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β-Adrenergic Receptor Subtype-Specific Signaling in Cardiac Myocytes from β_1 and β_2 Adrenoceptor Knockout Mice

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ABSTRACT

The sympathetic nervous system modulates cardiac contractility and rate by activating β-adrenergic receptors (βAR) expressed on cardiac myocytes and specialized cells in the sinoatrial node and the conduction system. Recent clinical studies have suggested that β-adrenergic receptors also play a role in cardiac remodeling that occurs in the pathogenesis of cardiomyopathy. Both β_1 and β_2 adrenergic receptors are expressed in human and murine hearts. We have examined the effect of βAR activation on the spontaneous contraction rate of neonatal myocyte cultures from wild-type and β receptor knockout (KO) mice (β_1AR-KO, β_2AR-KO and $\beta_1\beta_2$AR-KO mice). Stimulation of the β_1AR in β_2AR-KO myocytes produces the greatest increase in contraction rate through a signaling pathway that requires protein kinase A (PKA) activation. In contrast, stimulation of the β_2AR in β_1AR-KO myocytes results in a biphasic effect on contraction rate with an initial increase in rate that does not require PKA, followed by a decrease in rate that involves coupling to a pertussis toxin sensitive G protein. A small isoproterenol-induced decrease in contraction rate observed in $\beta_1\beta_2$AR-KO myocytes can be attributed to the β_3AR. These studies show that all three βAR subtypes are expressed in neonatal cardiac myocytes, and the β_1AR and β_2AR couple to distinct signaling pathways.

β-Adrenergic receptors (βAR) have been among the most extensively studied members of the G protein-coupled receptor (GPCR) family. βAR activation of adenylyl cyclase is one of the first hormone-activated GPCR pathways to be characterized, and these receptors are therapeutic targets for a variety of clinical conditions including hypertension, coronary artery disease, heart failure, and asthma. Three βAR subtypes have been cloned (β_1AR, β_2AR, and β_3AR). The β_1AR and β_2AR are pharmacologically more similar to each other than they are to the β_3AR. All three receptor subtypes are highly conserved across species, suggesting that both sequence similarities and differences between subtypes are physiologically important. The close structural and functional properties of the β_1AR and β_2AR are paradigmatic of many other GPCR families where two or more receptor subtypes respond to the same hormone and couple to the same effector systems. However, although β_1AR and β_2AR have very similar signaling properties when expressed in undifferentiated cell lines (Green et al., 1992), there is a growing body of experimental evidence suggesting that they have different signaling properties in differentiated cells in vivo (Aprigliano et al., 1997; Zhou et al., 1997). Moreover, these receptors may differ in other functional parameters such as desensitization (Michel et al., 1990). Thus, characterizing the functional differences between these highly homologous receptors in the context of differentiated cells will shed light on their physiologic function and the consequence of pharmacologic manipulation in vivo.

We have been interested understanding the specific role of β_1 and β_2AR subtypes in regulating cardiac myocyte function. Both subtypes are expressed in human and murine hearts. Our previous in vivo studies on β_1AR knockout (β_1AR-KO) mice, β_2AR knockout (β_2AR-KO) mice and $\beta_1\beta_2$AR double knockout ($\beta_1\beta_2$AR-KO) mice demonstrated that the β_1AR is primarily responsible for catecholamine-induced changes in heart rate and contractility (Rohrer et al., 1996, 1999; Chruscinski et al., 1999). In an effort to understand the functional role of β_2AR in the heart, we chose to study receptor subtype-specific signaling in cultured neonatal myocytes. This experimental system has several experimental advantages. These cells contract spontaneously and cultures can be maintained for up to 1 week. The contraction rate of cultured myocytes is responsive to catecholamines added to the culture medium. Thus, by studying receptor-modulated contraction rate, we are able to examine the integrated response to all of the signaling pathways activated by the complement of β adrenergic receptors natively expressed in these cells. By examining the effect of a nonselective βAR agonist in myocytes from β_1AR-KO, β_2AR-KO, and $\beta_1\beta_2$AR-

ABBREVIATIONS: βAR, β-Adrenergic receptor; GPCR, G protein-coupled receptor; KO, knockout; IBMX, isobutyl methylxanthine; PKI, protein kinase inhibitor; PTX, pertussis toxin; PKA, cAMP-dependent protein kinase A.

KO mice, we have been able to identify distinct signaling pathways for the β_1AR, β_2AR, and the β_3AR.

Materials and Methods

Mice. β_1AR-KO (Rohrer et al., 1996), β_2AR-KO (Chruscinski et al., 1999) and $\beta_1\beta_2$AR-KO (Rohrer et al., 1999) mice were all constructed by gene targeting as described.

