High Affinity Binding of β-Adrenergic Receptor Kinase to Microsomal Membranes

MODULATION OF THE ACTIVITY OF BOUND KINASE BY HETEROTRIMERIC G PROTEIN ACTIVATION*

(Received for publication, September 14, 1995, and in revised form, October 30, 1995)

Cristina Murga§, Ana Ruiz-Goméz, Irene García-Higuera†, Chong M. Kim, J effrey L. Benovic, and Federico Mayor, Jr.**

From the Department of Pharmacology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and the Centro de Biología Molecular “Severo Ochoa,” Consejo Superior de Investigaciones Científicas-Universidad Autónoma, 28049 Madrid, Spain

The β-adrenergic receptor kinase (βARK) modulates β-adrenergic and other G protein-coupled receptors by rapidly phosphorylating agonist-occupied receptors at the plasma membrane. We have recently shown that βARK also associates with intracellular microsomal membranes both "in vitro" and "in situ" (García-Higuera, I., Penela, P., Murga, C., Egea, G., Bonay, P., Benovic, J. L., and Mayor, F., Jr. (1994) J. Biol. Chem. 269, 1348–1355), thus suggesting a complex modulation of the subcellular distribution of βARK. In this report, we used recombinant [35S]methionine-labeled βARK to show that this kinase interacts rapidly with a high affinity binding site (Kd of 20 ± 1 nM) present in salt-stripped rat liver microsomal membranes. Although βARK binding is not modulated by membrane preincubation with G protein activators, the activity of bound βARK toward rhodopsin or a synthetic peptide substrate was markedly enhanced upon stimulation of the endogenous heterotrimeric G proteins present in the microsomal membranes by AlF4 or mastoparan/guanosine 5'-[3-O-thio]triphosphate, thus strongly suggesting a functional link between these proteins and membrane-associated βARK. Interestingly, βARK association with microsomal membranes is not significantly affected by a fusion protein derived from the carboxyl terminus of βARK1 (the proposed location of the βγ subunit binding site), whereas it is markedly inhibited by fusion proteins corresponding to the amino-terminal region of the kinase. The main determinants of binding appear to be localized to a ~60-amino acid residue stretch (residues 88 to 145). Our results further indicate that functional relationship between βARK and heterotrimeric G proteins in different intracellular organelles, and suggest that additional proteins may be involved in modulating the cellular localization of the kinase through a new targeting domain of βARK.

A general feature of G protein-coupled receptors is that exposure to agonists often leads to a rapid loss of receptor responsiveness, a process termed desensitization or tolerance. Such regulatory mechanisms are triggered every time a receptor is activated and play a key role in signal integration and plasticity at the cellular level. The β-adrenergic receptor (βAR)3-coupled adenylyl cyclase system has provided an important model for the study of the molecular mechanisms of desensitization (Benovic et al., 1988; Dohlman et al., 1991; Koblika, 1992; Lohse, 1993). Work from several laboratories has shown that rapid agonist-specific desensitization of the βAR is due to functional uncoupling from the transducing G protein, which is initiated by phosphorylation of the βAR by β-adrenergic receptor kinase (βARK), a serine-threonine kinase that specifically phosphorylates the agonist-occupied form of the receptor (Palczewski and Benovic, 1991; Lefkowitz, 1993; Haga et al., 1994a). βARK-mediated phosphorylation allows the interaction with the βAR of an additional regulatory protein, β-arrestin, which precludes receptor interaction with G proteins and blocks signal transduction (Lohse et al., 1990; Koblika, 1992). The uncoupling process is followed by transient receptor internalization away from the plasma membrane. Interestingly, emerging evidence indicates that βARK may be able to modulate a variety of G protein-coupled receptors (Benovic et al., 1987; Kim et al., 1993b; Kwatra et al., 1993; Richardson et al., 1993). This, together with the recent characterization of different βARK-related kinases, which constitute the G-protein-coupled receptor kinase (GRK) multigene family, strongly suggests that very similar mechanisms of regulation operate for all G protein-coupled receptors (Inglesé et al., 1993; Haga et al., 1994a; Loudon and Benovic, 1994; García-Higuera et al., 1994b).

However, there is little understanding of the protein-protein interactions of this complex regulatory network, and of the changes in the subcellular distribution of these proteins that take place upon receptor activation. βARK has been described as a soluble, cytosolic enzyme that transiently translocates to the plasma membrane when the receptor is occupied by an agonist (Strasser et al., 1986; Mayor et al., 1987; García-Higuera and Mayor, 1992; Chuang et al., 1992). Recent data indicate that βγ subunits of heterotrimeric G proteins are able to enhance βARK activity toward different G protein-coupled receptors (Haga and Haga, 1992; Pitcher et al., 1992; Kam...

*This work was supported in part by Dirección General Investigacín Científica y Tecnica PB920135, EC Biotech CT-930083–2·Boehringer Ingelheim and Fundación Ramón Areces (to F. M., Jr.), and National Institutes of Health Grant GM 44944 (to J. L. B). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**To whom correspondence should be addressed. Tel.: 341-397-4965; Fax: 341-397-4799.

§Supported by a predoctoral fellowship from Ministerio de Educación y Ciencia.

†Current address: Dept. of Medicine, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115.

∥To whom correspondence should be addressed. Tel.: 341-397-4865; Fax: 341-397-4799.

The abbreviations used are: βARK, β-adrenergic receptor; βARK-β-adrenergic receptor kinase; G protein, guanine nucleotide binding protein; GRK, G protein-coupled receptor kinase; GST, glutathione S-transferase; GTPyS, guanosine 5'-[3-O-thio]triphosphate; PAGE, polyacrylamide gel electrophoresis; App(NH)p, 5'-adenylyl-βγ-imidodiphosphate.
eyama et al., 1993; Kim et al., 1993b), and both purified G proteins and isolated βγ subunits can interact with βARK in vitro (Pitcher et al., 1992; Kim et al., 1993b). Therefore, it has been proposed that the interaction of βγ subunits with βARK would help to target the kinase to the periphery of the plasma membrane and to increase its activity toward the activated receptor. The βγ-binding domain of βARK has been localized to a 125-amin acid stretch in the COOH-terminal region of the enzyme (Kameyama et al., 1993; Koch et al., 1993); this region partially overlaps with a pleckstrin homology domain present in βARK (Touhara et al., 1994). Moreover, we have recently reported that, in addition to being a soluble enzyme that transfers βγ subunits into the membrane by means of a tetrameric protein component of the microsomal membranes by means of electrostatic interactions (García-Higuera et al., 1994a), in a way reminiscent of its transient interaction with the plasma membrane.

In this paper, we have used a direct binding assay with [35S]methionine-labeled βARK (Kim et al., 1993b) to further characterize the kinase binding site in the microsomes and investigated the functional consequences of such interaction. Our data support a functional link between βARK and microsomal heterotrimeric G proteins and suggest the existence of a new targeting domain of βARK involved in the modulation of the complex subcellular distribution of this important regulatory kinase.

