Time-dependent fading of the activation of K_{ATP} channels, induced by aprikalim and nucleotides, in excised membrane patches from cardiac myocytes

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1 The effects of the potassium channel opener (KCO) aprikalim (RP 52891) on the nucleotide-induced modulation of ATP-sensitive K^+ (K_{ATP}) channels in freshly dissociated ventricular myocytes of guinea-pig heart, were studied by use of the inside-out patch-clamp technique. The internal surface of the excised membrane patch was initially bathed with a standard solution (Mg^{2+} -free with EDTA), then sequentially superfused with solutions containing nucleoside diphosphates (NDPs: 200 μM ADP and 50 μM GDP) and NDPs plus 1 mM $MgCl_2$ (with EGTA; referred to as Mg-NDP solution).

2 The normalized concentration-response (channel closing) relationship to ATP was shifted to the right when the standard solution was replaced by the Mg-NDP solution. Hence, the internal concentration of ATP ($[ATP]_i$) inhibiting the channel activity by half (K_i) increased from 56 μM to 180 μM , with an apparently constant slope factor ($s = 2.37$). NDPs in the absence of Mg^{2+} did not decrease the sensitivity of the channels to ATP.

3 In standard solution, aprikalim (100 μM) activated K_{ATP} channels in the presence of a maximally inhibitory $[ATP]_i$ (500 μM). This effect was strongly enhanced when aprikalim was applied to patches exposed to Mg-NDP solution, as demonstrated by the 9 fold increase in K_i for $[ATP]_i$ (from 180 μM to 1.5 mM and $s = 2.37$).

4 The ability of aprikalim to overcome the channel closing effects of ATP in Mg-NDP solution waned rapidly. Similarly, the NDP-induced activation of ATP-blocked channels was also time-dependent. Both activation processes disappeared before the channel run-down phenomenon appeared in ATP-free conditions.

5 In conclusion, aprikalim is much more potent in opening K_{ATP} channels in membrane patches bathed in Mg-NDP solution than in standard solution. However, under the former experimental conditions, the effect of aprikalim waned rapidly. It is proposed that the waning phenomenon results from changes in the intrinsic enzymatic activity of the K_{ATP} channel protein (possibly linked to the experimental conditions) which lead to the channel closure.

Keywords: ATP-sensitive K^+ channel; aprikalim (RP 52891); patch-clamp; potassium channel opener (KCO); magnesium (Mg^{2+}); nucleoside diphosphates (NDPs); adenosine triphosphate (ATP); guinea-pig heart; ventricular myocytes

Introduction

Potassium channel openers (KCOs) constitute a class of chemically diverse compounds (Edwards & Weston, 1990) which increase cell membrane permeability to K^+ in various tissues (for review see Longman & Hamilton, 1992). The primary action of KCOs is to overcome the closure of K_{ATP} channels produced by intracellular ATP concentrations ($[ATP]_i$), although these agents may alter other K^+ currents (for review see Ashcroft & Ashcroft, 1990; Longman & Hamilton, 1992; Quast, 1993). In experiments with excised inside-out patches, micromolar concentrations of KCOs shift the $[ATP]_i$ -response (closing channel) curve towards the right. Hence, in the presence of the KCO, RP 49356 (30 μM), the $[ATP]_i$ necessary to close 50% of the K_{ATP} channels increases by approximately 9 fold (from 56 to 500 μM ; Thuringer & Escande, 1989). While this provides evidence that KCOs open K_{ATP} channels, it is not immediately evident how, in isolated heart preparations in which physiological $[ATP]_i$ is 5–10 mM (Elliot *et al.*, 1989), RP 49356 and other KCOs at this concentration can markedly shorten action potential duration and depress contractile activity. Several proposals have been made to account for this apparent paradox. Firstly, the activation of a very small fraction of cellular K_{ATP} channels is sufficient to shorten markedly the action potential duration (Findlay *et al.*, 1989; Carmeliet *et al.*, 1990; Faivre

& Findlay, 1990; Nichols *et al.*, 1991). Secondly, catabolites of high energy phosphates, in addition to or independently of ATP, modulate channel activity in intact cells (for review see Ashcroft & Ashcroft, 1990; Deutsch *et al.*, 1991; Standen, 1992; Deutsch & Weiss, 1993; Weiss & Venkatesh, 1993); indeed, cytosolic ADP or GDP increases channel activity in the presence of Mg^{2+} (Findlay, 1988a; Lederer & Nichols, 1989; Tung & Kurachi, 1991) by shifting towards the right the concentration-response curve for closing channels induced by a change in $[ATP]$ (Nichols & Lederer, 1990; Tung & Kurachi, 1991). Thirdly, there may be an intracellular compartmentalization of ATP. Finally, some unknown channel regulatory proteins may exist in intact cells, and these may be lost following patch excision (Noma & Shibasaki, 1985; Elliot *et al.*, 1989). The identification of any of these possible modulators might help to understand not only the paradox that KCOs can open K_{ATP} channels at physiological $[ATP]_i$, but also some discrepant observations that have been made with inside-out patches, such as that KCOs do (Escande *et al.*, 1989; Thuringer & Escande, 1989; Shen *et al.*, 1991; Tung & Kurachi, 1991) or do not (Ripoll *et al.*, 1990) activate channels following run-down. Thus, differences in channel response to KCOs might be linked to some as yet uncontrollable changes in channel modulatory mechanisms which take place after the membrane is excised from the cell.

