β-Adrenergic Receptor Kinase: Primary Structure Delineates a Multigene Family

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The β-adrenergic receptor kinase (β-ARK), which specifically phosphorylates only the agonist-occupied form of the β-adrenergic and closely related receptors, appears to be important in mediating rapid agonist-specific (homologous) desensitization. The structure of this enzyme was elucidated by isolating clones from a bovine brain complementary DNA library through the use of oligonucleotide probes derived from partial amino acid sequence. The β-ARK cDNA codes for a protein of 689 amino acids (79.7 kilodaltons) with a protein kinase catalytic domain that bears greatest sequence similarity to protein kinase C and the cyclic adenosine monophosphate (cyclic AMP)–dependent protein kinase. When this clone was inserted into a mammalian expression vector and transfected into COS-7 cells, a protein that specifically phosphorylated the agonist-occupied form of the β_2-adrenergic receptor and phosphorylated, much more weakly, the light-bleached form of rhodopsin was expressed. RNA blot analysis revealed a messenger RNA of four kilobases with highest amounts in brain and spleen. Genomic DNA blot analysis also suggests that β-ARK may be the first sequenced member of a multigene family of receptor kinases.

REGULATION OF CELLULAR SENSITIVITY TO SENSORY, NEUrotransmitter, and hormonal stimuli is observed in diverse signaling systems. Attenuation of cellular responsiveness, generally termed desensitization, has been most extensively studied for cell surface receptors that signal to the interior of the cell via their interaction with guanine nucleotide regulatory proteins or G proteins. The prototypic model for these studies has been the β-adrenergic receptor (β-AR), which mediates the stimulatory effects of catecholamines on the adenylyl cyclase system and thereby regulates intracellular cyclic AMP levels (*1*). Desensitization in such systems may be mediated by regulation at several different points including the receptors, the G proteins, and the effector enzyme.

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Several different mechanisms of desensitization that operate at the receptor have been identified (*2*). These pathways either remove receptors from the plasma membrane by internalization within the cell or functionally inactivate the receptors within the plasma membrane. Such "uncoupling" of receptors appears to be mediated by receptor phosphorylation (*3*). Several different protein kinases appear to participate in this receptor phosphorylation, including the cyclic AMP–dependent protein kinase (*4*) and a novel cyclic AMP–independent kinase termed the β-adrenergic receptor kinase (β-ARK) (*5*). β-ARK has the unusual property of phosphorylating its substrate (the β-AR) only when the receptor is stimulated by agonist, thus coupling receptor activation and desensitization.

Purified preparations of β-ARK phosphorylate in vitro the agonist-occupied forms of several G protein–coupled receptors including the β_2-AR, α_2-adrenergic receptor, and the muscarinic cholinergic receptor, all of which either stimulate or inhibit adenylyl cyclase (*6*). Such preparations also weakly phosphorylate light-bleached rhodopsin (*7*). Moreover, stimulus-dependent receptor phosphorylation, occurring in association with attenuation of receptor function, is also seen in the α_1-adrenergic (*8*) and muscarinic cholinergic receptors (*9*), the cyclic AMP receptor from *Dictyostelium discoideum* (*10*), the α–mating factor receptor from yeast (*11*), and the "light receptor" rhodopsin in the retina (*12*). The phosphorylation of rhodopsin is effected by the enzyme rhodopsin kinase, which is, in a number of respects, analogous to β-ARK (*7, 13, 14*).

The wide occurrence of such stimulus-dependent receptor phosphorylation events in organisms from yeast to mammals and in response to signals as divergent as pheromones, photons of light, and neurotransmitters suggests a general role for such receptor kinases in controlling cellular responsiveness. Moreover, it raises numerous questions about the number and diversity of such kinases, their specificity, tissue and cellular distribution, biological roles, and evolutionary relation to other regulatory kinases. In order to examine these and related issues experimentally, we have cloned the cDNA for the β-ARK and now report its complete deduced amino acid sequence. Our results also indicate the presence of several other closely related genes, thus suggesting the existence of a multigene family of related receptor kinases.

Isolation of cDNA clones for β-ARK. The β-ARK was purified approximately 20,000-fold from bovine brain (*13*). The purified protein migrated as a single band of 80 kD on SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1A). Attempts at sequencing the intact protein were unsuccessful, suggesting that the NH_2-terminus is blocked. Thus, approximately 50 μg of purified β-ARK was lyophilized and digested with cyanogen bromide in 70 percent

formic acid. The peptides resulting from this hydrolysis were resolved by reversed-phase high-performance liquid chromatography (HPLC) (Fig. 1B). Peaks 1 through 5 yielded distinct amino acid sequences when subjected to gas-phase sequencing. In an attempt to generate a polyclonal antibody, we synthesized one of the peptide sequences (peak 2) with an NH_2-terminal cysteine and coupled it to chicken ovalbumin (*15*). Rabbit antiserum to the ovalbumin-coupled peptide specifically recognized β-ARK in an immunoblot analysis (Fig. 1C). The antiserum binding to β-ARK is specifically blocked by the synthetic peptide but is not blocked by ovalbumin, demonstrating that this peptide sequence is indeed from β-ARK

