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# 4-Aminopyridine affects rat arterial smooth muscle $BK_{Ca}$ currents by changing intracellular pH

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**1** The hypothesis whether or not 4-AP can affect vascular smooth muscle $BK_{Ca}$ currents was tested using the patch-clamp technique, pH- and calcium-fluorimetry, and freshly isolated rat arterial smooth muscle cells.

**2** Application of 4-AP reversibly inhibited $BK_{Ca}$ currents at an intracellular calcium ($[Ca]_i$) of 250 nM with a half-block of 2.5 mM at +50 mV.

**3** The presence of 2 $\mu$M thapsigargin, 10 $\mu$M heparin, and 10 $\mu$M ryanodine did not alter the effect of 4-AP on $BK_{Ca}$ currents at $[Ca]_i$ 250 nM.

**4** At $[Ca]_i < 100$ nM 4-AP did not inhibit $BK_{Ca}$ currents.

**5** Application of 4-AP to the intracellular or extracellular side of excised $BK_{Ca}$ channels did not alter channel activity or channel amplitude.

**6** Replacement of the pH-sensitive calcium buffer EGTA by the pH-insensitive calcium buffer BAPTA in the intracellular solution turned the 4-AP-induced inhibition of $BK_{Ca}$ currents into a stimulation at $[Ca]_i$ 250 nM.

**7** Application of 4-AP to single cells increased intracellular pH, which was accompanied by a reduction of $[Ca]_i$ in EGTA-loaded cells and a stable $[Ca]_i$ in BAPTA-loaded cells.

**8** Thus, these results suggest that in isolated vascular smooth muscle cells at $[Ca]_i > 100$ nM 4-AP affects $BK_{Ca}$ currents *via* an alteration of intracellular pH.

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**Keywords:** Arteries; smooth muscle; calcium-activated potassium channel; voltage-dependent potassium channel; 4-aminopyridine; intracellular pH; intracellular calcium

**Abbreviations:** 4-AP, 4-aminopyridine; $BK_{Ca}$, calcium-activated potassium channel; $[Ca]_i$, intracellular calcium concentration; $K_v$, voltage-dependent potassium channel

## Introduction

Systemic blood pressure is determined by the contractile state of small arteries. One of the major factors affecting the contractile state of small arteries is the smooth muscle cell membrane potential (Daut *et al*., 1994). A large number of studies has shown that potassium channels are particularly important for the regulation of the membrane potential (for review see Nelson & Quayle, 1995).

Various potassium channel types are expressed in vascular smooth muscle cells. Best described are voltage-dependent potassium channels ($K_v$ channels), inward rectifier potassium channels, ATP-sensitive potassium channels, and calcium-activated potassium channels of high conductance ($BK_{Ca}$ channels) (Nelson & Quayle, 1995). Ion channel inhibitors are widely used for pharmacological discrimination between the different channel types as well as for the determination of their functional role. Unfortunately, only a few inhibitors act on one channel type exclusively. For example, iberiotoxin is considered to interact only with $BK_{Ca}$ channels (Galvez *et al*., 1990; Giangiacomo *et al*., 1992). In contrast, most of the inhibitors affect several channel types. Fortunately, quantitative differences often exist, i.e. the concentration ranges for inhibiting certain channel types are different. Thus, tetraethylammonium binds to several potassium channel types, where half-block appeared, for example, at 0.2 mM for $BK_{Ca}$ channels (Langton *et al*., 1991), at 10 mM for $K_v$ currents (Robertson & Nelson, 1994), and at 7 mM for ATP-sensitive potassium currents (Beech *et al*., 1993). Thus, in order to be able to use an ion channel inhibitor for the determination of the role of a certain potassium channel in vessel contractile responses quantitative data on its effect on different vascular smooth muscle potassium channels are required.

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), which is often used as a tool to isolate $K_v$ currents from other potassium currents. Concerning vascular smooth muscle $BK_{Ca}$ channels, it has been reported that 4-AP in millimolar concentrations did not alter the activity and the amplitude of excised channels from rabbit portal vein (Beech & Bolton, 1989), human pulmonary artery (Peng *et al*., 1996), and rat renal arterioles (Zou *et al*., 1996). In contrast, 4-AP was shown to reduce the activity of excised $BK_{Ca}$ channels from porcine coronary arteries (Fujino *et al*., 1991). Furthermore, a recent study showed that 4-AP can alter intracellular pH in Jurkat T-lymphocytes (Guse *et al*., 1994). This is an interesting finding because the activity of vascular smooth muscle $BK_{Ca}$ channels has been shown to be modulated by intracellular pH (Hayabuchi *et al*., 1998; Schubert & Gagov, 1997). Thus, the hypothesis was tested whether or not 4-AP does affect vascular smooth muscle $BK_{Ca}$ channels by recording whole-cell $BK_{Ca}$ currents and single $BK_{Ca}$ channel activity of cells isolated from rat tail and basilar arteries.

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## Methods

The methods used in this study will be described only briefly, because they have been presented in detail previously (Schubert *et al*., 1999).

### *Cell isolation*

Male Wistar-Kyoto rats were killed by stunning and subsequent decapitation. The basilar artery, the main tail artery, and first order branches of the latter were dissected free. A piece of artery was placed into a microtube containing 1 ml of an enzyme solution consisting of (in mM): NaCl 110, KCl 5, $CaCl_2$ 0.16, $MgCl_2$ 2, HEPES 10, $NaHCO_3$ 10, $KH_2PO_4$ 0.5, $NaH_2PO_4$ 0.5, glucose 10, EDTA 0.49, and taurine 10 at pH 7.0, as well as 1.5 mg $ml^{-1}$ papain, 1.6 mg $ml^{-1}$ albumin and 0.4 mg $ml^{-1}$ DL-dithiothreitol and stored there overnight at 4°C. The next day the microtube with the vessel was incubated for 5–20 min at 37°C. Single cells were released by trituration with a polyethylene pipette. Because there were no differences between the effects observed on smooth muscle cells from tail or basilar arteries, results have been pooled. The following solutions have been used:

