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Expression of a Putative Vesicular Acetylcholine Transporter Facilitates Quantal Transmitter Packaging

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Summary

A putative vesicular acetylcholine transporter (VAChT) was overexpressed in developing Xenopus spinal neurons by injection of rat VAChT cDNA or synthetic mRNA into Xenopus embryos. This resulted in a marked increase in the amplitude and frequency of miniature excitatory postsynaptic currents at neuromuscular synapses, reflecting an over 10-fold increase in the vesicular packaging of acetylcholine (ACh). The effect appeared in developing neurons even before synaptogenesis and was blocked by L-vesamicol, a specific blocker of ACh uptake into synaptic vesicles. Mutational studies showed that two highly conserved aspartate residues within putative transmembrane domains 4 and 10 are essential for the transport activity. These results provide direct evidence for the physiological function of a putative VAChT and demonstrate that quantal size can be regulated by changes in vesicular transporter activity.

Introduction

Synaptic transmission depends on the packaging of neurotransmitter into synaptic vesicles and the synchronous exocytosis of these vesicles upon arrival of an action potential in the nerve terminal. Studies of cholinergic vesicles purified from the electric organ of Torpedo have identified a vesicular transport activity for acetylcholine (ACh) (Parsons et al., 1993). This activity depends on a proton electrochemical gradient generated by a vacuolar type H^+–ATPase and is selective for ACh. The compound vesamicol blocks cholinergic neurotransmission by interfering with vesicular ACh storage (Prior et al., 1992), and binding studies suggest that vesamicol interacts directly with the transporter (Parsons et al., 1993). Recent studies of C. elegans mutants that are defective in neuromuscular function have led to the identification of the gene *unc-17* (Alfonso et al., 1993). Based on its expression in cholinergic nerve endings and the striking sequence similarity to a vesicular transporter for monoamines (Liu et al., 1992; Erickson et al., 1992), the *unc-17* gene product was proposed to be the vesicular ACh transporter (VAChT). Homologous genes were later cloned from Torpedo, rat, and human (Bejanin et al., 1994; Erickson et al., 1994; Roghani et al., 1994; Varoqui et al., 1994). Sequence analysis of the putative VAChT cDNA predicts a protein with 12 putative transmembrane domains. In situ hybridization has demonstrated specific localization of the putative VAChT mRNA in cholinergic cell groups, and immunostaining has shown the expected localization of VAChT protein in cell bodies and terminals of cholinergic neurons (Schafer et al., 1994; Gilmor et al., 1996). Interestingly, specific promoters for the putative VAChT gene reside within the first intron of the gene for choline acetyltransferase (ChAT), and coordinated expression of VAChT and ChAT has recently been observed in sympathetic superior cervical neurons (Berse and Blusztajn, 1995; Berrard et al., 1995; Cervini et al., 1995; Misawa et al., 1995). Despite strong circumstantial evidence for its identity as the ACh transporter, the physiological function of the putative VAChT has been difficult to demonstrate directly. Nonneural cell lines that support heterologous expression of the vesicular monoamine transporters do not support significant VAChT function (Erickson et al., 1994). However, high level expression of VAChT in the pheochromocytoma cell line PC12 appears to confer ACh transport into membrane vesicles (Varoqui and Erickson, 1996). In the present study, we have examined the function of VAChT in a neuronal environment by expressing the putative rat VAChT cDNA or synthetic mRNA in developing Xenopus spinal neurons before the quantal transmitter secretion had reached maturity.

During the first few days in culture, cholinergic Xenopus spinal neurons establish functional synaptic transmission with cocultured myocytes (Evers et al., 1989). At these developing neuromuscular synapses, action potential–independent miniature excitatory postsynaptic currents (MEPCs) exhibit a wide variation in the peak amplitude. The amplitude distribution of the quantal events is skewed toward smaller amplitudes (Kidokoro et al., 1980; Evers et al., 1989). In contrast, the amplitude distribution of MEPCs at mature neuromuscular junctions assumes a Gaussian profile around the mean amplitude (bell-shaped distribution), which defines the quantal size (del Castillo and Katz, 1954; Boyd and Martin, 1956). The transition from a skewed to a bell-shaped distribution occurs during the first week in Xenopus nerve–muscle cultures, a process that appears to reflect both a maturation of vesicular ACh packaging and an increase in receptor density at the subsynaptic membrane. Previous studies have shown that in 1-day-old Xenopus nerve–muscle culture the variation in the MEPC amplitude is primarily due to a variation in the amount of ACh contained in the ACh packets, rather than differences in the postsynaptic ACh sensitivity or the amount of acetylcholinesterase at the synapse (Evers et al., 1989). In particular, the amplitude distribution of MEPCs, which is highly skewed toward smaller values, suggests incomplete ACh filling of most synaptic vesicles. In the present study, we took advantage of the immature status of ACh packaging in developing neurons to examine the function of VAChT by overexpressing the protein. A shift in the amplitude distribution toward larger sizes would indicate higher levels of ACh per vesicle, and the

transition from a skewed to a bell-shaped amplitude distribution would suggest quantal maturation. The elevated ACh transport activity may also increase the frequency of MEPCs, since more vesicles loaded with sufficient ACh would be available to generate detectable current events after exocytosis.

The rat VAChT cDNA or synthetic mRNA was expressed in Xenopus neurons by injection into the early blastomeres of Xenopus embryos. Previous studies have shown that introducing exogenous cDNA or mRNA into the blastomeres of early Xenopus embryos results in the expression of exogenous proteins in the progeny neurons and muscle cells derived from the injected blastomere. Presynaptic functions of spinal neurons were found to be affected by the presence of these foreign proteins at the neuromuscular junction (Alder et al., 1995). In the present study, we found that the expression of rat VAChT by injection of either cDNA or mRNA leads to a marked enhancement of ACh packaging at developing synapses, an effect which is observed only when VAChT is expressed in the presynaptic neurons, not in the postsynaptic muscle cells. This effect can be seen in developing neurons prior to synaptogenesis and is blocked by L-vesamicol, a specific blocker of ACh uptake into synaptic vesicles. Mutational studies further indicate that two conserved aspartate residues predicted to reside in the putative transmembrane domains 4 and 10 are essential for its function. Taken together, these results provide strong evidence that this protein acts as the vesicular ACh transporter in neurons. Furthermore, expression of VAChT during development appears to be a limiting factor in determining the transmitter quantal size. Regulation of VAChT activity may thus provide a means of modulating the efficacy of synaptic transmission.

Results

Expression of Putative VAChT in Developing Xenopus Neurons

Rat VAChT cDNA or synthetic mRNA prepared from the cDNA was injected into one of the early blastomeres of Xenopus embryos at the 2-cell stage. Dextran conjugated with fluorescein isothiocyanate (FITC–dextran) was coinjected with the cDNA or mRNA as a fluorescence marker for the identification of progeny cells derived from the injected blastomere. Cultures of dissociated spinal neurons and muscle cells were prepared from these embryos 1 day after injection (see Experimental Procedures). The expression of putative rat VAChT in the Xenopus cells was confirmed first by Western analysis of the embryos injected with VAChT cDNA or mRNA, using polyclonal antibodies raised against the C-terminus of the rat VAChT protein, which has a predicted MW of 56.5 kDa (Gilmor et al., 1996). Figure 1A shows a much higher level of the protein at ~55 kDa in the injected embryos as compared with the noninjected embryos 1 day after injection of the cDNA or mRNA for VAChT. The weak staining of noninjected embryos may reflect expression of endogenous Xenopus VAChT protein at a lower level, or a poor recognition of Xenopus VAChT by the antibodies raised against the rat protein. Interestingly, the endogenous Xenopus protein appeared to have a similar MW as the rat protein. The presence of VAChT protein in cultured Xenopus cells was also examined by immunocytochemical staining of 1-day-old cultures, using antibodies to rat VAChT. In two separate cultures, 52 of 55 dextran-positive cells showed strong immunostaining for VAChT, whereas 54 of 60 dextran-negative cells revealed very weak staining. We also performed double immunofluorescence staining with antibodies to the synaptic vesicle protein synaptophysin as well as VAChT. As depicted by the examples in Figures 1B–1D, the level of VAChT staining in neurons derived from injected blastomeres showed substantial variation, while the level was much higher compared with that of neurons derived from noninjected blastomeres in the same culture. In contrast, a similar level of punctuate staining of synaptophysin was observed in different neurons. Double immunostaining of the same neurons showed that the expressed VAChT molecules are largely colocalized with synaptophysin, particularly those associated with punctuate staining, suggesting the expression of VAChT in synaptic vesicles.

