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Regulation Of Choline Acetyltransferase In Rat Hippocampal Synaptosomes

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REGULATION OF CHOLINE ACETYLTRANSFERASE IN RAT HIPPOCAMPAL SYNAPTOSOMES

by

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ABSTRACT

Choline acetyltransferase (ChAT; EC 2.3.1.6) catalyses the biosynthesis of acetylcholine (ACh) from choline and acetylCoenzyme A in cholinergic neurons. Within nerve terminals, this enzyme exists in both cytosolic and membrane-bound forms, although the role of the latter fraction is not clear. ChAT is clearly subject to long-term regulation (over periods of hours to days), likely through alterations in protein synthesis, however very little is known about short-term regulation (seconds to minutes). Therefore, the goals of this study were to determine the mechanisms of short-term regulation of ChAT activity and to elucidate the physiological role of membrane-bound ChAT in ACh biosynthesis.

Using synaptosomes prepared from rat brain hippocampus as a model of intact nerve terminals, approximately 80% of total ChAT activity was found in the cytoplasm and could be further divided into a water-soluble fraction (20%) and sodium phosphate-soluble fraction (60%), while the remaining ChAT (15-20%) appeared to be membrane-bound. Interestingly, only the specific activity of membrane-bound ChAT was significantly increased, in a calcium-dependent manner, following depolarization of intact synaptosomes by both 40 mM KCl and 50 μM veratridine, agents which induce ACh release and subsequent choline uptake. Since W-7, an inhibitor of calcium-calmodulin kinase II, attenuated the depolarization-induced activation of membrane-bound ChAT, protein phosphorylation was examined as a mechanism of short-term regulation. Under

resting conditions, only cytosolic ChAT appeared to exist as a phosphoprotein, the abundance of which was calcium-dependent. Furthermore, the pattern of phosphorylation neither correlated with enzyme activity, nor was altered by nerve terminal depolarization. Since the specific activity of cytosolic ChAT was not altered by any experimental manipulation, it was subdivided into water-soluble and sodium phosphate-soluble enzyme fractions. Subsequently, it was found that reduction of intracellular calcium concentration, by lowering the extracellular calcium concentration, reduced membrane-bound ChAT and water-soluble ChAT activities and concomitantly increased sodium phosphate-soluble ChAT activity. These alterations in specific activity were accompanied by parallel changes in both the V_{max} and the amount of ChAT-immunoreactive protein in each fraction. suggesting translocation of the enzyme between cytosolic and membrane-bound pools and within cytosolic pools. Finally, to assess the physiological role of membrane-bound ChAT, an experimental strategy was developed based upon the observation that enzyme activity was sensitive to alterations in chloride ion concentration. Under these conditions membrane-bound ChAT did not appear to regulate basal ACh biosynthesis.

In summary, translocation of enzyme protein may be involved in the short-term regulation of ChAT activity within cholinergic nerve terminals. Furthermore, cytosolic ChAT appeared to play the predominant role in the regulation of basal ACh synthesis.

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ACh	Acetylcholine
BSA	Bovine Serum Albumin
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
DBH	Dopamine Beta-Hydroxylase
DIDS	4,4'-Diisothiocyanatostilbene-2,2'-disulfonic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol-bis(β-aminoethyl Ether)
GAD	Glutamic Acid Decarboxylase
HEPES	N-2-Hydroxyethyl Piperazine-N'-2-Ethanesulfonic Acid
HRP	Horseradish Peroxidase
KR	Krebs-Ringer Buffer
LDH	Lactate Dehydrogenase
PMSF	Phenylmethylsulfonyl Fluoride
SDS-PAGE	Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis
SITS	4-Acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic Acid
TBS-T	Tris-Buffered Saline with 0.2% Tween-20
TCA	Trichloroacetic Acid
TH	Tyrosine Hydroxylase

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Acetylcholine (ACh) is a key neurotransmitter in mammalian peripheral and central nervous systems (CNS) responsible for communicating a variety of physiological messages in most body tissues. A number of experimental methods, including immunohistochemistry, electrophysiology, and bioassays, have been used to demonstrate the location and function of cholinergic neuronal pathways.

In the central nervous system, cholinergic neurons comprise about 10% of the total neuron population and play roles in learning and memory, sleep and wakefulness, and motor behaviour. Dysfunction of specific cholinergic pathways have been implicated in disorders such as Parkinson's disease, Huntington's chorea, schizophrenia, mania, Alzheimer's disease, and myasthenia gravis (Nordberg, 1992; Karson *et al.*, 1993). CNS cholinergic neurons appear to be organized into two patterns: those neurons which project from the site of origin to innervate other brain areas and those which are intrinsically organized, local circuit interneurons. At present, six major cholinergic cell groups providing cholinergic innervation in the rat brain have been identified. These include projections to the cerebral cortex and hippocampus from the medial septal nucleus and the nucleus of the diagonal band of Broca, some of which appear to play important roles in learning and memory function. ACh is also the neurotransmitter of a large population of intrinsic neurons in the caudate-putamen (striatum), involved in control of motor function (Butcher and Woolf, 1986; McGeer *et al.*, 1986).

In the periphery, motor neurons of the ventral horn of the spinal cord drive the voluntary musculature of the vertebrate body via the nicotinic cholinergic neuromuscular junction. In addition, in the autonomic nervous system preganglionic parasympathetic and sympathetic neurons and postganglionic parasympathetic fibers innervate a wide range of organ systems and glands using ACh as their neurotransmitter (Dodd and Role, 1991).

1.2 Regulation of Acetylcholine Synthesis

Although ACh was the first chemical neurotransmitter described (Loewi, 1921), surprisingly little is known about the physiological mechanisms regulating its biosynthesis. ACh is formed in cholinergic neurons from the co-substrates choline and acetylCoenzyme A through a reaction catalyzed by the enzyme choline acetyltransferase (ChAT; EC 2.3.1.6). The enzymatic reaction is reversible, but the equilibrium is shifted to the right thus favouring ACh formation with the equilibrium constant, K_{eq}, being close to 13 (Tucek, 1985). Histochemical and immunocytochemical evidence indicates that ChAT is distributed throughout all parts of the cholinergic neuron including perikarya, neurites, and dendrites, but it is generally agreed that the majority of synthesis of ACh occurs in presynaptic nerve terminals where the sodium-coupled high-affinity choline transporter appears to be selectively localized (Ross *et al.*, 1983). Although the precise mechanisms are unclear, the rate of ACh synthesis is regulated in such a manner that stores of transmitter in the nerve terminals are maintained at a near constant level under

a variety of physiological conditions, with synthesis of transmitter keeping pace with its release (Birks and MacIntosh, 1961; Browning and Schulman, 1968; Collier and MacIntosh, 1969).

It appears that within nerve terminals, newly synthesized ACh exists primarily in the cytoplasm and initially adds to the cytoplasmic pool. From this, a proportion of the transmitter is transported into synaptic vesicles and is subsequently available for depolarization-evoked quantal release. This movement of transmitter into synaptic vesicles is against a concentration gradient and utilizes energy arising from accumulation of protons within the storage vesicles (Toll and Howard, 1979; Anderson *et al.*, 1982).

The acetyl moiety used in ACh synthesis originates from glucose and pyruvate (Tucek and Cheng, 1974). AcetylCoenyzme A is formed from pyruvate by the intramitochondrial pyruvate dehydrogenase complex, but it is not clearly understood how this substrate then translocates to the cytoplasm. Three possible mechanisms have been proposed. First, and most likely, it is commonly believed that incorporation of the acetyl group into citrate facilitates movement across the mitochondrial membrane. Second, a more hypothetical proposal is the direct passage of acetylCoenzyme A through this barrier, although the inner mitochondrial membrane is generally impermeable to bulky charged molecules similar to acetylCoenzyme A (Ricny and Tucek, 1983). Lastly, it has been postulated that extramitochondrial pyruvate dehydrogenase may exist either in membranes or in the cytoplasm, thus allowing the direct utilization of pyruvate-

generated acetylCoenzyme A without permeability problems (Lefresne *et al.*, 1978; reviewed by Tucek, 1985).

A large body of evidence indicates that, under experimental conditions, most of the choline that is used for the synthesis of ACh in cholinergic neurons is derived from the extracellular fluid surrounding them. Choline originates from free choline in blood plasma, from phospholipase-mediated hydrolysis of choline containing membrane phospholipids and from extracellular acetylcholinesterase-mediated hydrolysis of ACh released into cholinergic synapses (for review, see Ansell and Spanner, 1982). Choline is taken up into nerve cells by two functionally separate transport systems located in the presynaptic plasma membrane. A low-affinity facilitated diffusion process appears to supply choline which is directed toward synthesis of membrane phospholipids. Choline taken in by sodium-coupled high-affinity carriers is destined, perhaps by cytosolic compartmentation, for ACh synthesis and appears to be the rate-limiting step in this process. This latter transport process is dependent upon the presence of extracellular sodium and chloride ions, is highly sensitive to inhibition by low concentrations of hemicholinium-3 ($IC_{50}=10^{-7}M$) (O'Regan, 1988), and is localized exclusively to cholinergic presynaptic nerve terminals (Suszkiw and Pilar, 1976). The main driving forces for the transport of choline by the high-affinity carrier appears to be the electrical potential difference across the neuronal plasma membrane and the sodium electrochemical gradient. Although physiological regulation of activity of the high-affinity choline carrier is poorly understood at

present, its capacity appears to be coupled to neuronal activity (Jope, 1979; Ducis and Whittaker, 1985). Although ACh is not transported by this carrier, the transmitter appears to be a potent inhibitor of synaptosomal choline uptake, presumably by binding to carriers and immobilizing them (Marchbanks *et al.*, 1981). It has been proposed that availability and activity of choline transporters may be regulated, at least in part, by the cytoplasmic ACh level in the nerve terminal (Jenden *et al.*, 1976; Weiler *et al.*, 1978; Marchbanks *et al.*, 1981).

1.3 General Characteristics of ChAT

ChAT, first characterized in 1943 by Nachmansohn and Machado, is viewed primarily as a neuron-specific gene product whose function is the biosynthesis of ACh (reviewed by Salvaterra and Vaughn, 1989). Although catalytic activity ascribed to this enzyme has been observed in a number of nonneuronal tissues including placenta (Hersh *et al.*, 1978), spermatozoa (Bishop *et al.*, 1976), plants (Barlow and Dixon, 1973) and bacteria (White and Cavallito, 1970), its functional significance in these tissues remains unknown. The precise mechanisms involved in control of expression of ChAT are not yet known, but Ibanez and Persson (1991) determined that the region distal to the ChAT promoter contains a cell-specific silencer-element to restrict expression of the ChAT gene to cholinergic cells. In addition, they found that the gene contains several AP1 sites allowing the possibility that transcription factors of the AP1 complex may be involved in control of expression by hormones or growth factors, in particular nerve growth factor.

Following synthesis in the cholinergic neuronal perikaryon, ChAT is transported, unassociated with intra-axonal organelles, by slow axonal transport (4 mm/day) to nerve terminals (for review see Dahlstrom, 1983). ChAT appears to be a relatively stable protein as Wenthold and Mahler (1975) estimated its half-life to be 5.2 days, and more recently, Hersh (1992) estimated it to be greater than 24 hours in the cell line NG108-15. Purification of ChAT has proven difficult because of its low content in tissues and the presence of other proteins of similar size and shape. The enzyme, however, does appear to be a simple globular protein with a comparatively high positive surface charge (Fonnum, 1970) and apparent molecular mass (M_r) close to 68,000 daltons (Rossier, 1976; Crawford *et al.*, 1982; Eckenstein and Thoenen, 1982; Bruce *et al.*, 1985). Confirmation of this molecular size has come from the cloning of the cDNA for rat brain ChAT (Berrard *et al.*, 1986). It is generally considered that this enzyme is largely cytosolic at physiologic pH and ionic strength, although a small percentage of the total enzyme appears to be associated with the plasma membrane under these conditions (Fonnum, 1968; Benishin and Carroll, 1983; Badamchian and Carroll, 1985; Eder-Colli *et al.*, 1986), as discussed in detail in Chapter 1, Section 1.4.

Although short-term regulation of ChAT has not been studied extensively, experimental conditions which affect the activity of the enzyme have been examined since levels of acetylcholine in neural tissue remain relatively constant under a range of physiological conditions (for review, see Mautner, 1977). Based upon the work of Hersh and Peet (1978), it is believed that the reaction catalyzed

by ChAT follows a random Theorell-Chance mechanism. Although the binding of substrates and the subsequent release of products are random, the predominant pathway is that in which acetylCoenzyme A binds to the enzyme before choline and ACh dissociates from the enzyme before Coenzyme A.

It appears that direct inhibition of ChAT by ACh is involved in the regulation of biosynthesis of the transmitter. Following nerve stimulation, the ACh concentration in the nerve terminal falls due to release of the transmitter, thus decreasing product inhibition of the enzyme (for review, see Tucek, 1985). ChAT activity, at least for the solubilized enzyme, is also increased by sodium ions, the intracellular concentration of which is increased by nerve depolarization (Birks, 1963). Birks suggested that this salt effect may serve "as a means of coupling cellular activity to metabolism". Schuberth (1966) first suggested that chloride ions may also specifically activate the enzyme isolated from human placenta, and showed that the initial velocity of the ChAT-catalysed reaction increased in the presence of this anion. Prince and Hide (1971) noted, however, that binding of both choline and acetylCoenzyme A to the enzyme was looser as the ionic strength of the medium increased. Rossier and collegues (1977) confirmed the activating effects of chloride on the acetylation reaction in their study on crude and purified forms of rat brain ChAT. They proposed that ChAT may be "part of a presynaptic membrane-bound multi-enzyme complex under direct control of ion fluxes promoted by nerve impulses". Although salt appears to increase ChAT activity, Hersh (1978) suggested that these effects may be related more to ionic

strength, rather than being ion specific. It should also be noted that salt effects differ greatly depending upon the purity of the ChAT preparation studied.

Investigators who have examined regulation of this enzyme generally have reported only the specific activity of a "total" pool of partially purified ChAT. This is now considered to represent only the cytosolic pool of ChAT since it is now generally agreed that a small fraction of the enzyme is also associated non-ionically with neuronal membranes (see Section 1.4). The biochemical properties of membrane-bound ChAT appear to differ from those of its cytosolic counterpart in several respects (Smith and Carroll, 1980; Benishin and Carroll, 1981, 1983; Badamchian *et al.*, 1986), and it is presently unknown whether the above observations regarding regulation of enzyme activity also apply to membrane-bound ChAT.

1.4 Characteristics of Membrane-Bound ChAT

A membrane-bound pool of ChAT (membrane-bound ChAT, 5-20% of total ChAT) has been described in nerve terminals from rodent (Malthe-Sorenssen, 1976; Atterwill and Prince, 1978; Smith and Carroll, 1980; Massarelli *et al.*, 1988; Rylett, 1989), bovine (Ryan and McClure, 1979), and human brain (Peng *et al.*, 1986), and *Torpedo* electric organ (Eder-Colli and Amato, 1985; Eder-Colli *et al.*, 1986), as well as the neuronal cell line NS-20Y (Barochovsky *et al.*, 1988). The detergent-soluble pool of the enzyme differs from its cytosolic counterpart in several respects, including pH profile, sensitivity to the ChAT inhibitor

naphthylvinylpyridine, heat inactivation and the ability to acetylate the choline analogue homocholine (Benishin and Carroll, 1983). It has also been suggested that the nitrogen mustard analogue of choline, choline mustard, transported on the high-affinity choline carrier may be directed to a membrane-associated form of the enzyme (Rylett, 1989). Dietz and Salvaterra (1980) have shown 3 closely spaced but distinct protein bands with apparent molecular weight of approximately 67 kDa after SDS-PAGE of "total" ChAT purified from rat brain. Since the tryptic peptide maps for each of these proteins were nearly identical with only minor differences, they were interpreted to represent different molecular forms of the enzyme.

It has been postulated that the membrane-bound pool of enzyme may play a critical role in the synthesis of ACh required for calcium-dependent quantal release of transmitter following nerve activity (Benishin and Carroll, 1983; Mykita and Collier, 1989). Evidence is accumulating in other biological systems to indicate that membrane-associated forms of amphotropic proteins play important roles in several cellular pathways (Jamil *et al.*, 1990). Some investigators have suggested that membrane-bound ChAT may be linked functionally, or even structurally, to the high-affinity choline carrier to create a multienzyme complex responsible for ACh synthesis during repetitive neuronal activity (Atterwill and Prince, 1978; Burgess *et al.*, 1978; Jope, 1979), but direct experimental evidence to support this model has not been obtained. Indirect support for this theory has been given by this laboratory using choline mustard aziridinium ion which

irreversibly inhibits ChAT and high-affinity choline uptake by rat forebrain synaptosomes (Rylett and Colhoun, 1980; Rylett, 1989).

It is likely that membrane-bound ChAT arises either through non-covalent attachment of this enzyme to an integral membrane protein which serves as an anchor, or through covalent attachment to a membrane lipid. Bradford and coworkers (Docherty *et al.*, 1982; Barochovsky *et al.*, 1988) have suggested, however, that 1% of total ChAT may be a transmembrane protein based upon the observed lysis of cholinergic synaptosomes following exposure of the intact nerve terminals to anti-ChAT antibodies and complement. The physiological significance of this finding is unclear at present and may simply be the result of antibody binding to membrane-bound ChAT exposed on synaptosomes which resealed "inside out". Those authors, however, proposed that an antigenic determinant may be present on the plasma membrane which may be a portion of the membrane-bound form of the enzyme accessible at the extracellular surface of the synaptosome. To date, there have been no experimental studies which have examined directly the physiological role of membrane-bound ChAT and the relative contribution of each enzyme pool to ACh biosynthesis. In terms of possible differential regulation of the two pools of ChAT, several studies have shown that binding of ChAT to membranes is reversible and dependent upon the pH and ionic strength of the medium (Fonnum, 1968; Docherty and Bradford, 1988), both of which may change during propagation of a nerve impulse. Thus, binding and

release of the enzyme from the plasma membrane may be an important regulatory process in ACh synthesis by controlling ChAT activity or subcellular distribution.

1.5 Rationale

The co-substrates for ACh synthesis, acetylCoenzyme A and choline, are also used in many other cellular processes such as carbohydrate metabolism, lipid biosynthesis and the formation of membrane components. However, if converted by active ChAT to ACh, these metabolic intermediates are no longer free to be used in other metabolic pathways. Therefore, biosynthesis of ACh by ChAT is a committed step in a cellular pathway and is likely regulated, at least in part, at the level of the enzyme to ensure adequate neurotransmitter production. During the past decade, research efforts have focussed on both the short- and long-term regulation of this enzyme.

With regard to long-term regulation occurring over a time span of hours to days, it appears that enzyme levels within CNS neurons can be modulated by a number of hormones and growth factors including thyroid hormone (Hefti *et al.*, 1986; Giordano *et al.*, 1992), estrogen (Luine, 1985), 1,25-dihydroxyvitamin D_3 (Sonnenberg *et al.*, 1986), basic fibroblast growth factor (Knusel *et al.*, 1990), brain-derived neurotrophic factor (Knusel *et al.*, 1991), and nerve growth factor (Honegger and Lenoir, 1982; Gnahn *et al.*, 1983), and in sympathetic neurons and pheochromocytoma cells by retinoic acid (Casper and Davies, 1989; Matsuoka *et al.*, 1989) and ciliary neurotrophic factor (Saadat *et al.*, 1989; Kalberg *et al.*, 1993).

Results of these studies generally suggest that long-term regulation of ChAT activity is most likely achieved by changes in gene transcription and/or alterations in synthesis of the protein.

While there is considerable information on the long-term regulation of ChAT, there is only limited evidence that this enzyme is subject to short-term regulation occurring within seconds or minutes, similar to that observed for neurotransmitter-synthesizing enzymes in monoaminergic neurons. This form of regulation of enzyme catalytic activity would seem likely to occur since during synaptic transmission the ionic composition of the nerve terminal alters transiently as a result of the opening and closing of ion channels, and it is known that ChAT activity is affected by several physiologically important ions including Na^+ and Cl^- (Rossier *et al.*, 1977; Hersh and Peet, 1978). Therefore, catalytic activity of the enzyme or its subcellular distribution may be controlled, at least in part, by ionic fluxes following nerve depolarization and this may result in alterations in transmitter synthesis. Possible mechanisms of short-term enzyme regulation could include: (1) post-translational protein modification such as kinase-mediated phosphorylation, (2) changes in subcellular distribution, and (3) alterations in protein turnover rate.

ChAT operates at only a fraction of its maximal catalytic capacity within cholinergic nerve terminals as choline availabili' · appears to be rate-limiting in ACh synthesis. Therefore, it has been widely held that neuronal activity had no effect upon enzyme activity. However, this dogma was challenged by Carroll and

coworkers when they characterized the various subcellular pools of ChAT biochemically and demonstrated that neuronal depolarizing agents veratridine and elevated extracellular potassium, which augment ACh release, also selectively stimulate certain subcellular fractions of ChAT via a calcium-dependent mechanism in rat brain cholinergic neurons (Benishin and Carroll, 1983; Carroll and Benishin, 1984; Carroll, 1987). The mechanism of this selective activation was not determined.

Indeed, there is now widespread speculation that membrane-bound ChAT may be subject to different regulatory control than its cytosolic counterpart. However, early studies examined "total" nerve terminal ChAT activity, thereby masking possible changes in the relatively small membrane-associated pool. In practice, it has proven technically difficult to examine the regulation and physiological role of each subcellular ChAT fraction because no specific blockers of membrane-bound ChAT versus cytosolic ChAT have been identified to allow selective inhibition of one enzyme form. In view of this, it has become necessary to develop alternative experimental strategies to modulate selectively the activity of one ChAT pool, without changing activity of another pool of enzyme or provision of the neurotransmitter precursors, and evaluate the effects on ACh synthesis.

Using synaptosomes prepared from rat brain hippocampus as a model of intact nerve terminals, the overall goals of this study were to determine the mechanisms of short-term regulation of ChAT activity and the role of membrane-

bound ChAT in ACh biosynthesis. Specific objectives were:

1. to confirm the existence of membrane-bound ChAT in rat hippocampal synaptosomes, and to find experimental conditions in which the activities of cytosolic ChAT or membrane-bound ChAT could be selectively and acutely altered,

2. to determine whether the subcellular pools of ChAT may be substrates for phosphorylation by protein kinases *in situ*, and to determine whether this post-translational protein modification is associated with changes in enzyme catalytic activity,

3. to determine whether translocation of ChAT protein within cholinergic nerve terminals represents a mechanism of short-term enzyme regulation, and

4. to design an experimental paradigm in which the physiological role of membrane-bound ChAT in basal (resting) ACh biosynthesis may be elucidated.

1.6 Experimental Model

It is generally believed that the hippocampus plays a fundamental role in the process of learning and memory, and that cholinergic neurons are critically involved in this process. The observation that hippocampal ChAT activity is diminished in the normal process of aging and in Alzheimer's disease (Norberg, 1992) has renewed interest in the regulation of this enzyme, especially in this brain area. Most of the extrinsic cholinergic input to the hippocampus is derived from cells in the basal forebrain, and particularly from the medial septal nucleus and the nucleus of the diagonal band of Broca (Malthe-Sorenssen *et al.*, 1980). Recent data, using *in situ* hybridization to localize ChAT-producing neuronal cell bodies, indicate that cholinergic neuronal perikarya do not exist within the hippocampus (Butcher *et al.*, 1992). Because of this extrinsic innervation, isolated hippocampal

tissue contains cholinergic nerve terminals only, thus eliminating the confounding contribution of cholinergic cell bodies to ACh biosynthesis and serving as an excellent model system for studies on the regulation of cholinergic nerve terminal function.

During homogenization of brain tissue in media iso-osmotic to plasma, presynaptic nerve terminals are sheared from axons while resisting disruption, and reseal, retaining mitochondria, synaptic vesicles and enzymes (Whittaker *et al.*, 1964). These synaptosomes maintain a resting membrane potential like most intact resting neurons (approximately -55 mV) (Tamkun and Catterall, 1981). Mammalian brain synaptosomes have been characterized extensively and found to be a very good model of nerve endings *in situ* since they contain specific mechanisms for the transport of Ca^{2+}, Na^{+}, K^{+}, and Cl^{-} across the plasma membrane (Lust and Robinson, 1970; Blaustein 1975; Blaustein and Oborn, 1975; Marchbanks and Campbell, 1976; Nachshen *et al.*, 1986; Stuenkel, 1990; Xiang *et al.*, 1990; Duarte *et al.*, 1991). Additionally, they exhibit oxidative phosphorylation (Abdel-Latif *et al.*, 1968), metabolism of phospholipids (Abdel-Latif *et al.*, 1968; Schacht and Agranoff, 1972) and uptake, synthesis and release of neurotransmitters (Marchbanks, 1969; de Belleroche and Bradford, 1972).

CHAPTER 2

SELECTIVE ACTIVATION OF MEMBRANE-BOUND ChAT FOLLOWING DEPOLARIZATION OF HIPPOCAMPAL SYNAPTOSOMES.

1. INTRODUCTION

In addition to membrane-bound ChAT (Benishin and Carroll, 1983), the existence of membrane-bound forms of other neurotransmitter-related enzymes including tyrosine hydroxylase (Kuczenski and Mandell, 1972; Kuhn *et al.*, 1990), dopamine beta-hydroxylase (Lamouroux *et al.*, 1987; Oyarce and Fleming, 1991), histidine decarboxylase (Toledo *et al.*, 1991), and glutamate decarboxylase (Erlander *et al.*, 1991), has also been clearly demonstrated. The physiological roles which these forms of the enzymes serve are presently unknown. In cholinergic neurons it has been postulated that the membrane-bound pool of enzyme may be responsible for the formation of ACh required for calcium-dependent quantal release following nerve activity (Benishin and Carroll, 1983). In support of this theory, Mykita and Collier (1989) suggested that membrane-bound ChAT may play a critical role in the activation of ACh synthesis during stimulation of cat sympathetic ganglia.

Indeed, neuronal activity in the presence of extracellular calcium ions appears to be an important component of short-term regulation of other neurotransmitter synthesizing enzymes and may exert its actions in a variety of ways. For example, potassium-depolarization of synaptosomes results in an influx of extrasynaptosomal calcium ions (Adam-Vizi and Ligeti, 1986) and induces phosphorylation of tyrosine hydroxylase, decreasing its K_m for the pterin cofactor in catecholaminergic neurons (Haycock, 1987). Furthermore, Yanigihara *et al.*

(1984) reported that although basal or resting tyrosine hydroxylase activity was not dramatically changed in pheochromocytoma (PC12) cells treated with different calcium concentrations, omission of Ca^{2+} from the incubation medium abolished K^+-induced activation and phosphorylation. Depolarization-induced calcium influx also appeared to result in significant solubilization of membrane-associated histidine decarboxylase activity in rat brain synaptosomes; reduced membrane-bound activity was accompanied by a concommitant increase in cytosolic enzyme activity (Toledo *et al.*, 1991).

In cholinergic systems, Carroll and collegues (Carroll *et al.*, 1986; Carroll, 1987) first examined the short-term modulation of ChAT activity by depolarization using minces prepared from mouse and rat brain. These studies demonstrated that elevated extracellular potassium and veratridine, both of which augment ACh release, selectively stimulated the activity of membrane-bound ChAT in the presence of extracellular calcium. Although these investigators suggested that stimulation of membrane-bound ChAT may be linked with neurotransmitter release, others have suggested that this enzyme pool may be associated, instead, with the sodium dependent high-affinity choline transporter (Atterwill and Prince, 1978).

In this Chapter, I present the results of experiments designed to assess the subcellular distribution of ChAT activity in rat hippocampal synaptosomes, and to determine the effect of depolarization upon the activity of each enzyme pool. The demonstration of subcellular location of cytosolic and membrane-bound enzyme

isoforms has been achieved by a variety of experimental methods. For example, parallel measurement of the activity of lactate dehydrogenase, a cytosolic enzyme, has often been used as an indication of whether the protein in question is soluble in nature. In contrast, nonionic detergents have been used widely for the solubilization and characterization of integral membrane proteins since water-soluble proteins show little or no hydrophobic interaction with these detergents (Helenius and Simons, 1975). Particularly useful has been the technique of Triton X-114 phase partitioning which exploits the low cloud point of this non-ionic detergent to permit physical separation of detergent-soluble and water-soluble proteins at 20°C (Bordier, 1981).

The pharmacological agents veratridine, a sodium channel agonist, and elevated KCl were used to induce depolarization of intact hippocampal synaptosomes. In my hands, membrane-bound ChAT activity was selectively increased in a calcium-dependent manner.

2. METHODS

2.1 Preparation and Incubation of Synaptosomes

Synaptosomes were prepared from hippocampi of brain of female Sprague-Dawley rats (150-200 g) by the procedure of Gray and Whittaker (1962) with some modifications. All manipulations were performed at 4°C. Tissue (about 300 mg) was homogenized in 0.32 M sucrose containing 5 mM Tris-HCl buffer, pH 7.4, using 12 up and down motions (900 rpm). Centrifugation of the homogenate at 1,000 g for 10 min yielded a pellet (P1) containing nuclei and unbroken cells and the supernatant (S1). The pellet P1 was washed once with buffered sucrose to give the supernatant S1'. The two supernatants, S1 and S1', were pooled, then centrifuged at 17,000 g for 20 min to obtain the crude synaptosomal pellet (P2). The supernatant was discarded and the P2 washed twice with cold buffered sucrose solution. The synaptosomal pellet (washed P2) was then resuspended in 2 ml of oxygenated Krebs-Ringer (KR) buffer, pH 7.4 (comprised in mM of: NaCl, 124; KCl, 5.0; $MgSO_4$, 1.3; $CaCl_2$, 1.5; glucose, 10; Hepes-NaOH, 20). Following equilibration to 37°C, experimental incubations were begun with the addition of specific treatments (for incubation times and treatments, see Table 1). Control samples received an equal volume of the appropriate vehicle. In those experiments examining the calcium dependence of ChAT activity, synaptosomes were resuspended in 2 ml of nominally calcium-free oxygenated KR, pH 7.4 (no added calcium plus 0.1 mM EGTA). Following incubation, samples were

centrifuged at 17,000 g for 20 min and the pellets washed twice with 0.32 M sucrose buffered with 5 mM Tris-HCl buffer, pH 7.4 to remove salts.

Table 1. Experimental treatments and additions made to synaptosomal incubation solutions. Crude synaptosomal pellets were resuspended in 2 ml of normal oxygenated Krebs-Ringer buffer (pH 7.4) ± calcium, and warmed to 37°C. Incubations were begun with the addition of the following treatments, shown as final concentration. Vehicle solutions are shown in parentheses.

