From single K ATP channel recordings, we excluded a direct subsequent declining phase (t5 0.88 min), ATP hydrolysis, had little immediate effect on rapidly switches mitochondria from net ATP synthesis to net or the mitochondrial uncoupler dinitrophenol (DNP), which perturbation. In rabbit ventricular myocytes, either pinacidil index of subsarcolemmal energy state during mitochondrial perturbation. In rabbit ventricular myocytes, either pinacidil or the mitochondrial uncoupler dinitrophenol (DNP), which rapidly switches mitochondria from net ATP synthesis to net ATP hydrolysis, had little immediate effect on K,ATP. In contrast, in the presence of pinacidil, exposure to 100 μM DNP rapidly activated K,ATP with complex kinetics consisting of a quick rise [time constant of K,ATP increase (τ) = 0.13 ± 0.01 min], an early partial recovery (τ = 0.43 ± 0.04 min), and then a more gradual increase. This DNP-induced activation of K,ATP was reversible and accompanied by mitochondrial flavoprotein oxidation. The F1F0-ATPase inhibitor oligomycin abolished the DNP-induced activation of K,ATP. The initial rapid rise in K,ATP was blunted by atracyloside (an adenine nucleotide translocator inhibitor), leaving only a slow increase (τ = 0.66 ± 0.17 min, P < 0.01). 2,4-Dinitrofluorobenzene (a creatine kinase inhibitor) slowed both the rapid rise (τ = 0.20 ± 0.01 min, P < 0.05) and the subsequent declining phase (τ = 0.88 ± 0.19 min, P < 0.05). From single K,ATP channel recordings, we excluded a direct effect of DNP on K,ATP channels. Taken together, these results indicate that rapid changes in F1F0-ATPase function dramatically alter subsarcolemmal energy charge, as reported by pinacidil-primed K,ATP channel activity, revealing cross-talk between mitochondria and sarcolemma. The effects of mitochondrial ATP hydrolysis on sarcolemmal K,ATP channels can be rationalized by reversal of F1F0-ATPase and the facilitation of coupling by the creatine kinase system.

dinitrophenol; ATP hydrolysis; patch-clamp; ATP-sensitive K+ channels

MITOCHONDRIAL F1F0-ATPASE generates ATP from ADP using the proton gradient established by the electron transport chain of the inner membrane. Mitochondrial uncouplers such as 2,4-dinitrophenol (DNP) or carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) are chemical protonophores that dissipate the mitochondrial matrix NADH (8, 22). Mitochondrial uncoupling can also rapidly induce net ATP hydrolysis as a result of reverse-mode F1F0-ATPase activity (6, 17). Thus mitochondrial ATP consumption is likely to play a significant role in the cellular response to mitochondrial permeability transitions (6, 10), redox oscillations (20), and ischemia (25, 26), conditions that dissipate the mitochondrial inner membrane potential. Exposure to a mitochondrial uncoupler changes cardiac muscle contractility before activation of sarcolemmal ATP-sensitive K+ (K,ATP) channels (1). In isolated ventricular myocytes, FCCP immediately decreases the cytosolic Ca2+ transient and increases diastolic Ca2+ concentration, suggesting that impaired Ca2+ handling precedes significant activation of sarcolemmal K,ATP channels (9).

Two factors are likely to be involved in preventing early K,ATP channel activation in the face of impaired mitochondrial function. First, ATP derived from nearby glycolytic enzymes may provide effective spatiotemporal inhibition of K,ATP channels (27, 28). Second, the creatine kinase (CK) system is an important buffer of high-energy phosphate (14), maintaining the cytosolic ATP level despite perturbations of mitochondrial energy metabolism (12). On the other hand, the CK system also acts as an ATP shuttle, connecting the sites of energy production to the sites of energy consumption including sarcolemma (14). If the mitochondrial membrane potential collapses and the mitochondria switch from ATP generation to ATP consumption, this change could also be rapidly transmitted back to sarcolemma by the CK system even if the bulk ATP concentration were above that required to inhibit K,ATP channels.

The present study provides novel evidence for close coupling between mitochondrial energy production and/or consumption and subsarcolemmal energy charge. To assay energetics in the subsarcolemmal space, we converted K,ATP channels into sensitive reporters of local energy metabolism. Such channels normally require extreme ATP depletion (and/or ADP accumulation) to open (19), but their nucleotide sensitivity can be shifted into the physiological range by exposure to K,ATP channel agonists such as pinacidil (13). Thus our strategy was to manipulate mitochondrial uncoupling by exposure to mitochondrial uncouplers such as pinacidil or dinitrophenol. ATP hydrolysis, had little immediate effect on rapidly switches mitochondria from net ATP synthesis to net ATP hydrolysis, had little immediate effect on mito-
drial energetics while measuring drug-primed $K_{\text{ATP}}$ current ($I_{K_{\text{ATP}}}$) as an indicator of nucleotide concentrations just under the surface membrane.

