Vol. 103, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1981 Pages 422-428

UNCOUPLING OF ACETYLCHOLINE UPTAKE FROM THE TORPEDO CHOLINERGIC SYNAPTIC VESICLE ATPase

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Received October 5,1981

SUMMARY

Cholinergic synaptic vesicles isolated from the electric organ of Torpedo californica are confirmed to exhibit energy-linked uptake of [^{3}H] acetylcholine. [^{3}H]Acetylcholine is concentrated in the vesicles by a factor of 10-14 in the presence of MgATP and bicarbonate. This active uptake can be completely inhibited by the mitochondrial uncouplers 3-t-butyl-5-Cl-2'-Cl-4'-nitro-salicylanilide (S-13) and p-nitrophenol. The vesicle-associated ATPase is stimulated by S-13 in the same concentration range which inhibits [^{3}H]acetylcholine active uptake. The ATPase also is stimulated by valinomycin. Both S-13 and valinomycin effects are independent of exogenous Ca^{2+}. Thus, a proton gradient generated by the vesicle-associated ATPase appears to be coupled to active [^{3}H]acetylcholine uptake.

INTRODUCTION

Synaptic vesicles isolated from the electric organ of Torpedo californica contain about 0.6 M ACh[1], which is exocytotically released into the synapse upon stimulation of the electric organ (1). Since vesicles are recycled (2) it is of considerable interest to study how they are loaded with ACh. Passive uptake of ACh has been demonstrated with these vesicles (3-6). Concentrative energy-linked uptake of [^{3}H]ACh into vesicles also has been demonstrated. This uptake required HCO_3^- and MgATP or CaATP (7,8), requires a functional protein system containing a critical sulfhydryl group (9), and is saturable by ACh with an apparent dissociation constant of 0.4 mM (8).

An ATPase also is associated with Torpedo synaptic vesicles (10-12). It is stimulated in a saturable fashion by bicarbonate ion in the presence of MgATP or CaATP and is independent of Na^+, K^+ or Cl^- ions (13). Both active

[1]Abbreviations used: ACh, acetylcholine; S-13, 3-t-butyl-5-Cl-2'-Cl-4'-nitro-salicylanilide; hepes, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.

0006-291X/81/220422-07$01.00/0

[^{3}H]ACh uptake and the ATPase activity are inhibited by similar concentrations of 4-chloro-7-nitrobenzo-oxadiazole and dicyclohexylcarbodiimide (9,11). In view of the suggested involvement of proton-pumping ATPases in the uptake of other neurotransmitters by storage vesicles, we have examined the effects of uncouplers on ACh transport and ATPase activity in Torpedo electric organ synaptic vesicles.

MATERIALS AND METHODS

The isolation and preparation of diethyl-p-nitrophenylphosphate-treated and [^{14}C]mannitol-equilibrated Torpedo californica electric organ synaptic vesicles, and the protocol for [^{3}H]ACh uptake experiments is described in (7). Ethanol solutions of S-13 and p-nitrophenol were dried in a clean test tube under N_2 gas before addition of vesicle suspensions 1 hour before initiation of [^{3}H]ACh uptake. Standard double-channel liquid scintillation counting was performed as described (4). The uptake ratio gives the ratio of the concentration of [^{3}H]ACh inside the vesicles compared to the concentration outside.

The vesicle-associated ATPase was assayed as follows. Vesicles (5-20 µl) were added to 1 ml solutions containing 0.4 mM NADH, 2 mM potassium phosphoenolpyruvate, 50 units of pyruvate kinase (Sigma type III rabbit muscle), 45 units of lactate dehydrogenase (Sigma type XI rabbit muscle) and 2 mM MgATP. The oxidation of NADH was observed at 340 nm. Valinomycin, MgATP, potassium phosphoenolpyruvate, K_2ATP, and $MgCl_2$ were obtained from Sigma Chemical Company. Potassium bicarbonate and p-nitrophenol were obtained from MCB. The p-nitrophenol was recrystallized from toluene before use. S-13 was a gift from Dr. Paul Boyer. [^{3}H]Acetylcholine chloride (250 mCi/mmol) and D-[1-^{14}C]mannitol (56 mCi/mmol) were obtained from Amersham Searle.

