Immunogold Localization of Inositol 1, 4, 5-Trisphosphate (InsP₃) Receptor in Mouse Cerebellar Purkinje Cells Using Three Monoclonal Antibodies

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ABSTRACT. Ultrastructural localization of InsP₃ receptor in mouse cerebellar Purkinje cells was investigated by immunogold technique using three monoclonal antibodies (mab 10A6, 4C11 and 18A10). The epitopes of the three antibodies were numerously detected on the smooth endoplasmic reticulum (ER) (especially, on the stacks of flattened smooth ER, subsurface cisterns and spine apparatus), scantily on the rough ER and on the outer nuclear membrane, but were not detectable on either the plasmalemma, synaptic densities, mitochondria or Golgi apparatus. Not only mab 4C11 and 10A6 which bind to the N-terminal region of the receptor but also 18A10 which binds to the C-terminal region were localized on the cytoplasmic surface of the ER membranes. This indicates that the C terminus of InsP₃ receptor is localized on the cytoplasmic surface of the ER. We noticed that gold particles are usually localized on the fuzzy structure of the cytoplasmic surface of smooth ER, which is suggested to correspond to the feet structure of the ryanodine receptor. In the Nissl body, gold particles were found not only on the ER membranes but also in the cytoplasmic matrix between the rough ER cisterns. We suggest that the peculiar structure of Nissl body, which is composed of parallel cisterns of rough ER, sandwiching a number of free polyribosomes between the cisternal elements, is due to the fact that the major proteins like InsP₃ receptor are synthesized mostly on the free polyribosomes and become membrane bound only at the later stage of the biosynthesis.

Inositol 1, 4, 5-trisphosphate (InsP₃) mediates the effects of various neurotransmitters, hormones and growth factors by initiating calcium release from intracellular calcium stores (2). The endoplasmic reticulum (ER) has been suggested to be the site of InsP₃ action by a number of studies (1, 12, 20, 26, 27). In addition, the plasma membrane (10, 14) and a smooth vesicle termed calciosome (11, 30), have been also suggested.

Recently immunocytochemical localization of InsP₃ receptor has been reported from the laboratories of Mikoshiba (15), Snyder (24) and Südhof (18). Using pre-embedding avidin-biotin labeling and monoclonal antibodies against P₄₀₀ protein, which has been identified as the InsP₃ receptor protein (16), this receptor was localized on the ER, plasma membrane and postsynaptic density of Purkinje cells by Maeda et al. (15).

Using pre-embedding avidin-biotin labeling and monoclonal antibodies directed against a purified InsP₃-receptor, Ross et al. (24) have localized the receptor to ER, including portions of the rough ER, a population of smooth ER, a portion of subsurface cisterns, the nuclear membrane and cis-most cisterns of Golgi apparatus. They suggested that in cerebellar Purkinje cells, the InsP₃-induced intracellular calcium release is not the properties of a single organelle but is effected by specialized portions of both rough and smooth ER, and possibly by other smooth surfaced structures (calciosomes).

Mignery et al. (18) prepared a peptide antibody against residues 482-500 of a synthetic peptide PCD6, which correspond to the residues 2731-2749 of the primary structure of mouse InsP₃ receptor deduced from its cDNA sequenced by Furuichi et al. (7). Using pre-embedding immunogold labeling of agarose-embedded fragments of rat cerebellar and of ultrathin frozen sections (data not shown), they showed that the protein is present in all parts of ER.

However, the exact subcellular localization of the InsP₃ receptor in mouse cerebellar Purkinje cells is still controversial and has not yet been settled.

In this article we analysed the subcellular localization of the InsP₃ receptor in cerebellar Purkinje cells by immunogold labeling of ultrathin frozen sections and by pre-embedding immunogold labeling of the subcellular
fractions of mouse cerebellum.

MATERIALS AND METHODS

Animals. Adult female Balb/C mice were used.

Monoclonal antibodies. The monoclonal antibodies (mab) against P<sub>600</sub> protein were prepared by injecting partially purified mouse P<sub>600</sub> protein into a rat and fusing the spleen cells with mouse Sp2 myeloma cells as described elsewhere (17). The hybridomas were cultured in Nissui SFM 101 medium, and the culture supernatant concentrated with an Amicon YM10 membrane was dialyzed against phosphate buffered saline (PBS), then applied to a Biogel HPHT column preequilibrated in 10 mM sodium phosphate, pH 6.8. The proteins were eluted with linear gradient of sodium phosphate (10-0.25 M, pH 6.8). IgG fractions were collected and concentrated with an Amicon YM10 membrane. After dialysis against PBS, the antibody solution was stored at -80°C. Three monoclonal antibodies (mab 10A6, 4C11 and 18A10) were prepared, the probable epitope sequence of which are shown by dashed lines in Fig. 1 of Reference 7.

