mental fibres into four segments (2 mm long) from the proximal end, where the fast endplate was located. Although fast MHC are found in all fibres 3 or 14 months after surgery (Fig. 2*C–D*; Table 1), they are detected almost exclusively in the segment of fibre that bears the fast endplate and no fast MHC is ever detected in the segment containing the slow endplate or that located more distally. Only the proximal segment of six 14-month dually innervated fibres is stained by histochemical myofibrillar ATPase reaction, after precincubation at *p*H 10.4, that is, after inhibition of slow myosin ATPase activity (data not shown). Normal soleus fibres contained only slow MHC, even in the segment corresponding to the proximal end (Fig. 2*f*). By contrast, a fibre from a single 14-month operated soleus muscle, in which only the foreign endplate at the proximal end was demonstrated by the α-bungarotoxin labelling, contained both fast and slow MHC, but the relative amounts of the two isoforms were constant throughout the entire fibre length (Fig. 2*g*).

We conclude that this fibre is a slow fibre 'cross-innervated' by the fast nerve that undergoes a partial slow-to-fast transformation, in agreement with previous results[14]. The possibility that the additional band seen in experimental fibres could have been contributed by synthesis of embryonic myosin induced by denervation was excluded because the heavy chains of purified rat embryonic myosin show an electrophoretic mobility intermediate between those of heavy chains of adult fast and slow myosin, and closer to that of slow MHC (Fig. 2, lane 4) and, therefore, it cannot co-migrate with the additional band. These findings demonstrate that the phenotypic expression of myosin isoforms may differ along the length of these single muscle fibres and suggest that this localization is related to the presence of a specific neural input. Clearly this trophic influence may only be exerted over a rather short distance from the endplate.

It is not known what is the message transmitted from nerve to muscle, nor which mediators in the muscle cell activate gene expression. Electrical activity may be the main message transmitted by a nerve[5,16], but there may be certain chemical factors (trophic factors)[17,18], some of which appear to control specific electrical characteristics of the muscle surface membrane[19]. The pattern of electrical activity seems important because the trains of action potentials propagate all along the fibre and could induce formation of mediators that control in a coordinated manner the activation of either 'fast' or 'slow' genes in all nuclei of the fibre. Moreover, direct stimulation by short high-frequency bursts of impulses (fast pattern of stimulation) is more effective than more prolonged trains of low-frequency impulses (slow pattern) in restoring normal membrane properties after denervation[20]. But the pattern of electrical activity cannot fully explain why the expression of fast genes is restricted to the region around the endplate.

Nonetheless, the localized synthesis of fast myosin in a slow fibre indicates that only a few nuclei are affected by the fast endplate, and that specific chemical mediators must be formed in the muscle cell, but near the endplate. A preferential localization of a muscle membrane antigen (5.1.H11) near the nucleus responsible for its production has been reported in muscle heterokaryons *in vitro*[21]. On the other hand, 'mosaic' skeletal muscle fibres from mouse chimaeras show no spatial phenotypic heterogeneity related to genotypically distinct myonuclei[22]. These findings are not inconsistent with our results, because in mosaic fibres genotypically distinct myonuclei are randomly distributed, whereas in our experimental model it is the cytoplasmic mediator(s) which have a limited range of activity.

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The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next

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The tight junction (zonula occludens) links epithelial cells into a monolayer by forming a continuous belt of sealing contacts around the apex of each cell. They appear in thin sections as if they were 'fusions' between the apposed plasma membranes[1] and in freeze-fracture replicas as patterns of complementary strands and furrows[2]. These images have led to the proposal that the core of the tight junction is formed by a hexagonal cylinder of lipids[3,4]. In this model, the cytoplasmic leaflet of the apical and basolateral plasma membrane domains would be continuous, whereas the exoplasmic leaflets of the two plasma membrane domains of the same cell would be separated at the tight junction and are instead predicted to be continuous between the plasma membranes of neighbouring cells. We demonstrate here that this prediction does not hold true. An endogenous glycolipid (Forssman antigen), present in the exoplasmic leaflet of the apical membrane of MDCK strain II cells[5,6], is unable to pass to MDCK strain I cells (which lack this glycolipid) under conditions where these cells are connected by tight junctions. In addition, fluorescent lipids which have been fused into the plasma membrane[7,8] of one MDCK cell do not diffuse to neighbouring cells while the tight junctions between the cells are intact.

