Activation of ATP-dependent K^+ channels by hypoxia in smooth muscle cells isolated from the pig coronary artery

C. Dart and N. B. Standen

Ion Channel Group, Department of Cell Physiology and Pharmacology, University of Leicester, PO Box 138, Leicester LE1 9HN, UK

1. The perforated patch technique with amphotericin B was used to record whole-cell currents activated by hypoxia in smooth muscle cells, isolated enzymatically from pig coronary arteries.

2. Superfusion with hypoxic solution (O_2 partial pressure, 25–40 mmHg) activated an inward current at −60 mV in 143 mM extracellular K^+. The reversal potential of the current induced by hypoxia shifted with extracellular $[K^+]$ as expected for a K^+ current, while its current–voltage relation was consistent with the channels showing little voltage dependence.

3. The hypoxia-induced current was inhibited by glibenclamide (10 μM), but was unaffected by charybdotoxin (50 nM).

4. In whole-cell recordings at −60 mV in 143 mM K^+ solution, openings of single channels passing a current close to −2 pA could sometimes be detected in normoxic solution. Openings became more frequent during the onset of the response to hypoxia, when several levels could be detected. Channels with a similar conductance were activated by hypoxia in cell-attached patches.

5. Our results suggest that hypoxia activates ATP-dependent K^+ channels. We discuss possible mechanisms by which this activation may occur.

In the normal myocardium, coronary blood flow is regulated so that oxygen delivery to the working heart precisely matches its metabolic needs. A local fall in oxygen tension, for example, as a result of increased oxygen consumption in the heart muscle, causes the coronary arteries to dilate, thereby increasing both blood flow and oxygen supply. The ability of the heart to regulate blood flow in the coronary circulation is of fundamental importance. Any significant imbalance between oxygen delivery and oxygen demand can lead to conditions such as angina pectoris and, in extreme cases, myocardial infarction.

There are several routes by which a reduction in oxygen tension could cause coronary arteries to dilate. (i) The oxygen tension could act directly on smooth muscle cells of the coronary arteries, leading to relaxation. (ii) Reduced intravascular O_2 tension could cause the release of vasodilator substances such as nitric oxide or prostaglandins from endothelial cells lining the arteries. (iii) Cardiac myocytes could release vasodilator metabolites, such as adenosine (Berne, 1980), which then act on coronary smooth muscle (or endothelium) leading to dilatation. Clearly, these routes are not mutually exclusive, and each could exert part or all of its dilator effect through membrane hyperpolarization caused by activation of K^+ channels.

Several studies using isolated hearts have suggested that the activation of ATP-dependent K^+ channels (K^+_{ATP} channels) provides a major mechanism for hypoxic vasodilatation, since such vasodilatation is blocked by the sulphonylurea K^+_{ATP} channel blocker glibenclamide and mimicked by K^+ channel openers (Daut, Maier-Rudolph, von Beckerath, Mehrke, Günther & Goedel-Meinen, 1990; von Beckerath, Cyrys, Dischner & Daut, 1991; Nakhostine & Lamontagne, 1993). K^+_{ATP} channels form targets for a number of vasodilators, their activation leading to membrane hyperpolarization and a reduction in contractile tone (Standen, Quayle, Davies, Brayden, Huang & Nelson, 1989; Quayle & Standen, 1994). We have recently shown that adenosine can activate K^+_{ATP} channels of isolated pig coronary smooth muscle cells (Dart & Standen, 1993), and adenosine release is suggested to play a major role in hypoxic vasodilatation of the rabbit heart (Nakhostine & Lamontagne, 1993). In guinea-pig heart, however, though adenosine may contribute to hypoxic vasodilatation, it appears not to be the principal mediator during the crucial early phase of hypoxia, while

release of endothelial vasoactive compounds also does not contribute significantly to hypoxia-induced vasodilatation (von Beckerath *et al.* 1991). Therefore, von Beckerath *et al.* (1991) suggest that early hypoxic vasodilatation results from activation of K^+_{ATP} channels as a direct consequence of changes in energy metabolism within the coronary smooth muscle cells.

In the present study, we have investigated directly whether hypoxia can activate K^+_{ATP} channels in isolated coronary smooth muscle cells where effects of substances released from either endothelial or cardiac muscle cells are eliminated. We have used the perforated patch technique to record currents from single smooth muscle cells isolated from small-diameter vessels of the pig coronary artery. We have found that reduced oxygen levels are in themselves sufficient to activate a K^+ current, and that this effect can be inhibited by glibenclamide. Our results provide evidence, at the single-cell level, that hypoxia directly causes the activation of K^+_{ATP} channels within the coronary circulation.

A brief report of some of these findings has been presented to the Physiological Society (Dart & Standen, 1994).

METHODS

Preparation and cell isolation

Fresh pig hearts were collected from a local abattoir and transported to the laboratory in ice-cold buffer of composition (mM): NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; glucose, 10; pH adjusted to 7·4 at 4 °C using NaOH. The left descending coronary arteries were dissected out and cleaned of blood and connective tissue. By following the branching of the arteries that run parallel to the heart surface, it was possible to successfully dissect out vessels of relatively small diameter. The coronary arteries used in the present study had diameters in the range 200–500 μm. After dissection, the artery was placed in a cold physiological salt solution of composition (mM): NaCl, 110; KCl, 5; $CaCl_2$, 1·8; $MgCl_2$, 2; NaH_2PO_4, 0·5; KH_2PO_4, 0·5; $NaHCO_3$, 10; Hepes, 10; glucose, 10; Phenol Red, 0·04; pH adjusted to 7·4 at 4 °C using NaOH. The preparation was then stored at 4 °C.