**MATERIALS AND METHODS**

**Materials.** Collagenase (type II) was purchased from Worthington (Lakewood, NJ). DNP, FCCP, NaCN, glibenclamide, oligomycin, atractyloside, and 2,4-dinitrofluorobenzene (DNFB) were obtained from Sigma (St. Louis, MO). Pinacidil was purchased from Research Biochemicals International (Natick, MA). Pinacidil, glibenclamide, oligomycin, and atractyloside were dissolved in DMSO before being added into experimental solutions. The final concentration of DMSO was <0.3%.

**Cell isolation.** New Zealand White rabbits of either sex (1–2 kg) were anesthetized by intravenous injection of pentobarbital (30 mg/kg) until consciousness was lost. After the absence of a corneal reflex was confirmed, hearts were rapidly removed and mounted on a Langendorff apparatus. Ventricular myocytes were isolated by conventional enzymatic dissociation as described previously (18, 23). Hearts were perfused with a constant flow (12–14 ml/min at 37°C) for 5 min with modified Tyrode solution (containing (in mM) 140 NaCl, 5 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose (pH 7.4 with NaOH)) and then 5 min of Ca$^{2+}$-free Tyrode, 20 min of Ca$^{2+}$-free Tyrode containing collagenase (1 mg/ml), and 5 min of Ca$^{2+}$-free Tyrode solution sequentially. Cells were then cultured on laminin-coated coverslips in M199 culture medium with 2% fetal bovine serum at 37°C. Experiments were performed 24–48 h after isolation.

**Electrophysiology and flavoprotein fluorescence measurement.** For whole cell patch-clamp recordings, the internal pipette solution contained (in mM) 120 potassium glutamate, 25 KCl, 1 MgCl$_2$, 10 EGTA, 10 HEPES, and 3 MgATP (pH 7.2 with KOH). The composition of the external solution was the same as the Tyrode solution used for cell isolation. The K$^+$-free external solution (Fig. 4A) was made by omitting K$^+$ from the Tyrode solution. Currents were elicited every 6 s from a holding potential of −80 mV by consecutive steps to −40 mV for 100 ms and then to 0 mV for 380 ms. To quantify $I_{K_{\text{ATP}}}$ currents were measured 200 ms into the second pulse.

Single-channel recordings were performed in high-K$^+$ pipette and bath solutions containing (in mM) 120 potassium glutamate, 125 KCl, 1 MgCl$_2$, 10 EGTA, 10 HEPES, and 10 glucose (pH 7.2 with KOH) in either the cell-attached mode or excised inside-out mode. In some excised patch experiments, 1 mM MgATP was added to the intracellular (bath) solution as indicated. Steady-state single-channel currents were recorded every 5 s for 800 ms at a holding potential of −80 mV. To quantify single $K_{\text{ATP}}$ channel activation from such recordings, we calculated the product of the channel number ($N$) and open probability ($P_o$) from each trace recorded.

In some whole cell clamp experiments (Fig. 3, A and B), flavoprotein fluorescence was measured simultaneously with membrane current to monitor mitochondrial redox state. Endogenous flavoprotein fluorescence was excited with a xenon arc lamp with a band-pass filter centered at 480 nm, but only during the 100-ms step to −40 mV to minimize photobleaching. Emitted fluorescence was recorded at 530 nm by a photomultiplier tube and digitized (18, 23). All experiments were performed at room temperature (22–23°C).

**Quantitative analysis of $I_{K_{\text{ATP}}}$ activation.** To quantify the kinetics of activation of $K_{\text{ATP}}$ channels induced by DNP in the presence of pinacidil, the rapid rising phase and the subsequent partial recovery phase were fitted by exponential functions (Fig. 5, A and B).

**RESULTS**

DNP rapidly activates $I_{K_{\text{ATP}}}$ in presence of pinacidil. To test for coupling between mitochondria and basal energetics in the subsarcolemmal space, we measured whole cell $I_{K_{\text{ATP}}}$ during exposure to the mitochondrial inhibitor DNP. Exposure to DNP alone did not induce any acute change, but after a considerable delay, it did slowly activate outward current at 0 mV. This current was identified as $I_{K_{\text{ATP}}}$ because of its sensitivity to glibenclamide inhibition (Fig. 1A). We then checked whether pinacidil-primed channels might be able to report energetic changes to which $K_{\text{ATP}}$ channels are normally oblivious. Pinacidil (100 μM) alone failed to activate $I_{K_{\text{ATP}}}$ (Fig. 1C). In contrast, in the presence of pinacidil, exposure to DNP rapidly turned on $I_{K_{\text{ATP}}}$, and this activation was readily reversed by washout of DNP (Fig. 1C). These observations can be rationalized as indicative of DNP-induced changes in local energet-
ics just below the surface membrane, suggesting rapid coupling between mitochondria and sarcolemma.

