Modulation of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Activity and Genistein Binding by Cytosolic pH\textsuperscript{[S]}

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Potentiators are molecules that increase the activity of the cystic fibrosis transmembrane conductance regulator (CFTR). Some potentiators can also inhibit CFTR at higher concentrations. The activating binding site is thought to be located at the interface of the dimer formed by the two nucleotide-binding domains. We have hypothesized that if binding of potentiators involves titratable residues forming salt bridges, then modifications of cytosolic pH (pH\textsubscript{i}) would alter the binding affinity. Here, we analyzed the effect of pH\textsubscript{i} on CFTR activation and on the binding of genistein, a well known CFTR potentiator. We found that pH\textsubscript{i} does modify CFTR maximum current (I\textsubscript{m}) and half-activation concentration (K\textsubscript{a}): I\textsubscript{m} = 127.7, 185.5, and 231.8 \mu A/cm\textsuperscript{2} and K\textsubscript{a} = 32.7, 56.6 and 71.9 \mu M at pH 6, 7.35, and 8, respectively. We also found that the genistein apparent dissociation constant for activation (K\textsubscript{a}) increased at alkaline pH\textsubscript{i}, near cysteine pK\textsubscript{a} (K\textsubscript{a} = 1.83, 1.81 and 4.99 \mu M at pH 6, 7.35, and 8, respectively), suggesting the involvement of cysteines in the binding site. Mutations of cysteine residues predicted to be within (Cys-491) or outside (Cys-1344) the potentiator-binding site showed that Cys-491 is responsible for the sensitivity of potentiator binding to alkaline pH\textsubscript{i}. Effects of pH\textsubscript{i} on inhibition by high genistein doses were also analyzed. Our results extend previous data about multiple effects of pH\textsubscript{i} on CFTR activity and demonstrate that binding of potentiators involves salt bridge formation with amino acids of nucleotide-binding domain 1.

The cystic fibrosis transmembrane conductance regulator (CFTR)\textsuperscript{2} is a Cl\textsuperscript{-} channel expressed principally in the apical membrane of epithelial cells, where it forms a pathway for anion movement and controls the rate of fluid flow across epithelia (1, 2). The crucial role of CFTR in transepithelial fluid and electrolyte transport is dramatically highlighted by the consequences of the absence of functional CFTR in cystic fibrosis (CF). CF, caused by mutations in the corresponding gene, is a life-threatening disease that affects several epithelia, in particular the airways, intestine, exocrine pancreas, and reproductive tract.

The CFTR protein is formed by two homologous halves, each composed of a membrane-spanning domain containing six transmembrane motifs followed by a nucleotide-binding domain (NBD). The two protein halves are linked by a unique regulatory domain, which contains multiple consensus sites for protein kinase A- and C-dependent phosphorylation, which is a prerequisite for channel opening. Following phosphorylation, ATP binding and hydrolysis at the NBDs permit opening and closing of the CFTR channel (3, 4). It is believed that ATP binding in the interface between the two NBDs, forming a head-to-tail conformation, produces a structural change that is transmitted to the transmembrane domain to gate the channel (5). The phosphorylation state modulates allosterically CFTR gating by ATP.

In the last few years, the idea of a protein-directed pharmacologic therapy for CF has received considerable attention. Rational drug design and high-throughput screening approaches have permitted the identification of a large number of agents with very different chemical structure that are able to potentiate the activity of those mutant CFTR channels that are present in the plasma membrane bearing a gating defect (6–17). These compounds have been termed potentiators because they can only enhance the activity of previously phosphorylated CFTR. By themselves, they are unable to activate the channel. It is worth noting that besides increasing the channel function, at high concentrations, most potentiators inhibit CFTR activity, indicating that they probably bind to at least two sites, an activating site and an inhibitory site (18–20). Several observations suggest that, to potentiate CFTR, most compounds act by binding probably to a common activating site in the CFTR protein. First, CFTR carrying NBD mutations, such as G551D and G1349D, exhibit a lower affinity for several potentiators, indicating that mutations are close to the binding site (21–23). Second, competition has been described between genistein and benzimidazolones (24), between 7,8-benzoflavones and benzimidazolones (25, 26), and between genistein and capsaicin (6). Hence, despite important structural differences between potentiators of different chemical classes, most of them probably bind to the same site at the NBDs to favor the chloride-permeable state of the protein. Third, our previous measurements of the binding free energy of 18 potentiators for WT-CFTR and mutants on a NBD molecular model pointed to the...
**PH Modulates CFTR and Genistein-CFTR Interaction**

NBDs as the regions containing the activating binding site. In that study, a good correlation was found between experimental located and theoretical binding free energy for a site placed in a cavity within NBD1 and in contact with the surface of NBD2 (12). Finally, mutations produced on residues assumed to form part of this site were found to modify the affinity for potentiators (20).

