Phosphorylation of a Vesicular Monoamine Transporter by Casein Kinase II*

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The vesicular monoamine transporters (VMATs) package monoamine neurotransmitters into secretory vesicles for regulated exocytotic release. One isoform occurs in the adrenal gland (VMAT1) and another in the brain (VMAT2). To assess their potential for regulation, we have investigated the phosphorylation of the VMATs. Using heterologous expression in Chinese hamster ovary, PC12, and COS cells, we find that rat VMAT2, but not VMAT1, is constitutively phosphorylated. Phosphoamino acid analysis indicates that this phosphorylation occurs on serine residues, and the analysis of VMAT1-VMAT2 chimeras and site-directed mutagenesis localize the phosphorylation sites to serines 512 and 514 at the carboxyl terminus of VMAT2. Since these residues occur in an acidic region, we tested the ability of the acidotropic kinases casein kinase I (CKI) and casein kinase II (CKII) to phosphorylate bacterial fusion proteins containing the carboxyl terminus of VMAT2. Purified CKI and CKII phosphorylate the wild-type carboxyl terminus of VMAT2, but not a double mutant with both serines 512 and 514 replaced by alanine. The protein kinase inhibitor CKI-7 and unlabeled GTP both block in vitro phosphorylation by cell homogenates, indicating a role for CKII and possibly CKI in vivo. Both kinases phosphorylate the VMAT2 fusion protein to a much greater extent than a similar fusion protein containing the carboxyl terminus of VMAT1, consistent with differential phosphorylation of the two transporters observed in intact cells. These results provide the first demonstration of phosphorylation of a vesicular neurotransmitter transporter and a potential mechanism for differential regulation of the two VMATs.

For classical neurotransmitters such as the monoamines, synaptic transmission involves two distinct transport activities. Transport across the plasma membrane removes neurotransmitter from the synaptic cleft, thereby terminating its action on the postsynaptic cell and recycling it for another round of exocytosis (1, 2). Transport across the membrane of secretory vesicles serves to package newly synthesized as well as recycled transmitter for regulated release by exocytosis (3, 4). Plasma membrane transport and vesicular neurotransmitter transport differ in their bioenergetic mechanism and sensitivity to drugs. Plasma membrane transport uses the sodium gradient across the plasma membrane and involves cotransport of sodium with the transmitter (2). Vesicular transport uses the H+ electrochemical gradient across the vesicular membrane generated by a H+ ATPase (5, 6) and, in the case of the monoamines, involves the exchange of two luminal protons for each molecule of cytoplasmic transmitter (7). Monoamine transport across the plasma membrane also differs from vesicular transport in terms of pharmacology. Whereas cocaine and antidepressants inhibit plasma membrane transport, the antihypertensives reserpine and tetrabenazine inhibit vesicular transport (8). Interestingly, reserpine can cause a syndrome resembling depression (9), indicating the importance of vesicular transport activity for the control of mood and behavior. The psychostimulant amphetamine also disrupts the storage of amines in secretory vesicles (10), further indicating that alterations in vesicular monoamine transport can affect behavior.

Molecular cloning has recently identified several transport proteins responsible for packaging classical neurotransmitters including the monoamines into secretory vesicles (3, 4). The vesicular monoamine transporters (VMATs)1 protect against the parkinsonian neurotoxin N-methyl-4-phenylpyridinium (MPP+) by transporting it into vesicles, thereby sequestering it away from its primary site of action in mitochondria (11, 12). We took advantage of this property to isolate the cDNA for a vesicular monoamine transporter expressed in the adrenal gland (VMAT1) (13). We (13) and another group (14) then isolated the cDNA for a highly related protein expressed in the brain (VMAT2). Both of the VMATs have 12 predicted transmembrane domains and a large luminal loop between transmembrane domains 1 and 2 (13). Consistent with the observed differences in bioenergetics and pharmacology, the VMATs show no sequence similarity to the plasma membrane transporters (3, 13). Transport and drug binding assays have recently identified differences in substrate affinity and drug sensitivity between the two VMATs (15), and analysis of VMAT1-VMAT2 chimeras has begun to elucidate the regions responsible for these differences (16). VMAT1 and VMAT2 also differ in their subcellular localization (17)2 and in the number and type of potential phosphorylation sites (13), suggesting possible differences in regulation.

