Discrimination by valinomycin K-selective surface microelectrodes of a sulphonylurea-sensitive and a distinct sulphonylurea-, barium-, TEA- and cinnamate-insensitive component of K-efflux from isolated pig coronary arteries during simulated ischaemia

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Discrimination by valinomycin K-selective surface microelectrodes of a sulphonylurea-sensitive and a distinct sulphonylurea-, barium-, TEA- and cinnamate-insensitive component of K-efflux from isolated pig coronary arteries during simulated ischaemia

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Ischaemia was simulated in isolated pig coronary artery strips by immersing the preparation in paraffin oil. Ion-selective microelectrodes recorded surface potassium (K^+_s), -pH (pH_s) and -Na^+ (Na^+_s) in the thin film of Tyrode solution trapped in the artificially created reduced extracellular space (ARECS) between the hydrophobic liquid ion-selective sensor and the surface of the preparation.

The paraffin oil model reproduced two salient characteristics of myocardial ischaemia: surface acidification and K^+_s, accumulation – both features have not so far been described in arterial tissue. Sulphonylurea compounds such as glibenclamide ($20 \, \mu M$, $50 \, \mu M$, $200 \, \mu M$) and tolbutamide (0.5 mM, 1 mM) as well as a non-sulphonylurea type blocker of K^+_{ATP}-channels, tetramethyl-pyrazine (1 mM), reduced ischaemia-induced K^+-accumulation by 66 %. A fraction of the K^+-accumulation (33 %) was not sensitive to sulphonylureas and was not affected by Ba^{2+} (0.3 mM, 1 mM) or tetraethylammonium (TEA; 1 mM).

Ischaemia-induced K^+-accumulation was unaffected by the known inhibitor of lactic acid transport, alpha-cyano-4hydroxy-cinnamic acid (4 mM). The removal of external Cl^- (leading to the depletion of internal Cl^-; Cl^- replaced by glucuronate) had no effect upon ischaemia-induced K^+ accumulation. During simulated ischaemia there was a fall in pH_s. The degree of this acidification was reduced by both amiloride (1 mM) and alpha-cyano-4hydroxycinnamic acid (4 mM). Our results suggest that in isolated pig coronary preparations under conditions of simulated ischaemia, K^+-accumulation is partially mediated by a sulphonylurea-sensitive pathway. We consider it likely that this pathway involves the opening of ATP-dependent K^+ channels. The source of the sulphonylurea insensitive fraction of K^+-accumulation remains unidentified. Furthermore, we conclude that extracellular acidification in these preparation is mediated by both Na^+/H^+ exchange and the lactic acid carrier. *J Clin Bas Cardiol 1998; 1: 43–51.*

Key words: Smooth muscle, ischaemia, sulphonylurea, glibenclamide, K-channel

The opening of K^+-channels in coronary smooth muscle is believed to underlie the coronary vasodilation that occurs in response to hypoxia and ischaemia [1]; for review see [2]. It has been suggested that the outward current flowing through these K^+-channels produces a membrane hyperpolarisation which acts to limit Ca^{2+} entry into the arterial smooth muscle cells by inhibiting voltage-dependent Ca^{2+} channels. The subsequent drop in intracellular free Ca^{2+} causes smooth muscle relaxation and thus coronary vasodilation [3–5].

The extracellular accumulation of K^+ ions has long been recognised as a salient feature of myocardial ischaemia [6], and ischaemic K^+ efflux has been a much studied phenomenon in heart muscle [7–9]; for review see [10–12]. To date, however, no direct measurement has been made of K^+ efflux from vascular smooth muscle under ischaemic conditions. In the present study, we used ion-selective microelectrodes to record extracellular/surface changes in the concentrations of K^+ and H^+ ions in isolated strips of pig coronary artery during episodes of simulated ischaemia. We find that simulated ischaemia provokes the extracellular accumulation of K^+ ions and that this effect can largely be inhibited by the sulphonylurea compounds glibenclamide and tolbutamide, known blockers of the ATP-sensitive potassium channel [13-17]. We show that this ischaemia-induced K^+ efflux is not linked to Cl^- or lactate extrusion. Our results are consistent with those of Daut et al. [1] who have shown that hypoxia/ischaemia induced vasodilation in the isolated guinea-pig heart can be inhibited by glibenclamide. This group suggested that hypoxia/ischaemia

leads to the activation of K^+_{ATP} channels. They further support the finding that metabolic (ATP) depletion leads to an efflux of K^+ from aortic smooth muscle that is sensitive to glibenclamide [18].

We find that a component of the ischaemia-induced K^+ accumulation is not blocked by the sulphonylureas and is insensitive to both Ba^{2+} and tetraethylammonium (TEA).

We also measure surface pH during episodes of simulated ischaemia and find that acid extrusion in coronary smooth muscle is linked to both Na^+/H^+ exchange and the alpha-cyano-4-hydroxycinnamate sensitive lactic acid carrier.

Methods

Preparations
Experiments were performed in pig coronary artery strips. Pig hearts were collected from a local abattoir. Proximal segments of the coronary arteries of several cm length were carefully excised from the hearts, and trimmed free of adhering fat and connective tissue in a cool (4–10 °C) Tyrode bath solution bubbled with 100 % O_2. Preparation was performed using forceps and a fine ophthalmo-surgical pair of scissors under a binocular microscope. Arteries were helically cut, approximately 2.5 mm in width and 2 cm in length. These strips were ligated with a thin thread and cut into shorter segments, each measuring approximately 7 mm in length. These specimens, which were ligated on both ends (Fig. 1), were then vertically fixed with stainless steel pins in the bath containing the nutrient Tyrode solution (see below). The
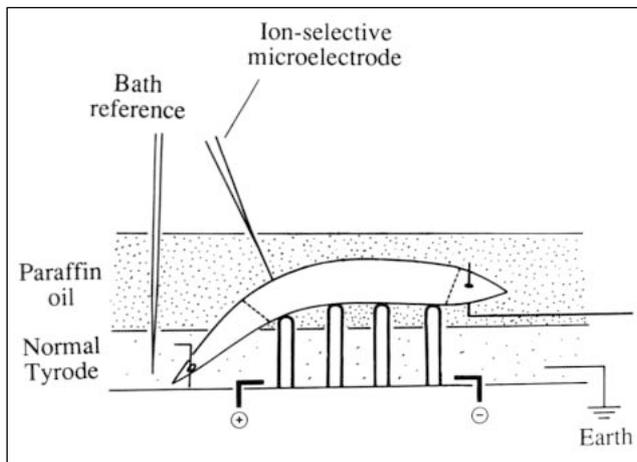
Figure 1: Experimental assembly used for simulating ischaemia. Fig. redrawn from Gasser & Vaughan-Jones (1990). Schematic diagram of perfusion chamber. Ion-selective microelectrode (in our experiments e.g. Na+, pH, K+- selective microelectrode) pressed gently on the surface of a pig coronary artery strip and recording specific ion concentration changes during ischaemia. Preparation is laid across four supports (micropins, 100 μm in diameter) and pinned on one end and attached to a force transducer at the other. Also shown are two electrodes for field Stimulation. The diagram illustrates an episode of simulated ischaemia, with the preparation immersed almost completely in a stationary pool of paraffin oil (heavy stippling) while perfusion of warm (37 °C) Tyrode (light stippling) continues at the base of the chamber. Dashed lines represent ligated areas as described in the Methods.

preparation was partially immobilised by stretching it lightly over a narrow bridge made of four fine micropins (100 μm diameter; see [19]; *cf.* Fig. 1) so that the luminal side of the artery is upwardly directed. A pH-, K+- or Na+ sensitive microelectrode was pressed on the surface of the preparation (Fig. 1; for further details see [9]).