In nucleotide-bound crystal structures of bacterial ATP-binding cassette transporters, interactions between NBDs involve van der Waals contacts and hydrogen bonds (27, 28). In a CFTR NBD molecular model, interactions between potentiators and the residues forming the cavity where potentiators bind also seem to require hydrogen bond formation (12). In particular, the putative binding site seems to involve two protonable residues, Cys-491 and His-1348.

Here, we analyzed the contribution of salt bridge formation to the binding of potentiators by modifying the pH of the intracellular solution. We confirmed and extended previous work showing that cytosolic pH affects CFTR activity in multiple ways (29–31). In addition, we found that intracellular pH does modify the binding affinity of genistein, a well known CFTR potentiator. We observed that only alkaline intracellular medium (near cysteine pK) reduces potentiator binding affinity, suggesting the involvement of cysteines (but not histidines) in the binding site. Mutations of cysteine residues introduced in WT-CFTR indicated that Cys-491 (within the putative binding site for potentiators), but not Cys-1344 (outside this site), abolishes the effect of alkaline pH on potentiator binding.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Fisher rat thyroid (FRT) cells stably transfected with WT-CFTR or with CFTR carrying the C491A or C1344A mutation were grown as described previously (23). For electrophysiology experiments, FRT cells were seeded on permeable supports at a density of 500,000 cells/cm² and maintained at 37 °C in 5% CO₂ and 95% air. Experiments were done 8–12 days after seeding.

**Short-circuit Current Recordings**—Apical membrane current (I_{apical}) was measured from FRT epithelia as described previously (22). The basolateral membrane was permeabilized with 250 μg/ml amphotericin B, and a transepithelial Cl⁻ gradient was applied to provide the driving force for Cl⁻ secretion. The basolateral solution contained 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM NaHEPES, and 10 mM glucose. The apical solution was modified by replacing half of the NaCl with sodium gluconate and increasing the CaCl₂ to 2 mM. The hemichambers were connected to a DVC-1000 voltage clamp (World Precision Instruments, Inc., Sarasota, FL) via Ag/AgCl electrodes and agar bridges. Measurements were done at 37 °C. Data were analyzed as described previously (20). See supplemental “Experimental Procedures” for details.

**Statistics**—All data are shown as raw data or as means ± S.E. Statistical significance was established using Student’s two-tailed t test for unpaired data.

**RESULTS**

**CFTR Cl⁻ Currents Are Modulated by pH**—It was first established that it is possible to control the pH in FRT cells permeabilized with basolateral amphotericin B by modifying the pH of the basolateral solution (see supplemental “Experimental Procedures” and “Results”). Next, the effect of pH changes on CFTR-mediated apical Cl⁻ currents (I_{apical}) was measured. We observed that, after the addition of small volumes of NaOH, CFTR current increased (supplemental Fig. S1). These preliminary experiments indicated that CFTR activity was indeed dependent on pH. To obtain a more precise analysis of this relationship, we next studied the effect of pH on the apparent equilibrium constant of 8-(4-chlorophenylthio)-cAMP (CPT-cAMP).

In the presence of increasing concentrations of CPT-cAMP, FRT epithelia showed dose-dependent increases in I_{apical} at three pH values, i.e. 6 (near histidine pK), 7.35, and 8 (near cysteine pK) (Fig. 1, A and B). Dose-response relationships were fitted to Equation 1 (Table 1).

\[
\frac{I}{I_m} = \frac{[c]}{(K_d + [c])}
\]  
(Eq. 1)

We found that, at pH 6, the maximum current (I_m) was lower and the apparent dissociation constant (K_d) was reduced with respect to pH 7.35. In contrast, the K_d and the maximum current response to CPT-cAMP at pH 8, although higher, were not statistically different from the response at pH 7.35.

