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Peptide inhibitors of G protein-coupled receptor kinases

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Abstract

G protein-coupled receptor kinases (GRKs) are regulatory enzymes involved in the modulation of seven-transmembrane-helix receptors. In order to develop specific inhibitors for these kinases, we synthesized and investigated peptide inhibitors derived from the sequence of the first intracellular loop of the β_2-adrenergic receptor. Introduction of changes in the sequence and truncation of N- and C-terminal amino acids increased the inhibitory potency by a factor of 40. These inhibitors not only inhibited the prototypical GRK2 but also GRK3 and GRK5. In contrast there was no inhibition of protein kinase C and protein kinase A even at the highest concentration tested. The peptide with the sequence AKFERLQTVTNYFITSE inhibited GRK2 with an IC_{50} of 0.6 μM, GRK3 with 2.6 μM and GRK5 with 1.6 μM. The peptide inhibitors were non-competitive for receptor and ATP. These findings demonstrate that specific peptides can inhibit GRKs in the submicromolar range and suggest that a further decrease in size is possible without losing the inhibitory potency.
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Keywords: G-protein-coupled receptor kinases; Receptor desensitization; Phosphorylation; Non-competitive inhibition

1. Introduction

Exposure of G protein-coupled receptors (GPCRs) to agonists activates catalytic cascades of intracellular mediators which greatly amplify the response to an extracellular stimulus. The same event often triggers counter-regulatory pathways which attenuate receptor signalling [1]. This phenomenon referred to as desensitization is thought to adapt responsiveness of the cell to continuous or to successive multiple stimuli. Over the past years it has become clear that desensitization is a multistep process [1]. First, phosphorylation initiates uncoupling of the receptor from the G proteins. Second, the receptor is sequestered and removed from the cell surface. While this prevents further access of the ligand to the receptor or of the receptor to the G-protein, it appears that this step is also responsible for the resensitization of recycled receptors [2–5]. Third, upon chronic stimulation by agonist the receptors are downregulated by several mechanisms on the transcriptional, posttranscriptional and protein levels [6,7]. Even though these processes are important for the physiological control of homeostasis, they may also contribute to pathological conditions and may limit the effectiveness of therapeutic agonists [8,9].

Two protein kinase classes are involved in the rapid step of receptor phosphorylation: second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs). The latter class is an especially interesting target for new drugs, inhibitors or activators. The GRKs are a small family of serine/threonine protein kinases whose best characterized function is to phosphorylate activated GPCRs. Seven members have been cloned and are termed GRK1–GRK7 [10]. By phosphorylation of activated GPCRs they facilitate binding of members of another protein family, the arrestins, which thereby block signalling to the G protein [11,12]. Arrestins also act as adaptors for the localization of GPCRs to clathrin-coated pits and are therefore essential for sequestration [1] and mediate a variety of non-classical signals [13]. Thus, the proteins which lead to desensitization of GPCRs also initiate their resensitization.

Abbreviations: $G_{\beta\gamma}$, βγ subunits of heterotrimeric GTP-binding proteins; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; IBMX, 3-isobutyl-1-methylxanthine; PKA, protein kinase A; PKC, protein kinase C; ROS, rod outer segments

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In a number of diseases, GRK activity and/or mRNA levels are elevated, for example in heart failure [14–16], myocardial ischemia [17] and in hypertension [18,19]. In these diseases a GRK-specific inhibitor might be of interest. This hypothesis is supported by reports on the restoration of β-adrenergic signalling in several heart failure models via adenoviral-mediated gene transfer of the GRK2 C-terminus which acts as a GRK2 inhibitor [20,21].

Few inhibitors of the G protein-coupled receptor kinases have been identified [22]. Polyanions such as heparin and dextran sulphate are still the most potent inhibitors of the GRKs (in the nanomolar range, see [23]); however, they inhibit a broad spectrum of other enzymes and are quite unspecific because their inhibitory potency is proportional to the number of negative charges on the molecule. Palczewski et al. [24] showed that adenosine analogs can compete very efficiently with ATP for the binding pocket of GRK1. The clinically more relevant GRK2, however, is inhibited only weakly. More recently, first attempts have been reported to design competitive inhibitors of ATP [25]. However, these substances must possess high affinity to their substrates to overcome the high intracellular ATP concentration, and they have the inherent problem of cross-inhibition of many other kinases reducing their usefulness as specific tools.

