

Substrate Specificities of G Protein-Coupled Receptor Kinase-2 and -3 at Cardiac Myocyte Receptors Provide Basis for Distinct Roles in Regulation of Myocardial Function

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ABSTRACT

The closely related G protein-coupled receptor kinases GRK2 and GRK3 are both expressed in cardiac myocytes. Although GRK2 has been extensively investigated in terms of regulation of cardiac β -adrenergic receptors, the substrate specificities of the two GRK isoforms at G protein-coupled receptors (GPCR) are poorly understood. In this study, the substrate specificities of GRK2 and GRK3 at GPCRs that control cardiac myocyte function were determined in fully differentiated adult cardiac myocytes. Concentration-effect relationships of GRK2, GRK3, and their respective competitive inhibitors, GRK2ct and GRK3ct, at endogenous endothelin, α_1 -adrenergic, and β_1 -adrenergic receptor-generated responses in cardiac myocytes were achieved by adenovirus gene transduction. GRK3 and GRK3ct were highly potent and efficient at the endothelin receptors (IC_{50} for GRK3, 5 ± 0.7 pmol/mg of protein; EC_{50} for GRK3ct, 2 ± 0.2 pmol/mg of protein). The α_1 -adrenergic re-

ceptor was also a preferred substrate of GRK3 (IC_{50} , 7 ± 0.4 pmol/mg of protein). GRK2 lacked efficacy at both endothelin and α_1 -adrenergic receptors despite massive overexpression. On the contrary, both GRK2ct and GRK3ct enhanced β_1 -adrenergic receptor-induced cAMP production with comparable potencies. However, the potency of GRK3ct at β_1 -adrenergic receptors was at least 20-fold lower than that at endothelin receptors. In conclusion, this study demonstrates distinct substrate specificities of GRK2 and GRK3 at different GPCRs in fully differentiated adult cardiac myocytes. As inferred from the above findings, GRK2 may play its primary role in regulation of cardiac contractility and chronotropy by controlling β_1 -adrenergic receptors, whereas GRK3 may play important roles in regulation of cardiac growth and hypertrophy by selectively controlling endothelin and α_1 -adrenergic receptors.

G protein-coupled receptor kinases (GRK) are enzymes that catalyze phosphorylation and cause desensitization of agonist-activated G protein-coupled receptors (GPCR). GRK-catalyzed phosphorylation of GPCRs promotes binding of arrestins, which sterically interdict receptor coupling to its cognate G protein. Previous investigations have revealed that several GRK isoforms are expressed in myocardial tis-

sue (Pitcher et al., 1998; Vinge et al., 2001). We have previously reported that the cellular distribution of GRK3 (also called β ARK2) in rat, porcine, and human myocardial tissue is restricted to cardiac myocytes (Vinge et al., 2001). This is in sharp contrast to the cellular distribution of myocardial GRK2 (also called β ARK1) and GRK5, which are ubiquitously expressed. Although cardiovascular GRK2 is predominantly expressed in endothelial cells, it can also be detected in cardiac myocytes (Vinge et al., 2001). The nonuniform distribution of GRK2 and GRK3 may suggest that these isoforms subserve different roles in regulation of myocardial function. Distinct cellular distribution of the GRK isoforms

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ABBREVIATIONS: GRK, G protein-coupled receptor kinase; GPCR, G protein-coupled receptor; β ARK, β -adrenergic receptor kinase; PH, pleckstrin homology; GST, glutathione transferase; PAGE, polyacrylamide gel electrophoresis; IP, inositol phosphate; ET-1, endothelin-1; m.o.i., multiplicity of infection; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; AR, adrenergic receptor; ET-R, endothelin receptor.

may also indicate that the different isoforms regulate different cardiovascular GPCRs. Although the role of GRK2 in regulation of cardiac β -adrenergic receptors (β -ARs) has been extensively studied, the substrate specificities of the different GRK isoforms toward cardiovascular GPCRs are poorly understood.

GRK2 and GRK3 constitute a subfamily among the different GRK isoforms and consist of three domains: an amino-terminal RGS homology domain, a central protein kinase domain, and a carboxyl-terminal pleckstrin homology (PH) domain. The PH domain is unique to GRK2 and GRK3 and constitutes the mechanism targeting these kinases to the plasma membrane (Pitcher et al., 1992; Koch et al., 1993). Analysis of the crystal structure of GRK2 bound in complex with $G\beta\gamma$ revealed that the PH domain provides binding interfaces with $G\beta\gamma$, the plasma membrane constituent phosphatidylinositol-bisphosphate, as well as the cytoplasmic surface of the receptor (GPCR) to bring the kinase in proper orientation for catalysis of phosphorylation of the ligand-bound GPCR (Lodowski et al., 2003). It seems that this complex trimeric interaction also determines the substrate specificities of GRK2 and GRK3. Thus, hypothetically, GRK2 and GRK3 interact with different GPCRs on cardiac myocytes through specificity mediated to large extent by the divergent primary structures of the carboxyl-terminal regions of these kinases.

Competitive inhibition of GRK2 by minigene-directed expression of a polypeptide from the carboxyl-terminal PH domain of GRK2 has provided substantial insights into regulation of β -AR function (Koch et al., 1995; Drazner et al., 1997). These studies revealed that endogenous GRK2 controls β_1 -AR signaling in cardiac myocytes. Although data from over-expression studies indicate that GRK2 may regulate several GPCRs (Freedman et al., 1995, 1997; Diviani et al., 1996), little information is available regarding the substrate specificities of this receptor kinase. Even less is known about the substrate specificities of GRK3, as well as the role of GRK3 in the regulation of cardiac myocyte function. Thus, the principal aim of the present study was to determine the substrate specificities of GRK2 and GRK3 at three important GPCRs in fully differentiated adult rat cardiac myocytes [i.e., β_1 -ARs, α_1 -adrenergic receptors (α_1 -ARs) and endothelin receptors (ET-Rs)]. The study provides novel data demonstrating distinct substrate specificities of GRK2 and GRK3 at GPCRs. The implications of the study are that the two kinases exert distinct roles in regulation of cardiac myocyte function.

Materials and Methods

Isolation of Cardiac Myocytes and Maintenance of Primary Cell Cultures. Cardiac myocytes were isolated from adult rat hearts (male Wistar rats ~ 250 g) by Ca^{2+} -free retrograde perfusion and enzymatic digestion as described previously (Vinge et al., 2001). Ca^{2+} was reintroduced in successive steps to 0.5 mM, and the cardiac myocytes were plated in wells precoated with mouse laminin (Invitrogen, Carlsbad, CA) and maintained in Joklik's minimum essential medium supplemented with 23.8 mM sodium bicarbonate, 0.6 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1 mM DL-carnitine, 10 mM creatine, 20 mM taurine, 0.1 mg/ml bovine serum albumin, 0.1 μ M insulin, and 0.1 nM thyroxine in humidified atmosphere containing 5% CO_2 . The homogeneity of these primary isolates was assessed by immunocytochemical analysis as described previously (Vinge et al., 2001). Less

than 2% of the cells of the cardiac myocyte preparations were non-cardiac myocytes (data not shown). Cardiac myocytes were plated at a density of 100,000 cells per 35-mm culture dish and infected with recombinant adenovirus 2 h after seeding onto cell culture dishes. All assays were performed 48 h after infection. The experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Generation of Recombinant Adenoviruses Encoding GRK2, GRK3, and Their Respective Competitive Inhibitors GRK2ct and GRK3ct or LacZ. All recombinant adenoviral clones were derived from replication-deficient human adenovirus type 5 that had been altered by deleting the E1 and E3 regions of the viral genome. Generation of recombinant adenovirus encoding rat GRK2 (β ARK1) or a peptide inhibitor of GRK2 (GRK2ct/ β ARK1ct) has previously been described (Drazner et al., 1997; Peppel et al., 2000). Recombinant adenovirus encoding rat GRK3 was generated using the Adeno-X Expression System (Clontech, Mountain View, CA). In brief, the open reading frame of rat GRK3 was ligated into the expression cassette of pShuttle. The expression cassette of pShuttle was subsequently transferred and ligated into the Adeno-X viral DNA using the unique restriction sites PI-SceI and I-CeuI. A minigene encoding a peptide inhibitor of GRK3 (GRK3ct/ β ARK2ct) was designed similar to the GRK2ct/ β ARK1ct minigene. In brief, a carboxyl-terminal fragment of GRK3 (amino acids 495–687) with preceding Kozak consensus sequence for initiation of transcription was amplified by PCR and subcloned between the restriction sites XbaI and KpnI of pShuttle. The amplified DNA fragment was verified by DNA sequence analysis. The expression cassette was subsequently transferred and ligated into Adeno-X viral DNA. Recombinant adenoviral DNA encoding prokaryotic LacZ cDNA was generated from the pShuttle-LacZ plasmid provided with the Adeno-X Expression System. Recombinant adenoviruses were obtained by transfection of adenoviral DNA into HEK293 cells (American Type Culture Collection, Manassas, VA) with subsequent amplification and purification of virus as described previously (Graham and Prevec, 1995). For all adenoviral clones, infectious titers were determined simultaneously using the Adeno-X Rapid Titer Kit (Clontech).

