An increase in intracellular calcium concentration that is induced by basolateral CO2 in rabbit renal proximal tubule

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Bouyer, Patrice, Yuehan Zhou, and Walter F. Boron. An increase in intracellular calcium concentration that is induced by basolateral CO2 in rabbit renal proximal tubule. Am J Physiol Renal Physiol 285: F674–F687, 2003—Working with isolated perfused S2 proximal tubules, we asked whether the basolateral CO2 sensor acts, in part, by raising intracellular Ca2+ concentration ([Ca2+]i), monitored with the dye fura 2 (or fura-PE3). In paired experiments, adding 5% CO2/22 mM HCO3− (constant pH 7.40) to the bath (basolateral) solution caused [Ca2+]i to increase from 57 ± 3 to 97 ± 5 nM (n = 8, P < 0.002), whereas the same maneuver in the lumen had no effect. Intracellular pH (pHi), measured with a low initial pH i, thus it cannot be a pHi change that whereas an OOE solution containing 0% CO2/22 mM HCO3− (endo) plasmic reticulum Ca2+ pump secrete H+, whereas para- (endo) plasmic reticulum Ca2+—ATPase inhibitor or 4 mM rotenone (mitochondrial inhibitor) had no effect on [Ca2+]i, whereas adding 5% CO2/22 mM HCO3− to the lumen, pH fell by 0.55 ± 0.11 in 9 with a high initial pHi, but rose by 0.28 ± 0.07 in the other 5 with a low initial pHi. Thus it cannot be a pHi change that triggers the [Ca2+]i increase. Introducing to the bath an out-of-equilibrium (OOE) solution containing 20% CO2/no HCO3−/pH 7.40 caused [Ca2+]i to rise by 62 ± 17 mM (n = 10), whereas an OOE solution containing 0% CO2/22 mM HCO3−/pH 7.40 caused only a trivial increase. Removing Ca2+ from the lumen and bath, or adding 10 μM nifedipine (L- and T-type Ca2+—channel blocker) or 2 μM thapsigargin [sarcoplasmic reticulum Ca2+—ATPase inhibitor] or 4 μM rotenone (mitochondrial inhibitor) to the lumen and bath, failed to reduce the CO2-induced increase in [Ca2+]i. Adding 10 mM caffeine (ryanodine-receptor agonist) had no effect on [Ca2+]i. Thus basolateral CO2, presumably via a basolateral sensor, triggers the release of Ca2+ from a nonconventional intracellular pool.

intracellular pH; carbon dioxide; out-of-equilibrium solutions; fura 2; ions; transport; kidney

A MAJOR ROLE OF THE KIDNEY is to maintain the pH of the extracellular fluid within normal limits. The proximal tubule actively participates in this activity by reabsorbing ~80% of the NaHCO3 filtered at the glomeruli and also by generating “new” HCO3− to neutralize non-volatile acids generated by metabolism. Bicarbonate reabsorption (JHCO3) occurs as the apical Na/H exchanger and H+ pump secrete H+, and as this acid titrates luminal HCO3− to CO2 and H2O under the influence of apical carbonic anhydrase (2, 20). The newly formed CO2 and H2O then diffuse into the cells, where soluble carbonic anhydrase regenerates H+ and HCO3− in the cytosol. Finally, the aforementioned H+ extruders recycle H+ to the lumen, while the basolateral Na-HCO3 cotransporter moves HCO3− to the blood (8). The generation of new HCO3− is similar to the reabsorption of HCO3− except that the H+ secreted into the lumen titrates a buffer (e.g., HPO42−) other than HCO3−, and the intracellular CO2 and H2O derive from the blood rather than the luminal fluid.

The rate of H+ secretion by the proximal tubule, which is nearly identical to JHCO3, is under the control of several hormones. For instance, angiotensin II (36, 71) and nitric oxide (70) increase JHCO3, whereas parathyroid hormone (PTH) has the opposite effect (21, 41). Another potent regulator of JHCO3 is the acid-base status of blood. For example, respiratory acidosis (i.e., an increase in blood PCO2 that causes a decrease in blood pH and small increase in blood HCO3− concentration ([HCO3−]) raises JHCO3 (1, 11, 22). To determine whether it is a change in PCO2, pH, or [HCO3−] that is responsible for the increase in JHCO3 during respiratory acidosis, the laboratory developed a technique for making out-of-equilibrium (OOE) CO2/HCO3− solutions. Using this approach it is possible to generate solutions having physiological levels of CO2 concentration ([CO2]) and pH but virtually no HCO3− (i.e., a “pure CO2” solution), or solutions having physiological levels of [HCO3−] and pH but virtually no CO2 (i.e., a “pure HCO3−” solution).

OOE solutions were first used to study K-HCO3 cotransport in squid giant axons (75). More recently, our laboratory adapted this technique to mammalian cells and found that removing HCO3− from the basolateral or “bath” solution (pure CO2) caused JHCO3 to increase, whereas removing CO2 from the basolateral solution (pure HCO3−) had the opposite effect (76). In other experiments, our laboratory used the OOE approach to vary basolateral [CO2], [HCO3−], and pH one at a time, while holding the other two parameters constant. The most surprising result was that JHCO3 was totally insensitive to wide changes in basolateral pH, even though these changes in basolateral pH were associated with rather wide changes in intracellular pH.
pH (pHᵢ). Nevertheless, J₇HCO₃ increased markedly in response to increases in basolateral [CO₂] (77). These results led to the hypothesis that renal proximal tubule cells have a mechanism at or near the basolateral membrane for sensing CO₂ independently of pH. This hypothesis is consistent with earlier work with equilibrated CO₂/HCO₃ solutions that showed that adding CO₂/HCO₃ to the bath, but not to the lumen, causes steady-state pHᵢ to rise in proximal tubule cells (46) and stimulates luminal acid extruders (17, 18).

In the present study, we investigated one of the potential intracellular signaling pathways of the basolateral CO₂ sensor by monitoring intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ). Intracellular Ca²⁺ is a common second messenger for numerous stimuli (19). For example, a rise in [Ca²⁺]ᵢ is a key step in the response of the chemosensitive cells in the carotid body to hypoxia, metabolic acidosis, respiratory acidosis, or isohydric hypercapnia (13). Based on these findings, we felt that a rise in [Ca²⁺]ᵢ was a good candidate as a signaling pathway for the CO₂ sensor. Some authors working on proximal tubule cells have reported that increases in [Ca²⁺]ᵢ raise J₇HCO₃ (38), whereas others have reported that increases in [Ca²⁺]ᵢ lower J₇HCO₃ (16). Here, using a Ca²⁺-sensitive fluorescent dye, we found that we could trigger a significant increase in [Ca²⁺]ᵢ; by introducing equilibrated CO₂/HCO₃ to the basolateral (but not luminal) side of the tubule, or by introducing basolateral pure CO₂ (but not pure HCO₃). Also, we found that basolateral CO₂ does not increase [Ca²⁺]ᵢ; by lowering pHᵢ and that the source of the Ca²⁺ is a thapsigargin (Tg)-insensitive intracellular store. Our results are thus consistent with the hypothesis that an increase in [Ca²⁺]ᵢ might be involved in the proximal tubule cell’s response to basolateral CO₂.

