Cytosolic Protein Concentration Is the Primary Volume Signal for Swelling-induced [K-Cl] Cotransport in Dog Red Cells

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ABSTRACT Chloride-dependent K transport ([K-Cl] cotransport) in dog red cells is activated by cell swelling. Whether the volume signal is generated by a change in cell configuration or by the dilution of some cytosolic constituent is not known. To differentiate between these two alternatives we prepared resealed ghosts that, compared with intact red cells, had the same surface area and similar hemoglobin concentration, but a greatly diminished volume. Swelling-induced [K-Cl] cotransport was activated in the ghosts at a volume (20 fl) well below the activation volume for intact cells (70 fl), but at a similar hemoglobin concentration (30–35 g dry solids per 100 g wet weight). Ghosts made to contain 40% albumin and 60% hemoglobin showed activation of [K-Cl] cotransport at a concentration of cell solids similar to intact cells or ghosts containing only hemoglobin. [K-Cl] cotransport in the resealed ghosts became quiescent at a dry solid concentration close to that at which shrinkage-induced Na/H exchange became activated. These results support the notion that the primary volume sensor in dog red cells is cytosolic protein concentration. We speculate that macromolecular crowding is the mechanism by which cells initiate responses to volume perturbation.

INTRODUCTION

Most animal cells respond to volume perturbations by altering their solute content. Water movements follow until the perturbation is corrected. The effector mechanisms of volume regulation have been well described. They consist of inorganic ion transport systems (e.g., Na/H exchange, chloride channels) and/or the synthesis and transport of organic osmolytes (e.g., sorbitol, taurine). On the other hand, very little is known about the "afferent loop" of the volume response—how cells detect a change in their water content. Some authors think the volume signal originates with stretching or displacement of the membrane or the cell skeleton. Others hypothesize

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that the activation of volume regulatory transporters or enzymes begins with dilution/concentration of some cell constituent (Sarkadi and Parker, 1991).

In a previous communication we showed that shrinkage-activated Na/H exchange in dog red cells was correlated with cytosolic protein concentration, not with cell volume per se (Colclasure and Parker, 1991*a*). We therefore rejected the notion that cell volume is perceived by a change in cell shape and thus aligned ourselves with Jennings and Schulz (1990), who had reached a similar conclusion on different grounds. We speculated that Na/H exchange is triggered by a shrinkage-induced rise in cytosolic protein concentration and cited the work of Minton (1983, 1990) on macromolecular crowding. According to this theory, the kinetics and equilibria of enzymes can be greatly influenced by small changes in the concentration of ambient, inert macromolecules. These effects are important in concentrated protein solutions like the cytosol of red cells, which contain as much as 30–35 g/dl hemoglobin.

Whereas our previous report dealt with a shrinkage-activated response, Na/H exchange, this study describes observations on a swelling-induced transporter, [K-Cl] cotransport.

A preliminary account of the results was published as an abstract (Colclasure and Parker, 1991*b*).

METHODS

Preparation of Resealed Ghosts Unsupplemented with Albumin

Venous blood was collected from mongrel dogs into heparinized syringes within 30 min of processing. After discarding the plasma and buffy coat the cells were washed three times in 10 vol of ice-cold 0.16 M NaCl and pelleted at 20,000 *g* for 1 min at 0°C. Ghosts were made by a modification of methods previously reported (Colclasure and Parker, 1991*a*). The cells were drawn into a chilled syringe and injected into four times their volume of a swirling, 0–0.5°C solution that contained 10 μM $CaCl_2$ and 13 mM acetic acid. The hemolysate was stirred at 0–0.5°C for 1 min, after which its pH was ~6.5. The hemolysate was supplemented with (final added concentrations) 150 μM ATP, 325 μM $MgCl_2$, 500 μM glutathione, 3 mM phosphocreatine, and 0.02 IU creatine kinase/ml (Sigma Chemical Co., St. Louis, MO). These additions increased the fluid volume of the hemolysate by < 1%. Crystalline Tris base was added to a final concentration of ~1.5 mM, at which point the hemolysate pH was 6.8. Note that no salt was added before the resealing phase. After these additions the hemolysis mixture had an osmolality of 50–60 mosM as determined by freezing point depression (Advanced Instruments, Inc., Needham Heights, MA). After a further 5 min stirring at 0–0.5°C the hemolysis mixture was placed in a 37°C water bath and incubated for 30 min to promote resealing. The ghosts were then centrifuged, washed three times at room temperature with warm, 0.1 M $NaNO_3$, and transferred to other media for the experiments described.

