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# Subcellular localization of ryanodine receptors in rat brain

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**Abstract**

Subcellular fractions of rat brain were used to determine the subcellular localization of ryanodine receptors. [$^3$H]Ryanodine binding in purified cortical, cerebellar and hippocampal synaptosomes was up to 3.6-fold higher compared with mitochondrial fractions. The density of sites ($B_{max}$) in hippocampal mossy fiber synaptosomes (249 fmol/mg protein) was 3.7-fold greater than in cortical synaptosomes (67 fmol/mg protein) and binding affinity was approximately 2-fold greater in the former ($K_D$, 6.1 nM) than the latter ($K_D$, 3.1 nM). At single sub-saturating concentrations of [$^3$H]ryanodine, binding was 1.6-fold higher in mossy fibers compared with total hippocampal synaptosomes. [$^3$H]Ryanodine binding sites were distributed similarly in subfractions of cortical synaptosomes and microsomes from discontinuous sucrose density gradients. An enrichment of sites was found in the lightest fractions containing the lowest activities of plasma membrane (5'-nucleotidase) and endoplasmic reticulum (glucose 6-phosphatase) enzyme markers when data for microsomal and synaptosomal subfractions were expressed as activity/binding per mg protein and when data for synaptosomal subfractions were expressed as a percentage of total activity/binding in collected fractions. Thus, ryanodine receptors appear to be concentrated in presynaptic terminals where they may play a major role in neurotransmitter release, and appear to be localized either in a specialized endoplasmic reticulum subcompartment or a distinct subcellular organelle.

*Keywords:* Ryanodine receptor; Synaptosome; Hippocampus; $Ca^{2+}$, intracellular; Subcellular localization

## 1. Introduction

Two functionally distinct non-mitochondrial intracellular $Ca^{2+}$ pools in neurons have been described; release of $Ca^{2+}$ from these pools is mediated by either inositol 1,4,5-trisphosphate ($IP_3$) or ryanodine receptors – the latter is thought to be caffeine-sensitive and activated via $Ca^{2+}$-induced $Ca^{2+}$ release (Pozzan et al., 1994). Ryanodine receptors have been pharmacologically, biochemically and physiologically characterized (Henzi and MacDermott, 1992) and are reported to be heterogeneously distributed in the CNS (Padua et al., 1991; Padua et al., 1992; Stein et al., 1992; Ouyang et al., 1993; Sharp et al., 1993; Furuichi et al., 1994). Studies of intracellular organelles that contain $IP_3$ and ryanodine receptors indicate that endoplasmic reticulum subfractions rich in $IP_3$ receptors are distinct from those containing ryanodine receptors, that these subfractions do not segregate with endoplasmic reticulum markers, and that caffeine stimulates $Ca^{2+}$ release from multiple $Ca^{2+}$ stores (Villa et al., 1992; Martone et al., 1993; Sah et al., 1994; Pozzan et al., 1994). These findings suggest the release of $Ca^{2+}$ from functionally distinct organelles or specialized subdomains of endoplasmic reticulum. We have previously demonstrated [$^3$H]ryanodine binding to crude subcellular fractions of rat brain nuclei, synaptosomes and microsomes as well as autoradiographic evidence for [$^3$H]ryanodine binding sites in hippocampal nerve terminals (Padua et al., 1991, 1992). Ryanodine receptor-regulated $Ca^{2+}$ sequestration and release in nerve terminals may be of particular physiological importance as suggested by reports of caffeine-sensitive $Ca^{2+}$ stores in rat brain synaptosomes (Martinez-Serrano and Satrustegui, 1989) and induction of catecholamine

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secretion by intracellular $Ca^{2+}$ via ryanodine receptors in PC12 cells (Avidor et al., 1994). Thus, we determined whether [$^3$H]ryanodine binding sites were enriched in synaptosomal and microsomal subfractions prepared from rat brain regions and compared the distributions of these sites in subcellular fractions with those of enzyme markers of plasma membrane and endoplasmic reticulum.

