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Research report

Choline's phosphorylation in rat striatal slices is regulated by the activity of cholinergic neurons

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Abstract

The mechanism by which populations of brain cells regulate the flux of choline (Ch) into membrane or neurotransmitter biosynthesis was investigated using electrically stimulated superfused slices of rat corpus striatum. [*Me*-^{14}C]Ch placed in the superfusion medium for 30 min during a 1-h stimulation period was incorporated into tissue [^{14}C]phosphorylcholine (PCh) and [^{14}C]phosphatidylcholine (PtdCh). Stimulation also caused a profound inhibition of PCh synthesis and a 10-fold increase in [^{14}C]ACh release into the medium; it failed to affect tissue [^{14}C]ACh levels. This effect was not explained by changes in ATP levels nor in the kinetic properties of Ch kinase (E.C. 2.7.1.32) or Ch acetyltransferase (ChAT) (E.C. 2.3.1.7). To investigate the mechanism of these effects, Ch uptake studies were performed with and without hemicholinium-3 (HC3), a selective inhibitor of high affinity Ch uptake. A two-compartment model accurately fit the observed data and yielded a K_m for Ch uptake of 5 μM into cholinergic structures and 72 μM into all other cells. Using this model it was estimated that cholinergic neurons account for 60% of observed uptake of Ch at physiologic Ch concentrations, even though they represent fewer than 1% of the total cells in the slice. The model also predicts that an increase in Ch uptake within cholinergic neurons, reported to be associated with depolarization [4,27,32], would significantly inhibit Ch uptake into all other cells, and would account for the observed decrease in PCh synthesis.

Keywords: Acetylcholine; Choline; Choline uptake; Phosphatidylcholine; Phosphocholine; Phospholipid; Choline acetyltransferase; Choline kinase; Rat striatum

1. Introduction

All cells utilize choline (Ch) as a precursor for phosphatidylcholine (PtdCh), a major phospholipid component. However only cholinergic neurons also acetylate Ch to form the neurotransmitter acetylcholine (ACh). The enzyme Ch kinase catalyzes the phosphorylation of Ch, the initial step in the most prominent route of brain PtdCh synthesis, the cytidyldiphosphocholine (CDP-Ch) pathway [2,3,14,30]. Cholinergic neurons uniquely express the enzyme choline acetyltransferase (ChAT) which catalyzes the synthesis of ACh. Numerous studies suggest that Ch levels can be rate limiting for ACh synthesis [6,20,22,38,39,43] and that brain PtdCh constitutes a reservoir of Ch that can be used for ACh synthesis [16,20,24,36]. Earlier work from this laboratory had shown that stimulated brain slices maintain ACh release at the expense of membrane PtdCh levels (the reduction in PtdCh could be prevented by exogenous Ch) [36]. The factors that regulate the flux of brain Ch into phospholipids or acetylcholine remain unclear.

A mechanism that might regulate both PtdCh and ACh synthesis was proposed by Ando et al. [1] based on experiments in which the relative activities of Ch kinase and ChAT were dependent upon extracellular Ch levels [1]. A limitation of this study was the lack of data demonstrating that the alterations in enzyme kinetics observed in the homogenized tissue preparation correlated with changes in the synthesis of ACh or PtdCh in the intact preparation.

Alternatively, regulatory control of Ch uptake into cholinergic neurons but, not other brain cells, might obviate the need to control Ch's utilization by altering the kinetic properties of ChAT and Ch kinase. To facilitate the synthesis of ACh, cholinergic neurons express a high affinity Ch uptake transporter that has an approximately a 10-fold greater affinity for Ch than that observed in any

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other cells [12]. In numerous preparations it has been shown that neuronal activity enhances this Ch uptake [4,27,32]. However, the effect of this enhancement on Ch's availability to other than cholinergic neurons has not been examined. This is especially important in more physiologic preparations, like brain slices, that contain multiple cell types.

We have utilized superfused slices of rat corpus striatum to study the relationships between neuronal activity and ACh synthesis in cholinergic cells, and the metabolism of PCh and PtdCh synthesis in all other cells. This preparation contains intact cholinergic interneurons (dendrites, cell bodies and axons) and not simply cholinergic terminals. Ch metabolism was studied by: (1) labeling the slices with [^{14}C]Ch under various conditions, and subsequently examining the accumulation of [^{14}C]Ch-containing compounds (Ch, ACh, PCh, and PtdCh) and (2) examining the kinetics of Ch uptake, Ch kinase and ChAT.

2. Materials and methods

[*Me*-^{14}C]Ch chloride (53 Ci/mol), [*Me*-^{3}H]Ch chloride (86.7 Ci/mmol) and [*Acetyl*-^{3}H]acetyl coenzyme A (AcCoA) (200 Ci/mol) were purchased from DuPont – New England Nuclear. Unless otherwise noted, all chemicals were purchased from Sigma Chemical (St. Louis, MO).

2.1. Animals, slices, chambers and stimulating system

Experiments utilized male Sprague–Dawley rats (Charles River) weighing 250–300 g and exposed to a 12/12 h light/dark cycle. The animals were given free access to water and food and treated in accordance with the guidelines established by the MIT Committee on Animal Care. Animals were anesthetized with ketamine (85 mg/kg body weight i.m.) and decapitated, and slices were prepared from striata as previously described [7]. The slices were loaded into chambers (Warner Instruments, CT) that were maintained at 37°C [7]. In some experiments slices were first incubated (37°C) for 7 min in 1 ml of buffer containing 5 μCi [*Me*-^{3}H]Ch chloride (56 nM from the label and 0.1–1.0 μM Ch released from the tissue), then washed with buffer containing 100 μM Ch prior to chamber loading. Chambers were continually superfused with warmed, oxygenated Krebs–Ringer buffer [7] containing eserine salicylate (20 μM). In all experiments, slices were superfused (0.8 ml/min) for either 45 min (pulse-chase studies) or 60 min (all other studies) prior to the start of sample collection and/or stimulation.

