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BICARBONATE AND MAGNESIUM ION-ATP DEPENDENT STIMULATION OF ACETYLCHOLINE UPTAKE BY TORPEDO ELECTRIC ORGAN SYNAPTIC VESICLES

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SUMMARY Cholinergic synaptic vesicles isolated from the electric organ of Torpedo californica rapidly take up [^{3}H]acetylcholine in a passive process. The internal concentration achieved was equal to the external concentration as determined by the ratio of [^{3}H]acetylcholine to preloaded [^{14}C]mannitol. Addition of bicarbonate had little effect, whereas addition of MgATP suppressed uptake by two fold. Simultaneous addition of HCO_3^- and MgATP stimulated uptake over five fold compared to the suppressed condition and resulted in net concentrative uptake. All effects depended on no other inorganic ions, were not ionic strength dependent, and other tested anions would not substitute for HCO_3^-. Thus, concentrative uptake of [^{3}H]acetylcholine linked to HCO_3^- and MgATP per se is exhibited by cholinergic synaptic vesicles.

INTRODUCTION Recent studies in Torpedo electric organ tissue blocks strongly suggest that neurally evoked release of acetylcholine is mediated by exocytosis of synaptic vesicle contents (1-3). A model postulating the presence in the nerve terminal of depot vesicles, active vesicles, and demobilized vesicles has been formulated for the electric organ (4). The model suggests that active vesicles near the presynaptic membrane undergo repetitive exocytosis, endocytosis, and reloading with newly synthesized acetylcholine. Storage of newly synthesized ATP, which is the other major small organic component of cholinergic vesicles, can be uncoupled from acetylcholine storage in recycled vesicles (5). Thus much evidence obtained from electric organ tissue blocks strongly suggests that cholinergic synaptic vesicles are capable of acetylcholine transport and storage.

Despite this, all early attempts to demonstrate concentrative acetylcholine uptake by synaptic vesicles in vitro

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have failed or have been ambiguous (6-11). However, these early studies suffered from severe disadvantages. Either brain vesicles which are heterogeneous as to neurotransmitter type, or relatively small amounts of but partially pure electric organ vesicles were studied, thus reducing sensitivity to uptake. Only recently have large amounts of highly purified homogeneously cholinergic synaptic vesicles from electric organ been available. We report here the observation of MgATP linked concentrative uptake of [^{3}H]acetylcholine by Torpedo electric organ synaptic vesicles.

MATERIALS AND METHODS Synaptic vesicles were isolated from Torpedo californica (obtained locally) similarly to the methods of Carlson et. al. (12) and Nagy et. al. (13) in 800 mOsm glycine and glycine-sucrose media at pH 7.0. Briefly, this method involves differential sedimentation velocity pelleting, equilibrium buoyant density centrifugation and controlled pore glass bead gel filtration of vesicles. [^{3}H]Acetylcholine Chloride (250mCi/mmol) and D-[^{14}C]mannitol (56.8mCi/mmol) were both from Amersham Searle. Diethyl-p-nitrophenylphosphate (Paraoxon) was from Sigma.

Isolated vesicles were centrifuged for 3h. at 90,000xg (25,000rpm) in an SW25.1 rotor at 4°C. Pellets were each resuspended in 0.5ml of 0.8M Glycine, 5mM HEPES, 1mM EDTA, 1mM EGTA, 0.02%(w/v) KN_3, pH 7.0 with KOH (glycine buffer) and pooled with 0.5ml more of glycine buffer. Pooled vesicles were added to an equal volume of [^{14}C]mannitol (14μM final concentration) in glycine buffer, and equilibrated at 25°C for 7h, after which time Paraoxon (0.44mM final concentration) was added, and then allowed to incubate 60 min. Uptake of [^{3}H]acetylcholine at 25°C was initiated by addition of an equal volume of glycine buffer which contained 100μM [^{3}H]acetylcholine, 14μM [^{14}C]mannitol, and twice the desired final concentration of salts and/or ATP. Radioisotopes were added to vesicle solutions by first drying them in a clean tube under N_2 gas, then adding glycine buffer (containing salts and ATP if applicable) and vortexing. Paraoxon was similarly dried under N_2, and the vesicle solution was added directly to the tube containing dried Paraoxon.