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The purpose of the present work was to analyse the activation of cardiac K_{ATP} channels by aprikalim (the eutomer of RP 49356) in order to provide new insights into the mechanisms regulating channel function. We have shown previously that in Mg^{2+} -free high- K^+ solution containing EGTA, cardiac K_{ATP} channels need to be partially closed either by ATP or by run-down before RP 49356 exerts its effect (Thuringer & Escande, 1989). Since that time, much evidence has come to light pointing to the amplification of KCO-induced channel activation by NDPs and Mg^{2+} (Shen *et al.*, 1991; Tung & Kurachi, 1991; Larsson *et al.*, 1993). The Mg^{2+} -dependent activation site proposed for NDPs (Findlay 1988a; Lederer & Nichols, 1989; Shen *et al.*, 1991; Tung & Kurachi, 1991) has been recently advanced for KCOs in skeletal muscle (Hussain *et al.*, 1994). The aim of this investigation was to assess whether the same basal mechanism might be involved in NDP- and KCO-induced activation of K_{ATP} channels in excised membrane patches from guinea-pig cardiac myocytes. To this end, we examined drug-channel interactions for aprikalim in the absence (EDTA with no $MgCl_2$) or presence of ADP, GDP and Mg^{2+} (EGTA), before and during channel run-down.

Methods

Cell isolation

Ventricular myocytes were isolated from hearts of guinea-pig weighing 200–300 g by a standard dissociation technique. Briefly, the heart was mounted on a Langendorff-type apparatus and successively perfused (at 37°C) with Ca^{2+} -free Tyrode solution (see below) and the digesting Tyrode solution containing 1 mg ml⁻¹ collagenase (Type I, Sigma) plus 0.28 mg ml⁻¹ protease (Type XIV, Sigma). The ventricles were then separated from the heart, cut into small pieces in standard Tyrode solution containing additional $CaCl_2$ (200 µM). Myocytes were dispersed mechanically, reseeded in Petri dishes filled with the latter solution and then kept at room temperature (22°–24°C) until their use.

Solutions and drugs

The Ca^{2+} -free Tyrode solution contained (in mM): NaCl 136, KCl 5.4, $MgCl_2$ 1.0, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid) 10, NaH_2PO_4 0.33, glucose 10, adjusted to pH 7.3 with NaOH. The composition of the pipette solution was (in mM): KCl 140, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10, adjusted to pH 7.3 with KOH. Inside-out patches were formed in the standard solution containing (in mM): KCl 130, KOH 10, HEPES 10, EDTA (ethylenediamine tetraacetic acid) 3, glucose 10, adjusted to pH 7.3 with KOH. The Mg-NDP containing solution was prepared by adding 200 µM ADP and 50 µM GDP in the form of K^+ salts, plus 1 mM $MgCl_2$ to the standard solution in which 1 mM EGTA (ethylene glycol-*bis*-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid) was substituted for 3 mM EDTA (Nichols & Lederer, 1990). A flow of solution from one of a series of five pipette outlets continuously superfused the inside-out membrane patch. The flow rate of perfusion solutions was 50–100 µl min⁻¹. Thus, the solution exchange can be considered quasi-instantaneous in the patch area. In all experiments, ATP was used as Mg^{2+} -salt. A solution of 2 mM aprikalim in 1% DMSO (dimethyl sulphoxide) was prepared daily. Nucleotides and drugs were added at various concentrations to bath solutions. The pH of these solutions was always adjusted to 7.3 with KOH just before use. All chemical compounds were purchased from Sigma Chemical (St. Louis, MO, U.S.A.) except for aprikalim, which was provided by Rhône-Poulenc Rorer (Vitry-sur-Seine, France).

Patch-clamp recordings and data analysis

Patch-clamp experiments were performed at room temperature. Membrane currents were recorded in the inside-out patch-clamp configuration with a RK300 Bio-Logic amplifier (Bio-Logic, Echirrolles, France). Patch pipettes were pulled from Pyrex capillaries (Corning 7740) and had resistances ranging between 5 and 8 MΩ. Current traces recorded on tapes (Bio-Logic DTR 1202 recorder) were retrieved on a Brush recorder (TA240, Gould Inc.) and filtered at 300 Hz using a five-pole Tchebicheff filter (Bio-Logic, Echirrolles, France) or digitalized at a minimum sampling rate of 2 kHz using software Acquis1 (G.Sadoc, CNRS-URA 1121, Orsay, France). For each period of drug application, the current flowing through the patch was calculated with respect to the zero current level determined in the presence of an ATP concentration high enough to produce complete closure of all active K_{ATP} channels in the patch area. The mean patch current amplitude per time unit (calculated over periods of 20–30 s) is expressed as a fraction of the current measured in ATP-free standard solution in order to obtain relative current values. Data for the dose-response curve were fitted with the following general equation:

$$\text{Relative current} = 1/[1 + ([ATP]_i/K_i)^s]$$

where K_i is the $[ATP]_i$ causing half-maximal inhibition (50%) and s is the slope factor (pseudo Hill coefficient). When possible, data are given as mean ± standard deviation (s.d. vertical bar) of n different experiments.

