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Lectin cytochemistry of carbohydrates on cell membranes of rat cerebellum

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Summary

Wheat germ agglutinin (WGA), *Ricinis communis* agglutinin (RCA) and *Lens culinaris* (LC) lectins have been used to characterize carbohydrates of neuronal membrane systems in rat and chick cerebellum. WGA and RCA both label Golgi membrane cisternae on the side of the membrane facing the cisternal space but they do not label other elements of the granular and agranular endoplasmic reticulum (ER). WGA labels the plasma membrane generally and WGA binding sites are concentrated within the synaptic cleft and at specialized sites along the axolemma at the node of Ranvier. RCA labelling of plasma membranes is sparse. Neuraminidase treatment of tissue slices prior to lectin cytochemistry does not alter the Golgi membrane distribution of WGA and RCA binding sites and other ER membranes remain unlabelled. The plasma membrane staining with WGA is decreased and that with RCA is increased after neuraminidase pretreatment. The pattern of lectin labelling with LC resembles that seen with concanavalin A (con A) in that all elements of the ER within cell bodies label on the side of the membrane facing the cisternal space. A major difference when compared to con A labelling is that the hypolemmal cisternae lying adjacent to the plasma membrane of Purkinje cell axons and dendrites do not label with LC. Collectively, these results suggest that elements of the ER of Purkinje cells are heterogeneous with respect to their lectin binding properties in a manner which is consistent with the formation of more complex oligosaccharides on membranes of the cell periphery.

Introduction

Some of the smooth membrane cisternae in neurons of the rat cerebellum contain concanavalin A (con A) binding sites, apparently on glycoproteins (Wood *et al.*, 1974; Wood & McLaughlin, 1976). In the axons and dendrites of cerebellar Purkinje cells these con A binding sites are located on the side of the membrane facing the cisternal

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space of cisternae which lie just inside the plasma membrane (Wood *et al.*, 1974; Wood & McLaughlin, 1976). Other smooth membrane profiles in the axons and dendrites are not labelled with con A suggesting that the populations of membrane profiles within neuronal processes are not homogeneous (see Holtzman, 1977). The presence of a specialized smooth membrane 'channel' lined with carbohydrates adjacent to the plasma membranes of neurons is of interest because there is evidence that smooth membrane profiles in this position within the axolemma of chicken ciliary ganglion neurons are a part of the morphological substrate for fast axoplasmic transport (Droz *et al.*, 1973, 1975). In the present studies we have extended our characterization of these internal membrane systems as well as the neuronal plasma membranes using peroxidase conjugates of several affinity purified lectins. Part of this work has appeared in abstract form (Wood *et al.*, 1977b).

Materials and methods

Wheat germ agglutinin (WGA) and *Ricinis communis* agglutinin (RCA) lectin were purified as described previously (Gurd & Mahler, 1974; Gurd, 1977). *Lens culinaris* (LC) lectin was obtained from Sigma Chemical Co. (St Louis, Missouri) and from EY Laboratories (San Mateo, California). Concanavalin A was obtained from Miles Laboratories (Elkhart, Illinois). Neuraminidase was obtained from Worthington (10–15 units/mg) and from Sigma (Type IX). Glutaraldehyde (8%) was purchased from Polysciences, Inc. Horseradish peroxidase (Sigma, Type VI) and 3',3'-diaminobenzidine (Sigma, no. 8126) were used in the cytochemical procedures.

Preparation of conjugates

The two-step glutaraldehyde method of Avrameas & Ternynck (1971) was used to prepare conjugates of horseradish peroxidase and the WGA, RCA and LC lectins. The peroxidase was dissolved in 0.15 M NaCl and added with constant gentle agitation to the activated 24 h. The 'activated' peroxidase was purified over a Sephadex G-25 (coarse) column (19 × 450 mm) equilibrated and eluted with 0.15 M NaCl. The lectins to be conjugated were dissolved in 0.15 M NaCl and added with constant gentle agitation to the activated peroxidase. The pH of this solution was elevated by adding 1/20 volume of 1 M bicarbonate buffer pH 9.5. After two days the reaction was terminated by addition of an excess of lysine and the conjugates were dialysed against phosphate-buffered saline (PBS), pH 7.2, containing 0.01% sodium azide. The approximate ratio of peroxidase to lectin in the conjugate was 2–3 : 1.