Standard double channel liquid scintillation counting techniques were used to determine dpms of radioisotopes after assaying 0.25ml portionsof the uptake solution at 4°C by the method of Neal and Florini (14). Aliquots of the original uptake solution were transferred to the same counting medium (10ml of Aquasol-2, 0.6ml H_2O) in order to determine dpms in solution. The uptake ratio was calculated as follows:

$$\text{uptake ratio} = \frac{\text{dpm } ^3H_v/\text{dpm } ^{14}C_v}{\text{dpm } ^3H_s/\text{dpm } ^{14}C_s}$$

where v=assayed vesicle sample
s=reaction solution.

RESULTS Uptake of [^{3}H]acetylcholine by Torpedo electric organ synaptic vesicles preloaded with [^{14}C]mannitol was studied. This approach was taken in order to facilitate accurate and unambiguous determination of the extent of [^{3}H]acetylcholine uptake. Mannitol will distribute at equal concentrations between the external solvent volume and the vesicle internal solvent volume. After addition of [^{3}H]acetylcholine, vesicles were rapidly separated in the cold from the external incubation medium by a centrifugation gel filtration technique. By comparing the [^{3}H]acetylcholine to [^{14}C]mannitol ratio inside of the separated vesicles to the ratio outside of the vesicles one obtains an "uptake ratio" for [^{3}H]-acetylcholine.

Figure 1 shows the time courses for uptake of [^{3}H]acetylcholine by vesicles under four sets of conditions in terms of the uptake ratio. The reference condition is that la-

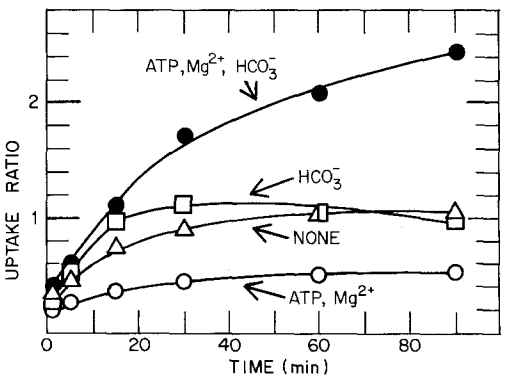

Figure 1: Effect of additions on the time course of uptake of [^{3}H]acetylcholine by Torpedo electric organ synaptic vesicles. Synaptic vesicles (1.65ml) of resuspended pellets in glycine buffer were incubated in [^{14}C]mannitol and Paraoxon as described in methods. Portions of 0.4ml were taken and uptake of 50 μM [^{3}H]acetylcholine was initiated for each indicated addition (5mM K_2ATP, 7mM $MgCl_2$, 40mM $KHCO_3$) as described in methods. Final vesicle protein concentration was 1.1mg/ml in a volume of 1.65mls per condition at 25°. Uptake was assayed periodically and the dpms taken up per datum varied from 7900 to 95000 for [^{3}H]acetylcholine and from 2900 to 4100 for [^{14}C]mannitol, with backgrounds of about 100 cpm. Uptake ratio gives the concentration of [^{3}H]acetylcholine inside the vesicle relative to its concentration outside.

TABLE 1.

Suppression of [^{3}H]Acetylcholine Uptake by MgATP

Dependency	Addition[a]	Relative[b] Uptake ratio
MgATP dependent?		
	None	1.0
	2mM Na_2ATP	0.95
	4mM $MgCl_2$	0.81
	2mM Na_2ATP, 4mM $MgCl_2$	0.61
Cl^- dependent?		
	None	1.0
	2mM Na_2ATP, 4mM $MgCl_2$	0.60
	2mM Na_2ATP, 4mM $MgSO_4$	0.60
Na^+ or K^+ dependent?		
	None	1.0
	2mM Na_2ATP, 4mM $MgCl_2$	0.63
	2mM K_2ATP, 4mM $MgCl_2$	0.68[c]
Ionic Strength dependent?		
	None	1.0
	2mM Na_2ATP, 4mM $MgCl_2$	0.61
	2mM Na_2ATP, 4mM $MgCl_2$, 100mM KCl	0.53[d]
	2mM Na_2ATP, 4mM $MgCl_2$, 100mM NaCl	0.45[d]