**Potentiation of WT-CFTR by Genistein Is Reduced at pH 8 but Unchanged at pH 6**—We hypothesized that if cysteines or histidines are involved in the binding of potentiators, then changes in pH from 7.35 to more alkaline or more acidic pH values would alter the apparent affinity (K_a) of genistein for the activating effect. The interaction of genistein with WT-CFTR at different pH values was studied on partially phosphorylated CFTR channels. As observed previously, at pH 7.35 genistein, first produced a dose-dependent I_{apical} increase, followed by inhibition at higher concentrations. As illustrated in Fig. 1C, a similar behavior was observed at the other two pH values, although with some quantitative differences. Dose-response relationships (Fig. 1D) were fitted to Equation 2 (see Table 2 and supplemental “Experimental Procedures” for a complete description).

\[
I = \left( [c] \left( f_A [g] + K_a K_i \right) / (K_d K_b K_i + [c] ([g] + [g] K_i + K_d K_b K_i) \right)
\]  
(Eq. 2)

We found that f_A, the amount of extra current that genistein activates in addition to the maximum current reachable with CPT-CAMP alone, behaves as I_m, the maximum current obtained with CPT-CAMP, in that both increased with pH. In particular, at pH 8, f_A was significantly higher than at pH 7.35. Regarding the affinity for the activating site, the genistein K_a was ~3-fold higher at pH 8 than at pH 7.35. In contrast, the K_a at pH 6 was not different from the K_a at pH 7.35. These results indicate that a cysteine (not a histidine) is involved in the activating effect of CFTR potentiators. The affinity for the inhibitory site (K_i) was independent of the intracellular pH.
To validate the role of this amino acid in docking to make contacts with CFTR potentiators, we identified Cys-491 (12). To determine the modulation by pH 8, we mutated this cysteine to alanine. As a control, we mutated to alanine also Cys-1344, a NBD2 cysteine located outside the putative binding site for potentiators. Both C491A and C1344A were produced on a wild-type CFTR background. Stably transfected clones were obtained on FRT cells, and the level of protein expression was detected by Western blotting (Fig. 2A and supplemental “Experimental Procedures”).

Dose-response relationships to CPT-cAMP showed that the maximum current attainable from mutant C491A was 5–10-fold lower than that from WT-CFTR at all pH values (Table 1). This is not surprising because the clone expressed less CFTR protein than WT-CFTR (Fig. 2A). In addition, it has been shown that mutating this cysteine residue leads CFTR to gate with shorter openings and to open almost exclusively to a small subconductance state of 3 pico Siemens compared with the 9-pico Siemen full conductance of WT-CFTR (32). Nevertheless, the sensitivity to acidic pH was conserved (Table 1). As for the WT protein, the $I_m$ and $K_a$ were significantly smaller at pH 6 than at pH 7.35 and 8.

The response to genistein of C491A-CFTR was bimodal, as in the WT channel, with dose-dependent current increase at low concentrations and inhibition when the genistein concentration was further raised (Fig. 2, B and C). Fitting the dose-response relationship to Equation 2 (Table 2) showed that, although the $K_a$ was higher for this mutant compared with WT-CFTR, it remained constant in the pH range studied here. In fact, removal of Cys-491 determined a loss of the sensitivity of $K_a$ to alkalinization that was present in the WT channel. The $K_a$ values at pH 6 and 7.35 were significantly smaller than for WT-CFTR, whereas at pH 8, the difference was not statistically significant. In other words, removal of Cys-491 made the K of the mutant protein pH-sensitive.

**Genistein Potentiation of C1344A-CFTR Maintains the Sensitivity to pH 8**—The cysteine mutant outside the putative binding site for potentiators, C1344A, showed a CPT-cAMP maximum current even smaller than C491A, but the equilibrium constant ($K_d$) was very close to the equilibrium constants of C491A and WT-CFTR (Table 1). Similar to the other two proteins, also C1344A-CFTR showed a higher apparent affinity (lower $K_d$) for CPT-cAMP at pH 6.


