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# Choline increases acetylcholine release and protects against the stimulation-induced decrease in phosphatide levels within membranes of rat corpus striatum

Ismail H. Ulus[1], Richard J. Wurtman[2], Charlotte Mauron[2] and Jan Krzysztof Blusztajn[3]

*[1]Department of Pharmacology, Uludag University Medical School, Bursa (Turkey), [2]Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139 (U.S.A.) and [3]Department of Pathology, Boston University School of Medicine, Boston, MA 02118 (U.S.A.)*

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*Key words:* Choline; Acetylcholine; Phospholipid; Phosphatidylcholine; Precursor; Striatum

This study examined the possibility that membrane phospholipids might be a source of choline used for acetylcholine (ACh) synthesis. Slices of rat striatum or cerebellum were superfused with a choline-free or choline-containing (10, 20 or 40 μM) physiological solution with eserine, for alternating 20 min periods of rest or electrical stimulation. Superfusion media were assayed for choline and ACh, and slice samples taken before and after stimulation were assayed for choline, ACh, various phospholipids, protein and DNA. The striatal slices were able to sustain the stimulation-induced release of ACh, releasing a total of about 3 times their initial ACh contents during the 8 periods of stimulation and rest. During these 8 cycles, 885 pmol/μg DNA free choline was released from the slices into the medium, an amount about 45-fold higher than the initial or final free choline levels in the slices. Although repeated stimulation of the striatal slices failed to affect tissue levels of free choline or of ACh, this treatment did cause significant, dose-related (i.e., number of stimulation periods) stoichiometric decreases in tissue levels of phosphatidylcholine (PC) and of the other major phospholipids; tissue protein levels also declined significantly. Addition of exogenous choline to the superfusion medium produced dose-related increases in resting and evoked ACh release. The choline also fully protected the striatal slices from phospholipid depletion for as many as 6 stimulation periods. Cerebellar slices liberated large amounts of free choline into the medium but did not release measurable quantities of ACh; their phospholipid and protein levels did not decline with electrical stimulation. These data show that membrane phospholipids constitute a reservoir of free choline that can be used for ACh synthesis. When free choline is in short supply, ACh synthesis and release are sustained at the expense of this reservoir. The consequent reduction in membrane PC apparently is associated with a depletion of cellular membrane. The use of free choline by cholinergic neurons for two purposes, the syntheses of both ACh and membrane phospholipids, may thus impart vulnerability to them in situations where the supply of free choline is less than that needed for acetylation.

## INTRODUCTION

Brain slices and minces continue to make and, upon depolarization, to release large amounts of acetylcholine (ACh) without exhibiting reductions in their contents of free choline or ACh even when they are superfused with a choline-free medium[4,10,26,34,49,50]. However, addition to the choline-free medium of hemicholinium-3 (HC-3), a drug which blocks choline's high-affinity uptake into cholinergic terminals, stops the release of acetylcholine from nerve terminals, and decreases tissue acetylcholine levels[26]. These findings were interpreted as suggesting that some of the choline used by the slices for acetylcholine synthesis originated in the phosphatidylcholine (PC) of neuronal membranes[26]: depolarization would cause a net hydrolysis of this PC, and the choline thus released into extracellular fluid would be taken up into cholinergic terminals for acetylation[5].

*Correspondence:* R.J. Wurtman, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

If this explanation were correct, it might be predicted that prolonged stimulation of cholinergic neurons superfused without choline would also reduce their PC content: moreover, this reduction might be blocked by adding choline to the medium. Studies described below tested these predictions.

## MATERIALS AND METHODS

Male Sprague–Dawley rats (280–320 g) were decapitated, and their striata or cerebella were rapidly dissected and kept in ice-cold physiological solution. Slices 0.3 mm thick (40–70 mg) were prepared with a McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Gomstall, U.K.) and collected in cold medium. The slices were washed several times with ice-cold medium to remove most of the membrane debris and were then transferred to a superfusion chamber (volume 1.0 ml) and placed between two $Ag/AgCl_2$ stimulation electrodes. A nylon gauze disk prevented direct contact of the tissue with the electrodes. The chamber was maintained at 37 °C in a water-bath, the slices were superfused (0.6 ml/min) with a physiological solution (mM: NaCl, 120; KCl, 3.5; $CaCl_2$, 1.3; $MgSO_4$, 1.2; $NaH_2PO_4$, 1.2; $NaHCO_3$, 25; glucose, 10; eserine salicylate, 0.02). The physiological solution was constantly bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. Choline was sometimes added to the superfusion medium as indicated in the text.

Following a 20 min equilibration period, some slices were removed for determination of ACh, choline, phospholipids, protein and DNA contents (referred to as 'tissue initial'). The remaining slices were then superfused for 90–340 min. They were alternately maintained at rest for 20 min and stimulated electrically (1 ms pulses at 15 Hz with an amplitude of 30 V), also for 20 min, for 2, 4, 6 or 8 stimulation periods. Perfusates representing each entire 20 min rest or stimulation period were collected in glass tubes that were kept on ice, and later were assayed for ACh and choline. The slices were taken from the superfusion medium 10 min after their last stimulation period and assayed for ACh, choline, phospholipids, protein and DNA (referred to as 'tissue final').

