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Apparent Competition Between ATP and the Potassium Channel Opener RP 49356 on ATP-Sensitive K^+ Channels of Cardiac Myocytes

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SUMMARY

The mechanism whereby RP 49356, a novel potassium channel opener, activates ATP-sensitive K^+ channels (K^+-ATP channels) in isolated cardiac cells was investigated with the patch-clamp technique. When directly applied onto the inner face of an inside-out membrane patch, RP 49356 (300 μM) had no effect on K^+ channels opened in an ATP-free solution. In contrast, the same concentration of the drug reactivated K^+-ATP channels that had experienced spontaneous "run-down" of their activity following long recording periods. In cell-attached experiments, externally applied RP 49356 (300 μM) opened K^+-ATP channels at 35° in spite of the high intracellular ATP concentration, which was sufficient to prevent channel openings in the absence of the drug. In control conditions, the dose-response relation for ATP closing the channels had a Hill coefficient of 2.37 and a half-inhibition concentration of 56 μM. With 30 μM RP 49356 present in the intracellular medium, the slope factor of this relation was unchanged but the curve was shifted to the right, with a half-inhibition concentration of 515 μM. Conversely, the dose-response relation of RP 49356 activating K^+-ATP channels was shifted to the right in a parallel manner under the influence of increasing concentrations of ATP. It is concluded that RP 49356 acts on cardiac K^+-ATP channels by decreasing their sensitivity to ATP. Our results are consistent with an apparent competition between ATP and RP 49356.

The PCOs form a novel class of vasodilating agents that are believed to relax vascular smooth muscle cells via a hyperpolarization of the cell membrane consequent to an increased K^+ conductance (1, 2). Although PCOs are more potent in the nonstriated musculature, it appears that these agents are also active on K^+ channels from both skeletal (3) and cardiac muscle (4–6). We have previously demonstrated that in cardiac myocytes the specific target for three chemically unrelated PCOs (i.e. cromakalim, pinacidil, and RP 49356) is the K^+-ATP channel (7–9). More recently, Standen *et al.* (10) have shown that PCOs also activate K^+-ATP channels in arterial smooth muscle.

In contrast to pancreatic β-cells, K^+-ATP channels in cardiac cells are believed to be blocked under physiological conditions by the high intracellular ATP concentration, $[ATP]_i$ (for a recent review on K^+-ATP channels, see Ref. 11), and to open only when $[ATP]_i$ is low because of metabolic exhaustion (e.g., metabolic inhibitors, hypoxia, or ischemia). Because externally applied PCOs activate K^+-ATP channels in intact cardiac cells (7–9, 12), it can be concluded that they either act indirectly via a decrease in $[ATP]_i$ or act directly on the channels by modifying their gating properties. Insomuch as PCOs are also active in cell-free membrane patches (7–9, 10) where the ATP concentration facing the internal surface of the membrane is adequately controlled, a direct action at the level of the channel protein seems likely. Thus, in intact cells, PCOs are capable of relieving the closing of the channels produced by $[ATP]_i$. In the present study, we have investigated the interaction between ATP and RP 49356, a novel PCO (9, 13), on cardiac K^+-ATP channels. Our results show the existence of an apparent competition between ATP and RP 49356 at the macroscopic level. A competitive mechanism between ATP and diazoxide, another vasodilator agent that exerts hyperglycemic side effects because of an activation of pancreatic K^+-ATP channels (14), has previously been suggested by Dunne *et a.* (15) in insulin-secreting cells. Some of our results have already appeared in abstract form (16).

Materials and Methods

Cell isolation. Single ventricular cells were dissociated at 37° by treating adult guinea pig hearts with both collagenase and protease (17). Briefly, hearts were mounted on a Langendorff-type apparatus and perfused with a Ca^{2+}-free Tyrode solution for 5 min, then with an enzymatic solution containing 2 mg/ml collagenase (Type I; Sigma

ABBREVIATIONS: PCO, potassium channel opener; K^+-ATP channels, ATP-sensitive K^+ channels; i_{K1} channels, inward rectifier K^+ channels; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; P_{open}, open probability of channels.

