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The inhibitory effect of propranolol on ATP-sensitive potassium channels in neonatal rat heart

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1 Whole cell and single channel recordings of ATP-sensitive K^+ current ($I_{K,ATP}$) were carried out in ventricular myocytes isolated from neonatal rat hearts.

2 (±)-Propranolol, a commonly used β-blocker, inhibited the whole cell $I_{K,ATP}$ in a concentration-dependent manner with a half-maximal concentration (IC_{50}) of 6.7±1.4 μM, whereas it blocked the inward rectifier K^+ current ($I_{K,1}$) only at much higher concentrations (IC_{50} = 102.4±20.2 μM). The inhibition was time- and voltage-independent.

3 In the outside-out patch configuration, (±)-propranolol inhibited $I_{K,ATP}$ (IC_{50} = 9.8±2.9 μM) by decreasing the open probability of the channel without inducing additional noise in the open-channel current or a decrease of single channel conductance. The single channel current of $I_{K,1}$ was also blocked by (±)-propranolol in the same way as $I_{K,ATP}$.

4 (+)-Propranolol, an optic isomer having no β-blocking effect, inhibited $I_{K,ATP}$ (IC_{50} = 5.8±1.0 μM), whilst atenolol, a selective β_1-blocker had no effect. Neither GDPβS (1 mM) nor GTPγS (200 μM) included in the pipette solution modulated the inhibitory effect of (±)-propranolol.

5 We concluded that the inhibitory effect of (±)-propranolol was not via the β-adrenergic signal transduction pathway, but by direct inhibition of $I_{K,ATP}$ channels.

Keywords: Propranolol; β-blocker; ATP-sensitive K^+ channel; patch clamp; rat heart

Introduction

The functional adenosine 5′-triphosphate (ATP)-sensitive K^+ channel ($I_{K,ATP}$ channel) is composed of at least two distinct subunits; sulphonylurea receptors (SURs, Aguilar-Bryan *et al*., 1995; Inagaki *et al*., 1996; Isomoto *et al*., 1997) and inwardly rectifying potassium channels (KIR6.1: Inagaki *et al*., 1995; KIR6.2: Inagaki *et al*., 1996; Takano *et al*., 1996). The pharmacological modulation of native $I_{K,ATP}$ channel activity has so far been accomplished by various drugs targeting SURs. Sulphonylureas, such as glibenclamide and tolbulamide bind to SUR and inhibit functional $I_{K,ATP}$ channels. It is also believed that K^+ channel openers such as pinacidil and nicorandil also bind to SURs. However, no pharmacological tool to modify pore subunits KIR6.1 or KIR6.2 is available. Although the native $I_{K,ATP}$ channel is blocked by classical non-organic K^+ channel blockers such as Ba^{2+} and Cs^+ (Quayle *et al*., 1988; Takano & Ashcroft, 1995), the blocking effect is not selective for the $I_{K,ATP}$ channel among the family of the inwardly rectifying K^+ channels (Hille, 1992).

In addition to the above compounds directly targeting $I_{K,ATP}$ channel, native $I_{K,ATP}$ channel activity could be modulated indirectly by oubain and β-adrenoceptor agonists. It is postulated that these drugs modulate the consumption of ATP by Na^+/K^+ ATPase and adenylate cyclase, and thereby change local ATP concentration under the surface membrane (Schackow & Ten Eick, 1994; Priebe *et al*., 1996; Horie *et al*., 1996). During our study on the relationship between β-adrenoceptor stimulation and $I_{K,ATP}$ channel activity in neonatal rat heart, we found that $I_{K,ATP}$ was inhibited by propranolol, the most commonly used β-adrenoceptor antagonist, at micromolar concentrations. In the present study, we examined the inhibitory effect of propranolol on the $I_{K,ATP}$ and demonstrated that the block is not mediated by the β-adrenoceptor, but the channel ionophore may be directly blocked by propranolol.

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Methods

Preparation of cardiac myocytes

The neonatal rats (post-natal day 1–3) were killed by decapitation and hearts were excised out. After the blood had been washed out with control physiological saline, the hearts were immersed in nominally Ca^{2+}-free solution for several minutes. After spontaneous contractions had ceased, the ventricle was minced in the nominally Ca^{2+}-free solution containing 0.5 mg ml^{-1} collagenase (Wako, Osaka Japan) and 0.1 mg ml^{-1} thermolysin (Sigma, St. Louis) and then stirred gently at 37°C for ~60 min. Subsequently the tissue was incubated for 30 min in KB solution (Isenberg & Klöcker, 1982) before the myocytes were dissociated. The dissociated myocytes were used within 8 h after their isolation. In some experiments, cardiac myocytes were isolated from adult rat hearts by the procedure described previously (Kakei *et al*., 1985; Isenberg & Klöcker, 1982), and were used for a comparison with the neonatal myocytes.

