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Signaling from β_1- and β_2-adrenergic receptors is defined by differential interactions with PDE4

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β_1- and β_2-adrenergic receptors (βARs) are highly homologous, yet they play clearly distinct roles in cardiac physiology and pathology. Myocyte contraction, for instance, is readily stimulated by β_1AR but not β_2AR signaling, and chronic stimulation of the two receptors has opposing effects on myocyte apoptosis and cell survival. Differences in the assembly of macromolecular signaling complexes may explain the distinct biological outcomes. Here, we demonstrate that β_1AR forms a signaling complex with a cAMP-specific phosphodiesterase (PDE) in a manner inherently different from a β_2AR/β-arrestin/PDE complex reported previously. The β_1AR binds a PDE variant, PDE4D8, in a direct manner, and occupancy of the receptor by an agonist causes dissociation of this complex. Conversely, agonist binding to the β_2AR is a prerequisite for the recruitment of a complex consisting of β-arrestin and the PDE4D variant, PDE4D5, to the receptor. We propose that the distinct modes of interaction with PDEs result in divergent cAMP signals in the vicinity of the two receptors, thus, providing an additional layer of complexity to enforce the specificity of β_1- and β_2-adrenoceptor signaling.

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Introduction

To meet the increased metabolic demands of stress or exercise, the sympathetic nervous system stimulates cardiac function through activation of the closely related β_1- and β_2-adrenergic receptors (β_1AR and β_2AR). Even though these highly homologous receptors both activate the G protein stimulatory for adenylyl cyclase (G_s), signaling through β_1AR and β_2AR produces clearly distinguishable biological effects (Xiang and Kobilka, 2003; Xiao *et al*, 2004). The β_1AR plays the dominant role in stimulating heart rate and strength of myocyte contraction, whereas β_2AR produces only modest chronotropic effects. Chronic stimulation of β_1AR produces myocyte hypertrophy and apoptosis, whereas β_2AR signaling promotes cell survival. The assembly of distinct macromolecular signaling complexes with transducer, scaffold, and effector proteins, which determine signaling properties and subcellular localization of the βARs, is thought to be at the core of the divergent properties of these receptors (Xiang and Kobilka, 2003; Xiao *et al*, 2004). Thus, understanding the differences of these receptor complexes has important pharmacological and clinical implications.

One of the emerging mechanisms that safeguard the specificity of G-protein-coupled receptor/cAMP signaling is the control of cAMP transients via degradation by cyclic nucleotide phosphodiesterases (PDEs) (Conti and Beavo, 2007). Biochemical, electrophysiological, and *in vivo* imaging studies are consolidating the idea that occupancy of different receptors generates a nonuniform pattern of activation of cAMP effector proteins such as PKA (cAMP-dependent protein kinase). PDEs play a critical role for the specificity in cAMP-signaling by preventing the free diffusion of cAMP, thus, effectively creating cyclic nucleotide microdomains and/or cAMP gradients that can be sensed by the cell (Zaccolo and Pozzan, 2002; Xiang *et al*, 2005; Fischmeister *et al*, 2006).

PDEs comprise a large group of over 20 genes that are divided into 11 PDE families based on their amino-acid sequence homology, substrate specificities, and pharmacological properties (Conti and Beavo, 2007). Each of the 11 PDE families encompasses one to four distinct genes. In addition, most PDE genes encode for multiple splicing variants through the use of multiple promoters and alternative splicing.

Previous studies indicated that occupancy of the β_2AR initiates the recruitment of a preformed complex consisting of β-arrestin and the cyclic AMP-specific PDE, PDE4D5 (Perry *et al*, 2002; Baillie *et al*, 2003). Conversely, no data are available on complexes between PDEs and the β_1AR even though it has been shown that PDE4 inhibitors potentiate cAMP accumulation induced by either β_1AR or β_2AR (Xiang *et al*, 2005). Here, we show that β_1AR forms a signaling

complex with a PDE4D splicing variant in a manner inherently different from the β_2AR/β-arrestin/PDE complex reported previously. Thus, this study challenges the assumption that the regulation of receptor signaling by PDEs described for the β_2AR also applies to β_1AR. We propose that the distinct modes of interaction with PDEs provide an additional layer of complexity to enforce the specificity of β_1- and β_2-adrenoceptor signaling.

Results

Detection of a β_1AR/PDE4D signaling complex in mouse neonatal cardiac myocytes

To probe for a possible signaling complex including the β_1AR and a PDE, mouse neonatal cardiomyocytes were infected with an adenovirus encoding a Flag-tagged β_1AR, and the receptor was subsequently immunoprecipitated using an antibody against the tag. A significant amount of endogenous PDE activity was recovered in the β_1AR immunoprecipitation (IP) pellet (Figure 1A). The PDE activity associated with the β_1AR was inhibited by the PDE4-selective inhibitor, Rolipram, identifying this activity as PDE4. Three PDE4 subtypes, PDE4A, PDE4B, and PDE4D, are expressed in neonatal cardiomyocytes at comparable levels (Figure 2B). The fourth gene, PDE4C, is not expressed in the heart and was not investigated. To assess which of the PDE4 subtypes contribute to the activity recovered in the β_1AR IP, cardiomyocytes deficient in PDE4A, PDE4B, and PDE4D were subjected to pull-down experiments. Whereas ablation of PDE4A or PDE4B had no effect, inactivation of the PDE4D gene prevented co-IP of PDE activity with the β_1AR (Figure 1B). Thus, PDE4D is the endogenous PDE recovered in complex with the receptor. This conclusion is further supported by western blot analysis of the immunoprecipitated PDE. A band immunoreactive with PDE4D-selective antibodies was consistently detected in the IP pellet (Figure 1C), and its mobility is consistent with that of a subset of PDE4D splicing variants that include PDE4D3, PDE4D8, and PDE4D9 (Richter *et al*, 2005). Together, these data suggest the presence of a signaling complex containing the β_1AR, a PDE4D isoform, and perhaps other components of the cAMP signaling pathway in cardiac myocytes.

Distinct PDE4D splice variants co-IP with the β_1AR

Through alternate splicing and the use of multiple promoters, nine different proteins, PDE4D1–9, originate from the PDE4D gene (Richter *et al*, 2005; Figure 2D). These proteins are identical in the catalytic domain and C-terminus but diverge at the N-terminus. Long forms contain a conserved UCR1/UCR2 (upstream conserved regions 1 and 2) motif, whereas short forms lack UCR1 and part of UCR2 (Conti *et al*, 2003; Houslay and Adams, 2003). Using antibodies raised against the unique N-terminus of each variant, we determined that PDE4D5, PDE4D8, and PDE4D9 are the splicing variants most abundant in cardiomyocytes, with trace amounts of PDE4D3 (Figure 2C). The co-IP of these PDE4D splice variants expressed exogenously in HEK293 cells identified PDE4D8 as the variant that most efficiently interacts with β_1AR. Other long PDE4D splice variants were also recovered in β_1AR IP pellets with the following rank order: PDE4D8 > PDE4D9 > PDE4D3 > PDE4D5 (Figure 3A and B). Conversely, the short PDE4D form, PDE4D2, did not co-IP with the β_1AR, indicating that the UCR domains unique to long PDE4 splice variants may contribute to the formation of the β_1AR/PDE4D complex.

