CHARACTERIZATION AND DISTRIBUTION OF RYANODINE RECEPTORS IN BRAIN

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by

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABSTRACT

Ryanodine, a plant alkaloid, binds to and influences the gating of ryanodine receptor/Ca^{2+} release channels in muscle sarcoplasmic reticulum (SR). The alkaloid also exhibits Ca^{2+}-related effects in neurons suggesting that Ca^{2+} release channels analogous to those in muscle are present in brain. Radioligand binding, autoradiographic, subcellular fractionation and enzyme assay techniques were employed in the present investigation to determine the properties, regional distribution, and cellular and subcellular localization of ryanodine receptors in rat and human brain. Analyses of [^{3}H]ryanodine binding data revealed the presence of high affinity sites in total and in various subcellular membrane fractions of rat and human neural tissues. Low affinity sites were observed in total membrane preparations. Binding was influenced by Ca^{2+}, Mg^{2+}, adenine nucleotide, caffeine and ionic strength. Distribution of receptor sites in rat and human brain, as determined by autoradiography and membrane binding, was widespread and heterogeneous. An extremely high density of sites was present in hippocampus, more specifically in the CA3 region. Results of rat hippocampal kainic acid lesion studies revealed that ryanodine receptors were preferentially localized in neurons and that these sites may be found in mossy fiber nerve terminals. This finding was supported by subsequent studies which showed the presence high affinity receptor sites in purified rat cortical and mossy fiber synaptosomes. Subfractionation of synaptosomes and microsomes yielded a separation of ryanodine receptors and enzyme markers of endoplasmic reticulum (ER) and plasma membrane which suggests that these receptors may be located either in a distinct intracellular organelle or a subcompartment of ER specialized for Ca^{2+} sequestration and release. These results suggest that ryanodine receptors are present in brain and that they may have a role in the regulation of neuronal Ca^{2+} concentration.

I. GENERAL INTRODUCTION

Over the past two decades, the general picture has emerged that cells, regardless of type or tissue origin, utilize several different mechanisms to maintain intracellular concentrations of free calcium within a range required for normal cellular functions. The demonstration that cellular organelles in neurons are capable of storing calcium suggests their involvement in calcium regulation. However, only in recent years have such calcium stores been recognized to play a role in intracellular calcium release and signalling events. In skeletal and cardiac muscle, identification of calcium release channels in the sarcoplasmic reticulum (SR), the intracellular calcium store of muscle, was greatly enhanced with the use of the plant alkaloid ryanodine. It has now been demonstrated that ryanodine receptor/calcium release channels directly contribute to excitation-contraction coupling in muscle. In neurons, the physiological role of intracellular calcium release channels remains to be determined. Establishment of calcium sequestration and release mechanisms, the morphological identity and the molecular composition of these stores are required in order to understand the function of these calcium storage compartments. Therefore, the aim of the work described in this thesis was to determine whether ryanodine receptor/calcium release channels analogous to those in muscle were present in brain and how they compared with their muscle counterparts with respect to regulatory properties and intracellular localization. The hypothesis was that neurons possess a specific intracellular calcium release channel referred to as the "ryanodine receptor/calcium release channel complex". As background, this introduction will focus on the literature describing: 1) properties and functions of SR ryanodine receptor/calcium release channels in muscle; 2) physiological actions of calcium in neurons; 3) neuronal mechanisms of intracellular calcium buffering; and 4) known properties of intraneuronal calcium compartments.

I.I. *Regulation and function of the muscle ryanodine receptor/calcium release channel*

I.I.I. *Excitation-contraction coupling in muscle*

Muscle contraction or excitation-contraction (E-C) coupling is initiated when depolarization of sarcolemmal transverse tubules (TT) activates dihydropyridine receptors (DHPR) located in the TT membrane. In skeletal muscle, the close relationship between DHPR and calcium release channels localized in the terminal cisternae (TC) of the SR forms the anatomical basis of E-C coupling (Schneider and Chandler, 1973). Electron microscopic analysis of the TC/TT junction reveals that DHPR and SR Ca^{2+} release channels are physically coupled (Block et al., 1988). However, direct interactions between DHPR and Ca^{2+} release channels have not been demonstrated. The DHPR is thought to serve as a voltage sensor (Rios and Brum, 1987) which, upon depolarization-induced activation, triggers an intracellular signal transduction process that results in the opening of SR Ca^{2+} release channels (Endo, 1977). Released Ca^{2+} interacts with muscle contractile proteins to initiate muscle contraction. In contrast, cardiac muscle contraction appears to be regulated through a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Fabiato and Fabiato, 1977). In this case, TT depolarization activates DHPR voltage-operated Ca^{2+} channels resulting in Ca^{2+} influx and subsequent activation of SR Ca^{2+} release channels. Whether CICR plays a role in skeletal muscle E-C coupling is currently uncertain.

I.I.2. *Calcium release from muscle sarcoplasmic reticulum*

Various methods and pharmacological agents have been employed to study calcium release from muscle SR (Palade et al., 1989). By far, the compound which has received the most attention in studies of SR Ca^{2+} release is the methylxanthine caffeine. Administration of caffeine in intact skeletal muscle potentiates the strength of muscle contraction, prolongs muscle activity and produces contractures independent of membrane depolarization and integrity of transverse tubule membranes (Axelson and

Thesleff, 1958; Gage and Eisenberg, 1967; Weber, 1968; Weber and Herz, 1968). As all these actions presumably occur through modulation of calcium uptake into or release from the SR, caffeine has frequently been used as a probe in subsequent studies of the mechanisms of SR calcium release.

Weber and Herz (1968) utilized subcellular fractionation techniques to isolate SR membrane vesicles and demonstrated that caffeine releases calcium from these vesicles. Heavy compared with lighter SR membranes were more sensitive to the actions of caffeine (Weber, 1968; Weber and Herz, 1968). The former are enriched in terminal cisternal membranes and the latter are mainly composed of longitudinal SR regions (Watras and Katz, 1984). Caffeine-induced calcium release is influenced by ionic strength, the degree of calcium loading and concentration of free ATP (Fairhurst and Hasselbach, 1970; Inesi and Malan, 1976; Katz et al., 1977; Kim et al., 1983; Miyamoto and Racker, 1981; Su and Hasselbach, 1984; Weber and Murray, 1973). In addition, Kim et al. (1983) demonstrated that the kinetics of calcium release from heavy SR membranes is consistent with that observed in intact muscle. Thus, muscle SR is endowed with a caffeine-sensitive mechanism to release sequestered calcium. However, the identity of the site at which caffeine acts on SR membranes remained uncertain for some time and was further complicated by other known actions of caffeine on adenosine receptors and on phosphodiesterase enzymes (Gould et al., 1984; Williams and Jarvis, 1988). More precise definition of SR calcium release sites followed the development of purified SR-TT preparations (Mitchell et al., 1983) coupled with the use of ryanodine as a probe for SR Ca^{2+} release sites (Sutko, 1985).

I.I.3. *Effects of ryanodine in muscle*

Ryanodine (Figure 1), an alkaloid first isolated from the stem of the Brazilian plant *Ryania speciosa* Vahl, was initially utilized for its insecticidal properties and was

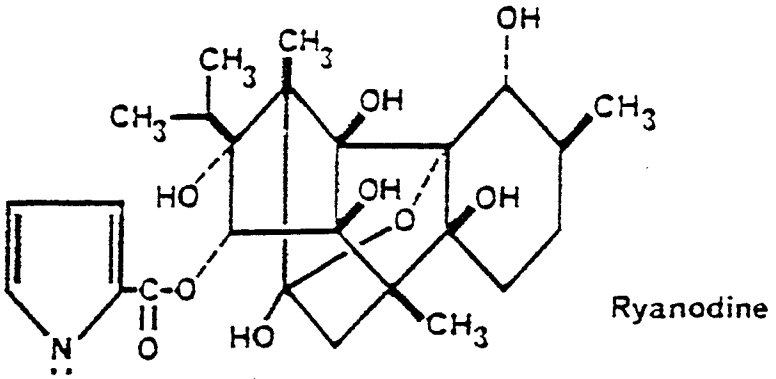

Figure 1. The chemical structure of ryanodine

shown to have extremely potent effects on the musculature of various mammalian species (Jenden and Fairhurst, 1969; Procita, 1958). Its effects are complex and highly dependent on muscle type, Ca^{2+} activity, pattern of muscle stimulation and ryanodine concentration (Jenden and Fairhurst, 1969). Ryanodine produces irreversible skeletal muscle contracture (Jenden and Fairhurst, 1969; Fairhurst and Hasselbach, 1970; Fairhurst, 1974) and has negative and positive inotropic effects in cardiac muscle (Ciofalo, 1973; Hillyard and Procita, 1959; Jenden and Fairhurst, 1969; Sutko et al., 1979, 1985). Ryanodine does not affect cardiac transmembrane action potentials and was suggested to have an intracellular site of action (Penefsky and Kahn, 1970). Besch (1985) and Jones et al. (1979, 1981) proposed that ryanodine increases SR Ca^{2+} accumulation and suggested that this is due to a decrease in the rate of Ca^{2+} efflux from SR vesicles rather than an increase in SR Ca^{2+} influx. Fabiato (1985) demonstrated a ryanodine-induced reduction in the rate of Ca^{2+} accumulation into cardiac SR and subsequently proposed that ryanodine blocks three types of SR Ca^{2+} release mechanisms: 1) CICR, 2) caffeine-induced Ca^{2+} release, and 3) cyclic release of Ca^{2+}. He suggested that all three release processes are mediated by the same channel (Fabiato, 1985). The apparent contradictory actions of ryanodine on muscle SR Ca^{2+} accumulation was suggested to be due to either multiple actions of ryanodine on a single site or multiple sites of ryanodine action (Fabiato, 1985; Sutko et al., 1985).

Several studies indicate that ryanodine acts directly at the SR Ca^{2+} release channel. First, the effects of ryanodine are localized at junctional or terminal SR regions believed to be sites of Ca^{2+} release (Fleischer et al., 1985; Lattanzio et al., 1987). Second, the flux rate of SR calcium release is consistent with that expected of a Ca^{2+} channel (Lattanzio et al., 1987; Meissner, 1984). Finally, ryanodine does not effect other components of the SR or sarcolemmal Ca^{2+} transport system (Besch, 1985; Lattanzio et al., 1987). In contrast to studies that demonstrate a block of SR Ca^{2+} release by

ryanodine (Sutko et al., 1985), ryanodine-induced increases in Ca^{2+} efflux from the SR has been reported (Hilgemann et al., 1983; Hunter et al., 1983). In skeletal and cardiac heavy SR fractions, nanomolar concentrations of ryanodine increases vesicular Ca^{2+} permeability whereas greater than 10 μM ryanodine inhibits Ca^{2+} efflux (Meissner, 1986). Hansford and Lakatta (1987) demonstrated that ryanodine at concentrations ranging from 10 nM to 1 μM causes a slow depletion of Ca^{2+} from cardiac SR. It was proposed that ryanodine directly binds to SR Ca^{2+} release channels and locks them in an open configuration (Fleischer et al., 1985).

In single channel conductance measurements, skeletal and cardiac SR vesicles reconstituted into planar lipid bilayers display functional Ca^{2+} channels with a unit conductance that decreases and an open probability that increases in the presence of ryanodine (Rousseau et al., 1987). The channel is activated by Ca^{2+} and ATP and inhibited by Mg^{2+} and ruthenium red (Rousseau et al., 1987). It was suggested that ryanodine, through a time- and use-dependent manner, reduces the ability of the SR to regulate Ca^{2+} flux through conversion of the channel to a long-lasting subconductance state (Rousseau et al., 1987).

A further complexity in the action of ryanodine is the lower sensitivity of SR membranes and skinned muscle preparations towards ryanodine compared with intact muscle. Intact muscle responds to low nanomolar concentrations of ryanodine (Sutko et al., 1979, 1985), while SR membranes and skinned cardiac myocytes are sensitive to concentrations in the micromolar range (Fairhurst, 1974; Fairhurst and Hasselbach, 1970; Jones et al., 1979, 1981). This difference in sensitivity may be due to metabolism of ryanodine to a more active form in intact muscle versus fragmented membrane preparations, disruption of cytoarchitectural or other factors required for ryanodine action, impurities in ryanodine preparations that may antagonize the effectiveness of

ryanodine in isolated membranes or the use-dependence binding of ryanodine to the open channel state (Sutko et al., 1985). In any case, ryanodine appears to act specifically on the SR Ca^{2+} release channel and this has led to its utility as a tool to study mechanisms of Ca^{2+} release from the SR.

I.I.4. *Identification of ryanodine receptors in muscle*

The development and use of radiolabeled ryanodine in studies of SR calcium release was reported over two decades ago (Fairhurst, 1971; Ciofalo, 1973). However, it was not until 1984 that preparations of this labeled compound became available with sufficiently high specific activity to allow direct examinations of ryanodine-sensitive sites in muscle (Waterhouse et al., 1984). The radiolabeled alkaloid was utilized to characterize ryanodine receptors in various cardiac and skeletal muscle membrane preparations (Alderson and Feher, 1987; Cambell et al., 1987; Fleischer et al., 1985; Fleischer and Inui, 1988; Hymel et al., 1988a,b; Imagawa et al., 1987; Inui et al., 1987 a,b; Lai et al., 1988 a,b; McGrew et al., 1989; Pessah et al., 1985, 1986, 1987; Pessah and Zimanyi, 1991; Zimanyi and Pessah, 1991). Ryanodine appears to bind directly to the SR Ca^{2+} release channel now referred to as the ryanodine receptor/Ca^{2+} release channel complex (Pessah et al., 1985, 1986, 1987; Pessah and Zimanyi, 1991; McGrew et al., 1989; Zimanyi and Pessah, 1991). Multiple affinity ryanodine binding sites are present in cardiac and skeletal muscle SR membranes and these sites are subject to complex regulatory mechanisms similar to those controlling SR calcium release (Lattanzio et al., 1987). Furthermore, [^{3}H]ryanodine binding may serve as an indicator for activation of the calcium release channel (Chu et al., 1990; Takasago et al., 1991).

I.I.5. *Skeletal muscle ryanodine receptor subtype*

In various skeletal muscle SR membrane preparations, the presence of high and low affinity [^{3}H]ryanodine binding sites sensitive to nanomolar and micromolar concentrations of ryanodine, respectively, has been demonstrated (Campbell et al., 1987; McGrew et al., 1989; Pessah et al., 1985, 1986, 1987). A wide range of affinity constants have been reported and may reflect the use of different preparations and assay conditions. The range of K_D values for the high and low affinity sites were 1.2 to 200 nM and 1 to 4 μM, respectively (Anderson et al., 1989; Chu et al., 1990; Fleischer et al., 1985; Imagawa et al., 1989; Lattanzio et al., 1987; McGrew et al., 1989; Michalak et al., 1988; Pessah et al., 1985, 1986, 1987; Pessah and Zimanyi, 1991; Takeshima et al., 1989; Zarka and Shoshan-Barmatz, 1992). In contrast to the monophasic profile exhibited by [^{3}H]ryanodine association with its receptor (McGrew et al., 1989; Pessah et al., 1987; Chu et al., 1990), the receptor displays a biphasic dissociation having both fast and slow components (Chu et al., 1990; McGrew et al., 1989; Pessah et al., 1987). This was suggested to be due to positive cooperativity where binding of ryanodine to low affinity sites decreases its dissociation from high affinity sites (McGrew et al., 1989). The apparent K_D value (micromolar range) of the low affinity site is comparable with the concentrations of ryanodine that enhance Ca^{2+} loading into SR vesicles (McGrew et al., 1989) which suggests that these sites are involved in closing the Ca^{2+} release channel, hence preventing release.

Based on receptor binding analyses and Ca^{2+} transport measurements of SR vesicles, Pessah and Zimanyi (1991) demonstrated the presence of four classes of ryanodine binding sites of differing affinites. Occupation by ryanodine at these sites produces sequential activation followed by inactivation of the SR Ca^{2+} channel. In contrast to the earlier suggestion of positive cooperativity between high and low affinity sites (McGrew et al., 1989), they proposed that negatively cooperative interactions occur

between ryanodine receptor binding proteins which may explain why high ryanodine concentrations irreversibly uncouple the native function of the receptor/channel complex (Pessah and Zimanyi, 1991). Ryanodine apparently promotes a long-lived conformational state which physically occludes the channel and prevents ryanodine from freely diffusing away from its binding site (Pessah and Zimanyi, 1991).

I.I.6. *Cardiac ryanodine receptor subtype*

Like skeletal muscle SR, cardiac SR exhibits high and low affinity [^{3}H]ryanodine binding sites. The range of K_D values reported are 1 to 36 nM for the high affinity site and 28 nM to 4 μM for the low affinity site (Alderson and Feher, 1987; Imagawa et al., 1989; Michalak et al., 1988; Pessah et al., 1985; Pessah and Zimanyi, 1991; Seifert and Casida, 1986). The cardiac ryanodine receptor is similar to the skeletal muscle receptor with respect to protein composition, morphology, chromatographic behaviour on heparin- and p-aminobenzamidine-agarose columns, and dependence of ryanodine binding on Ca^{2+} and salt. Moreover, low affinity sites are suggested to be responsible for calcium release channel closure (Alderson and Feher, 1987) similar to their proposed action in skeletal muscle (Pessah and Zimanyi, 1991). The significantly lower levels of cardiac ryanodine binding sites compared with Ca^{2+} transport pumps suggests that cardiac ryanodine receptors are distinct from SR Ca^{2+} pumps (Alderson and Feher, 1987).

In contrast to skeletal muscle ryanodine receptors, activation of cardiac receptors occurs via a Ca^{2+}-induced Ca^{2+} release mechanism (Endo, 1977; Fabiato and Fabiato, 1977). This hypothesis is supported by studies demonstrating the Ca^{2+} dependence of [^{3}H]ryanodine binding to cardiac SR vesicles and ryanodine actions on cardiac SR Ca^{2+} release (Alderson and Feher, 1987; Fabiato, 1985). Although triad junctions in both heart and skeletal muscle have similar mechanisms to initiate depolarization-induced SR Ca^{2+} release (Inui et al., 1987a,b), these junctions are more abundant in skeletal muscle and

sarcolemmal Ca^{2+} influx does not appear to be required for skeletal muscle SR Ca^{2+} release (Inui et al., 1987a). In addition, the stoichiometric distribution of DHPR and ryanodine receptors in cardiac tissues is approximately 1:9 which suggests that a physical link between the two receptors is not likely to be involved in the SR Ca^{2+} release process (Wibo et al., 1991).

Using laser scanning confocal microscopy and the fluorescent Ca^{2+} indicator fluo-3, Cheng et al. (1993) showed in cardiac muscle that local increases in intracellular calcium, referred to as "calcium sparks", occur through spontaneous opening of SR calcium release channels. Upon SR calcium loading, the frequency of channel opening increases to a level which triggers calcium waves. They suggest that calcium sparks may be the underlying mechanism of E-C coupling in mammalian heart and that CICR may by more sensitive to increases in intracellular calcium levels during periods of Ca^{2+} overload. Thus, it appears that ryanodine receptors in cardiac and skeletal muscle, although similar in many respects contribute differentially to E-C coupling.

I.I.7. *Regulation of ryanodine receptor/calcium release channel activity*

A wide variety of compounds affect SR Ca^{2+} release (Palade et al., 1989) and many of these appear to act directly at the ryanodine receptor. They alter [^{3}H]ryanodine binding to SR membranes and modify the conductance or open probability of channels in SR vesicles or purified ryanodine receptors incorporated into planar lipid bilayers (Fleischer and Inui, 1989). Ca^{2+} release channel activity is stimulated by Ca^{2+}, adenine nucleotides and caffeine, and inhibited by Mg^{2+}, ruthenium red and calmodulin (Fleisher and Inui, 1989; Lai and Meissner, 1989; Meissner and Henderson, 1987; Smith et al., 1988a,b). Similarly, these agents influence [^{3}H]ryanodine binding in both muscle types (Chu et al., 1990; Ogawa and Harafuji, 1990a; Pessah et al., 1985, 1986, 1987; Zimanyi and Pessah, 1991). Caffeine increases the maximum level of ryanodine binding by

enhancing the apparent affinity and cooperativity of Ca^{2+} activation of the receptor (Ogawa and Harafuji, 1990a; Pessah et al., 1987). Adenine nucleotides either increase the efficiency of channel gating or enhance the duration of channel opening following Ca^{2+} activation (Chu et al., 1990; Imagawa et al., 1989; Pessah et al., 1987). Caffeine and adenine nucleotides appear to act at different sites since their effects are additive (Ogawa and Harafuji, 1990a; Pessah et al., 1987). Calcium regulates SR Ca^{2+} release and ryanodine binding (Chu et al., 1990; Imagawa et al., 1989; Meissner et al., 1986; Pessah et al., 1986, 1987; Seifert and Casida, 1986; Su, 1992); low Ca^{2+} concentrations (nanomolar to micromolar range) activate Ca^{2+} release channels and enhance ryanodine binding (Meissner et al., 1986; Pessah et al., 1987; Seifert and Casida, 1986; Takasago et al., 1991) and high concentrations (millimolar) close channels and reduce the levels of binding (Chu et al., 1990; Imagawa et al., 1989; Seifert and Casida, 1986). This Ca^{2+} dependence supports the view that CICR plays a functional role in E-C coupling. Mg^{2+} inhibits the Ca^{2+} release channel and [^{3}H]ryanodine binding by competing with Ca^{2+} (Michalak et al., 1988; Ogawa and Harafuji, 1990b; Pessah et al., 1987). A model proposed by Pessah et al. (1987) showing the possible relationship of regulatory domains in the ryanodine receptor is presented in Figure 2. In this model, the ryanodine binding domain is proximal to the channel and rapidly occludes upon binding of ryanodine. The Ca^{2+} activator site occupies a peripheral position which allows accessibility of sulfhydryl groups. The caffeine and adenine nucleotide binding domains are adjacent and may overlap as indicated by their synergistic effects seen at suboptimal Ca^{2+} concentrations (Pessah et al., 1987).

Polyamines such as spermine modify calcium channel activity and enhance [^{3}H]ryanodine binding (Zarka and Shoshan-Barmatz, 1992). It was suggested that

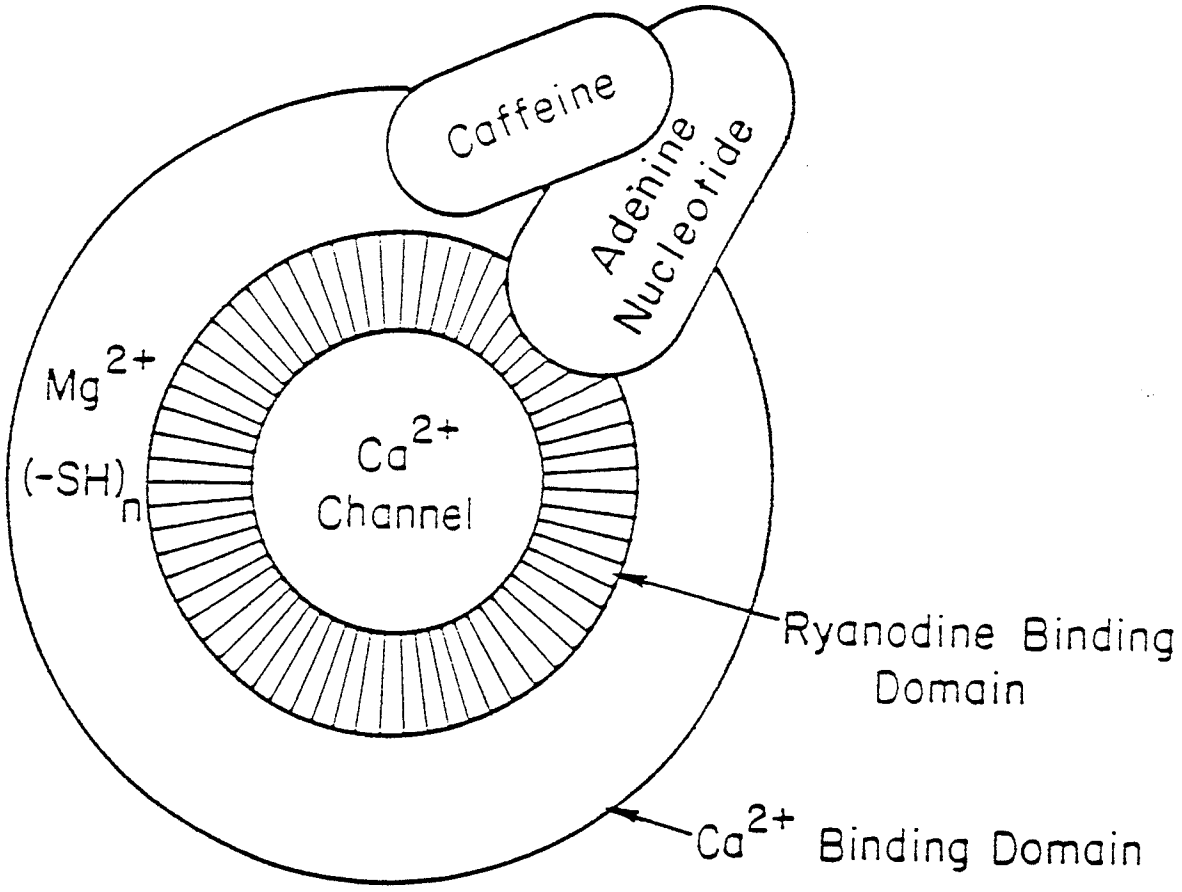

Figure 2. Functional domains of the ryanodine receptor/Ca^{2+} release channel as proposed by Pessah et al. (1987)

electrostatic interactions between polyamines and the ryanodine receptor may play an important role in their actions (Zarka and Shoshan-Barmatz, 1992).

High ionic strength increases [^{3}H]ryanodine binding to and Ca^{2+} release from skeletal and cardiac muscle SR vesicles (Chu et al., 1990; Hamilton et al., 1989; Imagawa et al., 1989; Meissner and El-Hashem, 1992; Michalak et al., 1988; Ogawa and Harafuji, 1990b; Seifert and Casida, 1986; Su and Hasselbach, 1983; Zarka and Shoshan-Barmatz, 1992; Zimanyi and Pessah, 1991). This occurs through an enhancement in the association rate of ryanodine binding and the affinity of the receptor for ryanodine (Chu et al., 1990; Hamilton et al., 1989; Meissner and El-Hashem, 1992; Ogawa and Harafuji, 1990b; Seifert and Casida, 1986; Zarka and Shoshan-Barmatz, 1992; Zimanyi and Pessah, 1991) and may be due to direct salt interactions with the ryanodine binding site to either stabilize the ryanodine receptor complex or to increase its sensitivity toward Ca^{2+} (Chu et al., 1990; Michalak et al., 1988).

SR Ca^{2+} release channels and ryanodine binding are also influenced by temperature and pH changes. Lower temperatures stimulate Ca^{2+} release, but decrease the affinity of the receptor for ryanodine (Ogawa and Harafuji, 1990a) and increases in pH stimulate Ca^{2+} release from SR vesicles and enhance ryanodine binding (Michalak et al., 1988). Ogawa and Harafuji (1990a) suggested that two phases are involved in ryanodine binding; a conformational change in the channel to an open state is followed by binding of ryanodine to its site once this favorable conformational state is produced.

I.I.8. *Biochemical properties of ryanodine receptors*

Skeletal and cardiac ryanodine receptors have been cloned (Takeshima et al., 1989; Otsu et al., 1990a; Nakai et al., 1990; Zorzato et al., 1990) and found to be 66% homologous (Nakai et al., 1990; Otsu et al., 1990a; Takeshima et al., 1989; Zorzato et al.,

1990). They consist of a tetramer of a single polypeptide with an apparent sedimentation coefficient of 30S (Lai et al., 1988a; Otsu et al., 1990a), are encoded by approximately 5000 amino acids and have an estimated molecular weight (M_r) of 565 kDa based on their deduced amino acid sequence (Nakai et al., 1990; Otsu et al., 1990a; Takeshima et al., 1989; Zorzato et al., 1990). The predicted secondary structure of the receptor contains: 1) four highly hydrophobic segments of approximately 20 amino acids in length each of which may form transmembrane regions, 2) a non-hydrophobic amino-terminal end which may act as a signal sequence on the cytoplasmic side of the SR membrane, 3) several regulatory sequences which house proposed binding sites for adenine nucleotides, calmodulin and other modulators, and 4) a large cytoplasmic region which comprises the foot structure of the receptor (Takeshima et al., 1989). The proposed transmembrane and cytoplasmic domains are similar in the skeletal and cardiac receptors (Nakai et al., 1990). At the electron microscopic level, the receptor exhibits a quatrefoil appearance which is morphologically similar to the receptor foot structure (Anderson et al., 1989; Lai et al., 1988b). The maximimum theoretical [^{3}H]ryanodine binding density of the purified receptor is estimated to be 2500 pmol/mg protein, but the actual observed density is 26% of this theoretical value (Lai et al., 1988b). This suggests the presence of one ryanodine binding site per four protein subunits (Lai et al., 1988b).

The modulatory sites of the ryanodine receptor appear to be localized in a region between amino acid residues 2619-3016 at the N-terminal portion of the first transmembrane domain (Otsu et al., 1990a; Takeshima et al., 1989). A Ca^{2+} binding site was identified in the regulatory region and antibodies directed against this site increase the probability and time of channel opening which suggests its involvement in regulation of channel conductance (Chen et al., 1992). Chen et al., (1993) recently generated several tryptic fragments by partial proteolytic digestion of the ryanodine receptor. Further

characterization of these fragments may reveal other regulatory binding sites, domain structures and subunit interactions in the ryanodine receptor.

The ryanodine receptor can be phosphorylated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), cGMP-dependent protein kinase (PKG) and Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) (Takasago et al., 1989; Yoshida et al., 1992). PKA, PKC and PKG phosphorlyation of the receptor increases ryanodine binding levels (Takasago et al., 1989; Yoshida et al., 1992). In contrast, phosphorylation by CaMKII results in a reduction in the levels of binding (Yoshida et al., 1992). PKA, PKC and PKG phosphorylate serine residues in the same phosphopeptide (Yoshida et al., 1992), while CaMKII phosphorylates a single serine residue found within the modulatory domain proposed by Otsu et al. (1990a). Phosphorylation of this residue results in activation of the Ca^{2+} release channel (Witcher et al., 1991). A 12kDa peptide sequence homologous to the human T-cell FK506-binding protein that functions as a PKC inhibitor is associated with skeletal muscle ryanodine receptors and may contribute to PKC modulation of SR Ca^{2+} release channels (Collins, 1991; Jayeraman et al., 1992).

Heavy metals and various SH oxidizing agents interact with sulfhydryl groups of the SR calcium release channel to promote rapid calcium release and this suggests that reduction and oxidation of channel sulfhydryl groups may regulate the gating of the channel (Abramson and Salama, 1989).

Triadin, a 95-kD protein located on the junctional face of terminal cisternae, is associated with both ryanodine receptors and DHPR (Brandt et al., 1990; Caswell et al., 1991; Kim et al., 1990). Expression of triadin follows ryanodine receptor expression which suggests a close association between the two proteins (Flucher et al., 1993). The bulk of triadin is localized in the SR interior and 47 amino acids are exposed to the

cytoplasm (Knudson et al., 1993a,b). McPherson and Campbell (1993) hypothesize that triadin may act as a link to allow mutual regulatory interactions between the ryanodine receptor and calsequestrin, an SR Ca^{2+} binding protein (McPherson and Campbell, 1993). This proposal is supported by a study conducted by Gilchrist et al. (1992) which showed that Ca^{2+} sequestration within the SR lumen is modulated by open and closed states of the Ca^{2+} release channel. A proposed molecular model of the ryanodine receptor/Ca^{2+} channel complex is presented in Figure 3 (McPherson and Campbell, 1993).

I.I.9. *Conductance states of ryanodine receptor /calcium release channels*

Reconstitution of purified cardiac and skeletal muscle ryanodine receptor/calcium release channels into planar lipid bilayers, followed by single channel measurements, indicate that these channel proteins form functional Ca^{2+} channels which are activated and inhibited by submicromolar and micromolar concentrations of Ca^{2+}, respectively, and exhibit a range of sub- or variable-conductance states (Anderson et al., 1989; Campbell et al., 1987; Fill et al., 1989; Hymel et al., 1988b; Imagawa et al., 1987; Inui et al., 1987a,b; Lai et al., 1988a,b; Liu et al., 1989; Ma et al., 1988; Smith et al., 1988a,b). The purified skeletal muscle ryanodine receptor complex exhibits at least four different Ca^{2+} conductance levels with a prominent conductance of approximately 100 pS (Liu et al., 1989; Smith et al., 1988a). The channel is selectively permeable to Ca^{2+} ions over monovalent cations, but in the absence of Ca^{2+}, it displays broad selectivity for monovalent species (Liu et al., 1989; Smith et al., 1988a). The main unitary Ca^{2+} conductance has been suggested to be composed of several sub-conductances (Liu et al., 1989). The slope conductance of the reconstitued purified cardiac receptor is 70-72 pS which is similar to that observed for native cardiac SR Ca^{2+} release channels (75-83 pS) (Anderson et al., 1989; Lai et al., 1988a). Ma et al. (1988) suggested that ryanodine receptor/Ca^{2+} release channels have similarities to gap junction channels based on their

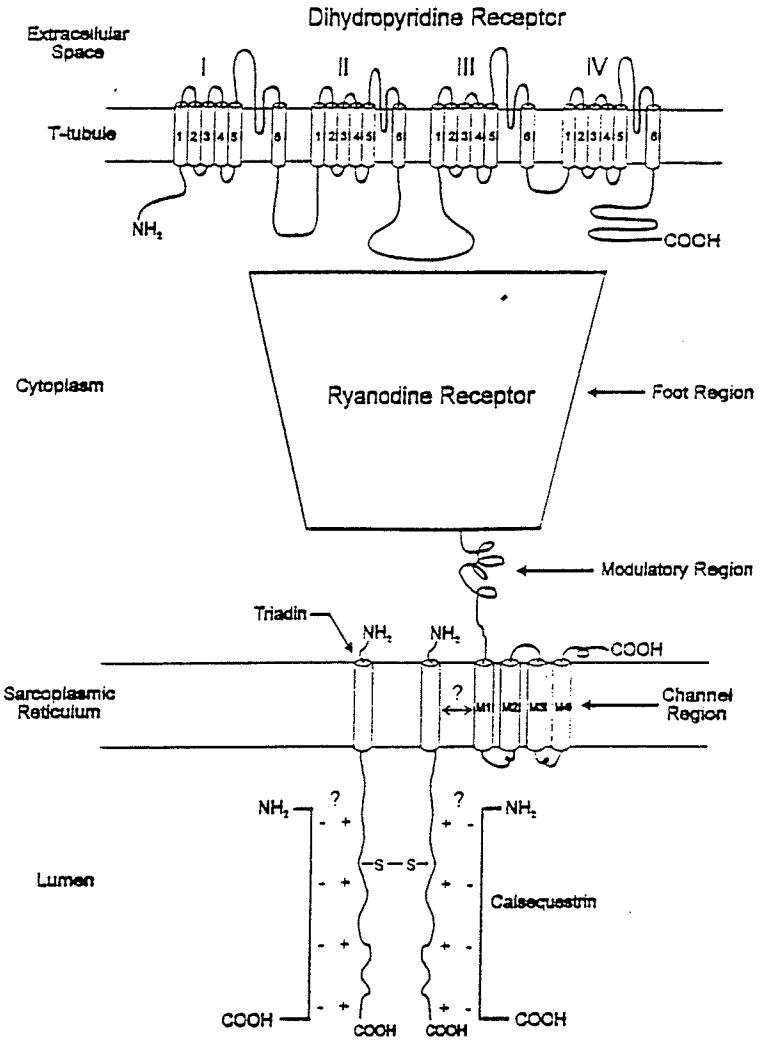

Figure 3. Model of the triad junction containing the ryanodine receptor/Ca^{2+} release channel as proposed by McPherson and Campbell (1993)

large voltage-dependent conductance and their sensitivity to inhibition by low pH, high concentrations of calcium and by agents known to uncouple gap junctions.

Ca^{2+} channel conductance is activated by ATP and inhibited by ruthenium red and Mg^{2+}, all of which are modulators of SR Ca^{2+} release (Hymel et al., 1988; Imagawa et al., 1987; Smith et al., 1986, 1988a,b). A reduced Ca^{2+} channel conductance is observed following addition of ryanodine prior to ruthenium red (Hymel et al., 1988b; Imagawa et al., 1987; Nagasaki and Fleischer, 1988) and this may be due to ryanodine-induced stabilization of an open conformational channel state (Hymel et al., 1988; Imagawa et al., 1987; Nagasaki and Fleischer, 1988). Ryanodine reduces channel conductance by approximately 40% (Nagasaki and Fleischer, 1988). These findings indicate that a Ca^{2+}-activated Ca^{2+} channel is a component of the ryanodine receptor. A model proposed by Nagasaki and Fleischer (1988) depicting the action of ryanodine and ruthenium red on the SR calcium release channel is illustrated in Figure 4. The utilization of antibodies that recognize specific regions on the ryanodine receptor protein may add a new dimension to functional analyses of ryanodine receptor/Ca^{2+} release channels. Fill et al. (1991) demonstrated that anti-ryanodine receptor antibodies directed against the C-terminal end of the ryanodine receptor protein alter the gating properties of the channel through an effect on regulatory sites of the receptor. Taken together, these studies indicate that the ryanodine receptor/Ca^{2+} channel complex regulates the release of Ca^{2+} stored within muscle SR and that the channel may play an important role in E-C coupling by controlling the intracellular levels of Ca^{2+}.

I.I.I0. *Summary of differences between skeletal and cardiac ryanodine receptors*

It is now clear that cardiac and skeletal muscle ryanodine receptors are very similar both in function and regulation. However, several important immunological, biochemical and regulatory differences exist which distinguish the two receptors.

