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Cytidine and Uridine Increase Striatal CDP-Choline Levels Without Decreasing Acetylcholine Synthesis or Release

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SUMMARY

Aims: Treatments that increase acetylcholine release from brain slices decrease the synthesis of phosphatidylcholine by, and its levels in, the slices. We examined whether adding cytidine or uridine to the slice medium, which increases the utilization of choline to form phospholipids, also decreases acetylcholine levels and release.

Methods: We incubated rat brain slices with or without cytidine or uridine (both 25–400 μM), and with or without choline (20–40 μM), and measured the spontaneous and potassium-evoked release of acetylcholine.

Results: Striatal slices stimulated for 2 h released 2650 ± 365 pmol of acetylcholine per mg protein when incubated without choline, or 4600 ± 450 pmol/mg protein acetylcholine when incubated with choline (20 μM). Adding cytidine or uridine (both 25–400 μM) to the media failed to affect acetylcholine release whether or not choline was also added, even though the pyrimidines (400 μM) did enhance choline`s utilization to form CDP-choline by 89 or 61%, respectively. The pyrimidines also had no effect on acetylcholine release from hippocampal and cortical slices. Cytidine or uridine also failed to affect acetylcholine levels in striatal slices, nor choline transport into striatal synaptosomes.

Conclusion: These data show that cytidine and uridine can stimulate brain phosphatide synthesis without diminishing acetylcholine synthesis or release.

KEY WORDS: cytidine; uridine; choline; phospholipid synthesis; acetylcholine release; CDP-choline.

INTRODUCTION

The conversion of free choline to membrane phosphatidylcholine (PC) involves its phosphorylation to phosphocholine; the reaction of phosphocholine with cytidine triphosphate (CTP) to yield 5′-cytidine diphosphocholine (CDP-choline); and

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the transfer of that compound's phosphocholine moiety to the free hydroxyl group of diacylglycerol (DAG) (Kennedy and Weiss, 1956). The rates of all three reactions can be influenced by substrate availability, i.e., choline administration elevates brain phosphocholine (Millington and Wurtman, 1982); CTP levels limit the formation of endogenous CDP-choline (Choy *et al.*, 1980); and DAG levels, increased when PC12 cells are differentiated by treatment with nerve growth factor (NGF), control the rate at which these cells convert CDP-choline to PC (Araki and Wurtman, 1997). The rate-limiting step in PC synthesis usually is the formation of CDP-choline from CTP and phosphocholine (Araki and Wurtman, 1997, 1998). Brain CTP levels are lower than those needed to saturate the enzyme (CTP: phosphocholine cytidylyltransferase) that catalyses this reaction, thus giving cytidine, which increases brain CTP, accelerates PC synthesis in both PC12 cells (Lopez-Coviella and Wurtman, 1992) and rat brain slices (Savci and Wurtman, 1995). Similarly, incubation of PC12 cells with uridine, which is converted to UTP and CTP (Wurtman *et al.*, 2000; Richardson *et al.*, 2003), also enhances the formation of CDP-choline (Richardson *et al.*, 2003), the immediate precursor of PC.

Cholinergic neurons are unique in that they both acetylate and phosphorylate choline, to form acetylcholine and phosphocholine (and, ultimately, phosphatidylcholine); the acetylation pathway is favored over phosphorylation when the neurons are depolarized (Ando *et al.*, 1987; Farber *et al.*, 1996). Studies on striatal slices have shown that addition of choline to the perfusion medium both increases acetylcholine synthesis and release (Maire and Wurtman, 1985; Ulus *et al.*, 1989; Wecker *et al.*, 1989) and enhances membrane phospholipid synthesis (Ulus *et al.*, 1989; Savci and Wurtman, 1995). However if cytidine was also added, phosphatidylcholine synthesis was increased (Savci and Wurtman, 1995), but the tissue acetylcholine content reportedly declined (Savci and Wurtman, 1995). These observations suggested that, in cholinergic neurons, increasing the conversion of choline to membrane phospholipids might limit its availability for acetylcholine synthesis.

Hence, the present study was designed to determine whether cytidine generally suppresses the choline-induced stimulation of acetylcholine synthesis in, and release from cholinergic neurons, i.e., in rat brain slices. Because uridine is the primary circulating pyrimidine in humans (Wurtman *et al.*, 2000), and is readily converted in brain to UTP and then CTP (Cansev *et al.*, 2005), we also tested the effects of uridine on acetylcholine synthesis and release.

MATERIALS AND METHODS

Preparation and Perfusion of Slices

Male Sprague-Dawley rats (250–300 g) were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and sacrificed by decapitation, and their brains were rapidly removed. Striatal, hippocampal and cortical tissues were dissected and 0.3 mm thick slices were prepared using a McIlwain tissue chopper (Brinkman

Instruments, Westbury, NY). Tissue slices from two rats were combined and washed with ice-cold medium to remove most of the membrane debris. Four to six striatal slices were then transferred into each of eight superfusion chambers (volume, 1 mL). When cortical or hippocampal slices were used, six to eight were placed in the acetylcholine superfusion chamber. The chambers were kept at 37°C in a water bath, and slices were equilibrated for 60 min with a physiological solution (millimolar: NaCl, 120; KCl, 3.4; $CaCl_2$, 1.3; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 10 and eserine salicylate, 0.02) at a constant flow rate of 0.6 mL/min, by means of a Rainin Rabbit eight-channel peristaltic pump (Rainin Instrument Co. Inc., Woburn, MA). This solution was bubbled continuously with a mixture of 95% O_2 and 5% CO_2.

