Chemical Cross-Linking of the Substance P (NK-1) Receptor to the α Subunits of the G Proteins G_q and G_{11}†

Susan G. Macdonald,‡ John J. Dumas,§ and Norman D. Boyd*,§

Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, Massachusetts 02118

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ABSTRACT: We have previously shown that the high-affinity binding of substance P (SP) to its receptor is dependent on an interaction with a PTX-insensitive G protein. This G protein couples SP receptor activation to stimulation of its effector, phospholipase C. In this study, we combined photoaffinity labeling, chemical cross-linking techniques, and immunological characterization using sequence-specific antibody probes to identify G proteins that couple to the SP receptor. First we covalently labeled the SP receptor present on rat submaxillary gland membranes with a radioiodinated photoreactive derivative of SP, *p*-benzoyl-L-phenylalanine[8]-substance P (^{125}I-[Bpa[8]]SP). Photoincorporation of this SP derivative was susceptible to guanine nucleotide inhibition, indicating that the receptor was coupled to its G protein during labeling. We then used a chemical cross-linking agent to covalently link the photoaffinity labeled SP receptor and its associated G protein. Cross-linking generated a 96 kDa product, formation of which was prevented by the addition of a guanine nucleotide, but not an adenine nucleotide, following photolabeling, but prior to cross-linking. Furthermore, the 96 kDa cross-linked complex was absent in membranes which had been depleted of G proteins by treatment with alkaline buffer prior to addition of the cross-linking agent. Reductive cleavage of the cross-link in the isolated 96 kDa complex yields two products: the 53 kDa SP receptor and a 42 kDa protein identified by immunoblot analysis as either $G\alpha_q$ or $G\alpha_{11}$. Antisera against a common sequence within $G\alpha_s$, $G\alpha_i$, and $G\alpha_o$ showed no immunoreactivity to the complex or its cleavage products. These results provide the first direct evidence of specific interaction between photoaffinity labeled SP receptor and the α subunits of G_q and G_{11}, members of a family of G proteins known to be associated with pertussis toxin-insensitive phospholipase C activation.

Upon binding to its receptor, the neurotransmitter substance P (SP)[1] elicits a wide variety of effects, including stimulation of salivary secretion, excitation of central and peripheral neurons, inflammation, contraction of smooth muscle, and plasma extravasation. The binding properties of the SP receptor, also termed the neurokinin-1 (NK-1) receptor, as well as the intracellular events stimulated by SP binding, are regulated by guanine nucleotides (Cascieri & Liang, 1983; Lee et al., 1983; Luber-Narod et al., 1990), implying that the SP receptor is coupled to a guanine nucleotide binding protein (G protein) which regulates agonist affinity. Indeed, the sequences of the SP receptor deduced from cDNA clones (Yokota et al., 1989; Hershey & Krause, 1990) show homology with sequences of other members of the superfamily of G protein-coupled receptors (Dohlman et al., 1991; Strader et al., 1995). The first direct demonstration that the SP receptor is functionally coupled to a G protein was provided by studies in which high-affinity SP binding was restored in G protein-depleted membranes from rat submaxillary gland by reconstituting the membranes with purified G proteins, G_o and G_i (Macdonald & Boyd, 1989). More recent studies have shown that addition of another G protein preparation, consisting of purified G_q/G_{11}, to rat SP receptor reconstituted in lipid vesicles, resulted in a conversion of receptors to a high-affinity state (Kwatra et al., 1993).

The objective of the present study was to identify G proteins which couple to the SP receptor under conditions which more closely approximate the physiological environment. SP receptors were photoaffinity labeled in a membrane preparation from rat submaxillary glands, then specific receptor−G protein α subunit interactions were identified using chemical cross-linking strategies and immunodetection techniques.

Receptor stimulation by SP leads to the activation of phospholipase C and thus the generation of inositol trisphosphate, diacylglycerol, and Ca^{2+} release (Hanley et al.,

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* Address correspondence to this author: Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, 80 E. Concord St., Boston, MA 02118.

‡ Present address: Onyx Pharmaceuticals, 3031 Research Drive, Richmond, CA 94806.

§ Boston University School of Medicine.

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[1] Abbreviations: SP, substance P; [Bpa[8]]SP, *p*-benzoyl-L-phenylalanine[8]-substance P; ^{125}I-[Bpa[8]]SP, ^{125}I-labeled Bolton−Hunter conjugate of [Bpa[8]]SP; GppNHp, 5′-guanylylimidodiphosphate; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); AppNHp, 5′-adenylylimidodiphosphate; G protein, guanine nucleotide binding protein; G_o, G_i, pertussis toxin-sensitive guanine nucleotide binding proteins; G_q, G_{11}, pertussis toxin-insensitive guanine nucleotide binding proteins; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DMSO, dimethyl sulfoxide; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); EGS, ethylene glycol bis(succinimidylsuccinate); sulfo-EGS, ethylene glycol bis(sulfosuccinimidylsuccinate); endoglycosidase F, endo-β-*N*-acetylglucosamine F, EC 3.2.1.96; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide.

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1980; Watson & Downes, 1983; Berridge & Irvine, 1984; Mantyh et al., 1984; Taylor et al., 1986, Merritt & Rink, 1987). There are at least two distinct pathways by which G proteins are able to stimulate receptor-mediated phospholipase C activation (Lo & Hughes, 1987; Ashkenazi et al., 1989): one that is susceptible to pertussis toxin-catalyzed ADP-ribosylation and one that is not. Pertussis toxin-catalyzed ADP-ribosylation of submaxillary gland membranes, which functionally uncouples G_o and G_i from receptors, has no effect on high-affinity SP binding (Macdonald & Boyd, 1989), implying that the SP receptor is physiologically coupled to a pertussis toxin-insensitive G protein. Pertussis or cholera toxin-catalyzed ADP-ribosylation, which had traditionally been used to identify G proteins, cannot be used to label the G proteins that couple to the SP receptor.