A size-selected (>4.4 kb), randomly primed cDNA library from bovine brain with 50,000 independent clones in λ ZAP was screened with two synthetic oligonucleotide probes derived from the amino acid sequences of peptides 1 and 2 (*16*). Seven distinct clones that hybridized with both radiolabeled oligonucleotide probes were isolated. The restriction map and sequencing strategy for two of these clones are shown (Fig. 2A). One clone, termed pβ-ARK3A, was 3949 bp in length and contained an open reading frame of 689 amino acids starting with a potential initiation codon (Fig. 2B). This open reading frame codes for a protein of 79.7 kD, similar to the size of purified β-ARK (*13*). A second clone, termed pβ-ARK4, was 2583 bp with an open reading frame identical to that in pβ-ARK3A and an additonal 56 bp of 5′ untranslated sequence. Each of the seven distinct clones isolated appears to be derived from messenger RNA (mRNA) transcribed from a single gene. However, at least one clone contains several inserts in its sequence that appear to have resulted from incomplete mRNA processing. These inserts occur at bp 642 and 1138 of the sequence

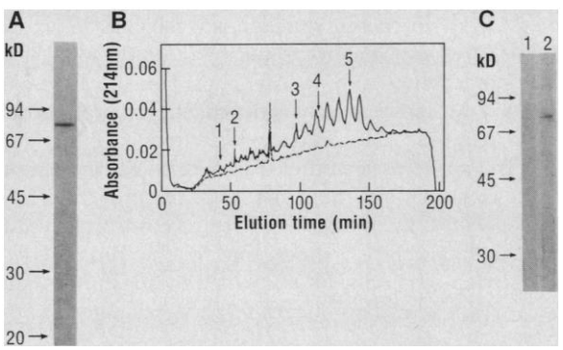

Fig. 1. Purification, cyanogen bromide cleavage, and immunoblotting of the β-ARK. (**A**) β-ARK was purified from bovine brain as described (*13*) with several minor modifications (*29*). The final material from the CM Fractogel column was reapplied to this column after dilution to remove residual Tween 20 used in the purification. One percent of the sample was run on a 10 percent SDS- polyacrylamide gel (*30*), which was then stained with Coomassie blue. (**B**) Reversed-phase HPLC profile of cyanogen bromide–treated β-ARK. Purified β-ARK (~50 μg) was lyophilized and subjected to treatment with 0.5*M* cyanogen bromide in 70 percent formic acid for 20 hours at 23°C. The sample, after dilution with water and lyophilization, was dissolved in 10 percent acetonitrile, 0.1 percent trifluoroacetic acid (TFA), and injected on a W-Porex C4 column (0.2 by 10 cm). Elution was accomplished with a linear gradient from 10 to 50 percent acetonitrile in 0.1 percent TFA at a flow rate of 0.5 ml/min. Shown is the elution of the β-ARK (solid line) and buffer (dashed line) samples. The arrows denote the peptides 1 through 5 that gave a distinct amino acid sequence. (**C**) Immunoblot analysis of β-ARK with rabbit antiserum to peptide 2. Peptide 2 was synthesized, purified by reversed-phase HPLC, and coupled to chicken ovalbumin (Sigma) with the use of the reagent *m*-maleimidobenzoate *N*-hydroxysuccinimide (*15*). Partially purified (10 percent) β-ARK was run on a 10 percent SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with preimmune (lane 1) or immune (lane 2) serum (1:100 dilution). The strips were then incubated with ^{125}I-labeled protein A and visualized by autoradiography (*31*).

in Fig. 2B and may suggest the presence of introns at these sites in the β-ARK gene.

Several lines of evidence suggest that the first ATG in the sequence serves as the initiation site for translation. That is, there is a Kozak consensus sequence (*17*), a GC-rich region (83 percent in the first 104 bp), and an open reading frame that codes for a protein of the predicted molecular size. In addition, in vitro translation studies demonstrate that this ATG can, in fact, be used for the initiation of translation.

In vitro translation of mRNA transcribed from pβ-ARK3A was used to assess the size of the polypeptide encoded by the β-ARK cDNA. In the absence of RNA (Fig. 3A, lane 1) or in the presence of antisense RNA (lane 2), no protein product is made. However, in the presence of sense RNA (lane 3), a major protein of about 80 kD is translated. In addition, several smaller peptides also appear to be translated from this RNA, possibly because of initiation at internal methionines. This has been observed in other in vitro translation systems as well [for example, protein kinase C (*18*)].

Messenger RNA was also made from a different clone (pβ-ARK7) that begins at bp 109; that is, it lacks the 5′ untranslated sequence and putative first eight amino acids. When this mRNA was translated in vitro, a slightly smaller protein product is produced compared to mRNA from clone pβ-ARK3A (Fig. 3B, lanes 1 and 2). This is further evidence that the first ATG in the sequence can be used to initiate translation.

Structure of β-ARK and homology with other proteins. The overall topology of β-ARK suggests an NH_2-terminal domain of 197 amino acids, a central catalytic domain of 239 amino acids, and a COOH-terminal domain of 253 amino acids. The function of the NH_2- and COOH-terminal domains is unknown; however, at least one, if not both domains might participate in mediating the remarkable specificity of the kinase for phosphorylating only agonist-occupied receptors.

The predicted amino acid sequence of β-ARK reveals that the catalytic domain has substantial homology with the catalytic domains of other protein kinases. Thus, β-ARK has a Gly-X-Gly-X-X-Gly-$(X)^{10-16}$-Lys stretch, which is found in all adenosine triphosphate (ATP)–binding proteins (*19*). The first Gly in this stretch is at amino acid 198 and delineates an NH_2-terminal domain of 197 amino acids that bears no significant homology with any other sequenced proteins. One potentially interesting difference between the catalytic domain of β-ARK when compared to those of other protein kinases is in the sequence Asp-Phe-Gly, which appears to be involved in ATP binding (*20*) and which is found in virtually every protein kinase catalytic domain (*19*). The β-ARK sequence contains a Leu in place of the Phe. The conserved Arg at amino acid 436 separates the end of the catalytic domain from a COOH-terminal stretch of 253 amino acids. This COOH-terminal region has no significant homology with any other sequenced protein.

