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ATP-SENSITIVE K⁺ CHANNEL MODIFICATION BY METABOLIC INHIBITION IN ISOLATED GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. ATP-sensitive K^+ (K^+_{ATP}) channels are believed to make an important contribution to the increased cellular K^+ efflux and shortening of the action potential duration (APD) during metabolic inhibition, hypoxia, and ischaemia in the heart. The mechanisms by which the activity of the K^+_{ATP} channel is regulated during conditions of metabolic impairment are not completely clear. *Extrinsic* factors such as increased $[ADP]_i$, acidosis, and stimulation of adenosine receptors appear to decrease the K^+_{ATP} channel's sensitivity to closure by $[ATP]_i$. The purpose of this study was to determine whether the K^+_{ATP} channel itself is *intrinsically* altered by the processes associated with metabolic impairment.

2. Isolated guinea-pig ventricular myocytes were metabolically inhibited in glucose-free 1·8 mM Ca^{2+} Tyrode solution containing 9 μM rotenone and 0·9 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) while recording unitary currents through K^+_{ATP} channels in cell-attached patches. When K^+_{ATP} channel activity became maximal, the patch was excised (inside-out) into 150 mM K^+ bath solution containing different ATP concentrations. The K_d for suppression by $[ATP]_i$ ($[ATP]_i$ causing half-maximal suppression of current through K^+_{ATP} channels) was markedly increased to 305 μM ($n = 9$) compared to patches excised from control myocytes not exposed to metabolic inhibitors ($K_d = 46$ μM, $n = 28$).

3. A $[Ca^{2+}]_i$-dependent process was involved in K^+_{ATP} channel modification during metabolic inhibition. Removal of extracellular Ca^{2+} during metabolic inhibition led to an intermediate decrease in the ATP sensitivity of the K^+_{ATP} channels ($K_d = 120$ μM, $n = 6$). In myocytes that were pretreated with 10 μM ryanodine in addition to removing extracellular Ca^{2+}, the reduction in ATP sensitivity was completely prevented ($K_d = 23$ μM, $n = 6$).

4. In inside-out membrane patches excised from control non-metabolically inhibited myocytes, elevated free $[Ca^{2+}]_i$ (2 μM) did not alter the sensitivity of the K^+_{ATP} channel to closure by $[ATP]_i$, suggesting that in metabolically inhibited myocytes elevated $[Ca^{2+}]_i$ acted indirectly. K^+_{ATP} channel run-down was found to increase the sensitivity of K^+_{ATP} channels to closure to $[ATP]_i$ ($K_d = 16$ μM, $n = 13$).

5. Inside-out membrane patches excised from control non-metabolically inhibited myocytes were also exposed to various proteases, phospholipases and other reagents that may be activated during metabolic inhibition. Trypsin and chymotrypsin

treatment increased the K_d from 39 to 213 μM ($n = 8$) and 110 μM ($n = 5$), respectively. Calpain I had no apparent effect on the K_d. Phospholipases A_2, C and D were all found to modestly desensitize K^+_{ATP} channels to closure by $[ATP]_i$. Treatment of excised membrane patches with the free radical generating system, H_2O_2 + ferric chloride, or with the glycolytic inhibitor and sulfhydryl-modifying agent, iodoacetate, did not significantly affect the ATP sensitivity of the K^+_{ATP} channel.

6. In conclusion, we have found that in isolated ventricular myocytes subjected to severe metabolic inhibition a Ca^{2+}-dependent process *intrinsically* modified the K^+_{ATP} channel to decrease its sensitivity to closure by $[ATP]_i$. We speculate that this process may contribute to persistent cellular K^+ loss and failure of APD to recover fully in reperfused myocardium after prolonged ischaemia.

INTRODUCTION

The ATP-sensitive K^+ (K^+_{ATP}) channel has been extensively studied in cardiac tissue. Although its role in normal cardiac function is not well understood, during conditions of metabolic impairment such as ischaemia and hypoxia, accumulating evidence suggests that activation of K^+_{ATP} channels makes an important contribution to shortening of the action potential duration (APD) and increased cellular K^+ efflux (Noma, 1983; Gasser & Vaughan-Jones, 1990; Kantor, Coetzee, Carmeliet, Dennis & Opie, 1990; Wilde, Escande, Schumacher, Thuringer, Mestre, Fiolet & Janse, 1990; Deutsch, Klitzner, Lamp & Weiss, 1991; Venkatesh, Lamp & Weiss, 1991). During ischaemia, the resulting $[K^+]_o$ accumulation exacerbates electrophysiological alterations predisposing the heart to re-entrant ventricular arrhythmias (Janse & Wit, 1989), the leading cause of death from coronary artery disease (Goldman, Cook, Hashimoto, Stone, Muller & Loscalzo, 1982). However, the role of K^+_{ATP} channels in these phenomena is still controversial because of the large discrepancy between the typically millimolar levels of cytosolic [ATP] during early ischaemia in intact heart and the low concentrations of ATP_i needed to suppress K^+_{ATP} channels in excised membrane patches, with half-maximal suppression occurring at $[ATP]_i$ from 15 to 100 μM (Noma, 1983; Findlay, 1987; Nichols & Lederer, 1990; Weiss, Venkatesh & Lamp, 1992). Recent evidence suggests that activation of only a small fraction of K^+_{ATP} channels (< 1%) are needed to account quantitatively for the degree of APD shortening and increased K^+ efflux observed during early ischaemia and hypoxia (Carmeliet, Storms & Vereecke, 1990; Faivre & Findlay, 1990; Nichols, Ripoll & Lederer, 1991; Weiss *et al.* 1992). Furthermore, in addition to the modest fall in $[ATP]_i$ during ischaemia, it is likely that additional *extrinsic* factors such as the rapid rise in free cytosolic $[ADP]_i$, acidosis, stimulation of adenosine receptors and lactate accumulation can activate K^+_{ATP} channels to the required degree by decreasing their sensitivity to inhibition by intracellular ATP (Lederer & Nichols, 1989; Kirsch, Codina, Birnbaumer & Brown, 1990; Nichols & Lederer, 1990; Cuevas, Bassett, Cameron, Furukawa, Myerburg & Kimura, 1991; Keung & Li, 1991; Weiss *et al.* 1992). The possibility that K^+_{ATP} channels themselves may be *intrinsically* modified by processes associated with metabolic inhibition, such that their sensitivity to closure by intracellular ATP is altered, has not been systematically explored. To examine this possibility, we characterized the ATP sensitivity of K^+_{ATP} channels in inside-out membrane patches excised from guinea-pig ventricular myocytes after they had been subjected to metabolic inhibition. The results indicate that a Ca^{2+}-dependent process associated with metabolic inhibition dramatically decreased the sensitivity of the K^+_{ATP} channels to $[ATP]_i$. Furthermore, treatment of inside-out patches excised from non-metabolically inhibited myocytes with proteases such as trypsin, and, to a lesser extent, with phospholipases, caused similar reductions in intracellular ATP sensitivity. Portions of this work have been published previously in abstract form (Deutsch & Weiss, 1991).

