Association of 14-3-3 Proteins to β_1 -Adrenergic Receptors Modulates Kv11.1 K⁺ Channel Activity in Recombinant Systems^D

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We identify a new mechanism for the β_1 -adrenergic receptor (β_1 AR)-mediated regulation of human ether-a-go-go-related gene (HERG) potassium channel (Kv11.1). We find that the previously reported modulatory interaction between Kv11.1 channels and 14-3-3 ϵ proteins is competed by wild type β_1 AR by means of a novel interaction between this receptor and 14-3-3 ϵ . The association between β_1 AR and 14-3-3 ϵ is increased by agonist stimulation in both transfected cells and heart tissue and requires cAMP-dependent protein kinase (PKA) activity. The β_1 AR/14-3-3 ϵ association is direct, since it can be recapitulated using purified 14-3-3 ϵ and β_1 AR fusion proteins and is abolished in cells expressing β_1 AR phosphorylation-deficient mutants. Biochemical and electrophysiological studies of the effects of isoproterenol on Kv11.1 currents recorded using the whole-cell patch clamp demonstrated that β_1 AR phosphorylation-deficient mutants do not recruit 14-3-3 ϵ away from Kv11.1 and display a markedly altered agonist-mediated modulation of Kv11.1 currents compared with wild-type β_1 AR, increasing instead of inhibiting current amplitudes. Interestingly, such differential modulation is not observed in the presence of 14-3-3 inhibitors. Our results suggest that the dynamic association of 14-3-3 proteins to both β_1 AR and Kv11.1 channels is involved in the adrenergic modulation of this critical regulator of cardiac repolarization and refractoriness.

INTRODUCTION

The human ether-a-go-go-related gene (HERG) channel (Kv11.1) is the pore-forming subunit of the voltage-gated potassium channel underlying the rapidly activating delayed rectifier current (IKr), which is critical for the kinetics of cardiac action potential repolarization (Sanguinetti *et al.*, 1995; Tamargo *et al.*, 2004). Mutations in Kv11.1 or drug channel blockade can produce an excessive prolongation of the action potential duration and the QT interval. These effects are considered to be proarrhythmic, because they might produce polymorphic ventricular tachycardia known as “torsades de pointes” (Roden, 2004; Rosen and Cohen, 2006). Such disturbances are often stress-induced, consistent with a functional link between adrenergic stimulation and Kv11.1 activity. Indeed, altered β_1 adrenergic receptor (β_1 AR) regulatory and/or signaling mechanisms are associated to high incidence of cardiac arrhythmias (Olson, 2004).

On agonist binding β_1 AR stimulates the Gs-adenylyl cyclase/cAMP/protein kinase A pathway (AC/cAMP/PKA), which results in a complex regulation on Kv11.1 channels

not completely understood (Karle *et al.*, 2002; Thomas *et al.*, 2004). An increase of Kv11.1 currents by the β -agonist isoproterenol (Iso) has been reported (Cui *et al.*, 2000, 2001), although an inhibition is more often observed (Karle *et al.*, 2002; Thomas *et al.*, 2004). These discrepancies might be due to the existence of differential modulatory factors. Indeed, cAMP can directly bind to the cyclic nucleotide-binding domain of Kv11.1, favoring an increase of the current by shifting the activation curve of the channels to more negative potentials (Cui *et al.*, 2000, 2001). In addition, cAMP-dependent protein kinase (PKA) phosphorylates Kv11.1 and promotes an acceleration of the deactivation with a shift in the activation curve to more positive potentials, thus reducing the current (Karle *et al.*, 2002; Thomas *et al.*, 2004).

14-3-3 proteins are a family of regulatory molecules expressed in all eukaryotic cells that modulates multitude of signaling proteins by binding to a prototype phosphorylated serine or threonine recognition motifs (Fu *et al.*, 2000). Recently, it has been reported that PKA-phosphorylated Kv11.1 channels can associate to 14-3-3 ϵ protein, the most abundant 14-3-3 isoform in the heart (Kagan *et al.*, 2002). 14-3-3 ϵ binding to Kv11.1 channels accelerates the time course of channel activation and shifts the activation curve to more hyperpolarized potentials, resulting in an increase of outward and tail current amplitudes at negative potentials (Kagan *et al.*, 2002). However, the modulation of the Kv11.1/14-3-3 ϵ interaction by β_1 AR stimulation has not been investigated, and an integrated view of how all the reported mechanisms cooperate in modulating Kv11.1 channel activity is lacking.

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Here we describe that β_1 AR competes with Kv11.1 for the interaction with 14-3-3 ϵ by directly associating to 14-3-3 ϵ proteins in a PKA-dependent manner and that this new level of control has important functional consequences on the activity of Kv 11.1 channels expressed in CHO cells. The dynamic association of 14-3-3 to both β_1 AR and Kv11.1 emerges as a novel mechanism in the regulation of cardiac repolarization in response to adrenergic stimulation.

MATERIALS AND METHODS

HEK-293 and CHO cells were from the American Type Culture Collection (Manassas, VA). Culture media and Lipofect-AMINE were from Invitrogen (Carlsbad, CA). Alprenolol and forskolin were from Sigma (St. Louis, MO). Iso and the kinase inhibitors H-89, PD98059 and Ro 31-8220 were purchased from Calbiochem (La Jolla, CA). Purified PKA and GST-14-3-3 ϵ were purchased from Sigma and Biomol (Plymouth Meeting, PA), respectively. Betaxolol was a generous gift from Synthelabo (Sanofi-Aventis, Bridgewater, NJ). HA-14-3-3 ϵ was kindly provided by Dr. R. Pulido (Universidad de Valencia). Plasmids encompassing GST fusion proteins of the entire β_1 AR third intracellular loop (pGEX-2TK-i3loop β_1 AR; Tang *et al.*, 1999) or the first 80 amino acids of the β_1 AR carboxyl-terminus (pGEX-4T1- β_1 ARCT), were kindly provided by Dr. R. Hall (Emory University School of Medicine, Atlanta, GA). HERG and β_1 AR-S412D cDNA were a gift from Dr. Sanguinetti (University of Utah, Salt Lake City, UT) and Dr. Bahouth (University of Tennessee Health Sciences Center, Chattanooga, TN), respectively.

