ORIGINAL ARTICLE

Resting microglial cells exhibit tubular membrane protrusions

Ulrike Gimsa[1], Veronika Kralj-Iglič[2], Jan Gimsa[3], and Aleš Iglič[4]

Abstract

Gimsa, U., Kralj-Iglič, V., Gimsa, J., and Iglič, A.
Resting microglial cells exhibit tubular membrane protrusions
Songklanakarin J. Sci. Technol., 2002, 24(Suppl.) : 771-776

Nano- and microtubular structures have recently become a subject of increasing interest due to their importance in biology and medicine as well as their technological potential. Such structures have been observed in anorganic (Iijima, 1991) as well as in organic (Schnur 1993; Oda *et al.* 1991) systems. Micro- and nanotubular protrusions of bilayer membranes have been found in cells (Kralj-Iglič *et al.* 1998; Kralj-Iglič *et al.* 2001a) and phospholipid vesicles (Kralj-Iglič *et al.* 2002; Kralj-Iglič *et al.* 2001b). In this work we describe membrane protrusions in microglial cells.

Key words : microglia, shape, tubular structure, bilayer couple model

[1]Ph.D, University of Rostock, Department of Neurology, Gehlsheimerstr. 20, D-18055 Rostock, Germany, [2]Ph.D., D.Sc, Institute of Biophysics, Faculty of Medicine, Lipičeva 2, SI-1000 Ljubljana, Slovenia, and Laboratory of Applied Physics, Faculty of Electrical Engineering, Tržaška 25, SI-1000 Ljubljana, Slovenia, [3]Ph.D., D.Sc., Prof., University of Rostock, Department of Biology – Chair of Biophysics, Gertrudenstr. 11A, D-18057 Rostock, Germany, [4]Ph.D., D.Sc., Prof., Laboratory of Applied Physics, Faculty of Electrical Engineering, Trzaska 25, SI-1000 Ljubljana, Slovenia
Corresponding e-mail : ulrike.gimsa@med.uni-rostock.de
Received, 16 January 2003 Accepted, 18 April 2003

Microglial cells are small, non-neuronal interstitial cells of mesodermal origin that form part of the supporting structure of the central nervous system. They are migratory and act as phagocytes to waste products of nerve tissue. Thus, microglial activation is a feature that commonly accompanies neurodegeneration, neuroinflammation and demyelination. Recently, a study has been performed investigating the ability of brain-invading T cells to induce axonal destruction and the involvement of microglial cells in this process (Gimsa *et al*. 2000). We utilized a murine entorhinal-hippocampal slice culture to analyze the influence of T cells on microglial cells in an organotypic setting. The slice culture has proven feasible for analysis of glial cell activation. This reaction was accompanied by upregulation of activation markers of microglial cells such as major histocompatibility complex (MHC) class II and intercellular adhesion molecule 1 (ICAM-1) expression and phagocytosis of axonal material induced by T cells. Microglial activation was also characterized by shape changes from a resting to an activated morphology involving thin tubular membraneous structures.

The mechanisms that are important for the stability of thin tubular protrusions of the cell membrane are not fully understood so far. According to the fluid mosaic model (Singer and Nicholson 1972), the membrane is composed of a matrix formed by the phospholipid molecules in which various molecules such as membrane proteins are embedded. The matrix may be considered as a two dimensional fluid with inclusions that are more or less free to move within the membrane plane. There is no preferred orientation of the inclusions with respect to the membrane normal. Such a view is consistent with a mathematical model based on an isotropic membrane elasticity. Within this model the stable shape will correspond to the minimum of the membrane free energy F_b

$$F_b = k_c/2 \int (2H)^2 \, dA + k_G \int (C_1 C_2) \, dA + k_n A \, (<H> - H_o)^2 , \quad (1)$$

where k_c stands for the isotropic bending constant, $H = (C_1 + C_2)/2$ for the local mean curvature, C_1 and C_2 for the principal membrane curvatures ($C_1 > C_2$), dA for an area element, k_G for the Gaussian constant, k_n for the non-local bending constant, and $<H>$ for the average mean curvature of the membrane:

$$<H> = 1/A \int H \, dA , \quad (2)$$

H_o is a constant that reflects the asymmetry of the composition of the two membrane leaflets and their interactions with the respective surrounding solutions. The above model, expressed by the local and non-local terms proposed by Canham (1970), Helfrich (1974), Evans (1974), and Evans and Skalak (1980) is an acknowledged model for determination of the membrane shape. However, it has been recently shown (Kralj-Iglic *et al*. 2002) that this model can not explain the stability of thin anisotropic structures such as micro- and nanotubes attached to a mother cell. In order to explain these structures, the above model has been extended by the assumption that membrane constituents at strongly anisotropically curved regions such as tubular protrusions, exhibit a preferred in-plane orientation (Kralj-Iglič *et al*. 1999). The consequent orientational ordering of the membrane constituents gives rise to the so-called deviatoric elasticity.