ACh and choline were extracted from the superfusate by a liquid cation exchange procedure[14]. One ml of tetraphenylboron in 3-heptanone (10 mg/ml) was added to each 2 ml fraction of collected effluent. The tubes were shaken and then centrifuged (1500 *g*, 10 min), and 0.75 ml of the upper heptanone layer was transferred into 0.4 ml of 0.4 N HCl. The samples were mixed and briefly centrifuged, and the upper organic layer was discarded by aspiration. Measured aliquots of the HCl solution were transferred to small plastic tubes and dried under a vacuum (Savant Instruments Vacuum Centrifuge, Hicksville, NY). Standards for ACh and choline (0–1.6 nmol) were prepared in 2 ml of the same solution as that used for superfusing the slices, and were processed in parallel with the samples.

When exogenous choline was added to the superfusion medium, ACh was extracted from it using a silica column procedure[16]. Two ml of the superfusate were applied to a silica column (5 × 8 mm bed of Bio-Sil A, 200–400 mesh, Bio-Rad Laboratories, CA). The column was then washed successively with 1 ml of 0.001 N HCl, 0.8 ml of 0.075 N HCl and 0.7 ml of 0.03 N HCl in 10% (v/v) 2-butanone. Subsequently, the latter fraction (0.030 N HCl in 10% 2-butanone) was collected in glass tubes and dried under vacuum. The dried samples were resuspended in 2 ml of water and the column procedure was repeated once again. The recovery of ACh in the final HCl-in-butanone fraction was about 65%, and that of choline less than 2%. These final fractions were dried under vacuum and stored at −20 °C until they could be assayed for ACh. ACh standards (0–1.6 nmol) 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.

Tissue ACh and choline were extracted as described by Torn and Aprison[38]. The 'initial' and 'final' tissue samples were homogenized in 1 ml of cold water, and 0.2 ml of the homogenate were mixed with 2 ml of 1 M formic acid in acetone (15:85, v/v). The mixtures were allowed to stand for about 2 h in a cold room and then centrifuged (1500 *g*, for 10 min). The supernatant fluid was transferred to a glass tube and dried under vacuum. The residue was dissolved in 2 ml of the same choline-free medium as that used for superfusing the slices. ACh and choline were then extracted from this solution as

described above. Standards for ACh and choline (0–1.6 nmol) were prepared in 0.2 ml of water and processed in parallel with the samples.

The ACh and choline contents of the dried samples were determined by the radioenzymatic method of Goldberg and McCaman[17]. In brief, choline was phosphorylated by choline kinase in the presence of [$^{32}$P-$\gamma$]ATP, and the labelled phosphocholine was then separated from excess ATP and quantitated. For the determination of ACh, the endogenous choline was first converted to unlabelled phosphocholine; the ACh was then hydrolyzed by acetylcholinesterase and the choline resulting from this hydrolysis was assayed as above.

For the extraction of lipids[13], 0.4 ml of the homogenate were mixed with 9 ml of chloroform/methanol (2:1) and 1.8 ml of 0.75% aqueous KCl (w/v), and allowed to stand overnight in a cold room; aliquots of the lower phase (containing phospholipids) were used for the determination of phosphorus[37] and for further separation of the individual phospholipid classes. These classes 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[39]. The individual phospholipids were scraped off the plates and quantified based on the phosphorus contents.

Aliquots of tissue homogenates were assayed for protein[24] and DNA[23].

## RESULTS

The striatal slices released ACh and choline into the medium during superfusion with the choline-free medium (Fig. 1). The rate of ACh release was 4.2 ± 0.8 pmol/$\mu$g DNA/20 min (mean ± S.E.M., $n$ = 11) during the first collection period; this remained constant for 3 h among slices superfused at rest (Fig. 1). When the slices were stimulated, ACh release increased about 4-fold, to 15.4 ± 2.1 pmol/$\mu$g DNA/20 min (mean ± S.E.M., $n$ = 11); this quickly returned to basal levels when the stimulation was terminated (Fig. 1).

Initial studies used slices stimulated for 4 20-min periods. Slices continued to release ACh at the higher rate for all 4 periods (Fig. 1). The rate of choline efflux was 66.0 ± 8.0 pmol/$\mu$g DNA/20 min (mean ± S.E.M., $n$ = 14) during the first collection period; this gradually fell, during the next 3 h, to 40.0 ± 8.0 pmol/$\mu$g DNA/20 min (mean ± S.E.M., $n$ = 4) in unstimulated slices, or to 36.0 ± 4.0 pmol/$\mu$g DNA/20 min (mean ± S.E.M., $n$ = 9) in stimulated slices. The rate of choline efflux showed a tendency to increase while the tissue was being stimulated (Figs. 1 and 2). This tendency was not statistically significant.