**EXPERIMENTAL PROCEDURES**

Preparation of Rat Liver Subcellular Fractions—Subcellular fractionation of rat liver was performed essentially as described (García-Higuera and Mayor, 1994; García-Higuera et al., 1994a). Briefly, adult male Wistar rats were sacrificed by decapitation, and their livers were rapidly excised, weighed, minced and homogenized with a motorized Teflon pestle in 4 volumes of ice-cold buffer A (20 ml Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml benzamidine) plus 250 mM sucrose. All subsequent manipulations were performed at 4°C. The supernatant of a low-speed centrifugation (25,000 g) was centrifuged at 10,000 g for 20 min to obtain the crude mitochondrial pellet, the microsomal membrane pellet was obtained by centrifugation of the postmitochondrial supernatant for 60 min at 300,000 g of protein) by electrophoresis on 11% SDS-PAGE and Coomassie Blue staining. The protein subunits were isolated by chromatography on heptylamine-Sepharose columns exactly as described previously (Kim et al., 1993a). For radiolabeling, infected Sf9 cells were incubated for 20 h in methionine-free Grace media containing 1-2 μCi of [35S]methionine (1190 Ci/mmol) and then harvested and purified exactly as previously reported (Kim et al., 1993b). Purity of radiolabeled βARK was >95%, as judged by SDS-polyacrylamide gel electrophoresis. The radiolabeled βARK was stored at −20°C and used at a specific activity of 350-1000 cpm/μg.

Purification of βARK Subunits—After purification of G and Gs, from bovine brain membranes as described (Strenweiß and Robishaw, 1984), βγ subunits were isolated by chromatography on heptacylamide-Sepharose in the presence of AMF (30 μM AIC8, 6 mM MgCl2, and 10 mM NaF) (Katada et al., 1984) followed by anion exchange chromatography on Mono Q column to obtain βγ subunits free of AMF cholate (Kim et al., 1993b). The purified βγ subunits was stored in 20 ml Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, and 0.05% Lubrol at −80°C.

**Analysis of βARK Subunits in Rat Liver Membrane Fractions—** The presence of heterotrimeric G proteins in the plasma and microsomal membrane fractions used in our βARK binding and activity experiments was investigated by [35S]GTPγS binding as previously reported (Northup et al., 1982). The presence of α and βγ subunits was also investigated by immunoblot analysis after resolving the membrane fractions (30-70 μg of protein) by electrophoresis on 11% SDS-polyacrylamide gels and electroblotting to nitrocellulose filters, using as probes the G protein α subunit antibody AS2B (1:300 dilution, a gift from Drs. G. Mayor and K. Schatz, Max-Planck-Institut, Berlin), the β subunit antibody M-14 (5 μg/ml, Santa Cruz Biotechnology) or the β subunit antibody MS/1 (1:300, DuPont NEN) as described (Koch et al., 1993). Similar results were obtained with the different β subunit antibodies. The blots were developed using a chemiluminescence method (ECL, Amersham Corp.). In the latter case, an approximate quantitation of the presence of βγ subunits was performed by simultaneously probing known amounts (20–40 ng) of purified bovine βγ subunits (see above) resolved in the same gel, and densitometric analysis using a Molecular Dynamics laser densitometer.

Preparation of GST-βARK Fusion Proteins—Fusion proteins containing amino acids 50–145 (FP1) or 437–689 (FP2) of bovine βARK1 (Koch et al., 1993) were prepared essentially as reported (Koch et al., 1993). Specific 5′ and 3′ polymerase chain reaction primers (containing BamHI and EcoRI restriction sites, respectively) were used to amplify the desired cDNA fragments. The amplified fragments were ligated in frame between the BamHI and EcoRI sites of the glutathione S-transferase (GST) gene fusion vector pGEX-2T (Pharmacia). Additional GST-βARK constructs comprising amino acids 1–63, 1–88, 1–122, or 1–147 were prepared in a similar way. Selected clones were verified by dideoxy sequencing using T7 DNA-Polymerase (Sequenase, Stratagene). The fusion protein constructs were used to transform the Escherichia coli strain AG1 induced with isopropyl-thio-β-D-galactopyranoside, and proteins were purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia). The integrity of the purified fusion proteins (or GST alone) was confirmed by SDS-PAGE on 12% polyacrylamide gels. Analysis of βγ Binding to GST-βARK Fusion Proteins—Detection of possible interactions between purified G protein βγ subunits and our fusion proteins was performed as described (Koch et al., 1993; Ingelse et al., 1994). Briefly, purified bovine brain βγ subunits (150 μg) were incubated for 30 min at 4°C with the desired βARK fusion proteins (or GST as a negative control) at a final concentration of 1 μg in 50 μl of 20 ml Tris-HCl, pH 7.4, 1 mM MgCl2, 0.01% Lubrol. Then, 20 μl of a 50% slurry of glutathione-Sepharose 4B were added, and incubation continued for 90 min in ice with gentle mixing. The beads were washed three times with 1 ml of buffer containing 0.01% Lubrol, and retained proteins were analyzed by SDS-PAGE on 11% acrylamide gels, followed by Western blot analysis with antibodies against β subunits as described above, using purified βγ subunits run in the same gel as a positive control.

**βARK Binding to Microsomal Membranes—** [35S]Labeled βARK (5–80 nm) was incubated in buffer A or in 20 ml Tris-HCl, pH 7.4, in reaction volumes of 100–300 μl (Kim et al., 1993b) with 0.4–0.75 μg/ml salt-containing microsomal membranes (which do not contain βARK). In some experiments, microsomal membranes were pre-incubated for 15–30 min at 37°C in 20 ml Tris-HCl, pH 7.4, 2 mM MgCl2 alone, or in the presence of different concentrations of MgCl2, CaCl2, heparin, AIF2, GDP, mastoparan, and GTPγS (as indicated in the figure legends), or different concentrations (5–30 μM) of bovine serum albumin, GST, or GST-βARK fusion proteins. The binding mixtures were incubated at 37°C for 0.5–15 min, and membrane-associated βARK was collected by centrifugation at 250,000 × g for 10–30 min at 4°C in a Beckman TL-100 centrifuge. The microsomal membrane pel-
lets were carefully washed with buffer and then solubilized with 1% SDS and counted with scintillation fluid. In some experiments, the radioactivity of the supernatant containing unbound βARK was also quantitated. Nonspecific binding was defined by performing the binding reaction in the presence of 200 mM NaCl, an experimental condition in which βARK association with a protein component of the microsomal membrane has been shown to be completely inhibited (García-Higuera et al., 1994a). 35S-Labeled βARK binding can also be competed by unlabeled recombinant βARK (data not shown). In other experiments, unlabeled recombinant βARK was used as the ligand, and bound βARK was determined as described by immunoblot analysis with specific antibodies raised against βARK (García-Higuera and Mayor, 1994; García-Higuera et al., 1994a). Alternatively, the extent of βARK binding under different experimental conditions was assessed by quantitating the kinase activity remaining in the soluble fraction after high-speed centrifugation using the rhodopsin phosphorylation assay as described (García-Higuera et al., 1994a), also see below.

