

Coupling Function of Endogenous α_1 - and β -Adrenergic Receptors in Mouse Cardiomyocytes

Abdelkarim Sabri, Elena Pak, Sasha A. Alcott, Brenda A. Wilson, Susan F. Steinberg

Abstract—Genetically altered mouse models constitute unique systems to delineate the role of adrenergic receptor (AR) signaling mechanisms as modulators of cardiomyocyte function. The interpretation of results from these models depends on knowledge of the signaling properties of endogenous ARs in mouse cardiomyocytes. In the present study, we identify for the first time several defects in AR signaling in cardiomyocytes cultured from mouse ventricles. β_1 -ARs induce robust increases in cAMP accumulation and the amplitude of the calcium and cell motion transients in mouse cardiomyocytes. Selective β_2 -AR stimulation increases the amplitude of calcium and motion transients, with only a trivial rise in cAMP accumulation in comparison. β_2 -AR responses are not influenced by pertussis toxin in cultured mouse cardiomyocytes. α_1 -ARs fail to activate phospholipase C, the extracellular signal-regulated protein kinase, p38-MAPK, or stimulate hypertrophy in mouse cardiomyocytes. Control experiments establish that this is not due to a lesion in distal elements in the signaling machinery, because these responses are induced by protease-activated receptor-1 agonists and phospholipase C is activated by *Pasteurella multocida* toxin (a G_q α -subunit agonist). Surprisingly, norepinephrine activates p38-MAPK via β -ARs in mouse cardiomyocytes, but β -AR activation of p38-MAPK alone is not sufficient to induce cardiomyocyte hypertrophy. Collectively, these results identify a generalized defect in α_1 -AR signaling and a defect in β_2 -AR linkage to cAMP (although not to an inotropic response) in cultured mouse cardiomyocytes. These naturally occurring vagaries in AR signaling in mouse cardiomyocytes provide informative insights into the requirements for hypertrophic signaling and impact on the value of mouse cardiomyocytes as a reconstitution system to investigate AR signaling in the heart. (*Circ Res.* 2000;86:1047-1053.)

Key Words: receptors, adrenergic ■ cardiomyocytes ■ cAMP ■ phospholipase C ■ mitogen-activated protein kinases

Catecholamines regulate cardiomyocyte contractility and induce hypertrophy through interactions with cardiomyocyte adrenergic receptors (ARs). Catecholamine-dependent increases in heart rate and contractility are mediated primarily by the predominant β_1 -AR subtype (75% to 80% of total β -ARs in the hearts of most mammalian species), which acts exclusively through a cAMP-dependent mechanism to enhance cardiac contractility. Nevertheless, functional responses to catecholamines can also be mediated by β_2 -ARs that modulate contractile function through traditional cAMP-dependent mechanisms, as well as distinct cAMP-independent signaling pathways.^{1,2} The importance of cardiac β_2 -ARs is reported to increase in the context of human heart failure and after heart transplantation.³

Catecholamines also induce morphological changes in the heart. The initial studies by Simpson laid the groundwork for the use of cultured neonatal rat cardiomyocytes to dissect the diverse signaling properties of α_1 -ARs and determine their role in hypertrophic growth responses. These studies have established that through the pertussis toxin (PTX)-insensitive G_q protein, α_1 -ARs stimulate phospholipase C

(PLC) and the formation of inositol phosphates (IPs) and diacylglycerol and activate protein kinase C, Ras, and the extracellular signal-regulated protein kinase (ERK) subfamily of mitogen-activated protein kinases (MAPKs). Transgenic mice that overexpress the G_q α -subunit or certain protein kinase C isoforms develop hypertrophy, which underscores the importance of these signaling molecules in hypertrophic signaling.^{4,5} Nevertheless, the controversy lingers as to whether the ERK cascade represents an obligatory or a sufficient component of the cellular signaling machinery through which α_1 -ARs induce hypertrophy, because α_1 -AR agonists also activate stress-activated protein kinases (JNK and p38-MAPK), Rho, Rac, Ca^{2+} /calmodulin-dependent protein kinase, and calcineurin⁶; each has been deemed obligatory in the transduction of hypertrophic signals. In recent years, β -ARs also have been implicated in the acquisition of some features of the hypertrophic phenotype and the development of myocardial dysfunction due to cardiomyocyte loss through apoptosis.⁷ The molecular mechanisms that contribute to these β -AR actions are as yet incompletely understood.

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Despite considerable progress, efforts to obtain a precise understanding of the determinants of AR function in cardiomyocytes continue to be stymied by the lack of drugs with sufficient pharmacological selectivity or specificity. Accordingly, many laboratories have turned to transgenic and gene-targeted knockout mouse models. Mice with targeted cardiac overexpression of various ARs, G protein-coupled receptor kinases, and G protein α -subunits have been generated; the genes for several components of the AR complex also have been disrupted with knockout technology. The correct interpretation of data from these models requires a detailed understanding of the signaling properties of ARs endogenous to the mouse heart. The conclusions of most studies have been predicated on the untested assumption that ARs recruit identical signaling pathways in cardiomyocytes from rodent hearts. However, some experimental results in mouse cardiomyocytes are difficult to reconcile with models of AR signaling derived from studies in the rat and suggest species-dependent differences in AR signaling. Accordingly, the goal of the present study was to compare endogenous AR signaling pathways in rat and mouse cardiomyocytes. The results identify important differences between AR actions in cardiomyocytes cultured from mouse and rat ventricles that are germane to the interpretation of studies in transgenic/knockout mouse models and provide novel insights into the determinants for cardiomyocyte growth responses.