Cerebellar slices failed to release detectable amounts of ACh into the superfusate at rest or

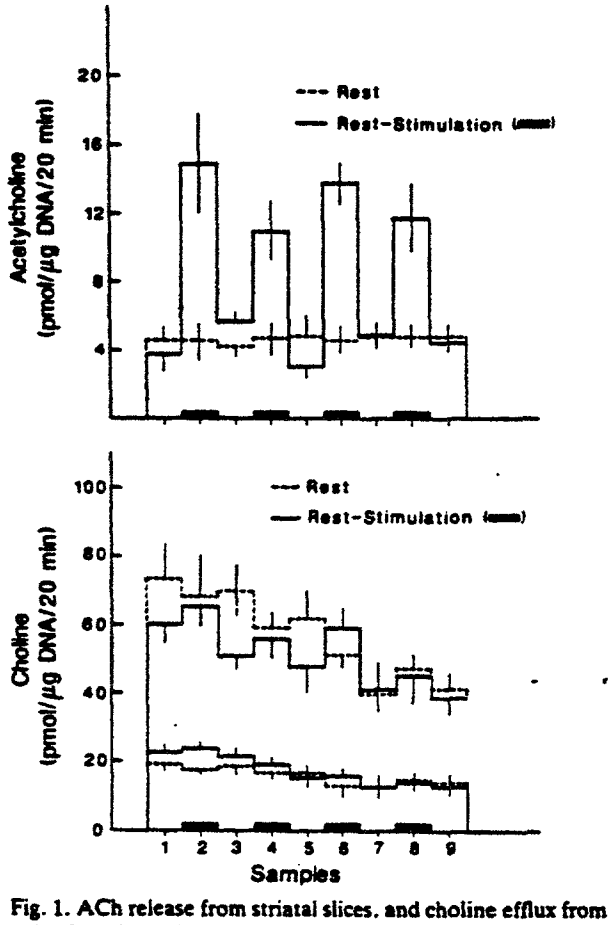


Fig. 1. ACh release from striatal slices, and choline efflux from striatal and cerebellar slices, at rest and during electrical stimulation. Slices of rat striatum or cerebellum were superfused (0.6 ml/min) with a choline-free physiological solution. After a 20 min equilibration period, the superfusate was collected for 20 min; the slices in one of the two chambers were then stimulated electrically (15 Hz) for 20 min, after which they were allowed to rest for 20 min and the rest–stimulation cycle was repeated for a total of 4 times. The slices in the other chamber were superfused at rest for the same total duration. ACh release and choline efflux are expressed as pmol/$\mu$g DNA/20 min. Each point represents the mean of 6–11 separate experiments. Vertical bars represent S.E.M. Upper panel shows ACh release for striatal slices; lower panel shows choline release for striatal or cerebellar slices.

TABLE I

*Effect of electrical stimulation on phospholipid contents of striatal and cerebellar slices superfused with a choline-free medium*

Slices of rat striatum or cerebellum were superfused (0.6 ml/min) with a choline-free physiological solution. After a 20 min equilibration period, samples of the slices were taken for determination of initial phospholipid and protein contents. The remaining tissues were superfused at rest for 180 min, or stimulated electrically (15 Hz) for 4 20 min periods, separated by 20 min intervals. At the end of the experiments, slices were assayed for phospholipids and protein, and corrected for their DNA contents. The final tissue contents of protein and phospholipids were then expressed as percents of the initial levels. The initial levels of phospholipid in striatum and cerebellum were (mean ± S.E.M.) 808 ± 17 ng/μg DNA ($n$ = 27) and 671 ± 30 ng/μg DNA ($n$ = 8); the initial levels of protein were 63.6 ± 2.1 μg/μg DNA ($n$ = 22) and 58.4 ± 2.0 g/μg DNA ($n$ = 8). Data are given as means ± S.E.M. Numbers of determinations are shown in parentheses. Data were subjected to analysis of variance followed by Newman–Keuls multiple comparison test.

| *Slice and condition* | *Phospholipid (% initial level)* | *Protein (% initial level)* |
|---|---|---|
| Striatal slices | | |
| Superfusion at rest | 102.1 ± 3.2 (11) | 98.1 ± 3.8 (11) |
| Stimulated | 86.1 ± 2.6 (22)** | 89.7 ± 3.5 (22) |
| Cerebellar slices | | |
| Superfusion at rest | 98.0 ± 4.0 (8) | 97.7 ± 4.1 (8) |
| Stimulated | 96.0 ± 5.0 (8) | 95.9 ± 5.4 (8) |

*$P$ < 0.05 significantly differs from the corresponding value obtained from superfusion at rest.

during periods of electrical stimulation. They did, however, release free choline (Fig. 1), at an initial rate of 22.0 ± 2.0 pmol/μg DNA/20 min (mean ± S.E.M., $n$ = 12) and a final rate of 12.0 ± 2.0 pmol/μg DNA/20 min (mean ± S.E.M., $n$ = 12). Choline efflux from cerebellar slices was not altered by electrical stimulation (Fig. 1).

