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THE IMPENETRABILITY OF 5-(N-HEXADECANOYL)AMINOFLUOROSCEIN THROUGH ENDOTHELIAL CELL MONOLAYERS IS DEPENDENT UPON ITS SOLUTION PROPERTIES, NOT THE PRESENCE OF TIGHT JUNCTIONS

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SUMMARY: The solution properties of two fluorescent lipophilic analogues were examined in conjunction with their ability to penetrate the tight junctions of bovine aortic endothelial cell monolayers. 5-(N-dodecanoyl) aminofluoroscein was shown to label both the apical and basolateral plasma membrane domains of confluent monolayers at 4°C and pH 7.3, but 5-(N-hexadecanoyl) aminofluoroscein was shown to label only the apical membrane domain. When used under more soluble conditions at 20°C and pH 8.5, both probes labeled apical and basolateral plasma membrane domains more equally. This indicates that solubility conditions, and not tight junctions, dictate the penetration of 5-(N-hexadecanoyl) aminofluoroscein from the apical to the basolateral plasma membrane domain.

It has been documented that epithelia possessing occluding junctions restrict the lateral movement of membrane lipids between the apical (AP) and basolateral (BL) plasma membrane (PM) domains in cells in vitro and therefore maintain a polarized lipid population in each domain (1-5). Recently, fluorescent probes have been used to determine that bovine aortic endothelial cells (BAEC) also maintain a polarized monolayer in culture with respect to lipid movement (6,7). 5-(N-dodecanoyl) aminofluoroscein (AFC12) and 5-(N-hexadecanoyl) aminofluoroscein (AFC16) are two fluorescent lipid analogues which reportedly display different properties in confluent monolayers due to the size of their lipid tails. AFC12 has a 12-carbon tail and it has been proposed (1,2) that this truncated length allows the molecule to "flip-flop" from the outer to the inner lipid leaflet and cross any occluding junctions which interrupt the lipid bilayer. AFC16 has a 16-carbon tail and it is thought that the longer acyl side chain prohibits "flip-flop" and its subsequent lateral

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Abbreviations used: BAEC: bovine aortic endothelial cells, AFC12: 5-(N-dodecanoyl) aminofluoroscein, AFC16: 5-(N-hexadecanoyl) aminofluoroscein, AP: apical membrane domain, BL: basolateral membrane domain, IM: internal membrane, PM: plasma membrane, HBSS: Hank's balanced salt solution.

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diffusion across the occluding junctions in the plane of the membrane. We show here that penetration properties characteristic of each probe is the result of different solubility properities exhibited by each probe under specific labeling conditions.

MATERIALS AND METHODS

Cells and Cell Culture. All BAEC culture techniques were carried out as described previously (8,9). Subconfluent monolayers were prepared by trypsin/EDTA subculturing and cells were seeded at low density (usually 1:10 split ratio) onto 1% gelatin adsorbed to tissue culture dishes. Cells were allowed to recover by culturing for at least 2 days prior to labeling and domain isolation. At the time of labeling and subcellular fractionation, monolayers were visibly preconfluent as little to no cell-cell contact was evident between cells when examined by light microscopy.

