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Rat Beta-Adrenergic Receptor Kinases 1 and 2 in Mouse Neuroblastoma X Rat Glioma NG 108-15 Hybrid Cells

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ABSTRACT. Beta-adrenergic receptor kinase (βARK, EC 2.7.1.-) has been implicated in the phosphorylation of G protein-coupled receptors, including opioid receptors. Since δ-opioid receptors of mouse neuroblastoma × rat glioma hybrid cells (NG 108-15) desensitize upon activation, this investigation was designed to find out whether NG 108-15 cells contain βARK activity. Using the reverse transcription polymerase chain reaction technique, we identified two mRNAs, one coding for rat βARK1 and the other for rat βARK2. No hint was found for the presence of mouse βARK. Examining the cytosolic βARK activity in these hybrid cells using rhodopsin as substrate, we found a strict functional dependence on the presence of exogenous G protein subunit Gβγ. This relationship reflects a characteristic for βARK1 and 2 out of the known G protein-coupled receptor kinases. Finally, highly purified recombinant βARK1 proved active to phosphorylate enriched δ-opioid receptor preparations in an opioid agonist-dependent manner. The results reported here provide the basis to study more closely the molecular function of G protein-coupled receptor kinases in a cell line (NG 108-15) most frequently used to investigate acute and chronic opioid actions. BIOCHEM PHARMACOL **55**;1:65–70, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. rat beta-adrenergic receptor kinase; βARK; δ-opioid receptor; receptor phosphorylation; NG 108-15 cells

G protein-coupled receptors (GPRs)‖ desensitize upon activation, which is accompanied by uncoupling from their respective G protein subunit [1]. This process is known to involve multiple biochemical mechanisms, including phosphorylation of the receptor protein [2]. Opioid receptors belong to the GPR-family [3] and are well known to desensitize or develop tolerance when stimulated by an agonist [4]. Very recent investigations into biochemical processes underlying adaptation of the opioidergic system suggest a major function of beta-adrenergic receptor kinases (βARKs). Their overexpression is associated with opioid receptor phosphorylation [5, 6], and opioid tolerance causes increased kinase levels [7]. This experimental outcome as well as the rather low substrate specificity of βARK and related kinases [8] strongly support the notion that at least certain G protein-coupled receptor kinases (GRKs) [2], e.g. βARK1 and 2, own the potency to phosphorylate opioid receptors.

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‖ *Abbreviations:* βARK, beta-adrenergic receptor kinase (EC 2.7.1.-); GPR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; PCR, polymerase chain reaction; DPDPE, [D-penicillamine^2, D-penicillamine^5]enkephalin; bp, base pair.

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Neuroblastoma × glioma hybrid cells (NG 108-15) [9] have been widely used in opioid research to study acute and chronic opioid actions [10]. They exhibit δ-opioid receptors [11], which may be phosphorylated upon activation [6], and subsequently uncouple from their corresponding G proteins [12]. Thus, opioid receptors share characteristics of GPRs interacting with βARK. These findings caused us to hypothesize that desensitation of δ-receptors in NG 108-15 cells is brought about by distinct GRKs. Verification of this notion requires documentation of βARK activity in this clonal cell line. When searching for their mRNA, one has to consider that the NG 108-15 cell line is derived from mouse neuroblastoma fused with rat glioma cells [9]. βARK isoforms have been published for both species, namely rat βARK1 and 2 [13] and a truncated βARK of the mouse [14]. We used the reverse transcription polymerase chain reaction (PCR) technique to examine whether the messages of these rat and mouse βARKs are present in NG 108-15 cells. As messages for rat βARKs could be identified, studies were carried out to search for βARK activity [15]. We found kinase activity to be strictly dependent on the presence of G protein βγ subunits, indicating the presence of βARK1 and 2 [16, 17] in NG 108-15 cells. Finally, βARK was found to efficiently phosphorylate δ-opioid receptors in an agonist-dependent manner.

