Selective Photoaffinity Labeling of the Inositol Polyphosphate Binding C2B Domains of Synaptotagmins*

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Synaptotagmin (Syt) II, a synaptic vesicle protein containing two copies of highly conserved protein kinase C homology regions known as the C2A and C2B domains, acts as a Ca\(^{2+}\) sensor and provides both phospholipid and inositol polyphosphate (IP\(_n\)) recognition domains important in endo- and exocytosis. Four photoaffinity analogues of IP\(_3\), IP\(_4\), and IP\(_6\) containing a P-1- or P-2-linked 4-benzoyldihydrocinnamidyl (BZDC) photophore were used to label glutathione S-transferase (GST) fusion constructs of the Syt II-C2A and C2B domains. The P-2-linked [\(^3\)H]BZDC-IP\(_6\) showed efficient, IP\(_6\)-displaceable labeling of the GST-Syt II-C2B domain. The rank order of photoactive modification paralleled the order of competitive displacement: IP\(_6\) (P-2-linked) > IP\(_4\) > IP\(_3\). The P-1-linked [\(^3\)H]BZDC-IP\(_6\) failed to label the C2B domains. The GST-Syt III-C2B domain, which lacks IP\(_6\) binding affinity, also failed to undergo labeling by P-2-linked [\(^3\)H]BZDC-IP\(_6\). When mixtures of the 32-amino acid basic peptide corresponding to the essential IP\(_6\) binding region of the Syt II-C2B domain and GST-Syt II-C2B were labeled by a stoichiometric amount of P-2-linked [\(^3\)H]BZDC-IP\(_6\), the two polypeptides showed equivalent affinity for the photolabel. Although the CD spectrum of this 32-mer at two pH values showed a random coil, the photoaffinity analogue of IP\(_6\) appeared to induce a binding-compatible structure in the short peptide.

The synaptotagmins (Systs)\(^1\) are synaptic vesicle proteins that play essential roles in nucleating the clathrin coat during endocytosis and in acting as Ca\(^{2+}\) sensors (1–3) and phosphoinositide sensors (4) during exocytosis. They are a critical part of a complex machinery of intracellular protein transport (5, 6) and the synaptic vesicle cycle (7). In addition to a short N-terminal intravesicular region and single transmembrane domain, Syts have two copies of highly conserved repeats, known as the C2A and C2B domains, which are homologous to the C2 regulatory region of protein kinase C (8). In particular, the C2B domain appears to play several roles. First, the C2B domain of mouse Syt II shows specific binding to high polyphosphate inositol somitors (IP\(_{n}\)) (9), a feature that is not shared by the highly homologous C2A domain nor with the C2 domains of other proteins such as rabphilin. Moreover, the C2B domain is necessary but not sufficient for IP\(_3\) binding. Although Syt II and IV show high affinity binding of IP\(_4\) and IP\(_6\), the Syt III-C2B domain shows negligible binding, despite a high sequence identity with the Syt II-C2B domain, including the 32-residue region examined by mutational analysis (10). Inhibition of C2B function in the squid giant axon disrupts synaptic vesicle release and recycling (11). Similarly, Caenorhabditis elegans mutants lacking Syt or simply the C2B domain cannot recycle synaptic vesicles (12), and the presence of Syt I in cerebrospinal fluid of Alzheimer’s patients (13) may provide clues to the disruption of synaptic function in humans. The C2B domain acts as a high affinity receptor for clathrin assembly protein AP-2 (14), and Ca\(^{2+}\) appears to mediate Syt dimerization via the C2B domain (15).

Synaptotagmin II isolated from mouse cerebellum shows high affinity binding to Ins(1,3,4,5)P\(_4\) with a K\(_D\) of 30 μM (16) and 117 nM for the GST-Syt II-C2B construct (9). Curiously, the rank order of IP\(_{n}\) binding for native Syt II was Ins(1,3,4,5,6)-P\(_n\) > (1,3,4,5,6)-P\(_n\) > Ins(1,2,3,4,5,6)-P\(_n\) > Ins(1,4,5,6)-P\(_n\), whereas for the GST-Syt II-C2B domain the order was IP\(_4\) > IP\(_6\) > IP\(_3\) (9). Deletion mutants allowed mapping of the IP\(_{n}\) binding site to the central region of the mouse Syt II-C2B domain, specifically residues 315–346 (HILMQNGKRLKKKTTVKKKTLNPVFENSSFS) (9). In order to obtain direct evidence for the binding site of IP\(_{n}\) in the C2B domain, to examine the K\(_D\) selectivity of the domains, to explore the Ca\(^{2+}\) dependence of binding, to determine the role of the central 315–346 peptide, and to evaluate the reason for the failure of Syt III-C2B domain to bind IP\(_{n}\), we undertook a series of photoaffinity labeling experiments using four tritium-labeled, benzophenone-containing derivatives of IP\(_3\), IP\(_4\), and IP\(_6\) (17).

