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THE EFFECTS OF ETHANOL AND FATTY ACIDS
ON INTRASYNAPTOSOMAL CALCIUM CONCENTRATION

BY

MEGUMI ADACHI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
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MASTER OF SCIENCE THESIS
OF
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1994

ABSTRACT

The present study is composed of two relatively independent analyses of ethanol and fatty acids as potential effectors of neuronal Ca^{2+} homeostasis. Nerve ending preparations (synaptosomes) from rat brains were used to examine the effects of ethanol and fatty acids on intrasynaptosomal free Ca^{2+} ($[\text{Ca}^{2+}]_i$). $[\text{Ca}^{2+}]_i$ was measured using Fura2, a fluorescent Ca^{2+} indicator dye.

Ca^{2+} has been proposed as a possible mediator of some of the effects of ethanol on the mammalian brain. The present study evaluates the action of ethanol on $[\text{Ca}^{2+}]_i$ and the relationship between the change in $[\text{Ca}^{2+}]_i$ caused by ethanol and the activation of a calnaktin-like inhibitor of Na^+ , K^+ -ATPase. A small increase in synaptosomal $[\text{Ca}^{2+}]_i$ was observed in the presence of ethanol. In addition, ethanol released Ca^{2+} from microsomes in dose dependent manner. However, the concentrations of ethanol required for significant effects on $[\text{Ca}^{2+}]_i$ were not relevant to alcohol intoxication and greater effects on $[\text{Ca}^{2+}]_i$ would be needed to increase calnaktin activity. Therefore, these results suggest that pharmacologically relevant concentrations of ethanol ($< 200 \text{ mM}$) do not elevate $[\text{Ca}^{2+}]_i$ to levels necessary for the inhibition of Na^+ , K^+ -ATPase.

Two mechanisms have been proposed for the inhibition of neurotransmitter amino acid uptake by unsaturated fatty acids: the uncoupling of Na^+ /amino acid symport and the depolarization of the plasma membrane. The present study demonstrated a potent decrease in $[\text{Ca}^{2+}]_i$ by two unsaturated fatty acids, but not by a saturated fatty acid. This result is not consistent with the depolarization mechanism and supports the proposed uncoupling mechanism. The target site for the decrease in $[\text{Ca}^{2+}]_i$ by unsaturated fatty acids appears to be distinct from the site for inhibition of

amino acid uptake. Protein kinase C (PKC) does not seem to be required since a PKC inhibitor, H-7, did not alter the decrease in $[Ca^{2+}]_i$ caused by unsaturated fatty acids. Unsaturated fatty acids also increased Ca^{2+} uptake into microsomes. The increased microsomal Ca^{2+} uptake was observed even in the presence of the Ca^{2+} -ATPase inhibitor, orthovanadate, but was partially inhibited in the presence of a physiological concentration of ATP. The reduction in synaptosomal $[Ca^{2+}]_i$ by unsaturated fatty acids is most likely due to enhanced mechanisms for extrusion of Ca^{2+} across the plasma membrane.

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I. INTRODUCTION

The involvement of Ca^{2+} as a second messenger had been suggested for a variety of cellular physiological roles (Campbell, 1987, 1988). It is important to understand how cellular calcium homeostasis is maintained and is selectively elevated as a response to specific stimuli. While not alone in their use of Ca^{2+} in signaling, neurons have been extensively studied with respect to Ca^{2+} homeostasis.

One of the central roles for Ca^{2+} in neurons is the trigger for exocytotic release of neurotransmitters from synaptic vesicles. The "Ca hypothesis" of transmitter release proposed by Katz (1969) is now widely accepted and a great deal of information has established the molecular processes involved in neurotransmitter release (reviewed in Augustine *et al.*, 1987 and Smith and Augustine, 1988). Membrane depolarization opens voltage-sensitive Ca^{2+} channels and allows Ca^{2+} entry into the cytoplasm. Elevated Ca^{2+} activates Ca^{2+} /calmodulin dependent protein kinase, which phosphorylates a synaptic vesicle associated protein, synapsin I (Huttner *et al.*, 1981). Synapsin I is thought to link synaptic vesicles to other cytoskeletal elements, such as microtubules and spectrin (Navone *et al.*, 1984). Phosphorylation of synapsin I causes dissociation of synapsin I from the synaptic vesicle (Huttner *et al.*, 1983) and may result in the fusion of the synaptic vesicle to the plasma membrane. This fusion releases neurotransmitters stored within the vesicle lumen. By microinjection of either Ca^{2+} /calmodulin dependent protein kinase or dephosphorylated synapsin I into squid giant nerve terminals, Llinas *et al.* (1985) provided the direct evidence that Ca^{2+} -dependent phosphorylation of synapsin I modulates the release of neurotransmitters.

In order to return to a responsive state, the increased Ca^{2+} is extruded, sequestered, or buffered back to the low resting level (Blaustein, 1988). Several

coordinated systems maintain the intracellular calcium concentration ($[Ca^{2+}]_i$) in the nanomolar range against a 1 ~ 2 mM extracellular calcium concentration (reviewed in Carafoli, 1987; Meldlesi *et al.*, 1990). Neurons have two independent transporters in their plasma membrane for the extrusion of Ca^{2+} : Na^+ / Ca^{2+} exchanger and Ca^{2+} -ATPase. The movement of Na^+ down its electrochemical gradient drives the efflux of Ca^{2+} from the cytosol through Na^+ / Ca^{2+} exchanger (Blaustein *et al.*, 1982, Dipolo, 1987). Ca^{2+} -ATPase also extrudes Ca^{2+} to the extracellular space but in this case Ca^{2+} transport is coupled to ATP hydrolysis (Carafoli, 1991).

Sequestration of Ca^{2+} in intracellular organelles is another important mechanism to reduce elevated cytosolic Ca^{2+} . Ca^{2+} -ATPase located in the membrane of endoplasmic reticulum (ER) mainly serves to accumulate Ca^{2+} into the ER which serves as an intracellular Ca^{2+} store (Verma *et al.*, 1990). The Ca^{2+} pump of the ER is calmodulin insensitive unlike that of the plasma membrane and has a slightly larger molecular weight. Synaptic vesicles also sequester Ca^{2+} concomitant with ATP hydrolysis. Because of low affinity for Ca^{2+} and small luminal volume, their capacity is thought to be much less than that of ER and, therefore, it is unlikely that they contribute appreciably to the control of $[Ca^{2+}]_i$.

In early studies, mitochondria were thought of as a main internal store of Ca^{2+} and it was assumed that they played a key role in intracellular calcium homeostasis (Lehninger, 1970). However, mitochondria are now considered to play a role only when $[Ca^{2+}]$ reaches very high concentrations. When $[Ca^{2+}]_i$ rises to an abnormally high level, mitochondria function as a high capacity safety apparatus to prevent or at least postpone cell death caused by high $[Ca^{2+}]_i$ (Carafoli, 1987).

In the mammalian brain, there are a number of Ca^{2+} -binding proteins, such as calmodulin (CaM), parvalbumin, calbindin-D28K, calretinin, and annexins

(Baimbridge, *et al.*, 1992). These bind Ca^{2+} with high affinity and rapidly buffer Ca^{2+} in the cytosol as Ca^{2+} diffuses away from specific sites following its entry from the extracellular space or its release from ER. Some of these proteins serve additional roles in cytoskeletal organization, cell-motility and differentiation, and cell cycle regulation. Overall, these Ca^{2+} binding proteins are limited in their capacity for Ca^{2+} buffering and may fine-tune $[\text{Ca}^{2+}]_i$, while extrusion and/or sequestration systems serve to reduce elevated $[\text{Ca}^{2+}]_i$. Regardless of secondary importance for maintenance of Ca^{2+} homeostasis, it is notable that changes in the expression of Ca^{2+} buffering proteins are associated with neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, ischemia, and epilepsy (Heizmann and Braun, 1992).

In neurons, there are two major mechanisms by which $[\text{Ca}^{2+}]_i$ can be increased. One is mediated by voltage sensitive Ca^{2+} channels in the plasma membrane (Tsien, 1990). Depolarization, which characterizes the electrical signal for the communication between neurons, causes the inward movement of extracellular Ca^{2+} through voltage sensitive channels.

The other mechanism is Ca^{2+} mobilization from the internal ER store, through the inositol 1,4,5-trisphosphate (IP_3) receptor, a ligand-gated Ca^{2+} channel (reviewed in Berridge, 1993). Released neurotransmitters, such as glutamate and adrenaline, bind to their specific receptors on adjacent neurons, resulting in the activation of phospholipase C- β (PLC) via interaction with one or more G-proteins (Taylor *et al.*, 1991). Another isozyme, PLC- γ , can be activated through phosphorylation by a tyrosine kinase such as those associated with growth factor receptor (Carpenter, 1992). Both PLC- β and PLC- γ hydrolyze phosphatidylinositol 4,5-bisphosphate to generate two second messengers, diacylglycerol (DG) and IP_3 . IP_3 , in turn, binds to the IP_3 receptor on ER and releases luminal Ca^{2+} from ER (Berridge, 1987; Berridge and Irvine, 1989).

As part of signal transduction, Ca^{2+} mobilization mediates activation of a variety of cell activities. In neurons, targets for the increased $[\text{Ca}^{2+}]_i$ can be divided into two groups, those in the plasma membrane and those in the cytosol (Kennedy, 1989). Among the Ca^{2+} targets in the plasma membrane are Ca^{2+} dependent channels selective for K^+ (Blatz and Magleby, 1987), for monovalent cations (Partridge and Swandulla, 1988), or for Cl^- . Ca^{2+} generally increases the probability of opening of these channels. Another target is phospholipase A_2 (PLA_2), which cleaves fatty acids, principally arachidonic acid, from phospholipids (Van Den Bosch, 1980; Irvine, 1982). A rise in $[\text{Ca}^{2+}]_i$ can activate PLA_2 . While it is involved in Ca^{2+} mobilization, PLC is also activated by Ca^{2+} at higher Ca^{2+} concentration (Majerus *et al.*, 1986; Lo *et al.*, 1993).