Preparation of tissues

Adult Sprague–Dawley rats or hatched White Leghorn chicks were anaesthesized by intraperitoneal injection of 35% chloral hydrate (1 ml/kg body weight) and then perfused through the heart for 20 min with a fixative (Wood *et al.*, 1976) containing freshly prepared 4.0% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M phosphate buffer, pH 7.2 (Millonig, 1961). After perfusion the cerebellum was removed and stored overnight at 4° C in 4.0% phosphate-buffered paraformaldehyde without glutaraldehyde. The fixed cerebellum was cut parasagitally into 50–75 μm slices with a Sorvall TC-2 tissue sectioner (Ivan Sorvall, Inc.,

Newton, Connecticut). The slices were collected in PBS, pH 7.2 and the best sections were chosen under the dissecting microscope for lectin cytochemistry.

Cytochemical procedures

All steps in the lectin labelling procedure were performed with constant gentle agitation at room temperature, except where indicated. The tissue slices were incubated in the various lectin conjugates diluted from 1 : 10 to 1 : 40 in PBS for 30–45 min. The slices were next washed in five changes of PBS over a 3 h period. Control slices were treated and washed exactly the same except that appropriate hapten inhibitors (*N*-acetylglucosamine for WGA, D-galactose for RCA and α-methyl-mannoside for LC) were included at concentrations of 0.2 M in lectin incubation steps. The washed slices were treated for 10 min at ice-bath temperature with a solution containing 30 mg 3, 3′-diaminobenzidine (DAB) and 10 μl of 30% hydrogen peroxide in 50 ml of PBS. They were next washed for 30 min in PBS, postfixed in 2.0% osmium tetroxide in 0.12 M Millonig's phosphate buffer for 30 min, and dehydrated for embedding in Epon–Araldite. Ultrathin sections were cut parallel to the parasagittal face and superficial sections were examined in a Philips EM 301 electron microscope, both with and without prior staining in lead citrate.

Results

Slices of rat or chicken cerebellum treated with WGA-peroxidase reveal extensive labelling of neuronal plasma membranes and labelling of the Golgi apparatus within neurons, but no labelling of the granular or agranular endoplasmic reticulum (Fig. 1A).The Golgi membrane labelling is restricted to the side of the membrane facing the cisternal space (Fig. 1A) and the plasma membrane labelling is restricted to the external surface of the membrane (Fig. 1C). Slices of cerebellum pretreated with neuraminidase and then stained with WGA-peroxidase show considerable diminution of the neuronal plasma membrane staining and a slight decrease in the staining within Golgi cisternae (Figs. 1B, D).

Several interesting examples of WGA labelling of specialized regions of neuronal plasma membrane are seen in these studies. The synaptic cleft region of synapses with both symmetrical and asymmetrical specializations contain reaction product for peroxidase indicating the presence of WGA binding sites within the cleft (Figs. 2A, B). The axolemma of myelinated axons also contains specialized regions that bind WGA in that the nodal axolemma labels with this lectin but the internodal axolemma does not (Fig. 2C).

Cerebellar slices treated with RCA–peroxidase show less labelling of neuronal plasma membranes but equivalent labelling within the Golgi membrane cisternae compared to WGA treatment (Fig. 3A). The rough and smooth membrane cisternae are not labelled (Fig. 3A). Neuraminidase pretreatment enhances the plasma membrane labelling with RCA but causes no apparent change in the Golgi membrane labelling (Figs. 3B, C).

Cerebellar slices treated with LC–peroxidase conjugates show extensive staining within the cisternae of the endoplasmic reticulum of Purkinje cell somata and other

neurons as well (Fig. 3D). This staining is seen within the nuclear envelope, the granular and agranular endoplasmic reticulum and the Golgi apparatus. No staining of the hypolemmal cisternae (Palay & Chan-Palay, 1974) is seen, even though we know from con A cytochemical studies that these cisternae contain mannose or glucose-like sugar residues which should bind LC. The staining of Purkinje cell somata is consistently more intense in those elements of the granular and agranular endoplasmic reticulum nearest the nucleus and less intense in these elements at the cell periphery. The hypolemmal cisternae contain very little if any product for peroxidase (Fig. 3D). The staining of Purkinje cell plasma membranes with LC is very light (Fig. 3D).

Figs. 1–3. Electron micrographs showing lectin labelling of membrane systems in rat cerebellar cortex.

Fig. 1A. WGA-peroxidase labelling within Golgi cisternae (curved arrows) of Purkinje neurons. × 30 400.

Fig. 1B. WGA-peroxidase labelling of Purkinje neurons after neuraminidase pretreatment of the tissue. The label is restricted to Golgi cisternae (curved arrows). × 20 300.