## 2. Materials and methods

Washed subcellular fractions of cerebral cortex, hippocampus and cerebellum from Sprague-Dawley rats were prepared as previously described (Padua et al., 1991; Whittaker et al., 1964). All animal protocols were approved by the Animal Care Committee of the University of Manitoba in conformity with Canadian Council on Animal Care guidelines. Tissues were homogenized (25 strokes) in 9 volumes of 0.32 M sucrose containing 5.0 mM Na-Hepes (pH 7.4) and 1.0 mM PMSF (medium 1) using a glass-teflon homogenizer with a loose-fitting pestle and following centrifugations at 1000, 12000 (crude synaptosomal pellet, P2) and $100000 \times g$ (crude microsomal pellet P3) fractions were either washed and taken for binding assays or further fractionated. 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) and centrifuged at $10000 \times g$ for 30 min. Supernatants were loaded onto discontinuous sucrose gradients composed of 0.4, 0.6 and 0.8 M sucrose containing 5.0 mM Hepes (pH 7.4) and centrifuged for 2 h at $53300 \times g$. 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 $100000 \times g$ for 2 h. To obtain microsomal subfractions, P3 pellets were suspended in 2.0 ml of a buffer consisting of 0.25 M sucrose, 5.0 mM Hepes and 1.0 mM PMSF (Medium 2), loaded onto discontinuous sucrose gradients and centrifuged as above. Tissue at interfaces 0.25/0.4, 0.4/0.6 and 0.6/0.8 were collected, diluted, pelleted and taken for binding and enzyme assays. Purified synaptosomes were prepared from cerebral cortex and hippocampal P2 pellets using discontinuous gradients consisting of layers of 10% and 16% Percoll as previously described (Nagy and Delgado-Escueta, 1984); the 10/16% Percoll interface (synaptosomes) and the pellet (mitochondria) were taken for binding assays. Mossy fiber synaptosomes were prepared from hippocampus as previously described (Terrian et al., 1988). Pellets containing nuclei and mossy fiber terminals were suspended in homogenization buffer containing 18% (w/v) Ficoll, centrifuged and the mossy fiber synaptosomes remaining in suspension were collected. 5'-Nucleotidase and glucose-6-phosphatase activities were determined spectrophotometrically (Heymann et al., 1984; Baginski et al., 1974). All binding and protein assays were conducted as previously described (Padua et al., 1991, 1992) using 15

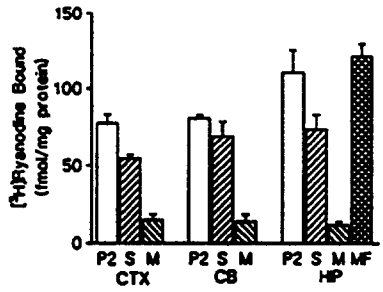


Fig. 1. Comparison of [$^3$H]ryanodine binding levels in crude synaptosomal/mitochondrial (P2), and purified synaptosomal (S), mitochondrial (M) and mossy fiber synaptosomal (MF) fractions of rat cerebral cortex (CTX), cerebellum (CB) and hippocampus (HIP). Data represent means ± S.E.M. from three to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

nM [$^3$H]ryanodine (total binding) or 15 nM [$^3$H]ryanodine plus 50 $\mu$M unlabeled ryanodine (non-specific binding). In saturation experiments, [$^3$H]ryanodine concentrations ranged from about 0.8 to 40 nM. Affinity constants ($K_D$), maximum number of receptor sites ($B_{max}$) and partial $F$-tests were obtained with the computer program LIG-

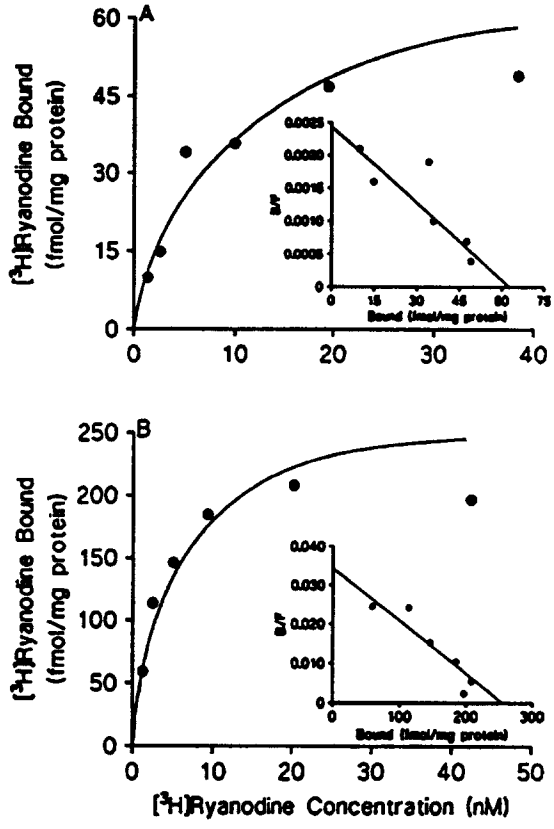


Fig. 2. Labeled titration curves of [$^3$H]ryanodine binding in (A) cerebral cortical and (B) hippocampal mossy fiber synaptosomes. Synaptosomes were incubated with concentrations of [$^3$H]ryanodine ranging from about 0.8 to 40 nM. Scatchard plots (insets) show binding to a single class of high affinity binding sites (values for $K_D$ and $B_{max}$ are indicated in Table 1). Data shown are representative of experiments replicated at least 2 times.