Solutions
The preparations were continuously superfused with Tyrode solution. The solution was pumped to the experimental

chamber (about 1.5 ml/min; bath volume 150 μl), passing through a heat exchanger, before reaching the preparation. The heating configuration was such that, in any experiment, temperature was maintained constant at 37.0 $\pm$ 0.5 °C (see below). The superfusate was equilibrated with either 100 % O_2 in 20 mM-HEPES-buffered solution or with 5 % CO_2 plus 95 % O_2 in 23 mm bicarbonate buffered solution. For details of the chamber see [9].

The normal Tyrode solution consisted of (in mM): NaCl (140), KCl (4.5), $CaCl_2$ (2.5), $MgCl_2$ (1.0), glucose (10), HEPES (20). The solution was adjusted to pH 7.4 by titration using 4 M NaOH. CO_2 bicarbonate buffered solutions were modified from our normal Tyrode solution and contained: NaCl (127), $NaHCO_3$ (23), HEPES (0); all other constituents were unchanged.

Tolbutamide (SIGMA) was dissolved in 0.3 ml dimethyl sulphoxide (DMSO) to make a solution (0.33 M) which was added to the Tyrode solution such that the concentration of DMSO did not exceed 0.3 % in the final solution. The same procedure was applied when glibenclamide (received as a gift from Hoechst, Germany) and tetramethylpyrazine (1 mM; Aldrich) were used. Alpha-cyano-4-hydroxycinnamic acid (SIGMA) was dissolved (4 mM) in Tyrode solution by warming it to approximately 50 °C, pH was then readjusted after 2 h. Bumetanide (2 mM; Leo Pharmaceuticals), amiloride (1 mM; SIGMA), Tetramethylammonium (TEA; 1 mM; SIG-MA), and Ba^{2+} (1 mM) were simply added to the solution shortly before use.

Ionselective microelectrodes
Micropipettes used for construction of ion-selective micro-electrodes were pulled from borosilicate tubing, 1.5 mm o.d., 1 mm i.d. (GC150 Clark Electromedical). They were constructed exactly as described previously ([9], *cf.* 20). Briefly, electrodes were silanised using trimethyl-dimethyl silylamine, they were then right-angle dry-bevelled (flat tip; *cf.* Fig. 2 c) to an outer tip diameter of approximately 1.5–2 um for surface use (for review of construction of ion-selective microelectrodes see also [21, 22]).
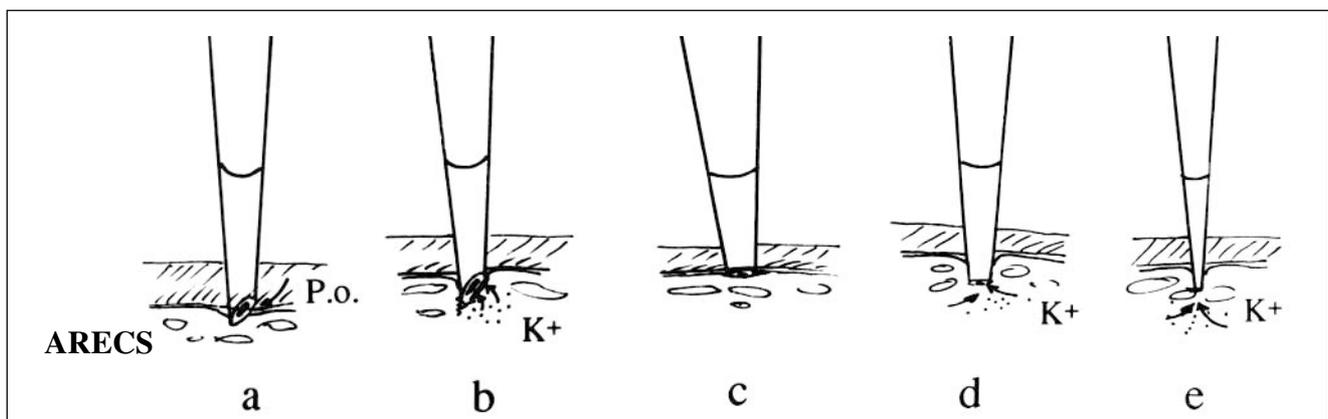
Figure 2: Simplified diagram of different microelectrode tips during simulated ischaemia. Hatched area represents paraffin oil (P.o.), area underneath, marked with oval rings, represents myocardial or smooth muscle tissue (T). Between tissue and P.o. one can see the artificially created reduced extra-cellular space (ARECS). **a.** Electrode tip bevelled in a 45° angle not yielding ideal contact with the tissue surface, thus making contact with the paraffin oil. Under these conditions the hydrophobic carrier will instantly escape from the tip and the electrode does not give further readings. **b.** Elec-trode tip bevelled in a 45° angle pushed too deeply into the tissue causing cell damage. Cells will be either damaged or destroyed causing a constant leak of K+ (K+ ions represented by stippling) into the ARECS which, in turn, leads to faulty measurements: instead of ischaemia-induced K+ efflux caused by changed K+ conductance of the membrane the electrode measures changes resulting from damaged cells (c.f. Fig. 3). **c.** Electrode tip bevelled in a 90° angle (flat tip) ideally positioned in the ARECS. Only under these circumstances correct measurements of transmembrane K+ efflux induced by ischaemia are possible (c.f. Fig. 3). **d.** Ideally bevelled surface microelectrode, impaled too deeply into the tissue. Stippling represents K+ ions leaking from damaged tissue – readings will be misleading. **e.** Too fine-tipped microelectrode, pushed too deeply into the tissue. Leaking K+ from the damaged tissue like in b and d, represented by stippling and small arrows.
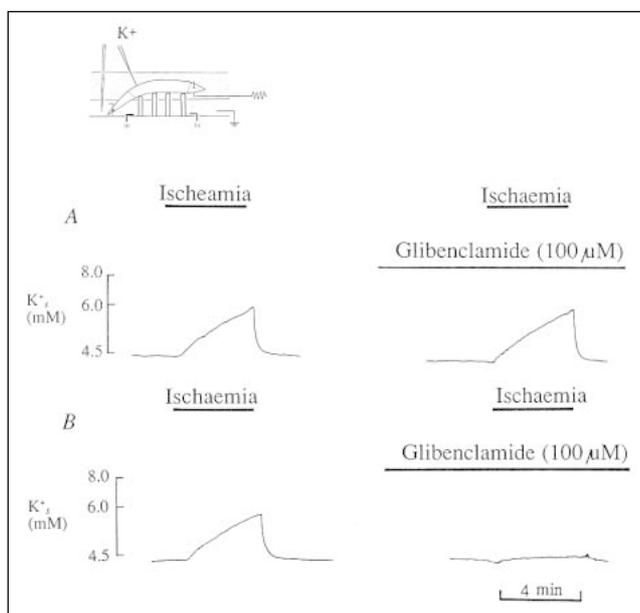
Figure 3: Guinea pig papillary muscle. Typical results when measuring K^+ during an ischaemic episode. A. Experiment performed using an electrode bevelled with a 45° angle pushed too deeply into the preparation – penetrating approximately two or three layers of myocardial cells. Right handside panel: lack of effect of glibenclamide upon ischaemic K^+ efflux in the same experiment, when microelectrode pushed too deeply into the tissue. B. Ideal experimental conditions: flat-tipped surface microelectrode gently touching the preparation like in Figure 2. Glibenclamide (100 uM) prevents early ischaemia-induced K^+ efflux.

