Real-time Visualization of the Cellular Redistribution of G Protein-coupled Receptor Kinase 2 and β-Arrestin 2 during Homologous Desensitization of the Substance P Receptor*

(Received for publication, September 14, 1998, and in revised form, December 3, 1998)

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The substance P receptor (SPR) is a G protein-coupled receptor (GPCR) that plays a key role in pain regulation. The SPR desensitizes in the continued presence of agonist, presumably via mechanisms that implicate G protein-coupled receptor kinases (GRKs) and β-arrestins. The temporal relationship of these proposed biochemical events has never been established for any GPCR other than rhodopsin beyond the resolution provided by biochemical assays. We investigate the real-time activation and desensitization of the human SPR in live HEK293 cells using green fluorescent protein conjugates of protein kinase C, GRK2, and β-arrestin 2. The translocation of protein kinase C βII-GFP, PKCβII-GFP, or β-arrestin 2-GFP, β-arrestin 2 tagged with green fluorescent protein to and from the plasma membrane in response to substance P indicates that the human SPR becomes activated within seconds of agonist exposure, and the response desensitizes within 30 s. This desensitization process coincides with a redistribution of GRK2 from the cytosol to the plasma membrane, followed by a robust redistribution of β-arrestin 2 and a profound change in cell morphology that occurs after 1 min of SPR stimulation. These data establish a role for GRKs and β-arrestins in homologous desensitization of the SPR and provide the first visual and temporal resolution of the sequence of events underlying homologous desensitization of a GPCR in living cells.

The substance P or neurokinin 1 receptor is a member of the G protein-coupled receptor superfamily that regulates signaling for pain perception in both the peripheral and central nervous systems (1). Its role in pain transmission has been convincingly demonstrated in animal models using genetic techniques. Disruption of either the gene for the SP1 precursor preprotachykinin A or the gene for the SPR in the mouse dramatically reduces the animal’s response to painful stimuli (2, 3). Therefore, a pharmacological blockade of SPR activation or an enhancement of mechanisms that attenuate SPR signaling may ultimately lead to novel therapies for pain.

The SPR is coupled to a Gs-mediated signaling cascade. Thus, the binding of SP to the receptor results in the hydrolysis of membrane phosphoinositides, the formation of inositol trisphosphate and diacylglycerol, the mobilization of intracellular calcium, and the activation of protein kinase C (PKC) and translocation to the plasma membrane (4–6). Biochemical and electrophysiological studies on cells expressing either endogenous or transfected SPR indicate that SPR signaling diminishes within seconds of agonist exposure (7–11). The molecular events that lead to this agonist-dependent desensitization in cells remain ill-defined. Studies performed over the last several years on GPCRs such as β2-adrenergic receptor (β2AR) and rhodopsin have proposed that agonist-dependent desensitization of GPCRs requires phosphorylation by G protein-coupled receptor kinases (GRKs) followed by the binding of β-arrestin proteins (12–14). The applicability of this homologous desensitization paradigm to the SPR is suggested by recent in vitro studies demonstrating that GRKs efficiently phosphorylate the human and rat SPR (15, 16).

The goal of the present study was to ascertain the extent and kinetics of GRK and β-arrestin involvement in SPR signaling in live cells. To this end, HEK293 cells were transfected with human SPR and green fluorescent protein conjugates of PKCβII, GRK2, or β-arrestin 2. The effects of human SPR activation on the cellular distribution of these proteins were then visualized in real time by confocal microscopy. Our data demonstrate that during SP-mediated homologous desensitization of the human SPR, the translocations of GRK2 and β-arrestin 2 from the cytosol to the plasma membrane closely follow the activation of PKC. Interestingly, whereas redistribution of β-arrestin 2 to activated GPCRs has previously been observed (17), the SPR is the first GPCR whose activation led to the observable translocation of GRK2 to the plasma membrane in real time. Hence, these observations in live cells represent the first visual confirmation that the temporal sequence of biochemical events underlying homologous GPCR desensitization does indeed occur.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and physiological buffers were obtained from Life Technologies, Inc. Substance P was obtained from Sigma. H32PO4 (specific activity, 8500–9120 Ci/mmol) was from New England Nuclear (Boston, MA). Plasmid constructs of green fluorescent protein conjugated to PKCβII, GRK2, and β-arrestin 2 were made and characterized as described previously (17–19).