The α subunit of the heterotrimeric G protein G_q, α_q, has been cloned (Strathmann & Simon, 1990), and it has been shown by several independent investigators that partially purified preparations of this protein activate phospholipase C (Smrcka et al., 1991; Taylor et al., 1991; Waldo et al., 1991). Members of this family lack the site for PTX-catalyzed ADP-ribosylation, a cysteine which is located four residues from the carboxy terminus. A mixture of the α subunits of two members of this family, G_q and G_{11}, was purified from bovine brain by $\beta\gamma$-agarose affinity chromatography (Pang & Sternweis, 1990). These α subunits from bovine brain, as well as an apparently similar 42 kDa α subunit purified from rat liver, have been shown to activate polyphosphoinositide-specific phospholipase C (Smrcka et al., 1991; Taylor et al., 1991). Similarly, stimulation of activation of the β isoform of phospholipase C was reconstituted by addition of a chromatographically purified cholate extract from turkey erythrocytes which is immunoreactive to α_q antisera (Waldo et al., 1991). More recently, recombinant $G\alpha_q$ and $G\alpha_{11}$ have been expressed in Sf9 cells, purified, and shown to specifically and selectively stimulate β isoforms of phospholipase C (Hepler et al., 1993). Currently, there are many receptors that appear to activate phospholipase C via $G\alpha_q/G\alpha_{11}$. These include receptors for thromboxane A2, bradykinin, angiotensin, histamine, and vasopressin, as well as muscarinic acetylcholine receptors (Shenker et al., 1991; Wange et al., 1991; Gutowski et al., 1991; Berstein et al., 1992). These particular α subunits, and α subunits of the other members of the G_q family with which they share homology, are therefore excellent candidates for involvement in the SP receptor signal transduction pathway.

Previously, we have demonstrated the usefulness of a radioiodinated photoreactive analogue of SP for labeling SP receptors with high (>70%) efficiency. Preliminary evidence was also obtained which indicated that photolabeling occurred when the receptors were coupled to their associated G protein (Boyd et al., 1991a). Chemical cross-linking strategies have previously been used to characterize ligand−receptor interactions in many systems (Pilch & Czech, 1980; Massague et al., 1981; Payan et al., 1986; Resek & Ruoho, 1988) and to determine nearest neighbor relationships among membrane proteins (Wang & Richards, 1974), including receptors and G proteins (Negishi et al., 1987; Kermode et al., 1992). In this report, we describe the chemical cross-linking of the photoaffinity labeled SP receptor to a 42 kDa polypeptide and provide evidence that it represents the α subunits of G_q and G_{11}.

EXPERIMENTAL PROCEDURES

Materials. *p*-Benzoyl-L-phenylalanine (Bpa) was prepared as described by Kauer et al. (1986), and synthesis of the [Bpa^8]SP derivative was prepared as described previously (Boyd et al., 1991b). In this study a radioactive derivative of ^{125}I-[Bpa^8]SP was prepared by conjugation with monoiodinated ^{125}I-labeled Bolton−Hunter reagent (2200 Ci/mmol). GppNHp, AppNHp, DTT, EDTA, HEPES, DMSO, and protein A-conjugated Sepharose CL-4B beads were purchased from Sigma Chemical Co. DTSSP, EGS, and sulfo-EGS were obtained from Pierce. Substance P was purchased from Peninsula Laboratories (San Carlos, CA). Endoglycosidase F was from Boehringer Mannheim. All other biochemicals were purchased from Bio-Rad Laboratories. Centricon 30 microconcentrator units were obtained from Amicon Corporation (Danvers, MA). Antisera raised against α_q-specific and α_{11}-specific internal amino acid sequence, W082 (Pang & Sternweis, 1990) and B825 antisera, respectively, was generously provided by Dr. Paul C. Sternweis (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX). Antiserum generated against synthetic peptide with sequence corresponding to SP receptor residues 273−287, the presumed third extracellular domain of the receptor, was kindly provided by Dr. James E. Krause (Department of Anatomy and Neurobiology, Washington University, St. Louis, MO). Antiserum raised against a common sequence within α subunits of G_q and G_{11} and antiserum against a common sequence within the GTP-binding site of α subunits of G_s, G_o, and G_i were purchased from DuPont-NEN.

Photoaffinity Labeling and Chemical Cross-Linking of the SP Receptor. Membranes were prepared as previously described from rat submaxillary glands (Macdonald & Boyd, 1989). Membrane preparations at a concentration of 5 mg of membrane protein per mL were incubated in the dark with 0.3 nM ^{125}I-[Bpa^8]SP for 30 min at room temperature in 25 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 1 mM EDTA (HME buffer). After the binding had reached equilibrium, the membranes were washed three times with ice-cold HME (pH 7.4) to remove unbound ligand. Membranes were then resuspended at 2 mg of membrane protein per mL in HME (pH 8.0) at 4 °C and distributed in 0.5 mL aliquots into chilled, 35 mm tissue culture dishes. All dishes were placed on ice and irradiated for 15 min at a distance of 4 cm from a 100 W long-wave (365 nm) UV lamp (Blak-Ray).

To achieve chemical cross-linking, the photoaffinity labeled membranes were warmed to room temperature and a 100-fold concentrated solution of sulfo-EGS in DMSO was then added to give a final concentration of 1 mM. The membranes were incubated on a shaker at room temperature for 15 min when using sulfo-EGS and for 30 min when using DTSSP. The cross-linking reaction was quenched by adding two volumes of 50 mM Tris-HCl and 10 mM EDTA, pH 7.4 (TE buffer). When DTSSP was used, 20 mM NEM was included in the quenching buffer to alkylate any reactive sulfhydryl groups. In some instances, membranes were incubated at room temperature for 30 min with 10 μM GppNHP or 10 μM AppNHp before chemical cross-linking. The photoaffinity labeled membranes were treated before or

after chemical cross-linking with 50 mM sodium phosphate buffer (pH 11.5) for 30 min at 4 °C. Previously, we have shown that these conditions deplete salivary gland membranes of functional endogenous G proteins (Macdonald & Boyd, 1989). Following alkaline treatment, membranes were resuspended in HME buffer prior to addition of the cross-linking agent.

SDS/PAGE and Autoradiography. Following the photoaffinity labeling and cross-linking procedures, membranes (3 mg of protein per mL) were solubilized in SDS sample buffer (Laemmli, 1970) without β-mercaptoethanol for 10 min at 60 °C. Samples (100−300 μg of membrane protein) were electrophoresed on 10% SDS/polyacrylamide slab gels according to the methods of Laemmli (1970).