(1) Whole-cell, bath solution (in mM): NaCl 140, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 0.1, glucose 10, taurine 10, Na-pyruvate 5, and HEPES 10 at pH 7.4 containing 3 $\mu$M nicardipine.
(2) Outside-out, bath solution (in mM): NaCl 135, KCl 6, $MgCl_2$ 1, $CaCl_2$ 0.1, EGTA 3 (purity 96%) and HEPES 10 at pH 7.4 having a free calcium concentration ($[Ca]_i$) $<100$ nM.
(3) Whole-cell, pipette solution (in mM): KCl 122, NaCl 10, $MgCl_2$ 1, EGTA 3 (purity 96%), HEPES 10 and an appropriate amount of $CaCl_2$ to get a $[Ca]_i$ of 4, 100 and 250 nM at pH 7.4; or (in mM): KCl 102, NaCl 10, $MgCl_2$ 1, EGTA 10 (purity 96%) or BAPTA 10 (purity 98%), HEPES 10 and an appropriate amount of $CaCl_2$ to get a $[Ca]_i$ of 250 nM at pH 7.4.
(4) Outside-out, pipette solution and inside-out, bath solution (in mM): KCl 140, $MgCl_2$ 1, EGTA 3 (purity 96%), HEPES 10 and an appropriate amount of $CaCl_2$ to get a $[Ca]_i$ of 2.5 $\mu$M at pH 7.4. $[Ca]_i$ was calculated with the following apparent reaction constants at pH 7.4: log $K_{CaEGTA} = 7.17$, log $K_{MgEGTA} = 1.93$ (Schubert, 1996) and checked using FURA-2 fluorescence and calcium standard solutions (WPI, Germany).
(5) Inside-out, pipette solution (in mM): NaCl 135, KCl 6, $MgCl_2$ 1, EGTA 3 (purity 96%), HEPES 10 and an appropriate amount of $CaCl_2$ to get a $[Ca]_i$ of 300 nM at pH 7.4.
(6) Physiological bath solution for single cell fluorescence (in mM): NaCl 135, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1.6 and HEPES 10 at pH 7.4.
(7) High potassium bath solution for single cell fluorescence (in mM): KCl 135, NaCl 6, $MgCl_2$ 1, $CaCl_2$ 1.6 and HEPES 10 at pH 7.4.

### *Patch-clamp recording*

All experiments were performed at room temperature. Patch pipettes had resistances of 2–6 MOhm. Recordings were made with an Axopatch 200 or EPC-7 amplifier with the whole-cell, inside-out and outside-out patch-clamp configurations. In the whole-cell experiments, stimulation of currents with pulse and ramp protocols, data sampling at a rate of 1 kHz, and data analysis were done with the software package ISO2 (MFK, Germany) or SWT (Shkodrov, 1995). Stability of the access resistance was tested regularly during the course of the experiment. Single channel data were stored on DTR-1800 data recorder and later replayed for analysis, where they were filtered at 1 kHz with use of an eight-pole Bessel filter and digitised at 5 kHz. Thereafter, they were analysed off-line with the software package ASCD (G. Droogmans, Lab. Fysiologie, KU Leuven, Belgium).

### *Single cell fluorescence*

Single cells were loaded with 2 $\mu$M BCECF-AM, a pH-sensitive dye, or 50 nM $DiBAC_4(3)$, a membrane potential-sensitive dye, or 5 $\mu$M FLUO-3-AM, a calcium-sensitive dye, respectively at room temperature for 20 min. During measurements the dyes were excited with light at 455 and 493 nm (BCECF) or 493 nm ($DiBAC_4(3)$ and FLUO-3) with use of a filter wheel and a xenon arc lamp (Luigs Neumann, Germany). The emission from the cells was filtered at 505 nm, detected by a photomultiplier and sent to a computer. There, during intracellular pH measurements the ratio of emission at the two excitation wavelengths after subtraction of the background fluorescence was calculated. The calibration of the ratio in terms of pH was performed with use of nigericin. Thus, at the end of the experiment, the cell was incubated in a solution consisting of (in mM) KCl 144.5, NaCl 10, $MgCl_2$ 1, EGTA 1, glucose 5.5, and HEPES 10 at pH 7.4 and 2 mg $l^{-1}$ nigericin for 15 min (Aalkjaer & Cragoe, 1988). Then, the cell was superfused with solutions having a pH of 7.0, 7.4, and 7.8. From these data a calibration curve was calculated. The parameters of this curve were entered into the analysis program, which transformed the ratio signal into intracellular pH values. During membrane potential and calcium concentration measurements the fluorescence signal was normalized by dividing the emission at the excitation wavelength during the experimental interventions by the emission just before the first intervention. The membrane potential-sensitive dye $DiBAC_4(3)$ partitions between the cell membrane and the cytosol as a function of the membrane potential, where fluorescence intensity increases upon depolarization (Epps *et al*., 1994). The non-ratiometric dye FLUO-3 had to be used for the measurement of calcium concentration changes because 4-AP had significant autofluorescence when excited with light at the excitation wavelengths of the ratiometric dye FURA-2. A calibration of the fluorescence signal of the single wavelength dyes was not performed. In order to simulate the conditions of the patch-clamp experiments, cells were loaded with 1 mM EGTA-AM or 30 $\mu$M BAPTA-AM, respectively and bathed in 135 mM KCl. Higher concentrations of BAPTA-AM could not be achieved because of limitations in solubility.

### *Drugs*

Albumin, DL-dithiothreitol, TEA, heparin, nigericin, and the salts for the solutions were obtained from Sigma (Deisenhofen, Germany). Papain was from Ferak (Berlin, Germany). BCECF, $DiBAC_4(3)$, FLUO-3, EGTA-AM, BAPTA-AM, and 4-bromo-A23187 were purchased from Molecular Probes (Leiden, The Netherlands) and iberiotoxin, thapsigargin, 4-aminopyridine, and ryanodine were from RBI (Cologne, Germany).