Treatment	Incubation Time	Additions
40 mM KCl (water)	30 sec 10 min	± 1.5 mM calcium ± 100 μM W-7
50 μM Veratridine (DMSO)	10 min	± 1.5 mM calcium ± 1 μg/ml tetrodotoxin
0 Calcium KR plus 0.1 mM EGTA	10 min	± 40 mM KCl ± 50 μM Veratridine

2.2 Isolation of Subcellular ChAT Pools

Cytosolic and membrane-associated pools of ChAT were isolated by subcellular fractionation of synaptosomes following the method of Badamchian *et al.* (1986), with some modifications. Following experimental manipulations, synaptosomes were washed with buffered sucrose to remove salts, then synaptosomal pellets were resuspended in 1 ml cold deionized water. To ensure maximum lysis of the synaptosomes, samples were incubated on ice for at least 20 min prior to 30 sec homogenization by polytron. An aliquot of this homogenate (usually 200 μl) was retained for determination of "total" synaptosomal ChAT activity.

The remaining homogenate was centrifuged at 100,000 g for 60 min to yield the water-soluble pool of ChAT in the supernatant. Pellets were resuspended in 1 ml of 100 mM sodium phosphate buffer, pH 7.4, and homogenized again by polytron, then centrifuged at 100,000 g for 30 min. The resulting supernatant contained ChAT which associates ionically with membranes under experimental conditions of low ionic strength and pH, but is thought to be cytoplasmic under physiologic conditions. For this reason, the water-soluble and sodium phosphate-soluble fractions were pooled for further assay. The pellet was washed a second time with 1 ml of 100 mM sodium phosphate buffer, pH 7.4, but as this supernatant yielded negligible ChAT activity, it was discarded. The final washed membrane pellet, containing ChAT which associates non-ionically with membranes, was suspended in 1 ml of 100 mM sodium phosphate buffer, pH 7.4, containing 1.5% (final concentration, w/v; detergent:protein ratio of 10) of the non-ionic detergent Triton X-100; at this concentration the detergent does not appear to alter catalytic activity of the enzyme. This homogenate was placed on ice for about 30 min with intermittent mixing before determination of enzyme activity; catalytic activity of ChAT in this homogenate was equal to that determined in the supernatant following 30 min centrifugation of the homogenate at 100,000 g.

2.3 Determination of ChAT Activity

ChAT activity was measured in the total homogenate, the pooled cytosolic fraction and the detergent-soluble pool by the radiometric method of Fonnum

(1968), with some modifications. An aliquot of enzyme sample (20 μl) was incubated at 37°C with 80 μl reaction buffer (containing NaCl, 234 mM; bovine serum albumin, 0.375%; $MgCl_2$, 17 mM; sodium phosphate buffer, 84 mM (pH 7.4); eserine, 150 μM; choline, 5 mM). Blank values were obtained by incubating samples in the absence of the substrate choline. Reactions were begun with the addition of 20 μl ^{3}H-acetylCoenzyme A (200 μM; specific activity: 1.44 Ci/mmol). The incubation was stopped after times up to 60 min with the addition of 300 μl of 3-heptanone containing sodium tetraphenylboron (75 mg/ml) at 4°C to extract ^{3}H-ACh. After shaking for 1 min and centrifuging for 2 min at 8000 rpm, an aliquot (usually 200 μl) of the upper organic phase was added to 5 ml scintillation cocktail and radioactivity determined. Enzyme specific activity was normalized to sample protein content of each subfraction rather than to original tissue weight, and is expressed as nmol / mg protein / hr. Based upon original tissue weight, cytosolic ChAT and membrane-bound ChAT represent approximately 80% and 20% of total ChAT activity, respectively. ChAT assays were performed on the same day as the experimental procedure.

2.4 Determination of Choline Uptake

To measure high-affinity choline uptake by synaptosomes, the crude synaptosomal fraction (P2) was prepared as described in Chapter 2, Section 2.1. Following a 10 minute exposure to either 40 mM KCl or 50 μM veratridine, an aliquot of synaptosomal suspension was removed and centrifuged at 8000 rpm for

5 min. The supernatant was discarded, then the pellet was resuspended in normal oxygenated KR buffer and warmed to 37°C. Since Yamamura and Snyder (1973) provided evidence that activity of the low-affinity choline transporter was not dependent upon temperature, whereas the sodium-coupled high-affinity choline transport was temperature-sensitive, parallel samples were incubated at 4°C to allow correction for accumulation of choline by diffusional processes. ^{3}H-choline (1 μM; specific activity: 1.87 Ci/mmol) was added to synaptosomes and incubated for 4 min. The incubation was stopped by placing warmed samples on ice and centrifuging in a Savant microcentrifuge at 8,000 rpm for 5 min. Pellets were washed twice with normal KR buffer and then resuspended in 1 ml of 0.1 N NaOH for several hours to allow protein digestion. An aliquot (500 μl) was removed and mixed with 5 ml of scintillation cocktail for the determination of total ^{3}H-choline accumulation. The remainder of sample digests was used for measurement of protein content. Choline uptake activity was expressed as pmol choline / mg protein / 4 min.

2.5 Determination of LDH Activity

Lactate dehydrogenase (LDH) activity was quantified spectrophotometrically by following the rate of oxidation of NADH at 340 nm in the presence of sodium pyruvate as substrate and Triton X-100 to ensure release of enzyme contained in subcellular particles and prevent revesiculation of membrane fragments. Samples contained Tris-HCl buffer (50 mM), pH 7.4,

NADH (70 μg/ml), sodium pyruvate (40 μg/ml), Triton X-100 (0.1%) and enzyme in a final volume of 1.1 ml. Initial reaction rates were determined over 4 min at 25°C and expressed as μmoles NADH oxidized / gram of tissue / minute using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$. LDH assays were performed on the same day as the experimental procedure.

2.6 Trion X-114 Partition of ChAT Activity

The Triton X-114 phase separation technique was also used to elucidate the subcellular distribution of synaptosomal ChAT. Below its cloud point temperature of 20°C, Triton X-114 forms micellar solutions and is an efficient solubilizer of membrane proteins. At temperatures above 20°C, however, solutions of Triton X-114 separate into two phases. Bordier (1981) demonstrated that hydrophobic or integral membrane proteins partition into the detergent phase, whereas hydrophilic or peripheral proteins partition into the aqueous phase.

Prior to use, commercial Triton X-114 was precondensed to remove hydrophilic molecules and free radicles. Detergent (2 g) was dissolved at 4°C in 98 ml of a solubilization buffer comprised of Tris-phosphate (10 mM, pH 7.4), NaCl (150 mM), and butylated hydroxytoluene (1.6 mg) as an antioxidant. Following incubation at 37°C for 16 hours to allow separation of detergent and aqueous phases, the top (aqueous) phase was removed and the lower (detergent) phase was redissolved in 98 ml of fresh solubilization buffer at 4°C. The separation procedure was repeated twice more, then the optical absorbance of a

solution of Triton X-114 was determined at wavelength 275 nm to allow calculation of the actual detergent final concentration.

To determine the phase partitioning of synaptosomal ChAT activity, the crude synaptosomal pellet was suspended in solubilization buffer containing 1% of precondensed Triton X-114 at 4°C. To ensure solubilization of the pellet, the suspension was incubated on ice for at least 15 min. Following centrifugation at 10,000 g for 10 min at 4°C, aliquots of supernatant and pellet (following suspension in solubilization buffer) were retained for the determination of ChAT activity. An aliquot (200 μl) of the remaining supernatant was layered onto 300 μl of a 6% sucrose cushion (solubilization buffer containing 6% sucrose) and incubated at 37°C for 4 min to allow phase partitioning. The sample was centrifuged at 10,000 g for 3 min at 25°C, and 200 μl of the upper aqueous phase was mixed with 200 μl of solubilization buffer containing 1% of precondensed Triton X-114. This was relayered over the same 6% sucrose cushion and incubated for 3 min at 37°C. Following centrifugation at 10,000 g for 3 min at 25°C, the supernatant was removed and combined directly with 2% of precondensed Triton X-114 (no sucrose cushion). Following a 3 min incubation at 37°C, the sample was centrifuged at 10,000 g for 3 min at 25°C. Both the supernatant and the detergent phase were retained for determination of ChAT activity.

Because each aliquot of detergent phase contained different volumes of Triton X-114 which dissolves in aqueous solutions only at 4°C, most of the

detergent was removed from the samples using Biobeads (BioRad, SM2). Samples containing Biobeads (0.3 g/ml) were gently mixed on a circular rotator at 4°C for 2 hours before centrifugation in a Savant microcentrifuge for 5 min at 8,000 rpm. Finally, 0.5% of Triton X-100 (final concentration) was added to 400 μl of the supernatant to ensure the cloud point was raised above 37°C prior to determination of ChAT activity (described in Chapter 2, Section 2.3).

2.7 Determination of Sample Protein Content

The protein content of each sample was measured by the method of Markwell *et al.* (1978) to allow for the presence of detergent, with bovine serum albumin used as the standard.

2.8 Materials

β-Nicotinamide adenine dinucleotide (reduced form), choline iodide, crystalline bovine serum albumin (fraction 5), dithiothreitol, EGTA, eserine sulphate, HEPES, sodium tetraphenylboron, Triton X-100, Triton X-114 and veratridine were purchased from Sigma Chemical Co., St. Louis, Missouri. 3-Heptanone was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. AcetylCoenzyme A, lithium salt was from Boehringer-Mannheim, Laval, Quebec. THAM (Tris buffer) was obtained from Fisher Chemical Co., Mississauga, Ontario. [^{3}H]acetylCoenzyme A (3.7 Ci/mmol) and [^{3}H]choline chloride (78 Ci/mmol) were purchased from Amersham Radiochemical Corp., Oakville, Ont.

2.9 Statistics

Experimental data are calculated as the mean ± SEM. Within each experiment, measurements were made in duplicate or triplicate, with values averaged to give a single n value. Coefficient of variation within individual measures was always less than 10%. Statistical differences were determined by paired t-test or one-way analysis of variance with repeated measures and Duncan's multiple range post-hoc test. The criterion for statistical significance was $P \leq 0.05$.

3. RESULTS

3.1 Characterization of Membrane-Bound ChAT

The subcellular localization of ChAT within cholinergic nerve terminals has been a subject of some controversy. Although the presence of a large cytosolic pool of the enzyme is accepted, there is some question as to the nature of the smaller membrane-bound fraction of ChAT. Because it has been suggested that this small membrane-bound pool may represent cytosolic ChAT occluded within membrane fragments rather than true membrane-bound enzyme (Bruce and Hersh, 1987), the subcellular distribution of ChAT activity in synaptosomes was compared to that of the cytosolic enzyme lactate dehydogenase (LDH). However, both enzymes appeared to partition similarly following subcellular fractionation of synaptosomes (Table 2) suggesting cytosolic contamination of the membrane fraction. Therefore the distribution of ChAT activity following phase partitioning in the nonionic detergent Triton X 114 was also examined. A similar percentage of ChAT activity was also extracted in the detergent phase of Triton X-114 as shown in Table 3, thus confirming the hydrophobic nature of a subcellular pool of this enzyme, designated to be membrane-bound ChAT.

3.2 Depolarization of Synaptosomes

Carroll and coworkers (Carroll *et al.*, 1986; Carroll, 1987) have demonstrated that membrane-bound ChAT activity is selectively increased

following 10 min depolarization of synaptosomes by elevated extracellular KCl.

Table 2. Subcellular distribution of ChAT and lactate dehydrogenase (LDH) activities in hippocampal synaptosomes. Enzyme activities were determined following subfractionation of synaptosomes. Data are mean ± SEM of 5 separate experiments and are expressed as percentage of total enzyme activity.

	ChAT Activity (% total)	LDH Activity (% total)
Aqueous Phase	71.9 ± 7.5	78.3 ± 2.3
Detergent Phase (1.5% Triton X-100)	13.2 ± 0.6	15.3 ± 1.8

Table 3. Triton X-114 phase separation of ChAT activity. The distribution of synaptosomal ChAT activity in Triton X-114 was determined as described in Section 2.6. Data are shown as mean ± SEM of 4 separate experiments and are expressed as percentage of total enzyme activity.

ChAT Activity Distribution (Triton X-114) (% total)	
Aqueous Phase	Detergent Phase
60.3 ± 2.8	16.8 ± 2.1

A duration of 10 min was used to induce depolarization in synaptosomes as it allowed easier manipulation of samples and has been shown to lead to depolarization-induced enzyme stimulation within the adrenergic system. It was of interest, however, to determine whether the activity of a subcellular pool of ChAT was enhanced following a shorter interval of depolarization of nerve terminals. A 30 sec depolarization time was chosen as this was the shortest incubation interval which could allow accurate manipulation of samples. As shown in Figure 1, depolarization of nerve terminals by elevated extracellular potassium

(40 mM KCl) had no effect upon cytosolic ChAT activity. Membrane-bound ChAT activity, however, was increased to 142.9 ± 14.6% of control following 30 sec depolarization. In comparison, a 10 min depolarization of nerve terminals increased membrane-bound ChAT activity selectively to 126.7 ± 12.8% of control.

The activation of synaptosomal membrane-bound ChAT induced by 10 min depolarization with 40 mM KCl appeared to be dependent upon the presence of calcium ions in the extracellular medium, as illustrated in Figure 2. Under non-depolarizing conditions (5 mM KCl), cytosolic ChAT activity was not altered by incubation of synaptosomes in calcium-free KR buffer, whereas membrane-bound ChAT activity was reduced to 62.4 ± 12.3% of control. Depolarization-induced activation of nerve terminal membrane-bound ChAT was prevented in calcium-free KR buffer, and interestingly, activity of the membrane-bound enzyme was decreased to an even greater extent than when incubated in calcium-free KR buffer in the absence of depolarizing agents (Figures 2 and 3, compare ■ and ■ for membrane-bound ChAT). An elevation in extracellular potassium ions (above 5 mM) causes depolarization of nerve terminals by passively shifting the resting membrane potential to more positive values. Although frequently used experimentally, potassium-mediated depolarization does not resemble physiological nerve activation via voltage-dependent sodium channels. Therefore depolarization was also induced by veratridine, an agent which binds to open sodium channels and maintains their open, conducting conformation. Similar to the results obtained with potassium-depolarization, there was no change in cytosolic ChAT activity

following 10 min depolarization by 50 μM veratridine, and membrane-bound ChAT activity was selectively increased to 132.3 ± 5.7% of control, in a calcium-dependent manner, as shown in Figure 3. The effect of veratridine on membrane-bound ChAT activity was prevented by the presence of the sodium channel blocker tetrodotoxin (Figure 4), confirming that veratridine depolarizes nerve terminals by acting on sodium channels.

The effects of the calcium ionophores A23187 and ionomycin were also examined in several experiments. It was reasoned that these agents would raise the intracellular calcium ion concentration and therefore could selectively enhance membrane-bound ChAT activity in a manner similar to that seen following depolarization of synaptosomes. However, results obtained in experiments using 3 different lot numbers of A23187 and 2 lot numbers of ionomycin (purchased from Sigma Chemical Co.) were conflicting and, therefore, are not presented.

3.3 Membrane-Bound ChAT and High-Affinity Choline Uptake

It has been suggested that membrane-bound ChAT may be closely associated with the high-affinity choline transporter in cholinergic nerve terminals, and may be responsible for ACh synthesis during repetitive neuronal activity (Jope, 1979). The present series of experiments provided some indirect support for this theory; when synaptosomes were pretreated for 10 min with either depolarizing agent followed by measurement of high-affinity choline transport activity in normal KR buffer, choline uptake was stimulated similar to the observed increases in

membrane-bound ChAT activity (Table 4, Figures 2 and 3).

Table 4. Synaptosomal high-affinity choline uptake following incubation with depolarizing agents. High-affinity choline uptake (HACU) by synaptosomes preincubated in normal KR buffer is shown following 10 min incubation at 37°C with the depolarizing agents KCl and veratridine (shown as final concentrations). Data are shown as mean ± SEM of 4 separate experiments, each with duplicate determinations. *a* indicates significantly different from control at $P \leq 0.05$.

PREINCUBATION TREATMENT	HACU (pmol/mg protein/4 min)	% CONTROL
KR buffer	32.5 ± 2.9	100
KR buffer + 40 mM KCl	40.2 ± 4.6[a]	124
KR buffer + 50 μM Veratridine	40.7 ± 5.5[a]	125

3.4 Mechanism of Depolarization-induced Activation of Membrane-Bound ChAT

Bruce and Hersh (1989) demonstrated that purified placental ChAT is a substrate for phosphorylation by a calcium-dependent protein kinase. At least two distinct types of calcium-dependent kinases are known to exist in rat brain, namely the calcium / calmodulin-dependent kinases and the calcium / phospholipid-dependent kinases. Experimentally, selective inhibition of a specific protein kinase could provide indirect support for the involvement of that kinase in enzyme phosphorylation. As calmodulin kinase II is the predominant calcium-dependent protein kinase in neurons of mammalian cortex and hippocampus, constituting up to 2% of the total protein (Erondu and Kennedy, 1985; Scott and Soderling, 1992), the effects of the potent *in vitro* antagonist of this enzyme, W-7 (Calbiochem),

upon the depolarization-induced activation of synaptosomal membrane-bound ChAT were examined. Although cytosolic ChAT activity was not affected (data not shown), high concentrations of W-7 (100 μM) appeared to attenuate the depolarization-induced activation of membrane-bound ChAT (Figure 5) suggesting that post-translational modification of the protein involving phosphorylation may be involved in the short-term regulation of ChAT activity.

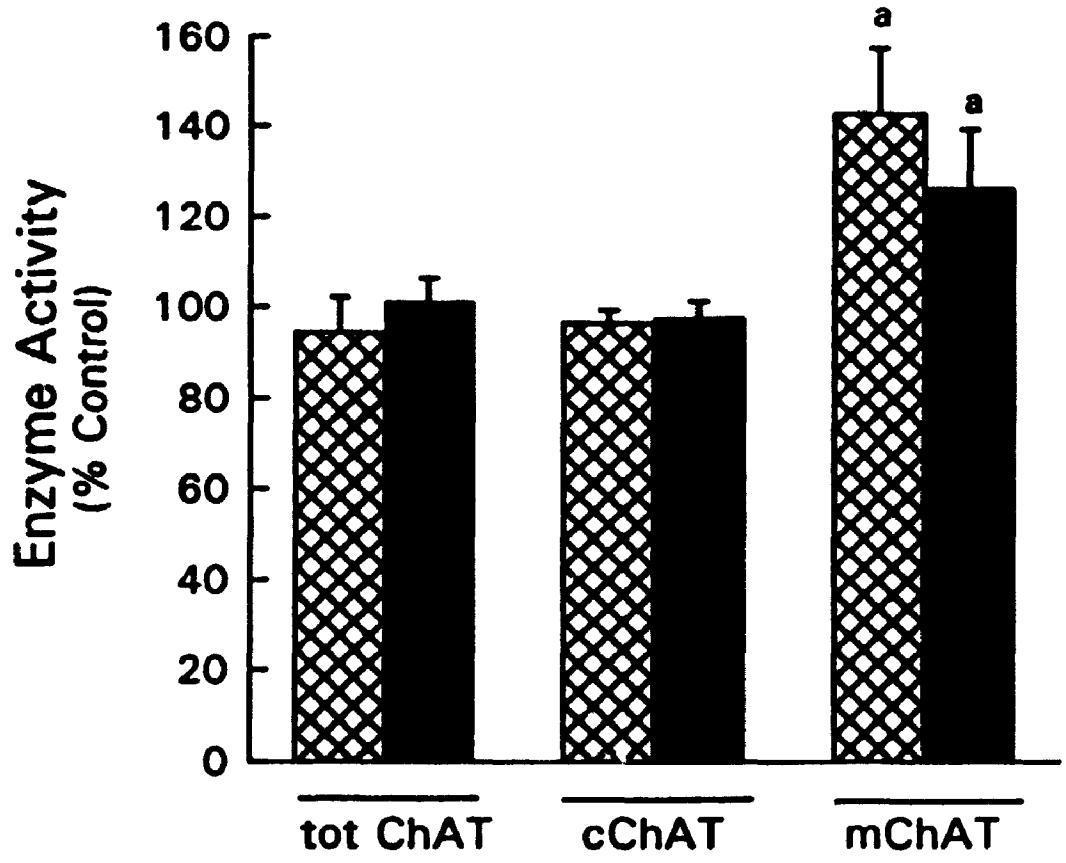


Figure 1. Membrane-bound ChAT activity was selectively increased following depolarization with 40 mM KCl. Synaptosomes were incubated at 37°C for 30 sec (▩) or 10 min (■) in KR buffer containing 40 mM KCl. Parallel control samples were incubated in the absence of added KCl. Membrane-bound ChAT (mChAT) and cytosolic ChAT (cChAT) pools were then isolated by subcellular fractionation and assayed for enzyme activity. *a* denotes significantly different from control at $P \leq 0.05$. Control values for total ChAT, cytosolic ChAT and membrane-bound ChAT activity were 62.6 ± 11.1, 527.7 ± 41.8, and 5.2 ± 0.5 nmoles ACh formed/mg protein/hour, respectively. Data are mean ± SEM of 3 separate experiments, each with duplicate determinations.

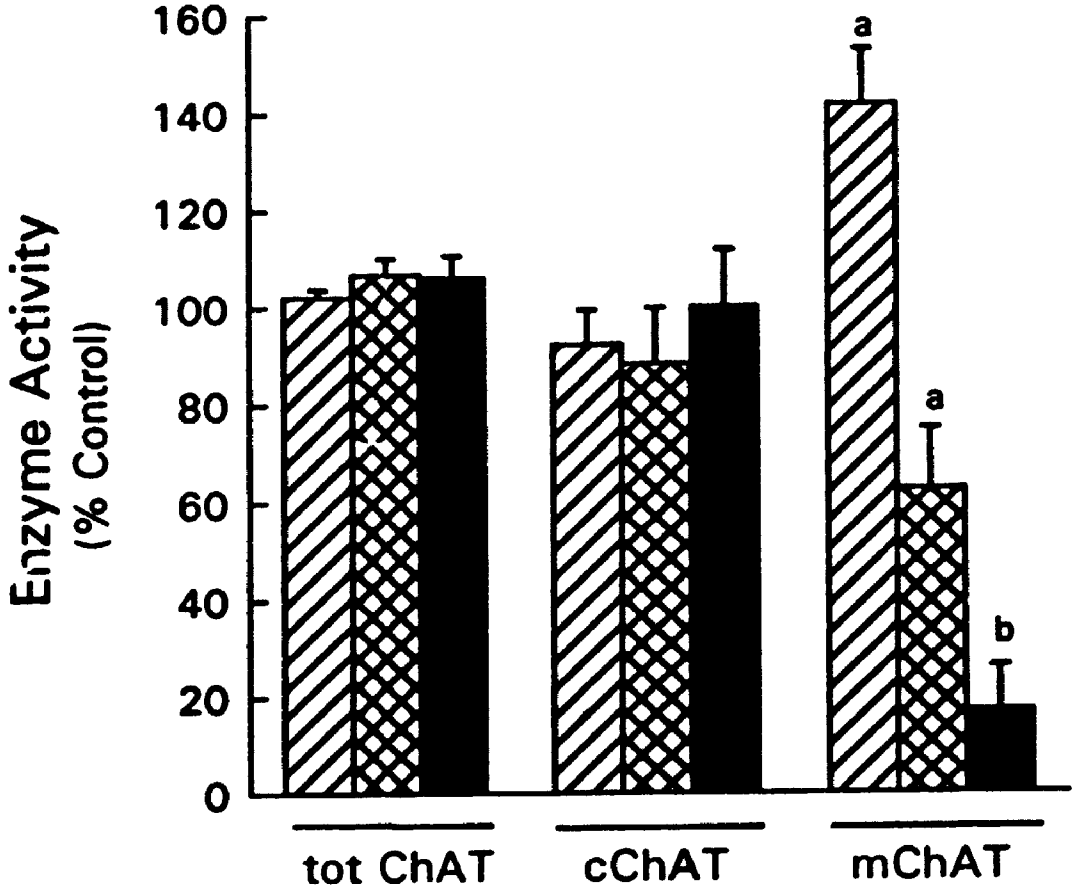


Figure 2. Depolarization-induced activation of membrane-bound ChAT was dependent upon the presence of extracellular calcium ions. Synaptosomes were warmed to 37°C in KR buffer in the presence (control) or absence of 1.5 mM calcium (▩) before 40 mM KCl was added to calcium-containing (▨) or calcium-depleted (■) samples. Following a 10 min incubation, membrane-bound ChAT (mChAT) and cytosolic ChAT (cChAT) pools were isolated by subcellular fractionation and assayed for enzyme activity. *a* denotes significantly different from control at $P \leq 0.05$. *b* denotes significantly different from control at $P \leq 0.01$. Control values for total ChAT, cytosolic ChAT and membrane-bound ChAT activity were 78 ± 10.6, 714.2 ± 35, and 7.8 ± 0.9 nmoles ACh formed/mg protein/hour, respectively. Data are mean ± SEM of 4 experiments, each with duplicate determinations.

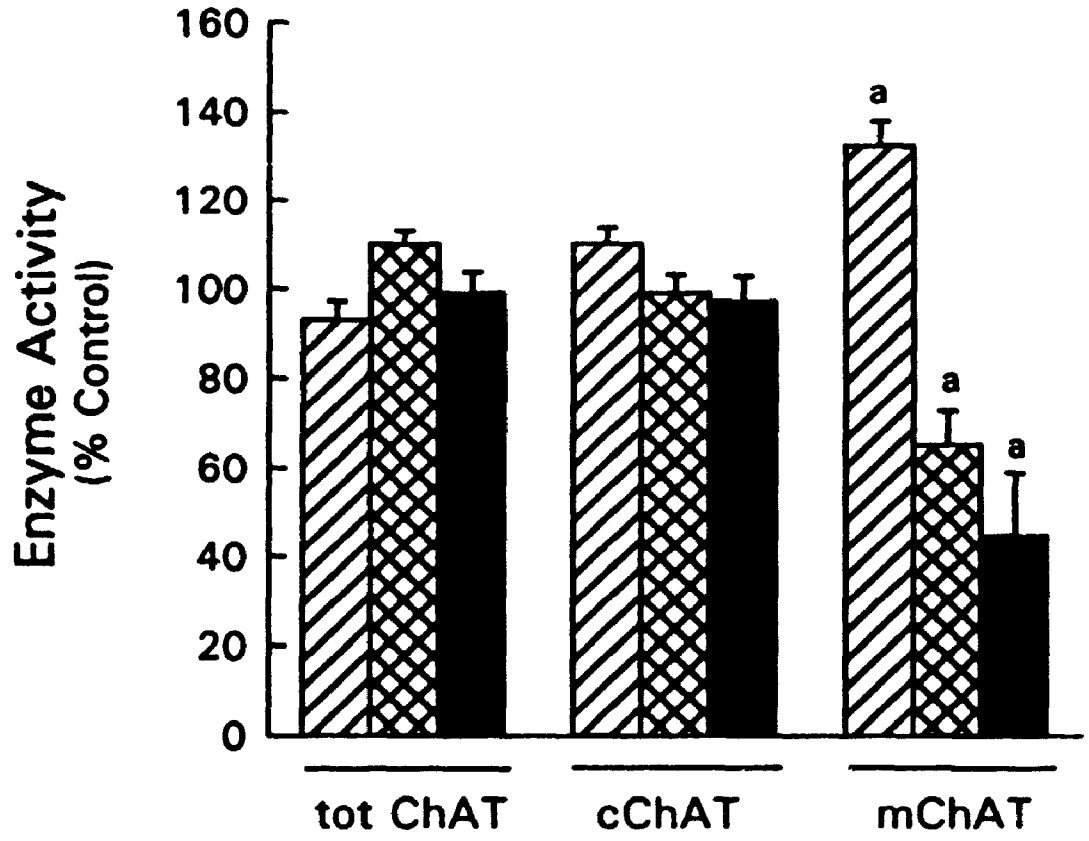


Figure 3. Depolarization of synaptosomes by veratridine selectively increased membrane-bound ChAT activity in a calcium-dependent manner. Synaptosomes were warmed to 37°C in KR buffer in the presence (control) or absence of 1.5 mM calcium (■) before 50 μM veratridine was added to calcium-containing (▨) or calcium-depleted (■) samples. Following 10 min incubation, membrane-bound ChAT (mChAT) and cytosolic ChAT (cChAT) pools were isolated by subcellular fractionation and enzyme activity was determined. *a* denotes significantly different from control at $P \leq 0.05$. Control values for total ChAT, cytosolic ChAT and membrane-bound ChAT were 68.8 ± 8.8, 556.4 ± 96.6, and 2.2 ± 0.3 nmoles ACh formed/mg protein/hour, respectively. Data are mean ± SEM of 4 experiments, each with duplicate determinations.

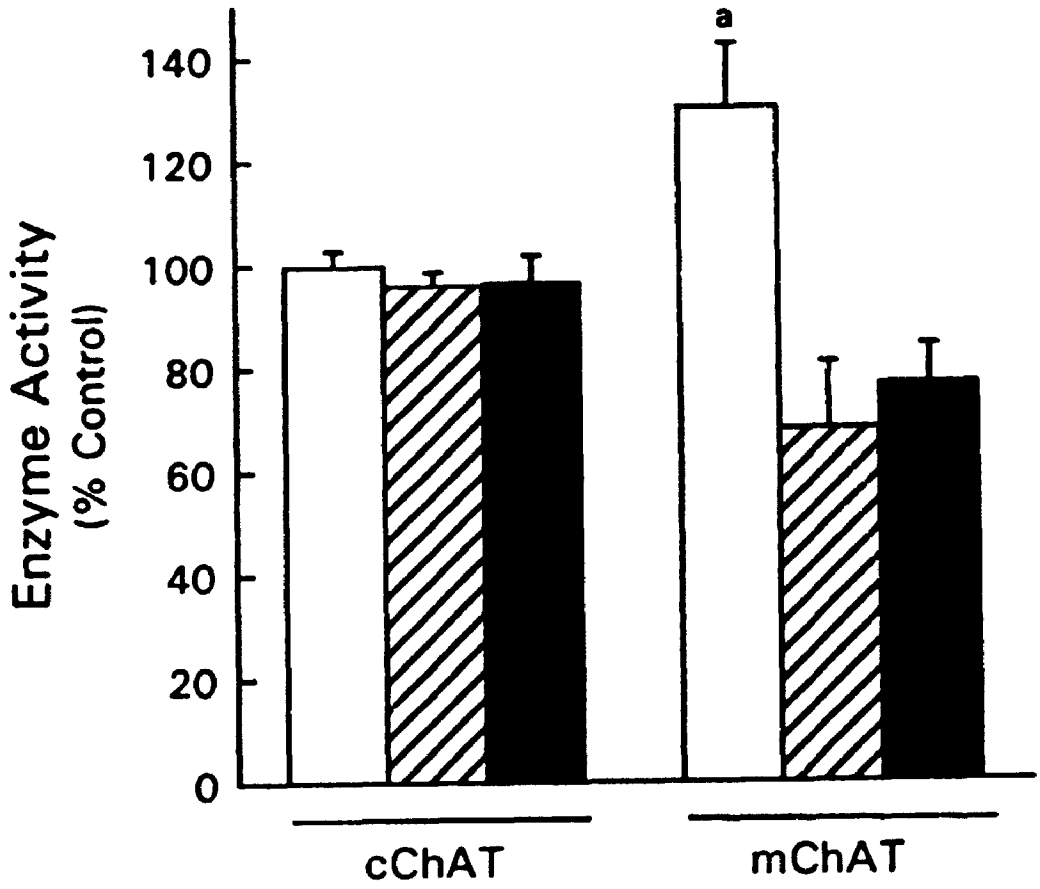


Figure 4. Tetrodotoxin blocked the veratridine-induced activation of membrane-bound ChAT. Synaptosomes were warmed to 37°C in normal oxygenated KR buffer in the presence (▨ and ■) or absence (control and □) of 1 μg/ml tetrodotoxin, then a 10 min incubation was begun with the addition of 50 μM veratridine to samples with (■) and without (□) tetrodotoxin. Membrane-bound ChAT (mChAT) and cytosolic ChAT (cChAT) were isolated by subcellular fractionation and assayed for enzyme activity. *a* denotes significantly different from control at $P \leq 0.05$. Control values for cytosolic ChAT and membrane-bound ChAT activity were 418.8 ± 70.9 and 2.2 ± 0.3 nmoles ACh formed/mg protein/hour, respectively. Data are mean ± SEM of 3 experiments, each with duplicate determinations.