PDE4D binds directly to the β_1AR

As previously reported, tethering of PDE4D5 to the β_2AR signaling complex is mediated by β-arrestins (Baillie *et al*, 2003). However, the co-IP of exogenous PDE4D and β_1AR from extracts of mouse embryonic fibroblasts (MEFs) deficient in β-arrestin 1 and 2 (Kohout *et al*, 2001) was not decreased compared with wild-type controls, suggesting that formation of the β_1AR/PDE4D complex is independent of β-arrestins (Figure 3C). To further characterize the interaction, we performed IPs using PDE4D and βARs that were purified from a baculovirus expression system to >90% purity (see Supplementary Figure 1 for the characterization of the purified proteins). In this paradigm, PDE4D shows robust association with β_1AR but not with β_2AR (Figure 3D

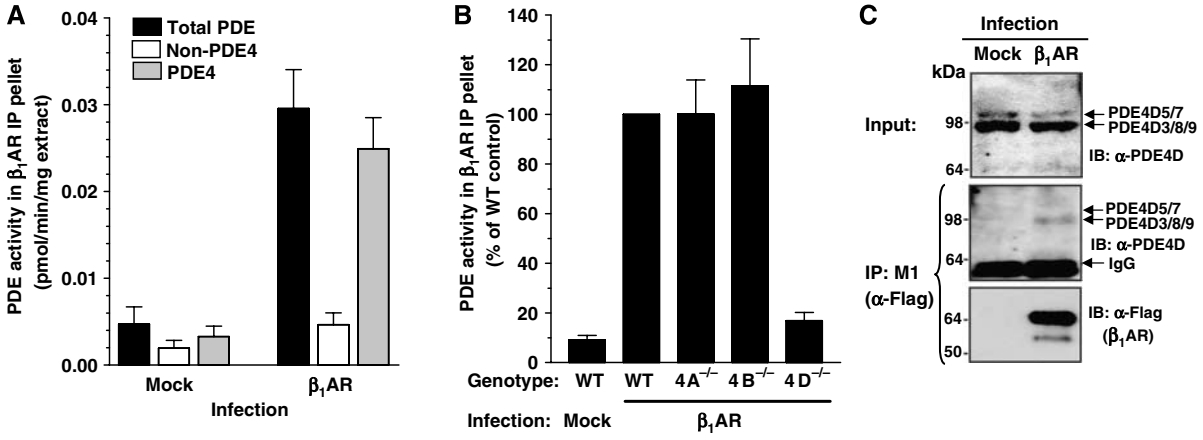

Figure 1 A β_1AR/PDE4D signaling complex in mouse neonatal cardiomyocytes. Shown are IPs of a Flag-tagged β_1AR from detergent extracts of mouse neonatal cardiomyocytes. (**A**) Total, non-PDE4, and PDE4 activity in the IP pellet. (**B**) Co-IP of β_1AR and PDE activity from cardiomyocytes deficient in PDE4A, PDE4B, or PDE4D, and wild-type controls. (**C**) Western blot with the β_1AR IP from wild-type myocytes. The migration of the PDE4D-immunoreactive band in the IP pellet corresponds to that of PDE4D splicing variants PDE4D3, PDE4D8, and PDE4D9. Data shown represent the means ± s.e.m. (A, B) or are representative (C) of at least three experiments performed.

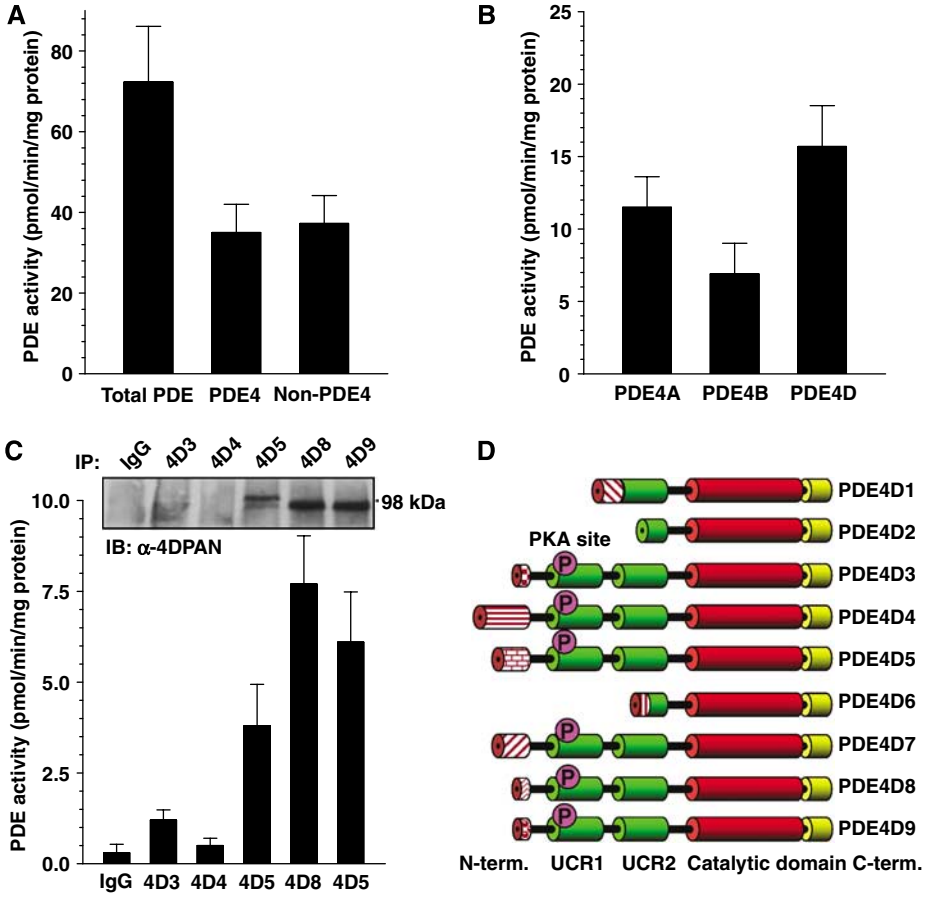

Figure 2 PDE4 subtypes and splice variants expressed in mouse neonatal cardiac myocytes. (**A**) Total, PDE4, and non-PDE4 activity in detergent extracts of cultured neonatal cardiac myocytes. PDE3 is the major non-PDE4 subtype expressed in these cells contributing 24 ± 5 pmol/min/mg to the total PDE activity. (**B, C**) Detergent extracts from neonatal cardiac myocytes were immunoprecipitated with PAN-selective antibodies for the PDE4 subtypes, PDE4A, PDE4B, and PDE4D (B), or with splice variant-selective anti-PDE4D antibodies (C). PDEs recovered in the IP pellet were detected by PDE activity assay (B, C) or western blotting (C). All results are expressed as the means $\pm$ s.e.m. of at least three experiments performed. (**D**) Schematic representation of the domain organization of PDE4D splice variants. Domains are depicted as barrels connected by wires (putative linker regions). The variants are distinguished into long forms (PDE4D3, 4, 5, 7, 8, and 9) and short forms (PDE4D1, 2, and 6) by the complete or partial presence of the UCR1/2 (upstream conserved regions 1 and 2) module (green barrels), respectively. The PKA phosphorylation site conserved among long splice variants is indicated with red circles.

and E). This confirms that β-arrestins are not required for the β_1AR/PDE4D complex. More importantly, this approach clearly indicates that PDE4D binds directly to the β_1AR but has no significant, or a much reduced affinity for β_2AR.