Preparation of Resealed Ghosts Supplemented with Albumin

The procedures for albumin-supplemented ghosts were in general the same as those outlined above, with the following exceptions: Fatty acid–free bovine serum albumin that had been dialyzed against distilled water and lyophilized was dissolved in the hemolysis solution at a concentration of 43 mg/ml, before the addition of packed cells. The hemolysis solution contained 5.7 mM acetic acid and 10 μM $CaCl_2$. 1 vol of cells was added to 8.5 vol (instead of 4 vol as noted above) of hemolysis solution at 0°C. 1 min later the ATP, magnesium, glutathione,

phosphocreatine, and creatine kinase were added at the final concentrations noted above. 6 min after hemolysis, sufficient solid NaCl was slowly added to adjust the osmolality to ~110 mosM. The ghost suspension was brought to pH 7.2 with crystalline Tris base. It was incubated for 20 min at 0°C with stirring and 20 min in an ice bath without stirring before being transferred to the 37°C bath for resealing.

These methods for preparing ghosts differ from our previous account in that phosphocreatine and creatine kinase were added to the hemolysates before resealing. In preliminary studies we found that swelling-induced, chloride-dependent K flux in resealed ghosts depended on this modification, in agreement with Sachs (1988) (Table I).

Measurement of Cell and Ghost Dry Solid Content

Cells/ghosts were washed twice in appropriate media containing 0.5% fatty acid–free bovine serum albumin. Dialyzed ^{125}I-labeled albumin (0.1 μCi/ml) was added to the second wash, and the cells/ghosts pelleted by centrifugation at 28,000 *g*. The pellet was weighed before and after drying for 20 h at 95°C, and the result was corrected for trapped medium using the radioactive albumin as an extracellular marker.

TABLE I

Chloride-dependent K Flux in Resealed Ghosts in the Presence and Absence of an ATP-regenerating System

Added ghost contents (final concentrations)	K influx		
	Cl	NO_3	Cl minus NO_3
	amol/ghost per 30 min		
150 μM ATP only	20.4	19.2	1.2
150 μM ATP 3 mM phosphocreatine 0.02 IU creatine kinase/ml	52.2	28.5	23.7

Ghosts were prepared as noted in Methods, with and without the phosphocreatine/creatine kinase regenerating system. After resealing, rubidium uptake was determined in hypotonic media (80 mM NaCl or $NaNO_3$) designed to activate [K–Cl] cotransport. This study is representative of two others.

Measurement of Rb and Na Influx

Cells/ghosts were suspended in 10 times their volume of incubation media, which covered a range of tonicities and consisted of 80 mM NaCl or $NaNO_3$, 4 mM KNO_3 10 mM HEPES buffer (pH 7.7 at 25°C), 5 mM glucose, and 0.5 gm/dl bovine serum albumin, with or without 0.5 mM amiloride. For albumin-loaded ghosts the basal medium was 40 mM NaCl or $NaNO_3$, with KNO_3, buffer, glucose, and pH as noted above. Solutions were adjusted to the desired range of osmolalities with *N*-methyl-*d*-glucamine sulfamate. The flux was begun by adding ^{86}Rb or ^{22}Na (1 μCi/ml) to each suspension at 37°C and sampling at 30 min. The influx was calculated as the number of counts taken up by the cells/ghosts in 30 min, divided by the specific activity of extracellular Rb or Na, and normalized to the number of cells/ghosts per suspension as determined with a model B Coulter counter (Coulter Electronics, Inc., Hialeah, FL). To convert flux values in amol/cell per 30 min to the more conventional dimensions (mmol/kg dry cell weight per h), multiply by $1.8/VD$, where V is the cell volume in femtoliters and D is the dry

solid fraction of the cell pellet. For example, a 60-fl cell with 63% water (dry weight fraction of 0.37) has a K influx of ~9 amol/cell per 30 min (Fig. 1) or 0.7 mmol/kg dry weight per h. This conversion factor is derived as follows: two 30-min periods/h × 10^{-15} mmol/amol × $(V)^{-1}$ 10^{15} cells/liter cells × 0.9 liter cells/kg cells × $(D)^{-1}$ kg cells/kg dry cell weight.

