

Astrocyte Membrane Structure: Changes after Circulatory Arrest

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ABSTRACT Membranes of the astrocytic processes investing small blood vessels and the surface of the brain contain numerous arrays of orthogonally packed particles as revealed by the freeze-fracture technique. The structure of these particle arrays, which we have termed "assemblies," is the same whether tissue is prepared for freeze-fracture by conventional fixation or by quick excision and rapid freezing. However, assemblies are progressively replaced by amorphous clumps and then disappear as the interval between decapitation and rapid freezing increases. Nearly normal numbers of assemblies may be maintained in cerebellar slices *in vitro*, but there too they disappear at low P_{O_2} or in the presence of dinitrophenol. No other neuronal or glial membrane specialization exhibits a comparable lability.

Astrocytic processes in the mammalian central nervous system surround blood vessels, form the surface of the brain (the glial limitans), and invest many neuronal structures. We have previously found with freeze-fracture techniques that astrocytic processes facing the basal lamina of blood vessels or the cerebrospinal fluid have a distinctive intramembrane specialization, consisting of numerous aggregates of small intramembrane particles packed in orthogonal arrays (14). These particle aggregates, which we have termed "assemblies," are much less common in astrocytic membranes apposed to neuronal cell bodies and processes, and are virtually absent in the plasmalemma of the astrocyte cell body. The distribution of assemblies in astrocytic processes facing the cerebrospinal fluid and vascular structures suggests that they are involved in transport into or out of those compartments.

Substances moving from capillaries to the extracellular space around neurons must pass through or by three structures: the endothelial cell of the blood vessel, the surrounding basal lamina, and the astrocytic sheath. The system of tight junctions that seals endothelial cells forms a barrier between the brain and blood which blocks the transit of molecules from the vascular to the brain extracellular space (4). The plasma membrane of the endothelial cells contains several stereospecific, facilitated diffusion systems which regulate transport of amino acids, glucose, and other substances into the brain (16). What role the astrocytic sheath might have in the blood-brain barrier is unknown.

In an effort to define the properties of assemblies in astrocytic membranes, we have examined them in tissue prepared for freeze-fracture by direct freezing, without conventional chem-

ical fixation (20, 21, 11, 9). We now report that assemblies exhibit a lability unprecedented among the various intramembrane particle specializations in that they progressively are replaced by amorphous clumps and disappear within minutes after circulatory arrest *in vivo* or incubation at low P_{O_2} *in vitro*.

MATERIALS AND METHODS

Rapid Freezing

C57Bl/6 mice, 30–60 d of age, were decapitated and a hemispherical slice of cerebellar cortex 2–3 mm in diameter and 0.5–1.0 mm in thickness was rapidly excised. The slice was placed on the stage of a rapid-freezing device, mounted on a filter paper soaked in HEPES buffer, and rapidly frozen by impact against a copper block cooled by liquid helium to a surface temperature of 10°–15°K (9, 11). The interval between decapitation and freezing was <30 s. The rapidly frozen slice was then freeze-fractured in a Balzers 301 apparatus (Balzers Corp., Hudson, N. H.) fitted with electron beam guns and a quartz-crystal monitor.

Circulatory Arrest

C57Bl/6 mice were decapitated and the heads maintained in a moist chamber at 37°C for intervals of 5, 10, 15, or 30 min. A slice of cerebellar cortex was then rapidly removed and frozen within 30 s of the onset of dissection. The same procedure was followed to obtain other slices, which were fixed by immersion in HEPES buffer containing 5% glutaraldehyde for 1 h at 37°C, and then rapidly frozen.

