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SPECIFICITY OF ASSOCIATION OF A Ca^{2+}/Mg^{2+} ATPase WITH CHOLINERGIC SYNAPTIC VESICLES FROM TORPEDO ELECTRIC ORGAN

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SUMMARY: Cholinergic synaptic vesicles from the electric organ of Torpedo californica have been subjected to analytical scale separation techniques not utilized in the isolation procedure, and the ATPase activity of separated fractions determined. Most of the ATPase activity migrated with the vesicles. Sensitivity of the ATPase activity to 16 potential inhibitors also was determined. Most of the ATPase activity was inhibited by low concentrations of 4-chloro-7-nitrobenzo-oxadiazole (NBD-Cl) and dicyclohexylcarbodiimide (DCCD), but not by a water soluble carbodiimide. The close association of the ATPase with the vesicles and the pattern of inhibition obtained provide further support for the authentic presence of a membrane bound Ca^{2+}/Mg^{2+} ATPase in the cholinergic synaptic vesicle.

INTRODUCTION

Synaptic vesicles can be isolated in a high degree of purity from the electric organ of the elasmobranch Torpedo (1-3). These purely cholinergic vesicles are enclosed by a lipid bilayer, contain very high concentrations of acetylcholine and ATP, and contain a number of polypeptides (2-4). Recently Breer, et al. (5) reported that purified vesicle ghosts from Torpedo marmorata had associated with them a Ca^{2+}/Mg^{2+} ATPase activity which was stimulated 20 percent in the presence of exogenous acetylcholine. This observation suggested that the Ca^{2+}/Mg^{2+} ATPase might be involved in the uptake and storage of acetylcholine by the cholinergic synaptic vesicle.

Uptake and storage of norepinephrine and serotonin by brain synaptic vesicles and of catecholamines by chromaffin granules involves vesicle or granule associated ATPase activities (6-10). Thus, ample precedent for the plausibility of a transport ATPase in the cholinergic synaptic vesicle

Abbreviations used: NBD-Cl, 4-chloro-7-nitrobenzo-oxadiazole, which is also abbreviated Nbf-Cl; DCCD, dicyclohexylcarbodiimide; CMCD, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid; HC-3, hemicholinium-3.

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exists. However, ATP stimulated uptake of acetylcholine by cholinergic vesicles has not yet been demonstrated. In view of the rather modest stimulation of the ATPase activity by acetylcholine and the failure to demonstrate a reciprocal effect of ATP on acetylcholine uptake, additional evidence that a Ca^{2+}/Mg^{2+} ATPase indeed is endogenous to the vesicles and is not a contaminant of the preparation seems desirable. We report here studies which subjected the isolated vesicles to two higher resolution analytical separation techniques, which were developed by Carlson, et al. (1), in an attempt to separate contaminating ATPase from the vesicles. Also, sensitivity of the vesicular ATPase to a number of compounds which inhibit known ATPases was determined.

MATERIALS AND METHODS

Synaptic vesicles were isolated from the electric organ of Torpedo californica similarly to the method of Carlson, et al. (1). In brief, the method relies on differential sedimentation velocity pelleting, equilibrium buoyant density centrifugation, and CPG 3000 filtration of synaptic vesicles in glycine and glycine-sucrose media at pH 7.0.

Purified vesicles were pelleted 2 hr at 4° in the SW50.1 rotor to concentrate them for all experiments. Vesicles (1 mg protein) resuspended in 300 µl of 0.8 M glycine, 5 mM HEPES, 0.02 percent sodium azide, pH 7.0 with NaOH were layered on a 10-50 percent continuous glycerol gradient containing 0.4 M NaCl, 3 mM HEPES, 3 mM EGTA, pH 7.0 with NaOH, and centrifuged to their equilibrium buoyant density as described (1). Similarly prepared vesicles were subjected to sedimentation velocity analysis on a similar 5 to 25 percent glycerol density gradient as described (1). Fractions from both experiments were assayed for ATP content, protein and ATPase activity using firefly luciferase, Coomassie Blue, and coupled pyruvate kinase-lactate dehydrogenase techniques, respectively, as described (1, 11, 12). Before measuring ATPase activity, the vesicles were hypoosmotically shocked by diluting with 2 volumes of water at 0° in the presence of 3.5 mM oubain.

