Passive Uptake of Acetylcholine and Other Organic Cations by Synaptic Vesicles from *Torpedo* Electric Organ†

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ABSTRACT: Homogeneously cholinergic synaptic vesicles isolated from the electric organ of *Torpedo californica* were depolarized by high external sodium ion concentrations in the presence of gramicidin and by external carbamoylcholine, as detected by triphenyl[^{14}C]methylphosphonium ion partitioning. Vesicles resuspended in low sodium ion media containing acetylcholine immediately hyperpolarized upon addition of gramicidin but then spontaneously depolarized as indicated by 3,3′-dipropylthiadicarbocyanine [diS-C_3-(5)] fluorescence, with greater depolarization at higher acetylcholine concentrations. Eleven other monovalent organic cations showed similar behavior, but some depolarized the vesicles more slowly and some more rapidly than acetylcholine did, with over a 200-fold variation in rate. Vesicles resuspended in sucrose and buffer exhibited spontaneous hyperpolarization due to net electrogenic efflux of one or more endogenous cations, as seen by diS-C_3-(5) fluorescence. Vesicles resuspended in low sodium ion media containing either 210 mM acetylcholine, 210 mM choline, or 210 mM *dl*-lysine underwent electroneutral exchange between the external organic cation and one or more endogenous gramicidin-releaseable cations, as seen by diS-C_3-(5) fluorescence. Vesicles preloaded with [^{14}C]mannitol took up [^{3}H]acetylcholine with a half-life of 8 min at 25 °C. Comparison of the ratio of ^{3}H to ^{14}C inside of the vesicles to the ratio outside gave an equilibrium concentration for [^{3}H]acetylcholine inside relative to that outside of ~1. Uptake was slowed fourfold at 4 °C, was directly proportional to the external [^{3}H]acetylcholine concentration up to 50 μM, and was osmotically labile. [^{3}H]Choline behaved similarly. Thus, acetylcholine and other monovalent organic cations are taken up by isolated cholinergic vesicles into the internal solution via an electrogenic process which can be driven by efflux of one or more endogenous gramicidin-releasable cations under the experimental conditions.

Acetylcholine released by exocytosis from the stimulated cholinergic nerve terminal in *Torpedo californica* electric organ is stored in membrane-enclosed synaptic vesicles (Whittaker & Dowdall, 1975; Waser, 1975; Zimmermann & Whittaker, 1974). The depleted vesicles are recovered locally in the nerve terminal and appear to undergo a maturation process involving loading with acetylcholine (Zimmermann & Denston, 1977a,b). A synaptic vesicle recycling model postulating the presence in the nerve terminal of depot vesicles, active vesicles, and demobilized vesicles has been formulated for the electric organ (Suszkiw et al., 1978). The model suggests that active vesicles near the presynaptic membrane undergo repetitive exocytosis, endocytosis, and reloading with newly synthesized acetylcholine. Depot vesicles resupply the active pool as it becomes demobilized for unknown reasons.

The isolated vesicles are membrane-enclosed spherical organelles ~850 Å in diameter. Lipid and protein compositions (Ohsawa et al., 1979; Tashiro & Stadler, 1978; Wagner et al., 1978) and protein accessibilities (Wagner & Kelley, 1979) have been partially characterized. The vesicles contain about 0.6 M acetylcholine, 0.16 M ATP, and common inorganic ions (Schmidt et al., 1976) and behave like perfect osmometers at physiological osmolarities (Breer et al., 1978). They establish a transmembrane electrical potential which depends on the external sodium ion concentration when exposed to gramicidin (Carpenter & Parsons, 1978).

The vesicles contain a Ca^{2+},Mg^{2+}-ATPase, very modestly stimulated by acetylcholine, which might participate in acetylcholine storage (Breer et al., 1977; Rothlein & Parsons, 1979). As background for seeking the mechanism of energy-linked specific uptake of acetylcholine, we have studied the characteristics of the passive uptake of acetylcholine which occurs with highly purified cholinergic vesicles obtained from the electric organ of *T. californica*.

Materials and Methods

Live *T. californica* was obtained locally, and the electric organs were stored at −100 °C until used. [^{14}C]TPMP[1] (36 Ci/mol) was a generous gift from Dr. Frederick W. Dahlquist, Institute of Molecular Biology, University of Oregon, Eugene, OR. The dye diS-C_3-(5) was synthesized as described (Sims et al., 1974). [^{3}H]Acetylcholine chloride (250 mCi/mmol), D-[1-^{14}C]mannitol (56.8 mCi/mmol), and N-[propionyl-^{3}H]propionylated α-bungarotoxin (58 Ci/mmol) were from Amersham Searle. Nonradioactive α-BuTx was supplied by Boehringer/Mannheim Biochemicals, and gramicidin (Dubos), Triton X-100, and diethyl *p*-nitrophenyl phosphate (Paraoxon) came from Sigma Chemical Co. All other chemicals were of the highest grade commercially available.