Preparation of Cultured Neonatal Mouse Ventricular Myocytes. Spontaneously beating neonatal cardiac myocytes were prepared from hearts of 1- to 2-day-old mouse pups (from wild-type mice and from β_1AR-KO, β_2AR-KO, and β_1/β_2AR-KO mice). Briefly, hearts were quickly excised, the atria were cut off, then the ventricles were minced and digested at 37°C for 45 min in calcium-free HEPES-buffered Hanks' solution, pH 7.4, plus 100 μg/ml collagenase type II and 1X pancreatin (Invitrogen, Carlsbad, CA). To reduce the contribution of nonmyocardial cells, cells are preplated for 1 h. The myocyte-enriched cells remaining in suspension were plated in 35-mm tissue culture dishes for the contraction studies, or 12-well plates for cAMP accumulation assay. Culture dishes are precoated with 1.5% gelatin for 30 min. Myocytes were cultured in Dulbecco's modified Eagle's medium containing 10% horse serum, 5% fetal bovine serum, and antibiotics (1× gentamycin; Roche Molecular Biochemicals, Indianapolis, IN). Although the culture technique includes a preplating step that effectively decreases fibroblast contamination, myocytes are cultured in presence of 1× cytosine-β-D-arabinofuranoside (Sigma, St. Louis, MO) to block the cardiac fibroblast proliferation. Contraction rate and cAMP accumulation assays are performed in culture media containing serum and buffered with 20 mM HEPES, pH 7.4.

Measurement of Spontaneous Rate of Cardiac Myocyte Contraction. Measurement of spontaneous contraction rate was carried out as described previously (Johnson and Mochly-Rosen, 1995) with some modifications. Briefly, about 3×10^5 cardiac cells were cultured in 35-mm Petri dishes (Corning, Palo Alto, CA, as described above) to obtain a uniformly beating syncytium. On day 4, the culture dishes were placed in a temperature-regulation apparatus positioned on the stage of an inverted microscope (Nikon, Tokyo, Japan) connected to a video camera. Cells were equilibrated at 37°C for 10 min before monitoring the contraction rate. Contraction rates of cells within the syncytium were determined at 2- to 5-min intervals for 10 min before and 30 min after the addition of isoproterenol or CL 316243. All assays were recorded on videotape.

Measurement of cAMP Accumulation. To measure intracellular cAMP, myocytes were cultured in 12-well plates (5×10^5 cardiac cells per well). Cells were incubated for 30 min at 37°C with 1 mM isobutyl methylxanthine (IBMX; Sigma) immediately before the addition of the agonist isoproterenol. Cells were treated with different concentrations of isoproterenol for 5 to 15 min at room temperature. The assay was terminated by the aspiration of the incubation buffer and addition of 1 ml of 100% ethanol to each well. The cell lysates were then collected, boiled for 5 min, cooled, and stored at −80°C. Aliquots were dried in a spin-vacuum, and cAMP in the residue was determined using a radioimmunoassay (Amersham Pharmacia Biotech, Piscataway, NJ).

PTX Treatment. For contraction and cAMP measurement, cells were preincubated with PTX (0.75 mg/ml) at 37°C for at least 3 h as described previously (Xiao et al., 1995). Successful inactivation of inhibitory G proteins (Gi/Go) in PTX-treated cells was verified by the loss of the ability of adenosine (at 10^{-6} M) to decrease the basal contraction rate or to reverse the positive chronotropic effect of βAR stimulation by isoproterenol in WT myocytes.

PKI Treatment. Cells were preincubated with *myr* PKI_{14-22} (20 μM) at 37°C for 10 min before isoproterenol exposure.

Drugs. Isoproterenol, adenosine, and PTX were obtained from Sigma, CL-316243 was a kind gift of Wyeth Ayerst Laboratories (Philadelphia, PA), and *myr* PKI_{14-22} was obtained from Calbiochem (San Diego, CA).

Data Analysis. Time course experiments, two-way analysis of variance corrected for repeated measures was used to test for significance ($p < 0.05$) between groups. If the analysis of variance was significant, a *t* test using Bonferroni's method was used to compare responses at multiple time points of interest where maximal effects of treatments were observed. Analysis was done using Prism (GraphPad Software, Inc., San Diego, CA).

Results

Contraction Rate Response to Isoproterenol in Myocytes from Wild-Type and βAR Knockout Mice. Neonatal myocytes were harvested and maintained in culture for 4 days before contraction rate studies. Figure 1A shows the response of wild-type neonatal myocytes to increasing doses of the βAR selective agonist isoproterenol. We observed the maximal response with 10 μM isoproterenol. We also examined cAMP accumulation in cultures of wild-type, neonatal myocytes. In contrast to the relatively high concentrations of isoproterenol needed to stimulate contraction rate, elevations of intracellular cAMP could be observed with as little as 0.01 μM isoproterenol (Fig. 1B). This may be caused in part by the

