The characteristics of ATP-dependent transport of acetylcholine (ACh) in homogenates of pheochromocytoma (PC-12) cells stably transfected with the human vesicular acetylcholine transporter (VACHT) cDNA are described. The human VACHT protein was abundantly expressed in this line and appeared as a diffuse band with a molecular mass of ~75 kDa on Western blots. Vesicular [3H]ACh accumulation increased ~20 times over levels attained by the endogenous rat VACHT, expressed at low levels in control PC-12 cells. The transport of [3H]ACh by human VACHT was dependent upon the addition of exogenous ATP at 37 °C. Uptake was abolished by low temperature (4 °C), the proton ionophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (2.5 μM) and bafilomycin A1 (1 μM), a specific inhibitor of the vesicular H⁺-ATPase. The kinetics of [3H]ACh uptake by human VACHT were saturable, exhibiting an apparent Kₘ of 0.97 ± 0.1 mM and Vₘₐₓ of 0.58 ± 0.04 nmol/min/mg. Maximal steady-state levels of vesicular [3H]ACh accumulation were directly proportional to the concentration of substrate present in the medium with saturation occurring at ~4 mM. Uptake was stereospecifically inhibited by L-vesamicol with an IC₅₀ of 14.7 ± 1.5 nM. The apparent affinity (Kₗ) of [3H]vesamicol for human VACHT was 4.1 ± 0.5 nM, and the Bₘₐₓ was 8.9 ± 0.6 pmol/mg. The turnover (Vₘₐₓ/Bₘₐₓ) of the human VACHT was ~65/min. This expression system should prove useful for the structure/function analysis of VACHT.

Active Transport of Acetylcholine by the Human Vesicular Acetylcholine Transporter*  
(Received for publication, August 20, 1996, and in revised form, September 12, 1996)  
Hélène Varoqui and Jeffrey D. Erickson‡  
From the Section on Molecular Neuroscience, Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892

Acetylcholine (ACh) is synthesized in the cytoplasm of cholinergic neurons by choline acetyltransferase (ChAT) and transported into cholinergic synaptic vesicles by a vesicular acetylcholine transporter (VACHT) (1–3). Recently, Rand and colleagues (4) cloned a putative VACHT cDNA (unc-17) from the nematode Caenorhabditis elegans. We obtained the unc-17 homolog from the marine ray Torpedo and demonstrated that it possessed a high affinity binding site for vesamicol (5), a drug which blocks in vitro and in vivo ACh accumulation in cholinergic synaptic vesicles (2). Subsequently, we showed that the rat homolog of the Torpedo vesamicol-binding protein was a functional vesicular transporter for ACh (6). Expression of the rat VACHT in fibroblasts enabled intact cells to sequester ACh in a vacuolar ATPase-containing intracellular compartment by a process which was inhibited by l-vesamicol (6). Kinetic analysis of the ACh transport system was not possible, however, using an intact fibroblast cell assay, and has yet to be demonstrated following transfection of VACHT cDNA.

The characteristics of vesicular ACh transport have been extensively studied in highly purified synaptic vesicles from the electric organ of Torpedo (reviewed in Ref. 2). In mammalian synaptic vesicle preparations, however, ATP-dependent transport of ACh is very low, and kinetic parameters have not been determined (7, 8). The PC-12 cell line synthesizes, stores, and secretes low levels of ACh in addition to dopaminergic (9–11). Early studies showed that when PC-12 cells were incubated with [3H]choline, part of the [3H]ACh synthesized was seques terted in acidic intracellular storage organelles that contained an H⁺-ATPase (12–14). Low levels of specific ACh transport and vesamicol binding reported in PC-12 cells have been associated with membrane fractions containing markers for small synaptic vesicles (7, 15). A preferential association of VACHT with small synaptic vesicles in nerve growth factor differentiated PC-12 cells and in cholinergic nerve terminals in situ has been observed by immunoelectron microscopy (16–18).

Since rat PC-12 cells possess storage vesicles of the type that bear VACHT, we transfected these cells with human VACHT cDNA. Using species-specific antisera, we selected human VACHT expressing cells by immunocytochemistry and identified the human VACHT protein by Western blotting. Active transport of [3H]ACh by, and binding of [3H]vesamicol to, human VACHT in storage organelles of PC-12 cells was significantly higher than levels mediated by the endogenous rat VACHT. This ATP-dependent uptake of ACh mediated by human VACHT was vesamicol-sensitive and was dependent on the proton gradient generated by the vesicular H⁺-ATPase. These results provide the first kinetic analysis of mammalian VACHT and demonstrate that ATP-dependent vesicular ACh transport can be studied in a cell-free assay following transfection of VACHT cDNA.

