NEURONAL ACTIVITY AND MEMBRANE TURNOVER IN RAT BRAIN

by

Steven A. Farber

B.S. Electrical and Biomedical Engineering
Rutgers University, 1986

S.M. Technology and Policy
Massachusetts Institute of Technology, 1991

Submitted to the Department of Brain and Cognitive Sciences in
Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

at the

Massachusetts Institute of Technology

June 1993

Signature of Author_____
Department of Brain and Cognitive Sciences
April 15, 1993

Certified by_____
Richard J. Wurtman, Professor
Department of Brain and Cognitive Sciences
Thesis Advisor

Accepted by_____
William Quinn, Professor
Chair, Department Graduate Committee

MEMBRANE TURNOVER IN RAT BRAIN

by

Steven A. Farber

Submitted to the Department of Brain and Cognitive Sciences on
April 15, 1993 in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in Neurobiology

ABSTRACT

We investigated the interaction between neurotransmitter release, and membrane composition and phospholipid turnover, specifically, the mechanism by which brain structures rich in cholinergic neurons utilize choline (Ch) to synthesize acetylcholine (ACh) and phosphatidylcholine (PC). These studies included the development of a brain slice superfusion system with associated electronics to electrically stimulate the slices, and novel methods of both stimulating and perfusing rat corpus striatum *in vivo*.

Superfused rat striatal slices can release large amounts of ACh for periods greater than 1 hour and without the addition of exogenous Ch. However, the enhanced release is at the expense of both PC and phosphorylcholine (PCh). The loss of membrane PC during stimulation can be prevented by superfusion with medium containing Ch (40 μM) or tetrodotoxin (1 μM). The parameters (frequency and pulse duration) that evoke ACh from this brain structure *in vitro are* similar to those observed in the intact animal using our hybrid microdialysis probe. When slices are superfused with [^{14}C]Ch and stimulated, there is a marked reduction in labeled PCh synthesis comparable in magnitude to the increase in ACh release. This reduction in Ch phosphorylation was not accompanied by changes in the Km for Ch of either Ch kinase or Ch acetyltransferase, as assayed in slice homogenates. While these studies support the view that the tissue Ch pool is the precursor for PCh and PC synthesis, ACh seems to be synthesized from a smaller, more highly labeled pool. A comparison of the specific activities of released and tissue ACh suggests that the most recently synthesized pool is the first released.

Our microdialysis studies were used to selectively stimulate a group of neurons while simultaneously measuring the release of neurotransmitters *in vivo*. We developed a novel method of determining the strength-duration curve of this response. This curve relates the level of current to the pulse duration for a given increase in neurotransmitter release. Using this method we stimulated striatal cholinergic neurons and not dopaminergic terminals. We also demonstrated that both evoked and basal ACh release was significantly enhance by peripheral Ch administration.

Additionally, we applied the *in vitro* slice system to assess the effect of electrical stimulation on the release of the amyloid β-protein precursor from hippocampal slices. This was the first demonstration that this protein could be released from brain slices and that the release was enhanced by neuronal activity. The stimulated release of the amyloid β- protein precursor was prevented by superfusion with tetrodotoxin and was not associated with an increase in lactate dehydrogenase activity in the medium.

Thesis Supervisor: Dr. Richard J. Wurtman
Title: Professor of Neuroscience

TABLE OF CONTENTS

The effect of increased neuronal activity on choline-containing phospholipid intermediates in rat corpus striatum

Steven A. Farber, Barbara E. Slack, Alice Wei and Richard J. Wurtman
Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology Cambridge, MA 02139

Key Words: Acetylcholine; Choline; Glycerophosphocholine; Phosphatidylcholine; Phosphocholine; Phospholipids; Striatum

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

ABSTRACT

We investigated the effect of enhanced neuronal activity, in the absence of exogenous choline (Ch), on choline-containing phospholipid intermediates in superfused slices of rat corpus striatum. In order to establish parameters that maximally evoked acetylcholine (ACh) release, the effect of pulse duration, current and frequency were studied. ACh release from superfused slices was increased to greater than 7 times basal levels by exposure to alternating polarity field stimulation (125 mA, 15 Hz and 1.0 ms pulse duration). Total protein and lactate dehydrogenase (LDH) activity released by the slices into the superfusion medium were unaffected by stimulation, suggesting that the slice viability was not compromised by our stimulation paradigm. High levels of ACh release were maintained for 1 hr during maximal conditions of stimulation (125 mA). Under these conditions Ch release decreased (from 77.7 $\pm$ 5.0 to 36.0 $\pm$ 4.9 pmol/g DNA/hr [$p < 0.01$]) while ACh release increased (from 19.4 $\pm$ 3.5 to 136.4 $\pm$ 7.2 pmol/g DNA/hr [$p < 0.01$]). The increase in ACh release was accompanied by reductions in cellular phosphatidylcholine (PC) (from 7.5 4 $\pm$ 0.07 to 6.43 $\pm$ 0.20 nmol/g DNA [$p < 0.01$]), phosphatidylethanolamine (PE) (from 8.21 $\pm$ 0.49 to 5.5 $\pm$ 0.36 nmol/g DNA [$p < 0.05$]) and phosphatidylserine (PS) (from 2.39 $\pm$ 0.13 to 1.82 $\pm$ 0.08 nmol/g DNA [$p < 0.05$]) levels. These reductions in individual phospholipids were also reflected in a decrease in total lipid phosphate (from 24.7 $\pm$ 1.3 to 19.2 $\pm$ 1.2 nmol/g DNA [$p < 0.01$]) that was linearly related to duration of stimulation ($r = 0.995$, $p < 0.01$), and was blocked by the addition of tetrodotoxin (TTX) or Ch to the superfusate during the stimulation period. Significant reductions in tissue ACh, Ch, glycerophosphocholine (GPCh) and phosphocholine (PCh) levels were also observed after 1 hr stimulation. These data suggest that ACh release prevails over phospholipid synthesis under conditions of enhanced neuronal activity, in the presence of low exogenous Ch concentrations, and that

the decrease in phospholipids levels observed after stimulation is related to a decrease in synthesis.

INTRODUCTION

Brain Ch is not only a substrate for the formation of ACh by the enzyme Ch acetyltransferase (E.C. 2.3.2.6) but serves also as the initial precursor for PC synthesis. While PC can be synthesized by base-exchange enzymes (Ch replaces either the serine in PS or the ethanolamine in PE) (Kanfer et al. 1988) or by successive methylations of PE (Blusztajn et al. 1985; Hitzemann, 1982), most of the PC in the brain is synthesized via the Kennedy pathway (Ansell and Spanner, 1968) the first step of which is the phosphorylation of Ch by Ch kinase (E.C. 2.7.1.32). It remains unclear how cholinergic neurons, especially under conditions of low Ch, "choose" whether to acetylate Ch to form ACh or phosphorylate Ch to eventually form PC.

There have been numerous studies of Ch metabolism following injection directly into the brain, or blood stream (Karlen et al. 1982; Arienti et al. 1976; Freeman et al. 1975; Ansell and Spanner, 1968), or after oral administration (Jope and Jenden, 1979; Cohen and Wurtman, 1975). When labeled Ch is injected intracerebrally the label is rapidly incorporated into PCh (Ansell and Spanner, 1968). In fact, it is the phosphorylation of Ch that keeps the concentration of free Ch low in the brain. Klein et al. have shown that a bolus intraperitoneal injection of radioactive Ch (60 mg/kg) to rats resulted in a net uptake of Ch label into the brain with only a minor change in brain free Ch. However, lasting changes in brain PCh levels were observed, accompanied by an increase in PC labeling (Klein et al. 1992). It is the buffering capacity of this PC synthesis pathway that has made it difficult to elevate brain ACh with the administration of low doses of Ch (Millington and Wurtman, 1982). However, *in vivo* studies using microdialysis (Farber et al. 1993) and *in vitro* brain slice studies (Maire and Wurtman, 1985; Trommer et al. 1982) have shown that Ch administration can increase ACh synthesis and release, especially when neurons are rapidly firing.

Previously, work from our laboratory demonstrated that electrical stimulation of slices of rat corpus striatum, in the presence of an acetylcholinesterase (AChE) inhibitor and in the absence of exogenous Ch, resulted in a decrease in tissue PC levels which correlated with the number of stimulation intervals as well as the total ACh released (Ulus et al. 1989). This finding was anticipated when Maire et al. found that the increased Ch and ACh efflux from stimulated slices could not be accounted for by the observed reductions in tissue levels of these compounds (Maire and Wurtman, 1985). The authors argued that there is an endogenous pool of bound Ch that can sustain high levels of ACh release and that membrane PC is the most likely source. The present study was designed to elucidate the mechanism responsible for the reductions in membrane phospholipids observed in electrically stimulated brain tissue. Stimulation parameters resulting in a reduction of membrane phospholipids were characterized, and Ch-containing phospholipid intermediates within the tissue were measured. The changes observed in these intermediates suggest that stimulation might decrease lipid synthesis by providing additional Ch for ACh synthesis. Some of this data was previously presented in a preliminary form (Farber et al. 1991).

MATERIAL AND METHODS

All chemicals were purchased from Sigma Chemical (St. Louis, MO). TTX was dissolved in water upon arrival and frozen (at -70°C) in 1 mM aliquots. Likewise, 1 mM standard solutions of Ch, ACh, GPCh and PCh were frozen (at -20°C) in aliquots. [*Me*-^{14}C]Ch chloride (53 Ci/mol) was purchased from DuPont - New England Nuclear. The bactericide, Kathon CG, was obtained from Rohm & Hass (Philadelphia, PA).

Animals

Experiments utilized male and female Sprague-Dawley rats (Charles River, Cambridge, MA) weighing 250 - 300 g and exposed to a 12 hr light-dark cycle. The animals were given access to water and food (Charles River Rat, Mouse and Hamster Original Formula) ad libitum 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.*), decapitated and their brains were removed and placed in chilled physiologic buffer containing ketamine (1 mM). Striata were rapidly dissected and sliced (300 mm) using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Gomstall, Surrey, U.K.). Slices were washed three times to remove debris with cold ketamine containing medium and randomly sorted into eight groups (4-7 slices/group). The entire dissection, slicing and washing procedure was performed in a cold room at 4°C in less than 10 min.

Chambers and Stimulating System

The slices were then loaded into custom-designed chambers (Warner Instrument Corp., Hamden, CT) that were maintained at 37°C by immersion in a water bath. Chambers were continually superfused with warmed, oxygenated Krebs-Ringer buffer (in mM: 120 NaCl, 3.5 KCL, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 0.02 eserine salicylate, pH 7.4) that was constantly bubbled with a mixture of 95% O_2 and 5% CO_2. In all experiments, slices were superfused for 1 hr (chamber volume of 0.7 ml @ 0.8 ml/min) prior to the start of sample collection and/or stimulation. In some experiments TTX (1mM) or Ch (40 mM) were added to the superfusate after 45 min equilibration, 15 min before the onset of stimulation.

The chambers were designed such that the slices were entirely immersed in buffer while resting on a nylon mesh support located in the middle of the chamber. This represents a modified version of the chambers previously described (Maire and Wurtman, 1985). Fine silver screens fixed at each end of the chamber and separated by a distance of 1.5 cm were used to stimulate the slices. Under conditions of maximal stimulation (125 mA, 1.0 ms and 15 Hz), the current density was 4.95 mA/mm^2 (30 - 40 V). Control groups were analyzed under identical conditions but were not electrically stimulated.

We developed a unique polarity reversal and resistance monitoring system to stimulate the slices (Warner Scientific). Electrical pulses originating from a square-wave

stimulator (Model S88, Grass Instruments, Quincy, MA) were passed through a device that switched the polarity of every second pulse and simultaneously sampled the current and voltage 50 ms after the onset of each pulse. The current and voltage were monitored to maintain uniform chamber resistance and detect the accumulation of bubbles (typically formed as the oxygen rich buffer leaves the narrow inlet tubing and enters the chamber). The chambers were designed with a small port that allowed the removal of any bubbles and a cap that enabled the easy collection of superfusates.

At the completion of the experiment, slices were removed from the chambers and placed on ice in cold physiologic buffer. The buffer was then carefully removed and replaced with 1ml of a second buffer (230 mM sucrose, 1 mM $ZnSO_4$ and 20 mM Hepes pH 7.4) in which the tissue was homogenized (10x Teflon/glass). Immediately following homogenization, 1 ml of methanol was added to the mixture.

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 (chloroform/methanol/water 2:1:1), vortexed for 60 sec and briefly centrifuged (Folch J. et al., 1957). The upper aqueous phase containing ACh, Ch, GPCh and PCh was separated from the lower lipid containing phase. After extraction, the separate phases were dried under vacuum. For the determination of ACh and Ch, aliquots of dried aqueous phase were resuspended in water and subjected to HPLC analysis on a polymeric reversed-phase column (BAS, West Lafayette, IN) with a mobile phase of 50 mM phosphate (pH 8.5) containing 0.005 % Kathon CG as a bactericide. Once separated, ACh and Ch were converted to hydrogen peroxide by a post-column enzymatic reactor (BAS) containing AChE and choline oxidase. The hydrogen peroxide was detected electrochemically using a platinum electrode (500 mV *vs.* Ag/AgCl) (model 200a, BAS).

Determination of GPCh and PCh were separated by a second HPLC system (Liscovitch et al. 1985) and dried under vacuum. To liberate Ch from these fractions

GPCh samples were heated in 6 N HCl for 3 hr while the PCh fraction was exposed to alkaline phosphatase (0.3 U/tube) for 3 hr then centrifuged with methanol to precipitate the added enzyme. Samples were then dried under vacuum, resuspended in HPLC buffer and subjected to HPLC analysis for Ch as previously described (post-column enzymatic reactor system).

Total lipid phosphate was determined from a dried aliquot of the organic phase by perchloric acid digestion (Svanborg and Svennerholm, 1961) utilizing a vapor trapping device. Individual phospholipids were separated by thin-layer chromatography on silica gel G, with chloroform: ethanol: triethylamine: water (30:34:30:8; v:v) as a mobile phase (Touchstone et al. 1980). The individual phospholipids were scraped off the plates and assayed for their total phosphorous content.

In some experiments, after stimulation, slices were incubated in 1 ml of physiologic buffer containing 2.4 mCi of [^{14}C]Ch for 1 hr. The medium was replaced after 30 min with fresh buffer containing the same amount of labeled Ch. Slices were then homogenized as described above. Labeled PCh was determined on the HPLC system previously described except that the radioactivity was determined with an on-line monitor.

All measurements were normalized to total tissue DNA using the method of Labarca and Paigen (1980) to adjust for variations in the amount of tissue loaded into each chamber.

Analysis of Superfusates:

After the slices had equilibrated, superfusates were collected from each chamber into ice cold tubes. The released ACh and Ch was determined by HPLC analysis of 0.2 ml of filtered (0.2 mm) superfusate. Total superfusate protein was determined by vacuum ultrafiltration against water at 4^{o}C using cellulose dialysis tubing. This procedure yielded a final volume of 4 ml which was lyophilized (vacuum dried while frozen) and reconstituted in water. Protein levels were measured using the bicinchoninic acid assay

(Pierce, Rockford, IL). LDH activity in 0.5 ml of superfusate was assayed using a modified version of the commercial kit available from Sigma Chemical (Sigma # 500).

Statistics:

Data are expressed as means ± S.E.M. A t-test was used to compare the effects of Ch and TTX on total phospholipid levels while ANOVA with a *post-hoc* Newman-Keuls tests were used for all other comparisons. ANOVA and a Pearson correlation were used to demonstrate the relationship between stimulation duration and total phospholipid levels.

RESULTS

Stimulation Parameters

Since this experiment utilized a new type of chamber design and stimulation electronics it was necessary to assess the effect of various stimulation parameters on ACh release. In a typical experiment, the striatal slices from one rat were divided into four chambers. An acetylcholinesterase inhibitor (eserine) was added to the medium in all experiments to prevent the breakdown of released ACh and the subsequent reutilization of the Ch. Following equilibration of the slices, superfusates were collected for 10 min to determine basal ACh release, then all slices were stimulated with varying current intensities for 10 min while pulse duration and frequency were kept constant at 1.0 ms and 15 Hz respectively. The effect of current intensity was expressed as the ratio of the stimulated *vs.* basal release. ACh release increased linearly with increasing current up to 125 mA (Fig. 1A). Likewise, when current intensity and frequency were held constant, ACh release increased linearly up to pulse durations of 0.5 ms and then leveled off (Fig. 1B). The half maximal response was evoked at pulses of 0.18 ms. Stimulation frequencies greater than 15 Hz were unable to release any additional ACh when current and pulse duration were kept at 100 mA and 1.0 ms respectively (Fig. 1C).

Once establishing that our stimulation system could reliably evoke ACh release, the reproducibility of the response over longer repeated stimulation periods was assessed.

Slices were stimulated with either 30, 60 or 100 mA of current at a pulse duration of 1.0 ms and a frequency of 15 Hz. In all experiments a set of chambers, processed concurrently, were unstimulated. The superfusates from each chamber were collected during three time intervals (0 - 20, 20 - 40 and 40 - 60 min) during stimulation. The slices consistently released high amounts of ACh at all currents tested throughout the 1 hr stimulation period (Fig. 2). To correct for different amounts of tissue in each chamber, the release during each interval was normalized to the total tissue DNA determined at the end of the experiment.

In a final series of experiments the effect of stimulation current intensity was assessed in slices stimulated for periods of 1 hr. The release of ACh was dependent on the stimulation current and was increased by more than 7-fold relative to basal values (from 19.4 ± 3.6 to 136.4 ± 7.2 pmol/g DNA/hr [$p<.01$])(Fig. 3A) by a stimulation current of 125 mA . Since basal release tends to rise slightly over a 1 hr period the net stimulation-induced increase in ACh release over a 1 hr period was somewhat less than that observed during 10 min of stimulation (Fig. 1A). Ch release, from the same samples, decreased linearly with increasing stimulation current in the range of 0 to 100 mA after which Ch release reached a plateau at approximately 39 pmol/g DNA/hr, a decrease of 58.7 % from unstimulated slices ($p< 0.01$) (Fig 3B). Both the ACh and Ch release curves show an inflection point at 100 mA.

Tissue Viability

In order to assess whether field stimulation compromised the viability of the slices we measured both neurotransmitter release over time (a complex energy-dependent process) and the release of tissue protein into to the medium. Neither tissue protein remaining in the slices after the experiment nor protein recovered in the medium were affected by stimulation (Table 1). A more specific indicator of cell death and lysis is release of the cytosolic enzyme, LDH. The LDH activity in both the medium and the

tissue was unchanged by stimulation. The released LDH activity represented less than 0.05 % of the activity remaining in the tissue.

Stimulation Induced Changes in Phospholipids and Ch-Containing Metabolites

Field stimulation, in the absence of exogenous Ch resulted in a significant reduction in total phospholipids (from 24.7 $\pm$ 1.3 to 19.1 $\pm$ 0.7 nmol/g DNA at 100 mA, 15 Hz and 1.0 ms [$p<.05$]) after 1 hr of stimulation (Fig. 4A). This decrease was not enhanced by further increases in the current and occurred at the same current at which Ch release, reached a plateau (see Fig. 3B). In order to determine whether the decrease in total lipids was gradual or occurred rapidly, tissue samples were collected at various time periods. The loss of total lipid phosphate was dependent on the stimulation time ($p< 0.01$), and in a related series of experiments, reached significance only after 90 min of stimulation ($p< 0.05$) (Fig. 4B). Phospholipid levels were significantly correlated with the duration of the stimulation period ($r = 0.995$, $p< 0.01$). The changes in total phospholipids observed at 100 mA were a result of reductions in each individual phospholipid species. Levels of PC, PE and PS were reduced by 14.7 % ($p< 0.01$), 32.7 % ($p< 0.05$) and 23 % ($p< 0.05$) respectively (Fig. 4C).

Tissue Ch metabolites were measured in both control and stimulated (125 mA, 15 Hz and 1.0 ms) tissues. After 1 hr of stimulation, significant reductions in PCh (from 806 $\pm$ 33 to 655 $\pm$ 38 [$p< 0.05$]), GPCh (from 388 $\pm$ 40 to 225 $\pm$ 25 [$p< 0.05$], Ch (from 94.0 $\pm$ 4.6 to 52.6 $\pm$ 1.8 [$p< 0.01$]) and ACh (from 56.9 $\pm$ 1.1 to 26.3 $\pm$ 2.0 [$p< 0.01$]) (Fig. 5). The levels of Ch metabolites are consistent with those reported in the literature (Arienti et al. 1976; Ansell and Spanner, 1968). In slices that were incubated with [^{14}C]Ch, stimulation reduced the radioactivity recovered in tissue PCh by 26.3 $\pm$ 4.6 % as compared with controls ($p< 0.05$).

The Effect of Superfusion with Ch or TTX

In order to test whether the reduction in both individual phospholipids and total lipid phosphate required neuronal depolarization, TTX (1mM) was used to prevent action

potential generation. Slices were maximally stimulated (125 mA, 15 Hz and 1.0 ms) for 90 min in the presence or absence of TTX. TTX reduced evoked ACh release approximately 64 % during the first 20 min collection period (from 107.6 $\pm$ 8.6 to 38.1 $\pm$ 6.0 pmol/g DNA [$p < 0.01$]) and was similarly effective throughout the experiment. Basal release was significantly reduced by TTX only during the first collection period (from 8.44 $\pm$ 0.31 to 5.24 $\pm$ 0.61 pmol/g DNA/hr [$p < 0.05$]) (Fig. 6). The stimulation induced decrease in total lipid phosphate was blocked by TTX superfusion while phospholipid levels in unstimulated tissues were not changed (Fig. 7A). Superfusion with exogenous Ch (40 μM) during stimulation also prevented the reduction in phospholipids induced by stimulation in the absence of Ch (Fig 7B).

DISCUSSION

In this study we developed both superfusion chambers and field stimulation electronics to more reliably evoke ACh release from striatal slices. Utilizing this system we characterized the electrical parameters that influence ACh release and studied the effect of field stimulation on intracellular Ch-containing compounds. We found that stimulation, in the absence of exogenous Ch and in the presence of an AChE inhibitor, resulted in sustained ACh release for periods greater than 1 hr while reducing the tissue levels of ACh, Ch, GPCh, PCh and PC. The decrease in membrane phospholipids was TTX and Ch sensitive.

The superfusion system allowed us to carefully study some electrical parameters that influence ACh release from striatal slices. ACh release was dependent on both current and pulse duration with a half-maximal pulse duration of 0.18 ms (Fig. 1A and 1B). At a frequency of 15 Hz, ACh release was maximally evoked (Fig. 1C). The response of striatal neurons to stimulation *in vivo* measured by microdialysis, yields strikingly similar results, with a half maximal pulse duration of 0.20 ms and a maximal frequency of 20 Hz (15 Hz was not tested in that study) (Farber et al. 1993).

Electrophysiologic recordings showed that identified cholinergic striatal interneurons fired spontaneously at rates of 3 - 10 Hz and in bursts of up to 23 Hz (Wilson et al. 1990). Taken together, the microdialysis and intracellular recording data suggest that the electrical properties of cholinergic interneurons are preserved in the superfused slice system.

The consistent evoked release of ACh for 1 hr, at 7 - 10 times the basal rate, and low levels of released LDH activity (a well established marker of cell death and injury [Koh and Choi, 1987]) and protein; further support the viability of the slices used in the study. Moreover, the pioneering slice studies of Yamamoto and McIlwain that established the *in vitro* slice approach, found that only under conditions of field stimulation did the respiratory rate approach that seen in the intact brain (Ghajar et al. 1982; Yamamoto and McIlwain, 1966). This finding might suggest that stimulated slices better model the *in vivo* condition.

The depletion in phospholipids observed after electrical stimulation was consistent with earlier reports (Buyukuysal and Wurtman, 1990; Ulus et al. 1989; Blusztajn et al. 1987). The present study extends the former work by characterizing the amount of current needed to induce a reduction in membrane phospholipids. It is interesting to note that the depletion in total lipids occurs at the same current where Ch release has reached a plateau (100 mA) (Fig. 4a & Fig. 3b). There is also a shift in the rate of ACh release at this current (Fig. 3a). The decrease in total lipids was linearly related to the amount of stimulation ($r = 0.995$, $p < 0.01$) and could be blocked by TTX. The change reflected a decrease in the individual phospholipids, PC, PE and PS (Fig. 4b & Fig 4c). Ulus et al. found a similar pattern, the changes in total lipids reflected concomitant changes in PC, PS and PE, and that the decrease was proportional to the number of 20 min stimulation intervals (Ulus et al. 1989).

Several studies suggest that neuronal PC can serve as a reservoir of Ch for ACh synthesis. Parducz et al. found that preganglionic stimulation of the cat superior cervical

ganglion, in the presence of hemicholinium-3, resulted in an 18 % decrease in PC levels along with a reduction in the number of synaptic vesicles. The vesicles reappeared within two minutes after reperfusion with Ch-containing buffer (Parducz et al. 1986). Experiments by Maire and Wurtman, utilizing a similar superfused slice system as the present study, found that the amount of ACh and Ch recovered in the medium in response to stimulation and in the presence of an AChE inhibitor, exceeded the reduction in the tissue levels of the compounds (Maire and Wurtman, 1985).

It has been proposed that the reductions in PC resulting from neuronal activity are due to an increase in breakdown that liberates Ch for ACh synthesis (Billah and Anthes, 1990). This model poses a few problems. While there is enough newly synthesized PC on the inner leaflet to account for the increase in Ch, a majority of PC is predominately localized on the outer membrane surface (Bishop and Bell, 1988; Butler and Morell, 1982). To liberate Ch, lipases would have to cleave either newly synthesized PC or older PC on the outer surface (requiring high affinity transport to retrieve the Ch). It could be hypothesized that cholinergic cells contain some mechanism to determine the level of intracellular Ch and activate lipases when that level drops below some set amount. This is because stimulation in the presence of Ch prevented phospholipid depletion in this study (Fig. 7B) and in the studies by Ulus et al. (1989).

Alternatively, electrical stimulation might reduces membrane synthesis and hence Ch utilization by this pathway. Under conditions of high activity and low Ch the neuron inhibits membrane synthesis by inhibiting Ch kinase activity or by reducing the flow of Ch into a pool that is available for phosphorylation. If membrane turnover continues at its usual rate, the result would be a reduction in total lipids. This hypothesis requires much fewer regulatory events to explain the observed depletion in PC. Studies by Ando et al. in rat superior cervical ganglion showed that at low Ch levels, Ch kinase activity is reduced and Ch acetyltransferase activity is enhanced, while at high Ch levels the relationship is reversed (Ando et al. 1987). While the prevailing view holds that PCh: CTP

cytidylyltransferase (E.C. 2.7.7.15) is the rate limiting step in the synthesis of PC via the Kennedy pathway (Tercé et al. 1991; Hatch et al. 1991; Jamil et al. 1990; Bishop and Bell, 1988; Lim et al. 1986; Pelech and Vance, 1984) under some conditions Ch kinase might play a regulatory role. There is evidence that elevated PCh levels result in increased PC synthesis (Teegarden et al. 1990; Bishop and Bell, 1988; Warden and Friedkin, 1985).

In studies of newborn rat septal slice cultures preincubated with [^{3}H]Ch, potassium increased ACh synthesis while decreasing the incorporation of label into PCh and lipids (Keller et al. 1987). However, growing cholinergic sympathetic neurons exhibit the reverse pattern of labeling. Suidan et al. found that under conditions of low Ch, lipid synthesis is maintained at the expense of ACh synthesis suggesting that regenerating neurons relinquish ACh synthesis to support neuronal outgrowth (Suidan and Tolkovsky, 1993). While these experiments illustrate that Ch utilization can be cell type specific, they both point towards a regulatory system that controls Ch phosphorylation and not lipid turnover to adjust for changes in Ch availability.

The stimulation induced decrease in both the incorporation [^{14}C]Ch in to PCh and PCh mass most likely reflect decreased Ch phosphorylation and possibly PC synthesis. Since total PC levels were reduced and GPCh levels were not increased it is unlikely that phospholipase A (PLA) activity was enhanced by stimulation. GPCh can be elevated by increased PLA activity and once formed, can only be degraded. It is unlikely that it can be reacylated to form PC. Despite early evidence to the contrary by Infante (Infante, 1987), Veldhuizen et al. was unable to demonstrate the existence of a "GPCh-dependent" pathway of PC synthesis in liver or lung mitochondria (Veldhuizen et al. 1991). Therefore, a reduction of GPCh reflects either less lipase activity or increased GPCh degradation to Ch, both of which would argue against the hypothesis that the depletion was mediated by enhanced lipid breakdown. The changes observed in Ch-containing compounds and the reduction of PCh labeling support the hypothesis that PC synthesis is

reduced and that striatal cholinergic neurons favor ACh synthesis over Ch phosphorylation under conditions of low Ch and enhanced activity.

Acknowledgments: These studies were supported in part by a research grant (MH28783) from the National Institute of Mental Health. In addition, Mr. Farber was supported by training grant MH15761. The authors would like to acknowledge the helpful discussions with Dr. J.K. Blusztajn, Dr. A. Lander and Dr. F. Solomon. We thank Ms. Rajivi Pothiraj for excellent assistance with phospholipid assays.

REFERENCES

Ando M., Iwata M., Takahama K. and Nagata Y. (1987). Effects of extracellular choline concentration and K+ depolarization on choline kinase and choline acetyltransferase activities in superior cervical sympathetic ganglia excised from rats. *J. Neurochem.* **48**, 1448-1453.

Ansell G.B. and Spanner S. (1968). The metabolism of [Me-^{14}C]Choline in the brain of the rat in vivo. *Biochem. J.* **110**, 201-206.

Arienti G., Corazzi L., Woelk H. and Porcellati G. (1976). Biosynthesis of rat brain phosphatidylcholines from intracerebrally injected choline. *J. Neurochem.* **27**, 203-210.

Billah M.M. and Anthes J.C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* **269 Suppl.**, 173-183.

Bishop W.R. and Bell R.M. (1988). Assembly of phospholipids into cellular membranes: Biosynthesis, transmembrane movement, and intracellular translocation. *Ann. Rev. Biochem.* **4**, 579-610.

Blusztajn, J.K., Liscovitch, M., Mauron, C., Richardson, U.I. & Wurtman, R.J (1987). Phosphatidylcholine as a precursor of choline for acetylcholine synthesis. In *Cellular and molecular basis of cholinergic function* (Dowdall, M.J. and Hawthorne, J.N., eds.), Ellis Harwood, Chichester (Sussex) U.K., pp. 341-346.

Blusztajn J.K., Zeisel S.H. and Wurtman R.J. (1985). Developmental changes in the activity of phophatidylethanolamine N-methyltransferases in rat brain. *Biochem. J.* **232**, 505-511.

Butler M. and Morell P. (1982). Sidedness of phospholipid synthesis on brain membranes. *J. Neurochem.* **39(1)**, 155-164.

Buyukuysal R.L. and Wurtman R.J. (1990). 4-Aminopyridine inceases acetylcholine release without diminishing membrane phosphatidylcholine. *J. Neurochem.* **54**, 1302-1309.

