To determine the intracellular signaling mechanism of the 5-HT2c receptor endogenously expressed in choroid plexus epithelial cells, we employed a strategy of targeted disruption of protein-protein interactions. This strategy entails the delivery of conjugated membrane-permeable peptides that disrupt domain interaction at specific steps in the signaling cascade. As proof of concept, two peptides targeted against receptor-G protein interaction domains were examined. Only GαCT, which targets the receptor-G protein interacting domain, disrupted 5-HT2c receptor-mediated phosphatidylinositol hydrolysis. GαCT, targeting the receptor-G protein, disrupted β2 adrenergic receptor-mediated activation of cAMP but not 5-HT2c receptor-mediated phosphatidylinositol turnover. However, both peptides disrupted Gβγ-mediated α2A adrenergic receptor activation of mitogen-activated protein kinase. These results provide the first direct demonstration that active Gα subunits mediate endogenous 5-HT2c receptor activation. Moreover, peptides Gβγ-mediated α2A adrenergic receptor activation of mitogen-activated protein kinase. These results provide the first direct demonstration that active Gα subunits mediate endogenous 5-HT2c receptor activation of PLCβ1 resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol (12). In addition, activation of the 5-HT2c receptor has been observed to release arachidonic acid (13), increase cyclic GMP (14), and regulate potassium channels and Ca2+-activated chloride channels (15–18). These observations suggest that 5-HT2c receptor activation results in the induction of multiple signaling pathways. However, it is unclear how each individual intracellular signaling pathway contributes to modulation of the cell as a whole. In this paper, we employed a novel strategy to dissect intracellular signaling pathways, which combines a newly developed peptide synthesis technology with the application of targeted disruption of protein-protein interactions. This strategy is applied to examine PI hydrolysis signaling, a well defined pathway associated with 5-HT2c receptor activation.

The current model of 5-HT2c receptor signaling suggests that Gα11 heterotrimers are the immediate G protein mediators of receptor signaling based on two indirect observations. First, activation of PLCβ1 predominantly occurs through the Gα11 family. Second, 5-HT2c receptor-mediated PI hydrolysis is largely pertussis toxin (PTX)-insensitive, which suggests that Gα11 heterotrimers, which activate PLC via their βγ subunits, are not involved (19). However, previous studies that directly examined the identity of the G protein-mediating 5-HT2c receptor signaling were all conducted in artificial systems (20–22) where the receptors may have promiscuous interactions with various heterotrimeric G proteins. To address this question in a native environment, we examined the G protein mediator of 5-HT2c receptor signaling in primary cultures of choroid plexus epithelial cells and further assessed the role of Gα and Gβγ subunits.

The 5-HT2 receptor family consists of three members, 5-HT2A, 5-HT2B, and 5-HT2C. All three receptors belong to the G protein-coupled serpentine receptor superfamily. Their pharmacological profiles are very similar, leading to difficulty in defining their functional roles. 5-HT2 receptors have been implicated in behaviors such as sleep, feeding, aggression, pain, and anxiety and are thought to play a role in a number of central nervous system disorders including affective disease, schizophrenia, and epilepsy (1). In addition, 5-HT2 receptors may play a major role in mediating the actions of hallucinogenic drugs (2) as well as antipsychotic drugs (3, 4). Mice expressing nonfunctional 5-HT2c receptors exhibit epileptic and obese phenotypes (5, 6), suggesting that these receptors play a crucial role in moderating central nervous system function.

Expression of the 5-HT2c receptor is exceptionally high in the choroid plexus (7, 8), where it plays a role in the regulation of production and composition of cerebrospinal fluid (9–11). Initial studies of 5-HT2c receptor signaling showed that these receptors activate the downstream intracellular effector, phospholipase Cβ (PLCβ) resulting in the hydrolysis of phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol (12). In addition, activation of the 5-HT2c receptor has been observed to release arachidonic acid (13), increase cyclic GMP (14), and regulate potassium channels and Ca2+-activated chloride channels (15–18). These observations suggest that 5-HT2c receptor activation results in the induction of multiple signaling pathways. However, it is unclear how each individual intracellular signaling pathway contributes to modulation of the cell as a whole. In this paper, we employed a novel strategy to dissect intracellular signaling pathways, which combines a newly developed peptide synthesis technology with the application of targeted disruption of protein-protein interactions. This strategy is applied to examine PI hydrolysis signaling, a well defined pathway associated with 5-HT2c receptor activation.

