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# Extracellular calcium modulates the inhibitory effect of 4-aminopyridine on Kv current in vascular smooth muscle cells

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ABSTRACT

4-Aminopyridine is widely used as a Kv channel blocker. However, its mechanism of action is still a matter of debate. Extracellular calcium as well as 4-aminopyridine have been reported to interact with the activation kinetics of particular Kv channels. The objective of the present study was to investigate whether extracellular calcium could modulate the inhibition of Kv current by 4-aminopyridine in vascular myocytes. Kv current was recorded by using whole-cell patch-clamp in freshly isolated smooth muscle cells from rat mesenteric artery. Macroscopic properties of Kv current were not affected by change in extracellular calcium from 0 to 2 mM. During a 10 s depolarizing pulse, 4-aminopyridine inhibited the peak current without affecting the end-pulse current. The concentration–effect curve of 4-aminopyridine was shifted to the left in the presence of 2 mM calcium compared to 0 calcium. After 4-aminopyridine washout, current recovery from block was slower in the presence than in the absence of calcium. Inhibition of Kv current by 4-aminopyridine (0.5 mM) and the Kv2 blocker stromatoxin (50 nM) was additive and stromatoxin did not alter the potentiation of 4-aminopyridine effect by extracellular calcium. These results showed that extracellular calcium modulated the inhibitory potency of 4-aminopyridine on Kv current in vascular myocytes. The component of Kv current that was inhibited by 4-aminopyridine in a calcium-sensitive manner was distinct from Kv2 current.



## 1. Introduction

Voltage-gated potassium channels (Kv) contribute to the regulation of membrane potential in vascular smooth muscle cells and are involved in several vascular pathologies (Ko et al., 2008; Nelson and Quayle, 1995). These channels are tetramers of four α subunits, each subunit contributing to the formation of the pore selective for $K^+$ ions. Twelve families of α subunits have been identified, Kv1.x to Kv12.x. Kv1.x and Kv2.x seem to be major components of Kv current in vascular smooth muscle cells (Cox, 2005; Cox et al., 2001; Davies and Kozlowski, 2001; Lu et al., 2001; McDaniel et al., 2001; Ohya et al., 2000; Xu et al., 1999), Kv1.5 and Kv2.1 expression being demonstrated in mesenteric arterioles (Hald et al., 2012). Kv1 channels have been proposed to play an important role in the regulation of myogenic tone in middle cerebral artery (Chen et al., 2006) but the precise contribution of Kv1 and Kv2 families in the control of the membrane potential in vascular smooth muscle cell is not elucidated. In addition, Kv7 channels have been proposed to contribute to the regulation of vascular tone (Joshi et al., 2006; Mackie et al., 2008; Zhong et al., 2010).

4-Aminopyridine (4-AP) is a selective inhibitor of Kv channels. It presents different affinities according to the nature of the α subunit involved in the current with a high affinity (0.05–1 mM) for Kv1.x subunits and a low affinity (0.5–18 mM) for Kv2.x channel family (Coetzee et al., 1999; Gelband et al., 1999; Grissmer et al., 1994; Judge et al., 2002; Kerr et al., 2001; Kirsch and Drewe, 1993; Lang et al., 2004; Schmalz et al., 1998; Stuhmer et al., 1989; Yao and Tseng, 1994). Its mechanism of action is still a matter of debate: open and closed channel block have been described (Bouchard and Fedida, 1995) and different reports point out the fact that the binding of 4-AP promotes the activated-not-open channel conformation, blocking the access to the pore (Armstrong and Loboda, 2001; Claydon et al., 2007; del Camino et al., 2005).

The effect of 4-AP on Kv current is most often recorded in the absence of external calcium to prevent the activation of calcium-activated $K^+$ current. However, external calcium ions have been shown to modulate the gating kinetics of Shaker potassium channel by accelerating the closing of the activation gate (Gomez-Lagunas et al., 2003). This phenomenon has never been recorded with native channels, probably because activation kinetics is better studied after

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deletion of the inactivation ball of the $K^+$ channel (Gomez-Lagunas et al., 2003). The potential interaction between external calcium and 4-AP has not been investigated yet.

The objective of the present study was to investigate the effect of physiological concentration of external calcium on the action of 4-AP on Kv current in freshly isolated myocytes from mesenteric artery. The results revealed that calcium modulates the inhibitory potency of submillimolar concentration of 4-AP on a component of Kv current that is distinct from Kv2 current.

## 2. Material and methods

Male Wistar rats (weight 220–250 g) were anesthetized and killed by decapitation in accordance with international guidelines and the local ethic committee. The superior mesenteric artery was removed, cleaned of connective tissue and used for enzymatic cell isolation.

### 2.1. Composition of solutions and drugs

Composition of physiological salt solution (PSS) solution was (in mM): NaCl 137, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 2, glucose 10, Hepes 10, pH was adjusted to 7.4 with Tris. Nominally $Ca^{2+}$-free PSS had the same composition as PSS but $CaCl_2$ was omitted. The pipette solution containing 8 nM free $Ca^{2+}$ (Maxchelator, Stanford, USA) had the following composition (in mM): KCl 137, $MgCl_2$ 5, $CaCl_2$ 0.3, EGTA 10, ATP 5, Hepes 10, pH adjusted to 7.2 with KOH. All the chemicals were obtained from Sigma, including 4-aminopyridine (4-AP) and recombinant stromatoxin. Stock solution of 4-AP 0.5 M was prepared in $Ca^{2+}$-free PSS solution and pH was adjusted to 7.4.

