Activation of muscarinic K^+ current by β-adrenergic receptors in cultured atrial myocytes transfected with β_1 subunit of heterotrimeric G proteins

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Abstract Muscarinic K^+ channels ($I_{K(ACh)}$) in native atrial myocytes are activated by βγ subunits of pertussis toxin (Ptx)-sensitive heterotrimeric G proteins coupled to different receptors. βγ subunits of Ptx-insensitive G_s, coupled to β-adrenergic receptors, do not activate native $I_{K(ACh)}$. In atrial myocytes from adult rats transfected with rat brain β_1 subunit $I_{K(ACh)}$ can be activated by stimulation of β-adrenergic receptors using isoprenaline. This effect is insensitive to Ptx. These findings demonstrate for the first time promiscuous (Ptx-insensitive) coupling of $G_{s\beta\gamma}$ to GIRK channels in their native environment.

Key words: Potassium channel; G protein; Beta receptor; GIRK; Cardiac cell

1. Introduction

Heterotrimeric G proteins coupled to 7-helix receptors dissociate into an α subunit and a dimeric βγ subunit upon receptor activation. [1]. Both subunits can affect target molecules. The first identified target of βγ subunits was the muscarinic K^+ ($K_{(ACh)}$) channel expressed in supraventricular cells of the heart [2,3] which is composed of two different subunits (GIRK1/GIRK4) [2,4]. Opening activity of $K_{(ACh)}$ channels in atrial myocytes is increased by direct interaction of GIRK1/GIRK4 with βγ subunits (see [2] for review) of pertussis toxin (Ptx)-sensitive G proteins coupled to different receptors, such as M_2, A_1 [5] and a novel sphingolipid receptor [6,7]. It is generally assumed that βγ dimers derived from Ptx-insensitive G proteins, such as G_s, coupled to β-adrenergic receptors, do not activate $K_{(ACh)}$ channels in atrial cells [2]. This contrasts with two findings. (i) Various combinations of different β and γ monomers are rather unselectively capable of activating $K_{(ACh)}$ channels from the cytosolic side of inside-out membrane patches from atrial myocytes [8]. (ii) If expressed in *Xenopus* oocytes, $K_{(ACh)}$ channels can be activated in a Ptx-insensitive fashion by co-expressed β-adrenergic receptors [9]. This raises the question of how targeting of $K_{(ACh)}$ channels by $G_{s\beta\gamma}$ is suppressed in native atrial myocytes. We hypothesized that βγ sequestering by other proteins could serve as one mechanism how $G_{s\beta\gamma}$ are prevented from interacting with a 'wrong' target in a native cell. In this case an antagonist for binding of dimeric βγ to other proteins should impair target specificity of β-adrenergic receptors.

In the present study we overexpressed a β subunit (β_1) by transient transfection in cultured rat atrial myocytes using co-transfected eGFP as reporter protein [10]. It has been shown that the binding site of βγ dimers to target proteins is located on the β subunit. In β_1 transfected myocytes we found, apart from a reduction in $I_{K(ACh)}$, activated by M_2 receptors, a significant Ptx-insensitive activation of $I_{K(ACh)}$ by the β-receptor agonist isoprenaline. This study for the first time demonstrates activation of $K_{(ACh)}$ channels by βγ subunits derived from G_s in native myocytes.

2. Materials and methods

2.1. Isolation and culture of atrial myocytes

Wistar rats of either sex (around 200 g) were anesthetized by i.v. injection of urethane (1 g/kg). The chest was opened and the heart was removed and mounted on the cannula of a sterile Langendorff apparatus for coronary perfusion at constant flow. The method of enzymatic isolation of atrial myocytes has been described elsewhere [11]. The culture medium was FCS-free bicarbonate-buffered M199 (Gibco, Dreieich, Germany) containing gentamicin and kanamycin (Sigma, Deisenhofen, Germany). Cells were plated at a low density (several thousand cells per dish) on 36 mm culture dishes. Medium was changed 24 h after plating and then every second day.

