Biochemistry

 Volume 21, Number 13 June 22, 1982

# Proton Gradient Linkage to Active Uptake of [$^3$H]Acetylcholine by *Torpedo* Electric Organ Synaptic Vesicles†

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ABSTRACT: It has been confirmed that cholinergic synaptic vesicles isolated from the electric organ of *Torpedo californica* exhibit adenosine 5′-triphosphate (ATP) dependent active uptake of [$^3$H]acetylcholine. Active uptake can be completely inhibited by low concentrations of the mitochondrial uncouplers carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone, nigericin, gramicidin, valinomycin, and A 23187. Under similar conditions uncouplers stimulate the vesicle adenosinetriphosphatase (ATPase) by from 40 to 80%. ATP-supported uptake of [$^3$H]acetylcholine increases greatly as the external pH is increased from 6.6 to 7.6 and remains approximately constant from pH 7.8 to pH 8.6. The uptake also becomes more selective for [$^3$H]acetylcholine compared to [$^{14}$C]choline as the pH is increased from 6.6 to 7.6, achieving 12-fold selectivity, in a manner similar to the increase in the amount of [$^3$H]acetylcholine taken up. Bicarbonate stimulates both the amount and selectivity of [$^3$H]acetylcholine uptake over the lower pH range, but it has no effect over the higher pH range. Exogenous ammonium ion completely inhibits active [$^3$H]acetylcholine uptake, with lower concentrations of ammonium ion required at higher pH values in a manner consistent with ammonia being the active species. Adenosine 5′-diphosphate and a nonhydrolyzable ATP analogue do not support active [$^3$H]acetylcholine uptake. It is concluded that an ATPase pumps protons into the cholinergic synaptic vesicle to produce an internally acidic and positively charged proton gradient that is linked to [$^3$H]acetylcholine uptake.

Synaptic vesicles in *Torpedo* electric organ nerve terminals store acetylcholine (AcCh),[1] which is released by exocytosis upon nerve terminal stimulation (Zimmermann & Whittaker, 1974; Whittaker & Dowdall, 1975). After exocytotic release the vesicles are reloaded with AcCh to make them available for reuse (Suszkiw et al., 1978). This is a formidable task since vesicles isolated from rested fish contain about 0.6 M AcCh that is osmotically active (Breer et al., 1978; Stadler & Füldner, 1980). Thus, transport of AcCh into synaptic vesicles in vivo almost surely proceeds by an energy-linked process.

Highly purified vesicles obtained from *Torpedo* contain an ATPase (Breer et al., 1977; Rothlein & Parsons, 1979; Michaelson & Ophir, 1980) that is stimulated modestly by AcCh (Breer et al., 1977) and more by bicarbonate (Rothlein & Parsons, 1980; J. E. Rothlein and S. M. Parsons, unpublished observations). Both basal and bicarbonate-stimulated activities can be supported by $Mg^{2+}$ or $Ca^{2+}$, and neither activity requires any other exogenous ion such as $Na^+$, $K^+$, or $Cl^-$.

The properties of the vesicle ATPase suggest that it might be involved in AcCh uptake. Indeed, concentrative uptake of [$^3$H]AcCh by *Torpedo* synaptic vesicles recently was demonstrated in the presence of MgATP or CaATP (Koenigsberger & Parsons, 1980; Parsons & Koenigsberger, 1980; Parsons et al., 1982). Under the conditions of the experiments, active uptake required bicarbonate and did not require any other exogenous ion such as $Na^+$, $K^+$, or $Cl^-$. It also required a system that could be inhibited by protein-modification reagents, was inhibited by low temperature, was saturable with an apparent AcCh dissociation constant of 0.4 mM, was specific for AcCh compared to Ch, and was inhibited by reagents such as dicyclohexylcarbodiimide and 4-chloro-7-nitrobenzoxadiazole, which inhibit the ATPase. This behavior strongly implicates the vesicle ATPase in active AcCh uptake.

Passive uptake of AcCh also has been characterized (Carpenter & Parsons, 1978; Carpenter et al., 1980; Michaelson & Angel, 1981; Diebler & Morot-Gaudry, 1981; Giompres & Luqmani, 1980). It is not substantially con-

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[1] Abbreviations: AcCh, acetylcholine; Ch, choline; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; PEP, phosphoenolpyruvate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; PK, pyruvate kinase; $APPCH_2P$, adenosine 5′-(β,γ-methylenetriphosphate); val, valinomycin; NADH, reduced nicotinamide adenine dinucleotide; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; ATPase, adenosinetriphosphatase.

0006-2960/82/0421-3037$01.25/0 

centrative, is relatively nonspecific for AcCh compared to Ch, and is electrogenic in the sense that AcCh is taken up with net positive charge.

In this paper we confirm the occurrence of active AcCh uptake by *Torpedo* synaptic vesicles and extend these studies to examine the mechanism of energy linkage. Of particular interest here was to determine the role of the vesicle ATPase in active uptake and to determine whether an ion gradient arising from protons or bicarbonate is coupled to [$^3$H]AcCh uptake. A preliminary study of these questions has been reported (Anderson et al., 1981).

