Expression, Purification, and Characterization of the
G Protein-coupled Receptor Kinase GRK5*

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G protein-coupled receptor kinases (GRKs) such as rhodopsin kinase and the β-adrenergic receptor kinase (βARK) play an important role in agonist-specific phosphorylation and desensitization of G protein-coupled receptors. GRK5 is a recently identified member of the GRK family that has greater homology with rhodopsin kinase than with βARK. To further characterize the activity of GRK5, it has been overexpressed in Sf9 insect cells and purified by successive chromatography on S-Sepharose and Mono S columns. GRK5 phosphorylates the β2-adrenergic receptor (β2AR), m2 muscarinic cholinergic receptor, and rhodopsin in an agonist-dependent manner to maximal stoichiometries of ~2.5, 1.5, and 1 mol of phosphate/mol of receptor, respectively, with $K_\text{m}$ values of ~0.5 μM for the β2AR, ~16 μM for rhodopsin, and ~24 μM for ATP. Peptide phosphorylation studies suggest that in contrast to βARK and rhodopsin kinase, GRK5 preferentially phosphorylates nonacidic peptides with a $K_\text{m}$ of ~1.3 mM. Heparin and dextran sulfate were found to be potent inhibitors of GRK5 with IC_{50} values of ~1 mM, thereby being at least 150-fold more potent on GRK5 than on βARK. GRK5 can also be activated by polyacrylamide gels, which promotes an ~2.6-fold activation. Overall, these studies demonstrate that GRK5 has unique properties that distinguish it from other members of the GRK family and that likely play an important role in modulating its mechanism of action.

Many transmembrane signalling systems consist of specific G protein-coupled receptors that transduce the binding of extracellular ligands into intracellular signalling events (1, 2). G protein-coupled receptors mediate the activity of a wide variety of effector molecules including adenylyl cyclase, cGMP phosphodiesterase, phospholipase C, phospholipase A2, and various ion channels. Two of the best characterized G protein-coupled receptors are the β2-adrenergic receptor (β2AR), which mediates catecholamine stimulation of adenylyl cyclase (3), and the visual “light receptor” rhodopsin, which mediates phototransduction in retinal rod cells (4). In both of these systems, a rapid loss of responsiveness occurs following receptor activation (3, 4). This rapid activation-dependent loss of receptor responsiveness appears to be mediated by specific G protein-coupled receptor kinases (GRKs) that have the unique ability to recognize and phosphorylate their receptor substrates when they are in their active conformations (5). The β-adrenergic receptor kinase (βARK) (6, 7) and rhodopsin kinase (8, 9) have been implicated as the major kinases involved in the stimulus-dependent phosphorylation of the β2AR and rhodopsin, respectively. The subsequent uncoupling of the receptor and G protein is then mediated by arrestin proteins that specifically bind to the phosphorylated and activated forms of the receptor (8–10).

To date, six mammalian and two Drosophila GRKs have been identified. In addition to βARK (11) and rhodopsin kinase (12), the other members of the GRK family include bovine βARK2 (13), human IT11 (14), GRK5 (15) and GRK6 (16), and Drosophila GPRK-1 and GPRK-2 (17). A comparison of the amino acid sequences of the GRKs suggests that there are two major branches of the GRK family tree. While βARK2 and Drosophila GPRK-1 appear to be most similar to βARK, with amino acid identities of 84% and 64%, respectively, rhodopsin kinase, IT11, GRK5, GRK6, and Drosophila GPRK-2 have significantly lower homology with βARK (35–40% amino acid identity) and form a separate branch of the family tree. The phylogenetic classification of the GRK family is further supported by functional analysis of the various GRKs. In vitro studies have demonstrated that both βARK and βARK2 share a very similar substrate specificity at the level of amino acid preference (they phosphorylate serine-containing peptides with amino-terminal acidic residues) (18), receptor phosphorylation (β2AR, m2 muscarinic cholinergic, and substance P receptors are good substrates in vitro) (18–20), and potential mechanism of cellular activation (interaction with G protein βγ subunits) (18), βARK, βARK2, and Drosophila GPRK-1 also appear to be ubiquitous proteins being expressed in a variety of tissues (11, 13, 17).

