PARTICLE ARRAYS ON INSECT NERVE MEMBRANES

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SUMMARY

Freeze-fracture studies of Lepidopteran antennae have revealed the presence of orthogonal arrays of particles on antennal nerve membranes. Initial impressions were that several different arrays were present. Optical diffraction was used to examine the arrays on the electron micrograph negatives. This technique showed that all the arrays were derived from the same basic structure, suggesting that the superficial differences in appearance were due to shadowing effects. The particles are arranged in a lattice with spacings of 10-7 nm x 9-1 nm. The arrays are not clear-cut but tend to break up, producing a disorganized region around their edges. The particles are shown to have depressions in them. However, the evidence available suggests that the arrays do not have the other characteristics of gap junctions. The arrays appear not to be present on most of the nerve membrane faces, occurring only in localized regions of the nerve membranes where they are present in large numbers. This suggests that the arrays may have a specialized local function.

INTRODUCTION

Fracture faces of membranes revealed by the freeze-fracture technique almost always contain randomly distributed particles. Their numbers can range from only a few per \( \mu m^2 \) to many thousands per \( \mu m^2 \). In nearly all membranes the particles are predominantly on the P face, while only in a few membranes are the particles more abundant on the E face (Flower, 1972; Dempsey, Bullivant & Watkins, 1973). Usually these particles are randomly distributed on the membrane, and only occasionally do the particles form recognizable linear or two-dimensional arrays. By far the most common two-dimensional particle array found in animal tissues is the gap junction. These junctions vary in detailed structure depending on the tissue type and origin, but are readily recognizable because of their particle arrangement, characteristic appearances and the involvement of both membranes in the junction.

There are now a growing number of other types of particle arrays reported in the literature. These have been found in such diverse tissues as bacterial membranes (Tauschel, 1971; Usukura, Yamada, Tokunaga & Yoshizawa, 1980), plant membranes (Staehelin, 1976) and animal membranes. Some of the types found in animal tissue seem to be of wide distribution but most seem to be confined to one type of tissue. The function of some of these arrays can be inferred from their location and the findings of other lines of research. A large number of the types reported in animal tissue have been found on excitable membranes, or on other types of cell membranes apposed to excitable membranes. These include systems where the two membranes involved are both nerves (Wiley & Ellisman, 1980); or where a nerve membrane is apposed to a Schwann cell membrane (Dermietzel, Schünke & Leibstein, 1978; Wiley & Ellisman, 1980; Peracchia, 1974), a muscle cell membrane (Heuser, Reese &
Landis, 1974; Rash & Ellisman, 1974; Ellisman, Rash, Stachelin & Porter, 1976; Rheuben & Reese, 1976), or an electroplax membrane (Cartaud, 1974; Orci, Perrelet & Dunant, 1974). The present study was undertaken to investigate fully a two-dimensional particle array found on the membrane faces of nerves within the antennae of Lepidoptera and to investigate the relationship of this array with types previously reported in other tissues in order to ascertain the functional implications of the array.

MATERIALS AND METHODS

Electron microscopy

Adults of a number of local pest species of moths, mainly Graphania species, were gathered by light trapping. Antennae were removed and placed in either insect Ringer solution or a 6% glutaraldehyde solution buffered at pH 7.4 with 0.1 M cacodylate. The nerve bundles running along the antennae were carefully dissected out. Unfixed tissue for freeze-fracturing was placed into 25% glycerol buffered at pH 7.4 with 0.1 M cacodylate for 30 min. Fixed tissue from nerve bundles for freeze-fracturing were left in the fixative solution for 1 h after dissection and then 25% glycerol was added to the solution and the samples left in this solution for 30 min. Specimens were frozen in Freon 12 at −150°C and freeze-fractured in a Balzers BA500 machine.

Specimens for sectioning were fixed in glutaraldehyde as above, post-fixed in 1% OsO4, dehydrated in an ethanol series and embedded in Spurr's resin. Sections were stained with uranyl acetate and lead citrate.