The homology of the catalytic domain of β-ARK with other protein kinases is greatest with the cyclic AMP–dependent protein kinase (33.1 percent identity in an overlap of 347 amino acids) and with protein kinase C (33.7 percent identity in an overlap of 303 amino acids). In Fig. 4 the catalytic domain of β-ARK is compared with the catalytic domains of other protein kinases by construction of a phylogenetic tree. These results suggest that β-ARK is a unique enzyme that is structurally most closely related to the cyclic nucleotide–dependent and protein kinase C subfamilies. It may, in fact, represent the first member of a new subfamily of receptor kinases.

Expression of β-ARK in COS-7 cells. A mammalian expression plasmid for β-ARK was constructed by inserting a Hind III fragment of clone pβ-ARK3A (3023 bp) into the Hind III site of the expression vector pBC12BI (*21*). The corresponding vector, termed pBCβ-ARK, was used to transfect COS-7 cells by the DEAE

dextran procedure (*22*). After a 72-hour incubation period, cells transfected with either pBC12BI alone or pBCβ-ARK were tested for β-ARK activity. Cells transfected with pBC12BI have a low basal β-ARK activity as assessed by the ability of a lysed cell supernatant fraction to phosphorylate light-bleached rhodopsin (Fig. 5A, lane 2). This basal activity corresponds to 1.2 pmol of phosphate per minute per milligram of protein, a value similar to that found in bovine brain (*13*). In contrast, COS-7 cells transfected with pBCβ-ARK show a 43-fold increase in β-ARK activity (Fig. 5A, lane 3). This corresponds to a specific activity of 51 pmol min^{-1} mg^{-1}.

The specificity of the expressed kinase was then determined with hamster β_2-AR and bovine rhodopsin as potential substrates. The purified β_2-AR is a substrate for the kinase preparation in an agonist-specific fashion (Fig. 5B, lanes 1 and 2). Background phosphorylation from the crude kinase preparation was minimal under the conditions of the experiment (lane 3). The kinase preparation also phosphorylates rhodopsin in a light-dependent manner (lanes 4 and 5). The preference for the β_2-AR as a substrate as compared to rhodopsin is also apparent in Fig. 5B (compare lane 2, which contains 1.1 pmol of β_2-AR, to lane 5, which contains 40 pmol of rhodopsin). Clearly, the β_2-AR is the preferred substrate for the expressed kinase. It remains to be determined whether the expressed kinase preferentially phosphorylates the β_2-AR as compared to other adenylyl cyclase–coupled receptors.

Localization of β-ARK and evidence for a multigene family. In an effort to examine the β-ARK mRNA size and distribution, we used RNA blot analysis with a 720-bp fragment from pβ-ARK3A (bp 630 to 1347). This fragment was labeled with ^{32}P by nick-translation and was then used to probe total RNA from various bovine tissues (Fig. 6A). The major mRNA species is a 4-kb band that appears at highest levels in brain and spleen. Heart and lung have about 40 percent the signal of brain, whereas ovary and kidney have only about 20 percent. There was no distinguishable mRNA band seen in liver, muscle, and adrenal gland. These results suggest that β-ARK mRNA may be localized in tissues that have a high degree of sympathetic innervation. In particular, brain, spleen, and heart, which have the highest amounts of β-ARK mRNA, are also densely innervated with high concentrations of norepinephrine-containing fibers. In contrast, liver and muscle, in which β-ARK mRNA is not detectable, show much less adrenergic innervation. In addition to the 4-kb mRNA there also appears to be a 2.5-kb species, predominant in heart and spleen. This may represent a

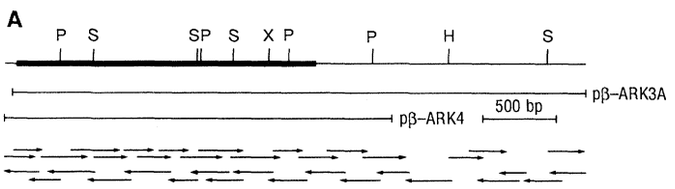

Fig. 2. Restriction mapping of the β-ARK clones and nucleotide and deduced amino acid sequence of bovine brain β-ARK. (**A**) Restriction map and sequencing strategy for the β-ARK clones. The restriction sites on clones pβ-ARK3A and pβ-ARK4 are indicated for the enzymes Sac I (S), Pst I (P), Hind III (H), and Xho I (X). Both clones contained the identical 689–amino acid open reading frame indicated by the heavy line. DNA sequencing was performed with the dideoxynucleotide chain termination method of Sanger (*32*). The extent and direction of the sequence reactions are shown by the arrows. Both clones were sequenced in their entirety. (**B**) The nucleotides are numbered on the left-hand side beginning with the 5′ region of clone pβ-ARK4 and ending at bp 2520. Clone pβ-ARK3A begins at bp 59 and is missing bp 73 to 79 (CGCCGCC) found in clone pβ-ARK4. The amino acid sequence shown begins at the first ATG in the nucleotide sequence and is numbered on the right-hand side. The sequenced peptides derived from the cyanogen bromide cleavage of purified β-ARK are underlined. The residues predicted to be involved in formation of the ATP binding site are marked by asterisks [Gly-X-Gly-X-X-Gly-$(X)^{10-16}$-Lys]. The 3′ 1485 bp of clone pβ-ARK3A is not shown in this figure; however, it does not contain a polyadenylation signal or polyadenylated tail.