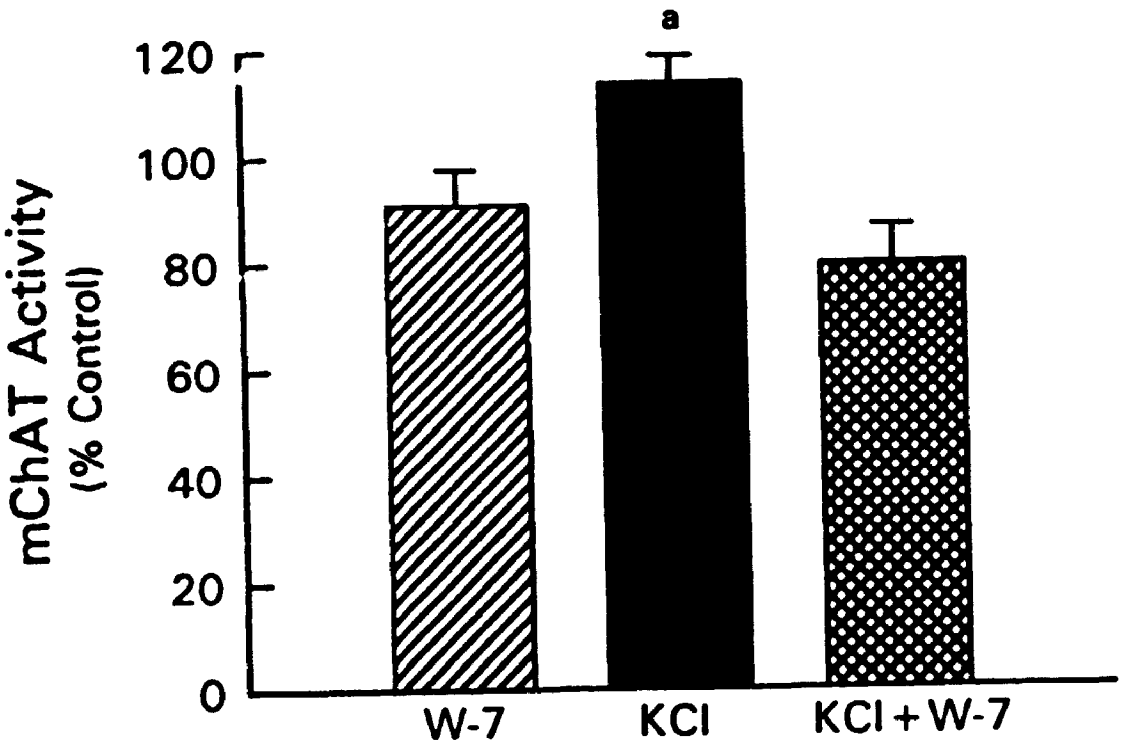


Figure 5. W-7 attenuated the depolarization-induced activation of membrane-bound ChAT. Synaptosomes were warmed to 37°C in normal oxygenated KR buffer and 100 μM of W-7 was added (▨, ■). Parallel control samples received an equal volume of distilled H_2O. 10 min depolarization was begun with the addition of 40 mM KCl to samples containing 100 μM W-7 (■) or an equal volume of distilled H_2O (■). Membrane-bound ChAT (mChAT) was then isolated by subcellular fractionation and assayed for enzyme activity. *a* denotes significantly different from control at $P \leq 0.05$. Control membrane-bound ChAT specific activity was 4.8 nmoles ACh formed/mg protein/hour, respectively. Data are mean ± SEM of 3 experiments, each with duplicate determinations.

4. DISCUSSION

The objectives of experiments described in this chapter were to assess the relative distribution of cytosolic and membrane-bound ChAT in rat brain hippocampal synaptosomes and to determine the effect of depolarization upon enzyme activity. I observed that: (1) approximately 15% of total nerve terminal ChAT activity appeared to be associated with synaptosomal membranes, (2) depolarization of intact synaptosomes with 40 mM KCl or 50 μM veratridine selectively increased membrane-bound ChAT activity, (3) enhanced membrane-bound ChAT activity observed following nerve terminal depolarization was dependent upon the presence of calcium ions in the extracellular medium, and (4) the depolarization-induced activation of membrane-bound ChAT was attenuated by W-7, an inhibitor of calcium-calmodulin kinase II.

Release of the cytosolic enzyme lactate dehydrogenase (LDH) has often been used to assess the efficiency of synaptosomal lysis (Adam-Vizi and Marchbanks, 1983). Previous studies in our laboratory (Rylett, 1989) demonstrated that the percentage of total synaptosomal ChAT activity recovered in the final membrane pellet always exceeded that of LDH, suggesting that ChAT associated with this pellet was a membrane-bound form of enzyme. In the present studies, LDH activity was determined in each subcellular fraction assessed for ChAT activity, but the relative distribution of LDH activity appeared to parallel that of ChAT. This suggested cytosolic contamination of the membrane fraction,

therefore partitioning of ChAT activity in the nonionic detergent Triton X-114 was also used to assist in the determination of the nature of the association of ChAT with synaptic membranes.

Non-ionic detergents have been used routinely for the solubilization and characterization of integral membrane proteins (Bordier, 1981). Within the Triton X series of detergents, Triton X-114 exhibits a cloud point at 20°C (compared to the cloud point for Triton X-100 of 64°C) thus making it convenient for reversible condensation and separation of the detergent from the aqueous phase. Bordier (1981) has shown that hydrophilic proteins are recovered in the aqueous phase and amphiphilic integral membrane proteins are found in the detergent phase after phase separation of Triton X-114 detergent. I determined that an equivalent proportion of synaptosomal ChAT activity was recovered in the Triton X-114 detergent phase as that recovered in the washed membrane fraction following subcellular fractionation, and, since by definition this is an integral membrane protein, it has been designated membrane-bound ChAT. As shown in Table 3, full recovery of ChAT activity was not achieved following Triton X-114 phase partitioning. This was likely due to the loss of enzyme during the removal of detergent with Biobeads and/or the partitioning of ChAT into the detergent-insoluble pellet which was not measured. The classification of membrane-bound ChAT based upon Triton X-114 phase separation has also been used by other laboratories (Eder-Colli *et al.*, 1986; Docherty and Bradford, 1988; Massarelli *et al.*, 1988; Eder-Colli *et al.*, 1992).

The physiological role of membrane-bound ChAT in cholinergic nerve terminals is not clear at present. Carroll (1987) showed that membrane-bound ChAT activity was selectively enhanced following 10 min depolarization of synaptosomes by elevated extracellular KCl or veratridine, agents which are also known to elicit calcium-dependent ACh release. In the study by Carroll, veratridine-induced depolarization was accompanied by an increase in the V_{max} of membrane-bound ChAT, suggesting an enlarged pool of enzyme which may have some regulatory importance. Although a 10 min depolarization interval resulted in altered membrane-bound ChAT activity, this long duration may obviate events such as post-translational modifications of the enzyme. For example, in the adrenergic system, phosphorylation of tyrosine hydroxylase in rat brain synaptosomes is demonstrable within 30 sec of depolarization by elevated KCl (Haycock, 1987). Therefore, it was important and of interest to determine that membrane-bound ChAT activity was significantly enhanced following a 30 sec depolarization interval. However, as 10 min depolarization intervals allowed easier manipulation of samples and increased membrane-bound ChAT activity up to about 120-140% of control, subsequent studies employed this time frame. Furthermore, in support of this choice, Haycock (1987) demonstrated that depolarization-induced phosphorylation of tyrosine hydroxylase was maintained relatively constant for the time frame of several minutes, and Yanagihara *et al.* (1984) observed that depolarization-induced activation of tyrosine hydroxylase persisted for 10 min in PC12 cells. These investigators also reported that

phosphorylation of the enzyme was maintained during 5 min depolarization, however they did not examine its level after 10 min depolarization. Therefore, based upon these findings, it was believed that 10 min depolarization of synaptosomes could be used with confidence to study the presynaptic regulation of ChAT activity.

Depolarization-induced activation of membrane-bound ChAT by both 40 mM KCl and 50 μM veratridine was dependent upon the presence of extracellular calcium ions. Although the basal specific activity of synaptosomal cytosolic ChAT was not altered in the absence of extracellular calcium, the basal specific activity of membrane-bound ChAT was significantly reduced to about 60% of control. The different sensitivities of the two forms of ChAT to manipulation of cytosolic calcium ion composition suggest that each pool of enzyme may be subject to different regulatory controls and may be responsible for synthesizing different pools of releasable neurotransmitter.

ACh is released from nerve terminals by at least two different processes. Cytosolic ChAT may catalyse the formation of a cytosolic pool of ACh which is spontaneously released by a calcium-independent mechanism and could involve membrane transport processes (O'Fallon *et al.*, 1981; Vyskocil *et al.*, 1983). Depolarization of nerve terminals, however, causes the quantal exocytotic release of ACh by a calcium-dependent mechanism which in turn stimulates high-affinity choline uptake required to replenish precursor for resynthesis of neurotransmitter stores. Since membrane-bound ChAT activity also appeared to be enhanced

similarly under these conditions, it is tempting to speculate that this enzyme pool is closely associated with the high-affinity choline transporter and is responsible for the synthesis of vesicular ACh involved in evoked release.

The mechanism(s) by which membrane-bound ChAT activity was selectively altered is presently unknown. Because of the short time required to enhance membrane-bound ChAT activity (30 sec), some post-translational protein modification may be involved. In preliminary studies, I demonstrated that W-7, an antagonist of calcium-calmodulin kinase II, prevented the depolarization-induced activation of membrane-bound ChAT suggesting the involvement of calcium-dependent protein phosphorylation as a possible mechanism of short-term enzyme regulation in cholinergic neurons. In support of this, Lapchak and Collier (1988) demonstrated a calcium-dependent increase in ChAT activity in hippocampal homogenates following exposure to vasoactive intestinal peptide. Subsequently, Bruce and Hersh (1989) demonstrated that purified human placental ChAT was a substrate for phosphorylation by calcium-dependent kinases. Interestingly they also demonstrated the presence of phosphorylated ChAT in rat brain synaptosomes, however they did not investigate the subcellular distribution of the phosphoprotein. The physiological significance of protein phosphorylation as a mechanism of short-term regulation of ChAT activity is addressed in the next chapter.

CHAPTER 3

RAT BRAIN ChAT EXISTS AS AN ENDOGENOUS PHOSPHOPROTEIN ONLY IN THE CYTOSOLIC POOL

1. INTRODUCTION

At present, little is known about the short-term regulation of ChAT since early investigations indicated that the enzyme was not maximally active under normal physiological conditions and, therefore, unlikely to assume a regulatory role in ACh biosynthesis in cholinergic nerve terminals (Tucek, 1985). However, despite strong evidence that choline availability is rate-limiting in ACh synthesis, regulation of ChAT must be recognised as important as it is at a metabolic branch point for the common components acetylCoenzyme A and choline (Salvaterra and Vaughn, 1989).

One common short-term regulatory mechanism for proteins in the central nervous system is covalent modification by phosphorylation (Browning *et al.*, 1985). A variety of data indicate that protein phosphorylation regulates the efficacy of synaptic transmission by modulating turnover and release of neurotransmitter from the presynaptic nerve terminal, and by modifying the sensitivity of neurotransmitter receptors (reviewed by Huganir and Greengard, 1987; Hemmings *et al.*, 1989; Walaas and Greengard, 1991). Phosphorylation-dependent regulation of enzymes involved in neurotransmitter synthesis has been studied extensively in a number of systems and is commonly believed to involve a structural change in the protein resulting in altered function (reviewed by Barford, 1991). Tyrosine hydroxylase, the rate-limiting biosynthetic enzyme in catecholaminergic neurons, is a substrate for phosphorylation by multiple protein kinases; regulation of enzyme catalytic

activity involves a complicated interplay between phosphorylation pathways (Haycock, 1987; Haycock *et al.*, 1988). There is also evidence to indicate that tryptophan hydroxylase activity is also regulated by kinase-mediated phosphorylation (Ehret *et al.*, 1989). In cholinergic neurons, indirect evidence suggests that ChAT activity may be increased under conditions which could favour its phosphorylation (Luine *et al.*, 1984; Lapchak and Collier, 1988), and Bruce and Hersh (1989) provided direct evidence that the purified placental enzyme may serve as a substrate for purified calcium-dependent kinases from rat brain. More recently, Mallet and coworkers demonstrated the existence of phosphorylated recombinant rat ChAT in baculovirus-infected Sf9 cells (Habert *et al.*, 1992).

Whereas the rate of synthesis of catecholamines, and perhaps indolamines, is controlled by depolarization-regulated phosphorylation of their biosynthetic enzymes (Haycock, 1987), a physiological role for phosphorylation of ChAT is unclear. Unlike tyrosine hydroxylase, ChAT is not generally considered to represent the rate-limiting step for transmitter synthesis under physiological conditions. The objectives of research reported in this chapter were to determine whether cytosolic ChAT and membrane-bound ChAT exist as phosphoproteins in rat brain synaptosomes, to investigate conditions for modulation of ChAT phosphorylation, and to examine the relationship between phosphorylation and ChAT activity. I detected the presence of a phosphorylated form of ChAT in cytosolic fractions of rat hippocampal synaptosomes under basal, unstimulated conditions, and observed that the abundance of the phosphoprotein was apparently

dependent upon the resting level of cytosolic calcium. Depolarization of nerve terminals with veratridine did not appear to alter the pattern of phosphorylation of ChAT, although the activity of membrane-bound ChAT was selectively increased.

2. METHODS

2.1 Determination of Optimal Immunoprecipitation Conditions

Preliminary studies were done to determine the incubation time and dilution of antibody required for optimal immunoprecipitation of ChAT. Following the preparation of hippocampal synaptosomes from up to 20 rats (Chapter 2, Section 2.1) and the isolation of each subcellular pool of ChAT (Chapter 2, Section 2.2), 300 µl of each sample including 1% Triton X-100 (final concentration) was precleared for 20 min at 4°C with prewashed Pansorbin cells (Staph. aureus cells bearing Protein A on the outer surface; 100 µl/ml of prewashed 10% (w/v) Pansorbin cells) to minimize nonspecific binding, then incubated with one of the following treatments: (1) polyclonal rabbit anti-ChAT antibody (Chemicon) of various dilutions (1:300 - 1:1600), (2) nonimmune rabbit sera (NIS; 1:600 dilution) or, (3) an equal volume of 100 mM sodium phosphate buffer, pH 7.4 and mixed gently on a circular rotator at 4°C for 1 hour, 2 hours or overnight (16 hours). Following the incubation, 25.0 µl of prewashed 10% Pansorbin cells was added per ml of sample and gently mixed on a circular rotator at 4°C for 45 min to allow the Protein A to form complexes with rabbit anti-ChAT antibody-antigen reaction products. To precipitate the ChAT/antibody complexes, samples were centrifuged in a Savant microcentrifuge for 2 min at 8,000 rpm. Supernatants were assayed for ChAT activity (described in Chapter 2, Section 2.3) to monitor the efficiency of immunoprecipitation.

2.2 Determination of Phosphate Incorporation by ChAT

The washed synaptosomal pellet prepared from up to 20 rats was suspended in either 3 ml of normal KR buffer, pH 7.4 (mM: NaCl, 124; KCl, 5.0; $MgSO_4$, 1.3; $CaCl_2$, 1.5; glucose, 10; Hepes, 20 pH 7.4 with NaOH) or 3 ml of calcium-free oxygenated KR buffer, pH 7.4 (no added calcium plus 0.1 mM EGTA). The homogenate was warmed to 37°C and oxygenated for 5 min. At that time, 300 μCi $^{32}P_i$ (carrier-free, DuPont NEN) was added, and the solution was oxygenated again briefly. To allow labelling of endogenous ATP pools, incubation continued for 45 min at 37°C since it has been shown that isotopic equilibrium between these pools is likely to occur within this time (Robinson and Dunkley, 1983). Phosphorylation was stopped by placing samples on ice prior to centrifugation at 17,000 g for 10 min. The supernatant was discarded, and the pellet washed twice with 4 ml of the appropriate KR buffer containing 50 mM NaF and 0.1 mM PMSF to inhibit endogenous phosphatases and proteases.

Cytosolic and membrane-bound pools of ChAT were isolated by subcellular fractionation of synaptosomes as described in Chapter 2, Section 2.2 with the following modifications. Following the labelling of ATP pools, all solutions contained 50 mM NaF and 0.1 mM PMSF. Washed synaptosomal pellets were resuspended in 1 ml of cold deionized water. To ensure lysis of the synaptosomes, samples were left on ice for at least 20 min before freeze-thawing twice in an ethanol/dry ice bath instead of homogenization by polytron to prevent the formation of radioactive aerosols. Homogenates were centrifuged at 100,000 g for

60 min to yield the water-soluble pool of ChAT in the supernatant. Pellets were resuspended in 1 ml of 100 mM sodium phosphate buffer, pH 7.4, before freeze-thawing again. Samples were centrifuged at 100,000 g for 30 min. The final pellet was resuspended in 2 ml of 100 mM sodium phosphate buffer, pH 7.4 containing 1.5% Triton X-100 (final concentration; detergent:protein ratio 10) in addition to NaF and PMSF. This homogenate was kept on ice for 30 min with intermittent mixing before centrifugation at 100,000 g for 30 min. The resulting supernatant was used for immunoprecipitation.

2.3 ChAT Immunoprecipitation and Determination of Phosphate Incorporation

All fractions containing ChAT were normalized to 1.5% Triton X-100 (final concentration), and precleared for 30 min at 4°C with prewashed Pansorbin cells (100 µl of 10% cell suspension/ml sample). Samples were incubated for 2 hours at 4°C with one of the following: (1) polyclonal rabbit anti-ChAT antibody (1:500 dilution, Chemicon), (2) nonimmune rabbit sera (1:500 dilution), or (3) an equal volume of 100 mM sodium phosphate buffer, pH 7.4. Following incubation, Pansorbin cells (200 µl/ml sample) were added to samples and mixed gently at 4°C for 45 min to allow binding between Protein A on Staph. aureus and the antigen-antibody complexes. Samples were centrifuged at 8,000 rpm for 2 min, then the pellet containing Pansorbin cells, anti-ChAT antibody and ChAT was washed 3 times with resuspension buffer (SDS, 0.5%; Nonidet P-40, 0.5%; deoxycholate, 0.25%; EGTA, 1 mM in phosphate buffered saline, pH 7.4) to remove

contaminants. The final pellet was solubilized in 100 μl of 100 mM sodium phosphate buffer, pH 7.4 plus an equal volume of Laemmli double-strength sample buffer (containing SDS, 4.6% (w/v); glycerol, 20% (w/v); Tris, 124 mM; dithiothreitol, 200 mM; bromophenol blue, 1%).

Samples were denatured by boiling for 5 min and centrifuged at 8,000 rpm for 10 min to remove insoluble material, then supernatants were electrophoresed on denaturing polyacrylamide gels (5% stacking gel, 11% separating gel) according to the method of Laemmli (1970). When dry, gels were subjected to autoradiography (exposure time 7 days with Kodak X-OMAT AR film and intensifying screen) to assess phosphate incorporation into proteins.

2.4 Materials

Choline iodide, crystalline bovine serum albumin, dithiothreitol, EGTA, eserine sulphate, HEPES, PMSF, sodium tetraphenylboron and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Missouri. 3-Heptanone was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. AcetylCoenzyme A, lithium salt was from Boehringer-Mannheim, Laval, Quebec. Sodium fluoride and THAM (Tris buffer) were obtained from Fisher Chemical Co., Mississauga, Ontario. Acrylagel and Bisacrylagel were purchased from National Diagnostics, Manville, New Jersey. SDS was from Bio-Rad Laboratories, Richmond, California. Deoxycholic acid, sodium salt and Pansorbin cells (10% cell suspension) were purchased from Calbiochem, La Jolla, California. Electrophoretic low molecular

weight standards were obtained from Pharmacia Inc., Piscataway, New Jersey. Polyclonal rabbit anti-human ChAT Ig was obtained from Chemicon International Inc., Temecula, California. Nonidet P40 was from BDH Chemicals Ltd., Pool, England. [^{3}H]acetylCoenzyme A (3.7 Ci/mmol) was purchased from Amersham Radiochemical Corp., Oakville, Ontario. [$^{32}P_i$] carrier-free (30 Ci/mmol) was purchased from Dupont-NEN, Mississauga, Ontario.

3. RESULTS

3.1 Immunoprecipitation of ChAT

ChAT is an extremely low abundance protein; Eckenstein *et al.* (1981) estimated its abundance at less than one part in several million in mammalian brain, and, more recently, Ostermann and coworkers purified the enzyme to homogeneity and estimated its concentration to be 75 μg/kg porcine brain (Ostermann *et al.*, 1990). Based upon observations made in a number of laboratories, when enzyme specific activity is normalized to tissue weight, membrane-bound ChAT represents about 10-20% of total ChAT. Therefore to isolate and concentrate this protein in the present experiments, ChAT was efficiently immunoprecipitated from total, cytosolic and membrane-associated synaptosomal fractions by a polyclonal rabbit anti-human ChAT antibody coupled with Protein A-bearing Staph. aureus (Pansorbin) cells. At an anti-ChAT antibody dilution of 1:500, almost all ChAT activity was immunoprecipitated (Figure 6; 93±1, 94±1 and 91±2 % ChAT activity precipitated from total, cytosolic and solubilized membrane fractions, respectively), whereas nonimmune rabbit serum at a similar dilution precipitated little enzyme activity under identical conditions. Incubation of enzyme fractions with antibody, but not precipitated by Pansorbin cells, had no effect on enzyme activity as shown in Figure 7. Because about 95% of ChAT activity was consistently removed from supernatant following 2 hours incubation (Figure 8), this duration was used for all subsequent studies.

3.2 Basal Phosphorylation of ChAT

Bruce and Hersh (1989) demonstrated the presence of phosphorylated ChAT in rat forebrain synaptosomes using an immunoaffinity purification procedure. I have confirmed this finding, and extended it by determining the subcellular pool in which the phosphoprotein originated. As illustrated in Figure 9, phosphorylated ChAT was recovered from hippocampal synaptosomes incubated under resting conditions. Immunoprecipitation of synaptosomal subfractions with anti-ChAT antibody, followed by SDS-PAGE and autoradiography revealed a phosphoprotein in the cytosolic fraction with an apparent molecular mass of 67,000 ± 730 daltons (n=4; Figure 9, lane 3), corresponding to the apparent molecular mass for ChAT purified from rat brain (Dietz and Salvaterra, 1980). A phosphoprotein with this molecular mass was not observed in parallel control samples incubated with nonimmune serum, however a band with apparent molecular mass 37,000 was seen in some experiments (Figure 9, lane 4). It is interesting to note that, unlike the cytosolic fraction, a phosphorylated form of ChAT was not recovered from the solubilized synaptosomal membrane fraction which was prepared from the hippocampi of at least 10 rats (n=11) (Figure 10, lane 7).

Initially, it was considered that the low abundance of membrane-bound ChAT in cholinergic neurons may have prevented the detection of a phosphorylated form by autoradiography. To evaluate this, I assessed the sensitivity of the assay procedure by immunoprecipitating phosphorylated cytosolic

ChAT from between 1 and 5 hippocampi and constructed a sensitivity curve following densitometric scanning of the autoradiographs. Figure 11 demonstrates that phosphorylated cytosolic ChAT immunoprecipitated from the cytosolic fraction of one hippocampus could be detected by this method. Cytosolic enzyme activity in one hippocampus (44 nmoles ACh syn/ml/hour) is roughly equivalent to that isolated from membranes of 10 hippocampi (54 nmoles ACh syn/ml/hour). Visualization of phosphorylated cytosolic ChAT suggests, therefore, that this detection system would be sensitive enough for detection of phosphorylated membrane-bound ChAT under these experimental conditions if it were present in the same abundance.

3.3 Calcium Dependence of Basal ChAT Phosphorylation

Bruce and Hersh (1989) observed that purified human placental ChAT could be phosphorylated by calcium-dependent protein kinases. As cytosolic ChAT appears to exist, at least in part, as a phosphoprotein in the unstimulated nerve terminal, it was of interest to extend this observation and determine whether the abundance of phosphorylated ChAT was related to the level of synaptosomal cytosolic free calcium. Incubation of synaptosomes in nominally calcium-free solutions (no added calcium with the addition of 0.1 mM EGTA) significantly lowers intracellular free calcium content within minutes (Komulainen and Bondy, 1986; Brammer and Weaver, 1989). I found that lowering synaptosomal cytosolic calcium in this manner resulted in diminished [$^{32}P_i$] incorporation into cytosolic

ChAT as shown in Figure 10, lane 4. Quantitative comparison of autoradiographic films by densitometric scanning revealed that [$^{32}P_i$] incorporation into cytosolic ChAT in preparations incubated in nominally calcium-free medium was only 43 ± 7% (n=3) of control.

Although phosphorylation of cytosolic ChAT appeared to be dependent, in part, upon the level of cytosolic calcium, the corresponding enzyme activity did not. As illustrated in Figures 2 and 3 (Chapter 2, Results), preincubation of synaptosomes in nominally calcium-free KR did not alter the specific activities of total synaptosomal or cytosolic ChAT relative to controls incubated in KR medium containing 1.5 mM calcium. Removal of extracellular calcium did, however, significantly decrease the activity of membrane-bound ChAT to about 60% of control.

3.4 Effect of Depolarization on ChAT Phosphorylation

Within catecholaminergic neurons of the central and peripheral nervous systems, the catalytic activity of cytosolic tyrosine hydroxylase is enhanced by a depolarization-dependent increase in enzyme phosphorylation (Haycock, 1987; Zigmond *et al.*, 1989). This covalent modification appears to result, in part, from calcium-dependent protein kinases, which are activated by the depolarization-induced increase in intracellular calcium. I have demonstrated that ChAT exists as a phosphoprotein in the cytosol of nondepolarized nerve terminals and that the abundance of cytosolic phosphoprotein, but not the corresponding enzyme activity,

was dependent upon the level of cytosolic calcium. The physiological significance of phosphorylation of ChAT remains to be determined, but may be involved in its short-term regulation within the cholinergic neuron. In Chapter 2, I provided evidence that neuronal depolarization, which increases calcium influx, selectively increased the activity of membrane-bound ChAT. It was of interest, therefore, to determine whether depolarization-induced activation of membrane-bound ChAT was associated with phosphorylation of the protein, and whether the level of phosphorylation of cytosolic ChAT was altered by depolarization-induced increases in calcium influx. Using the sodium channel agonist veratridine to depolarize hippocampal synaptosomes prior to isolation of subcellular pools, I found that veratridine-induced depolarization did not appear to result in detectable [^{32}P]phosphate incorporation into membrane-bound ChAT, nor did it appear to alter the extent of phosphorylation of cytosolic ChAT, with the relative incorporation of [$^{32}P_i$] into cytosolic ChAT following depolarization being 101 ± 11% of control (n=3).

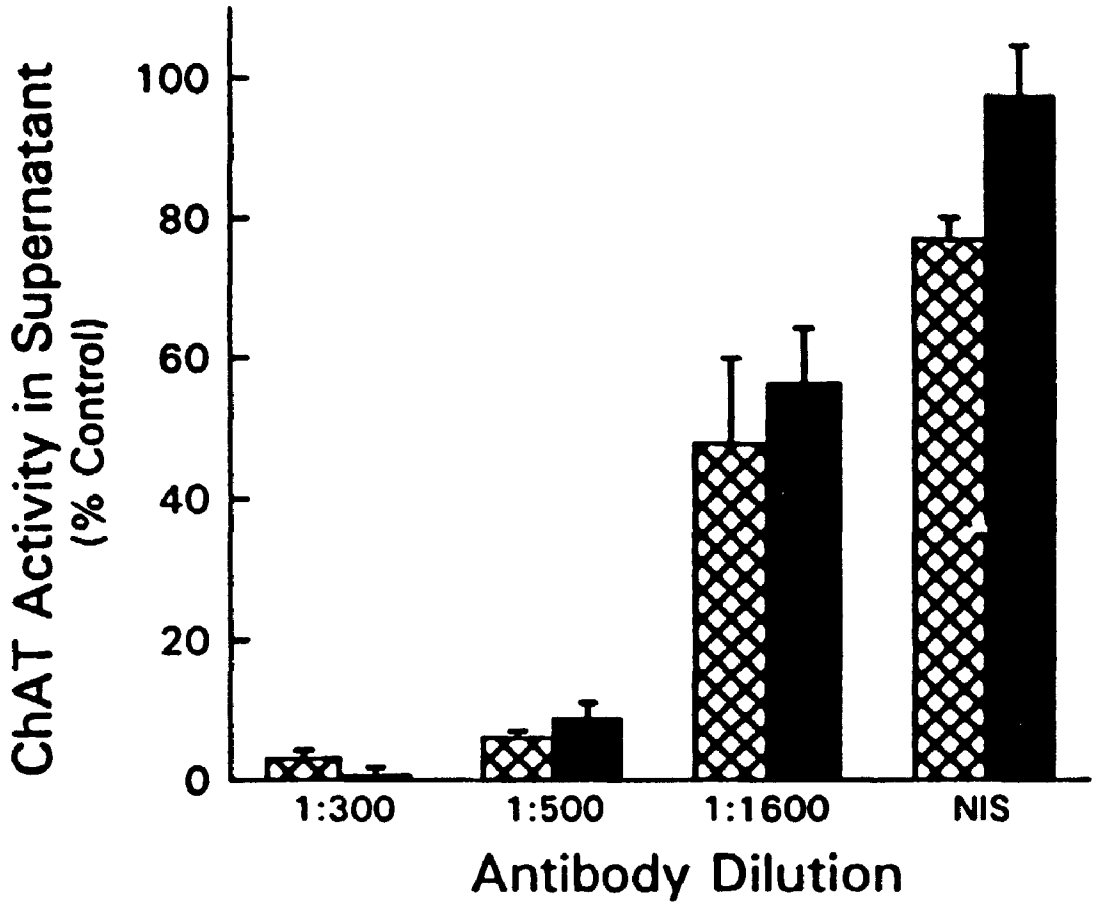


Figure 6. Low concentrations of rabbit anti-ChAT antibody precipitate ChAT activity in the presence of Pansorbin cells. Subcellular pools of cytosolic ChAT (■) and membrane-bound ChAT (■) were isolated, precleared and gently mixed on a circular rotator at 4°C with various dilutions of polyclonal rabbit anti-ChAT antibody (1:300 - 1:1600), non-immune rabbit serum (NIS; 1:600) or an equal volume of 100 mM sodium phosphate buffer, pH 7.4 (control) for 2 hours. Prewashed Pansorbin cells (250 µl/ml of 10% cell suspension) were then added and incubation continued for 45 min. Following centrifugation at 8,000 rpm for 10 min, enzyme activity remaining in the supernatant was determined. Data are mean ± SEM of 3 separate experiments, with duplicate determinations.