**METHODS**

**Biological Preparation**

All the experiments were carried out in “pathogen-free” female rabbits (New Zealand White, Elite, Covance, Denver, PA) weighing 1.4–2.0 kg. The methods for preparing the animals, harvesting the kidneys, and perfusing the tubules were similar to those originally described by Burg et al. (14) and subsequently modified in our laboratory (47, 76). The Yale Animal Care and Use Committee approved all the procedures. Briefly, an animal was euthanized by intravenous injection of pentobarbital sodium; an incision of the abdominal wall was performed to expose the left kidney, which was rapidly removed. The kidney was then cut into coronal slices and kept in cold (4°C) modified Hanks’ solution (solution 1 in Table 1). The microdissection of the slice was carried out in the same solution under a dissecting microscope, using a pair of fine forceps to grasp a portion of a medullary ray and gently tear it from the rest of the slice, starting from the inner medulla and proceeding toward the cortex. Our initial landmark was the junction between the thin descending limb of Henle’s loop and the S3 segment (i.e., distal portion of the proximal straight tubule). We isolated a portion of the S2 segment that consisted of the distal-most 600–800 μm of the proximal convoluted tubules plus 200–300 μm of the proximal-most part of the proximal straight tubule. After transferring the tubule to a chamber (adapted for rapid mixing of OOE solutions; see below), we perfused the distal-most 400–500 μm of the proximal convoluted tubule. Tubules were perfused and bathed at 37°C.

**Table 1. Physiological solutions**

<table>
<thead>
<tr>
<th>(Dissection, Hanks')</th>
<th>(Lumen/Bath, HEPES)</th>
<th>(Bath, “Pure CO₂” OOE solution)</th>
<th>(Bath, “Pure HCO₃” OOE solution)</th>
<th>(Bath, EGTA)</th>
<th>(Bath, High Ca²⁺)</th>
<th>(Bath, MnCl₂)</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>137</td>
<td>130.7</td>
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<td>A</td>
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<td>137</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>5</td>
<td>B</td>
<td>B</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.3</td>
<td>0</td>
<td>A</td>
<td>a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0</td>
<td>2.0</td>
<td>B</td>
<td>B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.2</td>
<td>1.0</td>
<td>A</td>
<td>A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.8</td>
<td>1.2</td>
<td>B</td>
<td>B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MgCl₂</td>
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<td>0</td>
<td>A</td>
<td>A</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Glucose</td>
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<td>10.5</td>
<td>B</td>
<td>B</td>
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<td>0</td>
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<tr>
<td>Glutamine</td>
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<td>2</td>
<td>B</td>
<td>B</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>L-Lactic acid</td>
<td>2</td>
<td>2</td>
<td>B</td>
<td>B</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CO₃%</td>
<td>2</td>
<td>2</td>
<td>B</td>
<td>B</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0</td>
<td>22</td>
<td>A</td>
<td>A</td>
<td>0</td>
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<td>MnCl₂</td>
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<td>0</td>
<td>B</td>
<td>B</td>
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<td>0</td>
</tr>
<tr>
<td>Tri-HCl</td>
<td>10</td>
<td>0</td>
<td>A</td>
<td>A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HEPES</td>
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<td>B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 at 4°C</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>Mixed to pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

The concentrations are in mM, except for CO₂ (given both in mM and %). Except for solution 1, all solutions were titrated to the indicated pH at 37°C. Osmolalities were adjusted to 300 ± 3 mosM. Each of the 2 out-of-equilibrium (OOE) solutions was generated by rapidly mixing their respective A and B components in a 1:1 ratio. Solutions 4B and 5A were vigorously gassed with 100% O₂ to render them free of CO₂, H₂CO₃, HCO₃, and CO₃⁻. Solution 5B was also gassed with 100% O₂.
Fluorescence Measurements

Measurement of fluorescence of Ca\(^{2+}\)-sensitive dyes. Measurements of \([\text{Ca}^{2+}]\), were performed by loading the isolated perfused tubule with 5 µM of either fura 2-AM (Molecular Probes, Eugene, OR) or fura-P3-AM (TefLabs, Austin TX) in our HEPES-buffered solution (solution 2) along with 0.5% (vol/vol) pluronic F-127 (Molecular Probes). We dye-loaded the tubules at room temperature for 20–30 min for fura 2 and 40–50 min for fura-P3. We added the dye precursors as 2 mM stock solutions in DMSO and added the pluronic F-127 as a 20% w/vol stock solution in DMSO. Before the fluorescence recordings, we washed the tubule by flowing a large volume of solution 2 through the chamber.

In tubules loaded with fura 2, dye leakage led to a gradual loss of fluorescence that often prevented us from performing longer experiments. Therefore, in lengthy experiments (>25 min), we used fura-P3, which is more resistant to dye leakage, has the same absorbance spectrum as fura 2 (69), and has been used successfully in proximal tubule cells by others (53). However, we used fura 2 in most of our experiments because fura-P3 required a longer period of dye loading, which reduced the number of experiments we could perform per rabbit and also increased our failure rate. Therefore, unless our experimental protocol required that we use fura-P3, we preferred fura 2 over fura-P3.

The microscope was an Olympus IX70 inverted microscope, equipped with a ×40 oil-immersion objective (1.35 numerical aperture, with a ×1.5 magnification selector knob) and apparatus for epi-illumination. The light source was a 75-W xenon arc lamp. We generated light at two excitation wavelengths by using a filter wheel (Ludl Electronic Products, Hawthorne, NY) to alternate the placement of two filters, 340 ± 15 and 380 ± 15 nm (Omega Optical, Brattleboro, VT), in the excitation light path. Appropriate neutral-density filters (Omega Optical) mounted on a second wheel were used to avoid overillumination of the specimen, which could cause photobleaching, and to equalize as nearly as possible the emitted fluorescent light intensities obtained while excitation occurred at the two wavelengths. The excitation light was directed to the tubule via a 415-nm long-pass dichroic mirror (DM 415, Omega Optical) and the aforementioned objective. The emitted light was collected by the same objective and, via a band-pass filter (510 ± 40 nm, Omega Optical), was directed to an intensified CCD camera (model 350F, Video Scope International, Dulles, VA).