At the completion of each experiment, slices were placed in ice cold buffer which was then carefully removed and replaced with 1 ml of a second buffer (230 mM sucrose, 1 mM $ZnSO_4$ and 20 mM Hepes pH 7.4). The tissue was then homogenized (10 × Teflon/glass) and 1 ml of methanol was added to the mixture. In experiments were enzyme activities were to be determined, methanol was omitted and instead homogenates where rapidly frozen (−70°C).

2.2. Analysis of tissue homogenates

For the extraction of lipids and choline-containing compounds, 1.0 ml of homogenate was mixed with 2 ml of chloroform [8]. To determine the radioactivities of water-soluble Ch-containing compounds, concentrated samples of the aqueous fraction were separated by HPLC and counted using an on-line radioactivity monitor [17]. For the determination of ACh and Ch mass, aliquots of dried aqueous phase were resuspended in water and subjected to HPLC analysis using an immobilized post-column enzyme reactor containing Ch oxidase and ACh esterase (BAS). ATP, ADP and AMP levels were determined as described [26].

To measure tissue contents of PCh, the PCh faction was collected and treated with alkaline phosphatase (0.3 U/tube) for 3 h, then centrifuged with methanol. Samples were then dried under vacuum, resuspended in HPLC buffer and subjected to HPLC analysis for Ch (immobilized post-column enzyme reactor system). In some experiments, water-soluble Ch-containing compounds were separated by thin-layer chromatography [28] and scraped into scintillation vials. Total lipid phosphate was determined from a dried aliquot of the organic phase by perchloric acid digestion [31]. Individual phospholipids were separated by thin-layer chromatography [34]. The individual phospholipids were scraped off the plates and assayed for total phosphorous content and/or radioactivity.

2.3. ChAT and Ch kinase assays

ChAT was assayed according to the method of Fonnum [9]. Briefly, 5 μl of tissue homogenate (20 μg) were mixed with 10 μl of ice-cold reaction buffer (in mM: 2 AcCoA, 20 EDTA, 2 eserine, 50 $NaH2PO_4$, 300 NaCl) with 7.5 μCi/ml of [*Acetyl*-^{3}H]AcCoA and varying amounts of Ch. Ch kinase was assayed using a modification of the method of Spanner and Ansell [29]. A reaction buffer was prepared (final concentration in mM: 40 $MgCl_2$, 0.1 PCh, 3 ATP, 10 Hepes pH 7.4). Typically, 2.5 μCi of [*Me*-^{14}C]Ch/ml was added to the reaction buffer. To determine the kinetic properties of Ch kinase the final Ch concentration was varied. The reaction was initiated by the addition of 40 μl of reaction buffer to 10 μl of tissue homogenate (20 μg) and the tubes were incubated at 37°C. After 10 min the reaction was stopped by the addition of 0.5 ml of 50% v/v of methanol, followed by 1 ml of 0.001 HCl.

Labeled Ch was separated from PCh by retention on a weak cation exchange resin (0.5 × 7.0 cm Amberlite CG-50, 200–400 mesh). The resin was rinsed approximately

20 × with water to remove fine particles. The sample and then a wash of 0.9 ml of water were applied to columns. The entire eluate was collected into a scintillation vial and counted.

2.4. Analysis of superfusates

After the slices had equilibrated, superfusion fluids from each chamber were collected in ice-cold tubes. The amounts of ACh and Ch released were determined by HPLC analysis of 0.2 ml of each such fluid. In samples that contained 5 μM Ch and 0.25 μCi/ml of Ch it was not possible to separate the ACh from the Ch on either HPLC system. To circumvent this problem the Ch levels in the fluids were reduced by greater than 100-fold by two purification steps. Superfusion fluids (1 ml) were first applied to a silica column to elute mostly ACh [36]. The eluates from these columns were dried and subject to thin-layer chromatography as described above. Samples scraped from the plate were either counted or analyzed by HPLC for Ch or ACh mass. Total protein and LDH activity in each fluid were determined as previously described [7].

2.5. Normalization and data analysis

All measurements were normalized to total tissue protein. Data are expressed as means ± S.E.M. All time-course data were analyzed by ANOVA with repeated measures and/or with a post-hoc Newman–Keuls test. *t*-Tests were used to compare the effects of stimulation at a given time point.

3. Results

3.1. Effect of electrical stimulation on ACh release and slice viability

Electrical stimulation of slices superfused without Ch was associated with sustained increase in ACh release. Basal secretions during the initial three 20-min collection periods were 0.40 ± 0.03, 0.38 ± 0.06 and 0.40 ± 0.05 nmol/mg/h; stimulated slices released 4.04 ± 0.54, 3.61 ± 0.35 and 3.24 ± 0.28 nmol/mg/h (evoked release was significantly different than basal release at all time points, $P < 0.01$). Ch release was unaffected by stimulation or by the duration of superfusion: basal secretions were 3.78 ± 0.14, 3.94 ± 0.07 and 4.20 ± 0.06 nmol/mg/h; those from stimulated slices were 4.86 ± 0.47, 4.38 ± 0.27 and 3.88 ± 0.56 nmol/mg/h.