Other than rhodopsin kinase, which is predominantly expressed in rod and possibly cone outer segments (4), the expression pattern of the various GRKs does not coincide with any particular G protein-coupled receptor. While βARK is a ubiquitous protein that likely plays a major role in the phosphorylation and desensitization of the agonist-activated β2AR, it may well have a general role in phosphorylating and regulating the activity of multiple receptors (18–23). The expression and in vitro substrate specificity of βARK2 demonstrates that it shares many properties with βARK (13, 18–20). However, recent findings suggest that βARK2 may also be localized in olfactory cilia in the rat and may play a role in regulating odorant receptor function (24). In general, the tissue distribution of human GRK6 also appears to be very similar to that of βARK with the highest levels for both in brain, skeletal muscle, and pancreas (16). In contrast, the tissue distribution of IT11

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1. The abbreviations used are: β2AR, β2-adrenergic receptor; βARK, β-adrenergic receptor kinase; G protein, guanine nucleotide binding protein; GRK, G protein-coupled receptor kinase; H-7, 1-(5-isooquinolinyl)-2-methylpiperazine; m2 mAChR, m2 muscarinic cholinergic receptor; ROS, rod outer segments.

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and GRK5 are quite distinct from that of βARK, βARK2, and GRK6. IT11 is expressed at highest levels in the testis and at lower levels in a number of other tissues (14) while GRK5 is expressed at highest levels in the heart, placenta, and lung (15). While at present little is known about the function of GRK5, GRK6, IT11, and Drosophila GRPK-2, the similarities between these four proteins suggest that they may well share common roles in the cell. In this study, we present the results of the first successful purification and characterization of the properties of GRK5, including an analysis of its ability to phosphorylate three functionally distinct G protein-coupled receptors. The results highlight striking differences that exist in the properties of GRK5 and βARK.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chromatography resins S-Sepharose and Mono S were purchased from Pharmacia LKB Biotechnology Inc. Frozen bovine retinas were from George A. Hormel & Co. Wild type Spodoptera frugiperda (Sf9) cells were from the American Type Culture Collection. 1-[(2-Aminoethyl)amino]cyclopentane-1-carboxylic acid (H-7), heparin (average M, \( M_s = 4,000-6,000 \)), dextran sulfate (average M, \( M_s = 5,000 \)), poly-c-aspatic acid (M, \( M_s = 5,000-15,000 \)), poly-n-glutamic acid (M, \( M_s = 2,000-15,000 \)), spermine, spermidine, poly-L-lysine (M, \( M_s = 1,000-4,000 \)), dextran (average M, \( M_s = 5,000-15,000 \)), heparin (average M, \( M_s = 5,000-15,000 \)), spermine, spermidine, poly-L-lysine (M, \( M_s = 1,000-4,000 \)), histones (type II), phosvitin, (1-isoproterenol, and carbachol) were from Sigma. Tissue culture reagents were from Life Technologies and Sigma. [γ-32P]ATP (3000 cpm/pmol) was obtained from DuPont-NEN. The catalytic subunit of the cAMP-dependent protein kinase was purchased from Promega.

**Expression and Purification of GRK5 from Sf9 Cells**—Previously, we isolated a baculovirus containing the GRK5 cDNA (pBacPAR-GRK5) and used it to express human GRK5 in Sf9 insect cells (15). To purify GRK5, Sf9 cells were infected with the baculovirus and then harvested by scraping in 3 M, pH 7.2, 2 mM EDTA, 6 mM concentration of the peptide RRRASASAA or RRRASASAASAA, 10 mM MgCl₂, 6 mM \( \gamma-\)32P[ATP (1 cpm/pmol), and 7 μg of cAMP-dependent protein kinase catalytic subunit. After a 6-h incubation at room temperature, 32P incorporation was determined by spotting 10-μl aliquots onto P81 paper followed by six washes with 75 mM phosphoric acid (28). The phosphoserine-containing peptides were then purified on a 1-mL Mono S column using a linear gradient from 0–1 M ammonium acetate as described (29). This step effectively separated the more acidic phosphorylated peptide from the nonphosphorylated peptide. All peptide stock solutions were adjusted to pH 7.0 with 50 mM Tris and then used in phosphorylation reactions.

