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SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 1001–1008
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Peptide inhibitors of G protein-coupled receptor kinases

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Received 11 May 2005; accepted 16 June 2005

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.

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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0006-2952/$ – see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.bcp.2005.06.015

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,