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FREEZE-FRACTURE STUDIES OF FROG ATRIAL FIBRES

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SUMMARY

The freeze-fracturing technique was used to characterize the junctional devices involved in the electrical coupling of frog atrial fibres. These fibres are connected by a type of junction which can be interpreted as a morphological variant of the 'gap junction' or 'nexus'. The most characteristic features are rows of 9-nm junctional particles forming single or anastomosed circular profiles on the inner membrane face, and corresponding pits on the outer membrane face. Very seldom aggregates consisting of few geometrically disposed 9-nm particles are found. The significance of the junctional structures in the atrial fibres is discussed, with respect to present knowledge about junctional features of gap junctions in various tissues, including embryonic ones.

INTRODUCTION

In mammalian myocardial fibres, there is considerable experimental evidence that the facilitated passage of electrical current between cardiac cells is associated with the presence of a 'gap junction' (Dreifuss, Girardier & Forssman, 1966; Goshima, 1970). The 'gap junction', also called 'nexus' is defined as close contact between plasma membranes of 2 adjacent cells (Revel & Karnovsky, 1967). Similarly, propagation of action potentials across inactive portions of atrial bundles in sucrose demonstrates electrical coupling between amphibian atrial cells. In other words, junctions between these cells are of sufficiently low resistance to allow transmission of action potentials with a considerable margin of safety (Barr, Dewey & Berger, 1965). However, in the case of frog atrial fibres, it has not yet been demonstrated whether or not the electrical coupling is dependent upon the existence of close contact between the plasma membranes ('gap junction' or 'nexus'). The existence of nexus has been claimed by Barr *et al.* (1965) utilizing potassium permanganate fixation of this tissue. More recent observations, of specimens fixed in glutaraldehyde-osmium tetroxide, have not confirmed these conclusions (Sommer & Johnson, 1969; Denoit-Mazet & Vassort, 1971). In a previous study (Mazet, 1975) we showed that the appearance of gap junctions as 7-layered junctions is dependent upon fixation technique, as already suggested with nervous tissue (Brightman & Reese, 1969). Staining with uranyl acetate 'en bloc', according to Farquhar & Palade (1965) and Revel & Karnovsky (1967) has provided a more appropriate tool for definition of junctions. In a preliminary study using this technique we observed that frog atrial fibres are connected by

punctate close contacts of plasma membranes, as has been found in other tissues (Pinto da Silva & Gilula, 1972; Revel, Yip & Chang, 1973).

In this paper, freeze-fracture studies of frog auricular fibres have provided confirmation of a type of junction which may be considered as a morphological variant of the nexus or gap junction.

MATERIAL AND METHODS

The atrial fibres were dissected from the auricular wall of the frog heart in ringer solution and gently stretched between 2 needles before fixation. For thin sectioning, the stretched fibres were fixed in 1·12 % $KMnO_4$ in veronal acetate buffer (0·14 M) at pH 7·4 for 1 h (Luft, 1956). The fixed material was subsequently dehydrated through a graded series of ethanols (up to absolute) and 2 washes with pure acetone which had been dehydrated by Actigel, and then embedded in Araldite.

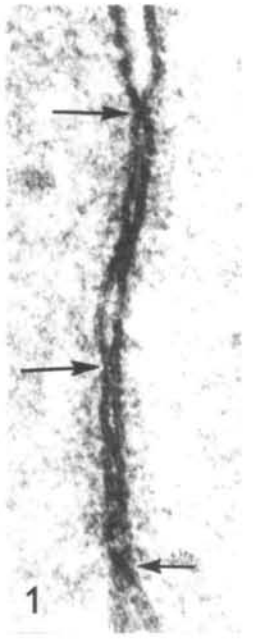
For freeze-fracturing, atrial fibres were fixed 3–4 h in 3 % glutaraldehyde in 0·2 M cacodylate buffer. They were then washed overnight in the same buffer, infiltrated for 2 h with 10 % glycerol, then for another 2 h with 25 % glycerol at 4 °C. Small fragments of trabeculae were mounted on gold disks, and rapidly frozen in Freon 22, and stored in liquid nitrogen. Freeze-fracturing and platinum-carbon shadowing were performed with a Balzer's apparatus. During the whole operation, the specimen temperature was maintained at −140 °C. The cleaned replicas were studied with a Siemens IA and a Philips EM 301 electron microscope. Micrographs are mounted with the direction of shadowing from bottom to top. Shadows appear white.