Regarding the effect of the potentiator, we found that the two phases of the genistein effect, activation at low and inhibition at higher doses, were preserved in this mutant (Fig. 3, A and B). The $K_i$ was found to be higher than that of the WT-CFTR, but in the WT channel, the $K_i$ of C1344A-CFTR increased when pH$_i$ was set at a value of 8. This indicates that, in contrast to what was found with C491A, the C1344A mutation did not modify the sensitivity of genistein binding to the activating site at alkaline pH$_i$ (Fig. 3C). As with C491A, the $K_i$ values at pH$_i$ 6 and 7.35 were significantly smaller than for WT-CFTR, whereas at pH 8, the $K_i$ did not change. Similarly to removal of Cys-491, also removal of Cys-1344 rendered the $K_i$ of the mutant protein pH-sensitive.

**DISCUSSION**

Recent data indicate that drugs able to recover Cl$^-$ channel function, like CFTR potentiators, will be of value in the treatment of CF patients. Potentiators may be useful to increase the activity of those mutant CFTR channels that are present in the plasma membrane carrying a gating defect, the so-called class III mutations (6–16). Potentiators might also be employed to augment the activity of channels delivered to the membrane by other treatments, like processing mutations rescued by correctors or WT-CFTR delivered through gene therapy. A recent report on phase II clinical trials using potentiator VX-770 in patients carrying at least one G551D allele (severe gating mutation) indicated that short-term treatment (2–4 weeks) improved nasal potential difference and lung function (clinicaltrials.gov/ct2/results?int=rn=%22VX-770%22). Therefore, a better insight into the mechanisms involved in the CFTR binding of potentiators may be important to understand how these substances function, may help to understand the malfunction of gating mutants, and could be useful to design better and more potent compounds.

A putative binding site for potentiators has been identified in the interface between NBD1 and NBD2 (12). The site seems to involve at least two protonable residues, Cys-491 and His-1348 (12). Binding of potentiators to NBDs probably relies on van der Waals forces and hydrogen bonds, as van der Waals contacts and salt bridges might be involved in the formation and stabilization of the NBD dimer during the gating cycle (27, 28). We hypothesized that modifications of surface partial charges of CFTR by pH titration would change the activity of CFTR as well as the binding of potentiators. To study the effect of pH on the binding of genistein, we first had to ana-

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**FIGURE 2. Western blot of cysteine mutants and response of C491A-CFTR to genistein.** A, Western blot showing CFTR protein expression in untransfected FRT cells (FRT-null) and in cells stably transfected with WT-, C491A-, and C1344A-CFTR. A mouse anti-CFTR monoclonal primary antibody (clone M3A7, Millipore) and a horseradish peroxidase-conjugated anti-mouse secondary antibody were used. Detection of the proteins was done using the chemiluminescent LiteAblot TURBO detection kit. The first lane contains molecular weight markers. The same amount of total lysate (30 µg) was loaded for WT-CFTR and null cells, whereas for the low-expression mutants, the protein load was increased to 80 µg. The mature CFTR band is indicated by an arrow. Correction for the amount of loaded protein indicated that expression of both mutant CFTR proteins was ~30-fold lower than that of WT-CFTR. B, representative traces showing the response of mutant C491A-CFTR to the application of genistein (indicated by arrows) after activating CFTR with 20 µM CPT-CAMP. Dashed lines under the curves indicate the zero current level.

**FIGURE 3. Dose-response relationship of C1344A-CFTR to genistein.** A, representative traces showing the response of mutant C1344A-CFTR to increasing doses of genistein (indicated by arrows). Dashed lines under the curves indicate the zero current level. B, normalized dose-response relationships in experiments on mutant C1344A at pH 6 (n = 4), 7.35 (n = 4), and 8 (n = 4). C, course of $K_i$ measured on WT-, C491A-, and C1344A-CFTR at different pH$_i$ values. Symbols are means ± S.E. of 4–16 different experiments. Data were normalized to the value at pH 7.35. The $K_i$ at pH 6 was very similar to the value at pH 7.35 for the three proteins. However, at pH 8, the $K_i$ for WT and C1344A-CFTR increased significantly (p < 0.05), whereas it remained unchanged for mutant C491A. D, molecular model of the NBDs based on the outward-facing conformation of Mormon et al. (36), showing NBD1 in pink, NBD2 in yellow, ATP molecules in green, and the two cysteine residues mutated here as blue balls. The residues that possibly interact with potentiators, i.e. Gly-551, Arg-553, Cys-491, and Pro-574 in NBD1 and Val-1293 in NBD2 (12, 20, 23), are also shown as balls.
lyze if and how the activation of CFTR by CPT-cAMP was altered by pH.

