Test of a two-pathway model for small-solute exchange across the capillary wall

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Fu, B. M., R. H. Adamson, and F. E. Curry. Test of a two-pathway model for small-solute exchange across the capillary wall. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H2062–H2073, 1998.—We previously proposed a two-pathway model for solute and water transport across vascular endothelium (Fu, B. M., R. Tsay, F. E. Curry, and S. Weinbaum. J. Biomech. Eng. 116: 502–513, 1994) that hypothesized the existence of a continuous slit 2 nm wide along tight junction strands within the interendothelial cleft in parallel with 20 × 150-nm breaks in tight junctions. We tested this model by measuring capillary permeability coefficients (P) to a small solute (sodium fluorescein, radius 0.45 nm), assumed to permeate primarily the 2-nm small pore, and an intermediate-sized solute (FITC-α-lactalbumin, radius 2.01 nm) excluded from the small pore. Mean values of the paired diffusive permeability coefficients, P(sodium fluorescein) and P(FITC-α-lactalbumin), were 34.4 and 2.9 × 10⁻⁶ cm/s, respectively, after corrections for solvent drag and free dye (n = 26). These permeabilities were accounted for by transport through the large-break pathway without the additional capacity of the hypothetical 2-nm pathway. As a further test we examined the relative reductions of P(sodium fluorescein) and P(FITC-α-lactalbumin) produced by elevated intracelluar cAMP. Within 20 min after the introduction of rolipram and forskolin, P(sodium fluorescein) and P(FITC-α-lactalbumin) decreased to 0.67 and 0.64 times their respective baseline values. These similar responses to permeability decrease were evidence that the two solutes were carried by a common pathway. Combined results in both control and reduced permeability states did not support the hypothesis that a separate pathway across tight junctions is available for solutes with a radius as large as 0.75 nm. If such a pathway was present, then its size must be smaller than that of sodium fluorescein.

Quantitative fluorescence microscope photometry; paired measurements on single capillaries; three-dimensional junction-pore matrix model for interendothelial cleft; rolipram; forskolin

The cleft between adjacent endothelial cells is the primary pathway for water and hydrophilic solutes across the wall of endothelial barriers. Within the cleft, a major barrier to the movement of water and hydrophilic solutes is the junctional strand (5, 14–16). Using serial section reconstructions in individually perfused capillaries of frog mesentery, Adamson and Michel (4) demonstrated that the junctional strands were not continuous but were interrupted by infrequent breaks that, on average, were 150 nm long, spaced 2–4 μm apart along the strand, and accounted for up to 10% of the length of the strand. At these breaks, the space between adjacent endothelial cells (average 20 nm) was as wide as that in regions of the cleft between adjacent cells with no strands. Adamson and Michel (4) were able to account for the measured hydraulic conductivities of continuous capillaries of frog mesentery by modeling water flow through the breaks when they considered the two-dimensional spreading of the water flows on either side of the breaks. Fu et al. (10, 11) extended this approach and developed a three-dimensional model of water and solute flows through the strand breaks in the presence of a surface fiber matrix (Fig. 1). They found that the observed break frequency was sufficient to account for the measured water flows and the permeability coefficients of solutes with a radius larger than 0.75 nm, but that the observed break frequency was too small to account for the measured permeability coefficients for solutes with a Stokes radius smaller than 0.75 nm. Fu et al. (10, 11) suggested that, in addition to the breaks described by Adamson and Michel (4), solutes with a radius smaller than 0.75 nm could cross through the strands via a very small pore system within the junctional strand, which excluded solutes with a radius larger than 0.75 nm. They suggested that this pathway may be formed by a narrow slit pathway 2 nm wide, which may represent a continuous 2-nm translucent narrow slit along the outer leaflets in the tight junction revealed in the investigation of strand structure on a tilting stage. The model describing both large breaks and the putative pathway for small solutes is called the two-pathway model to distinguish it from the large-break model.

The aim of the present experiments was to test the hypothesis proposed by Fu et al. (10, 11) that a pathway for small solutes, separate from a pathway through larger breaks in the junctional strand, makes a measurable contribution to the transcapillary flux of the small solutes. One difficulty with the comparison made by Fu et al. (10, 11) was that the permeability coefficients used to test their models for solutes ranging in size from that of sodium chloride to that of albumin were not measured on the same population of microvessels. Furthermore, the values were measured using several different techniques. Thus one aim of these experiments was to make paired permeability measurements on each microvessel of frog mesentery for a small fluorescent solute (sodium fluorescein; mol wt 376, Stokes radius 0.45 nm), which would be representative of solutes assumed to cross the wall mainly through the putative very small pore pathway, and a larger solute (α-lactalbumin; mol wt 14,176, Stokes radius 2 nm) labeled with FITC, which should not penetrate the very small pore pathway but should penetrate the vessel wall via the pathway formed by the larger breaks in the junctional strand. Another aim of the experiments was to determine whether experimental conditions that raised endothelial cell cAMP concentrations and low-
fibers is cylindrical fibers. Radius of these fibers is 3. Glycocalyx structures are represented by a periodic square array of large junctional breaks. At entrance of cleft on luminal side, surface when the vessels were exposed to conditions that observe a reduction in the permeability for both solutes, we should argued that if both small and intermediate-sized solutes shared a common pathway with water, we should measure for water and larger solutes.

The solute permeability coefficient (P) was measured by using quantitative fluorescence microscope photometry (1, 12, 13). All experiments have been performed on individually perfused venular microvessels in frog mesentery. Each capillary was perfused via two micropipettes to enable the perfusate to be switched rapidly from a clear (washout) perfusate to one containing the fluorescently labeled test solute. This method enabled us to repeat measurements of the permeability coefficient of the capillary wall to more than one solute and to the same solute under more than one chemical treatment on each capillary. In addition, the permeability was measured by perfusing segments of the microvessels with fluorescently labeled solutes under conditions in which the transcapillary differences of solute concentration and hydrostatic pressure were known (13).

MATERIALS AND METHODS

General

All in vivo experiments reported in this paper were performed on male leopard frogs (Rana pipiens; 2.5–3 in. in length), supplied by J. M. Hazen (Alburg, VT). The methods used to prepare the frog mesentery, perfusates solutions, and micropipettes for microperfusion experiments have been described in detail elsewhere (1, 9, 12, 13). A brief outline of the methods is given with emphasis on the special features of the current experiments.

