Role of a tyrosine kinase in the CO2-induced stimulation of HCO3⁻ reabsorption by rabbit S2 proximal tubules

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Zhou, Yuehan, Patrice Bouyer, and Walter F. Boron. Role of a tyrosine kinase in the CO2-induced stimulation of HCO3⁻ reabsorption by rabbit S2 proximal tubules. Am J Physiol Renal Physiol 291: F358–F367, 2006; doi:10.1152/ajprenal.00520.2005.—A previous study demonstrated that proximal tubule cells regulate HCO3⁻ reabsorption by sensing acute changes in basolateral CO2 concentration, suggesting that there is some sort of CO2 sensor at or near the basolateral membrane (Zhou Y, Zhao J, Bouyer P, and Boron WF Proc Natl Acad Sci USA 102: 3875–3880, 2005). Here, we hypothesized that an early element in the CO2 signal-transduction cascade might be either a receptor tyrosine kinase (RTK) or a receptor-associated (or soluble) tyrosine kinase (sTK). In our experiments, we found, first, that basolateral 17.5 μM genistein, a broad-spectrum tyrosine kinase inhibitor, virtually eliminates the CO2 sensitivity of HCO3⁻ absorption rate (JHCO3). Second, we found that neither basolateral 250 mM nor basolateral 2 μM PP2, a high-affinity inhibitor for the Src family that also inhibits the Bcr-Abl sTK as well as the Kit RTK, reduces the CO2-stimulated increase in JHCO3. Third, we found that either basolateral 35 mM PD168393, a high-affinity inhibitor of RTKs in the erbB (i.e., EGF receptor) family, or basolateral 10 nM BPIQ-I, which blocks erbB RTKs by competing with ATP, eliminates the CO2 sensitivity. In conclusion, the transduction of the CO2 signal requires activation of a tyrosine kinase, perhaps an erbB. The possibilities include the following: 1) a TK is simply permissive for the effect of CO2 on JHCO3; 2) a CO2 receptor activates an sTK, which would then raise JHCO3; 3) a CO2 receptor transactivates an RTK; and 4) the CO2 receptor could itself be an RTK.

RENAL TUBULE CELLS PLAY A central role in whole body acid-base balance by J secreting H⁻ into the tubule lumen, thereby titrating NH₃ to NH₄⁺ and also creating titratable acid; and 2) moving an equivalent amount of “new HCO₃⁻” into the blood, ~70 mmol/day in humans, and thereby titrating the fixed acid produced by metabolism and generated by the gastrointestinal tract. At the same time, the tubules must also reabsorb nearly all of a much larger amount of HCO₃⁻ filtered in the glomeruli. The proximal tubule (PT) is the site of generation of ~60% of the new HCO₃⁻ and the site of reabsorption of ~80% of the filtered HCO₃⁻ using the same transporters in both processes. The cell uses cytosolic carbonic anhydrase II (51, 52) to convert CO₂ + H₂O to H⁺ + HCO₃⁻, and then extrudes the H⁺ across the apical membrane (3, 10, 50) via Na/H exchangers (6, 7, 34) and H⁺ pumps (22) and exports the HCO₃⁻ across the basolateral membrane, mainly via the electrogenic Na-HCO₃ cotransporter (11, 16, 47, 48). For the system to work properly, the PT cell must be able to respond to changes in whole body acid-base status by appropriately adjusting transporters activities.

To study how the PT senses changes in whole body acid-base status, our laboratory developed out-of-equilibrium (OEE) CO2/HCO₃⁻ solutions for altering, one at a time, basolateral [CO₂], [HCO₃⁻], or [H⁺] (brackets denote concentration) (58, 59). We found that, at least in regard to acute acid-base disturbances, the PT responds not to changes in either basolateral (BL) pH (pHBL) or intracellular pH (pHi) but to changes in [CO₂]BL and [HCO₃⁻]BL (61). In the case of [CO₂]BL, increases cause HCO₃⁻ reabsorption to rise, whereas decreases have the opposite effect. Moreover, the data suggest that the PT cell senses [CO₂]BL directly, utilizing some sort of CO₂ sensor at or near the basolateral membrane. The key questions concern the nature of the CO₂ sensor and the mechanisms by which the cell transduces the CO₂ signal to an increase in HCO₃⁻ reabsorption.

The past two decades have seen major advances in understanding how organisms sense dissolved gases. For instance, nitric oxide binds to a heme moiety of soluble guanylyl cyclase (45), oxygen binds to a two-component receptor in bacteria, and ethylene binds to a receptor in Arabidopsis thaliana. The bacterium Rhizobium meliloti senses oxygen using a two-component system, consisting of FixL and FixJ (20). FixL is a transmembrane protein with a COOH-terminal histidine kinase domain and a NH₂-terminal heme-binding domain that blocks the histidine kinase when O₂ binds to the heme. When the [O₂] falls to microaerobic levels, the histidine kinase autophosphorylates a conserved His within its catalytic core, thereby activating FixJ, which, in turn, induces transcription of genes involved in nitrogen fixation (21, 45).