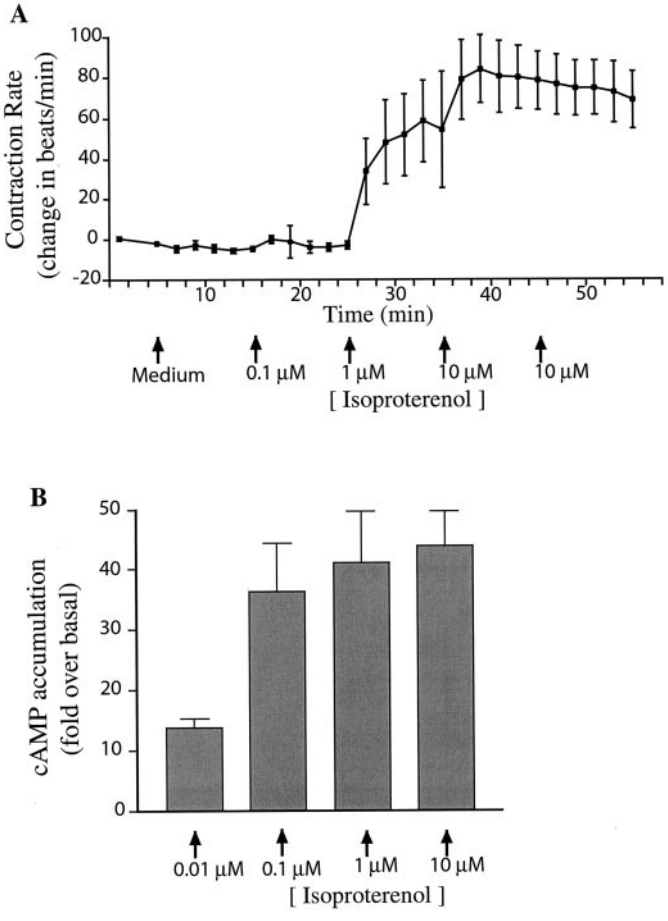

Fig. 1. Contraction rate of neonatal myocytes in culture. Myocytes from wild-type (WT), β_1AR-KO, β_2AR-KO, and $\beta_1\beta_2$AR-KO mice were cultured and intrinsic contraction rate was measured as described under *Materials and Methods*. A, neonatal myocytes from WT mice were exposed to increasing concentrations of isoproterenol. The maximum change in contraction rate relative to basal was observed with 10 μM isoproterenol. The data represent the mean ± S.E. of four experiments. B, cAMP accumulation in cultured neonatal myocytes from wild-type mice after a 5-min treatment with different concentrations of isoproterenol. The data represent the mean ± S.E. of three experiments done in triplicate.

inclusion of the phosphodiesterase inhibitor IBMX in the cAMP accumulation assays.

Figure 2A shows the change in contraction rate as a function of time after adding 10 μM isoproterenol to cultures of myocytes from wild-type, β_1AR-KO mice, β_2AR-KO mice and $\beta_1\beta_2$AR-KO mice. The maximal change in contraction rate induced by isoproterenol is shown in Fig. 2B. The baseline contraction rates of neonatal myocytes from the different strains are given in Table 1. As expected from our in vivo studies, we observe the greatest increase in myocyte contraction rate in wild-type myocytes and myocytes from β_2AR-KO mice, in which contraction rate reaches a maximum after 10 min of isoproterenol exposure then gradually declines toward baseline over the next 30 min. Most of the decline in contraction rate seems to be caused by desensitization to the agonist because a second addition of 10 μM isoproterenol at 30 min had little additional stimulatory effect (data not shown).

The increase in contraction rate of β_2AR-KO myocytes induced by isoproterenol is only slightly smaller than that observed for wild-type mice and the overall profile of contraction rate as a function of time is similar. In contrast, the effect of isoproterenol on the contraction rate from β_1AR knockout myocytes is relatively small and biphasic with an initial increase that peaks at 5 min followed by a sustained decrease. Not obvious from Fig. 2A is a small but reproducible decrease in contraction rate lasting 10 min in myocytes from $\beta_1\beta_2$AR-KO mice. This is examined in more detail below.

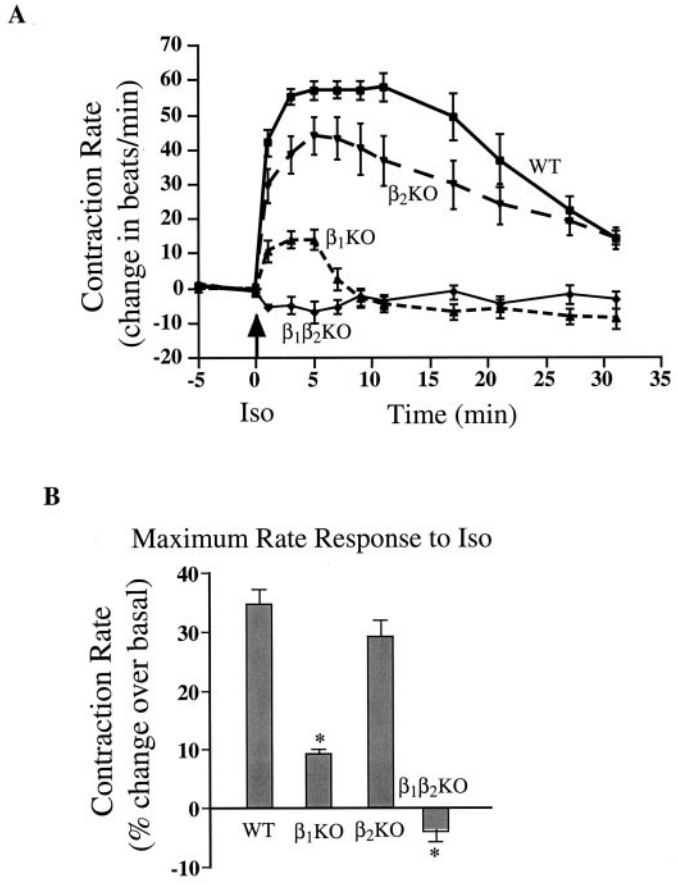

Fig. 2. A, the change in contraction rate in response to 10 μM isoproterenol in neonatal myocytes from WT, β_1AR-KO, β_2AR-KO, and $\beta_1\beta_2$AR-KO mice. The data represent the mean ± S.E. of n experiments (WT, $n = 8$; β_1AR-KO, $n = 10$; β_2AR-KO, $n = 9$; $\beta_1\beta_2$AR-KO, $n = 8$) from at least five different myocyte preparations. Individual myocyte cultures were used only once. B, maximum responses to 10 μM isoproterenol in neonatal myocytes from WT, β_1AR-KO, β_2AR-KO, and $\beta_1\beta_2$AR-KO mice. Maximal response were taken from experiments shown in Fig. 2A and compared by unpaired Student t test. $^*p < 0.05$, significant differences from wild-type.