Solutions and drugs

The composition of control saline was (in mM): NaCl 140, KCl 5.4, NaH_2PO_4 0.33, $CaCl_2$ 1.8, $MgCl_2$ 0.5, glucose 5,5 and N-2-hydroxyethylpiperazine N′-2-ethanesulphonic acid (HEPES)5; the pH was adjusted to 7.4 with NaOH. The glucose was omitted when it was used as the bath solution in recording $I_{K,ATP}$. The nominally Ca^{2+}-free bath solution had the same composition, except $CaCl_2$ was omitted. The modified KB solution contained: taurine 10, glutamic acid 70, KCl 25, KH_2PO_4 10, glucose 11, ethylene glycol-bis(b-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) 0.5 and HEPES 10 (pH = 7.3 with KOH). The bath solution used in the outside-out recording of the inward rectifier K^+ current ($I_{K,1}$) contained: KCl 140, HEPES 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5; the pH was adjusted to 7.4 with NaOH.

The ATP-free pipette solution used in the whole-cell or outside-out patch recording of $I_{K,ATP}$ contained: aspartic acid 130, KCl 10, $MgCl_2$ 1, EGTA 5 and HEPES 5, (pH = 7.4 with KOH). In some experiments, 200 μM GTPγS or 1 mM GDPβS was added. The pipette solution used in the whole-cell or outside-out patch recording of the $I_{K,1}$ contained: aspartic acid 120, KCl 10, Mg-ATP 5, Na_2-creatine phosphate 5, EGTA 5, $CaCl_2$ 1 and HEPES 5 (pH 7.4 with KOH).

(±)-Propranolol hydrochloride, (+)-propranolol hydrochloride, (±)-atenolol, alprenolol hydrochloride and bromoacetyl alprenolol menthane (BAAM) were purchased from RBI (MA, U.S.A.), Iodoacetic acid (IAA) were from Sigma. All drugs were dissolved in the bath solution and the pH was readjusted.

All experiments were carried out at room temperature (22–25°C). The Y-tube apparatus was used to enable rapid exchange of bathing solutions (Ogata & Tatebayashi, 1991; Takano & Noma, 1997).

Voltage clamp experiments and data analysis

The whole-cell voltage clamp (Hamill *et al*., 1981) was conducted by use of a patch-clamp amplifier (Axopatch200B, Axon, CA U.S.A.). The tip resistance of patch pipettes ranged from 6 to 10 MΩ for neonatal myocytes and from 3 to 5 MΩ for adult myocytes. Recorded membrane potentials were corrected for the liquid junction potential (assumed to be 10 mV) between the low Cl^- pipette solution and the control bath solution. The whole-cell *I*-V relationship was measured by applying ramp or square pulses from the holding potential of −50 mV. The ramp pulses (±200 mV s^{-1}) were applied every 6 s. Initial positive slope from −50 to +50 mV was followed by a negative slope to −150 mV, and the *I*-V relationship was determined from this negative limb of the ramp pulse. Data were recorded on both an on-line computer (PC-98, NEC, Tokyo Japan) and digital tape recorder (RD-120, TEAC, Tokyo Japan).

The single channel current was recorded in the outside-out configuration of the patch-clamp technique (Hamill *et al*., 1981). The exterior of the patch pipette was made hydrophobic by applying N,N-dimethyltrimethylsilylamine (Fluka, Switzerland) to reduce the electrical capacitance. The resistance of electrode was 4 - 6 MΩ. The current signal was stored on a digital tape recorder and played back for computer analysis with pClamp software (Axon). The signal was filtered at 2 kHz and digitized at 1 kHz by use of an A/D converter (Digidata 1200 interface, Axon).

Results are expressed as mean ± s.e. and statistical analyses were performed by means of unpaired or paired Student's *t* test with $P<0.05$ being considered significant. To obtain the concentration-inhibition relationship, the mean percentage inhibitions were fitted to the Hill equation:

$$\%\text{inhibition} = 100/\{+(IC_{50}/[\text{drug}])^{n_H}\},$$

where IC_{50} is the drug concentration causing half-maximal inhibition, and n_H the Hill coefficient.