The experiments reported here were designed to provide

TABLE 1. Oligonucleotide primer sequences for rat β-adrenergic receptor kinase1 (βARK1), β-adrenergic receptor kinase2 (βARK2), and mouse β-adrenergic receptor kinase

mRNA	Primer	Position	bp
rat βARK1			
forward	5′ gtc gac gag gcg gta ctg gcc gat gtg 3′	157	987
reverse	5′ gaa ttc gag att ctc aca tgg cca tgc 3′	1144	
rat βARK2			
forward	5′ gtc gac gag gcc gtg ctg gcc gac gtc 3′	16	992
reverse	5′ gaa ttc gat atc ctc aca tgc cca tat 3′	1008	
mouse βARK			
forward	5′ gtc gac gag gcg gta ctg gcc gat gtg 3′	3	473
reverse	5′ gaa ttc gaa cac atc cct tcg gag gc 3′	476	

"Position" indicates location of individual primers in mRNA. "bp": length of base pair sequences of PCR products. Primers of rat βARK 1 and 2 are deduced from Arriza et al. [13], mouse βARK from Marra et al. [14]. cDNAs can be accessed under "Houston Database" (M87854, Ratbark1; M87855, Ratbark2), and "Wisconsin Database" (W48962, mousebark). Restriction sites (underlined): forward, *SalI*; reverse, *EcoRI*.

basic information as to whether NG 108-15 cells can also be used to investigate distinct GRKs and their interaction with opioid receptors. The outcome is likely to initiate detailed studies in this classical hybrid cell line regarding the functions of GRKs with respect to acute and chronic opioid actions.

MATERIALS AND METHODS

Cell Culture

NG 108-15 cells and A431 cells were cultured as described [18, 19]. They were harvested at 70% confluency, washed twice in ice-cold phosphate-buffered saline, and stored at −86° until use.

HIGH 5 insect cells were employed to overexpress δ-opioid receptors as previously detailed [20]. These cells exhibit highest receptor concentrations on the third day after infection (1.5 to 2 pmol/mg membrane protein), when they were harvested by low speed centrifugation. Cells were washed (phosphate-buffered saline, PBS) and stored at −86°.

RNA Preparation and Reverse Transcription

Total RNA was prepared from frozen NG 108-15 cells using Trizol® (Life Technologies) according to the manufacturer's instructions, and the obtained mRNA (1 μg) was used for reverse transcription into cDNA. Briefly, denatured RNA (3 min, 65°) was incubated (final volume 20 μL) for 90 min at 42° in the presence of dNTP (0.5 mM), dithiothreitol (0.01 mM), random hexamer primer (10 pmol), 1 U M-MuLV reverse transcriptase (MBI Fermentas), and 1 U RNAsin (Promega) in Tris (50 mM, pH 8.3), containing KCl (75 mM) and $MgCl_2$ (3 mM). The reaction was terminated with 50 μL Tris (10 mM, pH 8), containing 1 mM ethylenediaminetetraacetic acid (EDTA), and heating (70°) for 10 min.

PCR

Primers (Gibco) were specifically designed to amplify βARK cDNAs of defined lengths of rat and mouse [13, 14]. Rat βARK1 (2683 base pairs, bp) primers span a sequence of 987 bp, rat βARK2 (2464 bp) primers amplify 992 bp, and primers recognizing the mouse βARK fragment (633 bp) span 473 bp. For position of individual primers within the respective mRNAs, see Table 1.

PCRs were conducted with cDNA transcripts corresponding to 100 ng RNA. The mixture consisted of 0.8 μM of the appropriate primers, 1.25 U Vent DNA polymerase (New England Biolabs), 250 μM dNTP, and Vent-polymerase-buffer in 100 μL final volume. Samples were overlayed with paraffin-pellets. Amplifications were performed in a thermal cycler (Perkin–Elmer 480). Denaturation was carried out at 96° for 1 min (3 min for the first cycle), followed by an annealing step at 50° or 55°, depending on the primer used, for 1 min, and an extension step at 72° for 1.5 min. Thirty cycles were performed, warranting sufficient amplification. PCR-products were digested with *SalI* and *EcoRI*, and were purified on 0.6% TAE LMP-Agarose-Gel (Biozym). The 987.992 bp bands were cut off the gel and subcoloned into the *SalI*/*EcoRI*-site of the vector pBluescript II SK (+) by means of In-Gel Ligation. The obtained βARK1/2 inserts were verified by sequencing (MediGene).