## Materials and Methods

Live *Torpedo californica* were obtained locally, and the excised electric organ was stored at −95 °C until used. Synaptic vesicles were isolated as described (Koenigsberger & Parsons, 1980). Diethyl *p*-nitrophenyl phosphate, MgATP, adenosine 5′-($\beta,\gamma$-methylenetriphosphate), valinomycin, and gramicidin D were from Sigma Chemical Co. FCCP was obtained from Boehringer-Mannheim. A 23187 was from Calbiochem. Nigericin was a gift from W. E. Scott of Hoffmann-La Roche Inc. Tetramethylammonium chloride was from Eastman Kodak and was recrystallized from ethanol before use. [$^3$H]AcChCl (250 mCi/mmol) and D-[1-$^{14}$C]-mannitol (56 mCi/mmol) were from Amersham/Searle. All other chemicals were of the highest commercial grade from usual sources.

Vesicles pelleted in the ultracentrifuge in an SW28 rotor at 28 000 rpm and 4 °C were resuspended in indicated buffers for uptake ratio measurements and equilibrated 7 h at 25 °C with [$^{14}$C]mannitol. Trace acetylcholinesterase was inhibited by addition of diethyl *p*-nitrophenyl phosphate to 0.4 mM final concentration 1 h before experiments were performed. Uncouplers were added 1 h before experiments were performed by drying ethanol solutions of them in a clean test tube with $N_2$ gas, followed by addition of the [$^{14}$C]mannitol-equilibrated vesicle suspension. Vesicles were added to equal volumes of the indicated solutions containing 100 $\mu$M [$^3$H]AcCh and either [$^{14}$C]mannitol at the equilibrium concentration for uptake ratio determinations or 100 $\mu$M [$^{14}$C]Ch for the selectivity determination. Uptake at 25 °C was terminated by centrifugation–gel filtration at 4 °C, and the $^3$H to $^{14}$C ratios inside and outside of the vesicles were measured by standard double-channel liquid scintillation spectrometry. The uptake ratio was calculated as described in Carpenter et al. (1980) and gives the concentration of [$^3$H]AcCh inside the vesicles compared to its concentration outside. When [$^{14}$]Ch was used, the uptake of [$^3$H]AcCh relative to [$^{14}$C]Ch was determined. Where indicated, [$^3$H]AcCh uptake is presented as the percent of the maximal active uptake beyond the passive amount in order to compare different vesicle preparations and is given by percent active uptake = (uptake ratio with uncoupler − passive uptake ratio) × 100/(uptake ratio with no uncoupler − passive uptake ratio). In some experiments active [$^3$H]AcCh uptake was enhanced by the use of an ATP regenerating system consisting of 5 mM PEP (Sigma), 5 mM $MgCl_2$ (Sigma), and 25 units of pyruvate kinase (Sigma, type III) for each experimental point of 0.3-mL volume before centrifugation–gel filtration.

The vesicle ATPase was assayed at 25 °C by using the procedure of Pullman et al. (1960). Oxidation of NADH was observed at 340 nm after addition of vesicles to 1 mL of 0.4 mM NADH, 2 mM PEP, 50 units of pyruvate kinase, 45 units of lactate dehydrogenase (Sigma, type XI), and 2 mM MgATP in 0.10 M Hepes and 0.70 M glycine adjusted to pH 7.40 with 0.80 M KOH. Ethanol from FCCP and valinomycin stock solutions never exceeded 0.5% (v/v) in the assays and had no significant effect.

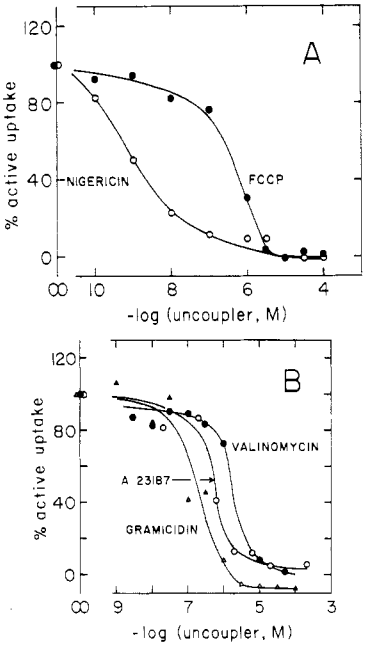


FIGURE 1: Inhibition of active [$^3$H]AcCh uptake by uncouplers. (A) Synaptic vesicles in 0.20 M Hepes and 0.60 M glycine containing 1 mM each of EDTA and EGTA and 0.02% (w/v) $KN_3$ adjusted to pH 7.40 with 0.80 M KOH were treated with nigericin or FCCP at the indicated concentrations. The uptake of [$^3$H]AcCh was allowed to proceed 30 min at 25 °C in 5 mM MgATP and 40 mM $KHCO_3$. The uptake ratio in the absence of uncoupler was 7.1 for both FCCP and nigericin for 0.6 mg of protein/mL final concentration. (B) Synaptic vesicles were resuspended either in 0.20 M Hepes, 0.60 M glycine, 1 mM EDTA, 1 mM EGTA, and 3 mM $KN_3$ adjusted to pH 7.40 with 0.80 N KOH (gramicidin experiment), in the same buffer without EDTA and EGTA (A 23187 experiment), or in the same buffer with $K^+$ replaced by $Na^+$ (valinomycin experiment). Resuspended vesicles were treated with the indicated final concentrations of uncouplers. The uptake of [$^3$H]AcCh at 25 °C in the presence of 5 mM MgATP and 40 mM $KHCO_3$ was terminated after 30 min. The external bicarbonate concentration in the valinomycin experiment thus was 20 mM. In the valinomycin and gramicidin experiments ATP was regenerated with PEP as described under Materials and Methods. The final vesicle protein concentrations were 0.3, 0.5, and 0.2 mg/mL with uptake ratios of 11.3, 10.8, and 10.1 in the absence of uncoupler for the valinomycin, A 23187, and gramicidin experiments, respectively.