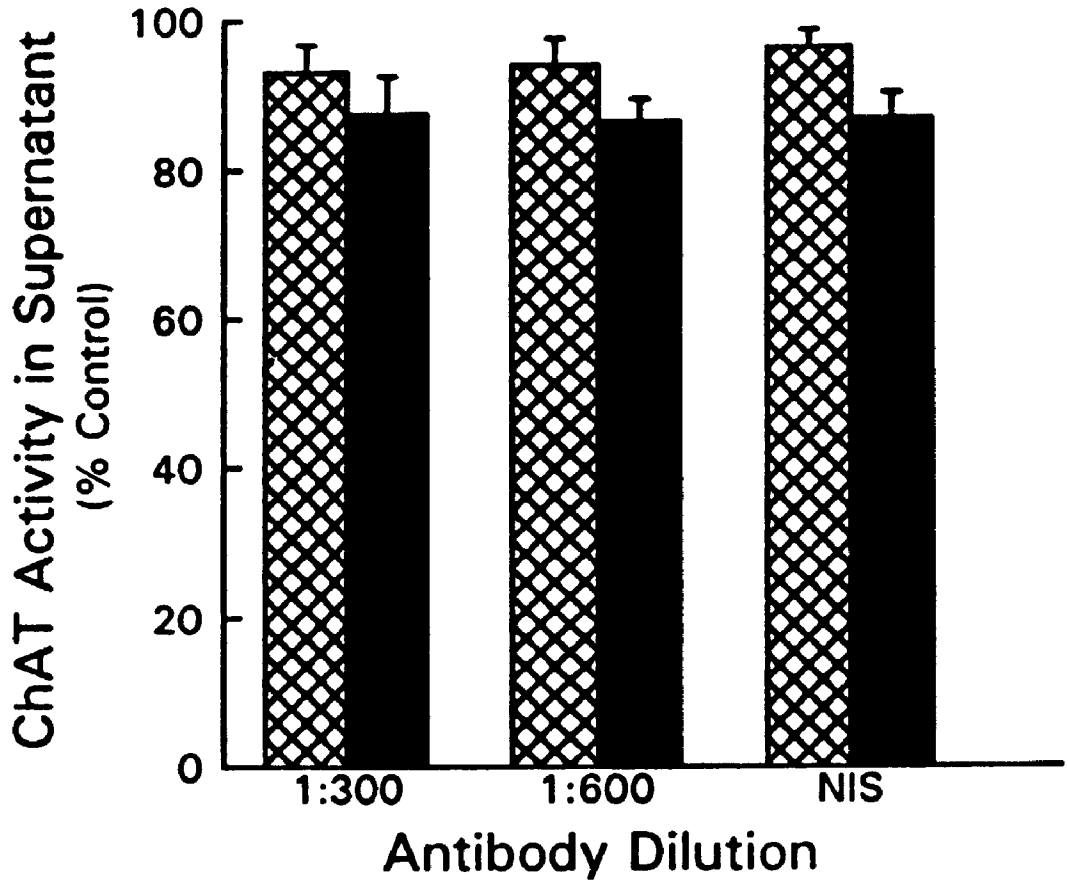


Figure 7. The effect of polyclonal rabbit anti-ChAT antibody on enzyme activity. Various dilutions of rabbit anti-ChAT antibody did not precipitate or inhibit ChAT activity in the absence of Pansorbin cells. Subcellular pools of cytosolic ChAT (▩) and membrane-bound ChAT (■) were isolated, precleared and gently mixed on a circular rotator at 4°C with various dilutions of polyclonal rabbit anti-ChAT antibody (1:300 and 1:600), non-immune rabbit serum (NIS; 1:600) or an equal volume of 100 mM sodium phosphate buffer, pH 7.4 (control) for 2 hours. Following centrifugation at 8,000 rpm for 10 min, enzyme activity remaining in the supernatant was determined. Data are mean ± SEM of 3 separate experiments, with duplicate determinations.

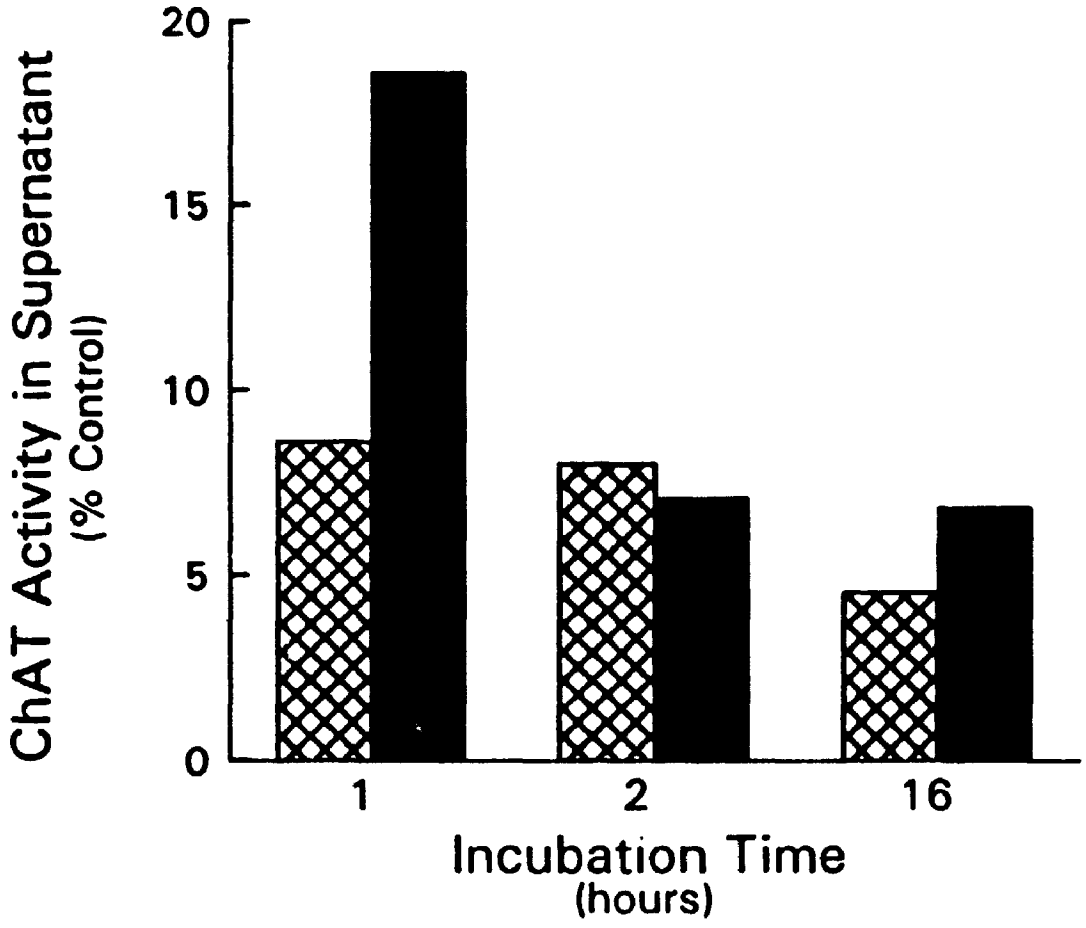


Figure 8. Immunoprecipitation of ChAT activity. Most ChAT activity was precipitated by a 2 hour incubation with rabbit anti-ChAT antibody and the addition of Pansorbin cells. Subcellular pools of cytosolic ChAT (▩) and membrane-bound ChAT (■) were isolated, precleared and gently mixed on a circular rotator at 4°C with 1:500 dilution of polyclonal rabbit anti-ChAT antibody or an equal volume of 100 mM sodium phosphate buffer, pH 7.4 (control) for 1, 2 or 16 hours. Prewashed Pansorbin cells (250 μl/ml sample of 10% cell suspension) were then added and the incubation continued for 45 min. Following centrifugation at 8,000 rpm for 10 min, enzyme activity remaining in the supernatant was determined. Data are the average of 2 experiments, with duplicate determinations.

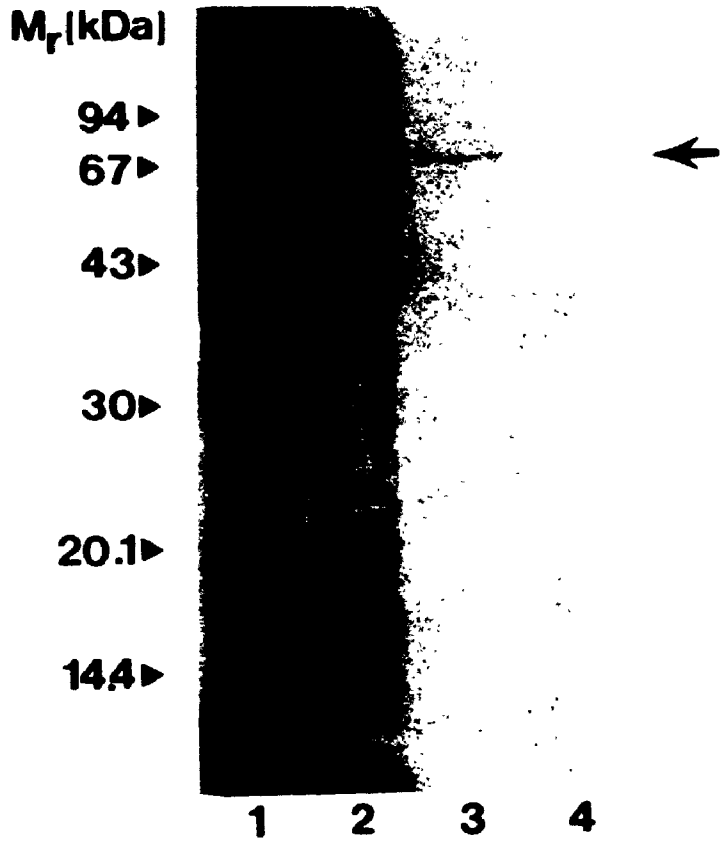


Figure 9. Autoradiograph of ^{32}P-labelled phosphorylated ChAT. Phosphorylated ChAT was isolated by immunoprecipitation from the cytosolic pool of rat hippocampal synaptosomes. The molecular weights (kDa) of known standards are shown at left. Lane 1: [$^{32}P_i$] incorporation into the total synaptosomal homogenate before immunoprecipitation. Lane 2: [$^{32}P_i$] incorporation into the cytosolic fraction following preclearing with Pansorbin cells. Lane 3: incubation of the precleared sample with anti-ChAT antibody isolated a phosphoprotein with the apparent molecular weight of 67,000 ± 730 Da. Lane 4: incubation of cytosolic ChAT with non-immune rabbit serum.

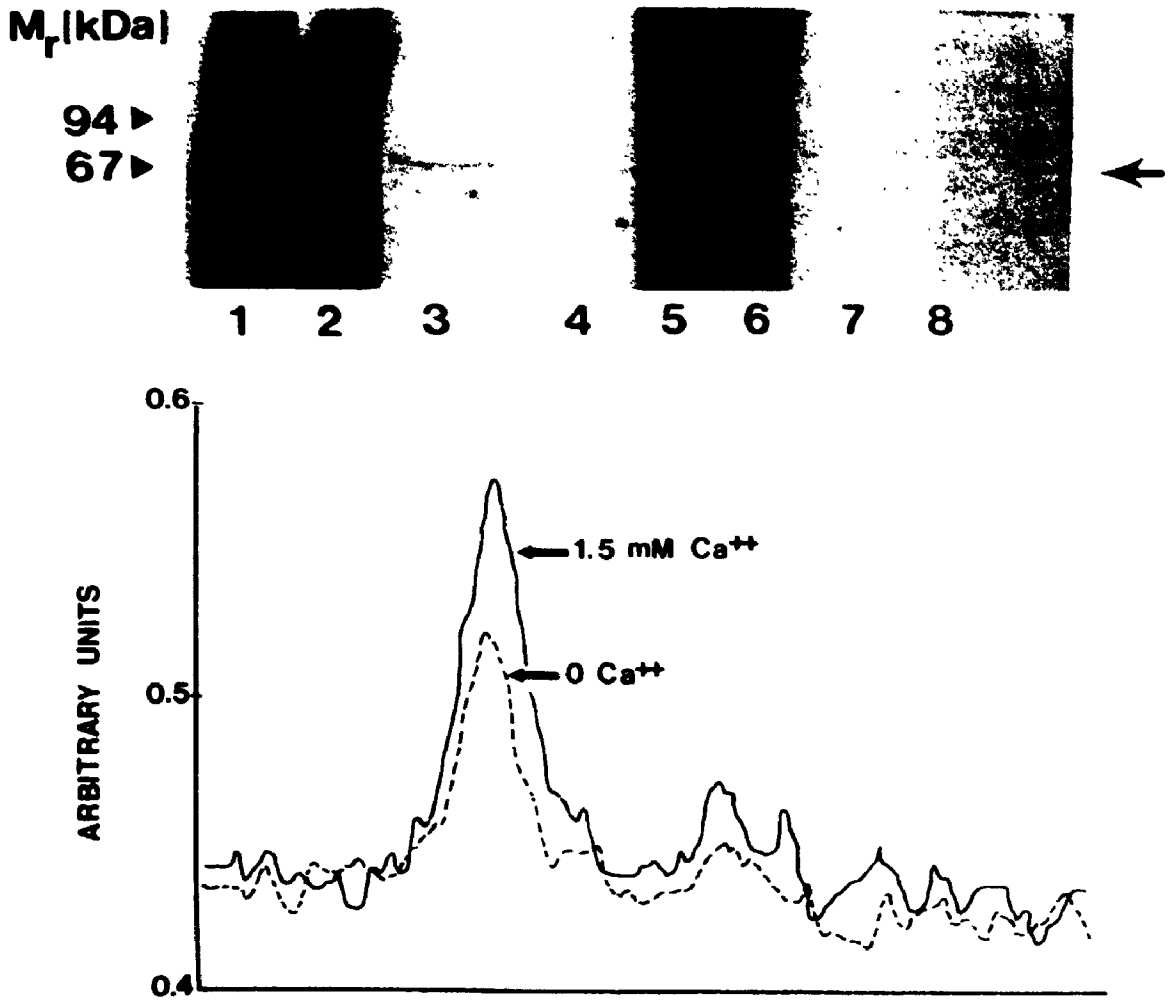


Figure 10. Calcium dependence of ChAT phosphorylation. The abundance of phosphorylated ChAT in rat hippocampal synaptosomes is dependent in part upon intracellular calcium levels. Upper: autoradiograph illustrating calcium dependence. The molecular weights (kDa) of known standards are shown at left. [$^{32}P_i$] incorporation into the cytosolic fraction following preclearing with Pansorbin cells is shown in the presence (Lane 1) and absence (Lane 2) of extracellular calcium. Incubation of the precleared cytosolic fraction with anti-ChAT antibody in the presence of calcium (Lane 3) isolated a phosphoprotein with greater phosphate incorporation than when extracellular calcium was absent from the media (Lane 4). [$^{32}P_i$] incorporation into the precleared detergent-solubilized membrane fraction is shown in the presence (Lane 5) and absence (Lane 6) of extracellular calcium. Incubation of the solubilized precleared membrane fraction with anti-ChAT antibody revealed no phosphoprotein in either the presence (Lane 7) or absence (Lane 8) of calcium. Lower: Representative densitometric scanning of phosphate incorporation into the cytosolic fraction following immunoprecipitation illustrating calcium-dependence. Similar results were obtained in three experiments.

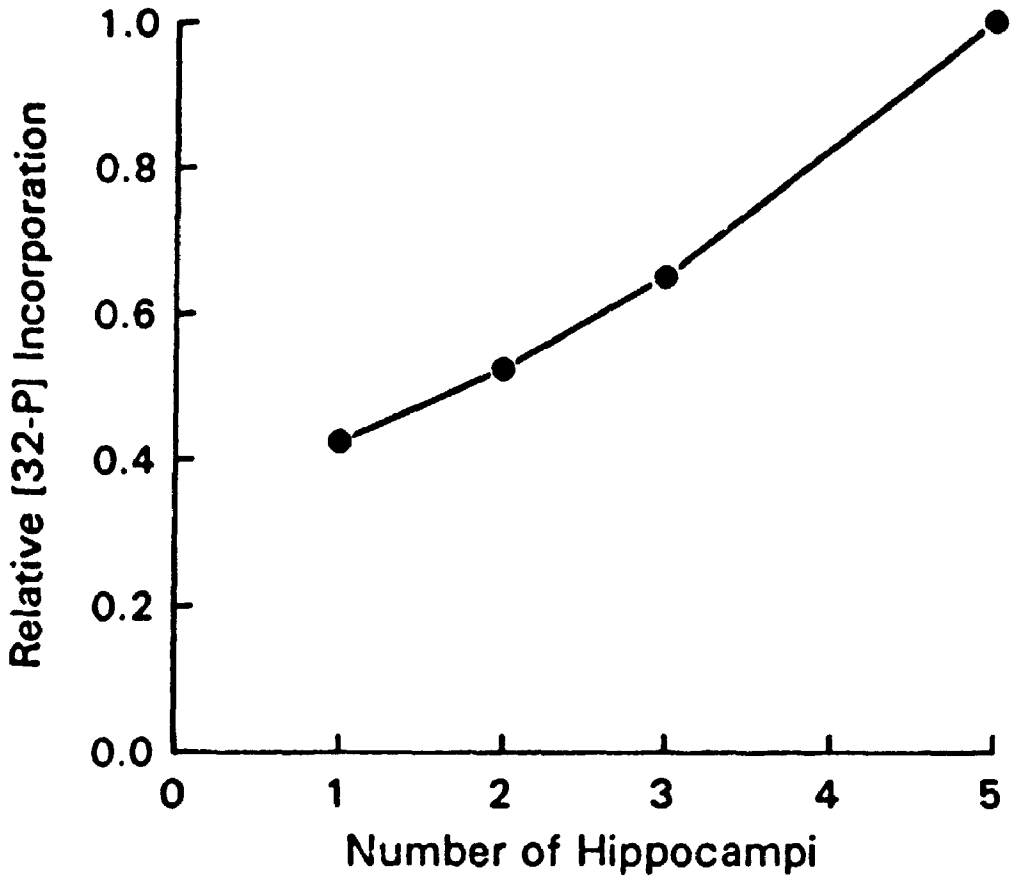


Figure 11. Detection sensitivity for ^{32}P-phosphate incorporation into ChAT. To assess the detection sensitivity of [$^{32}P_i$] incorporation by autoradiography, cytosolic ChAT was immunoprecipitated from 1, 2, 3 or 5 hippocampi. Relative [$^{32}P_i$] incorporation into cytosolic ChAT was then normalized to [$^{32}P_i$] incorporation into 5 hippocampi. Data are the average of 2 separate experiments.

4. DISCUSSION

The objectives of the present chapter were to examine modulation of phosphorylation of ChAT in intact rat hippocampal synaptosomes, and to study the relationship between phosphorylation of the protein and alterations in enzymatic activity. Using experimental protocols in which rat hippocampal synaptosomes were incubated under conditions which would allow protein phosphorylation, I determined that (1) a portion of cytosolic ChAT, with apparent molecular mass of 67,000 ± 730 daltons, was phosphorylated under resting conditions, (2) [^{32}P]phosphate did not appear to be incorporated into membrane-bound ChAT under these experimental conditions, (3) the abundance of [^{32}P]phosphate-labelled cytosolic ChAT, but not its enzymatic activity, was decreased when synaptosomal cytosolic calcium concentration was reduced, and (4) depolarization of synaptosomes with 50 μM veratridine did not appear to alter the phosphorylation pattern of either pool of enzyme, although membrane-bound ChAT specific activity was selectively increased.

Luine and coworkers (1984) proposed that ChAT may serve as a substrate for phophorylation by protein kinases to explain a small but consistent increase in activity of the enzyme observed following the addition of vasoactive intestinal peptide to rat brain hippocampal homogenates. Subsequently, Lapchak and Collier (1988) determined that activation of the enzyme and ACh synthesis in rat hippocampal slices and homogenates was not mediated by either protein kinases

A or C, but may be associated with another calcium-requiring mechanism. Elucidation of the primary sequence of the enzyme from porcine, rat and human ChAT cDNA revealed 6, 4 and 2 serine and threonine residues, respectively, which could serve as potential phosphorylation sites on the protein (Berrard *et al.*, 1987; Brice *et al.*, 1989, Oda *et al.*, 1992). Bruce and Hersh (1989) have since demonstrated that purified human placental ChAT can be phosphorylated *in vitro* at a single serine residue by two purified calcium-dependent kinases, Calcium-Calmodulin kinase II and to a lesser extent Protein Kinase C, which are present in rat brain. In addition, a phosphorylated form of ChAT has been isolated from rat forebrain synaptosomes by immunoaffinity purification (Bruce and Hersh, 1989), and from Sf9 cells (Habert *et al.*, 1992).

My findings confirmed those of Bruce and Hersh (1989) by demonstrating that ChAT can exist as a phosphoprotein in rat brain synaptosomes and extended their observation by showing that [^{32}P]-labelled ChAT was isolated only from the cytosolic pool, thereby suggesting differential regulation or physiological function of the two enzyme subfractions. The functional significance of a membrane-associated form of ChAT in the regulation of ACh synthesis has not been elucidated, although it has been suggested that membrane-bound ChAT may be closely associated with the sodium-coupled, high-affinity choline transporter and may be maximally activated during repetitive neuronal firing to meet the demand for synthesis of readily-releasable ACh (Barker *et al.*, 1978; Jope, 1979; Carroll and Aspry, 1980; Carroll, 1983). Indeed, Carroll (1987) demonstrated that neuronal

depolarization by veratridine selectively increased membrane-bound ChAT activity, with no change in cytosolic ChAT activity. I also demonstrated a similar depolarization-induced increase in membrane-bound ChAT activity, however, this did not appear to correlate with an altered phosphorylation state of the enzyme; [^{32}P]phosphate was not incorporated into membrane-bound ChAT and the amount of [^{32}P]-labelled cytosolic ChAT did not change following 5 min or 10 min depolarization of intact synaptosomes by 40 mM KCl and 50 μM veratridine. This suggests that, in the cholinergic system, phosphorylation of the neurotransmitter biosynthetic enzyme may not correlate directly with catalytic activity of the enzyme and demand for transmitter synthesis, as is the case in catecholaminergic neurons.

Similarly, examination of the calcium-dependence of [^{32}P]phosphate incorporation into cytosolic ChAT in the present study revealed that alterations in the abundance of [^{32}P]-labelled cytosolic ChAT did not correlate with changes in specific activity of the enzyme. Lowering intraterminal free calcium content by incubation of synaptosomes in nominally calcium-free medium decreased [^{32}P]phosphate incorporation into cytosolic ChAT to 43% of control, but cytosolic ChAT specific activity was unchanged. It is unlikely that the decreased phosphorylation of cytosolic ChAT in synaptosomes incubated in calcium-free medium was a consequence of decreased specific activity of synaptosomal [^{32}P]ATP, as it has been demonstrated in other studies that incubation of synaptosomes in calcium-free medium does not alter phosphorylation reactions or incorporation of [^{32}P]phosphate into high energy phosphate pools (Robinson *et al.*,

1987; Brammer and Weaver, 1989; Yip and Kelly, 1989). It would be of interest to determine the effect of this marked reduction of phosphorylation of cytosolic ChAT on synaptosomal ACh synthesis, but interpretation of this measurement would be complicated by the sustained and marked reduction in membrane-bound ChAT activity at lowered calcium levels. It was demonstrated in Chapter 2 that removal of calcium during the incubation does not alter total or cytosolic ChAT activities, but membrane-bound ChAT activity was significantly reduced to less than 60% of control under the same calcium-free conditions. From the present study, it is also interesting to note that synaptosomal cytosolic ChAT may be maximally phosphorylated under resting conditions. Whereas decreased intraterminal calcium levels resulted in reduced [^{32}P]phosphate-labelling of cytosolic ChAT, depolarizing conditions which are known to increase intracellular calcium content by calcium influx through voltage-gated calcium channels did not increase the level of [^{32}P]phosphate incorporation into cytosolic ChAT, or lead to detectable [^{32}P]phosphate labelling into membrane-bound ChAT. While it cannot be excluded that phosphorylation of cytosolic ChAT may be enhanced, or that membrane-bound ChAT may become phosphorylated, by other physiologically important stimuli, it does not appear that this occurs in response to neuronal depolarization.

Although no [^{32}P]phosphate incorporation into the membrane-bound enzyme fraction was detected, it is unknown to what extent, if any, membrane-bound ChAT may have already been phosphorylated. Indeed, if membrane-bound

ChAT was stably phosphorylated during the time frame of the experimental manipulations, or if the phosphoprotein was inaccessible to phosphatases with the rate of turnover of phosphate longer than the 45 minutes used to label synaptosomal energy pools with $^{32}P_i$, incorporation of [^{32}P]phosphate into this enzyme subfraction would not have occurred. It would also be of interest to determine what proportion of cytosolic ChAT exists as a phosphoprotein under resting conditions, to assess more completely the role of phosphorylation in regulation of enzyme activity. This is addressed in Section 6.2.

A functional role for phosphorylation of ChAT in cholinergic neurons is unclear. In addition to altering enzyme catalytic activity, protein phosphorylation has been shown to influence processing and turnover of proteins by serving as a signal for degradation or prevention of degradation by proteases. For example, activation of Mg(II)-ATP-dependent phosphoprotein phosphatase appears to result from a phosphorylation-induced conformational change in this protein which resists degradation by reverting only slowly back to the inactive state following dephosphorylation (Jurgensen *et al.*, 1983; DePaoli-Roach, 1984). Using a synthetic heptapeptide analogous to the endogenous phosphorylation site of pyruvate kinase, Benore-Parsons and coworkers (1989) demonstrated that phosphorylation could alter susceptibility of the enzyme to proteolytic cleavage by two proteases. However, phosphorylation-induced degradation of other isozymes of pyruvate kinase appears to occur from the breakdown of the enzyme to less active subunits (Bergstrom *et al.*, 1978; Eigenbrodt *et al.*, 1992).

Alternatively, phosphorylation could alter the subcellular compartmentation of an enzyme; Bruce and Hersh (1989) provided limited evidence that phosphorylated ChAT had a lower affinity than the native protein for binding to rat synaptosomal membranes. Phosphorylation-dependent regulation of subcellular translocation and compartmentation of proteins has been demonstrated in other systems. For example, Sanghera and Vance (1989) observed that phosphorylation of purified cytidylyltransferase appeared to reduce the activity of the enzyme in particulate fractions by decreasing its affinity for membranes, and more recently, Watkins and Kent (1991) determined that dephosphorylation of soluble cytidylyltransferase was required for translocation of the enzyme to the membrane in Chinese hamster ovary cells. The possibility that ChAT may be regulated by translocation between its subcellular pools is examined in Chapter 5.

The physiological significance of ChAT phosphorylation remains unresolved, but it may be involved in its short-term regulation within the cholinergic system. Additionally, the kinases mediating phosphorylation of ChAT within the cholinergic nerve terminal remain to be determined, although at least one calcium-dependent mechanism appears to be involved. Similarly, the phosphatases involved in regulating turnover of the phosphoprotein are unknown. In addition, the physiological stimuli which may alter the level of phosphorylation of the enzyme must be investigated. It appears from the present study that modulation of [^{32}P]phosphate incorporation into ChAT did not correlate with changes in catalytic activity of the enzyme, and that this was not regulated by depolarization, at least

in hippocampal synaptosomes.

The observation that membrane-bound ChAT does not appear to incorporate [^{32}P]phosphate under resting conditions provides further support for the possibility that each enzyme pool is subject to different short-term regulatory control. In the next chapter, I examine the physiological role of cytosolic ChAT and membrane-bound ChAT in basal ACh biosynthesis.

CHAPTER 4

BASAL SYNTHESIS OF ACh IN RAT HIPPOCAMPAL SYNAPTOSOMES IS NOT REGULATED BY MEMBRANE-BOUND ChAT

I. INTRODUCTION

The physiological role which membrane-bound ChAT may play in the regulation of ACh biosynthesis is poorly understood at present, although Carroll and coworkers have reported that, relative to cytosolic ChAT, membrane-bound ChAT has distinct biochemical and kinetic properties (Benishin and Carroll, 1983; Carroll and Benishin, 1984; Badamchian and Carroll, 1985). It has been proposed that membrane-bound ChAT may form part of a presynaptic membrane-bound complex which is functionally or structurally associated with the sodium-dependent, high-affinity choline carrier (Barker *et al.*, 1978; Jope, 1979).

Tissue ACh levels are maintained at relatively constant levels, with resynthesis of the neurotransmitter being tightly coupled to its release. Considerable experimental evidence indicates that there is a close association between the uptake of choline and its subsequent acetylation; most of the choline taken up into the nerve terminal by the sodium-coupled transport system is converted to ACh (Yamamura and Snyder, 1973). Furthermore, the amount of ACh synthesized is proportional to the amount of choline transported (Guyenet *et al.*, 1973), and disruption of high-affinity choline uptake into synaptosomes permeabilized with low concentrations of Triton X-100 blocked ACh synthesis in a manner which was not restored by addition of choline to the partially disrupted system (Lefresne *et al.*, 1975). The apparent obligatory nature of the relationship between the high-affinity choline transporter and ChAT has been demonstrated

most clearly by the acetylation of the choline analogue homocholine only following its translocation into nerve terminals, and not during incubation with homogenates enriched with ChAT activity (Collier *et al.*, 1977; Benishin and Carroll, 1981). This latter observation suggests that choline, or a choline analogue, may be directed towards a particular pool of the enzyme, perhaps a membrane-associated form, for acetylation subsequent to its uptake.

The objective of experiments reported in this chapter was to investigate the role of membrane-bound ChAT in ACh biosynthesis. Presently no specific blockers of either membrane-bound ChAT or cytosolic ChAT have been identified to inhibit only one form of the enzyme to test its role in ACh synthesis under different physiological conditions. However, it has been reported previously that synaptosomal membrane-bound ChAT was more sensitive to inhibition by choline mustard transported into nerve terminals by the high-affinity choline carrier, but cytosolic ChAT activity was also decreased to a small extent (Rylett, 1989). It has, therefore, been necessary to develop an alternative strategy to modulate the activity of membrane-bound ChAT without changing the activity of cytosolic ChAT or provision of the precursors, thereby allowing evaluation of the effects of increased or decreased membrane-bound ChAT activity on ACh synthesis.