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The abbreviations used are: PLCβ, phospholipase Cβ; PI, phosphoinositide; PTX, pertussis toxin; MPS, membrane-permeable sequence; PLCβ1M, phospholipase Cβ1-mimicking peptide; Phos, phospholin-like peptide; PhLP, phospholipin-like protein; Fmoc, fluorenlymethyloxycarbonyl; HPLC, high performance liquid chromatography; CFE, choroid plexus epithelial; HBSS, Hank’s balanced salt solution; DMEM, Dulbecco’s modified Eagle’s medium; MAP, mitogen-activated protein; CHAPS, 3-[3-cholamidopropyl]dimethylammonionitropropanesulfonic acid; mGluR, metabotropic glutamate receptor.

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Dissecting Endogenous 5-HT\textsubscript{2C} Receptor Signaling Pathways

**Table I**

| Name      | Protein          | Residue | Peptide target                  | Sequence                  | Solubility |
|-----------|------------------|---------|----------------------------------|---------------------------|------------|
| MPS       | Kaposi fibroblast growth factor signal sequence |         |                                  | AVALLPAVLLALLAK(S\textsuperscript{a}) | >100 µg/ml |
| G\textsubscript{CT} | G\textsubscript{q11} C terminus | 350–359 | Receptors coupling to G\textsubscript{q11} heterotrimers | CQLNLKEYLN | 5 µg/ml |
| G\textsubscript{CT} | G, C terminus | 385–394 | Receptors coupling to G\textsubscript{q11} heterotrimers | CRMHLRQYELL | 50 µg/ml |
| PLC\textsubscript{b1M} | Phospholipase C\textsubscript{b1} | 1053–1084 | Active G\textsubscript{q} subunits | CQLKKLKEICKEKKKMKKKMKKQKKEIKEAK | >100 µg/ml |
| PLC\textsubscript{b2M} | Phospholipase C\textsubscript{b2} | 564–583 | Free G\textsubscript{b} subunits | CNRSVYISSPTELKADLS | 10 µg/ml |
| Phos      | Phosducin-like protein C terminus | 186–195 | Free G\textsubscript{b} subunits | CVTDQGLGDFDAYDLEAFQEGGLPEKE | 40 µg/ml |

\textsuperscript{a}This lysine is attached to the carboxyl terminus of the serine by its side-chain group (see Figure 1).

\textsuperscript{b}The C-terminal cysteine moiety is added to facilitate chemical conjugation through thiazolidine formation.

**Experimental Procedures**

**Materials**—Most peptides used were synthesized in our laboratory; G\textsubscript{CT}, G\textsubscript{CT}, G\textsubscript{CT}, and PLC\textsubscript{b2M} were also synthesized by Genosys (The Woodlands, Texas). Antibodies against PLC\textsubscript{b1} isozymes and G\textsubscript{q11} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). G\textsubscript{q} antibodies were a kind gift of Dr. Tom Martin (University of Wisconsin-Madison, WI).

**Peptide Design**—The sequence of the various peptides and their proposed targets are presented in Table I. The membrane-permeable sequence (MPS) peptide was based on a hydrophobic membrane-permeable sequence described previously (23–25). Lysine and serine residues, as a pseudo-dipeptide, were added at the carboxyl terminus to serve as a linker and a masked aldehyde to facilitate conjugation. The lysine was attached to the carboxyl terminus of the MPS sequence by its primary amine, and then serine was attached at the side chain amine (Fig. 1).

Phospholipase C\textsubscript{b1} mimicking peptide (PLC\textsubscript{b1M}) was derived from amino acids 1053–1084 of the PLC\textsubscript{b1} enzyme. This design stems from the observation that loss of the last 10 kDa from the carboxyl terminus of PLC\textsubscript{b1} results in the loss of interaction with active G\textsubscript{q} (28). Additional work, using a series of deletion mutants, defined region 1030–1142 as the domain required for interaction with G\textsubscript{a} subunits (27). A specific segment within this region (amino acids 1053–1084) was observed to dose dependently inhibit GTP\textsubscript{S}-dependent activation of PLC using either purified PLC\textsubscript{b1} or a crude membrane assay.