Protein A-gold technique on frozen ultrathin sections. Frozen ultramicrotomy was performed as described by Keller et al. (13) with some modifications. Mice were fixed by perfusion with 4% paraformaldehyde containing 1% glutaraldehyde. The cerebellum was incubated overnight in 2.3 M sucrose in 0.1 M sodium phosphate buffer (pH 7.4), containing 20% polyvinyl pyrrolidone (29), cut into small pieces, and rapidly frozen in liquid propane at -180°C. Frozen ultrathin sections were cut with Reichert Ultracut-N with a cryoattachment (FC-4D) at -115°C to the (approximate thickness of 70 nm). The sections were picked up on formvar-carbon-coated nickel grids and were incubated with 2% gelatin in PBS containing 10 mM glycine, then reacted for 15 min with a monoclonal antibody (10 μg/ml), washed 6 times with gelatin solution, then 15 min with rabbit IgG (10 μg/ml) against rat IgG, subsequently for 15 min with protein A-gold complex (4 and 8 nm in diameter, OD<sub>525</sub>nm=0.023 and 0.08, respectively). As shown by dashed lines in Fig. 1 of Reference 7.

RESULTS

Ultrastructural localization of InsP<sub>3</sub> receptor in the Purkinje cells. Figs. 1-5 show cryoimmunogold localization of InsP<sub>3</sub> receptor in the perikaryon of Purkinje cells by mab 4C11. Gold particles are observed on almost all cisterns and tubules of the smooth and rough ER. Especially stacks of flattened smooth cisterns are heavily labeled as shown in Fig. 4. These structures are not always homogeneously labeled, and gold particles are frequently found in clusters as shown in Figs. 1-5. In the Nissl body, these clusters are usually observed not only on or near the cytoplasmic surface of the ER membranes, but also in the cytoplasmic matrix between the cisterns (Fig. 3).

Another subcellular structure which is labeled by gold particles is the outer membranes of the nuclear envelope. The labeling of the nuclear envelope was slight and quite heterogeneous, as is clearly shown in Figs. 1 and 2. Mitochondria were not labeled at all (Figs. 1, 2, 4), which is in marked contrast to the heavily labeled stack of flattened cisterns which are occasionally in close association with mitochondria (Figs. 1 and 4). Neither the trans nor the cis cisterns of the Golgi apparatus, which are numerously found in the perikaryon of Purkinje cells, are labeled as shown in Figs. 2 and 5. This finding is again in marked contrast to the heavy labeling of the stacks of flattened cisterns. Although the flattened cisterns of smooth ER and the Golgi apparatus are similar in appearance, the lumina of the former structures are usually narrower than those of the Golgi apparatus and the cisterns of the former are neither fenestrated nor branched, and are separated by a constant interval of 10 to 50 nm.

Fig. 6 shows a stack of flattened cisterns in a dendritic process, which is heavily labeled with gold particles in cluster. Subsurface cisterns as described by Rosenbluth...
Immunogold Localization of \textit{insP}_3\textsubscript{1} Receptor

1

2

3

ER

M

N

GO

ER

N

ER

ER

M

165
Immunogold Localization of insP₃ Receptor

Fig. 8. Labeling density (gold particles/µm membrane) of various intracellular membrane structures. Monoclonal antibody used was 4C11.


(23) are enriched in the Purkinje cells and are also heavily labeled.

Fig. 7 shows axon-dendrite synapses where dendritic spine apparatus are heavily labeled. In marked contrast, neither the plasmalemma nor the post-synaptic density are labeled.

Essentially similar distribution patterns of gold particles were obtained with the other two mab, 10A6 and 18A10 (data not shown).

Semiquantitative analyses of the particle densities on the various subcellular membrane structures. The labeling density of various subcellular membrane structures in the Purkinje cells was shown in Fig. 8. It is evident that smooth ER is most heavily labeled while rough ER and nuclear envelope were slightly labeled. The labeling density of the Golgi apparatus, plasmalemma and mitochondria was negligible and at a control level. In the Purkinje cells, smooth ER exist either as solitary tubules or vesicles, or as stacks of flattened cisterns and subsurface cisterns. When the labeling density of the stacked and non-stacked smooth ER were compared, it was seen that stacked smooth ER was much heavily labeled than nonstacked one (Fig. 8).