Size as well as shape of the tip seem to play a decisive role for the accuracy of measurements. Figure 2 shows various problems we have observed with ion-selective microelectrodes during ischaemic episodes using the paraffin oil model: a. Electrode bevelled at 45° angle; this type of tip may easily make contact with the paraffin oil when not pressed tightly enough on the surface – subsequently, the hydrophobic ion-selective carrier escapes and no further reading is possible. b. Same type of electrode as in a., pushed too deeply into the tissue – in this case, myocardial cells are likely to be damaged and K^+-ions leak from the cytoplasm, leading to false readings, which can look very similar to correct measurements. For example, Figure 3A shows a typical reading of surface potassium during an ischaemic episode in a guinea pig papillary muscle (for preparation and other details see [9] using an electrode bevelled with a **45°** angle pushed into the preparation penetrating approximately two or three layers of myocardial cells. The right hand panel shows an ischaemic episode in the *same preparation* in the presence of $100 \mu M$ glibenclamide: no effect of glibenclamide on the potassium accumulation. Figure 3 B shows a similar experiment with a flat bevelled microelectrode gently pressed on the surface of the preparation as illustrated in 2 c) where the electrode rests in the artificially created reduced extracellular space (ARECS; for RECS properties see [23]) in this experiment glibenclamide completely prevents K^+ accumulation. Similar observations we made when using pig coronary artery preparations. We conclude that both very fine-tipped (e) and angled microelectrodes (b) as well as microelectrodes too deeply impaled into the tissue (d) may cause damage to myocardial cell membranes thus leading to continuous K^+ leakage from the cells. During perfusion these K^+ ions are washed away, thus not effecting ion-selective readings. During arrest of perfusion, however, leaking cells may thus release K^+ ions which accumulate and lead to false readings of the electrode. In such experiments, K^+ accumulation secondary

to cell damage is therefore not prevented by blocking K^+_{ATP} channels as shown in Figure 3. This could be a reason why Wilde et al. [8] see only partial inhibition by sulphonylureas of K^+ accumulation in isolated guinea pig hearts during arrest of perfusion after they have inserted a K^+ selective microelectrode deeply into the preparation. Hence, it is of major importance that experiments are performed with flat bevelled microelectrodes, pressed gently on the surface (upon contact with the surface of the preparation there is a small transient rise in the K^+ selective signal; as soon as this rise (–2 mV) is seen the electrode should not be advanced further).

The bevelled electrodes were then filled with ion-selective carrier plus electrode-filling solution (in mM: NaCl (100), KCl (4.5), HEPES (10), buffered to pH 7.5 with NaOH). For pH electrodes, we used the H^+ selective ionophore as used by Ammann et al. [24]. For K^+ selective microelectrodes, we used a valinomycin cocktail [25]. Short-column electrodes were used to obtain low noise recordings [26]. Electrodes were calibrated before the experiments and those that produced less than 57 mV for one pH unit or one decade change in K^+-activity were discarded.

Electrical arrangements

For surface ion-activity measurements, single barrelled microelectrodes were used. The ion-selective microelectrode signal was referred to a blunt (bevelled) 3 M-KCl-filled microelectrode located in a corner of the experimental chamber (Fig. 1). The ion-selective electrode signal was monitored with a varactor bridge diode (Analogue Devices 311J) and the bath reference (–1 M Ohm) was connected to a FET unity-gain, operational amplifiers (Signetics NES36H). All connections between electrodes and amplifiers were made via Ag-AgCl wires.

Statistics

Mean values are quoted ± standard error of mean (± SEM), paired T-test was used in Figure 7, n = 3 for each quoted experiment if not shown otherwise.

Simulated ischaemia

We used a modified version of the model first described by de Hemptinne et al. [27, 28]. Ischaemia was simulated by immersing the preparation in paraffin oil as shown in Figure 1. This was achieved by floating a drop of paraffin oil on top of the meniscus in the experimental chamber, while maintaining a constant solution flow. The level of the aqueous-oil interface in the bath was then reduced electronically using a servo-controlled level device [29] until the bulk of the preparation was immersed in the oil. A small area of contact between fibre and Tyrode solution remained, as shown in Figure 1. This was essential in order to maintain electrical contact between the surface-ion microelectrode and the bath reference electrode. The flowing Tyrode solution at the base of the chamber also maintained total bath temperature at 37 °C (this was monitored in some experiments using a bead thermistor touching the preparation surface; surface temperature changes upon paraffin immersion were < 1 °C): The small area of Tyrode solution-fibre contact during simulated ischaemia was considered acceptable since surface-O_2 reading with an O_2 sensitive electrode gave relative O_2-readings similar to those in complete anoxia (100 N_2 in Tyrode; for technical details see [28]). With the specific features and validity of this model has been dealt in further detail elsewhere [9, 28]. In order to simulate reperfusion, the level of aqueous-oil interface in the bath was raised again electronically until the fibre was completely re-immersed in well-oxygenated flowing Tyrode solution.
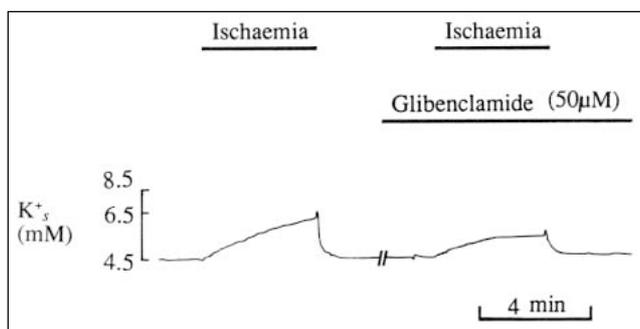
Figure 4. Effect of glibenclamide during simulated ischaemia in an isolated pig coronary artery preparation. Glibenclamide (50 μm) reduces rate and extent of K^+efflux from the tissue. Left and right hand panel from the same preparation. Glibenclamide added 20 minutes before beginning of trace on the right hand side panel. Gap in trace represents 30 min.

