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# Activation of High-Affinity Choline Uptake *in Vitro* by Depolarizing Agents

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SUMMARY

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Incubation of rat brain hippocampal synaptosomes under depolarizing conditions, i.e., with veratridine or high concentrations of potassium, resulted in activation of sodium-dependent, high-affinity choline uptake. The depolarizing incubation did not affect the sodium-dependent uptake of octopamine, serotonin, or γ-aminobutyric acid. Omission of calcium during the incubation period with depolarizing agents, or addition of a calcium antagonist, Bay a 1040, prevented the increase in choline uptake. Omission of magnesium was without effect. Partial replacement of sodium with either lithium or sucrose, treatments which also enhance calcium influx into synaptosomes, resulted in an increase in choline uptake. These data *in vitro* suggest that calcium influx is an important feature of the activation of sodium-dependent, high-affinity choline uptake *in vivo*. Furthermore, the characteristics of the activation *in vitro* are similar to those observed *in vivo;* thus the system *in vitro* should be useful in exploring further the mechanisms involved in the activation of sodium-dependent, high-affinity choline uptake caused by neuronal activity *in vivo*.

## INTRODUCTION

The high-affinity choline transport system (1-4) is highly, perhaps selectively, localized to cholinergic nerve terminals in the central and peripheral nervous systems (5-8). It efficiently serves the formation of releasable acetylcholine (9) and appears to be capable of supporting acetylcholine turnover (10). The structure-activity relationships and the ion and energy dependence of the transport system have been reported (11, 12). This high-affinity uptake may be a rate-limiting step in the synthesis of acetylcholine (13).

Recently it has been observed that the $V_{max}$ of the transport system is coupled to neuronal activity *in vivo* (14, 15). Thus it has been proposed that choline uptake is a regulatory step in the synthesis of acetylcholine *in vivo*. Additional experiments *in vivo* could be interpreted to support this proposal (16). Since increased neuronal activity results in increased sodium-dependent, high-affinity choline uptake (15), and since the mechanisms underlying this effect are unknown, in this study we have examined, by methods *in vitro,* whether depolarization and ions are involved in this activation of uptake.

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## METHODS

*Tissue preparation*. Male Sprague-Dawley albino rats were decapitated, and the whole bilateral hippocampal formation was dissected. Hippocampal tissues were used in this study because our previous studies *in vivo* involved the well-defined cholinergic septal-hippocampal tract. Hippocampi were homogenized in 20 volumes of ice-cold 0.32 M sucrose, using a Teflon pestle. The homogenates were centrifuged at 1000 × *g* for 10 min at 4°. The pellet ($P_1$) was discarded. The supernatants were centrifuged at 17,000 × *g* for 15 min at 4° to obtain crude mitochondrial pellets ($P_2$). The supernatant fluid was discarded.

*Uptake assay*. The procedure used in this study was modified from procedures previously reported (1–4, 9, 15). A preliminary incubation step was added to expose synaptosomes to different media before uptake assay. The pellets ($P_2$) were resuspended in 40 volumes of normal or modified Krebs-Ringer-phosphate buffer, pH 7.4. These suspensions were then incubated at 37° for 10 min unless otherwise stated, and then centrifuged at 17,000 × *g* for 15 min at 4°. The supernatants were discarded. The pellets were resuspended in 20 volumes of ice-cold 0.32 M sucrose and briefly homogenized, and the resulting synaptosomal suspensions were used for uptake studies.

The synaptosomal uptake assay employed in these studies was similar to that used in many previous studies (1–4, 9, 15). Our present experiments studied sodium-dependent uptake (15). The synaptosomal suspension was added to both normal and sodium-free Krebs-Ringer-phosphate buffer, pH 7.4. Uptake values obtained with the latter medium represented sodium-independent uptake and were subtracted from the values using normal Krebs-Ringer-phosphate medium.