A third class of GRK inhibitors are peptides derived from the first intracellular loop of the β-adrenergic receptor. These receptor-derived peptides appeared to be a promising starting point for the development of GRK inhibitors. The main problem concerning these peptides is their low affinity; the most potent inhibitor of GRKs identified so far has an IC_{50}-value of ~40 μM [26]. Furthermore, the solubility of these inhibitors was modest. Therefore we set out to improve the affinity of these peptide inhibitors and to test their specificity versus other kinases relevant to GPCRs and also within the GRK family.

2. Materials and methods

2.1. Materials

SP-Sepharose and Heparin-Sepharose were purchased from GE Healthcare, Freiburg. [γ^{32}-P]ATP was obtained from Amersham Life Sciences, Braunschweig. Amino acids and resins were purchased from Nova-Biochem, 1-hydroxybenzotriazole and *N*,*N*-diisopropylcarbodiimide from Fluka, Deisenhofen. All other chemicals were obtained from Merck or Sigma–Aldrich, Deisenhofen.

2.2. Protein purification

G-protein βγ-subunits were purified from bovine brain according to Sternweis and Robishaw [27]. Bovine GRK2, bovine GRK3 and human GRK5 were expressed in Sf9 cells and purified according to [28,29], respectively. The concentrations of these proteins were determined with the Biorad protein assay as described by the manufacturer (Biorad, Munich). Rod outer segments (ROS) were prepared according to Wilden and Kühn [30]. Rhodopsin kinase-free membranes were obtained by urea-treatment as described [31]. Urea-treated ROS showed negligible endogenous kinase activity or functional G-protein βγ subunits and consisted of over 95% rhodopsin.

2.3. Peptide synthesis

The peptides were synthesized using 9-fluorenylmethoxycarbonyl chemistry on a Zinsser Analytics SMPS A multiple peptide synthesizer using *N*,*N*-diisopropylcarbodiimide/1-hydroxybenzotriazole activation with a 10-fold excess of amino acids. Coupling and deprotection times were 60 and 25 min, respectively. We used Wang-resins preloaded with the first amino acid for the peptide acids and Rink-resins for the peptide-amides. Side chain protection was *tert*-butyl for Ser, Thr, Tyr and Glu, *N*-*tert*-butoxy-carbonyl for Lys, pentamethylchroman-6-sulfonyl for Arg and S-trityl for Gln and Asn. The peptides were cleaved from the resin by a 2 h treatment with reagent K (82.5% TFA, 5% thianisole, 5% phenole, 5% water, 2.5% ethanedithiol) and then precipitated with ether using standard procedures. The peptides were lyophilised from *tert*-butylalcohol/water 4:1. The purity and identity was checked by reversed phase-HPLC and electrospray mass spectrometry.

2.4. Solubilization of peptides

The peptides (Table 1) were dissolved in 100% DMSO to a concentration of 10 mM. Shortly before the assay the stock solutions of the peptides were diluted with DMSO in a way that 1 μl of the dilution in a 100 μl assay volume gave the desired concentration. All controls and samples of one set of experiments contained the same amount of DMSO (1%) and it was verified that 1% DMSO did not inhibit the kinases by itself.

2.5. Phosphorylation of rhodopsin

To obtain the optimal phosphorylation conditions (signal/noise ratio, linear kinetics) the activity was tested as described previously [32]. Briefly, ROS (0.6 μM or as indicated) were incubated with GRK2 (5–40 nM) and βγ-subunits of heterotrimeric G proteins ($G_{\beta\gamma}$) (120 nM) in a buffer containing 20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 8 mM $MgCl_2$, 30–100 μM [γ^{32}-P] ATP (0.2–0.5 cpm/fmol) at different temperatures for 5–60 min. The reaction was stopped with ice-cold buffer (25 mM Tris pH 7.4, 15 mM EDTA, 15 mM EGTA). After centrifugation for 15 min at 20,000 × *g* the supernatant was removed and discarded, the pellet was resuspended with 15–30 μl SDS-sample buffer (0.1 M Tris pH 6.8, 2% SDS,

