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Characteristics of (^{3}H)-Choline Uptake into Synaptosomes from Rat Hippocampus

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Certain basic characteristics of choline uptake in nerve terminals were studied with synaptosomes from rat hippocampus. Synaptosomal [^{3}H]-choline uptake was clarified as specific and high affinity by low K_m value (2.2 μM), Na^+-dependency and high sensitivity to hemicholinium-3, a competitive inhibitor of choline uptake. Choline uptake into synaptosomes was linearly related to Na^+ concentration and membrane potential. Extracellular Ca^{2+} modulated the choline uptake, but probably not through increase of intracellular Ca^{2+}, because this modulation was not affected the by high K^+-depolarization. EGTA (2 mM) added for Ca^{2+}-free condition had a peculiar effect of decreasing choline uptake. These results suggest that Ca^{2+} may play an important role in regulating the metabolism of acetylcholine in the nerve terminals directly through the increase of acetylcholine release.

Neurons make and store neurotransmitters and release them through their nerve terminals to transduce the signals to other neurons or tissues. To relay the transduction of nerve signals, neurons should make their own neurotransmitters according to the amount required during the transduction. Acetylcholine, one of the neurotransmitters, is synthesized from choline and acetyl-CoA in the cholinergic nerve terminals by choline acetyltransferase. Acetylcholine should be made continuously to compensate the released acetylcholine.

The regulatory mechanisms of acetylcholine synthesis have been studied successively. It has been found that the acetylcholine synthesis is continuously regulated to prevent the depletion of acetylcholine in nerve terminals (Birks and Macintosh, 1961; Browning and Schulman, 1968; Collier and MacIntosh, 1969). According to Vaca and Pilar (1979), trains of electric stimulation on nerve cell did not result in acetylcholine depletion and increased acetylcholine synthesis in nerve terminals. Acetylcholine in extracellular space can also regulate the acetylcholine synthesis (Yamamura and Snyder, 1973). For the regulation of acetylcholine synthesis, choline acetyltransferase was proposed to be an important rate-limiting step but more recently some studies suggested that the velocity of choline uptake into nerve terminals might be the more important step (Jope, 1979; Vaca and Pilar 1979; Ducis, 1988).

Choline uptake by nerve terminals is used for acetylcholine synthesis. Choline uptake of neurons is known to be carrier-mediated. Two kinds of carriers are known: high affinity-choline uptake carrier and low affinity-choline uptake carriers (Jope, 1979; Murrin, 1980). The high affinity-choline uptake is dependent on extracellular Na^+ concentration while low affinity choline uptake is not (Haga, 1971; Yamamura and Snyder, 1973; Kuhar and Zarbin; 1978). Most high affinity carriers of choline seem to be distributed on membrane of terminals and the low affinity carrier are concentrated on the cell body of neurons (Suszkiw and Pilar, 1976; Massarelli et al., 1974). Choline acetyltransferase, the acetylcholine-making enzyme, is also distributed mainly in the nerve terminals (Hebb and Whittaker, 1958; Ross et al., 1983). The majority of choline uptake and acetylcholine synthesis, therefore, seems to occur in nerve terminals.

We examined in this study the various characteristics of choline uptake of nerve terminals, importance of membrane potentials, and effects of divalant ions in synaptosomal choline uptake. We chose rat hippocampus for isolation of synaptosomes because it is known that cholinergic nerve terminals are abundantly distributed in hippocampus.

Some of preliminary data have been presented in abstract form (Lee and Hong, 1995).

Materials and Methods

Materials

[^{3}H]-Choline (specific acitivity: 80 *Ci*/mmol) was purchased from Amersham International. Dithiothreitol (DTT), ethylenediaminetetra-acetic acid (disodium salt; EDTA), ethylene glycol-bis (b-aminoethylether) N,N,N,N-tetra-

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acetic acid (EGTA), hemicholinium-3, Percoll, Triton X-100 (t-octyl phenoxypolyethoxyethanol), and N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid] (HEPES) were from Sigma. All general reagents used were analytical grade.