Our previous communication gave methods for determination of cell/ghost volumes by counting the number of cells/ghosts in a pellet of known volume, and also for quantitation of ghost albumin and hemoglobin contents by gel electrophoresis and scanning densitometry (Colclasure and Parker, 1991*a*).

RESULTS

As in a previous communication (Colclasure and Parker, 1991*a*), values for flux are given as a function of dry solids to highlight the relationship between transport and

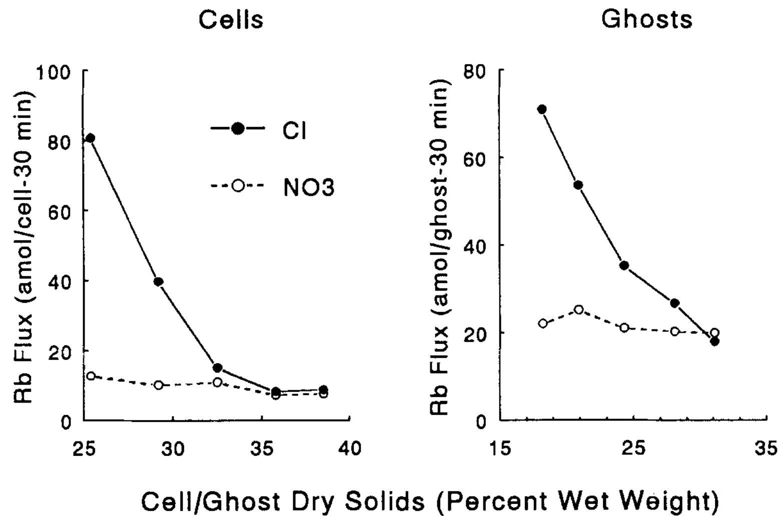

FIGURE 1. Rb influx in cells (left) and resealed ghosts (right) as a function of cell/ghost dry solids. Flux values in chloride media are shown by filled circles and solid lines. Values in nitrate media are shown in open circles and dashed lines.

protein concentration. Dry solids is expressed as a percentage and equals 100 × (dry weight/wet weight).

Prior studies demonstrated that ghosts prepared as described here have a lower osmotic fragility, and thus a lower volume-to-surface ratio than intact cells (Colclasure and Parker, 1991*a*), confirming that the process of hemolysis and resealing is not associated with a loss of cell surface area (Hoffman, Eden, Barr, and Bedell, 1958).

Fig. 1 compares intact cells (left panel) and resealed ghosts (right panel). The term "[K-Cl] cotransport" is operationally defined as the difference between the Rb influx in chloride versus non-halide (nitrate) media. Activation of [K-Cl] cotransport in both cell and ghost preparations occurs as the value for dry solids falls below 30–35% of wet weight.

As reported in human red cell ghosts by Sachs (1988), the volume responsiveness and chloride dependence of the Rb flux in the ghosts were dependent on the addition to the hemolysis medium of an ATP-regenerating system (Table I).

Fig. 2 presents two separate experiments comparing the volume at which [K-Cl] cotransport is activated in the cell and ghost preparations. Whereas the ghosts show activation above a cell volume of 20 fl, the intact cells show no chloride-dependent K influx below a volume of 70 fl. Therefore, resealed ghosts and intact cells manifest [K-Cl] cotransport at greatly different volumes.

When the flux values from the experiments in Fig. 2 are plotted as functions of the dry solid content, activation of [K-Cl] cotransport in both ghost and cell preparations occurs at 30–35% of wet weight (Fig. 3). Activation of the transporter is more closely correlated with dry weight content than volume.