Table 1
Sequences of the peptides used in this study

	Peptide	Sequence	IC_{50} (μM) for GRK2
Previously published peptides [26]	56–74	TAIAKFERLQTVTNYFITS	40
	57–71	AKFERLQTVTNYF	62
	59–69	AKFERLQTVTN	1600
	60–66	KFERLQT	2600
Acylated and amidated peptides	Ac56–74NH_2	Ac-TAIAKFERLQTVTNYFITS-$CONH_2$	–
	Ac57–71NH_2	Ac-AKFERLQTVTNYF-$CONH_2$	–
	Ac59–69NH_2	Ac-AKFERLQTVTN-$CONH_2$	–
Modified full-length peptides	K56–74K	KTAIAKFERLQTVTNYFITSK	37
	E56–74K	ETAIAKFERLQTVTNYFITSK	52
	K56–74E	KTAIAKFERLQTVTNYFITSE	4
Modified and truncated peptides	K56–69	KTAIAKFERLQTVTN	6
	59–74K	AKFERLQTVTNYFITSK	32
	59–74E	AKFERLQTVTNYFITSE	0.6

The top four peptides characterized as GRK2 inhibitors by Benovic et al. [26] are given as reference compounds. The peptides are derived from the first intracellular loop of the hamster β_2-adrenergic receptor. The numbers denoting the peptides correspond to the first and last position of the corresponding protein sequence.

20% glycerol, 0.002% bromophenolblue), and the sample electrophoresed on 12.5% SDS polyacrylamide gels. The gels were dried and analyzed with a phosphorimager.

To determine the dependence of GRK2 on the substrates rhodopsin and ATP, either ROS (0.5–20 μM) or ATP (5–170 μM) were varied and the assays carried out as described above using 7.2 nM GRK2 and 60 nM $G_{\beta\gamma}$.

2.6. Phosphorylation of synthetic peptide

The phosphorylation of a soluble synthetic peptide (RRREEEEESAAA) was determined according to [33]. A 5 mM stock solution of the purified synthetic peptide was prepared, and the pH adjusted to 7.4 by the addition of Tris base. The peptides were incubated with GRK2 (100 nM) in a buffer containing 20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 0.1 mM [γ^{32}-P] ATP (~0.4 cpm/fmol) and 10 mM $MgCl_2$ in a final volume of 50 μl at 30 °C. The inhibitor was added in the indicated amount. The reaction was stopped after 45 min by transferring the reaction mixture to a 2 cm × 2 cm square of P81 paper. After six washes in 75 mM phosphoric acid (10 ml/sq) the incorporated radioactivity was measured by Cerenkov counting.

2.7. Histone phosphorylation by second messenger-dependent kinases

Phosphorylation of histone type III-SS (Sigma) by protein kinase Cδ (PKC) expressed in Sf9 cells (provided by H. Mischak, Hanover; described in [34]) and by the catalytic subunit of protein kinase A purified from bovine heart according to Sugden et al. [35] were determined. This was done by incubating 0.24 μg of the PKC preparation or 6 ng of purified PKA with the substrate for 10 min at 30 °C in a buffer containing 20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 8 mM $MgCl_2$ and 100 μM [γ^{32}-P] ATP (~0.2 cpm/fmol). To activate the PKC, 500 nM phorbol 12-myristate 13-acetate and 85 ng lipid vesicles were added. The vesicles were prepared from L-α-phosphatidylcholine type II-S [36]. Inhibitors were added in the indicated concentrations. The reactions were stopped by adding SDS-sample buffer followed by SDS polyacrylamide gel electrophoresis. The gels were dried and analyzed with a phosphorimager.

2.8. cAMP accumulation in permeabilized A431 cells

A431 cells were permeabilized with digitonin by a procedure described earlier [37]. In brief, 2×10^5 cells/well were incubated in Dulbecco's modified Eagle's medium supplemented with the phosphodiesterase inhibitor IBMX (100 μM) for 30 min. The cells were then washed with phosphate-buffered saline, followed by two washes with 150 mM potassium glutamate, pH 7.1, 5 mM EGTA, 7 mM $MgCl_2$, 5 mM glucose, 2 mM ATP, and 100 μM IBMX. 1 ml of this buffer was added per well and the cells were permeabilized by stepwise addition of digitonin to a final concentration of ~0.015%. Verification of the degree of permeabilization was done by trypan blue staining. The tested inhibitors were added immediately after the permeabilization in the indicated concentrations. (−)-Isoproterenol or vehicle was added 10 min later. After 20 s the incubation was stopped by addition of 2 ml of boiling water. The cells were then scraped off the plates and separated from soluble material by centrifugation at 5000 × *g* for 10 min.

The cAMP content in the supernatant was determined by radioimmunoassay according to Harper and Brooker [38]. This was done using a rabbit antiserum raised against cAMP coupled to bovine serum albumin via a succinyl moiety in the 2′-position. Samples and standards were 2′-*O*-acetylated with acetic anhydride and then measured using ^{125}I-cAMP-2′-*O*-succinyltyrosylmethylester (DuPont NEN) as the radioligand.