The other category of Ca^{2+} targets (cytosolic targets) includes protein kinase C (PKC), CaM-dependent protein kinase (CaM kinase), and calpain (Kennedy, 1989). PKC plays an important role in receptor desensitization (Huganir and Greengard, 1990). PKC also enhances neurotransmitter release, which occurred in Ca^{2+} independent manner (Nishizuka, 1984; Malenka *et al.*, 1987). In addition, modulation of ion channels by PKC has been demonstrated in a variety of tissues (Shearman *et al.*, 1989). K^+ , Cl^- , and Ca^{2+} channel activities are most commonly reduced although the mechanism and the nature of reduction in these ion channel activities are not established yet.

CaM kinase plays important roles in neurotransmitter release and synthesis. It regulates ion channels, cytoskeletal function and gene expression (Hanson, 1992). Gene expression also appears to be modulated by PKC (Kikkawa and Nishizuka, 1986).

Calpain is a Ca^{2+} -dependent cysteine proteinase. It seems to act in regulation of membrane proteins and cytoskeleton and may be involved in neuronal degeneration (Melleni and Pontremoli, 1989). The expression of these cytosolic

target proteins for Ca^{2+} is altered in the brain of patients with Alzheimer's disease (Iwamoto and Emson, 1991).

Impaired calcium homeostasis in cells is observed under a variety of pathological conditions, such as ischemia and seizures (Chien *et al.*, 1979). In these necrotic tissues, Ca^{2+} is abnormally elevated and eventually causes cell-death. Intracellular calcium homeostasis is also disrupted by many toxic compounds which alter membrane integrity and cytoskeletal structure (Schanne *et al.*, 1979; Jewell *et al.*, 1982). Disruption of intracellular calcium homeostasis is also observed during immunological responses (Poenie *et al.*, 1987). Throughout the process of killing by cytotoxic T cell lymphocytes (CTL), intracellular free calcium ($[\text{Ca}^{2+}]_i$) is elevated in target cells and may contribute to cytolysis of target cells.

Previous work in this laboratory has included two independent studies of modifiers of synaptic function, ethanol and fatty acids. Each of these may involve Ca^{2+} as a mediator of their neuroactivity. In these and other studies, nerve ending preparations (synaptosomes) have been extensively applied as a model for understanding nerve function (Gray and Whittaker, 1962). Since synaptosomes resemble the intact cells enclosing a variety of organelles, they are still able to maintain calcium homeostasis (Jones, 1975). Synaptosomes were also used in the present study.

Ethanol has a variety of behavioral effects such as hypnosis, anesthesia, and intoxication. It alters the properties of the lipid-bilayer of cell membranes (Miceli and Ferell, 1973; LaDroite *et al.*, 1984), resulting in changes in numerous peripheral or integral membrane proteins. Among these, Na^+ , K^+ ATPase (Sun and Sun, 1983), a ubiquitous protein in eukaryotic plasma membrane responsible for the maintenance of osmolarity, transmembrane gradients of Na^+ and K^+ and membrane electrical potential, has been extensively studied. Stimulation of Na^+ , K^+ -ATPase

by ethanol may decrease intracellular Ca^{2+} . Na^+ , K^+ -ATPase provides the Na^+ electrochemical potential required for Ca^{2+} extrusion via $\text{Na}^+/\text{Ca}^{2+}$ exchanger. However, ethanol has been reported to increase intracellular Ca^{2+} (Hoek *et al.*, 1987; Rubin *et al.*, 1988) and to affect Na^+ , K^+ -ATPase activity in biphasic manner. High concentrations of ethanol (> 200 mM) inhibit Na^+ , K^+ -ATPase activity (Israel *et al.*, 1965, 1966, Marques and Guerri, 1988; Nhamburo *et al.*, 1987), while low concentrations (< 100 mM) stimulate Na^+ , K^+ -ATPase (Marques and Guerri, 1988; Swann, 1990; Foley and Rhoads, 1994). Since Ca^{2+} itself has been demonstrated to inhibit Na^+ , K^+ -ATPase (Skou, 1957), Ca^{2+} could be a mediator of the action of ethanol on Na^+ , K^+ -ATPase. In red blood cells, calnaktin has been reported as an endogenous inhibitory protein for Na^+ , K^+ -ATPase. Calnaktin inhibits Na^+ , K^+ -ATPase in a Ca^{2+} dependent manner that increases the sensitivity of the enzyme to inhibition by Ca^{2+} (Yingst, 1988). A calnaktin-like inhibitor is present in a rat brain 6/50 protein fraction (molecular weight between 6 and 50 kD)(Wang, 1993). This calnaktin-like protein inhibits Na^+ , K^+ -ATPase up to 70 % in a Ca^{2+} dependent manner. In the presence of the 6/50 fraction, inhibition of Na^+ , K^+ -ATPase was observed at a Ca^{2+} concentration as low as 100 nM and was half maximal at $3\mu\text{M}$ Ca^{2+} . There was no effect of the 6/50 fraction on Na^+ , K^+ -ATPase in the absence of Ca^{2+} . Also, there was no effect of Ca^{2+} on semi-purified Na^+ , K^+ -ATPase in the absence of the calnaktin-like inhibitor.

The reported elevation of intracellular Ca^{2+} by ethanol and the presence of a calnaktin-like protein that inhibits Na^+ , K^+ -ATPase when intracellular Ca^{2+} reaches the micro molar level raises the possibility that Ca^{2+} /calnaktin modulates the inhibition of Na^+ , K^+ -ATPase by ethanol. Three related questions need to be addressed to test this possibility. (1) Compared to the minimum requirement of calnaktin for Ca^{2+} , what is the resting Ca^{2+} level, i.e., is there basal inhibition of Na^+ , K^+ -ATPase? (2) Compared to the Ca^{2+} -sensitivity of calnaktin, to what

extent does ethanol elevate intracellular Ca^{2+} ? (3) Are the concentrations of ethanol that elevate intracellular Ca^{2+} relevant to alcohol intoxication?

In the present study, the intrasynaptosomal Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured under physiological conditions and the effect of ethanol on $[\text{Ca}^{2+}]_i$ was examined. The key question was whether ethanol elevated $[\text{Ca}^{2+}]_i$ enough to activate the calnaktin-like inhibitor of Na^+ , K^+ -ATPase. In addition, the mechanism by which ethanol alters calcium homeostasis in synaptosomes was partially addressed.

Fatty acids have also been shown to alter synaptic activity in a manner that could involve elevation of $[\text{Ca}^{2+}]_i$. The actions of neurotransmitters released from presynaptic compartment are terminated by two mechanisms. One mechanism is that neurotransmitter is metabolized by the enzymes present in the synaptic cleft. The other is mediated by Na^+ / amino acid symport, through which the released neurotransmitters are transported back into the presynaptic terminal (reuptake) in a Na^+ -dependent manner (Iversen, 1974). Previous work by Rhoads *et al.* (1982, 1983) demonstrated that unsaturated fatty acids, but not saturated fatty acids, inhibited the uptake of amino acid neurotransmitters in synaptosome preparations. During ischemia, elevated levels of free fatty acids (FFA), especially unsaturated fatty acids such as arachidonic acid and oleic acid, were observed in the brain (Galloc, 1976). These FFA were also elevated in brain tissue following induced seizures (Bazan and Spagnuol, 1970; Siesjo *et al.*, 1982).

The mechanism whereby unsaturated fatty acids inhibit amino acid uptake has been partially elucidated. Rhoads *et al.* (1983) presented evidence that unsaturated fatty acids cause the uncoupling of Na^+ / amino acid symport. Inhibition of uptake and stimulation of amino acid release occurred under conditions where Na^+ permeability was not altered by unsaturated fatty acids. This can be explained if amino acid transport is uncoupled from the Na^+ concentration

gradient in the presence of unsaturated fatty acids. It was suggested that perturbation of the lipid or lipid-protein interaction by unsaturated FFA in the vicinity of the carrier proteins was responsible for this uncoupling. In contrast, based on the change in the transmembrane electrical potential and the total intrasynaptosomal Ca, measured by a radiolabeled $^{45}\text{Ca}^{2+}$ equilibration technique, another group of investigators (Troeger *et al.*, 1984) proposed the involvement of depolarization of the plasma membrane by unsaturated fatty acids. These investigators suggested depolarization resulted in the release of neurotransmitters and inhibition of amino acid uptake .

Of the two proposed mechanisms, the uncoupling proposal is independent of a change in $[\text{Ca}^{2+}]_i$, whereas the depolarization proposal has elevated $[\text{Ca}^{2+}]_i$ as an absolute requirement. In order to elucidate the mechanism involved in the inhibition of amino acid uptake by unsaturated fatty acids, the present study was also aimed to examine if fatty acids, especially unsaturated fatty acids, cause the membrane depolarization, which would be indicated as an increase in $[\text{Ca}^{2+}]_i$.

Historically, the very low level of the intracellular Ca^{2+} concentration was an obstacle for its accurate measurement. However, Grynkiewicz *et al.* (1985) developed the measurement of cytosolic Ca^{2+} using fluorescent dyes. The second generation of these Ca^{2+} -sensitive dyes included Fura2. Fura2 has four carboxyl groups in its backbone similar to those of EGTA, a well-known Ca^{2+} chelator. Fura2 is able to chelate Ca^{2+} with high affinity and specificity. The Ca^{2+} binding specificity of Fura2 is 10^5 times that of EGTA and the dissociation constant is in the nanomolar range and is close to the physiological intracellular Ca^{2+} concentration. This great improvement enables us to measure quantitatively cytosolic Ca^{2+} . The important characteristic of Fura2 is that the binding of Ca^{2+} shifts the excitation spectrum of Fura2 to shorter wavelength. Ca^{2+} -bound Fura2 and Ca^{2+} -unbound Fura2 are monitored at 340 and 380 nm, respectively.