The frog brain was destroyed by pithing, leaving the spinal cord intact. The abdominal cavity was opened, and the mesentery was gently arranged on the surface of a polished quartz pillar (1 cm in diameter; Heraeus-Amersil, Fairfield, NJ) to maintain the circulation to the gut and mesentery of the animal. The upper mesentery was continuously superfused with frog Ringer solution at 14–18°C. Venular capillaries, generally 20–30 μm in diameter, were chosen for study. All vessels had brisk blood flow immediately before cannulation and had no marginating white cells. Each of the two arms of a Y-branched microvessel were cannulated with beveled glass micropipettes containing perfusion solutions. This arrangement allowed alternate perfusion of the downstream vessel with a washout solution (containing no fluorescent solute) or the test solution (containing the fluorescent solute). Each pipette was connected to a water manometer that enabled perfusion at known pressures. For these experiments the pressure in the capillaries was 5–8 cmH2O as determined by balancing the interface between fluorescent and nonfluorescent solution within the nonflowing branch of the Y (13). This pressure range was chosen to minimize convectively coupled solute flux. In each vessel, P was determined for straight segments, 300–500 μm long, at least 100 μm downstream from the Y-branch junction point.

Frog Ringer solution was used for all dissections, perfusates, and superfusates. The solution composition was (in mM) 111 NaCl, 2.4 KCl, 1.0 MgSO4, 1.1 CaCl2, 0.195 NaHCO3, 5.5 glucose, and 5.0 HEPES. The pH was balanced to 7.4 by increase intracellular cAMP. On the other hand, if the pathway available for larger solute accounted for less than one-half the flux of small solutes, and if the main effect of increased cAMP was on the common solute-water pathway, then the proportional reduction in permeability for small solutes in vessels treated with increased cAMP was expected to be less than that measured for water and larger solutes.

Fig. 1. (A) Plane view of junction-orifice-matrix entrance layer model for interendothelial cleft (10, 11). Junction strand with periodic openings lies parallel to luminal front. L is total depth of cleft, L2 is depth of pores in junction strand, and L1 and L3 are depths between junctional strand and luminal and abluminal fronts, respectively. L2 is thickness of fiber matrix at cleft entrance. Distance between 2 adjacent breaks in junctional strand is 2D, and d is one-half width of large junctional breaks. At entrance of cleft on luminal side, surface glycocalyx structures are represented by a periodic square array of cylindrical fibers. Radius of these fibers is a, and gap spacing between fibers is Δ. B: 3-dimensional sketch of model. Large-break only model: only 2D × 2B = 150 × 20-nm breaks in junction strand; 2-pathway model: a continuous small slit with width 2b2 = 1.5 nm existing in parallel with 2D × 2B = 150 × 20-nm large breaks in junction strand, where B is one-half cleft width.

...er the basal hydraulic conductivities would also reduce the permeability coefficients to small and larger solutes. Specifically, Adamson et al. (3) demonstrated that elevation of endothelial cell intracellular cAMP concentrations by simultaneous adenylylate cyclase activation ( forskolin) and phosphodiesterase (PDE 4) inhibition (rolipram) reduced capillary hydraulic permeability (P) to 43% of baseline values within 20 min. We argued that if both small and intermediate-sized solutes shared a common pathway with water, we should observe a reduction in the permeability for both solutes when the vessels were exposed to conditions that...
adjusting the ratio of HEPES acid to base. In addition, both the clear washout solution and the fluorescent dye solution contained BSA (A4378, Sigma) at 10 mg/ml.

**Fluorescent Test Solute Preparation**

Sodium fluorescein. Sodium fluorescein (F6377, Sigma; mol wt 376, Stokes-Einstein radius ~0.45 nm) was dissolved at 0.1 mg/ml in frog Ringer solution containing 10 mg/ml BSA. The solution was made fresh on the day of use to avoid binding to the serum albumin (2).

FITC-labeled α-lactalbumin. α-Lactalbumin (L6010, Sigma; mol wt 14,176, Stokes-Einstein radius ~2 nm) was labeled with FITC (F7250, Sigma; mol wt 389.4) as follows (revised from Ref. 13). Protein (90 mg) was dissolved in 15 ml of borate buffer (0.05 M, pH 9.3, 20°C) containing 0.4 M NaCl. The solution was placed in 18-mm-diameter dialysis tubing with a 3,500-mol wt cutoff (Spectrum Medical Industries) and was dialyzed for 12 h with constant stirring at 15°C against 50 ml of borate buffer containing FITC (0.5 mM). The labeled protein then was dialyzed against 2 liters of glucose-free frog Ringer solution twice, each time for 12 h. The dialysis procedure was repeated twice further with 2 liters of normal frog Ringer solution until there was no free dye. The influence of free dye on measured permeability to a labeled protein will be discussed in the Appendix. The FITC-labeled α-lactalbumin was stored frozen and was used within 2 wk of preparation. On the day of use, unlabeled BSA was added to aliquots of the labeled protein. The final FITC-α-lactalbumin dye concentration used in the experiment was 2 mg/ml in frog Ringer solution. For this preparation, the fluorescence intensity of the free FITC dye was 1% of the solution, which was checked using the photometer at the same instrument settings used in our experiments.

Dye solutions with either sodium fluorescein or FITC-α-lactalbumin and BSA were kept chilled until just before use and were discarded at the end of the day. The pH of all solutions was adjusted to 7.4 at 23°C.

**Microscope and Photometer Preparation**

A detailed description of the method used to measure P of fluorescently labeled solutes has been published (13). In the current experiments we used a different but similar experimental setup. A Nikon Diaphot inverted fluorescence microscope was used to observe the mesentery. A ×10 lens (Nikon, NA 0.3) gave a field of view of ~2 mm in diameter. The tissue was observed with either transmitted white light from a light pipe held suspended above the preparation or with fluorescent light from a xenon lamp (Nikon, 75 W) with an appropriate filter set for fluorescein. The set consisted of an excitation filter (460–500 nm), a dichroic mirror (DM505), and a bandpass filter (510–560 nm). A neutral density filter (ND = 1.0) in the light path reduced the excitation light intensity, which prevented tissue damage. Further protection was provided by using an experimental protocol in which the time of tissue exposure to the excitation light was kept as short as possible for the permeability measurement. Generally, the exposure time for an individual measurement was 10–30 s. The light was off when measurements were not in progress. The fluorescence intensity (I₀) in the capillary lumen and surrounding tissue was measured by aligning the vessel segment within an adjustable measuring window consisting of a rectangular diaphragm in the light path. The maximum size of the window is 250 µm wide and 650 µm long. In our experiment, the dimensions of the measuring window were generally 100–200 µm wide (roughly 5 times the capillary diameter) and 300–500 µm long. The measuring window was set at least 100 µm from the base of the Y to avoid solute contamination from the sidearm. I₀ measured by a photometer (P101, Nikon), was continuously recorded on a strip chart (model 17500A, Hewlett-Packard).

P was calculated from the relationship

\[ P = \frac{1}{\Delta I_0} \left( \frac{dI}{dt} \right)_0 \frac{1}{r^2} \]

where \( \Delta I_0 \) is the step increase in fluorescent light intensity as the test solute fills the capillary lumen, \( (dI/dt)_0 \) is the initial rate of increase in fluorescence light intensity after solute fills the lumen and begins to accumulate in the tissue, and \( r \) is the capillary radius (13).