Quantal ACh Secretion at Developing Synapses

Within 1 day following cell plating, many neurons and muscle cells formed functional synapses (Buchanan et al., 1989; Evers et al., 1989). Spontaneous quantal transmitter release at the neuromuscular synapse was assayed electrophysiologically by whole-cell recording of synaptic currents from the postsynaptic muscle cell. In 1-day-old cultures, MEPCs recorded from postsynaptic muscle cells exhibited variable amplitude and frequency. These current events were due to spontaneous quantal release of ACh from the presynaptic neuron, as they were insensitive to tetrodotoxin (TTX), an agent known to block action potentials in this culture (Xie and Poo, 1986). The mean frequency and mean amplitude of MEPCs after treatment with TTX (10 μM) were 109% $\pm$ 10% and 95% $\pm$ 9% (SEM, n = 10), respectively, of the values observed at the same synapse before the treatment. However, when the properties of MEPCs were examined at synapses made by neurons derived from Xenopus blastomeres injected with VAChT cDNA [VAChT(+)] or from noninjected blastomeres [VAChT(−)] in the same culture, a marked difference was observed. As shown by the pair of recordings depicted in Figure 2A, both the mean amplitude and the mean frequency of MEPCs were much higher at synapses made by the VAChT(+) neuron. Since both the presynaptic neuron (N) and the postsynaptic myocyte (M) may express (+) or may not express (−) the exogenous VAChT, we have analyzed the mean peak amplitude and frequency of MEPCs for four possible combinations: N+M+, N+M−, N−M+, or N−M−. As shown in Figure 2B, higher values were observed when the neuron was VAChT(+), irrespective of the expression in the postsynaptic muscle cell. A relative assessment of the overall amount of spontaneous vesicular secretion of ACh was also made by integrating the current over time to obtain the total charge associated with all MEPCs within a unit of time (min) at each synapse. This measure integrates changes

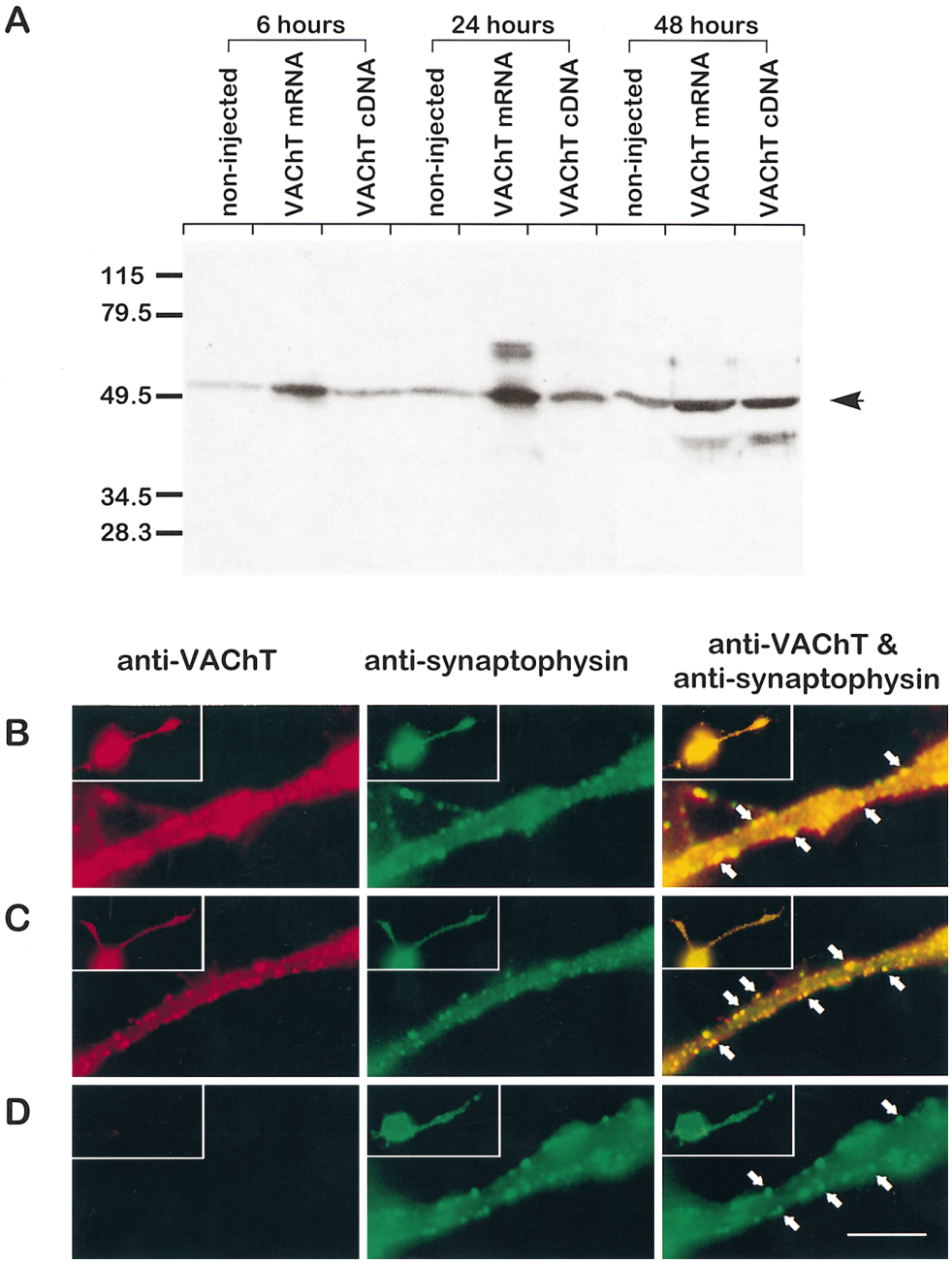

Figure 1. Expression of Putative Rat VAChT in Xenopus Embryos after Blastomere Injection

(A) Western blot analysis of rat VAChT in noninjected and injected embryos at the indicated time after fertilization, using polyclonal antibodies against the putative rat VAChT. Putative VAChT cDNA or mRNA was injected into one of the early blastomeres of Xenopus embryos at the 2-cell stage. Proteins from one embryo were loaded into each lane. The size of molecular weight markers is shown on the left side. The arrow indicates a band at ~55 kDa, which is likely to represent the VAChT protein (Gilmor et al., 1996).