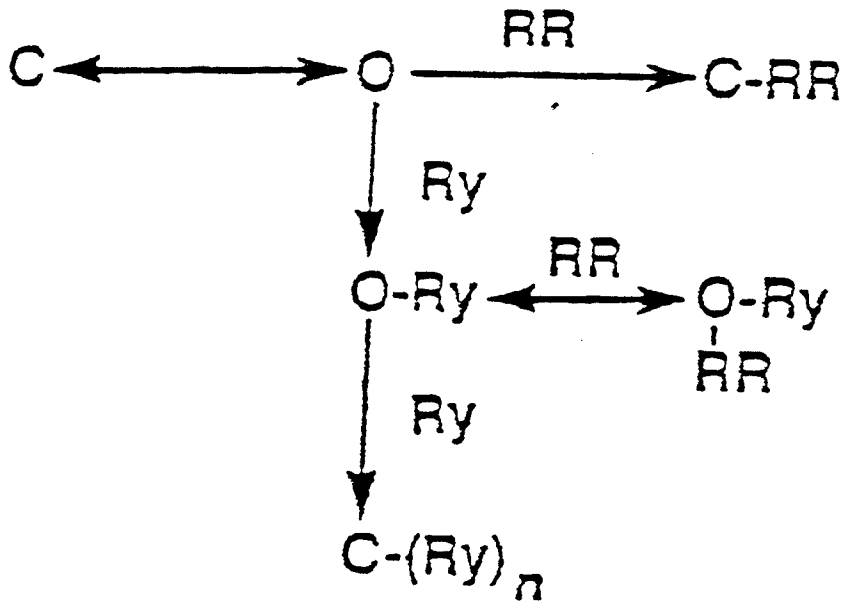

Figure 4. Diagramatic representation of the actions of ryanodine (Ry) and ruthenium red (RR) on the open (O) and closed (C) conformational states of the SR calcium release channel as proposed by Nagasaki and Fleischer (1988). In the absence of drugs, the Ca^{2+} channel alternates between O and C states. Ca^{2+} and ATP enhance the O state, whereas Mg^{2+} promotes the formation of the C state. RR acts to completely close the channel (C-RR) and Ry at submicromolar concentrations locks the channel in a long open sub-conductance state (O-Ry). If RR is added prior to Ry, the channel closes and the alkaloid cannot reopen the channel. Micromolar concentrations of Ry promotes channel closure ($C-(Ry)_n$).

Northern blot analyses conducted on cardiac and skeletal muscle ryanodine receptors reveal that each is the product of a separate gene (Nakai et al., 1990; Otsu et al., 1990a; Takeshima et al., 1989; Zorzato et al., 1990). The gene encoding the cardiac ryanodine receptor is localized to chromosome 1 (Otsu et al., 1990a). Antibodies generated against the purified cardiac ryanodine receptor fail to recognize epitopes on the skeletal receptor (Imagawa et al., 1989). The cardiac receptor displays a slightly higher mobility as determined by polyacrylamide gel electrophoresis compared with the skeletal muscle form which suggests that the cardiac protein is a slightly smaller molecule (Inui et al., 1987b; Lai et al., 1988a; Imagawa et al., 1989). [^{3}H]Ryanodine binding to solubilized cardiac receptors is dependent on the presence of phospholipid which is not a requirement for binding to the skeletal muscle protein (Anderson et al., 1989). Cardiac Ca^{2+} release channel activity is highly dependent on Ca^{2+} and less sensitive to ruthenium red inhibition compared with the skeletal muscle channel (Anderson et al., 1989; Zimanyi and Pessah, 1991). The greater Ca^{2+} sensitivity expressed by cardiac SR channels supports the proposed dominance of CICR in initiating cardiac E-C coupling and its lesser role in skeletal muscle contractions (Zimanyi and Pessah, 1991).

Ryanodine binding to skeletal muscle SR vesicles is enhanced by ionic strength, adenine nucleotides, increased pH and micromolar concentrations of Ca^{2+}, while binding to cardiac SR is less sensitive to these conditions (Zimanyi and Pessah, 1991). High Ca^{2+}, Mg^{2+}, ruthenium red and neomycin all have greater inhibitory effects on the skeletal compared with the cardiac ryanodine receptor. The skeletal receptor is also less sensitive to activation by caffeine and is not phosphorylated as efficiently by CaMKII and PKA compared with the cardiac receptor (Witcher et al., 1991; Zimanyi and Pessah, 1991). Thus, the observed differences in size, stability, function, regulation and expression between the cardiac and skeletal muscle ryanodine receptors suggest that different E-C coupling mechanisms are operative in the two tissues. Moreover, the

presence of more than one subtype of ryanodine receptor in the regulation of Ca^{2+} release from intracellular compartments in muscle suggest the possibility of the existence of the same or different subtypes of ryanodine receptors in the regulation of cytosolic calcium in other tissues and cell types including neurons.

I.2 *Intracellular Ca^{2+} release channels in neurons*

I.2.I. *Role of Ca^{2+} in neurons*

Neurons have a resting intracellular Ca^{2+} concentration of approximately 10^{-7} M. During periods of depolarization- or receptor-mediated stimulation, cytosolic Ca^{2+} increases to a level that is dependent on the stimulus strength and regional localization in the neuron (Kennedy, 1989a) thereby allowing concentration- and site-dependent intracellular actions of Ca^{2+}. In addition to the involvement of Ca^{2+} in neurotransmitter release, a diversity of studies have examined the role of Ca^{2+}-dependent processes in the regulation of ion channels, regulatory enzymes, gene expression, growth cone movements and long-term potentiation (Kennedy, 1989a).

Membrane proteins that are targets of regulation by Ca^{2+} alone or in concert with other intracellular messengers include three classes of ion channels. These are Ca^{2+}-dependent potassium channels, monovalent cation-selective channels and chloride channels (Marty, 1989). Ca^{2+}-regulated K^+ channels are heterogeneous and differ from each other with respect to their pharmacological, biochemical and electrophysiological profiles. Ca^{2+} inhibits or activates channel conductance directly or indirectly as a result of PKA activation (Marty, 1989). Similar regulatory influences of Ca^{2+} on cation-selective and chloride channels have been reported (Maruyama and Petersen, 1984; Paulais and Teulon, 1989, 1990; Sturgess et al., 1987). Ca^{2+} modulation of ion channels can regulate neuronal excitability through the shaping of action potentials and firing

patterns (Marty, 1989). In addition, Ca^{2+} can activate membrane phospholipases which leads to the synthesis of arachidonic acid by phospholipase A and/or the production of diacylglycerol and inositol 1,4,5-trisphosphate by phospholipase C (Kennedy, 1989a). The generated products are themselves second messengers which affect other neuronal functions.

In the cytosol, Ca^{2+} regulates the activities of three classes of proteins. The first group comprise the C-kinases which are activated by the synergistic actions of Ca^{2+} and diacylglycerol. Activated C-kinases translocate from the cytosol to the plasma membrane where they influence electrophysiological activities and synaptic transmission through phosphorylation of membrane proteins (Malenka et al., 1986; Strong et al., 1987) and are somehow linked with synaptic events underlying long-term potentiation, the putative substrate of memory formation in the hippocampus (Kennedy, 1989b; Malinow et al., 1988; Reymann et al., 1988). Calpains or neutral proteases, which form the second group, appear to be involved in regulation of membrane proteins and cytoskeletal dynamics (Melloni and Pontremoli, 1989). The third class, calmodulin (CaM), is normally inactive at resting Ca^{2+} concentrations (Kennedy, 1989a), but as cytosolic Ca^{2+} increases towards 1 μM, the four Ca^{2+} binding sites located in the CaM EF-hand domain become successively occupied resulting in CaM activation (Kennedy, 1989a). Activated CaM regulates various neuronal proteins including Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), calcineurin, adenylate cyclase and phosphodiesterase (Kennedy, 1989a). CaMKII is the predominant Ca^{2+}-dependent protein kinase in the cortex and hippocampus of mammalian brain (Erondu and Kennedy, 1985) and phosphorylates proteins involved in such functions as neurotransmitter release and long-term potentiation (Huttner et al., 1983; Kennedy, 1989a,b; Malinow et al., 1989). Calcineurin is a protein phosphatase involved in the inactivation of voltage-gated L-type Ca^{2+} channels (Armstrong, 1989; Chad and Eckert, 1986). CaM modulation of adenylate cyclase and

phosphodiesterase regulates cAMP levels and therefore indirectly influences the activity of PKA (Kennedy, 1989a). These examples of the importance of Ca^{2+} in neuronal activity indicate that neurons likely possess elaborate systems to regulate cytosolic Ca^{2+} concentrations.

Under steady-state conditions, uptake and efflux pathways at the plasma membrane appear to be the main determinants of cellular Ca^{2+} content (Thomas et al., 1992). Plasma membrane voltage- and receptor-operated Ca^{2+} channels regulate the influx of extracellular Ca^{2+} into cells, while Na^{+}/Ca^{2+} exchange and Ca^{2+}-ATPase pumps reduce cytoplasmic Ca^{2+} levels through Ca^{2+} extrusion. Intracellular Ca^{2+} binding proteins and internal Ca^{2+} storage organelles buffer cytosolic Ca^{2+}. Release of Ca^{2+} from intracellular Ca^{2+} stores can increase the levels of free intracellular Ca^{2+}. The limited capacity for Ca^{2+} storage in and release from non-mitochondrial intracellular Ca^{2+} pools most likely restricts the contribution of these compartments in Ca^{2+} regulation under steady-state conditions (Thomas et al., 1992). The role of each of these mechanisms in intraneuronal Ca^{2+} homeostasis are discussed in more detail below.

I.2.2. *Voltage- and receptor-operated Ca^{2+} channels*

Four voltage-activated Ca^{2+} channel subtypes are categorized into two main classes based on their biophysical and pharmacological properties. "T" (transient)-type channels constitute a low voltage-activated (LVA) or low-threshold class and "L" (long lasting)-, "N" (neuronal)- and "P" (Purkinje cells)-type channels are placed in the high voltage-activated (HVA) or high-threshold class (Bertolino and Llinas, 1992; Nowycki et al., 1985; Tsien et al., 1988).

T-channels, activated at more negative potentials, are relatively insensitive to elevations in cytosolic Ca^{2+} compared with the other Ca^{2+} channel subtypes and rapidly

inactivate during maintained depolarizations (Bertolino and Llinas, 1992). These currents may be involved in the generation of synaptic activity-independent spontaneous membrane potential fluctuations (Bertolino and Llinas, 1992; Tsien et al., 1988) which may play a role in motor coordination and wakefulness regulation (Llinas, 1988). Selective pharmacological T-channel ligands are presently lacking. N-type Ca^{2+} channel currents are difficult to discern from L-type currents due to their similar biophysical properties. N-type channels display slow inactivating and long-lasting components (Plummer et al., 1989), carry the majority of the whole cell Ca^{2+} current (Plummer et al., 1989; Aosaki and Kasai, 1989) and may play a role in eliciting neurotransmitter release (Hirning et al., 1988; Miller, 1987; Stanley and Atrakchi, 1990). They are insensitive to DHPs and are inhibited by the snake venom ω-conotoxin (ω-CgTx) (McCleskey et al., 1987; Plummer et al., 1989). Functional P-type channels are widely distributed throughout brain and may contribute to synaptic transmission, neuronal integration, generation of neuronal intrinsic activity, and cell death (Bertolino and Llinas, 1992; Llinas et al., 1992; Mintz et al., 1992). These channels are insensitive to both DHPs and ω-CgTx (Bertolino and Llinas, 1992), but are blocked by the funnel web spider poison, FTX, cadmium, cobalt and several synthetic polyamines (Cherksey et al., 1991; Llinas et al., 1989). L-type channels are activated by large depolarizations, are slow to inactivate (Tsien et al., 1988) and are localized in cell bodies and proximal dentrites of neurons in the CNS (Bertolino and Llinas, 1992) where they appear to be involved in the generation of action potentials and in signal transduction mechanisms (Bertolino and Llinas, 1992). They are potently inhibited by Ca^{2+} antagonists such as dihydropyridines (DHP) and phenylalkylamines and are activated by Bay K8644 (Tsien et al., 1988). L-type Ca^{2+} channels also known as dihydropyridine receptors (DHPR) have been sequenced and are composed of five subunits termed α_1, α_2, β, γ and δ (Bertolino and Llinas, 1992; Campbell et al., 1988). Partial cDNA clones homologous to skeletal and cardiac DHPR were isolated and, based on the hybridation patterns of brain mRNA species, classified as

A to D (Miller, 1992; Snutch et al., 1990; Tsien et al., 1991). Southern blot analyses further reveal that each class represents a distinct gene or gene family (Tsien et al., 1991).

Ca^{2+} influx into neurons can also be mediated by activation of receptor-operated Ca^{2+} channels by neurotransmitters or hormones. A Ca^{2+} channel appears to be an integral component of some receptors including the nicotinic acetylcholine, $GABA_a$ and N-methyl-D-apartate (NMDA) receptors (Atlas, 1990). These receptors have been cloned and are permeable to Ca^{2+} ions (Atlas, 1990). The much studied NMDA receptor is selective for Ca^{2+} unlike other glutamate receptor subtypes which selectively permits the passage of monovalent cations (Buhrle and Sonnhof, 1983; Choi and Rothman, 1990; Collingridge, 1987; Daw et al., 1993; Dingledine, 1983; Mayer et al., 1987; Mayer and Miller, 1990).

I.2.3. *Ca^{2+} buffering proteins, membrane Na+/Ca^{2+} exchangers and Ca^{2+} pumps*

Resting levels of cytoplasmic Ca^{2+} is maintained by several different processes. One mechanism involves Ca^{2+} binding to cytosolic Ca^{2+} binding proteins (CaBPs) which belong to the 'EF-hand' family of CaBPs (Baimbridge et al., 1992). Of the major CNS CaBPs, parvalbumin, calbindin-D28 and calretinin, which are present in distinct subpopulations of neurons, appear to be the most prominent with respect to neuronal Ca^{2+} buffering (Baimbridge et al., 1992; Celio, 1990; Rogers, 1987; Winsky et al., 1989). Their distribution and properties suggest an involvement in modulation of neuronal excitability (Baimbridge et al., 1992). CaBPs may influence the duration of action potentials, promote neuronal bursting activity and protect neurons against Ca^{2+} neurotoxicity (Baimbridge et al., 1992; Kawaguchi et al., 1987; Kohr et al., 1991; Mattson et al., 1991; Nitsch et al., 1989; Scharfman and Schwartzkroin, 1989; Sloviter, 1989; Weiss et al., 1990).

Na^+/Ca^{2+} exchangers in the plasmalemma appear to play two major roles in intraneuronal Ca^{2+} regulation. First, the energy inherent within the trans-plasmalemmal Na^+ electrochemical gradient is used by the exchanger to rapidly extrude Ca^{2+} from the cytosolic space. Second, they regulate the level of Ca^{2+} sequestered within intracellular compartments (Blaustein et al., 1991). They operate in a bidirectional manner depending on the extent of the Na^+ and Ca^{2+} gradients across the membrane as well as the membrane potential (Lagnado and McNaughton, 1990; Rahamimoff, 1990). The stoichiometric ratio of Na^+ to Ca^{2+} ions necessary to maintain the concentration of Ca^{2+} below 1 μM varies depending on tissue type and, in synaptic plasma membranes, this ratio is 4-5 Na^+ per 1 Ca^{2+} (Barzilai and Rahamimoff, 1987). Under normal resting conditions, the exchanger is relatively inactive and therefore is probably not involved in the maintenance of resting free cytosolic Ca^{2+} levels (Blaustein et al., 1991). It becomes active at free cytosolic Ca^{2+} concentrations of approximately 750 nM (Miller, 1991) and is stimulated by K^+ ions (Rahamimoff et al., 1991). Recently, the cDNA for the cardiac Na^+/Ca^{2+} exchanger was used to identify mRNA species present in brain (Komuro et al., 1992; Nicoll et al., 1990; Wroblewski, 1992).

Plasma membrane Ca^{2+}-ATPase pumps (PMCA) extrude Ca^{2+} against large Ca^{2+} concentration gradients from the cytosol into the extracellular space or endoplasmic reticulum (ER) and are most likely the primary mechanism for restoring or maintaining basal levels of intracellular Ca^{2+} (Carafoli, 1991). PMCA are activated by Ca^{2+}, dependent on the presence of Mg^{2+}, and regulated by calmodulin (CaM) (Carafoli, 1991). CaM increases the affinity of the pump by about 20-fold (Carafoli, 1991) which allows the pump to be active at physiological concentrations of Ca^{2+}. PMCA of brain has been cloned, has a molecular weight of approximately 140 kDa, and is structurally similar to other ion translocating membrane ATPases (Carafoli, 1991). Thus, neurons possess

two different plasma membrane mechanisms to extrude intracellular Ca^{2+} which are critical for the fine regulation of cytosolic Ca^{2+} concentrations.

I.2.4. *Intracellular Ca^{2+} compartments*

In neurons, calcium is sequestered in various organelles including nuclei, mitochondria, ER, and the putative calciosome (Andrews et al., 1987, 1988; Parducz et al., 1987; Volpe et al., 1988). Baker and Schlaepfer (1978) and Blaustein et al. (1978a,b) demonstrated that intraneuronal membrane vesicles of non-mitochondrial origin can sequester Ca^{2+} in an ATP-dependent fashion. These Ca^{2+} stores can potentially buffer cytosolic Ca^{2+} to approximately 100 nM which indicates that they have a role in maintaining the resting levels of intracellular Ca^{2+}. In electron microscopic and X-ray microprobe analyses, it was shown that intracellular compartments, most likely ER, sequesters Ca^{2+} during neuronal stimulation (Henkart et al., 1978). Dendrites, cell bodies and axon terminals also accumulate Ca^{2+} within similar intracellular stores (Miller, 1991). An analogous functional role as muscle SR in the uptake and storage of Ca^{2+} was suggested for ER. Although it is still uncertain whether ER functions as muscle SR in the release of sequestered Ca^{2+}, it is now believed that neurons have two major Ca^{2+} stores, namely, those sensitive to actions of inositol 1,4,5-trisphosphate (IP_3) and those insensitive to IP_3, but affected by the methylxanthine caffeine.

Mammalian presynaptic nerve terminals have at least two major ATP-dependent Ca^{2+} sequestering systems; a large capacity, low Ca^{2+} affinity store located in mitochondria and a high Ca^{2+} affinity but considerably lower capacity Ca^{2+} pool believed to be localized in smooth endoplasmic reticulum (SER) (Blaustein and Rasgado-Flores, 1981; McGraw et al., 1980). Although mitochondria may play a prominent role in regulating intracellular Ca^{2+} levels under normal physiological conditions (Miller, 1991), much of the recent literature on intracellular Ca^{2+} pools has centered on the function and

regulation of non-mitochondrial stores. In view of the focus of the present thesis work, the remainder of this discussion will concentrate on the physiological relevance of non-mitochondrial intracellular compartments in nerve terminals.

Studies conducted by Blaustein et al. (1978a,b) and others (Gill et al., 1981; Martinez-Serrano and Satrustegui, 1989; Michaelis et al., 1987; Mekhail-Ishak et al., 1987) demonstrated the presence of a non-mitochondrial intracellular ATP-dependent Ca^{2+} uptake mechanism in synaptosomes and intact nerve terminals. Synaptosomes are essentially "pinched off" presynaptic nerve terminals which retain the morphological and chemical properties of intact nerve terminals (Gray and Whittaker, 1962). The uptake requires Mg^{2+}, is active at Ca^{2+} concentrations less than 400 nM and was suggested to have an important role in Ca^{2+} buffering following neural activity (Blaustein et al., 1978a,b; Martinez-Serrano and Satrustegui, 1989; Rasgado-Flores and Blaustein, 1987). Ultrastructural and X-ray microprobe analyses reveal that the non-mitochondrial store is most likely the SER (McGraw et al., 1980; Blaustein et al., 1980). Ca^{2+} uptake into brain microsomal membranes and in permeabilized synaptosomes can be distinguished from that in synaptic plasma membrane vesicles (SPM) through their differences in vanadate (Ca^{2+} pump inhibitor) sensitivity and by the presence of functional Na^{+}/Ca^{2+} exchangers in SPM (Martinez-Serrano and Satrustegui, 1989; Michaelis et al., 1987). This suggests that two distinct Ca^{2+} transport sytems are present in synaptic terminals; one functioning at the plasma membrane and the other in intraterminal non-mitochondrial membranes (Michaelis et al., 1987). Caffeine biphasically influences synaptosomal SER Ca^{2+} uptake; less than 30 mM activates and 60 mM inhibits uptake (Mekhail-Ishak et al., 1987).

IP_3- and caffeine-sensitive Ca^{2+} pools appear to be present in nerve terminals (Gandhi and Ross, 1987; Martinez-Serrano and Satrustegui, 1989; Mekhail-Ishak et al.,

1987). In saponin-permeabilized synaptosomes, IP_3 does not release all the stored Ca^{2+} which indicates the presence of an IP_3-insensitive Ca^{2+} pool (Gandhi and Ross, 1987; Gill et al., 1981; Mekhail-Ishak et al., 1987). This is supported by a study conducted by Martinez-Serrano and Satrustegui (1989) who demonstrated that caffeine induces Ca^{2+} release from a non-mitochondrial IP_3-insensitive compartment in permeabilized synaptosomes.

Although Ca^{2+} buffering is a likely function for these intraterminal compartments, the demonstrated release of sequestered Ca^{2+} suggests a wider role in presynaptic Ca^{2+} regulation. In this regard, it was shown that the rate of uptake into SER is too slow to be involved in terminating neurotransmitter release (Rasgado-Flores and Blaustein, 1987). Although influx of Ca^{2+} from the extracellular space into the nerve terminal is believed to be the primary trigger for transmitter secretion (Augustine et al., 1987; Atlas, 1990; Llinas et al., 1992), several reports describe transmitter release independent of extracellular Ca^{2+}. For example, [^{3}H]noradrenaline release in hippocampal slices and ATP secretion appear to require some contribution of Ca^{2+} released from intracellular stores (Etcheberrigaray et al., 1991; Huang et al., 1991). In PC12 cells which express neuronal properties, caffeine-induced Ca^{2+} release stimulates dopamine release (Avidor et al., 1994). At the neuromuscular junction, ryanodine facilitates the release of acetylcholine (Nishimura et al., 1990). Thus, Ca^{2+} release from intraterminal Ca^{2+} pools influences some component of the neurosecretory pathway which initiates transmitter release.

I.2.5. *Inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} pool*

Hokin and Hokin (1953) reported that acetylcholine enhances the incorporation of radiolabelled phosphates into cellular phospholipids. Numerous studies have now shown that binding of various hormones, growth factors, neurotransmitters, and neuropeptides to G-protein-coupled receptors results in phospholipase C-mediated hydrolysis of

phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Berridge, 1981, 1983, 1987; Berridge and Irvine, 1989; Charest et al., 1985; Downes, 1982; Weiss et al., 1982; Williamson et al., 1985). Neurotransmitter-stimulated Ca^{2+} release from internal stores is associated with enhanced hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Michell, 1981, 1986) and neural tissues contain high concentrations of PIP_2 (Hawthorne, 1979). A variety of cell types including neurons mobilize intracellularly stored Ca^{2+} via IP_3 (Authi and Crawford, 1985; Biden et al., 1984; Burgess et al., 1984; Gandhi and Ross, 1987; Hirata et al., 1984; Joseph et al., 1984; Prentki et al., 1984; Streb et al., 1983). The action of IP_3 is rapidly terminated either by conversion of IP_3 to the non-Ca^{2+} mobilizing metabolites, myoinositol-bisphosphate (IP_2) or monophosphate (IP), by 5-phosphatase or to inositol 1,3,4,5-tetraphosphate (IP_4) by 3-kinase. IP_4 is suggested to function as a second messenger that acts either independently or in concert with IP_3 (Berridge and Irvine, 1984, 1989; Irvine, 1992).

IP_3-induced intracellular Ca^{2+} release appears to be 'quantal' in nature such that each successive increment of IP_3 releases precise amounts of sequestered Ca^{2+} (Bootman, 1994; Brown et al., 1992; Ferris et al., 1992). The mechanism by which this occurs is uncertain, but two models are proposed which describe either a steady-state mechanism or an all-or-none process of IP_3-induced release (Bootman, 1994). In kinetic measurements of Ca^{2+} release elicited by caged Ca^{2+} and IP_3, Ca^{2+} appears to mediate positive feedback control of IP_3-induced release at Ca^{2+} concentrations below 300 nM (Iino and Tsukioka, 1994); release is inhibited at concentrations greater than 300 nM (see next section). It is clear from these studies that IP_3 acts directly or indirectly on an intracellular channel which is involved in gating the release of sequestered Ca^{2+}. The molecular mechanisms by which IP_3 activates Ca^{2+} release has been forthcoming with studies of the biochemical and pharmacological characteristics, the immunohistochemical

and autoradiographical distribution, the molecular cloning and expression, and the functional properties of putative IP_3 receptors.

I.2.6. *Inositol 1,4,5-trisphosphate receptor*

Studies have demonstrated that peripheral and neural tissues bind [^{3}H]IP_3 with high affinity (nanomolar K_d values) (Baukal et al., 1985; Garlind et al., 1994; Guillemette et al., 1987; Spat et al., 1986; Worley et al., 1987a) and that this affinity parallels IP_3 potency in activating calcium release (Ferris et al., 1989; Stauderman et al., 1988; Worley et al., 1987b). This suggests that IP_3 directly binds to an IP_3-activated Ca^{2+} release channel or to a protein coupled to the channel. Various agents and conditions influence [^{3}H]IP_3 binding in brain membrane preparations. Concentrations of Ca^{2+} greater than 300 nM inhibit IP_3 binding (IC_{50} = 300 nM) (Worley et al., 1987b; Supattapone et al., 1988b) and this appears to be mediated through a Ca^{2+} binding protein termed 'calmedin' which allows negative feedback of IP_3 actions (Danoff et al., 1988). Physiological elevations of intracellular pH (between 7.5 and 8.5) which occur as a result of growth factors and hormone stimulation increases IP_3 binding by 3-fold and enhances the IP_3-induced release of Ca^{2+} (Joseph et al., 1989; Macara, 1985; Siffert and Akkerman, 1987; Worley et al., 1987b). Heparin, a potent antagonist of IP_3 binding and IP_3-mediated Ca^{2+} release (Ghosh et al., 1988; Guillemette et al., 1989; Hill et al., 1987; Joseph and Rice, 1989; Worley et al., 1987b) is extensively employed as a tool in examinations of IP_3 receptor function (Harootunian et al., 1991; Wakui et al., 1990). The high potency of heparin inhibition of IP_3 binding has been used as the basis for purification of the IP_3 receptor (Supattapone et al., 1988b).

IP_3 enhances flux of $^{45}Ca^{2+}$ in lipid vesicles incorporated with the purified IP_3 receptor and heparin blocks this action (Ferris et al., 1989). Single channel measurements of the reconstituted receptor in planar lipid bilayers revealed the presence of a Ca^{2+}

channel with a conductance of 26 pS (Maeda et al., 1991). ATP regulates the actions of IP_3 and biphasically influences Ca^{2+} flux in reconstituted vesicles (Ferris et al., 1990; Maeda et al., 1991). Low (1 to 10 μM) and high (0.1 to 1.0 mM) concentrations of ATP cooperatively enhance and inhibit, respectively, Ca^{2+} flux (Ferris et al., 1990) and micromolar concentrations of ATP significantly enhance the open probability of the channel (Maeda et al., 1991). A high affinity ATP binding site on the IP_3 receptor was subsequently identified (Ferris et al., 1990; Maeda et al., 1991).

The cloned IP_3 receptor has a highly conserved sequence between species and is encoded by at least four different genes designated types 1-4 (Blondel et al., 1993; Ferris and Snyder, 1992; Furuichi et al., 1989; Mignery et al., 1989, 1990; Nordquist et al., 1988; Ross et al., 1992; Sudhof et al., 1991). Alternatively spliced forms of the IP_3 receptor have been identified and differ with respect to their patterns of receptor phosphorylation by PKA (Danoff et al., 1991; Nakagawa et al., 1991). This processing may influence ATP regulation of the IP_3 receptor since splicing occurs near the ATP binding site (Ferris and Snyder, 1992). Type 1 IP_3 receptor expression predominates in cerebellar tissues and at least two other subtypes are co-expressed in lower quantities (Ross et al., 1992; Takei et al., 1994). This suggests a possible differential regulation of IP_3 receptor function within some of these cells. Short and long forms of the receptor, which differ by about 40 amino acids, were identified in adult and fetal brain (Danoff et al., 1991). The long form is mainly found in neurons, while the short form is restricted to non-neuronal tissues (Danoff et al., 1991).

The native IP_3 receptor is a homotetramer consisting of four 260 kDa subunits (Maeda et al., 1991; Supattapone et al., 1988b). It is composed of four or eight transmembrane domains located at the carboxyl terminal end of the receptor and both the amino and carboxy termini face the cytoplasm (Ferris and Snyder, 1992). The last four

transmembrane regions appear to form the Ca^{2+}-permeable pore (Furuichi et al., 1989; Mignery et al., 1990) and the IP_3 binding domain is localized in the extreme portion of the N-terminus where binding of IP_3 can elicit a conformational change in the receptor protein (Mignery and Sudhof, 1990). IP_3 receptors are phosphorylated by PKA, PKC and CaMKII at different sites on the receptor protein (Ferris et al., 1991a,b; Ferris and Snyder, 1992; Supattapone et al., 1988a). Putative adenine nucleotide binding and PKA phosphorylation sites appear to be located between the IP_3 and Ca^{2+} binding domains (Ferris et al., 1991a; Ferris and Snyder, 1992). PKA phosphorylation of the receptor reduces the potency of IP_3 to release sequestered Ca^{2+} and enhances Ca^{2+} accumulation by ER membranes (Supattapone et al., 1988a). Phosphorylation of IP_3 receptors by PKC and CaMKII provides a means of feedback regulation of the phosphoinositide signal transduction system. Agonist stimulation of phosphoinositide turnover gives rise to DAG and IP_3. DAG activation of PKC results in phosphorylation of the IP_3 receptor. IP_3 mobilizes Ca^{2+} which directly activates CaMKII and provides feedback phosphorylation of the IP_3 receptor (Ferris and Snyder, 1992).

It has been shown that a major portion of the the IP_3 receptor is homologous to the ryanodine receptor especially in their putative pore forming regions (Furuichi et al.,1989; Takeshima et al., 1989). Both receptors are homotetramers which visually display a fourfold symmetry. One key difference between the two receptors is that Ca^{2+}, which activates and inhibits ryanodine receptors at micromolar and millimolar concentrations, respectively (Smith et al., 1986, 1988a,b), inhibits IP_3 receptor binding at physiological (sub-micromolar) concentrations (Danoff et al., 1988; Worley et al., 1987b). Moreover, ATP directly activates ryanodine-sensitive Ca^{2+} channels (Lai et al., 1988b; Smith et al., 1986, 1988a,b), while influencing IP_3 receptor channels only in the presence of IP_3 (Ferris et al., 1990).

Autoradiographic, immunohistochemical and *in situ* hybridization analyses of IP_3 receptor distribution in brain revealed a high concentration of sites in the molecular layer of the cerebellum where they appeared to be closely associated with Purkinje cells (Ross et al., 1989; Satoh et al., 1990; Sharp et al., 1993; Worley et al., 1987a; 1989). High density of sites was also observed in the hippocampus, more specifically in the CA-1 subfield. Immunohistochemical analyses conducted at the electron microscopic level revealed labelling in rough and smooth ER elements (Mignery et al., 1989; Otsu et al., 1990b; Ross et al., 1989; Satoh et al., 1990) and particularly in stacks of ER cisternae where electron-dense projections appear to connect cytoplasmic faces of adjacent cisternae (Mignery et al., 1989; Otsu et al., 1990; Satoh et al., 1990; Takei et al., 1992). These dense projections appear to be comparable with the SR-TT foot structures in muscle and may represent the cytoplasmic domains of the IP_3 receptor (Satoh et al., 1990; Takei et al., 1992, 1994). Dense labelling was also seen near and in the nuclear membrane, while plasma membrane, mitochondria and golgi cisternae were unlabelled (Ferris and Snyder, 1992; Mignery et al., 1989; Otsu et al., 1990b; Ross et al., 1989; Satoh et al., 1990).

I.2.7. *Caffeine-sensitive intracellular Ca^{2+} stores*

The inability of IP_3 to release the maximum amount of sequestered Ca^{2+} in the presence of mitochondrial inhibitors led to the idea that neurons possess an intracellular Ca^{2+} store which is IP_3-insensitive (Henzi and MacDermott, 1992; Miller, 1991). Analyses of ^{45}Ca uptake as determined by autoradiography of brain slices and by radioligand measurements in brain microsomes reveals that IP_3-induced reductions of ^{45}Ca levels are not homogeneously distributed throughout brain and suggests that IP_3-insensitve compartments may account for the ineffectiveness of IP_3 in some regions (Verma et al., 1990a,b). Pharmacological and physiological studies conducted on the IP_3-insensitive Ca^{2+} pool reveals that this Ca^{2+} store has distinct properties from those of the

IP_3-senstitive compartment. Ca^{2+} movements from the IP_3-insensitive pool are influenced by caffeine and ryanodine. Thayer et al. (1988a,b) demonstrated that in sensory and sympathetic neurons, caffeine mobilizes Ca^{2+} from an IP_3-insensitive store and this effect was inhibited by ryanodine. Caffeine-induced Ca^{2+} release was demonstrated in PC-12 cells, hippocampal cell cultures and permeabilized synaptosomes, and found to be distinct from IP_3-mediated release (Glaum et al., 1990; Martinez-Serrano and Satrustegui, 1989; Murphy and Miller, 1989). In autoradiographic analyses of ^{45}Ca uptake in brain slices, caffeine and IP_3 act synergistically to reduce the levels of uptake (Verma et al., 1992). Moreover, in brain microsomal fractions, IP_3-induced reductions in ^{45}Ca uptake is activated at lower concentrations of Ca^{2+} compared with the caffeine-mediated response (Verma et al., 1992). Further demonstrations of either facilitative or distinct effects of IP_3 and caffeine in releasing sequestered Ca^{2+} support the view that neurons have multiple Ca^{2+} stores (Fasolato et al., 1991; Kobayashi et al., 1986; Robinson and Burgoyne, 1991).

The possible role of caffeine-sensitive Ca^{2+} stores in neurotransmitter release has been alluded to in Section I.2.4. However, several studies indicate that these pools may regulate other neuronal functions. A component of the slow afterhyperpolarization (AH) that results from activation of Ca^{2+}-dependent K^+ channels and regulates the rate of neuronal firing is mediated by intracellular Ca^{2+} release in sympathetic neurons (Kuba et al., 1983). Caffeine enhanced and ryanodine reduced the frequency of the slow component of AH (Jobling et al., 1993; Kawai and Watanabe, 1989, 1991). Moreover, ryanodine abolished the caffeine effect (Kawai and Watanabe, 1989, 1991). This suggests that release of Ca^{2+} from caffeine-sensitive pools modulates the activity of Ca^{2+}-dependent K^+ channels. Ca^{2+}-dependent chloride currents appear to be regulated by a caffeine and ryanodine-sensitive mechanism (Ivanenko et al., 1993). Furthermore, caffeine-evoked elevations of intracellular Ca^{2+} activates an outward current which is

attenuated following addition of ryanodine (Marrion and Adams, 1992). This current apparently results from an increase in frequency of small miniature outward currents (SMOC) (Marrion and Adams, 1992) that appear to be activated by punctate release of intracellular Ca^{2+} presumably from caffeine-sensitive stores (Marrion and Adams, 1992). In dorsal root ganglion neurons, caffeine-induced Ca^{2+} release depressed $GABA_A$-activated chloride currents (Desaulles et al., 1991) and suggests a possible role of caffeine-sensitive stores in regulating presynaptic inhibition. An involvement of these stores in fast axonal transport was indicated by a ryanodine-induced reduction in the rate of retrograde fast axonal transport (Breuer et al., 1992). Thus, neurons may possess distinct intracellular calcium stores which are activated by caffeine and insensitive to IP_3. Ca^{2+} release from these stores may be mediated by a Ca^{2+} release channel comparable with that found in muscle SR as indicated by the sensitivity of the release mechanism to caffeine and ryanodine.

I.2.8. *Molecular composition of intraneuronal Ca^{2+} stores*

It has been suggested that in order for a compartment to be classified as a rapidly exchanging intracellular Ca^{2+} store, it must contain a Ca^{2+} uptake system, a mechanism to release sequestered Ca^{2+}, and low affinity/high capacity Ca^{2+} binding proteins (Abe et al., 1992). In neurons, Ca^{2+} uptake into Ca^{2+} stores is mediated by Ca^{2+}-ATPase pumps of the SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) family. Three different SERCA genes have been identified and designated as SERCA1, SERCA2 and SERCA3. The transcripts of SERCA1 and SERCA2 each have two isoforms as a result of alternative processing of their primary transcripts. The SERCA2b subtype is a 115 kDa protein encoded by 1042 amino acids and is the predominant form in brain, while SERCA3 mRNA appears in more discrete sites such as in cerebellar Purkinje cells (Hanley, 1993; Miller et al., 1991; Wuytack et al., 1992). Thapsigargin, a sesquiterpene lactone tumor promotor, inhibits SERCA Ca^{2+} pump activity, which results in a rapid

discharge of stored Ca^{2+} (Hanley, 1993) or it may act to unmask an as yet undefined Ca^{2+} leak from the store (Hanley, 1993).

The major Ca^{2+} binding protein in ER is calreticulin (CR) (Milner et al., 1991). CR is highly enriched in brain compared with other tissues (Abe et al., 1992; Fliegel et al., 1989; Johnson et al., 1992; Treves et al., 1990), has a very acidic C-terminal domain and posseses an ER retention signal (KDEL) which indicates it may have a homogeneous distribution in ER cisternae (Treves et al., 1992). Multiple isoforms of CR were identified in the CNS suggesting a differential regulation of Ca^{2+} binding (Treves et al., 1992). Abe et al. (1992) found by *in situ* hybridization that the distribution of CR mRNA is widely and differentially expressed in brain. A high level of expression was seen in hippocampal pyramidal cells, cells of the piriform cortex and in the choroid plexus (Abe et al., 1992). Moreover, CR is distributed evenly in subcellular fractions and correlates well with the localization of the general ER markers BiP and calnexin (Nori et al., 1993). Up-regulation of CR mRNA synthesis during long-term sensitization in *Aplysia* has been reported (Kennedy et al., 1992) which suggests an enhancement of internal Ca^{2+} storage sites during long-term adaptive synaptic events (Hanley, 1993). Authentic calsequestrin was identified in chick cerebellar Purkinje cells but not in other neurons and non-muscle cells (Takei et al., 1992; Villa et al., 1991; Volpe et al.1990, 1991a,b). It was suggested that CR is most likely the CaBP in intracellular Ca^{2+} pools in non-muscle cells (Treves et al., 1992).

Ca^{2+}-ATPase pumps are co-localized with IP_3 receptors in cerebellar Purkinje cell bodies and dendrites (Mignery et al., 1989; Ross et al., 1989; Takei et al., 1992) and the distribution of CR resembles that of SERCA2b (Miller et al., 1991). Taken together the above findings support the proposed existence, in neurons, of distinct intracellular Ca^{2+} stores which function in a similar capacity as the SR of muscle. However, whereas the cytological organization of SR is highly defined, neuronal ER is a more heterogenous

entity especially with respect to the distribution of specific ER proteins and internal Ca^{2+} pools (Sitia and Meldolesi, 1992; Villa et al., 1993).