After the 60-min equilibration period, slices were perfused for 120 min with a choline-free or choline-containing (20–40 μM) high-potassium (52 mM) solution in the presence or absence of cytidine (25, 100, 200 or 400 μM) or uridine (25, 100, 200 or 400 μM). During this stimulation period, perfusates representing 20-min fractions or the entire 120-min period were collected in beakers on ice, and later assayed for acetylcholine. At the end of the stimulation period, slices were removed from the perfusion chamber and their choline and acetylcholine contents were determined.

Extraction of Acetylcholine from Perfusates

Acetylcholine was extracted from the perfusates using a silica column procedure (Gilberstat and Russell, 1984) as described previously (Ulus *et al.*, 1989). Briefly, 2 mL of the perfusates were applied to a silica column (5 mm × 8 mm bed of Bio-Sil A, 200–400 mesh, Bio-Rad Laboratories, CA). The column was then washed successively with 1 mL of 0.001N HCl, 0.8 mL of 0.075N HCl and 1 mL of 0.003N HCl in 10% (v/v) 2-butanone. The latter fraction (0.003N HCl in 10% 2-butanone) was collected in glass tubes (13 mm × 75 mm) and dried under vacuum. To remove any remaining choline the dried samples were then resuspended in 0.1 mL of Tris–HCl buffer (50 mM; pH 8.00) containing $MgCl_2$ (100 mM) and 2 U of choline oxidase (Sigma Chemical Co.) and incubated at 37°C in water bath for 60 min. The reaction was terminated by adding 1.9 mL of ice-cold distilled water, and then the column procedure was repeated once again. The recovery of acetylcholine in the final HCL-in-butanone fraction was about 60%, and that of choline less than 1%. These final fractions were dried under vacuum and stored at –20°C until assay for acetylcholine. Acetylcholine standards (0–800 pmol) were prepared in 2 mL of the same choline-containing medium as that used for superfusing the slices, and processed in parallel with the samples.

Extraction of Acetylcholine from the Tissues

Slices were removed from the chamber and washed twice with ice-cold physiological solution and then homogenized in 1 mL of cold water containing 0.02 M eserine salicylate. Aliquots (0.2 mL) of the homogenates were mixed with 1 mL of

ice-cold methanol; the mixtures were then vortexed and allowed to stand for about 2 h in a cold room after which they were centrifuged (1500 × *g* for 10 min). Supernatant fluids were transferred to glass tubes and dried under vacuum. Acetylcholine standards (0–800 pmol) were prepared in 0.2 mL of water and processed in parallel with the samples.

Determination of Acetylcholine

Acetylcholine contents of dried samples obtained from perfusates or tissues were determined by the radioenzymatic method (Gilberstat and Russell, 1984) described previously (Ulus *et al.*, 1989). In brief, any remaining choline in the samples was first converted to unlabelled phosphocholine; the acetylcholine was then hydrolyzed by acetylcholinesterase; and the choline resulting from this hydrolysis was phosphorylated by choline kinase (Sigma–Aldrich Corp., MO, USA) in the presence of [^{32}P-γ] ATP (Amersham Biosciences Corp., NJ, USA). Labeled phosphocholine was then separated from excess ATP by column chromatography, and quantitated using a liquid scintillation spectrometer (LS 6500, Beckman Coulter Inc., Fullerton, CA).

Determination of CDP-Choline in Striatal Slices

Slices were removed from the chamber, washed twice with ice-cold physiological solution, and then homogenized in 1 mL of cold water. Aliquots (0.5 mL) of the homogenates were mixed with 2 mL of ice-cold methanol; the mixtures were vortexed and allowed to stand for about 2 h in a cold room and then centrifuged (1500 × *g* for 10 min). Supernatant fluids were transferred to glass tubes and dried under vacuum. The dried samples were reconstituted in 100 μL water and then analyzed for CDP-choline by HPLC on an anion-exchange column (Alltech Hypersil APS-2, 5 μm, 250 mm × 4.6 mm) using a modification of the method of Simmonds *et al.* (1991), as described by Richardson *et al.* (2003). CDP-choline standards (0–200 pmol) were prepared in 0.5 mL cold water and processed in parallel with the samples.

Measurement of Choline Uptake in Striatal Synaptosomes

Male Sprague-Dawley rats (250–300 g) were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and sacrificed by decapitation; their brains were then removed rapidly. Striatal tissues were dissected and homogenized in 10 volumes of cold sucrose (0.32 M). The homogenates were then centrifuged at 1000 × *g* for 10 min; pellet was discarded and supernatant fluid was centrifuged again at 10,000 × *g* for 10 min. The crude synaptosomal pellets were resuspended in 50 volumes of sucrose (0.32 M) and used to examine high-affinity choline uptake. Aliquots (50 μL) of the synaptosomal suspension were transferred to eppendorf tubes (1.5 mL) containing 950 μL of ice-cold physiological medium (millimolar: NaCl, 120; KCl, 3.4; $CaCl_2$, 1.3; $MgSO_4$,

