Pharmacological and Histochemical Distinctions Between Molecularly Defined Sarcolemmal $K_{\text{ATP}}$ Channels and Native Cardiac Mitochondrial $K_{\text{ATP}}$ Channels

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ABSTRACT

A variety of direct and indirect techniques have revealed the existence of ATP-sensitive potassium ($K_{\text{ATP}}$) channels in the inner membranes of mitochondria. The molecular identity of these mitochondrial $K_{\text{ATP}}$ (mito$K_{\text{ATP}}$) channels remains unclear. We used a pharmacological approach to distinguish mito$K_{\text{ATP}}$ channels from classical, molecularly defined cardiac sarcolemmal $K_{\text{ATP}}$ (surface$K_{\text{ATP}}$) channels encoded by the sulfonylurea receptor SUR2A and the pore-forming subunit Kir6.2. SUR2A and Kir6.2 were expressed in human embryonic kidney (HEK)293 cells, and their activities were measured by patch-clamp recordings of membrane current. Surface$K_{\text{ATP}}$ channels are activated potently by 100 $\mu$M pinacolide but only weakly by 100 $\mu$M diazoxide; in addition, they are blocked by 10 $\mu$M glibenclamide, but are insensitive to 500 $\mu$M 5-hydroxydecanoate. This pharmacology, which was confirmed with patch-clamp recordings in intact rabbit ventricular myocytes, contrasts with that of mito$K_{\text{ATP}}$ channels as indexed by flavoprotein oxidation. Mito$K_{\text{ATP}}$ channels in myocytes are activated equally by 100 $\mu$M diazoxide and 100 $\mu$M pinacolide. In contrast to its lack of effect on surface$K_{\text{ATP}}$ channels, 5-hydroxydecanoate is an effective blocker of mito$K_{\text{ATP}}$ channels. Glibenclamide’s effects on mito$K_{\text{ATP}}$ channels are difficult to assess, because it independently activates flavoprotein fluorescence, consistent with a previously described primary uncoupling effect. Confocal imaging of the subcellular distribution of expressed fluorescent Kir6.2 in HEK cells and in myocytes revealed no targeting of mitochondrial membranes. The differences in drug sensitivity and subcellular localization indicate that mito$K_{\text{ATP}}$ channels are distinct from surface $K_{\text{ATP}}$ channels at a molecular level.

In 1986, Murry and coworkers (Murry et al., 1986) described a paradoxical form of cardioprotection whereby brief ischemic insults can reduce the damage to the heart caused by subsequent, prolonged ischemia. This phenomenon, known as ischemic preconditioning, appears to involve ATP-sensitive potassium ($K_{\text{ATP}}$) channels as the end effectors. Such a key role was first attributed to the classical sarcolemmal $K_{\text{ATP}}$ (surface$K_{\text{ATP}}$) channels (Gross and Auchampach, 1992; Speegley-Dick et al., 1995; Liang, 1997; Kloner et al., 1998), but recent evidence implicates the more nebulous mitochondrial $K_{\text{ATP}}$ (mito$K_{\text{ATP}}$) channels (Inoue et al., 1991; Garlid et al., 1997; Liu et al., 1997; Sato et al., 1998).

Native cardiac mito$K_{\text{ATP}}$ channels share some pharmacological properties with their classical, sarcolemmal counterparts. Both channels are activated by pinacolide and inhibited by glibenclamide (Pacek et al., 1992; Garlid et al., 1997). Thus, specific inhibitors and activators are in demand for the study of cardiac mito$K_{\text{ATP}}$ channels; 5-hydroxydecanoate (5HD) and diazoxide are candidates for such specific reagents (Garlid et al., 1997; Liu et al., 1998; Sato et al., 1998). Nevertheless, there is controversy as to whether 5HD blocks surface$K_{\text{ATP}}$ channels (Notsu et al., 1992; Garlid et al., 1997; Liu et al., 1998). Likewise, the extent to which the activator diazoxide opens surface$K_{\text{ATP}}$ channels is a matter of some dispute (Inagaki et al., 1996; Garlid et al., 1997; Liu et al., 1998).