Cells were isolated using a method essentially identical to that described previously (Dart & Standen, 1993). Briefly, a section of artery, typically 5 mm in length, was cut open along its length and dissected into strips about 1 mm wide. These were placed in 5 ml of cold isolation buffer of composition (mM): NaCl, 110; KCl, 5; $CaCl_2$, 0·16; $MgCl_2$, 2; NaH_2PO_4, 0·5; KH_2PO_4, 0·5; $NaHCO_3$, 10; Hepes, 10; glucose, 10; Phenol Red, 0·04; EDTA, 0·49; taurine, 10; containing 0·02% bovine serum albumin (essentially fatty acid free; Sigma, Poole, Dorset, UK), 0·8 mg ml^{-1} collagenase (Type I; Sigma) and 0·2 mg ml^{-1} elastase (Type IIA; Sigma). The strips were incubated in this solution for 20–25 min at 4 °C, allowing the enzymes to diffuse into the extracellular matrix of the tissue. The solution was then warmed to 37 °C in a shaking water bath for 60–70 min, after which time the muscle strips were removed into fresh, enzyme-free isolation buffer and gently triturated with a wide-bore Pasteur pipette. The isolation procedure produced relaxed coronary smooth muscle cells of dimensions ~100 μm long by ~10 μm maximum diameter. The muscle strips and dispersed cells were stored in isolation buffer at 4 °C and used over the next 8–10 h.

Solutions

Single isolated myocytes were allowed to settle in isolation buffer on the bottom of a small plastic Petri dish and were continuously superfused with a bath solution containing (mM): NaCl, 140; KCl, 5; $CaCl_2$, 1·8; $MgCl_2$, 1; Hepes, 10; glucose, 10; pH adjusted to 7·4 at 22 °C using NaOH. During the course of an experiment, a cell could be superfused with any one of up to seven different solutions using a flow system in which seven lines fed a common outflow placed near the cell (Langton, 1993). Each line was controlled by an electrically switched valve (Lee, Westbrook, CT, USA). The diameter of the flow system aperture was about 200 μm and the dead volume was < 5 μl, thus facilitating the rapid change of solutions. The flow solutions used were either a 143 mM K^+ solution containing (mM): KCl, 143; $CaCl_2$, 1·8; $MgCl_2$, 1; Hepes, 10; glucose, 10; pH adjusted to 7·4 at 22 °C using KOH, or a 40 mM K^+ solution in which 103 mM KCl was replaced by NaCl, and NaOH was used to adjust the pH.

To obtain hypoxic flow solutions, the 143 mM K^+ or the 40 mM K^+ solutions were continuously bubbled with 100% N_2 in glass reservoirs for at least 30 min before the start of each experiment. The partial pressure of oxygen (P_{O_2}) of these solutions, measured using an oxygen electrode (Oxygen Meter POM2; Jenway, Dunmow, Essex, UK), was found to be in the range 25–40 mmHg. The 'normoxic' control flow solutions that had not been equilibrated with N_2 had a P_{O_2} in the range 150–170 mmHg. The measured pH of the N_2-bubbled solution was the same as that of the normoxic flow solution. The small diameter of the flow system aperture (see above) meant that a single cell could be superfused with the hypoxic flow solution at a high flow rate, so obviating the problems of maintaining low oxygen levels in the whole bath. Due to the small volume of solution leaving the flow system, it was impossible to measure directly the P_{O_2} of the flow solution superfusing the cell. We assume, however, that since all connections from the reservoir were either of glass or low-permeability tubing (i.d. 0·5 mm; o.d. 3·7 mm; PharMed, Cole-Palmer, Bishop's Stortford, Herts, UK) and that the flow rate was high, minimal levels of oxygen were likely to have been taken up by the solution between leaving the continuously bubbled reservoir and coming in contact with the cell.

Glibenclamide (Sigma) was dissolved in dimethyl sulphoxide (DMSO; Sigma) to give a 10 mM stock solution. Charybdotoxin (a gift from Zeneca Ltd, Macclesfield, Cheshire, UK) was dissolved in distilled water to give a 0·1 mM stock solution. All stock solutions were stored as aliquots at −20 °C. Both glibenclamide and charybdotoxin were diluted into the flow solution immediately prior to use. The final concentrations are given throughout.

The pipette-filling solution had the following composition (mM): KCl, 143; $MgCl_2$, 1; EGTA, 0·5; Hepes, 10; pH adjusted to 7·2 at 22 °C using KOH.

Recording methods

Recordings were made using the perforated patch method with amphotericin B (Rae, Cooper, Gates & Watsky, 1991). Amphotericin B was made up as a stock solution (6 mg 100 μl^{-1}) in DMSO (tissue culture grade; Sigma) and stored at −20 °C. Twenty microlitres of the stock solution were diluted into 5 ml of the pipette-filling solution immediately prior to use.

Patch pipettes for whole-cell recording were pulled from thin-walled borosilicate tubing (o.d. 1·5 mm; Clarke Electromedical, Pangbourne, Berks, UK), coated with dental sticky wax (Kemdent, Swindon, Wilts, UK), and fire polished. Their resistance when filled with pipette solution was in the range 5–10 MΩ. Patch pipettes for cell-attached recording were pulled from thick-walled 1·5 mm borosilicate tubing. After fire polishing, their resistance was 10–20 MΩ. Seals, formed by the application of negative pressure, were in the order of 10 GΩ. Whole-cell access was achieved by the antibiotic within 5–15 min of seal formation. Series resistance (R_s), input resistance and whole-cell capacitance were measured using a 10 mV depolarization. Series resistance was 19·2 ± 1·5 MΩ ($n = 25$). Since the currents measured were generally <100 pA, the voltage error caused by R_s would be <2 mV so we did not routinely compensate for R_s. In experiments in which voltage ramps were used, however, up to 80% compensation for R_s was used.

Whole-cell and single-channel currents were recorded using a List EPC-7 amplifier and stored on a modified Sony digital audio tape (DAT) recorder. Current records in response to voltage ramps were digitized at 10 kHz, using a TL-125 Labmaster interface (Axon Instruments, Foster City, CA, USA) and a Dell 325 microcomputer. A suite of programs developed using the AxoBASIC library (Davies, 1993) was used both to apply voltage clamp command potentials and for analysis of whole-cell and single-channel currents. Single-channel current amplitudes were measured from histograms formed from records digitized at 2 kHz (CED 502 interface, PDP 11/73 computer) and fitted with Gaussian curves using a Marquardt algorithm as described previously (Standen *et al.* 1985). For display purposes, whole-cell currents have been low-pass filtered at 500 Hz (8-pole Bessel) unless otherwise stated. Membrane potentials are expressed as inside (of membrane) relative to outside, and outward currents are defined as positive and plotted upwards. Experiments were performed at room temperature, 18–22 °C, and results are given as means ± S.E.M.

