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Reconstitution in Lipid Bilayers of an ATP-Sensitive K^+ Channel from Pig Coronary Smooth Muscle

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Abstract. A K^+ channel with a main conductance of 29 pS was recorded after the incorporation of coronary artery membrane vesicles into lipid bilayers. This channel was identified as an ATP-sensitive K^+ channel (K_{ATP}) because its activity was diminished by the internal application of 50–250 μM ATP-Na_2. Moreover, it was opened when 10–50 μM pinacidil was externally applied. Single-channel records revealed the existence of several (sub)conductance states. At 0 mV and with a 5/250 KCl gradient, the main conductance of the K_{ATP} channel was 29 pS. The other (sub)conductance states were less frequent and had discrete values of 12, 17 and 22 pS. Pinacidil stabilized the channel open state primarily in the 29 pS conductance level; whereas ATP inhibited all the conductance levels. In general, K_{ATP} channels were characterized by brief openings followed by long closings (open probability, $P_o \approx 0.02$); only occasionally (3 out of 12 experiments) did the K_{ATP} channels have a high open probability ($P_o \geqslant 0.7$). Channel activity could be increased or rescued by adding 2.5–10 mM UDP-TRIS and 0.5–2 mM $MgCl_2$ to the internal side of the channel.

Key words: Vascular smooth muscle — Pinacidil — UDP — Subconductances

Introduction

After the discovery of K_{ATP} channels in vascular smooth muscle [16], there was a common interest in trying to understand their role in this tissue. K_{ATP} channels are essential in maintaining basal coronary vascular tone [15]. They are the physiological target of endogenous (calcitonin gene-related peptide, norepinephrine and vasoactive intestinal peptide) and synthetic vasodilator agents (pinacidil, diazoxide, nicorandil and cromakalim) [1, 10, 12, 13, 14]. The latter belong to the class of pharmacological ''K channel openers,'' which exert potent hypotensive action by changing the K^+ permeability with the subsequent relaxation of the smooth muscle.

Although all K_{ATP} channels from different tissues share common pharmacological characteristics, such as sensitivity to ATP and activation by ''K^+ channel openers,'' this class of K^+ channels shows different conductance values. Channels of both large and small conductances have been reported and, in some cases, multiconductance states have been observed. For example, in rabbit mesenteric artery, a 135 pS channel was recorded using a 60/140 mM K^+ gradient [16]; while in pig coronary artery, a channel with a conductance of 30 pS in a 2.7/140 mM K^+ gradient has been described [8]. Few studies report the presence of (sub)conductance levels in these channels. Kakei [9] described multiple conductance states of a K_{ATP} channel from guinea-pig ventricular cells. The amplitudes of these sublevels varied between 20% and 90% of the main level. In another study, Weik [21] demonstrated that K_{ATP} channels from skeletal muscle have subconductances at 1/4, 1/2, 3/4, 1/6, 1/3 and 2/3 of the main level. Later, Babenko [2] observed subconductance states of K_{ATP} channels from human ventricular myocytes. Inoue [8] reported, in abstract form, the presence of subconductance states in explants of coronary smooth muscle. However, little is known about the single channel properties of K_{ATP} channels from vascular smooth muscle and the mechanisms of drug action on this class of potassium channels.

In this work, we have been able to reconstitute an ATP-sensitive K^+ channel from pig coronary smooth muscle which is characterized by its small conductance (29 pS at holding potential of 0 mV, 5/250 mM KCl, T = 20°C) and by the presence of different (sub)conductance levels. We show that pinacidil stabilizes channel opening in the 29 pS substate, and that UDP plus Mg^{2+} are

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able to rescue channel activity after rundown. These results show that reconstitution of K_{ATP} channels in lipid bilayers should be useful for studying the mechanisms of drug action at the single-channel level.

Materials and Methods

Membrane vesicles from pig coronary smooth muscle were collected from a 20:25 sucrose gradient, as previously described [17]. We used two independent membrane preparations in this study (about 100 coronaries). Lipid bilayers were cast from a phospholipid solution in n-decane containing a mixture 5:2:3 of phosphatidylethanolamine/phosphatidylserine/phosphatidylcholine (25 mg/ml). The vesicles were applied to the preformed bilayer from the *cis* side, which was the voltage-controlled side, while the *trans* side was referred to ground.