I.2.9. *Organellar identity of intraneuronal Ca^{2+} stores*

The ER is the proposed major site of Ca^{2+} storage in neurons (Rossier and Putney, 1991). The initial basis for this assertion was mainly from ultrastructural and X-ray microprobe analyses of Ca^{2+} uptake into intracellular compartments (McGraw et al., 1980). The advent of more refined biochemical fractionation techniques, the development of specific antibodies that recognize epitopes on organelle-specific proteins and the utilization of immunocytochemical methods at the light and electron microscopic levels has enhanced capabilities to investigate the molecular constitution and organellar identity of the intracellular Ca^{2+} stores in neurons (Meldolesi et al., 1992). Because of the lack of specific antibodies and ligands to study the cellular localization of the caffeine-sensitive store, much of what is inferred about the intracellular distribution and identity of Ca^{2+} compartments has come from biochemical and immunocytochemical studies conducted on the IP_3-sensitive Ca^{2+} store.

Oxalate-mediated Ca^{2+} precipitation is a property generally attributed to ER (Rossier and Putney, 1991). Gandhi and Ross (1987) found that ATP dependent Ca^{2+} uptake in permeabilized synaptosomes is stimulated in the presence of oxalate and suggested that Ca^{2+} is sequestered in the ER. Subcellular fractionation studies where the distribution of IP_3-mediated Ca^{2+} release was compared with that of known subcellular biochemical markers revealed a microsomal localization of Ca^{2+} release and uptake (Shah et al., 1987). Since ER is the major vesicular constituent of microsomes, this organelle was considered to be the Ca^{2+} storage compartment. In similar experiments performed on subfractionated microsomal vesicles, a positive correlation between IP_3-induced Ca^{2+} release, enzyme markers for ER, and ATP-dependent Ca^{2+} uptake was

reported (Rossier and Putney, 1991). Furthermore, as analyzed by electron microscopy immunogold labelling, IP_3 receptors and the general ER markers BiP and calnexin were co-localized in cerebellar Purkinje neurons (Villa et al., 1992). Thus, based on the above studies, ER appears to be the intracellular Ca^{2+} storage organelle.

A second line of research clearly indicates a contrasting view to an exclusively ER location for intracellular Ca^{2+} storage. A number of studies have demonstrated a segregation between the various enzyme markers for ER and Ca^{2+} uptake, IP_3 binding and IP_3-induced Ca^{2+} release in various cell types and subcellular fractions (Meldolesi et al., 1992; Rossier and Putney, 1991). Volpe et al. (1988) proposed the existence of a distinct Ca^{2+} store physically separated from the ER which they refer to as calciosomes. These structures were originally identified in non-muscle cells based on their immunoreactivity to muscle calsequestrin (CS) antibody and possess Ca^{2+}-ATPase pumps and IP_3 receptor proteins. In brain, authentic CS was identified only in cerebellar Purkinje cells (Volpe et al., 1990, 1991a). More recently, a three-dimensional study of SER in cerebellar Purkinje cell dendrites did not show any evidence of physically separate membraneous vesicles not in continuity with the ER (Martone et al., 1993). Thus, the physiological existence of these structures in neurons including cerebellar Purkinje cells remains controversial. The more widespread view is that the ER as a whole is molecularly heterogeneous and contains specialized subcompartments some of which are devoted to Ca^{2+} handling (Martone et al., 1993; Nori et al., 1993; Sitia and Meldolesi, 1992; Villa et al., 1992). An important question in this regard is whether IP_3- and caffeine-sensitive Ca^{2+} stores occupy the same, different or overlapping subcompartments.

I.3. *Specific objectives*

The documented actions of caffeine in mediating Ca^{2+} efflux from intracellular IP_3-insensitive, non-mitochondrial compartments in sensory and sympathetic neurons, the effects of ryanodine and caffeine in affecting specific electrophysiological events in sympathetic neurons, and the facilitative actions of ryanodine in releasing neurotransmitters at the neuromuscular junction prompted us to investigate whether the actions of caffeine and ryanodine were mediated through a protein analogous to the ryanodine receptor/Ca^{2+} release channel complex found in striated muscle. Thus, the objectives of Part I of this research project were to investigate, through radioligand binding methods, whether ryanodine receptors were present in rat brain, to determine the conditions necessary to obtain optimal [^{3}H]ryanodine binding to brain membranes and to examine the distribution of ryanodine receptors in brain. This led to the demonstration that brain ryanodine receptors possess similar pharmacological properties as those in striated muscle and are present in various subcellular membrane fractions. The main objectives in Part II of the project were to determine the distribution of ryanodine receptors in rat brain by autoradiography and to examine, through the use of kainic acid lesions, the cellular localization of receptors in hippocampus. Results from these studies demonstrated that ryanodine receptors were heterogeneously dispersed among brain regions. The kainic acid studies confirmed not only the neuronal localization of receptors in hippocampus but also indicated the presence of a population of receptors in mossy fiber terminals. In Part III, the main objective was to demonstrate and characterize ryanodine receptors in human brain. This study led to the determination that ryanodine receptors in human brain possess similar pharmacological and distributional properties as those seen in rat brain and also served to provide relevance to our studies of rat brain ryanodine receptors. In Parts IV and V, the objectives were to study in more detail the pharmacological and regulatory properties of brain ryanodine receptors as well as to identify the subcellular membranes associated with these receptors in synaptosomes and

microsomes. It was demonstrated that synaptosomes and microsomes exhibit high affinity ryanodine receptors and that these receptors do not segregate with enzyme markers of ER and plasma membrane. Overall, the research described in this thesis has led to the enhancement of knowledge regarding intracellular Ca^{2+} stores in neurons through the demonstrated presence and characterization of ryanodine receptor/Ca^{2+} release channels.

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Part I

[^{3}H]Ryanodine Binding Sites in Rat Brain Demonstrated by Membrane Binding and Autoradiography

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Abstract-High affinity [^{3}H]ryanodine binding sites were characterized in P1 (crude nuclear), P2 (mitochondrial/synaptosomal) and P3 (microsomal) subcellular fractions of rat brain. Binding in each of the fractions was highest at 37°C and pH 8-9, optimal in the presence of 100 μM Ca^{2+}, 550 μM ATP and 1.0 M KCl, and increased linearly as a function of protein. Saturation analyses revealed a single class of binding sites with mean K_D values (nM) of 8.9, 1.6 and 5.7 and Bmax values (fmol/mg protein) of 122, 69 and 106 for the P1, P2 and P3 fractions, respectively. The levels of [^{3}H]ryanodine binding in P1 and P2 fractions of four brain regions were fairly uniform while those in P3 fractions were five-fold greater in cerebral cortex than in the other areas examined. By autoradiography, a high concentration of [^{3}H]ryanodine binding sites was seen in the dentate gyrus and CA3 subregions of the hippocampus. The results suggest that [^{3}H]ryanodine binding sites, perhaps similar to [^{3}H]ryanodine receptors in muscle, are associated with various subcellular structures and are heterogeneously distributed in the CNS.

Ryanodine, a plant alkaloid, influences Ca^{2+} release from the sarcoplasmic reticulum (SR) of cardiac and skeletal muscle (Lattanzio et al., 1987; Meissner, 1986) through an action on what has been termed the "Ca^{2+} channel/ryanodine receptor complex" which has been localized to the SR terminal cisterns and identified as a component of the cisternal endfoot structures (Anderson et al., 1989; Fleischer et al., 1985; Inui et al., 1987; Lai and Meissner, 1989). The high affinity binding of [^{3}H]ryanodine to this complex has allowed purification of the ryanodine receptor (Anderson et al., 1989; Lai et al., 1988) and subsequent elucidation of the cDNA sequence from clones of the forms in rabbit cardiac and skeletal muscle (Marks et al., 1989; Otsu et al., 1990; Penner et al., 1989). Although most studies to date have focused on the molecular properties of the ryanodine receptor in muscle and its characteristics with respect to biochemical interactions with potential endogenous regulators (Michalak et al., 1988; Pessah et al., 1986; Pessah et al., 1987), actions of ryanodine have been described in several other tissues (Procita, 1958; Procita et al., 1968) including neurons. In rat sympathetic neurons, ryanodine inhibited caffeine-induced Ca^{2+} release from intracellular Ca^{2+} stores and altered certain electrophysiological properties of these cells (Kawai and Watanabe, 1989; Thayer et al., 1988a,b). In mouse neuromuscular junctions ryanodine potentiated the Ca^{2+}-stimulated release of transmitter suggesting a possible influence of the drug on Ca^{2+} movements in nerve terminals (Nishimura et al., 1990). A recent report has shown the presence of high affinity [^{3}H]ryanodine binding sites in rat brain microsomes (Ashley, 1989). In addition a ryanodine binding protein has been detected immunohistochemically in cerebellar Purkinje cells of chick (Ellisman et al., 1990). These findings strongly suggest that a Ca^{2+} channel/ryanodine receptor complex similar to that in muscle may be present in CNS. In order to further characterize ryanodine interactions with potential receptor sites in brain and to develop the utility of ryanodine as a probe for Ca^{2+} regulatory mechanisms in neural tissues, we examined the properties of [^{3}H]ryanodine binding in subcellular fractions of rat brain and determined

by membrane binding and autoradiographic methods the distribution of [^{3}H]ryanodine binding sites in various CNS regions.

METHODS

Adult male Sprague-Dawley rats (150-200g) were decapitated, cerebral cortex, cerebellum, striatum, hippocampus and spinal cord were dissected, and subcellular fractions of each region were prepared as previously described (Yamamoto et al., 1987). Tissues were homogenized by disruption (20 strokes, 500 rpm) in 10 volumes (w/v) of 0.32 M sucrose containing 1 mM PMSF and 10 μg/ml trypsin inhibitor, the homogenates were centrifuged at 1000 x g for 10 min, and the pellets representing a crude nuclear fraction (P1) were kept on ice. The supernatants were centrifuged at 10,000 x g for 20 min, the resultant pellets (P2) containing mitochondria and synaptosomes were placed on ice and the supernatants were centrifuged at 105,000 x g for 60 min to obtain the final pellet (P3) enriched in microsomes. The fractions were either taken immediately for assay or stored at -20°C. No loss of [^{3}H]ryanodine binding was seen in tissues stored for up to one week. Pellets were suspended to a final protein concentration of approximately 2 mg/ml in Buffer A consisting of 20 mM PIPES-KOH (pH 8.0), 1.0 M KCl, 550 μM ATP, 100 μM $CaCl_2$ and 1.0 mM PMSF. Binding assays were conducted in a total volume of 1.0 ml of Buffer A containing 3 nM [^{3}H]ryanodine (60.0 Ci/mmol, Dupont-New England Nuclear, Boston, MA) and 0.1 ml of tissue. Specific binding was calculated by subtracting non-specific binding defined as that seen in the presence of 3 nM [^{3}H]ryanodine plus 50 μM unlabeled ryanodine (Research Biochemicals Inc., Wayland, Mass.) from total binding obtained with 3 nM [^{3}H]ryanodine alone. For optimization of conditions, the concentrations of $CaCl_2$, ATP or KCl in Buffer A were varied from 0 to 250 μM, 0 to 2000 μM, and 0 to 1000 mM, respectively. In saturation experiments, [^{3}H]ryanodine concentrations ranged from 0.05 nM to 48 nM. Tissues were incubated for 1 hr at 37°C and all assays were performed in duplicate. Reactions were

terminated by rapid filtration under reduced pressure through GF/C glass-fiber filters using a Brandell Cell Harvester M-24R. Filters were washed rapidly three times with 5 ml of ice-cold buffer containing 20 mM PIPES-KOH (pH 8), 1.0 M KCl, and 100 μM $CaCl_2$, placed in vials and taken for liquid scintillation spectroscopy in 4.5 ml of Beckman Ready Solvent. Protein was determined by the method of Lowry et al. (1951). Dissociation constants (K_D), maximum number of receptor sites (Bmax), and partial F tests for best fit by a one- or two-site model were derived from analyses of data by the nonlinear multipurpose curve-fitting program LIGAND (Munson and Rodbard, 1980). Significance was considered at the $p < 0.05$ level.

In autoradiographic studies, 4 rats (300 to 400 g) were deeply anaesthetized with pentobarbital and perfused transcardially with 200 ml of ice-cold saline (0.9%). Brain, cardiac and gastrocnemius muscles were removed, mounted onto cryostat chucks and 10 μm transverse sections of brain and longitudinal sections of cardiac and gastrocnemius were collected onto subbed slides. Sections were washed twice in Buffer A for 15 min and incubated with 15 nM [^{3}H]ryanodine for 15 min at 25°C. Reactions were terminated by sequential 5 min washes in ice-cold Buffer A, Buffer A diluted 1:10 (v/v) with distilled water, and ice-cold distilled water. Non-specific binding was determined in adjacent sections processed as above but incubated with [^{3}H]ryanodine in the presence of 10 μM unlabeled ryanodine. Slides were apposed to Hyperfilm-H^3 (Amersham) for one month, and the film was developed in D-19 developer, fixed, washed and dried. For competition studies, tissue sections of brain or gastrocnemius muscle were processed on subbed slides as above but with concentrations of ryanodine ranging from 0.4 nM to 10 μM. After drying, sections were scraped into scintillation vials, digested in 0.1 N NaOH, and taken for liquid scintillation spectroscopy. Inhibition constants (K_I) were calculated by the Cheng-Prusoff equation ($K_I = IC_{50}/(1 + [L]/K_D)$) where IC_{50} values were derived from competition curves.

RESULTS

The conditions for [^{3}H]ryanodine binding were optimized initially in P3 fractions of cerebral cortex and subsequent tests of the effects of various agents in each of the subcellular fractions varied according to these conditions. [^{3}H]Ryanodine binding reached equilibrium within 15 min at 25° and 37°C (data not shown), increased at temperatures ranging from 4° to 37°C (Fig. 1), and maximal binding was obtained at a pH range of 8 to 9 (Fig. 2). Addition of Ca^{2+} increased ligand binding up to five-fold over basal levels (Table I) while EGTA (1 mM) virtually eliminated binding in the three subcellular fractions tested (Table I). ATP at a concentration of 550 μM increased binding up to three-fold over basal levels in P1 and P3 fractions, but had little effect on [^{3}H]ryanodine binding in P2 fractions (Table I). Binding increased up to four-fold over basal levels with the addition of 1.0 M KCl (Table I) or NaCl (data not shown). [^{3}H]Ryanodine binding in P1, P2, and P3 fractions of cerebral cortex was linear (r^2 = 0.995) with protein concentration over a range of 0.01 to 0.3 mg/ml.

[^{3}H]Ryanodine bound to a single class of high affinity sites in all three subcellular fractions of cerbral cortex. K_D values (nM) of 8.9 ± 2.4 in P1 and 5.7 ± 1.8 in P3 fractions were not significantly different from each other. However, K_D values of 1.9 ± 0.7 in P2 fractions were significantly lower than those observed in the other two fractions. Similarly, Bmax values (fmol/mg protein) of 122 ± 11 in P1 and 106 ± 12 in P3 fractions were not significantly different from each other but were significantly higher than the value of 69 ± 23 obtained in P2 fractions. The distribution of [^{3}H]ryanodine binding in subcellular fractions of the five CNS regions examined are listed in Table II. In all fractions, the levels of binding were highest in cortex and lowest in spinal cord. In P3 fractions, binding in cortex was 5-fold greater than in hippocampus, striatum, and spinal cord, while in P1 and P2 fractions binding levels were more evenly distributed among brain regions. In autoradiographic studies (Fig. 3), high density

[^{3}H]ryanodine binding appeared throughout in cardiac and skeletal muscle tissue sections. In brain, levels of autoradiographic grains were barely above background in subcortical structures, were present at a slightly higher density in the amygdala as well as cerebral cortex where moderate levels were observed in superficial and deep layers, and were most concentrated in the dentate gyrus and the CA3 subfield of the hippocampus. Analysis of the distribution of [^{3}H]ryanodine binding sites in other CNS regions is currently in progress. In tissue sections of brain and skeletal muscle, K_I values calculated from competition experiments were 22 and 24 nM, respectively (Fig. 4). Similar values were obtained from such experiments conducted with membrane fractions (data not shown) indicating the detection of high affinity [^{3}H]ryanodine binding sites by the autoradiographic method.

DISCUSSION

The present study provides further evidence for the presence of putative [^{3}H]ryanodine receptor sites in rat CNS. A comparison of [^{3}H]ryanodine binding properties in brain with various characteristics of the ryanodine receptor in cardiac and skeletal muscle indicate both similarities and differences among the three tissues. In both muscle types, [^{3}H]ryanodine binding exhibited Ca^{2+}-dependency, was optimal in the presence of high salt and alkaline pH (Imagawa et al., 1989; Michalak et al., 1988; Pessah et al., 1987; Pessah et al., 1985) and was shown to display both high (K_D=3.6 to 36 nM) and low affinity (K_D=0.34 to 3 μM) components (McGrew et al., 1989; Michalak et al., 1988; Pessah et al., 1985). In contrast, Mg^{2+} and supra-optimal Ca^{2+} concentrations did not inhibit binding in cardiac muscle but did so in skeletal muscle (Michalak et al., 1988), binding in cardiac compared with skeletal muscle was less sensitive to adenine nucleotides (Imagawa et al., 1989; Michalak et al., 1988), the cardiac receptor complex exhibited a lower M_r compared with the skeletal complex (Inui et al., 1987), and antibodies against the cardiac ryanodine receptor failed to recognize the

skeletal muscle form (Imagawa et al., 1989). Furthermore, the amino acid sequences of the cardiac and skeletal muscle ryanodine receptor proteins deduced from their respective cDNA sequences displayed only 66% homology (Otsu et al., 1990). These differences strongly indicate the presence in muscle of at least two forms of the ryanodine receptor. In brain, [^{3}H]ryanodine binding sites exhibited characteristics common to the ryanodine receptor in cardiac and skeletal muscle as well as features distinct from one or the other or both. The greater levels of binding seen in brain under high salt conditions was also found in muscle where it was suggested that the high salt either stabilized the receptor (Michalak et al., 1988) or had an osmotic influence (Ogawa and Harafuji, 1990). The pH optimum for [^{3}H]ryanodine binding in brain was comparable to that seen in muscle. Although binding in brain was Ca^{2+}-dependent, being abolished in the presence of EGTA, it was not inhibited by greater than optimal Ca^{2+} concentrations as in skeletal muscle. In addition, binding was optimal at micromolar concentrations of ATP in brain, but at millimolar concentrations in muscle (Chu et al., 1990; Imagawa et al., 1989; Pessah et al., 1987) suggesting that the putative [^{3}H]ryanodine receptors in the CNS may be more sensitive to adenine nucleotides. In brain, only a single class of high affinity binding sites was found in contrast with high and low affinity receptor sites observed in cardiac and skeletal muscle (McGrew et al., 1989; Michalak et al., 1988; Pessah et al., 1985). An indication that the ryanodine binding protein in brain may be more closely related to the ryanodine receptor in cardiac than skeletal muscle is suggested by the observation that a cDNA probe corresponding to the cardiac ryanodine receptor sequence recognized mRNA species on Northern blots of brain but not skeletal muscle extracts (Otsu et al., 1990). The existence of two forms of ryanodine receptors in muscle and the differences in [^{3}H]ryanodine binding properties to putative receptors in brain and muscle raises the possibility of the presence of yet other forms of ryanodine receptors in the CNS.

The presence of [^{3}H]ryanodine binding sites in P3 fractions is consistent with previous demonstrations of such sites in rat brain microsomal fractions (Ashley, 1989) and with the localization of ryanodine binding proteins on membranous structures within avian cerebellar Purkinje cells (Ellisman et al., 1990). If putative ryanodine receptors in CNS serve a similar role in the regulation of intracellular Ca^{2+} release as those in SR of muscle, and if the SR in muscle and the intracellular Ca^{2+} store in neurons which is thought to be endoplasmic reticulum (ER) (Somlyo, 1984) exhibit analogous Ca^{2+} regulatory properties as previously suggested (Somlyo, 1984), then Ca^{2+} release from ER may be mediated through Ca^{2+} channel/ryanodine receptor complexes in the CNS. However, such a role of CNS ryanodine receptors remains to be demonstrated. The binding seen in P2 fractions suggests that putative receptors may be associated with nerve terminals. This is supported indirectly by demonstrated actions of ryanodine on Ca^{2+} movements in axon terminals at the neuromuscular junction (Nishimura et al., 1990). Regional analysis of [^{3}H]ryanodine binding in rat CNS regions indicated a heterogeneous distribution of binding sites with a variation of up to 5-fold between areas displaying the highest and lowest levels. Moreover, in autoradiographic studies, high density binding was shown in discrete areas of the hippocampus. Several possibilities may be considered for the observed heterogeneity of [^{3}H]ryanodine binding. First, detection of different receptor isoforms or states may not have been possible under the present conditions used to measure binding. Second, ryanodine receptors may be more concentrated in some cells of the CNS which have greater requirements for intracellular Ca^{2+} mobilization. Third, although all cells may exhibit intracellular Ca^{2+} release, only some cells may utilize a ryanodine receptor associated Ca^{2+} channel to govern such release.

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FIGURE LEGENDS

Fig. 1. The effect of temperature on [^{3}H]ryanodine binding in subcellular fractions of cerebral cortex. Values represent means ± S.E.M. of results from 3 to 5 separate experiments. P1, closed circles; P2, open circles; P3, closed triangles.

Fig. 2. The effect of pH on [^{3}H]ryanodine binding in subcellular fractions of cerebral cortex. Values represent means ± S.E.M. of results from 3 to 5 experiments. P1, closed circles; P2, open circles; P3, closed triangles.

Fig. 3. Autoradiographic localization of [^{3}H]ryanodine binding sites in various tissues. Adjacent sections of skeletal muscle (A,B), cardiac muscle (C,D) or brain (E,F) were processed in the presence of 15 nM [^{3}H]ryanodine alone (A,C,E) or in the presence of 15 nM [^{3}H]ryanodine and 10 μM unlabeled ryanodine (B,D,F). Both ventricular (arrow) and atrial (arrowhead) muscle are shown in C. In brain, note the the greater concentration of binding sites in the dentate gyrus (arrow) and CA3 region (arrowhead) compared with the CA2 and CA1 regions of the hippocampus. Magnification, X 5.

Fig. 4. Competition curves of [^{3}H]ryanodine binding to slide-mounted sections of skeletal muscle (A) and brain (B). Sections were incubated in the presence of 15 nM [^{3}H]ryanodine and concentrations of unlabeled ryanodine ranging from 0.4 nM to 10 μM. Non-specific binding was defined as the degree of competition obtained in the presence of 10 μM ryanodine. Points represent means of duplicate determinations.

Table 1. Effect of $CaCl_2$, KCl and ATP on [^{3}H]ryanodine binding in subcellular fractions of cerebral cortex.

Additions to Incubation Buffer	[^{3}H]Ryanodine Binding (fmol/mg protein) P1	P2	P3
A. $CaCl_2$ (μM)			
0 + 1.0 mM EGTA	2 ± 2	ND	4 ± 2
0	16 ± 5	17 ± 1	33 ± 8
25	71 ± 3	66 ± 7	81 ± 11
50	50 ± 6	48 ± 3	94 ± 6
100	63 ± 5	54 ± 5	95 ± 8
250	82 ± 10	59 ± 11	90 ± 4
B. ATP (μM)			
0	21 ± 9	40 ± 6	33 ± 11
100	70 ± 13	47 ± 8	71 ± 6
250	66 ± 8	53 ± 11	67 ± 34
550	63 ± 5	54 ± 5	95 ± 8
1000	64 ± 16	41 ± 16	42 ± 8
2000	61 ± 11	29 ± 3	40 ± 17
C. KCl (mM)			
0	15 ± 2	22 ± 11	22 ± 7
50	23 ± 7	27 ± 9	36 ± 15
150	31 ± 8	32 ± 11	37 ± 23
250	32 ± 8	34 ± 6	54 ± 26
500	50 ± 17	45 ± 13	69 ± 36
1000	63 ± 5	54 ± 5	95 ± 8

Subcellular fractions were suspended in 20 mM PIPES-KOH-PMSF buffer (pH 8.0) containing either 1.0 M KCl and 550 μM ATP to test the effects of $CaCl_2$, 100 μM $CaCl_2$ and 1.0 M KCl to test the effects of ATP, or 100 μM $CaCl_2$ and 550 μM ATP to test the effects of KCl. Values represent means ± S.E.M. of results from at least three separate experiments performed in duplicate. ND, not detectable.

Table 2. Specific binding of [^{3}H]ryanodine to CNS subcellular fractions.

Tissue	Subcellular Fraction		
	P1	P2	P3
Cortex	63 ± 5	54 ± 5	95 ± 8
Cerebellum	33 ± 8	38 ± 2	35 ± 12
Hippocampus	64 ± 5	54 ± 9	27 ± 9
Striatum	35 ± 3	36 ± 5	22 ± 6
Spinal Cord	5 ± 3	3 ± 2	17 ± 9

Values (fmol/mg protein) represent means ± S.E.M. of five separate experiments performed in duplicate.

Figure 1

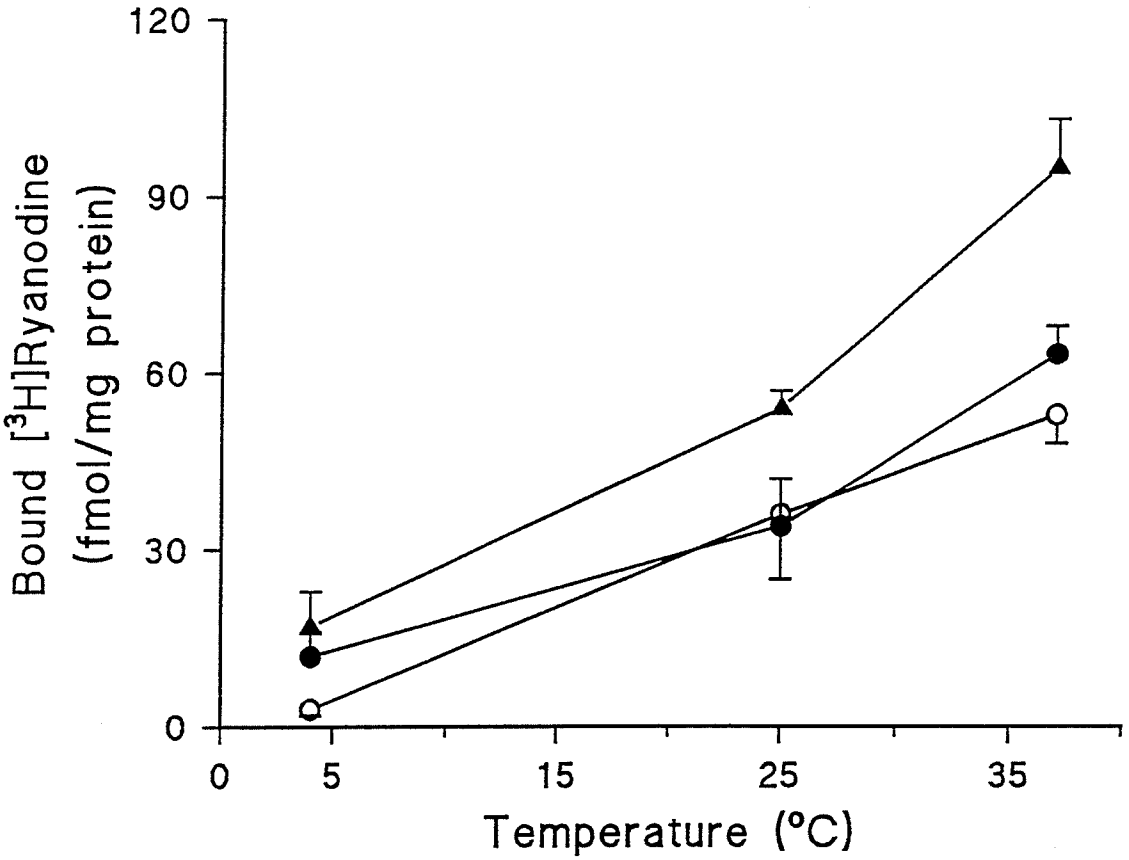

120
90
60
30
0
Bound [3H]Ryanodine
(fmol/mg protein)
0
5
15
25
35
Temperature (°C)

Figure 2

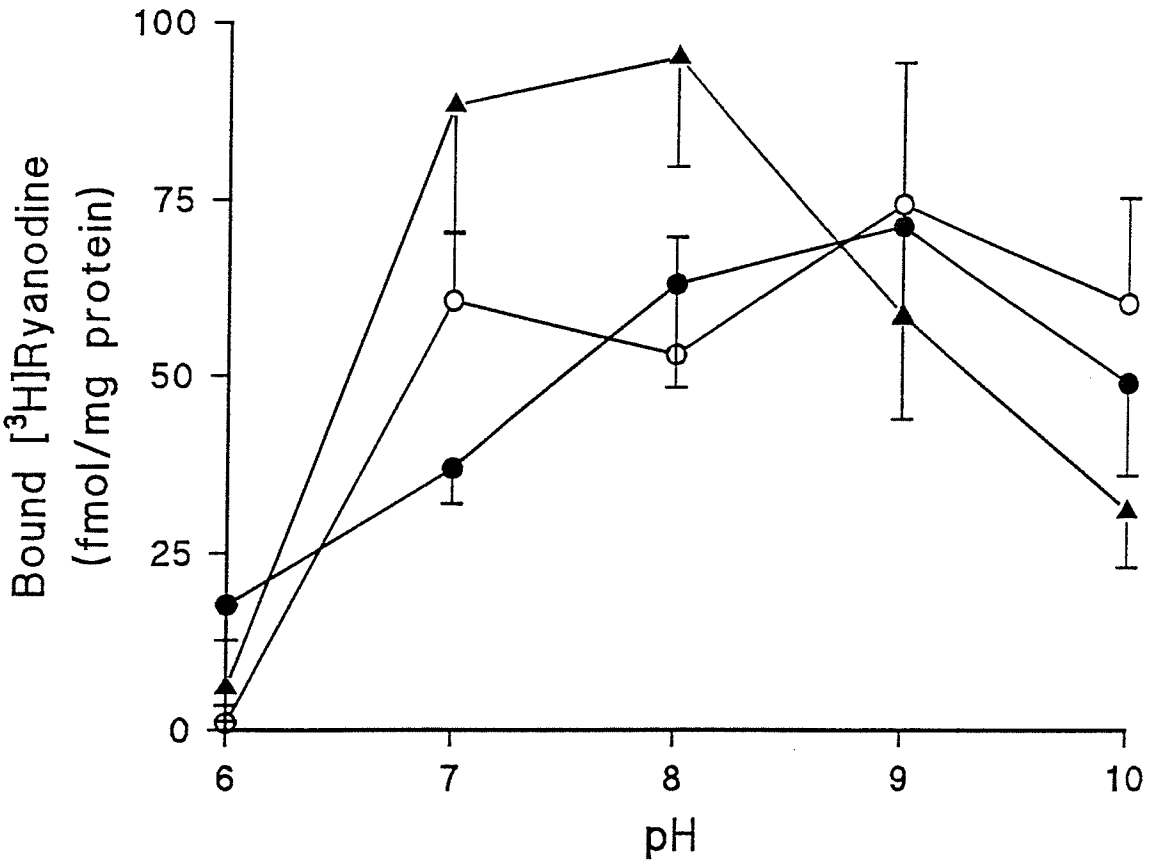
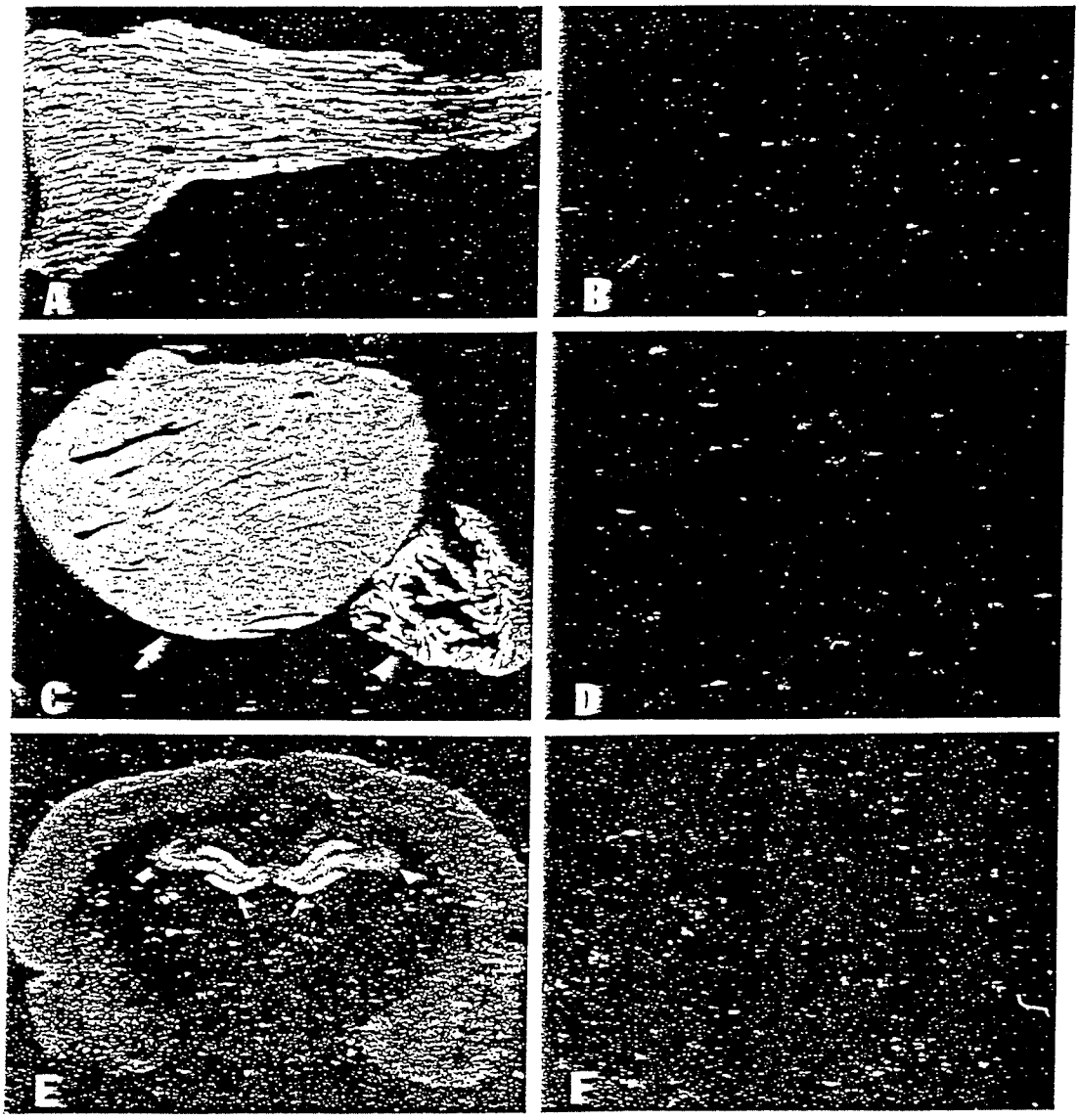


Figure 3



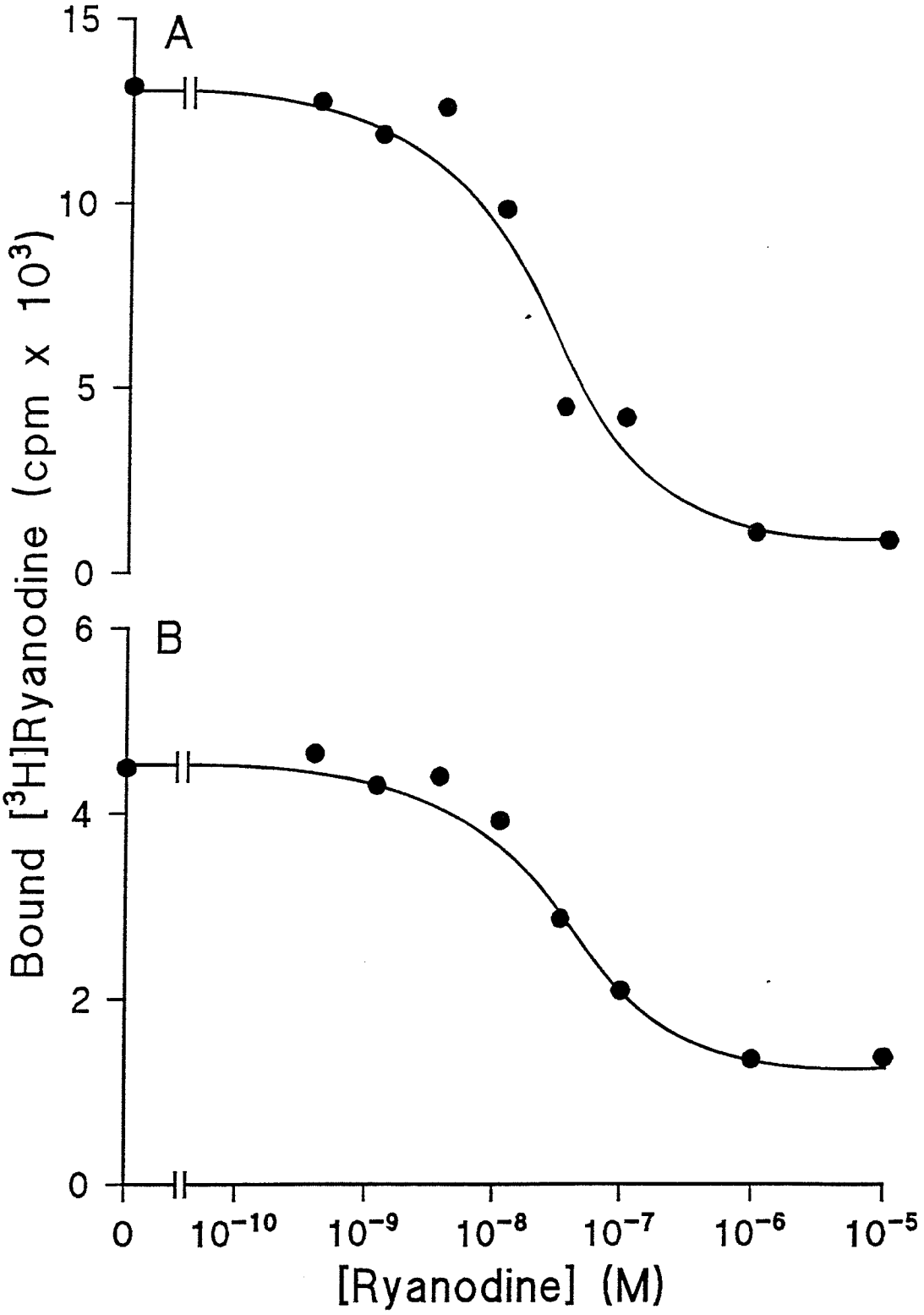

A
B
15
10
5
0
6
4
2
0
Bound [3H]Ryanodine (cpm x 10^3)
0
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
[Ryanodine] (M)

Figure 4

Part II

Autoradiographic analysis of [^{3}H]ryanodine binding sites in rat brain: regional distribution and the effects of lesions on sites in the hippocampus

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Abstract--Quantitative and qualitative autoradiographic methods together with lesion approaches were used to determine the distribution of [^{3}H]ryanodine binding sites in rat brain and the neuronal localization of these sites in the hippocampus. In normal animals, levels of ^{3}H-ryanodine binding sites ranged from a low of about 1 fmol/mg tissue in subcortical structures to a high of 12 to 18 fmol/mg tissue in subregions of the hippocampus and the olfactory bulb. Relatively high densities of sites (5 to 9 fmol/mg tissue) were also seen in the olfactory tubercle, most areas of the cerebral cortex, accumbens nucleus, striatum, lateral septal nuclei, pontine nucleus, superior colliculus and granule cell layer of the cerebellum. Specific binding was undetectable in white matter. In experimental animals, intracerebral injections of kainic acid caused neuronal degeneration and a near total depletion of [^{3}H]ryanodine binding sites in the dentate gyrus and in fields CA1, CA2 and CA3 of the hippocampus. Injections of kainic acid that left dentate granule cells largely intact while destroying all neurons in field CA3 had no effect on binding sites in the dentate gyrus. However, these lesions substantially reduced the density of binding in field CA3 leaving a narrow band of sites outlining the position of the degenerated CA3 pyramidal cells. Mechanical knife cut lesions that severed the granule cell mossy fiber input to field CA3 reduced the density of binding sites in the CA3 region. The results indicate that [^{3}H]ryanodine binding sites in brain are heterogeneously distributed and suggest that a proportion of these sites in the hippocampus may be contained in mossy fiber terminals where a presumptive calcium channel/ryanodine receptor complex may be involved in the regulation of calcium mobilization and/or neurotransmitter release.