Determination of Expression Levels of Recombinant Proteins. Cardiac myocytes infected with recombinant adenovirus were harvested in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), homogenized by ultrasonication, and heat-denatured (85°C/5 min). To quantify the expression levels of GRK2ct and GRK3ct, standard curves with known amounts of affinity-purified glutathione transferase (GST) fusion proteins of the same carboxyl-terminal segments of GRK2 (GST-GRK2ct) and GRK3 (GST-GRK3ct), respectively, were employed. The purity of the fusion proteins (glutathione-Sepharose affinity purified) was assessed by SDS-PAGE and Coomassie blue staining. Western blot analysis of GST-GRK2ct and GST-GRK3ct with anti-GST-antiserum was employed to estimate the relative amounts of fusion protein per unit of purified protein. Non-homologous tissue extracts (fish liver lysate) were employed as carrier on SDS-PAGE. The expression levels of GRK2ct and GRK3ct in infected cardiac myocytes were determined by Western blot analysis using standard curves with known amounts of standard (GST-GRK2ct or GST-GRK3ct) and specific IgG antibodies (anti-GRK2 or anti-GRK3; Santa Cruz Biotechnology, Santa Cruz, CA), as described previously (Vinge et al., 2001).

Determination of Endogenous Levels of GRK2 and GRK3 in Cardiac Myocytes. Cardiac myocytes were harvested in radioimmunoprecipitation assay buffer and detergent solubilized as described previously (Vinge et al., 2001). The solubilized extract (1 mg of protein) was incubated at 4°C overnight with the common epitope-directed anti-GRK2/3 IgG_{2ak} (Upstate Biotechnology, Charlottesville, VA). The immune complexes were then captured with protein A agarose, washed in ice-cold phosphate-buffered saline, and separated by 10% SDS-PAGE. Western blot analysis using purified anti-GRK2-

or anti-GRK3-specific IgG was performed as described previously (Vinge et al., 2001). Standards containing serial dilutions of affinity purified GST-GRK2ct and GST-GRK3ct fusion proteins were also included in the same SDS-PAGE to allow quantitative determination of endogenous anti-GRK2 and anti-GRK3 immunoreactivities using the standard curve method.

Phosphoinositide Hydrolysis. Agonist-stimulated phosphoinositide hydrolysis was performed as described previously (Bremnes et al., 2000), in cardiac myocytes metabolically labeled for 18 to 24 h in Joklik's minimum essential medium (including the supplements as indicated above) containing 3 μ Ci of [*myo*-2-³H]inositol (14 Ci/mmol; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The cells were preincubated for 10 min in medium containing LiCl (20 mM) before initiation of assay. The assay was stopped after 30 min unless otherwise indicated. Total inositol phosphates (IP) were separated by ion exchange chromatography (AG 1 \times 8, formate form; Bio-Rad Laboratories) and determined by liquid scintillation spectrometry.

Assay of p42/p44 MAPK Activities. Assay of p42/p44 MAPK activity was performed after incubation of cardiac myocytes in the absence or presence of endothelin-1 (ET-1; 5 nM) or phenylephrine (1 μ M) for 5 min. The reactions were stopped and the cells harvested in sample buffer (described above) containing 1 mM sodium orthovanadate. The lysates (10 μ g of protein) were analyzed by Western blot analysis probing with phospho-p42/p44 MAPK-specific IgG (anti-phosphothreonine-202/phosphotyrosine-204 p42/p44 MAPK; Cell Signaling Technology Inc., Danvers, MA) according to the manufacturer's instructions. To confirm similar levels of total p42/p44 MAPK in the samples, the membranes were stripped and reprobed with anti-p42/p44 MAPK IgG (Cell Signaling Technology).

Radioimmunoassay of cAMP. Cardiac myocytes were incubated with 3-isobutyl-1-methylxanthine (0.5 mM) for 10 min, and then stimulated with isoproterenol (10 μ M) in medium containing 3-isobutyl-1-methylxanthine and 0.1 mM ascorbic acid. The reactions were stopped after 10 min by addition of ice-cold trichloroacetic acid to a final concentration of 5% (v/v). Cellular cAMP content was determined by a radioimmunoassay of cAMP as described previously (Skomedal et al., 1980).

Assay of GRK Activities. GRK activities in extracts (25 μ g of sample protein) of cardiac myocytes infected with AdGRK2, AdGRK3, or AdLacZ were determined by assay of light-induced phosphorylation of rhodopsin in dark-adapted, urea-stripped rod outer segment membranes as described previously (Choi et al., 1997). The extracts were prepared from cardiac myocytes 48 h after infection in ice-cold homogenization buffer (25 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM EGTA, 20 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), homogenized with a Dounce glass-glass homogenizer using 3 \times 10 strokes, and centrifuged at 40,000g (10 min; 4°C).

Statistical Analysis. All data are presented as mean \pm S.E.M., unless otherwise indicated. Curve fitting was performed using Prism version 4.0 (GraphPad Software, Inc. San Diego, CA) and the indicated algorithms. The data in Fig. 4 were assessed by unpaired *t* test analysis. *P* < 0.05 was considered statistically significant.

Results

Quantification of Endogenous Levels of GRK2 and GRK3 and Determination of Their Subcellular Localization in Adult Rat Cardiac Myocytes. Immunoprecipitation of GRK2 and GRK3 from cardiac myocyte lysates with a nondiscriminatory anti-GRK2/anti-GRK3 antibody and subsequent immunoblot analysis were performed to determine endogenous levels of the respective kinases (Fig. 1B). Anti-GRK2 and anti-GRK3 immunoreactivities in the immunoprecipitates were related to standard curves of immunoreactivities (identical epitopes) from increasing amounts of purified GST-GRK2ct and GST-GRK3ct fusion proteins, re-

spectively (Fig. 1A). Densitometric analysis of the blots revealed that the endogenous levels of GRK2 and GRK3 were approximately 30 and 5 fmol/mg of total cellular protein, respectively. Figure 1C demonstrates Western blot analysis of extracts of adult rat cardiac myocytes immunoblotted with the anti-GRK2-specific IgG and the anti-GRK3-specific IgG employed for immunofluorescence microscopy. As demonstrated, the antibodies are highly specific for their respective GRK immunogen. Immunofluorescence microscopy of GRK2 and GRK3 in cardiac myocytes revealed similar distribution of anti-GRK2 and anti-GRK3 immunoreactivities, respectively (Fig. 1D). Immunofluorescence was concentrated at the

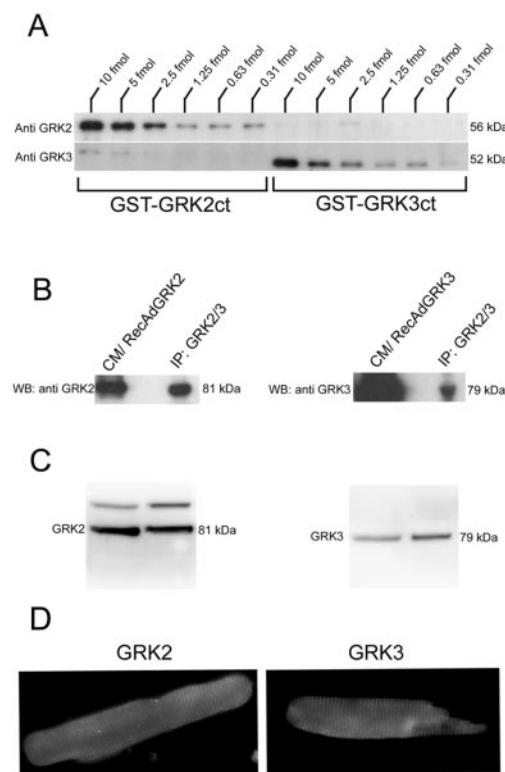


Fig. 1. Determination of cellular levels and subcellular distribution of endogenous GRK2 and GRK3 in adult rat cardiac myocytes. **A**, immunoblot analysis of increasing amounts of purified GST-GRK2ct or GST-GRK3ct fusion proteins. The indicated amounts of protein were separated by SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, and incubated with purified anti-GRK2-specific or anti-GRK3-specific IgG as described under *Materials and Methods*. **B**, immunoblot of endogenous GRK2 and GRK3 immunoprecipitated from adult rat cardiac myocytes. The immunoreactive bands migrated similar to the immunoreactive bands of lysates from cardiac myocytes infected with AdGRK2 or AdGRK3, respectively. Results of **A** and **B** are from the same immunoblot and autoradiographic procedures. Immunoreactive bands were analyzed by densitometry using the ImageMaster software package (GE Healthcare). The data from **A** were used to create standard curves to quantify the levels of GRK2 and GRK3 in cardiac myocytes (**B**). **C**, photomicrographs of Western blot analysis of extracts of adult rat cardiac myocytes immunoblotted with anti-GRK2-specific IgG or anti-GRK3-specific IgG. The anti-GRK2 IgG detects an 81-kDa band corresponding to GRK2, whereas the anti-GRK3 IgG detects only a 79-kDa band corresponding to GRK3. **D**, photomicrographs of adult rat cardiac myocytes subjected to immunofluorescence analysis. Cardiac myocytes were immunostained with anti-GRK2 or anti-GRK3 IgG and subsequently goat anti-rabbit IgG labeled with Alexa Fluor 488 (Invitrogen), mounted onto object glasses using Mowiol (Hoechst), and analyzed using the Zeiss Axiovert-100 fluorescence microscope with 100 \times , 1.3 numerical aperture, oil immersion objective. Images were digitally captured and processed using the Metamorph Imaging system (Molecular Devices, Sunnyvale, CA).