Binding of β-adrenergic agonists induces dissociation of the β_1AR/PDE4D complex

To determine whether receptor occupancy affects the β_1AR/PDE4D complex, HEK293 cells expressing exogenous β_1AR and PDE4D8 were incubated with different ligands. Treatment with the physiological β_1AR agonist, (−)-Norepinephrine (NorEpi), caused dissociation of the β_1AR/PDE4D complex (Figure 4A and B), whereas the stereoisomer, (+)-norepinephrine, which is a poor β_1AR ligand, had no effect. Dissociation of the β_1AR/PDE4D complex was observed also in cardiac myocytes and β-arrestin-deficient MEFs (Supplementary Figures 3 and 4) and occurred whether the overexpressed PDE4D was catalytically active or inactive (Supplementary Figure 5). Dissociation of the β_1AR/PDE4D complex by NorEpi binding is rapid ($T_{1/2} < 1$ min; Figure 4C and D) and dose-dependent (Figure 4E and F), reaching maximum at approximately 100 μM NorEpi. Thus, the concentration-dependence of dissociation of the β_1AR/PDE4D complex is comparable to that of receptor occupancy by NorEpi rather than that of receptor-induced cAMP production, which is in the nanomolar range. In addition, washout of the agonist results in β_1AR/PDE4D reassociation (data not shown). This dynamic, receptor occupancy-dependent regulation of β_1AR/PDE4D complex formation may explain why β_1AR/PDE4D dissociation is not complete and some portion of receptor/PDE complexes (~30%) remain at any given time point. Treatment with the β-adrenergic agonists, isoproterenol (ISO; 10 μM; see Supplementary Figure 5) or Epinephrine (100 μM; data not shown), also promoted dissociation of the β_1AR/PDE4D complex.

Selective activation of PDE4D splice variants upon stimulation of β_1AR and β_2AR

All PDE4 long forms are activated by phosphorylation at a conserved PKA consensus site in UCR1 (see Figure 2D); this mechanism provides a ubiquitous negative-feedback loop critical for cAMP signaling (Conti *et al*, 2003). Accordingly, stimulation of cultured neonatal cardiac myocytes with

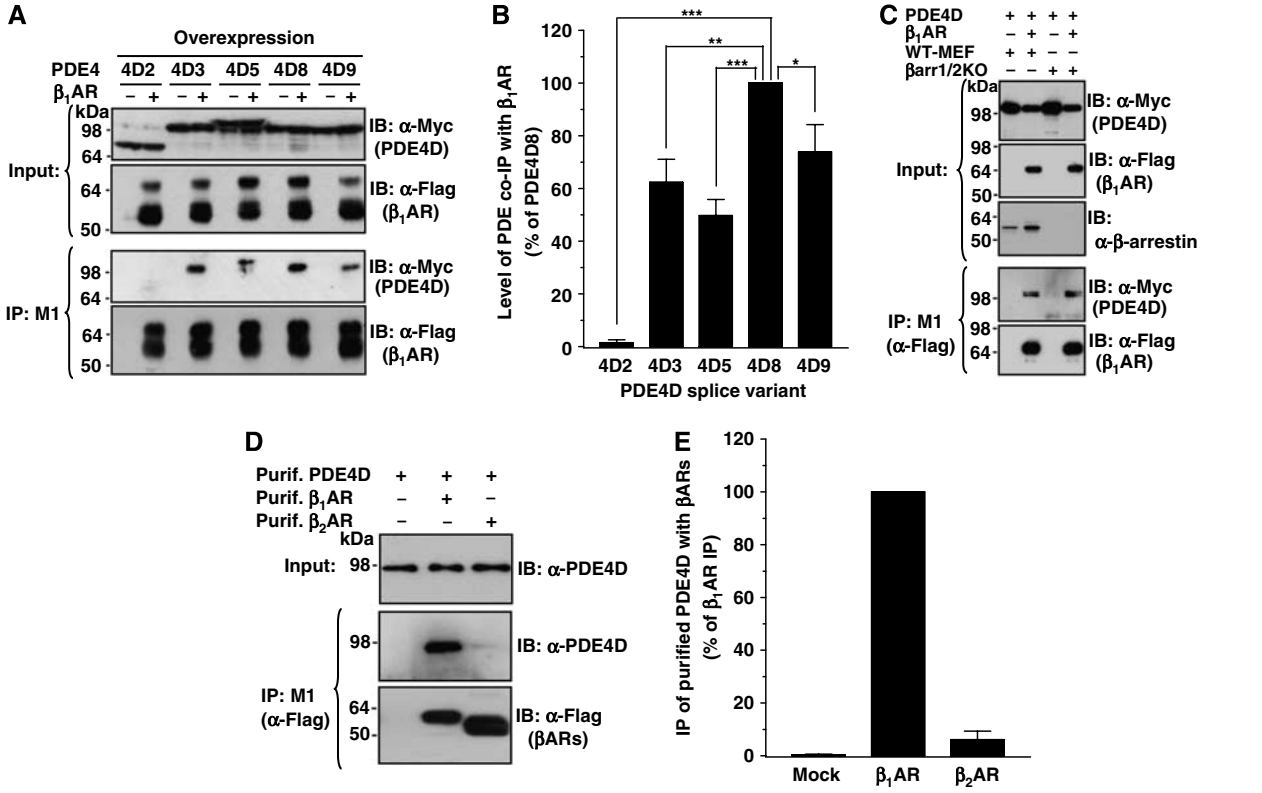

Figure 3 Interaction of exogenous β_1AR and PDE4D. (**A, B**) Co-IP of exogenous β_1AR and Myc-tagged PDE4D splice variants expressed in HEK293 cells. The efficiency with which β_1AR pulls down the different PDE4D splice variants is quantified in (B). (**C**) Shown is the co-IP of exogenous β_1AR and PDE4D8-Myc from extracts of MEFs derived from mice deficient in β-arrestin 1 and 2 (βarr1/2KO) or from wild-type controls (WT-MEF). (**D, E**) PDE4D3, and Flag-tagged receptors, β_1AR and β_2AR, were affinity purified after baculovirus expression (see Supplementary Figure 1). Purified PDE and (βARs) were then combined and the βARs immunoprecipitated. Quantification of PDE4D recovered in the βAR IP pellet is shown in (E). All data shown are representative of (A, C, D) or are expressed as the means ± s.e.m. (B, E) of at least three experiments performed. *($P<0.05$); **($P<0.005$); ***($P<0.0005$).