Optical diffraction

A negative of the electron micrograph to be analysed was placed in the expanded beam from a helium/neon laser (wavelength 633 nm, power output 25 mW, beam diameter 5 mm). The negative was arranged to be normal to the laser beam and was moved in its own plane until the area of interest intercepted the laser beam. The far-field (Fraunhofer) diffraction pattern was observed on a screen at a distance of about 2 m and could be photographed merely by replacing the screen with photographic film. This simple technique is frequently used in the field of laser speckle photography, where it is known as the optical-transform technique (Archbold, Burch & Ennos, 1970; Briers & Angus, 1979). In order to prevent the complete swamping of the film by the undiffracted portion of the laser beam, the zero-order component was removed by placing an opaque disc in the beam immediately before the film. The presence of this disc can be seen in Fig. 6.

RESULTS

Most moth antennae are composed of a number of segments. Each segment is covered by a large number of sensors of several different types. The numbers of each type on a single segment varies from one to hundreds (Flower & Helson, 1976). Underlying each of these sensors is a number of cells that transverse the sense into an electrical impulse for transmission along the nerve network to the brain. Within the body of the antenna, large nerve bundles run along the antenna. A cross-section of such a bundle is shown in Fig. 1. As is apparent in this micrograph, the nerves within

Fig. 1. A transverse section of a small portion of a nerve bundle running along an antenna. ×20000.

Fig. 2. Freeze-fracture replica of an unfixed glycerinated antennal nerve bundle. The P face particles (P) are grouped in small clusters rather than being dispersed. The E faces (E) have few particles on them. ×24000.
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Figs 1 and 2
such bundles are always closely packed. It is apparent, especially at higher magnifications, that in most regions the membranes of apposed cells are regularly and closely spaced. The separation of the apposing membranes usually appears to be less than 8 nm.

When these nerve membranes are examined by the freeze-fracture technique, most replicas reveal membrane P faces that have large numbers of particles on them, usually grouped together in clusters (Fig. 2). In contrast, relatively few particles are seen on the E faces in these replicas and those present appear to be randomly distributed. The replica shown in Fig. 2 was made from unfixed but glycerinated tissue. Replicas of fixed material are similar, except that particles on the P faces usually appear to be in larger clumps than are generally found in replicas of unfixed tissue (see bottom half of Fig. 3). However, in a few replicas of fixed material, and even then only in limited areas, regular arrays of particles have been seen on the P faces (large arrowhead in Fig. 3). The lack of these arrays in unfixed tissue may not be significant because of the relatively few times such groups of arrays have been found in replicas and the difficulty of processing unfixed nerves. In the regions where arrays of particles are present on the P faces, grooves or arrays of holes can be seen also on E faces (small arrowheads in Fig. 3).

Initially, the most surprising thing about these arrays was their apparent variability in the freeze-fracture replicas. Thus the arrays present in Figs 3 and 4 vary in appearance from each other in particle spacings and in the angles between reinforcement directions. The latter are most easily seen when the micrographs are viewed at glancing angles. The higher magnification micrograph of Fig. 5 shows an array having a smaller repeat structure than is visible in the arrays in Figs 3 and 4. Such variability led to an initial impression that more than one type of array may exist. In order to investigate this possibility it was decided to subject areas of some negatives to optical diffraction examination. The very simple optical diffraction technique used allowed quick examination of a number of negatives and proved capable of providing the required information about the structure of the arrays. Orientation of individual micrographs was not carried out other than to ensure that micrographs were perpendicular to the laser beam and that the area of interest was illuminated. Thus the orientations of the diffraction patterns on the optical diffraction negatives were random. However, by noting the orientation of the micrograph and the negative on any occasion, correlations between the two could be made readily. When doing this it must be remembered that any reflection in the diffraction pattern arises from a structure at right angles to the reflection.

Fig. 3. Freeze-fracture replica of a fixed and glycerinated antennal nerve bundle. The P face particles (P) in such replicas are in larger clumps but do not usually appear to be regularly arrayed (see lower P face). In some areas of such replicas, however, regular arrays of particles can be identified on the P faces (large arrowhead). Arrays of grooves or holes (small arrowheads) are present on the E faces (E). X70,000.

