Human substance P receptor expressed in Chinese hamster ovary cells directly activates $G_{\alpha q/11}$, $G_{\alpha s}$, and $G_{\alpha o}$

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Abstract Substance P receptor (SPR) stably expressed in Chinese hamster ovary (CHO) cells stimulates at least three second messenger systems including phosphoinositide hydrolysis, cyclic AMP (cAMP) formation, and arachidonic acid release. Whether these second messenger systems are activated via single or multiple G proteins is not known. Therefore, in the present study we examined whether human SPR (hSPR) stably expressed in CHO cells activates multiple G proteins. This was achieved by photoaffinity labeling of G_α-subunits with [^{32}P]azidoanilido-GTP ([^{32}P]AA-GTP) upon hSPR stimulation in CHO-hSPR membranes followed by immunoprecipitation of the labeled G_α-subunits with antibodies specific for various G_α-subunits. These experiments reveal that hSPR directly activates $G_{\alpha q/11}$, $G_{\alpha s}$ and $G_{\alpha o}$. While hSPR is known to couple $G_{\alpha q/11}$, the present study provides the first evidence that hSPR can also activate $G_{\alpha s}$ and $G_{\alpha o}$ in a mammalian system.

Key words: Human substance P receptor; G protein; Chinese hamster ovary cell

1. Introduction

Substance P receptor (SPR; also known as NK-1 receptor) mediates the effects of substance P (SP), an 11 amino acid neuropeptide that plays a key role in several physiological processes including pain transmission and inflammation [1]. SPR is a member of a superfamily of receptors known as G protein-coupled receptors (GPCRs) which function through heterotrimeric G proteins consisting of α, β, and γ-subunits. Stimulation of GPCRs dissociates G proteins into G_α and $G_{\beta\gamma}$, which in turn activate various effector enzymes and ion channels [2,3]. Although SPR in most tissues and cell lines couples to phosphoinositide hydrolysis (PI) [4,5], recent studies on SPR stably transfected in Chinese hamster ovary (CHO) cells indicate that SPR activates multiple cellular responses, including PI hydrolysis, cAMP formation, and arachidonic acid release [6–9]. The G proteins that mediate these responses have not been identified.

Based on the characteristics of their α-subunits, G proteins have been classified into four families: G_s, G_i, G_q and G_{12}. The Gα-subunits of different families activate different effectors. For example: G_s family members ($G_{\alpha s}$ and $G_{\alpha olf}$) activate adenylyl cyclase isozymes, leading to increased cAMP levels; while members of the G_q family (α_q, α_{11}, α_{14}, and α_{16}) activate phospholiase C-β (PLC-β) isozymes which hydrolyze membrane phosphoinositides into inositol triphosphate and diacylglycerol. Although knowing which effector is stimulated by a receptor could identify the involved G_α-subunit, recent studies indicate that a given effector (for example, PLC-β) can be activated by both G_α and $G_{\beta\gamma}$-subunits [3,10,11].

Thus, SPR mediated PI hydrolysis may occur either through G_α-subunits of the G_q family or through $G_{\beta\gamma}$. However, it is likely that the process is mediated through $G_{\alpha q}$ since a direct interaction between $G_{\alpha q/11}$ and SPR has been demonstrated in a number of systems [12–14]. Similarly, SPR-mediated increase in cAMP formation in CHO cells could occur through $G_{\alpha s}$, a known activator of adenylyl cyclase [15]. However, a direct interaction between SPR and $G_{\alpha s}$ has not yet been demonstrated in mammalian systems. In addition, there are conflicting reports concerning the coupling of SPR to $G_{\alpha s}$ in other systems. A recent study indicated that in Sf9-insect cells, stimulation of human SPR (hSPR) does not lead to activation of coexpressed $G_{\alpha s}$ [16]. In contrast, our laboratory observed that hSPR overexpressed in Sf9 cells activates endogenous $G_{\alpha s}$ [17]. Since stimulation of adenylyl cyclase almost always requires activation of $G_{\alpha s}$ [15], the question of whether or not SPR can activate adenylyl cyclase through $G_{\alpha s}$ deserves investigation.