Isolation of synaptosomes

Synaptosomes were isolated from rat hippocampi by methods of Harrison et al. (1988) and Dunkley et al. (1988) with modifications. Male Sprague-Dawley rats were sacrificed by decapitation and the brains were rapidly removed and dipped in air-saturated cold homogenization buffer (sucrose 0.32 mM, EDTA 1.0 mM, DTT 0.25 mM, pH 7.4). Hippocampi were isolated and homogenized in cold homogenation buffer with Teflon-glass homogenizer and centrifuged at 1,000 x g for 10 min at 4℃. The supernatants (S1 fraction) were collected. S1 fraction (2 ml) was laid onto Percoll gradient (3, 10, 15, and 23% in homogenization buffer adjusted to pH 7.4). Two ml of each fraction was centrifuged at 32,500 x g for 5 min at 4℃. Fractions 4 (between 15% and 23% Percoll) and 5 (precipitates in 23% Percoll) were collected and washed three times by centrifugation at 15,000 x g for 15 min in air-saturated HEPES- buffered saline (HBS: HEPES 10, NaCl 145 mM, KCl 3 mM, glucose 10 mM, pH 7.4). The precipitant was suspended in HBS.

Uptake assays

Uptake of [^{3}H]-choline into synaptosomes was measured by a modified procedure of Thorne et al. (1991). Synaptosomal fraction (0.4-0.5 μg of protein) was preincubated for 10 min at 37℃ before addition of [^{3}H]-choline (final concentration 0.567 μM, 0.5 μ*Ci*) to the reaction mixture. The reaction was started at the point of addition of [^{3}H]-choline and stopped at the point of filtering. The volumes of the final solution was 0.5 ml in all experiments.

After the reaction, the solution including synaptosomes was immediately filtered through Whatmann GF/F filter using vacuum pump (15 psi) and washed 3 times with 5 ml of cold HBS. To confirm the specific choline uptake, hemicholinium-3 (0.1-50 μM) was added to the reaction solution 10 min before addition of [^{3}H]-choline.

In NaCl subtraction (from 145 mM, to 125, 105, 85, 65, 45, and 25 mM) experiments, LiCl or sucrose were added to HBS as equinormal concentration of NaCl subtracted (LiCl 20, 40, 60, 80, 100, and 120 mM; sucrose 40, 80, 120, 160, 200, and 240 mM). In the case of KCl addition, the same amount of NaCl as KCl added was subtracted. All other reagents used in this study were treated 10 min before addition of [^{3}H]-choline.

Determination of ^{3}H-choline uptake

After washing, the filters were put in the scintillation vials and ^{3}H was extracted from filter by dipping in the extraction solution (0.1 N HCl and 1% Triton X- 100) for at least 12 h at room temperature. Then 10 ml of scintillation cocktail solution (ScINT-A XF^{TM}) was added. After 2 days, ^{3}H was determined using scintillation beta-counter (LKB Comp.).

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951).

Data analysis

Data were calculated and expressed as pmol/mg protein and statistical analysis were performed with those values. One way ANOVA was used first and Student *t*-test was accompanied.

Results

Uptake of [^{3}H]-choline into synaptosomes

The choline uptake into synaptosomes from rat hippocampus was propotionally increased dependending on the incubation time and the quantity of synaptosomes and [^{3}H]-choline (Figs. 1A, 1B, and 2A).

Based on these results, the choline uptake was determined under the following condition: 0.02-0.04 mg protein, 0,567 μM [^{3}H]-choline (final concentration), for 20 min at 37℃.