Cell Culture and Transfection—HEK293 cells were maintained in minimum essential medium with 10% fetal bovine serum and 1:100 penicillin/streptomycin in a 5% CO2 incubator at 37 °C. Cells were transfected with 5–10 μg of plasmid pcDNA1.1 containing human SPR cDNA and 1–2.5 μg of plasmid containing cDNA for either PKCβII-GFP, GRK2-GFP, or β-arrestin 2-GFP using coprecipitation with calcium phosphate as described previously (17).
Fig. 1. Activation and desensitization of the human substance P receptor as visualized by the redistribution of PKCβII. HEK293 cells expressing both PKCβII-GFP and the human SPR were exposed to 100 nM substance P at time 0. The redistribution of PKCβII was followed by confocal microscopy using repetitive 3-s scanning intervals. The activation of the receptor and its subsequent desensitization coincide with the increased plasma membrane fluorescence that occurs by 3 s and the subsequent loss of membrane fluorescence that is evident after 30 s. Bar = 10 μm.

Whole Cell Phosphorylation—Human SPR-transfected HEK293 cells (106 cells/well) were labeled with 0.3 mCi of 32P, in 1 ml of HEPES/Krebs buffer (10 mM HEPES, pH 7.4, 118 mM KCl, 1.17 mM MgSO4, 1.3 mM CaCl2, 0.34 mM NaHCO3, and 11.7 mM glucose) for 90 min at 37 °C. The cells were then stimulated with 1 nM SP for various times. After this treatment, cells were washed with cold buffer and then lysed with 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.1 mM Na3VO4, 10 mM Na4P2O7, 1 mM EGTA, 10 mM NaF, 0.5% Nonidet P-40, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 0.1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged, precleared by incubating with protein A-Sepharose, and incubated for 1 h with 5 μl of a polyclonal antibody raised against the C terminus of human SPR (16). The mixture was then incubated for 1 h at 4 °C. The protein A-Sepharose was collected by centrifugation, washed with lysis buffer containing 1 mM NaCl, resuspended in 50 μl of SDS-polyacrylamide gel electrophoresis sample buffer (2% SDS, 60 mM Tris-HCl, pH 6.8, 10% glycerol, 10% β-mercaptoethanol, and 0.025% bromphenol blue), and the phosphorylated proteins were visualized by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Confocal Microscopy—Transfected cells were split into 35-mm plastic dishes containing a centered, 1-cm, glass-bottomed well (MaTEK, Inc., Ashland, MA). Cells were placed in 1 ml of minimum essential medium buffered with 20 mM HEPES and treated with 100 nM SP. The cells were then stimulated with 1 μM SP for various times. After this treatment, cells were washed with cold buffer and then lysed with 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 0.1 mM Na3VO4, 10 mM Na4P2O7, 1 mM EGTA, 10 mM NaF, 0.5% Nonidet P-40, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 0.1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged, precleared by incubating with protein A-Sepharose, and incubated for 1 h with 5 μl of a polyclonal antibody raised against the C terminus of human SPR (16). The mixture was then incubated for 1 h at 4 °C. The protein A-Sepharose was collected by centrifugation, washed with lysis buffer containing 1 mM NaCl, resuspended in 50 μl of SDS-polyacrylamide gel electrophoresis sample buffer (2% SDS, 60 mM Tris-HCl, pH 6.8, 10% glycerol, 10% β-mercaptoethanol, and 0.025% bromphenol blue), and the phosphorylated proteins were visualized by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