To test whether lipids can diffuse from one epithelial cell to another through continuous exoplasmic leaflets of their apical plasma membranes, we studied the behaviour of endogenous glycolipids. Strain II MDCK cells, a subline of the MDCK cell line[9], possess a series of glycolipids, the globo series, which are not found in MDCK strain I cells, a different MDCK subline[5,6]. Forssman antigen, one glycolipid of this series [GalNAc(α1-3)GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer], constitutes 21% of the total neutral glycosphingolipids of MDCK strain II cells[5]. When a monolayer of these cells was labelled

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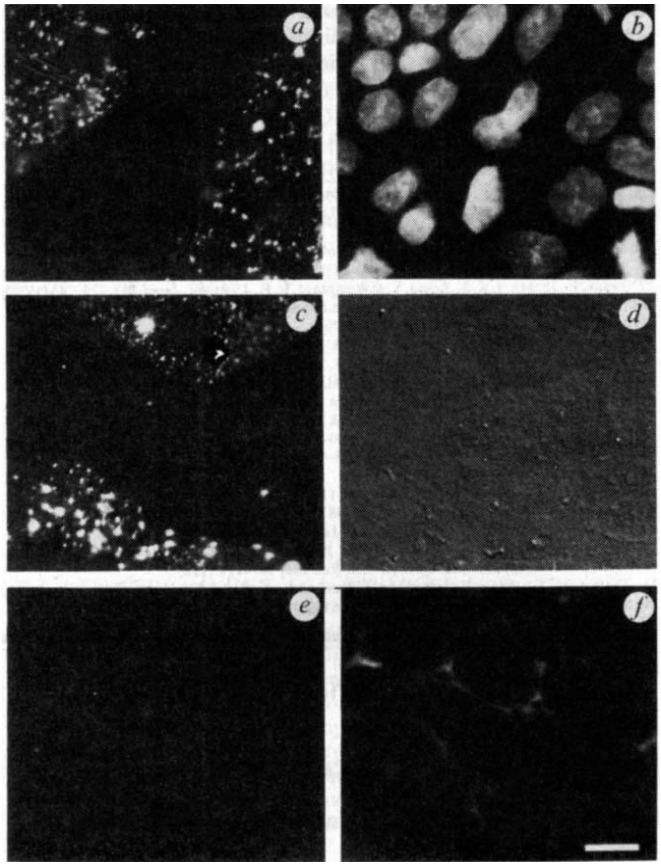

Fig. 1 Forssman antigen does not move from MDCK strain II cells to MDCK strain I cells during 96 h of co-culture. *a*, A mixed monolayer of MDCK strain I and strain II cells on nitrocellulose filters was fixed with 3% formaldehyde and incubated on the apical surface with monoclonal IgGs against Forssman antigen. Clustering of the fluorescent anti-Forssman antibodies in Fig. 1*a* is prevented if unfixed cells are incubated with the antibody at 0 °C and postfixed before addition of the second antibody (C. M. B. Butor and J. Davoust, unpublished results). As a second antibody we used rhodamine anti-mouse IgG. *b*, The same field of cells after staining the DNA in the nuclei by Hoechst dye 33258. *c*, A mixed monolayer grown on a glass coverslip stained apically against Forssman antigen as described for *a*. *d*, Nomarski optics micrograph of the field shown in *c*. *e*, Parallel monolayers on coverslips were incubated on the apical surface with a monoclonal antibody against uvomorulin, a lateral membrane marker in these cells[10]. As a second antibody we used rhodamine anti-mouse IgG. *f*, Staining with the anti-uvomorulin antibody after opening of the junctions by incubation with 2 mM EGTA for 5 min at 37 °C (ref. 10). Scale bar, 10 μm.