Table 1
[$^3$H]Ryanodine binding affinity ($K_D$) and density ($B_{max}$) in subcellular fractions of rat cerebral cortex and hippocampus

| Fraction | $K_D$ (nM) | $B_{max}$ (fmol/mg protein) |
|---|---|---|
| Synaptosomes | | |
| Cerebral cortical | 6.1 ± 0.5 | 67 ± 5.3 |
| Hippocampal mossy fiber ($n$ = 2) | 3.1 (2.5–3.7) | 249 (242–256) |
| Cerebral cortical synaptosomes | | |
| 0/0.4 interface | 8.3 ± 2.6 | 394 ± 68 |
| 0.4/0.6 interface | 15.3 ± 5.6 | 196 ± 27 |
| Cerebral cortical microsomes | | |
| 0.25/0.4 interface | 7.7 ± 2.6 | 508 ± 135 |
| 0.4/0.6 interface | 6.1 ± 1.1 | 318 ± 140 |

$K_D$ and $B_{max}$ values were obtained from analyses of labeled titration/equilibrium binding data. Data are mean ± S.E.M. values from three experiments except for hippocampal mossy fiber synaptosomes where two experiments were performed ($n$ = 2) and mean values for $K_D$ and $B_{max}$ were listed as well as the range of values (numbers in parentheses).

AND (Biosoft). Analyses of variance (ANOVA) and Tukey's multiple comparison tests were conducted using INSTAT (Biosoft).

## 3. Results

The levels of [$^3$H]ryanodine binding at a single concentration of [$^3$H]ryanodine were compared in P2, purified synaptosomes and mitochondria (Fig. 1). In cortical, cerebellar and hippocampal synaptosomes, binding levels, expressed as fmol [$^3$H]ryanodine bound per mg protein, were slightly less (no statistically significant differences) than in P2, but were 3.6-, 4.9- and 6.1-fold greater than in mitochondria of these regions, respectively (Fig. 1). Binding in mossy fiber synaptosomes was comparable to that in hippocampal P2 fractions, but was significantly greater than synaptosome and P2 fractions from cerebral cortex and cerebellum. Saturation analyses revealed high affinity [$^3$H]ryanodine binding sites in cortical and mossy fiber synaptosomes (Fig. 2A,B, insets) with the latter exhibiting an approximately 2-fold greater affinity than the former (Table 1). The apparent $B_{max}$ of sites in mossy fiber synaptosomes was 3.7-fold greater than in cortical synaptosomes (Fig. 2A,B, insets; Table 1). Upon further fractionation of cortical samples, subfractions of synaptosomes at the 0/0.4 and microsomes at the 0.25/0.4 interfaces exhibited a 1.7-fold or greater enrichment in [$^3$H]ryanodine binding sites compared with membranes of higher density (Fig. 3C). In contrast, the 5′-nucleotidase and glucose 6-phosphatase activities were greater in higher density membranes (Fig. 3A,B). A similar pattern was evident when the total activity/binding data for each fraction were expressed as a percentage of total enzyme

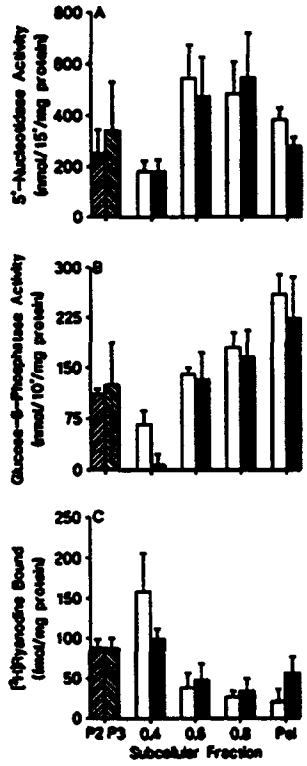


Fig. 3. Distribution of cerebral cortical (A) plasma membrane 5′-nucleotidase activity, (B) endoplasmic reticulum glucose 6-phosphatase activity, and (C) [$^3$H]ryanodine binding levels in subfractions of synaptosomes (open bars) and microsomes (closed bars) obtained by discontinuous gradient centrifugation. Data represent means ± S.E.M. of three to six experiments. Binding levels in total P2 (left hatched bars) and P3 (right hatched bars) fractions are shown for comparison. Numbers refer to gradient interface and Pel indicates pellet at the bottom of the gradient.