**Assay for GRK5**—GRK5 was routinely assayed using 2-6 μM rhodopsin (urea-treated ROS), 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, 0.1 mM \( \gamma-\)32P[ATP (specific activity of 700–1500 cpm/pmol) in a total reaction volume of 20–40 μl. Rhodopsin phosphorylation reactions were incubated at 30 °C for 1–3 min in the presence of fluorescent room light. The GRK migrates at \( M_s = 67.7 \) kDa.

**RESULTS**

Previously, we demonstrated that human GRK5, expressed in Sf9 insect cells using the baculovirus system, was able to phosphorylate rhodopsin in a light-dependent fashion (15). In an effort to more extensively characterize the activity of GRK5, here we have purified the recombinant human GRK5 from Sf9 cells and tested the kinase for its reactivity toward several G protein-coupled receptors. The ability of GRK5 to phosphorylate rhodopsin was used to assay the kinase activity at various stages of the purification. A time course of expression following infection of Sf9 cells with the pBacPAR-GRK5 virus reveals that ~48 h postinfection yields the highest expression of GRK5 (data not shown). Thus, cells were harvested 48 h postinfection, homogenized, and centrifuged, and the high speed supernatant fraction was then chromatographed on an S-Sepharose column. Elution of GRK5 from the S-Sepharose column with a linear salt gradient yielded ~80% recovery of the kinase activity (Table I). The pooled S-Sepharose fractions were ~60% pure, as judged by SDS-polyacrylamide electrophoresis and Coomassie Blue staining (Fig. 1, lane 2). GRK5 migrates at ~66 kDa, similar to its predicted molecular mass of 67.7 kDa. The pooled S-Sepharose fractions were then chromatographed on a Mono S column. An overall ~34-fold purification and 25% recovery were obtained from the purification (Table I). This purified kinase preparation appears to be >95% homogeneous.
GRK5 was purified as described under "Experimental Procedures." Briefly, three 150 x 25-mm dishes of monolayer Sf9 cells were infected with the pBacPAK-GRK5 virus and harvested 48 h postinfection. After homogenization of the harvested cells and several centrifugations, the high speed supernatant was chromatographed on S-Sepharose and Mono S columns. At each step of the purification, GRK5 was assayed using 3-5 μM rhodopsin and 0.1 mM (γ-32P)ATP (700-1000 cpm/pmol) in buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 5 mM MgCl2, as described under "Experimental Procedures." Protein concentrations were determined by the method of Bradford (38). The means ± S.E. from four separate purifications are presented.

### Purification of GRK5 from Sf9 Cells

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein mg</th>
<th>Specific activity nmol P/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>5.0 ± 0.7</td>
<td>4.5 ± 2.0</td>
</tr>
<tr>
<td>S-Sepharose</td>
<td>0.2 ± 0.02</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>Mono S</td>
<td>0.03 ± 0.01</td>
<td>84 ± 4</td>
</tr>
</tbody>
</table>

#### Purification and Characterization of GRK5

Fig. 1. *Purification of GRK5 from Sf9 cells.* Aliquots from each step of purification were electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. Lane 1, crude supernatant obtained after high speed centrifugation of pBacPAK-GRK5-infected Sf9 cells (10 μg of protein); lane 2, pooled peak fractions after chromatography on an S-Sepharose column (2 μg of protein); lane 3, pooled peak fractions obtained after chromatography on Mono S column (0.8 μg of protein).

(Fig. 1, lane 3) and was used for all further characterizations of GRK5.