from the apical side with a monoclonal antibody against Forssman antigen, apical staining was observed (see also Fig. 4 of ref. 5). This glycolipid was therefore present in the exoplasmic leaflet of the apical plasma membrane. We co-cultured MDCK strain II cells with MDCK strain I cells which do not express Forssman antigen. The presence of intact tight junctions between the two cell types was demonstrated by adding a monoclonal antibody which recognizes uvomorulin, a protein present on the lateral membrane of MDCK cells[10], and showing that it did not label the lateral membrane unless the tight junctions had been opened by treatment with 2 mM EGTA for 5 min at 37 °C before fixation (Fig. 1). Therefore, tight junctions were present between strain I and II cells grown together on nitrocellulose filters or glass slides. Staining with 33B12, a monoclonal IgG2c against Forssman antigen[11], demonstrated Forssman antigen on the apical surface of about 50% of the cells in the mixed monolayer under both growth conditions. The boundary between stained and unstained cells was sharp. Thus, the endogenous glycolipid

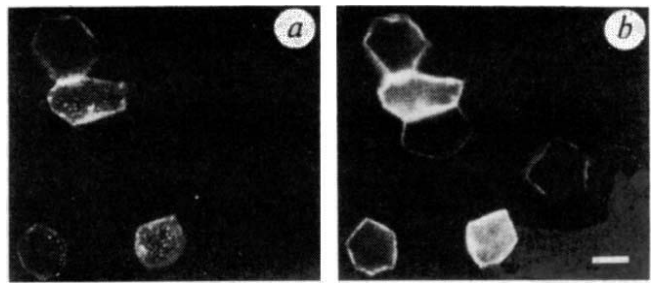

Fig. 2 The fluorescent phospholipid *N*-rhodamine phosphatidylethanolamine (N-Rh-PE) does not diffuse to neighbouring cells in a monolayer of MDCK strain I cells grown on nitrocellulose filters. N-Rh-PE, a water-insoluble lipid[12], was fused into the apical plasma membrane[7,8] using unilamellar liposomes containing the fluorescent lipid equally distributed over both bilayer leaflets[16]. The cells were infected with 4 plaque-forming units per cell of influenza N virus and infection allowed to proceed for 4 h at 37 °C. The liposomes were prepared by reverse-phase evaporation[19] from egg phosphatidylcholine, egg phosphatidylethanolamine, cholesterol, ganglioside G_{D1a} and N-Rh-PE (25:25:50:5:1, mol/mol). 20 nmol total lipid was added in 500 μl of serum-free medium to a monolayer of 3×10^6 cells. After 30 min at 0 °C, fusion was initiated by treatment with a serum-free medium buffered at *p*H 5.0 by 20 mM succinate, at 37 °C for 60 s. The cells were then returned to *p*H 7.4 medium and put on ice. Treatment of the cell surface with trypsin, necessary to cleave the haemagglutinin to its fusogenic form[7,8], was omitted because in strain I MDCK cells the haemagglutinin is cleaved spontaneously by a secreted protease (G.vanM., unpublished observations). Electrical resistance before infection was $6 \pm 2 \times 10^3\ \Omega\ cm^2$ ($n = 3$), and after the complete procedure it was $1.2 \pm 0.2 \times 10^3\ \Omega\ cm^2$ ($n = 6$). The microscope, equipped with a water immersion objective, was focused at the apical surface of the living cells (*a*) or halfway down the lateral surface (*b*), after liposome-cell fusion. Scale bar, 10 μm.

could not pass from MDCK strain II cells to the apical surface of neighbouring MDCK strain I cells over a period of 96 h of co-culture at 37 °C.