2.2. Solutions and chemicals

For the patch clamp measurements an extracellular solution of the following composition was used (mM): NaCl 120; KCl 20; $CaCl_2$ 2.0; $MgCl_2$ 1.0; HEPES/NaOH 10.0, pH 7.4. The solution for filling the patch clamp pipettes for whole cell voltage clamp experiments contained (mM): K-aspartate 110; KCl 20; NaCl 10, $MgCl_2$ 1.0; MgATP 2,0; EGTA 2,0; GTP 0.01; HEPES/KOH 10.0, pH 7.4. K^+. Standard chemicals were from Merck (Darmstadt, Germany). EGTA, HEPES, MgATP, adenosine GTP and ACh-chloride were from Sigma (Deisenhofen, Germany).

2.3. Current measurement

Membrane currents were measured using whole-cell patch clamp. Pipettes were fabricated from borosilicate glass and were filled with the solution listed above (DC resistance 4–6 MΩ). Currents were measured by means of a patch clamp amplifier (List LM/EPC 7, Darmstadt, Germany). Signals were analog filtered (corner frequency of 1–3 kHz), digitally sampled at 5 kHz and stored on a computer, equipped with a hardware/software package (ISO2 by MFK, Frankfurt/Main, Germany) for voltage control and data acquisition. Experiments were performed at ambient temperature (22–24°C). Cells were voltage-clamped at −90 mV, i.e. negative to E_K, resulting in inward K^+ currents. Current–voltage relations were determined by means of voltage ramps between −120 mV and +60 mV. Rapid superfusion of the cells for application and withdrawal of different solutions was performed by means of a solenoid-operated flow system that permitted switching between up to six different solutions ($t_{1/2} \leq 100$ ms).

2.4. Transfection

Myocytes were cultured overnight to allow attachment. For transfection 2.5 μg/plate of the reporter IRES-GFP vector (Clontech) and 2.5 μg/plate pCMV5-β1 construct (β1 subunit derived from human brain; kindly provided by Dr. A.G. Gilman), both under CMV promoter control, were used. Transfection was performed by means of

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Lipofectamine/Plus reagent (Life Technologies Inc.) according to the manufacturer's instructions.

Electrophysiological recordings were made on days 3 and 4 after transfection.

3. Results and discussion

A representative recording of membrane current from a myocyte transfected with IRES-GFP vector is illustrated in Fig. 1. Fig. 1A represents current at −90 mV holding potential. Brief (5 s) exposure to ACh (2 μM) in line with previous studies using similar conditions (e.g. [11]) caused rapid and reversible activation of inward $I_{K(ACh)}$. The voltage dependence of the ACh-activated current (Fig. 1B) is characterized by strong inward-going rectification and a reversal close to E_K (−50 mV). Superfusion with isoprenaline (Iso, 10 μM) did not