The K_M value of [^{3}H]-choline uptake was 2.27±0.26

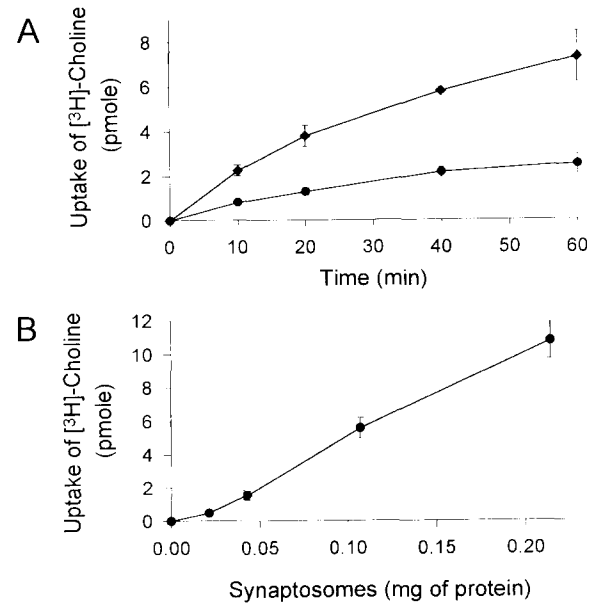

Fig. 1. Time (A)- and synaptosomal amount (B)-dependency of [^{3}H]-choline uptake into rat hippocampal synaptosomes. Synaptosomes were incubated at 37℃ for indicated time or 20 min with [^{3}H]-choline (0.567 μM, 0.5 μ*Ci*) after preincubation for 10 min at 37℃. Data were expressed as mean±S.E. (n=6) in total picomoles uptake by synaptosomes.

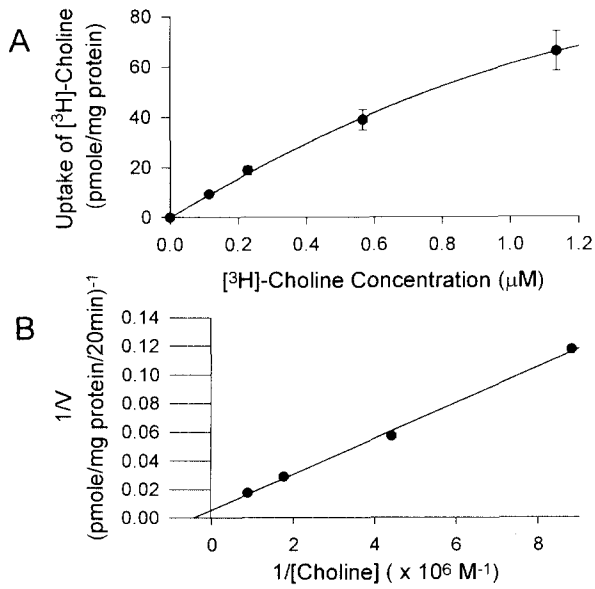

Fig. 2. Concentration-dependency (A) and its reciprocal plot (B) of [^{3}H]-choline uptake into rat hippocampal synaptosomes. Synaptosomes were incubated at 37℃ for 20 min with [^{3}H]-choline after preincubation for 10 min at 37℃. [^{3}H]-Choline concentrations in HBS were 0, 0.142, 0.284, 0.567 and 0.134 μM. Data were expressed as mean±S.E. (n=6) in picomoles of [^{3}H]-choline/mg protein of synaptosomes. K_m value of [^{3}H]-choline uptake was 2.27±0.26 μM and V_{max} was 181±25.79 pmole/mg of protein/20 min.

μM (mean±S.E., n=7) of [^{3}H]-choline and V_{max} was 181.49 pmole/mg for 20 min at 37℃ (Fig. 2B). Hemicholinium-3, a choline uptake inhibitor, added to 0.2 μM in final concentration inhibited [^{3}H]-choline uptake into synaptosomes (from 27.25±0.87 to 3.04±0.12 pmole/mg protein, 86% inhibition, mean±S.E., n=6) and inhibition was saturated at 2.0 μM of hemichlinium-3 (Fig. 3). These results showed that [^{3}H]-choline uptake into synaptosomes from rat hippocampus occured through high affinity-choline carrier. Under the concentration of [^{3}H]-choline (0.567 μM) used, above 90% of total [^{3}H]-choline uptake was due to high affinity-choline carrier. So the data were not subtracted with the value in the presence of hemicholinium-3 for high affinity-choline transport.