Ethylene acts like a hormone in plants, regulating such events as seed germination, fruit ripening, and leaf senescence (8, 18). In 1993 Chang et al. (13), working on the plant A. thaliana, found that mutations in the ETR1 gene block ethylene signaling. The deduced amino acid sequence of the COOH-terminal half of ETR1 is homologous to both components of the two-component systems. Later work showed that ethylene binds to an NH₂-terminal hydrophobic domain of ETR1 (49) and that the binding involves copper as a cofactor (46). Downstream of ETR1, which is a histidine kinase, the ethylene signaling cascade involves the Raf-like kinase CTR1 (15, 29) and MAPK (37).

Because, like O₂ and ethylene, CO₂ is a small volatile molecule, we entertained the hypothesis that PT cells sense CO₂ using a comparable mechanism. Because mammalian cells do not have histidine kinases, we postulated that the sensor might
be either a receptor tyrosine kinase (RTK) or a receptor-associated (i.e., soluble) tyrosine kinase (sTK). Furthermore, previous studies showed that both EGF and TGF-α, both of which bind to receptors in the erbB family of RTKs, stimulate \( \text{HCO}_3^- \) and phosphate reabsorption, with a higher potency for TGF-α on the \( \text{HCO}_3^- \) absorption rate (\( J_{\text{HCO}_3^-} \)) (41, 42). As a first step, in the present study we have examined the effect of tyrosine kinase inhibitors on the \( \text{CO}_2 \)-induced increase in \( J_{\text{HCO}_3^-} \) by the PT cell.

Our approach was to use OOE solutions to vary basolateral \([\text{CO}_2]\) from 0 to 20% while keeping basolateral \([\text{HCO}_3^-]\) and pH fixed near their physiological values in isolated, perfused rabbit S2 PTs. We found that basolateral 17.5 μM genistein, a broad-spectrum tyrosine kinase inhibitor (1, 24), virtually removes \( \text{CO}_2 \) sensitivity. Thus the transduction of BPIQ-I, which blocks erbB RTKs by competing with ATP at the cysteine residue in the ATP binding pocket (19), blocks the RTKs in the erbB (i.e., EGF receptor) family by alkylating a member of the erbB family. On the other hand, basolateral 35 nM PD168393, which blocks the Src family (26) sTK, the Bcr-Abl sTK, as well as the Kit (53) RTK, is without effect at either 250 nM or 2.5 mM. Moreover, 10 nM basolateral BPIQ-I, which blocks erbB RTKs by competing with ATP (44), also eliminates \( \text{CO}_2 \) sensitivity. Thus the transduction of the \( \text{CO}_2 \) signal requires activation of a tyrosine kinase, possibly a receptor tyrosine kinase (RTK) or a receptor-

**METHODS**

The methods are similar to those in our previous studies (60, 61).

**Biological Preparation**

According to procedures approved by the Yale Animal Care and Use Committee, we perfused the PTs isolated from "pathogen-free" female rabbits (New Zealand White, Elite, Covance, Denver, PA) using methods similar to those described by Burg et al. (12) and later modified by Baum et al. (5) and by our laboratory (36, 59–61). To summarize, a rabbit weighing 1.4–2.0 kg was euthanized by a single overdose of 3 ml (~20 mg) of intravenous pentobarbital sodium. An incision of the abdominal wall exposed the left kidney, which we rapidly removed and cut into coronal slices that we kept in cold (4°C) modified Hanks’ solution (solution I in Table 1). We hand-dissected a slice to obtain individual midcortical S2 segments of a PT. We cannulated the perfusion end of the tubule using concentric holding, perfusion, and exchange pipettes, and drew the collection end into a holding pipette. We randomized the orientation of the tubule between the two pipettes. However, the extreme proximal portion of the proximal straight tubule was always inside one pipette, the most distal portion of the proximal convoluted tubule was exposed to the bath solution between the two pipettes, and a more proximal portion of the proximal convoluted tubule was inside the second pipette. On the collection end, we used a calibrated collection pipette (volume = 55 nl) to obtain samples of fluid. The mean length of perfused tubules in our \( J_{\text{HCO}_3^-}/\text{fluid} \) absorption rate (\( J_{\text{V}} \)) experiments, as measured with an eyepiece micrometer, was 1.23 ± 0.02 mm (n = 96 tubules). The mean luminal collection rate was 12.1 ± 0.2 nl/min (n = 192 collection periods). We perfused the basolateral side of the tubule (i.e., “bath”) at 7 ml/min with a solution at 37°C.

**Experimental Protocol and Solutions**

Table 1 lists the compositions of the solutions, all of which were identical to those used in the aforementioned studies (60, 61).

