Biochimica et Biophysica Acta, 943 (1988) 297–304
Elsevier

BBA 74106

Calcium-dependent inactivation of the ATP-sensitive K^+ channel of rat ventricular myocytes

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(Received 29 March 1988)

Key words: Ventricular myocyte; Patch clamp; Potassium channel; ATP; Calcium dependent inactivation; (Rat)

Single-channel currents were recorded from ATP-sensitive K^+ channels in inside-out membrane patches excised from isolated rat ventricular myocytes. Perfusion of the internal surface of excised membrane patches with solutions which contained between 5 and 100 μM free calcium caused the loss of K^+_{ATP} channel activity which was not reversed when the membranes were washed with Ca-free solution. K^+_{ATP} channel activity could be recovered by bathing the patches in Mg · ATP. The loss of K^+_{ATP} channel activity provoked by internal calcium was a process which occurred over a time scale of seconds. Channel closure evoked by internal ATP was essentially instantaneous. The speed of K^+_{ATP} channel inactivation increased with the concentration of calcium. Neither a phosphatase inhibitor (fluoride ions) nor a proteinase inhibitor (leupeptin) had any effect upon the loss of K^+ channel activity stimulated by internal calcium.

Introduction

A class of potassium-selective ion channels whose opening was inhibited by intracellular adenosine 5′-triphosphate (ATP) were first recorded in membrane patches which had been excised from isolated cardiac muscle cells [1]. Similar ATP-sensitive potassium channels (K^+_{ATP} channels) have since been recorded from skeletal muscle [2] and insulin-secreting B cells of the Islets of Langerhans [3].

It has been a frequent observation that the activity of K^+_{ATP} channels declined after a membrane patch had been excised from the intact cell [4–9]. Channels could then be reactivated by exposing their internal surface to Mg · ATP [10]. The failure of ATP^{4-} and ATP analogs to reactivate 'run-down' K^+_{ATP} channels led to the suggestion that they may be phosphorylated [11–14]. That a biochemical process might underly such reactivation was also suggested when it was found that the extent of K^+_{ATP} channel reactivation depended upon the time for which the channels were bathed in Mg · ATP [10,12].

A number of studies have shown that K^+_{ATP} channels could be blocked by the application of divalent cations to the internal surface of excised membrane patches [3,4,6,13–15]. It was not recorded whether this blockade had a noticeable latency but in nearly every case the blockade was reversible. Kakei and Noma [4] noticed that blockade of the ion channel by internal calcium was not reversible. It was subsequently found that this 'blockade' could be reversed if the membrane patches were bathed in Mg · ATP though not if

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid.

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they were bathed in ATP^{4-} or ATP analogs [14]. These results suggest that the 'run-down' of channel activity and the 'irreversible' loss of channel activity which was provoked by internal calcium might reflect aspects of the same process.

The present report examines the time course of the inactivation provoked by internal calcium of K^+_{ATP} channels from rat ventricular myocytes. It has been found that the loss of channel activity was a process which could proceed relatively slowly (over 10's of seconds) and that a complex relationship existed between channel activity, the concentration of calcium, and the duration for which the channels were exposed to the calcium.

Materials and Methods

Individual myocytes were obtained from rat hearts by standard methods which have been described previously [14]. All experiments were conducted at room temperature (20–22 °C).

Single-channel currents were recorded with the methods of Hamill et al. [16]. Single-channel currents were recorded with a Dagan 8900 patch-clamp amplifier and stored on video-cassette (Sony PCM-701ES digital audio processor and SL-HF100F video cassette recorder). Recorded experiments were replayed through an 8-pole Bessel filter (Frequency Devices Inc. 902LPF) at 1000 Hz and digitised at 4000 Hz into a BBC Master Microcomputer (Acorn Computers Ltd.). Two forms of analysis were performed upon this data. First the patch K^+_{ATP} channel current was calculated by averaging 65536 analog-digital conversions (16.36 seconds of continuous record) recorded in the absence of ATP and then subtracting the average value which had been recorded in the presence of a supramaximal concentration of ATP. These two values were used to define the arbitrary levels of patch current 100 and 0, respectively. The second analysis involved sequentially storing the means of 256 conversions (64 ms of continuous record) in the computer memory. 768 of these values represented 49.15 s of continuous experimental record. These values were stored on floppy disc. They were subsequently used to reconstruct an approximation of the experimental record (see Fig. 2). The conversion of raw A/D values to a standard form enabled results which had been obtained from different membrane patches to be averaged (see Figs. 4, 5 and 9).

