Activation by intracellular GDP, metabolic inhibition and pinacidil of a glibenclamide-sensitive K-channel in smooth muscle cells of rat mesenteric artery

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1 Single-channel recordings were made from cell-attached and isolated patches, and whole-cell currents were recorded under voltage clamp from single smooth muscle cells obtained by enzymic digestion of a small branch of the rat mesenteric artery.

2 In single voltage-clamped cells 1 mM uridine diphosphate (UDP) or guanine diphosphate (GDP) added to the pipette solution, or pinacidil (100 μ M) a K-channel opener (KCO) applied in the bathing solution, evoked an outward current of up to 100 pA which was blocked by glibenclamide (10 μ M). In single cells from which recordings were made by the 'perforated patch' (nystatin pipette) technique, metabolic inhibition by 1 mM NaCN and 10 mM 2-deoxy-glucose also evoked a similar glibenclamide-sensitive current.

3 Single K-channel activity was observed in cell-attached patches only infrequently unless the metabolism of the cell was inhibited, whereupon channel activity blocked by glibenclamide was seen; pinacidil applied to the cell evoked similar glibenclamide-sensitive channel activity. If the patch was pulled off the cell to form an isolated inside-out patch, similar glibenclamide-sensitive single-channel currents were observed in the presence of UDP and/or pinacidil to those seen in cell-attached mode; channel conductance was 20 pS (60:130 K-gradient) and openings showed no voltage-dependence and noisy inward currents, typical of the nucleoside diphosphate (NDP) activated K-channel (K_{NDP}) seen previously in rabbit portal vein.

4 Formation of an isolated inside-out patch into an ATP-free solution did not increase the probability of channel opening which declined with time even when some single-channel activity had occurred in the cell-attached mode before detachment. However, application of 1 mM UDP or GDP, but not ATP, to inside-out patches evoked single-channel activity. Application of ATP-free solution to isolated patches, previously exposed to ATP and in which channel activity had been seen, did not evoke channel activity.

5 It is concluded that small conductance K-channels (K_{NDP}) open in smooth muscle cells from this small artery in response to UDP or GDP acting from the inside, or pinacidil acting from the outside; the same channels open during inhibition of metabolism presumably mainly due to the rise in nucleoside diphosphates, but a fall in the ATP concentration on the inside of the channel did not by itself evoke channel activity. Failure to respond to a fall in ATP concentration upon formation of an inside-out patch could not be due to dephosphorylation of the channel because sometimes it had been active previously during cell-attached recording. NDPs, instead of ATP, are more important regulators of K_{NDP} channels. It is suggested that the K_{NDP} is the main target K-channel for KCOs.

Keywords: Metabolic inhibition; cyanide; 2-deoxyglucose; pinacidil; GDP; uridine diphosphate; K_{NDP}

Introduction

K_{ATP} channels, by definition, are channels inhibited by intracellular ATP ($[ATP]_i$). If $[ATP]_i$ is the primary regulator of the channels, they should open when inside-out patches are made into ATP-free solution from a high- $[ATP]_i$ or normal- $[ATP]_i$ circumstances. This is true in the cases of both cardiac and pancreatic β -cells (Cook & Hales, 1984; Trube & Hescheler, 1984; Shen *et al.*, 1991). Standen *et al.* (1989) observed remarkable channel activity in inside-out patches in the absence of ATP in mesenteric artery smooth muscle cells which was clearly inhibited by ATP and reactivated by cromakalim. However, channel activity has not been observed by many groups when isolated inside-out patches were formed from smooth muscle cells into ATP-free solution (Kajioka *et al.*, 1991; Nakao & Bolton, 1991; Robertson *et al.*, 1992; Beech *et al.*, 1993a; Bonev & Nelson, 1993), unless GDP is present (Kajioka *et al.*, 1991; Beech *et al.*, 1993a,b). Furthermore, the channel conductance reported by Standen *et al.* (1989) is much higher than that in other tissues (Noma, 1983; Trube & Hescheler, 1984; Rorsman & Trube, 1985) and that in smooth muscle reported by Kajioka *et al.* (1991).

While the channel conductance is quite similar in cardiac and pancreatic β -cells, the channel conductance of K_{ATP} channel in smooth muscle cells has been variously described as 258 pS (Lorenz *et al.*, 1992), 100–135 pS (Standen *et al.*, 1989; Kovacs & Nelson, 1991), 30 pS (Inoue *et al.*, 1989; Miyoshi & Nakaya, 1993), 24 pS (Beech *et al.*, 1993a), 15 pS (Kajioka *et al.*, 1991), 10 pS (Kajioka *et al.*, 1990) and 7 pS (Bonev & Nelson, 1993). Only a few of these differences can be explained by the different K-gradients used. As a consequence it is still unclear whether smooth muscle has a different type of K_{ATP} channel from other tissues and whether other factors rather than $[ATP]_i$ are more important in determining channel opening.

