Kinetic, Thermodynamic, and Developmental Consequences of Deleting Creatine Kinase Isoenzymes from the Heart

REACTION KINETICS OF THE CREATINE KINASE ISOENZYMES IN THE INTACT HEART*

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Creatine kinase (CK, EC 2.7.3.2) plays a central role in the energetics of excitable cells, catalyzing the transfer of a phosphoryl moiety between creatine and ADP in the reaction PCr + ADP + H+ ⇌ ATP + creatine (K_eq of 1.66 × 10^9 mol/l^{-1}). CK exists as a family of five isoenzymes, three dimers located in the cytoplasm (MM, MB, and BB-CK) and two multimers that connect the inner and outer mitochondrial membranes (sarcomorphic and ubiquitous Mt-CK). The distribution of these CK isoenzymes is tissue-specific, developmentally regulated, and changes in disease, especially in the heart (1, 2). During development in the heart, BB-CK activity decreases while MM-CK, and later Mt-CK, activities increase. The capacity for ATP synthesis via oxidative phosphorylation also increases during this time. Because the CK reaction is coupled to ATP synthesis, the developmental changes in CK may also be linked to substrate preference for ATP synthesis of the developing heart.

Despite the fact that CK is one of the most abundant enzymes in the heart and plays a central role in the energetics of cardiac muscle, many questions about the biochemistry of CK isoenzymes remain unanswered. Several of these long-standing questions regarding the function of CK isoenzymes in the heart can be directly addressed by studying the hearts from mice bioengineered to lack either the most abundant muscle isof orm (MM-CK) or the two most abundant isof orms (MM-CK and Mt-CK).

Here we address four questions central to the role of CK isoenzymes in the heart. The first question is whether a feedback system exists linking changes in the amount of one isoenzyme with a change in the amounts of other isoenzymes. Such a system is suggested by the observations that a reciprocal relationship exists between BB-CK and MM-CK during both development and heart failure (1, 2). The second question is, what are the kinetic properties of each of the CK isoenzymes in the intact beating heart? The properties of the individual isoenzymes determine whether the changes in isoenzyme distribution that occur during development and disease confer a kinetic advantage or disadvantage for maintaining high [ATP]. The third issue is whether the rate of ATP synthesis via the CK reaction can fall below the rate of ATP synthesis from oxidative phosphorylation. It has been suggested that localization of specific CK isoenzymes in the mitochondria and sites of ATP utilization form an "energy shuttle" whereby PCr serves as an efficient energy transfer molecule. Whether this shuttling of phosphoryl moieties is obligatory remains controversial (3–6) but can be directly tested in hearts lacking CK isoenzymes. The fourth issue addressed here is the role of specific CK isoenzymes in determining the thermodynamics of ATP hydrolysis in the heart. It is well established that the amount of free energy released from ATP hydrolysis (∆G_{ATP}) varies in the heart depending on which substrates for oxidative phosphorylation are present (7). What role do the CK isoenzymes play in this modulation of ∆G_{ATP}? is not known.

To address these questions, hearts from wild type mice and mice lacking genes for M-CK (8–10) (MCK–/– mice) or both M-CK and Mt-CK (11, 12) (M/MtCK–/– mice) were studied. In addition to measuring CK isoenzyme distribution and V_{max}^{31}P NMR spectroscopy was used to measure ∆G_{ATP}.
[ADP], $\Delta G_{\text{ATP}}$, and pH in the isolated, beating hearts. Additionally, the unidirectional flux through the CK reaction (rate of ATP synthesis from PCr or CK velocity) was measured using $^{31}$P magnetization transfer in isolated, beating hearts.

**EXPERIMENTAL PROCEDURES**

*Animals and Experimental Groups—MCK $^-$/$^-$ and M/MtCK $^+$/$^+$ mice were provided by Dr. Bé Wieringa, University of Nijmegen, The Netherlands (8, 11). Hearts from fetal (20, 24, and 27 days post-conception), neonatal (1, 3, and 7 days post-partum), young (15 and 25 days post-partum), and adult mice (25–40 weeks of age) were rapidly excised and frozen to define the developmental pattern of change in CK V$_{\text{max}}$ and CK isoenzyme distribution, confirming the genotype. 185 hearts were studied, 5–15 at each time point for wild type, MCK $^-$, and M/MtCK $^-$.