Phospholipase C\textsubscript{b2} mimicking peptide (PLC\textsubscript{b2M}) is based on residues 564–583. The domain of PLC\textsubscript{b2} interacting with G\textsubscript{b} subunits has been determined utilizing a peptide fragment strategy. Two twenty amino acid segments of PLC\textsubscript{b2} (564–583 and 574–593) were defined as the domains binding to G\textsubscript{b} (28). The segment with the optimal interaction with G\textsubscript{b} subunits was observed to span amino acids 564–583. Synthetic peptide of this region exhibited specific binding to G\textsubscript{b} subunits as well as specific inhibition of G\textsubscript{b}-effector interactions.

Phosducin-like peptide (Phos) was derived from carboxyl-terminal residues 168–195 of phosducin-like protein (PhLP). PhLP isolated from rat brain (29) was determined to be an ubiquitous inhibitor of G\textsubscript{a} subunits that has been recognized as a site of interaction between G\textsubscript{a} subunits and the G\textsubscript{q} subunits that have limited solubility (Table I), and these were used in our experiments at the maximum soluble concentration.

**Cell Culture**—Primary cultures of choroid plexus epithelial (CPE) cells were prepared as described previously (34). Briefly, choroid plexus, removed from 20-day-old Harlan Sprague-Dawley rats, were treated with Pronase (333 µg/ml) containing DNase (7.5 µg/ml) in Hanks’ balanced salt solution (HBSS) (pH 7.4) for 10 min at 37 °C to dissociate cells. Dissociated cells were resuspended in 10% dialyzed calf serum or 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium (DMEM) + D-Val (Life Technologies, Inc.). Cells were plated in 48-well plates for 3–5 days.

Primary cultures of astrocytes were prepared as described previously (35). Brains were removed from postnatal day 2–5 rats, the meninges were carefully removed, and the cerebral cortex was dissected using visual landmarks. Cells were dissociated in horse serum by mechanical trituration, collected by centrifugation, and resuspended in 1 ml of fetal bovine serum. Dissociated cells were cultured in DMEM containing 10% fetal bovine serum in 75 cm\textsuperscript{2} culture flasks coated with poly-D-lysine. After 7 days, cells were shaken in an orbital shaker at 37 °C overnight to remove nonastrocytic cells. The cells were then replated in 24- or 48-well plates for functional assays.

Human embryonic kidney-293 cells stably expressing the rat adrenergic \(\alpha\textsubscript{2A} \) receptor (HEK-\(\alpha\textsubscript{2A} \)) were a generous gift from Dr. Lee Limbird (Vanderbilt University). Cells were cultured in DMEM containing 10% fetal bovine serum (100 units penicillin/ml, 100 mg of streptomycin/ml) under 5% CO\textsubscript{2} at 37 °C.

**Phosphoinositide (PI) Hydrolysis Assay**—CPE cells plated in 48-well...
plates were incubated for 16–20 h with 2 μCi/ml myo-[3H]inositol (20–25 Ci/mmol, NEN Life Science Products) in serum-free, inositol-free DMEM to label phospholipid pools. Labeling medium was aspirated, and the cells washed twice with HBSS containing 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$. Cells were treated with peptides solubilized in HBSS ($1\text{Ca}^{2+}/\text{Mg}^{2+}$) at 37 °C for 30 min. Subsequently, 10 mM lithium chloride and 10 μM pargyline were added to the cells for 10 min prior to agonist activation for 30 min at 37 °C. The reaction was stopped by aspirating the solution and fixing with 25 ml of methanol/well. [3H]Inositol monophosphates were isolated as described previously (36).

Dissecting Endogenous 5-HT$_{2C}$ Receptor Signaling Pathways

**Fig. 1.** A representative scheme for functionalizing MPS and subsequent conjugation with the cargo peptide PLC$\beta$2M to yield the membrane-permeable product MPS-PLC$\beta$2M.

**Fig. 2.** Example of sample preparation. A, products from the conjugation of PLC$\beta$1M with MPS were separated by preparative HPLC. B, matrix-assisted laser desorption ionization-mass spectrometry was performed on the fractions collected in A. Results shown are from the fraction in A denoted by an asterisk. C, the fraction identified to be the MPS-PLC$\beta$1M peptide (denoted by * in A) is checked with analytical HPLC to verify purity.

Western Blot—Protein extraction from cells and separation on acryl-
amide gel was done as described previously (38). The molecular masses were determined using Sigma high molecular weight markers.