Cohen E.L. and Wurtman R.J. (1975). Brain acetylcholine: Increase after systemic choline administration. *Life Sci.* **16**, 1095-1102.

Farber S.A., Buyukuysal R.L. and Wurtman R.J. (1991). Why do phospholipid levels decrease with repeated stimulation? A study of choline-containing compounds in rat striatum following electrical stimulation. *Annals of the New York Academy of Sciences* **640**, 114-117.

Farber S.A., Kischka U., Marshall D.L. and Wurtman R.J. (1993). Potentiation by choline of basal and ellectrically-evoked acetylcholine release, as studied using a novel device which both stimulates and perfuses rat corpus striatum. *Brain Res.* **In press,**

Folch J., Lees M. and Sloane-Stanley G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-503.

Freeman J.J., Choi R.L. and Jenden D.J. (1975). Plasma choline: Its turnover and exchange with brain choline. *J. Neurochem.* **24**, 729-734.

Ghajar J.B.G., Plum F. and Duffy T.E. (1982). Cerebral oxidative metabolism and blood flow during acute hypoglycemia and recovery in anesthetized rats. *J. Neurochem.* **38**, 397-408.

Hatch G.M., Tsukitani Y. and Vance D.E. (1991). The protein phosphatase inhibitor, okadaic acid, inhibits phosphatidylcholine biosynthesis in isolated rat hepatocytes. *Biochim. Biophys. Acta* **1081**, 25-32.

Hitzemann R. (1982). Developmental regulation of phospholipid methylation in rat brain synaptosomes. *Life Sci.* **30**, 1297-1303.

Infante J.P. (1987). Denovo CDP-Choline-Dependent Glycerophosphorylcholine Synthesis and Its Involvement As an Intermediate in Phosphatidylcholine Synthesis. *FEBS. Lett.* **214**, 149-152.

Jamil H., Yao Z. and Vance D.E. (1990). Feedback regulation of CTP:phosphocholine cytidyltransferase translocation between the cytosol and endoplasmic reticulum by phosphatidylcholine. *J. Biol. Chem.* **265**, 4332-4339.

Jope R.S. and Jenden D.J. (1979). Choline and phospholipid metabolism and the synthesis of acetylcholine in rat brain. *J. Neurosci. Res.* **4**, 69-82.

Kanfer J.N., McCartney D. and Hattori H. (1988). Regulation of the Choline, Ethanolamine and Serine Base-Exchange Enzyme-Activities of Rat-Brain Microsomes by Phosphorylation and Dephosphorylation. *FEBS. Let.* **240**, 101-104.

Karlen B., Lundgren G., Lundin J. and Holmstedt B. (1982). On the turnover of acetylcholine in mouse brain: Influence of dose size of deuterium labelled choline given as precursor. *Biochem. Pharmac.* **31**, 2867-2872.

Keller F., Rimvall K. andWaser P.G. (1987). Choline and Acetylcholine Metabolism in Slice Cultures of the Newborn Rat Septum. *Brain Res.* **405**, 305-312.

Klein J., Köppen A., Löffelholz K. and Schmitthenner J. (1992). Uptake and metabolism of choline by rat brain after acute choline administration. *J. Neurochem.* **58**, 870-876.

Koh J.Y. and Choi D.W. (1987). Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase assay. *J. Neurosci. Meth.* **20**, 83-90.

Labaraca C. and Paigen K. (1980). A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102**, 344-352.

Lim P., Cornell R. and Vance D.E. (1986). The Supply of Both CDP-Choline and Diacylglycerol Can Regulate the Rate of Phosphatidylcholine Synthesis in HeLa-Cells. *Biochem. Cell Biol.-Biochemi. et Bio.* **64**, 692-698.

Liscovitch M., Freese A., Blusztajn J.K. and Wurtman R.J. (1985). High performance liquid chromatography of water-soluable choline metabolites. *Anal. Biochem.* **151**, 182-187.

Maire J.C. and Wurtman R.J. (1985). Effects of electrical stimulation and choline availability on the release and contents of acetylcholine and choline in superfused slices from rat striatum. *J. Physiol. ,Paris* **80**, 189-195.

Millington W.R. and Wurtman R.J. (1982) Choline administration elevates brain phosphorylcholine concentrations. *J. Neurochem.* **38**, 1748-1742.

Parducz A., Joo F. and Toldi J. (1986). Formation of Synaptic Vesicles in the Superior Cervical-Ganglion of Cat - Choline Dependency. *Experimental Brain Research* **63**, 221-224.

Pelech S.L. and Vance D.E. (1984). Regulation of phosphatidylcholine biosynthesis. *Biochim. Biophys. Acta* **779**, 217-251.

Suidan H.S. and Tolkovsky A.M. (1993). Sympathetic neurons expressing cholinergic properties are poised to allocate choline symmetrically between acetylcholine and the phosphatidylcholine-generating pathway in growing neurites. *J. Neurosci.* **13**, 1190-1201.

Svanborg A. and Svennerholm L. (1961). Plasma total lipids, cholesterol, triglycerides, phospholipids and free fatty acids in a healthy Scandinavian population. *Acta. Med. Scand.* **169**, 43-69.

Teegarden D., Taparowsky E.J. and Kent C. (1990). Altered phosphatidylcholine metabolism in C3H10T1/2 cells transfected with the Harvey-ras oncogene. *J. Biol. Chem.* **265**, 6042-6047.

Tercé F., Record M., Tronchère H., Ribbes G. and Chap H. (1991). Cytidylyltransferase translocation onto endoplasmic reticulum and increased de novo synthesis without phosphatidylcholine accumulation in Krebs-II ascite cells. *Biochim. Biophys. Acta* **1084**, 69-77.

Touchstone J.C., Chen J.C. and Beaver K.M. (1980). Inproved separation of phospholipids on thin layer chromatography. *Lipids* **15**, 61-62.

Trommer B.A., Schmidt D.E. and Wecker L. (1982). Exogenous choline enhances the synthesis of acetylcholine only under conditions of increased cholinergic neuronal activity. *J. Neurochem.* **39**, 1704-1708.

Ulus I.H., Wurtman R.J., Mauron C. and Blusztajn J.K. (1989). Choline increases acetylcholine release and protects against the stimulation-induced decrease in phosphatide levels within membranes of rat corpus striatum. *Brain Res.* **484**, 217-227.

Veldhuizen R.A.W., Thompson W. and Possmayer F. (1991). Rat liver and lung mitochondria do not incorporate radioactivity from glycerol-3-phosphate or CDP-choline into glycero-3-phosphocholine. *Biochim. Biophys. Acta* **1083**, 211-216.

Warden C.H. and Friedkin M. (1985). Regulation of Choline Kinase-Activity and Phosphatidylcholine Biosynthesis by Mitogenic Growth-Factors in 3T3-Fibroblasts. *J. Biol. Chem.* **260**, 6006-6011.

Wilson C.J., Chang H.T. and Kitai S.T. (1990). Firing patterns and synaptic potentials of identified giant aspiny interneurons in the rat neostriatum. *J. Neurosci.* **10**, 508-519.

Yamamoto C. and McIlwain H. (1966). Electrical activities in thin sections from the mammalian brain maintained in chemically-defined media in vitro. *J. Neurochem.* **13**, 1333-1343.

FIGURE LEGENDS

FIG. 1. Effect of stimulation parameters on ACh release from striatal slices. Slices were equilibrated for 1 hr following which superfusates were collected for 10 min (basal release). All chambers were then stimulated with various currents, frequencies and pulse durations for 10 min. Release was expressed as a percent of basal from each chamber. Data represent the mean ± S.E.M. (n = from 3 - 5 rats per point, each rat provided enough slices for 8 different groups). A: Current was varied while frequency and pulse duration were set at 15 Hz and 1.0 ms respectively. B: Pulse duration was varied while current and frequency were set at 100 mA and 15 Hz respectively. C: Frequency was varied while current and pulse duration were set at 100 mA and 1.0 ms.

FIG. 2. ACh release remains high throughout stimulation period. Data are the result of a typical experiment by which slices were stimulated with varying amounts of current for 1 hr. Superfusates were collected for 20 min intervals. ACh levels in the perfusates were determined and corrected for the total DNA in the slices from the corresponding chamber.

FIG. 3. ACh and Ch release are inversely affected by stimulation current. Slices were stimulated (15 Hz and 1.0 ms) with varying amounts of current for 1 hr. The entire superfusate from one chamber was collected in a single ice-cold tube and assayed for ACh (**A**) and Ch (**B**). Data were normalized to the total tissue DNA in each chamber and expressed as means ± S.E.M. (n= 3 - 7 rats, each rat provided enough slices for 8 different groups). Statistics were performed using an ANOVA with a *post-hoc* Newman-Keuls test (* $p < 0.05$, ** $p < 0.01$ compared to basal levels)

FIG. 4. Total and individual tissue phospholipid levels are decreased by stimulation. **A**: Slices from each rat were divided into 5 chambers that received varying amounts of current for 1 hr (15 Hz and 1.0 ms). Total tissue lipids were then determined and corrected for total tissue DNA. Data are expressed as means ± S.E.M. from 4 - 7 experiments. Statistics were performed using an ANOVA with a *post-hoc* Newman-Keuls test (* $p < 0.05$ compared to basal levels). The difference between the means was

significant to $p < 0.01$. **B**: The decrease in total lipids was dependent on the length of the stimulation period. Slices from each rat were divided into eight groups, four of which were stimulated (125 mA, 15 Hz and 1.0 ms) for varying amounts of times. Unstimulated slices were run concurrently. Data represent means $\pm$ S.E.M. from seven experiments. Open circles represent unstimulated slices and filled circles stimulated slices. Statistics were performed using by ANOVA with a *post-hoc* Newman-Keuls test (* $p < 0.05$ compared to basal levels). The difference between the means was significant at $p < 0.01$. A Pearson correlation was performed to assess the relationship between stimulation duration and total lipid levels ($r = 0.995$, $p < 0.01$). **C**: Individual lipids were decreased in approximately the same proportion as total lipids. Slices from each rat were divided into four groups, two stimulated (100 mA, 15 Hz and 1.0 ms) and two control groups. After 1 hr of stimulation, total and individual phospholipid levels were determined and corrected for total DNA. Data are expressed as means $\pm$ S.E.M. of the mean from each experiment (n=4). A t-test was used to compare the stimulated to the control groups (* $p < 0.05$, ** $p < 0.01$).

FIG. 5. Electrical stimulation resulted in significant reductions in tissue Ch-containing compounds. Slices from each rat were divided into four groups, two stimulated (100 mA, 15 Hz and 1.0 ms) and two control groups. After 1 hr of stimulation, tissue levels of ACh, Ch, PCh and GPCh were determined and corrected for total DNA. Data are expressed as means $\pm$ S.E.M. of the mean from each experiment (n=4). A t-test was used to compare the stimulated to the control groups (* $p < 0.05$, ** $p < 0.01$).

FIG. 6. TTX inhibited the stimulation induced increase in ACh release. Slices from each rat were divided into four groups, stimulation (125 mA, 15 Hz and 1.0 ms) with and without TTX (1 mM) and unstimulated controls with and without TTX. Superfusates were collected over various intervals throughout 90 min of stimulation and assayed for ACh. Released ACh was corrected for total DNA. Data are expressed as means $\pm$

S.E.M. from each experiment (n=5). A t-test was used to compare the groups (* $p< 0.05$, ** $p< 0.01$).

FIG. 7. Superfusion with TTX or Ch prevented the depletion in tissue phospholipids caused by stimulation. Slices from each rat were divided into four groups, stimulation (125 mA, 15 Hz and 1.0 ms) with and without drug and unstimulated controls with and without drug. After 90 min of stimulation, total phospholipid levels were determined and corrected for total DNA. Data are expressed as means $\pm$ S.E.M. of the mean from each experiment (n=5). A t-test was used to compare the groups (* $p< 0.05$, ** $p< 0.01$). Superfusion with TTX (1mM) (**A**) or Ch (40 mM) (**B**) prevented the stimulation induced reduction in total lipid phosphate.

TABLE LEGEND

TABLE 1. Slices were stimulated (125 mA, 15 Hz and 1.0 ms) for 1 hr and the superfusates were collected (n = 5). Tissue protein and DNA levels were determined after stimulation. Most of the superfusate (40 ml of 48 ml) was then subjected to vacuum ultrafiltration against water. The total released protein was calculated from measurements on aliquots of the dialyzed samples and corrected for the total tissue DNA. LDH activity was determined from both an aliquot of superfusate and tissue homogenate and corrected for the total tissue DNA.

Figure 1

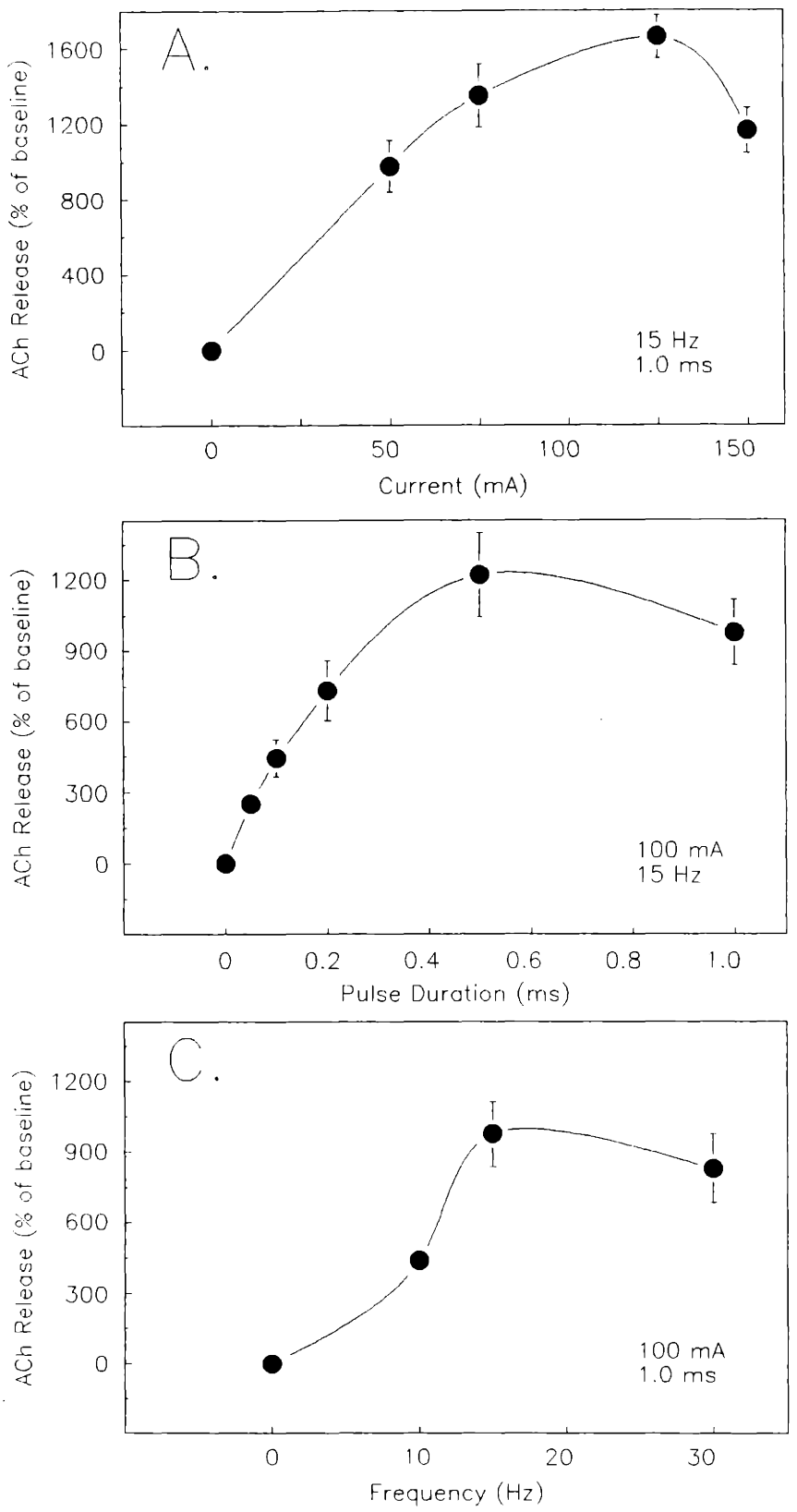

A.
ACh Release (% of baseline)
1600
1200
800
400
0
15 Hz
1.0 ms
0
50
100
150
Current (mA)
B.
ACh Release (% of baseline)
1200
900
600
300
0
100 mA
15 Hz
0
0.2
0.4
0.6
0.8
1.0
Pulse Duration (ms)
C.
ACh Release (% of baseline)
1200
900
600
300
0
100 mA
1.0 ms
0
10
20
30
Frequency (Hz)

Figure 2

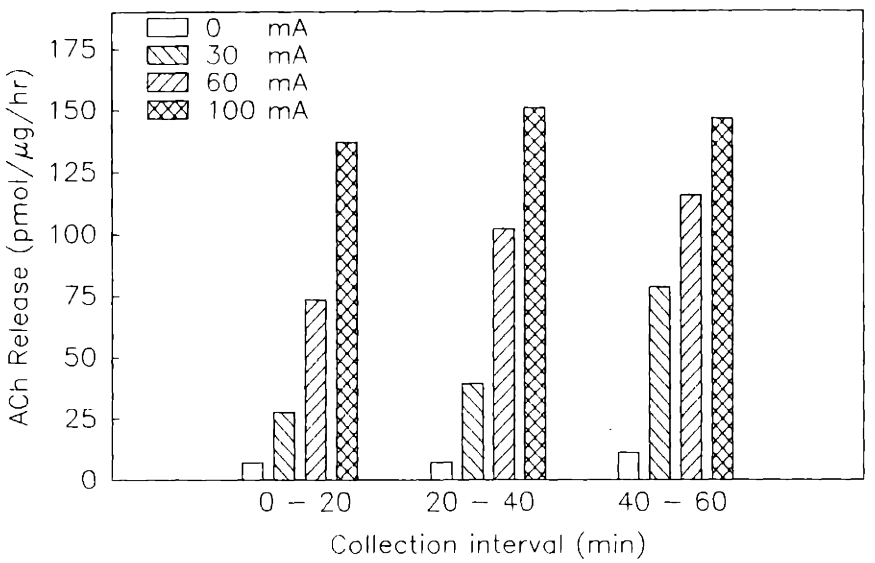

0 mA
30 mA
60 mA
100 mA
ACh Release (pmol/μg/hr)
175
150
125
100
75
50
25
0
0 – 20
20 – 40
40 – 60
Collection interval (min)

Figure 3

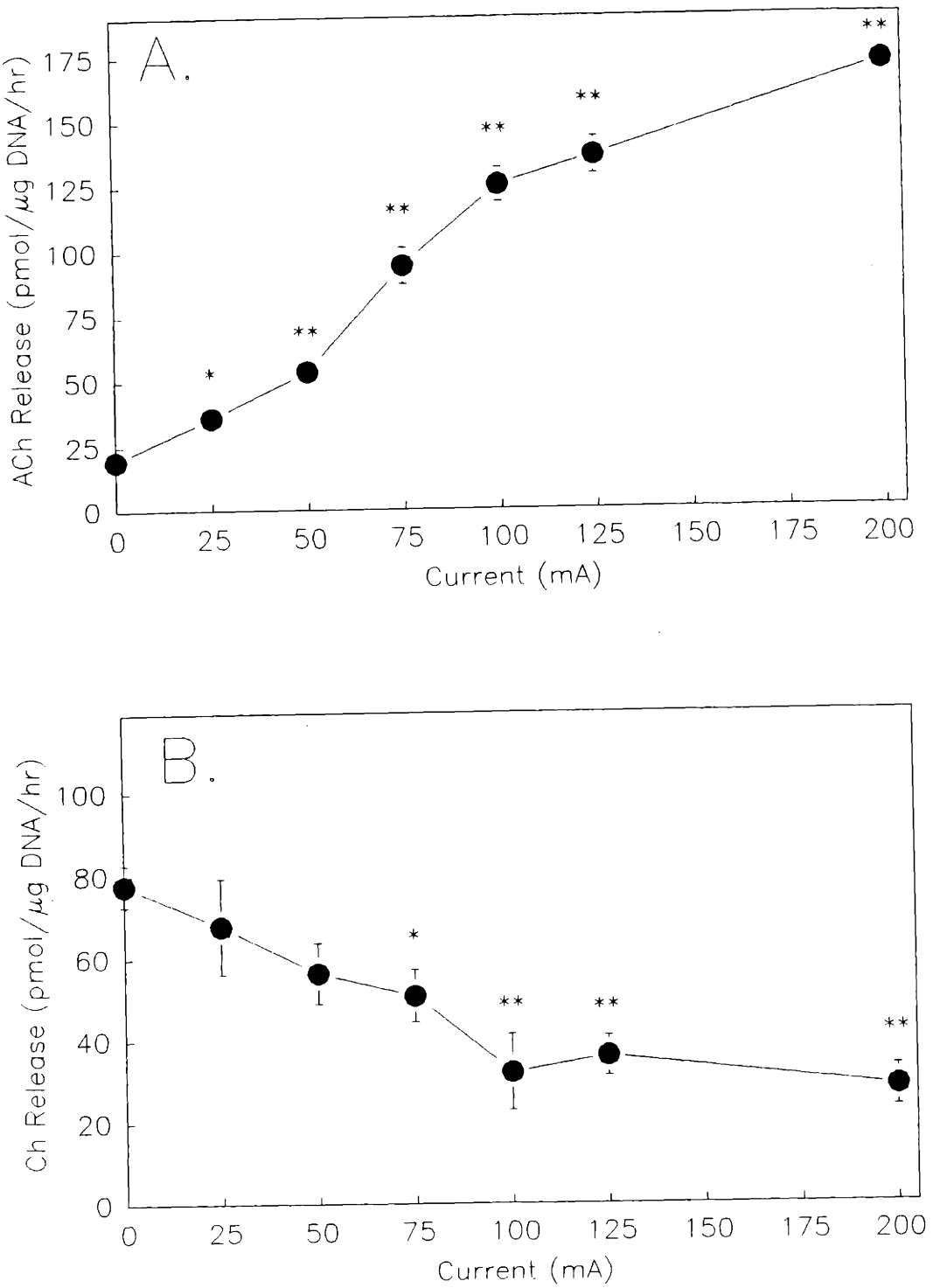

A.
ACh Release (pmol/μg DNA/hr)
0
25
50
75
100
125
150
175
0
25
50
75
100
125
150
175
200
Current (mA)
*
**
B.
Ch Release (pmol/μg DNA/hr)
0
20
40
60
80
100
Current (mA)

Figure 4

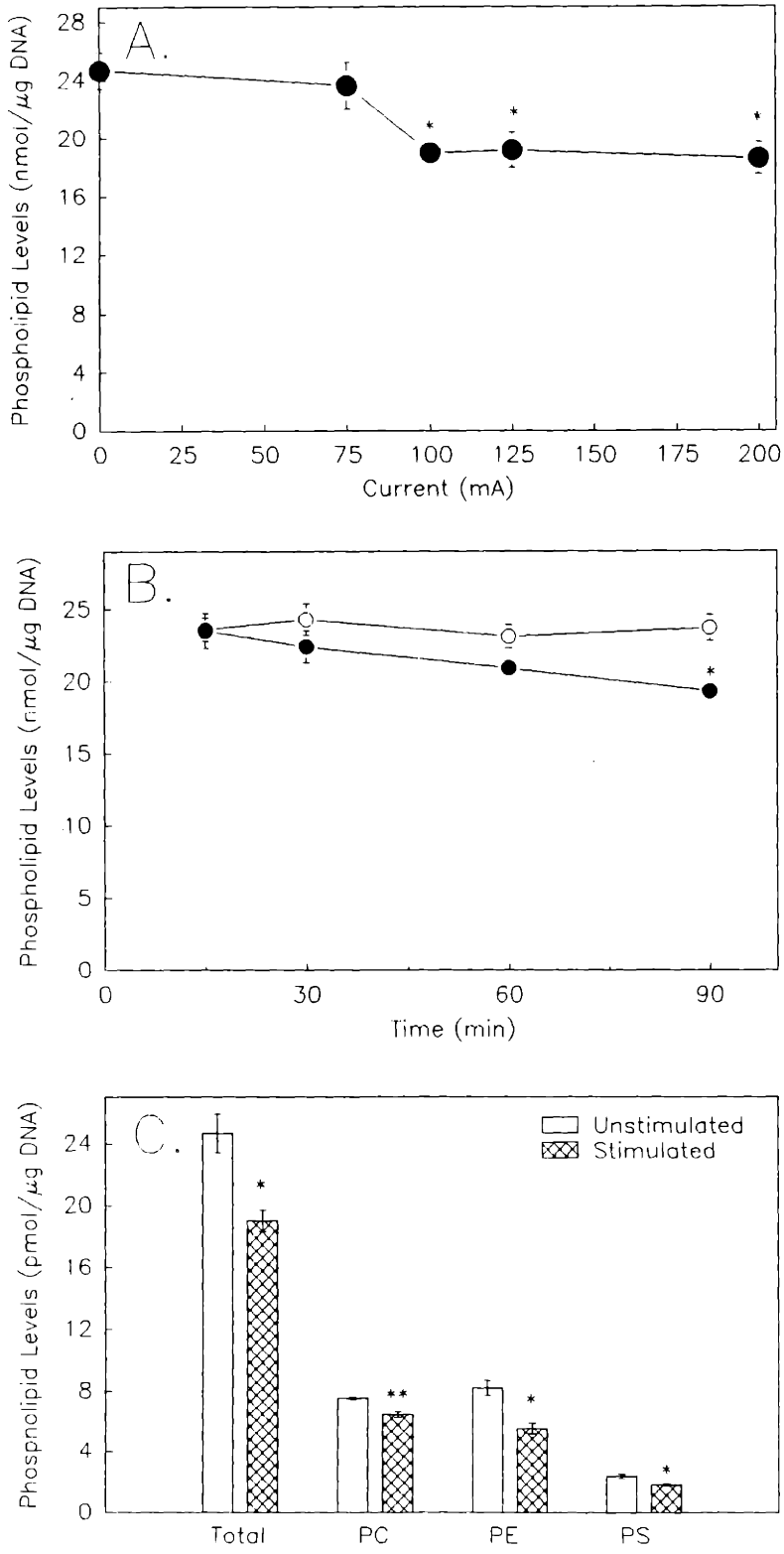

A.
Phospholipid Levels (nmol/μg DNA)
28
24
20
16
12
8
4
0
0
25
50
75
100
125
150
175
200
Current (mA)
B.
Phospholipid Levels (nmol/μg DNA)
25
20
15
10
5
0
0
30
60
90
Time (min)
C.
Phospholipid Levels (pmol/μg DNA)
Unstimulated
Stimulated
24
20
16
12
8
4
0
Total
PC
PE
PS

Figure 5

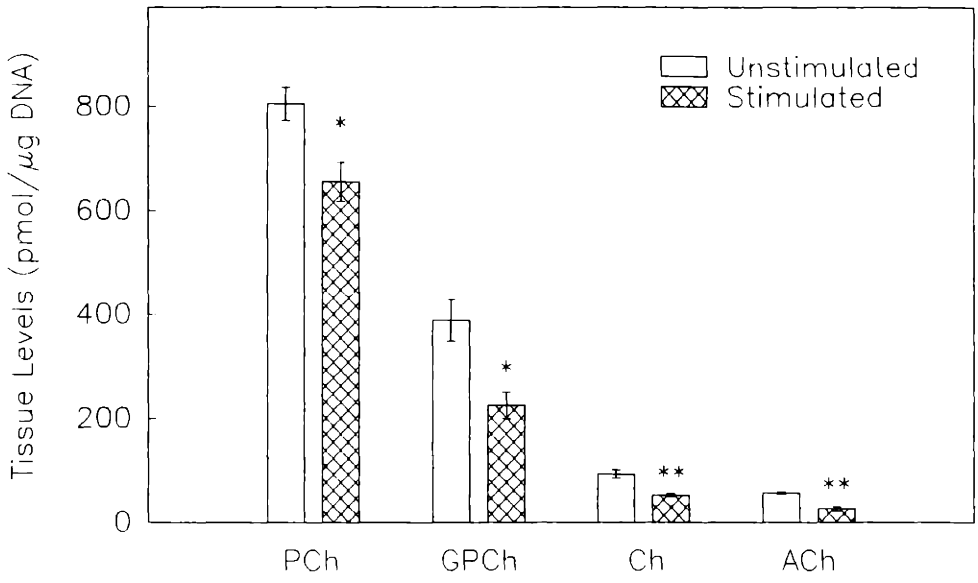

Tissue Levels (pmol/μg DNA)
800
600
400
200
0
Unstimulated
Stimulated
*
*
**
**
PCh
GPCh
Ch
ACh

Figure 6

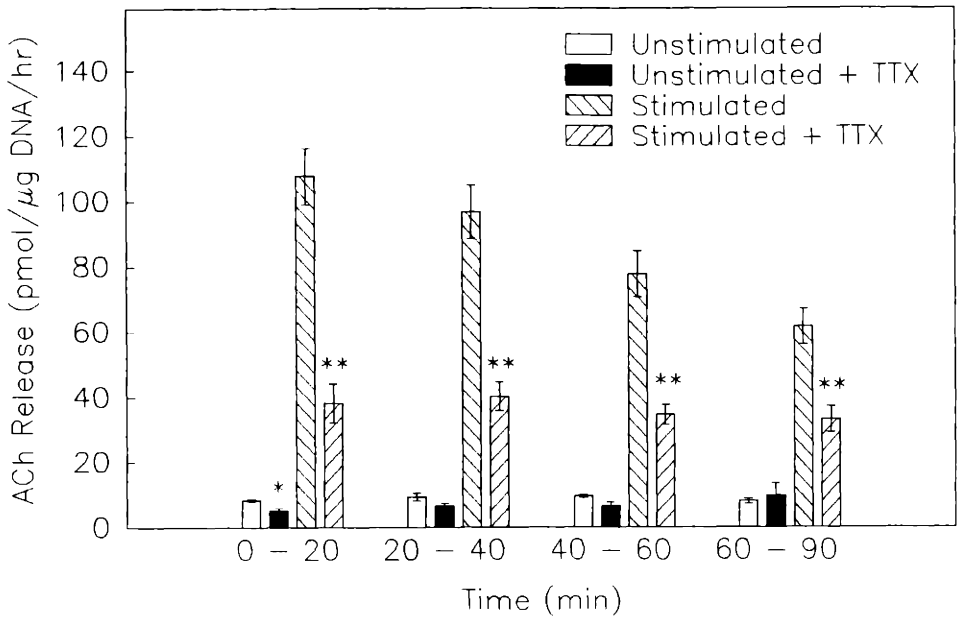

Unstimulated
Unstimulated + TTX
Stimulated
Stimulated + TTX
ACh Release (pmol/μg DNA/hr)
0
20
40
60
80
100
120
140
*
**
**
**
**
0 – 20
20 – 40
40 – 60
60 – 90
Time (min)