### *Statistics*

All data are means ± s.e.mean, $n$ is the number of cells. Statistical analysis was performed using repeated measures ANOVA, paired samples and independent samples $t$-test as appropriate (SPSS 9.0 for Windows).

## Results

### *Effect of 4-aminopyridine on $BK_{Ca}$ currents*

The application of 500-ms voltage steps from a holding potential of −40 mV to test potentials in the range from −50 to +50 mV evoked large, non-inactivating, and fluctuating outward currents at an intracellular calcium ($[Ca]_i$) of 250 nM (Figure 1a,b). These currents were inhibited considerably by 300 nM iberiotoxin ($P<0.01$, $n=4$) (Figure 1a, c(i)), the specific blocker of $BK_{Ca}$ channels (Galvez *et al*., 1990; Giangiacomo *et al*., 1992), e.g. the outward current at +30 mV was reduced by 84 ± 6% ($n=4$). Thus, under these conditions the outward current was dominated by the $BK_{Ca}$ current. Consequently, all experiments on $BK_{Ca}$ currents were performed using these conditions. Application of 4-aminopyridine (4-AP) also suppressed the outward current in the voltage range studied ($P<0.001$, $n=10$ for 1, 5, and 20 mM 4-AP, respectively) (Figure 1b(i), (ii), and c(ii)–(iv)). Concentration-response relationships have been derived from these data and $IC_{50}$-values of 1.8 mM at +10 mV, 2.2 mM at +30 mV, and 2.5 mM at +50 mV were determined.

### *Effect of 4-AP on $K_v$ currents*

The application of 4000-ms voltage steps from a more negative holding potential of −90 mV to test potentials in the range from −70 to +30 mV evoked small, fast-inactivating as well as slowly-inactivating outward currents in the presence of 300 nM iberiotoxin at $[Ca]_i$ 250 nM (Figure 2a). At a holding potential of −20 mV these currents were abolished (data not shown). Thus, under these conditions the outward current was dominated by the $K_v$ current. Application of 5 mM 4-AP inhibited these outward currents in the voltage range studied ($P<0.01$, $n=5$ for the slowly-inactivating current and for the fast-inactivating current, respectively) (Figure 2a(i), (ii), b(i), (ii)).

### *Effect of 4-AP on $BK_{Ca}$ currents during inhibition of $[Ca]_i$ release and uptake*

In the presence of 2 μM thapsigargin, the inhibitor of the sarcoplasmic reticulum calcium pump, 10 μM heparin, the inhibitor of $IP_3$-induced calcium release, and 10 μM ryanodine, the inhibitor of calcium-induced calcium release, the application of 500-ms voltage steps from a holding potential of −40 mV to test potentials in the range from −50 to +50 mV evoked large, non-inactivating, and fluctuating outward currents at $[Ca]_i$ 250 nM. These currents were suppressed considerably by 5 mM 4-AP in the voltage range studied ($P<0.001$, $n=5$) (Figure 3a). This effect of 4-AP did not differ from the effect of the drug when applied alone ($P=0.38$, $n=5$).