Dual Coupling of β_2AR in Neonatal Myocytes. The biphasic contraction rate response after stimulation of β_2AR in neonatal myocytes from β_1AR KO mice suggests sequential coupling to different signaling pathways. We therefore examined the contribution of pertussis toxin-sensitive G proteins to isoproterenol-induced changes in contraction rate. Figure 3A shows the effectiveness of the pertussis toxin treatment protocol in wild-type myocytes. We observed no response to adenosine, either before or after exposure to 10 μM isoproterenol, in pertussis toxin-treated cells. We used this protocol to study the role of pertussis toxin-sensitive G proteins in isoproterenol-induced changes in contraction rate in β1AR knockout myocytes (Fig. 3B). Compared with control myocytes, the magnitude of the rate increase is greater and stimulatory phase is prolonged in pertussis toxin-treated myocytes. No decrease below baseline is observed. Interestingly, the increase in contraction rate after isoproterenol stimulation in β_1AR-KO myocytes in the presence of pertussis toxin attenuates more rapidly than the response in β_2AR-KO myocytes, suggesting that the β_2AR desensitizes more rapidly than does the β_1AR under these experimental conditions. Stimulation of contraction rate by β_1AR in β_2AR-KO myocytes was not significantly altered by pertussis toxin treatment (Fig. 3C).

Evidence for β_3AR in Neonatal Myocyte. We observe a small but reproducible decrease in contraction rate in myocytes from $\beta_1\beta_2$AR-KO mice. In myocytes treated with pertussis toxin, isoproterenol induced a brief (~10 min) and small stimulation of contraction rate (Fig. 4A). This observation suggested a possible role for the β_3AR. This was confirmed by treatment of $\beta_1\beta_2$AR-KO myocytes with the β_3AR selective agonist CL-316243, which produced a greater decrease in contraction rate in untreated myocytes than did isoproterenol. Moreover, CL-316243 stimulated an increase in contraction rate in pertussis toxin-treated myocytes similar to that stimulated by isoproterenol (Fig. 4B). Comparable inhibitory effects of CL-316243 could also be observed in myocytes from wild-type mice (Fig. 4C), indicating that the β_3AR response in $\beta_1\beta_2$AR-KO myocytes is not caused by up-regulation of β_3AR receptors as a consequence of the loss of β_1AR and β_2AR expression.

Differential Effect of PKA Inhibition on β_1AR and β_2AR Signaling in Neonatal Myocytes. Stimulation of both β_1AR and β_2AR increases contraction rate in neonatal myocytes and both β_1AR and β_2AR stimulate adenylyl cyclase in cultured myocytes. To determine the role of cAMP-dependent protein kinase A (PKA) in modulating contraction

TABLE 1

Basal contraction rate of neonatal myocytes in culture

Contraction rate was determined as described under *Materials and Methods* after myocytes had equilibrated on the heated microscope stage for 10 min.

Genotype	Basal Contraction Rate ± SEM	*N*
Wild-Type	167 ± 8	11
β_1AR-KO	192 ± 10	10
β_2AR-KO	168 ± 7	9
$\beta_1\beta_2$AR-KO	176 ± 9	9

rate, we examined the effect of the PKA specific inhibitor PKI. As shown in Fig. 5A, PKI had a dramatic inhibitory effect on isoproterenol-stimulated contraction rate in β_2AR-KO myocytes. In contrast, the stimulation of contraction rate was slightly increased and more prolonged in β_1AR-KO myocytes after PKI treatment (Fig. 5B). Surprisingly, the magnitude of the inhibition of contraction rate was also more profound in PKI-treated β_1AR-KO myocytes at 30 min after ISO addition. PKI had no significant effect on the response to isoproterenol in $\beta_1\beta_2$AR-KO myocytes (Fig. 5C).

cAMP Levels Do Not Predict the Coupling Behavior of the β_2AR in β_1AR-KO Myocytes. The biphasic response of β_1AR-KO myocyte contraction rate to stimulation by isoproterenol suggests that the β_2AR initially couples predominantly to Gs, but switches predominantly to Gi after ~15 min. To determine the role of cAMP in the biphasic modulation of contraction rate, we examined cAMP levels in β_1AR-KO myocytes at 5 and 15 min after isoproterenol stimulation. We were unable to detect cAMP accumulation in the absence of the phosphodiesterase inhibitor IBMX; therefore, the cAMP accumulation experiments do not exactly reproduce the conditions used for the contraction rate experiments. Nevertheless, we expected to see differences in accumulation following pertussis toxin treatment. As shown in Fig. 6, in control myocytes, the cAMP accumulation at 15 min was only slightly greater than at 5 min, consistent with a switch in coupling from Gs to Gi at 15 min. However, pertussis toxin treatment did not increase the cAMP accumulation at 15