Considerable evidence indicates that the biosynthesis and transport of neurotransmitters undergo regulation by protein

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1 The abbreviations used are: VMATs, vesicular monoamine transporters; CHO, Chinese hamster ovary; HA, hemagglutinin; GST, glutathione S-transferase; CKI, casein kinase I; CKII, casein kinase II.

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In the case of catecholamines, tyrosine hydroxylase is the rate-limiting enzyme in transmitter biosynthesis and is regulated by several kinases, including cyclic AMP-dependent protein kinase and Ca\(^{2+}\)- and calmodulin-dependent kinase II (18, 19). Transport of monoamines across the plasma membrane also undergoes regulation by phosphorylation. Activation of Ca\(^{2+}\)- and phospholipid-dependent kinase by phorbol esters inhibits plasma membrane serotonin transport (20, 21). Conversely, activation of a GMP-regulated kinase by way of the adenosine receptor and nitrogen-oxide synthase increases serotonin transport (22). Although the phosphorylation of a plasma membrane transporter for monoamines has not yet been directly demonstrated, metabolic labeling of a plasma membrane transporter for glutamate has revealed phosphorylation-dependent changes in activity (23). The transport of neurotransmitter into synaptic vesicles may also be regulated by phosphorylation. Indeed, stimulation of protein kinases A and C in rat pheochromocytoma (PC12) cells has been suggested to inhibit vesicular transport activity (24–26). However, the mechanism of inhibition remains unclear, and the phosphorylation of a vesicular neurotransmitter transporter has not yet been demonstrated.

To assess the potential for regulation of the VMATs by phosphorylation, we have determined their phosphorylation state in cultured cells under a variety of conditions. Surprisingly, we observed phosphorylation of rat VMAT2 under all tested conditions, suggesting constitutive phosphorylation. Using mutational analysis, we have identified sites for constitutive phosphorylation. In vitro assays using purified kinases as well as cellular homogenates suggest the involvement of casein kinase II and possibly casein kinase I. Interestingly, rat VMAT1 does not show this pattern of constitutive phosphorylation, suggesting that the two VMATs may be regulated differently.

MATERIALS AND METHODS

Cell Culture—Chinese hamster ovary (CHO) fibroblast cells were maintained in Ham’s F-12 medium containing 5% calf serum (Hyclone Laboratories), penicillin, and streptomycin. Rat pheochromocytoma PC12 cells were maintained in Dulbecco’s modified Eagle’s medium containing 5% calf serum, 10% equine serum, penicillin, and streptomycin. Monkey kidney COS cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% calf serum, penicillin, and streptomycin. For transient transfections, COS cells were electroporated with 15–30 μg of DNA using a Bio-Rad Gene Pulser apparatus as described previously (15).