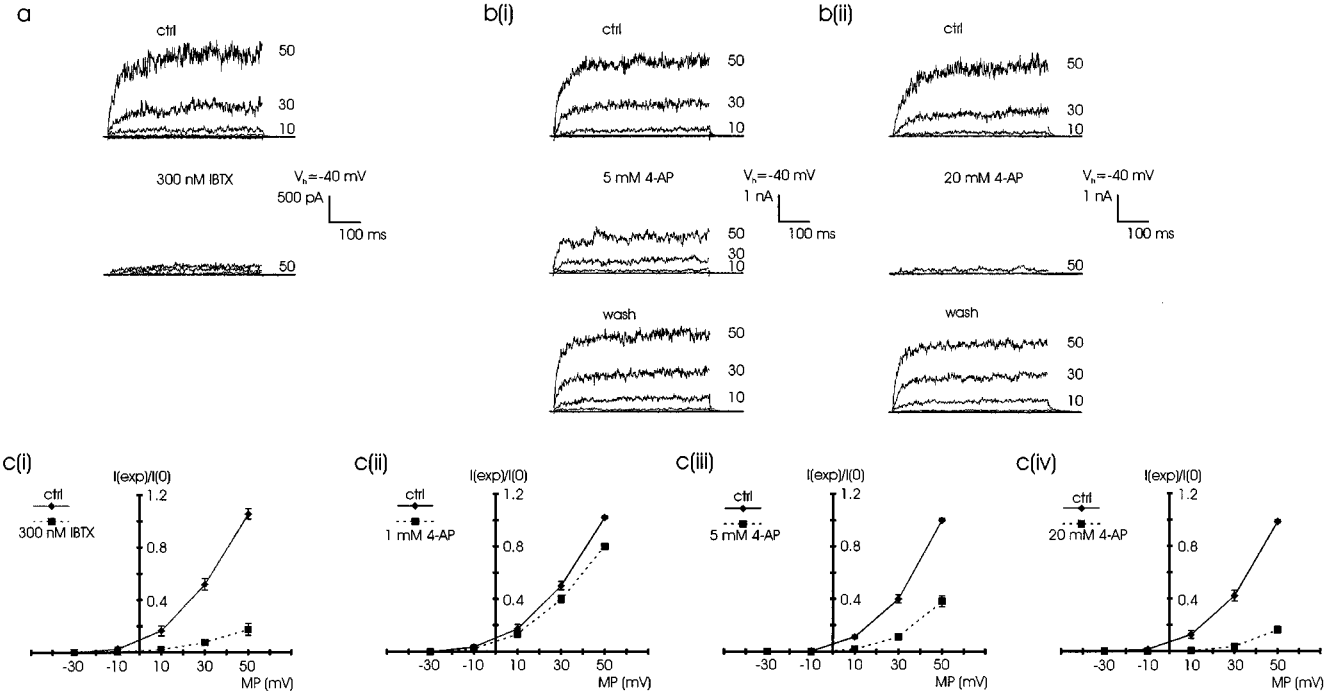


**Figure 1** Effect of 4-aminopyridine on rat vascular smooth muscle $BK_{Ca}$ currents. (a) Examples of traces of outward current elicited with use of 500-ms voltage steps from a holding potential ($V_h$) of −40 mV to test potentials in the range from −50 to +50 mV before (ctrl) and in the presence of iberiotoxin (IBTX). (b) Examples of traces of outward current elicited with use of 500-ms voltage steps from a holding potential of −40 mV to test potentials in the range from −50 to +50 mV before (ctrl), in the presence of 5 mM (b(i)) and 20 mM (b(ii)) 4-aminopyridine (4-AP), and following washout (wash) of 4-AP. (c) Summarized data of the current-voltage (I-MP) relationship of outward currents like those shown in (a) and (b) for the application of IBTX (c(i)), 1 mM 4-AP (c(ii)), 5 mM 4-AP (c(iii)), and 20 mM 4-AP (c(iv)). Data are expressed as ratio of current under specified experimental conditions (I(exp)), i.e. before (ctrl) and in the presence of inhibitors at selected membrane potentials, to current at +50 mV determined 30–60 s before starting the control (ctrl) measurement (I(0)). This presentation was selected in order to reduce inter-cell variability. Significant changes of the current-voltage relationship were observed with 300 nM IBTX ($P<0.01$, $n=4$), 1 mM 4-AP ($P<0.001$, $n=10$), 5 mM 4-AP ($P<0.001$, $n=10$), and 20 mM 4-AP ($P<0.001$, $n=10$).

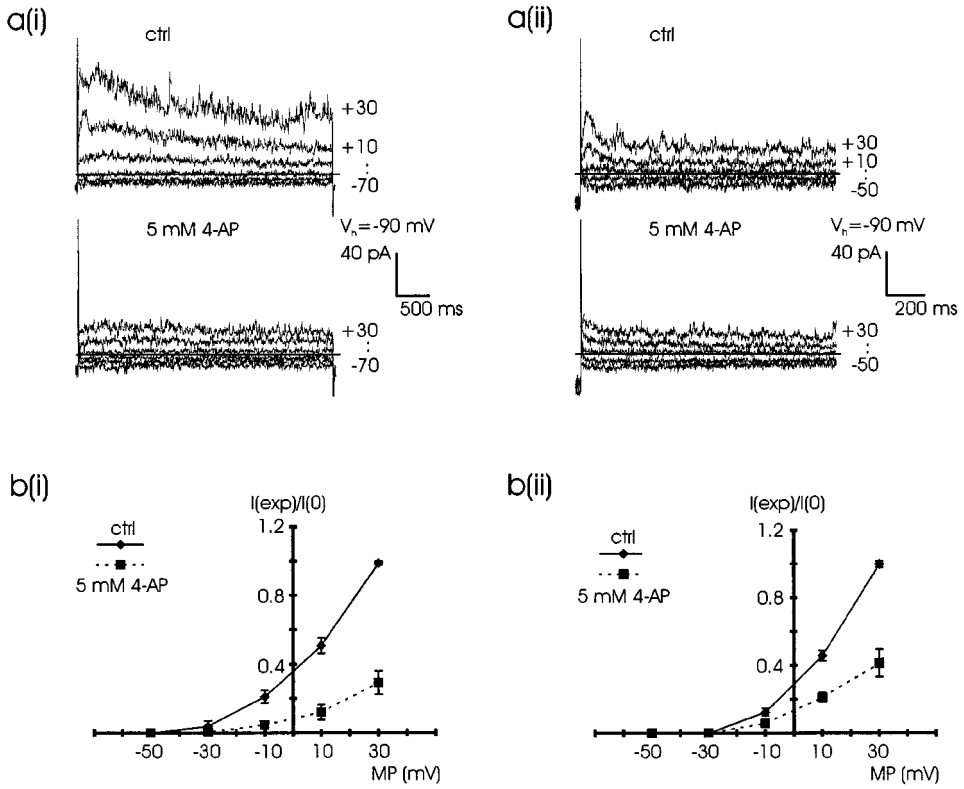


**Figure 2** Effect of 4-aminopyridine on rat vascular smooth muscle $K_v$ currents. (a) Examples of traces of outward current elicited with use of 4000-ms voltage steps from a holding potential ($V_h$) of −90 mV to test potentials in the range from −70 to +30 mV before (ctrl) and in the presence of 4-AP showing slowly-inactivating (a(i)) and fast-inactivating (a(ii)) currents. Note different y-axis scaling compared to Figure 1. In a(ii) only part of the trace is shown to demonstrate the fast-inactivating current more clearly. (b) Summarized data of current-voltage (I-MP) relationship of outward currents like those shown in a (after leak substraction) for the application of 5 mM 4-AP to slowly-inactivating (b(i)) and fast-inactivating (b(ii)) currents. Current was measured at the peak of the current trace. Data are expressed as ratio of current under specified experimental conditions (I(exp)), i.e. before (ctrl) and in the presence of 4-AP at selected membrane potentials, to current at +30 mV determined 30–60 s before starting the control (ctrl) measurement (I(0)). This presentation was selected in order to reduce inter-cell variability. Significant changes of the current-voltage relationship were observed with 5 mM 4-AP for the slowly-inactivating ($P<0.01$, $n=5$) and for the fast-inactivating ($P<0.01$, $n=5$) current.