All of the experiments described in this study involved excised inside-out membrane patches where K^+-rich solutions bathed both sides of the membrane. Each excised membrane patch was voltage-clamped at −50 mV membrane potential. The K^+-rich extracellular (pipette) solution contained (mM): 140 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes; the pH was adjusted to 7.4 with KOH. The K^+-rich intracellular (bath) solution contained (mM): 140 KCl, 10 glucose, 10 Hepes; the pH was adjusted to 7.4 with KOH. The K^+-rich intracellular solutions also contained a divalent cation buffer. Control K^+-rich solution contained 5 mM of either EDTA or HEDTA and no added calcium or magnesium. For solutions which required a known concentration of calcium ions mixtures of either EGTA and $CaCl_2$ (for 1 μM free Ca^{2+}) or HEDTA and $CaCl_2$ (for either 5 or 10 μM free Ca^{2+}) were used as calculated from the stability constants of Martell and Smith [17] and Sillén and Martell [18], respectively. 100 μM free Ca^{2+} was provided by adding 100 μM $CaCl_2$ to a K^+-rich solution which did not contain a divalent cation buffer. Adenosine 5′-triphosphate (ATP: Mg-salt: Sigma, St. Louis, MO, U.S.A.) was added to K^+-rich solution which contained 5 mM EGTA and 1.4 mM $MgCl_2$. 20 mM potassium fluoride replaced 20 mM KCl in the K^+-rich intracellular solutions for some experiments. Leupeptin (acetyl-Leu-Leu-Arg-Al: hemisulphate salt: Sigma, St. Louis, MO, U.S.A.) was added to K^+-rich intracellular solutions when required.

Excised membrane patches were continuously perfused by a stream of solution from one of a series of piped outlets. Changes of solution were performed manually under visual control and identified vocally upon the video recording. Triggering of data collection by the computer was performed manually.

Results

When either ATP or Ca^{2+} was applied to the internal surface of an excised inside-out membrane patch K^+_{ATP} channel activity was inhibited (Fig. 1). This record shows that there were signifi-

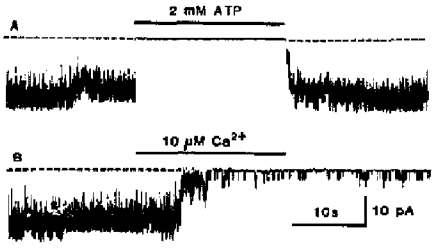

Fig. 1. The effects of internal ATP and Ca^{2+} upon the activity of K^+_{ATP} channels. (A) and (B) are records of unitary currents which were obtained from one excised inside-out membrane patch. Downward deflections of the traces represent inwardly directed membrane currents. The dotted lines represent the patch current level which was recorded when all channels in the patch were closed. Except for the periods indicated by the bars above each trace the internal surface of the membrane was bathed in control K^+-rich solution. (A) This record commences 45 s after the membrane patch had been excised from the cell. The bar above the trace indicates a period of 20 s for which the internal surface of the membrane was perfused with K^+-rich solution which contained 2 mM ATP. (B) The bar above the trace indicates a period of 20 s for which the internal surface of the membrane was perfused with K^+-rich solution which contained 10 μM Ca^{2+}. Five seconds of continuous recording have been omitted between traces (A) and (B).