The initial characterization of GRK5 involved determining the optimal buffer conditions for the kinase. Similar to most other protein kinases, GRK5 prefers Mg2+ as the divalent metal cofactor (Fig. 2). The optimum concentration was found to be 2–3 mM free Mg2+, while higher concentrations were inhibitory to the kinase. This is very similar to the Mg2+ requirements for βARK (18, 31). The optimum Mn2+ concentration was 1–2 mM; however, Mn2+ gave only ~23% the activity of Mg2+. The optimum pH for GRK5 was found to be in the range of 5.5 to 7.5 (data not shown). At a rhodopsin concentration of 10 μM, the K_m of GRK5 for ATP was ~24 μM (Table II). This is similar to the value previously measured for βARK (37–60 μM) (18, 31) but significantly higher than the K_m for ATP determined for rhodopsin kinase (~2 μM) (32).

The ability of GRK5 to phosphorylate different G protein-coupled receptors was studied next. Our previous work had established that GRK5 phosphorylates rhodopsin in a light-dependent manner, albeit not as well as βARK (15). In addition, in contrast to the ability of βARK to be activated by G protein βγ subunits, rhodopsin phosphorylation by GRK5 was not enhanced by βγ subunits (15). In this study, we have compared the ability of GRK5 and βARK to phosphorylate the βγ-adrenergic receptor, the m2 muscarinic cholinergic receptor, and rhodopsin. As shown in Fig. 3A, the β2AR serves as the best substrate for GRK5, being rapidly phosphorylated to a stoichiometry of 2–2.5 mol of P_i/mol of receptor. Rhodopsin is also rapidly phosphorylated by GRK5, albeit to a stoichiometry of only ~1 mol/mol under these conditions. In contrast, the m2 mACHr appears to be the poorest receptor substrate tested for GRK5 since both the rate of phosphorylation and final stoichiometry (1–1.5 mol/mol) are significantly lower than that for the β2AR (Fig. 3A). However, for each receptor, the phosphorylation by GRK5 was agonist-dependent. In the absence of the appropriate agonist, GRK5 phosphorylated the β2AR, m2 mACHr, and rhodopsin to maximal stoichiometries of only 0.4, 0.24, and 0.07 mol/mol, respectively. The pattern of phosphorylation observed with GRK5 was strikingly different from that observed with βARK. In experiments performed under identical conditions, the β2AR and m2 mACHr were both rapidly and extensively phosphorylated (4–5 mol/mol) while rhodopsin was also rapidly phosphorylated but to a stoichiometry of only ~2 mol/mol under these conditions (Fig. 3B). While previous studies have demonstrated that G protein βγ subunits significantly enhance both the rate and stoichiometry of phosphorylation of each of these receptor substrates by βARK (18, 19), G protein βγ subunits did not affect GRK5-mediated phosphorylation (Ref. 15 and data not shown). Previous kinetic studies have shown that βARK phosphorylates the β2AR with a K_m of 0.15–0.25 μM and rhodopsin with a K_m of 6–14 μM (18, 31). A similar series of kinetic studies demonstrates that GRK5 phosphorylates the β2AR with a K_m of ~0.5 μM while the K_m for rhodopsin was ~16 μM (Table II). Our initial efforts at determining a K_m for phosphorylation of the m2 mACHr by GRK5 were unsuccessful, possibly reflecting a low affinity for the kinase.

