Phosphorylation of the rat vesicular acetylcholine transporter.

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Running Title: Phosphorylation of rat vesicular acetylcholine transporter.

Abbreviations used: VACHT-vesicular acetylcholine transporter; ACh - acetylcholine; ChAT- choline acetyltransferase; VMAT- vesicular monoamine transporter, PBS- phosphate buffered saline; PKA- protein kinase A; TGN- trans golgi network.
ABSTRACT

Metabolic labeling of a mutant PC12 cell line, A123.7, expressing recombinant rat VACHT with radiolabeled inorganic phosphate was used to demonstrate phosphorylation of the transporter on a serine residue. Mutational analysis was used to demonstrate that serine 480, which is located on the C-terminal cytoplasmic tail, is the sole phosphorylation site. Phosphorylation of serine 480 was attributable to the action of protein kinase C. Using a permanently dephosphorylated form of rat VACHT, S480A rVACHT, it was shown that this mutant displays the same kinetics for the transport of acetylcholine and the binding of the inhibitor vesamicol as does the wild type transporter. However, sucrose gradient density centrifugation showed that, unlike wild type VACHT, the S480A mutant did not localize to synaptic vesicles. These results suggest that phosphorylation of serine 480 of VACHT is involved in the trafficking of this transporter.
Acetylcholine is synthesized in the cytoplasm of cholinergic neurons through the action of the enzyme choline acetyltransferase, and is then transported into small synaptic vesicles by the vesicular acetylcholine transporter (VACHT) (1). Acetylcholine (ACh) transport involves the exchange of two luminal protons for each molecule of cytoplasmic transmitter transported into the vesicle (2-4). The driving force for transport is derived from a proton electrochemical gradient across the vesicular membrane generated by a vacuolar type H\(^+\)-ATPase located on the synaptic vesicle (5, 6). The filled vesicles release their contents through exocytosis, producing an action potential at the nerve terminal (7). As a consequence of this process VACHT is transported with the synaptic vesicle to the plasma membrane where it fuses with the plasma membrane. Synaptic vesicles and their component proteins, including VACHT, are recycled from the plasma membrane back to the synaptic vesicle by endocytosis via an endosomal compartment (8). This cycle is critical for neurotransmission, and it appears that newly synthesized synaptic vesicle proteins are transported to the plasma membrane even before they reach the synaptic vesicle (9). Evidence has been obtained that the cytoplasmic tail of VACHT contains the signal(s) which traffic it to the synaptic vesicle (10).

VACHT cDNAs have recently been cloned from a number of sources including the nematode Caenorhabditis elegans (11), the electric marine ray Torpedo (12), rat brain stem, and rat neuroendocrine PC12 cells, and the human neuroblastoma SK-N-SH cell line (13, 14). The amino acid sequence of VACHT has revealed that it belongs to a family of transporters which includes the monoamine transporters VMAT1 and VMAT2. This family of
transporters can be characterized as containing 12 transmembrane domains, an N-linked glycosylated loop located between transmembrane domains 1 and 2, and cytoplasmic N and C terminal domains (13). The twelve transmembrane domains are highly conserved and maintain native conformation of the transporters (4). In contrast, the cytoplasmic amino and carboxyl terminal domains are rather divergent (4).

It has recently been shown that VMAT2 can be phosphorylated by casein kinase I, and possibly casein kinase II, on two serine residues, serine 512 and serine 514, located on the C-terminal cytoplasmic tail (15). These serine residues are not conserved in VACHT, and thus the question of whether or not VACHT is also phosphorylated on the C-terminal cytoplasmic tail or elsewhere has been addressed in this study. We utilized a mutant PC12 cell line, PC12A123.7, which has reduced levels of protein kinase A type I and type II activity (16), and as a consequence VACHT gene transcription is reduced (17) such that VACHT protein is undetectable. On the other hand, PC12A123.7 contains synaptic vesicles which can be reconstituted with recombinant VACHT to produce a transport competent vesicle (18). In this study this system has been used to demonstrate that rVACHT, like VMAT2, is phosphorylated on its C-terminal cytoplasmic domain. However, VACHT phosphorylation occurs at a distinct site and appears to be catalyzed by protein kinase C, rather than casein kinase II as is the case for VMAT2. Studies of a VACHT species in which the phosphorylation site is removed by site-directed mutagenesis suggests that phosphorylation of rVACHT may play a role in trafficking of the transporter.
Materials and Methods

Cell Lines and Cell Culture: We used for these studies a parental PC12 cell line, a protein kinase A (PKA) deficient variant of the parental PC12 cell referred to as $\text{PC12}^{A123.7}$ (16), and a derivative of $\text{PC12}^{A123.7}$ stably transfected with a rat VAChT cDNA, $\text{PC12}^{A123.7/VAChT}$ (18). PC12 cells were maintained at 37°C in 10% CO$_2$ in Dulbecco's modified Eagle's medium containing 5% horse serum, 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The PKA deficient PC12 cell line $\text{PC12}^{A123.7}$ was maintained in F-12 medium containing 5% horse serum, 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. $\text{PC12}^{A123.7/VAChT}$ was cultured in the same media with 100 µg/ml of G418 added.

