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

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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 βARK1 in Sf9 cells and purified them to apparent homogeneity in milligram quantities. The K_m-values of the enzyme were 3.8 μM for rhodopsin and 22 μM for ATP; the V_{max}-value was 9.9 mol phosphate/mol βARK/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 βARK.

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 (βARK) [1–3]. Phosphorylation of the receptors by βARK, 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, βARK can also phosphorylate α_2-adrenergic and muscarinic receptors as well as the 'light receptor' rhodopsin [8–10]. βARK 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 βARK and, therefore, agonist-occupancy of receptors and βARK-dependent phosphorylation occur in parallel [6,11]. Second, the cytosolic βARK appears to require G protein βγ-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 βARK have been cloned to date, of which βARK1 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 βARK2 [15]. Previous studies on βARK 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 βARK1 using the baculovirus expression system [16].

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Abbreviations. βARK, 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.

2. MATERIALS AND METHODS

2.1. *Plasmid constructions*

A *Bam*HI site was created in the cDNA for bovine βARK1 [3] immediately 5′ to the start codon by ligating into the *Bal*I site two complementary oligonucleotides (47 and 51 nucleotides) corresponding to the βARK1 sequence. The 2.1 kbp *Bam*HI–*Nhe*I fragment containing the whole coding region of βARK was introduced into the *Bam*HI–*Xba*I linearized baculovirus vector pVL1393 [16] to generate the transfer vector pVL-bovβARK1 for the expression of bovine βARK1 under the control of the polyhedrin promoter.

Cloning of the human βARK1 cDNA was performed essentially as described for bovine βARK1. A 2.8 kbp *Eco*RI fragment coding for human βARK1 [17], provided by Dr. J. Benovic, was complemented with a PCR product from A431 cell cDNA to reconstitute the 5′-end

of βARK. A 1.0 kbp *Sac*I fragment located within the βARK1 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-huβARK1.

2.2. *Production of recombinant baculovirus and expression of βARK 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 βARK*

The cell pellets were resuspended in lysis buffer (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 100 μM PMSF, 10 μg/ml leupeptin and 10 μg/ml benzamidine; 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 × *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 βARK activity using rod outer segments as substrate. The concentration of purified βARK was measured using the protein determination method of Bradford [19]

2.4. *βARK assay*

Activity of βARK 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 $MgCl_2$, 2 mM EDTA, and 100 μM [γ-^{32}P]ATP (0.4–2 cpm/fmol) Unless otherwise indicated, substrate concentrations were 0.6 μM for rhodopsin, or 10 nM human β_2-adrenergic receptor, the latter purified from Sf9 cells (Hekman et al., in preparation) and co-reconstituted

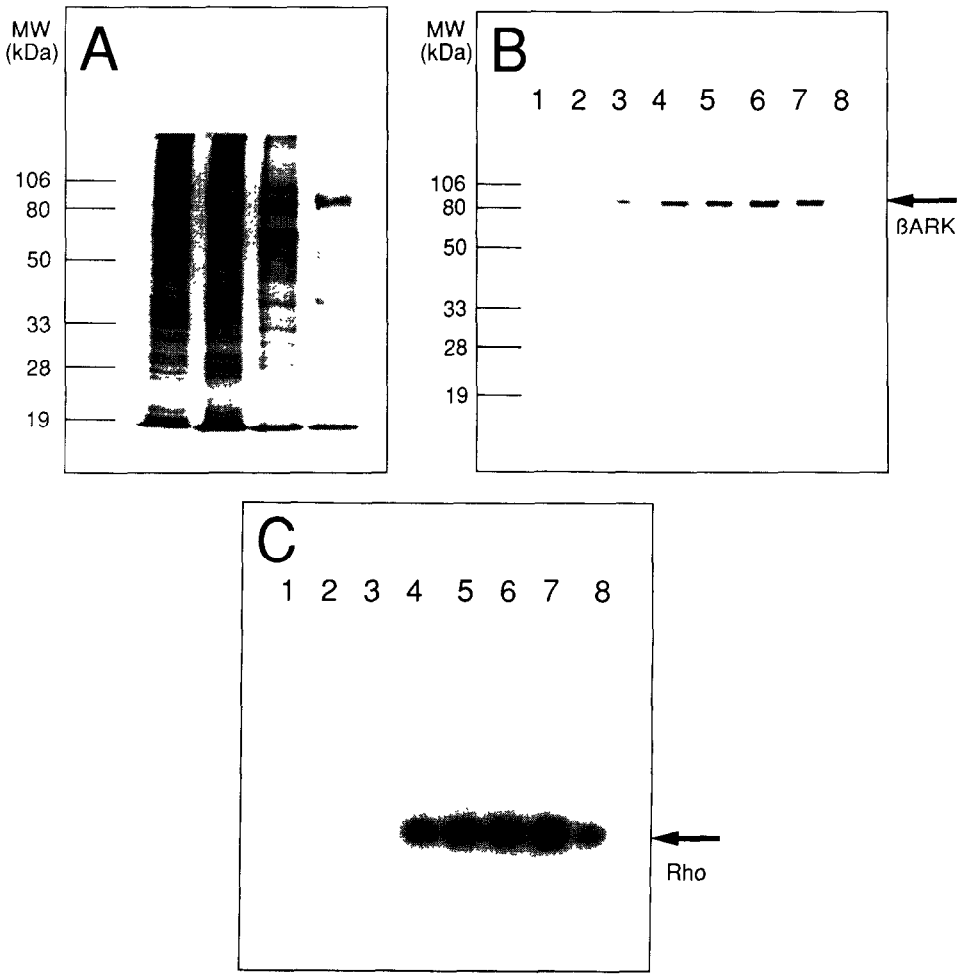

Fig. 1. Purification of bovine βARK from infected Sf9 cells. Aliquots of non-infected Sf9 cells (panel A, lane 1), or cells infected with recombinant baculovirus (lane 2), of the cytosol from infected cells (lane 3), of the DEAE-Sephacel pass-through of the cytosol (lane 4) and of the fractions eluting 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 βARK-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 μM. Purified βARK 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 βARK1 resulted in a time-dependent expression of βARK as revealed by determination of βARK-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, βARK was seen in Coomassie-stained gels as the major soluble protein in infected Sf9 cells (Fig. 1).