Fig. 4 shows three separate studies in which albumin was included in the

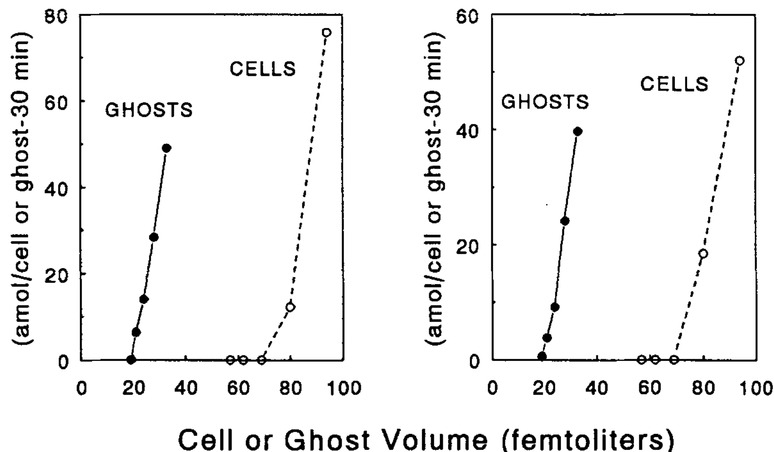

FIGURE 2. Chloride-dependent K transport as a function of volume in intact cells (open circles, dashed lines) and resealed ghosts (filled circles, solid lines). The two panels show duplicate studies.

hemolyzing medium so that the resealed ghosts contained ~40% albumin and 60% hemoglobin as previously described (Parker and Colclasure, 1991*a*). The relationship between chloride-dependent K flux and ghost dry solid content is similar to that of intact cells and ghosts containing only hemoglobin.

Fig. 5 shows the results of Na and K flux determinations on a single preparation of resealed ghosts (containing no added albumin). The dry solid content at which [K-Cl] cotransport is inactivated is similar to the value at which Na/H exchange is activated (~30% wet weight).

DISCUSSION

These experiments and those of a previous report (Colclasure and Parker, 1991*a*) deal with the activation of ion transport by cell volume changes. Compared with intact cells, the resealed ghosts had the same surface area and a similar *concentration*

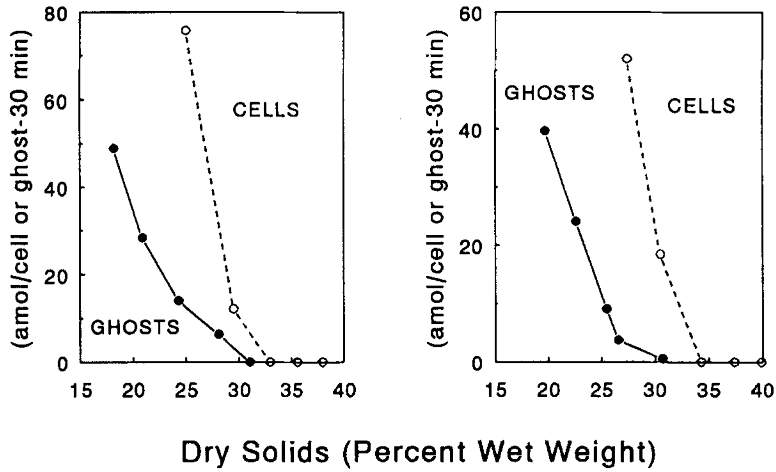

FIGURE 3. Plot of the influx data from Fig. 2 as a function of dry solids in intact cells (open circles, dashed lines) and resealed ghosts (filled circles, solid lines).

of dry solids. However, because their *amount* of dry solids was lower, the resealed ghosts were smaller in volume than intact cells suspended in the same medium. Like the intact cells, the resealed ghosts behaved as osmometers (Colclasure and Parker, 1991*a*).

The volume at which [K-Cl] cotransport was induced was much smaller in resealed ghosts than in intact cells, but when expressed as a function of dry solid content, the flux responded similarly in the two preparations. Therefore, the activation of cation transporters by swelling and shrinking in dog red cells is not initiated by a change in volume per se, but rather by concentration and dilution of the cytosol.

In ghosts made to contain roughly half albumin and half hemoglobin, swelling-activated [K-Cl] cotransport occurred at a similar dry solid concentration, but a much lower hemoglobin concentration than in ghosts containing only hemoglobin (Fig. 4).

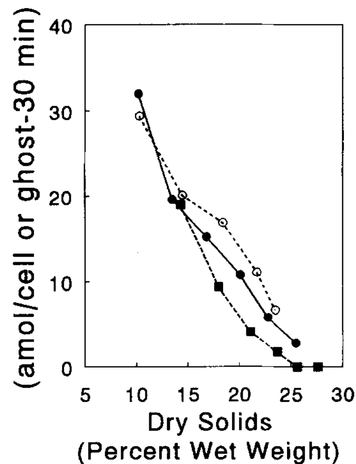

FIGURE 4. Chloride-dependent K transport as a function of dry solids in three different resealed ghost preparation containing a mixture of 40% bovine serum albumin and 60% hemoglobin.