Materials and Methods

Cardiomyocytes were dissociated from the ventricles of 2-day neonatal Wistar rats and 18-day embryonic ICR mice via a trypsin digestion protocol that incorporates a differential attachment procedure and irradiation (24 hours after culture) to halt the proliferation of residual fibroblasts, as described previously.⁸ Mouse cultures were prepared from embryonic ventricles (which contain few fibroblasts) rather than neonatal ventricles because the β_2 -AR-containing mouse cardiac fibroblasts displayed unusual refractoriness to maneuvers that readily curtail rat cardiac fibroblast proliferation (irradiation, bromodeoxyuridine treatment). However, pilot experiments on 2-day neonatal mouse and 18-day embryonic mouse cultures established that differences in AR responses are species rather than age dependent.

Measurements of cAMP and IP accumulation and the simultaneous photometric measurement of calcium (with Fura-2) and cell shortening were made according to published methods.⁸ ERK activation was measured with in-gel kinase assays with myelin basic protein as substrate⁹ or with an antibody selective for the phosphorylated form of the kinase (New England Biolabs). p38-MAPK activation was detected with immunoblot analysis with an antibody selective for the phospho-p38-MAPK (New England Biolabs). Immunoblot analysis of β_2 -ARs was made with an affinity-purified polyclonal antiserum (1:100; Santa Cruz Biotechnology); epitope-specific immunoreactivity was established in preliminary experiments. Bound primary antibodies were visualized with enhanced chemiluminescence (Amersham) and quantified with laser scanning densitometry.

Cardiomyocyte growth was assessed by planimetry with 7 to 10 frames per dish captured at $\times 40$ magnification and 30 to 50 cells analyzed for each treatment. For measurements of [¹⁴C]phenylalanine incorporation into protein, cells were stimulated in serum-free medium with agonists (or vehicle as control) for 48 hours at 37°C. Medium was supplemented with [¹⁴C]phenylalanine (0.1 μ Ci/mL) plus 0.3 mmol/L nonradioactive phenylalanine during the final 24 hours of stimulation. Cells were rinsed with PBS and incubated in 10% trichloroacetic acid for 30 minutes on ice. Precipitates were washed twice with ice-cold 10% trichloroacetic acid and solubilized

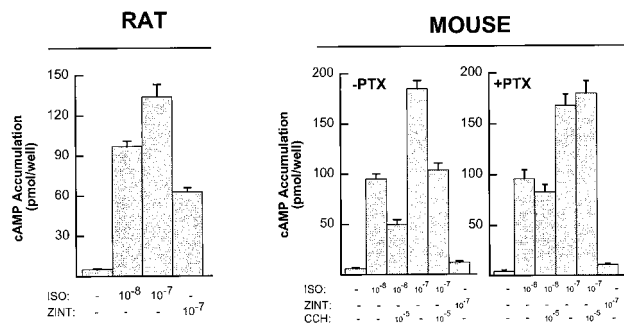


Figure 1. β_2 -ARs promote cAMP accumulation in rat, but not mouse, cardiomyocytes. Cardiomyocytes were incubated with isoproterenol (ISO) or zinterol (ZINT) for 5 minutes, and cAMP accumulation was measured by radioimmunoassay (n=6). cAMP accumulation is maximal at 10^{-7} mol/L isoproterenol in rat and mouse cardiomyocytes. Where indicated, carbachol (CCH) was added 5 minutes before isoproterenol. Pretreatment with 100 ng/mL PTX was for 24 hours.

in 1% SDS (1 mL/well) at 37°C for 1 hour. Duplicate aliquots from each sample were assayed for radioactivity and DNA content.