Transient transfections: For transient transfection of $\text{PC12}^{A123.7}$, cells were suspended at a density of ~6x10$^7$ cells/ml in cold phosphate buffered saline. To the cell suspension was added 100 µg of plasmid DNA and following a 10 min incubation on ice, cells were electroporated in a 0.4 cm gap cuvette (Bio-Rad) at 0.2 kV, 950 microfarads using a Bio-Rad Gene Pulser apparatus. The cells were then transferred to DMEM/F12 medium and cultured. Cells were harvested 40 to 60 hrs after transfection. VAChT expression was determined by Western blot analysis.

For transient transfection of CHO cells, 2 µg of plasmid DNA was incubated with 8 µl of LipofectAMINE (GIBCO BRL) in 200 µl of Opti-MEM media (GIBCO BRL) for 15 min at room temperature. 800 µl of Opti-MEM media was then added and the lipid-DNA complex was transferred to 1x10$^5$ cells grown on a coated coverslip in a six well plate. The cells were incubated with the LipofectAMINE/plasmid mixture for 4 hours. After washing the
cells with 3 ml of PBS, 3 ml of media was added and the cells were incubated for an additional 24 hours. Following this time period cells were used for immunofluorescence studies.

**Mutagenesis:** Site-directed mutagenesis was performed on subcloned regions of the VAChT cDNA using either the “Quick Site” mutagenesis kit from Stratagen employing Pfu DNA polymerase or according to the method of Kunkel (19) using the Escherichia coli strain BD23399 to prepare single-stranded uracil containing DNA. The mutagenized DNA fragments were subcloned into the expression vector pcDNA3 (Invitrogen) containing the wild-type rVAChT cDNA using EcoRI and XbaI restriction sites. The dideoxy sequencing method (20) was used to verify mutations.

**Metabolic Labeling:** PC12<sup>A123.7</sup> cells, PC12<sup>A123.7</sup> cells transiently expressing rVAChT mutants, or PC12<sup>A123.7/VAChT</sup> cells were cultured in phosphate-free medium (ICN) for 1 hour. After washing with TBS (150 mM NaCl in 100 mM Tris-HCl, pH7.5), cells were metabolically labeled with 0.5 mCi/ml <sup>32</sup>P-inorganic phosphate (H<sub>3</sub>PO<sub>4</sub>) (ICN) in 4 ml of phosphate free medium. After labeling for 2 hours, cells were rinsed with TBS, detached by scraping with a rubber policeman into lysis buffer (1% nonidet P-40, 0.15 M NaCl, 10 mM sodium phosphate pH 7.2, 2 mM EDTA) containing 50 mM sodium fluoride, 0.2 mM sodium vanadate and a protein inhibitor cocktail (Boehringer Mannheim). Sodium vanadate and protein inhibitor cocktail were added fresh before use. After incubation for 30 min at 4°C, the cell debris and nuclei were removed by centrifugation at 12,000xg for 30 min at 4°C. Radiolabeled VAChT was isolated by immunoprecipitation and analyzed by SDS-PAGE.
**Immunoprecipitation & Western blot analysis:** For immunoprecipitation of VACHT the supernatant of a cell lysate was first precleared by incubation for 1 h at 4°C with protein-G Sepharose (Sigma) containing 25 µl of rabbit serum. Following centrifugation, the cleared cell lysate was incubated for 1 h at 4°C with a rabbit anti-rVACHT anti-sera (21) at a dilution of 1:500, and precipitated by incubation for 1 h at 4°C with 30 µl of prewashed protein G-Sepharose. The precipitate was washed two times in lysis buffer and one time in 10 mM Tris-HCl (pH 7.5) containing 0.1% nonidet P-40. The washed precipitate was resuspended in 30 µl of SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol, 100 mM dithiothreitol), and subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Following electrophoresis the gel was dried and exposed to a phosphorImager screen (Kodak).

