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K channel activation by nucleotide diphosphates and its inhibition by glibenclamide in vascular smooth muscle cells

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1 Whole-cell and inside-out patch recordings were made from single smooth muscle cells that had been isolated enzymatically and mechanically from the rabbit portal vein.

2 In whole-cells the inclusion in the recording pipette solution of nucleotide diphosphates (NDPs), but not tri- or monophosphates, induced a K-current that developed gradually over 5 to 15 min. Intracellular 1 mM guanosine 5'-diphosphate (GDP) induced a slowly developing outward K-current at − 37 mV that reached a maximum on average of 72 ± 4 pA ($n = 40$). Half maximal effect was estimated to occur with about 0.2 mM GDP. Except for ADP, other NDPs had comparable effects. At 0.1 mM, ADP was equivalent to GDP but at higher concentration ADP was less effective. ADP induced its maximum effect at 1 mM but had almost no effect at 10 mM.

3 In 14% of inside-out patches exposed to 1 mM GDP at the intracellular surface, characteristic K channel activity was observed which showed long (> 1 s) bursts of openings separated by longer closed periods. The current-voltage relationship for the channel was linear in a 60 mM:130 mM K-gradient and the unitary conductance was 24 pS.

4 Glibenclamide applied via the extracellular solution was found to be a potent inhibitor of GDP-induced K-current ($I_{K(GDP)}$) in the whole-cell. The K_d was 25 nM and the inhibition was fully reversible on wash-out.

5 $I_{K(GDP)}$ was not evoked if Mg ions were absent from the pipette solution. In contrast the omission of extracellular Mg ions had no effect on outward or inward $I_{K(GDP)}$.

6 Inclusion of 1 mM ATP in the recording pipette solution reduced $I_{K(GDP)}$ and also attenuated its decline during long (25 min) recordings.

7 When perforated-patch whole-cell recording was used, metabolic poisoning with cyanide and 2-deoxy-D-glucose induced a glibenclamide-sensitive K-current. This current was not observed when conventional whole-cell recording was used. Possible reasons for this difference are discussed.

8 These K channels appear similar to ATP-sensitive K channels but we refer to them as nucleotide diphosphate-dependent K channels (K_{NDP}) to emphasise what seems to be a primary role for nucleotide diphosphates in their regulation.

Keywords: Smooth muscle; ATP-sensitive K channel; glibenclamide

Introduction

Potassium channels that are inhibited by adenosine 5'-triphosphate (ATP) acting at the cytoplasmic side of the plasma membrane were first observed in cardiac muscle (Noma, 1983). ATP was subsequently found to inhibit K channels in other types of cell (reviewed by Ashcroft & Ashcroft, 1990) and the common theme of ATP-sensitivity has led to a grouping of the channels under the name ATP-sensitive K channels, or K_{ATP}. The name implies a particular significance of ATP but other intracellular factors such as pH, Ca, Mg, nucleotide diphosphates, pyridine nucleotides, adenosine 3':5'-cyclic monophosphate (cyclic AMP) and GTP-binding proteins can also modulate K_{ATP} to varying degrees in different cells (reviewed by Ashcroft & Ashcroft, 1990; Davies *et al.*, 1991). The antidiabetic sulphonylureas (e.g. tolbutamide and glibenclamide) inhibit K_{ATP} and the K channel opener (KCO) drugs (e.g. diazoxide, pinacidil and cromakalim) activate K_{ATP} in some cells (reviewed by Ashcroft & Ashcroft, 1990; 1992; Nichols & Lederer, 1991; Lazdunski *et al.*, 1992).

Although K_{ATP} were not found in smooth muscle when single channel and whole-cell patch-clamp recordings were first introduced (reviewed by Tomita, 1988; Bolton & Beech, 1992) the observation that the smooth muscle relaxant effects of KCOs, an emerging class of drugs (reviewed by Robertson & Steinberg, 1990; Weston & Edwards, 1992), were inhibited by glibenclamide (Quast & Cook, 1989) suggested that K_{ATP} might after all exist in smooth muscle. On the basis that K_{ATP} characteristically open simply in the absence of ATP several attempts were made to identify K_{ATP} in smooth muscle by depleting $[ATP]_i$ in single cells or by forming inside-out patches into ATP-free solution. Glibenclamide-sensitive K-current or hyperpolarization has been observed in single cells expected to be depleted of $[ATP]_i$, suggesting the presence of K_{ATP} (Clapp & Gurney, 1992; Silberberg & van Breemen, 1992; Noack *et al.*, 1992). Single K channel activity has been observed in the absence of $[ATP]_i$ in patches and in some studies clear inhibition by $[ATP]_i$ has been seen (Standen *et al.*, 1989). The channels are infrequently observed, however, and have widely varying properties (see Discussion). Thus it is unclear whether there are different types of K_{ATP} in smooth muscle or if other factors have led to an apparent heterogeneity in experiments so far described.

This study was prompted by our own inability to observe ATP-sensitive K-currents in either whole-cells or isolated patches from smooth muscle cells (Nakao & Bolton, 1991) and by the results of Kajioka *et al.* (1991) which suggested that K channels which did not open simply in the absence of $[ATP]_i$ could be activated by guanosine diphosphate (GDP), but only if pinacidil was present. It is significant that GDP, and other nucleotide diphosphates (NDPs), activate the K_{ATP} of other cell types but in the absence of a KCO drug (Dunne

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& Petersen, 1986; Tung & Kurachi, 1991; Allard & Lazdunski, 1992). We decided to investigate the action of NDPs on smooth muscle at both whole-cell and single channel levels and in doing so we have developed a working hypothesis where K_{ATP}-like channels in smooth muscle do not open simply in the absence of intracellular ATP but depend on the presence of a NDP at the intracellular surface of the membrane. Inability of the channels to open in the absence of $[ATP]_i$ does not exclude them from the K_{ATP} class but we refer to the channels as K_{NDP} to avoid a seemingly undue emphasis on the action of ATP and to recognise the particularly critical role of NDPs in the control of these smooth muscle K channels.

Methods

Single smooth muscle cells were isolated from the rabbit portal vein by a method similar to that previously described (Beech & Bolton, 1989), except the enzymatic incubation medium included collagenase (1 mg ml^{-1}; Sigma type XI) with papain (4 mg ml^{-1}; Sigma; no dithiothreitol added) and the solution was magnesium-free. Cells were stored at 4°C in a quasi-physiological medium containing Ca and Mg (both at 0.2 mM).

Patch-clamp recordings (Hamill *et al.*, 1981) were made from cells within 10 h of the isolation procedure. All recordings were made at room temperature (20–24°C). The patch-clamp amplifier was either an Axopatch-1D (Axon Instr.) or an RK300 (Biologic). Patch pipettes were made from borosilicate glass (Clark Electromedical or Plowden & Thompson); they had resistance of 2 to 4 MΩ after fire-polishing, and were coated with Sylgard (Dow Corning) for single channel recordings. Data were either stored on FM-tape (Racal) or captured directly to a 386 PC after digitization (CED interface and software). All whole-cell current records were filtered at 500 Hz (Bessel) and single channel records as described in the legend of Figure 4. Capacity currents were partially cancelled in the whole-cell recordings.

