Pharmacological opening of mitochondrial cardiac ATP-sensitive potassium (K\textsubscript{ATP}) channels has the chance to be a promising but still controversial cardioprotective mechanism. Physiological roles of mitochondrial K\textsubscript{ATP} channels in the myocardium remain unclear. We studied the effects of diazoxide, a specific opener of these channels, on the function of rat mitochondria in situ in saponin-permeabilized fibers using an ionic medium that mimics the cytosol. In the presence of NADH-producing substrates (malate + glutamate), neither 100 \( \mu \text{M} \) diazoxide nor 100 \( \mu \text{M} \) glibenclamide (a K\textsubscript{ATP} channel blocker) changed the mitochondrial respiration in the absence or presence of ADP. Because the K\textsubscript{ATP} channel function could be modified by changes in adenine nucleotide concentrations near the mitochondria, we studied the effects of diazoxide and glibenclamide on the functional activity of mitochondrial kinases. Both diazoxide and glibenclamide did not change the in situ ADP sensitivity in the presence or absence of creatine (apparent \( K_m \) values for ADP were, respectively, 59 ± 9 and 379 ± 45 \( \mu \text{M} \)). Similarly, stimulation of the mitochondrial respiration with AMP in the presence of ATP due to adenylate kinase activity was not affected by the modulators of K\textsubscript{ATP} channels. However, when succinate was used as substrate, diazoxide significantly inhibited basal respiration by 22% and maximal respiration by 24%. Thus, at a cardioprotective dose, the main functional effect of diazoxide depends on respiratory substrates and seems not to be related to K\textsubscript{ATP} channel activity.

A number of studies have shown that substances that are able to open ATP-sensitive potassium (K\textsubscript{ATP}) channels (diazoxide, chromakalim) have cardioprotective effects (for recent review, see Refs. 1 and 2). These substances have beneficial effects on the myocardium in various models of ischemia and induce preconditioning, which is able to protect the cardiac tissue against subsequent episodes of ischemia. This protection may be blocked by antagonists of K\textsubscript{ATP} channels (glibenclamide, 5-hydroxydecanoate).

The precise cellular mechanisms by which K\textsubscript{ATP} openers exert their cardioprotective effects still remains under debate. Initially, it was proposed (3) that opening of sarcolemmal K\textsubscript{ATP} channels would enhance the shortening of the cardiac action potential thus inhibiting calcium entry into the cell. However, numerous studies (4–6) have shown an absence of correlation between action potential duration and cardioprotective action of K\textsubscript{ATP} channel openers.

Another site of action that has been proposed for K\textsubscript{ATP} channel openers is the recently identified mitochondrial K\textsubscript{ATP} channel (7, 8). It was demonstrated that the K\textsuperscript{+} channel opener diazoxide that is selective for the mitochondrial channels (9) produced a cardioprotective effect in isolated rat hearts and that this protection was independent of sarcolemmal K\textsubscript{ATP} channels. In addition, the protection could be reversed by the channel antagonists, glibenclamide and 5-hydroxydecanoate. A similar protective effect of diazoxide was shown in rabbit myocytes killed in a model of simulated ischemia (10). These results suggested that diazoxide and perhaps other K\textsubscript{ATP} openers were interacting with the mitochondrial K\textsubscript{ATP} channels to produce cardioprotection.

It is unclear, however, why opening of mitochondrial K\textsubscript{ATP} channels would be cardioprotective. It has been suggested (10) that K\textsuperscript{+} influx into the mitochondrial matrix would dissipate the potential across the inner membrane and uncouple electron transfer. In addition, K\textsuperscript{+} influx would increase matrix volume (mitochondrial swelling) (11). The physiological significance of the K\textsubscript{ATP} channels opening for the cellular energetics remains obscure.

Although intracellular ATP is the endogenous inhibitor of K\textsubscript{ATP} channels and a low ADP concentration serves as a channel activator (12), it is still a matter of debate whether changes in the intracellular concentrations of ATP, ADP, and/or in the ATP/ADP ratio could account for the transition between the ATP- (closed) and ADP-liganded (open) states. The microenvironment of the K\textsubscript{ATP} channel harbors phosphotransfer enzymes (creatine kinase, adenylate kinase) that are able to transfer phosphohydrils between ATP and ADP and in such a way potentially regulate K\textsubscript{ATP} channel activity (for review, see Dzeja and Terzic (13) and references therein). It has been suggested that this activity could be regulated through interactions between different kinases (13). However, to our knowledge, effects of modulators of K\textsubscript{ATP} channels on the cellular phosphotransfer systems have not been studied yet.

