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Developmental Changes in β_2-Adrenergic Receptor Signaling in Ventricular Myocytes: the Role of Gi proteins and Caveolae Microdomains

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ABSTRACT

Cardiomyocyte β_2-adrenergic receptors (β-ARs) provide a source of inotropic support and influence the evolution of heart failure. Recent studies identify distinct mechanisms for β_2-AR actions in neonatal and adult rat cardiomyocytes. This study examines whether ontogenic changes in cardiac β_2-AR actions can be attributed to altered Gi expression or changes in the spatial organization of the β_2-AR complex in membrane subdomains (caveolae). We show that β_2-ARs increase cAMP, calcium, and contractile amplitude in a pertussis toxin (PTX)-insensitive manner in neonatal cardiomyocytes. This is not caused by lack of Gi; Gαi expression is higher in neonatal cardiomyocytes than in those of adult rats. β_2-ARs provide inotropic support without detectably increasing cAMP, in adult cardiomyocytes. This cannot be attributed to dual coupling of β_2-ARs to Gs and Gi, because β_2-ARs do not promote cAMP accumulation in PTX-pretreated adult cardiomyocytes. Spatial segregation of β_2-ARs, Gαs/Gαi, and adenylyl cyclase to distinct membrane subdomains also is not a factor, because all of these proteins copurify in caveolin-3-enriched vesicles isolated from adult cardiomyocytes. However, these studies demonstrate that enzyme-based protocols routinely used to isolate ventricular cardiomyocytes lead to proteolysis of β-ARs. The functional consequences of this limited β-AR proteolysis is uncertain, because truncated β_1-ARs promote cAMP accumulation and truncated β_2-ARs provide inotropic support in adult cardiomyocytes. Collectively, these studies indicate that components of the β_2-AR signaling complex compartmentalize to restricted membrane subdomains in adult rat cardiomyocytes. Neither compartmentalization nor changes in Gi expression fully explain the ontogenic changes in β_2-AR responsiveness in the rat ventricle.

β-Adrenergic receptors (β-ARs) play key roles in the rapid modulation of cardiomyocyte contractile function and the long-term induction of a gene program that leads to cardiomyocyte hypertrophy and cardiac failure (Steinberg, 1999; Xiao, 2001). Under normal physiological conditions, catecholamine actions are mediated by the predominant β_1-AR acting via the Gs-adenylyl cyclase (AC) pathway. The contribution of the more minor β_2-AR subtype to catecholamine responsiveness is most prominent in neonatal ventricles (which lack sympathetic innervation) and in failing/aged hearts (in which β_1-ARs are selectively down-regulated). Recent studies identify a striking developmental change in β_2-AR actions in the rat ventricle. β_2-ARs promote cAMP accumulation, activate protein kinase A, and induce positive inotropic and lusitropic responses in neonatal rat cardiomyocytes; β_2-ARs augment contractile function without hastening the kinetics of relaxation in adult rat cardiomyocytes (Steinberg, 1999;Xiao, 2001). Some investigators have attributed this age-dependent difference in β_2-AR signaling to the developmental acquisition of a pertussis toxin (PTX)-sensitive Gi protein (Xiao, 2001). According to this model, β_2-ARs couple to the Gs/cAMP pathway and promote cAMP accumulation in all cardiomyocyte preparations (neonatal and adult). In adult cardiomyocytes (with higher levels of functional Gi expression than neonatal rat cardiomyocytes), β_2-ARs also couple to a Gi-dependent pathway that activates an intracellular protein phosphatase and functionally confines the cAMP/protein kinase A-dependent signal to targets at the surface membrane (Steinberg, 1999; Xiao et al., 1999). However, certain aspects of this model, including whether β_2-ARs induce a global elevation in cAMP levels in adult rat cardiomyocytes (which can be measured by conventional radioimmunoassay

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ABBREVIATIONS: AR, adrenergic receptor; AC, adenylyl cyclase; PTX, pertussis toxin; PAGE, polyacrylamide gel electrophoresis; mAChR, muscarinic acetylcholine receptor; CGP20712A, [2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulfonate; ICI 118,551, *erythro*-(±)-3-isopropylamino-1-(7-methylindan-4-yloxy)butan-2-ol; PNGF, peptide-*N*-glycosidase F.

techniques) remain disputed in the literature (Laflamme and Becker, 1998; Steinberg, 1999; Xiao et al., 1999). Moreover, a model that places the locus of regulatory control by Gi proteins downstream at the level of an intracellular protein phosphatase is at odds with studies that view Gi proteins as inhibitors of AC; there is still little consensus as to whether the β_2-AR-Gi pathway inhibits AC in cardiomyocytes (Kuschel et al., 1999; Kilts et al., 2000). Finally, recent studies identify compartmentation of β_2-ARs and cardiac AC isoforms to caveolin-3-enriched membrane subdomains in neonatal rat cardiomyocytes. (Rybin et al., 2000; Ostrom et al., 2001; Steinberg and Brunton, 2001). These microdomains are formed as a result of the expression of caveolins (a multigene family of immunologically distinct isoforms that self-assemble to form high molecular mass oligomers and drive caveolae biogenesis); caveolae have been implicated as sites that sequester and regulate signal transduction molecules (Smart et al., 1999; Galbiati et al., 2001). The recent observation that β_2-ARs target to caveolae in neonatal cardiomyocytes but are excluded from caveolae membranes in vascular smooth muscle cells (Ostrom et al., 2002) has been taken to suggest that cell-specific differences in β_2-AR partitioning (between caveolin-enriched and noncaveolae membrane fractions) could alter the spatial relationship between β_2-ARs and their binding partners/downstream effectors and thereby alter the intensity of (or influence the mechanism for) β_2-AR signaling (in the absence of any change in β_2-AR, G protein, or AC expression). Given the pivotal role for β-ARs in the normal regulation of cardiac contractile function and in the evolution of heart failure syndromes, this study examines whether changes in Gi expression and/or β_2-AR compartmentation contribute to the developmental change in β_2-AR responsiveness in the rat ventricle.

Materials and Methods

Preparation of Cultured Neonatal and Acutely Disaggregated Adult Rat Ventricular Myocytes. Cardiomyocytes were isolated from the hearts of 2-day-old Wistar rats by a trypsin dispersion procedure according to a protocol that incorporates a differential attachment procedure to enrich for cardiomyocytes followed by irradiation as described previously (Kuznetsov et al., 1995). The yield of cardiomyocytes typically is 2.5 to 3×10^6 cells per neonatal heart. Cells were plated at a density of 0.5×10^6 cells/ml on protamine sulfate-coated culture dishes (for biochemical assays) or glass coverslips (for calcium and cell motion experiments). Experiments were performed after 5 to 6 days of culture in minimal essential medium (Invitrogen, Carlsbad, CA) with 10% fetal calf serum, 5×10^{-6} M hypoxanthine, and 12 mM $NaHCO_3$. Adult rat ventricular myocytes were disaggregated according to methods described in detail previously (Kuznetsov et al., 1995). Cells were used within 1 to 6 h of isolation.

Measurements of cAMP. Intracellular cAMP accumulation was measured in neonatal myocytes grown in 22.1-mm multiwell dishes or in rod-shaped adult myocytes as described previously (Kuznetsov et al., 1995). Preincubation was with 10 mM theophylline for 60 min at room temperature and was followed by assays performed for 5 min at room temperature in the absence or presence of isoproterenol (10^{-7} M), carbachol (10^{-5} M, starting 5 min before isoproterenol), or forskolin (10^{-7} M) as indicated. Assays were terminated by removal of the incubation buffer and addition of 1 ml of ethanol, with each condition performed on three wells and assayed for cAMP in quadruplicate. Aliquots of the alcohol-fixed cell extracts were dried under a stream of nitrogen, and cAMP in the residue was determined by radioimmunoassay (PerkinElmer Life Sciences, Boston, MA).

Pertussis Toxin-Dependent ADP-Ribosylation of G Proteins. A crude membrane fraction was prepared by scraping cells into a buffer containing 0.25 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris, pH 7.6, followed by homogenization with a Polytron homogenizer and centrifugation at 43,000*g* for 45 min. Membranes were resuspended in 50 mM Tris buffer, pH 7.6, containing 2 mM $MgCl_2$ and 1 mM EDTA at a concentration of 2 to 3 mg/ml. ADP-ribosylation assays were performed as follows: membranes were incubated in 20 μl of a 50 mM Tris-chloride buffer, pH 8.0, containing 2 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 0.1% Lubrol PX, 10 mM thymidine, 10 μM [^{32}P]NAD (1.5 μCi per assay), and 20 μg/ml PTX for 1 h at 37°C. The reaction was terminated by addition of SDS-PAGE sample buffer and boiling for 5 min. Electrophoresis was performed on vertical slab gels (resolving gel, 12%; stacking gel, 3.9% acrylamide) and was followed by autoradiography.