RESULTS

PKC Translocation after Human SPR Exposure to SP—The translocation of PKC to the plasma membrane occurs upon agonist stimulation of Gq-coupled GPCRs (19). Fig. 1 shows how the temporal distribution of PKCβII-GFP changes in HEK293 cells upon activation of SPRs by 100 nM SP. As seen in Fig. 1, the increase in PKCβII-GFP fluorescence at the plasma membrane, which separates the two cells, becomes evident within 3 s. This redistribution of PKC reflects SPR activation, which probably occurs even more rapidly. However, our ability to measure shorter time intervals is limited because there is image degradation upon employing more rapid scanning rates. As can be observed in Fig. 1, PKC translocation remains stable for 20–30 s, begins to wane after 30 s, and disappears by 60–90 s. To demonstrate that the reversibility of PKC translocation upon SPR activation is consistent with receptor desensitization and is not simply a generalized waning of the PKC response, cells were also exposed to phorbol 12-myristate 13-acetate. This compound persistently activates PKC by a receptor-independent mechanism (20, 21). Fig. 2 shows that the exposure of the cells to 100 nM phorbol 12-myristate 13-acetate results in a translocation of PKC to the plasma membrane that shows no sign of decreasing even after 5 min. These data indicate that the waning of PKC upon SPR activation is due to an upstream event which probably occurs at the level of the receptor. A similar conclusion was reached in a recent study by Feng et al. (19). It was found that stimulation of Gq-linked angiotensin or endothelin receptors also produces a single transient pulse of PKC translocation. However, the sequential activation of angiotensin receptors followed after a few minutes by the activation of endothelin receptors produced a second independent pulse of PKC translocation. This indicated that the desensitization occurred at the level of the receptor itself and was not due to the desensitization of downstream Gq-mediated signaling. The 60-s time period after SPR activation thus defines a window in which changes in the distributions of the cellular proteins responsible for homologous SPR desensitization should become apparent.

Translocation of GRK2 to the Plasma with SP Treatment—The cell cytoplasm is a major reservoir of GRK2 (18). If GRK2 plays a major role in initiating human SPR desensitization, then its physical translocation from the cytoplasm to the plasma membrane should be apparent within the temporal...
window defined by the reversible translocation of SP-activated PKC. To investigate the kinetics of GRK2 behavior during SP stimulation, GRK2-GFP was coexpressed along with the human SPR in HEK293 cells (Fig. 3). Previous studies have documented that GRK2-GFP is a functional kinase because it phosphorylates rhodopsin in both a Gβγ-independent and -dependent manner (18), facilitates β2AR internalization (18), and phosphorylates β2AR in intact cells.2 As seen in Fig. 3, the increase in fluorescence from the plasma membrane after SP treatment reflects the agonist-dependent translocation of GRK2-GFP. Using 3-s scanning intervals, it is seen that GRK2 accumulates at the membrane interface between two cells within 6–9 s of agonist treatment. This translocation peaks at between 15 and 21 s and wanes thereafter but does not entirely disappear, even after 1 min. These results visually demonstrate that human SPR activation recruits GRK2 to the plasma membrane during the period in which the receptor desensitizes. These data represent the first demonstration in living cells of a physical translocation of GRK2 to the plasma membrane after GPCR activation.

Agonist Stimulation of Human SPR Results in Its Phosphorylation—Because GRK2 translocates to the plasma membrane after SP stimulation, we examined whether this event leads to receptor phosphorylation. HEK293 cells expressing human SPR were labeled with 32P, and the receptor was immunoprecipitated from control and SP-treated cells. The results, which are shown in Fig. 4, indicate that SP causes a rapid phosphorylation of the receptor within 30 s of SP exposure. Thus, SP-induced translocation of GRK2 correlates with SP-induced phosphorylation of the SPR. A similarly rapid (t1/2 < 1 min) SP-dependent phosphorylation of the human SPR also occurs in Chinese hamster ovary cells that stably express the receptor.3

Fig. 3. Redistribution of GRK2 to the plasma membrane after SPR activation. HEK293 cells containing GRK2-GFP and human SPR were treated with SP at time 0 (top left panel). The kinetics of GRK2 translocation are followed using 3-s scanning intervals on the confocal microscope. GRK2 translocation occurs within 6 s (top panels), rapidly peaks, and then begins to wane (bottom panels) during the first minute after SPR activation. Bar = 10 μm.