When the effects of different pipette solutions were compared in whole-cell experiments we carried out a series of experiments where the pipette solutions were used in strict rotation or we observed the effect of 1 mM GDP (with Mg) to evaluate the responsiveness of the cells on a given day. All averages are expressed as mean ± s.e.mean and *t* tests were used to assess statistical significance. 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 all experiments. Junction potentials between path and pipette solutions were measured using a 3 M KCl reference electrode and were < 3 mV; correction for these potentials was not made. The volume of the bath was 150 µl and the flow rate through it about 2 ml min^{-1}. The start of the application of substances is indicated in the figures as the time when the solution reservoirs were switched and complete exchange of the bath was not until about 30 s later. In all figures, except Figure 9b, zero time is when recording in whole-cell mode began.

All solutions (Table 1) were titrated to the specified pH using the hydroxide of the dominant cation. The total K concentration in solution Y with 1 mM GDP added and after titration to pH 7.4 with KOH was 171 mM and the final osmolarity was measured as 290 M Osmol. Nucleotides were added as the Na-salt (2.5 mM Na for 1 mM GDP) and the solution was titrated to pH 7.4, filtered (pore size 0.2 µm; Whatman) and frozen in aliquots at − 20°C. For the inside-out patch experiments 1 mM GDP was added to the bath solution by dilution immediately prior to the application from a 100 mM stock solution of Na-GDP dissolved in bath solution which had been titrated to pH 7.4 and frozen at − 20°C. Nystatin was prepared as a 5 mg ml^{-1} stock in methanol and used at final concentration of 100 µg ml^{-1}. Glibenclamide was prepared as 10 mM stock solution in dimethylsulphoxide (DMSO). The final concentration of DMSO was 0.2% for 20 µM glibenclamide and less for other concentrations. These dilutions of DMSO had no effect on $I_{K(NDP)}$.

The following were used: ADP (adenosine diphosphate), GDP (guanosine diphosphate), TDP (thymidine diphosphate), UDP (uridine diphosphate), IDP (inosine diphosphate), CDP (cytidine diphosphate), GMP (guanosine monophosphate), GTP (guanosine triphosphate), ATP (adenosine triphosphate), AMP (adenosine monophosphate), EGTA (ethylglycol-*bis*-(β-aminoethyl)-N,N,N′,N′-tetraacetic acid), EDTA (ethylenediaminetetraacetic acid), BAPTA (*bis*-(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid) and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid). Most salts were from BDH and other chemicals from Sigma.

Results

Outward current induced by purine and pyrimidine diphosphates (NDPs)

We have concentrated on a search for K channels at a membrane potential of − 37 mV and thus have assumed that they can open near the resting potential. The experimental design was to compare whole-cell recordings under voltage-clamp with or without a nucleotide in the recording pipette solution and determine if current was either induced or inhibited. Pipette solution X or Y included 5 mM EGTA or 10 mM BAPTA respectively and 20 mM HEPES and so it is expected that intracellular Ca and H ions were strongly buffered.

Figure 1 shows membrane current recorded from 12 cells under the same conditions except for the inclusion of different nucleotides in the pipette solution. All cells were voltage-clamped at − 37 mV, about 50 mV positive of the K equilibrium potential (E_K), and averages for all of these recordings are shown in Figure 2. With no nucleotide present the current record usually remained constant at a level within a few picoamperes of zero current, suggesting that K-current did not develop. This result is surprising if it is assumed that K_{ATP} in smooth muscle behave as they do in cardiac muscle and open simply when $[ATP]_i$ decreases (reviewed by Nichols & Lederer, 1991), which is to be expected as endogenous $[ATP]_i$ and substrates for metabolism diffused into the pipette solution (see Discussion). However, also on the basis of results on the cardiac K_{ATP}, it might be that $[ATP]_i$ in the cells was already so low that the channels were dephos-

Table 1 Composition of solutions

Solution	*NaCl*	*KCl*	*$CaCl_2$*	*$MgCl_2$*	*HEPES*	*Glucose*	*EGTA*	*BAPTA*	*pH*
A (bath)	130	5	1.7	1.2	10	10	0	0	7.4
B (bath)	126	6	1.7	1.2	10.5	14	0	0	7.2
X (pipette)	0	130	0	2	20	0	5	0	7.4
Y (pipette)	0	110	0	2	20	0	0	10	7.4
Z (pipette)	0	134	0	1.2	10.5	14	3	0	7.2
E (bath or pipette)	80	60	1.7	1.2	10	10	0	0	7.4
I (bath or pipette)	9	117	0	3	18	0	9	0	7.4

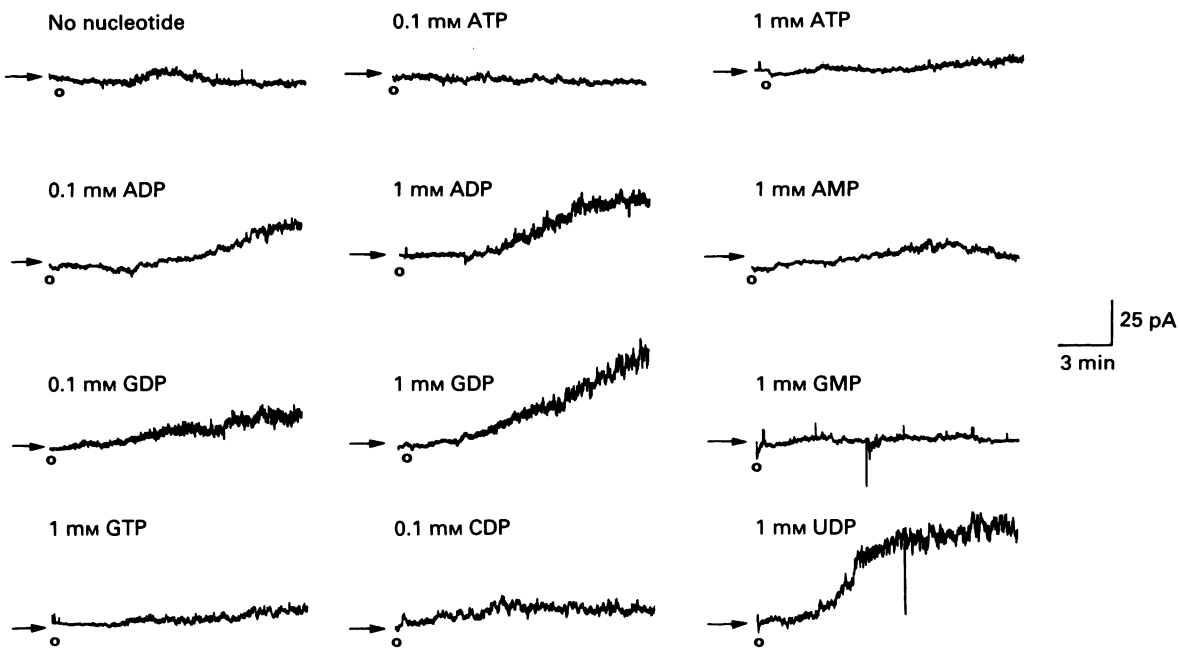

Figure 1 Effects of intracellular nucleotides on membrane current in whole-cells at a holding potential of − 37 mV. The cells were smooth muscle cells isolated from the rabbit portal vein and each recording was from a separate cell. The cell-attached patch was formed, − 37 mV applied to the pipette and then the patch was ruptured to provide access of the whole-cell (time marked as 0). Pipette solution X was used with nucleotides included as indicated and the external solution was A. The arrows mark the position of zero current.

phorylated and thus closed. This explanation was investigated by including 0.1 mM ATP in the pipette which is sufficient to phosphorylate K_{ATP} but not block them completely (Noma & Shibasaki, 1985; Ashcroft & Ashcroft, 1990). Again no appreciable outward current was evoked, the average current was 0.7 ± 0.7 pA ($n = 3$; an example is shown in Figure 1).