Many experiments have shown that relaxations of smooth muscle caused by potassium channel opener (KCO) drugs are blocked by glibenclamide (e.g. Winkler *et al.*, 1989; Standen *et al.*, 1989) which has been interpreted as indicating that the K_{ATP} channel is the target channel. In single cell and single channel experiments, glibenclamide, in micromolar concentrations blocked the whole cell currents induced by various KCOs including levcromakalim, pinacidil, nicorandil, diazoxide, minoxidil sulphate, RP 49356 (Noack *et al.*, 1992a;

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Silberberg & van Breemen, 1992; Beech *et al.*, 1993b; Zhang & Bolton, unpublished) and the increase in probability of opening of K-channels produced by KCOs (Standen *et al.*, 1989; Kajioka *et al.*, 1991; Beech *et al.*, 1993b). However, the Ca-activated large conductance K-channel (BK_{Ca} channel) (Gelband & McCullough, 1993) and delayed rectifier K-channel (Edwards *et al.*, 1993) have been suggested as target channels for KCOs and the effect of KCOs on these are also inhibited by glibenclamide.

In our previous paper, we showed that GDP and other nucleoside diphosphates (NDPs) activated a K-channel with small (24 pS) conductance in rabbit portal vein cells (Beech *et al.*, 1993a). The action of NDPs on the channel was sensitive to the blocking action of glibenclamide and channel open probability was increased by the KCO, levcromakalim (Beech *et al.*, 1993b). We designated this channel K_{NDP} because nucleoside diphosphates needed to be present for the channel to open and [ATP]_i could inhibit channel activity only when increased by NDPs; thus NDPs, and not ATP, seemed to be the primary regulator of channel activity because channel activity did not appear when isolated patches were formed into ATP-free solution if NDP was not present. In this paper, we use single smooth muscle cells isolated from rat mesenteric artery to test whether the channel activated by NDPs is also present in resistance arteries and whether there is a relation to the K_{ATP} channel which has been described in this tissue (Standen *et al.*, 1989). We also try to clarify whether KCO and metabolic inhibition can open the same channel as that opened by NDPs.

Methods

Single cell dispersion

Single smooth muscle cells were isolated from small branches of rat mesenteric artery. Adult, male Wistar rats (200–300 g) were killed by cervical dislocation. The second and third order branches of the artery were cut open along their longitudinal axis after careful dissection and incubated in a low-Ca (10 µM) and Mg-free physiological salt solution (PSS) at 37°C for 20 min. Tissue was then moved to the same solution which contained 0.4 mg ml⁻¹ collagenase (type XI, Sigma), 0.4 mg ml⁻¹ pronase (type E, Sigma) and 1 mg ml⁻¹ bovine albumin (Sigma) at 37°C for 30 min. The tissue was washed with enzyme-free solution and triturated by sucking in and out of a wide bore, smooth-tipped pipette. The cells were used for experiments within 6 h of separation during which time they were stored at 4°C in low-Ca (10 µM) solution. All experiments were performed at room temperature (22°C). The solutions used for the whole procedure of the dispersion were oxygenated (100% O₂) vigorously and continuously and ice-cold solution was used for the tissue dissection to reduce as far as possible the reduction of ATP levels in the cells.

Electrophysiology

Conventional whole-cell, cell attached and isolated patch recording (Hamill *et al.*, 1981) and 'perforated patch' recording (Horn & Marty, 1988) were used in the experiments. The patch amplifier was a RK300 (Biologic). Patch pipettes were made from borosilicate glass (Plowden & Thompson); they had resistance of 2–5 MΩ after fire-polishing, and were coated with Sylgard (Dow Corning) for single channel recordings. Data were stored of FM-tape (Racal) (3.75 in s⁻¹, low-band pass 1250 Hz) and captured later to a 386 PC for data analysis after digitization using CED interface and software, or replayed using a Gould pen recorder (Gould Electronic Ltd.) for illustrations. All whole-cell current records were filtered at 500 Hz (4-pole Bessel filter, Barr and Stroud) and single channel records filtered as described in the legends of Figures 2 to 7. The input resistance was monitored in

whole-cell experiments by square or ramp hyperpolarizing commands. The series resistance was estimated by the rate of decay of the capacity current after an instantaneous step in the command voltage and was estimated to be < 10 MΩ in conventional whole-cell and < 20 MΩ in 'perforated patch' experiments. Results are given as means ± standard error of the mean (s.e.mean).

Data analysis

Unitary current amplitude was determined by fitting amplitude histograms with Gaussian distributions. The channel open-state probability (*P*_o) was calculated using the following equation

$$P_o = \sum_{i=1}^N t_i / (TN)$$

where the *t_i* is the time spent with *i* = 1, 2, 3, ..., *N* channels open, *N* is the number of the channels, *i* is unitary current and *T* is the sample length. The calculation assumes the maximum number of unitary current levels observed in a patch to be equal to the number of active channels, *N*, in the patch. All data were expressed as means ± s.e.mean.

Solutions

Low-Ca and Mg-free solution (mM): NaCl 130, KCl 5, CaCl₂ 0.01, HEPES 10, glucose 10, pH 7.4 with NaOH. Solutions for whole-cell recording mode: physiological salt solution (PSS) in bath (mM): NaCl 130, KCl 5, CaCl₂ 1.7, MgCl₂ 1.2, HEPES 10, glucose 10, pH 7.4 with NaOH. Pipette (mM): KCl 130, MgCl₂ 2, EGTA 5, HEPES 20, pH 7.4 with KOH. Nystatin (Sigma) 100 µg ml⁻¹ was added to the solution to make a 'perforated patch'. Solutions for single channel recording mode: Bath (mM): NaCl 9, KCl 117, MgCl₂ 3, HEPES 18, EGTA 5, pH 7.4 with KOH. Pipette (mM): NaCl 80, KCl 60, CaCl₂ 1.7, MgCl₂ 1.3, HEPES 10, glucose 10, pH 7.4 with NaOH. Glucose was replaced by 10 mM 2-deoxy-glucose (2DG) and sodium cyanide (CN) (1 mM) was added to the solution when metabolic inhibition was used. UDP, GDP and ATP were added as Na-salts and 100 mM stock solutions of these were prepared and frozen at -20°C. Dilution was made immediately prior to the application. Nystatin was prepared as a 5 mg ml⁻¹ stock in methanol and diluted to the final concentration. Glibenclamide was prepared as a 10 mM stock solution in dimethylsulphoxide (DMSO). The final concentration of DMSO was 0.1% for 10 µM glibenclamide.