Routine assays were performed as follows. A 0.1-ml aliquot of the synaptosomal suspension was added to 0.9 ml of incubation medium in a Corex glass centrifuge tube. This was incubated for 5 min at 37°. To begin uptake, [$^3$H]choline (Amersham/Searle, 10 Ci/mmole) was added to produce a final concentration of 0.04 μM, and the incubation was continued for 4 min. Under these conditions uptake was linear for 4 min. In some uptake experiments L-[$^3$H]-glutamate (20 Ci/mmole), L-[$^3$H]phenylalanine (5.6 Ci/mmole), [$^3$H]serotonin binoxalate (1.3 Ci/mmole), DL-[$^3$H]octopamine (3.7 Ci/mmole), or [$^3$H]GABA[3] (37 Ci/mmole) (all from New England Nuclear) was used in place of [$^3$H]choline, each at 0.04 μM. Uptake was terminated by the addition of 2 ml of ice-cold Krebs-Ringer-phosphate buffer to the assay tubes and transferral of the tubes to an ice-water bath. The samples were then centrifuged at 3000 × *g* at 4° for 15 min. Under these conditions all the synaptosomes were pelleted. The medium was poured off, the pellet was gently washed with 4 ml of ice-cold 0.9% NaCl, the walls of the test tube were dried, and 1.0 ml of Protosol (New England Nuclear) was added to each tube. After digestion was complete, the Protosol solution was transferred to a scintillation vial, and the sample tubes were rinsed with 10 ml of toluene-based scintillation fluid (LSC–complete, Yorktown Research), which was added to the scintillation vial. Radioactivity was determined by liquid scintillation spectrometry. Protein was measured by the method of Lowry *et al*. (17). Under these conditions the sample to blank ratio (i.e., ratio of sodium-dependent to sodium-independent uptake) was about 5:1.

Kinetic parameters of choline uptake were determined by plotting $v$ vs. $v/S$ and $1/v$ vs. $1/S$ (18). The choline concentration range used for kinetic studies was 0.12–2.0 μM.

Statistical analyses were carried out using Student's two-tailed *t*-test.

*Solutions and media*. The Krebs-Ringer-phosphate medium contained NaCl, 122 mM; KCl, 4.9 mM; $CaCl_2$, 1.3 mM; $Na_2HPO_4$, 15.8 mM; $MgSO_4$, 1.2 mM; and dextrose, 11.1 mM. The composition of the $Na^+$-free medium was the same, except that NaCl and $Na_2HPO_4$ were replaced by sucrose (252 mM) and Tris-phosphate (15.8 mM), respectively. The high-potassium (62 mM KCl), 62 mM rubidium (62 mM RbCl),

[3] The abbreviations used are: GABA, γ-aminobutyric acid; TTX, tetrodotoxin.

62 mM lithium (62 mM LiCl), and 124 mM sucrose media were maintained isosmotic by reducing NaCl to 64.9 mM. When $Ca^{2+}$ and $Mg^{2+}$ were omitted, they were replaced isosmotically with $Na^+$.

Veratridine (Aldrich Chemical Company), tetrodotoxin (Calbiochem), and Bay a 1040 [4-(2'-nitrophenyl)-2,6-dimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine] were added to the Krebs-Ringer-phosphate medium at various times in small volumes of ethanol (1–5% of the total volume) to produce the proper concentrations. Addition of ethanol alone in small volumes was found to have no effect on uptake. Experiments involving Bay a 1040 were carried out in semidarkness.

## RESULTS

*Effects of depolarizing agents on sodium-dependent, high-affinity synaptosomal choline uptake.* In a separate preliminary incubation step, hippocampal synaptosomes were subjected to treatments which depolarize the synaptosomal membrane. Sodium-dependent, high-affinity choline uptake was then examined in normal medium (Table 1). Following incubation of synaptosomes with 62 mM potassium or rubidium, treatments which have been shown to depolarize membranes and increase calcium influx (19, 20), there was an increase in sodium-dependent, high-affinity choline uptake. If sucrose or LiCl was added to the preliminary incubation medium to replace NaCl to the same extent as KCl or RbCl, choline uptake increased to about half the uptake found with high potassium ($p < 0.05$ compared with high potassium). Thus not all of the activation of uptake in the high-potassium and rubidium experiments could be due solely to a reduction in sodium. Incubation of synaptosomes with 75 μM veratridine, another depolarizing agent (19, 20), also resulted in activation of uptake. Incubation with 62 mM $K^+$ or 75 μM veratridine had no effect on sodium-independent choline uptake (data not shown).