1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 10). This medium also contained 3.5 μM of (^{3}H-methyl)-choline (about 30,000 dpm). Solutions were equilibrated with a mixture of 95% O_2 and 5% CO_2 and drugs to be tested were added to the mixture prior to incubation. Tubes were then transferred to a Dubnoff shaker and incubated for 2.5, 5, 10 or 20 min at 37°C, under 95% O_2 and 5% CO_2. Reactions were stopped by transferring tubes to an ice bath; tubes were then centrifuged at 10,000 × *g* for 10 min at 4°C. Pellets were washed in 1 mL of ice-cold physiological medium twice, and their radioactivity was extracted by homogenization in 0.5 mL of distilled water using an ultrasonic homogenizer (Sonicator Model W-225R, Heat Systems-Ultrasonics Inc., Plainview, NY). The homogenates were then transferred to a small scintillation vial. Following addition of 4 mL of Optifluor® and vigorous shaking, radioactivity was measured using a liquid scintillation spectrometer (LS-6500, Beckman Coulter, Fullerton, CA). Blank values were obtained by incubating the mixture at 4°C or by incubating the mixture in the presence 5 μM hemicholinium-3.

Measurement of the Effect of Cytidine or Uridine on [^{3}H-Methyl]-Choline Incorporation Into Membrane Phospholipids

Striatal slices were prepared and perfused as described previously, with omission of eserine salicylate. After the equilibration period, the slices were transferred to incubation tubes and were incubated for 120 min in 1 mL of Krebs buffer containing 3.2 μCi of [^{3}H-methyl]-choline (final concentration of choline was 40 μM) in the presence or absence of cytidine (400 μM) or uridine (400 μM) for 120 min. At the end of the incubation period, slices were rinsed with 10 mL of ice-cold Krebs buffer three times, and homogenized in 1 mL of ice-cold water. Aliquots (4 × 20 mL) of the homogenate were taken for protein assay. The remaining homogenates were mixed and vortexed sequentially with 1 mL of methanol and 2 mL of chloroform. The organic (lower) and aqueous (upper) phases of the mixtures were separated by centrifugation (10 min at 4°C; 2000 rpm). Aliquots (0.5 mL) of the organic phase (containing phospholipids) were used for further separation of the individual phospholipid classes followed by determination of their [^{3}H-methyl]-choline contents by liquid scintillation counter (LS-6500, Beckman Coulter, Fullerton, CA). Briefly, aliquots (0.5 mL) of the lower phase were dried under vacuum, and choline-containing individual phospholipid classes (phosphatidylcholine, lysophosphatidylcholine and sphingomyelin) were purified using thin layer chromatography on silica gel G, with chloroform/ethanol/triethylamine/water (30:34:30:8, v/v) as the mobile phase (Touchstone *et al.*, 1980; Ulus *et al.*, 1989). Bands representing the choline-containing phospholipid classes were scraped off the plates and extracted with 1 mL of methanol. Aliquots (0.8 mL) of the methanol extracts were transferred to a large scintillation vial and, following addition of 15 mL of Optifluor®, quantified using a liquid scintillation counter (LS-6500, Beckman Coulter, Fullerton, CA), and an automated quench correction program.

Statistical Analysis

Data are expressed as means ± SEM. Statistical analyses were performed by one- or two-way ANOVA followed by Tukey test. Values of p less than 0.05 were considered to be significant.

RESULTS

Effects of Cytidine and Uridine on Acetylcholine Release from Brain Slices

In the first set of experiments, acetylcholine release from brain slices at rest and during stimulation were examined in the presence or absence of exogenous choline added to perfusion medium. The rates of acetylcholine release during superfusion at rest, without or with exogenously added choline (20 μM), were 86 ± 10 versus 138 ± 18 pmol/20 min/mg protein ($n = 6$; $p < 0.05$) for striatal slices; 45 ± 8 versus 78 ± 12 pmol/20 min/mg protein ($n = 6$; $p < 0.05$) for hippocampal slices and 28 ± 5 versus 48 ± 6 pmol/20 min/mg protein ($n = 6$; $p < 0.05$) for cortical slices, respectively (Fig. 1). When slices were stimulated with 52 mM K^+, acetylcholine release increased several folds during the first 20 min stimulation period and then decreased gradually, approaching basal rates during the next five 20 min stimulation periods (Fig. 1A, C and E). In the presence of exogenously added choline (20 μM), acetylcholine release from the slices was further increased, and was better maintained during the 2 h stimulation period than the release observed in the absence of exogenous choline (Fig. 1). For striatal, hippocampal or cortical slices (Fig. 1B, D and F), the total amounts of acetylcholine released into the medium during the entire 2 h stimulation period were increased by about 111, 121 or 67% after addition of 20 μM of choline, respectively.

The effects of cytidine, uridine, choline, and their combinations on acetylcholine release from rat striatal, hippocampal and cortical slices were tested in a separate set of experiments. As seen in Fig. 2, neither cytidine (25 μM) nor uridine (25 μM) altered the stimulated release of acetylcholine from striatal, hippocampal or cortical slices, regardless of whether choline also was added. Similarly, though, acetylcholine release was augmented when choline (20 μM) was added to the medium (Fig. 2), this elevation also was unaffected by including cytidine or uridine in the medium (Fig. 2).