EXPERIMENTAL PROCEDURES

Chromatography and the P-1-Tethered [\(^3\)H]BZDC-IP\(_6\) was prepared by coupling the 1-O-(3-aminophenyl)-n-myo-Inositol(1,4,5)P\(_4\) (18, 19) with [\(^3\)H]BZDC-NHS ester (20). P-1-tethered [\(^3\)H]BZDC-IP\(_6\) (21) was prepared analogously from 1-O-(3-aminophenyl)-n-myo-Inositol(1,3,4,5)P\(_4\) (22). P-1-O-(6-aminophenyl)-IP\(_6\) and its [\(^3\)H]BZDC photoinfinity label were prepared.

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The abbreviations used are: Syt, synaptotagmin; AP-2, assembly protein-2; BZDC, 4-benzoyldihydrocinnamimoiy; GST, glutathione S-transferase; Ins, inositol; IP\(_n\), n-myo-Inositol(1,4,5)P\(_n\); IP\(_3\), n-myo-Inositol(1,3,4,5)P\(_3\); IP\(_4\), n-myo-Inositol(1,2,3,4,5,6)P\(_4\); IP\(_5\), n-myo-inositol polyphosphate; IP\(_6\), phosphatidylinositol (4,5)-bisphosphate; IP\(_6\), phosphatidylglycerol (3,4,5)-trisphosphate; IP\(_6\), phosphoinositol polyphosphate; HPLC, high pressure liquid chromatography.

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as described elsewhere (23). Specific activities for all BZDC derivatives were 35–42 Ci/mmol, depending on the lot of [3H]BZDC-NHS ester used. d-myo-IP₃ and IP₆ were synthesized (18, 22), and IP₆ was obtained from Sigma. All other chemicals were commercial products of reagent grade. Solutions were made in nanopure (ultrafiltered, distilled, and deionized) water. The peptide HLMQNGKRLKKKTIVKKTLNPFL- FNESFSF was synthesized by the Peptide Institute (Osaka, Japan) and was >98.1% homogeneous by HPLC, YMC-Pack ODS-AM, 10–60% CH₃CN in 0.1% trifluoroacetic acid.

Synthesis of P-2-tethered [3H]BZDC-IP₆—2-O-(6-Aminohexyl)-meso-Ins(1,2,3,4,5,6)P₆ was prepared as described (24) and coupled with [3H]BZDC-NHS in aqueous N,N-dimethylformamide (20). The following independent procedure was used for photoaffinity ligands. [3H]BZDC-NHS (2 mCi, 0.05 μmol) was dissolved in N,N-dimethylformamide (50 μl) and a solution of 1 molar equivalent of aminohexyl-IP₆ (50 μg, 0.05 μmol) in 0.25 M triethylammonium bicarbonate buffer (50 μl, pH 8.0) was added. The reaction was stirred overnight, concentrated in vacuo, redissolved in water, and loaded onto a DEAE-cellulose column (HCO₃⁻ form). The column was eluted with a step gradient of 0.1 to 1.0 M triethylammonium bicarbonate buffer; the [3H]BZDC-IP₆ was eluted at 0.4–0.5 M triethylammonium bicarbonate buffer. Its purity was analyzed by reverse phase HPLC (Aquapore RP-300, 7 μm, 4.5 × 250 mm, Brownlee column) using UV (254 nm) and radiochemical detection. The pure [3H]BZDC-IP₆ eluted at a retention time of 13 min using 0.05 M potassium phosphate in 15% acetonitrile water.

Expression and Purification of Fusion Proteins—JM109 Escherichia coli cells were electroporated and transformed with purified plasmids as described (9). Cells were grown in LB-ampicillin and induced with isopropyl-1-thio-β-D-galactopyranoside, and expressed proteins were isolated as described (9, 10). GST fusion proteins were purified by chromatography on glutathione-Sepharose 4B (Pharmacia Biotech Inc.) as specified by the supplier.