In the present work we investigate the effects of diazoxide and a K\textsubscript{ATP} channel antagonist, glibenclamide, on mitochondrial activity in permeabilized cardiac fibers in situ in ionic medium mimicking the cytosol. Using such a model appears to be important because factors that are able to change mitochondrial volume could potentially have different effects in isolated mitochondria and in mitochondria kept in their natural environment including the cytoskeleton. Indeed, a cardioprotective effect of diazoxide could be abolished by disruption of the cytoskeleton (14). Because some natural compounds in the cytosol like taurine (15) or sulfhydryl group reducers (12) may affect the K\textsubscript{ATP} channel state, whereas proteins can bind diazoxide (16), in the present work we have tried to mimic, as closely as possible, the cellular conditions at which diazoxide exerts its...
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**Table I**

| Parameter                     | $V_0$ | $V_{max}$ | ACR | $K_{m(app)}$ (Cr) | $K_{m(app)}$ (+Cr) |
|-------------------------------|-------|-----------|-----|------------------|-------------------|
|                               | µmol O$_2$/min/g dry weight | µmol O$_2$/min/g dry weight | µM  | µM               | µM                |
| Me$_2$SO (8–10)               | 4.04 ± 0.40 | 24.7 ± 1.5 | 6.66 ± 0.96 | 374 ± 41 | 61 ± 10 |
| Diazoxide (8–10)              | 3.55 ± 0.50 | 23.5 ± 2.2 | 7.68 ± 1.22 | 370 ± 48 | 69 ± 9  |
| Control (9–12)                | 5.18 ± 0.67 | 28.6 ± 1.0 | 8.72 ± 1.05 | 316 ± 45 | 86 ± 7  |
| Glibenclamide (9–12)         | 5.59 ± 0.84 | 28.4 ± 2.0 | 5.53 ± 0.85 | 236 ± 25 | 93 ± 15 |

Table I shows the respiratory parameters of mitochondria in the presence of diazoxide or glibenclamide on respiratory parameters of the total mitochondrial population at various ADP concentrations in the presence and absence of creatine. In the latter case, the absence of ADP-regenerating creatine kinase activity near the inner mitochondrial membrane allowed us to decrease the local ADP/ATP ratio in the intermembrane space. We also investigated effects of the $K_{ATP}$ channel modulators when another system (adenylate kinase), which regulates the local ATP/ADP ratio, was activated.

**EXPERIMENTAL PROCEDURES**

**Functional Properties of Mitochondria—**Respiratory parameters of the total mitochondrial population were studied in situ in saponin-permeabilized fibers using the method described earlier (17) with minor changes (18). Male Wistar rats were anesthetized with pentobarbital, and thin fiber bundles (100–250 µm in diameter) were excised from the subendocardial surface of the left ventricle. The bundles were incubated with intense shaking for 30 min in solution S (see below) containing 50 µg/ml saponin to selectively destroy the integrity of the sarcolemma. The bundles were then transferred into solution R (see below) for 10 min to wash out adenine nucleotides and phosphocreatine. All procedures were carried out at 4 °C.

Respiratory rates were determined by a Clark electrode (Strathkelvin Instruments, Glasgow, United Kingdom) in an oxygengraphic cell containing 15–20 fiber bundles in 3 ml of solution R at 22 °C with continuous stirring. The solubility of oxygen was taken to be 230 mmol of O$_2$/liter. After measurement, the bundles were removed and dried. Respiration rates were expressed as nanomoles of O$_2$/min/mg dry weight.

To obtain respiratory parameters at various ADP concentrations, cardiac fibers were exposed to increasing ADP in the presence (20 mM) or in the absence of creatine. The ADP-stimulated respiration above the basal oxygen consumption was plotted to determine the apparent $K_{m}$ for ADP and $V_{max}$. Adenylate kinase functional activity was evaluated by an increase in the respiration rate after addition of 2 mM AMP in the presence of 0.2 mM ATP.