Isolated perfused beating hearts from adult mice were studied to measure CK velocity using $^{31}$P magnetization transfer (5 wild type, 5 MCK $^-$, and 5 M/MtCK $^+$.). This group consisted of relatively old mice (60–70 weeks) because they had larger hearts, which improved the signal to noise ratio and provided the sensitivity necessary to detect the very low level of CK velocity in M/MtCK $^-$ hearts. A second set of hearts was perfused with glucose as the sole exogenous metabolic substrate (20 wild type, 13 MCK $^-$, and 12 M/MtCK) or with both glucose and pyruvate (10 wild type, 10 MCK $^-$, and 9 M/MtCK $^+$). This group consisted of both male and female mice 25–40 weeks old. For each heart isovolumic contractile function and energetics (using $^{31}$P NMR spectroscopy) were measured simultaneously.

The experimental protocols were approved by the Standing Committee on Animals of Harvard Medical Area and followed the recommendations of the National Institutes of Health and American Physiological Society guidelines for the use and care of laboratory animals.

*Biochemical Assays—Total CK activity (CK V$_{\text{max}}$) and the amount of activity attributable to each isoenzyme of CK was measured (12, 13). Since 5–10 mg of tissue was needed for this measurement, where necessary, tissue from 3 to 4 fetal hearts was pooled for analysis. CK activities were measured in units of IU per mg of protein and converted to nmol/mg using the measured concentrations of cardiac protein. All values are expressed as nmol/mg at 37 °C. To determine whether B-CK (i.e., the monomer peptide) expression is affected by loss of M-CK or the combined loss of M-CK and Mt-CK, direct comparison of the amounts of BB-CK in the three types of hearts is inadequate. Some B-CK monomers exist as MB-CK in wild type hearts, whereas in M/MtCK $^+$ hearts, all B-CK is in the form of BB-CK. Therefore, for wild type hearts we calculate the amount of BB-CK that would form if M-CK was not present (0.5 × MB-CK), and we added this to the measured amount of BB-CK.

The myocardial contents of ATP (via high pressure liquid chromatography), creatine (14), and protein (15) were determined in a separate group of freeze-clamped hearts (5 wild type, 5 MCK $^-$, and 5 M/MtCK). For ATP, content was converted to concentration using the measured values for protein concentration, which average 0.16 mg of protein/mg wet weight, and the literature value for the ratio of intracellular volume to total cell volume of 0.48 (16). The concentrations of ATP (mM) were 9.6 ± 0.1, 9.4 ± 0.2, and 9.3 ± 0.2 for wild type, MCK $^-$, and M/MtCK $^+$ hearts, respectively (not significant among groups). Total creatine content was 28.2 ± 0.5 mM in wild type, 32.1 ± 0.7 mM in MCK $^-$, and 30.5 ± 0.6 mM in M/MtCK $^+$ hearts (not significant among groups). Myocardial oxygen consumption was calculated from the relationship we previously established between rate-pressure-product and myocardial oxygen consumption for wild type, MCK $^-$, and M/MtCK $^+$ hearts (12).

*Isolated Perfused Heart Preparation—Hearts were isolated and perfused in the Langendorff mode (12). The coronary perfusate consisted of Krebs-Henseleit buffer containing (mM) NaCl (118), KCl (5.3), CaCl$_2$ (2.0), MgSO$_4$ (1.2), EDTA (0.5), and NaHCO$_3$ (25), equilibrated with 95% O$_2$ and 5% CO$_2$ yielding a pH of 7.4. Either glucose alone (10% or glucose in combination with pyruvate (0.5) was added as described above. All hearts, except those undergoing magnetization transfer, were paced at 7 Hz.