Catalytic activity of the enzyme may be controlled, at least in part, by alterations in the intraterminal ionic composition arising during neuronal activity (Rossier *et al.*, 1977; Hersh and Peet, 1978). In particular, V_{max} for purified ChAT appears to be markedly increased at higher sodium and chloride concentrations

(Rossier *et al.*, 1977; Hersh and Peet, 1978). Docherty and Bradford (1988) determined that membrane-binding of ChAT to synaptosomal membranes appears to be inversely related to Cl^- concentration; they interpreted this to mean that chloride has a chaotropic, or disruptive action causing the release of membrane-bound ChAT from membranes.

Based upon these observations, a novel approach of changing intrasynaptosomal Cl^- ion concentration was developed to selectively alter membrane-bound ChAT specific activity, without changing cytosolic ChAT activity or choline uptake. Interestingly, it appeared that increased or decreased membrane-bound ChAT activity was not reflected as a change in basal ACh synthesis.

2. METHODS

2.1 Incubation of Synaptosomes

Synaptosomes were prepared as described in Chapter 2, Section 2.1. The crude synaptosomal pellet (P2) was suspended in 3 ml of oxygenated KR buffer, pH 7.4, containing various chloride concentrations and preincubated for 30 min at 37°C to allow full equilibration of chloride ions (Marchbanks, 1975; Marchbanks and Campbell, 1976). When the chloride concentration was changed (as shown in Results), an equimolar concentration of Na-Isethionate, NaBr or NaI was substituted for NaCl. In some experiments, the Cl^- channel blockers niflumic acid or SITS, or the $GABA_A$ agonist muscimol, were added to synaptosomes resuspended in regular KR buffer during the final 5 min of the incubation. Stock solutions of niflumic acid were prepared in absolute ethanol at a concentration of 50 mM, with the final concentration of ethanol in experimental samples being less than 0.5%. SITS and muscimol stock solutions were made in KR buffer and water, respectively.

Following the incubation step, an aliquot of synaptosomal suspension was removed for determination of high-affinity choline uptake and ACh synthesis activity. The remaining synaptosomes were centrifuged at 20,000 g for 5 min. The resulting pellet was washed twice with 0.32 M sucrose buffered with 5 mM Tris-HCl, pH 7.4, prior to the isolation of subcellular ChAT pools (Chapter 2, Section 2.2) and determination of ChAT activity (Chapter 2, Section 2.3). At least 90%

of "total" ChAT activity was recovered after subfractionation of synaptosomes in each experiment presented.

2.2 Determination of High-Affinity Choline Uptake

Following the incubation in KR buffer containing various Cl^- concentrations, an aliquot (1 ml) of synaptosomal suspension was removed for determination of high-affinity choline uptake activity as described in Chapter 2, Section 2.4 with some modifications. Synaptosomes were not resuspended in fresh KR buffer. Following a 4 min incubation with ^{3}H-choline, samples were centrifuged in a Savant microcentrifuge at 8,000 rpm for 5 min and pellets were washed twice with the appropriate fresh KR buffer (identical to the incubation buffer), then resuspended in 1 ml of 5% trichloroacetic acid (TCA). Samples were mixed by vortex and incubated on ice for 30 min before centrifugation in the microcentrifuge for 10 min at 8,000 rpm. Aliquots (50 μl) of the acidic supernatant were mixed with 5 ml scintillation cocktail for the determination of total ^{3}H-choline accumulation. Pellets were resuspended in 1 ml of 0.1 N NaOH overnight to allow digestion before determination of sample protein content. High-affinity choline uptake activity was determined as the difference in ^{3}H-choline accumulation at 37°C and 4°C, and expressed as pmol / mg protein / 4 min. Within each experiment, measurements were made in duplicate, and data was averaged to give a single n value.

2.3 Determination of Acetylcholine Synthesis

The remainder (800 μl) of the acid extract of synaptosomes was used for the determination of ^{3}H-ACh synthesis. TCA was removed from the aqueous solution by shaking vigorously three times with 4 volumes of water-saturated diethyl-ether. ^{3}H-Choline and ^{3}H-ACh were separated from other metabolites in the aqueous solution by liquid cation extraction as a salt with tetraphenylboron into 3-heptanone (10 mg/ml). ^{3}H-Choline and ^{3}H-ACh were extracted back into aqueous solution by shaking an aliquot of the organic phase with an equal volume of 0.4 N HCl. This aqueous phase, containing ^{3}H-choline and ^{3}H-ACh, was dried in a vacuum centrifuge.

To allow separation of ^{3}H-choline and ^{3}H-ACh, choline was phosphorylated using a modification of the radioenzymatic assay described by Goldberg and McCaman (1975). To the dried samples, 100 μl of choline kinase reaction mixture (comprised of choline kinase, 0.2 U/ml; ATP, 15.5 mM; dithiothreitol, 12 mM; and $MgCl_2$, 18.5 mM, in glycylglycine buffer, 93 mM, pH 8.3) was added and incubated at 37°C for 30 min. The reaction was stopped by the addition of 150 μl of sodium tetraphenylboron in 3-heptanone (10 mg/ml) and mixed by vortex to extract ^{3}H-ACh into the organic phase. Aliquots of the organic phase (^{3}H-ACh) and aqueous phase (^{3}H-choline as phosphorylcholine) were added to scintillation cocktail for quantification. ACh synthesis was determined as the difference in ACh produced at 37°C and 4°C, normalized to sample protein content and expressed as

pmol / mg protein / 4 min. Sample protein content was determined as outlined in Chapter 2, Section 2.7.

2.4 Materials

Choline iodide, choline kinase (from baker's yeast), crystalline bovine serum albumin, dithiothreitol, eserine sulphate, HEPES, isethionic acid (sodium salt), muscimol, niflumic acid, SITS, sodium ATP, sodium tetraphenylboron and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Missouri. Sodium bromide and sodium iodide were purchased from BDH Inc., Toronto, Ontario. 3-Heptanone was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. AcetylCoenzyme A, lithium salt was from Boehringer-Mannheim, Laval, Quebec. THAM (Tris buffer) was obtained from Fisher, Mississauga, Ontario. ^{3}H-Choline chloride (78 Ci/mmol) and ^{3}H-acetylCoenzyme A (3.7 Ci/mmol) were purchased from Amersham Radiochemical Corp., Oakville, Ontario.

3. RESULTS

3.1 Effects of Changing Extracellular Chloride Ion Concentration

Docherty and Bradford (1988) provided indirect evidence that variations in intrasynaptosomal Cl^- concentration may influence membrane-association of ChAT within the nerve terminal. In the present experiments, intact synaptosomes were equilibrated in media containing various concentrations of Cl^-, then subfractionated to examine the effect on subcellular distribution and activity of ChAT; intrasynaptosomal Cl^- concentration was lowered by replacement of extracellular NaCl with an equimolar concentration of sodium isethionate. Isethionate was chosen as the anion of substitution as it does not substantially alter cytosolic pH, it is essentially membrane impermeant, and it does not chelate calcium as some other anions do (see Ashford and Wann, 1983).

Incubation of synaptosomes in Cl^--depleted extracellular media resulted in a selective decrease in membrane-bound ChAT activity. As shown in Figure 12, membrane-bound ChAT activity was significantly decreased in a graded manner as the medium Cl^- concentration was decreased from 131 mM (control) to 90, 48 or 8 mM. It should be noted that changes in membrane-bound ChAT activity were maintained throughout the subfractionation procedure, since several hours elapse between the experimental manipulations and the isolation of each pool of ChAT prior to determination of enzymatic activity. Importantly, under these experimental conditions, cytosolic ChAT activity was not changed, with the specific

activity of the enzyme being 1093 ± 74, 1107 ± 94, 999 ± 40 and 1040 ± 53 nmoles ACh synthesized / mg protein / hour at Cl^- concentrations of 131, 90, 48 and 8 mM, respectively (n=4 experiments). The effect of reduced Cl^- concentrations on ACh biosynthesis was monitored in the same samples; high-affinity choline uptake was also measured to determine whether reduced extracellular Cl^- concentration altered accumulation of precursor. As illustrated in Figure 13, choline uptake and ACh biosynthesis were not changed compared to control by incubation of synaptosomes in medium containing 90 mM and 48 mM Cl^-, conditions where membrane-bound ChAT activity was significantly decreased to about 50% of control. Reduction of extracellular Cl^- to 8 mM, however, resulted in significant decreases in choline uptake and acetylation to about 40% of control, with membrane-bound ChAT being reduced to 15% of control.

3.2 Effects of Anion Substitution

To determine whether the cholinergic neuronal parameters measured, and membrane-bound ChAT in particular, have a specific requirement for Cl^-, NaCl in normal KR buffer was replaced by the sodium salts of I^-, Br^- and isethionate; 8 mM Cl^- remained in the extracellular solutions as potassium and calcium salts of chloride were not substituted. The effect of extracellular medium anion substitution on synaptosomal membrane-bound ChAT activity is displayed in Figure 14. In agreement with data in Figure 12, equilibration of nerve terminals in KR solution comprised of 124 mM Na isethionate and 8 mM Cl^- resulted in

over 80% reduction in membrane-bound ChAT activity. Br^- appeared to substitute completely, and I^- partially, for Cl^- with membrane-bound ChAT activity being 103 ± 8 and 60 ± 10% of the Cl^- controls, respectively. Synaptosomal cytosolic ChAT activity appeared to be unaffected by equilibration of nerve terminals in any of the Cl^- substituted KR solutions, with specific activities of 940 ± 128, 902 ± 115, 912 ± 95,and 809 ± 110 nmoles ACh synthesized / mg protein / hr in the presence of Cl^-, I^-, Br^- and isethionate, respectively (n=5 experiments).

Responses of synaptosomal high-affinity choline uptake and ACh synthesis to extracellular medium anion substitution paralleled that of membrane-bound ChAT activity, as shown in Figure 15. Rank orders for the anions were $Cl^- = Br^- > I^-$ > isethionate; choline accumulation and acetylation, as well as membrane-bound ChAT activity, were all reduced to approximately 60% of Cl^- controls in the I^--substituted samples, and choline uptake and ACh synthesis were reduced to about 40% of control, compared to a reduction to 15% of control for membrane-bound ChAT activity, in the isethionate-substituted samples.

3.3 Effects of Chloride Channel Blockers

Neurons are highly permeable to Cl^-, with the anion traversing the plasma membrane through a variety of channels and transport systems (Hille, 1992). Recently, a number of compounds, including the disulphonic stilbene derivatives SITS and DIDS and the nonsteroidal anti-inflammatory agent niflumic acid, have

been characterized as relatively broad spectrum blockers of anion channels and transporters in a number of cell types, including neurons (Greger, 1990).

Based upon the findings reported above, it was hypothesized that blockade of nerve terminal Cl^- channels would decrease intraterminal Cl^- concentration and result in decreased membrane-bound ChAT activity; this hypothesis was tested by the use of two inhibitors SITS and niflumic acid. Incubation of synaptosomes with the anion channel blocker niflumic acid caused a selective reduction of membrane-bound ChAT specific activity. As illustrated in Figure 16, at the concentrations of niflumic acid used (100 and 250 μM), high-affinity choline uptake activity and ACh synthesis were not different from control; cytosolic ChAT specific activity was not altered by incubation of nerve terminals with niflumic acid. Importantly, ACh synthesis appeared to parallel high-affinity choline uptake, and was not altered even though membrane-bound ChAT activity was significantly decreased to less than 30% of control.

Similar results were obtained when synaptosomes were incubated with SITS to block chloride channels and transporters. As shown in Figure 17, membrane-bound ChAT specific activity was decreased in a concentration-dependent manner by SITS over the concentration range 50 to 250 μM, with no change in high-affinity choline uptake and ACh synthesis. It is interesting to note that ACh synthesis was unaltered when membrane-bound ChAT activity was reduced to about 10% of control. Cytosolic ChAT activity was not changed relative to control at any of the concentrations of SITS used. High concentrations of this inhibitor,

up to 2 mM, abolished all membrane-bound ChAT activity, and lowered cytosolic ChAT activity, high-affinity choline uptake and ACh synthesis (data not shown).

3.4 Effects of Increased Cytosolic Chloride Concentration

As lowering the intraterminal Cl^- concentration resulted in selective inhibition of membrane-bound ChAT activity without altering choline accumulation and acetylation, it was reasoned that membrane-bound ChAT activity might be enhanced by elevation of synaptosomal cytosolic Cl^- concentration. Intrasynaptosomal Cl^- content was raised by incubating the synaptosomes with muscimol, an agonist of the $GABA_A$ receptor-operated chloride channels; it has been demonstrated that low concentrations of muscimol cause Cl^- influx into rat hippocampal synaptosomes without mediating release of ACh (Bonnano and Raiteri, 1987a, b). As predicted, membrane-bound ChAT specific activity was increased in synaptosomes incubated in low concentrations of muscimol; enzyme activity was 132 ± 9 ($P \leq 0.01$) and 140 ± 16% ($P = 0.065$) of control following treatment with 12.5 and 50 μM muscimol, respectively, as shown in Figure 18. Interestingly, the response to muscimol was biphasic with membrane-bound ChAT activity not different from control at higher concentrations of the agonist; membrane-bound ChAT activity in samples incubated with 50 μM muscimol was sigr.ificantly greater than samples incubated with 100 μM muscimol. The activity of synaptosomal cytosolic ChAT was not altered by exposure to any concentration

of muscimol (Figure 19). Similarly, high-affinity choline uptake activity and ACh synthesis were not altered by $GABA_A$ receptor activation, as illustrated in Figure 19.

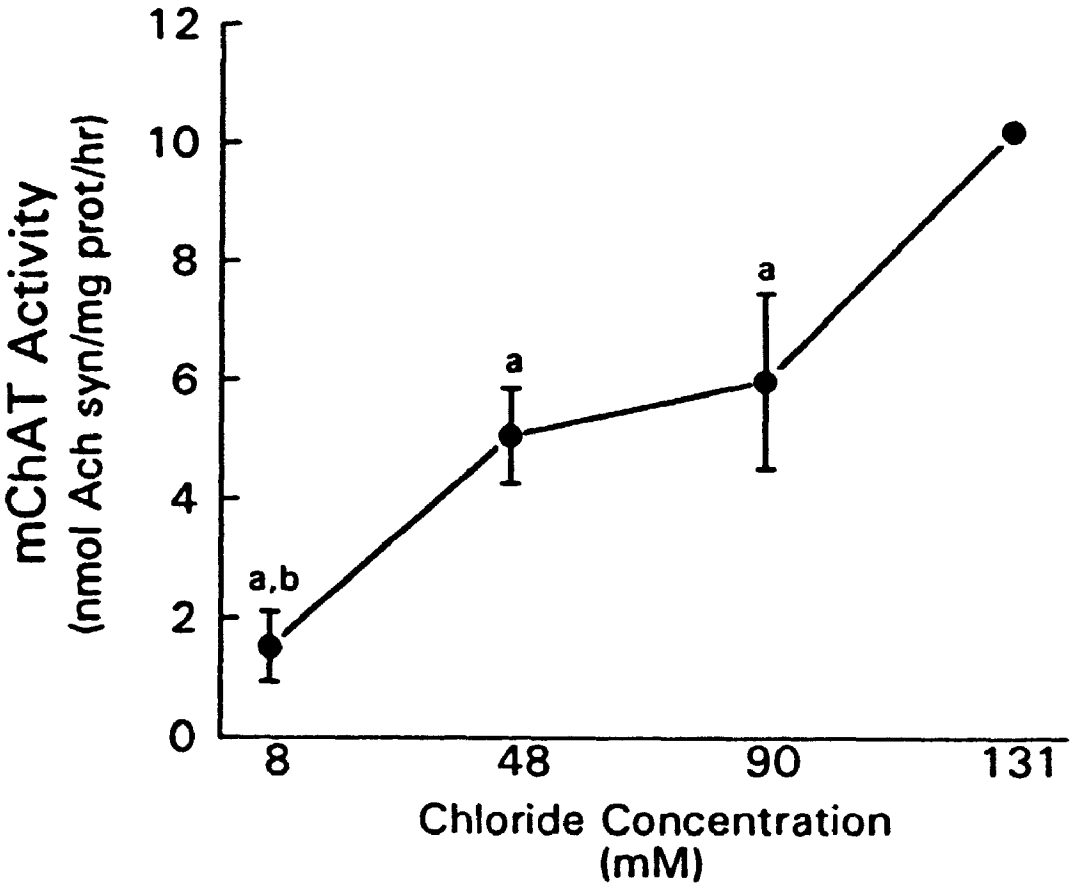


Figure 12. Effect of extracellular chloride ion concentration on synaptosomal ChAT activity. Synaptosomal membrane-bound ChAT activity decreased in a graded manner with decreased extracellular chloride ion concentration. Intact hippocampal synaptosomes were incubated for 30 min at 37°C in Krebs-Ringer buffer in which various concentrations of NaCl were replaced with equimolar concentrations of sodium isethionate to change intraterminal chloride levels, then subcellular fractions of ChAT were isolated. Whereas cytosolic ChAT activity was not altered in this experimental paradigm, membrane-bound ChAT activity was significantly decreased at 8, 48 and 90 mM Cl^- concentrations relative to control (131 mM Cl^-); *a* denotes significantly different from 131 mM Cl^- at $P \leq 0.01$, *b* denotes significantly different from 48 and 90 mM Cl^- at $P \leq 0.05$. Data is the mean ± S.E.M. of 4 separate experiments, with duplicate determinations.

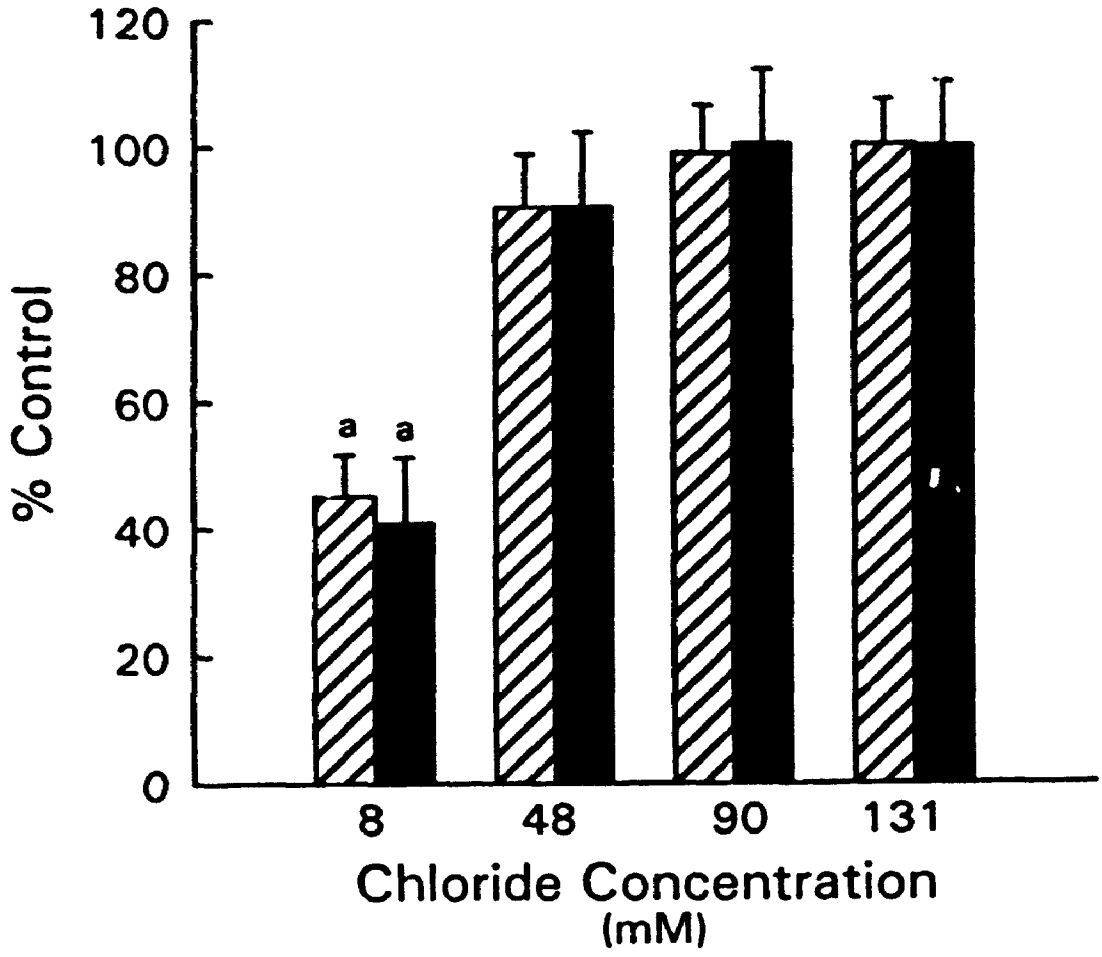


Figure 13. Effect of extracellular chloride ion concentration on cholinergic nerve terminal functional parameters. Synaptosomal high-affinity choline uptake and ACh synthesis were decreased only at low extracellular chloride ion concentrations. Following the experimental treatment described in Figure 13, an aliquot of synaptosomal suspension was removed for determination of choline uptake (▨) and acetylation (■). Choline uptake activity and ACh synthesis were maintained except at the lowest Cl^- concentration tested (8 mM); *a* denotes significantly different from 48, 90 and 131 mM Cl^- at $P \leq 0.01$. Control values for high-affinity choline uptake and ACh synthesis were 20.8 ± 1.5 pmol/mg protein/4 min and 8.2 ± 0.8 pmol ACh syn/mg protein/4 min, respectively. Data are the mean ± SEM of 4 separate experiments, with duplicate determinations.

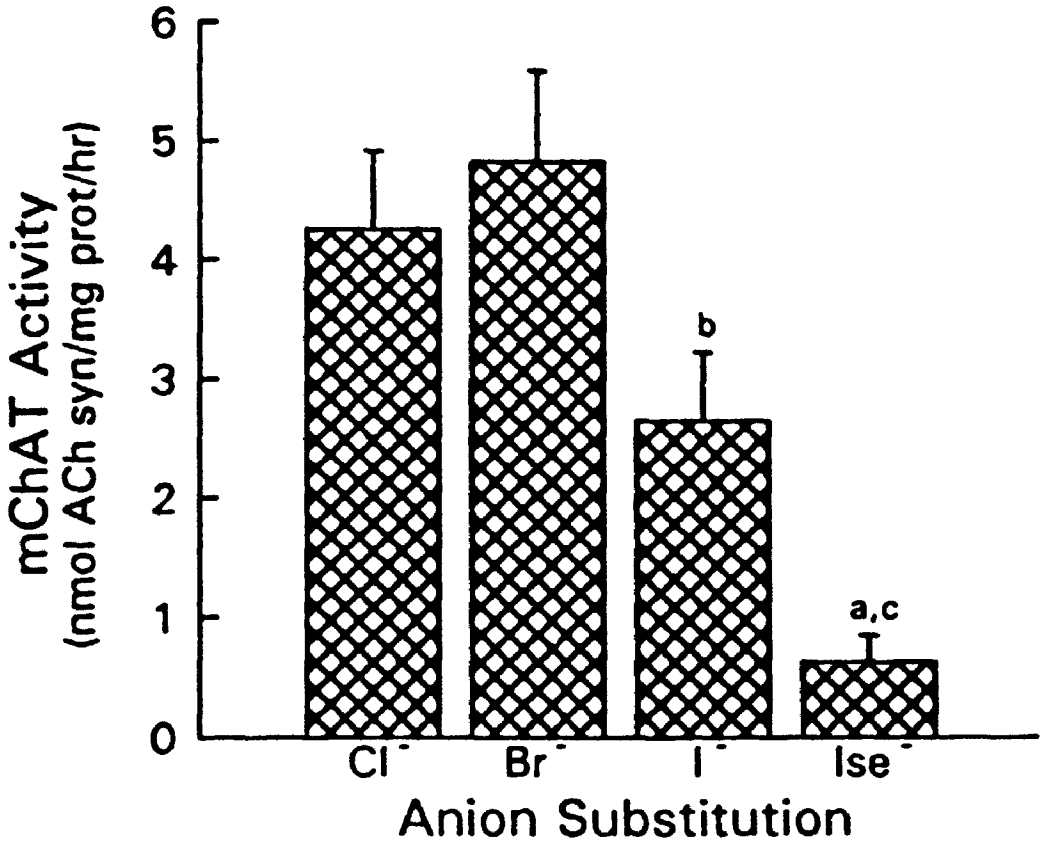


Figure 14. Extracellular chloride ion replacement and synaptosomal ChAT activity. Membrane-bound ChAT (mChAT) activity did not appear to have a specific requirement for Cl^-, as Br^- substituted fully to maintain enzyme activity. Synaptosomes were incubated for 30 min at 37°C in Krebs-Ringer buffer containing the sodium salt of Br^-, I^-, or isethionate (Ise^-) (124 mM) in place of NaCl, then subcellular fractions of ChAT were isolated. Although the activity of cytosolic ChAT was unaltered relative to control by anion substitution, synaptosomal membrane-bound ChAT activity was sensitive to medium anion composition with the rank order of anions for maintenance of membrane-bound ChAT activity being $Cl^- \approx Br^- > I^- > Ise^-$; *a* denotes significantly different from Cl^- and Br^- at $P \leq 0.01$, *b* denotes significantly different from Cl^- and Br^- at $P \leq 0.05$, and *c* denotes significantly different from I^- at $P \leq 0.05$. Data are the mean ± SEM of 5 separate experiments, with duplicate determinations.

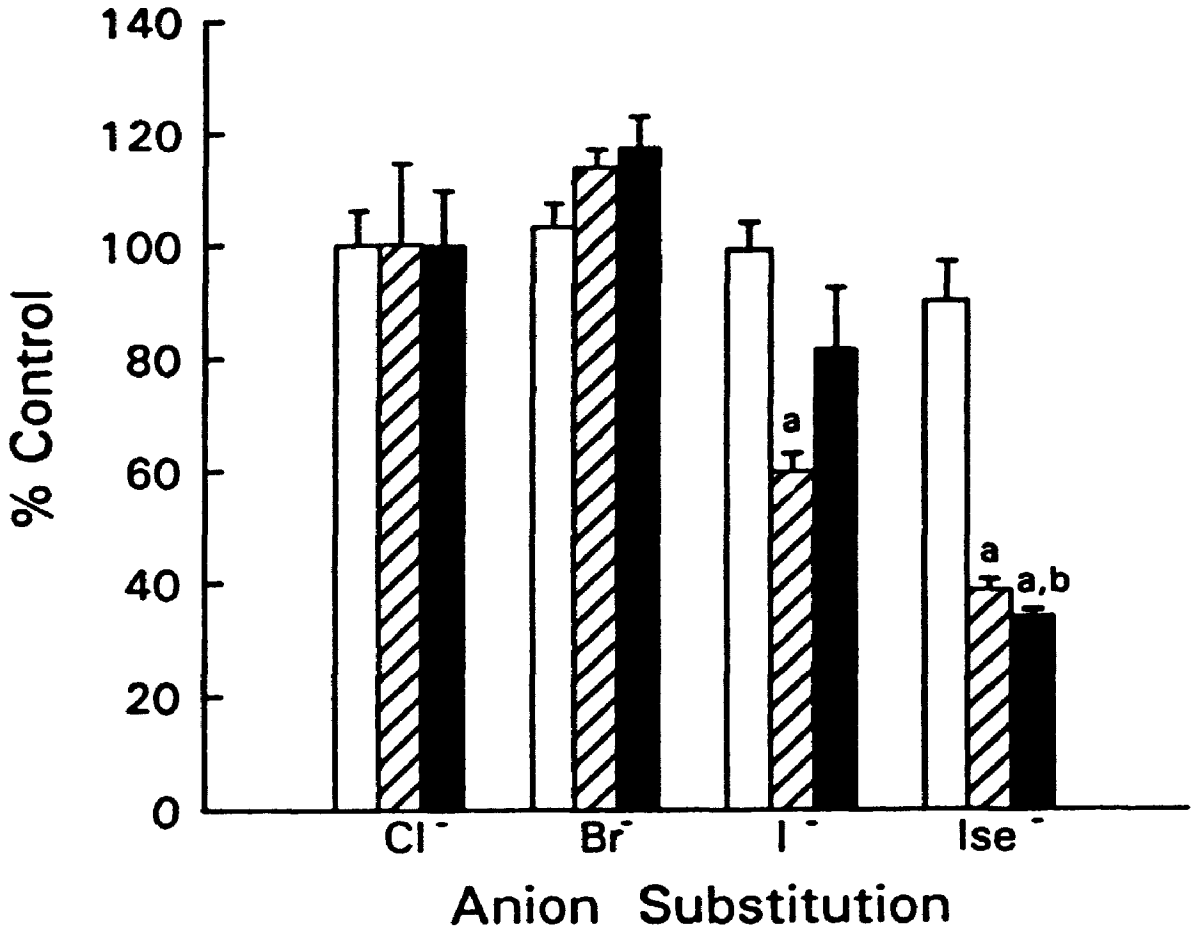


Figure 15. Extracellular chloride ion replacement and cholinergic neuronal functional parameters. The effects of extracellular anion substitution on high-affinity choline uptake activity and ACh synthesis paralleled the effects on membrane-bound ChAT activity. Following the experimental treatment described in Figure 15, an aliquot of synaptosomal suspension was removed for determination of cytosolic ChAT activity (□), choline uptake (▨) and acetylation (■); *a* denotes significantly different from Cl^- and Br^- at $P \leq 0.01$, and *b* denotes significantly different from I^- at $P \leq 0.01$. Control values for cytosolic ChAT activity, high-affinity choline uptake and ACh synthesis were 818.7 ± 44.5 nmol ACh syn/mg protein/hour, 25.5 ± 3.4 pmol/mg protein/4 min and 9.3 ± 0.8 pmol ACh syn/mg protein/4 min, respectively. Data are mean ± SEM of 5 separate experiments, with duplicate determinations.