plasma membrane including the transverse tubuli. Cardiac myocytes stained with nonspecific IgG, which served as control, did not display specific fluorescence patterns (data not shown).

Cellular Viability and Expression of Transgene after Adenoviral Infection. To investigate the efficiency of gene transduction with adenoviral infection and to elucidate putative cytotoxic effects, adult rat cardiac myocytes were infected with AdLacZ (encoding the β -galactosidase reporter gene) at increasing multiplicity of infection (m.o.i. 0.5–500). Forty-eight hours after adenoviral infection, the cardiac myocytes were fixed in 0.5% glutaraldehyde and stained for β -galactosidase activity (LacZ activity) with X-gal as described previously (Drazner et al., 1997). As shown in Fig. 2, the percentage of cardiac myocytes demonstrating LacZ activity (blue cellular staining with X-gal) increases with increasing m.o.i. Indeed, infection with AdLacZ at m.o.i. of 100 or higher essentially resulted in gene transduction (infection) of virtually all cardiac myocytes. As evidenced by the intensity of X-gal staining, transgene expression (LacZ activity) also increased with increasing m.o.i. of AdLacZ. As shown in Fig. 2, the cardiac myocytes also maintained the elongated, rod-shaped morphology upon adenoviral infection at high m.o.i. To elucidate putative cytotoxic effects of adenoviral infection, particularly at the higher m.o.i. (500) employed in the study, the number of cardiac myocytes that maintained rod-shaped morphology 48 h after infection were determined for AdLacZ-, AdGRK2-, AdGRK3-, AdGRK2ct-, or AdGRK3ct-infected cells, and compared with noninfected control cells. As shown in Table 1, the number of cardiac myocytes that maintained rod-shaped morphology 48 h after infection (m.o.i. 500) was not statistically different from that of noninfected controls for any of the viruses employed. Thus, even the highest m.o.i. employed in the present study does not seem to be associated with signs of adenoviral toxicity (i.e., reduced viability or contracture with loss of rod-shaped morphology). As shown in Fig. 4D, Western blot analysis of extracts from cardiac myocytes infected with AdGRK2, AdGRK2ct, AdGRK3, or AdGRK3ct also confirmed expression of recombinant proteins with molecular mass characteristic of the respective proteins.

Substrate Specificity of GRK2 and GRK3 at Endogenous ET-Rs in Adult Rat Cardiac Myocytes. ET-1 (0.1 μ M) elicited robust increases of IP accumulation in adult rat

cardiac myocytes (Fig. 3 and 4). However, ET-1-stimulated IP generation per time unit declined throughout the time span of the study approaching the rate of basal IP accumulation. Such time kinetics is typical of receptor-generated responses undergoing desensitization. To substantiate to what extent the declining rates of ET-1-stimulated IP generation were due to homologous desensitization, cardiac myocytes subjected to time course analysis of ET-1-stimulated IP generation were exposed to subsequent stimulation with phenylephrine (10 μ M) or ET-1 (0.1 μ M). As demonstrated in Fig. 3, subsequent stimulation with phenylephrine elicited further elevations of IP generation with similar initial rates as that of the primary ET-1- or phenylephrine-stimulated responses. Cardiac myocytes that received repeated stimulation with ET-1 (0.1 μ M), however, did not respond with increased rates of IP generation compared with myocytes stimulated with ET-1 only once. Thus, the declining rates of IP generation upon prolonged stimulation with ET-1 were due to homologous desensitization and not substrate consumption or degradation of ET-1.

To determine whether GRK2 or GRK3 were mechanisti-

TABLE 1

Adenovirus infection of cardiac myocytes and maintenance of rod-shaped morphology

Number of rod-shaped cardiac myocytes 48 h after infection with AdLacZ, AdGRK2, AdGRK3, AdGRK2ct, or AdGRK3ct. For all recombinant adenoviruses, infection was performed at same m.o.i. (500). The viral titers of all recombinant adenoviruses were determined simultaneously in cells seeded from same preparation of isolated cardiac myocytes. Same number of cells were seeded in cell culture dishes (100,000 per 35-mm well), infected 2 h after plating, and assayed 48 h after infection. Data represent number of rod-shaped myocytes per dish (mean \pm S.E.M.; $n = 6$ dishes). Rod-shaped myocytes were defined as those in which the length of the cell was at least 2 times its width with an overall linear morphology.

| Recombinant Adenovirus (m.o.i. 500) | Rod-Shaped Cardiac Myocytes/Dish ($\times 10^4$) |
|-------------------------------------|--|
| NI | 8.3 \pm 0.8 |
| AdLacZ | 8.3 \pm 1.2 |
| AdGRK2 | 8.5 \pm 1.2 |
| AdGRK3 | 8.4 \pm 1.1 |
| AdGRK2ct | 8.9 \pm 1.4 |
| AdGRK3ct | 8.8 \pm 1.0 |

NI, noninfected cardiac myocytes assayed at same time point.

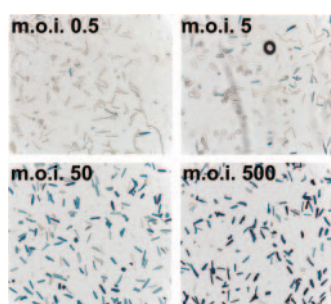


Fig. 2. Adenovirus infection, β -galactosidase (LacZ) expression, and cellular morphology of adult rat cardiac myocytes. Adult rat cardiac myocytes 48 h after infection with recombinant adenovirus encoding the LacZ (β -galactosidase) reporter gene. Adult rat cardiac myocytes were infected at increasing m.o.i. (0.5 – 500; A–D) of AdLacZ to test the efficiency of gene transduction and the maintenance of viable, rod-shaped morphology. Myocytes were stained for LacZ activity (blue staining) and morphology was analyzed by phase contrast microscopy.

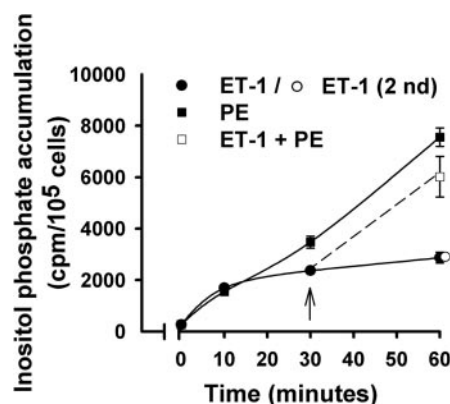


Fig. 3. Homologous desensitization of endogenous ET-Rs in adult rat cardiac myocytes. Time course of ET-1-stimulated (●; 0.1 μ M) and phenylephrine-stimulated (■; 10 μ M) IP accumulation in noninfected cardiac myocytes. Thirty minutes after initiation of the assay of ET-1-stimulated IP generation, parallel wells were exposed to costimulation with phenylephrine (□; 10 μ M), second addition of ET-1 (○; 0.1 μ M), or no further additions (●). The assay was stopped after 60 min, and total inositol phosphates were determined. Data are total accumulated IP levels per 10^5 cardiac myocytes. The data are mean of triplicate \pm S.D. and representative of three independent experiments.

cally involved in desensitization of endogenous ET-R, cardiac myocytes were infected with recombinant adenovirus encoding the competitive inhibitors of GRK2 or GRK3, GRK2ct or GRK3ct, respectively. Recombinant adenovirus encoding LacZ served as control. As shown in Fig. 4A, GRK3ct enhanced ET-1-stimulated IP generation, whereas GRK2ct did not alter IP generation above that seen for AdLacZ-infected cardiac myocytes. To determine to what extent these findings were due to differences in efficacy and/or potency of GRK2ct versus that of GRK3ct, concentration—effect relationships of GRK2ct and GRK3ct at ET-1-stimulated IP generation were investigated. Cardiac myocytes were infected with AdGRK2ct or AdGRK3ct at increasing m.o.i., and ET-1-stimulated IP accumulation was assayed 48 h after infection and related to the cellular contents of GRK2ct and GRK3ct, respectively. As demonstrated in Fig. 4B, increasing levels of

GRK3ct caused concentration-dependent elevations of ET-1-stimulated IP generation (EC_{50} , 2 ± 0.2 pmol/mg of cardiac myocyte protein; R_{max} , $139 \pm 1.7\%$; $n = 3$). On the other hand, comparable levels of GRK2ct displayed hardly any capacity at all to enhance ET-1-stimulated IP generation.