Fluorescent Lipid Probe Labeling of Membranes. Plasma membranes of confluent and subconfluent BAEC monolayers were labeled from the AP domain side with the fluorescent lipid analogues AFC12 or AFC16 (Molecular Probes, Eugene OR). Monolayers were washed twice with 37°C Hank's balanced salt solution (HBSS) with Ca^{2+} and Mg^{2+} then incubated in a 10 μg/ml solution of probe in HBSS at the specified temperature and pH for 3-5 min. (Probes were stored as a 10 mg/ml stock in dimethylformamide at -20°C in the dark). Low Ca^{2+} tight junction disruption was attained by incubating confluent monolayers in 20°C Ca^{2+}/Mg^{2+}-free HBSS for 15 min. This visibly disrupted cell-cell interactions. Monolayers were then labeled with 10 μg/ml probe in HBSS at 4°C for 3-5 min. AP and BL PM domains were isolated following labeling using the cationic colloidal silica technique (10) modified for endothelial cells (9) except that the metrizamide spin was not used as the density gradient material was found to interfere with subsequent fluorescence measurements. The fluorescence in the AP domain was determined from the 900 *g* pellet and the internal membrane (IM) fraction was determined from a high speed pellet (48,000 *g*) derived from the 900 *g* supernatant shown to be enriched in IM marker enzymes (9). Relative fluorescence in each domain was quantitated by dissolving each isolated domain in 2 ml of a 2% SDS, 50% methanol, 0.05 N NaOH solution and measured on a spectrofluorometer (Aminco-Bowman). For fluoromicrograph preparation, monolayers and isolated domains were fixed in 3% (w/w) paraformaldehyde (Tousimis, Rockville, MD) in HBSS pH 7.0, then washed in HBSS twice. To inhibit quenching of the fluorescence, a N-propyl gallate solution (3% (w/v) N-propyl gallate in a solution of 10 mM K_2HPO_4 and 90% (v/v) glycerol, pH 8.0) was added to the cells or membranes before viewing under epi-fluorescence. Labeled membranes were visualized using standard fluoroscein excitation:emission filters on a Dialux 20 EB fluorescence microscope (Leitz). Fluoromicrographs were obtained using an Orthomat-W (Leitz) photon counting camera.

Solubility characteristics of the probes were investigated by centrifuging working solutions (10 μg/ml) of AFC12 or AFC16 in HBSS at pH 7.3 and 8.5 and temperatures of 4°C, 20°C and 37°C. The resulting 14,000 *g* supernatant and pellets were solubilized in 2 ml of a 2% SDS, 50% methanol, 0.05 N NaOH solution and measured on a spectrofluorometer (Aminco-Bowman) and the relative fluorescence of each fraction was determined.

RESULTS

When confluent monolayers of BAECs were incubated with AFC16 at 4°C and pH 7.3, and the domains isolated, the probe was visibly excluded from the BL domain when examined by fluorescence microscopy (**Fig.1 D-F**). The probe could, however, penetrate the BL domain in subconfluent monolayers (**Fig.1 I, J**). AFC12 was found in both domains whether the monolayer was confluent or not (**Fig.1 A-C, G,H**). This experiment was repeated on confluent monolayers and monolayers whose tight junctions had been temporarily disrupted in low Ca^{2+} medium (11, 12) (**Fig.2**). In 5 d post-confluent monolayers, AFC12 was found to be equally distributed between AP and BL domains. The distribution of AFC16 was found to be overwhelmingly in the AP domain. The small amount of AFC16 in the BL domain is most likely