## Results

*Effects of Uncouplers on Active [$^3$H]AcCh Uptake.* Purified *Torpedo* electric organ synaptic vesicles were incubated with wide concentration ranges of different classes of mitochondrial uncouplers to examine their effects on active [$^3$H]AcCh uptake. Figure 1A shows the effects of FCCP and nigericin. The electroneutral $H^+$–$K^+$ or $H^+$–$Na^+$ exchanger nigericin acts as a potent total inhibitor of active uptake with a half-inhibitory concentration of about $10^{-9}$ M. The electrogenic proton carrier FCCP also completely inhibited active uptake with a half-inhibitory concentration of about $4 \times 10^{-7}$ M. Thus, whether electroneutral or electrogenic, low concentrations of protonophores, which are fairly specific as to the species transported, completely inhibit active [$^3$H]AcCh uptake.

The effects of the other mitochondrial uncouplers A 23187, valinomycin, and gramicidin also were examined with the results shown in Figure 1B. The $H^+$–divalent cation exchanger A 23187 gave concentration-dependent total inhibition of active [$^3$H]AcCh uptake with a half-inhibitory concentration of about $6 \times 10^{-7}$ M. The result was independent of the presence of 100 $\mu$M $Ca^{2+}$ outside of the vesicles, suggesting

Table I: Effect of External $K^+$ Concentration on Valinomycin Uncoupling

| $K^+$ (mM) | [$^3$H]AcCh uptake ratio[a] no val | [$^3$H]AcCh uptake ratio[a] 1 μM val | % ATPase stimulation,[b] 5 μM val |
|---|---|---|---|
| 1 | 7.1 | 3.1 | |
| 10 | 7.2 | 2.9 | 87 ± 14 |
| 50 | 7.5 | 2.5 | 62 ± 17 |
| 200 | 5.8 | 2.7 | 60 ± 4 |

[a] Vesicles were in 0.4 mM diethyl *p*-nitrophenyl phosphate, 0.20 M Hepes, and 0.60 M glycine adjusted to pH 7.40 with 0.80 M NaOH. They were mixed with an equal volume of [$^3$H]AcCh and [$^{14}$C]mannitol in potassium-containing buffer made by mixing the above buffer with variable volumes of 0.40 M KCl and 5 mM Hepes adjusted to pH 7.40 with 0.80 M KOH. The final concentrations of $K^+$ are given, and MgATP and $NaHCO_3$ were present at 5 and 40 mM, respectively, with the vesicle protein concentration being 0.2 mg/mL. Uptake was terminated after 30 min. The uptake ratio under passive conditions was 2.8 in the presence of 40 mM $K^+$. [b] Assays were performed at 25 °C and constant ionic strength by using the coupled enzyme system described under Materials and Methods. Sodium buffer containing 50 mM Hepes and 0.375 M NaCl was titrated with 0.80 M NaOH to pH 7.40 to yield 0.385 M sodium ion. A potassium buffer was prepared similarly. The proportions of sodium and potassium buffer were varied to give the indicated concentration of $K^+$. The specific activity of the ATPase was 250 nmol $min^{-1}$ (mg of protein)$^{-1}$ in all solutions. The percent stimulation by 5 μM valinomycin is the mean and one standard deviation of three determinations at each $K^+$ concentration done in random order.

that external $Ca^{2+}$ plays no direct role in active [$^3$H]AcCh uptake. The $K^+$ carrier valinomycin gave inhibition of uptake with a half-inhibitory concentration of about $2 \times 10^{-6}$ M. Since valinomycin does not transport protons but will allow electrogenic movement of $K^+$ to collapse a transmembrane electrical potential gradient, a substantial part of the energy driving active [$^3$H]AcCh uptake probably is electrical in nature. The channel former gramicidin, which would allow free movement of $H^+$, $K^+$, and $Na^+$, also inhibited active [$^3$H]AcCh uptake with a half-inhibitory concentration of about $2 \times 10^{-7}$ M.

For further examination of the valinomycin effect, active uptake of [$^3$H]AcCh was studied in different concentrations of external $K^+$ in the absence and presence of valinomycin. The results shown in Table I demonstrate that the external $K^+$ concentration over a 200-fold range had little effect on either coupled or uncoupled uptake of [$^3$H]AcCh.

*Stimulation of Vesicle ATPase by Uncouplers.* If the vesicle ATPase is involved in the generation of a transmembrane proton electrochemical gradient coupled to [$^3$H]AcCh uptake, uncouplers might stimulate the activity of the enzyme. The results of an experiment probing the effects of FCCP and valinomycin on the ATPase activity are shown in Figure 2. The lower curve shows that concentration-dependent stimulation of the ATPase activity by FCCP occurs. The stimulation was saturated at about 40% above the basal ATPase activity and occurred with a half-stimulation FCCP concentration of about $4 \times 10^{-7}$ M. This concentration is in good agreement with that observed for uncoupling of active [$^3$H]-AcCh uptake. The upper curve in Figure 2 shows the effect of adding valinomycin after FCCP in the same assay. In the absence or presence of very low FCCP concentrations micromolar valinomycin stimulated the ATPase by about 30%. At higher concentrations of FCCP the valinomycin had no effect. Valinomycin and FCCP stimulations were not additive but rather exhibited a maximal stimulation of about 40%. This behavior did not depend on the absence or presence of AcCh