Results

NDPs decrease the ATP sensitivity of channels

In most inside-out patch experiments, inward unitary K^+ currents flowing through K_{ATP} channels were recorded at negative potentials. These channels rapidly fluctuate between open and closed states and are characterized by an elementary conductance of 76.1 ± 2.5 pS ($n = 5$) with 140 mM K^+ on both sides of the patch membrane. In all experiments, a patch superfused with the standard solution (NDP- and Mg^{2+} -free with EDTA) was used as control. Concentrations of NDPs used are probably in the upper limits of those proposed to occur during an ischaemic stress (Lederer & Nichols, 1989; Nichols & Lederer, 1990) because we wanted to obtain maximal effects on the K_{ATP} channel activity. Indeed, the effects of NDPs are concentration-dependent (Lederer & Nichols, 1989; Tung & Kurachi, 1991).

In the first series of experiments, the concentration-dependence of the channel inhibition by internal ATP in standard solution and solutions containing NDP was investigated. The effects of two or three short ATP applications were checked rapidly after patch excision to minimize the channel run-down normally observed during single-channel current recordings (Thuringer & Escande, 1989). An example of current traces from three different patches is shown in Figure 1. Under ATP-free standard conditions, K_{ATP} channels were fully active (Figure 1a). The application of 300 µM ATP rapidly suppressed this activity almost totally and revealed an additional type of channel activity of smaller current amplitude (about –0.9 pA) with long-lasting openings identified as the inwardly-rectifying K^+ current flowing through iK1 channels. The removal of ATP routinely reactivated quickly and completely all K_{ATP} channels present in the patch. For patches bathed in the Mg-NDP solution (200 µM ADP, 50 µM GDP, 1 mM $MgCl_2$ with EGTA), 300 µM ATP also inhibited the K_{ATP} channel activity, but did so only partially (Figure 1b; $n = 3$ different experiments). Comparable results were obtained at positive potentials ($n = 10$), except that the unitary current amplitudes of both iK1 and K_{ATP} channels decreased, probably due to the fast voltage-dependent block by internal Mg^{2+} (Horie *et al.*, 1987;

Figure 1 Effects of intracellular nucleotides and Mg^{2+} on single K_{ATP} channel currents in inside-out membrane patches. Current traces recorded from three different patches. The inhibitory effect of $300 \mu M$ ATP was tested in standard solution (Mg^{2+} -free with EDTA; a), in Mg -NDP solution ($200 \mu M$ ADP, $50 \mu M$ GDP, $1 mM$ $MgCl_2$ with EGTA; b) and in NDP solution ($200 \mu M$ ADP, $50 \mu M$ GDP, Mg^{2+} -free with EDTA; c) as indicated above each trace. The K_{ATP} channel activation appears as fast openings superimposed on slow current fluctuations resulting from iK_1 channel openings, as observed in both (a) and (c) traces during ATP exposure. At least 10 simultaneous openings of K_{ATP} channels were observable on each trace in ATP-free conditions. Internal NDPs reduced markedly the blocking effect of ATP on K_{ATP} channels only in the presence of Mg^{2+} (b). In Mg -free solution, NDPs appear to be slightly inhibitory upon ATP application (c). In this and subsequent figures, the patch membrane potential (E_m) was $-30 mV$ and downward deflections represent inwardly directed unitary K^+ currents (symmetrical high K^+ conditions). Traces were filtered at $300 Hz$. Dotted lines mark the current level when all channels were closed. Rectangular boxes indicate the kind of solution used and lines mark drug applications. Experiments were performed at $22^\circ-24^\circ C$.

Matsuda, 1991). ADP and GDP activated K_{ATP} channels exclusively in the presence of internal Mg^{2+} . In the absence of this cation (EDTA with no $MgCl_2$), the effectiveness of ATP in blocking K_{ATP} channels was slightly increased by purine diphosphates, as shown in Figure 1 (c; $n = 3$ different experiments), a result that is in line with previous reports (Findlay, 1988a; Lederer & Nichols, 1989; Nichols & Lederer, 1990; Nichols *et al.*, 1991; Tung & Kurachi, 1991). The concentration-response curves for ATP closing K_{ATP} channels in standard and Mg -NDP solutions are illustrated in Figure 2. In standard solution, the equation fitting data provided a K_i of $56 \mu M$ and a slope value of 2.37 (see Methods). These results are in good agreement with previous observations of ventricular myocytes (Findlay, 1988b; Thuringer & Escande, 1989). In Mg -NDP solution, data were also well fitted by the equation which provided a K_i of $180 \mu M$ and a slope value of 2.37. Therefore, the inefficiency of $300 \mu M$ ATP in blocking all channels in the presence of ADP, GDP and Mg^{2+} , as illustrated in Figure 1 (middle trace), results from a parallel shift towards substantially higher $[ATP]_i$ of the ATP-dependence of channel activity (Figure 2).

