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Monoclonal antibodies reveal receptor specificity among G-protein-coupled receptor kinases

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ABSTRACT **Guanine nucleotide-binding regulatory protein (G protein)-coupled receptor kinases (GRKs) constitute a family of serine/threonine kinases that play a major role in the agonist-induced phosphorylation and desensitization of G-protein-coupled receptors. Herein we describe the generation of monoclonal antibodies (mAbs) that specifically react with GRK2 and GRK3 or with GRK4, GRK5, and GRK6. They are used in several different receptor systems to identify the kinases that are responsible for receptor phosphorylation and desensitization. The ability of these reagents to inhibit GRK-mediated receptor phosphorylation is demonstrated in permeabilized 293 cells that overexpress individual GRKs and the type 1A angiotensin II receptor. We also use this approach to identify the endogenous GRKs that are responsible for the agonist-induced phosphorylation of epitope-tagged β_2-adrenergic receptors (β_2ARs) overexpressed in rabbit ventricular myocytes that are infected with a recombinant adenovirus. In these myocytes, anti-GRK2/3 mAbs inhibit isoproterenol-induced receptor phosphorylation by 77%, while GRK4–6-specific mAbs have no effect. Consistent with the operation of a βAR kinase-mediated mechanism, GRK2 is identified by immunoblot analysis as well as in a functional assay as the predominant GRK expressed in these cells. Microinjection of GRK2/3-specific mAbs into chicken sensory neurons, which have been shown to express a GRK3-like protein, abolishes desensitization of the α_2AR-mediated calcium current inhibition. The intracellular inhibition of endogenous GRKs by mAbs represents a novel approach to the study of receptor specificities among GRKs that should be widely applicable to many G-protein-coupled receptors.**

Many receptors that signal through guanine nucleotide-binding proteins (G proteins) are regulated by mechanisms that cause rapid attenuation of signaling in the continued presence of agonist. This homologous desensitization of G-protein-coupled receptors appears to be mediated, in part, by G-protein-coupled receptor kinases (GRKs) that have the unique property of phosphorylating their receptor substrates only when they are in their activated or agonist-occupied forms (1, 2). Receptor phosphorylation by GRKs subsequently promotes the binding of regulatory arrestin proteins that are thought to prevent further receptor coupling to G proteins.

To date, six distinct mammalian GRKs have been identified that can be grouped into three subfamilies on the basis of sequence and functional similarities (2). Expression of GRK1 (rhodopsin kinase) is confined almost exclusively to retinal photoreceptor cells. This observation makes rhodopsin kinase the only GRK with unequivocal receptor substrates, i.e., the retinal opsins. Likewise, GRK4 is also expressed in significant levels only in testis, implying limited receptor substrate specificity for this kinase. In contrast, two other closely related kinases, GRK5 and GRK6, as well as the two β-adrenergic receptor kinases βARK1(GRK2) and βARK2(GRK3) are expressed throughout the body. Given the disparity between the large number of G-protein-coupled receptors and the relative paucity of GRKs, it is likely that individual GRKs have broad and possibly overlapping receptor substrate specificities.

Direct evidence for a role of GRKs in receptor desensitization and phosphorylation has been obtained only for a limited number of G-protein-coupled receptors. Moreover, most of the data regarding GRK substrate specificity derive from experiments with purified GRKs and receptors that were either purified and reconstituted into phospholipid vesicles (3) or prepared as plasma membrane fractions from cells overexpressing receptor protein (4). More recently, cotransfection of cells with both epitope-tagged receptors and GRKs has allowed testing for effects of individual GRKs on receptor phosphorylation under more physiological conditions in intact cells. These experiments have shown that several GRKs can augment the agonist-induced phosphorylation of β_1-adrenergic (5), α_{1B}-adrenergic (6), δ-opioid (7), dopamine D1A (8), and type 1A angiotensin II receptors (AT_{1a}-Rs) (9).

While these studies have demonstrated that individual GRKs are capable of phosphorylating certain receptors, they do not address the question of which endogenously expressed kinases actually mediate receptor phosphorylation and desensitization in a given cell. In earlier studies, heparin was used to inhibit GRK-mediated receptor phosphorylation and desensitization in permeabilized cells (10). More recently, a dominant negative GRK2 mutant was used to antagonize GRK activity in whole cells (5–7, 9). However, neither heparin nor dominant negative GRK mutants are highly specific inhibitors of individual GRKs. Thus, these experimental approaches are not ideally suited to address the question of receptor specificities among different GRKs. In a recent report, β_2AR desensitization was affected in a cell-type-specific manner by oligonucleotides antisense to GRK2 mRNA (11). A different approach was taken in two previous studies that showed that affinity-purified polyclonal antibodies specific for GRK3, but not GRK2, completely prevented rapid desensitization of olfactory receptors and inhibited agonist-induced phosphorylation of ciliary proteins (12, 13). In the current report, we describe the generation of GRK-specific monoclonal antibodies (mAbs) and demonstrate the feasibility of utilizing these reagents to inhibit GRK-mediated phosphorylation and desensitization in several receptor systems.