Fig. 1C. WGA-peroxidase labelling in the cerebellar molecular layer. All plasma membranes are labelled including those of the Purkinje dendrites (D) and presynaptic terminals (T). The smooth membrane profiles within Purkinje dendrites are not labelled. × 38 200.

Fig. 1D. WGA-peroxidase labelling of membranes in the molecular layer after neuraminidase pretreatment. Essentially no label is associated with Purkinje dendrites (D) or presynaptic terminals (T). × 27 300.

Fig. 2A. WGA-peroxidase labelling of membranes in the cerebellar molecular layer. The label is present on all plasma membranes and is heavy in the synaptic cleft regions (arrows). T, presynaptic terminals. × 56 600.

Fig. 2B. WGA-peroxidase labelling of a mossy fibre terminal (Mo) in the cerebellar granular layer. Label is heavy in the synaptic cleft area (arrows). × 25 500.

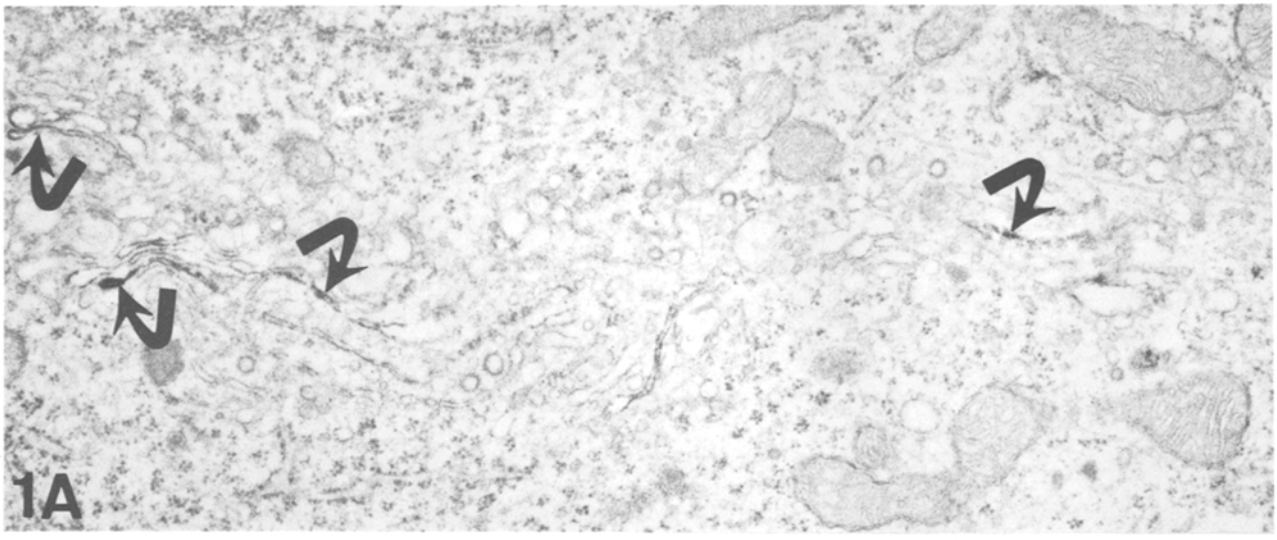
Fig. 2C. WGA-peroxidase labelling of the axolemma (large arrows) of an obliquely sectioned node of Ranvier (A). The axolemma underlying the paranodal glial loops is unlabelled (short arrows). × 39 300.

Fig. 3A. RCA-peroxidase labelling within Golgi membrane cisternae (curved arrows) of a Purkinje neuron. The rough and smooth endoplasmic reticulum is not labelled. The plasma membrane is lightly labelled (straight arrows). × 11 400.

Fig. 3B. RCA-peroxidase labelling of a Purkinje neuron is present only within Golgi membrane cisternae (curved arrows) after neuraminidase pretreatment. × 23 900.

Fig. 3C. RCA-peroxidase labelling of the Purkinje neuron plasma membrane is enhanced after neuraminidase pretreatment (straight arrows). The Golgi membrane labelling is unaffected (curved arrow). × 14 500.