Figs. 1–5. Immunogold localization of InsP₃ receptor in frozen sections of mouse cerebellar Purkinje cells by using a monoclonal antibody (4C11).

ER: endoplasmic reticulum, GO: Golgi apparatus, M: mitochondria, N: nucleus. Bars, 0.2 µm.

Figs. 1 and 2 show perikaryon of the cells. Rough and smooth ER are heterogeneously labeled with gold particles (Fig. 1: 4 nm, Fig. 2: 8 nm), occasionally in cluster. The outer nuclear membranes (arrows) are scantily labeled with gold particles. Clusters of gold particles are also found in the cytoplasmic matrix. Fig. 1 × 60,000, Fig. 2 × 25,000.

Fig. 3 shows a Nissl body stained with gold particles (4 nm). Clusters of gold particles are usually on the cytoplasmic surface of rough ER cisterns. Similar clusters were also found in the cytoplasmic matrix between the cisterns. × 60,000.

Fig. 4. A stack of four smooth ER cisterns sandwiched by two mitochondria were heavily labeled with gold particles (8 nm). × 30,000.

Fig. 5. Golgi apparatus, obliquely sectioned, is not labeled at all. This finding is in marked contrast with heavy labeling of smooth ER vesicles in cluster. 4 nm gold particles were used. × 60,000.

Fig. 6. A stack of smooth ER in a dendritic process of a Purkinje cells is heavily labeled with gold particles (4 nm). This stack is in close association with mitochondria. Subsurface cisterns (arrow heads) are also labeled with gold particles. × 75,000. Figs. 6 and 7 were labeled with mab 4C11.

Fig. 7. Spine apparatus (arrows) in an axo-dendritic synapse are heavily stained with gold particles (4 nm), whereas neither plasmalemma nor postsynaptic densities are labeled at all. It is also noted that presynaptic processes in Figs. 6 and 7 (stars) are not stained with gold particles. × 75,000.
Immunogold Localization of \( \text{InsP}_3 \) Receptor

**Topology of \( \text{InsP}_3 \) receptor in the ER membranes.**
By applying three monoclonal antibodies (mab 10A6, 4C11 and 18A10), which recognize three different epitopes of \( \text{InsP}_3 \) receptor protein (7), we tried to estimate the membrane topology of the receptor molecule in the ER membranes.

First we used the protein A-gold technique on frozen ultrathin sections. As shown in Fig. 9, gold particles were observed not only on the cytoplasmic side, but also on the luminal side of the ER membranes. This is probably due to the overlapping effect. The lumens of the flattened cisterns in the Purkinje cells are quite narrow (10-50 nm), whereas the section-thickness is \( \sim 70 \) nm, and usually only the surface of frozen ultrathin sections are labeled by the cryoimmunogold method (3).

In order to estimate the sidedness of the receptor protein more precisely, we selected the cross-sectional profiles of flattened smooth ER and rough ER (Nissl body) and measured the distance from the center of ER membranes to that of gold particles. Figs. 9A and 9B show the frequency distribution curve of gold particles of smooth and rough ER, respectively, when stained with mab 4C11 (4 nm). Similar distribution curve was obtained by labeling with 10A6 (4 nm) (data not shown). It is evident that the center of the distribution curves of the former two mab are on the cytoplasmic side of the ER membranes, suggesting that the epitopes to the two mab (4C11 and 10A6) are located on the cytoplasmic side of the ER membranes. When labeled with 18A10, however, Purkinje cells were only slightly labeled so

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Figs. 10-13. Pre-embedding immunogold (4 nm) localization of \( \text{InsP}_3 \) receptor in a subcellular fraction of mouse cerebella by using monoclonal antibodies 4C11 (Figs. 10 and 11), 10A6 (Fig. 12) and 18A10 (Fig. 13). Bars, 0.2 \( \mu \)m.

Fig. 10. An axo-dendritic synapse. The cisterns composing of the spine apparatus are heavily labeled with gold particles (stars). The spine apparatus localized deeply within the dentritic synaptic vesicles was not labeled. This is presumably because of difficulties in accessibility of antibodies and/or protein A-gold particles. \( \times 120,000 \).

Fig. 11. Numerous gold particles bind to the fuzzy structures (arrows) that are associated with the cytoplasmic surface of the smooth vesiculotubular structures which are presumably derived from spine apparatus. M: mitochondria C: coated vesicles. \( \times 80,000 \).