For Western blot analysis, the dried gels were rehydrated in 50 mM ammonium bicarbonate for 30 min and the proteins were electrophoretically transferred onto a PVDF membrane (Millipore). The membrane was blocked for 1 hour in TBS-T (20 mM Tris-HCl (pH 7.6), 135 mM NaCl, 0.1% Tween 20) containing 10% non-fat dry milk, washed three times with TBS-T, and incubated for 1 h at room temperature with a goat anti-VACHT polyclonal antibody (Chemicon Intern.) at a dilution of 1:500 in TBS-T containing 5% nonfat dry milk. Following removal of the primary antibody and washing, a peroxidase conjugated swine anti-goat IgG (Sigma) at a dilution of 1:4,000 in 5% nonfat dry milk was added. Following incubation for 1 h at room temperature and washing, immune complexes were visualized by ECL (Amersham Pharmacia Biotech) on x-ray film.
Phosphoamino Acid Analysis: Phosphoamino acid analysis was performed as described (22) with minor modification. Briefly, extracts prepared from PC12A123.7/VaChT cells metabolically labeled with 32P-inorganic phosphate were immunoprecipitated with rabbit anti-rVaChT antisera as described above. Immunoprecipitated VaChT was separated by electrophoresis on a 10% SDS-polyacrylamide gel. The radiolabeled VaChT band was excised from the gel and rehydrated in 50 mM ammonium bicarbonate, and eluted overnight in 0.2% SDS, 2%-mercaptoethanol. The eluate was then precipitated with 20% trichloroacetic acid and hydrolyzed in 6 M HCl for 2 hours at 110°C. The hydrolysates were evaporated on a speed-vac, resuspended in a mixture of phosphoserine, phosphothreonine, and phosphotyrosine at a final concentration of 0.3 µg/ml each, and spotted onto a thin-layer cellulose plate (Merck). Electrophoresis was performed for 20 min at 1.5 kV using pH 1.9 buffer (0.58 M formic acid, 1.36 M glacial acetic acid) for the first dimension and for 16 min at 1.3 kV using pH 3.5 buffer (0.87 M glacial acetic acid, 0.5% pyridine) for the second dimension. After drying the plates, standards were stained with ninhydrin and the plates were exposed to a phosphorImager screen.

Isolation of Postnuclear supernatants and synaptic vesicles: Postnuclear supernatants were isolated by differential centrifugation as previously described (18). Briefly, cell pellets were harvested by centrifugation and suspended in 10 mM HEPES-KOH buffer, pH 7.4, 0.32 M sucrose, 5 mM EGTA (pH8.0), 5 mM MgCl2, 4% of protease inhibitor cocktail (Boehringer Mannheim), and 0.2 mM phenylmethylsulfonyl fluoride (Sigma). Cells were disrupted at 4°C with a Potter-Elvejham homogenizer, and the
postnuclear supernatant collected by centrifugation at 800xg for 10 min. Protein concentration was measured with the Coomassie Plus protein assay reagent based on the Bradford method (Pierce).

Synaptic vesicles were isolated from postnuclear supernatants by centrifugation for six hours at 30,000 rpm in an SW40 rotor in a Beckman LE-80 centrifuge at 4°C through a 0.6-1.6 M linear sucrose gradient (18).

**In Vitro phosphorylation:** The postnuclear supernatant from PC12A123.7/VACHT or control PC12 cells was centrifuged at 100,000xg for 1 hour at 4°C. The supernatant was carefully removed without disturbing the pellet. The pellet was then washed three times with kinase buffer (20 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 50 mM KCl, 5 mM DTT) and resuspended into kinase buffer. The resuspended pellet (10 µg) was preincubated for 20 min at 30°C with either 7 nM protein kinase A inhibitor (H-Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-OH), 45 µM protein kinase C inhibitor (H-Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn-OH), or 18 µM casein kinase II inhibitor (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole). To the reaction mixture was added 400 µM [γ-³²P]-ATP (specific radioactivity = 500 µCi/µmol), and incubation was continued for 20 min at 30°C. Radiolabeled VACHT was immunoprecipitated as described above, subjected to SDS-PAGE, followed by electrophoretic transfer to PVDF paper, and autoradiography. Protein kinase inhibitors were obtained from CalBiochem.

**In vitro protein kinase assays:** Protein kinase C and calcium kinase II activity was measured with 10 µg of postnuclear supernatant protein from PC12A123.7 cells. Assays involved measurement of the
incorporation of $^{32}$P-from 0.1 mM [$\gamma^{32}$P] ATP (specific radioactivity = 200 cpm/pmol) in reaction mixtures containing kinase buffer and 20 $\mu$M peptide substrate. For measurement of protein kinase C activity the specific peptide substrate H-Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys-OH was used while for measurement of casein kinase II activity the specific peptide substrate H-Arg-Arg-Arg-Ala-Asp-Asp-Ser-Asp-Asp-Asp-Asp-OH was used. Reactions were terminated by addition of 50 $\mu$l of 7.5 mM phosphoric acid, and an aliquot was spotted onto a P81 filter (Whatman) and washed 5 times in 75 mM phosphoric acid with vigour shaking. $^{32}$P-incorporation was determined by scintillation counting.