(B–D) Immunostaining of Xenopus neurons with both antibodies against rat VAChT (left, by red fluorescence) and against rat synaptophysin (middle, by green fluorescence). Colocalization of VAChT with synaptophysin was indicated as yellow in the superimposed images (right). Arrows point to putative synaptic vesicle clusters. Shown in the insert are images of the neurons at a lower magnification. Note the different levels of expression of rat VAChT in two cells derived from the same injected blastomere ([B] and [C]) and the absence of staining in the neuron derived from the noninjected blastomere (D) in the same culture. Scale, 10 μm (60 μm for the insert).

in both the frequency and the amplitude of MEPCs, and we have found a >10-fold increase in the total amount of ACh released from VAChT(+) neurons. These results are consistent with the expectation that exogenous rat VAChT facilitates ACh packaging into synaptic vesicles, which only occurs with VAChT expression in the presynaptic neuron. Since expression in the muscle cells had no effect on the properties of MEPCs, we have grouped

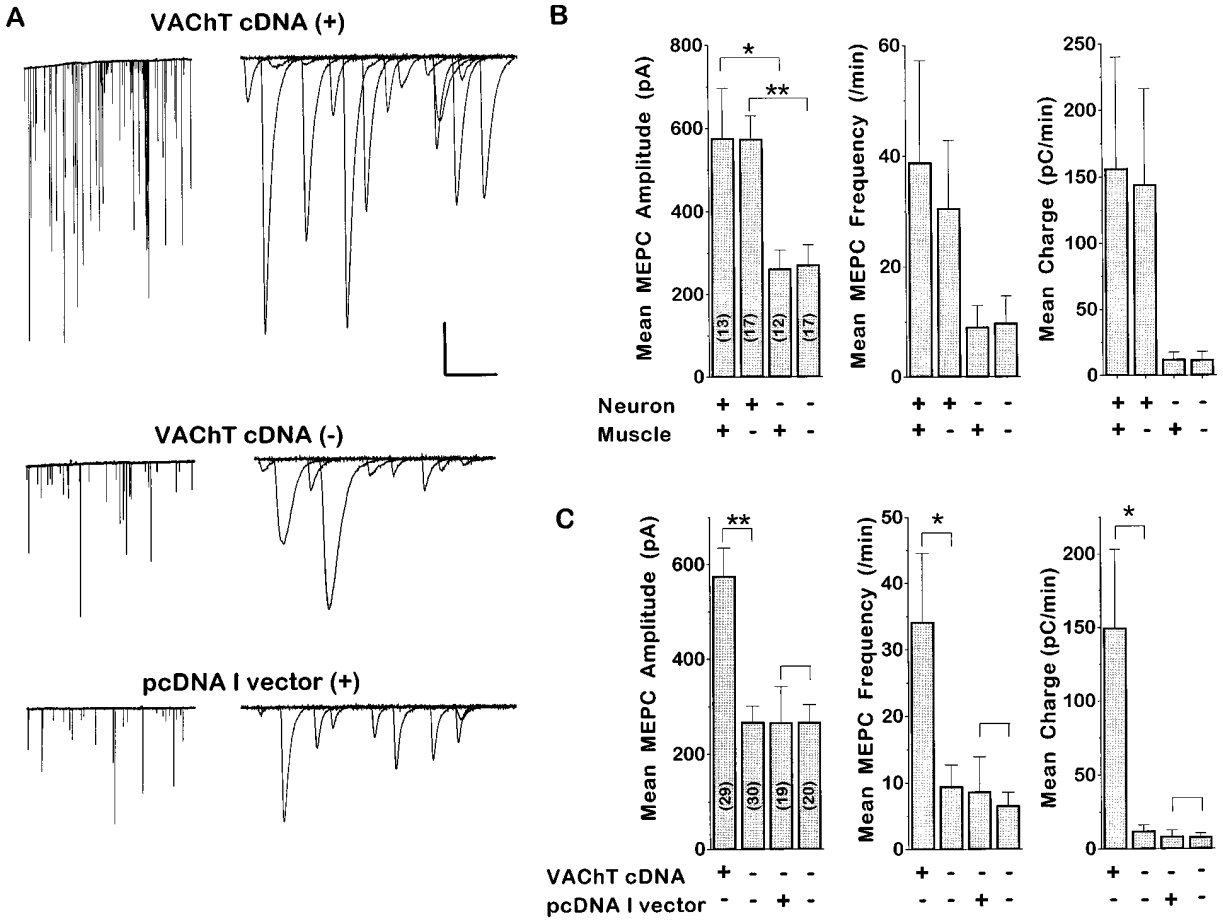

Figure 2. Effect of Expressing Putative VAChT on Spontaneous MEPCs Observed at Developing Xenopus Neuromuscular Synapses in Culture

(A) Membrane currents recorded from a whole-cell voltage-clamped myocyte that was innervated by a neuron expressing rat VAChT (upper traces), or a control neuron not expressing rat VAChT (middle traces), or a neuron expressing the empty vector (bottom traces), as identified by the presence or absence of FITC–dextran fluorescence. Traces on the left are recordings of membrane currents at a slow timescale; downward deflections represent MEPCs. Traces on the right are overlapped oscilloscopic sweeps of membrane currents at a faster timescale observed over 1 min. Scale bars, 0.5 nA, 1 min and 0.5 nA, 20 ms for the slow and fast traces, respectively.

(B) Average values of MEPC amplitude, frequency, and total charge at neuromuscular synapses in cultures obtained from embryos injected with rat VAChT cDNA. Data are grouped according to the expression of VAChT in the presynaptic neuron (N) and/or the postsynaptic myocyte (M), as determined by the presence of FITC–dextran fluorescence. Numbers associated with the bar refer to the total number of synapses examined. The same set of data was used for the analyses of amplitude, frequency, and charge. Error bars = SEM. Significance level was indicated by '*' ($p < 0.05$) and '**' ($p < 0.01$), using a Student's *t*-test.

(C) Similar to (B), except that the data are grouped according to whether the presynaptic neuron was expressing VAChT or an empty vector pcDNA I used for carrying the VAChT cDNA. Values for VAChT(+) differ significantly from control values obtained from the same cultures, as marked by '**' ($p < 0.01$) or '*' ($p < 0.05$, *t*-test). In contrast, values for empty vector(+) were not different from their own control values (unmarked bracket, $p > 0.05$, *t*-test). The same set of data as that in (B) was used for the VAChT group.

the results regardless of the status of muscle expression in the summary histograms shown in Figure 2C. In contrast to the marked effect on amplitude and frequency, the rise and decay times of MEPCs showed no significant difference in all cases (data not shown), suggesting no obvious effects of VAChT expression on the distribution, density, or conductance properties of postsynaptic ACh channels. Finally, to determine whether the microinjection of DNA alone perturbed secretion, we have introduced a pcDNA I vector without the rat VAChT cDNA into Xenopus embryos. MEPCs in cultures prepared from the injected embryos were recorded as described above. We found that synapses made by neurons derived from the blastomeres injected with the empty vector [pcDNA I(+)] showed no detectable change in the measurements of MEPCs compared with control synapses [pcDNA I(−)] in the same set of cultures. The results are summarized in the histograms shown in Figure 2C.

Amplitude Distribution of MEPCs

To determine the effect of VAChT on ACh packaging, further analysis of the MEPC amplitude distribution was performed. The increase in the mean amplitude could result from a general shift of quantal size toward larger amplitudes or from the appearance of a subpopulation of quanta with large amplitudes. Figure 3 depicts typical examples of MEPC amplitude distributions at synapses