RESULTS

Inhibition of Active [^{3}H]ACh Uptake by Protonophores

Torpedo electric organ synaptic vesicles achieved a 10-14 fold concentrative uptake of exogenous [^{3}H]ACh in the presence of MgATP and bicarbonate in two experiments shown in Figure 1. The active uptake was inhibited by S-13 above 10^{-8} M, with total inhibition by 10^{-6} M. In this concentration range of S-13 the effect was specific for active uptake, since passive uptake remained unaffected. Para-nitrophenol also totally inhibited active uptake of [^{3}H]ACh, but about a 1000 fold higher concentration was required. These results suggest that active ACh uptake is linked to a vesicle proton gradient which is generated in the presence of MgATP and bicarbonate.

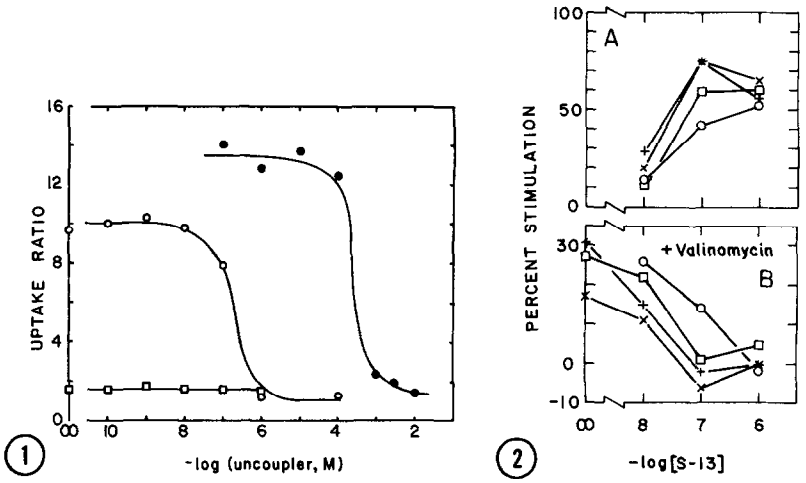

Figure 1. Inhibition of active [^{3}H]ACh uptake by S-13 and p-nitrophenol. For S-13, active (—o—) and passive (—□—) uptake, the experiment was at 25°C in 5 mM hepes -0.8 M glycine buffer at pH 7.0 containing 1 mM each of EDTA and EGTA and 0.02% (w/v) KN_3. For p-nitrophenol (—●—) the buffer was 0.2 M hepes -0.6 M glycine at 25° and pH 7.4 containing EDTA, EGTA and KN_3 as above. For S-13, [^{14}C] mannitol-equilibrated vesicles were added to [^{3}H]ACh, K_2ATP, $MgCl_2$ and $KHCO_3$ to respective final concentrations of 50 μM, 5 mM, 7 mM and 40 mM. For p-nitrophenol, [^{14}C]mannitol-equilibrated vesicles were added to [^{3}H]ACh, MgATP, $MgCl_2$, and $KHCO_3$ to respective final concentrations of 50 μM, 5 mM, 7 mM, and 40 mM, and ATP was recycled by 25 units of pyruvate kinase and 5 mM potassium phosphoenolpyruvate. The latter solutions contained [^{14}C]mannitol at the same concentration as the vesicle suspensions. Uptake was terminated 30 min later by centrifugation-gel filtration at 4°, and the [^{3}H] to [^{14}C] ratio inside of the vesicles was compared to the ratio outside of the vesicles to obtain the uptake ratio.

Figure 2. Stimulation of ATPase activity by S-13 and valinomycin. Vesicles were pelleted in the ultracentrifuge and resuspended overnight at 4°C in 5 mM hepes -0.8 M glycine buffer at pH 7.0 containing either 50 μM $CaCl_2$ plus 5 mM $MgCl_2$ (— + —), 5 μM $CaCl_2$ plus 5 mM $MgCl_2$ (—□—), 5 mM $MgCl_2$ alone (— x —) or no divalent cations (—O—). The coupled ATPase assay was performed in buffers identical to those used in the overnight incubations. ATPase activity was measured sequentially with addition of the synaptic vesicles, followed by addition of 20 mM $KHCO_3$, S-13 and valinomycin to the indicated concentrations. The average (4 trials) bicarbonate stimulation of MgATPase activity was 14% (— + —), 23% (—□—), 4% (— x —) and 24% (—O—). A. Percent stimulation by S-13 was calculated relative to the rate in bicarbonate medium. B. Percent stimulation by 1 μM valinomycin was calculated relative to the S-13 stimulated rate immediately preceeding the addition of valinomycin.