Aprikalim counteracts the inhibitory effect of ATP

Under standard conditions, aprikalim was weakly activating in the presence of ATP (Figure 3). Aprikalim ($10 \mu M$) failed to activate significantly the K_{ATP} channel activity in the presence of $500 \mu M$ ATP. The iK_1 channel activity was still present after ATP had inhibited K_{ATP} channels. A modest activation of K_{ATP} channels was observed when the concentration of aprikalim was increased to $100 \mu M$; this activation was suppressed when the solution was replaced by one containing $10 \mu M$ aprikalim. That the level of activity induced by aprikalim was low cannot be attributed to the run-down of K_{ATP} channels, because the level of simultaneous channel openings recorded after ATP removal (Figure 3d) was similar to that recorded at the beginning of the experiment (Figure 3a). Similar results were obtained in six other experiments.

Because the extent of KCO effects in some circumstances depends on the degree of channel activity under nucleotide-free conditions (Thuringer & Escande, 1989; Fan *et al.*, 1990; Allard & Lazdunski, 1993), we also tested aprikalim in the absence of internal ATP (Figure 4). A few seconds after the

Figure 2 Concentration-response relationships for the closing of K_{ATP} channels by ATP in standard solution (Mg^{2+} -free with EDTA; $\square$) and in Mg-NDP solution (200 μM ADP, 50 μM GDP, 1 mM $MgCl_2$ with EGTA; $\blacktriangle$). The relative current represents the mean patch current in the presence of ATP as a fraction of that in ATP-free solution. Values are mean $\pm$ s.d. ($n = 3$ to 5 in each case). The dashed lines are fitted by regression analysis with $K_i = 56 \mu M$ (standard) or 180 μM (NDPs + Mg) and a slope factor $s = 2.37$.

patch was formed in standard solution, the high level of channel activity generally recorded in all patches was neither increased nor reduced by the internal application of 100 μM aprikalim (Figure 4a; $n = 5$ experiments). Moreover, after several minutes of recording, during which the channel activity decreased spontaneously, 100 μM aprikalim did not activate more K_{ATP} channels (Figure 4b; $n = 4$ experiments).

Whether aprikalim would open ATP-blocked channels in patches maintained in the Mg-NDP solution was also tested in the presence of 500 μM ATP, which reduces channel activity by $\approx 90\%$ (see Figure 2). As shown in Figure 5a, only one K_{ATP} channel briefly opened during ATP superfusion. Returning to ATP-free standard conditions invariably reactivated K_{ATP} channels present in the patch area to a maximal extent (13 simultaneous openings). This high level of activity persisted when the patch was superfused with Mg-NDP solution containing 100 μM aprikalim. Under the latter conditions, exposing the patch to 500 μM ATP reduced channel activity only slightly (10–12 simultaneous openings; Figure 5b). Decreasing the concentration of aprikalim to 10 μM was accompanied by a substantial decrease in activity and channels attained a new steady-state level (1–3 levels of activity) in approximately 15 s (Figure 5c). The removal of aprikalim but not ATP resulted in a rapid and almost complete loss of K_{ATP} channel activity (only one channel opened from time to time). The maximal sustained activity of K_{ATP} channels could be restored by removing ATP and adding 100 μM aprikalim, indicating that no run-down occurred dur-

Figure 3 Effect of aprikalim on K_{ATP} channels in NDP- and Mg^{2+} -free conditions. Continuous current traces recorded from one inside-out patch containing at least four K_{ATP} channels. Patch was perfused with standard solution (NDP- and Mg^{2+} -free, EDTA) initially, then both ATP (500 μM) and aprikalim (10 μM then 100 μM) were added, before returning to standard solution. Aprikalim (10 μM) did not induce K_{ATP} channel activity in presence of ATP. Increasing the dose of aprikalim (100 μM) caused a slight and reversible activation of ATP-blocked channels. Comparison of channel activity in drug-free standard solution at the beginning (a) and at the end (d) of current recording indicates the absence of channel run-down.

Figure 4 Effect of aprikalim on K_{ATP} channels in nucleotide-free standard conditions. Current traces recorded from two separate inside-out patches continuously superfused with the standard solution (NDP- and Mg^{2+} -free, EDTA). The effect of $100\ \mu M$ aprikalim added to the standard solution was tested in a patch with high activity (a; few seconds after its excision) and in a patch with low activity (b; after 5 min of recording). In ATP-free conditions, aprikalim did not affect the K_{ATP} channel activity.

Figure 5 Effect of aprikalim on K_{ATP} channels in the presence of NDPs and Mg^{2+} . Current traces recorded from one inside-out patch. Patch was excised in standard solution (NDP- and Mg^{2+} -free; not shown), then superfused with Mg-NDP solution containing $500\ \mu M$ ATP, before returning to standard solution. Aprikalim ($100\ \mu M$ then $10\ \mu M$) was added to the Mg-NDP solution ($200\ \mu M$ ADP, $50\ \mu M$ GDP, $1\ mM$ $MgCl_2$ with EGTA) with or without $500\ \mu M$ ATP. Change of successive drug applications is marked by arrow. At least fifteen simultaneous openings of K_{ATP} channels can be detected in the standard solution as in the Mg-NDP solution containing aprikalim.

ing the course of this experimental procedure. Similar results were obtained with four additional inside-out membrane patches from different myocytes. Therefore, aprikalim activated K_{ATP} channels despite the continued presence of almost maximally inhibitory [ATP].