While activation of PI hydrolysis and cAMP formation through SPR may occur through $G_{\alpha q/11}$ and $G_{\alpha s}$, less is known about the G proteins involved in SPR-mediated arachidonic acid release. It has been reported that SPR-mediated arachidonic acid release in CHO cells is blocked by pretreatment with pertussis toxin [9], implicating a member of the G_i family of G proteins. However, although we have observed that hSPR in Sf9-insect cells activates endogenous $G_{\alpha o}$ [17], direct coupling of SPR to a member or members of the G_i family of G proteins has not been demonstrated in a mammalian system. Therefore, the present study was undertaken to determine whether SPR's coupling to multiple second messengers in CHO cells is due to activation of multiple G proteins.

2. Materials and methods

2.1. Materials

Aprotinin, bacitracin, chymostatin, leupeptin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, and substance P were from Sigma (St. Louis, MO). [^{32}P]α-GTP (3000 Ci/mmol), [^{3}H]SR140,333 (60 Ci/mmol), antibodies QL, RM/1, AS/7, EC/2 and GC/2 were obtained from New England Nuclear (Boston, MA) (see Table 1 for details). An antibody GK/1 against the peptide sequence CTLSAEERAALERGK from the N-terminus of human $G_{\alpha o}$ [18] was raised in rabbits

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Abbreviations: [^{32}P]AA-GTP, P^3-(4-azidoanilido)-[P^1-^{32}P]-5′-guanosine triphosphate; cAMP, cyclic adenosine 3′:5′-cyclic monophosphate; CHO, Chinese hamster ovary; CHO-hSPR, Chinese hamster ovary cell stably transfected with hSPR; CHO-K1, Chinese hamster ovary cells, strain K1; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SP, substance P; SPR, substance P receptor; hSPR, human substance P receptor

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by Quality Controlled Biochemicals, Inc. (Hopkinton, MA). Recombinant G protein subunits $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$ and $G_{\alpha s}$ (raised in *E. coli*) were obtained from Calbiochem (La Jolla, CA). Protein A Sepharose and unstained low molecular weight markers were from Pharmacia (Piscataway, NJ). The non-peptide SPR antagonists CP99,994 and CP96,345 were gifts from Dr. Saul Kadin, Pfizer, Inc., Groton, CT. These antagonists are used interchangeably in our laboratory. The cDNA of hSPR in pBluescript (hSPR-pBluescript) was kindly provided by Dr. J.E. Krause, Washington University School of Medicine, St. Louis, MO. Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in appropriate media at the cell culture facility of the Duke Comprehensive Cancer Center, Durham, NC.

2.2. Stable expression of hSPR in CHO-K1 cells

CHO-K1 cells were maintained in Ham's F-12 media containing 10% (v/v) heat-inactivated fetal calf serum at 37°C in a 5% CO_2 incubator. The cells were transfected with a full length cDNA of hSPR in pcDNA3 (Invitrogen, Carlsbad, CA) using a calcium phosphate method [19,20]. Clones expressing hSPR were selected by growth in 400 μg/ml G418 and were screened both by immunoblotting with hSPR-Ab and by ligand binding using [^{125}I]-BHSP. A clone (CHO-hSPR) was selected for further study which expressed about 2×10^5 receptors/cell, as measured with the antagonist radioligand [^{3}H]SR140,333 [17,21]. Receptor number was found to decrease with passage; therefore, new flasks of CHP-hSPR cells were started from frozen stock every 1–2 months in order to maintain high levels of receptor expression.

2.3. Photoaffinity labeling of CHO-hSPR membranes with [^{32}P]AA-GTP

CHO-hSPR cells were harvested from 175-cm^2 tissue culture flasks by incubation with PBS+0.5 mM EDTA, followed by centrifugation at $1800\times g$ at 4°C. A plasma membrane-enriched fraction derived from the CHO-hSPR cell pellet was prepared as described by Nishimura [17]. In these membranes, receptor density, measured with radioligand [^{3}H]SR140,333 [17,20], varied between 5–10 pmol/μg membrane protein. The membranes were either used the same day for photoaffinity experiments or were stored at −70°C until needed. Optimal photoaffinity labeling was observed with fresh membranes.