The release of Ca^{2+} from the sarcoplasmic reticulum (SR) of muscle is thought to be mediated by a Ca^{2+} channel/ryanodine receptor complex which constitutes the endfoot structure localized at the SR terminal cistern-transverse tubule junction (Imagawa et al., 1987; Hymel et al., 1988; Lai et al., 1988a, 1988b; Anderson et al., 1989; Lai and Meissner, 1989). Ryanodine, a plant alkaloid that binds to this SR complex with high affinity (Pessah et al., 1985, 1987; Alderson and Feher, 1987; Michalak et al., 1988) prevents closure of these channels and consequently depletes the SR of its Ca^{2+} store (Fleischer et al., 1985; Nagasaki and Fleischer, 1988; Fill and Coronado, 1988). The binding characteristics of ryanodine to this complex are modified by agents such as Ca^{2+}, Mg^{2+}, ATP and caffeine which are known to influence Ca^{2+} release through the SR Ca^{2+} channel (Kim et al., 1983; Pessah et al., 1987; Rousseau et al., 1987; Chu et al., 1990). There are now several lines of evidence suggesting that ryanodine receptors analogous to those in muscle are present in neurons and that such receptors may be involved in the regulation of intraneuronal Ca^{2+} mobilization. Thus, ryanodine was shown to inhibit caffeine-induced release of Ca^{2+} from intraneuronal storage compartments (Thayer et al., 1988a, 1988b) and to block slow after-hyperpolarizations which are thought to arise from activation of K^+ channels by intracellularly released Ca^{2+} (Kawai and Watanabe, 1989). In brain microsomal membranes, the alkaloid partially inhibited Ca^{2+} channel conductance (Ashley, 1989). Ligand binding studies have shown the presence of high affinity ^{3}H-ryanodine binding sites in various membrane preparations of rabbit and rat brain (Ashley, 1989; McPherson and Campbell, 1990; Kawai et al., 1991; Padua et al., 1991) and a number of substances modulated binding to these sites in a fashion similar to that seen in muscle (Kawai et al., 1991). Immunohistochemical and Western blot approaches using antibodies directed against the ryanodine receptor in skeletal muscle have demonstrated that these antibodies recognize epitopes in chick and rabbit brain (Ellisman et al., 1990; McPherson and Campbell, 1990). In rabbit brain, a solubilized ryanodine binding protein was found to

have a M_r of about 400 kDa, a value similar to that of the muscle receptor (McPherson and Campbell, 1990). Although the physiological role and molecular identity of the protein complex with which ryanodine interacts in neurons is as yet uncertain, among the above provocative findings is a report showing that ryanodine facilitates the release of acetylcholine at the neuromuscular junction (Nishimura et al., 1990) thereby implicating a contribution of ryanodine receptors in the regulation of neurotransmitter release.

Previously, we demonstrated the presence of high affinity [^{3}H]ryanodine binding sites in subcellular fractions of rat brain, determined the optimal conditions for measurements of ^{3}H-ryanodine binding sites with respect to Ca^{2+}, ATP and KCl concentrations, and provided preliminary data indicating markedly different levels of expression of these sites among brain regions (Padua et al., 1991). In the present study, we utilized quantitative and qualitative autoradiography to determine in greater detail the anatomical distribution of ^{3}H-ryanodine binding sites and to demonstrate, through lesion methods, the specific localization of these sites in neurons of the hippocampus.

METHODS

[^{3}H]Ryanodine Autoradiography

[^{3}H]Ryanodine autoradiography was performed as previously described (Padua et al., 1991). Male Sprague-Dawley rats (250-300g) were anesthetized with chloral hydrate and perfused transcardially with 200 ml of ice-cold 0.9% saline. Brains were removed, blocked, frozen on dry ice, and mounted onto cryostat chucks. Transverse sections were cut at a thickness of 10 μm and thaw-mounted onto gelatinized slides. Sections were washed twice for 15 min in buffer A (20 mM PIPES, 100 μM $CaCl_2$, 1.0 M KCl and 1.0 mM PMSF) and incubated for 15 min at 25°C in buffer A containing 15 nM [^{3}H]ryanodine (NEN/Dupont, Boston, 95 Ci/mmol). Under these conditions, binding was at equilibrium (Data not shown). Reactions were terminated by sequential washes

for 5 min each in ice-cold buffer A, ice-cold buffer A diluted (1:10) in distilled water, and ice-cold distilled water. The sections were then dried for a period of 2 min and apposed to ^{3}H-Hyperfilm (Amersham) for 8 weeks. The films were developed in Kodak D-19 developer, fixed, washed and dried. Assessment of non-specific binding on adjacent sections was conducted as above except that sections were incubated in buffer A containing 15 nM [^{3}H]ryanodine and 10 μM unlabelled ryanodine (Polysciences, Warrington, PA). Levels of ^{3}H-ryanodine binding sites in brain regions were analyzed by quantitative microdensitometry of autoradiographic images using a RAS-1000 receptor image analysis system equipped with a receptor analysis program (Amersham). Optical density (OD) readings were converted to total binding (fmol/mg tissue) with the aid of a best fit polynomial standard curve (OD vs radioactivity counts) generated from measurements of ODs in films exposed to whole brain pastes mixed with varying concentrations of ^{3}H-2-deoxyglucose (ARC Inc., 45 Ci/mmol). The specific binding of [^{3}H]ryanodine in each brain region examined was calculated by subtraction of non-specific binding from total binding in adjacent sections. Data obtained from 4 animals were pooled and the mean±s.e.m. values for specific binding were determined using the computer program STATA.

We have previously shown that [^{3}H]ryanodine bound to a single class of high affinity sites (K_D, 1.9 to 8.9 nM) in brain membrane fractions (Padua et al., 1991). In competition experiments performed with unlabelled ryanodine, it was demonstrated that the binding characteristics in homogenates were comparable to those obtained in brain sections (data not shown). This confirmed the selective binding of [^{3}H]ryanodine to specific sites in brain sections taken for autoradiographic analysis.

Hippocampal Lesions

Adult male Sprague-Dawley rats were anesthetized with pentobarbital and were subjected to two lesion procedures performed under stereotaxic guidance with coordinates determined from the rat brain atlas of Paxinos and Watson (1986). Kainic acid (Sigma, 5 nmol in 1 μl saline) was used to destroy selectively neurons of the hippocampus and unilateral mechanical knife cuts were used to transect mossy fiber projections from the dentate gyrus to the hippocampal field CA3. For kainic acid lesions, animals were positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA) with the incisor bar set at -3.3 mm. The toxin was administered with a Hamilton microsyringe over a period of 10 to 15 min. Separate animals were injected at different locations. In three rats, the injection was aimed at a dorsolateral hippocampal region at the interaural coordinates: AP, +5.7 mm; ML, +2.5 mm; and DV, +7.1 mm. In another three rats, the injection was aimed at the hilus at the interaural coordinates: AP, +5.7 mm; ML, +1.6 mm; and DV, +6.3 mm. Mossy fiber transections in three rats were performed with a Scouten adjustable wire knife (Kopf Instruments, Tujunga, CA) as previously described (Okazaki and Nadler, 1988). Animals were positioned in a stereotaxic frame as above except that the incisor bar was placed at +5.0 mm. Two knife cuts were made with the blade pointed 45° towards the midline. Coordinates for the first knife placement were: AP, -3.8 mm from bregma; ML, +3.05 mm from the midline suture; DV, 3.6 mm below the dura. Coordinates for the second knife placement were: AP, -2.6 mm from bregma; ML, +1.85 mm from the midline suture; DV, 3.6 mm below the dura. The knife blade was extended 2.5 mm, lifted dorsally 3.6 mm and this position was maintained for 5 min. The blade was then lowered to its original position and retracted. Animals were sacrificed 10 days after the kainic acid lesions and 12 days after the knife cut lesions. Brains were taken for [^{3}H]ryanodine autoradiography as described above. Histological examinations of adjacent brain sections stained with thionin were conducted to confirm the extent of the kainic acid lesions. Sections of brain from animals given knife cut

lesions were examined by the Timm's sulfide silver staining method (Danscher, 1981) to confirm the knife placement and the transection of mossy fibers.

RESULTS

Regional distribution

The anatomical distribution of [^{3}H]ryanodine binding sites in sections at various levels through the rat brain is shown in Figure 1Á-L and the results of quantitative measurements are shown in Table I. Anatomical nomenclature for brain structures was according to the atlas of Paxinos and Watson (1986). The levels of [^{3}H]ryanodine binding sites in qualitative terms and in units of fmol/mg tissue ranged from: extremely dense, >12 ; very dense, 8-12; dense, 4-8; moderate, 2-4; and sparse, <2. Areas of extremely dense binding sites were few and included the external plexiform layer of the olfactory bulb (Fig. 1A), the molecular and granule cell layers of the dentate gyrus and field CA3 of the hippocampus (Fig. 1H and I). A high density of sites was seen in other layers of the olfactory bulb as well as in related areas including the anterior olfactory nuclei and the olfactory tubercle (Fig. 1A-D). The concentration of sites in the cerebral cortex bordered between dense and very dense in most areas with the exception of the temporal cortex where levels tended to be slightly lower. Although not analyzed in detail due to resolution limitations, it was clear that the density of sites in virtually all areas of neocortex was higher in the outer and inner third of the cortical layers than the middle third (Fig. 1D-I). Whether these density differences correspond to cytoarchitectonically defined cortical layers remains to be determined. It should be noted that the present density measurements encompassed the full thickness of the neocortical areas and more precise analysis of the outer and inner layers may yield higher levels than indicated in Table I. In the hippocampus there appeared to be no rostro-caudal differences in binding density and the fields CA1 and CA2 exhibited consistently lower densities of sites than the other subregions mentioned above. In the septum, [^{3}H]ryanodine binding sites were

more heavily concentrated in the lateral than in the medial or posterior septal nuclei. Interestingly, the pars intermediate of the lateral septal nucleus contained a much higher density of sites at anterior than at posterior levels (Fig. 1E and F). In the amygdala, binding density was distinctly higher in the basolateral than either the medial or central amygdaloid nuclei (Fig. 1H). Structures in the basal ganglia exhibited heterogeneous levels of sites; binding was dense in the anterior and ventrolateral portion of the head of the striatum as well as in the tail of the striatum, moderate in the medial part of the striatum (Fig. 1D-G), and sparse in the globus pallidus and substantia nigra (Fig. 1G and I). Structures in diencephalic and mesencephalic areas were generally unremarkable in that they exhibited rather uniform levels of sites which ranged from sparse to moderate. Elevated levels were, however, seen in the superior colliculus, central gray area and the pontine nucleus (Fig. 1J and K). In the cerebellum, binding sites were dense throughout the cortical layers and, although not evident in Figure 1L, there tended to be a greater concentration of sites in the granule cell layer. Analyses of binding sites in brain stem and spinal cord were not conducted, but preliminary results in the latter have shown that the superficial layers I and II exhibit greater densities of sites than the rest of the spinal gray matter.

Hippocampal lesions

In view of the very high levels and subregional heterogeneity of [^{3}H]ryanodine binding sites in the hippocampus, the neuronal localization of binding sites in this structure was investigated by lesion methods. As seen in Nissl-stained sections from two of three animals where kainic acid was successfully delivered at the center of the hilus, there occurred a total loss of neurons throughout the hippocampus at the level of the injection, except for the sparing of a very small portion of the medial dentate gyrus (Fig. 2A). In adjacent sections taken for autoradiography, [^{3}H]ryanodine binding sites were depleted in the lesioned area and there was a slight reduction of sites in the medial part of

the dentate gyrus that remained intact (Fig. 3A). In addition, binding sites were depleted in areas of the cerebral cortex to which kainic acid had diffused (Fig. 3A). Injections of kainic acid that were placed in the dorsolateral quadrant of the hippocampus caused a total loss of neurons in field CA3, CA2 and the lateral portion of CA1 (Fig. 2B and C). Granule cells in the lateral tip of the dorsal blade of the dentate gyrus were also destroyed, while those in the ventral blade were largely intact. Autoradiographic analysis of [^{3}H]ryanodine binding in sections adjacent to those in Fig. 2B revealed extensive depletion of binding sites in fields CA1 and CA2, in the extreme lateral tip of the dorsal blade of the dentate gyrus, and again in cortical regions straddling the needle tract (Fig. 3B). There was also a substantial reduction of binding sites in field CA3. There remained, however, a narrow band of sites the location of which matched the normal position of the CA3 pyramidal cell layer. This result was seen in each of three animals. The thickness of the region of very dense binding sites along the horizontal limb of field CA3 (regio inferior) on the control side was about 400 μm, suggesting that this region encompassed the stratum pyramidale and large portions of the flanking stratum oriens and radiatum. On the lesioned side, the thickness of the remaining band of sites in this same region of field CA3 was reduced to about 125 μm (Fig. 3B).

The above results suggested that some proportion of [^{3}H]ryanodine binding sites in field CA3 are present in structures other than neurons intrinsic to this hippocampal subregion. The possibility that the sites spared after kainic acid-induced neuron loss in this area may be contained in mossy fiber projections from dentate granule cells was tested by creating knife-cut lesions between the dentate gyrus and the lateral hippocampus. As can be seen in the Nissl-stained section shown in Fig. 2D, the knife-cuts employed successfully separated these two regions of the hippocampus, at least over the rostrocaudal length of the knife blade (about 4 mm). Specifically, the cut was located just lateral to the lateral extreme of the dentate granule cells. In adjacent sections taken

for [^{3}H]ryanodine autoradiography, the lesion had no effect on the density of sites in hippocampal areas medial to the cut, but based on qualitative observations reduced that in field CA3 lateral to it (Fig. 3C). Similar results were seen in two other animals that received the same treatment. In order to confirm that these lesions depleted field CA3 of mossy fiber terminals, sections adjacent to those taken for autoradiography were processed by the Timm's sulfide silver method which heavily stains the mossy fibers and their terminals. As shown in Figure 4, the knife-cuts depleted Timm's staining in the CA3 field lateral to the cut. On the control side, the thickness of the band of Timm's staining along the horizontal limb of field CA3 was about 110 μm.

DISCUSSION

The present observations extend our previous findings (Padua et al., 1991) on [^{3}H]ryanodine binding sites in rat brain by showing quantitatively and qualitatively the global distribution of these sites in some major brain areas. In general, the results indicate that these sites have a highly heterogeneous distribution among brain regions as well as within some individual structures such as the olfactory bulb, hippocampus and striatum. Most striking was the distinct difference in the overall high levels of sites in, for example, the cerebral cortex, hippocampus and a few other forebrain structures and the uniformly lower densities of sites in nearly all diencephalic, mesencephalic and pontine structures. The significance of these relatively broad demarcations in the levels of sites must await determination of the nature and function of the receptor protein or proteins with which ryanodine interacts in brain. However, it appears that [^{3}H]ryanodine binding sites in the CNS may mediate intracellular calcium mobilization events in a fashion analogous to the actions of ryanodine in muscle (Ashley, 1989; Ellisman et al., 1990; McPherson and Campbell, 1990; Kawai et al., 1991; Padua et al., 1991) and in some peripheral neurons (Thayer et al., 1988a, 1988b; Kawai and Watanabe, 1989). In view of this, it might be tentatively inferred that, in contrast to other brain areas,

functions associated with a considerable proportion of rhinencephalic and telencephalic as well as some cerebellar cortical structures have a greater dependence on whatever special calcium mobilization properties are endowed by intracellular calcium channels presumed to be associated with [^{3}H]ryanodine binding sites. A more detailed consideration of how these properties might be manifested will require analyses of the cell types that express and the intracellular organelles that contain the high levels of binding sites in these structures. In this regard, antibodies directed against the skeletal muscle ryanodine receptor have been recently used to investigate immunohistochemically the localization of cross-reacting proteins in the cerebellum (Ellisman et al., 1990). It was found that these antibodies recognize epitopes localized on intracellular membranes in cerebellar Purkinje cells. These results are consistent with our finding of a relatively high density of sites in the cerebellar cortex. However, the correspondence of the material detected immunohistochemically to binding sites detected autoradiographically remain to be investigated by anatomical comparisons on a larger scale.

Our results concerning the localization of [^{3}H]ryanodine binding sites in the hippocampus raise several points for consideration. First, the total depletion of these sites after the large kainic acid lesions which destroyed nearly all the neurons at the hippocampal injection area suggests that [^{3}H]ryanodine binds to sites contained in or on neurons and that resident glial cells as well as reactive astrocytes are largely devoid of these sites. Second, the massive depletion observed after these lesions also suggests that the vast proportion of binding sites at least at the anterior hippocampal levels studied here are contained in neurons intrinsic to the dentate gyrus and the CA1, CA2 and CA3 fields of this structure rather than in axons or terminals associated with afferent projections which are thought to remain largely intact at kainic acid lesion sites. Thus, major projections to the hippocampus that appear to make a negligible contribution to

binding sites in this structure include entorhinal, commissural and septal afferents. And third, the persistence of a narrow band of binding sites in field CA3 after the more restricted kainic acid lesions that destroyed nearly all cells in this area but left granule cells in the dentate gyrus intact suggests a dual neuronal localization of sites in this hippocampal subfield. A proportion of sites is contained probably in the cell bodies of CA3 pyramidal neurons as well as their apical and basal dendrites which span the roughly 400 μm thick region of dense binding along field CA3. The remainder of sites appear to be contained in axons projecting to this area and a likely candidate for these are the mossy fiber terminals that are known to arise from dentate granule cells (Swanson et al., 1978). In support of this notion is the observation that the thickness of the remaining band of sites (about 125 μm) corresponds to the length over which mossy fiber terminals are distributed on the initial dendritic segments of CA3 pyramidal cells (Blackstad and Kjaerheim, 1961; Blackstad et al., 1970; Gaarskjaer, 1978). As we have previously shown (Padua et al., 1991), although less evident here, a dense band of [^{3}H]ryanodine binding sites of similar thickness embedded within field CA3 can occasionally be detected in autoradiograms of normal hippocampus and this band of higher density may correspond to that remaining after the laterally placed kainic acid lesions. Although not quantitatively assessed, the reduction in the density of binding sites in the CA3 area lateral to knife-cut lesions that severed the axons of mossy fiber terminals would also indirectly suggest the presence of binding sites in these terminals. Further support for this is provided by our observations of significant levels of [^{3}H]ryanodine binding sites in crude P2 synaptosomal (nerve terminals plus mitochondria) fractions (Padua et al., 1991) and more recently by our findings that purified synaptosomal fractions exhibit much higher levels of binding than purified mitochondrial fractions (unpublished observations).

A causal relationship of an increase in cytoplasmic free Ca^{2+} to neurotransmitter release at presynaptic terminals has been well described (Augustine et al., 1987). However, evidence that Ca^{2+} released from intracellular Ca^{2+} compartments contributes to this process is currently uncertain. In nerve terminals, Ca^{2+} accumulation in a non-mitochondrial organelle, thought to be smooth endoplasmic reticulum (SER), was demonstrated (Blaustein et al., 1980; Hartter et al., 1987; Martinez-Serrano and Satrustegui, 1989) and caffeine was shown to mobilize Ca^{2+} from these stores (Martinez-Serrano and Satrustegui, 1989). In certain depolarized states, intracellular Ca^{2+} release from presumed internal stores was shown to increase the release of acetylcholine from synaptosomes and at neuromuscular junctions (Rahamimoff et al., 1978; Adam-Vizi and Ligati, 1984). Moreover, ryanodine has been demonstrated to exert a facilitatory action on acetylcholine release at the neuromuscular junction (Nishimura et al., 1990). These findings together with our results suggesting the presence of [^{3}H]ryanodine binding sites in mossy-fiber terminals point to the possibility that Ca^{2+} release through a ryanodine receptor/Ca^{2+} channel complex in terminals may be involved in the regulation of neurotransmitter release or in other actions of Ca^{2+} that have been attributed to its release from intracellular storage pools (Brown and Higashida, 1988; Kuba et al., 1983; Dutar and Nicoll, 1988; Fink et al., 1988; Kawai and Watanabe, 1989).

In addition to possible regulation of intracellular Ca^{2+} through ryanodine receptor gated channels, it is now well established that an inositol 1,4,5-trisphosphate (IP_3) receptor mediates intracellular Ca^{2+} release through activation of Ca^{2+} channels localized on SER in neurons (Ferris et al., 1989; Satoh et al., 1990; Ross et al., 1989). Pertinent here is that although the IP_3 receptor has some sequence homology with the ryanodine receptor of muscle (Mignery et al., 1989), these two receptors are different with respect to molecular weight, immunological characteristics and interactions with

other Ca^{2+} releasing agents (McPherson and Campbell, 1990; Thayer et al., 1988a, 1988b). A comparison of the anatomical distribution of IP_3 (Worley et al., 1989) and ryanodine binding sites in rat brain indicates that whereas IP_3 and ryanodine receptors are co-localized in some regions, their distribution differs in others. For example, IP_3 receptors are concentrated in field CA1 and ryanodine binding sites in field CA3 of the hippocampus. This suggests that Ca^{2+} mobilization in these subregions may in part involve different mechanisms. The relative contribution of each type of receptor to the release of Ca^{2+} from intracellular stores and to specific intracellular Ca^{2+}-mediated events remains to be determined.

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FIGURE LEGENDS

Figs. 1A-L. Photomicrographs showing the distribution of ^{3}H-ryanodine binding sites in transverse sections of rat brain. Digitized autoradiograms were displayed as bright-field images on a video monitor of an image analysis system and photomicrographs were produced directly from the monitor screen. Scale bars for A-L are indicated in A and G, 3 mm. Brain areas indicated by numbers are listed in the text.

Fig. 2. Photomicrographs of Nissl-stained transverse sections showing the placement and effect of unilateral kainic acid and knife-cut lesions in the rostral hippocampus. **A:** An injection of kainic acid in the region of the hilus. With reference to the contralateral control side (left), note the large depletion of cells in both the pyramidal (PCL) and granule cell (GCL) layers on the lesioned side (right). Only a portion of medially located granule cells is left intact (arrow). **B:** An injection of kainic acid in the dorsolateral region of the hippocampus (right side) resulting in the distruction of pyramidal cells in fields CA3 (small arrows) and a portion of those in fields CA1/2 (large arrow), but sparing granule cells in the dentate gyrus (arrowheads). **C:** Higher magnification of the lesion site in B showing the loss of pyramidal cells and intense gliosis (arrows) in field CA3. **D:** A knife-cut lesion (arrow) severing the lateral portion of field CA3 from the dentate gyrus. Scale bars: 500 μm.

Fig. 3. Photomicrographs showing the effect of kainic acid and knife-cut lesions on ^{3}H-ryanodine binding sites in the hippocampus. Digitized autoradiograms were displayed as dark-field images on a video monitor of an image analysis system and photographed on the monitor screen. **A:** Hilus kainic acid injection showing a loss of binding sites throughout the lesioned area (large arrow) including fields CA1 and CA3, and the dorsal (d) and ventral (v) blades of the dentate gyrus. Binding sites are still evident in the region of the dentate gyrus where some granule cells were left intact (small arrow) **B:**

Dorsolateral hippocampus kainic acid injection showing normal binding in the dorsal (d) and ventral (v) blades of the dentate gyrus on the lesioned side (right) and loss of all but a narrow band of sites in field CA3 (arrow). **C:** Knife-cut between the dentate gyrus and the lateral region of field CA3 showing a reduction of sites in the latter (arrow). The sections taken for ^{3}H-ryanodine binding in A, B and C are adjacent to the Nissl-stained sections shown in Figures 2A, B and D, respectively. Scale bar: 3 mm.

Fig. 4. Photomicrogragh showing the normal appearence of Timm's sulfide silver staining in field CA3 of the hippocampus (left control side, small arrow) and the absence of this staining after transection (large arrow) of the mossy fiber projection from the dentate granule cells to the CA3 pyramidal cells (right side, arrowhead). Scale bar: 500 μm.

List of structures labelled by numbers in Figure 1

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1 External plexiform layer

2 Internal granular, plexiform, and mitral cell layers

3 Frontal cortex

4 Anterior olfactory nucleus

5 Olfactory tubercle

6 Striatum

7 Tenia tecta

8 Lateral septal nucleus, dorsal part

9 Medial septal nucleus

10 Cingulate cortex

11 Accumbens nucleus

12 Parietal cortex

13 Medial preoptic area

14 Bed nucleus of stria terminalis

15 Lateral septal nucleus, intermediate part

16 Dorsal thalamus

17 Globus pallidus

18 Anterior hypothalamic area

19 Lateral hypothalamic area

20 Internal capsule

21 Basolateral amygdaloid nucleus

22 Medial amygdaloid nucleus

23 Central amygdaloid nucleus

24 Dentate gyrus

25 Hippocampus

26 Perirhinal cortex

27 Ventromedial hypothalamic nucleus

28 Retrosplenial granular/agranular cortex

29 Temporal cortex

30 Medial geniculate nucleus

31 Mammillary body

32 Substantia nigra

33 Superior colliculus

34 Occipital cortex

35 Entorhinal cortex

36 Inferior colliculus

37 Pontine reticular nucleus

38 Pontine nucleus

39 Central gray substance

40 Cerebellum

Table 1. Regional distribution of [^{3}H]ryanodine binding sites in rat brain.

Structure	Specific Binding fmol/mg of tissue
Olfactory areas	
Internal granular, plexiform, mitral cell layers	9.0 ± 1.3
External plexiform layer	17.9 ± 3.3
Glomerular layer	7.5 ± 1.4
Anterior olfactory nuclei	10.6 ± 1.1
Olfactory tubercle	9.3 ± 0.7
Cerebral cortex	
Cingulate cortex	8.0 ± 0.4
Frontal (motor) cortex	8.3 ± 0.5
Parietal cortex	8.6 ± 1.0
Occipital cortex	7.8 ± 1.1
Retrosplenial, granular/agranular cortex	6.5 ± 0.9
Temporal cortex	4.8 ± 0.6
Entorhinal cortex	5.7 ± 0.7
Hippocampal formation	
Whole hippocampus	8.8 ± 0.8
Dentate gyrus	14.8 ± 1.3
Field CA3	12.1 ± 1.0
Field CA1 and CA2	4.4 ± 0.4

(Table 1, con't)

Septum	
Bed nucleus of stria terminalis	3.0 ± 0.6
Medial septum	3.5 ± 0.4
Lateral septum	6.6 ± 0.6
Amygdala	
Medial amygdaloid nucleus	2.7 ± 0.5
Basolateral amygdaloid nucleus	4.0 ± 0.4
Central amygdaloid nucleus	1.5 ± 0.3
Basal ganglia	
Accumbens nucleus	6.1 ± 0.8
Head of striatum, lateral	6.9 ± 0.7
Head of striatum, medial	4.0 ± 0.4
Tail of striatum	4.1 ± 0.6
Globus pallidus	2.0 ± 0.3
Substantia nigra	2.0 ± 0.5
Diencephalon	
Ventromedial hypothalamic nucleus	2.9 ± 0.5
Lateral hypothalamus	1.1 ± 0.2
Mammillary nucleus, medial and lateral	1.2 ± 0.2
Dorsal thalamus	2.0 ± 0.2
Medial habenula	3.3 ± 0.3
Lateral habenula	0.8 ± 0.1

(Table 1, con't)

Midbrain	
Central gray substance	4.1 ± 0.7
Interpeduncular nucleus	2.4 ± 0.5
pontine nucleus	3.8 ± 0.7
Superior colliculus	5.4 ± 0.4
Inferior colliculus	2.7 ± 0.4
Cerebellum	
Molecular layer	4.3 ± 0.5
Granule layer	7.0 ± 0.5
Deep nuclei	0.9 ± 0.1

Values represent the mean ± S.E.M. of measurements from 4 animals.

Figure 1

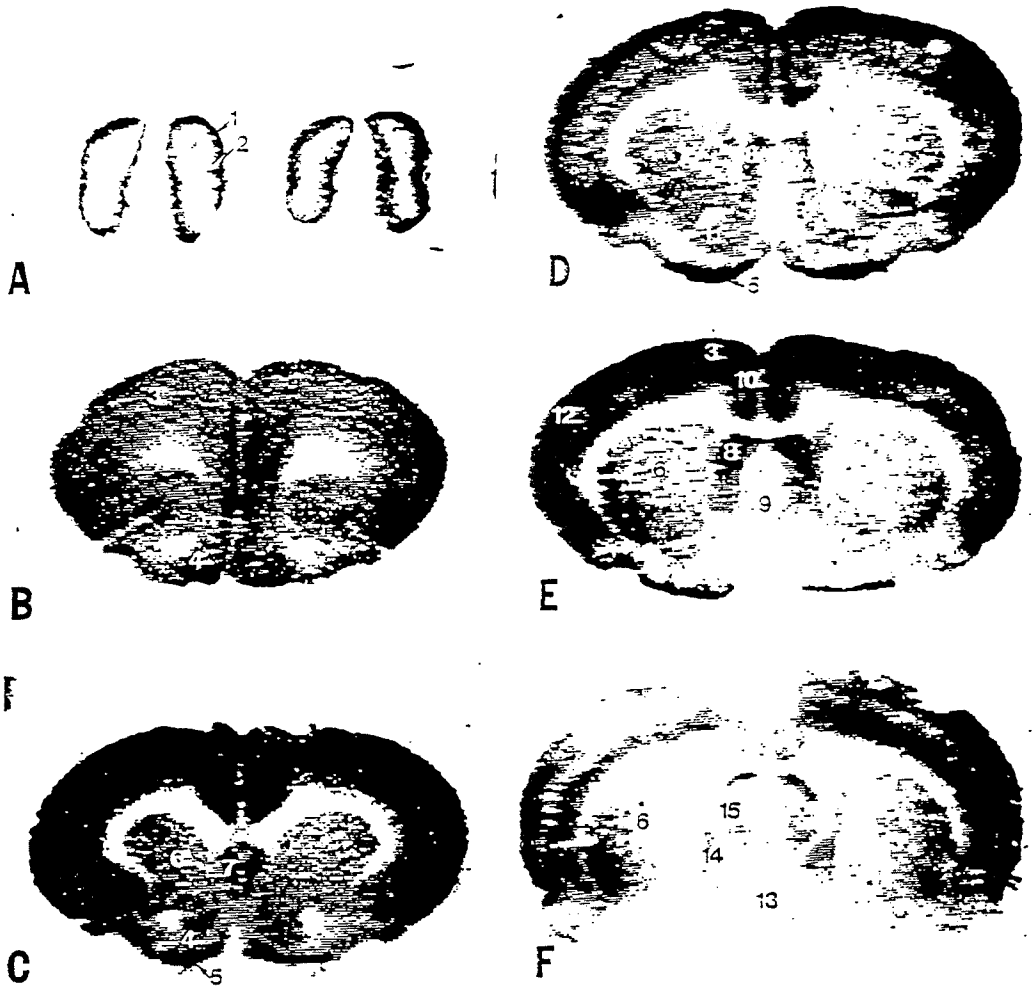

Figure 1 (con't)

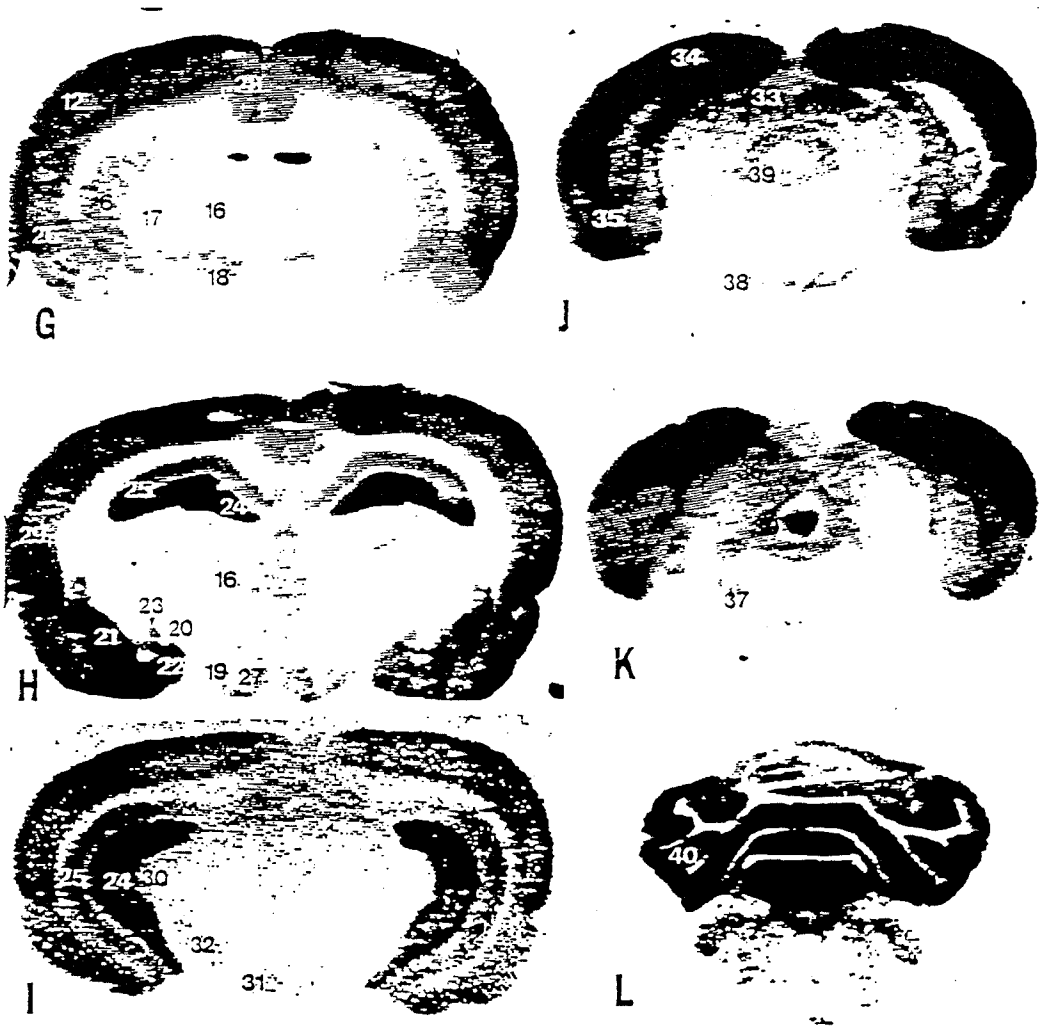

Figure 2

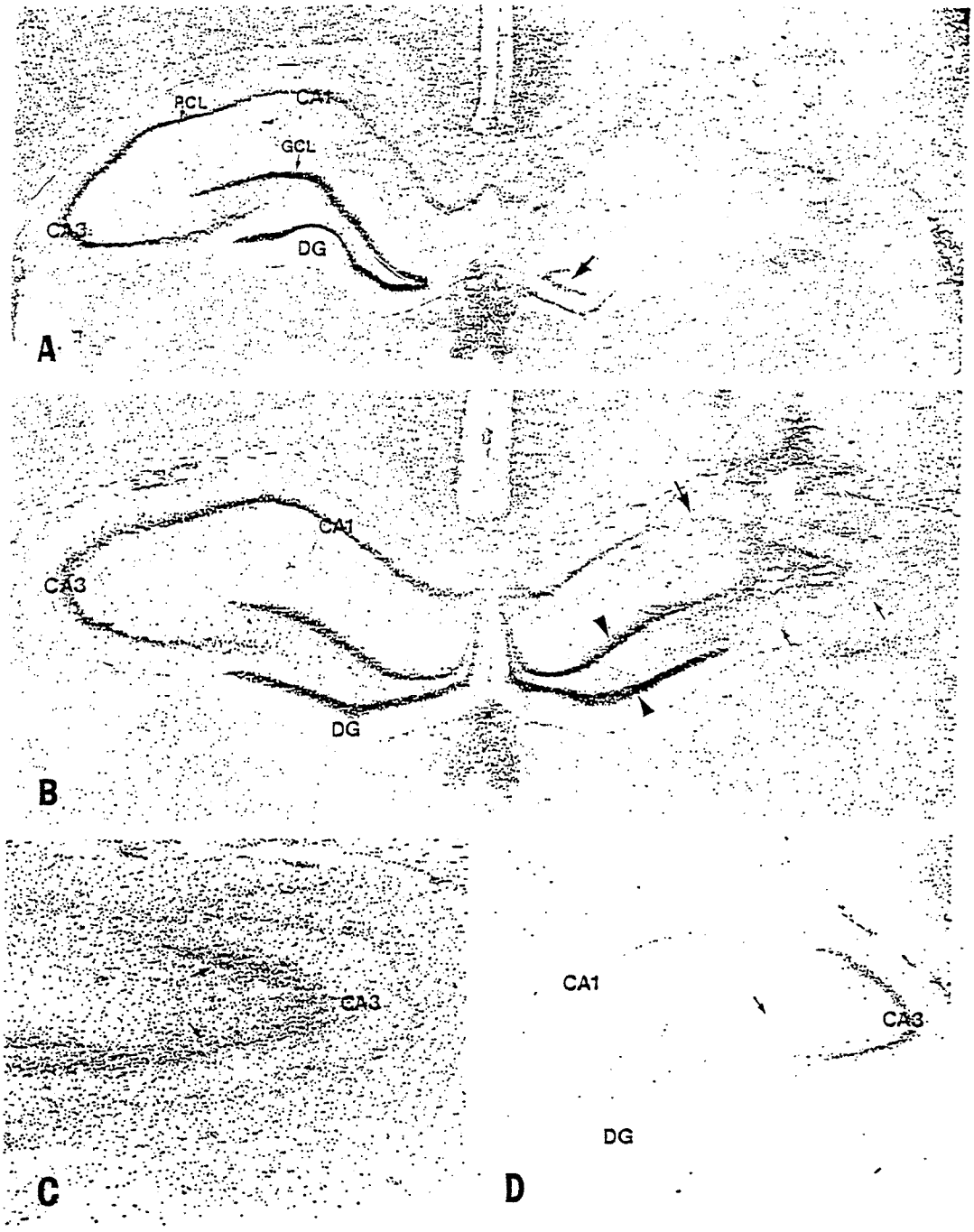

Figure 3

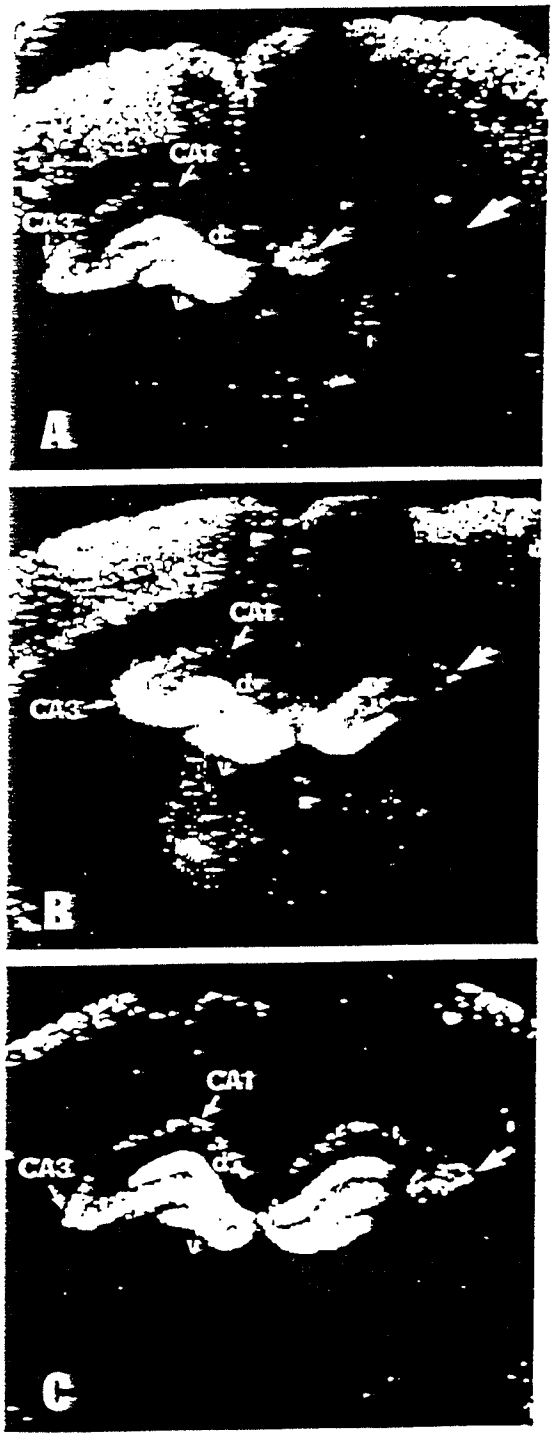

Figure 4

Part III

High Affinity [^{3}H]Ryanodine Binding Sites in Postmortem Human Brain: Regional Distribution and Effects of Calcium, Magnesium and Caffeine.