βARK Assay

βARK activity of NG 108-15 cells was measured analogous to the method described [21, 22]. Cells were resuspended in lysis buffer (25 mM Tris, pH 7.5, 5 mM EDTA, 5 mM EGTA, 20 μg/mL leupeptin, 20 μg/mL benzamidine, and 40 μg/mL phenylmethyl sulfonylfluoride). After homogenization (Polytron) and centrifugation (30 min, 400,000 × g, 4°), the supernatant was subjected to DEAE-Sephacel chromatography. Protein was eluted (20 mM Tris, pH 7.4,

containing 2 mM EDTA, 50 mM NaCl) and the flow-through was used for measurement of βARK activity.

The βARK activity was determined by the use of urea-treated rod outer segments, containing >95% rhodopsin as substrate. Briefly, phosphorylation reactions (100 μL) contained urea-treated rod outer segments (400 pmol rhodopsin) in 20 mM Tris (pH 7.5), containing 2 mM EDTA, 4–7 mM $MgCl_2$, and 50–100 μM [γ^{32}P]ATP (1–5 cpm/fmol). Samples were incubated at 30° for 20 min for light-activated rhodopsin phosphorylation. If not stated otherwise, reactions were conducted in the presence of 150 nM bovine Gβ/γ subunits. Reactions were stopped with 500 μL ice-cold stop-buffer (20 mM Tris, pH 7.6), containing 5 mM EDTA and 5 mM EGTA. After centrifugation, the supernatant was discarded and the pellet resuspended in Laemmli buffer [23] for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To test for concentrations of rhodopsin, bands were visualized by Coomassie blue, destained, and the gel subjected to autoradiography. For quantification of incorporated ^{32}P, the bands indicative for phosphorylated rhodopsin were cut and counted for radioactivity. Enzyme activities were calculated as ^{32}P incorporation per minute per milligram of cytosolic protein. Rhodopsin phosphorylated by recombinant βARK served as standard.

Enrichment of Opioid Receptors

Enrichment of δ-opioid receptors was carried out by sucrose gradient centrifugation [16]. HIGH 5 cells were homogenized (Polytron) in a hypotonic buffer "A" (20 mM Tris, pH 7.4, 4°), containing 5 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin and 0.1 mM benzamidine. The homogenate was passed five times through a 27-gauge needle and centrifuged (15,000 × g, 20 min, 4°). The crude membranes were taken up in 12 mL buffer, and 3 mL were placed carefully on sucrose gradient (40%) for centrifugation (100,000 × g, 4°, 90 min, SW 41 rotor). The resulting interphase contained purified membrane material, which was collected in buffer "A", containing 250 mM NaCl, and centrifuged (40,000 × g, 10 min, 4°). The pellet was suspended in 5 M urea, kept on ice (30 min), subsequently sonicated (ice bath) and centrifuged (20,000 × g, 10 min). The pellet was washed with buffer "A", centrifuged and the sedimented membrane material, which contains the highly enriched opioid receptors, was resuspended in 50 mM Tris buffer (pH 7.4), containing 2 mM EDTA and 5 mM $MgCl_2$. Protein concentration was determined as described by Lowry et al. [24], and opioid receptor concentrations were quantified by means of [^{3}H]diprenorphine as described [20]. Opioid receptor enrichment reached 5 pmol/mg protein.

Opioid Receptor Phosphorylation

Recombinant purified bovine βARK1 [21] was used for phosphorylation [22] of opioid receptors. Briefly, the reaction mixture (Hepes-buffer, pH 7.5, 40 μL) consisted of enriched δ-receptors (0.75 and 1.5 pmol, respectively), 100 nM βARK1, 500 nM G protein β/γ complex, 10 mM $MgCl_2$, 2 mM EDTA and 50 μM [γ-^{32}P]ATP. Incubations were conducted at 30° for 8 min. Tests were conducted in the presence of 0.1 μM [D-penicillamine^2,D-penicillamine^5]enkephalin (DPDPE) and 0.1 μM DPDPE plus naloxone (1 μM), respectively. Thereafter, the samples were analyzed by electrophoresis (10% polyacrylamidgel) and autoradiographed.