For the MAP kinase assay, HEK-α2 cells in 24-well plates, containing serum-free medium, were treated with appropriate peptides for 30 min at 37 °C prior to activation with 100 μM epinephrine for 2 min. Supernatant was aspirated, and cells were solubilized with 1X sample buffer (62.5 mM Tris, 2% SDS, pH 6.8, containing 10% (v/v) glycerol). Proteins were separated in 12% SDS-polyacrylamide gels. Active MAP kinase was detected using the Promega anti-phospho-MAP kinase antibody at 1:1000 dilution with an overnight incubation at 4 °C. Total MAP kinase was detected using NEB total MAP kinase antibodies at 1:500 dilution with an overnight incubation at 4 °C. Secondary peroxidase-conjugated donkey anti-rabbit antibodies were used at 1:2000 dilution with incubation at room temperature for 30 min. Immunoreactive protein bands were visualized by treatment of blots with NEB ECL reagent and subsequent exposure to Kodak Biomax film.

For the detection of PLCβ isoforms and G protein isoforms, CPE were removed from Spargue-Dawley rats and solubilized in Tris buffer containing 10 mM CHAPS (39). The CHAPS-soluble fraction was fractionated in 7.5% SDS-polyacrylamide gels. Detection of PLCβ isoforms was performed using PLCβ isozyme-specific antibodies as per the manufacturer’s recommendations (Santa Cruz Biotechnology). Gq, detection was achieved using affinity purified polyclonal antibodies provided by Dr. Tom Martin.

cAMP Assay—Primary cultured astrocytes plated in 48-well plates were labeled for 16–20 h with 2 μCi/ml [3H]adenosine in serum-free DMEM. Peptides solubilized in HBSS (+Ca2+/Mg2+) were added to cells and incubated at 37 °C for 30 min prior to initiation of the assay. Agonist was added and the incubation continued for 30 min at 4 °C in presence of 1 mM isobutylmethylxanthine. The reaction was stopped with 10% trichloroacetic acid containing 2 mM ATP and 2 mM cAMP. Accumulated [3H]cAMP was separated on alumina columns as described previously (40).

RESULTS

PLCβ Signaling Machinery in CPE Cells—Immunobots were utilized to evaluate signaling molecules including the PLCβ isozymes, which have differential specificity for activation either by Gq or Gβγ subunits. Three isoforms of PLCβ were detected in the choroid plexus (Fig. 3A). Using anti-PLCβ1 antibodies two bands at approximately 140 and 100 kDa were detected; the latter is an expected degradation product of PLCβ1 (Santa Cruz antibody protocol). PLCβ2 and PLCβ3 were detected with apparent masses of approximately 100 and 140 kDa, respectively. However, PLCβ4 was not present at a detectable level. We also probed CPE extracts with anti-Gi/o as well as anti-Gq, antibodies to verify the potential for Gβγ- and Gq-mediated signaling (Fig. 3B). These results demonstrated that Gq, as well as Gi/o, are expressed in CPE.

G Protein Mediators of Endogenous 5-HT2C Receptor Signal in CPE Cells—Given that both PLCβ2 and Gi/o exist in CPE cells, the possibility that activation of PLCβ in CPE cells is mediated by Gβγ subunits released from Gi/o heterotrimers was examined using an indirect method based on PTX sensitivity. The 5-HT2C receptor PI hydrolysis response in CPE cells is predominantly insensitive to PTX (Fig. 3C), which ADP-ribosylates Gi/o heterotrimers leading to their inactivation. As a control for PTX activity, CPE cells were pretreated overnight with PTX and then subjected to an in vitro ADP-ribosylation assay. Cells treated overnight with PTX were not ADP-ribosylated by PTX added in vitro (Fig. 3D), whereas non-PTX-treated cells were ADP-ribosylated. These results suggest that Gi/o heterotrimers are predominantly not involved in the endogenous 5-HT2C receptor PI signal.

To determine directly the heterotrimeric G protein mediator of endogenous 5-HT2C receptor signaling, we introduced the membrane-permeable MPS-Gq,CT peptide to cultured CPE cells. This peptide is designed to disrupt receptor coupling to Gi/o, heterotrimers (Fig. 4A). MPS-Gq,CT, at 5 μM, was able to block PI hydrolysis resulting from treatment of CPE cells with 100 nM serotonin (Fig. 4B). A peptide designed to disrupt receptor coupling to Gq heterotrimer (MPS-Gq,CT) was ineffective in perturbing endogenous 5-HT2C receptor-mediated PI hydrolysis. Signal sequence (MPS) peptide alone was also ineffective demonstrating that the domain conferring permeability does not attenuate the observed PI signal. Additionally, membrane-impermeable, nonconjugated Gq,CT peptide was unable to disrupt 5-HT2C receptor signaling, which is consistent with the idea that without membrane permeability the peptide is not functional. This is also an indication that the effect of the Gq,CT peptide is not due to general toxicity during
the course of the experiment. The observation that neither MPS nor the Gq-CT peptide alone was an effective inhibitor of receptor-Gq coupling validates the MPS-importing strategy.