Fig. 12. Immunogold localization of the epitopes to mab 10A6 which bind to the N-terminal region of \( \text{InsP}_3 \) receptor similar to mab 4C11. \( \times 80,000 \).

Fig. 13. Immunogold localization of the epitopes to mab 18A10 which binds to the C-terminal region of \( \text{InsP}_3 \) receptor molecules. Cytoplasmic surfaces of the smooth vesicles were heavily labeled similar to the immunogold localization with mab 4C11 and 10A6. \( \times 80,000 \).
Fig. 14. A high magnification electron micrograph of fuzzy materials on the cytoplasmic surface of smooth vesicles. Taken by a Hitach HF-2000 field emission microscope. Fuzzy materials are shown by arrows. Monoclonal antibody used was 4C11. ×250,000. Bar, 0.1 μm.

Fig. 15. Control experiment. In place of the monoclonal antibodies, cerebellar subcellular fraction was incubated with IgG from preimmune serum. Fuzzy materials are shown by arrows. ×80,000. Bar, 0.2 μm.

that we could not get the distribution curve for this mab.

Comparing the distribution curve of the rough ER (Fig. 9B) with that of the smooth ER (Fig. 9A), it is evident that the former is skewed toward the matrix space between the cisterns, clearly indicating the localization of the immunoreactive sites of the InsP₃ receptor in the matrix space. This finding is discussed in detail later.

As another way of determining topology of InsP₃ receptor, we used pre-embedding immunogold labeling of agarose-embedded fragments of mouse cerebella by the three monoclonal antibodies. When incubated with mab 4C11 and 10A6, the outer surfaces of small vesicles, tubules and lamellae were heavily labeled with gold particles (Figs. 10, 11 and 12). This result is consistent with the above finding that the center of the distribution curve of gold particles, when labeled with mab 4C11 and 10A6, is situated on the cytoplasmic side of the smooth ER membranes. In Fig. 10, it is evident that the heavily labeled cisterns are derived from a spine apparatus in a dendritic spine.

However, when labeled with mab 18A10, gold particles bound also to the cytoplasmic surface of the smooth vesicles as shown in Fig. 13, indicating that the epitopes against 18A10 are also located on the cytoplasmic surface of the ER membranes.

Gold particles barely bound to the smooth vesicles, when incubated with control IgG from preimmune serum (Fig. 15).

Feet-like structures on the immunoreactive sites of the InsP₃ receptor. When observing a number of micrographs of the agarose-embedded fragments of mouse cerebella, we noticed that gold particles are usually localized on fuzzy structure on the cytoplasmic surface of smooth vesicles or cisterns as shown in Figs. 10 and 11. In Fig. 11, especially, colocalization of fuzzy structure with gold particles is quite evident.

Figure 14 is a high magnification view of a smooth vesicles taken by a Hitachi field emission transmission microscope HF-2000. Fuzzy structure clearly visible on the cross sectional profile of a smooth vesicle is labeled with gold particles. Existence of such fuzzy structure was also evident in the control specimens (Fig. 15).

DISCUSSION

Subcellular localization of InsP₃ receptor in the Purkinje cells. In the present experiment the epitopes of the three monoclonal antibodies were detected numerously on the smooth ER, especially on the stacked smooth ER, and scantily on the rough ER and outer nuclear membrane, but they were not detectable either on the plasmalemma, mitochondria or Golgi apparatus.

The positive reaction at the plasmalemmal region demonstrated in the previous observation (15) might be due to the diffusion of the reaction product from the InsP₃ receptors located on the ER just beneath the plasmalemma (subsurface cisterns). However, we can not
deny the possibility that another type of InsP₃ receptor, or related receptors might exist on the plasmalemma. Further immunological and immunocytochemical studies should be carried out in order to clarify these points.

In Fig. 8, we showed that the stacked smooth ER is extremely heavily labeled with gold particles, suggesting that such flattened ER cisterns are the apparatus specialized for signal transduction by InsP₃. Existence of specialized smooth ER systems such as stacked ER membranes (22), subsurface cisterns (23) and dendritic spine apparatus (8) have been described in nerve cells, especially in Purkinje cells. We have shown that InsP₃ receptor is highly concentrated in these flattened cisterns. We strongly suggest that these structures play very important roles in the Ca²⁺-signal transduction in nerve cells.

It is also quite interesting that some of the stacked smooth ER cisterns are in close association with mitochondria (Figs. 1, 4, and 6), suggesting direct interaction of these two organelles in the process of intracellular Ca²⁺-signalling.

