the results in Fig. 2 might have underestimated the inhibitory effect of 4-AP on BK_{Ca}, since depolarization itself is a strong activator of BK_{Ca} open probability.

Depolarization together with inhibition of STHPs by 4-AP

Nelson et al. (1995) and Bae et al. (1999) described that the activation of BK_{Ca} by spontaneous Ca^{2+} release from caffeine- and ryanodine-sensitive Ca^{2+} store (Ca^{2+} spark) contributes to the spontaneous hyperpolarizations of the resting membrane potential and vasorelaxation. Since we observed that 4-AP inhibited the STOCs and single channel BK_{Ca} currents in the present study, it is quite probable that the drug can depolarize the resting membrane potential of PASMCs by inhibiting BK_{Ca} currents. The result in Fig. 3 shows the effect of 4-AP on the membrane potential of PASMCs: The membrane potential was recorded in the current-clamp mode. Application of 4-AP inhibited of the spontaneous transient membrane hyperpolarizations (STHPs), resulting in membrane depolarizations (4 cells out of 8 cells tried). This result shows that 4-AP could depolarize the PASMCs by inhibiting the BK_{Ca} currents under the physiological conditions.

DISCUSSION

Under the present experimental condition (temperature of ~35°C and use of nystatin-perforated or cell-attached patch clamp technique), cells' intrinsic mechanisms for pH_i and $[Ca^{2+}]_i$ regulations are intact. Hence, the present study shows that 4-AP can affect the BK_{Ca} currents of intact vascular smooth muscle cells isolated from rabbit pulmo-

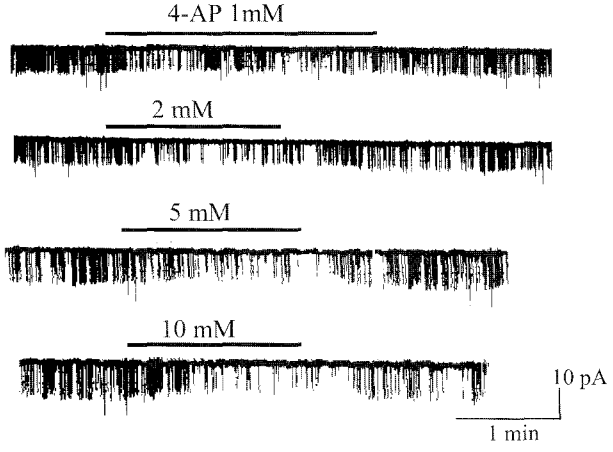

Fig. 2. Inhibition of single BK_{Ca} current by 4-AP. Representative chart records of single channel BK_{Ca} currents under cell-attached mode and their dose-dependent inhibition by 4-AP. Patch pipette potential was held at +7 mV. Results from the same cell.

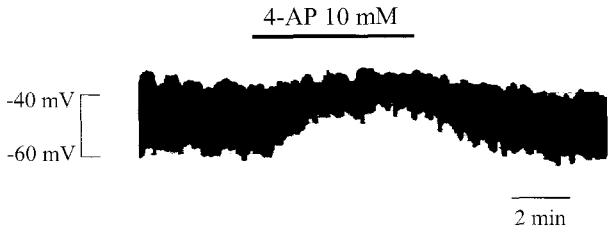

Fig. 3. Effects of 4-AP on the V_m. Representative chart record of V_m showing spontaneous transient hyperpolarizations (STHPs) and their inhibition by bath application of 4-AP.

nary artery.

4-AP has been a useful and powerful tool for the isolation of K_V channels in the study of vascular smooth muscle. Recently, however, the use of 4-AP for evaluation of the role of K_V current in vascular smooth muscle physiology has started to be reconsidered. The mechanism of 4-AP on the BK_{Ca} inhibition was shown to be not direct on the BK_{Ca}, but rather due to alterations in pH_i (Petkova-kirova et al, 2000, see next). 4-AP has one amino-group, which is protonated in solution. Although the pH is usually adjusted to 7.4, 4-AP molecules enter the cell and reach intracellular site of action in their uncharged, non- protonated form causing intracellular alkalinization after protonation inside the cell (Guse et al, 1994;. Petkova-Kirova et al, 2000). Consequently to intracellular alkalinization, the Ca^{2+}-binding capacity of EGTA increases, resulting in $[Ca^{2+}]_i$ decrease. This is a simplified summary mechanism for 4-AP inhibition of BK_{Ca} currents (Petkova-Kirova et al, 2000).

The above mechanism, however, is certainly restricted to the experimental condition, under which the $[Ca^{2+}]_i$ is mainly buffered by a pH-sensitive molecules such as EGTA. The situation, in which intracellular Ca^{2+} and pH are regulated by the intrinsic systems, is quite different from these experimental conditions. In contrast to EGTA-loaded cells, the intracellular alkalinization has been reported to increase the $[Ca^{2+}]_i$ in intact vascular smooth muscle cells (Siskind et al, 1989; Batlle et al, 1993; Petkova-Kirova et al, 2000). Provided that $[Ca^{2+}]_i$ is held constant, the intracellular alkalinzation itself is expected to activate BK_{Ca} current (Hayabuchi et al, 1998; Schubert et al, 2001). Taking these facts into accounts, it is not easy to predict the effect of 4-AP on the BK_{Ca} of intact smooth muscle cells. Therefore, the present observation that 4-AP inhibited the STOCs and single BK_{Ca} currents of intact vascular smooth muscle cells might contribute to correctly evaluating the role of each type of K^+ channels in further studies.

It is not certain in the present study whether mechanisms other than $[Ca^{2+}]_i$ decrease following intracellular alkalinization contributed to the 4-AP inhibition of BK_{Ca} currents. Actually, pH_i or $[Ca^{2+}]_i$ regulation mechanisms in intact smooth muscle cells must be multifactorial, and some cells, where BK_{Ca} currents were not affected by 4-AP application in the present investigation, could be explained by such a multifactorial modulations, i.e. activation of BK_{Ca} by increased pH_i and inhibition of BK_{Ca} by decreased $[Ca^{2+}]_i$ canceling (offsetting) each other. At any rate, the present investigation certainly demonstrated that 4-AP inhibited the BK_{Ca} currents under the physiological conditions. Therefore, the use of 4-AP as a selective K_V channel blocker for the study of vascular smooth muscle function must be carefully evaluated.

ACKNOWLEDGEMENT

This work was supported by the Faculty Fund of Konkuk University in 2001.

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