Preparation of Caveolae Membranes. Fractions enriched in the muscle-specific caveolin-3 isoform were prepared according to a detergent-free purification scheme essentially as described previously (Rybin et al., 2000). All steps were carried out at 4°C. Briefly, cells from five 100-mm diameter dishes were washed twice with ice-cold phosphate-buffered saline and then scraped into 0.5 M sodium carbonate, pH 11.0 (0.5 ml/dish). Cells from five dishes were combined (total volume, 2.5 ml) for each preparation. The extract was sequentially disrupted by homogenization with a loose-fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts), and a tip sonicator (three 20-s bursts). The homogenate was then adjusted to 40% sucrose by adding an equal volume of 80% sucrose prepared in MES-buffered saline (25 mM MES, pH 6.5, and 0.15 M NaCl), placed on the bottom of an ultracentrifuge tube, overlaid with a 5 to 30% continuous sucrose gradient, and centrifuged at 38,000 rpm for 16 to 18 h in a SW40 rotor (Beckman Coulter, Palo Alto, CA). Preliminary studies established that caveolin-3–enriched vesicles are optimally isolated from adult rat cardiomyocytes using a 5 to 30% continuous sucrose gradient. This was based upon the observation that the floating vesicle fractions isolated by 5 to 30% continuous and 5 to 35% discontinuous sucrose gradient centrifugation contain identical amounts of caveolin-3 immunoreactivity, but fractions 4 and 5 were isolated using the 5 to 35% discontinuous sucrose gradient centrifugation containing 3- to 4-fold more protein (i.e., they are contaminated with a substantial amount of noncaveolin-3-containing protein material). Contaminating proteins are separated into the heavier fractions 7 to 9 using a 5 to 30% continuous sucrose gradient centrifugation scheme. Although the recovery of caveolin-3 immunoreactivity and total protein from neonatal rat cardiomyocytes was similar using either discontinuous 5 to 35% or continuous 5 to 30% sucrose gradient centrifugation schemes, the continuous sucrose gradient centrifugation purification scheme (required to optimize purification of caveolin-3-containing membrane from adult cardiomyocytes) was used in studies on neonatal cardiomyocytes to maintain a consistent method throughout the study and facilitate comparisons. After centrifugation, thirteen 1-ml fractions were collected and aliquots were subjected to SDS-PAGE and immunoblotting.

Immunoblot Analysis. Samples were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose for immunoblot analysis with antibodies for β_1-ARs, β_2-ARs, $G\alpha_s$, $G\alpha_{i1/2}$, $G\alpha_{o/i3}$, Type V/VI AC, caveolin-3, and clathrin according to methods described in detail previously (Rybin et al., 2000). The specificity of immunoblot analysis with each of these antibodies was established in the previous study (Rybin et al., 2000). Anti-ryanodine receptor monoclonal antibodies were from Affinity BioReagents, Inc. (Goldne, CO). In some experiments, samples were treated with PNGF, to deglycosylate β-ARs, according to methods published previously (Rybin et al., 2000). Bound primary antibodies were visualized with enhanced chemiluminescence according to manufacturer's instructions, with each in each figure representing results from a single gel exposed for a uniform duration.

Immunoprecipitation. Cardiomyocytes from four 100-mm diameter dishes (~4 mg of protein) were rinsed with ice-cold phosphate-buffered saline and harvested by addition of 3.6 ml of extraction buffer (50 mM Tris-Cl, pH 8, 150 mM NaCl, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml benzamidin, 2 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin A, and 1% Igepal) followed by centrifugation at 4°C for 15 min at 10,000*g*. For immunoprecipitation, supernatants were incubated with 15 μg of anti-caveolin-3 antibodies for 1 h at 4°C followed by addition of 150 μl of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and incubation overnight at 4°C. The beads were washed three times with extraction buffer and bound proteins were eluted with SDS sample buffer and boiled for 5 min. Samples were subjected to SDS-PAGE and immunoblotting with caveolin-3- and $G\alpha_s$-specific antibodies.

Measurements of Intracellular Calcium and Contraction. Simultaneous measurements of cytosolic calcium and cell motion in fura-2–loaded cardiomyocytes were performed as published previously (Kuznetsov et al., 1995). In brief, myocytes were loaded with the acetoxymethyl ester form of fura-2 by incubation with 3 μM fura-2 AM and 1.5 μl of 25% (w/w in dimethyl sulfoxide) Pluronic F-127 (BASF Wyandotte Corp., Wyandotte, MI) dissolved in 1.0 ml of Tyrode's solution for 20 min at 37°C. Cytosolic free calcium ion concentration is reported as the fura-2 fluorescence ratio, which was measured with a device (Photon Technologies, Inc., Princeton, NJ) that alternately illuminates the cells with 340- and 380-nm light while measuring emission at 520 nm (100-Hz sampling rate). To monitor cell motion, cells were simultaneously illuminated with red light and a dichroic mirror (630-nm cut-off) in the emission path deflected the cell image to a video optical system (Crescent Electronics) which tracked motion of a high contrast microsphere attached to the myocyte surface (for neonatal cardiomyocytes) or the motion of the cell edges (for adult cardiomyocytes) along raster line segments of the image during electrically stimulated contractions. The analog voltage output from the motion detector was calibrated to convert to microns of motion. The motion signal was obtained at a rate of 60 Hz and reflected the motion of the same myocyte simultaneously monitored with fura-2 for calcium. The signal was digitized and stored along with the fluorescence data. For measurements of calcium and cell motion, six successive transients were superimposed and averaged. The amplitude of the calcium transient is defined as the difference between the peak systolic and diastolic fura-2 fluorescence rations. The amplitude of the twitch is defined as the difference in cell length before electrical stimulation and at peak contraction.

Results

Development Is Associated with Changes in Cardiomyocyte G Protein α Subunit Expression. Initial studies explored whether developmental changes in Gi expression could contribute to the maturation of β_2-AR responsiveness in cardiomyocytes. G protein α subunit expression was compared in neonatal and adult ventricular myocardium as well as in cardiomyocytes isolated from neonatal and adult ventricles [using the preparations typically used by us and others for signaling studies (Xiao and Lakatta, 1993; Kuznetsov et al., 1995; Chesley et al., 2000), because contaminating cell types in intact ventricular myocardium could contribute to Gα subunit measurements and themselves may be subject to important regulatory control during development. The studies focused on $G\alpha_{i2}$ and $G\alpha_{i3}$, the PTX-sensitive $G\alpha_i$ subunits expressed by cardiomyocytes, using a selective anti-$G\alpha_{i2}$ antibody and an antibody that recognizes $G\alpha_{i3}$ but also cross-reacts with $G\alpha_o$. Figure 1 shows that $G\alpha_{i2}$ and $G\alpha_{i3/o}$ are readily detected in neonatal cardiomyocyte cultures; both PTX-sensitive Gα subunits are detected at substantially lower levels in acutely isolated adult cardiomyocytes preparations. The developmental decline in $G\alpha_{i2}$ immunoreactivity is also identified in intact myocardial tissue. In contrast, levels of $G\alpha_{i3/o}$ immunoreactivity are similar in intact neonatal and adult ventricular preparations. The discrepancy between the relative abundance of $G\alpha_{i3/o}$ in tissue and cell preparations is probably attributable to the presence of $G\alpha_o$-enriched neuronal contaminants in tissue samples and/or the induction of $G\alpha_o$ in neonatal ventricular myocytes during culture (Foster and Robishaw, 1991).

Gαs exists as short ($G\alpha_{sS}$) and long ($G\alpha_{sL}$) splice variants; $G\alpha_{sL}$ contains an additional 15 amino acid insert in the first of two linker sequences connecting the Ras-like and α-helical domains [a region close to two of the three "switch" regions in the Gs nucleotide binding pocket (Seifert et al., 1998)] (Walseth et al., 1989). $G\alpha_{sL}$ expression is prominent in neonatal cardiomyocytes, whereas Gαs is expressed largely as $G\alpha_{sS}$ in adult cardiomyocytes; little age-dependent difference in total Gαs expression is detected. Hence, the developmental change in Gα subunit expression (in intact tissue and isolated cardiomyocytes) is predicted to result in an age-dependent decrease in Gi function.