Photoaffinity Labeling of GST Fusion Proteins—GST fusion proteins (1 μM) were incubated with 30 μl of phosphate-buffered saline (pH 7.4) containing 0.5 μCi (0.25 μM) of [3H]BZDC-IP₆ (n = 3, 4, or 6). To determine the affinity and specificity of binding, unlabeled IP₆ (0.25 mM) was included in the solution. Samples were equilibrated at 4 °C for 15 min in a 96-well plate. Wells in the plate were aligned with the axis of a UV light with a 2-cm distance between the bottom of the wells and the bulb and irradiated (360 nm at 1900 μW/cm²) for 45 min at 0 °C. Then 6 × SDS sample buffer was added to the samples, and the proteins were separated by SDS-polyacrylamide gel electrophoresis on 12.5% Laemmli gels. The gels were stained with Coomassie Blue, destained, and impregnated with En³Hance (DuPont NEN) according to the manufacturer’s instructions, dried, and exposed to XAR-5 x-ray film for 5–30 days at ~80 °C. No covalent incorporation of the photo label occurred in the absence of UV irradiation. All of the photo labeling experiments were performed in at least three independent replicates. Fluorograms were digitized on a UMAX-UC 840 scanner using Adobe Photoshop, and the densities of the bands were determined by NIH IMAGE 1.59 to find the relative incorporation.

Circular Dichroism—CD spectra were recorded using an AVIV model 62DS spectropolarimeter. Far-UV CD spectra of 50 μg peptide solutions were recorded at 11 °C using a 1-mm quartz cell.

RESULTS

Synthesis of Photoaffinity Labels—Four photoaffinity probes (Fig. 1) were used to probe the selectivity and specificity of the GST-Syt II-C2B interaction with IP₆’s. Each inositol polyphosphate photoaffinity label (25) was freshly prepared for this study from the corresponding 1- or 2-O-(α-aminoalkyl) IP₆ derivative with the heterobifunctional reagent [3H]BZDC-NHS ester (20). The P-1-linked 3-aminopropylphosphodiesters of d-myo-IP₃ (18) and IP₆ (22), and the P-2-linked 6-aminohexylphosphodiester of meso-IP₆ (24) were prepared as described previously. The racemic P-1-linked 6-aminohexyl derivative of IP₆ was prepared by modification of the protecting group strategy developed for the P-2-linked probe (23). Each probe had the same nominal specific activity, between 35 and 42 Ci/mmol, as determined by the specific activity of the lot of [3H]BZDC-NHS ester employed. This ensured that a given level of radioactivity (in dpm) corresponded to an equivalent concentration of each probe, so that direct comparisons of relative efficiency of photoattachment would be meaningful. The homogeneity of each probe was assessed by reverse phase HPLC following periodic repurification of [3H]BZDC-IP₆’s by ion exchange chromatography.

Photolabeling of Crude Fusion Proteins—To determine the selectivity of the BZDC-IP₆ probes for Syt II domains, the crude supernatant proteins from transformed E. coli were photolabeled with [3H]BZDC-IP₃ and [3H]BZDC-IP₆. To ascertain whether any nonspecifically or specifically labeled protein was derived from plasmid or from E. coli, we also photolabeled a crude supernatant containing a GST fusion to a truncated human thrombin receptor.² Fig. 2 shows the initial labeling experiments. Neither the 40-kDa GST-C2B nor the 57-kDa GST-C2A+C2B protein bands (arrows) were labeled by [3H]BZDC-IP₃ in the crude supernatants. Interestingly, an ~45-kDa protein present in all supernatants, including the GST-NTR control, showed efficient and specific IP₆-displaceable labeling. This observation suggests the presence of a hitherto unknown bacterial IP₆-binding protein. In contrast, [3H]BZDC-IP₆ efficiently and selectively labeled both the 40-kDa GST-Syt II-C2B and 57-kDa GST-Syt II-C2A+C2B proteins, with no detectable labeling of proteins in the GST-NTR control. It is noteworthy that despite the low abundance of the

² J. T. Elliott, unpublished results.
GST-Syt II-C2B constructs in the crude supernatants, virtually no other proteins were labeled. The specificity of the labeling was confirmed by the absence of labeling in the presence of 1000-fold excess IP₆ as a competitor. Initial data supported a higher affinity of the Syt II-C2B constructs for [³H]BZDC-IP₆ relative to [³H]BZDC-IP₄ consistent with binding affinity measurements (9).