Therefore, the measurement is carried out at two wavelengths, 340 and 380 nm, giving a ratio at two wavelengths for the calculation of Ca^{2+} concentration. One more advantage of Fura2 is that the carboxyl groups on Fura2 can be esterified with acetoxymethyl groups, so that Fura2 becomes permeable to the plasma membrane. This allows it to be loaded into the cytoplasm without any puncture of plasma membrane. After esterified Fura2 is loaded into the cells, it is hydrolyzed by cytoplasmic esterases to regenerate and to trap the dye into the cytosol (Tsien, 1988). The method using Fura2 has been well established in a variety of cells and Fura2 is now most widely used for studying the physiological role of Ca^{2+} where accurate measurements of Ca^{2+} levels are required.

In summary, the present study is composed of the following three objectives:

- (1) To use Fura2 to measure intrasynaptosomal Ca^{2+} concentrations under physiological conditions.
- (2) To determine how ethanol effects the intrasynaptosomal Ca^{2+} concentration and if ethanol elevates Ca^{2+} to a level sufficient for the action of a calnaktin-like inhibitor of Na^+ , K^+ -ATPase.
- (3) To determine the effects of representative fatty acids on intrasynaptosomal Ca^{2+} concentration in the context of two proposed mechanisms for the inhibition of neurotransmitter amino acid uptake by unsaturated fatty acids.

II. MATERIALS AND METHODS

1. *Preparation of Crude Synaptosomes and Microsomes*

Adult male rats (Charles River, Wilmington, MA) were sacrificed by decapitation using thin plastic cones for restraint (Decapicones; Braintree Scientific Inc., Braintree, MA). Whole brains were rapidly removed and dissected on ice into two parts, cerebellum and cerebrum. The crude synaptosome fraction (P_2) was prepared by a method adapted from that of Gray and Whittaker (1962). Each tissue was homogenized in 10 volumes of ice-cold homogenization buffer (0.32M sucrose and 10 mM Tris base adjusted to pH 7.0 with 1N HCl) with 4 - 5 strokes in a Teflon glass homogenizer. Centrifugations were carried out at 4°C in a Sovall RC-2 or RC-5B centrifuge. First, the homogenate was centrifuged for 20 min at $1000 \times g$. The resulting supernatant was removed and centrifuged for 20 min at $12,000 \times g$. From this centrifugation, the supernatant (S_2) was discarded or used for microsomal preparation (see below), while the pellet (P_2) was removed and washed twice by resuspension in homogenization buffer and centrifugation for 10 min at $12,000 \times g$. The washed pellet was designated crude synaptosomes.

In experiments requiring an endoplasmic reticulum rich fraction, a classical microsomal fraction (P_3) was prepared from ultracentrifugation of the supernatant (S_2) at $100,000 \times g$ for 30 min in Beckman L2-65B ultracentrifuge by a method adapted from that of Daniell and Harris (1989). The microsomal fraction was resuspended in a microsomal assay buffer (20 mM Hepes, 2 mM $MgCl_2$, 150 mM KCl, and 0.5 mM sodium azide with the pH adjusted to 7.0 with Tris base) and used immediately for Ca^{2+} measurements or stored frozen at -20 °C.

2. Purification of Synaptosomes

To obtain a highly enriched synaptosome fraction, the P₂ fraction was centrifuged on a discontinuous Ficoll gradient (Booth and Clarke, 1978) as modified previously (Foley and Rhoads, 1994). The P₂ fraction was resuspended in ice-cold 12 % Ficoll in 0.32 M sucrose. Equal volumes of 6 % Ficoll in 0.32 M sucrose and homogenization buffer were layered sequentially on top of the 12 % Ficoll in an ultracentrifuge tube. After ultracentrifugation (SW41Ti rotor) for 45 min at 55,000 × g, the synaptosomal band was collected from the interface of the 6 and 12 % Ficoll layer, diluted with homogenation buffer and centrifuged for 10 min at 15,000 × g. The pellet was resuspended in 20 volumes of homogenation buffer (supplemented with 1 mM EGTA) homogenized manually with 3 strokes and incubated on ice for 30 min. This step was repeated twice. The final synaptosome fraction was collected by centrifugation for 20 min at 40,000 × g, resuspended in synaptosomal assay buffer (10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 1 mM CaCl₂, and 10 mM glucose with pH adjusted to 7.5 with Tris base) and used immediately after preparation.

3. Protein Determination

Protein concentration of synaptosomal and microsomal fractions was determined using Bio-Rad Protein Assay Kit, which is based on the Bradford dye binding procedure (1976). Bovine serum albumin was used as standard.

4. Measurement of intrasynaptosomal Ca²⁺ concentration

Intrasynaptosomal Ca²⁺ concentration was measured by using a fluorescent dye, its acetoxymethyl ester form (Fura2-AM) (purchased from Sigma Chemical Co., St.Louis, MO). As described by Grynkiewicz *et al.* (1985), fluorescence intensity was monitored at two excitation wavelengths, 340 nm and

380 nm with emission intensity measured at 505 nm using a Perkin-Elmer LS-5B spectrophotometer operated with an IBM PC. The data was collected and analyzed using FURA2 software supplied by Perkin-Elmer for IBM PC. The loading conditions for Fura2-AM were as described for synaptosomes by Daniell *et al.* (1987) and Thomas and Delaville (1991) with slight modification. Fura2-AM (0.5 nmol Fura2-AM / mg of synaptosomal protein), the membrane permeable form of Fura2, was loaded into synaptosomes during a 40 min incubation at room temperature. The concentration of Fura2-AM during incubation was adjusted to 5 μ M using synaptosomal assay buffer. After incubation, synaptosomes were washed three times by microcentrifugation and resuspension in synaptosomal assay buffer, and then divided into aliquot amounts containing about 100 μ g of synaptosomal protein. Each aliquot of synaptosomes was diluted to 2 ml with assay buffer in a quartz cuvette for measurement of fluorescence.

The intrasynaptosomal Ca^{2+} concentration was calculated according to the equation described by Grynkiewicz *et al* (1985):

$$[\text{Ca}^{2+}] = K_d \times \frac{R - R_{\min}}{R_{\max} - R} \times \frac{S_{f\ 380}}{S_{b\ 380}}$$

where K_d is the dissociation constant, 224 nM at 37 °C, R is the fluorescence ratio $[\text{Ca}^{2+} \text{ bound (340nm)} / \text{Ca}^{2+} \text{ free (380nm)}]$, $R_{\max}$ and $R_{\min}$ are the fluorescence ratio under saturating Ca^{2+} and Ca^{2+} -free conditions, respectively, and $S_{f\ 380} / S_{b\ 380}$ refers to the fluorescence ratio at 380 nm for Ca^{2+} free / saturating Ca^{2+} conditions. $R_{\max}$ was determined in the presence of 0.1 % sodium dodecyl sulfate and $R_{\min}$ was determined in the presence of 50 mM Tris base plus 5 mM EGTA, which is equivalent to 5 times the medium Ca^{2+} content and has been shown to eliminate the free Ca^{2+} .

The leak of Fura2 or unhydrolyzed Fura2-AM from synaptosomes was measured by addition of MnCl_2 (final concentration, 40 μM). Mn^{2+} quenches Fura2 outside synaptosome because it binds to Fura2 with a higher affinity than Ca^{2+} . Because Mn^{2+} does not cross the synaptosomal membrane, it quenches extrasynaptosomal Fura2 only. The decrease in fluorescence intensities in the presence of MnCl_2 was less than 3 % of total fluorescence intensity, was considered as the leak of Fura2 or Fura2-AM and was subtracted from the data before the calculation of intrasynaptosomal Ca^{2+} concentration.

5. The Effect of Ethanol on Intrasynaptosomal Ca^{2+} Concentration

The effect of ethanol on intrasynaptosomal Ca^{2+} concentration was monitored at concentrations of ethanol from 25 to 800 mM. At 3 min intervals, aliquots of ethanol (100 %) were added to a cuvette, containing synaptosomes, and any change in fluorescence intensity was measured over a total period of 24 min. Following each addition, the fluorescence intensity was found to stabilize within 3 min. The concentration of ethanol was started at 25 mM and ended at 800 mM over a 24 min time period. As a control, another cuvette containing synaptosomes had the same volume of H_2O (instead of ethanol) added at 3 min intervals and the fluorescence intensity was monitored. This served as a control for the small volume changes and for any mixing artifacts.

Other experiments examined the effect of a single concentration of ethanol. Before the addition of ethanol or H_2O (controls), the resting Ca^{2+} concentration was measured. After the addition of ethanol or H_2O , synaptosomes were incubated for 10 min in 36 °C water bath. Intrasynaptosomal Ca^{2+} concentration was then measured. All measurements were carried out at room temperature.

6. Ca^{2+} Release from Microsomes

The effect of ethanol on microsomes was also examined as described by Daniell and Harris (1989). In these experiments, the microsomal fraction was used and extra-microsomal Ca^{2+} concentrations were measured using Fura2, its free form (purchased from Sigma Chemical Co., St. Louis, MO). No loading with Fura2-AM, acetoxymethyl ester form, was needed. Fluorescence was monitored at the two excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 505 nm. R_{max} was determined in the presence of excess free Ca^{2+} (1.4 mM CaCl_2 , final concentration) to saturate the Fura2 and R_{min} was determined in the presence of 6 mM EGTA plus 13 mM Tris base to chelate the free Ca^{2+} . These parameters were applied to the method of Grynkiewicz *et al.* (1985) and extra-microsomal Ca^{2+} concentration was calculated by the ratioing method described above.

Each sample was analyzed independently by a method adapted from that of Daniell and Harris (1989). An aliquot amount of the microsomal fraction (100 μg microsomal protein) was placed in a quartz cuvette, followed by mitochondria inhibitors [antimycin (0.06 μg / ml), oligomycin (2 μg / ml), and rotenone (1 μM)] and Fura2 (0.14 μM final concentration). The total volume was adjusted to 2 ml using the microsomal assay buffer.