Synaptosomal uptake studies in the presence of depolarizing agents (rather than in a separate preliminary incubation step) resulted in a large reduction in uptake (Table 1), as observed previously (12, 21).

*Specificity of increased uptake produced by depolarization.* In order to examine whether this activation of choline uptake by depolarization is a unique occurrence in transport systems, the effects of preliminary incubation with high potassium on the synaptosomal sodium-dependent uptake of [$^3$H]choline, [$^3$H]serotonin, [$^3$H]-glutamate, [$^3$H]GABA, [$^3$H]phenylalanine, and [$^3$H]octopamine was examined (Table 2). In these experiments a 45% increase in [$^3$H]choline uptake was observed after incubation in the presence of high

TABLE 1

*Effects of depolarizing agents on synaptosomal choline uptake*

See METHODS for details. Choline uptake data are presented as means ± standard errors of the number of experiments shown in parentheses.

| Preliminry incubation medium | Incubation medium | Choline uptake | Percentage of control |
|---|---|---|---|
| | | *pmoles/4 min/mg protein* | % |
| KRP[a] (control) | KRP | 2.63 ± 0.12 (30) | 100 |
| 62 mM $K^+$ | KRP | 4.21 ± 0.13[b] (26) | 160 |
| 62 mM $Rb^+$ | KRP | 3.61 ± 0.21[b] (4) | 137 |
| 124 mM sucrose | KRP | 3.28 ± 0.16[c] (3) | 124 |
| 62 mM $Li^+$ | KRP | 3.30 ± 0.15[c] (3) | 124 |
| 75 μM veratridine | KRP | 4.03 ± 0.22[b] (6) | 153 |
| KRP | 62 mM $K^+$ | 0.24 ± 0.14[b] (4) | 9 |
| KRP | 75 μM veratridine | 0.52 ± 0.02[b] (4) | 20 |

[a] Krebs-Ringer phosphate buffer, pH 7.4.

[b] $p < 0.01$ compared with controls.

[c] $p < 0.05$ compared with controls in the same experiments (2.65 ± 0.10, paired $t$-test) and compared with tissue incubated in 62 mM $K^+$.

TABLE 2

*Specificity of increase in sodium-dependent uptake produced by depolarization*

See METHODS for details. Uptake data are presented as means ± standard errors of the number of experiments shown in parentheses. All substrates were used at a concentration of 0.04 μM. Hemicholinium-3 was used at a concentration of 0.1 μM.

| Substrate | KRP[a] KRP (control)[b] | 62 mM $K^+$; KRP | KRP; KRP + HC-3[c] | 62 mM $K^+$; KRP + HC-3 |
|---|---|---|---|---|
| | *pmoles/4 min/mg protein* | | | |
| [$^3$H]Choline | 3.11 ± 0.14 (17) | 4.51 ± 0.15[d] (14) | 0.02 ± 0.01[d] (7) | 0.05 ± 0.02[d] (4) |
| [$^3$H]Glutamate | 51.4 ± 2.3 (10) | 66.9 ± 5.0[d] (7) | 51.6 ± 2.6 (6) | 57.9 ± 2.9[e] (3) |
| [$^3$H]Phenylalanine | 1.29 ± 0.10 (12) | 1.71 ± 0.13[d] (9) | 1.21 ± 0.13 (6) | |
| [$^3$H]GABA | 44.4 ± 2.1 (4) | 49.6 ± 1.5 (4) | | |
| [$^3$H]Serotonin | 5.76 ± 0.60 (4) | 5.00 ± 0.52 (4) | | |
| [$^3$H]Octopamine | 0.87 ± 0.14 (4) | 0.84 ± 0.12 (4) | | |

[a] Krebs-Ringer-Phosphate buffer, pH 7.4.

[b] The preliminary incubation medium precedes the semicolon; the subsequent incubation medium follows it.

[c] Hemicholinium-3.

[d] $p < 0.02$ compared with controls.