Synaptic vesicles used in fluorescence studies were isolated from *T. californica* electric organ by the zonal centrifugation procedure of Whittaker et al. (1972b). This procedure uses 800 milliosmolar NaCl–sucrose medium for the isolation at the buoyant density of vesicles. Synaptic vesicles used in the [^{14}C]TPMP and [^{3}H]AcCh partitioning experiments and in lysis experiments were isolated similarly to the methods of Nagy et al. (1976) and Carlson et al. (1978) in 800 millios-

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[1] Abbreviations used: diS-C_3-(5), 3,3′-dipropylthiadicarbocyanine iodide; [^{14}C]TPMP, triphenyl[^{14}C]methylphosphonium bromide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; AcCh (ACh in the figures), acetylcholine; α-BuTx, α-bungarotoxin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; ATP, adenosine 5′-triphosphate.

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molar glycine and glycine–sucrose medium at pH 7.0. The method involves differential sedimentation velocity pelleting, equilibrium buoyant density centrifugation, and controlled-pore glass-bead filtration of vesicles. These vesicles were found to be contaminated by 1–4% acetylcholine receptor when assayed in Triton X-100 (Schmidt & Raftery, 1973), with an average of 2.6% (six determinations). The moles of receptor of M_r 270000 were determined as half of the moles of nonradioactive α-BuTx required to completely block binding of [^{3}H]-α-BuTx added 120 min later. The small amount of receptor significantly affected [^{3}H]AcCh uptake results only at concentrations below 25 μM. The nonradioactive α-BuTx was found to contain a large quantity of contaminating phospholipase activity, as assayed by the method of Boehringer/Mannheim Co. (1970), and it was purified by gel filtration on Sephadex G-50 Fine (Pharmacia Co.). The purified toxin was shown to cause an irreversible neuromuscular blockade of the rat phrenic nerve–diaphragm preparation. Protein was determined as described (Bradford, 1976).

Fluorescence experiments were conducted as described (Carpenter & Parsons, 1978). Acetylcholine solutions contained 30 μM eserine sulfate to inhibit acetylcholine esterase. Uptake of [^{14}C]TPMP by synaptic vesicles was determined for vesicles which were transferred into the indicated solutions by centrifugal pelleting, resuspension, and dilution in a manner similar to that of the [^{3}H]AcCh experiments, except that gramicidin and [^{14}C]TPMP ethanol stock solutions resulted in 0.5% v/v final ethanol concentrations.

For [^{3}H]AcCh-uptake experiments, isolated vesicles were centrifuged 3 h at 25000 rpm (90000g) in an SW 25.1 rotor (Beckman) at 4 °C. The supernatant was poured off, and each pellet was resuspended in 0.5 mL of 800 mM glycine, 5 mM Hepes, 1 mM EDTA, 1 mM EGTA, and 0.02% (w/v) NaN_3, pH 7.0, with NaOH (referred to as glycine buffer) and pooled with the others. Resuspended vesicles were mixed with an equal volume of glycine buffer containing a two-fold excess of phospholipase-free α-Butx, as determined by prior titration of acetylcholine receptor, and 28 μM [^{14}C]mannitol and incubated 7 h at 25 °C. Absence or presence of a tenfold excess of α-BuTx had no effect on the uptake of 50 μM [^{3}H]AcCh. The solution was made 0.44 mM in Paraoxon and incubation continued for 60 min. Uptake of [^{3}H]AcCh was initiated by mixing the vesicle suspension with an equal volume of glycine buffer containing 14 μM D-[1-^{14}C]mannitol and twice the desired final molarity of [^{3}H]AcCh. The [^{14}C]mannitol concentration thus did not change. Solutions of radioisotopes were made by drying the desired radioisotope(s) in a test tube under a flow of nitrogen gas, adding glycine buffer, and vortexing prior to adding the radioactive solution to vesicles. The addition of Paraoxon and gramicidin was accomplished by drying them under N_2 in a clean test tube and transferring the vesicle solution to the same tube. Thus, no organic solvents were added to these vesicle suspensions.

Uptake of radioactive isotopes by vesicles was assayed for 0.25-mL portions by centrifugation gel filtration (Neal & Florini, 1973) at 4 °C on 2-mL columns of Sephadex G-50 medium equilibrated in glycine buffer and prepared in 3-mL plastic syringe barrels, except for the hypoosmotic shock experiment. The excluded volume containing separated vesicles was quantitatively transferred with three 0.2-mL washes of water to polyethylene scintillation vials, 10 mL of Aquasol 2 (New England Nuclear) was added, and the radioactivity was determined in a Beckman Model LS-3155T scintillation spectrophotometer. For double-label experiments, 20 μL of the total uptake solution was transferred to a similar counting medium to determine total ^{3}H and ^{14}C disintegrations per minute (dpm) in solution.

