

Purification and functional characterization of β -adrenergic receptor kinase expressed in insect cells

Peter Söhlemann, Mirko Hekman, Claudia Buchen, John S. Elce and Martin J. Lohse

Laboratory of Molecular Biology, University of Munich, Max-Planck-Institute of Biochemistry, 8033 Martinsried, Germany

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The β -adrenergic receptor kinase mediates agonist-dependent phosphorylation of β -adrenergic receptors, which is thought to represent the first step of homologous desensitization. We have expressed bovine and human β ARK1 in Sf9 cells and purified them to apparent homogeneity in milligram quantities. The K_m -values of the enzyme were 3.8 μ M for rhodopsin and 22 μ M for ATP; the V_{max} -value was 9.9 mol phosphate/mol β ARK/min. These data indicate that the two recombinant kinases were at least as active as preparations previously obtained from bovine brain. There were no differences in the functional activity of human and bovine β ARK.

β -Adrenergic receptor kinase; β_2 -Adrenergic receptor; Rhodopsin; Receptor phosphorylation; Baculovirus; *Spodoptera frugiperda* cell

1. INTRODUCTION

Signal transduction by G protein-coupled receptors is subject to regulation by a multitude of mechanisms, and one of the most prominent of these is a process called desensitization. Desensitization is defined as a decline of receptor-mediated effects in response to continuous or repeated agonist exposure. This process has been studied most extensively for the β_2 -adrenergic receptor/G_s/adenylylcyclase system [1,2]. β -Adrenergic receptors can become desensitized via phosphorylation by protein kinase A or the β -adrenergic receptor kinase (β ARK) [1–3]. Phosphorylation of the receptors by β ARK, followed by binding of the inhibitor protein β -arrestin to the phosphorylated receptors [3–6] represents the most rapid and quantitatively most important mechanism causing agonist-induced, homologous desensitization of β -adrenergic receptors [7]. In addition to phosphorylating β_2 -adrenergic receptors, β ARK can also phosphorylate α_2 -adrenergic and muscarinic receptors as well as the 'light receptor' rhodopsin [8–10]. β ARK phosphorylates (and thereby desensitizes) only receptors which are occupied by agonists apparently for two reasons. First, only the agonist-occupied receptors are good substrates for β ARK and, therefore, agonist-occupancy of receptors and β ARK-dependent phos-

phorylation occur in parallel [6,11]. Second, the cytosolic β ARK appears to require G protein $\beta\gamma$ -subunits, presumably liberated upon G protein activation by receptors, in order to bind to the membranes in close vicinity to the receptors [12–14].

The cDNAs of two isoforms of β ARK have been cloned to date, of which β ARK1 appears to be the major isoform responsible for phosphorylating β_2 -adrenergic receptors, since it is more widely distributed and more active towards β_2 -receptors than β ARK2 [15]. Previous studies on β ARK have been done mostly with preparations partially purified from bovine brain, or with cytosolic extracts from transiently transfected COS-cells. These preparations were neither purified to homogeneity nor, in the case of the most commonly used bovine brain preparations, defined in their isoform composition, and were limited in quantity. In order to overcome these problems we undertook the expression of the bovine and human major isoform β ARK1 using the baculovirus expression system [16].

2. MATERIALS AND METHODS

2.1. Plasmid constructions

A *Bam*HI site was created in the cDNA for bovine β ARK1 [3] immediately 5' to the start codon by ligating into the *Bal*I site two complementary oligonucleotides (47 and 51 nucleotides) corresponding to the β ARK1 sequence. The 2.1 kbp *Bam*HI–*Nhe*I fragment containing the whole coding region of β ARK was introduced into the *Bam*HI–*Xba*I linearized baculovirus vector pVL1393 [16] to generate the transfer vector pVL-bov β ARK1 for the expression of bovine β ARK1 under the control of the polyhedrin promoter.

Cloning of the human β ARK1 cDNA was performed essentially as described for bovine β ARK1. A 2.8 kbp *Eco*RI fragment coding for human β ARK1 [17], provided by Dr. J. Benovic, was complemented with a PCR product from A431 cell cDNA to reconstitute the 5'-end

Correspondence address: P. Söhlemann, Laboratory of Molecular Biology, University of Munich, Max-Planck-Institute of Biochemistry, 8033 Martinsried, Germany. Fax: (49) (89) 8578-3795.

