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Letters to the Editor

The Sodium Concentration of Lateral Intercellular Spaces

Sir,

In their recent paper entitled, "The Sodium Concentration of the Lateral Intercellular Spaces of MDCK Cells: A Microspectrofluorimetric Study," Chatton and Spring [1] criticize our work [5, 6]. We used video-enhanced epifluorescence microscopy to investigate the Na^+ distribution in the interstitial space surrounding descending colonic crypts perifused with Tyrode and the Na^+ fluorescence probe SBFI(20μM) (free acid, cell impermeant form).

In rat, rabbit and sheep mucosa Na.SBFI accumulated in the extracellular pericryptal regions to a concentration 3–5-fold above that in the surrounding bathing solution. Ouabain, or metabolic inhibitors abolished pericryptal Na.SBFI accumulation, indicating a very high pericryptal Na^+ concentration.

In a closed single phase the Law of Mass Action predicts that Na^+ complexes with SBFI as follows:

$$[Na^+] \cdot [SBFI] = [Na.SBFI] \cdot K_d \text{ (where } K_d \text{ is the dissociation constant of the reaction).} \quad (1)$$

$$\text{Since } SBFI_{(\text{Total})} = Na.SBFI_{(\text{complex})} + SBFI_{(\text{uncomplexed})} \quad (2)$$

from Eqs. 1 and 2 it follows that:

$$[SBFI_{(\text{Total})} - SBFI_{(\text{uncomplexed})}] \cdot [Na^+] = [Na.SBFI]_{(\text{complex})} \cdot K_d \quad (3)$$

$$\text{hence, } [Na.SBFI]_{(\text{complex})} = [Na^+] \cdot [SBFI_{(\text{Total})}]/(K_d + [Na^+]). \quad (4)$$

Because in a single closed phase any increase in $[Na.SBFI]_{(\text{complex})}$ occurs at the expense of $[SBFI]_{(\text{uncomplexed})}$, the relationship between $[Na^+]$ and $[Na.SBFI]$ is hyperbolic (half saturation concentration of *SBFI* for $Na^+ = K_d = 18$ mM [4]). Consequently, were the extracellular solution surrounding colonic mucosa, a single closed phase, the change in fluorescence excitation ratio 340/380 nm of SBFI resulting from a change in $[Na^+]$ from 140 to 560 mM would be a mere 10%, so monitoring changes in this range of extracellular Na^+ concentration with the probe would be unrealistic.

However, because of adjacent membrane pump activity, extracellular subcompartments exist where $[Na^+]$ or $[H^+]$ are higher than in the bulk solution [1, 2, 5, 7]; so the extracellular solution *cannot* be considered as a *single* uniform phase.

As the extracellular distribution of Na^+ is nonuniform, so at steady state $[Na.SBFI]_{(\text{complex})}$ is not homogeneously distributed either.

However, because $[SBFI]_{\text{uncomplexed}}$ is freely diffusible, at steady state it will not be uniformly distributed in all phases to which it has access, so:

$$[SBFI]^{(\text{local})} = [SBFI]^{(\text{bulk})}. \quad (5)$$

The Law of Mass Action requires:

$$[Na^+]^{(\text{local})} \cdot [SBFI]^{(\text{local})} = [Na^+.SBFI]^{(\text{local})} \cdot K_d. \quad (6)$$

$$\text{and } [Na^+]^{(\text{bulk})} \cdot [SBFI]^{(\text{bulk})} = [Na^+.SBFI]^{(\text{bulk})} \cdot K_d. \quad (7)$$

Hence, at steady state, from Eqs. 5, 6 and 7, and assuming similar K_ds for SBFI in the bulk and local solutions, phase equilibrium requires:

$$[Na^+]^{(\text{local})}/[Na^+.\text{SBFI}]^{(\text{bulk})} = [\text{Na}^+.\text{SBFI}]^{(\text{local})}/[\text{Na}^+.\text{SBFI}]^{(\text{bulk})} \quad (8)$$

The linear relationship between $[Na^+]^{(\text{local})}$ and $[Na^+.SBFI]^{(\text{local})}$ in Eq. 8, instead of the hyperbolic predicted on the basis of the single closed extracellular phase, Eq. 4 [1, 2], results from the microscopic size of the *open* local phase in proportion to the bulk phase; so rises in $[Na^+.SBFI]^{(\text{local})}$ occur without significant changes in $[SBFI]^{(\text{local})}$ or $[SBFI]^{(\text{bulk})}$.

Equation 8 explains how a 400% increase in local fluorescence arises when the pericryptal $[Na^+]$ is increased from 140–560 mM. This large increase in local fluorescence can readily be demonstrated, even with a low magnification (16× Zeiss multi-immersion Planar Achromat, N.A. 0.8.) lens [5, 6]. These findings have been confirmed using a confocal microscope with a Ni-

kon 60× lens, N.A 1.4. and the impermeant fluorescence probe Sodium Green (Molecular Probes) [7].