METHODS

Cell isolation

Guinea-pigs of either sex weighing 200–300 g were anaesthetized with an intraperitoneal injection of a lethal dose of sodium pentobarbitone. The heart was rapidly excised through a thoracotomy incision and mounted on a Langendorff perfusion apparatus. Single ventricular myocytes were isolated by enzymatic digestion with collagenase and protease (Mitra & Morad, 1985).

Patch clamp methods

Cells were placed in a 0·5 ml capacity experimental chamber mounted on the stage of an inverted microscope and were continuously perfused at a rate of 1–4 ml/min. Patch electrodes were fabricated from 8161 glass (Corning Glass Inc., Corning, NY, USA) and had a tip resistance of 2–4 MΩ when filled with the standard patch electrode solution. Patch electrodes were mounted on the head-stage of an Axopatch 1D or 200 amplifier (Axon instruments, Burlingame, CA, USA). Membrane current and voltage signals were recorded on a chart recorder and on a hard disk using Axotape software (Axon instruments) for later computer analysis with customized software. A six-channel multibarrelled rapid-perfusion device with a common opening was used to facilitate rapid solution changes at the cytoplasmic surface (facing the bath) of the inside-out patches. The 90% exchange time of the bath solution surrounding the patch electrode was typically < 200 ms (Weiss *et al.* 1992). All patch-clamp experiments were performed at room temperature (22–24 °C).

Solutions and experimental procedures

The standard patch electrode solution contained (mM): KCl, 4; NaCl + NaOH, 145; and Hepes, 5; pH 7·35. In some experiments, Na^+ was replaced by equimolar K^+. Myocytes were initially superfused with a modified Tyrode solution containing (mM): NaCl, 136; NaOH, 9; NaH_2PO_4, 0·33; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 1; Hepes, 10; dextrose, 10; pH 7·25. Before excising inside-out membrane patches, the bath solution was changed to a high-K^+, low-Ca^{2+} solution consisting of (mM): KCl + KOH, 150; $CaCl_2$, 0·5; EGTA, 2; $MgCl_2$, 2; MgATP, 2; and Hepes, 5; pH 7·25. The sensitivity of the K^+_{ATP} channels to closure by $[ATP]_i$ was tested by varying the [ATP] of this solution from 0 to 2 mM. The magnesium salt of ATP along with 2 mM $MgCl_2$ was always used to maintain a free $[Mg^{2+}]$ of ≈ 2 mM in all bath solutions. The free Ca^{2+} concentration was estimated at ≈ 0·04 μM.

With 4 mM K^+ in the patch electrode, the patch electrode potential was held at 0 mV to record outward currents through K^+_{ATP} channels in order to minimize contamination of the current tracings by inwardly rectifying K^+ channels. In experiments with 150 mM K^+ in the patch electrode, the patch electrode was generally held at either +40 or −40 mV to examine inward or outward currents through K^+_{ATP} channels. Because of the large number of channels (> 10) in most patches, recording membrane current for 10–12 s was adequate to assess the effects of a given $[ATP]_i$ on channel activity while minimizing channel run-down. Each intracellular ATP concentration tested was bracketed by exposure to ATP-free bath solution and data were accepted for analysis only if the current in the ATP-free solution returned to > 80% of the pre-test value.

Metabolic inhibition

Metabolic inhibition was produced using 0·9 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler which also increases ATP utilization through induction of mitochondrial ATPase activity, and 9 μM rotenone, an inhibitor of mitochondrial NAD-linked respiration (Haworth, Nicolaus, Goknur & Berkoff, 1988). These agents were added to either dextrose-free modified Tyrode solution containing 1·8 mM Ca^{2+} or to dextrose-free high-K^+, low-Ca^{2+} (EGTA-buffered) bath solution described above. In some experiments 10 μM ryanodine was included in the high-K^+, low-Ca^{2+} solution.

Proteases, phospholipases and other treatments

To test the effects of various interventions on the ATP sensitivity of K^+_{ATP} channels, patches were excised in the standard high-K^+, low-Ca^{2+} bath solution and the dose response to various intracellular ATP concentrations was first defined under control conditions. The patches were then exposed to various reagents in the presence of 100 or 300 μM $[ATP]_i$ (with 2 mM free $[Mg^{2+}]$ present). This intracellular ATP concentration range was high enough to minimize channel run-down but low enough to observe increases in K^+_{ATP} channel activity if their ATP sensitivity decreased. At various time points until the patch ruptured, the dose response to $[ATP]_i$ was redetermined in the absence of the reagent. The following reagents were investigated: trypsin (Sigma type III, 2 mg/ml), α-chymotrypsin (Sigma type II, 2 mg/ml), calpain I (1 unit/ml), phospholipase A_2 (Sigma P9279, 2 units/ml), phospholipase C (Sigma type I, 2 units/ml), phospholipase D (Calbiochem, La Jolla, CA, USA, 10 units/ml), hydrogen peroxide (1 mM) plus $FeCl_3$ (0·1 mM), iodoacetate (1 mM), and elevated free $[Ca^{2+}]_i$ (2 μM). The reagents were added directly to the standard high-K^+, low-Ca^{2+} (EGTA-buffered to $\approx$ 0·04 μM) bath solution except for calpain and the phospholipases which were suspended in the same solution buffered to a free $[Ca^{2+}]_i$ of 2 μM.

Drugs and chemicals

All chemicals were obtained from Sigma Chemicals (St Louis, MO, USA) unless otherwise indicated. Calpain I (8 units/ml), isolated from human erythrocytes, was kept frozen in 50% glycerol buffer at −70 °C and was thawed and dialysed in bath solution prior to use. FCCP and rotenone were dissolved in dimethyl sulphoxide (DMSO) to make stock solutions (10 mM) and then added to bath solution to achieve the desired final concentration. Ryanodine (Progressive Agri Systems, Wind Gap, PA, USA) was dissolved in H_2O to make a 2 mM stock solution and then added to the bath solution to achieve the desired concentration.

Data analysis

Statistical analysis was performed by analysis of variance (ANOVA). $P < 0.05$ was considered significant. All results are presented as means ± 1 S.E.M.