We dissected PTs in Hanks’ solution (solution I) at 4°C. The luminal perfusate always was solution 2, which contained dialyzed \( ^{[3\text{H}]\text{methoxyinulin}} \) (MW ~7,146, NET-086L, PerkinElmer Life Sciences, Boston, MA) as the volume marker. During a 20- to 30-min warm-up period, solution 3, which contained 2% albumin, flowed through the bath at 37°C. Following this warm-up period, we switched the bath to solution 4, 5, or 6, containing no drugs or DMSO, for the

**Table 1. Physiological solutions**

<table>
<thead>
<tr>
<th>Component</th>
<th>Dissection, Hanks*</th>
<th>Lumen*, Equilibrated 5% ( \text{CO}_2 ), 22 mM ( \text{HCO}_3^- )</th>
<th>Bath†, Warm-Up, Equilibrated 5% ( \text{CO}_2 ), 22 mM ( \text{HCO}_3^- )</th>
<th>Bath‡, Standard, Equilibrated 5% ( \text{CO}_2 ), 22 mM ( \text{HCO}_3^- )</th>
<th>Bath§, OOE‡ Solution 0% ( \text{CO}_2 ), 22 mM ( \text{HCO}_3^- )</th>
<th>Bath¶, OOE Solution 20% ( \text{CO}_2 ), 22 mM ( \text{HCO}_3^- )</th>
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<td>NaCl</td>
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<td>115</td>
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<td>5</td>
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<td>0</td>
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<td>1</td>
<td>1.2</td>
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<tr>
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<td>10.5</td>
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<td>2</td>
<td>4</td>
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<tr>
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<td>0</td>
<td>5</td>
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<td>l-Lactic acid</td>
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<td>1.2</td>
<td>0</td>
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<tr>
<td>%</td>
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<td>5%</td>
<td>5%</td>
<td>5%</td>
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<td>0</td>
<td>0</td>
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<td>6.99</td>
<td>9.40</td>
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Concentrations are in mM except for \( \text{CO}_2 \) (given both in mM and %) and albumin (g/l). Except for solution 1, all solutions were titrated to the indicated pH at 37°C. Tris·HCl and HEPESE were titrated with \( \text{NaOH} \). *Solution was used as a luminal perfusate. †Solution was used as a basolateral perfusate. ‡Out-of-equilibrium (OOE) solutions were generated by rapidly mixing their respective A and B components in a 1:1 ratio. Solutions 5A, 5B, and 6B, were vigorously gassed with 100% \( \text{O}_2 \) to render them free of \( \text{CO}_2 \).
first of two collection periods. During the first 5–8 min of the first collection period, we discarded the first two collected samples before allowing the collected fluid to accumulate in the collection pipette. Subsequently, we began a series of three timed and calibrated collections, two samples for analysis of \(^{3}H\) methoxypreninulin and one for analysis of total CO\(_2\). We then switched to a second bath solution (solution 4, 5, or 6) containing genistein (345834, Calbiochem, La Jolla, CA); PP2 (529573, Calbiochem); PD168393 (513033, Calbiochem); or BPI-I (203696, Calbiochem) plus 1:20,000 DMSO (D-5534, Sigma, St. Louis, MO). We then repeated the procedure outlined for the first collection periods. We generated OEE CO\(_2/\)HCO\(_3\) solutions (solution 5 and 6) by rapidly mixing streams of two dissimilar solutions (i.e., mixing solutions 5A and 5B to yield solution 5 and mixing solutions 6A and 6B to yield solution 6) (58, 61) and delivering the newly mixed solution to the tubule within ~200 ms. All solutions had osmolalities of 300 ± 2 mosmol/kgH\(_2\)O.

**Measurement of \(J_{HCO_3}\) and \(J_V\)**

Our measurement of \(J_{HCO_3}\) (pmol·min\(^{-1}\)·mm tubule length\(^{-1}\)) and \(J_V\) (nl·min\(^{-1}\)·mm\(^{-1}\)) was similar to that used by McKinney and Burg (32) and nearly identical to our previous approach (59–61). We determined total CO\(_2\) in aliquots of the perfusate and collected fluid (32) and nearly identical to our previous approach (59–61). We had osmolalities of 300 ± 2 mosmol/kgH\(_2\)O.

**RESULTS**

**Evaluation of DMSO**

In this study, we added the inhibitors, predissolved in DMSO, to the bath solution at a final DMSO concentration of 1:20,000. Therefore, we first examined the effect of basolateral 1:20,000 DMSO on \(J_{HCO_3}\) and \(J_V\). Figure 1, A and B, summarizes experiments in which DMSO was present in the bath during both collection periods. During the first collection period, with equilibrated 5% CO\(_2/22\) mM HCO\(_3\), in the lumen (solution 2) and in the bath (solution 4), the \(J_{HCO_3}\) and \(J_V\) values (grey bars) were similar to the historical averages (61). However, during the second collection period, when we switched the bath to an out-of-equilibrium solution (solution 6) containing 20% CO\(_2/22\) mM HCO\(_3\), and pH 7.40 (filled bars), the 20% CO\(_2\) failed to increase \(J_{HCO_3}\). Thus we suspected that an extended exposure to DMSO, even at a dilution of 1:20,000, reduces \(J_{HCO_3}\) during the second collection period.

Figure 1, C and D, summarizes a study identical to that above, except that DMSO was present in the bath only during the second collection period. In these experiments, the switch from 5 to 20% CO\(_2\) increased \(J_{HCO_3}\) (\(P = 0.0032\), 2-tailed t-test, unpaired) to its historical value, (61) which is ~50% higher than the value in the first collection period.

As a final check, we examined the effect of adding DMSO during the second collection period when the bath contained 5% CO\(_2\) throughout the experiment. Figure 1, E and F, shows that the addition of DMSO had no effect on either \(J_{HCO_3}\) or \(J_V\). Thus, in our remaining experiments, we added DMSO only in the second collection period, where its effects appear to be negligible.