**Calibration Experiments**

The primary assumption in the calculation of \( P \) with the use of fluorescent solutes is that \( I_0 \) is a linear function of the number of solute molecules in the measuring field. In both in vivo and in vitro calibrations, we used the same instrument settings used for the permeability experiments. The settings were the same for sodium fluorescein and FITC-α-lactalbumin test solutes to allow comparison of their permeabilities in the paired measurements on the same individual vessel.

The results of experiments performed to test the assumption follow. In vitro calibrations were performed using a simple chamber constructed from coverslips (2). A large 24 × 50-mm coverslip formed the base of the chamber. Two small 22-mm² coverslips were laid on top of this base ~1 cm apart, and a third small coverslip was placed on top of those to form a chamber ~170 µm deep. Solutions of fluorescein were applied to the edge of the opening, and the chamber filled by capillarity. A new chamber was made for each concentration. \( I_0 \) was measured from each concentration. These in vitro calibrations showed that the relationship between the concentration and fluorescence intensity was linear over the concentration range from 0.025 to 0.15 mg/ml for sodium fluorescein and from 0.5 to 3 mg/ml for FITC-α-lactalbumin.

In the in vivo calibration experiment, a capillary was cannulated and perfused as if for measurement of \( P \). We recorded the step increase in \( I_0 \) as the solute was perfused into the capillary. Perfusion time was ~5 s during each run to allow the solute to accumulate in the surrounding tissue. The procedure was repeated on the same capillary at each of four sodium fluorescein solute concentrations: 0.05, 0.1, 0.15 and 0.2 mg/ml. Step increase was plotted as a function of concentration. In a separate experiment, the step increase was recorded on a single vessel at each of four FITC-α-lactalbumin concentrations: 1, 2, 2.5, and 3 mg/ml. We also checked, in an in vivo experiment whether \( I_0 \) was independent of the width of the measuring window when the capillary was aligned along the center line of the window. In vivo calibration results for the same concentration ranges as in the in vitro experiment are shown in Fig. 2. Figure 2A shows the results of an experiment to perfuse a capillary with four concentrations of sodium fluorescein. The capillary diameter was 30 µm, and the measuring window was 240 µm wide and 350 µm long. Figure 2B shows the results for FITC-α-lactalbumin in a capillary of 40-µm diameter. The measuring window was also 240 µm wide and 350 µm long. Both the 0.1 mg/ml sodium fluorescein and the 2 mg/ml FITC-α-lactalbumin concentrations used in all our measurements fell within the linear range of the calibration. In addition, the linear relationship between the fluorescence intensity and the concentration held for both solutes under the same instrument settings. We can therefore directly compare the results from different solutes on each capillary.
achieved with the use of the neutral density filter in our experiments. If this method were used for a larger molecule or in a tighter and smaller vessel, the exposure time to the fluorescence light for the individual permeability measurement would be longer than 30 s.

Experimental Protocol

During the interval between measurements using the test solutes (30–120 s), the microvessel was perfused with the washout solution and the test solute was washed out of the measuring window. This arrangement minimized solute accumulation within the measuring window and enabled repeated permeability measurements of the same test solute under various conditions and/or a series of test solutes on the same microvessel segment to be performed.

To test the effects of rolipram and forskolin on sodium fluorescein and FITC-α-lactalbumin permeabilities, for each test solute, after making several control measurements when the washout pipette was filled with Ringer perfusate containing BSA (10 mg/ml) and the dye pipette was filled with the same perfusate, to which the test solute was added, we replaced both washout and test pipettes with new pipettes that also contained rolipram (10 µM) and forskolin (5 µM). The concentrations of rolipram and forskolin were chosen to be consistent with those used in Lp measurements by Adamson et al. (3). The test measurement during the treatment with rolipram and forskolin was performed once for every 2- to 5-min interval. The duration of the fluorescent light exposure was 10–30 s for each measurement. The treatment lasted ~20 min.

We also performed paired measurement of sodium fluorescein and FITC-α-lactalbumin permeabilities on single capillaries. In one set of measurements, we first measured the sodium fluorescein permeability and then changed the dye pipette to FITC-α-lactalbumin to measure its permeability under control conditions. Replacing washout and test pipettes with pipettes containing rolipram and forskolin, we measured sodium fluorescein permeability for 20 min and then changed the dye pipette to measure FITC-α-lactalbumin permeability for another 20 min. In the second set of these measurements, we used exactly the same procedure as in the first set but changed the order of measurements to FITC-α-lactalbumin first and sodium fluorescein second.

Reagents

Rolipram (supplied by Eisai London Laboratories) and forskolin (Biomol) were prepared as 50 mM and 25 mM stock solutions in ethanol, respectively. These stock solutions were kept at –20°C and were not used for more than 1 mo. Final test solutions containing both forskolin (5 µM) and rolipram (10 µM) were made by diluting the stock using 10 mg/ml BSA frog Ringer solution. The ethanol concentration in the final test solution was 6.8 mM (i.e., 0.04% vol/vol). All other perfusates without rolipram and forskolin also contained ethanol as a vehicle.

Analysis and Statistics

P measurements during the control period in a vessel were averaged to establish a single value for control P. This value was then used as a reference for all subsequent measurements on that vessel. To present data at a specific time, individual measurements were averaged during the period from 3 min before to 3 min after measurements. The within-experiment averaged P was then presented at time 0 (control); at 5, 10, 15, and 20 min for single solute permeability measurements; and at 25, 30, 35, and 40 min for paired
RESULTS

Rollipram and Forskolin Effect on P to Sodium Fluorescein and FITC-α-Lactalbumin

In a series of preliminary experiments we tested whether solutions containing both forskolin (5 µM) and rolipram (10 µM) caused a decrease in capillary permeabilities to sodium fluorescein (P<sub>sodium fluorescein</sub>) and FITC-α-lactalbumin (P<sub>FITC-α-lactalbumin</sub>). The permeability coefficients to these solutes were measured on separate microvessels. Continuous measurements of either P<sub>sodium fluorescein</sub> or P<sub>FITC-α-lactalbumin</sub> during treatment with rolipram and forskolin in individual vessels are shown in Figs. 3 and 4. For each test solute, after making several measurements by perfusing the control solution to establish a baseline P<sub>control</sub>, we recannulated the same capillary with perfusates containing rolipram and forskolin to increase the CAMP level and made measurements once for every 2-5 min interval. Figure 3A shows that in one typical vessel, P<sub>sodium fluorescein</sub> started to fall within 5 min and fell further with time. P<sub>sodium fluorescein</sub> fell to 52% of its control value at ~20 min in this capillary. Figure 3B shows the control experiment in which the vessel was reperfused with the control perfusate containing no forskolin or rolipram. Measured values of permeability showed a small fluctuation around the mean value but did not fall over a period of ~60 min. Results for FITC-α-lactalbumin in similar experiments are demonstrated in Fig. 4, A and B.