In an effort to assess whether GRK5 phosphorylates a particular consensus amino acid sequence, we studied the ability of GRK5 to phosphorylate various peptide substrates. Previous studies have demonstrated that βARK preferentially phosphorylates peptides containing acidic residues amino-terminal.
The kinetic parameters of GRK5 for ATP were determined by incubating 10 µM rhodopsin, 60 nm GRK5, and 10-100 µM [γ-32P]ATP (1250 cpm/pmol) for 1 min at 30 °C in buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 5 mM MgCl₂. The kinetic parameters for the βAR were determined by incubating 0.025-0.45 µM reconstituted βAR and 60 nm GRK5 for 10 min at 30 °C in buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, and 0.1 mM [γ-32P]ATP (1400 cpm/pmol). The kinetic parameters for the βAR were determined by incubating 2.5-40 µM rhodopsin and 15 nm GRK5 for 2 min at 30 °C in buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, and 0.1 mM [γ-32P]ATP (1000 cpm/pmol). All reactions were stopped by the addition of SDS sample buffer, followed by electrophoresis on a 10% SDS-polyacrylamide gel, and autoradiography. The 32P incorporation was determined by counting and cutting the receptor bands in a scintillation counter. The kinetic parameters for the peptides RRRASASAA and RRRAEASAA were determined by incubating 0.05-5 µM peptide and 60 nm GRK5 for 30 min at 30 °C in buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, and 0.1 mM [γ-32P]ATP (1000 cpm/pmol). Reactions were stopped by the addition of trichloroacetic acid to a final concentration of 15%. The denatured protein was removed by centrifugation of the samples for 10 min at 16,000 x g, and the supernatants were then spotted on P81 paper and washed six times with 75 mM phosphoric acid. The 32P incorporation into the peptide was determined by counting the P81 paper in a scintillation counter.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol P/min/mg)</th>
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<tbody>
<tr>
<td>ATP</td>
<td>23.8 ± 1.3</td>
<td>1148 ± 167</td>
</tr>
<tr>
<td>βAR</td>
<td>0.54 ± 0.15</td>
<td>580 ± 142</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>15.6 ± 4.0</td>
<td>1001 ± 180</td>
</tr>
<tr>
<td>RRRASASAA</td>
<td>1500 ± 500</td>
<td>1.34 ± 0.4</td>
</tr>
<tr>
<td>RRRAEASAA</td>
<td>1700 ± 310</td>
<td>0.50 ± 0.12</td>
</tr>
</tbody>
</table>

To a serine or threonine, while rhodopsin kinase prefers peptides containing acidic residues on the carboxyl-terminal side of a serine or threonine (33). Two of the best peptide substrates for βARK, RRRDDDDDSAAA and RRREEESSG, were not phosphorylated by GRK5 when tested at a concentration of 1 µM. Similarly, the best identified peptide substrates for rhodopsin kinase, RRRAAASEEE and RRREEESSEE, were also not substrates for GRK5. Of all of the peptide substrates tested, RRRASASAA, a poor substrate for βARK (29), was found to be the best substrate for GRK5 (Fig. 4). However, this peptide was a very poor substrate ($K_m$ of ~1.5 µM, $V_{max}$ of ~1.3 nmol/min/mg) compared to the remaining receptors tested with an ~100,000-fold lower $V_{max}/K_m$ ratio (Table II). When the first serine in the peptide RRRASASAA was changed to an acidic residue (either glutamic acid or phosphoserine), the resulting peptides were poorer substrates (Fig. 4). This was also reflected in an ~3-fold decreased $V_{max}/K_m$ ratio for RRRAEASAA phosphorylation by GRK5 ($K_m$ of ~1.7 µM, $V_{max}$ of ~0.5 nmol/min/mg) compared to RRRASASAA (Table II). This may be due to the loss of the first serine in the peptide or due to the creation of an acidic environment near the remaining serine. The peptide RRRAAASASSA was also a poorer substrate compared to RRRASASAA (Fig. 4). This might suggest that the second serine in these peptides prefers being nearer the basic arginine residues at the amino terminus of the peptide. However, RRASLG (Kemptide), a basic peptide substrate for the cAMP-dependent protein kinase, was a poor substrate for GRK5 (Fig. 4). While these studies do not delineate any consensus sequence for GRK5 phosphorylation, they do suggest that GRK5, in contrast to βARK and rhodopsin kinase, does not phosphorylate serine residues in an acidic environment.