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Chemical, St. Louis, MO) and 0.28 mg/ml protease (Type XIV; Sigma). After an 8-min digestion, the heart was rinsed with a 0.2 mM Ca^{2+} solution for 5 min and then cut with scissors into small pieces. Gentle agitation of ventricular chunks with fire-polished Pasteur pipettes released isolated cells which were stored at room temperature in a high K^+/low Cl^- storage solution.

Solutions and drugs. Isolated cells placed in Petri dishes were continually perfused with an internal-type medium containing (in mM): KCl, 127; HEPES, 10; KOH, 13; EGTA, 5; and glucose, 10; pH 7.4 with KOH. Patch pipettes were filled with a medium containing (in mM): KCl, 140; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; and glucose, 10; pH 7.4 with KOH. Drugs diluted in the bath solution to the desired final concentration were directly applied onto the membrane patch by means of a micropressure ejection system (Miniframe-PPS2; Medical System). ATP (Sigma) was used as the Mg^{2+} salt. RP 49356 (*N*-methyl-2-[3-pyridil]-tetrahydrothiopyran-2-carbothioamide-1-oxide), synthesized by Rhône-Poulenc Laboratories (Vitry-Sur-Seine, France), was dissolved as a stock solution in 0.1 N HCl at a concentration of 10^{-2} M. The pH of each solution containing Mg^{2+}-ATP or RP 49356 was readjusted to 7.4 with KOH.

Recordings and data analysis. Unitary current recordings were performed in both the cell-attached and cell-free inside-out configurations of the patch-clamp technique (18) by using an EPC7 List patch-clamp amplifier (Darmstadt, FRG). On-cell recordings were conducted either at 33–35° or at room temperature, whereas inside-out experiments were performed at 19–22°. Patch pipettes were pulled with a BBCH vertical puller from borosilicate capillary tubes (Preciver, Creteil, France) and had resistances ranging between 6 and 10 MΩ. Recordings were stored on videocassettes (Sony PCM-701 digital audio processor and Sony SL-T50 ME videocassette recorder) and then subsequently replayed through an eight-pole Bessel low pass filter (Frequency Devices Inc. 902 LPF), at 300–500 Hz, onto either a Nicolet 3091 digital oscilloscope or a Brush recorder (2400S; Gould Inc.).

For the analysis of K^+-ATP channel activity, unitary currents recorded over many seconds at a constant membrane potential were digitized at 1 KHz with an eight-bit analog-to-digital converter and then stored directly on disk for later analysis with an IBM-AT personal computer. For each 500-msec recording segment, the current flowing through the patch was calculated with regard to the zero current level, which was determined when all the channels were closed. The open probability of K^+-ATP channels was determined over recording periods of 20 to 30 sec according to:

$$P_{open} = I/(N_i)$$

where I is the average channel current calculated as the integrated current divided by the total time of the sample, N is the total number of channels active in the patch, and i is the unit amplitude of the single K^+-ATP channel current. Using this quantitative analysis, openings of i_{K1} channels provide a portion of the average current measured. However, the error induced by i_{K1} channel activity, which was not affected either by RP 49356 or ATP, can be considered as a constant during the recording periods analyzed.

Results

RP 49356 reactivates "run-down" K^+-ATP channels. The exposure of the inner side of an excised membrane patch to an ATP-free solution invariably led to the opening of several large K^+ channels (elementary conductance of 66 pS), which could be completely and reversibly blocked by 2 mM ATP (Fig. 1). The activity of these K^+-ATP channels usually masked elementary events of smaller amplitude, which were identified as openings of i_{K1} channels (elementary conductance of 21 pS in symmetrical 140 mM K^+). These latter channels were clearly visible either in the cell-attached mode where intracellular ATP prevents K^+-ATP channels from opening (Fig. 1A) or in inside-out patches when the internal solution contained 2 mM ATP (Fig. 1B).