Mutagenesis—Site-directed mutagenesis was performed as described previously (27,28) and used to insert the hemagglutinin (HA) epitope (29) as well as to introduce point mutations into the rat VMAT2 cDNA. After sequence analysis to verify the mutations and to exclude unbound nucleotide changes (30), a fragment containing the mutated region was subcloned into the expression vector pcDNA1-Amp (Invitrogen) containing the wild-type VMAT2 cDNA. Glutathione S-transferase (GST)-VMAT2 fusion proteins (31) were constructed by subcloning EcoRI fragments encoding the carboxyl termini of wild-type and mutant rat VMAT2 cDNAs into the SmaI site of the pGEX-3X bacterial expression vector (Pharmacia Biotech Inc.). GST-VMAT1 was constructed by amplification of the region of rat VMAT1 cDNA encoding the carboxyl-terminal domain of the protein using the polymerase chain reaction (32) and the high-fidelity Pfu DNA polymerase (Stratagene). Oligonucleotides used for polymerase chain reaction amplification encoded in-frame EcoRI and Xhol cleavage sites, allowing isolation of the amplified fragment between the EcoRI and Xhol sites of the pGEX-5X-1 bacterial expression vector (Pharmacia Biotech Inc.). To facilitate cloning, the first amino acid of VMAT1 after the GST moiety was changed from serine to aspartate. Metabolic Labeling—For metabolic labeling with \(^{32}P\), cells were washed three times in medium lacking phosphate and then incubated for 2 h at 37°C in the presence of 0.5–1.0 mM \(^{32}P\)-labeled medium. After labeling, cells were washed with ice-cold 10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl (HEPES-buffered saline) and then frozen on dry ice. The frozen cells were harvested by scraping into HEPES-buffered saline; pelleted by centrifugation at 5000 × g for 5 min at 4°C; and then resuspended by trituration in 1 ml of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 0.2 mM NaVO\(_4\), 10 mM EDTA, 5 mM EGTA, 10 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 μg/ml Nonidet P-40 detergent (homogenization buffer). After removal of the cell debris and nucleic acid by centrifugation at 4°C, SDS was added to the supernatant to a final concentration of 0.2%. For immunoprecipitation, the mixture was incubated overnight at 4°C with protein A-Sepharose (Sigma) prebound either to affinity-purified polyclonal antibodies to VMAT1 or VMAT2 or to monoclonal antibody 12CA5 to HA (Boehringer Mannheim) (29). Immune complexes were washed three times in homogenization buffer containing 0.5% Triton X-100, resuspended in 2 × Laemmlie sample buffer (33), and the proteins were separated by electrophoresis through 10% polyacrylamide. The gels were then fixed in 10% acetic acid, 50% methanol; dried; and submitted to autoradiography.

For metabolic labeling with \(^{35}S\)methionine/cysteine, cells were washed three times in medium lacking cysteine and methionine and then incubated for 2 h at 37°C in the presence of 0.05–0.1 μCi/ml \(^{35}S\)Met/Cys (Trans-35S-label, ICN). Cell harvesting, immunoprecipitation, and electrophoresis were performed as described above for labeling with \(^{32}P\), except that the gels were incubated in 1 mM sodium salicylate as a fluid prior to drying. Western analysis of HA-tagged protein was performed as described previously (28), with a primary rabbit polyclonal antibody to HA (Boehringer) diluted 1:1000, followed by a secondary antibody conjugated to horseradish peroxidase and visualization by enhanced chemiluminescence (Amersham Corp.).

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described (34). Briefly, extracts prepared from cells metabolically labeled with \(^{32}P\) were immunoprecipitated with antibody to HA or VMAT2, and the immunoprecipitates were separated by electrophoresis through polyacrylamide. Following autoradiography, the radiolabeled band was excised from the gel and rehydrolyzed in 50 mM ammonium bicarbonate, and protein was eluted overnight in 0.2% SDS, 2% β-mercaptoethanol. The eluate was then precipitated with 20% trichloroacetic acid and partially hydrolyzed by boiling in 5.7 M HCl for 60 min. The hydrolysate was washed with distilled water and pH 1.9 buffer (7.8% acetic acid, 2.2% formic acid), resuspended in a minimal volume of pH 1.9 buffer containing phosphoamino acid standards, and spotted onto thin-layer cellulose plates. Electrophoresis was performed at 4°C using pH 1.9 buffer for the first dimension and pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension. The standards were then stained with ninhydrin, and the plates were submitted to autoradiography to visualize radiolabeled material.