The protocol for alternately exciting the tubule with wavelengths of light, and for subsequently acquiring the fluorescence images, was described previously (76). Briefly, a typical data-acquisition cycle consisted of a ~370-ms period of illumination with 340-nm light, followed immediately by an identical period with 380-nm light. For each excitation wavelength, we averaged four successive video frames using an image-processing board (DT3155, Data Translation, Marlboro, MA) and thereby obtained the emitted light intensity for an excitation of either 340 nm (I\(_{340}\)) or 380 nm (I\(_{380}\)). This pair of excitation cycles was repeated at intervals ranging from 2.5 to 8 s; between excitation cycles, a shutter on the filter wheel protected the tubule from the light. Software developed in our laboratory using the Optimas (Media Cybernetics, Silver Spring, MD) platform controlled data acquisition and analysis on an Intel-based computer running Windows 98SE. We identified an area of interest that represented ~30% of the tubule length. The sum of the I\(_{340}\) values of the pixels in the area of interest, for the background (see below), was divided by the sum of the corresponding background-subtracted I\(_{380}\) values to yield the fluorescence excitation ratio (I\(_{340}\)/I\(_{380}\) or R\(_{340/380}\), which strongly depends on [Ca\(^{2+}\)], but is relatively insensitive to factors such as dye concentration. Because it was our impression that sudden...
increases in the rate of dye loss were associated with sudden increases in [I_340]/[I_380], we discarded experiments in which I_340 and I_380 declined rapidly.

**Intracellular calibration of Ca^{2+}-sensitive dyes.** The generally accepted approach for converting R_340/380 values into [Ca^{2+}], values is that of Grynkiewicz et al. (27), in which one determines R_min (the minimum R_340/380 when [Ca^{2+}]_i → 0) and R_max (the maximum R_340/380 when [Ca^{2+}]_i → ∞) for each cell, and computes [Ca^{2+}], on the assumption that the dissociation of dye is the same inside the cell as it is in vitro:

\[
[\text{Ca}^{2+}] = K_d \frac{R_{340/380} - R_{\text{min}}}{R_{\text{max}} - R_{340/380}} S_{fb}
\]

where S_{fb} is the I_380 measured when [Ca^{2+}]_i → 0 divided by I_380 when [Ca^{2+}]_i → ∞. R_{min} is typically determined by exposing the cell to a Ca^{2+}-free solution containing EGTA and a Ca^{2+} ionophore. Similarly, R_{max} is typically determined by exposing the cell to a solution containing a high concentration of Ca^{2+} plus the ionophore.

Unfortunately, as reported by several groups, the above calibration approach is difficult to apply to isolated proximal tubules because of problems with dye leakage during the prolonged calibration procedure. Thus most [Ca^{2+}]_i studies on proximal tubules, and the associated calibrations, have been done on collapsed tubules (9, 40, 74). Another group performed their physiological experiments in perfused tubules but obtained the values for R_{min}, R_{max}, and S_{fb} by performing calibrations on collapsed tubules (4). We know only one study in which the authors calibrated a Ca^{2+}-sensitive dye (i.e., fura-PE3) in a limited number of perfused proximal tubules (53).

Despite our attempts to minimize dye loss and cell damage, we found it impossible to perform physiological experiment and then routinely obtain R_{min}, R_{max}, and S_{fb} values on the same perfused tubule at 37°C. For example, although probenecid (an inhibitor of organic anion transporters) reduces the loss of flu 2 from neurons (44), we did not find probenecid (300–1,000 μM) useful in the proximal tubule. Similarly, neither lowering the ionomycin concentration to 1 μM, nor switching from ionomycin to 4-bromo A-23187 was helpful. Instead, we adopted the following procedure.

First, we obtained a mean S_{fb} as well as mean, normalized values of R_{min} and R_{max} on a subset of 30 tubules perfused at 37°C for 7.00 min. Then we performed physiological experiments on these tubules. At the end of the experiment, we switched successively to bath solutions containing 1) 0 mM Ca^{2+} plus 5 mM EGTA and 5 μM of the Ca^{2+} ionophore ionomycin (solution 6 in Table 1), 2) 5 mM Ca^{2+} plus 5 μM ionomycin (solution 7), and 3) 5 mM Mn^{2+} (solution 8). This last maneuver allowed us to determine the autoluminescence of the tubule by quenching the fluorescence of the dye. We subtracted these quenched values of I_340 and I_380 from all respective I_340 and I_380 values in the experiment and used these background-subtracted values to compute R_340/380 values for each data point. Finally, we identified a segment of data at the beginning of the experiment in which the R_340/380 values were stable with the HEPES-buffered solution (solution 2) present in the lumen and bath, calculated the mean initial R_340/380 value, and divided all R_340/380 values in the experiment by this mean initial R_340/380 value. The mean quotient during the calibration period with 0 mM Ca^{2+} was thus the normalized R_{min}, and the mean quotient during the calibration period with 5 mM Ca^{2+} was the normalized R_{max}. In the 30 tubules, R_{min} was 0.65 ± 0.05, R_{max} was 6.07 ± 0.65, and S_{fb} was 3.35 ± 0.51.

Second, we used the above values of R_{min}, R_{max}, and S_{fb} to compute [Ca^{2+}], values in each of our experiments, including the 30 described above. In each of these experiments, we normalized all R_340/380 values to the mean initial R_340/380 value obtained with the HEPES-buffered solution present in the lumen and bath (see above). We then used Eq. 1 to compute [Ca^{2+}], values at each time point, employing the aforementioned mean value of S_{fb}, the mean normalized values of R_{min} and R_{max}, and a K_d for fura 2 of 224 nM (27) or a K_d for fura-PES of 290 nM (69).

**Measurement of pH.** The ratiometric optical technique used to measure pH, was similar to that described above for [Ca^{2+}]_i. Briefly, isolated microperfused tubules were loaded with the acetoxymethyl ester of the pH-sensitive dye BCECF-AM (Molecular Probes) at 10 μM final concentration, dissolved in the HEPES-buffered Ringer (solution 2 in Table 1). The excitation band-pass filters were centered at 440 ± 5 and 495 ± 5 nm (Omegagical Optical). We also used a 510-nm long-pass dichroic mirror and a 530-nm long-pass emission filter (Omega Optical). We identified areas of interest as outlined above for the [Ca^{2+}]_i measurements, subtracted the background (~0.3% of the signal) from the BCECF-loaded tubules) from the I_490 and I_500 values as described previously (76), and computed the time course of I_490/I_440. We discarded experiments in which the rate constant for the decrease in the I_490 signal (−k) exceeded 0.05 min⁻¹ (6).

We computed pH values from the I_490/I_440 ratios using a variation of the high-K⁺/nigericin technique (66), in which one performs a one-point calibration at pH 7.00 (10). At the end of each experiment, we drove pH toward 7.00 by introducing a pH-7.00 high-K⁺/nigericin solution (54) into the bath. We normalized the I_490/I_440 ratios of the entire experiment by dividing them by the I_490/I_440 ratio obtained at pH 7.00 and then used the following equation (10) to calculate pH:

\[
I_{490}/I_{440} = 1 + b \left( \frac{1}{1 + 10^{pK - pH}} \right)
\]

From a separate series of 64 fluorescence measurements in a total of 10 tubules, we obtained values for pK and b by using a nigericin-containing solution to alter pH, as described elsewhere (54). We used a nonlinear least-squares method to fit the parameters in the above equation, which forces the best-fit curve to pass through unity at pH_i = 7.00, to the calibration data. The best-fit values were pK = 7.24 ± (SD)0.01 and b = 1.79 ± (SD)0.02.