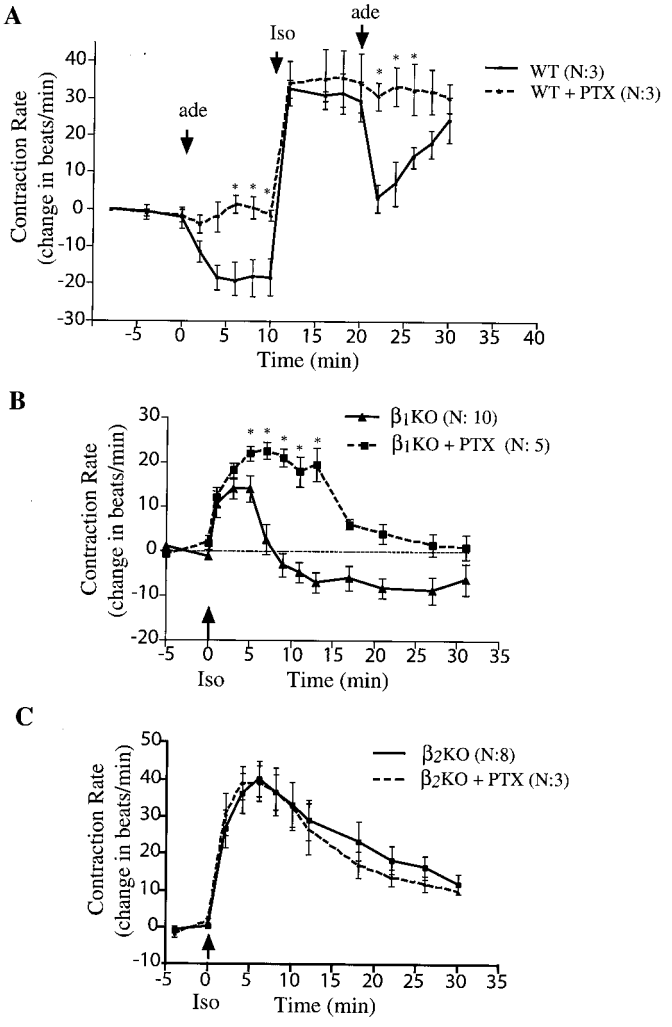

Fig. 3. The effect of pertussis toxin treatment on the isoproterenol-stimulated contraction rate of neonatal myocytes. Neonatal myocytes were cultured and treated with pertussis toxin (PTX) 3 h before contraction rate experiments as described under *Materials and Methods*. The basal contraction rate of myocytes was not altered significantly by PTX treatment. A, experiments on wild-type myocytes show that pertussis toxin treatment effectively blocks the inhibitory effect of adenosine (ade) on contraction rate before and after exposure to isoproterenol (Iso). B, the effect of pertussis toxin treatment on isoproterenol-induced changes in contraction rate in myocytes from β_1AR-KO mice. C, the effect of pertussis toxin treatment on isoproterenol-induced changes in contraction rate in myocytes from β2AR-KO mice. The data represent the mean ± S.E. of N experiments from at least three different myocyte preparations. $*p < 0.05$; a Bonferroni's t test was performed on curves that were found to be significantly different by a two-way analysis of variance corrected for repeated measures.

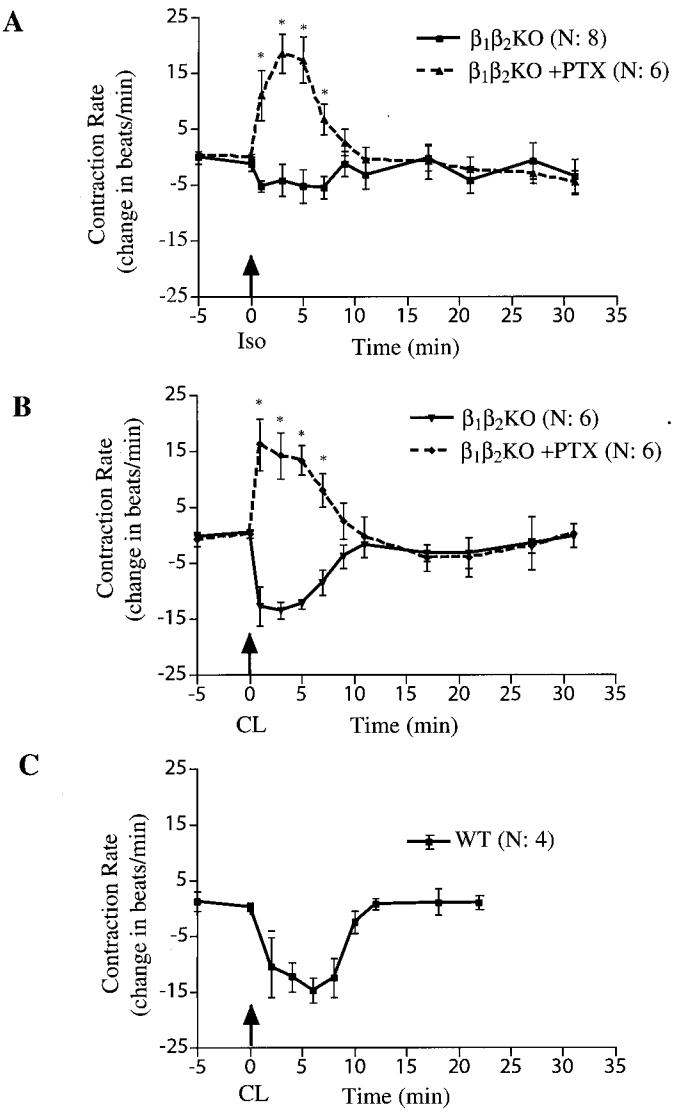

Fig. 4. Evidence for β_3AR receptor signaling in wild-type and $\beta_1\beta_2$AR-KO mice. A and B, the effect of pertussis toxin treatment on the contraction rate of neonatal myocytes from $\beta_1\beta_2$AR-KO mice. Cultures were treated with 10 μM isoproterenol (A) or 10 μM CL316243 (B, a β_3AR-selective agonist) at time = 0 min. C, The effect of CL316243 on the contraction rate of neonatal myocytes from wild-type mice. The data represent the mean ± S.E. of N experiments from at least two different myocyte preparations. $*p < 0.05$; a Bonferroni's t test was performed on curves that were found to be significantly different by a two-way analysis of variance corrected for repeated measures.