Stimulation failed to affect the release of LDH activity, a marker of cell injury [15]: activities in media from control and stimulated slices were 0.44 ± 0.05 mU/mg and 0.49 ± 0.06 mU/mg respectively. The total LDH activity released into the media was only 0.04% of that

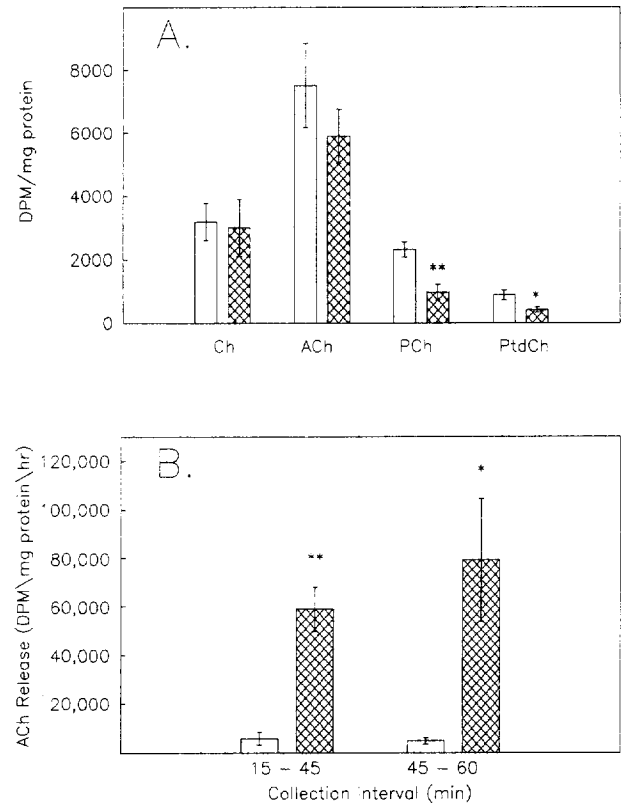

Fig. 1. Effect of electrical stimulation on levels of [*Me*-^{14}C]Ch-containing compounds in tissue slices and media. A: striatal slices were stimulated (4.95 mA/mm^2, 1 ms and 15 Hz) for 1 h: between the 15th and 45th min they were also exposed to [*Me*-^{14}C]Ch (0.25 μCi/ml; 4.7 μM). Bars indicate levels of [*Me*-^{14}C]Ch-containing compounds in stimulated (hatched) and control (open) slices. Values represent means ± S.E.M. from groups of eight rats; * $P < 0.01$; ** $P < 0.001$ (values significantly different from unstimulated values). B: media from these tissues were collected and assayed for labeled ACh. (Media collected from the first 15 min were excluded since the label was not present during this inverval.) Values represent means ± S.E.M. from four rats; * $P < 0.05$; ** $P < 0.01$ (values significantly different from unstimulated values).

present in the tissue at the end of the experiment. Stimulation also failed to affect either tissue protein levels or protein release into the medium (approximately 5% of total tissue protein).

3.2. Effect of stimulation on incorporation of [Me-^{14}C]Ch into Ch-containing compounds

To determine if Ch utilization is altered by stimulation, [*Me*-^{14}C]Ch was added to superfusion media of striatal slices, between the 15th and the 45th min of the 1-h stimulation period, and the slices were assayed at the end of that period. Stimulation reduced the accumulation of labeled Ch as PCh (from 2320 ± 236 to 971 ± 256 d.p.m./mg) and as PtdCh (from 99.1 ± 14.1 to 35.3 ± 8.9 d.p.m./mg) without affecting the labeling of tissue ACh or Ch (Fig. 1a). Stimulation increased the release of [^{14}C]ACh during the half-hour labeling period and the subsequent