EXPERIMENTAL PROCEDURES  
Cell Transfections and Selection of Stable Lines—Rat PC-12 cells and monkey kidney fibroblasts (CV-1 cells) were maintained at 37 °C in an atmosphere of 95% air, 5% CO₂ in Dulbecco's modified Eagle's medium containing 7% fetal bovine serum, 7% heat-inactivated horse serum, 10% fetal bovine serum (CV-1), penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (4 μg/ml). The full-length human VACHT cDNA (6) was subcloned into ReCMV (Invitrogen) at the T7 promoter-driven RCCMV vectors using the transient vaccinia-T7 expression system (22) as described previously (5). Briefly, cells were plated at 2 × 10⁶ per plate (10 cm) and infected the following day with

---

*This work was supported by the National Institute of Mental Health Intramural Research Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.
‡To whom correspondence should be addressed: Laboratory of Cell Biology, Bldg. 36, Room 3A-17, Bethesda, MD 20892. Tel.: 301-496-2573; Fax: 301-402-1748; E-mail: disbrow@codon.nih.gov.

The abbreviations used are: ACh, acetylcholine; ChAT, choline acetyltransferase; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; PC-12, rat pheochromocytoma cell line; TBZOH, dihydrotetranabenzene; VACHT, vesicular acetylcholine transporter; VMAT2, neuronal isoform of the vesicular monoamine transporter.
recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (40 mCi/ml) for 45 min at 20°C. The cells were harvested, and postnuclear supernatants were prepared.

Preparation of Postnuclear Supernatants—Control or human VAChT-expressing PC-12 cells and human VAChT or human VMAT2 expressing CV-1 fibroblasts were rinsed with phosphate-buffered saline and solubilized in phosphate-buffered saline containing 10 mM EDTA (pH 7.4). The cell suspensions were centrifuged at 800 × g for 10 min, and the cell pellets were homogenized (Dounce, type B pestle) in ice-cold buffer containing 80 mM potassium tartrate, 20 mM HEPES, 0.5 mM EGTA, 1 mM ascorbic acid, protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin), and 50 µl of the eukaryotic transfection reagent (1 µl of Hybond-ECL, Amersham). Following a 1-h preincubation in TBS (0.2 mM Tris-HCl, pH 7.5) the cell pellets were pelleted by centrifugation at 100,000 rpm for 45 min. The high speed pellets (100,000 rpm for 45 min) were subjected to SDS-polyacrylamide gel electrophoresis and immuno-blotted with an anti-human VAChT polyclonal antiserum (1:500). Human VAChT appears as a diffuse band with a molecular mass of ~75 kDa. The low molecular mass species (~40 kDa) probably arises from proteolysis.

RESULTS AND DISCUSSION

Initially, the human VAChT cDNA was transiently expressed in CV-1 fibroblasts by the recombinant vaccinia/T7 polymerase system to ensure that VAChT protein was made and capable of binding [3H]vesamicol as described for the rat and Torpedo homologs as well as for unc-17 (5, 6). Human VAChT-expressing CV-1 cells contained a high affinity binding site for [3H]vesamicol which displayed a Kd of ~3 nM and a Bmax of 2.4 pmol/mg (n = 2). The level of human VAChT expression in CV-1 fibroblasts was relatively low when compared with the level of expression of VMAT2 using the recombinant vaccinia virus assay. While the affinity of [3H]TBZOH for human VMAT2 was similar (Kd ~6.6 nM), the abundance of human VMAT2 expressed in this assay (Bmax ~12 pmol/mg) was approximately 5 times greater than that observed with human VAChT. Uptake of [3H]ACh by permeabilized fibroblasts or in postnuclear supernatants from CV-1 cells expressing the human VAChT cDNA was less than 2-fold greater than uptake observed in the presence of vesamicol (data not shown). This is in contrast to the ATP-dependent transport of monoamines by VMAT2 where specific uptake in permeabilized fibroblasts (21, 25) and in postnuclear supernatants (26) is approximately 10-50 times greater than uptake observed in the presence of reserpine or tetrabenazine.

Western blot analysis of the human VAChT protein. Membrane proteins from postnuclear supernatants (40 µg of protein) of human VAChT-expressing PC-12 cells (A) and control PC-12 cells (B) were subjected to SDS-polyacrylamide gel electrophoresis and immuno-blotted with an anti-human VAChT polyclonal antiserum (1:500). Human VAChT appears as a diffuse band with a molecular mass of ~75 kDa. The low molecular mass species (~40 kDa) probably arises from proteolysis.

The time course of vesamicol-sensitive vesicular [3H]ACh accumulation by human VAChT-expressing PC-12 cells and control PC-12 cells is shown in Fig. 3. Specific uptake mediated by human VAChT cDNA was linear for approximately 10 min with maximal steady-state levels attained by 30–60 min. Human VAChT-dependent uptake of [3H]ACh was completely inhibited by 2 µM t-vesamicol. Approximately 20 times more [3H]ACh uptake was observed in the stable PC-12 cell line expressing human VAChT cDNA than in control PC-12 cells which only express the endogenous rat VAChT protein. Uptake...
mediated by the endogenous rat VAChT protein was less than 2-fold greater than that observed in the presence of 2 μM l-vesamicol at 4 °C.