**Effects of Basolateral Genistein on Basolateral CO\(_2\) Dependence of \(J_{HCO_3}\) and \(J_V\)**

To test the hypothesis that the PT’s CO\(_2\)-sensing mechanism may require a RTK or an sTK, we examined the effects of basolateral genistein (1, 24) on \(J_{HCO_3}\) and \(J_V\). We used OEE solutions to vary basolateral [CO\(_2\)] from 0 to 20%, while keeping [HCO\(_3\)]\(_{BL}\) fixed at 22 mM and pH\(_{BL}\) fixed at 7.40.

In the first group of experiments, we examined effect of basolateral 7 \(\mu\)M genistein with equilibrated 5% CO\(_2/22\) mM HCO\(_3\) in both the lumen (solution 2) and the bath (solution 4). During the first collection period, no drug was present (\(\bigcirc\) in Fig. 2). During the second collection period, we added 7 \(\mu\)M genistein to solution 4 (\(\nabla\) in Fig. 2). Although basolateral 7 \(\mu\)M genistein reduced the mean \(J_{HCO_3}\) by ~25%, from 55 ± 3 to 41 ± 7 pmol·min\(^{-1}\)·mm\(^{-1}\) (\(n = 4\) paired experiments), the difference was not statistically significant (\(P = 0.07\), 2-tailed t-test, paired). Then, we increased the concentration of basolateral genistein to 17.5 \(\mu\)M and repeated the above protocol in six paired experiments. Basolateral 17.5 \(\mu\)M genistein reduced the mean \(J_{HCO_3}\) by ~45%, from 55 ± 3 to 30 ± 6 pmol·min\(^{-1}\)·mm\(^{-1}\) in analyzing the effects of basolateral 17.5 \(\mu\)M genistein in basolateral 5% CO\(_2\), we employed a one-way ANOVA for three groups: 1) control data (\(\bigcirc\) in Fig. 2A), 2) 7 \(\mu\)M genistein (\(\nabla\) in Fig. 2A), and 3) 17.5 \(\mu\)M genistein (filled pentagon in Fig. 2A). The overall \(P\) value was...
Dunnett’s multiple comparison shows that although the effect of basolateral 7 μM genistein on \( J_{\text{HCO}_3} \), was not statistically significant \( (P = 0.48, n = 4) \), the effect of basolateral 17.5 μM genistein did not significantly affect \( J_{\text{HCO}_3} \) which changed \( J_{\text{HCO}_3} \), from 28 ± 4 to 19 ± 2 pmol·min⁻¹·mm⁻¹ \( (P = 0.072, \text{2-tailed t-test, paired}) \). In contrast, at a \([\text{CO}_2]_{\text{BL}}\) of 20%, 17.5 μM genistein significantly reduced \( J_{\text{HCO}_3} \), from 72 ± 2 to 29 ± 2 pmol·min⁻¹·mm⁻¹ \( (P = 0.000007, \text{2-tailed t-test, paired}) \). An ANOVA shows that, in the presence of 17.5 μM genistein, the \( J_{\text{HCO}_3} \) data at \([\text{CO}_2]_{\text{BL}}\) values of 0, 5, and 20% \( (i.e., \text{the 3 filled pentagons in Fig. 2A}) \) are not statistically significant \( (\text{overall P value was} 0.13) \); Dunnett’s multiple comparison shows \( P = 0.11 \) at \([\text{CO}_2]_{\text{BL}}\) levels of 0% and \( P = 0.96 \) at \([\text{CO}_2]_{\text{BL}}\) levels of 20%. Thus basolateral 17.5 μM genistein eliminates the stimulatory effect produced by basolateral \([\text{CO}_2]_{\text{BL}}\) in the range 0–20%.

As far as \( J_V \) is concerned, at a \([\text{CO}_2]_{\text{BL}}\) of 5%, the overall \( P \) value in a one-way ANOVA was 0.28, and Dunnett’s multiple comparison indicates that, relative to the control condition, neither basolateral 7 μM genistein \( (\text{filled pentagon in Fig. 2B}, P = 0.66) \) nor basolateral 17.5 μM genistein \( (\text{filled pentagon in Fig. 2B}, P = 0.45) \) had a significant effect on \( J_V \). In paired, two-tailed \( t \)-tests, the effects of basolateral 17.5 μM genistein on \( J_V \) were not statistically significant at \([\text{CO}_2]_{\text{BL}}\) values of 0% \( (P = 0.26) \) or 20% \( (P = 0.80) \).

