Evidence That an ATPase and a Protonmotive Force Function in the Transport of Acetylcholine into Storage Vesicles*

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PC12, a clonal line of rat pheochromocytoma, accumulates newly synthesized acetylcholine in storage granules. The accumulation of acetylcholine in PC12 granules, but not acetylcholine synthesis, was inhibited by treatment of the cells with any of several inhibitors of energy metabolism. These included nigericin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone, di-cyclohexylcarbodiimide, and iodoacetate. Valinomycin alone and oligomycin were without effect. Except for iodoacetate, these agents did not exert their effects on acetylcholine storage by depleting the cells of ATP.

Little is known about the mechanism by which acetylcholine is transported into synaptic vesicles of cholinergic nerve terminals. However, recent studies have demonstrated that cholinergic synaptic vesicles from the eel have intrinsic ATPase activity (1) with kinetic properties intriguingly similar to those of the ATPase that functions in the transport of catecholamines into catecholaminergic vesicles (2). The transport of catecholamines into synaptic vesicles obtained from neurons and into chromaffin granules obtained from the adrenal medulla is driven by a trans-vesicle pH gradient or membrane potential, or both, which are established by the activity of a proton-translocating ATPase associated with the vesicles or granules (3-8). This ATPase activity and the ATP-stimulated uptake of catecholamines into isolated synaptic vesicles and chromaffin granules are inhibited by DCCD1 and NBD-Cl, but not by oligomycin or efrapeptin, two inhibitors of mitochondrial ATPase (3, 6, 8). Catecholamine transport into the vesicles and granules is also blocked by ionophores that make membranes permeable to protons (3, 5, 8).

The ATPase of Torpedo cholinergic vesicles also is inhibited by DCCD and NBD-Cl, but not by oligomycin or efrapeptin.

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The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; NBD-Cl, 4-chloro-7-nitrobenzofurazan; AH5183, 2-(4-phenylpiperidino)cyclohexanol; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ΔΨ, transmembrane proton concentration gradient; Δρ, transmembrane potential.

(2). Unfortunately, since specific loading of acetylcholine into isolated synaptic vesicles has not been demonstrated, it is not possible at present to examine the effects of the ATPase inhibitors and ionophores on acetylcholine loading using preparations of isolated vesicles. Rebois et al. (9) described the uptake of acetylcholine into storage granules in intact cells of PC12, a clonal cell line of rat pheochromocytoma that stores and secretes both acetylcholine and catecholamines, primarily dopamine (9-12). In an extension of that work, we have examined the effect of various inhibitors of energy metabolism on acetylcholine synthesis and uptake into storage granules in PC12 cells. We report here that the net uptake of acetylcholine into the PC12 granules is blocked by agents known either to inhibit the ATPase activity of Torpedo synaptic vesicles or to dissipate proton electrochemical gradients. These effects do not seem to be attributable to a decrease by the agents of cellular stores of ATP.

MATERIALS AND METHODS

Chemicals—Isodiacetate, oligomycin, reserpine, and valinomycin were purchased from Sigma Chemical Co.; DCCD was from Eastman Kodak; and NBD-Cl was from ICN. [3H]Choline chloride (84 Ci/mmol) was obtained from New England Nuclear. AH5183, FCCP, and nigericin were gifts from Allen and Hanbury, DuPont, and Hoffman-La Roche, respectively.

Cell Culture—The clonal rat pheochromocytoma line, PC12, was obtained from Dr. D. Schubert. The cells were grown at 37°C under an atmosphere of 11% CO2, 88% air in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 5% horse serum, 50 µg/ml of streptomycin, and 50 units/ml of penicillin on plastic culture dishes or flasks. The cells were used for the various experiments 4 to 5 days after subculturing, just prior to becoming confluent.