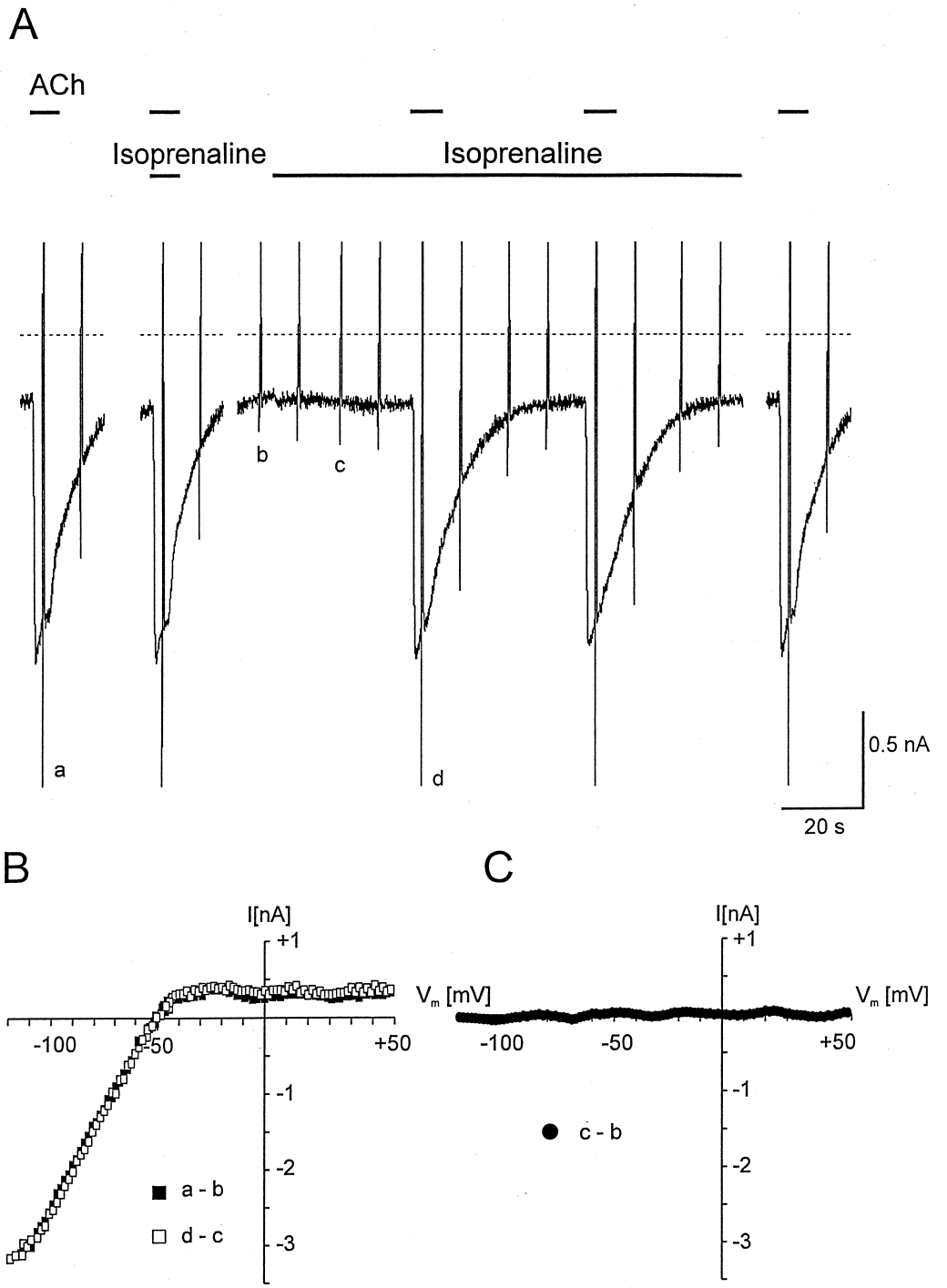

Fig. 1. Isoprenaline does not activate $I_{K(ACh)}$ in control myocytes. A: Low-speed recording of membrane current at −90 mV holding potential. ACh (2 μM) and Iso (10 μM) were applied as indicated by horizontal bars. The rapid deflections represent changes in membrane current caused by voltage ramps. B: Current–voltage relations of ACh-induced currents in the absence and in the presence of Iso; background current was subtracted as indicated by the lowercase letters. C: Difference I/V relation of background current in the presence minus background current in the absence of Iso.