Site-directed Mutagenesis

The cDNA coding for human β_1 AR in the pSP95 vector, kindly provided by Dr. Kobilka (Stanford University), was subcloned into a flag-epitope tagged eukaryotic expression vector. To this end, site-directed mutagenesis (Quick-Change Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) was performed with the primer 5'-CCTCCGAATTCGCCATGGCGCGGGGTGCTC 3' to introduce an NcoI restriction site (underlined) encompassing the ATG codon at the initiation of the open reading frame. The resultant product was digested with NcoI and XbaI and subsequently ligated into a pcDNA3 plasmid bearing an epitope flag. Single point mutations changing serine 312 or 412 to alanine were engineered by the PCR using the primers 5'-GGTAAGCGGCGGCGCGCGCTCGTGGCCCTACGC 3' and 5'-GGAGACCGCGCGGCGGCGCGGCTGTCTGGCC 3', respectively, and the pcDNA3-Flag- β_1 AR construct as template. The double mutant receptor S312,412A was obtained by restriction digestion of the S312A construct with PvuII/XmnI and ligation of the corresponding insert into the S412A plasmid. A dominant negative construct of 14-3-3 proteins (DN 14-3-3) was engineered by mutation of arginine residues to alanines at position 56 and 60 in the 14-3-3 ζ isoform. These residues are highly conserved in all 14-3-3 proteins, and they are essential for supporting binding of 14-3-3 proteins to their partners. When overexpressed in cells, the 14-3-3 ζ -R56, 60A mutant efficiently blocks the interaction of 14-3-3 proteins with its targets as a result of its heterodimerization with endogenous 14-3-3 (Xing *et al.*, 2000). Site-directed mutagenesis was performed as above using the primer 5'-GTTGTAGGAGCCCGTGGCTCATCTTGGCGGTCTCAAGT 3' and its complementary pair and the pcDNA3-myc-14-3-3 ζ construct as template, gently provided by Dr. A. Aitken (University of Edinburgh). The sequence of all constructs was confirmed using an automated ABI DNA sequencer (Centro de Biología Molecular, Madrid).

Cell Culture and Transfection

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere. CHO cells stably expressing the Kv11.1 channel protein were cultured in Ham's F12 medium (GIBCO, Gaithersburg, MD) supplemented with L-glutamine and 10% (vol/vol) fetal bovine serum in the presence of geneticin 0.5 mg/ml (Calbiochem). Transfections (5–10 μ g of plasmid constructs) were performed in serum-free OPTIMEM plus LipofectAMINE reagent on 70% confluent monolayers in 100-mm dishes for adenylyl cyclase assays or 60-mm dishes for immunoprecipitation experiments. Empty vector was added as needed to keep the total amount of DNA constant. Similar expression of wild-type and mutated proteins was confirmed by immunoblot analysis of whole-cell lysates with specific antisera.

Cell Treatment

Cell stimulation was performed 48 h after transfection and was carried out in normal culture medium supplemented with 20 mM HEPES, pH 7.5, and 1 mM ascorbic acid (Sigma). Iso was used at the concentration indicated in the figure legends and forskolin at 30 μ M. Treatments with kinase inhibitors of PKA (H-89, 10 μ M), PKC (Ro 31-8220, 5 μ M), and MEK (PD98059, 50 μ M), as well as with the receptor antagonist betaxolol (10 μ M) were initiated 1 h before Iso stimulation and maintained thereafter.

Immunoprecipitation and Western Blotting

Cells were washed and lysed in ice-cold lysis buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 5 mM EDTA, 0.05% NP-40, 20% glycerol) supplemented with 1% *n*-docecyl- β -D-maltoside plus protease inhibitors for 60–90 min at 4°C. After centrifugation (15,000 $\times$ g, 15 min), cellular extracts were diluted to 0.33% *n*-docecyl- β -D-maltoside and immunoprecipitated with the agarose-conjugated monoclonal anti-HA or monoclonal M2 anti-flag epitope antibodies (dilution 1/100, Sigma) by overnight incubation at 4°C. After incubation with protein A-agarose and extensive washing with lysis buffer, immunoprecipitates were resolved by SDS-PAGE, and the gel was transferred to nitrocellulose membranes to be probed (1/500) with specific polyclonal anti-Flag antibody (ABR, Golden, CO), anti-14-3-3 common antibody (K19, Santa Cruz Biotechnology, Santa Cruz, CA), anti-HA antibody, anti-14-3-3 ϵ antibody (CT-161, Santa Cruz), or anti-Kv11.1 antibody (H-175 dilution 1/200, Chemicon, Temecula, CA). Lysate aliquots were taken to assess protein overexpression of the different protein constructs transfected. Blots were developed using a chemiluminescent method (ECL, Amersham, Piscataway, NJ). Band density was quantitated by laser densitometric analysis.

β_1 AR Purification by Affinity Chromatography

Guinea-pig hearts were minced and subjected to isoproterenol (10 μ M) treatment for different periods of time as reported (Caballero *et al.*, 2003). Membrane preparations were then obtained after whole tissue homogenization in 10 vol (wt/vol) of buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, and a cocktail of protease inhibitors) using a Polytron device. Extracts were centrifuged at 4,000 $\times$ g for 5 min and the obtained supernatants at 19,000 $\times$ g for 20 min. The precipitated was recentrifuged three times in the same conditions. β_1 AR present in the membrane-enriched fractions was solubilized by adding 1% *n*-docecyl- β -D-maltoside and purified by its selective binding to an alprenolol-conjugated Sepharose 4B column. After loading the sample, the column was washed with 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.05% *n*-docecyl- β -D-maltoside, and 100 mM NaCl. Elution was performed at low ionic strength in the presence of 0.1 mM alprenolol. The fractions obtained were analyzed by immunoblotting with a specific anti- β_1 AR polyclonal antibody (V-19, dilution 1/250, Santa Cruz) and a specific anti-14-3-3 ϵ antibody as indicated above.