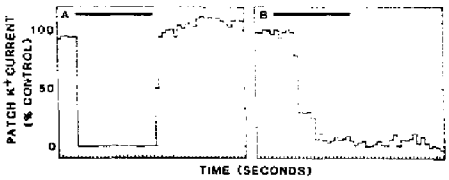

Fig. 2. Graphic representation of the membrane patch unitary current records which were shown in Fig. 1. These graphs were constructed by averaging the data collected by the computer over consecutive periods of 1024 ms. The polarity of the recorded patch current has been inverted. Inwardly directed membrane currents are now represented as upwards deflections of the record. In this way a reduction in the patch K^+_{ATP} current evoked either by ATP (A) or Ca^{2+} (B) is represented by a lower value on the graph. The vertical axis represents the patch K^+_{ATP} current as a standard score which was calculated from the patch current recorded first in the absence of ATP or Ca^{2+} (100) and then in the presence of a supramaximal concentration of ATP (0). The horizontal axis represent time where the short vertical lines mark seconds. The horizontal bars indicate the periods for which the internal surface of the excised membrane patch was perfused with a K^+-rich solution which contained either 2 mM ATP (A) or 10 μM Ca^{2+} (B).

cant differences between the inhibition which was evoked by ATP (Fig. 1A) and that which was evoked by Ca^{2+} (Fig. 1B). First, whereas ATP evoked an almost instantaneous closure of the 7 K^+_{ATP} channels which were recorded in this patch the effect of Ca^{2+} was more gradual. In this experiment at least 5 s elapsed after the application of Ca^{2+} before channel activity began to decline and a further 3–4 s passed before there remained only the occasional opening of 1–2 single-channel current levels. Secondly, whereas K^+_{ATP} channel activity recovered when ATP was washed away from the membrane (Fig. 1A), the washout of Ca^{2+} was without effect, channel activity remained at a low level (Fig. 1B).

To be able to compare results from different experiments it was necessary to standardise not only the experimental protocol, as will be described below, but also the expression of the experimental results. The form that this analysis took is illustrated in Fig. 2 as it was performed for the experimental records shown in Fig. 1. It can be seen that even though the data have been converted into a standard score format, inverted, and averaged over sequential periods which lasted for 1024 ms the graphs closely follow the form of the raw experimental records.

The protocol which was used for each experiment is illustrated in schematic form in Fig. 3. Except for the periods indicated by the bars above this schematic record the excised membrane

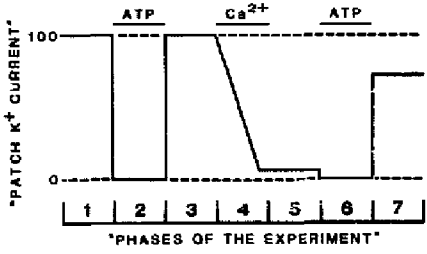

Fig. 3. An schematic representation of the experimental protocol which was used to compare the effects of ATP and Ca^{2+} upon K^+_{ATP} channel activity recorded from excised inside-out membrane patches of rat ventricular muscle. This figure should only be regarded as a guide to the explanation of the experimental results. It does not accurately reflect either the time scale or the result of an experiment.

patches would be bathed in control K^+-rich solution which contained neither ATP nor divalent cations. The protocol has been divided into a number of 'phases' which will be used to facilitate the description of the experimental results.

First of all, membrane patches were excised from rat ventricular myocytes into a stream of control K^+-rich solution. For those membranes which directly formed an open inside-out membrane patch the experiment began. Those membranes which instead formed a closed vesicle in the mouth of the pipette were passed repetitively through the surface of the bath solution until the vesicle opened [16]. This was indicated by the appearance of full sized and undistorted openings of K^+_{ATP} channel currents which could be inhibited by the perfusion of the patch with ATP. It was frequently, if not usually, observed that K^+_{ATP} channel activity in such 'opened vesicles' was at a low and irregular level when compared with the high open probability and often large number of channel currents which were recorded in membranes which excised directly as open inside-out patches. These 'opened vesicles' were perfused with K^+-rich solution which contained Mg · ATP to reactivate channel activity. This was continued until the activity of the K^+_{ATP} channels reached an apparently maximal and stable level [12]. The experiment then began.