Results

Inhibition of the whole-cell $I_{K,ATP}$ by propranolol in neonatal myocytes

Since the $I_{K,ATP}$ channel may be preferentially sensitive to ATP-production via glycolysis rather than the oxidative phosphorylation (Weiss & Lamp, 1989), $I_{K,ATP}$ was activated by superfusing neonatal ventricular myocytes with the glucose-free bath solution containing 5 mM IAA, a glycolysis inhibitor. After a delay of 3–4 min during the IAA superfusion, the membrane conductance gradually increased, as measured by applying the ramp pulse every 6 s (Figure 1). The *I*-V curve obtained before the IAA treatment showed a slight but clear inward rectification due to $I_{K,1}$ at potentials more negative than −90 mV (curve (a) in Figure 1B). At the maximal activation of IAA-induced current, the *I*-V curve crossed the control *I*-V curve at the expected reversal potential (−85 mV) for K^+. Furthermore, the IAA-induced current was inhibited by 1 or 10 μM glibenclamide (g,h) in Figure 1A and Figure 1C). Thereby, we concluded that the IAA-induced current was due to the activation of the $I_{K,ATP}$ channel (Xie *et al*., 1997). The activation of $I_{K,ATP}$ might be slightly underestimated by the inhibition of $I_{K,1}$ during the metabolic inhibition (Kakei *et al*., 1985; Xie *et al*., 1997). Unexpectedly the IAA-induced current, $I_{K,ATP}$ was blocked by various concentrations of propranolol in a dose-dependent manner (c,d,e) in Figure 1A and Figure 1C). In the *I*-V diagram, propranolol equally scaled down the magnitude of current at all membrane potentials examined, showing that the inhibitory effect of propranolol was voltage-independent.

The voltage-independent block of $I_{K,ATP}$ was further examined by recording the whole cell current induced by square pulses. In the control condition, no time-dependent current change was observed except for the time-dependent inactivation of $I_{K,1}$ during hyperpolarizing pulses (Figure 2a, Control). The IAA-induced current did not show clear time-dependent change which was typical for the whole cell $I_{K,ATP}$. The application of propranolol did not induce an obviously time-dependent current change at all the potentials examined.

To measure the dose-response relationship, the amplitude of the IAA-induced current at −40 mV was measured before and during the application of various concentrations of propranolol. The degree of inhibition (%inhibition) was determined according to,

$$\%\text{inhibition} = (I_{cont} - I_{drug})/(I_{cont}),$$

where I_{cont} is the amplitude of the IAA-induced current immediately before the application of a given concentration of IAA, and I_{drug} is during the drug application. Figure 2b shows the relationship between %inhibition and drug concentration. The best fit of Hill equation to the mean data ($n>5$) gave an IC_{50} of 6.7 ± 1.4 μM and a n_H of 1.16 ± 0.38.

The blocking effect was also examined for other β-adrenoceptor blockers. The β_{1+2}-blocker, alprenolol 10 μM inhibited the whole cell $I_{K,ATP}$ by 69 ± 5% and the selective β_2-blocker, BAAM 10 μM blocked $I_{K,ATP}$ by 70 ± 6%. However, atenolol, a β_1 selective blocker, failed to block $I_{K,ATP}$.

Effects of propranolol on whole cell $I_{K,1}$

Propranolol could also inhibit $I_{K,1}$, but only at much higher concentrations. In the experiment shown in Figure 3a, the whole cell $I_{K,1}$ current was recorded by applying hyperpolarizing pulses. Clearly 300 μM propranolol inhibited $I_{K,1}$ and no additional time-dependent change of $I_{K,1}$ was found during hyperpolarization. The inhibitory effect was readily reversible. In the *I*-V curve (Figure 3b), $I_{K,1}$ was inhibited by 70–80% by 300 μM propranolol at all the potentials examined.

The dose-%inhibition response relationship was measured at −110 mV. The best fit of the Hill equation to the mean data ($n>5$) gave an IC_{50} of 102.4 ± 20.2 μM, which was ~15 times higher than that for $I_{K,ATP}$. The n_H was 1.15 ± 0.08.