The molecular identity of cardiac mito$K_{\text{ATP}}$ channels remains unclear, although cardiac surface$K_{\text{ATP}}$ channels have been molecularly defined as an octameric complex of four pore-forming Kir6.2 subunits and four SUR2A sulfonylurea receptors (Inagaki et al., 1996; Clement et al., 1997). To

ABBREVIATIONS: 5HD, 5-hydroxydecanoate; DNP, 2,4-dinitrophenol; GFP, green fluorescent protein; EGFP, enhanced GFP; HEK, human embryonic kidney; Kir, inwardly rectifying potassium channels; $K_{\text{ATP}}$ channels, ATP-sensitive potassium channels; mito$K_{\text{ATP}}$, inner mitochondrial membrane $K_{\text{ATP}}$; SUR, Sulfonylurea receptor; surface$K_{\text{ATP}}$, sarcolemmal (surface membrane) $K_{\text{ATP}}$. 

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distinguish unambiguously between surface\(K_{\text{ATP}}\) and mito\(K_{\text{ATP}}\) channels, we compared native cardiac mito\(K_{\text{ATP}}\) channels to molecularly defined and native surface\(K_{\text{ATP}}\) channels. Differences in pharmacology and subcellular distribution reveal clear distinctions between cardiac mito\(K_{\text{ATP}}\) and surface\(K_{\text{ATP}}\) channels.

Materials and Methods

Functional Expression of \(K_{\text{r},6.2} + \text{SUR2A Channels}\). The transient transfection method in human embryonic kidney (HEK/293 cells was modified from Hu et al. (1997). Briefly, HEK293 cells were maintained in Dulbecco’s minimal Eagle’s medium with glucose and l-glutamine, supplemented with 10% fetal calf serum (GIBCO-BRL, Gaithersburg, MD) and 1% penicillin and streptomycin (GIBCO-BRL). Cells were plated on 35-mm Petri dishes at a density of 0.2 million cells/dish 1 day before transfection, and maintained in a 37°C incubator. Cells were then transfected by calcium phosphate precipitation (Graham and van der Eb, 1973; Calcium Phosphate Transfection System, GIBCO-BRL) with 0.5 to 1 \(\mu\)g/dish of equimolar plasmid DNAs of the rabbit cardiac Kir6.2 subunit (Hu et al., 1999) and the rat SUR2A sulfonylurea receptor (Inagaki et al., 1996), supplemented with 0.2 \(\mu\)g/dish of mitochondrial targeted green fluorescent protein, which enabled visual identification of transfected cells (Marshall et al., 1995). The calcium phosphate-DNA mixture was left on cells for 5 to 6 h before washing with PBS and the addition of fresh media.

Electrophysiology of Expressed \(K_{\text{r},6.2} + \text{SUR2A Channels}\). For functionally expressed surface\(K_{\text{ATP}}\) channels, electrophysiological recordings were made 18 to 48 h after transfection. A coverslip with cells was placed in a 0.3-ml perfusion chamber connected to a gravity-driven perfusion system. Flow was maintained throughout the experiment at rates of 2 to 3 ml/min. Membrane current was recorded using the whole-cell patch configuration (Hamill et al., 1981), with bath solution containing (in mM): 140 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), and 10 HEPES, pH 7.4 titrated with NaOH. The pipette solution contained: 120 mM K-glutamate, 5 mM K-EGTA, 25 mM KCl, 3 mM MgATP, and 10 mM HEPES, pH 7.2, titrated with KOH (21–22°C). Junction potential was corrected. In voltage-clamp recordings, a ramp voltage command from \(-100\) mV to +50 mV was applied over 100 ms every 10 s, from a holding potential of \(-80\) mV. Currents were recorded using a patch-clamp amplifier (Axopatch 200, Axon Instruments, Inc.), and sampled at 10 kHz after analog filtering at 2 to 5 kHz. Acquisition and analysis of the data were performed with custom software. For data analysis, the current at 0 mV was measured to assay \(K_{\text{ATP}}\) channel activity. The holding current at \(-80\) mV (near \(E_{\text{K}}\)) was also measured to verify the gigaseal stability. In the study of the effect of 5HD on membrane current, 1 min of \(K_{\text{ATP}}\) currents before drug application were averaged as the control, and the currents 5 to 6 min into drug application were averaged for comparison with the control. The effect of reagent diazoxide was assayed in a similar way, except that steady-state currents (typically achieved within 5–10 min) were measured.