**Specificity of Competitive Displacement of [³H]BZDC-IP₆ by IPₙ**—To study the relative specificity for different IPₙs, the GST-Syt II-C2B fusion protein was purified by glutathione affinity chromatography and incubated with 0.25 μM [³H]BZDC-IP₆ in the presence of increasing concentrations of IP₃, IP₄, and IP₆. The unlabeled IPₙs should compete for the same binding pocket with the labeled BZDC-IP₆. After a 10-min preincubation, irradiation (45 min, 4 °C, 360 nm), followed by electrophoresis and fluorography, provided the result shown in Fig. 3A. The data show substantial qualitative differences. At 1000-fold excess, IP₃ fails to displace the labeling, IP₄ shows ~60% reduction of labeling, and IP₆ completely displaces labeling by [³H]BZDC-IP₆; over 90% reduction in labeling is seen with 250-fold excess IP₆.

**Time Course of Photolysis and Relative Affinities of Selected IPₙ Photoprobes**—The fusion protein GST-Syt II-C2B was irradiated for 20, 30, 45, 60, and 90 min at 4 °C with [³H]BZDC-IP₆ to determine the optimal time for covalent attachment of the ligand to the protein. As expected from previous studies with benzophenone (26) and specifically [³H]BZDC-modified-(P)IPₙs (17), the covalent binding reached saturation at 45 min. After 45 min, the specific binding sites were saturated and only nonspecific labeling occurred. This validated the selection of 45 min for the screening studies described above; in addition, all subsequent photolabeling experiments employed 45-min irradiation times.

To determine the specificity of the Syt II-C2B domain for different IPₙs, the purified GST-Syt II-C2B fusion protein was photoaffinity labeled with each of the three [³H]BZDC-IPₙ probes (Fig. 3B). Consistent with data from the photolabeling of crude supernatants from transformed cells, the purified fusion protein showed maximal binding for P-2-tethered [³H]BZDC-IP₆ with somewhat reduced labeling by [³H]BZDC-IP₄. Label-
Central lysine residues (underlined) were confirmed as crucial for binding of inositol polyphosphates to Syt II-C2B domain (9). These photoaffinity labeled with P-1-tethered [3H]BZDC-IP3, P-1-tethered [3H]BZDC-IP4, P-2-tethered GST-Syt II-C2B. Purified GST-Syt II-C2B was photoaffinity labeled at 4°C, 360 nm) in the absence (−) or the presence (+) of 50-, 250-, or 1000-fold molar excess of IP3, IP4, or IP6 relative to the concentration of photoaffinity label. B, relative specificity of [3H]BZDC-IP6 labeling of GST-Syt II-C2B. Purified GST-Syt II-C2B was photoaffinity labeled with P-1-tethered [3H]BZDC-IP3, P-1-tethered [3H]BZDC-IP4, P-2-tethered [3H]BZDC-IP6, and P-1-tethered [3H]BZDC-IP6 (each, 0.4 μCi, 0.2 μM). The photoaffinity labeling was quantified using NIH IMAGE 1.59 and is expressed as a percentage of binding to P-2-tethered [3H]BZDC-IP6. The asterisk indicates that no labeling was observed with the P-1-tethered [3H]BZDC-IP6 under these conditions.

**Photolabeling of Different Domains of Syt II and Syt III with [3H]BZDC-IP6**—Synaptotagmins have two C2 domains, C2A and C2B, but only the C2B domain binds to IP6. The C2A domain appears to acts a Ca2+-sensor (1, 3) and binds to phospholipids in a Ca2+-dependent manner (27). To confirm the selectivity of [3H]BZDC-IP6 for the C2B domain, three GST fusion constructs (9) containing the C2B, the C2A, and the C2A+C2B domains of Syt II (Fig. 6A) were expressed, purified, and photoaffinity labeled. Consistent with the known specificity of the C2B, but not C2A, domain for high affinity binding of [3H]IP6 and higher IP6s (9), only the C2B and C2A+C2B domains were covalently modified with [3H]BZDC-IP6. No photoaffinity labeling of the C2A domain was detected.