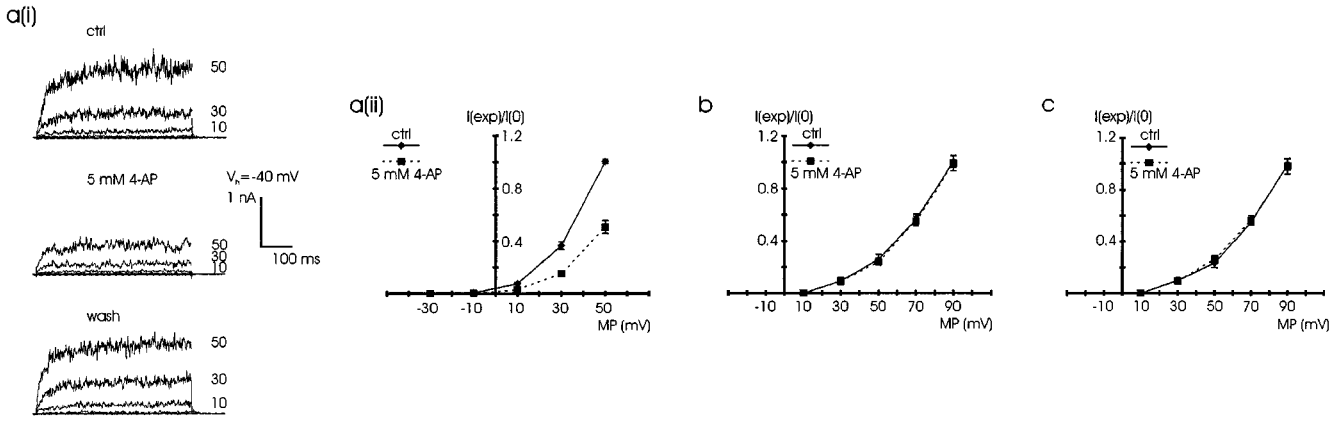


**Figure 3** Effect of 4-AP on $BK_{Ca}$ currents during blockade of $[Ca]_i$ homeostasis and at low $[Ca]_i$. Example traces (a(i)) and summarized data (a(ii)), (b), (c) of current-voltage (I-MP) relationship of outward current elicited with use of a 500-ms voltage steps from a holding potential of −40 mV to test potentials in the range from −50 to +50 mV and +90 mV for the application of 5 mM 4-AP in the presence of 2 μM thapsigargin, 10 μM heparin, and 10 μM ryanodine at 250 nM $[Ca]_i$ (a), and without inhibitors at 4 nM $[Ca]_i$ (b) as well as 100 nM $[Ca]_i$ (c). Data are expressed as ratio of current under specified experimental conditions (I(exp)), i.e. before (ctrl) and in the presence of 4-AP at selected membrane potentials, to current at +50 mV determined 30–60 s before starting the control (ctrl) measurement (I(0)). This presentation was selected in order to reduce inter-cell variability. Significant changes of the current-voltage relationship were observed with 5 mM 4-AP only in (a) ($P<0.001$, $n=5$), but not in (b) ($P=0.68$, $n=5$) and (c) ($P=0.74$, $n=5$).

### *Effect of 4-AP on $BK_{Ca}$ currents at low $[Ca]_i$*

At low $[Ca]_i$ of 4 and 100 nM, the application of 500-ms voltage steps from a holding potential of −40 mV to test potentials in the range from −30 to +90 mV evoked small, non-inactivating, and fluctuating outward currents, which were largely blocked by iberiotoxin. The iberiotoxin-sensitive, i.e. $BK_{Ca}$, current at +70 mV had a similar density of $9.2\pm2.3$ pA $pF^{-1}$ at $[Ca]_i$ 4 nM and $9.1\pm1.3$ pA $pF^{-1}$ at

$[Ca]_i$ 100 nM ($P=0.95$, $n=7$ and 6, respectively) compared to a density of $40.6\pm11.2$ pA $pF^{-1}$ at $[Ca]_i$ 250 nM ($n=8$). Thus, under these conditions the outward current was dominated by $BK_{Ca}$ currents, which were regulated only by the membrane potential. Application of 4-AP did not affect the outward current in the voltage range studied ($P=0.68$, $n=5$ for 4 nM $[Ca]_i$ and $P=0.74$, $n=5$ for 100 nM $[Ca]_i$) (Figure 3b, c).

### *Effect of 4-AP on single $BK_{Ca}$ channels*

The properties of the predominantly observed single channel in excised patches have been described by us previously and found to be characteristic for the vascular smooth muscle high conductance calcium-activated ($BK_{Ca}$) channel (Serebryakov *et al*., 1997). During application of 5 mM 4-AP to the intracellular side of $BK_{Ca}$ channels at 0 mV the activity and the unitary current amplitude of $BK_{Ca}$ channels in inside-out patches changed $0.95\pm0.14$ fold ($n=4$) and $0.99\pm0.03$ fold ($n=4$), respectively, which is not significantly different from the $1.00\pm0.19$ fold ($P=0.86$, $n=4$) and $0.97\pm0.02$ fold ($P=0.64$, $n=4$) change, respectively during time control measurements. Further, during application of 5 mM 4-AP to the extracellular side of $BK_{Ca}$ channels at 0 mV the activity and the unitary current amplitude of $BK_{Ca}$ channels in outside-out patches changed $1.04\pm0.15$ fold ($n=4$) and $1.03\pm0.03$ fold ($n=4$), respectively, which is not significantly different from the $0.98\pm0.13$ fold ($P=0.63$, $n=4$) and $0.99\pm0.02$ fold ($P=0.75$, $n=4$) change, respectively during time control measurements.

### *Effect of 4-AP on $BK_{Ca}$ currents using different $[Ca]_i$-buffers*

All previous experiments were performed in the presence of 3 mM of the pH-sensitive calcium buffer EGTA in the pipette solution. In this experimental series, buffer capacity was increased using 10 mM EGTA or 10 mM BAPTA, a pH-insensitive calcium buffer. In the presence of both EGTA and BAPTA the application of 500-ms voltage steps from a holding potential of $-40$ mV to test potentials in the range from $-50$ to $+50$ mV evoked large, non-inactivating, and fluctuating outward currents at $[Ca]_i$ 250 nM. In the presence of 10 mM EGTA, these currents were suppressed considerably by 5 mM 4-AP in the voltage range studied ($P<0.01$, $n=5$) (Figure 4a). This effect was not significantly different from the effect of 5 mM 4-AP in the presence of 3 mM EGTA ($P=0.58$, $n=5$). In contrast, in the presence of 10 mM BAPTA, the outward currents were stimulated considerably by 5 mM 4-AP in the voltage range studied ($P<0.01$, $n=5$) (Figure 4b). However, in the presence of 300 nM iberiotoxin there was no stimulation of outward currents but rather an inhibition of iberiotoxin-insensitive currents suggesting that the 4-AP-stimulated current is the $BK_{Ca}$ current.