3. Results

To define the sites of interaction between GRK2 and its receptor substrate Benovic et al. [26] had characterized peptides of the intra- and extracellular loops of the hamster β_2-adrenergic receptor. While the carboxyl terminus acted as a substrate for GRK2, all other loops inhibited receptor phosphorylation by the kinase. Truncation of the most potent inhibitory peptide, the first intracellular loop (amino acids 56–74), from both ends had resulted in a reduction of the inhibition in all cases (Table 1).

We wondered whether removal of non-physiological charges at the ends of the peptides would increase the interaction of peptide and kinase. N-terminally acetylated and C-terminally amidated derivatives of the peptides 56–74, 57–71 and 59–69 were synthesized (Table 1). However, all three peptides were not soluble in significant quantities in any physiological buffer, preventing the testing of this hypothesis.

Therefore, we tested the opposite modification, i.e. an increase in charges by introducing an additional glutamate or lysine in the first and last positions (Table 1). Under our experimental conditions we obtained a signal/noise ratio of about 10:1 for the phosphorylation reaction, with the phosphorylation of rhodopsin in the presence of 30 μM heparin (as the ''reference'' GRK inhibitor [23]) accounting for background. Great care was taken to remain in the linear range of the phosphorylation reaction. The results for the inhibition of GRK2 by the first generation of modified peptides are shown in Fig. 1A. In comparison to the prototype of GRK peptide inhibitors (56–74 with an IC_{50}-value of 40 μM) our newly synthesized ones were of similar or higher potency (Table 2). In particular, the peptide K56–74E showed a significant (8-fold) increase in potency with an IC_{50}-value of 4.4 μM.

A comparison with the peptide inhibitors of GRK2 described earlier (Table 1) suggested that the sequence AKFERLQTVTN defines a kind of inactive core region of the peptide. Only two additional amino acids at the N- and C-terminus decrease the IC_{50}-value by a factor of 26. Therefore we next shortened the peptides at either the N- or the C-terminus up to the inactive core, in order to define which of the two ends of the peptide conferred most of the inhibitory activity. In addition, since K56–74E was more potent than K56–74K we wanted to characterize the influence of the C-terminal glutamate. Three new peptides: K56–69, 59–74E and 59–74K were synthesized and tested for their inhibitory activities towards GRK2. To our surprise the removal of either N- or C-terminus increased the potency (Fig. 1B and Table 1). K56–69 (IC_{50}-value of 5.6 μM) and 59–74E (IC_{50}-value of 0.6 μM) had both a significantly lower IC_{50}-value than the longer peptides from which they were formally derived. A typical autoradiogram for inhibition of rhodopsin phosphorylation by 59–74E is shown in Fig. 1C.

Since there are several isoforms of GRKs that appear to be involved in receptor phosphorylation, we tested whether other GRKs were also inhibited by these peptides. As further representatives of this kinase family we used recombinant GRK3 and GRK5. GRK3 is closely related to GRK2 (84.9% identity in amino acid sequence) and