The experimental solutions were (mM): 250 KCl, 10 MOPS, 1 HEDTA, 0.67 $CaCl_2$ (pH 7.2, pCa 5.25), for the *cis* chamber, and 5 KCl, 10 MOPS, 1 HEDTA, 0.670 $CaCl_2$ (pH 7.2, pCa 5.25), 245 sucrose (after incorporation), for the *trans* chamber. Variations to these solutions will be indicated in the figure legends. The laterality of channel incorporation was determined by the sensitivity to ATP and, in some cases, by the simultaneous incorporation of Ca^{2+}-dependent K^+ channels. In the latter occasions, Ca^{2+}-dependent K^+ channel activity was avoided by lowering calcium with chelators.

Pinacidil was a kind gift from Lilly Research Laboratories, Indianapolis, IN. Stock solutions (100 mM) were made in 70% ethanol.

The majority of the recordings were filtered at 500 Hz using an eight-pole Bessel filter and acquired at 400 μs/point for further analysis. Single-channel analysis was performed using TRANSIT. This program allows the simultaneous idealization of different (sub)conductance levels with a new algorithm that uses a slope threshold and a relative amplitude threshold [19], and not the 50% amplitude threshold algorithm. Open probability (P_o) was obtained from total point histograms. Values are means ± SEM (n ⩾ 3) or means ± SD if $n = 2$.

Results

K_{ATP} Channel Activity after Reconstitution into Lipid Bilayers

K_{ATP} channels in vascular smooth muscle have been difficult to study at the microscopic level. This difficulty may be related to a low open probability in intact cells, rundown in excised patches, a small conductance, and/or the presence of a few channels per cell. As a consequence, single-channel behavior of this class of channels is poorly understood. Conductances of very different magnitudes, ranging from 135 pS [16] to 7 pS [3], have been reported. Thus, to further characterize the single channel properties of K_{ATP} channels, we decided to test the possibility that these channels could survive after incorporation into lipid bilayers.

As previously reported, fusion of purified plasma membrane vesicles from coronary smooth muscle mainly led to the activity of large-conductance calcium-activated potassium (K_{Ca}) channels [17]. Besides K_{Ca} channel activity other small outward conductances were observed: (i) a seldom seen (12 out of 76 channel incorporations) K^+ channel characterized by a main conductance of 29 pS, that was not activated by internal Ca^{2+}, and was inhibited by internal ATP-Na_2, and (ii) a small conductance channel of about 8 pS which was difficult to resolve from the noise level, but was insensitive to Ca^{2+} or ATP (*not shown*).

We decided to study the 29 pS channel and determine its identity as a K_{ATP} channel using the following criteria: (i) channels were tested for their insensitivity to internal calcium; (ii) they were inhibited by ATP-Na_2; and (iii) activated by pinacidil. K_{ATP} channels could be recorded independently, however, most of the times (70%) they were incorporated together with K_{Ca} channels. In this case, the activity of K_{Ca} channels was diminished or abolished by lowering $[Ca^{2+}]_i$ as shown in Fig. 1*A*.

To maximize bilayer stability, we performed experiments at 0 mV in a K^+ gradient of 5/250 KCl made isosmotic with sucrose. Under these conditions, K_{ATP} channels could be observed with the following characteristics: (i) channels with ''spiky'' activity without considerable ''rundown''' ($n = 8$); (ii) channels with ''stable'' high open probability ($n = 3$); and (iii) channels that lasted a few seconds and then ''rundown'' to near or at zero level ($n > 2$).

Channel activity was usually less than 0.8 (open probability, P_o). Occasionally, K_{ATP} channels were ''fully'' opened with openings lasting hundreds of msec, which produced a mean open probability of 0.99 ($n = 3$). In 8 out of 12 experiments, P_o was about 0.02 and characterized by brief openings (≈3 msec) separated by long closings (≈200 msec) (Fig. 1*B*). In some cases, the open probability was even lower than 0.02 ($P_o = 0.003 \pm 0.001$) with closings longer than 500 msec ($n = 2$). As described below, channels with low open probability could be opened with UDP.