K^+_{ATP} channel inhibition evoked by ATP (Phases 1–3)

Phase 1 (duration 60–90 s). After excision, or the establishment of a stable and apparently maximal level of K^+_{ATP} channel activity, inside-out membrane patches remained in the control K^+-rich solution. The average patch current carried by K^+ passing inwards through K^+_{ATP} channels at −50 mV membrane potential in the 57 separate membrane patches which make up this study was 25.10 ± 3.49 pA (S.E.).

Phase 2 (duration 20 s). Each patch was perfused with K^+-rich solution which contained 2 mM ATP. In each experiment K^+_{ATP} channel activity was completely inhibited. This section of each record was therefore used to measure the basal patch membrane current recorded under voltage-clamp at −50 mV membrane potential. This value was then subtracted from all of the other sections of the patch current record and provided the zero-current base line for each of the figures and calculations.

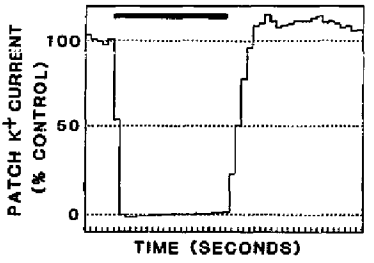

Fig. 4. The effect of internal ATP upon excised inside-out membrane patches. The graph represents the average K^+_{ATP} current, in standard form (see Fig. 2), recorded from 32 separate membrane patches which were perfused with K^+-rich solution containing 2 mM ATP for 20 s (bar).

Phase 3 (duration 40–50 s). Each patch was returned to control K^+-rich solution and the mean patch current was measured. Since the ATP applied to the patches was the Mg-salt this post-ATP patch current could achieve values slightly higher than the previous control level, particularly if the patch concerned had been an 'opened vesicle'. The K^+_{ATP} channel current which was recorded at this time averaged 107.70 ± 2.74% (S.E.: $n = 57$) of the current which had been recorded prior to the application of ATP (Phase 1).

Part of this section of the experimental record (the final 5 s of Phase 1; all of Phase 2; and the first 24 s of Phase 3) was reconstructed for each of 32 separate membrane patches. The average of these 32 records is shown in Fig. 4. There is little obvious difference between this 'average' record and that which had been reconstructed from an individual membrane patch (Fig. 2A).

K^+_{ATP} channel inactivation evoked by calcium (Phases 4 and 5)

Phase 4 (duration 20 s). After perfusion with control K^+-rich solution (Phase 3) each membrane patch was perfused with a K^+-rich solution which contained a known concentration of free calcium. The results recorded during this section of the protocol (which included the final 5 s of Phase 3; all of Phase 4; and the first 24 s of Phase 5) were

reconstructed. Fig. 5 illustrates the averages of these reconstructions and shows the time course of the reduction in K^+_{ATP} channel current which was evoked by calcium. It was found that the application of 1 μM Ca^{2+} for 20 s had little obvious effect upon the K^+_{ATP} current (Fig. 5A). The application of 5 μM Ca^{2+}, on the other hand, resulted in a gradual decline of the K^+_{ATP} channel current (Fig. 5B). The rate of decline of the K^+_{ATP} channel current was clearly increased when patches were perfused with 10 μM Ca^{2+} (Fig. 5C). 100 μM Ca^{2+} (Fig. 5D) evoked a rapid and virtually complete inhibition of the K^+_{ATP} channel current well within the 20 s period of its perfusion. The time taken for the K^+_{ATP} channel current to be completely inhibited when patches were perfused with 100 μM Ca^{2+} was 2.45 ± 0.51 s (S.E.: $n = 7$). This was significantly slower than the inhibition evoked by ATP, 0.62 ± 0.07 s (t-test: $P > 0.01$), recorded in the same experiments.