MATERIALS AND METHODS

Materials. All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD). Human embryonic

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Abbreviations: AR, adrenergic receptor; AT_{1A}-R, type 1A angiotensin II receptor; βARK, β-adrenergic receptor kinase; G protein, guanine nucleotide-binding regulatory protein; GRK, G-protein-coupled receptor kinase; GST, glutathione *S*-transferase; mAb, monoclonal antibody.

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kidney (293) cells and X63-Ag8.653 myeloma cells were from the American Type Culture Collection. Protein A- and protein G-Sepharose CL4B were purchased from Pharmacia. Angiotensin II, (−)-isoproterenol bitartrate and norepinephrine were from Sigma. Reduced streptolysin O was obtained from Murex (Norcross, GA). [γ-^{32}P]ATP came from DuPont/NEN. cDNA constructs in pcDNAI (Invitrogen) for the 12CA5 epitope-tagged rat AT_{1A}-R and for GRKs have been described (5, 9). Bovine GRK2, GRK3 (4), and GRK5 (14) as well as human β_2-adrenergic receptor (β_2AR) (15) were overexpressed and purified from baculovirus-infected Sf9 cells as described. G-protein $\beta\gamma$ subunits ($G\beta\gamma$) were purified from bovine brain (16).

Cell Culture, Transfection, and Adenoviral Infection. The 293 cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and were transiently transfected with pcDNAI expression vectors for GRKs and AT_{1A}-R as described (9). A 16-amino acid signal sequence from influenza hemagglutinin and the octapeptide FLAG epitope were added to the 5′ end of human β_2AR cDNA by cassette PCR, in a modification of the procedure described by Guan *et al.* (17). A recombinant adenovirus encoding the FLAG-β_2AR [Adeno-(FLAG)β_2AR] was created as described. Rabbit ventricular myocyte isolation, cell culture, and radioligand binding studies have been described in detail (M.H.D., K.C.P., A. O. Grant, W. J. Koch, and R.J.L., unpublished results). Myocytes were infected with Adeno-(FLAG)β_2AR or β-galactosidase adenovirus (18) at 100 plaque-forming units per cell. Embryonic chicken sensory neurons were grown in culture as described elsewhere (19). Cells were plated at a density of 50,000 cells per collagen-coated 35-mm tissue culture dish and studied after 1–3 days of *in vitro* cultivation.

mAbs. BALB/c mice were immunized with fusion proteins of glutathione *S*-transferase (GST) and the C-terminal fragments of rat GRK3 (20) or bovine GRK5 (14). After the fusion of splenocytes with myeloma cells, positive hybridoma clones were identified by ELISA on the basis of their reactivities toward recombinant bovine GRK2, GRK3, or GRK5. A hybridoma clone (O3/5; IgG1/κ) derived from the same fusion secreted antibodies reactive with GST and served as a control antibody in this study.

Receptor Phosphorylation Assays. GRK activities in myocyte extracts were detected by a rhodopsin phosphorylation assay as described (21). Agonist-induced receptor phosphorylation in permeabilized 293 cells or cardiac myocytes was determined by a modification of previously described procedures (9, 22). Cells were washed with KG buffer (22) and permeabilized by incubation for 5 min with 0.5 unit of streptolysin O per ml of KG buffer. After the incubation for 10 min with mAb (500 μg/ml) in KG buffer containing 10 μM ATP and 50 μM GTP, [γ-^{32}P]ATP (75 μCi/ml; 1 Ci = 37 GBq) and the indicated stimuli were added. After 5 min, cells were washed with PBS and scraped into lysis buffer. The 293 cells were solubilized in RIPA lysis buffer (9), and immunoprecipitation of receptors was performed with 12CA5 IgG and protein A-Sepharose beads as described (9). Cardiac myocytes were solubilized in modified lysis buffer that contained 1% Triton X-100 and 0.05% SDS as detergents. Receptors were immunoprecipitated using the anti-FLAG M2 antibody (IBI) and protein G-Sepharose.

Electrophysiological Recording and Microinjection. Standard tight-seal whole-cell recording methods were performed as described (23). Norepinephrine was diluted to the desired concentration in the external solution and applied by pressure ejection from a blunt-tipped (0.5 μm diameter) pipette positioned close to the cell of interest. Antibodies were pressure injected into the cytosol by using an automated Eppendorf 5272 microinjector. For these experiments, injection pipettes were back-filled with 3 μl of antibody at 300 μg/ml and mounted on an Eppendorf 5171 microprocessor-controlled micromanipulator.