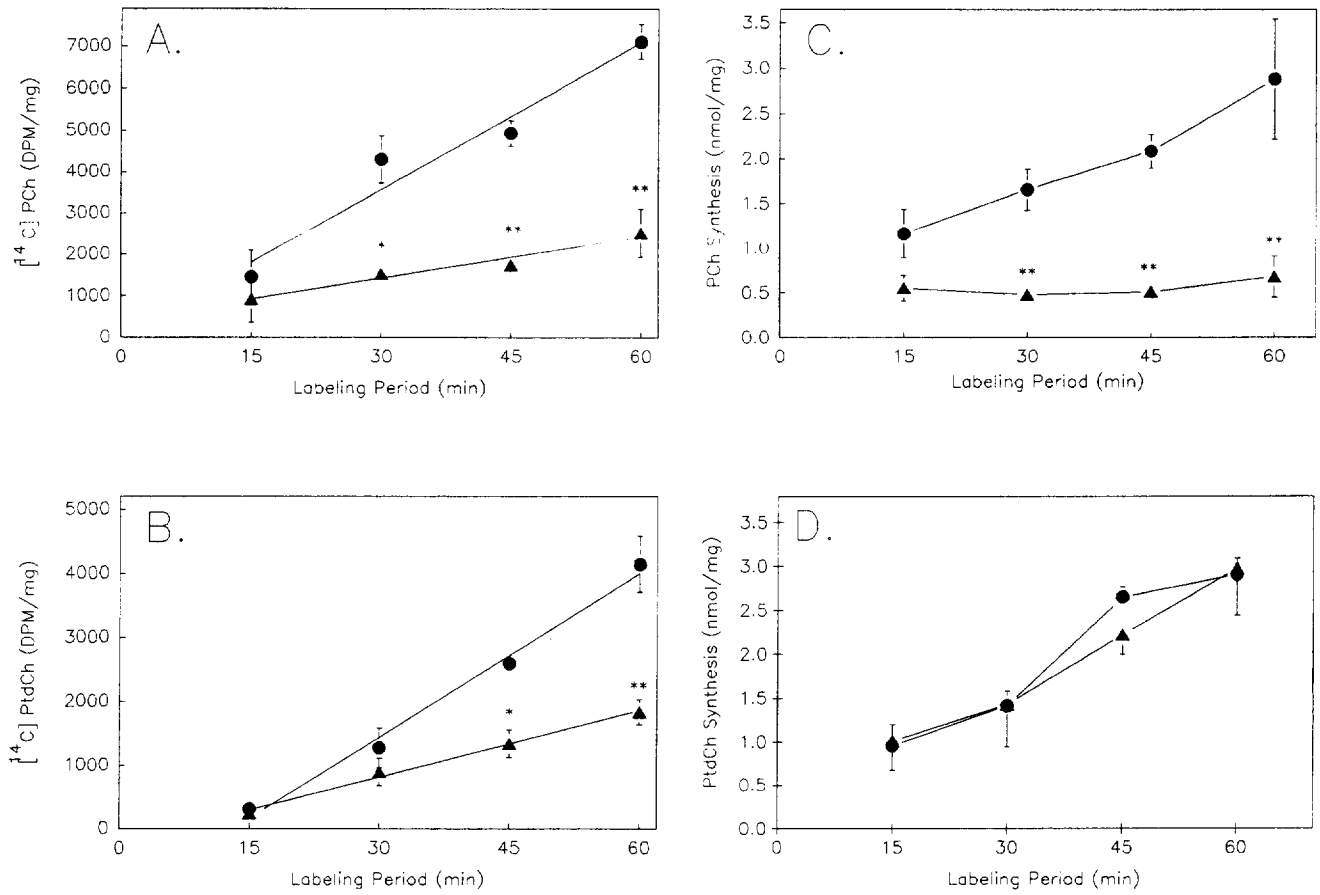

Fig. 2. Effect of stimulation on [^{14}C]PCh and [^{14}C]PtdCh levels and synthesis. Both striata were dissected, divided into multiple slices, and incubated with (triangles) or without (circles) stimulation as in Fig. 1 except that [*Me*-^{14}C]Ch (0.25 μCi/ml; 4.7 μM) was present throughout the stimulation period. Slices were taken at designated intervals and assayed for labeled and unlabeled PCh and PtdCh. A: levels of [^{14}C]PCh. B: levels of [^{14}C]PtdCh. C: estimated synthesis of PCh calculated by adding the radioactivity in PCh and PtdCh and dividing this sum by the specific activity of tissue Ch determined in each sample. D: estimated synthesis of PtdCh calculated by dividing the radioactivity in PtdCh by the specific activity of tissue PCh determined from the total tissue PCh radioactivity and the total PCh mass determined in a different set of samples that had been treated identically. In all panels: filled circles represent control slices, filled triangles designate stimulated slices, values represent means ± S.E.M. from four rats. * $P < 0.05$; ** $P < 0.01$ (values significantly different from corresponding controls at the same time point).

Table 1
Specific activities of Ch-containing compounds labeled by superfusion with [^{14}C]Ch. Specific activities for Ch, PCh and tissue ACh were calculated from measurements of mass and radioactivity as described in Fig. 2. Specific activities for released ACh were calculated from the data described in Fig. 1. Data represent means ± S.E.M. from four rats

Compound	Treatment	Specific activity of choline-containing compounds (d.p.m./nmol)			
		Labeling time (min)			
		15	30	45	60
Ch	Control	1860 ± 150	3350 ± 170	3670 ± 280	4750 ± 1260
	Stimulation	2580 ± 210 [a]	5440 ± 690 [a]	6000 ± 670 [a]	5410 ± 1400
ACh	Control	1140 ± 240	2640 ± 170	3500 ± 250	8370 ± 3030
	Stimulation	3530 ± 130 [a]	8360 ± 1150 [a]	21130 ± 6610 [a]	9420 ± 2700
PCh	Control	388 ± 119	862 ± 115	987 ± 63	1423 ± 83
	Stimulation	268 ± 105	424 ± 64 [a]	484 ± 59 [a]	701 ± 164 [a]
ACh	Control	–	15200 ± 6670 [b]	12340 ± 3320 [c]	–
Release	Stimulation	–	14610 ± 2250 [b]	18830 ± 7670 [c]	–

[a] Significantly different from control values.
[b] Release collected from the first 30 min after the influx of label.
[c] Release collected from 30–45 min after the influx of label.

wash period by 10–15 fold (Fig. 1b). We were unable to detect any radioactivity in either CDP-Ch or in the PtdCh degradation product, glycerophosphocholine (GPCh).

3.3. Effect of stimulation on tissue Ch and ACh levels and on [^{14}C]Ch incorporation into tissue PCh and PtdCh

Concomitant measurements of Ch and ACh mass and label were made at 15-min intervals in stimulated and unstimulated slices continuously perfused with [*Me*-^{14}C]Ch. Tissue Ch decreased over the course of the experiment ($P < 0.001$) and was significantly reduced by stimulation at all time points ($P < 0.05$) consistently decreasing by about 20%. After the first interval Ch was decreased from 2360 ± 90 to 1930 ± 140 pmol/mg ($P < 0.05$); after the last, Ch levels were 1840 ± 120 pmol/mg in control and 1370 ± 140 pmol/mg ($P < 0.05$) in stimulated slices. Tissue ACh levels were also decreased by stimulation after 15 min (from 1940 ± 180 to 1310 ± 100 pmol/mg; $P < 0.05$) and remained stable thereafter.

PCh labeling in these slices was inhibited by stimulation, an effect that was significantly more pronounced over time ($P < 0.01$) (Fig. 2a). PtdCh labeling followed the same pattern as PCh. After 1 h, [^{14}C]PtdCh levels were 56% lower in stimulated than in control slices (Fig. 2b).

3.4. Effect of stimulation on the specific activities of Ch-containing compounds

The mass and radioactivity measurements from the preceding experiments were used to calculate the specific activities of tissue Ch in stimulated and unstimulated slices. Stimulation increased the specific activity of tissue [^{14}C]Ch over the course of the experiment (Table 1). The synthesis of PCh was determined by adding the radioactivity in PtdCh (it was assumed that the precursor of [^{14}C]PtdCh was [^{14}C]PCh) to the total radioactivity in PCh and dividing by the specific activity of Ch at each time interval. Net PCh synthesis was 70% lower in stimulated slices over the 1-h test period (Fig. 2c). Using PCh mass measurements from parallel experiments described below, we determined the specific activity of PCh and found it to be significantly reduced by (51%) after 30, 45 and 60 min of stimulation (Table 1). These reductions were associated with reductions in PtdCh labeling, so that when total PtdCh synthesis was calculated based on the specific activity of PCh, no effect of stimulation on net PtdCh synthesis during the 1-h test period (Fig. 2d).

