British Journal of Pharmacology (2004) 143, 872–880
www.nature.com/bjp

K_{ATP}-channel-induced vasodilation is modulated by the Na,K-pump activity in rabbit coronary small arteries

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1 The purpose of the study was to evaluate the importance of the Na,K-pump in relaxations induced by K_{ATP}-channel openers in rabbit coronary small arteries.

2 Arterial segments were mounted in myographs for recording of isometric tension. Whole-cell patch clamp was used to assess K_{ATP}-channel currents in isolated smooth muscle cells from the arteries.

3 In arteries preconstricted with the thromboxane A_2 analogue U46619 pinacidil and cromakalim induced concentration-dependent relaxations. In arteries preconstricted with potassium (124 mM) only high concentrations of pinacidil had a small relaxant effect.

4 In arteries preconstricted with U46619 pinacidil-induced relaxations were unaffected by pretreatment with N_ω-nitro-L-arginine (L-NNA) and only slightly reduced after mechanical removal of the endothelium.

5 Pinacidil induced relaxations were not significantly affected by 1 μM glibenclamide. However, the relaxations were partly inhibited in potassium-free media and by 1 μM ouabain.

6 In contrast, the concentration-dependent relaxation to cromakalim was partly blocked by 1 μM glibenclamide and partly by 1 μM ouabain and when both drugs were present the inhibition increased.

7 Ouabain (1 μM) and glibenclamide (1 μM) each partly inhibited an ATP-sensitive current induced by pinacidil and cromakalim. In the presence of both inhibitors a greater inhibition was seen. When the solution in the patch pipette was sodium-free the current was reduced and ouabain had no effect.

8 The study suggests that the relaxation to cromakalim and most likely pinacidil is mediated through opening of K_{ATP} channels. Inhibition of the Na,K-pump, however, may change the local environment for the K_{ATP} channels (i.e. increases the ATP/ADPratio and/or decreases the transmembrane potassium gradient), which partly prevents the activation of the K_{ATP}-channel current.
British Journal of Pharmacology (2004) **143,** 872–880. doi:10.1038/sj.bjp.0706016

Keywords: Pinacidil; ouabain; cromakalim; glibenclamide; rabbit coronary artery; K_{ATP} channel; Na,K-pump

Abbreviations: cAMP, cyclic AMP; K_{ATP}, ATP-sensitive K^+ channel; L-NAME, N^G-nitro-L-arginine methyl ester; L-NNA, N_ω-nitro-L-arginine; U46619, (15S)-hydroxy-11_a,9_a-(epoxymethano) prosta-5Z,13 E-dienoic acid

Introduction

The ATP-sensitive K^+ channel (K_{ATP}) is found in vascular smooth muscle cells (Standen *et al*., 1989). Opening of these channels is stimulated by a fall in intracellular ATP and causes potassium efflux leading to hyperpolarization of the cell membrane and vasodilatation (Quast, 1993; Nielsen-Kudsk *et al*., 1996). Thereby, these channels are an important link between metabolism and bloodflow through regulation of vascular tone, not least in the coronary circulation (Daut *et al*., 1990).

The drugs pinacidil and cromakalim have been shown to open K_{ATP} channels, thereby causing hyperpolarization and vasodilatation. However, pinacidil also has vasorelaxant effects unrelated to K_{ATP} channel opening (Wilson *et al*., 1988; Kuromaru & Sakai, 1996), which are not well defined. In some preparations the effect of pinacidil seems to be inhibited by nitric oxide (NO) from vascular endothelium. This might be due to competition between NO and pinacidil for opening of K_{ATP} (McCulloch & Randall, 1996).

Also, the Na,K-pump may play a role for the relaxation to the potassium channel openers. In pig retinal arteries the relaxation to pinacidil is completely blocked by the Na,K-pump inhibitor ouabain (Jeppesen *et al*., 2002). Furthermore, in rabbit femoral and ear arteries ouabain impairs cromakalim-induced relaxation (Garcia-Villaon *et al*., 1996) and in human mesenteric arteries, ouabain inhibits relaxation induced by low concentrations (<0.1 μM) of cromakalim (Hong *et al*., 1993).

The aim of this study was to examine the mode of action of pinacidil and cromakalim in isolated rabbit coronary resistance arteries with the specific aim to evaluate the importance of the Na,K-pump.

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Advance online publication: 25 October 2004

Methods

Animals

All investigations were performed on arteries obtained from 32 weeks old, nonpregnant female rabbits (albino Danish Land race) from The State Serum Institute, Copenhagen. The study was approved by the National Committee on Animal Research Ethics.

Isolation of coronary arteries The rabbits were killed by a blow on the neck. The heart was removed immediately and placed in ice-cold physiological salt solution (for composition, see below) that had been gassed with a mixture of 95% oxygen and 5% carbon dioxide, and the sample was transported to the laboratory.

The left anterior descending artery was isolated under a stereo microscope and arterial segments were taken from third-order branches. During removal of the heart and the subsequent dissection procedure, the tissues were continuously rinsed with ice-cold oxygenated physiological salt solution.

Myographic procedure

The arteries were prepared as ring preparations and threaded onto two parallel stainless-steel wires (diameter 40 μm) and mounted in a wire-myograph (model 500A, Danish Myotechnology, Aarhus, Denmark), allowing recording of isometric wall tension while the internal circumference was controlled (Mulvany & Halpern, 1977). The vessels were mounted in ice-cold physiological salt solution whereafter the temperature was adjusted to 37°C. After a 30 min equilibration period the vessel preparations were gradually stretched to characterize the relation between the vessel circumference and the passive wall tension. The circumference that the vessel would have *in vivo* when relaxed and under a transmural pressure of 100 mmHg (L_{100}) was found using the Laplace law (pressure = tension radius^{-1}). The vessel circumference was adjusted to the normalized internal circumference $L_1 = 0.9 \times L_{100}$ since active force production is found to be near maximal at this circumference.

After an equilibration period of 10 min, contractions were induced by potassium (124 mmol l^{-1}) until reproducible (<10% variation between two successive contractions).