**Data Analysis and Statistics**

Except for the curve fitting discussed above, all the values are means ± SE, with n being the number of observations. The statistical significance of the data was assessed by two-tailed Student’s t-tests on paired or unpaired data as indicated, using the Analysis Toolpack of Microsoft Excel. Mean steady-state [Ca^{2+}]_i values were obtained by averaging [Ca^{2+}]_i values over a period of ~1 min.

**RESULTS**

In 30 tubules in which we obtained individual R_{min}, R_{max}, and S_{fb} values in each tubule and used...
these values to compute \([Ca^{2+}]_i\) in each tubule, the mean, steady-state \([Ca^{2+}]_i\) was 63 ± 10 nM. As discussed in METHODS, we also used the data from the above 30 tubules to compute mean values for \(R_{\text{min}}\), \(R_{\text{max}}\), and \(S_{\text{fb}}\) and then used these mean calibration parameters to compute initial \([Ca^{2+}]_i\) values in a total of 131 tubules, including the 30 noted above. The average steady-state \([Ca^{2+}]_i\) for these 131 tubules was 61 ± 1 nM (n = 131), which is not significantly different from the mean value for the 30 tubules (P = 0.8, unpaired t-test).

**Effect on \([Ca^{2+}]_i\) of Applying CO\(_2\)/HCO\(_3^-\) Unilaterally**

Our first approach in studying the effect of CO\(_2\)/HCO\(_3^-\)-buffered solutions on \([Ca^{2+}]_i\) was to measure \([Ca^{2+}]_i\) while exposing either the luminal or the basolateral side of the tubule, but not both, to 5% CO\(_2\)/22 mM HCO\(_3^-\) at a fixed extracellular pH of 7.40. A typical recording is shown in Fig. 1A. At the beginning of the experiment, we bilaterally perfused the tubule with a solution buffered to pH 7.40 with HEPES (solution 2). After we switched the luminal solution from one buffered with HEPES to one buffered with 5% CO\(_2\)/22 mM HCO\(_3^-\) (solution 3), \([Ca^{2+}]_i\) slowly drifted upward by a small amount (segment ab). On the other hand, after we removed the CO\(_2\)/HCO\(_3^-\) from the lumen (bc) and then introduced the CO\(_2\)/HCO\(_3^-\)-buffered solution to the bath, \([Ca^{2+}]_i\) increased to a new and substantially higher steady-state value (cd). Switching back to the CO\(_2\)/HCO\(_3^-\)-free bath solution caused \([Ca^{2+}]_i\) to return close to baseline (de). Figure 1B shows that we obtained the same result when we made the luminal and basolateral solution changes in the opposite order.

The histogram in Fig. 1C represents the mean paired changes in \([Ca^{2+}]_i\); \((\Delta[Ca^{2+}]_i)\) elicited in eight tubules by switching the solution in either the lumen (filled bar, corresponding to segment ab in Fig. 1A and cd in Fig. 1B) or the bath (stippled bar, corresponding to segment cd in Fig. 1A and ab in Fig. 1B) from HEPES-buffer to 5% CO\(_2\)/22 mM HCO\(_3^-\); \(\Delta[Ca^{2+}]_i\) was not statistically significant when we applied CO\(_2\)/HCO\(_3^-\) to the lumen (P = 0.8) but was significant when we applied CO\(_2\)/HCO\(_3^-\) to the bath (P < 0.002).

**Effect on pH\(_i\) of Applying CO\(_2\)/HCO\(_3^-\) Unilaterally**

To test the hypothesis that the increase in \([Ca^{2+}]_i\) was caused by a change in pH\(_i\), we repeated the above protocol while measuring pH\(_i\) in a total of 14 different tubules. Because we included lactate in our luminal solutions to mimic the conditions in other parallel experiments in our laboratory, we anticipated that the tubules would have a high initial pH\(_i\). Previous work has shown that adding lactate to the lumen of the salamander proximal tubule, or adding acetate to the lumen of the rabbit S3 segment, raises pH\(_i\) by ~0.2 due to the coupled apical entry of Na\(^+\) and monocarboxylate followed by the coupled exit of H\(^+\) and lactate (or lactate/OH exchange) across the basolateral membrane (45, 59). Indeed, in 9 of the 14 tubules, the initial pH\(_i\) was relatively high (averaging 7.54 ± 0.08). However, for unknown reasons, in the other five tubules, the initial pH\(_i\) in HEPES was lower (averaging 7.23 ±
BASOLATERAL CO2-INDUCED [Ca2++] INCREASE

Table 2. Effect of 5% CO2/22 mM HCO3 on intracellular pH

<table>
<thead>
<tr>
<th>Tubules that acidified with bath CO3/HCO3</th>
<th>Lumen</th>
<th>Bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 9)</td>
<td>HEPES</td>
<td>5% CO2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.64 ± 0.11</td>
</tr>
<tr>
<td>Tubules that alkalized with bath CO3/HCO3</td>
<td></td>
<td>7.29 ± 0.07</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of tubules. pH; Δ, change.

0.07). Regardless of whether the initial pHi in HEPES was high or low, introducing 5% CO2/22 mM HCO3 to the lumen caused a sustained decrease in pHi. On the other hand, the initial pHi in HEPES had a major impact on the pHi response when we added CO2/HCO3 to the bath. In the nine tubules with a high initial pHi, introducing 5% CO2/22 mM HCO3 to the bath caused a sustained acidification, whereas in the five other tubules with a lower initial pHi, introducing 5% CO2/22 mM HCO3 induced an alkalinization. The results are summarized in the Table 2. As noted in the DISCUSSION, the divergent response to the addition of basolateral CO2/HCO3 is consistent with observations made in other preparations.

Thus in all of the experiments in which we monitored pHi, introducing luminal CO2/HCO3 caused an acidification; in all experiments in which we monitored [Ca2+], introducing luminal CO2/HCO3 had no effect. The relationship between ΔpHi and Δ[Ca2+] was just the opposite in two-thirds of the experiments in which we added CO2/HCO3 to the bath. In 9 of 14 tubules in which we monitored pHi, introducing basolateral CO2/HCO3 caused an acidification, just as if we had added CO2/HCO3 to the lumen. However, in all experiments in which we monitored [Ca2+], introducing basolateral CO2/HCO3 caused an increase in [Ca2+]. Therefore, a change in pHi cannot be the cause of the [Ca2+] increase elicited by basolateral CO2/HCO3.

Effect on [Ca2+]i of Applying Pure HCO3 or Pure CO2 Basolaterally

The above experiments ruled out a role for pHi in the increase of [Ca2+]i elicited by bath CO2/HCO3 but did not discriminate between bath CO2 and bath HCO3. Next, we used OOE solutions to investigate separately the effect of pure CO2 (solution 4) and pure HCO3 (solution 5) on [Ca2+]. We chose to use 20% CO2 (nominally no HCO3, pH 7.40) because this basolateral PCO2 causes a substantially larger stimulation of JHCO3 in the S2 proximal tubule than does 5% CO2 (77). A HEPES-buffered solution continuously perfused the lumen. As shown in Fig. 2A, introducing 22 mM pure HCO3 to the bath caused, at most, a trivial increase in

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3 One reason for this relatively low pHi is that these tubules could have relatively low activities of either the apical or basolateral monocarboxylate transporters.