Na^+-dependency of [^{3}H]-choline uptake

Na^+-dependency of [^{3}H]-choline uptake into synaptosomes was tested by substitution of Na^+ with sucrose in HBS. [^{3}H]-Choline uptake into synaptosomes was reduced by decrease of Na^+ concentration by LiCl substitution (from 35.76±10.89 to 18.62±4.13 pmol/mg protein [52.1% of control] at 60 mM replacement of NaCl, mean±S.E., n=9) (Fig. 4A) and sucrose substitution (from 16.83±0.85 to 9.85±0.35 pmol/mg protein [56.9% of control] at 60 mM replacement of NaCl, mean±S.E., n=9) (Fig. 4B). For adjustment of osmolarity in low Na^+-HBS, sucrose was added two times equimolar concentration of subtracted NaCl.

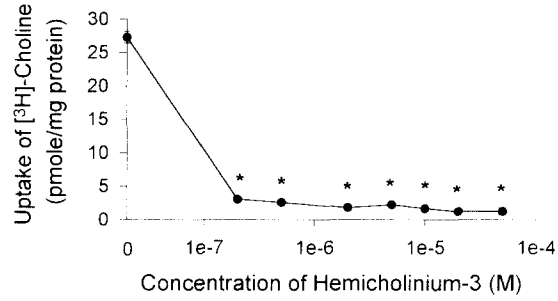

Fig. 3. Inhibition of [^{3}H]-choline uptake by hemicholinium-3. Synaptosomes were incubated with [^{3}H]-choline at 37℃ for 20 min after preincubattion with hemicholinium-3 (0, 0.2, 0.5, 2, 5, 10, 20 and 50 μM) for 10 min. Data were expressed as mean±S.E. (n=3~6) in picomoles of [^{3}H]-choline/mg protein. ** P<0.01.

Effect of high K^+-concentrations on [^{3}H]-choline uptake

The composition of HBS was changed for the increase of K^+ in the HBS. According to the increase of KCl, NaCl was reduced at equal amount of KCl added. High concentration of K^+ decreased [^{3}H]-choline uptake into synaptosomes more than low Na^+-HBS by replacing NaCl with sucrose or LiCl (from 19.81±2.53 pmol/mg protein to 9.54±2.52 (48% of control) and 4.73±0.41 (23%), at 20 and 40 mM of KCl added, respectively, mean±S.E., n=6) (Fig. 5A). The concentration of K^+ is one of the major components in determining membrane potential and it is known that membrane potential is proportional to logarithmic value of K^+ concentra-

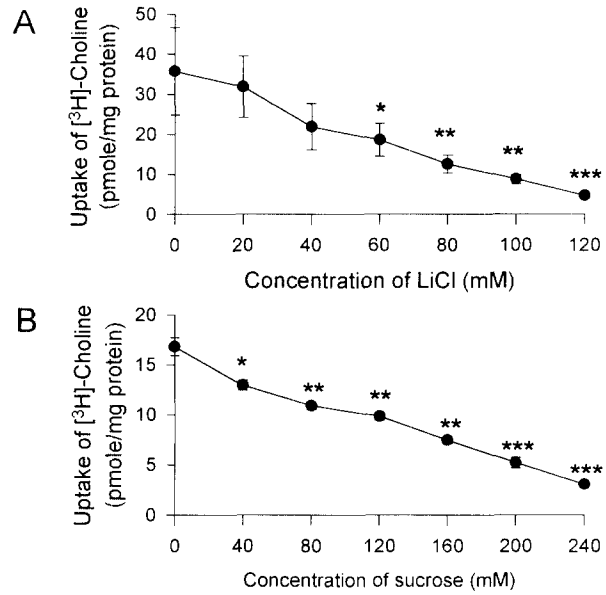

Fig. 4. Decrease of [^{3}H]-choline uptake according to substitution of NaCl. Several concentrations of LiCl (A) or sucrose (B) were added to HBS to prevent the decrease of osmolarity by subtraction of NaCl (20, 40, 60, 80, 100 and 120 mM). After 10 min preincubation of synaptosomes in these modified HBS solutions, addition of [^{3}H]-choline to HBS were followed by further incubations for 20 min at 37℃. Data were expressed as mean±S.E. (n=6) in picomoles of [^{3}H]-choline/mg protein. * P<0.05. ** P<0.01.