Different isoforms of synaptotagmin have been found in mammals (2, 28, 29). Curiously, despite significant sequence similarity in the C2B domains and particularly in the central base-rich IP6 binding region (Fig. 6B), the Syt III-C2B domain did not show high affinity binding of [3H]IP6 (10). This appeared to involve masking of the active site by the C-terminal region, because truncation of the C-terminal one-third of Syt III-C2B domain resulted in recovery of 30% of the [3H]IP6 binding. Moreover, a chimeric fusion protein, GST-Syt III-IIIC2B, containing the C-terminal third of Syt II-C2B domain fused to the N-terminal two-thirds of the Syt III-C2B domain also recovered 70% of the [3H]IP6 binding (10). Thus, these two GST fusion proteins (see Fig. 6A) were expressed, purified, and photoaffinity labeled with [3H]BZDC-IP6 (Fig. 6C). Consistent with the presence of 0, 1, 2, 4, 8, and 16 molar equivalents of this peptide (Fig. 4). At equimolar concentrations of GST-Syt II-C2B and the peptide, labeling of the complete domain and the central 32-mer are essentially equal. Labeling of the C2B fusion protein decreases and the labeling of the 32-mer increases as the peptide concentration is increased. At 4 equivalents of the peptide, nearly 95% of the [3H]BZDC-IP6 is sequestered by the 32-mer, thereby “protecting” the C2B fusion protein from labeling. At 8 and 16 equivalents of peptide (>20 μM), aggregation was observed during electrophoresis (data not shown).
with its failure to bind \( ^3\text{H}\text{IP}_4 \) under equilibrium conditions, GST-Syt III-C2B domain showed negligible photoaffinity labeling by \( ^3\text{H}\text{BZDC-IP}_6 \). The truncated GST-Syt III-C2B(ΔC) construct showed modest but readily detectable labeling (Fig. 6C). Essentially no covalent modification was observed for the GST-Syt III/II-C2B chimeric protein, perhaps attributable to the inaccessibility of the sterically bulky BZDC moiety to the IP\(_6\) binding site of this chimera.

**DISCUSSION**

The availability of tetherable analogues of the inositol polyphosphates and phosphoinositide polyphosphates (25), including affinity purification resins and benzophenone-containing photoaffinity labels (17, 26) has enabled the identification of new PIP\(_2\)- and IP\(_6\)-binding proteins and the characterization of the ligand binding sites within these proteins. For example, four IP\(_4\) binding proteins were affinity-purified from rat brain...
(30) and photoaffinity labeled with \(^{[3]H}\)BZDC-IP\(_3\) (22, 31). Two have been further characterized; the \(\alpha\) subunit of clathrin assembly protein (AP-2) (32) had the highest affinity for IP\(_3\), whereas the novel cytoskeletal linker protein centaurin-\(\alpha\) had the highest affinity forPIP\(_3\) (21). The IP\(_3\) binding site of the affinity-purified rat brain IP\(_3\) receptor (19) has been identified by sequencing a proteolytic fragment of the \(^{[3]H}\)BZDC-IP\(_3\) photoaffinity-labeled protein (33). The availability of two regioisomeric-tethered derivatives of IP\(_3\) (23, 24) has facilitated the further study of kinases (34, 35) and other proteins that interact with IP\(_3\). The P-2-tethered \(^{[3]H}\)BZDC-IP\(_3\) prepared in this work was employed to establish the location of the IP\(_3\) binding site in the C2B region of the synaptotagmin and to confirm the ligand specificity of this domain by an independent method. In the process, the site specificity and ligand selectivity found fully validates the use of the photoaffinity labeling approach in the search for and active-site mapping of additional PIP\(_n\)- and IP\(_3\)-binding proteins.

Six GST-Syt C2 domain-containing fusion proteins were examined in this study, using four photoaffinity probes corresponding to modifications of IP\(_3\), IP\(_4\), and IP\(_6\). The most important findings are summarized as follows. First, we observed extremely high selectivity and ligand specificity of labeling of the C2B-containing proteins in crude extracts. No other proteins were significantly labeled with the high specific activity \(^{[3]H}\)BZDC-IP\(_3\) derivatives even when the target proteins represented less than 2% of the total protein. The only exception was a hitherto unknown 45-kDa \(E.\ coli\) protein that showed high selectivity and specificity for binding of IP\(_3\). This observation may provide the basis for purification of this protein and the determination of its role in the biochemistry of this common bacterium. For all proteins and ligands, labeling followed an expected time course, with a maximum efficiency at 45 min.