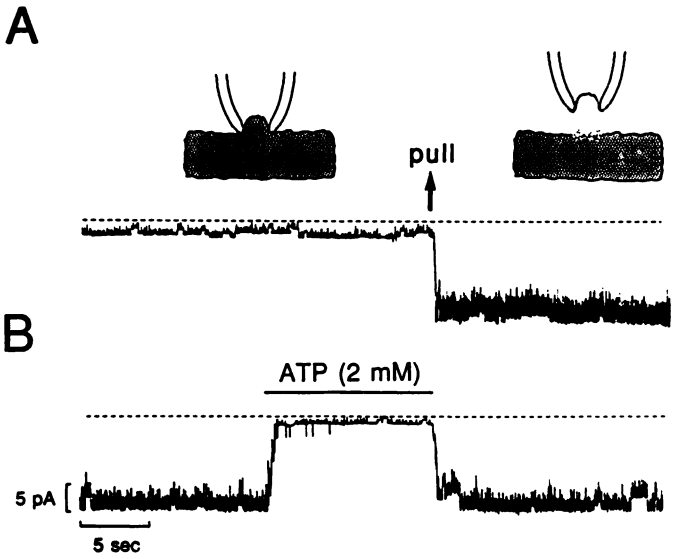

Fig. 1. Single K^+ channel currents recorded in the cell-attached and inside-out configurations. A, Unitary K^+ currents recorded in a patch clamped at −50 mV and excised from the cell at the time indicated by the *arrow*. B, Selective blockade by internal ATP of K^+-ATP channels. The inside-out patch contained at least six different K^+-ATP channels. In this figure (as in following ones), the *dotted lines* represent the current level where all channels were closed; *downward deflections* are inward unitary currents. Holding potential, −50 mV; *traces* were filtered at f = 500 Hz.

It is well known that the activity of K^+-ATP channels recorded in cell-free patches spontaneously declines over relatively short recording periods (19, 20). This "run-down" phenomenon, whose exact mechanism remains open to discussion (11), was also observed in our cell-free preparations, although its magnitude and time course markedly varied from patch to patch. In 12 inside-out patches, we investigated the action of RP 49356 on K^+-ATP channels recorded immediately after patch excision so as to minimize disturbances arising from the run-down. Under these conditions, RP 49356 lacked effects on opened K^+-ATP channels (Fig. 2A) or on small i_{K1} channels recorded in the presence of 3 mM ATP (not shown). In contrast, when RP 49356 was applied after a longer period of recording, it reactivated K^+-ATP channels that had experienced run-down. The reactivation by RP 49356 of run-down channels was observed in all the experiments performed (n = 35) although, as illustrated in Fig. 2B, where channels had reached complete inactivation the channel activity was usually only partially restored by the drug. Both number and burst duration of channel openings were increased by RP 49356, whereas the unitary current amplitude determined at either negative or positive membrane potentials remained unchanged (not shown).

RP 49356 activates K^+-ATP channels in cell-attached patches. The effects of RP 49356 were further explored using the cell-attached configuration, in which the channels are not prone to run-down. In these experiments, the membrane potential was expressed relative to the resting potential, which is assumed to be 0 mV with 140 mM K^+ in the extracellular solution. As previously observed by others (19, 21–23), K^+-ATP channel activity was rarely observed from cell-attached patches and only openings of small i_{K1} channels were recorded. This

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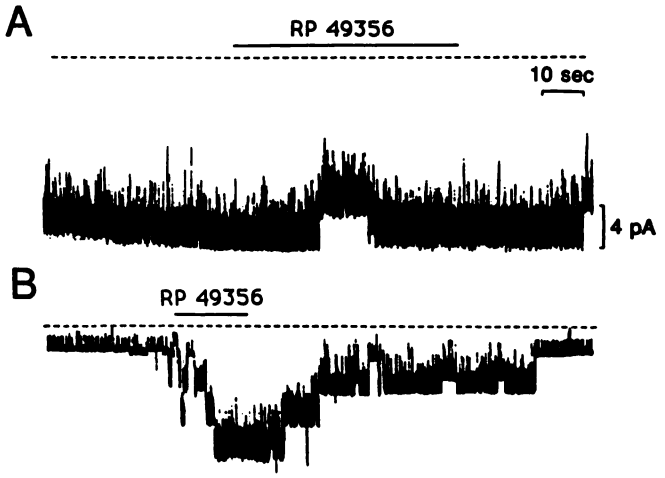