**Topology of InsP₃ receptor protein.** In the previous paper (7) a schematic model for the topology of InsP₃ receptor was proposed in which the probable sites of epitopes for the three mab have been localized; mab 4C11 and 10A6 on the cytoplasmic surface, whereas mab 18A10 on the luminal surface of the ER membrane. Although the immunogold localization of 4C11 and 10A6 were consistent with the previous prediction, that of 18A10 was not supported experimentally.

The present experiment suggests strongly that the epitope for mab 18A10 on the C-terminus of the receptor molecule is localized on the cytoplasmic side of the ER membranes, similar to those for mab 10A6 and 4C11. This suggestion is also supported by the inhibition of Ca release from microsome fraction from mouse cerebella by mab 18A10 (Nakade, M., Maeda, N., and Mikoshiba, K. Manuscript in preparation). Thus the membrane topology of the InsP₃ receptor and that of the ryanodine receptor is likely to be very similar as pointed out by Furuichi et al. (7) and De Camilli et al. (6). To orient both N- and C-termini to cytoplasmic side, InsP₃ receptor should spin around the membrane an even number of times; 2, 4 of 6 times. The exact topology of the receptor could be determined by high resolution immunocytochemistry.

**A fuzzy structure on the cytoplasmic surface of the smooth ER membranes.** We found a fuzzy structure on the cytoplasmic surface of smooth ER, which is apparently similar to the feet structure found in the ryanodine receptor of sarcoplasmic reticulum (4, 25). Presumably this structure corresponds to the dense intermediate line between each cistern in the spine apparatus (8, 23) and a faint, discontinuous intermediate line between subsurface cisterns and plasmalemma (23). We strongly suggest that such a structure plays a pivotal role in the signal transduction in the Purkinje cells.

There is a possibility, however, that the fuzzy structure might simply show antibody molecules connecting the epitopes and protein A gold particles. We could rule out this possibility, because similar fuzzy structure was observed even in the control experiment (Fig. 15). It is very probable, however, that such connecting IgG molecules might contribute to some of the contrast of the fuzzy structure.

Further extensive immunocytochemical and biochemical studies should be carried out to determine the biological significance of the fuzzy structure and to show structural and functional relationships between the InsP₃ receptor and the ryanodine receptor.

**Significance of immunoreactivity in the cytoplasmic matrix of the Nissl bodies.** We have shown that in the Nissl bodies, gold particles are observed not only on the ER membranes but also in the cytoplasmic matrix between the rough ER cisterns (Fig. 3). IP₃ receptor is certainly an integral transmembrane protein of ER (7) and it is rather surprising that the immunoreactivities were localized in such cytoplasmic matrix where a number of free polyribosomes exist.

According to the cDNA sequence, the predicted amino-acid sequence and structure model of InsP₃ receptor (7), the protein lacks a definable signal sequence with a hydrophobic stretch at the N-terminus and the first hydrophobic segment which presumably spun the ER membrane is localized at residue No. 2276–2294. It is suggested, therefore, that the protein is mostly synthesized on the free polyribosomes in the cytoplasmic matrix between the cisternal elements, then binds to the rough ER membrane when more than 82.7% (2276/total 2749 residues) of the molecule is synthesized on the free polyribosomes.

InsP₃ receptor protein is especially abundant in the Purkinje cells and is definitely one of the major proteins synthesized there (19). It has been reported recently that ryanodine receptor, another Ca²⁺ channel protein, consists of a very large cytoplasmic N-terminal region which apparently correspond to the feet structure and the small C-terminal channel region (7, 28).

Nissl body, a very characteristic structure found in the perikaryon of neurons, is composed of parallel cisterns of rough ER sandwiching a number of free polyribosomes between the cisternal elements (22). We suggest that this peculiar structure of Nissl body is due to the fact that the major proteins synthesized in the body proceeds mostly on the free polyribosomes in the cytoplasmic matrix and become membrane bound only at the later stage of biosynthesis.

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REFERENCES


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Note added in Proof. After writing this paper, we received a preprint of an article entitled "The inositol 1, 4, 5-trisphosphate receptor in cerebellar Purkinje cells" by Satoh, T., Ross, C.A., Villa, A., Supattapone, S., Possan, T., Snyder, S.H., and Meidolesi, J., in which essentially similar quantitative intracellular distribution of InsP$_3$ receptor has been reported. According to Dr. Meidolesi, their article is to be published in J. Cell Biol.