RESULTS AND DISCUSSION

Characterization of mAbs. We sought to identify the specific GRKs that mediate receptor phosphorylation and desensitization in several different receptor systems by applying mAbs as intracellular inhibitors of GRKs. A major prerequisite for these studies was the generation of mAbs that specifically react with individual GRKs and that also inhibit GRK activity. To create these mAbs, we exploited the C-terminal GRK domains, which most clearly distinguish the GRKs from one another. We used GST fusion proteins with either the last 221 amino acids of GRK3 (GRK3ct) or the last 128 amino acids of GRK5 (GRK5ct) as immunogens. After the immunization of mice with GST–GRK3ct, 16 hybridoma clones were identified that produced mAbs that reacted with purified GRK3. While none of these antibodies significantly cross-reacted with GRK1, GRK4, GRK5, or GRK6, they also recognized GRK2. Similarly, none of six mAbs that were obtained after immunization with GST–GRK2ct fusion proteins discriminated between GRK2 and GRK3. A different set of mAbs with complementary specificities was generated by immunization with GST–GRK5ct fusion proteins. Five hybridoma clones were identified that secreted mAbs with specificities for GRK4, GRK5, and GRK6. These mAbs did not react with GRK1, GRK2, or GRK3. The GRK2/3-specific mAb C5/1 (IgG2a/κ) and the mAb A16/17 (IgG1/κ), which binds GRK4, GRK5, and GRK6, were characterized by immunoblot analysis (Fig. 1) and were used in all subsequent experiments. The anti-GRK2/3 mAb reacts with rat, murine, human, bovine, and porcine GRK2 and/or GRK3. The mAb with specificity for GRK4–6 recognizes all four GRK4 splice variants (24).

The finding that antibody reactivities were restricted to either GRK2/3 on the one hand or GRK4–6 on the other reflects the pronounced degree of homology within these two GRK subfamilies (2). While the overall amino acid identity between GRK2 and GRK3 is 85%, the other GRKs share only

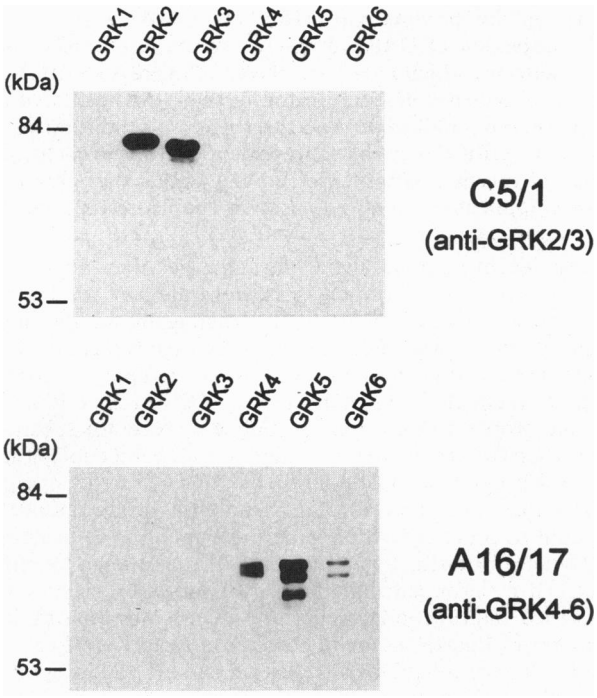

FIG. 1. Immunoblot analysis showing the specificity of the mAbs C5/1 and A16/17. The 293 cells were transfected with pcDNAI expression vectors for GRKs 1 to 6 and were scraped into 20 mM Tris·HCl (pH 7.4) containing protease inhibitors. Whole cell protein extracts (6 μg per lane) were resolved by SDS/PAGE on 10% gels and then subjected to Western blot analysis with the mAbs C5/1 or A16/17.

35–40% sequence identity with the βARK subfamily. Within the GRK4 subfamily, kinases show generally about 70% sequence homology. GRK1 (rhodopsin kinase) is intermediate in similarity between GRK2/3 and GRK4–6 and forms a separate subfamily. None of the tested mAbs cross-reacted with GRK1. Thus, a categorization of GRKs according to their antigenic properties as determined by these antibodies agrees with the previously defined classification of GRKs on the basis of similarity in sequence and function (2).