Bovine βARK was purified from the cytosol of these cells by a simplified procedure adapted from the protocol used for the purification of βARK 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 βARK activity was recovered in the pass through fractions from this column (not shown). βARK was by far the major protein in these eluates (Fig. 1). Subsequent chromatography of the DEAE-eluates on a

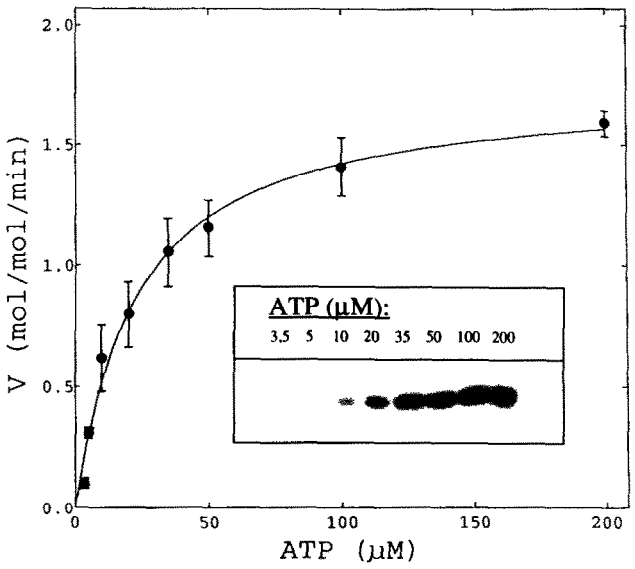

Fig. 2. ATP-dependence of the activity of purified recombinant βARK. Rod outer segments (0.6 μM rhodopsin) were incubated for 5 min at 30°C under bright light with purified bovine βARK (6 nM) and 3.5–200 μM [γ-^{32}P]ATP (≈ 0.2 cpm/fmol). The rod outer segments were pelleted at 4°C and subjected to 12.5% 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 ± 5 μM and V_{max} = 1.72 ± 0.14 mol phosphate/mol βARK/min.

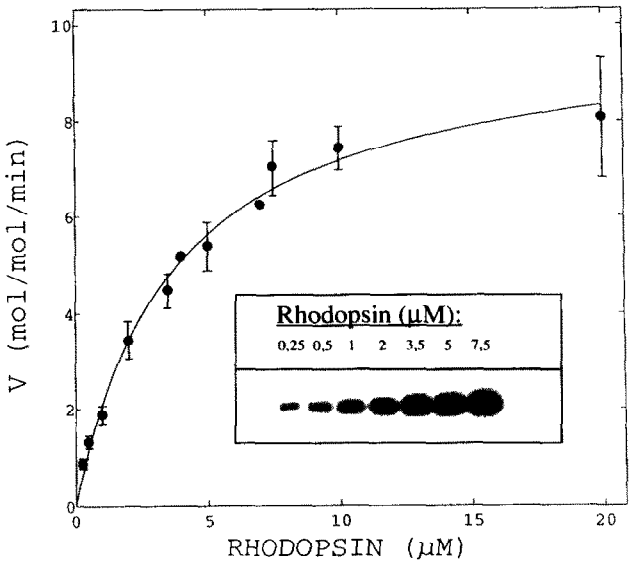

Fig. 3. Concentration-dependent phosphorylation of rhodopsin by purified recombinant βARK. Rod outer segments (0.2–20 μM rhodopsin) were phosphorylated for 5 min at 30°C under bright light with bovine βARK (3 nM). ATP was used at a saturating concentration of 300 μM. Non-linear curve-fitting [23] yielded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

CM-Fractogel cation exchange column resulted in purification of βARK to apparent homogeneity. The amounts of βARK in the eluates determined by SDS-PAGE were exactly paralleled by βARK-activity (Fig. 1, panels B and C). The yields of purified kinase were about 1–2 mg (≈ 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 μM rhodopsin, the apparent K_m of purified recombinant bovine βARK for the cofactor ATP was 22 ± 5 μM, and the apparent V_{max} was 1.72 ± 0.14 mol phosphate/mol βARK/min (Fig. 2). This K_m-value for ATP is similar to the value of 33–37 μM of the kinase purified from bovine brain [22].

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

The purification of human βARK 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 βARK

βARK (nM)	mol P_i/mol β_2-adrenergic receptor	
	Bovine βARK	Human βARK
3.7	2.0 ± 0.02	2.5 ± 0 02
37.0	3.7 ± 0.2	4.7 ± 0.2

βARK 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 βARK was assayed after co-reconstitution with G protein $\beta\gamma$-subunits, which have been reported to be required for full activity of βARK 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 βARK displayed only minor differences in their activity with respect to the human receptor as a substrate.

4. DISCUSSION

Our data indicate that βARK 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.

βARK 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 βARK we analysed whether human βARK differs from bovine βARK in its specific activity towards its natural substrate, the human β_2-adrenergic receptor. Bovine and human βARK 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 inhibitors of βARK, 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 βARK 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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