In subsequent experiments, the effects of 100 μ M aprikalim on K_{ATP} channels were more extensively studied by determin-

ing a concentration-response curve to [ATP]. The results obtained from 3 to 7 experiments for each [ATP] are shown in Figure 6. The aprikalim-induced activation of K_{ATP} channels occurred at millimolar [ATP]. Fitting of experimental data obtained by superfusing the patch with Mg-NDP solution containing 100 μ M aprikalim yield a K_i for [ATP] of 1.5 mM with a slope value of 2.37 (unbroken line; Figure 6).

Figure 6 Concentration-response relationships for the closing of K_{ATP} channels by ATP in Mg-NDP solution in the absence (dotted curve redrawn from Figure 2) and the presence of 100 μ M aprikalim (● and continuous curve). The relative current represents the mean patch current in test [ATP] as a fraction of that in ATP-free conditions. Values are mean $\pm$ s.d. ($n = 3$ to 7 in each case). The continuous line is fitted by regression analysis ($K_i = 1.5$ mM and $s = 2.37$).

Activating action of aprikalim depends on time after patch excision

In preliminary experiments, we observed that the activating effect of aprikalim, which was strong during the first few minutes that the patches were bathed in Mg-NDP solution, tended to vanish rapidly with time. Specific experiments ($n = 10$) were then carried out to determine whether this decay differed from the run-down process. Just after inside-out patch formation, the channel activity in standard solution was briefly recorded, then the standard solution was replaced by the Mg-NDP solution in which 100 μ M aprikalim were added alone or with ATP (at one or several concentrations). This protocol was repeated several times and the channel run-down estimated by returning the patches to standard solution after each drug application. To quantify these effects more precisely, we calculated the current through the patch as the ratio of the mean current amplitude to a time unit (averaged over 20–30 s after reaching a steady level). This ratio was determined both for patches in test solutions and for patches in the standard solution at the onset of the current recording. A typical experiment is illustrated in Figure 7, where 100 μ M aprikalim was applied in the absence of ATP and then in the presence of 1 mM ATP. Excision of the patch in the standard medium maximally activated K_{ATP} channels present in the patch area (9–10 levels of activity, patch current taken as unity; Figure 7a). The internal app-

Figure 7 Time-dependent decline of aprikalim-induced channel activity. Current traces recorded from one inside-out patch. Trace (a) started 20 s after patch excision in standard solution (NDP- and Mg^{2+} -free) whereas trace (b) was obtained 4 min later. Traces (b) and (c) represent a continuous recording. ATP (1 mM) was added to Mg-NDP solution (200 μ M ADP, 50 μ M GDP, 1 mM $MgCl_2$) containing aprikalim (100 μ M), as indicated above each trace. Comparison of channel activity in standard solution at the beginning (a) and at the end (c) of the current recording indicates the absence of channel run-down.

lication of $100\text{ }\mu\text{M}$ aprikalim (in Mg-NDP solution) did not affect this sustained channel activity; further addition of 1 mM ATP inhibited it partially and reversibly (3–10 simultaneous channel openings; relative current = 0.724). However, after ATP was removed, the number of active channels progressively decreased despite the presence of aprikalim (4–8 levels of activity about 4 min following the patch excision; relative current = 0.797; Figure 7b), and a second addition of 1 mM ATP further reduced the channel activity to 0–4 levels (relative current = 0.145). This gradual loss of aprikalim-induced activation might be attributed to a concur-

rently arising run-down phenomenon which routinely occurs in isolated patches with kinetics depending on the patch tested. However, this was clearly not the case, since, when patches were returned to standard solution, the channel activity attained a new steady-state level of 9 simultaneous openings (relative current = 0.908), as shown in Figure 7c. Although the kinetics of this time-dependent process varied greatly from patch to patch, a comparable lack of effect of aprikalim on ATP-blocked channels was observed after a few minutes in all the patches ($n=9$), even in the presence of micromolar [ATP]. A more complete example of such a

Figure 8 Comparative evolution with time after patch excision of aprikalim-induced channel activity and channel run-down. (a) Current traces recorded from one inside-out patch: 30 s after patch excision (i), 5 min later (ii), and 10 min later (iii). Just after patch excision, the K_{ATP} channel activity was recorded in standard solution, then in Mg-NDP solution in which aprikalim ($100\text{ }\mu\text{M}$) and/or ATP ($500\text{ }\mu\text{M}$) were added, as indicated. After each series of drug applications, the channel run-down was estimated by returning to standard solution. (b) Time course of the relative current measured during repetitive applications of $100\text{ }\mu\text{M}$ aprikalim ($\blacktriangle$) and $100\text{ }\mu\text{M}$ aprikalim plus $500\text{ }\mu\text{M}$ ATP (Δ) in Mg-NDP conditions, and during exposure to standard solution ($\bullet$). Relative current is the mean patch current measured in the different conditions expressed as a fraction of that measured in standard solution immediately after patch excision. Data in (i), (ii) and (iii) are obtained from the corresponding traces in (a).