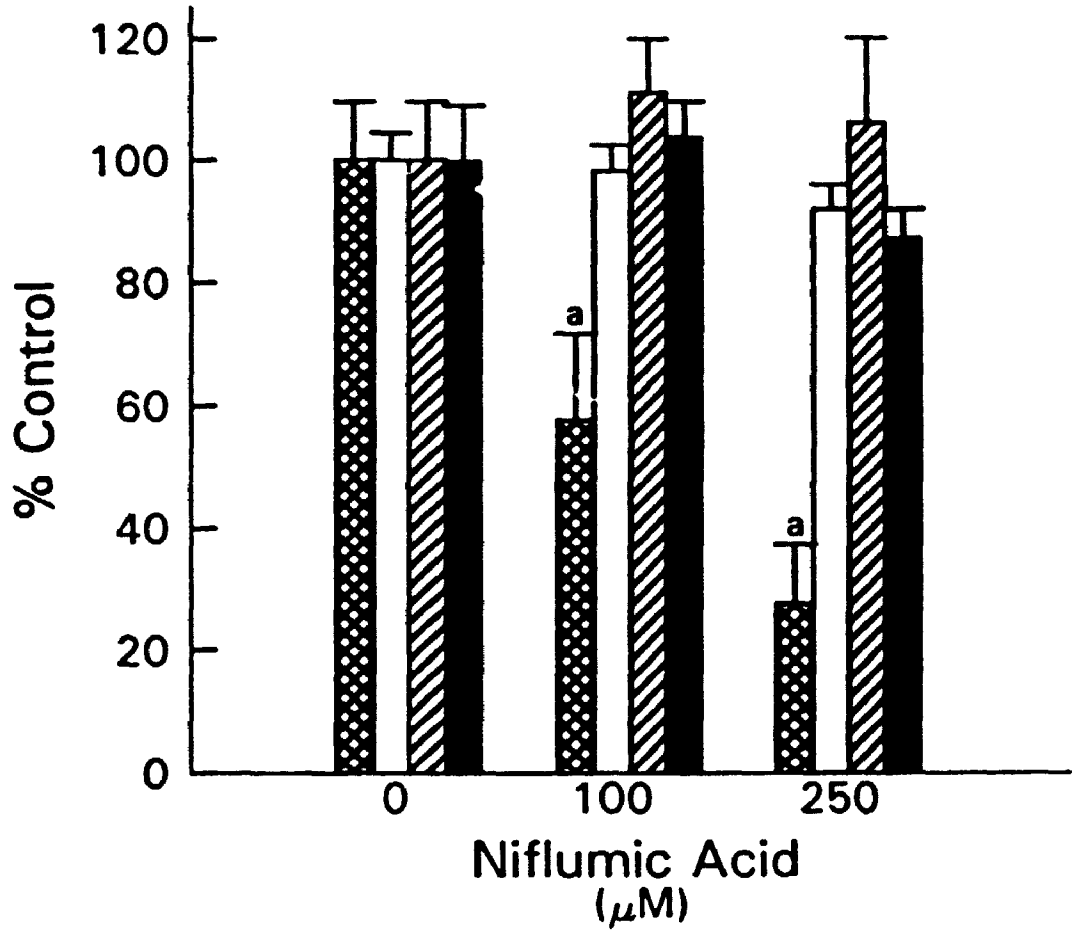


Figure 16. Cholinergic functional parameters following incubation of nerve terminals with niflumic acid. Membrane-bound ChAT activity was selectively reduced following exposure to the Cl^- channel blocker niflumic acid. Synaptosomes were incubated in regular Krebs-Ringer buffer (131 mM Cl^-) for 30 min at 37°C, with the addition of niflumic acid during the final 5 minutes. Cytosolic ChAT activity (□), high-affinity choline uptake (▨) and ACh synthesis (■) were not changed relative to control, whereas membrane-bound ChAT activity (■) was significantly decreased; *a* denotes significantly different from control at $P \leq 0.01$. Control values for membrane-bound ChAT activity, cytosolic ChAT activity, high-affinity choline uptake and ACh synthesis were 4.1 ± 0.4 nmol ACh syn/mg protein/hour, 674.8 ± 30.2 nmol ACh syn/mg protein/hour, 32.2 ± 3.1 pmol/mg protein/4 min and 9.2 ± 1.4 pmol ACh syn/mg protein/4 min, respectively. Data are mean ± SEM of 4 separate experiments, with duplicate determinations.

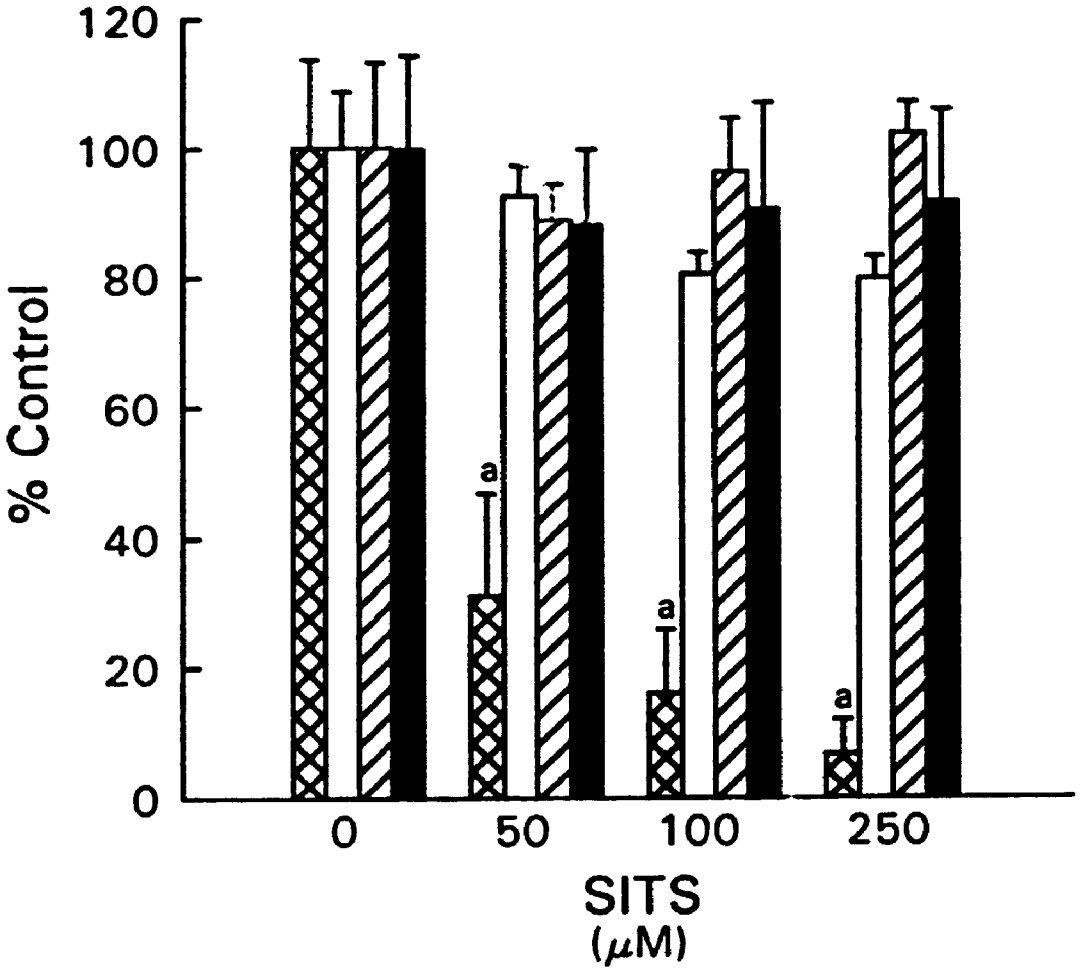


Figure 17. Cholinergic functional parameters following incubation of nerve terminals with SITS. Membrane-bound ChAT activity was selectively decreased following incubation of hippocampal synaptosomes with the Cl^- channel blocker SITS. Synaptosomes were incubated in regular Krebs-Ringer buffer (131 mM Cl^-) for 30 min at 37°C, with the addition of SITS during the final 5 min. Cytosolic ChAT activity (□), high-affinity choline uptake (▨) and ACh synthesis (■) were not changed relative to control, whereas membrane-bound ChAT activity (■) was significantly decreased; *a* denotes significantly different from control at $P \leq 0.01$. Control values for membrane-bound ChAT activity, cytosolic ChAT activity, high-affinity choline uptake and ACh synthesis were 2.7 ± 0.4 nmol ACh syn/mg protein/hour, 732.6 ± 68.8 nmol ACh syn/mg protein/hour, 42.9 ± 5.7 pmol/mg protein/4 min and 9.9 ± 1.4 pmol ACh syn/mg protein/4 min, respectively. Data are mean ± SEM of 6 separate experiments, with duplicate determinations.

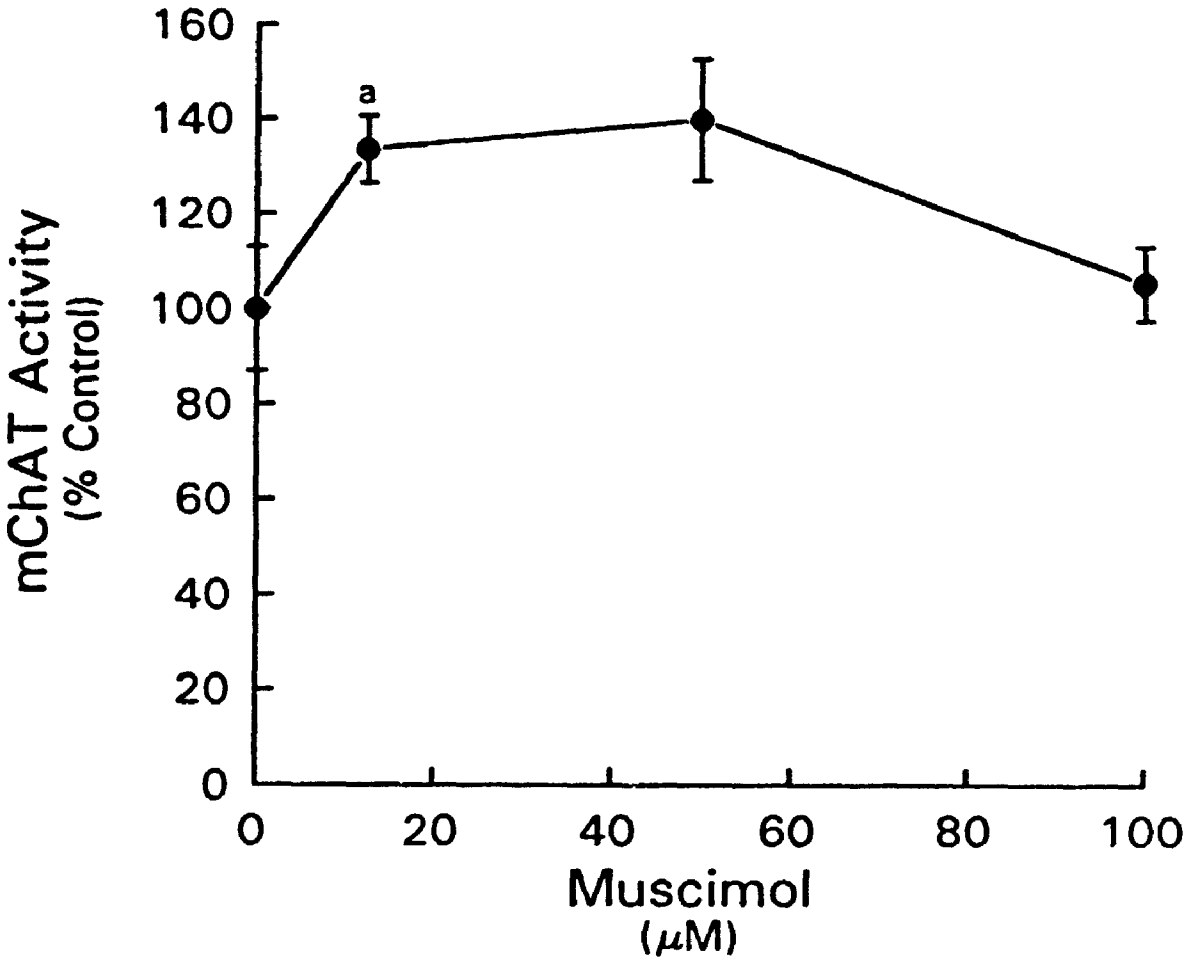


Figure 18. Membrane-bound ChAT activity following incubation of nerve terminals with muscimol. Low concentrations of the $GABA_A$ agonist muscimol selectively enhanced the activity of membrane-bound ChAT. Synaptosomes were incubated for 5 min at 37°C with various concentrations of muscimol, then subcellular fractions of ChAT were isolated. Membrane-bound ChAT (mChAT) activity in synaptosomes incubated with 50 μM muscimol was increased relative to control, with a statistical significance of $P = 0.065$. The effect of muscimol was biphasic, with membrane-bound ChAT activity in samples incubated with higher concentrations of the agonist being the same as control. *a* denotes significantly different from control at $P \leq 0.05$. The control value for membrane-bound ChAT activity was 2.6 ± 0.3 nmol ACh syn/mg protein/hour. Data is mean ± SEM of 3 separate experiments, with duplicate determinations.

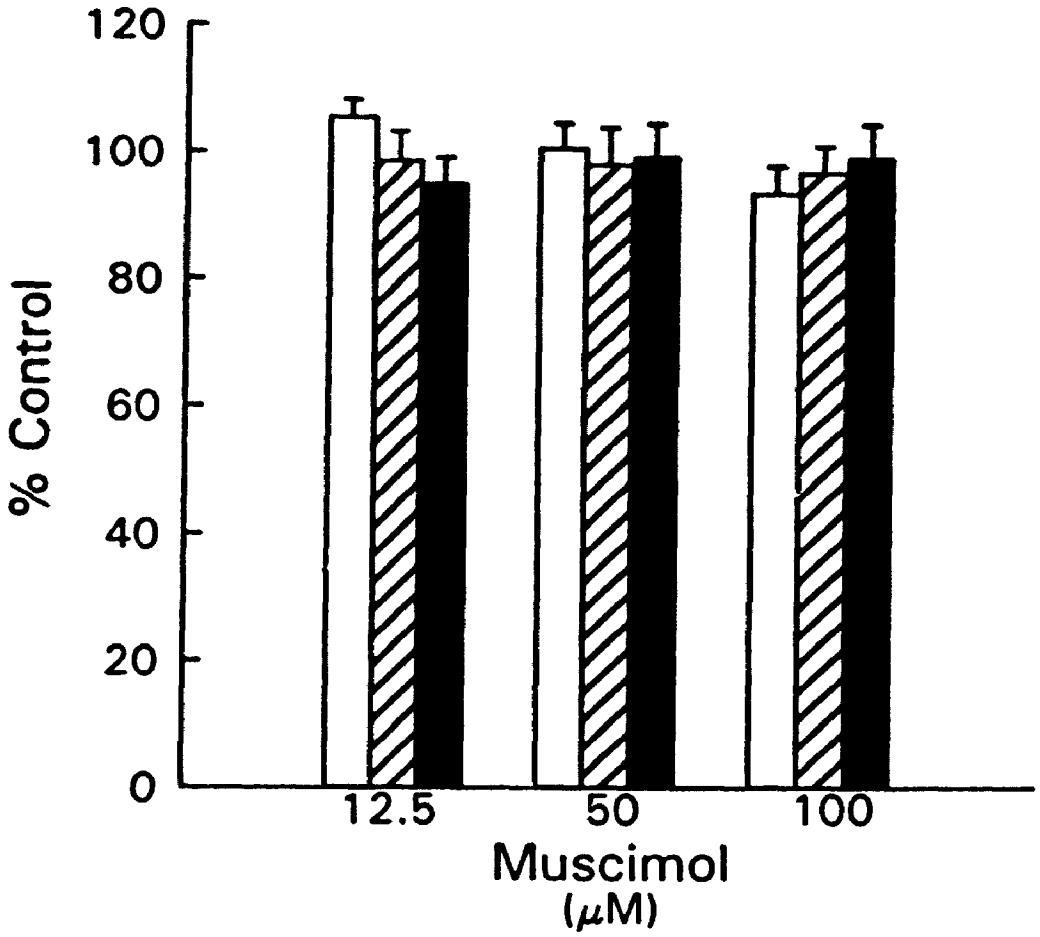


Figure 19. Cholinergic functional parameters following incubation of nerve terminals with muscimol. Cytosolic ChAT activity, high-affinity choline uptake and ACh synthesis were unchanged relative to control in synaptosomes incubated with the $GABA_A$ receptor agonist muscimol. Following the experimental treatment described in Figure 19, an aliquot of synaptosomal suspension was removed for determination of cytosolic ChAT activity (□), high-affinity choline uptake (▨) and acetylation (■). Control values for cytosolic ChAT activity, high-affinity choline uptake and ACh synthesis were 628.9 ± 74.3 nmol ACh syn/mg protein/hour, 27.1 ± 4.5 pmol/mg protein/4 min and 8.2 ± 1.4 pmol ACh syn/mg protein/4 min, respectively. Data are mean ± SEM of 3 separate experiments, with duplicate determinations.

4. DISCUSSION

Characterization of a membrane-associated pool of ChAT with distinct biochemical and kinetic properties (Benishin and Carroll, 1983; Carroll and Benishin, 1984; Badamchian and Carroll, 1985; Eder-Colli *et al.*, 1986) has led to suggestions that this pool of enzyme might be of regulatory importance in cholinergic neurotransmission (Salvaterra and Vaughn, 1989). As ChAT is present in kinetic excess within cholinergic nerve terminals, it is generally not considered to be rate-limiting in ACh synthesis, hence short-term regulation of this enzyme has not been examined extensively. The physiological role of membrane-bound ChAT in ACh synthesis is controversial, and has proven difficult to examine experimentally. In the present experiments, I developed an experimental strategy based upon findings that ChAT activity is sensitive to Cl^- concentration. I observed that: (1) membrane-bound ChAT specific activity was selectively decreased when intrasynaptosomal Cl^- concentration was lowered by incubation of nerve terminals in Cl^--depleted buffers and with Cl^- channel / transporter blockers SITS and niflumic acid, (2) cytosolic ChAT specific activity was unaffected by decreased intraterminal Cl^- concentration, (3) membrane-bound ChAT specific activity was selectively increased when Cl^- influx into synaptosomes was stimulated by the $GABA_A$ receptor agonist muscimol, and (4) basal ACh synthesis was not changed when membrane-bound ChAT activity was either increased to 140% of control, or decreased to about 10% of control.

Multiple steps in the cholinergic neurotransmission process appear to exhibit Cl^--dependence. First, high-affinity choline uptake into rat brain synaptosomes has a requirement for Cl^- (Simon and Kuhar, 1976; Kuhar and Zarbin, 1978). The requirement for Cl^- does not appear to be absolute; Br^-, the crystal radius of which is closest to Cl^- (Edwards, 1982), appeared to substitute for Cl^-, and be better than either I^- or isethionate ions in maintaining choline accumulation (Figure 14), giving a rank order of $Cl^- = Br^- > I^-$ > isethionate. These findings are in general agreement with those of Kuhar and Zarbin (1978). The nature of the requirement of a number of transporters, including choline, for Cl^- is unclear, although it has been proposed that Cl^- may act at a specific site on the transport protein to alter solute binding kinetics (Kuhar and Zarbin, 1978). Direct binding of Cl^- to the transporter may be involved in the translocation of choline into rat brain synaptosomes (Kuhar and Zarbin, 1978), GABA into nerve terminals (Radian *et al.*, 1986) and serotonin into porcine platelets (Nelson and Rudnick, 1982). It seems unlikely, however, that electrogenic Cl^- flux instead is required to create a membrane potential (interior negative), or to prevent accumulation of internal positive charge as permeant anions such as nitrate (Kuhar and Zarbin, 1978; Ducis and Whittaker, 1985) do not substitute for Cl^-, and as shown in the present study, neither SITS nor niflumic acid (Figures 15 and 16) had an effect on choline uptake at concentrations commonly used to block Cl^- channels (White and Aylwin, 1990).

Second, ChAT in brain homogenates (Hebb *et al.*, 1972) and purified from placenta (Hersh and Peet, 1978) appears to be activated by elevated concentrations of salt, and there is some evidence to suggest that catalytic activity of this enzyme may be increased specifically by Cl^- (Rossier *et al.*, 1977). Whereas the effect of extracellular medium anion composition on ACh synthesis and release has been examined (Ksiezak-Reding and Goldberg, 1982), the effect on nerve terminal ChAT activity *in situ* was not measured. Docherty and Bradford (1988) investigated the effect of Cl^- concentration on the solubilization and distribution of synaptosomal membrane-bound ChAT between hydrophilic and hydrophobic phases of Triton X-114, and observed an inverse relationship between the amount of membrane-bound (hydrophobic) ChAT and medium Cl^- concentration. Their use of isolated synaptosomal membranes allowed exposure of membrane-bound ChAT to varying concentrations of Cl^-, but did not permit simultaneous determination of ACh synthesis activity. They also determined that with decreased Cl^- concentrations there was increased susceptibility of cholinergic synaptosomes to immunolysis by anti-ChAT antibodies in the presence of complement, suggesting an enlarged pool of membrane-bound ChAT.

Observations made in this chapter that manipulations designed to increase or decrease neuronal Cl^- concentration caused parallel, long-lasting changes in membrane-bound ChAT activity disagree with the findings of Docherty and Bradford (1988), and suggest alternative interpretations. Based upon the data of those investigators, I predicted initially that incubation of synaptosomes in media

that should lower intraterminal Cl^- concentration would lead to an increase in activity of membrane-bound ChAT since more enzyme protein would partition into the lipid bilayer. Indeed, the opposite result was observed, suggesting that changes in membrane-bound ChAT activity can not be explained by a chaotropic action of Cl^- modifying binding of the protein to the membrane. However, as the changes in membrane-bound ChAT activity endured the subcellular fractionation process, it is probable that this represents variations in the amount of membrane-bound enzyme protein; alternatively, changes in membrane-bound ChAT activity could be due to persistent modifications in enzyme kinetics.

The mechanisms by which membrane-bound ChAT activity was changed by alterations in intraterminal Cl^- homeostasis are unclear, and remain to be elucidated in further studies. For example, the mechanism of action of the anion channel blockers, SITS and niflumic acid, upon membrane-bound ChAT activity was not apparent. Since synaptosomes have a large surface area to volume ratio, it was reasoned that blockade of anion channels would lead to a reduction in the intracellular chloride ion concentration, however this was parameter not measured directly. It is clear that various treatments which are believed to alter cytosolic Cl^- levels resulted in dissociation of cholinergic neurochemical parameters and selective modulation of membrane-bound ChAT activity, thus permitting the first direct evaluation of the role of membrane-bound ChAT in basal ACh synthesis. While it has been proposed that the high-affinity choline carrier is directly coupled to a membrane-associated pool of ChAT to coordinate efficient acetylation of

translocated choline (Simon and Kuhar, 1976), the present data do not appear to support this model; if the uptake of a substantial portion of choline was directly coupled to acetylation by membrane-bound ChAT under resting conditions, then ACh synthesis would have been expected to parallel the alterations in membrane-bound ChAT activity. As the uptake and subsequent acetylation of choline are known to be tightly coupled to ACh release from cholinergic nerve terminals, it was of concern that changes in membrane-bound ChAT activity simply reflected changes in release of the transmitter. For example, Carroll (1987) found that depolarization of isolated nerve terminals increased the release of ACh and was accompanied by the selective enhancement of membrane-bound ChAT activity. In the present study, changes in membrane-bound ChAT activity which followed extracellular Cl^- depletion or incubation with muscimol did not appear to be associated with changes in ACh release since this would have altered choline uptake, and neither high-affinity choline uptake nor ACh synthesis were changed under conditions where membrane-bound ChAT activity was either increased or decreased.

Therefore, based upon the protocol developed in the present series of experiments, membrane-bound ChAT did not appear to play a significant role in the regulation of basal ACh synthesis in hippocampal synaptosomes prepared from adult rat. It remains to be determined, however, whether this pool of enzyme performs a primary function in the increased demand for synthesis of neurotransmitter during repetitive neuronal firing. This question is difficult to

address experimentally since depolarization-evoked neurotransmitter release results in simultaneous activation of high-affinity choline uptake, ACh synthesis and membrane-bound ChAT activity. To date, it has not been possible to dissociate the contribution which increased membrane-bound ChAT activity may make to enhanced ACh synthesis from the increased provision of precursor on the sodium-coupled choline carrier following depolarization. It seems likely that it is under this condition that functional coupling of the high-affinity choline transporter and membrane-bound ChAT would be maximal. Alternatively, this enzyme pool may be of developmental importance as it has been shown that in the developing mouse brain membrane-bound ChAT activity is low until postpartum days 15-20 when its activity increases about 4-fold then gradually decreases to a lower level found in adult brain (Benishin, 1984). Similar developmental changes in the subcellular distribution of glutamic acid decarboxylase also occurs in GABAergic neurons (Greif *et al.*, 1991).

Throughout this dissertation, it has become increasingly evident that several different experimental manipulations result in selective changes in membrane-bound ChAT activity with no apparent change in the activity of the larger pool of cytosolic ChAT. Possible mechanisms responsible for short-term regulation are examined in the next chapter.

CHAPTER 5

ROLE OF CALCIUM IN REGULATION OF ChAT ACTIVITY

1. INTRODUCTION

ChAT appears to be predominantly a cytosolic protein in the nerve terminal at physiologic pH and ionic strength, but according to Carroll and coworkers, this cytosolic pool of enzyme may be further subdivided into water-soluble and salt-soluble (ionically-bound) ChAT (Smith and Carroll, 1980; Benishin and Carroll, 1983; Badamchian *et al.*, 1986). It is presently unknown whether these enzyme pools play distinct regulatory roles in ACh biosynthesis. In addition, approximately 10-20% of the total enzyme appears to be associated non-ionically with synaptic membranes as discussed previously; several studies have proposed that membrane-bound ChAT may be an integral membrane protein as it is only solubilized efficiently in the presence of non-ionic detergents (Docherty and Bradford, 1988; Eder-Colli *et al.*, 1992).

Findings by our laboratory and others that the specific activity of membrane-bound ChAT can be altered selectively and acutely by changes in the extracellular, and consequently intracellular, composition of such physiologically important ions as K^+, Cl^-, and Ca^{2+} suggest that each pool of enzyme may have regulatory importance in neurotransmitter synthesis (Benishin and Carroll, 1983; Carroll, 1987; Chapters 2, 3, 4 of this thesis). Short-term regulation of enzyme activity may arise by various mechanisms such as post-translational modification by protein phosphorylation, altered substrate availability, product inhibition, or by changes in the proteolysis or spatial distribution of the enzyme. Although the

precise intracellular mechanisms involved in short-term ChAT regulation have not been elucidated, indirect evidence suggests that translocation of the protein between hydrophilic (cytosolic) and hydrophobic (membrane-bound) compartments may be involved. Carroll (1987) demonstrated that depolarization of hippocampal slices with high potassium and veratridine was accompanied by a significant increase in the V_{max} for water-soluble ChAT and membrane-bound ChAT; one possible interpretation of the increase in V_{max} could be an increase in enzyme protein in each of these pools in the depolarized nerve terminal. Furthermore, Docherty and Bradford (1988) suggested that membrane-bound ChAT could be released from synaptic plasma membranes by increasing concentrations of chloride ions, and Bruce and Hersh (1989) provided limited evidence that phosphorylated ChAT had a lower affinity than the native unphosphorylated protein for binding to rat synaptosomal membranes. More recently, Eder-Colli and coworkers (1992) demonstrated in *Torpedo* electric organ, that ChAT activity can be released from synaptic plasma membranes in the presence of Proteinase K, a serine protease with a fairly broad substrate specificity (Lebherz *et al.*, 1986). Because catalytic activity was retained following solubilization, these investigators suggested that membrane-bound ChAT may not be deeply embedded in the membrane.

Intracellular calcium ions appear to play a critical role in the short-term regulation of ChAT activity. Studies presented in Chapter 2 of this thesis demonstrated that depolarizing conditions, known to raise the intrasynaptosomal calcium concentration and result in ACh release (Adam-Vizi and Ligeti, 1986;

Satoh and Nakazato, 1989; Stuenkel, 1990), stimulated membrane-bound ChAT activity selectively. In Chapter 3 of this thesis, the studies revealed that reduction of the intracellular calcium concentration by incubation of intact synaptosomes in nominally calcium-free solutions containing 0.1 mM EGTA (Komulainen and Bondy, 1987; Xiang *et al.*, 1990) significantly reduced membrane-bound ChAT activity and decreased the amount of [^{32}P]phosphate incorporated into cytosolic ChAT (pooled water-soluble and sodium phosphate-soluble ChAT) under basal or resting conditions. However, the mechanism by which calcium ions exert their effect upon ChAT activity is not understood.

In the present series of experiments, I tested the hypothesis that translocation of enzyme protein between subcellular pools is a mechanism which underlies short-term regulation of ChAT activity following exposure of synaptosomes to experimental manipulations designed to lower intraterminal calcium ion concentration.

2. METHODS

2.1 Incubation of Synaptosomes

For each experiment, brains of 4-6 female Sprague-Dawley rats (150-200 g) were removed rapidly following decapitation and hippocampi were dissected in the cold. Synaptosomes were prepared as described in Chapter 2, Section 2.1, and the washed synaptosomal pellet was resuspended in 6 ml normal oxygenated KR buffer, pH 7.4, or 6 ml nominally calcium-free oxygenated KR buffer, pH 7.4 (no added calcium plus 0.1 mM EGTA). Following incubation for 30 min at 37°C, samples were placed on ice, then centrifuged at 17,000 g for 10 min. The supernatant was discarded, and the pellet washed twice with 2 ml of ice-cold 0.32 M sucrose buffered with 5 mM Tris-HCl, pH 7.4, prior to subcellular fractionation.

2.2 Isolation of Subcellular Pools of ChAT

Cytosolic and membrane-bound pools of ChAT were isolated by subcellular fractionation of synaptosomes as described in Chapter 2, Section 2.2 with the following modifications. The water-soluble and sodium phosphate-soluble pools of ChAT were not combined for analysis but kept separate, and the final pellet, containing ChAT which associates non-ionically with membranes, was resuspended in 1 ml of 100 mM sodium phosphate buffer, pH 7.4, containing 10 mM EDTA and 1 mM PMSF and incubated on ice for 15 min to remove peripheral proteins

and inhibit serine and metallo-proteases. This membrane homogenate was then centrifuged at 100,000 g for 30 min, the supernatant discarded and the pellet resuspended in 1 ml of 100 mM sodium phosphate buffer, pH 7.4 and 1.5% Triton X-100 (final concentration; detergent:protein ratio 10) to solubilize membrane-bound ChAT. This homogenate was kept on ice for 30 min with intermittent mixing before centrifugation at 100,000 g for 30 min. An aliquot (usually 150 μl) of the resulting supernatant was removed for determination of ChAT activity and protein concentration, and the remainder (850 μl) was used for immunoblotting (see Section 2.4)

2.3 Determination of ChAT Activity and Kinetic Analysis

ChAT activity was measured in the water-soluble, sodium phosphate-soluble and detergent-soluble pools as described in Chapter 2, Section 2.3, except the incubation was stopped after 30 min. The apparent Michaelis affinity constant (K_m) for choline was determined in the presence of 200 μM acetylCoenyzme A and 0.17-10 mM choline iodide (6 concentrations). The K_m for acetylCoenzyme A was determined in the presence of 5 mM choline iodide and 10-200 μM acetylCoenzyme A (6 concentrations). Kinetic values were determined from double-reciprocal plots according to the method of Lineweaver and Burk (1934).