To investigate to what extent overexpression of GRK2 or GRK3 would cause the converse effects of their respective inhibitors, GRK2ct and GRK3ct, ET-1-stimulated IP accumulation was assayed in cardiac myocytes infected at increasing m.o.i. of recombinant adenovirus encoding full-length, active GRK2 or GRK3, i.e., AdGRK2 or AdGRK3. As shown in Fig. 4C, GRK3 caused substantial expression-dependent attenuation of ET-1-stimulated IP generation (IC_{50} , 5 ± 0.7 pmol/mg of cardiac myocyte protein; $n = 3$). To a lesser extent, GRK2 also elicited significant attenuation of ET-1-stimulated IP generation. At maximally effective con-

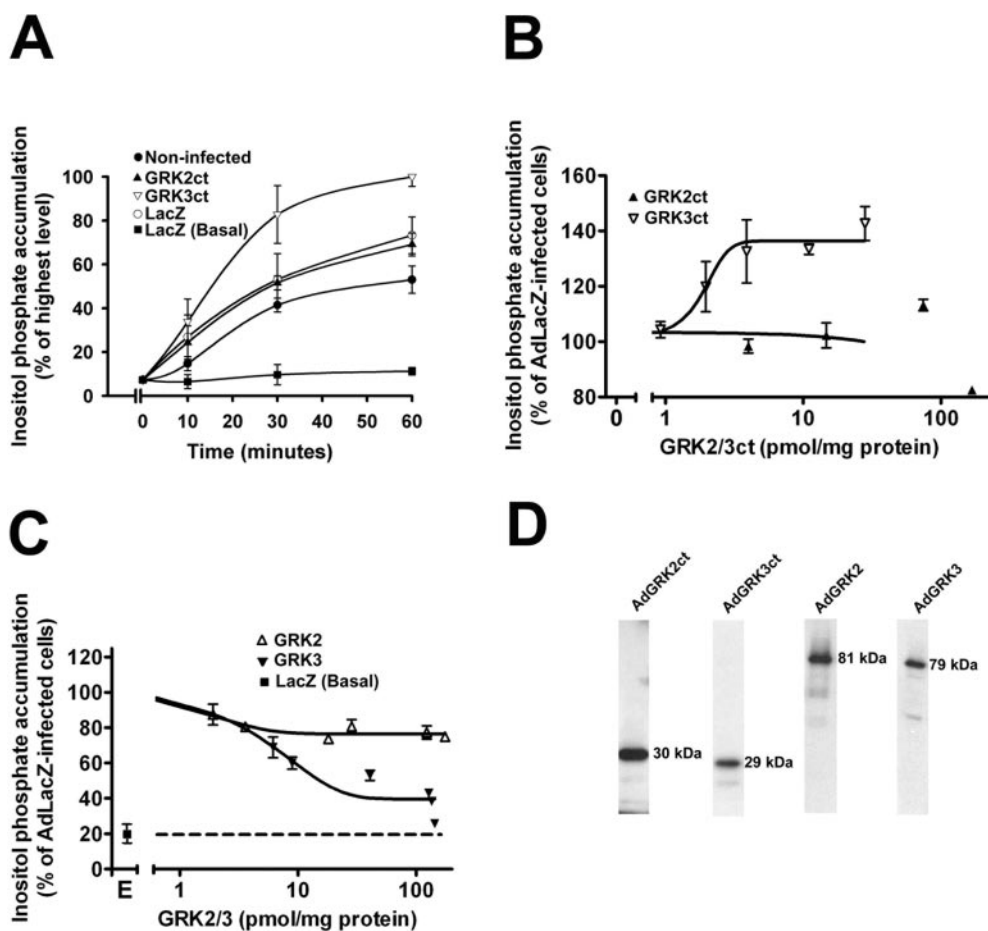


Fig. 4. Potency and efficacy of GRK2 versus GRK3 at ET-R-mediated IP generation in adult rat cardiac myocytes. **A**, time course of ET-1-stimulated IP accumulation in noninfected cardiac myocytes (●) or cardiac myocytes infected (m.o.i. ~500) with AdGRK2ct (▲), AdGRK3ct (▼), or AdLacZ (○). Cardiac myocytes infected with AdLacZ (■, m.o.i. ~500), but not stimulated with agonist were included to provide basal levels of activity. Data are total accumulated IP levels plotted as percent of highest observed levels after 60 min of agonist stimulation. **B** and **C**, concentration-effect curves of increasing amounts of GRK2ct (▲) or GRK3ct (▼) (**B**), and GRK2 (▲) or GRK3 (▼) (**C**) on ET-1-stimulated IP generation. Cardiac myocytes were infected with AdGRK2ct, AdGRK3ct, AdGRK2, AdGRK3, or AdLacZ at increasing m.o.i. (10–500) and subjected to assay of ET-1-stimulated IP generation 48 h after infection. The IP accumulation assay was terminated after 30 min of stimulation with ET-1 (0.1 μ M). Parallel cell culture dishes for each titer of infection were subjected to analysis of contents of recombinant protein according to the standard curve method outlined in the legend to Fig. 1. Data are presented relative to ET-1-stimulated IP levels in AdLacZ-infected control cells at corresponding m.o.i. and plotted as function of increasing amounts of GRK or GRKct in a logarithmic x-axis format. Basal IP generation is assayed in the absence of agonist in cells infected (m.o.i., 500) with AdLacZ (**E**, dashed line). Data in **A–C** represent mean of triplicates \pm S.D. and are representative of three independent experiments. The GRK3ct and GRK3 curve plots were fitted using a sigmoidal curve fit algorithm in GraphPad Prism 4.0 ($R^2 = 0.86$ and 0.89 for GRK3ct and GRK3, respectively). **D**, photographs demonstrating Western blot analysis of GRK2 and GRK2ct (anti-GRK2-specific IgG; Santa Cruz Biotechnology), and GRK3 and GRK3ct (anti-GRK3-specific IgG; Santa Cruz Biotechnology) in extracts from cardiac myocytes infected with AdGRK2, AdGRK2ct, AdGRK3, or AdGRK3ct, respectively (m.o.i. 500). Immunoblots were performed as outlined under *Materials and Methods*. Apparent molecular mass (kilodaltons) of the respective immunoreactive bands is indicated.

centrations (R_{\max}), GRK3 and GRK2 caused 77 and 28% desensitization, respectively, of the ET-1-stimulated response ($P < 0.05$, $n = 3$). Thus, compared with GRK2, GRK3 displayed substantially higher efficacy in desensitizing ET-1-elicited phosphoinositide hydrolysis, consistent with the superior potency and efficacy of GRK3ct versus that of GRK2ct.

Lysates of cardiac myocytes overexpressing recombinant GRK2 or GRK3 elicited expression-dependent, light-induced phosphorylation of rhodopsin. Indeed, the catalytic activities of GRK2 and GRK3 on light-stimulated rhodopsin were similar and increased proportionally with increased levels of enzyme expressed in the cardiac myocytes (Fig. 5). GRK activities in lysates from cardiac myocytes infected with AdLacZ control virus were not different from that of noninfected cardiac myocytes (data not shown).

To elucidate whether the selectivity of GRK3 at ET-R also reflected on downstream signaling responses, we analyzed the effects of the GRK inhibitors GRK2ct and GRK3ct on ET-1-stimulated phosphorylation of p42/p44 extracellular signal-regulated kinase (ERK) in cardiac myocytes. At comparable multiplicities of infection of AdGRK2ct, AdGRK3ct, or AdLacZ, only GRK3ct elicited significant elevations of ET-1-stimulated levels of phospho-p42/p44 ERK (Fig. 6A). Conversely, similar experiments in cardiac myocytes infected with AdGRK3 at increasing m.o.i. revealed dramatic, GRK3 expression level-dependent attenuation of ET-1-stimulated p42/p44 ERK phosphorylation (Fig. 6B). Similar m.o.i. of AdLacZ did not cause statistically significant alterations of ET-1-induced ERK phosphorylation compared with noninfected control cells.