Fig. 4. Freeze-fracture replica of a fixed and glycerinated antennal nerve bundle. This micrograph shows a number of arrays on both P and E faces (P and E). Measurements of these arrays showed different repeats. Optical diffraction studies were carried out on the arrays. Patterns obtained from areas A, B, C and D are shown in Fig. 6. X70,000.
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Figs 3 and 4
Fig. 5. Freeze-fracture replica of a fixed and glycerinated antennal nerve bundle. The particle array (arrowhead) has a smaller repeat than is present in most arrays. × 150,000.

Fig. 6. Optical diffraction patterns taken of both P and E face arrays. The patterns A, B, C and D were obtained from areas A, B, C and D in Fig. 4. It should be noted that larger spacings in optical diffraction patterns represent smaller spacings in micrographs.
Fig. 6 shows four optical diffraction patterns, A, B, C and D, obtained from the areas similarly marked in Fig. 4. Measurement of the reflection spacings in a number of different optical diffraction patterns showed that the same spacings were present in all the patterns, although not all reflections were present in any given pattern. The reflections present in a number of optical diffraction patterns were therefore plotted out onto a single diagram (Fig. 7) to confirm this finding. All reflections did in fact lie on a single lattice as shown in Fig. 7. This finding indicated that the structures present in each of the arrays on the micrographs were identical. It should be noted that the optical diffraction patterns shown in Fig. 6 have been orientated to give identical spacings along the same figure axis and so the reflections do not correspond in relative orientation with the orientations of the arrays in Fig. 4 from which they were obtained. However, by examining corresponding optical diffraction patterns and areas of array, we were able to make a number of observations. Such examination showed that in all of these optical diffraction patterns an area existed, lying about a line through the centre, where little information was contained. Although this is much more evident in the lower-contrast original negatives, in which a large number of random low-brightness background reflections existed, than in the micrographs shown in Fig. 4, the effect can still be seen in these micrographs. Thus in Fig. 4A this area lies from lower left to upper right, while in Fig. 4B and D it lies from lower right to upper left. In all cases this direction corresponded to a direction perpendicular to the direction of shadowing. This finding indicates that in the shadowed arrays little spatial information is present at right angles to the direction of shadowing. It also explains why the strong reflections in the optical diffraction patterns tend to lie along,
or close to, the shadowing direction, i.e. they are strong because they are reinforced by being in an optimal orientation for shadowing. Comparisons showed that the strongest reflections in the optical diffraction patterns always corresponded to the most obvious repeats in the arrays. As can be seen in Fig. 6, the strongest reflections in any given diffraction pattern varied, and so the most easily observable repeat in the various arrays also differed. Thus, it was this effect that led to the initial impression that more than one type of array could exist and that the optical diffraction patterns help to explain as arising from an enhancement of a repeat or number of repeats in a given array, depending on the orientation of that array compared to the shadowing direction.

It should be noted that spacings in optical diffraction patterns are inversely proportional to those in the micrographs. Thus, a larger spacing in the diffraction pattern represents a smaller spacing in the actual micrographs. The optical diffraction diagrams show that the arrays are almost orthogonal, with repeats of \(10^{-7}\) nm \(\times 9.1\) nm. The \(87^\circ\) angle between the two repeats was approximately constant in all the electron diffraction patterns taken from several negatives. The strong maxima present in some of the diffraction patterns at twice the spacing corresponding to \(10^{-7}\) nm (Figs 6b, 7) suggest that the basic \(10^{-7}\) nm repeat is often halved in the micrographs and thus that a repeat structure of \(5.35\) nm is present in some of the micrographs along this direction. The fact that these second-order maxima are sometimes stronger than the first-order maxima makes this halving a more likely explanation than that we are merely seeing a harmonic of the basic periodicity. Examination of the areas giving such repeats in the optical diffraction patterns revealed two apparently different structures giving rise to the halving. Thus in some arrays the \(5.35\) nm repeat could be identified in areas in which small, approximately \(3\) nm, particles were arranged in rows separated by \(5.35\) nm (arrowhead in Fig. 5). In general, these areas were continuous with the more usually observed larger particles showing the \(10^{-7}\) nm repeat in the same direction. In other arrays the \(5.35\) nm repeat appears to arise from the presence of holes or depressions in the centre of the large particles. The rows of holes in the centres of the particles lie approximately halfway between the 'shadows' caused by the rows of particles themselves, thus leading to a reflection in the optical diffraction pattern corresponding to this halving. This effect can be seen in Fig. 8, either by close examination of the areas circled or by viewing the array along the direction of the arrows and at a glancing angle. The rows of depressions are difficult to identify compared to the rows of particles and we were prompted to look for them only by the results of the diffraction experiments. A similar halving of the \(10^{-7}\) nm repeat can also be seen as a secondary effect between the arrays of holes in some of the E face arrays (Fig. 9).