Adenylyl Cyclase and MAPK Assays

HEK-293 cells transiently transfected with β_1 ARwt or different mutant constructs (β_1 AR-S321A, β_1 AR-S412A, and β_1 AR-S312, 412A) were subcultured (6×10^5 cells/P-6 well dishes) for 12 h before pretreatment with betaxolol for 2 min to lessen basal receptor activity. After extensive washing, cells were challenged with 1 μ M Iso or vehicle for 5 or 10 min. Lysates were obtained and assessed in triplicate for cAMP levels as described (Hough *et al.*, 1994). Data were corrected according to total protein amount in each sample and compared with values obtained in control. In similar experiments MAPK activation upon Iso challenge was assessed as reported (Elorza *et al.*, 2000).

In Vitro Phosphorylation and Association Assays

Purified GST fusion proteins encompassing the third intracellular loop (GST- β_1 AR₃ loop) of the β_1 AR or GST as a control were incubated (50 nM) in the presence or absence of purified PKA (7.5 nM) for 30 min at 30°C in 50 μ l of kinase reaction buffer (25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5 mM DTT, 50 μ M ATP, 4.5 mM NaF, 6 μ M cAMP, and 4,800–12,000 cpm/pmol [γ ³²-P]ATP). Proteins were resolved by SDS-PAGE and visualized by autoradiography. For association assays, fusion proteins (200 nM) were incubated in the presence or absence of purified PKA for 30 min at 30°C in 200 μ l of kinase reaction buffer (without [γ ³²-P]ATP). To remove PKA, GST-proteins were pulled down by incubation with 20 μ l of glutathione-Sepharose for 1 h at 4°C, eluted with glutathione, and recovered by ultrafiltration. GST proteins were then incubated for 3 h in the presence of purified GST 14-3-3 ϵ (400 nM) in immunoprecipitation buffer (50 mM Tris, pH 7.4, 300 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% glycerol, 5 mM NaF, and proteases inhibitors). After extensive washing, the association to the latter protein was analyzed by immunoprecipitation with specific 14-3-3 ϵ antibodies or a nonrelated IgG as a control. The immunoprecipitates were resolved by electrophoresis and blotted with 14-3-3 ϵ or anti-GST antibodies to detect fusion proteins.

Electrophysiological Studies

Kv11.1-stably transfected CHO cells were cultured as previously described (Caballero *et al.*, 2000, 2003) and perfused with an external solution containing (in mM): NaCl 130, KCl 4, CaCl₂ 1, MgCl₂ 1, HEPES 10, and glucose 10, pH 7.4. The internal solution contained (in mM): K-aspartate 80, KCl 42, KH₂PO₄ 10, MgATP 5, phosphocreatine 3, HEPES 5, and EGTA 5 (pH 7.2). Cells were transiently transfected with wild-type (β_1 ARwt) or mutated β_1 AR (S312A, S412A, S312, 412A, and S312D) and cDNA encoding the CD8 antigen (0.5 μ g of each) using Lipofect-AMINE. In some experiments, cells were cotransfected with 0.5 μ g of a dominant negative 14-3-3 construct (DN14-3-3). Alternatively, 14-3-3 function was disrupted by incubation with 14-3-3 inhibitor

Figure 1. β_1 AR disrupts Kv11.1/14-3-3 ϵ association and Iso inhibits Kv11.1 currents. (A) HEK-293 cells were cotransfected with Kv11.1 and HA-14-3-3 ϵ in the absence or presence of β_1 ARwt. After stimulation or not with Iso (10 μ M) for 5 min, the presence of Kv11.1 in HA-14-3-3 immunoprecipitates was assessed by using specific channel antibodies. The expression levels of the different proteins in cell lysates were analyzed by immunoblot analysis as detailed in *Materials and Methods*. Results are representative of three independent experiments. (B and E) Kv11.1 current traces obtained by applying 5-s pulses that were imposed in 20-mV increments between -80 mV and $+60$ mV. Deactivating Kv11.1 tail currents were recorded at -60 mV. Currents were obtained in the absence and the presence of 1 nM Iso in cells transfected (E) or not (B) with β_1 ARwt. (C and F) Mean Kv11.1 current-voltage relationship 5-s isochronal in the absence and presence of 1 nM Iso in the indicated experimental conditions. (D and G) Mean Kv11.1 activation curves in the absence and presence of 1 nM Iso in the indicated conditions. Each point represents the mean of ≥ 6 experiments and * $p < 0.05$ versus control.

(R18). Before use, cells were incubated with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450, Dynal Biotech, Oslo, Norway). Kv11.1 currents were measured using the whole-cell patch-clamp method. Recordings were performed at room temperature with 200B patch-clamp amplifiers (Axon Instruments, Foster City, CA). CHO cells capacitance and series resistance averaged 9.9 ± 1.2 pF and 4.5 ± 0.2 M Ω ($n = 33$), respectively. Maximum Kv11.1 tail-current amplitudes averaged 283.9 ± 30.9 pA. Typically, $\approx 80\%$ of capacitance and series resistance could be compensated, leading to mean uncompensated access resistances of 1.7 ± 0.1 M Ω . Thus, no significant voltage errors (< 5 mV) were expected with the electrodes used (tip resistance < 3 M Ω).

To obtain the current-voltage relationships, 5-s pulses, imposed in 10-mV increments between -80 and $+60$ mV were applied. Tail currents were recorded at -60 mV. The activation curves were constructed by plotting tail current amplitudes as a function of the membrane potential and fitted with a Boltzmann distribution. To describe the time course of current activation and deactivation, an exponential analysis was used as reported. Results are expressed as mean $\pm$ SEM. Data were compared by ANOVA followed by the Newman-Keuls test. $p < 0.05$ was considered significant.