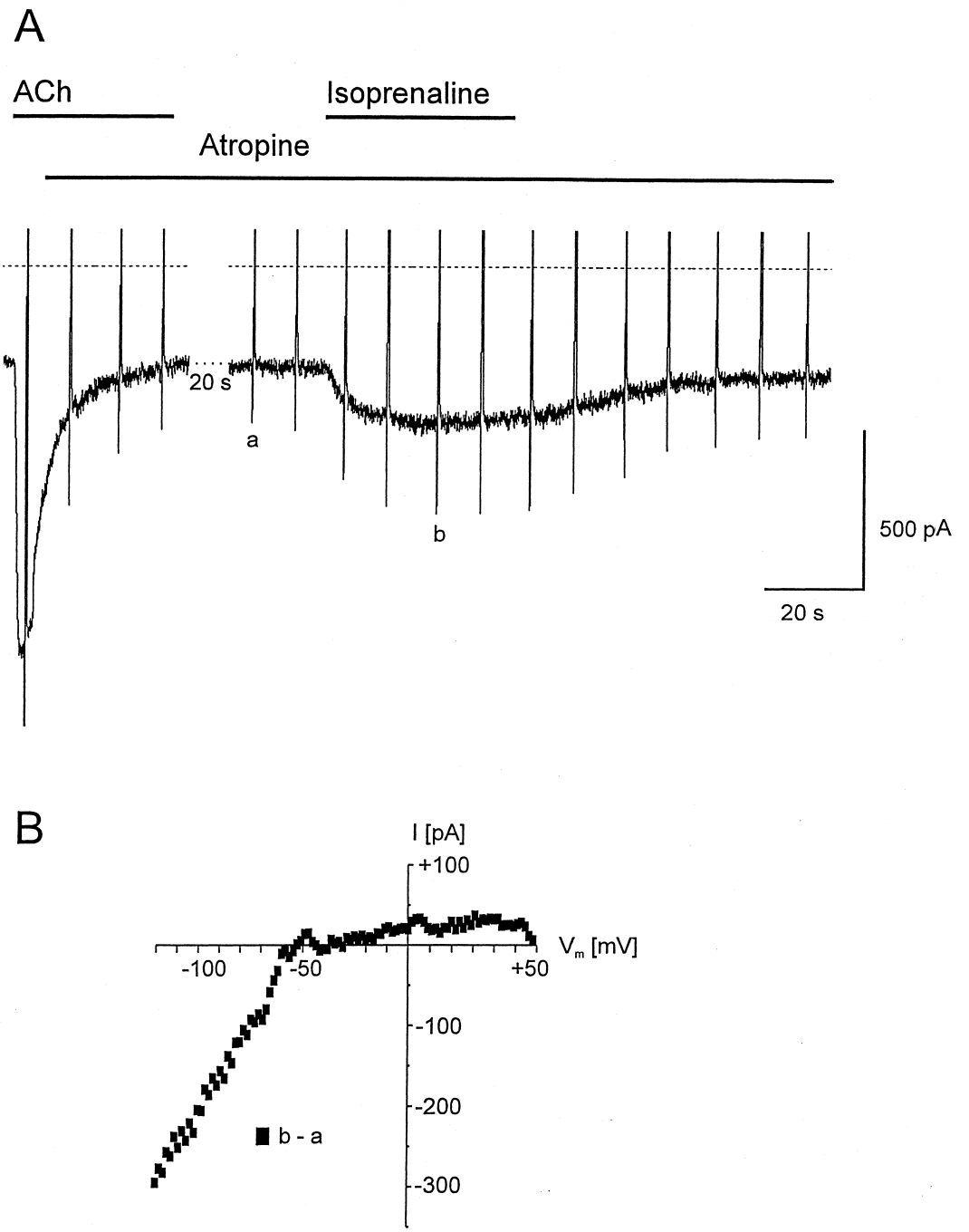

Fig. 2. Atropine-resistant activation of $I_{K(ACh)}$ by Iso (10 μM) in a β_1-transfected (eGFP-positive) myocyte. A: Low-speed recording of membrane current at −90 mV holding potential. ACh, atropine and Iso were applied as indicated by horizontal bars. B: Difference current–voltage relation of Iso-induced current (background subtracted).