As an additional proof-of-concept for the use of membrane-permeable peptides designed from the carboxyl terminus of Ga subunits, we examined the functional effect of MPS-Gq-CT on endogenous β2 adrenergic receptor signaling in cultured astrocytes. As seen in Fig. 4B, MPS-Gq-CT is effective in blocking, to almost basal levels, β2 adrenergic receptor-mediated activation of adenylate cyclase. In contrast, the peptide MPS-Gq-CT was not functionally disruptive in this system. Furthermore, at the same concentration, MPS-Gq-CT was ineffective in blocking 5-HT2C receptor-mediated PI hydrolysis in CPE (Fig. 4B). These results contribute collectively to demonstrate peptide specificity and their lack of toxicity.

Demonstration of Function and Specificity of Membrane-permeable Peptides Targeting G Protein Subunits Using HEK-α2A Cells—To assess directly whether the activation of PLCβ is mediated by active Gq subunits or free Gβγ subunits, we designed the following peptides: MPS-PLCβ1M targeted against the disruption of Gq-PLCβ interaction; and MPS-PLCβ2M and MPS-Phos, both designed to bind and sequester free Gβγ subunits thereby preventing subsequent activation of PLCβ. Because CPE cells lack the appropriate receptor-signaling pathways to determine peptide function, specificity and toxicity, we exploited HEK cells stably expressing α2A adrenergic receptors (HEK-α2A) for this purpose. HEK-α2A cells endogenously express thrombin receptors as well as transfected α2A adrenergic receptors, which signal through the Gq heterotrimeric proteins leading to Gβγ-mediated activation of MAP kinase (41); this serves as a suitable model to evaluate the effects of MPS-PLCβ2M and MPS-Phos peptides on free Gβγ-mediated signaling (Fig. 5A). Thrombin receptors have been observed to activate a PTX-insensitive PI signal postulated to be through the Gq heterotrimeric proteins (Fig. 5B). These two receptor systems provide divergent signaling pathways to test the aforementioned functional peptides.

Using antibodies directed against the phosphorylated, active form of MAP kinase or against a region of MAP kinase away from the phosphorylation site, we could discern active versus inactive forms of MAP kinase and visualize total MAP kinase. Activation α2A-adrenergic receptors in HEK-α2A cells with 100 μM epinephrine (Fig. 5A, control lanes) results in an increase in the level of active MAP kinase as compared with the untreated basal levels. Total MAP kinase labeling of the same blot indicates that the levels of MAP kinase are equal or even higher in basal versus control lanes. Pretreatment of cells with MPS-PLCβ2M and MPS-Phos peptides disrupted activation of MAP kinase by 100 μM epinephrine through the α2A receptors (Fig. 5A). However, the nonconjugated, membrane impermeant forms of Phos or PLCβ2M did not inhibit MAP kinase activation. MPS alone had no functional effect on MAP kinase activation. In addition, PTX pretreatment abrogated subsequent MAP kinase activation confirming that MAP kinase activation is through Gβγ subunits released from the Gq6 protein. When tested in the thrombin receptor PI hydrolysis pathway, the MPS-Phos and MPS-PLCβ2M peptides were shown to have no inhibitory effects (Fig. 5B) demonstrating the specificity and lack of toxicity of these peptides. These results validate the interpretation that MPS-Phos and MPS-PLCβ2M are functional and specific to target the sequestration and disruption of signaling by free Gβγ subunits.

Pretreatment of HEK-α2A cells with 100 μM MPS-PLCβ1M produced no disruptive effect on α2A receptor-mediated activation of MAP kinase, indicating that this peptide is apparently not toxic to the cells and does not nonspecifically disrupt Gβγ-mediated signaling (Fig. 5A). However, signaling of endogenous thrombin receptors in HEK-α2A cells was disrupted by MPS-PLCβ1M (Fig. 5B), demonstrating that in the same cells PI hydrolysis blockade can be achieved.