Effects of mAb on AT_{1A}-R Phosphorylation in 293 Cells. To determine whether GRK-specific antibodies inhibit receptor phosphorylation in cells, we established an assay for receptor phosphorylation in streptolysin O-permeabilized 293 cells that were cotransfected with the cDNAs encoding epitope-tagged AT_{1A}-R and GRKs 1 to 6. Fig. 2*A* shows the angiotensin II-induced AT_{1A}-R phosphorylation in GRK3-overexpressing cells. In permeabilized 293 cells that were incubated with a GST-specific control mAbs, overexpression of GRK1 resulted in a 9.8 ± 4.3-fold (mean ± SEM) increase in agonist-induced receptor phosphorylation compared with control cells with endogenous kinase levels. Similar results were obtained in cells that overexpressed GRK2 (8.2 ± 0.8-fold stimulation), GRK3 (9.8 ± 2.8), GRK5 (11.3 ± 1.1), or GRK6 (17.9 ± 4.9), whereas overexpression of GRK4 (1.3 ± 0.5-fold stimulation) did not significantly enhance agonist-induced receptor phosphorylation. These results confirm and extend our previous conclusions from an intact cell assay (9). The inability of overexpressed GRK4 to promote AT_{1A}-R phosphorylation indicates specificity among GRKs for this receptor that previously was not appreciated.

The permeabilized cell assay allowed us to investigate the ability of mAbs to inhibit GRK-mediated receptor phosphorylation in 293 cells (Fig. 2). Incubation of permeabilized 293 cells that expressed endogenous GRK levels with the anti-GRK2/3 mAb reduced the angiotensin II-induced AT_{1A}-R phosphorylation by 63 ± 4%, while the GRK4–6-specific mAb had virtually no effect. This result indicates that GRK2 and/or GRK3 are the endogenous GRKs in 293 cells that mediate phosphorylation of this receptor. In support of this notion, we identified a protein in 293 cellular extracts by immunoprecipitation with the anti-GRK2/3 mAb that comigrates with GRK2 (see below), but we could not find proteins with GRK4–6 immunoreactivities. The residual receptor phosphorylation in the presence of the anti-GRK2/3 mAb is probably due to other kinases that also phosphorylate the AT_{1A}-R, such as protein kinase C (9).

Incubation with the anti-GRK2/3 mAb inhibited the agonist-induced receptor phosphorylation in GRK2- or GRK3-overexpressing cells by more than 85% (Fig. 2). This result shows that this mAb efficiently blocks GRK2- or GRK3-mediated receptor phosphorylation in permeabilized cells. The ability of the GRK4–6-specific mAb to reduce receptor phosphorylation in cells that overexpress GRK5 or GRK6 was significant, yet less pronounced than inhibitory effects of the anti-GRK2/3 mAb. Even when cells were incubated with a combination of GRK2/3- and GRK4–6-specific mAbs, to inhibit both the endogenous and the overexpressed kinases, only 68–77% of the agonist-induced receptor phosphorylation was inhibited. This result suggests that membrane-associated GRKs, such as GRK5 or GRK6, may be less accessible to