Stimulation of the striatal slices for 4 20-min periods significantly decreased their contents of phospholipids (by 14%, compared with phospholipid levels in their tissue initials) and of proteins (by 10.5%) (Table I). In contrast, stimulation failed to affect the phospholipid or protein contents of the cerebellar slices (Table I). Superfusion alone did not affect phospholipid levels in either tissue (Table I).

When the striatal slices were stimulated for up to 8 periods, ACh release continued at unchanged rates (Fig. 2), the tissues liberating during the total 340 min an amount of ACh equivalent to about 3 times their initial ACh levels (Table III). Addition of exogenous choline (10–40 μM) to the superfusion medium enhanced ACh release, both basally and during stimulation (Table II). These increases were concentration-dependent and were statistically significant in the presence of 20 or 40 μM of choline (Table II). When the slices were superfused with a choline-free solution, their total phospholipid contents (examined initially and after 2, 4, 6 or 8 stimulation periods) declined at the rate of 2.7% per stimulation period (Fig. 3). This decrease was highly correlated with the number of stimulation periods ($r$ = −0.98; $P$ < 0.0001). Addition of exogenous choline (10–40 μM) to the superfusion medium partially or completely protected the slices from phospholipid depletion, for as many as 6 stimulation periods (Fig. 3; Table III). Moreover, the phospholipid contents of the slices were actually increased after two periods of electrical stimulation in the

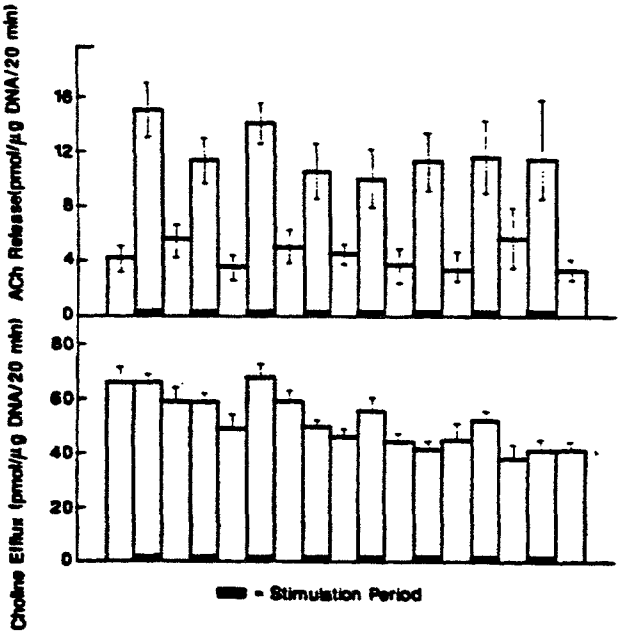


Fig. 2. ACh release and choline efflux from rat striatal slices during repeated rest–stimulation cycles. Slices of rat striatum were superfused (0.6 ml/min) with a choline-free physiological solution. After a 20 min equilibration period, the superfusate was collected for 20 min; the slices were then stimulated electrically (15 Hz) for 20 min after which they were allowed to rest for 20 min. This rest–stimulation cycle was repeated a total of 8 times. ACh release and choline efflux are expressed as pmol/period, corrected per μg of slice DNA. Each bar is the mean of 12 (ACh) or 6 (choline) separate experiments. Vertical bars represent S.E.M.

TABLE II

*Effect of choline on ACh release from the striatal slices*

Slices of rat striatum were superfused with a choline-free or choline (10–40 μM)-containing physiological solution. After a 20 min equilibration period, superfusates were collected for 20 min, and then the slices were stimulated electrically (15 Hz) for 20 min. This rest–stimulation cycle was repeated a total of 8 times, and superfusates collected during each period were assayed for ACh. Basal ACh release is expressed as total ACh (pmol) recovered from the superfusates collected during the 9 rest periods (9 × 20 min), corrected for the DNA contents of the slices. Data are given as means ± S.E.M. Numbers of determinations are shown in parentheses.

| *Choline concentration (μM)* | *ACh release* | |
|---|---|---|
| | *Basal* | *Evoked* |
| 0 (11) | 39 ± 8 | 107 ± 16 |
| 10 (6) | 56 ± 13 | 149 ± 37 |
| 20 (6) | 81 ± 9* | 239 ± 49* |
| 40 (7) | 139 ± 12** | 406 ± 37** |

*$P < 0.05$; **$P < 0.001$: significantly differs from the corresponding value obtained using the choline-free solution.

presence of 40 μM choline (Table III).

The major phospholipids in brain membranes are, besides PC, phosphatidylserine (PS) and phosphatidylethanolamine (PE); together, these 3 phosphatides account for 85% of total brain phospholipids[1]. Moreover, the choline in PC represents about 80% of the total membrane-bound choline in the brain[1], suggesting that PC would be the cellular phospholipid reservoir most likely to provide free choline for ACh synthesis. To determine whether the reduction in total brain phospholipids observed when stimulated slices were superfused without choline represented PC exclusively or also included other brain phospholipids, we fractioned and quantified these compounds in tissues obtained before or after 2, 4, 6 and 8 stimulation periods.