Heating

The heater control was based on a linear feed-back circuit which measured the bath temperature near the preparation with a small thermistor and supplied power to the heating coil (several turns of constantan wire around the glass tube) which delivers the normal Tyrode to the bath. The thermistor was supplied with a constant current from a PNP transistor. The desired temperature (37 °C) was set on a potentiometer. The voltage difference between the set value and the value sensed by the thermistor has been amplified and used to fuel the heater. The amplifier had a variable gain control which allowed the ideal adjustment according to the type of experiment (e.g. in experiments where the paraffin model was applied, a higher gain was used to allow quick adjustment to the new temperature conducting properties in the chamber after paraffin immersion).

Results

Simulated ischaemia in isolated pig coronary artery strips

Figure 4 shows the potassium accumulation measured with valinomycin K^+ selective microelectrodes on the surface of a pig coronary artery strip recorded during immersion of the preparation in paraffin oil. Within 4 min, there was a rise of K^+_s from 4.5 to 6.5 mM. In the same preparation, electrode readings of surface potassium have been reproducible in up to ten consecutive ischaemic episodes (not shown) suggesting that changes in rate and extent of ischaemic K^+ accumulation (e.g. upon addition of drugs) are not a result of degenerative changes in the tissue. No difference has been seen between experiments performed with or without endothelium (care-
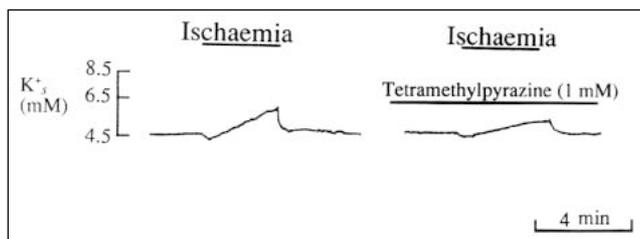
Figure 6. Effect of tetramethylpyrazine (a non-sulfonylurea compound known to block K^+_{ATP} channels in various tissues, in the literature also named ligustrazine) during simulated ischaemia in an isolated pig coronary artery preparation. Tetramethylpyrazine reduces rate and extent of K^+-efflux from the tissue. Left and right hand panel from the same preparation. Tetramethylpyrazine added 20 min before beginning of trace on the right hand panel. Gap represents 30 min.
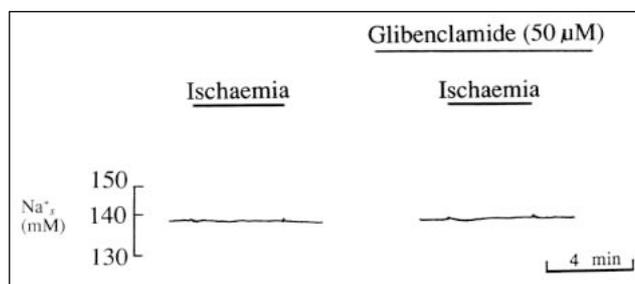

Figure 5. Measurement of Na^+ with Na^+ selective microelectrode during simulated ischaemia in an isolated pig coronary artery strip. Na^+_s did not change throughout the ischaemic episode, indicating a steady state of transmembrane Na^+-movement during early simulated ischaemia in this preparation. In the presence of glibenclamide (50 μM), Na^+ also remained unchanged. Left and right hand panel from the same preparation. Glibenclamide added 20 minutes before beginning of trace on the right hand panel. Like in Figure 4, left and right hand panel from the same preparation.

fully removed with a brush) as far as K^+ accumulation is concerned (not shown), indicating that endothelial cells neither modulate nor contribute to ischaemic K^+ efflux.

Figure 10 shows that simulated ischaemia also produced a fall in surface pH. Terminating the ischaemic period (by elevating the solution level in the chamber thus removing the layer of paraffin oil) reversed the changes in K^+, and pH.

In 13 experiments K^+_s rose within 4 min of simulated ischaemia from 4.5 to 6.6 ± 0.2 mM and pH fell within the same amount of time from 7.30 ± 0.01 to 7.12 ± 0.03. The changes are similar to those observed previously during various simulated ischaemic manoeuvres induced either in whole perfused heart [30, 31] or isolated cardiac muscle [11, 27, 32].

While, for convenience, our experiments were exclusively performed in HEPES buffered solutions, we have also attempted to perform the same type of experiment in CO_2-HCO_3^- buffered media. However, the changes of K^+_s and pH, were qualitatively similar during simulated ischaemia, although extracellular pH was ~40 % smaller when using CO_2-HCO_3^-buffered solutions. This observation is similar to data on cardiac tissue presented by Vanheel et al. [28] who reported the ischaemia induced fall in pH, and pH_i to be 50 % smaller when using CO_2-HCO_3^-buffered media, presumably because of an increase in intracellular and extracellular H^+ buffering power.
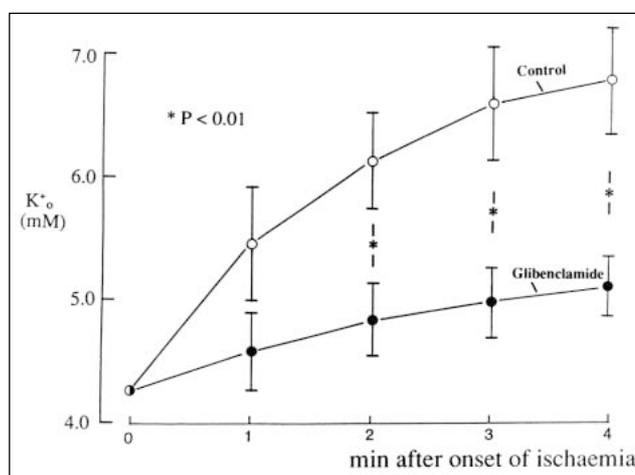
Figure 7. Ischaemia induced K^+ accumulation in 6 isolated pig coronary artery strips in the presence (closed symbols) and absence (open symbols) of glibenclamide (50 μM). Data from experiments similar to those in Figures 4 and 6.
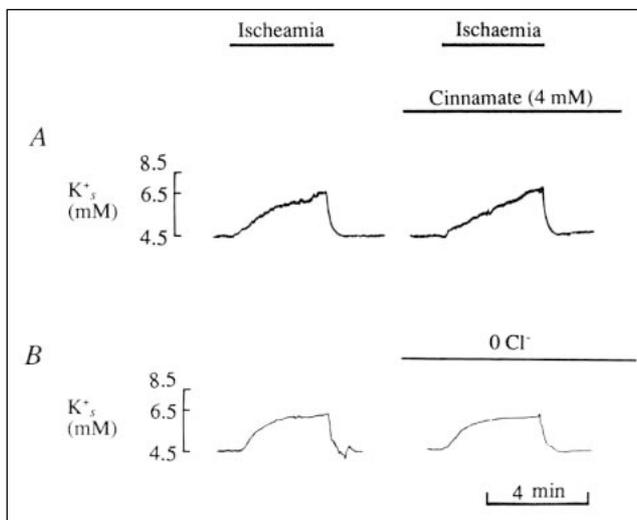
Figure 8. A. Lack of effect of alpha-cyano-4-hydroxycinnamate (cinnamate, a lactate transport inhibitor) on ischaemia induced K^+ efflux in an isolated pig coronary artery strip. Preparation has been incubated with 4 mM cinnamate for 15 minutes before beginning of trace in right hand panel. Left and right hand panel from the same preparation. Gap represents 20 min. B. In Cl^--free solution (Cl^- replaced by glucuronate one hour before testing the effect of simulated ischaemia) rate and extent of K^+ accumulation are the same as in the presence of Cl^-. Gap represents 60 min. Left and right hand panel from the same preparation (like in A).