Before the addition of ethanol, each sample was treated with H_2O (control) or ATP (1 mM final concentration) to activate Ca^{2+} -ATPase in microsomal membrane so that any leaked Ca^{2+} would be taken back into the microsomes. This is, in effect, reloading the microsomes with Ca^{2+} . Approximately 1 min after addition of H_2O or ATP, the Ca^{2+} -ATPase inhibitor, Na orthovanadate (50 μM final concentration) was added to prevent further uptake of subsequently released Ca^{2+} into microsomes. After the microsomes were prepared in this manner, ethanol was added to the cuvette and any change in extramicrosomal $[\text{Ca}^{2+}]$ was

determined. For a control experiment, the same volume of H₂O was added instead of ethanol. Released Ca²⁺ (the increase in extramicrosomal [Ca²⁺]) was expressed as nanomoles of Ca²⁺ per milligram of microsomal protein. For comparison, Ca²⁺ release from the microsome in the presence of inositol-1,4,5-trisphosphate (IP₃) (generously provided by Dr. C.-S.Chen) was examined using the same method.

The microsomal fraction was also exposed in the presence or the absence of ATP to ethanol ranging from 25 mM to 800 mM over a period of 24 min. Prior to the addition of ethanol, microsomes were treated with ATP and/or Na orthovanadate as described above. An aliquot of ethanol (100%) was added to the cuvette every 3 min and fluorescence intensity was monitored over time as described above.

All measurements were carried out at room temperature.

7. The Effect of Fatty Acids on Intrasynaptosomal Ca²⁺ concentration

After the measurement of resting intrasynaptosomal Ca²⁺ concentration for a 2 min period as described above, an appropriate amount of fatty acid was added directly in the cuvette. Fluorescence intensity was monitored and Ca²⁺ concentration was calculated as described above.

To examine the effect of bovine serum albumin (BSA) on fatty acids, synaptosomes were suspended in the assay buffer containing BSA (15 μM final concentration) and intrasynaptosomal Ca²⁺ concentration was measured before and after the addition of fatty acids as described above. The effect of BSA was also examined after synaptosomes were treated with fatty acids. A 1 ml aliquot of the synaptosome suspension from the cuvette where fatty acid was added was transferred to a test tube containing solid BSA and mixed gently. After BSA was dissolved, the synaptosome suspension was transferred back to the cuvette, mixed, and intrasynaptosomal Ca²⁺ concentration was measured again.

8. The Application of Protein Kinase C Inhibitor, H-7, to Synaptosomes

In order to examine the involvement of protein kinase C (PKC) in the effect of oleic acid on intrasynaptosomal Ca^{2+} concentration, a membrane permeable PKC inhibitor, H-7 (purchased from RBI, Natick, MA), was applied to synaptosomes. H-7 (dissolved in dH_2O) was added to the synaptosomes suspended in assay buffer (10 or 50 μM final concentration) after the measurement of resting intrasynaptosomal Ca^{2+} concentration for a two min period as described above. Two min after addition of H-7, an appropriate amount of oleic acid was added and intrasynaptosomal Ca^{2+} concentration was monitored for the following two min.

9. The Effect of Fatty Acids on Microsomes

The effects of fatty acids on microsomes were also examined. The extramicrosomal $[\text{Ca}^{2+}]$ was monitored before and after the addition of fatty acids using Fura2. The experimental conditions were the same as the ethanol experiments with microsomes described above. An aliquot amount of microsomes (100 μg) was placed in a quartz cuvette with mitochondria inhibitors [antimycin (0.06 $\mu\text{g}/\text{ml}$), oligomycin (2 $\mu\text{g}/\text{ml}$), and rotenone (1 μM)]. The measurement of fluorescence intensity was initiated by the addition of Fura2 (0.14 μM final concentration). Fluorescence intensity was monitored and extramicrosomal $[\text{Ca}^{2+}]$ was calculated as described above. Two min after the measurement of the resting $[\text{Ca}^{2+}]$ level, an appropriate amount of fatty acid was added directly in the cuvette. A change in fluorescence intensity was monitored for another 2 min. The average extramicrosomal $[\text{Ca}^{2+}]$ after the addition of fatty acids was compared to before the addition.

The effect of unsaturated fatty acid on extramicrosomal $[\text{Ca}^{2+}]$ was examined either in the presence of ATP or orthovanadate (VO_4^{3-}) in the same manner. The extramicrosomal $[\text{Ca}^{2+}]$ was measured throughout the addition of the

following compounds. Before the addition of unsaturated fatty acid, microsomes were treated with either ATP (1 mM final concentration) or orthovanadate (VO_4^{3-}) (50 μM final concentration). Two min after this treatment, an appropriate amount of unsaturated fatty acid was added and the extramicrosomal $[\text{Ca}^{2+}]$ after the addition of unsaturated fatty acid was compared to after the treatment with either ATP or VO_4^{3-} .

III. RESULTS

(1) Resting Intrasyntosomal Ca^{2+} Concentration

Intrasyntosomal free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) were measured under resting (no stimuli) conditions in physiological buffer. Average $[\text{Ca}^{2+}]_i$ levels were 304 nM and 250 nM in cerebrum and cerebellum, respectively (Table 1.). The difference in $[\text{Ca}^{2+}]_i$ between cerebrum and cerebellum was not significant, considering their errors. Values for $[\text{Ca}^{2+}]_i$ ranged between 150 and 400 nM for the individual determinations.

(2) The Effects of Ethanol on Intrasyntosomal Ca^{2+} Concentration

Syntosomes prepared from cerebrum were exposed to ethanol, beginning at 25 mM, which is considered indicative of intoxication, and ending, at 800 mM, which is much more than lethal concentration (Figure 1). The ethanol concentration was increased stepwise at 3 min intervals as indicated. After each addition of ethanol, there was a rapid and transient increase in $[\text{Ca}^{2+}]_i$, which returned back to a baseline level. Throughout the exposure to ethanol, the baseline of $[\text{Ca}^{2+}]_i$ gradually increased with increasing concentration of ethanol. In Figure 2, $[\text{Ca}^{2+}]_i$ at the peak point (highest point) after the addition of ethanol as well as the average baseline $[\text{Ca}^{2+}]_i$ between additions of ethanol were plotted versus concentration of ethanol. This analysis shows there was slight drift in the baseline during control experiments where the same volume of H_2O was added instead of ethanol. However, it reveals a greater increase in the baseline in the presence of ethanol. In examining the peak responses at each ethanol concentration, the maximum level of $[\text{Ca}^{2+}]_i$ that can be attained by ethanol is established. At the highest concentration of ethanol (800 mM), $[\text{Ca}^{2+}]_i$ of the baseline rose about 14 % to 280 nM with a peak $[\text{Ca}^{2+}]_i$ of 293 nM.

Enriched synaptosome preparations were obtained from rat cerebrum and cerebellum. These preparations were incubated with ethanol to determine if ethanol causes a differential increase in $[Ca^{2+}]_i$ in synaptosomes from two brain regions. As shown in Figure 3, only a 1-2 % increase in $[Ca^{2+}]_i$ was observed at a low concentration of ethanol (100 mM) in preparation from cerebrum. Relatively high concentrations of ethanol (350 and 700 mM) resulted in higher $[Ca^{2+}]_i$. Compared to control experiments where same volume of H_2O was added instead of ethanol, 350 and 700 mM ethanol increased $[Ca^{2+}]_i$ by 9 and 17 %, respectively in cerebrum. Enriched synaptosome preparations from cerebellum were even less responsive to ethanol, (Figure 3). There was essentially no change in $[Ca^{2+}]_i$ at 100 mM ethanol, while 3 and 10 % increases were caused by 350 and 700 mM ethanol, respectively. Although it seems that cerebral synaptosomes were more sensitive to ethanol than preparations from cerebellum, in both cases any significant increase required a very high concentration of ethanol relative to the lethal blood ethanol concentration (200 mM).

In order to examine if the increase in $[Ca^{2+}]_i$ caused by ethanol resulted from the internal Ca^{2+} store, endoplasmic reticulum (ER), and was perhaps attenuated by Ca^{2+} uptake of ER in the intact synaptosome, the release of Ca^{2+} from a microsomal fraction, which is an ER rich fraction, was monitored in the presence of ethanol. In the case of microsomes, extramicrosomal Ca^{2+} concentration ($[Ca^{2+}]$) was measured using Fura2 (free form) and a change in extramicrosomal $[Ca^{2+}]$ was quantified as Ca^{2+} release from microsomes.

Similar to the approach used with synaptosomes, microsomes were continuously exposed to ethanol starting at 25 mM and ending at 800 mM. Before the addition of ethanol, microsomes were treated with ATP to activate Ca^{2+} -ATPase allowing the accumulation of Ca^{2+} into ER. The addition of ATP resulted in a rapid decline in external $[Ca^{2+}]$ (Figure 4). Subsequent addition of

orthovanadate, which is a Ca^{2+} -ATPase inhibitor, to prevent further uptake of Ca^{2+} , stopped this decline and provided a steady baseline of extramicrosomal $[\text{Ca}^{2+}]$ (Figure 4). Every addition of ethanol caused an increase in extramicrosomal $[\text{Ca}^{2+}]$, interpreted as release of microsomal Ca^{2+} (Figure 4). This change in extramicrosomal $[\text{Ca}^{2+}]$ was not transient as it was in synaptosomes. There was essentially no change in extramicrosomal $[\text{Ca}^{2+}]$ in the control experiments. The difference in extramicrosomal $[\text{Ca}^{2+}]$ at the starting point (at time = 0) between control and ethanol experiments is simply because each starting extramicrosomal $[\text{Ca}^{2+}]$ value originated from the leak of Ca^{2+} initially present in the ER and varied considerably in each experiment.