Stimulation in the choline-free medium was associated with decreases not only in membrane PC but also, proportionately, in PE and PS (Table III). After 4 (or 8) stimulation–rest periods, the levels of total phospholipid, PC, PE, or PS were 83.2 ± 3.3 (or 77.5 ± 3.9%), 89 ± 5.2 (or 76.6 ± 2.5%), 84.7 ± 7.7 (or 77.3 ± 2.4%) and 89.2 ± 2.3 (or 78.2 ± 1.0%) of initial values, respectively. In 7 separate perfusion studies, the ratio of PC to the total phospholipid content [or to the sum of the 3 main structural phospholipids (PC + PE + PS)] was 0.33 ± 0.04 [or 0.41 ± 0.05] initially and 0.34 ± 0.04 or [0.43 ± 0.08], subsequent to 8 stimulation periods. Addition of choline to the superfusion medium protected the slices from the declines in the 3 phosphatides, i.e., PE and PS as well as PC (Fig. 3; Table III). The protective effect of choline on each phosphatide was apparently concentration-dependent. At 20 μM, none of their levels declined even after as many as 6 stimulation periods. Protection was partial and occurred for up to as many as 2–6 stimulation periods in the presence of 10 μM choline (Table III). Addition of 40 μM choline to the superfusion medium actually increased slice levels of PC, PE and PS by 19%, 24% and 23%, respectively (Table III), after two periods of electrical stimulation (i.e., compared with those in controls obtained at the beginning of superfusion). The small but significant depletion of protein caused by stimulation was also blocked by adding choline to the superfusion medium (Table III).

We examined the relationships between the net

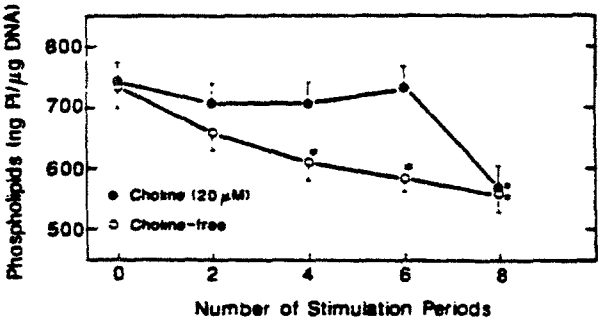


Fig. 3. Effect of electrical stimulation with or without choline on total phospholipid contents of rat striatal slices. Slices of rat striatum were superfused in a physiological solution containing no choline (open circles) or 20 μM choline (closed circles) and stimulated electrically as described in Fig. 2. Samples of the slices were removed, for determination of phospholipid phosphate, 20 min before the first stimulation and 10 min after the 2nd, 4th, 6th and 8th stimulation periods. Phospholipids were extracted and quantitated by a phosphate assay, and corrected for the DNA contents of the slices. Each point represents the mean of 8–14 determinations. Vertical bars represent S.E.M. Asterisks indicate the significance of differences (ANOVA with repeated measures followed by Tukey's procedure for pairwise comparison) from the corresponding initial (0 stimulation) values. Linear regression analysis of the data indicate an inverse relationship between the number of stimulation periods and the phospholipid contents of the slices when superfused with the choline-free medium ($y = -20.5x + 717$; $r = -0.98$; $P < 0.0001$).

TABLE III

*Effects of repeated electrical stimulations on choline, ACh, phospholipid and protein contents of striatal slices superfused with choline-free or choline-containing media*

Slices of rat striatum were superfused with a physiological solution containing no choline (0) or 10, 20, or 40 $\mu M$ choline and stimulated electrically as described in Fig. 2. Samples of the slices were removed, for determination of phospholipid phosphorus, choline, ACh, protein and DNA, 20 min before the first stimulation (tissue initial; TI) and 10 min after the 2nd (TF-2), 4th (TF-4), 6th (TF-6) and 8th (TF-8) stimulation periods. Data are given as means ± S.E.M. of ng P/$\mu$g DNA (phospholipids), or pmol/$\mu$g DNA (choline and ACh), or as $\mu$g/$\mu$g DNA (protein). $n$ = 4–6 tissue samples for choline and ACh, 4–7 for protein and 8–14 for phospholipids. Data were analyzed by ANOVA with repeated measures followed by Tukey's procedure for pairwise comparisons. N.D., not determined.