Results

β_1 -, but Not β_2 -, ARs Increase cAMP in Mouse Cardiomyocytes

Initial experiments discriminated the relative contributions of individual β -AR subtypes to cAMP accumulation in cardiomyocytes cultured from mouse ventricles. Figure 1 shows that the nonselective β -AR agonist isoproterenol at 10^{-7} mol/L induces 27- and 31-fold increases in cAMP accumulation in rat and mouse cardiomyocytes, respectively. The response to isoproterenol in rat ventricular cardiomyocyte cultures reflects its combined actions at β_1 - and β_2 -ARs. The response is attenuated by 66.5 \pm 3.6% with 10^{-7} mol/L CGP 20712A (β_1 -AR antagonist) and by 33.2 \pm 2.5% with 10^{-7} mol/L ICI 118,551 (β_2 -AR antagonist) and completely blocked in their combined presence (or with 10^{-7} mol/L propranolol, $P<0.05$; n=6). Zinterol at 10^{-7} mol/L also substantially increased cAMP accumulation in rat cardiomyocytes (13-fold) by activating β_2 -ARs (response abrogated 96.6 \pm 2.6% with ICI 118,551, n=6). In contrast, isoproterenol-induced increases in cAMP accumulation in mouse cardiomyocytes are mediated entirely by β_1 -ARs (response blocked 95.4 \pm 3.5% with 10^{-7} mol/L CGP 20712A but not with 10^{-7} mol/L ICI 118,551, n=6). Zinterol at 10^{-7} mol/L failed to induce more than a trivial increase in cAMP accumulation in cultured mouse cardiomyocytes (6.5% of the response to equimolar isoproterenol). Given recent reports that PTX-sensitive G proteins influence β_2 -AR signaling in cardiomyocytes from adult mouse ventricles,¹⁰ experiments also were performed in cultures pretreated with PTX according to a protocol that completely ADP-ribosylates and inactivates susceptible G protein α -subunits. Figure 1 shows that PTX completely abrogates muscarinic receptor-dependent inhibition of cAMP accumulation but fails to expose a β_2 -AR-dependent increase in cAMP accumulation. Collectively, these results indicate that only β_1 -ARs couple to cAMP accumulation in mouse cardiomyocytes and that the failure to detect β_2 -AR coupling to cAMP accumulation is not due to β_2 -AR coupling to G_i and an inhibitory pathway.

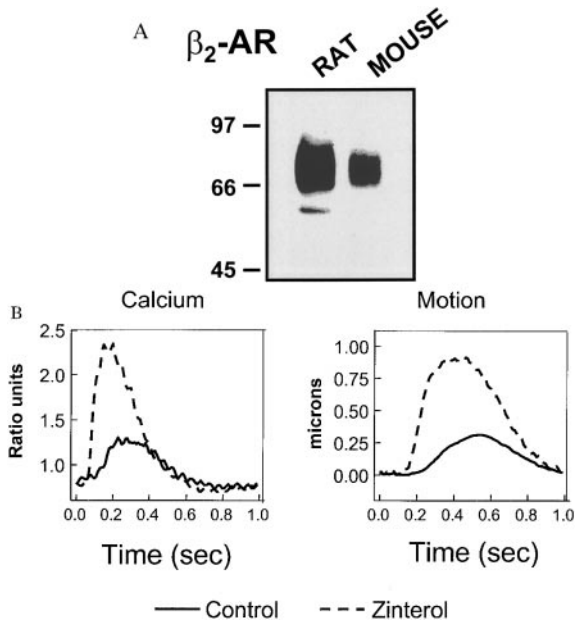


Figure 2. β_2 -AR expression and function in mouse cardiomyocytes. A, Representative immunoblots that provide a comparison of β_2 -AR expression in mouse and rat cardiomyocytes. β_2 -ARs are detected as broad glycosylated ≈ 64 -kDa bands; β_2 -ARs migrate as more distinct ≈ 47 -kDa bands after treatment with PNG-F. B, Representative tracings showing the effect of 10^{-7} mol/L zinterol on calcium and cell motion transients during continuous electrical field stimulation at 1 Hz. Signal-averaged transients during the control interval and at the peak of the response are superimposed.

Mouse Cardiomyocytes Express β_2 -ARs, Which Modulate Ca^{2+} and Contractile Function

The failure to detect β_2 -AR-dependent cAMP accumulation in mouse cardiomyocytes should not be construed as a generalized defect in β_2 -AR expression or signaling. Figure 2A shows that β_2 -ARs are readily detected by immunoblot analysis in rat and mouse cardiomyocytes, although the abundance of β_2 -ARs in mouse cardiomyocytes appears to be lower than that in their rat counterparts. Figure 2B establishes the functional integrity of β_2 -ARs in mouse cardiomyocytes. During electrical field stimulation at 1 Hz to maintain a constant contractile rate during agonist exposure, zinterol markedly increased the amplitude of calcium transients (from 0.62 ± 0.14 to 1.54 ± 0.33 ratio units) and motion transients (from 0.29 ± 0.05 to $0.82 \pm 0.09 \mu m$, $n = 14$, $P < 0.05$). Zinterol elicited a similar increase in the amplitude of the calcium transient (from 0.58 ± 0.12 to 1.44 ± 0.25 ratio units) and the motion transient (from 0.32 ± 0.05 to $0.88 \pm 0.08 \mu m$, $n = 6$, $P < 0.05$) in cultured mouse cardiomyocytes treated with 100 ng/mL PTX for 24 hours. Collectively, these results establish that β_2 -ARs enhance contractile function in mouse cardiomyocytes but that the response is not associated with a significant elevation of intracellular cAMP and is not detectably modulated by PTX-sensitive G proteins.