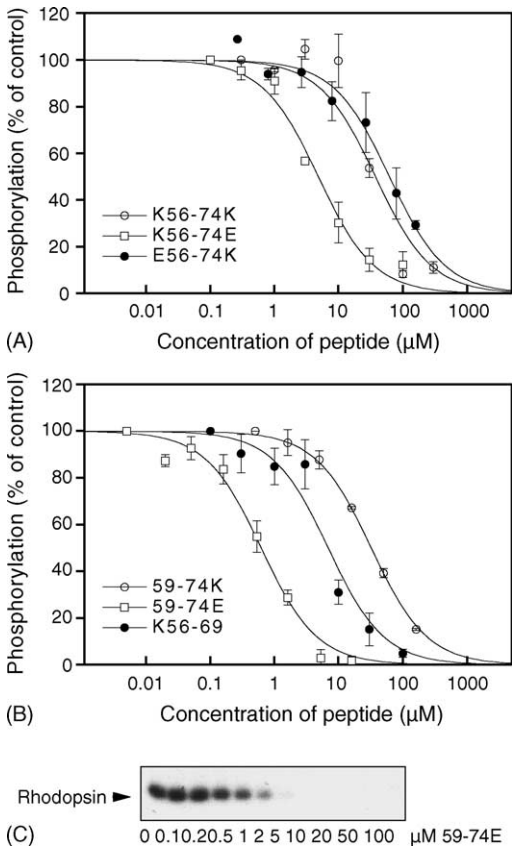

Fig. 1. Inhibition of GRK2 by the modified and truncated peptides. Urea-treated rod outer segments (30 pmol rhodopsin) were phosphorylated by GRK2 (2 pmol) in the presence of the cofactor $G_{\beta\gamma}$ (6 pmol) and increasing concentrations of the peptides as described under Section 2. Data are mean ± S.E.M. of three to four independent experiments. (A) Inhibition by the lysine and glutamate modified peptides K56–74K (open circles), K56–74E (open squares) and E56–74K (solid circles). (B) Concentration–response curves for the N- and C-terminally truncated peptides 59–74K (open circles), 59–74E (open squares) and K56–69 (solid circles). (C) Representative autoradiogram of the inhibition of GRK2-mediated rhodopsin phosphorylation by the peptide 59–74E.

Table 2
IC_{50}-value of peptide inhibitors for G protein-coupled receptor kinases

Peptide	GRK2 IC_{50} (μM)	GRK3 IC_{50} (μM)	GRK5 IC_{50} (μM)
K56–74K	36.5 (17.8–75.0)	30.4 (15.0–61.6)	61.3 (20.1–187.2)
K56–74E	4.4 (2.0–9.7)	6.1 (3.0–12.3)	3.9 (2.0–7.7)
E56–74K	52.2 (19.0–143.9)	22.6 (11.2–45.6)	7.4 (5.7–9.6)
K56–69	5.6 (2.3–14.0)	11.6 (6.8–19.9)	2.1 (0.4–10.9)
59–74K	32.2 (28.7–36.2)	55.5 (26.7–115.4)	9.4 (4.0–22.2)
59–74E	0.6 (0.2–1.7)	1.5 (0.5–4.7)	1.6 (0.9–2.6)

Phosphorylation of rhodopsin by GRK2, GRK3 and GRK5 in the presence of increasing concentrations of inhibitor was assessed as described under Section 2. The IC_{50}-values were estimated by non-linear curve-fitting and are presented as the mean, with the 95% confidence intervals shown in parenthesis. Experiments were performed three to six times.

shows very similar regulation and substrate specificity. GRK5 belongs to the GRK4 family of GRKs. It has many features which distinguish it from GRK2 and GRK3 [10] and can, because of its wide-spread tissue distribution, be regarded as the prototype of this family.

The results for all combinations are shown in Table 2. While differences in the IC_{50}-values of several peptides were observed between the three kinases, the selectivity was always below 10-fold. For all three GRKs, 59–74E showed the lowest IC_{50}-value of all tested peptides.

Since 59–74E was the most potent inhibitor, the mechanism of GRK2 inhibition by this peptide was further investigated. The substrate dependence of the GRK2-mediated phosphorylation in the absence and presence (1 μM) of 59–74E is shown in Fig. 2A. As expected the peptide was a non-competitive inhibitor with respect to ATP. The K_m-value of ATP was not significantly affected (15.3 ± 2.4 in the absence of peptide, 14.4 ± 0.5 μM in its presence) and agreed fairly well with previously published values (61 μM [28] and 22 μM [32]). To our surprise the peptide also behaved as a non-competitive inhibitor with respect to rhodopsin (Fig. 2B). The K_m-values for rhodopsin were 10.8 ± 2.5 and 6.8 ± 1.3 μM in the absence and presence of 59–74E, respectively. In order to assess whether this non-competitive type of inhibition was a general feature of the peptide inhibitors, similar