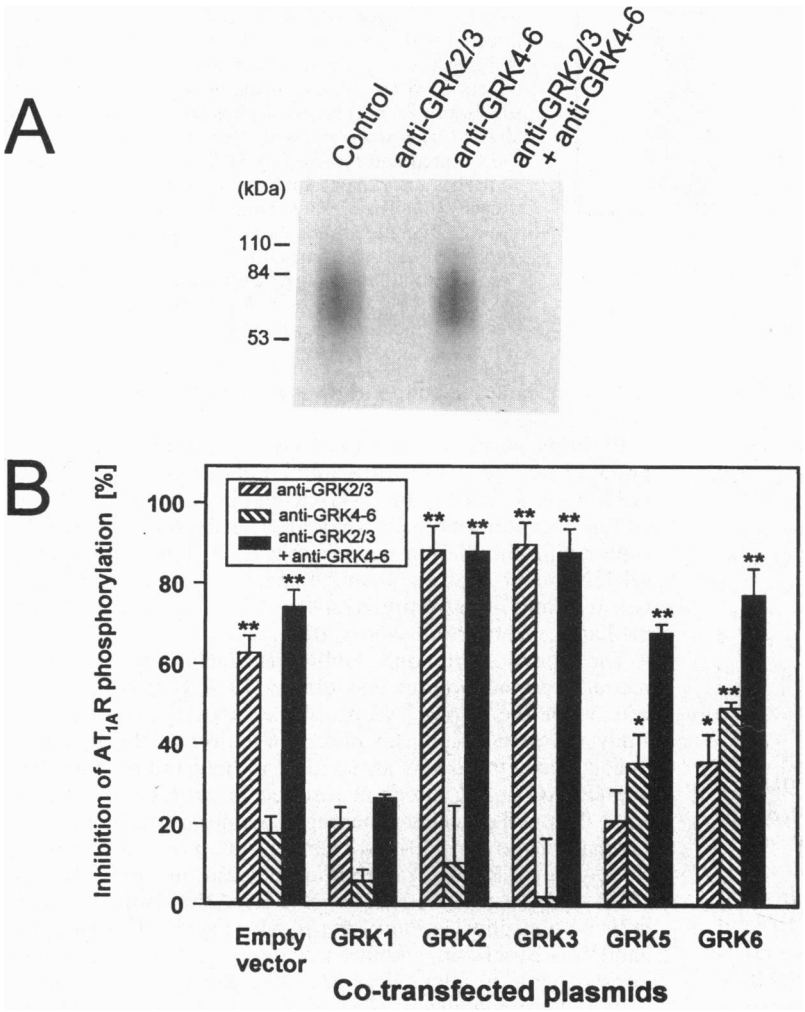

FIG. 2. Inhibition of angiotensin II-induced AT_{1A}-R phosphorylation in GRK-overexpressing 293 cells by GRK-specific mAbs. The 293 cells in 100-mm dishes were transfected with 10 μg of AT_{1A}-R-pcDNAI together with the empty vector pcDNAI or pcDNAI expression vectors for GRK1, GRK2, GRK3, GRK5, or GRK6 (5 μg per dish). The inhibition of agonist-induced (200 nM angiotensin II) receptor phosphorylation in permeabilized cells by the GRK2/3-specific mAbs, the GRK4/5/6-specific mAbs, or a combination of these antibodies is shown. (*A*) Autoradiogram from a single experiment with cells overexpressing GRK3. (*B*) Data represent the mean ± SEM of four experiments. **, $P <$ 0.001; *, $P < 0.05$ compared with control cells that were incubated with an irrelevant mAb.

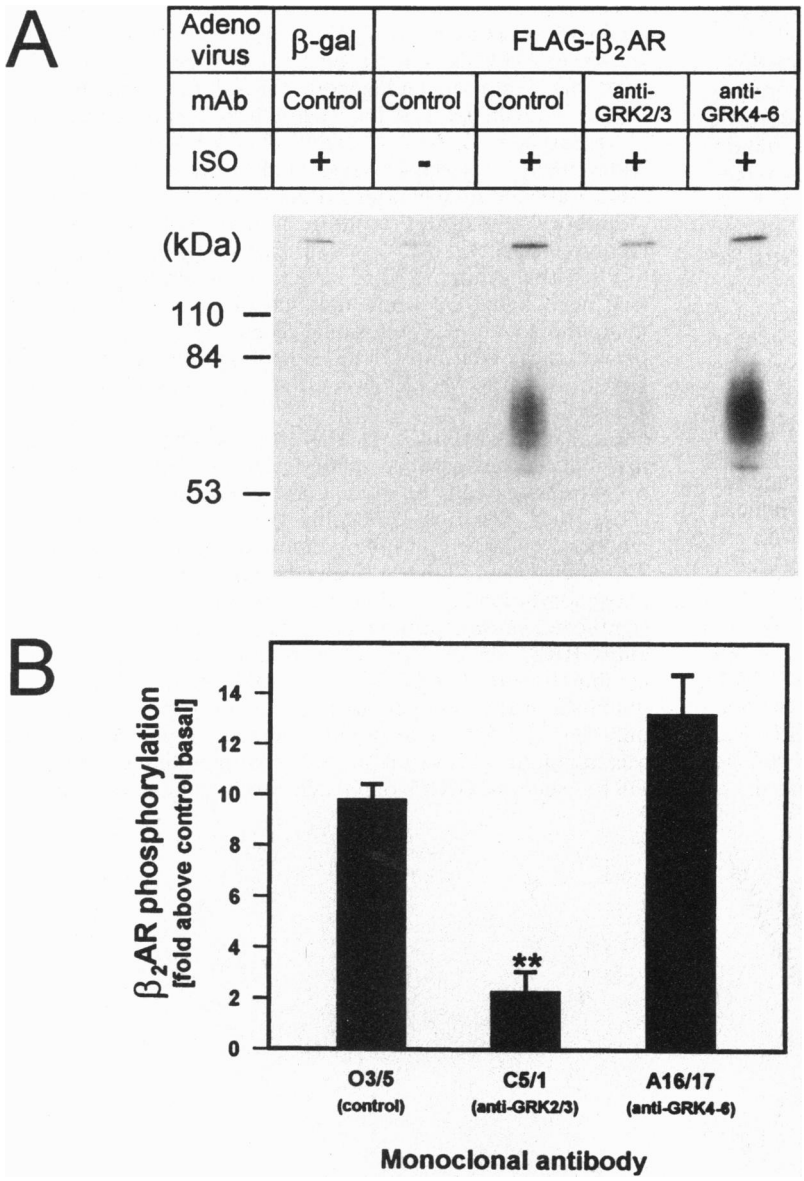

FIG. 3. Inhibition of isoproterenol-induced phosphorylation of the β_2AR in permeabilized rabbit ventricular myocytes by GRK-specific mAbs. Cells were infected with adenovirus that encoded either β-galactosidase (as a control) or epitope-tagged β_2AR. The effects of GRK-specific mAbs on agonist-induced (10 μM isoproterenol) receptor phosphorylation in permeabilized cells were assessed. Receptors were immunoprecipitated and resolved by SDS/PAGE on 10% gels. (*A*) The autoradiogram from a single experiment is shown. (*B*) The results from four experiments are shown. Receptor phosphorylation in isoproterenol-stimulated cells was normalized to β_2AR phosphorylation in unstimulated control cells (basal). Data represent the mean ± SEM. *, $P < 0.001$ compared with isoproterenol-stimulated control cells.

antibodies than cytosolic proteins like GRK2 or GRK3. None of the mAbs significantly affected AT_{1A}-R phosphorylation in cells that overexpressed GRK1. These results demonstrate the ability of mAbs to identify the GRKs that mediate receptor phosphorylation in permeabilized cells.