RESULTS

β_1 AR Disrupts Kv11.1/14-3-3 ϵ Association

Figure 1A shows that Kv11.1 coimmunoprecipitated with cotransfected 14-3-3 ϵ , a result that is consistent with the report of Kagan *et al.* (2002). Interestingly, coexpression of β_1 ARwt disrupts the interaction, both in basal and Iso stimulated conditions. A detailed electrophysiological analysis of Kv11.1 currents upon Iso β_1 AR stimulation was performed in CHO cells stably expressing Kv11.1 channels cotransfected or not with the cDNA encoding β_1 ARwt. Figure 1, B and E, shows families of current traces for control conditions and after 10-min exposure to 1 nM Iso, obtained by applying 5-s pulses, imposed in 20-mV increments between -80 and $+60$ mV. The holding potential was fixed at

-80 mV, and tail currents were recorded upon repolarization to -60 mV. In nontransfected cells (which express low levels of endogenous β AR, unpublished data), Iso accelerated the time course of activation but did not modify the maximum outward current and the time course of deactivation ($n = 6$; Figure 1B, Table 1). In cells expressing β_1 ARwt, Iso decreased both the outward and the tail current and accelerated the time course of the initial phase of deactivation, without modifying the time course of activation ($n = 6$; Figure 1E, Table 1). Panels C, F and D, G show the current-voltage plots of steady-state current at the end of the depolarizing step and the activation curves obtained in the presence and the absence of Iso in nontransfected (C and D) and in β_1 ARwt-transfected (F and G) cells. In nontransfected cells, Iso did not significantly modify either the steady state current or the slope of the activation curve (Figure 1, C and D), whereas it slightly shifted the midpoint of the activation curve to more negative potentials (Table 1). Such minor Iso-induced changes in channel activity in the absence of transfected β_1 AR appear to be not receptor-mediated, because it was also observed in the presence of 1 μ M propranolol (unpublished data). In cells expressing β_1 ARwt Iso significantly decreased the current amplitude at -20 and -10 mV (Figure 1F) and the tail current amplitude after pulses between -20 and $+60$ mV (Figure 1G), without modifying the voltage dependence of activation ($n = 6$, $p > 0.05$; Table 1).

β_1 AR Directly Interacts with 14-3-3 ϵ

A possible explanation for the inhibition of Kv11.1/14-3-3 ϵ interaction in the presence of β_1 AR may be a direct competition between Kv11.1 and β_1 AR for 14-3-3 ϵ proteins. In this

Table 1. Effects of 1 nM Iso on the time course of activation, fast phase of deactivation, and voltage dependence of activation of Kv11.1 currents recorded in CHO cells

	Time course of activation		Time course of deactivation		Voltage dependence of activation			
	τ_{CON} (ms)	τ_{ISO} (ms)	τ_{fCON} (ms)	τ_{fISO} (ms)	V_{hCON} (mV)	k_{CON} (mV)	V_{hISO} (mV)	k_{ISO} (mV)
Kv11.1	954 ± 77	773 ± 72 ^a	339 ± 21	346 ± 23	-14.6 ± 2.1	8.1 ± 1.3	-18.2 ± 2.5 ^a	8.3 ± 1.3
Kv11.1 + β_1 ARwt	1004 ± 174	900 ± 211	330 ± 35	259 ± 24 ^a	-16.2 ± 3.7	8.5 ± 1.6	-19.4 ± 4.3	8.9 ± 1.4
Kv11.1 + β_1 ARS312D	1473 ± 292	1251 ± 266	286 ± 31	234 ± 41 ^a	-15.5 ± 2.7	8.5 ± 0.6	-18.7 ± 2.9	9.4 ± 0.4
Kv11.1 + β_1 ARS312A	1213 ± 141	781 ± 135 ^a	321 ± 12	341 ± 19	-12.9 ± 2.5	8.1 ± 0.4	-21.3 ± 1.7 ^a	9.0 ± 0.8
Kv11.1 + β_1 ARS412A	1200 ± 62	808 ± 117 ^a	325 ± 38	293 ± 41	-14.5 ± 1.0	8.3 ± 0.3	-20.0 ± 1.6 ^a	8.2 ± 0.3
Kv11.1 + β_1 ARS312,S412A	1311 ± 250	1008 ± 193 ^a	313 ± 33	346 ± 48	-11.4 ± 3.0	8.9 ± 0.6	-15.8 ± 3.0 ^a	8.8 ± 1.7

τ , time constant of current activation at 0 mV; τ_{f} , time constant of the fast phase of tail current deactivation, recorded after pulses to +60 mV; V_{h} , midpoint of the activation curve; k , slope of the activation curve.

^a $p < 0.05$ vs. data obtained in control conditions.

regard, in the third cytoplasmic loop and the C-terminal region of the β_1 AR there are PKA phosphorylation sites (Rapacciuolo *et al.*, 2003; RRPSRLV and RPRASGCL, respectively) that exhibit certain homology with the consensus binding site of 14-3-3 proteins (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). Therefore, we explored whether 14-3-3 ϵ proteins bind to β_1 AR. To this end, HEK-293 cells were transiently transfected with tagged β_1 AR and 14-3-3 ϵ constructs. Figure 2A shows that upon immunoprecipitation of β_1 AR, HA-tagged 14-3-3 ϵ could be detected in the receptor complexes and that the presence of Iso promoted a marked increase in β_1 AR/14-3-3 ϵ coimmunoprecipitation at 5 min of stimulation. These results suggest that activation of β_1 AR promotes conformational changes and/or triggers signaling pathways that facilitate the recruitment of 14-3-3 proteins to the receptor complex. The basal association of β_1 AR and 14-3-3 ϵ in cotransfected cells is decreased upon incubation with the β -antagonist betaxolol (unpublished data), consistent with the idea that the reported basal β_1 AR activity associated with receptor overexpression (Engelhardt *et al.*, 2001) also triggers to certain extent the mechanisms underlying the association. To more clearly visualize the modulation of β_1 AR/14-3-3 binding by different agents, cells were pretreated with betaxolol to lower such basal recruitment.