**ACh Transport Assay:** ACh transport into synaptic vesicles was determined by a modification of the procedure of Varoqui and Erickson (23) as described in Kim et al (18). Briefly, ACh transport was measured with 500 $\mu$g of a postnuclear supernatant containing synaptic vesicles using a tartrate-HEPES-ascorbate buffer (110 mM potassium tartrate, 20 mM HEPES (pH 7.4), and 1 mM ascorbic acid), 5 mM Mg$^{2+}$-ATP, and [$^3$H]ACh (40-50 mCi/mmol, DuPont NEN) at the desired concentration. Reactions (200 $\mu$l) were allowed to proceed at 37°C for the desired time period. The reaction was terminated by collecting and washing the synaptic vesicles on a pre-wetted HAWP membrane (Millipore) using a vacuum manifold. Vesicular ACh was determined by scintillation counting. To determine non specific binding assays were conducted in the absence of ATP and in the presence of 4 $\mu$M vesamicol at 0°C. No ACh transport was observed with the postnuclear supernatant from PC12$^{A123.7}$ cells.
**Vesamicol binding assays:** Postnuclear supernatants containing 200 µg of protein were prepared in the tartrate-HEPES-ascorbate buffer and mixed with 20 nmol of [³H]vesamicol (31 Ci/mmol, DuPont NEN). Following incubation for 10 min at 37°C, the synaptic vesicles were collected as described above and bound vesamicol was determined by scintillation counting.

**Indirect Immunofluorescence:** PC12⁴³⁷/VACHT cells, pretreated with 50 ng/ml of NGF (Boehringer Mannheim) for 4 days, were grown on 5 µg/ml laminin (Sigma)-coated glass coverslips. After fixing the cells for 20 min at room temperature in a solution containing 3% formaldehyde, 490 µM MgCl₂, 410 µM MgSO₄, and 1.26 mM CaCl₂ in PBS, they were washed three times with 2 ml of PBS. Free aldehyde was quenched with 2 ml of 50 mM NH₄Cl in PBS for 10 min at room temperature and washed with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 4 min at room temperature and washed with a blocking buffer (5% calf serum, 5% horse serum in PBS) for 20 min at room temperature. Staining was performed using a goat anti-VACHT polyclonal antiserum diluted 1:800 or mouse anti-synaptophysin monoclonal antibody (Sigma) diluted 1:200 in blocking buffer. Primary antibodies were incubated for 20 min at room temperature. After three washes in 2 ml of blocking buffer, cells were incubated with Fluorescein conjugated rabbit anti-goat IgG antibodies (Vector Lab. Inc.) diluted 1:250 and Texas Red conjugated horse anti-mouse IgG antibodies (Vector Lab. Inc.) diluted 1:200 in PBS for 20 min at room temperature. Cells were then washed three times in blocking buffer, rinsed three times in PBS, and the
coverslips were mounted with 6 μl vectashield (Vector Lab. Inc.) and applied for fluorescence microscopy.
RESULTS

To investigate whether the vesicular acetylcholine transporter (rVAChT) can be phosphorylated, we utilized a PC12 cell line, PC12A123.7 which expresses a mutant regulatory subunit of protein kinase A (PKA) and thus exhibits low levels of PKA I and PKA II enzymatic activity (16). PC12A123.7 has been shown to express low levels of VACHT and ChAT mRNA and VACHT and ChAT protein (17, 24). This PC12A123.7 cell line produces small synaptic vesicles containing synaptophysin (P38), and as previously described, these vesicles can be reconstituted with recombinant VACHT (18).

We established a stably transfected PC12A123.7 cell line expressing recombinant rat VACHT, PC12A123.7/rVACHT (M.-H. Kim et al, manuscript in preparation). As shown in figure 1, VACHT expression in PC12A123.7/rVACHT exhibits a punctate staining pattern which is predominantly co-localized with the synaptic vesicle marker α-synaptophysin. Synaptic vesicles isolated from PC12A123.7 expressing recombinant rVACHT transport ACh and bind the specific VACHT inhibitor vesamicol (18). These findings provide evidence that recombinant rVACHT is expressed on synaptic vesicles in the PC12A123.7 cell line. Similar results have been obtained with wild type PC12 cells transfected with recombinant VACHT (4, 10).