Fig. 2. Effects of internal RP 49356 on channels recorded in an inside-out membrane patch containing at least five K^+-ATP channels. A, The internal application of 300 μM RP 49356 immediately after patch excision lacked effects on the channel activity. B, After spontaneous loss of K^+-ATP channel activity, only openings of small i_{K1} channels were observed. The addition of RP 49356 (300 μM) reversibly reactivated three run-down channels. Holding potential, −50 mV; *f*, 500 Hz.

channel activity was not affected by the addition of RP 49356 (300 μM) in the bath solution at room temperature (Fig. 3A). When the experimental temperature was increased to 33–35° (Fig. 3B), the same concentration of RP 49356 opened K^+-ATP channels, which were easily distinguished from i_{K1} channels because of their larger unitary current amplitude. Removal of the drug resulted in a slow decay in the activity of K^+-ATP channels to their complete closure. Experiments performed in cell-attached patches confirm the temperature dependence of the effects of externally applied RP 49356, a constant feature of PCOs in the whole-cell configuration (9, 12). They also strongly suggest that RP 49356 penetrates within the cell before activating K^+-ATP channels (9). These results also imply that RP 49356 is capable of relieving channel blockade by intracellular ATP. Further experiments were thus conducted in inside-out patches to determine whether the responsiveness of the channels to ATP differs depending on the presence of RP 49356.

RP 49356 modifies the ATP sensitivity of K^+-ATP channels. The blocking effects of ATP were quantified by measuring P_{open} during ATP application, expressed relative to its value in nucleotide-free solution. A major problem associated with the construction of a dose-response relation for ATP is the run-down of channel activity that follows patch excision. An illustrative example of this is shown in Fig. 4A. In this inside-out patch, a small dose of ATP was repeatedly applied while the channel activity rapidly declined. As illustrated in Fig. 4B, the relative P_{open} in the ATP solution gradually decreased with the run-down in such a way that the channels were more and more prone to ATP blockade along the recording period. Thus, because the run-down of the channels induced a bias in the measurement of their ATP sensitivity, the effects of a single ATP concentration were only explored in a given patch immediately after its excision.

In the concentration range 10 μM to 1 mM, ATP exerted a greater inhibition of the channels in the absence than in the presence of RP 49356 (Fig. 5A). From 81 patches, the dose-response relations for ATP closing the channels in the absence