Experimental protocols

Evaluation of the role of the endothelium in pinacidil-induced relaxation For relaxation experiments, all arteries were precontracted with 100 nM of the thromboxane A2 analogue U46619. One artery was first relaxed with 10 μM acetylcholine. Next, it was relaxed with pinacidil (1 nM–10 μM). The artery was now pretreated with 100 μM L-NNA for 20 min and the protocol was repeated in the presence of L-NNA supplemented with the addition of 100 μM NO on top of 10 μM pinacidil. Finally, the artery was pretreated with both 100 μM L-NNA and 1 μM ouabain for 30 min before precontraction with 100 nM U46619 and relaxation with pinacidil (1 nM–10 μM) and 100 μM NO in the continued presence of L-NNA and ouabain. A second artery (time control) was pretreated and precontracted in the same way as above, but no relaxing drugs were added (eight arteries were investigated in each experimental group). In a third vessel, pretreatment with L-NNA was replaced by removal of the endothelium. This was achieved by gently pulling a horse hair through the lumen of the relaxed vessel. Pretreatment with ouabain was left out in this vessel (six arteries were investigated).

Evaluation of the role of K_{ATP} channels for the effects of pinacidil One artery was pretreated with 1 μM ouabain for 30 min and ouabain was kept in the organbath throughout the experiment. A second artery was investigated without ouabain pretreatment, but apart from this, the protocols for the two arteries were identical (eight and seven arteries, respectively were investigated in the two goups). The relaxant effect of pinacidil (1 nM–10 μM) was investigated after precontraction with 100 nM U46619 and K^+ (124 mM), respectively. Glibenclamide was added for 20 min and the concentration–response (c–r) relations for pinacidil were repeated in the presence of glibenclamide. In a third artery the relaxant effect of cromakalim (1 nM–1 μM) was investigated after precontraction with 100 nM U46619 and before and after pretreatment with 1 μM glibenclamide for 20 min. Thereafter, the artery was treated with 1 μM ouabain for 30 min and the c–r relations for cromakalim (1 nM–1 μM) were re-examined before and after pretreatment with 1 μM glibenclamide for 20 min (six arteries were investigated). A fourth artery was investigated as the third artery except that ouabain-pretreatment was left out (six arteries were investigated). The washout of glibenclamide was tested by repeating the c–r relations for cromakalim after washout of glibenclamide (three arteries were investigated).

Evaluation of pinacidil-induced relaxation in potassium-free solution One artery was preconstricted with 100 nM U46619 and pinacidil (30 nM–10 μM) was added cumulatively. This procedure was repeated in potassium-free medium (six arteries were investigated).

Patch clamp recordings

For assessment of ATP-sensitive potassium current (K_{ATP}), smooth muscle cells were isolated from rabbits coronary arteries following previously described methods (Schubert *et al*., 1999; Peng *et al*., 2001). Arteries were placed in a papain enzyme solution (for composition see below) and stored overnight at 4°C. On the next day, the vessels were incubated in the same solution for 5–10 min at 37°C. The vessels were removed from the enzyme solution and stored in standard bath solution at 4°C. Single cells were released by trituration with a polyethylene pipette into the experimental bath solution.

All patch clamp recordings were made at room temperature (22–24°C). Whole-cell recordings were made with patch pipettes having resistances in the range of 2–5 MΩ.

Recordings were made with an Axopatch 200B amplifier (Axon Instruments, U.S.A.) in whole-cell configuration. Data were sampled at a rate of 2 kHz and filtered at 1 kHz. Data acquisition and analysis were carried out with the software package Clampex 7 for Windows (Axon Instruments) and analysis was carried out with Origin 5.0 (Microcal). Only cells with essentially no leak current (seal resistance higher than 2 GΩ) and a low access resistance (5–10 MΩ) were used, and the stability of these parameters was tested regularly during the course of the experiment. Series resistance and capacitive current were routinely compensated.

Current densities were recorded at a steady holding potential of −60 mV, and current–voltage characteristics were obtained from a ramp protocol. The voltage ramps were performed from −60 to +10 mV with duration of 80 ms.

Analysis of data

All results are expressed as mean ± s.e.m. Normality of data was tested using the Kolmogorow Smirnof's test. If normality was found, significances of differences were assessed by Student's *t*-test (paired or unpaired) or one-way analysis of variance on ranks (ANOVA) (multiple comparisons) followed by pairwise comparisons using Bonferroni's method. If normality was not found Mann–Whitney rank sum test or Kruskal–Wallis one-way analysis of variance on ranks were used followed by multiple comparison using Dunn's method. All values of $P<0.05$ were considered significant.

Relaxant responses are expressed as a percent of precontraction after correction for time-dependent effects on the precontraction. Maximal effect of a drug is expressed as E_{max}. The negative logarithm (pD_2) of the concentration (M) of the agonist producing half-maximum response (EC_{50}) was estimated if the complete c–r curve was established. Stability of the precontraction was controlled in time-matched controls.

Solutions

For mechanical experiments Physiological salt solution (mM): NaCl, 119; KCl, 4.6; $NaHCO_3$, 15; $CaCl_2$, 1.5; $MgCl_2$, 1.2; NaH_2PO_4 1.2; glucose, 11. High potassium solution (mM): KCl, 124; $NaHCO_3$, 15; $CaCl_2$, 1.5; $MgCl_2$ 1.2; NaH_2PO_4, 1.2; glucose, 11. Potassium-free solution (mM): NaCl, 123.6; $NaHCO_3$, 15; $CaCl_2$, 1.5; $MgCl_2$, 1.2; NaH_2PO_4 1.2; glucose, 11.

For patch clamp experiments Papain enzyme solution (mM): NaCl, 110; KCl, 5; $MgCl_2$, 2; KH_2PO_4, 0.5; NaH_2PO_4, 0.5; $NaHCO_3$, 10; $CaCl_2$, 0.16; EDTA, 0.49; Na-HEPES, 10; glucose, 10; 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. Standard bath solution (mM): NaCl, 80; KCl, 60; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 0.1, at pH 7.4. Sodium-containing pipette solution (mM): NaCl, 15; KCl, 92; KOH, 33; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 1; EGTA, 10; MgATP, 0.1 (unless otherwise stated), at pH 7.2. Sodium-free pipette solution (mM): KCl, 107; KOH, 33; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 1; EGTA, 10; MgATP, 0.1, at pH 7.2.

