Selective Recognition of Phosphatidylinositol 3,4,5-Trisphosphate by a Synthetic Peptide*

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The present study takes a novel approach to explore the mode of action of phosphoinositide 3-kinase lipid products by identifying a synthetic peptide W-NG28–43 (WAAKIQASFRGHMARKK) that displays discriminative affinity with phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3). This PtdIns(3,4,5)P3-binding peptide was discovered by a gel filtration-based binding assay and exhibits a high degree of stereochemical selectivity in phosphoinositide recognition. It forms a 1:1 complex with PtdIns(3,4,5)P3 with Kd of 2 μM, but binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) with substantially lower affinity (5- and 40-fold, respectively) despite the largely shared structural motifs with PtdIns(3,4,5)P3. Other phospholipids examined, including phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine, show low or negligible affinity with the peptide. Several lines of evidence indicate that this phosphoinositide-peptide interaction is not due to nonspecific electrostatic interactions or phospholipid aggregation, and requires a cooperative action among the hydrophobic and basic residues to exert the selective recognition. CD data suggest that the peptide acquires an ordered structure upon binding to PtdIns(3,4,5)P3. Furthermore, we demonstrate that PtdIns(3,4,5)P3 enhances the phosphorylation rate of this binding peptide by protein kinase C (PKC)-α in a dose-dependent manner. In view of the findings that this stimulatory effect is not noted with other PKC peptide substrates lacking affinity with PtdIns(3,4,5)P3 and that PKC-α is not susceptible to PtdIns(3,4,5)P3 activation, the activity enhancement is thought to result from the substrate-concentrating effect of the D-3 phosphoinositide, i.e. the presence of PtdIns(3,4,5)P3 allows the peptide to bind to the same vesicles/micelles to which PKC is bound. Moreover, it is noteworthy that neurogranin, a full-length protein of W-NG28–43 and a relevant PKC substrate in the forebrain, binds PtdIns(3,4,5)P3 with high affinity. Taken together, it is plausible that, in addition to PKC activation, PtdIns(3,4,5)P3 provides an alternative mechanism to regulate PKC activity in vivo by recruiting and concentrating its target proteins at the interface to facilitate the subsequent PKC phosphorylation.

Among various phospholipids in the plasma membrane, phosphatidylinositol 3-kinase lipid products have received much attention because of their pivotal role in transmembrane signal transduction (1, 2). Two major phosphoinositide-mediated signaling cascades have been characterized, both of which originate from phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) (3, 4). In the canonical pathway, phospholipase C activation leads to a rapid production of d-myoinositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol, which elicit Ca2+ release and protein kinase C (PKC) activation, respectively (3). The second pathway entails phosphoinositide 3-kinase (PI 3-kinase) of which the activation produces transient accumulations of two novel phosphoinositides, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) and phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2) (4, 5). This PI 3-kinase pathway has been implicated in diverse cellular responses to growth factors, including mitogenesis (6, 7), chemotaxia (8, 9), membrane trafficking (2), actin reorganization (10), receptor down-regulation (11), cell survival (12), and so forth. These PI 3-kinase lipid products are important cellular regulators, their molecular targets remain unclear. Recent evidence indicates that PtdIns(3,4,5)P3 and PtdIns(3,4)P2 activate Ca2+-independent PKC family members δ, ε, and η (14, 15). This stimulatory effect in conjunction with the action of diacylglycerol is thought to exert a sustained PKC activation (16). Another PI 3-kinase product, phosphatidylinositol 3-phosphate, was implicated in the activation of a protein kinase encoded by the Akt proto-oncogene via a Pleckstrin homology domain (17). In addition, PtdIns(3,4,5)P3 was shown to disrupt the association of PI 3-kinase with tyrosine-phosphorylated proteins by binding to the Src homology 2 (SH2) domains of the p85 subunit (18). Moreover, PtdIns(3,4,5)P3 and PtdIns(3,4)P2 exhibited high affinity with profilin, which provides a putative link between PI 3-kinase and actin rearrangement (19).