[^{32}P]AA-GTP was synthesized according to published procedures and purified by thin layer chromatography on PEI-cellulose (J.T. Baker, Phillipsburg, NJ) [17,22–25]. Photoaffinity labeling of CHO-hSPR membranes with [^{32}P]AA-GTP was performed as previously described [17]. Briefly, the reaction was performed in a total volume of 60 μl containing 20–50 μg CHO-hSPR membrane protein, 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 25 mM NaCl, 0.5 mM EDTA, and SPR ligands as indicated on figure legends. After incubation for 5 min at 30°C, 2 μCi of [^{32}P]AA-GTP was added and the samples were incubated at 30°C for the required time (3 min for members of the $G_{\alpha i}/G_{\alpha o}$ family, 5 min for $G_{\alpha s}$ and 10 min for $G_{\alpha q/11}$). The samples were then placed on ice and, after adding 8 μl of 20 mM DTT, irradiated at 254 nm with a UV lamp (model UVGL-25, UVP Inc., San Gabriel, CA) for 1 min from a distance of 6 cm. The mixture was solubilized by incubating for 10 min at room temperature with 15 μl of 10% SDS; diluted 1:1 with immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.5% SDS, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 μg/ml aprotinin, 0.2 mM PMSF); and centrifuged at $12\,000\times g$ for 10 min at 4°C. The pellet from this centrifugation was discarded and the supernatant was mixed with 3 μl of the appropriate antibody. The mixture was placed on a rotator for 1 h at 4°C and then 60 μl of 1:1 (v/v) Protein A Sepharose in immunoprecipitation buffer was added to each sample. After incubating the mixture for 1 h at 4°C, the Protein A Sepharose was pelleted by low speed centrifugation and washed twice with a buffer containing 50 mM Tris-HCl, pH 7.4, 0.6 M NaCl, 0.5% SDS, 1% Nonidet P-40, and twice with a buffer containing 100 mM Tris-HCl, pH 7.4, 0.3 M NaCl, 10 mM EDTA. The washed Protein A Sepharose was then resuspended in 100 μl SDS-PAGE sample buffer (2% SDS, 60 mM Tris-HCl pH 7.8, 10% glycerol, 10% β-mercaptoethanol, 0.025% bromophenol blue) and heated at 80°C for 5 min. The immunoprecipitated G proteins were visualized by SDS-PAGE/autoradiography.

3. Results and discussion

3.1. Second-messengers activated by hSPR in CHO-hSPR cells

As reported previously for rat SPR in CHO cells [6–9], we find that stimulation of hSPR in CHO-hSPR cells by SP leads to activation of multiple second messengers. A 20-min exposure to SP results in a 10-fold increase in inositol phosphate levels, a 4-fold increase in cAMP levels, and a 2-fold increase in arachidonic acid release relative to untransfected CHO-K1 cells (data not shown).

3.2. Identification of hSPR-activated G proteins in CHO-hSPR membranes

An elegant way of identifying receptor-activated G proteins is the covalent labeling of G_α-subunits with [^{32}P]AA-GTP in the presence or absence of an agonist followed by immunoprecipitation with antibodies specific for various G_α-subunits [17,22–25]. Using this technique, we show that stimulation of hSPR in CHO-hSPR membranes activates $G_{\alpha q/11}$ (Fig. 1). The activation of $G_{\alpha q/11}$ is receptor-mediated since it is blocked when hSPR is stimulated in the presence of the SPR antagonist CP99,994. These results suggest that hSPR-mediated PI hydrolysis in CHO cells may occur through $G_{\alpha q/11}$ since this G protein is a known activator of PLCβ, an enzyme known to catalyze PI-hydrolysis.