*31P NMR Spectroscopy—$^{31}$P NMR spectra were obtained at 161.94 MHz using a GE-400 wide-bore Omega spectrometer (Fremont, CA) (12). The measured ATP resonance areas/mg wet weight for wild type, MCK $^-$, and M/MtCK $^+$ hearts were not different, averaging 268 ± 11, 250 ± 10, and 282 ± 16, respectively. This confirmed our finding, made using high pressure liquid chromatography, that [ATP] was not different among groups. The ATP resonance area of each heart was used as an internal standard to convert resonance areas of PCr and P,

FIG. 1. Top panel, CK V$_{\text{max}}$ during development in wild type, MCK $^-$, and M/MtCK $^+$ hearts. In wild types the post-partum increase in CK V$_{\text{max}}$ was due primarily to an increase in the MM CK isoenzyme. Where S.E. bars cannot be seen, they are within the symbols. Middle panel, Mt-CK activity during development in wild type and MCK $^-$ hearts. The timing of the post-natal increase in Mt-CK was not affected by loss of MM-CK, but the final amount of Mt-CK was less that in wild types. Bottom panel, BB-CK activity during development in the three types of hearts. Note that neither loss of M-CK nor loss of both M-CK and Mt-CK caused an alteration in the normal developmental pattern of change in BB-CK observed in wild types.

to their respective concentrations. Intracellular pH was determined by comparing the chemical shift of the P, and PCr peaks in each spectrum to values from a standard curve.

Cytosolic free [ADP] was calculated using the equilibrium constant of the CK reaction (17) and from values obtained by NMR spectroscopy and biochemical assays (Equation 1),

$$[\text{ADP}] = ([\text{ATP}] / [\text{free Cr}]) / ([\text{PCr}] / [\text{Cr}]) / K_{\text{eq}}$$  
(1)

For purposes of clarity all values of $\Delta G_{\text{ATP}}$ are expressed as their absolute values as shown in Equation 2,

$$\Delta G_{\text{ATP}} = \Delta G^0 + RT \ln([\text{ATP}] / [\text{ADP}])$$  
(2)

where $\Delta G^0$ (−30.5 kJ/mol) is the value of $\Delta G_{\text{ATP}}$ under standard conditions of molarity, temperature, pH, and [Mg$^{2+}$]; $\Delta G_{\text{ATP}}^0$ is the gas constant (8.3 J/mol K), and $T$ is in Kelvin (18).

Magnetization transfer measurement of the forward velocity of the CK reaction, ADP + PCr $\rightarrow$ ATP + creatine, was made using the two-site chemical exchange (PCr $\rightarrow$ [γ-$^{31}$P]ATP) model of Forsen and Hoffmann, providing estimates of the pseudo first-order rate constant (k$_{\text{taur}}$) (19), as modified using a M$_M$ sequence developed in our laboratory (20). Each of the magnetization transfer spectra consisted of scans accumulated by repetitively cycling through the two different times of protonation (0 and 4.8 s). The integrated signal intensity of the PCR resonance peak decays from MT to M$_M$, (magnetization at zero and infinite saturation times, respectively) as [γ-$^{31}$P]ATP is saturated. k$_{\text{tau}}$ was calculated as k$_{\text{tau}}$ = (M$_M$ − M$_T$) / (T$_1$ × M$_T$) using the literature value for T$_1$ of 3.5 ± 0.2. Multiplying the rate constant k$_{\text{tau}}$ by the substrate (Pcr) concentration yields the forward velocity of the CK reaction,
CK\textsubscript{velocity} = k\textsubscript{for} [PCr]. Acquisition of these spectra required 5 h, during which time contractile function decreased by only 10–20%.

RESULTS

Regulation of CK Isoenzyme Expression during Development—In wild type hearts CK V\textsubscript{max} increased rapidly from day 27 to day 1 due to an increase in MM-CK (Fig. 1). From day 7 to day 15, CK V\textsubscript{max} continued to increase due to an increase in Mt-CK. In hearts containing only Mt-CK and BB-CK, CK V\textsubscript{max} did not increase prior to day 1; instead, V\textsubscript{max} increased only gradually after day 7 due to an increase in Mt-CK. Although Mt-CK increased after day 7 in these hearts, they had less Mt-CK activity from day 15 through adulthood than wild types (Fig. 1 and Table I). In hearts containing only BB-CK, CK V\textsubscript{max} did not increase after birth. Instead, V\textsubscript{max} gradually decreased. Since all three groups showed the same developmental pattern of change in BB-CK, it is apparent that the post-natal decrease in BB-CK is independent of accumulation of M-CK, Mt-CK, or total CK. Thus, the developmental pattern of change in BB-CK was not affected by deletion of CK isoenzymes nor was the timing of the increase in Mt-CK. However, the magnitude of the increase in Mt-CK was less in hearts lacking MM-CK than in wild types.