Figure 7

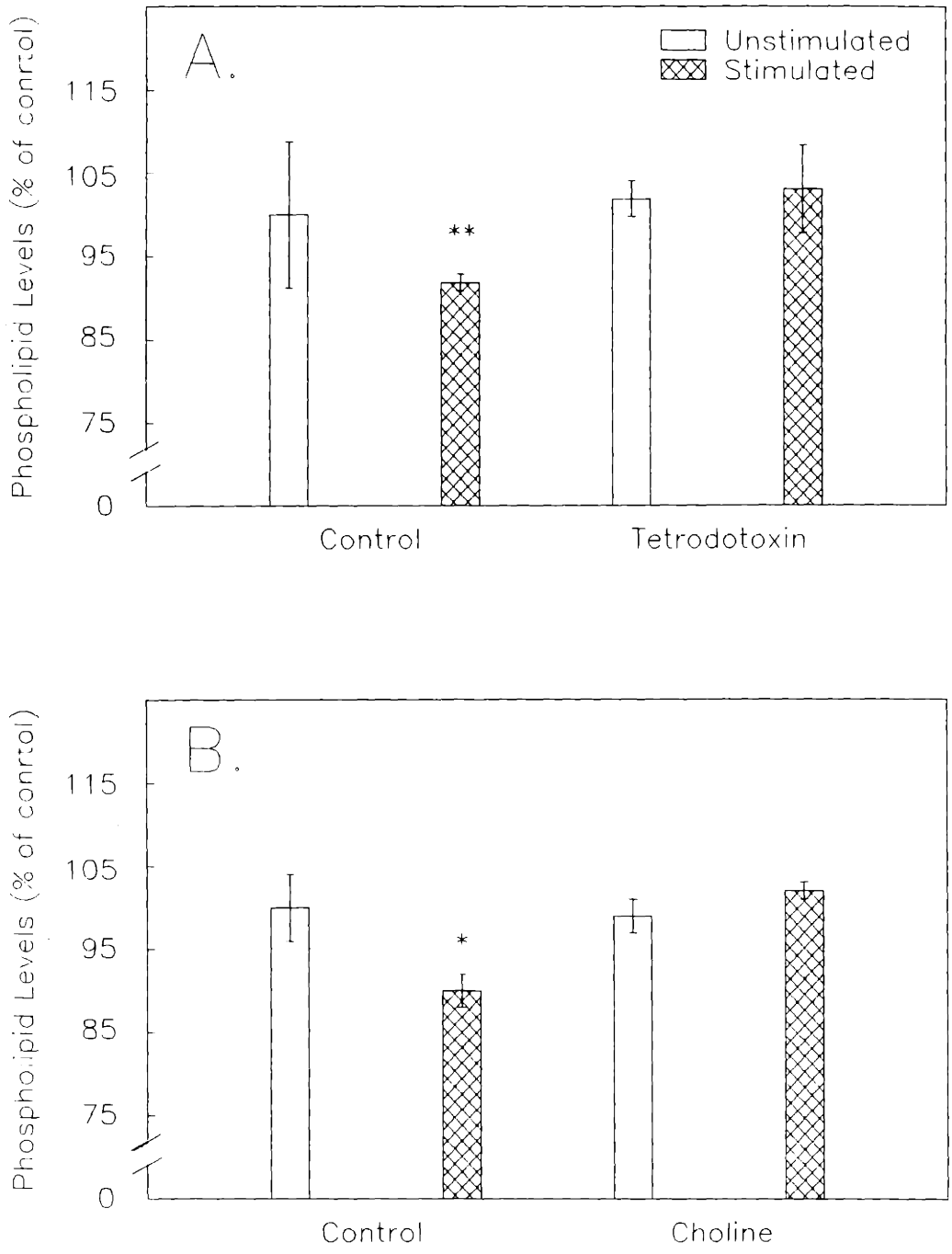

A.
Unstimulated
Stimulated
Phospholipid Levels (% of control)
115
105
95
85
75
0
**
Control
Tetrodotoxin
B.
Phospholipid Levels (% of control)
115
105
95
85
75
0
*
Control
Choline

Table I

Table 1. *Effect of field stimulation on lactate dehydrogenase activity and total protein*

		Control Slices	Stimulated Slices
		protein levels	
		ug protein/ ug DNA	
Protein	Tissue	42.9 ± 4.6	37.53 ± 1.74
	Medium	2.11 ± 0.3	1.55 ± 0.2
		mU/ug DNA	
LDH Activity	Tissue	62300 ± 8570	61800 ± 7620
	Medium	17.5 ± 2.0	18.9 ± 2.3

Neuronal activity inhibits choline phosphorylation while enhancing acetylcholine production and release: Choline flux and phosphatidylcholine turnover in superfused rat brain slices

Steven A. Farber, Vahide Savci, Alice Wei, Barbara E. Slack and Richard J. Wurtman
Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology
Cambridge, MA 02139

Key Words: Acetylcholine; Choline; Phosphatidylcholine; Phosphocholine; Phospholipids; Choline Acetyltransferase; Choline Kinase, Striatum

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

ABSTRACT

The mechanism by which cholinergic neurons regulate the flow of choline (Ch) towards membrane or neurotransmitter synthesis was investigated in stimulated superfused rat striatal slices. Electrical stimulation increased acetylcholine (ACh) release by 10-fold (i.e. from 0.34 ± 0.03 in the initial 20 min, from control slices, to 4.04 ± 0.55 nmol/mg/hr [n=4, $p< 0.01$] in stimulated tissues). High levels of release were sustained for 1 hr without the addition of exogenous Ch. The incorporation of [*Me*- ^{14}C]Ch into phosphorylcholine (PCh) (from 2320 ± 236 to 971 ± 256 DPM/mg [n = 8, $p< 0.001$]) and phosphatidylcholine (PC) (from 877 ± 161 to 416 ± 85 DPM/mg [n = 8, $p< 0.05$]) were markedly reduced by stimulation. The labeling of the ACh released into the superfusion medium was increased 10-fold (from 5890 ± 2580 to 59,000 ± 9060 DPM/mg/hr [n = 4, $p< 0.001$]) when compared with unstimulated controls. Stimulation failed to affect tissue [^{14}C]ACh and [^{14}C]Ch levels, but did significantly decrease the mass of these two compounds (for Ch ; 1840 ± 123 to 1370 ± 139 pmol/mg [n = 4, $p< 0.01$], for ACh; from 1910 ± 195 to 1100 ± 39 pmol/mg [n = 4, $p< 0.01$]. These decreases in Ch and ACh mass were entirely prevented by addition of Ch (100 mM) to the superfusate. The apparent synthesis of PCh, estimated using the specific activity of tissue Ch, was inhibited by stimulation. Thus, although PC labeling was reduced by stimulation, its net synthesis was unchanged (because the specific activity of the PC pool was decreased in proportion to the decrease in the PCh pool). The changes in the specific activities of tissue Ch, PCh and PC with time are consistent with the sequential conversion of Ch, to PCh, to PC, in that the specific activity of Ch was greater than that of PCh which in turn, was greater than that of PC. This was not the case for tissue Ch and ACh. In stimulated tissues, the specific activity of tissue ACh was higher than that of tissue Ch at 15, 30, and 45 min (e.g. the specific activities of Ch and ACh at 30 min were 5440 ± 690 DPM/nmol and 8360 ± 1150 DPM/nmol respectively [$p< 0.05$, n = 4]). Similarly, the specific activity of released

ACh was even higher than that of tissue ACh in both stimulated and control tissues, suggesting that a smaller more highly labeled Ch pool is the precursor for ACh, and newly synthesized ACh is preferentially released. The decrease in tissue PCh levels formed in stimulated tissues was approximately the same as the increase in ACh release into the medium. This effect was not associated with changes in the kinetics of Ch kinase (E.C.2.7.1.32) or Ch acetyltransferase (ChAT) (E.C. 2.3.1.7) activities as assayed in tissue homogenates. The turnover of PC was assessed using two experimental paradigms both involving a brief prelabeling with Ch followed by a chase period with or without unlabeled Ch (100 mM) in the superfusate. Under neither condition was PC turnover increased by stimulation.

INTRODUCTION

The metabolism of Ch by the brain has been investigated by a number of strategies. Intracerebrally injected Ch is rapidly taken up, phosphorylated and subsequently converted to CDP-Ch and then PC, a major lipid component (Ansell and Spanner, 1968). This pathway, first described by Kennedy and Weiss (1956), is a major route of PC synthesis in brain (Ansell and Spanner, 1968). Alternatively, labeled Ch can be incorporated by base-exchange with either phosphatidylserine (PS) or phosphatidylethanolamine (PE) (Kanfer et al. 1988; Arienti et al. 1976). A possible source of brain Ch is via sequential methylations of PE to PC followed by base-exchange or lipase activity to liberate free Ch (Blusztajn et al. 1987b; Yavin, 1985). While relative contributions of these various pathways in maintaining brain phospholipids remain to be discovered, the CDP-Ch pathway is the most prominent when labeled Ch is used a substrate (Klein et al. 1992).

Numerous studies have noted the there is a net efflux of Ch from the brain (Freeman et al. 1975; Dross and Kewitz, 1972) and that the process is reversed when

blood Ch levels exceed 16 mM (Klein et al. 1990). It has been proposed that the brain can store Ch in some other form based on the following observations: 1) The brain's capacity for *de novo* synthesis is low (Blusztajn and Wurtman, 1980). 2) Measurements of plasma Ch levels in brain often yield an artiovenous difference greater than negative 2 mM (Klein et al. 1992). 3) Ch is critical for ACh synthesis. 4) Brain free Ch levels are lower than those of most other Ch-containing compounds (Jope and Jenden, 1979). The rapid phosphorylation of Ch and its conversion to PC is thought to represent one mechanism for the storage of Ch in brain (Bertrand and Beley, 1990; Yavin et al. 1989; Millington and Wurtman 1982; Freeman et al. 1975). In Cholinergic neurons, this mechanism may be of particular importance because they allocate Ch between two distinct biochemical pathways: like all cells they must phosphorylate Ch to synthesize PC, and in addition, they acetylate Ch to form ACh.

The storage and regulation of brain Ch levels is important because ACh synthesis and release can be affected by changes in Ch availability (Yu and Van der Kloot, 1991; Maire and Wurtman, 1985; Weiler et al. 1983; Millington and Goldberg, 1982; Cohen and Wurtman, 1976), and Ch levels may be limiting; especially during periods of neuronal activity (Farber et al. 1993; Wecker, 1991; Wecker et al. 1989; Weiler et al. 1983; Trommer et al. 1982). The notion that there is a reciprocal relationship between PC and Ch levels is supported by *in vitro* studies. Preganglionic stimulation of the cat superior cervical ganglion, in the presence of an inhibitor of Ch uptake, hemicholinium-3 (HC3), resulted in an 18 % decrease in PC levels along with a reduction in the number of synaptic vesicles, an effect that was reversed by reperfusion with Ch (Parducz et al. 1986). Earlier work from this laboratory has shown that stimulated slices produced more ACh and Ch than can be accounted for by changes in the tissue levels of these compounds (Maire and Wurtman, 1985) and that PC levels in these slices are reduced (Blusztajn et al. 1987a). This reduction in PC was accompanied by concomitant reductions in PS and PE,

proportional to the number of stimulation periods, and was prevented by exogenous Ch (Ulus et al. 1989).

Because cholinergic neurons have a duel use for Ch, it is possible that decreases in Ch availability, or increases in neuronal activity might be associated with reductions in PC synthesis or increases in PC degradation in order to sustain the supply of Ch for ACh synthesis. There is evidence to support both possibilities. Studies in rat superior cervical ganglion suggest that at low Ch levels, ChAT activity is increased and Ch kinase activity is depressed, while at high Ch levels the relationship is reversed (Ando et al. 1987). This might indicate that Ch is diverted from PC by a reduction in Ch phosphorylation and thus in PC synthesis. In slice cultures of newborn rat septum, potassium depolarization resulted in an increased incorporation of [^{3}H]Ch into ACh and a decrease in PCh and PC labeling (Keller et al. 1987). While these studies are consistent with a stimulation-induced reduction in PC synthesis via a reduction in Ch phosphorylation, specific activities of the precursor pools were not measured; hence it is not clear whether the reduction in PC labeling reflected a true decrease in membrane synthesis.

There is a growing body of literature demonstrating that hydrolysis of PC by phospholipase D (PLD) and phospholipase C (PLC) can be regulated by extracellular signals (Conricode et al. 1992; Möhn et al. 1992; Qian and Drewes, 1991; Sandmann and Wurtman, 1991; Billah and Anthes, 1990; Qian and Drewes, 1990). Activation of these pathways could generate Ch that could conceivably be used for ACh synthesis. Muscarinic activation of rat brain synaptosomes resulted in enhanced PLD activity, Ch release, and its subsequent acetylation into ACh (Hattori and Kanfer, 1985).

A recent study of both PC and ACh synthesis in explants of sympathetic ganglion suggests that under conditions of impaired Ch uptake PCh synthesis is maintained at the expense of ACh synthesis (Suidan and Tolkovsky, 1993). This implies that developing neurites prioritize PC over ACh. The present study attempts to elucidate how neurons might "choose" between PC synthesis over that of ACh synthesis. We have addressed this

question by labeling superfused rat striatal slices with Ch under both basal and electrically stimulated conditions and by examining the effect of stimulation on the incorporation of label into Ch-containing compounds. Additionally, we assayed the effect of stimulation on the mass of the Ch-containing pools and on the activity of Ch kinase and ChAT.

EXPERIMENTAL PROCEDURES

Materials- [*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). Standard solutions (1 mM) of Ch, ACh, and PCh were frozen (at -20^{o}C) in aliquots. The bactericide, Kathon CG, was obtained from Rohm & Hass (Philadelphia, PA).

Animals- Experiments utilized male Sprague-Dawley rats (Charles River, Cambridge, MA) weighing 250 - 300 g and exposed to a 12/12 hr light/dark cycle. The animals were given free access to water and food (Charles River Rat, Mouse and Hamster Original Formula) 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 sacrificed by decapitation. Brains were removed and placed in chilled physiologic buffer and striata were rapidly dissected and sliced (300 mm) using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Gomstall, Surrey, U.K.). Slices were washed three times to remove debris with cold medium. The entire dissection, slicing and washing procedure was performed in cold room at 4^{o}C in less than 10 min.

Chambers and Stimulating System - The slices were then loaded into custom-designed chambers (1 striatum/chamber) (Warner Instrument Corp., Hamden, CT) that were maintained at 37^{o}C by immersion in a water bath. In some experiments slices were first incubated (37^{o}C) for 15 min in 1 ml of buffer containing either 3 mCi [*Me-*^{14}C]Ch chloride or 5 mCi [*Me-*^{3}H]Ch chloride then washed with buffer containing 100 mM Ch

prior to chamber loading. Chambers were continually superfused with warmed, oxygenated Krebs-Ringer buffer (in mM: 120 NaCl, 3.5 KCL, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 0.02 eserine salicylate) that was constantly bubbled with a mixture of 95% O_2 and 5% CO_2. In all experiments, slices were superfused (chamber volume of 0.7 ml at 0.8 ml/min) for either 1 hr or 45 min when slices had been preincubated prior to the start of sample collection and/or stimulation. In some experiments Ch (100 mM) or .25 mCi/ml [*Me*-^{14}C]Ch chloride were added to the superfusion medium.

The chambers represent a modified version of those previously described (Maire and Wurtman, 1985). Under conditions of maximal stimulation (125 mA, 1.0 ms and 15 Hz), the current density delivered by two fine silver screens located at the each end of the chamber was 4.95 mA/mm^2 (30 - 40 V). Slices were stimulated using a unique polarity reversal and resistance monitoring system (Warner Instrument Corp.). Electrical pulses originating from a square-wave stimulator (Model S88, Grass Instruments, Quincy, MA) were altered such that the polarity of every second pulse was reversed. The monitoring system simultaneously sampled the current and voltage 50 ms after the onset of each pulse to detect any changes in the chamber resistance (usually the result of bubbles formed as oxygen rich buffer leaves the narrow inlet tubing and enters the chamber expanse). A small access port in the side of the chamber facilitated the removal of bubbles and a 30 cm outlet tube attached to a screw cap that sealed the chamber enabled the easy collection of superfusates.

Labeling Paradigm- Three different labeling strategies were used in this study. The first approach was to equilibrate the slices for 1 hr then stimulate for an additional 1 hr. After 15 min of stimulation [*Me*-^{14}C]Ch chloride was introduced into the superfusion medium (25 mCi/100 ml buffer, 5 mM) for 30 min (Table 1). The second approach was similar to the first except the labeled Ch was present for the entire stimulation period and tissue samples were collected at 15 min interval after the initiation of labeling. In order to study

the turnover of PC a pulse-chase design was used. Slices were first incubated in 1 ml of buffer containing labeled Ch (3 mCi of [*Me*-^{14}C]Ch) for 15 min at 37°C then washed with buffer containing 100 mM Ch and placed into the chambers. For this series of experiments the superfusion buffer also contained 100 mM Ch (Table 1). In some experiments 5 mCi of[*Me*-^{3}H]Ch was used for the labeling and the 100 mM Ch chase was omitted. In all experiments concurrent unstimulated slices were utilized as controls.

At the completion of the experiment, slices were placed in ice cold buffer for 2 min. The buffer was then carefully removed and replaced with 1ml of a second buffer (230 mM sucrose, 1 mM $ZnSO_4$ and 20 mM Hepes pH 7.4). The tissue was then homogenized (10x Teflon/glass) and 1 ml of methanol was then added to the mixture.

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 (chloroform/methanol/water 2:1:1) (Folch et al. 1957), vortexed for 1 min and centrifuged (5 min 10,000 g). The upper aqueous phase containing ACh, Ch, cytidine diphosphate (CDP) - Ch, GPCh and PCh was separated from the lower lipid-containing phase. After extraction, the separate phases were dried under vacuum. For the determination of ACh and Ch mass, aliquots of dried aqueous phase were resuspended in water and subject to HPLC analysis using an immobilized post-column enzyme reactor containing Ch oxidase and ACh esterase (Farber et al. 1993).

To determine the radioactivity of water soluble Ch-containing compounds, concentrated samples of the aqueous fraction were separated and counted by HPLC with an on-line radioactivity monitor (Liscovitch et al. 1985). To determine unlabeled PCh content, the PCh faction was collected and treated with alkaline phosphatase (0.3 U/tube) for 3 hr, then centrifuged with methanol to precipitate the added enzyme. 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 (those in which the levels of radioactivity exceeded the HPLC sensitivity) samples were subject to

thin-layer chromatography on silica gel plates (Whatman LK5D) using a mobile phase of acetonitrile, ethanol, 30 % NH_4OH, 8.3 % NaCl (40:8:3:24, v/v) (Slack et al. 1991). Samples were scraped into scintillation vials containing 1 ml of 50 % methanol and counted.

Total lipid phosphate was determined from a dried aliquot of the organic phase by perchloric acid digestion (Svanborg and Svennerholm, 1961) utilizing a vapor trapping device. Individual phospholipids were separated by thin-layer chromatography on silica gel G, with chloroform: ethanol: triethylamine: water (30:34:30:8; v:v) as a mobile phase (Touchstone et al. 1980). The individual phospholipids were scraped off the plates and assayed for their total phosphorous content and/or radioactivity.

ChAT and Ch kinase assays - The ChAT assay was performed according to method of Fonnum (Fonnum, 1969). Briefly, frozen tissue homogenates were diluted in homogenization buffer to the desired concentration (usually 4 mg/ml). 5 ml of the homogenate were mixed with 10 ml of ice-cold reaction buffer (in mM: 2 AcCoA, 20 EDTA, 2 eserine, 50 NaH_2PO_4, 300 NaCl) with 7.5 mCi/ml of [*Acetyl*-^{3}H]AcCoA and varying amounts of Ch. The reaction was started by incubation at 37^{o}C and stopped after 10 min by placing the samples in ice-water. A boiled tissue sample was included in all experiments and subtracted. Labeled ACh was extracted by the addition of 1 ml 3-heptanone and 0.2 ml of NaH_2PO_4 (10 mM), pH 7.4 to each sample. An aliquot of the upper phase was then counted. The assay was linear with respect to tissue amount (from 0 - 30 mg protein) and incubation time (0 - 20 min).

Ch kinase was assayed using a modified method previously described (Spanner and Ansell, 1979). 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 mCi of [*Me*-^{14}C]Ch/ml was added to the reaction buffer. In some experiments final Ch concentration was varied. The reaction was initiated by the addition of 40 ml of reaction buffer to 10 ml of tissue homogenate (usually diluted to concentration usually 2 mg/ml) and the tubes were incubated at 37^{o}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 x 7.0 cm Amberlite CG-50, 200 - 400 mesh). A stock of resin was rinsed approximately 20 x with distilled water to remove fine particles. Columns were then poured and rinsed with 1 ml of water. The entire 1.5 ml of sample was applied to the column and the eluate was collected into a scintillation vial. An additional 0.9 ml of water was applied to the column and was collected into the same vial after which the column was removed and 19 ml of scintillation fluid was added (Hydroflour, National Diagnostics). The columns retained **all** of the radioactive Ch up to a concentration of 0.8 mM in the reaction buffer. A boiled sample blank for each Ch concentration (typically 50 DPM) was subtracted and the final activity was computed from the specific activity of the reaction buffer. We verified this column method by TLC as previously described above.

Analysis of Superfusates - After the slices had equilibrated, superfusates were collected from each chamber into ice-cold tubes. The amounts of ACh and Ch released were determined by HPLC analysis of 0.2 ml of filtered (0.2 mm) superfusate. In samples that contained 5 mM Ch and .25 mCi/ml of Ch it was not possible to separate the ACh from the Ch on either HPLC system. In order to circumvent this problem the Ch levels in the superfusates were reduced by greater than 100 fold by two purification steps. Superfusates (1 ml) were first applied to a silica column (5 x 60 mm bed of Bio-Sil A, 200-400 mesh; Bio-Rad Laboratories, Richmond, CA). The column was then washed with 3 ml of water, 3 ml of 0.03 M of HCl in 10 % (v/v) 2-butanone and then 1 ml of water. The last 3 ml were collected and dried. The dried samples were then 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. The total recovery for the entire process was approximately 30 %. Total superfusate protein was determined by vacuum ultrafiltration against water at 4^{o}C using cellulose dialysis tubing. This procedure

yielded a final volume of 4 ml which was lyophilized and reconstituted in water. LDH activity in 0.5 ml of superfusate and 2.0 ml of tissue homogenate was assayed using a modified version of the commercial kit available from Sigma Chemical (Sigma # 500).

Normalization - All measurements were normalized to total tissue protein using the bicinchoninic acid assay (Pierce, Rockford, IL) to adjust for variations in the amount of tissue loaded into each chamber. Total tissue DNA was measured according to the method of Labarca and Paigen (Labaraca and Paigen, 1980).

Statistics - Data are expressed as means $\pm$ S.E.M. All time-course data was analyzed by ANOVA with repeated measures to examine the effect of stimulation and time. ANOVA with a *post-hoc* Newman-Keuls test was used to assess the effect of time within a given group as compared with the initial time point. T-tests were used to compare the effects of stimulation at a given time point.

RESULTS

Continuous electrical stimulation (120 mA, 15 Hz and 1.0 ms pulse duration) of slices superfused in the absence of Ch resulted in a consistent release of ACh (Fig. 1). The entire superfusion medium was collected over the 1 hr period in three intervals. ACh release increased 10 fold in the first 20 min (from 0.34 $\pm$ 0.03 to 4.04 $\pm$ 0.55 nmol/mg/hr [$n = 4$, $p < 0.01$]) and was only slightly attenuated in the last 20 min (3.24 $\pm$ 0.28 nmol/mg/hr in stimulated slices). Ch release was not significantly affected by time or stimulation: In the first collection period Ch release was 3.78 $\pm$ 0.14 and 4.86 $\pm$ 0.47 nmol/mg/hr ($n = 4$) from control and stimulated slices respectively. Ch release in the last 20 min was 4.20 $\pm$ 0.06 and 3.88 $\pm$ 0.56 nmol/mg/hr from control and stimulated tissues, respectively.

In order to assess whether our stimulation paradigm might have compromised the viability of the slices, we measured the release of LDH from the slices during each 20 min collection interval. There was no significant difference between the LDH activity released from stimulated and control tissues during each time period. The average LDH activity

released into the medium over the entire stimulation period was 1.33 ± 0.15 mU/mg and 1.47 ± 0.17 mU/mg from control and stimulated tissues respectively (n = 4). There was also no difference in LDH activity in the tissue homogenates; less than 0.05 % of the LDH activity recovered in the tissue at the end of the experiment was released into the medium. Additionally, stimulation for 1 hr had no effect on tissue protein levels expressed per total tissue DNA (slices from groups contained approximately 40 mg protein/mg DNA) nor was there a change in the total protein released into the medium (approximately 5 % of total tissue protein).

Having established that stimulation profoundly enhanced ACh release without any measurable loss of cell viability, the utilization of Ch by the slices was assessed by adding [*Me*-^{14}C]Ch to the superfusion medium. Slices were equilibrated as usual and labeled as described in Table 1, series 1. Stimulation reduced the incorporation of labeled Ch into both PCh (from 2320 ± 236 to 971 ± 256 DPM/mg [n = 8, $p < 0.001$]) and PC (from 99.1 ± 14.1 to 35.3 ± 8.9 DPM/mg [n = 8, $p < 0.01$] while having no affect on tissue ACh and Ch labeling (Fig. 2A). Labeled ACh, like unlabeled ACh, increased approximately 10-fold during the half-hour labeling period (from 5860 ± 2590 to 59,000 ± 9060 DPM/mg/hr [n = 4, $p < 0.01$] and 15-fold during the 15 min wash out period (from 4940 ± 1330 to 79,100 ± 25,300 [n = 4, $p < 0.05$]) (Fig. 2B).

To more carefully study the stimulation-induced reduction in Ch phosphorylation, tissue levels of Ch and ACh were assayed over shorter time intervals to assess whether stimulation might alter the specific activity of Ch. ANOVA revealed both a significant effect of stimulation on tissue Ch mass at all time points ($p < 0.05$) and a significant effect of time in control and stimulated groups ($p < 0.001$) (Fig. 3A). Stimulation resulted in a consistent 20 % decrease in tissue Ch levels at all time points assayed (in the first interval Ch was decreased from 2360 ± 90 to 1930 ± 140 pmol/mg [n = 4, $p < 0.05$]). Tissue Ch levels decreased over the course of the experiment by 23 % in control tissues (to 1840 ± 120 [n = 4, $p < 0.05$]) and by 29 % in stimulated tissues (to 1370 ± 140 [n = 4, $p < 0.05$]).

The effect of both superfusion duration and stimulation were completely blocked by the addition of Ch (100 mM) to the superfusion medium (Fig. 3B). Tissue Ch levels were doubled by superfusion with Ch (to 4730 $\pm$ 120 pmol/mg [n = 4, $p < 0.01$].

Like Ch levels, tissue ACh was decreased by stimulation after 15 min (from 1940 $\pm$ 180 to 1310 $\pm$ 100 pmol/mg [n = 4, $p < 0.05$] (Fig. 3C). This effect was not significantly changed over the course of the experiment, in that ACh levels seem to decrease by an incremental amount immediately then remain constant in response to stimulation. Slices superfused with Ch (100 mM) showed no decrease in tissue ACh levels in response to stimulation. Unlike tissue Ch levels, tissue ACh levels did not increase with Ch superfusion (Fig. 3D).

PCh synthesis over time was studied in slices superfused with labeled Ch with and without stimulation (Table 1, labeling series 2). PCh labeling was affected by time ($p < 0.01$) and by stimulation ($p < 0.01$) and the effect stimulation also increased significantly over time ($p < 0.01$) (Fig. 4A). The incorporation of [^{14}C]Ch into PCh was dramatically reduced after 1 hr of stimulation (from 7120 $\pm$ 410 to 2520 $\pm$ 590 DPM/mg [n = 4, $p < 0.01$]). PC labeling followed the same pattern as PCh; there was an effect of stimulation ($p < 0.01$), and time ($p < 0.01$) and an interaction between the two ($p < 0.01$) (Fig. 4B). After 1 hr, PC labeling was decreased by 56 % in stimulated slices (from 4146 $\pm$ 432 to 1838 $\pm$ 199 DPM/mg [n = 4, $p < 0.01$]).

The specific activity of tissue Ch was calculated from measurements of both labeled and unlabeled Ch (Fig. 3A) from the same samples. Tissue Ch specific activity increased after 15 (28 %), 30 (38 %) and 45 (39 %) min of stimulation (Table II). The synthesis of PCh was determined by dividing the total radioactivity in PCh by the specific activity of Ch at each time interval. Stimulation resulted in a 70 % reduction in PCh synthesis over a 1 hr period (from 1810 $\pm$ 396 to 560 $\pm$ 175 pmol/mg/hr [n = 4, $p < 0.05$]) (Fig. 4C). Using PCh mass measurements collected from a different series of experiments in which slices were prepared and treated identically to those in the preceding experiment,

the specific activity of PCh was determined. PCh levels were uneffected after 40 min of stimulation and were reduced after 1 hr (from 15700 ± 3320 to 11296 ± 1184 pmol/mg). To calculate the specific activity of PCh at 15, 30, 45, 60 we interpolated from the mass measurements at 20, 40 and 60 min. The specific activity of PCh, as compared with controls, was significantly reduced after 30 (59 %) , 45 (57 %) and 60 (56 %) min of stimulation (Table II). The reductions in PCh labeling were paralleled by reductions in PC labeling with the result that when the total PC synthesis was calculated from the specific activity of PCh, there was no change in the rate of PC synthesis from stimulated tissues (Fig. 4D). The total PC synthesis over the 1 hr labeling period was 9.29 ± 0.97 and 14.67 ± 1.59 nmol/mg/hr in control and stimulated tissues respectively.

We also measured both ACh label and mass at each time point. Like Ch, the specific activity of ACh was significantly increased by stimulation after 15 (68 %), 30 (68 %) and 45 (83 %) min (Table II). In order to assess whether the ACh in tissue was derived from the tissue Ch pool, the mean specific activities at all times were determined for both compounds. The specific activity of ACh in stimulated tissues was twice that of tissue Ch (10600 ± 1210 as compared with 4760 ± 511 DPM/nmol [$p < 0.05$]) whereas the specific activity of released ACh was three times that of tissue Ch (16800 ± 3290 DPM/nmol [$p < 0.05$]). When comparing the specific activities over time, the specific activity of tissue ACh was approximately the same as tissue Ch in unstimulated tissues (Table II). In stimulated tissues, the specific activities of both tissue and released ACh were greater than the Ch specific activity all time points.

A pattern consistent with a precursor/product relationship was evident in the specific activities of Ch, PCh and PC. In contrast to ACh, the ratio of the specific activity of PCh to the specific activity of its precursor, Ch was less than one and increased over time from 0.052 to 0.074 (Table II). Likewise the ratio of the specific activity of PC to that of PCh also increased over time and ranged from 0.012 to 0.044. After 1 hr there

was a dilution of 300 times in the specific activity of PC compared to that of Ch in unstimulated tissues.