PTX-Sensitive G Proteins Do Not Modulate β_2-AR Responses in Neonatal Cardiomyocytes. The marked developmental change in $G\alpha_i$ expression in rat ventricular

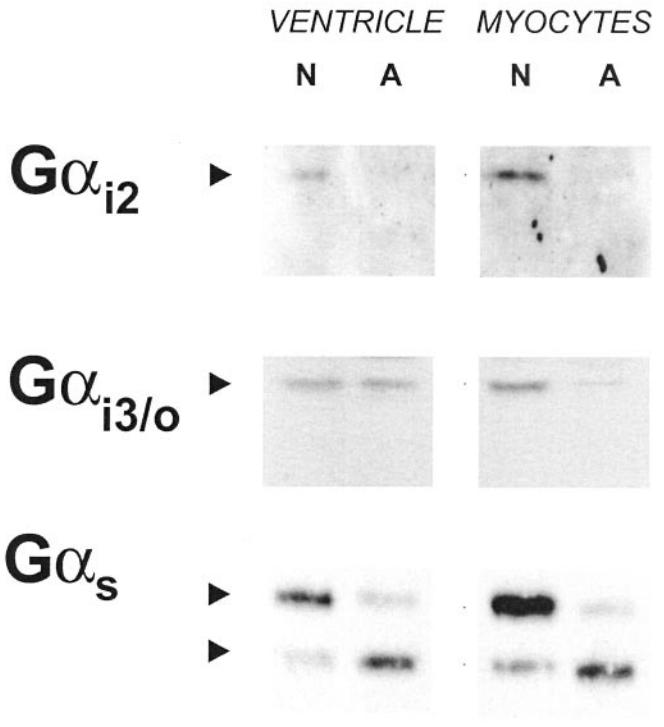

Fig. 1. Gα subunit expression in neonatal and adult cardiomyocytes and ventricles. Particulate fractions from neonatal (N) cardiomyocyte cultures, isolated adult (A) cardiomyocytes, neonatal ventricular myocardium, and adult ventricular myocardium (25 μg each) were subjected to immunoblot analysis with anti-$G\alpha_s$, anti-$G\alpha_{i2}$, and anti-$G\alpha_{i3/o}$ antibodies. Arrowheads denote the long and short splice variants of $G\alpha_s$, $G\alpha_{i2}$ and $G\alpha_{i3/o}$. In this particular experiment, the levels of $G\alpha_{i2}$ are decreased by 42% (whereas $G\alpha_{i3}$ is decreased only by 11%) in the adult, compared with the neonatal ventricular myocardial tissue. However, $G\alpha_{i2}$ and $G\alpha_{i3/o}$ levels are 91 and 87% lower in isolated adult cardiomyocytes, compared with neonatal cardiomyocytes. Similar results were obtained in two separate experiments performed on separate preparations.

myocytes provides a convenient assay system to examine the relationship between $G\alpha_i$ expression and $G\alpha_i$-dependent β_2-AR signaling. To address this issue, we examined the functional activity of $G\alpha_i$, and its role in β_2-AR responses, in neonatal rat cardiomyocytes.

Muscarinic cholinergic receptor (mAChR)-dependent inhibition of isoproterenol-activated cAMP accumulation was used as an assay to compare $G\alpha_i$ function in neonatal and adult cardiomyocytes. Table 1 shows that β-AR activation with isoproterenol elicits a similar large increase in cAMP formation in neonatal and adult rat cardiomyocytes. In each case, the stimulatory β-AR pathway is subject to inhibitory modulation by mAChRs; mAChR inhibition is mediated via Gi and it is inhibited by PTX. These results establish the functional competence of $G\alpha_i$ subunits in neonatal and adult cardiomyocytes as well as the efficacy of the PTX pretreatment protocol used to ADP-ribosylate/inactivate $G\alpha_i$ subunits. The adequacy of Gαi subunit ADP-ribosylation also was validated directly using a biochemical approach that relies upon the inverse relationship between the extent of ADP-ribosylation during the PTX pretreatment in vivo and the amount of [^{32}P]ADP-ribose that subsequently can be incorporated into control and PTX-pretreated cell membranes in an in vitro ADP-ribosylation reaction. Figure 2 shows that PTX pretreatment results in complete ADP-ribosylation/inactivation of $G\alpha_i$ subunits in neonatal and adult cardiomyocytes.

To explore the role of $G\alpha_i$ in the regulation of β_2-AR signaling to AC, it was necessary to first resolve the β_1- and β_2-AR pathways leading to cAMP accumulation. Figure 3A shows that isoproterenol (nonselective β-AR agonist) induces a robust increase in cAMP accumulation by activating both β_1- and β_2-ARs in neonatal rat cardiomyocytes. At low concentrations, isoproterenol primarily occupies the more abundant β_1-AR subtype [shown in a previous study, using radioligand binding techniques, to comprise ~84% of total β-ARs (Kuznetsov et al., 1995)]; cAMP accumulation in response to 10^{-9} M isoproterenol is completely blocked by the β_1-AR inhibitor CGP20712A. As the concentration of isoproterenol increases, a β_2-AR-component becomes more prominent; at 10^{-7} M [a concentration that maximally activates cAMP accumulation (Kuznetsov et al., 1995)], the effect of isoproterenol to increase cAMP levels is partially blocked by either CGP20712A or ICI 118,551 (β_1- and β_2-AR antagonists, respectively); 10^{-7} M isoproterenol induces only a trivial increase in cAMP accumulation in the combined presence of CGP20712A and ICI 118,551 (3.2% of the response to 10^{-7} M isoproterenol without β-AR inhibitors, $n = 5$, $p > 0.5$). The β_2-AR-dependent pathway for cAMP accumulation in neonatal cardiomyocytes also is exposed by zinterol (β_2-AR-selective agonist). Zinterol promotes a ~7-fold increase in cAMP levels by selectively activating β_2-ARs at 10^{-7} M (response completely inhibited by ICI 118,551); zinterol's selectivity for β_2-ARs progressively decreases at concentrations exceeding 10^{-7} M. Consistent with calculations (based upon published values for the affinity of zinterol at individual β-AR subtypes) that predict that 10^{-5} M zinterol occupies 100% of the β_2-ARs, but also 90% of the predominant β_1-AR population, approximately one third of the rise in cAMP levels induced by 10^{-5} M zinterol is blocked by the β_1-AR antagonist CGP20712A (not by ICI 118,551).

To determine whether the β_2-AR pathway leading to cAMP formation in neonatal rat cardiomyocytes is subject to inhibitory modulation by Gi proteins, cAMP was measured in parallel in neonatal cardiomyocyte cultures pretreated with vehicle or PTX. Figure 3B shows that PTX pretreatment neither increases nor decreases cAMP accumulation in response to isoproterenol, zinterol, or forskolin. In the context of control studies that establish functional Gi expression (and complete Gi inactivation by PTX; Fig. 2 and Table 1), these results exclude a significant role for Gi proteins in β-AR signaling to cAMP in neonatal cardiomyocytes.

Similar protocols were performed on adult cardiomyocytes. Here, isoproterenol promotes cAMP accumulation exclusively by activating β_1-ARs; the response is completely blocked by CGP20712A (Fig. 3A). cAMP levels also are not appreciably elevated by zinterol (although the absence of substantial promiscuous activation of β_1-ARs by high concentrations of zinterol is puzzling). The failure to detect a β_2-AR-dependent increase in cAMP levels in adult cardiomyocytes is not caused by dual β_2-AR actions via stimulatory Gs and inhibitory Gi proteins, with the stimulatory pathway obscured by a β_2-AR-Gi linkage. PTX pretreatment of adult cardiomyocytes (according to a regimen that reverses carbachol-dependent inhibition of β-AR-dependent cAMP formation) has no effect on cAMP accumulation in response to 10^{-9} to 10^{-8} M isoproterenol (Fig. 3B). Whereas PTX-treated cultures display slightly increased cAMP accumulation in response to 10^{-7} M isoproterenol, these actions of isoproterenol remain completely sensitive to inhibition by CGP20712A [not ICI 118,551 (data not shown)] and are accompanied by a significant increase in forskolin-dependent cAMP accumulation; zinterol does not elevate cAMP in PTX-treated adult cardiomyocytes. Collectively, these results suggest that there is a developmental increase in the inhibitory regulation of AC by PTX-sensitive G proteins (which can not be attributed to a

TABLE 1

$G\alpha_i$-mediated carbachol-dependent inhibition of isoproterenol-stimulated cAMP accumulation in neonatal and adult cardiomyocytes

Neonatal or adult cardiomyocytes were pretreated for 24 h at 37° C with vehicle or PTX (100 ng/ml for neonate and 1 μg/ml for adult). Incubations were for 5 min at room temperature in the absence or presence of isoproterenol (10^{-7} M) or carbachol (10^{-5} M, alone or starting 5 min before isoproterenol). cAMP was extracted from cells and measured by radioimmunoassay as described under *Materials and Methods*. Results are presented as mean ± S.E.M. ($n = 6$).