Drugs

GDP (guanosine diphosphate), UDP (uridine diphosphate), ATP (adenosine triphosphate), EGTA (ethylglycol-bis-(β-aminoethyl)-*N,N,N',N'*-tetraacetic acid), HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) and glibenclamide were from Sigma. Pinacidil monohydrate was from Leo Pharmaceutical Company (Denmark). Levcromakalim was a gift from Dr T. Hamilton (SKB). Nicorandil was from Chugai Pharmaceutical Company (Japan).

Results

Pinacidil, metabolic inhibition and intracellular GDP all induce a glibenclamide-sensitive K-current in whole-cell recordings

Figure 1 shows three recordings in single cells isolated from rat mesenteric artery. Cells a and d are conventional whole-cell recordings, and cell c is a 'perforated-patch' whole cell recording (see Methods). All the cells were held at -37 mV. In cell a, a small transient outward current (about 8 pA in peak) developed about 4 min after breaking through the cell membrane. Similar currents were seen from another two cells

out of 8 cells observed. The mean amplitude of the currents from these three cells was 6 ± 2 pA (means $\pm$ s.e.mean). When pinacidil ($100 \mu\text{M}$) was applied via bath, a large outward current with a peak amplitude of about 97 pA developed which decayed slowly after reaching a peak (Figure 1a). Glibenclamide ($10 \mu\text{M}$) totally blocked the current induced by pinacidil. In another three cells, pinacidil ($100 \mu\text{M}$) induced outward currents of average amplitude of 46 ± 12 pA which were all blocked by glibenclamide ($10 \mu\text{M}$). Ramp voltage changes were applied to the cell to determine the reversal potential of the current induced by pinacidil. The currents, induced by ramp voltages from -117 mV to 20 mV

before (i) and during (ii) application of pinacidil, intersected at -80 mV (Figure 1b), close to the calculated E_K (E_K was -88 mV), suggesting that most of the current induced by pinacidil was potassium current.

In Figure 1c, $100 \mu\text{g ml}^{-1}$ nystatin was included in the pipette to make a 'perforated patch'. After about 6 min when series resistance had fallen to less than $15 \text{ M}\Omega$, metabolic poison solution (CN + 2DG) was applied. An outward current developed slowly with peak amplitude of 20 pA at a holding potential of -37 mV. The current did not decay before pinacidil ($100 \mu\text{M}$) was bath-applied. Pinacidil induced an additional outward current of 40 pA (peak). Both currents

Figure 1 Pinacidil, metabolic inhibition and GDP, activate a glibenclamide-sensitive K-current in single smooth muscle cells isolated from the rat mesenteric artery. The current traces were constructed from whole-cell currents sampled every 10 s at a holding potential of -37 mV. Pinacidil and glibenclamide were applied via bath solution as indicated. (a) Conventional whole-cell recording mode with KCl 130 mM, EGTA 5 mM, MgCl_2 2 mM and HEPES 20 mM in the pipette solution; bath solution was 1.7 mM Ca PSS solution. The recording was started about 1 min after break-through to the whole cell recording mode. (b) Ramp change in voltage from -117 mV to $+20$ mV lasting 200 ms was applied during the recording shown at (a). (i) and (ii) indicate the responses to the ramp voltages before and at the peak of the response to pinacidil. (c) Perforated patch whole-cell recording mode. Nystatin ($100 \mu\text{g ml}^{-1}$) was included in the pipette solution which otherwise was same as in (a). The recording was started about 1 min after gaining electrical access to the interior of the cell which was indicated by a series resistance of less than $15 \text{ M}\Omega$. The PSS in the bath was replaced by PSS containing cyanide (1 mM) and 2-deoxy-glucose (10 mM) as indicated. (d) Conventional whole-cell recording mode with 1 mM GDP included in the pipette solution. The recording was started about 30 s after break-through to the whole-cell recording mode.

were blocked by glibenclamide ($10\ \mu\text{M}$). Similar results were seen in another three cells. However, we did not see currents developing in four cells during conventional whole-cell recordings either before or after metabolic inhibition, but pinacidil induced an average outward current in these cells of $36 \pm 11\ \text{pA}$ ($n = 4$).