In order to assess whether released ACh might be synthesized directly from Ch taken up from the medium prior to dilution by tissue Ch, we calculated the mass of ACh released by dividing the ACh radioactivity recovered in the medium by the specific activity of the Ch in the medium. The predicted amount of ACh release was seven time lower than the actual amount released by HPLC, suggesting that the medium specific activity was seven times greater than that of the actual precursor pool (Table III). On the other hand, the predicted ACh release calculated from the specific activity of the tissue Ch pool was three times higher than the actual release, indicating a Ch precursor pool of specific activity three times greater than the tissue overall Ch pool. The actual specific activity of the ACh precursor, inferred from the ACh specific activity determined in Table II, was approximately 16,000 DPM/nmol, a value in between the medium (116000 DPM/nmol) and the tissue (5440 DPM/nmol) Ch specific activities.

To study the effect of stimulation on the turnover of PC, a pulse-chase paradigm was used. Slices were first incubated with labeled Ch then washed and placed in the chambers. Initially, we used 5 mCi [*Me*-^{3}H]Ch/striatum with a specific activity 100 times greater than that of [^{14}C]Ch. Slices were washed with 100 mM Ch but not superfused with Ch. After equilibration some slices were stimulated. Aliquots of superfusates were counted since we had established that a majority of the released counts were found in ACh (Fig. 3). Most of the label was released within the first 20 min, and declined to control levels after 60 min (Fig. 5A). Despite this decline in released radioactivity, tissue Ch-containing compounds still contained significant amounts of label at this time.

The specific activity of PCh was calculated, as described earlier, and was not significantly affected by stimulation during the chase period (Fig. 5B). Likewise, there was no effect of stimulation on radioactivity in PC (Fig. 5C). While the slope of the PCh

specific activity verses time curve was negative throughout the chase, the specific activity of PC *vs* time, peaked or at least had a slope of zero. It has been stated that the specific activity of a precursor must equal that of the product when the product's specific activity reaches a maximum (Zilversmit et al. 1943). In our experiments, however, the PCh specific activity was considerably higher than that of PC. Using the radioactivity measured in the PC, we calculated the PC pool mass that would fulfill this criteria. The result was a PC pool size of approximately 20 -30 nmol/mg. .

In order to more effectively chase label out of the Ch-containing PC precursors (so that the PC specific activity slope was negative at all points) we superfused with 100 mM Ch after labeling with Ch of lower specific activity (53 Ci/mol as opposed to 86.7 Ci/mmol) (Table III, series 3). Using this paradigm, PCh levels were reduced close to the limit of detection after 50 min of equilibration and 1 hr stimulation (Fig. 6A). Stimulation had no effect on PCh levels at all time points during the chase period (Fig. 6B). The total PC labeling in both control and stimulated groups was averaged and a linear regression was performed ($r=0.884$). The estimated turnover of this labeled pool was approximately 40 % per hour. The downward trend in PC labeling was not significant and stimulation did not alter the turnover of labeled PC when the labeling occurred prior to stimulation.

The pronounced reduction in Ch phosphorylation elicited by stimulation was observed in a structure rich in cholinergic interneurons. To assess the regional specificity of this effect, we repeated our initial experiments (Table I, series 1) in the cerebellum, a structure with considerable less cholinergic innervation than the striatum. PCh labeling was reduced to a much lesser extent by stimulation than it was in the striatum (from 2790 $\pm$ 213 to 1670 $\pm$ 331 [$n = 3$, $p< 0.05$]) (Fig. 7). Moreover, in contrast to striatum, there was no change in PC labeling (Fig. 7) or tissue Ch levels (1100 $\pm$ 57 and 1130 $\pm$ 38 pmol/mg in control and stimulated tissue respectively). Ch release from these slices was 3508 $\pm$ 216 pmol/mg/hr from control and 4036 $\pm$ 147 pmol/mg/hr from stimulated tissues.

A possible mechanism for enhanced ACh synthesis and decreased Ch phosphorylation might be changes in the activity of Ch kinase or ChAT. To determine whether these enzymes might be altered in response to stimulation, the activity of both enzymes was assayed in homogenates from control and treated slices. We first verified the linearity of these assays with respect to protein and time (see methods). To thoroughly examine the kinetics of these enzymes, homogenates (20 mg of tissue) were incubated in various amounts of Ch at saturating concentrations of ATP, for Ch kinase assays, and AcCoA, for ChAT assays. Both enzymes exhibited classic Michaelis-Menton kinetics and were unaffected by stimulation (Fig. 8).

DISCUSSION

These data indicate the electrical stimulation of superfused striatal slices results in enhanced ACh release and reduced Ch phosphorylation that cannot be accounted for by alterations in Ch kinase or ChAT activities as assayed in tissue homogenates. We initially established that superfused slices can release large amounts of ACh in response to continuous electrical stimulation for over 1 hr without the addition of exogenous Ch (Fig. 1) a finding consistent with earlier reports (Buyukuysal and Wurtman, 1990; Ulus et al. 1989; Maire and Wurtman, 1985). Stimulation induced ACh release was not associated with decreased viability. LDH, an established marker of cell injury (Koh and Choi, 1987), and total protein release from equilibrated slices was minimal and unaffected by stimulation. .

Stimulation in the presence of [^{14}C]Ch markedly reduced PCh and PC labeling while leaving Ch and ACh pools unchanged (Fig. 2A). However, labeled ACh recovered in the medium was greatly enhanced and remained steady even during the 15 min washout period (Fig. 2B). While stimulation had no effect on labeled Ch and ACh pools the total levels of Ch and ACh mass within the tissue were significantly reduced (Fig. 3A & 3C), an effect that was prevented by the addition of Ch to the superfusate (Fig. 3B & 3D). This resulted in an increase in the specific activity of Ch within stimulated tissues, and a

reduction in PCh synthesis that was proportionately greater than the reduction in PCh labeling alone.

A more rigorous way to study the synthesis of PC and ACh using labeled Ch would require the measurement of specific activity of each Ch-containing pool over time (Zilversmit et al. 1943). In a second series of experiments (Table I, series II) we harvested tissues during the labeling period and determined both the total radioactivity and the mass of each pool. Both PCh and PC labeling was significantly enhanced by increased labeling time and, as we expected from the results of the first labeling series, reduced by stimulation (Fig. 4A & 4B). Unexpectedly, the mass of PC synthesized, calculated by dividing the radioactivity in PC by the specific activity of PCh, was increased by stimulation (Fig. 4D). The reduction in PC labeling simply reflected reduced labeling of the PCh pool in stimulated tissues. Given that PCh synthesis is almost entirely inhibited by stimulation (Fig. 4C) while PC synthesis continues unabated, it is probable that eventually the PCh pool would be depleted, ultimately resulting in a decrease in PC synthesis. However, within the time frame of this experiment (75 min of stimulation) this consequence was not observed.

In order to calculate the amount of PC synthesized we treated PCh as if it was the immediate precursor to PC, ignoring the intermediate 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 3680 pmol/mg (Arienti et al. 1976) as compared to 20,120 pmol/mg of PCh. Thus, it is likely that the specific activity in CDP-Ch would quickly equilibrate to that of PCh. In studies where rats were injected intracerebrally with [^{3}H]Ch and sacrificed at regular intervals, CDP-Ch radioactivity was low but attained the same specific activity as PCh as early as 10 s after the injection (Arienti et al. 1976).

An analysis of the specific activity over time of the various Ch-containing compounds revealed a number of interesting findings (Table II). The specific activities of

tissue ACh and Ch were significantly higher in stimulated tissues during the first three labeling intervals, This reflects a reduction in the pool size of these compounds (Fig. 3). In fact, the radioactivity recovered in Ch and ACh was unchanged at all time points (data not shown). After 1 hr of labeling, the specific activities of Ch, PCh and PC were consistent with a precursor-product relationship in that each product had a lower specific activity than its precursor (i.e. in unstimulated tissues the specific activity of Ch, PCh, and PC were 4750, 453, and 15.8 DPM/nmol respectively) (Table II). This is in agreement with *in vivo* studies that indicate that the CDP-Ch pathway is a major route of PC synthesis in the brain (Suidan and Tolkovsky, 1993; Arienti et al. 1976; Ansell and Spanner, 1968).

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 (Bishop and Bell, 1988; Pelech and Vance, 1984). If as is generally believed, cytidylyltransferase is the rate-limiting enzyme in this pathway, then changes in PCh levels might have little effect on PC synthesis. However, an increasing number of studies suggest that Ch kinase can also play a significant role in regulating PC synthesis (Bishop and Bell, 1988). Cell culture experiments using *Ras*-transfected C3H10T1/2 cells indicate that elevated PCh levels resulting from enhanced Ch kinase activity, can increase PC synthesis (Teegarden et al. 1990). A similar effect was reported 3T3 fibroblasts, serum treatment enhanced Ch kinase activity which subsequently increased PC turnover and decreased tissue Ch levels (Warden and Friedkin, 1985).

In cells that are synthesizing large amounts of membrane, Ch kinase can clearly play a regulatory role in PC synthesis. However, in brain slices, inhibition of Ch kinase might shunt Ch towards ACh synthesis when Ch levels are low. The total reduction in the mass of tissue PCh was almost exactly the same as the mass of ACh released from the slices. The total increase in ACh released during 1 hr was 3250 $\pm$ 340 pmol/mg (Fig. 1) and the reduction in PCh was 3780$\pm$ 150 pmol/mg ($p< 0.05$, $n = 4$) (data not shown).

Studies where rats were fed a Ch deficient diet found that while brain ACh was unchanged, PCh levels were reduced by 30 % (Millington and Wurtman, 1982).

A stimulation induced decrease in Ch phosphorylation was reported in slice cultures of newborn rat septum (Keller et al. 1987). In this study, potassium depolarization reduced both labeled PCh and PC by 32 %. The decrease in PCh labeling was approximately the same as the increase ACh label recovered in the medium. These data suggest, and the present study, suggest that lipid synthesis was not compromised during the potassium depolarization, since the decrease in PC label was proportionally the same as the decrease in PCh labeling. Studies using cultured sympathetic neurons indicate that neurites have a greater capacity for incorporating labeled Ch into ACh and PC then cell bodies (Suidan and Tolkovsky, 1993). The authors of this study found that inhibition of Ch uptake halted labeled Ch incorporation into ACh while leaving PCh synthesis unchanged, suggesting that developing neurites preferentially route Ch towards lipid synthesis. In contrast, in cortical synaptosomes inhibition of Ch uptake reduced incorporation of labeled ACh and PCh label incorporation (Meyer et al. 1982). These studies, while suggestive, are limited by their reliance solely on measurements of radioactivity without any analysis of the mass of Ch-containing compounds. Additionally, the reduction of ACh labeling during conditions of inhibited Ch uptake, observed in the previous studies, might reflect more on the association of ChAT with Ch uptake mechanisms and/or the presence of different Ch compartments than on the regulation of the flux of Ch into phospholipid and ACh synthesis.

To assess whether the decrease in Ch phosphorylation was specific to cholinergic structures we repeated the experiment in cerebellar slices (Table I, series 1). Stimulation reduced PCh labeling to a lesser extent than in the striatum (Fig. 7). However, PC labeling was unchanged suggesting that cytidylyltransferase activity might have been increased. This hypothesis could not be tested because PCh mass was not assayed in tissue samples from this structure. The weaker effect of stimulation on Ch

phosphorylation might reflect the sparse cholinergic inervation to this structure (Barmack et al. 1992; Ikeda et al. 1991).

The effect of stimulation of Ch kinase and ChAT - A mechanism by which stimulation reduced Ch phosphorylation might involve changes in the activity of Ch kinase or ChAT. Such a mechanism was suggested by studies in rat superior cervical ganglia where low Ch levels in the extracellular fluid resulted in decreased Ch kinase activity and increased ChAT activity (Ando et al. 1987). We attempted to determine if such a mechanism might regulate Ch metabolism in the brain slice. We found no effect of stimulation on the kinetics of Ch phosphorylation or acetylation (Fig. 8). We also found no effect of superfusing the slices with exogenous Ch on the activities of these enzymes (data not shown). These experiments do not rule out the possibility that some form of inhibition of Ch kinase occurs in the intact slices that is lost during homogenization.

Reported Ch Km values for ChAT typically range from 1000 to 20 mM depending on the ionic strength and the degree to which the enzyme is bound to membranes (Ando et al. 1987; Tucek, 1985; Blusztajn and Wurtman, 1983). The usual reported value for homogenates is 400 mM (Blusztajn and Wurtman, 1983). In the present study the Km for Ch was 130 $\pm$ 30 (assayed from 10 min incubations) When data from homogenates incubated for 30 min (the typical time reported) was used the Km was 332 $\pm$ 56 (data not shown). However, in our hands, the synthesis rate was only linear for 20 min. These data suggest that the large range of Km values reported in the literature may stem from variations in assay conditions.

Our calculated Km value for Ch kinase using a 30 min incubation was significantly higher, and typical of reported values (Haubrich, 1973), than the Km based on a shorter incubation time (7.13 $\pm$ 1.21 mM). It has been suggested that the increased Km at longer incubation times is due to "alternative pathways of ATP metabolism" that effectively deplete the ATP concentration to subsaturating levels (Burt and Brody, 1975). The Vmax

of Ch kinase obtained in the present study (4.5 $\pm$ 0.3 nmol/mg/hr) agrees with reported values from striatum using a 10 min incubation period(Wecker and Reinhardt, 1988).

On the issue of a single precursor Ch pool used for both ACh and PCh synthesis -The relationship between the specific activity of the tissue Ch and tissue ACh does not fit the criteria to establish a precursor product relationship (Zilversmit et al. 1943). In stimulated tissues, the specific activity of tissue ACh is significantly higher than that of tissue Ch at all but the last time points ($p< 0.05$) (Table II). Additionally the released ACh has an even higher specific activity than the tissue ACh pool (Table II). It might be the case that the released ACh is synthesized from the medium Ch which is significantly more radioactive than the tissue Ch pool. Ch could be taken up by the high affinity transporter and a fraction acetylated and released prior to mixing with the tissue Ch pool. However, we determined that the radioactivity in the medium must be diluted at least seven times with unlabeled Ch to achieve the specific activity measured in ACh, a level closer to the tissue Ch specific activity.

The notion that ACh release is coupled to Ch uptake is derived from experiments in which high affinity Ch uptake was blocked with HC3. Under these conditions, tissue Ch levels remained high while tissue ACh levels dropped to near zero (Suidan and Tolkovsky, 1993; Yavin et al. 1989; Maire and Wurtman, 1985). These data could also be explained by a selective effect of uptake block on cholinergic cells (only the cholinergic phenotype express the high affinity transporter) which in the striatum represent less than 5% by cell number (Gerfen et al. 1987). A blockade of only high affinity uptake would have a negligible effect on total tissue Ch levels, since non-cholinergic cells would contribute 95 % of the Ch pool while tissue Ch levels in cholinergic cells could be near zero, and producing little ACh. Thus, a direct coupling of Ch uptake to ACh synthesis is not required to explain the effect of HC3. If this is also the explanation for why the ACh specific activity is higher than the tissue Ch pool, then it must be assumed that in the non-cholinergic cells the Ch label has not equilibrated. This would result in a higher tissue Ch

specific activity in cholinergic cells (they equilibrate faster because they have high affinity uptake). This model would predict that the specific activity of tissue Ch should be increasing over time, which was not observed. (both stimulated and control slices equilibrated within 30 min [Table II]).

The proposal that ACh synthesis is directly coupled to uptake was seriously weakened by double labeling studies using [^{3}C]Glucose to prelabel the ACh pool via the incorporation of labeled AcCoA, and [^{3}H]Ch to label newly synthesized ACh via Ch uptake (Kessler and Marchbanks, 1982). These studies strongly suggest that a Ch molecule from within the cell is as likely to be acetylated as one transported. However, the authors noted the relation between the extracellular Ch specific activity and the specific activity of the ACh formed. Interpolating the ratio at 5 mM Ch, the concentration of Ch used in this study, the specific activity of the ACh in Kessler and Marchbanks study was six times less than the medium specific activity of the added Ch, a number close to the seven-fold difference seen in the present study.

The difference between the specific activity of the released ACh and the tissue ACh is easier to explain. The hypothesis that the "last synthesized is the first released" has been suggested by a number of labeling studies (Barker et al. 1972; Potter, 1970). It has been proposed that ACh synthesized near the synaptic region would attain a higher specific activity than that already stored in vesicles prior to labeling. This model would predict that over time, the specific activity of tissue ACh pool would approach the released ACh specific activity. In fact, this is exactly what we observed. At 30 and 45 min the specific activity of the released ACh was approximately the same while tissue ACh specific activity increased with time and only approached the released value after 1 hr (Table II). Our data is entirely consistent with a two compartment model for ACh storage, with a cytoplasmic compartment that contains the most recently synthesized ACh and a vesicular compartment that does not exchange readily with the cytoplasmic compartment (Kessler and Marchbanks, 1982).

The Turnover of PC - In order to assess whether stimulation might enhance phospholipid turnover we performed a series of labeling experiments in which slices were labeled for only 15 min and then equilibrated and stimulated in the presence and absence of Ch (Table I, series III). In the first experiment we labeled with [^{3}H]Ch (100x the specific activity of the [^{14}C]Ch) and did not chase with Ch. We found that stimulated slices released a majority of their labeled ACh and Ch during the first stimulation interval (Fig. 5a). The specific activity time relations for both PCh and PC were determined revealing no significant effect of stimulation (Fig. 5B & Fig. 5C).

Zilversmit et al. contributed greatly to the mathematical basis of pulse labeling studies and turnover rate calculations (Zilversmit et al. 1943). He first specified four hallmarks required to establish a precursor product relationship: 1) the immediate precursor must be a stable compound, 2) during the early interval after administration of the label, the precursor must have a higher specific activity of the product, 3) during a later interval the product must have a greater specific activity than the precursor and 4) at a point when the rate of change of the specific activity of the product becomes zero, (i.e. when the product specific activity reaches a peak), the specific activities of the precursor must equal that of the product (Zilversmit et al. 1943). Our labeling paradigm was neighborhood of such a region (the PCh slope was decreasing while the PC slope was flat or peaked) (Fig. 5C). As Zilversmit et al. outlined this is the point where the precursor (PCh) slope was negative; however, the specific activities were not equal.

If only a fraction of the total PC pool represented a newly synthesized compartment then the above criteria would be met. Assuming that the specific activities of PCh and PC were equal we solved for the size of the PC pool that would make that relation true and obtained values of between 20 - 30 nmol/mg of PC instead of the measured value 262 nmol/mg. These data suggest that a pulse of Ch labels only a pool representing 10 % of the total PC.

Since the specific activity of PC only started to decrease after 60 min (Fig. 5C) we could not conclude that the rate of disappearance of labeled PC was unaffected by stimulation. However, it was not technically feasible to extend the experiment to the times need to assess the disappearance rate. To address these concerns, the amount and the specific activity of the Ch label was reduced and Ch (100 mM) was present during the entire equilibration and stimulation periods. This resulted in a decreasing rate of PC labeling at all time points during the chase and revealed that stimulation had no effect on the disappearance rate of PC (Fig. 6B).

It is difficult to reconcile these findings with experiments, in our laboratory using the same slice paradigm, that suggest that stimulation activates phospholipases. Electrical stimulation enhanced phosphatidylinositol breakdown by six fold in striatal slices (data not shown) and in other cell culture experiments, treatments which enhance PLC also activate PLD (Qian and Drewes, 1991; Sandmann and Wurtman, 1991; Pelech and Vance, 1989). Given that the slices are releasing 10 times the basal rate of ACh and that these cells contain muscarinic receptors it likely that phospholipase A_2 is also activated (Conklin et al. 1988; Yamada et al. 1988). Yet, we could not detect any labeled glycerophosphocholine (GPCh), a breakdown product of PC generated by PLA activity. Labeled GPCh is usually not detected in acute labeling paradigms (Suidan and Tolkovsky, 1993; Jope and Jenden, 1979; Francescangeli et al. 1977). However, if labeled Ch is added to the diet, labeled GPCh is detectable after 2 days (Jope and Jenden, 1979). It could be that the pool of PC cleaved by lipases is different than the one labeled with a brief Ch pulse.

While the GPCh data from these experiments and those reported by others, are consistent with a two-compartment model (new and old PC), the lack of GPCh detection might simply reflect the lack of significant label in PC. Alternatively, the inability of stimulation to significantly alter labeled PC disappearance might indicate that the activation of lipases has a trivial effect on the overall mass of the PC pool. However, the

specific activity time curve data from this study and experiments examining the acylation patterns of PC support a distinction between old and new phospholipids. In experiments in which [2-^{3}H]glycerol was injected into the brains of young rats the PC composed of 16:0 - 18:1 incorporated the most label but, turned over more rapidly than 18:0 - 18:1(Ousley and Morell, 1992).

These experiments highlight the difficulties associated with the interpretation of labeling studies in a complex brain structure. However, taken as a whole, these data suggest that neuronal activity can significantly inhibit the conversion of Ch to PCh to a degree which equals the mass of Ch released as ACh. The changes in Ch phosphorylation cannot be explained by alterations in the activities of Ch kinase or ChAT as assayed in tissue homogenates. The turnover of labeled PC is not increased by stimulation, although the labeled pool probably represents only 10 % of the entire tissue PC. While the entire tissue Ch pool can conceivably represent the precursor for PC synthesis it cannot explain the high specific activities found in ACh.

REFERENCES

Ando M., Iwata M., Takahama K., and Nagata Y. (1987) Effects of extracellular choline concentration and K+ depolarization on choline kinase and choline acetyltransferase activities in superior cervical sympathetic ganglia excised from rats. *J. Neurochem.* **48(5)**, 1448-1453.

Ansell G.B. and Spanner S. (1968) The metabolism of [Me-14C]Choline in the brain of the rat in vivo. *Biochem. J.* **110**, 201-206.

Arienti G., Corazzi L., Woelk H., and Porcellati G. (1976) Biosynthesis of rat brain phosphatidylcholines from intracerebrally injected choline. *J. Neurochem.* **27**, 203-210.

Barker L.A., Dowdall M.J., and Whittaker V.P. (1972) Choline metabolism in the cerebral cortex of guinea pigs. *Biochem. J.* **130**, 1063-1080.

Barmack N.H., Baughman R.W., and Eckenstein F.P. (1992) Cholinergic innervation of the cerebellum of the rat by secondary vestibular afferents. *Annals of the New York Academy of Sciences* **656**, 566-579.

Bertrand N. and Beley A. (1990) Effect of oxotremorine, physostigmine, and scopolamine on brain acetylcholine synthesis: A study using HPLC. *Neurochem. Res.* **15**, 1097-1100.

Billah M.M. and Anthes J.C. (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* **269 Suppl.**, 173-183.

Bishop W.R. and Bell R.M. (1988) Assembly of phospholipids into cellular membranes: Biosynthesis, transmembrane movement, and intracellular translocation. *Ann. Rev. Biochem.* **4**, 579-610.

Blusztajn J.K., Liscovitch M., Mauron C., Richardson U.I., and Wurtman R.J. (1987a) Phosphatidylcholine As a Precursor of Choline for Acetylcholine Synthesis. *J. Neural Trans.* 247-259.

Blusztajn J.K., Liscovitch M., and Richardson U.I. (1987b) Synthesis of acetylcholine from choline derived from phosphatidylcholine in a human neuronal cell line. *Proc. Natl. Acad. Sci. USA* **84**, 5474-5477.

Blusztajn J.K. and Wurtman R.J. (1980) Choline biosynthesis by a preparation enriched in synaptosomes from rat brain. *Nature* **290**, 417-418.

Blusztajn J.K. and Wurtman R.J. (1983) Choline and cholinergic neurons. *Science* **221**, 614-620.

Burt A.M. and Brody S.A. (1975) The measurement of choline kinase activity in rat brain: The problem of alternate pathways of ATP metabolism. *Anal. Biochem.* **65**, 215-224.

Buyukuysal R.L. and Wurtman R.J. (1990) 4-Aminopyridine inceases acetylcholine release without diminishing membrane phosphatidylcholine. *J. Neurochem.* **54(4)**, 1302-1309.

Cohen E.L. and Wurtman R.J. (1976) Brain acetylcholine: Control by dietary choline. *Science* **191**, 561-562.

Conklin B.R., Brann M.R., Buckley N.J., Ma A.L., Bonner T.I., and Axelrod J. (1988) Stimulation of arachidonic acid release and inhibition of mitogenesis by cloned genes for muscarinic receptor subtypes stably expressed in A9 L cells. *Proc. Natl. Acad. Sci. USA* **85**, 8698-8702.

Conricode K.M., Brewer K.A., and Exton J.H. (1992) Activation of phospholipase D by protein kinase C. Evidence for a phosphorylation-independent mechanism. *J. Biol. Chem.* **267**, 7199-7202.

Dross K. and Kewitz H. (1972) Concentration and origin of choline in the rat brain. *Naunyn Schmiedebergs Arch. Pharmacol.* **274**, 91-106.

Farber S.A., Kischka U., Marshall D.L., and Wurtman R.J. (1993) Potentiation by choline of basal and ellectrically-evoked acetylcholine release, as studied using a novel device which both stimulates and perfuses rat corpus striatum. *Brain Res.* **In press,**

Folch J., Lees M., and Sloane-Stanley G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226**, 497-503.

Fonnum F. (1969) Radiochemical microassays for the determination of choline acetyltransferase activities. *Biochem. J.* **115**, 465-472.

Francescangeli E., Goracci G., Piccinin G.L., Mozzi R., Woelk H., and Porcellati G. (1977) The metabolism of labeled choline in neuronal and glial cells of the rabbit in vivo. *J. Neurochem.* **28**, 171-176.

Freeman J.J., Choi R.L., and Jenden D.J. (1975) Plasma choline: Its turnover and exchange with brain choline. *J. Neurochem.* **24**, 729-734.

Gerfen C.R., Herkenham M., and Thibault J. (1987) The neostraital mosaic: II Patch and matrix-directed mesostriatal dopaminergic and non-dopaminergic systems. *J. Neurosci.* **7**, 3915-3943.

Hattori H. and Kanfer J.N. (1985) Synaptosomal Phospholipase-D Potential Role in Providing Choline for Acetylcholine Synthesis. *J. Neurochem.* **45**, 1578-1584.

Haubrich D.R. (1973) Partial purification and properties of choline kinase (EC 2.7.1.32) from rabbit brain: measurement of acetylcholine. *J. Neurochem.* **21**, 315-328.

Ikeda M., Houtani T., Ueyama T., and Sugimoto T. (1991) Choline acetyltransferase immunoreactivity in the cat cerebellum. *Neuroscience* **45**, 671-690.

Jope R.S. and Jenden D.J. (1979) Choline and phospholipid metabolism and the synthesis of acetylcholine in rat brain. *J. Neurosci. Res.* **4**, 69-82.

Kanfer J.N., McCartney D., and Hattori H. (1988) Regulation of the Choline, Ethanolamine and Serine Base-Exchange Enzyme-Activities of Rat-Brain Microsomes by Phosphorylation and Dephosphorylation. *FEBS. Lett.* **240**, 101-104.

Keller F., Rimvall K., and Waser P.G. (1987) Choline and Acetylcholine Metabolism in Slice Cultures of the Newborn Rat Septum. *Brain Res.* **405**, 305-312.

Kennedy E.P. and Weiss S.B. (1956) The function of cytidine coenzymes in the biosynthesis for phospholipids. *J. Biol. Chem.* **222**, 193-214.

Kessler P.D. and Marchbanks R.M. (1982) The independency of choline transport and acetylcholine synthesis. *J. Neurochem.* **39**, 1424-1433.

Klein J., Koppen A., Schmitthenner J., and Loffelholz K. (1990) Small rises in plasma choline reverse the negative arterio-venous difference of brain choline. *J. Neurochem.* **55**, 1231-1236.

Klein J., Köppen A., Löffelholz K., and Schmitthenner J. (1992) Uptake and metabolism of choline by rat brain after acute choline administration. *J. Neurochem.* **58**, 870-876.

Koh J.Y. and Choi D.W. (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase assay. *J. Neurosci. Meth.* **20**, 83-90.

Labaraca C. and Paigen K. (1980) A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102**, 344-352.

Liscovitch M., Freese A., Blusztajn J.K., and Wurtman R.J. (1985) High performance liquid chromatography of water-soluable choline metabolites. *Anal. Biochem.* **151**, 182-187.

Maire J.C. and Wurtman R.J. (1985) Effects of electrical stimulation and choline availability on the release and contents of acetylcholine and choline in superfused slices from rat striatum. *J. Physiol. ,Paris* **80**, 189-195.

Meyer E.M., Engel D.A., and Cooper J.R. (1982) Acetylation and phosphorylation of choline following high or low affinity uptake by rat cortical synaptosomes. *Neurochem. Res.* **7(6)**, 749-759.

Millington W.R. and Goldberg A.M. (1982) Precursor dependence of acetylcholine release from rat brain in vitro. *Brain Res.* **243**, 263-270.

Millington W.R. and Wurtman R.J. (1982) Choline administration elevates brain phosphorylcholine concentrations. *J. Neurochem.* **38**, 1748-1742.

Möhn H., Chalifa V., and Liscovitch M. (1992) Substrate specificity of neutral phospholipase D from rat brain studied by selective labeling of endogenous synaptic membrane phospholipids *in vitro*. *J. Biol. Chem.* **267**, 11131-11136.

Ousley A.H. and Morell P. (1992) Individual molecular species of phosphatidylcholine and phosphatidylethanolamine in myelin turn over at different rates. *J. Biol. Chem.* **267**, 10362-10369.

Parducz A., Joo F., and Toldi J. (1986) Formation of Synaptic Vesicles in the Superior Cervical-Ganglion of Cat - Choline Dependency. *Experimental Brain Research* **63**, 221-224.

Pelech S.L. and Vance D.E. (1984) Regulation of phosphatidylcholine biosynthesis. *Biochim. Biophys. Acta* **779**, 217-251.

Pelech S.L. and Vance D.E. (1989) Signal transduction via phosphatidylcholine cycles. *Trends Biochem. Sci.* **14**, 28-30.

Potter L.T. (1970) Synthesis, storage and release of [14C]acetylcholine in isolated rat diaphram muscles. *J. Physiol.* **206**, 145-166.

Qian Z. and Drewes L.R. (1990) A novel mechanism for acetylcholine to generate diacylglycerol in brain. *J. Biol. Chem.* **265(7)**, 3607-3610.

Qian Z. and Drewes L.R. (1991) Cross-talk between receptor-regulated phospholipase D and phospholipase C in brain. *FASEB Journal* **5**, 315-319.

Sandmann J. and Wurtman R.J. (1991) Stimulation of phospholipase D activity in human neuroblastoma (LA-N-2) cells by activation of muscarinic acetylcholine receptors or by phorbol esters: Relationship to phosphoinositide turnover. *J. Neurochem.* **56**, 1312-1319.