	Neonate		Adult	
	−PTX	+PTX	−PTX	+PTX
	pmol/dish		*pmol/100,000 cells*	
Basal	10 ± 5	13 ± 8	12 ± 6	15 ± 7
Carbachol	9 ± 4	11 ± 6	9 ± 6	10 ± 5
Isoproterenol	268 ± 24*	261 ± 30*	333 ± 42*	329 ± 34*
Isoproterenol + Carbachol	73 ± 11*†	241 ± 19*	93 ± 12*†	284 ± 31*

* $P < 0.05$ versus corresponding basal.
† $P < 0.05$ versus corresponding isoproterenol.

developmental change in $G\alpha_i$ expression). This could be caused by a developmental increase in the expression of AC type V, which is more susceptible than AC type VI to inhibitory regulation by $G\alpha_i$ proteins (Okumura et al., 2002). Importantly, the results also indicate that a putative β_2-AR-Gi linkage does not obscure or attenuate cAMP accumulation through a stimulatory β-AR-Gs pathway.

β_2-ARs are reported to modulate intracellular calcium and contractile function via Gi proteins in adult cardiomyocytes, but the contribution of Gi proteins to inotropic support by β_2-ARs in neonatal cardiomyocytes has not been examined. Figure 4 shows typical records from control and PTX-treated cardiomyocytes electrically stimulated at 1 Hz during continuous monitoring of intracellular calcium and cell motion transients. In each case, superfusion with 10^{-7} M zinterol [a concentration previously identified as selective for β_2-ARs (Kuznetsov et al., 1995)] increases the amplitude and accelerates the kinetics of relaxation of the calcium transient and the contraction. The averaged results from a series of control and PTX-treated cells studied according to this protocol failed to reveal any difference between cells that had or had not been pretreated with PTX (Table 2). β_2-AR stimulation with isoproterenol in the presence of CGP20712A (in the β_2-mode) also is not influenced by PTX (data not shown). Collectively, these studies are most consistent with a Gi-independent mechanism for inotropic support by β_2-ARs in neonatal cardiomyocytes.

Components of the β_2-AR Signaling Complex Are Organized in Caveolae/Lipid Rafts in Neonatal and Adult Cardiomyocytes. Previous studies established that β_2-ARs cofractionate with AC in caveolin-3-enriched vesicles isolated from neonatal cardiomyocytes. Neonatal cardiomyocytes represent a preparation in which β_2-ARs promote cAMP accumulation. We reasoned that the colocalization of β_2-ARs and AC in caveolae might be required to 'launch' β_2-AR signaling to cAMP. According to this formulation, the failure of β_2-ARs to promote a detectable increase in cAMP accumulation in adult cardiomyocytes could result from a change in the localization of either β_2-ARs or the AC enzyme, restricting interactions between these two molecules. To compare β-AR and AC enzyme partitioning between caveolae and other cellular membranes in neonatal and adult rat ventricular preparations, caveolae were isolated by a protocol that relies upon their resistance to solubilization in detergent-free alkaline sodium carbonate buffer at low temperatures and buoyancy in sucrose density gradients. Figure 5A shows a representative example of this fractionation scheme applied to neonatal and adult rat cardiomyocytes. In each case, the light sucrose gradient fractions contain a minor fraction of total cell protein (no detectable contamination with clathrin or ryanodine receptor immunoreactivity; Fig. 5B), but the bulk of cellular caveolin-3 immunoreactivity. Over a series of experiments, protein recovery in the light vesicle fractions tended to be somewhat higher in neonatal cultures than in their adult counterparts. This result is consistent with a somewhat higher level of caveolin-3 expression in neonatal cultures than in isolated adult cardiomyocytes (Rybin et al., 2003), because caveolin-3 drives caveolae biogenesis (Smart et al., 1999; Galbiati et al., 2001).

Immunoblot analysis reveals an unanticipated difference in the mobility of β_2-ARs (but not such proteins as Gα subunits or caveolin-3, which are not exposed on the extracellular surface) in neonatal cardiomyocyte cultures versus isolated adult cardiomyocytes (Fig. 5A). The antibody to the β_2-AR intracellular C terminus detects a broad ~66-kDa, epitope-specific immunoreactive species in caveolae fractions from neonatal cardiomyocyte cultures; this diffuse immunoreactivity is blocked by competing antigen peptide [whereas the sharp band that comigrates with β_2-ARs and persists in the presence of competing peptide is nonspecific (Fig. 6)]. The diffuse appearance of β_2-ARs can be attributed to glycosylation, because β_2-ARs migrate as distinct smaller species after treatment with PNGF (Fig. 6). In contrast, β_2-ARs are recovered from acutely isolated adult rat cardiomyocyte caveolae as smaller ~55- and ~40-kDa bands, whose migration is not altered by PNGF treatment; both of these diffuse bands are epitope-specific [i.e., blocked when immunoblot analysis is performed with competing antigen peptide (Fig. 6, right)]. Figure 6 shows that β_2-ARs are recovered from caveolin-enriched fractions isolated from intact adult ventricular myocardial tissue as diffuse ~66-kDa (PNGF-sensitive) species, similar to the pattern observed in neonatal cardiomyocyte cultures; the more rapidly migrating species are not detected. These results suggest that the enzyme-based procedure used to isolate adult cardiomyocytes from the intact ventricle results in limited proteolysis of β_2-ARs (with cleavage of the N-terminal glycosylation sites). Although it is possible that the impairment in β_2-AR signaling to cAMP (identified in this and previous studies) represents an artifact, resulting from limited proteolysis of β_2-ARs during the enzyme-based cell isolation procedure, this is considered unlikely for three reasons:

1. The cleaved β_2-ARs can be activated by zinterol; it provides effective inotropic support. Figure 7 replicates results previously published demonstrating that β_2-AR activation with zinterol leads to an increase in the amplitude of the calcium transient and the twitch in adult rat ventricular myocytes (Kuznetsov et al., 1995; Laflamme and Becker, 1998).
2. Limited proteolytic cleavage is not limited to β_2-ARs; Fig. 6 shows that β_1-ARs, which show no obvious defect in signaling to cAMP, also are detected as truncated proteins in isolated adult cardiomyocytes, but not in intact adult ventricular myocardium. These results indicate that proteolysis does not necessarily lead to a defect in coupling to AC and the generation of cAMP and that the failure to observe an increase in cAMP should not be dismissed as an artifact of β_2-AR receptor cleavage during cell isolation.

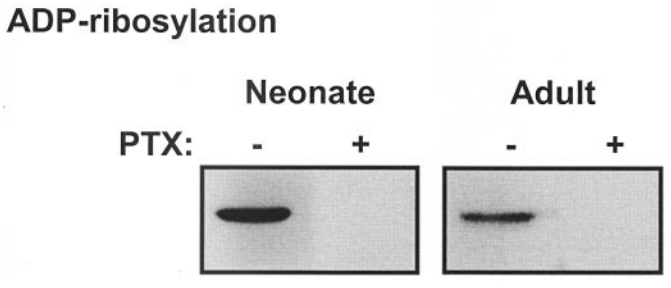

Fig. 2. PTX-dependent ADP-ribosylation of Gα subunits in neonatal and adult cardiomyocytes. Membranes from neonatal and adult cardiomyocytes pretreated for 24 h at 37°C with vehicle or PTX (100 ng/ml for the neonate and 1 μg/ml for the adult) were subjected to ADP-ribosylation.

3. Our previous studies identify β_2-ARs as full-length glycosylated proteins—which do not detectably increase cAMP accumulation—in embryonic mouse cardiomyocyte cultures (Sabri et al., 2000). Although preparative cleavage could induce subtle changes in β-AR responsiveness [perhaps by impairing higher-order events such as receptor oligomerization, which has been attributed to glycosylation and/or structural determinants in the extracellular domain of GPCRs (Rios et al., 2001; He et al., 2002)] that could represent somewhat of a liability for functional studies, the isolated cardiomyocyte preparation (freed of β_2-AR-containing cardiac fibroblasts) remains the preferred preparation to resolve catecholamine actions at cardiomyocyte β_2-ARs.