RESULTS

Metabolic inhibition reduces the intrinsic sensitivity of the K^+_{ATP} channels to closure by $[ATP]_i$

Figure 1*A* illustrates the activation of K^+_{ATP} channels in a cell-attached patch on an isolated guinea-pig ventricular myocyte during exposure to the metabolic inhibitors FCCP (0·9 μM) and rotenone (9 μM) in dextrose-free Tyrode solution containing 1·8 mM Ca^{2+}. With 4 mM K^+ in the patch electrode held at −40 mV, outward current through K^+_{ATP} channels became progressively larger during metabolic inhibition. After the level of current reached a plateau, the bath solution was changed to 150 mM K^+, low-Ca^{2+} solution to fully depolarize the myocyte and the patch electrode potential changed to 0 mV. The cell-attached patch was then excised into the bath to form an inside-out membrane patch. Subsequently, the patch was exposed to various $[ATP]_i$ (0, 10, 30, 100, 300 and 2000 μM) to define the ATP sensitivity of the K^+_{ATP} channels (Fig. 1*B*). In this example the K_d, i.e. the $[ATP]_i$ causing half-maximal suppression of current through K^+_{ATP} channels, was between 300 and 2000 μM. In nine patches excised from metabolically inhibited myocytes following the same protocol, the relative current at different $[ATP]_i$ was averaged and fitted to a Hill equation:

$$I/I_{max} = 1/\{1+([ATP]_i/K_d)^h\}.$$

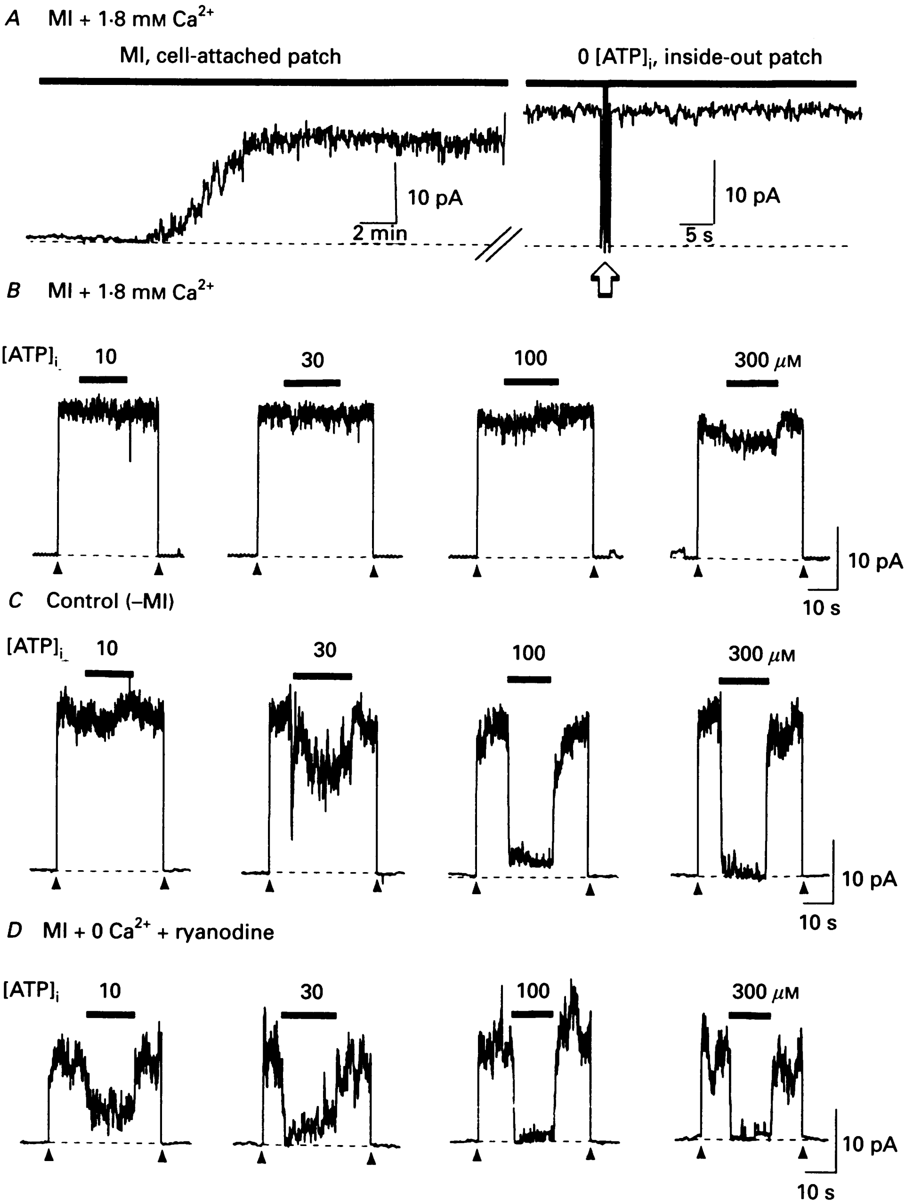

Fig. 1. Effect of metabolic inhibition (MI) on the ATP sensitivity of the K^+_{ATP} channel. *A*, outward current through K^+_{ATP} channels in a cell-attached patch on an isolated guinea-pig ventricular myocyte increased progressively during exposure to the metabolic inhibitors FCCP (0·9 μM) and rotenone (9 μM) in dextrose-free 1·8 mM Ca^{2+} Tyrode solution. After the current was maximal, the bath solution was changed to the 150 mM K^+, low-Ca^{2+}, ATP-free solution (at the break in the trace) and the patch was excised (at arrow). *B*, the same excised inside-out membrane patch was exposed to 10, 30, 100 and 300 μM $[ATP]_i$ (indicated by bars) after maximally activating the channels by removing 2000 μM $[ATP]_i$ between the small arrowheads. *C* and *D*, the $[ATP]_i$ sensitivity of representative inside-out patches excised from a control non-metabolically inhibited myocyte (*C*), and from a myocyte pretreated with ryanodine and subjected to metabolic inhibition in a Ca^{2+}-free bath solution (*D*). Zero current levels are indicated by the dashed lines. The patch

The best fit yielded a K_d of 305 μM, with a Hill coefficient (h) of 1·6 (Fig. 2*A*). In contrast, in inside-out membrane patches excised from control cells not exposed to metabolic inhibitors (Figs 1*C* and 2*A*), the K_d was 46 μM with a similar Hill coefficient of 1·7, within the range reported previously in ventricular myocytes (Noma, 1983; Findlay, 1987; Nichols & Lederer, 1990; Weiss *et al.* 1992). Since it has been previously noted that there is a large variation in the K_d values between different patches (Faivre & Findlay, 1990; Weiss *et al.* 1992), Fig. 2*B* compares the distribution of K_d values for patches excised from metabolically inhibited and non-metabolically inhibited cells. It is evident that the sensitivity of K^+_{ATP} channels to $[ATP]_i$ was shifted markedly to the right by metabolic inhibition. It is very unlikely that this shift was due to direct effects of FCCP and rotenone on the channels, since in membrane patches excised from control non-metabolically inhibited cells, these agents did not significantly affect the ATP sensitivity of K^+_{ATP} channels during up to 20 min of superfusion ($K_d = 50$ μM, $n = 6$, *versus* 46 μM, $n = 28$, for control patches). Also, the ATP sensitivity of K^+_{ATP} channels in patches from the metabolically inhibited cells was always tested after FCCP and rotenone had been washed from the bath, and under identical conditions to the patches excised from control non-metabolically inhibited myocytes.