Figure 5 summarizes the results from a series of individual measurements similar to those shown in Figs. 3 and 4. Mean results are shown for control measurements for P<sub>FITC-α-lactalbumin</sub> in four vessels and for P<sub>sodium fluorescein</sub> in five vessels. Test results are shown for both FITC-α-lactalbumin and sodium fluorescein when CAMP levels were elevated by rolipram and forskolin. P<sub>test</sub> represents the test permeability at a specific time, and results are expressed as the ratio for the mean control value (P<sub>test</sub>/P<sub>control</sub>) for each single vessel. After the introduction of rolipram and forskolin, P<sub>FITC-α-lactalbumin</sub> averaged over 17 vessels decreased from a mean control value of 3.5 (±1.5 SD) × 10<sup>-6</sup> cm/s to a mean value of 2.1 (±0.7 SD) × 10<sup>-6</sup> cm/s after 20 min, a reduction to 60%. This mean ratio was similar to the ratio P<sub>test</sub>/P<sub>control</sub> of 0.64 (±0.19 SD) measured on individual vessels. The range of individual ratios after 20 min was from 0.29 to 0.95. In 18 different vessels, P<sub>sodium fluorescein</sub> decreased from a mean control value of 28.5 (±11.6 SD) × 10<sup>-6</sup> cm/s to a mean value of 18.3 (±5.7 SD) × 10<sup>-6</sup> cm/s after 20 min. The mean ratio of 0.64 was also similar to the ratio P<sub>test</sub>/P<sub>control</sub> measured on individual vessels, which was 0.67 (±0.17 SD) after 20 min. The range of individual ratios was from 0.36 to 1.0. The falls in P<sub>FITC-α-lactalbumin</sub> and P<sub>sodium fluorescein</sub> were highly significant at all times and were different from their control values (P < 0.01, Wilcoxon signed-rank test). Figure 5 also indicates that the temporal patterns of decreasing permeability under the treatment of rolipram and forskolin are the same for FITC-α-lactalbumin and sodium fluorescein (P > 0.5, Mann-Whitney U test). Thus increased CAMP reduces both small-solute and large-solute permeability to nearly the same extent. This result would not be expected if most of the small solute crossed the vessel wall via a pathway that was regulated by a mechanism different from those modulating water and larger-solute permeability.

In four vessels in which control P<sub>sodium fluorescein</sub> was 25.1 (±12.3 SD) × 10<sup>-6</sup> cm/s, treatment with rolipram and forskolin decreased permeability to 17.3 (±10.6 SD) × 10<sup>-6</sup> cm/s in 20 min. After perfusion for 20 min with control solution, the final P<sub>sodium fluorescein</sub> was 17.9 (±10.0 SD) × 10<sup>-6</sup> cm/s. In a separate group of four vessels, rolipram and forskolin induced a fall in P<sub>FITC-α-lactalbumin</sub> from 3.7 (±1.4 SD) to 1.8 (±0.4 SD) × 10<sup>-6</sup> cm/s. Perfusion for a final 20 min with control
solution did not change \( P_{\text{FITC-\alpha-lactalbumin}} \) (1.8 \pm 0.3 SD) \times 10^{-6} \text{ cm/s}). Therefore, both groups exhibited a sustained and stable permeability decrease over the time course of our experiments.

**Paired Measurements of \( P_{\text{sodium fluorescein}} \) and \( P_{\text{FITC-\alpha-lactalbumin}} \) on Single Capillaries**

In the experiments described earlier, we measured \( P_{\text{sodium fluorescein}} \) and \( P_{\text{FITC-\alpha-lactalbumin}} \) in different groups of vessels. Because baseline vessel permeabilities can vary within groups, we extended the study to measure the permeabilities of both solutes on the same microvessels before and after treatment with rolipram and forskolin to increase intracellular cAMP concentrations.

We first report the results of paired measurements of \( P_{\text{sodium fluorescein}} \) and \( P_{\text{FITC-\alpha-lactalbumin}} \) in the control state (i.e., without rolipram or forskolin). Paired measurements of \( P_{\text{FITC-\alpha-lactalbumin}} \) and \( P_{\text{sodium fluorescein}} \) in 26 vessels are listed in Table 1. In 15 microvessels, FITC-\( \alpha \)-lactalbumin was the first test solute and sodium fluorescein was the second. In the other 11 vessels, the order of the perfusion was reversed. The mean values from 26 individual capillaries are \( P_{\text{FITC-\alpha-lactalbumin}} = 3.73 \pm 1.40 \) SD \times 10^{-6} \text{ cm/s} (range from 1.10 to 6.47 \times 10^{-6} \text{ cm/s}), \( P_{\text{sodium fluorescein}} = 34.4 \pm 14.5 \) SD \times 10^{-6} \text{ cm/s} (range from 12.36 to 60.70 \times 10^{-6} \text{ cm/s}), and the mean ratio \( P_{\text{sodium fluorescein}}/P_{\text{FITC-\alpha-lactalbumin}} = 10.55 \pm 6.20 \) SD (range from 3.10 to 26.45). There was some tendency for the ratio to increase with time.

**Table 1. Paired control measurements of \( P_{\text{FITC-\alpha-lactalbumin}} \) and \( P_{\text{sodium fluorescein}} \) on single capillaries**

<table>
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<tr>
<th>Vessel No.</th>
<th>( P_{\text{FITC-\alpha-lactalbumin}} )</th>
<th>( P_{\text{sodium fluorescein}} )</th>
<th>( P_{\text{sodium fluorescein}}/P_{\text{FITC-\alpha-lactalbumin}} )</th>
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<td>7.03</td>
</tr>
<tr>
<td>26</td>
<td>5.19</td>
<td>37.01</td>
<td>7.14</td>
</tr>
</tbody>
</table>

Mean (SD) \( 3.73 (1.40) \times 10^{-6} \text{ cm/s} \), \( 34.4 (14.5) \times 10^{-6} \text{ cm/s} \), \( 10.55 (6.20) \times 10^{-6} \text{ cm/s} \). Values are capillary permeabilities to FITC-\( \alpha \)-lactalbumin (\( P_{\text{FITC-\alpha-lactalbumin}} \)) and to sodium fluorescein (\( P_{\text{sodium fluorescein}} \)) given as \( \times 10^{-6} \text{ cm/s} \).
ratio of permeability coefficients to be lower in vessels in which sodium fluorescein was measured first, suggesting that there was some tendency to overestimate the second permeability measurement in a pair.