The importance of this observation is that it testifies to the nonspecificity of the volume perception mechanism. While the cytoplasm may contain specific enzymes and substrates (e.g., high-energy phosphate compounds) necessary for volume-induced activation of transporters, none of those ingredients were limiting in the ghosts with albumin-diluted cytoplasm.

Simultaneous measurements of amiloride-sensitive Na flux and chloride-dependent K flux in the same ghost preparation (Fig. 5) support the notion that the two volume-activated transporters involved—Na/H exchange and [K-Cl] cotransport—are coordinated (Parker, Colclasure, and McManus, 1991).

In preliminary studies we noted that the inclusion of creatine kinase and phosphocreatine in the ghosts was necessary for optimal demonstration of swelling-induced [K-Cl] cotransport (Table I). The ATP-regenerating system was not necessary for the demonstration of Na/H exchange (Colclasure and Parker, 1991*a*), nor was it inhibitory (Fig. 5). This result seems at variance with the suggestion that it is a dephosphorylation rather than a phosphorylation that activates swelling-induced [K-Cl] cotransport (Jennings and Schulz, 1990). However, ATP may be required to

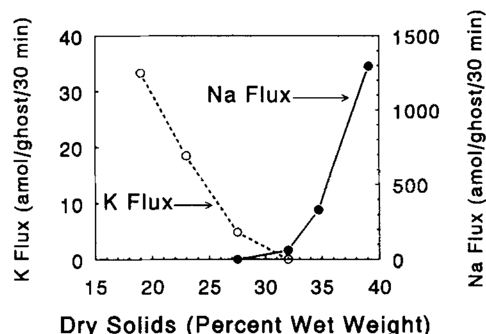

FIGURE 5. Chloride-dependent K transport (open circles, dashed lines, left axis) and amiloride-sensitive Na influx (filled circles, solid lines, right axis) as functions of dry solids in resealed ghosts. Both flux determinations were made on a single ghost preparation. The figure is representative of two additional studies.

activate, or de-inhibit, a phosphatase (Shenolikar and Nairn, 1991). The requirement for an ATP-regenerating system testifies that the fluxes measured were indeed from lysed and resealed cells and not from a minor intact cell population.

Previous Studies of [K-Cl] Cotransport in Ghosts

Several authors have studied [K-Cl] cotransport in resealed red cell membrane preparations. Dunham and Logue (1986) hemolyzed human red cells at a cell/medium ratio of ~ 1:10. Compared with intact cells the ghosts showed a total K influx that was 15 times greater and a furosemide-sensitive component that was two to six times greater; most of the flux was chloride dependent, and it was doubled by including ATP in the ghost interior. The hemoglobin content of the ghosts was not reported, but the finding that [K-Cl] cotransport is stimulated by dilution of the cytosol is consistent with our observations.

Sachs (1988) prepared hemoglobin-free resealed ghosts from human red cells by a gel filtration method. Provided that an ATP-regenerating system was included in the hemolysate, the ghosts showed a chloride-dependent K influx. When the osmolality

of the bathing medium was varied from 475 down to 210, the chloride-dependent flux increased about twofold. Complete inhibition of volume-dependent K flux by shrinkage, a phenomenon seen in intact cells and in the ghosts of Figs. 1–4, was not reported with either of the two ghost preparations cited above. Brugnara, Van Ha, and Tosteson (1988) made hemoglobin-free resealed ghosts by the gel filtration method from human red cells separated into three density fractions before lysis, and found a chloride-dependent K efflux that depended on ATP but was unresponsive to volume perturbation. Finally, Kracke and Dunham (1990) made inside-out vesicles from low-K sheep red cells and found that K efflux increased in progressively hypotonic bathing media. The volume-sensitive flux was not totally chloride dependent, however. In a chloride-containing medium the flux increased 2.7-fold as the osmolality was changed from 430 to 230 mosM; in a chloride-free medium the same osmotic perturbation was associated with a twofold increase in K efflux. There was no clearcut dependence of the volume-related fluxes on ATP.