Following electrophoresis, gels were dried and exposed to Kodak XAR-5 film at −80 °C using a Quanta III intensifying screen (Dupont-NEN). Bio-Rad low molecular weight standards and Bio-Rad prestained molecular weight markers were used to determine the molecular weights of the radiolabeled polypeptides.

Individual lanes of autoradiograms were analyzed densitometrically using the GS-365PS electrophoresis data reduction system, version 1.00 (Hoefer Scientific Instruments). The areas under the peaks corresponding to the SP receptor and the 96 kDa cross-linked receptor complex were determined, and the ratio of the amount of 96 kDa cross-linked receptor complex to the total amount of SP receptor in each lane was then calculated. In some experiments, the efficiency of cross-linking was also determined by the aligning of the dried gel with the autoradiogram, excising the regions of the gel corresponding to non-cross-linked SP receptor and the 96 kDa cross-linked complex, and comparing relative amounts of radioactivity associated with each region. Both methods of analysis yielded similar estimates of cross-linking efficacy.

Preparation of the Photoaffinity Labeled, Chemically Cross-Linked SP Receptor. ^{125}I-[Bpa^8]SP was covalently incorporated into 25 mg of rat submaxillary gland membranes and the photoaffinity labeled membranes chemically cross-linked as described above with the exception that the irradiation was performed at a membrane concentration of 5 mg of protein per mL in a 100 mm tissue culture dish. The membranes were then solubilized in SDS sample buffer, divided into two equal aliquots and loaded onto two 10% slab gels, each 3 mm in thickness. The location of the photoaffinity labeled, chemically cross-linked receptor complex was identified by removal of 5 mm pieces of a longitudinal strip from the side of each gel and determined by measurement of γ-emission. The horizontal band containing the higher molecular weight peak of radioactivity was then excised. The proteins were electroeluted using an ISCO concentrator, model 1750. If necessary, samples were further concentrated by centrifugation using a Centricon 30 unit (Amicon, Inc.). In some preparations, the region of the gel containing the photolabeled but non-cross-linked receptor was excised and subjected to the electroelution and concentration steps.

Endoglycosidase F Treatment of the Photoaffinity Labeled SP Receptor and Chemically Cross-Linked Receptor Complex. Aliquots of photoaffinity labeled SP receptor or photoaffinity labeled, chemically cross-linked receptor complex, obtained as described above by preparative SDS/PAGE, were diluted with two volumes of 0.2 M sodium phosphate (pH 8.2), 0.05 M EDTA, and 2% NP-40. Endoglycosidase F (6 units/120 μL) was then added to the samples to yield a final concentration of 4.5 units/mL. Following a 5 h incubation at 37 °C, the enzymatic reaction products were analyzed by SDS/PAGE and autoradiography.

Reductive Cleavage of the Photoaffinity Labeled, DTSSP-Cross-Linked SP Receptor Complex. Aliquots of isolated, photoaffinity labeled SP receptor or photoaffinity labeled, DTSSP-cross-linked receptor complex were incubated for 10 min at 60 °C in SDS sample buffer, which contained 250 mM DTT. Cleavage of the cross-link was confirmed by SDS/PAGE and autoradiography.

Gel Transfer and Immunoblotting. Chemically cross-linked 96 kDa complex was excised from slab gels, electroeluted, and concentrated as described above. Half of this eluted complex was subject to reductive cleavage by incubating in TE buffer containing a final concentration of 30 mM DTT at room temperature for 60 min. The 96 kDa complex and the products of its reduction were resolved by SDS/PAGE (10% polyacrylamide). Proteins were transferred onto nitrocellulose (MSI, Westboro, MA) in a Transphor Electrophoresis Unit (Hoefer Scientific Instruments) at a setting of 1 A for 4 h. Nonspecific antibody binding was blocked by incubating membrane for at least 2 h at 4 °C in phosphate buffered saline (PBS) with 5% nonfat dry milk. Membrane blots were then incubated overnight with primary antiserum (1:1000 dilution in 1% nonfat dry milk in PBS), followed by by three washes with 1% nonfat dry milk/0.3% Triton-100X in PBS at room temperature. Blots were subsequently incubated with secondary antibody (1:10 000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Life Science) in 1% nonfat dry milk in PBS) at room temperature for 2 h, followed by three washes with 0.3% Triton-100X in PBS. Renaissance Western Blot Chemiluminescence Reagent (DuPont-NEN) was used as the substrate for detection of immunoreactivity. Light emission was recorded on autoradiographic film.

Immunoprecipitation of SP Receptor Complex. Chemically cross-linked 96 kDa complex was electroeluted from SDS/PAGE gel slices as described above, and immunoprecipitation experiments were performed as described previously (Boyd et al., 1996). Aliquots of 96 kDa complex in electroelution buffer (5 mM Tris, 1 mM EDTA, 0.05% SDS, pH 8.0) were incubated at 4 °C for 1 h with antisera which recognize either G protein α subunits or the SP receptor. Following antisera incubation, protein A-conjugated Sepharose (50 μL of a 10% suspension in TE buffer) was then added and incubation continued for an additional 30 min. Immunoprecipitates were washed three times by alternate centrifugation and resuspension of the pellets. The amount of radiolabeled receptor complex precipitated was determined by measurement of γ-emission.

RESULTS

As has been described in detail previously (Boyd et al., 1991b), photoincorporation of ^{125}I-labeled *p*-benzoyl-L-phenylalanine^8-substance P (^{125}I-[Bpa^8]SP) into rat submaxillary gland membranes resulted in the specific labeling of two polypeptides possessing relative molecular masses of 53 and 46 kDa (Figure 1, lanes 1 and 2). Other work in this laboratory has shown that the 53 and 46 kDa SP receptor polypeptides contain similar amounts of N-linked carbohy-