Membrane inclusions of a given species that are free to move laterally in the membrane contribute to the membrane free energy (Kralj-Iglič *et al*. 1999)

$$F_d = -NkT \ln[1/A \int \exp(-\xi(H - H_m)^2/2kT - (\xi + \xi^*)(H^2 - C_1 C_2 + D_m^2)/4kT) \, I_0((\xi + \xi^*) D D_m/2kT) \, dA], \quad (3)$$

where N is the number of inclusions of that species, k is Boltzmann's constant, T is the temperature, ξ and ξ^* are the constants describing the interaction between the inclusion and the surrounding membrane, H_m is the mean curvature of the intrinsic shape of the inclusion, I_0 is the mo-

dified Bessel function, $D = (C_1 - C_2)/2$, is the curvature deviator and D_m is the curvature deviator of the intrinsic shape of the inclusion. If the inclusion is strongly anisotropic (D_m large) and located in the region of a large curvature deviator D like in a thin tubular protrusion, the modified Bessel function yields a large positive value and hence (as can be seen from Eq.(3)), the contribution of the inclusions becomes more negative. In other words, accumulation and orientational ordering of anisotropic membrane inclusions on thin tubular membrane protrusions cause a decrease of the membrane free energy resulting in a stabilization of these structures.

In this work we show that thin tubular protrusions of the microglial cell membrane are formed in the resting state while in the activated state they were not observed. We hypothesize that thin tubular protrusions are made stable by the mechanisms involving orientational ordering of the anisotropic membrane constituents on the protrusions.

Materials and Methods

Preparation of slice cultures

Entorhinal-hippocampal slice cultures were prepared from 11-day-old B10.PL mice (Jackson Laboratories, Boston, MA, USA). After decapitation of the animals, brains were rapidly removed under sterile conditions and placed in ice-cold preparation medium, consisting of minimum essential medium (MEM, Gibco Life Technologies) with 1% L-glutamine (Gibco) at pH 7.35. The frontal pole was removed and, beginning at the ventral surface, the brains were cut into 350µm thick horizontal slices on a vibratome (Technical Products International, St. Louis, MO, U.S.A.). The slices containing the hippocampi were cultured on Falcon cell culture inserts, pore size 0.4µm (Becton Dickinson, Franklin Lakes, NJ, U.S.A) in 6-well plates. The slices faced air on their top and medium (via the membrane of cell culture inserts) at the bottom. The medium for cultivation contained 50% MEM, 25% HBSS (Gibco), 25% normal horse serum (Gibco), 2% glutamine, 10µg/ml insulin-transferrin-sodium selenite supplement (Boehringer Mannheim, Germany), 2.64mg/ml glucose (Braun, Melsungen, Germany), 0.1 mg/ml streptomycin (Sigma, Deisenhofen, Germany), 100 U/ml penicillin (Sigma), and 0.8 µg/ml vitamin C (Sigma). The slice cultures were incubated at 35°C in humidified atmosphere with 5% CO_2.

T cell isolation and activation

Non-specific wildtype T cells: Spleens and lymph nodes of adult B10.PL mice were homogenized and washed. Erythrocytes were lysed by incubation in 0.83% NH_4Cl for 5 min at room temperature followed by washing in RPMI-1640. These cells were activated by incubation for 2 d at 37°C with phorbol myristate acetate (PMA) (5 ng/ml; Sigma) plus ionomycin (1 µg/ml;) in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin/ streptomycin (Sigma) and 2 mM L-glutamine (Gibco). MBP-specific T cells: Leucocytes were isolated as described above from transgenic mice on a B10Pl background, carrying a TCR for MBP peptide Ac1-11 (kind gift of David Wraith, Bristol [Liu *et al*. 1995]) and cultured with 0.3 µg/ml MBP peptide (Ac1-11) (Pepscan Immunoanalytic, Berlin, Germany) for 2 d in supplemented RPMI-1640 medium as described above.

Fixation and sectioning of slices

Cultures were fixed at 8 div in 4% paraformaldehyde / 15% picric acid / 0.1% glutaraldehyde / 0.1 M phosphate buffer (pH 7.4) for 15 min and washed. The procedure was repeated without glutaraldehyde. Fixed slices were incubated in phosphate buffered sucrose solution at 0.8 M to 1.4 M for 1 week before horizontal sectioning on a Jung cryostat 2800 Frigocut-E (Cambridge instruments, Nussloch, Germany) at -34°C. Slices were cut into 14µm sections for fluorescence and light microscopy.