**Effects of Basolateral PP2 on Basolateral \([\text{CO}_2]_{\text{BL}}\) Dependence of \( J_{\text{HCO}_3} \) and \( J_V \)**

The genistein data suggest that the transduction of the basolateral \([\text{CO}_2]_{\text{BL}}\) signal to an increase in \( J_{\text{HCO}_3} \) requires the activity of tyrosine kinase. Our next step was to examine the effect of PP2, a potent and relatively specific inhibitor of the Src family of sTKs \((26, 53)\), on the basolateral \([\text{CO}_2]_{\text{BL}}\) dependence of \( J_{\text{HCO}_3} \) (Fig. 3A). The protocol for this and the remaining series of experiments was somewhat different from that in Fig. 2. As before, we perfused the lumen with \( \text{solution 2} \) throughout the entire experiment. However, during the first collection period shown in Fig. 3, we always perfused the bath with \( \text{solution 4} \) (equilibrated 5% \([\text{CO}_2]_{\text{BL}}=22\text{mM HCO}_3^-\)) without any inhibitor \( (\text{at} [\text{CO}_2]_{\text{BL}} = 5\%) \). During the second collection period, we perfused the bath with \( \text{solution 5} \) \( (i.e., 0\% \text{ CO}_2) \) ± inhibitor, 4 \( (i.e., 5\% \text{ CO}_2) \) ± inhibitor, or 6 \( (i.e., 20\% \text{ CO}_2) \) ± inhibitor. The control \( (i.e., \text{drug-free}) \) data at \([\text{CO}_2]_{\text{BL}} = 0\% \) are from an earlier study \((61)\), as are 13 of the control points at \([\text{CO}_2]_{\text{BL}} = \text{5\%} \) \((61)\). The 5% control data are augmented by 59 points from the current study. Finally, seven of the control \( (i.e., \text{drug-free}) \) points at \([\text{CO}_2]_{\text{BL}} = \text{20\%} \) are from an earlier study \((61)\), augmented by six DMSO points from Fig.
A one-way ANOVA to analyze all of the data at 0% CO₂. In Figs. 3 and 4, we applied a one-way ANOVA to analyze all of the data at 5% and again at 20% CO₂. Similarly, we applied a one-way ANOVA to analyze all of the data at 5% and again at 20% CO₂ during the first collection periods and solutions 5, 4, 6 plus 7 or 17.5 μM genistein during the second collection periods. Values are means ± SE, with nos. of tubules in parentheses. At 0 and 20% CO₂, the statistical comparisons between ○ and filled pentagons at the same [CO₂]BL were made using a paired 2-tailed t-test (**P < 0.01). At 5% CO₂, the statistical comparison among ○, □, and the filled pentagon at the same [CO₂]BL was made using a 1-way ANOVA for 3 groups; Dunnett’s multiple comparison indicates that the only significant difference was between ○ and filled pentagon.

The J_HCO₃ data obtained in the presence of basolateral 250 nM PP2, 50-fold greater than the published in vitro Kᵢ (26), at [CO₂]BL levels of 0, 5, or 20% at a fixed [HCO₃]BL of 22 mM and a fixed pHBL of 7.4, are summarized in Fig. 3A. Because we used the same control data in our statistical analysis of the J_HCO₃ (or J_V) data in Figs. 3 and 4, we applied a one-way ANOVA to analyze all of the data at 0% CO₂ in Figs. 3 and 4. Similarly, we applied a one-way ANOVA to analyze all of the data at 5% and again at 20% CO₂ in Figs. 3 and 4. The overall P value for three J_HCO₃ groups was 0.87 for [CO₂]BL = 0% and was 0.018 for [CO₂]BL = 5%. For [CO₂]BL = 20%, the overall P value for five J_HCO₃ groups was <0.0001. For Fig. 3A, Dunnett’s multiple comparison indicates that basolateral 250 nM PP2 had no significant effect on J_HCO₃ at 0, 5 (P = 0.96), or 20% CO₂ (P = 0.48). Even when we increased its concentration eightfold to 2 μM, PP2 did not have a significant effect (P = 0.65) on J_HCO₃ at a [CO₂]BL of 20% (△ in Fig. 3A).

We analyzed the J_V data in the same way as the J_HCO₃ data described above. For the ANOVA, the overall P value for three J_V groups was 0.34 for [CO₂]BL = 0% and was 0.89 for [CO₂]BL = 5%. For [CO₂]BL = 20%, the overall P value for five J_V groups was 0.78. Compared with the control situation with no added inhibitor (○ in Fig. 3B), neither basolateral 250 nM PP2 (△ in Fig. 3B) nor 2 μM PP2 (▲ in Fig. 3B) had a significant effect on J_V at any value of [CO₂]BL.

Our data suggest that the J_HCO₃ response of the rabbit S2 PT to basolateral CO₂ does not involve Src. PP2 is also inhibiting the Bcr-Abl fusion protein (53), which forms as the Philadelphia 1C in the present study. As described in METHODS, the values of J_HCO₃ (or J_V) in the second collection period were normalized to the mean J_HCO₃ (or J_V) value computed from 72 experiments during the first collection period.