β-adrenergic agonists leads to a rapid PKA-mediated activation of PDE4D (Supplementary Figure 2A–C). If complexes composed of βARs and PDEs are present in these cells, phosphorylation should be biased toward the PDEs present in the vicinity of the occupied receptors. This is indeed the case when β_1AR- and β_2AR-stimulated phosphorylation of PDE4D isoforms was monitored. In cardiomyocytes lacking β_2AR, PDE4D8 was the PDE4D isoform predominantly activated after stimulation of β_1AR with ISO, with a limited activation of PDE4D9, and no significant effect on PDE4D5 (Figure 5A). Conversely, in myocytes lacking β_1AR, stimulation of β_2AR causes a selective increase in the activity of PDE4D5, with a less pronounced increase in PDE4D9, and no increase in PDE4D8 activity (Figure 5B). Importantly, upon stimulation with the adenylyl cyclase activator, Forskolin, all PDE4D isoforms show the same increase in activity in both cell types (Figure 5C), suggesting that loss of one βAR subtype or the other has not perturbed overall cAMP signaling. It also demonstrates that the spatial dimension of cAMP signaling is lost when generalized adenylyl cyclase activation is induced with Forskolin. The selective activation of PDE4D splicing variants by β_1AR and β_2AR signaling confirms the selectivity observed in the physical association of β_1AR with PDE4D8 (Figure 3A and B) and the preferential sequestration of PDE4D5 to the β_2AR by β-arrestin (Baillie *et al*, 2003). Because these experiments are with endogenous proteins, they strengthen our hypothesis of the presence of PDE4D variants in complex with β_1AR and β_2AR *in vivo*.

PDE4D controls the activity of PKA in the vicinity of the β_1AR

The presence of a PDE4D in the vicinity of the β_1AR should affect the activity of PKA localized with the receptor as well as the PKA-phosphorylation state of the receptor itself. This possibility was tested by blocking PDE activity with selective PDE4 inhibitors in cardiomyocytes (Figure 6A and B), by using MEFs deficient in PDE4D (Figure 6C and D), or by overexpressing a catalytically inactive PDE4D in cardiomyocytes, which acts as a dominant-negative construct (Perry *et al*, 2002; Baillie *et al*, 2003) by displacing endogenous PDE4D from the β_1AR complex (Figure 6E and F; Supplementary Figure 5). In all instances, blockage of PDE4 activity or, more specifically, ablation or displacement of PDE4D caused a significant increase in the phosphorylation of the transfected β_1AR in the absence of β-adrenergic agonists. It should be noted that inhibition of PDE3 activity or an overexpression of a dominant-negative PDE3A construct has no effect on β_1AR phosphorylation, confirming the specificity of the interactions. These findings indicate that PDE4D controls the access of cAMP to PKA localized with the β_1AR, effectively creating a domain with low basal cAMP/PKA activity. PDE4D also limits PKA-phosphorylation of β_1AR in response to low concentrations of β-adrenergic agonists that do not disrupt a large number of β_1AR/PDE4D complexes. This is likely due to the control of cAMP levels and PKA-activity in the vicinity of unoccupied, and thus, PDE4D-associated receptors in response to elevated cellular cAMP

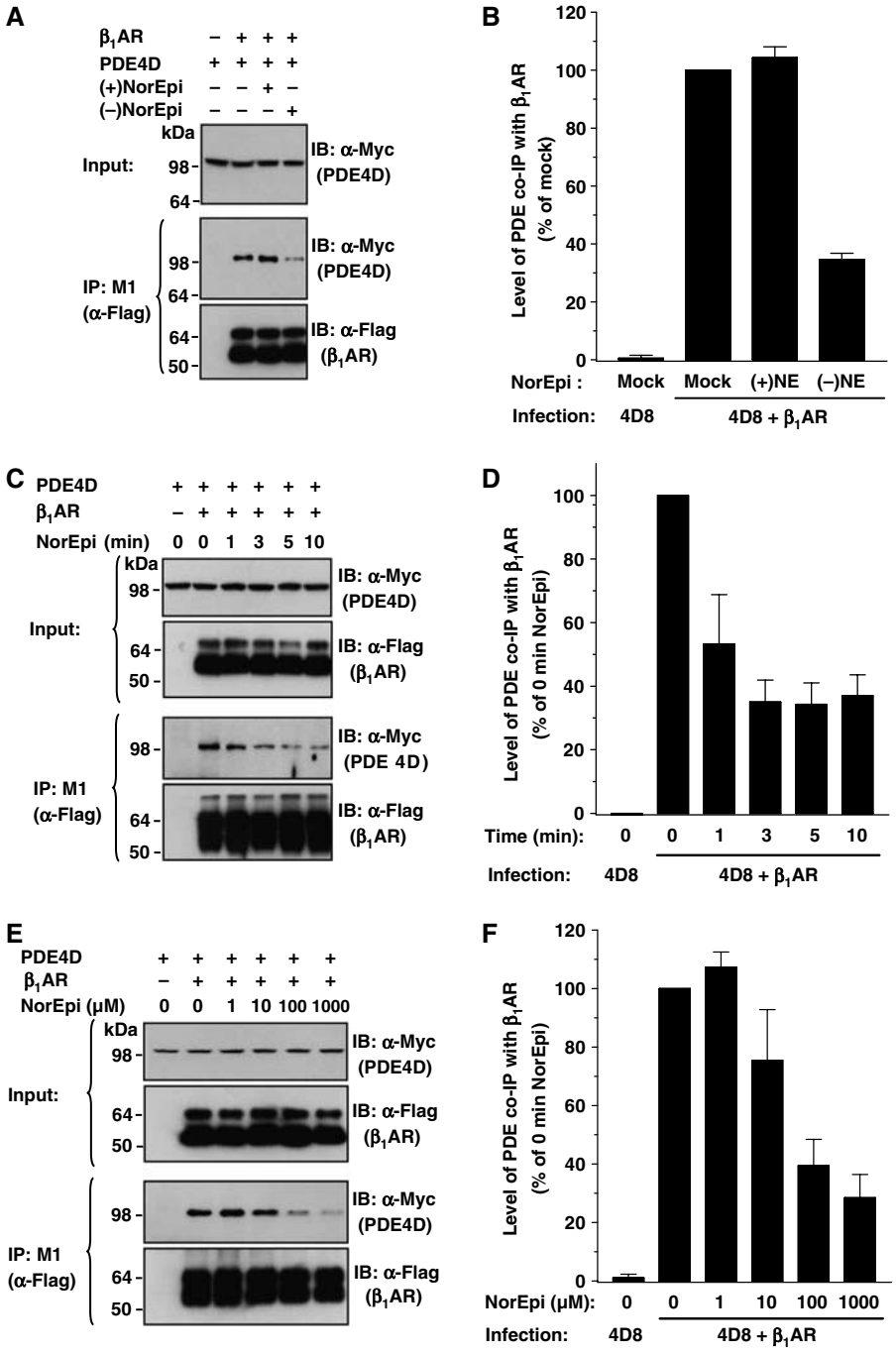

Figure 4 Binding of β-adrenergic agonists dissociates the β_1AR/PDE4D complex. HEK293 cells expressing exogenous β_1AR and PDE4D8-Myc were treated with β-adrenergic agonists before cell lysis and IP of the β_1AR. (**A**) Cells were treated for 10 min with 100 μM of the physiological β_1AR agonist (−)-Norepinephrine or the stereoisomer (+)-Norepinephrine, which is not an efficient ligand for the β_1AR. The amount of PDE4D recovered in the IP pellet is quantified in (**B**). (**C**–**F**) Time course and dose-dependency of the ligand-induced dissociation of the β_1AR/PDE4D complex. Cells were treated for various times with 100 μM NorEpi (C, D) or for 15 min with increasing concentrations of NorEpi (E, F) before cell lysis and β_1AR IP. The amount of PDE4D recovered in the IP pellet is quantified in (D, F). Data shown are representative of (A, C, E) or represent the means ± s.e.m. (B, D, F) of at least three experiments performed.