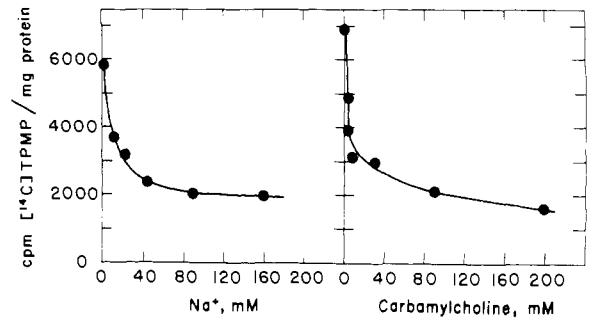

FIGURE 1: Uptake of [^{14}C]TPMP by synaptic vesicles as a function of external ion concentrations. Left frame: portions (150 μL) of synaptic vesicles (1.5 mg of vesicle protein/mL in 800 mM sucrose and 5 mM Hepes, pH 7.0 with NaOH; final Na^+ concentration 1 mM) were added to equal volumes of solutions containing increasing NaCl concentrations, enough sucrose to maintain the osmolarity at 800 mM, 56 μg/mL gramicidin, and 1.1×10^6 dpm [^{14}C]TPMP. Samples were incubated for 30 min at 25 °C before assay for bound [^{14}C]TPMP by centrifugation gel filtration. Separate experiments demonstrated that [^{14}C]TPMP uptake reached equilibrium in 10 min. The low concentration of [^{14}C]TPMP used should not affect the membrane potential. Right frame: similar solutions of vesicles were added to equal volumes of solutions containing increasing carbamoylcholine chloride concentrations, enough sucrose to maintain the osmolarity at 800 mM, gramicidin, and [^{14}C]TPMP. Samples were incubated for 2 h at 25 °C before assay for bound [^{14}C]TPMP by centrifugation gel filtration.

Double-channel liquid scintillation counting was done with the A channel set at 20–150 and the B channel set at 280–1000 utilizing automatic quench control (gain = 290; AQC = 0.500; ESCR = 0.750). Counting efficiencies determined with [^{3}H]- and [^{14}C]toluene standards (New England Nuclear) were 0.22 and 0.006 for ^{3}H and 0.05 and 0.69 for ^{14}C in channels A and B, respectively. Disintegrations per minute were calculated according to Hendee (1973). The uptake ratio was calculated from the obtained dpm by

$$\text{uptake ratio} = \frac{\text{dpm}(^3\text{H}_v)/\text{dpm}(^{14}\text{C}_v)}{\text{dpm}(^3\text{H}_s)/\text{dpm}(^{14}\text{C}_s)} \quad (1)$$

where v = separated vesicle sample and s = reaction solution.

Results

Fluorescence Validation with [^{14}C]TPMP. By use of a fluorescence approach, evidence was previously obtained that sodium ion diffusion potentials can be established for at least several minutes across the cholinergic vesicle membrane in the presence of gramicidin (Carpenter & Parsons, 1978). Uptake of the lipophilic cation [^{14}C]TPMP by synaptic vesicles resuspended in the presence of variable external sodium ion concentrations was determined in the presence of gramicidin to substantiate this conclusion. [^{14}C]TPMP partitions into organelles passively in response to the transmembrane electrical potential (Ramos & Kaback, 1977). The results in Figure 1 show that increased external sodium ion concentrations resulted in decreased [^{14}C]TPMP binding to synaptic vesicles. The result indicates relative depolarization of vesicles by higher external sodium ion concentrations and is consistent with the previous conclusion based on the fluorescence of diS-C_3-(5). Thus, in the following fluorescence experiments, a more negative (hyperpolarized) transmembrane electrical potential decreases the fluorescence by partitioning more dye into the bound state where the fluorescence is quenched (Sims et al., 1974). A more positive (depolarized) transmembrane electrical

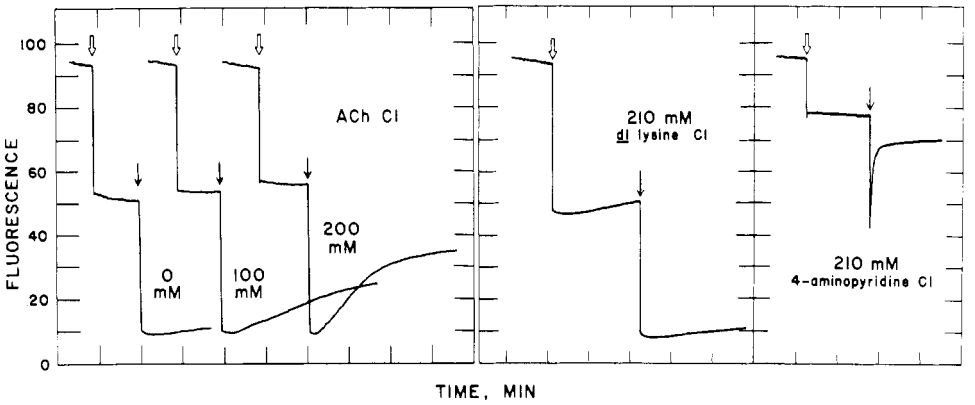

FIGURE 2: Left: fluorescence rebound as a function of external acetylcholine chloride concentration. The acetylcholine (ACh in the figure) chloride concentrations were 0, 100, or 200 mM with 200, 100, or 0 mM *dl*-lysine chloride and 4 mM sodium, 5 mM Hepes, and 380 mM sucrose present to maintain approximately constant ionic strength, osmolarity, and pH 7.2. Vesicles resuspended in the indicated solutions were added to the same solutions containing diS-C_3-(5) at the hollow arrow. Gramicidin was added at the line arrow. Time divisions are 1 min each. The low concentration of diS-C_3-(5) used should not affect the membrane potential significantly. Other concentrations of AcCh were studied at 25, 50, and 150 mM and gave the type of interpolated behavior expected from these selected curves. Right: fluorescence-rebound behavior for vesicles in 4-aminopyridinium and *dl*-lysine cation solutions. Synaptic vesicles were resuspended in 210 mM cation chloride containing 380 mM sucrose and 5 mM Hepes, pH 7.2. The pH was adjusted to 7.2 with NaOH for the *dl*-lysine chloride solution and with HCl for the 4-aminopyridinium chloride solution. Vesicles resuspended in the indicated solutions were treated similarly to those in the left frame.

potential increases the fluorescence by partitioning more dye into the free state where it fluoresces highly.