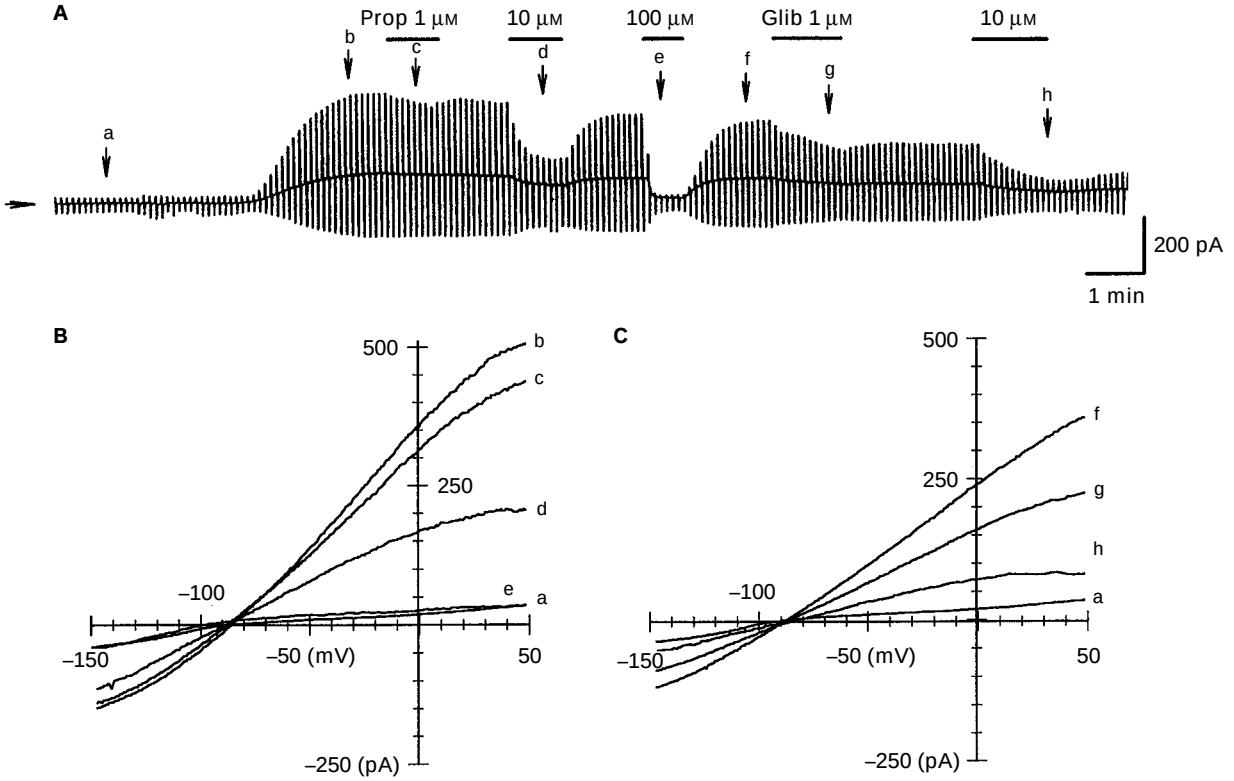

Figure 1 The inhibitory effect of propranolol on the whole-cell ATP-sensitive potassium current in the neonatal rat ventricular myocyte. (A) Chart recording of the whole cell current. The vertical deflections are due to ramp pulses applied every 6 s from the holding potential of −50 mV. The arrow to the left of trace indicates zero current level. (B, C) *I*-V relations obtained at times (a–h) indicated by arrows in (A). The perfusion of 5 mM iodoacetic acid was started about 1 min before the beginning of this segment of recording. The application of 1, 10, 100 mM propranolol (Prop) and 1, 10 mM glibenclamide (Glib) are indicated by the bars above the current recording in (A).

Effects of propranolol on single $I_{K,ATP}$ *and* $I_{K,1}$ *channels*

The block of $I_{K,ATP}$ was examined at the single channel level. In Figure 4, multi-channel recordings of $I_{K,ATP}$ were obtained at 0 mV in the outside-out configuration with 150 mM $[K^+]_i$ and 5.4 mM $[K^+]_o$. The *I*-V relationship showed a weak inward rectification and the single channel conductance was 22 pS at around −100 mV, which was in good agreement with the measurements obtained under comparable ionic conditions in rabbit or guinea-pig cardiac myocytes (Noma, 1983) (not shown). The channel activity was blocked by glibenclamide, confirming that the channel events were attributable to $I_{K,ATP}$ channels. The application of 1 μM propranolol markedly suppressed the channel activity, almost to the same extent as 10 μM glibenclamide. In the presence of 10 μM propranolol, the channels were mostly in the blocked state (bottom trace in Figure 4a).