α_1 -ARs Do Not Stimulate PLC in Mouse Cardiomyocytes

Previous studies from our laboratory and others established that norepinephrine (NE) promotes IP accumulation through

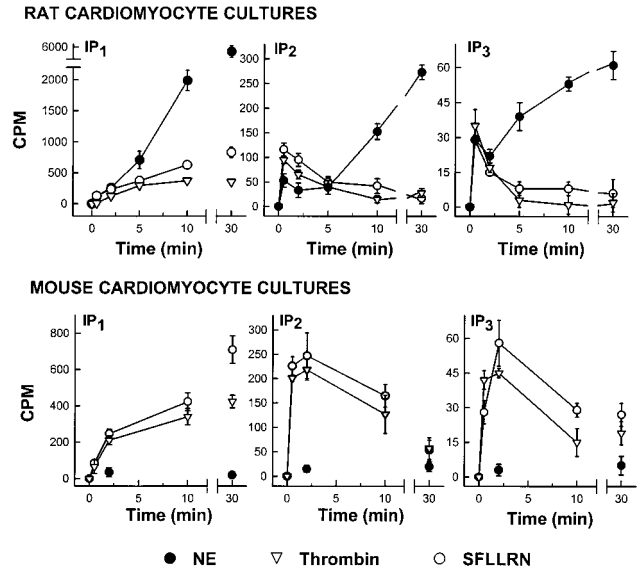


Figure 3. $[^3H]$ IP accumulation in response to α_1 -AR and PAR agonists in rat and mouse cardiomyocytes. The 3H -inositol-labeled myocytes were incubated with 300 μ mol/L SFLLRN, 1 U/mL thrombin, or 10^{-6} mol/L NE for the indicated intervals. Results are expressed as CPM over corresponding control values for triplicate determinations from a single experiment (mean \pm SEM), with similar results in 2 separate experiments on separate cultures.

actions at α_1 -ARs in neonatal rat cardiomyocytes. Figure 3 shows that NE induces sustained increases in IP_1 , IP_2 , and IP_3 accumulation in rat cardiomyocytes, but the identical stimulus evokes no response in mouse cardiomyocytes. The Table shows that G_q -coupled endothelin receptors also vigorously activate PLC in rat cardiomyocytes but fail to increase IPs in mouse cardiomyocytes. The failure to detect PLC activation by NE or endothelin in mouse cardiomyocytes cannot be attributed to technical issues (failure to adequately label membrane phosphoinositides and so on), because thrombin and SFLLRN (protease-activated receptor-1 [PAR-1] agonists) induce similar robust increases in PLC activity in rat and mouse cardiomyocytes. However, differences in the kinetics and magnitude of IP metabolite accumulation in rat cardiomyocytes challenged with NE/endothelin versus PAR-1 agonists (in the context of the selective lesion in PLC activation by NE/endothelin in mouse cardiomyocytes) suggest distinct activation mechanisms. In particular, PAR-1 agonists induce transient and pronounced elevations of IP_2/IP_3 , followed by relatively modest increases in IP_1 . In contrast, IP_1 , IP_2 , and IP_3 accumulate to high levels in a sustained fashion in rat cardiomyocytes exposed to NE/endothelin. The Table shows that PLC activation by PAR-1 agonists is severely curtailed by PTX. In contrast, PLC activation by NE/endothelin is PTX insensitive. The G_q , but not G_i , dependent pathway for PLC activation is selectively impaired in mouse cardiomyocytes. However, further experiments with *Pasteurella multocida* toxin (PMT, a G_q α -subunit agonist reported to activate the IP_3 signaling pathway by stimulating $PLC\beta^{11}$) suggest that this cannot be attributed to a deficiency in functional G_q proteins or a lesion in their ability to activate PLC. IP metabolites accumulate to high levels in mouse and rat

G Protein Dependence of PLC Activation in Mouse and Rat Cardiomyocytes

	Mouse				Rat			
	IP ₁		IP ₂ +IP ₃		IP ₁		IP ₂ +IP ₃	
	-PTX	+PTX	-PTX	+PTX	-PTX	+PTX	-PTX	+PTX
NE	25±18	NA	18±9	NA	3405±259*	3037±128*	86±11*	54±15*
Endothelin	33±20	NA	38±8*	NA	2545±235*	3136±373*	81±7*	63±14*
SFLLRN	523±28*	334±15*†	344±9*	112±5*†	517±85*	183±15*†	165±8*	26±8†
Thrombin	352±21*	155±12*†	272±8*	93±3*†	290±24*	46±13†	123±13*	9±8†

NA indicates not applicable.

* $P < 0.05$ vs corresponding control.

† $P < 0.05$ vs corresponding -PTX.

Cardiomyocytes were incubated with or without NE (10 μ mol/L), endothelin (100 nmol/L), SFLLRN (300 μ mol/L), or thrombin (1 U/mL), and IPs were measured according to standard methods. PTX treatment was 100 ng/mL for 24 hours at 37°C where indicated. Results are expressed as CPM over basal for IP₁ accumulation (at 30 minutes) or IP₂+IP₃ accumulation (at 2 minutes, n=6 to 9).

cardiomyocytes cultured for 24 hours with 200 ng/mL PMT (CPM over corresponding basal value: rat IP₁=1811±24, IP₂+IP₃=201±11; mouse IP₁=2690±151, IP₂+IP₃=188±8; n=6, $P < 0.05$). These results effectively exclude a lesion in the G_q pathway for PLC activation in mouse cardiomyocytes.