[a] Vesicles resuspended in glycine buffer in which K^+ was replaced with Na^+.
[b] The uptake ratio obtained for the stated additions relative to the uptake ratio for no additions.
[c] Vesicles resuspended in glycine buffer.
[d] Osmolarity was maintained at 0.8M by decreasing the glycine concentration to 0.6M.

beled "none" where no special additions were made. This passive uptake of [^{3}H]acetylcholine had a half life of about 7 min. and resulted in an equilibrium uptake ratio of about 1.0. That is, the concentration of [^{3}H]acetylcholine inside of the vesicle was the same as its concentration outside. It has been shown in data to be presented elsewhere that the bound [^{3}H]acetylcholine is osmotically labile, thus suggesting that it is transmembrane. Figure 1 shows that addition of bicarbonate ion by itself had little effect on the uptake process, whereas addition of magnesium ion and ATP suppressed the uptake by about two fold. However, when bicarbonate, magnesium ion and ATP all were included, over a two fold stimulation of [^{3}H]acetylcholine uptake was

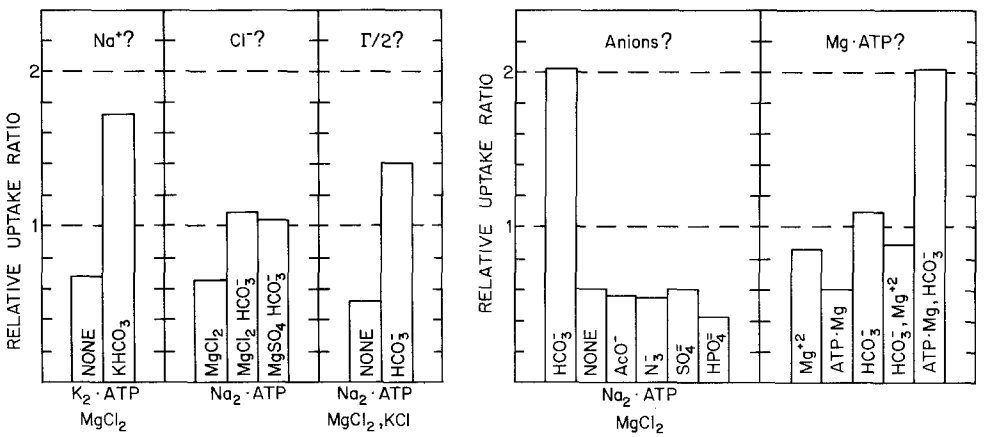

Figure 2: Dependence of bicarbonate MgATP stimulation of [^{3}H]acetylcholine uptake on ions. The question in the top of each frame indicates which ion or phenomenon was tested by the experiments in that frame. The label within each histobar designates additions, if any, beyond the common additions indicated below each frame. The relative uptake ratio is the uptake ratio obtained for the stated additions relative to the uptake ratio for no additions. Uptake of 50μM [^{3}H]acetylcholine was initiated similarly to before at a protein concentration of 0.56mg/ml for 0.6ml of solution. After 15 min uptake was assayed in duplicate. Answers agreed to within ± 2 per cent of the average which is reported. For all frames, Na_2ATP,K_2ATP=2mM, $MgCl_2$=4mM, HCO_3^-=10mM when present. Unless otherwise indicated, the sodium salt of HCO_3^- was used. Left Frames: For Na^+, good HCO_3^- stimulation was obtained in an all K^+ system, and this was equal to the stimulation obtained in Na_2ATP and $NaHCO_3$. Results for Cl^- were obtained in a preparation of vesicles that exhibited less HCO_3^- MgATP stimulation ([$MgSO_4$]=4mM). For ionic strength (Γ/2), 100mM KCl was used. Right Frames: For Anions, results were obtained glycine buffer with Na^+ replacing K^+. All anions (HCO_3^-=10mM, AcO^-=10mM, N_3^-=3.1mM, SO_4^{-2}=50mM, HPO_4^{-2}=62.5mM) were added to solution as Na^+ salts. For SO_4 and HPO_4^{-2} the amount of glycine in solution was adjusted to maintain osmolarity at 800mOsmolar. The MgATP frame experiments were done in glycine buffer with Na^+ substituted for all K^+.