Figure 2 UDP activates a K-channel in isolated inside-out patches. Recordings of two cells isolated from rat mesenteric artery. The cells were held at $-60\ \text{mV}$. KCl $117\ \text{mM}$, MgCl_2 $3\ \text{mM}$, EGTA $5\ \text{mM}$ (Ca-free) were in the bath solution and the pipette solution contained KCl $60\ \text{mM}$, CaCl_2 $1.7\ \text{mM}$. In cell a, UDP ($1\ \text{mM}$), applied via bath after ATP ($1\ \text{mM}$) was washed out, activated the channel with a unitary current amplitude of about $0.85\ \text{pA}$ at $+60\ \text{mV}$. Notice that a smaller unitary current ($\uparrow$) was also present. In cell b, UDP ($1\ \text{mM}$) also activated the channel. After washing out UDP, channel activity ceased in the presence of ATP. Only one brief channel opening was seen after washing out ATP during a period of 5 min. UDP reactivated the channel again. The current traces were recorded continuously on FM-tape ($3.75\ \text{s}^{-1}$) with a $0.5\ \text{kHz}$ low-pass filter ($-3\ \text{dB}$, 4-pole Bessel) and filtered for presentation at $100\ \text{Hz}$. The breaks in the records represented 3–4 min interruptions.

When $1\ \text{mM}$ GDP was included in the pipette solution, an outward current slowly developed (Figure 1d) after breaking through the cell membrane into the whole-cell mode and this reached a maximum, on average, of $35 \pm 7\ \text{pA}$ ($n = 4$), after 10 to 15 min. Once the response to GDP had reached a maximum, pinacidil ($100\ \mu\text{M}$), levcromakalim ($10\ \mu\text{M}$) or nicorandil ($100\ \mu\text{M}$) was bath-applied. These three KCOs induced an additional current of 40 (Figure 1c), 35 and $20\ \text{pA}$, respectively. Glibenclamide ($10\ \mu\text{M}$) blocked the currents induced by GDP and KCO. Ramp voltage was also applied to cell c and cell d; reversal potentials of -82 and $-84\ \text{mV}$ respectively, were obtained.

GDP and UDP activate a small conductance K-channel in inside-out patches

When inside-out patches were formed into zero-Ca EGTA solution in the absence of a nucleotide at the inner surface of the patch, no distinct channel activity was seen in most of the patches (35) held at $-60\ \text{mV}$ (Figure 2). However, some atypical channel activity did appear in two patches from two cells just once over a period of 2 to 5 min (an example is shown in Figure 3b; the unitary current is larger than that carried by K_{NDP} channels). When GDP (Figure 3) or UDP (Figure 2) ($1\ \text{mM}$) were applied in the bathing solution to the inner surface of the patches, a channel was activated that showed long bursts of openings (mean open time $3.8\ \text{s}$ in the case of Figure 3) separated by even longer closed periods (mean closed time $5.8\ \text{s}$ in the case of Figure 3). There was a pronounced fluctuation in the channel open-state when the current direction was inward (Figure 3; see later; cf. Figure 7). When the patches were held at $-60\ \text{mV}$, the unitary amplitude of the channel activated by UDP and GDP was

Figure 3 The K channel activated by metabolic inhibition in cell-attached patch and by GDP and pinacidil in inside-out patch can be blocked by glibenclamide. A patch from rat mesenteric artery was held at $-60\ \text{mV}$. The same pipette solution ($60\ \text{mM}$ K) and bath solution ($117\ \text{mM}$ K) were used as in Figure 2. Initially the patch was cell-attached and metabolic blocking agents, cyanide (CN, $1\ \text{mM}$) and 2-deoxy-glucose (2DG $10\ \text{mM}$), were applied via the bath; then an inside-out patch was formed in the ATP- and GDP-free, CN + 2DG solution. GDP ($1\ \text{mM}$), pinacidil ($100\ \mu\text{M}$) and glibenclamide ($10\ \mu\text{M}$) were applied to the inside-out patch via bath as indicated. Glibenclamide totally abolished the channel activity induced by GDP and pinacidil and the effect of glibenclamide could be washed out. Notice that some atypical channel activity (unusually larger unitary current) can be seen just after forming an inside-out patch. The current traces were recorded continuously on FM-tape ($3.75\ \text{s}^{-1}$) with a $0.5\ \text{kHz}$ low-pass filter ($-3\ \text{dB}$, 4-pole Bessel) and filtered for presentation at $100\ \text{Hz}$. The expanded sections of traces of cell-attached and inside-out patches showed that (a) and (c), but not (b), have the same unitary size and characteristics. The broken lines represented about 2–3 min interruption in each case.

0.85 ± 0.008 pA ($n = 9$). The conductance of the channel was 20 pS and the current reversed near E_K (-19.5 mV), suggesting K ions were the charge carrier (see later; cf. Figure 7). Pinacidil ($100 \mu\text{M}$) increased the channel activity and glibenclamide ($10 \mu\text{M}$) totally blocked it (Figure 3). All of these characteristics of channel activity are similar to those of the channel activated by GDP in rabbit portal vein smooth muscle cells described in our previous papers (Beech *et al.*, 1993a,b).

A low concentration of ATP, in the presence of Mg, is necessary to retain the ability of the K_{ATP} channel to open in both cardiac and pancreatic β -cells (Findlay & Dunne, 1986; Misler *et al.*, 1986; Findlay, 1987; Ohno-Shosaku *et al.*, 1987). So ATP (1 mM) in the presence of 3 mM MgCl_2 was applied to the inner surface of the patches in this experiment. In Figure 2a, no channel activity was observed before and after application of 1 mM ATP. However, when UDP (1 mM) was bath-applied after wash-out of ATP, the channel was

activated. UDP (1 mM) activated the channel in another inside-out patch (Figure 2b); after washing out UDP and applying 1 mM ATP, channel activity ceased. When ATP was washed out, a brief channel opening was seen during a period of 5 min. UDP (1 mM) was applied again in this cell and the channel was re-activated by UDP (Figure 2b). Experiments using the same protocol as that used in Figure 2b were performed in two other cells and no channel activity was observed after washing out ATP but UDP always activated the channel.