A pattern consistent with a precursor/product relationship was evident in the specific activities of Ch and PCh. The ratio of the specific activity of PCh to the specific activity of its precursor, Ch was less than one, and increased over time only from 0.21 to 0.30 (Table 1). Unlike PCh synthesis, ACh synthesis utilizes a pool of Ch that was different from the total slice Ch pool. The specific activity of ACh in stimulated slices was significantly greater than that for tissue Ch (Table 1). Likewise, released ACh had a higher specific activity that measured in the tissue.

3.5. Effects of stimulation on PtdCh and PCh turnover

A pulse-chase paradigm was used to study possible effects of stimulation on the disappearance of newly-formed PCh and PtdCh from the brain slices. Slices were equilibrated, incubated for 7 min with [*Me*-^{3}H]Ch, washed, placed in the chambers, harvested at various time intervals, and assayed. [^{3}H]PCh levels significantly decreased over time ($r = 0.92$, $P < 0.01$) at a rate of 36% per hour; this rate was unaffected by stimulation. PCh mass, assayed from the same samples, was unchanged over the course of the experiment in control samples but decreased significantly after 30 min of stimulation from (5.13 ± 0.17 to 3.73 ± 0.44 nmol/mg; $P < 0.05$). The specific activity of PCh, calculated from these data, was unaffected by stimulation although it did significantly decrease ($r = 0.89$, $P < 0.01$) during the chase period. Likewise, there was no significant effect of stimulation on [^{3}H]PtdCh levels or its rate of synthesis (4.15 ± 0.57 and 3.00 ± 0.81 nmol/h in control and stimulated tissues respectively).

3.6. Effect of stimulation on the kinetic properties of Ch-kinase and ChAT

Stimulation had no effect on the kinetic properties of Ch-kinase and ChAT observed in tissue homogenates from control and stimulated slices. The assays were performed with varying amounts of Ch and with saturating concentrations of ATP for Ch kinase, or of AcCoA for ChAT. Both enzymes exhibited classic Michaelis–Menten kinetics. The K_ms of Ch kinase for Ch from control and stimulated slices were 7.13 ± 1.21 and 5.92 ± 0.92 μM respectively. The K_ms of of ChAT for Ch were 130 ± 30 and 155 ± 21 μM from control and stimulated slices respectively. When the homogenates were incubated for 30 min (as opposed to 10 min), the K_m for Ch of ChAT was 332 ± 56 μM, a value consistent with previous reports [1,35].

In a separate experiment, possible effects of electrical stimulation on slice ATP levels were examined to determine if a reduction in cellular ATP stores might explain the observed inhibition of Ch phosphorylation. ATP, ADP and AMP levels in stimulated tissues did not differ significantly from those in controls (ATP, 1063 ± 141 and 998 ± 255; ADP 874 ± 110 and 1073 ± 170; AMP, 1418 ± 118 and 1968 ± 313 pmol/mg from control and stimulated slices respectively). However, ATP turnover was significantly increased by electrical stimulation in as much as the ATP/ADP ratio declined from 1.17 ± 0.02 to 0.91 ± 0.05 ($P < 0.01$).

3.7. A model of Ch flux in the striatal slice

To estimate the proportion of total brain Ch uptake due to uptake into cholinergic neurons, we performed a series

of uptake studies using Ch concentrations from 0.1–500 μM with and without the high affinity Ch uptake inhibitor, HC3 (1 μM). Despite the fact that cholinergic neurons represent less than 1% of all cells in the slice, at physiologic Ch concentrations more than 60% of the uptake was HC3 sensitive (i.e. uptake into cholinergic neurons) (Fig. 3a) [40]. A two-compartment model (cholinergic cells vs. all other cells) was designed in which uptake into all noncholinergic cells was modeled as follows:

$$\mathrm{FLUX}_0 = V_0/(1 + K_0/S) + K_{\mathrm{diff}} * S \quad (1)$$

where V_0 is the maximum uptake, K_0 is the K_m of this compartment, S is the Ch concentration and K_{diff} is a diffusion constant (Fig. 3b). Eq. (1) is a classic Michaelis–Menten equation with a term added for nonspecific diffusion. This equation was fit to the uptake data determined in the presence of HC3, the best fit was obtained when: $V_0 = 94$ pmol/mg/min, $K_0 = 72$ μM and $K_{\mathrm{diff}} = 0.22$ pmol/mg/min/μM. These values are consistent with those reported for low-affinity sodium-independent uptake [21,42].

The Ch uptake into only cholinergic neurons was estimated by subtracting the uptake with HC3 from the Ch uptake without HC3 for Ch concentrations less than 50 μM. The best fit curves used for the subtraction were calculated by the enzfitter computer program (Fig. 3c). Differences between the two curves in Fig. 3c, were fit to a second Michaelis–Menten equation:

$$\mathrm{FLUX}_C = V_C/(1 + K_C/S) \quad (2)$$

where V_C is the maximum uptake and K_C is the K_m of this compartment. The diffusion term was omitted since the contribution of nonspecific uptake is negligible at low Ch concentrations. The data fit best when $V_C = 17$ pmol/mg/min and $K_C = 5$ μM, values typical for sodium-dependent high affinity uptake [12,42]. The total