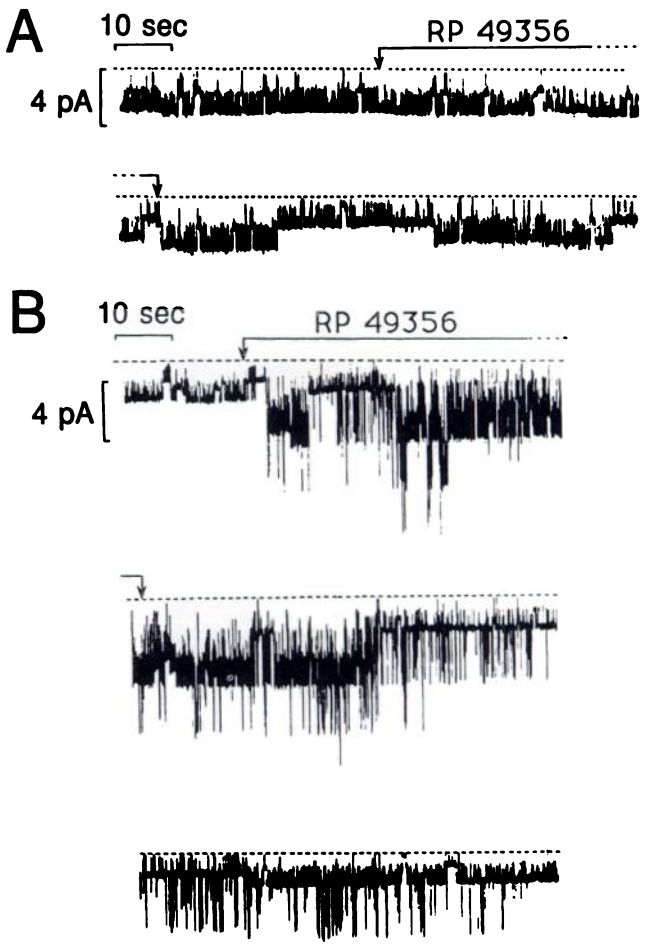

Fig. 3. Temperature dependence of the effects of RP 49356 in cell-attached patches. A, At room temperature, 300 μM RP 49356 added to the perfusion medium did not activate K^+-ATP channels in a patch that contained at least two i_{K1} channels. B, at 34°, RP 49356 (300 μM) reversibly activated two different K^+-ATP channels in a different patch from that in A. In A and B, holding potential, −40 mV; *f*, 300 Hz.

or presence of 30 μM RP 49356 were constructed (Fig. 5B). Experimental data were suitably fitted by a sigmoidal equation:

$$\text{relative } P_{open} = 1/1 + ([ATP]_i/K_A)n$$

where K_A is the ATP concentration evoking the half-maximal inhibition and n is the Hill coefficient. In control conditions, K_A and n were 56 μM and 2.37, respectively. In the presence of 30 μM RP 49356, the dose-response relation for ATP had the same slope as in its absence but was shifted to higher ATP concentrations by 1 order of magnitude (K_A = 515 μM). Moreover, we observed that RP 49356 affected the time course of the development of the ATP blockade. In the example shown in Fig. 6, 250 μM ATP produced a complete block of the channels in less than 3 seconds. When the same concentration of ATP was applied in sequence with 30 μM RP 49356, the maximum inhibition was delayed and evolved through two distinct phases; the first phase, which was similar to the ATP binding in control conditions, was followed by a slower phase leading to a complete inhibition within 20 sec. In the continuous presence of 30 μM RP 49356, 250 μM ATP was not sufficient to entirely suppress the channel activity and a new steady state was reached only after a 70-sec period of complex time course (Fig. 6C). The prolongation by RP 49356 of the onset of ATP

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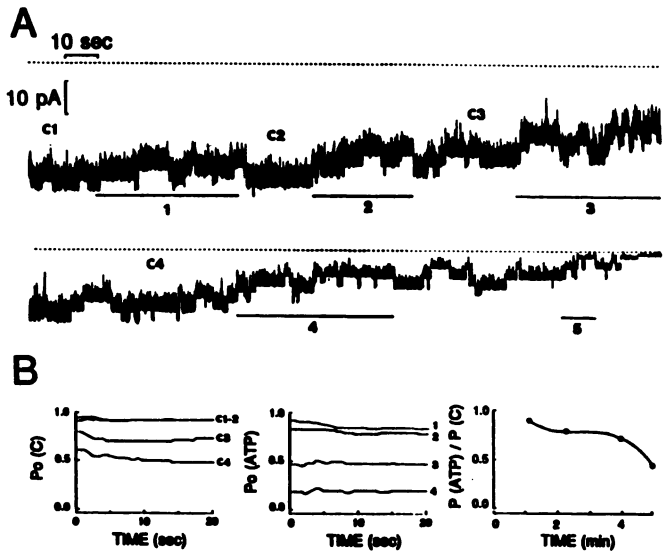

Fig. 4. The run-down of K⁺-ATP channels increases their ATP sensitivity. A, Continuous record of K⁺-ATP channels in a patch showing an extremely rapid, unusual run-down. Repetitive applications of ATP (30 μM), indicated by *solid bars* with *numbers* from *1* to *5*, were separated by control periods (*C1* to *C4*). Holding potential, −50 mV; *f*, 500 Hz. B, Plots of the cumulative open probability (P_o) against time in the absence (*left*) or presence (*right*) of 30 μM ATP for the different recording periods indicated in A. The ATP sensitivity of the channels, determined as the ratio between P_o(ATP) and P_o(C) for each successive ATP application, progressively declined as the run-down evolved (*right*).