Transport Assays—Transient transfection assays were performed essentially as described (13) with minor modifications. Briefly, transiently transfected COS cells were detached from the substrate with trypsin, resuspended in 320 mM sucrose, 10 mM HEPES-KOH, pH 7.4 (SH buffer), containing 2.5 mM MgSO\(_4\), 2.5 mM EGTA, 10 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 40 μg/ml bestatin, 2 μg/ml E-64, 50 mM NaF, and 0.2 mM NaVO\(_4\); and disrupted in the water-filled cup horn of an ultrasonic cell disrupter (Branson Ultrasonics Corp.) using 15 pulses at an intermediate setting. After removal of nuclei and cell debris by sedimentation at 1300 × g at 4°C for 5 min, 10–μl aliquots were added to SH buffer containing 2.5 mM MgSO\(_4\), 4 mM KCl, 100 mM ATP, 10 mM NaF, 0.2 mM NaVO\(_4\); and 20 μM [H]serotonin with varying concentrations of unlabeled serotonin (60–300 nM). After incubation for 2 min at 29°C, reactions were terminated by dilution into ice-cold SH buffer and filtration through 0.2-μm Supor 200 membranes (Gelman Instrument Co.). Filters were air-dried and added to scintillation fluid, and bound radioactivity was determined by scintillation counting in a Cytoscrint (ICN). Transport reactions were done in duplicate and repeated four times. The protein concentration of each postnuclear supernatant was determined by the Bradford assay (60) (Bio-Rad), and the transport assay values were normalized by dividing the amount of total protein (mg) added to the reaction. \(K_m\) and \(V_{max}\) values were determined by double-reciprocal plots of serotonin concentration and normalized transport activity.

In Vitro Phosphorylation—To purify GST fusion proteins, Escherichia coli cells were grown overnight in 1.6% tryptone, 0.1% yeast extract, 0.5% NaCl and inositol in 0.1 M isopropyl-β-D-thiogalactoside for additional 3–6 h at 37°C. Bacteria were added to phosphate-free 0.5 × richness buffer (10 mM NaHPO\(_4\), 1.8 mM KH\(_2 PO_4\), pH 7.3, 140 mM NaCl, 2.7 mM KCl (phosphate-buffered saline); and disrupted by vigorous sonication for 1–2 min at 0°C. The lysate was centrifuged at 14,000 × g to remove cell debris, and the resulting supernatant was used immediately or stored at −70°C. To partially purify the fusion protein, the cleared extract was bound to glutathione-Sepharose beads for 20 min at
room temperature in phosphate-buffered saline and washed twice in phosphate-buffered saline and once in 20 mM Tris, pH 7.5, 50 mM KCl, 5 mM dithiothreitol, 10 mM MgCl₂ (kinase buffer). Aliquots of fusion protein (0.2–8 µg) bound to glutathione-Sepharose (10–200 µl bed volume) were then incubated for 20 min at 30 °C in kinase buffer containing 200 µM ATP and [γ-32P]ATP to a final specific activity of 500 µCi/µmol with 100–1000 units of purified CKII (New England Biolabs Inc.), 25–500 units of purified CKI (New England Biolabs Inc.), or 1 µl (~10 µg of total protein) of a postnuclear supernatant from COS cells (see below). Reactions were stopped by washing with ice-cold phosphate-buffered saline containing 15 mM EDTA, and the phosphorylated proteins were eluted with 20 µl of 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. Eluates were added to an equal volume of 2 × Laemmli sample buffer, and proteins were separated by electrophoresis through 12.5% polyacrylamide. Gels were fixed and stained with Coomassie Blue and then dried and submitted to autoradiography. For quantitation of incorporated radiolabel, gel slices containing the stained radiolabeled bands were excised from the gel and bovine serum albumin standards were separated by electrophoresis in parallel with the fusion proteins. After staining with Coomassie Blue, the fusion proteins and bovine serum albumin standards were excised from the gel and eluted as described above for phosphoamino acid analysis, and the amount of protein was determined by measuring the absorbance of the bovine serum albumin at 280 nm. Preparation of a postnuclear supernatant was performed as described previously (13) with minor modifications. Briefly, COS or PC12 cells were detached from the substrate either with trypsin or with a cell scraper following one freeze-thaw cycle. Cells were resuspended in 10 mM HEPES-KOH, pH 7.4, 320 mM sucrose, 5 mM EGTA, 10 mM EDTA, 20 µg/ml phenylmethylsulfonyl fluoride, 25–500 units of purified CKI, 25–500 units of purified CKII, or 1 µl (~10 µg of total protein) of a postnuclear supernatant from COS cells (see below). To assess phosphorylation of the VMATs, we labeled the CHO cells with [35S]Met/Cys and detected phosphorylation under the same conditions (Fig. 1A). In contrast, the ~55-kDa form of VMAT1 showed no detectable phosphorylation under the same conditions (Fig. 1A). In addition to the ~55-kDa forms, metabolic labeling of VMAT2 metabolically labeled with [32P] in transfected COS cells. To reduce nonspecific background, we introduced an epitope tag from HA (29) into the large luminal loop between the first and second transmembrane domains of VMAT2. Expressed in COS cells, HA epitope-tagged VMAT2 (HA-VMAT2) showed protein expression, transport activity, and phosphorylation equivalent to wild-type VMAT2 (Fig. 3A and data not shown). Phosphoamino acid analysis of HA-VMAT2 metabolically labeled with [32P] in COS cells and immunoprecipitated with anti-HA antibody showed phosphorylation on serine, but not threonine or tyro-
that serine 514 also undergoes phosphorylation, albeit to a lesser extent than serine 512. To assess whether changes in expression of the mutant proteins might account for the reduced labeling, we performed both Western analysis (Fig. 4B) and metabolic labeling with \[^{35}S\]Met/Cys (data not shown). These analyses showed that the mutations did not affect expression of VMAT2 and indicate a selective effect of these mutations on protein phosphorylation.