We next tested whether hSPR in CHO cells activates $G_{\alpha s}$ since this G_α-subunit is a known activator of adenylyl cyclase, an enzyme that catalyzes cAMP formation. As shown in Fig. 2, stimulation of hSPR in membranes from CHO-hSPR cells activates $G_{\alpha s}$. This activation is receptor-mediated since it is blocked by SPR antagonist CP99,994 (Fig. 2). These results represent the first demonstration of the activation of $G_{\alpha s}$ by hSPR in a mammalian system. Since hSPR can activate $G_{\alpha s}$, it is likely that hSPR-stimulated cAMP formation in CHO cells is mediated through $G_{\alpha s}$. Consistent with this notion, we find that SP-stimulated adenylyl cyclase activity in CHO-hSPR membranes is specifically blocked by a polyclonal antibody against $G_{\alpha s}$ (data not shown).

Since hSPR-mediated arachidonic acid release is sensitive to pertussis toxin, we also examined the ability of hSPR to acti-

Table 1
Antibodies used in immunoprecipitation of various G_α-subunits

Antibody	Antigen sequence	Region of G_α	Specificity	Source
QL	QLNLKEYNLV	C-terminal	$G_{\alpha q/11}$	NEN
RM/1	RMHLRQYELL	C-terminal	$G_{\alpha s}$	NEN
AS/7	KENLKDCGLF	C-terminal	$G_{\alpha i1}$,$G_{\alpha i2}$	NEN
EC/2	KNNLKECGLY	C-terminal	$G_{\alpha i3}$, $G_{\alpha o}$	NEN
GC/2	GCTLSAEERAALERSK	N-terminal	$G_{\alpha o}$	NEN
GK/1[a]	CTLSAEERAALERGK	N-terminal	$G_{\alpha o} \approx G_{\alpha i1} \approx G_{\alpha i2} \gg G_{\alpha i3}$	Kwatra

[a]See Fig. 3.

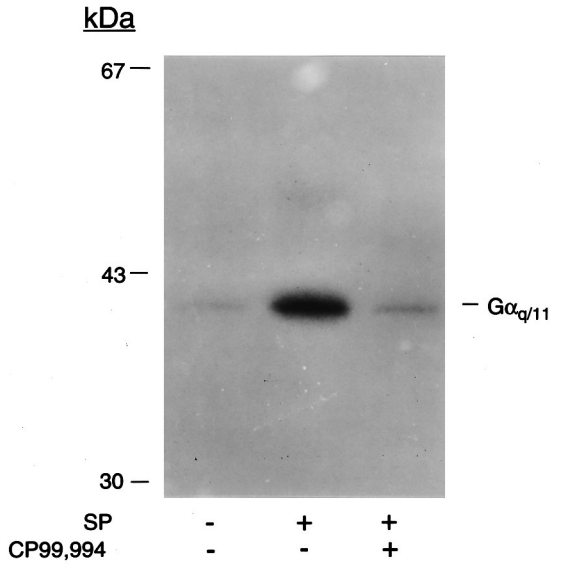

Fig. 1. Identification of hSPR-activated G_α-subunits in CHO-hSPR membranes by photoaffinity labeling with [^{32}P]AA-GTP followed by immunoprecipitation with antibodies specific for $G_{\alpha q/11}$. Enriched plasma membranes from CHO-hSPR cells were photoaffinity labeled with 2 μCi of [^{32}P]AA-GTP in a total volume of 60 μl, irradiated with UV light and processed for immunoprecipitation with QL antibody as described in Section 2. The concentrations of SP and CP99,994 were 1 μM and 1 mM, respectively. The experiment was repeated three times with similar results.