Effect of Na2CO3 Treatment on βARK Association with Microsomal Membranes—Aliquots of stripped microsomal membranes were resuspended in equal volumes of 100 mM Na2CO3, pH 11, or buffer A and incubated for 10 min at 4°C in order to extract peripheral membrane proteins, as described (Fujiki et al., 1982). The amount of β subunits of heterotrimeric G proteins remaining in the membranes or released by the treatment was determined by immunoblot analysis as described above. Control and treated membranes were subsequently washed in buffer A and its ability to interact with βARK was assessed as described (García-Higuera et al., 1994a). The presence of βARK in the soluble and particulate fractions was determined by the rhodopsin phosphorylation assay as reported (Mayor et al., 1987) and García-Higuera et al. (1994a), also see below, by using the direct 35S-labeled βARK binding assay as detailed above, or by utilizing recombinant unlabeled βARK followed by immunoblot analysis as described (García-Higuera et al., 1994a).

Determination of βARK Activity—βARK activity was assessed by using either a synthetic peptide substrate or purified urea-treated outer segments, as described previously in our laboratories (Benovic et al., 1986; Mayor et al., 1987; Kim et al., 1993b). Rhodopsin (100 μM), before purified rod outer segments consisting of >90% rhodopsin as indicated by Coomassie Blue staining of polyacrylamide gels were prepared in the dark as reported (Bovic et al., 1986), and aliquots containing 120 pmoles of rhodopsin were incubated for 20–30 min at 30°C in a buffer containing 27 mM Tris, pH 7.5, 1.4 mM EDTA, 1 mM EGTA, 5.5 mM MgCl2, 4.5 mM NaF, 57 μM γ[32P]ATP (2–3 cpmfmol) plus 20 nM recombinant bovine βARK in the presence or absence of 17–70 μg of microsomal or plasma membrane proteins. The final volume 60 μl. Before testing its effect on βARK activity toward rhodopsin, the desired amount of stripped microsomal or plasma membranes were preincubated (10 min at 37°C in 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2) alone or in the presence of the heterotrimeric G protein activators AlF3 (5 mM NaF, 50 μM AlCl3, 100 μM GDP) or mastoparan (100 μM) plus GTPγS (25–50 μM). Reactions were started by exposure to fluorescent laboratory lighting and stopped by 20-fold dilution with ice-cold 20 mM Tris-HCl followed by centrifugation. The resulting rhodopsin-containing pellets were resuspended in SDS-PAGE sample buffer and resolved by electrophoresis on 12% polyacrylamide gels followed by autoradiography. βARK activity was quantitated by measuring the radioactivity associated with the excised rhodopsin band in the dried gel by Cerenkov spectroscopy (Mayor et al., 1987). βARK activity was also investigated using a synthetic peptide as substrate. The peptide RRRREEEEEAAA was synthesized on a Applied Biosystems 431A synthesizer using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. The peptide (1 mM) was incubated for 30 min with βARK (20 nM) in the absence or presence of 0.6 μM, 30% trichloroacetic acid. The quenched reactions were centrifuged at 48,000 × g for 10 min and the resulting supernatants were transferred to a 2 × 2-cm square of P-81 paper before washed by fuses in 75 mM phosphoric acid and radioactivity quantitated as described (Kim et al., 1993b).

**RESULTS**

Previous studies from our laboratory have shown that βARK is associated with microsomal membranes in rat liver and many other tissues and cell lines (García-Higuera et al., 1994a). The kinase can be completely extracted from the membranes by mild salt treatment (200 mM NaCl), thus suggesting a peripheral association based on electrostatic interactions. Moreover, we reported (by using activity measurements and immunoblot analysis), that recombinant purified βARK1 was able to interact with a protein component of salt-stripped rat liver microsomal membranes in a cell-free system (García-Higuera et al., 1994a). In these experiments, membrane-associated and unbound βARK were separated by centrifugation at 250,000 × g followed by kinase quantitation in the pellet or the supernatant. In order to gain further insight into the mechanisms of association of βARK with microsomal membranes, we have utilized a direct binding assay using purified recombinant (145)metionine-labeled βARK1, which has recently been used for investigating the interaction of βARK with phospholipid vesicles containing purified protein subunits (Kim et al., 1993b). In the assays shown below, 35S-labeled βARK was incubated under different experimental conditions with stripped rat liver microsomal membranes, and the amount of membrane-associated βARK was then determined by measuring the radioactivity in the high-speed pellets. Nonspecific binding of βARK was estimated by performing parallel experiments in the presence of 200 mM NaCl, a condition known to completely inhibit the kinase binding to the microsomal vesicles (García-Higuera et al., 1994a). This has enabled a quantitative and detailed characterization of the interaction of βARK with this physiological membrane preparation.

Fig. 1A shows that βARK binding to stripped microsomal membranes is very rapid (half-maximal binding achieved in less than 1 min) and results in the association of most (~75%) of the kinase present in the assay. Scatchard analysis (Fig. 1B) indicates that the interaction process is saturable and appears to involve one population of affinity binding sites (Kd of 20 ± 1 nM). Similar results are obtained using lower concentrations of membranes. Interestingly, such kinetic and affinity data are similar to those reported for the interaction of βARK with purified heterotrimeric G proteins or βγ subunits (58 ± 14 and 32 ± 5 nM, respectively) (Kim et al., 1993b). In order to
better understand βARK association with microsomal membranes, we tried to obtain further information on the type of proteins responsible for the interaction, on the effect of binding on βARK activity, and on possible modulators of βARK association and function.

Since βARK binding to G protein subunits has been shown in vitro (Pitcher et al., 1992; Koch et al., 1993; Kim et al., 1993b), and the presence of heterotrimeric G proteins in intracellular organelles is mentioned in an increasing number of reports (see references in Balch (1992), García-Higuera et al. (1994a), and Neubig (1994)), we investigated the presence of endogenous G proteins in the salt-stripped microsomal membranes used in our studies. Specific β common and γ common subunit antibodies detect major bands of ~40- and 35-kDa, respectively (Fig. 2), thus confirming the presence of heterotrimeric G proteins in this preparation. G proteins can also be detected in the microsomal membranes by a 35S-labeled GTP-γS binding assay (data not shown). The identity of the subunit isoforms has not been investigated in detail, although additional protein bands in the 40–46 kDa range can be detected with the α common antibody at longer times of exposure. With regard to βγ subunits, comparison of the signal obtained in microsomal membranes with that of a known amount of βγ subunits purified from bovine brain (Fig. 2, lane 1) allows an approximate quantitation of at least 10 pmol of microsomal βγ per mg of stripped membrane protein, in the same order of magnitude as the Bmax detected for βARK binding (see Fig. 1B).