In both neonatal and adult cardiomyocyte preparations, β_2-ARs are confined to caveolae fractions; β_2-ARs are not detected in heavy fractions, even with 10-fold greater protein loading and long exposures of the gel. AC V/VI is recovered from light vesicular and (to a lesser extent) heavy sucrose fractions in the neonate but is much more confined to the light vesicular fractions in the adult (Fig. 5A). Hence, in both neonatal cardiomyocyte cultures (in which β_2-ARs promote cAMP accumulation) and adult cardiomyocytes (in which β_2-ARs do not promote a global increase in cAMP levels) β_2-ARs copurify with the bulk of the AC enzyme in caveolin-3–enriched light vesicles.

Because β_2-ARs and AC colocalize to caveolin-3–enriched light vesicles in neonatal and adult cardiomyocytes, subcellular compartmentalization of the G protein α subunits that couple receptors to the regulation of AC activity was considered. As reported previously, PTX-sensitive $G\alpha_{i2}$ subunits are highly localized to the caveolae fractions of neonatal cardiomyocytes (Fig. 5A; Rybin et al., 1999). $G\alpha_{i2}$ localizes similarly to caveolin-3-enriched light vesicles isolated from adult cardiomyocytes. In contrast, the two molecular species of $G\alpha_s$ partition differently across the gradient. In neonatal cardiomyocytes that express $G\alpha_{sS}$ and $G\alpha_{sL}$, $G\alpha_{sL}$ is recovered in both caveolae and heavy sucrose fractions, whereas $G\alpha_{sS}$ is much more localized to caveolae. In adult cardiomyocytes, Gαs is expressed largely as the short splice variant

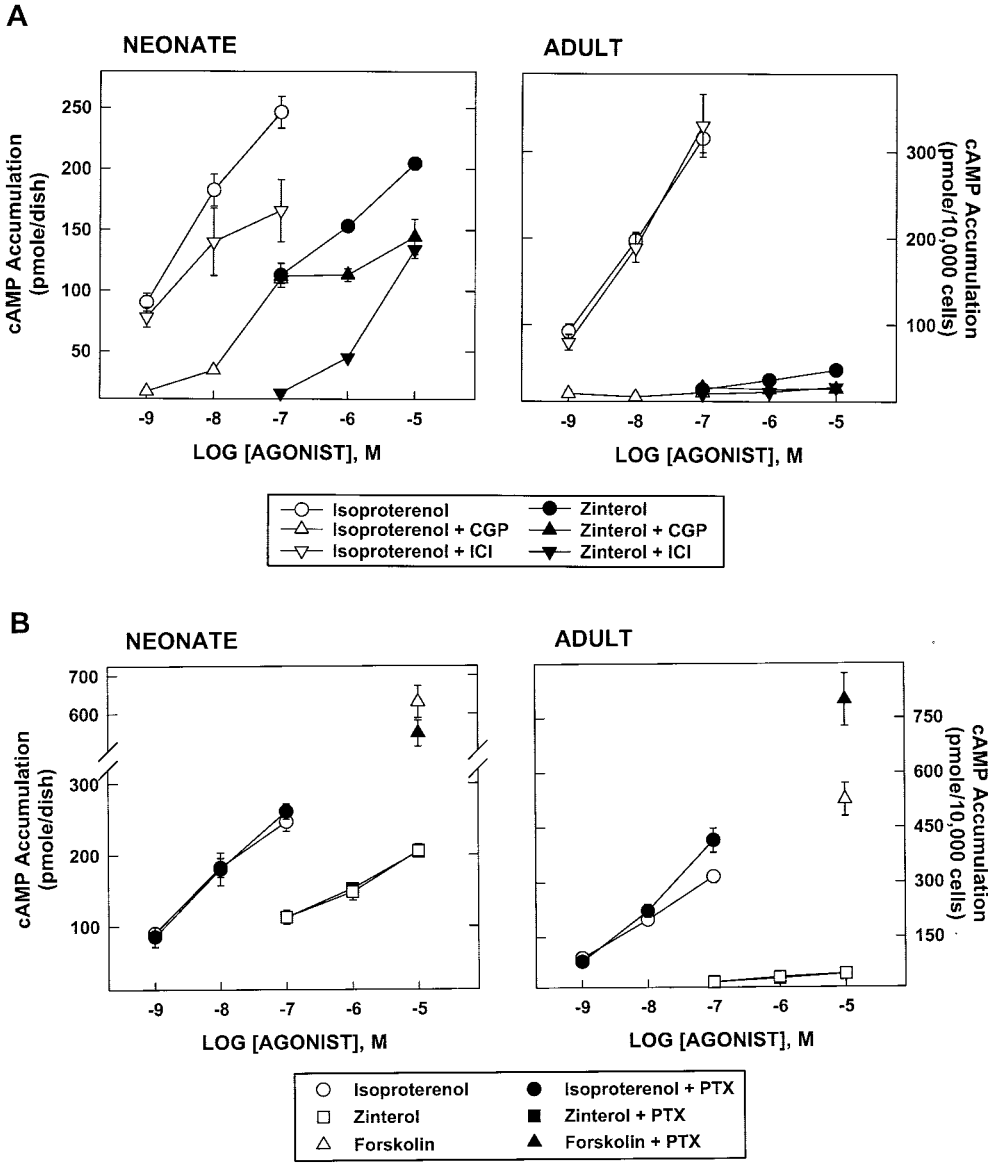

Fig. 3. β_2-ARs promote cAMP accumulation in neonatal, but not adult, cardiomyocytes; β_2-AR signaling to cAMP is not influenced by PTX-sensitive G proteins. A, neonatal or adult cardiomyocytes were challenged for 5 min with the indicated concentrations of isoproterenol or zinterol; cAMP accumulation (in the absence or in the presence of 10^{-7} M CGP20712A or 10^{-7} M ICI118,551, each starting 5 min before β-AR stimulation) was determined as described under *Materials and Methods*. Results are expressed as mean ± S.E.M. cAMP accumulation over basal (13.7 ± 2.3 pmol/well or 17.6 ± 0.7 pmol/10,000 cells for neonatal [n = 10] or adult cells [n = 9], respectively). Errors fall within some data points. B, neonatal or adult cardiomyocytes were pretreated for 24 h at 37°C with PTX (100 ng/ml for the neonate and 1 μg/ml for the adult) and then challenged for 5 min with the indicated concentrations of isoproterenol, zinterol, or forskolin before measurements of cAMP accumulation. Results are expressed as mean ± S.E.M. cAMP accumulation over basal cAMP accumulation (which was not altered by the PTX-treatment). Errors fall within some data points; on the scale used for this figure, the results for zinterol alone and zinterol + PTX are superimposed.

and is recovered largely in light vesicular fractions. These results suggest that the developmental shift from $G\alpha_{sL}$ to $G\alpha_{sS}$, in the context of the difference in $G\alpha_{sL}$ versus $G\alpha_{sS}$ partitioning across the gradient, contribute to the striking age-dependent difference in the subcellular $G\alpha_s$ targeting.

A mechanism for differences in $G\alpha_{sS}$ versus $G\alpha_{sL}$ targeting is not obvious. Because $G\alpha_s$ subunits are variably reported to interact with caveolin proteins (Huang et al., 1997; Oh and Schnitzer, 2001), we tested the hypothesis that caveolin-3 discriminates $G\alpha_{sS}$ versus $G\alpha_{sL}$ (perhaps explaining differ-