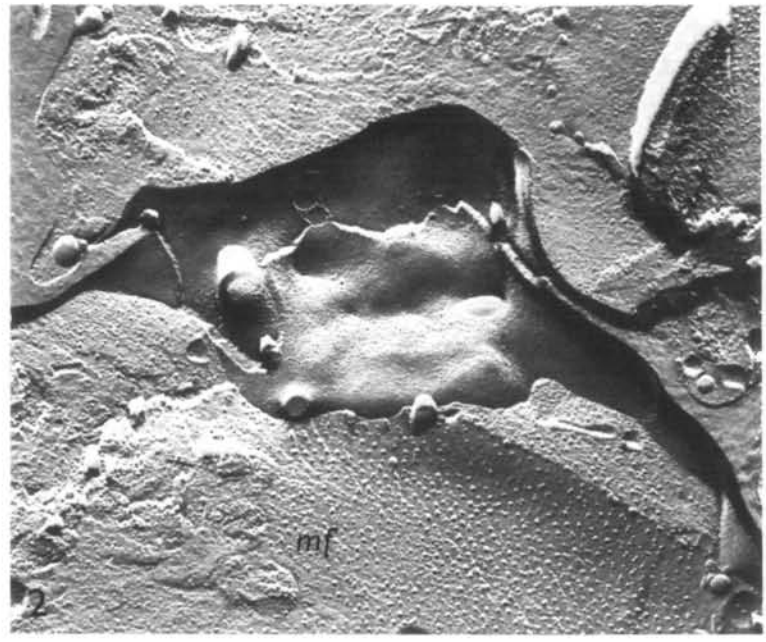
RESULTS

Electron-microscopical observations carried out on thin sections of frog atrial fibres show that neighbouring fibres are joined to one another by close contact of adjoining plasma membranes. These junctional regions are either focal (punctate junction) or extend over a larger area of the cell surface (Fig. 1).

Freeze-fracturing exposes the internal hydrophobic surfaces of the plasma membranes of adjacent myocardial cells (Fig. 2). The non-junctional fracture faces are characterized by a smooth area and by particulate entities of various sizes, from 4 to 8 nm. The 2 halves of the split plasma membrane are asymmetric. The fracture face close to the cytoplasm and outwardly directed (fracture face A or PF, protoplasmic fracture face) (Branton *et al.* 1975) contains a larger number of intramembranous particles (IMP) than the fracture face close to the intercellular space and inwardly directed (fracture face B or EF, extracellular fracture face).

Occasionally, in areas of fracture face A larger particles (9 nm) form small clusters (Fig. 4) or linear arrays in a smooth area almost completely devoid of smaller particles (Fig. 5). Particles of the same size forming small clusters or short arrays are also found in proximity to areas where the intercellular space, between 2 fractured adjoining fibres, appears to be abolished (inset Fig. 5). This latter region most probably corresponds to the focal close contact observed in thin-sectioned material and illustrated in Fig. 1. The most characteristic structures found in the cleaved plasma membrane of myocardial cells, in close contact, consist of rows of 9-nm particles which form anastomosed circular profiles, circumscribing smooth areas (Figs. 3–8).