Cytidine (25–400 μM) or uridine (25–400 μM) also failed to alter basal or stimulated acetylcholine release in the presence of a higher concentration (40 μM) of choline (Table I).

Effects of Cytidine and Uridine on Tissue Acetylcholine Contents in Striatal Slices

Tissue acetylcholine contents in striatal slices perfused at rest for 2 h in the absence or presence of exogenous choline were similar (Table II). When striatal slices were stimulated with 52 mM K^+ for 2 h, tissue levels of acetylcholine were

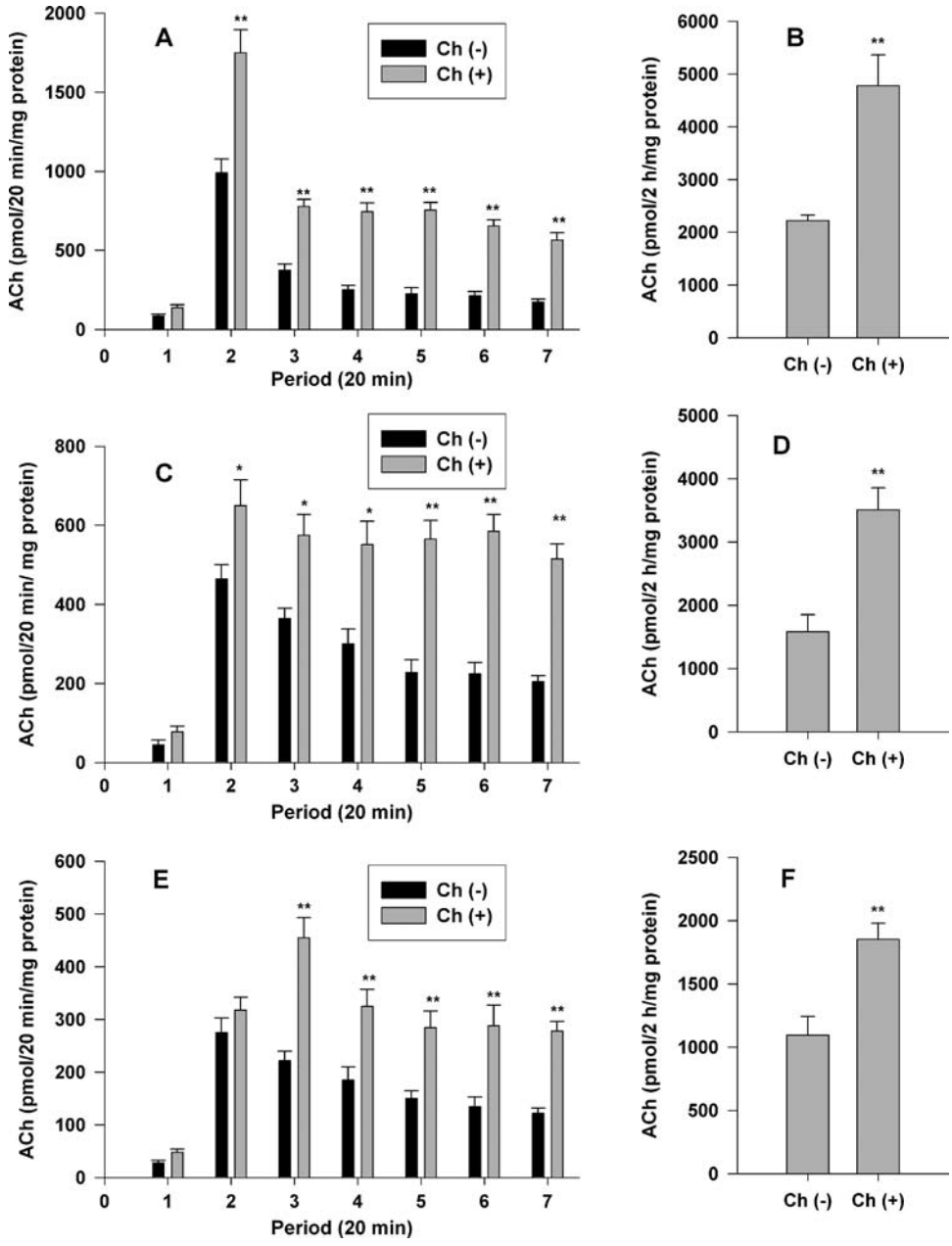

Fig. 1. Acetylcholine release from striatal, hippocampal and cortical slices during a 120-min stimulation period. Striatal (A and B), hippocampal (C and D) and cortical slices (E and F) were perfused with an eserine-containing normal Krebs medium at rest for 40 min. After the 40-min equilibration period, slices were perfused at rest for 20 min and then stimulated for 2 h with high K^+ (52 mM) in the presence [Ch(+)] or absence [Ch(−)] of choline (20 μM). Perfusates were collected for seven 20-min periods, starting at rest (1) and then during the 2-h stimulation (2–7) and assayed for acetylcholine. Values were normalized for the protein contents of each set of slices. Data are expressed as picomoles per milligram protein per 20 min (A, C and E) or per 2 h (B, D and F) and shown as means ± SEM; $N = 6$. $^*p < 0.01$; $^{**}p < 0.001$ higher than the respective values obtained with that in the absence of choline [Ch(−)].

