Cholinergic Vesicles: Ability to Empty and Refill Independently of Cytoplasmic Acetylcholine

Abstract. *Incubation of minced mouse-forebrain tissues in lithium Krebs solution reduces the acetylcholine content of the vesicular fraction 70 percent without altering that of the cytoplasmic fraction. Depleted vesicular-bound acetylcholine can be restored with newly synthesized acetylcholine (formed from extracellular choline) independently of the cytoplasmic pool. Depletion of vesicular-bound acetylcholine does not facilitate the movement of preformed extracellular acetylcholine into vesicles.*

Acetylcholine (ACh) in brain tissue is stored in two separate compartments within cholinergic nerve endings—the cytoplasm and the vesicles (*1, 2*). In the classical model of a cholinergic nerve ending, both of these pools of ACh are synthesized at a common site, the cytoplasm, and then released from a different site, the vesicles (*3, 4*). Recent evidence, however, indicates that this model may require modification. Acetylcholine appears to be synthesized (*4*) and released (*5*) from both the cytoplasm and the vesicles. In addition, the transfer of cytoplasmic ACh into vesicles has yet to be demonstrated. Therefore, the cytoplasm and vesicles of cholinergic nerve endings may represent not only separate storage sites for ACh but also independent synthesis and release sites.

The purpose of the investigation reported here was to ascertain whether the cytoplasmic and vesicular pools of ACh can empty and refill independently of each other. It was also of interest to determine whether only newly synthesized ACh can refill a depleted vesicular pool of ACh.

Male (CF-1) albino mice were killed by cervical dislocation in a cold room (4°C), where the brains (minus cerebellum, pons, and medulla) were removed and sectioned through the median sagittal fissure, and each hemisphere was weighed and placed in several hundred milliliters of ice-cold medium. Each half was removed from the washing medium, minced, and maintained in a petri dish on ice until the onset of incubation. In the initial set of experiments, minced tissues were incubated for two 15-minute periods in 10 ml of Krebs Ringer bicarbonate solution or lithium Krebs solution at 37°C under an atmosphere of 95 percent O_2 and 5 percent CO_2 (lithium replaced sodium, and potassium bicarbonate replaced sodium bicarbonate) and washed twice with ice-cold 0.32*M* sucrose, and subcellular fractions were prepared (*6*). The amount of ACh in the cytoplasmic (S_3) and vesicular (P_3) fractions was then determined (*7*). In the second set of experiments, minced tissues were preincubated in normal Krebs or lithium Krebs solution for 30 minutes, washed twice in the ice-cold normal Krebs solution, and then incubated for another 30 minutes at 37°C in normal Krebs solution containing either [^{14}C]choline (0.1 m*M*) or [^{14}C]ACh (0.1 m*M*). In both cases paraoxon (0.1 μM) was present during the second incubation. After the second incubation, the minced tissues were washed twice with ice-cold 0.32*M* sucrose and subcellular fractions were prepared. The amounts of labeled and total ACh present in the cytoplasmic and vesicular fractions of the minced tissues were then determined (*8*).

The results (Table 1) indicate that vesicular-bound ACh can empty independently of cytoplasmic ACh. Incubation of minced brain tissue in lithium Krebs solution reduces the level of vesicular-bound ACh approximately 70 percent without significantly altering the level of cytoplasmic ACh.

Subsequent incubation of minced tissues in normal Krebs solution with [^{14}C]choline refills the vesicular-bound pool (9.6 ± 1.7 nmole/g, N = 12) with newly synthesized ACh independently of the cytoplasmic pool (Table 2). Incubation of minced tissues in normal Krebs solution with [^{14}C]choline after incubation in lithium Krebs solution results in a significantly higher ratio of labeled to total vesicular-bound ACh (0.63) than is attained in the nondepleted vesicular fraction of minced tissues incubated in normal Krebs solution (0.35). The source of the ACh that refills the depleted vesicular-bound pool does not appear to be cytoplasmic ACh, since the ratio of labeled to total ACh in the cytoplasmic fraction after lithium treatment (0.33) is significantly below that attained in the vesicular fraction (0.63).