RESULTS

The smooth muscle cells isolated from small porcine coronary arteries and used in this study had input resistances of 7·8 ± 1·1 GΩ and membrane capacitances of 20·7 ± 1·2 pF ($n = 25$ in each case).

Hypoxia reversibly activates a K^+ conductance

Figure 1 shows the effect of hypoxia on the whole-cell current recorded from an isolated porcine coronary arterial cell. Throughout the experiment, the cell was held at −60 mV and was continuously superfused with a flow solution containing 143 mM K^+. Hypoxia was induced by changing rapidly from a normoxic flow solution with a P_{O_2} of 150–170 mmHg, to a flow solution of identical composition which had been bubbled with 100% N_2 and had a P_{O_2} in the range 25–40 mmHg (see Methods). Exposure to the hypoxic flow solution caused an inward current to develop which was associated with an increase in current noise ($n = 37$ cells). In the experiment of Fig. 1, the induced current began to develop 2 min after the switch to hypoxic solution, reaching a maximal value of −72 pA within 4 min. On switching the superfusing solution back to normoxic solution, the increased levels of both noise and current persisted for almost 60 s before gradually declining. It took 5–6 min following the removal of the hypoxic solution for the induced current and noise in this cell to recover to resting levels. In twenty-six experiments similar to that of Fig. 1, an inward current started to develop 2·1 ± 0·3 min after the onset of hypoxia, reaching a maximal value of 41·2 ± 6·9 pA after 3·5 ± 0·4 min. In three experiments in which the cell was then exposed to

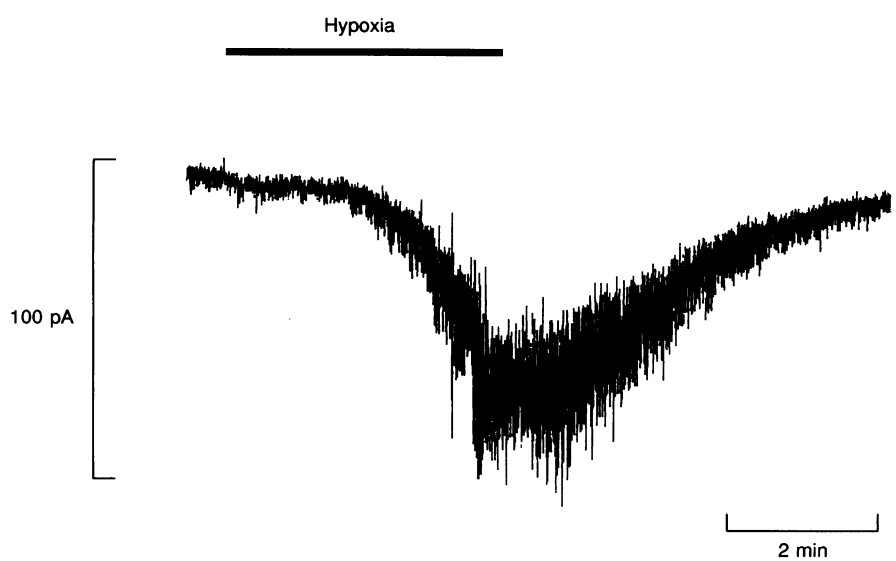

Figure 1. The effect of hypoxia on whole-cell current

The record shows the current recorded using the permeabilized-patch whole-cell method from a cell held at −60 mV and superfused with 143 mM K^+ solution throughout. The bar indicates a period when the superfusing solution was switched to solution bubbled with N_2 (hypoxic solution).

normoxic solution again, it took 4·4 ± 0·6 min for the activated current to return to its control level.

We used voltage ramps to investigate the reversal potential and voltage dependence of the current induced by hypoxia. Figure 2*A* shows the currents obtained in response to such ramps from a cell superfused with 143 mM K^+ solution both under normoxic control conditions and at the peak of the current response to hypoxia. The voltage was ramped from −60 to +40 mV over 80 ms and the currents shown are averages of the responses to six identical ramps in each case and are plotted against the membrane potential. Figure 2*B* shows the current–voltage relation for the hypoxia-activated current, obtained by subtracting the ramped current under normoxic conditions, from that under hypoxic conditions. The activated current reverses close to 0 mV, the calculated value for the equilibrium potential for K^+ ions (E_K) in symmetrical (143 mM) K^+. In experiments on six cells under the same ionic conditions the mean reversal potential was −3·7 ± 1·5 mV.