Fig. 3D. LC-peroxidase labelling within the nuclear envelope (block arrows), the granular endoplasmic reticulum (short arrows) and the Golgi membrane cisternae (curved arrows) of a Purkinje neuron. The label is heavier near the cell nucleus than towards the cell periphery. The plasma membrane is lightly labelled (long arrows). × 15 400.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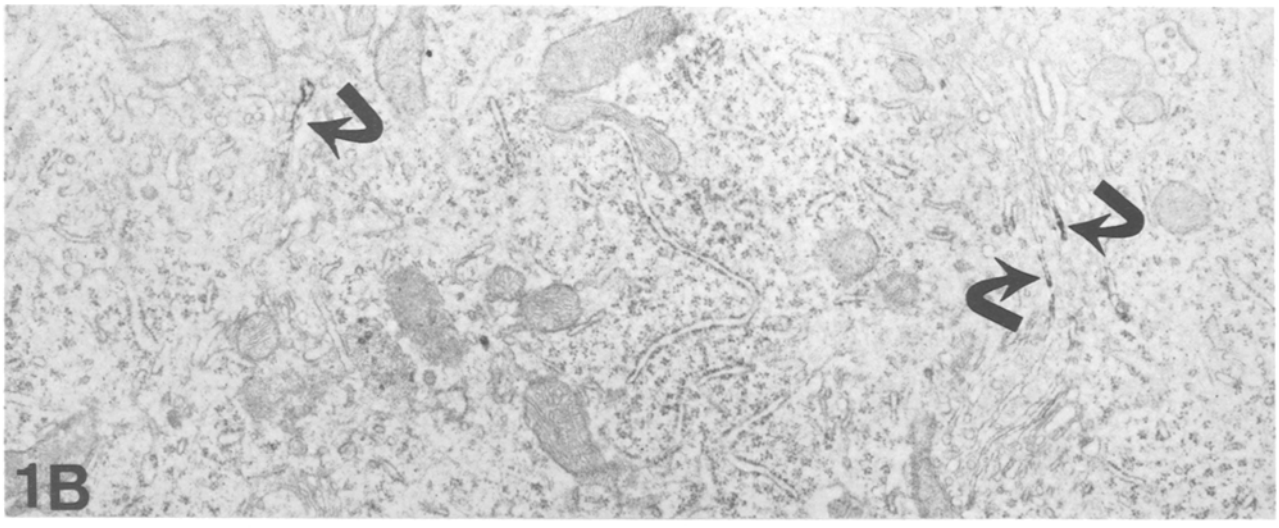
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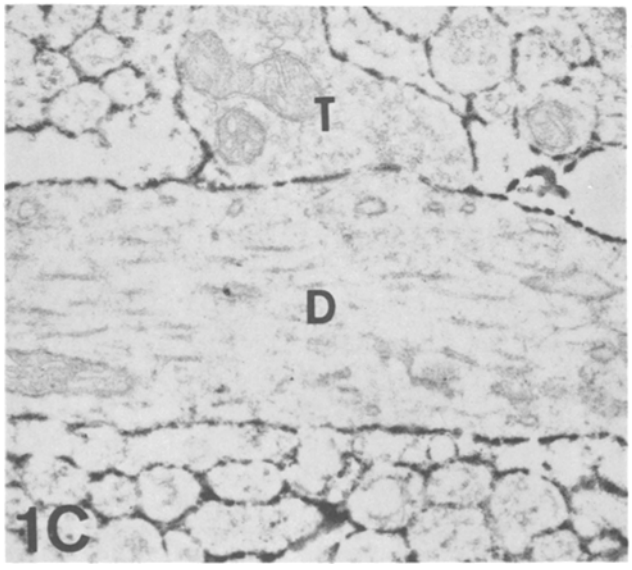
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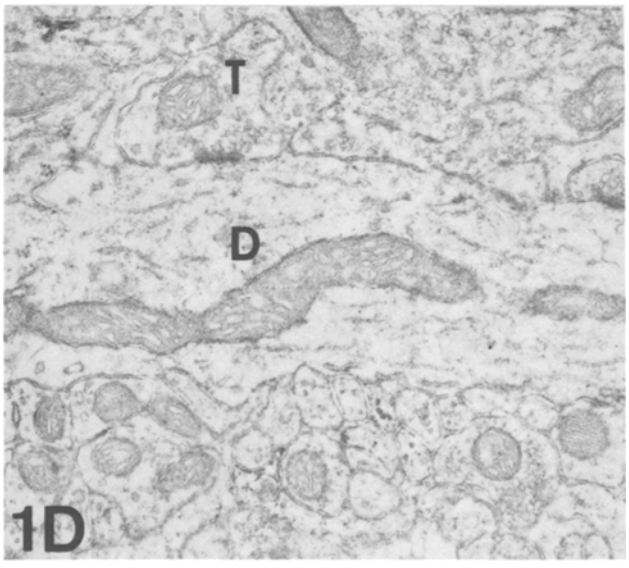
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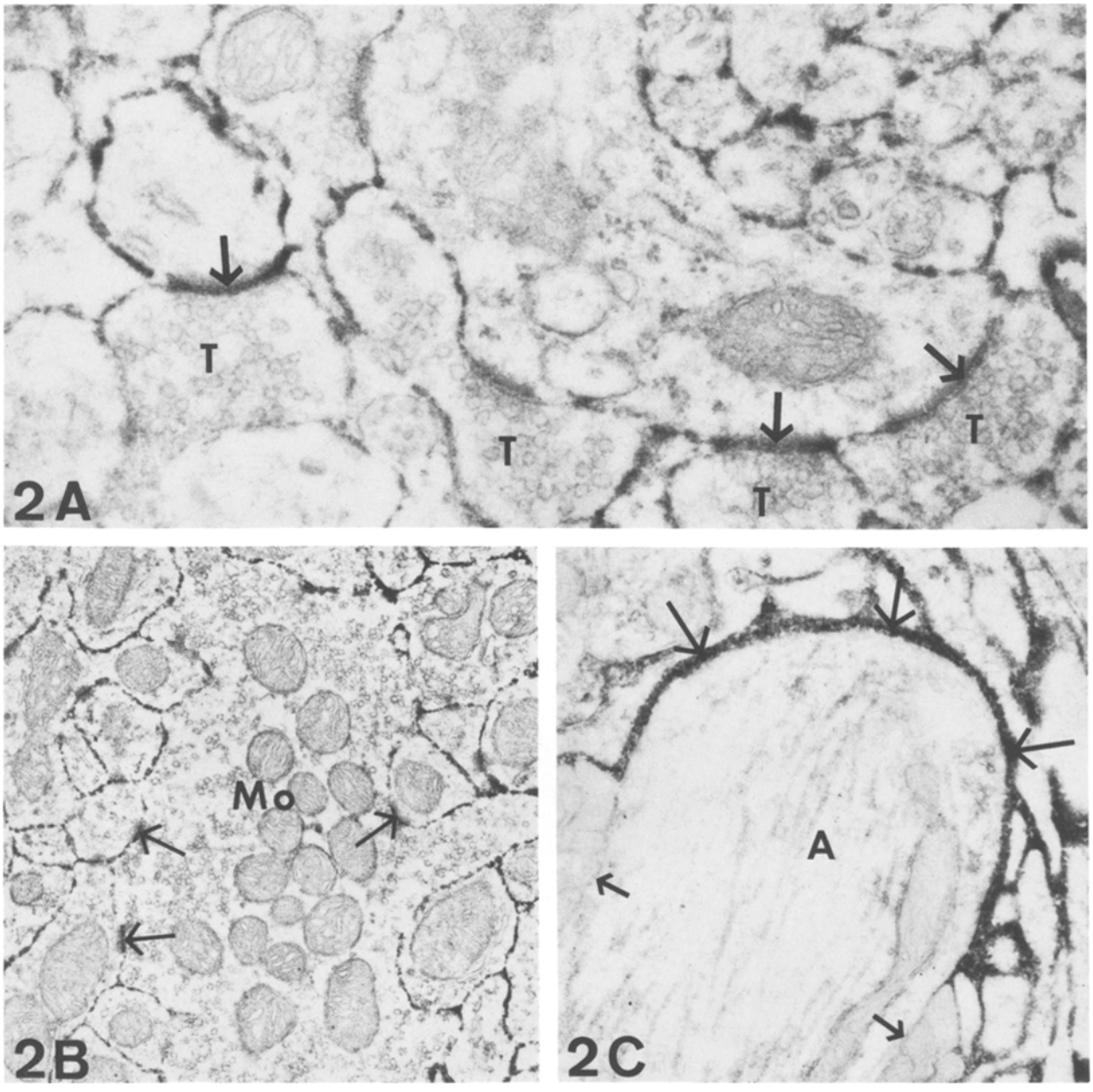