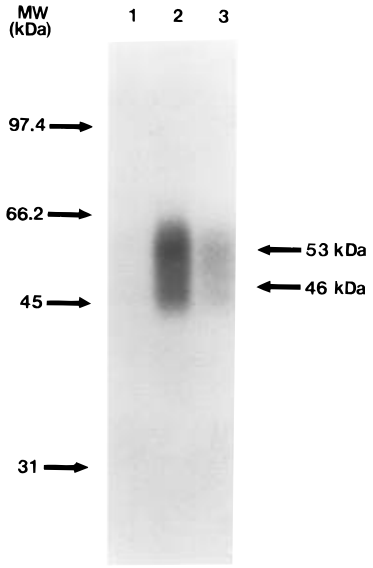

FIGURE 1: Photoaffinity labeling of the SP receptor. ^{125}I-[Bpa^8]SP (0.3 nM) was incubated with rat submaxillary gland membranes and photolyzed as described under Experimental Procedures. Following photolysis, membranes were subjected to SDS/PAGE and the labeled bands visualized by autoradiography. (Lane 1) ^{125}I-[Bpa^8]SP incubated in the presence of 1 μM SP before photolysis. (Lane 2) No further treatment. (Lane 3) Following equilibration of ^{125}I-[Bpa^8]SP, membranes were incubated for 30 min at room temperature with 10 μM GppNHp and then photolyzed.

drates but differ at the C-terminus in the length of their polypeptide chain (Kage et al., 1993). The labeling of the full-length and C-terminal truncated SP receptor polypeptides was inhibited by the addition of 10 μM GppNHp (Figure 1, lane 3), suggesting that the photolabeling of both forms of the SP receptor occurs when coupled to a G protein.

In an effort to characterize this G protein, chemical cross-linking agents were tested for their ability to cross-link the photoaffinity labeled SP receptor to subunits which comprise its associated G protein heterotrimer. Of the several cross-linking reagents tested, only the water-soluble cross-linking agents, sulfo-EGS and DTSSP, consistently generated a distinct radiolabeled complex of higher molecular weight. The results of a typical cross-linking experiment in which photolabeled SP receptors were treated with sulfo-EGS are shown in Figure 2A. Treatment of the photolabeled membranes with sulfo-EGS resulted in the appearance of an additional radiolabeled band at 96 kDa (compare lanes 1 and 2 in Figure 2A). Densitometric analysis of the autoradiogram indicated that about 14% of the photolabeled receptors underwent chemical cross-linking to yield the 96 kDa covalent complex. In similar cross-linking experiments, the average cross-linking efficiency was 13.6 $\pm$ 3.6% (n = 7). To provide evidence that the photolabeled SP receptor complex at 96 kDa was the result of a covalent linkage between the SP receptor and its G protein α subunit, the photoaffinity labeled membranes were incubated with 10 μM GppNHp for 30 min prior to the addition of sulfo-EGS. Prior incubation with the guanine nucleotide resulted in a marked reduction in the densitometric intensity of the 96 kDa band (Figure 2A, lane 3 and Table 1). In contrast, incubation with 10 μM AppNHp prior to cross-linking had little effect on the intensity of the 96 kDa band (Figure 2A, lane 4 and Table 1). The nucleotide specificity observed for inhibition of cross-linking reflected the nucleotide specificity observed for

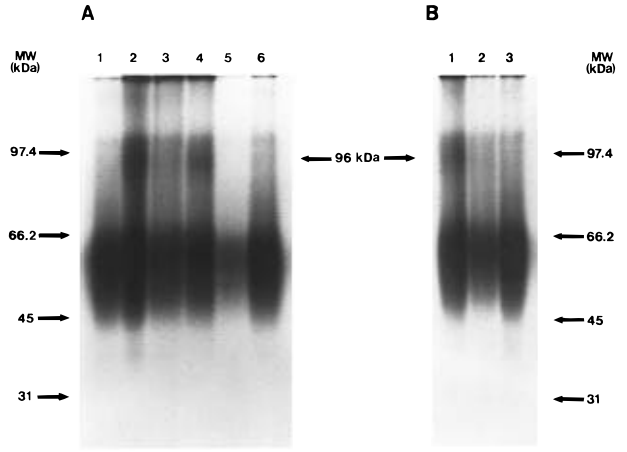

FIGURE 2: Chemical cross-linking of the photoaffinity labeled SP receptor. (Panel A) Rat submaxillary gland membranes were equilibrated with 0.3 nM ^{125}I-[Bpa^8]SP, photolyzed and incubated with 1 mM sulfo-EGS as described under Experimental Procedures. (Lane 1) Photoaffinity labeling followed by incubation without sulfo-EGS. (Lane 2) Photoaffinity labeling followed by incubation with sulfo-EGS. (Lane 3) Following photoaffinity labeling, membranes were incubated with 10 μM GppNHp for 30 min at room temperature prior to addition of sulfo-EGS. (Lane 4) Following photoaffinity labeling, membranes were incubated with 10 μM AppNHp for 30 min at room temperature prior to addition of sulfo-EGS. (Lane 5) Membranes equilibrated with ^{125}I-[Bpa^8]SP were incubated for 30 min at room temperature with 10 μM GppNHp prior to photolysis. (Lane 6) Membranes equilibrated with ^{125}I-[Bpa^8]SP were incubated for 30 min at room temperature with 10 μM AppNHp prior to photolysis. (Panel B) Photoaffinity labeled rat submaxillary gland membranes were treated with sodium phosphate (pH 11.5) before or after chemical cross-linking with 1 mM sulfo-EGS. (Lane 1) Membranes were photoaffinity labeled and chemically cross-linked prior to alkaline treatment. (Lane 2) Membranes were photoaffinity labeled and then alkaline treated. (Lane 3) Photoaffinity labeled membranes were alkaline treated before chemical cross-linking. (Note: The individual receptor bands are not distinguishable as the autoradiographs have been overexposed to show the position of the 96 kDa cross-linked complex.)

Table 1: Effect of Nucleotides and Alkaline Treatment on the Amount of Total Photolabeled Receptor That Undergoes Cross-Linking to form the 96 kDa Complex[a]

treatment[b]	% photolabeled receptor in 96 kDa complex[c]
0	13.6 $\pm$ 3.6% (n = 7)
10 μM GppNHp	3.3 $\pm$ 1.1% (n = 5)
10 μM AppNHp	11.8 $\pm$ 2.1% (n = 4)
pH 11.5	<1% (n = 2)

[a] Band intensities (in arbitrary densitometric units) were determined by densitometric scanning of autoradiograms. [b] Refers to the treatment of the photoaffinity labeled membranes prior to cross-linking with sulfo-EGS. [c] The fraction of the total photolabeled receptor that had undergone cross-linking to yield the 96 kDa complex was determined as described in Experimental Procedures. Values are given as mean $\pm$ SD for the indicated number of determinations.

inhibition of photoaffinity labeling of the SP receptor (Figure 2A, lanes 5 and 6). Thus, the well recognized ability of guanine, but not adenine nucleotides, to dissociate agonist-bound receptor−G protein complexes can account for both the reduction in photolabeling when added prior to the photoactivation step and the reduced formation of the photolabeled SP receptor−α subunit covalent complex when added following photolabeling, but prior to the addition of the cross-linker.