[e] $p < 0.05$ (paired $t$-test) compared with controls in the same experiments (50.2 ± 4.3), but not significantly different from depolarized tissue.

potassium. There was no increase in the sodium-dependent uptake of [$^3$H]serotonin, [$^3$H]GABA, or [$^3$H]octopamine after a depolarizing preliminary incubation. There was a significant increase in the uptake of [$^3$H]glutamate and [$^3$H]phenylalanine. Both normal and stimulated uptakes of choline were inhibited by 0.1 μM hemicholinium-3. The uptakes of [$^3$H]glutamate and [$^3$H]phenylalanine were not significantly affected by hemicholinium-3.

In the above experiments we also examined the choline acetyltransferase activity and protein content of the synaptosomal pellets following the incubation. The choline acetyltransferase activity and protein content were the same in all cases (data not shown), suggesting that the changes in choline uptake are not due to differential recovery of cholinergic synaptosomes after the various treatments.

*Time course, concentration, and ion dependence of veratridine depolarization.* In these experiments synaptosomes were first incubated for a total of 10 min, and veratridine (75 μM) was added at various times prior to transferral of the samples to the ice-water bath. In some cases veratridine was added immediately prior to transferral (zero time), or 1, 2, or 10 min prior to transferral. In the last case, veratridine was present during the entire preliminary incubation. Choline uptake was activated after veratridine exposure at all times (Table 3). Activation of uptake at zero time indicates that the action of veratridine is rapid and may occur even during cooling and centrifugation.

Different concentrations of veratridine were added to the preliminary incubation medium. Both 75 μM and 225 μM veratridine resulted in similar, significant activation of high-affinity choline uptake. However, addition of 750 μM veratridine caused a reduction in uptake (Table 3). This reduction seems most likely to be due to inefficient removal of veratridine from the pellets, since in further experiments, after repeated washing of the 750 μM veratridine-exposed pellets, activation of uptake was observed (data not shown). As mentioned above, the presence of veratridine during an incubation period will cause a reduction in uptake.

The action of veratridine on synaptosomes, which is to open sodium channels in the membrane (22), is blocked by tetrodotoxin (19, 23). Addition of TTX (4 μM) to the preliminary incubation medium had no effect by itself on high-affinity choline uptake, but did block the veratridine-induced increase in uptake (Table 3).

It was also examined whether divalent cations were necessary for activation of

TABLE 3

*Increased choline uptake due to veratridine depolarization*

See METHODS for details. All samples were first incubated for 10 min, and veratridine was added at various times. Exposure time refers to the time during which veratridine was present before the synaptosomes were removed from the water bath and placed on ice. Zero time of exposure indicates that veratridine was added at end of the preliminary incubation period. TTX (4 μM) and Bay a 1040 (120 μM) were present during the entire 10-min preliminary incubation. Uptake data are presented as the means ± standard errors of the number of experiments shown in parentheses.

| Veratridine | Exposure time | Choline uptake | Percentage of control |
|---|---|---|---|
| *μM* | *min* | *pmoles/4 min/mg protein* | *%* |
| 0[a] | | 2.86 ± 0.10 (25) | 100 |
| 75 | 0 | 4.11 ± 0.18[b] (9) | 144 |
| 75 | 1 | 4.23 ± 0.24[b] (9) | 148 |
| 75 | 2 | 4.11 ± 0.20[b] (11) | 144 |
| 75 | 10 | 4.20 ± 0.17[b] (21) | 147 |
| 225 | 10 | 3.91 ± 0.34[b] (3) | 137 |
| 750 | 10 | 1.42 ± 0.34[b] (3) | 50 |
| 0 + TTX | | 3.12 ± 0.15 (4) | 109 |
| 75 + TTX | 0 | 3.14 ± 0.11 (4) | 110 |
| 0, no $Ca^{2+}$ | | 2.11 ± 0.17[b] (12) | 74 |
| 75, no $Ca^{2+}$ | 10 | 2.69 ± 0.13[c] (4) | 94 |
| 0, no $Mg^{2+}$ | | 2.52 ± 0.09 (6) | 88 |
| 75, no $Mg^{2+}$ | 10 | 3.73 ± 0.25[b] (6) | 130 |
| 0 + Bay a 1040 | | 3.08 ± 0.31 (3) | 108 |
| 75 + Bay a 1040 | 0 | 3.43 ± 0.34[c] (3) | 120 |

[a] Control.