Modification of K^+_{ATP} channels by metabolic inhibition involves a $[Ca^{2+}]_i$-dependent process

To investigate the possible role of elevated $[Ca^{2+}]_i$ during metabolic inhibition as a cause of the reduced sensitivity of K^+_{ATP} channels to intracellular ATP, we removed extracellular Ca^{2+} from the bath solution containing FCCP and rotenone. Using the high-K^+, low-Ca^{2+} (EGTA-buffered to $\approx$ 0·04 μM) bath solution in place of modified Tyrode solution, the same protocol of metabolic inhibition was followed. In inside-out patches excised from these myocytes ($n = 6$), the ATP sensitivity was intermediate, with a K_d of 120 μM and Hill coefficient of 1·6 (Fig. 2*A* and *B*). When, in addition to removing extracellular Ca^{2+}, myocytes were pretreated with 10 μM ryanodine to deplete intracellular Ca^{2+} stores before exposure to metabolic inhibitors ($n = 6$), the reduction in ATP sensitivity was completely prevented (Figs 1*D* and 2*A* and *B*). The K_d under these conditions was 23 μM with a Hill coefficient of 1·3, which is lower than the K_d of 46 μM in excised membrane patches from control non-metabolically inhibited cells. This finding is consistent with the effects of run-down on the ATP sensitivity of K^+_{ATP} channels (see later).

Effects of elevated $[Ca^{2+}]_i$ and run-down in excised membrane patches

To determine whether elevated $[Ca^{2+}]_i$ may have directly reduced the sensitivity of K^+_{ATP} channels to $[ATP]_i$ during metabolic inhibition, inside-out patches were excised from control non-metabolically inhibited myocytes and their dose response

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electrode solution contained 4 mM K^+ in all cases, and the patch electrode was held at 0 mV except during superfusion of the myocyte with Tyrode solution in *A* when it was −40 mV (corresponding to an outward driving force for current through K^+ channels). Free Mg^{2+} was 2 mM in all solutions in *B–D*. See text for further details.

to various concentrations of intracellular ATP tested before and after increasing free $[Ca^{2+}]$ from $\approx 0{\cdot}04$ to 2 μM (in 150 mM K^+ bath solution). Elevated $[Ca^{2+}]_i$ had no significant effect on either the K_d (47 *versus* 46 μM) or Hill coefficient (1·5 *versus* 1·6) estimated from the averaged data from thirteen patches (Fig. 3*A*) or on the

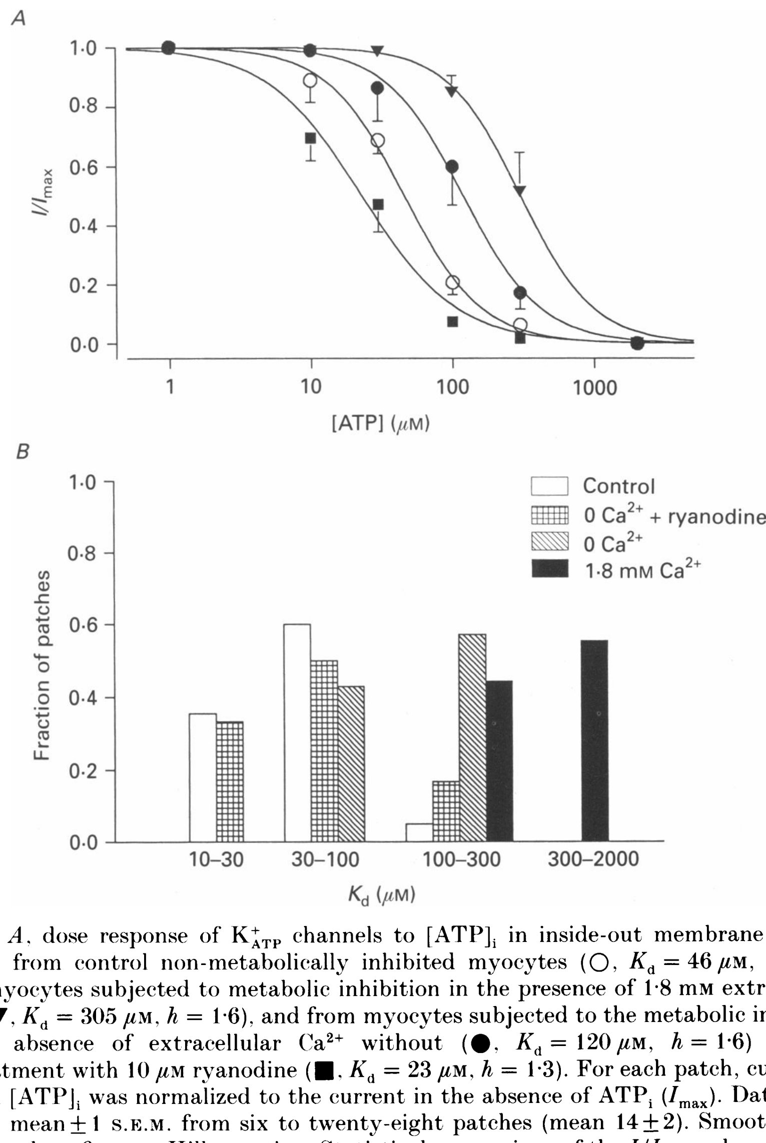

Fig. 2. *A*, dose response of K^+_{ATP} channels to $[ATP]_i$ in inside-out membrane patches excised from control non-metabolically inhibited myocytes (○, $K_d = 46\ \mu$M, $h = 1{\cdot}7$), from myocytes subjected to metabolic inhibition in the presence of 1·8 mM extracellular Ca^{2+} (▼, $K_d = 305\ \mu$M, $h = 1{\cdot}6$), and from myocytes subjected to the metabolic inhibition in the absence of extracellular Ca^{2+} without (●, $K_d = 120\ \mu$M, $h = 1{\cdot}6$) or with pretreatment with 10 μM ryanodine (■, $K_d = 23\ \mu$M, $h = 1{\cdot}3$). For each patch, current (I) at each $[ATP]_i$ was normalized to the current in the absence of ATP_i (I_{max}). Data points are the mean ± 1 S.E.M. from six to twenty-eight patches (mean 14 ± 2). Smooth curves represent best fits to a Hill equation. Statistical comparison of the I/I_{max} values over the [ATP] range of 10–300 μM were as follows: control *vs.* 1·8 mM Ca^{2+}, $P < 0{\cdot}001$; control *vs.* 0 Ca^{2+}, $P < 0{\cdot}01$; control *vs.* 0 Ca^{2+} + 10 μM ryanodine, $P > 0{\cdot}05$. *B*, distribution of K_d values among individual patches under the various conditions described above. Only individual patches in which a sufficient number of intracellular ATP_i concentrations were tested to estimate accurately the K_d within the ranges indicated are included. Experimental conditions were the same as described in Fig. 1. See text for further details.

distribution of K_d values among the individual patches (Fig. 3*B*). Since cytosolic Ca^{2+} and other divalent cations accelerate run-down of K^+_{ATP} channels (Findlay, 1987), we also examined whether run-down of K^+_{ATP} channels, which is likely to occur during severe metabolic inhibition, altered the ATP sensitivity of K^+_{ATP} channels.