4 CO2 passively enters the cell across the apical membrane, leading to the intracellular formation of H+ and CO3. Presumably, pHi fails to recover from this acid load because the unstimulated apical Na/H exchanger and H+ pump are unable to recover from the acidifying influence of HCO3 that exits across the basolateral membrane via the electronegic Na-HCO3 cotransporter.
[Ca\(^{2+}\)]\(_i\) (segment ab), whereas introducing 20% pure CO\(_2\) always caused a substantial and sustained increase in [Ca\(^{2+}\)]\(_i\) (Fig. 2A, segment cde). Removing the pure CO\(_2\) solution caused [Ca\(^{2+}\)]\(_i\) to decrease rapidly, but not all the way to the baseline. In a total of 10 similar experiments (Fig. 2B), pure HCO\(_3\) elicited a mean Δ[Ca\(^{2+}\)] of 7 ± 2 nM (n = 10; P < 0.01) from a mean steady-state [Ca\(^{2+}\)]\(_i\) of 76 ± 3 (n = 10). This small [Ca\(^{2+}\)]\(_i\) increase could be the result of a small CO\(_2\) contamination in our pure HCO\(_3\) solutions. On the other hand, measurements with a CO\(_2\) electrode did not detect CO\(_2\) in the pure HCO\(_3\) solutions exiting the mixing T of our OOE apparatus. In the same tubules, pure CO\(_2\) elicited a much larger Δ[Ca\(^{2+}\)]\(_i\) = 62 ± 17 nM (n = 10; P < 0.005) from a mean steady state [Ca\(^{2+}\)]\(_i\) of 78 ± 2 (n = 10). These results support the hypothesis that it is basolateral CO\(_2\), not HCO\(_3\), that is responsible for increasing [Ca\(^{2+}\)]\(_i\) in the proximal tubule.

One should recall that in Fig. 2A, [Ca\(^{2+}\)]\(_i\) did not fully return to its baseline value after removal of bath pure CO\(_2\) In a series of 9 tubules distinct from the 10 discussed above, we exposed the basolateral side of tubules to twin pulses of pure CO\(_2\), with a delay of ~5 min between pulses. The mean steady-state [Ca\(^{2+}\)]\(_i\) before the first pulse was 57 ± 1 nM. Because [Ca\(^{2+}\)]\(_i\) often did not return to the initial baseline, the mean steady-state [Ca\(^{2+}\)]\(_i\) before the second pulse was significantly higher, 107 ± 19 nM (P < 0.03). The Δ[Ca\(^{2+}\)] elicited by the first pure CO\(_2\) pulse was 85 ± 27 nM, whereas the Δ[Ca\(^{2+}\)]\(_i\) elicited by the second pure CO\(_2\) pulse (starting from a higher baseline) was only 48 ± 14, a difference that is on the verge of statistical significance (P = 0.052).

In six other experiments, we measured pH\(_i\) while switching the basolateral solution from HEPES (solution 2) to pure CO\(_2\) (solution 4). The mean pH\(_i\) in bilateral HEPES was 7.23 ± 0.02, whereas the mean pH\(_i\) during bath exposure to pure CO\(_2\) was 6.87 ± 0.08, a mean difference of 0.36 ± 0.09. Thus, even though the pH\(_i\) decrease elicited by basolateral pure CO\(_2\) was substantially less than that elicited by luminal CO\(_2\)/HCO\(_3\) (0.36 vs. the values of 0.55 and 0.53 shown in Table 2), basolateral pure CO\(_2\) triggered an increase in [Ca\(^{2+}\)]\(_i\), whereas luminal CO\(_2\)/HCO\(_3\) did not. This result thus provides additional support for the hypothesis that it is CO\(_2\) itself, and not the change in pH\(_i\), that is responsible for the [Ca\(^{2+}\)]\(_i\) increase in our experiments.

A technical question that arises is whether the large decrease in pH\(_i\) elicited by luminal CO\(_2\)/HCO\(_3\) may have affected the ability of fura 2 to report [Ca\(^{2+}\)]\(_i\). Although in their original paper Gryniewicz et al. (27) reported that fura 2 is poorly pH sensitive, others have reported that lowering the pH causes the K\(_d\) of fura 2 to increase (34, 39). In our experiments, we did not attempt to correct for this pH sensitivity of the K\(_d\) because we did not simultaneously measure pH\(_i\) and Ca\(^{2+}\). Thus we probably underestimated the rise in [Ca\(^{2+}\)]\(_i\) induced by the pure CO\(_2\) solution in tubules with a relatively high initial pH\(_i\).

**Mechanism of the [Ca\(^{2+}\)]\(_i\) Increase Induced by Basolateral CO\(_2\)**

Effect of bilateral Ca\(^{2+}\)-free solutions on the CO\(_2\)-induced [Ca\(^{2+}\)]\(_i\) increase. We next investigated the source of Ca\(^{2+}\) responsible for the CO\(_2\)-induced increase in [Ca\(^{2+}\)]\(_i\). Our first approach was to expose the tubule briefly to basolateral 20% pure CO\(_2\), as in the second half of Fig. 2A, first in the presence and then in the absence of Ca\(^{2+}\). Figure 3A shows such an experiment. Initially, the lumen and bath contained a HEPES-buffered solution (solution 2). A control pulse of 20% pure CO\(_2\) elicited a [Ca\(^{2+}\)]\(_i\) increase (segment ab) that averaged 16 ± 2 nM (n = 8) and was partially reversed in this experiment by removing the CO\(_2\) (bc). We then switched the luminal solution to a variant of solution 2 in which we omitted the Ca\(^{2+}\) and added 0.5 mM EGTA to chelate trace amounts of Ca\(^{2+}\). This removal (point c) reversed the slow upward drift in [Ca\(^{2+}\)]\(_i\) and caused [Ca\(^{2+}\)]\(_i\) to begin to decrease slowly. When we then similarly removed Ca\(^{2+}\) from the bath (point d), [Ca\(^{2+}\)]\(_i\) fell more rapidly (de). Because exposing tubules to Ca\(^{2+}\)-free solutions for long periods (~10 min) interfered with tubule integrity, we challenged the tubule with a second CO\(_2\) pulse even as [Ca\(^{2+}\)]\(_i\) continued to decline. We found that the second 20% pure CO\(_2\) pulse, in the continued bilateral absence of Ca\(^{2+}\), caused a [Ca\(^{2+}\)]\(_i\) increase (ef) that averaged 13 ± 2 nM (n = 8) and was indistinguishable from the first (P = 0.12). On removal of the bath pure CO\(_2\) solution, [Ca\(^{2+}\)]\(_i\) fell (fg) to a value that was lower than the value prevailing before we applied the CO\(_2\). Reintroducing Ca\(^{2+}\) to the lumen and bath restored [Ca\(^{2+}\)]\(_i\) to its initial level (gh). Figure 3B summarizes the mean Δ[Ca\(^{2+}\)]\(_i\) values in the presence and absence of extracellular Ca\(^{2+}\).