**DISCUSSION**

A large number of different two-dimensional particle arrays has been reported both in non-nerve tissue (Staehelin, Chlapowski & Bonneville, 1972; Cartaud, 1974; Orci, Perrelet, Malaise-Lagae & Vassalli, 1974; Wood, 1974; Skaer, Berridge & Lee, 1975;
Fig. 8. In this micrograph (at higher magnification) of a freeze-fracture replica, holes or depressions can be seen in the tops of some of the P face particles (ringed). The halving of the main repeat caused by these holes is difficult to observe, but can be seen if the micrograph is viewed at a glancing angle along the direction indicated by the arrowheads. ×150 000.

Fig. 9. Freeze-fracture replica showing an E face array. Although not easy to identify, the halving of the 10.7 nm repeat can be seen if the micrograph is viewed at a glancing angle along the direction indicated by the arrows. ×360 000.

Smith, Baerwald & Hart, 1975; Porvaznik, Johnson & Sheridan, 1976; Wood, 1977; Lane, 1979; Franzini-Armstrong, 1979; Kistler & Bullivant, 1980; Peracchia & Peracchia, 1980; Robertson & Vergara, 1980; Zampighi et al. 1982) and in tissue in which at least one nerve membrane is involved (Heuser, Reese & Landis, 1974; Landis & Reese, 1974; Peracchia, 1974; Rash & Ellisman, 1974; Rosenbluth, 1978;
Ellisman, Rash, Staehelin & Porter, 1976; Peracchia & Dulhunty, 1976; Rheuben & Reese, 1976; Dermietzel et al. 1978; Lane, 1979; Wiley & Ellisman, 1980). By far the most common type reported have been variations of the gap junction. Of the types found in non-nerve tissue, Lane (1979) working with insect rectum reported the only one with orthogonal packing and similar spacings to the present array. Of the types involving nerve membranes, the only array closely resembling the present one was reported by Peracchia (1974) in crayfish nerve. Although the spacings of this array were very similar to those in the present array, the particle size was much smaller. Furthermore, the array occurs where the nerve membrane abuts a Schwann cell membrane and the latter membrane is differentiated in the same area, although not with a complementary array but only with irregularly distributed particles. Considering the detailed differences and as the present array occurs in an area where there are only nerve membranes, it is unlikely that these two arrays are performing the same function as the array found during the present investigation.

The particle arrays observed on the membrane faces of nerves in this study were found only occasionally. On many replicas in which large areas of nerve membrane faces were revealed, no sign of such arrays was found. Even in those replicas in which particle arrays were found, the majority of the nerve membrane faces revealed were free of arrays and usually only one relatively small region was found in a replica in which the arrays were visible. In these regions relatively large numbers of arrays were seen on many neighbouring nerve membranes. As most of the nerve membrane P faces revealed in these replicas contained large numbers of particles and in view of the scarcity of the arrays, we wondered whether the arrays were being occasionally generated inadvertently from an 'in vivo' state of dispersed particles. Some experiments were carried out to test this hypothesis but they did not reveal any obvious variation in the occurrence or extent of the arrays.

The occurrence of two apparently different structures giving rise to the 5-35 nm repeat in the arrays is unusual. In most instances this repeat appears to arise from holes or depressions in the tops of the large particles making up the 10-7 nm \times 9.1 \text{nm} arrays. In a few cases only did the 5-35 \text{nm} repeat arise from an array of small particles as in Fig. 5. The fact that the two structures have identical repeats indicates that they both arise from the same basic structure. The most likely explanation of the small particle array is that it represents a structure underlying the large particles, as a number of large particles can often be seen standing proud of these arrays (Fig. 5). An alternative, though less likely, explanation is that the large particles could be made up of smaller subunits that can be visualized under some shadowing conditions.