Confocal Imaging Green Fluorescent Protein (GFP)-\(K_{\text{r},6.2}\), Mitochondrial GFP, and Mitochondria. The murine Kir6.2 cDNA (Inagaki et al., 1995) was amplified by the polymerase chain reaction and cloned into the vector pEGFP-C3 (CLONTECH, Palo Alto, CA) to make pEGFP-Kir6.2. This created a single open reading frame encoding a fusion protein between EGFP on the amino terminus and Kir6.2 on the carboxy terminus. Stable cell lines were created by transfecting HEK293 cells with linearized pEGFP-Kir6.2 using lipofectamine (Life Technologies, Gaithersburg, MD) followed by selection with genetinic sulfate at 500 \(\mu\)g/ml. Cells surviving selection were plated on glass coverslips and imaged. Mitochondrially targeted GFP (Rizzuto et al., 1995) was transiently transfected into HEK293 cells as described above, and the cells were imaged.

Terminally differentiated muscle cells such as heart cells are notoriously resistant to conventional transfection methods. To image the subcellular distribution of fluorescent Kir6.2 in cardiac myocytes, we created adenoviral vectors for injection of rabbit ventricular cells (isolated and cultured as described below). We first generated an isogenic Kir6.2.GFP fusion construct by amplifying the coding region of the rabbit Kir6.2 gene (Hu et al., 1999) by polymerase chain reaction to allow for in-frame fusion with EGFP in the vector pEGFP-N3 (CLONTECH) to create pRabKir6.2.GFP. The expression cassette was removed and cloned into the multiple cloning site of modified adenovirus shuttle vector pAdECD8I (Johns et al., 1999) to generate pAdECD8Kir6.2.GFP. The recombinant adenovirus containing the RabKir6.2.GFP fusion gene was generated by Cre-lox recombination as described previously (Johns et al., 1999). Isolated rabbit ventricular myocytes were coinfected with AdRabKir6.2.GFP and AdVgRXR (Johns et al., 1999) and plated on to coverslips in M199 culture medium with 5% fetal bovine serum at 37°C. Expression was induced by adding 5 \(\mu\)M ponasterone A for 72 h before imaging.

Confocal images were obtained using a 60× water immersion lens on a Diaphot 300 inverted fluorescence microscope with a PCM-2000 confocal scanning attachment (Nikon, Inc., Melville, NY). GFP was excited by the 488-nm line of an argon laser and the emission at 505 to 535 nm was imaged. Mitochondria were stained with tetramethylrhodamine ethyl ester (TMRE; added to the bath at a concentration of 100 nM and allowed to redistribute into the mitochondrial matrix for 15 min). TMRE was excited with the 543-nm line of a helium-neon laser and the 589–621-nm emission was imaged.

Flavoprotein Fluorescence and Electrophysiology of Rabbit Ventricular Myocytes. Ventricular myocytes were isolated from adult rabbit hearts by conventional enzymatic dissociation (Liu et al., 1996). Cells were then cultured on laminin-coated coverslips in M199 culture medium with 5% fetal bovine serum at 37°C. Experiments were performed over the next 2 days. Mitochondrial redox state was monitored by recording the fluorescence of flavin adenine nucleotide (FAD)-linked enzymes in the mitochondria and served as an index of mito\(K_{\text{ATP}}\) activity (Liu et al., 1998). Myocytes were superfused with external solution containing: 140 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 10 mM HEPES, pH 7.4, with NaOH (21–22°C). Endogenous flavoprotein fluorescence was excited for 100 ms every 6 s using a xenon arc lamp with a band-pass filter centered at 480 nm, and emitted fluorescence was recorded at 530 nm by a photomultiplier tube and digitized (Digidata 1200, Axon Instruments, Foster City, CA). By focusing on individual myocytes with a 40× objective, fluorescence was monitored from one cell at a time. The flavoprotein fluorescence signal was averaged during the excitation window and expressed as a percentage of the 2,4-dinitrophenol (DNP)-induced maximal oxidation.