The current recordings were strikingly different when a NDP was present in the pipette (Figures 1 and 2). GDP (1 mM), for example, induced a slowly developing outward current that reached a maximum, on average, of 72 ± 4 pA ($n = 40$) after 5 to 15 min. Half maximal effect was estimated to occur with 0.2 mM GDP in the pipette (Figure 2a). ADP was equivalent to GDP at 0.1 mM but was less effective at higher concentrations (Figure 2a). ADP evoked its maximum effect at 1 mM but had almost no effect at 10 mM. Figure 2b shows averages for a series of experiments where the actions of nucleotides were compared in strict rotation to avoid complications of cell-to-cell variation. (This was clearly important because in these experiments the K-current amplitudes were on average less than those observed in other sets of experiments.) ATP (1 mM), GTP (1 mM), AMP (1 mM) or GMP (1 mM) in the pipette did not induce any appreciable outward current (Figures 1 and 2). All NDPs tested induced appreciable outward current and all appeared equally effective at 1 mM except for ADP. Our experiments investigated the effects of NDPs at 0.1 mM and 14–15 min after starting the whole-cell recording outward current at − 37 mV averaged: 8.6 ± 2.8 pA (IDP, $n = 4$); 8.0 ± 2.2 pA (CDP, $n = 4$); 16.0 ± 5.8 pA (TDP, $n = 3$); 6.2 ± 2.8 pA (UDP, $n = 4$). These outward currents were not significantly different from controls (one-tail test, $P > 0.05$).

Figure 3 shows the evidence that the outward current induced by GDP was a K-current. Firstly, raising the external K concentration from 5 mM (E_K − 88 mV) to 60 mM (E_K − 26 mV) changed the current at a holding potential of − 37 mV from outward to inward. Secondly, ramp voltage changes from − 37 mV and − 107 mV in 5 mM external K gave a reversal potential for the GDP-induced current of − 82 mV (Figure 3b), close to the calculated E_K. The reversal potential in 60 mM K was not determined.

Single channel recordings

Single channel studies were carried out to investigate further if GDP did open K channels and to observe the properties of the channels. 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 64 patches held at − 80 mV (an example is shown in Figure 4a). This observation contrasts with that found in the heart where K_{ATP} open readily when patches are excised into ATP-free solution (Kakei *et al.*, 1985; Tung & Kurachi, 1991). However, in 14% of patches (15 out of 109) the application of a solution containing 1 mM GDP to the inner surface of the patch induced characteristic channel activity which showed long bursts of openings (many seconds) separated by even longer closed periods (Figure 4a). Channel activity at various voltages is shown in Figure 4b. It was noticed that in the open-state, current fluctuations were more pronounced when the current direction was inward. This effect resembled that observed for the cardiac K_{ATP} which may reflect a dependence of gating on ion flux (Zilberter *et al.*, 1988), but this phenomenon was not investigated in detail. In cell-attached patches this K channel was only rarely observed (a brief opening occurred in 2 out of 152 recordings). The current-voltage relationship was linear and reversed at − 20 mV (E_K − 19.5 mV), suggesting K ions were the charge carrier (Figure 4b, and Beech *et al.*, 1993). The mean unitary current was 1.5 ± 0.2 pA (13 patches) at − 80 mV, giving a conductance of 24 pS in the 60 mM: 130 mM K-gradient.

Inhibition of GDP-induced K-current $I_{K(GDP)}$ by glibenclamide

Glibenclamide inhibits K_{ATP} in pancreatic β-cells in the nanomolar concentration range and may be a specific inhibitor of this class of channel in several cell types (Ashcroft & Ashcroft, 1992). Figure 5a shows that bath application of glibenclamide did inhibit $I_{K(GDP)}$ in portal vein smooth muscle cells at very low concentrations; 50 nM glibenclamide in the bath solution caused the outward current to decline

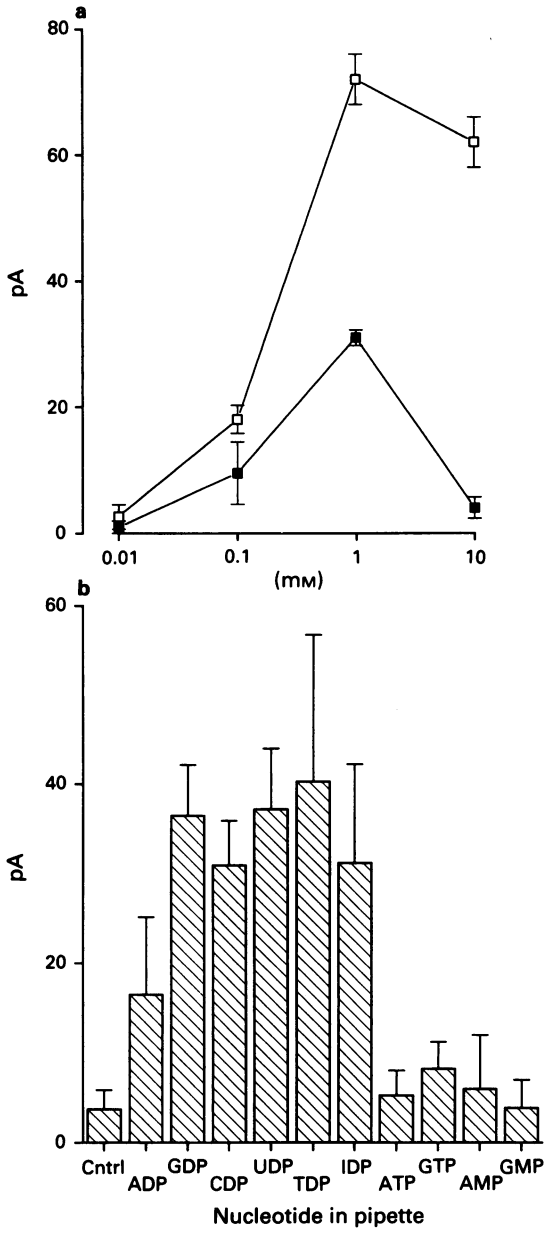

Figure 2 Averages (mean ± s.e.mean) for the outward current occurring in whole-cells loaded with different nucleotides and held at − 37 mV. The outward current was measured 14–15 min after break-through to the whole-cell mode and was defined as that which was blocked by 5 μM glibenclamide (see Figure 5): measurement of net outward current gave a similar result. (a) Concentration-response curves for the peak effects (glibenclamide-sensitive outward current at − 37 mV) of GDP (□) or ADP (■) in the pipette solution ($n = 4$–6). The pipette solution was X or Y (no difference was evident) and the bath solution was A. (b) The pipette solution X and bath solution A were used. The pipette solution included 1 mM of: ADP ($n = 4$); GDP ($n = 4$); TDP ($n = 3$); UDP ($n = 4$); IDP ($n = 4$); CDP ($n = 4$); GMP ($n = 4$); GTP ($n = 4$); ATP ($n = 4$); AMP ($n = 4$); control (no nucleotide, $n = 6$). The current amplitudes with GDP, TDP, UDP, IDP and CDP in the pipette were significantly different from the control at $P < 0.05$ and using a two-tailed test, the current amplitudes in cells held with ADP, ATP, GTP, AMP and GMP were not significantly different from control group.