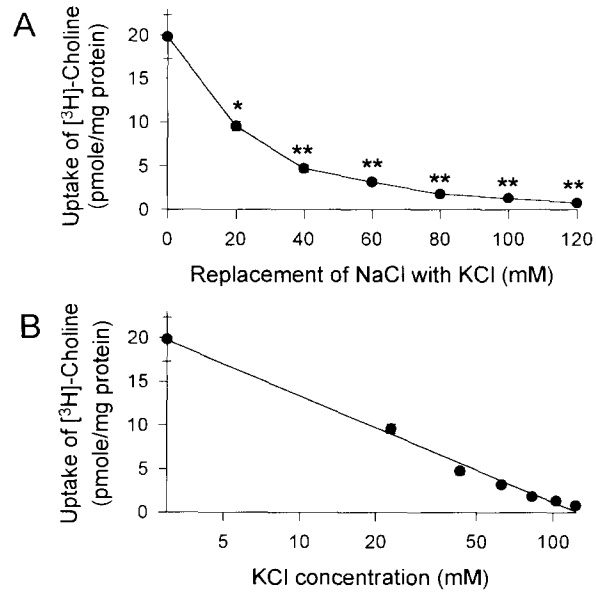

Fig. 5. Decrease of [^{3}H]-choline uptake by increase of KCl (A). Several concentrations of KCl were added to HBS and the same amounts of NaCl were subtracted to prevent the increase of osmolarity by adding KCl. And semilog plot of KCl concentration (B) shows the membrane potential-dependency of [^{3}H]-choline uptake into synaptosomes. Data were expressed as mean±S.E. (n=6) in picomoles of [^{3}H]-choline/mg protein. * P<0.05. ** P<0.01.

tion. So the data from Fig. 4A were replotted in semilog plot of KCl concentration. It showed the linear relationship between the velocity of [^{3}H]-choline uptake into synaptosomes and K^+ concentration (Fig. 5B).

Effects of Ca^{2+} on synaptosomal [^{3}H]-choline uptake

The various concentrations (0-5 mM) of $CaCl_2$ were tested for the function of these ions in the synaptosomal choline uptake. For Ca^{2+}-free medium, EGTA (2 mM) was added to HBS without $CaCl_2$. Decrease of Ca^{2+} concentration in HBS had little effect on [^{3}H]-choline uptake. But in Ca^{2+}-free HBS, synaptosomal [^{3}H]-choline uptake was reduced significantly (73.24±3.99 at 2 mM of $CaCl_2$ to 52.29±7.96 pmole/mg protein at Ca^{2+}-free HBS, mean±S.E., n=6, P<0.01) (Fig. 6A). Increase to 5 mM of $CaCl_2$ also reduced [^{3}H]-choline uptake (to 59.44±4.34 pmol/mg protein, mean±S.E., n=6, P<0.05) (Fig. 6B).

Neither decrease nor increase of $MgCl_2$ changed [^{3}H]-choline uptake of synaptosomes.

Ca^{2+}-dependency of reduction by high K^+-HBS in [^{3}H]-choline uptake

It was tested whether or not Ca^{2+} can modulate the high K^+-inhibition of [^{3}H]-choline uptake. Inhibitions by high K^+-concentration (40 and 80 mM) were similar regardless of absence or presence of Ca^{2+} (Fig. 7). In normal HBS, synaptosomal [^{3}H]-choline uptake was

