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# Rapid agonist-induced beta-adrenergic receptor kinase translocation in C6 glioma cells

Irene García-Higuera and Federico Mayor Jr.

*Departamento de Biología Molecular, Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, 28049 Madrid, Spain*

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Exposure of C6 glioma cells to 1 μM isoproterenol leads to fast desensitization of the β-adrenergic receptor/adenylyl cyclase system and transient receptor sequestration. It also triggers a very rapid and transient translocation to the plasma membrane of β-adrenergic receptor kinase (βARK), a specific cytoplasmic kinase that phosphorylates only the agonist-occupied form of several G protein-coupled receptors. βARK-mediated receptor phosphorylation appears to be a suitable mechanism for the rapid regulation of adrenergic receptor function in the nervous tissue.

β-Adrenergic receptor kinase; Desensitization; Translocation

## 1. INTRODUCTION

Desensitization is a general cellular process in which response to a continuous or repeated stimulus attenuates with time. The β-adrenergic receptor (βAR)/adenylyl cyclase system has been widely used as a model for such a phenomenon [1,2]. The molecular mechanisms underlying desensitization of βARs seem to involve both internalization of plasma membrane receptors and functional uncoupling of the receptor from the signal transducing protein, G's. Such uncoupling can be triggered by βAR phosphorylation by protein kinase A (PKA) or by β-adrenergic receptor kinase (βARK). βARK is a cytoplasmic kinase that specifically phosphorylates the agonist-occupied form of the βAR and other related G protein-coupled receptors [1,3,4], thus promoting the binding to the receptor of another protein, β-arrestin, which inhibits its coupling with G's and adenylyl cyclase [5].

Several studies performed with $\beta_2$-adrenergic receptors mutated at the presumed sites of phosphorylation by PKA or βARK [6], permeabilized A431 carcinoma cells treated with inhibitors of these kinases [7–9] and kin⁻ S49 lymphoma cells [10], have suggested that βARK-mediated receptor phosphorylation and desensitization requires relatively high agonist concentrations in the external medium. Thus, it has been proposed that the role of this kinase would be specially important in the nervous tissue, where neurons and glial cells are exposed to high and rapidly changing concentrations of cathecholamines released at the synapses [8]. In fact, the expression of βARK is higher in the brain and highly innervated tissues [11]. However, there is little knowledge about the mechanisms and kinetics of βARK translocation in nervous cells. In this report, we show that when C6 glioma cells are challenged with β-agonists, a strikingly rapid and transient process of translocation of βARK activity takes place within the same time-frame of βAR desensitization and internalization.

*Abbreviations:* βAR(s), β-adrenergic receptor(s); βARK, β-adrenergic receptor kinase; PKA, cAMP-dependent protein kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

*Correspondence address:* F. Mayor Jr., Departamento de Biología Molecular, Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, 28049 Madrid, Spain. Fax: (34) (1) 397 4799.

## 2. MATERIALS AND METHODS

Rat C6 glioma cells were grown as a monolayer on Falcon plastic dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% horse serum and 2.5% fetal calf serum at 37°C in a humidified atmosphere of 7% $CO_2$. Confluent cells were gently detached from the dish surface, centrifuged at 800 × g for 3 min and resuspended in DMEM supplemented with 20 mM HEPES, pH 7.4, and 1 mM ascorbic acid to a density of $3 \times 10^6$ cells/ml. Aliquots of the suspension were incubated for 1–10 min in the absence or presence of 1 μM (–)isoproterenol (Sigma). The incubations were stopped by diluting with ice-cold DMEM, 20 mM HEPES, and centrifugation (800 × g, 4 min). The cells were lysed in 5 mM Tris–HCl, pH 7.4, 2 mM EDTA, 3 mM $MgCl_2$, 1 mM phenylmethylsulfonil fluoride, 20 μg/ml benzamidine and 20 μg/ml leupeptin (cell lysis buffer) using a glass homogenizer. Unbroken cells and nuclei were discarded after centrifugation at 800 × g for 5 min, and crude plasma membrane and cytoplasmic fractions obtained by centrifugation at 12,000 × g for 30 min. These fractions were used for βAR binding or kinase activity assays (see below). For adenylyl cyclase assays, cell aliquots were incubated as above except that the cAMP phosphodiesterase inhibitor, isobutylmethylxanthine, was included (200 μM). After treatment, cells were lysed and cytoplasmic cAMP quantified by radioimmunoassay (Amersham) as described [12].