Phase 5 (duration 40–50 s). Each patch was returned to control K^+-rich solution and the mean patch K^+_{ATP} current was measured. Fig. 6 illustrates that the amount of K^+_{ATP} channel current which remained in control solution after the Ca^{2+} had been washed away was dependent upon the concentration of Ca^{2+} to which the patches had been exposed. The higher had been the concentration of Ca^{2+}, the less K^+_{ATP} channel current remained.

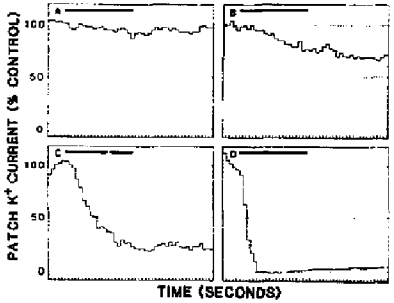

Fig. 5. The effect of internal calcium upon K^+_{ATP} channel currents. Each graph represents the average K^+_{ATP} current, in standard form (see Fig. 2), which had been recorded from a number of separate membrane patches. The bars indicate the period of 20 seconds for which the internal surface of the excised membrane patches was perfused with K^+-rich solutions containing a known concentration of free calcium. (A) 1 μM Ca^{2+} ($n = 10$). (B) 5 μM Ca^{2+} ($n = 7$). (C) 10 μM Ca^{2+} ($n = 8$). (D) 100 μM Ca^{2+} ($n = 7$). These results were obtained from the same membrane patches whose average response to internal ATP was shown in Fig. 4.

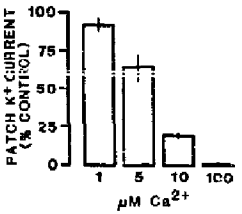

Fig. 6. The K^+_{ATP} channel current which remained after excised membrane patches had been exposed to Ca^{2+} for periods of 20 s. The patch K^+_{ATP} channel current is represented as that which was recorded in control solution after the washout of Ca^{2+} (Phase 5) relative to the current which had been recorded prior to exposure to Ca^{2+} (Phase 3). Mean values and S.E. bars are shown for patches which had been exposed to 1 ($n = 10$), 5 ($n = 7$), 10 ($n = 8$) and 100 ($n = 7$) μM free Ca^{2+}. These data were calculated from the same membrane patches whose response to Ca^{2+} perfusion was shown in Fig. 5.

A membrane patch had to survive this far into the experimental protocol before it was accepted for this study. Thus each patch was exposed to both a supramaximal concentration of ATP and a known concentration of free Ca^{2+}. Where it was possible the experiments were continued.

Reactivation of K^+_{ATP} channels evoked by Mg –ATP (Phases 6 and 7)

Phase 6 (duration 120 s). After perfusion with control K^+-rich solution (Phase 5) each patch was perfused with 2 mM Mg · ATP (Fig. 7A). The mean patch current was measured several times during this period and the results were used to reset the value of the basal patch current if this had drifted during the experiment.

Phase 7 (duration 60 s). Each patch was returned to control K^+-rich solution and the mean patch current was measured. This was significantly greater than that recorded after the washout of Ca^{2+} (t-test: $P > 0.05$ comparing data from Figs. 6 and 7B) and reflected the extent of reactivation of K^+_{ATP} channels which had been evoked by Mg · ATP. Fig. 7B illustrates that the weakest reactivation was associated with the strongest inactivation.