Role of Active Gqα and Free Gβγ Subunits in Mediating Endogenous 5-HT2C Receptor Signaling—To examine the direct contribution of active Gqα subunits mediating 5-HT2C receptor signals, MPS-PLCβ1M, designed to disrupt the Gqα-PLCβ interaction, was applied to cultured CPE cells. This peptide blocked, down to basal levels, serotonin-induced PI
hydrolysis (Fig. 6A). However, in the same assay, the Gβγ-sequestering peptides, MPS-PLCβ2M and MPS-Phos, at levels that blocked MAP kinase activation, did not significantly inhibit 5-HT2C receptor-mediated PI signaling. Because evidence suggests that the 5-HT2C receptor in CPE is the sole mediator of serotonin stimulation (12), these results indicate that active Gaq subunits are involved in 5-HT2C receptor signaling. The effect of MPS-PLCβ1M was dose-dependent, as seen in Fig. 6B, with an IC50 of 55 µM. Additionally, concentration response studies showed that MPS-PLCβ1M decreased the maximal signal produced by 5-HT without altering the EC50 (Fig. 6C). Although this type of effect may be attributed to general toxicity, results observed in earlier experiments do not support this conclusion.

Role of Membrane-permeable Peptides in Endogenous Receptors Expressed in Primary Cultures Astrocytes—To further demonstrate that the effects of these peptides are not receptor- or cell type-specific, we analyzed their effects on metabotropic glutamate receptor (mGluR) signaling in primary cultures of astrocytes. Type 1 mGluR, consisting of mGluR1 and mGluR5, activate PLCβ (42). Overexpression of Gaq subunits augments PI hydrolysis of mGluR1a transfected into HEK-293 cells, suggesting that this receptor couples through Gaq heterotrimeric protein (43). Furthermore, in astrocytes, the activation of mGluR5 signaling appears to be PTX-insensitive (44–46). However, the exact identity of the G protein mediating endogenous mGluR5 signal remains unclear. We addressed this question by analyzing primary cultures of astrocytes, which express mGluR5 but not mGluR1 (47). (R,S)-3,5-Dihydroxyphenylglycine, a mGluR1- and mGluR5-specific agonist, was used to activate mGluR5 in these cells. In the presence of MPS-PLCβ1M (300 µM), mGluR5-mediated PI hydrolysis was significantly inhibited. In contrast, treatment with MPS-PLCβ2M (100 µM) or MPS-Phos (100 µM) peptides did not decrease signaling relative to controls (Fig. 7). These results suggest that Gβγ subunits are not involved in mediating PLCβ activation of mGluR5, affirming the observed lack of PTX sensitivity and presumed lack of involvement of Gαi heterotrimers. These results indicate that signaling of mGluR5 receptors in astrocytes is mediated by active Gaq subunits, which is consistent with the current consensus of the involvement of Gaq heterotrimeric.

DISCUSSION

Most studies of receptor function involve the use of agonists and antagonists to modulate receptor activity in an attempt to understand how these receptors regulate cell physiology. Many G protein-coupled receptors activate multiple independent intracellular signaling cascades, and it is equally important to
understand how these various signals contribute to the physiological actions of drugs and to whole cell function. Current tools available for studying multicomponent intracellular signaling pathways are limited in comparison to the plethora of available receptor ligands. Contemporary methods for assessing the contribution of specific signaling molecules generally involve molecular strategies such as antisense oligonucleotides to knockdown expression of a specific protein or transfection experiments to overexpress the protein of interest. However, these approaches disrupt the stoichiometry of the protein within the signal transduction system and are not always applicable to native cell systems. For signaling proteins with enzymatic activity, chemical inhibitors or toxins may be available to provide a more temporally controlled dissection of signaling. The drawbacks of these compounds are that many of them have specificity problems and may be toxic. Currently, there is no systematic broadly applicable approach to the dissection of intracellular signaling events in native cells or tissues.