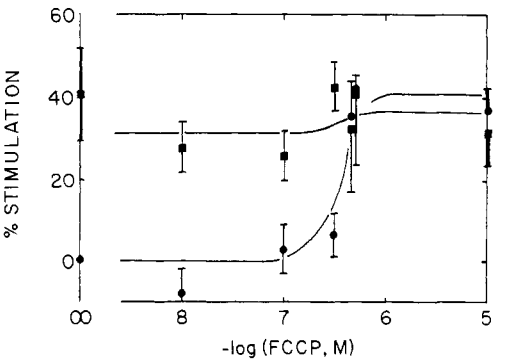


FIGURE 2: Stimulation of vesicle ATPase by uncouplers. The ATPase was assayed as described under Materials and Methods in the presence of 20 mM AcChCl with 30 μg of vesicle protein treated 1 h with 0.4 mM diethyl *p*-nitrophenyl phosphate. The activity was linear with time and was determined for each assay after addition of vesicles, after addition of FCCP (●) to the indicated concentrations and after addition of valinomycin to 1 μM (■). The experiments were performed in triplicate in random order of FCCP concentrations. The percent stimulation is relative to the original ATPase activity [80 nmol $min^{-1}$ (mg of protein)$^{-1}$] before addition of uncouplers. Error bars represent one standard deviation and are shown only on one side of some points for clarity. Similar results were obtained in the absence of AcCh and diethyl *p*-nitrophenyl phosphate.

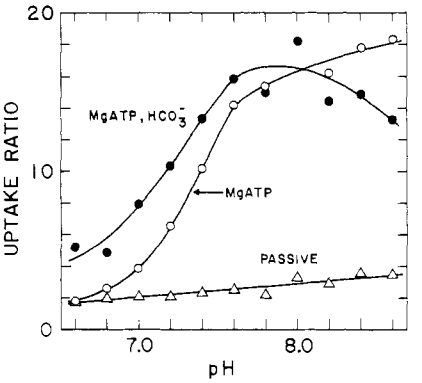


FIGURE 3: Effects of external pH and bicarbonate on extent of [$^3$H]AcCh uptake. Synaptic vesicles in 5 mM Hepes and 0.80 M glycine adjusted to pH 7.0 with 0.80 M KOH were mixed with an equal volume of [$^3$H]AcCh and [$^{14}$C]mannitol in 0.40 M Hepes and 0.40 M glycine that also contained either no other additions (△), 10 mM MgATP (○), or 10 mM MgATP plus 80 mM $KHCO_3$ (●) adjusted to the indicated pH with 0.80 M KOH. The pH values of the final vesicle suspensions (0.35 mg of protein/mL) were confirmed by measurement. The uptake of [$^3$H]AcCh was terminated after 30 min.

or bicarbonate. Table I also demonstrates that it did not depend on the external $K^+$ concentration. Similar results were obtained with gramicidin and the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, not shown). Thus, the vesicle ATPase can be uncoupled from an opposing electrochemical proton gradient, and neither AcCh nor bicarbonate significantly affects this behavior.

*Effects of External pH and Bicarbonate on Extent of [$^3$H]AcCh Uptake.* A transmembrane proton gradient obviously should be affected by the external pH. The effects of variable external pH on passive [$^3$H]AcCh uptake, as well as on ATP-stimulated [$^3$H]AcCh uptake, in the absence and presence of bicarbonate were studied with the results shown in Figure 3. There was a slight increase in passive [$^3$H]AcCh uptake at higher pH. However, the increase is not strongly linked to the external pH under these conditions since a 100-fold decrease in the external proton concentration led to a less than 2-fold increase in passive [$^3$H]AcCh uptake.

In contrast to the passive case the ATP-stimulated uptake

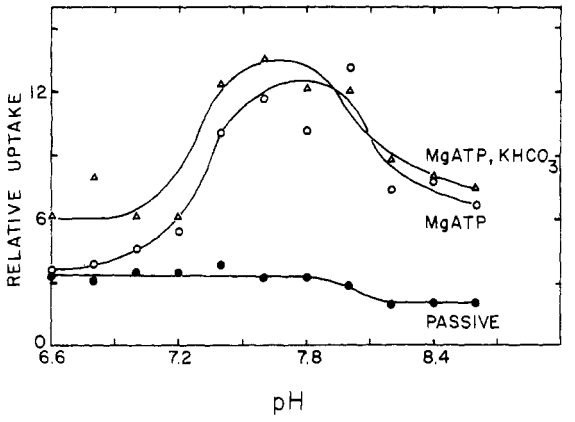


FIGURE 4: Effects of external pH and bicarbonate on selectivity of uptake. Synaptic vesicles in 5 mM Hepes and 0.80 M glycine adjusted to pH 7.0 with 0.80 M KOH were mixed with an equal volume of [$^3$H]AcCh and [$^{14}$C]Ch in 0.40 M Hepes and 0.40 M glycine that also contained either no additions (●), 10 mM MgATP (○), or 10 mM MgATP plus 80 mM $KHCO_3$ (△) adjusted to the indicated pH with 0.80 M KOH. The pH values of the final vesicle suspension (0.6 mg of protein/mL) were confirmed by measurement. The uptake was terminated after 30 min. The relative uptake was calculated from (dpm of [$^3$H]AcCh/dpm of [$^{14}$C]Ch)$_v$/(dpm of [$^3$H]AcCh/dpm of [$^{14}$C]Ch)$_s$, where v is for vesicles and s is for total solution, and gives the selectivity of uptake for [$^3$H]AcCh vs. [$^{14}$C]Ch.