Figure 5 shows a recording of Na^+_s, using a Na^+-selective microelectrode (*cf.* Methods). There were no changes in surface Na^+ during simulated ischaemia, indicating a steady state of transmembrane Na^+-movement during early simulated ischaemia in this type of preparation. A slowing of the Na^+/K^+ pump seems therefore unlikely to be the cause of extracellular K^+ accumulation, because in this case one would expect a decrease of Na^+_s in the reduced extracellular space created between the electrode tip and the surface of the preparation. This observation is in accordance with data reported on cardiac tissue by Rau et al [33, *cf.* 34]. Furthermore, glibenclamide (50 μM) had no effect on Na^+_s during simulated ischaemia suggesting that, in pig coronary artery, it has no effect on transmembrane Na^+ transport.

Effect of sulphonylureas on K^+ accumulation
Figure 3 shows that in isolated pig coronary artery, the sulphonylurea drug, glibenclamide (50 μM) partially inhibited the ischaemia induced K^+_s rise. The remaining glibenclamide insensitive fraction of K^+-accumulation was not abolished by higher concentrations of glibenclamide of up to 200 μM (not shown). Partial inhibition of K^+ efflux was also found using 200 μM and 1 mM of tolbutamide, another sulphonylurea compound (not shown). Tetramethylpyrazine (1 mM), a known blocker of K^+_{ATP} channels in pancreatic β-cells [35], which has also been studied in hypoxic rats [36] and in hypoxic isolated guinea pig hearts [37], reduced K^+_s accumulation by 50 % (Fig. 6). Figure 7 shows a plot of the time course of K^+_s-accumulation observed in six preparations in the presence and absence of 50 μM glibenclamide. In the same preparations, the rise in K^+ (from 4.5 to 6.8 ± 0.4 mM) was markedly reduced in the presence of glibenclamide, where K^+ rose from 4.5 to 5.1 ± 0.2 mM (± SEM; P < 0.01, p. T-test).

Lactate linked K^+ efflux
In heart, K^+ efflux has been suggested to be linked to lactate efflux which also increases during ischaemia [38]. In cardiac
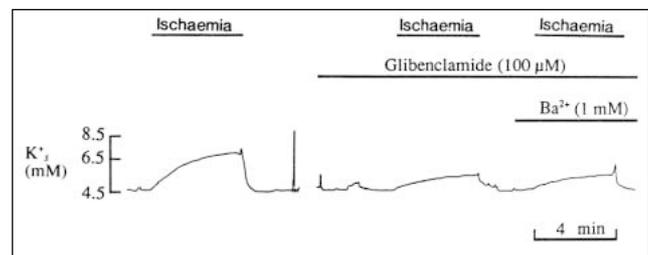
Figure 9. Glibenclamide partially inhibits ischaemia induced K^+-efflux in an isolated pig coronary artery strip. Left and right hand panel from the same preparation. Glibenclamide added 20 minutes before beginning of trace on the right hand panel. Gap in trace represents 30 minutes. The addition of 1 mM Ba^{2+} does not inhibit the glibenclamide-insensitive component of ischaemia induced K^+ efflux.

Purkinje fibres, lactate linked K^+ efflux could, however, not be verified [9]. Lactate efflux occurs to a large extent via a transmembrane carrier inhibitable by alpha-cyano-4-hydroxycinnamic acid (cinnamate; see below; *cf.* [39, 27]). On the one hand, although cinnamate (4 mM) reduced ischaemic pH changes in pig coronary arteries (Fig.10), glibenclamide did not (n = 6; not shown), suggesting that lactate- and K^+ movement must occur via independent pathways (*cf.* also [9]). On the other hand, cinnamate (4 mM) does not inhibit K^+ accumulation during simulated ischaemia as shown in Fig. 8 A. We conclude therefore that ischaemia induced K^+-efflux is not linked directly to lactate efflux.

Cl^--linked K^+ efflux
Some authors suggested that in heart ischaemia-induced K^+-efflux could be linked to Cl^- efflux (*cf.* [10, 11, 34]), but no evidence has been provided for such a co-transport. In fact, neither a very high dose of bumetanide, a high-affinity inhibitor of Na^+-K^+-2Cl co-transport with some inhibitory potency for KCl transport, nor complete removal of Cl^- from the preparation had an effect on K^+ accumulation in the sheep cardiac Purkinje fibre [9].

In the present work, we find that in the isolated pig coronary artery, K^+ accumulation can proceed in the total absence of Cl^- as shown in Fig. 8 B. In this experiment, Cl^- has been replaced by glucuronate for 1 h before testing the effect of simulated ischaemia, a time sufficient for complete removal of intracellular Cl^- [40]. We conclude therefore that ischaemia induced K^+-efflux is not directly linked to transmembrane Cl^- extrusion.

K^+ efflux via another K^+ channel
As we have shown above, there is a glibenclamide insensitive fraction of ischaemia induced K^+ efflux in pig coronary artery. Since most K^+ channels are blocked by Ba^{2+} or tetraethylammonium ions (TEA) we have simulated ischaemia in the presence of both glibenclamide (100 μM) and Ba^{2+} (1 mM) as shown in Figure 9. There has been no difference between K^+ accumulation in presence of glibenclamide plus Ba^{2+} and glibenclamide alone, suggesting that no Ba^{2+} sensitive channel other than the K^+_{ATP} channel is involved in ischaemia induced K^+ efflux in pig coronary artery. Similar results have been found with TEA (1 mM; not shown).