Single-Channel Conductance

Wakatsuki [20] reported a frequently seen K_{ATP} channel in explants from pig coronary smooth muscle with a conductance value of 30 pS. However, the K_{ATP} channel in freshly isolated cells, from the same source and in the cell-attached configuration, was rarely seen even under conditions where activity was higher such as hypoxia [5]. Dart [5] observed unitary currents in only 5 out 120 patches. This extremely low activity has prevented the study of single channel properties, some of which we now address.

Consistent with patch clamp studies, K_{ATP} channels incorporated into lipid bilayers were not as nearly abundant as calcium-activated K channels. Nevertheless, the rate of successful incorporation of vesicles containing functional K_{ATP} channels was about three times (12 out

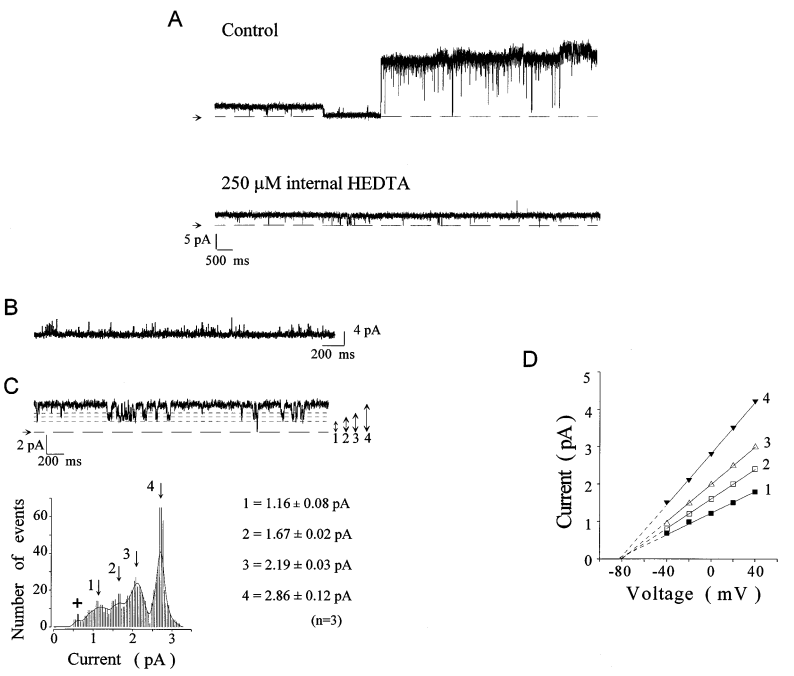

Fig. 1. K_{ATP} channel activity after reconstitution into lipid bilayers. (*A*) Simultaneous recordings of K_{ATP} and K_{Ca} channels from pig coronary smooth muscle incorporated into lipid bilayers. Activity of K_{Ca} channels was inhibited by addition of 250 μM HEDTA (2 μM Ca^{2+} free) to the internal side. (*B*) Example of low channel activity (P_o = 0.017). EGTA (1 mM) was added to the internal solution (*see* Materials and Methods) to lower free Ca^{2+} to 433 nM and reduce maxi K_{Ca} channel activity. V_H = 0 mV. (*C*) Example of high channel activity and corresponding total point histogram. In the trace, the broken lines and the double arrows mark the different (sub)conductance levels. The total point histogram shows the distribution of several current amplitudes. Arrows mark those that correspond to the K_{ATP} channel (sub)conductances (1 to 4) (mean pA values for three experiments are shown). The continuous line is the probability density function. The conductance values are 11 pS, 17 pS, 22 pS and 29 pS (5/250 mM KCl, T = 20°C, V_H = 0 mV). The plus sign (+) marks the amplitude of a small conductance channel (8 pS) insensitive to ATP. In this experiment, internal calcium was reduced to 1.3 μM by addition of 0.45 mM EGTA to the internal solution. (*D*) *I-V* curves for the four (sub)conductance levels. In this and in the following figures, arrows indicate the closed state of the channel.

of 76 experiments) as much as that reported by Dart [5] in cell-attached patches.