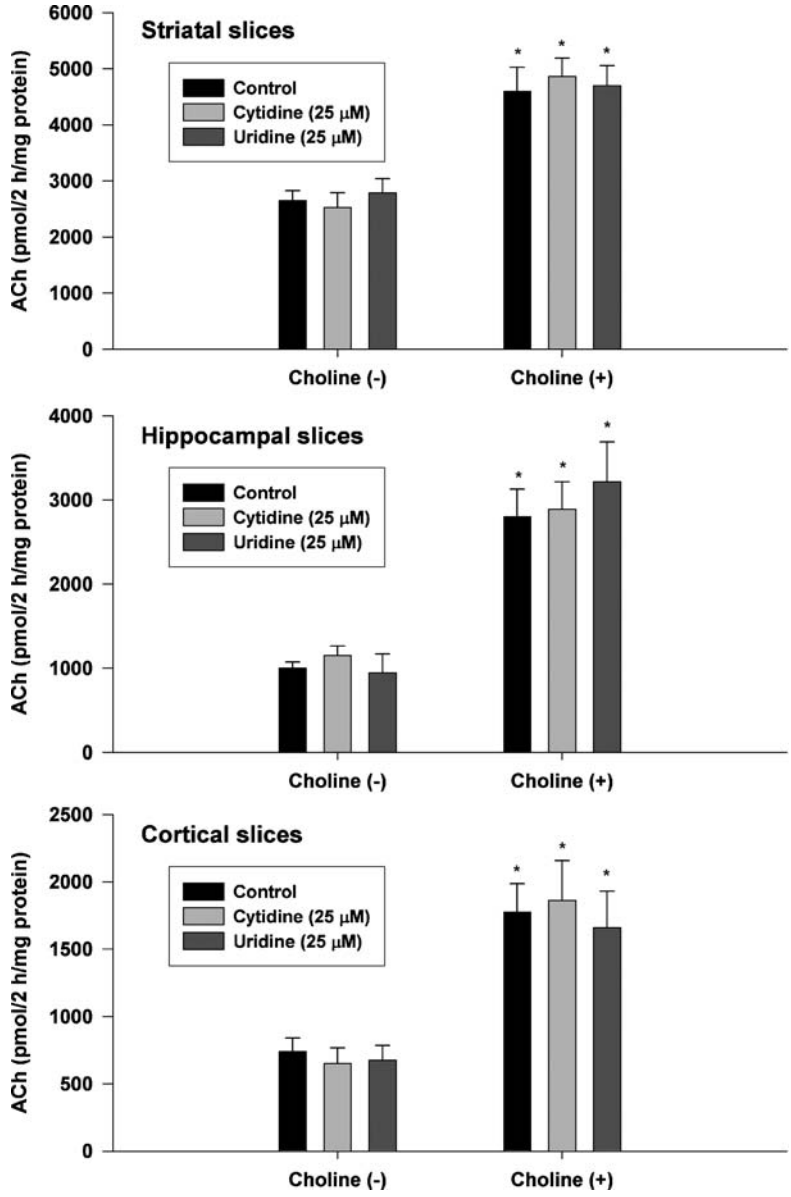

Fig. 2. Effects of cytidine or uridine on stimulated acetylcholine release from striatal, hippocampal and cortical slices in the presence or absence of choline in the medium. Brain slices were perfused with an eserine-containing normal Krebs medium. After a 60-min equilibration period, slices were perfused with an eserine-containing high K^+ (52 mM) Krebs medium for 2 h in the presence [Ch(+)] or absence [Ch(−)] of choline (20 μM). During this latter period, cytidine (25 μM) or uridine (25 μM) was added into the medium. Perfusates were collected during the entire 2-h period and assayed for acetylcholine. Values were normalized for the protein contents of each set of slices. Data are expressed as picomoles per milligram protein per 2 h, and shown as means $\pm$SEM; $N = 8$. $^*p < 0.001$ higher than the respective values obtained with that in the absence of choline [Ch(−)].

Effects of Addition of Cytidine and Uridine on Acetylcholine Release

Table I. Effects of Cytidine or Uridine on Acetylcholine Release from Striatal Slices at Rest and During Stimulation in the Presence of 40 μM Exogenously Added Choline in the Medium

		Acetylcholine release (pmol/2 h/mg protein)	
Medium	*N*	Rest	Stimulation
Cytidine (0)	6	2200 ± 400	10150 ± 860*
Cytidine (25 μM)	6	2225 ± 525	10600 ± 450*
Cytidine (100 μM)	6	2200 ± 400	10600 ± 440*
Cytidine (200 μM)	6	2000 ± 400	8500 ± 350*
Cytidine (400 μM)	6	2200 ± 350	8900 ± 670*
Uridine (0)	6	2650 ± 380	8600 ± 450*
Uridine (25 μM)	6	2500 ± 475	8850 ± 750*
Uridine (100 μM)	6	2400 ± 400	10500 ± 950*
Uridine (200 μM)	6	2600 ± 300	9500 ± 625*
Uridine (400 μM)	6	2200 ± 400	10000 ± 875*

Note. Striatal slices were perfused with an eserine-containing normal Krebs medium. After a 60-min equilibration period, slices were perfused for 2 h at rest or during stimulation (with 52 mM of K^+) in the absence (0) or presence of cytidine (25–400 μM) or uridine (25–400 μM) with exogenously added choline (40 μM) in the medium. Perfusates were collected during the entire 2-h period and assayed for acetylcholine. Values were normalized for the protein contents of each set of slices. Data are expressed as picomoles per milligram protein per 2 h, and shown as means ± SEM; $N = 6$.
*$p < 0.001$ (when compared with the respective values at rest).