[b] $p < 0.005$ compared with controls.

[c] $p < 0.005$ compared with tissue incubated in 75 μM veratridine with $Ca^{2+}$, but not significantly different from controls (paired *t*-test).

uptake during preliminary incubation in the presence of 75 μM veratridine (Table 3). Omission of $Ca^{2+}$ from the medium resulted in a small reduction in choline uptake, while omission of $Mg^{2+}$ was without significant effect. Omission of $Ca^{2+}$ during the incubation with veratridine blocked the increase in uptake due to veratridine, while omission of $Mg^{2+}$ did not. Addition of 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid to the $Ca^{2+}$-free medium produced no further effect.

Since this experiment suggested the necessity of calcium for the depolarization-induced increase in sodium-dependent choline uptake, we tested the effect of a proposed calcium channel antagonist, Bay a 1040 (23–25). Addition of Bay a 1040 (120 μM) to the preliminary incubation medium had no effect by itself on choline uptake, but did block the veratridine-induced increase in uptake (Table 3).

*Time course, concentration, and ion dependence of high-potassium-activated uptake.* In these experiments the duration of the preliminary incubation period was varied (Table 4). With the normal concentration of potassium in the medium (4.9 mM), uptake was unchanged whether the preliminary incubation was carried out for 5 or 10 min. However, after 15 min there appeared to be a small decrease in choline uptake. When high-potassium (62 mM) media were utilized for the preliminary incubation, a small increase in choline uptake was observed at 5 min, but the activation of uptake was even greater after both 10 and 15 min. When 35 mM potassium was utilized for a 10-min preliminary incubation, the high-affinity choline uptake was also activated, but was consistently

TABLE 4

*Increased choline uptake due to potassium depolarization*

See METHODS for details. Choline uptake data are presented as means ± standard errors of the number of experiments shown in parentheses.

| Preliminary incubation $[K^+]$ | Preliminary incubation time | Choline uptake | Percentage of Control |
|---|---|---|---|
| *mM* | *min* | *pmoles/4 min/mg protein* | *%* |
| 4.9 | 5 | 2.61 ± 0.16 (4) | 96 |
| 4.9[a] | 10 | 2.72 ± 0.10 (27) | 100 |
| 4.9 | 15 | 1.99 ± 0.17 (4) | 73 |
| 35 | 10 | 3.98 ± 0.41[b] (7) | 146 |
| 62 | 5 | 3.31 ± 0.22 (4) | 122 |
| 62 | 10 | 4.27 ± 0.14[b] (20) | 157 |
| 62 | 15 | 4.16 ± 0.22[b] (4) | 153 |
| 4.9, no $Ca^{2+}$ | 10 | 2.11 ± 0.17[b] (12) | 78 |
| 4.9, no $Mg^{2+}$ | 10 | 2.52 ± 0.09 (6) | 93 |
| 62, no $Ca^{2+}$ | 10 | 2.59 ± 0.20[c] (8) | 95 |
| 62, no $Mg^{2+}$ | 10 | 3.57 ± 0.30[b] (4) | 131 |

[a] Control.

[b] $p < 0.05$ compared with controls.

[c] $p < 0.001$ compared with tissue incubated in 62 mM $K^+$ with $Ca^{2+}$, but not significantly different from controls.

less than with 62 mM potassium.

The effect of divalent cations on the potassium-induced increase in choline uptake was studied. Omission of calcium during the high-potassium preliminary incubation reduced the increase in uptake, while omission of magnesium from the high-potassium medium had no effect (Table 4). Omission of $Ca^{2+}$ also reduced the rubidium-induced and 124 mM sucrose-induced increases in uptake. Increasing the calcium concentration (8-fold) in the normal preliminary incubation medium had no effect on uptake (data not shown).