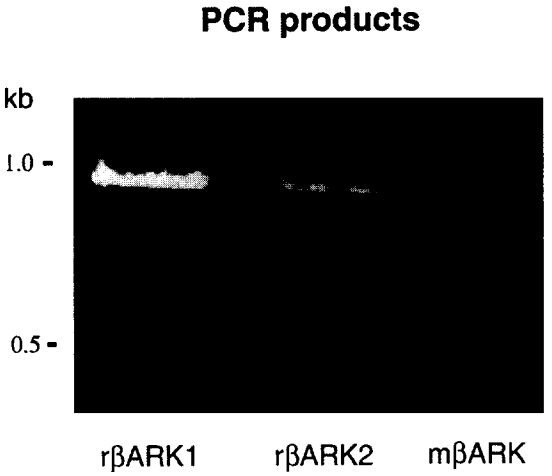

FIG. 1. PCR amplification products (*SalI/Eco*RI digests) for rat β-adrenergic receptor kinase 1 (rβARK1) and rat β-adrenergic receptor kinase 2 (rβARK2). RNA was prepared from cytosol of NG 108-15 hybrid cells. The individual primers employed are given in Table 1. DNA standard (number of base pairs) is indicated. Mouse β-adrenergic receptor kinase (mβARK) was not identified.

Western Blot of δ-Opioid Receptors

Recognition of δ-opioid receptors by Western blot analysis followed essentially the method described by Wehmeyer and Schulz [25].

Data Analysis

Sequences of PCR products were compared with known βARK-cDNAs, using the "Best Fit" program (Wisconsin Genetics Computer Group). Primer design for PCR was based on sequences obtained from "Houston Database" and "Wisconsin Database".

RESULTS

Detection of βARK Transcripts in NG 108-15 Cells

Total RNA from frozen hybrid cells was submitted to reverse transcription, and cDNA transcripts corresponding to 100 ng RNA were used for PCR. The sense and antisense primers designed to match the DNA sequences of rat βARK1 and 2 as well as of mouse βARK were employed to screen for their corresponding mRNAs. Fig. 1 demonstrates the PCR products visualized in ethidium bromide contain-

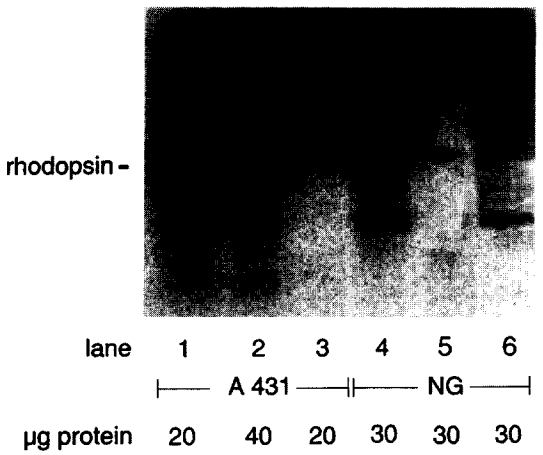

FIG. 2. Autoradiography of β-adrenergic receptor kinase activity, using light-activated rhodopsin as substrate in a βARK assay. Tests were conducted with cytosol of A431 cells (lanes 1 to 3) and with cytosol of NG 108-15 cells (NG, lanes 4 to 6). The µg cytosolic protein submitted to the βARK assay is indicated (µg protein). Cytosolic reaction products displayed at lanes 3 and 5 were heated (95°, 10 min) prior to the assay. Lane 6 displays βARK assay reaction products obtained in the absence of exogenous Gβ/γ subunits. Phosphorylated rhodopsin band is indicated ("rhodopsin").

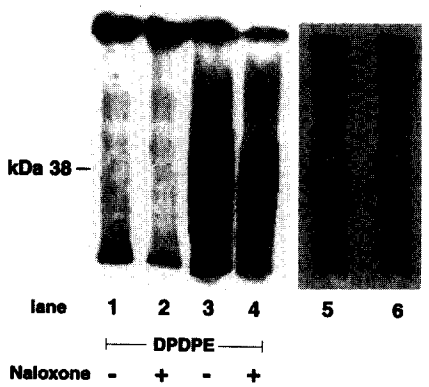

FIG. 3. Autoradiogram of δ-opioid receptors submitted to phosphorylation by recombinant bovine βARK 1 (lanes 1 to 4). Lanes 1 and 2 display results obtained with 750 fmol receptor in the presence of 1 µM DPDPE (lane 1) and in the presence of 1 µM DPDPE plus 10 µM naloxone (lane 2). A clear sign of phosphorylated receptor material was not detected under these conditions. Increasing the amount of δ-receptors to 1.5 pmol, phosphorylation became apparent. This mechanism occurred in a strictly agonist-dependent manner (1 µM DPDPE, lane 3). Phosphorylation failed when DPDPE was prevented from activating the receptor in the presence of naloxone (lane 4). A Western blot of δ-opioid receptor-enriched material (lane 5) indicates the position of receptor immunoreactivity at *ca.* 38 kDa (molecular mass standard). Lane 6 represents the pattern of SDS lysate from nontransfected HIGH 5 cells (identical amounts of protein submitted to lanes 5 and 6), missing immunoreactive staining at 38 kDa. Electrophoresis (lanes 1 to 6) was conducted under identical experimental conditions.