Drugs

Stock solutions of the drugs were prepared as follows: (15S)-hydroxy-11_a,9_a-(epoxymethano) prosta-5Z,13 E-dienoic acid (*U46619,* Upjohn, MI, U.S.A.) was prepared in ethanol and diluted with 0.9% sodium chloride. *Ouabain* (Sigma Chemical Co., St Louis, U.S.A.) was prepared in 0.9% sodium chloride containing 1.0 mM ascorbic acid. *Acetylcholine* (Sigma Chemical Co., St Louis, U.S.A.) and *N_ω-nitro-L-arginine (L-NNA)* (Serva, Heidelberg, Germany) were prepared in water. *Pinacidil* (Leo Pharma, Ballerup, Denmark), *cromakalim* (Beecham Pharmaceuticals, U.K.), g*libenclamide* (Sigma Chemical Co., St Louis, U.S.A.) were prepared in ethanol.

Nitric oxide NO solutions were made before every experiment as previously described (Palmer *et al*., 1987; Glavind-Kristensen *et al*., 1998).

Concentrations of drugs and NO are given as final bath concentrations.

Results

Evaluation of the role of the endothelium in pinacidil-induced relaxation

Effect of L-NNA and ouabain on pinacidil-induced relaxation Ten μM acetylcholine induced 74.1 ± 9.0% relaxation of U46619-induced tone. After pretreatment with 100 μM L-NNA, the effect of acetylcholine was only 50.1 ± 11.9% ($P=0.04$, paired *t*-test).

Cumulative addition of pinacidil (1 nM–1 μM) induced a concentration-dependent relaxation of U46619-induced tone (Figures 1 and 2). The maximal relaxation and the pD_2 value of the relaxation were 95.6 ± 1.1% and 6.79 ± 0.24, respectively.

Pretreatment with L-NNA was without effect on the pinacidil-induced relaxation. Thus, the maximal relaxation and the pD_2 after L-NNA were 96.2 ± 1.2% and 6.37 ± 0.10, respectively.

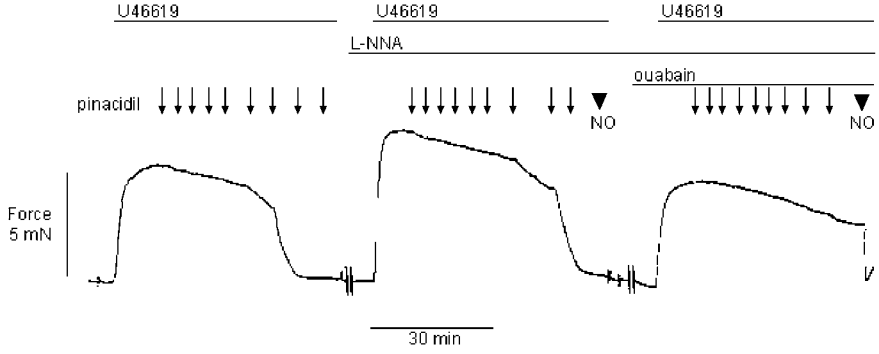

Figure 1 Trace from an experiment with an isolated rabbit coronary small artery. The trace shows the concentration-dependent effect of pinacidil and the effect of 100 μM L-NNA, 1 μM ouabain and 100 μM NO on the tension development to 100 nM of the tromboxane analogue U46619. The arrows indicate increasing concentrations of pinacidil from 1 nM to 10 μM (in half log units). The gaps in the trace were about 15 min.

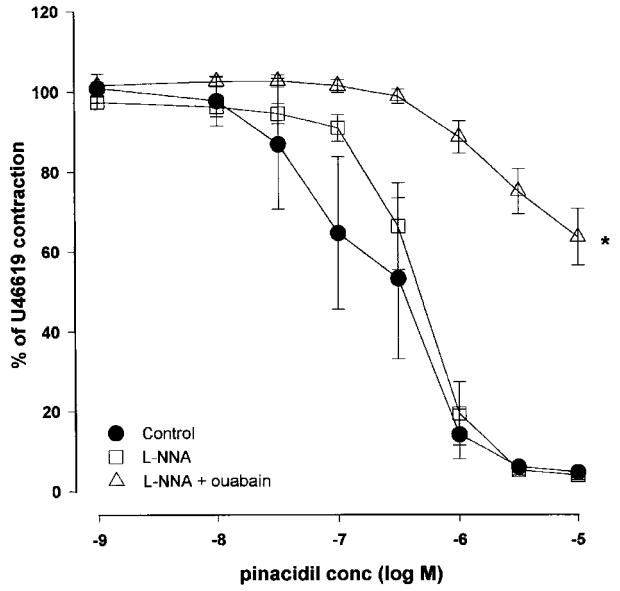

Figure 2 Concentration–response curves for pinacidil in isolated rabbit coronary arteries after precontraction with 100 nM U46619. Values are means ± s.e.m. $n = 8$. E_{max}: $P < 0.001$ (Kruskal–Wallis ANOVA on ranks). $^*P < 0.05$ L-NNA + Ouabain vs control and L-NNA (all pairwise comparison, Dunn's method).

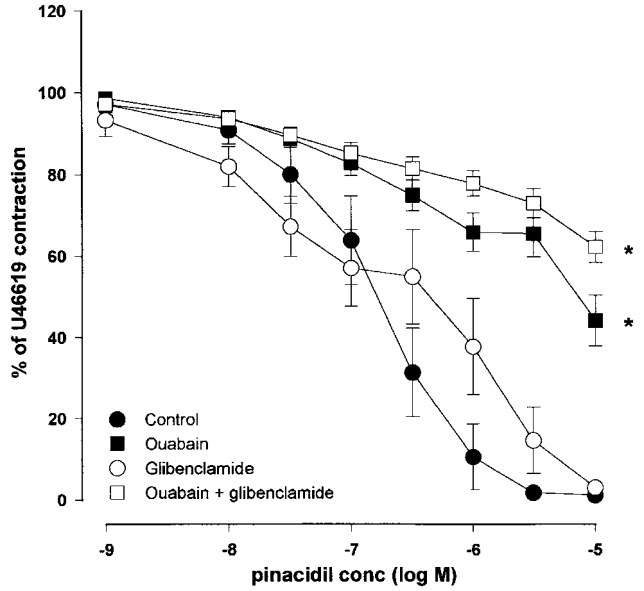

Figure 3 Pinacidil-induced relaxation of U46619 precontracted rabbit coronary arteries with and without pretreatment with glibenclamide, ouabain, and glibenclamide + ouabain. Control: $E_{max} = 98.8 \pm 0.4\%$ ($n = 7$); 1 μM glibenclamide: $E_{max} = 97.0 \pm 0.8\%$ ($n = 7$); 1 μM ouabain: $E_{max} = 55.8 \pm 6.3\%$ ($n = 8$); 1 μM ouabain + 1 μM glibenclamide: $E_{max} = 37.8 \pm 3.8\%$ ($n = 8$). Mean ± s.e.m. E_{max}: $P < 0.001$ (Kruskal–Wallis ANOVA on ranks). $^*P < 0.05$ vs control (multiple comparison, Dunn's method).

