THE HIGH AFFINITY CHOLINE UPTAKE SYSTEM WITH
RESPECT TO THE MUSCARINIC RECEPTORS USING
A SYNAPTOSOMAL FRACTION ABUNDANT
IN PRESYNAPTIC MEMBRANES

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Kinetic studies on choline (Ch) uptake into synaptosomes have shown the existence of two different uptake systems, a sodium-dependent high affinity uptake system and a sodium-independent low affinity system, the former system supplying most of the Ch necessary for synthesis of acetylcholine (ACh) in nerve terminals (1, 2). Recently, it has been found that presynaptic muscarinic receptors seem to modulate ACh release and Ch uptake (3, 4, 5, 6).

We examined the possible roles of the high affinity Ch uptake system in relation to muscarinic receptors in the presynaptic membranes of guinea pig brain cortex synaptosomes *in vitro*.

The high affinity uptake system is quite distinct from the low affinity uptake system present in many tissues: e.g., the former has a K_m value of $1.4 \mu\text{M}$ whereas in the corpus striatum the latter has a K_m value of $93 \mu\text{M}$ (2). Therefore, many workers have studied the high affinity Ch uptake into synaptosomes using Ch concentrations of $0.04\text{--}2 \mu\text{M}$ (7, 8, 9). However, the average Ch concentration is reported to be 40 nmole/g in brain tissue (10) and $10\text{--}45 \mu\text{M}$ in serum (11). We therefore used a concentration of $20 \mu\text{M}$ in the incubation medium, considering that such was about the same physiological concentration *in vivo*.

Guinea pigs weighing 300—350 g were decapitated and the cerebral cortex rapidly removed and homogenized in 10 volumes of 0.32 M sucrose. Fraction B described by Gray and Whittaker was used as the synaptosomal fraction (12). The synaptosomal fraction used in this experiment was examined by electron microscope (13, 14). The protein content of this fraction was measured by the method of Lowry *et al.* (15). The following procedures were carried out; 1) experiments after *in vivo* treatments, 2) experiments *in vitro*.

Experiments after in vivo treatments. Test animals were administered atropine sulfate (Iwaki Pharmaceutical Co. 5 mg/kg i.p.) or oxotremorine sesquifumarate (Aldrich Pharmaceutical Co. 0.3 mg/kg i.p.) 30 min before death, while control rats were given the same volume of saline (*i.p.*). Uptake of Ch was measured by a modification of the method of Haga and Noda (1) using ^3H -Ch (New England Nuclear, specific activity, 84 Ci/m mol). The synaptosomal B fraction mentioned above (ca. $1.2 \text{ mg protein/0.35 ml}$) was preincubated with 2 ml of modified Locke's solution (140 mM NaCl , 5 mM KCl , 1.2 mM MgCl_2 , 0.8 mM

CaCl₂, 10 mM glucose, 100 μ M neostigmine, 20 mM Tris-HCl and 1 mM phosphate buffers, pH 7.4) at 37°C for 10 min. Half the samples was incubated in sodium-free incubation medium made by replacing sodium salts by an osmotically equivalent amount of sucrose. High affinity Ch uptake was defined as the net accumulation of tritium in standard solution minus that in sodium-free solution, and was expressed as p moles/5 min/mg protein. A final Ch concentration of 20 μ M in the medium was obtained by adding 50 μ l of unlabelled and labelled Ch (1 μ Ci) to each tube. After 5 min the reaction was stopped by transferring the tubes to an ice-bath and adding 3 ml of ice-cold stopping solution (standard medium containing 10 mM Ch), and the suspensions were then centrifuged at 15,000 $\times$ g for 20 min. The resulting pellets were extracted with 0.5 ml of acidic ethanol solution (95% ethanol, 0.3% acetic acid, 1.5 mM ACh, 100 μ M neostigmine) overnight and the extract was mixed with 11 ml of scintillation mixture containing omunifluor (New England Nuclear) (10 ml toluene-1 ml ethanol).

Experiments in vitro: The synaptosomal fraction prepared from normal guinea pig brain was preincubated in modified Locke's solution containing 10–100 μ M of atropine or oxotremorine for 10 min. Subsequent procedures were the same as in the above-mentioned experiment after *in vivo* treatments.