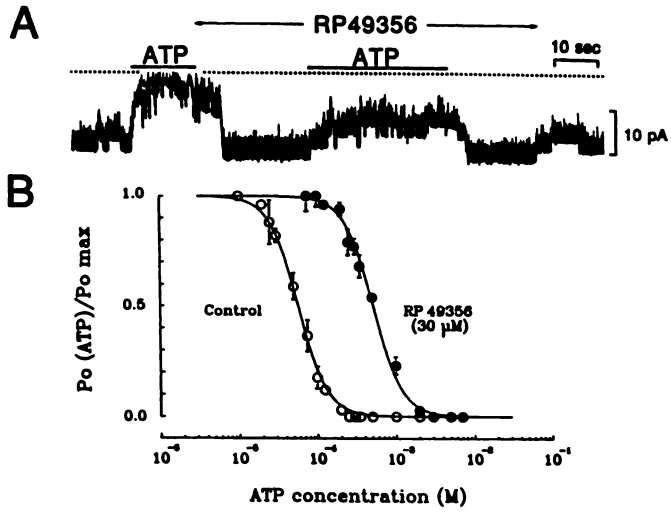

Fig. 5. RP 49356 decreases the ATP sensitivity of K⁺-ATP channels. A, Effects of 100 μM ATP on K⁺-ATP channels from an inside-out patch recorded before and upon their stimulation by 30 μM RP 49356. Holding potential, −40 mV; *f*, 500 Hz. B, Dose-response relations for ATP blocking K⁺-ATP channels in the absence (○) or presence (●) of RP 49356 (30 μM). The open probability in each ATP solution, P_o(ATP), was normalized by referring to its value, $P_o$max, in nucleotide-free solution. Values are means ± standard errors. Data were obtained from 81 inside-out patches.

blockade was observed in all the experiments performed. However, these effects were not further quantified because of their variability and complexity.

ATP modulates the activation by RP 49356 of K⁺-ATP channels. The antagonism between RP 49356 and ATP was further evaluated by determining dose-response curves for RP 49356 (0.3 μM to 3 mM) in the continuous presence of ATP. In

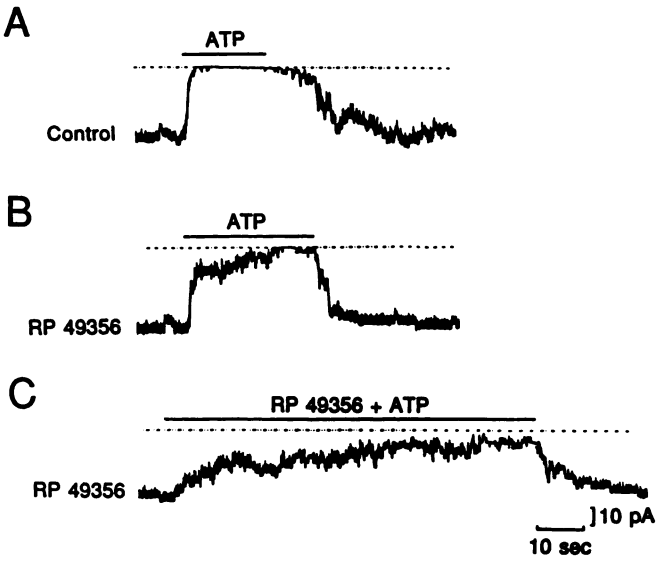

Fig. 6. RP 49356 modifies the onset of ATP blockade. Continuous record obtained from a single inside-out patch. A, 250 μM ATP was applied following a recording period in the control intracellular solution. B, the same concentration of ATP was internally perfused after a 3-min period with RP 49356 (30 μM). C, ATP was applied simultaneously with RP 49356 following a 5-min period with RP 49356 alone in the intracellular medium. Holding potential, −40 mV; *f*, 500 Hz.