For inhibitor studies pelleted vesicles were resuspended either in glycine-HEPES medium or in sucrose-HEPES medium at pH 7.0 as described below. Vesicle ghosts were prepared by hypoosmotic shock in the presence of inhibitors to allow access of inhibitors to both sides of the membrane and the suspension then incubated 1 hr at 25°. When present, alcohol concentrations arising from the inhibitor stock solutions never exceeded 1.25 percent (v/v) and were shown not to affect ATPase activity. The ATPase reaction was initiated by addition of 20 µl of 0.06 M vanadium-free disodium ATP to 380 µl of inhibited vesicle ghosts to give final concentrations of 5 mM Mg^{2+}, 3 mM ATP, and 50 µg protein. Incubation was continued for 20 to 60 min at 25° before determination of released phosphate as described (13). Blank phosphate values were determined for the ATP and buffer solutions and the vesicles. The effect of each inhibitor on color yield in the phosphate assay also was determined with standard curves, and appropriate corrections made in reported data.

NBD-Cl was from K and K Division of ICN Pharmaceuticals, Inc. DCCD, CMCD, HC-3 and trimethyltin chloride were from Aldrich. Quercetin, oubain, N-ethylmaleimide, oligomycin, and propanolol were from Sigma. Chlorpromazine was from S.K.F., desimipramine was from Ciba-Geigy, and efrapeptin was a generous gift from Dr. Hamill of Lilly Research.

RESULTS

Early work confirmed that highly purified vesicles obtained from controlled pore glass chromatography exhibited Ca^{2+}/Mg^{2+} ATPase activity. These purified synaptic vesicles were centrifuged to their equilibrium buoyant density in a glycerol density gradient which subsequently was fractionated. Figure 1 shows that vesicle-bound ATP and protein banded in the middle of the density gradient. Within the resolution of the experiment most of the ATPase activity banded coincidentally with the vesicles. The vesicle isolation procedure involves a step where vesicles are centrifuged to neutral buoyancy in a sucrose density gradient in which their density is between 1.045 to 1.059 g cm^{-3} (4). In glycerol their density changes to about 1.110 g cm^{-3}. This is due to permeation of the vesicle by glycerol with subsequent equilibration of the internal solvent volume with glycerol (4). Thus, the ATPase in Figure 1 has changed its buoyant density in glycerol solution similarly to synaptic vesicles.

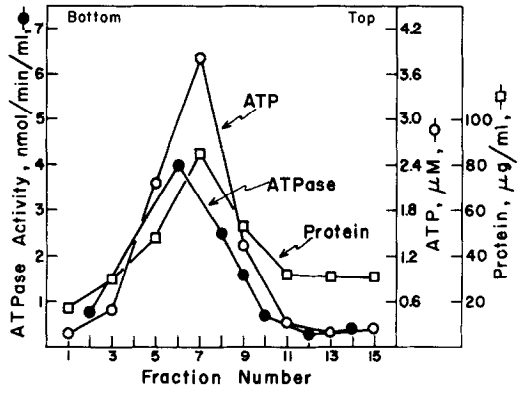

Figure 1. Equilibrium Buoyant Density Banding of Purified Vesicles in Glycerol. The top of the glycerol density gradient is to the right. Vesicles were centrifuged 11 hr at 38,000 rpm at 4° in the SW50.1 rotor. Ten drop fractions were collected from the bottom of the tube and fractions were assayed for ATP, protein, and total magnesium dependent ATPase activity.