To validate this association in a more physiological setting, we searched for the occurrence of these molecular complexes in endogenous conditions. After exposure of guinea pig heart samples to Iso for different periods of time, β_1 AR were partially purified by affinity chromatography using the antagonist alprenolol. On elution of bound receptors and their associated proteins, fractions were analyzed for the presence of β_1 AR and 14-3-3 ϵ proteins by using specific antibodies. Figure 2B indicates that 14-3-3 ϵ proteins coeluted with purified endogenous receptors and that such coelution is increased upon agonist stimulation. This result confirms that β_1 AR and 14-3-3 ϵ proteins associate "in situ" in the heart in an agonist-dependent way.

Consistent with the idea that stimulation of the cAMP/PKA pathway is involved in triggering β_1 AR/14-3-3 ϵ binding, their coimmunoprecipitation is rapidly promoted by incubating cells with forskolin, a well-known AC activator (Figure 3A). Conversely, both basal and agonist-induced association is blocked in the presence of the PKA inhibitor H-89 (Figure 3B), but not by protein kinase C or mitogen-activated protein kinase/ERK kinase (MEK) inhibitors (Ro-31-8220 and PD98059, respectively). Overall, these data clearly establish that PKA activity is directly or indirectly

Figure 2. β_1 AR stimulation increases β_1 AR/14-3-3 ϵ association in HEK-293 cells and guinea pig heart. (A) HEK-293 cells transiently transfected with Flag β_1 AR and HA-14-3-3 ϵ and pretreated with betaxolol for 1 h were challenged with Iso, and the association between β_1 AR and 14-3-3 proteins was investigated by immunoprecipitation and Western blot analysis as detailed in *Materials and Methods*. Data indicate the amount of coprecipitated 14-3-3 ϵ proteins, normalized by receptor immunoprecipitation and referred to association in basal (i.e., nonstimulated) conditions, and are mean $\pm$ SEM of four independent experiments. Representative gels are shown left. (B) Endogenous β_1 AR/14-3-3 ϵ complexes are present in guinea pig heart extracts. After incubation of dissociated heart tissue with Iso 10 μ M for the indicated periods of time, β_1 AR were partially purified from solubilized heart membrane extracts by affinity chromatography using alprenolol-Sepharose columns. After elution, fractions were assayed for the presence of receptor and 14-3-3 ϵ proteins by Western blot using specific antibodies as detailed in *Materials and Methods*. In the case of the β_1 AR, two bands are apparent in some fractions likely due to different glycosylation patterns. Gels are representative of two independent experiments.

Figure 3. The association between β_1 AR and 14-3-3 proteins is direct and dependent on PKA activity. HEK-293 cells transiently transfected with Flag β_1 AR and HA-14-3-3 ϵ were stimulated with forskolin (A) or Iso in the absence or presence of the indicated protein kinase inhibitors (B). The receptors were then immunoprecipitated and the presence of 14-3-3 ϵ proteins analyzed and quantitated as in Figure 1. Gels representative of three independent experiments are shown. The fold-stimulation of β_1 AR/14-3-3 ϵ association in each experimental setting with respect to wild-type receptor in basal conditions, normalized by the amount of β_1 AR present in the immunoprecipitates, is shown in Supplementary Figure 3, A and B. (C) Phosphorylation of β_1 AR cytoplasmic domain by PKA. The indicated GST fusion protein, encompassing the third intracellular loop of the β_1 AR, or GST as a control was incubated with PKA as detailed

in *Material and Methods*, resolved by electrophoresis and analyzed by Coomassie blue staining or autoradiography (32 P). (D) The β_1 AR GST fusion protein (or GST as a control) was incubated in the absence or presence of PKA as in C. After washing, the GST proteins were incubated for 3 h in the presence of purified GST 14-3-3 ϵ , and association to the latter protein was analyzed by immunoprecipitation with specific 14-3-3 ϵ antibodies or a nonrelated IgG as a control. The immunoprecipitates were resolved by electrophoresis and blotted with 14-3-3 ϵ or anti-GST antibodies to detect β_1 AR fusion protein, migration of which (including a GST-3il- β_1 AR proteolytic fragment) is shown with asterisks. Results are representative of two independent experiments.

required for triggering the presence of β_1 AR and 14-3-3 ϵ proteins in the same molecular complex.

To test if PKA phosphorylated β_1 AR would directly recruit 14-3-3 proteins, we next performed *in vitro* assays with purified 14-3-3 ϵ and a β_1 AR cytoplasmic domain fusion protein. As shown in Figure 3C, a GST-fusion protein encompassing the third intracellular loop of the β_1 AR (GST-3il- β_1 AR) was readily phosphorylated in the presence of purified PKA. The GST- β_1 AR construct were subsequently incubated with purified GST-14-3-3 ϵ , followed by immunoprecipitation with specific 14-3-3 ϵ antibodies (or control nonrelated antibodies). Figure 3D shows a robust, specific interaction of 14-3-3 ϵ with GST-3il- β_1 AR in a PKA phosphorylation-dependent way.

Mutation of PKA Phosphorylation Sites Renders β_1 AR Unable To Associate to 14-3-3 ϵ and To Compete Its Interaction with Kv11.1

Overall, these results demonstrated a direct interaction between 14-3-3 ϵ and PKA-phosphorylated β_1 AR. To further explore this point, we generated different β_1 AR mutants (β_1 ARS312A, β_1 ARS412A, and the double mutant β_1 ARS312,412A) in which serine residues 312 and 412 were substituted for alanines, a modification reported to prevent their phosphorylation by PKA upon receptor stimulation (Rappaciuolo *et al.*, 2003). All these receptor constructs showed a similar pattern of adenylyl cyclase stimulation upon expression of comparable levels in HEK-293 cells and challenge with Iso (see Supplementary Figure S1). Despite such efficient activation of the cAMP signaling pathway, the ability of these mutants to associate to cotransfected 14-3-3 ϵ isoforms, both in basal and agonist-stimulated conditions is completely lost in the double mutant β_1 ARS312,412A (Figure 4A) or markedly decreased for the individual mutants (Figure 4B), in comparison with β_1 ARwt. These results clearly indicate that PKA phosphorylation of the β_1 AR at these residues is required for the association of 14-3-3 proteins. In contrast, mutation of S312 to aspartate, which would mimic receptor phosphorylation, leads to an increased β_1 AR/14-3-3 ϵ interaction even in basal conditions (Figure 4C).