the experiment shown in Fig. 7A, 3, 30, and 300 μM RP 49356 were alternately added to the bath solution containing 350 μM ATP. RP 49356 dose-dependently activated K⁺-ATP channels, in spite of the continuous presence of ATP at a concentration sufficient to prevent channel openings in the absence of the drug (see Fig. 5). From 15 different patches, dose-response curves for RP 49356 were constructed in the presence of a constant concentration of ATP, i.e. 175, 350, and 700 μM (Fig. 7B). Data were fitted by the following equation:

$$\text{relative } P_{open} = 1/1 + (K_{RP}/[\text{RP 49356}]_i)^m$$

where K_{RP} is the RP 49356 concentration that produced half-maximum activation and m is the Hill coefficient. The dose-response curve for RP 49356 determined in the presence of 175 μM ATP (K_{RP} = 2 μM; m = 0.5) was shifted to the right in a parallel manner when the ATP concentration was increased to 350 μM (K_{RP} = 16 μM; m = 0.5) and to 700 μM (K_{RP} = 400 μM; m = 0.5). The Hill coefficient for these relations suggested a complex mechanism of interaction between RP 49356 and K⁺-ATP channels blocked by ATP.

Discussion

Our results demonstrate that the PCO RP 49356 opens cardiac K⁺-ATP channels that are blocked either by intracellular ATP or because of the run-down phenomenon but is inactive on "healthy" K⁺-ATP channels that had not experienced the run-down. In two previous articles (7, 9), we reported that RP 49356, cromakalim, and pinacidil, three PCOs with very different molecular structures (9), activate K⁺-ATP channels in inside-out patches bathed in an ATP-free solution because both the number of available channels and the time spent by the channels in the open state were markedly enhanced by the drugs. However, in the light of the results presented here, it is likely that what we interpreted as an

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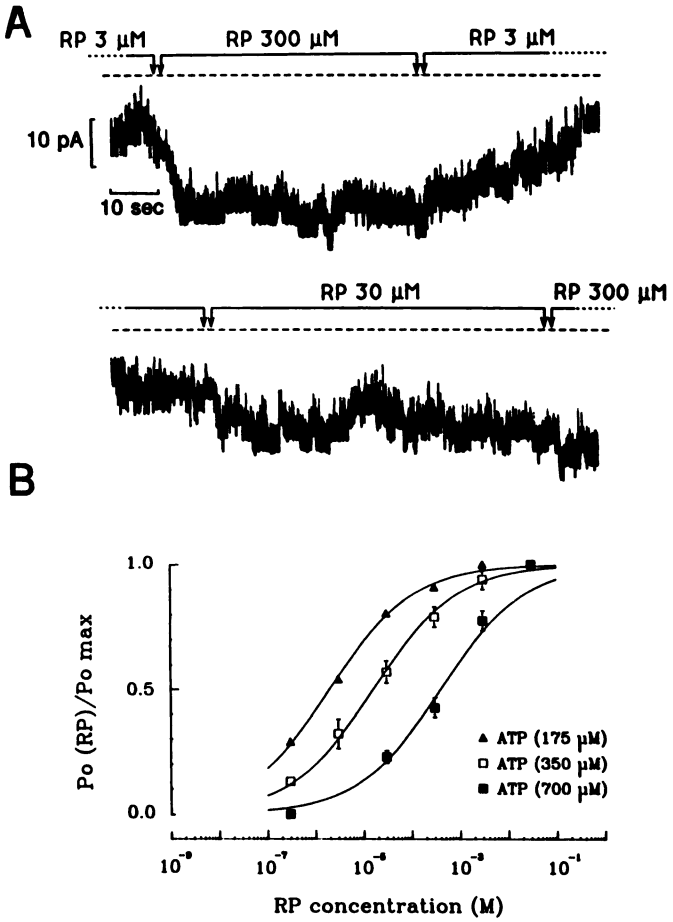