**Solutions and Reagents—**Solutions S and R contained 10 mM EGTA-Ca$^{2+}$EGTA buffer (free Ca$^{2+}$ concentration, 100 nM), 1 mM free Mg$^{2+}$, 20 mM taurine, 0.5 mM dithiothreitol, and 20 mM imidazole. Ionic strength was adjusted to 0.16 M by addition of potassium methanesulfonate. Solution S (pH 7.0) also contained 5 mM MgATP and 15 mM phosphocreatine. Solution R (pH 7.1) contained 3 mM phosphate and 2 mg/ml fatty acid-free bovine serum albumin instead of high energy phosphates. 5 mM glutamate + 2 mM malate or 10 mM succinate + 10 mM rotenone were used as substrates.

All reagents were purchased from Sigma, except phosphocreatine (Neaton, Schiaparelli Farmaceutica SEARLE, Turin, Italy) which was a kind gift from Prof. E. Strumia.

**Statistical Analysis—**The data are expressed as the mean ± S.E. Mean values were compared using one-way analysis of variance and Dunnett post hoc analysis. A difference was considered statistically different when the $p$ value was less than 0.05. Nonlinear fits to Michaelis-Menten kinetics were computed by a nonlinear least square routine.

**RESULTS**

Table I shows the respiratory parameters of mitochondria in permeabilized fibers using NADH-generating substrates (glutamate + malate) in the solution $R$ containing 116 mM K$^+$ in the presence of either 100 µM diazoxide or its solvent 0.1% dimethyl sulfoxide (Me$_2$SO). Such a concentration of diazoxide was shown to exert a prominent cardioprotective effect (9, 10). Besides, Garlid (11) testing five different diazoxide concentrations found that the optimal cardioprotection took place when 100 µM diazoxide was used. Basal respiration in the absence of adenine nucleotides ($V_0$) was considerably (about 7-fold) augmented by stepwise increases in [ADP] (Fig. 1), thus showing a good coupling between oxidation and phosphorylation. Diazoxide changed neither basal nor maximal ADP-stimulated ($V_{max}$) oxygen consumption rate. The respiratory activity of mitochondria at intermediate ADP concentrations was not modified by diazoxide as can be seen from the same values of Michaelis constant for ADP in the presence and in the absence of the $K_{ATP}$ channel opener. Creatine considerably decreased $K_{m}$ for ADP due to local ADP regeneration catalyzed by mitochondrial creatine kinase. Diazoxide did not change the respiratory kinetic parameters in the presence of creatine thus indicating the insensitivity of creatine kinase to this substance (Table 1).

Under our conditions, diazoxide did not exert any uncoupling action on the mitochondria in situ as can be seen from unchanged values of $V_0$ and acceptor control ratio ($V_{max}/V_0$). We did a positive control experiment to check if uncoupling effect could be demonstrated in permeabilized fibers. Indeed, 1 µM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, significantly increased the basal respiration rate from 6.12 ± 0.41 to 27.8 ± 3.7 µmol of O$_2$/min/mg dry weight ($n = 6; p < 0.001$).

We also checked the hypothesis that under our conditions, the $K_{ATP}$ channels are already opened so that diazoxide could not have any effect. Table I shows the respiratory parameters of the permeabilized fibers in the presence of 100 µM glibenclamide, a $K_{ATP}$ channel antagonist. Such a concentration was chosen because it was shown that the $K_i$ for glibenclamide’s specific inhibitory effect on the $K_{ATP}$ channels was in the micromolar range (19). As can be seen from the table, glibenclamide did not have any effect on the mitochondrial respiration in situ. A higher glibenclamide concentration of 500 µM led to a significant inhibition of the maximal oxygen consumption rate (22.3 ± 0.2 µmol of O$_2$/min/dry weight; $p < 0.02$ versus control) due, most probably, to nonspecific effects on the mitochondrial respiration as suggested by Garlid’s group (19).