Single-channel currents activated by pinacidil or metabolic inhibition in cell-attached and inside-out patches

The cell membrane was quiescent most of the time in cell-attached patches from rat mesenteric artery held at -60 mV. Occasionally, a channel with the same characteristics as the

Figure 4 Metabolic block and pinacidil both activate a glibenclamide-sensitive K channel in cell-attached patch from a smooth muscle cell isolated from rat mesenteric artery. The cell was held at -60 mV. KCl 117 mM, MgCl_2 3 mM and EGTA 5 mM (Ca-free) were in the bath solution and the pipette solution contained KCl 60 mM and CaCl_2 1.7 mM. Cyanide (CN, 1 mM), 2-deoxy-glucose (2-DG, 10 mM), pinacidil ($100 \mu\text{M}$) and glibenclamide (Glib, $10 \mu\text{M}$) were applied via the bath. (a–e) Recording of the current in a cell-attached patch. The control trace (a) was just before the application of CN + 2DG; later traces (b,c,d,e) were 2 to 3 min after the applications of CN + 2DG, pinacidil, or glibenclamide respectively; then pinacidil and glibenclamide were washed out. All-points histograms were constructed from a period of 4 min in each case with a sample frequency of 2 kHz and after filtering at 0.5 kHz. Notice the peak at about 0.8 pA representing activity of a single open channel.

channel activated by UDP and GDP in inside-out patches was seen in cell-attached patches but generally the patch was quiescent under control conditions (Figure 4a). However, when metabolic poisons (CN + 2DG) were applied to the cell, a channel was activated with an average unitary amplitude of 0.84 ± 0.02 pA ($n = 7$) at -60 mV. This channel also had open-state noise in an inward direction (Figure 4b; see later, cf. Figure 7), and was apparently similar to the type of channel activated by UDP and GDP in inside-out patches. Pinacidil ($100 \mu\text{M}$) added alone increased the channel activity and the open probability of the channel was increased from 0.014 to 0.025 (channel activity in control was 0.00093) (Figure 4c). However, application of glibenclamide ($10 \mu\text{M}$) completely abolished the channel activity induced by both CN + 2DG and pinacidil (histogram in Figure 4d was constructed just after the application of glibenclamide; channel activity was abolished 30 s after the application of glibenclamide). The effect of glibenclamide could be partly washed out (Figure 4e). The channel open probabilities were 0.0012 and 0.0063 in the presence and absence of glibenclamide, respectively. Figure 5 shows data from the same cell as Figure 4. The arrow in Figure 5 shows when an isolated inside-out patch was created after glibenclamide had been washed out. The channel activity declined after the inside-out patch was formed (5 min); one longer lasting opening (Figure 5b) was observed, presumably because the inhibition of the channel by ATP in the cell-attached mode was abolished in the inside-out patch (Kajioka *et al.*, 1991). After channel activity ceased, UDP (1 mM) and UDP (1 mM) plus pinacidil ($100 \mu\text{M}$) re-activated the channel with much greater channel activity and occasionally 3 channels were open simultaneously (Figure 5c). The sections of current record on a faster time base show that the characteristics of the channels open in the absence (Figure 5b) and presence (Figure 5c) of UDP and pinacidil, and in the cell-attached patch (Figure 4b,c and Figure 5a) were quite similar. The open probability of the channel in the presence of UDP + pinacidil was 0.16 and mean open time (one active channel in patch) was 1.8 s, compared with a mean open time of 164 ms in a cell-attached

patch in the presence of pinacidil. In another 4 cells, the channel activity activated by CN + 2DG always ceased within 40 s after an inside-out patch was formed; UDP or GDP (1 mM) always re-activated the channel.

Although channel activity was not normally seen in cell-attached patches, in one particular cell pronounced channel activity was found even without CN + 2DG or KCO. In this cell, the channel which had an amplitude of 0.83 pA at -60 mV, opened frequently, and two channels could be seen to be open simultaneously. The channel activity decreased and ceased after about 1 min when inside-out patch was formed. This channel, with characteristic unitary current size, long open time and noisy open-state, was apparently the same type of channel as that activated by NDPs.

In another series of experiments, only pinacidil ($10 \mu\text{M}$) was applied to cell-attached patches and in three cells, pinacidil alone activated the channel with an average amplitude of 0.80 ± 0.02 pA at -60 mV. Figure 6a(i)(ii), b(i)(ii), shows a typical example of results from these cells. When an inside-out patch was formed in the presence of pinacidil (alone, no nucleoside diphosphate), channel open probability was increased from 0.093 to 0.28 and two channels were seen to be open simultaneously (Figure 6a(iii), b(iii)). These channels also showed similar characteristics to those of the channel activated by CN + 2DG or NDPs. In another cell, pinacidil did not activate channels in a cell-attached patch. However, a channel with similar characteristics to those described above was activated by pinacidil alone after an inside-out patch was formed. So, it seems that pinacidil can open the K_{ATP} channel in the absence of any nucleotide in rat mesenteric artery, which is different from cardiac cells (Shen *et al.*, 1991) and pancreatic β -cells (Dunne, 1990; Dunne *et al.*, 1990a,b) in which the effect of KCO needs the presence of ATP or NDP. However, in at least two cells, pinacidil ($100 \mu\text{M}$), applied after, rather than before an inside-out patch was formed, failed to activate the channel, although subsequently application of UDP proved the existence of the channel in the patches.