Previously, we have shown that alkaline treatment of a membrane preparation from rat submaxillary gland can be

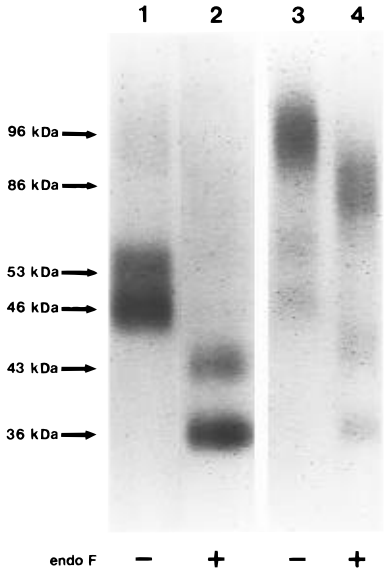

FIGURE 3: Endoglycosidase F treatment of the photoaffinity labeled SP receptor and cross-linked photoaffinity labeled receptor complex. The SP receptor and cross-linked receptor complex were obtained by preparative SDS/PAGE and electroelution and then treated with 4.5 units/mL endoglycosidase F as described under Experimental Procedures. Products of the enzymatic reaction were analyzed by SDS/PAGE and autoradiography. (Lane 1) Photoaffinity labeled SP receptor incubated in buffer without endoglycosidase F. (Lane 2) Photoaffinity labeled SP receptor incubated in buffer containing 4.5 units/mL endoglycodisase F. (Lane 3) Cross-linked receptor complex incubated in buffer without endoglycosidase F. (Lane 4) Cross-linked receptor complex incubated in buffer containing 4.5 units/mL endoglycosidase F.

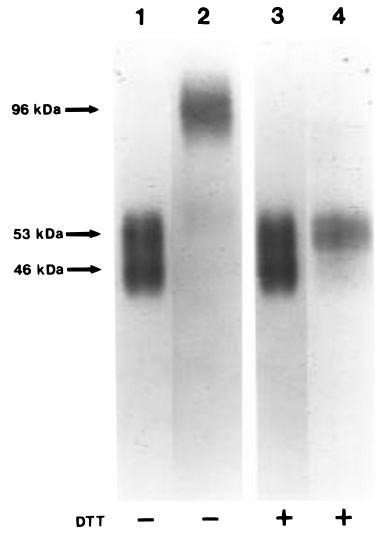

FIGURE 4: Reductive cleavage of the photoaffinity labeled, DTSSP-cross-linked receptor complex. The photoaffinity labeled SP receptor and the DTSSP-cross-linked complex were obtained by preparative SDS/PAGE under nonreducing conditions and then treated with 50 mM DTT as described. Following reduction samples were then analyzed by SDS/PAGE and autoradiography. (Lane 1) Photoaffinity labeled receptor in the absence of DTT. (Lane 2) DTSSP-cross-linked complex in the absence of DTT. (Lane 3) Photoaffinity labeled receptor following treatment with DTT. (Lane 4) DTSSP-cross-linked complex following treatment with DTT.

used to deplete these membranes of endogenous G proteins while leaving the SP receptor functionally intact (Macdonald & Boyd, 1989). This use of alkaline buffers to obtain G protein-depleted membrane preparations has also been described in studies of other G-protein coupled receptors, including β-adrenergic receptors in turkey erythrocyte membranes (Citri & Schramm, 1980) and α_2-adrenergic receptors in human platelet membranes (Kim & Neubig, 1985, 1987). To provide additional evidence supporting the identification of the protein cross-linked to the SP receptor as a G protein α subunit, photolabeled membranes were briefly treated with pH 11.5 buffer prior to the addition of sulfo-EGS (Figure 2B). This alkaline treatment, which has been shown previously to produce a >90% reduction in GTPγS binding sites, completely abolished the appearance of the 96 kDa band (Figure 2B, lane 3, and Table 1). On the other hand, alkaline treatment had no effect on the electrophoretic mobility of the photolabeled SP receptor (Figure 2B, lane 2) or the 96 kDa covalent complex (Figure 2B, lane 1).

Previous work in our laboratory has shown that the SP receptor is a glycoprotein containing about 10 kDa of N-linked carbohydrates (Boyd et al., 1991a). To provide information about the glycosylation state of the protein covalently linked to the SP receptor, the photoaffinity labeled SP receptor and the 96 kDa cross-linked receptor complex were resolved by preparative SDS/PAGE and individually treated with endoglycosidase F (Figure 3). When analyzed by SDS/PAGE and autoradiography, the photoaffinity labeled SP receptor ran as two polypeptides of 53 and 46 kDa (Figure 3, lane 1), and each was decreased by 10 kDa following endoglycosidase F treatment (Figure 3, lane 2).[2] Treatment of the 96 kDa cross-linked receptor complex with endoglycosidase F also caused a 10 kDa decrease in its relative molecular weight (Figure 3, lane 4) indicating that the protein covalently linked to the SP receptor lacks N-linked carbohydrate residues. This is the expected result if the photoaffinity labeled SP receptor is covalently linked to a G protein α subunit, since the amino acid sequence data for the known α subunits show that they all lack sites for the attachment of N-linked sugars (Simon et al., 1991).