The finding of holes or depressions in the tops of the large particles in the arrays suggests that the arrays could represent unusual gap junctions. This would be a very surprising finding, as all previous gap junctions in which regular structure has been apparent have been hexagonally packed. However, if these arrays are analogous to gap junctions they should show continuity of the array from P face to E face across membrane transitions. P face particle arrays are often found closely associated with arrays of holes on E faces. Unfortunately, because of the shape of the nerves not many fractures occur from one membrane face to the apposing one actually on the nerve
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membrane. Instead, in most cases either a P face or an E face of a membrane is revealed. Thus few fractures occur in such a position that one could hope to see continuity between the two arrays. This difficulty is confused further by the woolly-edged appearance of the arrays. Unlike gap junctions, which tend to terminate at a well-defined line, these arrays break up to produce a confused region around the edge. This makes the positive identification of continuity between the two faces even more difficult. Although large numbers of arrays have been observed, in only a few does a fracture occur either through or at the edge of an array. In none of these cases can complementarity be demonstrated. Certainly, two common occurrences in freeze-fractured gap junctions have not been observed in these arrays: the fracture plane has not been observed to step between membrane faces within the junction, nor does it appear to break into the cytoplasm within the junction (Wood, 1977). It should be noted at this point that a similar square array has been observed in eye lens tissue. Some authors (Peracchia & Peracchia, 1980) have argued that this structure is a variant of the gap junction structure produced by the preparation procedures used, while others (Kistler & Bullivant, 1980; Zampighi et al. 1982) have claimed that they are nothing to do with gap junctions. Kistler & Bullivant (1980) have claimed further that the lens arrays are single-layered, i.e. that similar structures do not occur in register in adjacent membranes. There appear to be, therefore, some similarities between the lens arrays and the arrays described in this paper. However, the lens arrays have a much smaller repeat of 6.5–7 nm, the particles in them show no sign of having a central hole or depression (Kistler & Bullivant, 1980) and the arrays are much more regular in structure.

Several sectioning studies of insect antennae have shown that sufficient axons run within the antennal nerve bundles to account for every axon leaving the sensors (Borg & Norris, 1971; Lewis, 1970; Dethier, 1971; McIver, 1978). Although this finding does not rule out inter-axon communication, it does suggest that no significant fusion or termination of axons is likely to be occurring between the sensors and brain, and that communication between axons may therefore not be necessary. Furthermore, apparently hollow particles have been reported in a number of systems in which function does not seem to involve direct cell-to-cell communication. In some of these cases the hollow particles have been found in membranes that do not appose other membranes closely (Orci et al. 1977), while in other cases no equivalent particles have been found in the other membrane (Cartaud, 1974; Peracchia, 1974). It has been suggested that these particles could be involved in trans-membrane transport of ions or molecules. However, apparently hollow particles have also been reported in vertebrate bladder membrane (Hicks & Ketterer, 1970; Staehelin et al. 1972; Robertson & Vergara, 1980), which is known to have low permeability. Thus the presence of apparently hollow particles in membranes is not a good indication of cell–cell communication or of membrane permeability.

There appears to be no need for structural rigidity of the nerve membranes in insect antennae, as there is in vertebrate bladder membranes, so that it is possible that the arrays found in antennal nerves may be involved in trans-membrane transport. Orci et al. (1977) interpreted the function of the dispersed hollow particles they found on
several membrane types as generalized transport pathways. If they were correct, then, considering the large number of dispersed particles on the antennal nerve membranes it is difficult to see why arrays would be needed for general transport. The fact that the arrays have been found in confined regions only, and then in fairly large numbers, suggests a need for a specialized membrane transport for a local purpose. Unfortunately, no obvious differences have been observed in such regions of the nerve bundles in the freeze-etch replicas examined.

On the balance of probability, therefore, the particle arrays reported do not appear to provide direct cell–cell interlinks but could be involved in some type of transmembrane transport of ions or molecules. The relatively large numbers of arrays that have been found in limited regions only suggests that they have a specific function that is needed in certain regions only. No information is available from the present study, or from previous studies of similar arrays, to suggest either the need for such arrays or why they occur over such limited regions of the nerve membranes.

REFERENCES


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(Received 15 April 1982)