In 17 of the 26 vessels in which paired measurements of solute permeability were performed, we also measured paired permeability coefficients after exposure to rolipram and forskolin. Results for our paired measurements of sodium fluorescein and FITC-α-lactalbumin on single capillaries are shown in Fig. 6, A and B. As demonstrated in one representative capillary (Fig. 6A), we first measured sodium fluorescein and then sodium fluorescein under control conditions. After both washout and dye pipettes were replaced with perfusates containing rolipram and forskolin, we continuously measured sodium fluorescein at intervals of 2–5 min for 20 min. Replacing the dye pipette with sodium fluorescein solution containing rolipram and forskolin, we measured the permeability coefficient for sodium fluorescein for another 20 min in the same manner. We successfully completed eight experiments in the order shown in Fig. 6A. We also completed an additional nine experiments with the order of the solute perfusion reversed (Fig. 6B). The time-dependent decreasing patterns of sodium fluorescein and FITC-α-lactalbumin in these representative experiments are similar to those in nonpaired measurements.

A summary of these paired permeability measurements is shown in Fig. 7. Results are described as Ptest/Pcontrol for the same capillary. The mean results are shown for measurement of sodium fluorescein first and sodium fluorescein second in eight vessels. The results are shown for measurement of sodium fluorescein first and FITC-α-lactalbumin second in 9 vessels. After 20–25 min, Ptest/Pcontrol with paired measurements on 17 vessels was 0.62 (±0.16 SD) for FITC-α-lactalbumin and 0.64 (±0.16 SD) for sodium fluorescein. These values are similar to those (0.64 and 0.67) measured in the nonpaired experiments as previously described. Statistical analysis of these data shows that there is no significant difference in decreasing patterns of sodium fluorescein and FITC-α-lactalbumin over time when the order of the measurement is switched (P > 0.4, Mann-Whitney’s U test). Furthermore, as shown in Fig. 8, for paired permeability measurements before and after treatment with rolipram and forskolin, there was no systematic variation in the magnitude of the reduction in permeability with the magnitude of the initial permeability coefficient.

Evaluation of Methods: Free Dye Associated With FITC-Labeled α-Lactalbumin

We chose FITC as the labeling fluorophore for the α-lactalbumin mainly to obtain high quantum yield (ratio of the number of fluorescence photons emitted to the number of photons absorbed) with low light excitation. However, FITC (mol wt 389.4) diffuses through capillary walls much faster than FITC-α-lactalbumin (mol wt 14,176). A small amount of the free FITC will cause a large overestimation of the permeability to FITC-α-lactalbumin molecules. We therefore measured the amount of free dye in our labeled α-lactalbumin solutions. After being ultrafiltered by a clinical centrifuge (1,750 rpm, 444 g) through a centricon filter (Millipore, 3,000 mol wt cutoff) from the 2 mg/ml FITC-α-lactalbumin solution used in our experiments, the filtrate was checked for fluorescence intensity due to free FITC (Iₘ). The method for measuring Iₘ is the same as that described for Iₘ in the in vitro calibration, and the instrument settings are the same as those used for the permeability measurements. We also measured the fluorescence intensity of the original solution containing a mixture of the pure labeled protein FITC-α-lactalbumin and free FITC (Iₖ). The percentage of the free dye intensity F = Iₘ/Iₖ is thus determined. F was ~1% in our solution of 2 mg/ml FITC-α-lactalbumin. The molecular weight of the labeling fluorophore FITC (389.4) is similar to that of sodium fluorescein (376). It should have a similar P to sodium fluorescein, provided that P is determined by solute size. In our experiments...
sodium fluorescein was measured as $34.4 \times 10^{-6}$ cm/s and $P_{\text{FITC-\alpha-lactalbumin}}$ was 3.73 $\times 10^{-6}$ cm/s, which are represented by $P_f$ and $P_m$ in the APPENDIX, respectively.

Substituting the values of $F$, $P_f$, and $P_m$ into Eq. A4, we obtain a true value for $P$ of the pure labeled protein $\text{FITC-\alpha-lactalbumin}$ of $3.42 \times 10^{-6}$ cm/s, which is 90% of the measured $P_{\text{FITC-\alpha-lactalbumin}}$.

DISCUSSION

The results of these experiments do not appear to support the hypothesis that the small fluorescent test solute, sodium fluorescein, crosses the walls of frog mesenteric microvessels via a pathway different from that available to the large solute, $\alpha$-lactalbumin. The first argument against a significant contribution from a very small pore pathway is that, on average, the permeability coefficients for sodium fluorescein were only 10.6 times larger than permeability coefficients for $\alpha$-lactalbumin measured on the same capillary. This difference is within the range of values predicted by the model described by Fu et al. (10, 11) for diffusion through breaks in the junctional strand observed by Adamson and Michel (4) and when there was a fiber matrix at the cleft entrance. Thus the contribution of a pathway available to sodium fluorescein, but not to $\alpha$-lactalbumin (e.g., a pathway formed by a narrow slit, 2 nm wide along the junction strand), was smaller than could be detected with these measurements. We will evaluate this result in more detail below. The second line of evidence against a separate pathway for sodium fluorescein is that the permeability coefficients for both sodium fluorescein (Stokes radius 0.45 nm) and $\alpha$-lactalbumin (Stokes radius 2 nm) are reduced to the same extent relative to their controls in microvessels exposed to conditions that lower basal permeabilities. We expected this result when the same mechanisms reduced permeabilities to both solutes. Some possible mechanisms to change the permeability coefficients for both solutes are evaluated below. As we shall discuss, these results do not definitively rule out the presence of a second pathway for solutes smaller than sodium fluorescein, but they place much tighter constraints on the properties of such a pathway than were available from the previous analysis (10, 11). Before we discuss these results further, it is useful to evaluate the possible errors in the method used to measure permeability coefficients.

Free Dye Influence on $P_{\text{FITC-\alpha-lactalbumin}}$

We estimated in RESULTS that free dye would lead to an overestimation of the permeability coefficient for $\alpha$-lactalbumin by $\sim$10%. The effect of free dye on the estimate of the fractional reduction in $\alpha$-lactalbumin permeability due to forskolin and rolipram is much smaller. This is seen from Eq. A5 in the APPENDIX. Substituting a value of the measured ratio of sodium fluorescein permeability to $\alpha$-lactalbumin permeability (g in Eq. A5, equal to 10.55), the decrease by rolipram and forskolin in permeability to the pure labeled protein $\text{FITC-\alpha-lactalbumin}$ is estimated to be 63.6%, which is only 0.4% less than the measured decrease of 64% in the presence of a small amount of free dye.