gradual decrease in the effectiveness of aprikalim, different from the run-down process, is shown in Figure 8: diminishing responses to successive applications of $100\ \mu\text{M}$ aprikalim were observed in the presence of $500\ \mu\text{M}$ ATP. The maximal level of channel activity normally recorded for patches in the standard solution (11 simultaneous openings) during the first minutes after inside-out patch formation (upper trace) was the same as that observed for patches in Mg-NDP solution containing aprikalim. Applying ATP alone to patches in Mg-NDP solution drastically inhibited K_{ATP} channel activity (1 level of activity), whereas applying ATP in the presence of aprikalim only slightly inhibited it (8–9 levels of activity), as already described (see Figures 4 to 6). Five minutes after patch excision (Figure 8a(ii)), all K_{ATP} channels were maximally activated upon returning the patches to standard solution (11 levels of activity), and the subsequent application of ATP in Mg-NDP solution still strongly reduced channel activity (1 level of activity). The second application of aprikalim did not reactivate all K_{ATP} channels (8–10 simultaneous channel openings) and the concomitant addition of ATP even more drastically reduced channel activity (1–4 levels of activity). Ten minutes after patch excision (Figure 8a(iii)), a modest run-down of K_{ATP} channel activity was noted when patches were returned to standard solution (5–8 levels of activity). A stronger inhibition of this activity was induced by ATP alone in Mg-NDP solution, probably as a consequence of the spontaneous loss of active channels (one opening). The subsequent application of aprikalim partially activated the channels (3–6 levels of activity), an activation which was almost entirely suppressed by ATP (1–2 brief openings). Quantitative analysis of the data (Figure 8b) indicated that, despite the presence of aprikalim, the patch current fell rapidly upon successive ATP applications during the first 10 min of recording (by 11.5% at the first application and by 69% at the second) whereas its value was reduced in the absence of ATP only slightly and only at the second application by 8%. Thereafter, the channel activity recorded in standard solution waned (run-down; reduction of 22% at the third application) at a rate somewhat slower than

that found for patches in Mg-NDP solution containing aprikalim (reduction of 38.5% at the third application). Aprikalim lost its ability to overcome ATP inhibition much faster, since ATP almost completely inhibited the patch current within 14 min (reduction of 96% at the third application), whereas reductions of 54% and 69% were subsequently observed for patches bathed in standard solution and in Mg-NDP solution containing aprikalim, respectively. Therefore the progressively developing ineffectiveness of aprikalim in counteracting the inhibitory effect of ATP was faster than, and different from, the spontaneous loss of channel activity due to run-down.

Activating action of Mg-NDPs wanes rapidly

The time-dependence of K_{ATP} channel activation induced by NDPs was also tested on inside-out patches ($n = 7$) successively superfused with standard solution then with Mg-NDP solution containing ATP at various concentrations. Before studying low [ATP], the patch was exposed to 1 mM ATP for at least 20–30 s to delay channel run-down. As observed in Figure 9a, 1 mM ATP inhibited strongly K_{ATP} channel activity when applied together with Mg^{2+} and NDPs. This basal activity was enhanced by decreasing the [ATP] to $200\ \mu\text{M}$, as already illustrated in Figure 2. Following a 5 min period of recording, during which a loss of four levels of channel activity was observed in the standard solution (Figure 9b), an inhibition of channel openings, much stronger than that shown in the upper trace, was produced by $200\ \mu\text{M}$ ATP. Some rare single-channel openings could still be detected in the presence of 1 mM ATP. The run-down of channel activity took place 3 min later under standard conditions. The time-course of the NDP-induced modulation of K_{ATP} channel activity recorded from another inside-out patch is illustrated in Figure 10. After patch formation in standard solution, the membrane was exposed to 1 mM, $200\ \mu\text{M}$, $100\ \mu\text{M}$ then $50\ \mu\text{M}$ ATP in Mg-NDP conditions, before being re-exposed to the standard solution. It can be seen that (i) some channel activity persisted throughout the

Figure 9 Time-dependent decline of NDP-induced channel activity. Current traces recorded from one inside-out patch at different times after patch excision: 10 s (a), 5 min (b) and 8 min (right part of b). Just after patch excision, the K_{ATP} channel activity was recorded in standard solution initially, then in Mg-NDP solution containing ATP at two different concentrations (1 mM then $200\ \mu\text{M}$) as indicated. Change of [ATP] is marked by arrow. After each series of ATP applications, the channel run-down was estimated by returning to standard solution.