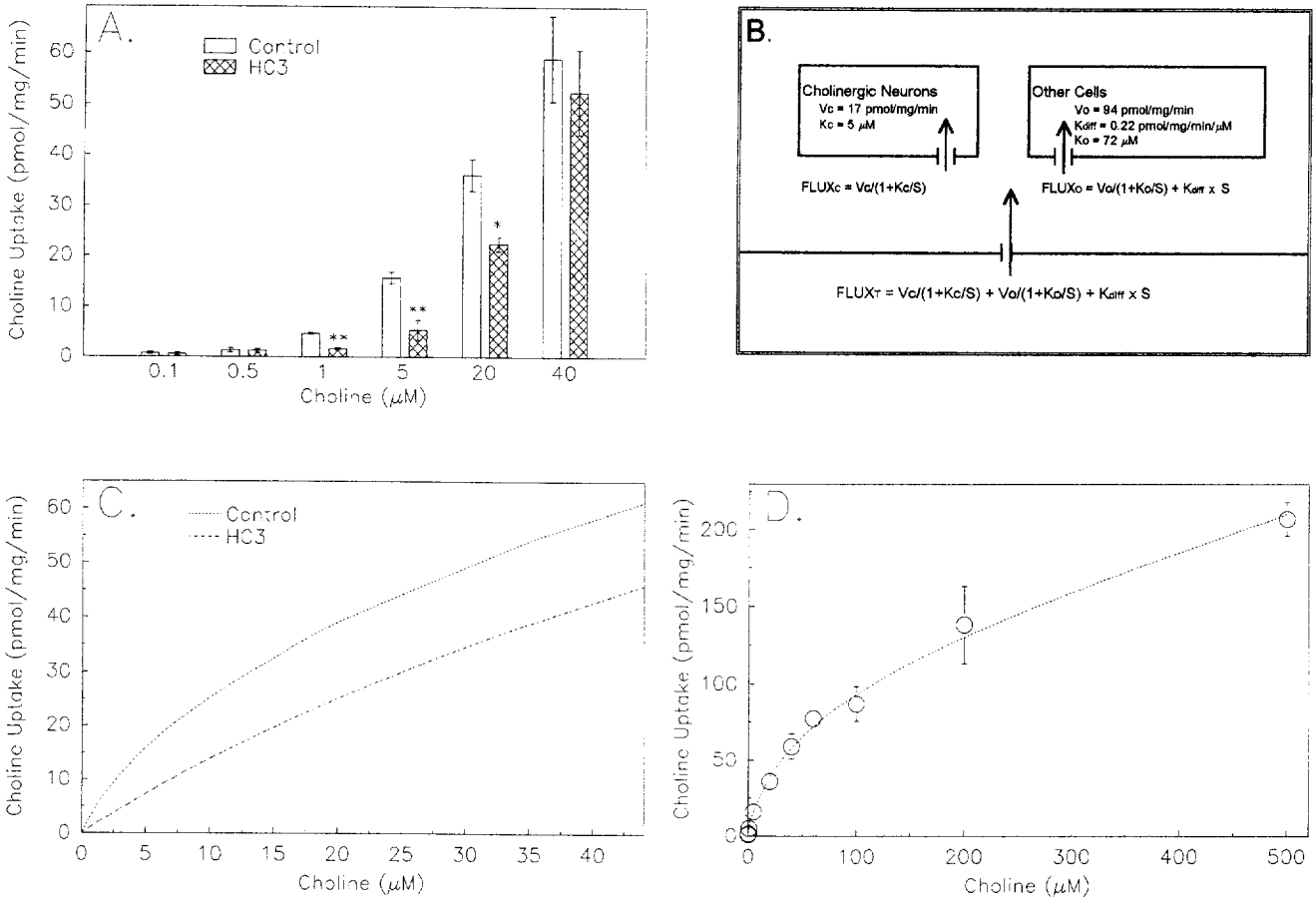

Fig. 3. Choline uptake into striatal slices. Following equilibration, slices were superfused for 10 min with medium contain various concentrations of Ch and [*Me*-^{3}H]Ch (0.1 Ci/mol; 0.1–500 μM Ch) with and without hemicholinium-3 (HC3). The slices were then rinsed with standard Krebs buffer for 5 min, homogenized in cold methanol and counted. A: HC3 inhibited as much as 70% of the Ch uptake into the slices. Values represent means ± S.E.M. from 3–6 rats. Statistics were performed using a Student's *t*-test with * $P < 0.01$, ** $P < 0.005$. B: a two-compartment model of rat striatum with kinetic values determined form uptake data in panel A and additional uptake data for Ch concentrations from 60–500 μM with and without HC3. Data were fit with the Enzfitter computer program. C: best fit lines for uptake with and without HC3. The differentials between Ch uptake with and without HC3 were used to determine the kinetic properties of high-affinity Ch uptake. The kinetic values are displayed in the model described in panel B. D: data represent means ± S.E.M. from 3–6 rats of Ch uptake into slices without HC3. Dashed line is curve described by the equation, $\mathrm{FLUX}_T = V_0/(1 + K_0/S) + K_{\mathrm{diff}} * S + V_C/(1 + K_C/S)$; where $V_0 = 94$ pmol/mg/min, $K_0 = 72$ μM, $K_{\mathrm{diff}} = 0.22$ pmol/mg/min/μM, $V_C = 17$ pmol/mg/min and $K_C = 5$ μM.

Ch flux into the slice could then be described as the sum of Ch uptake into cholinergic cells (Eq. (2)) and that into all other cells (Eq. (1)):

$$FLUX_T = V_0/(1 + K_0/S) + K_{diff} * S + V_C/(1 + K_C/S) \quad (3)$$

The total Ch uptake into slices (without HC3) determined experimentally at Ch concentrations from 0.1–500 μM is precisely fit by Eq. (3); where $V_0 = 94$ pmol/mg/min, $K_0 = 72$ μM, $K_{diff} = 0.22$ pmol/mg/min/μM, $V_C = 17$ pmol/mg/min and $K_C = 5$ μM (the dashed line in Fig. 3d is drawn using Eq. (3)).