In some experiments, whole-cell currents were recorded from myocytes. The internal pipette solution contained: 120 mM K-glutamate, 25 mM KCl, 0.5 mM MgCl\(_2\), 10 mM K-EGTA, 10 mM HEPES, and 1 mM MgATP (pH 7.2 with KOH). Whole-cell currents were elicited every 6 s from a holding potential of \(-80\) mV by two consecutive steps to \(-40\) mV (for 100 ms) and 0 mV (for 380 ms). Currents at 0 mV were measured 200 ms into the pulse.

Chemicals. Diazoxide and glibenclamide were purchased from Sigma Chemical Co. (St. Louis, MO). Pinacidil and 5HD were purchased from Research Biochemical International (Natick, MA). TMRE was obtained from Molecular Probes (Eugene, OR). To prepare stock solutions, 5HD and DNP were dissolved in water, and the other drugs were dissolved in dimethyl sulfoxide. Ponasterone A was purchased from Invitrogen (San Diego, CA) and used according to the manufacturer’s instructions.

Statistical Analyses. Pooled data are presented as mean ± S.E.M. Statistical comparison was evaluated by the two-tailed paired or unpaired Student’s \(t\) test where appropriate, with \(P < .05\) considered significant.
5HD Has Little Effect on Expressed Cardiac SurfaceKATP Channels. Figure 1 shows the effect of 5HD on currents elicited by 100 μM pinacidil in a HEK293 cell expressing K₆.2 + SUR2A, which forms cardiac-type surfaceKATP channels (Inagaki et al., 1996). Figure 1A shows a time course of pharmacological responses in a representative experiment quantified from membrane currents elicited by ramp pulses (as exemplified in Fig. 1B); these data illustrate that 500 μM 5HD did not convincingly inhibit the current, but subsequent application of glibenclamide (10 μM) did so completely. Fig. 1C shows the pooled results in a box graph, presented as the ratio of the current in the presence of both 5HD and pinacidil, to that originally induced by pinacidil alone. Overall, 5HD imposed no significant inhibitory effect (3 ± 3%, n = 5).

Diazoxide Weakly Activates Expressed Cardiac SurfaceKATP Currents. Diazoxide weakly activated expressed Kₓ₆.2 currents, as illustrated in Fig. 2. Upon washout of diazoxide, a subsequent application of 100 μM pinacidil activated much more current. Overall, 100 μM diazoxide elicited only 4 ± 1% (n = 26, P < 0.03) of the current activated by 100 μM pinacidil, which is a nearly saturating concentration in this expression system (Hu et al., 1998). Diazoxide-induced currents were completely suppressed by glibenclamide (n = 3, not shown).

Given that 100 μM diazoxide activates mitoKATP, but not surfaceKATP channels in native cardiomyocytes (Garlid et al., 1997; Liu et al., 1998), we questioned whether the minor activation of the expressed channels might have actually reflected cross talk between the mitochondrial and the sarcotemmal channels. We thus examined the effect of 5HD on diazoxide-induced currents in the expression system. Figure 2 shows the effect of 100 μM diazoxide and 500 μM 5HD on cells expressing K₆.2 + SUR2A channels. Diazoxide reversibly activated a small outward current at 0 mV. In the presence of 5HD, a second challenge with diazoxide induced a current comparable with the first one. After washing out diazoxide and 5HD, pinacidil activated a robust current, verifying that the channels were richly expressed. The inset shows pooled data of the effect of 5HD, presented as the ratio of the second current (diazoxide + 5HD) versus the first one (diazoxide only). Overall, 5HD had no effect on diazoxide-induced currents. Thus, diazoxide weakly activates surfaceKATP channels encoded by K₆.2 and SUR2A.

Surface Membrane KATP Currents in Heart Cells. The pattern of pharmacologic responsiveness observed for heterologously expressed channels encoded by K₆.2 and SUR2A also applies to the native channels in rabbit cardiac myocytes. We have previously shown that such channels are resistant to diazoxide but sensitive to pinacidil (Liu et al., 1998; Sato et al., 1998). Figure 3 shows that pinacidil readily elicits outward membrane current in a rabbit ventricular cell, an effect that cannot be suppressed by 5HD. The pooled data (Fig. 3B) extend our previous observations (Sato et al., 1998); taken together, the data in Figs. 1–3 verify that the molecularly defined surfaceKATP channels mimic the behavior of native cardiac surfaceKATP channels.