Conceivably, these D-3 inositol phospholipids initiate downstream signaling by rapidly recruiting and/or activating target proteins. Evidence suggests that the different lipid products of PI 3-kinase activate different molecular targets responsible for diverse physiological consequences (16, 18). Considering the presence of various phosphatidylinositol phosphates and other phospholipids in the plasma membrane, selective recognition of these phospholipids by phosphoinositide-specific phosphoinositide-binding peptides should provide a novel approach to explore the different modes of action of phosphoinositide lipid products.

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1 The abbreviations used are: PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdEA, phosphatidylethanolamine; PtdCho, phosphatidylcholine; Ins(1,4,5)P3, d-myoinositol 1,4,5-trisphosphate; Ins(1,3,4,5)P4, d-myoinositol 1,3,4,5-tetraakisphosphate; Ins(1,3,4,5,6)P5, d-myoinositol 1,3,4,5,6-pentaakisphosphate; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; SH2 domains, Src homology domains.
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RESULTS

Interfacial Recognition of PtdIns(3,4,5)P3 by a Synthetic Peptide—In this study, a gel filtration-based binding assay was employed to search for PtdIns(3,4,5)P3-binding peptides. Individual peptides were incubated with micelles consisting of pure PtdIns(3,4,5)P3, and the mixture was applied onto a short path Sephacryl S-200 column. In principle, binding to PtdIns(3,4,5)P3 would be indicated by the co-elution of the tested peptide with the micelles in the void volume. This experi-

range of phosphoinositides used, the bulk solution remained clear.

Circular Dichroism Spectroscopy—CD spectra were recorded with a JASCO J720 spectropolarimeter at room temperature in a 20 mm path length cell. The solution contained 25 μM W-NG29–43 and 50 μM individual phosphoinositides in 25 mM Tris/HCl and 100 mM KCl, pH 7.5. The following settings were used: wavelength range, 200–250 nm; bandpass, 1 nm; step resolution, 0.5 nm; scan speed, 10 millidegree/ min. Each spectrum represented an average of 10 scans with base-line subtraction.

31P NMR Spectroscopy—Decoupled 31P NMR spectra were recorded at 25 °C in 10-mm tubes on a Varian VXR400 spectrometer at 161.9 MHz. Aliquots of a W-NG29–43 solution were introduced into a 1-ml solution containing 0.8 mM PtdIns(3,4,5)P3 and 10%/v/v deuterium oxide. The spectrum width was 20 kHz with 10-μs pulse width and 0.8-s acquisition time. For each spectrum, 5,000 acquisitions were obtained over a 4,000-s period. External H3PO4 and deuterium oxide were employed as a chemical shift reference and a locking signal, respectively.

Protein Kinase C Assay—Effect of PtdIns(3,4,5)P3 on PKC-mediated phosphorylation of NG28–43 and other PKC substrates was examined using a colorimetric method developed by Pierce (25). The reaction mixture (25 μl) contained 30 mM Tris/HCl, pH 7.4, 50 mM NaCl, 2 mM ATP, 10 mM MgCl2, 0.1 mM CaCl2, 0.002% Triton X-100, PtdSer (1 mg/ml), 1,2-diacytanol-sn-glycerol (20 μg/ml), PKC-α (0.1 unit), 300 μM dye-labeled PKC substrate, and varying amounts of PtdIns(3,4,5)P3. After incubating at 30 °C for 20 min, 20 μl of the mixture were applied to a thin-layer affinity mat to purify the phosphorylated peptide. The membrane was washed under reduced pressure with 750 μl of a phosphopeptide-binding buffer consisting of 0.1 M sodium citrate, pH 5.0, 0.5 M NaCl, and 0.02% sodium azide to remove the unreacted peptide. The phosphopeptide was then eluted by washing the membrane with 600 μl of 15% formic acid. Quantitation of the phosphorylated product was accomplished by measuring its absorbance at 570 nm in reference to a standard curve derived from known amounts of the phosphopeptide generated from the reaction.