To assess the effect of enhancing Ch's uptake into cholinergic cells, total Ch uptake was fixed at the level determined experimentally and fit by Eq. (3) for a given Ch concentration (i.e. 10.9 pmol/mg/min @ 3 μM Ch). The K_m for high affinity Ch uptake (K_C) was then decreased (i.e. from 5 to 2 μM) and the Ch uptake into cholinergic cells recalculated using the new K_m using Eq. (2) (i.e. @ 3 μM Ch and a K_m of 2 μM the uptake would be 10.3 pmol/mg/min). The percent of total Ch uptake that was into cholinergic cells was determined by dividing the Ch uptake into cholinergic cells at the new K_m by the total uptake into both compartments (i.e. $10.3/10.9 \times 100 = 95\%$). Thus, at an extracellular Ch concentration of 3 μM, a reduction in the K_m from 5 to 2 μM would result in 95% of all Ch uptake passing through cholinergic cells (Fig. 4). Similarly, at a 3 μM extracellular Ch concentration, a shift in the maximum velocity from 17 to 27 pmol/mg/min would accomplish the same effect. Fig. 4 illustrates the effect of changes in the K_m for high affinity Ch uptake, on the proportion of Ch taken up by cholinergic cells.

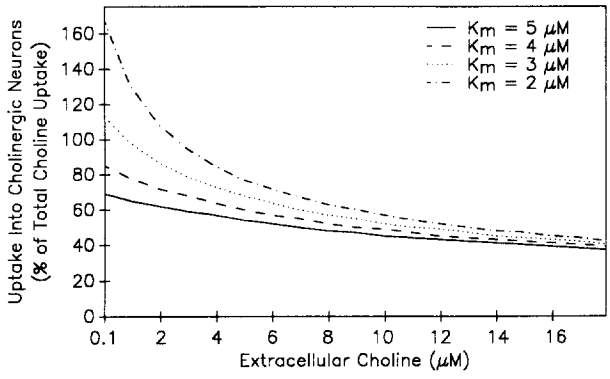

Fig. 4. Effect of decreased K_m of high affinity Ch uptake on the percent of all Ch uptake entering cholinergic cells. High-affinity uptake (described by Eq. (2)) and total uptake (described by Eqs. (2) and (3)) were determined experimentally as previously described (Fig. 3). Ratios of high affinity uptake to total uptake into the slices are displayed as the 5 K_m line (the K_m calculated from the data). Using Eq. (3), the ratios at other K_m values for high-affinity uptake are predicted (K_m equal to 4, 3 and 2).

4. Discussion

These data indicate that electrical stimulation of superfused striatal slices results in enhanced ACh release and reduced Ch phosphorylation which cannot be accounted for by alterations in Ch kinase or ChAT activities, nor in ATP levels.

4.1. PCh and PtdCh synthesis and turnover

Radioactive labeling studies revealed that stimulation markedly reduced conversion of [*Me*-^{14}C]Ch to [^{14}C]PCh and [^{14}C]PtdCh without attenuating tissue [^{14}C]Ch and [^{14}C]ACh levels (Fig. 1a and Fig. 2). However, the release of labeled ACh into the medium was greatly enhanced, and remained elevated throughout the stimulation period (Fig. 1b). After 1 h of labeling, the relative specific activities of Ch and PCh were consistent with the sequential conversion of Ch, to PCh (Table 1). This is in agreement with in vivo evidence that the CDP-Ch pathway is a major route of PtdCh synthesis in the brain [2,3,14,30]. Stimulation only marginally reduced PCh synthesis in cerebellar slices (unpublished observations), a structure containing few cholinergic neurons.

To determine whether the observed reductions in radioactive PCh and PtdCh were due to impaired synthesis or enhanced breakdown, additional experiments were performed in which slices were labeled, equilibrated and then stimulated. The radioactivities of PCh and PtdCh significantly decreased with time but were not affected by stimulation, suggesting that the observed decrease in PCh radioactivity was due to reduced phosphorylation of Ch.

The synthesis of PtdCh was calculated by dividing its radioactivity by the specific activity of PCh, its precursor. In both of our labeling paradigms (stimulation in the presence of label, or stimulation during the chase period) no effect of stimulation on PtdCh synthesis was observed, and approximately 3 nmol/h of PtdCh were synthesized (Fig. 2d). PCh synthesis in unstimulated slices was also approximately 3 nmol/h (Fig. 2c) consistent with the observation that the CDP-Ch pathway predominates when Ch is used as a substrate and the slices are quiescent. The reduction in PtdCh radioactivity observed during the stimulation period when the [*Me*-^{14}C]Ch was maintained in the medium presumably reflected the reduced labeling of the PCh pool in stimulated tissues. Given that PCh synthesis is almost entirely inhibited by stimulation (Fig. 2c) while PC synthesis continues unabated, it is probable that, had our experiment been extended sufficiently, the PCh pool would eventually have been depleted beyond the 25% loss observed, ultimately resulting in a decrease in PC synthesis. However, within the time frame of this experiment no such decrease was observed.

Our calculations of PtdCh synthesis assume that PCh is the immediate precursor to PtdCh, ignoring the intermediate metabolite CDP-Ch. We justified this calculation on

the following grounds: firstly, we could not detect any radioactivity in CDP-Ch. Secondly, the CDP-Ch pool is significantly smaller than the PCh pool, containing approximately 3.68 nmol/mg as compared with 20.12 nmol/mg of PCh [3]. Thus, the specific activity of CDP-Ch is likely to quickly approach that of PCh. In confirmation of this expectation it was shown that in rats injected intracerebrally with [^{3}H]Ch and sacrificed at regular intervals, CDP-Ch radioactivity was low, and rapidly (within 10 s) attained the same specific activity as PCh [3].