*Kinetics of choline uptake.* A kinetic analysis of choline uptake was performed, utilizing synaptosomes with various preliminary incubation conditions (Table 5). These conditions included preliminary incubation in normal and calcium-free Krebs-Ringer-phosphate medium, high-potassium medium with and without calcium, and medium with 75 $\mu$M veratridine added. The $K_m$ values obtained were not significantly different after any of these conditions and were approximately 0.5 $\mu$M, which is in agreement with our earlier studies (12, 14, 15). On the other hand, the increased uptake observed in the above experiment after incubation in depolarizing media was accounted for by an increased $V_{max}$ (Table 5), an increase which was blocked by omission of $Ca^{2+}$ from the preliminary incubation. Preliminary incubation in $Ca^{2+}$-free Krebs-Ringer-phosphate medium produced a small reduction in $V_{max}$.

*Efflux studies.* Since a decreased efflux after high-potassium exposure could cause an "apparent" increase in uptake, we resuspended synaptosomes loaded with [$^3$H]choline in regular Krebs medium at 37° for 4 min. The same percentage of radioactivity [25.5% ± 1.2% for controls, 24.3% ± 2.4% for potassium depolarization (means ± SE)] was found in the medium after centrifugation whether or not the synaptosomes had been "activated" by high potassium. Thus reduced efflux of radioactivity from stimulated synaptosomes did not appear to occur. These results agree with our earlier studies, in which homoexchange and efflux were not found to be involved in the various states of activation of choline uptake (15).

## DISCUSSION

Hippocampal synaptosomes were subjected to various preliminary incubation conditions to test the effect of membrane depolarization and its ion dependence on subsequent alterations of sodium-dependent, high-affinity choline uptake. Replacing a large portion of the sodium in normal medium with either potassium or rubidium during preliminary incubation produces a depolarization of the synaptosomes (19) and causes a significant increase in choline uptake. In other experiments, the ionic composition of the medium was not altered, but depolarization was achieved by addition of veratridine, an alkaloid which depolarizes synaptosomes by opening sodium channels (22). Veratridine also increased high-affinity choline uptake. Since different ionic alterations (that is,

TABLE 5

*Kinetics of choline uptake*

See METHODS for details. Data are presented as means ± standard errors of the number of experiments shown in parentheses. Except as noted, $K_m$ and $V_{max}$ values were not significantly different from controls.

| Preliminary incubation medium | Choline uptake | | Percentage of control $V_{max}$ |
|---|---|---|---|
| | $K_m$ | $V_{max}$ | |
| | *μM* | *pmoles/4 min/mg protein* | % |
| KRP[a] (control) | 0.53 ± 0.03 | 48.9 ± 1.4 (10) | 100 |
| KRP, no $Ca^{2+}$ | 0.58 ± 0.07 | 40.2 ± 4.1[b] (4) | 82 |
| 62 mM $K^+$ | 0.47 ± 0.02 | 76.6 ± 1.5[b] (4) | 157 |
| 62 mM $K^+$, no $Ca^{2+}$ | 0.56 ± 0.03 | 50.6 ± 3.3 (7) | 103 |
| 75 μM veratridine in KRP | 0.48 ± 0.02 | 63.8 ± 3.0[b] (6) | 130 |

[a] Krebs-Ringer-phosphate buffer, pH 7.4.

[b] $p < 0.05$ compared with controls.

with high potassium), as well as no ionic alterations (that is, with veratridine), were used to activate uptake, it seems that the activation is due specifically to depolarization rather than to some nonspecific ionic effect. The increase in choline uptake following potassium depolarization has also been observed by Barker (26), who found it to be temperature-dependent.

Not all sodium-dependent uptakes were activated by depolarization. For example, the sodium-dependent uptakes of serotonin, GABA, and octopamine were unaltered by high potassium. Interestingly, the uptakes of glutamic acid and phenylalanine were increased. As far as we are aware, these effects have not previously been reported. Since one could consider glutamate and phenylalanine to be neurotransmitter "precursors," it may be that the sodium-dependent uptake of these compounds is coupled to neuronal activity in a manner similar to that for choline. Hemicholinium-3, a very potent blocker of sodium-dependent, high-affinity choline uptake (1, 3), had no effect at 0.1 $\mu$M on the uptake of glutamate or phenylalanine. This indicates that, although the uptake of several compounds is increased by depolarization, the uptake of choline is a distinct and separate system, since it is the only one affected by hemicholinium-3. The observed activation of choline uptake is not due to nonspecific loss of synaptosomes or differential recovery of cholinergic synaptosomes, because not all uptakes tested were altered, and because the choline acetyltransferase activity and the protein contents of the synaptosomal pellets after various preliminary incubation conditions were similar.