K_{ATP} channels were distinguished by the presence of at least four different (sub)states, which were sensitive to ATP (*see below*). The larger of these substates had a conductance of 29 pS (V_H = 0 mV); whereas the other three had conductances of 11 pS, 17 pS and 22 pS. See, for example, the broken lines in the channel illustrated in Fig. 1*C*. The total point histogram in Fig. 1*C* demonstrates the distribution of the different conductances during the recording period. The continuous line is the fitted probability density function. The fitted mean values from three different experiments, at 0 mV, were: 1.16 ± 0.08 pA, 1.67 ± 0.017 pA, 2.2 ± 0.03 pA and 2.86 ± 0.12 pA. Fig. 1*D* shows the *I-V* curves for the four different conductances. Extrapolation to zero current (broken lines) gives a reversal potential of about −80 mV for all (sub)conductance levels, which is near E_K (−98 mV).

The frequent transition detected in all recordings between the closed state and the fully open state (2.8 pA, corresponding to the 29 pS conductance) suggests that the 2.8 pA transition is the main conductance level of the K_{ATP} channels.

Sensitivity to ATP

K_{ATP} channels from β-cells, ventricular myocytes and vascular smooth muscle are characterized by their inhibition by ATP. Figure 2 shows that K_{ATP} channels from coronary smooth muscle, after incorporation into lipid bilayers, are also inhibited by micromolar concentrations of ATP. Panel *A* illustrates an example of a single K_{ATP} channel with high activity (open arrows mark substates) and its corresponding amplitude histogram. Note that the amplitude histogram could be well fitted to four Gaussian curves with values of 1.06 ± 0.1 pA, 1.63 ± 0.4 pA, 2.3 ± 0.2 pA, and 2.8 ± 0.1 pA. In the absence of ATP-Na_2, the channel was open most of the time. With the addition of 100 μM ATP-Na_2 to the internal side of the channel, the activity of all the four conductance levels was markedly decreased (Fig. 2*B*). This result supports the view that indeed all the four conductances belong to the K_{ATP} channel. The percentage of inhibition caused by this ATP concentration was near 100%. The inhibition of K_{ATP} channels by ATP-Na_2 was dose-dependent. Open probability was reduced by 23 ± 10% (n = 3) with a concentration of 50 μM ATP and was almost abolished with concentrations higher than 85 μM (n = 3). As illustrated in Fig. 3, ATP-Na_2 exerted its inhibitory effect regardless of the presence of pinacidil to open the channel.

Opening of K_{ATP} Channels by Pinacidil

To confirm the nature of this channel as a K_{ATP} channel, we decided to explore its sensitivity to pinacidil, a ''K

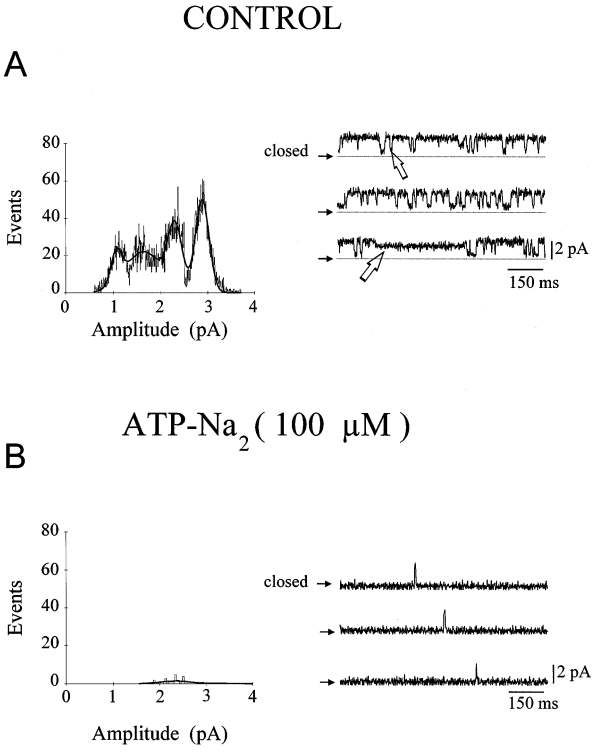

Fig. 2. Sensitivity of K_{ATP} channel reconstituted into lipid bilayers to ATP. (*A*) Amplitude histograms and corresponding records of single-channel activity. Amplitude histograms were fitted to four Gaussian curves in the control and to one Gaussian curve after addition of internal ATP. Values in control conditions are 1.06 ± 0.1 pA (% total area: 8%), 1.63 ± 0.4 pA (% total area: 37%), 2.3 ± 0.2 (% total area 23%) and 2.8 ± 0.1 (% total area 32%). Note the presence of different substates (open arrows). (*B*) Application of 100 μM ATP-Na_2 to the internal side of the channel decreased channel activity by inhibiting all (sub)conductance levels (the fitted value is 2.34 ± 0.4 pA with a corresponding % area of 2%). V_H = 0 mV.