Pretreatment with both L-NNA and ouabain significantly inhibited the effect of pinacidil (Figures 1 and 2). Thus, the relaxation to 10 μM pinacidil (maximal concentration tested) was only 42.2 ± 6.0% ($P < 0.05$, Kruskall–Wallis ANOVA).

In the arteries pretreated with L-NNA or ouabain + L-NNA, addition of 100 μM NO on top of the highest pinacidil concentration induced 98.0 ± 1.0 and 91.5 ± 1.6% relaxation, respectively ($P < 0.001$, paired t-test), indicating that the arteries can relax in the presence of ouabain (Figure 1).

Effect of removal of the endothelium on pinacidil-induced relaxation The effectiveness of endothelial removal was assessed by comparing acetylcholine-induced relaxation before and after. Removal of the endothelium changed the acetylcholine-induced relaxation from 70.6 ± 10.6% relaxation of U46619-induced tone to a contraction with a maximum of 130.3 ± 18.1% of U46619 induced tone ($P < 0.005$, paired t-test).

The effect of pinacidil was inhibited slightly but significantly by removal of the endothelium. Before and after the removal of the endothelium E_{max} were 96.2 ± 1.0 and 75.5 ± 4.1%, respectively ($P = 0.01$, Mann–Whitney rank sum test). There was no effect on pD_2 (6.27 ± 0.19 (before) and 6.5 ± 0.17 (after) ($P = 0.47$ Student's t-test)).

Effects of ouabain, glibenclamide and potassium-free conditions on pinacidil-induced relaxations

Pretreatment with 1 μM glibenclamide for 20 min had no significant effect in eight arteries on pinacidil-induced relaxation of U46619-induced tone. In contrast to this, pretreatment with 1 μM ouabain for 30 min significantly impaired the pinacidil-induced relaxations (Figure 3). The combination of glibenclamide and ouabain caused a slight further inhibition of the response to 10 μM pinacidil (Figure 3).

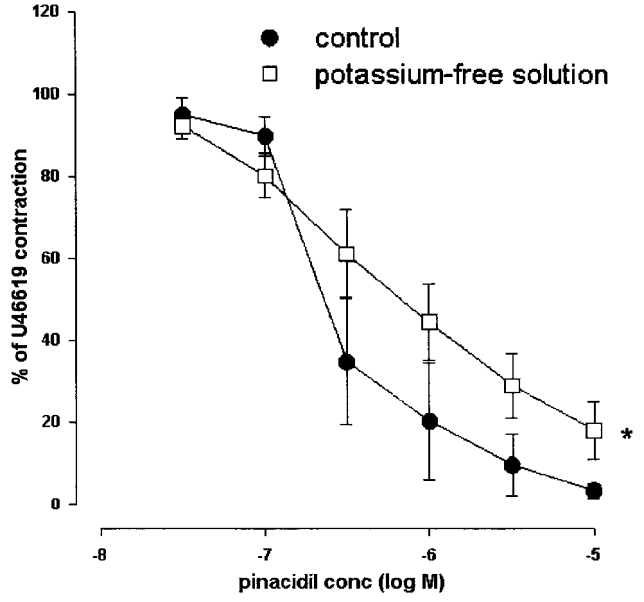

Figure 4 Relaxation induced by pinacidil after precontraction with U46619 in rabbit coronary arteries with and without potassium in the solution. Mean ± s.e.m., $n = 6$. E_{max}: $^*P < 0.05$ (paired t-test).

Cumulative addition of pinacidil (1 nM–10 μM) induced a minor relaxation of K^+ (124 mM)-induced tone with a maximum of 22.5 ± 2.1%. The relaxation was only seen with pinacidil concentrations higher than 1 μM.

Figure 4 shows that the concentration-dependent relaxation to pinacidil of U46619-induced tone was reduced in a

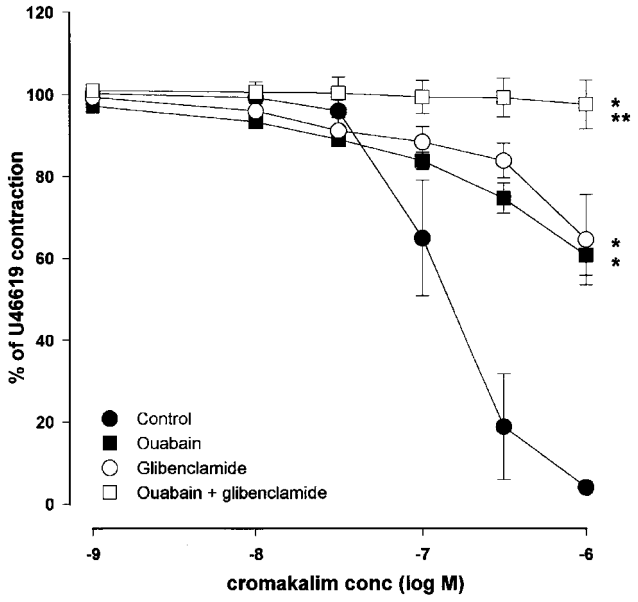

Figure 5 Cromakalim-induced relaxation of U46619 precontracted rabbit coronary arteries with and without pretreatment with glibenclamide, ouabain, and glibenclamide + ouabain. Control: $E_{max} = 95.9 \pm 1.3\%$ ($n = 6$); 1 μM glibenclamide: $E_{max} = 35.4 \pm 11.0\%$ ($n = 6$); 1 μM ouabain: $E_{max} = 39.4 \pm 4.9\%$ ($n = 3$); 1 μM ouabain + 1 μM glibenclamide: $E_{max} = 4.2 \pm 4.5\%$ ($n = 3$). Mean ± s.e.m. E_{max}: $P < 0.001$ (ANOVA). *$P < 0.001$ vs control. **$P < 0.05$ vs ouabain-pretreatment (multiple comparison, Bonferroni t-test).