The detection of an apparently single radiolabeled covalent complex either before or after deglycosylation by endoglycosidase F raised the question of whether both the full-length and truncated forms of the SP receptor can undergo covalent linkage to the G protein α subunit to form covalent adducts which, although differing in M_r, cannot be resolved by SDS/PAGE autoradiography, or whether only one form of the SP receptor undergoes cross-linking. To address this question, the composition of the 96 kDa complex was analyzed in further detail. Photoaffinity labeled membranes were treated with 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP), a cross-linking reagent that contains a cleavable disulfide bond within its spacer arm. The resulting 96 kDa covalent complex was resolved by preparative SDS/PAGE and subjected to compositional analysis by reductive cleavage of the covalent cross-link with dithiothreitol (DTT) followed by SDS/PAGE autoradiography (Figure 4). Reductive cleavage of the covalent cross-link in the 96 kDa complex yielded the 53 kDa, but not the 46 kDa, form of the SP receptor (Figure 4, lane 4). Treatment of the photoaffinity labeled SP receptor with DTT had no effect on the electrophoretic mobility of either receptor isoform (Figure 4, lane

[2] Since endoglycosidase F treatment of the isolated 53 kDa SP receptor yields a 43 kDa polypeptide and endoglycosidase F treatment of the isolated 46 kDa SP receptor yields a 36 kDa polypeptide, we have concluded that the 43 and 36 kDa bands represent deglycosylated forms of the 53 and 46 kDa SP receptor, respectively (Kage et al., 1993).

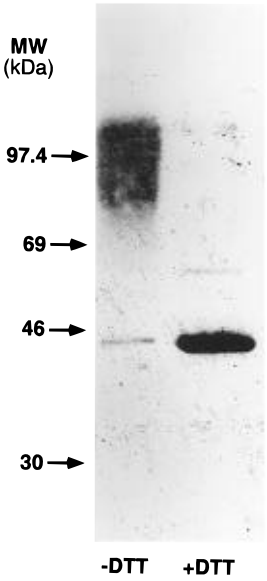

FIGURE 5: Immunoblot of the receptor complex and products of reductive cleavage using $G\alpha_q/G\alpha_{11}$-specific antisera. The DTSSP-cross-linked complex was obtained by preparative SDS/PAGE and electroelution. A portion of the complex was subject to reduction by incubation in buffer with 30 mM DTT as described. Immunoblots were labeled using α_q/α_{11}-specific antisera. (Lane 1) DTSSP-cross-linked complex in the absence of DTT. (Lane 2) DTSSP-cross-linked complex in the presence of DTT.

3). These results indicate that only the 53 kDa form of the SP receptor undergoes cross-linking to yield a 96 kDa complex. Thus the polypeptide cross-linked to the SP receptor has, by subtraction, an apparent molecular mass of about 42 kDa, a value within the range typically found for α subunits of known G proteins.

In order to identify the 42 kDa protein which we have cross-linked to the SP receptor, antisera that had been generated against specific G protein α subunits were used for immunoblot analysis. The 96 kDa SP receptor complex was formed by treatment of photolabeled membranes with DTSSP, and the band containing this complex was partially purified by preparative gel electrophoresis and electroelution. This partially purified protein complex and the products derived by reductive cleavage of the covalent cross-link were resolved by SDS/PAGE and then transferred to nitrocellulose for immunoblot analysis. Blots were probed using antisera generated against an internal amino acid sequence specific to either α_q or α_{11}, and antisera against a common α_q/α_{11} sequence, as well as antisera against a common sequence within α_s, α_i, and α_o. $G\alpha_q/G\alpha_{11}$-specific immunoreactivity was detected as a diffuse band in the 96 kDa region (Figure 5, lane 1), indicating that the 96 kDa complex contains this α subunit. The band representing radiolabeled 96 kDa complex, visualized autoradiographically as in Figure 3, was seen to superimpose on the band which corresponds to α_q/α_{11}-specific immunoreactivity. Immunoreactivity and radioactivity is diffuse in the 96 kDa region due to the variable degree of glycosylation of the population of SP receptors contained within complexes (Kage et al., 1993). Following reduction of the disulfide bond within DTSSP, a single, sharp band of α_q/α_{11}-specific immunoreactivity was visualized at 42 kDa, the predicted molecular mass of these α subunits (Figure 5, lane 2). Immunoblots probed with either α_q-specific or α_{11}-specific antisera showed results similar to those seen with α_q/α_{11} antisera (data not shown), indicating that both of these α subunits were cross-linked to the photolabeled SP receptor. In contrast, using antisera generated against a common amino acid sequence found within the GTP-binding site of α subunits of G_s, G_o, and G_i, immunoreactivity was not detected in either the 96 kDa region of the uncleaved complex or, following reduction, within the predicted molecular weight range for these α subunits.

Table 2: Summary of Immunoprecipitation Experiments

antisera specificity	immunoprecipation of 96 kDa complex
α_q	+
α_{11}	+
α_q/α_{11}	++
α_{common}	−
SP receptor	++

Further evidence that the 96 kDa complex represents a cross-linked species consisting of photolabeled SP receptor covalently attached to either α_q or α_{11} subunits was obtained by immunoprecipitation of the complex with a variety of antisera, including antisera generated against specific α subunits and the SP receptor (Table 2). Antisera which recognize α_q and α_{11}, but not antisera which recognize α_s, α_o, and α_i isoforms, immunoprecipitate the photolabeled receptor complex.

DISCUSSION

Activation of the SP receptor leads to stimulation of phospholipase C activity by a pathway that appears to involve a PTX-insensitive G protein. The initial evidence that a G protein is involved in SP receptor signaling was provided by Lee et al. (1983), who showed that the binding of radiolabeled SP to rat salivary gland membranes was inhibited by guanine nucleotides. Subsequently, we have shown that this effect of guanine nucleotides was through a PTX-insensitive G protein that was required for the expression of high-affinity agonist binding to the SP receptor (Macdonald & Boyd, 1989). Thus, as for other G protein-coupled receptors, SP binding in the absence of guanine nucleotides gives rise to a stable, high-affinity ternary complex of SP, SP receptor, and G protein. The addition of a guanine nucleotide causes a rapid dissociation of the G protein that is accompanied by a marked loss in affinity for SP (Luber-Narod et al., 1990). The G protein that couples to the SP receptor stimulates phospholipase C in a pertussis toxin-insensitive manner (Merritt et al., 1986; Laniyonu et al., 1988; Nakajima et al., 1988). Of the currently known G protein α subunits, only members from the G_q family have been shown to activate phospholipase C *in vitro* in a PTX-insensitive manner (Pang & Sternweis, 1990; Taylor et al., 1990, 1991; Rhee & Choi, 1992; Hepler et al., 1993).

In the present studies, we investigate the feasibility of using a combination of photoaffinity labeling and chemical cross-linking techniques to identify G protein α subunits which couple to the SP receptor. Results from immunoblot and autoradiograph analysis indicate that the complex consists of a radiolabeled full-length SP receptor isoform cross-linked to either a G_q or G_{11} α subunit. Identification is confirmed by a number of characteristics, including molecular size, lack

of glycosylation, sensitivity to guanine nucleotides, and sequence-specific immunoreactivity.