We next tested the ability of different compounds to modulate βARK association with microsomal membranes using the direct binding assay. Particularly, we investigated whether βARK interaction would be modulated by known G protein activators. In initial experiments, preincubation of stripped microsomal membranes with the selective heterotrimeric G protein activator AlF4− (Finazzi et al., 1994) in the presence of excess Mg2+ and GDP promoted a marked increase in subsequent βARK binding (Fig. 3A). However, this effect seems to be due only to the presence of Mg2+ and not to G protein activation, since a similar increase in βARK interaction is observed when only Mg2+ is added during the preincubation (Fig. 3A); GDP alone has no effect on βARK binding (data not shown). A more detailed investigation on the effect of Mg2+ was performed. Fig. 3B shows a dose-dependent effect of submillimolar concentrations of Mg2+ on βARK association with the microsomal membranes, which reaches an ~4-fold increase over control binding at ~1 mM Mg2+. Such fold-increase is similar to that observed in Fig. 3A at 2.5 mM Mg2+, thus suggesting that the Mg2+ effect attains a maximum in such concentration range. The presence of Mg2+ promotes an increase in the apparent Bmax (65 pmol/pmol of protein with 1 mM Mg2+) without changing the affinity of βARK for its binding sites. The divalent cation Mn2+ can substitute for Mg2+ (70% of Mg2+ effect at 1 mM Mn2+). In order to optimize βARK binding and to approach physiological intracellular concentrations, 1 mM MgCl2 was routinely included in all subsequent experiments.

The fact that βARK binding to microsomal membranes is not modulated by known G protein activators is further confirmed by the lack of effect on βARK association of mastoparan, a tetradecapeptide which stimulates guanine nucleotide exchange and activates heterotrimeric G proteins (Higashijima et al., 1988; Colombbo et al., 1992) or GTP-γS, a non-hydrolyzable GTP analog which is a general activator of G proteins (Colombo et al., 1992; Tsai et al., 1992) (data not shown). The presence of other nucleotides such as GDP, ATP, GTP, or ApNH(p) (0.5–1 mM) did not have any effect on βARK binding, and did not promote the release of previously bound βARK from the microsomal membranes (data not shown). Other possible modulatory compounds, such as cAMP or heparin, a βARK inhibitor (Benovic et al., 1989a), do not significantly modulate βARK interaction. The association of the kinase with the stripped microsomal membranes is reduced by ~50% when tested in the presence of 100 mM potassium gluconate, an ionic condition similar to the intracellular medium (not shown, see also García-Higuera et al. (1994a)). In summary, our data indicate that despite its presence in microsomal membranes and the previously reported interaction with βARK in vitro (Pitcher et al., 1992) G protein activation was not required and did not modulate βARK binding in our model.

We have previously shown (García-Higuera et al., 1994a) that βARK association to microsomal membranes is reversible, i.e. that previously extracted or recombinant βARK1 can interact with salt-stripped microsomes, thus indicating that the microsomal component involved in the interaction is not removed during the mild stripping procedure. Fig. 4A shows that when salt-stripped membranes are further treated with 0.1 M Na2CO3 at pH 11, a commonly used method for removing
Blotted to nitrocellulose membranes, and probed with a
MgCl₂ and incubated for 5 min at 37°C with 30 nM recombinant
ARSubunits. Extract remaining peripheral proteins. After one wash in buffer A,
100 mM Na₂CO₃, pH 11, and incubated for 30 min at 4°C in order to
activity assay) is not affected, thus suggesting that
interaction of recombinant bARK with Na₂CO₃-stripped membranes (Fig. 4
C) indicates the position of phosphorylated rhodopsin. B, immunoblot analysis of bARK association.
Aliquots of salt-stripped microsomal membranes were treated in
the absence (lane 1) or presence (lanes 2 and 3) of Na₂CO₃, pH 11 as
indicated under “Experimental Procedures” and extracted proteins sep-
parated by high-speed centrifugation. The same volume of microsomal
pellets (corresponding to 50 µg of protein in the initial, non Na₂CO₃-
treated membranes) were resuspended in 20 mM Tris-HCl, pH 7.5, 2 mM
MgCl₂ and incubated for 5 min at 37°C with 30 nM recombinant bARK
in the absence (lanes 1 and 2) or presence (lane 3) of 200 mM NaCl. After
centrifugation at 250,000 × g for 10 min, the amount of bound bARK in
each sample was assessed by immunoblot analysis as detailed under
“Experimental Procedures.” 80 ng of recombinant bARK were directly
resolved and analyzed in the same gel as a control (lane 4). Results are
representative of two independent experiments. C, presence of G pro-
tein β subunits in microsomal membranes subjected to different strip-
ing procedures. Salt-stripped purified microsomal membranes (800 µg of
protein) were treated in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of Na₂CO₃, pH 11 as indicated under “Experimental Proce-
dures” in a final volume of 800 µL. After high-speed centrifugation to
separate membranes and extracted proteins, equivalent aliquots of the
membrane pellets (60 µg of proteins, lanes 2 and 4) and extracted supernatants (60 µL, lanes 3 and 5) were resolved by 11% SDS-PAGE,
bliotted to nitrocellulose membranes, and probed with a β subunit
antibody (M14, Santa Cruz Biotechnology, 5 µg/ml) as described under
“Experimental Procedures.” 60 µg of unstripped microsomal mem-
branes (lane 1) and 20 ng of purified βγ subunits obtained from bovine
brain (lane 6) were resolved and blotted in the same gel and probed as
above.

Strongly-attached peripheral membrane proteins (Fujiki et al.,
1982; Yu et al., 1992), bARK association (as assessed by an activity assay) is not affected, thus suggesting that bARK inter-
acts with a microsomal component which behaves as an
integral membrane protein. A more detailed and quantitative
analysis of the interaction of bARK with Na₂CO₃-stripped membranes was performed. Immunoblot analysis indicates that the interaction of recombinant bARK with a defined amount of salt-stripped microsomal membranes (Fig. 4B, lane
1) is not altered after removal of additional peripheral proteins
with the Na₂CO₃ pH 11 treatment (lane 2); the association with the Na₂CO₃-stripped membranes is blocked in the presence of
200 mM NaCl (lane 3). Direct binding studies using 50 nM
³⁵S-labeled bARK show that a given amount of microsomal
membranes retains 86 ± 9% (mean ± S.D. of two independent
experiments performed in triplicate) of specific kinase binding
after the Na₂CO₃ pH 11 treatment, thus leading to an −1.5-fold
increase in binding specific activity (up to 82 ± 5 pmol/mg protein).
Similar results were obtained in other sets of assays
using 25 nM labeled bARK. It is worth noting that the Na₂CO₃
3 treatment leads to the loss of −50% of the G protein β subunits
from the membranes, as assessed by immunoblot analysis (Fig. 4C).
The fact that such −50% decrease in G protein subunits does not alter bARK association suggests that G proteins may
not be the main anchor for bARK in the microsomal mem-
branes (also see below).