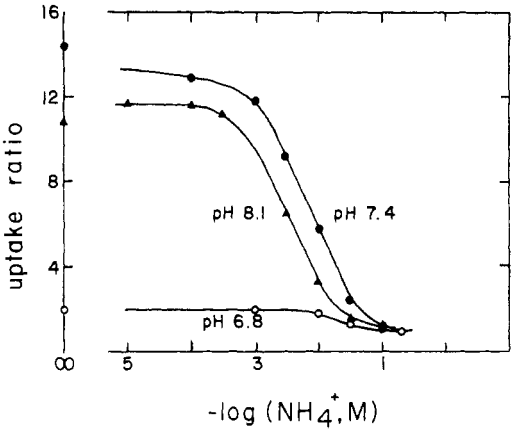


FIGURE 5: Inhibition of active [$^3$H]AcCh uptake by $NH_4Cl$. Synaptic vesicles in 5 mM Hepes and 0.80 M glycine adjusted to pH 7.00 with 0.80 M KOH were mixed with an equal volume of [$^3$H]AcCh and [$^{14}$C]mannitol in 0.20 M Mes and 0.60 M glycine, 0.20 M Hepes and 0.60 M glycine, or 0.20 M Taps and 0.60 M glycine, which were adjusted to pH 6.80, 7.40, or 8.10, respectively, with 0.80 M KOH. The latter solutions also contained 10 mM MgATP, twice the indicated concentrations of $NH_4Cl$, and the ATP regeneration system. The pH values of the final vesicle suspensions (0.3 mg of protein/mL) were confirmed by measurement. The uptake of [$^3$H]AcCh was terminated after 30 min.

of [$^3$H]AcCh exhibited a strong pH dependence both in the absence and in the presence of bicarbonate (Figure 3). Uptake dramatically increased at increasing pH values from 6.6 to about 7.6 where uptake reached an approximate plateau. In the lower pH range, bicarbonate stimulated [$^3$H]AcCh uptake by about 2-fold beyond that obtained in the presence of ATP alone and was in fact necessary for any uptake beyond the passive amount at pH 6.6. In the plateau range above pH 7.6 there was no significant effect of bicarbonate. Thus, the stimulatory effect of bicarbonate on active [$^3$H]AcCh uptake is conditional and not obligatory.

*Effect of External pH and Bicarbonate on Selectivity of Uptake.* Previous results have shown that bicarbonate increased the selectivity of active uptake to favor AcCh in an experiment where [$^3$H]AcCh and [$^{14}$C]Ch were present simultaneously (Parsons & Koenigsberger, 1980). Here we extend that experiment to determine the effects of variable external pH on the selectivity of the uptake process. The results are shown in Figure 4. Under passive conditions there was only a 3-fold preference for [$^3$H]AcCh compared to [$^{14}$C]Ch uptake from pH 6.6 to pH 7.8. At higher pH values the preference for [$^3$H]AcCh decreased slightly with an approximate midpoint for the transition at pH 8.1.

In contrast to the passive case the ATP-stimulated preference for [$^3$H]AcCh uptake exhibited a strong biphasic pH dependence both in the absence and in the presence of bicarbonate (Figure 4). The preference dramatically increased at increasing pH values from 6.6 to about 7.6 where it reached a maximal 12–13-fold preference. At pH values above 8.0 the preference for [$^3$H]AcCh decreased to about 7-fold with an approximate midpoint for the transition at pH 8.1, which is similar to the transition range in the passive case. At pH 6.6 bicarbonate gave about 2-fold increased selectivity for [$^3$H]AcCh uptake beyond that obtained either with ATP alone or with no ATP. Above pH 7.8 bicarbonate had no effect on the selectivity of uptake. Thus, with the added feature of a high pH transition, the selectivity of the vesicle transport system for [$^3$H]AcCh compared to [$^{14}$C]Ch responds to variable pH and bicarbonate in a fashion similar to the response of [$^3$H]AcCh uptake.

*Effect of $NH_3$ on Active [$^3$H]AcCh Uptake.* Ammonia is freely diffusible across biological membranes whereas ammonium ion is not. Ammonium ion added externally to synaptic vesicles equilibrates with ammonia, which can diffuse into vesicles to make them more alkaline inside. This technique has been used to collapse an internally acidic proton gradient in chromaffin granules (Pollard et al., 1979). If the proton gradient driving active [$^3$H]AcCh uptake is internally acidic, ammonium ion should inhibit the uptake, whereas it should not inhibit if the gradient is internally alkaline.

Figure 5 shows that external ammonium ion completely inhibits active uptake in a concentration- and pH-dependent manner. Inhibition was most potent at pH 8.1, less so at pH 7.4, and least potent at pH 6.8 in terms of the concentrations of ammonium ion required for half-inhibition, which were 3, 5, and 30 mM, respectively. The different maximal uptake ratios in the absence of external ammonium ion simply reflect the variation in [$^3$H]AcCh uptake as a function of pH (Figure 3).