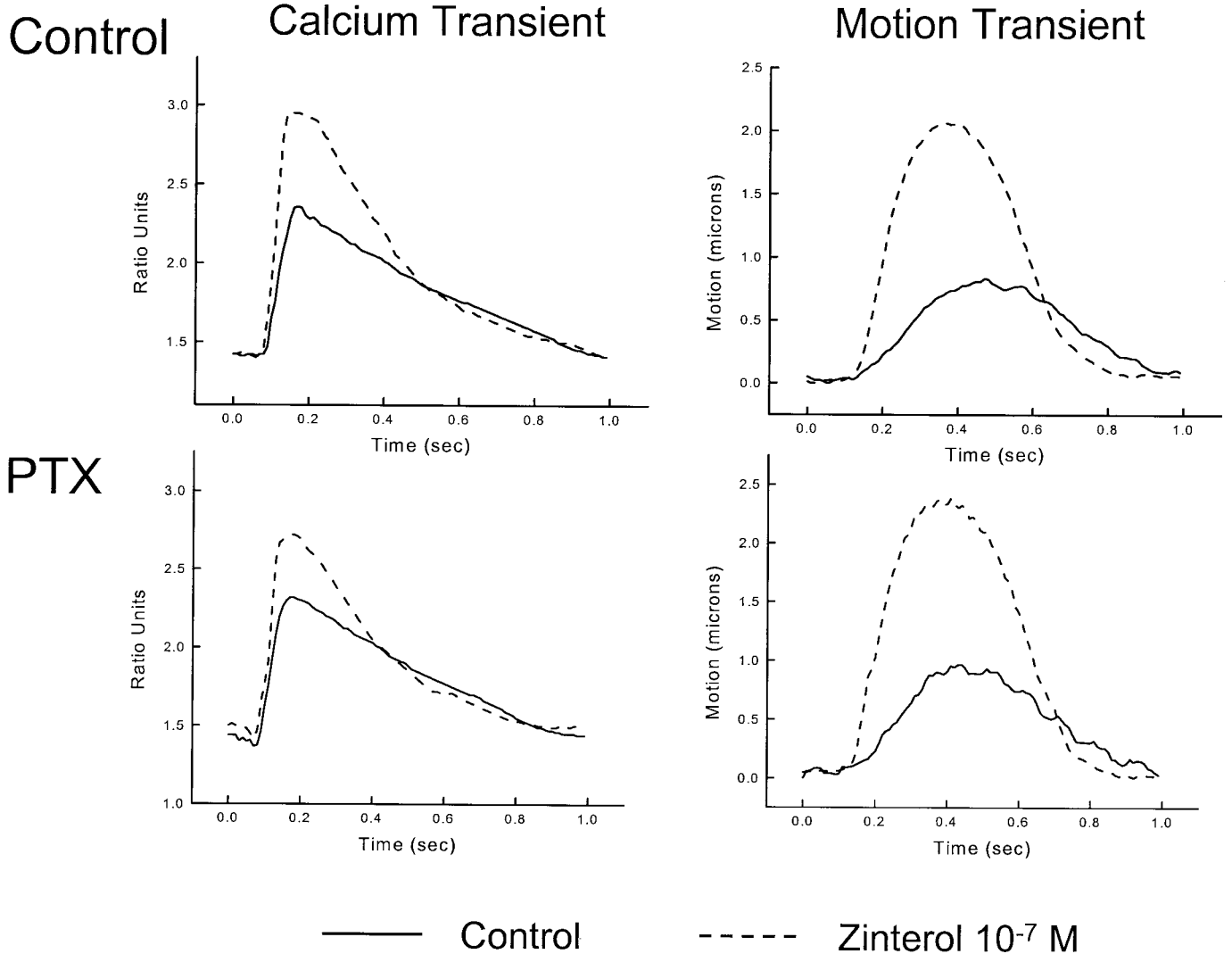

Fig. 4. β_2-AR modulation of calcium transients and cell shortening is not influenced by PTX in neonatal rat cardiomyocytes. Representative tracings from cardiomyocytes cultured with vehicle or PTX (100 ng/ml) for 24 h and then electrically driven at 1 Hz during challenge with 10^{-7} M zinterol. Cardiomyocyte shortening was recorded as micrometers of motion of a glass microsphere on the cell surface. Because the motion of only one portion of the neonatal myocytes is monitored (rather than total cell length), the position of the microsphere before electrical stimulation is set to zero (diastole) and motion relative to the diastolic position is reported. Signal averaged motion transients during the control interval and at the peak of the response to zinterol (which was at 3 min in both vehicle and PTX-treated cardiomyocytes) are superimposed for comparison.

TABLE 2

β_2-AR-dependent modulation of calcium transients and cell shortening is not influenced by PTX in neonatal cardiomyocytes

Calcium or motion amplitudes are defined as the differences between ratio values (for calcium) or the position of a microsphere attached to the cell surface (for motion) before electrical stimulation and at peak contraction, respectively. Duration is measured at half-maximal amplitude. Results represent the mean ± S.E.M. for determinations in cardiomyocytes exposed to vehicle or 100 ng/ml PTX for 24 h at 37° C and then challenged with 10^{-7} M zinterol. Effects of zinterol to increase the amplitude and shorten the duration of calcium and cell-shortening transients in vehicle and PTX-treated cells are statistically significant and not distinguishable between vehicle- and PTX-treated cultures.

	−PTX (n = 16)		+PTX (n = 16)	
	Basal	Zinterol	Basal	Zinterol
Calcium amplitude (ratio units)	0.94 ± .09	1.47 ± .11*	0.97 ± .08	1.50 ± .08*
Calcium duration (ms)	423 ± 16	322 ± 12*	433 ± 14	333 ± 7*
Motion amplitude (μm)	.89 ± .13	2.23 ± .17*	.96 ± .11	2.12 ± .16*
Motion duration (ms)	530 ± 23	477 ± 19*	523 ± 26	467 ± 23*

* $P < 0.05$ versus corresponding basal.

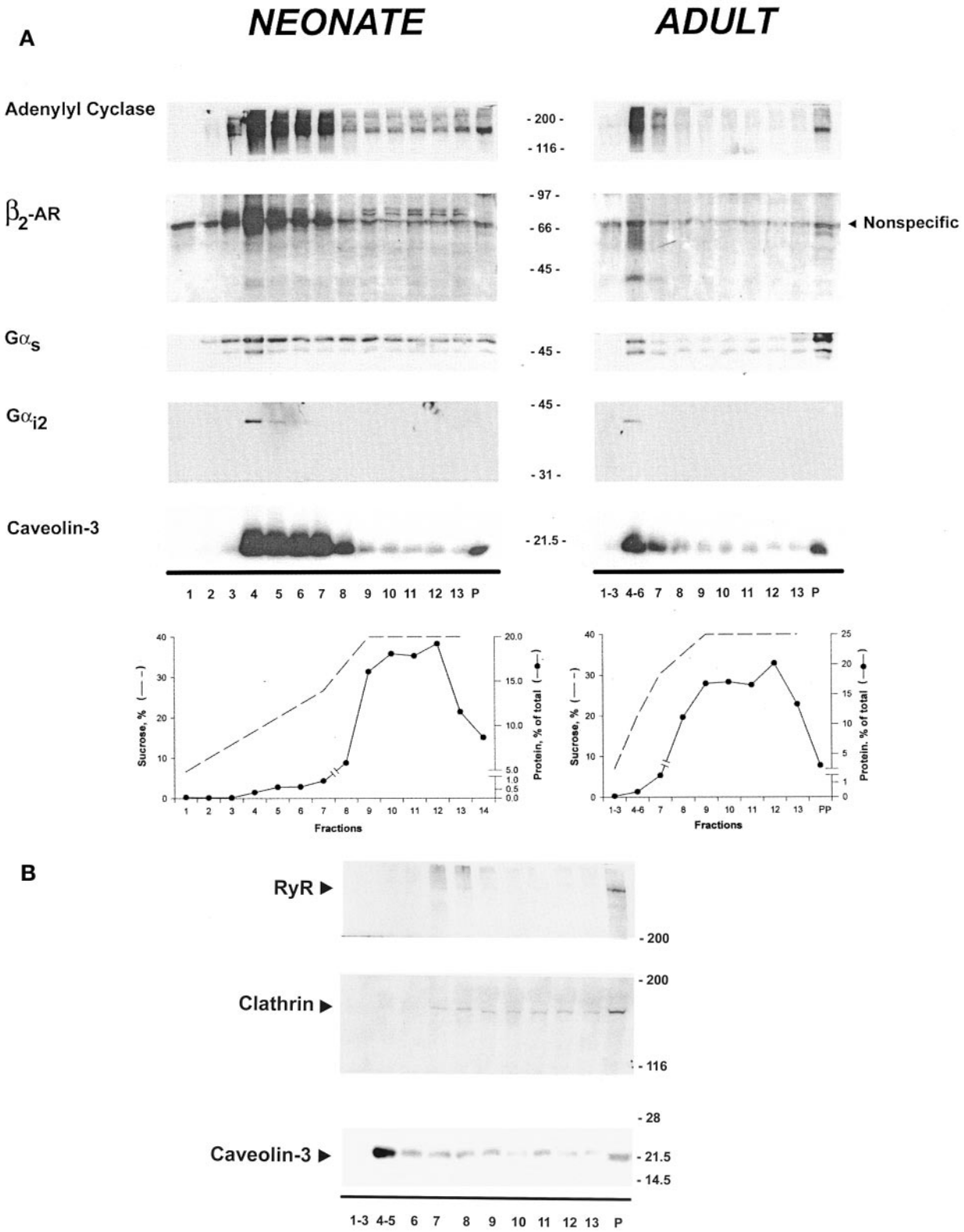

Fig. 5. β_2-ARs, G protein α subunit, and AC partitioning between caveolin-3–enriched vesicles and heavy sucrose fractions in neonatal and adult cardiomyocytes. Neonatal or adult cardiomyocytes were homogenized in sodium carbonate buffer and fractionated by relative buoyancy in sucrose gradients as described under *Materials and Methods*. Fractions were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblot analysis with anti-AC V/VI, anti-β_2-AR, anti-$G\alpha_s$, anti-$G\alpha_{i2}$, and anti-caveolin-3 antibodies (A). A separate profile from adult rat cardiomyocytes was used to document that ryanodine receptors and clathrin are not recovered in the caveolin-3-enriched fractions (B). Protein recovery in gradient fractions is reported as a percentage of total protein. Fractions 1 to 3 and 4 to 6 (in A, or fractions 1 to 3 and 4 to 5 in B) in profiles from adult cardiomyocytes were pooled, because of the limiting amounts of protein recovered in these fractions. Results are representative of data from three separate experiments.