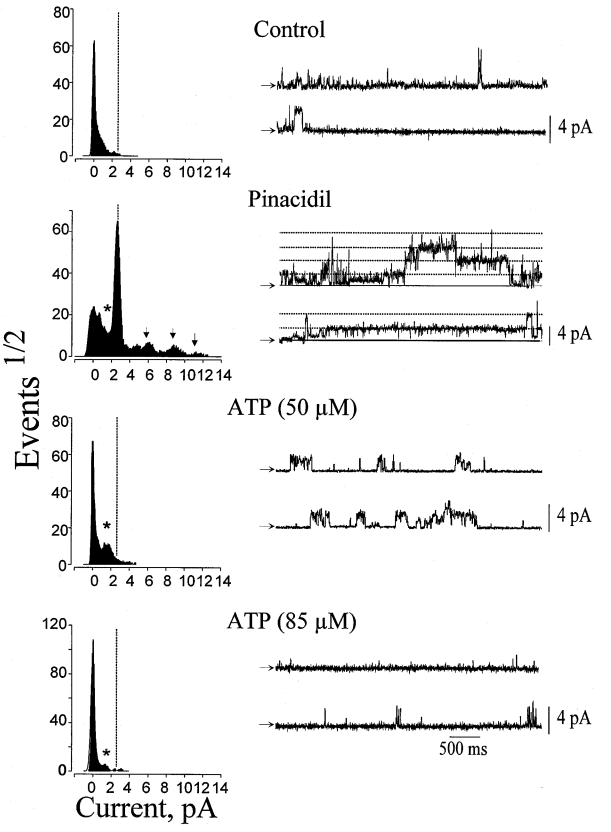

Fig. 3. Pinacidil activates K_{ATP} channels. Total point histograms and examples of channel recordings of a single experiment. Histograms were fitted to the probability density function. Broken line marks the 2.8 pA amplitude. The % of total area of this peak in control conditions was 0.4%, after pinacidil was 69%, after 50 μM ATP-Na_2 was 0.7%, and after 85 μM ATP-Na_2 was 0.1%. Application of 50 μM pinacidil activated another three channels (arrows in total point histogram). Traces clearly show at least 4 conductance steps (broken lines) and long openings induced by pinacidil. The entire experiment was performed in the presence of 1 mM UDP and 2 mM HEDTA (free $[Ca^{2+}]$ = 1.4 μM) in the internal solution. V_H = 0 mV.

channel opener'' [1]. In Fig. 3, the sequence of total point histograms and corresponding channel recordings illustrate how K_{ATP} channels, reconstituted in lipid bilayers, are affected by pinacidil and ATP-Na_2.

In control conditions, the activity of the channel(s) was low. In contrast, channel activity was markedly enhanced when we added pinacidil (50 μM) to the external solution. The histogram demonstrates that the 2.8 peak (corresponding to the 29 pS conductance) was affected to a greater extent after the application of pinacidil. This peak contributed only 0.4% to the total area in control conditions; whereas after pinacidil, in contributed 69% to the total area. In other words, the probability that the channel opened to the 29 pS level increased from 0.004 to 0.69. The record also shows that pinacidil promoted long openings (≈128 msec) of this 29 pS state and uncovered the presence of other channels (at least three) that opened to the same conductance level. This is also quantified in the total point histogram, showing additional peaks at 5.9 pA, 8.7 pA and 11 pA (arrows), which closely correspond to multiples of the 2.8 pA peak. The histogram shows an additional peak at 4.3 pA that may represent the main conductance level in addition to a substate. These results support the idea that pinacidil stabilizes the channel in the larger conductance value (2.8 pA peak, 29 pS).