Isolation of Storage Granules—A cell homogenate was obtained by 15 up and down passes of a tight fitting pestle in a Dounce homogenizer. The homogenate was centrifuged at 1000 x g for 10 min at 4°C. The supernatant suspension was centrifuged at 20,000 x g for 30 min. Almost all of the acetylcholine in the resulting pellet (P2 fraction) was found to bind in a sucrose density gradient at regions determined by Schubert and Klier (12) to be enriched in granules and transmitter. Therefore, we have assumed that the bound acetylcholine in the P2 fraction is in storage granules and the P2 fraction was used without further purification for the determination of acetylcholine content of isolated granules.

Incubation of Cells with [3H]Choline—The cells were incubated at 37°C while still attached to plastic dishes or flasks in a cell incubation buffer consisting of 60 mM sucrose, 10 mM glucose, 130 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM Hepes, pH 7.3. To measure newly synthesized acetylcholine, cells were preincubated for 15 min in incubation buffer containing drugs as indicated. The buffer was replaced with 5 ml/plate of fresh buffer containing the appropriate drug and 2 µCi of [3H]choline (84 Ci/ml). After an additional 30 min of incubation, the cells were washed twice with 5 ml/plate of ice cold incubation buffer containing 40 µM eserine and once with 5 ml of 0.32 M sucrose, 40 µM eserine. The cells were scraped off the plate with a rubber policeman and the plates were washed with an additional 1.5 ml of 0.32 M sucrose, 40 µM eserine. After adjusting the volume of the cell suspension to 2.0 ml with 0.32 M sucrose, 40 µM eserine, a 0.4-ml sample was taken for measurement of [3H]acetylcholine. The remaining cell suspension was homogenized and the granule fraction was prepared as described above.

Determination of [3H]Acetylcholine—[3H]Acetylcholine was separated from [3H]choline by thin layer chromatography after extraction of these compounds initially into 1 N formic acid/acetone (15:85) and subsequently into CH2Cl2 with dipicrylamine as described (13). [3H]Acetylcholine served as an internal standard. After drying the CH2Cl2 under nitrogen, the acetylcholine was redissolved in acetone and spotted on cellulose thin layer plates. The plates were developed with...
with a solvent system of ethyl acetate/formic acid/H_2O (70:20:10). The spots were visualized with iodine and scraped into scintillation vials containing 0.5 ml of 0.1 N HCl. To each vial was added 10 ml of 3aHBO liquid scintillation mixture (Research Products International) and the radioactivity was counted by liquid scintillation spectrometry.

**Determination of ATP and Protein—**ATP was measured as described (14) and protein was measured by the method of Lowry et al. (15).

**RESULTS**

Under the conditions of incubations used for our studies, [^3]H]acetylcholine is rapidly taken up by the PC12 cells and acetylated (Fig. 1A). The cellular level of [^3]H]acetylcholine reaches a plateau by 10 to 15 min after the addition of [^3]H]choline. However, the loading of [^3]H]acetylcholine into granules occurs comparatively slowly as shown in Fig. 1B. (Note the difference in scales between A and B of Fig. 1.) The amount of [^3]H]acetylcholine in granules increases linearly with time for at least 120 min; at that time, [^3]H]acetylcholine in granules is about 30% of total cell [^3]H]acetylcholine (9).

Fig. 1 also shows the effect on acetylcholine metabolism of the ionophore nigericin, which causes a H^+ /K^+ exchange. The presence of nigericin (0.2 μg/ml) during a 30-min incubation with [^3]H]choline did not appreciably affect the amount of [^3]H]acetylcholine formed in the cells during this time (Fig. 1A). However, nigericin almost completely prevented the accumulation of [^3]H]acetylcholine in the granule fraction (Fig. 1B).