α_1 -ARs Do Not Stimulate ERK in Mouse Cardiomyocyte Cultures

We next compared the effects of NE to activate ERK in rat and mouse cardiomyocytes. Figure 4A (left) shows robust ERK activation by NE, phorbol-12-myristate-13-acetate (PMA), thrombin, and SFLLRN in cultured rat cardiomyocytes. The effects of NE are mediated predominantly by α_1 -ARs; only a minor β -AR component to ERK activation by NE is detected. ERK activation is detected with the pure β -AR agonist isoproterenol (but with a relatively high agonist concentration of 10⁻⁵ mol/L) or with zinterol at a concentration that retains β_2 -AR selectivity (10⁻⁷ mol/L; Figure 4C). The observation that zinterol activates ERK almost as effectively as isoproterenol (89.0±7.2% of the response to 10⁻⁵ mol/L isoproterenol, n=3) indicates that a major component of β -AR activation of ERK in rat cardiomyocytes is mediated by β_2 -ARs. Figure 4B (left) shows that ERK activation by PMA and PAR-1 agonists (SFLLRN and thrombin) is vigorous in mouse cardiomyocytes, whereas ERK activation by NE is not detectable. Pretreatment with PTX to inactivate G_i proteins failed to disclose α_1 - or β -AR components to ERK activation by NE in mouse cardiomyocytes (data not shown).

NE Activates p38-MAPK Through β -ARs in Mouse Cardiomyocytes

Analyses of SAPK activation in mouse cardiomyocytes first focused on JNK. However, measurements of JNK activation were precluded by the high basal JNK activity in mouse cardiomyocytes, which tended to obscure any stimulatory effect of agonist-activated receptors or sorbitol (data not shown). In contrast, p38-MAPK activation by NE and PAR-1 agonists is readily detected in rat and mouse cardiomyocytes (Figures 4A and 4B, right). Unexpectedly, the effect of NE to activate p38-MAPK in mouse cardiomyocytes is not prevented by the α_1 -AR blocker prazosin but rather is completely prevented with β -AR inhibition with propranolol (Figure 5).

Further studies demonstrate that p38-MAPK is activated in both mouse and rat cardiomyocytes by isoproterenol (detectable at 10⁻⁹ mol/L and maximal at 10⁻⁷ mol/L, Figure 4C, right). Of note, the isoproterenol concentrations required to activate p38-MAPK, stimulate cAMP, and enhance contractile function correspond closely to each other and are 100-fold lower than those required to activate ERK (Figure 4C). Figure 5 shows that p38-MAPK also is weakly activated by 10⁻⁷ mol/L zinterol. Collectively, these results support the conclusion that the effect of NE to increase p38-MAPK activity in mouse cardiomyocyte cultures is mediated exclusively by β -ARs, with β_2 -ARs contributing to this process. In contrast, the effect of NE to activate p38-MAPK in rat cardiomyocytes is mediated by both α_1 - and β -ARs.

α_1 -ARs Do Not Promote Hypertrophic Growth of Mouse Cardiomyocytes

Each MAPK family member has been implicated in cardiomyocyte growth responses, although their precise roles remain controversial.¹² We reasoned that mouse cardiomyocytes might represent a unique system to probe the functional consequences of p38-MAPK activation, because here, NE selectively activates p38-MAPK and not ERK (ie, NE should elicit a hypertrophic response in mouse cardiomyocytes only if p38-MAPK is sufficient to induce hypertrophic growth). Figure 6 shows that NE does not promote [¹⁴C]phenylalanine incorporation or increase mouse cardiomyocyte cell size. Serum-deprived mouse cardiomyocytes tended to be larger than their rat counterparts grown under the same conditions, but this inherent enlargement did not obscure the growth-stimulatory effects of SFLLRN or serum. Agonists for the α_1 -AR and PAR-1 induce hypertrophy in cultured rat cardiomyocytes. Collectively, these results identify a generalized defect in α_1 -AR signaling in cardiomyocytes cultured from mouse ventricles and indicate that p38-MAPK activation alone cannot support the full cardiomyocyte hypertrophic growth response.