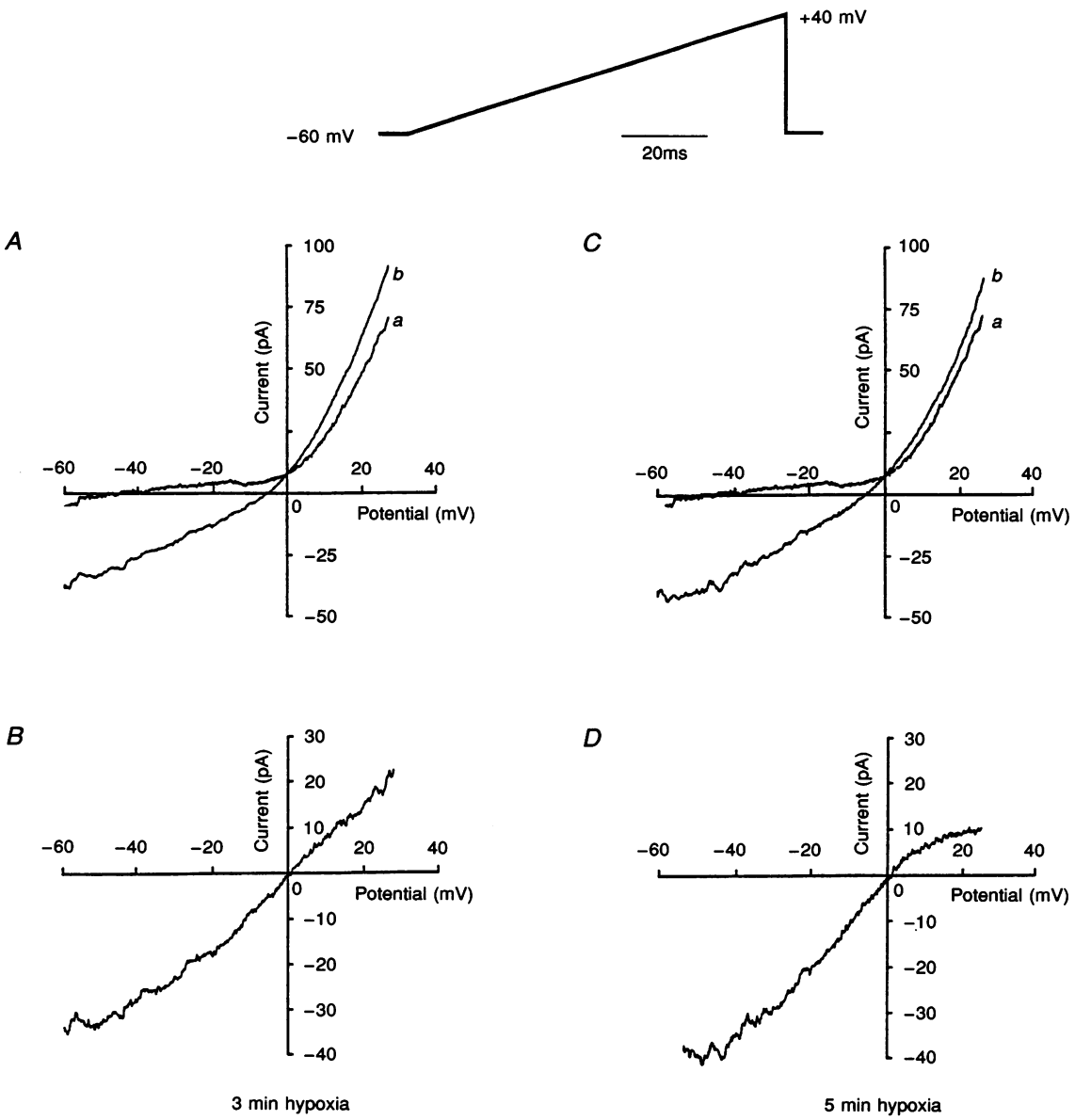

Figure 2. Current–voltage relations for the hypoxia-activated current

A, averaged currents recorded in response to a linear voltage ramp (shown above) from a cell superfused with control solution (*a*) and 3 min after the superfusing solution was switched to hypoxic solution (*b*). The solutions contained 143 mM K^+ and each current trace is the average of the response to 6 identical ramps. Current traces are offset from 0 on the current axis owing to the time-invariant capacity current in response to the rapid voltage ramp. *B*, current–voltage relation for the hypoxia-activated current obtained by subtracting *a* from *b* in *A*. *C*, averaged ramp currents from the same cell as in *A*, in control solution (*a*) and after 5 min in hypoxic solution (*b*). *D*, subtracted *I–V* relation (*b* − *a*) showing inward rectification of the hypoxia-activated current.

When an extracellular perfusing solution with a $[K^+]$ of 40 mM was used, switching to hypoxic solution still led to an inward current at −60 mV. Under these conditions, we used voltage ramps from −90 to +40 mV over 80 ms, and measured a mean reversal potential for the hypoxia-induced current of −27·2 ± 3·4 mV ($n = 4$), quite close to the calculated E_K of −32 mV. This correspondence of the reversal potential with E_K, together with the glibenclamide sensitivity described below, suggests that the current activated by hypoxia flows through K^+ channels.

The current–voltage relation of Fig. 2*B* suggests that the open-state probability of the channels activated in hypoxia show little voltage dependence, since the relation is linear over the voltage range investigated. It was found, however, that the shape of the current–voltage relation sometimes changed over time during hypoxia. Figure 2*C* shows the currents obtained in response to voltage ramps identical to those shown in Fig. 2*A* and from the same cell, but recorded 2 min after the currents shown in Fig. 2*A*. The change in current–voltage relation is illustrated more clearly in the subtracted current shown in Fig. 2*D*. Whilst the reversal potential remains at 0 mV and the current in the range −60 to 0 mV is virtually unchanged, the outward current at membrane potentials positive to E_K is clearly reduced, so that the current–voltage relation for hypoxia-activated current now shows some inward rectification. It is possible that this effect, which we saw in five cells, may result from a rise in intracellular Mg^{2+} leading to block of outward K^+ current (see Discussion).