ences in $G\alpha_s$ splice variant targeting as well as inconsistencies regarding caveolin-3-$G\alpha_s$ interactions in the literature). Figure 8 shows that extracts prepared with an Igepal-containing buffer from neonatal rat cardiomyocytes contain all of the cellular caveolin-3 and a substantial fraction of total cellular $G\alpha_{sS}$ and $G\alpha_{sL}$. Immunoprecipitation of caveolin-3 from these extracts (according to conditions that essentially clear caveolin-3 immunoreactivity from the Igepal extract) leads to the coimmunoprecipitation of a minor portion of total $G\alpha_{sL}$ but not $G\alpha_{sS}$. These results indicate that the distinct $G\alpha_s$ splice variants differ in their interactions with caveolin-3 (which could potentially explain discrepancies in previous literature on this issue), but these immunoprecipitation experiments do not provide an explanation for the apparent preferential targeting of $G\alpha_{sS}$ to caveolin-enriched vesicles.

Discussion

The mechanisms whereby β_2-ARs provide inotropic support and influence the evolution of cardiac hypertrophy and failure remain incompletely understood. This study expands upon current models of β_2-AR signaling to show that β_2-ARs promote cAMP accumulation, modulate calcium cycling, and enhance contractile function in a completely PTX-sensitive, Gi protein-independent manner in neonatal cardiomyocytes. β_2-ARs also increase the amplitude of contraction but without a detectable increase in cAMP levels in adult rat cardiomyocytes. The failure to detect a β_2-AR–dependent increase in cAMP in adult rat cardiomyocytes can not be attributed to spatial segregation of β_2-ARs, $G\alpha_s/G\alpha_i$ subunits, and AC to distinct membrane subdomains; β_2-ARs, $G\alpha$ subunits, and AC all copurify with buoyant caveolin-3–enriched membranes isolated from neonatal and adult rat cardiomyocytes. Dual coupling of β_2-ARs to Gs and Gi also does not provide an obvious mechanism to preclude β_2-AR signaling to AC, because β_2-ARs do not promote cAMP accumulation in PTX-pretreated adult rat cardiomyocytes. These later results are noteworthy, because there is still only limited information on the nature of the Gi-dependent pathway(s) emanating from β_2-ARs in adult rat cardiomyocytes. Because Gi proteins typically link GPCRs to inhibition of AC, some investigators have inferred that β_2-ARs exert dual regulation on AC activity through reciprocal actions mediated by Gs and Gi proteins. However, these studies provide clear evidence that $G\alpha_i$ subunits do not mitigate cAMP formation by either β_1- or β_2-ARs. The mechanism whereby β_2-ARs provide inotropic support in adult rat cardiomyocytes, without detectably elevating cAMP levels, remains uncertain. Our previous study identified intracellular alkalinization (which would enhance myofibrillar calcium sensitivity) as a cAMP-independent inotropic mechanism for β_2-ARs in adult cardiomyocytes (Jiang and Steinberg, 1997). However, an additional contribution from a cAMP signal that is too small (or too highly localized to the plasma membrane) to be detected by a typical cAMP radioimmunoassay that measures a rise in cAMP in the bulk cytosol cannot be excluded. Future studies with newer technologies that resolve localized changes in cAMP levels in subcellular microdomains in cardiomyocytes should be revealing (Zaccolo and Pozzan, 2002).

This study demonstrates that the standard enzyme-based procedure typically used to isolate cardiomyocytes from intact rat ventricles results in limited proteolysis of β-ARs. This has not been appreciated previously but might have been predicted based upon previous results suggesting that proteolysis of the extracellular tethered-ligand domain of protease-activated receptor-1 (PAR-1, the receptor for thrombin) during enzyme-based cell disaggregation renders adult

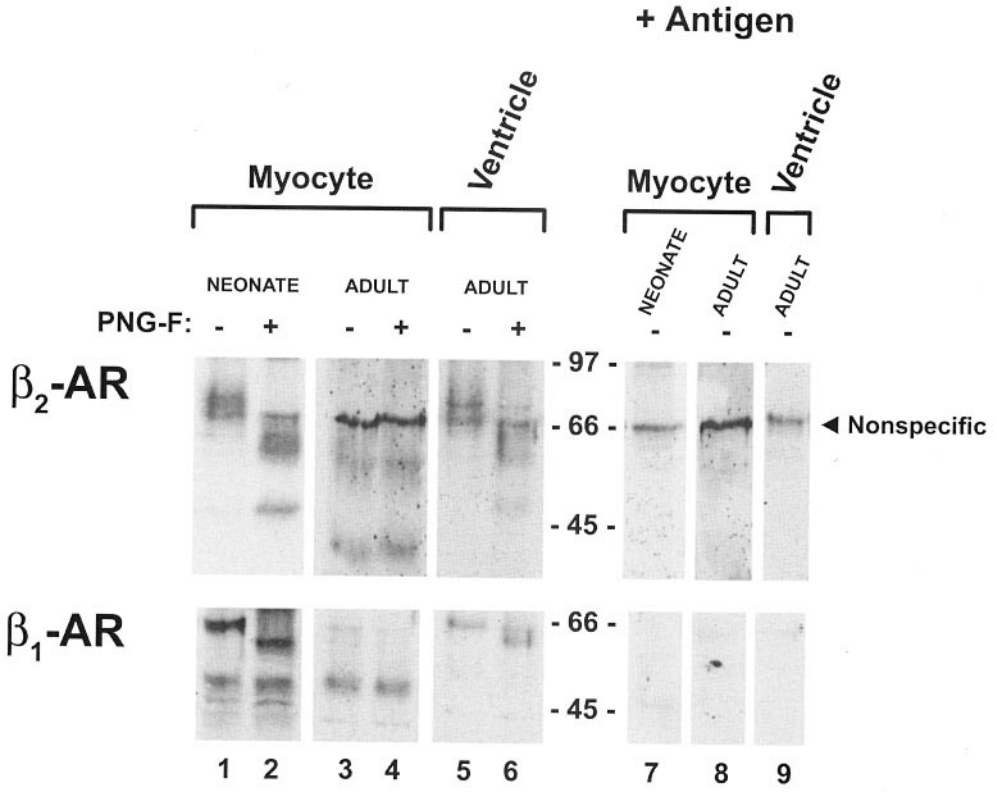

Fig. 6. Immunoblot analysis of β-ARs in neonatal and adult cardiomyocyte and cardiac tissue preparations. Membranes from neonatal cardiomyocyte cultures (lanes 1, 2, and 7), acutely isolated adult cardiomyocytes (lanes 3, 4, and 8), and adult ventricular myocardial tissue (5, 6, and 9) were subjected to immunoblot analysis with antibodies that recognize the C terminus of the β_2- and β_1-ARs (without and with preblocking with antigen peptide to distinguish β-ARs from nonspecific immunoreactivity). Samples were treated with PNGF to deglycosylate the receptor as indicated.

rat cardiomyocytes relatively refractory to thrombin's actions (Jiang et al., 1998). Proteolytic cleavage of GPCRs is probably an intrinsic (and heretofore unnoticed) feature of standard adult rat cardiomyocyte preparations used for biochemical and electrophysiologic studies. However, the significance of limited β-AR proteolysis during cell isolation is uncertain, because truncated β_1-ARs promote a robust increase in cAMP accumulation and truncated β_2-ARs effectively provide inotropic support. Nevertheless, recent evidence that higher-order events such as β_1-AR receptor oligomerization may be influenced by N-terminal glycosylation events suggests that limited β-AR proteolysis could introduce subtle changes in the regulation of β-AR responsiveness that deserve further study (He et al., 2002).