From Eq. 8 it can be deduced (i) that a low affinity Na^+ probe is not required to demonstrate changes in extracellular [Na^+]; SBFI or Sodium Green will do as well as SBFO, and (ii) estimates of local increases in extracellular [Na^+] or [H^+] from calibration curves based on the assumption that the same hyperbolic relationships of the ligands with their probes that hold in a *single closed phase* system are equally appropriate in an *open two phase system,* are erroneous [1, 2].

A second problem in Chatton and Spring's paper [1] and an earlier paper [2] relates to their claim, based on epifluorescence signals of SBFO and BCECF, that [Na^+] and pH are uniform along the 7.0 μm length of lateral intercellular spaces of MDCK cells.

Transforming epifluorescence signals into concentrations at any depth requires removal of contributions from out of focus fluorescence. The deconvolution procedure applied [1, 2] depends on the observed Point Spread Function, PSF, of ***resolved*** images obtained from 1 μm diameter fluorescent beads. The deblurring algorithm improves the bead resolution to ±1–2 μm perpendicular to the focal plane and gives impressive 3-D reconstruction images of the LIS in MDCK cells [2].

However, the fluorescence signals arising from the dye solution within the LIS, [1, 2] are ***unresolved.*** The transfer function obtained using a 63× lens N.A.1.3 from unresolved subresolution 0.1 μm particles is much wider than from a resolved source i.e., 50% decrease at ±10 μm; [3] Hence, application of a "deblurring" routine based on a PSF from ***resolved*** images to determine the Na^+ and H^+ from ***unresolved*** dye sources within the LIS, may be inappropriate, as insufficient out of focus fluorescent light will be removed to ascertain the concentration gradients with any precision along the length of the LIS.

Sincerely,

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Reply to: The Sodium Concentration of Lateral Intercellular Spaces

Sir,

Drs. Naftalin and Pedley voice two concerns in their letter of April 26, 1995: (1) that our criticisms of their interstitial sodium measurements [1] are unfounded; (2) that we incorrectly utilized and interpreted digital deblurring of our microscope images. We respond to these issues in the same order.

(1) Validity of measurements by Naftalin and Pedley: Their letter reiterates the algebraic manipulations of the equations previously published in 1990 and 1993. Their imaginative approach requires two contradictory assumptions — that free SBFI is uniformly distributed by diffusion between the bulk and local solutions, and that the Na-SBFI complex does not freely diffuse. In addition, their sodium concentration calculations critically depend on the incorrect assumption that the binding constant (K_d) of SBFI for Na is the same in tissue as measured by Minta and Tsien [3] in a cuvette. Our study employing a related dye, SBFO, and those of Minta and Tsien on both SBFI and SBFO show dramatic shifts in K_d as a function of the local environment. They compound the uncertainty of their measurements with this ratiometric indicator by utilizing a single wavelength for excitation (340 nm), a low power (16×) objective lens, failing to calibrate their measurements *in situ* or even to validate their new method *in vitro.* Indeed, their choice of spectroscopic approach appears to have been dictated by the limitations of their equipment rather than theory as indicated by the following statement in the Methods section of their paper in the *Journal of Physiology* [4]:

"Because of the operational delays between obtaining successive whole screen images, each with 256,000 pixels, with the present equipment, the fluorescence intensity of whole-screen images could only be conveniently monitored at a single wavelength. Because of the low intensity of the free SBFI signal at 380 nm, which was exaggerated by the low magnification lens system used and also because of the uneven background intensity of the video camera image, it was impractical to obtain a continuous time series of full screen ratio-images at 340/380 nm."

(2) Their point about the appropriateness of the point spread function used in our paper in the *American Journal of Physiology* [2] is based on a misreading of the paper. On page C74, we state:

"Because the commercial nearest-neighbor algo-

rithm employed a theoretical point spread function rather than an experimentally determined function as had been used by others (20), an evaluation of the accuracy and efficacy of the deblurring algorithm was performed. A z-axis resolution test object was constructed from three layers of 1-μm-diameter fluorescent beads immersed in a background solution of fluorescein sulfonate (Fig. 2, top)."

To our knowledge there is no theoretical basis for the determination of a point spread function from a resolved object. Our previous efforts were devoted to testing the validity of the deblurring algorithm with an object which simulated our biological preparation. In any case, our conclusions about the lack of a gradient along the lateral intercellular spaces of fluorescent dye, pH, Na or Cl do not depend on the efficacy of the deblurring algorithm. In the case of pH measured by the fluorescent dye BCECF, recent measurements using high speed ratio imaging on a confocal microscope also indicated a lack of gradient in dye or pH along the lateral intercellular space (R. Nitschke and K.R. Spring, Electro-optical wavelength selection enables confocal ratio imaging at low light levels. *J. Microsc. Soc. Am.* 1:1–11, 1995).

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