cause a measurable shift in holding current. No effect of Iso on ACh-activated $I_{K(ACh)}$ was observed (Fig. 1B). A complete lack of effect of β-adrenergic stimulation on $I_{K(ACh)}$ is representative of 6/6 freshly isolated myocytes, 10/10 non-transfected myocytes cultured for 4–5 days and 10/10 myocytes successfully transfected with eGFP only. Comparable results were obtained in 12/12 native guinea pig atrial myocytes cultured for 1–5 days. Fig. 2A illustrates a recording of membrane current from an eGFP-positive cell co-transfected with the vector encoding the β_1 subunit. Isoprenaline, in the presence of a saturating concentration of atropine, caused activation of a current with strong inward-rectifying properties characteristic of $I_{K(ACh)}$ (Fig. 2B), amounting to 20% of peak $I_{K(ACh)}$ elicited by 2 μM ACh. In four out of 19 eGFP-positive myocytes Iso failed to cause measurable activation of $I_{K(ACh)}$. In those cells which responded to Iso, the mean current (at −90 mV) was 18.9 ± 1.2% of peak ACh-induced $I_{K(ACh)}$ ($n = 15$; five cultures). Supplementation of the pipette filling solution with cAMP at a concentration (100 μM) which in the same type of cell caused substantial effects on other membrane currents susceptible to β-adrenergic stimulation [12] did not affect the signalling pathway under