Table 2
Total enzyme activity and [$^3$H]ryanodine binding in synaptosomal and microsomal subcellular subfractions

| Fraction | Enzyme recovery (%) | | Binding recovery (%) |
|---|---|---|---|
| | 5′-N | G-6-P | [$^3$H]Ryanodine binding |
| *Microsomal* | | | |
| 0.4 | 3.2 ± 0.4 | 0.2 ± 0.2 | 8.5 ± 3.9 |
| 0.6 | 8.0 ± 1.4 | 3.3 ± 0.9 | 8.2 ± 5.6 |
| 0.8 | 14.7 ± 6.6 | 7.0 ± 1.1 | 10.2 ± 4.7 |
| Pel | 74.1 ± 3.1 | 89.5 ± 2.0 | 73.2 ± 13.7 |
| *Synaptosomal* | | | |
| 0.4 | 6.4 ± 1.5 | 1.6 ± 1.6 | 63.6 ± 4.6 |
| 0.6 | 16.2 ± 2.0 | 7.3 ± 2.6 | 11.5 ± 1.4 |
| 0.8 | 22.7 ± 1.5 | 13.2 ± 2.2 | 7.9 ± 0.9 |
| Pel | 54.7 ± 4.4 | 78.0 ± 5.6 | 17.6 ± 5.5 |

Data were expressed (means ± S.E.M.) as a percentage of total enzyme activity or binding levels in fractions collected from 0.4, 0.6 and 0.8 interfaces and pellets. Total 5′-nucleotidase (5′-N) activity (nmol/15 min) recovered in microsomal and synaptosomal subfractions was 2811 ± 356 and 1436 ± 337, respectively. Total glucose-6-phosphatase (G-6-P) activity (nmol/10 min) recovered in microsomal and synaptosomal subfractions was 1561 ± 80 and 677 ± 144, respectively. Total [$^3$H]ryanodine binding (fmol) recovered in microsomal and synaptosomal subfractions was 386 ± 124 and 280 ± 20, respectively.

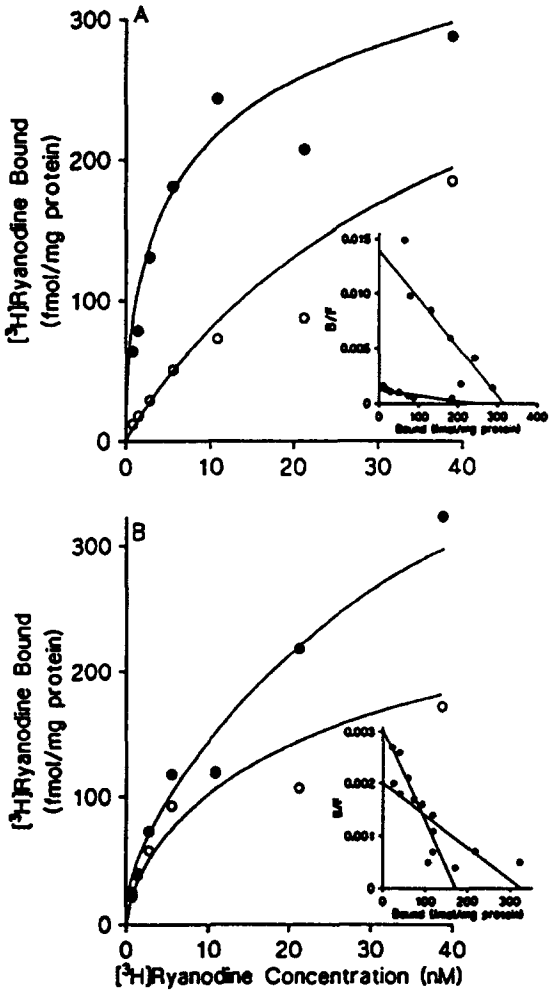


Fig. 4. Labeled titration curves of [$^3$H]ryanodine binding in purified (A) synaptosomal and (B) microsomal subfractions at the 0.4 M (open circles) and 0.6 M (closed circles) interface. Scatchard plots (insets) generated with [$^3$H]ryanodine concentrations ranging from about 0.8 to 40 nM show binding to a single class of high affinity sites (values for $K_D$ and $B_{max}$ are indicated in Table 1). Data shown are representative of experiments replicated at least 2 times.