To determine whether rVACHT is phosphorylated in neuroendocrine cells, we metabolically labeled PC12A123.7/rVACHT with 32P-inorganic phosphate. Following metabolic labeling, cells were disrupted and VACHT immunoprecipitated with a polyclonal rabbit anti-VACHT antisera. The immunoprecipitate was subjected to SDS-PAGE, dried, and analyzed by radioautography. As shown in figure 2A, lane 2, a 70 kDa phospho-protein
was observed. Applying the same protocol to the parental PC12$^{A123.7}$ cell line which does not express detectable VACHT, no phosphorylated protein at 75 kDa was observed, figure 2A, lane 1. The gel was rehydrated, proteins were then electrophoretically transferred to PDVF paper, and submitted to Western blot analysis. As shown in figure 2B, lane 2, the phosphorylated protein at 70 kDa reacted with a goat anti-VACHT antiserum different from the one used for immunoprecipitation. No band was detected with the parental cell line, figure 2B, lane 1. Although not shown phosphorylation of recombinant VACHT was also observed with wild type PC12 cells expressing recombinant VACHT, and as noted below cytosolic extracts from wild type VACHT can phosphorylate recombinant VACHT on vesicles derived from PC12$^{A123.7/rVACHT}$.

Phosphoamino acid analysis of the phosphorylated VACHT was performed to identify the nature of the phosphorylated residue. PC12$^{A123.7/rVACHT}$ was metabolically labeled with $^{32}$P-inorganic phosphate and VACHT immunoprecipitated as described above. The immunoprecipitated VACHT was purified by electrophoresis through a polyacrylamide gel, localized by autoradiography, excised from the gel, eluted, and hydrolyzed in 6 M HCl at 110°C. Phosphoamino acid analysis of the hydrolysate by two dimensional electrophoresis on cellulose thin layer chromatography plates showed phosphorylation of serine, but not threonine or tyrosine, figure 3. Thus, the phosphorylation of rVACHT in intact cells occurs on one or more serine residues.

The cDNA sequence of rVACHT predicts a protein of 530 amino acids with 12 transmembrane domains (13), related in structure to the vesicular
monoamine transporters VMAT1 (25) and VMAT2 (26). Phosphorylation of VMAT2 has been observed on its C-terminal cytoplasmic tail (15), thus this domain represents a potential phosphorylation site of VACChT. The C-terminal cytoplasmic tail of rat VACChT contains five serine residues, figure 4. Of these, serine 478 and serine 480 represent consensus casein kinase II sites, while serine 480 is also a consensus protein kinase C site (27). These potential phosphorylation sites are highly conserved in all mammalian VACChTs obtained so far, figure 4. In order to determine whether any of the five serines on the C-terminal cytoplasmic tail represent a VACChT phosphorylation site, we used site-directed mutagenesis to replace each with an alanine residue. The rVACChT cDNAs containing these point mutations were expressed in PC12A123.7 cells by transient transfection and metabolically labeled with $^{32}$P-inorganic phosphate. VACChT was immunoprecipitated, subjected to SDS-PAGE, and the $^{32}$P content determined by radioautography. As shown in figure 5, replacement of serine 478 with alanine (lane 2) had no effect on VACChT phosphorylation. However, replacement of serine 480 with alanine (lane 3), or the double mutant in which both serine 478 and serine 480 were replaced with alanine (lane 4), completely eliminated VACChT phosphorylation. To prove that the lack of VACChT phosphorylation with S480A and the S478/S480A double mutant was not due to a reduced expression of the mutant proteins, we performed Western blot analysis on the immunoprecipitated proteins. This analysis showed that each mutant VACChT was expressed to the same level as the wild type protein, figure 5. Although not shown, the ability to phosphorylate VACChT mutants S512A, S528A, and S530A was unaffected by conversion of the appropriate serine to
alanine. These results indicate that only the serine at position 480 of VACHT undergoes phosphorylation within the intact PC12 cell.

To assess which kinase is involved in rVACHT phosphorylation, we measured phosphorylation of the transporter in a post nuclear supernatant fraction of PC12A123.7/rVACHT cells in which VACHT is present on synaptic vesicles. The cytosolic fraction from PC12A123.7 cells was used as a source of kinase activity. We tested the effect on VACHT phosphorylation of the protein kinase A inhibitor peptide (H-Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-OH) at 7 nM (Ki = 2.3 nM) (28), the protein kinase C inhibitor peptide (H-Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn-OH) at 45 µM (Ki = 15 µM) (29), and the casein kinase II inhibitor 5,6-dicholoro-1-B-D-ribofuranosylbenzimidazole (DRB) at 18 µM (Ki = 6 µM) (30, 31). Using the cytosol from PC12A123.7 cells, complete inhibition was afforded by the protein kinase C inhibitor, figure 6, lane 3; however partial inhibition by the casein kinase II inhibitor was also observed, figure 6, lane 4. The cytosolic fraction of wild type PC12 cells was also able to phosphorylate VACHT. The wild type PC12 extract contains higher levels of protein kinase A activity than the PC12A123.7 protein kinase A deficient cell line. However, as noted with the cytosol from PC12A123.7 cells, the protein kinase A inhibitor H-Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-OH was ineffective in preventing VACHT phosphorylation.