ing agarose gel. Thus, amplification of cDNA synthesized from total RNA of NG 108-15 cells resulted in PCR signals representing both βARK 1 and 2 DNAs with predicted molecular lengths (see Table 1). DNA sequences of PCR amplification products were probed for homology to rat and mouse βARKs, respectively, using the "Best Fit" program. These tests revealed a sequence homology of 96.9% between the PCR-rat βARK1 product and the corresponding rat βARK1 fragment. The homology between rat βARK2 and the respective PCR product was 93.5%.

Screening for cDNA encoding mouse βARK ("Houston Database") utilized a forward primer, which was identical to the rat βARK forward oligonucleotide sequence. In contrast, the reverse sequence used specifically primes to the mouse kinase cDNA. Using these specifically designed primers, no PCR product was detected. Thus, there is no hint of the existence of mouse βARK cDNA in NG 108-15 cells.

βARK Activity

Cytosol of NG 108-15 cells as well as of A431 cells was examined for its activity to catalyze light-activated rhodopsin phosphorylation (βARK assay). A431 cells were included, as these cells were described to exhibit a rather high βARK activity [26]. As expected, cytosol (20 and 40 µg protein) of A431 cells phosphorylated light-activated rhodopsin (Fig. 2, lanes 1 and 2), while no reaction product was observed upon heating of the sample (lane 3). Using cytosol of NG 108-15 cells, an almost identical pattern of phosphorylated rhodopsin is displayed by cytosol of NG 108-15 cells (lane 4), while heating prevents appearance of a band where rhodopsin migrates (lane 5). Rhodopsin phosphorylation (lanes 1, 2 and 4) was most efficiently conducted in the presence of 150 nM Gβγ. Using lower βγ concentrations (10, 50 nM), the phosphorylation was strongly reduced (data not shown). Lane 6 displays the outcome of rhodopsin phosphorylation by NG 108-15 cytosol in the absence of exogenous Gβγ. Under these conditions, the autoradiogram exhibits a faint band where rhodopsin migrates, indicating the strict requirement of Gβγ for the cytosolic receptor kinase to phosphorylate its substrate.

The rhodopsin bands served for a rough estimate of βARK activities, using purified recombinant βARK as standard. Based on counted ^{32}P-radioactivity we found an enzyme activity of 0.68 pmol/mg protein for cytosol (40 µg protein) of A431 cells and *ca.* 0.78 pmol/mg protein for NG 108-15 cytosol.

Opioid Receptor Phosphorylation by βARK

The presence of mRNA for rat βARK 1 and 2 in NG 108-15 cells as well as the documentation that cytosol phosphorylates rhodopsin caused us to test whether the δ-opioid receptor can be phosphorylated by βARK. The recombinant receptor material used originates from HIGH 5 insect cells, where they are suggested to interact with G_o [25]. Figure 3 demonstrates the agonist-dependent phosphorylation of the δ-opioid receptor by highly purified

recombinant bovine βARK1. Using 1.5 pmol receptor protein (quantified by means of [^{3}H]diprenorphin binding) activated by 1 μM DPDPE, electrophoresis of the phosphorylated material and subsequent autoradiography revealed an increased staining at 38 kDa (lane 3). Staining was prevented in the presence of 1 μM naloxone (lane 4), indicating that receptor phosphorylation occurs in an agonist-dependent manner. Use of only 750 fmol receptor protein failed to produce a clear phosphorylation signal in the presence of DPDPE (lane 1). Relation of the visualized phosphorylation band to the δ-opioid receptor was conducted by means of the Western blot technique. Lane 5 displays immunoreactivity of the enriched δ-opioid receptor material (SDS lysate) visualized by enhanced chemoluminiscence (Amersham), which is missing in nontransfected HIGH 5 insect cells (lane 6) [25]. Apparently, the immunoreactive receptor material comigrates with the phosphorylated receptor (lane 3).