It was clear from Figure 4a, that no obvious increase of noise (flickery block) was noticed in the open channel current, and the amplitude histograms shown in Figure 4b clearly resolved a unitary amplitude of 1.5 pA both in the control and during the application of propranolol. Thus, it seems unlikely that propranolol acts as an open channel blocker with very fast kinetics.

The dose-inhibition relationship (Figure 5b) obtained by calculating the mean patch current at different doses of propranolol was well fitted with the Hill equation with an IC_{50} of 9.8 ± 2.9 μM and a n_H of 0.94 ± 0.22, which were in good agreement with the data from the whole cell current experiments.

The inhibitory effect of propranolol on $I_{K,1}$ was also examined at the single channel level. In the outside-out patch recordings in which the symmetrical high K^+ solutions were used, the single channel conductance was approximately 32 pS. The application of 100 μM propranolol markedly decreased the open probability of the channel; the mean patch current decreased from -2.58 ± 0.92 pA to -1.63 ± 0.74 pA ($n = 5$) without a decrease in the unitary amplitude (1.30 ± 0.05 pA at −40 mV). No additional noise was observed in the open-channel currents in the presence of propranolol (data not shown). These findings suggest that the members of the inwardly rectifying K^+ channel family were inhibited by propranolol by a similar mechanism, but with different sensitivities.

The inhibition of $I_{K,ATP}$ *was not via a β-receptor and G-proteins*

The involvement of β-adrenoceptor-dependent reactions was examined in the propranolol block of $I_{K,ATP}$. It is well known that (+)-propranolol, the optical isomer of (−)-propranolol, does not block β-adrenoceptors. However, (+)-propranolol was almost equally potent as (±)-propranolol in inhibiting the whole cell $I_{K,ATP}$. The dose-inhibition curve (Figure 6) determined an IC_{50} of 5.8 ± 1.0 μM and n_H of 0.92 ± 0.14, which were comparable with those for (±)-propranolol, thus excluding the involvement of β-adrenoceptor-dependent reactions in this effect of propranolol.

In support of the above view, the inhibitory effect of propranolol was not affected by blocking the activity of the

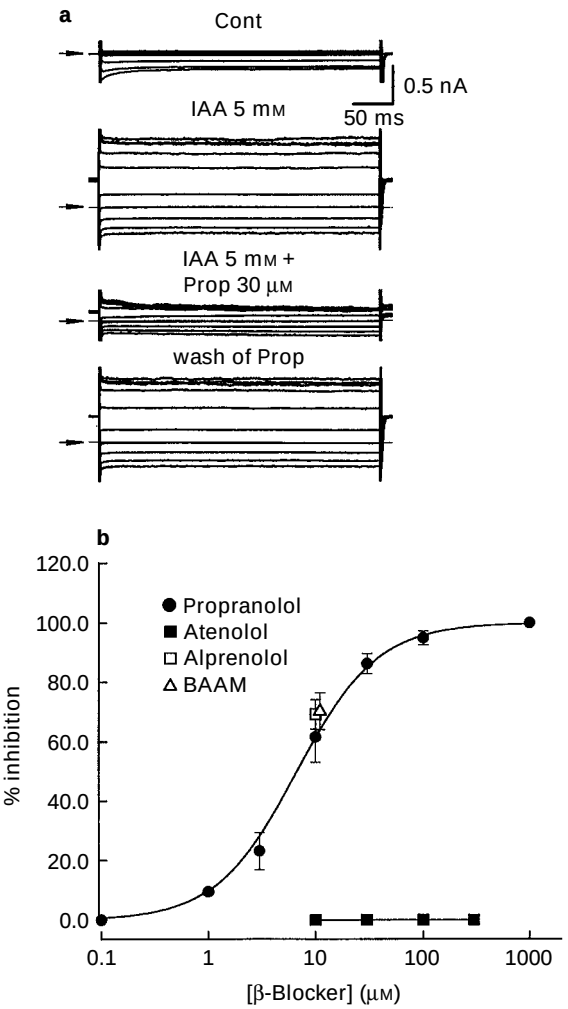

Figure 2 (a) Voltage and time-independence of the blockage on $I_{K,ATP}$ by propranolol. The currents elicited by applying a set of voltage pulses (−150–50 mV) in 20 mV steps from a holding potential of −50 mV were superimposed. The recordings were obtained in the control, after the activation by iodoacetic acid (IAA), during the block by propranolol and after washing out of propranolol. The arrows to the left of traces indicate zero current level. (b) Concentration-inhibition relationships for the effects of various β-blockers on $I_{K,ATP}$ in neonates. The % inhibition is plotted against various concentrations of propranolol. Each symbol represents the mean value ($n>5$). Vertical lines indicate s.e. The smooth curve for propranolol was the best fit to the Hill equation, as described in the Methods; IC_{50} for propranolol = 6.7 μM.