The BK_{Ca} channel was rarely seen because of the negative

Figure 5 The same cell as in Figure 4. An inside-out patch was formed by pulling away the pipette from the cell ($\downarrow$) into cyanide + 2-deoxy-glucose solution and held at -60 mV. The current with the same unitary size and the same characteristics (noisy open-state) as seen in the cell-attached mode (a) but with much longer open times was seen in the inside-out patch (b). The channel activity ceased in the trace (b) and was not seen for at least 5 min. The channel was reactivated by the application of UDP (1 mM) and pinacidil ($100 \mu\text{M}$) (c). The expanded section shows that as many as three channels were open simultaneously.

holding potential (-60 mV) and zero Ca in the bathing solution. However, when the holding potential was made positive ($+60$ mV), the BK_{Ca} channel was activated. Some cells were held at $+60$ mV but pinacidil and metabolic block had no effect on the BK_{Ca} channel. The conductance of the BK_{Ca} channel under the conditions of the present experiments ($60:130$ mM K-gradient) was about 150 pS in inside-out patches (see later, Figure 7).

The conductance of the channel activated by NDPs, metabolic inhibition and pinacidil

The current-voltage relationship was linear for the channel activated by pinacidil, CN + 2DG or NDPs (Figure 7c).

Figure 7a is a record from a cell-attached patch in the presence of CN + 2DG and pinacidil and Figure 7b is an inside-out patch in the presence of UDP and pinacidil. A noisy open-state was observed only when the current direction was inward in both cases (Figure 7a,b). Two kinds of channels can be seen in Figure 7a, (i) BK_{Ca} channel with a conductance of 150 pS; (ii) the channel activated by CN + 2DG and pinacidil with a conductance of 20 pS. In Figure 7b, three different channels can be seen, (i) BK_{Ca} channel, (ii) 20 pS channel activated by UDP and pinacidil, (iii) 10 pS channel activated by UDP and pinacidil. The small conductance channel (10 pS) may be a sub-conductance state of the 20 pS channel, since it had similar characteristics apart from its conductance. However, current transfers from the subcon-

Figure 6 Pinacidil alone activated a K channel in cell-attached and inside-out patches in smooth muscle cells isolated from rat mesenteric artery. (a) Recordings, (b) all-point histograms. (i) The cell was held at -60 mV. KCl 117 mM, MgCl_2 3 mM, and EGTA 5 mM (Ca-free) were in the bath solution and KCl 60 mM, and CaCl_2 1.7 mM were in the pipette solution. (ii) Pinacidil ($100\ \mu\text{M}$) was applied via bath in the cell-attached recording mode and (iii) an inside-out patch was formed in the presence of pinacidil. Pinacidil activated the channel with an unitary current amplitude of about 0.8 pA at -60 mV in the cell-attached mode and the channel activity was increased after an inside-out patch was formed. All points histograms were constructed from periods of 4 min in each case with a sample frequency of 2 kHz after filtering at 0.5 kHz.

Figure 7 The conductance of the K channel activated by metabolic inhibition and pinacidil in cell-attached patches, and by UDP and pinacidil in inside-out patches was about 20 pS. Two cells from rat mesenteric artery were held at different voltages ranging from -100 mV to $+60$ mV. KCl 117 mM, MgCl₂ 3 mM and EGTA 5 mM (Ca-free) were in the bath solution and KCl 60 mM and CaCl₂ 1.7 mM were in the pipette. (a) Cell-attached patch from one cell in the presence of cyanide (CN) (1 mM) + 2-deoxy-glucose (2-DG) (10 mM) and pinacidil (100 μ M). (b) Inside-out patch from another cell in the presence of UDP (1 mM) and pinacidil (100 μ M). (c) The current-voltage relationship of the channel in both cases. Notice that the 20 pS channel was activated by CN + 2DG-pinacidil in the cell-attached patch (○) and by UDP + pinacidil in the inside-out patch (Δ). A smaller conductance channel (□) in inside-out patch, and the BK_{Ca} channel (◇) were also seen. The current traces were recorded continuously on FM-tape (3.75 in s⁻¹) with a 0.5 kHz low-pass filter (-3 dB, 4-pole Bessel) and filtered for presentation at 300 Hz.

ductance to fully conducting state, or *vice versa* were not seen. All three current-voltage relationships reversed near E_K (-19.5 mV). The subconductance state of channels activated by pinacidil were also seen in a cell-attached patch. In Figure 2a, as well as the 20 pS channel activated by UDP in an inside-out patch, channel activity with a half-size unitary current was also present. The voltage-dependence of the 20 pS channel activated by pinacidil and UDP was checked over the voltage range from -80 mV to $+40$ mV. No modification was found in channel probability of opening upon changing the holding potential.

Discussion

In this paper, we have demonstrated that metabolic block, pinacidil and intracellular NDPs can induce K-currents in rat mesenteric artery cells, which are apparently from the same target channel. The channel activated by metabolic block, pinacidil and intracellular NDPs has similar characteristics to those of the channel we described in rabbit portal vein cells in our previous papers (Beech *et al.*, 1993a,b); it is a small conductance channel (probably about 15 pS in quasi-physiological K-gradient, Kajioka *et al.*, 1991). NDPs are a crucial regulator of these channels, which suggests that they should be designated K_{NDP} channels (Beech *et al.*, 1993a).