Table I describes the effect of other agents on cell and granule levels of [^3]H]acetylcholine 30 min after addition of [^3]H]choline. Several of these agents caused a substantial decrease in the granule store of [^3]H]acetylcholine without affecting total cellular [^3]H]acetylcholine. One of them was the proton ionophore FCCP. The K^+ ionophore valinomycin by itself had no effect on cell or granule levels of [^3]H]acetylcholine; however, it potentiated the effect of FCCP. Valinomycin also potentiates the ability of FCCP to decrease the storage of catecholamines in isolated synaptic vesicles, chromaffin granules, and PC12 granules (7, 9, 16) and to dissipate the proton gradient across chromaffin granule membranes (17). In these latter cases, the effect can be attributed to a valinomycin-induced influx of K^+, which facilitates the FCCP-induced efflux of protons from the catecholamine-storing vesicles or granules (17).

The net loading of [^3]H]acetylcholine into PC12 granules was also inhibited by DCCD, but it was not affected by oligomycin (Table I). Neither of these ATPase inhibitors significantly altered total cell levels of [^3]H]acetylcholine. We did not study the effect of NBD-CI on acetylcholine metabolism because a brief exposure of the PC12 cells to 100 μM NBD-CI proved to be extremely cytotoxic.

We have previously reported that if PC12 cells were incubated with [^3]H]choline for 60 min as described for Table I and then treated for 10 min with 0.2 μg of nigericin/ml, there occurred a large efflux (65%) from the storage granules of previously accumulated [^3]H]acetylcholine (9). We have repeated this experiment with the modification that, following the 60-min incubation with [^3]H]choline, the cells were exposed for 10 min to 40 μM DCCD. Under these conditions, granules isolated from the DCCD-treated cells contained as much of the previously accumulated [^3]H]acetylcholine as granules from control cells (data not shown). Thus, the effects of DCCD on acetylcholine storage in PC12 granules are similar to its effects on norepinephrine storage in synaptic vesicles from rat brain (8); DCCD inhibits the ATP-stimulated uptake of norepinephrine into the synaptic vesicles but does not cause an efflux of previously accumulated norepinephrine.

All of the ionophores and ATPase inhibitors listed in Table I can inhibit mitochondrial ATP synthesis. However, under the incubation conditions used for the experiments discussed above, none of these agents, with the exception of nigericin, caused any depletion of cellular stores of ATP (Table I). Nigericin caused only a very slight decrease in cellular ATP. Thus, it is unlikely that any of the ionophores or ATPase inhibitors exerted their effects on granular storage of acetylcholine by altering cellular pools of ATP. The inability of these agents to reduce cellular ATP can be explained by the fact that a major portion of the ATP in PC12 is derived from glycolysis rather than oxidative phosphorylation.2

Iodoacetate, an inhibitor of glycolysis, did markedly reduce cellular ATP (Table I). Unexpectedly, the iodoacetate treatment substantially increased the level of [^3]H]acetylcholine in the PC12 cells. We have not investigated the mechanism of this effect, but it may relate to an alteration in the uptake of choline; iodoacetate does increase choline uptake into PC12

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1. E. Reynolds, unpublished observations.

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**TABLE I**


The cells were incubated with [^3]H]choline and the agents listed as described under "Materials and Methods." After the incubation, [^3]H]acetylcholine was measured in samples of whole cells and a granule fraction was obtained from a cell homogenate. ATP was measured in extracts of unfractionated cells after a similar incubation but without [^3]H]choline. The results, which are expressed as percentage of control values, are the means ± S.D. for the number of incubations given in parentheses. Control acetylcholine values were 30,200 ± 630 dpm/mg of cell protein and 16,000 ± 1,500 dpm/mg of granule protein for whole cells and granules, respectively. Control cellular ATP was 24.8 ± 1.1 nmol/mg of protein. Abbreviation used: ND, not determined.