We next investigated the functional consequences of bARK binding to the microsomal membranes by comparing the activity
of the free and bound kinase toward exogenous substrates. Fig. 5 shows that, compared to free bARK (lane 1), a marked
inhibition of light-dependent rhodopsin phosphorylation is ob-
served in the presence of increasing amounts of microsomal
membranes (lanes 3–5), which do not show any residual bARK activity (lane 2). This result indicates that bound bARK is less
able to interact with rhodopsin and/or to phosphorylate this
membrane-bound specific substrate. It is worth noting that the
same effect is observed when a synthetic peptide substrate is used to test the activity of bARK (Fig. 7A), thus showing that
the observed effect is not restricted to the rhodopsin phos-
phorylation assay. Interestingly, it has been reported that rhodop-
sin phosphorylation by the homologous, if not identical, mus-
carinic acetylcholine receptor kinase was strongly inhibited by
heterotrimeric G proteins in the absence of guanine nucleotides
in a dose-dependent way (Haga and Haga, 1992).

Since purified βγ subunits or stimulators of G protein acti-
vation and dissociation such as GTP-γS have been shown to
stimulate bARK activity toward different substrates (Haga and
Haga, 1992; Pitcher et al., 1992; Müller et al., 1993; Pei et al.,
1994), we next investigated whether preincubation of stripped
microsomal membranes with specific agents that would acti-
vate endogenous heterotrimeric G proteins may modulate the
activity of bound bARK. Fig. 6A shows that the marked inhibi-
tion of bARK activity observed in the presence of microsomal
membranes (compare lanes 1 and 3) is relieved when the mi-
crosomes were previously preincubated with the G protein activators AlF₄⁻ (lane 4) or mastoparan plus GTPγS (lane 5). This effect cannot be simply ascribed to a release of bound βARK, since such compounds do not show any effect on βARK binding to the microsomal membranes (Fig. 3 and data not shown), and the same result is obtained when the microsomal membranes are pelleted and then resuspended in phosphorylation buffer in the presence of rhodopsin (data not shown), thus indicating a direct effect on bound βARK as a consequence of G protein activation and subunit dissociation. The same effect can be observed when βARK functionality is assessed using a synthetic peptide substrate (Fig. 7A). It is interesting to note that similar results were apparent when we used stripped plasma membranes instead of microsomal membranes for these experiments: bound βARK activity toward either rhodopsin or a peptide substrate was markedly inhibited and was only relieved in the presence of heterotrimeric G protein activators (Figs. 6B and 7B). These data suggest a general feature of βARK interaction with cellular membranes and indicate, for the first time using physiological membrane preparations, that the activity of βARK is regulated by endogenous G proteins in different intracellular locations.

The fact that G protein activation can modulate the activity of bound βARK clearly demonstrates a functional link between these proteins in the microsomal membranes. Such a functional link could be the consequence of a direct association of βARK to G proteins under basal conditions, which would result in kinase activation in the presence of specific G protein stimulators. Alternatively, or in addition, βARK could be binding to a different protein in the stripped microsomes, but its activity may be modulated by additional interactions with G protein βγ subunits released upon G protein activation.

Since attempts to directly identify the microsomal protein(s) involved in βARK interaction using different cross-linkers were unsuccessful, we tried another approach based on investigating the ability of fusion proteins containing different βARK domains to displace βARK activity toward a synthetic peptide substrate (Fig. 6). The data suggest a general feature of βARK interactions with βγ subunits (Pitcher et al., 1992; Inglese et al., 1994), which implies that the βγ binding site domain of βARK is localized in the COOH-terminal portion of the kinase (Koch et al., 1993; Kameyama et al., 1993) and GST fusion proteins containing the last 222 amino acids of βARK have been recently used to characterize βARK interactions with βγ subunits (Pitcher et al., 1992; Inglese et al., 1994; Touhara et al., 1994). Therefore, we prepared a similar fusion protein construct (FP2) containing amino acids 437–689 of βARK Subcellular Localization

![Fig. 6. Activation of endogenous heterotrimeric G proteins present in stripped microsomal or plasma membrane fractions modulate the phosphorylation of rhodopsin by βARK. Recombinant βARK (20 nm) was preincubated in the presence of 0.7 μg of protein/μl of either stripped microsomal (A) or stripped plasma membrane fractions (B) as detailed under "Experimental Procedures" and in Fig. 5, in the absence or presence of AlF₄⁻ (5 mM NaF, 50 μM AlCl₃, 100 μM GDP) or mastoparan (100 μM) plus GTPγS (50 μM), as indicated. The activity of bound βARK was assessed by the rhodopsin phosphorylation assay as described in the legend to Fig. 5. Results are representative of three independent experiments.]

![Fig. 7. The activity of βARK toward a synthetic peptide substrate is modulated by the presence of stripped microsomal or plasma membrane fractions and by heterotrimeric G protein activators. Stripped microsomal (panel A) or plasma (panel B) membrane fractions (0.6 μg of protein/μl) were preincubated for 15 min at 37 °C in the presence of 20 nm βARK in 20 mM Tris-HCl pH 7.5, 1 mM MgCl₂, alone or in the presence of AlF₄⁻ (5 mM NaF, 50 μM AlCl₃, 100 μM GDP) or mastoparan (20 or 100 μM) and GTPγS (50 μM). The activity of bound βARK toward the synthetic peptide substrate RRREEEESAAA was subsequently assessed for 30 min at 30 °C, after the sequential addition of peptide substrate and phosphorylation buffer to give the final concentrations detailed under "Experimental Procedures." After determination of the radioactivity incorporated into the peptide, the activity of βARK in the presence of stripped microsomal membranes (0.7 ± 0.17 pmol of phosphate incorporated) or stripped plasma membranes (0.6 ± 0.28 pmol of phosphate incorporated) alone were taken as the basal values to which all the other experimental conditions were referred. Data are mean ± S.E. of two to four independent experiments performed in duplicate.]