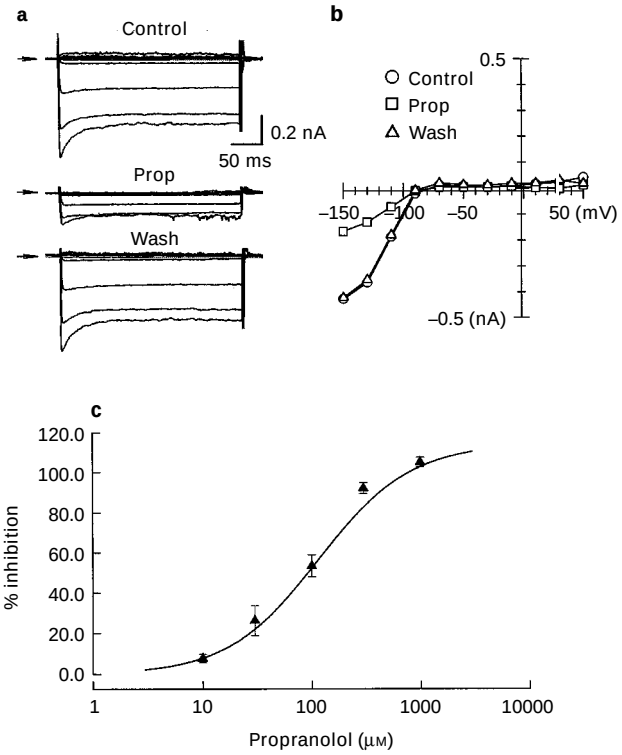

Figure 3 The inhibition of $I_{K,1}$ at a higher concentration of propranolol in neonatal rats. (a) Representative whole-cell $I_{K,1}$ recorded in control in the presence of 300 μM propranolol (Prop) and after washing out propranolol (Wash). The current traces were obtained by subtracting the background currents, which were recorded after suppressing $I_{K,1}$ by superfusing the myocytes with the K^+-free solution. The arrow to the left of trace indicates zero current level. (b) Steady-state *I*-V relationships for the same cell as in (a). (c) Concentration-inhibition relationship of propranolol effect on $I_{K,1}$. Each symbol represents the mean value ($n>5$). Vertical lines indicate s.e. The smooth curve is the best fit to the Hill equation described in the text; IC_{50} = 102.4 μM.

GTP-binding protein. In the experiment shown in Figure 6b, the pipette solution contained 1 mM GDPβS which completely inhibits GTP-binding proteins. Even under this condition, 10 μM propranolol clearly inhibited $I_{K,ATP}$. The magnitude of inhibition was 62±10%, which was not significantly different from the control experiments (61±9%, Figure 2b). Activation of GTP binding proteins by an additional 200 μM GTPγS in the pipette solution also failed to modify $I_{K,ATP}$ block by propranolol. These results strongly suggested that neither β-adrenoceptors nor GTP-binding proteins are involved in this inhibitory effect of (±)-propranolol on $I_{K,ATP}$.

Effects of propranolol in adult ventricular myocytes

Propranolol also inhibited $I_{K,ATP}$ in the adult myocytes. However, the inhibition of the whole cell $I_{K,ATP}$ required more than 10 times higher concentration (IC_{50} of 73.1±4.3 μM, data not shown).

Discussion

Direct block of $I_{K,ATP}$ channel by propranolol

When we first observed the block of $I_{K,ATP}$ by propranolol, two alternative mechanisms were proposed. One was that the channel closure was induced indirectly by an increase in the ATP concentration under the cell membrane, and the other was a direct channel block by the drug. According to the 'empty adrenoceptor' hypothesis (Mewes *et al*., 1993), adenylate cyclase might have a basal activity in the absence of β-adrenoceptor agonists and the binding of propranolol to the β-adrenoceptor might prevent this basal activity of adenylate cyclase. If the local concentration of ATP ($[ATP]_i$) was slightly increased by this mechanism, $I_{K,ATP}$ channel activity might be depressed. Such a modulation of the $I_{K,ATP}$ channel activity through a change in $[ATP]_i$ has been suggested as a mechanism underlying the channel activation induced by β-adrenoceptor stimulation (Schackow & Ten Eick, 1994) and underlying the effect of stimulating or inhibiting the Na^+/K^+ pump on channel activity (Priebe *et al*., 1996). However, the findings in the present study clearly indicate that neither the β-adrenoceptor nor the GTP-binding protein is involved in the