Acetylcholine-Induced Depolarization of Vesicles. The diS-C_3-(5) fluorescence response for vesicles isolated in a high sodium ion medium and resuspended in solutions containing a low sodium ion concentration and variable acetylcholine concentration was studied to characterize the effect of acetylcholine on the membrane potential of vesicles. Figure 2 shows that immediately after addition of gramicidin, a large decrease in fluorescence occurred consistent with hyperpolarization of the vesicles. This was followed by a spontaneous fluorescence rebound whose rate increased at higher external acetylcholine concentrations. The extent of fluorescence quenching occurring immediately after addition of gramicidin was constant and independent of the acetylcholine concentration, as was expected since the external sodium ion concentration was held approximately constant. Also, the external ionic strength and chloride ion concentration were held constant by using *dl*-lysine chloride to replace acetylcholine chloride. Therefore, the fluorescence rebound effect is not related to chloride or nonspecific ionic strength effects on the membrane structure. Thus, the acetylcholine-dependent fluorescence rebound is consistent with depolarization of the vesicles as a result of acetylcholine uptake.

Specificity of Depolarization. The specificity of the depolarization process was examined with other organic cations, and the very different responses observed for the two cations exhibiting opposite extremes of behavior also are shown in Figure 2. Vesicles resuspended in *dl*-lysine chloride showed a very slow spontaneous increase in fluorescence after hyperpolarization, suggesting little depolarization due to *dl*-lysine uptake. In sharp contrast, vesicles that were resuspended in 4-aminopyridinium chloride showed a very rapid spontaneous increase in fluorescence after hyperpolarization, suggesting rapid depolarization due to 4-aminopyridinium uptake. The fluorescence level obtained in the presence of 4-aminopyridinium before gramicidin addition was significantly higher than that in *dl*-lysine, possibly indicating that 4-aminopyridinium depolarized the vesicles even before gramicidin addition. The 4-aminopyridinium results demonstrate that the fluorescence response is rapidly reversible. Therefore, fluorescence rebound occurring at moderate or slow rates as in the cases of other cations is not limited by the dye desorption rate from the vesicles. The fluorescence rebound rate can be taken as accurately reflecting relative vesicle depolarization rates in these cases.

Table I: Depolarization Rates for Organic Monovalent Cations

cation[a]	rate rel to AcCh[b]	cation[a]	rate rel to AcCh[b]
lysine[c]	0.04	Tris[d]	0.9
carbamoylcholine	0.1	acetylcholine	1
arginine	0.1	guanidinium	2
tetramethylammonium	0.2	triethanolamine[e]	4
tetraethylammonium	0.2	morpholine[f]	4
choline	0.4	4-aminopyridine[g]	>10

[a] Cation at 210 mM at pH 7.2 with chloride present as the counterion. [b] Determined from maximal recording slope of the spontaneous fluorescence rebound after gramicidin-induced hyperpolarization in low sodium ion media. [c] $pK_1 = 2.2$, $pK_2 = 9.0$, and $pK_3 = 10.5$. [d] $pK = 8.3$. [e] $pK = 7.8$. [f] $pK = 8.5$. [g] $pK = 9.2$.

The fact that the depolarization rate was strongly dependent on the nature of the external organic cation further suggests that depolarization is due to uptake of the external cation, as was concluded from the acetylcholine concentration studies, rather than to efflux of an endogenous anion. Thus, the relative depolarization rates listed in Table I for 12 organic cations can be taken as measures of their relative permeabilities in the hyperpolarized synaptic vesicle membrane. None of the cations tested was found to be totally impermeable under these conditions, but their apparent rates varied by over 200-fold.

Confirmation of Carbamoylcholine-Induced Depolarization with [^{14}C]TPMP. [^{14}C]TPMP partitioning was utilized to confirm the occurrence of carbamoylcholine-induced depolarization of vesicles. The addition of 25 mM carbamoylcholine chloride to vesicles equilibrated at 25 °C with [^{14}C]TPMP in low sodium ion medium resulted in a time-dependent decrease of bound [^{14}C]TPMP (half-life of 15 min, not shown). The results in Figure 1 show that increasing external carbamoylcholine concentrations decreased the amount [^{14}C]TPMP taken up, which is consistent with depolarized synaptic vesicles, in agreement with the fluorescence results.

Exchange of an Endogenous Gramicidin-Releasable Cation with External Organic Cations. The effect of delaying the time of addition of gramicidin to the vesicles isolated in a high

sodium ion medium was investigated as shown in Figure 3. Vesicles in sucrose containing no external organic cation showed a large spontaneous decrease in fluorescence in the absence of gramicidin. Subsequent gramicidin addition resulted in some additional fluorescence decrease. Early addition of gramicidin resulted in immediate fluorescence quenching to a stable value similar to that achieved spontaneously. Similar spontaneous hyperpolarization results were obtained in isoosmotic 210 mM betaine and succinylcholine chloride, except that in succinylcholine rebound depolarization also occurred. These results demonstrate that in the absence of external monovalent organic cations net electrogenic efflux of endogenous cations occurred spontaneously.