### *Effect of 4-AP on intracellular pH and calcium*

Dissolving 4-AP in the whole-cell pipette solutions having an initial pH of 7.4 to a final concentration of 5 mM increased pH by $0.73\pm0.02$ units ($n=10$, $P<0.001$). Therefore, in all 4-AP-containing solutions applied to single cells in the experiments described in this and the previous paragraphs, pH was checked and readjusted to 7.4. Nevertheless, application of a 5 mM 4-AP-containing solution with pH 7.4 to single cells increased intracellular pH by $0.77\pm0.15$ units ($n=5$, $P<0.01$) (Figure 5a), which was not significantly different from the pH change obtained in solutions ($P=0.79$). In these and the following single cell experiments, the cells were loaded with EGTA-AM or BAPTA-AM, respectively to simulate the calcium buffering conditions of the patch-clamp experiments and were bathed in a high potassium solution to simulate the voltage-clamp of the patch-clamp experiments. Indeed, application of a 5 mM 4-AP-containing solution with pH 7.4 to single cells did not alter membrane potential ($n=5$, $P=0.43$) (Figure 5b).

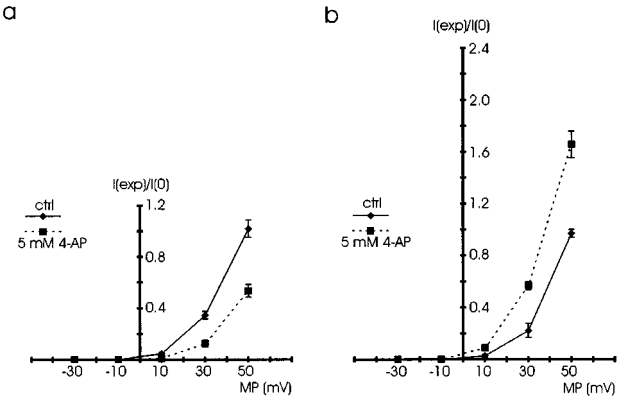


**Figure 4** Effect of 4-AP on $BK_{Ca}$ currents using different $[Ca]_i$-buffers. Summarized data of current-voltage (I-MP) relationship of outward current elicited with use of 500-ms voltage steps from a holding potential of $-40$ mV to test potentials in the range from $-50$ to $+50$ mV in the presence of 10 mM EGTA (a) or 10 mM BAPTA (b) in the pipette solution at 250 nM $[Ca]_i$. Data are expressed as ratio of current under specified experimental conditions (I(exp)), i.e. before (ctrl) and in the presence of 4-AP at selected membrane potentials, to current at $+50$ mV determined 30–60 s before starting the control (ctrl) measurement (I(0)). This presentation was selected in order to reduce inter-cell variability. Significant changes of the current-voltage relationship were observed with 5 mM 4-AP in (a) ($P<0.01$, $n=5$) and in (b) ($P<0.01$, $n=5$).

A calculation with a computer program showed that an increase of pH by 0.77 units from an initial pH of 7.40 should decrease the free calcium concentration in the EGTA-containing pipette solution from 250 to 16 nM, but should not alter the free calcium concentration in the BAPTA-containing pipette solution. Indeed, dissolving 4-AP in the EGTA-containing pipette solution to a final concentration of 5 mM decreased the free calcium concentration to $14.2\pm0.4$ nM ($n=5$), but did not change it in the BAPTA-containing pipette solution. Application of a 5 mM 4-AP-containing solution with pH 7.4 to single cells pre-treated with 10 $\mu$M 4-bromo-A23187 in order to increase the intracellular calcium concentration reduced fluorescence intensity of the calcium-sensitive dye FLUO-3 in EGTA-loaded cells to $48.9\pm7.3\%$ of the initial fluorescence indicating a fall in the intracellular calcium concentration, which is a significant change compared to the control application of bath solution ($n=5$, $P<0.01$) (Figure 5c), but did not change fluorescence intensity in BAPTA-loaded cells ($n=5$, $P=0.92$) (Figure 5d).

## Discussion

The investigation of the action of 4-AP on vascular smooth muscle potassium currents required the selection of experimental conditions enabling separation of each potassium current component. Under the conditions used in the present study, 1 $\mu$M glibenclamide, the blocker of ATP-sensitive potassium channels, and 10 $\mu$M barium, the blocker of