The pH dependence of the potencies for ammonium ion inhibition is consistent with the expectation that ammonia is the active species. For confirmation of this, tetramethylammonium ion, which cannot generate a base, was tested for inhibition at the same pH values. It is an inhibitor of [$^3$H]-AcCh uptake with a half-inhibition concentration of 25 mM, which is not pH dependent (data not shown). Tetramethylammonium ion probably binds directly to the AcCh porter as an AcCh analogue. These results suggest that an internally acidic proton gradient is linked to active [$^3$H]AcCh uptake.

*Effects of ADP and Adenosine 5′-($\beta$,$\gamma$-Methylenetriphosphate) on [$^3$H]AcCh Uptake.* If ATP hydrolysis by the ATPase is required to generate a transmembrane proton gradient, one would expect that ADP and the nonhydrolyzable analogue of ATP, adenosine 5′-($\beta$,$\gamma$-methylenetriphosphate), would not support active [$^3$H]AcCh uptake. The results of such a study are shown in Table II. Adenosine diphosphate by itself had no significant effect on passive [$^3$H]AcCh uptake, but it inhibited active uptake. Half-inhibition occurred at approximately equimolar ADP and ATP (5 mM), suggesting that ADP may bind to the ATPase about as well as ATP. The inhibitory effect of ADP was confirmed by use of an ATP

Table II: Effects of ADP and Adenosine 5′-($\beta$,$\gamma$-Methylenetriphosphate) on [$^3$H]AcCh Uptake

| conditions[a] | uptake ratio | conditions[b] | uptake ratio |
|---|---|---|---|
| passive | 1.5 | passive | 2.3 |
| passive + 10 mM MgADP | 1.8 | active[d] | 4.4 |
| active[c] | 12.5 | 10 mM MgAPPCH$_2$P + 40 mM NaHCO$_3$ | 1.1 |
| active + 5 mM MgADP | 6.0 | | |
| active + 10 mM MgADP | 2.6 | | |
| active + 5 mM MgPEP | 13.4 | | |
| active + 16 mM MgPEP | 15.5 | | |
| active + 5 mM MgPEP + PK[e] | 20.5 | | |
| active + 16 mM MgPEP + PK | 22.1 | | |

[a] The experiment was in 0.20 M Hepes and 0.60 M glycine adjusted to pH 7.00 with 0.80 M KOH. The final vesicle protein concentration was 0.35 mg/mL. The uptake of [$^3$H]AcCh was terminated after 30 min. [b] The experiment was in 5 mM Hepes, 0.20 M NaCl, and 0.40 M glycine adjusted to pH 7.00 with 0.80 M NaOH. The uptake of [$^3$H]AcCh was terminated after 30 min. [c] Five millimolar MgATP and 40 mM $KHCO_3$ were present. [d] Five millimolar MgATP and 40 mM $NaHCO_3$ were present. [e] Pyruvate kinase was present at 187 units/mL.

recycling system composed of phosphoenolpyruvate and pyruvate kinase. In the presence of the recycling system active uptake of [$^3$H]AcCh was increased by nearly 2-fold.

Table II also shows the effect on passive [$^3$H]AcCh uptake of adenosine 5′-($\beta$,$\gamma$-methylenetriphosphate). It did not support active uptake and actually appeared to inhibit passive uptake. These results clearly suggest that hydrolysis of ATP is required to support active uptake of [$^3$H]AcCh.

## Discussion

This paper confirms our previous reports of ATP-linked stimulation of [$^3$H]AcCh uptake by *Torpedo* electric organ synaptic vesicles. This is an active process that leads to concentrative uptake of [$^3$H]AcCh. Although the concentrative factor (uptake ratio) varies with different vesicle preparations, factors of greater than 10 are easily attained. The active uptake is distinct from the passive process that occurs in the absence of exogenous ATP and that under most comparable conditions results in much less uptake of [$^3$H]AcCh. Some reports in the literature regarding the passive process appear to have confused it with the active.

Here we present evidence that active [$^3$H]AcCh uptake by *Torpedo* electric organ synaptic vesicles is linked to an inwardly acidic electrochemical gradient, which is established by a proton pumping ATPase. Several observations support this hypothesis. First, active uptake can be inhibited completely by low concentrations of many different types of mitochondrial uncouplers. The different types include examples that carry only protons electrogenically, that exchange protons with $K^+$ or $Na^+$ electroneutrally, that exchange protons for divalent cations, that channel any small univalent ion including protons, and that carry only $K^+$ (Pressman, 1976). The only ion gradient that would be altered by all of these uncouplers is a charged proton gradient. Second, the vesicle ATPase is stimulated by diverse mitochondrial uncouplers, suggesting that it is normally at least partially inhibited by an opposing electrochemical gradient of protons. Third, active uptake of [$^3$H]AcCh can be inhibited completely by externally added ammonium chloride, suggesting that internal protons drive [$^3$H]AcCh uptake. Fourth, the large increase in uptake at higher pH values is most readily reconciled with involvement of an internally acidic (rather than internally alkaline) vesicle in active [$^3$H]AcCh uptake since the transmembrane gradient would increase at higher external pH. Fifth, hydrolysis of ATP is required since ADP and adenosine 5′-($\beta$,$\gamma$-methylenetriphosphate) do not support active uptake. These results clearly implicate an internally acidic gradient, but a number of additional conclusions and some speculations and reservations can be stated.