The depolarization-induced increase in high-affinity choline uptake was dependent on the presence of calcium, but not of magnesium. Omission of calcium from the depolarizing preliminary incubation medium caused a much larger reduction in choline uptake than did omission from normal medium. Preliminary incubation with the calcium channel antagonist Bay a 1040 also blocked the increased uptake after the depolarizing preliminary incubation, without affecting uptake after normal preliminary incubation. Some other ionic alterations performed here which alter uptake could also indirectly involve calcium fluxes. Partial replacement of sodium with lithium or sucrose in the preliminary incubation medium produced small increases in choline uptake. Both these treatments have been shown to enhance synaptosomal calcium influx (27), as does depolarization with high potassium concentrations or rubidium (19, 20). These findings suggest that calcium influx may be responsible in some way for the activation of high-affinity choline uptake. One explanation is that release of transmitter consequent to calcium influx must occur before this activation of uptake takes place. This raises the possibility that intracellular concentrations of acetylcholine are involved in the regulation of choline uptake (15). However, at present, perhaps not all observations are compatible with this point of view (15, 26). Yet another possibility is that calcium influx is required to activate the uptake for some reason unrelated to release. Calcium dependence of the potassium-induced increase in choline uptake was not observed in another study (26). There is no obvious reason for this difference, as the methods utilized appear similar.

A kinetic analysis of the changes in sodium-dependent, high-affinity uptake revealed alterations in $V_{max}$. Thus the changes observed here *in vitro* are similar to those found in our studies *in vivo* (14, 15), in which $V_{max}$ rather than $K_m$ changes were observed. These changes do not appear to be due to homoexchange or efflux mechanisms (data here and ref. 15). As previously mentioned (12), the $V_{max}$ increase could be due to an increased number of transport sites, an increased rate of transport (i.e., an increased turnover number), or both. The results presented here support our earlier proposals that sodium-dependent, high-affinity choline uptake is at least one of the regulatory steps in the synthesis of nerve terminal acetylcholine (15) and could be used as a relative measure of cholinergic activity (28). We hope that this model *in vitro* will be useful in further studies of the mechanism of uptake modulation.

The increases in uptake observed in this

study *in vitro* and in our earlier studies *in vivo* (14, 15, 28) are similar, that is, in the range of 30–60%. Our earlier studies *in vivo* also showed decreases in uptake consequent to decreases in neuronal activity (14, 15, 28). A reasonable question is: Is the range of uptake modulation sufficient to account for the actual range of acetylcholine synthesis rates observed *in vivo*? The capability of cholinergic neurons to synthesize releasable acetylcholine is definitely limited, since experiments with the septal-hippocampal tract (29), neuromuscular junction (30), ileum (31, 32), superior cervical ganglia (33), and cerebral cortical slices (34) showed that the amount of acetylcholine released per impulse decreased several fold with increasing frequencies of stimulation. However, over-all synthesis rates could increase markedly. Birks and MacIntosh (33) estimated that the synthesis rate of acetylcholine in superior cervical ganglia increased 7-fold after preganglionic stimulation in an anesthetized cat. In our previous studies, when comparing anesthetized with convulsant-treated animals (15), we observed a 2.5–6-fold increase in choline uptake in rat hippocampus, depending on assay conditions. The turnover rates of acetylcholine in brain, the tissue used in our studies, show a similar or slightly larger range of alterations than we have found in choline uptake (see ref. 28 for references). A difficulty in making these comparisons is that there may be some postmortem decline in activated states of choline uptake,[4] and therefore the range of changes in uptake may be underestimated. In any case, it appears from the above discussion that the observed range of choline uptake modulation may be compared with the range of changes of acetylcholine synthesis rates. However, at this point one cannot eliminate the possibility that there are mechanisms in addition to high-affinity choline uptake, such as intrasynaptosomal availability of acetyl-CoA or choline in strategic compartments, that can regulate acetylcholine synthesis under various conditions. This is an important question and worthy of further study.

[4] L. C. Murrin, unpublished observations.

A preliminary report of this work has appeared (35).

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