The results shown in Table 2 indicate that a lithium-induced reduction in vesicular-bound ACh does not significantly facilitate the movement of preformed ACh into vesicles. Prior incubation of the minced tissues with lithium does not alter the ratio of labeled to total ACh attained in the vesicular fraction after incubation in normal Krebs solution with [^{14}C]ACh (0.20 ± 0.05) compared with the ratio attained when the first incubation is in normal Krebs solution (0.14 ± 0.03).

Table 1. Differential effect of incubation in lithium Krebs solution on the emptying of cytoplasmic and vesicular-bound ACh. Minced tissues prepared from mouse forebrain were incubated in normal Krebs or lithium Krebs solution as described in the text. They were then washed twice with 5 ml of 0.32*M* sucrose and subcellular fractions were prepared (*6*). The amounts of cytoplasmic and vesicular-bound ACh were then determined (*7*). Each value represents the mean ± standard error for 12 brains.

Incubation medium	ACh (nmole/g, wet weight)	
	Cytoplasmic fraction	Vesicular fraction
Normal Krebs	11.8 ± 1.6	8.7 ± 0.9
Lithium Krebs	12.8 ± 2.5	2.5 ± 0.4*

*Significantly different at $P < .05$ from the value obtained after incubation in normal Krebs solution (paired Student's t-test).

Table 2. Differential effect of incubation in lithium Krebs solution on the refilling of cytoplasmic and vesicular pools with newly synthesized and preformed ACh. Minced tissues prepared from mouse forebrain were incubated in normal Krebs or lithium Krebs solution as described in the text. One group of minced tissues was then incubated for another 30 minutes in normal Krebs solution with 100 μM [^{14}C]choline (specific activity, 6.34 mc/mmole) and paraoxon (0.1 μM), while another group was incubated the second time in normal Krebs solution with 100 μM [^{14}C]ACh (specific activity, 1.2 mc/mmole) and paraoxon (0.1 μM). The minced tissues were then washed twice with 5 ml of 0.32*M* sucrose and subcellular fractions were prepared (*6*). The amounts of labeled and total ACh present in the subcellular fractions were then determined (*7, 8*). Each value represents the mean ± standard error for the ratio of labeled to total ACh found in subcellular fractions for 12 brains.

Incubation 1	Incubation 2	[^{14}C]ACh/total ACh	
		Cytoplasmic fraction	Vesicular fraction
Normal Krebs	Normal Krebs + [^{14}C]choline	0.35 ± 0.05	0.35 ± 0.07
Lithium Krebs	Normal Krebs + [^{14}C]choline	0.33 ± 0.05	0.63 ± 0.09*
Normal Krebs	Normal Krebs + [^{14}C]ACh	0.46 ± 0.04	0.14 ± 0.03
Lithium Krebs	Normal Krebs + [^{14}C]ACh	0.39 ± 0.03	0.20 ± 0.05

*Significantly different at $P < .05$ from the value obtained when the first incubation was in normal Krebs solution (paired Student's t-test).

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This study shows that the vesicular-bound pool of ACh is capable not only of emptying but also of replenishing its contents independently of the cytoplasm. Depleted vesicular ACh is refilled with newly synthesized ACh formed from extracellular choline but not from preformed or newly synthesized cytoplasmic ACh; therefore, it appears that cholinergic vesicles may be supplied with ACh by vesicular choline acetyltransferase (E.C. 2.3.1.6) activity.

Lithium treatment reduces the level of vesicular-bound ACh by 70 percent without altering the level of the cytoplasmic ACh, which suggests that cytoplasmic ACh may not be mobilized into vesicles even when their ACh pools are expandable. This observation does not dismiss the possibility that ACh enters vesicles by diffusion, since lithium treatment could conceivably reduce the ACh content of all vesicles by 70 percent which might be inadequate to permit diffusion from the cytoplasm into the vesicles.