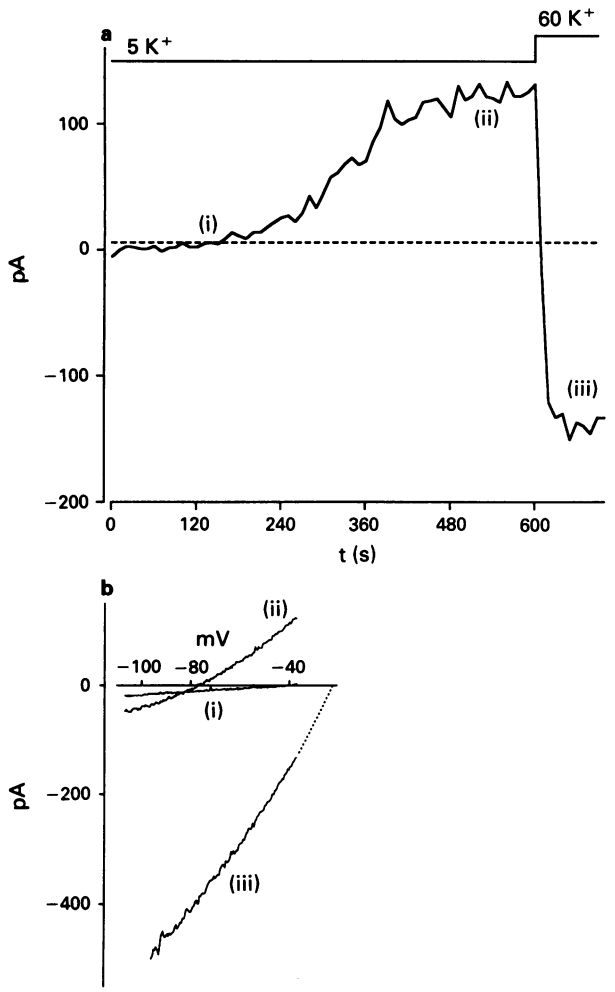

Figure 3 The outward current induced by GDP is a K-current. (a) Whole-cell recording of current at − 37 mV when the pipette solution Y contained 1 mM GDP. The bath solution was A initially and then the external [K] was raised as indicated (solution E). (b) Current in response to linear ramp (80 ms duration) changes in membrane voltage from the holding potential of − 37 mV to − 107 mV: (i) an average of 3 current records captured at 10 s intervals starting 1.3 min after break-through to the whole-cell mode; (ii) a single current record taken 9.5 min after break-through. Current records (i) and (ii) intersected at − 82 mV. Assuming a − 3 mV junction potential the reversal potential for the GDP-induced current was − 85 mV (calculated E_K was − 88 mV). Record (iii) was in 60 mM $[K]_o$ and the extrapolation (dotted line) is the Goldman-Hodgkin-Katz current equation.

from 62 pA to 34 pA and raising the concentration to 5 μM reduced the current to about zero (− 3 pA). At 20 μM glibenclamide had no further effect and wash-out of glibenclamide from the bath allowed a recovery of the current to 58 pA. When nucleotides were omitted from the pipette, glibenclamide had only small effects on membrane current (Figure 2b). A concentration-response curve is shown in Figure 5b for glibenclamide inhibition of $I_{K(GDP)}$. The Hill equation was fitted by eye with a slope of 1 and the IC_{50} (K_d) was 25 nM.

Dependence on Mg ions

Although the work of Kajioka *et al.* (1991) suggested that GDP opened K channels in the absence of Mg ions we investigated this possibility because the action of NDPs on K_{ATP} in other types of cell does require Mg ions (e.g. Tung & Kurachi, 1991) and because quite low concentrations of Mg are required for the activity of some enzymes (Sun *et al.*, 1990).

Three 1 mM GDP-containing pipette solutions were compared alternately in different cells: (Ya) included 3 mM Mg; (Yb) included 4 mM Mg and 2 mM EDTA; (Yc) had no Mg added and included 2 mM EDTA. All solutions contained 10 mM BAPTA and no added Ca. Figure 6a shows typical recordings for pipette solutions Yb and Yc, and Figure 6b

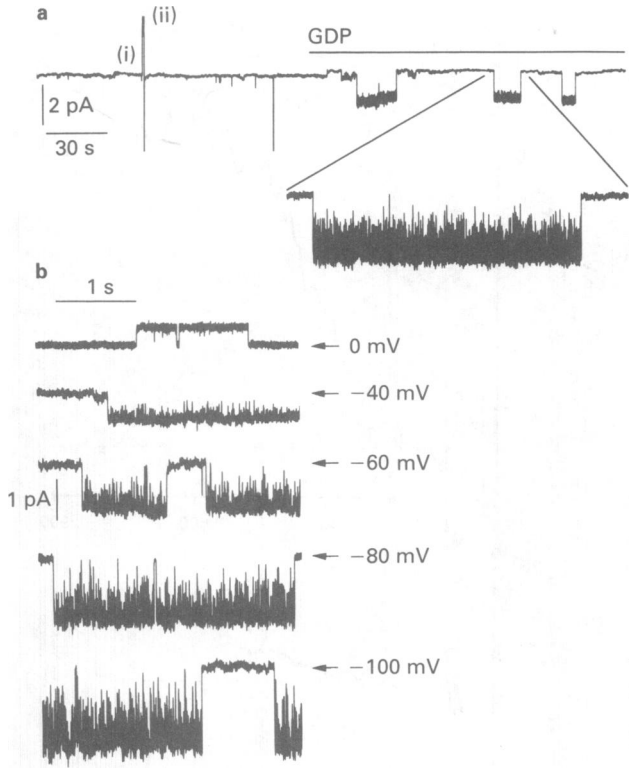

Figure 4 Inside-out patch recordings showing K channels activated by GDP. (a) A continuous recording of current in a patch held at − 80 mV with solution E in the pipette and solution I in the bath. Initially the patch was cell-attached and then it was excised (i) into the ATP- and GDP-free solution and brief test voltage steps applied to 0, 80 and 0 mV (ii) to test for activity of large Ca-activated K-channels and confirm the patch was inside-out. GDP (1 mM) was applied via the bath solution as indicated and 3 long bursts of channel openings were observed. The continuous trace was recorded 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 50 Hz. The expanded segment was amplified 2 times and filtered again at 0.5 kHz. (b) In a different patch, the GDP-activated channel at 0, − 40, − 60, − 80, − 100 mV. The current level when the channel was not open is marked with arrows. A plot of unitary current amplitude against voltage (not shown, but see the following paper) showed that the current reversed at − 20 mV, suggesting the channels were K-selective. The unitary conductance of the channel was 24 pS.