In Vivo CK Reaction Velocity (CK\textsubscript{velocity})—\textsuperscript{31}P magnetization transfer provides a measure of CK reaction velocity in the intact beating heart. Representative \textsuperscript{31}P magnetization transfer spectra for hearts containing all CK isoenzymes, only Mt-CK and BB-CK and only BB-CK are shown in Fig. 2. Mean values for \( k \) for and CK\textsubscript{velocity} are shown in Table II. The decrease in the PCr resonance area during selective saturation was less in hearts containing only BB-CK and Mt-CK than in wild types, reflecting a decreased rate of ATP synthesis from PCr (CK\textsubscript{velocity}) secondary to loss of MM-CK. In hearts containing only BB-CK there was a small but detectable CK\textsubscript{velocity}. CK\textsubscript{velocity} was 6.2 and 2.9 times the rate of oxidative ATP synthesis in wild type hearts and those lacking MM-CK, respectively. In hearts with only BB-CK, CK\textsubscript{velocity} was 9% the

| TABLE I | Creatine kinase isoenzyme distribution and activity (V\textsubscript{max}) in adult mouse hearts |
|-----------------|-----------------------------------------------|
|                 | Wild type | MCK\textsuperscript{−/−} | M/MtCK\textsuperscript{−/−} |
| Total CK (mM/s) | 46.9 ± 1.0 | 12.2 ± 1.0\textsuperscript{a} | 1.0 ± 0.1\textsuperscript{a} |
| MMCK (mM/s)     | 27.6 ± 2.6 | 0 | 0 |
| MBCK (mM/s)     | 1.5 ± 0.3 | 0 | 0 |
| BBCK (mM/s)     | 0.7 ± 0.1\textsuperscript{b} | 0.8 ± 0.1 | 1.0 ± 0.1 |
| MtCK (mM/s)     | 17.0 ± 1.9 | 11.4 ± 0.9\textsuperscript{a} | 0 |
| MMCK (% total)  | 58.9 ± 1.4 | 0 | 0 |
| MBCK (% total)  | 3.3 ± 0.3 | 0 | 0 |
| BBCK (% total)  | 1.5 ± 0.2 | 6.5 ± 0.5 | 100 |
| MtCK (% total)  | 36.3 ± 1.3 | 93.5 ± 0.5 | 0 |

\textsuperscript{a} Significantly different from wild types.

\textsuperscript{b} If BCK monomers from MBCK are added to BBCK, as described under "Experimental Procedures," the total is 1.5 ± 0.1.

Fig. 2. Representative \textsuperscript{31}P magnetization transfer spectra showing the rate of transfer of a high energy phosphate group between PCr and the \( \gamma \)-phosphate of ATP. The three spectra on the left are without saturation (\( M_{0} \)), and those on the right were obtained with a saturation time of 4.8 s (\( M' \)). In the wild type hearts (top two panels) high energy phosphate transfer is rapid as evidenced by the large decrease in the PCr resonance area during \( M' \). In MCK\textsuperscript{−/−} hearts this rate was much slower as indicated by the modest decrease in the PCr resonance area during \( M' \). In M/MtCK\textsuperscript{−/−} hearts there was a small but detectable transfer of high energy phosphates between PCr and ATP, indicating a very slow rate of CK\textsubscript{velocity}. Under base-line conditions (\( M_{0} \)) the concentration of PCr is significantly lower in M/MtCK\textsuperscript{−/−} hearts than in the other two groups as reported previously (12).
rate of oxidative ATP synthesis (Table II).

The values for CK velocity and CK \( V_{\text{max}} \) for the three types of hearts can be used to calculate the ratio of CK velocity/CK \( V_{\text{max}} \) for each of the pure isoenzymes (BB-CK, MM-CK, and Mt-CK). For BB-CK this ratio was 0.09, since this was the measured ratio for hearts with only BB-CK. Based on our finding that hearts that were 94% Mt-CK and 6% BB-CK had a CK velocity/CK \( V_{\text{max}} \) of 0.25, the CK velocity/CK \( V_{\text{max}} \) of Mt-CK was calculated to be 0.26. By using the values of 0.09 for BB-CK and 0.26 for Mt-CK, we calculated that MM-CK in wild type hearts had a CK velocity/CK \( V_{\text{max}} \) of 0.10. Thus, BB-CK and MM-CK have the same CK velocity/CK \( V_{\text{max}} \), whereas the ratio for Mt-CK is \( \sim 2.5 \) times higher.