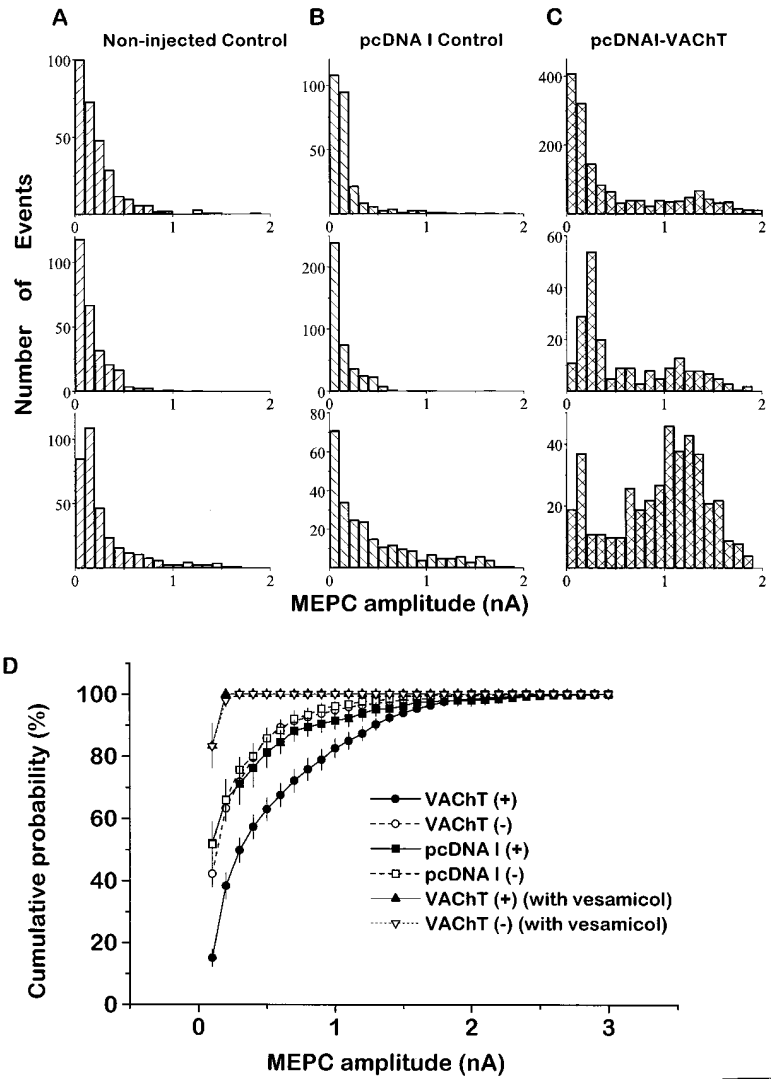

Figure 3. Amplitude Distribution of MEPCs

The top three rows depict representative histograms of MEPC amplitude distribution obtained from individual myocytes innervated by neurons derived from noninjected blastomeres (A), or from blastomeres injected with an empty pcDNA I vector (B) or a vector containing VAChT cDNA (C).

(D) Comparison of the amplitude distribution of MEPCs. The cumulative probability refers to the percentage of all events with amplitudes smaller than or equal to a given value. Data are obtained from synapses made by (1) neurons expressing VAChT (n = 28), (2) control neurons not expressing VAChT in the same group of embryos as in (1) (n = 29), (3) neuron expressing empty vector pcDNA I (n = 16), (4) control neurons not expressing the empty vector in the same group of embryos as in (3) (n = 17), (5) neurons expressing VAChT but treated with 2 μM L-vesamicol (n = 9), and (6) control neurons not expressing VAChT in the same group of embryos as in (5) (n = 11). Bar = SEM. The data for VAChT(+) neurons are significantly different from all other groups ($p < 0.01$, Kolmogorov–Smirnov test). The data for VAChT(+) and VAChT(−) with vesamicol are also significantly different from all other groups ($p < 0.01$, Kolmogorov–Smirnov test), but are not significantly different from each other ($p > 0.05$, Kolmogorov–Smirnov test).

made by control neurons derived from noninjected blastomeres and neurons derived from blastomeres loaded with the empty vector alone or with VAChT cDNA. The noninjected control and empty vector control synapses showed a monotonic skewed distribution of MEPCs, as described previously, at these developing synapses (Xie and Poo, 1986; Evers et al., 1989). In 35 of 42 noninjected control and in 17 of 19 empty vector(+) synapses, the smallest amplitude bin (0–100 pA) contained the highest number of events. In contrast, only 5 of 29 VAChT(+) synapses exhibited the most frequent events at this bin. Furthermore, 10 of 29 synapses made by VAChT(+) neurons showed a distinct Gaussian profile at higher amplitudes, reminiscent of the amplitude distribution of MEPCs observed at more mature synapses in these cultures (Lu et al., 1992). In contrast, such a Gaussian profile of MEPC amplitude was rarely observed at noninjected control and empty vector(+) synapses (1 of 42 and 1 of 19, respectively).

Quantitative comparison of the amplitude distribution between various groups of synapses was made by constructing the cumulative probability plot shown in Figure 3D. The distribution of MEPC amplitudes for synapses made by VAChT(+) neurons was found to be significantly different from that for synapses made by pcDNA I(+), VAChT(−), or pcDNA I(−) neurons ($p < 0.01$, Kolmogorov-Smirnov test). In a separate series of experiments, in vitro transcribed mRNA made from cDNA encoding rat VAChT, rather than the cDNA, was injected into Xenopus blastomeres. Similar enhancement of MEPC amplitude and frequency was observed at synapses made by neurons derived from mRNA-injected blastomeres (Figure 4A). The cumulative probability plot for the amplitude distribution of MEPCs was found to be indistinguishable from that observed with cDNA injection (Figure 4B).

Effect of L-(−)-vesamicol

Previous pharmacological studies have shown that L-vesamicol specifically inhibits ACh packaging into synaptic vesicles (Prior et al., 1992). In addition, L-vesamicol binding has been used as evidence for the physiological function of the putative VAChT (Erickson et al., 1994). If the effects that we observed on quantal secretion were due to increased transporter activity, L-vesamicol

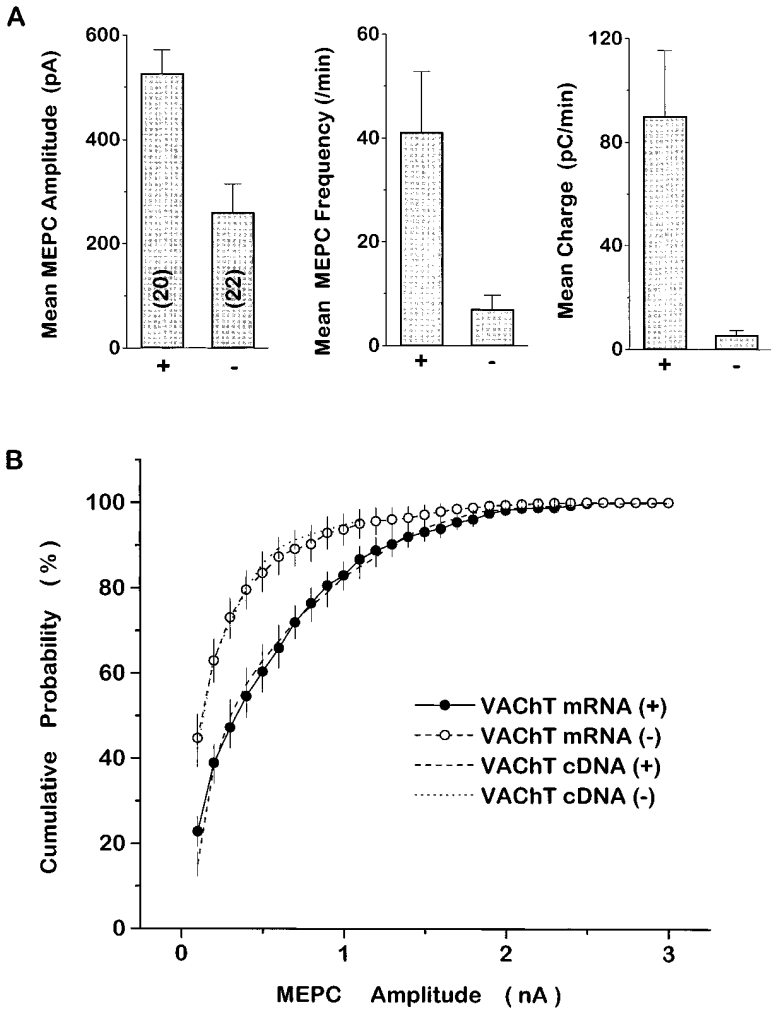

Figure 4. Effect of Injection with Putative Rat VAChT mRNA

(A) Average values of MEPC amplitude, frequency, and total charge at synapses made by neurons derived from blastomeres injected with synthetic rat VAChT mRNA(+) and neurons from noninjected blastomeres in the same group of embryos (−). Data are grouped according to the expression of VAChT mRNA in the presynaptic neuron, regardless of the expression in the postsynaptic myocyte. Numbers associated with the bar refer to the total number of synapses examined. Error bars = SEM. Data for (+) are significantly different from those for (−) ($p < 0.01$; *t*-test).