Substrate Specificities of GRK2 and GRK3 at Endogenous α_1 -ARs in Adult Rat Cardiac Myocytes. In contrast to the IP accumulation kinetics observed with ET-1, the

rate of IP generation observed upon stimulation of endogenous α_1 -AR with its selective agonist phenylephrine did not wane during the time span studied (Fig. 3 and 7A). Furthermore, expression of GRK2ct or GRK3ct did not enhance phenylephrine-stimulated IP generation above that in AdLacZ-infected control cells (Fig. 7A). Thus, it seems that endogenous α_1 -ARs in cardiac myocytes are not prone to extensive desensitization. On the other hand, as demonstrated in Fig. 7B, increasing levels of GRK3 elicited dramatic, concentration-dependent attenuation of phenylephrine-stimulated IP generation (R_{\max} , $94 \pm 1.1\%$ desensitization) with half-maximal inhibition (IC_{50}) at 7 ± 0.4 pmol/mg of cardiac myocyte protein (mean \pm S.E.M.; $n = 3$). Likewise, AdGRK3 infection also blunted phenylephrine-stimulated phosphorylation of p42/p44 ERK at very low m.o.i., consistent with the potent action of GRK3 at α_1 -ARs

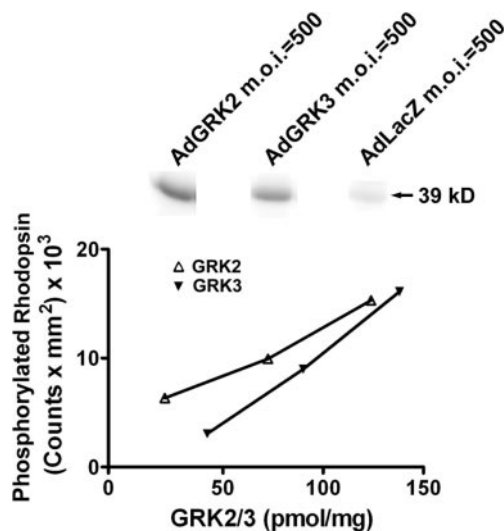


Fig. 5. Assay of GRK activities in cardiac myocytes infected with increasing viral amounts (m.o.i. 40, 200, and 1000) of AdGRK2 or AdGRK3. Dark-adapted rod outer segments were incubated with [γ - 32 P]ATP and cell lysate under illumination (white light) for 15 min. The reactions were quenched, and phosphorylated rhodopsin was separated by SDS-PAGE. The gel was subjected to autoradiography on storage phosphor plates and densitometric analysis of phosphorylated rhodopsin. Parallel wells were processed for quantification of GRK2 and GRK3 as outlined in Fig. 1B. Data are presented in densitometric units (counts \times mm 2) as function of cellular contents of expressed GRK2 (Δ) or GRK3 (\blacktriangledown), respectively, and are mean of duplicate wells.

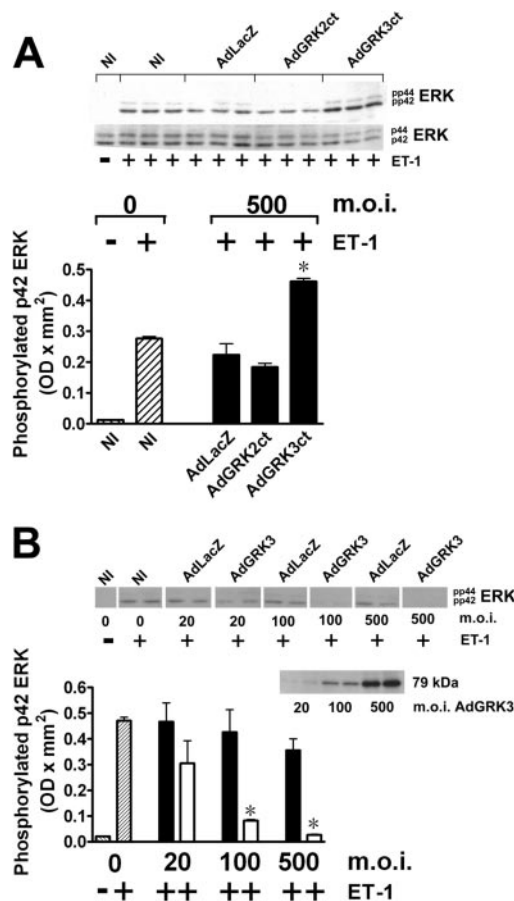


Fig. 6. Effects of GRK2ct, GRK3ct, and GRK3 on ET-1-stimulated p42/p44 ERK activities in adult rat cardiac myocytes. A, immunoblot of ET-1-stimulated phospho-p42/p44 ERK levels in cardiac myocytes infected with AdLacZ, AdGRK2ct, or AdGRK3ct (all at m.o.i. 500) assayed 48 h after infection as described under *Materials and Methods* (NI, noninfected cells). Immunoblot of total p42/p44 ERK levels in the same samples serves as control. The histogram demonstrates densitometric analysis of immunoreactive phospho-p42 ERK bands. The values (optical density \times mm 2) are mean of triplicates \pm S.E.M. and are representative of three independent experiments. B, immunoblot of ET-1-stimulated phospho-p42/p44 ERK levels in cardiac myocytes infected at increasing m.o.i. with AdLacZ or AdGRK3 (immunoblot of GRK3 levels is shown below) assayed 48 h after infection as described. The histogram demonstrates densitometric analysis of immunoreactive phospho-p42 ERK bands (\square , noninfected cells; \blacksquare , AdLacZ-infected cells; \square , GRK3-infected cells). The values are mean of duplicates \pm S.E.M. and are representative of three independent experiments. *, $P < 0.05$ versus AdLacZ-infected cardiac myocytes.

(data not shown). It is noteworthy that GRK2 completely lacked the capacity to attenuate phenylephrine-stimulated IP generation (Fig. 7B). Indeed, GRK2 did not cause statistically significant alterations of phenylephrine-stimulated IP generation over the concentration span, providing maximal effect of GRK3.

Substrate Specificities of GRK2 and GRK3 at Endogenous β_1 -ARs in Adult Rat Cardiac Myocytes. As demonstrated in Fig. 8A, isoproterenol-stimulated cAMP production per time unit declined throughout the time-span of the study as typical of receptor-generated responses undergoing desensitization. For isoproterenol-stimulated cAMP generation, both GRK2ct and GRK3ct enhanced cAMP production (Fig. 8A). To investigate the substrate specificities of GRK2 and GRK3 at cardiac myocyte β_1 -ARs, increasing concentrations of the respective competitive inhibitors GRK2ct or GRK3ct were obtained by infection of adult rat cardiac myocytes at increasing m.o.i. with AdGRK2ct or AdGRK3ct. Infection of cardiac myocytes at corresponding m.o.i. of AdLacZ served as control. As demonstrated in Fig. 8B, increasing levels of GRK2ct and GRK3ct caused concentration-depen-

dent elevations of isoproterenol-stimulated cAMP production; i.e., both GRK2ct and GRK3ct displayed efficacy at the β_1 -AR. Although the maximally effective concentrations of GRK3ct could not be confidently determined (because of limiting expression of GRK3ct), GRK3ct enhanced cAMP accumulation in the same range of concentrations as that of GRK2ct, indicating similar potencies at the β_1 -AR (R_{max} , GRK3ct, $205 \pm 10\%$; GRK2ct, $230 \pm 16\%$; EC_{50} , GRK2ct, 43 ± 1 pmol/mg of cardiac myocyte protein; mean \pm S.E.M., $n = 3$). Although isolated adult rat cardiac myocytes apparently only possess β_1 -adrenergic receptors, we performed competition studies of isoproterenol-stimulated cAMP generation to assess to what extent the isoproterenol-stimulated response were only mediated through β_1 -ARs. Isoproterenol-stimulated cAMP generation was assayed in the presence of increasing concentrations the β_1 -AR-selective antagonist CGP20712A or the β_2 -AR-selective antagonist ICI118551 (Fig. 8C). The inhibition curves revealed that both antagonists competed with isoproterenol at a single site. CGP20712A and ICI118551 inhibited cAMP generation with pK_i values of 9.0 and 6.3, respectively, which is in agreement with previously reported values for the β_1 -AR (Levy et al., 1993).

Discussion

This study is the first demonstration of GPCR substrate specificity between the closely related GRKs, GRK2 and GRK3, in cardiac myocytes. Analysis of adult rat cardiac myocytes demonstrated that GRK2 and GRK3 display striking specificities at GPCRs controlling different aspects of cardiac function. Overall, the present data reveal for the first time that GRK3 has substantially higher potency and efficacy than GRK2 at endogenous ET-R and α_1 -AR. This does not seem to be the case for the β_1 -AR, in that GRK3ct potency at this receptor seems much weaker than for the ET-R and was equipotent with GRK2ct. Thus, GRK3 emerges as a primary regulator of ET-R and α_1 -AR signaling, which may have important implications in cardiac function.