K₆.2 Does Not Target Mitochondrial Membranes. To address whether expressed K₆.2 subunits are targeted to mitochondrial membranes, we imaged HEK293 cells stably expressing the fusion construct GFP-K₆.2. Figure 4A–C shows the distribution of expressed K₆.2 subunits (Fig. 4A, green), the mitochondria of the cells (Fig. 4B, labeled red by TMRE), and the overlay image (Fig. 4C). Green signals are distributed in both the surface and internal membranes of cells, but this distribution does not overlap that of the red mitochondria. As a positive control, we imaged HEK293 cells transiently expressing mitochondrially targeted GFP in the same manner. The GFP shows up in the same organelles (Fig. 4D, green) that are labeled red by TMRE (Fig. 4E), making them yellow in the overlay image (Fig. 4F).

Expression of isogeneic fluorescently labeled Kir6.2 in cardiac myocytes revealed a similar lack of concordance between the subcellular distribution of the channel (Fig. 5A) and the
mitochondria (Fig. 5B). From these experiments, we conclude that Kir6.2 subunits do not target mitochondrial membranes.

5HD Inhibits mitoK$_{\text{ATP}}$ in Intact Rabbit Ventricular Myocytes. Having studied the effects of diazoxide and 5HD on expressed surfaceK$_{\text{ATP}}$ channels, we then examined their effects on the native mitoK$_{\text{ATP}}$ in rabbit cardiomyocytes. We previously established single-cell methodologies to assay surfaceK$_{\text{ATP}}$ and mitoK$_{\text{ATP}}$ channel activities by measuring membrane current and flavoprotein fluorescence simultaneously (Liu et al., 1998; Sato et al., 1998); in the present study, we verified the effects of the reagents on mitoK$_{\text{ATP}}$ channels in intact rabbit ventricular myocytes not invaded by patch pipettes.

Figure 6 shows that diazoxide (100 μM) reversibly increased the flavoprotein oxidation, and the oxidative effect was inhibited by 500 μM 5HD. DNP was introduced at

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\caption{Pharmacological sensitivity of native surfaceK$_{\text{ATP}}$ channels in rabbit ventricular myocytes. A, time course of membrane current elicited by pinacidil (100 μM) in the absence and presence of 5HD (500 μM). B, pooled data for pinacidil-induced membrane current measured in the absence and presence of 5HD. $P = \text{N.S.}$}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{fig4}
\caption{Confocal fluorescent images of HEK293 cells stably expressing Kir6.2 fused with GFP (left column, A–C), and HEK293 cells transiently expressing mitochondrial GFP (right column, D–F). Green signals indicate the locations of GFP-Kir6.2 (A) and mitochondrially targeted GFP (D), and red signals reveal the locations of mitochondria as the cells were loaded with TMRE (B and E). C and F, overlay images of A and B, and D and E, respectively.}
\end{figure}
end of each experiment to determine the maximal level of oxidation. As summarized in Fig. 6B, diazoxide increased flavoprotein oxidation to 38 ± 6% of that induced by DNP (n = 5), a value equivalent to the 34 ± 4% induced by 100 μM pinacidil (Sato et al., 1998). 5HD inhibited the effect of diazoxide to 7 ± 2% of the DNP level (n = 5, P < .005 versus diazoxide alone). These results indicate that diazoxide activates, and 5HD inhibits, mitoK_{ATP} channels in intact rabbit ventricular myocytes.

Glibenclamide Independently Activates Flavoprotein Fluorescence in Intact Rabbit Cardiomyocytes. Glibenclamide, a well-known sulfonylurea K_{ATP} channel blocker, has also been reported to be an uncoupler of mitochondria (Szweczyk et al., 1997). On uncoupling respiration from ATP synthesis, the mitochondrial redox potential is oxidized like that induced by DNP. We therefore examined the effect of glibenclamide on flavoprotein fluorescence. Figure 7 illustrates one such experiment. Glibenclamide (100 μM) induced reversible oxidation of the flavoprotein. This effect was concentration dependent. As summarized in Fig. 7B, 10 and 100 μM glibenclamide increased mitochondrial oxidation to 15 ± 4% (n = 4) and 27 ± 4% (n = 5), respectively. Thus, glibenclamide is not a useful probe of mitoK_{ATP}−induced mitochondrial oxidation.