Slack B.E., Breu J., and Wurtman R.J. (1991) Production of diacylglycerol by exogenous phospholipase C stimulates CTP:phosphocholine cytidylyltransferase activity and phosphatidylcholine synthesis in human neuroblastoma cells. *J. Biol. Chem.* **266**, 24503-24508.

Spanner S. and Ansell G.B. (1979) Choline kinase and ethanolamine kinase in the cytosol of nerve endings from rat forebrain. *Biochem. J.* **178**, 753-760.

Suidan H.S. and Tolkovsky A.M. (1993) Sympathetic neurons expressing cholinergic properties are poised to allocate choline symmetrically between acetylcholine and the phosphatidylcholine-generating pathway in growing neurites. *J. Neurosci.* **13(3)**, 1190-1201.

Svanborg A. and Svennerholm L. (1961) Plasma total lipids, cholesterol, triglycerides, phospholipids and free fatty acids in a healthy Scandinavian population. *Acta. Med. Scand.* **169**, 43-69.

Teegarden D., Taparowsky E.J., and Kent C. (1990) Altered phosphatidylcholine metabolism in C3H10T1/2 cells transfected with the Harvey-ras oncogene. *J. Biol. Chem.* **265(11)**, 6042-6047.

Touchstone J.C., Chen J.C., and Beaver K.M. (1980) Inproved separation of phospholipids on thin layer chromatography. *Lipids* **15**, 61-62.

Trommer B.A., Schmidt D.E., and Wecker L. (1982) Exogenous choline enhances the synthesis of acetylcholine only under conditions of increased cholinergic neuronal activity. *J. Neurochem.* **39(6)**, 1704-1708.

Tucek S (1985) Regulation of acetylcholine synthesis in the brain. *J. Neurochem.* **44(1)**, 11-24.

Ulus I.H., Wurtman R.J., Mauron C., and Blusztajn J.K. (1989) Choline increases acetylcholine release and protects against the stimulation-induced decrease in phosphatide levels within membranes of rat corpus striatum. *Brain Res.* **484**, 217-227.

Warden C.H. and Friedkin M. (1985) Regulation of Choline Kinase-Activity and Phosphatidylcholine Biosynthesis by Mitogenic Growth-Factors in 3T3-Fibroblasts. *J. Biol. Chem.* **260**, 6006-6011.

Wecker L., Cawley G., and Rothermel S. (1989) Acute choline supplementation in vivo enhances acetylcholine synthesis in vitro when neurotransmitter release is increased by potassium. *J. Neurochem.* **52(2)**, 568-575.

Wecker L. (1991) The synthesis and release of acetylcholine by depolarized hippocampal slices is increased by increased choline available in vitro prior to stimulation. *J. Neurochem.* **57**, 1119-1127.

Wecker L. and Reinhardt R.R. (1988) Adenosine inhibits choline kinase activity and decreases the phosphorylation of choline in striatal synaptosomes. *J. Neurochem.* **50(6)**, 1945-1951.

Weiler M.H., Bak I.J., and Jenden D.J. (1983) Choline and acetylcholine metabolism in rat neostriatal slices. *J. Neurochem.* **41(2)**, 473-480.

Yamada K., Saltarelli M.D., and Coyle J.T. (1988) Involvement of phospholipase A2 in the regulation of [3H]hemicholinium-3 binding. *Biochem. Pharmac.* **37**, 4367-4373.

Yavin E. (1985) Polar head group decarboxylation and methylation of phospholipids: An alternate route for phosphatidylcholine formation in cultured neuronal cells. *J. Neurochem.* **44**, 1451-1458.

Yavin E., Tanaka Y., and Ando S. (1989) Phospholipid-derived choline intermediates and acetylcholine synthesis in mouse brain synaptosomes. *J. Neurosci. Res.* **24**, 241-246.

Yu S.P. and Van der Kloot W. (1991) Increasing quantal size at the mouse neuromuscular junction and the role of choline. *J. Physiol.* **433**, 677-704.

Zilversmit D.B., Entenman C., and Fishler M.C. (1943) On the calculation of "turnover time" and "turnover rate" from experiments involving the use of labeling agents. *J. Gen. Physiol.* **26**, 325-331.

FIGURE AND TABLE LEGENDS

Fig. 1. **Electric field stimulation induces ACh release with little attenuation over time**

Stimulated rat striatal slices were exposed to alternating polarity pulses (120 mA, 15 Hz and 1.0 ms duration) while being superfused. Medium was collected into three vials during the 1 hr stimulation period and assayed for ACh. Data are expressed as means ± S.E. from four rats.

Fig. 2. **Stimulation of slices superfused with [^{14}C]Ch enhanced release of [^{14}C]ACh and decreased tissue [^{14}C]PCh and [^{14}C]PC.** Slices were treated as described in Table I, series 1. After superfusion the slices were homogenized. *Upper,* the radioactivity in tissue Ch-containing compounds were determined by HPLC (see "Experimental Procedures"). Values represent the means ± S.E. from eight rats, crosshatched bars refer to stimulated tissues; *, $p < 0.05$; **, $p < 0.01$(values significantly different form unstimulated values). *Lower,* medium from these same tissues was collected and assayed for labeled ACh (see "Experimental Procedures") the medium from the first 15 min was excluded since the label was not yet administered. Values represent the means ± S.E. from four rats, crosshatched bars refer to stimulated tissues; *, $p < 0.05$; **, $p < 0.01$(values significantly different form unstimulated values).

Fig. 3. Effects of stimulation on tissue Ch and ACh levels. One striatum from each rat was stimulated and the other served as the control. Aliquots of tissue were taken for assay after 15, 30, 45 and 60 min and assayed for Ch and ACh (see "Experimental Procedures"). *A,* tissue Ch levels. *B,* tissue Ch levels from control and stimulated slices that were superfused with 100 mM Ch. *C,* tissue ACh levels . *D,* tissue ACh levels from control and stimulated slices that were superfused with 100 mM Ch. In all panels: filled

circles represent control slices, filled triangles designate stimulated slices, values represent the means ± S.E. from four rats. *, $p < 0.05$; **, $p < 0.01$(values significantly different from corresponding controls at the same time point).

Fig. 4. Effect of stimulation on PCh and PC labeling and synthesis. One striatum from each rat was stimulated and the other served as the control. Aliquots of tissue were assayed for both labeled and unlabeled PC and PCh (see "Experimental Procedures"). *A*, incorporation of [^{14}C]Ch into PCh. *B*, incorporation of [^{14}C]Ch into PC. *C*, PCh synthesis was calculated from the specific activity of tissue Ch determined from the total tissue Ch radioactivity and total Ch mass measured in each sample . *D*, PC synthesis was calculated from 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 were treated identically. In all panels: filled circles represent control slices, filled triangles designate stimulated slices, values represent the means ± S.E. from four rats. *, $p < 0.05$; **, $p < 0.01$(values significantly different from corresponding controls at the same time point).

Fig. 5. Effect of stimulation on PCh and PC turnover: In this study no Ch chase was used. One striatum from each rat was stimulated and the other served as the control. Tissues were first labeled for 15 min, washed and placed in superfusion chambers (see Table III, series 3). However, unlike series 3, slices were labeled with [^{3}H]Ch and were not superfused with unlabeled Ch. Aliquots of tissue were taken for assay after 15, 30, 45 and 60 min and assayed for labeled PC and PCh (see "Experimental Procedures"). *A*, the total released [^{3}H] into the superfusion medium by both control and stimulated slices. *B*, the specific activity of tissue PCh. *C*, specific activity of tissue PC. In all panels: filled circles represent control slices, filled triangles designate stimulated slices, values represent the means ± S.E. from four rats.

Fig. 6. The effect of stimulation on PCh and PC: This study utilized a Ch chase. One striatum from each rat was stimulated and the other served as control. Tissues were first labeled for 15 min, washed and superfused with 100 mM unlabeled Ch (see Table III, series 3). Tissue samples were collected at designated intervals from both control and stimulated striata, homogenized and assayed for labeled PC and PCh (see "Experimental Procedures"). *A*, the turnover PCh labeled with a brief pulse of [^{14}C]Ch. *B*, the turnover PC labeled with a brief pulse [^{14}C]Ch. In all panels: filled circles represent control slices, filled triangles designate stimulated slices, values represent the means $\pm$ S.E. from four rats.

Fig. 7. **Stimulation of cerebellar slices superfused with [^{14}C]Ch results in reduced PCh and not PC radioactivity.** Slices were treated as described in Table I, series 1. After superfusion the slices were homogenized and the radioactivity in tissue Ch-containing compounds were determined by HPLC (see "Experimental Procedures"). Values represent the means $\pm$ S.E. from three rats, crosshatched bars refer to stimulated tissues; *, $p< 0.05$ (values significantly different form unstimulated values).

Fig. 8. The effect of stimulation on the activity of Ch kinase and ChAT. Both striatum were dissected from each rat; one striatum served as control and the other stimulated. Slices were prepared and equilibrated for 1 hr. Then some slices were stimulated for a period of 1 hr. Slices were then homogenized and aliquots frozen (see "Experimental Procedures") *A*, the activity of ChAT. Different amounts of Ch were added to the enzyme reaction mixture and the best fit Km (mM) and Vmax (nmol/mg/hr) were determined (Enzfitter, Biosoft, Cambridge, UK); In control tissues, 130 $\pm$ 30 and 674 $\pm$ 167 respectively; In stimulated tissues, 155 $\pm$ 21 and 725 $\pm$ 86 respectively. *B*, the activity of Ch kinase. Different amounts of Ch were added to the enzyme reaction mixture and the best fit Km (mM) and Vmax (nmol/mg/hr) were determined (Enzfitter); In control tissues,

7.13 ± 1.21 and 4.50 ± 0.31 respectively; In stimulated tissues, 5.92 ± 0.92 and 5.18 ± 1.20 respectively. In both panels: Values represent the means ± S.E. from four rats.

Table I. *Slice equilibration, labeling and stimulation paradigms*

Solid bars represent the time (min) used for each aspect of the experiment. In all studies controls were treated identically as stimulated tissues except that no current was applied to the chamber. Time 0 is the time the animal was sacrificed. Equilibration refers to the time slices were left undisturbed in the chambers while being superfused with buffer. In experiments that involved tissue collection, chambers were opened and tissue removed at the designated intervals.

Table II. *Specific activities of Ch-containing compounds labeled by superfusion with [^{14}C]Ch*

Specific activities were calculated from measurements of mass and radioactivity as described (see "Experimental Procedures"). Data represent the means ± S.E. from four rats

Table III. *The mass of ACh release determined directly and from the specific activity of Ch*

Total ACh was determined by HPLC analysis of an aliquot of superfusate. The radioactivity recovered in ACh was divided by the specific activity of tissue Ch calculated and summarized in Table II, or the medium specific activity derived solely from the labeled Ch added to the medium (116 DPM/pmol). Data represent the means ± S.E. from four rats.

Figure 1

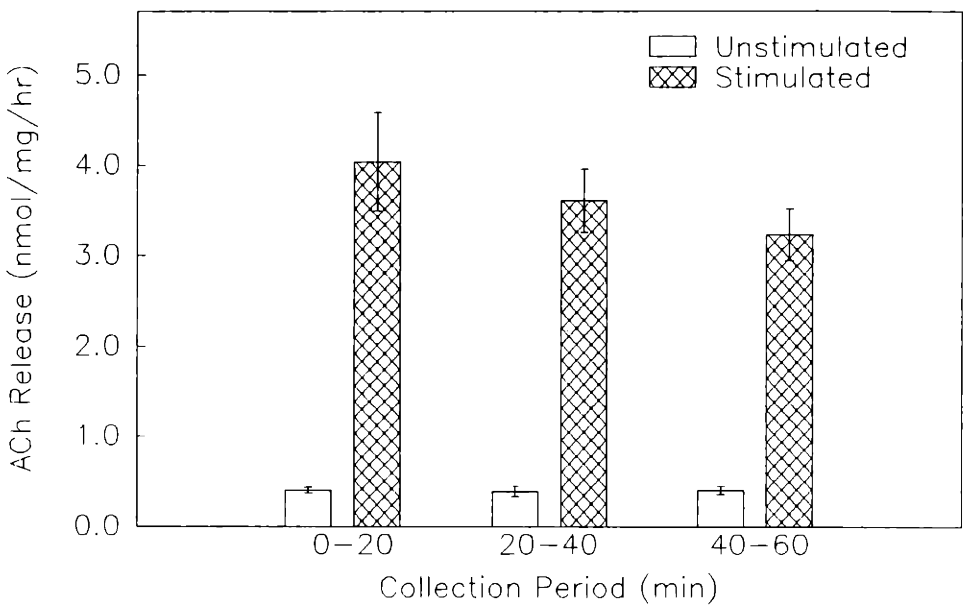

Unstimulated
Stimulated
ACh Release (nmol/mg/hr)
5.0
4.0
3.0
2.0
1.0
0.0
0–20
20–40
40–60
Collection Period (min)

Figure 2

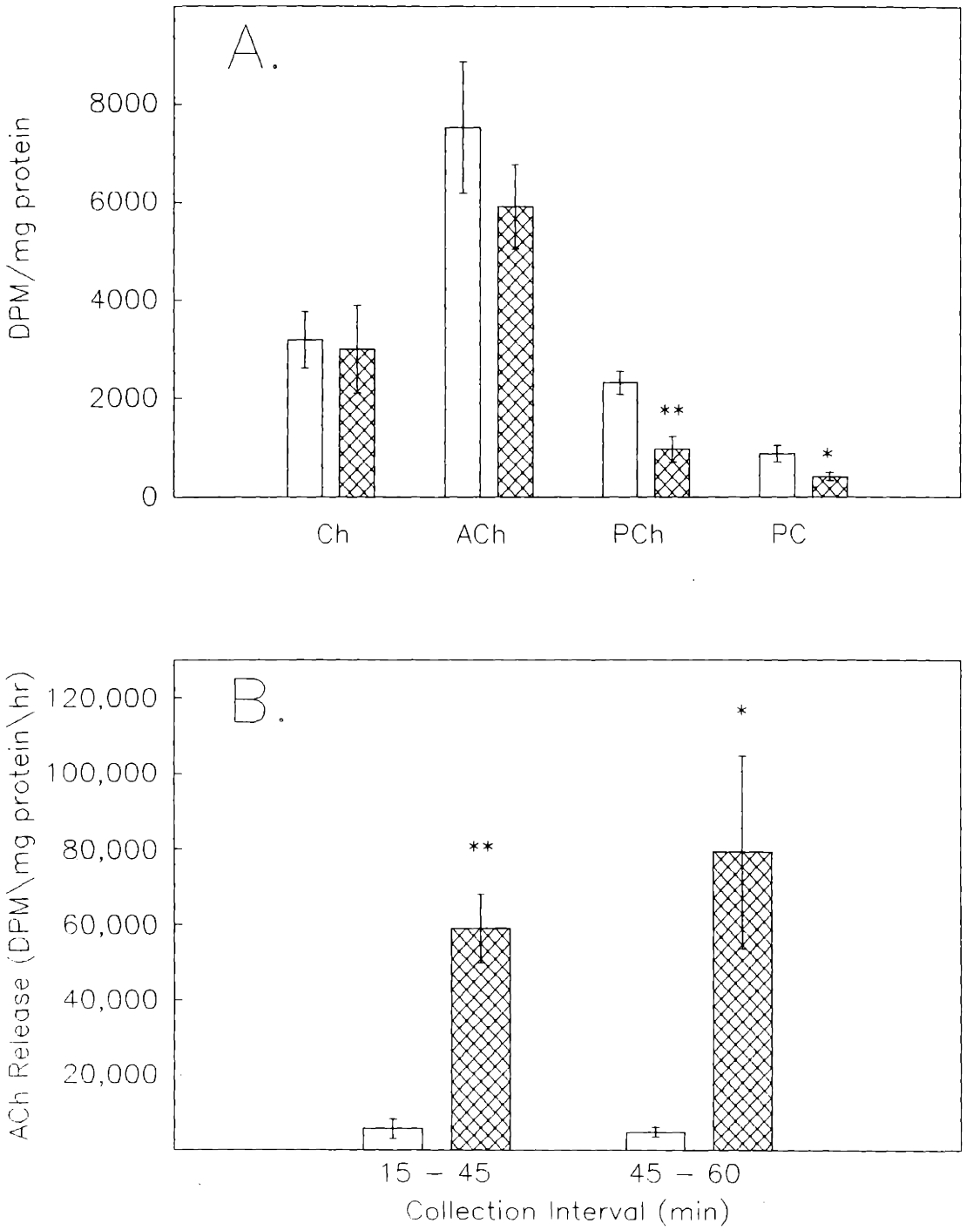

A.
DPM/mg protein
8000
6000
4000
2000
0
Ch
ACh
PCh
PC
**
*
B.
ACh Release (DPM\mg protein\hr)
120,000
100,000
80,000
60,000
40,000
20,000
**
*
15 – 45
45 – 60
Collection Interval (min)

Figure 3

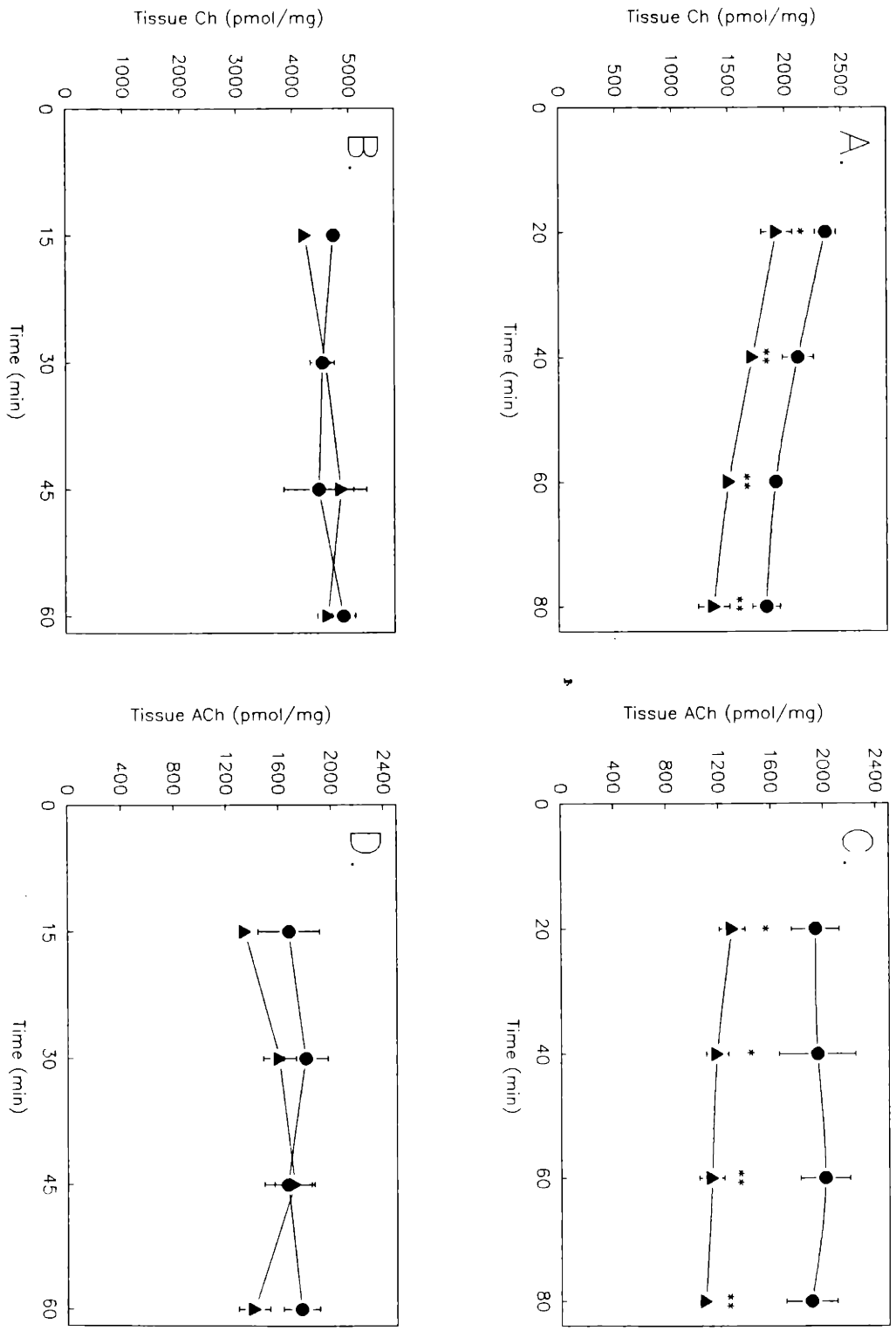

A.
Tissue Ch (pmol/mg)
Time (min)
B.
Tissue Ch (pmol/mg)
Time (min)
C.
Tissue ACh (pmol/mg)
Time (min)
D.
Tissue ACh (pmol/mg)
Time (min)

Figure 4

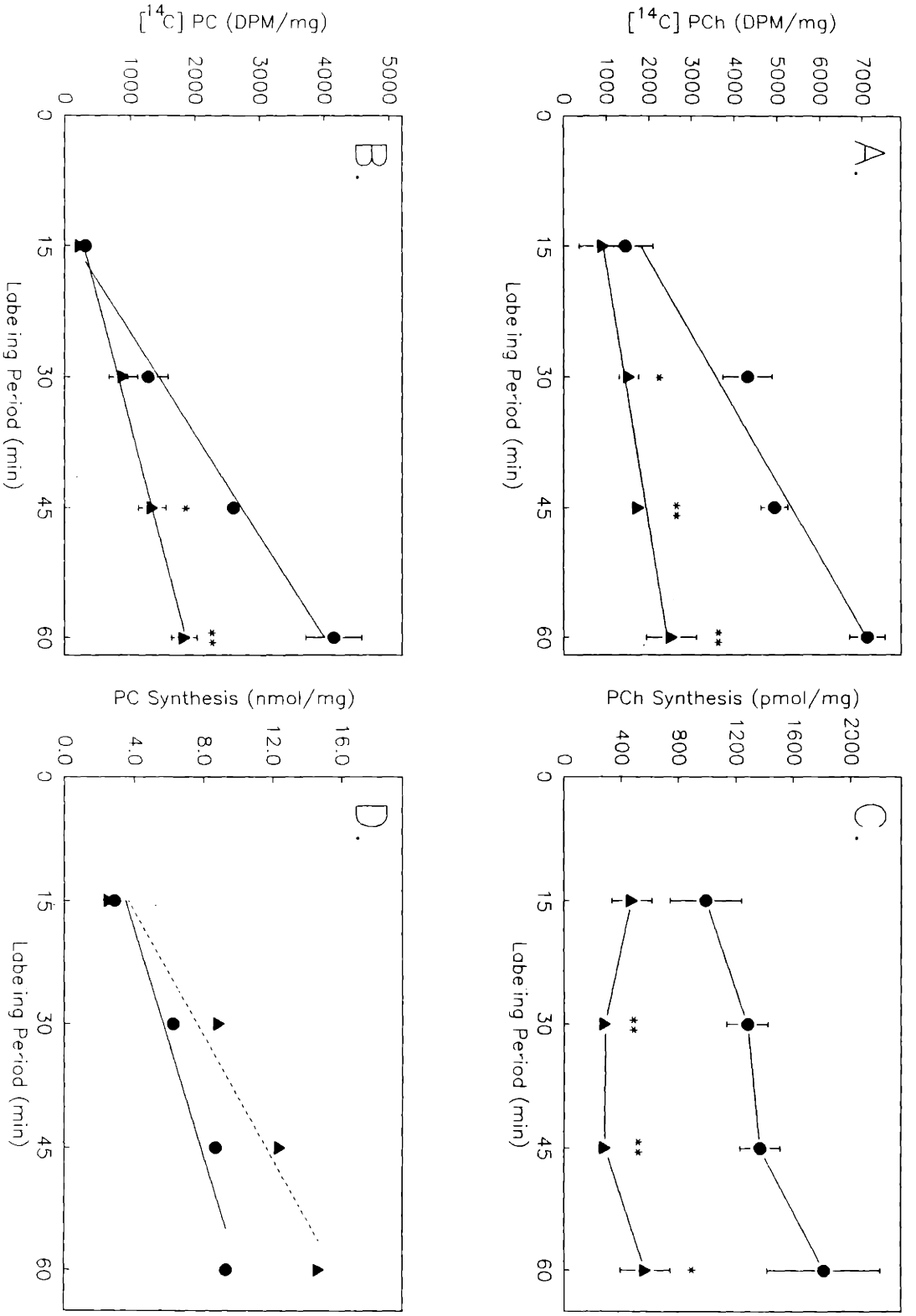

A.
[14C] PCh (DPM/mg)
Labeling Period (min)
B.
[14C] PC (DPM/mg)
Labeling Period (min)
C.
PCh Synthesis (pmol/mg)
Labeling Period (min)
D.
PC Synthesis (nmol/mg)
Labeling Period (min)

Figure 5

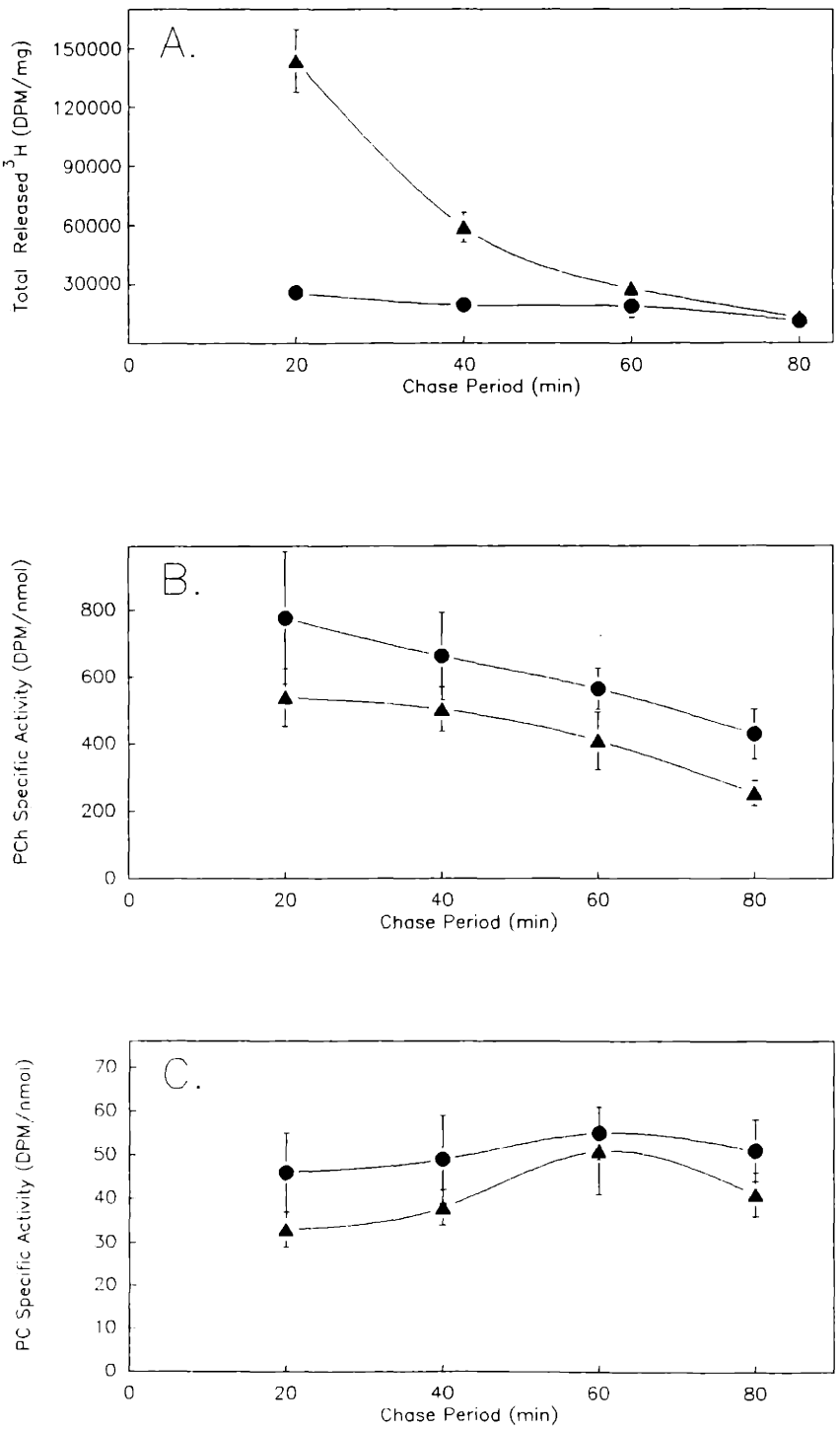
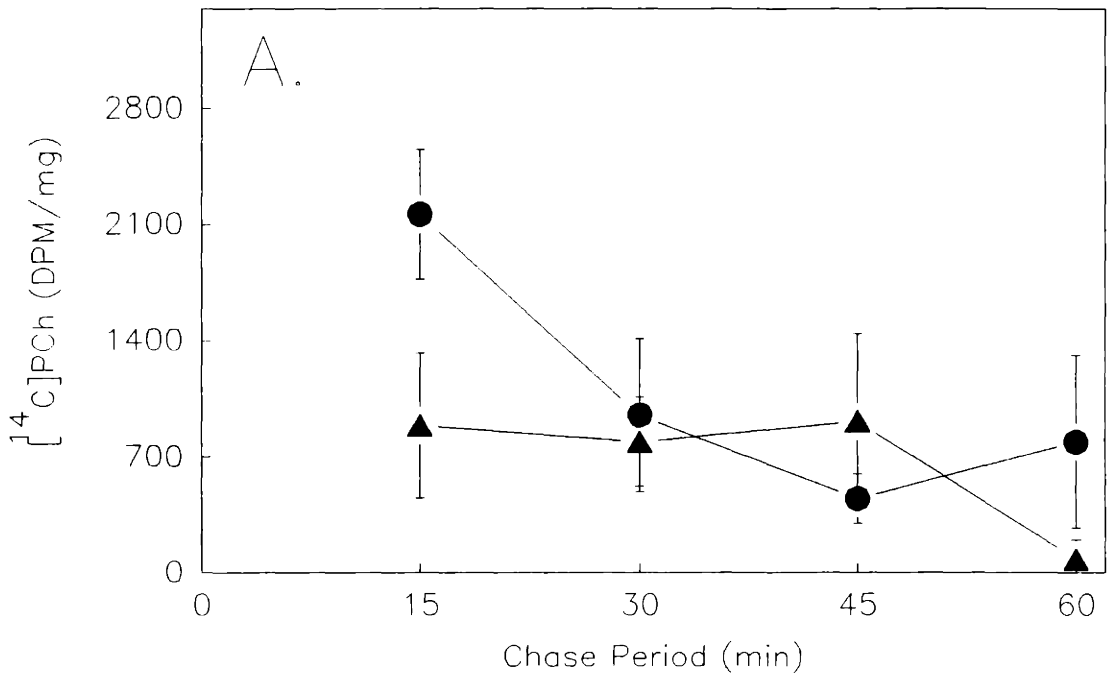