When recordings were made from the cells of rat mesenteric artery in the conventional whole-cell recording mode with ATP-free pipette solution, only a few cells developed a small transitory outward current at -37 mV (E_K was -88 mV). No further currents developed when metabolic inhibition was applied to these cells. However, when either GDP was added to the pipette solution, or metabolic block was applied during 'perforated patch' whole-cell recording, a sustained outward current developed consistently. Similar results were observed in rabbit portal vein cells where not only GDP but several other NDPs could induce the current. The simplest explanation for these observations is that the concentration of NDPs may rise, as well as that of ATP fall, during 'perforated patch' whole-cell recordings because of the metabolic block; ATP and NDPs may decline simultaneously in conventional whole-cell mode as a result of diffusion into the pipette, although at some stage of diffusion the ratio of NDPs to ATP might rise high enough for the channels to open and cause a small transient current. Hence, the effects of ATP in closing the channel are only possible once NDP has opened it. Pinacidil could induce an outward current during conventional whole-cell recording whether a transitory current had developed or not; pinacidil also induced an additional current after current had been induced by metabolic block or by intracellular GDP (Figure 1). Noack *et al.* (1992b) found a transient outward current (I_{met}) during whole-cell recording in rat portal vein cells held at -50 mV in the absence of ATP and metabolic substrates. After I_{met} had developed, no response to levromakalin was observed. However, we always found that the responses to KCO and NDP were not exclusive (Beech *et al.*, 1993b; present results and unpublished results). The responses to NDP, metabolic inhibition and pinacidil were all blocked by glibenclamide, suggesting that the K_{NDP} channel was involved, since glibenclamide at this concentration had no significant effect on voltage-dependent K-current (Beech *et al.*, 1993b).

Although the K-channels in smooth muscle cells which can be inhibited by ATP have been observed to be active in the absence of ATP in inside-out patches in several vascular muscles, viz: in rabbit and rat mesenteric artery (Standen *et al.*, 1989), in the afferent artery of rabbit kidney (Lorenz *et al.*, 1992) and in porcine coronary artery (Wakatsuki *et al.*, 1992; Miyoshi *et al.*, 1992; 1993), we did not see such channels in inside-out patches in our previous experiments in rabbit portal vein smooth muscle cell (Beech *et al.*, 1993a,b). In the experiments on rat mesenteric artery cells in the absence of prior activation by metabolic inhibition or pina-

cidil in the cell-attached patch mode, when inside-out patches were created into nucleotide-free solution, no channel activity was observed unless NDPs were applied to the inner surface of the patches (Figure 2). However, when the channels were already activated by metabolic inhibition and/or pinacidil in cell-attached patches, channel activity could still be observed briefly after inside-out patches were created (Figure 5b, Figure 6c). However, these channel openings in inside-out patches were unlikely to be due to the absence of ATP since the channels had already been activated by metabolic inhibition and pinacidil in the cell-attached patch and the channel activity in Figure 5b did not increase when an inside-out patch was excised but declined with time. Other groups have also failed to identify K-channel activity, inhibitable by $[ATP]_i$, in inside-out patches in the absence of ATP and NDP in rabbit portal vein cells (Kajioka *et al.*, 1991; Kamouchi *et al.*, 1993) and in rat pulmonary artery cells (Robertson *et al.*, 1992). This is surprising because the K_{ATP} channel is supposed to be closed by ATP in normal cells, and should open in the absence of ATP; this characteristic can be used to define a K_{ATP} channel, in contradistinction to our K_{NDP} channels which do not open simply in the absence of ATP.

One explanation which has been offered for the lack of channel activity in ATP- and NDP-free solution is that the K_{ATP} channel rapidly 'runs-down' due to dephosphorylation of the channel, after patches are excised from the cell, a phenomenon which occurs in cardiac and pancreatic β -cells (Terzic *et al.*, 1994). However, K_{ATP} channels in cardiac and pancreatic β -cells always showed dramatic channel activity immediately after inside-out patches were formed into ATP-free solution, which could last several minutes before the channels ran down (Trube & Hescheler, 1984; Kakei *et al.*, 1985; Findlay & Dunne, 1986; Lederer & Nichols, 1989; Tung & Kurachi, 1991). It is difficult to imagine that the K_{ATP} channels in smooth muscle run down so fast (milliseconds) that we were unable to see their activity.

A second explanation which might be offered is that the K_{ATP} channels in smooth muscle have already been dephosphorylated in the intact cell because $[ATP]_i$ in these cells, for some reason, have become very low. This is unlikely to be true, at least in the present experiments, as we tried very carefully to prevent reduction of the $[ATP]_i$ level and the energy supply in the cells by oxygenating during the whole procedure of cell dispersion and by making the dissection solution ice-cold. The ratio of ATP to ADP in these cells (kindly measured using high performance liquid chromatography (h.p.l.c.) by S.A. Prestwich) was similar to that in fresh tissues which were taken immediately after death (the relative concentrations of ATP and ADP in fresh tissues were 13.3 and 4 nmol mg^{-1} protein respectively; the relative concentrations of ATP and ADP in the cells were 6.13 and 1.27 nmol mg^{-1} protein respectively). Even stronger evidence came from the effects of metabolic poisons applied to whole cells during cell-attached patch recording, which caused the development of a glibenclamide-sensitive K-channel activity in many patches which were otherwise quiescent. This result showed that ATP in these cells was high enough to maintain the K-channels closed and sufficiently phosphorylated (if that is necessary) to allow them to become active upon metabolic inhibition, so they should also open upon forming isolated patches into ATP- and NDP-free solution.