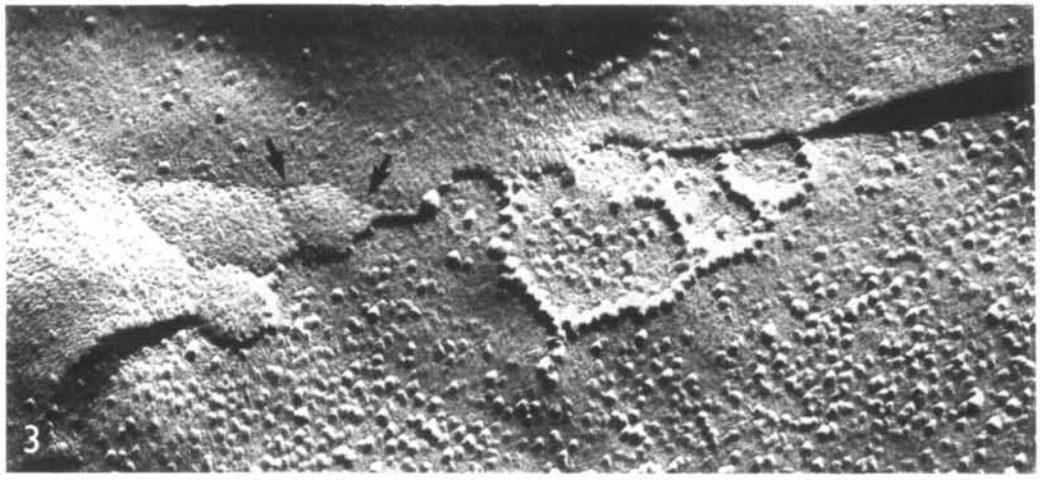
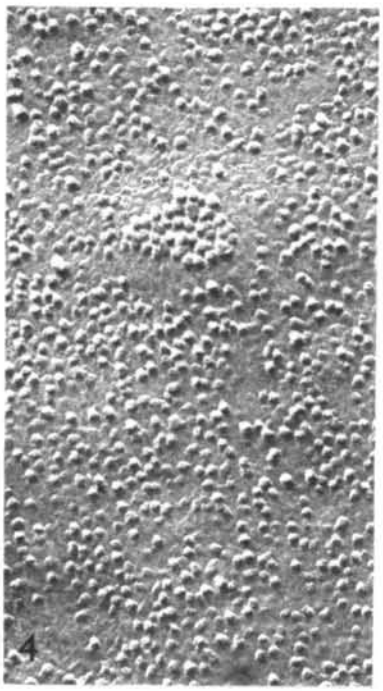
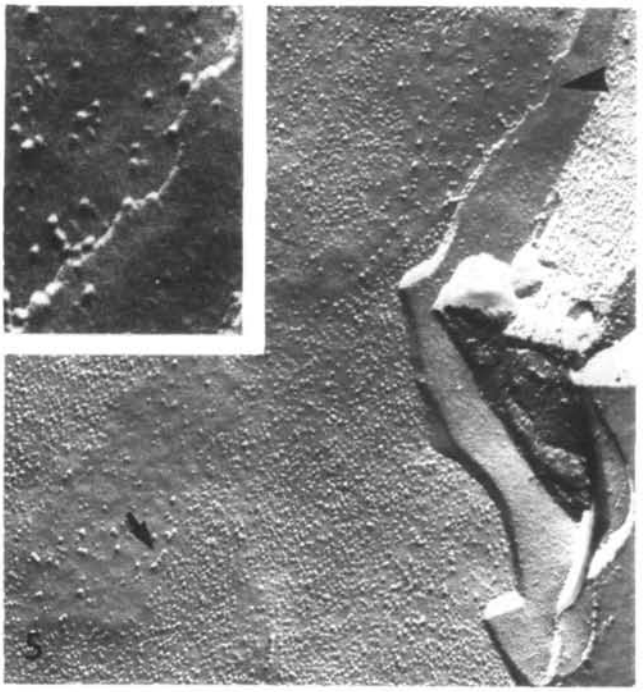


Fig. 1. Thin section of frog atrial fibre, showing a junctional complex between 2 adjacent plasma membranes. Arrows point to focal contacts. × 160000.

Fig. 2. Freeze-fracture of 2 adjacent myocardial cells of frog atrial fibre. A junctional complex is present on the fracture face A of the plasma membrane, in a region of close contact between 2 myocardial cells. *mf*, myofilaments in the cytoplasm. × 40000.

Fig. 3. A-to-B fracture-face transition, showing circular profiles of 9-nm particles on the A face in register with an array of pits or depressions on the B face (arrows). The arrangement of these circular profiles may represent the freeze-fracture appearance of the junction in Fig. 1. × 150000.