In the presence of acetylcholine, choline, or *dl*-lysine solutions, delayed gramicidin addition resulted in a nearly unchanging fluorescence, indicating little net electrogenic ion flux (Figure 3). However, addition of gramicidin to vesicle suspensions after longer incubation times produced substantially less hyperpolarization in acetylcholine and choline media but not in lysine medium. The time-dependent decrease in gramicidin-induced fluorescence quenching in acetylcholine and choline is consistent with the following interpretation. Since gramicidin addition establishes a sodium ion diffusion potential across the vesicle membrane and since the external sodium ion concentration was constant during the incubations, the internal concentration of an endogenous gramicidin-releasable cation apparently decreased upon prolonged incubation in acetylcholine and choline solutions. Because the endogenous cation was lost under electroneutral conditions before gramicidin addition, it apparently was spontaneously exchanged with external acetylcholine or choline during the incubation.

The extent of the fluorescence quenching produced by gramicidin as a function of incubation time is plotted in Figure 3. Since the fluorescence quenching is a logarithmic function of the transmembrane sodium ion gradient (Carpenter & Parsons, 1978), Figure 3 is similar to a first-order plot of the loss of endogenous gramicidin-releasable cation from vesicles. Lysine apparently slows this rate greatly, whereas acetylcholine stimulates it by a factor of ~2 over the rate in choline.

The possibility that the high external acetylcholine concentrations caused lysis of synaptic vesicles and thus loss of gramicidin-induced hyperpolarization was examined by monitoring the ATP content of vesicles treated similarly to those of Figure 3. No loss of vesicular ATP was found for vesicles in acetylcholine, carbamoylcholine, or *dl*-lysine chloride, thus eliminating vesicle lysis under these conditions (not shown).

[^3H]*Acetylcholine Uptake by Vesicles.* Whether vesicles took up [^{3}H]acetylcholine was studied in the presence of [^{14}C]mannitol by using a double-label counting technique. This approach was taken in order to facilitate accurate and unambiguous determination of the concentrative extent of [^{3}H]acetylcholine uptake. Mannitol will distribute at equal concentrations between the external solvent volume and the vesicle internal solvent volume. Uptake of [^{14}C]mannitol was found to reach equilibrium in ~6 h at 25 °C, to be proportional to the concentration of vesicles present, and to correspond to 5.3 ± 0.8 μL of internal solvent volume/mg of vesicle protein (5 determinations, ±1σ). In typical experiments reported below, ~0.1% of the total solution volume was present inside of the vesicles. By comparing the [^{3}H]acetylcholine to [^{14}C]mannitol ratio inside of vesicles separated by centrifugation gel filtration to the ratio outside of the vesicles, one obtains an "uptake ratio" for [^{3}H]acetylcholine. At a [^{3}H]-