Second, labeling was highly dependent on the choice of ligand. Although P-2-linked \(^{[3]H}\)BZDC-IP\(_6\) gave the strongest labeling of C2B-containing constructs, P-1-linked \(^{[3]H}\)BZDC-IP\(_4\), \(^{[3]H}\)BZDC-IP\(_3\), and \(^{[3]H}\)BZDC-IP\(_6\) showed modest, weak, and no labeling, respectively. The failure of the P-1-tethered IP\(_3\) to label C2B domains was unexpected but an important "negative control." It is important to recognize that the aminopropyl-tethered photophore adds significant "unnatural" lipophilicity to the soluble IP\(_3\) recognition elements as well as masking one of the phosphates. The importance of the number and the positions of phosphates and the position of the linker were critical factors determining the photolabeling efficiency, not simply nonselective hydrophobic interactions of the photophore with the protein.

Nonetheless, the observation that purified but not crude C2B domains could be weakly labeled by \(^{[3]H}\)BZDC-IP\(_3\) emphasizes two important points. When only a single protein is present in a photoaffinity labeling experiment, even relatively low affinity sites may become covalently modified. The ligand may poorly associate with the target, but in the absence of alternative sites for occupancy, the irreversible nature of the photoaffinity labeling experiment allows labeled protein to accumulate.

The photoaffinity probe may also mimic a different ligand. In BZDC-IP\(_3\), the presence of the P-1-O-(3-aminopropyl) linker bearing the hydrophobic tritiated 4-benzoyldihydrocinnamide photophore allows the photoaffinity ligand to resemble a 2-desacyl phosphoinositide 4,5-bisphosphate, PIP\(_2\). For example, profilin, a PIP\(_2\)-binding cytosolic protein important in interactions of the actin cytoskeleton with membrane phospholipids (36) has been labeled with \(^{[3]H}\)BZDC-IP\(_3\), but displacement of labeling requires PIP\(_2\), not IP\(_3\) (17). The PH domain of the phospholipase C \(\delta\) isoenzyme can also be labeled efficiently\(^3\) with \(^{[3]H}\)BZDC-IP\(_3\). Indeed, the C2B domain of Syt I has been recently implicated in binding of PIP\(_3\) at high calcium concentrations (37), providing further explanation for the affinity of the photoaffinity probes for the Syt II-C2B domains employed in this study.

Similarly the \(^{[3]H}\)BZDC-IP\(_3\) probe effectively mimics PIP\(_3\). This observation in fact enabled the isolation and purification of centaurin-\(\alpha\), a novel multi-domain protein with both homology regions, suggesting its role in communication of membrane lipid changes to the cytoskeleton (21). Very recently, the N-terminal region of the \(\alpha\) subunit of AP-2 was found to have high affinity for PIP\(_3\) (38), a finding foreshadowed by the highly selective and efficient photoaffinity labeling of AP-2 by \(^{[3]H}\)BZDC-IP\(_3\) (31, 32). In unpublished work from our laboratories, \(^{[3]H}\)BZDC-IP\(_4\) showed efficient labeling of assembly protein AP-3, already documented to have high affinity for the higher IP\(_5\), IP\(_4\), IP\(_3\), IP\(_2\), and IP\(_3\) (39), which inhibited assembly of the clathrin coat. Interestingly, PIP\(_3\) has now been shown to be more effective than the soluble IP\(_5\), IP\(_4\), IP\(_3\), IP\(_2\), and IP\(_3\) in inhibiting AP-3-mediated clathrin coat assembly. Finally, the labeling of purified Syt II-C2B domains by \(^{[3]H}\)BZDC-IP\(_3\) can be understood in terms of the affinity of Syt C2 domains for PIP\(_3\) at low calcium concentrations (37).

The relative importance of the phosphate groups in binding to the C2B domains was examined in an independent set of NMR studies (40). The relative rate enhancements, which depend on increases in motional correlation time, were determined for clearly resolved phosphorus and proton signals of the photoaffinity ligand in the presence and the absence of GST-Syt II-C2B. Thus, \(^1\)H and \(^31\)P NMR of 10 mM BZDC-IP\(_4\) were first determined at 298 K for 20 mM sodium acetate buffer containing 0.1 mM EDTA, pH 5.5; next, 0.04 molar equivalent of purified GST-Syt II-C2B (0.4 mM) was added to the solution, and the chemical shifts and relaxation times were remeasured. The signals exhibiting the largest rate enhancements included P-5, H-5, and H-6, whereas the P-1 and side chain aminopropyl relaxation rates were essentially unaffected by the binding to the C2B domain. Thus, the 4,5,6 region of the inositol ring is intimately involved in the binding to C2B, whereas the aminopropyl tether retains motional freedom even in the bound state (40).