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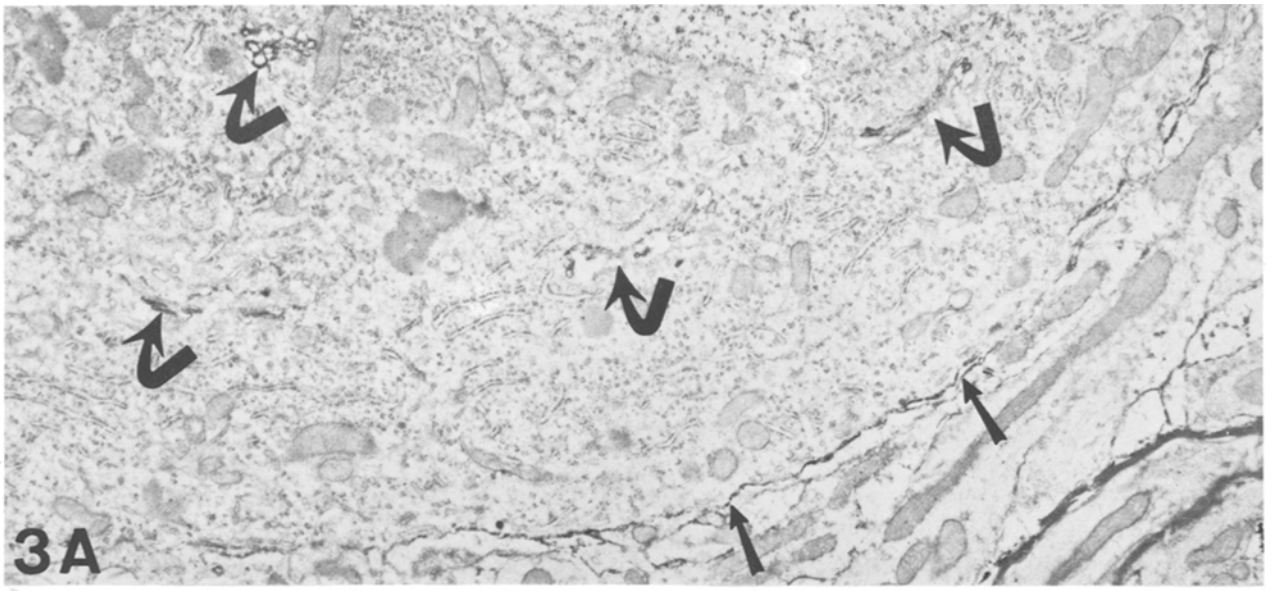
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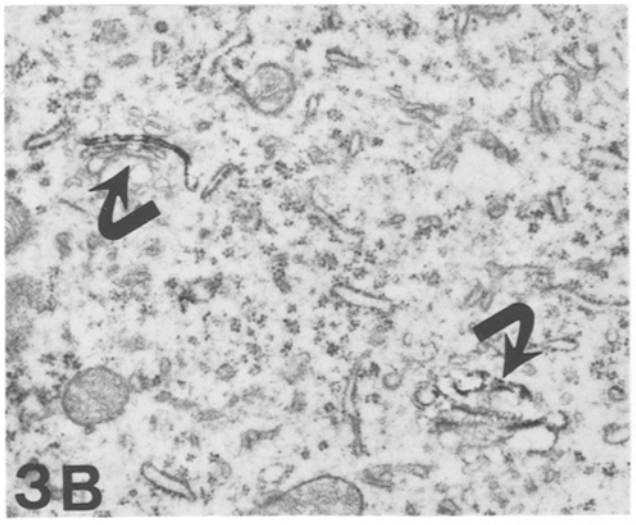
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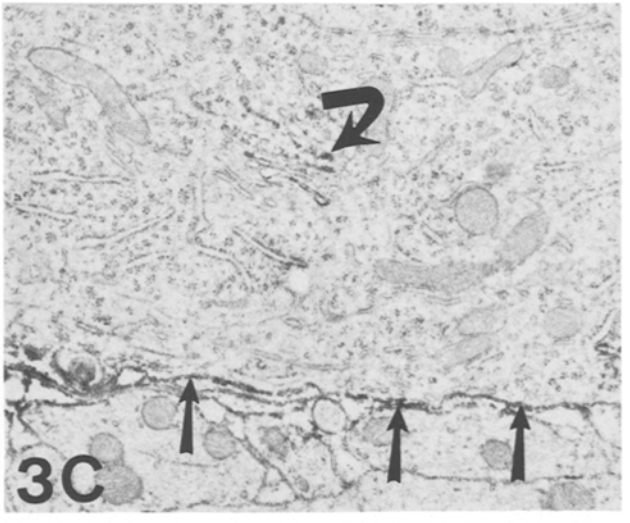
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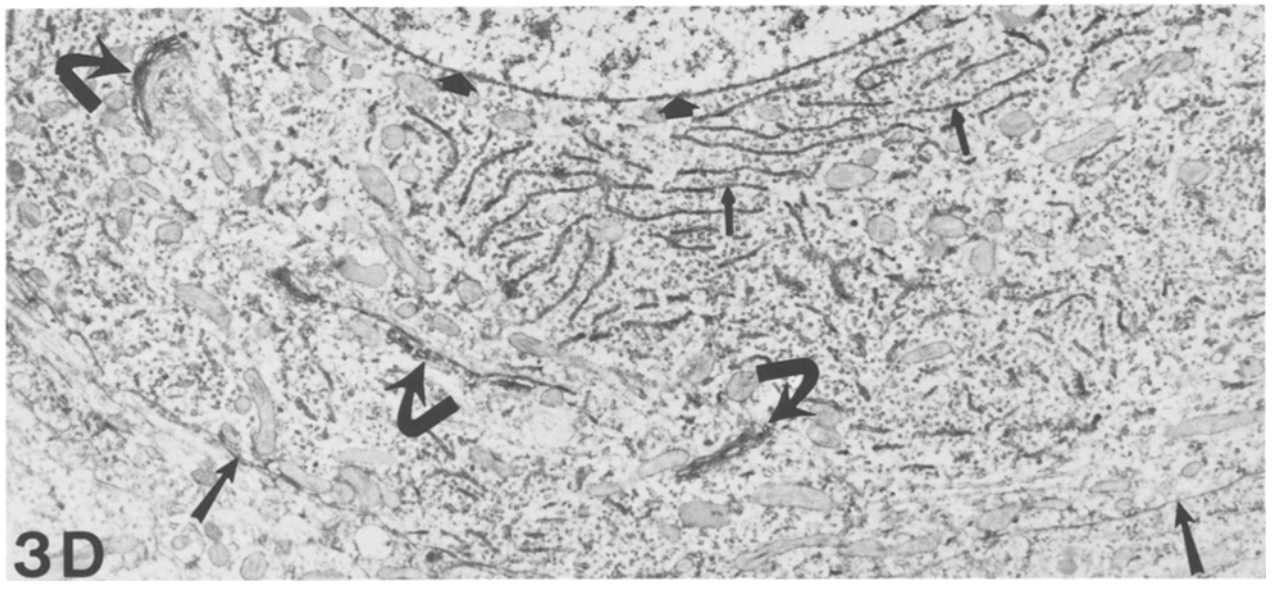
3D