β_1 ARwt and Mutants Unable To Interact with 14-3-3 ϵ Display a Different Pattern of Modulation of Kv11.1

Our data suggested that β_1 AR stimulation would sequentially lead to PKA activation, receptor phosphorylation, and recruitment of 14-3-3 ϵ proteins to the complex, which would

Figure 4. Mutation of residues critical for PKA-mediated phosphorylation render β_1 AR unable to associate to 14-3-3 ϵ proteins. HEK-293 cells were transiently transfected with HA-tagged 14-3-3 ϵ and different Flag-tagged β_1 AR constructs. Cells were stimulated with Iso (10 μM) for the time indicated, and the association between the different receptors and 14-3-3 ϵ proteins assessed as in previous figures. Gels are representative of 3–4 independent experiments. The fold-stimulation of β_1 AR/14-3-3 ϵ association in each experimental setting with respect to the wild-type receptor in basal conditions, normalized by the amount of β_1 AR present in the immunoprecipitates, is shown in Supplementary Figure 3, C–E.

Figure 5. Modulation of Kv11.1/14-3-3 ϵ interaction and Kv11.1 currents by expression of different β_1 AR mutants. (A) HEK-293 cells were cotransfected with Kv11.1 and HA-14-3-3 ϵ in the absence or presence of the indicated β_1 AR constructs. After stimulation or not with Iso (10 μ M) for 5 min, the presence of Kv11.1 in HA-14-3-3 immunoprecipitates was assessed as in Figure 1A. The expression levels of the different proteins in cell lysates were analyzed by immunoblot analysis as detailed in *Materials and Methods*. Results are representative of three independent experiments. (B and E) Kv11.1 current traces were obtained and analyzed as detailed in Figure 1 in the absence and the presence of 1 nM Iso in cells transfected with β_1 ARS312,412A or β_1 ARS312D as indicated. (C and F) Mean Kv11.1 current-voltage relationship 5 s isochronal in the absence and presence of 1 nM Iso in cells transfected with the indicated receptor constructs. (D and G) Mean Kv11.1 activation curves in the absence and presence of 1 nM Iso in cells transfected with the indicated receptor constructs. Each point represents the mean of ≥ 6 experiments and * $p < 0.05$ versus control.

displace these molecules from their binding sites in Kv11.1 channels. To explore this hypothesis, we tested whether the presence of β_1 AR mutants, differing in their ability to recruit 14-3-3 ϵ , had different effects on the interaction between Kv11.1 and 14-3-3 ϵ .

In contrast to the disruption of binding observed with β_1 ARwt, the Kv11.1/14-3-3 ϵ association is preserved upon expression of similar levels of β_1 ARS312,412A (Figure 5A). In this case the presence of Iso leads to an enhanced association, in agreement with the expected increase in PKA-phosphorylated Kv11.1. Consistently, the β_1 ARS312A mutant, which barely displays association with 14-3-3, is also unable to disrupt the Kv11.1/14-3-3 binding, whereas β_1 ARS312D markedly inhibits Kv11.1/14-3-3 coimmunoprecipitation, as the β_1 ARwt does (Figure 5A, right panel).

Interestingly, in cells expressing β_1 ARS312,412A (Figure 5B) Iso increased the maximum outward current and accelerated the time course of activation but did not modify the time course of deactivation (Table 1). In contrast to the results obtained with β_1 ARwt (see Figure 1, E–G), Iso significantly increased the maximum outward current amplitude at negative potentials (Figure 5C) and markedly shifted the voltage-dependence of activation (Figure 5D), which may explain the increase in the tail and maximum outward currents observed at negative potentials ($n = 6$ – 9 ; $p < 0.05$ vs. control; Table 1). It should be stressed that in cells expressing β_1 ARS312A or β_1 ARS312,412A the effects produced by Iso on current amplitude, as well as on the time- and voltage-dependent properties of the channels, were almost identical to those produced in cell expressing β_1 ARS312,412A

(Table 1). On the contrary, in cells expressing the β_1 ARS312D mutant (Figure 5, E–G), Iso accelerated the time course of the initial phase of deactivation (Table 1) and decreased the maximum outward current at potentials between -20 and $+20$ mV and the tail current elicited by applying pulses positive to -30 mV, without modifying the voltage-dependence of the activation (Figure 5G and Table 1), a situation reminiscent of the effects observed in cells expressing β_1 ARwt (Figure 1, E–G).

To better compare the effects induced by Iso, in Figures 6, I–K the percentages of change in the maximum outward current that elicited at -20 mV and in the amplitude of tail currents elicited after pulses to $+60$ mV or to -20 mV are shown. In cells expressing β_1 ARwt or β_1 ARS312D, Iso decreased the current amplitude at -20 mV by $19.9 \pm 5.4\%$ ($n = 6$) and by $23.5 \pm 1.8\%$ ($n = 7$), respectively (panel I), whereas in cells not transfected with β_1 AR, Iso increased the current by $13.9 \pm 3.2\%$ ($n = 6$). In cells expressing β_1 ARS312A, β_1 ARS412A, or β_1 ARS312,412A, Iso markedly increased the current amplitude ($n = 6$ – 9 , $p < 0.05$ vs. increase in nontransfected cells; panel I). In cells expressing β_1 ARwt or β_1 ARS312D, Iso inhibited the tail current after pulses to $+60$ mV (panel J) by 30.1 ± 5.3 and $32.1 \pm 12.3\%$, respectively. In contrast, the reduction of the tail currents induced by Iso in nontransfected cells and in cells expressing β_1 ARS312A, β_1 ARS412A, or β_1 ARS312,412A was significantly lower ($p < 0.01$, $n = 6$ – 9 ; panel J). Moreover, in these mutants again in contrast to the $\approx 20\%$ inhibition observed in cells expressing β_1 ARwt or S312D, Iso significantly in-