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Brain Res. (1992) 585, 349-354

Abstract--The pharmacological properties, regional distribution and autoradiographic localization of [^{3}H]ryanodine binding sites were examined in postmortem human brain. Analyses of binding data from labeled ryanodine titration experiments conducted in frontal cortex revealed a single class of high affinity binding sites with a K_d value of 3.6 nM and a B_{max} value of 99 fmol/mg protein. In unlabeled ryanodine titration experiments, K_d and B_{max} values were 6.5 nM and 132 fmol/mg protein, respectively. Binding was found to be dependent on free Ca^{2+} (ED_{50} value, 89 μM) and was decreased by 35% in the presence of 5 mM Mg^{2+}. This Mg^{2+} inhibition was abolished by the addition of 10 mM caffeine. Analysis of the regional distribution of [^{3}H]ryanodine binding in membrane preparations revealed high levels of sites in putamen and caudate nucleus, intermediate levels in hippocampus and cortex, and low levels in cerebellum. Autoradiographically, the hippocampus displayed a high density of binding sites in the CA3 region and the dentate gyrus. Ryanodine binding sites in human brain exhibit similar, but not identical binding and pharmacological characteristics to ryanodine receptors previously identified in muscle and more recently in rat and rabbit brain and accordingly may be involved in the regulation of intracellular calcium.

Ryanodine (RYD), a plant alkaloid, has been widely used as a probe to study the role of Ca^{2+} release from muscle sarcoplasmic reticulum (SR) in excitation-contraction coupling. It binds with high affinity to the Ca^{2+} channel/ryanodine receptor complex localized on terminal cisterns of SR and at low concentrations, it prevents Ca^{2+} channel closure thereby depleting the SR Ca^{2+} store (Fill and Coronado, 1988; Lai and Meissner, 1989). Several lines of evidence suggest that RYD receptors may be involved in regulating intraneuronal Ca^{2+}. In sympathetic neurons, RYD blocked caffeine-induced Ca^{2+} release from intracellular compartments and interfered with spike after-hyperpolarizations which are thought to be caused by Ca^{2+} release from these stores (Kawai and Watanabe, 1989; Thayer et al., 1988). At mouse neuromuscular junctions, RYD facilitated the release of acetylcholine (Nishimura et al., 1990). High affinity [^{3}H]RYD binding sites have been demonstrated in various membrane preparations of rat, rabbit and chick brain (Ashley, 1989; Kawai et al., 1991; McPherson and Campbell, 1990; Padua et al., 1991; Walton et al., 1991; Zimanyi and Pessah, 1991a). Immunohistochemical and Western blot analyses of the binding protein have shown that antibodies directed against the RYD receptor in skeletal muscle recognize the form of the receptor expressed in rabbit and chick brain (Ellisman et al., 1990; McPherson and Campbell, 1990; Walton et al., 1991). Purified ryanodine receptor proteins from brain form active caffeine- and ryanodine-sensitive calcium release channels when reconstituted into planar lipid bilayers (Ashley, 1989; Ellisman et al., 1990; McPherson et al., 1991). Taken together, these studies suggest that the Ca^{2+} channel/ryanodine receptor complex is present in neurons and that it may have physiological and pharmacological significance with respect to intracellular Ca^{2+} mediated events in neurons. Although the mechanisms by which intraneuronal Ca^{2+} is regulated are more difficult to study in human brain, defects in these mechanisms have been implicated in certain pathological states (Warsh et al., 1991). In order to develop the use of RYD as a neurochemical tool to facilitate studies of intraneuronal Ca^{2+} regulation in neurons of

human CNS, we have utilized membrane binding and autoradiographic techniques to show that [^{3}H]RYD binding sites can be detected in postmortem human brain and have examined the pharmacological properties and the anatomical localization of these sites.

METHODS

Postmortem human brains from 5 males and 1 female (average age, 66 ± 6 years) were obtained within 24 hours after death. All patients were free of neurological disease and all died of complications secondary to peripheral vascular or pulmonary diseases. Brains were bisected on an ice-cold metal tray; the left hemisphere was taken for neuropathological analyses and the right hemisphere was dissected on ice. Frontal cortex, cerebellum, hippocampus, putamen, globus pallidus and caudate nucleus were removed and frozen at -80°C until taken for assay. Tissues were homogenized with a glass-teflon homogenizer (20 strokes, 500 rpm) in 1:10 (w/v) of buffer A containing 20 mM PIPES-KOH (pH 8.0), 1.0 M KCl, 1.0 mM PMSF, and 100 μM $CaCl_2$ and centrifuged at 100,000 x g for 60 min at 4°C. These crude membrane pellets were either frozen at -20°C for up to 1 week or resuspended in buffer and assayed immediately. All binding assays were conducted using crude cortical membranes except in regional distribution experiments where crude membranes prepared from the other regions were used as well. Binding assays were performed in duplicate in a total volume of 1.0 ml of buffer A containing 0.3-0.4 mg of protein and 3 nM [^{3}H]RYD (60.0 Ci/mmol, Dupont-NEN, Boston, MA) as previously described (Padua et al., 1991). Specific binding was defined as the difference between total binding in the presence of 3 nM [^{3}H]RYD alone and nonspecific binding in the presence of 3 nM [^{3}H]RYD plus 50 μM unlabeled RYD (Research Biochemicals, Wayland MA). In labeled titration experiments, [^{3}H]RYD concentrations were varied over a range of 0.05 to 24.0 nM. In unlabeled titration experiments, RYD concentrations were varied from 1 pM to 50 μM. In tests of the effects of Ca^{2+}, free Ca^{2+} concentrations in the assay system were varied in the

presence of 0.2 mM EGTA and calculations of free Ca^{2+} were conducted using an EGTA-Ca^{2+} dissociation constant of 39 μM. The effects of 5 mM $MgCl_2$ or 10 mM caffeine, alone or in combination with each other, were tested at low (4 nM) and high (100 μM) concentrations of free Ca^{2+}. Incubations were conducted at 37°C for 60 min and were terminated by rapid vacuum filtration through GF/C glass-fiber filters using a Brandel Cell Harvester M-24R. Filters were washed 3 times with 5 ml of ice-cold buffer A, placed in vials and taken for liquid scintillation counting in 4.5 ml of Beckman Ready Solvent. Dissociation constants (K_d), maximum number of receptor sites (B_{max}), and partial F-tests for best fit by a one or two-site model were derived from analyses of data by the non-linear multipurpose curve-fitting program LIGAND (Munson and Rodbard, 1980). Protein was determined by the method of Lowry et al. (1951) with BSA as standards.

For autoradiographic localization of [^{3}H]RYD binding sites, hippocampus from postmortem human brain obtained at the time of autopsy was removed at the level of the midbrain posterior to the optic chiasm and anterior to the mammillary bodies; the surrounding white matter was carefully stripped away. Hippocampal tissue was cut into 1 cm blocks, frozen on dry ice, mounted onto cryostat chucks and 10 μm sections were thaw-mounted onto gelatinized slides. Sections were washed twice in buffer A for 15 min and incubated with 15 nM [^{3}H]RYD for 15 min at 25°C, conditions under which binding equilibrium was reached. Reactions were terminated by consecutive washes of 5 minutes in ice-cold buffer A, ice-cold buffer A diluted 1:10 (v/v) with distilled water, and ice-cold distilled water. Non-specific binding was determined in adjacent sections processed as above except that sections were incubated in buffer A with 15 nM [^{3}H]RYDin the presence of 10 μM unlabeled RYD. Sections were dried and then apposed to ^{3}H-Hyperfilm (Amersham) for 2 months. The film was developed in Kodak D-19 developer, fixed, washed and dried.

RESULTS

In labeled titration experiments, [^{3}H]RYD bound to a single class of high affinity sites with a K_d value of 3.6 ± 1.0 nM and a B_{max} value of 99 ± 15 fmol/mg protein (Fig. 1). In unlabeled titration experiments, using a wide range of RYD concentrations, unlabeled RYD was found to compete dose dependently with an IC_{50} value of 12 ± 6 nM at the high affinity binding site (Fig. 2). Computer analyses of the unlabeled titration binding data revealed a single class of high affinity binding sites with a K_d value of 6.5 ±2.8 nM and a B_{max} value of 132 ± 11 fmol/mg protein both of which were not significantly different from those values obtained from labeled titration experiments. High concentrations (up to 40 μM) of unlabeled RYD were able to compete for approximately 95% of the specific binding. High affinity [^{3}H]RYD binding was found to be highly dependent on the concentration of free Ca^{2+} such that when Ca^{2+} was varied from 10 nM to 20 mM, [^{3}H]RYD binding increased from a baseline level of between 0 to 15% of maximal binding to 100% of maximal binding over a very narrow range of free Ca^{2+}. Calculation of the ED_{50} from the steep curve shown in Figure 3 yielded a value of 89 ± 18 μM. Maximal binding was observed at a Ca^{2+} concentration of 100 μM and binding decreased to approximately 70% of this level as Ca^{2+} was raised to 20 mM. In order to further test the Ca^{2+} dependency of binding as well as interactions between Ca^{2+} and other regulatory agents known to have effects on [^{3}H]RYD binding at SR Ca^{2+} channels in muscle, experiments were conducted with added Mg^{2+} and/or caffeine at low (4 nM free Ca^{2+}) and high (100 μM free Ca^{2+}) calcium levels (Fig. 4). In the presence of 5 mM Mg^{2+}, binding was reduced by 35% in low calcium ($p<0.01$) and by 15% in high calcium ($p<0.0005$). In both high and low Ca^{2+}, the addition of 10 mM caffeine reversed the Mg^{2+} inhibition and restored binding to levels that were not significantly different from those observed in the absence of Mg^{2+}. Caffeine alone had no significant influence on [^{3}H]RYD binding.

In a limited analysis of the levels of [^{3}H]RYD binding sites in some major brain regions, a highly heterogenous distribution of these sites was found; high levels of binding were observed in putamen and caudate nucleus, intermediate levels in cortex, hippocampus and globus pallidus, and low levels in cerebellum (Fig. 5). Autoradiographic analysis of binding within the hippocampus (Fig. 6A) revealed a high density of binding sites in the CA3 hippocampal region and in the granule cell layer of the dentate gyrus, intermediate levels of binding in fields CA1, CA2 and the subiculum and low levels within the hilus. No detectable binding was observed when sections were incubated with [^{3}H]RYD in the presence of 10 μM unlabeled RYD (Fig. 6B).

DISCUSSION

The binding properties and anatomical distribution of [^{3}H]RYD binding sites in human brain, described here for the first time, exhibit both similarities and differences to those in rat, rabbit and chick brain and in muscle. The interactions of these sites with agents known to affect Ca^{2+} release from sarcoplasmic reticulum (SR) and [^{3}H]RYD binding in muscle (Ca^{2+}, Mg^{2+} and caffeine) (Pessah et al., 1987; Zimanyi and Pessah, 1991b) suggest that they may be analogous to the ryanodine receptor/Ca^{2+} channel complex of muscle SR. Scatchard analyses of labeled and unlabeled titration data revealed a single class of high affinity [^{3}H]RYD binding sites with K_d values similar to those obtained for rodent and chick brain (Kawai et al., 1991; McPherson and Campbell, 1990; Padua et al., 1991; Walton et al., 1991; Zimanyi and Pessah, 1991a) and in muscle (Michalak et al., 1988; Pessah et al., 1987; Pessah and Zimanyi, 1991; Zimanyi and Pessah, 1991b). However, our labeled and unlabeled titration experiments failed to reveal a second class of low affinity binding sites which have been observed in some tissues (Kawai et al., 1991; McGrew et al., 1989; Pessah and Zimanyi, 1991). It is noteworthy in this regard that the addition of high concentrations of unlabeled RYD caused a near total displacement of the radiolabeled ligand resulting in less than 5% non-

specific/non-displaceable binding indicating that [^{3}H]RYD binds to highly specific sites in human brain membranes and that low affinity sites were not simply obscured by high non-specific binding. Although this suggests an absence of low affinity sites in human brain, the possibility of a postmortem loss of this site cannot be excluded.

It has been proposed that the ryanodine receptor/Ca^{2+} channel complex in muscle contains regulatory domains which bind Ca^{2+}, adenine nucleotides and caffeine. These domains are thought to modulate the Ca^{2+} release channel and the interaction of ryanodine within the complex (Pessah et al., 1987). In this model, ryanodine binding sites localized near the channel are activated by Ca^{2+} causing the channel to open. Binding of ryanodine requires a concentration of free Ca^{2+} in the micromolar range (Pessah et al., 1987; Zimanyi and Pessah, 1991b). In human brain, [^{3}H]RYD binding exhibited a similar Ca^{2+} dependency which suggests that ryanodine receptors in human brain possess a Ca^{2+} regulatory site. That this is a physiologically relevant effect of Ca^{2+} is suggested by the ability of Mg^{2+} to decrease the amount of [^{3}H]RYD bound in the presence of optimal and suboptimal free Ca^{2+} concentrations. In muscle, Mg^{2+} at physiological concentrations was shown to inhibit [^{3}H]RYD binding by competing with Ca^{2+} (Pessah et al., 1987; Zimanyi and Pessah, 1991b). Binding of caffeine to apparently another domain reverses the inhibitory effects of Mg^{2+} and increases the sensitivity of the complex to activation by Ca^{2+} (Pessah et al., 1987; Zimanyi and Pessah, 1991b). In human, rat and rabbit brain, the modulatory action of caffeine on [^{3}H]RYD binding appears to occur in a similar fashion as that in muscle (Kawai et al., 1991; Zimanyi and Pessah, 1991a).

A comparison of the distribution of [^{3}H]RYD binding sites in human brain with that of rodent brain showed that whereas high levels of binding were found in rat and rabbit hippocampus and cerebellum (McPherson and Campbell, 1990; Padua et al.,

1991b), the highest levels of sites in human brain were observed in structures of the basal ganglia (putamen and caudate nucleus). Autoradiographic analysis of [^{3}H]RYD binding sites in the human hippocampus revealed a high density of these sites in the dentate gyrus and CA3 subfield similar to that seen in rat (Padua et al., 1991a,b). Although the physiological function of ryanodine receptors in brain remain to be determined, the high levels of [^{3}H]RYD binding sites in basal ganglia prompt speculation regarding the possible role of these sites within dopaminergic neurons localized in these structures. In particular, we are considering the possibility that these sites may be relevant to the neurological disorder neuroleptic malignant syndrome (NMS). NMS is a potentially lethal disorder induced through the use of neuroleptic drugs or the withdrawal of antiparkinsonian agents (Guze and Baxter, 1985; Keyser and Rodnitzky, 1991). A seemingly similar disorder, namely malignant hyperthermia (MH), appears to arise from an abnormality in the release of Ca^{2+} from the SR of skeletal muscle and probably involves the ryanodine receptor (Carrier et al., 1991; Ervasti et al., 1991; Nelson et al., 1991). Patients with NMS and MH exhibit several of the same symptoms including muscular rigidity, elevated creatine phosphokinase levels and hyperpyrexia. Dantrolene, a drug known to block Ca^{2+} release from SR and ryanodine-induced contractures of muscle (Fairhurst et al., 1980; Ohta et al., 1990), is used in the treatment of both NMS and MH (Fairhurst et al., 1980; Granato et al., 1983). Furthermore, in the treatment of NMS, bromocriptine, a dopamine agonist, has been used successfully and appears to be as effective as dantrolene in decreasing the clinical response time when administered concurrently with supportive measures (Rosenberg and Green, 1989). Given these observations, the possibility exists that an abnormality in the ability of dopaminergic neurons in the basal ganglia to regulate intracellular Ca^{2+} via ryanodine receptors may be involved in the development of neuropathological disorders such as NMS. Furthermore, a heretofore unexplored hypothesis is that alterations in brain ryanodine receptors, analogous to that observed in skeletal muscle (Carrier et al., 1991; Ervasti et

al., 1991; Nelson et al., 1991), may account for some of the CNS symptoms seen in the MH syndrome. The physiologic significance of ryanodine receptors and their relevance to CNS disease states should be further studied.

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FIGURE LEGENDS

Figure 1. Saturation isotherm and Scatchard plot (inset) of [^{3}H]RYD binding in a membrane preparation of cerebral cortex. Membranes were incubated with [^{3}H]RYD concentrations ranging from 0.05 to 24.0 nM. Non-specific binding was defined as binding in the presence of labeled RYD plus 50 μM unlabeled RYD. [^{3}H]RYD bound to a single class of high affinity sites with K_d and B_{max} values of 3.6 nM and 99 fmol/mg protein, respectively. Data shown are representative of four separate experiments performed in duplicate.

Figure 2. Unlabeled titration curve of [^{3}H]RYD binding in a membrane preparation of cerebral cortex. Membranes were incubated in the presence of 3 nM [^{3}H]RYD and concentrations of unlabeled RYD ranging from 1 pM to 50 μM. Non-specific binding was defined as that remaining in the presence of 50 μM RYD. The K_d and B_{max} values were 6.5 nM and 132 fmol/mg protein, respectively (IC_{50} value was 12 nM). Data shown are representive of three separate experiments performed in duplicate.

Figure 3. The effects of free Ca^{2+} concentration on [^{3}H]RYD binding in a membrane preparation of cerebral cortex. Free Ca^{2+} concentration was determined by titration with 0.2 mM EGTA (K_D, 39 μM) and was varied from 10 nM to 20 mM. [^{3}H]RYD binding was maximal at a Ca^{2+} concentration of 100 μM and half maximal at 89 μM. Data shown are representative of four separate experiments performed in duplicate.

Figure 4. The effects of 5 mM $MgCl_2$ and 10 mM caffeine alone or in combination with Mg^{2+} on [^{3}H]RYD binding under conditions of low (4 nM, open bars) and high (100 μM, closed bars) free calcium concentrations in membrane preparations of cerebral cortex. Data shown are means ± S.E.M. of at least three separate experiments performed in duplicate. Mg, $MgCl_2$; Caf, caffeine. *Significantly different from control, $p<0.01$. **Significantly different from control, $p<0.0005$. +Significantly different from 5 mM Mg (low Ca^{2+} concentration), $p<0.05$. ++Significantly different from 5 mM Mg (high Ca^{2+} concentration), $p<0.05$.

Figure 5. Regional distribution of [^{3}H]ryanodine binding in membrane preparations of six brain regions. Data shown are means ± S.E.M. of 4 or 5 separate experiments performed in duplicate. CTX, cortex; CB, cerebellum; H, hippocampus; P, putamen; GP, globus pallidus; CN, caudate nucleus.

Figure 6. Autoradiographic localization of [^{3}H]ryanodine binding sites in the hippocampus. A and B are adjacent transverse sections incubated in the presence of 15 nM [^{3}H]RYD alone (A) or in the presence of 15 nM [^{3}H]RYD and 10 μM unlabeled RYD (B). Note the elevated levels of binding sites in the granule cell layer of the dentate gyrus (arrowheads) and in field CA3 (arrows). Magnification, X 5.

Figure 1

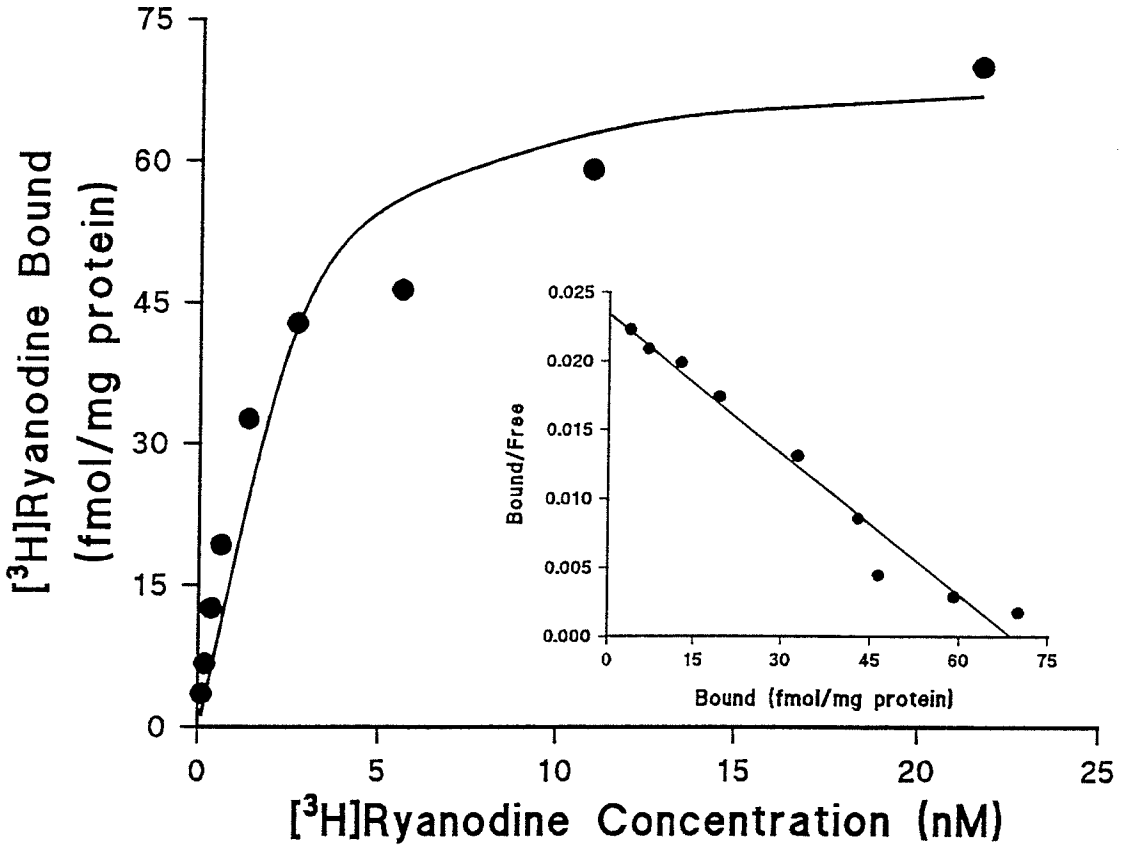

[3H]Ryanodine Bound (fmol/mg protein)
75
60
45
30
15
0
0
5
10
15
20
25
[3H]Ryanodine Concentration (nM)
Bound/Free
0.025
0.020
0.015
0.010
0.005
0.000
0
15
30
45
60
75
Bound (fmol/mg protein)

Figure 2

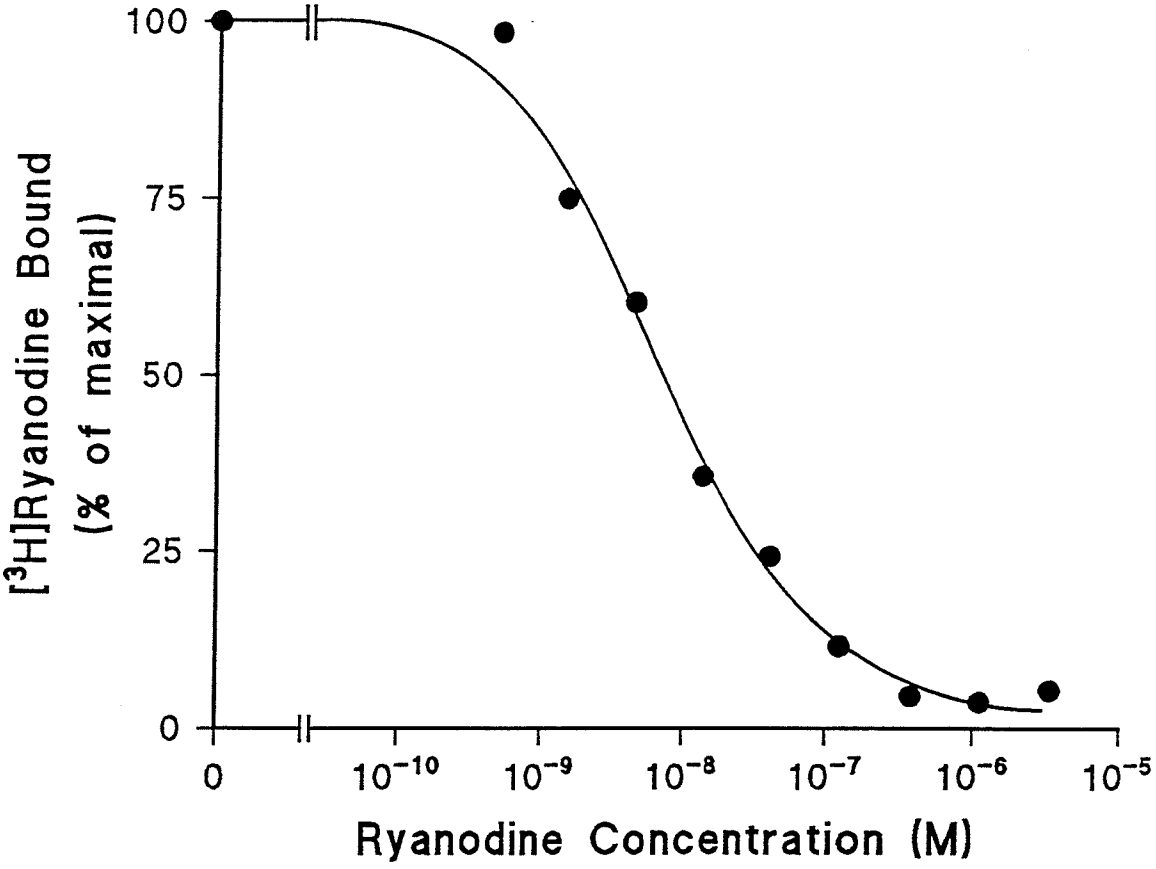

100
75
50
25
0
[3H]Ryanodine Bound
(% of maximal)
0
10-10
10-9
10-8
10-7
10-6
10-5
Ryanodine Concentration (M)

Figure 3

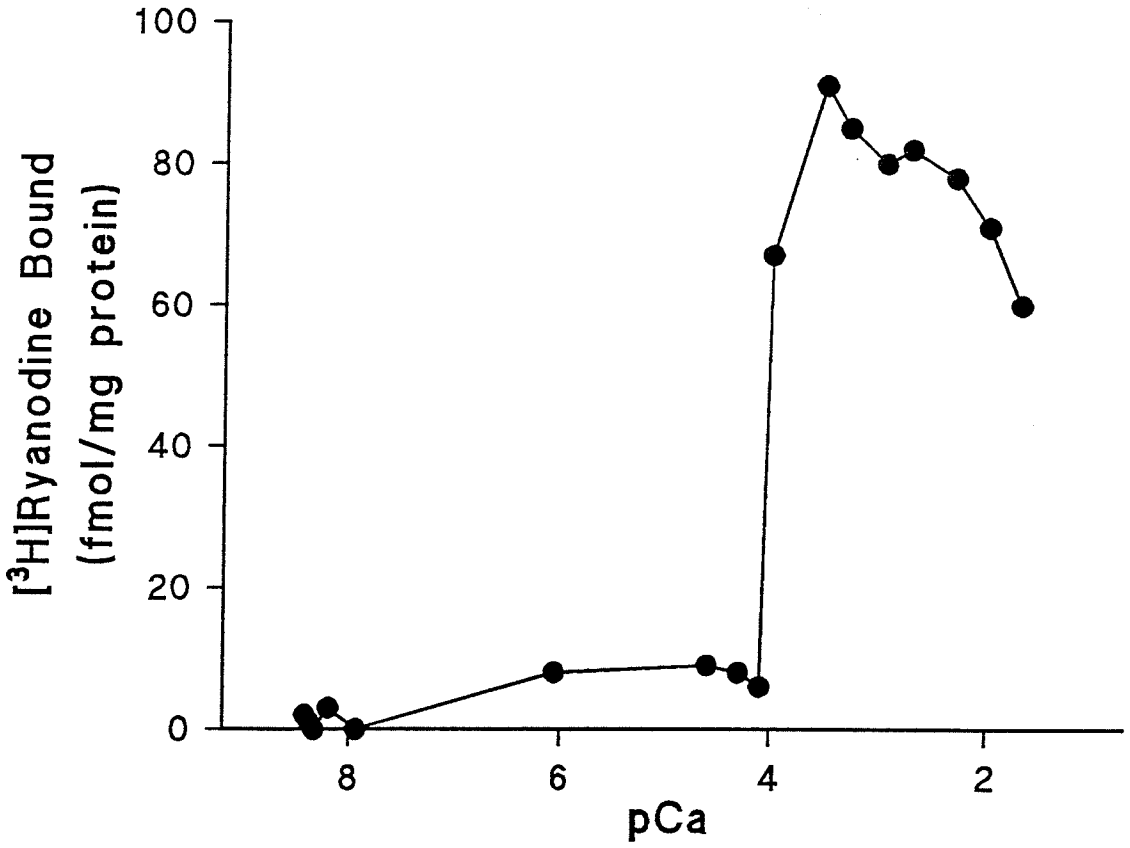


Figure 4

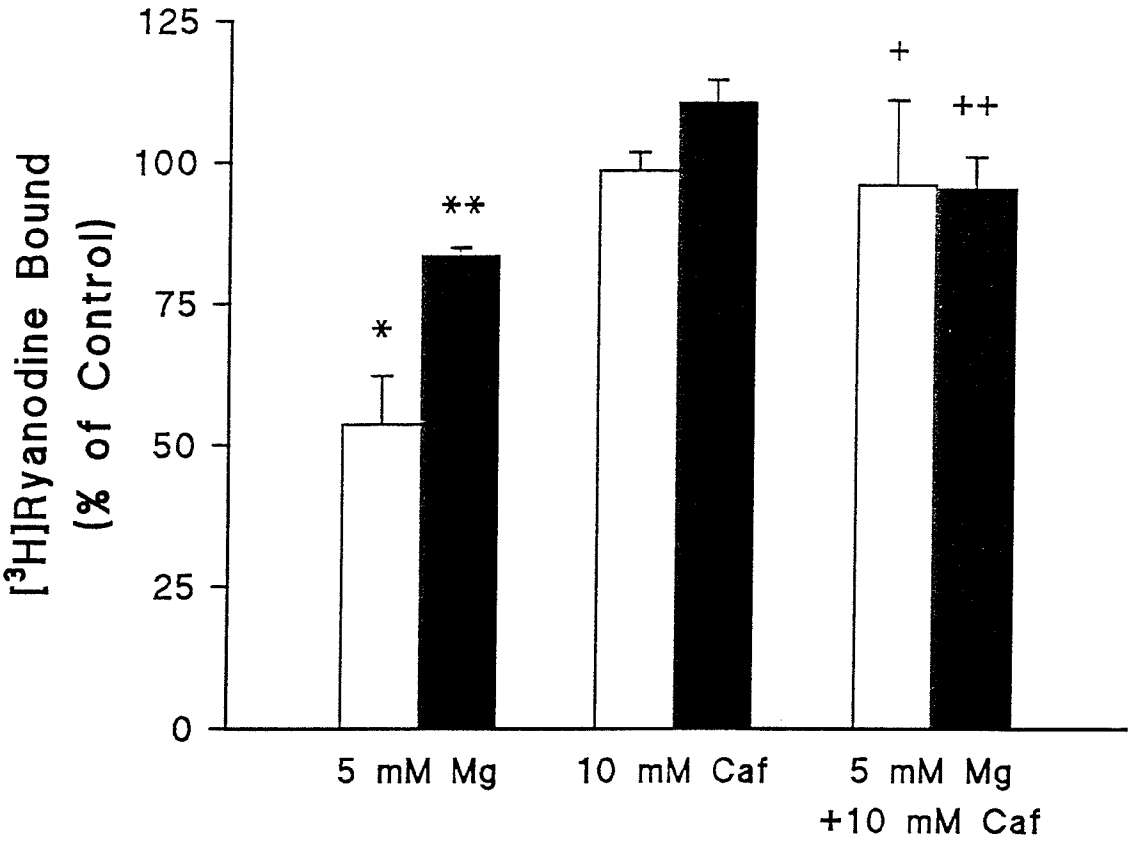

125
100
75
50
25
0
[3H]Ryanodine Bound
(% of Control)
*
**
+
++
5 mM Mg
10 mM Caf
5 mM Mg
+10 mM Caf

Figure 5

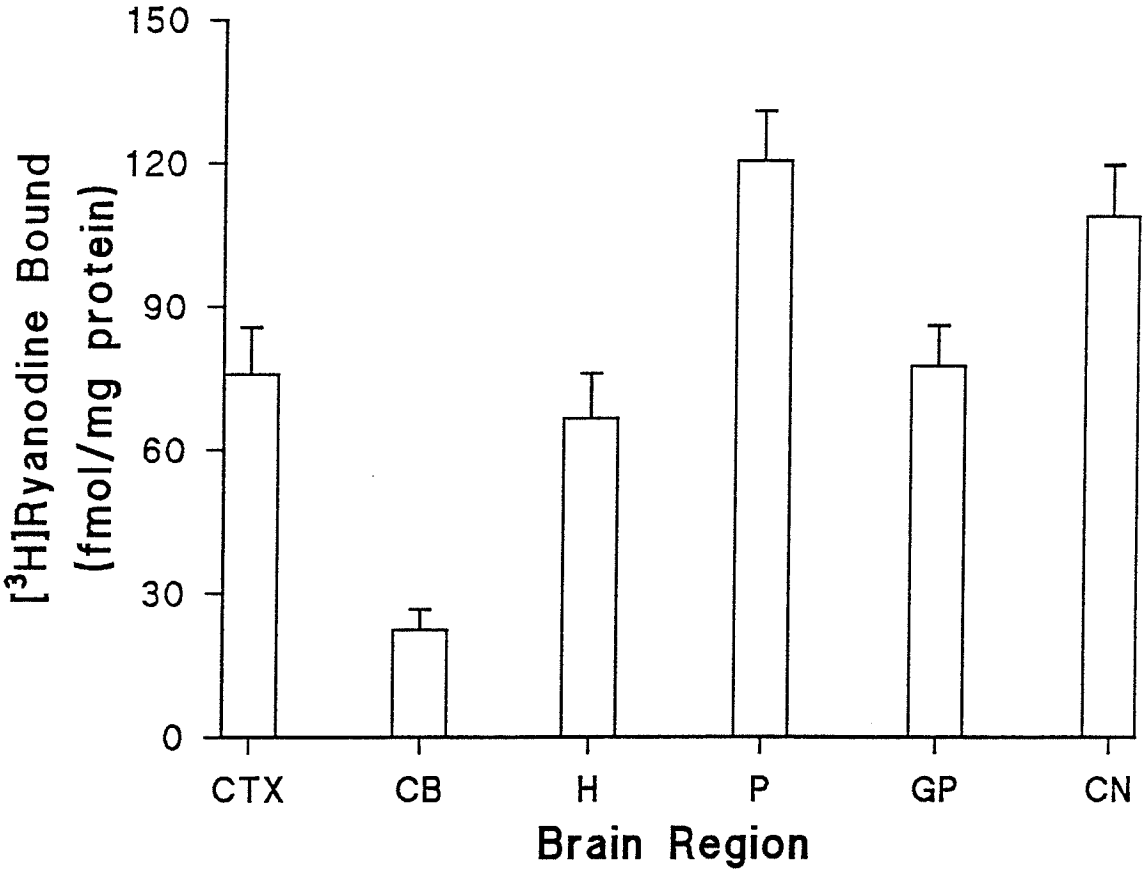
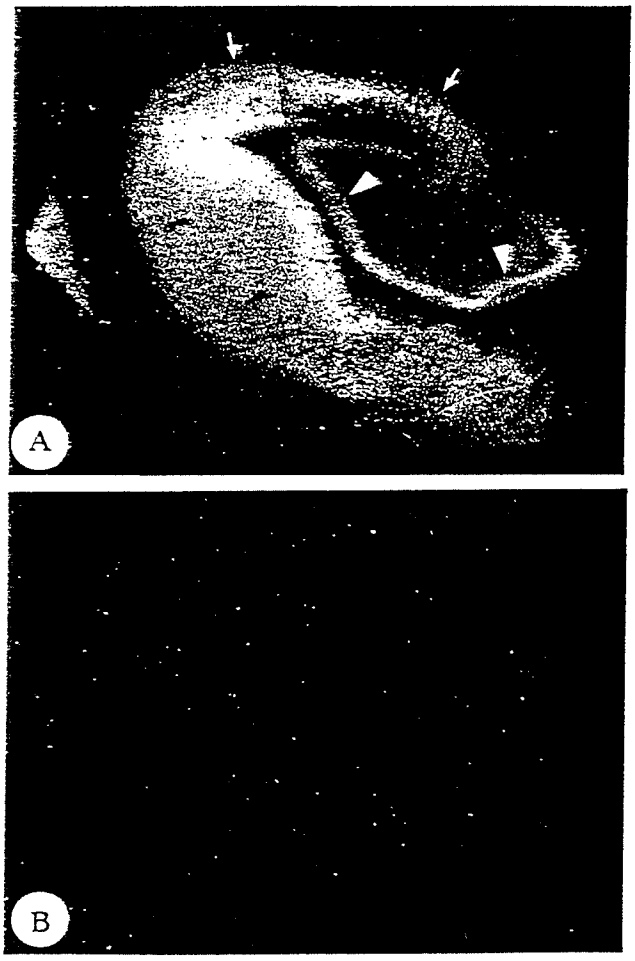

150
120
90
60
30
0
[3H]Ryanodine Bound
(fmol/mg protein)
CTX
CB
H
P
GP
CN
Brain Region

Figure 6



Part IV

Ionic strength-dependence of calcium, adenine nucleotide, magnesium and caffeine actions on ryanodine receptors in rat brain.