Discussion

Results presented in this paper indicate that elements of the endoplasmic reticulum as well as the plasma membrane are not uniform with regard to the presence or availability for binding to lectin of specific saccharides (see also Palay & Chan-Palay, 1974; Holtzman, 1977). The WGA labelling pattern indicates an enrichment of either *N*-acetylglucosamine (GLcNAc) or *N*-acetylneuraminic acid derivatives within the Golgi cisternae and on the plasma membrane of neurons since it is known that this lectin will interact with both saccharides (Bhavanandan & Katlic, 1979). Since neuraminidase pretreatment virtually eliminates the plasma membrane staining and causes little change in the Golgi staining it is likely that the WGA is bound to *N*-acetylneuraminic acid derivatives on the cell surface and to GlcNAc on the inner surface of the Golgi membranes. Although we cannot rule out the possibility that neuraminidase will not penetrate to the Golgi region work in the paper and in our previous work (Wood & McLaughlin, 1976) indicates that this region is accessible to a variety of protein probes. Unlike our previous results (Wood *et al.*, 1974) with con A, other elements of the endoplasmic reticulum are not stained with WGA.

Several interesting WGA labelling patterns are observed on neuronal membranes. The lectin exhibits a neuraminidase-sensitive labelling of the synaptic cleft region that is at least as intense as the general labelling of axons and dendrites. Since the intact synaptic cleft is relatively inaccessible to reagents compared to other regions of synaptic membrane (Kelly *et al.*, 1976) this result suggests a considerable enrichment in WGA binding sites in the intact synaptic cleft of adult animals. Developmental studies of photoreceptor synapses also show an enrichment of WGA binding in the cleft of mature synapses (McLaughlin *et al.*, 1980). The WGA staining of the axolemma of myelinated axons is restricted to the node of Ranvier. This pattern is remarkably similar to that seen after antibody cytochemistry to localize the $Na^+ + K^+$-ATPase in knifefish brain (Wood *et al.*, 1977). The ATPase is a glycoprotein and it may be that the high concentration of WGA labelling at the Ranvier node reflects the apparent sequestering of the enzyme at the site where it is required to participate in the process of impulse conduction of the neuron (Wood *et al.*, 1977a).