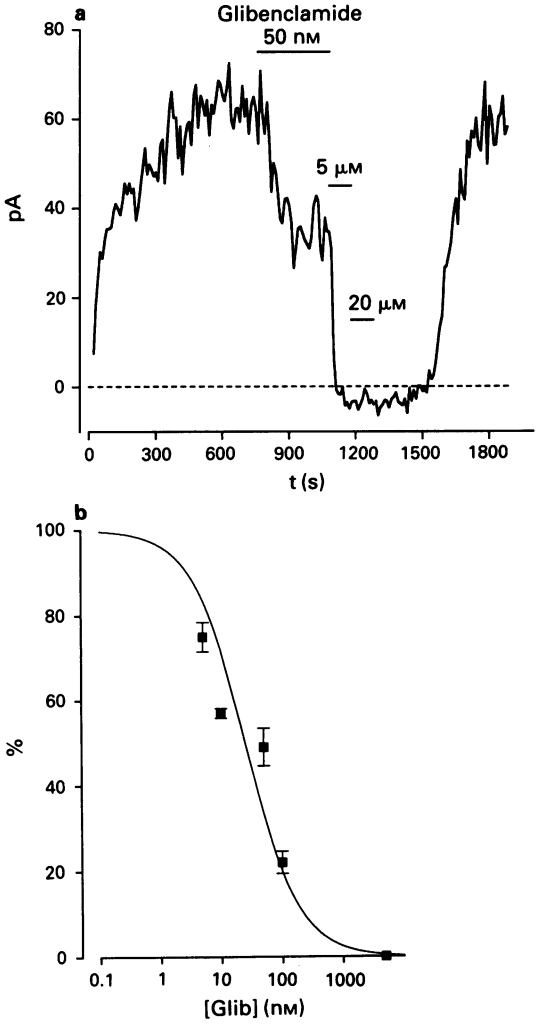

Figure 5 Inhibition of $I_{K(GDP)}$ by glibenclamide. (a) Plot of membrane current sampled every 10 s at − 37 mV. GDP (10 mM) was in the pipette solution X. Application of glibenclamide (50 nM, then 5 and 20 μM) inhibited $I_{K(GDP)}$ and after returning to glibenclamide-free solution current returned. Ramp voltage changes (as in Figure 3b) were used to measure the reversal potential of the glibenclamide-sensitive current and it was found to be close to − 80 mV (not shown) and thus close to E_K. (b) Plot of the mean (± s.e.mean; $n = 3$–16) percentage of $I_{K(GDP)}$ remaining in the presence of various concentrations of glibenclamide. The smooth function is the Hill equation with slope 1 and mid-point 25 nM (fitted by least squares method).

shows the averages for all of the experiments. It seemed that Mg ions were required for $I_{K(GDP)}$ and that EDTA did not have a blocking action when Mg ions were present in excess. In contrast, external Mg ions seemed of no importance for $I_{K(GDP)}$. Figure 7a shows that $I_{K(GDP)}$ occurred normally in the absence of Mg from the bath solution and that addition of Mg ions did not block the current. Mg ions were also unimportant when $I_{K(GDP)}$ was inward and thus more likely to be affected by an external cation (Figure 7b).

Effects of intracellular ATP

Although the K channels we observed did not open simply in the absence of $[ATP]_i$ in inside-out patches, which contrasts with the observations made on K_{ATP} for example in the heart (Kakei *et al.*, 1985), we looked for an effect of $[ATP]_i$ when the channels were opened by GDP in the whole-cell. To investigate if an effect of ATP might occur because Mg was chelated (the pK for Mg binding to ATP is near 4; Sillen & Martell, 1964), three pipette solutions were compared alternately: (YA) 2 mM Mg and 1 mM GDP; (YB) 3 mM Mg and 1 mM GDP; (YC) 3 mM Mg, 1 mM GDP and 1 mM ATP. This experimental design relies on the assumption that 1 mM ATP reduces free Mg ions from 3 mM to not less than 2 mM and considers principally the effect of Mg-ATP. *In vivo*, $[Mg]_i$ may be lower than in our experiments, perhaps 0.3 mM (Nakayama & Tomita, 1991), and so more of the ATP may be free. In cardiac and skeletal muscle cells K_{ATP} channels are roughly equally susceptible to inhibition by free ATP and Mg-ATP but in pancreatic β-cells free ATP seems a more potent inhibitor (Ashcroft & Ashcroft, 1990).

Figure 8 shows typical experiments with the YB and YC pipette solutions. Both outward (5 mM external K) and inward (60 mM external K) $I_{K(GDP)}$ were measured in the absence and presence of 5 μM glibenclamide. $I_{K(GDP)}$ measured about 10 min after starting the whole-cell recording was less when ATP was in the pipette and the averages suggested $I_{K(GDP)}$ was reduced by 64% (Figure 8c). Reducing Mg from 3 to 2 mM (in the absence of ATP) did not affect the amplitude of outward $I_{K(GDP)}$ (100 ± 18 pA to 94 ± 9 pA; $n = 4$ and 5 respectively) or inward $I_{K(GDP)}$ (122 ± 8 pA to 127 ± 12 pA; $n = 4$ and 5 respectively). It was noted that $I_{K(GDP)}$ declined

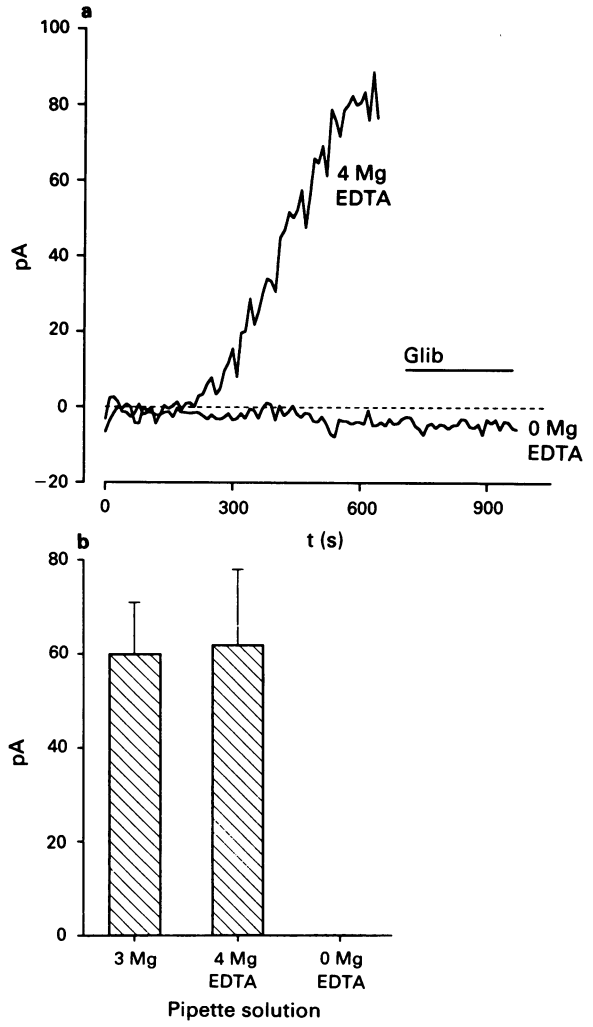

Figure 6 Dependence of $I_{K(GDP)}$ on intracellular Mg ions. Whole-cell current was sampled every 10 s at a holding potential of − 37 mV. The bath solution was A and the pipette solution Y with: (Ya) 1 mM GDP plus 1 mM extra $MgCl_2$ (total Mg = 3 mM); (Yb) 2 mM extra $MgCl_2$ and 2 mM EDTA; (Yc) 2 mM EDTA and with $MgCl_2$ omitted. (a), An experiment with pipette solution Yb compared with one using solution Yc. Glibenclamide (5 μM) was bath-applied as indicated. (b) Means (± s.e.mean, $n = 4-5$) for the three pipette solutions.