Effect of bilateral nifedipine on the CO\(_2\)-induced [Ca\(^{2+}\)]\(_i\) increase. In experiments similar to that shown in Fig. 3A, we examined the effect of adding 10 μM nifedipine, which blocks dihydropyridine-sensitive (L- and T-type) Ca\(^{2+}\) channels (62), to both the lumen and the bath (not shown). We found that control 20% pure CO\(_2\) pulses elicited a mean Δ[Ca\(^{2+}\)]\(_i\) of 10 ± 3 nM (n = 6), a value that was not significantly different from the Δ[Ca\(^{2+}\)]\(_i\) of 13 ± 1 nM elicited by 20% pure CO\(_2\) in the presence of bilateral nifedipine (P = 0.4). The results of the experiments in this and the previous paragraph indicate that an influx of extracellular Ca\(^{2+}\) is not directly responsible for the CO\(_2\)-induced increase in [Ca\(^{2+}\)]\(_i\).

Effect of Tg on CO\(_2\)-induced [Ca\(^{2+}\)]\(_i\) increase. If CO\(_2\) causes the release of Ca\(^{2+}\) from an intracellular store, then blocking the reuptake of Ca\(^{2+}\) into this store ought to deplete the store and reduce the size of the CO\(_2\)-induced increase [Ca\(^{2+}\)]\(_i\). Tg is a well-known inhibitor of SERCA, the Ca\(^{2+}\) pump responsible for the uptake of Ca\(^{2+}\) into the sarco- and endoplasmic reticulum (52, 65). Figure 4A shows an experiment in which we tested the effect of Tg on the CO\(_2\)-induced increase in [Ca\(^{2+}\)]\(_i\). As a control, we first exposed the basolateral side of the tubule to 20% pure CO\(_2\), observing a revers-
produced a small but statistically significant increase in the CO2-induced increase in [Ca2+]i.

To verify that Tg was indeed blocking the sarcoplasmic Ca2+ pump, we performed a positive control experiment in which we used extracellular ATP to activate the P2Y purinergic receptor and thereby release Ca2+ from Tg-sensitive stores (9, 74). As shown in Fig. 4B, basolateral ATP (0.5 mM) caused a very large but transient rise in [Ca2+]i, and Tg virtually eliminated this effect. As summarized in Fig. 4C for a total of eight similar experiments, the inhibition by Tg was statistically significant.

One might argue that a desensitization of the P2Y receptor may have been responsible for the absence of a [Ca2+]i increase during the second ATP pulse in Fig. 4B, an effect that would have led us to overestimate the blockade by Tg. We therefore performed a separate series of experiments (not shown) in which we exposed tubules to two ATP pulses (~5 min apart) in the absence of inhibitors. The first exposure of the basolateral side of the tubule to 0.5 mM ATP caused a mean Δ[Ca2+]i of 99 ± 20 nM, whereas the second induced a mean Δ[Ca2+]i of 110 ± 22 nM (n = 7); this difference is not statistically significant (P = 0.6).

Finally, we also performed two experiments (not shown) similar to the one in Fig. 4B, but in which, in the presence of Tg, we first pulsed the tubule with 0.5 mM ATP and then with 20% pure CO2. Even though ATP had a minimal effect, CO2 still elicited an increase in [Ca2+]i. The results of these three series of Tg experiments thus indicate that CO2 does not cause the release of Ca2+ from Tg-sensitive Ca2+ stores.

**Effect of caffeine.** To explore the possibility that a ryanodine receptor might be involved in the CO2-induced increase in [Ca2+]i, we assessed the ability of caffeine, a well-known agonist of this receptor (32, 78), to raise [Ca2+]i in proximal tubule cells. In a total of four experiments similar to the one shown in Fig. 5, we exposed the proximal tubule to 10 mM caffeine for ~2 min. The mean [Ca2+]i value measured before application of caffeine was 57 ± 1 nM; adding caffeine caused a mean Δ[Ca2+]i of 1 ± 2 nM, a value not statistically different from the baseline value (P = 0.8). On the other hand, applying ATP always caused a transient increase in [Ca2+]i. In the same four experiments, from a mean baseline [Ca2+]i of 58 ± 1 nM, adding ATP caused a mean Δ[Ca2+]i of 128 ± 29 nM. We conclude from these experiments that S2 proximal tubules have no demonstrable ryanodine receptor activity and that it is unlikely that these receptors play a role in the CO2-induced increase in [Ca2+]i.

**Effect of rotenone on CO2-induced [Ca2+]i.** To explore the possibility that CO2 causes the release of Ca2+ from the mitochondria, we examined the effect of rotenone on the CO2-induced [Ca2+]i increase. Our protocol was the same as for Tg (see Fig. 4A). Because rotenone blocks electron transport, we would expect that rotenone would cause Ca2+ to leak out of the mitochondria. Indeed, applying 4 μM rotenone caused the baseline [Ca2+]i to increase from 100 ± 14 to 155 ± 24 nM (P < 0.02, n = 5). Nevertheless, as summarized in Fig. 6,
pulsing with 20% pure CO₂ produced, if anything, a larger [Ca²⁺] increase in the presence of rotenone than in its absence, although the difference was not statistically significant (P > 0.05, n = 5). An unavoidable complication in these experiments is that rotenone undoubtedly disturbed cellular energy metabolism. If these changes in energy metabolism did not affect the mechanism by which CO₂ releases Ca²⁺ from internal stores, we would conclude that the mitochondria are not the source of the Ca²⁺ released in response to CO₂.

**DISCUSSION**

By increasing the rate at which they transport HCO₃⁻ into the blood, the kidneys play an important role in the response to respiratory acidosis. Although work from our laboratory indicates that the trigger for increased HCO₃ transport is basolateral CO₂ per se, rather than the accompanying acidosis, the underlying intracellular signals have not yet been resolved. We performed the present experiments to evaluate whether a rise in [Ca²⁺], might be an element in one of the signaling pathways by which basolateral CO₂ acts on renal proximal tubule cells.

**Influence of Initial pH i in HEPES on the pH i Response to Bath CO₂/HCO₃⁻**

As noted in RESULTS and summarized in Table 2, 9 of the 14 tubules that we tested had a relatively high initial pH_i in HEPES and underwent a sustained acidification when we introduced CO₂/HCO₃⁻ to the bath.
The other five tubules had a relatively low initial pH; and underwent an alkalinization when we introduced CO\textsubscript{2}/HCO\textsubscript{3} to the bath. This dependence on the initial pH is consistent with three previous observations made using other preparations.