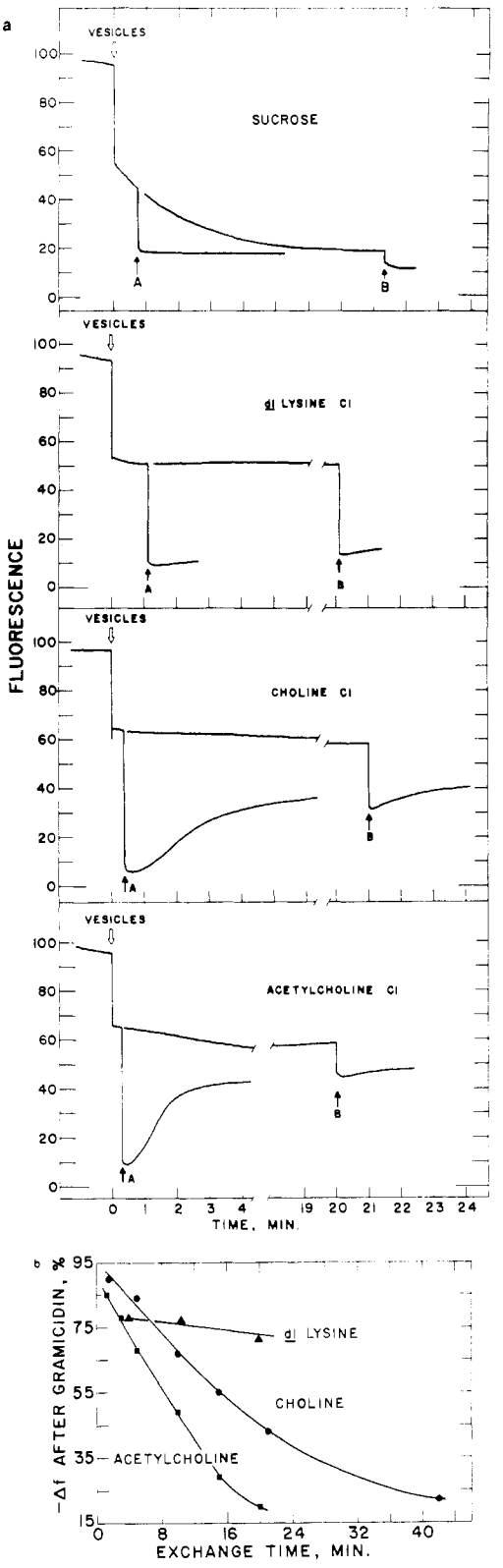

FIGURE 3: Fluorescence behavior for delayed gramicidin addition. (a) Vesicles resuspended in 800 mM sucrose and 5 mM Hepes or in 210 mM acetylcholine, 210 mM choline, or 210 mM *dl*-lysine chloride containing 380 mM sucrose and 5 mM Hepes, pH 7.2 with NaOH, were added immediately to the same solution containing diS-C_3-(5) at the hollow arrow. Gramicidin was added shortly after resuspension at time A or after a longer incubation period at time B. Each time division is 1 min. (b) The gramicidin-induced fluorescence decrease in organic cation solutions is graphed vs. time. The half-life for loss of the fluorescence decrease after the addition of gramicidin was 7 min in acetylcholine chloride, 14 min in choline chloride, and very long in *dl*-lysine chloride solutions.

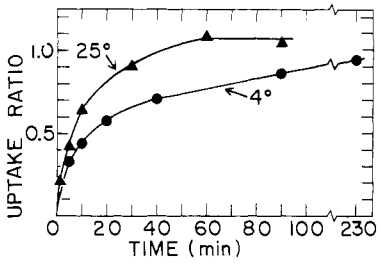

FIGURE 4: Time-dependent uptake of [^{3}H]ACh by *Torpedo* electric organ synaptic vesicles at 25 and 4 °C. Synaptic vesicles (2.0 mL of resuspended pellets in glycine buffer) were treated with excess α-BuTx (2.0 nM) and Paraoxon and equilibrated with [^{14}C]mannitol as described under Materials and Methods. One milliliter of vesicles was transferred to 4 °C and uptake of 50 μM external [^{3}H]ACh initiated at 25 and 4 °C. The final vesicle protein concentration was 1.34 mg/mL in a total of 2.0 mL at each temperature. The uptake of isotopes was determined periodically by centrifugation gel filtration. The dpm taken up per datum varied from 10000 to 58000 for [^{3}H]AcCh and from 4300 to 4700 for [^{14}C]mannitol with ~100 cpm of background for each isotope found for similar samples not containing synaptic vesicles. The uptake ratio gives the concentration of [^{3}H]AcCh inside the vesicle relative to its concentration outside as calculated by eq 1.

acetylcholine concentration inside the vesicle equal to that outside, the uptake ratio would equal 1, whereas concentrative acetylcholine uptake would give an uptake ratio >1. This method significantly reduces data scatter since losses of bound [^{3}H]acetylcholine due both to variable recovery of vesicles and to potential lysis of vesicles are corrected by normalization to the internal [^{14}C]mannitol.

Figure 4 shows the time course for uptake of [^{3}H]acetylcholine by vesicles which were not subjected to sodium-induced hyperpolarization. Uptake at 25 °C occurred rapidly with a half-life of ~8 min, which was similar to the rate determined by fluorescence (Figure 3). The equilibrium uptake ratio reached in ~60 min was 1.1. At 4 °C the uptake rate slowed by about fourfold but appeared to approach the same equilibrium uptake ratio at long time periods. The time dependence and low-temperature inhibition are consistent with transmembrane uptake of [^{3}H]acetylcholine. Similar results also were obtained for [^{3}H]choline uptake at 25 °C (not shown).

Concentration Dependence of [^{3}H]Acetylcholine Uptake. The uptake of [^{3}H]acetylcholine was studied as a function of the external [^{3}H]acetylcholine concentration at both 25 and 4 °C as shown in Figure 5. Uptake was directly proportional to the external concentration in both cases, with a small but significant inhibition due to low temperature again evident. No indication of a saturable component in [^{3}H]acetylcholine uptake over the 0–50 μM concentration range was evident, thus making it unlikely that binding of [^{3}H]acetylcholine to specific vesicular proteins caused uptake. This is again consistent with uptake into the vesicle interior solution. Similar direct proportionality of [^{3}H]choline uptake at 25 °C also was observed (not shown).

Osmotic Lability of Bound [^{3}H]Acetylcholine. Loaded vesicles were placed for variable time periods on either isoosmotic gel filtration columns or hypoosmotic columns before they were centrifuged for assay to further examine the nature of vesicle-bound [^{3}H]acetylcholine. In this way increasing time periods of exposure of loaded vesicles to hypoosmotic conditions readily could be obtained along with isoosmotic controls. The results in Figure 6A indicate that osmotic lysis was quite rapid since much of the [^{14}C]mannitol was immediately lost from shocked vesicles. If the vesicles reseal after lysis, as has been suggested (Breer et al., 1978),