All these experiments were carried out in the absence of added ATP, although some ATP existed near the mitochondria due to oxidative phosphorylation. This could be shown by the effect of creatine on respiration. Thus, we tested the effects of $K_{ATP}$ channel modulators on the (glutamate + malate)-supported respiration in the presence of 200 µM exogenous ATP. This induced an increase in respiration due to endogenous ATP production by intrafiber ATPas. Diazoxide did not change the

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1 The abbreviations used are: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; PKC, protein kinase C.
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Fig. 1. Permeabilized fiber respiration in the presence of 100 μM diazoxide. A, oxygraph traces of respiratory activities at different ADP concentrations (indicated in μM). Upper curve, fibers (0.40 mg dry weight) in the presence of 0.1% Me2SO (DMSO). Lower curve, fibers (0.39 mg dry weight) in the presence of 100 μM diazoxide. B, oxygen consumption rate values fitted by a Michaelis-Menten equation. ■, Me2SO; □, diazoxide.

Fig. 2. Effects of glibenclamide or glibenclamide + diazoxide on the permeabilized fiber respiration rate in the presence of 200 μM ATP (V_ATP) and 200 μM ATP + 2 mM ADP + 20 mM creatine (V_max). *p < 0.05; **p < 0.01. DMSO, Me2SO.

The oxygen consumption rate (10.4 ± 0.6 μmol of O2/min/mg dry weight; n = 14) as compared with its control, 0.1% Me2SO (11.0 ± 1.0 μmol of O2/min/mg dry weight; n = 13). However, glibenclamide significantly decreased mitochondrial respiration (Fig. 2). Without glibenclamide, addition of 200 μM ATP increased the oxygen consumption rate from 5.23 ± 0.6 to 10.8 ± 0.7 μmol of O2/min/mg dry weight (n = 12), but in the presence of the KATP channel antagonist, the respiration increased to only 8.86 ± 0.45 μmol of O2/min/mg dry weight (n = 12; p < 0.05). To check if this effect was related to the closing of KATP channels, we investigated it in the presence of 100 μM diazoxide. The results are presented in Fig. 2. Respiration in the presence of 0.1% Me2SO was taken as control. Glibenclamide markedly decreased the respiration in the presence of only 200 μM ATP and in the presence of ATP + 2 mM ADP. Diazoxide did not reverse this effect.

We studied the effects of the KATP channel modulators on the functional activity of adenylate kinase in situ, another enzyme regulating the ADP/ATP ratio near the mitochondria and potentially capable of influencing the channel activity. Addition of 200 μM ATP induced a submaximal respiration rate that was considerably augmented by a subsequent addition of 2 mM AMP. This augmentation was evidently induced by an intrafiber ADP production due to adenylate kinase fixed to intracellular organelles like mitochondria and myofibrils. The percentage of this respiration rate increment could be taken as an index of the functional activity of cellular adenylate kinase. Table II shows that this parameter was not changed by the modulators of KATP channels. To check that respiration in the presence of ATP + AMP did not reach the saturation level, a high ADP concentration (2 mM) and 20 mM creatine were added at the end of the experiment. After this addition, the oxygen consumption rate was further increased. Note that glibenclamide in the presence of exogenous ATP consistently decreased the respiration both in the presence and in the absence of AMP or ADP.

All these experiments were performed in the presence of NADH-generating substrates, glutamate and malate. To test if the modulators of KATP channels could affect mitochondrial function when the respiratory chain used other reduced equivalents, this function was studied in the presence of 10 mM succinate. Complex I activity was blocked by 10 μM rotenone. Oxygen consumption rate in the absence (Vₕ) and in the presence of 2 mM ADP + 20 mM creatine (V_max) are presented in Fig. 3. Diazoxide significantly diminished the succinate-supported basal respiration by 22% and the maximal ADP-stimulated respiration by 24%. To test if such an effect is related to the KATP channel opening, 100 μM glibenclamide was added into the medium. Surprisingly, the KATP channel antagonist appeared to accentuate the diazoxide effect at least on the maximal respiration rate. Glibenclamide itself had no influence on the permeabilized fiber oxygen consumption (Fig. 3).

Diazoxide inhibited also uncoupled succinate-supported respiration. In the presence of 1 μM FCCP, addition of 0.1% Me2SO decreased the respiration rate by 38 ± 4% (n = 8) whereas diazoxide + Me2SO diminished this parameter by 51 ± 2% (n = 8; p < 0.02).