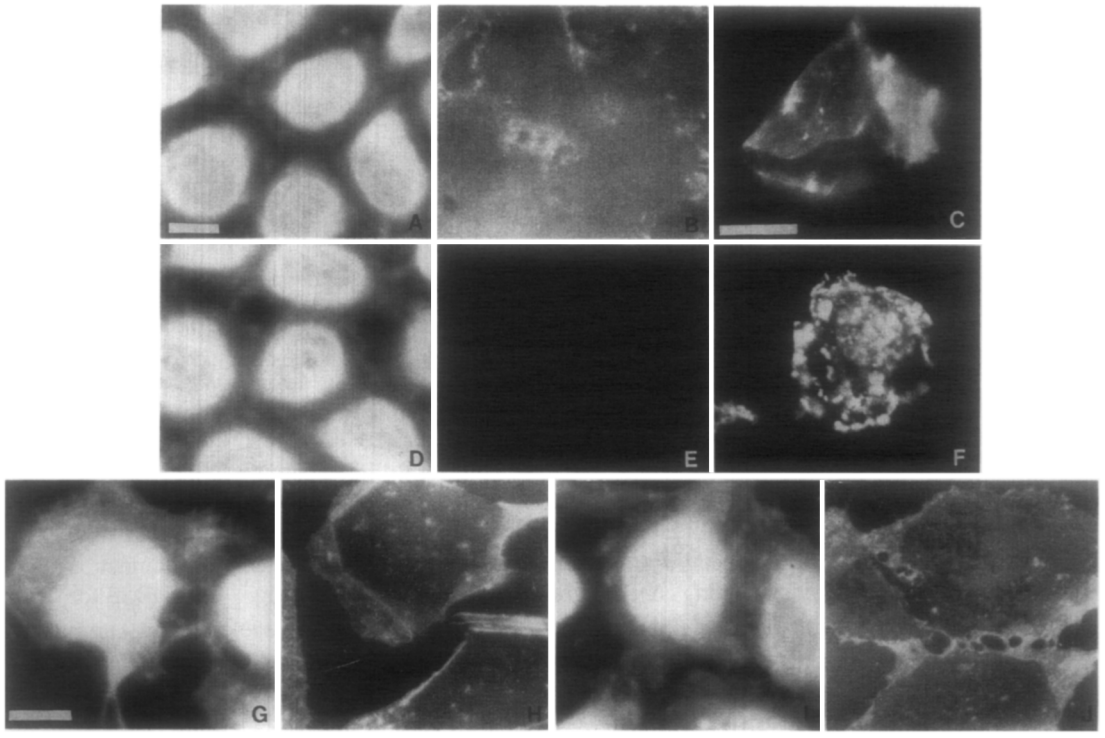


Figure 1. Epi-fluorescence micrographs of BAEC monolayers labeled with lipophilic fluorophors. **A)** Confluent BAEC monolayer labeled with AFC12. **B)** BL and **C)** AP membrane domains isolated from AFC12 labeled monolayers. **D)** Confluent monolayers of BAECs labeled with AFC16. **E)** BL and **F)** AP membrane domains isolated from AFC16 labeled monlayers. Above experiment repeated using subconfluent monolayers: **G)** Subconfluent monolayer labeled with AFC12. **H)** BL domain isolated from AFC12 labeled cells. **I)** Subconfluent monolayer labeled with AFC16. **J)** BL domain isolated from AFC16 labeled cells. Due to the absence of intercellular tight junctions in the subconfluent monolayer, AFC16 is now accessable to the BL membrane domain. All bars indicate 10 μm. Bar in **A** represents panels **A, B, D,** and **E**. Bar in **C** represents panels **C** and **F**. Bar in **G** represents **G-J**.

due to the small number of unlysed whole cells (usually 5 to 10%) present in the BL domain preparation (9) since no fluorescence was directly observed in the BL domain (**Fig.1E**). The IM fraction, which has previously been shown to contain enrichments of IM markers enzymes (9), was also found to consistantly contain some fluorescence. We attribute this to the loss of probe aggregates from the monolayer and PM surface during the membrane isolation procedure, and not to internalization of the probes. Under low Ca^{2+} conditions both probes became more equilibrated between the AP and BL PM domains indicating that disruption of cell-cell junctions had occured and mimicked the subconfluent state (**Fig.1 G-J**).

The results discussed above, though not unexpected since others had reported similar findings using AFC12 and AFC16, did raise a few questions. We had observed that when small PM surface-selective protein-labeling agents, like biotin derivatives (1 mm sulfo-NHS-biotin) and radio-iodine, were applied to the AP PM domain, they crossed the intact junctional structures of