Production of Recombinant Neurogranin—The bacterial expression vector for rat neurogranin pDGRC3 (a kind gift from Dr. Dan D. Gerendas) was transformed into Escherichia coli BL21 (DE3) (pLysS), and recombinant neurogranin was produced and purified according to a procedure described by Kenet et al. (9). Recombinant neurogranin was grown for another 6 h. The cells were collected by centrifugation and were suspended in 10 ml of cold lysis buffer consisting of 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM EGTA, and 50 mM dithiothreitol. The suspension was frozen and thawed, and sonicated. After removing cell debris by centrifugation, the crude homogenate was treated with percoll acid (final concentration, 2.5% w/v), followed by trichloroacetic acid (final concentration, 15% w/v). The percoll acid-soluble, trichloroacetic-insoluble material was dissolved in 50 mM Tris/HCl, pH 7.5, containing 200 mM NaCl, 2 mM EDTA, 1 mM EGTA, and 50 mM dithiothreitol. The suspension was frozen and thawed, and sonicated. After recovering by centrifugation, the supernatant was subjected to a standard curve. The bound material was used as a chemical shift reference and a locking signal, respectively. The resulting pellet was washed twice with 15 ml of calmodulin-Sepharose 4B. The column was washed with 5 column volumes of washing buffer and the adsorbed protein was eluted with 50 ml of elution buffer consisting of 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 7 mM CaCl2, and 50 mM dithiothreitol. Fractions were collected throughout, and the pooled solution was subjected to the aforementioned acid treatments. The resulting pellet was washed twice with ethanol-ether (1:1) and dissolved in 10 mM Tris/HCl, 75 mM KCl, and 1 mM dithiothreitol. The homogeneity of the purified protein was indicated by a single band on SDS-PAGE with silver staining, and the identity was confirmed by N-terminal sequencing. The sequence of the first 15 amino acids at the N terminus was DDCCTEASACSKPDDD, which was identical to that reported in the literature (26). Concentrations of neurogranin were determined by the BCA method with bovine serum albumin as a standard.

The D-3 phosphoinositides at the interface represents a crucial issue in addressing their mode of action. Thus, to better understand the molecular basis by which the PI 3-kinase lipid products regulate cellular functions, our effort has focused on delineating the mode of recognition of these phosphoinositides. In this study, by using a gel filtration-based binding analysis, we have identified a synthetic 17-mer peptide W-NG29–43 that displays discriminative affinity with PtdIns(3,4,5)P3. It is worth-
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**FIG. 1.** Gel filtration assay for the binding of W-NG$_{28–43}$ to PtdIns(3,4,5)P$_3$-containing micelles. The solid line with full circles indicates the elution profile of W-NG$_{28–43}$ (125 μM) in the presence of one molar equivalent of PtdIns(3,4,5)P$_3$ on a Sephacryl S-200 column (1 × 10 cm) at room temperature. The column was eluted with 10 mM Tris/HCl, pH 7.5, containing 75 mM KCl, and fractions of 0.45 ml were collected. The dotted line represents the elution profile of the free peptide. The inset indicates the amounts of remaining free peptides as a function of PtdIns(3,4,5)P$_3$ concentrations, in which the slope of the line represented the binding stoichiometry. The experimental conditions were the same as described above except that the PtdIns(3,4,5)P$_3$ concentration varied. Due to the interference of phospholipids in the protein assay, the concentrations of eluted peptide were expressed in arbitrary units.

**FIG. 2.** Tryptophan fluorescence emission spectra of W-NG$_{28–43}$ in the presence of varying amounts of A, PtdIns(3,4,5)P$_3$; B, PtdIns(4,5)P$_2$; and C, PtdIns(3,4)P$_2$. Spectra were recorded with 7 μM W-NG$_{28–43}$ with an excitation wavelength set at 292 nm according to the method described under “Experimental Procedures.” Molar ratios of the phosphoinositide to W-NG$_{28–43}$ were A, for PtdIns(3,4,5)P$_3$ (top to bottom): 0, 0.1, 0.11, 0.27, 0.66, 0.65, 0.53, and 1:1 (reached saturation); B, for PtdIns(4,5)P$_2$ (top to bottom) 0, 0.1, 0.11, 0.3, 0.65, 0.81, 1.21, and 1.61 (reached saturation); C, for PtdIns(3,4)P$_2$ (top to bottom) 0, 0.1, 0.37, 0.91, 1.67, 2.6, 3.9, 4.5, and 6.0 (reached saturation).
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Differential Recognition of Phospholipids by W-NG28–43—