The labelling pattern observed with RCA is similar to that seen with WGA in that the Golgi cisternae and plasma membrane of neurons are labelled. The result of enzyme treatment on the plasma membrane staining is reversed, however, since RCA labelling is sparse before enzyme treatment and heavy afterwards. This would be consistent with the presence of oligosaccharides on the cell surface which are enriched in a terminal *N*-acetylneuraminic acid attached to an underlying galactose residue (Nicolson *et al.*, 1975; Rosen & Hughes, 1977). The Golgi cisternae labelling with RCA is unaffected by neuraminidase pretreatment suggesting that the galactose residue of oligosaccharides lining the membrane system is not penultimate to a neuraminic acid. One alternate possibility is that the RCA labelling of the Golgi membrane is to *N*-acetylgalactosamine rather than galactose since the lectin does have some affinity for this saccharide (Lis & Sharon, 1973).

The cytochemical labelling profile of Purkinje somata obtained with LC is virtually identical to our previous results obtained with con A in that all elements of the ER are labelled on the side of the membrane facing cisternal spaces. A major difference is apparent, however, in the labelling of the hypolemmal cisternae; con A strongly labels this cisternae (Wood *et al.*, 1974) while LC does not label it at all. Con A and LC both bind to mannose residues (Lis & Sharon, 1973) but con A has a fifty-fold higher binding constant (Stein *et al.*, 1971). Although it is possible that con A is recognizing oligosaccharides of the hypolemmal cisternae that are not recognized by LC (Gurd, 1977), this result suggests that the mannose residues of the hypolemmal cisternae in neuronal processes are less available to lectins than the mannose residues of the ER cisternae present in cell somata.

One possible explanation for the results of our lectin cytochemical experiments to date is that the differences in staining represent pools of oligosaccharides of varying degrees of complexity. The glycoproteins of the rough ER, at least, are thought to contain primarily core oligosaccharides consisting of GLcNAc-mannose chains in which the GlcNAc is linked to an amino acid (Schachter *et al.*, 1979). Such glycoproteins would be expected to label with mannose binding lectins, and perhaps GlcNAc binding lectins but not other lectins. Our results with con A, LC and RCA are consistent with the possibility. The failure of WGA to label GlcNAc of the ER is not consistent with this possibility. At present we have no explanation for this unless the proximity of the GlcNAc to the protein portion of glycoproteins hinders the lectin–sugar interaction. In this regard it should be noted that the binding of WGA to internal GLcNAc residues of oligosaccharide chains may be variable (Bhavanandan & Katlic, 1979). The Golgi cisternae are thought to be a major site of elongation of glycoprotein oligosaccharides (cf. Schachter *et al.*, 1979), a process which includes addition of GlcNAc, galactose and *N*-acetylneuraminic acid residues. The Golgi should, therefore contain populations of oligosaccharides at various stages of elongation in which these sugar residues are terminal. This could explain the selective labelling with WGA and RCA of the Golgi. The WGA labelling may be due to both GlcNAc and *N*-acetylneuraminic acid but most of the label must be to GlcNAc since neuraminidase treatment causes only a slight loss of WGA Golgi staining. Con A and LC would label the Golgi as well since presumably this organelle contains core oligosaccharides with available mannose residues.

As expected the plasma membrane compared to ER membrane labelling reflects an increased complexity of cell surface carbohydrates, particularly due to addition of sialic acid residues leading to enhanced WGA staining. The other lectins used in this study bind to sugars which presumably are largely internal in the cell surface oligosaccharide chain, since all three lectins do not label the cell surface well.

Our results indicate that elements of the ER of Purkinje cells are heterogeneous with respect to their lectin binding properties. We are particularly interested in the possibility that the hypolemmal cisternae observed in Purkinje cell axons and dendrites might serve as a morphological substrate for axoplasmic transport (Wood *et al.*, 1974; Wood & McLaughlin, 1976). The lectin cytochemical results suggest that

we might utilize the lectins to identify selectively components of the hypolemmal cisternae using polyacrylamide electrophoresis together with lectin staining of the gels or using lectin affinity chromatography to isolate glycoproteins from microsomal fractions obtained from regions of cerebellum enriched in these specialized cisternae.

Acknowledgements

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