Immunohistochemistry

Microglial cells were stained with *Griffonia simplicifolia* isolectin-B_4 (GFS-IB_4; 1:40; Sigma) which was conjugated with FITC. Following immunohistochemistry, 20 mm sections were studied under a Leica DM IRBE inverse confocal laser scanning microscope (Leica Camera AG, Solms, Germany) equipped with an argon/krypton laser. Images were acquired using ScanWare 5.10.

Results and Discussion

The entorhinal-hippocampal slice culture provides a model system of organotypic tissue in which functionally relevant processes involving neuronal as well as non-neuronal cells can be studied (Diekmann *et al*. 1994; Hailer *et al*. 1996; Heppner *et al*. 1998). An important feature of the model is that microglial cells return to a resting state after a brief activation phase following explantation. Among other parameters, this is demonstrated by a ramified shape directly after explantation, an amoeboid morphology during the activation phase of about 1-5 d *in vitro* and reassumption of a ramified, i.e. resting, morphology at about 6 d *in vitro*. We used this model system to investigate the influence of nonspecifically activated T cells on the activation of microglial cells. Taking into account that the microglial cells close to the sectioned surfaces of the slices remain activated (Hailer *et al*. 1996) we only analyzed the middle zone of the slices. In control cultures without T cells, microglial cells had a ramified appearance with long cellular processes (Figure 1a) while their morphology in cultures with activated T cells was amoeboid (Figure 1b). It can be seen that the resting cells exhibit numerous protrusions and blebs of the plasma membrane while in activated cells the membrane contour is much smoother. There is no evidence that the protrusions contain rigid structures composed of protein (such as microtubular rods [Iglič *et al*. 2001]), however, the protrusions and blebs may be stable also without such structures.

In the introduction we have presented the onset of deviatoric elasticity due to the anisotropic membrane inclusions of a single species. Generalization of these principles to more than one species of inclusions is straightforward and the contributions to the free energy of the respective species can be summed up. Further, the same principles, though elaborated by a different statistical mechanical derivation, can also be applied to the matrix formed by phospholipid molecules which are anisotropic due to their chemical structure (two tails and a head) (Kralj-Iglič *et al*. 2002).

Recently, it has experimentally been proven

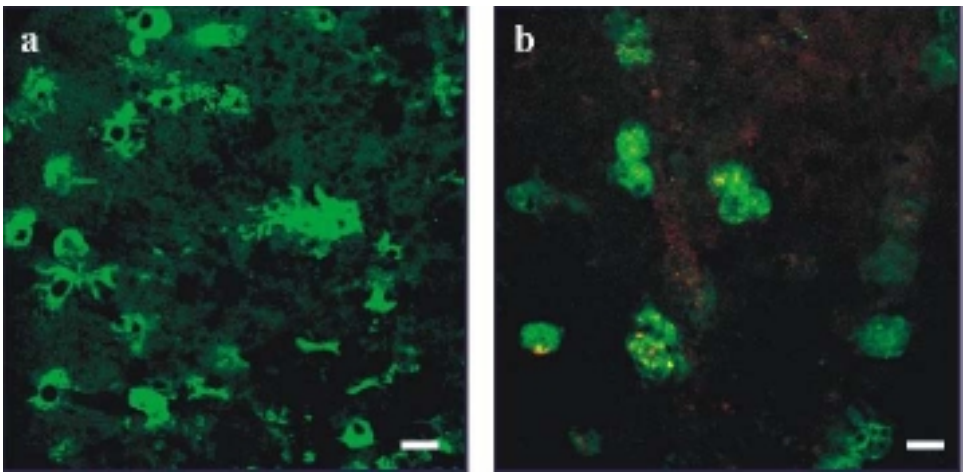

Figure 1. Resting microglial cells (a) and activated microglial cells (b). Scale bar=20 μm.

that stable nanotubes formed of phospholipids are commonly attached to giant POPC vesicles (Mathivet *et al*. 1996; Kralj-Iglic *et al*. 2001b). These structures are so thin that they cannot be directly observed by optical microscopy and have therefore been overlooked for a long time (Mathivet *et al*. 1996). As the phospholipid matrix is the predominant structure of the cell membrane, with inclusions additionally supporting the proposed mechanism, the question can be posed whether such nanostructures are a common feature of biological cells. If yes, activated microglial cells may be a model system for these structures. The process of the formation of tubular protrusions of microglial cells and their structure have not been described, yet. However, since they can be stained by lectins, their visible part must contain protein inclusions (see Figure 1a). The stained protrusions can be estimated to have a diameter of less than 1µm. Due to the staining technique used in our study and the optical resolution of our system we cannot exclude that the protrusions proceed into longer and thinner structures composed entirely of phospholipids. To further elaborate this problem, the transformation of microglial cells from their resting (Figure 1a) to their activated state (Figure 1b) has to be followed microscopically, preferably by video-microscopy (Weiss 1986).