levels. These findings suggest a function of PDE4D in complex with the β_1AR in the intact cell.

PDE4D ablation promotes β_1AR desensitization in vivo

To assess the role of PDE4D in β_1AR function in a more physiological context, changes in the heart rate of mice in response to β-adrenergic stimulation were measured, as it is established that *in vivo* contraction rate is primarily controlled by β_1AR (Rohrer *et al*, 1996, 1999; Devic *et al*, 2001). Wild-type and PDE4DKO mice, matched by age, sex, and genetic background, were sedated using isoflurane. While their heart rate was continuously measured using a mouse pulse oximeter sensor, the mice were then injected with a submaximal concentration of ISO. An additional group of

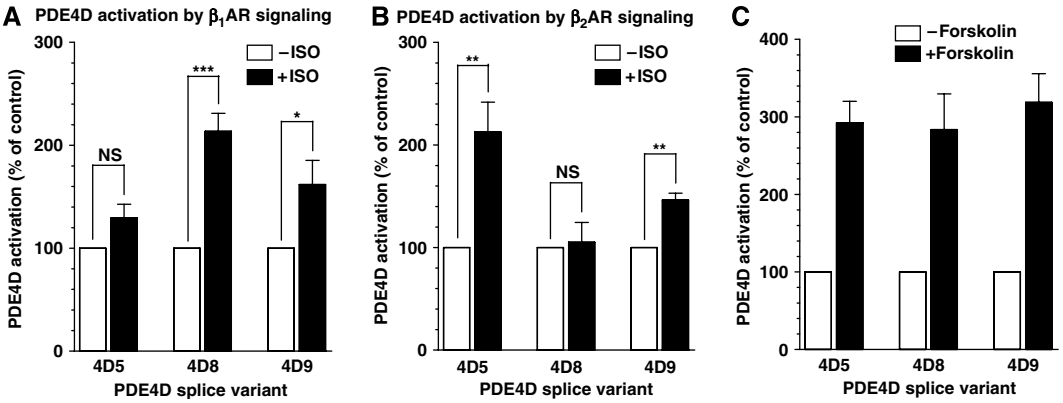

Figure 5 Selective activation of PDE4D splicing variants after stimulation of β_1AR and β_2AR. (**A, B**) Neonatal cardiac myocytes derived from mice deficient in β_2AR were stimulated for 3 min with 100 nM ISO (A) and cells deficient in β_1AR were treated for 3 min with 10 μM ISO (B). At the end of incubation, cells were lysed, PDE4D5, 8, and 9 were immunoprecipitated with variant-specific antibodies, and the PDE activity recovered in the IP pellet was measured. Data shown are expressed as the means ± s.e.m. of at least three experiments performed. (**C**) Activation of PDE4D splice variants after treatment of neonatal cardiac myocytes with 100 μM Forskolin for 20 min. Shown is the average of five experiments; three experiments performed using myocytes deficient in β_2AR and two experiments using cells deficient in β_1AR. NS ($P \geqslant 0.05$); *($P < 0.05$); **($P < 0.005$); ***($P < 0.0005$).

mice was first injected with glucagon-like peptide 1 (GLP1) to enhance the heterologous desensitization of β_1AR before the ISO injection. Wild-type and PDE4DKO mice showed no significant differences in basal heart rate (WT = 410 ± 52 and 4DKO = 386 ± 46 beats/min, means ± s.e.m.), the maximal heart rate after ISO injection (WT = 544 ± 34 and 4DKO = 515 ± 32 beats/min), the maximal heart rate after GLP1 injection (WT = 487 ± 14 and 4DKO = 448 ± 19 beats/min), or the maximal heart rate after sequential injection of GLP1 and ISO (WT = 539 ± 24 and 4DKO = 581 ± 17 beats/min). The rate of return to basal heart rate after the initial response to ISO was slightly faster in PDE4DKO mice compared with wild-type controls (Figure 7A); however, this effect was greatly magnified by pretreatment of mice with GLP1 (Figure 7B; $P < 0.0001$). The faster decrease in heart rate is in agreement with our stated hypothesis that elevated levels of cAMP/PKA activity in the vicinity of the β_1AR, due to absence of PDE4D in this compartment, causes an increased phosphorylation and heterologous desensitization of β_1AR (see Figure 6).

Discussion

With the above findings, we have identified a novel signaling complex that distinguishes β_1AR from β_2AR. Although both receptors are in complexes with PDEs, their interactions differ in terms of the PDE4D splice variant recruited to the receptor, the mode of interaction with the PDE4D variant, and the effect of receptor agonists on the complex (see the illustration in Figure 8). β_1AR preferentially associates with PDE4D8 in cardiomyocytes as shown by co-IP of endogenous PDE with the β_1AR (Figure 1C), as well as the selective activation of PDE4D8 in intact cells (Figure 5A). This preference of β_1AR for PDE4D8 was confirmed by co-IP experiments with exogenous proteins (Figure 3A and B). Conversely, PDE4D5 is the variant tethered to the β_2AR/β-arrestin complex (Baillie *et al*, 2003) concurring with the preferential activation of PDE4D5 upon β_2AR signaling (Figure 5B). In pull-down experiments using purified proteins (Figure 3D and E), β1AR efficiently interacts with PDE4D, whereas β_2AR has negligible affinity for PDE4D, underscoring the direct mode of PDE4D–β_1AR interaction versus the indirect, β-arrestin-dependent mode of PDE4D–β_2AR interaction. The most important difference regarding the function of βARs is the effect of receptor occupancy on the PDE4D complexes. The β_1AR/PDE4D complex is present in the absence of agonist and dissociates after receptor occupancy, whereas agonist binding to the β_2AR is a prerequisite for the recruitment of the β-arrestin/PDE4D complex to the receptor. Thus, under basal conditions, PDE4D is poised to control local cAMP concentration and PKA activity in the vicinity of the β_1AR (see Figure 6), whereas it affects β_2AR signaling only after ligand binding and β-arrestin recruitment. These differences likely impact the time course of cAMP accumulation in the vicinity of the receptors. We propose that these divergent interactions with PDE4D variants specify the property of the signals emanating from the two receptors.