**CFTR Gating and Phosphorylation Levels Are Sensitive to pH** — Despite knowing that a number of successive steps are involved in CFTR activation by CPT-cAMP, including binding of cAMP to PKA, phosphorylation of several residues in the regulatory domain, ATP binding at the NBDs, and dephosphorylation by phosphatases, we used a hyperbolic function (Equation 1) to fit the current response to CPT-cAMP, treating the dose-response relationship as a first-order reaction. This allowed us to obtain a relationship between the CPT-cAMP level and CFTR activity, and the apparent $K_d$ linking CPT-cAMP and CFTR-mediated currents was used in the analysis of potentiator effects at different pH values.

We found that the activity of CFTR was sensitive to shifts in pH. In general, variations of cytosolic pH might modify CFTR activity through several mechanisms. First, H+ may titrate surface charges on the internal face of the membrane, which could change the local anionic concentration near the pore entry and therefore modify the channel current (34). Second, pH could affect kinase and phosphatase activities and the number of phosphate charges of phosphorylated CFTR (30). Third, titration of charges on the surface of NBDs could alter the interaction between NBDs during conformational changes of the dimer. Four, pH changes the ionization state of ATP (35).

Analysis of previous reports on the effects of pH on CFTR suggests that most of these mechanisms may be involved in the pH effect recorded here (29–31). We found that the maximum $I_{apical}$ was significantly smaller at acidic and higher at alkaline pH, compared with pH 7.35. These results are in agreement with those of Reddy et al. (30), who found similar pH dependence in sweat ducts and established that phosphorylation was prevented at acidic pH, and that basic pH prevented endogenous phosphatases from dephosphorylating CFTR. Nevertheless, these authors also found that some of the effects of pH were independent of phosphorylation and dephosphorylation of CFTR and probably involved an inhibitory effect of acidic pH on ATP interaction with CFTR.

Our data are also in agreement with single CFTR channel recordings in lipid bilayers, where reduction of cytosolic pH from 7.4 to 4.5 was found to decrease the open probability ($P_o$) of CFTR through a reduction of the mean open time (31). Results apparently contrasting with ours have been recently reported in a study about the effects of pH in excised membrane patches (29). The authors found that the $P_o$ of CFTR channels, but not the single channel amplitude nor the number of active channels under the pipette tip, was increased at pH 6.3 compared with pH 7.3 and that this effect was, at least in part, mediated by an augmented apparent affinity of MgATP for CFTR. In contrast, pH 8.3 was found to decrease the single channel amplitude and $P_o$. We have no explanation for these discrepancies, but, as mentioned above, in the apical membrane current measurements are included several steps that cannot be discriminated in detail and that might be modulated by pH in opposite directions. In this regard, it is interesting to note that, in this study, the equilibrium constant for CPT-cAMP of WT-CFTR was found to be higher at pH 6, indicating that the free energy needed to activate CFTR is lower at this pH.

In addition to pH-dependent changes in the equilibrium constant for CPT-cAMP of WT-CFTR, we found that mutating either of the two cysteine residues, Cys-491 or Cys-1344, leads to a further decrease in the equilibrium constant for CPT-cAMP at pH 6 (Table 1). These results suggest that some of the pH-dependent effects found here might be due to modification of amino acid residues in regions of the NBDs involved in the gating mechanism.