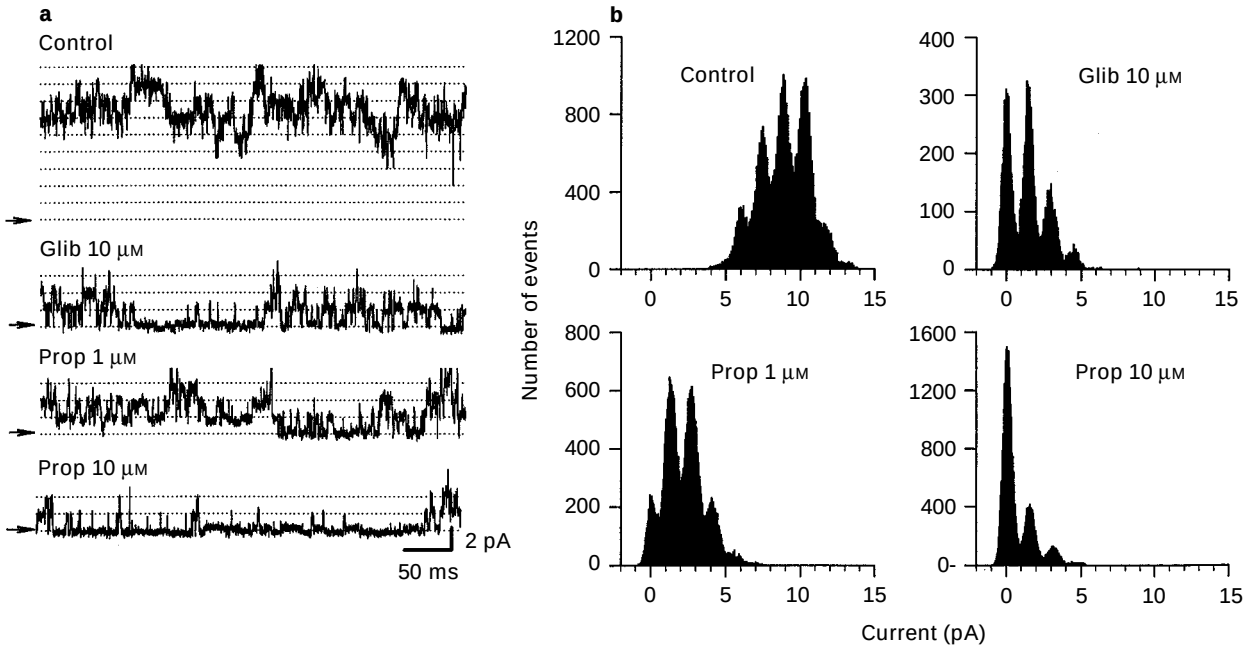

Figure 4 Decrease of the open probability of single $I_{K,ATP}$ channels induced by propranolol. (a) Expanded current traces at the various conditions indicated. Zero current level is indicated by the arrow in each trace. The dotted line indicated the multiple opening levels of the channels. (b) Histograms gained from current traces shown in (a).