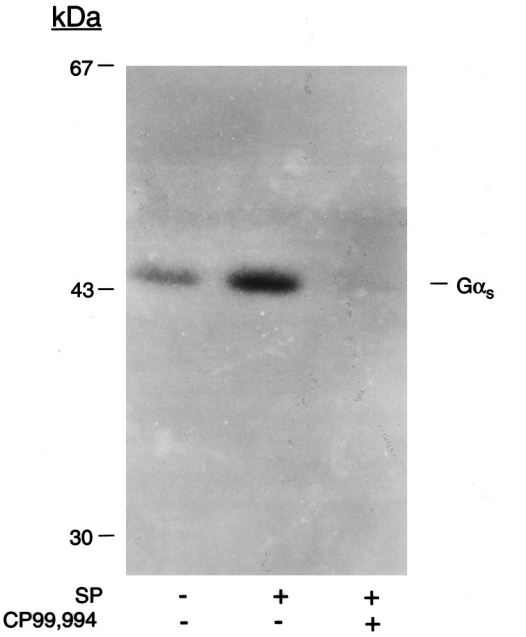

Fig. 2. Identification of hSPR-activated G_α-subunits in CHO-hSPR membranes by photoaffinity labeling with [^{32}P]AA-GTP followed by immunoprecipitation with antibodies specific for $G_{\alpha s}$. Enriched plasma membranes from CHO-hSPR cells were photoaffinity labeled with 2 μCi of [^{32}P]AA-GTP in a total volume of 60 μl that also contained 1 μM GDP, irradiated with UV light and processed for immunoprecipitation with RM/1 antibody as described in Section 2. The concentrations of SP and CP99,994 were 1 μM and 1 mM, respectively. The experiment was repeated three times with similar results.

vate G proteins of the G_i family. To this end, we used several different antibodies specific for various G_α-subunits of the G_i family (Table 1). The specificities of antibodies AS/7, EC/2, and GC/2 are provided by the manufacturer (New England Nuclear, Boston, MA), while the specificity of antibody GK/1 against various G_α-subunits was determined using G_α-subunits standards purchased from commercial sources (Calbiochem, La Jolla, CA). This antibody recognizes $G_{\alpha o}$, $G_{\alpha i1}$, and $G_{\alpha i2}$ equally well (Fig. 3, lanes 1–3) but poorly recognizes $G_{\alpha i3}$ (Fig. 3, lane 4) and does not react at all with $G_{\alpha s}$ (Fig. 3, lane 5). Fig. 4 shows the G proteins immunoprecipitated by these antibodies after [^{32}P]AA-GTP labeling. As can be seen, immunoprecipitation with GK/1 indicates that stimulation of hSPR in CHO-hSPR membranes activates a G_α-subunit of the G_i family. This G_α-subunit could either be one of the several $G_{\alpha i}$'s or $G_{\alpha o}$ since these subunits are recognized by GK/1. However, the immunoprecipitated G_α-subunit is probably not $G_{\alpha i1}$ or $G_{\alpha i2}$ since no labeled G_α-subunits are immunoprecipitated with AS/7 antibody. The activated G_α-subunit appears to be $G_{\alpha o}$ since it is also immunoprecipitated with the $G_{\alpha o}$-specific antibody GC/2. It should be pointed out that a different G_α-subunit is labeled and immunoprecipitated with EC/2; this G protein could be $G_{\alpha i3}$. However, this G_α-subunit does not exhibit hSPR-mediated activation.

The results of the present study indicate that hSPR in CHO cells activates $G_{\alpha q/11}$, $G_{\alpha s}$, and $G_{\alpha o}$. Therefore, activation of these G_α-subunits appears to be the underlying mechanism for SPR-mediated activation of PI-hydrolysis, cAMP formation, and arachidonic acid release, respectively. While coupling of SPR to $G_{\alpha q/11}$ has been demonstrated in several systems, the present study is the first report to demonstrate the coupling of hSPR to $G_{\alpha s}$ and $G_{\alpha o}$ in a mammalian system. These findings are similar to our previous demonstration of coupling of hSPR to $G_{\alpha q/11}$, $G_{\alpha o}$, and $G_{\alpha s}$ in Sf9 cells expressing high levels (65 pmol/mg membrane protein) of hSPR [17]. However, while our data strongly support that hSPR activates $G_{\alpha s}$, the inability of Barr et al. to demonstrate interactions between hSPR and exogenously introduced $G_{\alpha s}$ in Sf9 cells [16] remains to be explained.