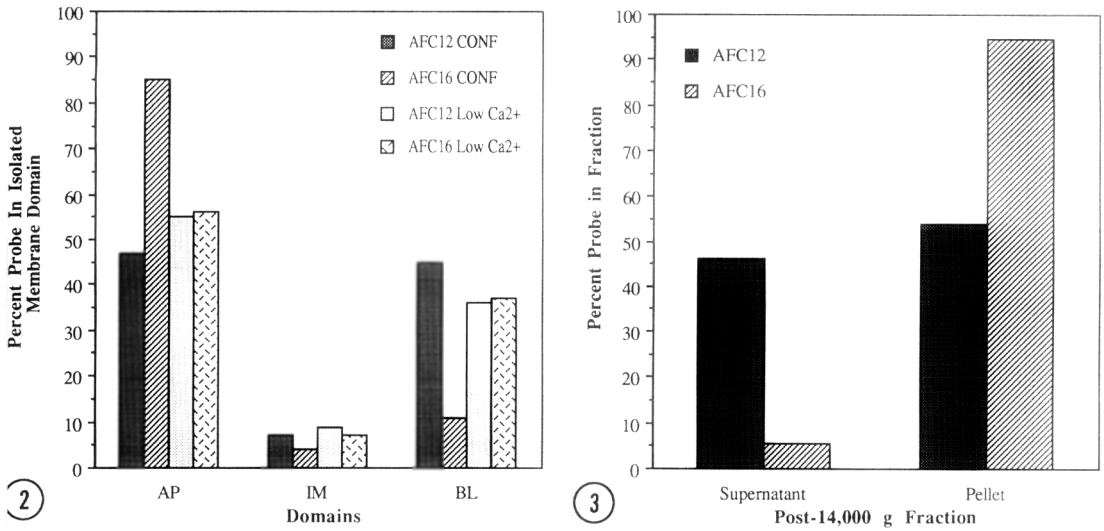
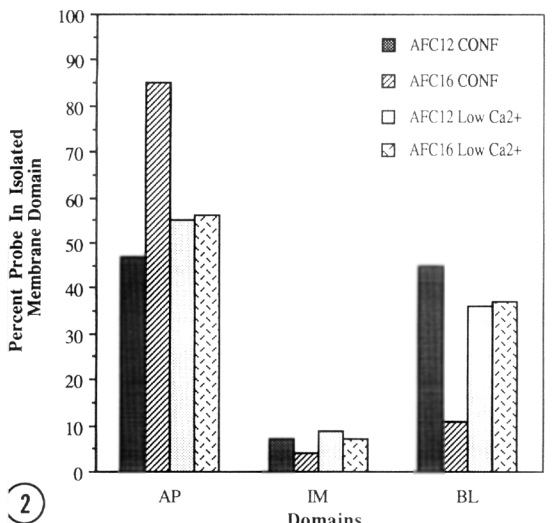
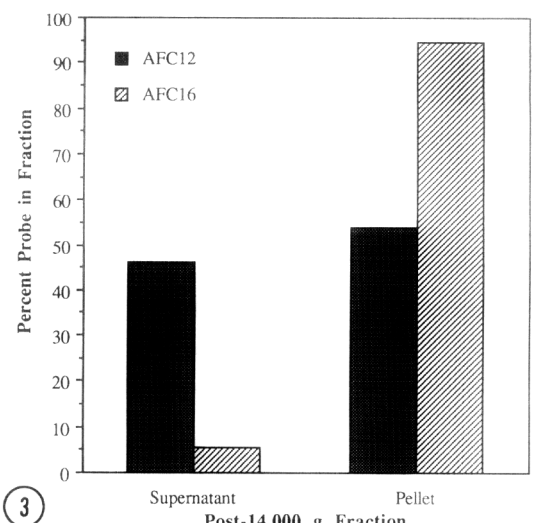


Figure 2. Histogram indicating the partitioning of the fluorescent lipophilic probes AFC12 and AFC16 into AP, IM and BL membrane domains of confluent monolayers and confluent monolayers whose junctions have been disrupted by low Ca^{2+} concentrations. Relative fluorescence measurements are the average of two separate experiments. The results coincide with the data obtained using fluorescence microscopy (Fig. 1) that AFC12 penetrated through the tight junctions and labeled the BL membrane, while AFC16 did not cross the junctional complexes to label the BL domain in confluent monolayers. Both probes labeled both AP and BL domains in low Ca^{2+} medium, indicating that intercellular junctions had been disrupted.