Figure 6. The differential effect of Iso on Kv11.1 currents mediated by β_1 ARwt or β_1 AR S312,412A is abolished in the presence of 14-3-3 inhibitors. (A, C, E, and G) Mean Kv11.1 current-voltage relationship 5 s isochronal in the absence and presence of 1 nM Iso for cells transfected with β_1 AR wt or β_1 ARS312,412A in the presence of R18 (A and C) or in cells cotransfected with DN14-3-3 mutant (E and G). (B, D, F, and H) Mean Kv11.1 activation curves in the absence and presence of 1 nM Iso for cells transfected with β_1 ARwt or β_1 ARS312,412A in the presence of R18 (B and D) or in cells cotransfected with DN14-3-3 mutant (F and H). (I-K) Percentage of change induced by 1 nM Iso in the different conditions in the amplitude of Kv11.1 currents elicited after 5-s pulses to -20 mV (I) and tail currents elicited on repolarization to -60 mV after 5-s pulses to +60 mV (J) and -20 mV (K). In I * p < 0.05 versus Kv11.1. In J, * p < 0.05 versus data obtained in β_1 ARwt transfected cells, # p < 0.05 versus data obtained in β_1 ARS312,412A-transfected cells. Each point or bar represents the mean $\pm$ SEM of ≥ 6 experiments.

Table 2. Effects of 1 nM Iso on the time course of activation, fast phase of deactivation, and voltage-dependence of activation of Kv11.1 currents recorded in CHO cells

	Time course of activation		Time course of deactivation		Voltage dependence of activation			
	τ_{CON} (ms)	τ_{ISO} (ms)	τ_{fCON} (ms)	τ_{fISO} (ms)	V_{hCON} (mV)	k_{CON} (mV)	V_{hISO} (mV)	k_{ISO} (mV)
R18								
Kv11.1 + β_1 ARwt	933 $\pm$ 186	856 $\pm$ 172	365 $\pm$ 85	378 $\pm$ 80.9	-17.6 $\pm$ 3.5	10.6 $\pm$ 1.2	-20.2 $\pm$ 3.7	10.4 $\pm$ 1.3
Kv11.1 + β_1 ARS312,412A	841 $\pm$ 97	802 $\pm$ 179	361 $\pm$ 96	295 $\pm$ 59	-17.6 $\pm$ 3.7	11.8 $\pm$ 2.2	-20.5 $\pm$ 4.4	10.2 $\pm$ 0.8
DN 14-3-3								
Kv11.1 + β_1 ARwt	996 $\pm$ 194	802 $\pm$ 179	422 $\pm$ 100	524 $\pm$ 130	-17.3 $\pm$ 3.3	9.5 $\pm$ 0.5	-20.2 $\pm$ 1.1	8.8 $\pm$ 0.5
Kv11.1 + β_1 ARS312,412A	1058 $\pm$ 213	1183 $\pm$ 257	301 $\pm$ 76	273 $\pm$ 68	-13.1 $\pm$ 3.3	9.9 $\pm$ 0.7	-13.6 $\pm$ 3.0	9.2 $\pm$ 0.4

τ , time constant of current activation at 0 mV; τ_f , time constant of the fast phase of tail current deactivation, recorded after pulses to +60 mV; V_h , midpoint of the activation curve; k , slope of the activation curve.

creased the tail current amplitude after pulses to -20 mV ($n = 6-9$; Figure 6K).

To confirm that 14-3-3 proteins are involved in these differential effects of the β_1 AR phosphorylation deficient mutants on Kv11.1 current modulation, we next performed similar experiments in conditions where endogenous 14-3-3 function was inhibited. We first studied the effects of Iso in the presence of R-18 (200 nM), an unphosphorylated peptide that binds to all 14-3-3 isoforms with equal affinities producing a competitive inhibition (Wang *et al.*, 1999). Because it can be presumed that the membrane diffusion of R-18 would be limited, this inhibitor was added to the internal solution that dialyzed the cells when the patch seals were broken. Kv11.1 currents recorded in R-18 dialyzed cells were of similar amplitude to those recorded in not dialyzed cells ($p > 0.05$) and exhibited the same time- and voltage-dependent properties. Interestingly, under these conditions, the differential effects produced by Iso on Kv11.1 currents in β_1 ARwt- and β_1 ARS312,412A-transfected cells were not observed. Iso similarly inhibited the maximum outward current elicited in cells expressing either β_1 ARwt or β_1 ARS312,S412A (Figure 6, A and C), as well as the tail currents elicited on return to -60 mV (Figure 6, B and D). Furthermore, Iso did not modify either the time course of channel activation and deactivation, or the voltage-dependence of activation (Table 2). Similar results were obtained when a different strategy of inhibition of 14-3-3 function based on cotransfection with a DN14-3-3 mutant was used (Figure 6, E-H, and Table 2). The effects of Iso on Kv11.1 channels in cells expressing β_1 AR wt or β_1 ARS312,S412A in the presence of 14-3-3 inhibitors, were qualitatively identical and not quantitatively different to those produced in cells expressing β_1 ARwt in the absence of inhibitors.

DISCUSSION

β_1 -adrenergic stimulation can exert a complex regulation on Kv11.1 channels by acting at different levels, including direct binding of cAMP (Cui *et al.*, 2000, 2001) and PKA phosphorylation (Karle *et al.*, 2002; Thomas *et al.*, 2004). In this report we describe a novel regulatory mechanism by showing that PKA-dependent phosphorylation of the β_1 AR promotes its association with 14-3-3 ϵ proteins, which disrupts the interaction between Kv11.1 and 14-3-3 and contributes to the adrenergic modulation of Kv11.1 channel activity in a heterologous cell model.