Accumulation of extracellular ACh by brain tissue, unlike that of extracellular choline, is believed to be nonspecific for cholinergic nerve endings (*9*). Consequently, the ratios of labeled to total ACh found in subcellular compartments after incubations with [^{14}C]ACh cannot be compared with those attained after incubations with [^{14}C]choline since more of the extracellular [^{14}C]ACh would be expected to enter noncholinergic nerve endings. That lithium pretreatment facilitates the movement of newly synthesized ACh (formed from extracellular [^{14}C]choline) but not preformed [^{14}C]ACh into vesicles suggests that newly synthesized and not preformed ACh replaces ACh that has been released from vesicles. Whether extracellular ACh enters the vesicles directly or by way of the cytoplasm and then the vesicles cannot be determined from this study.

PAUL T. CARROLL
STEPHEN H. NELSON
Department of Pharmacology and Toxicology, University of Rhode Island, Kingston 02881

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Choline Administration: Modification of the Central Actions of Atropine

Abstract. *The anticholinergic agent atropine decreases acetylcholine concentrations and increases high-affinity choline uptake in cortical and hippocampal regions of rat brain. Administration of choline 1 hour before atropine prevents both of these atropine-induced alterations. These findings suggest that alterations in acetylcholine precursor availability may modify the effects of centrally active anticholinergic agents.*

Recent investigations indicate that there is a positive relationship between choline availability and brain concentrations of acetylcholine (ACh) (*1*). Dose- and time-related increases in ACh concentrations have been noted after both injection and dietary administration of choline (*2, 3*). There is also a positive correlation between dietary choline administration and the concentration of nicotinic receptors in brain (*4*). To elucidate the pharmacological significance of these findings, we investigated the effects of increased choline availability on atropine-induced alterations in central cholinergic neurons. The effects of atropine on ACh concentrations and on Na^+-dependent high-affinity choline uptake in rat brain hippocampus and cortex were studied and compared in naive and choline-treated animals.

Male Sprague-Dawley rats (175 to 225 g) were maintained on a 12-hour light-dark cycle with food (Standard Purina Rat Chow, 9.5 nmole of choline per kilogram) and water freely available. Drugs were dissolved in saline and administered (0.1 ml per 100 g of body weight) between 9 and 11 a.m. Rats were injected with either saline or atropine sulfate (5 mg/kg, intraperitoneally) 1 hour after the administration of either saline or choline chloride (60 mg/kg, intraperitoneally) and killed 30 minutes after the second injection. Results in our laboratory have shown that the maximum ACh depletion is achieved 30 minutes after the administration of atropine (*5*). Furthermore, 1 hour was allowed to elapse after choline administration to minimize any transient alterations induced by this agent (*2*). Animals were killed by microwave irradiation (2.4 seconds, 2750 Mhz, 1250 watts) focused on the head for ACh and choline determinations (*6*) or by decapitation for studies of Na^+-dependent high-affinity choline uptake.

For ACh and choline analyses, brains were removed, chilled in ice-cold pentane, and dissected into discrete brain areas by using a brain slicing apparatus (*7*). Slices from the hippocampus and midbrain cortex were isolated, homogenized in 2 ml of acetonitrile (containing 2

Table 1. Effects of choline pretreatment on atropine-induced ACh depletion in rat cortex and hippocampus. Values are expressed as nanomoles of ACh or choline per gram of brain tissue ± the standard error of the mean. The number of determinations is given parentheses. The procedure and doses are described in the text.

Treatment	Cerebral cortex		Hippocampus	
	ACh (nmole/g)	Choline (nmole/g)	ACh (nmole/g)	Choline (nmole/g)
Saline (controls)	14.3 ± 0.67 (7)	14.9 ± 1.09 (7)	24.9 ± 1.20 (5)	19.5 ± 2.43 (5)
Choline	13.1 ± 1.17 (7)	15.8 ± 1.61 (7)	22.9 ± 1.82 (6)	26.1 ± 2.97 (5)
Atropine	11.4 ± 0.84*(7)	17.4 ± 1.38*(6)	19.4 ± 1.00*(7)	28.6 ± 2.57*(7)
Atropine following choline	13.0 ± 1.13 (6)	18.4 ± 2.26 (6)	22.5 ± 1.60 (6)	26.9 ± 5.36 (5)

*Significantly different from control ($P < .05$) by two-tailed Student's t-test.

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