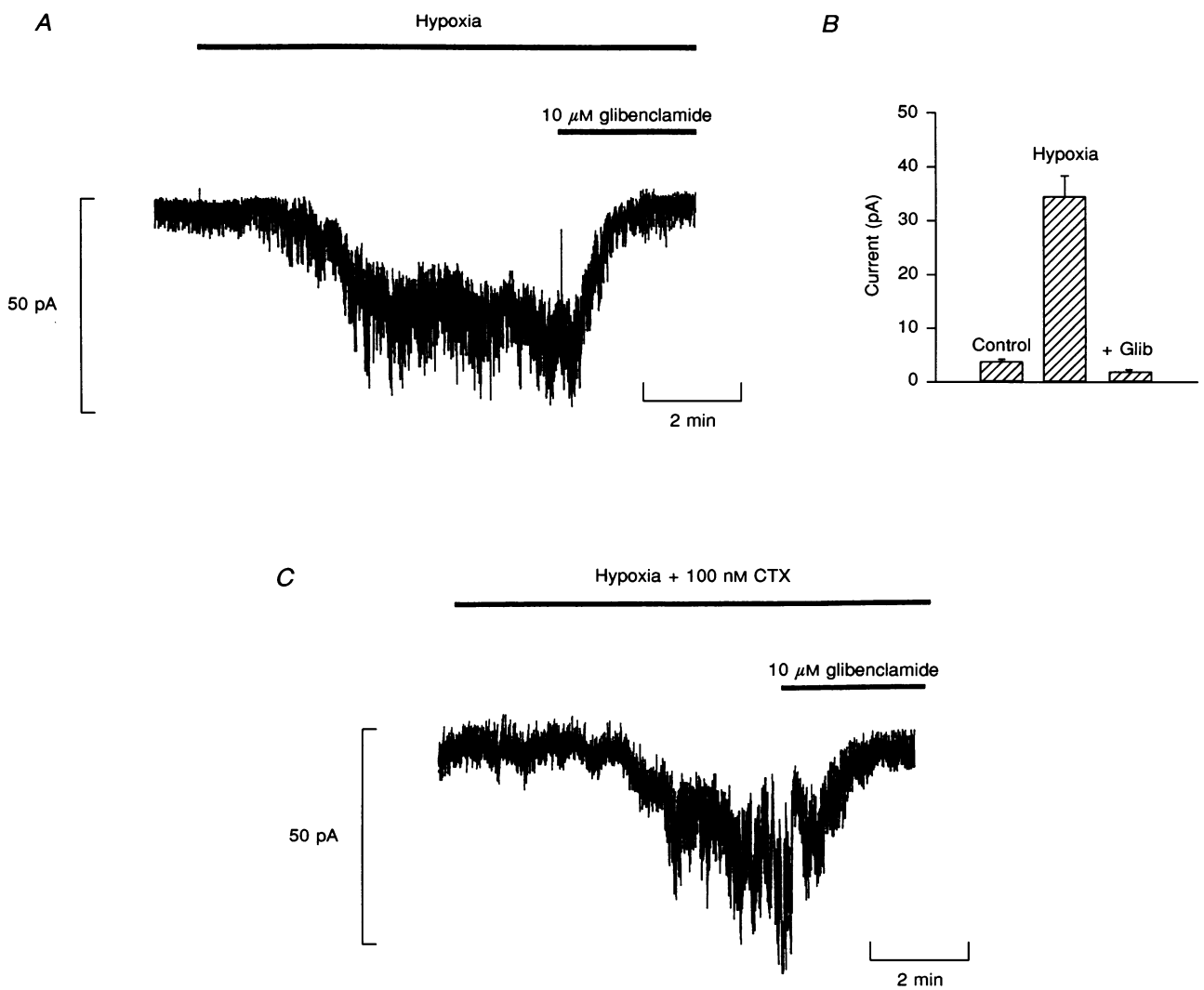

Figure 3. Block of hypoxia-induced current with glibenclamide

A, current recorded from a cell bathed in 143 mM K^+ and held at −60 mV. Where indicated, the solution superfusing the cell was switched to hypoxic solution, and to hypoxic solution containing 10 μM glibenclamide. *B*, mean currents recorded from 5 cells in control 143 mM K^+ solution at −60 mV, in hypoxic solution, and in hypoxic solution with 10 μM glibenclamide (Glib). *C*, lack of effect of charybdotoxin (CTX) on hypoxic activation of current in a cell under the same recording conditions as *A*. Where indicated, the superfusing solution was switched to hypoxic solution with 100 nM CTX, or to the same solution plus 10 μM glibenclamide.

It has been proposed that adenosine is released from cardiac muscle cells during hypoxia, and leads to vasodilatation of coronary arteries (Berne, 1980). Since we have previously shown that adenosine can activate K^+ channels in the isolated coronary myocytes used here (Dart & Standen, 1993), we considered the possibility that the K^+ current we measured in response to hypoxia results from the action of adenosine released by the smooth muscle cell on receptors on its own membrane. Such an effect seems unlikely as we used single isolated cells exposed to a constantly flowing extracellular solution, so we would expect any adenosine to be washed away rapidly from the cell membrane. This seems to be the case, since we found that the response to hypoxia persisted in four cells in which the enzyme adenosine deaminase (1–2 units ml^{-1}), which should rapidly break down adenosine, was added to all extracellular solutions.

The whole-cell current activated by hypoxia is sensitive to glibenclamide but not charybdotoxin

The antidiabetic sulphonylurea glibenclamide has been shown to reduce the open probability of ATP-sensitive K^+ channels in a variety of tissues (e.g. Cook & Quast, 1990). Glibenclamide has also been reported to prevent hypoxic vasodilatation in the guinea-pig heart (Daut *et al.* 1990). We therefore tested the effect of this sulphonylurea on the K^+ current activated in coronary myocytes by hypoxia.

Figure 3*A* shows such an experiment. The cell was continuously superfused with 143 mM K^+ solution and held at −60 mV. Two minutes after the normoxic superfusing solution was replaced by the hypoxic solution, an inward current developed which was associated with an increase in current noise. In this experiment, the induced current reached a maximal value of −32 pA and remained relatively stable at this current level and noise. The application of 10 μM glibenclamide to the hypoxic flow solution caused both the noise and inward current to decline rapidly to resting levels. In five cells, 10 μM glibenclamide reduced the current activated by hypoxia from −34·5 ± 3·6 to −1·62 ± 0·54 pA (Fig. 3*B*). In two cells to which we applied 5 μM glibenclamide, we observed a similar block of the hypoxia-induced current.

Large-conductance Ca^{2+}-activated K^+ channels (BK channels) are readily detected in patches excised from most smooth muscle cells. Such channels are activated by an increase in cytoplasmic free Ca^{2+} and are also voltage