(B) Comparison of amplitude distribution of MEPCs. Cumulative probability was calculated as described in Figure 3D. Data are obtained from synapses made by (1) neurons expressing rat VAChT mRNA (n = 19), (2) control noninjected neurons in the same group of embryos as in group one (n = 21), (3) neurons injected with VAChT cDNA (n = 28), and (4) control noninjected neurons in the same group of embryos as in group three (n = 29). Data for groups three and four are the same as those shown for groups one and two in Figure 3D. Error bar = SEM. The data for VAChT mRNA(+) and VAChT cDNA(+) are significantly different from their respective noninjected controls ($p < 0.01$, Kolmogorov–Smirnov test), but are not different from each other ($p > 0.05$, Kolmogorov–Smirnov test).

should block them. At control VAChT(−) synapses derived from noninjected Xenopus blastomeres, we found that treatment with 2 μM of L-(−)-vesamicol markedly reduced the amplitude, frequency, and total charge of MEPCs, without apparent effect on the time course of the individual events (Figure 5). This effect on the synaptic currents was presumably due to reduced ACh loading by endogenous VAChT. With the same vesamicol treatment, we found that the mean amplitude and frequency of MEPCs at synapses made by VAChT(+) neurons were reduced to the same levels as those from synapses made by control VAChT(−) neurons treated with vesamicol (Figure 5; also see Figure 3D). Both the rise and decay times of MEPCs were unaffected (data not shown). The residual amount of quantal ACh secretion in vesamicol-treated cultures may result from either incomplete inhibition of VAChT by vesamicol or VAChT-independent mechanisms of vesicular ACh accumulation.

To confirm that vesamicol did not affect postsynaptic sensitivity to ACh, we directly assayed the effect of vesamicol on the ACh sensitivity of the muscle cells, using iontophoretic application of identical ACh pulses at the muscle surface. We found that in the presence of 2 μM of L-(−)-vesamicol the average peak amplitude of ACh-induced currents was 108% ± 10% (SEM, n = 10 cells) of that observed at myocytes in the absence of the vesamicol treatment. Thus, it is unlikely that vesamicol had any significant effect on the postsynaptic ACh sensitivity under these experimental conditions.

Increased ACh Packaging in Isolated Neurons prior to Synaptogenesis

It is well known that postsynaptic cells exert retrograde influence on the differentiation of presynaptic neurons, including determination of transmitter phenotype (Landis, 1990). Although the presence of VAChT in the postsynaptic myocyte did not affect the enhancement of ACh packaging in VAChT(+) neurons, it is nonetheless possible that the action of presynaptically expressed VAChT in ACh packaging depends on the presence of postsynaptic muscle cells. We have therefore determined whether increased ACh packaging can occur in isolated neurons prior to the contact with the myocyte. In Xenopus cultures, previous studies have shown that spontaneous and evoked ACh secretion can occur prior to synaptogenesis, using a myocyte or an excised patch of muscle membrane as a detector of the released ACh (Young and Poo, 1983; Sun and Poo, 1987). In the present study, we examined MEPCs that occurred immediately following the contact with a manipulated myocyte, assuming that spontaneous quantal release at this early time involves the exocytosis of vesicles packaged prior

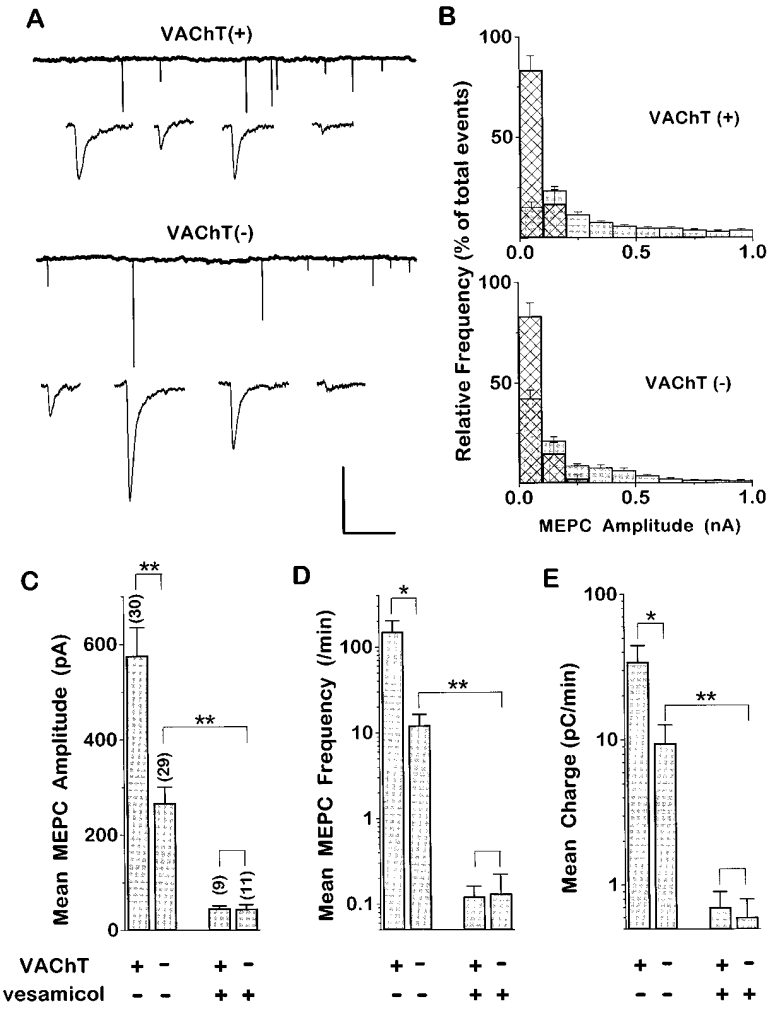

Figure 5. Effect of L-(−)-Vesamicol on Quantal ACh Secretion

(A) Continuous traces depict examples of membrane currents recorded from a whole-cell voltage-clamped myocyte (Vc = −70 mV) that was innervated by a VAChT(+) neuron (upper trace) or by a VAChT(−) neuron (lower trace) in the presence of 2 μM L-(−)-vesamicol, which was added to the culture medium at the time of cell plating. Samples of MEPCs at various times are shown below at a higher time resolution. Scale bars, 100 pA, 1.5 min and 100 pA, 20 ms for slow and fast time-scales, respectively.

(B) Histograms for the amplitude distribution of MEPCs at synapses made by VAChT(+) and VAChT(−) neurons in the presence (crossed) and absence (shaded) of L-(−)-vesamicol (2 μM). Relative frequency refers to the percentage of events associated with each amplitude bin. Error bar = SEM.

(C–E) Averaged values of the amplitude, frequency, and total charge of MEPCs at synapses made by VAChT(+) and VAChT(−) neurons in the presence and absence of L-(−)-vesamicol (2 μM). The number associated with the bars refers to the total number of synapses examined. Error bar = SEM. Values from synapses in the presence of vesamicol were significantly different from those in the absence of vesamicol ('**', $p < 0.01$, *t*-test). However, values from VAChT(+) and VAChT(−) in the presence of vesamicol are not significantly different from each other (unmarked bracket, $p > 0.05$, *t*-test).

to the myocyte contact. After achieving whole-cell recording of an isolated spherical myocyte, the cell was manipulated into contact with growth cones of isolated spinal neurons in the same culture (Figure 6A). The immediate appearance of MEPCs, previously shown to be induced by contact (Xie and Poo, 1986), was assayed during the first 5 min following the initial contact of the myocyte. To minimize the effect due to the variation in ACh sensitivity of the myocyte, the same myocyte was used to measure ACh release from several growth cones of both VAChT(+) and VAChT(−) neurons in the same culture in a random order. An example of recordings made by a myocyte from two different growth cones is shown in Figure 6B. We found that the mean amplitude, frequency, and total charge of MEPCs were much higher for VAChT(+) neurons (Figure 6C). The higher level of quantal ACh secretion during the initial phase of neuron–muscle contact supports the notion that the elevated ACh packaging had occurred prior to the muscle contact. The differences between the VAChT(+) and VAChT(−) neurons, however, were slightly smaller than those observed at natural synapses in 1-day-old cultures, suggesting that further enhancement of ACh packaging had occurred after synapse formation. Some inductive action of the postsynaptic muscle cell may directly or indirectly affect further maturation of the quanta.