| *Compounds* | *Choline ($\mu M$)* | *TI* | *TF-2* | *TF-4* | *TF-6* | *TF-8* |
|---|---|---|---|---|---|---|
| Choline | | | | | | |
| | 0 | 16 ± 3 | 22 ± 6 | 16 ± 5 | 17 ± 3 | 15 ± 2 |
| | 10 | N.D. | N.D. | N.D. | N.D. | N.D. |
| | 20 | 16 ± 3 | 43 ± 8 | 34 ± 3** | 26 ± 4 | 16 ± 4 |
| | 40 | N.D. | N.D. | N.D. | N.D. | N.D. |
| ACh | | | | | | |
| | 0 | 49 ± 3 | 60 ± 13 | 42 ± 11 | 49 ± 9 | 22 ± 3* |
| | 10 | 47 ± 3 | 36 ± 8 | 37 ± 12 | 42 ± 12 | 30 ± 14 |
| | 20 | 47 ± 2 | 42 ± 4 | 45 ± 2 | 44 ± 2 | 20 ± 3* |
| | 40 | 49 ± 3 | 64 ± 14 | 54 ± 8 | 39 ± 3 | 25 ± 4* |
| Protein | | | | | | |
| | 0 | 65 ± 2 | 62 ± 2 | 61 ± 1* | 58 ± 1* | 54 ± 1* |
| | 10 | 62 ± 2 | 61 ± 2 | 63 ± 2 | 60 ± 5 | 56 ± 2* |
| | 20 | 63 ± 2 | 66 ± 7 | 57 ± 2 | 61 ± 3 | 54 ± 3* |
| | 40 | 66 ± 3 | 61 ± 5 | 63 ± 4 | 61 ± 3 | 52 ± 2* |
| Total phospholipid | | | | | | |
| | 0 | 735 ± 12 | 666 ± 41 | 621 ± 27* | 585 ± 22* | 570 ± 31* |
| | 10 | 686 ± 21 | 672 ± 46 | 650 ± 46 | 716 ± 54 | 574 ± 47* |
| | 20 | 739 ± 23 | 704 ± 37 | 704 ± 31 | 730 ± 46 | 573 ± 20* |
| | 40 | 750 ± 14 | 828 ± 22** | 726 ± 65 | 700 ± 37 | 616 ± 21* |
| PC | | | | | | |
| | 0 | 256 ± 14 | 225 ± 24* | 223 ± 20* | 222 ± 27* | 196 ± 15* |
| | 10 | 248 ± 9 | 228 ± 10 | 237 ± 26 | 243 ± 16 | 206 ± 19* |
| | 20 | 246 ± 27 | 267 ± 12 | 247 ± 15 | 241 ± 8 | 210 ± 8* |
| | 40 | 248 ± 15 | 295 ± 29** | 264 ± 12 | 292 ± 18** | 209 ± 14* |
| PE | | | | | | |
| | 0 | 248 ± 8 | 206 ± 8* | 203 ± 12* | 201 ± 21* | 187 ± 18* |
| | 10 | 243 ± 19 | 184 ± 30 | 186 ± 11* | 204 ± 24 | 171 ± 13* |
| | 20 | 251 ± 18 | 240 ± 20 | 243 ± 11 | 245 ± 32 | 175 ± 34* |
| | 40 | 242 ± 17 | 304 ± 32** | 232 ± 9 | 258 ± 24 | 199 ± 18* |
| PS | | | | | | |
| | 0 | 97 ± 8 | 92 ± 8 | 83 ± 8* | 78 ± 6* | 77 ± 8* |
| | 10 | 92 ± 4 | 86 ± 14 | 84 ± 13 | 85 ± 6 | 81 ± 12 |
| | 20 | 95 ± 10 | 98 ± 4 | 99 ± 7 | 92 ± 7 | 82 ± 5* |
| | 40 | 92 ± 6 | 117 ± 6** | 105 ± 5 | 104 ± 6 | 83 ± 6* |

Significantly lower (*$P < 0.05$) or higher (**$P < 0.05$) than corresponding TI value.

loss in PC from a tissue and the recovery of choline (as free choline + ACh) from its superfusate, using data from 6 separate perfusion studies (Table IV). One pmol of choline recovered from the superfusate was associated with the loss of 5 pmol of total phospholipid, or 1.6 pmol of PC (Table IV). The net losses in PE and PS were also proportional to their tissue contents.

## DISCUSSION

These data show that striatal slices continue to make large amounts of ACh when superfused with a choline-free solution (Figs. 1 and 2). Under these conditions, tissue contents of PC and other structural membrane phospholipids decrease significantly (Fig. 3, Table III). Addition of increasing concentrations

(10–40 μM) of choline to the superfusion medium increases the release of ACh both at rest and during electrical stimulation, in proportion to the choline concentration (Table II). Moreover, the exogenous choline also protects the slices from losses of PC, PE, PS, and protein (Table III).

These results confirm and extend recent reports from our laboratory[26] showing that ACh release is enhanced by superfusing striatal slices with choline, both during electrical stimulation and at rest. Increases in ACh release in the presence of exogenous choline have also been described in brain slices incubated with high potassium concentrations, which depolarize the neurons, but not in slices at rest[28,50]. This discrepancy may result, in the latter studies, from the accumulation of exogenous choline, liberated from the slices, in the incubation media; i.e., this endogenous choline may have raised effective tissue choline concentrations well above those at which choline acetyltransferase was capable of responding to additional choline. (Choline concentrations in the media reportedly rise to 20–40 μM, causing slices to accumulate very high free choline concentrations[4,28,49,50].) Under our experimental conditions, the liberation of choline from the slices elevated choline concentrations in the superfusate only to 0.5 μM, or even less; and the choline contents of the slices (Table III) did not exceed those usually seen in vivo.