Solvent Drag Contribution to $P_{\text{FITC-\alpha-lactalbumin}}$

We kept hydrostatic pressures in the microvessels low during permeability measurements. However, because solute flux can couple to water flow (solvent drag), the permeability coefficient $P$ measured in our experiments (apparent permeability) tends to overestimate the true diffusive permeability coefficient ($P_d$) of intermediate-sized molecules. The solvent drag may account for an increase in apparent permeability of
α-lactalbumin of close to $0.24 \times 10^{-6}$ cm/s for each 1 cmH2O of effective pressure across the vessel wall (13). Furthermore, because exposure of microvessels to rolipram and forskolin at the same concentrations used in the present experiment reduces the $L_p$ of frog mesenteric microvessels to 43% of control values under the same conditions (3), we examined the possibility that some of the reduction in the apparent permeability to α-lactalbumin was the result of a decrease in the solvent drag component of transport and not a true change in permeability coefficient. The relationship between $P$ and $P_d$ was determined by

$$P = \frac{P_e}{\exp (P_e) - 1} + L_p (1 - \sigma) \Delta P_{eff} \tag{2}$$

where $\sigma$ is the solute reflection coefficient of the capillary wall to FITC-α-lactalbumin (8) and $P_e$ is the Péclet number. $\Delta P_{eff}$ is the effective filtration pressure across the capillary wall, which can be expressed as

$$\Delta P_{eff} = \Delta P - \sigma^{\text{albumin}} \Delta P^{\text{α-lactalbumin}} - \sigma^{\text{FITC-α-lactalbumin}} \Delta P^{\text{FITC-α-lactalbumin}} \tag{3}$$

$\Delta P$ and $\Delta \sigma$ are the hydrostatic and osmotic pressure drops across the capillary wall, respectively. $P_e$ in Eq. 2 is defined as

$$P_e = \frac{L_p (1 - \sigma) \Delta P_{eff}}{P_d} \tag{4}$$

In our experiments, $\Delta P$ ranges from 5 to 8 cmH2O; $\sigma^{\text{albumin}}$ is 0.83, and $\Delta P^{\text{α-lactalbumin}}$ is 3.6 cmH2O for 10 mg/ml BSA. $\sigma^{\text{FITC-α-lactalbumin}}$ is 0.35, and $\Delta P^{\text{FITC-α-lactalbumin}}$ is 3.1 cmH2O for 2 mg/ml α-lactalbumin (13). We estimated the rolipram and forskolin effect on $L_p$ to decrease from $4 \times 10^{-7}$ to $1.72 \times 10^{-7}$ cm$^2$ s$^{-1}$ cmH2O$^{-1}$ on the basis of results of Adamson et al. (3). We used the apparent permeability to α-lactalbumin measured before ($3.5 \times 10^{-6}$ cm/s) and after ($2.1 \times 10^{-6}$ cm/s) rolipram and forskolin treatment as described in RESULTS. Table 2 summarizes the calculations and shows that the contribution from the solvent drag ranges from 4% when $\Delta P_{eff} = 1$ cmH2O to 16% when $\Delta P_{eff} = 4$ cmH2O under control conditions. Table 2 also shows that when $L_p$ is reduced with the treatment of rolipram and forskolin, the solvent drag contribution to the decreased apparent $P$ (3% at $\Delta P_{eff} = 1$ cmH2O, 11% at $\Delta P_{eff} = 4$ cmH2O) is almost the same as that in the control conditions. If the apparent test and control permeability ratio $P_{test}/P_{control}$ is 2.1/3.5 = 0.6, the true test and control diffusive permeability ratio $P_{test}/P_{control}$ is 0.605 at $\Delta P_{eff} = 1$ cmH2O and 0.63 at $\Delta P_{eff} = 4$ cmH2O. Thus we conclude that, although solvent drag may overestimate true diffusive permeabilities $P_{control}$ or $P_{test}$ by 3–16% under our experimental conditions, the measured apparent permeability ratio $P_{test}/P_{control}$ underestimates the true permeability ratio by only 1–6%. Under our experimental conditions, $P_{test}$ for sodium fluorescein is <0.05. The solvent drag contribution to $P_{sodium fluorescein}$ is negligible.

Taking into account both the free dye and solvent drag effects on measured $P_{FITC-α-lactalbumin}$, we estimated a corrected mean $P_{FITC-α-lactalbumin}$ of $2.85 \times 10^{-6}$ cm/s. This value is only slightly larger than the previously measured data for TRITC-α-lactalbumin, which was $2.1 \times 10^{-6}$ cm/s (1, 13). One reason for the slightly larger value may be that all microvessels used in our study were venular capillaries, which tend to have higher permeability than the population of venular, true, and arterial capillaries used previously (1, 13).

Table 2. Solvent drag effect on $P_{FITC-α-lactalbumin}$ under control and rolipram/forskolin treatment conditions

<table>
<thead>
<tr>
<th>$\Delta P_{eff}$</th>
<th>$L_p$</th>
<th>$P_e$</th>
<th>$P_d$</th>
<th>$P_{test}$</th>
<th>$P_{control}$</th>
<th>$P_{test}/P_{control}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>cmH2O</td>
<td>cm$^2$ s$^{-1}$ cmH2O$^{-1}$</td>
<td>cm/s</td>
<td>cm/s</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4 $\times$ 10$^{-7}$</td>
<td>3.5 $\times$ 10$^{-6}$</td>
<td>0.08</td>
<td>3.37 $\times$ 10$^{-6}$</td>
<td>96.3</td>
<td>96.3</td>
</tr>
<tr>
<td>1</td>
<td>4 $\times$ 10$^{-7}$</td>
<td>3.5 $\times$ 10$^{-6}$</td>
<td>0.35</td>
<td>2.95 $\times$ 10$^{-6}$</td>
<td>84.3</td>
<td>84.3</td>
</tr>
</tbody>
</table>

Rolipram and forskolin in effect:

| 1               | 1.72 $\times$ 10$^{-7}$ | 2.1 $\times$ 10$^{-6}$ | 0.06  | 2.04 $\times$ 10$^{-6}$ | 97.1          | 97.1                   |
| 4               | 1.72 $\times$ 10$^{-7}$ | 2.1 $\times$ 10$^{-6}$ | 0.24  | 1.87 $\times$ 10$^{-6}$ | 89.0          | 89.0                   |

Values are calculated from Eq. 2 (8). $\Delta P_{eff}$, effective filtration pressure across capillary wall; $L_p$, hydraulic conductivity; $P$, apparent diffusive permeability coefficient; $P_e$, Péclet number; $P_o$, true diffusive permeability coefficient; $P_{test}/P_{control}$, contribution of diffusion.