We show that agonist-dependent association between the β_1 AR and 14-3-3 ϵ can be detected by coimmunoprecipitation experiments in both a heterologous cell model and in endogenous conditions upon affinity chromatography purification of β_1 AR from guinea-pig heart, supporting the occurrence of a physiological interaction between these proteins.

We demonstrate that 14-3-3 ϵ binding to β_1 AR is direct, strictly dependent on PKA activity, and requires defined PKA phosphorylation sites in the receptor. The association between β_1 AR and 14-3-3 ϵ proteins is rapidly promoted by the AC activator forskolin, whereas PKA inhibition completely abrogates 14-3-3 binding to the receptor. Consistently, *in vitro* association of purified 14-3-3 ϵ to a β_1 AR cytoplasmic domain fusion protein is dependent on previous incubation with PKA. Finally, we demonstrate that β_1 AR serines 312 and 412, described as PKA phosphorylation sites (Rapacciuolo *et al.*, 2003), are critical residues for supporting 14-3-3 ϵ binding. Despite efficiently stimulating cAMP production, the double mutant β_1 ARS312,412A is totally unable to recruit 14-3-3 ϵ in basal conditions or upon

receptor stimulation. The analysis of the single mutants also reveals a marked decrease in their interaction, suggesting that both sites are involved in 14-3-3 ϵ recruitment and probably required in a synergistically manner, in agreement with a dual site-recognition mechanism (Aitken *et al.*, 2002). On the contrary, β_1 ARS312D, which would mimic PKA phosphorylation, readily interacts with 14-3-3 ϵ .

Moreover, we provide evidence that β_1 AR phosphorylation by PKA and its ability to interact with 14-3-3 ϵ is critical for modulating the Kv11.1/14-3-3 ϵ interface and Kv11.1 channel activity. When β_1 ARwt or β_1 ARS312D is coexpressed with Kv11.1, the association between Kv11.1 and 14-3-3 ϵ is markedly reduced. In contrast, expression of phosphorylation deficient mutants, which are unable to associate to 14-3-3 proteins, does not display this effect. It is worth noting that these mutant receptors display similar ability to promote cAMP generation, as well as to trigger MAPK activation, (Supplementary Figures S1 and S2, respectively), compared with wild-type receptors. Thus, although the contribution of other potential differences in receptor functionality beyond 14-3-3 binding cannot be completely ruled out, the latter seems to be a strong candidate for causing the observed difference in Kv 11.1 regulation.

Overall, these results suggest that the β_1 AR and Kv11.1 would compete for the same pool of 14-3-3 ϵ proteins in the vicinity of the plasma membrane, providing a mechanism for the dynamic modulation of this association. In cells stably expressing Kv11.1 channels and transiently transfected with β_1 ARwt, Iso inhibits Kv11.1 outward and tail currents and accelerates the time course of deactivation, without modifying either the activation kinetics or the voltage-dependence of current activation. These results are in line with those described by others and consistent with the notion that the overall effect of β_1 AR stimulation is to decrease the IKr/Kv11.1 current (Karle *et al.*, 2002; Thomas *et al.*, 2004). However, in cells expressing similar levels of β_1 AR PKA phosphorylation-deficient mutants, Iso does not reduce but increases Kv11.1 maximum and tail currents at negative potentials. Furthermore, Iso promotes an acceleration of the activation time course and markedly shifts the midpoint of the activation curve to more negative potentials, without modifying the deactivation kinetics. These effects on Kv11.1 currents strikingly resemble those reported upon 14-3-3 ϵ protein overexpression (Kagan *et al.*, 2002). Therefore, these results strongly suggest that β_1 AR mutants would favor a more sustained binding of these proteins to Kv11.1 and thus an increase in Kv11.1 current. Consistent with this idea, inhibition of 14-3-3 by R18 or overexpression of DN14-3-3 abolishes the differential effect of Iso between β_1 AR and phosphorylation-deficient mutants on Kv11.1, confirming the involvement of 14-3-3 proteins in this process.

The association between some G-proteins-coupled receptors and 14-3-3 proteins has been recently reported. The α_2 -adrenergic receptor interacts via its third intracellular loop with 14-3-3 ξ in its basal or inactive state, but not upon receptor stimulation (Prezeau *et al.*, 1999). The C-terminal tail of GABA_B receptors binds to 14-3-3 ϵ and ξ , and this association appears to modulate receptor dimerization (Couve *et al.*, 2001). However, this is the first report clearly establishing a PKA-dependent association of 14-3-3 proteins to a G-protein-coupled receptor. Moreover, the requirement of a macromolecular signaling complex for β_1 AR modulation of another cardiac K⁺ channel has been previously described (Marx *et al.*, 2002). Indeed, β_1 AR modulation of Kv7.1+minK channels requires targeting of PKA and protein phosphatase 1 to Kv7.1 through the protein yotiao.

Therefore, we hypothesize that, after agonist activation of β_1 AR in the heart, activated PKA would be sequentially targeted, among other cellular substrates, to Kv11.1 channels and β_1 AR, via specific A-kinase-anchoring proteins (AKAPS; Kagan *et al.*, 2002). Phosphorylated Kv11.1 could then interact with 14-3-3 ϵ proteins that may modulate the channel, probably by shielding Kv11.1 phosphates from cellular phosphatases, as suggested (Kagan *et al.*, 2002). In turn, association of 14-3-3 ϵ proteins to nearby phosphorylated β_1 AR would lower local concentrations of free 14-3-3 and favor their displacement from Kv11.1. The lack of this latter level of modulation, as in the case of the presence of phosphorylation deficient mutants, alters the overall effect of Iso stimulation on Kv11.1 currents in our experimental system, suggesting its functional relevance. However, the establishment of the precise physiological implications of the reported β_1 AR/14-3-3 ϵ association on Kv 11.1 function in the heart would require a better knowledge of the interaction dynamics, local stoichiometry, and compartmentalization of these proteins in cardiomyocytes.

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