Stimulation of the ATPase by Uncouplers

If it is responsible for creation of the proton gradient, the vesicle-associated ATPase might be stimulated by mitochondrial uncouplers. The results in the upper part of Figure 2 establish the occurrence of this behavior

with S-13. The stimulation was dependent on the concentration of S-13, beginning around 10^{-8} M and saturating at about 10^{-6} M with 50-75 percent stimulation. The absence or presence of exogenous Ca^{2+} had no large effect on the result. However, overnight incubation of vesicles in the presence of divalent cations led to more tightly coupled vesicles since S-13 gave somewhat greater stimulation in these cases.

Valinomycin was able to stimulate the vesicle ATPase up to 30 percent in the absence of S-13 (Figure 2, bottom). At high S-13 concentrations, however, valinomycin produced no additional stimulation above that already produced by the S-13. These results also were substantially independent of exogenous Ca^{2+}. Stimulation of the ATPase by valinomycin suggests that the ATPase generates a significant transmembrane electrical gradient which can be collapsed by valinomycin mediated K^+ movement.

DISCUSSION

Cholinergic synaptic vesicles exhibit uptake of [^{3}H]ACh which is stimulated by bicarbonate and either MgATP or CaATP. The active uptake of [^{3}H]ACh exhibits so many parallels with the properties of the vesicle-associated ATPase that it has seemed probable that the two processes are linked. However, the nature of the linkage has not been explored until this study.

The mitochondrial uncoupler S-13, which acts by collapsing proton gradients, completely inhibits active [^{3}H]ACh uptake. The concentration range over which S-13 becomes effective is similar to that reported for other neurotransmitter storage systems (14,15). The inhibition appears to be specific for active [^{3}H]ACh uptake since passive uptake was unaffected. This eliminates the unlikely possibility that S-13 acted directly on the ACh porter. Thus, a transmembrane proton gradient probably is linked to active [^{3}H]ACh uptake.

The uncoupler p-nitrophenol can be generated as a hydrolysis product of the diethyl-p-nitrophenylphosphate used to inhibit acetylcholinesterase

in our experiments. It also abolished active [^{3}H]ACh uptake into synaptic vesicles. It is much less potent than S-13, however, having no effect up to 10^{-4} M. To avoid excessive p-nitrophenol we are careful to use only low concentrations of fresh paraoxon which is not allowed to hydrolyze for long periods in the vesicle solutions before experiments are begun. We continue to use paraoxon despite the required precautions because it is the most effective acetylcholinesterase inhibitor without other potential problems which we have found to date.

The proton gradient linked to active [^{3}H]ACh uptake appears to be generated by the ATPase, since in the same concentration range over which S-13 inhibits active [^{3}H]ACh uptake the ATPase is stimulated by up to 75 percent. Stimulation by S-13 is not due to the anionic form acting as a bicarbonate analog since in the presence of bicarbonate uncouplers inhibit the mitochondrial ATPase (16). The observed extent of ATPase stimulation is comparable to or greater than those seen for the mitochondrial and chromaffin granule ATPases (15, 17) but about a 10-fold higher concentration of S-13 was required here compared to that required for mitochondrial uncoupling (18). This is not surprising, however, since the lipid compositions of the two membrane systems differ considerably, particularly in the cholesterol content which is 33 mol percent in vesicles and about 3 mol percent in mitochondria (19-21).

Valinomycin also stimulates the ATPase in a fashion complementary with S-13 in that the maximal stimulations are not additive. Since valinomycin cannot carry protons across the membrane, but can collapse an electrical gradient by allowing movement of K^+ across the membrane, a substantial part of the energy in the proton gradient generated by the ATPase could be electrical in nature.

Stimulation of the vesicle-associated ATPase by both valinomycin and S-13 appears to be independent of exogenous Ca^{2+}. This independence is not consistent with the involvement of Ca^{2+} in a primary active transport

process catalyzed by the ATPase.

Other workers have obtained indirect evidence consistent with the importance of a transmembrane proton gradient in ACh storage. Melega and Howard have shown that in vivo ACh storage in PC12 cells is blocked by uncouplers (22). Michaelson and coworkers have shown that endogenous ACh contained in isolated Torpedo electric organ synaptic vesicles is lost in the presence of uncouplers, and isolated vesicles are internally acidic (23-24). The experiments reported here provide direct evidence that an electrochemical proton gradient generated by an ATPase is linked to active [^{3}H]ACh uptake by synaptic vesicles.

ACKNOWLEDGMENTS

We thank Marianne L. Henry for preparation of the synaptic vesicles. This investigation was supported by grant NS15047 from the National Institute of Neurological and Communicative Disorders and Stroke and a grant from the Muscular Dystrophy Association to SMP and by a Public Health Service National Research Service Award to DCA.

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