Influence on $P_{sodium fluorescein}$ from Fluorescein Concentration at Close Exit

Two important assumptions implicit in the calculation of $P$ (Eq. 1) are that the flux remains constant during the measurement and that the concentration difference across the capillary wall remains constant. In all experiments, the flux (proportional to the rate of change in intensity (dI/dt)) was constant for at least 15 s, and we used only those initial values to calculate permeability, thereby satisfying the requirement of constant flux. Although the luminal concentration is stepped from zero to that in the perfusate in less than 1 s, the calculation of $P$ is based on the assumption that the solute concentration in the interstitial space (cliff exit concentration) is negligibly low and remains so as solute diffuses rapidly into the surrounding tissue. These assumptions are valid for the permeability measurement of intermediate-sized FITC-α-lactalbumin molecules, which cross the microvessel quite slowly. However, the use of the confocal microscope to measure the local solute concentration and development of tissue-solute gradients shows the limitation of this second assumption for measurement of sodium fluorescein permeability. Specifically, Adamson et al. (2) observed that after 23 s of perfusion, the sodium fluorescein concentration immediately outside the capillary wall rose to as high as 0.5–0.75 times the luminal concentration. Such high interstitial concentrations should have significantly reduced the flux across the capillary wall due to the reduction of the gradient for diffusion within a few seconds of beginning the perfusion. A worst-case error due to such tissue gradients would thus lead to an underestimation of the permeability to sodium fluorescein by up to a factor of 2 in the control condition.

$P_{sodium fluorescein}$ decreased in response to treatment...
with rolipram and forskolin. Because the degree of underestimation of P_{sodium fluorescein} will always be larger for the control case when permeability is larger than that when cAMP was elevated in the test case, the corrected ratio \( \frac{P_{\text{test}}}{P_{\text{control}}} \) should be smaller than the measured value of 67%.

It follows from the above that the measured mean \( P_{sodium fluorescein} \) of 34.4 (±14.5 SD) × 10^{-6} cm/s (range 12.4 to 60.7 × 10^{-6} cm/s) would increase after the concentration increase in the extravascular space is taken into account. If the underestimation of \( P_{sodium fluorescein} \) due to buildup of extracellular solute is as high as a factor of 2, the corrected mean \( P_{sodium fluorescein} \) will be roughly 68.8 × 10^{-6} cm/s. With this estimate, the mean ratio of corrected \( P_{sodium fluorescein} \) to corrected \( P_{FITC-\alpha-lactalbumin} \) in single capillaries is increased from the measured value of 10.6 to a value of 27.6.

**Previous Models for Capillary Permeability and Hydraulic Conductivity**

At 20°C, the free diffusion coefficient of sodium fluorescein (\( D_{sodium fluorescein} \)) is 5.40 × 10^{-6} cm²/s (10) and the free diffusion coefficient of FITC-\( \alpha \)-lactalbumin (\( D_{FITC-\alpha-lactalbumin} \)) is 1.07 × 10^{-6} cm²/s (1). The ratio of \( D_{sodium fluorescein} \) to \( D_{FITC-\alpha-lactalbumin} \), 5.05, is about one-fifth the ratio of corrected \( P_{sodium fluorescein} \) to \( P_{FITC-\alpha-lactalbumin} \).

This indicates that the interendothelial path pathway provides much more steric exclusion and restriction to the diffusion of FITC-\( \alpha \)-lactalbumin than to sodium fluorescein through its size-limiting structural components. These structural components of the interendothelial cleft are plasma membranes composing the cleft wall, the surface glycocalyx of endothelial cells, and the tight junction strands inside the cleft.

Figure 9 shows the permeability coefficients predicted by a model of either the junctional strand containing only the large breaks or the junctional strand containing large breaks plus an additional 2-nm-wide narrow slit pathway as a function of solute radius (10, 11). Also shown are the current experimental data using quantitative fluorescence microscope photometry to measure \( P_{sodium fluorescein} \) and \( P_{FITC-\alpha-lactalbumin} \) on the same single capillaries, with uncorrected and corrected P values. The relationship between the measured permeability P and the structural components of the interendothelial cleft pathway is described by a three-dimensional model proposed previously (11) as

\[
P = \frac{4D^{(1)}D^{(3)}}{D^{(1)} + D^{(3)}} \frac{K[(1 - \alpha^{-2})^{\frac{1}{2}}]L_{j}}{2D} + P_{s}
\]

\[\alpha = \cosh \left( \frac{\pi d}{L} \right)\]

where \( D^{(1)} \) and \( D^{(3)} \) are the effective solute diffusion coefficients in regions 1 and 3 of the interendothelial cleft (see Fig. 1A). \( D^{(1)} \) and \( D^{(3)} \) include the influences from the cleft wall and the fiber matrix. B is the half cleft width, L is the cleft depth, d is one-half the width of the large junctional break, and 2D is the spacing between adjacent large breaks. \( L_{j} \) is the total cleft length (total cell perimeter) per unit capillary surface area. K is a complete elliptic integral of the first kind.

The first term in Eq. 5 is the contribution to P from the large breaks; the second term, \( P_{s} \), which has a complicated form, is the contribution from the possible small continuous ~2-nm slit in the tight junction strand. The first subterm in the large-break contribution term (Eq. 5) is dependent on the fiber matrix composition (fiber radius \( a \), volume fraction \( S_{f} \), and its ordering manner), the cleft width, and the solute properties. The second subterm is the cleft depth. The third subterm is dependent on the break size and the cleft depth. The fourth subterm is dependent on the frequency of the breaks in one cleft and the frequency of the clefts per unit capillary surface area.

Figure 9 shows that both the corrected and uncorrected measured \( P_{sodium fluorescein} \) and \( P_{FITC-\alpha-lactalbumin} \) fall close to the curve predicted by the large-break model. We do not need an additional small slit to explain the data from the paired experiment for \( P_{sodium fluorescein} \) and \( P_{FITC-\alpha-lactalbumin} \). Furthermore, because we measured a proportional reduction in the permeability coefficients to sodium fluorescein and\( \alpha \)-lactalbumin in the presence of elevated cAMP, we could account for the data in the lower-permeability state induced by rolipram and forskolin simply by sliding the curve vertically without changing its shape. According to Eq. 5, P should be changed in the same proportion for different-sized solutes as long as the first subterm, which is determined by the fiber matrix properties, and the second term, the cleft depth, are unchanged under the treat-
SOLUTE PERMEABILITY AND cAMP EFFECT

Adamson et al. (3) have begun to investigate ultrastructural changes in the cleft of microvessels treated with rolipram and forskolin under the same experimental conditions used in the present experiments. In the vessels studied, Adamson et al. (3) found that the mean decrease in Lp of frog mesenteric microvessels after 20 min of exposure to rolipram and forskolin was 43%. These investigators have not yet made detailed analyses of changes in the size and frequency of breaks in the junctional strand, but they have observed that reduced permeability is associated with an increase in the number of junctional strands per cleft from an average of 1.7 to 2.2 (3). This change occurred with no change in the average cleft length. Although the change in number of strands is small, the addition of strand is expected to reduce the effective area available for diffusion through breaks in the junctional strand and to increase the diffusion distance for solutes within the cleft (3). The actual reduction in Lp is larger than the 33–36% reduction measured for the diffusible solutes. The reason for this difference has not been investigated in detail and may not be significant because of the variation from vessel to vessel. However, it is instructive to compare the expression for the Lp of the junctional pore-fiber matrix model with the corresponding relationship for diffusive exchange in Eq. 5. The Lp is given by the relationship (11)

$$L_p = \frac{4}{3} \left( \frac{B^2}{k} \right) \frac{\mu(1) + \mu(3)}{\mu(1)} \left( 1 - \frac{a^2}{2} \right) L_{jl} \frac{\mu(3)}{2D + \mu(3) ps}$$

Here $\mu(1)$ and $\mu(3)$ are effective fluid viscosities in regions 1 and 3 of the cleft. $L_{ps}$ is the contribution from the small slit. It is noted that the terms describing the effects of breaks in the junctional strand are the same in Eqs. 5 and 6. The biggest difference is that $L_p$ depends on mean cleft width to the third power and not as a simple proportion, as for diffusion. Thus, in the simplest case, a change in break size or frequency is expected to have similar effects on $L_p$ and solute permeabilities. However, a larger reduction in $L_p$ relative to diffusion would not be unexpected if there were a small reduction in mean cleft width after exposure to cAMP.