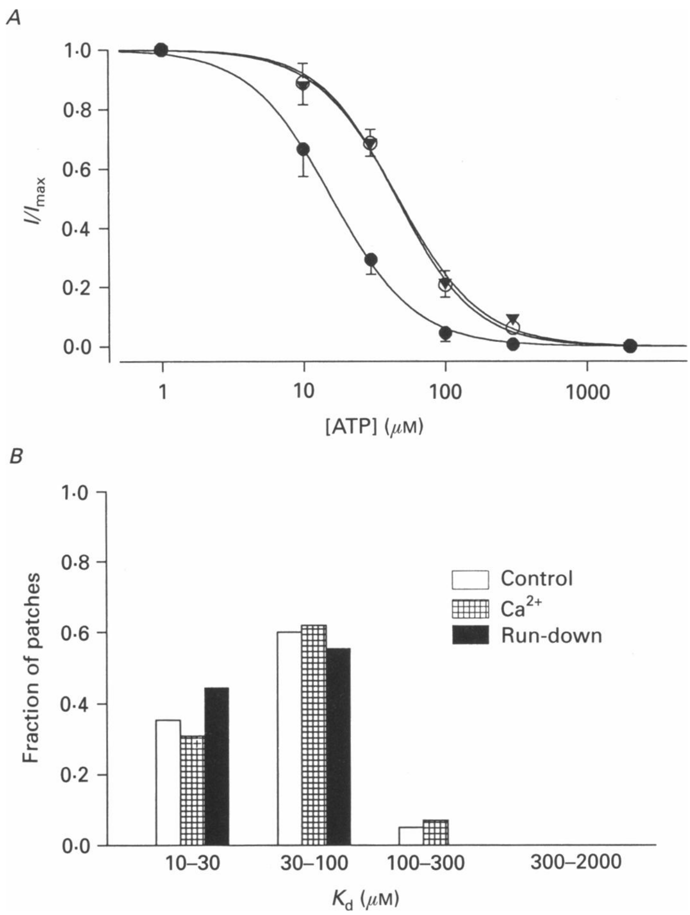

Fig. 3. Dose–response curve of K^+_{ATP} channels to $[ATP]_i$ in inside-out membrane patches excised from control non-metabolically inhibited myocytes before and after exposure to elevated free $[Ca^{2+}]_i$ (2 μM), and before and after channel run-down by > 50%. For graphic illustration the control $[ATP]_i$ dose–response curves were pooled. *A*, averaged data from nine to twenty-eight patches (mean 17 ± 4) for each $[ATP]_i$ under control conditions (0·04 μM free Ca^{2+}, ○, $K_d = 46$ μM, $h = 1·6$), with 2 μM free Ca^{2+} (▼, $K_d = 47$ μM, $h = 1·5$) and after run-down (●, $K_d = 16$ μM, $h = 1·5$). Smooth curves are best fits to a Hill equation. Statistical comparison of the I/I_{max} values over the [ATP] range of 10–300 μM were as follows: control *vs.* 2 μM free Ca^{2+}, $P > 0·05$; control *vs.* run-down, $P < 0·001$. *B*, distribution of K_d values among individual patches under the various conditions, as indicated in the key. See text and legend to Fig. 2 for further details.

After testing their dose response to $[ATP]_i$, inside-out patches excised from control non-metabolically inhibited myocytes were exposed to ATP-free bath solution (with 2 mM free Mg^{2+} present) until the current ran down to less than half the initial value,

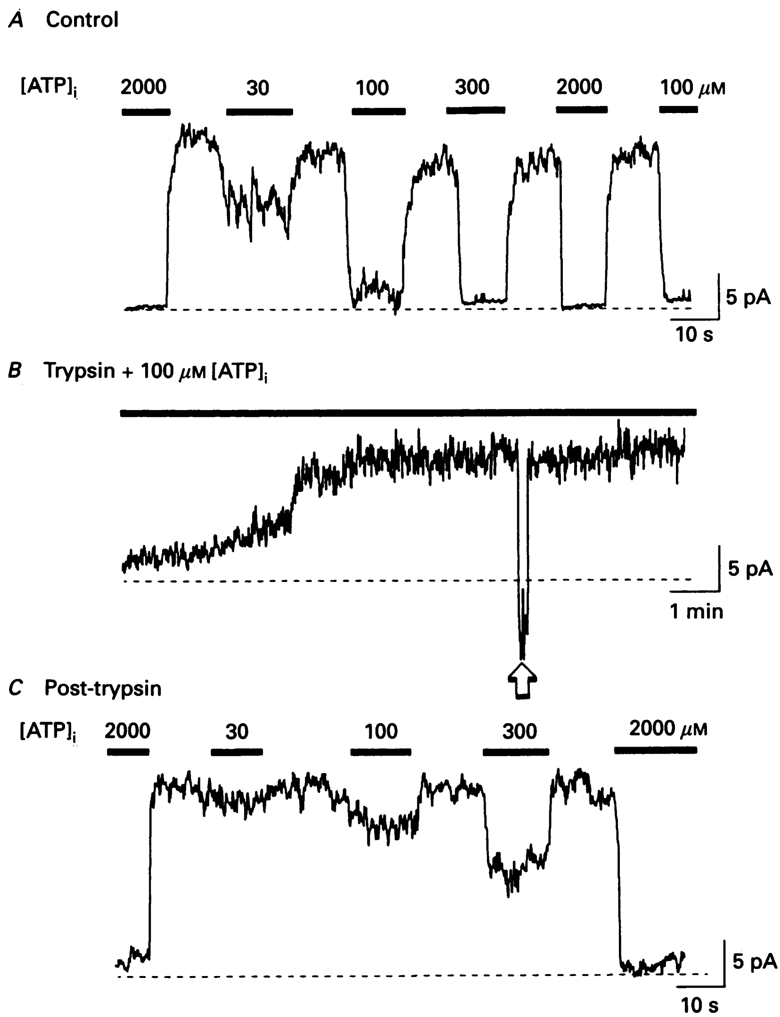

Fig. 4. Effect of trypsin on the sensitivity of K^+_{ATP} channels to closure by intracellular ATP. *A*, an inside-out patch was excised from a control non-metabolically inhibited myocyte and exposed to the different ATP concentrations as indicated above the bars. *B*, in the presence of 100 μM $[ATP]_i$, the same patch was exposed to trypsin (2 mg/ml), resulting in a progressive increase in outward current through K^+_{ATP} channels despite constant $[ATP]_i$. The dip in current trace was due to briefly changing the patch electrode potential from 0 to −80 mV. *C*, after washing out the trypsin, the patch was re-exposed to the various $[ATP]_i$ indicated and demonstrated a markedly reduced sensitivity to closure by intracellular ATP. The patch electrode solution contained 4 mM K^+, and the patch electrode was held at 0 mV except for the brief period in *B*. Free Mg^{2+} was 2 mM in all solutions. The zero current levels are indicated by the dashed lines.

(mean time 5·6 ± 1·1 min). The sensitivity of the K^+_{ATP} channel to closure by $[ATP]_i$ was then retested. After run-down, the K^+_{ATP} channels became more sensitive to closure by $[ATP]_i$, with the K_d decreasing to 16 μM and the Hill coefficient remaining at 1·5 (Fig. 3*A*). The distribution of K_d values among the individual patches also shifted to the left after run-down (Fig. 3*B*).