**Contribution of the Individual CK Isoenzymes to Changes in \( \Delta G_{\text{ATP}} \)—** In wild type hearts, including pyruvate in the perfusate as a substrate for oxidative phosphorylation caused the expected changes in energetics as [PCr] increased, whereas [P\(_i\)] and [ADP] decreased, resulting in an increased \( \Delta G_{\text{ATP}} \) (Table III). In hearts that contained only MtCK and BBCK, including pyruvate in the perfusate affected the thermodynamics of ATP hydrolysis in the same way as wild type. In contrast, hearts that contained only BB-CK failed to increase \( \Delta G_{\text{ATP}} \) in response to pyruvate. Therefore, the combined loss of MM-CK and Mt-CK (but not loss of only MM-CK) prevented hearts from increasing \( \Delta G_{\text{ATP}} \) when administered pyruvate as a substrate for oxidative phosphorylation.

**DISCUSSION**

The main findings of this study were as follows. First, loss of M-CK and loss of both M-CK and Mt-CK had little or no effect on either the normal developmental change in the amount of the remaining isoenzymes or the final amounts present in adult hearts. Second, the CK velocity/CK \( V_{\text{max}} \) of BB-CK and MM-CK were very similar, which suggests that isoenzyme shifts during development and disease do not confer any obvious kinetic advantage. Third, hearts containing only BB-CK the rate of CK velocity was only a small fraction of the rate of ATP synthesis from oxidative phosphorylation, demonstrating that phosphorl shuttling is not obligatory. Finally, the combined loss of M-CK and Mt-CK, but not loss of only Mt-CK, prevented hearts from significantly increasing \( \Delta G_{\text{ATP}} \) when perfused with pyruvate as a substrate for oxidative phosphorylation.

**Compensatory Response to Loss of CK Isoenzymes in the Developing and Mature Myocardium—** Expression of the isoenzymes of CK in the heart is developmentally regulated (2). Both the developmental and tissue-specific regulation of CK expression can be explained by cis-acting elements (21). Since the genes for the CK isoenzymes are on different chromosomes, it may also be that trans-acting elements are involved in the coordinated expression of CK isoenzymes. During late fetal and neonatal development, expression of both MM-CK and Mt-CK increases dramatically, whereas BB-CK peaks at birth and then declines (Fig. 1) (2). What triggers and coordinates these changes is largely unknown. One possibility is that a change in the amount of one isoenzyme triggers the appropriate changes in the other isoenzymes. Our data indicate that this is not the case, since loss of either MM-CK or both MM-CK and Mt-CK had no effect on the normal post-partum decrease in BB-CK. Also demonstrating the independence of CK isoenzyme regulation during development is the observation that the timing of post-natal increase in Mt-CK was not affected by loss of MM-CK. However, the magnitude of the increase in Mt-CK was less in hearts lacking MM-CK than wild type for unknown reasons.

This independence of isoenzyme regulation during development is consistent with the finding in adult heart and skeletal muscle that loss of MM-CK does not cause a compensatory increase in Mt-CK (9). Less clear, due to the difficulty in quantifying the small amount of cardiac BB-CK, is whether loss of MM-CK causes an increase in BB-CK (9). Here we demonstrated that no compensatory increase in BB-CK occurred in hearts lacking MM-CK and furthermore that even loss of both MM-CK and Mt-CK did not alter the amount of BB-CK. Overall, our results in the developing and mature myocardium demonstrate that the amount of each CK isoenzyme is influenced surprisingly little, if at all, by changes in the amounts of the other isoenzymes.