Another way to analyze these results is to note that, according to the model in which solutes with a radius smaller than 0.75 nm cross the capillary wall via both the large breaks and a narrow slit pathway, nearly 70% of the flux of sodium fluorescein might have been expected to cross the vessel wall via the very small pathway, whereas 100% of the $\alpha$-lactalbumin and close to 75% of the water was expected to cross via the larger breaks. If these values were correct, and if the primary effect of raising cAMP was through an action on the large breaks, the reduction in $L_p$ of sodium fluorescein to only 85% of control. Alternatively, if increased cAMP were to effectively close the narrow slit as well as modify the larger breaks, the reduction in $L_p$ of sodium fluorescein would have been much larger, to as low as 15% of control values. These extreme values were not observed. We note, however, that theoretically it may be possible for a mechanism that effectively closes part of both pathways to cause reductions in the permeability of both small- and large-pore pathways within the ranges we measured. One such mechanism may involve additional junctional strands, as observed by Adamson et al. (3), which change the geometry of the diffusion pathways for both solutes.

An important caveat to the conclusion that our results do not support the hypothesis that a very small pore pathway is present is that sodium fluorescein may not be as good a probe of the putative small-pore pathway as we originally expected. We note that the corrected value of $L_p$ of sodium fluorescein, $-69 \times 10^{-6}$ cm/s, is close to the value measured by Adamson et al. (2) using confocal microscopy. However, it is only one-half the mean value of $L_p$ of sodium fluorescein (143 $\times 10^{-6}$ cm/s) measured previously using osmotic transients (sucrose mol wt 342 vs. sodium fluorescein mol wt 376) (7). Furthermore, for even smaller solutes, Na+ and K+, the measured permeability coefficients are $440 \times 10^{-6}$ (7) and $670 \times 10^{-6}$ cm/s (6), respectively. For the smallest solutes the large-break model underestimates the measured permeabilities by up to one order of magnitude. Thus a conservative interpretation of our results is that, although they do not support the hypothesis that there is a significant flux of sodium fluorescein across a very small pore pathway, they do not rule out such a pathway for solutes smaller than sodium fluorescein. This would be the case if the additional pathway for smaller solutes had a size cutoff smaller than the 0.75-nm radius threshold proposed by Fu et al. (10, 11). This possibility needs further exploration with the use of both venular microvessels, as used in the present experiments, and true capillaries. One problem with such experiments is that it will be difficult to design experiments in which the same method is used to measure the permeability coefficients to small and large solutes because sodium fluorescein is one of the smallest fluorescent solutes available for use within the visible spectrum. Thus more refined methods to investigate the modulation of permeability properties of the capillary wall to very small solutes are needed to overcome possible limitations of the effectiveness of sodium fluorescein as a probe of putative pathways for very small solutes across the junctional strands.

In summary, the combined results from experiments using the best-understood methods to measure sodium fluorescein and $\alpha$-lactalbumin permeability coefficients on microvessels in both the control state and after permeability is reduced do not support the hypothesis that a separate pathway across the tight junction is available for solutes with a radius as large as 0.75 nm. If such a pathway is present, its cut-off size is smaller than the size of sodium fluorescein. Furthermore, the proportional reduction in the permeability of both
sodium fluorescein and α-lactalbumin is also consistent with the hypothesis that a mechanism involving a change in the number of tight junction strands within the cleft reduces the permeability of microvessels under conditions in which intracellular cAMP levels are increased.

APPENDIX

We evaluated the free dye contribution to the labeled protein permeability measured using quantitative fluorescence microscopy photometry. If \( P_f \) represents the free dye permeability coefficient, \( P_p \) is the protein permeability coefficient and \( P_m \) is the permeability coefficient of the mixture of free dye and the labeled protein. From Eq. 1 we have

\[
P_p = \frac{1}{\Delta I_{fp0}} \frac{dI_{fp}}{dt} \frac{r}{2}
\]

(A1)

\[
P_f = \frac{1}{\Delta I_{fo0}} \frac{dI_{fo}}{dt} \frac{r}{2}
\]

(A2)

\[
P_m = \frac{1}{\Delta I_{mol} + \Delta I_{fp0}} \frac{dI_{mol} + dI_{fp}}{dt} \frac{r}{2}
\]

(A3)

where \( \Delta I_{io0} \), in which \( i = f, p, f \), are step increases in fluorescence intensity when the dye is perfused into the vessel lumen; \( dI_{i}/dt \), in which \( i = p, f \), are the initial rates of increase in fluorescence intensity after the solute fills the lumen and begins to accumulate in the tissue; \( r \) is the microvessel radius. Here, \( P_p \) and \( P_m \) are measured in the experiment. If we define \( F = I_{mol}/(\Delta I_{mol} + \Delta I_{fp0}) \) as the percentage of the free dye intensity to the total mixture fluorescence intensity, from Eqs. A1, A2, and A3, the true value of the pure labeled protein permeability \( P_p \) can be determined using \( P_f \) and \( P_m \) as

\[
P_p = \frac{1}{1 - F} P_m - \frac{F}{1 - F} P_f
\]

(A4)

The percentage of the free dye intensity \( F \) can also be written as \( I_f/I_m \). Here, fluorescence intensities correspond to \( I_f \) for the free dye and \( I_m \) for the mixture of free dye plus labeled protein in solution. Both \( I_f \) and \( I_m \) can be measured in vitro experiments as described in the calibration section.

Using treatment with rolipram and forskolin, we can directly measure the changes in \( P_p \) and \( P_m \). The change in \( P_p \) is thus described as

\[
\frac{p_{test}}{p_{control}} \frac{A_f}{A_m} = \frac{1 - A_f}{1 - F_g} \frac{F_g}{A_m}
\]

(A5)

\[
g = \frac{p_{test}}{p_{control}}\frac{A_f}{A_m} = \frac{p_{test}}{p_{control}}\frac{A_f}{A_m}
\]

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