Abbreviations. β ARK, β -adrenergic receptor kinase; CM, carboxymethyl; DEAE, diethyl aminoethyl; MOI, multiplicity of infection (plaque forming units/cell); PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

of β ARK. A 1.0 kbp *SacI* fragment located within the β ARK1 coding region was replaced by a second PCR product from human A431 cell cDNA to correct mutations in the clone. The resulting cDNA sequence was shown to be identical with that published by Chuang et al. [18] and was cloned into pVL1393 to yield pVL-hu β ARK1.

2.2. Production of recombinant baculovirus and expression of β ARK in Sf9 cells

Recombinant baculoviruses were produced by co-transfection of *Spodoptera frugiperda* Sf9 cells, with 200 ng of the transfer vector and 20 ng of baculovirus DNA containing a lethal deletion (BaculoGold, Dianova). Single recombinant plaques were isolated from the pool of recombinant viruses and amplified in Sf9 cells. A large batch of recombinant viruses was obtained after three rounds of amplification (MOI = 0.1). For large scale expression, Sf9 cells were grown in suspension (200 ml) and infected at a cell density of 2×10^6 /ml (MOI = 10). Cells were harvested after 3 days and washed three times in PBS.

2.3. Purification of β ARK

The cell pellets were resuspended in lysis buffer (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 100 μ M PMSF, 10 μ g/ml leupeptin and 10 μ g/ml benzamide; 10 ml/pellet of a 200 ml suspension culture). The cells were disrupted with an ultraturrax six times for

20 s at 4°C and centrifuged for 30 min at $250,000 \times g$. NaCl (10 mM) was added to the supernatant which was then adsorbed to DEAE-Sephacel (Pharmacia-LKB, 1 ml/5 ml supernatant) for 30 min at 4°C. The slurry was poured into a column, and the pass-through plus a 15 ml wash with lysis buffer containing 10 mM NaCl were collected. These fractions from the DEAE-Sephacel column were diluted 4-fold with 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.02% Triton X-100 and applied onto a 8 ml CM-Fractogel (Merck) column. The column was washed with 50 ml of 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.02% Triton X-100, and eluted stepwise with the same buffer containing 20 to 100 mM NaCl. All column fractions were analyzed by SDS-polyacrylamide gel electrophoresis and were assayed for β ARK activity using rod outer segments as substrate. The concentration of purified β ARK was measured using the protein determination method of Bradford [19].

2.4. β ARK assay

Activity of β ARK was measured as described earlier [11,20] using urea-treated rod outer segments as substrate. Standard conditions were incubations for 10 min at 30°C in 20 mM Tris-HCl, pH 7.5, 8 mM MgCl₂, 2 mM EDTA, and 100 μ M [γ -³²P]ATP (0.4–2 cpm/fmol). Unless otherwise indicated, substrate concentrations were 0.6 μ M for rhodopsin, or 10 nM human β_2 -adrenergic receptor, the latter purified from Sf9 cells (Hekman et al., in preparation) and co-reconstituted