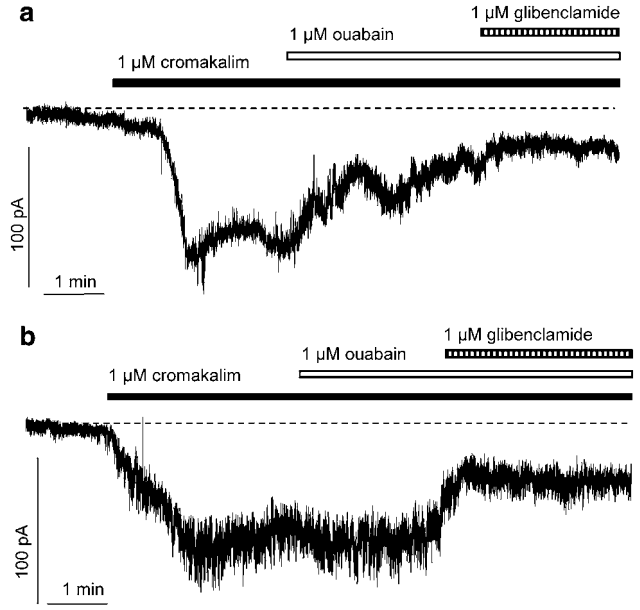

Figure 6 Trace of current in single smooth muscle cell from a rabbit coronary small artery. The current was recorded at a steady holding potential of −60 mV. Bath [K^+] was 60 mM. The pipette contained 0.1 mM ATP. Dashed line indicates the pre-drug current level. In (a) the pipette [K^+] was 125 mM ($E_K = -19$ mV) and [Na^+] 15 mM. In (b) pipette [K^+] was 140 mM ($E_K = -22$ mV) and [Na^+] 0 mM. Under these conditions, activation of K_{ATP} channels will cause an inward current at −60 mV holding potential.

potassium-free solution. Thus, the E_{max} was 96.8 ± 1.8 and $82.2 \pm 7.1\%$ in normal and potassium-free solution, respectively ($P = 0.05$, paired t-test).

Effects of ouabain and glibenclamide on cromakalim-induced relaxations

Cromakalim (1 nM–1 μM) induced concentration-dependent relaxation of U46619-induced tone with a maximum of $95.9 \pm 1.3\%$ (Figure 5). Pretreatment with 1 μM glibenclamide for 20 min or 1 μM ouabain for 30 min significantly inhibited the effect of cromakalim (Figure 5). When both drugs were present the inhibition was enhanced and almost complete. In three experiments the reversibility of glibenclamide was tested. E_{max} of cromakalim were 95.5 ± 2.8 and $95.0 \pm 2.3\%$ before addition and after washout of glibenclamide, respectively.

Effect of pinacidil, cromakalim, glibenclamide and ouabain on ion current in isolated smooth muscle cells

Pinacidil and cromakalim each induced an inward current at membrane potentials negative to the potassium equilibrium potential (E_K), which was −19 or −22 mV in our experiments (Figures 6 and 7). Figure 6 represents original traces from the recording in steady conditions at −60 mV. Application of 1 μM cromakalim evoked a significant inward current. In the presence of 0.1 mM ATP in the pipette, the density of the current evoked with 10 μM pinacidil was -1.63 ± 0.20 A F^{-1} (9) at −60 mV. When pipette ATP was increased to 1 mM the density of the pinacidil-induced current at −60 mV was significantly lower ($-0.38 \pm$ A F^{-1} (5)). The pinacidil-induced current was inhibited by glibenclamide and the inhibition was enhanced with 10 μM glibenclamide compared with 1 μM (Figures 7a and 8a).

A concentration of 1 μM ouabain caused a partial inhibition of the current induced with both pinacidil and cromakalim (Figures 6a, 7b and 9). In the presence of ouabain, 1 μM glibenclamide caused a further inhibition of the current (Figures 6a, 7b and 9). A concentration of 10 μM ouabain and 10 μM glibenclamide caused a complete inhibition of the pinacidil induced current (Figure 8b).

To assess the effect of ouabain and glibenclamide under conditions where the Na,K-pump was inactivated experiments were made with sodium-free solution in the patch pipette. Under these conditions, the current induced by pinacidil and cromakalim was reduced (Figure 10). In the absence of sodium, ouabain did not inhibit the current induced with pinacidil (Figure 9a) or cromakalim (Figure 6b and 9b). However, sodium-free conditions did not prevent the effect of 1 μM glibenclamide on the currents induced by pinacidil or cromakalim (Figure 9). Sodium-free conditions significantly reduced the effect of ouabain plus glibenclamide on the pinacidil-induced current (Figure 9a) and also reduced ($P = 0.051$) the effect of ouabain plus glibenclamide on the cromakalium-induced current (Figure 9b).

Discussion

In the present study, pinacidil and cromakalim were found to be potent vasodilators in small coronary arteries from rabbits, and they induced nearly complete relaxation of U46619-induced contraction. The surprising observation was that these vasodilator responses were substantially reduced after