Alternatively, in the presence of Ba^{2+} without glibenclamide, K^+ efflux is reduced to the same extent as in the presence of glibenclamide (not shown), suggesting that Ba^{2+} ions inhibit ischaemia induced K^+ efflux via the same pathway as does glibenclamide. Similar results have been already reported on isolated smooth muscle membrane patches by Nelson et al. [4].
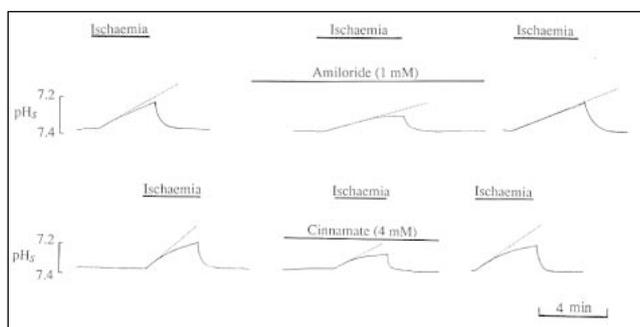
Figure 10. A. Pig coronary artery strip. Simulated ischaemia produces a fall in surface pH, which is reversed upon terminating the ischaemic episode. Amiloride (1 mM) reduces rate and extent of surface acidification. Amiloride added to the solution 20 minutes before beginning of the second trace. Third trace: control, 30 minutes after amiloride has been washed off. Gap between traces represents 30 minutes. B. Experiment similar to the one shown in A, cinnamate (4 mM) reduces rate and extent of surface acidification. Gap in traces represents 30 min. Third panel on the right hand side represents control, 30 minutes after cinnamate has been washed off.

Measurement of pH during simulated ischaemia

Figure 10 displays changes in surface pH during simulated ischaemia in the isolated pig coronary artery strip. It can be seen that pH_s falls from 7.36 to 7.28 (Fig. 10 A) and from 7.38 to 7.23 (Fig. 10 B) in the two experiments shown (left hand panel).

(i) Effect of amiloride: In cardiac muscle, the key mechanism in regulating intracellular pH is Na^+/H^+-exchange (for review see [41, 42, 43]). This acid extrusion mechanism is strongly activated by intracellular acidification and can be blocked by amiloride. Therefore, in our experiments, the effect of this drug on transmembrane H^+-transport during simulated ischaemia has been tested in HEPES buffered solutions, where Na^+/H^+ exchange activity may be expected to be larger than in CO_2-HCO_3- buffered media, due to more extensive intracellular acidosis as observed in cardiac tissue by Vanheel et al. [28]. In their experiments, these authors have shown that amiloride reduces surface acidification during paraffin-oil simulated ischaemia in isolated guinea pig papillary muscle.

In the presence of 1 mM amiloride, the rate and extent of surface acidification was considerably slower (Fig. 10 A). An ischaemic control episode shows the recovery of rate and extent of surface acidification in the same preparation 30 min after amiloride has been washed off (Fig. 10 A, left hand panel). From these experiments we conclude that Na^+/H^+ exchange, in vascular smooth muscle too, plays a role in acid extrusion during conditions like ischaemia.

(ii) Effect of alpha-cyano-4-hydroxy-cinnamic acid (cinnamate): In cardiac tissue, acid extrusion is also mediated by a transmembrane lactic acid co-transporter, which can be inhibited by 4 mM cinnamate [20, 27, 39, 42]. Under the assumption that this transport system is effective also in coronary artery smooth muscle, we have measured ischaemia induced surface acidification in the presence and absence of this drug as shown in Fig. 10 B. A clear reduction in rate and extent of acidification can be seen in the presence of cinnamate, suggesting that during ischaemic conditions, in vascular smooth muscle, the lactic acid transport mechanism participates in acid extrusion.

In this work, we have not attempted to measure intracellular pH during simulated ischaemia in vascular smooth muscle simply for technical difficulties (small size of smooth muscle cells). A complete assessment of acid extrusion mechanisms in smooth muscle during simulated ischaemia would involve more complex measurements, including the testing for Cl-/

HCO_3--exchange, intracellular buffering power, intracellular Ca^{2+}-concentrations etc and shall be attempted in further work.

Discussion

Our main finding is that ischaemia induced extracellular K^+ accumulation is not a typical feature of myocardial tissue alone but can also be seen in arterial preparations. We also find that this K^+ efflux is, to some extent, linked to a sulphonylurea (specific inhibitors of K^+_{ATP}-channels) sensitive pathway. The lack of effect of cinnamate on ischaemia-induced K^+ accumulation indicates that this K^+ accumulation is not mediated by the lactic acid carrier, nor is it linked to Cl- efflux either, since K^+ accumulation proceeds well in the complete absence of Cl-. Thus, in pig coronary artery, ischaemia-induced K^+ efflux is unlikely to be linked obligatorily with anion extrusion, a conclusion which has been previously drawn for myocardial K^+ efflux [38].

Furthermore, we find that ischaemia induced extracellular acidification, a feature also known from ischaemic myocardium, can be seen in arterial tissue, too. We show that, in arterial tissue, acid extrusion during conditions of simulated ischaemia is mediated by both Na^+/H^+ exchange and the lactic acid carrier.

Simulated ischaemia and K^+_s accumulation

The validity of this particular experimental approach to simulate ischaemia (paraffin oil model) has been discussed extensively in earlier publications and shall not be repeated here [9, 27, 28]. The method is technically difficult. Special problems observed with ion-selective microelectrodes during paraffin simulated ischaemia are shown and discussed in Methods.

In cardiac tissue, the paraffin oil model produces the salient characteristics of myocardial ischaemia, extra- and intracellular acidification, action potential shortening and K^+ accumulation (see also Fig. 3) with well correlating concomitant diastolic membrane depolarisation. These phenomena have been witnessed clinically [11, 30], experimentally in whole hearts (eg, [8]), in isolated multicellular tissue (eg, [28, 31]) and in isolated cardiac myocytes (eg, [16, 44]).

In arterial tissue, the paraffin oil model also produces extracellular acidification and K^+ accumulation during simulated ischaemia. The rate and extent of both H^+ and K^+ accumulation are similar in arterial and myocardial tissue. Unfortunately, in arterial tissue, no comparable data have been obtained with other techniques so far. However, evidence for the opening of K^+_{ATP} channels in vascular smooth muscle during hypoxia and metabolic inhibition has been provided directly (eg, [3, 4]) and indirectly [1, 4].

Inhibition by sulphonylureas of extracellular K^+-accumulation during simulated ischaemia in artery

Since ischaemia induced K^+ accumulation in arterial tissue has not been described so far, the underlying mechanism has remained unspecified too.