DISCUSSION

The main result of this work is that neither diazoxide nor glibenclamide at relatively high concentrations (100 μM) affected the functional activity of cardiac mitochondria in situ by modulating the KATP channel activity (that is by inducing or blocking the uncoupling effect). However, these substances have other effects on mitochondria that depend on the substrate used for oxidation.

The beneficial effects of KATP channel openers was proposed to be related to dissipation of the mitochondrial membrane potential (10). Opening of potassium-selective ion channels in the energized inner mitochondrial membrane, in contact with a
K⁺-rich extramitochondrial milieu, would induce an inward potassium flux. As a consequence, the membrane potential established by the proton pump would decrease. This depolarization would accelerate electron transfer and diminish the ADP phosphorylation rate, in other words, produce an uncoupling effect. It has been suggested (10) that such a depolarization would accelerate electron transfer and diminish the respiration rate due to regeneration of ADP from ATP in the creatine kinase reaction. Such an absence of uncoupling effect on the mitochondrial under various conditions makes unlikely the possibility that diazoxide-induced K⁺ flux into the mitochondrial matrix could induce a significant dissipation of the membrane potential. Interestingly, 100 μM diazoxide slightly depolarized the isolated liver mitochondria (20). However, in intact pancreatic B-cells this compound at the same concentration produced only minimal changes in the inner membrane potential. In intact cardiomyocytes, a dissipation of mitochondrial membrane potential was recorded when a higher diazoxide concentration (300 μM) was used (20).

It is noteworthy that even in isolated mitochondria the K<sub>ATP</sub> channel openers do not always have an uncoupling action. Szewczyk et al. (22) tested five various K<sub>ATP</sub> channel openers, and only one of them was able to induce depolarization of rat liver mitochondria. However, other K<sub>ATP</sub> channel openers (pinacidil, cromakalim, and levcromakalim) were shown to markedly decrease the mitochondrial membrane potential (23). Garlid et al. (9, 24) have demonstrated that K<sub>ATP</sub> channel openers including diazoxide are efficient only when transmembrane mitochondrial K⁺ flux is inhibited by ATP and magnesium. Other authors have shown that diazoxide is able to affect the membrane potential and consequently Ca<sup>2+</sup> uptake by isolated mitochondria in the absence of both ATP and Mg<sup>2+</sup> (21).

Our results clearly suggest that in the absence of exogenous ATP, modulators of K<sub>ATP</sub> channels do not change the maximal glutamate + malate supported activity of in situ mitochondria. These substances apparently do not affect the functional activity of cellular kinases (creatine kinase and adenylate kinase) that control the ATP/ADP ratio in the vicinity of the mitochondria and that are theoretically also able to modulate the activity of the K<sub>ATP</sub> channels. One must take into account, however, that the methods used to assess the functional activity of these kinases in situ were semiquantitative, and it is possible that these enzymes, being nonlimiting for respiration, were somehow affected by the substances tested. Even if this were the case, however, such a hypothetical inhibition would have no significant physiological effect.

In the presence of exogenous ATP, however, glibenclamide significantly inhibited ATP-stimulated respiration. Evidently, this effect does not seem to be related to the activity of specific K<sub>ATP</sub> channels because diazoxide did not block such an effect of glibenclamide. As oxygen consumption by permeabilized fibers depends on the ADP/ATP ratio, in the presence of exogenous ATP any inhibition of cellular ATPase activity would slow down the respiration rate. Indeed, glibenclamide was shown to inhibit Na⁺/K⁺-ATPase activity (25). This could play an important role in total cellular ATPase activity when myofibrillar and sarcoplasmic reticulum ATPases are inhibited due to low free Ca<sup>2+</sup> concentration.