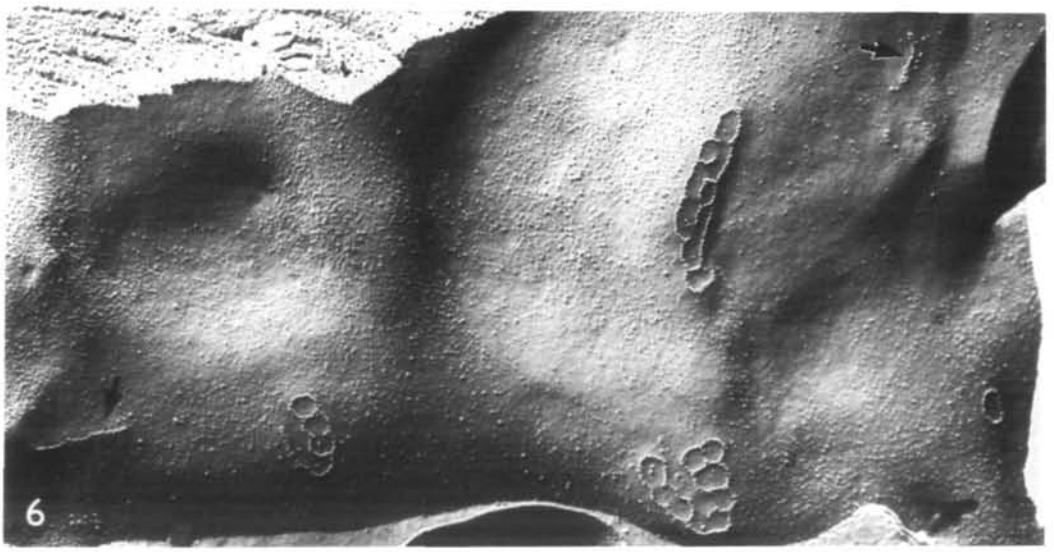


Fig. 4. Fracture face A of myocardial cell showing an exceptionally small cluster of 9–10-nm particles in a junctional array. × 150000.

Fig. 5. Fracture face A of myocardial cells. In a smooth area almost completely devoid of particles, small clusters or linear arrays of large particles (9 to 10 nm) are observed (arrow). In close proximity to this region the intercellular space between 2 adjoining fibres appears to be abolished (arrowhead). × 60000; inset, × 150000.

Fig. 6. Fracture face A of myocardial cell showing the polymorphism of the particle arrangements. Single rows of 9-nm particles forming isolated or anastomosed circular profiles are visible. A few linear rows are also to be observed (arrows). × 60000.

At high magnification each circular profile consists of about 20–30 particles (Fig. 7). The number of circular profiles of particles, for each junctional region, seems to be no more than 10 (Fig. 6). When the linear rows merge small clusters of geometrically packed particles may be found. On the fracture face close to the intercellular space arrays of pits or depressions, also forming circular profiles, represent the location of particulate structures, most of which remain associated with the outwardly directed fracture face A during cleavage. Only a few 9-nm particles remain attached to fracture face B along the array of pits (Fig. 8).

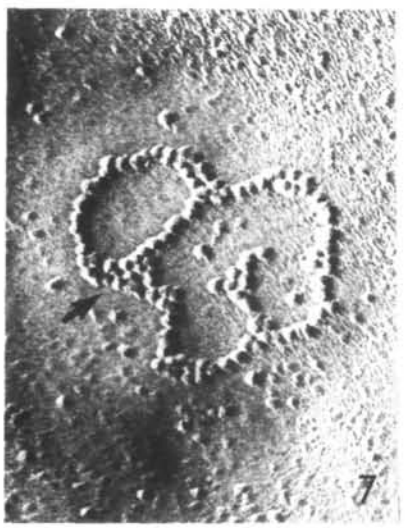


Fig. 7. Detailed view of a junctional complex showing that each circular profile on the fracture face A consists of about 20–30 regular particles. Where these rows merge small clusters of geometrically packed particles may be found (arrow). Note that a smooth area is circumscribed by a circular profile of particles. × 150000.

Fig. 8. Typical aspect of the fracture face B of the junction. Arrays of pits or depressions represent the location of junctional particles which, on cleavage, remain associated with the complementary fracture face A. × 150000.

At low magnification the junctional structures appeared to be scattered over a large area of fracture face (Fig. 6). We never observed between myocardial cells smooth ridges of tight junction (Staehelin, 1974), such as can be found between endothelial cells which surround the myocardial fibres.