First, in the rabbit S3 segment, perfusing the lumen with a monocarboxylate-free solution results in a relatively low initial pH. Under these conditions, adding basolateral CO\textsubscript{2}/HCO\textsubscript{3} causes a transient pH fall followed by a large and sustained rise (46), reflecting a three- to fourfold stimulation of apical Na/H exchangers and H\textsuperscript{+} pumps (17, 18) that overcomes the acidifying influence of the basolateral Na-HCO\textsubscript{3} cotransporter. However, when the tubule lumen is perfused with acetate, the initial pH is relatively high, and adding basolateral CO\textsubscript{2}/HCO\textsubscript{3} causes a large and sustained fall in pH; (46).

Finally, in both hippocampal neurons (58) and hippocampal astrocytes (7), the effect of adding CO\textsubscript{2}/HCO\textsubscript{3} on steady-state pH critically depends on the initial pH. The induced alkalinization is greatest at the lowest initial pH values and gradually falls off (or even reverses in the case of the astrocytes) at progressively higher initial pH values. A general explanation for all three cases is that relatively high pH values stimulate acid loading but inhibit acid extrusion.

**Basolateral CO\textsubscript{2} Directly Triggers an Increase in \([\text{Ca}^{2+}]\text{ i}\)**

Ten years ago, Nakhoul et al. (46), working with the rabbit S3 proximal tubule (which always had a relatively low initial pH under the conditions of their experiments), showed that adding CO\textsubscript{2}/HCO\textsubscript{3} to the lumen causes a sustained pH decrease that is presumably due to 1) the rapid diffusion of CO\textsubscript{2} into the cell, followed by 2) the formation of H\textsuperscript{+} and HCO\textsubscript{3} and the sustained basolateral exit of HCO\textsubscript{3}. On the other hand, they found that adding CO\textsubscript{2}/HCO\textsubscript{3} to the bath induces only a transient pH decrease, followed by a sustained increase that is presumably due to an increase in net acid extrusion from the cell. Indeed, Chen and Boron (17, 18) showed that adding equilibrated CO\textsubscript{2}/HCO\textsubscript{3} to the lumen had no effect on rates of apical Na/H exchange and H\textsuperscript{+} pumping, whereas adding the same solution to the bath increased these rates by two- to fourfold. Here, we report a parallel observation: adding CO\textsubscript{2}/HCO\textsubscript{3} to the lumen never elicits a significant rise in \([\text{Ca}^{2+}]\text{ i}\), whereas adding CO\textsubscript{2}/HCO\textsubscript{3} to the bath always triggers an increase in \([\text{Ca}^{2+}]\text{ i}\). Thus it appears that basolateral, but not luminal, CO\textsubscript{2}/HCO\textsubscript{3} produces several unique effects: 1) an increase in steady-state pH when the initial pH is low, 2) an increase in apical H\textsuperscript{+} extrusion, and 3) an increase in \([\text{Ca}^{2+}]\text{ i}\).

One key question is whether it is CO\textsubscript{2} or HCO\textsubscript{3} that causes the above three effects. Other work from our laboratory shows that it is specifically basolateral CO\textsubscript{2}, and not basolateral HCO\textsubscript{3}, that increases \(J_{\text{HCO3}}\) (76, 77). In the present study, we have made an additional parallel observation: a 20% pure CO\textsubscript{2} solution in the bath can elicit a substantial rise in \([\text{Ca}^{2+}]\text{ i}\), whereas a 22 mM pure HCO\textsubscript{3} solution in the bath cannot. If CO\textsubscript{2}, and not HCO\textsubscript{3}, is indeed the trigger, why is it that CO\textsubscript{2} added to the lumen does not diffuse across the apical membrane and through the cytoplasm to exert a measurable effect at the basolateral membrane? We presume that, under the conditions of such an experiment, the CO\textsubscript{2} concentration near the basolateral membrane is too low to produce a measurable stimulation of some sort of a CO\textsubscript{2} sensor.

The observation that it is basolateral CO\textsubscript{2} and not HCO\textsubscript{3} that triggers the increase in \([\text{Ca}^{2+}]\text{ i}\), does not

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5 The cause of this acidification has not been investigated. One possible explanation is that the basolateral Na-HCO\textsubscript{3} cotransporter is more active at a higher pH, and thus exerts a larger acidifying influence. In addition, adding basolateral CO\textsubscript{2}/HCO\textsubscript{3} might inhibit monocarboxylate transport. The gain of acid-loading capacity and/or the loss of acid-extruding capacity might overwhelm the alkalinizing effect of stimulating the apical Na/H exchanger and H\textsuperscript{+} pump.

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*Fig. 5. Representative recording of \([\text{Ca}^{2+}]\text{ i}\), during sequential bath exposures to 10 mM caffeine and 0.5 mM ATP. The tubule lumen was continuously perfused with a HEPES-buffered, CO\textsubscript{2}/HCO\textsubscript{3}-free solution (solution 2, Table 1). At the indicated times, the bath solution was temporarily replaced twice, first with solution 2 supplemented with 10 mM caffeine and the second time with solution 2 supplemented with 0.5 mM ATP. In a total of 4 such experiments, caffeine elicited a mean Δ[Ca\textsuperscript{2+}]\text{ i}, (compared with paired baseline [Ca\textsuperscript{2+}]\text{ i}), of 128 ± 29 nM (P < 0.02, paired t-test), whereas ATP elicited a mean Δ[Ca\textsuperscript{2+}]\text{ i}, value just before the application of caffeine) of 1 ± 2 nM (P = 0.8, paired t-test), whereas ATP elicited a mean Δ[Ca\textsuperscript{2+}]\text{ i}, of 128 ± 29 nM (P < 0.02, paired t-test).*

*Fig. 6. Effect of rotenone on the [Ca\textsuperscript{2+}]\text{ i}, increase elicited by 20% pure CO\textsubscript{2}. Open bar, mean Δ[Ca\textsuperscript{2+}]\text{ i}, caused by the basolateral switch from the HEPES buffer to the pure CO\textsubscript{2} solution in the absence of rotenone; filled bar, comparable Δ[Ca\textsuperscript{2+}]\text{ i}, in the presence of 4 \(\mu\text{M}\) rotenone. See the text for details. The statistical analysis summarized in the figure is the result of a paired 2-tailed Student’s t-test.*
distinguish between the possibilities that basolateral CO2 I acts directly on the tubule to raise [Ca2+]i, or 2) acts indirectly by lowering pHj, which in turn leads to a rise in [Ca2+]i. A precedent for the latter hypothesis is that cytosolic acidification causes [Ca2+]i to rise in gastric parietal cells (67), platelets (67), and cultured collecting duct cells (60). On the other hand, cytosolic acidification causes [Ca2+]i to fall in squid giant axons (3), and cytosolic alkalization causes [Ca2+]i to rise in both HT 29 cells (48) and rat pancreatic acinar cells (61).