βARK + - + + +
Microsomal membranes - + + + +
AlF₄⁻ + - + - -
Mastoparan - - - - +
+GTPγS

βARK + + + +
Plasma membranes - + + +
AlF₄⁻ - - - -

Rhodopsin

Peptide Phosphorylation (pmol P)
bovine βARK1 indicating the regions from which fusion protein containing amino acids 50–145 (FP1) or 437–689 (FP2) were derived; the proposed location of the pleckstrin homology domain (PH) and the γ subunits binding region are also indicated. B, γ binding properties of GST-βARK fusion proteins. Purified bovine brain γ subunits were incubated with identical concentrations of GST, FP1, and FP2 and protein complexes pelleted using glutathione-Sepharose beads as indicated under “Experimental Procedures.” Bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and detected with a specific antibody; purified brain γ subunits (Gsγ) were used as a positive control. C, effect of GST-βARK fusion proteins FP1 and FP2 on rhodopsin phosphorylation by βARK in the presence of G protein γ subunits. The rhodopsin phosphorylation assay was performed as described under “Experimental Procedures”; after incubation for 40 min at 30°C in the presence of 20 nM recombinant βARK and 80 nM γ subunits alone or in the presence of 10 μM GST or the indicated GST-βARK fusion proteins the reaction was stopped by addition of SDS-PAGE buffer, and phosphorylated rhodopsin was resolved by 12% SDS-PAGE followed by autoradiography. D, dose-dependent inhibition of 35S-labeled βARK binding to microsomal membranes by GST-βARK fusion proteins. Stripped microsomal membranes (0.75 μg of protein/μl) were preincubated for 30 min at 37°C in 20 mM Tris-HCl, pH 7.5, 2 mM MgCl2 with the indicated concentrations of GST or the fusion proteins FP1 and FP2 or an equivalent amount of bovine serum albumin in the same vehicle (control conditions). Labeled βARK was then added at a final concentration of 15 nM. After 5 min at 37°C βARK specific binding was determined as detailed in Fig. 1 and under “Experimental Procedures.” Data are expressed as percentage of the binding detected in control conditions, and are means ± S.E. of 3–4 independent experiments performed in duplicate.

Fig. 8. Effect of GST-βARK fusion proteins on 35S-labeled βARK binding to microsomal membranes. A, domain structure of bovine βARK1 indicating the regions from which fusion protein containing amino acids 50–145 (FP1) or 437–689 (FP2) were derived; the proposed location of the pleckstrin homology domain (PH) and the γ subunits binding region are also indicated. B, γ binding properties of GST-βARK fusion proteins. Purified bovine brain γ subunits were incubated with identical concentrations of GST, FP1, and FP2 and protein complexes pelleted using glutathione-Sepharose beads as indicated under “Experimental Procedures.” Bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and detected with a specific antibody; purified brain γ subunits (Gsγ) were used as a positive control. C, effect of GST-βARK fusion proteins FP1 and FP2 on rhodopsin phosphorylation by βARK in the presence of G protein γ subunits. The rhodopsin phosphorylation assay was performed as described under “Experimental Procedures”; after incubation for 40 min at 30°C in the presence of 20 nM recombinant βARK and 80 nM γ subunits alone or in the presence of 10 μM GST or the indicated GST-βARK fusion proteins the reaction was stopped by addition of SDS-PAGE buffer, and phosphorylated rhodopsin was resolved by 12% SDS-PAGE followed by autoradiography. D, dose-dependent inhibition of 35S-labeled βARK binding to microsomal membranes by GST-βARK fusion proteins. Stripped microsomal membranes (0.75 μg of protein/μl) were preincubated for 30 min at 37°C in 20 mM Tris-HCl, pH 7.5, 2 mM MgCl2 with the indicated concentrations of GST or the fusion proteins FP1 and FP2 or an equivalent amount of bovine serum albumin in the same vehicle (control conditions). Labeled βARK was then added at a final concentration of 15 nM. After 5 min at 37°C βARK specific binding was determined as detailed in Fig. 1 and under “Experimental Procedures.” Data are expressed as percentage of the binding detected in control conditions, and are means ± S.E. of 3–4 independent experiments performed in duplicate.

βARK Subcellular Localization

Fig. 9. Analysis of the effect of amino-terminal GST-βARK fusion proteins on βARK association with microsomal membranes. A, schematic representation of the GST-βARK constructs used in the experiments. B, effect of GST-βARK fusion proteins on βARK binding to microsomal membranes. Stripped microsomal membranes (0.75 μg of protein/μl) were preincubated for 30 min at 37°C as detailed in Fig. 8D with the indicated fusion proteins (15 μM) or NaCl 200 mM, and recombinant βARK was then added at a final concentration of 10 nM. After 5 min at 37°C, bound and soluble βARK were separated by high-speed centrifugation as detailed under “Experimental Procedures,” and unbound βARK was quantitated in 5 μl of the supernatants using the rhodopsin phosphorylation assay. Results are representative of five independent experiments.
main determinants of βARK association are located in the region 88–145. Taken together, these results indicate that the interaction of this regulatory kinase with the microsomes preferentially involves a domain located within the amino-terminal portion of βARK, and suggest that G protein βγ subunits are not the main anchor for βARK in the microsomal membranes.

DISCUSSION

An early step in the regulation of βARK is the phosphorylation of the agonist-occupied receptor by βARK, which was initially described as a soluble enzyme that transiently translocates to the plasma membrane upon receptor activation (Lefkowitz, 1993; Inglese et al., 1994; Haga et al., 1994a). Recent data have shown that βARK can interact with purified βγ subunits or whole heterotrimeric G proteins in vitro (Pitcher et al., 1992; Kim et al., 1993b), thus suggesting possible anchors for the kinase in the plasma membrane. In addition, we have recently reported that βARK associates with internal microsomal membranes both in vitro and in situ (García-Higuera et al., 1994a). These data indicate that several βARK pools (microsome-bound, plasma membrane-bound, and cytosolic) exist inside the cell and suggest that complex mechanisms (probably involving specific interactions with different proteins) may operate in order to regulate the subcellular distribution and activation of βARK. In this report, we have further characterized the association of βARK with microsomal membranes using a direct 32S-labeled βARK binding assay, and investigated the modulation of the functionality of the microsome-bound kinase.

Our data indicate that βARK binds very rapidly to stripped microsomal membranes with a K_d of ~20 nm. The kinase appears to associate with only one population of high affinity binding sites, although the possibility of more than one population of sites with similar binding affinities cannot be completely ruled out. Such binding sites are suggested to be protein components of the microsomal membrane, since previous data from our laboratory showed that protease or heat pretreatment of the microsomes strongly inhibits βARK association (García-Higuera et al., 1994a). Furthermore, the lack of effect on βARK binding of Na_2CO_3 pH 11 treatment shown in the present report indicates that the kinase binding sites behave as integral membrane proteins. It is worth noting that the affinity of βARK interaction with microsomal membranes is higher than that reported for its association with agonist-activated β-AR (K_d > 100 nm) and similar to that obtained with purified G proteins (K_d ~ 56 nm) or isolated βγ subunits (K_d ~ 32 nm) (Kim et al., 1993b), consistent with a functionally relevant role. The present study provides the first report of a high affinity interaction of βARK with a physiological membrane preparation. Association with microsomal membranes is not unique for βARK since a number of other proteins (pp60^crk, PKC-γ, ADP-ribosylation factors, kinesin, and dynein) have been reported to interact in different ways with such types of preparations (Resh, 1989; Chida et al., 1994; Tsai et al., 1992; Yu et al., 1992; Thissen and Casey, 1993).