Two Aspartate Residues Essential for the Transporter Function

To explore the molecular mechanism of the VAChT function, we introduced two single mutations into the rat VAChT by site-directed mutagenesis. As illustrated in Figure 7A, mutation D193N involves a replacement of an aspartate residue in transmembrane domain 4 with asparagine. This residue shows conservation in all the VAChT cDNAs from different species, but not in the vesicular monoamine transporters, suggesting a possible role in substrate recognition. The second mutation D398N involves a similar replacement of aspartate 398 in transmembrane domain 10 with asparagine. This residue is conserved among all vesicular neurotransmitter transporters identified so far. Neutralization of the homologous residue in monoamine transporters disrupts the function (Steiner-Mordoch et al., 1996; Merickel et al., 1997). In vitro synthesized mRNA for the VAChT mutants was injected into Xenopus blastomeres. Western analysis shows that the mutant proteins were expressed in Xenopus embryos at a similar level to wild-type rat VAChT and exhibited the same electrophoretic mobility (Figure 7B). Using cultures prepared from the injected embryos, we found that D193N completely abolished the transport function of the introduced VAChT (Figure 7C). Interestingly, D398N reduced the mean amplitude, frequency, and total charge of MEPCs

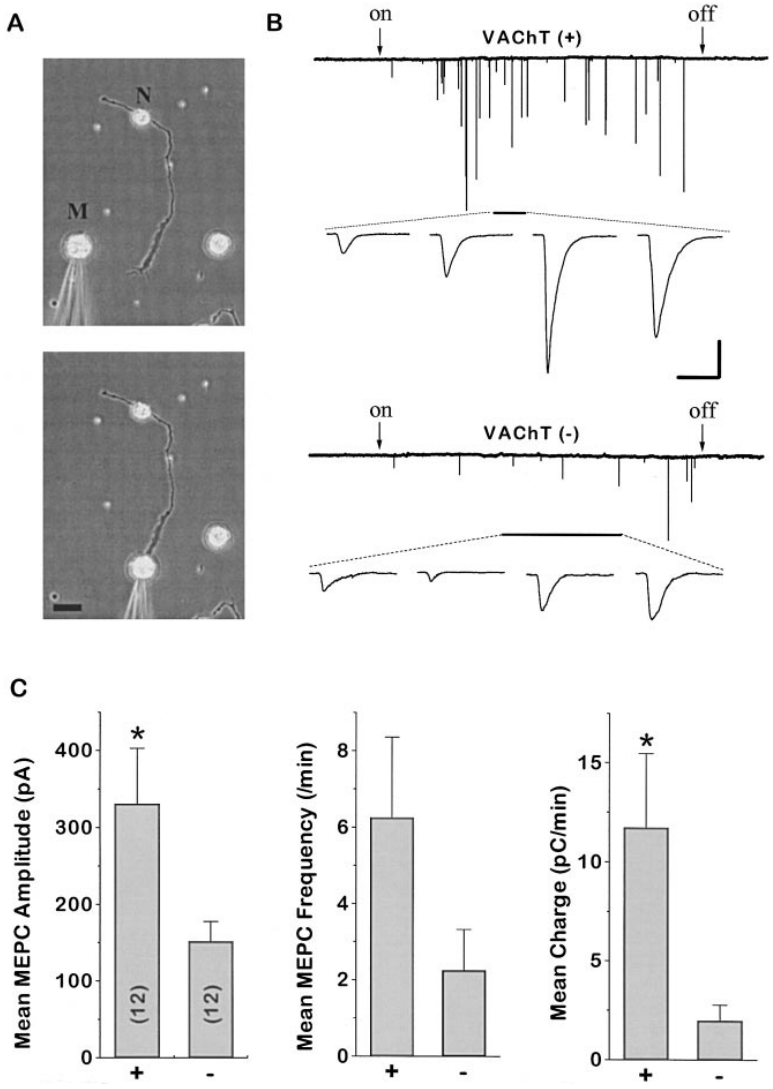

Figure 6. Detection of Increased ACh Packaging in Isolated Neurons

(A) Phase-contrast microscopic images of an isolated Xenopus spinal neuron (N) before (top) and after (bottom) a whole-cell voltage-clamped Xenopus myocyte (M) was manipulated into contact with its growth cone. Scale bar, 50 μm.

(B) Representative traces depict membrane currents recorded from a whole-cell voltage-clamped myocyte (Vc = −70 mV), after it was first manipulated into contact with the growth cone of a VAChT(+) neuron for 5 min (upper trace) and then manipulated into contact with the growth cone of a VAChT(−) neuron (lower trace). Samples of MEPCs at various times are shown below at a higher time resolution. Arrows indicate the time of nerve–muscle contact (on) and detachment (off). Scale bars, 300 pA, 45 s and 200 pA, 10 ms for slow and fast timescales, respectively.

(C) Mean values of MEPC amplitude, frequency, and total charge obtained from 12 pairs of VAChT(+) and VAChT(−) neurons. Only VAChT(−) myocyte was used in this assay. '*' marks significant difference ($p < 0.05$, *t*-test).

to a level significantly below that of noninjected controls. Again, the rise and decay times of MEPCs showed no significant differences in all cases (data not shown), suggesting no postsynaptic effects.

Discussion

Previous studies have indicated the existence of four distinct vesicular transporter activities for classical neurotransmitters, including those for monoamines, ACh, glutamate, and γ-aminobutyric/glycine (Sudhof and Jahn, 1991; Edwards, 1992; Schuldiner et al., 1995). Molecular cloning has identified two proteins involved in vesicular monoamine transport (VMAT1 and 2), and several biochemical assays have established the physiological function of these proteins in a variety of heterologous expression systems (Erickson et al., 1992; Liu et al., 1992; Schuldiner et al., 1993; Merickel et al., 1995). Similar to vesicular monoamine transport, vesicular ACh transport involves proton exchange (Parsons et al., 1993). The sequence similarity of *unc-17* and its vertebrate homologs to VMATs further supports their similarity in function. Despite the selective expression of VAChT by cholinergic neurons, however, the expression of VAChT cDNA in a variety of cell lines has failed to yield ACh transport activity using standard membrane preparations (E. F., E. B., and R. H. E., unpublished data). One group has reported ACh uptake into intact cells that shows inhibition by vesamicol (Erickson et al., 1994). Surprisingly, a semipermeabilized cell preparation more appropriate to demonstrate uptake by intracellular vesicles did not reveal ACh transport activity. Recently, the same group has reported vesamicol-sensitive ACh uptake by membranes prepared from PC 12 cells stably expressing VAChT (Varoqui and Erickson, 1996), suggesting that the transport activity may require expression of the transport protein in a neuroendocrine cell. Our observations now provide direct physiological evidence of vesicular ACh transport activity by rat VAChT expressed in a cholinergic neuron.