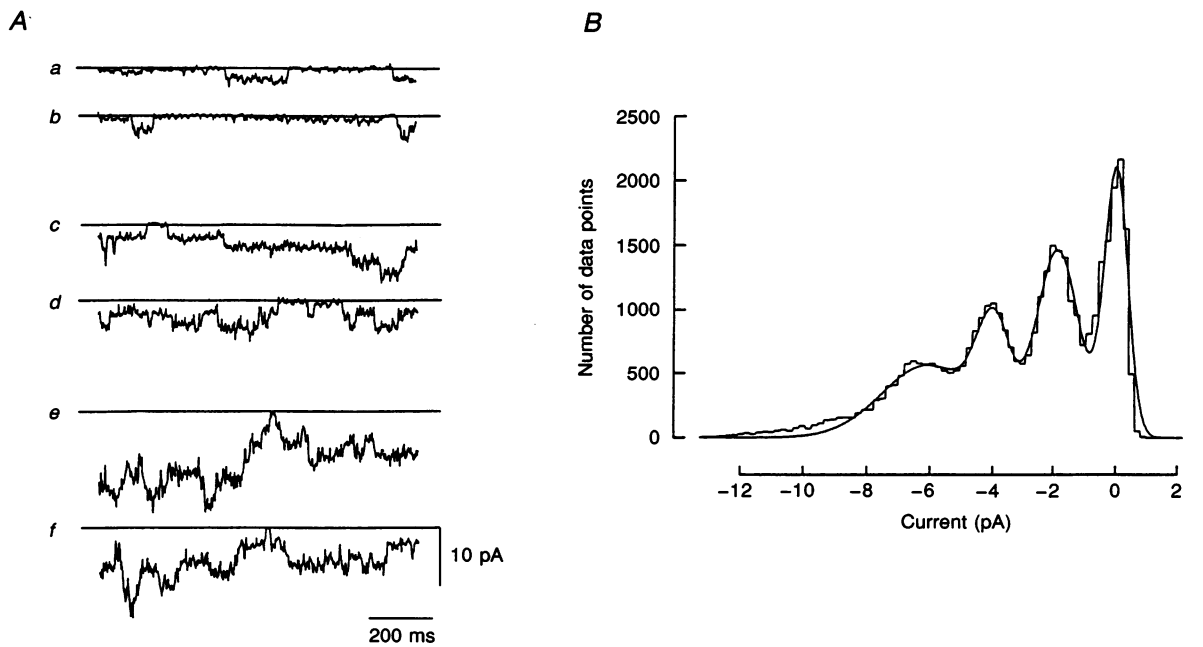

Figure 4. K^+ channels activated by hypoxia in whole-cell recording

A, whole-cell current recordings from a cell held at −60 mV and bathed in 143 mM K^+ solution. Records *a* and *b* were made while the cell was superfused with normoxic solution, records *c* and *d* at the onset of the response to hypoxia (2–3 min after the superfusing solution was switched to hypoxic solution), and records *e* and *f* later in the response. The straight line indicates the zero current level in each case, and the records were filtered at 200 Hz for display. *B*, histogram of current amplitude formed from recordings made during the onset of the response to hypoxia from the same cell as in *A*. The histogram has been fitted with the sum of 4 Gaussian curves with mean and S.D. values (pA) of 0, 0·38; −1·87, 0·69; −3·98, 0·55 and −6·08, 1·46, respectively.

dependent, their open-state probability increasing with membrane depolarization (Latorre, Oberhauser, Labarca & Alvarez, 1989). The lack of voltage dependence of the underlying channels, indicated by the current–voltage relation of the hypoxia-induced current (Fig. 2*B*), argues against the involvement of BK channels in this current, as does its complete blockade by glibenclamide. To confirm that BK channels do not contribute to the hypoxia-induced current in our experiments, we tested the effect of charybdotoxin, a peptide from scorpion venom which we have previously shown to be an effective blocker of BK channels in this preparation (Dart & Standen, 1993). We found, in experiments on four cells, that 50 or 100 nM charybdotoxin had no detectable effect upon the K^+ current induced by the exposure to hypoxia. One of these experiments is illustrated in Fig. 3*C*.

Single channels activated by hypoxia

Whole-cell recordings

In some whole-cell recordings, the current noise was found to be low enough for single-channel openings to be detected quite easily with appropriate filtering (usually in the range 200–400 Hz). Figure 4*A* shows whole-cell current recorded from a cell held at −60 mV in 143 mM K^+. The current record has been filtered at 200 Hz and replayed on a relatively fast time base. Occasional openings of single channels passing an inward current of about −2 pA could be observed under normoxic control conditions (records *a*