We mapped the C5/1 (anti-GRK2/3) epitope by mAb binding studies to GST fusion proteins that contained various GRK2 truncation mutants (25) and defined the minimal C5/1 binding region as a 44-amino acid residue stretch (Tyr^{466} to Pro^{510}) within the GRK2/3 C-terminal domain. The A16/17 (anti-GRK4–6) epitope on GRK5 is delimited by amino acid residues 463 and 512.

Effects of mAb on β_2AR Phosphorylation in Cardiac Myocytes. We next applied this experimental approach to the identification of those GRKs in rabbit ventricular myocytes that are responsible for the agonist-induced phosphorylation of the β_2AR. Our choice of this particular receptor system was based on the following rationale. (*i*) Mechanisms that bring about β_2AR phosphorylation and desensitization have been studied in great detail using other techniques. (*ii*) Unlike several other G-protein-coupled receptors, the β_2AR appears to be a promiscuous GRK substrate. Since GRK2, GRK3, GRK5, and GRK6 mRNA have been shown to be expressed in the heart (1), this experimental system appears well suited to investigate GRK substrate specificity.

By using purified β_2AR that was reconstituted into phospholipid vesicles as substrate, we first confirmed the ability of GRK-specific mAb to block GRK-mediated phosphorylation of this receptor (data not shown). The GRK2-mediated receptor phosphorylation was effectively inhibited by the anti-GRK2/3 mAb, but not by mAb specific for GRK4–6. Conversely, only the anti-GRK4–6 mAb prevented GRK5-mediated receptor phosphorylation.

The infection of adult rabbit ventricular myocytes with recombinant adenovirus encoding a (FLAG) epitope-tagged version of the β_2AR [Adeno-(FLAG)β_2AR] allowed us to study the agonist-induced phosphorylation of the β_2AR by endogenous kinases. As assessed by radioligand binding, Adeno-(FLAG)β_2AR infection resulted in expression levels of 8.2 ± 0.9 pmol of receptor per mg of membrane protein, which corresponds to an ≈50-fold overexpression over endogenous βAR levels. Receptor immunoprecipitation from Adeno-(FLAG)β_2AR-infected cells revealed the isoproterenol-induced phosphorylation of the β_2AR (Fig. 3). This receptor band was absent in immunoprecipitates from control cells infected with an adenovirus encoding β-galactosidase. Incubation of permeabilized cells with the anti-GRK2/3 mAb, but

not with the mAb specific for GRK4–6, significantly ($P < 0.001$) inhibited agonist-induced receptor phosphorylation by 77% compared with cells treated with control mAbs. A combination of the two GRK-specific mAb was not more effective than the GRK2/3-specific mAb alone (data not shown). This result identifies GRK2 and/or GRK3 as the kinases that primarily phosphorylate the β_2AR in ventricular myocytes following short-term agonist stimulation. The residual receptor phosphorylation observed in cells treated with anti-GRK2/3 mAb may be due to cAMP-dependent protein kinase that become activated in the course of β_2AR stimulation (10).

In previous reports, the myocardial expression of GRKs was investigated primarily by Northern blot analysis. These studies have found expression of various GRKs in tissue derived from whole rat, bovine, and human hearts (1). To document GRK expression in isolated rabbit ventricular myocytes, we performed immunoblot analysis of concentrated cellular lysates from myocytes by using GRK-specific mAbs. While no GRK4–6-immunoreactive proteins were identified, the mAb specific for GRK2/3 immunoprecipitated a single ≈80-kDa protein that comigrated with GRK2 (Fig. 4*A*). This result was corroborated in a functional assay that determined rhodopsin phosphorylation by kinases present in cytosolic extracts from these cells (Fig. 4 *B* and *C*). The anti-GRK2/3 mAb significantly inhibited rhodopsin phosphorylation either in the presence or in the absence of exogenous G$\beta\gamma$ subunits, whereas the anti-GRK4–6 mAb was ineffective. Finally, these results correspond with the finding of readily detectable levels of GRK2 mRNA in these cells, while GRK3 and GRK5 mRNA expression was either much weaker or even absent [F. T. Lin, personal communication].