Effect of proteases on excised inside-out membrane patches

To investigate the possibility that activation of an intracellular protease facilitated by an increase in $[Ca^{2+}]_i$ during metabolic inhibition might have been responsible for reducing the intrinsic sensitivity of K^+_{ATP} channels to $[ATP]_i$, we examined the effects

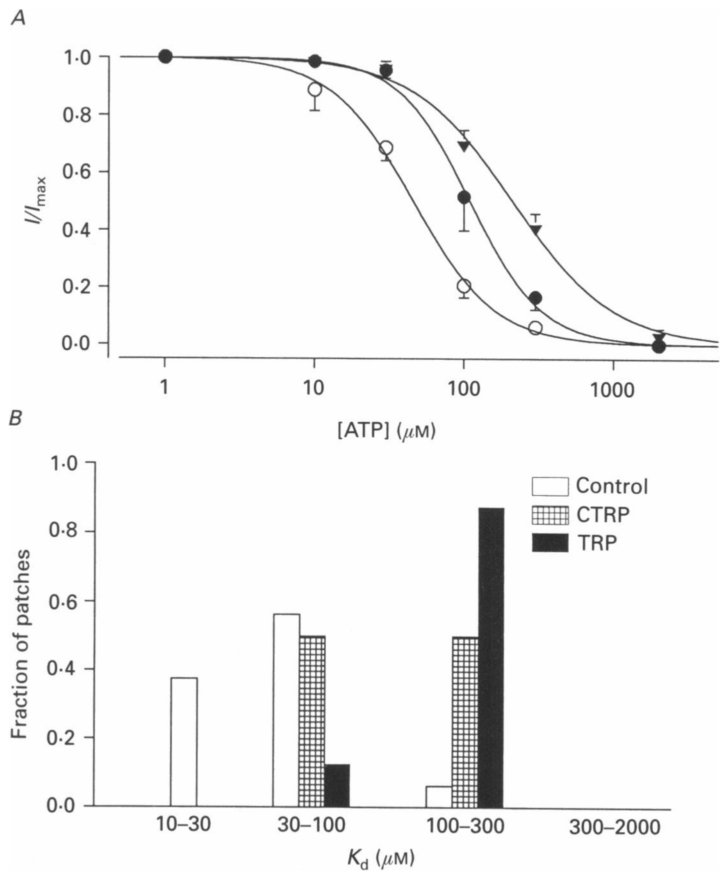

Fig. 5. Effects of trypsin and chymotrypsin treatment on the dose response of K^+_{ATP} channels to $[ATP]_i$ in inside-out membrane patches excised from control non-metabolically inhibited myocytes. For graphic illustration the control intracellular ATP dose–response curves before exposure to trypsin and chymotrypsin patches were pooled. *A*, averaged data from five to thirteen patches (mean 9 ± 2) for each $[ATP]_i$ before (○, $K_d = 39$ μM, $h = 1{\cdot}5$) and after treatment with trypsin (▼, $K_d = 213$ μM, $h = 1{\cdot}3$) or chymotrypsin (●, $K_d = 110$ μM, $h = 1{\cdot}8$), fitted to a Hill equation (smooth curves). Statistical comparison of the I/I_{max} values over the [ATP] range of 10–300 μM were as follows: control *vs.* trypsin, $P < 0{\cdot}001$; control *vs.* chymotrypsin, $P < 0{\cdot}01$. *B*, distribution of K_d values among individual patches before and after treatment with trypsin (TRP) or chymotrypsin (CTRP). See text and legend to Fig. 2 for further details.

of treating excised inside-out patches with several types of proteases, including trypsin (2 mg/ml), chymotrypsin (2 mg/ml) and calpain I (1 unit/ml). Figure 4 illustrates an example of the typical protocol in an inside-out patch excised from a control non-metabolically inhibited myocyte. Before treatment with trypsin, the patch was exposed to high-K^+, low-Ca^{2+} bath solution containing various ATP concentrations (in the presence of 2 mM free $[Mg^{2+}]_i$) to define the sensitivity of the

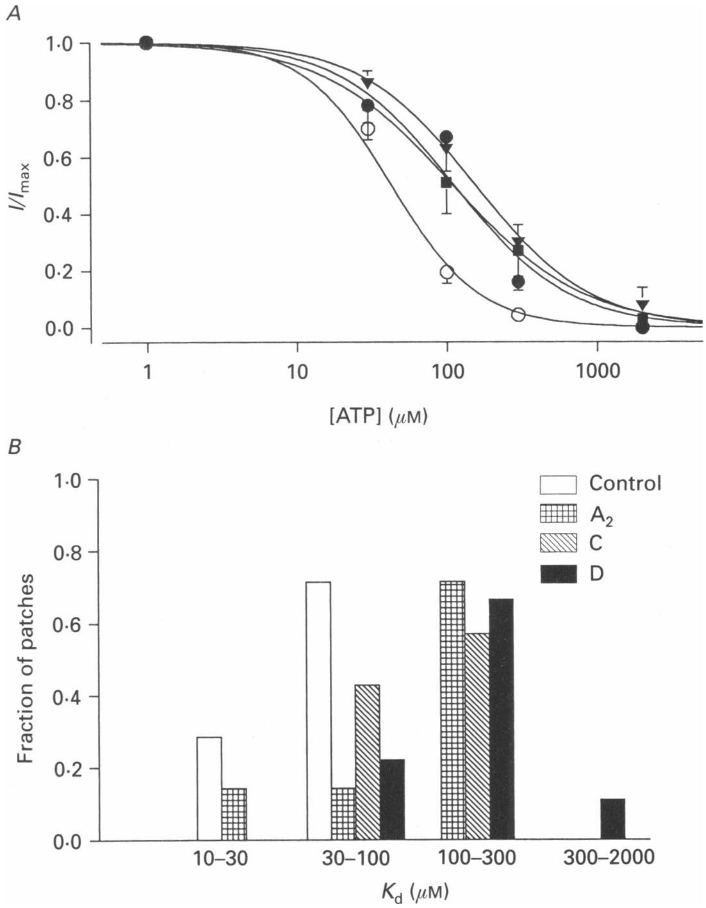

Fig. 6. Effect of phospholipase A_2, C, and D on the ATP sensitivity of K^+_{ATP} channels in inside-out patches excised from control non-metabolically inhibited myocytes. The free $[Ca^{2+}]$ of the bath solution containing each phospholipase was increased to 2 μM. For graphic illustration the control $[ATP]_i$ dose–response curves were pooled. *A*, averaged data from six to ten patches (mean 8 ± 2) for each $[ATP]_i$ before phospholipase treatment (○, $K_d = 43$ μM, $h = 1{\cdot}6$) and after treatment with phospholipase A_2 (●, $K_d = 113$ μM, $h = 1{\cdot}2$), phospholipase C (■, $K_d = 111$ μM, $h = 1{\cdot}0$), and phospholipase D (▼, $K_d = 157$ μM, $h = 1{\cdot}2$), fitted to a Hill equation. Statistical comparison of the I/I_{max} values over the [ATP] range of 30–300 μM were as follows: control *vs.* phospholipase A_2, $P < 0{\cdot}05$; control *vs.* phospholipase C, $P < 0{\cdot}01$; and control *vs.* phospholipase D, $P < 0{\cdot}001$. *B*, distribution of K_d values before and after treatment of patches with phospholipases A_2, C and D. See text and legend to Fig. 2 for further details.