Microsomes were also exposed to increasing concentrations of ethanol in the absence of ATP. Discrete increases in extramicrosomal $[\text{Ca}^{2+}]$ were not observed after each addition of ethanol (Figure 5). Nevertheless increased extramicrosomal Ca^{2+} was observed at high ethanol concentrations (< 400 mM).

The amounts of released Ca^{2+} from microsomal fraction upon each addition of ethanol, either in the presence or in the absence of ATP, were plotted versus concentrations of ethanol (Figure 6). This analysis showed the release of Ca^{2+} by ethanol was dose dependent. In the absence of ATP, 600 and 800 mM ethanol caused relatively large Ca^{2+} release from microsomes. The values obtained below 200 mM ethanol were very small and there appeared to be no effect of ATP.

In Table 2, the amount of Ca^{2+} released from the microsomal fraction by 100 mM ethanol, a high intoxicating dose of ethanol, was compared with that caused by 1 μM IP_3 , which is known specifically to mobilize Ca^{2+} from the ER. In the presence of ATP, 100 mM ethanol released 0.43 nmol Ca^{2+} per mg of microsomal protein. This level of release was much smaller than that by IP_3 which released 1.61 nmol Ca^{2+} / mg protein. In the absence of ATP (i.e., without

reloading of leaked Ca^{2+} from the microsomes), almost no release of Ca^{2+} was observed by ethanol, in contrast, IP_3 still released 0.67 nmol Ca^{2+} / mg protein.

(3) The Effects of Fatty Acids on Intrasyntosomal Ca^{2+} Concentration

Intrasyntosomal Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) were measured in the presence and in the absence of exogenous fatty acids. Prominent differences in effects of fatty acids between saturated and unsaturated fatty acids were observed (Figure 7). The saturated fatty acid, palmitic acid, had little effect at low concentrations (10 ~ 15 μM) and even a high concentration of palmitic acid (100 μM) caused only a 10 % decrease in resting $[\text{Ca}^{2+}]_i$. In contrast to palmitic acid, unsaturated fatty acids, oleic acid and arachidonic acid, significantly decreased resting $[\text{Ca}^{2+}]_i$ in dose dependent manner. This decrease occurred rapidly after the addition of unsaturated fatty acids. Although $[\text{Ca}^{2+}]_i$ was not decreased considerably at 5 ~ 10 μM of arachidonic acid, a decrease in resting $[\text{Ca}^{2+}]_i$ was evident as low as 15 μM . A maximal 36 % decrease was observed at a concentration of 75 μM arachidonic acid. Half-maximal decrease occurred at a concentration of about 30 μM arachidonic acid. Unlike arachidonic acid, decrease in resting $[\text{Ca}^{2+}]_i$ caused by oleic acid was apparent as low as 5 μM . The maximal decrease was 40 % at 50 μM oleic acid and half-maximal decrease was observed at a concentration of about 12 μM oleic acid.

These effects of unsaturated fatty acids were shifted in the presence of 15 μM BSA as shown as closed symbols in Figure 8. Decrease in $[\text{Ca}^{2+}]_i$ by arachidonic acid required a concentration of 75 μM in the presence of BSA. BSA also shifted the dose-dependent decrease in $[\text{Ca}^{2+}]_i$ caused by oleic acid to higher oleic acid concentration.

In order to examine if this effect of fatty acid is reversible, the same amount of BSA was added after syntosomes were treated with oleic acid. Table

3 clearly shows that BSA did not reverse the decreases in $[Ca^{2+}]_i$ caused by 15, 30, and 50 μM of oleic acid. This indicated that the effect of unsaturated fatty acids was not readily reversal.

(4) The Application of Protein Kinase C Inhibitor (H-7) to Synaptosomes

Since there was the potential involvement of protein kinase C (PKC) in the decrease in $[Ca^{2+}]_i$ by unsaturated fatty acids, synaptosomes were treated with PKC inhibitor, H-7, before the addition of oleic acid. Preincubation with 10 μM of H-7, which is close to K_i (6 μM) for purified PKC (Hidaka H, *et al.*, 1984), had little effect on $[Ca^{2+}]_i$ (Table 4). This pretreatment was ineffective in preventing 15, 30, and 50 μM of oleic acid from decreasing $[Ca^{2+}]_i$ compared to in the absence of PKC inhibitor. Relatively high concentration of H-7 (50 μM), which exhibited inhibitory effect on PKC in platelet (Hidaka and Kawamoto, 1984) and in HL-60 cells (Nishikawa *et al.*, 1986), reduced $[Ca^{2+}]_i$ approximately 5%. After the treatment with 50 μM of H-7, the addition of oleic acid apparently caused more decrease in $[Ca^{2+}]_i$ than that not treated with PKC inhibitor. Thus, the application of either low or high concentration of PKC inhibitor did not attenuate the decrease in $[Ca^{2+}]_i$ caused by oleic acid and appeared to augment it.

(5) The Effect of Fatty Acids on Microsomes

In order to examine if the decrease in $[Ca^{2+}]_i$ by unsaturated fatty acids results from the increased uptake of Ca^{2+} into ER, extramicrosomal $[Ca^{2+}]$ was measured in the presence and in the absence of exogenous fatty acids. Similar to the experiments with synaptosomes, a prominent difference was observed between unsaturated fatty acids and saturated fatty acid (Figure 9). Unsaturated fatty acids, oleic acid and arachidonic acid, apparently decreased extramicrosomal $[Ca^{2+}]$ in dose dependent manner, suggesting increased Ca^{2+} uptake into ER or the increased

permeability of ER membrane. Although less than 15 μM of arachidonic acid did not cause a significant decrease in extramicrosomal $[\text{Ca}^{2+}]$, 25 % decrease was observed as low as 50 μM of arachidonic acid. In contrast, oleic acid even as low as 5 μM showed 10 % decrease and a maximal decrease was observed at approximately 12 μM . Oleic acid seems to be more potent to decrease extramicrosomal $[\text{Ca}^{2+}]$ than arachidonic acid. In contrast, up to 30 μM the saturated fatty acid, palmitic acid, had little effect on extramicrosomal $[\text{Ca}^{2+}]$. Extramicrosomal $[\text{Ca}^{2+}]$ was slightly increased at 50 μM palmitic acid.

The involvement of Ca^{2+} -ATPase on ER was also examined. Prior treatment of microsomes with Ca^{2+} -ATPase inhibitor, orthovanadate, did not attenuate the effect of oleic acid. The decrease in extramicrosomal $[\text{Ca}^{2+}]$ in the presence of oleic acid was the same in the presence of orthovanadate except at 5 μM oleic acid where the decrease in extramicrosomal $[\text{Ca}^{2+}]$ was even greater (30 %) in the presence of orthovanadate.

When microsomes were treated with ATP before the addition of oleic acid, the decreases in extramicrosomal $[\text{Ca}^{2+}]$ were suppressed. The decrease was less than 5 % at 12 μM oleic acid, which corresponds to the half-maximal dose in the absence of ATP. A 19 % decrease was observed at high concentration of oleic acid (50 μM) in the presence of ATP, however, the decrease appeared to be substantially smaller than that in the absence of ATP.

IV. DISCUSSION

(1) Resting Intrasyntosomal Ca^{2+} Concentration

Resting intrasyntosomal Ca^{2+} concentration under physiological condition (without stimuli) observed in the present study was maintained in nano molar range in cerebrum as well as in cerebellum. These values are consistent with others measured using fluorescent dyes, Fura2 and Indo1, not only in syntosomes (Komulainen and Bondy, 1987; Lynch and Voss, 1991; Daniell *et al.*, 1987; Iredale *et al.*, 1993) but also in frog sympathetic intact neurons (Lipscombe *et al.*, 1988). Resting intrasyntosomal Ca^{2+} concentrations observed in these studies ranged between 180 and 500 nM for syntosomes, while somewhat lower cytosolic Ca^{2+} concentration (70-100 nM) was demonstrated in intact neurons.

Among enzymes regulated by intracellular Ca^{2+} , the integral protein, Na^+ , K^+ -ATPase has been shown to be inhibited by Ca^{2+} (Skou, 1957) and by one or more Ca^{2+} dependent proteins (Yingst, 1988). In addition, regulation of Na^+ , K^+ -ATPase has been proposed as a target for the action of ethanol in the brain function (Foley and Rhoads, 1994) and Ca^{2+} could be a mediator of the action of ethanol on Na^+ , K^+ -ATPase. Na^+ , K^+ -ATPase is composed of two subunits; the catalytic component (α subunit) and the small glycosylated protein (β subunit). Three isozyms (α_1 , α_2 , α_3) have been identified in mammalian brain (Sweadner, 1984). The α_2 and/or α_3 isozyms have been suggested to be inducible pool of Na^+ , K^+ -ATPase activity in brain (Brodsky, 1990). Further, stimulation of Na^+ , K^+ -ATPase by low concentration of ethanol (80 mM) is specific in the activity of α_2 and/or α_3 isozyms (Foley and Rhoads, 1994). A 6/50 fraction, originally derived from soluble protein fraction from EGTA treated syntosomes, has been demonstrated to inhibit α_2/α_3 isozyms as well as α_1 isozyme (Wang, 1993). The feature of inhibition of Na^+ , K^+ -ATPase by 6/50 fraction was Ca^{2+} dependence similar to

calnaktin, an endogenous inhibitory protein of Na^+ , K^+ -ATPase in red blood cells. It is of interest to investigate the extent to which calnaktin-like inhibitor is active in brain at resting intracellular Ca^{2+} level.

The observed intrasynaptosomal Ca^{2+} concentrations in the present study correspond to 15 % inhibition of total activity of semi-purified Na^+ , K^+ -ATPase by calnaktin-like inhibitor (6/50 fraction)(Wang, 1993). More importantly, this shows that intrasynaptosomal Ca^{2+} concentration is positioned under resting condition to be responsive to elevated Ca^{2+} for further activation of calnaktin-like inhibitor. It also suggests it is unlikely that calnaktin would be the endogenous inhibition of the α_2 and/or α_3 isozymes under resting conditions.