As an alternative approach, we fused[7,8] fluorescent lipids into the apical plasma membrane of 50% or less of MDCK cells in a confluent monolayer and used a fluorescence microscope to observe whether the fluorescent lipid would move to non-fluorescent neighbour cells. For this, the fluorescent lipids dioleoyl *N*-rhodamine phosphatidylethanolamine (N-Rh-PE) or octadecyl rhodamine B (R18), which do not spontaneously exchange through the aqueous phase[12,13], were incorporated into liposomes. These liposomes were then added at 0 °C to the apical surface of MDCK cells infected with influenza virus. They were bound to the viral haemagglutinin glycoprotein expressed on the apical surface of these cells by including a haemagglutinin receptor into the liposomal membrane, the G_{D1a} ganglioside. Fusion was induced by a short treatment (60 s) at low *p*H (5.0) and 37 °C[7,8], after which the medium was readjusted to *p*H 7.4 and 0 °C. Lipid insertion was limited to a fraction of the cells in the epithelial monolayer by infecting the cells with a low multiplicity of influenza virus; in this way, only part of the cells in the monolayer became infected. Only the infected cells subsequently bound and fused liposomes.

MDCK strain I cells were selected for one set of experiments because they develop monolayers possessing a high electrical resistance ($>2{,}000\ \Omega\ cm^2$) when grown on a permeable support[14], and this resistance can be used to assay the intactness of the tight junctions during the experiment[10,15]. The fluorescent phospholipid N-Rh-PE was fused into the apical plasma membrane of about 50% of the MDCK strain I cells in a confluent monolayer (Fig. 2). Fusion was evident from the fact that the fluorescent phospholipid reached the basolateral surface (Fig. 2*b*). This process will be demonstrated and discussed in more detail elsewhere[16]. Fusion was also assayed by the hydrolysis of liposomal cholesterol oleate by a cellular enzyme[8]. The

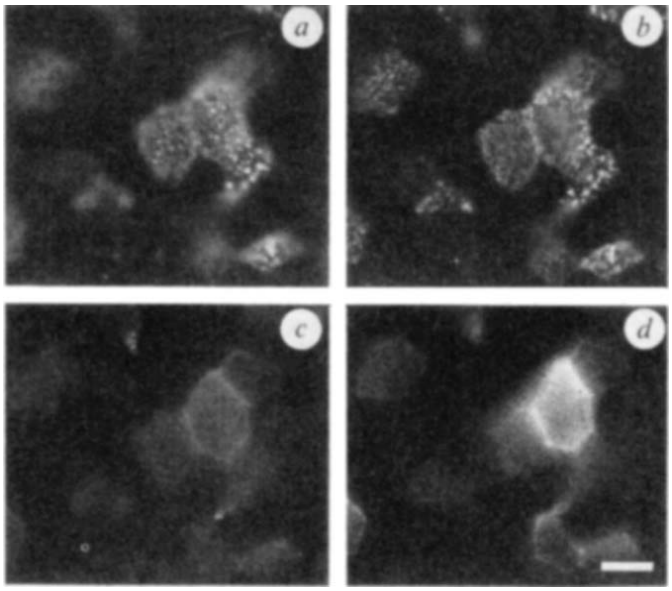

Fig. 3 The fluorescent lipid probe octadecyl rhodamine B (R18) does not pass from cell to cell in a monolayer of MDCK strain II cells on nitrocellulose filters. R18, a positively charged rhodamine lipid unable to exchange spontaneously between membranes[13], was fused into the apical plasma membrane as in Fig. 2 with the following differences. At 5 h after infection with 4 plaque-forming units per cell of influenza N virus, the cell monolayer was treated with trypsin as described elsewhere[7,8]. Large unilamellar liposomes were prepared by octyl β-D-glucoside dialysis from the same lipids as used in Fig. 2 but containing 1 mol % R18 instead of N-Rh-PE. The microscope was focused from the apical surface (*a*) down the lateral surface (*b–d*). The contorted orthogonal morphology of the columnar MDCK cells on nitrocellulose filters is clearly visible. Scale bar, 10 μm.