**TABLE II**

Effects of diazoxide and glibenclamide on the functional activity of adenylate kinase in situ

|        | V<sub>ATP</sub> | V<sub>ATP + AMP</sub> | %AMP | V<sub>max</sub> |
|--------|----------------|-----------------------|------|---------------|
|        | μmol O₂/min/g  | μmol O₂/min/g         | %AMP | μmol O₂/min/g |
|        | dry weight     | dry weight             |      | dry weight    |
| Me₂SO (14) | 11.0 ± 1.0   | 18.4 ± 0.9            | 75 ± 8  | 29.4 ± 1.9   |
| Diazoxide (13) | 10.4 ± 0.6  | 17.5 ± 0.7            | 69 ± 4   | 27.3 ± 1.1   |
| Control (12)    | 10.8 ± 0.7   | 18.8 ± 1.2            | 78 ± 7   | 28.1 ± 1.5   |
| Glibenclamide (12) | 8.9 ± 0.45a | 15.0 ± 0.9b           | 68 ± 6   | 21.8 ± 1.2b  |

* p < 0.05 compared to respective control.

**FIG. 3. Effects of K<sub>ATP</sub> channel modulators on the succinate-supported permeabilized fiber respiration.** A, effect of diazoxide in the absence or in the presence of glibenclamide on the basal (V<sub>o</sub>) and maximal (2 mM ADP + 20 mM creatine, V<sub>max</sub>) respiration. B, effect of glibenclamide on the basal (V<sub>o</sub>) and maximal (2 mM ADP + 20 mM creatine, V<sub>max</sub>) respiration. DMSO, Me₂SO.
In contrast to the data obtained when glutamate and malate were used as respiratory substrates, mitochondrial respiration supported by succinate was markedly inhibited by diazoxide. Interestingly, this inhibition was not dependent on the presence of ADP. Addition of glibenclamide did not block the effect of diazoxide thus suggesting again that it is not related to the KATP channel opening. Because diazoxide had no effect on (glutamate + malate)-supported respiration, it was reasonable to suggest that this substance could inhibit the oxidation of succinate. Our results are in line with those obtained by Marban’s group (10, 28) concerning the effect of diazoxide on mitochondria. These authors demonstrated a considerable increase in mitochondrial flavoprotein fluorescence in ventricular myocytes induced by diazoxide. This effect was blocked by a high concentration (500 μM) of 5-hydroxydecanoic acid, a KATP channel antagonist, but not by glibenclamide. The authors ascribed the effect of diazoxide to a dissipation of the inner mitochondrial membrane potential due to increased K+ influx. This was followed by an acceleration of the electron transfer, uncompensated by increased production of electron donors. However, our result showing inhibition of succinate-supported respiration in situ suggests that another mechanism of the flavoprotein redox state alteration may exist. By inhibiting the production of reduced equivalents, diazoxide could at least partially contribute to the elevated oxidized/reduced ratio for flavoprotein.

Actually, the mechanism of the beneficial effect of diazoxide as well as other KATP channel openers is not clear. Moreover, there is not yet a clear demonstration that it is mitochondrial KATP channel opening that induces cardioprotection. Generally, such a conclusion comes from the experiments with substances known as KATP channel modulators (diazoxide, glibenclamide, 5-hydroxydecanoate), where mitochondrial channel activity itself is not determined. However, these substances may have targets other than mitochondrial KATP channels. Diazoxide, for example, is also a known inhibitor of phosphodiesterase (29). It has been shown that blockade of Ca2+ entry during diazoxide treatment of rat hearts, by inhibiting L-type Ca2+ channels with verapamil or nifedipine, completely reverses the beneficial effect of diazoxide during ischemia and reperfusion (30). Furthermore, the effect of diazoxide is potentiated (28) or even mediated (30, 31) by activation of the cellular protein kinase C (PKC) signal pathway. Diazoxide induced translocation of PKC isoforms to different intracellular organelles, PKC-α to sarcolemma, PKC-δ to mitochondria, PKC-ε to intercalated discs. Administration of PKC inhibitors completely abolished diazoxide-induced cardioprotection thus indicating a crucial role of the PKC activation.

In general, our data demonstrate that besides inhibition of succinate oxidation, diazoxide does not directly modulate the mitochondrial functional activity. It can be suggested that the cardioprotective action of diazoxide is not mediated by simple changes in mitochondrial function produced by this substance. More likely, the effect of diazoxide on cardiac cells, at least that inducing the preconditioning, includes a complex interaction of several subcellular compartments. Does the mitochondrial KATP channel opening precede activation of the PKC cascade or conversely, does stimulation of the channel activity in living cells activate the PKC pathway that in turn mediates cardioprotection? This is a question that deserves an intensive study in the near future.

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