DISCUSSION

Intercellular junctions show great variation in morphology. Therefore it is not readily apparent which type of junctional organization might be considered responsible for the existence of intercellular communication and electrotonic coupling. The conclusion that the gap junction or nexus or 'communicating junction' (to use the more appropriate term proposed by Simionescu, Simionescu & Palade, 1975) is the most

probable candidate for the specialized structure responsible for electrical coupling is supported by the observation that this type of junction is always present in electrically coupled cells (Gilula, Reeves & Steinbach, 1972; Bennett, 1973). Freeze-etching and negative staining demonstrated that the most common feature of the gap junction consists of arrays, of various extension, of geometrically packed particles spanning the 2 plasma membranes in close contact.

However, in various tissues of vertebrate (Raviola & Gilula, 1973) and invertebrate (Flower, 1972) animals, electrical coupling and metabolic coupling appear to be associated with morphological variants of the gap junction. These variants are characterized by the fact that the junctional particles do not form maculae of packed arrays, but rather form rows of irregular circular profiles or interconnected aggregates. The major structural feature of the junctions connecting auricular fibres in frog heart is the presence of anastomosed circular profiles of 9-nm particles which very seldom form small geometrically packed aggregates. The junctional nature of the arrays of particles is demonstrated by the fact that they are found in regions where the intercellular space between 2 membranes is absent and that on the B fracture face complementry pits are visible. Gap junctions showing analogous features are found in the myocardium of another adult amphibian (*Ambystoma tigrinum*) (studies in progress). Similar types of gap junctions, also described in photoreceptor cells of vertebrate retina (Raviola & Gilula, 1973), and in mammalian kidney glomeruli, mesanglial and lacis cells (Pricam, Humbert, Perrelet & Orci, 1974), have been interpreted as a variant of the low-resistance junction.

On the other hand linear arrays or small clusters of junctional particles have been described also in developing or embryonic tissues (Revel *et al.* 1973; Benedetti, Dunia & Bloemendal, 1974; Decker & Friend, 1974; Montesano, Friend, Perrelet & Orci, 1975). Although the auricular myocardial fibres of adult animals may be considered as a fully developed type of tissue, it is not readily apparent whether or not turnover or disassembly and reassembly of plasma membrane junction constituents may occur. These structural features are probably most consistent with the observation that the adult amphibian myocardium can easily regenerate (Becker, Chapin & Sherry, 1974; Oberpriller & Oberpriller, 1971), in contrast to the mammalian heart which can regenerate only in early embryonic or postnatal stages of development. It is noteworthy that independently of the developed junctional region, we have found in large areas of cleaved atrial fibre plasma membrane, 10-nm particles located in extremely smooth areas without accompanying particles of smaller diameter. Similar observations have been reported in cells forming the somite region of young chick embryos, stage 8 (Revel *et al.* 1973). The junctional nature of these large particles, which have been also incidentally observed in reaggregated hepatoma cells (Raviola & Gilula, 1973; Johnson, Hammer, Sheridan & Revel, 1974) is not known. It has been proposed either that the large particles might split into smaller junctional particulate entities or represent specific recognition sites between adjoining plasma membranes during gap junction development. Further observations of regenerating or *in vitro* amphibian heart may provide important knowledge concerning the role of these large particles.

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REFERENCES

BARR, L., DEWEY, M. M. & BERGER, W. (1965). Propagation of action potentials and the structure of the nexus in cardiac muscle. *J. gen. Physiol.* **48**, 797–824.

BECKER, R. O., CHAPIN, S. & SHERRY, R. (1974). Regeneration of the ventricular myocardium in amphibians. *Nature, Lond.* **248**, 145–146.

BENEDETTI, E. L., DUNIA, I. & BLOEMENDAL, H. (1974). Development of junctions during differentiation of lens fibers. *Proc. natn. Acad. Sci. U.S.A.* **71**, 5073–5077.

BENNETT, M. V. L. (1973). Function of electrotonic junctions in embryonic and adult tissues. *Fedn Proc. Fedn Am. Socs. exp. Biol.* **32**, 65–73.