DISCUSSION

NG 108-15 cells represent a classical cell line to study mechanisms underlying opioid actions, including tolerance and dependence [4]. Use of these hybrid cells to study desensitization of opioid receptors by GRKs has attracted interest, and these kinases have been implicated in opioid receptor phosphorylation [5, 6, 7]. Since the literature provides no information concerning GRKs in NG 108-15 cells, this study was designed to obtain initial information as to whether this δ-opioid receptor carrying cell line can be utilized for investigations into β-adrenergic receptor kinases. We report here that NG 108-15 cells own mRNA coding for rat βARK1 and 2, that they exhibit βARK activity phosphorylating rhodopsin, and that purified bovine βARK phosphorylates enriched δ-opioid receptor material.

The suggestion that NG 108-15 cells contain the message for rat βARK1 is based on the detection of a PCR product consisting of 987 base pairs. This material exhibits a 97% identity to the corresponding sequence of rat βARK1, representing approximately half the size of the published rat kinase (2683 bp, [13]). The size of the fragment as well as the high identity to the rat enzyme leads us to propose the existence of mRNA coding for rat βARK1 for NG 108-15 cells. Similarly, the PCR product obtained with primers designed to detect rat βARK2 also represents a fragment (992 bp) of the rat enzyme (2464 bp), displaying an identity with rat βARK2 of 94%. Again, size and degree of homology to rat βARK2 [13] strongly suggests the presence of the corresponding mRNA in the cells. In fact, βARK1 may be expected to be present in NG 108-15 cells (mouse neuroblastoma fused with rat glioma, [9]), as this isoenzyme has been proposed to be present in rat glia cells [13].

Although no information is presently available for glia cells regarding additional GRKs, including βARK2, the presence of mRNA for rat βARK2 defines rat glia as origin for this material. We failed, however, to provide evidence for the presence of mouse βARK cDNA in NG 108-15 cells, although the PCR-primers used were designed to specifically recognize a 473 bp DNA out of a 633 fragment communicated for mouse "kinase" (βARK [14]). Although this fragment displays a high similarity (96.9%) to rat βARK1, distinction between mouse and rat βARK1 is rendered possible by use of the reverse primer oligonucleotide employed here. We assumed a specific mouse βARK in NG 108-15 cells, since one component of the hybrid cell originates from mouse neurons, and neurons, e.g. of the rat [13], are rich in mRNA for both βARK1 and 2 isoenzymes.

The existence of mRNAs for βARK 1 and 2 in NG 108-15 cells is highly suggestive of the expression of both enzymes. Indeed, their cytosol revealed a capacity to phosphorylate rhodopsin, a substrate for βARK [15]. A rough estimate suggests a similar GRK activity as described for A431 cells [26], and a 3-fold higher activity as compared with human heart myocytes [27]. The kinase activity determined for NG 108-15 cells does not distinguish between βARK 1 and 2, and does not inform about participation of additional receptor kinases. However, rhodopsin phosphorylation by NG 108-15 cytosol was strongly dependent on the presence of exogenous Gβγ. This finding links the cytosolic kinase activity to βARK 1 and 2, respectively, since out of the six GRKs known only βARK1 and 2 depend on the presence of Gβγ to bring about receptor phosphorylation [16, 17, 22].

The impact of the findings reported here with respect to the function of acute and chronic opioid actions is emphasized by the documentation that highly purified recombinant bovine βARK1 brings about phosphorylation of agonist-occupied δ-opioid receptor preparations. This kind of experiment was conducted with enriched recombinant opioid receptors obtained from HIGH 5 insect cells [25]. The technique provides sufficiently concentrated G protein-coupled receptor material to enable Gβ/γ-dependent phosphorylation studies with recombinant βARK1. The fact that βARK1 phosphorylates δ-opioid receptors is not surprising, as a number of G protein-coupled receptors are substrates of this enzyme [2], and opioid receptors are phosphorylated in HEK293 and COS-7 cells overexpressing βARK [5, 6]. The βARK activity found in cytosol of NG 108-15 cells appears high enough to postulate a major function in the mechanisms underlying opioid tolerance. To distinguish between βARK 1 and βARK 2 with respect to their biologic activities, experiments should be conducted with defined Gβγ combinations [22], an issue beyond the aim of the present investigation.

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