min. Therefore, the cAMP studies do not predict the contraction rate behavior observed in Fig. 3B.

Discussion

Our studies indicate that all three βAR subtypes are expressed in neonatal myocytes and demonstrate that each subtype couples to distinct signaling pathways that influence the rate of spontaneous beating of myocytes in culture. Stimulation of the β_1AR produces the greatest chronotropic effect through a PKA-dependent mechanism. The inhibition of contraction rate by the PKA inhibitor PKI suggests that PKA phosphorylation of the L-type calcium channel (Yue et al., 1990) is a likely mechanism for the increase in contraction rate mediated by the β_1AR. The difference in maximal contraction rate between wild-type and β_2AR-KO myocytes (Fig. 2) was not found to be significant, but the observed trend could be explained by the loss of the stimulatory component of β_2AR activation. The mechanism by which the β_2AR stimulates contraction rate during the first 10 min after agonist activation seems to differ from that used by the β_1AR. Although β_2AR stimulation causes a rise in intracellular cAMP when assayed using a cAMP accumulation assay, β_2AR stimulation of rate is insensitive to PKI (Fig. 5). The stimulation of contraction rate by the β_2AR may involve direct interactions between Gs and a channel (Imoto et al., 1988; Yatani and Brown, 1989; Yatani et al., 1990). It is also possible that the β_2AR stimulates contraction rate via the cAMP-sensitive, nonselective cation channel (pacemaker channel, I_f channel) (Ludwig et al., 1998). The lack of effect of PKI on β_2AR stimulated contraction rate suggests that the increase in cAMP induced by β_2AR stimulation is physically compartmentalized and either does not activate PKA or the PKA activated after β_2AR stimulation does not have access to the L-type calcium channel. Evidence for compartmentalization of cAMP signaling has been observed in adult rat myocytes (Zhou et al., 1997; Kuschel et al., 1999a) and adult canine myocytes (Kuschel et al., 1999b) where both β_1AR and β_2AR stimulate cAMP accumulation, but only β_1 activation leads to phosphorylation of phospholamban by PKA.

Several studies have implicated caveolae as potential signaling domains for β-adrenergic receptors. Both β_1AR and β_2AR co-purify with caveolin in sucrose gradient fractions from COS-7 cells overexpressing the receptors (Schwencke et al., 1999). These results suggest that caveolar localization would not be a mechanism for differential signaling by the β_1AR and β_2AR. Yet, this may be attributed to overexpression of recombinant receptors in a highly undifferentiated cell line. Studies in cultured cardiac myocytes provide more support for compartmentalization of signaling components. Adenylyl cyclase type 6 and β_1AR were both preferentially localized to caveolae when overexpressed by recombinant adenovirus in neonatal rat cardiac myocytes (Ostrom et al., 2000). However, studies of native receptors in neonatal rat