Third, labeling was well correlated with reported relative IP\(_n\) binding affinity as determined by equilibrium methods using displacement of \(^{[3]H}\)IP\(_4\) with unlabeled IP\(_5\) (9). Thus, using \(^{[3]H}\)BZDC-IP\(_3\) as the probe, displacement of 50% of the labeling required about a 150-fold molar excess of IP\(_5\), whereas little or no displacement was observed with IP\(_3\) or IP\(_5\) at six times this concentration of displacer. When \(^{[3]H}\)BZDC-IP\(_4\) labeling was examined in detail (data not shown), IP\(_3\) was again the most effective competing ligand.

Fourth, labeling was correlated with binding affinities of C2B domains for the functionally diverse Syt II and Syt III constructs. Consistent with the \(^{[3]H}\)IP\(_3\) binding affinities (10), the GST-Syt III-C2B domain was not covalently labeled upon irradiation with \(^{[3]H}\)BZDC-IP\(_3\). Moreover, purified GST fusion proteins in which \(^{[3]H}\)IP\(_5\) binding had been partially restored also recovered the ability to be photoaffinity labeled. Thus, the C-terminal truncated GST-Syt III-C2B(ΔC) showed partial unmasking of the IP\(_3\)-binding domain.

\(^{3}\) E. Tall, G. Dormán, P. García, S. Shah, J. Chen, A. A. Profit, Q.-M. Gu, A. Chaudhary, G. D. Prestwich, and M. J. Rebecchi, submitted for publication.

\(^{4}\) A. A. Profit, J. M. Rabinoch, B. Mehrotra, G. D. Prestwich, and E. M. Lafer, unpublished observations.

\(^{5}\) W. Hao, Z. Tan, K. Prasad, K. K. Reddy, J. Chen, G. D. Prestwich, J. R. Falck, S. B. Shears, and E. M. Lafer (1997) J. Biol. Chem. 272, in press.
Fifth, labeling by P-2-linked [3H]BZDC-IP₆ correlated with the known affinities of the two different C2 domains, which show different calcium-ion-dependent properties (41). The C2A domain acts as a calcium sensor (3) during endocytosis, and three-dimensional structures have been described (42, 43) for this critical domain. The phospholipid selectivities of several Syts have been described in detail, and the Syt IV C2A domain also appears to function as a calcium sensor (27). Consistent with the absence of high affinity [3H]IP₄ binding to C2A (10), the GST-Syt II-C2A domain was not labeled by [3H]BZDC-IP₆. Preliminary observations suggest Ca²⁺-dependent labeling of this domain with [3H]BZDC-IP₆, consistent with binding to other anionic phospholipids such as phosphatidylserine. In contrast, the C2B domain has been implicated in binding to IP₇₈ (9), PIP₇₈ (38), AP-2 (14), and other elements of the synaptic vesicle fusion machinery (44). The efficient labeling of the GST Syt II-C2B domain (and GST-Syt II-C2A+C2B domain) with [3H]BZDC-IP₆ is consistent with the different affinities of these two protein kinase C2 homology domains.

Sixth, a synthetic 32-residue peptide corresponding to the base-rich central region of the C2B domain, identified by mutational and truncation/deletion methods as crucial to high affinity [3H]IP₄ binding (9, 10), also showed high affinity for the [3H]BZDC-IP₆ photoaffinity probe. Indeed, when an equimolar amount of this photoaffinity label, the full-length C2B domain acts as a calcium sensor (3) during endocytosis, and three-dimensional structures have been described (42, 43) for this critical domain. The phospholipid selectivities of several Syts have been described in detail, and the Syt IV C2A domain also appears to function as a calcium sensor (27). Consistent with the absence of high affinity [3H]IP₄ binding to C2A (10), the GST-Syt II-C2A domain was not labeled by [3H]BZDC-IP₆. Preliminary observations suggest Ca²⁺-dependent labeling of this domain with [3H]BZDC-IP₆, consistent with binding to other anionic phospholipids such as phosphatidylserine. In contrast, the C2B domain has been implicated in binding to IP₇₈ (9), PIP₇₈ (38), AP-2 (14), and other elements of the synaptic vesicle fusion machinery (44). The efficient labeling of the GST Syt II-C2B domain (and GST-Syt II-C2A+C2B domain) with [3H]BZDC-IP₆ is consistent with the different affinities of these two protein kinase C2 homology domains.