Discussion

Genetically altered mouse models provide unique opportunities to identify the molecular determinants of AR action in the

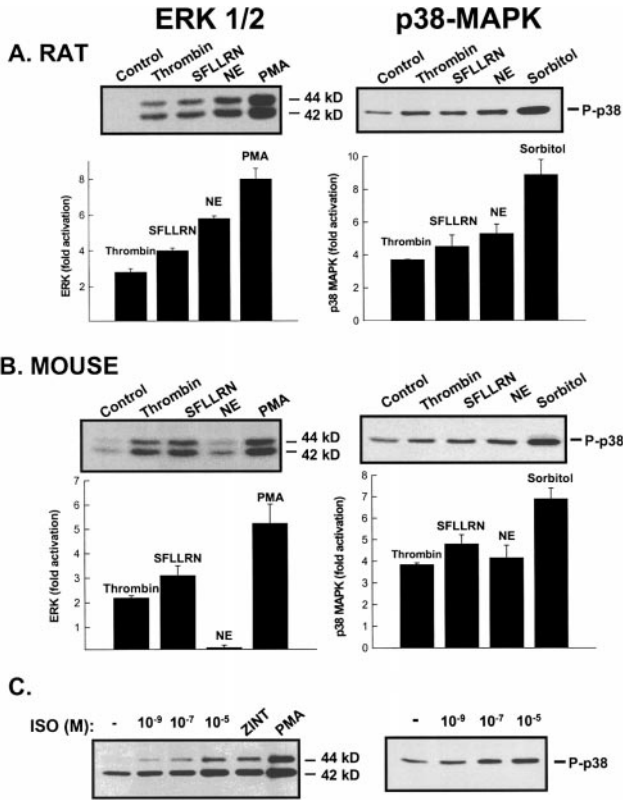


Figure 4. ERK and p38-MAPK activation by NE and PAR agonists in rat and mouse cardiomyocytes. A and B, Incubations were for 5 minutes without or with 300 μ M SFLLRN, 1 U/mL thrombin, 10^{-5} mol/L NE, 100 nmol/L PMA, or 0.5 mol/L sorbitol. Extracts were assayed for ERK activity with MBP as substrate (left) or were probed with polyclonal anti-phospho-p38-MAPK antibody (right). Top, Representative autoradiograms (with each lane from a single gel exposed for the same duration). Bottom, Quantification of each series of experiments (n=3 or 4). C, Activation of ERK (in rat cardiomyocytes) or p38-MAPK (in mouse cardiomyocytes) was for 5 minutes with the indicated concentrations of isoproterenol (ISO), zinterol (ZINT, 10^{-7} mol/L), or PMA (100 nmol/L) with similar results in 2 separate experiments. ERK and p38-MAPK activations were detected with antibodies that recognize phospho-ERK or phospho-p38-MAPK. Similar results for isoproterenol activation of p38-MAPK were obtained in rat cardiomyocytes. ERK activation by isoproterenol is not detected in mouse cardiomyocytes; it could not be studied in this manner.

heart. However, the correct interpretation of results derived from these models requires a detailed understanding of the signaling properties of ARs endogenous to mouse cardiomyocytes. Surprisingly, there has been limited progress in characterizing AR signaling pathways in mouse cardiomyocytes; none of the previous studies considered cardiomyocytes cultured from the mouse ventricle, the model that has been most predictive of the key molecular events that drive hypertrophic growth in the adult heart. The current report rectifies this deficiency and identifies key differences in the signaling properties of α_1 - and β_2 -ARs between rat and mouse cardiomyocytes in culture. Inherent species-dependent differences in AR signaling are represented in Figure 7.

Cultured mouse cardiomyocytes exhibit a generalized defect in α_1 -AR. The trivial explanation for this result is that cultured mouse cardiomyocytes are deficient in α_1 -AR ex-

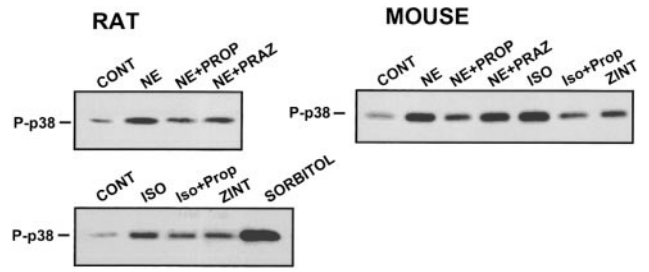


Figure 5. p38-MAPK activation by NE is mediated by α_1 - and β -ARs in rat cardiomyocytes; only β -ARs activate p38-MAPK in mouse cardiomyocytes. Cardiomyocytes were challenged for 5 minutes with NE (10^{-5} mol/L), isoproterenol (ISO, 10^{-7} mol/L), or zinterol (ZINT, 10^{-7} mol/L) after a 10-minute preincubation with vehicle, propranolol (PROP, 10^{-7} mol/L), or prazosin (PRAZ, 10^{-7} mol/L) as indicated. Assay of p38-MAPK was conducted as described in the legend to Figure 4. Data are from representative gels, with similar results in 3 separate experiments.

pression (ie, are a null background for α_1 -AR overexpression studies). However, this explanation does not account for the concomitant defect in endothelin-dependent activation of PLC and suggests that other mechanisms must be considered. Studies reported here demonstrate for the first time that cardiomyocyte G protein-coupled receptors activate PLC through distinct mechanisms that differ in their kinetics and G protein dependence. α_1 -ARs and endothelin receptors selectively activate PLC through a G_q -dependent pathway; this G_q -dependent pathway is impaired in mouse cardiomyocytes. In contrast, PAR-1 agonists recruit a distinct G_i -dependent mechanism for PLC activation, which is intact and vigorous in the mouse heart. Studies with PMT, a G_q α -subunit agonist, establish the functional integrity of elements distal to the receptor in the G_q -dependent pathway for PLC activation in mouse cardiomyocytes. With these constraints on the locus for the defect in PLC activation, mechanisms that might be considered in future studies could include G protein $\beta\gamma$ dimers (which through the γ -subunit could impart specificity to G protein interactions with receptors, effectors, or both¹³)