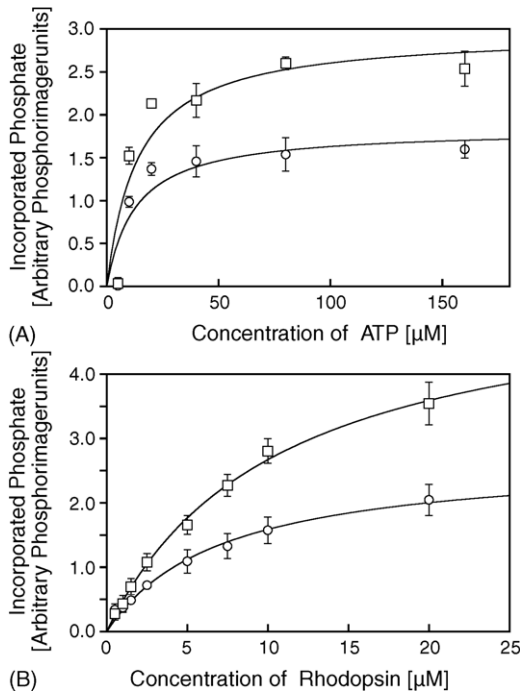

Fig. 2. Characterization of GRK2 inhibition by the peptide 59–74E. (A) ATP was varied in the assay from 2.5 to 160 μM. The substrate phosphorylation was tested in the absence (squares) and presence (circles) of 1 μM of the inhibitor peptide as described under Section 2. The incorporated phosphate is expressed in arbitrary phosphorimager units. (B) Rhodopsin was varied in the assay from 0.5 to 20 μM. The peptide concentration was again 0 μM (squares) and 1 μM (circles). The other conditions were the same as in (A). Data are mean ± S.E.M. of three experiments.

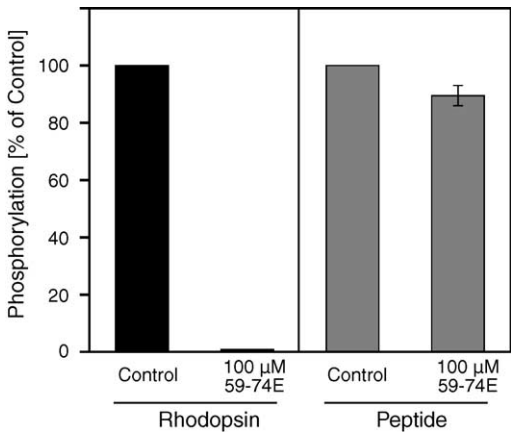

Fig. 3. Effects of the inhibitor peptide 59–74E on GRK2-mediated phosphorylation of membrane bound rhodopsin (left) and the soluble peptide substrate RRREEEEESAAA (right). Both substrates were phosphorylated in the absence or presence of 100 μM of the inhibitor peptide. The assays were carried out under optimized conditions as described under Section 2. Data are mean ± S.E.M. of 4 or 12 experiments, respectively.

experiments were also performed with the parent peptide 56–74. These revealed a non-competitive inhibition as well (data not shown).

The phosphorylation of a soluble peptide substrate by GRK2 was examined to discriminate if 59–74E interfered with the catalytic site or the recognition of the receptor substrate. Phosphorylation of the soluble peptide RRREEEEESAAA was only marginally inhibited by the peptide 59–74E (Fig. 3) even at concentrations 100-fold above the IC_{50}-value for the receptor substrate. This further supports our proposal that the inhibitor peptide competes neither with the binding of ATP nor with that of the substrate; it also shows that the peptide does not reduce the number of active GRK2 in a non-specific manner.

To find out how specifically these peptides inhibited GRK activity, we tested their effects on the activity of two prototypical second messenger-dependent kinases, PKC and PKA. Typical inhibitors of these kinases (staurosporine and protein kinase inhibitor (PKI), respectively) blocked the activity as expected (Fig. 4). However, we found no significant inhibition of either PKA or PKC with the most potent peptide 59–74E at concentrations up to 100 times its IC_{50}-value for GRKs.

Receptor phosphorylation is a prerequisite for desensitization. In order to test the ability of the GRK inhibitor peptides to inhibit agonist-induced receptor desensitization, we employed a permeabilized cell model described earlier [37,39]. For these experiments we used the human A431 cell line which expresses endogenous β_2-adrenergic receptors. The cells were permeabilized with digitonin, and then the β_2-adrenergic receptors were stimulated for 20 s with 10 μM (−)-isoproterenol (or not) in the absence or presence of 100 μM heparin or 50 μM of the peptide K56–74E. Since GRK-mediated desensitization in this model proceeds with a $t_{1/2}$ of less than 15 s we expected that inhibition of GRKs should result in greater accumulation