Conclusion

We have observed thin tubular membraneous structures in microglial cells. These structures mimic findings on artificial phospholipid vesicles. Our results strongly support the hypothesis that deviatoric elasticity induced by orientational ordering of anisotropic membrane constituents provides the mechanism explaining stability of these structures. Indeed, we found no evidence that would point to other mechanisms.

References

Canham, P.B. 1970. The minimum energy of bending as a possible explanation of biconcave shape of the human red blood cell. J. Theor. Biol., 26: 61-81.

Diekmann, S., Nitsch, R. and Ohm, T.G. 1994. The organotypic entorhinal-hippocampal complex slice culture of adolescent rats. A model to study transcellular changes in a circuit particularly vulnerable in neurodegenerative disorders. J. Neural Transm. Suppl., 44: 61-71.

Evans, E. 1974. Bending resistance and chemically induced moments in membrane bilayer. Biophys. J., 14: 923-931.

Evans, E. and Skalak, R. 1980. Mechanics and Thermodynamics of Biomembranes. CRC Press, Boca Raton .

Gimsa, U., Peter, S.A., Lehmann, K., Bechmann, I. and Nitsch, R. 2000. Axonal damage induced by invading T cells in organotypic central nervous system tissue *in vitro*: involvement of microglial cells. Brain Pathol., 10: 365-377.

Hailer, N.P., Järhult, J.D. and Nitsch, R. 1996. Resting microglial cells *in vitro*: analysis of morphology and adhesion molecule expression in organotypic hippocampal slice cultures. Glia, 18: 319-331.

Helfrich, W. 1974. Blocked lipid exchange in bilayers and its possible influence on the shape of vesicles. Z. Naturforsch., 29c: 510-515.

Heppner, F.L., Skutella, T., Hailer, N.P. and Nitsch, R. 1998. Activated microglial cells migrate towards sites of excitotoxic neuronal injury inside organotypic hippocampal slice cultures. Eur. J. Neurosci., 10: 3284-3290.

Iglič, A., Veranic, P., Batista, U. and Kralj-Iglič, V. 2001. Theoretical analysis of shape transformation of V-79 cells after treatment with cytochalasin B. J. Biomechanics, 34: 765-772.

Iijima, S. 1991. Helical microtubules of graphitic carbon, Nature, 345: 56-58.

Kralj-Iglič, V., Batista, U., Hägerstrand, H., Iglič, A., Majhenc, J. and Sok, M. 1998. On mechanism of cell plasma membrane vesiculation. Radiol. Oncol. 32: 119-124.

Kralj-Iglič, V., Heinrich, V., Svetina, S. and Žekš, B. 1999. Free energy of closed membrane with anisotropic inclusions. Eur. Phys. J. B., 10: 5-8.

Kralj-Iglič, V., Iglič, A., Bobrowska-Hägerstrand, M. and Hägerstrand, H. 2001a. Tethers connecting daughter vesicles and parent red blood cell may

be formed due to ordering of anisotropic membrane constituents, Coll. Surf. A , 179: 57-64.

Kralj-Iglič, V., Gomiscek, G., Majhenc, J., Arrigler, V. and Svetina, S. 2001b. Myelin-like protrusions of giant phospholipid vesicles prepared by electroformation, Coll. Surf. A, 181: 315-318.

Kralj-Iglič, V., Iglič, A., Gomiscek, G., Sevsek, F., Arrigler, V. and Hägerstrand, H. 2002. Microtubes and nanotubes of a phospholipid bilayer membrane, J. Phys. A: Math. Gen., 35: 1533-1549.

Liu, G.Y., Fairchild, P.J., Smith, R.M., Prowle, J.R., Kioussis, D. and Wraith, D.C. 1995. Low avidity recognition of self-antigen by T cells permits escape from central tolerance. Immunity, 3: 407-415.

Mathivet, L., Cribier, S. and Devaux, P.F. 1996. Shape change and physical properties of giant phospholipid vesicles prepared in the presence of an AC electric field. Biophys. J., 70: 1112-1121.

Oda, R., Huc, I., Schmutz, M., S.J. Candau S.J. and MacKintosh, F.C. 1999. Tuning bilayer twist using chiral counter-ions. Nature, 399: 566-569.

Schnur, J.M. 1993. Lipid tubules – a paradigm for molecularly engineered structures. Science 262, 1669-1676.

Singer, S.J. and Nicholson, G.L. 1972. The fluid mosaic model of the structure of cell membranes. Science, 175: 720-731.

Weiss, D.G. 1986. Visualization of the living cytoskeleton by video-enhanced microscopy and digital image processing. J. Cell Sci. Suppl., 5: 1-15.