The blastomere injection method has been used in several previous studies of synaptic vesicle proteins. Overexpression of rat synaptophysin and synaptotagmin increased and decreased the frequency of MEPCs, respectively (Alder et al., 1995; T. Morimoto and M. M. P., unpublished data), without affecting the mean amplitude

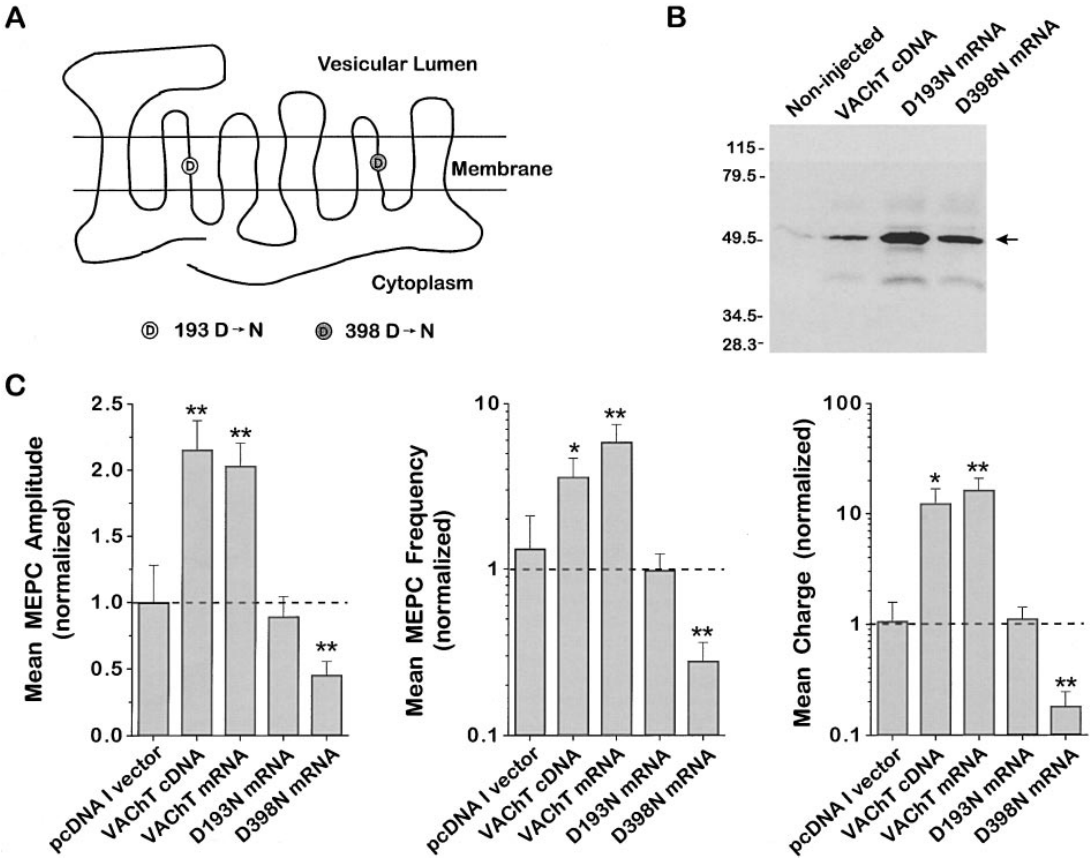

Figure 7. Mutational Studies of Putative VAChT

(A) Schematic drawing of proposed topology of the rat VAChT and the sites of mutations introduced. Mutation D193N has a single amino acid substitution from aspartate to asparagine at 193 in transmembrane domain 4, a site conserved in all VAChTs identified in different species. Mutation D398N has the same single amino acid substitution from aspartate to asparagine at 398 in transmembrane domain 10, a site conserved in all vesicular transporters.

(B) Western analysis of different rat VAChTs in 2-day-old noninjected and injected embryos. Different VAChT mRNAs were injected into one of the early blastomeres of Xenopus embryos at the 2-cell stage. Total proteins from one embryo 2 days after fertilization were loaded into each lane. Staining was performed using antibody against rat putative VAChT.

(C) Summary of the effects of various VAChTs on quantal ACh secretion. Data were normalized against the mean value obtained from neurons derived from noninjected blastomeres in the same group of embryos. Shown are data on MEPCs produced by neurons derived from embryos injected with empty pcDNA I vector ($n = 19$ for vector[+]; $n = 20$ for vector[−]), with vector containing VAChT cDNA (30[+]; 29[−]), with synthetic VAChT mRNA (20[+]; 22[−]), with mutated VAChT D193N mRNA (20[+]; 20[−]), and with VAChT D398N mRNA (18[+]; 21[−]). Error bar = SEM. Data exhibiting a significant difference from their respective noninjected controls are indicated ('*' $p < 0.05$; '**' $p < 0.01$; *t*-test).

or amplitude distribution of MEPCs. In contrast, overexpression of rat VAChT markedly increases both the mean amplitude and the frequency of MEPCs. These differences provide strong evidence that the effect on the quantal events found in the present study reflects a specific effect on ACh packaging. Thus, although immunostaining of cultured Xenopus neurons indicates that VAChT colocalizes with synaptophysin, the function of VAChT in the vesicle clearly differs from that of synaptophysin and synaptotagmins, proteins implicated in other aspects of the synaptic vesicle cycle.

Synapses made by VAChT(+) spinal neurons exhibited MEPCs of larger mean amplitude and higher frequency. The increased amplitude directly reflects larger amounts of ACh packaged into the synaptic vesicle. The increased ACh packaging may contribute in part to the increase in frequency, since the number of vesicles with sufficient ACh to produce detectable postsynaptic events (amplitude >10 pA) will be increased. On the other hand, VAChT may also promote maturation or the formation and stability of synaptic vesicles, resulting in more filled vesicles available for spontaneous release. It may also facilitate exocytosis, leading directly to a higher frequency of spontaneous ACh release. Nonetheless, a specific role for this VAChT protein in ACh packaging is strongly supported by the observation that vesamicol, a drug known to inhibit ACh transporter function in vivo and in vitro (Prior et al., 1992), completely blocked the effects of expressing this protein on MEPCs. A potential effect of vesamicol on the ACh sensitivity of the myocyte was excluded by iontophoretic tests of the ACh sensitivity of the myocyte after the drug treatment.

The observed effect of VAChT on MEPCs apparently requires its expression in the presynaptic neuron, while

expression in the postsynaptic muscle cell had no influence on MEPCs (Figure 2B). We found that the rise and decay times of MEPCs were unchanged in all experimental conditions, regardless of the site of VAChT overexpression, further arguing against any effect of the protein expression on postsynaptic ACh channel properties. Indeed, the postsynaptic muscle seems unlikely to have an inductive or permissive role for the presynaptic action of VAChT. Larger and more frequent quantal release was observed immediately following the contact of the muscle with the VAChT(+) neuron (Figure 6), suggesting that the effect of VAChT on ACh packaging had begun in the isolated neuron prior to synaptogenesis, in the absence of the muscle cell. This result, however, did not exclude the possibility that the muscle cell can exert retrograde influence on further maturation of ACh packaging following synaptogenesis.

The effect of VAChT on the amplitude distribution of MEPCs varies substantially. Although higher mean amplitude was observed in nearly all cases, only a fraction of synapses exhibited the bell-shaped distribution characteristic of mature quantal secretion. As indicated by immunostaining of VAChT (Figure 1), there was a substantial variation in the expression level among various neurons. Insufficient expression of the VAChT protein may account for the immature packaging of ACh. In addition, the clustering of ACh receptors in the postsynaptic muscle membrane contributes directly to the size of synaptic currents and takes many days to complete. In these Xenopus cultures, the increase in the amplitude of MEPCs and the appearance of a bell-shaped distribution of MEPC amplitudes correlate with the size and frequency of postsynaptic ACh clusters along the nerve contact sites during the first few days in culture (Kidokoro et al., 1980).