B

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   1 CGACGGAGCGGCCGGGCCGGGCCGAGCGCGCCGAGCGGAGCGGAGCGCAGCCGAGCGGCGGGAGAGGCC

  70 GCCGCCGCCGCCAAGATGGCGGACCTGGAGGCGGTGCTGGCCGACGTGAGCTACCTGATGGCCATGGAG
                  MetAlaAspLeuGluAlaValLeuAlaAspValSerTyrLeuMetAlaMetGlu 18

 139 AAGAGCAAGGCCACGCCGGCGGCGCGCGCCAGCAAGAAGATCCTGCTGCCCGAGCCCAGCATCCGCAGC
     LysSerLysAlaThrProAlaAlaArgAlaSerLysLysIleLeuLeuProGluProSerIleArgSer 41

 208 GTCATGCAGAAGTACCTGGAGGACCGGGGCGAGGTGACTTTTGAGAAGATCTTCTCCCAGAAGCTGGGG
     ValMetGlnLysTyrLeuGluAspArgGlyGluValThrPheGluLysIlePheSerGlnLysLeuGly 64

 277 TACCTGCTTTTCCGAGACTTCTGCCTGAAGCACCTGGAGGAGGCCAAGCCCTTGGTAGAGTTCTACGAG
     TyrLeuLeuPheArgAspPheCysLeuLysHisLeuGluGluAlaLysProLeuValGluPheTyrGlu 87

 346 GAGATCAAGAAATACGAGAAGCTGGAGACAGAGGAGGAGCGCCTGGTCTGCAGCCGAGAGATCTTCGAC
     GluIleLysLysTyrGluLysLeuGluThrGluGluGluArgLeuValCysSerArgGluIlePheAsp 110

 415 ACGTACATCATGAAGGAGCTGCTGGCCTGCTCACATCCTTTCTCGAAGAGCGCCATTGAGCACGTCCAG
     ThrTyrIleMetLysGluLeuLeuAlaCysSerHisProPheSerLysSerAlaIleGluHisValGln 133

 484 GGCCATCTGGTGAAGAAGCAGGTGCCTCCGGATCTCTTCCAGCCATATATTGAAGAAATTTGCCAGAAC
     GlyHisLeuValLysLysGlnValProProAspLeuPheGlnProTyrIleGluGluIleCysGlnAsn 156

 553 CTCCGAGGAGACGTGTTCCAGAAATTCATCGAGAGCGATAAATTCACACGATTTTGCCAGTGGAAGAAT
     LeuArgGlyAspValPheGlnLysPheIleGluSerAspLysPheThrArgPheCysGlnTrpLysAsn 179

 622 GTAGAGCTCAACATCCACCTGACCATGAACGACTTCAGTGTGCACCGCATCATCGGGCGAGCGGCTTC
     ValGluLeuAsnIleHisLeuThrMetAsnAspPheSerValHisArgIleIleGlyArgGlyGlyPhe 202

 691 GGTGAGGTCTACGGCTGCCGGAAGGCCGACACGGGCAAGATGTACGCCATGAAGTGTCTGGACAAGAAG
     GlyGluValTyrGlyCysArgLysAlaAspThrGlyLysMetTyrAlaMetLysCysLeuAspLysLys 225

 760 CGCATCAAGATGAAGCAAGGGGAGACTCTGGCCCTGAATGAGCGCATCATGCTGTCGCTCGTCAGCACC
     ArgIleLysMetLysGlnGlyGluThrLeuAlaLeuAsnGluArgIleMetLeuSerLeuValSerThr 248

 829 GGGGACTGCCCGTTCATCGTCTGCATGTCATACGCCTTCCACACACCGGACAAGCTCAGCTTCATCCTG
     GlyAspCysProPheIleValCysMetSerTyrAlaPheHisThrProAspLysLeuSerPheIleLeu 271

 898 GATCTCATGAACGGCGGGGACCTGCACTACCACCTGTCCCAGCACGGGGTCTTCTCCGAGGCCGACATG
     AspLeuMetAsnGlyGlyAspLeuHisTyrHisLeuSerGlnHisGlyValPheSerGluAlaAspMet 294
                                  2
 967 CGTTTCTACGCCGCCGAGATCATCCTGGGCCTGGAGCACATGCACAACCGCTTCGTGGTCTACCGGGAC
     ArgPheTyrAlaAlaGluIleIleLeuGlyLeuGluHisMetHisAsnArgPheValValTyrArgAsp 317
                              3
1036 CTGAAGCCGGCCAACATCCTGCTGGACGAGCACGGCCACGTGCGCATCTCAGACCTGGGCCTGGCCTGT
     LeuLysProAlaAsnIleLeuLeuAspGluHisGlyHisValArgIleSerAspLeuGlyLeuAlaCys 340

1105 GACTTCTCCAAGAAGAAGCCTCACGCCAGTGTGGGCACCCACGGGTACATGGCTCCCGAGGTTCTACAG
     AspPheSerLysLysLysProHisAlaSerValGlyThrHisGlyTyrMetAlaProGluValLeuGln 363

1174 AAGGGTGTGGCCTACGACAGCAGCGCCGACTGGTTCTCCCTGGGCTGCATGCTCTTCAAGCTGCTGCGA
     LysGlyValAlaTyrAspSerSerAlaAspTrpPheSerLeuGlyCysMetLeuPheLysLeuLeuArg 386