Figure 10 Comparative evolution with time after patch excision of NDP-induced channel activity and channel run-down. Data were obtained from one inside-out patch repeatedly exposed to various $[ATP]_i$: 1 mM (○), 200 μ M (◇), 100 μ M (△) and 50 μ M (□) in Mg-NDP solution, and then patch was re-exposed to standard solution (●) in order to estimate channel run-down. Ordinate scale shows patch current relative to the current recorded in standard solution immediately after patch excision.

experimental procedure when 1 mM ATP was applied to the patch under Mg-NDP conditions, (ii) the inhibitory effect of moderate ATP concentrations increased markedly as a function of time, and (iii) the channel activity recorded in the standard solution was consistently lower than that recorded prior to inhibition by a series of successive ATP applications. For instance, the patch current declined from 1 to 0.482 (i.e., 51.8%) 10 min after patch excision. Repeated applications of micromolar $[ATP]_i$ also caused progressively smaller responses, i.e. from 0.567 to 0.1 in 200 μ M ATP (82.4%), from 0.699 to 0.234 in 100 μ M ATP (66.5%) and from 0.786 to 0.257 in 50 μ M ATP (67.3%) after a comparable period of time. Although the kinetics of this time-dependent process varied from patch to patch, a comparable lack of effect of NDPs on ATP-blocked channels was observed in the six other experiments. Thus, the NDP-induced activation of ATP-blocked channels waned faster than the channel run-down appeared.

Discussion

Our study provides two major pieces of information about the electrophysiological profile of aprikalim (RP 52891, the eutomer of RP 49356), as observed with inside-out membrane patches from guinea-pig ventricular myocytes. First, aprikalim is much more potent in opening ATP-blocked K_{ATP} channels in the presence than in the absence of internal Mg-NDPs. Second, this channel activation induced by aprikalim wanes more rapidly than can be accounted for by channel run-down.

Many features of the interaction between aprikalim and K_{ATP} channels reported here are common to other KCOs (for review, see Ashcroft & Ashcroft, 1990): (i) the internal application of aprikalim reduced, in a concentration-dependent manner, the ATP sensitivity of cardiac K_{ATP} channels; (ii) the aprikalim-induced activation of K_{ATP} channels occurred even in the absence of internal Mg^{2+} ; and (iii) this effect was markedly enhanced by NDPs in the presence of Mg^{2+} . The ability of aprikalim and some other KCOs (Shen *et al.*, 1991; Tung & Kurachi, 1991; Allard & Lazdunski, 1993; Larsson *et al.*, 1993) to facilitate the opening of K_{ATP} channels and to hasten strongly their recruitment once this has been initiated by an endogenous event (i.e. increases in $[ADP]_i$ from 10 to 200 μ M; Allen *et al.*, 1985) may be of a particular relevance for the therapeutic potential of this class of compounds protecting the heart against an ischaemic/perfusion injury (Richer *et al.*, 1990; Auchampach *et al.*, 1991; Anderson, 1992; Escande & Caverio, 1992; Gross & Auchampach, 1992; Lynch *et al.*, 1992; Opie, 1993). Through the additional

stimulating effects of H^+ (Fan & Makielski, 1993), lactate (Han *et al.* 1994) and adenosine (Li *et al.*, 1993), KCOs might even further accelerate the opening of K_{ATP} channels when $[ATP]_i$ is higher than that generally required for such an opening to occur in the absence of a KCO. The beneficial consequence which may be expected from this effect of KCOs is to prolong cell viability during oxygen deprivation, since the cardiac myocyte can rapidly adopt a strategy of defence against ischaemia/reperfusion damage before the drastic fall of its $[ATP]_i$ (Auchampach *et al.*, 1991).

The mechanism of action of aprikalim seems to be somewhat different from that of the racemic compound, RP 49356 (Thuringer & Escande, 1989; Weik & Neumcke, 1990), since aprikalim required the presence of internal ATP for its effects and only weakly activated K_{ATP} channels in patches bathed in Mg^{2+} -free solution. Similar differences have also been reported for other KCOs (Escande *et al.*, 1989; Kozłowski *et al.*, 1989; Fan *et al.*, 1990; Takano & Noma, 1990; Shen *et al.*, 1991; Tung & Kurachi, 1991; Larsson *et al.*, 1993; Hussain *et al.*, 1994). The exact reason for these observations remains unclear. However, it is difficult to be sure that, for aprikalim and RP 49356, such discrepancies are entirely independent of the experimental conditions, since we did not study these two compounds at the same time. In the present experiments, the continuous and direct superfusion of the patch with Mg^{2+} -free standard solution containing EDTA very likely washed out any Mg^{2+} bound to the internal surface of the excised membrane. Levromakalim (formerly called lemakalim; the eutomer of cromakalim) has been recently proposed to have a Mg^{2+} -dependent mechanism of K_{ATP} channel activation in skeletal muscle (Hussain *et al.*, 1994); intracellular Mg^{2+} , therefore, may also be required for channel activation by KCOs in the heart. However, the possibility that aprikalim-induced channel activation is mediated through a Mg^{2+} -dependent binding site can be discarded in view of the observation that a high concentration of aprikalim still activated ATP-blocked channels in Mg^{2+} -free conditions, as also reported for pinacidil in cardiac myocytes (Fan *et al.*, 1990). A simple interpretation of these results would be that the inhibitory nucleotide binding site and the site for KCOs allosterically interact, as proposed for pinacidil in skeletal muscle cells (Allard & Lazdunski, 1993). Such an interaction could also account for the strongly facilitating effect of NDPs on aprikalim action in the presence of Mg^{2+} .