activity or binding levels in fractions collected from 0.4, 0.6 and 0.8 interfaces, and pellets (Table 2). For microsomes, 5′-nucleotidase activity was about 5 times lower, glucose-6-phosphatase was 35 times lower, and ryanodine binding was not significantly different in fraction 0.4 compared with fraction 0.8. For synaptosomes, 5′-nucleotidase activity was about 5 times lower, glucose-6-phosphatase was 8 times lower, and ryanodine binding was 8 times higher in fraction 0.4 compared with fraction 0.8 (Table 2).

Saturation analyses conducted on synaptosomal subfractions at the 0/0.4 and 0.4/0.6 interfaces and microsomal subfractions at the 0.25/0.4 and 0.4/0.6 interfaces yielded a single class of high affinity sites (Fig. 4A,B; Table 1). Although all subfractions exhibited a similar affinity for ryanodine, the $B_{max}$ of the 0/0.4 and 0.25/0.4 subfractions were up to 2-fold greater than the 0.4/0.6 subfractions (Table 1) which is consistent with observations seen using single concentrations of [$^3$H]ryanodine.

## 4. Discussion

These results demonstrate high affinity [$^3$H]ryanodine binding sites in purified synaptosomes and their enrichment in mossy fiber synaptosomes which is consistent with our earlier anatomical results and with molecular studies (Lai et al., 1992; Padua et al., 1991, 1992). Although influx of extracellular $Ca^{2+}$ triggers neurotransmitter release (Augustine et al., 1987), several reports suggest an influence on this process by $Ca^{2+}$ mobilization from ryanodine-sensitive intraterminal stores. Immunocytochemical and biochemical studies have demonstrated localization of ryanodine receptors in neuronal cell bodies, dendrites, and axons (Lai et al., 1992; Ouyang et al., 1993; Sharp et al., 1993; Villa et al., 1992; Walton et al., 1991). Synaptosomal secretion of acetylcholine and ATP in the absence of extracellular $Ca^{2+}$ was suggested to be due to intraterminal $Ca^{2+}$ release via ryanodine receptors (Etcheberrigaray et al., 1991) and ryanodine facilitates acetylcholine release at the neuromuscular junction (Nishimura et al., 1990). Moreover, caffeine acting at ryanodine receptors releases sequestered $Ca^{2+}$ in synaptosomes (Martinez-Serrano and Satrustegui, 1989) and caffeine-induced $Ca^{2+}$ release stimulates dopamine and noradrenaline release from PC12 cells and sympathetic nerves, respectively (Avidor et al., 1994; Toth et al., 1993). Thus, intracellular $Ca^{2+}$ release mediated by ryanodine receptors may play a major role in neurotransmitter release and the high levels of these receptors in hippocampal mossy fiber terminals suggest a greater contribution of ryanodine receptor-gated $Ca^{2+}$-dependent processes in these terminals.

The localization of [$^3$H]ryanodine binding sites and enzyme markers of either plasma membrane or endoplasmic reticulum subfractions of synaptosomes and microsomes is consistent with views that ryanodine receptors are localized to either a $Ca^{2+}$ storage compartment physically separate from endoplasmic reticulum or are contained within a subdomain of endoplasmic reticulum (Alderson and Volpe, 1989; Pozzan et al., 1994). Moreover, the similar distribution pattern of [$^3$H]ryanodine binding sites in synaptosomal and microsomal subfractions suggests a comparable intracellular membrane localization of these sites in the two preparations. Whether $IP_3$ and ryanodine receptors access the same or physically separated $Ca^{2+}$ stores is still uncertain. A study of smooth endoplasmic reticulum in cerebellar Purkinje cells demonstrated a continuous and interconnected intracellular membrane network suggesting that $IP_3$- and ryanodine-sensitive $Ca^{2+}$ stores are part of the same membrane system, but occupy different endoplasmic reticulum subdomains (Martone et al., 1993), the existence of which is suggested by the heterogeneous distribution of various endoplasmic reticu-

lum-specific proteins (Villa et al., 1992). The subcellular distribution of the different ryanodine receptor isoforms expressed in brain and the relative contribution of each in $Ca^{2+}$ regulation particularly in nerve terminals remains to be determined.

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