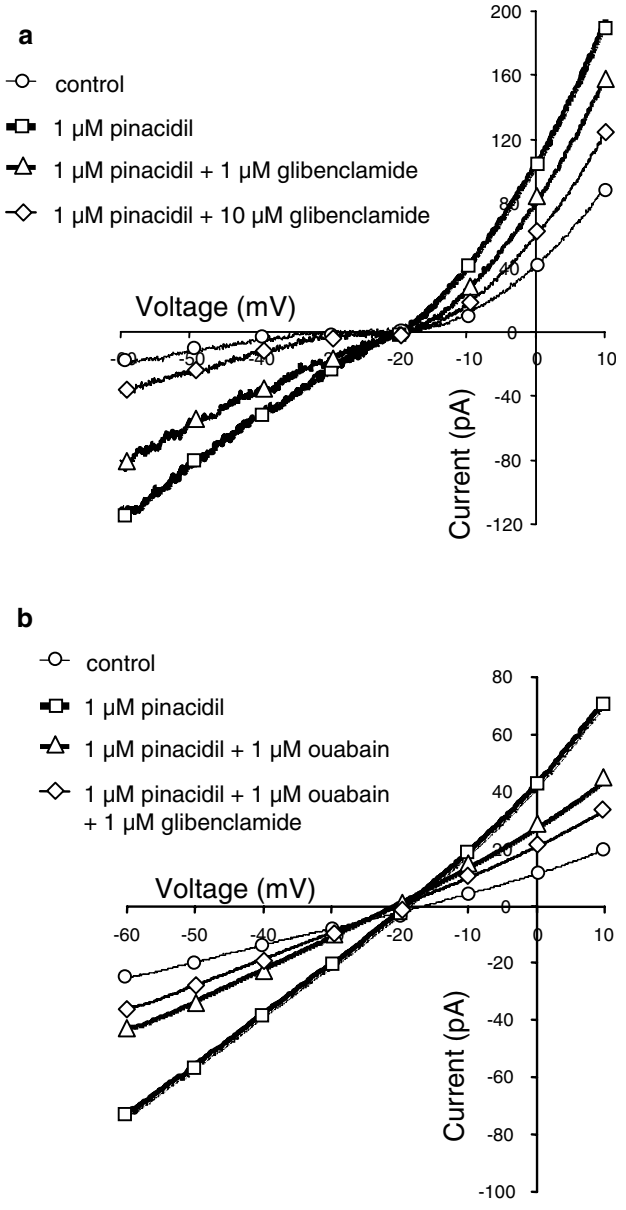

Figure 7 Current–voltage relationships of pinacidil-activated, glibenclamide-sensitive currents in single smooth muscle cells from rabbit coronary small arteries. The pipette contained 0.1 mM ATP. Pipette $[K^+]$ was 125 mM, $[Na^+]$ 15 mM and bath $[K^+]$ was 60 mM ($E_K = -19$ mV). Currents were recorded in response to 80 ms voltage ramp from −60 to 10 mV. Symbols are placed to ease definition of individual lines. In (a) the concentration-dependent effect of glibenclamide is shown. In (b) the effect of 1 μM ouabain is shown.

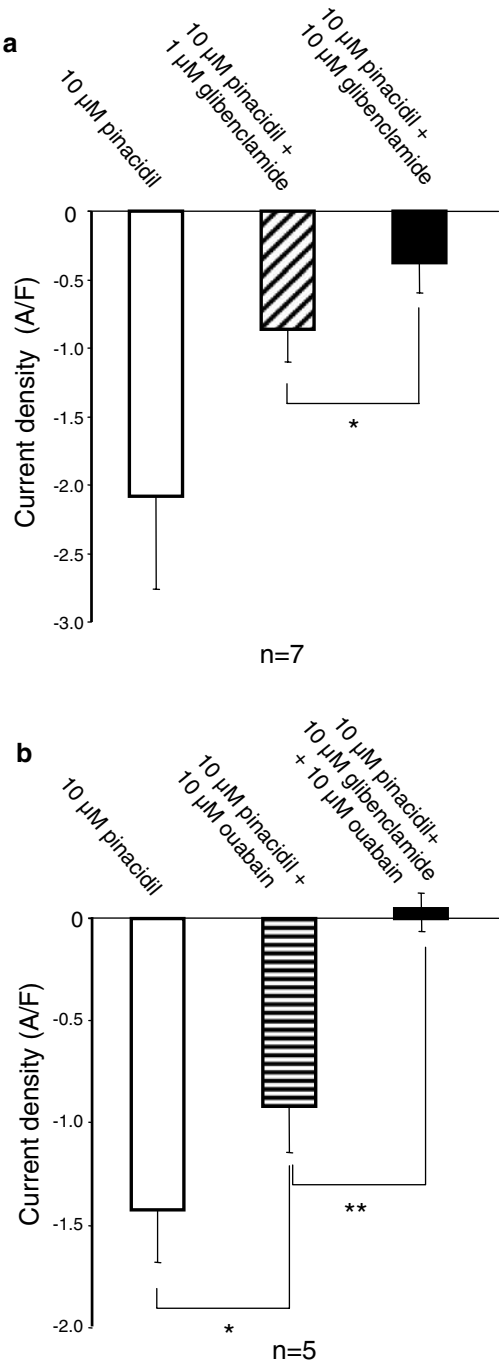

Figure 8 Mean current densities measured at steady state at −60 mV in experiments similar to the one shown in Figure 6. (a) Glibenclamide concentration-dependent inhibition of pinacidil activated current in isolated smooth muscle cells from rabbit coronary small arteries. The pipette contained 0.1 mM ATP. Pipette $[K^+]$ was 125 mM, $[Na^+]$ 15 mM and bath $[K^+]$ was 60 mM ($E_K = -19$ mV). (b) Effect of ouabain + glibenclamide on pinacidil activated current in isolated smooth muscle cells from rabbit coronary small arteries. The pipette contained 0.1 mM ATP. Pipette $[K^+]$ was 125 mM, $[Na^+]$ 15 mM and bath $[K^+]$ was 60 mM ($E_K = -19$ mV). Error bars indicate s.e.m., $*P<0.05$; $**P<0.01$.

inhibition of the Na,K-pump with ouabain and also reduced in potassium-free conditions, which also blocks the Na,K-pump. It was somewhat surprising that 1 μM glibenclamide had little effect on the relaxation to pinacidil although glibenclamide at this concentration caused a substantial inhibition of the relaxation to cromakalim.

Hypoxia induces dilatation of coronary arteries (Daut *et al.*, 1990; Jiang & Collins, 1994), but the mechanism is not completely known (Taggart & Wray, 1998). It has been suggested that the relaxation might be induced through endothelial and/or K_{ATP} channel-related mechanisms.

K_{ATP} channels have been demonstrated in vascular smooth muscle cells (Quale & Standen, 1994) and pinacidil seems to induce vasodilatation through opening of these channels in many different vascular preparations, and we have also