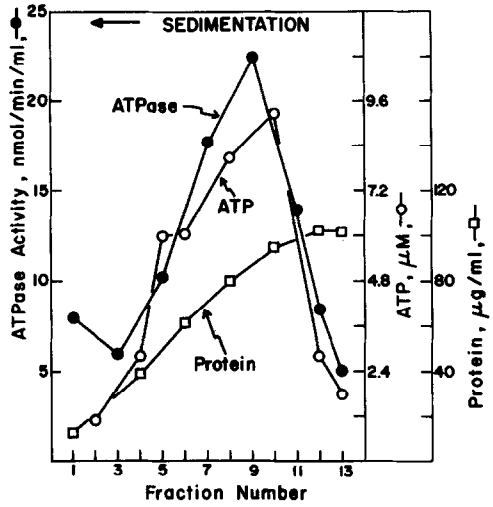

Figure 2. Band Sedimentation Velocity Centrifugation of Purified Vesicles in Glycerol. The top of the glycerol density gradient is to the right. Vesicles were centrifuged 1 hr at 38,000 rpm at 4° in the SW50.1 rotor. The gradient was analyzed as in Figure 1.

Synaptic vesicles also were centrifuged in a glycerol density gradient as a band under sedimentation velocity conditions. Figure 2 shows that most of the ATPase activity was coincident with the vesicle-bound ATP. A significant quantity of protein remained at the top of the gradient but it contained no ATPase activity. This protein apparently dissociated from the vesicles during centrifugation, possibly as a result of transfer of the vesicles from the relatively low ionic strength isolation buffer to the higher ionic strength sedimentation velocity buffer system. Recently, Ohsawa et al. (2) also found that protein is removed from vesicles during further purification. Thus, even though significant protein was removed from purified vesicles in Figure 2, no ATPase activity was lost, again suggesting intimate association of the two.

Another approach to characterizing the specificity of ATPase association with cholinergic synaptic vesicles is to determine whether the ATPase can be inhibited by compounds which act on known ATPases. Table 1 lists 12 tested compounds which had effects judged not to be significant. The table includes compounds known to inhibit Na^+/K^+, mitochondrial and sarcoplasmic reticulum

TABLE 1. Potential Inhibitors of the Vesicle ATPase.

Compound	Concentration	Percent Inhibition
Oligomycin[a]	40 µg/ml[d]	4
	250 µg/ml[d]	0
Efrapeptin[b]	60 µg/ml[d]	5
Quercetin[a]	75 µM[e]	7
Oubain[c]	0.7 mM[d]	6
	2.0 mM[e]	0
Sodium Azide[c]	3.0 mM[e]	0
HC-3[c]	1.0 mM[e]	2
Colchicine[a]	12.5 µM[e]	0
Trimethyltin[b]	125 µg/ml[d]	19
Sodium Vanadate[c]	0.1 µM[d]	14
	1.0 mM[d]	37
dl-Propanolol[b]	0.3 mM[d]	15
N-ethylmaleimide[c]	10.0 mM[d]	37
Desimipramine[c]	0.1 mM[f]	16
	1.0 mM[f]	24

[a]Concentrated stock solution prepared in methanol.

[b]Concentrated stock solution prepared in ethanol.

[c]Concentrated stock solution prepared in specified buffer.

[d]Final assay buffer concentrations were 3.75 mM HEPES, 0.6 M glycine, 5 mM magnesium acetate, pH 7.0.

[e]Final assay buffer concentrations were 37.5 mM HEPES, 0.56 M glycine, 5 mM $MgCl_2$, pH 7.0.

[f]Final assay buffer concentrations were 50.0 mM HEPES, 0.15 M sucrose, 5 mM $MgCl_2$, pH 7.0.

ATPases (14-17). The moderate inhibition by N-ethylmaleimide is not considered significant because the very high concentration required can be expected to produce nonspecific effects. Neither sodium azide, used as a microbialstat during isolation, nor hemicholinium-3, an inhibitor of high affinity uptake in the cholinergic nerve terminal (18), had any effect either.