Tethering of PDE4D to the β_1AR provides a means to target cAMP hydrolytic activity in the vicinity of the unoccupied receptor, thus, preventing a local increase in cAMP under basal conditions. This, in turn, protects the β_1AR from PKA-mediated phosphorylation and desensitization (Rapacciuolo *et al*, 2003; Gardner *et al*, 2006) and may control PKA-mediated phosphorylation of other localized substrates. Indeed, when PDE4 activity is inhibited in cardiomyocytes (Figure 6A and B), is absent, as in the PDE4D-deficient MEFs (Figure 6C and D), or is displaced, as in PDE4D-DN infected cardiomyocytes (Figure 6E and F), a substantial increase in basal β_1AR receptor phosphorylation is observed. In addition to controlling basal cAMP accumulation, targeting of PDE4D to the β_1AR may prevent heterologous desensitization of unoccupied receptors. Consistent with this idea, we report here that PDE4D deficiency accelerates the desensitization of β-adrenergic signals measured as changes in mouse heart rate in response to ISO injection (Figure 7). At the same time and given the observation that occupancy of the β_1AR causes dissociation of the complex, it is likely that the ligand-induced dissociation of PDE4D from the β_1AR cooperates to produce a localized increase in cAMP in the proximity of the occupied receptor. This event should decrease local cAMP

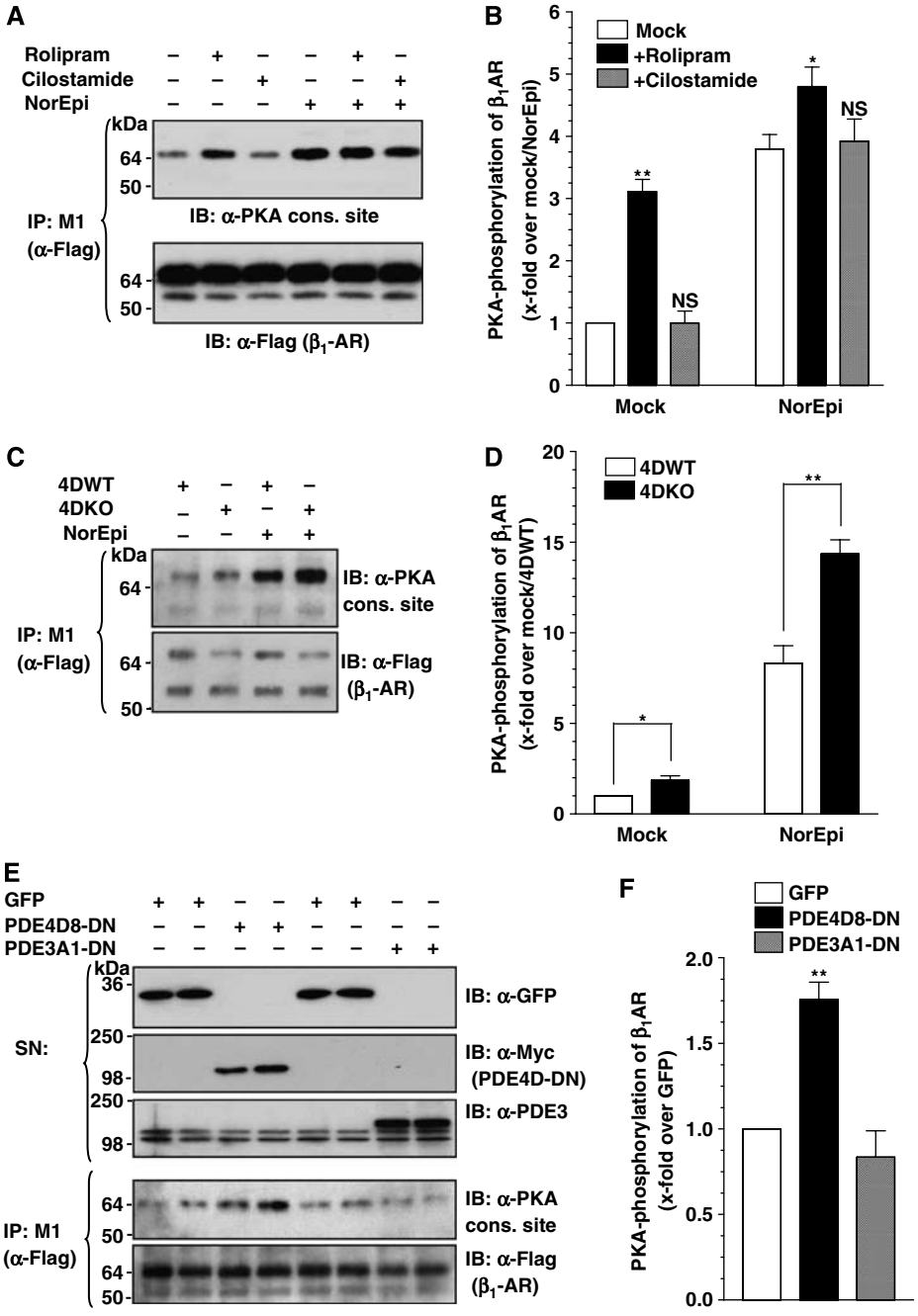

Figure 6 PDE4D in the β_1AR complex controls local PKA activity. (**A, B**) Neonatal cardiac myocytes expressing a Flag-tagged β_1AR were treated for 3 min with 100 nM Norepinephrine before cell lysis and IP with M1 (α-Flag) resin. The effect of a 5 min pre-treatment with 10 μM of the PDE4-specific inhibitor, Rolipram, or the PDE3-selective inhibitor, Cilostamide, on PKA-phosphorylation of the β_1AR is detected in IBs using a PKA-site-specific antibody. (**C, D**) MEFs derived from mice deficient in PDE4D or wild-type controls were infected with adenovirus to express a Flag-tagged β_1AR construct. At 40 h post-infection, cells were treated for 3 min with 100 nM Norepinephrine (NorEpi) before cell lysis and IP with M1 (α-Flag) resin. PKA-phosphorylation of the β_1AR is detected in IB using a PKA-site-specific antibody. (**E, F**) Neonatal cardiac myocytes coexpressing a Flag-tagged β_1AR and either GFP, a catalytically inactive PDE4D8 construct (PDE4D-DN; see also Supplementary Figure 5), or a catalytically inactive PDE3A1 (PDE3A1-DN) were subjected to α-Flag(M1)-IP, and the phosphorylation of the β_1AR was subsequently detected in IB using a PKA-substrate-specific antibody. Quantification of all results (B, D, F) is expressed as the means ± s.e.m. of three experiments performed. NS ($P \geqslant 0.05$; *($P < 0.05$); **($P < 0.005$).

degradation and therefore amplify the β_1-adrenergic signal locally. Consistent with this view, localized cAMP transients in the dyad space are elevated in the PDE4DKO mouse, whereas global cAMP signaling is not perturbed (Lehnart *et al*, 2005). The fate and function of the released PDE4D are to be determined and may provide a means to regulate a distinct pool of cAMP away from the membrane.