The partial inhibition observed by the casein kinase II inhibitor was not in keeping with complete inhibition by the protein kinase C inhibitor. If both casein kinase II and protein kinase C phosphorylated VACHT, both of
their inhibitors should have produced partial inhibition. In order to clarify this point we tested the effect of the casein kinase II inhibitor DRB on protein kinase C activity using a specific peptide substrate for protein kinase C (H-Pro-Leu-Ser-Arg-Thr-Leu-Ser-Bal-Ala-Ala-Lys-Lys-OH) (32). We found that the casein kinase II inhibitor afforded partial inhibition (35%) in this assay.

We also tested the effect of the protein kinase C inhibitor on casein kinase II activity using the specific casein kinase II peptide substrate H-Arg-Arg-Arg-Ala-Asp-Asp-Ser-Asp-Asp-Asp-Asp-Asp-Asp-OH (33). In this case there was no effect of the protein kinase C inhibitor on casein kinase II activity. This finding suggests that serine 480 is phosphorylated exclusively by protein kinase C.

We examined a number of potential functions of VAChT which might be affected by its phosphorylation. We first determined if phosphorylation might affect the kinetics of ACh transport by comparing the Km and Vmax for ACh transport between the wild type and S480A mutant. As shown in Table 1, wild type VAChT transported ACh at a maximal rate of 126 pmols/min/mg protein with a Km for transport of 2.3 mM. The S480A mutant transported ACh at a maximal rate of 143 pmols/min/mg protein with a Km for transport of 2.7 mM. We do not consider these differences significant. The wild type transporter bound 1.9 pmol of vesamicol per mg protein, while the S480A mutant bound 2.0 pmol of vesamicol per mg protein.

We compared the targeting of the S480A mutant to synaptic vesicles to that of wild type VAChT. As shown in figure 7A and B, during sucrose density gradient centrifugation wild type VAChT co-migrates with synaptophysin (P38) used as a marker for synaptic vesicles. Secretogranin II,
which was used as a marker for dense core vesicles, is clearly separated from synaptophysin. In contrast to the results obtained with wild type VACHT, the S480A mutant shows a broad distribution during sucrose density gradient centrifugation. The S480A VACHT mutant does not co-sediment with synaptophysin, but is found at a density intermediate between the synaptic vesicles and dense core vesicles, figure 7B. Although not shown, an S480D mutant behaved like wild type VACHT.
**Discussion**

We have shown that rat VACHT can be phosphorylated in intact PC12 cells. We as well as others (34) have also found VACHT phosphorylation in wild type PC12 cells. Phosphoamino acid analysis of VACHT metabolically labeled with $^{32}$P-inorganic phosphate shows that this phosphorylation occurs exclusively on a serine residue. Mutational analysis localized the site of phosphorylation to serine 480 of the cytoplasmic C-terminal tail. The absence of VACHT phosphorylation of an S480A VACHT mutant indicates that this is the only site at which VACHT is phosphorylated in PC12 cells. Serine 480 is conserved among all mammalian VACHT species suggesting that this is a common phosphorylation site of VACHT. Although serine 480 lies within a consensus sequence for phosphorylation by both protein kinase C and casein kinase II, phosphorylation was limited to the action of protein kinase C. This is in contrast to phosphorylation of VMAT2 which occurs at either of two serine residues, serine 512 and serine 514, and is catalyzed by casein kinase II and possibly casein kinase I (15). Interestingly, phosphorylation of VMAT1 was not observed under conditions in which VMAT2 was phosphorylated.

Studies utilizing the S480A VACHT mutant showed that phosphorylation of serine 480 is not required for acetylcholine transport activity or for vesamicol binding. On the other hand we found that the S480A VACHT mutant, which cannot be phosphorylated, did not co-sediment with synaptic vesicles nor did it co-sediment with dense core vesicles. Thus serine 480 appears to play a role in vesicle trafficking.
Krantz and Edwards, citing unpublished results in a paper by Tan et al. (34), noted that they had observed phosphorylation of serine 480 of VAChT. They predicted that phosphorylation of serine 480, in conjunction with the dileucine pair found at residues 485 and 486 of VAChT, might influence VAChT trafficking. Our experiments support their prediction, and provide evidence that phosphorylation of serine 480 plays a role in the targeting of VAChT to the synaptic vesicle.

Phosphorylation/dephosphorylation has previously been shown to play a role in clathrin dependent endocytosis of synaptic vesicles (35-37). Slepnev et al. (38) provided evidence that phosphorylation regulates the clathrin-dependent endocytic system and proposed that dephosphorylation may prime the nerve terminal for endocytosis following exocytosis. Chen et al. (39) showed that phosphorylation inhibited the interaction of Eps15 and Epsin, two proteins involved in the endocytosis of synaptic vesicles, with the clathrin adapter AP-2 subunit of α-adaptin.