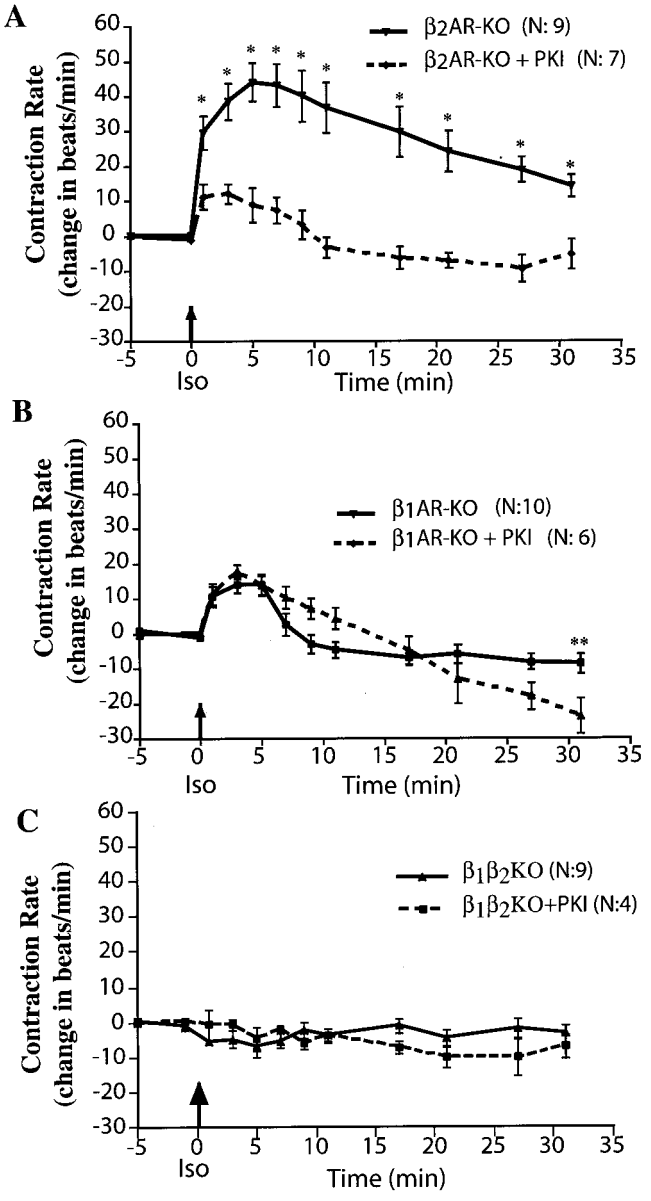

Fig. 5. The effect of the PKA inhibitor PKI on the contraction rate of neonatal myocytes from β_2AR-KO mice (A), β_1AR-KO mice (B), and $\beta_1\beta_2$AR-KO mice (C). Neonatal myocyte cultures were treated with 20 μM PKI 10 min before the addition of 10 μM isoproterenol as described under *Materials and Methods*. The data represent the mean ± S.E. of N experiments from at least two different myocyte preparations. *$p < 0.05$; a Bonferroni's t test was performed on curves that were found to be significantly different by a two-way analysis of variance corrected for repeated measures. **$p < 0.01$; significance by unpaired Student t test. Note that the curves in B were not found to be significantly different by two-way analysis of variance; however, the effect of PKI on the maximal isoproterenol-induced inhibition of contraction rate (**) in β_1AR-KO myocytes was examined by an unpaired Student t test and found to be significant ($p < 0.005$).

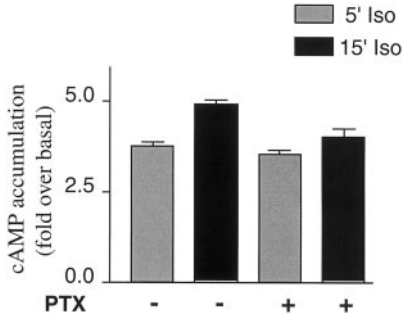

Fig. 6. cAMP accumulation in neonatal myocytes from β_1AR KO mice. Myocyte cultures were treated with pertussis toxin (PTX) or vehicle control for 3 h before the addition of 10 μM isoproterenol. cAMP accumulation was determined at 5 and 15 min as described under *Materials and Methods*. The data represent the average of three experiments done in triplicate.

cardiac myocytes provide evidence for preferential localization of β_2AR in caveolae, whereas the majority of β_1ARs were found in noncoveolar membrane fractions (Rybin et al., 2000).

The inhibitory effect of β_2AR activation on contraction rate can be abolished by pertussis toxin (Fig. 3), indicating that β_2ARs are capable of coupling to Gi/o proteins in cardiac myocytes. Other investigators have failed to detect evidence for β_2AR coupling to Gi proteins in neonatal murine cardiac myocytes as assayed by cAMP accumulation, calcium transients, and contractile function (Sabri et al., 2000). These conflicting results can be explained by the fact that different functional assays are used. More likely, Gi coupling may be observed only when β_2ARs are maximally stimulated by a full agonist. This is technically difficult to do in wild-type myocytes without also activating β_1ARs. Selective activation of only β_2ARs in wild-type mice requires submaximal doses of subtype-selective agonists such as zinterol, which is a partial agonist relative to isoproterenol. By using neonatal myocytes from β_1AR-KO mice, we are able to use maximal doses of the full agonist isoproterenol and observe the relatively subtle effects of β_2AR coupling to Gi on myocyte contraction rate.

Of particular interest is the biphasic coupling of the β_2AR, with predominant Gs coupling during the first 10 min of stimulation and predominant Gi coupling from 15 min onward. Dual coupling of the β_2AR to both Gs and Gi has been observed in 293 cells (Daaka et al., 1997) and in adult cardiac myocytes (Xiao et al., 1995, 1999a; Communal et al., 1999), but this is the first demonstration of sequential Gs, Gi coupling in cardiac myocytes. Moreover, the mechanism of the sequential coupling in 293 cells appears to differ from that in murine neonatal myocytes. In 293 cells evidence suggests that the β_2AR coupling to Gi requires PKA phosphorylation of the β_2AR (Daaka et al., 1997). Based on this model one would expect that treatment of neonatal myocytes from β1AR-KO mice with the PKA selective inhibitor PKI would reduce or abolish the inhibition of contraction rate by the β_2AR. However, PKI treatment resulted in an exaggeration of both stimulatory and inhibitory effects of isoproterenol on myocyte contraction rate in β_1AR-KO myocytes (Fig. 5B). Thus, it is unlikely that PKA phosphorylation plays a significant role in the switch of β_2AR coupling from Gs to Gi/o proteins in neonatal myocytes. The fact that PKI augments coupling to both Gs and Gi/o proteins suggests that PKA mediated receptor phosphorylation may impair coupling to both G proteins. The mechanism of this apparent switch in coupling from Gs to Gi/o has yet to be determined, but may be caused by differences in the relative abundance of Gi/o and Gs in myocytes. Although the β_2AR has a higher affinity for Gs, Gi may be more abundant. Precoupling of the β_2AR to Gs may account for the initial stimulation in contraction rate. However, after Gsα dissociates from the activated receptor, the receptor is more likely to encounter Gi than another Gs. The mechanism by which pertussis toxin-sensitive G proteins decrease contraction rate after β_2AR activation is not known. It could be due to inhibition of adenylyl cyclase or through a direct effect of the activated $Gi_{\beta\gamma}$ subunit on an inwardly rectifying potassium channel (IKACh) (Wickman et al., 1994).