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Abstract--[^{3}H]Ryanodine binding studies of ryanodine receptors in brain membrane preparations typically require the presence of high salt concentrations in assay incubations in order to yield optimal levels of binding. Here, radioligand binding measurements on rat cerebral cortical tissues were conducted under high (1.0 M KCl) and low (200 mM KCl) salt buffer conditions to determine the effects of ionic strength on receptor binding properties as well as on modulation of ligand binding by Ca^{2+}, Mg^{2+}, β,γ-methyleneadenosine 5'-triphosphate (AMP-PCP) and caffeine. In 1.0 M KCl buffer, labeled titration/equilibrium analyses yielded two classes of binding sites with apparent K_d (nM) and B_{max} (fmol/mg protein) values of 2.4 and 34, respectively for the high affinity site, and 19.9 and 157, respectively for the low affinity site. Unlabeled titration/equilibrium measurements gave a single high affinity site with a K_d value of 1.9 nM and a B_{max} value of 95 fmol/mg protein. The apparent K_d value derived from association and dissociation studies was 20 pM. Equilibrium binding was activated by Ca^{2+} (K_d/Ca^{2+} value, 14 nM), inhibited by Mg^{2+} (IC_{50} value, 5.0 mM), and unaffected by AMP-PCP or caffeine. In 200 mM KCl buffer conditions, labeled titration analyses gave only a single site with a K_d value similar to and a B_{max} value 1.8-fold greater than those obtained for the low affinity site in 1.0 M KCl buffer. In unlabeled titration measurements, the K_d value was 5-fold lower while the B_{max} value was unaffected. The K_d value derived from association and dissociation analysis was 2.4-fold greater in 200 mM KCl compared with 1.0 M KCl buffer conditions. In 200 mM compared with 1.0 M KCl, the potency with which Mg^{2+} inhibited binding was increased by 3.8-fold, while the affinity of the activation site for Ca^{2+} was reduced by 13-fold. Addition of caffeine in the presence of low salt increased the affinity of Ca^{2+} activation by 1.7-fold. The inhibitory effect of Mg^{2+} on [^{3}H]ryanodine binding in the presence of 200 mM KCl was reversed by AMP-PCP and caffeine with apparent EC_{50} values of 0.25 mM and 7.6 mM, respectively. Taken together, these results indicate that ionic strength is an important

consideration in binding studies of brain ryanodine receptors and their interactions with modulatory agents.

In neurons, at least two different intracellular Ca^{2+} pools contribute to elevations of cytosolic Ca^{2+} levels that occur as a consequence of neuronal depolarization or the actions of various neurotransmitter substances (Miller, 1991; Henzi and MacDermott, 1992). It has been suggested that the pool acted upon by the Ca^{2+} liberating agents ryanodine and caffeine is involved in the process of calcium-induced calcium release and that this release is mediated by multimeric proteins referred to as ryanodine receptor/Ca^{2+} channel complexes (Henzi and MacDermott, 1992). The presence of ryanodine receptor-regulated intracellular calcium release channels in neural tissues has been demonstrated by a variety of methods (Ashley, 1989; Ellisman et al., 1990; McPherson and Campbell, 1990; Kawai et al., 1991; McPherson et al., 1991; Padua et al., 1991, 1992; Walton et al., 1991; Stein et al., 1992). That Ca^{2+} release via these receptors is physiologically relevant and contributes to Ca^{2+}-mediated actions in the central nervous system (CNS) is indicated by the ability of ryanodine to block caffeine-induced Ca^{2+} release from intracellular Ca^{2+} stores, to interfere with spike after-hyperpolarizations, to facilitate neurotransmitter release, and to decrease the rate of retrograde transport in axons (Thayer et al., 1988a, 1988b; Kawai and Watanabe, 1989, 1991; Nishimura et al., 1990; Brorson et al., 1991; Breuer et al., 1992; Marrion and Adams, 1992). Regulatory mechanisms governing the activity of ryanodine receptors appear to be similar in brain and muscle. Receptor phosphorylation as well as modulatory actions of Ca^{2+}, Mg^{2+}, adenine nucleotide and caffeine on ryanodine receptor binding and the influence of these agents on the release of Ca^{2+} from ryanodine-sensitive pools in muscle also appear to occur in brain (Meissner and Henderson, 1987; Pessah et al., 1987; Chu et al., 1990; McPherson et al., 1991; Takasago et al., 1991; Zimanyi and Pessah, 1991a; Witcher et al., 1991, 1992; Yoshida et al., 1992; Smith and Nahorski, 1993).

Typically, [^{3}H]ryanodine binding assays of brain membrane preparations are conducted in the presence of high salt concentration or high ionic strength buffer systems (Ashley et al., 1989; McPherson and Campbell, 1990; Meszaros and Volpe, 1991; Padua et al., 1991; 1992; Volpe et al., 1991; Zimanyi and Pessah, 1991a; Stein et al., 1992; Smith and Nahorski, 1993). This methodological approach for at least CNS tissues is a result of the general observation that inclusion of up to 1.0 M KCl or NaCl in assay buffers is required in order to obtain optimal levels of [^{3}H]ryanodine binding under conditions conducted at sub-saturating [^{3}H]ryanodine concentrations (Padua et al., 1991). The levels of ryanodine receptors detected under such high salt conditions were found to be at least five-fold greater than those obtained with lower salt concentrations (Kawai et al., 1991; unpublished observations). Although progress has been made regarding identification of factors that influence interactions between [^{3}H]ryanodine and its receptor sites under conditions of high salt, little is known about the relevance of such conditions to ryanodine receptor regulation and function at more physiologically relevant salt concentrations. Of particular importance is recent evidence which showed that adenine nucleotide, Mg^{2+} and caffeine modulated [^{3}H]ryanodine binding under physiological buffer conditions (Kawai et al., 1991). In order to investigate relationships between ionic strength and properties of ryanodine receptor in brain, we analyzed the characteristics of [^{3}H]ryanodine binding in preparations of rat brain membranes under high and low ionic strength buffer conditions and examined the effects of salt concentration on the modulation of binding by Ca^{2+}, Mg^{2+}, adenine nucleotide and caffeine.

METHODS

Tissue preparation. Adult male Sprague-Dawley rats were sacrificed by decapitation, brains were rapidly removed, and cortices were dissected on an ice-cold metal tray. In most experiments, cortical tissues were placed in 20 mM PIPES-KOH (pH

8.0), 100 μM $CaCl_2$ and 1.0 mM PMSF (solution A) containing either 1.0 M KCl (high ionic strength buffer) or 200 mM KCl (low ionic strength buffer) and disrupted at 4°C in a glass-teflon homogenizer (20 strokes). In some cases involving investigations of the actions of Mg^{2+}, tissues were homogenized in solution A containing 200 mM KCl plus 1.2 mM $MgCl_2$ (low ionic strength buffer plus Mg^{2+}). In studies of the effects of Ca^{2+} on ryanodine receptors at high and low ionic strength, tissues were homogenized as above except in a Ca^{2+}-free buffer containing 0.2 mM EGTA. Homogenates were centrifuged at 100,000 x g for 60 min at 4°C and pellets were either frozen at -20°C or taken for immediate assay; no differences in results were observed in studies of fresh compared with frozen tissue. Pellets were resuspended in their respective buffers to yield final membrane protein concentrations of approximately 5 mg/ml. In typical equilibrium binding measurements with buffer containing 3.0 nM [^{3}H]ryanodine, total and specific binding levels expressed as dpm/mg protein were, respectively, 28,210 and 14,607 in 1.0 M KCl buffer conditions, 13,359 and 3,828 in 200 mM KCl buffer conditions and 7,948 and 2,411 in 200 mM KCl plus 1.2 mM $MgCl_2$ buffer conditions. Protein was determined by the method of Lowry et al. (1951) with BSA as standard.

Analysis of [^{3}H]ryanodine binding under equilibrium assay conditions. All binding assays were conducted in duplicate at 37°C in a final assay volume of 1.0 ml as previously described (Padua et al., 1991; 1992). In most experiments, 100 μl of membrane suspension were aliquoted into tubes containing 3.0 nM [^{3}H]ryanodine (total binding) or 3.0 nM [^{3}H]ryanodine plus 50 μM unlabeled ryanodine (non-specific binding). Specific binding was defined as the difference between total and non-specific binding. Reactions were terminated by rapid filtration through GF/C glass fibre filters using a Brandel Cell Harvester M24R. Filters were washed 3 times with 5.0 ml of ice-cold wash buffer consisting of 20 mM PIPES-KOH (pH 8.0), 100 μM $CaCl_2$ and 200 mM KCl, placed in vials with 4.5 ml of Beckman Ready Solvent, and analyzed by liquid

scintillation spectroscopy. No differences in binding levels were obtained when filters were washed in the above buffer compared with those washed in the same buffer containing 1.0 M instead of 200 mM KCl. In labeled titration experiments, [^{3}H]ryanodine was added to reaction mixtures at concentrations ranging from 0.09 to 78 nM. In unlabeled titration experiments, all tissue homogenates were incubated with 3.0 nM [^{3}H]ryanodine, and unlabeled ryanodine was added at concentrations ranging from 0.1 pM to 40 μM. In tests of the effects of Ca^{2+} on [^{3}H]ryanodine binding both in the presence and absence of 1.2 mM Mg^{2+}, free Ca^{2+} was buffered at concentrations ranging from 1 nM to 4 mM with 0.2 mM EGTA. EGTA/Ca^{2+} and EGTA/Mg^{2+} dissociation constants were 3.7 nM and 0.49 mM, respectively. In studies of the modulatory actions of $MgCl_2$, ß,γ-methyleneadenosine 5'-triphosphate (AMP-PCP) and caffeine on maximal levels of [^{3}H]ryanodine binding, these agents were included in assays at concentrations ranging from 80 nM to 60 mM, 55 nM to 2 mM and 0.8 to 30 mM, respectively.

Association and dissociation kinetics. In association experiments, tissue homogenates were incubated with 3.0 nM [^{3}H]ryanodine and reactions were terminated at various intervals over a period of up to 2 hrs. In dissociation experiments, incubations were conducted with 3.0 nM [^{3}H]ryanodine for 1 hr and reactions were terminated at times ranging from 0 to 24 hrs following the addition of unlabeled ryanodine at a concentration of 50 μM.

Data analyses. Kinetic rate constants (K_{+1} and K_{-1}), dissociation constants (K_d), maximum number of receptor sites (B_{max}), and partial F tests for best fit of binding to either one receptor site or two receptor sites with differing affinities were derived from analyses of data by the non-linear multipurpose curve-fitting program LIGAND (Munson and Rodbard, 1980). The rate constant, K_1, was determined from the equation

$K_1 = (K_{obs} - K_{-1})/[L]$ where K_{obs} is the observed association rate constant, K_{-1} is the dissociation rate constant and [L] is the final concentration of [^{3}H]ryanodine in the assay. Data obtained from tests of Ca^{2+} effects on [^{3}H]ryanodine binding under each buffer condition were analyzed by Hill plots as previously described (Pessah et al., 1987) and statistical analyses using ANOVA and Bonferroni tests were conducted for data points obtained at each concentration of Ca^{2+}. The apparent affinity of the activator site for Ca^{2+} (K_d/Ca^{2+}) and the degree of cooperativity were determined from linear regression analyses of log (B/B_{ns}-B) versus free [Ca^{2+}] where B is the level of [^{3}H]ryanodine bound and B_{ns} is the maximum specific binding obtained in the presence of 3 nM [^{3}H]ryanodine under each buffer condition tested.

RESULTS

General considerations. [^{3}H]Ryanodine binding in total cellular membrane preparations of rat brain was measured under high ionic strength conditions (1.0 M KCl) previously found to produce optimal binding at sub-saturating concentrations of ryanodine (Padua et al., 1991) and results were compared with those obtained under what is referred to here, on a relative basis, as low ionic strength (200 mM KCl) conditions. Since our aim in these studies was to investigate the properties of ryanodine receptors and their interactions with regulatory agents at salt concentrations as close to physiological as possible, various concentrations of KCl were first tested. The ratio of total over specific binding obtained with KCl concentrations below 200 mM was too low to provide confidence in the data (data not shown). Although the levels of specific binding of [^{3}H]ryanodine to receptor sites in membrane preparations incubated in 200 mM KCl buffer were about 4-times lower than the levels obtained in high ionic strength buffer, signal to noise ratios as indicated in Methods were sufficiently high to allow reliable analysis of specific binding at each concentration of [^{3}H]ryanodine tested. Therefore, this 5-fold lower concentration of KCl was chosen as being most suitable for

investigations of ryanodine receptors at the lower limits of sub-optimal ionic strength. Since we have shown previously that enhancement of [^{3}H]ryanodine binding with high salt concentration occurs equally well with either NaCl or with KCl (Padua et al., 1991), only the effects of the latter were presently tested. Replacement of 1.0 M KCl with an equivalent osmolar concentration of sucrose gave the same level of suboptimal binding as that seen in the presence of 200 mM KCl (data not shown).

Kinetic measurements of receptor sites. Analyses of saturation data obtained from labeled titration/equilibrium binding studies conducted in 1.0 M KCl buffer conditions revealed the presence of high and low affinity binding sites with K_d values (nM) of 2.4 ± 1.2 and 19.9 ± 8.1, respectively, and B_{max} values (fmol/mg protein) of 34 ± 17 and 157 ± 30, respectively. In 200 mM KCl, a single site was found with a K_d value of 21.9 ± 1.9 nM which was not significantly different than that of the low affinity site seen under 1.0 M KCl buffer conditions. The B_{max} (fmol/mg protein) in 200 mM KCl buffer (286 ± 30) was 45% greater ($p<0.01$) than the density of low affinity sites in 1.0 M KCl.

Unlabeled titration/equilibrium binding experiments were conducted in order to determine first whether receptor sites with different affinities or affinity states for ryanodine can be detected in brain membrane preparations, and second whether ionic strength differentially affects the ability of [^{3}H]ryanodine to bind to these sites. As shown in Figure 2, single high affinity binding sites were observed under each of the ionic strength buffer conditions. In 1.0 M KCl buffer, the K_d value for this site was 5-fold lower than that obtained in 200 mM KCl buffer (Table 1). No significant differences were found between B_{max} values at the two ionic strengths (Table 1).

The association of [^{3}H]ryanodine to receptor sites observed under conditions of high and low ionic strength displayed a monophasic profile and the data were best fit by a

single exponential equation that yielded observed association rate constants (K_{obs}) of 0.116 ± 0.011 min^{-1} in 1.0 M KCl and 0.052 ± 0.004 min^{-1} in 200 mM KCl buffer (Fig. 3). Maximal levels of binding were obtained within 60 min (Fig. 3). Similarly, the dissociation of [^{3}H]ryanodine from receptor sites in the presence of excess unlabeled ryanodine was monophasic; a tendency toward a biphasic dissociation was observed, but the data were best fit by a single exponential equation. The calculated dissociation rate constants (K_{-1}) were 5 ± 1 x 10^{-4} min^{-1} and 6 ± 1 x 10^{-4} min^{-1} in high and low ionic strength conditions, respectively. The rate constants (K_1) were 2.6 ± 0.3 x 10^7 M^{-1} min^{-1} in 1.0 M KCl and 1.2 ± 0.1 x 10^7 M^{-1} min^{-1} in 200 mM KCl buffer. The calculated K_d value ($K_d = K_{-1}/K_{+1}$) of 20 ± 5 pM under high ionic strength conditions was significantly lower ($p<0.05$) than that obtain under low ionic strength (K_d value, 48 ± 7 pM).

Modification of receptor binding by Mg^{2+}, AMP-PCP and caffeine. Regulatory interactions between ryanodine receptors and the agents caffeine, AMP-PCP and Mg^{2+}, which are known to occur at ryanodine receptor/calcium channel complexes, were tested under high and low ionic strength buffer conditions. In assays conducted at a single sub-saturating concentration of [^{3}H]ryanodine (3 nM), binding of this ligand was dose-dependently inhibited by Mg^{2+} and the inhibition curve was shifted to the left in the presence of low compared with high KCl buffer. The IC_{50} values (mM) for Mg^{2+} were 1.3 ± 0.1 and 5.0 ± 0.4 at the low and high salt concentrations, respectively (Fig. 4). In the 1.0 M KCl buffer system, neither 20 mM caffeine nor 1.0 mM AMP-PCP significantly affected levels of [^{3}H]ryanodine binding (Fig. 5A). In 200 mM KCl buffer, the levels of [^{3}H]ryanodine binding were significantly increased by 55% in the presence of caffeine and by 40% in the presence of AMP-PCP (Fig. 5B). In the same buffer, the inclusion of 1.2 mM $MgCl_2$ reduced ryanodine binding by 68% compared with that seen in the absence of magnesium ion (Fig. 5B and C). This reduction by Mg^{2+} was largely

reversed by the addition of either caffeine or AMP-PCP both of which restored levels of binding nearly to those seen with these agents in the absence of Mg^{2+}. Moreover, while the stimulatory effects of caffeine and AMP-PCP when tested individually were not significantly different than their combined effects in the absence of Mg^{2+} (Fig. 5B), their actions together appeared to be greater in the presence of Mg^{2+} (Fig. 5C) although this did not reach statistical significance. Since the inhibitory effect of Mg^{2+} on binding was seen at relatively greater, non-physiological concentrations (5 mM) in the presence of 1.0 M KCl, tests of interactions between Mg^{2+} and other agents at the higher ionic strength were not conducted.

Kinetic parameters of the effects of Mg^{2+} and caffeine on [^{3}H]ryanodine binding and the potencies with which caffeine and AMP-PCP alter binding in the presence of Mg^{2+} were determined. Table 2 shows the effects of Mg^{2+} and caffeine alone or in combination on [^{3}H]ryanodine binding in 200 mM KCl buffer conditions. Addition of Mg^{2+} under these conditions had no significant effect on B_{max} values but significantly increased K_d values by slightly more than 2-fold. Thus, the above results showing a Mg^{2+}-induced reduction of binding at a single sub-saturating concentration of [^{3}H]ryanodine appears to be due to the effect of this ion on affinity rather than receptor density. Similarly, addition of 20 mM caffeine under Mg^{2+} and Mg^{2+}-free binding conditions increased the apparent affinity of the receptor for [^{3}H]ryanodine by about 3-fold indicating that the caffeine-induced elevation of binding in the presence of Mg^{2+} (Fig. 5B and C) at non-saturating conditions (3 nM [^{3}H]ryanodine) was due to changes in the affinity of receptor/ligand interactions. In 200 mM KCl buffer containing Mg^{2+}, the apparent EC_{50} values for AMP-PCP and caffeine enhancement of [^{3}H]ryanodine binding were 0.25 ± 0.06 mM and 7.6 ± 1.9 mM, respectively (Fig. 6). Because the enhancing effects of these agents at low ionic strength conditions were relatively smaller in the absence of Mg^{2+}, their potencies were not determined under these conditions.

Influence of Ca^{2+} on binding at high and low ionic strength. The activation of [^{3}H]ryanodine binding by calcium under high and low ionic strength conditions is illustrated in Figure 7. The levels of [^{3}H]ryanodine binding were increased by free Ca^{2+} at low nanomolar concentrations and inhibited by millimolar concentrations (Fig. 7A). The apparent affinity of the activator site for Ca^{2+} in 1.0 M KCl (K_d/Ca^{2+} value, 14 ± 1 nM) was 2.1-fold greater than that obtained in 200 mM KCl buffer conditions (K_d/Ca^{2+} value, 29 ± 4 nM). The increase in apparent affinity for Ca^{2+} under high ionic strength is accompanied by an enhancement in cooperativity of Ca^{2+} activation of [^{3}H]ryanodine binding (n_H from 1.09 ± 0.14 to 1.86 ± 0.03) (Fig. 7B). Under low salt conditions, the effects of caffeine and Mg^{2+} on Ca^{2+} activation were also determined. Addition of 20 mM caffeine increased the apparent affinity for Ca^{2+} (K_d/Ca^{2+} value, 17 ± 1 nM) by 1.7-fold with a concomitant increase in the cooperativity of Ca^{2+} activation (n_H from 1.09 ± 0.14 to 1.40 ± 0.06). In contrast, addition of 1.2 mM Mg^{2+} significantly decreased the apparent affinity for Ca^{2+} and cooperativity of Ca^{2+} activation such that K_d/Ca^{2+} and n_H values were 180 ± 30 nM and 0.80 ± 0.05, respectively. Interactions between Ca^{2+} and caffeine in 1.0 M KCl buffer were not investigated since caffeine did not alter [^{3}H]ryanodine binding in this buffer system.

DISCUSSION

The release of calcium sequestered within ryanodine-sensitive intracellular compartments in a variety of tissues is subject to complex control mechanisms. For example, there are numerous reports indicating that ryanodine receptors in muscle are regulated by Ca^{2+}, Mg^{2+} and adenine nucleotides. Such regulation of brain ryanodine receptors has also been observed but has not been as thoroughly studied. It is thought that each of these agents act at particular regulatory domains on the receptor. In addition, it has been suggested that the calcium-liberating action of caffeine occurs through its interaction with such receptor domains. With the exception of caffeine, a modulatory

role of these other agents has been inferred largely from characterization of mechanisms whereby they interfere with or enhance the binding of radiolabeled ryanodine to its receptor site(s) in tissue membrane preparations (Pessah et al., 1987; Kawai et al., 1991; McPherson et al., 1991; Meszaros and Volpe, 1991; Padua et al., 1991; Zimanyi and Pessah, 1991a, 1991b). However, by virtue of the relatively low levels of ryanodine receptors in CNS tissues, nearly all studies that have been conducted with brain membrane preparations have required the use of high salt (1.0 M KCl) assay buffers which have been found to yield optimal ryanodine binding and consequently manageable signal to noise ratios. With the routine use of such high ionic strength buffers, the degree to which results generated are reflective of functional events *in vivo* remain uncertain. This has not been a problem in muscle where very high levels of receptors allow receptor analysis under suboptimal, more physiologically relevant salt concentrations. The present results indicate that ryanodine binding properties of brain ryanodine receptors and the influence on these properties by the above agents are, in fact, dependent on the ionic strength conditions under which binding assays are conducted.

Our saturation binding studies of brain tissue (Padua et al., 1991, 1992; Stein et al., 1992;) and those of others (Ashley, 1989; McPherson and Campbell, 1990; McPherson et al., 1991; Walton et al., 1991; Zimanyi and Pessah, 1991a; Smith and Nahorski, 1993), but with one exception (Kawai et al., 1991), have involved titrations of [^{3}H]ryanodine and were conducted in the presence of 1.0 M KCl buffer. The results of these have demonstrated the presence of a single high affinity binding site in brain membranes. Here, we showed that similar assays conducted in the presence of high and low KCl concentrations yields high and low affinity sites at 1.0 M KCl and a single low affinity site at 200 mM KCl, the apparent affinity of which is similar to that of the high affinity site reported by Kawai et al. (1991) under their low salt conditions. The failure to detect even lower affinity sites as was seen by Kawai et al. (1991) may be due to the smaller

concentration range of labeled ryanodine used in our assays. Furthermore, the lowest concentration of labeled ryanodine used by Kawai et al. (1991) was 10 nM which would exclude detection of high affinity sites reported here under high salt conditions. Examination of a wider range of ligand concentrations allowed by titrations of unlabeled ryanodine, revealed the presence of high affinity binding sites with K_d values 5-fold lower (greater binding affinity) in 1.0 M than in 200 mM KCl buffer. Lower affinity sites as detected in labeled titration assays were not seen possibly due to the high level of non-specific binding at higher concentrations of unlabeled ryanodine. The functional significance of different affinity sites or states of ryanodine receptors is at present unclear, but it is noteworthy that several different molecular forms of ryanodine receptors have been indentified in brain and other tissues (Otsu et al., 1990; Giannini et al., 1992; Hakamata et al., 1992). In any case, the presence of a high affinity site in high salt may explain the higher levels of receptors detected in assays conducted with sub-saturating concentrations of ryanodine. In view of this, it might be of value to conduct analyses of changes in receptor affinity after various treatments at both high and low salt concentrations. On the other hand, the lower B_{max} values observed with 1.0 M KCl indicates that high salt is less than optimal with respect to measurements of apparent maximal numbers of receptors.

The K_d of [^{3}H]ryanodine binding calculated from association/dissociation analyses in 1.0 M salt conditions were found to be roughly three orders of magnitude lower (20 pM) than that derived from labeled or unlabeled titrations under equilibrium binding conditions. Similar observations were previously reported in preparations of both muscle and brain by Pessah and colleagues (McGrew et al., 1989; Pessah and Zimanyi, 1991; Zimanyi and Pessah, 1991a) who suggested that this very high apparent affinity may originate from cooperative interactions between added unlabeled ryanodine and bound [^{3}H]ryanodine where, under non-equilibrium conditions, the former decreases the

dissociation of the latter from high affinity binding sites. In the presence of 200 mM salt conditions, the association/dissociation kinetics yielded an apparent K_d of 48 pM. While this lower apparent affinity is not necessarily a predictable outcome of the reduced K_d values seen in unlabeled titration/equilibrium assays conducted under low salt conditions, the slower association rate under these conditions would be consistent with a proportional reduction in affinity. According to the above interpretation offered by Pessah and Zimanyi (1991), our results would suggest that cooperative interactions between bound [^{3}H]ryanodine and added unlabeled ryanodine still occur, but to a lesser extent under reduced salt conditions. Whether such cooperativity reflects functional aspects of interactions between ryanodine receptor subunits remains to be determined.

In membrane preparations of skeletal muscle, calcium stimulates [^{3}H]ryanodine binding at low micromolar concentrations and inhibits binding at high millimolar concentrations (Michalak et al., 1988; Chu et al., 1990). It was previously shown in these preparations that high ionic strength increases the inhibitory potency of Ca^{2+} while having no effect on Ca^{2+} stimulation of binding (Seifert and Casida, 1986; Chu et al., 1990; Ogawa and Harafuji, 1990). Although we also found a biphasic effect of Ca^{2+} on [^{3}H]ryanodine binding in brain membranes, unlike the effects of high ionic strength in muscle, we observed an increased apparent affinity of the activator site for Ca^{2+} with high salt. This augmented affinity for Ca^{2+} may be related to the 3-fold reduced potency with which Mg^{2+} inhibits [^{3}H]ryanodine binding in brain membranes under high compared with low salt conditions. The inhibitory action of Mg^{2+}, for example, has been previously suggested to be due to competitive interactions between this ion and Ca^{2+} at the Ca^{2+} activator site on the ryanodine receptor (Pessah et al., 1987). Our results which show a decrease in the apparent affinity of the activator site for Ca^{2+} in low salt is consistent with this suggestion. Thus, the increased potency of Mg^{2+} inhibition of binding at low salt conditions may allow greater competitive effectiveness of Mg^{2+} at

the Ca^{2+} activator site and give rise to the presently observed ability of Mg^{2+} to significantly reduce the affinity of [^{3}H]ryanodine binding under these conditions. Conversely, the lower potency with which Mg^{2+} inhibits [^{3}H]ryanodine binding at high ionic strength would be consistent with its reduced ability to compete with Ca^{2+} when the receptor is more sensitized to Ca^{2+}under high salt conditions. It should be noted that the K_d/Ca^{2+} values for Ca^{2+} enhancement of [^{3}H]ryanodine binding in brain membranes under high salt conditions found here are substantially lower than that previously reported for brain under high ionic strength conditions (Zimanyi and Pessah, 1991a). In view of the forgoing points and based on interpretations offered by Pessah et al. (1987), this apparent discrepancy may result from assays conducted in the presence of Mg^{2+} (Zimanyi and Pessah, 1991a) which may have competitively decreased the sensitivity of Ca^{2+} activation of the receptor thereby shifting the Ca^{2+} dependency curve toward the right. Of relevance to the action of high salt may be a recent finding that polyanions, possibly through electrostatic interactions, increased the sensitivity of ryanodine receptors to low nanomolar concentrations of Ca^{2+} (Bezprozvanny et al., 1993).

It was found here that two known regulators of ryanodine receptors, namely AMP-PCP and caffeine, increased the levels of [^{3}H]ryanodine binding to brain membranes under low but not high salt buffer conditions. Others have also found that caffeine failed to enhance binding in optimal assay buffer (Smith and Nahorski, 1993) while caffeine and AMP-PCP stimulated binding in sub-optimal buffer or lower KCl buffer conditions (Kawai et al., 1991; McPherson et al., 1991; Smith and Nahorski, 1993). In contrast, caffeine and AMP-PCP in muscle enhanced binding at both high and low ionic strength (Ogawa and Harafuji, 1990). This may have some bearing on the mechanism whereby high salt promotes [^{3}H]ryanodine binding to receptors in brain membranes. In membrane preparations of muscle, it has been suggested by Chu et al. (1990) that salt directly

interacts with the Ca^{2+} binding site of ryanodine receptors. As both caffeine and AMP-PCP are known to affect calcium release and the calcium-sensitivity of ryanodine receptors, the absence of their modulatory actions on [^{3}H]ryanodine binding under high ionic strength conditions in membrane preparations of brain may be due, as discussed above, to an already maximized sensitivity of the receptor to Ca^{2+} in high salt. This possibility is consistent with the proposal that caffeine acts to increase Ca^{2+} sensitivity of the ryanodine receptor complex (Pessah et al., 1987) and our observations that at low ionic strength caffeine increases the affinity of the activator site for Ca^{2+} to a level approaching that seen at high ionic strength in the absence of caffeine.

In summary, it was previously suggested that high ionic strength or salt concentration promotes ryanodine binding in muscle membrane preparations by increasing receptor stability, solubilization or polymerization (Seifert and Casida, 1986; Ogawa and Harafuji, 1990), by removing putative endogenous ligand from the receptor (Chu et al., 1990) or by allowing non-specific ryanodine/receptor electrostatic interactions (Zarka and Shoshan-Barmatz, 1992). The effects of salt concentrations on ryanodine receptors may be physiologically relevant in view of findings that increased ionic strength enhances Ca^{2+} release from muscle sarcoplasmic reticulum (SR) as well as caffeine sensitivity of SR Ca^{2+} release channels (Su and Hasselbach, 1984; Meissner and El-Hashem, 1992). In neurons, ryanodine receptors may be influenced by changes in intracellular ionic milieu during, for example, periods of elevated activity and this may contribute to regulation of Ca^{2+} mobilization via ryanodine receptors.

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FIGURE LEGENDS

Fig. 1. Labeled titration curves of [^{3}H]ryanodine binding in membrane preparations of rat cerebral cortex. Membranes were incubated with varying concentrations of [^{3}H]ryanodine in buffer containing 1.0 M KCl (closed circles) or 200 mM KCl (open circles). Scatchard plots (inset) show binding of [^{3}H]ryanodine to high and low affinity binding sites in 1.0 M KCl and to a single low affinity site in 200 mM KCl with K_d and B_{max} values indicated in Table 1. Data shown are representative of five experiments performed in duplicate.

Fig. 2. Unlabeled titration curves of [^{3}H]ryanodine binding in cortical membrane preparations. Membranes were incubated in 1.0 M KCl (closed circles) or 200 mM KCl (open circles) buffer containing 3 nM [^{3}H]ryanodine and various concentrations of unlabeled ryanodine. Scatchard analyses (not shown) revealed the presence of single high affinity binding sites with K_d and B_{max} values indicated in Table 1. For clarity, concentrations of unlabeled ryanodine below 10^{-11} M were omitted from the Figure. Data shown represent means ± S.E.M. of five experiments performed in duplicate.

Fig. 3. Association and dissociation kinetics of [^{3}H]ryanodine binding in membrane preparations of rat cerebral cortex in 1.0 M KCl (closed circles) and 200 mM (open circles) KCl buffers. In association experiments, tissues were incubated with 3 nM [^{3}H]ryanodine and reactions were terminated at the times indicated. In dissociation experiments, membranes were incubated for 1 hr with 3 nM [^{3}H]ryanodine and then further incubated for the durations indicated following the addition (see illustrated arrows) of 50 μM unlabeled ryanodine. The K_d values as calculated from the association and dissociation rate constants (K_{+1} and K_{-1}) were 20 pM in 1.0 M KCl and 48 pM in 200 mM KCl buffer. Data shown are representative of five experiments performed in duplicate.

Fig. 4. Effects of Mg^{2+} on [^{3}H]ryanodine binding in membrane preparations of rat cerebral cortex. Membranes were incubated with 3 nM [^{3}H]ryanodine in either 1.0 M KCl buffer (closed circles) or 200 mM KCl buffer (open circles). IC_{50} values were 5.0 mM and 1.3 mM in high and low salt conditions, respectively. Data shown represent means ± S.E.M. of five experiments performed in duplicate.

Fig. 5. Effects of caffeine (Caf, 20 mM) and ß,γ-methlyeneadenosine 5'-triphosphate (AMP-PCP, 1 mM) on [^{3}H]ryanodine binding in membrane preparations of rat cerebral cortex. Membranes were incubated with 3 nM [^{3}H]ryanodine in (A) 1.0 M KCl buffer, (B) 200 mM KCl buffer, or (C) 200 mM KCl buffer plus 1.2 mM $MgCl_2$. Data represent means ± S.E.M. of five experiments performed in duplicate. **, $p<0.01$, treatment vs. control; *, $p<0.05$, treatment vs. control; ***, $p<0.01$, 200 mM KCl buffer vs. 200 mM KCl buffer plus 1.2 mM $MgCl_2$.

Fig. 6. Effects of AMP-PCP (open circles) and caffeine (closed circles) on [^{3}H]ryanodine binding in membrane preparations of rat cerebral cortex. Membranes were incubated with 3 nM [^{3}H]ryanodine in 200 mM KCl buffer containing 1.2 mM $MgCl_2$. Apparent EC_{50} values were 0.25 mM for AMP-PCP and 7.6 mM for caffeine. Data represent means ± S.E.M. of four experiments performed in duplicate.

Fig. 7. Effects of ionic strength, caffeine and Mg^{2+} on Ca^{2+} activation of [^{3}H]ryanodine binding in membrane preparations of rat cerebral cortex. Membranes were incubated with 3 nM [^{3}H]ryanodine in 1.0 M KCl buffer (open circles) or 200 mM KCl buffer (closed circles). Other additions in the presence of 200 mM KCl included 20 mM caffeine (closed triangles) or 1.2 mM Mg^{2+} (closed squares). (A) Plot of [^{3}H]ryanodine binding activation by Ca^{2+} under various conditions. (B) Determination of the apparent affinity of the activator site for Ca^{2+} (K_d/Ca^{2+}) and the degree of cooperativity (n_H) for

activation of [^{3}H]ryanodine binding by Ca^{2+} under various conditions. K_d/Ca^{2+} and n_H values were determined directly from linear regression analyses of log (B/B_{ns}-B) plotted against free [Ca^{2+}]. Data shown represent means ± S.E.M. of five experiments performed in duplicate.

Table 1. Effects of KCl on [^{3}H]ryanodine binding affinity (K_d) and density (B_{max}) as demonstrated by saturation analyses with labeled and unlabeled ligand titrations in membranes of rat cerebral cortex.

Assay	[KCl]	K_d (nM)	B_{max} (fmol/mg protein)
Labeled	200 mM	21.9 ± 1.9	286 ± 30**
	1.0 M (H)	2.4 ± 1.2	34 ± 17
	(L)	19.9 ± 8.1	157 ± 30
Unlabeled	200 mM	9.5 ± 2.4**	69 ± 12
	1.0 M	1.9 ± 0.4	95 ± 11

Membranes were suspended either in buffer containing 20 mM PIPES-KOH (pH 8.0), 100 μM $CaCl_2$, 1.0 mM PMSF and 200 mM KCl or in the same buffer except with 1.0 M KCl. K_d and B_{max} values were obtained from analyses of unlabeled titration/equilibrium binding data using the multipurpose curve-fitting program, LIGAND (Munson and Rodbard, 1980). Values represent means ± S.E.M. of 4 or more experiments performed in duplicate. **$p<0.01$, 200 mM vs 1.0 M (L); 200 mM vs. 1.0 M in unlabeled titrations. H, high affinity site; L, low affinity site.

Table 2. Effects of Mg^{2+} and caffeine on [^{3}H]ryanodine binding affinity (K_d) and density (B_{max}) measured in membranes of rat cerebral cortex in the presence of 200 mM KCl buffer system.