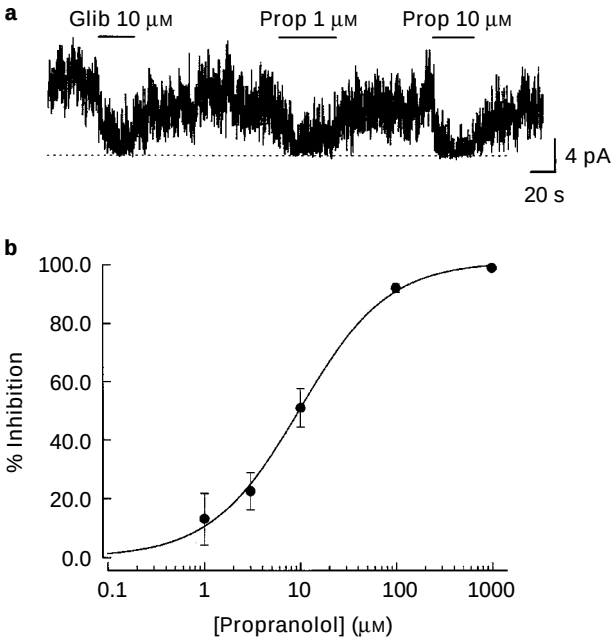

Figure 5 Propranolol inhibition of single $I_{K,ATP}$ channels measured in outside-out mode. (a) Slow chart record of single channel currents. The dotted line represents the current level when the channel is closed. The application of 10 μM glibenclamide, 1 μM and 10 μM propranolol are indicated above the chart record. (b) Concentration-inhibition relationship of propranolol effect on single $I_{K,ATP}$ channels. Values of inhibited mean patch currents measured at different propranolol concentrations were normalized to those in the control solution in individual patches. Each symbol represents the mean value ($n>5$). Vertical lines indicate s.e. Smooth curves were best fits to the Hill equation; $IC_{50}=9.8$ μM.

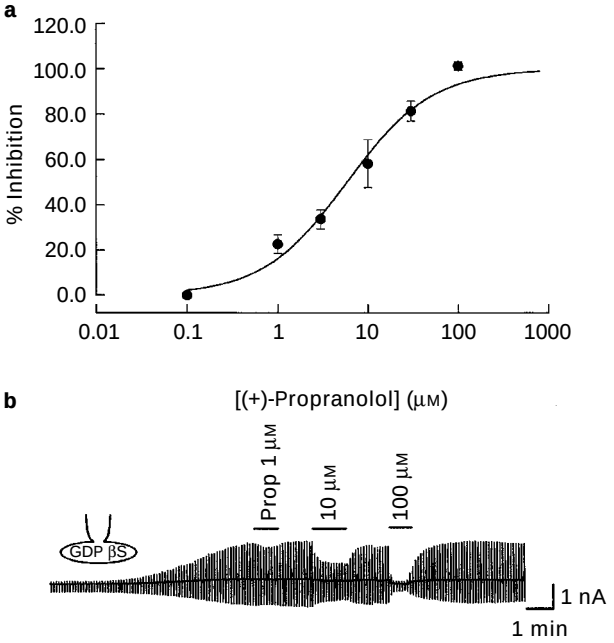

Figure 6 Blockade of $I_{K,ATP}$ was independent of β-receptors or G-proteins. (a) Concentration-inhibition relation for (+)-propranolol (a non-β-blocker). (b) The chart record of whole-cell current when the cell was internally dialysed with 1 mM GDPβS. Normal bathing solution containing 5 mM indoacetic acid (IAA) was perfused from the beginning of the recording. The applications of 1, 10 and 100 μM propranolol are indicated by the bars above the current recording.

propranolol block. The channel block in the absence of intracellular ATP (outside-out patch experiments) further supported this view.

The cardiac $I_{K,ATP}$ channel is composed of at least two classes of molecules; SUR and KIR6.2. In the present study, it was impossible to address readily which molecule was the target of propranolol. However, the block of $I_{K,1}$ channel, which is composed of KIR2.1 without SUR, may suggest that propranolol blocked the ionophore subunit. Basic structures of

KIR2.1 and KIR6.2 are closely related. In particular, the amino acid sequence of the pore-forming region (H5) share 76% identity (Isomoto *et al*., 1997). Furthermore, the single channel analysis in the present study revealed that the mode of inhibition of $I_{K,ATP}$ and $I_{K,1}$ is very similar. The inhibition was independent of the membrane potential. Neither additional noise in the open channel current nor a decrease in the single channel conductance was induced by the application of propranolol. Therefore, it seemed most likely that propranolol bound to and blocked directly the ionophore formed by the KIR subunits.

Pharmacological significance of the inhibitory effect of propranolol

β-Adrenoceptor blockers are widely used for the treatment of cardiovascular diseases, such as ischaemia, arrhythmia and hypertension. The doses of propranolol that are generally used clinically seem to have no direct effect on $I_{K,1}$. However, 1 μM propranolol as well as alprenolol and BAAM, but not atenolol, inhibited the $I_{K,ATP}$ channel by about 10% in both whole-cell and outside-out recording. If this finding is applicable to human heart, the inhibitory effect on $I_{K,ATP}$ should be considered as an important indicator for the choice of different β-blockers. Blockade of $I_{K,ATP}$ channels may reverse the shortening of the action potential during ischaemia and thereby increase the magnitude of contraction. This effect is the opposite of the negative inotropic effect expected when β-adrenoceptor blockers are used. In the case of arrhythmia enhanced by the shortening of the action potential, blockade of the $I_{K,ATP}$ channel as well as inhibition of the L-type Ca^{2+} channel by the β-adrenoceptor blockers may be beneficial in treating the arrhythmia.

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