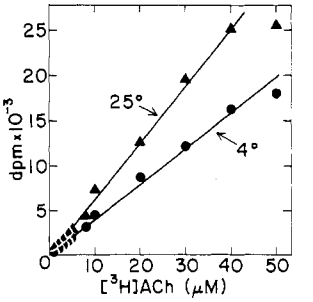

FIGURE 5: Uptake of [^{3}H]AcCh as a function of external [^{3}H]AcCh concentration at 25 and 4 °C. Synaptic vesicles in glycine buffer treated with excess α-BuTx (1.2 nM) and Paraoxon were equilibrated with [^{14}C] mannitol as described under Materials and Methods. [^{3}H]AcCh uptake was initiated at 25 and 4 °C. Uptake was allowed to proceed for 90 min before assay by centrifugation gel filtration. Final vesicle protein concentration was 0.44 mg/mL in total volumes of 0.30 mL for each [^{3}H]AcCh concentration at each temperature. The number of dpm of [^{3}H]AcCh taken up which is given on the ordinate has been normalized to a constant averaged amount of [^{14}C]mannitol. The uptake ratio at 25 °C was constant at 0.86 and at 4 °C was 0.54.

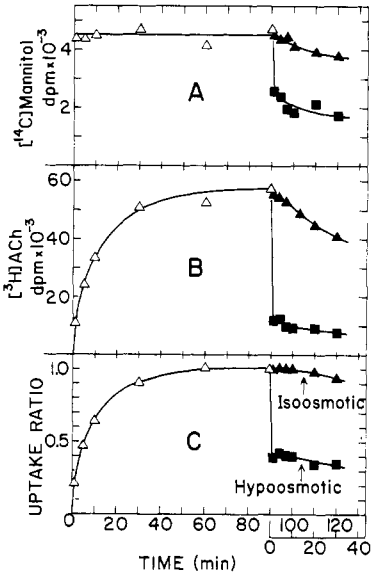

FIGURE 6: (A) Effect of hypoosmotic shock on retention of [^{14}C]-mannitol by synaptic vesicles. Synaptic vesicles in glycine buffer treated with excess α-BuTx (2.0 nM) and Paraoxon were equilibrated with [^{14}C]mannitol as described under Materials and Methods. Uptake of 50 μM external [^{3}H]AcCh was initiated at the first time 0 at the left at 25 °C. Final vesicle protein concentration was 1.34 mg/mL in a 6.0-mL volume. Vesicle content of [^{14}C]mannitol (△) and of [^{3}H]AcCh (B) was assayed periodically by immediate centrifugation gel filtration on isoosmotic columns. After 90 min samples were hypoosmotically shocked (■) by incubating 0.25-mL portions on centrifugation gel filtration columns equilibrated at 4 °C with 5 mM Hepes, 1 mM EDTA, 1 mM EGTA, and 0.02% (w/v) NaN_3, pH 7.0 with NaOH, for increasing time periods before centrifugation. The second time 0 at the right indicates the start of hypoosmotic shock. Similarly, treated controls were incubated for increasing time periods on isoosmotic centrifugation gel filtration columns equilibrated in glycine buffer before centrifugation (▲). (B) Effect of hypoosmotic shock on retention of [^{3}H]AcCh by synaptic vesicles. These results were obtained simultaneously with those in (A). Uptake of 50 μM external [^{3}H]AcCh at 25 °C was followed up to 90 min (▲), after which vesicles were incubated at 4 °C on hypoosmotic (■) or isoosmotic (▲) centrifugation gel filtration columns for increasing time periods before centrifugation. (C) Effect of hypoosmotic shock on the uptake ratio. The data of (A) and (B) are expressed as the uptake ratio.

total loss of the [^{14}C]mannitol is not expected. This method of osmotic shock results in only a two- to threefold dilution of the external [^{14}C]mannitol concentration since the incubation solution equilibrates only with the column solvent

volume within the gel particles with which it is in contact. Under isoosmotic conditions a slow loss of vesicular [^{14}C]-mannitol occurred, as expected (Figure 6A).

Figure 6B shows the [^{3}H]acetylcholine incorporation data for the same experiment. Uptake was again time dependent until equilibrium was reached. After osmotic shock most of the bound [^{3}H]acetylcholine was immediately lost whereas under isoosmotic conditions a relatively slow loss occurred. These data also are presented in Figure 6C in terms of the uptake ratio. Native vesicles achieved an uptake ratio of 1.0 whereas the shocked vesicles exhibited an uptake ratio of ~0.4. Vesicles transferred to the isoosmotic conditions nearly maintained their 1.0 uptake ratio. Thus, [^{3}H]acetylcholine taken up by synaptic vesicles behaves osmotically as if it is localized in the vesicle interior solution.

Discussion

Several groups have attempted to demonstrate acetylcholine uptake by brain or electric organ synaptic vesicles in vitro (Suszkiw, 1976; Mattson & O'Brien, 1976; Whittaker et al., 1972a; Guth, 1969; Kuriyama et al., 1968; Marchbanks, 1967). Except for our previous indirect demonstration (Carpenter & Parsons, 1978), these attempts were negative or inconclusive. However, the other previous attempts to examine acetylcholine uptake by synaptic vesicles suffered from serious disadvantages. In general, sensitivity was poor either because heterogeneous brain synaptic vesicles were used or because relatively small amounts of but partially pure *Torpedo* vesicles were used. Only recently have the rather large amounts of highly purified homogeneously cholinergic vesicles which were used here become available.