Recent efforts to decipher β_2-AR subtype function have relied heavily on the β_2-AR-selective agonist zinterol. This study presents evidence that zinterol's actions are faithfully mimicked by isoproterenol plus CGP (the more traditional β-AR agonist acting in the β_2-mode), largely eliminating significant concerns that zinterol exerts unusual properties as an agonist. However, the pharmacologic studies with β-AR subtype selective antagonists emphasize that zinterol displays only a modest level of selectivity for β_2-ARs over β_1-ARs. In neonatal cardiomyocytes, where β_2-AR responses are elicited by 10^{-7} M zinterol (a relatively low concentration that retains full β_2-AR selectivity), the use of zinterol without a β_1-AR antagonist is justified. However, for reasons that are still obscure, β_2-AR-dependent responses are optimally detected only at high zinterol concentrations in the adult cardiomyocytes [2 log orders higher than in the neonate (Steinberg, 1999; Xiao et al., 1999)]. The evidence that zinterol's β_2-AR-selectivity declines rapidly at concentrations $>10^{-7}$ M suggests caution in interpreting previous studies that used 10^{-5} M zinterol (without a β_1-AR inhibitor) to selectively activate β_2-ARs in adult rat cardiomyocytes.

This study identifies a developmental switch in $G\alpha_s$ subunits expression from $G\alpha_{sL}$ to $G\alpha_{sS}$. The functional significance of the distinct molecular forms of $G\alpha_s$ has never been adequately explained. Previous efforts to distinguish the signaling properties of $G\alpha_{sS}$ and $G\alpha_{sL}$ have relied largely on in vitro assays, where $G\alpha_{sS}$ reportedly shows a somewhat increased affinity for GDP [and activates AC somewhat more effectively than $G\alpha_{sL}$ (Seifert et al., 1998)]. However, this study shows that $G\alpha_{sL}$ and $G\alpha_{sS}$ differ markedly in their subcellular targeting; this property would be pertinent to studies in intact cells, but probably would be missed in studies that rely on in vitro assays to discriminate $G\alpha_s$ splice variant function. A mechanism that would permit $G\alpha_{sL}$ to distribute widely (in caveolae as well as other membrane domains) but restrict $G\alpha_{sS}$ to caveolae is not obvious. Although $G\alpha_s$ splice variants differ in their propensity to form complexes with caveolin-3, $G\alpha_{sL}$ (rather than $G\alpha_{sS}$, which is preferentially recovered in caveolin-3–enriched membranes) coimmunoprecipitates with caveolin-3. $G\alpha_s$ subunits anchor to plasma membranes (and partitioning to caveolae/lipid rafts) because of covalent lipid modifications and stable interactions with $\beta\gamma$ dimers (Moffett et al., 2000; Zaccolo and Pozzan, 2002). However, there is no reason to expect differences in N-terminal palmitoylation of $G\alpha_{sL}$ versus $G\alpha_s$. Rather, distinct interactions with $\beta\gamma$ dimers (with distinct subcellular distributions) are more likely, particularly as dramatic age-dependent differences in $\beta\gamma$ dimer expression have been reported (Hansen et al., 1995).

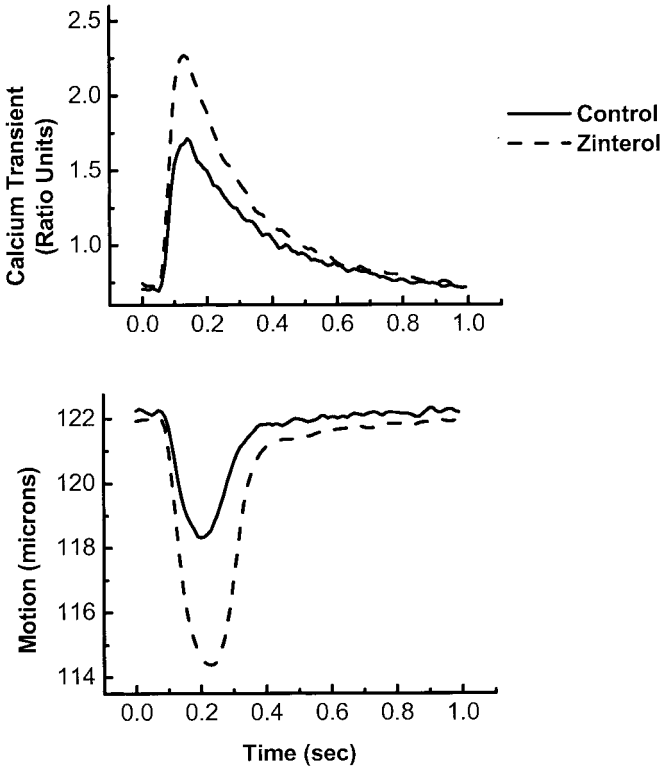

Fig. 7. β_2-ARs provide inotropic support in cardiomyocytes isolated from the adult ventricle. Representative tracings demonstrating that superfusion with zinterol (10^{-5} M, in the presence of 10^{-7} M CGP20712A) increases the amplitude of the calcium transient and twitch in adult rat cardiomyocytes.

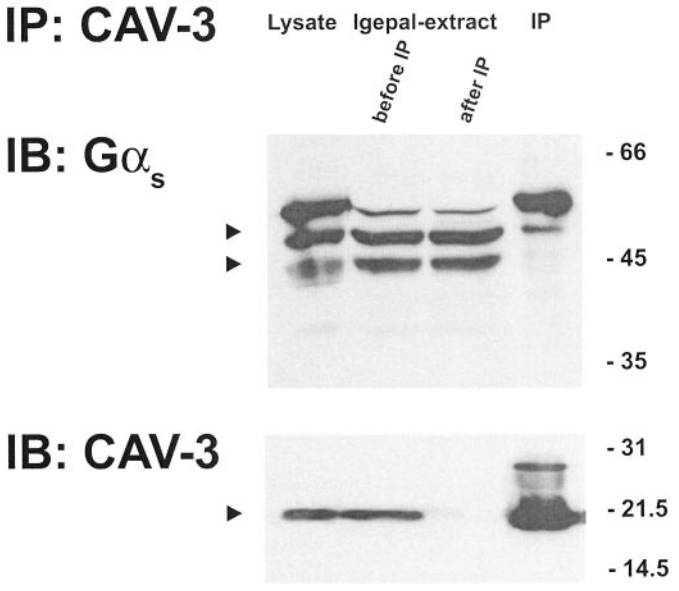

Fig. 8. Immunoprecipitation of caveolin-3 and $G\alpha_s$. Cardiomyocytes were lysed and subjected to immunoprecipitation with anti-caveolin-3 IgG. The starting lysate, Igepal extract before and after immunoprecipitation (IP) with anti-caveolin-3 and the anti-caveolin-3 immunoprecipitate was probed by Western analysis with anti-$G\alpha_s$ and anti-caveolin-3 IgGs. Under conditions that clear caveolin-3 from the Igepal-extract, we detect coimmunoprecipitation of $G\alpha_{sL}$ but not $G\alpha_{sS}$. The larger $G\alpha_s$ immunoreactive species also coimmunoprecipitates with caveolin-3; the significance of this observation is uncertain. The result was replicated in three separate experiments.

On the basis of studies that implicate PTX-sensitive G proteins in the regulation of cardiac β_2-AR responsiveness, current models generally have assumed that altered $G\alpha_i$ expression (as occurs in heart failure) would functionally affect β_2-AR signaling (Xiao, 2001). However, the relationship between $G\alpha_i$ expression and β_2-AR signaling via $G\alpha_i$ proteins has not been subject to formal scrutiny in previous studies. This study identifies a developmental decline in PTX-sensitive G protein α subunit expression in rat ventricular myocytes [similar to the results previously reported for rat and rabbit ventricle (Luetje et al., 1988; Kumar et al., 1994; Bartel et al., 1996)], identifying rat ventricles as a convenient assay system to test the hypothesis that changes in $G\alpha_i$ expression translate directly into changes in $G\alpha_i$-dependent β_2-AR signaling. The observation that β_2-AR responses are not modulated by PTX-sensitive G proteins in neonatal rat cardiomyocytes that express ample levels of functional $G\alpha_i$ proteins deserves emphasis. These results suggest that the intuitive assumption that up-regulation of $G\alpha_i$ proteins in human heart failure syndromes might be sufficient to shift the balance of β_2-AR signaling toward Gi pathways [and thereby constitute a cardioprotective mechanism (Xiao et al., 1999)] deserves more direct and careful scrutiny. The results also emphasize that even sophisticated models of signal transduction mechanisms that allow for localized differences in signaling molecule stoichiometry (imposed by structures such as caveolae/lipid rafts) may be inadequate to fully explain the complexity of signaling networks in differentiated cells such as cardiomyocytes.

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