Binding of the βAR ligand [$^3$H]CGP-12177 (Amersham) to plasma membranes resuspended in cell lysis buffer was performed essentially

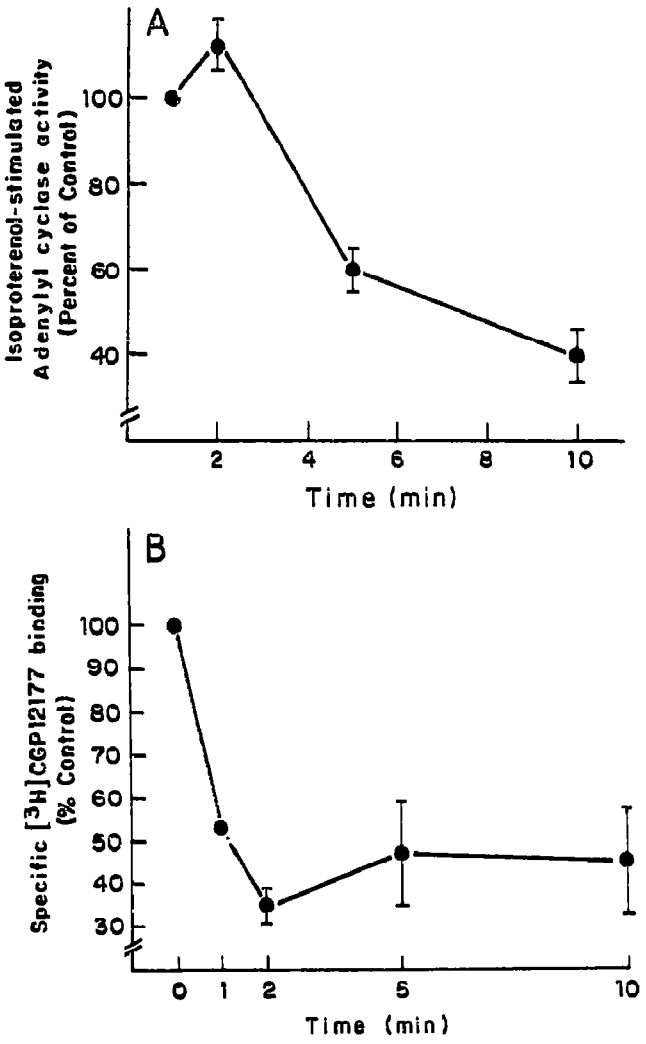


Fig. 1. (A) Rate of $\beta$-adrenergic agonists-stimulated adenylyl cyclase activity in whole C6 rat glioma cells incubated with 1 $\mu$M isoproterenol for the indicated periods of time. The results are means ± S.E.M. of 3–4 independent experiments with triplicate cAMP determinations. (B) Changes in $\beta$-adrenergic receptor number in the plasma membrane of C6 glioma cells stimulated with 1 $\mu$M isoproterenol for the indicated periods of time. Binding of [$^3$H]CGP-12177 was performed as detailed in section 2. The data are expressed as a percentage of the number of receptors present in the plasma membrane of cells not exposed to isoproterenol (73 ± 8 fmol per mg of protein). Results are means ± S.E.M. of 3–5 independent experiments.

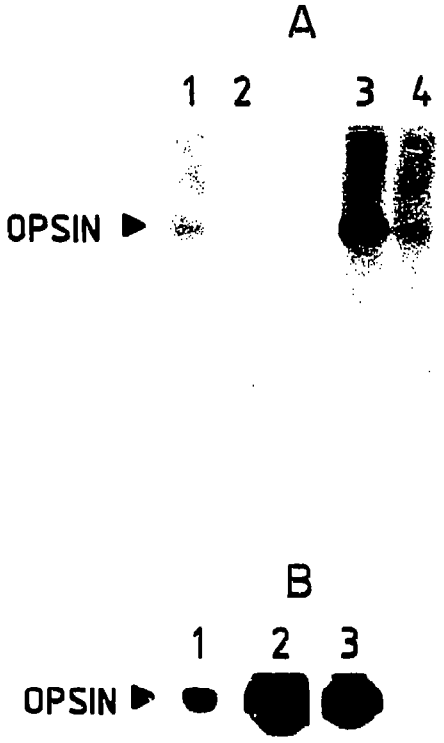


Fig. 2. (A) Presence of $\beta$ARK activity in soluble (lanes 1–2) and particulate (lanes 3–4) fractions of C6 glioma cells as demonstrated by light-dependent rhodopsin phosphorylation. Cell extracts were incubated with rhodopsin as detailed in section 2 either in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of light, followed by SDS-PAGE and autoradiography in order to detect opsin phosphorylation. (B) Extraction of membrane-associated $\beta$ARK activity by different methods. Membranes were treated as detailed in section 2 in the presence of Triton X-100 0.1% (lane 1), 200 mM NaCl (lane 2) or subjected to repeated freeze-thawing (lane 3). Extracted proteins were assayed for $\beta$ARK activity as above. The resulting rhodopsin phosporylation is shown.

as reported [13]. Radioligand concentration was 2 nM and non-specific binding was determined in the presence of 50 $\mu$M propanolol.