In heart, a number of mechanisms have been suggested as involved in ischaemic K^+ accumulation: inhibition of active K^+ uptake (*cf.* [11]), osmotic changes (eg, [47]), K^+ efflux linked to anion extrusion (eg, [34, 38, 48]), K^+ efflux by cation exchange for reasons of electro-neutrality [34, 49], association of K^+ efflux with altered cellular energy levels (eg. [33, 50]), involvement of a K^+ channel [8, 9, 33, 51] and a catecholamine activated pathway [52] and some other less likely possibilities. However, the mechanism of K^+ efflux from myocardial cells during ischaemia has not been completely identified to date. In our experiments we find that ischaemia induces K^+ accu-

mulation on the surface of isolated strips of pig coronary arteries. Firstly, this K^+ accumulation occurs equally in both preparations with and without endothelium, indicating that the measured K^+ accumulation does not derive from endothelial cells. The adventitial layer mainly consists of collagen fibres and thus is unlikely to be the source of potassium efflux. Hence, during ischaemia, the accumulating K^+ ions measured on the surface of pig coronary arteries are likely to derive exclusively from arterial smooth muscle cells. Secondly, we find that 50 μM glibenclamide (a known blocker of K^+_{ATP} channels in smooth muscle; *cf.* [3, 4]) reduce early ischaemic K^+ efflux from arterial preparations by ~66 % (Fig. 7). In these preparations, higher doses of glibenclamide (up to 200 μM; K_d for glibenclamide is 10 μM; see [45]) did not lead to complete inhibition of K^+ accumulation in contrast to observations in guinea pig papillary muscle (Fig 3 [9]). In three experiments, we used a dose as small as 20 μM, which also led to a similar inhibition of K^+ efflux. The relatively high dose of 50 μM has been chosen in order to assure that as many K^+_{ATP} channels as possible are blocked. Dose-response curves have not been attempted, because the experiments are extremely difficult and time consuming. Similar data were obtained using high concentrations of tolbutamide (0.5 mM and 1 mM; eg. in myocytes $K_d (K_{ATP}) = 0.5$ mM for tolbutamide – *cf.* [14]) and tetramethylpyrazine (1 mM). The latter has been shown to act as a weak blocker of K^+_{ATP}-channels ($K_d = 0.7$ mM) in pancreatic β-cells [35] and prevents ischaemia-induced coronary dilation in isolated Langendorff-perfused guinea pig hearts [37]. Furthermore, this interesting drug, which constitutes a purified and chemically identified component of a Chinese herbal remidy effects hypoxic pulmonary vasoconstriction [36].

K^+ loss by slowing of the Na^+/K^+ pump?

The net loss of K^+ during early ischaemia can theoretically be explained by either an increased efflux or by a decreased influx of K^+. Normally, intracellular K^+ levels are in a steady state with extracellular levels and passive K^+-efflux will be balanced by an equal K^+ uptake by the Na^+/K^+-pump. Thus, reducing the extracellular space by pressing a microelectrode on the surface of a preparation would not be expected to raise K^+, unless either K^+ efflux increased or K^+ re-uptake decreased (*cf.* [23]). The observed rise of K^+_s therefore suggests that either K^+ efflux or K^+-influx must change. A decreased K^+ re-uptake may result from an altered activity of the Na^+/K^+-pump (during ischaemia as a result of decreasing levels of intracellular ATP). In heart, however, experimental evidence has been provided that K^+ accumulation is not due to a failure of the Na^+/K^+-pump [33]. The latter authors have demonstrated, that K^+ loss during the first hour of hypoxia could be completely accounted for by an increase in K^+ efflux without evidence of suppressed K^+ influx. If, however, a failing Na^+/K^+ pump was the reason for K^+ accumulation in the artificially created RECS between ion-selective carrier and preparation, one would, at the same time, measure a concomitant decrease of Na^+ in this RECS. Using a Na^+ selective microelectrode in coronary artery, we did not measure even a small signal change that could be associated with a decrease in Na^+, on the surface of the preparation during an ischaemic episode (Fig. 5). Na^+/K^+ pump slowing thus seems not to be the mechanism of K^+ accumulation in the present study. Furthermore, sulphonylureas inhibit K^+ accumulation. If slowing of the Na^+/K^+ pump were the sole mechanism responsible for K^+ accumulation, one would have to propose a pump protective property of sulphonylureas during ischaemia, which seems inherently unlikely. Furthermore, at least in heart muscle, it has been shown that an increase in Na^+_i started only after 6–7 min of global ischaemia in the rat heart. Under the assumption that, in

smooth muscle cells too, Na^+_i does not rise earlier than 6–7 minutes after the onset of ischaemia, it is unlikely that a K^+ accumulation occurring within 15 seconds after the onset of ischaemia be linked to a failure of the Na^+/K^+ pump.

Is K^+ accumulation in artery linked to anion extrusion?

Transmembrane ionic flux is mediated by highly selective membrane proteins such as ion-channels or transport carriers. In heart, it has been suggested that anions (e.g. lactate) generated during anaerobic metabolism would be transported out of the cell in an electro-neutral form together with K^+ [34, 38, 48]. This idea has originally derived from a suggestion of Boyle and Conway [53] and Mainwood & Lucier [54] who assumed that the efflux of K^+ in fatigued frog skeletal muscle was linked to efflux of lactic acid. Castle and Haylett [55] showed in the same preparation that this was not the case. In fact, these authors showed that in fatigued frog skeletal muscle, enhanced K^+-efflux was predominantly due to opening of sulphonylurea sensitive K^+ channels. The hypothesis of lactate-linked cation-efflux is based on proportional efflux rates of K^+ and lactate from progressively ischaemic dog hearts [28, 48, 56] and a decreased H^+/K^+ efflux ratio with maintained lactate efflux from fatigued frog sartorius muscle [54]. For arterial smooth muscle under ischaemic conditions no concept of anion-linked K^+ efflux has been advanced so far. However, our data on artery do not support such a hypothesis fully: In heart, lactate efflux occurs to a large extent via a transmembrane carrier protein inhibitable by alpha-cyano-4-hydroxy-cinnamic acid (cinnamate) [39]. In pig coronary artery, the carrier is revealed by the fact that both rate and extent of surface acidification are reduced during an ischaemic episode in the presence of cinnamate (*cf.* Fig, 10). Glibenclamide does not influence the rate of acidosis during an ischaemic episode (not shown). Similarly, cinnamate does not inhibit K^+-accumulation during the first 4 minutes of simulated ischaemia as shown in Fig. 8. It is therefore not likely that the major fraction of early ischaemic K^+ efflux in artery smooth muscle is mediated by the lactic acid carrier. These findings are in accordance with those of Cascio, Yan & Kleber [57] who demonstrated that, during the first five minutes of myocardial ischaemia, cinnamate had no effect on K^+ efflux. Another interesting observation of these authors was that K^+ efflux to some extent depended on extracellular PCO_2. They conclude that accumulating CO_2 during ischaemic conditions affect pH_i and pH_s thus likely to affect transmembrane transfer of H^+ and /or anions and possibly K^+ efflux. This PCO_2 dependency, however, was significant only more than 5 min after the onset of ischaemia. In our experiments on arterial smooth muscle, which studied only the first four minutes of simulated ischaemia, we therefore did not attempt to investigate the possibility of an effect of CO_2-accumulation on K^+ efflux or upon intracellular and extracellular pH. Such changes in pH could also be of interest in the light of the known pH-sensitivity of K^+_{ATP} channel open probability [58].