Figure 3. The solubility of fluorescent lipophilic probes at 4°C and pH 7.3. AFC12 is 46% soluble and only 5.5% of AFC16 is in solution under these conditions. The measurements are the average of three separate experiments.

confluent BAEC monolayers and labeled proteins in the BL PM domain as well as the AP (9, 13). Others had also examined the ability of ferritin, which is much larger than either of these lipophilic probes, to cross the junctional structures of confluent monolayers of endothelial cells (14). Although no data was available on the solution properties of either AFC probe in physiological buffers and temperatures, we surmised they may possess different solubility properties resulting in different abilities to partition into the PM. **Fig.3** shows that at the working concentration (10 [illegible] of AFC12 and only 5.5% of





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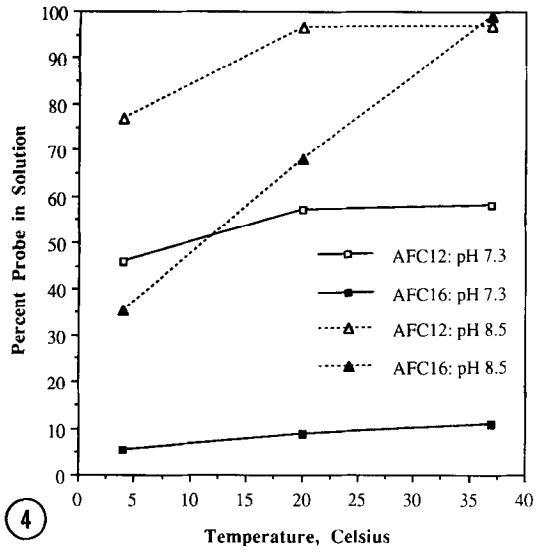

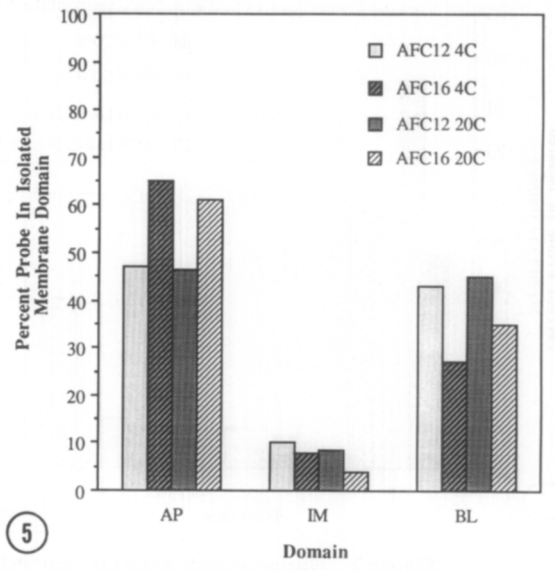

Figure 4. The solubility of fluorescent lipophilic probes at varying temperature and pH. Solubility measurements were examined on the probes at pH 7.3 and pH 8.5 after adding the stock probe solutions to solutions of HBSS at 4°C, 20°C or 37°C. Solubility was determined by the pelleting procedure described in the text. Measurements are the average of two separate experiments.

Figure 5. The partitioning of fluorescent lipophilic probes at pH 8.5 into membrane domains of confluent monolayers at 4°C and 20°C. AFC16, found to be nearly insoluble at pH 7.3 and 4°C (Fig. 3) and unable to label the BL domain under these conditions (Fig. 1 and 2), was found to label the AP and BL domains more equally under more solubilized conditions of pH 8.5. The measurements are the average of two separate experiments.

and AFC16 may also be temperature-dependent. We found that the solubility of both probes is only very slightly affected by temperature at pH 7.3, but was more affected by a simultaneous change in pH and temperature. **Fig. 4** shows the effect of pH 7.3 and 8.5 on the solubility of ACF12 and AFC16 at 4°C, 20°C and 37°C. Only at 37°C and pH 8.5 are both probes completely in solution. This suggested that at the temperatures and pH values used in the literature (1,2,6,7), about 50% of AFC12, and virtually non of AFC16, is in solution. We then tested the penetrability of AFC12 and AFC16 at pH 8.5 and at 4°C and 20°C on confluent BAEC monolayers. We chose these conditions since an equilibration of AFC16 between the AP and BL PM domains, similar to that seen for AFC12 at pH 7.3, should be seen. We avoided labeling at 37°C, since transcytosis is occuring at this temperature and should AFC16 appear on the BL membrane domain, it could be due to this event and not the result of the change in solubility of the probes. **Fig. 5** shows at the higher pH of 8.5, when confluent monolayers are labeled with AFC12 or AFC16 at either 4°C or 20°C, the AFC16 label does indeed appear to equilibrate more, but not completely, between the BL and AP PM domains than when the labeling procedure is carried out on confluent monolayers at 4°C at pH 7.3 (**Fig. 2**). Again, under these conditions, AFC16 is still not completely in solution, so equal labeling between the AP and BL domain was not expected. During the short, 3 min incubation period at pH 8.5, the monolayers displayed no visible disruption of tight junctional integrity.