Since $\beta$ARK has been shown to specifically phosphorylate rhodopsin in an agonist-dependent fashion [14], $\beta$ARK activities were assessed by utilizing urea-treated purified rod outer segments as substrate. This method has been previously used to determine $\beta$ARK activity in cellular fractions and in $\beta$ARK purification studies [4,15,16]. Briefly, purified rod outer segments (devoid of endogenous rhodopsin kinase activity), containing 300–500 pmol of rhodopsin, were incubated for 20 min at 30°C in the presence of light in a medium containing 25 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM EDTA, 4.5 mM NaF, 60 $\mu$M [gamma-$^{32}$P]ATP (2–3 cpm/fmol) and soluble extracts from cells pretreated in different conditions. Reactions were stopped by diluting 20-fold with ice-cold cell lysis buffer and centrifuging at 12,000 × $g$ for 15 min. The resultant pellets were resuspended in sodium dodecyl sulfate buffer and electrophoresed as described [14]. After autoradiography of the gels, rhodopsin bands were excised and radioactivity counted as reported [4] to compare the kinase activity in different preparations. For determining $\beta$ARK activity in membrane fractions, it first had to be extracted. Membranes resuspended in cell lysis buffer were treated for 15 min at 4°C either with 200 mM NaCl or Triton X-100 0.1%, or subjected to three cycles of freeze–thawing (see section 3). After treatments, membranes were pelleted and the supernatant containing the extracted proteins assayed for $\beta$ARK activity as described above.

## 3. RESULTS AND DISCUSSION

Fig. 1A shows that incubation of Cl glioma cells with 1 $\mu$M isoproterenol for increasing periods of time results in a marked decrease in the rate of cAMP synthesis stimulated by this $\beta$-adrenergic agonist, i.e. in functional uncoupling of the $\beta$AR/adenylyl cyclase system. Isoproterenol also induces a rapid decrease in the number of adrenergic receptors in the plasma membrane. Fig. 1B indicates that after 2 min of exposure to this drug, ≈60% of the $\beta$AR binding sites present in non-