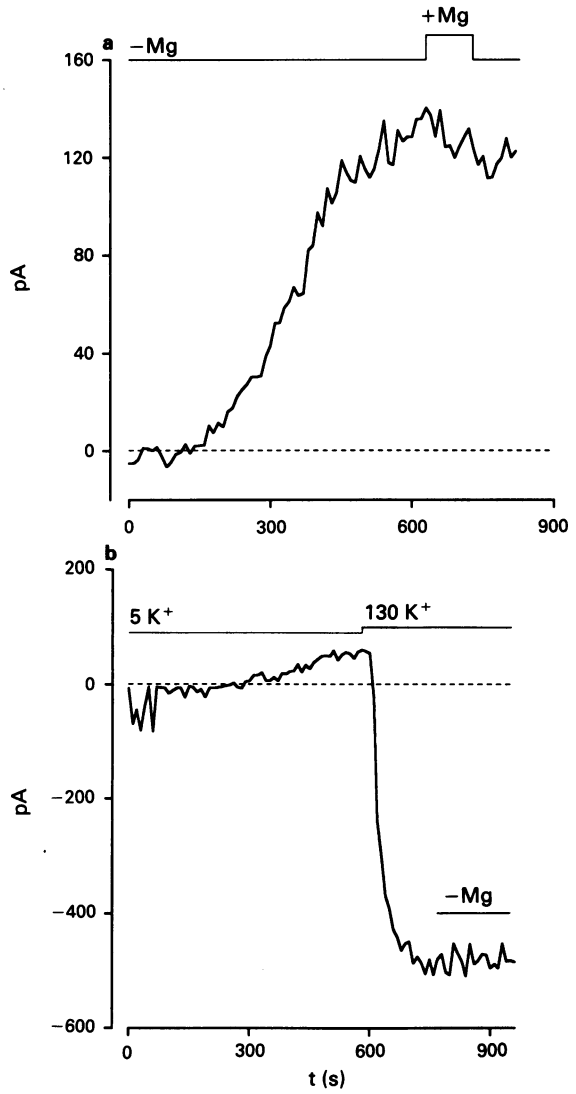

Figure 7 Dependence of $I_{K(GDP)}$ on external Mg ions (Mg was present in the pipette solution). (a) Whole-cell current sampled every 10 s at a holding potential of − 37 mV. Pipette solution Y contained 1 mM GDP plus 1 mM extra Mg (total Mg = 3 mM). Initially Mg was omitted from the bath solution A and then at 10 min $MgCl_2$ (1.2 mM) was added as indicated. (b) Holding current sampled every 10 s at − 37 mV. Pipette solution Y contained 1 mM GDP plus 1 mM extra Mg. The recording was initiated in the presence of 1.2 mM in bath solution A and then the external K concentration was raised from 5 mM to 130 mM (KCl replaced NaCl in solution A) to produce an inward current through the GDP-activated K channels. The same solution but without Mg ions was then applied and no change in current occurred. In all of these experiments Mg ions were simply omitted from the bath solution and EDTA was not included.

slowly after reaching a maximum in long (25 min) whole-cell recordings without ATP in the pipette solution but that with ATP present $I_{K(GDP)}$, although smaller, was better maintained (Figure 8a and b). Measured 25 min into the whole-cell recording $I_{K(GDP)}$ was 45.0 ± 5.6 pA ($n = 4$) for pipette solution YB and 53.3 ± 7.9 pA ($n = 4$) for pipette solution YC ($[K]_o$ was 5 mM).

Metabolic poisoning

In all of the experiments described so far glucose was present in the extracellular solution. Although the cells were expected to be able to produce ATP in this condition their ability to maintain a normal level may have been reduced when the whole-cell pipette solution contained a low concentration of ATP or no ATP (see Discussion). In an attempt to determine if production of ATP inside the cell was of consequence we removed glucose from the bath solution and replaced it with 2-deoxy-D-glucose (2-DG) to prevent glycolysis and added cyanide (CN) to inhibit aerobic metabolism.

Figure 9a shows a whole-cell recording where the pipette solution contained no NDP. This pipette solution had little effect on the holding current at − 37 mV and the subsequent removal of glucose and application of 10 mM 2-DG and 5 mM CN also did not alter the holding current. Separate experiments were carried out to compare the input resistance of cells under severe metabolic deprivation (a), or with a very high concentration of intracellular ATP (b). The conditions were: (a) pretreatment for more than 30 min with 6 mM CN and 14 mM 2-DG in the bath solution and recording with a pipette solution containing 14 mM 2-DG; (b) incubation of cells in 14 mM glucose and recording with a pipette solution containing 14 mM glucose and 10 mM Mg-ATP. Under condition (a) the input resistance was 3.18 ± 0.18 GΩ ($n = 9$) and under condition (b) it was 3.21 ± 0.23 GΩ ($n = 5$). In some cells under condition (a) 10 mM caged-ATP was included in the pipette solution (see Methods in the following

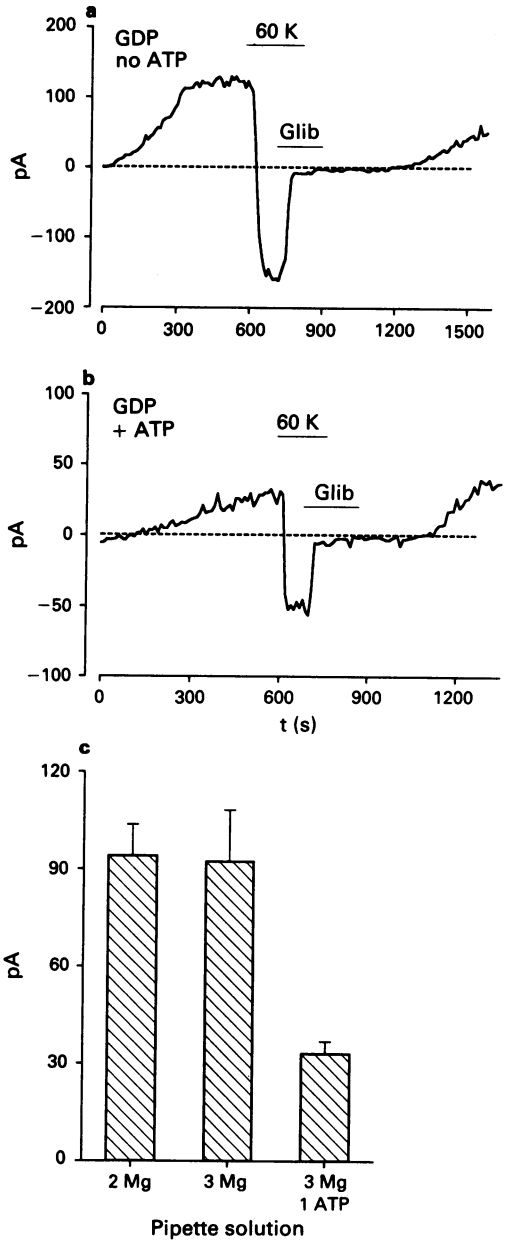

Figure 8 Effects of $[ATP]_i$ on $I_{K(GDP)}$. Whole-cell current was sampled every 10 s at − 37 mV with 1 mM GDP in pipette solution Y. (a) The pipette solution had added a total of 3 mM $MgCl_2$. The external K concentration was raised from 5 mM (solution A) to 60 mM (solution E) and 5 μM glibenclamide was applied in the bath as indicated. (b) The same experimental procotol was used as in (a) but the pipette solution included 1 mM Na-ATP in addition to the 1 mM GDP and 3 mM $MgCl_2$. (c) Averages (mean ± s.e.mean; $n = 5$) for a series of experiments on cells held at − 37 mV for 10 min with 1 mM GDP in the pipette plus: (YA) 2 mM Mg; (YB) 3 mM Mg; (YC) 3 mM Mg and 1 mM Na-ATP. Outward current recorded with pipette solution YC was significantly less than that recorded with pipette solution YA ($P < 0.01$, two-tailed test). Inward $I_{K(GDP)}$ (60 mM external K) was also inhibited by ATP: with pipette solution (YB) the current was − 122.3 ± 7.8 pA ($n = 4$) and with pipette solution (YC) it was − 47.5 ± 3.9 pA ($n = 4$).