Although cAMP plays a critical role in regulating myocyte function, we did not observe a strong correlation between cAMP accumulation and myocyte contraction rate in response to isoproterenol (Figs. 1 and 6). An increase in cAMP accumulation was detected after exposure to 0.01 μM isoproterenol, whereas 1 μM isoproterenol was needed to induce a detectable increase in contraction rate (Fig. 1). Moreover, the cAMP accumulation assay did not predict the biphasic effect of β_2AR stimulation on contraction rate in β_1AR-KO myocytes (Figs. 3 and 6). These discrepancies are most likely caused by technical differences in the assays. To detect cAMP accumulation in myocytes, cultures must be preincubated with a phosphodiesterase inhibitor (IBMX) to prevent hydrolysis of cAMP. This increases the sensitivity of the cAMP accumulation assay, and may make it difficult to observe a change in coupling from Gs to Gi. In addition, the myocyte cultures contain a small population of fibroblasts, which may contribute disproportionately to cAMP accumulation. This may be particularly difficult for myocytes from β_1AR-KO mice, in which we observe the lowest levels of isoproterenol-induced cAMP accumulation.

The physiologic role of β_2AR in the adult heart is not well understood and may depend on the species being studied (Steinberg 1999; Xiao et al., 1999b). The Steinberg lab has observed differences in β_2AR signaling between mice and rats (Sabri et al., 2000) and between neonatal and adult rat cardiac myocytes (Kuznetsov et al., 1995). In β_1AR-KO mice there is no detectable effect of β_2AR stimulation on heart rate or contractility in vivo (Rohrer et al., 1996). Although studies in humans suggest that the β_2AR plays a significant role in regulating contractile function (Brodde, 1991; Kaumann et al., 1996, 1999; Molenaar et al., 2000). There is a growing body of evidence linking β_2AR stimulation to mitogen-activated kinase signaling pathways suggesting a possible role in myocyte growth and apoptosis (Communal et al., 1999; Communal et al., 2000; Singh et al., 2000).

Analysis of β_2AR coupling in β_1AR-KO mice is complicated by the presence of β_3ARs; however, examination of β_1/β_2AR-KO mice allows us to estimate the contribution of β_3AR signaling in β_1AR-KO mice. Stimulation of β_3ARs has a very small and relatively brief inhibitory effect on contraction rate. Contraction rate returns to baseline after 10 min of isoproterenol exposure and there is no effect of a second addition of isoproterenol at 30 min (data not shown). Thus, β_3AR mediated inhibition of contraction rate may reduce the initial stimulatory effect of β_2AR activation on contraction rate in myocytes from β_1AR-KO mice, but probably has no significant effect on the subsequent inhibitory phase, which is greatest after 15 min of isoproterenol exposure. The activation of contraction rate by the β_3AR in pertussis toxin-treated myocytes from β_1/β_2AR-KO mice is also very brief, returning to baseline 10 min after exposure to either isoproterenol or CL-316243 (Fig. 4). In contrast, in β_2AR-KO myocytes and pertussis toxin-treated β_1AR-KO myocytes, the contraction rate is near maximally stimulated at 10 min after isoproterenol stimulation. Thus, β_3AR signaling through both Gs and Gi seems to desensitize more rapidly than does signaling by either the β_1AR or the β_2AR. This is somewhat unexpected in light of earlier studies that failed to detect desensitization in β_3AR expressed in CHW or LTK^- cells (Nantel et al., 1993), although others have observed β_3AR desensitization to be depend on the cell line used (Chaudhry and Granneman, 1994).

Another interesting observation regarding β_3AR signaling in β_1/β_2AR-KO myocytes is that the relative efficacy of the

agonists isoproterenol and CL-316243 depends on the signaling pathway. When coupling to Gi/o proteins, CL-316243 is more efficacious at inhibiting contraction rate than is isoproterenol (compare solid lines in Fig. 4, A and B), whereas CL-316243 and isoproterenol are equally efficacious at stimulating contraction rate in cells treated with pertussis toxin (compare dashed lines in Fig. 4, A and B). This suggests that agonist efficacy may be G protein-specific, as has been demonstrated for other GPCRs (Spengler et al., 1993; Kenakin 1995). Thus, isoproterenol may be a full agonist for the β_3AR when coupled to Gs but not when coupled to Gi.

In conclusion, we examined isoproterenol-stimulated changes in contraction rate in neonatal myocytes from mice having disruptions in the genes for the β_1AR, the β_2AR, and both β_1AR and β_2AR. This experimental system allows us to examine subtype-specific signaling in differentiated cells in which receptors are expressed at physiologic levels. Our results indicate that all three βAR subtypes are expressed in neonatal myocytes and activate distinct signaling pathways. Of particular interest is the biphasic effect of β_2AR stimulation on myocyte contraction rate and the fact that the β_2AR and the β_1AR stimulate contraction rate through different mechanisms. The different signaling pathways used by the β_1AR and the β_2AR suggest that these proteins are physically separated in the myocyte membrane providing further evidence for functional signaling microdomains.

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