Another candidate for K^+/anion co-transport would be Cl^-, perhaps via a KCl or even a Na^+-K^+-$2Cl^-$ co-transport system [10, 11, 34]. In our experiments on arterial preparations, we find that K^+ accumulation can proceed in the total absence of Cl^- as shown in Fig. 8B. In this experiment, Cl^- has been replaced by glucuronate 1 h before testing the effect of simulated ischaemia, a time sufficient for complete removal of intracellular Cl^- [40].

Thus, it appears unlikely that K^+ efflux be linked to Cl^- extrusion during ischaemia.

Other mechanisms of K^+ efflux

Despite the general agreement in the literature that early K^+ accumulation during myocardial ischaemia is not due to cell

membrane damage (eg, [10]) one must consider the possibility of cell damage in our experiments. Cell damage may be produced not only by ischaemic manoeuvres but also by methodological problems. Fig. 2 and Fig. 3 allude to the possibility of physical damage as a result of positioning the electrode. However, the fact that K^+_{ATP}-channel blockade inhibits K^+ accumulation during early ischaemia (in heart completely, in smooth muscle partially using this type of experimental assembly) naturally argues against physical damage as the source of K^+ efflux. On the other hand, late K^+ efflux is believed to be caused by irreversible cell damage, since intracellular H_2O and sodium increase [59]. In our experiments we have not investigated late K^+ efflux in artery. In heart, osmotic changes as a source of increased K^+ efflux [47] have been already rejected by Kleber [34] who calculated that an 80 % loss of extracellular fibre water would be needed in order to produce a rise in K^+ efflux of the magnitude observed experimentally. A similar calculation can be made for arterial smooth muscle.

Involvement of an ion-channel in ischaemic K⁺ efflux
The rapidity, reversibility and reproducibility of ischaemic K^+ efflux gives rise to the question whether or not one or several ion-channels might be involved. In heart this concept has been favoured by many authors (eg, [7, 8, 33, 51, 60]). In fact, the possibility of an enhanced K^+-outward current during simulated myocardial ischaemia has already been proposed in the 1950's [61, 62]. In arterial smooth muscle, possible candidates would be delayed rectifier channels, large conductance K^+_{Ca} channels and K^+_{ATP} channels. Delayed rectifier K^+ current has been studied at the whole cell level and using single channel recordings [63, 64]. The current is voltage dependent, activating with depolarisation, and also shows time- and voltage-dependent inactivation. The channel is quite insensitive to TEA and intracellular metabolic changes as well as to glibenclamide [45]. Thus, both the fact that glibenclamide inhibits ischaemia induced K^+ efflux and that open probability (P_{open}) of the channel is independent of metabolic changes argues against this channel being involved. The same applies for large conductance K^+_{Ca} channels, which are equally insensitive to glibenclamide and changes in intracellular ATP. These channels are activated by high levels of cytoplasmic ionised Ca^{2+}. The latter is not a typical feature of arterial smooth muscle which is known to dilate under conditions of hypoxia or metabolic inhibition (*cf.* [1, 3]). We therefore conclude that this channel does not contribute substantially to ischaemic K^+ efflux. The ATP-dependent K^+ channel, however, is thought to have multiple closely associated regulatory binding sites with which sulphonylureas interact specifically. In single, isolated smooth muscle cells as well as in membrane patches and whole heart preparations (coronary perfusion pressure as an equivalent to the contractile state of coronary arteries) this channel is operational during conditions of hypoxia and metabolic inhibition [1, 3, 4]. In our experiments K^+ efflux is markedly inhibited by specific blockers of K^+_{ATP} channels, suggesting that this channel is involved in ischaemic K^+ efflux. The main argument against opening of the K^+_{ATP} channel, at least in the initial stages of ischaemia, is that intracellular ATP (ATP_i)has not decreased sufficiently, so that the channels would remain inactivated. Resting ATP in cells is usually 5–10 mM and the Kd_{ATP} for the K^+_{ATP} channels in isolated patches is ~100 μM, a concentration of ATP certainly not reached after 15–30 seconds when K^+_s accumulation is at its rise. Although the K^+_{ATP} channel is defined by its sensitivity to intracellular ATP, ATP may not necessarily be the most important modulator of its activity in many tissues. Channel P_{open} has been reported to be affected by a number of factors in addition to ATP, including ADP,

GDP, GTP, pH, glycolytic by-products [16, 17, 58, 65]. K^+_{ATP} channels can also be activated by neuropeptides in intact cells [45, 65] with physiological levels of intracellular ATP and thus made insensitive to ATP. Phosphorylation appears to be required for the maintenance of channel activity in β-cells. Noteworthy in this context is a suggestion by Coburn, Grubb & Aronson [66] who favoured the concept of an O_2-sensing system in smooth muscle distinct from oxidative phosphorylation. Another possible explanation for early opening of K^+_{ATP}-channels is that compartmentalisation of $(ATP)_i$ may mean that the *subsarcolemmal* ATP concentration that *inhibits* the channel is much lower than the measured whole-cell level [67, 68], thus being more vulnerable to changes in cellular metabolism. Recently, a disturbance of the PCr shuttle mechanism (used for the transfer of the high-energy phosphate bond to ADP in the cell) has been addressed as causal for certain ischaemic phenomena [69]. An involvement of such a defect in subsarcolemmal high energy phosphate metabolism during ischaemia cannot be excluded.

Whatever the trigger for K^+ channel opening, it would appear to be rapid following the onset of simulated ischaemia. In our own work, sulphonylurea sensitive K^+ accumulation is detectable within 15–30 seconds, which is in consistent with observations on K^+_{ATP} channel opening in multicellular preparations upon hypoxia [1, 46].

The source of the sulphonylurea-insensitive component of ischaemic K^+ efflux, however, is difficult to identify. It is not sensitive to Ba^{2+} or TEA, excluding specific K^+ channels sensitive to these substances (eg, i_{KCa} is sensitive to TEA). Furthermore, it is insensitive to cinnamate and occurs in the complete absence of Cl^-, excluding therefore anion co-transport as a source. Thus, this glibenclamide-insensitive component of ischaemic K^+ efflux remains unspecified.

The possible role of K⁺ATP channel opening during ischaemia
The general function of K^+_{ATP}-channels seems to be to sense the metabolic state of the muscle cell in whose membrane they lie, and form part of an appropriate homeostatic response [3]. In vascular smooth muscle, the opening of these channels provides a means of regulating vascular tone. In fact, the increased transmembrane outward current resulting from K^+_{ATP} channel opening leads to hyperpolarisation (*cf.* Goldman equation) thus reducing the P_{open} state of voltage gated Ca^{2+} channels. A subsequently reduced Ca^{2+} influx, in turn, leads to vasodilation, a vital response to hypoxia or ischaemia, especially in the regulation of myocardial blood supply through the coronary arteries [1, 4].

For the future, specific openers of this channel with a high selectivity for coronary arteries could play a role in the treatment of ischaemic heart disease.

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