2.4 Immunoblotting of ChAT

Water-soluble and sodium phosphate-soluble ChAT fractions were concentrated 8-fold in Centricon-10 Microconcentrators (Amicon), and the membrane-bound ChAT fraction was concentrated by immunoprecipitation prior to electrophoresis. The supernatant containing membrane-bound ChAT (850 μl) was incubated with 1:500 dilution of polyclonal rabbit anti-human ChAT antibody for 90 min at 4°C (Chemicon, see Chapter 3 of this thesis). Immunobeads (200 μl; goat anti-rabbit immunoglobulin, BioRad) were then added to form immune-complexes with rabbit anti-ChAT antibody-antigen reaction products and the incubation continued at 37°C for 90 min with intermittent mixing. To precipitate the membrane-bound ChAT/antibody complexes, samples were centrifuged in a Savant microcentrifuge for 10 min at 8,000 rpm. Supernatants were discarded and pellets were resuspended in 75 μl of 100 mM sodium phosphate buffer, pH 7.4 plus an equal volume of Laemmli double strength sample buffer (containing SDS, 4.6% (w/v); glycerol, 20% (w/v); Tris, 124 mM; dithiothreitol, 200 mM; bromophenol blue, 1%). After boiling the samples for 5 min to separate ChAT and antibodies from the immunobeads, samples were centrifuged for 10 min in a Savant microcentrifuge at 8,000 rpm and the resulting supernatant was used for Western immunoblot analysis.

ChAT samples (water-soluble, sodium phosphate-soluble and membrane-bound pools) were then electrophoresed on denaturing polyacrylamide gels (5% stacking and 9% separating) and transferred to 0.45 μm nitrocellulose (Hybond-

ECL) by a semi-dry transfer unit (BioRad Transblot, 20 V for 60 min). After blocking for 30 min in Tris-buffered saline containing 0.2% Tween-20 detergent (TBS-T) and 3% bovine serum albumin (BSA), the nitrocellulose membrane was probed for ChAT protein with affinity-purified goat anti-human ChAT antibody (1:500 dilution in TBS-T with 0.3% BSA). Following 90 min incubation at 25°C, unbound antibody was washed from the membrane with several changes of fresh TBS-T. The ChAT protein / primary antibody complex was then localized by incubation of the membrane with affinity purified mouse anti-goat IgG conjugated to horseradish peroxidase (1:5000 dilution in TBS-T with 0.3% BSA) for 1 hour at 25°C. The membrane was then washed extensively and the secondary HRP-linked antibody visualized with the Amersham Chemiluminescent system (ECL, subpicomolar sensitivity). Immunoreactive ChAT protein in each lane was quantified by laser densitometry using an LKB ultroscan.

2.5 Materials

Crystalline bovine serum albumin, EGTA, eserine sulphate, HEPES, prestained low molecular weight standards, sodium tetraphenylboron and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Missouri. 3 Heptanone was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. AcetylCoenzyme A, lithium salt was from Boehringer-Mannheim, Laval, Quebec. THAM (Tris buffer) was obtained from Fisher Chemical Co., Mississauga, Ontario. Acrylagel and Bisacrylagel were purchased from National Diagnostics,

Manville, New Jersey. Goat anti-rabbit immunoglobulin linked immunobeads, Sodium Dodecyl Sulfate, Temed and Tween-20 detergent were from Bio-Rad Laboratories, Richmond, California. Electrophoretic low molecular weight standards were obtained from Pharmacia Inc., Piscataway, New Jersey. Polyclonal rabbit anti-human ChAT Ig and affinity-purified goat anti-human ChAT Ig was obtained from Chemicon International Inc., Temecula, California. Mouse anti-goat HRP-linked antibody was purchased from Pierce, Rockford, Il. Hybond-ECL 0.5 μm nitrocellulose, Hyperfilm-ECL, and Western blotting ECL detection reagents were obtained from Amersham Canada. [^{3}H]acetylCoenzyme A (3.7 Ci/mmol) was purchased from Amersham Radiochemical Corp., Oakville, Ontario.

3. RESULTS

3.1 Calcium Dependence of ChAT Activity

Previous studies demonstrated the activation of water-soluble ChAT and membrane-bound ChAT following exposure of rat hippocampal slices to the depolarizing agents veratridine and elevated extracellular KCl (Carroll, 1987; see Chapter 2 of this thesis). The response appeared to be calcium-dependent since omission of calcium in the extracellular medium attenuated the veratridine-induced increases in ChAT activity. However, the seemingly critical role which calcium ions play in the regulation of ChAT activity was not examined further.

In the present chapter, I report the results of experiments designed to lower the intracellular calcium concentration. Incubation of intact synaptosomes in nominally calcium-free KR buffer, followed by subcellular fractionation of the synaptosomes with pooling of the cytosolic ChAT fractions (water-soluble and sodium phosphate-soluble ChAT) revealed no detectable change in enzyme activity from control conditions (Figures 2 and 3). However, when the enzyme activity was measured in these two pools separately, the specific activity of ChAT in the water-soluble fraction was significantly reduced to 62% of control under calcium-free conditions while the specific activity of salt-soluble ChAT was significantly increased to 122% of control (Table 5). This did not appear to be a salt-dependent effect as incubation of water-soluble ChAT with 100 mM sodium-phosphate buffer following subcellular fractionation did not alter its specific

activity. Furthermore, reduction of extracellular calcium ions also resulted in a significant decrease in the specific activity of membrane-bound ChAT to 73% of control. When expressed as a percentage of total enzyme activity, reduction of extracellular calcium ions appeared to result in a shift of ChAT activity from the water-soluble and detergent-soluble pools to the sodium phosphate-soluble pool (Table 5).

Table 5. Subcellular distribution of synaptosomal ChAT activity following reduction of extracellular Ca^{2+} concentration. The specific activity of both water-soluble ChAT and membrane-bound ChAT was significantly decreased, whereas that of sodium phosphate (NaP)-soluble ChAT was significantly increased following 30 min incubation of intact synaptosomes in nominally calcium-free KR buffer (no added calcium plus 0.1 mM EGTA), pH 7.4. *a* indicates significantly different from control at $P \leq 0.003$; *b* indicates significantly different from control at $P \leq 0.0001$. Data are expressed as mean ± SEM of 10 separate experiments.

	Specific Activity (nmol ACh/mg prot/30 min)			Percent Total Enzyme Activity		
ChAT Pool	Control	Calcium-free	Per Cent Control	Control	Calcium-free	Per Cent Control
Water-soluble ChAT	43.1 ± 2.6	26.8 ± 1.5[a]	62	24.1 ± 1.9	14.0 ± 0.9[b]	58
NaP-soluble ChAT	268.3 ± 24.1	326.3 ± 23.8[a]	122	62.7 ± 1.7	76.2 ± 1.8[b]	125
Membrane-bound ChAT	7.5 ± 0.6	5.5 ± 0.2[a]	73	13.4 ± 0.7	9.9 ± 0.4[a]	74

3.2 Kinetics of ChAT Activity

To elucidate the mechanism of this altered distribution of ChAT activity, Michaelis-Menten kinetic parameters of each subcellular enzyme fraction were determined. Tables 6 and 7 list the X-intercepts, Y-intercepts and correlation

coefficients of the Lineweaver-Burk double reciprocal plots for the substrates choline (Figure 20) and acetylCoenzyme A (Figure 21). As shown in Tables 8 and 9, calcium-dependent changes in ChAT activity were reflected as parallel changes in the V_{max} of each subcellular fraction. The apparent Michaelis affinity constant (K_m) for the substrate choline was unchanged in all subcellular pools (Table 8). Interestingly, following incubation of synaptosomes in nominally calcium-free KR buffer, the K_m for acetylCoenzyme A was increased from control only in sodium phosphate-soluble ChAT, indicating slightly decreased affinity of this enzyme pool for this substrate (Table 9) under these experimental conditions.

The K_m of each subcellular pool of ChAT for the substrate choline were not significantly different (Table 8), however the K_m for acetylCoenzyme A was different for each enzyme subfraction as indicated in Table 9. Under control conditions, both water-soluble ChAT and sodium phosphate-soluble ChAT exhibited a significantly lower K_m for this substrate than membrane-bound ChAT, indicating greater affinity. Under calcium-free conditions, the affinity of water-soluble ChAT for acetylCoenzyme A was significantly lower than both sodium phosphate-soluble ChAT and membrane-bound ChAT.

Table 6. Intercepts and slopes derived from Lineweaver-Burk double-reciprocal plots (Figure 20) for the substrate choline. The statistical difference of each line's slope was determined by least squares regression analysis.

Enzyme Pool	Treatment	y = mx + b	r-value	p-value
Water-soluble ChAT	+ calcium	$y = (2.4 \times 10^{-2})x + 0.02$	0.9845	0.0004
	calcium-free	$y = (1.4 \times 10^{-2})x + 0.02$	0.9909	0.0001
NaP-soluble ChAT	+ calcium	$y = (2.3 \times 10^{-3})x + 0.003$	0.9957	<0.0001
	calcium-free	$y = (2.6 \times 10^{-3})x + 0.002$	0.9958	<0.0001
Membrane-bound ChAT	+ calcium	$y = (7.3 \times 10^{-2})x + 0.17$	0.9889	0.0002
	calcium-free	$y = (6.4 \times 10^{-2})x + 0.12$	0.9968	<0.0001

Table 7. Intercepts and slopes derived from Lineweaver-Burk double-reciprocal plots (Figure 21) for the substrate acetylCoenzyme A. The statistical difference of each line's slope was determined by least squares regression analysis.

Enzyme Pool	Treatment	y = mx + b	r-value	p-value
Water-soluble ChAT	+ calcium	y = 0.31x + 0.03	0.9888	0.0002
	calcium-free	y = 0.19x + 0.02	0.9894	0.0002
NaP-soluble ChAT	+ calcium	$y = (6.1 \times 10^{-2})x + 0.005$	0.9977	<0.0001
	calcium-free	$y = (6.2 \times 10^{-2})x + 0.005$	0.9963	<0.0001
Membrane-bound ChAT	+ calcium	y = 3.5x + 0.20	0.9930	<0.0001
	calcium-free	y = 2.8x + 0.16	0.9930	<0.0001

Table 8. Apparent Michaelis-Menten kinetics of ChAT for the substrate choline. The affinity constant (K_m) for choline of each subcellular pool of ChAT was unchanged following 30 min incubation of intact synaptosomes in nominally calcium-free KR buffer, pH 7.4. However, the maximal velocity (V_{max}) of both water-soluble ChAT and membrane-bound ChAT was significantly reduced while that of sodium phosphate (NaP)-soluble ChAT was significantly increased under the same experimental conditions. ChAT specific activities were determined in the presence of 200 μM acetylCoenzyme A and 0.17-10 mM choline. *a* indicates significantly different from control at $P \leq 0.05$ (paired t-test). Data are expressed as mean ± SEM of 5 separate experiments.

	V_{max} (nmol ACh/mg prot/30 min)			K_m Choline (mM)	
ChAT Pool	Control	Calcium-free	Per Cent Control	Control	Calcium-free
Water-soluble ChAT	58.3 ± 3.7	37.4 ± 2.9[a]	64	0.84 ± 0.08	0.81 ± 0.08
NaP-soluble ChAT	351.9 ± 41.9	442.8 ± 42.8[a]	126	0.68 ± 0.02	0.73 ± 0.03
Membrane-bound ChAT	9.2 ± 1.0	6.7 ± 0.4[a]	73	0.55 ± 0.05	0.53 ± 0.09

3.3 Translocation of ChAT-Immunoreactive Protein

In this chapter, I demonstrate that alterations in the subcellular distribution of ChAT activity in synaptosomes were accompanied by parallel changes in the absolute amount of immunoreactive ChAT protein in each fraction. Figure 22 shows that in all three subcellular fractions of ChAT, immunoreactive protein bands were seen with apparent molecular mass of about 67 kDa, corresponding to the apparent molecular weight for ChAT purified from rat brain (Dietz and Salvaterra, 1980). No bands with this molecular mass were visualized when immunoblotting was done in the presence of non-immune goat serum. Surprisingly, there were also two strongly immuno-reactive bands with apparent

Table 9. Apparent Michaelis-Menten kinetics of ChAT for the substrate acetylCoenzyme A. The affinity constant (K_m) for acetylCoenzyme A of only sodium phosphate (NaP)-soluble ChAT was increased following 30 min incubation of intact synaptosomes in nominally calcium-free KR buffer, pH 7.4. However, the maximal velocity (V_{max}) of both water-soluble ChAT and membrane-bound ChAT was significantly reduced while that of NaP-soluble ChAT was significantly increased under the same experimental conditions. ChAT specific activities were determined in the presence of 5 mM choline iodide and 10-200 μM acetylCoenzyme A. *a* indicates significantly different from control at $P \leq 0.05$ (paired t-test), *b* indicates significantly different from water-soluble ChAT at $P \leq 0.01$, *c* indicates significantly different from membrane-bound ChAT at $P \leq 0.01$ (*b* and *c*: one-way ANOVA with repeated measures, followed by Duncan's post-hoc test). Data are expressed as mean ± SEM of 5 separate experiments.

	V_{max} (nmol ACh mg prot/30 min)			K_m AcetylCo A (μM)	
ChAT Pool	Control	Calcium-free	Per Cent Control	Control	Calcium-free
Water-soluble ChAT	43.1 ± 4.3	25.5 ± 1.9[a]	59	9.3 ± 0.6[c]	8.3 ± 0.5
NaP-soluble ChAT	255.1 ± 33.3	331.2 ± 39.2[a]	130	11.3 ± 0.9[c]	15.3 ± 1.1[a,b]
Membrane-bound ChAT	6.6 ± 0.4	5.5 ± 0.3[a]	84	19.6 ± 1.5	19.9 ± 2.2[b]

molecular weight of greater than (>) 106 kDa and approximately 90 kDa in the water-soluble pool. The presence these water-soluble proteins is in contrast to recent findings of Docherty (1991) who reported the presence of a 120 kDa protein following immunoblotting of synaptic plasma membranes of rat cerebral cortex and speculated that it may represent the high- affinity choline carrier complexed with ChAT. These bands were not visualized in the sodium phosphate-soluble pool, but were present when the two pools were combined (Figure 23B). Therefore, the presence of 100 mM sodium phosphate buffer does not appear to induce proteolysis or dissociation of possible subunits of the higher molecular

weight proteins. Additionally, these proteins were not isolated by polyclonal rabbit anti-ChAT antibody, but remained in the supernatant following immunoprecipitation. The immunoreactive band with apparent molecular mass of approximately 55 kDa in the detergent-soluble enzyme fraction (Figure 22, Lane 3) is goat anti-rabbit immunoglobulin which was released from immunobeads following the boiling of samples in Laemmli sample buffer and subsequently detected during Western immunoblotting by the mouse anti-goat IgG conjugated to horseradish peroxidase.

The abundance of immuno-reactive ChAT protein in each subcellular enzyme fraction appeared to be dependent upon the presence of calcium ions as shown in Figure 22. Densitometric scanning of samples revealed that incubation of intact synaptosomes in calcium-free KR buffer significantly decreased the amount of ChAT-immunoreactive protein in the water-soluble fraction by 59%, while that in the sodium phosphate-soluble fraction significantly increased by 41% (Table 10).

Because of the low abundance of membrane-bound ChAT in cholinergic nerve terminals and the high lipid content of synaptic membranes, clear demonstration of this enzyme fraction by Western immunoblot technique was exceedingly difficult. Various techniques were used to resolve membrane-bound ChAT (see Appendix I) before immunoprecipitation of the enzyme with polyclonal rabbit anti-ChAT Ig and immunobeads proved successful.

Interestingly in two experiments using immunoprecipitated membrane-bound ChAT, reduction of calcium appeared to decrease the absolute amount of membrane-bound ChAT protein. A representative immunoblot of these experiments is shown in Figure 22 (Lane 3).

Table 10. Quantification by densitometry of ChAT-immunoreactive protein. The abundance of ChAT-immunoreactive protein detected by immunoblot was quantified by densitometry. The effect of calcium-depletion was determined by tracing scans onto paper and weighing the area beneath peaks (shown in grams). *a* indicates significantly different from control at $P \leq 0.05$ (paired t-test). Data are expressed as mean ± SEM.

Enzyme Pool	Control (grams)	Calcium-free (grams)	Per Cent Control	n-value
Water-Soluble ChAT	0.88 ± 0.09	0.36 ± 0.06[a]	41	5
NaP-Soluble ChAT	0.32 ± 0.09	0.45 ± 0.01[a]	141	4

Figure 20. The effect of reducing Ca^{2+} concentration on the kinetic parameters of various ChAT fractions for the substrate choline. Each point represents the average velocity for 2 determinations of catalytic activity of water-soluble ChAT, sodium phosphate (NaP)-soluble ChAT and membrane-bound ChAT from a single experiment in the presence (□) and absence (○) of extracellular calcium (repeated 5 times with similar results). The X-intercepts, Y-intercepts and r-values of these graphs are listed in Table 6. Apparent K_m and V_{max} values are shown in Table 8.

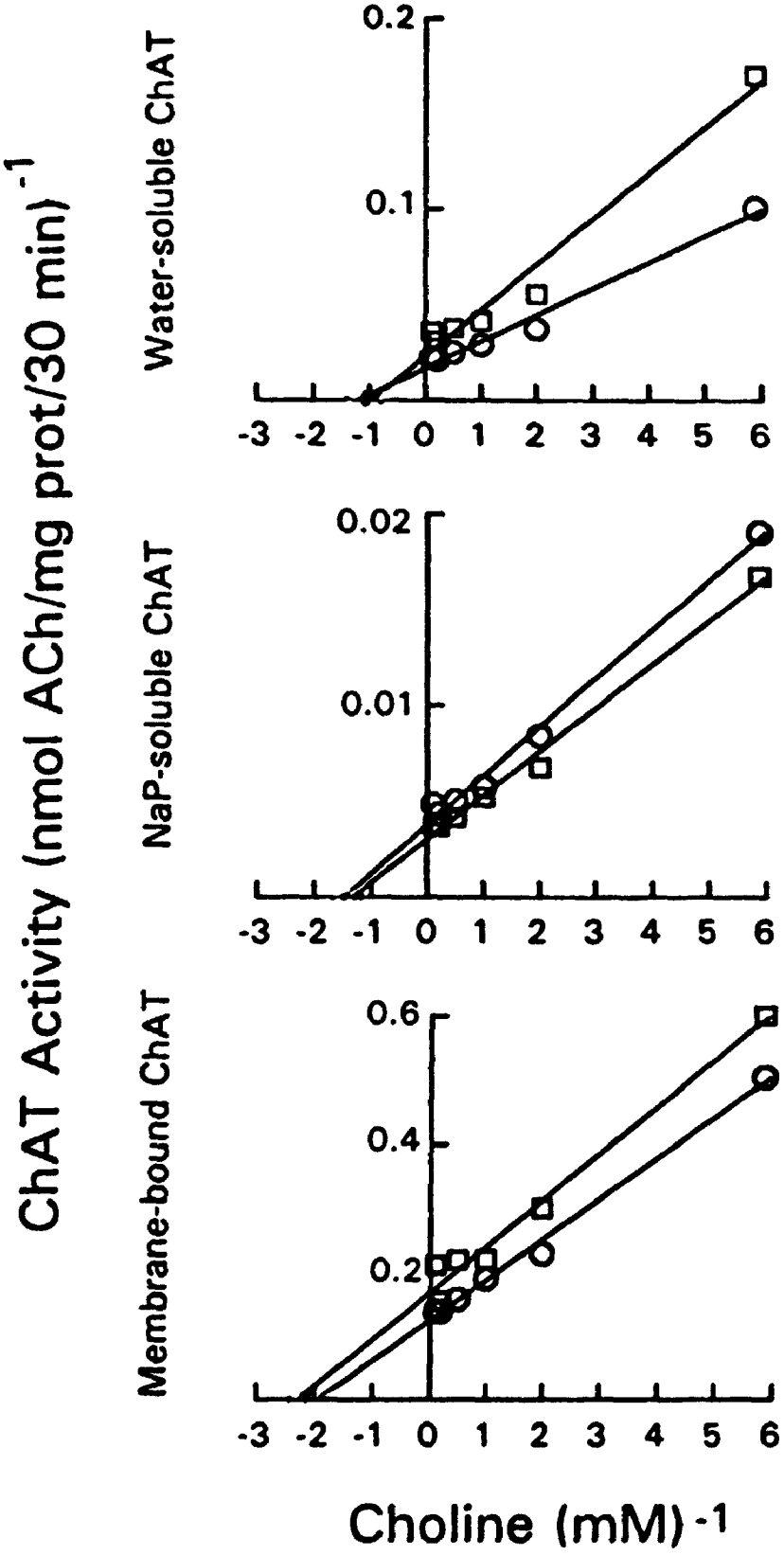
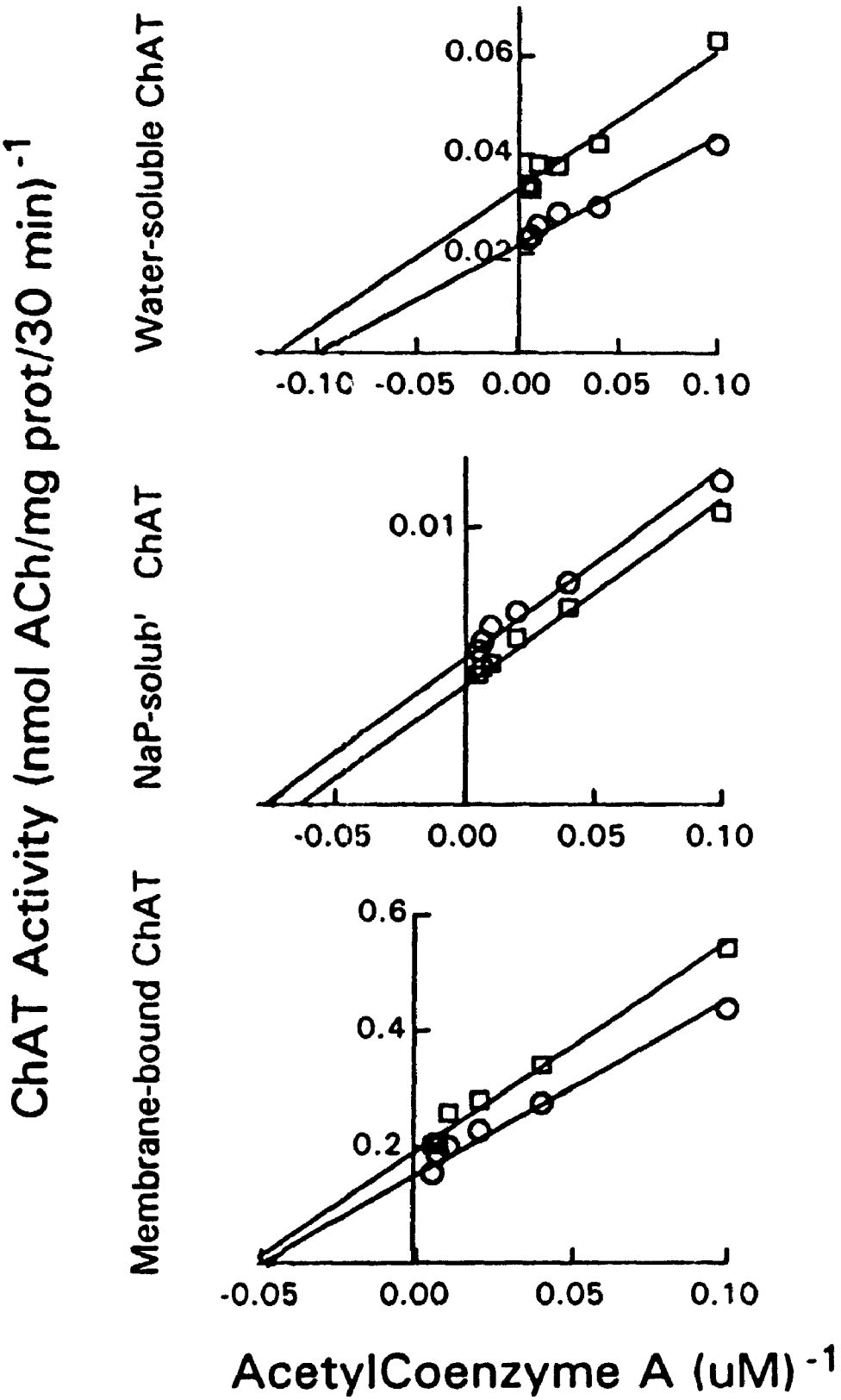

ChAT Activity (nmol ACh/mg prot/30 min)-1
Water-soluble ChAT
NaP-soluble ChAT
Membrane-bound ChAT
0.2
0.1
0.02
0.01
0.6
0.4
0.2
-3 -2 -1 0 1 2 3 4 5 6
Choline (mM)-1

Figure 21. The effect of reducing Ca^{2+} concentration on the kinetic parameters of various ChAT fractions for the substrate acetylCoenzyme A. Each point represents the average velocity for 2 determinations of catalytic activity of water-soluble ChAT, sodium phosphate (NaP)-soluble ChAT and membrane-bound ChAT from a single experiment in the presence (□) and absence (○) of extracellular calcium (repeated 5 times with similar results). The X-intercepts, Y-intercepts and r-values of these graphs are listed in Table 7. Apparent K_m and V_{max} values are shown in Table 9.


ChAT Activity (nmol ACh/mg prot/30 min)$^{-1}$
Water-soluble ChAT
0.06
0.04
0.02
-0.10 -0.05 0.00 0.05 0.10
NaP-soluh' ChAT
0.01
-0.05 0.00 0.05 0.10
Membrane-bound ChAT
0.6
0.4
0.2
-0.05 0.00 0.05 0.10
AcetylCoenzyme A (uM)$^{-1}$

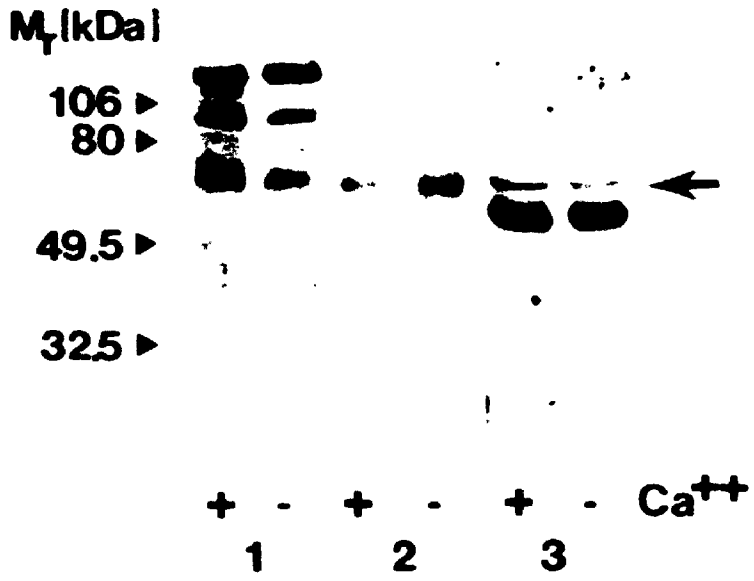


Figure 22. Immunoblots showing the presence of immunoreactive ChAT protein with apparent molecular mass of 67 kDa in each subcellular enzyme pool following incubation with polyclonal goat anti-ChAT antibody. The molecular weights (kDa) of known standards are shown at left. Right arrow indicates water-soluble ChAT (1; 70 μg total protein per lane), sodium phosphate-soluble ChAT (2; 8 μg total protein per lane), and membrane-bound ChAT (3; 20 μl supernatant following immunoprecipitation per lane, see Methods 2.4 page 107) in the presence (+) and absence (-) of extracellular calcium ions (15 minutes exposure).

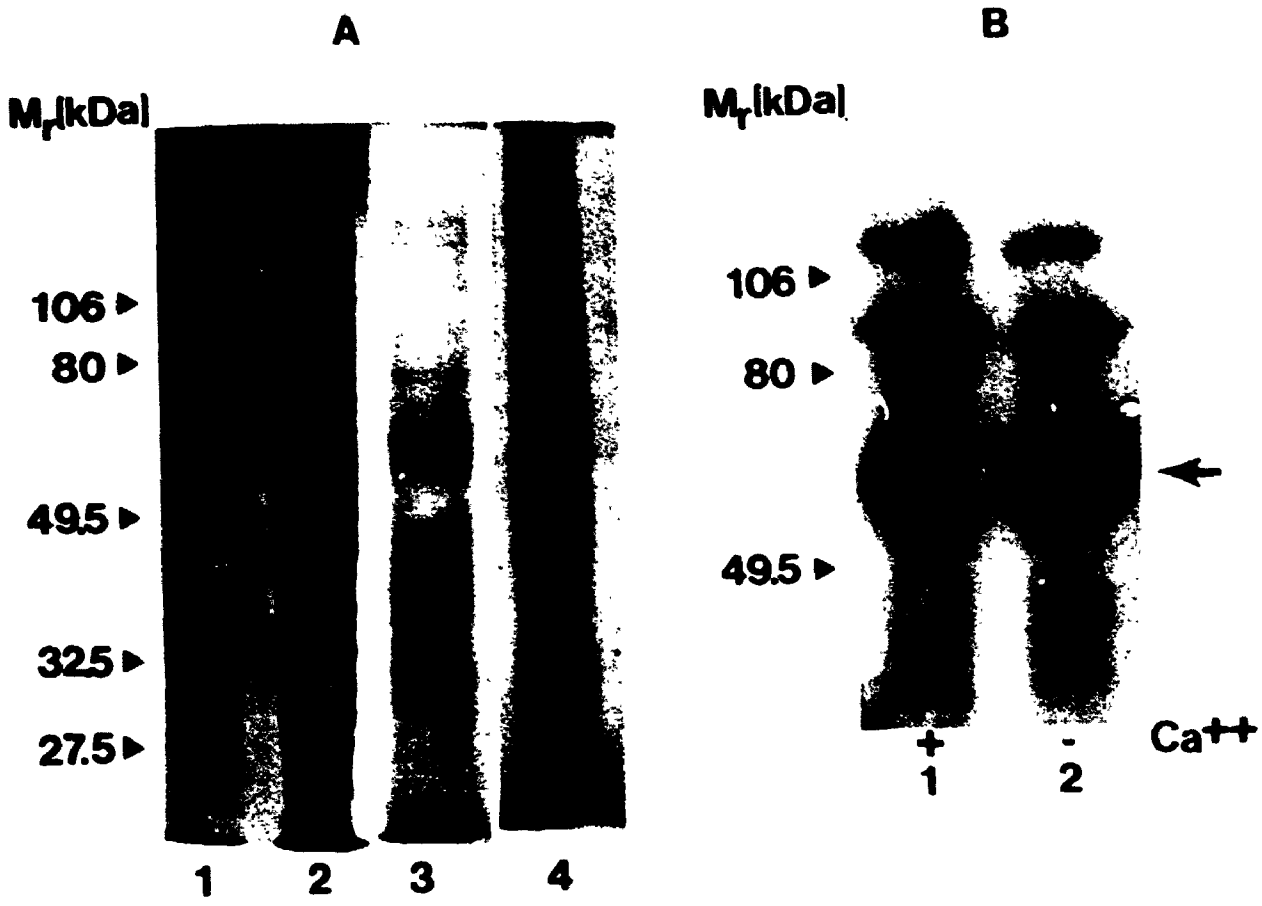


Figure 23. Coomassie Blue stain of total synaptosomal proteins and immunoblot of cytosolic ChAT. (A) Total proteins were isolated during subcellular fractionation of hippocampal synaptosomes, separated by SDS-PAGE and stained with Coomassie Blue. Known standards are shown in Lane 1, with molecular masses indicated at left. Total proteins from supernatants were isolated following water-lysis of synaptosomes (Lane 2, 50 μg), sodium phosphate wash (Lane 3, 10 μg), and Triton X-100 solubilization of synaptic membranes (Lane 4, 30 μg). (B) Following immunoblotting of cytosolic ChAT (ie. pooled water-soluble ChAT and sodium phosphate-soluble ChAT; 35 μg total protein per lane), 3 distinct protein bands of similar intensity were observed after incubation of synaptosomes in the presence (+) and absence (-) of extracellular calcium (30 sec. exposure).