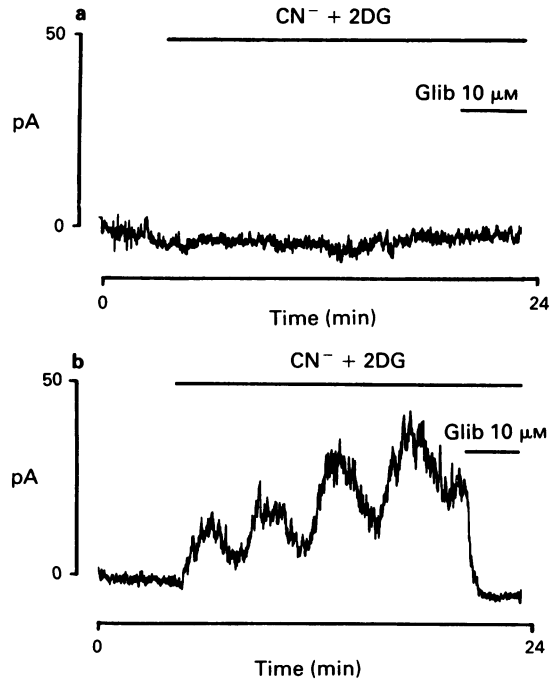

Figure 9 Effects of metabolic poisoning on membrane current. (a) A conventional whole-cell recording of membrane current at − 37 mV. Pipette solution X was used without a nucleotide and initially 10 mM glucose was present in the bath (solution A). As indicated, the bath solution was changed so that glucose was absent and 2 mM cyanide and 5 mM 2-deoxy-D-glucose were included. No outward current occurred and glibenclamide had no effect. (b) A nystatin-mode whole-cell recording at a holding potential of − 37 mV. As indicated, the bath solution was changed so that glucose was absent and 2 mM cyanide and 5 mM 2-deoxy-D-glucose were included. An undulating outward current developed that was inhibited by 10 μM glibenclamide.

paper for details). A standard pulse of u.v.-light from a xenon flash lamp was estimated to release about 1 mM free ATP from the caged precursor but the flash had no effect on membrane current in cells loaded with caged-ATP ($n = 4$; not shown).

In contrast, metabolic poisoning in perforated-patch whole-cell recordings (Horn & Marty, 1988) did affect membrane current. Figure 9b shows a typical perforated-patch whole-cell recording where there was no change in holding current in the presence of glucose but removal of glucose and the application of 2-DG and CN caused a pronounced outward current that undulated slowly. Bath application of 10 μM glibenclamide abolished this current. The same procedure induced a similar outward current in all 7 cells studied. Glibenclamide (100 nM) was applied to 3 of these cells and it was observed that the undulations ceased and that the current level was reduced to a steady level; 10 μM glibenclamide reduced the outward current further to near zero current. Although it proved difficult to quantify the effect of glibenclamide in these perforated-patch whole-cell recordings it seemed that the 2-DG/CN-induced current showed a sensitivity to glibenclamide that was similar to that of $I_{K(GDP)}$ recorded in the conventional whole-cell (Figure 5b), suggesting that K_{NDP} carried both currents.

Discussion

We have observed a class of small conductance K channels (K_{NDP}) that opened when NDPs were present with Mg ions at the intracellular surface of the plasma membrane of smooth muscle cells. K-current through K_{NDP} in the whole-cell was found to be sensitive to inhibition by nanomolar glibenclamide. It is our working hypothesis that NDPs are a crucial regulator of these channels and that ATP effects are only of consequence once NDPs have opened the channel. Although K_{NDP} did not open simply in the absence of $[ATP]_i$ in other regards they showed several similarities to the ATP-sensitive K channels (K_{ATP}) of other cell types.

The interpretation of our whole-cell experiments depends partly on the changes which might be supposed to occur in

the intracellular ATP concentration. We describe here our working hypothesis for the changes and absolute values of $[ATP]_i$ in whole-cells which is firstly consistent with our inside-out patch experiments that suggested K channels did not open simply in the absence of ATP, and secondly explains the observation that metabolic poisoning did not affect membrane current during conventional whole-cell recording. We cannot be certain of the total concentration of ATP in the cell or of the concentration close to a channel but we estimate here what the reasonable limits for $[ATP]_i$ might have been. We know that smooth muscle cells in the intact tissue have an $[ATP]_i$ in the region of 3 to 5 mM (van Breemen *et al.*, 1975; Ishida & Paul, 1990) and so the value in an isolated cell maintained in glucose may be similar and certainly more than 1 mM. Therefore, after breakthrough to the whole-cell recording mode without ATP in the pipette solution, $[ATP]_i$ may decline from 1 mM as ATP and some substrates for metabolism diffuse into the pipette. That $[ATP]_i$ was < 1 mM 10 min into a whole-cell recording was suggested by the observation that 1 mM ATP in the pipette solution inhibited $I_{K(GDP)}$ at this time (Figure 8). Therefore, we estimate that $[ATP]_i$ may have been between 1 and 100 μM after 10 min of whole-cell recording. Metabolic poisoning would cause $[ATP]_i$ to decline further and initially $[NDP]_i$ to increase relative to $[ATP]_i$. The maximum rise of $[NDP]_i$ relative to $[ATP]_i$ on metabolic poisoning is uncertain but $[NDP]_i$ could rise to 15% of $[ATP]_i$ (Ishida & Paul, 1990). On this basis, in our whole-cell recordings $[NDP]_i$ would not become more than 15 μM and $I_{K(NDP)}$ would be small or insignificant (Figure 2a). In perforated-patch whole-cell recordings $[ATP]_i$ would not decline as a result of diffusion into the pipette and metabolic poisoning might cause NDPs to rise to 150 μM or more and so induce $I_{K(NDP)}$. Silberberg & van Breemen (1992) also observed glibenclamide-sensitive K-current in response to metabolic poisoning of mesenteric artery smooth muscle cells from which recordings were made by the perforated-patch whole-cell method. Our result is similar except we observed undulations in the K-current, the mechanism of which is being investigated. The physiological significance of ATP effects on K_{NDP} is uncertain. The slope of the concentration-inhibition curve for Mg-ATP against K_{NDP} seems likely to be in the millimolar range (Figure 8) and so it might be that slight changes in the normal ATP concentration (3–5 mM) will have important effects on K_{NDP} channel activity if it has already been induced by a NDP. However, because $[ATP]_i$ tends to be quite resistant to change (van Breemen *et al.*, 1975) it might be that $[ATP]_i$ is relatively unimportant for regulation and instead it is changes in $[NDP]_i$ in the threshold region for channel activation that are of most significance. The latter interpretation also seems to be favoured by Pfründer *et al.* (1993) after a recent study on guinea-pig portal vein smooth muscle cells.

The K_{ATP} of cardiac muscle exhibit a high opening probability when patches are excised into ATP-free solution and a large K-current is induced in whole-cells when $[ATP]_i$ is depleted (Kakei *et al.*, 1985; Noma & Shibasaki, 1985). These effects did not occur in our recordings from smooth muscle or in those of others (Robertson *et al.*, 1992; Kamouchi *et al.*, 1993). With reference to work on other cell types it might be suggested that this difference occurred because K_{NDP} became dephosphorylated particularly quickly when $[ATP]_i$ was low. (Dephosphorylation is the proposed mechanism for 'run-down' of K_{ATP} which occurs over several minutes in inside-out patch excised from cardiac muscle in the absence of ATP.) However, we argue that the smooth muscle channels are closed in the absence of ATP not because they are dephosphorylated but because this is the state the channels adopt unless an agonist (e.g. GDP) is present. This is not only a simpler interpretation of our data but one that is supported by two key observations: (a) K channels did not open even within a few milliseconds after an inside-out patch was formed and patches formed in 10 μM ATP did not reveal channel activity (not shown); (b) whole-cell experiments with 100 μM ATP and no GDP in the pipette did not reveal K-current (Figure 1) and yet this concentration of ATP was not expected to inhibit $I_{K(GDP)}$ strongly (Figure 8) and is known to be sufficient for phosphorylation of K_{ATP} (Ashcroft & Ashcroft, 1990) so K_{ATP} channels should open.