A third explanation, which is the one we prefer and which we have suggested in our previous paper (Beech *et al.*, 1993a), is that intracellular NDPs, instead of ATP, are the crucial regulators of these K-channels in smooth muscle. NDPs as well as ATP are lost when inside-out patches are formed. ATP, more likely binding to the open-state of the channel, inhibits the channel opening once the channel is activated by NDPs. This explanation agrees very well with our results above and there is more evidence supporting this. Firstly, ATP alone had no effects in patches in which UDP was able to activate the channel (Figure 2). However, it is

known that ATP reactivates the K_{ATP} channel in cardiac and pancreatic β -cells and prevents channel run down (Findlay & Dunne, 1986; Misler *et al.*, 1986; Ohno-Shosaku *et al.*, 1987; Takano *et al.*, 1990). Also, the mean open time of the channel activity induced by metabolic poisons in cell-attached patches was much shorter than that in inside-out patches activated by UDP, presumably because the inhibition of the channel by ATP in cell-attached patches was absent in inside-out patches (see also Kajioka *et al.*, 1991). Thus, the channels we describe according to our definitions should be designated K_{NDP} not K_{ATP} .

In cardiac muscle the view is forming that NDPs are important regulators of K_{ATP} channel activity and that the effects of NDPs depend on the operative state of the channel (Terzic *et al.*, 1994). However, cardiac K_{ATP} channels differ from those described in this paper and elsewhere (Kajioka *et al.*, 1991; Robertson *et al.*, 1992; Beech *et al.*, 1993a,b; Kamouchi *et al.*, 1993) in that lowering ATP_i results in a pronounced increase in activity. It seems likely that cardiac K_{ATP} and smooth K_{NDP} channels have some structural similarities but also important differences. It is significant that rat heart K_{ATP} channel mRNA was found to be expressed in a variety of peripheral and central tissues but not in smooth muscles (Ashford *et al.*, 1994).

Working on the same tissue (rat mesenteric artery) and under almost identical conditions (60:120 mM K-gradient; negative holding potentials) as those used in this paper, Standen *et al.* (1989) found an ATP-sensitive K-channel with a conductance of 135 pS. This large conductance channel was sensitive to ATP, activated by cromakalim, and the action of cromakalim was inhibited by glibenclamide. We did not see this channel in the present experiments nor did we see it in rabbit portal vein cells in our previous experiments (Beech *et al.*, 1993a,b). Apart from channel conductance, there are other differences in our channel characteristics, such as long channel opening and noisy channel open state. Conductances of the K-channels sensitive to [ATP]_i in smooth muscle cells, ranging from 7 pA to 257 pA have been reported (Inoue *et al.*, 1989; Kajioka *et al.*, 1990; 1991; Kovacs & Nelson, 1991; Miyoshi & Nakaya, 1991; Lorenz *et al.*, 1992; Bonev & Nelson, 1993), with different channel characteristics such as Ca-sensitivity and voltage-dependence. Although many of

these channels show sensitivity to one or more of the agents ATP, glibenclamide, or KCO, which has led to their designation at K_{ATP} channels, it seems reasonable to suggest that they are not, after all, the same type of channel. An important question which should now be asked is, what are the roles of all these different K channels sensitive to ATP, glibenclamide, or KCOs in smooth muscle?

The results from the experiments of Kajioka *et al.* (1991), Kamouchi *et al.* (1993) and those obtained by us (Beech *et al.*, 1993b) suggest that the K_{NDP} is the target channel KCO in rabbit portal vein smooth muscle cells. These results agree well with the observations that KCO hyperpolarizations and relaxations of intact smooth muscle are blocked by glibenclamide and are unaffected by blockers of BK channels (charybdotoxin or low concentrations of TEA) (Standen *et al.*, 1989; Winquist *et al.*, 1989; Daut *et al.*, 1990; Fujii *et al.*, 1990; Nelson *et al.*, 1990; Grant & Zuzack, 1991; Brayden & Nelson, 1992). The conclusion that a small conductance (about 15 pS under quasi-physiological K gradient) channel is the target for KCOs is consistent with estimates of single channel conductance made from whole-cell current noise (Beech & Bolton, 1989; Noack *et al.*, 1992a,b; Clapp *et al.*, 1994; Criddle *et al.*, 1994). However, although K_{NDP} is most likely the target channel of KCO in smooth muscle, KCOs can also modulate BK_{Ca} channel in isolated patches of smooth muscle cells (Gelband *et al.*, 1989; Klöckner *et al.*, 1989; Carl *et al.*, 1992; Gelband & McCullough, 1993), although this modulation may not be responsible for the effects of KCO on the whole tissue. In the present experiments, we found that pinacidil activated a glibenclamide-sensitive K current in both whole-cells and in patches. The channel activated by pinacidil had apparently similar characteristics to those of the channel activated by NDPs and metabolic poisons. Further, pinacidil potentiated the channel activity activated by NDPs but had no effect on the BK channel. These results suggest that K_{NDP} is the target channel of pinacidil in rat mesenteric artery.

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