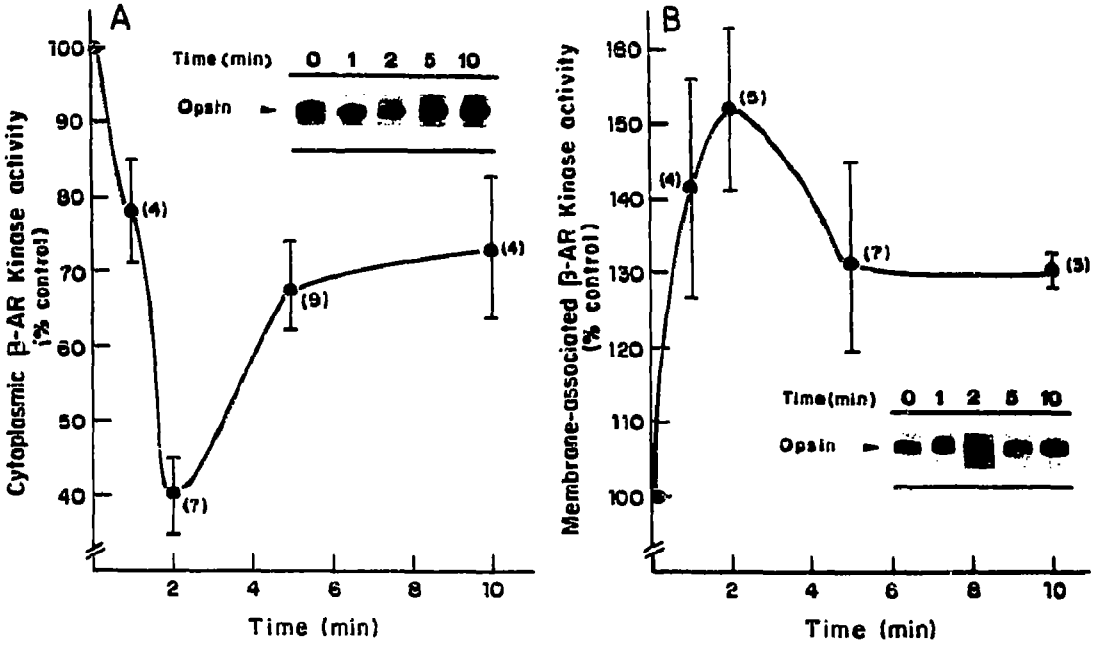


Fig. 3. Agonist-promoted changes in the subcellular distribution of βARK activity. After stimulation with 1 μM isoproterenol for the times indicated, cytoplasmic and membrane fractions were obtained from C6 glioma cells and assayed for βARK activity as described in section 2. The results are expressed as a percentage of βARK specific activities in non-stimulated control cells (specific activities of 1.8 ± 0.4 and 7.6 ± 0.4 pmol per min per mg of protein for the soluble and membrane fractions, respectively). Data are means ± S.E.M. of the number of independent experiments indicated in parentheses. Insets, autoradiograms showing patterns of βARK activity during agonist treatment in a representative experiment.

treated cells have been sequestered away from the plasma membrane. These results are indicative of an homologous desensitization process, in agreement with previously reported data [13,17,18]. Receptor disappearance reflects its transient redistribution to an internal vesicle fraction and is reversed once the stimulus is removed ([13] and data not shown).

We next sought to establish whether βARK was implicated in the mechanism of desensitization. Fig. 2A shows that βARK activity is present in both soluble and particulate fractions of this glial cell line, as demonstrated by light-dependent phosphorylation of rhodopsin. Membrane-associated activity had to be extracted prior to the rhodopsin assay. Since the βARK sequence does not include putative transmembrane domains, and its proposed mechanism of action suggests a transient, peripheral association with the plasma membrane, we screened several mild methods of extraction. Fig. 2B indicates that the removal of βARK activity is best attained after treatment with 200 mM NaCl; this method was thus used throughout the experiments.

Activation of C6 glioma cells βARs with isoproterenol promotes a very rapid change in the subcellular distribution of βARK. Fig. 3 displays the time-course of the kinase activity in cytoplasm (A) and plasma membrane (B) fractions. A decrease in cytoplasmic βARK is clearly detected within 1 min of exposure to isoproterenol and is maximal by 2 min of treatment, followed by a gradual return to control values. Conversely, membrane-associated βARK rapidly increases, reaches a peak by 2 min and declines thereafter. Interestingly, these changes take place within the same time-frame of βAR homologous desensitization. Although a detailed kinetic study has not been attempted, βARK translocation is parallel to βAR sequestration and slightly precedes the detection of βAR/adenylyl cyclase uncoupling. The precise relationships between these processes are still a matter of controversy [6,8,9] and remain to be elucidated. However, such a close temporal relationship strongly suggests a role for βARK-induced receptor phosphorylation in βAR desensitization in C6 glioma cells. It is worth noting that these cells express both $\beta_1$- and $\beta_2$-adrenergic receptors in a 2:1 ratio [19]. Given the differences in putative βARK phosphorylation sites between $\beta_1$- and $\beta_2$-receptors in several species (discussed in [19]), it would be of interest to determine if both receptor subtypes are regulated by the same mechanism and at the same rate.

Transient translocation of βARK activity has been previously reported in DDT1-MF2 hamster smooth muscle cells and S49 lymphoma cells in response to β-adrenergic, prostaglandin E1 and somatostatin agonists [4,20]. In these experiments, the time-course of kinase translocation was significantly slower than that observed in C6 glioma cells. On the other hand, our results tie in nicely with the rapidity of βARK-induced receptor phosphorylation and desensitization (half-maximal at ≈20 s) recently detected in permeabilized A431 epidermoid carcinoma cells [9]. Besides experimental differences, several factors may affect the extent

and rate of βARK translocation and βAR desensitization in different cells and tissues, including density of receptors per cell, the expression levels and isoforms of the kinase, cell shape and subcellular location of βARK under control conditions. The rapid translocation of βARK activity described in C6 glioma cells is consistent with its proposed key role in the rapid regulation of adrenergic receptor function in the nervous tissue [6,9], where neurons and glial cells are subjected to rapidly changing messenger environments. Finally, the experimental system reported herein could be of interest for investigating in more detail βAR desensitization mechanisms, as well as for testing the ability of agonists other than β-adrenergic ones in promoting βARK translocation in different cultured cells.

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