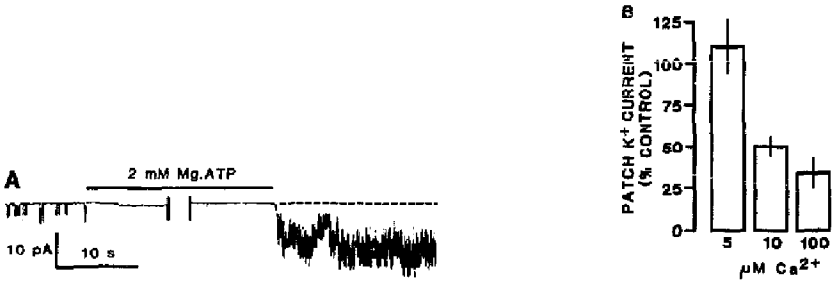

Fig. 7. Reactivation of Ca^{2+}-inactivated K^+_{ATP} channels evoked by Mg·ATP. (A) This single-channel current record is a continuation of the experiment which was illustrated in Fig. 1. This record commences 15 s following Fig. 1B. For the period indicated by the bar above the current record the internal surface of the membrane patch was perfused with K^+-rich solution which contained 2 mM Mg·ATP. One minute and 40 s of continuous recording have been omitted from this record (vertical bars). (B) The K^+_{ATP} channel current which was reactivated by 2 mM Mg·ATP after inactivation evoked by different concentrations of Ca^{2+}. The patch K^+_{ATP} channel current is represented as that which was recorded in control solution after the washout of Mg·ATP (Phase 7) relative to the current which had been recorded before the patches had been exposed to Ca^{2+} (Phase 3). Mean values and S.E. bars are shown for patches which had been exposed to 5 ($n = 4$), 10 ($n = 6$) and 100 ($n = 7$) μM free Ca^{2+}.

Experiments were conducted to examine whether either the fluoride ion, which may act as a phosphatase inhibitor, or the peptide leupeptin, which may act as a proteinase inhibitor, had any effect upon the inactivation of K^+_{ATP} channels evoked by internal Ca^{2+}. Neither 20 mM potassium fluoride (11 membrane patches) nor 150 μM leupeptin (7 membrane patches) reduced the inactivation of K^+_{ATP} channels which was evoked by 10 μM Ca^{2+}. The subsequent reactivation of the K^+_{ATP} channel current evoked by 2 mM Mg · ATP in the same experiments was likewise not affected by the presence of either fluoride or leupeptin.

Since 5 μM Ca^{2+} evoked only a gradual and incomplete reduction of the K^+_{ATP} channel current (Fig. 5B) it was decided to determine whether with a longer exposure to this concentration of Ca^{2+} the current would continue to decline or whether it would reach a steady-state level. The standard experimental protocol (Fig. 3) was used with little modification. Phase 4, the period for which the membrane patches were perfused with K^+-rich solution which contained 5 μM Ca^{2+}, was increased from 20 to 60 s. The results recorded from this section of each experiment (which included the final 10 s of Phase 3; all of Phase 4; and the first 28 s of Phase 5) were reconstructed. Fig. 8 illustrates the average of these reconstructions. Clearly the K^+_{ATP} channel current continued to decline throughout the period of perfusion of the patches with 5 μM Ca^{2+}. The K^+_{ATP} channel current which remained after the washout of Ca^{2+} (Phase 5) was measured and represented 34.5 ± 5.5% (S.E.: $n = 7$) of the current which had been measured in these patches before they had been exposed to Ca^{2+} (Phase 3). This was significantly less than the 63.4 ± 9.4% (S.E.: $n = 7$: data taken from Fig. 6) of control current which remained

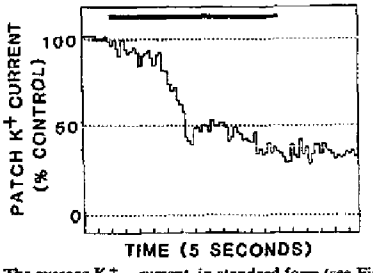

Fig. 8 The average K^+_{ATP} current, in standard form (see Fig. 2), recorded from seven separate membrane patches whose internal surface was perfused with K^+-rich solution which contained 5 μM Ca^{2+} for 60 s (bar). Graph format and axis labels are as for Fig. 2 except that the time marks indicate 5-s intervals.

after only 20 s of perfusion with 5 μM Ca^{2+} (*t*-test: $P > 0.05$).