To distinguish between specific peptide-phospholipid binding and phospholipid aggregation, fully activated PKC α was used to examine the phosphorylation of NG28–43 in the presence of varying amounts of PtdIns(3,4,5)P3. A number of PKC substrates that lacked affinity with PtdIns(3,4,5)P3 were also tested as control, which included myelin basic protein peptide4–14, glycerogen synthase peptide, PKC pseudosubstrate, epidermal growth factor receptor peptide, and e peptide (data not shown).

To examine the binding specificity of W-NG28–43, various phosphoinositides that included PtdIns(4,5)P2, PtdIns(3,4)P2, PtdIns, PtdSer, PtdCho, and PtdEA. The respective molecular stoichiometry and Kd values were estimated by the gel filtration assay summarized in Table I.

Differential Recognition of Phospholipids by W-NG28–43—

Among these phospholipids, PtdIns(4,5)P2 cross-reacted with W-NG28–43, however, with an affinity 5-fold lower than PtdIns(3,4,5)P3. This cross-reactivity was presumably due to their largely shared structural motifs. In contrast, the affinity with PtdIns(3,4)P2 was 40-fold weaker than that of PtdIns(3,4,5)P3. This structure-activity correlation suggests the importance of the 5-phosphate in the peptide binding, which was supported by the 31P NMR examination of the interaction between W-NG28–43 and PtdIns(3,4,5)P3 (Fig. 4).

The 31P NMR signals for the phosphates at positions 1, 3, 4, and 5 of the inositol ring of PtdIns(3,4,5)P3 were noted at 2.92, 3.08, 3.28, and 3.49 ppm, respectively (spectrum a). The peak assignment was achieved by comparing spectra of PtdIns(4,5)P2 and PtdIns(4)P as described in Harlan et al. (30).

The stoichiometry and binding affinity (Kd) of W-NG28–43 with various phospholipids is summarized in Table I. Both values were determined by a gel filtration method described under “Experimental Procedures.”

Fig. 4. 31P NMR spectra of PtdIns(3,4,5)P3 as a function of increasing concentrations of W-NG28–43 with a final peptide/lipid ratio of a, 0; b, 0.25; c, 0.5; and d, 0.75. Numbers above the peaks correspond to the phosphates at positions 1, 3, 4, and 5 of the inositol ring of PtdIns(3,4,5)P3. The peak assignment was achieved by comparing spectra of PtdIns(4,5)P2 and PtdIns(4)P as described in Harlan et al. (30).

Table I

| Phospholipids     | Stoichiometry (Per Peptide) | Kd (μM) |
|------------------|-----------------------------|---------|
| PtdIns(3,4,5)P3  | 1                           |         |
| PtdIns(4,5)P2    | 1.6                         | 9.8 ± 2.5 (n = 6) |
| PtdIns(3,4)P2    | 8                          | 80      |
| PtdIns           | 8                          | >100    |
| PtdSer           |                              | >500    |
| PtdCho           | No detectable interaction   |         |
| PtdEA            | No detectable interaction   |         |

* These values could not be accurately determined due to low binding affinity.

FIG. 3. Dose dependence of PKC-mediated phosphorylation of myelin basic protein peptide4–14 (open bars) and NG28–43 (shaded bars) on PtdIns(3,4,5)P3. Initial activity of PKC α was analyzed in the presence of elevated levels of PtdIns(3,4,5)P3 by using a colorimetric method described under “Experimental Procedures.” Each data point represents the average of three experiments. No modulatory effect of PtdIns(3,4,5)P3 was noted on myelin basic protein peptide4–14 and other PKC substrates including glycogen synthase peptide, PKC pseudosubstrate, epidermal growth factor receptor peptide, and e peptide (data not shown).