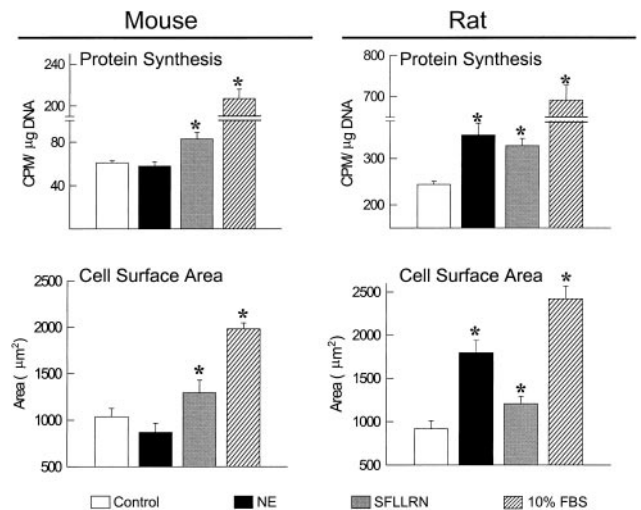


Figure 6. SFLLRN induces hypertrophy of mouse and rat cardiomyocytes; NE induces hypertrophy of rat, but not mouse, cardiomyocytes. Culture was with NE (5×10^{-5} mol/L), SFLLRN (300 μ M), or 10% FCS for 48 hours as indicated (n=6).

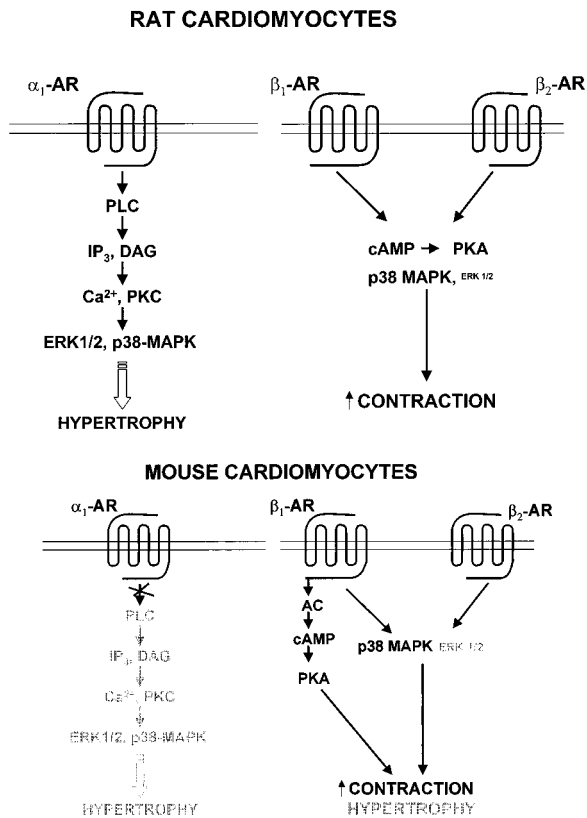


Figure 7. Schematic of distinct AR signaling pathways in rat and mouse cardiomyocytes. Black indicates intact signaling pathways; gray, signaling pathways that are defective in mouse cardiomyocytes; and smaller font, more minor signaling pathways (ie, β -AR activation of ERK). β -AR activation of ERK displays a prominent β_2 -AR component; p38-MAPK is effectively activated by β_1 -ARs (see text for details).

or RGS proteins (which can act as molecular switches and, at least in theory, impair signaling by receptors coupled to G_i while preserving signaling by receptors coupled to G_i^{14}).

Studies of β -AR subtype signaling identify another major species-dependent difference in AR responsiveness. β_1 -ARs elevate cAMP in rat and mouse cardiomyocytes. In contrast, β_2 -ARs increase contractile function in association with a substantial rise in cAMP only in rat cardiomyocytes; there is no detectable rise in cAMP in mouse cardiomyocytes. This result was surprising in view of previous studies in genetically engineered mouse models in which overexpression of human β_2 -ARs is reported to increase adenylyl cyclase activity.¹⁵ Because β_2 -ARs generally couple to G_s and the accumulation of cAMP, there was little reason to suspect that overexpressed receptors might not simulate the signaling properties of endogenous β_2 -ARs in mouse cardiomyocytes. However, the literature on endogenous β_2 -AR expression and function in murine cardiomyocytes is quite limited. To date, studies have been confined to adult murine tissue where $\approx 25\%$ of total β -ARs are characterized as β_2 -AR by radioligand binding analysis.^{16,17} However, these experiments were performed on membranes from intact ventricles; results were clouded by uncertainties as to whether β_2 -ARs reside on connective tissue or vessel contaminants, particularly because isoproterenol does not alter contractile function of isolated