Table II. Effects of Cytidine or Uridine on Acetylcholine Content in Striatal Slices at Rest and During Stimulation in the Absence or Presence of 40 μM Exogenously Added Choline in the Medium

Condition/treatment	*N*	Rest	Stimulation
Absence of choline			
Cytidine (0)	4	1700 ± 100	430 ± 25*
Cytidine (25 μM)	4	1690 ± 130	520 ± 65*
Cytidine (100 μM)	4	1725 ± 100	470 ± 25*
Cytidine (200 μM)	4	1850 ± 140	550 ± 20*
Cytidine (400 μM)	4	1750 ± 100	460 ± 20*
Uridine (0)	4	1780 ± 110	500 ± 75*
Uridine (25 μM)	4	1980 ± 100	550 ± 80*
Uridine (100 μM)	4	1830 ± 100	510 ± 55*
Uridine (200 μM)	4	1780 ± 120	550 ± 50*
Uridine (400 μM)	4	1690 ± 140	590 ± 40*
Presence of 40 μM choline			
Cytidine (0)	6	1800 ± 125	790 ± 65**
Cytidine (25 μM)	6	1825 ± 150	830 ± 80
Cytidine (100 μM)	6	1875 ± 230	810 ± 50**
Cytidine (200 μM)	6	1880 ± 180	850 ± 95**
Cytidine (400 μM)	6	1900 ± 150	795 ± 90**
Uridine (0)	6	1910 ± 120	830 ± 70**
Uridine (25 μM)	6	1890 ± 100	790 ± 80**
Uridine (100 μM)	6	2090 ± 220	765 ± 75**
Uridine (200 μM)	6	1960 ± 110	845 ± 45**
Uridine (400 μM)	6	2010 ± 220	815 ± 65**

Note. Striatal slices were perfused with an eserine-containing normal Krebs medium. After a 60-min equilibration period, slices were perfused with an eserine-containing high K^+ (52 mM) Krebs medium for 2 h in the absence or presence of choline (40 μM). During this latter period, cytidine (25–400 μM) or uridine (25–400 μM) was added into the medium. At the end of 2-h period, slices were removed from the chambers and assayed for protein and acetylcholine. Acetylcholine values were normalized for the protein contents of each set of slices. Data are expressed as picomoles per milligram protein, and shown as means ± SEM; $N = 4$–6.
*$p < 0.001$ (when compared with the respective values at rest); **$p < 0.01$ (when compared with the respective values observed in the absence of choline).

reduced by about 75% in the absence, or by 55% in the presence of 40 μM choline (Table II). Neither cytidine (25–400 μM) nor uridine (25–400 μM) affected striatal acetylcholine levels in slices at rest or after stimulation, whether or not choline (40 μM) was also added to the medium.

Effects of Cytidine and Uridine on Choline Uptake in Striatal Synaptosomes

Figure 3 illustrates effects dose- and time course studies on effects of cytidine or uridine on choline uptake into striatal synaptosomes. Cytidine or uridine, at 25–800 μM concentrations, failed to affect choline uptake into striatal synaptosomes (Fig. 3).

Effects of Cytidine and Uridine on [^{3}H-Methyl]-Choline Incorporation into Membrane Phospholipids

Cytidine significantly [$F(4, 35) = 3.92$; $p < 0.01$] enhanced [^{3}H-methyl]-choline incorporation into membrane phospholipids (Fig. 4). Uridine also tended to increase [^{3}H-methyl]-choline incorporation into membrane phospholipids; however this effect failed to attain statistical significance at any of the concentrations tested (100–800 μM).

Effects of Cytidine and Uridine on Tissue CDP-Choline Levels

Measurements of tissue CDP-choline accumulation have been shown to provide a more sensitive indication of the rate of PC synthesis than measuring changes in PC itself, since CDP-choline's pool size is orders of magnitude less than, and its overall turnover rate considerably faster than, that of PC. Hence, we examined the effect of cytidine or uridine supplementation on CDP-choline levels in striatal slices perfused with cytidine (400 μM) or uridine (400 μM) for 2 h in the presence of choline (40 μM). Cytidine and uridine both increased tissue CDP-choline levels, by about 88% ($p < 0.01$) and 62% ($p < 0.05$), respectively (Table III).