K^+_{ATP} channels in the patch to closure by $[ATP]_i$ (top panel). The patch was then exposed to 2 mg/ml trypsin in the continuous presence of 100 μM $[ATP]_i$ (middle panel). As has been reported previously by others, current gradually increased during exposure to trypsin (Trube, Hescheler & Schröter, 1989), and run-down was virtually eliminated (Furukawa, Zheng, Sawanobori & Hiroaka, 1992). After 12 min, trypsin was washed out and the ATP sensitivity of K^+_{ATP} channels in the patch retested (lower panel). As can be seen by comparing the upper and lower traces, the K_d increased from ≈ 30 to ≈ 300 μM after trypsin treatment in this patch. In eight patches exposed to the same protocol, treatment with trypsin for an average of $11{\cdot}3 \pm 1{\cdot}4$ min increased the K_d from 39 to 213 μM $[ATP]_i$, with Hill coefficients of 1·5 and 1·3 respectively (Fig. 5*A*). The distribution of K_d values in the individual patches was commensurately shifted to the right (Fig. 5*B*).

Chymotrypsin had a similar but quantitatively lesser effect than trypsin, with the K_d increasing from 39 to 110 μM $[ATP]_i$ and Hill coefficients of 1·5 and 1·8 respectively (Fig. 5*A*). The distribution of K_d values also shifted to the right in five patches (Fig. 5*B*). Prevention of run-down by chymotrypsin was not apparent, however.

In four patches, calpain I (1 unit/ml), a Ca^{2+}-activated protease present in heart (Croall & Demartino, 1991), had no apparent effect on the K_d to $[ATP]_i$. During exposure to calpain, the $[Ca^{2+}]$ of the high-K^+ bath solution was raised to 2 μM. The K_d of all four patches was between 30 and 100 μM $[ATP]_i$ both before and after calpain I treatment, although a subtle change within this range may have been missed.

Effects of phospholipases on excised inside-out membrane patches

Since phospholipases are also Ca^{2+} sensitive and may be activated during metabolic inhibition, we examined the effects of various phospholipases on the ATP sensitivity of K^+_{ATP} channels in excised inside-out patches. We used the same protocol as for the proteases, except that the free Ca^{2+} of the bath solution was raised to 2 μM to promote phospholipase activity. After treatment with phospholipases A_2, C or D, the K_d increased to 113, 111 and 157 μM $[ATP]_i$ with Hill coefficients of 1·2, 1·0 and 1·2, respectively (Fig. 6*A* and *B*). Unlike trypsin, the phospholipases tended to promote rather than prevent channel run-down.

Effects of other treatments on excised inside-out membrane patches

We tested several additional interventions using similar protocols. An oxygen free radical-generating system consisting of 1 mM H_2O_2 and 0·1 mM ferric chloride mixed just prior to superfusing the excised patch did not significantly affect the distribution of K_d values in six excised inside-out membrane patches. Iodoacetate (1 mM), a potent inhibitor of glycolysis and a sulfhydryl group modifying agent, also had no significant effect in four patches.

DISCUSSION

Effects of metabolic inhibition on the sensitivity of K^+_{ATP} channels to $[ATP]_i$

The role of K^+_{ATP} channels during myocardial ischaemia, hypoxia and other forms of metabolic inhibition remains controversial. However, a number of recent studies have provided evidence that because of the high density of K^+_{ATP} channels in heart, only a very small increase in their open probability to < 1% is sufficient to explain the degree of APD shortening and increased cellular K^+ efflux observed during early ischaemia or hypoxia (Carmeliet *et al.* 1990; Faivre & Findlay, 1990; Nichols *et al.* 1991; Weiss *et al.* 1992). In addition, *extrinsic mechanisms* have been found to promote activation of K^+_{ATP} channels during metabolic inhibition by reducing their sensitivity to $[ATP]_i$ (Lederer & Nichols, 1989; Kirsch *et al.* 1990; Nichols & Lederer, 1990; Cuevas *et al.* 1991; Keung & Li, 1991; Weiss *et al.* 1992). In the present study, we have identified a mechanism which reduces the *intrinsic* sensitivity of K^+_{ATP} channels to $[ATP]_i$ presumably by irreversibly modifying the channel protein in some way. We emphasize that the ATP sensitivity of K^+_{ATP} channels in inside-out membrane patches excised from metabolically inhibited myocytes was intrinsically reduced because their ATP sensitivity was tested under conditions identical to those in patches excised from control non-metabolically inhibited myocytes, i.e. in the absence of any soluble components which may have accumulated in the myocyte during the period of metabolic inhibition before the patch was excised.

Mechanism of the reduced intrinsic ATP sensitivity of K^+_{ATP} channels caused by metabolic inhibition

The process during metabolic inhibition which reduced the intrinsic sensitivity of K^+_{ATP} channels to $[ATP]_i$ appeared to be dependent on $[Ca^{2+}]_i$, which is well documented to rise during metabolic inhibition in isolated myocytes (Barry, Peeters, Rasmussen & Cunningham, 1987; Eisner, Nichols, O'Neill, Smith & Valdeolmillos, 1989; Li, Hohl, Altschuld & Stokes, 1989) and during ischaemia in intact heart (Marban, Kitakaze, Kusuoka, Porterfield, Yue & Chacko, 1987; Steenbergen, Murphy, Levy & London, 1987; Mohabir, Lee, Kurz & Clusin, 1991). Specifically, removal of extracellular Ca^{2+} and pretreatment with ryanodine to deplete intracellular Ca^{2+} stores before subjecting myocytes to metabolic inhibition completely prevented the $\approx$ 7-fold increase in K_d for suppression of the channels by $[ATP]_i$ observed in patches excised from myocytes subjected to metabolic inhibitors with 1·8 mM Ca^{2+} present. In patches from the Ca^{2+}-depleted metabolically inhibited myocytes, the K_d for $[ATP]_i$ was actually reduced after metabolic inhibition, probably due to channel run-down, which is likely to occur concurrently with K^+_{ATP} channel activation as metabolic inhibition progresses. This is supported by the finding that in patches excised from control non-metabolically inhibited myocytes, run-down increased the sensitivity of K^+_{ATP} channels to $[ATP]_i$ (Fig. 3), as has been noted previously (Thuringer & Escande, 1990; Nichols & Lederer, 1991). Ca^{2+} from either extracellular or intracellular compartments appeared to be important in desensitizing K^+_{ATP} channels to closure by intracellular ATP, since removal of extracellular Ca^{2+} alone only partially prevented the increase in K_d.