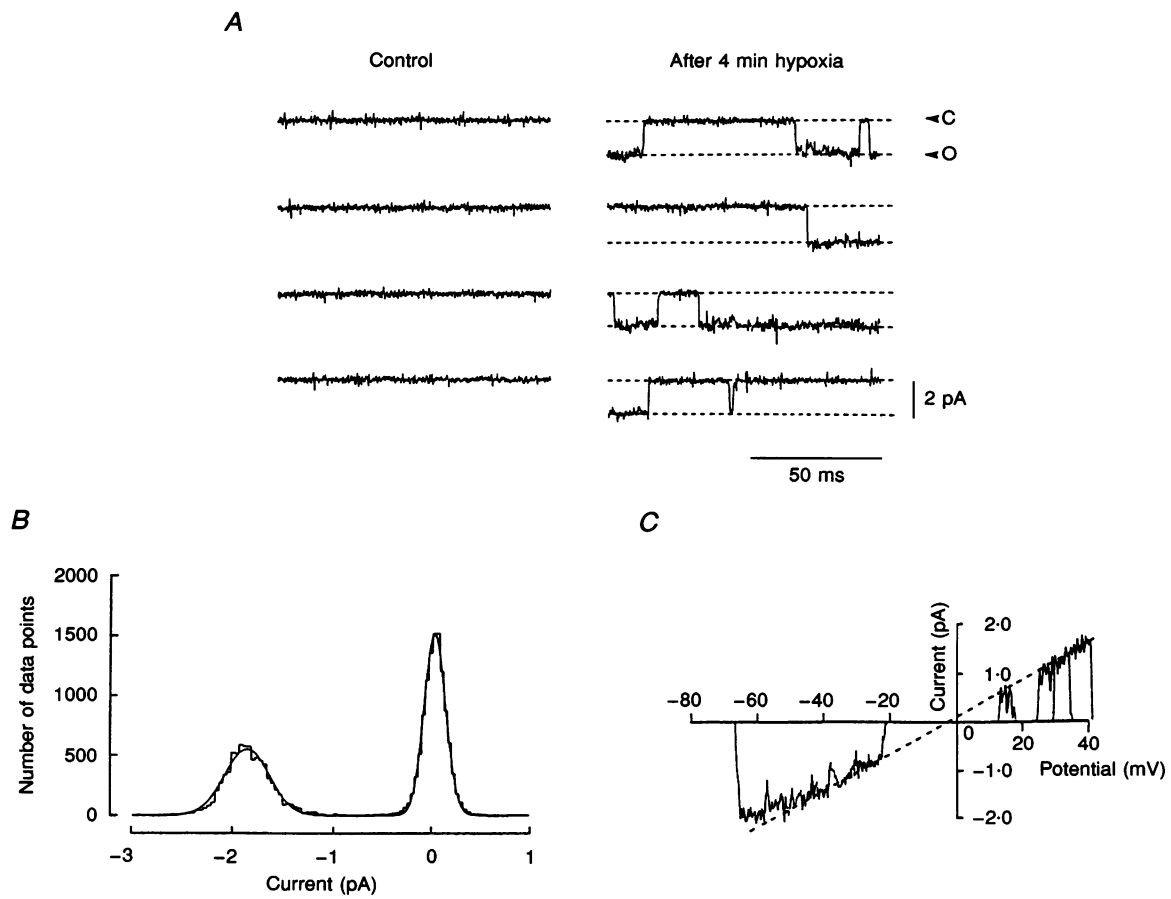

Figure 5. K^+ channel activation by hypoxia in cell-attached patches

A, current recordings from a cell-attached patch on a cell bathed in 143 mM K^+ solution. The pipette solution contained 143 mM K^+ and the pipette potential was clamped to +60 mV. Left panel, records made while the cell was superfused with control solution. Right panel, records made 4 min after the superfusing solution was switched to hypoxic solution. Inward currents appear as downward deflections. Records were filtered at 2 kHz and sampled at 10 kHz. C and O represent closed and open levels. *B*, histogram of current amplitude made during hypoxia from the cell shown in *A*. The histogram has been fitted with 2 Gaussian curves with mean and S.D. values (pA) of 0, 0·11 and −1·90, 0·22, respectively. *C*, single channel current in a different cell in response to a voltage ramp. The pipette potential was ramped from +60 to −40 mV over 80 ms. The voltage axis represents the potential across the membrane patch assuming that the resting potential of the cell is 0, and has been corrected for the measured junction potential at the pipette tip of −4 mV.

and *b*). After 2–3 min of exposure to hypoxic flow solution, the channel activity began to increase, as can be seen in records *c* and *d* which were taken at the onset of the whole-cell response to hypoxia.

Figure 4*B* shows a histogram of current amplitude made during the onset of the hypoxic response in the same cell as Fig. 4*A*. The histogram shows clear peaks corresponding to current levels with either zero, one or two channels open, and a broader peak corresponding to a third level. As the whole-cell response to hypoxia develops and the induced current increases (records *e* and *f*), the currents through more channels are superimposed and the underlying channel openings can no longer be clearly resolved. In seven experiments like that of Fig. 4, the mean unitary current of channels activated by hypoxia was $-2{\cdot}04 \pm 0{\cdot}07$ pA, not significantly different (Student's *t* test) from the value we measured previously in response to adenosine under the same conditions (Dart & Standen, 1993). Assuming a reversal potential of 0 mV (see Fig. 2), this corresponds to a unitary conductance of 34 pS.

Cell-attached patches

We made cell-attached patches on cells bathed in 143 mM K^+, in which the membrane potential should have been close to 0 mV. The pipette potential was clamped to +60 mV and we have assumed, therefore, that the potential of the patch would be −60 mV, and have used this value in our estimates on unitary conductance. In agreement with the high input resistance measured from isolated coronary myocytes, the majority of recordings showed no channel activity either under control or hypoxic conditions. In patches in which channel activity was observed, the channels recorded fell into one of three distinct groups, based on their unitary conductance.

The most frequently observed type of channel passed an inward current of 0·5 pA. A second channel type had a much larger conductance, passing an inward current of 13 pA, which corresponds to a conductance of 217 pS. Such channels were rarely observed and had a brief open time. We believe them to be large-conductance Ca^{2+}-activated K^+ channels since their unitary conductance was in the range reported for these channels in a variety of tissues (Latorre *et al.* 1989), and was the same as that of the charybdotoxin-sensitive K^+ channel which we have previously recorded in patches excised from pig coronary arterial cells (Dart & Standen, 1993). At −60 mV, and with physiological levels of Ca^{2+}, Ca^{2+}-activated K^+ channels would be expected to have a very low open probability. The activity of neither of the above channels was consistently influenced by exposure to hypoxic solutions, though the activity of the large conductance channel (217 pS) occasionally increased towards the end of a long recording. This was, however, always associated with the appearance on the cells of membrane blebs and a loss of phase brightness. We believe, therefore, that the increase in channel activity resulted from a deterioration in the condition of the cell, probably such that the cell membrane became more permeable to Ca^{2+}.

The activity of the third type of channel was linked to exposure to hypoxia, as is illustrated in Fig. 5, and its unitary current corresponded to that seen in whole-cell recordings. This channel type was rarely observed, being seen in 5 out of approximately 120 patches, probably reflecting a low channel density in the cell membrane. In addition, openings were seldom seen under normoxic conditions. In the patch shown in Fig. 5*A* there was no sign of any channel activity before exposure to hypoxia. After 4 min superfusion of the cell with hypoxic solution, the activity of a channel passing an inward current of just under −2 pA was observed. Figure 5*B* shows a histogram of current amplitude made from the channel activity in the cell of Fig. 5*A*, fitted with Gaussian curves to give a unitary current amplitude of −1·89. It was occasionally possible to measure the unitary current during a voltage ramp applied during the on-cell recording (Fig. 5*C*), confirming that the current through the hypoxia-activated channel reversed close to 0 mV, as expected for a K^+ channel. In five separate experiments, in which we formed amplitude histograms from channel activity induced by exposure to hypoxic solutions, the mean unitary amplitude was $1{\cdot}91 \pm 0{\cdot}03$ pA, corresponding to a unitary conductance of 32 pS, a value very close to the 34 pS we measured from single-channel activity measured in whole-cell recordings during the onset of the response to hypoxia.

DISCUSSION

Hypoxia and K^+ channel activation

In this paper, we have shown that superfusion of isolated coronary myocytes with a solution in which the P_{O_2} is reduced to 25–40 mmHg leads to activation of K^+ channels. Our results provide very strong evidence that hypoxia can act directly on coronary smooth muscle cells to activate K^+_{ATP} channels. This is in agreement with the suggestion of von Beckerath *et al.* (1991), that vasodilatation during early hypoxia in the perfused guinea-pig heart may involve a direct effect of hypoxia on smooth muscle. Our isolated myocytes are removed from any endothelial influences, and the channel activation we observed is also very unlikely to result from an effect of adenosine.