Although, the present study demonstrates that adult rat cardiac myocytes contain somewhat higher amounts of GRK2 than GRK3, the two kinases seem to have similar subcellular distribution. Thus, different regulatory roles of GRK2 and GRK3 would depend on the substrate specificities of the two kinases. It is noteworthy that reports on cardiac function in transgenic mice indicate that GRK2 and GRK3 are not functionally redundant, suggesting different substrate specificities for these kinases (Koch et al., 1995; Iaccarino et al., 1998; Eckhart et al., 2000). Although beyond the focus of this study, it ought to be kept in mind that GRK5, another GRK isoforms expressed in cardiac myocytes, has been shown to participate in regulation of β -ARs on cardiac myocytes (Rockman et al., 1996).

Expression of the PH domain of GRK2 (GRK2ct) has been extensively employed as competitive inhibitor of endogenous GRK2 activities in cardiac myocytes both in vitro and in vivo (Koch et al., 1995, 1996; Akhter et al., 1997; Drazner et al., 1997; Rockman et al., 1998). These studies have revealed that GRK2 regulates β -AR signaling and myocardial contractility. However, similar experiments using the PH domain of GRK3 to inhibit endogenous GRK3 activities have not been performed. Although GRK2 and GRK3 share a high degree of

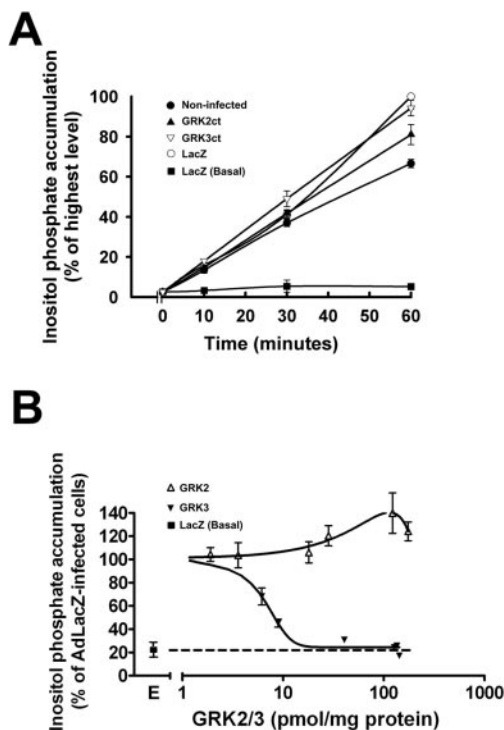


Fig. 7. Potency and efficacy of GRK2 versus GRK3 at α_1 -AR-mediated IP generation in adult rat cardiac myocytes. **A**, time course of phenylephrine-stimulated IP generation in noninfected cardiac myocytes (●) or cardiac myocytes infected with AdGRK2ct (▲), AdGRK3ct (▼), or AdLacZ (○) at m.o.i. 500. Cardiac myocytes infected with AdLacZ (■, m.o.i. 500) but not stimulated with agonist were included to provide basal levels of activity. Data are total accumulated IP levels plotted as percent of highest observed levels after 30 min of agonist stimulation ($10 \mu\text{M}$ phenylephrine). **B**, concentration-effect curves of increasing amounts of GRK2 (▲) or GRK3 (▼) on phenylephrine-stimulated IP generation. Cardiac myocytes infected with AdGRK2, AdGRK3 or AdLacZ at increasing m.o.i. (10–500) were assayed 48 h after infection. Data are presented relative to phenylephrine-stimulated IP levels in AdLacZ-infected control cells at corresponding m.o.i. and plotted as function of increasing amounts of GRK in a logarithmic x -axis format. Basal IP generation is assayed in the absence of agonist in cells infected (m.o.i., 500) with AdLacZ (E, dashed line). The data in A and B are mean of triplicates \pm S.D. and are representative of three independent experiments. The curve plots were fitted using sigmoidal curve fit ($R^2 = 0.97$ for GRK3).

overall amino acid identity, the PH domains of these isoforms are divergent (52% identity), suggesting specificity at binding to different $G\beta\gamma$ isoforms. Indeed, GST-fusion proteins with the carboxyl-terminal region of GRK2 and GRK3, respectively, displayed apparent differences of binding affinities for various $G\beta\gamma$ isoforms (Daaka et al., 1997). Furthermore, $G\beta\gamma$ isoforms have also been shown to display preference for different GPCRs (Kleuss et al., 1992, 1993). Thus, to the extent that GRK2 and GRK3 exhibit different affinities for distinct $G\beta\gamma$ isoforms, activation of GPCRs and release of specific $G\beta\gamma$ isoforms could lead to selective recruitment of either of the kinases. Accordingly, different functional roles of GRK2 and GRK3 in cardiac myocytes may reside in specificity mediated by the PH domain of the respective kinases.

It could be argued that inhibition of GRK2 or GRK3 by expression of their respective PH domain may be confounded

by signaling events because of sequestration of $G\beta\gamma$. Indeed, $G\beta\gamma$ regulates several signaling effectors as either a membrane anchor or an allosteric modulator. However, as revealed in our studies, GRK3ct apparently inhibited desensitization of cardiac myocyte ET-receptors by selectively inhibiting endogenous GRK3. This contention is supported by three lines of evidence: 1) although GRK3 itself desensitized ET-1-induced phosphoinositide hydrolysis and ERK activation, GRK3ct engendered enhancement of these signaling responses; 2) comparing the inhibitors GRK3ct and GRK2ct demonstrated specificity mirrored by corresponding analysis of the kinases GRK3 and GRK2; and 3) although GRK3ct enhanced ET-R-evoked phosphoinositide hydrolysis, it failed to enhance α_1 -AR-promoted phosphoinositide hydrolysis. The latter finding argues against nonspecific alterations of effector activities caused by sequestration of $G\beta\gamma$.