1243 GGGCATAGCCCTTTCCGGCAGCACAAGACCAAAGACAAGCATGAGATCGACAGAATGACATTGACAATG
     GlyHisSerProPheArgGlnHisLysThrLysAspLysHisGluIleAspArgMetThrLeuThrMet 409

1312 GCTGTGGAGCTGCCTGACTCCTTCTCCCCTGAGCTCCGCTCCTTGCTGGAGGGGCTGCTGCAGAGGGAT
     AlaValGluLeuProAspSerPheSerProGluLeuArgSerLeuLeuGluGlyLeuLeuGlnArgAsp 432

1381 GTCAACCGGAGGCTAGGCTGCCTGGGCCGAGGGGCCCAGGAGGTGAAGGAGAGCCCCTTCTTCCGTTCC
     ValAsnArgArgLeuGlyCysLeuGlyArgGlyAlaGlnGluValLysGluSerProPhePheArgSer 455

1450 CTGGACTGGCAGATGGTCTTTTTACAAAAGTACCCTCCCCCGTTGATCCCCCCACGAGGGGAGGTGAAT
     LeuAspTrpGlnMetValPheLeuGlnLysTyrProProProLeuIleProProArgGlyGluValAsn 478
                                  5
1519 GCAGCCGACGCCTTTGACATTGGCTCCTTCGATGAGGAGGACACAAAAGGAATCAAGCTACTGGACAGT
     AlaAlaAspAlaPheAspIleGlySerPheAspGluGluAspThrLysGlyIleLysLeuLeuAspSer 501

1588 GACCAGGAGCTCTACCGCAACTTCCCCCTGACCATCTCGGAGCGGTGGCAGCAGGAGGTAGCAGAGACT
     AspGlnGluLeuTyrArgAsnPheProLeuThrIleSerGluArgTrpGlnGlnGluValAlaGluThr 524

1657 GTCTTTGACACCATCAATGCTGAGACGGACCGGCTGGAGGCCCGCAAGAAAACCAAAAACAAGCAGTTG
     ValPheAspThrIleAsnAlaGluThrAspArgLeuGluAlaArgLysLysThrLysAsnLysGlnLeu 547

1726 GGCCACGAGGAAGACTACGCCCTGGGCAAGGACTGCATCATGCATGGCTACATGTCCAAGATGGGCAAC
     GlyHisGluGluAspTyrAlaLeuGlyLysAspCysIleMetHisGlyTyrMetSerLysMetGlyAsn 570

1795 CCCTTCCTGACCCAGTGGCAGCGGCGGTACTTCTACCTGTTCCCTAACCGGCTCGAGTGGCGGGGCGAG
     ProPheLeuThrGlnTrpGlnArgArgTyrPheTyrLeuPheProAsnArgLeuGluTrpArgGlyGlu 593

1864 GGCGAGGCCCCGCAGAGCCTGCTGACCATGGAGGAGATCCAGTCGGTGGAGGAGACGCAGATCAAGGAG
     GlyGluAlaProGlnSerLeuLeuThrMetGluGluIleGlnSerValGluGluThrGlnIleLysGlu 616
                                  4
1933 CGAAAGTGCCTCCTCCTCAAGATCCGAGGTGGCAAGCAGTTTGTCCTGCAGTGCGATAGTGACCCAGAG
     ArgLysCysLeuLeuLeuLysIleArgGlyGlyLysGlnPheValLeuGlnCysAspSerAspProGlu 639

2002 CTGGTGCAGTGGAAGAAGGAGCTTCGAGACGCCTACCGCGAGGCCCAGCAGCTAGTGCAGCGGGTGCCC
     LeuValGlnTrpLysLysGluLeuArgAspAlaTyrArgGluAlaGlnGlnLeuValGlnArgValPro 662

2071 AAGATGAAGAACAAGCCGCGCTCGCCCGTCGTGGAGCTGAGCAAGGTGCCACTGATCCAGCGCGGCAGT
     LysMetLysAsnLysProArgSerProValValGluLeuSerLysValProLeuIleGlnArgGlySer 685

2140 GCCAACGGCCTCTGACCCGCCCGCTAGCCCGGTCGCCCTCCTGCCCGCCCGCCTTTTATAAACCTCTAA
     AlaAsnGlyLeu .                                                        689

2209 TTTATTTTGTTGAATTTTTATTATTTGTTTTCCCGCCAAGCGGAAAAGGTTTTATTTTGTAATTATTGT

2278 GATTTCCTGTGGCCCCAGCCTGGACCAGCCCCCAGGGAGGGGCCCGCTTGCCTTGGCTCCTGCTGCCAC

2347 CCACCCAGCCACTGTCCAGCCACCTCACCCTGCCCCCAGGGGCCTCCGCTCCCGGTGTCTTCCCCACAC

2416 GGGGAGAGCAGCAGCCCCCAGCGCCCTCCCGCCCTTCCCCAGGGATGGTCGCCTGGAGTTGGGCCTGCC

2485 GTCCCCTCTCCCCAGCTCTGTGCCCACAGCACAGCT 2520
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distinct mRNA or, more likely, may be due to alternative RNA precursor processing.

Since β-ARK mRNA was highest in the brain, total RNA from various brain regions was also probed. We analyzed cortex, cerebellum, basal ganglia, brain stem, pituitary, and hypothalamus (Fig. 6B). Highest β-ARK mRNA levels were seen in cortex and cerebellum, with significantly lower levels in the other brain regions. Identical results were observed when a 3-kb Hind III fragment from clone pβ-ARK3A was used to probe the RNA blots.