The J_HCO₃ data obtained in the presence of basolateral 250 nM PP2, 50-fold greater than the published in vitro Kᵢ (26), at [CO₂]BL of 0, 5, or 20% at a fixed [HCO₃]BL of 22 mM and a fixed pHBL of 7.4, are summarized in Fig. 3A. Because we used the same control data in our statistical analysis of the J_HCO₃ (or J_V) data in Figs. 3 and 4, we applied a one-way ANOVA to analyze all of the data at 0% CO₂ in Figs. 3 and 4. Similarly, we applied a one-way ANOVA to analyze all of the data at 5% and again at 20% CO₂ in Figs. 3 and 4. The overall P value for three J_HCO₃ groups was 0.87 for [CO₂]BL = 0% and was 0.018 for [CO₂]BL = 5%. For [CO₂]BL = 20%, the overall P value for five J_HCO₃ groups was <0.0001. For Fig. 3A, Dunnett’s multiple comparison indicates that basolateral 250 nM PP2 had no significant effect on J_HCO₃ at 0, 5 (P = 0.96), or 20% CO₂ (P = 0.48). Even when we increased its concentration eightfold to 2 μM, PP2 did not have a significant effect (P = 0.65) on J_HCO₃ at a [CO₂]BL of 20% (△ in Fig. 3A).

We analyzed the J_V data in the same way as the J_HCO₃ data described above. For the ANOVA, the overall P value for three J_V groups was 0.34 for [CO₂]BL = 0% and was 0.89 for [CO₂]BL = 5%. For [CO₂]BL = 20%, the overall P value for five J_V groups was 0.78. Compared with the control situation with no added inhibitor (○ in Fig. 3B), neither basolateral 250 nM PP2 (△ in Fig. 3B) nor 2 μM PP2 (▲ in Fig. 3B) had a significant effect on J_V at any value of [CO₂]BL.
chromosome creates a fusion protein of Bcr and Abl (23), which is an sTK, and the Kit family (53) of RTKs. Thus our data also make it unlikely that these kinases are involved in transducing the CO₂ signal.

Effects of Basolateral PD168393 and BPIQ-I on the Basal CO₂ dependence of J

We next examined the effect of PD168393, which is a high-affinity inhibitor (K_i ≈ 0.7 nM) (19) of the erbB family of RTKs. The drug covalently reacts with a specific cysteine in the ATP binding pocket (see Table 2) and is thought to be rather specific. Our protocol was the same as in Fig. 3, and the control data in Fig. 4A (○) are the same as those presented in Fig. 3A. The dependence of J_HCO₃ on [CO₂]BL in the presence of basolateral 35 nM PD168393 is summarized in Fig. 4A (■). The statistical analysis of these J_HCO₃ data was part of the same J_HIKO ANOVA that we used to assess the PP2 data in Fig. 3A. Compared with the control condition, PD168393 did not significantly affect J_HCO₃ at [CO₂]BL of 0% (P = 0.998), according to Dunnett’s multiple comparison. However, at a [CO₂]BL of 5%, the drug significantly decreased J_HCO₃ from 64 ± 4 to 37 ± 3 pmol·min⁻¹·mm⁻¹ (P = 0.011). Moreover, at a [CO₂]BL of 20%, PD168393 significantly decreased J_HCO₃ from 94 ± 2 to 23 ± 3 pmol·min⁻¹·mm⁻¹ (P < 0.0001). Thus basolateral 35 nM PD168393 eliminates the PT’s J_HCO₃ response to basolateral CO₂ in the range 0–20%.

To further examine the possibility that an erbB or a related tyrosine kinase might be involved in the CO₂ signal-transduction cascade, we examined the effect of a second high-affinity inhibitor of the erbB family, BPIQ-I, which acts by competing with ATP in the binding pocket (K_i ≈ 25 pM) (44). At the single [CO₂]BL of 20%, basolateral 10 nM BPIQ-I (■ in Fig. 4A) significantly decreased J_HCO₃ from 94 ± 2 to 36 ± 6 pmol·min⁻¹·mm⁻¹ (P < 0.0001).

The statistical analysis of the J_V data for PD168393 and BPIQ-I was also part of the same J_HCO₃ ANOVA that we used to assess the PP2 data in Fig. 3B. Compared with the control condition with no added inhibitors (○ in Fig. 4B), neither PD168393 (■) nor 10 nM BPIQ-I (■) produced a significant effect on J_V at any level of [CO₂]BL according to Dunnett’s multiple comparison.

Table 2. Tyrosine kinases with a conserved cysteine residue in the ATP-binding pocket

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>First residue</th>
<th>Last residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>erbB1 (EGFR, HER1)</td>
<td>RTK</td>
<td>768 L M P F G C L L D Y V</td>
<td>778</td>
</tr>
<tr>
<td>erbB2 (HER2)</td>
<td>RTK</td>
<td>779 L M P Y G L C L L H D V</td>
<td>789</td>
</tr>
<tr>
<td>erbB3</td>
<td>RTK</td>
<td>789 Y L P L G S L L E D H V</td>
<td>799</td>
</tr>
<tr>
<td>erbB4</td>
<td>RTK</td>
<td>797 L M P H G C L L E Y V</td>
<td>807</td>
</tr>
<tr>
<td>TEK (Tie2)</td>
<td>RTK</td>
<td>444 F M E R G C L L N F L</td>
<td>454</td>
</tr>
<tr>
<td>EphB3 (Heck2)</td>
<td>RTK</td>
<td>713 F M E N C A L L S F</td>
<td>723</td>
</tr>
<tr>
<td>ITK (Tec family)</td>
<td>sTK</td>
<td>437 F M E H G C L S D Y L</td>
<td>447</td>
</tr>
<tr>
<td>BLK (Src family)</td>
<td>sTK</td>
<td>314 Y M A R G C L L D F L</td>
<td>324</td>
</tr>
<tr>
<td>JAK3 (Janus family)</td>
<td>sTK</td>
<td>904 Y L P S G C L R D F L</td>
<td>914</td>
</tr>
</tbody>
</table>

These sequences were identified in a search of human tyrosine kinases at the following Web site: http://pkr.sdsc.edu/html/pk_classification/pk_catalytic/pk_hanks_seq_align_long.html. RTK, receptor tyrosine kinase; sTK, soluble tyrosine kinase. First residue and last residue refer to the end points of the putative ATP-binding pocket, the single-letter sequences of which are provided.