The finding of osmolality-related [K-Cl] cotransport in human red cell ghosts containing no hemoglobin (Sachs, 1988, but not Brugnara et al., 1988) would appear to be inconsistent with the present results. However, the degree of responsiveness in the preparation cited was not as great as with the ghosts reported here, nor were indices of ghost volume or correlations with the behavior of intact cells (as in Figs. 1–3) presented. The results of Kracke and Dunham (1990) on inside-out membrane vesicles are not comparable with the present ones, since much of the volume responsiveness of their preparation was chloride independent. The putative [K-Cl] cotransporter was not totally inactivated by shrinkage, as we report in Figs. 1–3.

Macromolecular Crowding

Our evidence that cytoplasmic protein concentration mediates volume-activated transport might be explained by the concept of macromolecular crowding or volume occupancy (Minton, 1983, 1990). The osmotic activity coefficient of hemoglobin has been known since the studies of Adair (1928) to increase steeply with concentration. The relationship is evident at concentrations seen in normal red cells (Freedman and Hoffman, 1979). The rate of polymerization of sickle hemoglobin is exquisitely sensitive to its concentration (Eaton and Hofrichter, 1990). Minton (1983) pointed out that these influences of high hemoglobin concentration on the properties of hemoglobin itself might also be exerted among different species of macromolecules. According to the theory, effects of this sort would not be exerted on low molecular weight solutes and would be perfectly compatible with observations that the activity coefficients for small ions are the same in the cytoplasm as in the extracellular milieu (Freedman and Hoffman, 1979).

Minton (1983) hypothesized that the enzyme activities could be influenced by high ambient concentrations of "inert" macromolecules that were neither reactants nor products. He showed that glyceraldehyde-3-phosphate dehydrogenase underwent log changes in enzyme specific activity as a function of the concentration of ribonuclease, bovine serum albumin, and beta lactoglobulin. He suggested two mechanisms for effects of concentrated protein solutions on the properties of enzymes, based on the principle that crowding favors association of like and/or unlike macromolecules. The first mechanism would apply to an enzyme like glyceraldehyde-3-phosphate dehydro-

genase, which is more active as a monomer than a tetramer. Inhibition of enzymatic activity in progressively crowded solutions can be attributed to formation of the less active aggregate. A second mechanism applies to reactions in which both the enzyme and its substrate are macromolecules and in which the reaction catalyzed involves sterically minor modifications, e.g., kinases and phosphatases. In such cases the formation of the enzyme–substrate complex would be favored in a crowded solution. An example of this would be the binding of DNA polymerases to their template primers, which is enhanced in concentrated macromolecular solutions (Zimmerman and Harrison, 1987).

If the activation of transporters in response to volume perturbation involves phosphorylations and dephosphorylations, as recently proposed (Jennings and Al-Rohil, 1990; Pewitt, Hegde, Haas, and Palfrey, 1990; Cossins, 1991; Parker, Colclasure, and McManus, 1991; Jennings and Schulz, 1991), then it seems plausible that swelling and shrinking are translated into a chemical signal by the influence of macromolecular crowding on the relevant kinases and phosphatases.

Relation of These Findings to Other Cell Types

A possible example of the influence of cytosolic protein concentration on cell volume regulation can be found in a study of Baraona, Leo, Borowsky, and Lieber (1975), who reported that rats fed a high alcohol diet developed an impairment in the capacity of their livers to export proteins, a defect later shown to be associated with impaired glycosylation (Matsuda, Takada, Takase, and Sato, 1991). As a result, albumin and transferrin accumulated in the hepatocyte cytosol. There was an increase in cell volume with no change in the ratio of wet to dry weight in liver tissue: thus each cell accumulated water along with protein. Although the authors offered no explanation for this phenomenon, one could speculate that as the concentration of unsecreted protein increased, the cytosol became more crowded, and the cells behaved as though shrunken, switching on a transport system that allowed them to accumulate ions and water, thus diluting their cytosolic protein concentration back to normal. Like dog red cells, hepatocytes respond to shrinkage (or concentration of internal protein) by activating Na/H exchange (Haussinger and Lang, 1991). Thus the findings reported here may apply to a variety of cell types.

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