In Vitro Incubations

C57Bl/6 mice, 30–60 d of age, were decapitated and cerebellar slices removed within 35 s. The slices were immediately introduced into one of several media: (a) HEPES buffer adjusted to pH 7.3 (NaCl 150 mM, KCl 4 mM, CaCl₂ 1 mM, MgSO₄ 1 mM, glucose 10 mM, HEPES 10 mM) in the presence of bubbling 100% oxygen; (b) HEPES buffer adjusted to pH 7.3 and bubbled with 100%

nitrogen; (c) HEPES buffer, to which dinitrophenol had been added (100 μ M), adjusted to pH 7.3, and bubbled with 100% oxygen.

The slices were maintained at 37°C for 30 min, then rapidly frozen within 15 s of removal from the incubation medium. Other slices were removed and fixed by immersion in HEPES buffer containing 5% glutaraldehyde and bubbled with the gas of the particular incubation. These fixed slices were allowed to cool from 37° to 22°C over 1 h and then rapidly frozen.

RESULTS

Rapidly Frozen Slices

It proved possible to decapitate an adult C57Bl/6 mouse, remove the calvarium, dissect out a dome-shaped slab of superficial cerebellar cortex, and rapidly freeze the slab in <30 s. The superficial 10–40 μ m of the cerebellar cortex was frozen without visible distortion by ice crystals. Other studies employing the same rapid freezing device have shown that the freezing in this layer is complete in <2 ms, and so this method can capture rapidly evolving structural changes (9). In cerebellar cortex prepared in this manner, assemblies in the glial limitans were present in the same numbers as and had a substructure identical to those in tissue prepared by conventional perfusion with aldehyde fixatives and subsequent cryoprotection with glycerol (14, 15). Similarly, the structure of assemblies in rapidly frozen tissue was the same as in tissue rapidly frozen after immersion fixation in 5% glutaraldehyde.

Circulatory Arrest

If >30 s elapsed between decapitation (functionally equivalent to circulatory arrest) and the instant of freezing, the assemblies in the glial limitans progressively disappeared while large clumps of intramembrane particles appeared in their place (Fig. 1). In the 1st min after circulatory arrest, the extent of these changes varied from process to process and even in different regions of the same astrocytic process (Fig. 1A–C). At 5 and 10 min after decapitation, the loss of assemblies was more complete, and, if 15 min elapsed between decapitation and rapid freezing, only rare persisting assemblies could be found. In the first 10 min of anoxic interval of circulatory arrest after decapitation, large, irregular, pleomorphic particles appeared in astrocytic membranes as assemblies disappeared (Fig. 1A and B). Occasionally, the substructure of these novel large particles suggested that they might represent intermediate stages in the dissolution of assemblies (arrow, Fig. 1B). At 15 and 30 min after decapitation, the large amorphous particles were much less frequently encountered (Fig. 1D and E).

In Vitro Incubations

We wondered whether our approach of hasty dissection before rapid freezing produced mechanical effects that might unpredictably alter a labile membrane component. We therefore removed dome-shaped slices of mouse cerebellum within 35 s of decapitation and maintained them in vitro for 30 min before rapid freezing. Astrocytic membrane structure in cerebellar slices incubated for 25–30 min at 37°C in HEPES buffer adjusted to pH 7.3 (NaCl 150 mM, KCl 4 mM, CaCl₂ 1 mM, MgSO₄ 1 mM, glucose 10 mM, HEPES 10 mM) in the presence of continuously bubbling 100% O₂ and then rapidly frozen was indistinguishable from that in slices frozen within 25 s of decapitation. However, assemblies were virtually absent in the glial limitans of cerebellar slices maintained in HEPES buffer at pH 7.3 continuously bubbled with nitrogen (actual P_{O₂} not ascertained, but presumed to be low). Assemblies also disap-

peared from cerebellar slices incubated for 30 min in HEPES buffer bubbled with O₂ to which dinitrophenol (100 μ M) had been added. Similar results were observed when either the *in situ* or the *in vitro* incubation experiments were followed by aldehyde fixation and rapid freezing.