Additions	K_d (nM)	B_{max} (fmol/mg protein)
Control	21.9 ± 1.9	286 ± 30
Caffeine	8.7 ± 1.5**	272 ± 30
Mg^{2+} (1.2 mM)	48.9 ± 6.4*	245 ± 28
Mg^{2+} (1.2 mM) + Caffeine (20 mM)	12.1 ± 1.0**	203 ± 15

Membranes were suspended either in low ionic strength buffer containing 20 mM PIPES-KOH (pH 8.0), 100 μM $CaCl_2$, 1.0 mM PMSF and 200 mM KCl (control) or in the same buffer plus 1.2 mM Mg^{2+}. K_d and B_{max} values were obtained from analyses of binding isotherms using the multipurpose curve-fitting program, LIGAND (Munson and Rodbard, 1980). Values represent means ± S.E.M. of 4 or more experiments performed in duplicate. **$p<0.01$, control (no added Mg^{2+}) vs. caffeine, and Mg^{2+} vs. Mg^{2+} plus caffeine; *$p<0.05$, control vs. Mg^{2+}.

Figure 1

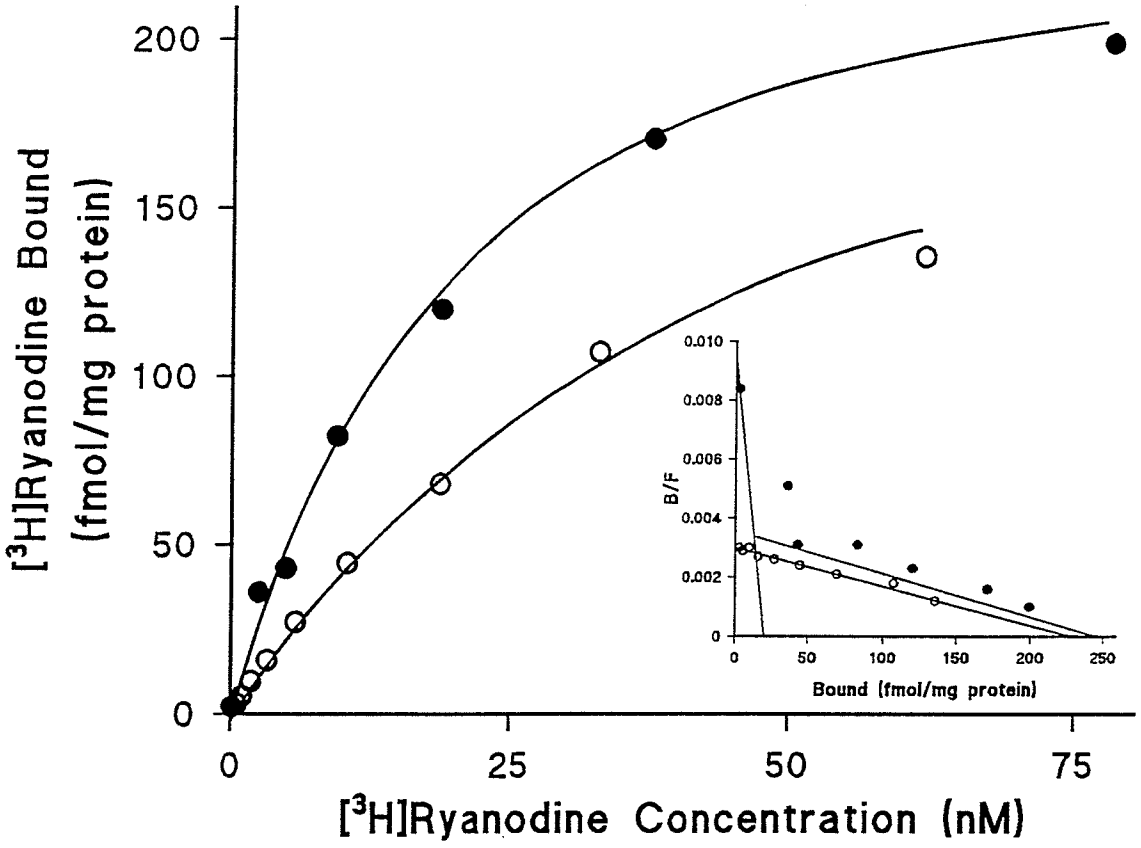

[3H]Ryanodine Bound
(fmol/mg protein)
200
150
100
50
0
0
25
50
75
[3H]Ryanodine Concentration (nM)
B/F
0.010
0.008
0.006
0.004
0.002
0
0
50
100
150
200
250
Bound (fmol/mg protein)

Figure 2

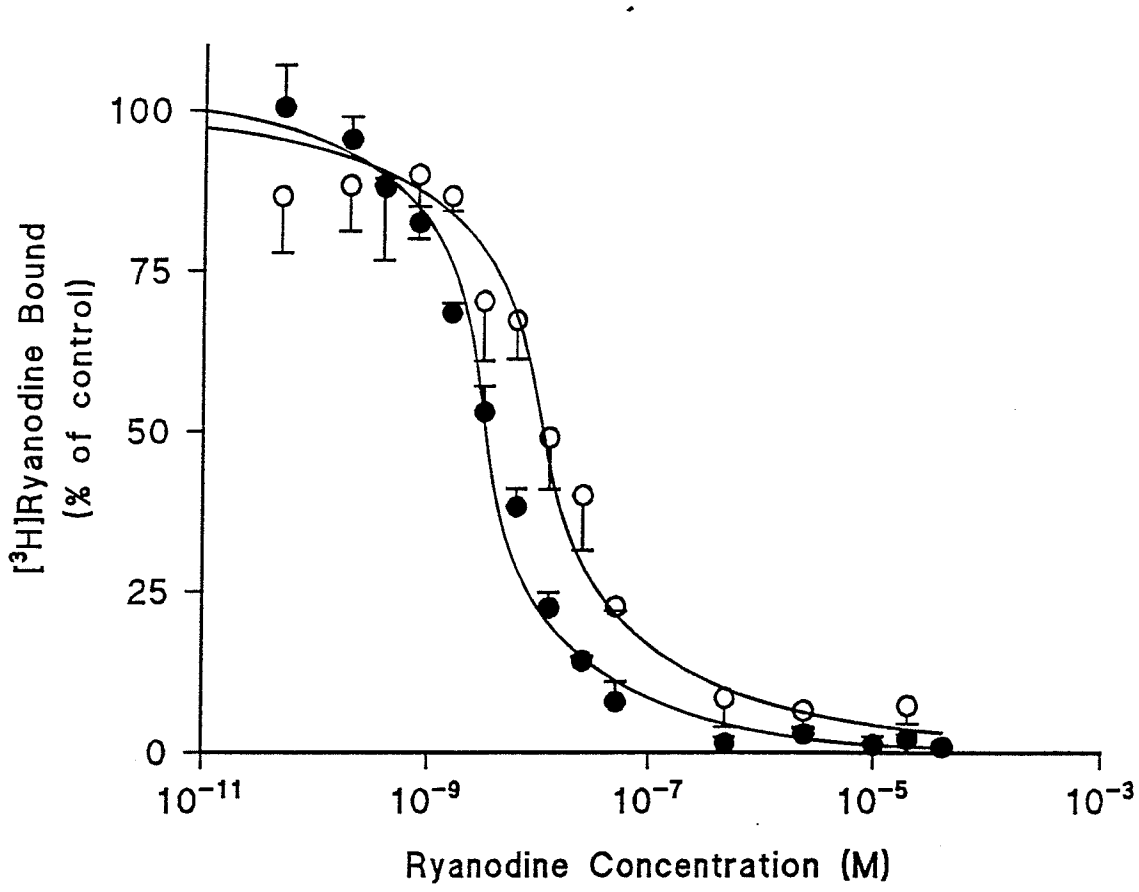

100
75
50
25
0
[3H]Ryanodine Bound
(% of control)
10-11
10-9
10-7
10-5
10-3
Ryanodine Concentration (M)

Figure 3

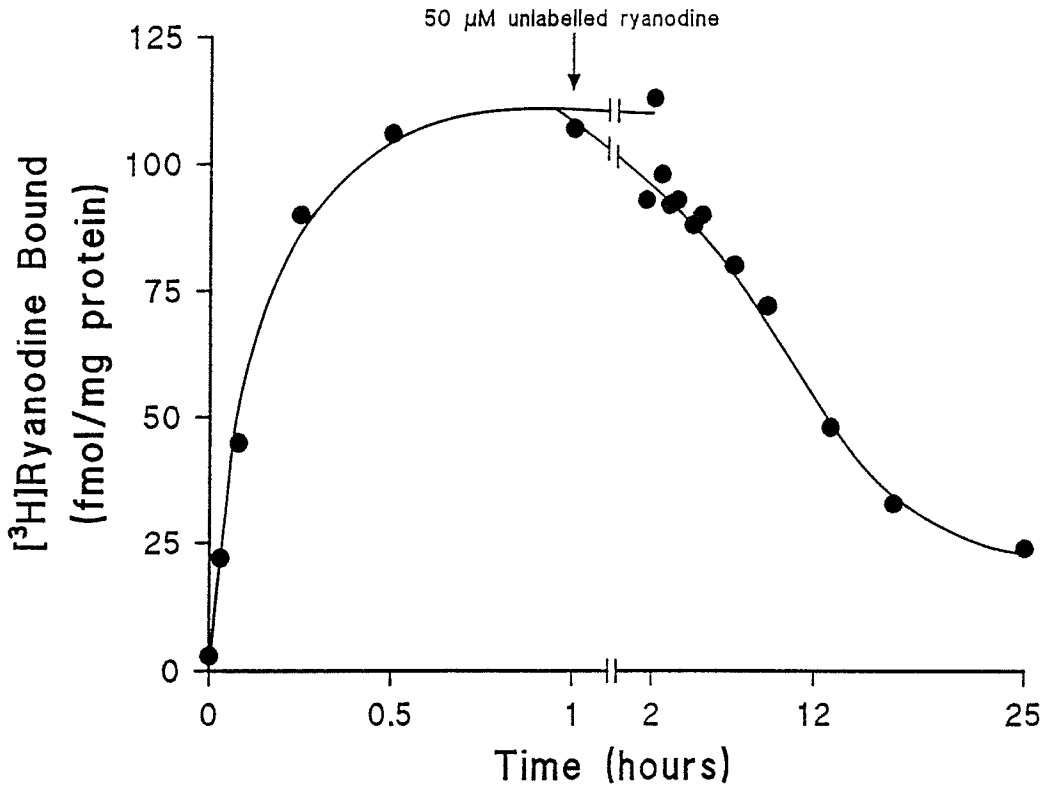
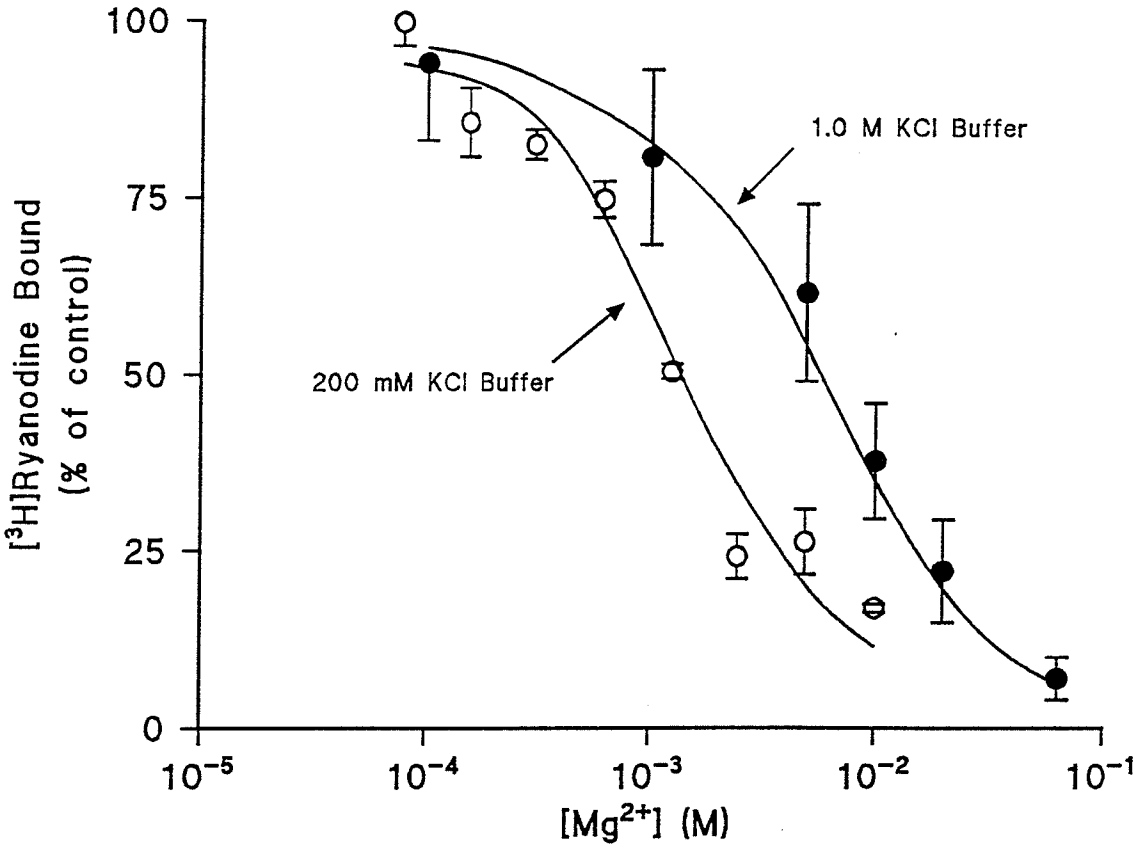

50 μM unlabelled ryanodine
125
100
75
50
25
0
[3H]Ryanodine Bound
(fmol/mg protein)
0
0.5
1
2
12
25
Time (hours)

Figure 4


100
75
50
25
0
[3H]Ryanodine Bound
(% of control)
1.0 M KCl Buffer
200 mM KCl Buffer
10-5
10-4
10-3
10-2
10-1
[Mg2+] (M)

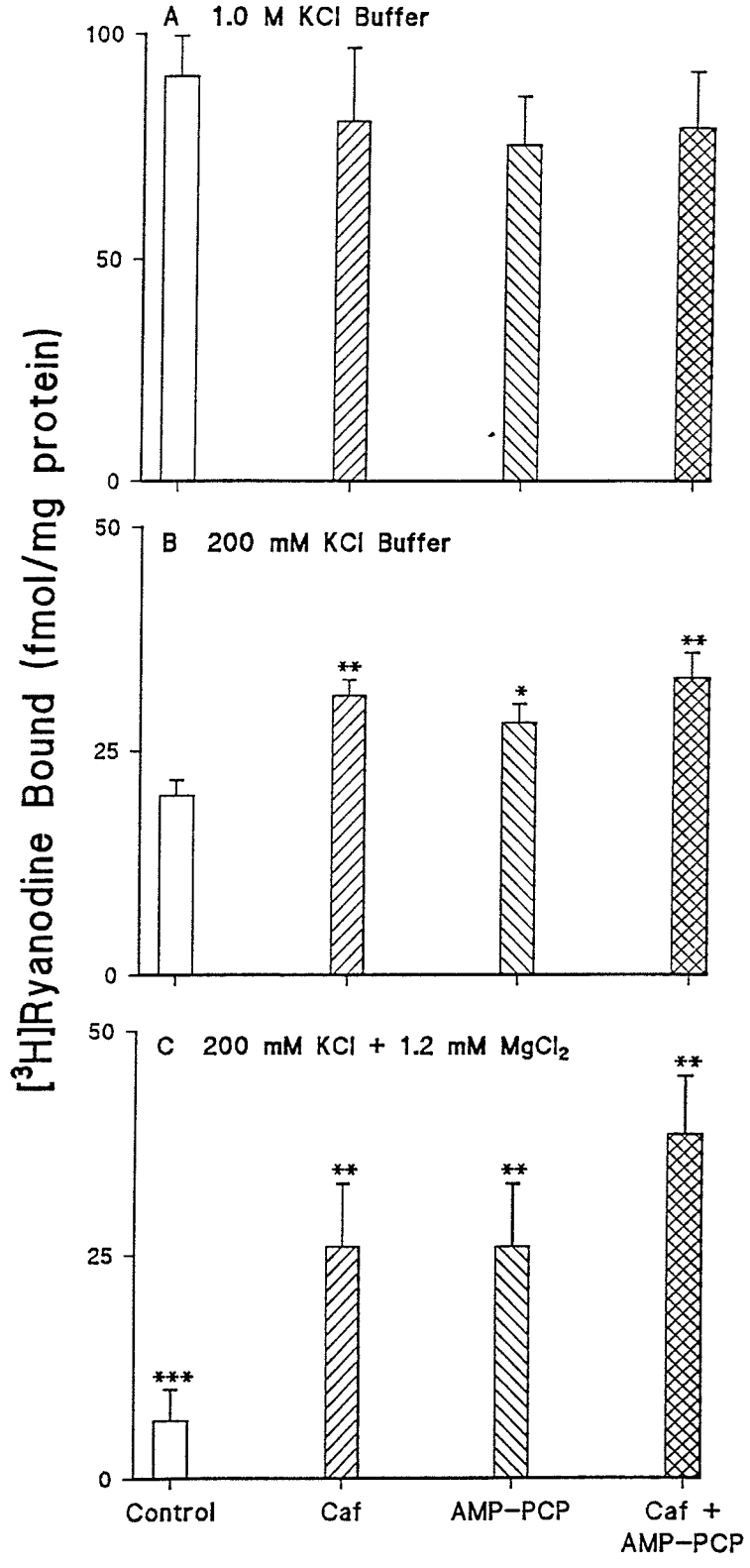
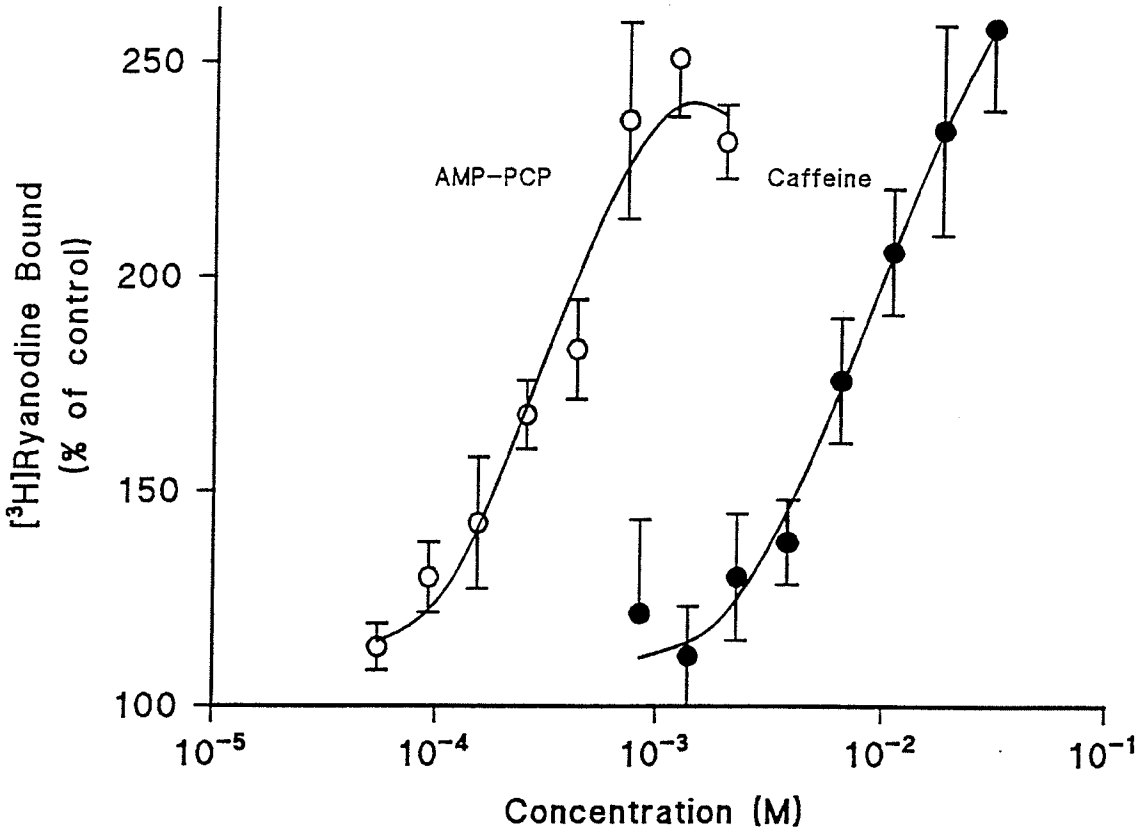

A 1.0 M KCl Buffer
B 200 mM KCl Buffer
C 200 mM KCl + 1.2 mM MgCl2
[3H]Ryanodine Bound (fmol/mg protein)
100
50
0
50
25
0
50
25
0
*
**
***
Control
Caf
AMP–PCP
Caf + AMP–PCP

Figure 5

Figure 6


250
200
150
100
[3H]Ryanodine Bound
(% of control)
AMP–PCP
Caffeine
10−5
10−4
10−3
10−2
10−1
Concentration (M)

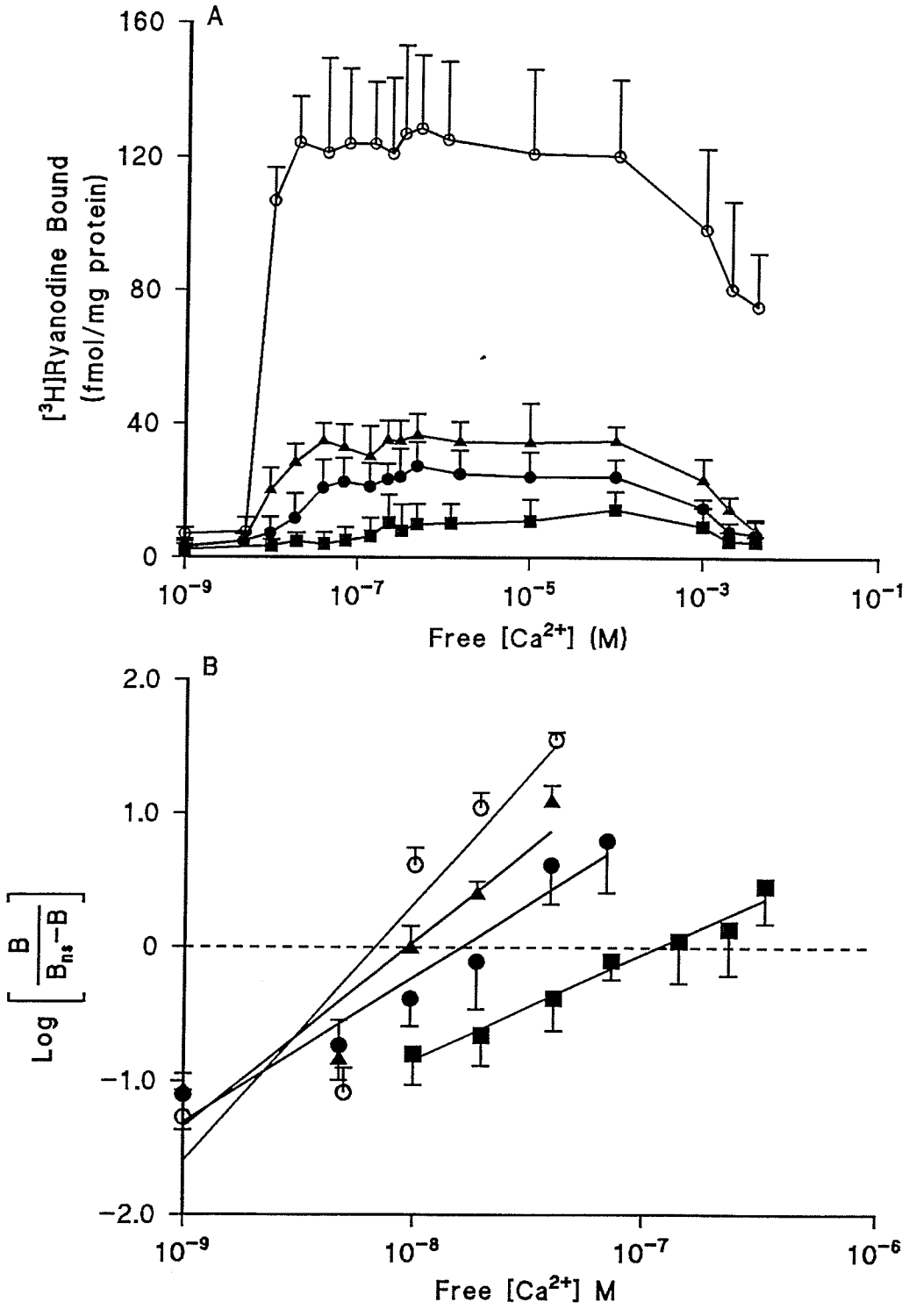

A
160
120
80
40
0
[3H]Ryanodine Bound
(fmol/mg protein)
10-9
10-7
10-5
10-3
10-1
Free [Ca2+] (M)
B
2.0
1.0
0
−1.0
−2.0
Log [B / Bns−B]
10-9
10-8
10-7
10-6
Free [Ca2+] M

Figure 7

Part V

Localization of ryanodine receptors in synaptosomal and microsomal fractions of rat brain

R.A. Padua , J.I. Nagy and J.D. Geiger

Abstract--The localization of ryanodine receptors was examined in subcellular fractions of rat brain. [^{3}H]Ryanodine binding was enriched in purified cortical, cerebellar and hippocampal synapotosomes where binding was 3.6-, 4.9- and 6.1-fold greater than that in mitochondrial fractions, respectively. Saturation analyses revealed a 3.7-fold greater maximum density (B_{max}) of sites in mossy fiber synaptosomes (249 fmol/mg protein) compared with cortical synaptosomes (67 fmol/mg protein). Binding at a single sub-saturating concentration of [^{3}H]ryanodine yielded 1.6-fold higher levels of sites in mossy fibers compared with total hippocampal synaptosomes. The affinity of binding sites for ryanodine in mossy fiber synaptosomes (K_D, 6.1 nM) was approximately 2-fold greater than those in cortical synaptosomes (K_D, 3.1 nM). Discontinuous sucrose density gradient centrifugation showed a similar distribution pattern of [^{3}H]ryanodine binding in subfractions of cortical synaptosomes and microsomes with 1.7-fold enrichment of binding sites in the lightest fractions compared with fractions of greater density. In contrast, enzyme markers of plasma membrane (5'-nucleotidase) and endoplasmic reticulum (glucose 6-phosphatase) exhibited the highest activity in the heaviest fractions. Saturation analyses of subsynaptosomal and microsomal fractions revealed the presence of a single class of high affinity sites with the lightest fractions exhibiting at least a 2-fold greater maximum number of sites than in heavier fractions. These results indicate that ryanodine receptors are concentrated in presynaptic terminals and suggest that these receptors are localized either in a specialized subcompartment of SER or in a distinct subcellular Ca^{2+} organelle.

Non-mitochondrial internal Ca^{2+} stores in neurons appear to play an important role in the regulation of intracellular Ca^{2+} concentration (Henzi and MacDermott, 1992). Two functionally distinct intracellular Ca^{2+} pools in neurons have been described (Sharp et al., 1993; Thayer et al., 1988; Verma et al., 1992). Release of Ca^{2+} from these pools are mediated by either inositol 1,4,5-trisphosphate (IP_3) or caffeine-sensitive ryanodine receptors (Henzi and MacDermott, 1992). The latter receptor is thought to be activated via a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism analogous to that found in striated muscle (Kuwajima et al., 1992; Lai et al., 1992; Sharp et al., 1993; Verma et al., 1992). The purified Ca^{2+} release channels comprising the ryanodine receptor has been pharmacologically, biochemically and physiologically characterized (Henzi and MacDermott, 1992) and localization studies have revealed a heterogeneous distribution of ryanodine receptors in brain and within individual cells (Hakamata et al., 1992; Lai et al., 1992; Ouyang et al., 1993; Padua et al., 1991; 1992; Sharp et al., 1993; Stein et al., 1992; Walton et al., 1991).

The majority of studies attempting to identify the non-mitochondrial organelles that contain Ca^{2+} release channels have focused on the organellar localization of IP_3 receptors (Meldolesi et al., 1992) and several comparing the subcellular distribution of [^{3}H]IP_3 binding, IP_3-induced Ca^{2+} release, and anti-IP_3 receptor immunoreactivity with that of organelle-specific immunological and biochemical (enzyme) markers have demonstrated a segregation of IP_3 receptors from known markers of endoplasmic reticulum (ER) (Meldolesi et al., 1992; Nori et al., 1993; Villa et al., 1992). This suggested the presence of either functionally distinct organelles or specialized subdomains of ER which are involved in Ca^{2+} sequestration and release (Meldolesi et al., 1992). Three recent investigations indicated a possible separation of ryanodine receptors, IP_3 receptors and several ER markers in cerebellar Purkinje cells (Martone et al., 1993; Nori et al., 1993; Villa et al., 1992).

Previously, we demonstrated that high affinity [^{3}H]ryanodine binding sites were present in crude subcellular fractions of rat brain (Padua et al., 1991) and that ryanodine receptors may be present in presynaptic nerve terminals (Padua et al., 1992) where they may be involved in presynaptic Ca^{2+} dependent processes such as neurotransmitter release (Henzi and MacDermott, 1992). As a step towards elucidation of the role and regulation of intracellular Ca^{2+} stores in presynaptic nerve terminals, the present study examined the localization of [^{3}H]ryanodine binding sites in synaptosomes and microsomes in relation to enzyme markers of plasma membrane and ER.

METHODS

Subcellular fractionation. Subcellular fractions were prepared essentially as previously described (Padua et al., 1991; Whittaker et al., 1964). All fractionation procedures were conducted at 4°C. Sprague-Dawley rats were decapitated, brains taken and cortices, hippocampi and cerebelli dissected on an ice-cold metal tray. Tissues were homogenized (25 strokes) in 9 volumes of 0.32 M sucrose containing 5 mM Na-HEPES (pH 7.4) and 1.0 mM PMSF (medium 1) using a glass-teflon homogenizer with a loose-fitting pestle. Homogenates were centrifuged at 1,000 x g for 10 min and pellets (P1) were suspended in medium 1 and re-centrifuged. The supernatants (S1) were combined and centrifuged at 12,000 x g for 20 min to obtain a crude synaptosomal fraction (P2). The resulting supernatant (S2) was centrifuged at 100,000 x g for 60 min to obtain a crude microsomal fraction (P3). P2 and P3 fractions were either taken for binding assays or subjected to further fractionations as described below.

To obtain synaptosomal subfractions, P2 pellets were suspended in hypoosmotic medium consisting of 5.0 mM HEPES (pH 7.4) (2.0 ml/g of original wet weight) for 30 min and centrifuged at 10,000 x g. Supernatants were loaded onto discontinuous sucrose gradients composed of 0.4 M, 0.6 M and 0.8 M sucrose containing 5.0 mM HEPES (pH 7.4) and centrifuged for 2 hours at 53,300 x g. Tissue at interfaces 0/0.4, 0.4/0.6 and

0.6/0.8 as well as pellets were collected, diluted 4-fold with assay buffer and sedimented at 100,000 x g for 2 hours. To obtain microsomal subfractions, P3 pellets were suspended in 2.0 ml of a buffer consisting of 0.25 M sucrose, 5 mM HEPES and 1.0 mM PMSF (Medium 2), loaded onto discontinuous sucrose gradients composed of the same sucrose layers as above and centrifuged for 2 hours at 80,000 x g. Tissue at interfaces 0.25/0.4, 0.4/0.6 and 0.6/0.8 as well as pellets were collected, diluted and centrifuged as above. Final pellets were taken for binding and enzyme assays.

Preparation of cortical and mossy fiber synaptosomes. Purified synaptosomes were prepared as previously described (Nagy and Delgado-Escueta, 1984). P2 pellets prepared from cortical or hippocampal tissues were suspended in 1.0 ml of medium 2 and diluted with 8.0 ml of 8.5% Percoll in medium 2. The suspension was layered onto discontinuous gradients consisting of layers of 10% and 16% Percoll in medium 2. Gradients were centrifuged for 15 min at 15,000 x g and tissue at the 10/16% Percoll interface (synaptosomes) and the pellet (mitochondria) were collected, diluted to 40 ml with a buffer consisting of 1.0 M KCl, 20 mM PIPES (pH 8.0), 100 μM $CaCl_2$ and 1.0 mM PMSF (buffer A), and centrifuged at 15,000 x g for 20 min. The synaptosomal pellets were washed once with an equal volume of buffer A, re-centrifuged and taken for binding assays.

Mossy fiber synaptosomes were prepared as previously described (Terrian et al., 1988). Hippocampi were homogenized in 9 volumes of 0.32 M sucrose, 1.0 mM $MgSO_4$ and 15 mM Na-HEPES (pH 7.4) (homogenization buffer) as above. Homogenates were passed through a series of nylon filters (130, 80 and 53 μm mesh opening) and centrifuged at 900 x g for 10 min. Pellets (P1) containing nuclei and mossy fiber terminals were washed once in an equal volume of homogenization buffer and re-centrifuged. Pellets were suspended in homogenization buffer containing 18% (w/v) Ficoll and centrifuged at 7500 x g for 40 min. Mossy fiber synaptosomes which were in

suspension were collected following dilution with 2 volumes of homogenization buffer and centrifugation at 13,000 x g for 20 min. The synaptosomal pellet was washed in an equal volume of buffer A, re-centrifuged and taken for binding assays. Protein was determined by the method of Lowry et al. (1951) with BSA as standard.

Enzyme assays. The activities of 5'-nucleotidase and glucose-6-phosphatase in subcellular fractions were determined as previously described (Heymann et al., 1984; Baginski et al., 1974). Pellets obtained from subcellular fractionation were suspended in 0.25M sucrose/1.0 mM EDTA. For determination of 5'-nucleotidase activity, 40 µl of each fraction were incubated in 1.5 ml tubes containing 40 µl of 30 mM $MgSO_4$ and 40 µl of 3.0 mM AMP. Control tubes contained AMP instead of assay buffer. The tubes were incubated for 15 min at 37°C and reactions were terminated by addition of 20 µl of 50% trichloroacetic acid. Following centrifugation at 16,000 x g for 2 min, supernatants were transferred to 5.0 ml glass test tubes containing 900 µl of H_2O to which was added 1.0 ml of a color reagent consisting of 1.2 N H_2SO_4, 2% ascorbic acid and 0.5% ammonium molybdate. Tubes were incubated at 37°C for 1 hr to allow color development. Spectrophotometric analyses at 820 nm were conducted using a Milton Roy Spectrophotometric 3000 Array Spectrophotometer. For calibration, 1.0 ml of color reagent was added to 1.0 ml of a KH_2PO_4 standard solution and tubes were treated as above. For determination of glucose 6-phosphatase activity, 50 µl of each fraction were placed in 1.5 ml tubes containing 50 µl of 0.1 M cacodylate buffer (pH 6.5) and 50 µl of 0.25 M sucrose/1.0 mM EDTA and incubated for 10 min at 37°C. Reactions were terminated with 1.0 ml of ascorbic acid/trichloroacetic acid (2%/10% w/v) and tubes were centrifuged at 16,000 x g for 2 min. Aliquots of supernatant (1.0 ml) were added to 0.5 ml of 1% (w/v) ammonium molybdate and 1.0 ml of sodium arsenite/sodium citrate (2%/2% w/v). Control tubes were treated the same except that incubations were performed in the presence of 1.0 ml of ascorbic acid/trichloroacetic acid. Tubes were vortexed, incubated

for 15 min at room temperature, and spectrophotometric analyses were performed as above at 840 nm. Calibrations were performed using standard solutions of KH_2PO_4 in 0.25 M sucrose/1.0 mM EDTA.

[^{3}H]Ryanodine binding. All binding assays were conducted in duplicate at 37°C in a final assay volume of 1.0 ml as previously described (Padua et al., 1991; 1992). In most experiments, 100 µl of membrane suspension were aliquoted into tubes containing 15 nM [^{3}H] ryanodine (total binding) or 15 nM [^{3}H]ryanodine plus 50 µM unlabeled ryanodine (non-specific binding). Specific binding was defined as the difference between total and non-specific binding. Reactions were terminated by rapid filtration through GF/C glass fibre filters using a Brandel Cell Harvester M24R. Filters were washed 3-times with 5.0 ml of ice-cold wash buffer consisting of 20 mM PIPES-KOH (pH 8.0), 100 µM $CaCl_2$ and 200 mM KCl, placed in vials with 4.5 ml of Beckman Ready Solvent, and analyzed by liquid scintillation spectroscopy. In saturation experiments, [^{3}H]ryanodine concentrations were varied from 0.9 to 90 nM.

Data analyses. Dissociation constants (K_d), maximum number of receptor sites (B_{max}), and partial F tests for best fit of data to either one or two receptor sites with differing affinities were derived from analyses of data by the non-linear multipurpose curve-fitting program LIGAND (Munson and Rodbard, 1980). Analyses of variance (ANOVA) and Tukey's multiple comparison tests were conducted using the computer statistics program INSTAT.

RESULTS

As a measure of the extent of enrichment of ryanodine receptors in purified synaptosomes, the levels of [^{3}H]ryanodine binding at a single concentration of [^{3}H]ryanodine were compared in P2, purified synaptosomes and mitochondria from rat

brain cortex, cerebellum and hippocampus (Fig. 1). Binding levels in synaptosomes and mitochondria were less than 87% of that observed in P2. However, in cortical, cerebellar and hippocampal synaptosomes compared with mitochondria, binding levels were 3.6-, 4.9- and 6.1-fold greater, respectively (Fig. 1). Binding in hippocampal mossy fiber synaptosomes was comparable to that observed in P2 fractions and was at least 1.6-fold greater than in other synaptosomes (Fig. 1). Saturation analyses revealed the presence of high affinity [^{3}H]ryanodine binding sites in cortical and mossy fiber synaptosomes (Fig. 2A and B, insets). The binding affinity in mossy fiber synaptosomes was approximately 2-fold greater than that in cortical synaptosomes (Table 1). The apparent maximum receptor density (B_{max}) in mossy fiber synaptosomes was 3.7-fold greater than in cortical synaptosomes (Fig. 2A and B, insets; Table 1). In comparison with the previously determined maximum receptor density in P2 fractions (Padua et al., 1991), mossy fibers terminals showed a similar enrichment of sites, while the number of sites in cortical synaptosomes was not significantly different.