The molar concentration $M$ of synaptic vesicles present in an experiment can be estimated from

$$M = Vp/(vA) \quad (1)$$

where $V$ is the internal volume of vesicles per gram of protein and is 5.3 ± 0.8 mL/g (Carpenter et al., 1980), $v$ is the calculated internal volume per vesicle ($1.8 \times 10^{-16}$ mL/vesicle), based on an average internal radius of 350 Å determined by electron microscopy, $A$ is Avogadro's number, and $p$ is the vesicle protein concentration (grams per liter). For the nigericin uncoupling experiment the vesicle molarity was about $3 \times 10^{-8}$ M. About this same concentration of nigericin sufficed to inhibit most of the active uptake of [$^3$H]AcCh. Thus, about one nigericin molecule per synaptic vesicle was all that was required for uncoupling.

An internally acidic proton gradient is consistent with the outward orientation of the vesicle ATPase (Michaelson & Ophir, 1980) if it is related to the mitochondrial ATPase. Elsewhere we demonstrate that the ATPase indeed has many kinetic similarities to the mitochondrial ATPase (J. E. Rothlein and S. M. Parsons, unpublished observations). On the assumption that the primary transport event under active conditions is the inward pumping of protons by the ATPase, it is likely that the "energized" vesicle has an internally positive electrical potential. Internal acidity and a positively charged vesicle both would increase the protonmotive force in the direction of proton efflux, and this could be used to drive secondary active uptake of [$^3$H]AcCh via antiport through a separate AcCh porter. The critical importance to active uptake of some additional factor such as the electrical potential is implied by the passive uptake results. Imposing up to a 100-fold difference in the pH gradient by varying the external pH had little effect on passive [$^3$H]AcCh uptake. Furthermore, the increase in the *selectivity* of the active uptake process, which parallels the increase in the amount of active uptake of [$^3$H]AcCh, suggests that in a more energized vesicle the conformation of the AcCh porter changes so as to become more specific for AcCh.

This behavior can be compared to the results of previous studies. Net positive AcCh and Ch are taken up passively equally well by hyperpolarized (internally negative) vesicles (Carpenter & Parsons, 1978; Carpenter et al., 1980). Consistent with this finding are the observations by Michaelson et al. (1979) and Angel & Michaelson (1981) that depolarization of the vesicles results in efflux of endogenous AcCh and that native vesicles that retain AcCh are hyperpolarized. Thus, under passive conditions AcCh spontaneously enters negatively polarized vesicles with little selectivity while under active conditions it enters positively polarized vesicles with high selectivity. The apparent contradiction can be resolved if the mechanism of transport and the AcCh porter structure depend on the vesicle transmembrane electrical potential. Modulation of the porter structure in response to changes in the transmembrane electrical potential or other factors could serve the useful purpose of turning off the AcCh porter during the period

following exocytosis when vesicle membrane is fused with the plasmalemma.

Active uptake of [$^3$H]AcCh by the vesicles is strongly dependent on the external pH. This is not due to a variable ATPase activity, however, since over the pH range investigated the vesicle ATPase has nearly constant maximal activity (J. E. Rothlein and S. M. Parsons, unpublished observations). This suggests that the pH dependencies seen here are due to phenomena associated with the AcCh porter. The large increase in [$^3$H]AcCh uptake and selectivity from pH 6.6 to pH 7.6 approximates a titration curve. It could be due either to titration of an externally exposed amino acid side chain in the porter or to an increase in the transmembrane pH gradient driving [$^3$H]AcCh uptake. Interestingly, uptake of 5-hydroxytryptamine into brain synaptic vesicles, which also is proton linked, exhibits a pH dependency similar to that of active [$^3$H]AcCh uptake (Maron et al., 1979).

At higher pH another transition appears in the specificity of [$^3$H]AcCh uptake under both passive and active conditions, but the transition does not clearly occur for the amount of [$^3$H]AcCh uptake. This might be due to deprotonation of an amino acid side chain with an apparent p$K$ of 8.1 in the AcCh porter, which leads to greater uptake of [$^{14}$C]Ch, that is, to a decrease in specificity for [$^3$H]AcCh.

Bicarbonate was shown both to stimulate active [$^3$H]AcCh uptake and to increase the selectivity of uptake by about 2-fold at low pH but not at high pH. This makes it unlikely that bicarbonate is cotransported into the vesicle to stimulate [$^3$H]AcCh uptake, for example, by making uptake electroneutral and by reacting with internal protons. Such an important role for bicarbonate likely would be obligatory. We show elsewhere that bicarbonate binds directly to the vesicle ATPase to stimulate its activity in the lower pH range, and there is no reason to invoke its transport (J. E. Rothlein and S. M. Parsons, unpublished experiments).

The bicarbonate effect might have physiological importance. The nerve terminal when active likely is more acidic and at higher $CO_2$ tension than when resting. During activity an increase in bicarbonate concentration could buffer the vesicle AcCh uptake system against inhibition by the low pH and help prevent depletion of the releasable AcCh pool. Our previous suggestion that decreases in the central nervous system bicarbonate level might contribute to decreased AcCh synthesis because of linkage to the storage step (Parsons & Koenigsberger, 1980) is consistent with the above scenario.

Under the conditions of our previous studies at pH 7.0 MgATP by itself suppressed uptake of [$^3$H]AcCh to below passive levels, and bicarbonate was required to achieve concentrative uptake. We have confirmed that result many times but do not see that behavior under the current conditions. Possibly, in the absence of bicarbonate the conformation and activity of the AcCh porter are sensitive to the solvent composition.