Our experiments identify GRK2 as the major kinase that mediates rapid agonist-induced phosphorylation of the β_2AR in rabbit ventricular myocytes as well as the predominant GRK present in these cells. This result contrasts with previous reports that had shown expression of other GRKs besides GRK2 in whole hearts and may indicate differential GRK expression in the various heart cell types. Other recent findings also suggest a central role for GRK2 in myocardial βAR regulation. Transgenic mice with cardiac overexpression of GRK2 showed a significant attenuation of isoproterenol-induced inotropy, thus demonstrating that cardiac βARs are *in vivo* substrates of GRK2 (21). In marked contrast, mice with overexpression of a βARK inhibitor that competes for G$\beta\gamma$ binding to GRK2 or GRK3 exhibited enhanced cardiac contractility and supersensitivity to exogenous isoproterenol (21). These observations were more recently corroborated in an *in vitro* model using rabbit ventricular myocytes in which adenovirus-mediated gene transfer of the βARK inhibitor significantly inhibited desensitization of βARs (M.H.D., K.C.P., A. O. Grant, W. J. Koch, and R.J.L., unpublished results). Finally, the finding that mice with a targeted disruption of the GRK2 gene display profound cardiac malformations points to an important developmental role for this kinase in the heart that, at least during embryogenesis, cannot be compensated by

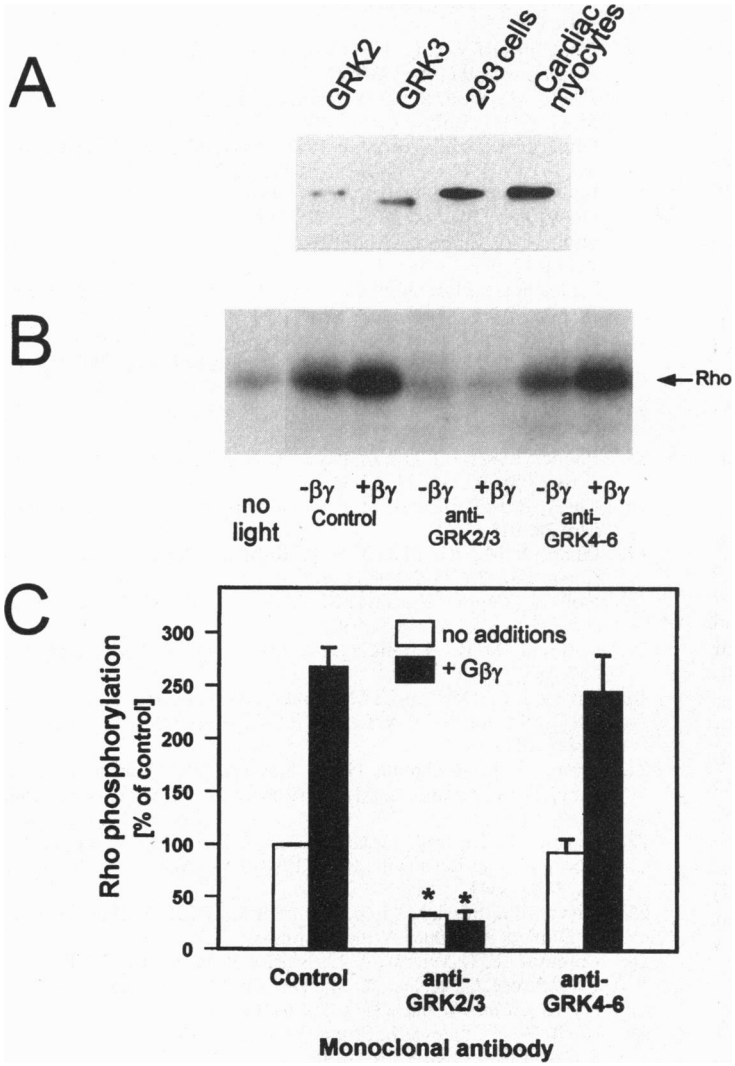

FIG. 4. GRK expression in 293 cells and in rabbit ventricular myocytes. (*A*) GRKs were concentrated by immune precipitation using anti-GRK2/3 mAbs from 500 μg of total cellular protein from 293 cells or cardiac myocytes and were examined by immunoblot analysis along with cellular lysates (1 μg of total cellular protein) from 293 cells that overexpress GRK2 or GRK3. Biotinylated GRK2/3-specific mAb was used for immunodetection in combination with horseradish peroxidase-conjugated streptavidin. (*B*) Assessment of GRK activity in extracts from rabbit ventricular myocytes. Cytosolic extracts (100 μg in a total volume of 75 μl) were preincubated for 30 min at 37°C with 37.5 μg of control mAbs (lanes 2 and 3), GRK2/3-specific mAbs (lanes 4 and 5), or GRK4–6-specific mAbs (lanes 6 and 7). Samples were assayed in a rhodopsin (Rho) phosphorylation assay either in the absence or in the presence of 28.5 pmol of G$\beta\gamma$. The first lane shows the control specimen that was not exposed to light. (*C*) Results (mean ± SEM) of three rhodopsin phosphorylation experiments. Results are expressed as a percentage of rhodopsin phosphorylation in cell extracts treated with control mAbs in the absence of G$\beta\gamma$. *, $P < 0.05$ compared with rhodopsin phosphorylation in the absence of antibodies.