The pinacidil-activated potassium current was inhibited by the subsequent addition of internal ATP-Na_2 (50 μM). By further increasing the concentration of ATP to 85 μM, the K_{ATP} current was almost abolished. Notice in the histograms of Fig. 3 that the component corresponding to the (sub)conductance levels (asterisk) is also diminished by ATP, supporting the idea that they belong to the K_{ATP} channels.

Similar results were obtained in three other experiments. The addition of 10 μM pinacidil enhanced the amplitude area from 0.016 ± 0.019 to 0.41 ± 0.13 (*n* =

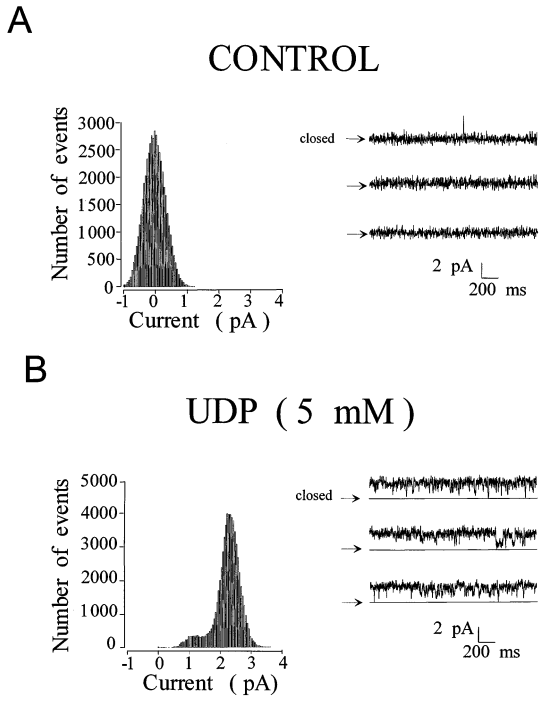

Fig. 4. UDP increases K_{ATP} channel activity. (*A*) Example of single K_{ATP} channel records and corresponding total point histograms obtained in control condition. P_o was 0.0008. (*B*) K_{ATP} current was activated after addition of 5 mM UDP and 1 mM $MgCl_2$ to the internal side. The open probability increased more than one thousandfold to a final P_o of 0.93. $V_H = 0$ mV.

2), and in another channel, 30 μM pinacidil increased the K_{ATP} activity from 0.007 to 0.154.

Rescue of K_{ATP} Channel Activity with UDP

K_{ATP} channel activity after incorporation was usually not very high. However, we were able to increase ATP-sensitive potassium current by adding UDP (uridine diphosphate) and $MgCl_2$ to the internal side of the channel. In this respect, K_{ATP} channels from coronary smooth muscle are similar to those of ventricular cells from guinea pig [18]. This property is exemplified in Fig. 4, where 5 mM internal UDP, in the presence of 1 mM $MgCl_2$, activated the K_{ATP} channel. In control conditions, only one event was observed during the whole recording ($P_o = 0.0008$), whereas in the presence of UDP the open probability increased to 0.93. Also in this case, the total point histogram indicates that UDP opened the channel preferentially to the 29 pS level.

Not only was UDP able to restore the channel activity of channels with very low P_o, but it was also capable of ''rescuing'' completely inactive channels. In three experiments, no activity was recorded in control conditions, but after adding UDP, the open probability increased to 0.43 (10 mM UDP, 2 mM $MgCl_2$), 0.034 and 0.06 (2.5 mM UDP, 500 μM $MgCl_2$). The presence of UDP did not prevent ATP from inhibiting the channel. UDP-induced K_{ATP} current was also completely abolished after the addition of 250 μM of internal ATP-Na_2 (*not shown*).

Discussion

In this work, we describe an ATP-sensitive K^+ channel from coronary smooth muscle reconstituted in lipid bilayers. This channel is characterized by several current levels, at least four (1.2 pA, 1.6 pA, 2.2 pA and 2.8 pA; at 0 mV) equally spaced at a value of 0.4 pA or multiples of this number.