Using changes in the heart rate of mice in response to β-adrenergic stimulation as a read-out, we show here that PDE4D deficiency promotes an accelerated desensitization of β_1AR signaling. Although additional steps in excitation contraction coupling may also be affected by the absence of PDE4D, this observation is consistent with the idea that a major function of PDE4D in the β_1AR signaling complex is to

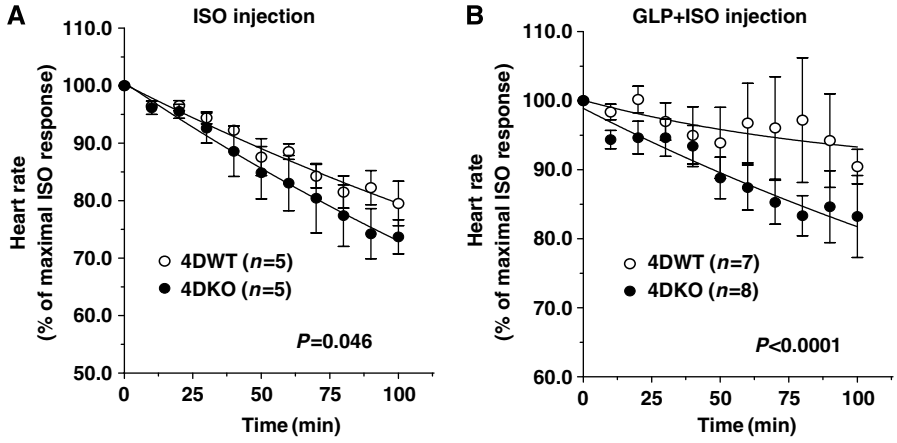

Figure 7 PDE4D ablation promotes desensitization of β_1AR signaling *in vivo*. Anesthetized mice were sequentially injected with GLP1 followed by a submaximal dose of ISO as described in Materials and methods and the heart rate of the animals was continuously recorded using pulse oximeter sensor (**B**). Control mice received ISO only (**A**). The decline in heart rate after ISO injection in PDE4DKO and wild-type control mice is reported. Data are expressed as percent of the initial, maximal heart rate in response to ISO injection. Number of mice used for each measurement is reported among brackets.

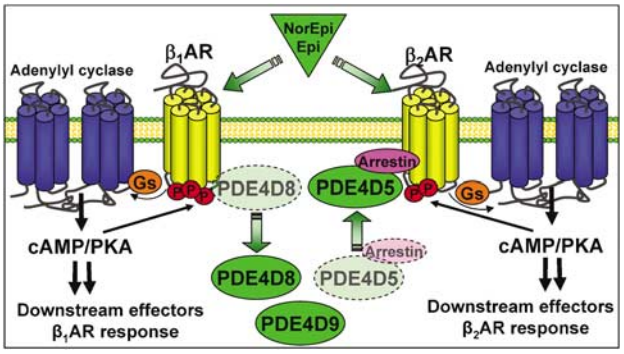

Figure 8 Schematic representation of the distinct modes of PDE4 interaction with βAR subtypes. Although both β_1AR and β_2AR form signaling complexes with PDE4D splice variants, the complexes formed by the two receptors are clearly distinguished by the different PDE4D variants recruited to the receptors (PDE4D8 to β_1AR versus PDE4D5 to β_2AR), the mode of interaction with PDE4D (direct for β_1AR versus β-arrestin mediated for β_2AR), and the effect of receptor agonists (induces dissociation or formation of the complex). In the case of β_1AR, a preformed complex with PDE4D8 that is likely responsible for controlling local cAMP concentration and PKA activity in the vicinity of the receptor under basal conditions is dissociated upon ligand binding. Conversely, β_2AR is not associated with PDE4D under basal conditions but a preformed complex consisting of β-arrestin and the PDE4D splice variant, PDE4D5, is recruited to the β_2AR after receptor occupancy. It remains to be determined to what extent PDE4D9, which is activated after both β_1AR and β_2AR stimulation (Figure 5) and which also showed interaction with β_1AR in co-IPs of exogenous proteins (Figure 3A and B), can substitute for interaction with the βARs *in vivo*.

protect the receptor from PKA-mediated heterologous desensitization. The direct interaction of PDE4D with the receptor, however, opens additional possibilities for a function of PDE4D in the β_1AR complex. These include a direct effect of PDE4D on the conformation of the receptor and a role of PDE4D in the assembly of macromolecular signaling complexes around the β_1AR. The latter may include the competition of PDE4D with other proteins for binding to the receptor or, in an opposite fashion, PDE4D may act as a scaffold by tethering additional proteins to the receptor complex such as the exchange protein activated by cAMP or PKA-anchoring proteins (Dodge-Kafka *et al*, 2005). These possibilities will be addressed in future studies.

Collectively, our findings demonstrate that stimulation of β_1AR and β_2AR has opposing effects on PDE4D recruitment in the membrane subdomain, with β_1AR occupancy causing a local decrease, whereas β_2AR promotes a local increase in PDE4. A critical role of PDE4s in the submembrane microdomain has been described using a modified CNG channel as a sensor for cAMP accumulation (Rich *et al*, 2001). The dynamic localization of different PDE4D splicing variants that we report, as well as their phosphorylation state, likely play a major role in controlling cAMP accumulation in this submembrane space. This view is consistent with the alterations in local cAMP accumulation observed in PDE4D-deficient cardiomyocytes (Lehnart *et al*, 2005). More importantly, the PDE4DKO mouse develops a late onset dilated cardiomyopathy and a propensity to arrhythmias during exercise (Lehnart *et al*, 2005). PDE4D-deficiency in the cardiac ryanodine receptor/calcium release channel (RyR2) complex has been associated with this phenotype. Lack of PDE4D causes PKA hyperphosphorylation of RyR2 and calstabin depletion from the channel, resulting in a 'leaky' RyR2 channel phenotype that, in turn, may contribute to cardiac arrhythmias. However, disruption of other PDE4D complexes in the PDE4DKO mouse most likely contributes to the development of the cardiac phenotype. The lack of PDE4D in the β_1AR complex and the aberrant β_1AR responses described in the present study may well contribute to the onset of hypertrophy and heart failure. The observed increased receptor phosphorylation that follows PDE4D deficiency (Figure 6) promotes progressive desensitization (Figure 7) and downregulation of this receptor, a hallmark in heart failure.

Materials and methods

Antibodies

Recombinant Flag-tagged βARs and Myc-tagged PDE4D splice variants were detected in western blots using antibodies against

their respective tags (mouse monoclonal α-Flag AB, Sigma Aldrich; mouse monoclonal α-Myc AB, Roche Applied Sciences). Endogenous (Figures 1C and 2C) and untagged PDE4D isoforms (Figure 3D; Supplementary Figures 1 and 2D) were detected using a mouse monoclonal PAN-PDE4D reactive antibody raised against the PDE4D C-terminus that is common to all splice variants (Icos4D). PAN-selective antibodies against PDE4A (AC55), PDE4B (K118), and PDE4D (M3S1), as well as splice variant-selective antibodies against PDE4D3, 4, 5, 8, and 9 (Richter *et al*, 2005), were used in IPs to determine the expression of the respective PDE4 subtype and splice variant in cardiac myocytes (Figure 2), as well as their PKA-dependent activation after β-adrenergic stimulation (Figure 5; Supplementary Figure 2). A PKA-site-specific antibody from Cell Signaling (Danvers, MA) was used to measure PKA phosphorylation of the β_1AR (Figure 6). Antibodies against β-arrestins and PDE3A were kindly provided by Dr R Lefkowitz and Dr C Yan, respectively.