(2) The Effect of Ethanol on Intrasynaptosomal Calcium Concentration

It has been generally agreed that high concentrations of ethanol (> 200 mM) inhibit Na^+ , K^+ -ATPase (Israel *et al.*, 1965, 1966; Marques A and Guerri, 1988; Nhamburo *et al.*, 1987) while lower concentrations of ethanol (50 ~ 100 mM) increases Na^+ , K^+ ATPase activity (Marques A and Guerri, 1988; Swann, 1990; Foley and Rhoads, 1994). While it is unlikely that stimulation of Na^+ , K^+ -ATPase involves Ca^{2+} (Wang, 1993), the inhibitory effect of ethanol on Na^+ , K^+ -ATPase could be mediated by Ca^{2+} . The present study examined the Ca^{2+} homeostasis of synaptosomes in the presence of ethanol.

Exposure of synaptosomes to ethanol resulted in an increase in intrasynaptosomal Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). However, the magnitude of increase in $[\text{Ca}^{2+}]_i$ was small in response to ethanol concentrations relevant to intoxication (25-100 mM). Relatively larger increases in $[\text{Ca}^{2+}]_i$ were observed at very high concentrations of ethanol which were far in excess of lethal blood alcohol concentrations. However, even under these conditions the maximum $[\text{Ca}^{2+}]_i$ was 293 nM, only a 20 % increase in $[\text{Ca}^{2+}]_i$ and raised $[\text{Ca}^{2+}]_i$ did not reach Ca^{2+}

concentration (20 μ M) necessary for the half-maximal inhibition of Na^+ , K^+ -ATPase by calnakin-like protein. Therefore, under pharmacologically relevant conditions as well as more than lethal conditions, ethanol does not appear to elevate Ca^{2+} in synaptosomes enough to alter most Ca^{2+} sensitive systems, including the brain calnakin-like protein, a Ca^{2+} sensitive inhibitor of Na^+ , K^+ -ATPase.

It was of interest to determine whether the increase in $[\text{Ca}^{2+}]_i$ resulted from the ER and the extent which the ER was able to attenuate the overall cellular (synaptosomal) response of Ca^{2+} to ethanol. Ethanol was reported to cause release of microsomal Ca^{2+} (Daniell *et al.*, 1987). We also observed a release of Ca^{2+} by ethanol from a microsomal fraction. These results suggest the existence of an internal Ca^{2+} store sensitive to ethanol. A relatively low concentration of ethanol (100 mM), which corresponds to intoxication or anesthetic conditions, mobilized Ca^{2+} from microsomal fraction to some extent. The amount was maximal in the presence of ATP, which as a substrate for ER Ca^{2+} -ATPase, allowed reloading of Ca^{2+} into microsomes. However the amounts of Ca^{2+} released by 100 mM ethanol was much less than that caused by IP_3 through a well characterized system to release Ca^{2+} from ER and to change cellular activities through mobilization of Ca^{2+} . In contrast, more than lethal levels of ethanol caused a larger release of Ca^{2+} from microsomal fraction in the presence of ATP. In this case, the amount of Ca^{2+} release was comparable to that caused by IP_3 .

In other studies, related to the effect of ethanol on neuronal calcium, voltage-sensitive calcium channels have been investigated as one of targets for the action of ethanol. The acute exposure to ethanol showed inhibition of depolarization dependent ^{45}Ca uptake, which is induced by high concentration of K^+ , in synaptosomes (Harris and Hood, 1980) and PC12 neuronal cell line (Messing *et al.*, 1986; Rabe and Weight, 1987). In addition, reduction in the

amplitude of T and L type Ca^{2+} currents by ethanol was demonstrated (Twombly *et al.*, 1990). These results seem to be opposing to the increase in cytosolic Ca^{2+} observed in the present study as well as others. However, depolarizing conditions were not used in the present study where the effects of ethanol were evaluated under resting conditions.

In conclusion, ethanol did increase $[\text{Ca}^{2+}]_i$ and did release Ca^{2+} from microsomal fraction. However, a large increase in $[\text{Ca}^{2+}]_i$ as well as a significant release of Ca^{2+} from ER required much higher concentrations of ethanol than are relevant to alcohol intoxication. Even an increase in $[\text{Ca}^{2+}]_i$ caused by very high concentration of ethanol did not reach Ca^{2+} concentration for the half-maximal inhibition of Na^+ , K^+ -ATPase mediated by calnaktin-like protein. Therefore it is unlikely that ethanol elevates intrasynaptosomal Ca^{2+} sufficiently to activate calnaktin-like protein. From these results, it is doubtful that Ca^{2+} is involved in the complex effects of ethanol on neuronal Na^+ , K^+ -ATPase. Ethanol does not elevate $[\text{Ca}^{2+}]_i$ sufficiently to inhibit Na^+ , K^+ -ATPase. Stimulation of Na^+ , K^+ -ATPase by ethanol has been reported to result from removal of the enzyme from an alcohol sensitive inhibitor (Foley and Rhoads, 1994). Resting $[\text{Ca}^{2+}]_i$ would appear to be too low to engage calnaktin in inhibition of Na^+ , K^+ -ATPase under resting condition and other recent work (Wang, 1993) has provided direct evidence that inhibition of Na^+ , K^+ -ATPase by calnaktin is not prevented by ethanol.

(3) The Effect of Fatty Acids on Intrasynaptosomal Calcium Concentration

Two mechanisms have been proposed for the inhibition by unsaturated fatty acids of amino acid neurotransmitter uptake via Na^+ / amino acid symport on synaptosomal plasma membrane. One is based on an uncoupling of Na^+ / amino acid symport resulting from the perturbation of membrane lipid-lipid or lipid-protein interactions in the vicinity of the carrier by unsaturated fatty acids (Rhoads, *et al.*,

1983). The other proposed mechanism involves membrane depolarization by unsaturated fatty acids, which would cause the release of neurotransmitters and the inhibition of amino acid neurotransmitter uptake (Troeger, *et al.* 1984). Because the uncoupling mechanism is independent of a change in $[Ca^{2+}]_i$, no change in $[Ca^{2+}]_i$ would be expected in the presence of unsaturated fatty acids. In contrast, depolarization results in elevated $[Ca^{2+}]_i$, so that unsaturated fatty acids would cause increase in $[Ca^{2+}]_i$. Therefore, based on these differences in the two proposed mechanisms, determining the effect of unsaturated fatty acids on $[Ca^{2+}]_i$ would clarify the mechanism involved in the inhibition of amino acid uptake by unsaturated fatty acids. Understanding the effects of free fatty acids on neuronal system is important because of their elevation in tissue during ischemia (Galloc and Spagnuolo, 1976) or seizures (Bazan, 1970; Siesjo *et al.*, 1982) and because of the potential contribution to the pathophysiology of these disorders.

The present study unexpectedly showed that unsaturated fatty acids decreased $[Ca^{2+}]_i$ significantly. A saturated fatty acid had no effect on $[Ca^{2+}]_i$. While raising new questions, this result is inconsistent with the proposal that unsaturated fatty acids cause membrane depolarization. Thus, the mechanism for inhibition of amino acid uptake by unsaturated fatty acids does not involve membrane depolarization. Effects of unsaturated fatty acids on amino acid transport occurred in the absence of Ca^{2+} (Rhoads *et al.*, 1983); however, effects on intracellular Ca^{2+} stores were possible. The results of the present study are also not consistent with significant mobilization of intracellular Ca^{2+} by unsaturated fatty acids to stimulate amino acids release. Lack of effect of unsaturated free fatty acids on $[Ca^{2+}]_i$ or, as shown previously (Rhoads *et al.*, 1983) on Na^+ permeability indicates that amino acid transporters fail to take up amino acids and catalyze efflux of amino acids under conditions where the ion concentration gradients are intact. Thus, these effects of unsaturated free fatty

acids are consistent with an uncoupling of the carriers from the Na^+ electrochemical gradient.

Prior treatment of synaptosomes with BSA greatly diminished the decrease in $[\text{Ca}^{2+}]_i$ caused by unsaturated fatty acids. This is because BSA has high affinity for binding fatty acids (Spector *et al.*, 1969, 1975) and because BSA present in the assay buffer is able to trap fatty acids, resulting in the loss of their activity. However, when synaptosomes were first treated with unsaturated fatty acids, subsequent addition of BSA did not reverse the decrease in $[\text{Ca}^{2+}]_i$. The effect of unsaturated fatty acids was irreversible within the time frame of experiments. This result is different from that observed by Rhoads *et al.* (1982 and 1983) where the effects of unsaturated fatty acids on the inhibition of neurotransmitter uptake was rapidly reversible upon addition of BSA. This suggests that the decrease in $[\text{Ca}^{2+}]_i$ may be an unrelated effect of unsaturated fatty acids and that the target sites for unsaturated fatty acids would be intrasynaptosomal, i.e., not accessible to extrasynaptosomal BSA. Alternatively, synaptosomes may be sufficiently impermeable to Ca^{2+} so that, if Ca^{2+} extrusion is enhanced by fatty acids, Ca^{2+} may not move back into the synaptosomes even if fatty acids are removed from the plasma membrane by BSA.