Given the previously reported interaction of βARK with purified G proteins and βγ subunits and the stimulation of βARK activity by free βγ subunits (see above), these proteins were obvious candidates for participating in βARK binding and for modulating the activity of microsomal βARK, since their presence in intracellular membranes has been increasingly appreciated (see references in García-Higuera et al. (1994a), Neubig (1994), and Nuoffer and Balch, 1994). We confirmed by ^32P(GTP-γS binding and immunoblot analysis the presence of heterotrimeric G proteins in our preparations, and its preliminary quantitative analysis was compatible with a role in βARK association, taking into account the apparent B_max of βARK binding. However, our results clearly show that the interaction of βARK with the microsomal membrane is not dependent on heterotrimeric G protein activation, since no significant changes can be observed under experimental conditions routinely used for G protein stimulation (Colombo et al., 1992; Tsai et al., 1992; De Almeida et al., 1993; Kim et al., 1993b). The only apparent modulators of βARK binding were low millimolar concentrations of divalent cations such as Mg^2+. Since Mg^2+ can modulate a variety of enzymatic reactions (including phosphorylation/dephosphorylation processes, although the involvement of proteins kinases as mediators of its effect is unlikely in our experimental conditions), the mechanisms of the Mg^2+ effect on βARK interaction and its possible functional relevance remain to be established.

Our studies on the modulation of the activity of microsome-bound βARK put forward two interesting findings: (i) the bound kinase is in an inactive state, as shown by its reduced ability to phosphorylate rhodopsin or a synthetic peptide substrate; and (ii) although βARK binding is not affected by stimulators of heterotrimeric G proteins such as mastoparan/GTP-γS or AlF_4^-, these agents promote a marked increase in the activity of bound kinase, indicating a functional link between endogenous microsomal G proteins and βARK.

It has been previously shown that the phosphorylation of rhodopsin or the muscarinic acetylcholine receptor by rhodopsin kinase or βARK-like kinases was strongly inhibited by phospholipid vesicles containing purified, non-activated heterotrimeric G proteins (Kelcheher and Johnson, 1988; Haga and Haga, 1990, 1992), and that this effect was relieved in the presence of GTP-γS (i.e. subunit dissociation) (Haga and Haga, 1992). It has been suggested that such an inhibitory effect is due to a competition between trimeric G proteins and receptor kinases for overlapping sites on the activated receptor. However, the fact that we observed inhibition of βARK activity toward both an activated receptor (rhodopsin) and a synthetic peptide substrate suggest that, in addition, the interaction of βARK with the membranes temporarily masks or alters functionally relevant domains of the kinase. The activation of microsomal heterotrimeric G proteins would favor the interaction of free βγ subunits with βARK, which will then adopt an active conformation. Interestingly, the same pattern of modulation of βARK activity can be detected when stripped plasma membranes were used instead of microsomal membranes, thus suggesting a general feature of βARK interaction with cellular membranes. Our results confirm, for the first time using physiological membrane preparations, that βγ subunits activate βARK-mediated phosphorylation (Haga and Haga, 1990, 1992; Pitcher et al., 1992; Kameyama et al., 1993; Kim et al., 1993b). Furthermore, we show that βARK activity can be modulated by endogenous G proteins in different intracellular locations. The fact that G protein stimulation also increases βARK activity toward a soluble peptide substrate is in line with recent in vitro results showing that phosphorylation of soluble substrates by βARK or related kinases is enhanced in the presence of βγ subunits (Haga et al., 1994b; Kim et al., 1993b). Interestingly, such results are obtained in the presence of putative synergistic modulators of the kinase (activated receptors, synthetic receptor fragments, mastoparan) but not in its absence (Pitcher et al., 1992; Kim et al., 1993b). Taken together, these data suggest that G protein βγ subunits stimulate the enzymatic activity of βARK, in addition to its possible role as a membrane anchor for the kinase (Haga et al. 1994a, 1994b; also see below).

Our results using either plasma or microsomal membranes support a clear distinction between the process of βARK association with cellular membranes and that of kinase activation. Since the association of βARK with cellular membranes does not require G protein stimulation, it could take place under
resting, basal conditions, whereas βARK activation leading to substrate phosphorylation would be dependent on the stimulation of G proteins by specific signals. This is consistent with previous data showing that βARK associates equally well to the heterotrimeric G protein or to the βγ dimer alone, thus leading to the suggestion that βARK targeting to the plasma membrane may take place prior to receptor activation (Kim et al., 1993b).

A key issue to be addressed is the identity of the protein(s) involved in the association of βARK with the microsomal membranes. To date, βARK1 has been reported to interact with the activated βγAR and with βγ dimers, which have been proposed to play a key role in the kinase targeting to the periphery of the plasma membrane (Pitcher et al., 1992; Kim et al., 1993b). The presence of βγ subunits in our salt-stripped microsomal membrane preparation argued for a role of these proteins in βARK association. The fact that G protein stimulators did not have any effect on 35S-labeled βARK binding did not rule out its participation, since it has been previously shown that the activation of purified G, proteins does not modulate the kinase binding to heterotrimeric G proteins, which could be an entity recognized by βARK (Kim et al., 1993b). On the other hand, the functional link between membrane-bound βARK and activated G proteins discussed above does not necessarily imply G proteins as the only anchors of βARK, since (as mentioned under "Results") βARK could also be binding to a different protein in the stripped microsomes, and its activity modulated by additional interactions with βγ subunits released upon G protein stimulation.

Two main lines of evidence support the notion of a binding site for βARK other than G protein subunits. First, the lack of effect on βARK association of membrane pretreatment with Na2CO3 pH 11, which leads to a −50% reduction in immuno-reactive G proteins (Kehlenbach et al., 1994) and Fig. 4C). Second, the effect of different GST-βARK fusion proteins on βARK binding. It has been described that the carboxyl-terminal portion of βARK-1 is responsible for the activation by G protein βγ subunits and that the minimal βγ-binding domain is localized to a 125-amino acid stretch (Koch et al., 1993; Kamayama et al., 1993). This region partially overlaps with a pleckstrin homology domain present in βARK (Touhara et al., 1994). It is worth noting that, although pleckstrin homology domains have been recently reported to interact with phospholipid vesicles rich in phosphatidylinositol 4,5-bisphosphate (Harlan et al., 1994), this does not appear to play a predominant role in the interaction of βARK with microsomal membranes, since we have previously shown that the association is heat and protease-sensitive and therefore involves a protein component of the microsomes (García-Higuera et al., 1994a). Interestingly, our results show that GST-FP2, a fusion protein containing the COOH terminus of βARK which has been used by others to characterize βARK interactions with βγ subunits (Pitcher et al., 1992; Inglese et al., 1994; Touhara et al., 1994), does not inhibit βARK1 binding to microsomal membranes, whereas it is able to interact with purified βγ subunits (Fig. 8B) and to inhibit the βγ effect on rhodopsin phosphorylation by βARK (Fig. 8C). In agreement with these results, recent experiments indicate that purified phosducin, which is able to interact with G protein βγ subunits (DebBurman et al., 1995) does not inhibit βARK binding to microsomal membranes in our experimental conditions (94 ± 12% of control binding at 300–450 nM phosducin, mean ± S.E. of four experiments). On the contrary, a fusion protein containing an NH2-terminal portion of the kinase (residues 50–145) which does not bind purified βγ subunits (FP1), strongly inhibits βARK association with a potency similar to other reported effects of βARK fragments (Koch et al., 1993, 1994; Inglese et al., 1994; Touhara et al., 1994). A similar inhibitory effect on βARK binding can be observed with a GST-βARK fusion protein comprising amino acids 1–147, whereas a 1–88 fragment is without effect, thus suggesting the βARK anchoring domain may reside within residues 88–145.