Design of expression vectors

Cloning of the open reading frames of the nine rat PDE4D splice variants, PDE4D1–9, has been described previously (Richter *et al*, 2005). In the present study, these constructs were subcloned into the pAd/CMV/V5-DEST vector to generate adenoviruses encoding for C-terminally Myc-tagged PDE4D variants using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA). Constructs encoding catalytically inactive PDE4D variants were generated by mutation of a critical histidine residue in the catalytic site of PDE4D to alanine (His326 in PDE4D3). Adenoviruses encoding Flag-tagged β_1AR and β_2AR were generated using the pAdenovator system (Q-Biogene, Irvine, CA) according to the manufacturer's instructions.

Cell culture and adenovirus infection

Ventricular cardiac myocytes were isolated from the excised hearts of 1–2 day old neonatal mice as described previously (Devic *et al*, 2001). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Nu Serum IV (BD Falcon), 5% fetal bovine serum (FBS), 1 mM glutamine, 20 μg/ml gentamycin, and 1 × ITS media supplement (Sigma) on plates precoated with 10 μg/ml laminin. Experiments were carried out on day 4 of culture. The use of animals for the experiments followed Stanford University guidelines and all experiments involving animals were approved by the Stanford University Administrative Panel on Laboratory Animal Care. HEK293 and MEF cells were cultured in DMEM supplemented with 10% FBS, 1 mM glutamine, 30 μg/ml penicillin, and 100 μg/ml streptomycin. All cells were cultured at 37°C and under a 5% CO_2 atmosphere. For expression of exogenous βARs and/or PDE4D constructs, cells were infected with adenoviruses 40 h before experimentation at an MOI of 2–6 (HEK293), 20–40 (cardiac myocytes), and 100 (MEFs), respectively. As 'mock' controls, cells were infected with comparable titers of an adenovirus encoding green fluorescent protein (GFP).

Immunoprecipitation of Flag-tagged receptors from cell lysates

After the respective cell treatment, cells were rinsed once with ice-cold PBS and then lysed in 500 μl of 20 mM HEPES, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% *N*-dodecyl-β-D-maltopyranoside (DDM, Anatrace), 1 μM microcystin-LR (Calbiochem), and Complete protease inhibitor cocktail (Roche). Lysates were rotated at 4°C for 1 h, followed by centrifugation in 14 000 r.p.m. at 4°C for 20 min. Soluble extracts were precleared by a 30-min incubation with 30 μl of ProteinG Sepharose. Flag-tagged receptors were then immunoprecipitated using M1-affinity resin (α-Flag antibody resin; Sigma Aldrich). After incubation for 4 h at 4°C, the resin was washed three times and proteins were eluted in 40 μl of elution buffer (200 μg/ml Flag peptide, 20 mM HEPES, 50 mM NaCl, 0.1% cholesterol, and 8 mM EDTA).

IP of purified PDE4D and βARs

Rat PDE4D3, expressed in Sf9 insect cells using a recombinant baculovirus, was purified to >90% purity using an anti-PDE4D antibody (M3S1) covalently coupled to ProteinG Sepharose as described previously (Salanova *et al*, 1998). Flag-tagged β_1AR and β_2AR were also expressed in Sf9 cells and subsequently purified in a two-step procedure consisting of an initial affinity chromatography using M1-resin (immobilized anti-Flag antibody; Sigma Aldrich), followed by an alprenolol-sepharose affinity column. For βAR/PDE4D IP, equal amounts of purified β_1AR and β_2AR (1 μg) were coupled to M1 resin and then incubated in 500 μl of 20 mM HEPES (pH 7.5), 100 mM NaCl, 0.1% DDM, 4 mM $CaCl_2$, and 0.01% cholesterol hemisuccinate with 0.5 μg of purified PDE4D under continuous rotation for 4 h at 4°C. Afterwards, the resin was washed three times and proteins were eluted with Flag peptide as described above.

IP of PDE4 subtypes and splice variants from cell extracts

After 4 days of culture and the respective cell treatment, neonatal cardiac myocytes were harvested in buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 1 μM microcystin-LR, Complete protease inhibitor cocktail (Roche Diagnostics), and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; Roche Diagnostics). Cell debris was pelleted (14 000 r.p.m. for 30 min), and soluble extracts were immunoprecipitated using 30 μl ProteinG Sepharose and the respective PDE4 subtype, or PDE4D splice variant antibodies, as well as IgG as a control. After incubation for 2 h at 4°C, the resin was washed three times, and PDE recovered in the pellet was detected by PDE activity assay or western blotting.

PDE assay

PDE activity was measured as described in detail previously (Richter and Conti, 2002). PDE4 and PDE3 activity are defined as the PDE activity inhibited by the PDE4-selective inhibitor, Rolipram (10 μM), or the PDE3-selective inhibitors, Cilostamide or Milrinone (both 10 μM), respectively.

Noninvasive measurement of heart rate in mice

Spontaneously breathing animals were induced with 2% isoflurane and anesthesia was maintained thereafter using 1.25% isoflurane. A mouse pulse oximeter sensor (Mouse Ox, Starr Life Science Corp., Allison Park, PA) was then placed on the thigh of the mouse, and baseline heart-rate data were recorded for 5 min. To stimulate heterologous βAR desensitization, GLP1 (3 μg in 250 μl saline; Sigma, St Louis, MO) was then given by tail vein injection. When the heart rate in response to GLP1 injection had peaked (determined as a return from maximal change in heart rate by maximal 30%), a submaximal dose of (−)-ISO bitartrate (1.5–3.0 μg in 250 μl saline; Sigma, St Louis, MO) was given by intraperitoneal injection. Data were then acquired until heart rate returned to the animal's baseline. The data collected was subsequently analyzed using Windaq waveform Browser software (DATAQ Instruments). PDE4D-KO and wild-type control mice used in this study were on the same genetic background and were matched by sex (six males and one female each) and age (average age = 7.3 ± 2.8 months).

Data analysis

Unless otherwise noted, all graphs show the mean ± s.e.m. of at least three experiments performed. Statistical significance was determined using Student's *t*-test and is indicated as follows: NS ($P \geqslant 0.05$); *($P < 0.05$); **($P < 0.005$); ***($P < 0.0005$). The GraphPad Prism program (GraphPad Inc., San Diego, CA) was used for all statistical analyses. For quantification of western blot bands, blots were scanned and the signal intensity of the immunoreactive bands was quantified as previously described (Richter and Conti, 2004) using the ScionImage software program (Frederick, MD).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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