DISCUSSION

These data show that although cytidine and uridine increase the rates at which rat brain slices incorporate choline into CDP-choline and then phosphatidylcholine, they do not alter basal or stimulated acetylcholine release from the slices, nor the effects of choline on this release (Fig. 2). Even though both cytidine and uridine (400 μM) increased CDP-choline levels in the slices (Table III), they had no effect on acetylcholine release from the brain slices (Fig. 2) nor on striatal acetylcholine levels (Table II). Cytidine or uridine also failed to modify tissue acetylcholine levels (Table II), or choline transport into cholinergic nerve endings by the high-affinity choline uptake system. Hence, using the pyrimidines to promote membrane

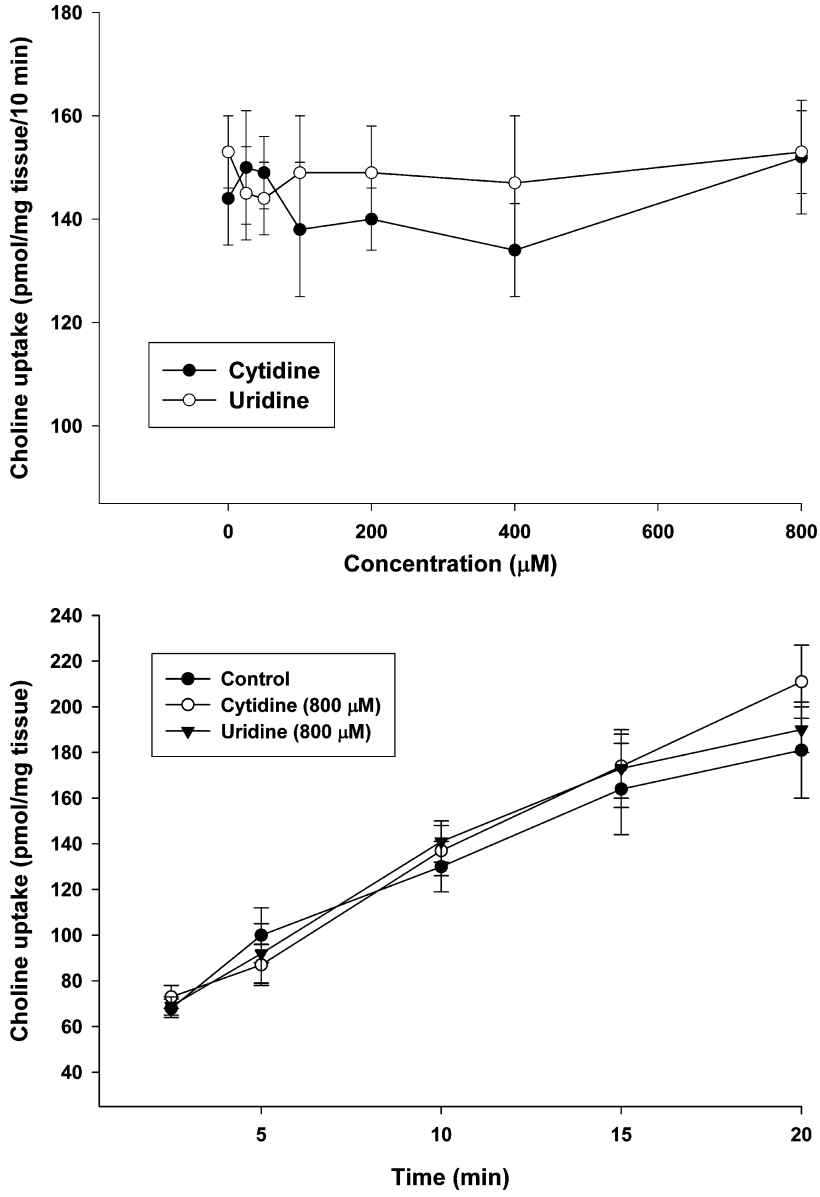

Fig. 3. Effects of cytidine or uridine on choline transport into the striatal synaptosomes by high affininity choline transporter. Upper figure: striatal synaptosomes were incubated with Krebs medium containing 3.5 μM (methyl-^{3}H)-choline for 10 min at 37°C in the absence (0) or presence of cytidine (25–800 μM) or uridine (25–800 μM). Lower figure: striatal synaptosomes were incubated with Krebs medium containing 3.5 μM (methyl-^{3}H)-choline for 2.5, 5, 10, 15 or 20 min at 37°C in the absence (control) or presence of cytidine (800 μM) or uridine (800 μM). At the end of the incubation period synaptosomes were collected by centrifugation and their radioactivity was extracted and measured. Data for choline uptake are expressed as picomole per milligram tissue per 10 min (upper figure) or picomole per milligram tissue (lower figure) and shown as means $\pm$ SEM; $N = 6$.

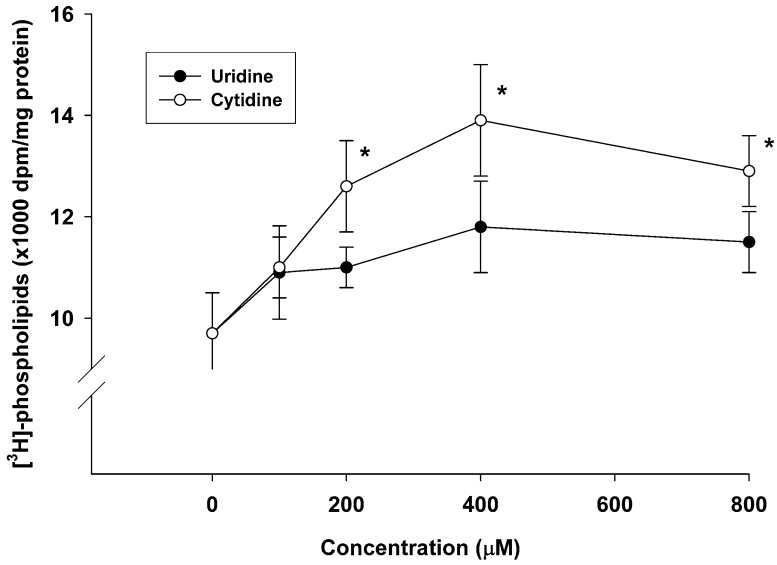

Fig. 4. Effects of cytidine or uridine on [^{3}H]-choline incorporation into membrane phospholipids in striatal slices. Striatal slices were incubated for 2 h in the presence of cytidine (100–800 μM) or uridine (100–800 μM) plus [^{3}H]-choline. Slices were washed and homogenized. Aliquots of homogenates were extracted in methanol/chloroform. Aliquots (0.5 mL) of the organic phase were used for determination of [^{3}H-methyl]-choline in phospholipids. $^*p < 0.05$ different from the value observed in the absence of cytidine (0 μM).