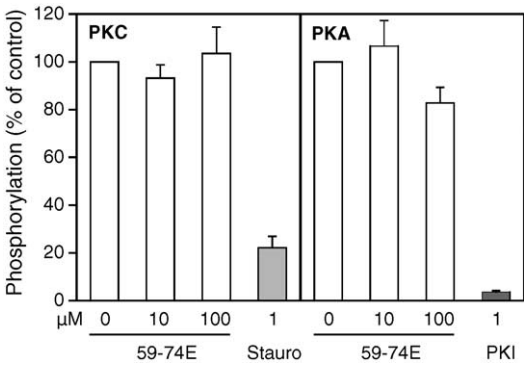

Fig. 4. 59–74E does not inhibit second messenger dependent kinases. Phosphorylation of histone by PKCδ and PKA was determined. Peptide 59–74E was added in concentrations of 10 or 100 μM corresponding to 10 or 100 times the IC_{50} of this peptide for GRK2. As a control, 1 μM staurosporine or PKI were added to one sample of PKC or PKA, respectively. The phosphorylation of the samples without inhibitor was set to 100%. The columns are mean ± S.E.M. of three independent experiments.

of cAMP. Fig. 5 shows that this was indeed the case: isoproterenol alone caused a >10-fold increase in cAMP-levels, and this stimulation was increased almost 2-fold in the presence of either heparin or the peptide K56–74E at a concentration of ∼10-fold above their IC_{50}-value for GRK2. Neither compound had an effect on basal cAMP-levels, indicating that they acted by enhancing the action of β_2-adrenergic receptor stimulation. These data show that the peptide GRK-inhibitors are capable of inhibiting β_2-adrenergic receptor desensitization in a cellular context.

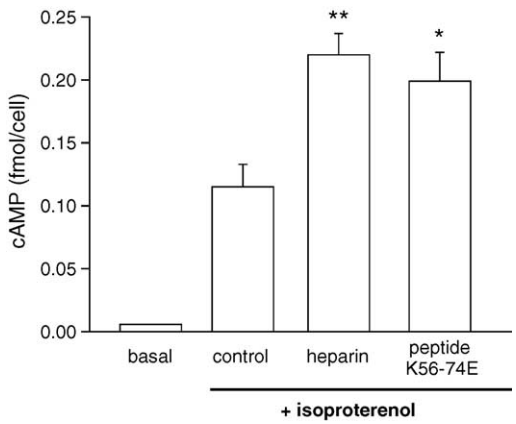

Fig. 5. Inhibition of β_2-adrenergic receptor desensitization by heparin and the peptide K56–74E. Permeabilized A431 cells were incubated with 100 nM heparin or 50 μM of the peptide K56–74E. cAMP levels were determined without isoproterenol (basal) or after stimulation of the cells for 20 s with 10 μM isoproterenol. The data are mean ± S.E.M. of six independent experiments with duplicate determinations. Statistical significance (ANOVA) is denoted as $^{**}p < 0.001$, $^{*}p < 0.01$ vs. isoproterenol alone. There was no statistically significant difference between (isoproterenol + -heparin) vs. (isoproterenol + peptide). Treatment of unstimulated, permeabilized cells with either heparin or peptide did not cause any significant changes in basal cAMP levels (data not shown).

4. Discussion

The role of GRKs in the signal transduction of eukaryotic cells becomes more and more complex as new substrates are found and the regulation of these kinases by cofactors and other kinases is investigated. In order to elucidate the many functions of these kinases specific inhibitors are urgently needed, because all currently known inhibitors have specific drawbacks: they suffer from either low affinity, poor selectivity or the inability to penetrate the cell membrane.

The polyanionic inhibitors like heparin and dextran sulphate show a wide range of activity from 1 nM (GRK5) to 200 μM (GRK1) [23,24,40–42] depending on the GRK. While meeting the criteria of high affinity for all GRKs except GRK1 (rhodopsin kinase) they lack selectivity – heparin inhibits e.g. casein kinase II and many other intra- and extracellular proteins – and membrane permeability. Studies of different polyanions and their inhibition mechanism indicate a correlation of activity and the number of sulphate groups in one molecule [26]. This excludes the modification of these inhibitors to gain cell permeability without losing the inhibition potential.

Many known protein kinase inhibitors compete with ATP for its binding site. Classical ones like H7 are ineffective versus GRKs while they cross-inhibit many other protein kinases [23]. Palczewski et al. found several nucleosides, nucleotides and isoquinolines that inhibit GRK1, sangivamycin (IC_{50} = 180 nM) being the most potent [24]. Inhibition of GRK2, however, was marginal (IC_{50}-value of sangivamycin: 67 μM) or non-existent. More recently, a number of other substances have been tested for their ability to inhibit GRK2. The IC_{50}-values obtained ranged from 30 to 45 μM [22] for well-known substances such as tamoxifen or chlorpromazine. However, these substances suffer from a lack of specificity. Two other substances which have been claimed to be specific GRK inhibitors are described in [25] (this substance has an IC_{50}-value of 126 μM) and [43] (this substance inhibits GRK2 activity by 30% at 30 μM). The selectivity profile of these substances is currently unknown.