To assess the effect of these mutations on transport activity, we performed in vitro transport assays using extracts from transiently transfected COS cells. The \(K_a\) and \(V_{\text{max}}\) values for wild-type VMAT2 were similar to those for transporters containing alanine replacements at serine 512 or 514 or both serines 512 and 514 (Table I). These results suggest that phos-
phosphorylation of these residues is not required for base-line transport activity and that phosphorylation at these sites does not dramatically alter either the affinity of the transporter for substrate or its base-line rate of transport.

To identify the kinase(s) responsible for phosphorylation of serines 512 and 514, we compared the sequence surrounding these sites with consensus sequences for phosphorylation by previously characterized kinases (35). The proximity of multiple acidic residues suggested the involvement of the acido tropic kinases CKI and/or CKII. The consensus sequence for CKI is (S/P)XX(D/E)(S/T) (in which the phosphorylated site is shown in boldface and S(P)/T represents a phosphoserine specificity determinant), and that for CKII is (S/T)XX(D/E) (36–38). Thus, serine 512 occurs in a consensus sequence for phosphorylation by both CKI and CKII, and serine 514 in a consensus sequence for CKI (see Fig. 4A). To assess the role of CKI and CKII in VMAT2 phosphorylation, we incubated GST fusion proteins (31) containing the carboxyl-terminal 55 amino acids of VMAT2 (GST-VMAT2) with the purified kinases in vitro (see Fig. 2B). Both CKI and CKII (Fig. 5A), but not protein kinase A or C or glycogen synthetase kinase-3 (data not shown), phosphorylated GST-VMAT2. Neither GST alone (Fig. 5A) nor a GST fusion protein containing the amino terminus of VMAT2 (data not shown) was phosphorylated by CKI or CKII, indicating that the phosphorylation of the carboxyl terminus was specific (Fig. 5A and data not shown). These data suggest that CKI and/or CKII may also mediate the phosphorylation of VMAT2 in vivo.

To determine whether purified CKI and CKII phosphory late the same sites in GST-VMAT2 that are phosphorylated in intact cells, we introduced point mutations into GST-VMAT2.

**Table I**

| VMAT2 mutation | $K_m$ (μM) | $V_{max}$ (pmol/min/mg) |
|----------------|------------|-------------------------|
| Wild-type      | 0.35 ± 0.18| 21.0 ± 11.5             |
| SS12A          | 0.44 ± 0.09| 17.8 ± 1.5              |
| SS14A          | 0.29 ± 0.13| 17.8 ± 4.3              |
| SS12A/SS14A    | 0.40 ± 0.04| 21.1 ± 10.3             |

Simultaneous mutation of serines 512 and 514 eliminated phosphorylation by purified CKII and greatly reduced phosphorylation by CKI (Fig. 5B). Mutation of serine 512 alone decreased the phosphorylation of GST-VMAT2 by CKI and, to a lesser extent, by CKII (Fig. 5B). These results suggest that CKI and CKII phosphorylate both serines 512 and 514 and support the relevance of phosphorylation in vitro to events observed in intact cells.