ventricles from β_1 -AR knockout mice.¹⁷ Recently, Xiao et al¹⁰ presented an alternative explanation for the absence of a baseline β_2 -AR response in adult murine ventricles. On the basis of evidence that PTX unmasks an effect of zinterol to increase calcium transient and contraction amplitude, these investigators concluded that myocytes isolated from adult mouse ventricles express endogenous β_2 -ARs but do not display β_2 -AR responses because signaling to mechanisms that enhance contractile performance is overwhelmed by simultaneous and highly efficient coupling of β_2 -ARs to an opposing inhibitory G protein-dependent pathway. Studies in this report broaden the analysis to consider cardiomyocytes cultured from immature mouse ventricles. Here, signaling by endogenous β_2 -ARs is readily detected, even in the absence of PTX treatment. β_2 -ARs agonists markedly increase calcium and cell motion transients through a mechanism that is neither associated with a rise in cAMP accumulation (as in neonatal rat cardiomyocytes) nor influenced by PTX (as is reported to occur in adult rat and mouse cardiomyocytes). The failure to detect a rise in cAMP accumulation could suggest that β_2 -ARs increase contractile function through a cAMP-independent mechanism, although a mechanism that involves localized cAMP elevations adjacent to the membrane (to selectively activate calcium channels) cannot be excluded. These findings reinforce the notion that species and developmentally regulated factors impart diversity to β_2 -AR responsiveness. Moreover, the evidence that β_2 -ARs display obvious differences in their coupling to cAMP accumulation in cultured rat and mouse cardiomyocytes, yet increase contractile function in both via a PTX-insensitive pathway, argues that not all of the diversity in β_2 -AR signaling can be attributed to differences in functional coupling to G_i proteins (as suggested by Xiao et al²). Finally, these studies interject a cautionary note in the extrapolation of results between models and emphasize the need to identify models that most closely resemble β_2 -AR signaling in human cardiomyocytes. Insofar as the preponderance of experiments to date on human tissue identify a cAMP-dependent mechanism for β_2 -AR action^{18,19} (and preliminary studies fail to detect human cardiomyocyte β_2 -AR coupling to G_i proteins²⁰), neonatal rat cardiomyocyte cultures, rather than any mouse model studied to date, emerge as potentially the most clinically relevant model of β_2 -AR function in the heart.

This study is the first to demonstrate that catecholamines activate p38-MAPK via a β -AR-dependent pathway in cardiomyocytes. Although there is ample precedent for β -AR activation of ERK, a linkage between cardiomyocyte β -ARs and the p38-MAPK cascade is novel. Two recent studies that attempted to identify such a pathway in other cell types met with mixed results. β_1 -AR overexpression in PC12 cells did not activate p38-MAPK,²¹ whereas stimulation of endogenous β -ARs in human embryonic kidney 293 cells is reported to activate p38-MAPK via a $G_{\beta\gamma}$ pathway.²² Although PTX-sensitive G proteins generally represent the source of activator $\beta\gamma$ dimers (and β_2 -ARs are reported to couple to G_i proteins in various cardiomyocyte preparations²), studies with PTX argue against any significant role for G_i proteins in the pathway for β -AR-dependent activation of p38-MAPK in cardiomyocytes. Studies with β -AR subtype-selective li-

gands indicate that p38-MAPK is activated primarily by β_1 -ARs, but a β_2 -AR subtype component to the response can be detected. In contrast, ERK activation is mediated preferentially by β_2 -ARs. This observation may be pertinent to the interpretation of recent studies in transgenic mouse models. Mice that overexpress β_2 -ARs display enhanced baseline cardiac function with no evidence of cardiotoxic effects unless overexpression is driven to very high levels or maintained for protracted intervals.¹⁵ The salutary effects of β_1 -AR overexpression are more transient, with progressive cardiac deterioration becoming prominent as the animals age. Studies of signaling mechanism or mechanisms that contribute to myopathic changes have focused on cAMP and calcium.²³ Studies reported here indicate that differential coupling to ERK and p38-MAPK also must be considered.

Current concepts of mechanisms that promote cardiomyocyte hypertrophy derive in large part from studies on cultured rat cardiomyocytes. Because these cells are replete with all elements of signaling cascades (ie, are not null backgrounds), most studies resort to molecular approaches, introducing constitutively active or dominant-negative mutants of various signaling molecules. Unfortunately, transfection approaches may markedly alter the stoichiometry of components of signaling cascades and produce systems that bear little resemblance to the physiological state. Perhaps as a result, studies that attempt to define the growth-regulatory properties of various MAPK subfamilies have been ambiguous. The literature has implicated p38-MAPK in hypertrophy, cell survival, or apoptosis.¹² Studies reported here identify an informative, naturally occurring species-dependent variation in AR signaling. The observation that NE activates p38-MAPK but fails to induce cell enlargement provides convincing evidence that p38-MAPK activation alone (without ERK or JNK) is not sufficient to promote cardiomyocyte hypertrophy.

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