In this paper, we describe a strategy that involves the use of functional peptides coupled to membrane-permeable peptides for dissecting intracellular signaling pathways. Signaling in many cases requires an activated protein to contact directly with its immediate downstream mediator. Thus, it is possible to disrupt protein-protein interactions of specific coupling domains by introducing into cells only the binding domain. Specificity is intrinsic to the amino acids encoding the peptide as well as their inherent binding properties. The blocking peptide strategy to disrupt protein-protein interactions has been applied sporadically by other investigators in signaling studies. Hamm and Rarick (31) as well as Taylor and Neubig (32) reviewed the use of peptides as probes for G protein signal transduction. Hamm and Rarick (31) analyzed the use of peptides to study receptor-G protein interaction and G protein-effector interaction and pointed out that, with a blocking peptide strategy, signaling pathways can be disrupted at any level, provided that protein-protein interactions exist. However, a limitation of this strategy lies in the intracellular import of peptides. Because most peptides do not readily penetrate the cell membrane, their use in disrupting intracellular signaling has been primarily limited to in vitro analyses, a major impediment to general use of this methodology. To alleviate this problem, we have adopted a noninvasive approach to introduce peptides into cells, the addition of a MPS to the blocking peptides. The feasibility of this approach has been demonstrated in experiments to prevent the inducible nuclear import of transcription factors in human monocytic, endothelial, and T lymphocyte cell lines (23, 25). We have also implemented a recently developed approach of modular peptide synthesis, which involves separate synthesis of the MPS and functional peptides with subsequent chemical ligation of the two peptides under mild aqueous conditions (24). The biological activity of the resulting modular peptides has been extensively documented by comparing modular peptides with those synthesized conventionally (23, 25, 48, 49). This modular approach not only provides versatility in preparing multiple MPS-coupled functional peptides for structure and function studies, but it also serves to increase yield and reduce the cost of synthesis.

The conjugation of MPS with blocking peptide has great potential as a versatile tool for studying intracellular signaling. This approach was validated in recombinant cell lines and then applied to cultured CPE cells for direct analysis of endogenous 5-HT2c receptor signaling at the receptor-G protein level and at the G protein-effector level. A decapeptide mimicking the carboxyl terminus of Goq subunits (MPS-GoqCT), a domain conferring specific interaction with receptors, profoundly disrupted 5-HT2c receptor-mediated PI hydrolysis in CPE cells. A PI hydrolysis assay of CPE cells pretreated for 30 min with various peptides before 30 min activation with 100 nM 5-HT. Concentrations of peptides used are those previously demonstrated to be maximally inhibiting: 100 μM MPS-PLCβ1M, 40 μM MPS-Phos, and 10 μM MPS-PLCβ2M. Statistical analysis was a one-way ANOVA using a nonparametric TUKEY test (α = 0.05). B, dose response of MPS-PLCβ1M peptide on 5-HT2c receptor PI signaling in CPE cells (IC50 = 55 μM). Graph is representative of three independent experiments. C, effects of MPS-PLCβ1M peptide on 5-HT dose response in CPE cells. Cells were untreated (solid line) or treated with 60 μM peptide (dashed line). EC50 values for Control and MPS-PLCβ1M were 124 and 67 nM, respectively. EC50 for control and MPS-PLCβ1M is 1602 and 990 cpm, respectively. The values plotted are means of triplicate determinations and are representative of three independent experiments each point with three replicates.
Dissecting Endogenous 5-HT$_{2C}$ Receptor Signaling Pathways

It is well documented that G$\beta\gamma$ subunits released from G$_{\alpha_0}$ heterotrimers have the ability to activate effectors, including PLC$\beta$2 (50–53). More recently, effector activation by G$\beta\gamma$ released from G$_{\alpha_0}$ heterotrimers has been reported (54). The possibility also exists for signaling mediated by G$\beta\gamma$ subunits released from G$_{\alpha_0}$ heterotrimers. For example, studies in Xenopus oocytes have shown that the M3 muscarinic receptor, which is G$_{q/11}$-coupled, activates PLC$\beta$ mainly through G$\beta\gamma$ subunits (55). However, transfected (COS) cells expressing the G$_{q/11}$-coupled parathyroid hormone or calcitonin receptor failed to show augmented PI hydrolysis when cotransfected with G$\beta\gamma$ subunits (56). Studies such as these in artificial systems may not represent the in vivo situation, therefore, we developed specific membrane-permeable peptides to evaluate the contribution of G protein subunits to the PI hydrolysis signal mediated by endogenous 5-HT$_{2C}$ receptors. To elucidate the function and specificity of the newly developed conjugated G$_{\alpha_0}$ and G$\beta\gamma$ peptides, we used an HEK-$\alpha_{2A}$ stable cell line expressing the cloned $\alpha_{2A}$ adrenergic receptor as well as endogenous thrombin receptors. $\alpha_{2A}$ adrenergic receptor activation of MAP kinase is mediated by G$\beta\gamma$ subunits released by G$_{\alpha_0}$ heterotrimer (41), whereas thrombin receptor signaling, in these cells, has been observed to be mediated by G$_{\alpha_q}$ protein (57). The addition of MPS-PLC$\beta$1M peptide into the HEK-$\alpha_{2A}$ cell line blocked the subsequent activation of PLC$\beta$ by thrombin receptor-activating peptide, consistent with the expected role for G$_{\alpha_q}$. The specificity of MPS-PLC$\beta$1M peptide was confirmed, because it had no effect on $\alpha_{2A}$-adrenergic receptor-mediated activation of MAP kinase, a G$\beta\gamma$-dependent response. In contrast, G$\beta\gamma$-sequestering peptides, MPS-Phos and MPS-PLC$\beta$2M, were both effective in disrupting $\alpha_{2A}$-adrenergic receptor activation of MAP kinase. The consistent results of both G$\beta\gamma$-sequestering peptides, which have different size and amino acid composition, provide converging support for their functionality and specificity. When the G$\beta\gamma$-sequestering peptides were tested on thrombin receptors in HEK-$\alpha_{2A}$ cells, they failed to block receptor-activated PI hydrolysis, providing evidence for the specificity of these constructs.