In an attempt to further characterize the substrate specificity of GRK5, a number of general protein kinase substrates such as histones, casein, and phosphotylin were studied. As shown in Fig. 5, GRK5 is able to phosphorylate the acidic proteins casein and phosphotylin. In contrast, GRK5 does not phosphorylate the basic protein histones (data not shown). The level of phosphorylation of casein and phosphotylin by GRK5 is comparable to that observed for several of the peptide substrates and again is significantly lower than the receptor phosphorylation. Similar results have previously been obtained for βARK (34). The ability of polyanions to modulate the activity of βARK and rhodopsin kinase has been studied previously (18, 34, 35). Similar studies performed with GRK5 demonstrate that heparin and dextran sulfate are very potent inhibitors of GRK5 with IC₅₀ values of ~1 and ~0.6 mM, respectively (Table III). Heparin and dextran sulfate are also the most potent inhibitors of βARK with IC₅₀ values of 150-2800 nm (18, 34); however, these compounds are at least 150-fold more potent at inhibiting GRK5 as compared to βARK. Heparin is also a potent inhibitor of casein kinase II (IC₅₀ of ~20-60 nm) (36), while it is a weak inhibitor of rhodopsin kinase (IC₅₀ of ~200 µM) (35). Comparable results were obtained when polyglutamic and polyaspartic acid were tested as inhibitors. Polyglutamic and polyaspartic acids were potent inhibitors of GRK5 (IC₅₀ values of ~25 nm and ~80 nm, respectively) (Table III), weaker inhibitors of βARK (IC₅₀ values of ~2000 and ~1300 nm) (34), and poor inhibitors of rhodopsin kinase (IC₅₀ values of ~700 µM and ~400 µM) (35). These results seem to be consistent with our analysis of the
Purification and Characterization of GRK5

Inhibition of GRK5 by protein kinase modulators

Phosphorylation reactions containing 3–5 μM rhodopsin and 15–25 μM GRK5 in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, and 0.1 mM (γ-32P)ATP (800 cpm/pmol) in the presence of increasing concentrations of various inhibitors were incubated at 30 °C for 3 min. The reactions were stopped by the addition of 10 μL of SDS sample buffer and then electrophoresed on a 10% SDS-polyacrylamide gel. The IC₅₀ values are presented as the means ± S.E. from 2–5 independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ for GRK5</th>
<th>IC₅₀ for βARK*</th>
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</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>1.05 ± 0.06 mM</td>
<td>150–1400 nm</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>0.58 ± 0.02 mM</td>
<td>150–2800 nm</td>
</tr>
<tr>
<td>Polysaccharic acid</td>
<td>80.0 ± 8.8 nm</td>
<td>1300 nm</td>
</tr>
<tr>
<td>Polyglutamic acid</td>
<td>25.0 ± 3.0 nm</td>
<td>2000 nm</td>
</tr>
<tr>
<td>H-7</td>
<td>170 ± 5 μM</td>
<td>300 ± 42 μM</td>
</tr>
<tr>
<td>NaCl</td>
<td>58 ± 1 μM</td>
<td>79 ± 12 μM</td>
</tr>
</tbody>
</table>

* Values were taken from Refs. 18 and 34.

Table III

Fig. 4. Peptide phosphorylation by GRK5. Phosphorylation reactions containing 1 mM peptide, pH 7.0, 25 nM GRK5, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, and 0.1 mM (γ-32P)ATP (800 cpm/pmol) in a total reaction volume of 20 μL were incubated at 30 °C. At 30 and 60 min, reactions were stopped by spotting the sample on P81 paper and washing six times with 78 mM phosphoric acid. The peptides used were: α, RRRASASAA; β, RRRAEASAA; γ, RRRApASAA; δ, RRRApASAA; ε, RRRApAASAA; δ, LLRASLG (Kemptide). At the 60-min time point, the stoichiometries of phosphorylation ranged from 0.7 x 10⁻⁴ for LLRASLG to 5 x 10⁻⁶ mol/mol for RRRApASAA. Under these conditions, no phosphorylation of the peptides RRDDDASAAA, DDRRSSAASA, RRRAAAAASEEE, RRREEESGGG, and RRREEESEEE was detected.