Although at present we cannot totally exclude the possibility that intact G proteins or specific βγ dimers play a role in βARK association with microsomal membranes, we feel that our data strongly suggest that βARK binding is preferentially mediated via a high affinity interaction with a currently unidentified microsomal protein. As shown in the model depicted in Fig. 10, such protein component (X) appears to interact with a region of the amino terminus of the kinase, thus suggesting a new targeting domain of βARK. Bound βARK would be inactive until G protein stimulation leads to additional interactions of the COOH terminus of βARK with βγ-subunits. In line with other recent results, our data suggest that the regulation of βARK activity and subcellular distribution will likely involve multiple interactions with G protein subunits, phospholipids (DebBurman et al., 1995), different domains of G protein-coupled receptors (Haga et al., 1994b; Ruiz-Gómez et al., 1994), and additional anchoring proteins. In this regard, the existence of anchor proteins has been previously reported for other protein kinases such as protein kinase A (Hausken et al., 1994) and references therein) and protein kinase C (Ron et al., 1994), and emerges as a general mechanism for regulating the subcellular distribution and activity of protein kinases (Mochly-Rosen, 1993).

Fig. 10. Proposed model for the interaction of βARK with microsomal membranes and the modulation of the activation of the bound kinase. The domain structure of βARK indicates the regions from which fusion proteins GST-βARK 50–145 (FP1) or GST-βARK 437–689 (FP2) are derived. X, putative anchor protein; α, β, and γ are heterotrimeric G protein subunits and α4 denotes G protein activation.
and the possible function(s) of complex subcellular distribution of this key regulatory kinase proteins, kinase anchors ...) and mechanisms governing the complex subcellular distribution of this key regulatory kinase and the possible function(s) of βARK in microsomal membranes (García-Higuera et al., 1994a).

Acknowledgments—We thank R. Sterne-Marr for providing some purified GST-βARK fusion proteins, M. Sanz for skillful secretarial assistance, Fundación Ramón Areces for institutional support, Prof. F. Mayor for continuous encouragement, and Dr. J. Avila for critical reading of the manuscript.

REFERENCES

Balch, W. E. (1992) Curr. Biol. 2, 157–160
Benovic, J. L., Mayor, F., Jr., Somers, R. L., Caron, M. G., and Lefkowitz R. J. (1986) Nature 321, 869–872
Benovic, J. L., Regen, J. W., Matsui, H., Mayor, F. Jr., Cotechis, S., Leeb-Lundberg I. M. F., Caron, M. G., and Lefkowitz Jr. (1987). J. Biol. Chem. 262, 17251–17253
Benovic, J. L., Bouvier, M., Caron, M. G., and Lefkowitz, R. J. (1988) Annu. Rev. Cell Biol. 4, 405–428
Benovic, J. L., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. (1989a) J. Biol. Chem. 264, 6707–6710
Benovic, J. L., Deblasi, A., Stone, W. C., Caron, M. G., and Lefkowitz, R. J. (1989b) Science 246, 235–240
Chida, K., Sagara, H., Suzuki, Y., Murakami, A., Osada, S-I., Ohno, S., Hiroasa, K., and Kuroki, T. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 10209–10212
Kwatra, M., M., Schwinn, D. A., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. G., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 8256–8260
Kwok, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197
Kunapuli, P., Gurevich, V. V., and Benovic, J. L. (1994) J. Biol. Chem. 269, 10217–10221
Kwatra, M., M., Schwinn, D. A., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. G., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 9161–9164
Lefkowitz, R. J. (1993) Cell 74, 409–412
Loose, M. J. (1993) Biochim. Biophys. Acta 1175, 171–188
Loehe, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) Science 248, 1547–1550
Lou, R. P., and Benovic, J. L. (1994) J. Biol. Chem. 269, 22691–22697
Mayor, F. Jr., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1987) J. Biol. Chem. 262, 6468–6471
Mochly-Rosen, D. (1995) Science 268, 247–251
Mueller, S., Hekman, M., and Loose, M. J. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10439–10443
Neubig, R. R. (1994) FASEB J. 8, 939–946
Northup, J. K., Smigel, M. D., and Gilman, A. G. (1982) J. Biol. Chem. 257, 11416–11423
Nuoffer, C., and Balch, W. E. (1994) Annu. Rev. Biochem. 63, 949–990
Palczewski, K., and Benovic, J. L. (1991) Trends Biochem. Sci. 16, 387–391
Palczewski, K., Buczylko, J., Lebioda, L., Crabb, J. W., and Polans, A. S. (1993) J. Biol. Chem. 268, 6004–6103
Pei, S., Tiberi, M., Caron, M. G., and Lefkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3633–3636
Pitcher, J. A., Inglese, J., Higgins, J. B., Arizzu, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) Science 257, 1264–1267
Reis, M. D. (1989) Cell 58, 281–286
Richardson, R. M., Kim, C., Benovic, J. L., and Hasey, M. M. (1993) J. Biol. Chem. 268, 13650–13656
Ron, D., Chen, C. H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 839–843
Ruiz-Gómez, A., Murga, C., de Carlos, J. A., and Mayor, F. Jr. (1994) Soc. Neurosci. Abstr. 20, 1086
Sternweis, P., and Robishaw, J. D. (1984) J. Biol. Chem. 259, 13806–13813
Strasser, R. H., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6362–6366
Thissen, J. A., and Casey, P. J. (1993) J. Biol. Chem. 268, 13780–13783
Touhara, K., Inglese, J., Kwatra, M. M., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 10217–10220
Tsai, S.-C., Adamik, R., Haun, R. S., Moss, J., and Vaughan, M. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 9272–9276
Yu, H., Toyoshima, I., Steuer, E. R., and Sheetz, M. P. (1992) J. Biol. Chem. 267, 20457–20464

994 βARK Subcellular Localization

Further research will strive to identify the βARK anchor protein in the microsomes, and to more precisely localize the βARK binding domain. Although it has been suggested that the NH₂ terminus of GRKs may interact with activated receptors (Palczewski et al., 1993), there is little knowledge on the role of this domain, which does not have significant sequence homology with other known GRKs, including GRK1, GRK6, and GRK5, which have been shown to display a different mechanism of membrane association (Kunapuli et al., 1994). Further investigation using different experimental approaches would be needed to ascertain the factors (expression of G protein-coupled receptors, specific combinations of heterotrimeric G proteins, kinase anchors ...).