The current studies of 5-HT$_{2C}$ receptors in CPE cells suggest that G$\beta\gamma$ does not contribute to the G$_{q/11}$ heterotrimer-mediated PI hydrolysis signal in this endogenous receptor system. This conclusion is based on results obtained with the specific peptides that block active G$_{\alpha_q}$ and free G$\beta\gamma$ subunits released from activated G$_{q/11}$ heterotrimers. MPS-PLC$\beta$1M blocked 5-HT$_{2C}$-mediated PI hydrolysis in CPE cells, whereas both G$\beta\gamma$-sequestering peptides, MPS-Phos and MPS-PLC$\beta$2M, had no effect at concentrations that eliminated $\alpha_{2A}$-adrenergic receptor-mediated MAP kinase activation. Analysis of mGluR5 in astrocytes, another putative endogenously expressed G$_{q/11}$-coupled receptor, using the same membrane-permeable peptides showed similar results. MPS-PLC$\beta$1M markedly decreased mGluR5-mediated PI hydrolysis, whereas both MPS-PLC$\beta$2M and MPS-Phos did not attenuate signaling in this pathway. The consistency of these results obtained with two different endogenous receptors in different native environments suggests that, unlike in artificial systems (55), endogenous G$\beta\gamma$ subunits released from G$_{q/11}$ heterotrimers may not contribute to the activation of PLC$\beta$ in native systems. However, these results do not rule out a role of G$\beta\gamma$ subunits released from G$_{q/11}$ heterotrimers in other receptor signaling cascades. For example, 5-HT$_{2C}$ receptors also induce the release of arachidonic acid via phospholipase A$_2$ activation (58, 59) as well as an increase in the intracellular level of cGMP (14). 5-HT$_{2C}$ receptor activation also modulates various potassium channels (17, 18). Currently, it is not known which intracellular pathways are involved in the activation of these various effectors. The availability of cell-permeant peptides that block at the level of receptor-G protein and G protein-effector should allow a more precise dissection of these various downstream signals.

In conclusion, we have directly demonstrated that PLC$\beta$ signaling of endogenously expressed 5-HT$_{2C}$ receptors in CPE cells is mediated by G$_{q/11}$ heterotrimeric protein and specifically by the G$_{\alpha_q}$ subunit. In addition, the G$_{\alpha_q}$ subunit is responsible for the metabotropic GluR5 receptor activation of PLC$\beta$ in primary cultures of astrocytes. These studies serve as the first direct demonstration that active G$_{\alpha_q}$ subunit released from G$_{q/11}$ heterotrimers mediates the downstream activation of PLC$\beta$ in native systems. We have also provided evidence that subsequent to receptor-G$_{q/11}$ activation, G$\beta\gamma$ subunits released from G$_{q/11}$ heterotrimers do not contribute to the activation of PI hydrolysis signal in natural systems where the stoichiometry of signaling molecules is undisturbed. These studies demonstrate that membrane-permeable peptides, generated by a chemical ligation strategy, are effective tools in the dissection of multiple intracellular signals of G protein-coupled receptors in their native environment.

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