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DISCUSSION

Use of Out-of-Equilibrium Solutions

Out-of-equilibrium solutions are a powerful tool for performing the experiments outlined in this paper. However, carbonic anhydrase (CA) activity at the extracellular surface of the basolateral membrane would tend to cause a local degradation of the OOE state. Blockade of this activity would independently reduce \( J_{\text{HCO}_3} \) and thus be impractical. On the other hand, as we raise \([\text{CO}_2]_\text{BL}\) from 0 to 20%, we clearly see major increases in \( J_{\text{HCO}_3} \) (e.g., see Fig. 3). Moreover, as shown previously, raising \([\text{HCO}_3]_\text{BL}\) from 0 to 44 mM causes major decreases in \( J_{\text{HCO}_3} \). Thus any degradation of the OOE state by basolateral CAs must be minor. Nevertheless, to the extent that some degradation of the OOE state does occur, our data underestimate the true magnitude of the response of the tubule to isolated changes in \([\text{CO}_2]_\text{BL}\). If a drug added to the PT inhibited all CAs, the result would be an enhancement of the OOE state but a decrease in \( J_{\text{HCO}_3} \). We are unaware of any reports that the drugs used in the present study inhibit CAs.

Inhibitor Specificity

Our previous study (61) demonstrated that PT cells somehow sense acute decreases in \([\text{CO}_2]_\text{BL}\) and respond by lowering \( J_{\text{HCO}_3} \). In addition, the PT cells change the reabsorption of other solutes (\( J_{\text{Other}} \)) in a direction opposite to that of \( J_{\text{HCO}_3} \), thereby minimizing changes in \( J_V \). A key question is how PT cells transduce the \( \text{CO}_2 \) signal. We hypothesized that an early element in the \( \text{CO}_2 \) signal-transduction cascade in PT cells is either a receptor tyrosine kinase or a receptor-associated (or soluble) tyrosine kinase.

Genistein is a broad-spectrum tyrosine-kinase inhibitor (24) that blocks both sTKs (e.g., Src family) and RTKs (e.g., erbB family) but exhibits no nonspecific inhibition with other kinases (PKA, PKC) at the concentration we used (24). As shown in Fig. 2A, genistein eliminates the ability of the PT to respond to increases in \([\text{CO}_2]_\text{BL}\). These data are consistent with the hypothesis that the \( \text{CO}_2 \) signal-transduction pathway in the PT cell requires either an sTK or an RTK.

PP2 was developed as a specific inhibitor of the Src family of sTKs (4), but it also inhibits Bcr-Abl (53), which is an sTK, as well as Kit (53) which is an RTK. Using PTs or PT-like cell lines, others have found that 1–10 \( \mu \text{M} \) PP2 can significantly reduce a variety of physiological responses (2, 31, 43, 56). As shown in Fig. 3A, even at a basolateral concentration that is 400-fold greater than its published \( K_i \), basolateral PP2 can significantly reduce the \( \text{CO}_2 \)-stimulated increase in \( J_{\text{HCO}_3} \).

PD168393 is a high-affinity, irreversible inhibitor of members of the erbB family of RTKs. It acts by alkylating a cysteine residue [i.e., Cys-773 in human EGFR receptor (EGFR)] in the ATP binding pocket (19). Figure 4A shows that, at a concentration 50-fold greater than its published \( K_i \), basolateral PD168393 totally eliminates the response to changes in \([\text{CO}_2]_\text{BL}\). Although many authors regard PD168393 as a specific erbB inhibitor, it is in fact impossible to know how specific it is without assaying all tyrosine kinases. A search of the human tyrosine kinases reveals a total of eight human tyrosine kinases that have a cysteine residue at a position comparable to Cys-773 in the ATP binding pocket of erbB1 (Table 2). In principle, each of these tyrosine kinases, five RTKs and three sTKs, is a potential target of PD168393. Of these, erbB1 and erbB2 are known to be present in the PT (33, 35), and mRNA transcripts for erbB4 (as well as erbB3) have been reported for the whole kidney (38, 39). TEK or Tie2 (17), the angiopoietin receptor (55), is expressed almost exclusively in endothelial cells as well as certain cancer cells. EphB3 or Hek2 (9), a receptor for the ephrin-B family, plays a key role in neural development but is also expressed in the kidney. ITK plays an important role in T cell activation (28). BLK, which is primarily expressed in hematopoietic cells (27), is a member of the Src family and thus should have been inhibited by PP2. Finally, JAK3 is expressed primarily in hematopoietic cells, where it interacts with cytokine receptors and plays a role in development and cell activation (54). Thus of the proteins listed in Table 2, erbB1, 2 and 4 as well as EphB3 are prime candidates as targets of PD168393 in our experiments.