One interesting observation we made is that aprikalim or NDPs in Mg^{2+} -containing solutions can lose their effects against the inhibitory action of ATP faster than K_{ATP} channels run down. Previous reports have shown that in cardiac myocytes KCOs alone (Escande *et al.*, 1989; Thuringer & Escande, 1989; Takano & Noma, 1990; Ashford *et al.*, 1994) or co-applied with Mg-NDPs (Shen *et al.*, 1991; Tung & Kurachi, 1991) can temporarily restore channel activity after complete run-down. However, the experimental conditions used in these studies differ substantially from ours, since we analysed the progressive inability of aprikalim to counteract the inhibitory action of internal ATP over time. In agreement with our results, Ripoll *et al.* (1990) have observed that cromakalim in NDP-free solution no longer counteracts the inhibitory action of ATP after the run-down process has taken place. In addition, diminishing responses to repeated applications of levromakalim in nucleotide-free solutions have also been reported to occur in skeletal muscle cells (Hussain *et al.*, 1994). Therefore, the time-dependent fading of the aprikalim-induced activation of K_{ATP} channels is probably not linked to the presence of NDPs. Like aprikalim, Mg-NDPs failed to activate partially run-down K_{ATP} channels in the presence of micromolar $[ATP]_i$. Such an increase in channel sensitivity to micromolar $[ATP]_i$ as channel activity runs down is in line with previous observations (Thuringer & Escande, 1989; Nichols & Lederer, 1991; Deutsch & Weiss, 1993). That the channel activity after the removal of Mg-NDPs and ATP was consistently lower than

the control activity prior to inhibition by ATP suggests that no phosphorylation occurred under our experimental conditions. In apparent contrast with our results, Ashford *et al.* (1994) have recently shown that, like native cardiac K_{ATP} channels (Shen *et al.*, 1991; Tung & Kurachi, 1991), the cloned cardiac channel (rcK_{ATP}-1) undergoing run-down can be reactivated by NDPs, exclusively at very high concentrations (10 mM) in the presence of 2 mM Mg²⁺ and that this effect is drastically increased when micromolar [ATP]_i is co-applied with Mg-NDP. However, it is possible that the high [NDP]_i used by these authors triggered an additional mechanism of channel opening. Indeed, the channel reactivation following run-down requires energy necessarily derived from high energy phosphates (Furukawa *et al.*, 1994). It may be speculated that millimolar [NDP]_i can also supply this energy to restore the channel protein to its active conformational state.

We have difficulties in offering a satisfactory interpretation for the time-dependent fading of K_{ATP} channel activation by aprikalim or NDPs for which the same basal mechanism (different from the run-down due to channel dephosphorylation; Findlay, 1988b; Furukawa *et al.*, 1994) may be responsible. Our data exclude the possibility that channel phosphorylation may be part of the mechanism underlying the action of aprikalim or NDPs in the presence of internal Mg²⁺. This, however, does not mean that the degree of channel phosphorylation and the potency of aprikalim or NDPs in activating channels are fully independent and cannot exert reciprocal influences. Interestingly, Shen *et al.* (1991) have reported that other KCOs (except nicorandil) can activate cardiac K_{ATP} channels only if the channels are not completely dephosphorylated. Our data, which are quite similar to theirs, perhaps could be explained on the basis of variable degrees of channel phosphorylation resulting from our experimental conditions. It may be speculated that, in our experimental conditions, K_{ATP} channels would become dephosphorylated over short periods of time (5–10 min) because the continuous superfusion of the patch with Mg-free standard solution (EDTA) would wash out any

superficial membranous ATP, Mg²⁺ or other regulatory molecules, such as kinases, associated with the channel protein (Larsson *et al.*, 1993; Hussain *et al.*, 1994). Whether any activating substances are actually lost during patch excision is still unknown. It is tempting to suggest that one of the G proteins proposed to prevent the ATP-dependent closure of K_{ATP} channels (Terzic *et al.*, 1994) might be involved in transducing the signal generated by the KCO or NDP binding to the channel protein. Presumably, this G protein could be washed out from the excised patch during the experimental procedure. Further studies are necessary to elucidate such possible regulatory mechanisms which are hinted at by our inside-out patch experiments.

In summary, we have shown that aprikalim is much more potent in opening ATP-blocked K_{ATP} channels in the presence than in the absence of internal Mg-NDPs, although its action wanes rapidly in excised membrane patches. This fading, like channel run-down, appears to be linked to the excised patch itself as well as to the experimental conditions. Therefore, the failure of aprikalim to produce in our experiments a sustained K_{ATP} channel activity cannot be considered an intrinsic property of the compound since it is not observed with intact tissues (Richer *et al.*, 1990; Auchampach *et al.*, 1991; Escande & Caverio, 1992). In conclusion, our results favour the hypothesis that cardiac myocytes suffering a transient ischaemic stress and treated with aprikalim (or more generally with KCO) are likely to adopt much sooner a strategy of protection and resume their contractile function more easily, upon timely reperfusion, than untreated cells (Caverio & Premmureur, 1994).

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