The observation of potent decrease in $[\text{Ca}^{2+}]_i$ by unsaturated fatty acids raises the question of how fatty acids exert their actions on $[\text{Ca}^{2+}]_i$. One of the possible explanations is the involvement of transporters for Ca^{2+} extrusion. Either the $\text{Na}^+/\text{Ca}^{2+}$ exchanger on the plasma membrane or Ca^{2+} -ATPase on either plasma membrane or ER could be involved. One or more of these transporters could be activated by unsaturated fatty acids. The involvement of PKC was also speculated since PKC has been reported to be activated by unsaturated fatty acids including oleic acid and arachidonic acid (McPhail *et al.* 1984; Murakami *et al.*, 1985, 1986). PKC is a known component of signal transduction and is a Ca^{2+}

requiring enzyme known to be abundant in the mammalian brain (Nishizuka, 1988). Further, plasma membrane Ca^{2+} -ATPase has been shown to be phosphorylated by PKC, resulting in the activation of ATP hydrolysis as well as Ca^{2+} translocation (Qu *et al.*, 1991; Wang *et al.*, 1991). However, the application of a PKC inhibitor, H-7 (Hidaka *et al.*, 1984), to synaptosomes before the addition of oleic acid did not suppress the decrease in $[\text{Ca}^{2+}]_i$ by unsaturated fatty acid. This result suggested that PKC does not mediate the effect of unsaturated fatty acids on $[\text{Ca}^{2+}]_i$. Previous studies have indicated that unsaturated fatty acids may act directly on $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Philpson and Ward, 1985) and Ca^{2+} -ATPase (Niggli *et al.*, 1981; Al-Jobore and Roufogalis, 1981).

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger is characterized as a low-affinity, high-capacity Ca^{2+} pumping system. The lack of potent selective inhibitors for $\text{Na}^+/\text{Ca}^{2+}$ exchanger has slowed an understanding of the precise physiological role of this transport system. The development of a preparation of plasma membrane vesicles derived from cardiac sarcolemma has provided a system for the measurement of $\text{Na}^+/\text{Ca}^{2+}$ exchange (Philpson, 1985). Using this cardiac sarcolemmal vesicle preparation, the activity of Na^+ , Ca^{2+} exchange has been shown to be stimulated by fatty acids. Unsaturated fatty acids were much more potent than saturated fatty acids (Philpson and Ward, 1985). The concentration applied for their experiments (30 μM for $\text{Na}^+/\text{Ca}^{2+}$ exchange activity) is similar to the experimental condition in the present study. Thus, the observation of decrease in intrasynaptosomal Ca^{2+} concentration by unsaturated fatty acids could be mediated by the activation of $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

In parallel to $\text{Na}^+/\text{Ca}^{2+}$ exchange, Ca^{2+} -ATPase is another Ca^{2+} extrusion mechanism on the plasma membrane. It is defined as a high-affinity and low-capacity Ca^{2+} pumping system (Carafoli, 1991). Ca^{2+} -ATPase on the plasma membrane is calmodulin sensitive unlike those on the ER membrane. The binding

of calmodulin to Ca^{2+} -ATPase has been shown to activate its function by increasing affinity for Ca^{2+} and V_{max} . Besides calmodulin, unsaturated fatty acids have been demonstrated to stimulate Ca^{2+} -ATPase (Niggli *et al.*, 1981; Al-Jobore and Roufogalis, 1981). The interaction between unsaturated fatty acids and Ca^{2+} -ATPase on synaptic plasma membrane would be a second possible target for decrease in intrasynaptosomal Ca^{2+} concentration. Effects on either the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger or plasma membrane Ca^{2+} -ATPase may not be reversed by BSA. While fatty acids would presumably partition into the plasma membrane to have these effects, Ca^{2+} transported out of the synaptosomes may not necessarily move back in immediately upon removal of plasma membrane bound fatty acids by BSA.

The effect of fatty acids on extramicrosomal Ca^{2+} concentration was examined. Unsaturated fatty acids, oleic acid and arachidonic acid, decreased extramicrosomal $[\text{Ca}^{2+}]$, while the saturated fatty acid, palmitic acid, did not. This observation suggests increased uptake of Ca^{2+} into microsomal vesicles. The decrease in extramicrosomal $[\text{Ca}^{2+}]$ was observed either in the absence of ATP or in the presence of Ca^{2+} -ATPase inhibitor, orthovanadate. Moreover unsaturated fatty acids caused less decrease in extramicrosomal $[\text{Ca}^{2+}]$ in the presence of ATP. Therefore, Ca^{2+} -ATPase on ER membrane does not seem to be involved in the action of unsaturated fatty acids at the level of the ER. If there is an effect of unsaturated fatty acids on Ca^{2+} -ATPase, it, like the effect of calmodulin, is specific to the plasma membrane form of Ca^{2+} -ATPase.

With respect to the possible contamination of mitochondria in the microsomal fraction, it is doubtful that only unsaturated fatty acids modulate Ca^{2+} sequestration into mitochondria. While the outer mitochondrial membrane is relatively permeable to many small molecules, the inner membrane regulates their transport. Ca^{2+} penetrates across the inner mitochondrial membrane driven by the negative transmembrane potential which is established by the respiratory chain

(Carafoli, 1982, 1987). It is unlikely that oxidation of fatty acids in mitochondria would occur appreciably in this preparation and, even if it did, the generation of the required electrical potential for Ca^{2+} uptake is unlikely in the presence of the mitochondrial inhibitors, antimycin and rotenone. It is also unlikely that unsaturated fatty acids would have this effect while saturated fatty acids would not.

Another possibility should be noted. Since fatty acids have the carboxyl group, which is also possessed by Fura2 where it mediates the formation of a complex with Ca^{2+} , it is possible that unsaturated fatty acids would form micelles with a capacity for binding Ca^{2+} . In order to examine this possibility, the binding of Ca^{2+} to fatty acids was examined in the absence of microsomes and synaptosomes in the physiological buffer containing a nano molar range of Ca^{2+} similar to cytosolic content. Ca^{2+} concentration in the buffer was monitored using Fura2 and compared to before and after the addition of fatty acids. Both unsaturated fatty acids and saturated fatty acid caused significant apparent decreases in Ca^{2+} concentration as measured by Fura2 (data not shown). This is consistent with the binding of Ca^{2+} to fatty acids but occurred equally with saturated and unsaturated fatty acids. This effect of saturated fatty acid was not seen in the presence of microsomes. The interaction of Ca^{2+} and palmitic acid has been studied previously using $^{45}\text{CaCl}_2$ and the Ca^{2+} indicator, arsenazo III, by Watras *et al.* (1984). The binding of Ca^{2+} to palmitic acid (10-80 μM) occurred slowly in the presence of 18 μM Ca^{2+} with approximately 3-7 min half time. The rate of Ca^{2+} binding increased as the concentration of Ca^{2+} was increased. These results might lead to the conclusion that our observation of decrease in $[\text{Ca}^{2+}]_i$ by unsaturated fatty acids simply resulted from the binding of Ca^{2+} to fatty acids. However, the observation that the saturated fatty acid, palmitic acid, exhibited no effect on extramicrosomal $[\text{Ca}^{2+}]_i$ is inconsistent with the binding of Ca^{2+} to saturated fatty acid as well as unsaturated fatty acids. In addition, the effect of

palmitic acid was not observed in intact synaptosomes or microsomes. In effect, palmitic acid serves as a control for direct interactions between fatty acids and Ca^{2+} that occur with both saturated and unsaturated fatty acids. Only unsaturated fatty acids had effects on Ca^{2+} in microsomal and synaptosomal preparations. It seems that unsaturated fatty acids have a specific mechanism to affect intrasynaptosomal Ca^{2+} and that this is distinct from an artificial effect due to direct binding of Ca^{2+} .

Since ATP-independent Ca^{2+} uptake into ER appears to be facilitated by unsaturated fatty acids, the possibility for the involvement of calreticulin, a major Ca^{2+} -storage protein in the ER lumen of non-muscle cells (Mihcalak *et al.*, 1992) should be considered. It is well established that calreticulin contributes to Ca^{2+} storage in the ER lumen and has high capacity and low affinity for Ca^{2+} that is transported through Ca^{2+} -ATPase. However, recent evidence indicates that calreticulin is a peripheral protein localized to the ER membrane. Thus, unsaturated fatty acids might modulate Ca^{2+} binding sites on calreticulin either on the ER membrane or inside ER to increase the storage capacity for the ER for Ca^{2+} .

In summary, unsaturated fatty acids, oleic acid and arachidonic acid, caused a significant decrease in intrasynaptosomal Ca^{2+} concentration. Therefore unsaturated fatty acids did not appear to depolarize the synaptic membrane and it is unlikely that depolarization is involved in the inhibition of amino acid neurotransmitter uptake by unsaturated fatty acids. On the other hand, this potential decrease in $[\text{Ca}^{2+}]_i$ by unsaturated fatty acids, but not by saturated fatty acid, is another neural effect of unsaturated fatty acids. The application of PKC inhibitor, H-7, argues against the involvement of PKC in the decrease in $[\text{Ca}^{2+}]_i$ by unsaturated fatty acids. The mechanism underlying the observed decrease remains to be defined. Three possible targets could be proposed : (1) increased $\text{Na}^+/\text{Ca}^{2+}$

exchange across the plasma membrane, (2) increased Ca^{2+} efflux via Ca^{2+} -ATPase on plasma membrane and/or (3) increase in Ca^{2+} uptake or storage in ER through some ATP-independent mechanism.

Table 1. Resting intrasynaptosomal Ca^{2+} concentrations

Synaptosomes were prepared from cerebrum or cerebellum and $[\text{Ca}^{2+}]_i$ was measured under physiological conditions as described in METHODS AND MATERIALS. Measurements were carried out at room temperature. Results are derived from the means $\pm$ S.E.M. of values from all experiments described in this study.

	Resting $[\text{Ca}^{2+}]_i$ (nM)
cerebrum	304 ± 40
cerebellum	250 ± 33

Table 2. The amount of released Ca^{2+} from microsome by ethanol and inositol-1,4,5-trisphosphate (IP_3)

Microsomes were prepared from cerebellum as described in MATERIALS AND METHODS. Extramicrosomal Ca^{2+} concentration was measured using Fura2 at room temperature. In the presence or absence of ATP (1 mM), microsomes were treated with Na orthovanadate (50 μM). 1 min after addition of Na orthovanadate, ethanol (100 mM) or IP_3 (1 μM) was added and change in extramicrosomal Ca^{2+} concentration was monitored. Each value represents nano moles of released Ca^{2+} per mg of microsomal protein. The data are the means $\pm$ S.E.M. for three or four independent experiments.