Fig. 5. Phosphorylation of casein and phospho-vitamin by GRK5. Phosphorylation reactions were performed as described under "Experimental Procedures" using 25 μg of casein (α) or phospho-vitamin (β) as the substrate and 20 nM GRK5 in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, and 0.1 mM (γ-32P)ATP (800 cpm/pmol) in a total reaction volume of 20 μL. Reactions were incubated at 30 °C and were stopped at the indicated times by the addition of 10 μL of SDS sample buffer. Samples were electrophoresed on a 10% polyacrylamide gel and autoradiographed, and the 32P incorporation was determined. At the 60-min time point, the stoichiometries of phosphorylation were 6 x 10⁻⁴ mol/mol for casein and 7.4 x 10⁻⁴ mol/mol for phospho-vitamin. Under similar conditions, no phosphorylation of histones was detected.

Peptide substrates, i.e. while βARK and rhodopsin kinase preferentially phosphorylate serine residues in an acidic environment, GRK5 appears to be inhibited by an acidic environment. Two other compounds were also tested for their ability to inhibit GRK5. H-7, a potent inhibitor of protein kinase C and the cAMP- and cGMP-dependent protein kinases, was found to be a weak inhibitor of GRK5 (IC₅₀ of ~170 μM) (Table III). Similarly, NaCl also appears to inhibit the activity of GRK5 with an IC₅₀ of ~60 mM, similar to the inhibition of βARK by NaCl (18, 31).

Since polyanions were potent inhibitors of GRK5, the effect of polycations on GRK5 activity was also studied. While polycations such as spermine, spermidine, and polysine are weak inhibitors of βARK (34), they are activators of rhodopsin kinase (35) and casein kinase II (36, 37). While tested for their ability to modulate the activity of GRK5, spermine, spermidine, and polysine were able to stimulate GRK5 (Fig. 6A). Spermine activated the activity of GRK5 to phosphorylate rhodopsin ~1.5-fold at an optimum concentration of 1 mM, while spermidine activated GRK5 ~1.8-fold at an optimum concentration of 0.1 mM. This activation is similar to that observed for casein kinase II at the same physiological concentrations of these compounds (37). Polysine was found to be the most potent activator of GRK5 resulting in an overall ~2.6-fold activation at an optimum concentration of 10 μM (Fig. 6A). In comparison, 0.1 μM pollysine results in an ~2-fold activation of casein kinase II (36), while 2 mM polysine promotes only an ~0.4-fold activation of rhodopsin kinase (35). Although polycations are weak inhibitors of βARK, at low concentrations they are also effective at reversing the ability of heparin to inhibit βARK (34). Similarly, low concentrations of the various polycations were also effective at reversing the heparin inhibition of GRK5 (Fig. 6B). At higher concentrations, the polycations activated GRK5 to a level similar to that observed in Fig. 6A while at still higher concentrations the polycations were inhibitory to GRK5 (Fig. 6B).

Discussion

G protein-coupled receptor kinases play an important role in regulating receptor function by their unique ability to specifically phosphorylate the activated form of various G protein-coupled receptors (5). While a significant number of studies have extensively characterized the properties of βARK and rhodopsin kinase, very little information is currently available on IT11, GRK5, and GRK6, the three most recently identified GRKs. To this end, this work focused on more extensive characterization of GRK5 utilizing the baculovirus expression system to overexpress and purify recombinant human GRK5. Purified GRK5 exhibited several properties that were similar to many other protein kinases including a preferential requirement for Mg²⁺ as the divalent cation, a pH optimum of 5.5–7.5, and a Kₘ for ATP of ~24 μM.