DISCUSSION

The only modification of membrane structure that we found under conditions of low P_{O₂} in vitro or circulatory arrest *in situ* is the disappearance of astrocytic assemblies. In contrast, post-synaptic particle arrays, gap junctions (astrocytic and meningeal), tight junctions (meningeal cells and vascular endothelia), and neuronal plasmalemma specializations at sites of subsurface cisterns persisted under our various experimental conditions and the single particles of astrocytic and neuronal processes remained uniformly distributed over fracture faces. Assemblies therefore appear to be a particularly labile membrane specialization.

As assemblies disappear from membranes in the first 5 min after circulatory arrest, there appears in their place a novel class of large, irregular particles. By 15 min, these clumps also have in large part disappeared from the fracture face. It is probable that assemblies are in fact replaced by large clumps, and that these subsequently become smaller.

The disappearance of assemblies is not an artifact of fixation by rapid freezing, because it is also observed in tissue fixed by immersion in aldehydes. The function of the proteins represented by assemblies is unknown, and so we cannot be sure whether the morphological change accompanies alteration of the physiological properties of assemblies. Assemblies have been found to vary in number in a given membrane under several metabolic conditions. Ellisman and his colleagues have shown that the number of assemblies (also referred to as “square arrays”) is different in fast-twitch and slow-twitch muscles (8), and that reinnervation of a slow-twitch muscle by a nerve previously associated with fast-twitch muscles would result in the appearance of new assemblies by 9–12 mo after reinnervation (7). Anders and Pagnanelli (2) describe gradual, total disappearance of assemblies from membranes of cultured primary astrocyte cells over a 3-h interval in the presence of an inhibitor of protein synthesis (cycloheximide, 10⁻⁶ M), and their recovery to control numbers in the subsequent 3 h. The time-course of the changes observed in the present study is very different, and it is presently uncertain whether disappearance of assemblies after anoxic insult involves the same mechanisms that subserve loss of assemblies in the presence of cycloheximide.

We are aware of no other components of astrocytes that are distributed in the same pattern as assemblies, and suppose that assemblies may support a hitherto unappreciated membrane function. Within the central nervous system, assemblies have been detected only in astrocytes and ependymal cells, and have never been found in neuronal membranes (1, 3, 5, 14, 15). Unfortunately, consideration of the distribution of assemblies in other tissues (6, 7, 12, 13, 18, 19) has not yet made clear their function in brain.

It is intriguing that the perivascular astrocytic processes that we have found to be characterized by large numbers of assemblies are also prone to swell after hypoxic/ischemic insult (17). Such astrocytic swelling may impede circulation after hypoxic/ischemic insult, and compromise neuronal function and recovery. We suspect that the disordering of assemblies in astrocytic membranes which we observed after circulatory arrest, incu-