DISCUSSION

We visually document in real time a rapid activation and desensitization of the human substance P receptor. The results indicate that SPR desensitization coincides with the active recruitment of GRK2 to the plasma membrane and phosphorylation of the receptor, followed by the recruitment of β-arrestins to the plasma membrane. Thus, these data establish a role for GRKs and β-arrestins in the homologous desensitization of SPRs. Moreover, these results represent the first direct confirmation in whole cells that the putative sequence of biochemical events underlying homologous GPCR desensitization (receptor activation and GRK-mediated phosphorylation followed by the binding of β-arrestins) actually occurs as postulated from a variety of in vitro and whole cell studies. Fig. 5 summarizes the temporal relationship of SPR activation and the redistribution of GRKs and β-arrestins.

Studies on the mechanism of homologous desensitization of GPCRs using the β2AR as a model have shown that increases in plasma membrane GRK activity occur after agonist treatment (22, 23). These findings did not necessarily require that a simultaneous recruitment of GRK from the cytosol also occurs. A significant finding of the present study is that a sustained physical translocation of GRK2 occurs with agonist stimulation of the human SPR. This type of GRK behavior in the presence

2 L. S. Barak, S. S. G. Ferguson, and M. G. Caron, unpublished results.
3 E. D. Roush and M. M. Kwatra, unpublished results.
of the substance P receptor has been suggested by immunochemical studies using fixed and extracted neuronal cells (24).

Similar studies using antibodies to assess GRK2 distribution in fixed HEK293 cells also seem to indicate that GRK2 is recruited to membranes during the early stages of desensitization and β2AR endocytosis (25). However, we have been unable to demonstrate any persistent observable physical translocation of GRK2-GFP to activated β2AR, despite results demonstrating that overexpressed GRK2-GFP enhances β2AR phosphorylation and sequestration. Similarly, we have been unable to document a physical translocation of GRK2-GFP to other types of GPCRs including the angiotensin II type IA receptor.

Thus, our finding of an observable physical GRK2-GFP translocation upon SPR stimulation is intriguing. A potential explanation for this may follow from the observation that the human SPR is an excellent substrate of GRK2. It can be nearly maximally phosphorylated in the absence of Gβγ with a K_m of 6 nM (16). In contrast, GRK2-mediated phosphorylation of the β2AR occurs with a K_m of 49 nM and requires Gβγ (26). The affinity of GRK2 for the β2AR is over 100-fold lower than the 0.3 nM K_d observed in vitro for β-arrestin 2 binding to the receptor (27). Therefore, for some GPCRs, β-arrestin may normally prevent persistent GRK/receptor interactions by displacing the kinase from the receptor, but GRK may remain at the membrane upon activation of GPCRs for which it has high affinity. This interpretation of the results also supports previous observations that GRKs facilitate but are not absolutely necessary for β2AR endocytosis (28).

An interesting observation made here is that SP causes a profound change in cell shape. This membrane remodeling appears to be more pronounced at the free surfaces of the cells, as shown in Fig. 7. Although the underlying mechanism of this phenomenon remains obscure, it may involve direct interactions of SP or GRK2 and tubulin (18, 29). It is interesting to speculate that the observed SP-induced changes in cell morphology may be due to the SPR’s high affinity for GRK2 (16), which would keep it localized on the membrane, where it can phosphorylate tubulin (18). Furthermore, whereas a role of the SPR in chemotaxis has been reported previously (30, 31), additional studies are needed to delineate the mechanism by which SP induces changes in cell shape.

4 L. S. Barak, unpublished data.
In summary, the present study visually illustrates the kinetic behavior of GRK2 and β-arrestin 2 during homologous desensitization of the human substance P receptor. The data provide direct evidence that the sequence of biochemical events proposed for homologous GPCR desensitization actually occurs in live cells. Moreover, the rapid but persistent membrane recruitment of GRK2 by the SPR suggests that the kinase may be involved in cell regulatory processes other than receptor desensitization.

Acknowledgments—We thank Drs. Eric D. Roush and Jie Zhang for helpful discussions and Susan R. S. Tumey for editorial assistance.

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