It seems clear from these results, which are based on three independent types of observations, that exogenous acetylcholine spontaneously permeates the cholinergic synaptic vesicle membrane under the experimental conditions and becomes incorporated into the vesicle internal solution. The uptake could be detected indirectly by its depolarizing effect on vesicles as seen by increased diS-C_3-(5) fluorescence. The uptake of the close analogue carbamoylcholine could be detected indirectly by its depolarizing effect on vesicles as seen by decreased [^{14}C]TPMP binding. Finally, direct detection of [^{3}H]acetylcholine uptake was possible. Some of the characteristics of acetylcholine uptake under these conditions are that it is electrogenic, is proportional to the external acetylcholine concentration, and can be driven by the hyperpolarizing efflux of one or more gramicidin-releasable endogenous cations (Myers & Haydon, 1972). It has long been known that endogenous acetylcholine rapidly leaks from isolated vesicles at 25 °C, whereas ATP does not (Whittaker, 1973; Marchbanks & Israel, 1971). This could be due to a cation-exchange mechanism similar to that observed in these studies, except that exchange would occur in the reverse manner.

Candidates for the endogenous gramicidin-releasable cations are the sodium ion and the proton. Vesicles have been reported to contain a large amount of sodium ion (Schmidt et al., 1976), and the vesicles used for the fluorescence studies were isolated in high sodium ion media. The proton is also a possible efflux candidate in view of the low pH found in the analogous neurosecretory chromaffin granules (Pollard et al., 1976). No attempts to quantitate the absolute membrane electrical potential of vesicles on the basis of diS-C_3-(5) and [^{14}C]TPMP measurements were made since the identity of all ions contributing to the potential is uncertain, and determination of zero-potential conditions was not attempted. However, relatively more hyperpolarizing or depolarizing membrane potentials unambiguously were achieved in sodium-isolated vesicles by the manipulations reported here. Electrical coupling of sodium ion efflux to acetylcholine uptake might contribute to vesicle recycling, since used vesicles recovered by endocytosis presumably are loaded with sodium ion obtained from the synaptic cleft, and they would be present in a relatively low sodium ion environment within the nerve terminal cytoplasm.

When uptake was not driven by imposed hyperpolarization of the vesicles, [^{3}H]acetylcholine was accumulated by the vesicles at approximately the same concentration as it was present in the outside solution, suggesting uptake into "deenergized" zero-potential vesicles. From the time course of uptake the permeability coefficient for [^{3}H]acetylcholine can be estimated from (Goldin & Seadner, 1975)

$$P_{AcCh} = 0.693(R/3)(1/t_{1/2(AcCh)}) \quad (2)$$

where P_{AcCh} is the acetylcholine permeability coefficient, R is the mean vesicle radius, and $t_{1/2(AcCh)}$ is the half-life required to achieve an equilibrium internal concentration equal to the external concentration. Taking R as 400 Å and $t_{1/2(AcCh)}$ as 480 s yields P_{AcCh} equal to 2×10^{-9} cm/s. This value compares, for example, with P_{Na^+} of 10^{-13}–10^{-12} cm/s for self-diffusion of sodium ion in various types of sonicated phospholipid vesicles (Papahadjopoulos et al., 1971). Determination of the structural significance of the high acetylcholine permeability coefficient must await further studies.

The vesicle membrane is permeable to a wide variety of exogenous organic monovalent cations in addition to acetylcholine under these conditions. This suggests that specific transport of acetylcholine is not being observed. While the external concentrations of acetylcholine and other organic cations used in parts of this study might seem high relative to typical concentrations used in membrane transport studies and thus might seem possibly to result in artifactual nonspecificity, they are low compared to the internal concentration of endogenous acetylcholine. Thus, the membrane in vivo must be relatively impermeable to even 0.6 M acetylcholine, and use of 0.2 M external concentrations of acetylcholine and other organic cations to study vesicle behavior in vitro is not inappropriate for the native membrane structure. No evidence for significant chloride permeability was seen, although this was not tested directly.

Some possible reasons for the lack of more substantial specificity in acetylcholine uptake follow. Protein factors modulating acetylcholine permeability in vivo and making the membrane more specific for acetylcholine might be removed from purified vesicles. Similarly, magnesium ion or other small cytoplasmic factors such as ATP might interact with the membrane lipid or a transport system to decrease its nonspecific organic cation permeability. Finally, damage to the membrane during isolation might allow it to maintain its integrity at 4 °C (to account for retention of acetylcholine and inorganic ions) but could result in cation leakiness at 25 °C. The membrane is not completely nonspecifically permeable to organic cations, however, since a greater than 200-fold difference in depolarization rates was found. Furthermore, even the in vivo vesicle storage system is not completely specific since acetyl-*N*-methyl-*N*-2-hydroxyethylpyrrolidinium ion, acetylhomocholine, and homocholine all can be taken up as "false transmitters" (Zimmermann & Dowdall, 1977; Luqmani et al., 1980).

In summary, these experiments demonstrate the utility of diS-C_3-(5) fluorescence and [^{14}C]TPMP, [^{3}H]acetylcholine, and [^{14}C]mannitol partitioning techniques for characterization of the electrogenic nature and the extent of acetylcholine uptake by vesicles. The experiments also establish passive

uptake behavior beyond which a specific energy-linked acetylcholine uptake system must perform.

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