A.
Total Released ^{3}H (DPM/mg)
150000
120000
90000
60000
30000
0
20
40
60
80
Chase Period (min)
B.
PCh Specific Activity (DPM/nmol)
800
600
400
200
0
20
40
60
80
Chase Period (min)
C.
PC Specific Activity (DPM/nmol)
70
60
50
40
30
20
10
0
20
40
60
80
Chase Period (min)

Figure 6


A.
2800
2100
1400
700
0
0
15
30
45
60
Chase Period (min)
[14C]PCh (DPM/mg)

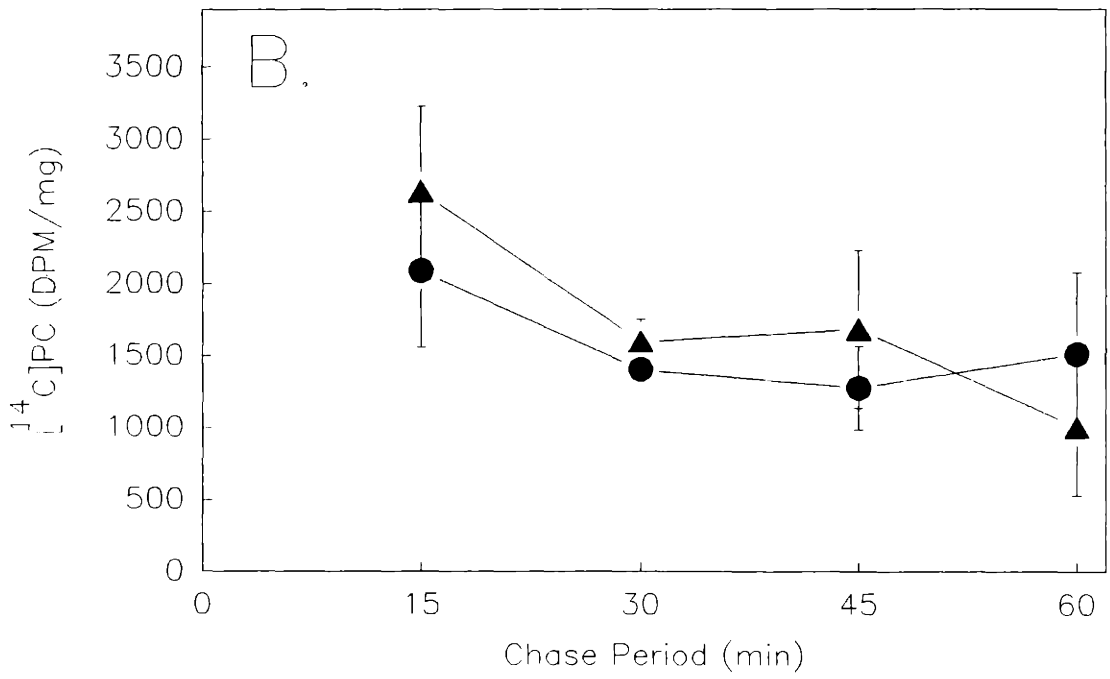

B.
3500
3000
2500
2000
1500
1000
500
0
0
15
30
45
60
Chase Period (min)
[14C]PC (DPM/mg)

Figure 7

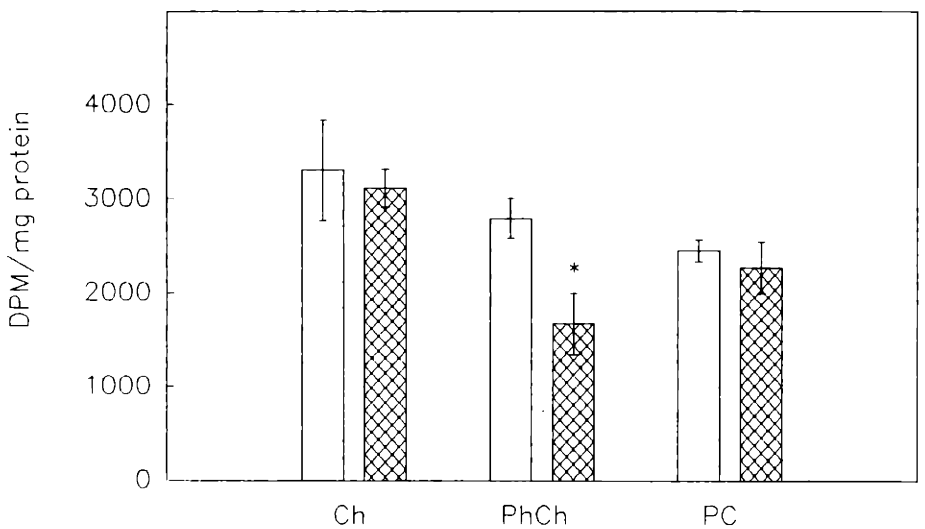


Figure 8

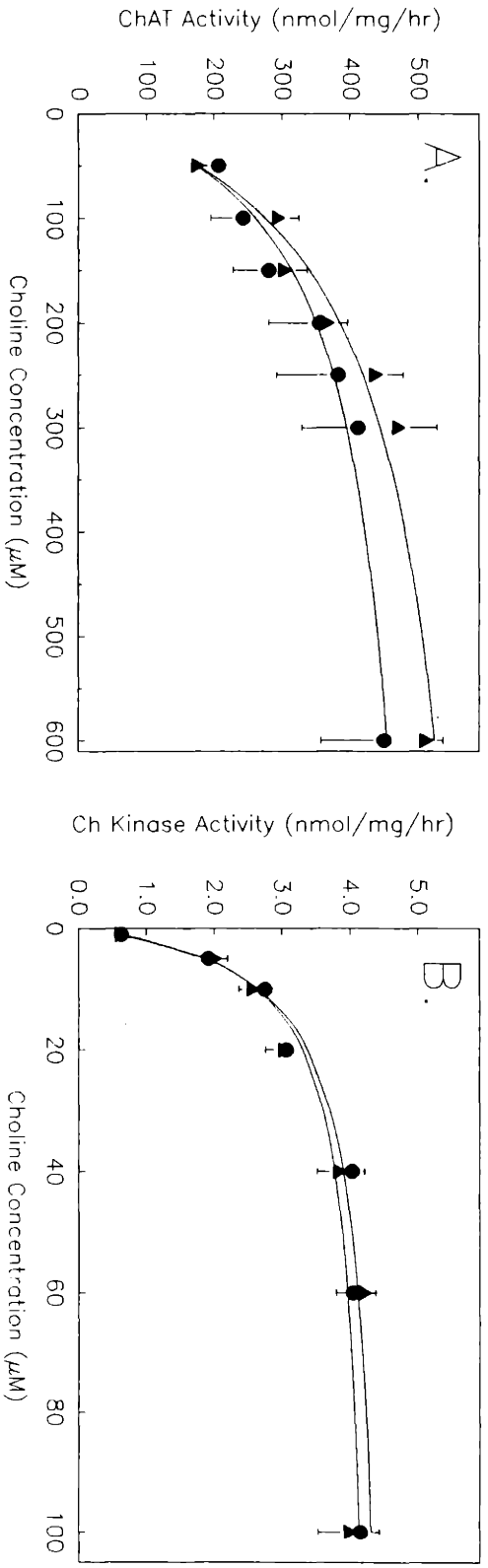
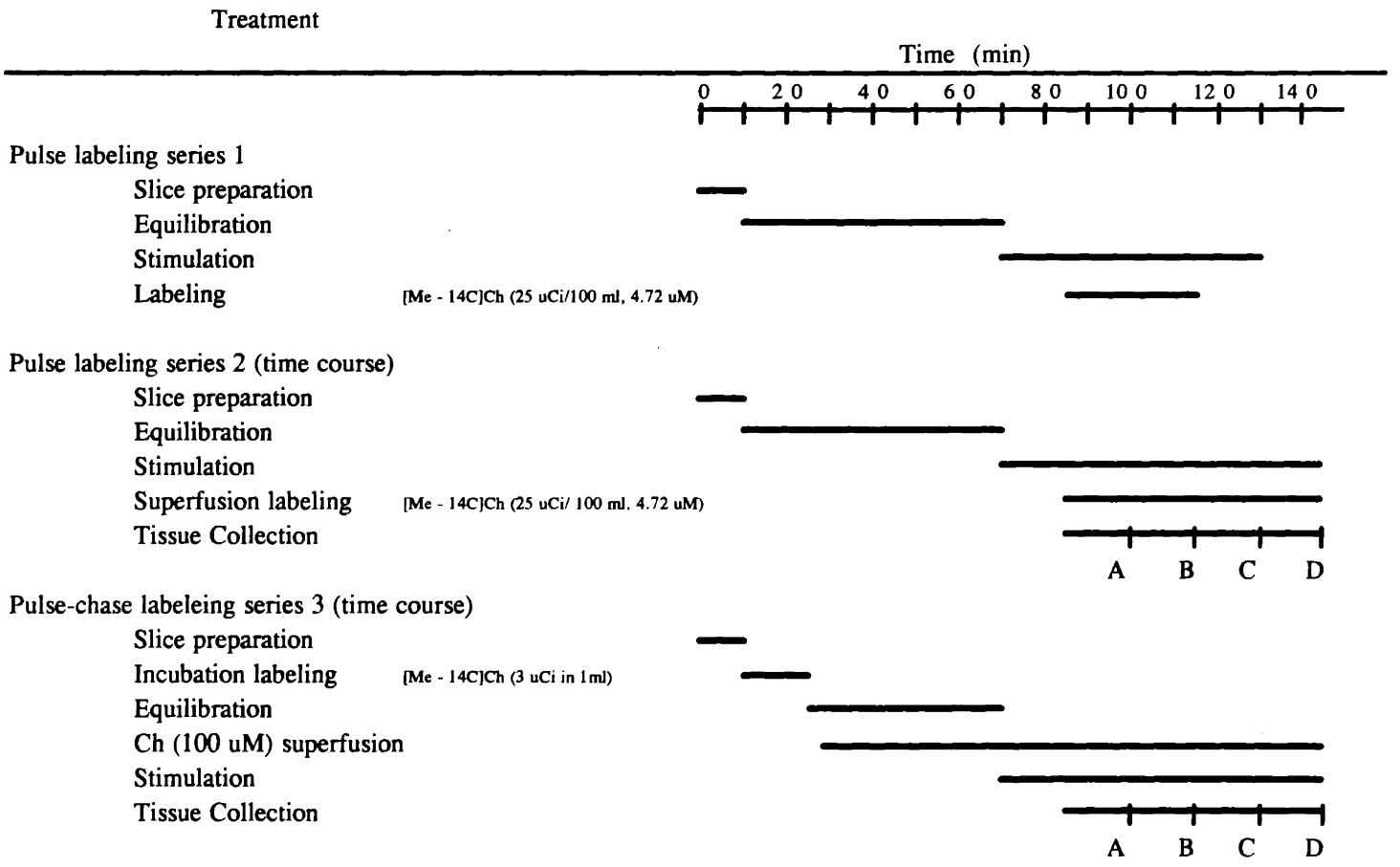

ChAT Activity (nmol/mg/hr)
0
100
200
300
400
500
A.
0
100
200
300
400
500
600
Choline Concentration (μM)
Ch Kinase Activity (nmol/mg/hr)
0.0
1.0
2.0
3.0
4.0
5.0
B.
0
20
40
60
80
100
Choline Concentration (μM)

Table I



A, B, C and D reperesent tissue collection periods

Table II

Compound	Treatment	Specific activity of choline-containing compounds (DPM/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 ± 6440 a	9420 ± 2700
PCh	Control	110 ± 34	300 ± 40	446 ± 28	453 ± 26
	Stimulation	94 ± 37	108 ± 16 a	125 ± 15 a	223 ± 52 a
PC	Control	1.29 ± 0.43	4.39 ± 1.08	10.1 ± 0.40	15.2 ± 1.57
	Stimulation	0.88 ± 0.13	3.43 ± 0.84	5.23 ± 0.83 a	6.48 ± 0.70 a
ACh Release	Control	-	14200 ± 3060 b, d	15000 ± 1100 c, d	-
	Stimulation	-	22100 ± 980 b, d	23690 ± 4670 c	-

a Significantly different from control values

b Release collected from the first 30 min after the influx of label (see Table 1 series 2)

c Release collected from 30 - 45 min after the influx of label (see Table 1 series 2)

d Significantly different from tissue levels under the same conditions

Table III

Calculation	Treatment	ACh release (pmol/mg/hr) Collection Interval (min) 15 - 45	45 - 60
Actual (HPLC)	Control	388 ± 59	400 ± 47
	Stimulated	3610 ± 351 a	3240 ± 283 a
DPM ACh / Specific Activity of Tisssue Ch	Control	1760 ± 773	1350 ± 362
	Stimulated	10800 ± 1670 a	13200 ± 4220 a
DPM ACh / Specific Medium Ch	Control	51 ± 22	43 ± 11
	Stimulated	508 ± 78 a	682 ± 218 a

a Significantly different from control values

Table III

Brain Research *in press*

Potentiation by choline of basal and electrically evoked acetylcholine release, as studied using a novel device which both stimulates and perfuses rat corpus striatum.

Steven A. Farber, Udo Kischka, David L. Marshall and Richard J. Wurtman

Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, (U.S.A.)

Key Words: Acetylcholine; Choline; Dopamine; Electrical Stimulation; Microdialysis; Neostigmine; Striatum

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

SUMMARY

We examined the release of acetylcholine (ACh) and dopamine (DA) using a novel probe through which striatal neurons could be both superfused and stimulated electrically in both anesthetized and freely moving awake animals. Optimal stimulation parameters for eliciting ACh release from cholinergic neurons differed from those required for eliciting DA release from dopaminergic terminals: at 0.6 ms pulse duration, 20 Hz and 200 μA., ACh release increased to 357 ± 30 % ($p < 0.01$) of baseline and was blocked by the addition of tetrodotoxin (TTX). Pulse durations of 2.0 ms or greater were required to increase DA release. Unlike ACh release, DA release showed no frequency dependence above 5 Hz. The maximal evoked releases of ACh and DA were 556 ± 94 % ($p < 0.01$) and 254 ± 38 % ($p < 0.05$) of baseline, respectively. Peripheral administration of choline (Ch) chloride (30 - 120 mg/kg) to anesthetized animals caused dose-related ($r = 0.994$, $p < 0.01$) increases in ACh release; basal release rose from 117 ± 7 % to 141 ± 5 % of initial baseline levels ($p < 0.05$) and electrically-evoked ACh release rose from 386 ± 38 % to 600 ± 34 % ($p < 0.01$) in rats given 120 mg/kg. However, Ch failed to affect basal or evoked DA release although neostigmine (10μM) significantly elevated basal DA release (from 36.7 fmol/10 min to 71.5 fmol/10 min [$p < 0.05$]). In awake animals, Ch (120 mg/kg) also elevated both basal (from 106 ± 7 % to 154 ± 17 % [$p < 0.05$]) and electrically-evoked (from 146 ± 13 to 262 ± 19 % [$p < 0.01$]) ACh release. These experiments demonstrate that ACh and DA release can both be reliably evoked from rat striatum, and that an intraperitoneal (i.p.) injection of Ch can increase both basal and evoked striatal ACh release.

INTRODUCTION

Numerous studies have applied the technique of *in vivo* microdialysis[29] to investigate the regulation of DA and ACh release from rat striatum[1,2,4,6,7,23,25]. Such studies have sometimes used high potassium concentrations within the perfusate to evoke neurotransmitter release by depolarizing neurons in the vicinity of the probe[2,23,25]. While this technique is useful for releasing large amounts of neurotransmitter[9], it is less physiologic than providing short-duration electrical pulses. Moreover, high potassium concentrations can produce permanent alterations in synaptic transmission[14], and can, with subsequent challenges, release diminishing quantities of ACh[19].

The present study used a novel custom-made microdialysis probe, containing a tungsten stimulating electrode, to depolarize neurons electrically in the direct vicinity of the microdialysis membrane. This treatment was found to produce more than a fourfold increase in ACh release, and similar responses could be elicited by subsequent stimulations. The use of the hybrid probe enabled us to characterize the electrical parameters that affect both ACh release from striatal interneurons and DA release from nigro-striatal terminals.

After establishing that ACh release could be reliably evoked and entirely suppressed by the infusion of TTX, we explored the effect of exogenous Ch on basal and electrically-evoked ACh release. Previous studies had established that dietary Ch could enhance tissue ACh and Ch levels in various regions of rat brain[3,15], and that addition of Ch to superfused neurons could produce dose-related increases in basal and evoked ACh release from striatal slices[17,28]. However, studies of the effects of Ch on basal ACh from rat striatum, as assessed by *in vivo* microdialysis, have provided contradictory data[4,16,32]. In the present study, we utilized the hybrid probe to examine the effect of Ch on both basal and electrically-stimulated ACh and DA release from rat striata.

MATERIALS AND METHODS

Stimulating Electrode and Microdialysis Probe

Stimulating electrodes were prepared from a 10 cm piece of teflon-coated tungsten wire (A-M Systems) with an uncoated diameter of 5μm. The teflon was stripped from the tip of the wire, using a dissecting microscope, to expose approximately 0.3 mm of the tungsten. Only electrodes that had an impedance of 50KΩ - 200KΩ were used. Each electrode was glued (with nail polish) to a custom-made microdialysis probe (5 mm membrane length) such that its tip extended to half the length of the microdialysis membrane (Fig.1).

Animals

Experiments were performed on both anesthetized and awake Male Sprague-Dawley rats (Charles River, Cambridge, MA) weighing 280-360 g. The animals were exposed to a 12 h light-dark cycle and given access to water and food (Charles River Rat, Mouse and Hamster Original Formula) ad libitum.

Anesthetized animal studies

Animals were anesthetized, placed in a stereotaxic frame, and kept on a heating pad maintained at 37°C. The anesthetic was prepared by mixing 1.25 g of urethane and 125 mg of chloralose in 10 ml of water. Throughout the experiment the solution was stirred and warmed in a sealed vial. An i.p. injection (8 ml/kg body wt.) of chloralose, 50 mg/kg and urethane, 500 mg/kg, was used to anesthetize the animals. The hybrid probe was placed stereotactically in the right corpus striatum at coordinates 0.4 mm anterior, -2.7 mm lateral from bregma, -6.5 mm ventral from dura[18] and then perfused (4 μl/min) using a microperfusion pump (model 100, CMA) with an oxygenated modified Ringer's solution (121 mM NaCl, 3.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4 and 25 mM $NaHCO_3$) containing 10μM neostigmine. Collection vials contained 5μl of 0.05 M perchloric acid to prevent DA breakdown. The positive lead of

an electrical stimulator (model 2100, A-M systems) was connected to the tungsten wire and the negative lead was attached to the metal probe holder (kept electrically isolated from the stereotaxic frame). After 3 h of equilibration, an additional injection of 0.2 - 0.4 ml of anesthetic was given prior to the start of the experiment. At the end of the experiment some brains were removed, fixed, and sectioned to verify probe placement. In all cases examined (n = 8), the probe was located in center of the striatum.

Awake freely moving animal studies

Animals were anesthetized with equithesin (3 ml/kg), placed in a stereotaxic frame and probes were placed as described above. A ground stud affixed to a gold plated post was placed in the cortex (2.0 mm anterior and -2.0 lateral from bregma). The tungsten electrode was also attached to a gold plated post. The probe and both posts were affixed to the skull with permabond 910 (Permabond International) and dental acrylic repair material (Healthco). The inlet and outlet tubes of the hybrid probe were cut 4 cm from the skull and sealed with epoxy. Animals were removed from the frame and allowed to recover. After 24 h, the epoxy was cut away and the probe was connected to longer inlet and outlet tubes. Prior to the start of the experiment, animals were perfused for 1 h with Ringer's solution as described above.

ACh and DA collection and determination

Experiments were started by collecting 3 - 4 consecutive 10 min dialysis samples for determination of baseline levels of ACh and/or DA. To characterize the excitability of striatal neurons, we stimulated with impulses of defined (and constant) current, duration and frequency for a period of 10 min. The TTX sensitivity of ACh release was determined by measuring release in both basal and stimulated (200μA, 20 Hz and 0.6 ms) samples prior to, and 1 h following, TTX (10μM) administration through the dialysis probe. To determine the effect of Ch on basal and evoked neurotransmitter release, we administered isotonic saline i.p. to 10 rats, and Ch chloride i.p. to 27 rats in the following doses: 30 mg/kg (n = 6), 60 mg/kg (n = 10), 120 mg/kg (n = 11). Three additional samples (10 min

each) were ms). Rats receiving 120 mg/kg Ch and respective control rats were stimulated for a second period, 30 min after the first stimulation.

Levels of ACh, Ch and DA were assayed by splitting the dialysate sample. Probe recoveries for ACh and Ch (tested in a 10μM solution) were 9.86 ± 0.37 % (mean ± S.E.M.; n = 28) and 10.81 ± 0.62 % (n = 21), respectively, ranging between 6.00 and 12.75 % for ACh, and between 6.36 and 16.00 % for Ch. Recovery for DA (tested in a 1 μM solution) was 8.60 ± 0.77 % (n = 13) and ranged between 5.15 and 13.04 %. Data values were not corrected for probe recoveries. ACh and Ch were determined by HPLC on a polymeric reversed-phase column (BAS) with a mobile phase of 50 mM phosphate (pH 8.5) containing 0.005 % Kathon CG as a bactericide. Once separated, ACh and Ch were converted to betaine and hydrogen peroxide in a post-column enzymatic reactor containing Ch oxidase and ACh esterase (BAS). The hydrogen peroxide was detected electrochemically using a platinum electrode set at 500 mV (*vs.* Ag/AgCl) (model 200a, BAS). The DA samples were assayed by HPLC on a C-18 (3.2 mm bore with 3.0 μm packing) column (ESA, HR-80). The mobile phase consisted of 70 mM NaH_2PO_4, 1 mM heptane sulphonic acid, 0.22 mM Na_2-EDTA and 4 % methanol v/v at pH 5.5. DA was detected electrochemically by oxidation at 360 mV (Coulochem II, ESA). Release data were expressed both as pmols released per 10 min and as percents of the 3 initial baseline values.

Strength-duration curve

Data relating the release of ACh to stimulation current (Fig. 2) and pulse duration (Fig. 3a) were used to calculate a strength-duration curve (Fig. 4). First, the equation describing the relation between current and percent ACh release was derived from data in Fig. 2 ($y = 0.00730x + 0.00391$, $r=.998$). This equation was then used to calculate the respective currents that would be required, at various pulse durations, to increase ACh release to 250 % of basal. For example, the release observed at 1 ms (494 ± 39 %) was approximately twice that observed at 0.2 ms (250 %). By extrapolating from the equation

relating release to current, it was determined that 109 μA would be needed to increase ACh release to 250 % of control at this pulse duration (1 ms). This calculation was repeated for each point in Fig 3a. The resulting curve (Fig. 4) fits the predicted equation $I = I_r(1+C/t)$ (I=current, I_r=the rheobase current [the current required at very long pulse durations], t=pulse duration and C=chronaxie [the time at twice the rheobase current]).

Statistics

Groups were compared using one way analysis of variance (ANOVA) with a post-hoc Newman Keul's test. Significance was assumed for $p< 0.05$. Data are expressed as mean ± S.E.M. from 4 - 12 experiments on individual animals. Data examining the effect of multiple stimulations were analyzed using an one way ANOVA and a multiple regression. The dose-response releationship was analyzed using a linear regression.

RESULTS

Effect of electrical stimulation on striatal ACh, Ch and DA release

Basal ACh release (i.e., prior to the first stimulation period) was 2.76 ± 0.18 pmol/10 min (n = 10); this remained stable throughout the experiment. Basal Ch release tended to decrease over time from a starting level of 14.40 ± 1.32 to 9.92 ± 0.90 pmol/10 min after 3 h (n = 10). When the striatum was stimulated using increasing currents with stimulation frequency fixed at 20 Hz and pulse duration at 0.2 ms (Fig. 2), ACh release exhibited a linear relationship to stimulation current between 50 and 300μA (r = 0.955). Using 200μA, 20 Hz and 4.0 ms pulse duration, electrical stimulation increased ACh release to 546 ± 32 % (n = 4) of baseline (Fig 3a). The half-maximal response was obtained with pulse durations of 0.2 ms and only a minor additional increase in ACh release was observed at durations greater than 1 ms. The effect on ACh release of increasing the stimulation frequency was studied by keeping the pulse duration and current constant at 200μA and 0.2 ms, respectively (Fig. 3b). ACh release reached a plateau at 20 Hz with a mean increase of 250 ± 30 % (n = 7) of baseline.

The response of dopaminergic terminals to electrical stimulation was markedly

different from that of cholinergic striatal interneurons. Basal DA release (0.0854 $\pm$ 0.0114 pmol/10 min) was affected in an all-or-none fashion by stimulation at the parameters tested (i.e., evoked release was not influenced by increasing frequency or pulse duration after reaching a threshold level). DA release was significantly increased at pulse durations of 2.0 ms or greater (Fig. 3a) or at frequencies of 20 Hz or greater (Fig. 3b); however, further increases in these parameters did not further augment DA release.

Electrophysiology of ACh release as determined by strength-duration relation

A strength-duration curve was calculated for the relationship between current and pulse duration, as described in the methods section. This calculation was possible because ACh release, as expressed as percent increase over basal, was found to be linearly related to current ($y = 0.00730x + 0.00391$, $r = .998$) in the range examined. The obtained curve fit the predicted equation $I = I_r (1+C/t)$. The chronaxie (C) (i.e., the pulse duration needed at twice the rheobase current) was 0.236 ms.

The effect of repeated stimulation on ACh release

Two experiments were performed to determine whether the release of ACh by the first stimulation period differed from that evoked by one or two subsequent stimulation periods, provided at intervals of 30 min. Eleven animals were stimulated using identical electrical parameters for two periods; three were stimulated for 3 periods (Fig. 5). ACh release during the second and third periods did not differ significantly from release during the first period.

In a second experiment, a multiple regression analysis was performed using all of the stimulation data (143 stimulation periods) to assess the effect of stimulation order on ACh release. In the regression analysis, ACh release was designated as the independent variable while current, duration, frequency and order were designated as dependent variables. In the resulting regression, duration, frequency and current all had p values of less than 0.001, whereas order had a p value of 0.23. The overall r-squared value (the percent of variance explained by the regression) was unaffected by the presence or

absence of the stimulation order variable (from 0.8361. to 0.8344), further demonstrating that the level of ACh evoked by the second or third stimulation did not differ from that evoked by the first.

TTX sensitivity of ACh release

Both basal and evoked ACh release were entirely TTX dependent. Basal ACh release (3.34 $\pm$ 0.33 pmol, n = 3) and electrically stimulated ACh release (9.86 $\pm$ 1.68 pmol, n = 3) were reduced by the addition of TTX (10μM) to levels below the limit of detection.

The effect of Ch on basal and evoked ACh and DA

Ch administration to anesthetized rats after collection of basal dialysate samples increased both basal and evoked ACh release. A typical experiment is illustrated in Fig. 6. Basal ACh release 30 min after injection of 120 mg/kg Ch chloride was significantly increased from a mean of 117 $\pm$ 7 to 141 $\pm$ 5 % of baseline levels ($p < 0.05$); lower doses were ineffective (Table I). Ch enhanced evoked ACh release in a dose-dependent manner ($r = 0.994$, $p < 0.01$); release after 120 mg/kg Ch chloride (600 $\pm$ 34 %, $p < 0.01$) and after 60 mg/kg (502 $\pm$ 49, $p < 0.05$) differed significantly from release after saline (386 $\pm$ 38 %). A potentiation of release was also observed in the second stimulation period, from 401 $\pm$ 33 % to 578 $\pm$ 52 % of basal levels in the 120 mg/kg group ($p < 0.05$). A statistically significant increase ($p < 0.01$) in brain dialysate Ch was observed immediately after the i.p. injection of Ch at all doses (Table I). Under these conditions, we saw no effect of Ch on DA release. However, the neostigmine in the perfusion medium elevated DA release from 36.6 $\pm$ 5.1 to 71.5 $\pm$ 15.0 fmol/10 min ($p < 0.01$, n = 15).

Effect of Ch on ACh release from awake animals

In awake animals, ACh release from the corpus striatum was approximately twice that observed in anesthetized animals (from 2.52 $\pm$ .20 to 4.10 $\pm$ .31 pmols/10 min, $p < 0.01$). Ch (120 mg/kg) significantly enhanced ($p < 0.05$) the basal ACh release to 154 $\pm$ 17 % (n = 4) of baseline as compared to a saline injection (105 $\pm$ 7 %, n = 6).

Electrically evoked release was also increased ($p< 0.01$) by Ch to 262 $\pm$ 19 % as compared to the saline group which was only increased 146 $\pm$ 13 % .

DISCUSSION

We incorporated a stimulating electrode into a standard microdialysis probe (Fig. 1) in order to characterize the release of transmitters from depolarized DA terminals and ACh interneurons within the rat striatum. Once the electrophysiological properties of such release were determined, we applied this apparatus to examining the effect of Ch administration on striatal ACh and DA release. We observed that Ch in the doses tested potentiated both basal and evoked ACh release, but had no apparent effect on DA release.

Our hybrid probe is similar to one described by Sandberg et al.[2,24], except that in our study the electrode was used for stimulating, and not recording from, neurons surrounding the probe. Using short-duration electrical pulses (0.6 ms, 20 Hz), we observed that striatal neurons can be stimulated for as much as 3 ten-minute periods without exhibiting attenuation of evoked ACh release (Fig. 5). In contrast, such release is rapidly attenuated when striata are perfused locally with high potassium solutions[19]. The long periods (10 - 20 min) of constant depolarization associated with exposure to high potassium concentrations make this method less physiologic than electrical stimulation.

Initially, we validated the use of the hybrid microdialysis probe by measuring ACh release in response to increasing amounts of current, using a frequency (20 Hz) and pulse duration (0.2 ms) typical of neurophysiologic studies involving brain stimulation. The basal and evoked ACh release was found to be TTX dependent, indicating that the release was indicative of physiologic processes and not the result of unspecific trauma. Basal ACh release (2.52 $\pm$ 0.29 pmol/10 min, using probes with approximately a 10 % recovery) was consistent with levels in previous reports, if the data in such reports are corrected for the lower recoveries (4 - 5 %) in those studies[1,6,33]. The release of ACh was found to

vary linearly with increasing current ($r = 0.955$; Fig. 2).

ACh release was profoundly affected by pulse durations in the range of 0.2 - 1.0 ms ($p < 0.01$; 20 Hz; 200μA) (Fig. 3a). A pulse duration of 0.2 ms increased ACh release to 251 $\pm$ 30 % of baseline while leaving DA release unchanged. DA release was significantly increased only at pulse durations of 2.0 ms or greater. This finding might suggest that the physiologic release of ACh within the striatum fails to affect DA release from nigrostriatal terminals. Alternatively, the presence of neostigmine (10μM) in our dialysis medium might, by elevating synaptic ACh levels, have rendered the dopaminergic terminals insensitive to additional ACh[6,13]. This is supported by our observation that basal DA levels are increased twofold by the addition of neostigmine.