Mutational studies of rat VAChT indicate the importance of specific amino acid residues in the transport mechanism. Replacement of aspartate 193 by asparagine completely abolished the enhancement of ACh packaging. This residue is conserved in all VAChT sequences isolated from different species but not in VMATs. Our results are consistent with its possible role in substrate recognition. In contrast, aspartate 398 shows conservation in VMATs as well as VAChTs. Interestingly, the D398N mutation not only blocked the enhancement of ACh packaging produced by VAChT overexpression, but also reduced endogenous Xenopus vesicular ACh transport, as reflected by the reduction in the amplitude and frequency of MEPCs (Figure 7C). The D398N mutation could exert such an effect through multiple distinct mechanisms. First, the mutant could dimerize with endogenous wild-type protein and prevent its activity. However, there is little evidence that any member of this family of proteins or any cotransporter or exchanger of either Na^+ or H^+ functions as an oligomer. Alternatively, the D398N mutant may interfere indirectly with the storage of ACh by dissipating the proton electrochemical driving force for ACh transport or by directly catalyzing the downhill movement of ACh out of synaptic vesicles. Interestingly, mutagenesis of the homologous residues in transmembrane domain 10 of both VMATs supports a selective defect in bioenergetic coupling to the driving force (Steiner-Mordoch et al., 1996; Merickel et al., 1997). Since the pH gradient across the vesicle membrane normally appears to prevent reverse flux by the VMATs (Maron et al., 1983), neutralization of aspartate 398 in VAChT may eliminate its ability to sense the gradient. This will promote ACh efflux, thus reducing the storage of ACh by endogenous Xenopus VAChT.

In summary, we have provided physiological evidence that a putative VAChT protein functions as the ACh transporter across the synaptic vesicle in cholinergic neurons. Furthermore, our results indicate that quantal synaptic transmission can be modulated directly by altering the level of expression of wild-type vesicular transporter or by expressing exogenous mutant proteins. The level of vesicular monoamine transporter content of chromaffin granules was found to be elevated by depolarization of the chromaffin cells (Desnos et al., 1995). Thus, regulation of vesicular transporter activity may provide a natural means of modulating the efficacy of synaptic transmission.

Experimental Procedures

Preparation of cDNA or mRNA for Injection

Rat VAChT cDNA was inserted into the pcDNA I amp vector (Invitrogen) at the EcoR I site. Capped mRNA for VAChT and mutant VAChTs were prepared from pcDNA I-VAChT, linearized with EcoR V, and transcribed in vitro using T7 RNA polymerase (mMESSAGE mMACHINE, Ambion).

Loading of VAChT cDNA or mRNA into Xenopus Embryos

Female Xenopus laevis (Xenopus I) were induced to lay eggs using human chorionic gonadotropin (Sigma), and the eggs were fertilized artificially with testis homogenates. The cDNA construct or mRNA was suspended in RNase-free H_2O mixed with FITC-conjugated dextran (15 mg/ml). The final concentration of the DNA construct is 2 $\mu g/\mu l$; for mRNA, it is 1 $\mu g/\mu l$. About 2 ng DNA or 1 ng mRNA was injected into one blastomere of Xenopus embryo at the 2-cell stage using a Picosprizer (General Valve, NJ), as described previously (Alder et al., 1995). The embryos were kept at room temperature for 24 hr prior to the preparation of dissociated cell cultures.

Immunostaining Analysis

For Western analysis, Xenopus embryos were collected at different time points after in vitro fertilization and homogenized by sonication in 1% SDS. Proteins from one embryo were loaded onto each lane, separated by SDS–polyacrylamide electrophoresis, and blotted onto nitrocellulose. The blot was blocked with 10% dry milk in 50 mM Tris-buffered saline (TBS) (pH 7.4), followed by incubation with polyclonal antibody raised against the C-terminus of the predicted rat VAChT (Gilmor et al., 1996) (1:1000 dilution) for 1 hr at room temperature. Membranes were rinsed and incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution) at room temperature. Chemiluminescent detection was performed with the ECL kit (Amersham) as instructed.

For immunostaining of cells in cultures, 1-day-old cultures were washed in a solution containing 10 mM Tris (pH 7.0), 150 mM NaCl, and 0.05% Tween-20 (TTBS) and blocked with 10% goat serum in TTBS overnight on a Nutator. They were then incubated with polyclonal anti-rat VAChT antibodies (rabbit; 1:1000) and monoclonal synaptophysin antibody (mouse; 1:400) in blocking solution at room temperature for 2 hr. Cultures were washed five times with TTBS, for 1 hr each time, and then incubated in anti-rabbit antibody conjugated to flurescensin (1:1000) and anti-mouse antibody conjugated to rhodamine (1:1000) in blocking solution at room temperature for 2 hr. Following five washes of cultures in TTBS for 1 hr each, the photographs were taken on a Nikon microscope at 40×.

Construction of Mutant VAChTs

Wild-type VAChT was subcloned into pBluescript II SK (Stratagene). Site-directed mutagensis was done by a 2-step PCR protocol. Four primers, including 5′ and 3′ completely overlapping synthetic oligonucleotide primers (28 bases with the desired mutation in the center) and two flanking primers (T3 and T7 primer), were used with wild-type rat VAChT cDNA as the template. Under standard PCR conditions, primers were extended in opposite directions using Pfu DNA polymerase (Stratagene) in two separate reactions. In the third PCR, the purified products from the first two PCR reactions were combined and extended to produce a single fragment with the desired mutation. To verify that the clone by this method contained only the desired mutation but no others, the region flanking the mutation was sequenced in its entirety by a DNA sequencer (Perkin Elmer, Applied Biosystems Model 373). Restriction fragments within this region were then subcloned back into the VAChT cDNA encoding the wild-type protein.

Culture Preparation

Xenopus nerve–muscle cultures were prepared according to previously reported methods (Spitzer and Lamborghini, 1976; Tabti and Poo, 1991) and used for experiments after 1 day of culturing at room temperature (20–22°C). Briefly, the neural tube and associated myotomal tissue of 1-day-old Xenopus embryos (stage 20–22, according to Nieuwkoop and Faber, 1976) were dissociated in Ca^{2+}/Mg^{2+}-free saline supplement with EDTA (115 mM NaCl, 2.6 mM KCl, 0.4 mM EDTA, and 10 mM HEPES [pH 7.4]) for 15–20 min. The cells were plated on clean glass coverslips and were used for experiments after 24 hr incubation at room temperature. The culture medium consisted of 50% (v/v) of Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, and 10 mM HEPES [pH 7.6]), 49% of Leibovitz medium (L15, GIBCO), and 1% fetal bovine serum (GIBCO).

Electrophysiology

Synaptic currents were recorded from innervated muscle cells by a whole-cell recording method (Hamill et al., 1981; Young and Poo, 1983). Muscle cells were voltage-clamped at −70 mV. The membrane currents were monitored by patch-clamp amplifiers (Axopatch-1D, Axon Instruments). The membrane currents were filtered at 5 kHz, digitized, and stored in a videotape recorder for later playback onto a storage oscilloscope (Tektronix 5113). Computer analysis was performed with the SCAN program kindly provided by Dr. J. Dempster (Strathclyde University, UK). The internal solution in the recording pipette for myocytes contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, 5 mM magnesium ATP, 0.5 mM sodium ATP, and 10 mM HEPES (pH 7.2). Recordings were made at room temperature in a solution containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). For L-(−)-vesamicol experiments, the recording solution contained 2 μM L-(−)-vesamicol. Iontophoresis of ACh was performed by methods previously described (Orida and Poo, 1978). To compare the ACh sensitivity on the myocyte surface with and without the vesamicol treatment, the tip of the iontophoretic pipette was positioned tangentially, in contact with the myocyte surface, and whole-cell membrane currents induced by identical 1 ms ACh pulses were measured. The ACh pipette was a conventional sharp micropipette pulled by a horizontal puller (Shutter Inst.), and filled with 3 M ACh chloride (resistance 100–300 MΩ). Repeated mapping of ACh responses at the same site on the cell surface usually yields reproducible membrane currents, with a variability of <10% (Orida and Poo, 1978).

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