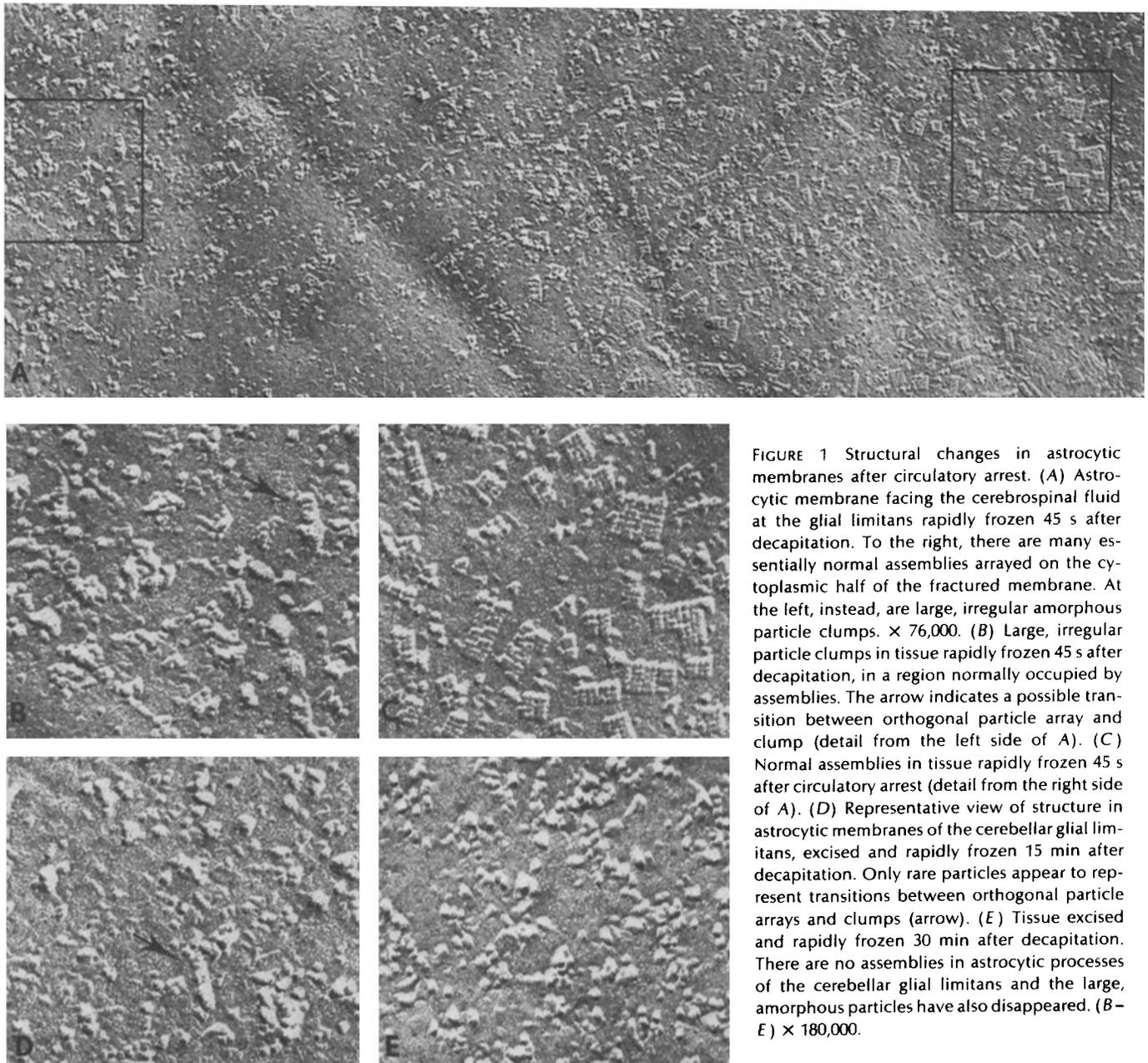


FIGURE 1 Structural changes in astrocytic membranes after circulatory arrest. (A) Astrocytic membrane facing the cerebrospinal fluid at the glial limitans rapidly frozen 45 s after decapitation. To the right, there are many essentially normal assemblies arrayed on the cytoplasmic half of the fractured membrane. At the left, instead, are large, irregular amorphous particle clumps. $\times 76,000$. (B) Large, irregular particle clumps in tissue rapidly frozen 45 s after decapitation, in a region normally occupied by assemblies. The arrow indicates a possible transition between orthogonal particle array and clump (detail from the left side of A). (C) Normal assemblies in tissue rapidly frozen 45 s after circulatory arrest (detail from the right side of A). (D) Representative view of structure in astrocytic membranes of the cerebellar glial limitans, excised and rapidly frozen 15 min after decapitation. Only rare particles appear to represent transitions between orthogonal particle arrays and clumps (arrow). (E) Tissue excised and rapidly frozen 30 min after decapitation. There are no assemblies in astrocytic processes of the cerebellar glial limitans and the large, amorphous particles have also disappeared. (B–E) $\times 180,000$.

bation at low P_{O_2} , and incubation in dinitrophenol is closely related to these disturbances of intracellular volume regulation.

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