Maximal ACh release was achieved at a frequency of 20 Hz (Fig. 3b). This frequency may be physiologic for striatal interneurons, inasmuch as Wilson et al. (1990) observed spontaneous firing at rates of 3 to 10 Hz, with bursts of up to 23 Hz, in intracellular recordings from such neurons[34]. Unlike ACh release, DA release was increased in an all-or-nothing fashion by intrastriatal stimulation (Figs. 3a and 3b). Similar findings have been reported for medial forebrain bundle (MFB) stimulation at frequencies of 6.67, 8.33, 12.5 and 25 Hz; all produced approximately the same (50 %) increase in DA release despite the fact that the number of action potentials recorded from the region surrounding the probe was increased in proportion to the stimulation frequency[26]. Our failure to observe any increase in DA release at frequencies below 20 Hz may reflect the differing calcium levels (1.2 mM *vs* 2.3 mM) used by us and by Tepper et al. (1991), or perhaps differences in the responsiveness of axons (MFB) and terminals (striata). Imperato et al. (1984) and Manley et al. (1992) both demonstrated a linear relationship between the number of pulses delivered and the increase in DA release from the rat striatum as determined by *in vivo* microdialysis, in the presence of elevated calcium levels (3.4 mM and 2.3 mM respectively)[12,18]. The calcium concentration is likely to play a critical role in regulating the release of DA and therefore comparisons to studies with

elevated calcium concentrations are tenuous. The finding that DA release exhibited all-or-none behavior in response to a range of stimulation frequencies may have resulted from autoreceptor-related mechanisms.

Neuron size, distance from the electrode, degree of myelination, neuronal region (perikaryon *vs.* axon *vs.* terminal) and various electrophysiological properties may all affect the excitability of a neuron in response to electrical stimulation[22,35]. The DA in rat striatum is localized predominantely in the nerve terminals - which are typically bulbous varicosities with a thickness of 0.4 - 2.0μm[34]. ACh is present in both terminals and cell bodies of aspiny striatal neurons with a calculated diameter of 20 - 30μm[10]. The larger cholinergic neurons, because of their greater length constant, would be expected to reach firing threshold at lower stimulation currents than the smaller dopaminergic terminals[22]. In our experiments, ACh release was, in fact, more easily evoked than DA, a finding consistent with anatomical data on the relative sizes of these two classes of neurons.

The responsiveness of neurons to electric current can be characterized by the strength-duration curve (Fig.4), which represents the amount of current and the duration of pulses that yield the same level of response (i.e., the evoked increase in ACh release). We calculated this curve in a novel fashion, using data relating ACh release to current level at a fixed duration, and using data relating ACh release to pulse duration at a fixed current (Figs. 2 and 3a). The calculated curve fit the theoretical strength-duration equation; moreover, the calculated chronaxie (0.236 ms), a measure of the excitability of cholinergic striatal neurons, was typical of CNS gray matter[22]. Thus, we have been able to relate neurotransmitter release *in vivo* to the electrophysiological properties of the cholinergic neurons.

Ch administration to anesthetized animals rapidly elevated brain Ch release and increased both basal and evoked ACh release, without affecting that of DA (Table I). This lack of response may have been an artifact of the use of neostigmine, an acetylcholinesterase inhibitor, in the dialysate, resulting in synaptic ACh levels which

saturated the responsiveness of DA terminals. This is supported by our findings that neostigmine significantly ($p< 0.01$) elevated basal DA levels. Ch *amplified* evoked ACh release in a dose-dependent fashion ($r = 0.994$, $p< 0.01$), but increased basal ACh release only at a dose of 120 mg/kg (Table I).

In order to establish that the effect of Ch on ACh release was not an artifact of the anesthetic used in this study which might have inhibited striatial glutamte receptors[5,8] thus enhancing Ch responsiveness[4] we examined the effect of Ch in awake, freely-moving rats. Our results indicate that the anesthetic significantly ($p< 0.01$) suppresses basal ACh release by approximately 40 %. Despite the higher basal ACh levels, Ch was still able to significantly elevate both basal ($p< 0.05$) and evoked ($p< 0.01$) ACh release at a dose of 120 mg/kg. In fact, the ratio of the evoked ACh release from rats receiving 120 mg/kg Ch *vs* saline controls, was 1.68 in anesthetized animals and 1.78 in awake animals.

Addition of exogenous Ch to striatal slices elevates tissue ACh and Ch levels and also increases ACh release during stimulation[17,27,28,30,31]. Prior *in vivo* studies, although contradictory, provided some evidence that Ch might act to potentiate ACh release *in vivo*. Studies by Consolo et al. (1990) failed to find an effect of i.p. Ch chloride (100 mg/kg) on basal ACh from sham operated rats, but did show an effect in rats that had been decorticated by interruption of the corticostriatal pathway[4]. Westerink and de Boer (1990) also found no effect on ACh release of i.p. Ch (100 mg/kg) despite a 30 fold increase in brain Ch release[33]. This is consistent with our finding that basal ACh release was increased only at doses of 120 mg/kg. However, we only observed a twofold increase in brain Ch release following Ch administration. Given that plasma Ch levels increase only 3- 4 fold following an i.p. Ch injection of 100 mg/kg[11], and that the macromolecular system which transports Ch across the blood-brain barrier operates by facilitated diffusion across a concentration gradient[20], it is difficult to understand the 30 fold increase observed by Westerink and de Boer (1990)[32]. While Koshimura et al. (1990) failed to find an effect of a low Ch dose (7.5 mg/kg, i.p.) on ACh release, they did demonstrate

increases after intracerebroventricular Ch (10μmol, 20μmol or 50μmol)[16]. Our data not only demonstrate that ACh and DA can be realiably evoked from rat striatum *in vivo*, but that changes in local Ch concentrations can affect ACh release.

ACKNOWLEDGEMENTS

These studies were supported in part by a research grant (MH28783) from the National Institute of Mental Health. In addition, Mr. Farber was supported by training grant MH15761. We thank ESA Corporation for the loan of a Coulochem II electrochemical detector. The authors gratefully acknowledge the helpful discussions with Dr. B. E. Slack and Ms. H. B. Parthasarathy. Drs. E. J. Tehovnik and S. Nelson provided advice on electrical stimulation. Dr. S. Kaakkola provided assistance with DA analysis. We would like to especially thank Dr. F. C. Liu for valuable advice on the striatal anatomy.

REFERENCES

1 Ajima, A. and Kato, T., Brain dialysis: detection of acetylcholine in the striatum of unrestrained and unanesthetized rats, *Neurosci. Lett.*, 81 (1987) 129-132.

2 Arbuthnott, G.W., Fairbrother, I.S. and Butcher, S.P., Dopamine release and metabolism in the rat striatum: an analysis by *in vivo* brain microdialysis, *Pharmacol. Ther.*, 48 (1990) 281- 293.

3 Cohen, E.L. and Wurtman, R.J., Brain acetylcholine: Control by dietary choline, *Science*, 191 (1976) 561-562.

4 Consolo, S., Salmoiraghi, P., Amoroso, D. and Kolasa, K., Treatment with oxiracetam or choline restores cholinergic biochemical and pharmacological activities in striata of decorticated rats, *J. Neurochem.*, 54 (1990) 571-577.

5 de Boer, P., Westerink, B.H.C. and Horn, A.S., The effect of acetylcholinesterase inhibition on the release of acetylcholine from the striatum *in vivo*: interaction with autoreceptors, *Neurosci. Lett.*, 116 (1990) 357-360.

6 During, M.J., Acworth, I.N. and Wurtman, R.J., Effects of systemic L-tyrosine on dopamine release from rat corpus striatum and nucleus accumbens, *Brain Res.*, 452 (1988) 378-380.

7 Gardier, A.M. and Wurtman, R.J., Persistent blockade of potassium-evoked serotonin release from rat frontocortical terminals after fluoxetine administration, *Brain Res.*, 540 (1991) 325-330.

8 Gerfen, C.R., Herkenham, M. and Thibault, J., The neostriatal mosaic: II. Patch- and matrix-directed mesostriatal dopaminergic and non-dopaminergic systems., *J. Neurosci.*, 7 (1987) 3915-3934.

9 Haubrich, D.R., Wang, P.F.L., Clody, D.E. and Wedeking, P.W., Increase in rat brain acetylcholine induced by choline or deanol, *Life Sci.*, 17 (1975) 1095-1102.

10 Imperato, A. and di Chiara, G., Trans-striatal dialysis coupled to reverse phase high performance liquid chromatography with electrochemical detection: A new method for the study of *in vivo* release of endogenous dopamine and metabolites, *J. Neurosci.*, 4 (1984) 966-977.

11 Kawashima, K., Hayakawa, T., Kashima, Y., Suzuki, T., Fujimoto, K. and Oohata, H., Determination of acetylcholine release in the striatum of anesthetized rats using *in vivo* microdialysis and a radioimmunoassay, *J. Neurochemi.*, 57 (1991) 882-887.

12 Kawaski, K., Czech, G. and Sojen, G.G., Prolonged exposure to high potassium concentrations results in irreversible loss of synaptic transmission in hippocampal tissue slices, *Brain Res.*, 457 (1988) 322-329.

13 Klein, J., Koppen, A. and Loffelholz, K., Uptake and storage of choline by rat brain: Influence of dietary choline supplementation, *J. Neurochem.*, 57 (1991) 370-375.

14 Koshimura, K., Miwa, S., Lee, K., Hayahi, Y., Hasegawa, H., Hamahata, K., Fujiwara, M., Kimura, M. and Itokawa, Y., Effects of choline administration on *in vivo* release and biosynthesis of acetylcholine in the rat striatum as studied by *in vivo* brain microdialysis, *J. Neurochem.*, 54 (1990) 533-539.

15 Maire, J.E. and Wurtman, R.J., Effects of electrical stimulation and choline availability on the release and contents of acetylcholine and choline in superfused slices from rat striatum, *J. Physiol., Paris,* 80 (1985) 189-195.

16 Manley, L.D., Kuczenski, R., Segal, D.S., Young, S.J. and Groves, P.M., Effects of frequency and pattern of medial forebrain bundle stimulation on caudate dialysate dopamine and serotonin, *J. Neurochem.*, 58 (1992) 1491-1498.

17 Marien, M.R. and Richard, J.W., Drug effects on the release of endogenous acetylcholine *in vivo*: Measurement by intracerebral dialysis and gas chromatography-mass spectrometry, *J. Neurochem.*, 54 (1990) 2016-2023.

18 Paxinos, G. and Watson, C., *The Rat Brain in Stereotaxic Coordinates*, Academic Press, New York, 1982.

19 Ranack, J.B. Jr., Extracellular stimulation. In M.M. Patterson & R.P. Kesner (Eds.), *Electrical Stimulation Research Techniques*, Academic Press, New York, 1981, pp. 1-35.

20 Robertson, G.S., Damsma, G. and Fibiger, H.C., Characterization of dopamine release in the substantia nigra by *in vivo* microdialysis in freely moving rats, *J. Neurosci.*, 11 (1991) 2209-2216.

21 Sandberg, M., Butcher, S.P. and Hagberg, H., Extracellular overflow of neuroactive amino acids during severe insulin induced hypoglycemia: *In vivo* microdialysis of the rat hippocampus, *J. Neurochem.*, 47 (1986) 178-184.

22 Shimoyama, M. and Shozo, K., Effect of cerulein on *in vivo* release of acetylcholine from rat striatum, *Brain Res.*, 492 (1989) 381-384.

23 Tepper, J.M., Creese, I. and Schwartz, D.H., Stimulus-evoked changes in neostriatal dopamine levels in awake and anesthetized rats as measured by microdialysis, *Brain Res.*, 559 (1991) 283-292.

24 Trommer, B.A., Schmidt, D.E. and Wecker L., Exogenous choline enhances the synthesis of acetylcholine only under conditions of increased cholinergic activity, *J. Neurosci.*, 39 (1982) 1704-1709.

25 Ulus, I.H., Wurtman, R.J., Mauron, C. and J.K., Blusztajn, Choline increases acetylcholine release and protects against the stimulation-induced decrease in phosphatide levels within membranes of rat corpus striatum, *Brain Res.*, 484 (1989) 217-227.

26 Ungerstedt, U., Measurement of neurotransmitter release by intercranial dialysis. In C.A. Marsden (Ed.), *Measurement of Neurotransmitter Release In Vivo*, Wiley & Sons, Chichester, 1984, pp. 81-105.

27 Wecker, L., Influence of dietary choline availability and neuronal demand on acetylcholine synthesis by rat brain, *J. Neurosci.*, 51 (1988) 497-504.

28 Weiler M.H., Bak, I.J. and Jenden, D.J., Choline and acetylcholine metabolism in rat neostriatal slices, *J. Neurosci.*, 41 (1983) 473-480.

29 Westerink, B.H.C. and de Boer, P., Effect of choline administration on the release of acetylcholine from the striatum as determined by microdialysis in awake rats, *Neurosci. Lett.*, 112 (1990) 297-301.

30 Westernick, B.H.C., de Boer, P. and Damsma, G., Dopamine-acetylcholine interaction in the striatum studied by microdialysis in the awake rat: some methodological aspects, *J. Neurosci. Methods*, 34 (1990) 117-124.

31 Wilson, C.J., Chang, H.T. and Kitai, S.T., Firing patterns and synaptic potentials of identified giant aspiny interneurons in the rat neostriatum, *J. Neurosci.*, 10 (1990) 508-519.

32 Yeomans, J.S.,*Principles of Brain Stimulation*, Oxford University Press, London, 1990, 36-54pp.

FIGURE LEGENDS

Fig. 1. Hybrid microdialysis probe [not drawn to scale]. A glass microcapillary (SP100170, Polymicron Technologies) (E) is passed through the dialysis tubing (C). The dialysis membrane and microcapillary are then slid through stainless steel 25 gauge tubing (B). A small drop of epoxy glue is used to seal the end of the microdialysis membrane and the interface between the membrane and the steel tube, such that the dialysis membrane extended 5 mm from the steel tube. After the epoxy has cured, the glass microcapillary is threaded through a hole in the side of the microline (i.d. 0.51 mm, Cole-Parmer) (F) and sealed with epoxy (D). The microcapillary is used to collect the microdialysate while the microline, attached to the perfusion pump, is used to provide the input fluid. The stainless steel tubing (B) is electrically isolated from the stereotaxic frame and utilized as the ground. The tungsten electrode (#7995, AM Systems) (A) is cut and tested prior to attachment to the probe.

Fig. 2. Electrically-evoked acetylcholine release from striatal interneurons as a function of stimulation current. Striata were stimulated with biphasic pulses (0.2 ms and 20 Hz) one, two, or three times and separated by at least 30 min to allow ACh release to return to baseline. Values represent pmols of ACh released per 10 min (mean $\pm$ S.E.M.) of 4 - 8 rats. Data were analyzed using an ANOVA with a post-hoc Newman Keul's test (* $p < 0.05$; ** $p < 0.01$ compared to basal levels).

Fig. 3. Electrically evoked acetylcholine and dopamine release as a function of stimulation frequency and pulse duration. Stimulation parameters were systematically varied to characterize the excitability of striatal neurons. DA values showed increased variability with repeated stimulations, so that only data from the first stimulation periods for each animal were used. Values represent percents of baseline levels (mean $\pm$ S.E.M.) of 4 - 8 rats. A: stimulation current and frequency were fixed at 200μA and 20 Hz, respectively, while pulse duration was varied. B: Stimulation current and pulse duration were fixed at

200μA and 4 ms respectively for DA, and 200μA and 0.2 ms respectively for ACh. ACh is represented by filled circles and DA by filled triangles. Statistics were performed using an ANOVA with a post-hoc Newman Keul's test (* $p < 0.05$, ** $p < 0.01$ compared to basal levels).

Fig. 4. Strength-duration curve for an increase to 250 % of basal acetylcholine release, calculated as described in methods. The curve fits the predicted equation $I=I_r(1+C/t)$, where I is the current, I_r is the rheobase current (the current required for very long duration pulses), t is the pulse duration and C is the Chronaxie (the time on the curve for twice I_r). C = 0.236 ms.

Fig. 5. Effect of repeated striatal stimulation on acetylcholine release. Animals were stimulated, utilizing the same parameters (0.6 ms, 20 Hz, 200μA) for two (n = 11) or three (n = 3) periods. Data are expressed as means ± S.E.M. S_1, S_2 and S_3 values did not differ significantly.

Fig. 6. Effect of choline administration on basal and evoked acetylcholine release from anesthetized rat corpus striatum. After three baseline samples, animals received isotonic saline i.p. (open bars) or choline chloride (120 mg/kg) (crossed hatched bars). Three additional samples were collected and then striata were electrically stimulated for 10 min (current 200μA, pulse duration 0.6 ms, frequency 20 Hz). After 30 additional min, the stimulation was repeated. Figure shows the results of a typical experiment.

Table Legend

Table I

Effect of choline administration on release of choline, acetylcholine and dopamine from anesthetized rat striatum

Baseline levels of ACh, Ch and DA were determined during the first three 10-min collection periods after which rats received either isotonic saline (n = 10) or choline chloride (30 [n = 6], 60 [n = 10], or 120 [n = 11] mg/kg) i.p. Three additional 10-min samples were collected and then the microdialysis electrodes were stimulated for 10 min (current 200μA, pulse duration 0.6 ms, frequency 20 Hz) and samples were collected for an additional 30 min. Resting levels represent the release immediately prior to stimulation. Data (means ± S.E.M.) are expressed both as pmols/10 min and percents of basal levels (i.e., levels during the initial three pretreatment collection periods). The normalized data were analyzed using an ANOVA with a post-hoc Newman Keul's test (* $p < 0.05$, ** $p < 0.01$).

Figure 1

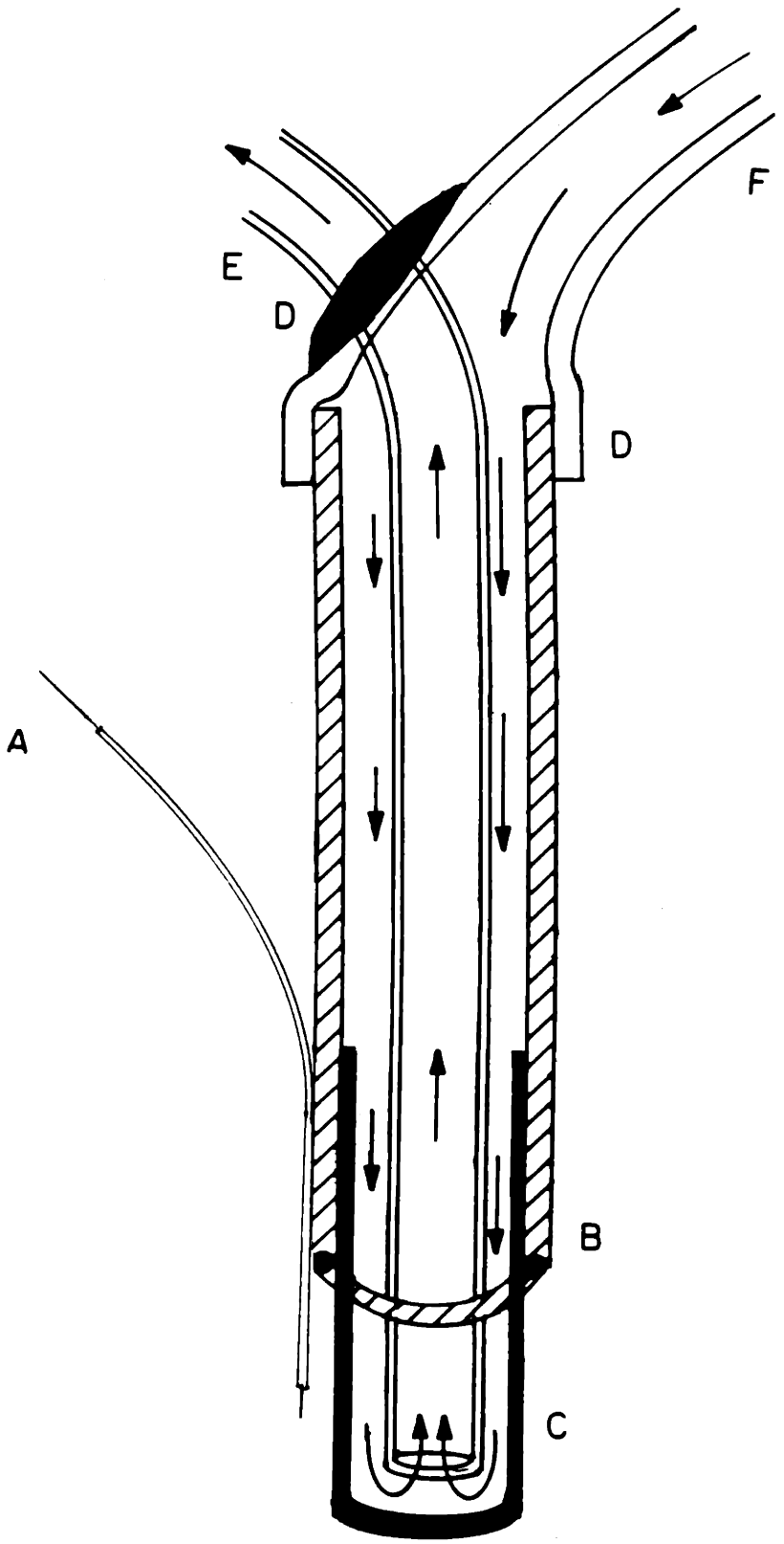


Figure 2

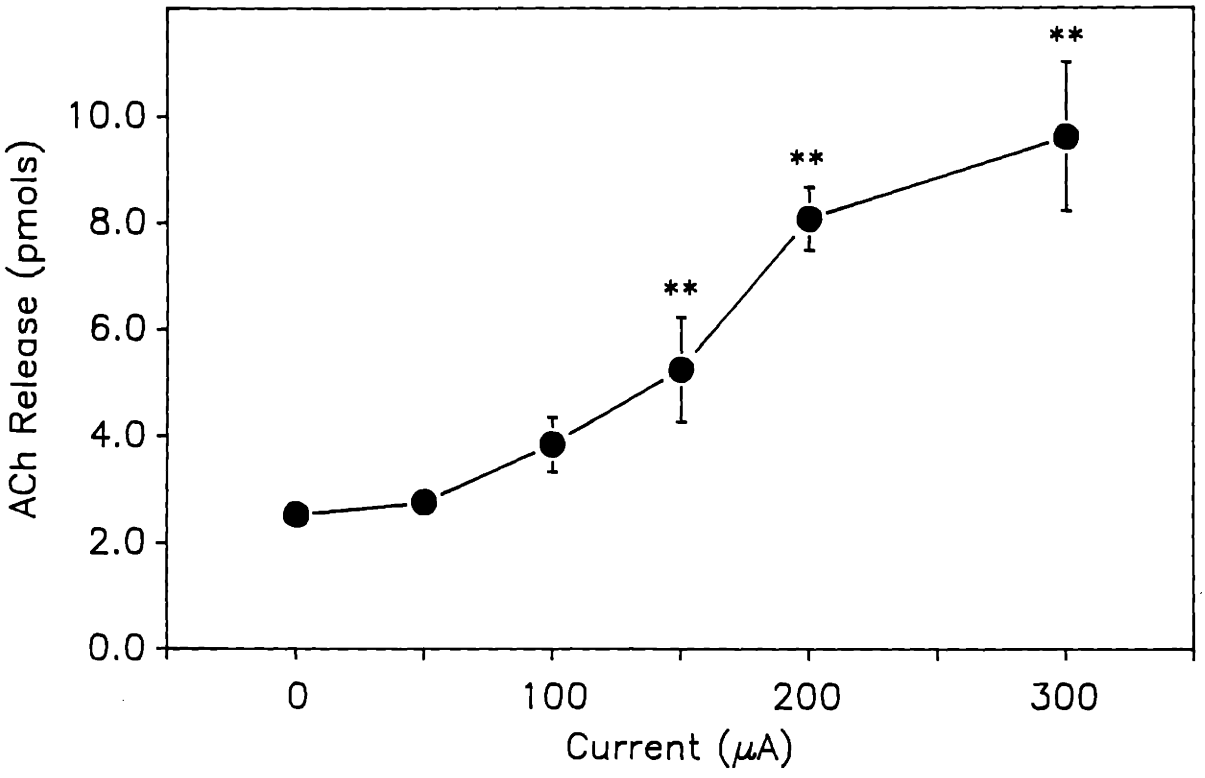
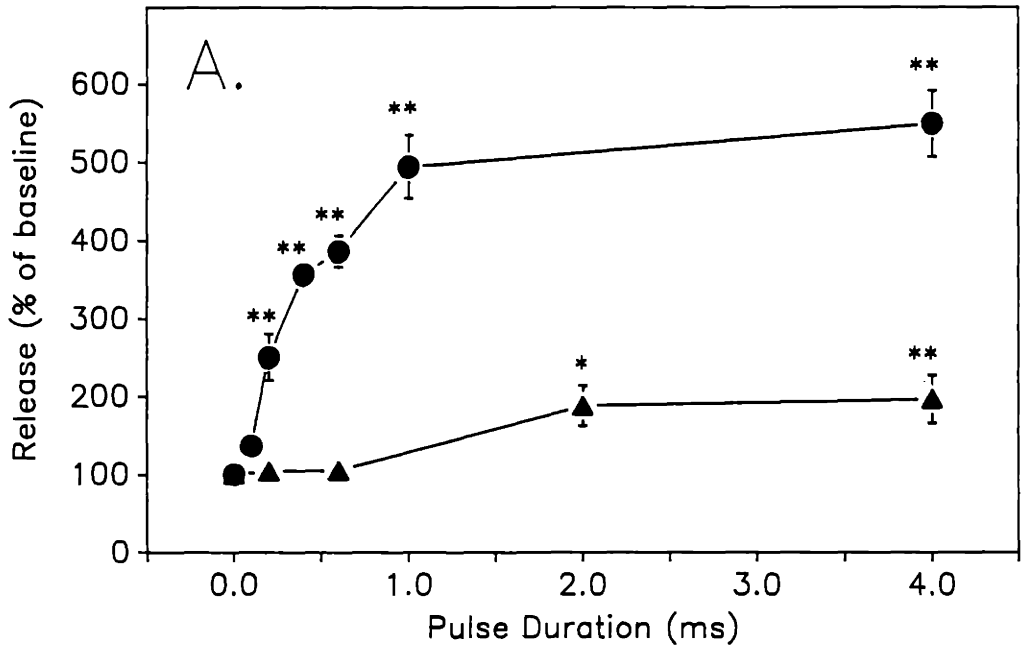


Figure 3


A.
Release (% of baseline)
600
500
400
300
200
100
0
0.0
1.0
2.0
3.0
4.0
Pulse Duration (ms)
**
*

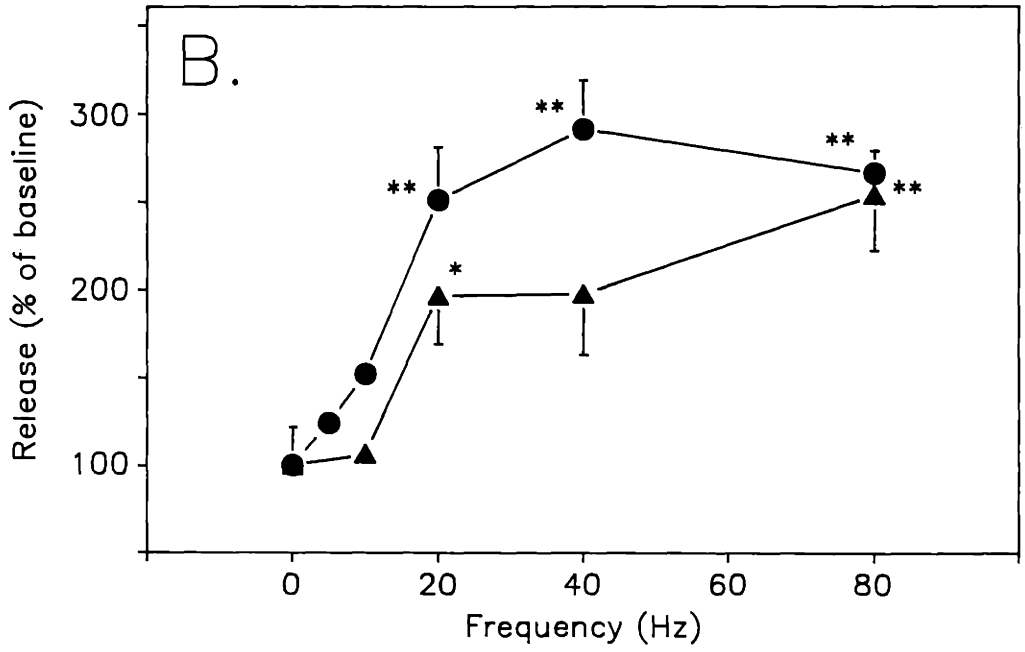

B.
Release (% of baseline)
300
200
100
0
20
40
60
80
Frequency (Hz)
**
*

Figure 4

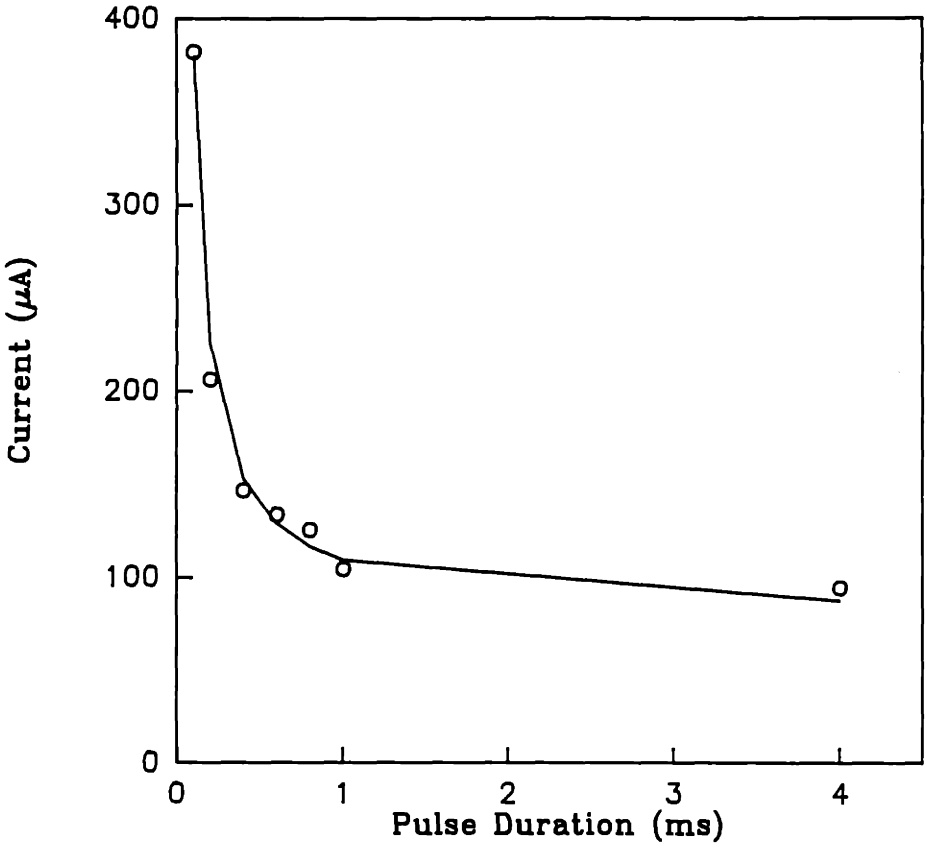


Figure 5

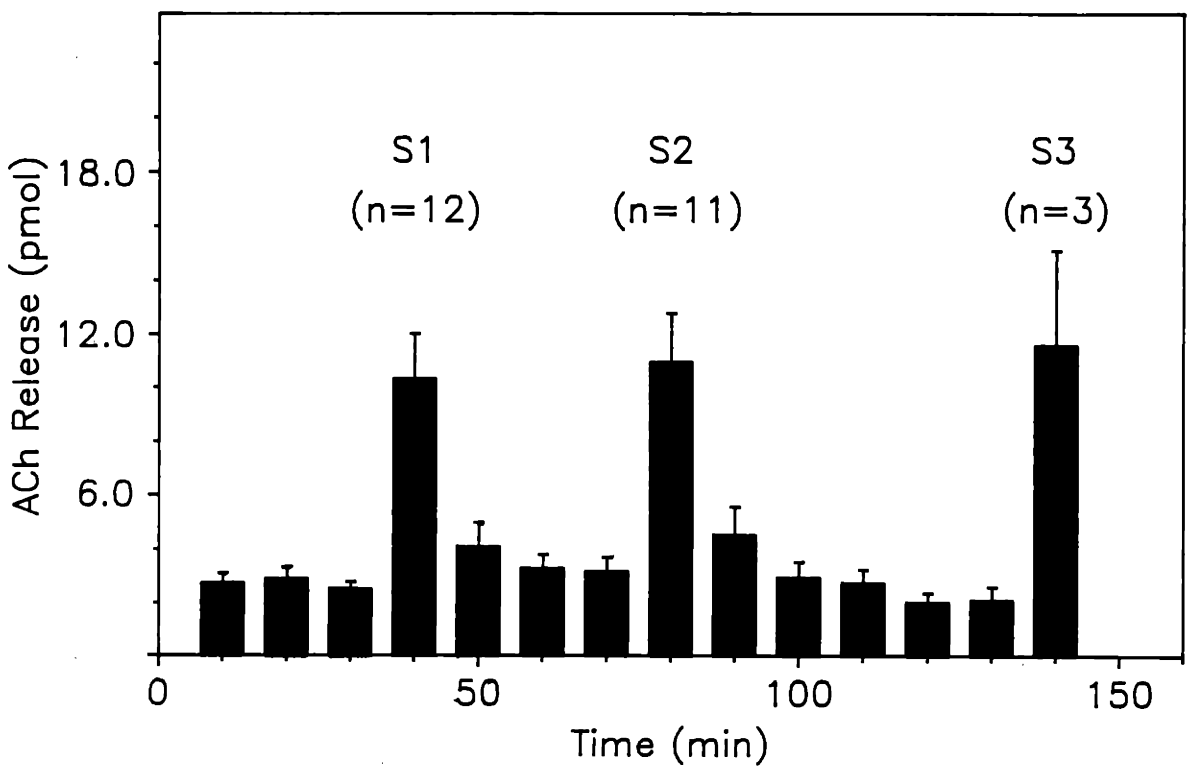

S1
(n=12)
S2
(n=11)
S3
(n=3)
ACh Release (pmol)
18.0
12.0
6.0
0
50
100
150
Time (min)