The concentration dependencies for additional compounds of particular interest are shown in Figure 3. The compound NBD-Cl was a very potent inhibitor with less than 50 µM being required for half-effect. DCCD also inhibited

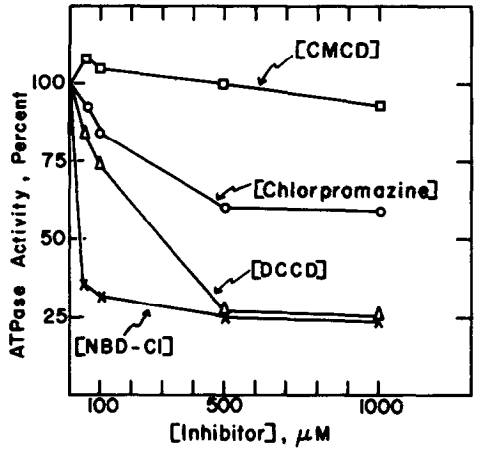

Figure 3. Percent Inhibition versus Concentration for Inhibitors of the Cholinergic Vesicle ATPase. NBD-Cl, DCCD, and chlorpromazine stock solutions were prepared in methanol. The volume of methanol in the assay was 1.25 percent for all concentrations of inhibitor tested. The water soluble carbodiimide CMCD stock solution was prepared in assay buffer and used immediately. Final assay buffer concentrations were 50 mM HEPES, 0.15 M sucrose, 5 mM $MgCl_2$, pH 7.0 with NaOH.

effectively but about 200 μM was required for half-effect. Since both reagents gave a similar maximal extent of inhibition it is probable that this represents total inhibition of a dominant ATPase. It is likely that one or more other ATPase activities which are not sensitive to these inhibitors were also present in a relatively minor amount. The less likely explanation for incomplete inhibition by NBD-Cl and DCCD is that both are noncompetitive inhibitors of a single ATPase, with both exhibiting the same maximal inhibition. In contrast, chlorpromazine inhibited more ATPase activity than represented by the NBD-Cl insensitive ATPase, but the maximal inhibition by chlorpromazine was less than that by NBD-Cl and DCCD. This suggests that chlorpromazine is an uncompetitive (or noncompetitive) inhibitor of the same ATPase inhibited by NBD-Cl and DCCD.

Figure 3 also shows that the water soluble carbodiimide CMCD did not significantly inhibit ATPase activity. Since carbodiimides react with carboxylic acids and NBD-Cl with nucleophiles, glycine and acetate species were removed from the buffer in these experiments. The inability of CMCD to

inhibit and the ability of lipid soluble DCCD to inhibit suggests that the dominant ATPase activity is membrane bound.

DISCUSSION

Most of the ATPase activity which purifies with cholinergic synaptic vesicles remained associated with them after fractionation by two additional separation techniques which were not used in the purification scheme. In all, the Ca^{2+}/Mg^{2+} ATPase remained associated with vesicles through sedimentation velocity steps in sucrose and glycerol, through neutral buoyant density banding in sucrose and glycerol, and through controlled pore glass bead filtration. The coincidence of ATPase activity and vesicles in sucrose and glycerol solvent systems is a particularly strong test of identity since the density change occurring on transfer from sucrose to glycerol is a function of the relative amounts of membrane and internal solvent volume in the particle (4). Thus, the ATPase is bound to a particle which has the same density as the synaptic vesicle and which has the same ratio of membrane to internal solvent volume as the synaptic vesicle.

The inhibition behavior obtained with different reagents suggests that more than one ATPase is present in the preparation but a single membrane bound ATPase activity dominates. The sensitivity of the dominant cholinergic synaptic vesicle ATPase to NBD-Cl and DCCD is similar to that of the brain synaptic vesicle ATPase involved in uptake of norepinephrine (6, 7). DCCD also inhibits the chromaffin granule ATPase involved in uptake of epinephrine (7). Both of these ATPases appear to pump protons into the vesicle or granule, and it is the resulting pH gradient which is utilized to drive the uptake and storage of catecholamines. NBD-Cl and DCCD also inhibit the mitochondrial ATPase (19, 20). Although these reagents thus often inhibit proton transporting ATPases, it cannot be assumed that the major cholinergic vesicle ATPase necessarily pumps protons since the sarcoplasmic reticulum ATPase also is inhibited by DCCD (21). In summary, a membrane-bound Ca^{2+}/Mg^{2+} ATPase inhibitable by NBD-Cl and DCCD indeed appears to be specifi-

cally associated with cholinergic synaptic vesicles, as originally reported by Breer, et al.

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