It is noteworthy that GRK2 and GRK2ct both failed to

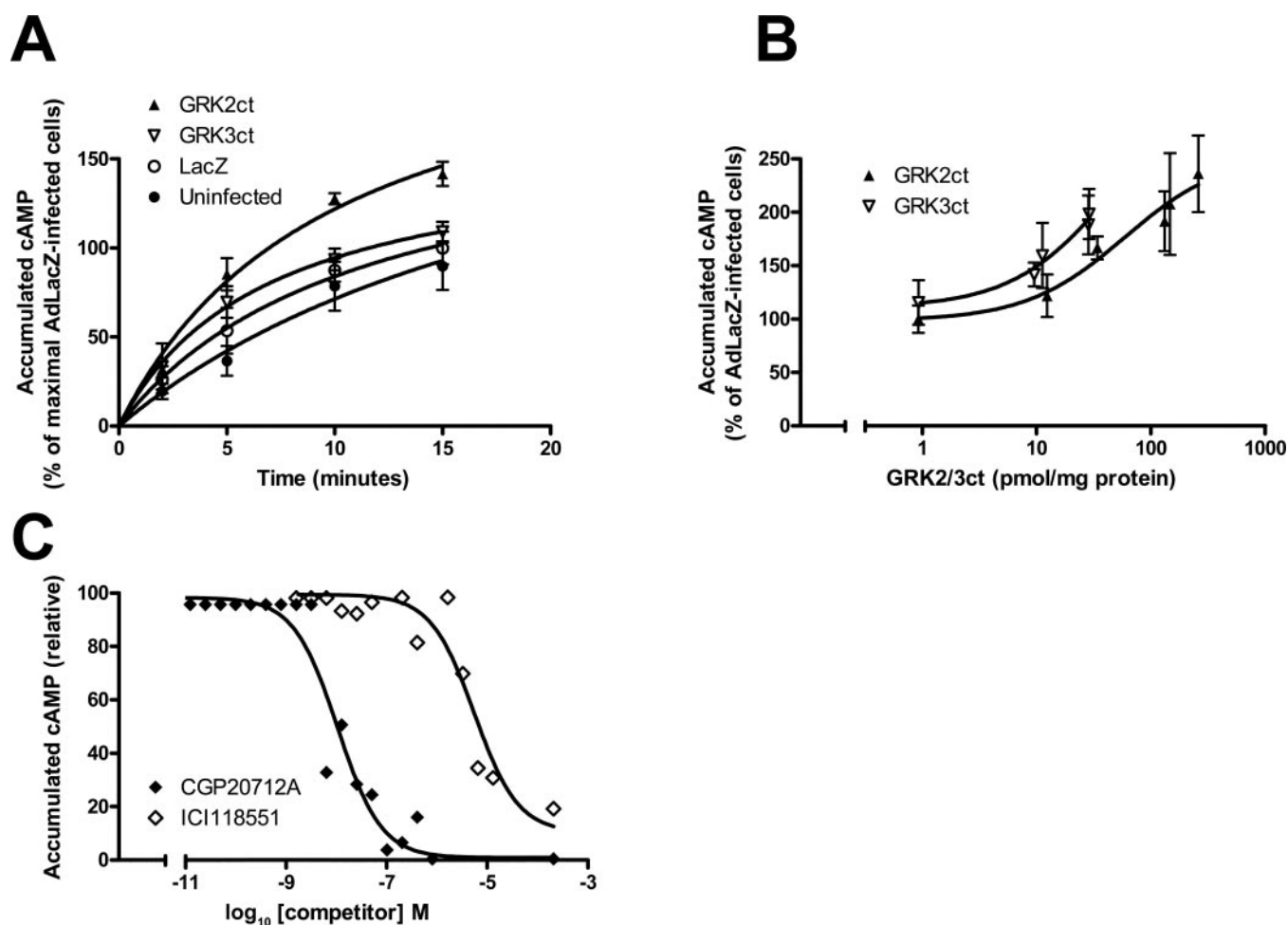


Fig. 8. Potency and efficacy of GRK2ct versus GRK3ct at β_1 -AR-stimulated cAMP production in adult rat cardiac myocytes. **A**, time course of isoproterenol-stimulated cAMP production in noninfected cardiac myocytes (●) or cardiac myocytes infected with AdGRK2ct (▲), AdGRK3ct (▼), or AdLacZ (○) at m.o.i. 500. Data are presented as percentage of maximal observed cAMP accumulation after 15 min of agonist stimulation in AdLacZ-infected cells. **B**, concentration-effect curves of increasing amounts of GRK2ct (▲) or GRK3ct (▼) on isoproterenol-stimulated cAMP production. Increasing expression of GRK2ct or GRK3ct was obtained by infection of cardiac myocytes at increasing m.o.i. (10–500) with AdGRK2ct or AdGRK3ct, respectively. cAMP levels are presented relative to isoproterenol-stimulated control cells (cardiac myocytes infected with AdLacZ at corresponding m.o.i.), and the value for AdGRK2ct at m.o.i. 10 (lowest m.o.i. employed) was given a value of 100%. The presented data are representative of three independent experiments. The curve plots were fitted using sigmoidal curve fit ($R^2 = 0.97$ for both GRK2ct and GRK3ct). **C**, inhibition curves of isoproterenol-stimulated cAMP production with increasing concentrations β_1 -AR-selective antagonist CGP20712A versus β_2 -AR-selective antagonist ICI118551. Adult rat cardiac myocytes were incubated with isoproterenol (1 μ M) and increasing concentrations of subtype-selective antagonist as indicated. Accumulated cAMP was then determined as described under *Materials and Methods*. Data are presented relative to cAMP levels generated in the presence isoproterenol (1 μ M) alone. Data in A–C are mean of triplicates \pm S.D. and are representative of three independent experiments.

affect signaling through ET-1 receptors in cardiac myocytes. These findings seem to contrast with results obtained in transiently transfected HEK293 cells, in which both GRK2 and GRK3 were found to phosphorylate and desensitize ET_A and ET_B receptors (Freedman et al., 1997). Overexpression of GRK2 also inhibited ET-R-stimulated IP generation more substantially in aortic smooth muscle cells than in the cardiac myocytes used in the present study (Peppel et al., 2000). It is possible that GRK expression levels in these earlier studies were higher than those achieved here. Alternatively, cell type-specific differences in GRK effects may derive from cell type-specific expression of signal transduction proteins (e.g., G $\beta\gamma$ subunits) or different compartmentation of signaling proteins.

As opposed to ET-1-stimulated IP generation, phenylephrine-stimulated IP generation was linear throughout the 60-min assay protocol. The most readily explicable interpretation of the latter observation would be that α_1 -ARs in cardiac myocytes are not undergoing desensitization. Accordingly, neither GRK2ct nor GRK3ct caused significant elevations of phenylephrine-stimulated phosphoinositide hydrolysis. Lack of desensitization of phenylephrine-stimulated responses in cardiac myocytes could be due to insufficient levels of GRK3. Indeed, the endogenous levels of GRK3 in cardiac myocytes reported in the present study would be too low to cause substantial desensitization of α_1 -ARs as judged from the GRK3 concentration-effect curve in Fig. 7. Lack of desensitization of cardiac myocyte α_1 -ARs, as demonstrated in the present study, is in concordance with maintained contractile responses to α_1 -AR agonists in rats with congestive heart failure, as opposed to β -ARs, which typically display reduced responsiveness in heart failure (Ungerer et al., 1993; Sjaastad et al., 2003). Limiting levels of endogenous GRK3 would also help explaining the profound sensitivity of phenylephrine-stimulated responses to overexpression of GRK3. The striking selectivity of GRK3 for α_1 -ARs, uncovered in this study, is in concordance with hybrid transgenic mice with cardiac-specific overexpression of the α_{1B} -AR and GRK2 or GRK3 (Eckhart et al., 2000). The predominant α_1 -AR subtypes in rat cardiac myocytes are α_{1A} - and α_{1B} -ARs. Although the striking selectivity of GRK3 in desensitization α_1 -AR responses in adult rat cardiac myocytes would indicate similar substrate specificities of α_{1A} - and α_{1B} -AR subtypes, detailed analysis of the substrate specificities of the distinct subtype are not feasible because of the lack of highly selective agonists for these subtypes.

Consistent with previous findings suggesting that β_1 -ARs do not discriminate between GRK2 and GRK3 in transfected cell models (Freedman et al., 1995), the present study demonstrates that GRK2ct and GRK3ct display similar potencies at β_1 -ARs in cardiac myocytes. It is noteworthy that regulation of cardiac β_1 -AR function by GRK2 is sensitive to both reduction and elevation of endogenous GRK2 levels (Rockman et al., 1998). Furthermore, myocardial GRK2 is up-regulated in heart failure and causes desensitization of cardiac β -ARs, a finding characteristic of this condition (Ungerer et al., 1993, 1994). Myocardial GRK3 levels, on the other hand, are not regulated in heart failure (Vinge et al., 2001). Thus, the revelation of substantially lower potencies of GRK3ct at β_1 -ARs compared with those at ET-Rs and α_1 -ARs, as demonstrated in this study, indicates that GRK2 exerts its primary role in regulation of cardiac contractility

and chronotropy by regulating cardiac myocyte β_1 -ARs. Selectivity of GRK3 for ET-Rs and α_1 -ARs suggests that GRK3 may play primary roles in regulation of cardiac growth and hypertrophy, as well as contractility. It is noteworthy that the reported findings on GRK2 and GRK3 substrate specificities are not affected by potential differences in receptor densities of the three receptors in cardiac myocytes, in that the α_1 -ARs, β_1 -ARs, and ET-Rs receptors have all been reported to be in the range of 1 to 2×10^5 sites per cell (Buxton and Brunton, 1985; Hilal-Dandan et al., 1994).

Several reports of transgenic mice overexpressing GRK isoforms or knockout mice with targeted disruption of specific GRKs, point to distinct functional roles of GRK isoforms (Rockman et al., 1996; Gainetdinov et al., 1999; Eckhart et al., 2000; Fong et al., 2002). However, the phenotypic findings may relate more to expression levels of individual GRKs than to specificity in its strictest sense. Thus, the current study is unique in that we describe strikingly different substrate specificities of two closely related GRK isoforms based on analysis of potency and efficacy at different receptors. It is noteworthy that emerging evidence also indicates that GRK specificity may not simply relate to extent phosphorylation of a given receptor. Rather, specificity may be caused by phosphorylation of distinct residues critical to receptor desensitization, for example by recruitment of β -arrestin isoforms (Violin et al., 2006). Although such distinct phosphorylation patterns have not yet been unequivocally demonstrated, extent of receptor phosphorylation not always correlates with extent of desensitization (Jewell-Motz and Liggett, 1996). Another feature is the existence of closely related receptor isoforms that display different levels of desensitization. For example, certain α_2 -AR isoforms apparently differ in their ability to undergo desensitization only as the result of differences in GRK phosphorylation sites (Jewell-Motz and Liggett, 1996). Thus, the mechanisms of GRK specificity are complex and still far from resolved.

In conclusion, this study has uncovered novel data on the substrate specificities of GRK2 and GRK3 at GPCRs controlling cardiac myocyte function. The study demonstrates that α_1 -ARs and ET-Rs on cardiac myocytes are preferred substrates of GRK3. Furthermore, GRK3 is more potent at ET-Rs and α_1 -ARs than at β_1 -ARs. Thus, the study provides biochemical evidence of different functional roles of the two receptor kinases in cardiac myocytes. The physiological and pathophysiological implications of these functional differences will be subject to future investigations.