Although stimulated slices release ACh at 10 times the basal rate, and express ACh receptors coupled to phospholipase A_2 [5,41], we could not detect any labeled GPCh, a breakdown product of PtdCh generated by PLA activity. This suggests that brain phospholipases act on a lipid pool that differs from the pool labeled after brief exposure to [*Me*-^{14}C]Ch. Others have also failed to detect labeled GPCh using acute Ch labeling paradigms [10,11,30]. However, if labeled Ch is added to the diet, labeled GPCh is detectable after 2 days [11].

4.2. The effect of stimulation on Ch kinase and ChAT

A large number of studies suggest that the CDP-Ch pathway is mostly regulated by the translocation of the PCh: CTP cytidylyltransferase (E.C. 2.7.7.15) from the cytosol to an active membrane-associated state [25]. If, as is generally believed, cytidylyltransferase activity is rate-limiting in this pathway, then changes in PCh levels might be expected to have little effect on PtdCh synthesis. However, numerous studies suggest that Ch phosphorylation can also affect the rate of PtdCh synthesis [33,37]. Inhibition of Ch kinase activity might increase the availability of Ch for ACh synthesis when Ch levels are low.

In studies using cultured slices of newborn rat septum, a stimulation-induced decrease in both Ch phosphorylation and PtdCh was associated with increased ACh release [13]. Those data and those in the present study suggest that although PCh synthesis is reduced by neuronal depolarization, the conversion of newly-formed PCh to PtdCh is not compromised, since the decrease in PtdCh label was proportionally the same as the decrease in PCh labeling. In contrast, Suidan and Tolkovsky found that inhibition of Ch uptake in neurites from developing brain halted the incorporation of labeled Ch into ACh while leaving PCh synthesis unchanged, suggesting that these immature structures preferentially route Ch towards lipid synthesis regardless of their electrical activity [30].

The kinetic properties of Ch kinase and ChAT were assayed to determine if alterations in their activities might explain the stimulation-induced reduction in PCh synthesis. However, unlike the changes reported in rat superior cervical ganglia [1], no effects of stimulation and/or media Ch levels were observed on the kinetics of Ch phosphorylation or acetylation in brain slices. These experiments do not rule out the possibility that some form of inhibition of Ch kinase occurs in the intact brain tissue which is lost during homogenization.

4.3. The effect of enhanced Ch uptake in cholinergic cells on total slice PCh synthesis

Having established that the reduced radioactive labeling of PCh in stimulated slices is not due to enhanced utilization of PCh or reductions in Ch kinase activity, the effect of stimulation on Ch uptake was indirectly assessed. (Direct measurement of Ch uptake during stimulation was not possible because the radioactivity released into the medium in the form of ACh would have to be added to that recovered in the tissue. A small amount of radioactive ACh would have to be resolved from a much larger amount of radioactive Ch, a task made more difficult when the medium's Ch concentration can range from 1–500 μM.) At present, there is little dispute that Ch uptake is enhanced under many conditions that increase ACh release [4,18,27,32], and inhibited while the neuron is depolarized [12]. However, the cellular mechanism underlying this effect remains unresolved. We performed Ch uptake studies, with and without HC3, to model the two major Ch uptake compartments within rat striatal slices. Using this model, we tested the hypothesis that under stimulated conditions the increase in Ch uptake by cholinergic cells starves the rest of the tissue of Ch.

The low affinity uptake compartment was studied using slices superfused with [*Me*-^{3}H]Ch and with HC3, a drug that blocks Ch uptake through the high affinity compartment (Fig. 3a,c). The data obtained closely fit the model equation (Eq. (1)), with a K_m for Ch of 72 μM. The uptake into the high affinity compartment was obtained by subtracting the uptake observed with HC3 from that obtained without HC3, yielding a K_m for Ch of 5 μM. The total uptake into the slice was then determined by summing these two compartments (Eq. (3)); this resulted in an equation that tightly fit the observed data (dashed line in Fig. 3d). Yamamura and Snyder observed some time ago that Ch uptake into homogenates of rat striatum was always fit by two components [42], however, we are not using these uptake equations to verify the existence of two uptake systems. The modeling of Ch flux into the striatal slice enabled us to examine the impact of an increase in high affinity uptake on the amount of Ch available for noncholinergic cells. We found that either a modest decrease in the K_m for Ch (Fig. 4) or an increase in its V_{max} would have a large effect on the proportion of the Ch available for uptake by the low affinity compartment, at physiologic Ch concentrations. The inhibition of PCh synthesis observed in this study occurred at Ch concentration of between 4–5 μM (Ch derived from the radioactive label), a value slightly higher than that observed in vivo [6]. At a Ch concentration of 5 μM, a shift in the K_m from 5 to 1 μM, well within the range reported for this compartment [23], would shunt virtually all of the Ch away

from the rest of noncholinergic cells (Fig. 4). The same effect is observed by a change in the V_{max}; thus, regardless of whether the reported increases in Ch uptake are due to changes in affinity or capacity, both can limit the available Ch to noncholinergic cells.

This model is supported by determinations of the specific activity of ACh, as compared with those of Ch (Table 1). The specific activities of released ACh were significantly higher than that of tissue ACh and both were higher than that of tissue Ch. These data suggest that cholinergic neurons increase their uptake for Ch and attain a specific activity of Ch greater than that observed in most other cells, it is also consistent with the hypothesis that the most recently synthesized ACh molecule is released first (a phenomenon reviewed by MacIntosh and Collier [19]).

Taken as a whole, these experiments suggest that neuronal activity greatly enhances ACh synthesis in, and release from, striatal cholinergic cells, and inhibits the conversion of Ch to PCh in most other cells. The reduction in PCh levels cannot be explained soley by its enhanced breakdown, by alterations in the activities of Ch kinase or ChAT, or by a reduction in ATP levels. At physiologic Ch concentrations it is probable that during periods of intense activity, the increase of Ch uptake into cholinergic cells severely limits the Ch available for low affinity uptake into the other cells of the brain.

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