BRANTON, D., BULLIVANT, S., GILULA, N. B., KARNOVSKY, M. J., MOOR, H., MÜHLETHALER, K., NORTHCOTE, D. H., PACKER, L., SATIR, B., SATIR, P., SPETH, V., STAEHELIN, L. A., STEERE, R. L. & WEINSTEIN, R. S. (1975). Freeze-etching nomenclature. *Science, N.Y.* **190**, 54–56.

BRIGHTMAN, M. W. & REESE, T. S. (1969). Junctions between intimately apposed cell membrane in the vertebrate brain. *J. Cell Biol.* **40**, 648–677.

DECKER, R. S. & FRIEND, D. S. (1974). Assembly of gap junctions during amphibian neurulation. *J. Cell Biol.* **62**, 32–47.

DENOIT-MAZET, F. & VASSORT, G. (1971). Ultrastructure des faisceaux sino-auriculaires de grenouille en relation avec leur activité électrophysiologique. *J. Microscopie* **12**, 413–424.

DREIFUSS, J. J., GIRARDIER, L. & FORSSMAN, W. G. (1966). Etude de la propagation de l'excitation dans le ventricule de rat au moyen de solutions hypertoniques. *Pflügers Arch. ges. Physiol.* **292**, 13–33.

FARQUHAR, M. G. & PALADE, G. E. (1965). Cell junctions in amphibian skin. *J. Cell Biol.* **26**, 263–291.

FLOWER, N. E. (1972). A new junctional structure in the epithelia of insects of the order Dictyoptera. *J. Cell Sci.* **10**, 683–692.

GILULA, N. B., REEVES, O. R. & STEINBACH, A. (1972). Metabolic coupling, ionic coupling and cell contacts. *Nature, Lond.* **235**, 262–265.

GOSHIMA, K. (1970). Formation of nexuses and electrotonic transmission between myocardial and FL cells in monolayer culture. *Expl Cell Res.* **63**, 124–131.

JOHNSON, R., HAMMER, M., SHERIDAN, J. & REVEL, J. P. (1974). Gap junction formation between reaggregated Novikoff hepatoma cells. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4536–4540.

LUFT, J. H. (1956). Permanganate. A new fixative for electron microscopy. *J. biophys. biochem. Cytol.* **2**, 799–801.

MAZET, F. (1975). Etude des jonctions des faisceaux sinoauriculaires de grenouille. *C. r. hebd. Séanc. Acad. Sci., Paris* **280**, 2873–2876.

MONTESANO, R., FRIEND, D. S., PERRELET, A. & ORCI, L. (1975). *In vivo* assembly of tight junctions in fetal rat liver. *J. Cell Biol.* **67**, 310–319.

OBERPRILLER, J. & OBERPRILLER, J. C. (1971). Mitosis in adult newt ventricle. *J. Cell Biol.* **49**, 560–563.

PINTO DA SILVA, P. & GILULA, N. B. (1972). Gap junctions in normal and transformed fibroblasts in culture. *Expl Cell Res.* **71**, 393–401.

PRICAM, C., HUMBERT, F., PERRELET, A. & ORCI, L. (1974). Gap junctions in mesangial and lacis cells. *J. Cell Biol.* **63**, 349–354.

RAVIOLA, E. & GILULA, N. B. (1973). Gap junctions between photoreceptor cells in vertebrate retina. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1677–1681.

REVEL, J. P. & KARNOVSKY, M. J. (1967). Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33**, C7.

REVEL, J. P., YIP, P. & CHANG, L. L. (1973). Cell junctions in the early chick embryo. A freeze-etch study. *Devl Biol.* **35**, 302–317.

SIMIONESCU, M., SIMIONESCU, N. & PALADE, G. E. (1975). Segmental differentiation of cell junctions in the vascular endothelium. The microvasculature. *J. Cell Biol.* **67**, 863–885.

SOMMER, J. R. & JOHNSON, A. E. (1969). Cardiac muscle. A comparative ultrastructural study with special references to frog and chicken heart. *Z. Zellforsch. mikrosk. Anat.* **98**, 437–468.

STAEHELIN, L. A. (1974). Structure and function of intercellular junctions. *Int. Rev. Cytol.* **39**, 191–283.

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