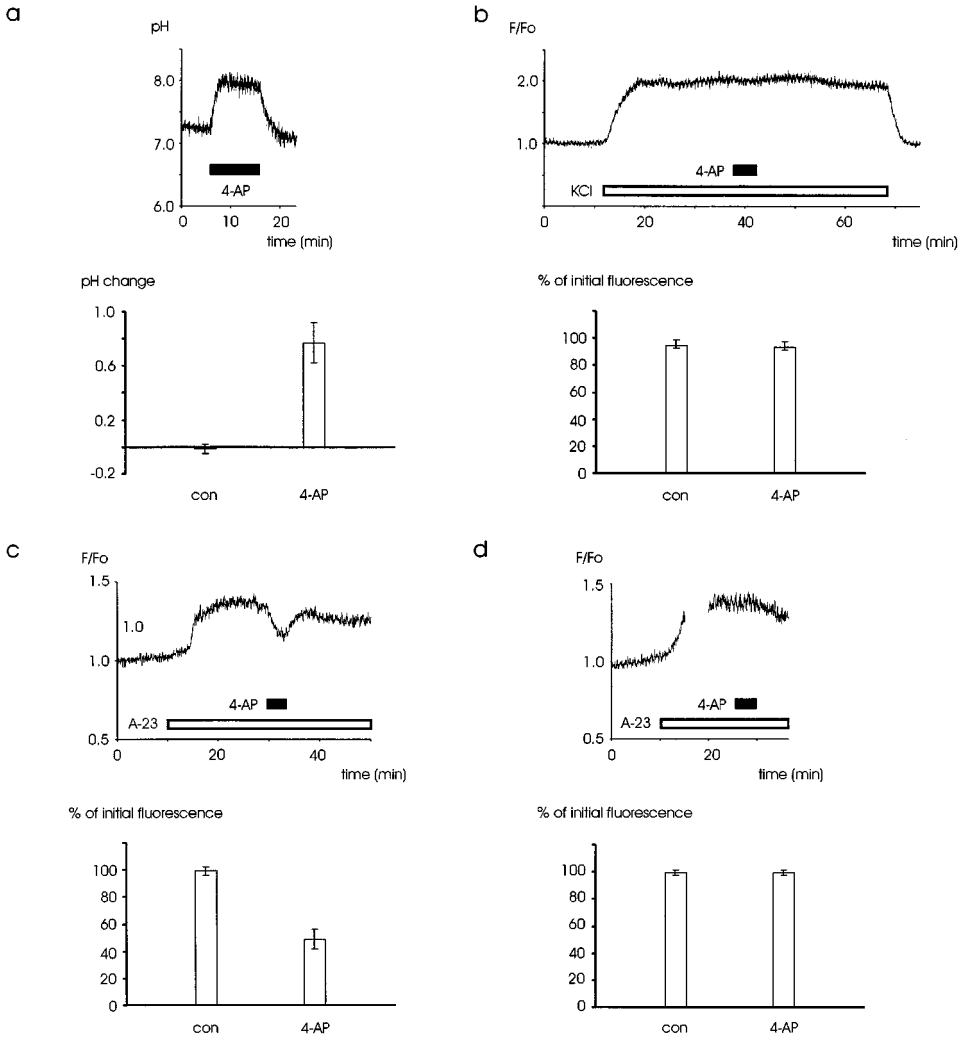


**Figure 5** Effect of 4-AP on intracellular pH and intracellular calcium in single cells. (a) Effect of 4-AP on intracellular pH; upper panel: example trace of the calibrated fluorescence change of BCECF, a pH-sensitive dye, during application of 5 mM 4-AP; lower panel: summarized data of the calibrated fluorescence change of BCECF during control (con) and addition of 5 mM 4-AP (4-AP), where a significant difference was observed ($P<0.001$, $n=5$). (b) Effect of 4-AP on membrane potential; upper panel: example trace of the fluorescence change of $DiBAC_4(3)$, a membrane potential-sensitive dye, during application of 5 mM 4-AP in the presence of a high potassium solution (135 mM KCl), which has been used to mimic a voltage clamp; lower panel: summarized data of the fluorescence change of $DiBAC_4(3)$ during control (con) and addition of 5 mM 4-AP (4-AP), where no significant difference was observed ($P=0.78$, $n=5$). (c, d) Effect of 4-AP on intracellular calcium in EGTA-loaded (c) and BAPTA-loaded (d) cells; upper panel: example trace of the fluorescence change of FLUO-3, a calcium-sensitive dye, during application of 5 mM 4-AP in the presence of the calcium-ionophore 4-bromo-A23187 (A-23), which has been used to elevate intracellular calcium; the discontinuity in the trace was caused by an interruption of the recording during a movement artifact-check; lower panel: summarized data of the fluorescence change of FLUO-3 in EGTA-loaded (c) and BAPTA-loaded (d) cells during control (con) and addition of 5 mM 4-AP (4-AP), where a significant difference was observed ($P<0.001$, $n=5$) in EGTA-loaded cells, but not in BAPTA-loaded cells ($P=0.91$, $n=5$).

inward rectifier potassium channels (Nelson & Quayle, 1995), had no effect on outward currents of the smooth muscle cells used (unpublished observations). Moreover, by stepping to positive potentials from a holding potential of $-40$ mV, a non-inactivating current with large fluctuations was observed. This current was blocked almost completely by iberiotoxin, the specific inhibitor of $BK_{Ca}$ channels (Galvez *et al*., 1990; Giangiacomo *et al*., 1992). In contrast, by stepping to positive potentials from a holding potential of $-90$ mV, having blocked $BK_{Ca}$ currents with iberiotoxin, an inactivating outward current was observed. Here, test potentials had been limited to $+30$ mV, because at more positive test potentials openings of single $BK_{Ca}$ channels appeared. This current was abolished to a large extent at a holding potential of $-20$ mV. Thus, under our experimental conditions the net outward current was flowing mostly through either $BK_{Ca}$ channels at a holding potential of $-40$ mV in the absence of iberiotoxin or through $K_v$ channels at a holding potential of $-90$ mV in the presence of iberiotoxin.

In the first part of this study, the effect of 4-AP on $BK_{Ca}$ currents was investigated. Under conditions where $BK_{Ca}$ currents predominate and EGTA was used to buffer $[Ca]_i$, 4-AP produced a concentration-dependent inhibition of outward currents. At 20 mM 4-AP the net outward current was suppressed by about 90%, i.e. to the same degree as with 300 nM iberiotoxin. The remaining iberiotoxin-insensitive part of the current consisted probably of a $K_v$ current. Since this current comprised not more than 10% of the net outward current, its inhibition by 4-AP couldn't explain the observed 90% inhibition of the current by 20 mM 4-AP. Thus, 4-AP obviously inhibits $BK_{Ca}$ currents. The 4-AP induced inhibition of the $BK_{Ca}$ current was characterized by

a half-block between 1 and 3 mM. The half-block of vascular smooth muscle $K_v$ currents by 4-AP was reported to be in the same concentration range (see review by Nelson & Quayle, (1995)). Indeed, in the present study under conditions where $K_v$ currents predominated, 4-AP caused a considerable suppression of both slow- and fast-inactivating $K_v$ currents. Moreover, the degree of inhibition induced by 5 mM 4-AP on $K_v$ and $BK_{Ca}$ currents was comparable. Thus, this study presents the novel observation that 4-AP affects vascular smooth muscle $BK_{Ca}$ currents in the same concentration range in which it acts on $K_v$ currents.