It should also be mentioned that while CHO-hSPR is an artificial expression system for hSPR, the expression of hSPR in these cells (200 000 receptors/cell) is comparable to the 150 000 hSPR molecules/cell found in the U373 and U11 human astrocytoma cell lines [26,27], and is only 10-fold higher than the 20 000 hSPR molecules/cell found in the IM-9 human lymphoblastoma cell line [28]. Although one cannot rule out the possibility that a heretofore undefined trait peculiar to CHO cells is responsible for the coupling of hSPR to these G_α-subunits, it seems more likely that the observed coupling between hSPR and Gα other than $G_{\alpha q/11}$ in CHO cells reflects

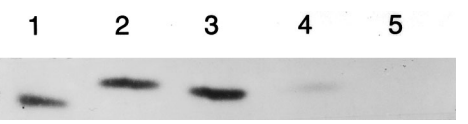

Fig. 3. Characterization of the polyclonal antibody GK/1 raised against the N-terminal sequence of $G_{\alpha o}$. 10-μl aliquots of G protein standards expressed in *E. coli* and partially purified (Calbiochem, La Jolla, CA) were run on a 10% SDS-PAGE gel and transferred to PVDF. The membrane was probed by a 1:200 dilution of the polyclonal antibody GK-1, followed by a 1:3000 dilution of goat-anti-rabbit secondary antibody coupled to alkaline phosphatase. The immunoblot was developed by BCIP/NBT. Lane 1: $G_{\alpha o}$; lane 2: $G_{\alpha i1}$; lane 3: $G_{\alpha i2}$; lane 4: $G_{\alpha i3}$; lane 5: $G_{\alpha s}$.

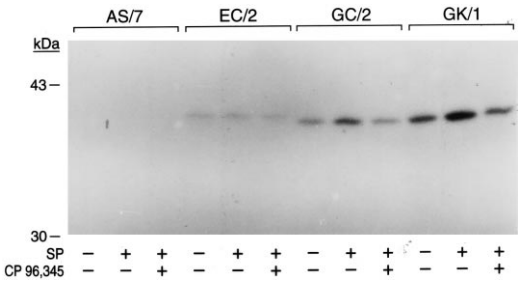

Fig. 4. Identification of hSPR-activated G_α-subunits in CHO-hSPR membranes by photoaffinity labeling with [^{32}P]AA-GTP followed by immunoprecipitation with antibodies specific for members of the G_i family. Enriched plasma membranes from CHO-hSPR cells were photoaffinity labeled with 2 μCi of [^{32}P]AA-GTP in a total volume of 60 μl that also contained 10 μM GDP, irradiated with UV light and processed for immunoprecipitation with commercially available antibodies against $G_{\alpha i1-i2}$ (lanes 1–3), $G_{\alpha i3}$ (lanes 4–6), and $G_{\alpha o}$ (lanes 7–9) as described in Section 2. Lanes 10–12 are immunoprecipitations of photoaffinity-labeled membranes using GK-1 (see Fig. 2). The concentrations of SP and CP96,345 were 1 μM and 1 mM, respectively. The experiment was repeated twice with similar results.

the ability of hSPR to interact with these G_α-subunits under physiological conditions. Consistent with this notion are the observations that (a) SP stimulates cAMP formation in thyroid tissue [29] and in neuroblastoma cells [30], and (b) treatment with pertussis toxin partially abolishes SP-stimulated arachidonic acid release in a lactotroph-enriched rat anterior pituitary cell culture [31] and completely abolishes an SP-stimulated guinea-pig mesenteric lymphatic contractile response driven by arachidonic acid metabolites [32].

In conclusion, we demonstrate that hSPR in CHO cells directly activates $G_{\alpha q/11}$, $G_{\alpha s}$, and $G_{\alpha o}$ and activation of these G_α-subunits appears to be an inherent property of hSPR.

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