Brain tissue is known to produce and release free choline in vivo[8,12,36] and during incubation in vitro[4,10,15,21,22,26,45,46,50,52]. In both situations, the source of the choline appears to be membrane phospholipids. When we superfused striatal slices in a choline-free medium, the slices lost a fraction of their PC, as well as of their PE and PS, during the course of the experiments (Table III). During the same period, the amount of ACh plus choline recovered in the superfusate was similar to the amount of choline lost from membrane PC (Table IV) but several orders of magnitude larger than the intracellular pools of choline and ACh (Table III).

The release of choline from a lipid-bound form can occur in all areas of the brain[22]: in vitro, the process is, apparently, more active in the striatum, which is rich in cholinergic activity, than in cortex[21] or cerebellum (Fig. 1). Other studies have suggested, however, that the release of tissue choline from a bound form is not related to the extent of its cholinergic innervation[15,22]. Also, as shown in the present study, the reductions in the PC and other membrane phospholipids were not simply related to the production of free choline but apparently also depended on an increased demand for free choline to sustain ACh synthesis. When the striatal slices were superfused with a choline-free medium at rest, they still released a large amount of free choline into the medium without reducing their phospholipid contents (Table I); in contrast, when stimulated electrically, they released slightly more choline and much more ACh (Figs. 1 and 2), and in that circumstance, depleted PC and other membrane phospholipids in proportion to the duration of stimulation (Fig. 3, Table III) and thus to the total amounts of ACh released. Moreover, when slices were provided with adequate free choline and stimulated electrically, they produced and released even *more* ACh (Table II), but without depleting their membrane of PC and other phosphatides (Fig. 3, Table III). Electrical stimulation failed to alter the phospholipid contents of the cerebellar slices (Table I), a tissue in which stimulation was not associated with ACh release.

Consistent with the view that neuronal PC is a reservoir of choline for ACh synthesis and that this

TABLE IV

*Relationships between the losses of phospholipids and of choline from striatal slices superfused with a choline-free medium and stimulated repeatedly*

Slices of rat striatum were superfused with a choline-free solution and stimulated electrically as described in Fig. 2. Losses in striatal phospholipids were expressed as the differences between their contents before and after the 8 stimulation periods (assuming that 31 pg P are equal to 1 pmol phospholipid). The net loss of choline from the slices was calculated by adding the total amounts of free choline and ACh recovered from the superfusates collected during the 9 rest and 8 stimulation periods. Data are given as means ± S.E.M. of 6 determinations.

| *Compounds* | *Net loss (pmol/μg DNA)* | *Ratio to choline* |
|---|---|---|
| Choline | 1050 ± 45 | |
| Total phospholipid | 5510 ± 669 | 5.05 ± 0.61 |
| PC | 1670 ± 281 | 1.57 ± 0.25 |
| PE | 1790 ± 422 | 1.68 ± 0.41 |
| PS | 620 ± 144 | 0.62 ± 0.21 |

reservoir can be depleted when more choline is needed for ACh synthesis, we find that the phospholipids contents of electrically-stimulated striatal slices do not decline if the stimulation-induced release of ACh is blocked by tetrodotoxin (unpublished observation), or if the cholinesterase inhibitor eserine is omitted from the superfusion medium[42]. (When cholinesterase is active, the breakdown of intrasynaptic ACh generates free choline that can be reused for ACh synthesis; this probably reduces the need for membrane PC to provide free choline.) The possibility that membrane constituents are depleted when neuronal tissues lack adequate free choline is supported by other data showing that PC levels and the number of synaptic vesicles in the cat's superior cervical ganglion fell significantly with stimulation of the preganglionic nerve trunk if the uptake of exogenous choline was blocked by HC-3[31], or if the ganglia were perfused with a choline-free Locke solution[32]. The vesicles reappeared immediately if the stimulated ganglia were provided with choline for 2 min[32].

The increase in ACh release associated with the increase in the free choline concentration of the superfusion medium was not accompanied by an increase in tissue ACh content (Table III). Superfusion with a choline-free medium also failed to alter the ACh contents of the slices during 6 stimulation-rest cycles (Table III), even though very large amounts of ACh were released into the medium during this period (Table II). However, the amounts of ACh that striatal slices released into the medium at rest or during electrical stimulation were highly correlated ($r = 0.99$; $P < 0.001$) with the choline concentration of the superfusion medium (Table II). Thus, cholinergic neurons may contain a regulatory mechanism which, perhaps by decreasing or increasing the amounts of ACh released into the medium, keeps intracellular ACh levels more or less constant. This mechanism might explain why increases in brain ACh levels have been an inconstant finding following choline administration, while increases in ACh release have been observed more consistently (see below).