Figure 6

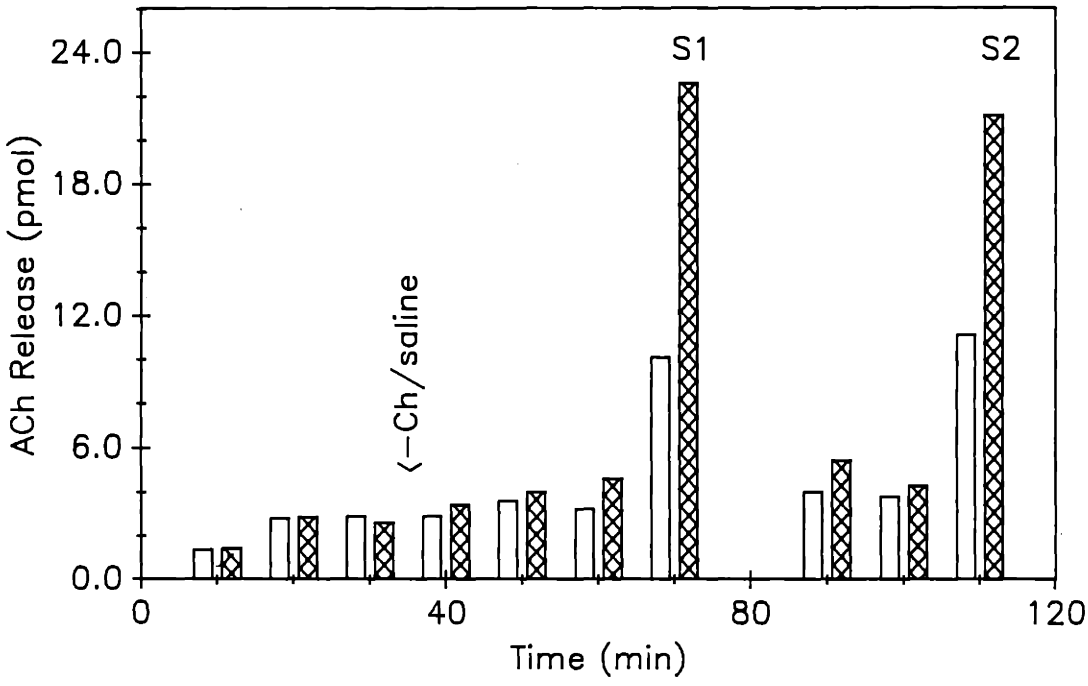

24.0
18.0
12.0
6.0
0.0
ACh Release (pmol)
S1
S2
<—Ch/saline
0
40
80
120
Time (min)

Table I

Choline (mg/kg)	Release/10 min Resting (pmols)	Resting (% basal)	Evoked (pmols)	Evoked (% basal)
		Choline		
0	10.9 ± 1.2	75 ± 5	11.4 ± 1.0	76 ± 3
30	15.0 ± 4.2	141 ± 26 *	13.9 ± 4.1	139 ± 22 *
60	17.0 ± 2.6	135 ± 14 *	16.1 ± 2.4	125 ± 13 *
120	20.9 ± 4.0	152 ± 16 **	17.4 ± 3.2	126 ± 11 *
		Acetylcholine		
0	2.76 ± .18	113 ± 7	10.7 ± 1.0	357 ± 30
30	2.92 ± .42	116 ± 5	10.2 ± 0.9	427 ± 30
60	2.20 ± .52	110 ± 5	13.2 ± 1.5	502 ± 49 *
120	3.54 ± .26	141 ± 5 **	15.2 ± 1.4	600 ± 34 **
		Dopamine		
0	.116 ± .024	107 ± 11	.152 ± .044	159 ± 26
30	.130 ± .022	131 ± 14	.242 ± .058	227 ± 24
60	.105 ± .008	99 ± 2	.160 ± .022	146 ± 25
120	.089 ± .011	107 ± 6	.152 ± .028	167 ± 16

PNAS *in press*

Release of amyloid β-protein precursor derivitives by electrical depolarization of rat hippocampal slices

(Alzheimer's Disease / APP / Electrical Stimulation / Neurotransmission)

ROGER M. NITSCH*,†,‡, STEVEN A. FARBER*, JOHN H. GROWDON†,
& RICHARD J. WURTMAN*.

*Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, E25-604, Cambridge, MA 02139; and †Department of Neurology, Massachusetts General Hospital and Harvard Medical School, ACC 830, Fruit Street, Boston, MA 02114

Communicated by Emilio Bizzi.

Abbreviations: APP, ß-amyloid protein precursor ; LDH, Lactate Dehydrogenase

‡To whom correspondence should be addressed

ABSTRACT

Proteolytic processing of the amyloid ß-protein precursor (APP) is regulated by cell-surface receptors. To determine whether neurotransmitter release in response to neuronal activation regulates APP processing in brain, we electrically depolarized superfused rat hippocampal slices and measured soluble APP derivatives released into the superfusate. Electrical depolarization caused a rapid increase in the release of both neurotransmitters and amino-terminal APP cleavage products. These derivatives lacked the APP carboxyl-terminus and were similar to those found in both cell culture media and human cerebrospinal fluid. Superfusate proteins including lactate dehydrogenase were not changed by electrical depolarization. The release of amino-terminal APP derivatives increased with increasing stimulation frequencies from 0 to 30Hz. The increased release was inhibited by the sodium-channel antagonist tetrodotoxin, suggesting that action potential formation mediates the release of large amino-terminal APP derivatives. These results suggest that neuronal activity regulates APP processing in the mammalian brain.

INTRODUCTION

Amyloid deposits in the brains of Alzheimer's disease patients consist of aggregates of ßA4 peptides (also Aß), which are 39-43 amino-acid proteolytic derivatives of the amyloid ß-protein precursors (APP) [for review see (1)]. Amyloid ß-protein precursors are members of a large family of integral transmembrane glycoproteins existing in various forms derived from alternative mRNA splicing [for review see (1)]. Mature APP can be processed by several alternative proteolytic pathways that generate different breakdown products. A secretory pathway generates non-amyloidogenic soluble amino-terminal derivatives following cleavage within the ßA4 segment (2,3). Alternatively, endosomal-lysosomal pathways yield carboxyl-terminal derivatives, some of which contain intact ßA4 sequences and thus are potentially amyloidogenic (4-6). An additional pathway results in the secretion of soluble ßA4 fragments (7-9). In cell culture, the release of soluble APP derivatives is rapidly enhanced by stimulation of muscarinic receptor subtypes m1 and m3 (10,11), which transiently activate protein kinase C (PKC) (12). The biochemical mechanisms regulating APP processing pathways in the brain are unknown.

To determine whether APP processing is regulated by neurotransmission in intact brain samples, we electrically stimulated superfused rat hippocampal slices and measured the release of soluble APP derivatives using Western blotting and densitometry.

MATERIALS AND METHODS

Animals and Hippocampal Slice Preparation. Male Sprague-Dawley rats (9 to 11 months) were anesthetized with ketamine (85 mg/kg body weight *i.m.*) and decapitated in a cold room at 4°C. Brains were rapidly removed and placed into chilled (4°C) oxygenated Krebs-Ringer buffer (see below) containing 1 mM ketamine. After removal of remaining meninges, hippocampal slices (300µm) were quickly prepared using a McIllwain tissue chopper, washed three times, and placed into custom-made superfusion chambers [a

modified version of those introduced by J.C.E. Maire and R.J. Wurtman (13)].

Superfusion and Electrical Stimulation. Slices were equilibrated for 50 min at 37°C by superfusing the chambers with oxygenated Krebs-Ringer buffer at a flow rate of 0.8 ml/min. Superfusion chambers contained two opposing silver mash electrodes which were connected to an electrical stimulator (Model S88, Grass Instruments). A custom-made polarity reversal device was used to prevent chamber polarization and also monitored both the current and the voltage 50μs after the onset of each pulse to ensure uniform chamber resistance. After the equilibration period, slices were stimulated electrically in the presence or absence of tetrodotoxin (1μM) at frequencies of 0 to 30Hz, 1ms pulse duration, and a current density of 4.95mA/mm^2. The typical voltage of a pulse was 40V at a current of 120mA. Control groups were analyzed in parallel under identical conditions but were not electrically stimulated.

Immunoblot Analysis. Superfusates were collected for 50 min in chilled tubes (4°C) in the presence of 250μM phenylmethylsulfonyl fluoride, centrifuged for 30 min at 10,000xg to remove debris, and subjected to ultrafiltration against water at 4°C using cellulose dialysis tubing. This ultrafiltration protocol yielded a final volume of 4 ml which was lyophylized and reconstituted in SDS-containing gel loading buffer, and boiled for 5 min. Total slice protein levels were measured using the bicinchoninic acid assay (Pierce), and were constant among all groups. Reconstituted superfusate proteins equivalent to 600μg slice protein were electrophoresed on linear 12% SDS-polyacrylamide mini gels and electro-blotted onto polyvinyldifluoride membranes (Immobilon, Waters) which were subsequently probed with either the monoclonal antibody 22C11 (Boehringer Mannheim) or the polyclonal antiserum anti-C_8. The secondary horseradish peroxidase-linked antibodies were visualized by chemiluminescence (ECL, Amersham) using linear (pre-flashed) films. Immunoreactive bands were compared densitometrically using a laser scanner (Pharmacia LKB UltroScan XL) set at 40μm vertical interval size and 2.4mm horizontal slit width. All densitometric measurements were performed in the linear range as

determined by standard dilution curves of rat brain protein extracts. Groups used for statistical comparisons were always determined within the same Western blot. Results were normalized to the control values set to 100% and compared statistically using analysis of variance and *post-hoc* Newman-Keuls tests.

Neurotransmitter and Lactate Dehydrogenase (LDH) Assays. Acetylcholine in the superfusates was determined by HPLC with electrochemical detection (Coulochem II, ESA) using a platinum electrode (300mV) and a post-column reactor containing immobilized acetylcholinesterase and choline oxidase (ESA). LDH activity in 1 ml superfusates was assayed using a modified version of a commercially available assay kit (Sigma no. 500).

RESULTS AND DISCUSSION

Immunoreactive material with apparent molecular masses ranging from 98kD to 130kD was detected in the superfusates with the monoclonal antibody 22C11 raised against a full-length APP fusion protein (14). The major band migrated at 117kD (Fig. 1A). These molecular masses are identical to those of secreted amino-terminal APP derivatives described in human cerebrospinal fluid and in conditioned cell culture media (14,15). Western blot analysis of the secreted proteins using an antibody against the carboxyl-terminal 20 amino acids of full-length APP (anti-C_8) failed to detect immunoreactive material in the same superfusates, while protein extracts from the hippocampal slices clearly showed immunoreactivity of the expected molecular mass (16) (Fig. 1B). These data suggest that hippocampal slices *in vitro* contain intact, full-length APP and release soluble amino-terminal APP derivatives that lack the APP carboxyl-terminus.

Electrical field stimulation increased the release of these APP derivatives within 50 minutes (Fig. 1A). Densitometric quantitation of this release indicated a significant twofold increase ($p<0.01$) (Fig. 2A). Similar increases in the release of soluble amino-terminal APP derivatives were observed after stimulation with receptor agonists in cell

culture (10,11).

The enhanced release of these APP derivatives evoked by electrical depolarization was prevented ($p<0.05$) by the addition of tetrodotoxin (Figs. 1A and 2A), which selectively blocks voltage-sensitive sodium channels necessary for the generation of action potentials (17). This finding indicates that neuronal activity can regulate APP processing in mammalian brain.

To ensure that interventions used in our study reliably modified neuronal activity, we monitored the efficacy of both electrical depolarization and tetrodotoxin by measuring the release of the neurotransmitter acetylcholine. Electrical stimulation increased acetylcholine release four-fold (72.1 $\pm$ 19.9 to 294.8 $\pm$ 44.0 pmol/hr/mg protein, means $\pm$ SEM, n=5 per group, $P<0.01$, *t*-test) and this effect was blocked by tetrodotoxin (121.9 $\pm$ 12.2 pmol/hr/mg protein, $P<0.01$).

To demonstrate cell viability and to control for the specificity of the release of APP derivatives, we measured the release of LDH (18) into the superfusate during electrical stimulation. The basal amount of LDH release was low, and was not increased by stimulation. These findings indicate that cells within the slices were intact during the experiment and that electrical stimulation did not affect viability (Fig. 2B). Moreover, stimulation did not change total release of protein into the superfusate: Coomassie blue staining of released proteins separated on linear 12% SDS-polyacrylamide gels showed that the overwhelming majority of bands were unaffected by electrical depolarization (Fig.1C).

Electrical depolarization via opening of voltage-gated sodium channels increases intracellular Ca^{++} concentrations which induces vesicular neurotransmitter release. It is unclear whether Ca^{++} is directly involved in APP cleavage and release. In cell culture, however, Ca^{++} was not necessary to stimulate the release of amino-terminal APP derivatives, nor were increases in intracellular Ca^{++} sufficient to alter this release (10), although there was a synergistic effect of Ca^{++} and activation of protein kinase C (R.M.N.

unpublished observations). In hippocampal slices, the role of Ca^{++} can not be addressed because the absence of Ca^{++} in the superfusate causes severe tissue damage leading to non-specific release of proteins including LDH (19). Pharmacological blockade of Ca^{++}-channels inhibits neurotransmitter release and thus also is not suited to address the role of Ca^{++} in regulating APP processing.

The increase in the release of soluble amino-terminal APP derivatives occurred within the first 50 minutes of stimulation, suggesting that these derivatives are likely to result from accelerated processing of pre-existing proteins and not from increased APP gene expression. This notion is compatible with results in cell culture that show that derivatives of newly synthesized APP do not appear in the culture medium until at least 50 minutes after the APP is synthesized (14). The present study did not address the possibility that APP gene expression may also be affected by neuronal depolarization.

The release of soluble amino-terminal APP derivatives induced by neuronal depolarization increased with increasing frequencies in the range of 0 to 30 Hz. The half-maximal response was reached at 17 Hz, and the maximum was attained at 30 Hz (Fig. 3). Electrophysiological evidence from intracellular recording experiments indicates that large hippocampal pyramidal cells fire in response to activation with frequencies ranging from 8 to 18 Hz, and can burst up to 150 Hz (20). Thus, the frequency-response curve for the release of soluble amino-terminal APP derivatives is consistent with the known electro-physiological properties of hippocampal neurons.

The cellular source of APP derivatives released from hippocampal slices is unclear and could include neurons, glia and cells from the cerebral vasculature. Unlikely sources are meninges, meningeal blood vessels and circulating blood because the meninges were carefully removed, slices were washed three times, and superfused for 50 minutes prior to the collection and stimulation period.

This study demonstrates for the first time that hippocampal slices *in vitro* release soluble amino-terminal APP derivatives similar to those found in human cerebrospinal

fluid, and indicates that neuronal depolarization modulates this release. The functional significance of coupling APP processing to neurotransmission awaits discovery.

ACKNOWLEDGMENTS

We thank Cindy Kim, Alice Wei and Rajivi Pothiraj for excellent assistance as well as Dennis J. Selkoe and Kurt Naujocks (Boehringer Mannheim) for providing antibodies. Supported by National Institute of Mental Health, National Institute of Aging, and the Center for Brain Science and Metabolism Charitable Trust. R.M.N. is the Hoffman Fellow in Alzheimer's disease at Massachusetts General Hospital.

REFERENCES

1. Kosik, K.S. (1992) Alzheimer's disease: A cell biological perspective. *Science* **256**, 780-783

2. Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A. & Price, D.L. (1990) Evidence that β-amyloid protein is not derived by normal processing. *Science* **248**, 492-495.

3. Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D. & Ward, P. (1990) Cleavage of amyloid β peptide during constitutive processing of its precursor. *Science* **248**, 1122-1124.

4. Golde, T.E., Estus, S., Younkin, L.H., Selkoe, D.J. & Younkin, S.G. (1992) Processing of amyloid protein precursor to potentially amyloidogenic derivitives. *Science* **255**, 728-730.

5. Estus, S., Golde, T.E., Kunishita, T., Blades, D., Lowery, D., Eisen, M., Usiak, M., Qu, X., Tabira, T., Greenberg, B.D. & Yonkin, S.G. (1992) Potentially amyloidogenic, carboxy-terminal derivitives of the amyloid protein precursor. *Science* **255**, 726-728.

6. Haass, C., Koo, E.H., Mellon, A., Hung, A.Y. & Selkoe, D.J. (1992) Targeting of cell-surface β-amyloid precursor to lysosomes: Alternative processing into amyloid-bearing fragments. *Nature* **357**, 500-503.

7. Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B. & Selkoe, D.J. (1992) Amyloid β-peptide is produced by cultured cells during normal processing. *Nature* **359**, 322-325.

8. Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M.G., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D.J., Lieberburg, I. & Schenk, D. (1992) Isolation and quantitation of soluable Alzheimer's β-peptide from biological fluids. *Nature* **359**, 325-327.

9. Shoji, M., Golde, T.E., Ghiso, J., Cheung, T.T., Estus, S., Shaffer, L.M., Cai, X.-D., McKay, D.M., Tintner, R., Frangione, B. & Younkin, S.G. (1992)Production of Alzheimer amyloid β-protein by normal proteolytic processing. *Science* **258**, 126-129.

10. Nitsch, R.M., Slack, B.E., Wurtman, R.J. & Growdon, J.H. (1992) Release of Alzheimer amyloid precursor derivitives stimulated by activation of muscarinic acetylcholine receptors. *Science* **258** 304-307.

11. Buxbaum, J.D., Oishi, M., Chen, H.I., Pinkas-Kramarski, R., Jaffe, E.A., Gandy, S.E. & Greengard, P. (1992) Cholinergic agonists and interleukin-1 regulate processing and secretion of the Alzheimer β /A4 amyloid protein precursor. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10075-10078.

12. Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 597-603.

13. Maire, J.C.E. & Wurtman, R.J. (1985) Effects of electrical stimulation and choline availability on the release and contents of acetylcholine and choline in superfused slices from rat striatum. *J. Physiol. (Paris)* **80**, 189-195.

14. Weidemann, A., König, G., Bunke, D., Fischer, P., Salbaum, J.M., Masters, C. L. & Beyreuther, K. (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57**, 115-126.

15. Palmert, M.R., Podlisny, M.B., Witker, D.S., Oltersdorf, T., Younkin, L.H., Selkoe, D.J. & Younkin, S.G. (1989) The β-amyloid protein precursor of Alzheimer disease has soluable derivitives found in human brain and cerebrospinal fluid. The *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6338-6342.

16. Selkoe, D.J., Podlisny, M.B., Joachim, C.L., Vickers, E.A., Lee, G., Fritz, L.C. & Oltersdorf, T. (1988) β-Amyloid precursor protein of Alzheimer disease occurs as 110 - 135 kilodalton membrane-associated proteins in neural and nonneural tissues. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7341-7345.

17. Jan, L. Y. & Jan, Y. N. (1989) Voltage-sensitive ion channels. *Cell* **56**, 13-25.

18. Koh, J.Y. & Choi, D.W. (1987) Quantitative determination of gluatamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase assay. *J. Neurosci. Meth.* **20**, 83-90.

19. Lonart, G. & Zigmond, M.J. (1991) Incuabation of tissue slices in the absence of Ca^{2+} and Mg^{2+} can cause nonspecific damage. *J. Neurochem.* **56**, 1445-1448.

20. Schwartzkroin, P.A. (1975) Characteristics of CA1 neurons recorded intracellularly in the hippocampal *in vitro* slice preparation. *Brain Res.* **85**, 423-426.

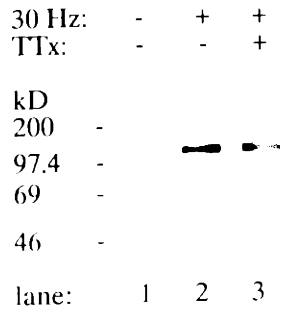
FIGURE LEGENDS

FIG. 1. Western blots of soluble amino-terminal APP derivatives released from hippocampal slices *in vitro* (*A,B*). The release of hippocampal APP derivatives detected with the monoclonal antibody 22C11 (*A*) is increased by electrical depolarization (*A*, lane 2), and is blocked by tetrodotoxin (*A*, lane 3). *30Hz*, electrical stimulation with 30Hz for 50 min; *TTx*, tetrodotoxin (1μM). APP derivatives released from superfused hippocampal slices *in vitro* lack the carboxyl terminus (*B*). Western blotting with the polyclonal antiserum anti-C_8 against the last 20 carboxyl-terminal amino acids of APP (*B*) did not react with the proteins detected by antibody 22C11, which was raised against a full-length APP fusion protein (*A*). Lane 4: protein extract from hippocampal slices used in the same experiment indicates the presence of full-length APP within the tissue. Coomassie blue staining of total proteins released into the superfusate (*C*).

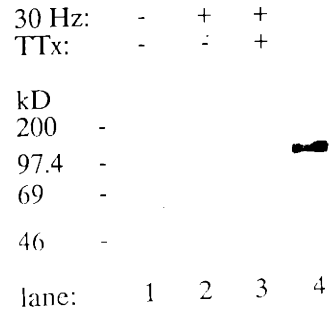
FIG. 2. Densitometric quantitation of released soluble amino-terminal APP derivatives from hippocampal slices (*A*). Bars indicate means ± SEM, n=4 animals per group, $^*P<0.01$ vs. unstimulated controls and $P<0.05$ vs. tetrodotoxin-treated group (ANOVA and *post-hoc* Neuman-Keuls test). LDH released from hippocampal slices in response to electrical stimulation (*B*). Data are expressed in μU/mg slice protein/ml superfusate. Bars indicate means ± SEM, n=4 animals. No statistical significance between the groups.

FIG 3. Electrically stimulated release of soluble amino-terminal APP derivatives from hippocampal slices is frequency-dependent. Values are normalized to basal, unstimulated release using the monoclonal antibody 22C11. Symbols represent means ± SEM; n = 3-5 animals per group. $^*P<0.01$ vs. 0 Hz (ANOVA and *post-hoc* Neuman-Keuls test).

A.


30 Hz: - + +
TTx: - - +
kD
200 -
97.4 -
69 -
46 -
lane: 1 2 3

B.


30 Hz: - + +
TTx: - - +
kD
200 -
97.4 -
69 -
46 -
lane: 1 2 3 4

C.

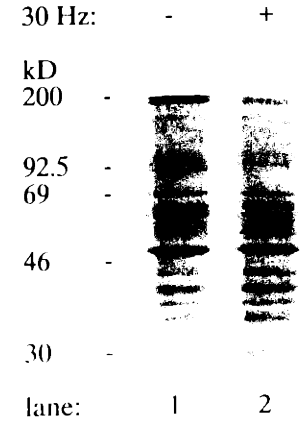

30 Hz: - +
kD
200 -
92.5 -
69 -
46 -
30 -
lane: 1 2

Figure 1

Figure 2

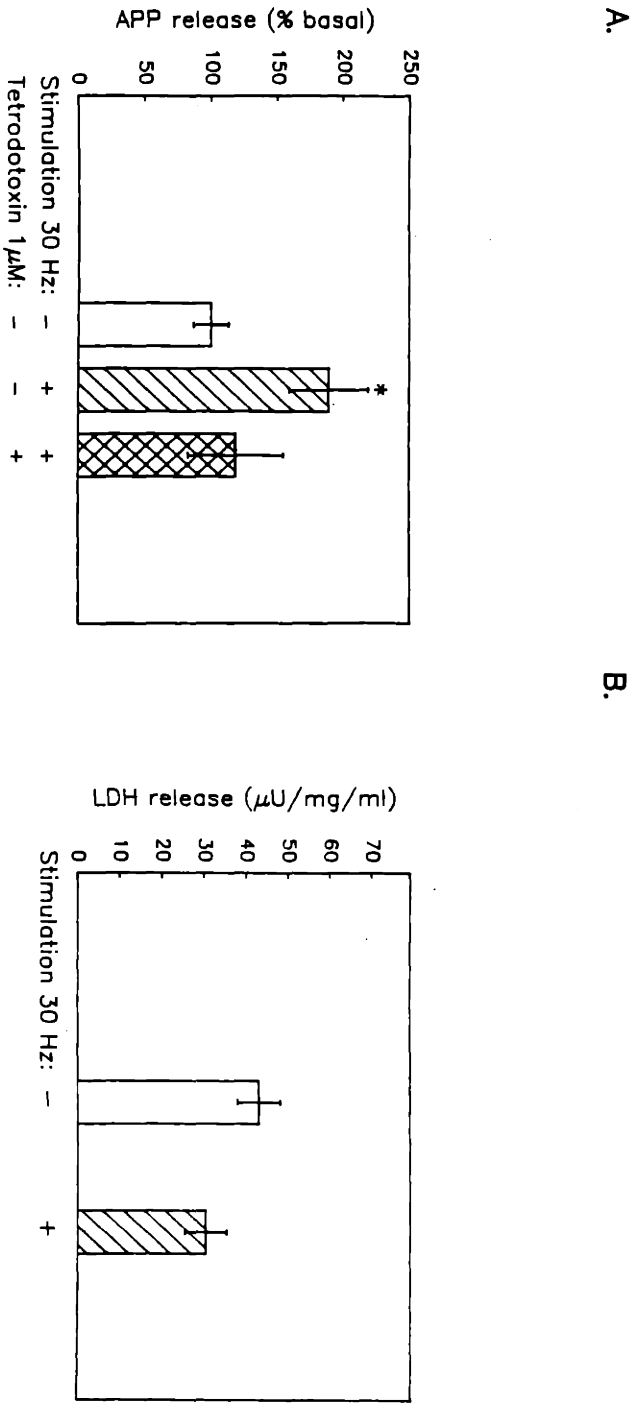
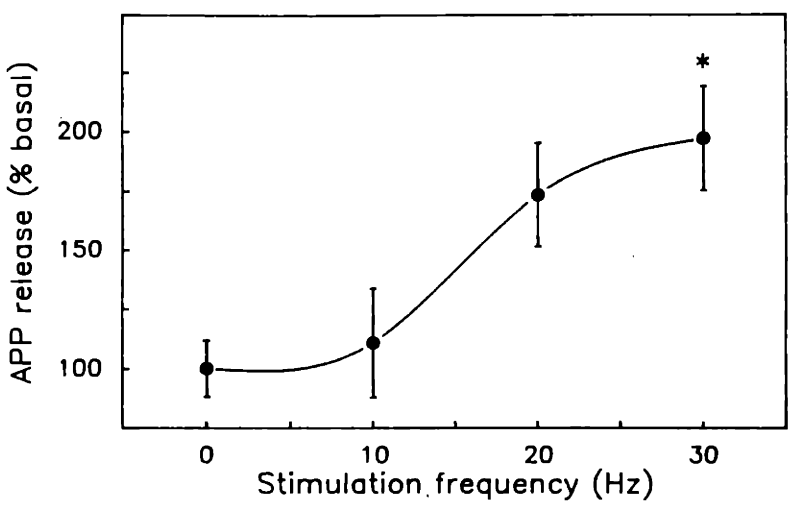

A.
APP release (% basal)
0
50
100
150
200
250
Stimulation 30 Hz: – + –
Tetrodotoxin 1μM: – – +
*
B.
LDH release (μU/mg/ml)
0
10
20
30
40
50
60
70
Stimulation 30 Hz: – +

Figure 3


APP release (% basal)
200
150
100
*
0
10
20
30
Stimulation frequency (Hz)