Reductive cleavage of the DTSSP-cross-linked receptor complex showed that only the 53 kDa form of the SP receptor participates in cross-linking. The protein cross-linked to the SP receptor has an apparent molecular mass of 42 kDa, a value that is within the molecular mass range of known G protein α subunits. Treatment of the complex with endoglycosidase F indicates that only a single receptor is present within the complex, thus eliminating the possibility that the cross-linked species consists of SP receptor dimers. Furthermore, 42 kDa proteins can be detected by immunoblot analysis following reductive cleavage of the cross-linked complex which are specifically identified as α_q and α_{11}. In addition to immunoblot analysis, immunoprecipitation experiments have demonstrated that the 96 kDa receptor complex possesses α_q/α_{11}-specific immunoreactivity. The 96 kDa complex, containing the ^{125}I-[Bpa^8]SP-photolabeled receptor, has been immunoprecipitated with antisera specifically generated against α_q and α_{11} peptide sequences, as well as SP receptor sequence. This result, together with immunoblot analysis, provides strong evidence that in membrane preparations isolated from rat salivary glands we have formed covalent complexes consisting of photoaffinity agonist, full-length receptor and a G protein α subunit, either α_q or α_{11}.

It is interesting that of the various cross-linking agents tested only sulfo-EGS and DSSTP produced the 96 kDa covalent complex in good yield. The importance of the water solubility of the cross-linker is apparent when the cross-linking patterns observed for EGS, which is relatively water-insoluble and readily partitions into the lipid bilayer, and a water-soluble derivative, sulfo-EGS, are compared. Higher molecular mass complexes (>200 kDa) predominated when EGS was used (data not shown), presumably as a result of formation of additional cross-links within the plane of the membrane. In contrast, the sulfonated derivative of EGS, sulfo-EGS, which is water-soluble and membrane impermeable (Staros, 1982), produced the 96 kDa photolabeled SP receptor complex with about a 14% cross-linking efficiency, while only small amounts of higher molecular weight complexes were formed. This striking difference in cross-linking patterns suggests that the amino functions of the SP receptor and the α subunit with which it forms a complex are located outside of the membrane in the aqueous environment. This conclusion is consistent with the results of studies that indicate the interactions of G protein-coupled receptors with their G proteins involves sequences located in their cytoplasmic domains (Ostrowski et al., 1992; Franke et al., 1992; Namba et al., 1993; Stefan & Blumer, 1994).

While both the full-length (53 kDa) and the C-terminal truncated (46 kDa) isoforms of the SP receptor are present in rat salivary gland membranes and couple to the G protein (Kage et al., 1993), only the full-length receptor can be covalently cross-linked to the α subunit. All cross-linkers used in this report are *N*-hydroxysuccinimidyl esters, which only react significantly with ϵ-amino functional groups in proteins (Cuatrecasas & Parikh, 1972). Since the truncated receptor lacks about 50−70 amino acids in its C-terminal cytoplasmic tail, it is reasonable to conclude that one or more of the four lysine residues present only within the full-length isoform, and not the truncated isoform, of the SP receptor participate in covalent linking to α subunits. Thus, while the distal end of the C-terminal cytoplasmic tail of the SP receptor is not an absolute requirement for interaction with G_a/G_{11}, nevertheless this region must be located in close proximity to the G protein α subunit. Such an arrangement is potentially of mechanistic significance since studies of the β-adrenergic receptor, which like the SP receptor possesses a serine- and threonine-rich C terminus, provide evidence that phosphorylation of these residues by a specific kinase plays an important role in desensitization, by interfering with the interaction between the receptor and its G protein (Hausdorff et al., 1991; Roth et al., 1991).

Since the G protein that couples to the SP receptor cannot be labeled by bacterial toxins, we have developed an alternative labeling approach which combines the use of the photolabeled SP receptor as a highly specific detection probe, with the use of recently available α subunit antisera for immunological detection. The successful application of this approach was dependent on the availability of an efficient photoaffinity agonist. The SP derivative, ^{125}I-[Bpa^8]SP, can be used to photolabel about 70% of the SP receptors in rat submaxillary gland membranes, and subsequently about 14% of these photolabeled receptors can be chemically cross-linked to their associated G protein(s). The efficiency of cross-linking is quite high considering that only full-length photolabeled receptors undergo cross-linking, which seems to indicate a close association between receptor and G protein. The concentration of SP receptor in crude membrane preparations of rat submaxillary gland is about 500 fmol/mg of membrane protein (Boyd et al., 1991b), and the yield of the 96 kDa covalent complex is typically between 20−50 fmol/mg of membrane protein, an amount that is sufficient for further analysis of SP receptor−G protein complexes.

The availability of α subunit-specific antibodies has enabled us to identify two α subunits, α_q and α_{11}, which can be cross-linked selectively to the SP receptor in salivary gland membranes. Results of analysis with α_{common} antiserum appear to rule out interactions with other α subunits known to be present in rat salivary glands, particularly α_s and α_i isoforms (Ali et al., 1992). For other G protein-coupled receptors, there is increasing, but as yet circumstantial, evidence that activation of multiple cellular pathways may involve interactions of single receptor types with multiple classes of G proteins (Offermanns & Schultz, 1994). The experimental approach described here should provide useful information for understanding the functional complexity of these G protein interactions.

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REFERENCES

Ali, N., Agrawal, D. K., & Cheung, P. (1992) *Mol. Cell. Biochem. 115*, 155−162.

Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J., & Capon, D. J. (1989) *Cell 56*, 487−493.

Berridge, M. J., & Irvine, R. F. (1984) *Nature 312*, 315−321.

Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C., Exton, J. H., & Ross, E. M. (1992) *J. Biol. Chem. 267*, 8081−8088.