The protective effect of exogenous choline on the stimulus-induced depletion of membrane phosphatides depends on its concentration. Choline (20 $\mu$M) in the superfusion medium completely protected the slices from phospholipid depletion for as many as 6 stimulation periods (Fig. 3; Table III), while 10 $\mu$M was less effective (Table III). In the presence of 40 $\mu$M choline, the phospholipid contents of the slices sometimes actually increased (Table III). After 4 stimulation-rest cycles, the levels of total phospholipids and of PC or PS in the striatal slices were highly ($r = 0.91$, 0.99 or 0.94, respectively) and significantly ($P < 0.001$) correlated with the choline concentration in the medium. It has recently been shown that the activities of choline kinase (which catalyzes the phosphorylation of choline to form phosphocholine in the first step of PC biosynthesis via the cytidyl-diphosphocholine pathway) and of choline acetyltransferase in superior cervical ganglia show reciprocal fluctuating changes as a function of the extracellular choline concentrations in the incubating medium[2]. Neuronal depolarization diminishes the activity of choline kinase and increases that of choline acetyltransferase[2], thus possibly shunting the choline into ACh synthesis. However, if the choline concentrations in the medium were 40–50 $\mu$M, neuronal depolarization diminished choline acetyltransferase activity and increased that of choline kinase[2]. Hence, the main determinant of the choline's actual fate in cholinergic neurons may be the relative activities of choline kinase and choline acetyltransferase which, in turn, depend upon extracellular choline concentrations. It has been shown that giving supplemental choline to laboratory animals can increase brain levels of phosphocholine[29], total phospholipids[45,46], and PC[33,45,46] while a choline-deficient diet significantly decreases PC levels[33]. Interestingly, the 19% increase in PC levels observed in slices superfused with 40 $\mu$M choline was associated with a 10% increase in total phospholipids (Table III). Our present results, taken with these in vivo observations, suggest that PC levels and even, conceivably, the total amounts of membrane in cholinergic neurons may be choline-dependent.

The above data are compatible with the view that PC's degradation provides choline for acetylcholine synthesis, and that this process is accelerated when neuronal activity is increased, thereby decreasing tissue phospholipid contents. However, they are also consistent with a slowing of PC synthesis, perhaps secondarily suppressing the incorporation of PS, PE and protein into membranes. Little information is

available about the processes that might mediate the accelerated PC breakdown or slowed PC synthesis. We have recently shown that PC in a human cholinergic cell line contributes choline used for acetylcholine synthesis[6]. However, in striatal slices it is likely that the decrease in PC and other major membrane phospholipids also reflects changes in membranes of non-cholinergic cells, perhaps in synaptic contact with cholinergic neurons. ACh reportedly can increase the availability of free choline by a mechanism mediated by muscarinic receptors[8,11].

In vivo, circulating, and presumably, extracellular choline levels are about 10 $\mu$M in fasting animals and humans[40] but can rise to 30–40 $\mu$M after ingestion of PC-rich foods[25,53]. Administration of choline to laboratory animals elevates plasma and brain choline levels, and sometimes[18,29], but not always[48,49], brain ACh; it also enhances ACh release[26] and can provide postsynaptic changes suggestive of enhanced cholinergic neurotransmission[19,41,43,44,47]. Our present data indicate that elevating choline concentrations from the fasting range (10 $\mu$M) to 20–40 $\mu$M enhances the release of ACh (Table II) without changing its tissue levels (Table III). Steady-state brain ACh concentrations are known to decrease when the activity of cholinergic neurons is increased pharmacologically, e.g., after administration of atropine, pentylenetetrazol, or fluphenazine[20,35,45–47]. Choline, administered either as the salt or as PC, prevents these decreases[20,35,45–47]. Moreover, choline administration augments cholinergic transmission during increased cholinergic activity[44]. The most parsimonious conclusion from these data is that under conditions of stimulated ACh release the normal supply of choline may not keep up with the demand for ACh synthesis, unless it is supplemented by giving choline or PC but that the increased levels of choline in the extracellular medium after choline treatment can be utilized to support ACh synthesis. In this way, cholinergic transmission can increase while steady-state ACh concentrations are protected from drastic changes.

In the absence of supplemental choline, extracellular free choline concentrations (10 $\mu$M) may not be sufficient to sustain membrane phospholipid levels in frequently firing cholinergic neurons. This propensity might be amplified among people taking cholinesterase inhibitors[42], or in old subjects with diminished choline transport across the blood–brain barrier[30].

Cholinergic neurons are unique in that they alone use choline for two purposes, i.e., the syntheses of their neurotransmitter, ACh, and of phospholipid constituents of their membranes. The selective vulnerability of certain cholinergic neurons in neurodegenerative disorders involving the brain (e.g., Alzheimer's disease) or motor neurons may result from this property, i.e., the over-utilization or slower production of membrane PC and the shunting of the choline towards ACh synthesis[51]. Consistent with this conclusion are recent reports describing elevations in the levels of phospholipid breakdown products within brains of untreated patients suffering from Alzheimer's disease[3,27].

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