Several lines of evidence suggest that the K^+ channels activated by hypoxia in our experiments were K^+_{ATP} channels. The hypoxia-induced whole-cell current was blocked by the sulphonylurea K^+_{ATP} channel blocker glibenclamide, but not by the blocker of large-conductance Ca^{2+}-activated K^+ channels, charybdotoxin. The current–voltage relation for the hypoxia-induced current was linear, at least early in hypoxia, consistent with channels the open-state probability of which shows little or no voltage dependence. Single-

channel currents, either resolvable in whole-cell recordings or recorded in cell-attached patches, showed that the channels activated by hypoxia had unitary currents of about −2 pA at −60 mV in symmetrical 143 mM K^+ solutions. The corresponding unitary conductance of 34 pS is very close to the value we previously measured for the glibenclamide-sensitive channels, activated by adenosine in these cells (35 pS; Dart & Standen, 1993) and to the 30 pS conductance reported for K^+_{ATP} channels in membrane patches of cells cultured from porcine coronary arteries (Miyoshi & Nakaya, 1991; Miyoshi *et al.* 1992).

How does hypoxia activate K^+_{ATP} channels of isolated myocytes?

The most obvious mechanism by which a reduced P_{O_2} could lead to activation of K^+_{ATP} channels is by causing a change in the energy metabolism of the coronary myocytes, so that K^+_{ATP} channels open in response to a fall in the submembrane concentration of ATP, or perhaps a change in the ADP/ATP ratio (Kajioka, Kitamura & Kuriyama, 1991; Beech, Zhang, Nakao & Bolton, 1993). Such a mechanism was suggested by von Beckerath *et al.* (1991) to explain the hypoxic vasodilatation of isolated heart, where vasodilatation could also be produced by the inhibitor of glycolysis, 2-deoxyglucose, or the mitochondrial uncoupler dinitrophenol. Inhibition of cellular metabolism using metabolic poisons has been shown to activate glibenclamide-sensitive K^+ currents in cells isolated from the mesenteric artery and portal vein (Silberberg & van Breemen, 1992; Beech *et al.* 1993), while conventional whole-cell recording with low ATP concentrations in the pipette solution can also lead to activation of K^+_{ATP} currents (Clapp & Gurney, 1992; Quayle, Bonev, Brayden & Nelson, 1994). Similarly, we have found that metabolic inhibition with deoxyglucose and dinitrophenol activates K^+_{ATP} current in the coronary myocytes used in the present study (C. Dart, unpublished observations).

While it seems clear that metabolic inhibition or a fall in intracellular [ATP] ($[ATP]_i$) can activate K^+_{ATP} channels, it is not certain that hypoxia will lead to a fall in $[ATP]_i$. In aortic smooth muscle, for example, hypoxia has been reported to cause little change in ATP content (Namm & Zucker, 1976; Post & Jones, 1991). It is possible that the metabolism of small arteries may differ from that of large vessels, or it might be the case that submembrane changes in ATP concentration during hypoxia are greater than changes in the bulk cytosol. There is some evidence for metabolic compartmentalization of this sort for the regulation of K^+_{ATP} channels in cardiac cells (Weiss & Lamp, 1987) and of ion transport in smooth muscle (Hardin, Raeymaeker & Paul, 1992). Since K^+_{ATP} channels in other tissues have been shown to be regulated by intracellular factors other than nucleotides, for example pH (Davies, Standen & Stanfield, 1992), it is also possible that hypoxia activates K^+_{ATP} channels by changing one of these. Finally, it may be that isolated coronary myocytes respond to hypoxia by means of an oxygen-sensing mechanism similar to that proposed for cells of the carotid body and airway mucosa. There, the membrane-bound enzyme NAD(P)H oxidase has been proposed to act as such a sensor, leading to production of oxygen free radicals, and thus a change in the glutathione redox state, leading to inhibition of K^+ channels (Cross, Henderson, Jones, Delpiano, Henschel & Acker, 1990; Youngson, Nurse, Yeger & Cutz, 1993).

Clearly, more experimental work will be needed to distinguish between the possibilities outlined above. We suspect, however, that a decrease in $[ATP]_i$ during relatively prolonged hypoxia (>5 min) may account for the rectification which developed in the current–voltage relation of the hypoxia-induced current (e.g. Fig. 2*D*). A fall in ATP would result in an increase in Mg^{2+} as MgATP is hydrolysed to ADP and Mg^{2+}. Intracellular Mg^{2+} is largely bound to intracellular buffers, particularly ATP, leaving only 0·5–3 mM free in the cytoplasm (Murphy, Freudenrich & Lieberman, 1991). In ventricular muscle, Buri & McGuigan (1990) found a 20% rise in intracellular Mg^{2+} during ATP_i depletion with cyanide and deoxyglucose. Block of outward K^+ current by millimolar concentrations of intracellular Mg^{2+} is known to cause a degree of inward rectification of K^+_{ATP} channels (Horie, Irisawa & Noma, 1987).

K^+_{ATP} channel activation and coronary vasodilatation

Activation of K^+_{ATP} channels in vascular smooth muscle should lead to membrane hyperpolarization and thus relaxation as we have previously discussed (Dart & Standen, 1993; see also Nelson, Patlak, Worley & Standen, 1990; Daut, Standen & Nelson, 1994). As described in the Introduction, there is evidence that K^+_{ATP} channel activation is an important mechanism underlying hypoxic vasodilatation in the coronary circulation (Daut *et al.* 1990; von Beckerath *et al.* 1991; Nakhostine & Lamontagne, 1993). It should also be noted that small coronary arteries and arterioles will normally experience oxygen tensions considerably lower than those in blood equilibrated with air. Partial pressure of O_2 has been found to decrease from 70 mmHg in distributing arterioles to 20 mmHg in terminal arterioles, and may also show considerable spatial and temporal variation (Duling & Berne, 1970; Olsson & Pearson, 1990). Thus, small arteries may often be exposed to P_{O_2} levels slightly higher than those we have used here. A number of functional studies have suggested that K^+_{ATP} channels contribute to the resting membrane potential of smooth muscle in coronary arteries, since glibenclamide increases basal coronary tone *in vivo* (Samaha, Heinemann, Ince, Fleming & Balaban, 1992; Duncker, Van Zon, Altman, Pavek & Bache, 1993) and can depolarize intact coronary arteries *in vitro* (Eckman, Frankovich & Keef, 1992). These findings imply a background activity of K^+_{ATP} channels that may result, in part, from a response to the P_{O_2} level, and probably also from the tonic presence of vasodilators, such as adenosine.

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