DNA blot analysis of bovine genomic DNA digested with either Hind III or Sac I was performed with the 717-bp Sac I restriction fragment from pβ-ARK3A. Low-stringency washing reveals multiple hybridizing bands in both the Hind III and Sac I digests (Fig. 7). The Hind III digest had two major bands of about equal intensity at approximately 4 and 3.5 kb; three minor bands (6.5, 6, 5.5 kb) were also observed. The Sac I digest also demonstrates the presence of multiple hybridizing species with bands seen at 2.3, 1.0, and 0.8 kb. Although restriction sites for Hind III and Sac I are not found within the 717-bp Sac I fragment used to probe this blot, it is possible that a site or sites might exist within an intron in the β-ARK gene. However, when the 717-bp fragment was cut with Ava I, yielding 530- and 187-bp fragments, each of these smaller fragments hybridized with all of the same bands as the 717-bp probe. These results suggest that the multiple hybridizing species are not due to the presence of restriction sites within an intron. Thus, these additional bands likely represent other genes that the β-ARK probe is recognizing and suggest that β-ARK is a member of a multigene family. In fact, we have recently isolated a cDNA species distinct from β-ARK by low-stringency hybridization. This cDNA encodes a protein of 688 amino acids with 85 percent amino acid identity with β-ARK (*23*).

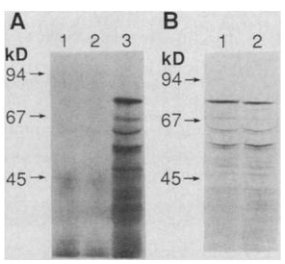

Fig. 3. In vitro translation of the β-ARK clone. (**A**) Clone pβ-ARK3A, which was contained in the vector Bluescript (Stratagene), was linearized with either Bam HI or Sal I and was then used for in vitro transcription with either T7 (Bam HI) or T3 (Sal I) polymerase. Capped RNA (1 μg) was translated in vitro with a rabbit reticulocyte lysate (*33*) for 30 min at 30°C in the presence of ~25 μCi of [^{35}S]methionine. The samples were then run on an 8 percent SDS-polyacrylamide gel, which was then fixed in 40 percent methanol, 10 percent acetic acid for 30 min before incubation in Enlightning (Dupont) for 30 min. The dried gel was subjected to autoradiography for 36 hours at room temperature. The various samples were no RNA (lane 1), antisense RNA transcribed with T3 polymerase (lane 2), and sense RNA transcribed with T7 polymerase (lane 3). (**B**) Clone pβ-ARK3A, linearized with Bam HI, and clone pβ-ARK7, linearized with Cla I, were used in in vitro transcription. The capped RNA was then translated in vitro as described above. Lane 1, RNA from clone pβ-ARK3A; lane 2, RNA from pβ-ARK7. Clone pβ-ARK7 contains the sequence beginning at bp 109 and ending at bp 2166. Most likely it is translated beginning at bp 127. This represents the first ATG in clone pβ-ARK and gives an open reading frame of 675 amino acids (78.2 kD), which coincides with the mobility of the major band in lane 2.

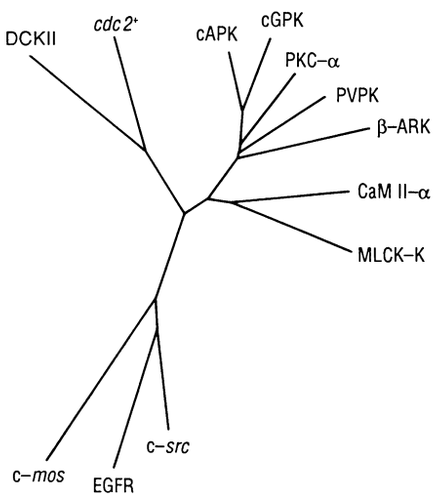

Fig. 4. Deduced phylogeny of the β-ARK catalytic domain. The catalytic domain of β-ARK (amino acids 180 to 436) was compared with the catalytic domains of several other protein kinases by construction of a phylogenetic tree. The methodology used to construct the tree has been described (*20*). The kinases used for the comparison were myosin light chain kinase (MLCK-K); Ca^{2+} calmodulin–dependent protein kinase type II; alpha subunit (CaMII α); dicot bean (*Phaseolus vulgaris L.*) protein kinase (PVPK) (*34*); protein kinase C, α form (PKC α); cyclic guanosine monophosphate–dependent protein kinase (cGPK); cyclic AMP–dependent protein kinase (cAPK); "cell-division-cycle" gene product in yeast (*cdc2*$^+$); *Drosophila* casein kinase II, α subunit (DCKII); c-*mos* protooncogene (c-*mos*); epidermal growth factor receptor (EGFR); and c-*src* protooncogene (c-*src*).

Biological implications. Several lines of experimental evidence have indicated the involvement of receptor phosphorylation in rapid desensitization of the β-adrenergic and related G protein–coupled receptors. The best studied enzymes involved in these reactions are the cyclic AMP–dependent protein kinase, which mediates heterologous or agonist nonspecific desensitization (*4, 24*), and β-ARK, which is involved in homologous or agonist-specific desensitization (*5*). The homologous nature of the effects of β-ARK on several different receptors is presumably explained by its specificity for only the agonist-occupied forms of these receptors. The evidence supporting the physiological relevance of these receptor phosphorylations includes the following:

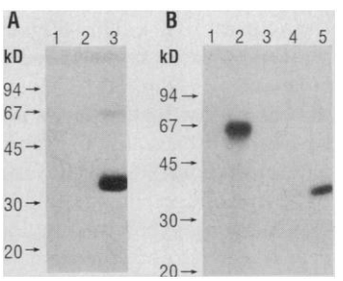

Fig. 5. Expression of β-ARK in COS-7 cells. (**A**) The Hind III fragment of clone pβ-ARK3A (3026 bp) was inserted into the Hind III site of the expression vector pBC12BI (*21*). COS-7 cells grown to ~75 percent confluence with Dulbecco's modified Eagle's medium (DMEM) containing 10 percent fetal calf serum were transfected with pBC12BI alone or pBCβ-ARK by the DEAE dextran procedure (*22*). The cells were scraped 72 hours after transfection into 2.5 ml of ice cold buffer [25 m*M* tris-HCl, *p*H 7.5, 5 m*M* EDTA, 0.1 m*M* phenylmethyl sulfonyl fluoride, pepstatin (5 μg/ml), leupeptin (10 μg/ml), and benzamidine (10 μg/ml)] and were lysed with a Brinkmann tissue disruptor followed by centrifugation at 300,000*g* for 20 min. The supernatant was then assessed for its ability to phosphorylate urea-treated rod outer segments, which were prepared as described (*35*). The incubations contained 20 m*M* tris-HCl, *p*H 7.5, 2 m*M* EDTA, 6 m*M* $MgCl_2$, 100 μ*M* ATP (~3 cpm/fmol), 400 pmol of rhodopsin, and either no supernatant (lane 1), 2 μl of pBC12BI supernatant (lane 2), or 2 μl of pBCβ-ARK supernatant (lane 3). The samples were incubated in room light for 20 min at 30°C and were quenched by the addition of 0.5 ml of cold 100 m*M* sodium phosphate, *p*H 7.5, and 10 m*M* EDTA. After centrifugation at 300,000*g* for 15 minutes the pellets were suspended in 100 μl of SDS sample buffer and separated by electrophoresis on a 10 percent homogeneous SDS-polyacrylamide gel. The dried gel was subjected to autoradiography for 2 hours at room temperature. (**B**) Specificity of the expressed kinase was determined with hamster lung β_2-AR purified as described (*36*) and urea-treated rod outer segments. The purified β_2-AR was reconstituted into phosphatidylcholine vesicles before being used as a substrate (*37*). The reactions contained 20 m*M* tris-HCl, *p*H 7.5, 2 m*M* EDTA, 6 m*M* $MgCl_2$, 6 m*M* sodium phosphate, 4 ng of synthetic cyclic AMP dependent–protein kinase inhibitor peptide (*38*), and 2 μl of pBCβ-ARK–transfected cell supernatant. The reactions also contained 1.1 pmol of reconstituted β_2-AR in the absence (lane 1) or presence (lane 2) of 50 μ*M* (−)isoproterenol or 40 pmol of rhodopsin in the absence (lane 4) or presence (lane 5) of room light. Lane 3 contains phospholipid vesicles without receptor. The samples were incubated for 20 minutes at 30°C and were quenched, centrifuged, and separated by electrophoresis as described above. After electrophoresis, the dried gel was subjected to autoradiography for 9 hours at room temperature. There was no phosphorylation of the receptor preparations in the absence of added kinase.

1) Phosphorylation of β-AR by either kinase in vitro reduces the functional ability of the receptor to activate the stimulatory G protein G_s in a reconstituted phospholipid vesicle system. In the case of β-ARK, demonstration of this effect requires the addition of the retinal protein arrestin (*25*). In the analogous retinal system, this protein binds to rhodopsin kinase–phosphorylated rhodopsin and uncouples it from transducin (*26*). It has been proposed that an analogous protein may function with β-ARK (*25*).

2) Mutagenesis of phosphorylation sites on the receptor for either the cyclic AMP–dependent protein kinase or β-ARK reduces receptor phosphorylation and attenuates desensitization whether tested either in whole cells or by adenylyl cyclase assays in membranes (*27*).

3) Specific inhibitors of the cyclic AMP–dependent protein kinase (PKI) or β-ARK (heparin) attenuate desensitization (*28*) with effects equivalent to those obtained by blocking receptor phosphorylation by mutagenesis.

The ability of the cyclic AMP–dependent kinase and β-ARK to mediate rapid desensitization occurs over quite different ranges of agonist concentrations. Since stimulation of cyclic AMP–dependent kinase activity approaches maximal levels at very low concentrations of catecholamine ($10^{-8}M$), desensitization mediated by this mechanism occurs at these low concentrations. In contrast, β-ARK–mediated phosphorylation occurs only with significant receptor occupancy (typically $10^{-8}M$ to $10^{-6}M$ catecholamine). These findings might suggest that the cyclic AMP–dependent protein kinase is primarily involved in mediating desensitization at peripheral receptors responding to the very low circulating levels of epinephrine. In contrast, β-ARK would seem better adapted to responding to the much higher neurotransmitter concentrations found at sympathetic synapses. Therefore, β-ARK mRNA is most prevalent in those tissues that have the densest sympathetic innervation and is less prevalent in the least innervated tissues (Fig. 6A).

There remain many unanswered questions about β-ARK and other putative related gene products, including the number of such kinases and their specificity and distribution. The genomic DNA blot and low-stringency hybridization analysis (*23*) already give evidence for at least one related gene. Rhodopsin kinase is an obvious additional candidate, as are ligand-dependent receptor kinases in *Dictyostelium discoideum* (*10*) and yeast (*11*). Various cross hybridization strategies should permit the cloning of these genes.