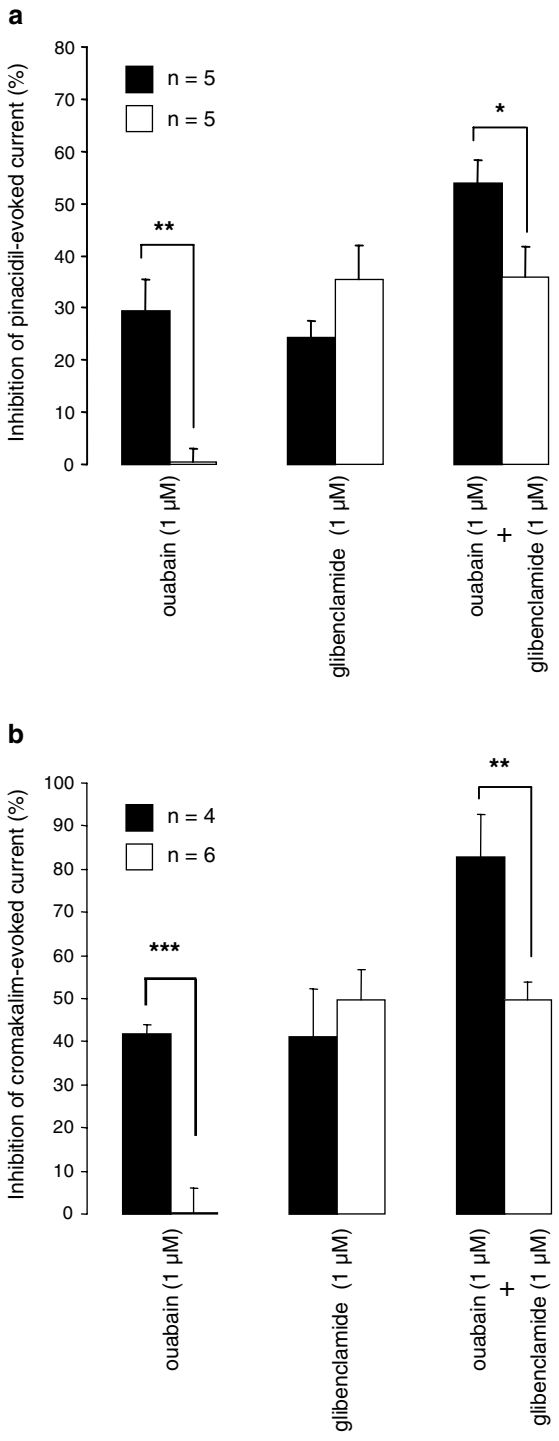

Figure 9 Mean current densities measured at steady state at −60 mV in experiments similar to the one shown in Figure 6. Inhibition of pinacidil (a) and cromakalim (b) induced current with ouabain, glibenclamide and ouabain + glibenclamide in isolated smooth muscle cells from rabbit coronary small arteries. The pipette contained 0.1 mM ATP. Closed bars: 15 mM Na^+ in the pipette (the pipette $[K^+]$ 125 mM ($E_K = -19$ mV)); open bars: 0 mM Na^+ in the pipette (the pipette $[K^+]$ 140 mM ($E_K = -22$ mV)). Error bars indicate s.e.m., $*P<0.05$; $**P<0.01$; $***P<0.005$.

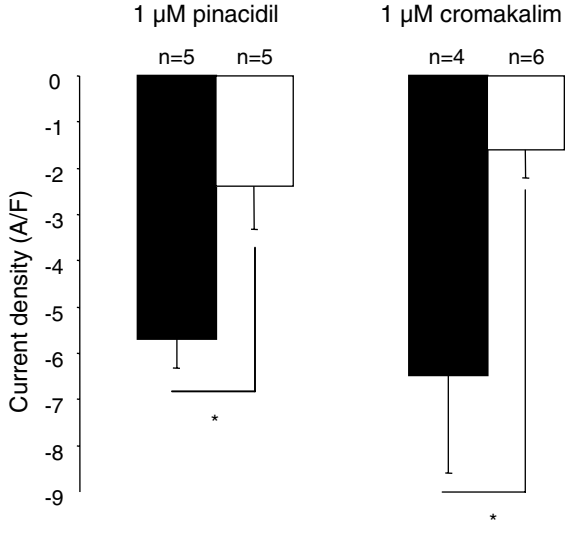

Figure 10 Mean current densities measured at steady state at −60 mV in experiments similar to the one shown in Figure 6. Effect of omission of sodium from the pipette solution on the pinacidil and cromakalim activated current. The pipette contained 0.1 mM ATP. Closed bars 15 mM Na^+ (the pipette $[K^+]$ 125 mM ($E_K = -19$ mV)), open bars 0 mM Na^+ (the pipette $[K^+]$ 140 mM ($E_K = -22$ mV)) in the pipette solution. Error bars indicate s.e.m., $*P<0.05$.

previously reported this in rat small mesenteric arteries (Videbaek *et al*., 1990). In this study, we first wanted to assess the role of the K_{ATP}-channels for the effect of pinacidil. To do this we used 1 μM of the K_{ATP} channel blocker glibenclamide, which, however was without significant effects on pinacidil-induced relaxation. This is surprising in view of the substantial evidence for an effect of glibenclamide on responses to pinacidil in a number of different vascular preparations (Standen *et al*., 1989; Mulvany *et al*., 1990; Belloni & Hintze, 1991; Nielsen-Kudsk & Bang 1991; Dart & Standen, 1993; Gollasch *et al*., 1995), and also in view of our patch clamp data, which suggested that 1 μM glibenclamide did reduce the pinacidil-induced current. A measure of 1 μM glibenclamide has previously been effective in mesenteric arteries from rabbits (Standen *et al*., 1989), rats (Lei *et al*., 1999) and dogs (Masuzawa *et al*., 1990a). We tried to increase the glibenclamide concentration to 3 and 10 μM. However, this induced a concentration-dependent inhibition of the U46619-induced contraction (unpublished observation) consistent with previous findings (Cocks *et al*., 1990). We therefore used another K_{ATP} channel opener, cromakalim (Wilson *et al*., 1988; Kuromaru & Sakai, 1996). In contrast to what we found with pinacidil the relaxation to cromakalim was significantly inhibited by 1 μM glibenclamide, indicating that with the protocol used glibenclamide gave the expected result against another opener of K_{ATP}-channels.