**CK velocity Relative to Rate of Oxidative ATP Synthesis—** In the healthy mammalian heart, the rate of ATP turnover via the CK reaction (CK velocity) is 5–10-fold higher than the net rate of ATP synthesis from glycolysis and oxidative phosphorylation combined. Therefore on average each molecule of ATP synthesized from glycolysis and oxidative phosphorylation is converted back and forth between ATP and PCr many times before it is finally hydrolyzed to ADP and P\(_i\). It has been suggested that this high CK velocity exists to facilitate movement of phosphoryl moieties from their primary site of synthesis (mitochondria), via the more easily diffusible PCr, to the primary site of hydrolysis (cytosolic ATPases) (3–6).
Here for the first time we were able to measure the low rate of CK\textsubscript{velocity} in hearts containing only BB-CK, and we found that the CK\textsubscript{velocity} is only 9% of the rate of oxidative ATP synthesis. This means that transport of ATP from the mitochondria to the cytoplasm is accomplished without the use of PCr as an obligatory intermediate in these hearts. The CK\textsubscript{velocity} measured in hearts containing only BB-CK, although low compared with wild type hearts, is still adequate to explain our previous finding that M/Mt-CK\textsuperscript{−/−} hearts can hydrolyze normal amounts of PCr during increased work and have no obvious contractile deficits (12). In that study approximately 4 mm PCr was hydrolyzed in 6 min. This would require a CK\textsubscript{velocity} of only 0.01 m\textsuperscript{M/s}, well below the measured value of 0.09 m\textsuperscript{M/s}. A similar situation likely exists in skeletal muscle from MCK and that the reaction rate is primarily under substrate control. Minimal non-substrate regulation occurs in the heart and for hearts containing normal CK substrate concentrations, this suggests that, at least under our perfusion conditions hearts are similar to those found for purified enzymes in solution (23).

Kinetic Properties of Individual CK Isoenzymes in Isolated, Beating Hearts—The ratio of CK\textsubscript{velocity} to CK\textsubscript{V\textsubscript{max}} indicates how fast the CK reaction is proceeding relative to its maximal capacity and has previously been reported for purified CK isoenzymes in solution (22). In solution, CK\textsubscript{velocity}/CK\textsubscript{V\textsubscript{max}} was 0.15 for MM-CK and 0.30 for Mt-CK. When purified isoenzymes are studied in solution, the CK\textsubscript{velocity}/CK\textsubscript{V\textsubscript{max}} ratio provides information about the intrinsic properties of the proteins and defines substrate control of enzyme velocity. When measured in living tissue, this ratio also reflects the influences of any additional regulatory systems (23). By using results obtained in the present study, we calculate the CK\textsubscript{velocity}/CK\textsubscript{V\textsubscript{max}} for the individual CK isoenzymes in beating hearts. The values we measured for the CK isoenzymes in intact beating hearts are similar to those found for purified enzymes in solution. This suggests that, at least under our perfusion conditions and for hearts containing normal CK substrate concentrations, minimal non-substrate regulation occurs in the heart in vivo and that the reaction rate is primarily under substrate control. Furthermore, the finding that the CK\textsubscript{velocity}/CK\textsubscript{V\textsubscript{max}} of BB-CK (0.09) is no larger that MM-CK (0.10) suggests that the isoenzyme shift toward the fetal pattern of expression during cardiac hypertrophy and heart failure confers no obvious kinetic advantage.

Effect of Changing Metabolic Substrates on |\Delta \textsubscript{G\textsuperscript{ATP}}|—Little is known regarding the mechanism(s) by which altering the availability of substrates for oxidative phosphorylation affects |\Delta \textsubscript{G\textsuperscript{ATP}}| (7). It is reasonable to expect that they depend at least in part on an intact CK system since the reaction catalyzed by CK maintains [ATP] high and the concentrations of its hydrolysis products (ADP and P\textsubscript{i}) low. Supporting a relationship between CK and |\Delta \textsubscript{G\textsuperscript{ATP}}| are data that demonstrated that severe acute inhibition of CK led to a fall in |\Delta \textsubscript{G\textsuperscript{ATP}}| (24). Here we report that wild type hearts and hearts containing MM-CK and Mt-CK increased |\Delta \textsubscript{G\textsuperscript{ATP}}| in response to supplying pyruvate. In contrast, hearts with only BB-CK failed to increase |\Delta \textsubscript{G\textsuperscript{ATP}}| when supplied with pyruvate. These results raise the possibility that substrate-dependent changes in |\Delta \textsubscript{G\textsuperscript{ATP}}| may depend on Mt-CK, the isoenzyme primarily responsible for PCr synthesis. Alternatively, it may be that the CK\textsubscript{velocity} rate in these hearts is inadequate to maintain [ADP] low and [PCr] high. We are not able to distinguish between these two possibilities.

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