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References

- Akhter SA, Skaer CA, Kypson AP, McDonald PH, Peppel KC, Glower DD, Lefkowitz RJ, and Koch WJ (1997) Restoration of β -adrenergic signaling in failing cardiac ventricular myocytes via adenoviral-mediated gene transfer. *Proc Natl Acad Sci U S A* **94**:12100–12105.
- Bremnes T, Paasche JD, Mehluum A, Sandberg C, Bremnes B, and Attramadal H (2000) Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem* **275**:17596–17604.
- Buxton IL and Brunton LL (1985) Direct analysis of β -adrenergic receptor subtypes on intact adult ventricular myocytes of the rat. *Circ Res* **56**:126–132.
- Choi DJ, Koch WJ, Hunter JJ, and Rockman HA (1997) Mechanism of β -adrenergic receptor desensitization in cardiac hypertrophy is increased β -adrenergic receptor kinase. *J Biol Chem* **272**:17223–17229.
- Daaka Y, Pitcher JA, Richardson M, Stoffel RH, Robishaw JD, and Lefkowitz RJ

- (1997) Receptor and G $\beta\gamma$ isoform-specific interactions with G protein-coupled receptor kinases. *Proc Natl Acad Sci U S A* **94**:2180–2185.
- Diviani D, Lattion AL, Larbi N, Kunapuli P, Pronin A, Benovic JL, and Cotechia S (1996) Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the α_{1B} -adrenergic receptor. *J Biol Chem* **271**:5049–5058.
- Drazner MH, Poppel KC, Dyer S, Grant AO, Koch WJ, and Lefkowitz RJ (1997) Potentiation of β -adrenergic signaling by adenoviral-mediated gene transfer in adult rabbit ventricular myocytes. *J Clin Invest* **99**:288–296.
- Eckhart AD, Duncan SJ, Penn RB, Benovic JL, Lefkowitz RJ, and Koch WJ (2000) Hybrid transgenic mice reveal in vivo specificity of G protein-coupled receptor kinases in the heart. *Circ Res* **86**:43–50.
- Fong AM, Premont RT, Richardson RM, Yu YRA, Lefkowitz RJ, and Patel DD (2002) Defective lymphocyte chemotaxis in β -arrestin2- and GRK6-deficient mice. *Proc Natl Acad Sci U S A* **99**:7478–7483.
- Freedman NJ, Liggett SB, Drachman DE, Pei G, Caron MG, and Lefkowitz RJ (1995) Phosphorylation and desensitization of the human β_1 -adrenergic receptor. Involvement of G protein-coupled receptor kinases and cAMP-dependent protein kinase. *J Biol Chem* **270**:17953–17961.
- Freedman NJ, Ament AS, Oppermann M, Stoffel RH, Exum ST, and Lefkowitz RJ (1997) Phosphorylation and desensitization of human endothelin A and B receptors. Evidence for G protein-coupled receptor kinase specificity. *J Biol Chem* **272**:17734–17743.
- Gainetdinov RR, Bohn LM, Walker JKL, Laporte SA, Macrae AD, Caron MG, Lefkowitz RJ, and Premont RT (1999) Muscarinic supersensitivity and impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice. *Neuron* **24**:1029–1036.
- Graham FL and Prevec L (1995) Methods for construction of adenovirus vectors. *Mol Biotechnol* **3**:207–220.
- Hilal-Dandan R, Merck DT, Lujan JP, and Brunton LL (1994) Coupling of the type A endothelin receptor to multiple responses in adult rat cardiac myocytes. *Mol Pharmacol* **45**:1183–1190.
- Iaccarino G, Rockman HA, Shotwell KF, Tomhave ED, and Koch WJ (1998) Myocardial overexpression of GRK3 in transgenic mice: evidence for in vivo selectivity of GRKs. *Am J Physiol - Heart Circ Physiol* **275**:H1298–H1306.
- Jewell-Motz EA, and Liggett SB (1996) G protein-coupled receptor kinase specificity for phosphorylation and desensitization of α_2 -adrenergic receptor subtypes. *J Biol Chem* **271**:18082–18087.
- Kleuss C, Scherubel H, Hescheler J, Schultz G, and Wittig B (1992) Different β -subunits determine G protein interaction with transmembrane receptors. *Nature* **358**:424–426.
- Kleuss C, Scherubel H, Hescheler J, Schultz G, and Wittig B (1993) Selectivity in signal transduction determined by γ -subunits of heterotrimeric G proteins. *Science* **259**:832–834.
- Koch WJ, Inglesse J, Stone WC, and Lefkowitz RJ (1993) The binding site for the $\beta\gamma$ -subunits of heterotrimeric G proteins on the β -adrenergic receptor kinase. *J Biol Chem* **268**:8256–8260.
- Koch WJ, Rockman HA, Samama P, Hamilton RA, Bond RA, Milano CA, and Lefkowitz RJ (1995) Cardiac function in mice overexpressing the β -adrenergic receptor kinase or a β ARK inhibitor. *Science* **268**:1350–1353.
- Koch WJ, Milano CA, and Lefkowitz RJ (1996) Transgenic manipulation of myocardial G protein-coupled receptors and receptor kinases. *Circ Res* **78**:511–516.
- Levy FO, Zhu X, Kaumann AJ, and Birnbaumer L (1993) Efficacy of β_1 -adrenergic receptors is lower than that of β_2 -Adrenergic Receptors. *Proc Natl Acad Sci U S A* **90**:10798–10802.
- Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ, and Tesmer JJ (2003) Keeping G proteins at bay: a complex between G protein-coupled receptor kinase-2 and G $\beta\gamma$. *Science* **300**:1256–1262.
- Poppel K, Jacobson A, Huang X, Murray JP, Oppermann M, and Freedman NJ (2000) Overexpression of G protein-coupled receptor kinase-2 in smooth muscle cells attenuates mitogenic signaling via G protein-coupled and platelet-derived growth factor receptors. *Circulation* **102**:793–799.
- Pitcher JA, Inglesse J, Higgins JB, Arriza JL, Casey PJ, Kim C, Benovic JL, Kwatra MM, Caron MG, and Lefkowitz RJ (1992) Role of $\beta\gamma$ subunits of G proteins in targeting the β -adrenergic receptor kinase to membrane-bound receptors. *Science* **257**:1264–1267.
- Pitcher JA, Freedman NJ, and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Ann Rev Biochem* **67**:653–692.
- Rockman HA, Choi DJ, Rahman NU, Akhter SA, Lefkowitz RJ, and Koch WJ (1996) Receptor-specific in vivo desensitization by the G protein-coupled receptor kinase-5 in transgenic mice. *Proc Natl Acad Sci U S A* **93**:9954–9959.
- Rockman HA, Choi DJ, Akhter SA, Jaber M, Giros B, Lefkowitz RJ, Caron MG, and Koch WJ (1998) Control of myocardial contractile function by the level of β -adrenergic receptor kinase 1 in gene-targeted mice. *J Biol Chem* **273**:18180–18184.
- Sjaastad I, Schiander I, Sjetnan A, Qvigstad E, Bøkenes J, Sandnes D, Osnes JB, Sejersted OM, and Skomedal T (2003) Increased contribution of α_1 - vs. β -adrenoceptor-mediated inotropic response in rats with congestive heart failure. *Acta Physiol Scand* **177**:449–458.
- Skomedal T, Grynné B, Osnes JB, Sjetnan AE, and Øye I (1980) A radioimmunoassay for cyclic AMP (cAMP) obtained by acetylation of both unlabeled and labeled (3 H-cAMP) ligand, or of unlabeled ligand only. *Acta Pharmacol Toxicol* **46**:200–204.
- Ungerer M, Böhm M, Elce JS, Erdmann E, and Lohse MJ (1993) Altered expression of β -adrenergic receptor kinase and β_1 -adrenergic receptors in the failing human heart. *Circulation* **87**:454–463.
- Ungerer M, Parruti G, Böhm M, Puzicha M, DeBlasi A, Erdmann E, and Lohse MJ (1994) Expression of β -arrestins and β -adrenergic receptor kinases in the failing human heart. *Circ Res* **74**:206–213.
- Vinge LE, Øie E, Andersson Y, Grøgaard HK, Andersen G, and Attramadal H (2001) Myocardial distribution and regulation of GRK and β -arrestin isoforms in congestive heart failure in rats. *Am J Physiol - Heart Circ Physiol* **281**:H2490–H2499.
- Violin JD, Ren XR, and Lefkowitz RJ (2006) G-protein-coupled receptor kinase specificity for β -arrestin recruitment to the β_2 -adrenergic receptor revealed by fluorescence resonance energy transfer. *J Biol Chem* **281**:20577–20588.

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