FIG. 4. 31P NMR spectra of PtdIns(3,4,5)P3 as a function of varying amounts of W-NG28–43 in the presence of elevated levels of PtdIns(3,4,5)P3, by using a colorimetric method described under “Experimental Procedures.” Each data point represents the average of three experiments. No modulatory effect of varying amounts of PtdIns(3,4,5)P3. A number of PKC preparations were virtually identical.

Among these phospholipids, PtdIns(4,5)P2 cross-reacted with W-NG28–43, however, with an affinity 5-fold lower than PtdIns(3,4,5)P3. This cross-reactivity was presumably due to their largely shared structural motifs. In contrast, the affinity with PtdIns(3,4)P2 was 40-fold weaker than that of PtdIns(3,4,5)P3. This structure-activity correlation suggests the importance of the 5-phosphate in the peptide binding, which was supported by the 31P NMR examination of the interaction between W-NG28–43 and PtdIns(3,4,5)P3 (Fig. 4).

The 31P NMR signals for the phosphates at positions 1, 3, 4, and 5 of the inositol ring of PtdIns(3,4,5)P3 were noted at 2.92, 3.08, 3.28, and 3.49 ppm, respectively (spectrum a). The peak assignment was achieved by comparing spectra of PtdIns(4,5)P2 and PtdIns(4)P as described in Harlan et al. (30).
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Between 200 and 250 nm (Fig. 5) for W-NG28–43 alone (fluorescence and CD spectroscopy. CD spectra were recorded in the far UV range determined by gel filtration. Although PtdIns(3,4,5)P3 binding to PtdIns(3,4)P2 (Fig. 2) PtdIns(3,4,5)P3 and the full-length protein was assessed by the binding domain of neurogranin, the interaction between PtdIns(3,4,5)P3 and the micelles-bound state at the interface and that the ligand-in-counterparts. These results suggest that the modes of binding with W-NG28–43 among these three phosphoinositides differed.

The same conclusion could be drawn by the results from CD spectroscopy. CD spectra were recorded in the far UV range between 200 and 250 nm (Fig. 5) for W-NG28–43 alone (curve a) or in the presence of PtdIns(3,4,5)P3 (curve c). The extent of fluorescence quenching by PtdIns(3,4,5)P3 was greater than the other two counterparts. These results suggest that the modes of binding with W-NG28–43 among these three phosphoinositides differed.

As shown, the CD spectrum of the free peptide is characterized by a weak, negative band centered at 204 nm, which might be attributed to a random coil conformation (29). The spectra underwent marked changes when phosphoinositides were added. This result indicates that W-NG28–43 exhibited different structural behaviors when moving from a free state to a micelle-bound state at the interface and that the ligand-induced conformational change was dependent upon the phosphoinositide structure. However, lack of significant absorption at 222 nm in these spectra indicates that the peptide did not acquire α-helical structures after interfacial binding. When inositol phosphates or other phospholipids that lacked affinity with the peptide (e.g. PtdCho and PtdEA) were added, no change in the CD spectrum was observed.

Neurogranin Binding to PtdIns(3,4,5)P3—In view of the fact that W-NG28–43 represented the PKC recognition/calmodulin-binding domain of neurogranin, the interaction between PtdIns(3,4,5)P3 and the full-length protein was assessed by the gel filtration assay. Fig. 6 illustrates the representative elution profiles of recombinant neurogranin (21.3 μM) alone (A) and with increasing amounts of PtdIns(3,4,5)P3. By using data determined at six different lipid/protein ratios, the Kd value and binding stoichiometry were estimated to be 2.2 ± 0.5 μM and 10, respectively.