Sixth, a synthetic 32-residue peptide corresponding to the base-rich central region of the C2B domain, identified by mutational and truncation/deletion methods as crucial to high affinity [3H]IP₄ binding (9, 10), also showed high affinity for the [3H]BZDC-IP₆ photoaffinity probe. Indeed, when an equimolar ratio of 32-mer to GST-Syt II-C2B was labeled with a stoichiometric amount of this photoaffinity label, the full-length C2B domain and 32-mer were equally labeled. Increasing amounts of peptide were able to completely sequester the photoprobe from the C2B domain. This observation is highly significant in that it represents the smallest minidomain known to demonstrate selective, high affinity binding to IP₆ or its analogues. Surprisingly, this peptide failed to exhibit secondary structure, other than random coil, in aqueous buffers at two pH values in the presence and the absence of IP₆. It is conceivable, in view of the dimerization of Syts via their C2B domains (15), that the peptide is only able to adopt an IP₆-binding conformation in the presence of a preorganized IP₆-C2B complex. However, the 32-mer peptide could be labeled successfully with [3H]BZDC-IP₆ even in the absence of the GST Syt II-C2B protein (data not shown), and this labeling can be reduced by addition of IP₆ as a competitor. This suggests that BZDC-IP₆ may induce a structure in the otherwise random coil polypeptide. The labeling was maximal with [3H]BZDC-IP₆ relative to the [3H]BZDC-IP₆ and IP₆ photoprobe, supporting the higher affinity of the 32-mer peptide for IP₆.

A related C2B domain has been identified in a novel IP₄-binding protein from rat brain and both human and porcine platelets (45); this protein also possesses GTPase-activating activity not found in other IP₄-binding proteins. This protein has high specificity for IP₆ with 1000-fold lower affinity for IP₅. The central IP₆ binding sequence would have missing or moved basic residues found in the IP₆ binding region of C2B with a central region of EAKKTKVKKK (mouse). A preliminary report suggested that a 25-mer peptide that includes this recognition site could bind IP₆ with high affinity via a “basket-like” structure, allowing the lysines or arginines to interact with the phosphates of the IP₆ (46). The data described herein constitute the experimental evidence in support of this hypothesis.

Synaptotagmin is essential for several aspects of synaptic vesicle dynamics. In particular, strong biological evidence now supports a critical role for the C2B domain in recycling of synaptic vesicles. This evidence is based on the accumulation of synaptic vesicles in C. elegans mutants lacking the C2B domain of their Syt (12) and from the inhibition of synaptic vesicle recycling in squid (Logrio pealei) giant synapse preterminals when injected with antibodies to the this C2B domain (11). The high inositol polyphosphates (IP₇₈, n = 4, 5, or 6) can block synaptic neurotransmitter release and may regulate the fusion step preceding exocytosis (11). The emergence of PIP₆ as a preferred ligand for inhibition of clathrin coating mediated by AP-2 (38) and AP-3 (35) may suggest that the IP₆₈ (n = 4, 5, or 6) may not be the actual physiological ligands involved. An important research area would now be studies directed at determining the nature and flux of PIP₆ and IP₇₈ ligands actually involved in the endo- and exocytotic events in vivo.

Conclusions—Four photoaffinity analogues of IP₆, IP₇₈, and IP₈ have been employed to document the selectivity and specificity of different inositol polyphosphates toward the C2 domains of two Syts. We made five key observations. First, labeling of the C2B-containing proteins in crude extracts showed extremely high selectivity and ligand specificity. Second, labeling was highly dependent on ligand, with [3H]BZDC-IP₆ showing the optimal labeling of C2B domains. Third, labeling was well correlated with reported relative IP₆₈ binding affinities as determined by equilibrium methods using displacement of [3H]IP₆ with unlabeled IP₆₈. This validates photolabeling as a methodology for identification of novel proteins that bind IP₆₈. Fourth, labeling was correlated with [3H]IP₄ binding affinities of C2B domains for the functionally diverse Syt II and Syt III constructs and for the C2A versus C2B domains of Syt II. Fifth, a 32-residue base-rich peptide located in the central region of the C2B domain exhibited high affinity binding to the photoaffinity label when employed in competition with the full-length C2B domain. This observation opens the door to use of peptides in physiological experiments to modulate IP₆₈-receptor binding in a wide variety of systems. Future studies on active site mapping and structure determination of domain-IP₆₈ complexes will also be realizable goals given these new data.

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