One of the crucial features of GRKs is their ability to specifically phosphorylate different G protein-coupled receptors in an agonist-dependent manner. In the past few years, the β₂AR and the m2 mACHR have been overexpressed in the baculovirus expression system, purified and reconstituted in phospholipid vesicles, and shown to be functionally active. The availability of such purified and reconstituted G protein-coupled receptors greatly facilitates detailed studies of the specificities of the GRKs. Using these purified receptors, βARK has been shown to phosphorylate both the agonist-occupied β₂AR and m2 mACHR in vitro (18, 19). Moreover, this phosphorylation is generally en-
phoresed on a 10% polyacrylamide gel, and, following autoradiography, the addition of EDTA, 5
S2P
Reactions were incubated at polylysine
reaction volume of 20
containing enhanced in the presence of G protein
and rhodopsin kinase compared to the β2AR and rhodopsin. A number of peptides tested here were also found to be substrates for GRK5. However, in contrast to βARK and rhodopsin kinase, GRK5 is a stronger inhibitor of rhodopsin kinase that do not contain acidic amino acids in the vicinity of a serine. The kinetic parameters for the peptides tested indicate that the presence of acidic residues predominantly decreases the rate of phosphorylation rather than the affinity of GRK5 for these peptides. The peptide studies contrast with the results obtained using several general protein kinase substrates. The acidic proteins casein and phosvitin were found to be weakly phosphorylated by GRK5 while the basic histones were not phosphorylated by GRK5. The results with these proteins are, in fact, very similar to those obtained for βARK (34). Overall, the results suggest that GRK5 has a complex substrate specificity that is not defined simply by the presence of acidic or basic residues in the vicinity of a serine or threonine.

A number of protein kinases such as casein kinase II, βARK, and rhodopsin kinase have previously been shown to be modulated by polyions (18, 34–37). The modulation of GRK5 activity by polyions reveals several interesting differences compared to βARK and rhodopsin kinase. Although heparin has been identified as the most potent inhibitor of βARK with an IC50 of 150–1400 nm (34), heparin is >150-fold more potent at inhibiting GRK5 (IC50 of ~1 nm). Heparin is also a potent inhibitor of casein kinase II (IC50 of ~20–60 nm) (36); however, it is only a weak inhibitor of rhodopsin kinase (IC50 of ~200 μM) (35). These results demonstrate that heparin should not be used as a “specific” inhibitor of βARK (7). Several other polyions such as polyaspartic and polyglutamic acid were also found to be more potent inhibitors (16–80-fold) of GRK5 as compared to βARK. These studies demonstrate a tremendous variance (~200,000-fold) in the ability of polyions to inhibit different members of the GRK family and suggest that specific inhibitors of these kinases could be engineered.

Previous peptide phosphorylation studies have revealed that βARK preferentially phosphorylates Ser/Thr residues in an acidic environment (either with acidic amino acids or a phosphoserine amino-terminal to the Ser/Thr), while rhodopsin kinase phosphorylates peptides with acidic amino acids carboxy-terminal to the Ser/Thr (29, 33). In general, peptides are very poor substrates for βARK and rhodopsin kinase compared to the β2AR and rhodopsin. A number of peptides tested here were also found to be substrates for GRK5. However, in contrast to βARK and rhodopsin kinase, GRK5 is a stronger inhibitor of rhodopsin kinase that do not contain acidic amino acids in the vicinity of a serine. The kinetic parameters for the peptides tested indicate that the presence of acidic residues predominantly decreases the rate of phosphorylation rather than the affinity of GRK5 for these peptides. The peptide studies contrast with the results obtained using several general protein kinase substrates. The acidic proteins casein and phosvitin were found to be weakly phosphorylated by GRK5 while the basic histones were not phosphorylated by GRK5. The results with these proteins are, in fact, very similar to those obtained for βARK (34). Overall, the results suggest that GRK5 has a complex substrate specificity that is not defined simply by the presence of acidic or basic residues in the vicinity of a serine or threonine.

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to heparin and thereby preventing its inhibition (34). A similar phenomenon was observed for GRK5, where spermine, spermidine, and polylysine effectively reversed the ability of heparin to inhibit GRK5. It is thus conceivable that in vivo polyamines or polycationic surfaces may play a role as physiological modulators of GRK5.

In conclusion, these studies have demonstrated that while GRK5 has the ability of polyanions to serve as potent inhibitors, it also has several unique properties compared to βARK and rhodopsin kinase. These include its preference for phosphorylating nonacidic peptides as well as the ability of polyamines to serve as potent inhibitors. Future studies will involve more rigorously addressing the substrate specificity of GRK5 as well as potential modes of regulation. The identification of heparin and dextran sulfate as extremely potent inhibitors of GRK5 should prove useful in further elucidating the role of GRK5 in the regulation of G protein-coupled receptors.

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REFERENCES