BPIQ-I is also a high-affinity inhibitor of EGFR (i.e., erbB1) as well as erbB2 and erbB4. It acts by competing with ATP (44). At a concentration 50-fold greater than its published \( K_i \), basolateral BPIQ-I decreased the \( \text{CO}_2 \)-sensitive component of \( J_{\text{HCO}_3} \) by about two-thirds (i.e., decreased total \( J_{\text{HCO}_3} \) by \( \sim 40\% \)) at a \([\text{CO}_2]_\text{BL}\) of 20% (data not shown). At a concentration 400-fold greater than its published \( K_i \), BPIQ-I blocked virtually 100% of the \( \text{CO}_2 \)-sensitive component of \( J_{\text{HCO}_3} \).

Our observation that both PD-168393 and BPIQ-I block the response to \( \text{CO}_2 \) increases the odds that, in our experiments, both drugs produce the observed effect by acting on a member of the erbB family.

Model

Previous work from our laboratory showed that simultaneously adding \( \text{CO}_2 \) and \( \text{HCO}_3^- \) to the basolateral, but not the luminal, side of the S3 segment of the rabbit PT triggers a fourfold increase in total \( \text{H}^+ \) extrusion (14). The results of our most recent study (61) indicate that this increase is due to basolateral \( \text{CO}_2 \). The data in the present study suggest that the tubule’s response to altered \([\text{CO}_2]_\text{BL}\) requires one of a small...
group of tyrosine kinases (see Table 2). Figure 5 summarizes several potential mechanisms by which the PT cell might transduce the CO2 signal. One possibility (Fig. 5, 1) is that an RTK is simply permissive for the effect of CO2 on JHCO3. Thus, blocking the RTK would eliminate the response to CO2 even though the RTK would not be downstream from the CO2 receptor. Second (Fig. 5, 2), a CO2 receptor could activate an PP2-insensitive sTK, which would then raise JHCO3. Third (Fig. 5, 3), a CO2 receptor could transactivate an RTK (57), which would, in turn, raise JHCO3. In a variant of this third pathway, the CO2 receptor could trigger a metalloproteinase to release an EGF-like proligand, which would, in turn, activate EGFR (40). In yet another variant of this third pathway, CO2 could block the ability of a receptor protein tyrosine phosphatase to transactivate an sTK or RTK. Finally (Fig. 5, 4), the CO2 receptor could itself be an RTK. Clearly, additional experiments would be required to establish the molecular identity of the tyrosine kinase involved in the response to CO2.

In the PT, the CO2 sensor triggers not only a compensatory rise in JHCO3 but also a decrease in the reabsorption of other solutes, thereby stabilizing JV (61). Thus we would predict that the PT cell can modulate acid-base transport without secondary effects on blood pressure. In Fig. 6A (○ connected by solid lines) are the control JHCO3 data (i.e., obtained in the absence of any drugs) in the present study, replotted from Fig. 3A. Also shown are the JHCO3 values that we computed from the corresponding JHCO3 and JV data (○ connected by dashed lines).

In Fig. 6B are the JHCO3 data obtained in the presence of 250 mM PP2, replotted from Fig. 3A (● connected by solid lines). Also shown are the JHCO3 values that we computed from the corresponding JHCO3 and JV data (● connected by dashed lines) and the data for 2,000 mM PP2 (adjacent ▲). For both control and PP2, the JHCO3 values at 0% are significantly greater than at 5% CO2, whereas those at 20% are not significantly different from those at 5%. Thus the trend is for JHCO3 to fall as [CO2]BL increases.

Finally, in Fig. 6C are the JHCO3 data obtained in the presence of 35 nM PD168393 (■ connected by solid lines) and 10 nM BPIQ-I (adjacent ●), respectively, replotted from Fig. 4A. Also shown are the corresponding JHCO3 values (■ connected by dashed lines and the adjacent ●). For PD168393, the JHCO3 value at 0% CO2 is not significantly different from that at 5%, whereas the JHCO3 value at 20% CO2 is significantly different from that at 5%. For BPIQ-I, the JHCO3 value at 20% CO2 (●) is similar to the PD168393 data at 0 and 5% CO2. Thus with blockade of the CO2 response, the trend is for JHCO3 to be relatively stable as [CO2]BL rises. In other words, the effective tyrosine kinase inhibitors not only blocked the JHCO3 response to CO2, they also tended to block the JHCO3 response. These results are consistent with the hypothesis that the tyrosine kinase targeted by PD168393 and BPIQ-I are relatively early in the signal-transduction cascade, that is, before the bifurcation to stimulate JHCO3 and inhibit JOther.

It is interesting to speculate that CO2 sensors similar to that in the PT may be present in other cells that perform large amounts of acid-base transport, as in the choroid plexus, ciliary body, stomach, pancreatic ducts, intestines, and male genital tract. Thus understanding how the PT transduces the CO2 signal may provide important clues about how such cells regulate acid-base transport.

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GRANTS

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