The assumption that the different conductance levels belong to the K_{ATP} channel is supported by the following facts: (i) All the four conductance levels were sensitive to ATP (*see* Figs. 2 and 3); (ii) The presence of the conductance levels was independent of the activity of the channel upon incorporation, suggesting that they belong to the same channel. Furthermore, in all incorporations of the K_{ATP} channel, we observed the four conductances; (iii) All the conductance levels assigned to the K_{ATP} channel had the same reversal potential (E_K) (Fig. 1*D*), indicating that all of them were permeable to potassium; (iv) We observed direct transition from one conductance level to another; and (v) The possible presence of four different channel proteins with different conductance levels should not only produce larger or intermediate conductance values due to their simultaneous openings, but also greater number of step levels. We never observed these different levels, even at very high open probability.

The presence of several K_{ATP} conductance levels in our records, can be explained by a fast flickering of the K_{ATP} channel filtered by our recording system; or else by the presence of (sub)conductance states. If the latter is the case, the regular conductance levels may be explained by considering the channel as an oligo-channel complex composed of several identical pores or as a single channel formed by homologous subunits assembled together around a central pore [4]. At present, we cannot discriminate between these two possibilities.

The molecular identity of K_{ATP} channels from pancreatic β cells has recently been resolved [7]. In β cells, the K_{ATP} channel is composed of at least two different subunits: the BIR, a member of the inward rectifier potassium channel family, and the sulfonylurea receptor (SUR), a member of the ATP-binding cassette superfamily. Nevertheless, mRNA of these two proteins was not detected in smooth muscle tissues using Northern blot analysis. It is possible that the SUR from β cells differs from the one in smooth muscle tissues, since glibenclamide is known to inhibit K_{ATP} channels in smooth

muscle including those from pig coronary [5, 11, 16, and one experiment in our reconstituted channels]. It seems therefore, that the molecular nature of K_{ATP} channels in smooth muscle differs from the β cell channel complex. This hypothesis is consistent with the fact that K_{ATP} channels from β cells have different conductance than those reported here.

The calculated main conductance for K_{ATP} channels from pig coronary reconstituted in lipid bilayers is 29 pS with 5/250 mM KCl. This value is in agreement with those reported for coronary smooth muscle measured in freshly isolated cells [5] and in tissue explants in culture [20], and differs from those encountered in other tissues. ATP-sensitive K^+ channels can be regrouped in three different classes (i) K_{ATP} channels with very large conductance (≥100 pS), present in human ventricular myocytes, rat tail artery and rat or rabbit mesenteric artery [2, 6, 16]; (ii) K_{ATP} channels characterized by low conductance (≤30 pS), present in urinary bladder, pig coronary artery and rabbit mesenteric artery [3, 12, 20]; and (iii) K_{ATP} channels of ≈70 pS, as those from guinea pig ventricular cells [9] and the recently cloned complex of pancreatic β cells [7]. However, the physiological significance of the heterogeneity of the K_{ATP} single channel conductances in different tissues is unclear.

Our results indicate that the K_{ATP} channels reconstituted in lipid bilayers share pharmacological properties as those found in other K_{ATP} channels from various tissues. In fact, K_{ATP} channels in lipid bilayers were activated by micromolar concentrations of pinacidil, which promoted very long openings, were inhibited by micromolar concentrations of ATP, and were activated by millimolar concentrations of UDP.

Channel activity frequently decreased immediately after incorporation. In these cases, we were able to restore the potassium current by adding UDP and Mg^{2+} to the internal solution. This nucleotide was also useful for evoking potassium currents when no activity was observed in the control. It has been assumed that rundown of K_{ATP} channels from heart is due to dephosphorylation of the channel, and that UDP opens dephosphorylated K_{ATP} channels [18]. Thus, it is possible that the different open probabilities we observed are a consequence of the phosphorylation status of the reconstituted channels.

The incidence of K_{ATP} channels incorporated into lipid bilayers was low. This result can at least be explained by: (i) the presence of few K_{ATP} channels in pig coronary smooth muscle; (ii) the loss of important factors during preparation and reconstitution that makes active channels; or (iii) modification of the channel complex. We support the first explanation, since the same type of frequency has been reported in studies using single cells and hypoxia-activated channels [5]. Furthermore, our results indicate that the main properties of the K_{ATP} channel remain intact after incorporation into lipid bilayers.

In conclusion, we have demonstrated that the K_{ATP} channel from pig coronary artery can be functionally incorporated into lipid bilayers. This method will be helpful to better understand the interactions between these important channels and vasoactive drugs.

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