Did pHj indirectly control [Ca2+]i, in our experiments? Although an isolated increase in bath [HCO3−] causes pHj to increase (76), we found that switching to a pure HCO3− solution causes only a trivial increase in [Ca2+]i (Fig. 2). Thus, if pHj controls [Ca2+]i, it would have to be a pHj decrease that causes [Ca2+]i to rise. Indeed, switching to a pure CO2 solution in the bath caused pHj to fall by ∼0.35 and consistently caused [Ca2+]i to increase, apparently supporting the pHj-[Ca2+]i hypothesis. However, we found that adding CO2/HCO3− to the lumen always causes pHj to fall by >0.5 (Table 2) but has no effect on [Ca2+]i (Fig. 1), ruling out the pHj-[Ca2+]i hypothesis. Finally, as noted in the presentation of Table 2 in RESULTS, introducing equilibrated CO2/HCO3− into the bath caused pHj to decrease by ∼0.3 in 9 of 14 tubules (i.e., the high-PHj, normal-PHj tubules) but caused [Ca2+]i to rise in 8 consecutive tubules. The chance of randomly choosing eight consecutive high-PHj tubules is only ∼3%. We conclude that a change in pHj is not the intermediary through which CO2 raises [Ca2+]i. This conclusion represents a third parallelism between the CO2-induced increase in [Ca2+]i and CO2-induced changes in acid-base transport: In the proximal tubule, the CO2-induced increase in \( J_{\text{HCO3}} \) does not occur via a decrease in pHj (77).

**Ca2+ Originates From an As Yet Unidentified Intracellular Pool**

Two pieces of evidence indicate that the immediate source of the Ca2+ for the CO2-induced increase in [Ca2+]i is an intracellular store. First, the CO2-induced increase in [Ca2+]i occurs even when Ca2+ is absent from the lumen and bath (Fig. 3). Second, the dihydropyridine derivative nifedipine fails to attenuate the CO2-induced increase in [Ca2+]i. We chose nifedipine because the proximal tubule has dihydropyridine-sensitive Ca2+ channels that mediate Ca2+ influx during volume regulation after a hypotonic shock (40), in response to PTH (63), or during hypoxia (49).

One of the classic types of Ca2+ stores in cells is the Tg-sensitive store, which often is triggered by inositol 1,4,5-trisphosphate (IP3). Indeed, the P2Y purinergic receptor on the basolateral membrane of the proximal tubule releases Ca2+ from a Tg-sensitive pool (9, 74). Although we confirmed that adding Tg blocks the rise in [Ca2+]i; stimulated by extracellular ATP (Fig. 4B), we found the drug to be ineffective in reducing the magnitude of the [Ca2+]i increase elicited by basolateral pure CO2 (Fig. 4A). In fact, in the presence of Tg, a pure CO2 pulse elicits a greater [Ca2+]i increase than a matched pulse in the absence of the drug (Fig. 4C). It is possible that, with Tg preventing the loading of Tg-sensitive stores, Tg-insensitive stores may accumulate extra Ca2+ that they release in response to CO2, resulting in a larger-than-normal CO2-induced increase in [Ca2+]i.

Ca2+ pools released by the ryanodine receptor are usually also Tg sensitive. However, we ruled out the possibility that ryanodine receptors are involved in the CO2-induced release of Ca2+ by demonstrating that millimolar concentrations of caffeine, which lead to a Ca2+-independent activation of the ryanodine channel (32, 78), do not elicit a rise in [Ca2+]i in the proximal tubule.

One Tg-insensitive Ca2+ pool is the mitochondria (23, 26, 29). However, our rotenone data are not consistent with the hypothesis that CO2 causes the release of Ca2+ from mitochondria. Thus our data are consistent with the hypothesis that, via a CO2 sensor at or near the basolateral membrane, CO2 triggers the release of Ca2+ from a nonconventional intracellular store.

Other investigators have demonstrated that multiple nonmitochondrial Ca2+ stores, functionally and spatially distinct, may coexist in the same cell (25, 43, 50) and have in particular demonstrated the presence of Tg-insensitive pools. For example, a variety of cell lines have a nonmitochondrial pool that can take up Ca2+ after maximal inhibition by Tg (51, 64). In goldfish somatotrophs, GnRH causes a release of Ca2+ from a Tg-insensitive store (30). Moreover, in sea urchin eggs, the second messenger nicotinic acid adenine dinucleotide causes the release of Ca2+ from a Tg-insensitive store that is distinct from that triggered by either IP3 or cADP-ribose (24, 35). The Ca2+ pumps responsible for accumulating Ca2+ in the Golgi apparatus are Tg insensitive. Certain agonists (e.g., arginine vasopressin, histamine) coupled to the generation of IP3 can partially release Ca2+ from this pool (43, 50). Thus several pools are candidates for the CO2-induced release of Ca2+.

**Potential Roles of the CO2-Induced Increase in [Ca2+]i**

Previous work has established conflicting precedents for the effects that increases in [Ca2+]i have on acid-base transport in the proximal tubule. Four lines of evidence suggest that an increase in [Ca2+]i is associated with an increase in acid-base transport and/or \( J_{\text{HCO3}} \). First, in experiments on in vivo microperfused proximal tubules, raising [Ca2+]i by the luminal addition of the Ca2+ ionophore A-23187 increases \( J_{\text{HCO3}} \) in a dose-dependent manner (38). Second, adding angiotensin II to the basolateral side of a proximal tubule leads to increases in both \( J_{\text{HCO3}} \) and [Ca2+]i (31). Third, carbachol triggers an increase in [Ca2+]i (42, 56) and stimulates the Na-HCO3 cosportransporter; conversely, the Ca2+ chelator BAPTA prevents the stimulation of the cotransporter (56). Fourth and finally, CO2 causes insertion of vesicles containing H+ pumps...
into the apical membrane of the proximal tubule (57). In the turtle bladder, the application of CO₂ triggers a rise in Ca²⁺ (15), and this rise in [Ca²⁺]j is required for the apical insertion of vesicles (68). A similar process may be at work in the rabbit outer medullary collecting duct (28).

Three lines of evidence suggest that an increase in [Ca²⁺]j is associated with a decrease in acid-base transport and/or JHCO₃ in the proximal tubule. First, increasing [Ca²⁺]j by adding ionomycin to the bath leads to a decrease in JHCO₃ (16). Second, PTH, a potent inhibitor of JHCO₃ (20, 21), also increases [Ca²⁺]j (63). And third, a rise in [Ca²⁺]j inhibits the apical Na/H exchanger (72, 73).

One explanation for the apparently divergent data discussed above is that the relevant changes in [Ca²⁺]j occur within microdomains, and local changes in [Ca²⁺]j are more important than global ones (25, 35, 55). Another explanation for these divergent effects is that they are the consequence of different frequencies of Ca²⁺ spikes or waves. In the context of these possibilities, it is difficult to predict the role that [Ca²⁺]j plays in the response of the proximal tubule to basolateral CO₂. We propose that CO₂ binds to a CO₂ sensor at or near the basolateral membrane and, independently of a change in pH, triggers the release of Ca²⁺ from a nonmitochondrial intracellular store that is insensitive to Tg. The released Ca²⁺ might (1) modulate cellular processes not directly related to JHCO₃ (2) be part of a signal-transduction pathway that results in an increase in JHCO₃, or (3) be part of a braking mechanism that helps prevent runaway JHCO₃ during CO₂ stimulation.

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DISCLOSURES

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