	Released Ca^{2+} n mol/mg protein	
	+ ATP	- ATP
IP_3	1.61 ± 0.17	0.67 ± 0.05
EtOH	0.43 ± 0.09	0.03 ± 0.03

Table 3. The effect of bovine serum albumin (BSA) on decrease in intrasynaptosomal Ca^{2+} concentration caused by oleic acid.

Cerebral synaptosomes were prepared as described in MATERIALS AND METHODS. The intrasynaptosomal Ca^{2+} concentration was measured under the different conditions and different concentration of oleic acid was added. As described in MATERIALS AND METHODS, oleic acid was added in the synaptosome suspended in assay buffer (Addition of oleic acid). Oleic acid was also added in the synaptosome suspended in assay buffer containing BSA (In the presence of BSA). After synaptosome was treated with oleic acid in the absence of BSA, solid BSA was added in the assay solution (Addition of BSA). Each value represents decrease in the percentage change compared to the resting intrasynaptosomal Ca^{2+} concentration. The data are the means $\pm$ S.E.M. for 4 independent experiments.

Concentration of oleic acid	Decrease in % compared to resting $[\text{Ca}^{2+}]_i$		
	Addition of oleic acid	In the presence of BSA	Addition of BSA
15 μM	24.1 ± 1.7	6.9 ± 2.5	25.2 ± 2.9
30 μM	28.3 ± 3.4	13.4 ± 1.3	32.0 ± 1.7
50 μM	38.6 ± 1.1	25.3 ± 1.7	40.8 ± 3.2

Table 4. The application of PKC inhibitor (H-7) before the addition of oleic acid to synaptosomes.

Cerebral synaptosomes were prepared as described in MATERIALS AND METHODS. Synaptosomes were treated with H-7 (10 or 50 μ M final concentration) before the addition of oleic acid and intrasynaptosomal Ca^{2+} concentration was monitored during addition of H-7, following addition of oleic acid as described in MATERIALS AND METHODS. Each value represents decrease in the percentage change compared to the resting intrasynaptosomal Ca^{2+} concentration (before the addition of H-7 and oleic acid). The data are the means $\pm$ S.E.M. derived from 3 independent experiments each performed in duplicate.

Concentration of oleic acid	Decrease in % compared to resting $[Ca^{2+}]_i$	
	Addition of H-7 (10 μ M) alone	Addition of oleic acid after H-7
15 μ M	1.8 $\pm$ 0.6	28.8 $\pm$ 2.7
30 μ M	0.5 $\pm$ 0.3	35.7 $\pm$ 3.7
50 μ M	1.2 $\pm$ 0.6	43.0 $\pm$ 2.1

Concentration of oleic acid	Decrease in % compared to resting $[Ca^{2+}]_i$	
	Addition of H-7 (50 μ M) alone	Addition of oleic acid after H-7
15 μ M	5.8 $\pm$ 1.2	36.2 $\pm$ 3.4
30 μ M	4.7 $\pm$ 0.6	44.5 $\pm$ 1.2
50 μ M	3.8 $\pm$ 0.7	49.2 $\pm$ 2.6

Figure. 1 Dose response of intrasynaptosomal Ca^{2+} concentration to ethanol.

Synaptosomes were prepared from cerebrum as described in MATERIALS AND METHODS. During measurement, ethanol was added every 3 min directly into the cuvette to reach the concentration indicated on each arrow. As for control, an amount of H_2O equal to the volume of ethanol was added after each time interval. Measurements were carried out at room temperature. Results are derived from the means of three separate experiments.

Figure. 2 Traces of peak and baseline $[Ca^{2+}]_i$ following addition of ethanol.

From the data of Figure 1, intrasynaptosomal Ca^{2+} concentration at the peak point after the addition of ethanol as well as the average intrasynaptosomal Ca^{2+} concentration between the additions of ethanol was plotted versus concentration of ethanol. From the control experiment, the average intrasynaptosomal Ca^{2+} concentration between the additions of H_2O was plotted against the equivalent volume of ethanol. In order to adjust the resting intrasynaptosomal Ca^{2+} concentrations of ethanol and control experiments at the same point on the axis, the difference in intrasynaptosomal Ca^{2+} concentration at 0 mM EtOH was subtracted from each actual value of control. Therefore the trace of base line from control experiment is shifted down from the actual line.

Figure 3. Change in intrasynaptosomal Ca^{2+} concentration in the presence of ethanol.

Synaptosomes were prepared from cerebrum and cerebellum as described in MATERIALS AND METHODS. After the resting Ca^{2+} concentration was measured, synaptosomes were incubated with ethanol for 10 min and then, intrasynaptosomal Ca^{2+} concentration was monitored. The data were quantified by the percentage change of the difference between intrasynaptosomal Ca^{2+} concentrations before and after the addition of ethanol. Same amounts of H_2O were added for controls. Measurements were carried out at room temperature. The data are the means $\pm$ S.E.M. derived from 3 experiments.

Figure 4. Time course of extramicrosomal Ca^{2+} concentration in the presence of ethanol

Microsomal fraction was prepared from cerebrum as described in MATERIALS AND METHODS and extramicrosomal Ca^{2+} concentration was determined by using Fura2. Microsomes were treated with ATP (1mM) and Na orthovanadate (50 μ M) before the addition of ethanol. ATP was added 1 min after the initiation of measurement and Na orthovanadate was added 1 min later. Then ethanol was started to add after a further 1 min, and kept adding every 3 min to reach the concentration as indicated on each arrow. As for control, same amounts of H_2O were added under the same condition and in the same manner. Measurements were carried out at room temperature. The results are the means of two separate experiments.

Figure 5. Time course of extramicrosomal Ca^{2+} concentration in the presence of ethanol without ATP.

Microsomal fraction was prepared from cerebrum as described in MATERIALS AND METHODS and experimental Ca^{2+} concentration was determined by using FURA2. Microsomes were only treated with Na orthovanadate ($50\mu\text{M}$) and not treated with ATP before the addition of ethanol. The same amount of H_2O instead of ATP in figure 4 was added 1 min after the initiation of measurement and Na orthovanadate was added 1 min later. Then ethanol was started to add after further 1 min and kept adding every 3 min to reach the concentration as indicated on each arrow. As for the control, same amounts of H_2O were added under the same condition and in the same manner. Measurements were carried out at room temperature. The results are the means of two separate experiments.

Figure. 6 The amount of Ca^{2+} released from microsomal fraction by ethanol in the presence and in the absence of ATP.

From the data of figure 4 and 5, the amounts of released Ca^{2+} from microsomal fraction after addition of ethanol was expressed as nano moles Ca^{2+} per milligrams of microsomal protein. They are plotted versus concentration of ethanol.

* The value for nano mole of released Ca^{2+} per mg of protein at 800 mM ethanol represents 2.2 ± 1.7

Figure 7. The effect of fatty acids on intrasynaptosomal Ca^{2+} concentration .

Cerebral synaptosomes were prepared as described in MATERIALS AND METHODS. After resting intrasynaptosomal Ca^{2+} was measured for 2 min, fatty acid was directly added into the cuvette and mixed using a pipette. Intrasynaptosomal Ca^{2+} concentration was then monitored for 2 min. The percentage change in intrasynaptosomal Ca^{2+} concentration was based on the difference between average of intrasynaptosomal Ca^{2+} concentrations before and after the addition of fatty acids. Measurements were carried out at room temperature. The data are the means $\pm$ S.E.M. derived from 2 or 3 independent experiments each performed in duplicate or triplicate.

Figure. 8 The effect of unsaturated fatty acids on intrasynaptosomal Ca^{2+} concentration in the presence or the absence of BSA.

Cerebral synaptosomes were prepared as described in MATERIALS AND METHODS. Synaptosomes was suspended in the buffer containing 15 μM BSA. After resting intrasynaptosomal Ca^{2+} was measured for 2 min, unsaturated fatty acid was directly added into the cuvette and mixed using a pipe pipette. Intrasynaptosomal Ca^{2+} concentration was then monitored for 2 min. The percentage change in intrasynaptosomal Ca^{2+} concentration were based on the difference between average of intrasynaptosomal Ca^{2+} concentrations before and after the addition of unsaturated fatty acids. Measurements were carried out at room temperature. The data are the means $\pm$ S.E.M. derived from 2 or 3 independent experiments, each performed in duplicate or triplicate.

Figure. 9 The effect of fatty acids on extramicrosomal Ca^{2+} concentration.

Cerebral microsomes were prepared as described MATERIALS AND METHODS. After resting extramicrosomal Ca^{2+} concentration ($[\text{Ca}^{2+}]$) was measured for 2 min, fatty acid was directly added into the cuvette and mixed using a pipette. Extramicrosomal $[\text{Ca}^{2+}]$ was then monitored for 2 min. The percentage change in extramicrosomal $[\text{Ca}^{2+}]$ was based on the difference between average of extramicrosomal $[\text{Ca}^{2+}]$ before and after the addition of fatty acids (right panel).

Before the addition of fatty acid, microsomes were treated with either ATP (1 mM) or orthovanadate (VO_4^{3-}) (50 μM) and extramicrosomal $[\text{Ca}^{2+}]$ was measured for 2 min. Fatty acid was then added and extramicrosomal $[\text{Ca}^{2+}]$ was measured for another 2 min. The change in extramicrosomal $[\text{Ca}^{2+}]$ after treatment with fatty acids was calculated as the percentage, compared to the average extramicrosomal $[\text{Ca}^{2+}]$ before the addition of fatty acids (i.e.; the average extramicrosomal $[\text{Ca}^{2+}]$ after either ATP or VO_4^{3-} was added).

Measurements were carried out at room temperature. The data are derived from a single experiment.

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