Fig. 7. ATP modifies the activation dose-response curve for RP 49356. A, Continuous traces showing single K^+-ATP currents in an inside-out membrane patch perfused, in the continuous presence of 350 μM ATP, with various concentrations of RP 49356 applied in the sequence shown. Holding potential, −50 mV; *f*, 500 Hz. B, Activation dose-response curves for RP 49356 in the continuous presence of three different ATP concentrations. The open probability for each RP 49356 concentration, P_o(RP), was normalized by referring to its value, $P_o$max, in nucleotide-free solution. Values are means ± standard errors (standard errors are only shown when $n > 3$) from several experiments similar to that illustrated in A. The *solid lines* were drawn from calculations that are described in the text.

activation by these agents of K^+-ATP channels was in fact a reactivation of a fraction of the channel population that underwent the run-down process. It has been suggested that, in pancreatic β-cells (24, 25) and in cardiac cells (20), the run-down of K^+-ATP channels in excised patches is due to a dephosphorylation of the channel protein because the phenomenon can be reversed by ATP. However, phosphorylation of the channels is unlikely to explain reactivation of the channels by PCOs such as RP 49356, which occurred in the complete absence of ATP or ADP. We and others (11) have found that the sensitivity to ATP of run-down channels is markedly increased in comparison with healthy channels. This means that the dose-response curve to ATP is shifted toward lower concentrations under the influence of the run-down process. By shifting this relation to the right, RP 49356 may normalize the sensitivity of the channels to ATP and thereby reactivate run-down channels.

Because RP 49356 induced a parallel shift of the ATP dose-response curve and because the dose-response curve for RP 49356 was shifted in a similar manner by increasing the internal ATP concentration, we propose that an apparent competition exists between RP 49356 and ATP for K^+-ATP channels. This interpretation is reinforced by the observation that the delay required for the channels to reach a new steady state in the presence of ATP was markedly enhanced by RP 49356. However, our data do not necessarily imply a direct competition for the same receptor site of the channel proteins, because the effects of RP 49356 could equally be explained by its binding to a site that is different from that of ATP but that may influence either the binding or the blocking effects of ATP. Finally, further studies with radioligands are needed to elucidate a possible direct competition between ATP and RP 49356 at a molecular level.

In line with previous observations (19, 26), the dose-response relation for ATP blockade of cardiac K^+-ATP channels was closely fitted by a sigmoidal curve with a Hill coefficient of more than 2, suggesting multiple ATP binding sites on each K^+-ATP channel. In contrast, in both skeletal muscle (27) and pancreatic β-cells (28, 29) only one molecule of ATP binds to each channel to close it. Moreover, the minimal concentration of internal ATP required to entirely block the channels is lower in pancreatic β-cells than in cardiac cells (11) or in skeletal muscle (27), which is in turn much lower than the ATP concentration required in central neurones (30). Finally, the effectiveness of the different configurations of the ATP molecule to block the channel activity differs depending on the tissues; in particular, the affinity of K^+-ATP channels for Mg^{2+}-bound forms of nucleotides is markedly increased in cardiac myocytes whereas free-acid forms are more effective in pancreatic β-cells (26). Taken together, these results suggest that the structure of the channel protein differs to some extent depending on the tissue. K^+-ATP channel and its activation by cromakalim or pinacidil have recently been reported in vascular smooth muscle cells (10). PCOs (1, 2, 10), and in particular RP 49356 (13), are at least 10-fold more potent in the vasculature than in the heart or in the pancreas (31). This difference in potency may well be explained by a higher density of the channels in the vascular smooth muscle membrane. Another explanation could be that different biophysical characteristics, particularly concerning the ATP binding to the channel, account for the different sensitivity of the tissues to PCOs and, therefore, for their tissue selectivity.

Acknowledgments

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