It has been established that leucine based sorting motifs (L-motifs), usually composed of two successive leucines, are involved in internalization of receptors from the plasma membrane as well as the sorting of receptors from the TGN to endosomes/lysosomes (40-43). It has also been found that an acidic amino acid four or five residues amino terminal to the L-based sorting motif can be important for sorting (44, 45). For the T-cell receptor subunit CD3γ it was shown that internalization was dependent on phosphorylation of a serine five residues amino terminal to this motif (44). Based on these studies it was proposed that there are three types of L-based sorting motifs; one that is directly accessible to adaptor proteins, one that is part of a multi-
subunit receptor accessible to adaptor proteins in the unassembled receptor, and inaccessible in the ligand free receptor, and an L-motif that becomes accessible to adaptor proteins upon phosphorylation of an adjacent serine residue (44). The C-terminal cytoplasmic tail of VAChT contains the sequence SDxxxLL, in which the serine residue is the site of phosphorylation. This is the sequence which is found in the latter category of L-based sorting motifs and suggests that phosphorylation of VAChT might produce structural changes in the transporter which permit interaction with adapter proteins.

Both VAChT and VMAT2 have a glutamate four residues amino terminal to their L-based sorting motif. In VMAT2 there is an additional glutamate five residues amino terminal to the L-based sorting motif, while in VAChT this residue is the phosphorylated serine 480. Thus VMAT2 has a motif of two constitutive negative charges upstream of the dileucine motif, while VAChT contain only one permanent negative charge at this position. In addition phosphoserine more often resembles an aspartate-like residue than a glutamate residue. The consequences of these differences may be two-fold. On the one hand phosphorylation of VAChT on a residue involved in sorting may be regulated through a kinase/phosphatase cycle. Furthermore, since VMAT2 is sorted to large dense core vesicles while VAChT is sorted to small synaptic vesicles, the C-terminal cytoplasmic serine-phosphate of VAChT and the corresponding C-terminal cytoplasmic glutamate of VMAT2 may serve as a part of a differential sorting signal. Le Borgne et al. (45) showed that serine phosphorylation on the cytoplasmic domain of the cation independent mannose 6-phosphate receptor is an essential feature for sorting this receptor to the TGN. Dephosphorylation of phosphorylated serine 480 of
VAChT in the TGN could be a part of a sorting signal for trafficking to the small synaptic vesicle, while retention of the negative charge as a glutamate in VMAT2 could be the signal to traffic to the large dense core vesicle. In support of this suggestion is the finding made in this study that the S480A mutant of VAChT, which cannot be phosphorylated, is mis-sorted in the mutant PC12 cell line. Whether this is a unique phenomena in PC12 cells or can be generalized to cholinergic neurons must await further experimentation.
Acknowledgements

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V. References


Table 1. ACh uptake and vesamicol binding assays with wild type and S480A rVACht.

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<th>VACht</th>
<th>ACh uptake</th>
<th>Vesamicol binding</th>
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<tr>
<td></td>
<td>Km (mM)</td>
<td>Vmax (pmol/min/mg)</td>
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<tr>
<td>Wild Type</td>
<td>2.3 ± 0.2</td>
<td>126 ± 14</td>
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<tr>
<td>S480A</td>
<td>2.7 ± 0.3</td>
<td>143 ± 9</td>
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Postnuclear supernatants of wild type or mutant VACht (S480A) expressed in PC12\(^{123,7}\) cells by transient transfection were assayed for ACh transporter activity and vesamicol binding as described in Methods. The level of expression of the transporter was assessed by Western blot analysis of the postnuclear supernatant and shown to be constant among individual transfections. Kinetic constants represent the average and standard error of three separate determinations.
Figure Legends

Figure 1. Stable expression and co-localization of rat VACHT with synaptophysin (p38) in PC12\textsuperscript{A123.7} cells.
PC12 A123.7 cells stably expressing rat VACHT were treated with 50 ng/ml NGF for 4 days and fixed with 3\% formaldehyde. Fixed cells were incubated with a mixture of goat anti-VACHT polyclonal and mouse anti-synaptophysin monoclonal antibodies. After removal of the unbound antibody, cells were treated with a fluorescein conjugated rabbit anti-goat IgG antibody and a Texas red conjugated horse anti-mouse IgG antibody. Shown in (A) is fluorescein fluorescence associated with rVACHT, while in (B) Texas red fluorescence associated with synaptophysin is shown. Panel (C) shows an overlay of panels A and B where the yellow color fluorescence represents coincidence of VACHT and synaptophysin.