Discussion

In this study every manipulation was performed manually and thus was subject to a certain degree of error. That a supramaximal concentration of ATP would inhibit every channel in the patches within 0.62 ± 0.07 (S.E.) s must not be taken to be an accurate estimate of the latency with which ATP binds to and closes the channel. But this value does provide a reasonable basis from which to make a comparison with the inactivation of the channels which was provoked by Ca^{2+} which was applied under the same conditions with exactly the same sources of error. The difference was sufficiently large for one to be able to exclude operator error as an explanation for the sluggish response of the channels to internal Ca^{2+}. It was clear that K^+_{ATP} channel inactivation provoked by Ca^{2+} was a process which takes time.

It was not possible to construct a dose-response curve for the Ca^{2+}-dependent inactivation of the K^+_{ATP} channels since the degree to which they were inactivated was found to depend upon the duration for which the internal surface of the membrane was perfused with any one concentration of Ca^{2+}. It was clear, however, that an increase in the concentration of Ca^{2+} increased the speed at which channel activity declined. The time which was required for the inactivation of approximately 50% of the K^+_{ATP} channel current was between 25–28 s for 5 μM Ca^{2+} (Fig. 8), 8–9 s for 10 μM Ca^{2+} (Fig. 5C) and 2–3 s for 100 μM Ca^{2+} (Fig. 5D). It was interesting that even after exposure to 100 μM Ca^{2+} for 20 s some K^+_{ATP} channel openings could still be observed. There would appear to be a portion of K^+_{ATP} channel behaviour which had not been affected, or perhaps some channels in the patches were Ca^{2+}-resistant to some extent.

It is tempting to think of the inactivation of K^+_{ATP} channels as a dephosphorylation of the channel protein. At the present time the evidence for this supposition is indirect and depends upon the observation that both channel run-down and Ca^{2+}-provoked inactivation are processes that may be reversed by exposure to Mg·ATP and not ATP^{4-} or ATP analogs [11–14]. Whether or not the K^+_{ATP} channel protein is phosphorylated remains to be proven. A structural component of the channel may also be involved in maintaining the integrity of the channel [6,19].

If, at least for the moment, one was willing to accept the idea that calcium stimulated an enzyme which was associated with or formed part of the channel protein and caused the dephosphorylation of the K^+_{ATP} channel, it would be interesting to determine what type of enzyme action might be involved. The fluoride ion, which had been generally thought to act as a phosphatase inhibitor, had no effect upon K^+_{ATP} channel inactivation. The protease inhibitor leupeptin, which has been shown to prevent the permanent loss of Ca^{2+} channel activity provoked by internal Ca^{2+} [20], had no effect on either the inactivation or subsequent reactivation of the K^+_{ATP} channels. The calcium-dependent inactivation of calcium channels and their phosphorylation via activation of cAMP-dependent protein kinases [20] could involve quite different mechanisms by which a channels activity may be regulated than those which are responsible for the inactivation and reactivation of K^+_{ATP} channels.

The evidence for a physiological function of the K^+_{ATP} channel in cardiac muscle is indirect. The channel is present in large numbers in each of the cardiac cell types that have been examined to date [1,4–6,14,15] yet it has not been recorded in intact isolated myocytes [1,5]. The intact isolated myocyte bears little relation to the same cell in a working heart. It has been suggested that the K^+_{ATP} channel may underly a K^+ current which is activated under quasi-ischaemic conditions in cardiac muscle cells [21–24]. This current is thought to play a cardio-protective role by shortening the action potential in ischaemic regions and inhibiting extraneous action potentials by preventing the spontaneous depolarisation of cardiac muscle cells [23,25]. However, extraneous action potentials do occur in such stressed hearts and cardiac muscle cells do depolarise under 'quasi-ischaemic' conditions [26]. A cardioprotective function for the K^+_{ATP} channel could be overcome if the channels were inactivated by an increase of the intracellular Ca^{2+} concentration in 'ischaemic' cardiac muscle cells [27,28]. Though the concentration of calcium which was required to evoke inactivation of the

K^+_{ATP} channels in this study was considerably greater than that which is known to cause maximal contracture of the cells [29].

Acknowledgements

I would like to thank Prof. E. Coraboeuf for critical reading of this manuscript. I would also like to thank Paulette Richer for preparing the cells.

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