An analysis of the energetics and specificity of [3H]ACh accumulation by human VAChT is shown in Table I. The transport of [3H]ACh by human VAChT was dependent on exogenous ATP at 37 °C with uptake reduced approximately 90% in its absence. Uptake was abolished by low temperature (4 °C), the proton ionophore FCCP (2.5 μM), and bafilomycin A1 (1 μM), a specific inhibitor of the vesicular H⁺-ATPase (27). Uptake was specifically inhibited by l-vesamicol, a noncompetitive inhibitor of vesicular ACh uptake in Torpedo (28, 29), exhibiting an IC₅₀ of 14.7 ± 1.5 nM (Fig. 3, inset). The (L) isoform of vesamicol is approximately 20 times more potent than the (D) isoform to inhibit active transport of ACh in vesicles isolated from Torpedo (30). d-Vesamicol at 0.5 μM concentration reduced [3H]ACh uptake by human VAChT by only 20%. [3H]ACh uptake was not significantly affected by 0.5 μM reserpine or tetrabenazine which are selective inhibitors of vesicular monoamine transport (31, 32). Furthermore, [3H]ACh accumulation was not affected by chloride ions (10 mM), which stimulate transport of monoamines and glutamic acid by mammalian synaptic vesicles (33–35).

A kinetic analysis of the uptake of [3H]ACh by human VAChT is shown in Fig. 4. The initial rate of [3H]ACh uptake was measured during the linear portion of the time course (6 or 10 min) and was saturable with an apparent K_m of 0.97 ± 0.1 mM and V_max = 0.58 ± 0.04 nmol/min/mg (n = 4). The turnover of human VAChT (V_max/B_max) was approximately 65/min. The turnover of ATP-dependent ACh transport in vesicles purified from Torpedo has been reported to be as high as 10/min (2). The turnover determined for VMAT1 and VMAT2 in postnuclear supernatants from transfected CHO endothelial cells is approximately 10/min and 40/min, respectively (26).

A comparison of the time course of active transport by human VAChT at various subsaturating concentrations of [3H]ACh in the medium reveal that different levels of maximal steady-state accumulation of [3H]ACh were attained at equilibrium (Fig. 5, inset). At each ACh concentration, uptake was linear for about 10 min and then leveled off by 30–60 min of incubation. Thus, the vesicles in human VAChT-expressing PC-12 cells in vitro do not simply accumulate [3H]ACh until the vesicular compartment is full but rather seems to reflect the concentration of [3H]ACh present in the medium. Using a wide range of ACh concentrations, we find that the maximal accumulation of [3H]ACh at 30 min increased linearly up to 1 mM exogenous [3H]ACh and saturated at approximately 4 mM (Fig. 5). The concentration of ACh in the cytoplasm of nerve terminals of mammalian brain is estimated to be at 0.2 to 1 mM (2). Given the K_m of ACh for VAChT (~1 mM), active transport of ACh by synaptic vesicles may not be saturated in vivo.

Vesicular levels of ACh in vivo may reflect the cytoplasmic concentration of ACh in cholinergic nerve terminals. Hence, treatments that increase brain choline levels result in increased synthesis and release of ACh in vivo (36–39). Furthermore, transfected rat PC-12 cells which overexpress the ACh biosynthetic enzyme ChAT show increased vesicular ACh levels and increased stimulated release of ACh following choline loading (40). Age-related memory loss and cognitive decline in Alzheimer’s disease correlate with the failure of cholinergic transmission in neurons of the basal forebrain (41, 42). It is possible that this results from a decrease in synthesis and vesicular storage of ACh. Recent studies indicate that [3H]vesamicol binding to VAChT and [3H]hemicholinium binding to the high affinity choline uptake transporter are unchanged or increased despite the marked reduction in ChAT mRNA and protein in surviving neurons of Alzheimer’s disease brain (43–45).
The Saturation isotherm of [3H]ACh (0.06–6.4 mM) accumulation at 30 min by human VAChT. Uptake of [3H]ACh by control PC-12 cells was subtracted. Inset, time courses of vesamicol-sensitive [3H]ACh uptake by human VAChT at four subsaturating concentrations of ACh. In all cases, the amount of ACh accumulated represents less than 1% of the total ACh added to the medium. The data are from a representative experiment performed in duplicate, and the experiment was repeated twice with essentially identical results.

46). The fact that the ChAT and VACHT genes represent a single genomic locus (6, 47, 48) where both mRNA and protein are coexpressed throughout the central and peripheral cholinergic nervous system (5, 6, 16, 17, 20, 49, 50) and coregulated by various extracellular factors (51–53) suggests that under normal physiological conditions the expression of ChAT and VAChT from this cholinergic “regulon” is tightly controlled. Uncoupling of ChAT and VAChT gene expression may lead to a reduction in vesicular ACh pools resulting in presynaptic cholinergic hypofunction. An analysis of the abundance of human VAChT mRNA and protein relative to the expression of ChAT in cholinergic neurons in normal and in pathological conditions may provide important information regarding the potential uncoupling of this “cholinergic” gene locus.

The in present study, we determined the functional parameters of ATP-dependent vesicular accumulation of ACh by human VAChT. The low apparent affinity of ACh for VAChT and the concentration-dependent steady-state levels of vesicular ACh accumulation attained in vitro support the notion that perturbation in the level of ACh synthesis would have corresponding effects on the storage and release of ACh in vivo.