Figure 2. Rat VACHT is phosphorylated in PC12 cells.
A. \textsuperscript{32}P labeling of rVACHT. The PC12\textsuperscript{A123.7} cell line (lane 1) and a stable transformant expressing rat VACHT, PC12\textsuperscript{A123.7/rVACHT} (lane 2), were metabolically labeled with \textsuperscript{32}P-Pi and immunoprecipitated with anti-rVACHT antisera.
B. Western blot of metabolically labeled VACHT. PC12\textsuperscript{A123.7} (lane 1) and PC12\textsuperscript{A123.7/rVACHT} (lane 2) cells were immunoprecipitated with anti-rVACHT antisera and subjected to SDS-PAGE followed by Western blot analysis.
Figure 3. Phosphoamino acid analysis shows VACHT is phosphorylated on a serine residue.

Two dimensional thin layer chromatography of a $^{32}$P-labeled amino acid hydrolysate of VACHT metabolically labeled in PC12$^{A123.7}$/VACHT cells. Phosphorylated VACHT was isolated by immunoprecipitation and SDS-PAGE and excised from gel and hydrolyzed in 6 M HCl. Chromatography was conducted as described in Methods.

Figure 4. Sequence of the C-terminal cytoplasmic domain of VACHT from various species.

Alignment of amino acid sequences of the VACHT C-terminal domain of Rattus novegicus (rVACHT; GenBank accession # U09838) compared to Homo sapiens (hVACHT; GenBank accession # U10554), Mus musculus (mVACHT; GenBank accession # AF019045), and Torpedo ocellata (tVACHT; GenBank accession # U05399). Amino acids conserved through all mammalian VACHTs are indicated by a dash type and the consensus sequence for phosphorylation by casein kinase II or protein kinase C (+) are indicated under the amino acid sequence of rVACHT.
Figure 5. VACHT is phosphorylated on serine 480.

PC12<sup>A123,7</sup> cells transiently transfected with a cDNA encoding the wild type (wild) VACHT protein (lane 1), or with the cDNA of mutants S478A (lane 2), S480A (lane 3), S478A/S480A (lane 4), or with no plasmid (lane 5). Transformants were metabolically labeled with 32P-Pi, immunoprecipitated with anti-rVACHT antisera, and subjected to SDS-PAGE and autoradiography (upper panel). The gel was rehydrated and used for Western blot analysis (lower panel).

Figure 6. rVACHT phosphorylation is blocked by a protein kinase C inhibitor.

Postnuclear supernatants from rVACHT stably expressed in PC12 A123.7 cells was used as a substrate for kinases from PC12<sup>A123,7</sup> cells as described in Methods. Lane 1 - no inhibitor; lane 2 - protein kinase A inhibitor; lane 3 - protein kinase C inhibitor; lane 4 - casein kinase II inhibitor.

Figure 7. Equilibrium sucrose density gradient fractionation of synaptic vesicles with wild type or mutant (S480A) VACHT transfected PC12<sup>A123,7</sup>.

A. Postnuclear supernatants of wild type (top) or mutant VACHT (S480A) (bottom) expressed in PC12<sup>A123,7</sup> by transient transfection were separated through a 0.6-1.6 M continuous sucrose gradient by ultra-centrifugation and collected in 0.6 ml fractions from the bottom of the tube. Fractions were
subjected to SDS-PAGE on a 10% acrylamide gel and transferred to a PVDF membrane. The membrane was probed with an anti-rVACht antibody, an anti-synaptophysin (Syn) antibody as a marker for synaptic vesicles, and an anti-secretogranin II (SgII) antibody as a marker for large dense core vesicles.

B. Quantitative distribution of wild type (top) or mutant VACht (S480A) (bottom) in the sucrose gradient. The autorad of the Western blot shown above was scanned and the density of each fraction determined using Kodak DC120 software. Shown is the relative distribution of the synaptic vesicle marker synaptophysin (▲), the dense core vesicle marker secretogranin II (●), and wild type or S480A VACht (■) as a function of the fraction number.
Phosphopeptides generated by partial hydrolysis

Phosphoserine
Phosphothreonine
Phosphotyrosine

Pi
hVACt → RNVGLLTRSRERDVLLEDEPPQGLYDAVRLRE-

mVACt → RNVGLLTRSRERDVLLEDEPPQGLYDAVRLRE-

fVACt → RNVCQMKPLSERHILLEDGPKGLYDTHIMEERKAKEPHGTSGHSVHAVLSDQEGYSE

rVACt → RNVGLLTRSRERDVLLEDEPPQGLYDAVRLRE-

***   *    **    *    ****   *    *

| 470 480 |                  | 512 | 520 530 |

Casein Kinase II  +  +
Protein Kinase C   +
Not for publication
Phosphorylation of the rat vesicular acetylcholine transporter
Goang-Won Cho, Myung-Hee Kim, Young-Gyu Chai, Michelle L Gilmor, Alan I Levey
and Louis B Hersh

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