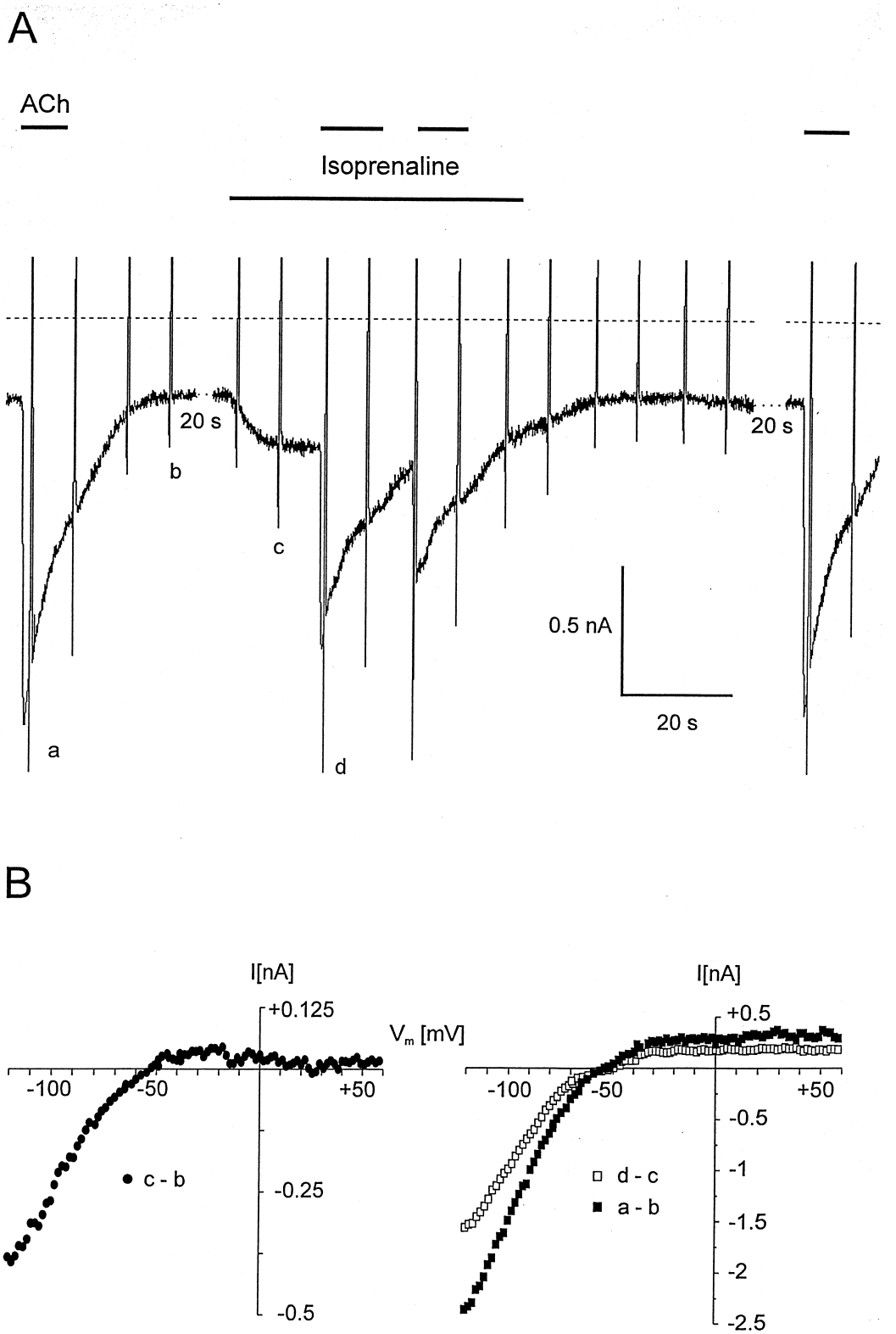

Fig. 3. Desensitization of ACh-induced $I_{K(ACh)}$ in the presence of Iso (β_1- transfected, eGFP-positive myocyte). A: Low-speed recording of membrane current at −90 mV holding potential. ACh and Iso were applied as indicated by horizontal bars. B: Current–voltage relations of ACh-induced and Iso-induced currents (background subtracted).

study. Neither in control ($n=4$) nor in β_1-transfected cells ($n=6$) did cAMP cause activation of $I_{K(ACh)}$. Sensitivity of $I_{K(ACh)}$ to Iso in transfected cells was not affected by prior intracellular loading with cAMP (data not shown). This is clear evidence that cAMP-dependent phosphorylation is not involved.

Apart from inducing sensitivity of $I_{K(ACh)}$ to β-adrenergic stimulation, overexpression of β_1 subunit caused a reduction