### 2.2. Cell isolation

Mesenteric artery was opened, endothelium was removed by gentle scrapping and the artery was cut into small segments immersed into an ice-cold Hanks solution (composition in mM: NaCl 140, KCl 4.2, $KH_2PO_4$ 1.2, Hepes 10, pH 7.4 with Tris) containing 0.45 mg $ml^{-1}$ DTT, 0.6 mg $ml^{-1}$ papain and 0.65 mg $ml^{-1}$ bovine serum albumin for 10 min followed by a 13 min incubation at 37 °C. The artery segments were then transferred into ice-cold Hanks solution supplemented with EGTA 0.1 mM, and myocytes were obtained by gentle trituration with a Pasteur pipette. They were stored at 4 °C and used on the same day.

### 2.3. Electrophysiological experiments

Whole-cell Kv current was measured by conventional whole-cell patch-clamp technique with the use of an Axopatch 200B amplifier and pClamp 9.2 software (Axon Instruments). Cells were continuously superfused with PSS solution containing or not the different pharmacological agents. Patch pipettes were made from thin-walled borosilicate glass (GC-150F-10, Harvard Apparatus) by using a DMZ Universal puller (Zeitz-Instrumente, Munich, Germany). The pipette filled with the intracellular solution had a resistance between 2 and 4 MΩ. No leak subtraction was applied. All patch-clamp experiments were performed at room temperature. The average membrane capacitance of the isolated vascular smooth muscle cells used in these experiments was $12.8 \pm 0.3$ pF ($n=90$).

The stimulation protocol was as follows: cells were held at −80 mV, prepulsed to 30 mV for 300 ms (test pulse 1) before being stepped back to −80 mV for 100 ms. Then, the cell was stimulated for 10 s to a voltage varying from −90 mV to 20 mV by steps of 10 mV. The cell was brought back to −80 mV for 20 ms before a second test pulse to 30 mV was applied for 300 ms (test pulse 2). One minute intervals were allowed between successive steps to allow for recovery from inactivation. The time-course of current inactivation during the 10 s stimulation pulse was fitted with a single exponential decay ($y=A\times\exp(-t/\tau)+B$, with $A$ the initial value of the current, $t$ the time, $\tau$ the time constant of the decay and $B$ the value of the current at the end of the decay). The $I$–$V$ curves were obtained from the peak current and the end-pulse current evoked by 10 s depolarizing pulses. Steady state inactivation curve was obtained by normalizing the current evoked by the second test pulse to the current evoked by the first test pulse, after correction for the non-inactivating fraction of the current. The curve was fitted either by a single Boltzmann function $y=1/(1+\exp[(V_{0.5}-V)/k])$ with $V_{0.5}$ the half-inactivation potential and $k$ the slope factor or a double Boltzmann function $y=A/(1+\exp[(V_1-V)/k_1]+(1-A)/(1+\exp[(V_2-V)/k_2]$ with A, the proportion of the first component, $V_1$ and $V_2$, the half-inactivation potential of the first and the second component and $k_1$ and $k_2$ the slope factor of the two components. Best fit was determined by a $F$ test. Steady state activation was obtained either from the global conductance $G$ calculated as follows: $G=I/(V-E_K)$ with $E_K$ equal to −78 mV, or by recording the tail current at −80 mV after the application of 250 ms pulses between −60 mV and +30 mV in 10 mV increments. Normalized conductance values or tail currents were fitted with a single Boltzmann function.

Determination of the time-course of 4-AP binding and dissociation was done by incubating cells with 4-AP for 10 min at a holding potential of −80 mV without stimulation. Current was then evoked by 150 ms depolarizing pulses to +20 mV from a holding potential of −80 mV every 10 s until stable effect on current was observed. Cells were thereafter perfused with bath solution without 4-AP for 10 min without stimulation before resuming the stimulation protocol. 4-AP-sensitive current (obtained by subtracting the current recorded in the presence of 4-AP from the current measured in the absence of 4-AP) was normalized to the control current recorded in the absence of 4-AP. Time-course of current block or unblock was fitted with a single exponential function.

### 2.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism 4.0. Data are expressed as mean ± SEM. The 4-AP concentration producing 50% inhibition of the current ($IC_{50}$) was calculated by non-linear curve fitting, using a classical sigmoïdal concentration–response function. Log values were used for statistical analysis. Comparisons between curves were made by two way ANOVA with a Bonferoni post test and comparisons between individual values were made with a t test or an ANOVA when more than 2 groups were involved in the comparison. Differences with P values smaller than 0.05 were considered significant.

## 3. Results

### 3.1. Extracellular calcium did not affect macroscopic properties of Kv current

Kv currents were recorded in vascular smooth muscle cells during perfusion with two different solutions: one containing 2 mM calcium and one without added calcium. Fig. 1A shows current traces recorded in a rat mesenteric artery cell in response to the triple pulse protocol with 10 s stimulation pulses from −60 mV to 20 mV. Depolarizing pulses higher than −50 mV evoked an outward current, characterized by a slow decrease with time, reflecting current inactivation. Inactivation did not lead to