Pinacidil has previously been shown to induce vasodilatation through mechanisms not related to K_{ATP} channels (Wilson *et al*., 1988; Kuromaru & Sakai, 1996). High concentrations of pinacidil (>10 μM) have been suggested to inhibit voltage-operated Ca^{2+} channels, thereby causing relaxation (Masuzawa *et al*., 1990b; Gollasch *et al*., 1995). In the present study we found some support for this, that is, 1 μM pinacidil induced a minor relaxation of high-potassium activated arteries. With high potassium the membrane potential is close to the potassium equilibrium potential of 0 mV and a relaxant effect cannot be mediated by opening of potassium channels, but could be mediated through a Ca^{2+} channel-blocking effect. In coronary arteries from pigs and humans, high concentrations of pinacidil might also interfere with the function of

intracellular Ca^{2+} stores, reduce the sensitivity of the contractile elements for Ca^{2+}, or increase the Ca^{2+}-efflux (Gollasch *et al*., 1995). Since the response to pinacidil was very limited in the potassium activated arteries, we do think it is unlikely that any of these mechanisms are explaining the insensitivity to glibenclamide. Another possibility is that pinacidil acts through another channel (i.e. different from K_{ATP}-channels) resulting in net increase of outward current. However, the reversal potential of the pinacidil-induced current in the patch clamp experiments was identical to potassium's equilibrium potential and the current was completely blocked with 10 μM glibenclamide. This excludes that pinacidil works through another membrane channel. We also investigated the role of the endothelium. The role of the endothelium in pinacidil-induced relaxation seems variable. In rat mesenteric arteries, pinacidil-induced relaxation is enhanced after block of NO production in the endothelium (McCulloch & Randall, 1996). In contrast to this pinacidil-induced relaxation of goat coronary arteries is partly mediated through release of NO from the endothelium. Thus, both L-NAME and mechanical removal of the endothelium caused a reduction of pinacidil-induced relaxation (Deka *et al*., 1998). On the other hand, pinacidil-induced relaxation of coronary arteries from pigs and humans seems to be independent of the endothelium (Gollasch *et al*., 1995). In this study, we found that even though L-NNA reduced the response to acetylcholine, L-NNA had no effect on the response to pinacidil. This makes a contribution from NO to the vasodilator response to pinacidil unlikely. In contrast, we found the response to pinacidil slightly but significantly reduced after removal of the endothelium. This is consistent with a small part of the relaxation to pinacidil being mediated through an endothelium dependent non-NO mechanism. If this is the case this may account for the relative glibenclamide insensitivity. However, it is also possible that the small effect of endothelium removal simply reflects that the pinacidil-induced relaxation is potentiated by an endothelium-dependent vasodilator action. We have therefore no clear explanation for the relative glibenclamide insensitivity, although an effect of pinacidil via the endothelium may explain some of it. However, since the pinacidil effect was dependent on the potassium gradient in the intact arteries, and only affected glibenclamide-sensitive currents in isolated cells, we believe that the most likely conclusion is that pinacidil mainly works through K_{ATP}-channels, despite the lack of effect of 1 μM glibenclamide in the intact arteries.

The most remarkable finding of this study is the substantial effect of ouabain on the responses to both pinacidil and cromakalim. To test whether this response was related to inhibition of the Na,K-pump we investigated the role of potassium-free media, which also blocks the Na,K-pump. The significant inhibitory action of potassium-free media was smaller than the inhibitory effect of ouabain. One explanation could be that the potassium-free solution only causes incomplete inhibition of the Na,K-pump due to minor amounts of K^+-ions remaining in the organ bath. Another explanation for the partial effect of potassium-free conditions is that this condition shifts the potassium equilibrium potential to very negative values and a leftward shift of the concentration–response curve to K_{ATP}-channel openers would be expected. The resultant effect could therefore be a balance between the opposing effects of inhibiting the Na,K-pump and shifting the potassium equilibrium potential to more negative values. Even so the data support a role for inhibition of the Na,K-pump for the effect of pinacidil.

The Na,K-pump has previously been suggested to play a role for the response to the K_{ATP}-channel openers. In pig retinal arteries the relaxation to pinacidil is completely blocked by the Na,K-pump inhibitor ouabain (Jeppesen *et al*., 2002) and in rabbit femoral and ear arteries ouabain impairs cromakalim-induced relaxation (Garcia-Villaon *et al*., 1996). In human mesenteric arteries, ouabain inhibits relaxation induced by low concentrations of cromakalim whereas relaxation induced by higher concentrations is inhibited by glibenclamide (Hong *et al*., 1993). Our patch clamp data provide a strong suggestion for the explanation of the ouabain (and potassium-free) effect on the responses to K_{ATP} channel openers. First of all they show that ouabain and omission of sodium from the cytosol block the pinacidil- or cromakalim-activated, ATP-sensitive current under voltage clamp conditions, which provides direct evidence for a link between the Na,K-pump activity and activation of the K_{ATP} current. Furthermore, the observation that ouabain had no effect on the pinacidil- and cromakalim-activated current after prior inhibition of the Na,K-pump by dialyzing the cell with a sodium-free solution strongly supports this interpretation. A possible explanation for this interaction is that the hydrolysis of ATP by the active pump reduces the ATP/ADP ratio and thus enhances the likelihood of pinacidil and cromakalim for opening the channel. Na,K-pump activity may not, however, affect bulk ATP concentration in smooth muscle cells (Hellstrand *et al*., 1984) and for this interaction to be relevant the changes in ATP concentration presumably must be confined to a sarcolemma near volume. A similar argument could be suggested for the local transmembrane potassium gradient, which could be modulated by the activity of the Na,K-pump to an extent which would be detected by the K_{ATP} channel. A similar interaction has previously been suggested for the response of cerebral arteries to an increased extracellular potassium, which through activation of the Na,K-pump would reduce the ATP/ADP ratio and thus enhance opening of K_{ATP} channels (Nguyen *et al*., 2000). A similar functional interaction between the K_{ATP} channel and the Na,K-pump has previously been suggested in the kidney (Tsuchiya *et al*., 1992) in pancreatic β-cells (Grapengiesser *et al*., 1993; Ding *et al*., 1996), in the heart (Haruna *et al*., 1998; Kabakov, 1998) and in skeletal muscle (Tricarico *et al*., 2003).

In conclusion, we have confirmed that the relaxation to cromakalim and probably pinacidil in rabbit coronary arteries is likely mediated through inhibition of the K_{ATP} channels. Importantly, we found that a functional interaction exists between the activity of the Na,K-pump and the K_{ATP}-channel current possibly through Na,K-pump induced changes in local ATP and or potassium concentrations. This could imply that the Na,K-pump and the K_{ATP}-channels are colocalized in the smooth muscle cells. The interaction demonstrated here could be of consequence for coronary dilation in response to hypoxia and opening of K_{ATP} channels in patients treated with digitalis.

We thank Mrs Kirsten Zeeberg Aggerholm and Mrs Kirsten Skaarup for technical assistance. The studies were supported by grants from the Institute of Experimental Clinical Research, University of Aarhus and the Danish Medical Research Council. Vladimir Matckov received a stipend from the Danish Heart Foundation.

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(Received March 22, 2003
Revised July 1, 2004
Accepted August 6, 2004)