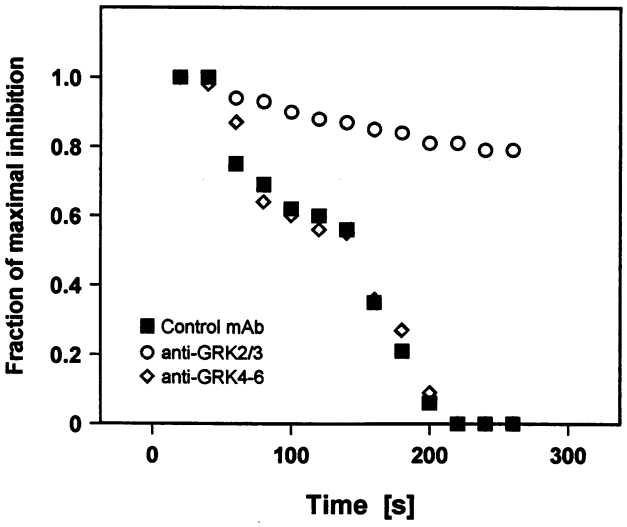

FIG. 5. Effect of GRK-specific mAbs on the desensitization of norepinephrine-induced inhibition of calcium current in sensory neurons. The fraction of norepinephrine-induced inhibition is plotted as a function of time for cells that were injected with control mAbs, anti-GRK2/3 mAbs, or anti-GRK4–6 mAbs. Norepinephrine (100 μM) was applied throughout the experiment. The plotted data begin at the time at which maximal inhibition was reached and represent the mean values for five cells. The SEM values fall within the symbol boundaries (range ± 0.03–0.09).

other GRKs (M. Jaber, W. J. Koch, H. Rockman, B. Smith, R. A. Bond, K. Sulik, J. Ross, R.J.L., M. G. Caron, and B. Giros, unpublished results). Our conclusion that GRK2 mediates agonist-induced phosphorylation of the β_2AR in rabbit ventricular myocytes is potentially relevant for a better understanding of the pathophysiology of cardiac diseases such as chronic heart failure, where GRKs have been implicated in the functional uncoupling of βARs.

These results demonstrate the ability of GRK-specific mAbs to inhibit agonist-induced receptor phosphorylation in permeabilized myocytes. Unfortunately, this same experimental system could not be used to investigate effects of mAbs on desensitization, since permeabilization with streptolysin O destroys the ability of cardiac myocytes as well as of many other cell types to attenuate receptor-mediated signaling over time. This is probably due to the loss or critical dilution of intracellular components that are essential for receptor desensitization.

Effects of mAb on α_2AR Desensitization in Sensory Neurons. It has been reported that the inhibition of voltage-dependent calcium channels in chicken embryonic sensory neurons, which is mediated by α_2AR, desensitizes with prolonged exposure to norepinephrine (23). Accordingly, we microinjected individual sensory neurons with GRK-specific mAbs under conditions that preserve the integrity of these cells. Microinjection of anti-GRK2/3 mAbs significantly slowed the rate of desensitization of norepinephrine-induced calcium current inhibition (Fig. 5). In contrast, injection of the GRK4–6-specific mAbs had no effect on desensitization when compared with control cells injected with irrelevant mAbs. Neither peak current measured prior to norepinephrine application nor its maximal inhibition by the transmitter was affected by mAb injection. These results are in accord with the hypothesis of a specific role for GRK3 in the desensitization of these receptors (23). It was previously shown that recombinant GRK3 enhances the rate of desensitization, while a GRK3-derived inhibitor peptide had the opposite effect. Moreover, a GRK3-like protein was detected by immunoblot analysis in these cells (23). The ability of GRK2/3-specific mAbs to block desensitization now directly demonstrates the involvement of a GRK3-like kinase in the regulation of transmitter-mediated calcium current inhibition in this system.

Conclusion. mAbs were generated that specifically react with and functionally inhibit GRK2/3 or GRK4–6. These reagents allowed us to identify kinases that are responsible for receptor phosphorylation or desensitization in three different receptor systems. While an essential role for members of the GRK4–6 subfamily in the regulation of G-protein-coupled receptors remains to be demonstrated, the results presented herein suggest that intracellular inhibition of endogenous GRKs by mAbs provides a novel approach that may be generally applicable to the identification of receptor specificities among the GRKs.

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