Another interesting issue relates to the evolutionary origin of β-ARK. As shown by the phylogenetic tree in Fig. 4, β-ARK appears to have evolved from the same progenitor as the cyclic AMP–dependent and Ca^{2+}-phospholipid–dependent protein kinase subfamilies. This is of particular interest since all three of these kinases function to mediate or modulate G protein–coupled receptor actions. Understanding the structure and function of this family of receptor kinases should provide fundamental insights into one of the most pervasive and basic types of biological phenomena, regulation of cellular sensitivity to environmental stimuli.

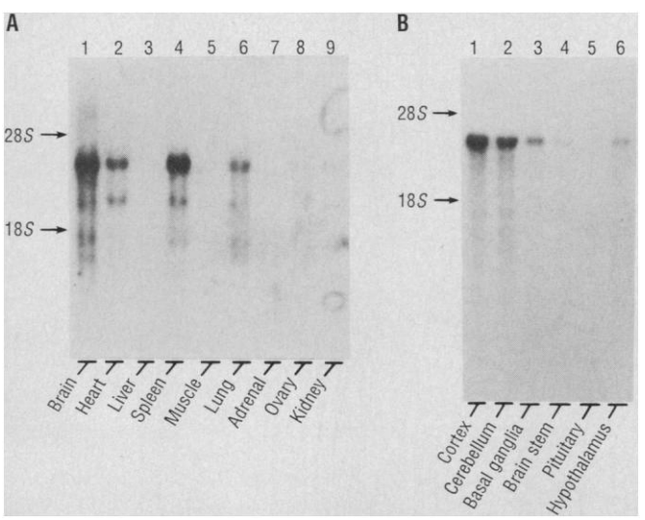

Fig. 6. RNA blot analysis of bovine mRNA from various tissues. (**A**) Total RNA (20 μg) from various bovine tissues were fractionated on a 1.2 percent agarose-formaldehyde gel (*39*) and transferred to Hybond-N (Amersham). The RNA blot was hybridized with a nick-translated radioactive Sac I fragment (bp 630 to 1347) in 50 percent formamide, 5× saline sodium citrate (SSC), *p*H 7, 50 m*M* Na phosphate, *p*H 6.5, 1× Denhardt's, 0.5 percent SDS, and denatured salmon sperm DNA (150 μg/ml) for 36 hours at 42°C. The blot was washed in 2× SSC at 50°C and was subjected to autoradiography for 72 hours at −80°C. (**B**) Total RNA (20 μg) from various bovine brain regions was fractionated and hybridized with a nick-translated Sac I fragment of clone pβ-ARK3A as described above. The blot was washed in 2× SSC at 50°C and was subjected to autoradiography for 24 hours at −80°C.

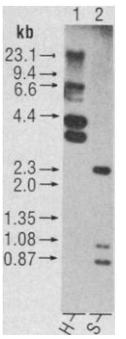

Fig. 7. DNA blot of bovine genomic DNA. DNA was prepared from frozen bovine brain by incubation for 16 hours at 50°C with 10 volumes of 100 m*M* NaCl, 10 m*M* tris-HCl, *p*H 8, 25 m*M* EDTA, 0.5 percent SDS, and proteinase K (0.1 mg/ml) (*40*). After extraction and precipitation, the DNA was dissolved at a concentration of 1 mg/ml. The DNA (~10 μg) was then digested with the enzymes Hind III (H) and Sac I (S) for 16 hours at 37°C. The DNA was fractionated on a 1 percent agarose gel, transferred to Hybond-N, and hybridized with a nick-translated radioactive Sac I fragment (bp 630 to 1347). The hybridization and washing conditions were the same as in Fig. 6.

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15. F.-T. Liu, M. Zinnecker, T. Hamaska, D. H. Katch, *Biochemistry* **18**, 690 (1979); N. Green *et al.*, *Cell* **28**, 477 (1982). The peptide coupled to ovalbumin was CMNGGDLHYRLSQARVF. This peptide contains three amino acids (underlined) that differ from the sequence shown in Fig. 2B. These differences appear to have been caused by a contaminant peptide in the sequencing run. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
16. The amino acid sequences of peptides 1 and 2 were MKQGETLALNERI (no. 1)

and MNGGDLHYXLSQAGVFSEAV (no. 2) The synthetic oligonucleotide probes used for screening the cDNA libraries were ATGAAGCAGGGCGAGAC-CTGGCCCTGAATGAGCGGAT (no. 1) and ATGAATGGCGGCGACCTG-CACTAGIIICTGTCCCAGGCCGGCGTCTTCTCCGAGGCTGT (no. 2). The oligonucleotides were predicted from codon bias tables [R. Lathe, *J. Mol. Biol.* **183**, 1 (1985)]. The inosines (I) in the oligonucleotide 2 sequence were inserted because the amino acid at this position could not be determined. The underlined bases delineate differences between the predicted and determined sequences (Fig. 2B). The mismatches were 4 of 38 for probe 1 and 8 of 59 for probe 2. The protein sequences from peptides 3, 4, and 5 were identical to those in Fig. 2B.
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41. We thank S. Hanks for analyzing the sequence of the β-ARK catalytic domain, R. Dixon for providing the bovine brain cDNA library, and B. Cullen for providing the vector pBC12BI. We also thank L. Obeid, J. Berry, J. Arriza, D. Stumpo, K. Daniels, C. Staniszewski, and G. Irons for their contributions to this work; R. Randall and C. Coonley for peptide sequencing; M. McAdams for peptide synthesis; K. Theisen for oligonucleotide synthesis; and M. Holben and D. Addison for secretarial assistance.

" The duck-pond could use some attention."