Discussion

We have shown that expressed cardiac K_{ATP} channels (Kir6.2 + SUR2A) are activated strongly by pinacidil but only weakly by diazoxide, while being blocked by glibenclamide and essentially insensitive to 5HD. This pharmacology contrasts with that of mitoK_{ATP} channels as indexed by flavoprotein oxidation in intact rabbit ventricular myocytes. MitoK_{ATP} channels are activated equally by diazoxide and pinacidil, and 5HD is an effective blocker of mitoK_{ATP} channels. These differences establish major pharmacological distinctions between the two channels at a functional level. In addition, confocal imaging reveals that expressed K_{6.2} subunits target sarcolemmal but not mitochondrial membranes. These key distinctions indicate that mitoK_{ATP} channels differ importantly from surfaceK_{ATP} channels at the molecular level.

In this study, we provide the first direct evidence that glibenclamide alone (10 μM or 100 μM) causes mitochondrial oxidation. This effect of glibenclamide precludes investigation in intact cells of its reported inhibition of mitoK_{ATP} channels in isolated mitochondria or reconstituted liposomes (Inoue et al., 1991; Paucek et al., 1992); nevertheless, the observations are consistent with the reported uncoupling effect of glibenclamide on mitochondria (Szweczyk et al., 1997). In addition, glibenclamide effectively blocks both native and expressed cardiac surfaceK_{ATP} channels (Edwards and Weston, 1993; Inagaki et al., 1996; Hu et al., 1999). Thus, glibenclamide is a less-than-optimal probe in the study of mitoK_{ATP} channels.

The results with glibenclamide illustrate one limitation of the methodology that we have used to detect mitoK_{ATP} activity. The method relies upon flavoprotein fluorescence to...
report the redox potential of the mitochondrial matrix. Although opening of inner membrane potassium channels will tend to dissipate the mitochondrial potential and lead to net oxidation of the matrix, so will uncouplers such as glibenclamide and DNP (Liu et al., 1998). The only other approaches devised so far to detect mitoK\textsubscript{ATP} activity use isolated mitochondria (Inoue et al., 1991; Garlid et al., 1997), which necessarily involves removing the organelles from their physiological surroundings. Thus, the two types of approaches have complementary strengths and limitations. It is therefore reassuring that similar conclusions regarding the pharmacology of mitoK\textsubscript{ATP} channels have been derived from studies of flavoprotein fluorescence in intact myocytes and potassium accumulation in isolated mitochondria (Garlid et al., 1997; Liu et al., 1998).

Identifying a specific mitoK\textsubscript{ATP} channel blocker is very important, especially given that mitoK\textsubscript{ATP} channels have been pinpointed as likely effectors of ischemic preconditioning (Garlid et al., 1997; Liu et al., 1998). Our data identify 5HD as a specific blocker of mitoK\textsubscript{ATP} channels. At 500 \textmu M, 5HD did not inhibit pinacidil-induced currents through expressed or native cardiac surfaceK\textsubscript{ATP} channels, nor did it suppress diazoxide-induced surfaceK\textsubscript{ATP} currents. In native cardiomyocytes, 5HD dramatically suppresses mitoK\textsubscript{ATP} channel activity, yet it does not affect surfaceK\textsubscript{ATP} currents (McCullough et al., 1991; Garlid et al., 1997; Liu et al., 1998; Sato et al., 1998), with the exception of one report that 5HD blocks surfaceK\textsubscript{ATP} channels in excised patches from guinea pig cardiomyocytes (Notsu et al., 1992). The reason for this contradictory observation remains unclear. In animal studies, 5HD has long been recognized to abolish cardioprotection (Auchampach et al., 1992; Hide and Thiemermann, 1996; Schultz et al., 1997). The establishment of its specificity further implicates mitoK\textsubscript{ATP} channels as key players in the mechanism of ischemic preconditioning.

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References


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