**Cys-491 Confers Sensitivity to pH 8 to the Genistein-Activating Site** — As mentioned above, two protonable residues, Cys-491 and His-1348, are present near the putative activating binding site for potentiators. However, analysis of WT currents showed that only alkalinization to pH 8, but not acidification to pH 6, caused the genistein dissociation constant for the activating site to change, indicating that only cysteines are involved in this effect. Consequently, we next mutated Cys-491 to alanine. As a control, we mutated to alanine also Cys-1344, a NBD2 cysteine situated outside the putative binding site for potentiators. Analysis of epithelium stably expressing these mutant CFTR proteins showed that, whereas C1344A-CFTR behaved as WT-CFTR, exhibiting reduced affinity for genistein at pH 8, the C491A mutation kept the same affinity for genistein at the three pH values (Fig. 3C and Table 2). These data support the hypothesis that Cys-491 is part of the genistein-binding site (Fig. 3D), as Gly-551, Arg-553, and Pro-574 in NBD1 and Val-1293 in NBD2 (12, 20). Because deprotonation of Cys-491 makes CFTR more difficult to potentiate by genistein, these results also suggest that protonated Cys-491 may act as hydrogen bond donor in the interaction with potentiators.

**Effects of pH on Genistein Binding to the Inhibitory Site** — The knowledge that we have about the inhibitory site is scarce. Although its location is still unknown, it is thought to be situated in the NBDs. This idea is supported by the inverse relationship that has been found between activation and inhibition by potentiators, which indicates that these sites are not completely independent from each other (20). The affinity of genistein for the inhibitory site is some orders of magnitude lower than that for the activating site (18–20). Here, we found that, at pH 7.35, the $K_i$ was $\sim 2 \mu M$, whereas the $K_i$ was $\sim 350 \mu M$. The low affinity of most potentiators for the inhibitory site hinders a better analysis of the inhibition phenomenon because potentiators are organic molecules with reduced solubility in water. Our results showed that the affinity of genistein for the inhibitory site was independent from pH on epithelia expressing WT-CFTR. Substitution of either Cys-491 or Cys-1344 with alanine resulted in an increased affinity for the inhibitory site at pH 6 (Table 2). This effect was more marked at pH 6 and for C1344A-CFTR ($K_i = 24 \mu M$). These results suggest that the inhibitory site is directly or indirectly related to the position of both cysteines, whose separation is $\sim 10 Å$ in the open state, as calculated on a recent molecular model of the complete CFTR protein (36).

**Effect of pH on Genistein** — One possibility that has to be taken into account is that pH might affect genistein directly and that small changes in the molecular conformation of the
pH Modulates CFTR and Genistein–CFTR Interaction

potentiator might be responsible for part of the effects found here. Genistein undergoes deprotonation reactions with three dissociation constants (pKᵄ = 7.2, 10, and 13.1), with only the lower being relevant for this study. Whereas at pH 6 genistein is expected to be almost completely protonated, at pH 7.35 and pH 8, part of it would lose one proton, probably 7-OH (37). Therefore, at these pH values, 57 or 54% of genistein, respectively, is expected to be monooanionic. The small protonation difference between pH 7.35 and 8 does not explain the affinity differences found here.

In conclusion, this work confirms and extends previous studies indicating that pH₇ has multiple effects on CFTR activity. These effects, which include modulation of the phosphorylation status and of CFTR gating, may determine a reduced function of the channel in acidic pH₇. Airway inflammation and infection are usually accompanied by acidification of the periciliary fluid and, as a consequence, also of the cytosol. We do not know how low the cytosolic pH could be under these pathological conditions. However, our data (Iₘ in Table 1) and those of Reddy et al. (see Fig. 4 in Ref. 30) indicate that there is a straight relationship between CFTR activity and pH₇ between pH 6 and 8. In this pH range, CFTR activity changes 2–4-fold. This suggests that even mild acidosis may reduce significantly CFTR function and worsen the activity changes 2–4-fold. This suggests that even mild acidosis may reduce significantly CFTR function and worsen the clinical conditions of CF patients. In addition, we have identified a cysteine (Cys-491) in the putative activating binding site for potentiators that undergoes redox-linked protonation. Under physiological conditions, Cys-491 may act as a hydrogen bond donor in the interaction with genistein. Under alkaline conditions and after deprotonation, the affinity of CFTR for genistein decreases, reducing the fraction of CFTR channel activity that may be recovered by using potentiators. Besides confirming the proposed binding site for potentiators, mostly involving amino acids from NBD1, our results indicate that binding to mutant CFTR and recovery of CFTR channel function by genistein, and probably by other potentiators, would not be hindered under acidic conditions.

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