DISCUSSION

In this study we demonstrate the selective recognition of PtdIns(3,4,5)P3 by a synthetic peptide, W-NG28–43. These two molecules form a 1:1 complex of which the binding affinity (Kd = 2 μM) is in line with that observed in many protein-phosphoinositide interactions (23, 24, 30). Moreover, this small peptide exhibited high degree of stereochemical selectivity in phospholipid binding. Despite largely shared structural motifs, PtdIns(4,5)P2 and PtdIns(3,4)P2 displayed substantially lower affinity (5-fold and 40-fold, respectively) with W-NG28–43 vis à vis PtdIns(3,4,5)P3. Other phospholipids examined, including PtdIns, PtdSer, PtdEA, and PtdCho, showed low or negligible binding affinity. Several lines of evidence rule out the possibility that the binding was due to nonspecific electrostatic interactions or phospholipid aggregation: 1) many unrelated polybasic peptides failed to exert appreciable binding to PtdIns(3,4,5)P3; 2) inositol phosphates such as Ins(1,3,4,5)P4 and Ins(1,4,5)P3 do not affect the binding; and 3) the Ser residue of W-NG28–43 was accessible to PKC phosphorylation after binding to the phospholipid.

Conceivably, this peptide model provides a useful tool to study the biomolecular recognition of phosphoinositides and to delineate PtdIns(3,4,5)P3-binding motifs in putative targets such as the nonconventional PKC isozymes and the SH2 domains on the p85 subunit. Structurally, this 17-mer peptide can be divided into two discrete regions composed largely of apolar and basic residues, respectively. It is noteworthy that the sequence of the C-terminal polybasic segment (e.g. RGH-MARKK) bears resemblance to the consensus sequences for PtdIns(4,5)P2-binding motifs deduced by Yin and co-workers.
it binds to micellar phosphoinositides. Secondly, a significant blue shift coupled with considerable attenuation of the fluorescence intensity shows that the N-terminal Trp physically interacts with the apolar environment at the interface. Consequently, it is reasonable to postulate that the PtdIns(3,4,5)P3-binding motifs consists of two contiguous segments, a polybasic region flanked by a hydrophobic segment for interfacial recognition. Previously, Cantley and co-workers reported that PtdIns(3,4,5)P3 interacted with the SH2 domain on the regulatory subunit of PI 3-kinase (18). It is interesting that the N-terminal SH2 domain on the p85 subunit of human PI 3-kinase (35) contains an internal peptide sequence that bears some resemblance to W-NG28–42 in the terms of the spacing of the stretch of basic residues (Scheme 1).

Moreover, in the inter-SH2 region, there is a partial sequence KQIEFKKR14 corresponding to the consensus sequence KXXXKK(K/R)(K/R)36. Investigations on the potential interaction between these SH2 internal peptides and PtdIns(3,4,5)P3 are currently in progress.

As mentioned, W-NG28–43 corresponds to the phosphorylation domain of neurogranin and closely resembles that of neurenomodulin (ATKIQASFRGHITRKK) (20). Both proteins are physiological relevant PKC substrates in the hippocampal region of central nerve system, and are not known to bind phospholipids. It is worthy to note that besides this homology, these two brain-specific proteins are not related over the rest of their sequences. Here, we provide evidence that neurogranin binds PtdIns(3,4,5)P3 with affinity comparable to that of the partial peptide, though with much larger binding stoichiometry. Thus, the physiological implication of this selective recognition is suggested by the dose-dependence of PKC α activity on PtdIns(3,4,5)P3. This enhancement is thought to result from the substrate-concentrating effect of PtdIns(3,4,5)P3-containing micelles based on the following rationales. First, it is known that PKC α is not susceptible to PtdIns(3,4,5)P3 activation (15). Secondly, the observed stimulatory effect was specific for W-NG28–43 and was not noted with other PKC substrates that lacked affinity with PtdIns(3,4,5)P3. This finding raises a possibility that PtdIns(3,4,5)P3 serves as a targeting site on the plasma membrane for neurogranin and neurenomodulin to facilitate their phosphorylation. The investigation of this hypothesis is currently underway in this laboratory.

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Selective Phosphoinositide Recognition
Selective Recognition of Phosphatidylinositol 3,4,5-Trisphosphate by a Synthetic Peptide
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