electrical resistance of the monolayer was $1,200 \pm 160\ \Omega\ cm^2$ ($n = 6$) after the experiment, indicating that the tight junctions between the cells were essentially intact. In comparison, the electrical resistance across an intact polarized monolayer of strain II MDCK cells without virus infection was $160\ \Omega\ cm^2$. Under these circumstances, no spreading of the fluorescent phospholipid to the adjacent non-infected cells was observed. Bright and dark cells remained juxtaposed in the cell monolayer for hours.

Similar experiments were performed on low-resistance MDCK strain II cells using another lipid probe (Fig. 3). The fluorescent lipid R18 was fused into the apical plasma membrane. R18 differs from N-Rh-PE in that it carries one positive charge at neutral *p*H. As a criterion for intactness of the tight junction, the impermeability of the tight junction to antibodies was monitored as described above. The antibody rr1 against the lateral protein uvomorulin[10] was unable to label its antigen throughout the experiment. Like N-Rh-PE, R18 remained confined to the cells into which it had been fused (Fig. 3*a–d*). No spreading from bright to dark cells was observed for hours at 0 °C.

We have presented evidence that three different types of lipids present in the apical plasma membrane domain are unable to diffuse into the apical membrane of adjacent cells. Dragsten *et al.*[17] earlier observed that after partitioning water-soluble fluorescent probes into a monolayer of epithelial cells and bleaching one cell completely, no fluorescence returned into this black cell from the surrounding bright cells. In our experiments we used endogenous glycolipids and water-insoluble probes introduced by fusion, which we know are present in the outer leaflet of the apical membrane. We avoided the potentially damaging effects of photobleaching by observing mixed populations of cells containing and lacking the lipid studied. Finally, we also demonstrated that the tight junctions between relevant cells were intact according to two criteria, both the transepithelial electrical resistance and the impermeability of the cell monolayer to antibodies. Moreover, we have tested the behaviour of the lipids at both 37 °C and 0 °C. Our observations argue against a continuity between the external leaflets of the apical plasma membrane in adjacent epithelial cells. The results are thus difficult to reconcile with the hexagonal lipid model of tight junction structure where the outer leaflets of the plasma membranes of neighbouring cells are fused[3,4]. It seems more likely that proteins which are part of the tight junction structure[10,18] bring the plasma membranes of adjacent cells very close together but do not induce a partial fusion. This would also be more consistent with the fact that the tight junction, as a barrier to ion diffusion between cells, displays ion selectivity[15].

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Synthetic peptides as nuclear localization signals

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The nuclear envelope defines a compartment boundary which is penetrated by pores that mediate a remarkable transport process. Precursor RNAs are retained in the nucleus, while processed messenger RNA[1], transfer RNA[2] and ribosomal subunits[3] are transported to the cytoplasm. Proteins destined for the nucleus become localized soon after synthesis and again following mitosis, while cytoplasmic proteins are excluded[4]. The process is highly specific: a single base change in vertebrate initiator $tRNA^{Met}$ ($tRNA_i^{Met}$) reduces the rate of export 20-fold[5]; a point mutation within the simian virus 40 (SV40) large-T antigen, converting Lys 128 to Thr (ref. 6) or Asn (ref. 7), prevents import. Lys 128 lies within a short 'signal' sequence which, when fused to large non-nuclear proteins, causes their accumulation in nuclei[6–8]. Regions of other eukaryotic proteins also seem to contain nuclear localization signals, although a single consensus sequence has not emerged[9–13]. We report here that a synthetic peptide containing 10 residues of large-T antigen sequence serves as a nuclear localization signal when cross-linked to bovine serum albumin (BSA) or immunoglobulin G (IgG) and microinjected in *Xenopus* oocytes. Substitution of Thr at the position of Lys 128 in this peptide