To assess further the relationship between phosphorylation of GST-VMAT2 in vitro and phosphorylation of the transporter in intact cells, we examined the phosphorylation of the fusion protein by COS and PC12 cell extracts. Concomitant replacement of both serines 512 and 514 by alanine or of serine 512 alone decreased phosphorylation of GST-VMAT2 by both extracts (Fig. 5C), similar to the effect of these mutations in intact cells. To determine whether the kinase(s) in the extracts responsible for phosphorylating GST-VMAT2 are CKI and/or CKII, we first used the protein kinase inhibitor CKI-7. CKI-7 inhibits CKI and CKII with $K_i$ values of 9.5 and 90 μM, respectively (39). In contrast, CKI-7 inhibits other kinases such as Ca$^{2+}$- and calmodulin-dependent kinase II and protein kinases A and C with $K_i$ values of 195, 550, and >1000 μM, respectively (39). Using purified CKI and CKII to test the effects of this inhibitor in our in vitro system, 5–10 μM CKI-7 inhibited phosphorylation of GST-VMAT2 by purified CKI, and 50–100 μM CKI-7 inhibited phosphorylation by CKII (Fig. 6). Using the COS cell extract, 10–50 μM CKI-7 inhibited phosphorylation of GST-VMAT2, consistent with a role for CKI and/or CKII in the phosphorylation of VMAT2. Furthermore, at 50 μM, the extract was inhibited at an intermediate level between CKI and CKII, suggesting that both kinases may be involved.

To examine further the involvement of CKII in VMAT2 phosphorylation, we determined the sensitivity of the in vitro phosphorylation reaction to GTP. Unlike CKI and other known kinases, CKII uses GTP as a phosphate donor almost as well as ATP, with $K_m$ values for ATP and GTP of ~10 and ~20 μM, respectively (40). Indeed, GTP potently inhibited the labeling of GST-VMAT2 with [γ-32P]ATP by purified CKII, but not CKI (Fig. 7). Using the COS cell extract, we found that GTP partially inhibited phosphorylation of GST-VMAT2 by [γ-32P]ATP, indicating that CKII contributes to the phosphorylation of VMAT2 (Fig. 7). Similar results were obtained using the PC12 cell extract (data not shown). However, GTP inhibited the extracts less potently than it inhibited purified CKII, suggesting the involvement of one or more additional kinases such as CKI.

Although the presence of consensus sites for both CKI and
CKII in VMAT1 suggests the potential for phosphorylation by these enzymes (see Fig. 4A), the phosphorylation of VMAT2 greatly exceeds that of VMAT1 in intact cells (see Fig. 1). We therefore determined whether purified CKII could phosphorylate a GST-VMAT1 carboxyl-terminal fusion protein similar to the GST-VMAT2 fusion protein described above (see Fig. 4A). Although GST-VMAT1 is a substrate for CKII, it was phosphorylated 10-fold less than comparable amounts of GST-VMAT2 at each substrate concentration tested (Fig. 8). In vitro phosphorylation using purified CKI yielded similar results (data not shown). These observations indicate that CKII and possibly CKI are responsible for the selective constitutive phosphorylation of VMAT2 and not VMAT1.

**DISCUSSION**

To assess the potential for regulation of vesicular monoamine transport, we have studied the phosphorylation state of the transport proteins and determined that the ~55-kDa form of the brain monoamine transporter VMAT2, but not the adrenal gland transporter VMAT1, undergoes constitutive phosphorylation in intact cells. Phosphoamino acid analysis of VMAT2 indicates that phosphorylation occurs on serine residues, and metabolic labeling of VMAT1-VMAT2 chimeras and VMAT2 point mutants maps the phosphorylation sites to serines 512 and 514 at the carboxyl terminus of the protein, with serine 512 phosphorylated to a greater extent than serine 514.