4. DISCUSSION

The main objective of the present series of experiments was to determine whether protein translocation is one mechanism which underlies short-term calcium-dependent changes in subcellular fractions of nerve terminal ChAT activity. Using an experimental protocol in which intact rat hippocampal synaptosomes were incubated in nominally calcium-free KR buffer to reduce the intracellular calcium concentration, I determined that (1) the specific activity of ChAT in both the water-soluble and membrane-bound pools decreased significantly by approximately 38% and 27% of control activity, respectively, (2) the specific activity of ChAT in the sodium phosphate-soluble pool increased significantly by approximately 22% of control, (3) these changes in enzyme activity were reflected as parallel changes in the maximal velocity (V_{max}) of each enzyme pool, (4) although the K_m for choline was not different between the subcellular enzyme fractions, the K_m for acetylCoenzyme A was significantly higher for membrane-bound ChAT than cytosolic ChAT, and (5) the amount of immunoreactive protein in each subcellular pool appeared to change in parallel with changes in enzyme specific activity, suggesting a change in the spatial distribution of enzyme protein.

In this series of experiments, water-soluble ChAT was not pooled with sodium phosphate-soluble ChAT for assay of enzyme activity as it was in Chapters 2, 3, and 4 of this thesis. Although most investigators believe that both these

enzyme fractions are located in the cytoplasm *in situ*, Carroll and coworkers demonstrated distinct biochemical characteristics of each pool (Smith and Carroll, 1980; Carroll and Benishin, 1984; Badamchian *et al.*, 1986). They suggested that water-soluble ChAT is truly cytosolic, whereas sodium phosphate-soluble ChAT is prone to ionic association with synaptosomal membranes when solubilized under experimental conditions of low ionic strength. As no experimental condition tested previously in this thesis altered the activity of (combined) cytosolic ChAT, it was of interest to examine the activity of each pool separately. Indeed each enzyme fraction did appear to behave differently following cytosolic calcium ion reduction, with a decrease in the specific activity of water-soluble ChAT and an increase in that of sodium phosphate-soluble ChAT.

Although several investigators have demonstrated that purified ChAT can be activated by increasing salt concentrations (Rossier *et al.*, 1977 ; Hersh *et al.*, 1978; Hersh and Peet, 1978), it is not believed that the calcium-dependent decrease in water-soluble ChAT activity can be attributed to lower salt content of this fraction for the following reasons: (1) the specific activity of each enzyme fraction was determined in the presence of equal salt concentration, (2) incubation of water-soluble ChAT with 100 mM sodium phosphate buffer, pH 7.4 did not alter enzyme specific activity, (3) the recovery of total enzyme activity was always greater than 90%, suggesting that little was lost from each subcellular pool during the isolation procedure, and (4) reduction of extracellular calcium consistently decreased the activity of water-soluble ChAT irrespective of the time interval

before the enzyme specific activity was measured.

Interestingly, the pattern of ChAT-reactive protein banding detected by Western immunoblotting paralleled the changes in enzyme specific activity observed following incubation of intact synaptosomes in calcium-free buffer. This experimental manipulation resulted in an increase in the specific activity of sodium phosphate-soluble ChAT and a similar increase in the amount of ChAT protein present in that fraction. Likewise, the reduction in specific activity of both water-soluble ChAT and membrane-bound ChAT were accompanied by a similar reduction of ChAT protein in those pools. These observations strongly suggest movement of enzyme protein between the subcellular pools.

It is possible, but not likely, that the high molecular weight proteins (M_r 90 and >100 kDa) observed following immunoblotting of water-soluble ChAT were degraded or dissociated by 100 mM sodium phosphate buffer, pH 7.4, and therefore not detected in the sodium phosphate-soluble fraction. However, mixing of the two enzyme pools prior to detection still revealed the presence of these high molecular weight bands. As indicated in Figure 23A (Lane 2), the total amount of protein isolated following water-lysis of the synaptosomes was much greater than that obtained following the washing of synaptosomal membranes with 100 mM sodium phosphate buffer (Lane 3). Therefore, it seems likely instead that the water-soluble pool contains other proteins which express epitopes similar to those recognized by the goat anti-ChAT antibody used for immunoblotting. This was supported by the observation that incubation of the water-soluble fraction with

anti-ChAT antibody did not result in the immunoprecipitation of these high molecular weight proteins. Alternatively, and more speculatively, these proteins may represent other forms of ChAT which would likely be catalytically inactive based upon the following observations: (1) calcium reduction did not alter the amount of immunoreactive protein in these bands although the band with M_r 67 kDa was significantly reduced, and (2) water-soluble ChAT accounts for only about 20% of total ChAT activity, however the bands with M_r 90 kDa and >106 kDa stained more intensely than those detected in the sodium phosphate-soluble fraction.

In my hands, the differences in both specific activity and pattern of protein banding on immunoblots between water-soluble and sodium phosphate-soluble ChAT do not appear to be artifacts. Indeed, Fonnum and Malthe-Sorenssen first demonstrated that these pools of ChAT appeared to be different as they had distinct isoelectric points which correlated with membrane affinity (Fonnum and Malthe-Sorenssen, 1972a, b). However, the mode of subcellular localization of these two pools remains unclear. Sodium phosphate-soluble ChAT may associate ionically with specific anchoring proteins of the cytoskeleton such as microtubule-associated protein 2 (MAP-2) as does Protein Kinase A (Luo *et al.*, 1990; Scott *et al.*, 1990); in comparison, water-soluble ChAT is likely free in the cytoplasm.

Furthermore, the physiological relevance of the distinct subcellular pools of cytosolic ChAT is presently unclear, although it has been suggested that water-soluble ChAT may be primarily responsible for synthesis of cytosolic ACh which

is released spontaneously from the nerve terminal (Carroll and Benishin, 1984; Carroll, 1983). The function of membrane-bound ChAT is even more uncertain, although its subcellular localization and catalytic activity appear to be dependent upon the level of cytosolic calcium.

As demonstrated by the present series of experiments, ChAT appears to bind reversibly to synaptic membranes, suggesting that protein translocation may be one mechanism for short-term regulation of enzyme activity in cholinergic nerve terminals. A variety of experimental conditions have been shown to alter enzyme activity in other cell types, presumably by altering subcellular distribution. For example, in rat brain synaptosomes, calcium (1-100 μM) significantly decreased membrane-bound histidine decarboxylase activity with a concomitant increase in the activity of its soluble counterpart (Toledo *et al.*, 1991). Additionally, *in vitro* studies have provided evidence that soluble purified dopamine β-hydroxylase (Taylor and Fleming, 1989), Protein Kinase C (Huang, 1989), bovine adrenal tyrosine hydroxylase (Kuhn *et al.*, 1990), phosphatidate phosphohydrolase of rat hepatocytes (Gomez-Munoz *et al.*, 1992) and phospholipase A_2 (Channon and Leslie, 1990) can interact with membranes in a specific and reversible manner.

Although ChAT is likely a substrate for phosphorylation by calcium-dependent protein kinases and phosphorylation can affect binding of the protein to membranes (Bruce and Hersh, 1989), other mechanisms may also be responsible for determining the subcellular distribution of ChAT. Membrane association of proteins can result from electrostatic or hydrophobic interactions,

or through fatty acylation which occurs by covalent modification of proteins including esterification, N-myristylation and glypiation (C-terminal glycosylphosphatidylinositol (GPI) attachment, for review see Schultz *et al.*, 1988). Indeed, Carroll and Smith (1990) suggested that rat brain membrane-bound ChAT may be associated with the plasma membrane through a GPI linkage; as most GPI-linked proteins are extracellularly oriented, the physiological relevance of this finding is unclear. More recently, Eder-Colli *et al.* (1992) demonstrated in *Torpedo* electric organ, that ChAT activity could be released from synaptic plasma membranes in the presence of Proteinase K, but not by phospholipase C. These observations refute Carroll's suggestion of GPI linkage since phospholipase C frees inositol from the membrane. It remains to be determined precisely how ChAT interacts with membranes.

CHAPTER 6

GENERAL DISCUSSION

6.1 Discussion

Although ChAT was first detected more than 40 years ago in cholinergic neurons, its low abundance in tissues has made progress in characterizing the enzyme and understanding the mechanisms of its regulation slow. In 1968, Fonnum provided the first evidence that ChAT may associate with membranes in addition to existing in a soluble form within the cytoplasm. However, only during the past decade has this observation become accepted as having potential physiological relevance. Since technical difficulties had precluded elucidation of the function of membrane-bound ChAT in cholinergic systems, I undertook this study initially to confirm its presence in rat hippocampal synaptosomes and to develop an experimental paradigm which would allow determination of its role in basal ACh synthesis. As the study evolved, it became apparent that short-term regulation of ChAT activity was indeed a complicated interplay of cellular pathways, and the following observations were made:

(a) following the partitioning of enzyme activity in the nonionic detergents Triton X-100 and Triton X-114, approximately 80% of total ChAT activity appeared to be cytosolic, while the remaining 15-20% was membrane-bound,

(b) only membrane-bound ChAT activity was significantly increased following depolarization of intact synaptosomes by both 40 mM KCl and 50 μM veratridine, in a calcium-dependent manner,

(c) the basal activity of cytosolic ChAT was not altered when synaptosomes

were incubated in nominally calcium-free buffer, whereas the basal activity of membrane-bound ChAT was significantly reduced,

(d) under basal (nondepolarized) conditions phosphorylated ChAT, with an apparent molecular weight of 67 kDa, was observed only in the cytosol of isolated rat brain nerve terminals; membrane-bound ChAT did not appear to incorporate [^{32}P]phosphate,

(e) the abundance of [^{32}P]phosphate-labelled cytosolic ChAT, but not its enzymatic activity, appeared to be dependent in part upon the level of intracellular calcium,

(f) membrane-bound ChAT did not appear to play a regulatory role in the biosynthesis of ACh following alteration of nerve terminal Cl^- ion homeostasis,

(g) although cytosolic ChAT activity was not apparently altered following experimental manipulations, it could be further separated into water-soluble ChAT (20% of total enzyme activity), and sodium phosphate-soluble ChAT (60% of total enzyme activity), the activities of which were sensitive to the presence of calcium ions, and

(h) calcium-dependent changes in the specific activity of all three subcellular ChAT fractions (water-soluble, sodium phosphate-soluble and membrane-bound) were accompanied by parallel changes in both the V_{max} of the enzyme and the absolute amount of ChAT-immunoreactive protein in each subcellular pool.

These findings suggest that each subcellular fraction of ChAT may be independently regulated and that although membrane-bound ChAT did not appear to regulate basal ACh synthesis, it remains to be determined whether it may play an important role during neuronal activity.

In addition to ChAT, a variety of other neurotransmitter biosynthetic enzymes such as tyrosine hydroxylase (TH), glutamic acid decarboxylase (GAD) and dopamine beta-hydroxylase (DBH) appear to exist in both hydrophilic (cytosolic) and hydrophobic (membrane-associated or membrane-bound) forms. Different isoforms of a given enzyme may result from proteolytic or post-translational processing of a single polypeptide, translation of alternatively spliced mRNA or transcription of distinct genes. For example, Kuhn *et al.* (1990) have described a form of TH representing 5-7% of total TH activity bound to the membrane of secretory granules in bovine adrenal medulla. Multiple forms of TH are encoded by different mRNAs generated by alternative splicing of a single gene (Le Bourdelles *et al.*, 1988). Alternatively, in GABAergic neurons, various subcellular forms of GAD appear to be encoded by two separate genes (Erlander and Tobin, 1991; Erlander *et al.*, 1991), however it is unclear whether soluble and membrane-bound isoforms of GAD within a single subcellular compartment are encoded by distinct genes (Wu *et al.*, 1991). Finally, there is speculation that in adrenergic neurons a membrane-bound form of DBH may result from noncovalent binding of soluble DBH to phosphatidylserine (Taylor and Fleming, 1989). The functional role and physiological regulation of the membrane-bound forms of these

enzymes is not known.

Within cholinergic neurons, ChAT appears to be the product of a single gene which contains a number of potential transcription factor binding sites. Recent evidence indicates that several different ChAT mRNAs exist in rat (Ishii *et al.*, 1990; Ibanez *et al.*, 1991) and human brain (Lorenzi *et al.*, 1992), mouse spinal cord (Misawa *et al.*, 1992), testes (Ibanez *et al.*, 1991) and PC12 cells (Hahn *et al.*, 1992). The physiological significance of various ChAT mRNAs whose translation may be induced independently is unclear at present, but may represent a mechanism to generate the different subcellular pools of the enzyme and suggests potential points of long-term regulation of ChAT activity.

Indeed, most studies on ChAT regulation have focussed upon modulation of enzyme activity over long periods of time of hours to days; several have found that a variety of peptides, hormones and growth factors enhance total ChAT activity both *in vivo* and in cultured neurons, most likely through enhanced gene expression or stimulation of protein synthesis (Ishida and Deguchi, 1983; Hefti *et al.*, 1986; Sonnenberg *et al.*, 1986; Hofmann, 1988; McManaman *et al.*, 1988; Alderson *et al.*, 1989; Barotte *et al.*, 1989; Casper and Davies, 1989; Hiramatsu *et al.*, 1989).

There is now increasing evidence, including studies presented in this thesis, to support a theory of short-term regulation of ChAT activity within the time frame of seconds to minutes also. For example, nerve terminal depolarization resulted in the selective stimulation of membrane-bound ChAT activity, suggesting

a coupling between neurotransmitter release and subsequent resynthesis by a specific subcellular pool of enzyme. Similarly, in catecholaminergic neurons, depolarization leads to enhanced activity of cytosolic tyrosine hydroxylase (Zigmond *et al.*, 1989). Since this enzyme is rate limiting in catecholamine biosynthesis, much effort has been spent defining the regulatory role of protein phosphorylation in its depolarization-dependent activation (Haycock, 1987). Interestingly, serine-40 of tyrosine hydroxylase can be phosphorylated by three different protein kinases (cAMP-dependent protein kinase, protein kinase C, and calcium-calmodulin kinase II) but, curiously, only phosphorylation by cAMP-dependent kinase results in remarkable activation of the enzyme (Funakoshi *et al.*, 1991). This indicates that a protein may be a substrate for kinase-mediated phosphorylation with no apparent change in its catalytic activity, or, possibly, physiological function. It is presently not known whether membrane-bound tyrosine hydroxylase is also a substrate for phosphorylation.

Purified ChAT can also be phosphorylated at a serine residue by calcium-dependent protein kinases (Bruce and Hersh, 1989). This finding, however, has little physiological relevance unless existence of the phosphoprotein and its regulation can be demonstrated *in situ* or *in vivo*, since many proteins contain the hydroxylated amino acids serine, threonine and tyrosine all of which can be potential sites for phosphorylation. Therefore, when Bruce and Hersh (1989) isolated a phosphorylated form of cytosolic ChAT from rat brain, I reasoned that protein phosphorylation may indeed represent one mechanism of short-term ChAT

regulation, and during the course of these studies, I provided the first demonstration of the subcellular location of phosphorylated ChAT. Only cytosolic ChAT appeared to be phosphorylated under resting conditions; it was not surprising that membrane-bound ChAT did not incorporate [^{32}P]phosphate, since phosphorylation would likely lead to a net negative charge on the protein thereby lowering its affinity for the membrane. Indeed, Bruce and Hersh (1989) found that purified ChAT appeared to have less affinity for synaptic membranes when phosphorylated.

Interestingly, ChAT activity appeared to be very sensitive to alterations in the intracellular calcium concentration; in fact calcium appeared to play a pivotal role in the short-term regulation of ChAT activity. Although the precise mechanism of action of calcium ions in regulation of ChAT function was not elucidated protein translocation between the cytoplasm and membrane and within cytoplasmic pools appeared to be involved. Komulainen and Bondy (1986) have estimated the resting intrasynaptosomal free calcium concentration ($[Ca^{2+}]_i$) to be about 300-400 nM using the indicator dyes fura-2 and quin-2, and it appears to respond rapidly to changes in the ionic composition of the extrasynaptosomal medium. For example, depolarization of the plasma membrane can increase $[Ca^{2+}]_i$ to near submicromolar levels within seconds, making alterations in the intracellular free calcium concentration an ideal tool for manipulation of calcium-dependent regulatory mechanisms (Thomas and Almers, 1992).

In addition to activating calcium-dependent protein kinases, for which

ChAT does appear to be a substrate, free calcium ions may act directly by binding to a protein such as calmodulin, or indirectly by altering the activity of other enzymes such as protein phosphatases or proteases. In cholinergic neurons, it is unlikely that calcium binds directly to ChAT, since the amino acid sequence of the enzyme does not appear to contain "calcium hands" or binding sequences similar to calmodulin or EGTA (see Williams, 1992). The involvement of calcium-dependent protein phosphatases and/or proteases in the short-term regulation of ChAT activity is presently not known.

In the present study, following the reduction of intracellular calcium ions, ^{32}P-phosphate incorporation by cytosolic ChAT was significantly reduced although the specific activity and V_{max} of sodium phosphate-soluble ChAT (which comprises the majority of *cytosolic* ChAT) were significantly increased. These changes apparently resulted from a change in the spatial distribution of the protein. Although accumulated evidence strongly suggests that ChAT may be a substrate for phosphorylation by calcium-requiring kinases *in situ*, there is presently no information about possible regulation of enzyme activity by phosphorylation and its mechanism. However, it is intriguing to speculate that protein phosphorylation may govern the subcellular localization of the enzyme by decreasing its affinity for, and therefore interaction with, membranes and/or inhibit the activity of some proportion of ChAT. The latter calcium-dependent mechanism has been found to be important in the regulation of glycogen synthase activity (Villar-Palasi, 1991; Fernandez-Novell *et al.*, 1992a, 1992b).

It is also plausible that the calcium effect is indirect and is mediated by alterations in membrane potential. In Chapter 2 of this thesis, it was demonstrated that depolarization of intact synaptosomes by either potassium or veratridine in the absence of extracellular calcium resulted in a greater reduction of membrane-bound ChAT activity than that observed following incubation of synaptosomes in calcium-free KR buffer without depolarizing agents. This suggests the involvement of some mechanism which is sensitive to a voltage change across the membrane and/or other ions which move during the course of nerve activity.

Although membrane-bound ChAT did not appear to be necessary for ACh synthesis in the basal or resting neuronal state as demonstrated in Chapter 4, its physiological function in the active neuron remains unknown. Since depolarizing conditions appeared to stimulate membrane-bound ChAT activity exclusively, in a calcium-dependent manner and to a degree comparable to the enhancement of precursor (choline) uptake (see Chapter 2 of this thesis), it is tempting to speculate that this enzyme subfraction may be essential in regulating synthesis of neurotransmitter required to replenish stores during repetitive neuronal activity. In fact, it has been proposed that membrane-bound ChAT may form part of a presynaptic membrane-bound complex associated with the sodium dependent, high-affinity choline carrier (Barker *et al.*, 1978; Jope, 1979). Contrary to this hypothesis, evidence presented in Chapter 4 of this thesis suggests that membrane-bound ChAT is not functionally associated with the choline transporter, at least under resting conditions. However, to date it has not been possible to examine

the relationship of these cholinergic neuronal parameters following depolarization, a condition in which functional association would likely be maximal.

Indirect evidence in support of the theory of depolarization-induced coupling was provided in Chapter 2 of this thesis and elsewhere (Rylett, 1989), but due to technical difficulties, the theory has never been tested directly. To speculate though, since Knipper *et al.* (1992) recently suggested that the high-affinity choline transporter may be subject to kinase-mediated phosphorylation, it is possible that membrane-bound ChAT contains a high-affinity site which favours binding to the phosphorylated transporter, thereby creating a multiprotein complex. Similarly, other cytoplasmic signaling proteins, including phospholipase C, can bind to tyrosine-phosphorylated growth factor receptors at the membrane through the Src homology (SH) domain 2 (Koch *et al.*, 1991)

During the course of this study, several different experimental conditions were found to alter the activity of membrane-bound ChAT without affecting the activity of the larger pool of cytosolic ChAT, until that pool was further subdivided into water-soluble and sodium phosphate-soluble ChAT in the final series of experiments. Variations in enzyme activity may be the result of persistent modifications in enzyme kinetics or altered subcellular location of the protein, or could be the result of post-translational protein modification such as phosphorylation-dephosphorylation. Since changes in the catalytic activity of each subcellular pool of ChAT endured the subcellular fractionation procedure of several hours, it seemed plausible that these changes in enzyme activity

represented variations in the amount of active enzyme protein. In support of this theory, kinetic analysis and Western immunoblot technique indicated that alterations in ChAT activity following incubation of synaptosomes in calcium-free media were associated with parallel changes in the V_{max} and the absolute amount of ChAT-immunoreactive protein in each enzyme subfraction. These findings may be interpreted as an alteration in the turnover rate of these subcellular pools of ChAT, a variation in the spatial distribution of the protein, or activation or inactivation of existing protein. Each of these mechanisms of short-term regulation could result from a post-translational modification such as protein phosphorylation.

It is clear that ChAT exists in kinetic excess within cholinergic nerve terminals, but it is not known whether some portion of the enzyme is inhibited by a regulatory mechanism such as phosphorylation, subcellular compartmentation or end-product inhibition. The last possibility exists in catecholaminergic neurons; several studies have demonstrated that native tyrosine hydroxylase is isolated from nerve terminals already tightly bound with catecholamines (Almas *et al.*, 1992; Haavik *et al.*, 1990). Almas *et al.* (1992) suggested that activation of the enzyme by phosphorylation and inactivation by binding of catecholamines are related events, with phosphorylation first causing the release of catecholamines followed by enzyme activation.

Throughout this study, I have assumed that the majority of membrane-bound ChAT is associated with the plasma membrane and have not focussed on possible interaction of the enzyme with intracellular membranes such as synaptic

vesicles. Historically, the subject of membrane-bound ChAT localization has been quite controversial (for review see Rossier, 1977). Although several other investigators have also focussed upon the activity of ChAT that is associated with synaptic plasma membranes prepared from a variety of sources including rat (Docherty and Bradford, 1988) and *Torpedo* electric organ (Eder-Colli and Amato, 1985), it should be noted that several studies have used immunocytochemical techniques to demonstrate the presence of ChAT-reactive staining associated with both plasma membranes and some intracellular membranes in electron micrographs (Martinez-Murrillo *et al.*, 1989a,b).

6.2 Future Studies

The present study has added much new information to our understanding of the short-term regulation of ChAT activity within cholinergic neurons. It has provided the first demonstration of subcellular localization of phosphorylated ChAT in the resting nerve terminal, demonstrated that membrane-bound ChAT does not play a regulatory role in the synthesis of basal levels of ACh, and has provided evidence that protein translocation between subcellular pools of the enzyme may play an important role in its short-term regulation. It has also raised many questions regarding cholinergic function, including the following:

1. What is the physiological role of membrane-bound ChAT during repetitive neuronal activity?

It is unclear whether selective activation of membrane-bound ChAT following depolarization represents a key regulatory step in ACh synthesis during repetitive neuronal firing. Since depolarization triggers quantal ACh release (Katz, 1969) with subsequent enhancement of choline uptake, thereby confounding the effect of enzyme activity on transmitter biosynthesis, it will be necessary to develop an alternative paradigm in which transmitter release is prevented but an adequate level of substrate for resynthesis is maintained.

In view of this, some use may be made of *Spodoptera frugiperda* Sf9 cells which can synthesize and accumulate ACh in the presence of external choline when infected with a cDNA containing the coding sequence for ChAT, but do not release transmitter in response to a calcium influx (Habert *et al.*, 1992). Although these authors did not detect membrane-bound ChAT activity, it is unclear whether this system could be optimized for its detection.

2. What proportion of ChAT is phosphorylated in situ?

Protein phosphorylation may be one mechanism which governs the subcellular distribution of ChAT. Although the presence of ^{32}P-labelled cytosolic ChAT was clearly demonstrated in this study, it is unknown what proportion of cytosolic ChAT exists as a phosphoprotein under resting conditions. This would require physical separation followed by quantification of phosphorylated cytosolic

ChAT from the unphosphorylated form of the protein, which theoretically could be accomplished by two methods. First, isoforms of the enzyme could be separated by two-dimensional gel electrophoresis with isoelectric focusing as the first dimension if the isoelectric point of the phosphoprotein was significantly decreased by the addition of acidic phosphate group(s). This procedure, however, requires knowledge of the coordinates of the dephosphoprotein and each phosphorylated derivative (Cooper, 1991), information which is not yet known for ChAT. Second, antibodies which distinguish between phosphorylated and dephosphorylated forms of the enzyme could be used for selective immunoprecipitation. Preparation of phosphorylation state-specific antibodies requires immunization with peptides containing the established phosphorylation sites for the protein (Czernik *et al.*, 1991). Although Bruce and Hersh (1989) have demonstrated that a serine residue appears to be phosphorylated, the exact residue is not yet known, and it is unclear whether physiological stimuli such as neuronal depolarization lead to changes in the site(s) or number of phosphate groups added to the enzyme.

Additionally, it must be determined whether membrane-bound ChAT is phosphorylated and not incorporating new radiolabel under the present experimental conditions.

3. How is ChAT bound to synaptosomal membranes?

In studies utilizing phase partitioning of ChAT into Triton X-114, it appeared that there are both amphiphilic and hydrophilic forms of the enzyme (Docherty and Bradford, 1988; Eder-Colli *et al.*, 1992: Chapter 2 of this thesis), with the amphiphilic enzyme exhibiting hydrophobic characteristics of an integral membrane protein (Eder-Colli *et al.*, 1992). Examination of the amino acid sequence deduced from cDNAs, however, does not reveal obvious hydrophobic regions in the protein which may serve as anchors in the lipid bilayer (Brice *et al.*, 1989).

Another mechanism of attachment of proteins to membranes can be through tight, but noncovalent, association with particular membrane lipids; an example is dopamine β-hydroxylase which exists in a soluble and a membranous form anchored noncovalently to phosphatidylserine (Taylor and Fleming, 1989). Using a similar technique, incubation of partially purified ChAT with phospholipid vesicles of varying lipid composition could be used to study the binding of this enzyme to membranes.

Association of ChAT with fatty acids may represent another mechanism by which membrane-bound ChAT could associate functionally with the choline transporter. Saltarelli *et al.* (1990) determined that unsaturated fatty acids regulate activity of the transporter as measured by ^{3}H-hemicholinium-3 binding. The fluidity of biological membranes is altered by changes in its fatty acid content and can affect the function of many membrane-localized enzymes, including Na^{+}/K^{+}

ATPase (Swann, 1984) and perhaps membrane-bound ChAT.

4. What is the physiological role of membrane-bound ChAT in vivo?

Functional identification of ChAT cDNA has been performed in various systems, including rabbit reticulocyte lysate (Berrard *et al.*, 1987), *Xenopus laevis* oocytes (Brice *et al.*, 1989) and mammalian cells (Ishii *et al.*, 1990). These models which resemble the physiological system more closely than synaptosomes may prove useful in elucidating the functional role of membrane-bound ChAT. However, finally the question of physiological relevance of membrane-bound ChAT during nerve activity must be addressed *in vivo*. No central model presently exists to address this question, therefore efforts will likely turn toward the neuromuscular junction. Peripherally, the phrenic nerve-hemidiaphragm and the ciliary nerve-iris muscle preparations, which can be repetitively stimulated, have been used to characterize the sodium-dependent high-affinity choline uptake system and ACh synthesis (Vaca and Pilar, 1979; Ruch *et al.*, 1982) and may prove useful in the characterization of ChAT regulation.

7. APPENDIX I

7. Methods Used to Detect Membrane-Bound ChAT by Western Immunoblot Technique

Because the precise mechanism of interaction between membrane-bound ChAT and the lipid bilayer is unknown, the most appropriate experimental strategy for solubilizing this subcellular enzyme pool for Western immunoblotting was not readily apparent. Several different approaches were attempted, and although unsuccessful, are listed below.

1. Various protease inhibitors including soybean trypsin inhibitor (100 μg/ml), leupeptin (1 μg/ml), aprotinin (9 K units), benzamidide-HCl (1 μg/ml), PMSF (0.1 mM), EGTA (0.1 mM), and EDTA (0.1 - 10 mM) were included in all solutions to minimize proteolysis of ChAT and provide evidence that the protein bands with apparent molecular masses less than 67 kDa were proteolytic fragments of membrane-bound ChAT. However, inclusion of these agents did not alter the pattern of protein banding.

2. Synaptic membranes were washed extensively with 500 mM NaCl before solubilization with Triton X-100 detergent to remove proteins that were ionically associated. There was, however, no change in the number of protein bands.

3. The high lipid content of membrane samples has posed difficulties to many investigators interested in isolating membrane-bound proteins by SDS-PAGE. I attempted to extract the lipid into acetone prior to Triton X-100 solubilization.

4. Membrane samples were suspended in various concentrations of Laemmli sample buffer, then centrifuged to remove lipid. This did not appear to alter the pattern of protein separation by SDS-PAGE. Furthermore, the drawback that SDS detergent inactivates enzyme proteins prohibited subsequent determination of recovery of membrane-bound ChAT activity.

5. CHAPS (3-[(3-chloramidopropyl)dimethylammonio]-1-propane sulfonate; 1% final concentration) was used to solubilize membrane samples instead of Triton X-100. Although CHAPS is a zwitterionic detergent and migrates like Triton X-100 in SDS-PAGE, its high CMC (critical micelle concentration) and low micelle weight allow easier removal of the detergent from samples prior to protein separation by SDS-PAGE (see 7). Since I did not observe much improvement in resolution, the use of Triton X-100 detergent was continued.

6. The supernatant which remained following Triton X-100 solubilization of membranes and ultracentrifugation at 100,000 g for 30 min was concentrated in Centricon-10 microconcentrators (Amicon), however the presence of detergent interfered with the procedure.

7. Triton X-100 and CHAPS detergents were removed from membrane samples using PD-10 columns and BioRad Biobeads, however too much membrane-bound ChAT activity was lost during the procedures.

8. Detection of membrane-bound ChAT protein on Western immunoblots was attempted with a polyclonal rabbit anti-human ChAT antibody (Chemicon), however background binding to nitrocellulose was too great for adequate resolution.

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