DISCUSSION

The two fluorescent probes that have been used extensively in polarity studies, AFC12 and AFC16, have been examined here in greater detail in order to elucidate discrepancies we have observed using surface-selective labeling techniques (9). We have found that the domain-specific labeling protocols that work extremely well on tight epithelial cell systems (e.g. biotinylation, radio-iodination), consistently perform less optimally on endothelial cell systems in vitro. The sum of the data presented above indicates that the tight junctions of cultured BAEC monolayers do not restrict the passage of fluorescent lipid probes from the AP to the BL PM domain via intercellular junctional complexes.

Under the conditions used in the literature (1,2,6,7), AFC16 is restricted to the AP PM domain because it is nearly insoluble and present in large aggregates, possibly several tens to several hundred nanometers in size. This is comparable or larger than the cationic colloidal silica microbeads (20-50 nm) used to isolate the AP and BL PM domains (15) and these have been shown not penetrate the tight juctions of epithelial cells (16) or endothelial cells (9). As a result, we now assume that the asymmetric labeling of AFC16 is not due to its inability to "flip-flop" from the external leaflet to the cytoplasmic leaflet in the lipid bilayer of the membrane, as is thought to be the case with AFC12, but due to the fact that it is essentially insoluble at pH 7.3 at 4°C. Interestingly, the experiments performed by Dragsten et al. (1) on epithelial cells indicated that differences in trans-epithelial electrical resistance from a "tight" 5,000 ohms·cm^2 to a "leaky" 100 ohms·cm^2 did not change the penetrability of AFC16 to the BL domain, but did not comment on this observation further. Since endothelial cells have not been shown to have electrical resistances greater than 69 ohms·cm^2 in vitro (14,17), they also fit into a "leaky" epithelial category. A difference in solubility properties between the two probes could explain the the reason that we and others found no difference in the AFC16 labeling among tight and leaky epithelial cells under the conditions used. This is consistant with the idea that the large AFC16 particles could not penetrate between cells, but free molecules or small micelles of AFC12 could gain access across the tight junctions of endothelial cells. Another interesting finding by Dragsten et al. (1) involves the differences in diffusion rates of AFC12 and AFC16 on the surface of epithelial cells. A three-fold slower diffusion coefficient of the AFC16 (4×10^{-9} cm^{2}sec^{-1}) was found when compared to that of the AFC12 (13×10^{-9} cm^{2}sec^{-1}). This observation also argues for the presence of large aggregates of AFC16 on the AP surface.

The data described above conform to the idea that the AP surface-selective labeling of AFC16 seen on confluent BAEC monolayers, as well as "leaky" epithelium, is due primarily to the solution properties of the probe. The results obtained using AFC12, especially on "tight" epithelia, are less clear. Previous results (1,2) indicate that the "flip-flop" of AFC12 from the AP to the BL PM domain of tight epithelia occurs at 20°C, but not at 5°C, within 10 min. This does support the idea that AFC12 may translocate to the BL domain via such a mechanism since "flip-flop" is known to be temperature-dependent and we observed no great solubility differences in AFC12 at 4°C and 20°C at physiological pH. The fact that tight epithelium can restrict the movement of ions across the monolayer can account for the "flip-flop" theory of symmetric AFC12 labeling in this specific system. It does not, however, conclude that AFC16 is thermodynamically prohibited from

achieving this same feat due to its longer acyl side chain. It should be noted that membrane lipid "flip-flop" is primarily restrictricted by the polar head group which is the same in both AFC12 and AFC16 -- the probes only differ by four methyl groups in the acyl chain.

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