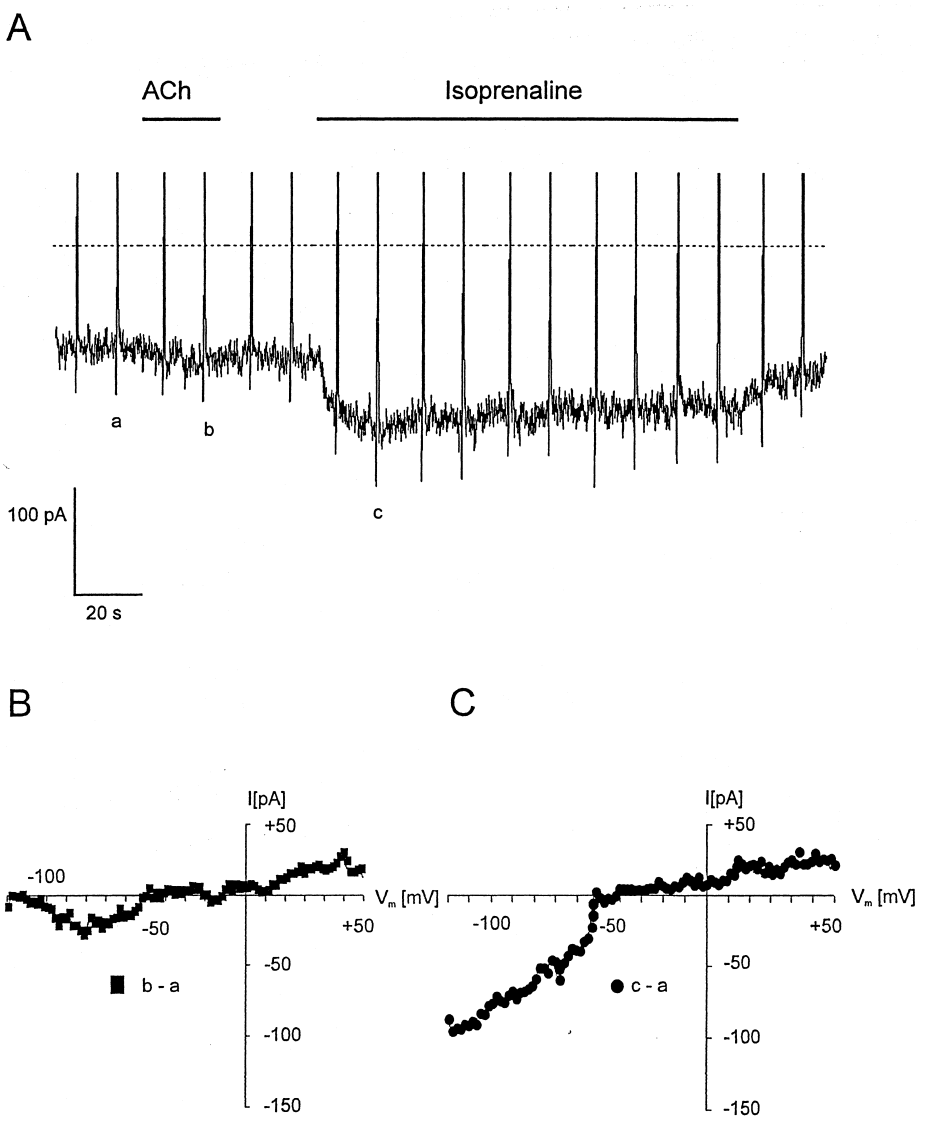

Fig. 4. Ptx-resistant activation of $I_{K(ACh)}$ by Iso. A: Low-speed recording of membrane current at −90 mV holding potential. ACh and Iso were applied as indicated. B: Current–voltage relation of Iso-induced current (background subtracted).

of ACh-induced $I_{K(ACh)}$. At the standard concentration of ACh (2 μM) peak ACh densities amounted to 39.6 ± 7.2 pA/pF (eGFP-transfected; $n = 9$) and 23.95 pA/pF (eGFP- and β_1-transfected; $n = 10$; $P < 0.005$). Although the similarity of the current–voltage relations of ACh- and Iso-induced currents is striking, it does not exclude a contribution of other inward-rectifying currents to the Iso-induced current, such as I_{K1} (e.g. [13]). Evidence against this is presented by the result illustrated in Fig. 3, which shows fast cross-desensitization between Iso and ACh. This behavior, which is a peculiarity of the $I_{K(ACh)}$ signalling pathway, does not represent a phenomenon related to the activating receptors but reflects a property either of the G protein [14] or, more likely, of the $K_{(ACh)}$ channels [15]. The result shown in Fig. 3, which is representative of six out of nine cells, supports the notion that the current activated by β-adrenergic stimulation is flowing through $K_{(ACh)}$ channels.

Whether activation of $I_{K(ACh)}$ by Iso in cells overexpressing β_1 subunit proceeds via G_s was tested by means of Ptx. A representative recording of membrane current from a β_1-transfected myocyte after incubation with Ptx (2 μM, ∼6 h) is illustrated in Fig. 4. Whereas the standard challenge by 2 μM ACh failed to evoke a measurable current, Iso still caused activation of an inward rectifying K^+ current. Activation of $I_{K(ACh)}$ by Iso was found in 5/6 Ptx-treated β_1-transfected cells, whereas in 4/4 Ptx-treated non-transfected myocytes no sensitivity to Iso was found. This clearly demonstrates that activation of $I_{K(ACh)}$ by β-adrenergic receptors in β_1-transfected myocytes proceeds via subunits released from the type of G protein (G_s) which normally couples to these receptors. Ptx insensitivity of the response to the β-agonist safely rules out that in the present condition a cAMP-dependent switch in the receptor coupling from G_s (Ptx-insensitive) to G_i (Ptx-sensitive) is responsible for the response of $I_{K(ACh)}$ to β-agonist (compare [16]).