In the second part of this study, the mechanism of the effect of 4-AP on the $BK_{Ca}$ current was investigated. It has been suggested that the effect of 4-AP on spontaneous transient outward currents of rabbit portal vein smooth muscle cells is caused by an action of 4-AP on calcium stores (Beech & Bolton, 1989). Thus, cells were pre-treated with ryanodine, the blocker of calcium-induced calcium release, heparin, the blocker of $IP_3$-induced calcium release, and thapsigargin, the blocker of the sarcoplasmic reticulum calcium pump at concentrations, at which these substances acting in concert eliminate calcium release and uptake by these mechanisms (Taylor & Broad, 1998). This procedure, however, did not affect the 4-AP-induced inhibition of the $BK_{Ca}$ current, showing that 4-AP acts independently of sarcoplasmic reticulum calcium release and uptake mechanisms. In contrast, after reduction of $[Ca]_i$ below the threshold for calcium regulation of the $BK_{Ca}$ current, 4-AP failed to influence this current. This finding implies that 4-AP may not have a direct effect on $BK_{Ca}$ channels. Indeed, single channel experiments showed that neither extracellularly nor intracellularly applied 4-AP could modify the activity or the unitary current amplitude of $BK_{Ca}$ channels in excised patches. A lack of effect of 4-AP on excised $BK_{Ca}$ channels has also been reported previously for channels from rabbit portal vein (Beech & Bolton, 1989), human pulmonary artery (Peng *et al*., 1996), and rat renal arterioles (Zou *et al*., 1996). In addition, the lack of effect of 4-AP on $BK_{Ca}$ channels in outside-out patches and on $BK_{Ca}$ currents at low $[Ca]_i$ suggests, that 4-AP does not act from the extracellular side on the $BK_{Ca}$ current, as was already suggested for the effect of 4-AP on $K_v$ currents (Chandy & Gutman, 1995). Thus, the data discussed here suggest that 4-AP, acting intracellularly, may produce its effect by a calcium store-independent effect on $[Ca]_i$.

To investigate this hypothesis the calcium buffering properties of the intracellular solution were varied. Upon replacement of the intracellular calcium buffer substance EGTA with BAPTA the inhibiting effect of 4-AP on the $BK_{Ca}$ current reversed into a stimulating one. Two important facts concerning the properties of EGTA, BAPTA and 4-AP guide to an explanation of the mechanism of action of 4-AP on $BK_{Ca}$ currents. Firstly, the calcium binding to EGTA is pH-dependent, whereas that for BAPTA is not (Schubert, 1996). Secondly, 4-AP has one amino-group which is protonated when 4-AP is dissolved in solution, i.e. the pH of a freshly prepared 4-AP solution is highly basic. Consequently, the pH of the 4-AP solutions used in the present study was carefully adjusted to 7.4. Nevertheless, when 4-AP molecules are entering the cell to reach their intracellular site of action, they have to cross the membrane in their uncharged, i.e. non-protonated form (Choquet & Korn, 1992) and will re-bind protons inside the cell. The latter reaction gives 4-AP the potential to make the cell interior more basic and indeed this has been observed in Jurkat T-lymphocytes (Guse *et al*., 1994) and in vascular smooth muscle cells in the present study. In this respect 4-AP behaves similar to ammoniumchloride (Aalkjaer & Poston, 1996). Considering these facts, the experimental findings may be explained as follows. Having the pH-dependent calcium buffer EGTA in the intracellular pipette solution and getting a more basic intracellular environment following application of 4-AP, the equilibrium between protons, free EGTA and the different protonated EGTA species is shifted to the less protonated forms of EGTA. The species of EGTA having lost protons now bind calcium producing a considerable decrease in $[Ca]_i$. Indeed, such a fall in $[Ca]_i$ was observed in the present study and seems to account for the observed reduction of the $BK_{Ca}$ current when EGTA is used as the intracellular calcium buffer and $[Ca]_i$ is initially high enough to be able to regulate $BK_{Ca}$ channel activity. This mechanism may also explain the decrease of spontaneous transient outward currents seen in rabbit portal vein smooth muscle cells (Beech & Bolton, 1989). In contrast, having the pH-independent BAPTA in the intracellular solution and getting a more basic intracellular environment following application of 4-AP, intracellular calcium is not expected to change. Indeed, the absence of such a change has been confirmed by measurements of $[Ca]_i$ in the present study. However, a change of pH to basic values is known to activate vascular smooth muscle $BK_{Ca}$ channels, probably by a competitive interaction of protons and calcium ions at the channel, i.e. pH will be effective only under conditions when $[Ca]_i$ is high enough to be able to regulate $BK_{Ca}$ channel activity (Hayabuchi *et al*., 1998; Schubert & Gagov, 1997). Thus, the 4-AP-induced pH enhancement seems to account for the observed increase of the $BK_{Ca}$ current when BAPTA is used. In summary, the results of the present study suggest that in isolated vascular smooth muscle cells 4-AP affects the $BK_{Ca}$ current by an alteration of intracellular pH under conditions when $[Ca]_i$ is high enough to regulate $BK_{Ca}$ currents.

The results of the present study have some practical applications for experimental work using 4-AP. In patch-clamp experiments on isolated cells, the selectivity of the effect of 4-AP is limited by its ability to alter intracellular pH. Thus, the presented data show that a non-selective action of an inhibitor may not only be caused by its direct interaction with several types of ion channels, but also by changes of the intracellular environment, like pH, which are able to alter the activity of ion channels indirectly. This has to be taken into account, for example, for $K_v$ currents, which recently have been shown to be regulated by intracellular pH (Berger *et al*., 1998).

In conclusion, this study presents the novel observation that 4-AP affects vascular smooth muscle cell $BK_{Ca}$ currents when $[Ca]_i$ is high enough to regulate them in the same concentration range as $K_v$ currents from the same cells. The data presented suggest that under these conditions the effect of 4-AP, acting intracellularly, is mediated by an alteration of intracellular pH producing a calcium store-independent reduction of $[Ca]_i$ due to calcium uptake to EGTA in EGTA-loaded cells and a displacement of calcium from the channel in BAPTA-loaded cells.

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