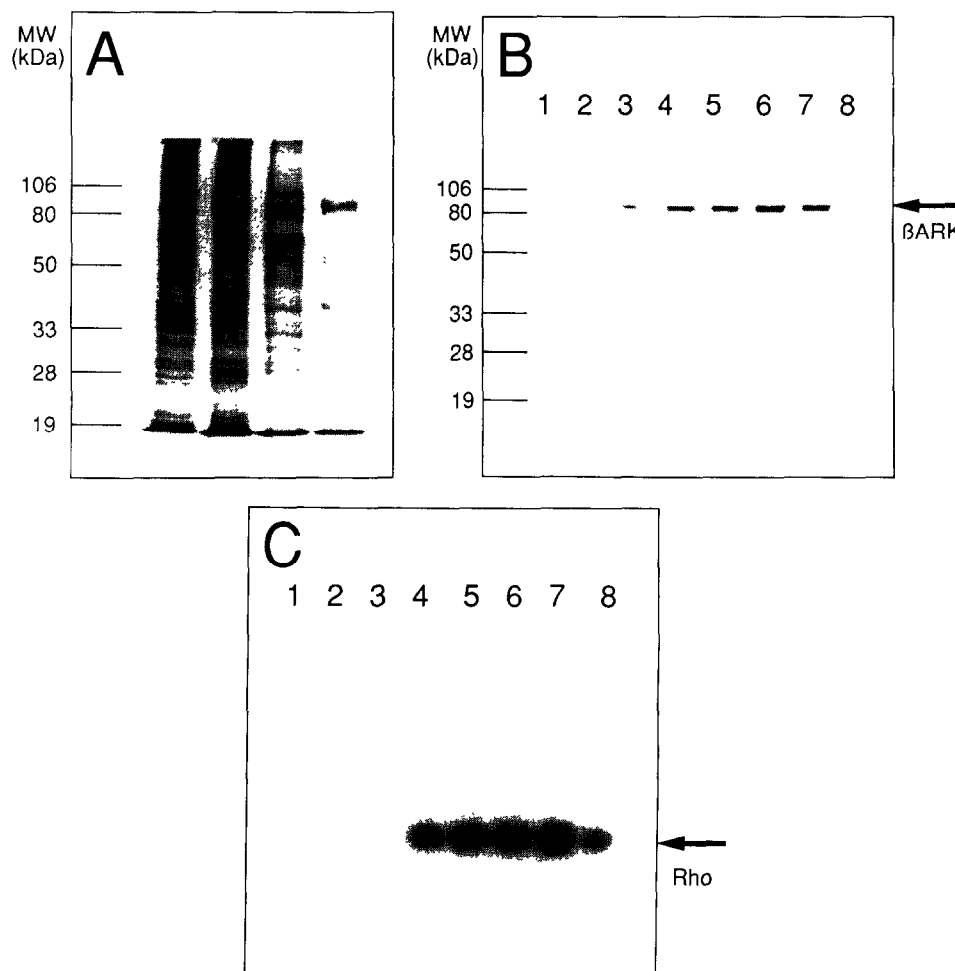


Fig. 1. Purification of bovine β ARK from infected Sf9 cells. Aliquots of non-infected Sf9 cells (panel A, lane 1), or cells infected with recombinant baculovirus (lane 2), of the cytosol from infected cells (lane 3), of the DEAE-Sephacel pass-through of the cytosol (lane 4) and of the fractions eluting from the CM-Fractogel column at 20, 30, 40, 50, 60, 70, 80 and 100 mM NaCl (panel B, lanes 1–8) were subjected to SDS-PAGE, and proteins were visualized by Coomassie blue staining. The β ARK-activities of the CM-Fractogel fractions shown in panel B (lanes 1–8) were determined by measuring rhodopsin phosphorylation (panel C, lanes 1–8).

with 50 nM G protein $\beta\gamma$ -subunits purified from bovine brain [21]. For determination of K_m and V_{max} of the enzymes for the receptor substrates, the cofactor [γ - 32 P]ATP was used at a saturating concentration of 300 μ M. Purified β ARK was present at 3 nM unless otherwise indicated. At the end of the incubation, the phosphorylated substrates were resolved by SDS-PAGE and identified by autoradiography. [32 P]Phosphate incorporation was measured by excision of the substrate bands and determination of their 32 P content.

3. RESULTS

Infection of Sf9 cells with recombinant baculovirus coding for either bovine or human β ARK1 resulted in a time-dependent expression of β ARK as revealed by determination of β ARK-activity, or by SDS-PAGE and Coomassie stains. Maximal expression was obtained 3 days after infection with a MOI of 10 (not shown). Under these conditions, β ARK was seen in Coomassie-stained gels as the major soluble protein in infected Sf9 cells (Fig. 1).