observed compared to the no additions condition, or nearly five fold stimulation campared to the suppressed level. This result represents concentrative uptake of [^{3}H]acetylcholine since the uptake ratio approached 2.5 at 90 min. Other data shows that this too is osmotically labile [^{3}H]acetylcholine.

Specific ion dependencies for these effects were investigated. Inspection of Table 1 reveals that no other inorganic ions were required for the MgATP suppression effect, and suppression was not ionic strength dependent. Figure 2 shows that the

bicarbonate MgATP stimulation also depended on no other inorganic ions, such as Na^+, K^+, or Cl^-, and it was not ionic strength dependent. Furthermore, no other anions tested, including Cl^-, acetate, azide, sulfate, and phosphate could substitute for bicarbonate in the stimulation reaction. Thus, stimulation of [^{3}H]acetylcholine uptake depends on HCO_3^- and MgATP per se.

DISCUSSION Precedent for energy linked transport in storage granules exists for other neurotransmitters in vitro. Dopamine, catecholamines, and serotonin have been shown to be taken up by brain synaptic vesicles or renal medullary chromaffin granules in MgATP driven processes (15-18). A similar uptake mechanism involving acidification of the vesicle or granule interior by an ATPase driven proton pump might be operating in all these cases. Protonation of bound neurotransmitter can then account thermodynamically for uptake. An ATPase which is modestly stimulated by exogenous acetylcholine is associated with cholinergic synaptic vesicles[1] (19,20). This might suggest a similarity in acetylcholine and other neurotransmitter storage systems. However, since acetylcholine cannot be protonated, the cholinergic vesicle uptake system must be different. Furthermore, for substantial accumulation of acetylcholine by vesicles, a way to neutralize the transported positive charge must be present. These considerations suggested that biologically important anions should be tested for their potential effects on MgATP driven [^{3}H]acetylcholine uptake by vesicles.

The effect of MgATP in suppressing [^{3}H]acetylcholine uptake might at first seem surprising. However, under similar control conditions involving no special additions, experiments

[1] ATPase activity stimulated by HCO_3^- also is present. J.E. Rothlein and S.M. Parsons, unpublished observations.

to be reported elsewhere demonstrate that the vesicle membrane is substantially (but not totally) nonspecifically permeable to cations. The equilibrium uptake ratio of 1 obtained for [^{3}H]acetylcholine under these conditions probably indicates that the vesic was in a permeable "nonenergized" state. Magnesium ion and ATP might decrease nonspecific vesicle membrane permeability, and thus passive [^{3}H]acetylcholine uptake, by binding to lipid or to a transport system to stabilize them. Magnesium ion is well known to bind to model bilayer membranes (21), and EDTA increases the permeability of the inner mitochondrial membrane to cations (22). Thus MgATP suppression of [^{3}H]acetylcholine uptake is reasonable, and might indicate that the acetylcholine uptake mechanism differs from that of other neurotransmitters even as to the thermodynamic driving force.

The stimulated [^{3}H]acetylcholine uptake observed here represents the average relative [^{3}H]acetylcholine concentration for all vesicular structures. If functional heterogeneity is present, as has been suggested (1-4), then the actual magnitude of concentrative [^{3}H]acetylcholine ahieved by the active population is in fact greater than seen here. Such a situation would be consistent with variable bicarbonate MgATP stimulation in different vesicle preparations, as is the case.

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