Although a proton gradient clearly is involved and influx of no other exogenous ion is required, the possibility that another ion such as $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, ATP, or AcCh contained inside of the vesicle also is linked to [$^3$H]AcCh uptake by synaptic vesicles cannot be excluded. This is because native vesicles contain large concentrations of these ions (Schmidt et al., 1980). One of these species could engage in linked efflux, along with $H^+$, or could act allosterically. Resolution of these possibilities must await the ability to control the internal composition of synaptic vesicles.

Several other studies have implicated a proton gradient in AcCh storage. PC12 cells store AcCh in granules, and Melega & Howard (1981) have shown that this process is inhibited in the intact cell by uncouplers. Michaelson & Angel (1980) found that isolated *Torpedo* vesicles are internally acidic and release endogenous AcCh in the presence of uncouplers. In addition to storage of catecholamines in chromaffin granules and 5-hydroxytryptamine in brain synaptic vesicles, catecholamine storage in brain and heart synaptic vesicles (Toll et al., 1977; Toll & Howard, 1978; Angelides, 1980), 5-hydroxytryptamine storage in platelet granules (Rudnick et al., 1980), and peptide storage in neurohypophysis secretory vesicles (Russell & Holz, 1981) are linked to proton gradients. Proton-linked storage thus appears to be common to a wide range of neural secretory systems.

Acknowledgments

We thank Marianne Henry for preparation of synaptic vesicles and Joan E. Rothlein for early observations on ATPase activation by uncoupling.

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# Rabbit Muscle Phosphorylase Derivatives with Oligosaccharides Covalently Bound to the Glycogen Storage Site†

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ABSTRACT: Linear maltooligosaccharides, e.g., maltoheptaose or terminal 4-*O*-methylmaltoheptaose, activated by cyanogen bromide, react covalently with rabbit muscle phosphorylases *b* and *a* (EC 2.4.1.1). Site-specific modification prevents further binding to glycogen and shifts the phosphorylase *a* tetramer–dimer equilibrium in favor of the dimer. Use was made of these properties to separate by affinity chromatography and gel filtration phosphorylase *a* dimers with specifically bound oligosaccharide from unspecifically modified products. The phosphorylase *a*–maltoheptaose derivative carries one oligosaccharide residue per monomer and can be distinguished from the native enzyme by its electrophoretic mobility in polyacrylamide gels or by affinity electrophoresis. Phosphorylase *a* preparations with covalently bound maltooligosaccharides are enzymatically active in the presence of a primer and α-D-glucopyranose 1-phosphate (glucose-1-P). Methylation of the nonreducing chain terminus of the bound oligosaccharide has no effect on glycogen synthesis. These findings exclude the participation of bound oligosaccharides in chain elongation. Purified covalent phosphorylase *a*–maltoheptaose complexes are stable dimers. They are no longer activated by glycogen. The properties of covalently modified phosphorylase–oligosaccharides are consistent with and provide direct evidence for the existence of a glycogen storage site in rabbit muscle phosphorylases. Covalent occupation of the storage site renders the affinity of glucose-1-P to phosphorylase *a* independent of modulation by glycogen, supporting the assumption that the glycogen storage site is involved in interactions with the catalytic site.

Crystallographic studies of rabbit muscle phosphorylases *a* and *b* (α-1,4-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) in their T conformational states have localized a binding site for linear maltooligosaccharides some 25–30 Å distant from the catalytic site (Fletterick et al., 1976; Weber et al., 1978; Johnson et al., 1980; Fletterick & Madsen, 1980). It has been suggested that this oligosaccharide binding site provides the means to anchor the enzyme to glycogen particles in the living cell (Meyer et al., 1970; Fletterick et al., 1976) and accordingly was named the glycogen storage site. It was further proposed that dissociation of tetrameric phosphorylase *a* to dimers by glycogen or maltoheptaose (Wang et al., 1965; Metzger et al., 1967) results from binding of the poly- or oligosaccharide to the storage site (Kasvinsky et al., 1978). Finally, so that the high affinity for branched polysaccharides and heterotropic cooperativity could be explained (Kasvinsky et al., 1978), the role of an activator site was ascribed to this crystallographically localized oligosaccharide binding site.

The failure to visualize two oligosaccharide sites crystallographically (Fletterick & Madsen, 1980; Johnson et al., 1980) was rationalized on the basis of kinetic studies with maltoheptaose, which indicated that the glycogen storage site has at least a 20 times greater affinity for oligosaccharides ($K_m \simeq 1$ mM) than the catalytic site (Kasvinsky et al., 1978). It was therefore reasoned that the concentration of maltoheptaose (up to 240 mM) used in crystallographic studies might not have been large enough to saturate the catalytic site. In addition, the active site might not be accessible for oligosaccharides in crystals of phosphorylase *a* in the glucose-inhibited T conformation.

Additional experiments are presented to prove more directly the roles ascribed to the glycogen binding site in phosphorylase. We have attacked the problem by covalently coupling cyanogen bromide activated oligosaccharides to phosphorylase and have studied the properties of the covalent phosphorylase–oligosaccharide derivatives, hoping to clarify two points: (1) to check whether phosphorylase preparations covalently modified at the glycogen storage site with oligosaccharides are catalytically active or not and (2) to check more directly whether structural and functional properties of phosphorylases altered by covalent occupation of the glycogen storage site support the anticipated role of an activator site.

## Materials and Methods

Glycogen (oyster, type II) was purchased from Sigma Chemical Co. Molar concentrations of glycogen were calculated by assuming 1 out of every 12 glucose residues is an end group (Hu & Gold, 1975). Maltodextrin 19 obtained from

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0006-2960/82/0421-3043$01.25/0 