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<td>Control</td>
<td>100 ± 2 (4)</td>
<td>100 ± 2 (4)</td>
<td>96 ± 2 (3)</td>
</tr>
<tr>
<td>FCCP, 4 μM</td>
<td>106 ± 8 (2)</td>
<td>23 ± 1 (2)</td>
<td>84 ± 2 (3)</td>
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<tr>
<td>FCCP, 1 μM</td>
<td>108 ± 21 (4)</td>
<td>49 ± 1 (4)</td>
<td>98 ± 9 (3)</td>
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<tr>
<td>Valinomycin, 3 μg/ml</td>
<td>96 ± 14 (6)</td>
<td>111 ± 15 (6)</td>
<td>98 ± 8 (3)</td>
</tr>
<tr>
<td>Valinomycin, 3 μg/ml, FCCP, 1 μM</td>
<td>78 ± 16 (6)</td>
<td>18 ± 5 (5)</td>
<td>99 ± 9 (3)</td>
</tr>
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<td>DCCD, 40 μM</td>
<td>88 ± 14 (6)</td>
<td>43 ± 8 (6)</td>
<td>112 ± 2 (3)</td>
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<td>Oligomycin, 4 μg/ml</td>
<td>123 ± 23 (6)</td>
<td>100 ± 18 (6)</td>
<td>104 ± 9 (3)</td>
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<td>Iodoacetate, 0.5 mM</td>
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<td>61 ± 9 (6)</td>
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<td>Reserpine, 1 μM</td>
<td>84 ± 2 (2)</td>
<td>157 ± 8 (2)</td>
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<td>AH5136, 10 μM</td>
<td>83 ± 4 (2)</td>
<td>12 ± 3 (2)</td>
<td>ND</td>
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**FIG. 1.** Effect of nigericin on acetylcholine synthesis (A) and accumulation by granules (B). The cells were incubated with [^3]H]choline and in some cases with nigericin (0.2 μg/ml) as described under "Materials and Methods." After the incubation, [^3]H]acetylcholine was measured in samples of whole cells (A) and a granule fraction (B) obtained from a cell homogenate. The data points represent the means for triplicate incubations and the error bars indicate the S.D. ○, control; ●, nigericin treated.
cells under some conditions of incubation. In spite of the increase in total cell $[^3H]$acetylcholine, the level of $[^3H]$acetylcholine in granules was decreased by the treatment with iodoacetate.

We have found that two other compounds produced interesting effects on acetylcholine storage in PC12 granules. One of the compounds was reserpine, which inhibits the loading of catecholamines into synaptic vesicles, chromaffin granules, and PC12 granules (8, 9, 18, 19), presumably by blocking the carrier for catecholamines (19). As shown in Table I, reserpine caused a slight decrease in total cell $[^3H]$acetylcholine and, surprisingly, a marked increase in the level of $[^3H]$acetylcholine in granules. A repeat experiment performed under slightly different conditions gave essentially the same results.

The compound AH5183 has been reported to cause neurotransmitter blockade by inhibiting the evoked release of acetylcholine from motor nerve terminals (21). The electrophysiological characteristics of this blockade have been interpreted to indicate an AH5183-induced decrease in synaptic vesicle stores of acetylcholine (21). In PC12 cells, AH5183 markedly decreased the level of $[^3H]$acetylcholine in granules while having only a slight effect on total cell $[^3H]$acetylcholine (Table I). A 10-min incubation of PC12 cells with 10 pg of AH5183/ml did not cause an efflux from granules of $[^3H]$acetylcholine (21). The electrochemical and catecholamine-storing synaptic vesicles from Torpedo contain an ATPase that is sensitive to DCCD but the PC12 cholinergic granules have not been sufficiently purified to determine whether they also contain a DCCD-sensitive ATPase (9).

We do not understand how reserpine increases the accumulation of acetylcholine by PC12 granules. If acetylcholine and dopamine were competing for transport into common granules in PC12, reserpine could be thought to exert its effects on acetylcholine storage by blocking the loading of dopamine into the granules. However, Schubert and Klier (12) concluded that dopamine and acetylcholine are stored in different granules because dopamine-containing granules have a buoyant density different from that of acetylcholine-containing granules. We have confirmed their finding. Further studies of this reserpine effect are in progress.

**REFERENCES**

Evidence that an ATPase and a protonmotive force function in the transport of acetylcholine into storage vesicles.

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