In order to determine the intracellular localization of ryanodine receptors in presynaptic terminals, the levels of [^{3}H]ryanodine binding in subfractions of synaptosomes and microsomes were compared with activities of enzyme markers of plasma membrane (5'-nucleotidase) and endoplasmic reticulum (glucose 6-phosphatase). The 0.4 subfractions of both synaptosomes and microsomes exhibited at least a 1.7-fold greater enrichment in [^{3}H]ryanodine binding sites compared with membranes of higher density (Fig 3C). In contrast, the distribution profile of 5'-nucleotidase and glucose 6-phosphatase activities in these subfractions did not correspond with the binding distribution (Fig. 3A and B). Enzyme activities were greater in higher density membranes than in 0.4 subfractions. Saturation analyses conducted on 0.4 and 0.6 synaptosomal and microsomal subfractions yielded a single class of high affinity sites (Fig. 4A and B, insets) with similar K_D and B_{max} values (Fig. 4A and B; Table 1). Although all

subfractions exhibited a similar affinity for ryanodine, maximum binding densities (B_{max}) in 0.4 subfractions were at least 2-fold greater than in 0.6 subfractions (Table 1) which is consistent with that seen in studies conducted at a single concentration of [^{3}H]ryanodine.

DISCUSSION

Our results demonstrate the presence of high affinity [^{3}H]ryanodine binding sites in purified synaptosomes and a particularly high enrichment of such sites in mossy fiber synaptosomes. Previous immunocytochemical and biochemical studies have demonstrated the intraneuronal localization of ryanodine receptors in cell bodies, dendrites, dendritic shafts, and axonal processes (Lai et al., 1992; Ouyang et al., 1993; Sharp et al., 1993; Villa et al., 1992; Walton et al., 1991). Of relevance to the present study were indications that ryanodine receptors may be specifically concentrated in mossy fiber nerve terminals (Lai et al., 1992; Padua et al., 1991, 1992) and that caffeine, which acts on the ryanodine-sensitive pool, releases sequestered Ca^{2+} in synaptosomes (Martinez-Serrano and Satrustegui, 1989). Together, the above results suggest that ryanodine receptors may be involved in Ca^{2+} mobilization in nerve terminals.

Although influx of extracellular Ca^{2+} has been implicated as the trigger for neurotransmitter release (Augustine et al., 1987), several reports indicate an involvement of Ca^{2+} mobilization from ryanodine-sensitive intraterminal stores. Secretion of acetylcholine and ATP in the absence of extracellular Ca^{2+} from brain synaptosomes was suggested to be due to intraterminal Ca^{2+} release (Adam-Vizi and Ligeti, 1984; Fiedler et al., 1992) presumably from ryanodine-sensitive Ca^{2+} pools (Etcheberrigaray et al., 1991) and ryanodine was shown to facilitate acetylcholine release at the neuromuscular junction (Nishimura et al., 1990). Moreover, caffeine-induced Ca^{2+} release stimulated the release of dopamine and noradrenaline from PC12 cells and sympathetic nerves, respectively

(Avidor et al., 1994; Toth et al., 1993). The above findings together with our present results strongly suggest that intracellular Ca^{2+} release mediated by ryanodine receptors plays a role in neurotransmitter release. In this regard, the high concentration of these receptors observed in hippocampal mossy fiber terminals may indicate a greater need for ryanodine receptor-gated Ca^{2+} movements in specific Ca^{2+}-dependent processes associated with these terminals.

The morphological identity of intracellular Ca^{2+} stores at which IP_3 and ryanodine act has been a topic of considerable attention (Meldolesi et al., 1992; Villa et al., 1992). We found a segregation of [^{3}H]ryanodine binding and enzyme markers of plasma membrane and endoplasmic reticulum (ER) in subfractions of synaptosomes and microsomes, and these findings are consistent with the view that ryanodine receptors are localized to either a Ca^{2+} storage compartment physically separate from ER or contained within a subdomain of ER (Nori et al., 1993; Villa et al., 1992). Moreover, the distribution pattern observed for [^{3}H]ryanodine binding in synaptosomal and microsomal subfractions were similar and suggests a comparable endomembrane localization of ryanodine receptors in these preparations. Taken together, our results are consistent with previous observations showing that subcellular fractions containing high levels of ER markers exhibit low levels of IP_3-mediated Ca^{2+} release and uptake activities, [^{3}H]IP_3 and [^{3}H]ryanodine binding, and anti-IP_3 and anti-ryanodine receptor immunoreactivity (Alderson and Volpe, 1989; Nori et al., 1993; Villa et al., 1992). The question still remains as to whether IP_3 and ryanodine receptors access the same or physically separated Ca^{2+} stores.

In cerebellar Purkinje cells, an intracellular IP_3-releasable Ca^{2+} store was found to co-purify with a calsequestrin-like protein and these stores were termed, calciosomes (Takei et al., 1992; Villa et al., 1991). These compartments were proposed to be

specialized organelles which are analogous to SR and whose function is to store and release Ca^{2+} (Volpe et al., 1988). Although the existence of such discrete organelles has been questioned (Martone et al., 1993), ryanodine receptors appear to be expressed on calsequestrin-rich intracellular membranes (Takei et al., 1992; Villa et al., 1991; Volpe et al., 1991). A three-dimensional study of smooth ER in cerebellar Purkinje cell dendrites showed that this endomembrane system forms a complex network which may be continuous and interconnected suggesting that IP_3- and ryanodine-sensitive Ca^{2+} stores are part of the same membrane system, but occupy different ER subdomains (Martone et al. 1993). The ER appears to be composed of multiple subregions based on the distribution of various ER-specific proteins (Villa et al., 1992).

Definition of the physiological role of ryanodine receptor/Ca^{2+} release channels in neurons has become more complex by the demonstrated presence of at least three subtypes of ryanodine receptors encoded by different genes (Giannini et al., 1992; Hakamata et al., 1992; Kuwajima et al., 1992; McPherson and Campbell, 1993; Sorrentino and Volpe, 1993). The cardiac receptor subtype appears to be the major form expressed in brain (McPherson and Campbell, 1993) which suggests that Ca^{2+}-induced Ca^{2+} release may mediate Ca^{2+} efflux from these pools. The presence of the skeletal muscle type in cerebellar Purkinje cells suggests the involvement of a voltage-regulated Ca^{2+} mobilization mechanism in these cells (Kuwajima et al., 1992). The third subtype is not well characterized, but may be expressed as a result of the actions of TGF-β (Giannini et al., 1992; Hakamata et al., 1992). The cellular distribution of these different receptor isoforms and the relative contribution of each in Ca^{2+} regulation is not known and awaits further development of specific antibodies and ligands.

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FIGURE LEGENDS

Fig. 1. Comparison of [^{3}H]ryanodine binding levels in crude synaptosomal/mitochondrial (P2), synaptosomal (S), mitochondrial (M) and mossy fiber synaptosomal (MF) fractions of rat brain cortex (CTX), cerebellum (CB) and hippocampus (HIP). Data shown represent means ± S.E.M. of three to six experiments performed in duplicate.

Fig. 2. Labeled titration curves of [^{3}H]ryanodine binding in (A) cortical and (B) mossy fiber synaptosomes. Synaptosomes were incubated with concentrations of [^{3}H]ryanodine ranging from 0.7 to 38 nM. Scatchard plots (insets) show binding of [^{3}H]ryanodine to a single class of high affinity binding sites with K_D and B_{max} values as indicated in Table 1. Data shown were representative of two or three experiments performed in duplicate.

Fig. 3. Comparison of the distribution of enzyme markers of (A) plasma membrane (5'-nucleotidase activity) and (B) endoplasmic reticulum (glucose 6-phosphatase activity) with (C) [^{3}H]ryanodine binding levels in synaptosomal (open bars) and microsomal (closed bars) subfractions. Fractions were prepared from rat cortical tissues as in Materials and Methods. Data shown represent means ± S.E.M. of three to six experiments performed in duplicate. CP represents P2 (open bar) and P3 (closed bar) fractions; Pel, pellet.

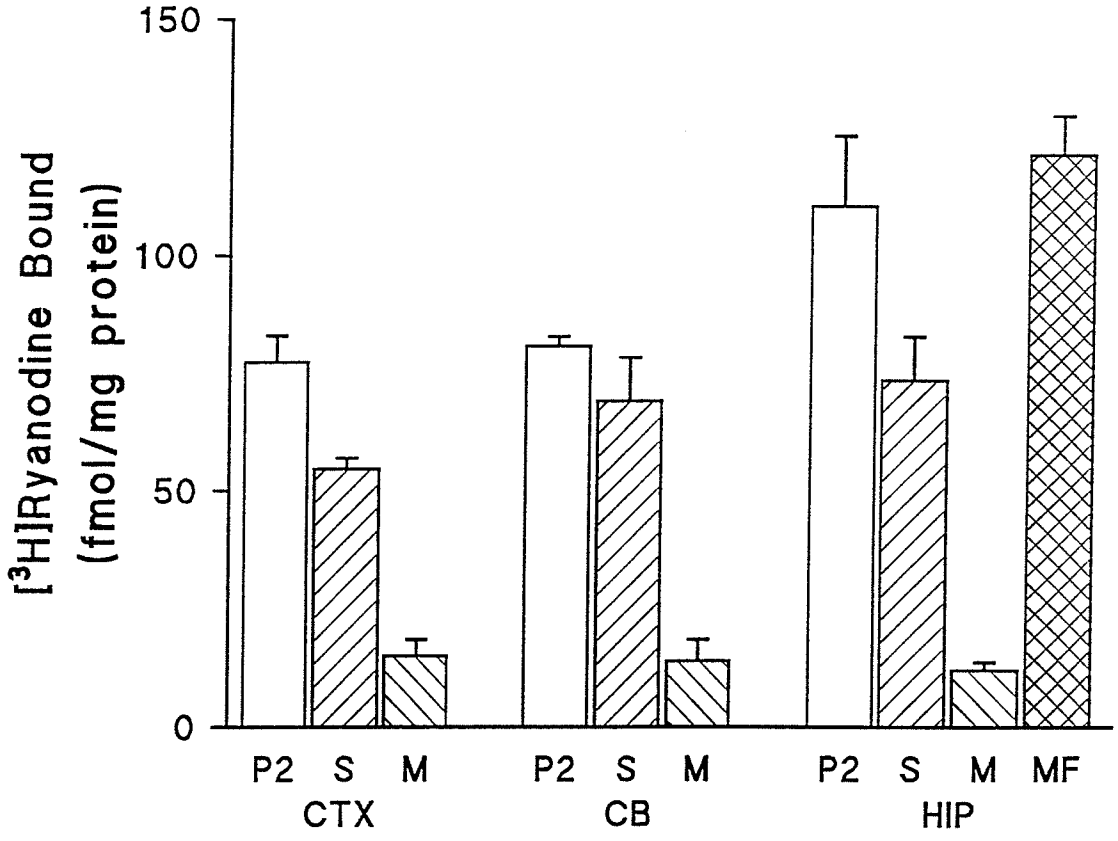
Fig. 4. Labeled titration curves of [^{3}H]ryanodine binding in 0.4 (open circles) and 0.6 (closed circles) subfractions of (A) synaptosomes and (B) microsomes. Subfractions were prepared as in Materials and Methods and were incubated with concentrations of [^{3}H]ryanodine ranging from 0.8 to 90 nM. Scatchard plots (insets) show binding of [^{3}H]ryanodine to a single class of high affinity binding sites with K_D and B_{max} values as indicated in Table 1. Data shown were representative of three experiments performed in duplicate.

Table 1. [^{3}H]Ryanodine binding affinity (K_D) and density (B_{max}) as demonstrated by saturation analyses with labeled ligand titrations in subcellular membrane fractions of rat cerebral cortex.

Fraction	K_D (nM)	B_{max} (fmol/mg protein)
Synaptosomes		
Cortical	6.1 ± 0.5	67 ± 5.3
Mossy Fiber (n=2)	3.1	249
Subfractions of synaptosomes		
0/0.4 interface	8.3 ± 2.6	394 ± 68
0.4/0.6 interface	15.3 ± 5.6	196 ± 27
Subfractions of microsomes		
0.25/0.4 interface (n=2)	6.4	377
0.4/0.6 interface (n=2)	5.0	178

Synaptosomes and subfractions were prepared as described in Materials and Methods. K_D and B_{max} values were obtained from analyses of labeled titration/equilibrium binding data using the multipurpose curve-fitting program LIGAND (Munson and Rodbard, 1980). Data are mean ± S.E.M. values from 3 experiments unless otherwise indicated. n, number of experiments.

Figure 1


150
100
50
0
[3H]Ryanodine Bound
(fmol/mg protein)
P2
S
M
CTX
P2
S
M
CB
P2
S
M
MF
HIP

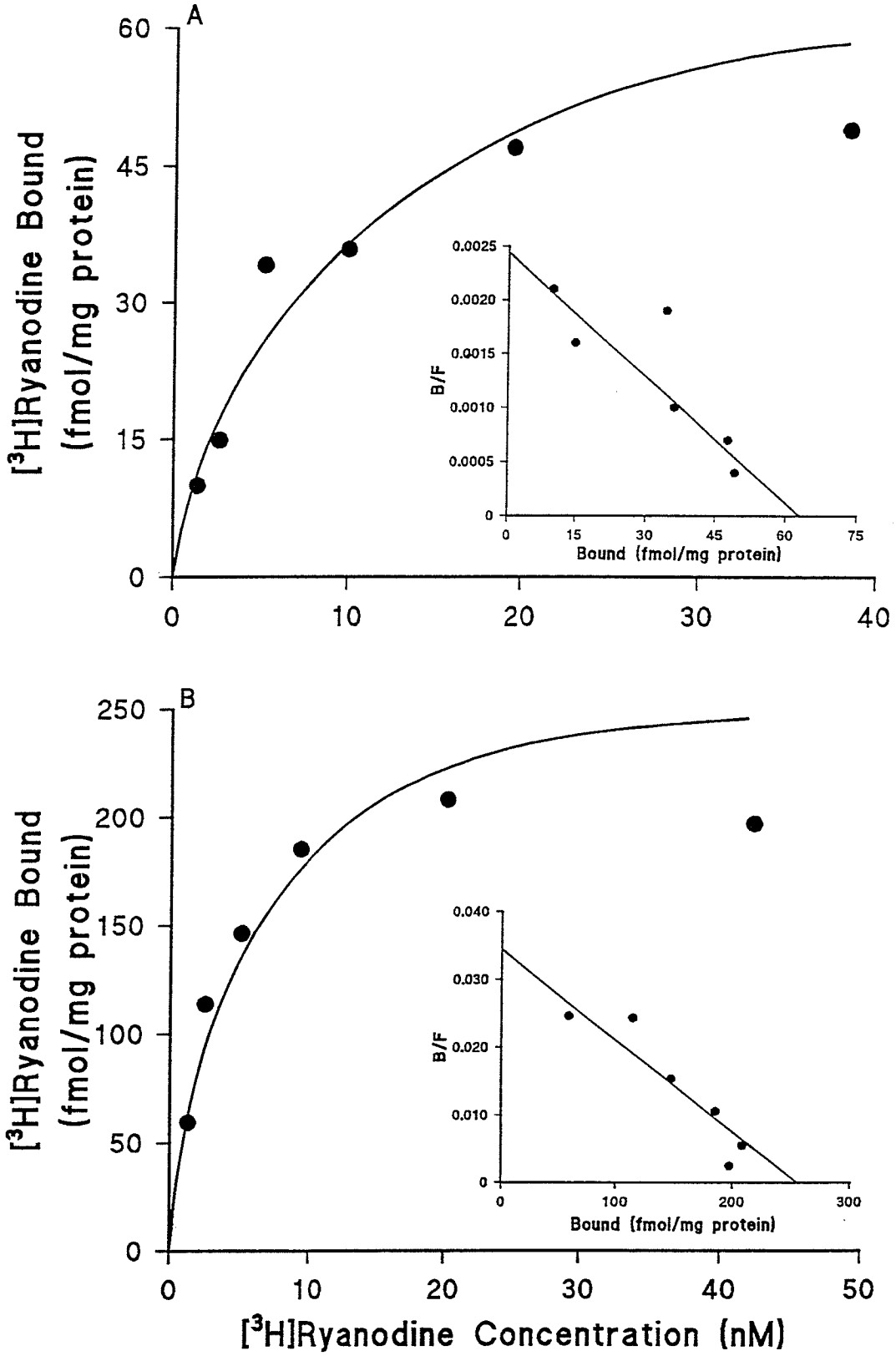

A
60
45
30
15
0
[3H]Ryanodine Bound
(fmol/mg protein)
0
10
20
30
40
0.0025
0.0020
0.0015
0.0010
0.0005
0
B/F
0
15
30
45
60
75
Bound (fmol/mg protein)
B
250
200
150
100
50
0
[3H]Ryanodine Bound
(fmol/mg protein)
0
10
20
30
40
50
[3H]Ryanodine Concentration (nM)
0.040
0.030
0.020
0.010
0
B/F
0
100
200
300
Bound (fmol/mg protein)

Figure 2

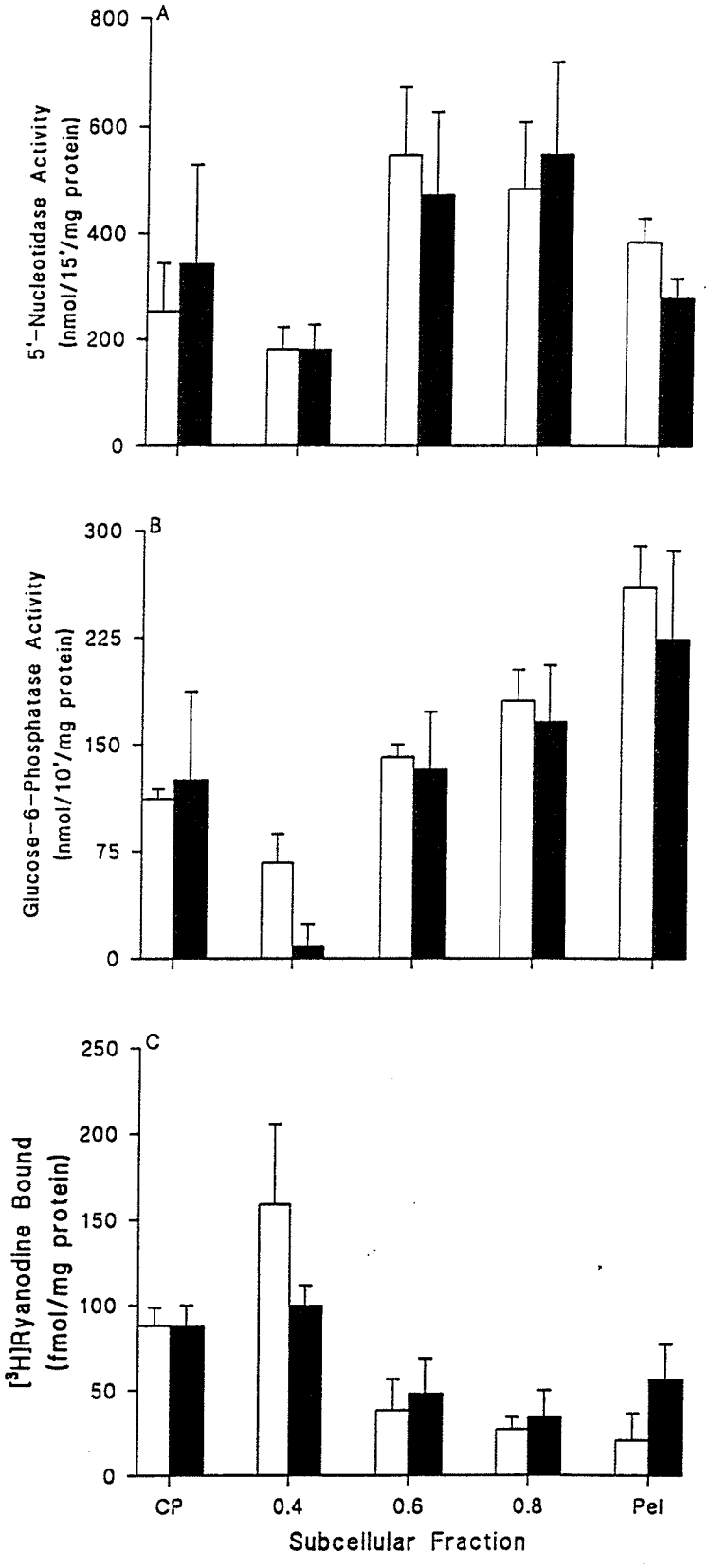

A
800
600
400
200
0
5'-Nucleotidase Activity
(nmol/15'/mg protein)
B
300
225
150
75
0
Glucose-6-Phosphatase Activity
(nmol/10'/mg protein)
C
250
200
150
100
50
0
[3H]Ryanodine Bound
(fmol/mg protein)
CP
0.4
0.6
0.8
Pel
Subcellular Fraction

Figure 3

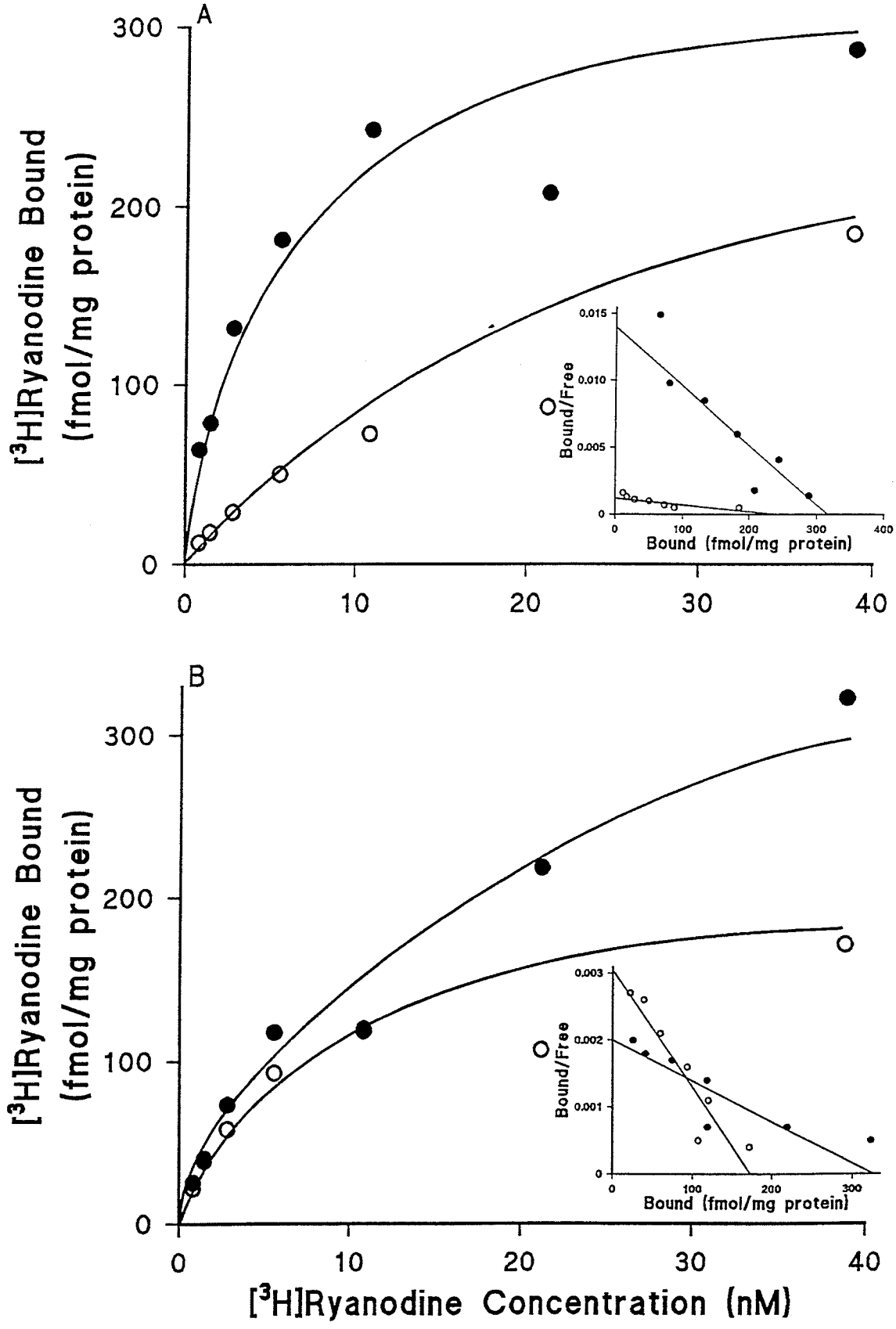

A
300
200
100
0
[3H]Ryanodine Bound
(fmol/mg protein)
0
10
20
30
40
0.015
0.010
0.005
0
Bound/Free
0
100
200
300
400
Bound (fmol/mg protein)
B
300
200
100
0
[3H]Ryanodine Bound
(fmol/mg protein)
0
10
20
30
40
[3H]Ryanodine Concentration (nM)
0.003
0.002
0.001
0
Bound/Free
0
100
200
300
Bound (fmol/mg protein)

Figure 4

GENERAL DISCUSSION

The research presented in this thesis described the pharmacological characteristics, regional distribution and cellular localization of ryanodine receptors in mammalian brain. Radioligand binding methodology was employed to study [^{3}H]ryanodine binding sites in various membrane preparations of neural tissues. Optimal conditions for binding of labeled ryanodine to rat brain membranes were determined and we showed that crude subcellular membrane fractions from several rat brain regions exhibited high affinity binding sites for ryanodine. Such sites were also present in membrane preparations of human brain. These receptors in rat and human brain appear to be regulated by agents such as Ca^{2+}, Mg^{2+}, ATP and caffeine; agents that influence ryanodine receptors in striated muscle. Moreover, high ionic strength conditions stimulated ryanodine binding possibly through an increase in the Ca^{2+} sensitivity of the receptor. The distribution of ryanodine receptors in whole brain, as judged by autoradiographic analyses of brain slices, was widespread and heterogeneous with some regions such as hippocampus and cortex displaying a high density of sites, while subcortical areas expressed lower levels of receptors. Furthermore, as demonstrated by kainic acid lesions in hippocampus, ryanodine receptors appear to be localized preferentially in neurons. Our autoradiographic results also suggest that a high concentration of these sites are present within mossy fiber nerve terminals of the hippocampus. [^{3}H]Ryanodine titration analyses of purified cortical and mossy fiber synaptosomes revealed the presence of high affinity binding sites in both preparations. Mossy fiber terminals had an approximately 4-fold higher enrichment of sites compared with cortical synaptosomes. In subfractions of synaptosomes and microsomes, a separation of [^{3}H]ryanodine binding and enzyme marker activities of plasma membrane (5'-nucleotidase) and endoplasmic reticulum (ER) (glucose-6-phosphatase) was observed. This suggested that ryanodine receptors may be localized to a distinct organelle or to a subcompartment of ER specialized for Ca^{2+} release and sequestration. Taken together,

the results indicate that ryanodine receptors are expressed in brain and have properties similar to those in striated muscle. Although the contribution of these receptors to the overall function of neurons remains to be determined, they may have an important role in several Ca^{2+}-mediated processes in neurons as discussed in more detail below in relation to findings in the literature.

In Parts II and V of the thesis, evidence is presented that ryanodine receptors are contained in presynaptic terminals. This localization suggests a role of ryanodine-sensitive calcium stores in presynaptic Ca^{2+}-dependent processes including modulation of synaptic transmission (Henzi and MacDermott, 1992). This possibility is supported by the results of Martinez-Serrano and Satrustegui (1989) and Fujimoto et al., (1980) who showed that caffeine mobilizes sequestered Ca^{2+} in permeabilized synaptosomes and in regions near presynaptic active zones of bullfrog sympathetic neurons, respectively. Moreover, it was previously suggested that release of Ca^{2+} from intracellular pools contribute to a portion of the tetanic and post-tetanic potentiation at the neuromuscular junction presumably through an influence on miniature end-plate potential (MEPP) frequency (Rahamimoff et al., 1978). Adam-Vizi and Ligeti (1984) further demonstrated secretion of acetylcholine from rat brain synaptosomes in the absence of extracellular Ca^{2+} and proposed a role for intraterminal Ca^{2+} release in the mediation of transmitter release. This view is supported by a study in which ryanodine facilitated neurotransmitter release at the neuromuscular junction (Nishimura et al., 1990). Depolarization-induced ATP secretion from mouse brain synaptosomes in the absence of extracellular Ca^{2+} was also suggested to be due to Ca^{2+} release from intracellular stores, more specifically from ryanodine-sensitive Ca^{2+} pools (Etcheberrigaray et al., 1991; Fiedler et al., 1992). In addition, caffeine stimulated dopamine release apparently through mobilization of Ca^{2+} from ryanodine-sensitive stores in PC12 cells (Avidor et al., 1994), facilitated K^{+}-induced secretion of GABA from growth cones (Lockerbie and Gordon-Weeks, 1986)

and promoted noradrenaline release from sympathetic nerves (Toth et al., 1990). Moreover, caffeine-induced Ca^{2+} release caused a depression of $GABA_A$-activated chloride currents in dorsal root ganglion neurons (Desaulles et al., 1991) and ryanodine decreased the activity of spontaneous inhibitory post-synaptic potentials of dissociated rat neurons (Akaike et al., 1992).

Induction of long-term potentiation (LTP) in the hippocampus is another Ca^{2+}-dependent process in which intracellular Ca^{2+} release may be involved. In CA1 pyramidal neurons, NMDA receptor-mediated Ca^{2+} influx is required for LTP induction (Collingridge and Bliss, 1987), but in other synapses such as mossy fiber/CA3 pyramidal cell synapses that express LTP and have few NMDA receptors, induction of LTP is resistant to NMDA antagonists (Harris and Cotman, 1986). In these synapses, elevations in cytosolic calcium may be achieved through release from internal stores or Ca^{2+} influx via voltage-dependent Ca^{2+} channels (Henzi and MacDermott, 1992). There is evidence that suggests the possible involvement of IP_3-mediated Ca^{2+} release in LTP at mossy fiber/CA3 synapses where increased levels of IP_3 have been observed during LTP (Lynch et al., 1988). In addition, dantrolene, a blocker of ryanodine receptor-mediated Ca^{2+} release (Ohta et al., 1990) has been shown to block induction of LTP in hippocampal slices (Obenaus et al., 1989) which indicates a possible role of these receptors in LTP. In dendritic spines of hippocampal pyramidal cells, the concentration of free cytosolic Ca^{2+} can be elevated to greater levels than in the dendritic shaft and can remain at a high level for several minutes (Muller and Connor, 1991). In these neurons, ryanodine receptors have been shown to be preferentially localized in dendritic spines compared with shafts (Sharp et al., 1993). Miller (1992) suggested that CICR, presumably mediated through ryanodine receptors, may boost the magnitude and duration of Ca^{2+} signals in spines during Ca^{2+} activated processes such as in synaptic plasticity.

Ryanodine-sensitive Ca^{2+} pools may contribute to the generation of Ca^{2+} oscillations observed in a variety of cell types including neurons (Friel and Tsien, 1992; Henzi and MacDermott, 1992; Kuba and Takeshita, 1981; Meyer and Stryer, 1991). Ca^{2+} oscillations appear to involve the alternating release of Ca^{2+} from and re-sequestration into internal Ca^{2+} stores (Henzi and MacDermott, 1992). Caffeine has been shown to elicit Ca^{2+} oscillations in rat superior cervical ganglion, snail neurons and frog sympathetic neurons (Friel and Tsien, 1992; Kawai and Watanabe, 1989; Kostyuk et al., 1989; Kuba, 1980; Lipscombe et al., 1988; Nohmi et al., 1992) and ryanodine blocks caffeine-induced oscillations (Kawai and Watanabe, 1989). Are ryanodine receptors solely responsible for the generation of neuronal Ca^{2+} oscillations? It is possible that in cases where both IP_3- and ryanodine-sensitive Ca^{2+} stores are present and depending on the proximity of Ca^{2+} pools, Ca^{2+} released from the IP_3-sensitive pool can generate release of the ion via ryanodine receptors through a CICR mechanism (Berridge, 1991; Meyer and Stryer, 1991). In addition, Ca^{2+} waves may be generated by activation and propagation of Ca^{2+} release by CICR from closely adjacent Ca^{2+} compartments (Meyer and Stryer, 1991). This mechanism may be important for the transport of Ca^{2+} signals to more interior cellular compartments such as nuclei or between cells via gap junctions (Meyer and Stryer, 1991). Furthermore, the rate of Ca^{2+} diffusion through the cytosol is much slower than that of IP_3 diffusion (Meyer and Stryer, 1991) which suggests that different patterns of Ca^{2+} oscillations or waves may be generated depending on the presence of one or both Ca^{2+} stores within a neuron. The physiological relevance of Ca^{2+} oscillations is uncertain, but has been speculated to be a mechanism for encoding information or to regulate non-cyclical cellular processes such as the activation state of protein kinases (Berridge et al., 1988; Goldbeter et al., 1990; Meyer and Stryer, 1991).

Three different subtypes of ryanodine receptors have been identified in brain as judged by immunohistochemical analyses with specific ryanodine receptor antibodies; a

cardiac muscle type which appears to be the predominant form in brain (Lai et al., 1992; McPherson and Campbell, 1993), a skeletal muscle type which is localized primarily in cerebellar Purkinje cells (Kuwajima et al., 1992) and a brain subtype which is expressed mainly in corpus striatum, thalamus and hippocampus (Hakamata et al., 1992). In part II of the thesis, autoradiographic analyses revealed that ryanodine receptors were heterogeneously distributed in brain. Although it is uncertain which receptor subtype is labeled in this study, the distribution pattern appears to correspond more to that of the cardiac receptor subtype (Lai et al., 1992; McPherson and Campbell, 1993). The significance of the expression of three different subtypes of ryanodine receptor is unknown, but suggests that Ca^{2+} regulation by intracellular stores that contain these receptors may vary depending on which subtype is expressed. The preponderance of the cardiac subtype supports the view that most ryanodine receptors in brain are activated via a Ca^{2+}-induced Ca^{2+} release mechanism similar to the activation process in heart (McPherson and Campbell, 1993; Verma et al., 1992). The skeletal subtype may provide an alternative form of rapid Ca^{2+} signalling, whereby Ca^{2+} release from internal stores is triggered by plasma membrane depolarization (Kuwajima et al., 1992). Further diversity in Ca^{2+} signalling may be achieved in neurons where both cardiac and skeletal receptor types are co-expressed; rapid release of Ca^{2+} through skeletal type receptors may promote CICR via the cardiac subtype. The third subtype has been suggested to be involved in pathological states due to their localization in regions succeptible to delayed neuronal death (Hakamata et al., 1992).

In parts I, III and IV of the thesis, Ca^{2+}, ATP, caffeine and Mg^{2+} were shown to modulate ryanodine receptor binding to brain membranes. Similarly, these agents influenced Ca^{2+} conductance of the purified brain receptor channel incorporated into lipid bilayers (McPherson et al., 1991) suggesting that this receptor functions as a Ca^{2+} channel analogous to that in striated muscle. Another compound referred to as cyclic

ADP-ribose (cADPR) has been shown to mobilize intracellularly stored Ca^{2+} in various cell types including neurons (Currie et al., 1992; Galione et al., 1991; Koshiyama et al., 1991; Takasawa et al., 1993). This agent has been suggested to be a novel endogenous regulator of the cardiac subtype ryanodine receptor which is the predominant form in brain (McPherson and Campbell, 1993; Meszaros et al., 1993) and appears to function as a second messenger in sea urchin eggs where it may be involved in the generation of Ca^{2+} waves (Galione, 1994). Production of cADPR from nicotinamide adenine dinuclueotide (NAD^+) is mediated by the enzyme, ADP-ribosyl cyclase which has been shown to have a widespread distribution in mammalian and invertebrate tissues including brain (Lee and Aarhus, 1993). The endogenous cADPR concentration in brain ranges between 20 and 100 nM (Walseth et al., 1991) which is within its reported effective concentration range for Ca^{2+} release from brain microsomes (White et al., 1993). Brain microsomal Ca^{2+} release is inhibited by ryanodine, but not affected by the IP_3 receptor antagonist, heparin, which suggests selective activation of ryanodine receptors (White et al., 1993). Furthermore, cADPR mimics caffeine in eliciting Ca^{2+} oscillations in sympathetic neurons (Currie et al., 1992). The extracellular factors involved in signalling the production of cADPR are unknown, but cGMP appears to be involved in stimulating ADP-ribosyl cyclase activity (Galione et al., 1993) which indicates that activators of guanylate cyclase may contribute to the initiation of cADPR production. Thus, the pathway leading to formation of cADPR may function as the phophoinositide pathway in regulating release of sequestered Ca^{2+} from intracellular stores and together, the two processes may act in concert to generate complex intracellular Ca^{2+} signalling patterns (Galione, 1994).

In part III of the thesis, it was demonstrated that ryanodine receptors are present in human brain. Because these receptors were studied in post-mortem tissues, the results were interpreted taking into account possible post-mortem effects. Ryanodine receptors in

human brain were similar to those in brain of other mammals and in striated muscle with respect to ryanodine binding properties and the influence of Ca^{2+}, Mg^{2+}, and caffeine on binding (Kawai et al., 1991; Pessah et al., 1987; McPherson and Campbell, 1993; Zimanyi and Pessah, 1991). The distribution of receptor sites in human and rat hippocampus was comparable. It is interesting to note that the levels of receptors associated with intracellular Ca^{2+} stores were reduced in brain tissues obtained from persons afflicted with Alzheimer's, Huntington's and Parkinson's diseases, as well as Olivopontocerebellar atrophy (Kish et al., 1989; Tanaka et al., 1993; Warsh et al., 1991; Young et al., 1988). Furthermore, as discussed in Part III, ryanodine receptor/Ca^{2+} release channels may be involved in the development of the neurological disorder, Neuroleptic Malignant Syndrome (NMS). Dantrolene, a drug that is used in the treatment of NMS (Granato et al., 1983), appears to protect against glutamate receptor-mediated neurotoxicity in cultured neurons (Frandsen and Schousboe, 1991) and delayed neuronal death associated with ischemia in gerbil hippocampus (Zhang et al., 1993) presumably through inhibition of intracellular Ca^{2+} release. Taken together, these observations suggest that ryanodine-sensitive Ca^{2+} stores may play a role in cell death resulting from neurodegenerative conditions. Characterization of ryanodine receptors in human brain provides a first step in determining the function of these receptors in normal and pathological Ca^{2+}-dependent processes in human CNS.

In summary, the experimental results presented in the body of this thesis support the hypothesis that neurons express ryanodine receptor/Ca^{2+} release channels. Our data combined with those in the literature establish an important role of ryanodine receptors in the regulation of intracellular Ca^{2+} concentration in neurons. Future research work in this area should focus on the further characterization and elucidation of the role of ryanodine receptor subtypes in brain, the determination of possible interactions between various intraneuronal Ca^{2+} pools and how each of these may contribute to Ca^{2+} signalling in

neurons, and the involvement of these pools in neuropathological states. Results from such studies may lead to the development of new pharmaceutical strategies in the treatment of various neuropathic disorders and diseases.

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