Boyd, N. D., Macdonald, S. G., Kage R., Luber-Narod, J., & Leeman, S. E. (1991a) *Ann. N.Y. Acad. Sci. 632*, 79−93.
Boyd, N. D., White, C. F., Cerpa, R., Kaiser, E. T., & Leeman, S. E. (1991b) *Biochemistry 30*, 336−342.
Boyd, N. D., Kage, R., Dumas, J. J., Krause, J. E., & Leeman, S. E. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 433−437.
Cascieri, M. A., & Liang, T. (1983) *J. Biol. Chem. 258*, 5158−5164.
Citri,Y., & Schramm, M. (1980) *Nature 287*, 297−300.
Cuatrecasas, P., & Parikh, I. (1972) *Biochemistry 11*, 2291−2299.
Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.
Franke, R. R., Sakamar, T. P., Graham, R. M., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 14767−14774.
Gutowski, S., Smrcka, A., Nowak, L., Wu, D., Simon, M., & Sternweis, P. C. (1991) *J. Biol. Chem. 266*, 20519−20524.
Hanley, M. R., Lee, C. M., Jones, L. M., & Michell, R. H. (1980) *Mol. Pharmacol. 18*, 78−83.
Hausdorff, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., & Lefkowitz, R. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2979−2983.
Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., & Gilman, A. G. (1993) *J. Biol. Chem. 268*, 14367−14375.
Hershey, A. D., & Krause, J. E. (1990) *Science 247*, 958−962.
Kage, R, Leeman, S. E., & Boyd, N. D. (1993) *J. Neurochem. 60*, 347−351.
Kauer, J. C., Erickson-Viitanen, S., Wolfe, H. R., Jr., & DeGrado, W. F. (1986) *J. Biol. Chem. 261*, 10695−10700.
Kermode, J. C., DeLuca, A. W., Zilberman, A., Valliere, J., & Shreeve, S. M. (1992) *J. Biol. Chem. 267*, 3382−3388.
Kim, M. H., & Neubig, R. R. (1985) *FEBS Lett. 192*, 321−325.
Kim, M. H., & Neubig, R. R. (1987) *Biochemistry 26*, 3664−3672.
Kwatra, M. M., Schwinn, D. A., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. G., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 9161−9164.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Laniyonu, A., Sliwinski-Lis, E., & Fleming, N. (1988) *FEBS Lett. 240*, 186−190.
Lee, C. M., Javitch, J. A., & Snyder, S. H. (1983) *Mol. Pharmacol. 23*, 563−569.
Lo, W. W. Y., & Hughes, J. (1987) *FEBS Lett. 224*, 1−3.
Luber-Narod, J., Boyd, N. D., & Leeman, S. E. (1990) *Eur. J. Pharmacol. 188*, 185−191.
Macdonald, S. G., & Boyd, N. D. (1989) *J. Neurochem. 53*, 264−272.
Mantyh, P. W., Pinnock, R. D., Downes, C. P., Goedert, M., & Hunt, S. P. (1984) *Nature 309*, 795−797.
Massague, J., Guillette, B. J., & Czech, M. P. (1981) *J. Biol. Chem. 256*, 2122−2125.
Merritt, J. E., & Rink, T. J. (1987) *J. Biol. Chem. 262*, 14912−14916.
Merritt, J. E., Taylor, C. W., Rubin, R. P., & Putney, J. W. (1986) *Biochem. J. 236*, 337−343.
Nakajima, Y., Nakajima, S., & Inoue, N. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3643−3647.
Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., & Narumiya, S. (1993) *Nature 365*, 166−170.
Negishi, M., Ito, S., Tanaka, T., Yokohama, H., Hayashi, H., Katada, T., Ui, M., & Hayaishi, O. (1987) *J. Biol. Chem. 262*, 12077−12084.
Offermanns, S., & Schultz, G. (1994) *Arch. Pharmacol. 350*, 329−338.
Ostrowski, J., Kjelsberg, M. A., Caron, M. G., & Lefkowitz, R. J. (1992) *Annu. Rev. Pharmacol. Toxicol. 32*, 167−183.
Pang, I.-H., & Sternweis, P. C. (1990) *J. Biol. Chem. 265*, 18707−18712.
Payan, D. G., McGillis, J. P., & Organist, M. L. (1986) *J. Biol. Chem. 261*, 14321−14329.
Pilch, P. F., & Czech, M. P. (1980*) J. Biol. Chem. 255*, 1722−1731.
Resek, J. F., & Ruoho, A. E. (1988) *J. Biol. Chem. 263*, 14410−14416.
Rhee, S. G., & Choi, K. D. (1992) *J. Biol. Chem. 267*, 12393−12396.
Roth, N. S., Campbell, P. T., Caron, M. G., Lefkowitz, R. J., & Lohse, M. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6201−6204.
Shenker, A., Goldsmith, P., Unson, C. G., & Spiegel, A. M. (1991) *J. Biol. Chem. 266*, 9309−9313.
Simon, M. I., Strathmann, M. P., & Gautam, N. (1991) *Science 252*, 802−808.
Smrcka, A. V., Hepler, J. R., Brown, K. O., & Sternweis, P. C. (1991) *Science 251*, 804−807.
Staros, J. V. (1982) *Biochemistry 21*, 3950−3955.
Stefan, C. J., & Blumer, K. J. (1994) *Mol. Cell. Biol. 14*, 3339−3349.
Strader, C. D., Fong, T. M., Graziano, M. P., & Tota, M. R. (1995) *FASEB J. 9*, 745−754.
Strathmann, M., & Simon, M. I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9113−9117.
Taylor, C. W., Merritt, J. E., Putney, M. W., Jr., & Rubin, R. P. (1986) *Biochem. Biophys. Res. Commun. 136*, 362−368.
Taylor, S. J., Smith, J. A., & Exton, J. H. (1990) *J. Biol. Chem. 265*, 17150−17156.
Taylor, S. J., Chae, H. Z., Rhee, S. G., & Exton, J. H. (1991) *Nature 350*, 516−518.
Waldo, G. L., Boyer, J. L., Morris, A. J., & Harden, T. K. (1991) *J. Biol. Chem. 266*, 14217−14225.
Wang, K., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 8005−8018.
Wange, R. L., Smrcka, A. V., Sternweis, P. C., & Exton, J. H. (1991) *J. Biol. Chem. 266*, 11409−11412.
Watson, S. P., & Downes, C. P. (1983) *Eur. J. Pharmacol. 93*, 245−253.
Yokota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, M. R., Kakizuka, A., Ohkub O. H., & Nakanishi, S. (1989) *J. Biol. Chem. 264*, 17649−17652.

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