In previous studies using the oocyte expression system, it has been demonstrated that $K_{(ACh)}$ channels in principle can be activated by βγ subunits derived from non-Ptx-sensitive G proteins coupled to co-expressed β-adrenergic [9] or $5HT_{1A}$ receptors [17]. These publications and others (e.g. [18]) dem-

onstrated a highly promiscuous coupling of receptors via their coupling G proteins to expressed $K_{(ACh)}$ channels. Together with the finding that selectivity of different combinations of βγ subunits for activating $K_{(ACh)}$ channels in excised membrane patches is low [8], this raises the question of how in the native system activation of $I_{K(ACh)}$ by βγ subunits released from G_s following β-adrenergic stimulation is prevented. An earlier study by Kim [19] claimed a cAMP-dependent activation of $I_{K(ACh)}$ in atrial myocytes. So far this has not been confirmed by any other study. In a detailed investigation of β-adrenergic/muscarinic interactions on $I_{K(ACh)}$, no evidence for a cAMP-dependent or a direct (G protein-mediated) effect of β-adrenergic stimulation on $I_{K(ACh)}$ was found [20]. No other studies in native myocytes so far have explicitly addressed this issue. Our data on native or mock-transfected myocytes confirm that β-adrenergic receptors are indeed excluded from activating $I_{K(ACh)}$.

Evidence has been provided recently that the site of interaction of βγ dimers with target proteins is located on the β subunit [21,22], whereas for activation of the target the intact βγ dimer is required. Thus the overexpressed β-subunit potentially acts as a 'βγ antagonist'. Using an in vitro expression system it has been shown that proper folding of monomeric β subunits in the absence of γ is impaired [23]. However, this does not exclude an affinity of overexpressed β to βγ targets via its N-terminal binding domain and thus a βγ-antagonistic action. Alternatively it is conceivable that expressed β_1 subunits might form heterodimers with intrinsic γ subunits resulting in an excess of this signalling element. Although this hypothesis would be compatible with the finding that the system gets promiscuous, it cannot provide a simple explanation for the reduction of M_2 receptor-induced $I_{K(ACh)}$ following β_1 transfection. Hypothesizing that overexpressed β_1 is able to bind to βγ targets, the most obvious sites of such an antagonism are the βγ binding domains on the GIRK subunits of the $K_{(ACh)}$ channel [24,25]. This is in line with the reduction of ACh-induced $I_{K(ACh)}$ in β_1-transfected cells observed in the present study. In a given cell type an array of proteins may compete for binding to βγ subunits released from a pool of heterotrimeric G protein complexes, such as the GIRK subunits, receptor kinases (GRK2; GRK3) and free G_α subunits. The net result of expressing a βγ antagonist (in terms of activation/inhibition) for a given βγ target cannot be predicted a priori. For example, the inhibitory effect on $I_{K(ACh)}$ of antagonizing βγ binding to the GIRK subunits could be partly overcome by simultaneously antagonizing re-association of βγ with the α subunits. Moreover, it is conceivable that saturating βγ sites on other binding proteins, such as GRK2, which is activated and targeted to the membrane by βγ binding [26], can result in an increased concentration in free βγ. This hypothesis implies that all components of a signalling pathway have to be expressed at a delicately balanced relation as a condition for ensuring target selectivity. This is likely to represent one of the reasons why in expression systems target selectivity of receptors is sometimes absent, or can be switched by manipulating the expression level of one of the signalling elements (e.g. [18]). Other factors, such as spatial distributions of G_s/G_i-coupled receptors and downstream signalling elements, are assumed to be important for specificity of signals [27]. This is not contradictory to our hypothesis, since buffering of $G_{\beta\gamma}$ is likely to affect the spatial distribution of this signalling element.

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