Bovine β ARK was purified from the cytosol of these cells by a simplified procedure adapted from the protocol used for the purification of β ARK from bovine brain [22]. The particulate fraction was removed by centrifugation, and the supernatant was applied at low ionic strength onto a DEAE-Sephacel anion exchange column. All β ARK activity was recovered in the pass through fractions from this column (not shown). β ARK was by far the major protein in these eluates (Fig. 1). Subsequent chromatography of the DEAE-eluates on a

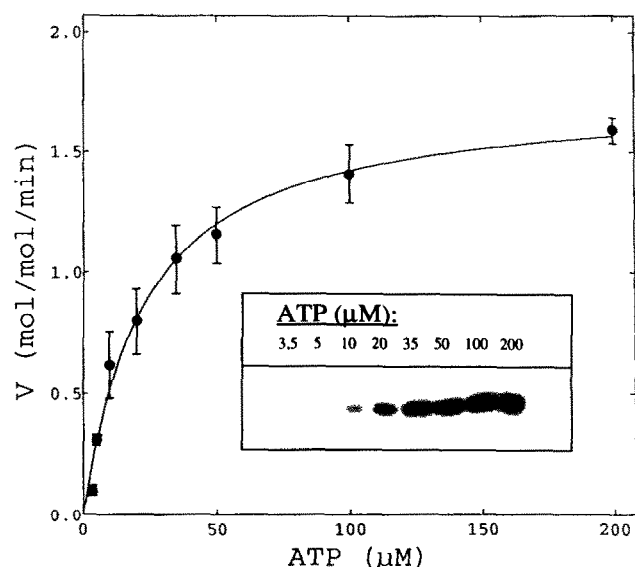


Fig. 2. ATP-dependence of the activity of purified recombinant β ARK. Rod outer segments (0.6 μ M rhodopsin) were incubated for 5 min at 30°C under bright light with purified bovine β ARK (6 nM) and 3.5–200 μ M [γ - 32 P]ATP ($\approx$ 0.2 cpm/fmol). The rod outer segments were pelleted at 4°C and subjected to 12.5% SDS-PAGE. 32 P-labeled rhodopsin was visualized by autoradiography (inset) and the 32 P-content determined by Cerenkov-counting of the excised bands. Non-linear curve-fitting [23] yielded values for $K_m = 22 \pm 5$ μ M and $V_{max} = 1.72 \pm 0.14$ mol phosphate/mol β ARK/min.

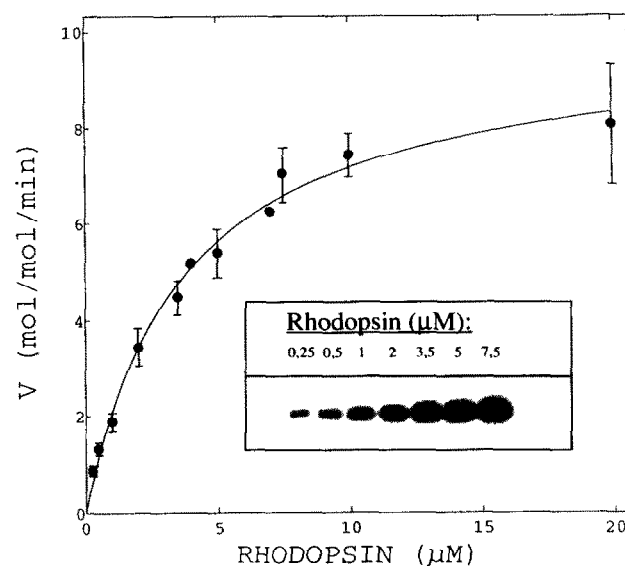


Fig. 3. Concentration-dependent phosphorylation of rhodopsin by purified recombinant β ARK. Rod outer segments (0.2–20 μ M rhodopsin) were phosphorylated for 5 min at 30°C under bright light with bovine β ARK (3 nM). ATP was used at a saturating concentration of 300 μ M. Non-linear curve-fitting [23] yielded values for $K_m = 3.8 \pm 0.7$ μ M and $V_{max} = 9.9 \pm 0.8$ mol phosphate/mol β ARK/min.

CM-Fractogel cation exchange column resulted in purification of β ARK to apparent homogeneity. The amounts of β ARK in the eluates determined by SDS-PAGE were exactly paralleled by β ARK-activity (Fig. 1, panels B and C). The yields of purified kinase were about 1–2 mg ($\approx$ 12–24 nmol) per 200 ml suspension culture.