Serines 512 and 514 occur in consensus sequences for phosphorylation by the acidotropic kinases CKI and CKII (35, 37). We have found that both purified CKI and CKII specifically phosphorylate serine 512 and, to a lesser extent, serine 514 of a bacterial fusion protein containing the carboxyl terminus of VMAT2. More important, the VMAT2 fusion protein is a much better substrate for CKI and CKII than an equivalent VMAT1 fusion protein, consistent with the prominent phosphorylation of VMAT2 relative to VMAT1 in intact cells. To assess the...
The 36-kDa bands were excised, and incorporated radioactivity was measured. Increasing concentrations of GST-VMAT2 fusion proteins. The 36-kDa bands were excised, and incorporated radioactivity was measured. The amount of 32Pi incorporated into GST-VMAT2 was 10-fold greater than that for GST-VMAT1 at all comparable protein concentrations. However, we cannot exclude this possibility entirely since we do not know the proportion of wild-type VMAT2 constitutively phosphorylated in vivo. If only a small fraction of total steady-state VMAT2 is phosphorylated, mutation of the acceptor sites might not show a difference in activity even if phosphorylation significantly affected transport function.

In addition to regulating intrinsic protein activity, phosphorylation by CKII may influence subcellular localization. Phosphorylation of both cation-dependent and -independent membrane 6-phosphate receptors by CKII may activate binding to the clathrin adaptor protein AP-1 and thereby influence membrane trafficking (54–56), although these results remain controversial (57, 58). Similarly, mutation of a CKII phosphorylation site appears to alter the subcellular localization of the endoprotease furin (59). These results raise the possibility that phosphorylation of VMATs and PKA. Indeed, differential phosphorylation of VMAT1 and VMAT2 may contribute to differences in the localization of the two transporters that we have observed in PC12 cells.2

In summary, we have found that VMAT2, but not VMAT1, undergoes constitutive phosphorylation by casein kinase II and possibly casein kinase I on two carboxyl-terminal serine residues, providing the first direct evidence for phosphorylation of a vesicular neurotransmitter transporter or any monoamine transporter. The identification of this phosphorylation event will now enable us to assess its role in the membrane trafficking and regulation of VMAT2 function, with the attendant implications for modulating the release of monoamine transmitters.

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FIG. 8. Differential phosphorylation of VMAT1 and VMAT2 fusion proteins. Increasing concentrations of GST-VMAT2 fusion protein (lanes 1–4) and GST-VMAT1 fusion protein (lanes 5–8) were phosphorylated in vitro using CKII. Samples were separated by electrophoresis, stained with Coomassie Blue to determine total protein levels (lower panel), and submitted to autoradiography (upper panel). The 36-kDa bands were excised, and incorporated radioactivity was determined by Cerenkov scintillation counting. The quantity of fusion protein (µg) present in each 36-kDa band is listed above the lanes. The amount of 32P incorporated into GST-VMAT2 was 10-fold greater than that for GST-VMAT1 at all comparable protein concentrations.

| µg | 0.7 | 1.8 | 2.6 | 3.9 | 0.8 | 1.9 | 2.7 | 3.8 |
|---|---|---|---|---|---|---|---|---|
| Lane 1 | △ | △ | △ | △ | △ | △ | △ | △ |
| Lane 2 | △ | △ | △ | △ | △ | △ | △ | △ |
| Lane 3 | △ | △ | △ | △ | △ | △ | △ | △ |
| Lane 4 | △ | △ | △ | △ | △ | △ | △ | △ |
| Lane 5 | △ | △ | △ | △ | △ | △ | △ | △ |
| Lane 6 | △ | △ | △ | △ | △ | △ | △ | △ |
| Lane 7 | △ | △ | △ | △ | △ | △ | △ | △ |
| Lane 8 | △ | △ | △ | △ | △ | △ | △ | △ |
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