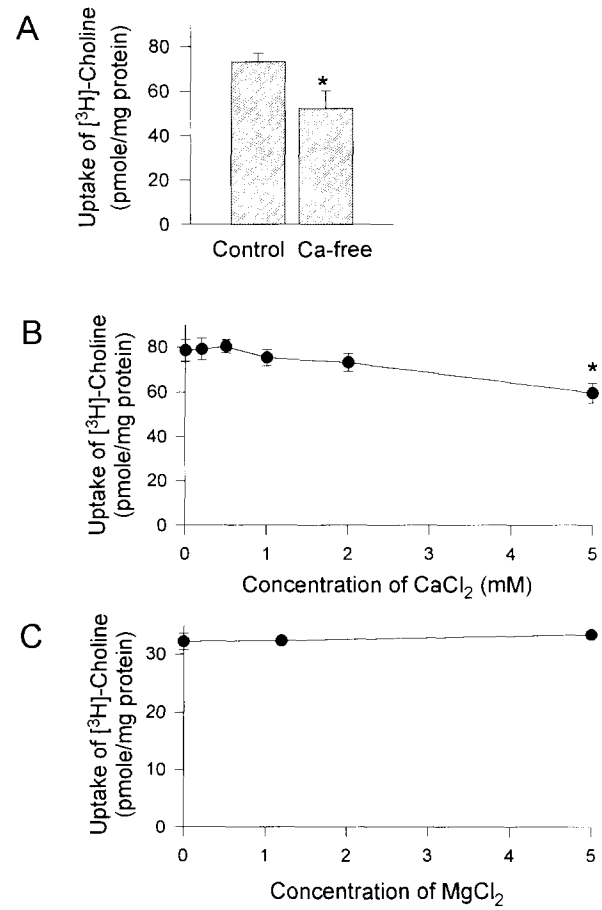

Fig. 6. Effect of divalent ionic concentrations on [^{3}H]-choline uptake into hippocampal synaptosomes. Changes of [^{3}H]-choline uptake into rat hippocampal synaptosomes were tested in Ca^{2+} free-HBS (EGTA 2 mM) (A) and various concentrations of $CaCl_2$ (B) or $MgCl_2$ (C). Data were expressed as mean±S.E. (n=6) in picomoles of [^{3}H]-choline/mg protein. * P<0.05.

decreased to 24.5 and 6.0% of control by adding KCl 40 mM and 80 mM. In Ca^{2+}-free HBS and HBS without $CaCl_2$, synaptosomal [^{3}H]-choline uptake also decreased similarly.

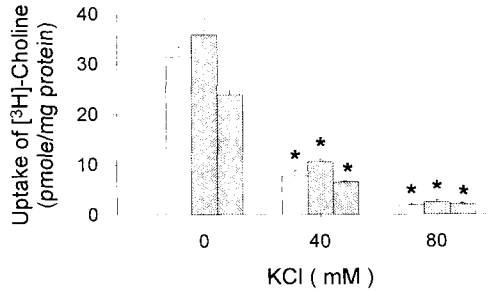

Fig. 7. Effect of $CaCl_2$ on decrease of [^{3}H]-choline uptake by high K^+-HBS. KCl was added to HBS with 2 mM $CaCl_2$ (□) or without (▨), or with 2 mM EGTA (▩). Data were expressed as mean±S.E. (n=6) in picomoles of [^{3}H]-choline/mg protein. ** P<0.01.

Discussion

Choline uptake of nerve cells in brain tissues occurred through high affinity and low affinity choline carrier. High affinity choline uptake is discriminated from low affinity in certain characteristics. High affinity choline uptake has low K_T value (< 5 μM) and is inhibited by hemicholinium-3 at submicromolar concentration. But low affinity choline uptake has high K_T value (>30 μM) and is inhibited by high concentrations of hemicholinium-3. And high affinity choline uptake is dependent on Na^+ concentration but low affinity choline uptake is less dependent (Haga, 1971; Yamamura and Snyder, 1973; Kuhar and Zarbin, 1978). In this study, high affinity choline uptake was confirmed by low K_m value (2.27 μM) of <5 μM and potent inhibition of hemicholinium-3 at submicromolar concentration (85% inhibition at 0.2 μM). It is well known that hemicholinium-3 as competitive inhibitor of choline uptake inhibit both high and low affnity choline uptake but at low concentration (less than 1 μM) inhibit only high affinity choline uptake not low affinity choline uptake (need more than 30 μM) (Guyenet et al., 1973). And Na^+-dependency of choline uptake in this study also suggested high affinity choline uptake. Kuhar and Zarbin (1978) suggested that the decrease in extracellular Cl^--concentration could reduce choline uptake. We did not discriminate the Cl^- effect but no difference was observed between LiCl and sucrose substitution instead of NaCl.