The catalytic properties of the recombinant kinases were investigated by determination of their K_m - and V_{max} -values for rhodopsin, and for ATP. The studies were performed with rhodopsin (in the form of rod outer segments) as substrate, both because it is more readily available than β_2 -receptors, and because it has been shown that the V_{max} -values of the brain enzyme for rhodopsin and β_2 -receptor were identical [22]. In the presence of 0.6 μ M rhodopsin, the apparent K_m of purified recombinant bovine β ARK for the cofactor ATP was 22 ± 5 μ M, and the apparent V_{max} was 1.72 ± 0.14 mol phosphate/mol β ARK/min (Fig. 2). This K_m -value for ATP is similar to the value of 33–37 μ M of the kinase purified from bovine brain [22].

At a saturating concentration of ATP (300 μ M), the K_m for rhodopsin was 3.8 ± 0.7 μ M and the V_{max} was 9.9 ± 0.8 mol phosphate/mol β ARK/min (Fig. 3), values which are similar to those reported for the bovine brain enzyme of 6 μ M and 5.8 mol phosphate/mol β ARK/min, respectively [22].

The purification of human β ARK was achieved in an identical manner. No differences in the elution profiles from anion or cation exchanger columns were detectable. The kinetic constants of the recombinant human

Table I

Phosphorylation of human β_2 -adrenergic receptor by bovine or human β ARK

β ARK (nM)	mol P _i /mol β_2 -adrenergic receptor	
	Bovine β ARK	Human β ARK
3.7	2.0 $\pm$ 0.02	2.5 $\pm$ 0.02
37.0	3.7 $\pm$ 0.2	4.7 $\pm$ 0.2

β ARK were identical to those of the bovine enzyme with respect to the substrate rhodopsin or the cofactor ATP (not shown).

Phosphorylation of β_2 -adrenergic receptors by either bovine or human β ARK was assayed after co-reconstitution with G protein $\beta\gamma$ -subunits, which have been reported to be required for full activity of β ARK towards receptors [12–14]. In contrast to earlier data [22], we did not observe any significant phosphorylation of β_2 -receptors in the absence of $\beta\gamma$ -subunits (not shown). Maximal phosphorylation stoichiometries for β_2 -receptors in the presence of $\beta\gamma$ -subunits were up to 5 mol phosphate/mol receptor (Table I). Bovine and human β ARK displayed only minor differences in their activity with respect to the human receptor as a substrate.

4. DISCUSSION

Our data indicate that β ARK can be expressed and purified in substantial quantities using the baculovirus system. The kinases purified from Sf9 cells appear to be fully functional. This is indicated by affinities for ATP and rhodopsin, and $V_{\max}$ -values, that compare favourably with those of the enzyme prepared from bovine brain. In fact, the $V_{\max}$ -values for the bovine brain preparation, which were extrapolated from preparations of 10% purity [22], were about 2-fold lower than those obtained with the recombinant enzyme. This difference may be due to difficulties in the extrapolation, or to protein degradation during the lengthy purification procedure. In any case, the data suggest that the recombinant proteins have essentially the same activity and the same properties as the native protein.

β ARK purified from bovine brain [22] was the first kinase preparation available for the study of agonist-specific phosphorylation of the β_2 -adrenergic receptor. In addition to the characterization of recombinant bovine β ARK we analysed whether human β ARK differs from bovine β ARK in its specific activity towards its natural substrate, the human β_2 -adrenergic receptor. Bovine and human β ARK show 98% protein sequence identity [17] and no significant differences of their activities were detected using either rhodopsin or the β_2 -adrenergic receptor as substrates (Table I). Thus the amino acid exchanges do not appear to be functionally relevant with respect to the functional properties of the recombinant kinases. However, in order to develop in-

hibitors of β ARK, the availability of the human enzyme is clearly an advantage, since the problem of species-differences of such inhibitors can be avoided.

In summary, expression of β ARK in Sf9 cells permits the purification of large quantities of the kinase to apparent homogeneity which will help in the elucidation of the mechanisms and physiological functions of this key enzyme in the control of signalling by β -adrenergic and probably many other G protein-coupled receptors.

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