Clapp & Gurney (1991) and Noack *et al.* (1992) have attempted to deplete $[ATP]_i$ in smooth muscle cells during whole-cell recording and have found evidence for glibenclamide-sensitive K-current or hyperpolarization. Although these experiments were on different smooth muscles from the one we used and so the channel properties may be different it is possible to interpret their data using our hypothesis for the control of K_{NDP}. Clapp & Gurney (1991) recorded from pulmonary artery smooth muscle cells with 11 mM glucose in the external solution and compared the effects of pipette solutions with and without ATP (1–3 mM) on membrane potential. When ATP was omitted the cells were more hyperpolarized and 1 μM glibenclamide depolarized them on average by 15 mV. We calculate that 3 pA of glibenclamide-sensitive K-current would have occurred at −40 mV and find it plausible that residual NDPs in the cell or GDP formed from the GTP (see Kajioka *et al.*, 1991) loaded into the cell from the whole-cell pipette (0.5 mM GTP was present) could have been enough to induce $I_{K(GDP)}$. Working on rat portal vein smooth muscle Noack *et al.* (1992) found a transient outward current (I_{met}) in some whole-cells held at −50 mV in the absence of ATP and metabolic substrates. A small transient outward current resembling I_{met} occurred in some of our whole-cell recordings from rabbit portal vein smooth muscle cells without nucleotide in the pipette (in the 9 cells in which it occurred its maximum amplitude was 10 ± 11 pA at 6 min after break-through to the whole-cell; Figure 1). In guinea-pig portal vein smooth muscle cells, the absence of metabolic substrates and of ATP in the pipette did not induce glibenclamide-sensitive outward current (Pfründer *et al.*, 1993). I_{met} in rat portal vein smooth muscle was inhibited by 1 μM but not 100 nM glibenclamide (Noack *et al.*, 1992). An explanation for I_{met} could be that $[NDP]_i$ was high before break-through to the whole-cell because the cells were already metabolically compromised and the transient time-course of I_{met} might have reflected complex changes in intracellular K, ATP, ADP and other NDPs. Dephosphorylation of the K channels underlying I_{met} seems unlikely, however, because the decline in I_{met} was unaffected by 18.7 μM ATP in the pipette. A comparison between rat and rabbit portal veins should be made cautiously because the underlying channels may be different (Kajioka *et al.*, 1990; 1991; see below).

The single channel observations suggest ATP-sensitive K channels in smooth muscle can be divided into 3 groups: (i) large conductance channels; (ii) small conductance channels opening without NDPs; (iii) small conductance channels that require NDPs. (i) Standen *et al.* (1989) observed 135 pS K channels (60 mM:120 mM K-gradient) in inside-out patches from mesenteric artery smooth muscle cells. These channels were clearly inhibited by ATP with an IC_{50} of 50–300 μM and were Ca- and voltage-insensitive. They were not demonstrated to be sensitive to glibenclamide in the absence of cromakalim. Similar K channels from the aorta have been observed in lipid bilayers (Kovacs & Nelson, 1991). The K channels observed by Lorenz *et al.* (1992) were also of large conductance but they were activated by depolarization and were inhibited by 1 μM glibenclamide. (ii) Kajioka *et al.* (1990) observed 10 pS K channel activity (6 mM:138 mM K-gradient) in outside-out and inside-out patches from rat portal vein smooth muscle cells. NDPs and glibenclamide were not tested but the channel opened in the absence of $[ATP]_i$ if 1 μM Ca_i was present and this activity could then be inhibited by 5 mM Na-ATP_i but not by Mg-ATP_i. These channels appear similar to those observed by Wakatsuki *et al.* (1992) in patches from cultured coronary artery smooth muscle cells ($[Ca]_o$ 0.1 mM), which were inhibited by 30 μM glibenclamide.

(iii) Kajioka *et al.* (1991) and Kamouchi *et al.* (1993) observed a Ca- and voltage-insensitive 15 pS K channel (6 mM:140 mM K-gradient) in cell-attached and inside-out patches from rabbit portal vein smooth muscle cells. This channel only opened when >3 μM pinacidil (or LP-805) was present and was inhibited completely by 100 μM glibenclamide. Even in the presence of pinacidil the channel activity disappeared on forming an inside-out patch and or an open-cell patch into ATP-free solution and could not be reactivated by 1 mM Mg-ATP_i. However, the channels were clearly activated in inside-out patches (pinacidil present) if 1 mM GDP_i was applied. Subsequent application of Na-ATP_i inhibited the channels potently (IC_{50} 29 μM), despite the presence of pinacidil (cf. Fan *et al.*, 1990); Mg-ATP was found to be a less effective inhibitor, producing about 65% inhibition at 1 mM.

The small conductance GDP-dependent K channel we observed seems most similar to that described by Kajioka *et al.* (1991). However, there are a number of differences in properties between the whole-cell K-currents we observed and those of the single K channel currents studied by Kajioka *et al.* (1991), viz: ADP evoked whole-cell current in our experiments but not channel openings in theirs; our GDP-induced current required Mg ions but their K channel activity did not; GDP alone evoked whole-cell current or single channel activity in our experiments but single channel opening was seen in theirs only when pinacidil was also present, our GDP-evoked currents were shown to be 1000 times more sensitive than theirs to glibenclamide. The explanations for these differences are not clear but they may be due to differences between the whole-cell and isolated patch recording modes and reflect difficulties experienced in detecting the channels in patches and then studying their regulation in detail. Nevertheless, our results and those of Kajioka *et al.* (1991) and of Kamouchi *et al.* (1993) support the conclusion that the K_{ATP}-like channels of these smooth muscle cells do not open simply in the absence of ATP and that the reason for this absence of activity is not because the channels have become dephosphorylated but because they adopt the closed state unless NDPs are present.

Physiological roles for K_{NDP} channels are indicated by the action of glibenclamide. On the assumption that low concentrations of glibenclamide (we suggest <1 μM) specifically inhibit K_{NDP} channels it seems that the channels may underlie some of the effects on smooth muscle of hypoxia (Daut *et al.*, 1990; Lydrup & Hellstrand, 1991), endothelium-derived hyperpolarizing factor (Brayden, 1990), nitric oxide (Garland & MacPherson, 1992) and peptide neurotransmitters (Nelson *et al.*, 1990). The effects of metabolic poisoning or deprivation on electrical activity in smooth muscle appear complex, perhaps because many membrane proteins are affected by changes in nucleotide levels. In the rat portal vein glucose-removal caused a small depolarization but if cyanide was applied in addition there was a pronounced hyperpolarization (Ekmeharg, 1989). The significance of K_{NDP} channels will need to be addressed carefully and in the context of other membrane effects in a given smooth muscle type. Pharmacologically the identification of K_{NDP} could be important if the channels are the target for the hyperpolarizing action of KCO drugs (see the following paper, Beech *et al.*, 1993). In addition, however, it is not inconceivable that as the properties of K_{NDP} channels are better understood and their role more clearly defined that a wider therapeutic potential will be realised for selective inhibitors and activators of these channels.

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