In this study we report the characterization of new peptide inhibitors of G protein-coupled receptor kinases. The known inhibitor peptide derived from the first intracellular loop of the hamster β_2-adrenergic receptor was modified by (i) the addition of charged amino acids and (ii) truncation. The most active resulting peptide inhibited GRK2 with an IC_{50}-value of below 1 μM, which is an improvement by a factor of 40 compared to the starting peptide.

The inhibitors we describe here represent a third group of GRK inhibitors. They showed a distinct inhibition mechanism compared to heparin or the ATP analogues. While those are competitive for either substrate or ATP [23,24], the peptides described in this study showed a

non-competitive mechanism of GRK inhibition for both ATP and the receptor substrate. This was true for the most potent peptide 59–74E and the unmodified parent peptide 56–74 and seems to be a property of this inhibitor class. The non-competition towards ATP was expected because peptides derived from the first intracellular loops should not interfere with the ATP binding pocket [26]. A more surprising observation is that the peptides were non-competitive versus the receptor substrate. The easiest explanation for this behaviour is that there are multiple contact points between the kinases and the substrate receptor. This is also suggested by the ability of the kinases to distinguish between the active and inactive conformations of the receptors. The first intracellular receptor loop might constitute one such contact point, and the peptides might inhibit receptor phosphorylation by imitating this contact. This view is supported by the observation that the peptides inhibited receptor phosphorylation by GRKs but not phosphorylation of a small soluble substrate. The latter finding indicates that the peptides do not inhibit the catalytic activity of GRKs but interfere with binding to receptors. Alternatively, the peptides could compete with G-protein βγ subunits for activation of GRK2. However, GRK5 was inhibited by the peptides with similar potencies (Table 2), although it does not require G-protein βγ subunits for activation [29]. This suggests that an interference of the peptides with G-protein βγ subunit binding to GRKs is unlikely to be their mechanism of action.

Polar residues added at both ends of the most potent peptide inhibitor found previously [26], 56–74, resulted in a markedly increased solubility. In the case of a C-terminal glutamate there was, in addition, a ~10-fold increase in potency. A similar increase in potency, but only for GRK5, was seen with a C-terminal lysine, provided there was no lysine at the N-terminus. Similar conclusions can be drawn from the shorter peptide. Considering that the ''core'' region 59–69 is essentially inactive it is interesting to see that an extension at either side results in potent GRK inhibition. Again, a single lysine at the end resulted in highest potency for GRK5, while a C-terminal glutamate increased the potency for all three GRKs tested.

The peptide inhibitors were quite selective for GRKs compared to PKA or PKC: at concentrations 100-fold above their K_m-values for GRKs they caused no significant inhibition of PKA or PKC. This selectivity is compatible with the hypothesis developed above that the peptides act by interfering with the specific mode of receptor binding by GRKs. Among the different GRKs, the selectivity of the peptide inhibitors was modest. The only fairly consistent pattern was that the presence of a single lysine at the N- or more so at the C-terminus resulted in relatively high affinity for GRK5. This suggests that there is a potential for selective inhibition of GRK isoforms.

While a kinase inhibitor may be a useful biochemical tool, its applicability is obviously much wider if it can be shown to inhibit also a kinase-mediated biological process. Due to their size and high water solubility the GRK-inhibitors described here are unlikely to penetrate cell membranes. Therefore, we permeabilized cells to allow access of the peptides to the cell interior. This approach was previously used to inhibit GRK activity by low concentrations of heparin [37]. We could show that in this experimental paradigm, the most potent peptide had an effect similar to heparin, i.e. both substances increased β_2-adrenergic receptor stimulation of cAMP production to the same extent. This suggests that by inhibiting GRKs they can enhance signalling by a G-protein-coupled receptor. Thus, these kinase inhibitors may, for example, be able to produce enhancement of β-adrenergic receptor signalling in cardiomyocytes, an effect which has been achieved previously by overexpression of the GRK2 C-terminus via adenovirus-mediated gene transfer [20,21].

Taken together, we report here the development of peptide inhibitors for GRKs that are active already in submicromolar concentrations, have no relevant effects on the second-messenger kinases PKA and PKC, and show some selectivity between isoforms of GRKs. Further studies will have to address the question of membrane permeability to permit the study of in vivo effects of such compounds.

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