phosphatide synthesis will probably not diminish acetylcholine-mediated neurotransmission.

In contrast, when the use of choline in brain slices to form acetylcholine was increased by repeated depolarization, both PC synthesis (Farber *et al.*, 1996) and its levels declined significantly (Ulus *et al.*, 1989). If the striatal slices were stimulated electrically for four to eight 20min periods, with 20min resting intervals, the greatly increased release of acetylcholine into the perfusion medium was accompanied by a decrease in tissue PC content by 13–25% ("autocannibalism"; Ulus *et al.*, 1989). Addition of choline (10–40 μM) to the perfusion medium protected

Table III. Effects of Cytidine or Uridine on Tissue CDP-Choline Levels

Treatment	*N*	CDP-choline (pmol/mg protein)
Control	8	20.2 ± 2.2
Cytidine (400 μM)	8	$38.2 \pm 4.1^{**}$
Uridine (400 μM)	8	$32.6 \pm 3.7^{*}$

Note. Striatal slices were incubated for 2 h in Krebs buffer containing cytidine (400 μM) or uridine (400 μM) in the presence of exogenously added choline (40 μM). Slices were washed and homogenized. Aliquots of the homogenates were extracted in methanol/chloroform. Aliquots of the aqueous phases were used for determination of CDP-choline.
$^*p < 0.01$; $^{**}p < 0.01$ (different from control).

against the stimulus-induced decrease in membrane phosphatide levels, and increased the release of acetylcholine (Ulus *et al.*, 1989). The present results are in accord with previous reports (Maire and Wurtman, 1985; Wecker *et al.*, 1989; Ulus *et al.*, 1989), and show that brain slices do continue to synthesize and release the neurotransmitter acetylcholine even when exogenous choline has not been added to the perfusion medium, and that when they are depolarized (e.g., with high K^+; 52 mM) they release larger amounts of acetylcholine. The addition of choline (20 μM) to the perfusion medium in this circumstance further increased acetylcholine release (Fig. 1). But the addition of cytidine or uridine to the medium—which promoted CDP-choline (Table III) and thus PC synthesis—failed to diminish acetylcholine release from striatal (Fig. 2, Table I), cortical (Fig. 2) or hippocampal (Fig. 2) slices, at rest or during stimulation and with or without exogenous choline.

Our earlier publication (Savci and Wurtman, 1995) described evidence that the presence of cytidine (200 μM) could decrease striatal acetylcholine content by about 18%; in contrast, in the present study, we found no effect of cytidine or uridine (both 25–400 μM) on striatal acetylcholine levels or release, whether or not choline had been added to the incubation medium (Table II). The apparent difference between the two sets of findings probably reflects methodological differences between them: In our earlier study (Savci and Wurtman, 1995), the striatal slices had been incubated in a static medium for an hour prior to measurement of acetylcholine, while in the present study the slices were continuously perfused at the rate of 0.6 mL/min. It is known that acetylcholine accumulates in striatal slices and its levels increase over physiological concentrations, during periods of static incubation (Weiler *et al.*, 1979). Indeed the reported value for tissue acetylcholine content in the previous study, 3037 ± 214 pmol/mg protein, was in good aggrement with values reported by Weiler *et al.* (1979) under static incubation condition but much higher than the values observed in the present study (Table I) or previous reports (Maire and Wurtman, 1985; Ulus *et al.*, 1989, 1992) from our laboratory in the perfused striatal slices. We now find that in a perfused slice system, which is more physiological than a static incubation and less likely to be associated with accumulation of metabolic products in the medium, added pyrimidines do elevate phospholipid synthesis without concurrently lowering tissue acetylcholine contents (Table II).

The transport of choline from the extracellular space to cholinergic neuronal terminals through the high-affinity choline uptake transporter is essential for maintaining normal acetylcholine synthesis and, therefore, normal cholinergic transmission (Ferguson and Blakely, 2004). In the present study, we observed that neither cytidine nor uridine, at 25–800 μM concentrations, affected [^{3}H]-choline uptake into striatal synaptosomes. These observations are consistent with the failures of cytidine or uridine to reduce the synthesis or release of acetylcholine from striatal slices (Fig. 3).

In conclusion, we demonstrate that cytidine or uridine supplementation, which increases CDP-choline levels and membrane phospholipid synthesis does not reduce acetylcholine synthesis in or release from brain slices in the presence or absence of exogenously added choline (20–40 μM). These data suggest that

giving supplemental cytidine or uridine with choline might increase the membrane reservoir of bound choline without compromising acetylcholine levels.

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