Identification of labelled compounds taken up by the synaptosomal pellets was carried out by thin layer chromatography with a modification of the method of Haga (16).

Intraperitoneal administration of atropine caused a significant increase in Ch uptake (136% increase over Ch uptake observed in control rats confirming the results by Jenden et al. (17) and Wecker et al. (5)). On the other hand, pretreatment with oxotremorine, muscarinic agonist, failed to show any significant decrease of Ch uptake (data not shown). These results differ from those by Simon et al. (7) and Jenden et al. (17). The discrepancy between ours and theirs might be due to the species difference in sensitivity for oxotremorine.

Furthermore we also examined the effects of atropine and oxotremorine *in vitro* on high

TABLE 1. Effects of atropine and oxotremorine *in vitro* on high affinity Ch uptake by synaptosomes from cerebral cortex

Treatment		Choline Uptake (pmoles/5 min/mg protein)	% Change from control
<i>Effect of Atropine</i>			
Control		17.4 $\pm$ 2.5 (4)	
Atropine	10 μ M	26.2 $\pm$ 1.6* (4)	+51
	100 μ M	27.2 $\pm$ 2.6* (4)	+56
<i>Effect of Oxotremorine</i>			
Control		25.8 $\pm$ 6.3 (4)	
Oxotremorine	10 μ M	17.8 $\pm$ 5.9 (4)	–31
	100 μ M	26.0 $\pm$ 3.4 (4)	0

Synaptosomal fractions were preincubated for 10 min in modified Locke's solution containing 10 μ M or 100 μ M of atropine or oxotremorine, respectively. Values are expressed as means $\pm$ SEM for the number of experiments shown in parentheses.

* Significantly different from control ($p<0.05$).

FIG. 1. Proportions of ³H-ACh and ³H-Ch in synaptosomes from cerebral cortex. The radioactive compounds in synaptosomal pellets were separated by thin layer chromatography. Results are expressed percentages of total radioactivity taken up *in vitro*.

affinity Ch uptake using a synaptosomal B fraction abundant in presynaptic membranes. Table 1 shows the effects of various concentrations of atropine and oxotremorine directly added to the incubation medium on sodium-dependent high affinity Ch uptake by synaptosomes. Atropine at 10 and 100 μM increased the high affinity Ch uptake (51% and 56% increase, respectively over the control value). Oxotremorine at 10 and 100 μM did not alter the uptake significantly.

The insensitivity to the higher concentration of oxotremorine in these experiments may be due to the presence of 100 μM neostigmine in the Locke's solution used as medium, since preparations of synaptosomes obtained by the present method contained varying amounts of endogenous ACh. It was demonstrated that the affinity of atropine to the neuronal muscarinic receptors in ileum was higher than that of oxotremorine (18). The present finding with oxotremorine was a 31% decrease and that by atropine a 51% increase (Table 1).

The labelled compounds accumulated in synaptosomes were separated by thin layer chromatography, and the amounts of ³H-Ch and ³H-ACh were measured (Fig. 1). The conversion of ³H-Ch to ³H-ACh was increased by 10 and 100 μM atropine through the high affinity Ch uptake system. A similar increase of ACh release has been demonstrated using rat brain slices (4).

These findings suggest that there are muscarinic receptors in the presynaptic membranes of synaptosomes and that these receptors regulate the high affinity Ch uptake. Furthermore, the ³H-ACh formed from ³H-Ch accumulated in synaptosomes and its percentage of total radioactivity was increased about 2-fold by 10 or 100 μM atropine *in vitro* under conditions which allowed spontaneous release of ACh. It remains to be elucidated whether or not the activity of choline acetyltransferase is changed by these muscarinic agents.

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ADENOSINE-GUANOSINE INTERACTIONS IN THE ISOLATED DOG ATRIUM

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Since the initial investigations of Drury and Szent-Györgyi (1) purine nucleosides has been reported to have both positive and negative inotropic and chronotropic properties. Adenosine produces negative chronotropic and inotropic effects while guanosine induces positive effects (2). It is also well documented that adenine nucleotides and nucleosides are released by sympathetic nerve stimulation (3, 4). On the other hand, parasympathetic