In patches excised from control non-metabolically inhibited myocytes, the inability of either elevated $[Ca^{2+}]_i$ or channel run-down to decrease the sensitivity of K^+_{ATP} channels to $[ATP]_i$ suggests that in metabolically inhibited myocytes $[Ca^{2+}]_i$ acted indirectly with other factors to reduce channel sensitivity to $[ATP]_i$. Since the activity of many proteases and phospholipases are increased by $[Ca^{2+}]$, we studied the effects of these enzymes on patches excised from control non-metabolically inhibited myocytes. Treating patches with trypsin decreased the ATP sensitivity of K^+_{ATP} channels to a similar extent as metabolic inhibition. Trube *et al.* (1989) observed that trypsin treatment of patches excised from pancreatic β-cells decreased the sensitivity of K^+_{ATP} channels to closure by $[ATP]_i$, in agreement with our results.

Furukawa *et al.* (1992) found that trypsin prevented channel run-down in patches excised from guinea-pig ventricular myocytes, but did not significantly increase the K_d for suppression by intracellular ATP (although a modest statistically insignificant increase was observed). The reason for this discrepancy with our findings is unclear.

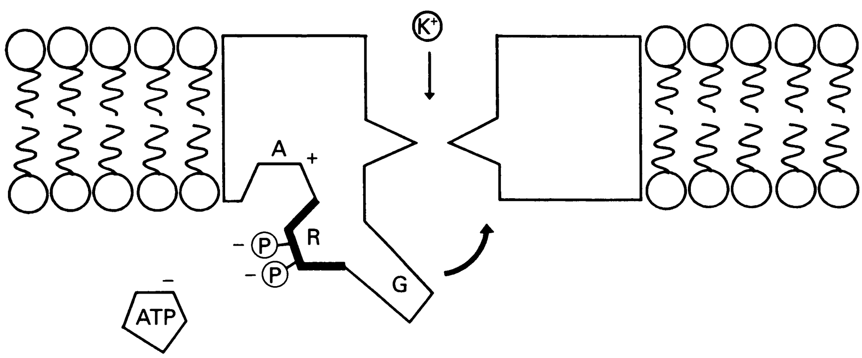

Fig. 7. Hypothetical model of the K^+_{ATP} channel. A, ATP binding site; R, region important for regulating interaction between ATP binding region and ATP-regulated gate; G, ATP-regulated gate; P, negatively charged phosphate group; K^+, potassium ion. See text for details.

Chymotrypsin and phospholipases A_2, C and D had more limited, but directionally similar effects to trypsin on the ATP sensitivity of K^+_{ATP} channels, whereas calpain I, a Ca^{2+}-activated protease, had no apparent effect. The ability of these different interventions to reduce the intrinsic sensitivity of K^+_{ATP} channels to $[ATP]_i$ suggests that the ATP-binding site is influenced by proteolysis of cytoplasmic domains of the channel protein, and is sensitive to lipid–protein interactions as well. Thus it seems plausible that during metabolic inhibition, activation of intracellular proteases and/or phospholipases promoted by increases in $[Ca^{2+}]_i$ could cause the reduced intrinsic sensitivity of K^+_{ATP} channels to $[ATP]_i$ in this setting. Whether a specific enzyme or multiple enzymes are involved, however, is completely speculative at this point. It is also possible that other factors in the milieu of severe metabolic inhibition may contribute to K^+_{ATP} channel desensitization. We examined the effects of an oxygen-free radical-generating system and the sulfhydryl group and glycolytic inhibitor iodoacetate, but neither significantly affected the ATP sensitivity of K^+_{ATP} channels.

A hypothetical model of the K^+_{ATP} channel which might account for some of these observations is shown in Fig. 7. We propose that a region of the channel protein (indicated by the heavy line) may be important for regulating the interaction between the ATP-binding region and the ATP-regulated gate. When this regulatory region is phosphorylated, ATP gating of the channel operates efficiently. As the region is progressively dephosphorylated, however, the gate tends to become 'stuck' in the closed position, manifest as channel run-down. The increased sensitivity of partially dephosphorylated (i.e. run-down) channels to $[ATP]_i$ could be due to loss of negatively charged phosphate groups in the vicinity of the ATP binding site which electrostatically repulse negatively charged ATP molecules. Proteolysis of the regulatory region by trypsin is postulated to remove the inhibitory effect of dephosphorylation on the ATP-regulated gate, thereby 'unsticking' the gate and reversing run-down. More extensive proteolysis of the channel protein by trypsin may directly alter the properties of the ATP binding site and its interaction with the gate, accounting for the reduced sensitivity to $[ATP]_i$. Similarly, if the ATP binding site is near the lipid–protein interface, alterations in the lipid environment by phospholipases may reduce ATP binding affinity. Variations in the average state of phosphorylation or proteolysis of K^+_{ATP} channel could thus account for the significant variability in K_d values between different patches noted experimentally (Findlay & Faivre, 1991; Weiss *et al.* 1992). Although this model is very hypothetical and oversimplified in many respects, (e.g. the issue of whether run-down is related to dephosphorylation remains controversial (de Weille, Muller & Lazdunski, 1992), only one ATP binding site is illustrated for simplicity, important interactions with Mg^{2+} and nucleotide diphosphates are not considered, etc.), certain elements may be testable with well-designed experiments.

Possible relevance to $[K^+]_o$ accumulation and electrophysiological alterations during myocardial ischaemia

If our findings in isolated ventricular myocytes can be related to ischaemia or metabolic inhibition in the intact heart, it is likely that they apply only to advanced stages, such as during the secondary rise in ischaemic $[K^+]_o$ accumulation associated with irreversible injury (Hill & Gettes, 1980; Weiss & Shine, 1982*a*). In our experiments, we excised the membrane patches to test their $[ATP]_i$ sensitivity after profound metabolic inhibition at a point when cytosolic ATP levels were probably severely depleted, analogous to advanced rather than early ischaemia in the intact heart. We did not investigate whether the intrinsic $[ATP]_i$ sensitivity of K^+_{ATP} channels was reduced at time points before they had became maximally activated. However, in the intact heart, it is unlikely that a reduction in the intrinsic sensitivity of K^+_{ATP} channels to $[ATP]_i$ occurs during early ischaemia or hypoxia, since APD and cellular K^+ balance rapidly return to normal upon reperfusion or reoxygenation (Weiss & Shine, 1982*b*; Benndorf, Friedrich & Hirche, 1991). This would not be likely if K^+_{ATP} channels had been irreversibly desensitized to $[ATP]_i$. However, when the ischaemic heart is reperfused during the secondary rise in $[K^+]_o$, APD often does not recover fully and net cellular K^+ loss persists. It has been traditionally assumed that these phenomena are manifestations of non-specific sarcolemmal damage associated with irreversible reperfusion injury. However, the present findings suggest that a persistent increase in the open probability of K^+_{ATP} channels due to a reduction in their intrinsic sensitivity to closure by intracellular ATP could also contribute to these abnormalities. Based on previous findings (Nichols *et al.* 1991; Weiss *et al.* 1992), it can be estimated that even if cytosolic [ATP] and free [ADP] remained normal, an increase in the K_d from 46 to 305 μM would increase the open probability of K^+_{ATP} channels from $\approx 0{\cdot}02$ to $\approx 0{\cdot}7$%, sufficient to shorten APD by ≈ 50%.

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