KCl substitution of NaCl decreased choline uptake more than sucrose and LiCl. K^+ is one of the critical components determining membrane potential and the increase of extracellular K^+ can depolarize the membrane potential (Vaca and Pilar, 1979). So the reduction of choline uptake by KCl substitution of NaCl was due to decrease of membrane potential but the effect by decrease of Na^+ could not be excluded. This result is similar to the report that the driving force of choline uptake is the Na^+ gradient and membrane potential across plasma membrane (Vaca and Pilar, 1979; Beach et al., 1980; O'Regan et al., 1984). Depolarization by ouabain, a Na^+, K^+-ATPase inhibitor, also inhibited the choline uptake (Haga and Noda, 1973). The decrease by high K^+-HBS seemed not to be due to enhancement of release of acetylcholine (Meyer and Cooper, 1982). It is also consistent with the result that choline uptake in high K^+-HBS was not different between with or without Ca^{2+}.

The increase of Ca^{2+} and Mg^{2+} showed no obvious effect. Only 5 mM $CaCl_2$ reduced the choline uptake slightly compared to normal concentration (2 mM). This result was similar to the report of Haga and Noda (1973) that Ca^{2+} reduced choline uptake of synaptosomes from rat brain but opposite to Silbergeld (1977) that choline uptake was decreased according to diminishing Ca^{2+} concentration. Another interesting result was the reduction of choline uptake by adding EGTA. The addition of EGTA, a chelating agent for divalent ions, especially for Ca^{2+}, may decrease Ca^{2+} in the medium extremely. This result was opposite to another result that high concentration of Ca^{2+} reduced choline uptake. We cannot explain why these results are different. One possibility is the cytotoxic effect of high concentration of Ca^{2+}. If this cytotoxic effect is applicable to synaptosomes, the decrease of choline uptake in high Ca^{2+}-HBS might be due to decrease of functional synaptosomes. But the result from the Ca^{2+}-free HBS excluded this possibility. Moreover there is no evidence about the cytotoxic effect of Ca^{2+} to synaptosomes. Another possibility is the regulation of choline metabolism by Ca^{2+}. According to Chatterjee and Bhatnagar (1990) the high-affinity and low-affinity states of choline carrier is interconvertible. They suggest the addition of ATP to media converted the choline carrier from low-affinity to high-affinity to hemicholinium-3 and this phenomenon requires divalent ions. They said the functional state is low-affinity state. From their report, it can be deduced that at high concentration Ca^{2+} can convert the functional low-affinity choline carrier to non- functional high-affinity choline carrier and reduce the synaptosomal choline uptake. Ferguson and Collier (1994) reported that EDTA treatment shifted the hemicholinium-3 binding site from a low-affinity state to a high-affinity state. Our result from EGTA treatment can also be explained similarly. Now, however, we are unable to explain how extracellular Ca^{2+} and EGTA or EDTA induce the changes of states of choline carrier. The results of high K^+ and Ca^{2+} combination suggested that these changes might not be from intracellular Ca^{2+} increase and that extracellular Ca^{2+} can also regulate some functions of neurons.

From these results we concluded that extracellular choline is uptaken through Na^+-dependent high-affinity carrier and Na^+ gradient and membrane potential across plasma membrane are essential for choline uptake. Extracellular Ca^{2+} seems to be able to regulate the choline uptake into nerve terminals and this phenomenon seems unlikely to be due to increase of acetylcholine release by intracellular Ca^{2+} increase.

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