The DNP-induced activation of $I_{K_{ATP}}$ in the presence of pinacidil had complex kinetics consisting of a rapid rise, an early partial recovery, and then a more gradual increase. When the order was reversed and DNP was applied first, pinacidil activated $I_{K_{ATP}}$ monotonically, an effect that was reversed on pinacidil washout (Fig. 1E). Brisk activation of $I_{K_{ATP}}$ (i.e., within seconds of drug application) always required the simultaneous presence of DNP and pinacidil. These phenomena were also observed in experiments using FCCP, another mitochondrial uncoupler, as an alternative to DNP (data not shown). Taken together, these findings can be explained by the combination of two effects: the ATP depletion induced by DNP and the shift of the ATP sensitivity of $K_{ATP}$ channels induced by pinacidil (13).

In other words, we could detect a mitochondrially mediated rapid decrease of subsarcolemmal ATP by measuring $I_{K_{ATP}}$ in the presence of pinacidil.

Figure 2 presents, in schematic form, a framework for the interpretation of our experimental findings. Under physiological conditions (Fig. 2, left), ATP synthesized by $F_{1}F_{0}$-ATPase using the proton gradient is transferred from the mitochondrial matrix to sarcolemma through an adenine nucleotide translocator (ANT) and two types of CK, i.e., cytosolic and mitochondrial CK (14, 24, 29). Several lines of evidence indicate that ANT is functionally coupled with mitochondrial CK (24, 29). The CK system, however, is not essential for the bulk diffusion of ATP between membranes, because many cell types lack the CK system. Nevertheless, in excitable tissues with high metabolic demands (notably neurons, skeletal muscle, and cardiac muscle), the CK system expedites the transport of high-energy metabolites by shuttling creatine phosphate in an energetic chain reaction. In addition, the maintenance of low ADP levels is important for preserving a high free energy of ATP hydrolysis at energy-consuming sites (14).

In contrast, in the case of mitochondrial uncoupling (Fig. 2, right), $F_{1}F_{0}$-ATPase hydrolyzes ATP and pumps protons out in the reverse mode. Such mitochondrial perturbation may be rapidly transferred to the sarcolemma through the ANT and the CK systems, which can be demonstrated by examining the activation kinetics of $I_{K_{ATP}}$

Fast activation of $I_{K_{ATP}}$ requires rapid dissipation of mitochondrial potential. To confirm that DNP actually affected mitochondria even when no $I_{K_{ATP}}$ is elicited, we measured flavoprotein fluorescence (an indicator of matrix redox state) and membrane current simultaneously. Figure 3A demonstrates that exposure to DNP alone rapidly increased flavoprotein oxidation; nevertheless, $I_{K_{ATP}}$ was activated with a considerable latency. In contrast, Fig. 3B shows that, in the presence of pinacidil, additional application of DNP rapidly increased both $I_{K_{ATP}}$ and flavoprotein oxidation. DNP-induced mitochondrial oxidation could be observed not only during dialysis in the whole cell patch-clamp configuration but also in untouched cells (18, 22, 23). Thus the lack of rapid $I_{K_{ATP}}$ activation in the absence of pinacidil is not due to the lack of an uncoupling effect of DNP on mitochondria.

Figure 3C demonstrates that, in the presence of pinacidil, exposure to $2 \text{mM} \text{CN}^-$(an electron transport chain inhibitor) did not induce rapid activation of $I_{K_{ATP}}$. Furthermore, the DNP-induced rapid activation of $I_{K_{ATP}}$ was not inhibited by $\text{CN}^-$, although $\text{CN}^-$

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**Figure 2.** Scheme of subcellular energy transport between mitochondria and the sarcolemma. **Left:** physiological condition; **right:** mitochondrial uncoupling. $P$, required in almost all steps is abbreviated. CK, creatine kinase; mtCK, mitochondrial CK; PCr, phosphocreatine; Cr, creatine; ANT, adenine nucleotide translocator; Q, coenzyme Q; c, cytochrome c; FCCP, carbonyl cyanide $p$-(trifluoromethoxy)phenylhydrazone; DFNB, 2,4-dinitrofluorobenzene.

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1. Pinacidil shifts nucleotide sensitivity here
2. DNP, FCCP uncouple here
3. $\text{CN}^-$ blocks electron transport here
4. Oligomycin inhibits $F_{1}F_{0}$ ATPase here
5. Atractyloside inhibits ANT here
6. DNFB inhibits CK here
could inhibit the oxidative effect of DNP on mitochondria (data not shown). These results indicate that acute activation of $I_{KATP}$ in the presence of pinacidil requires rapid dissipation of mitochondrial inner membrane potential, which is induced by uncouplers but not by CN$^-$.  

**DNP-induced $I_{KATP}$ activation is inhibited by oligomycin.** Mitochondrial uncouplers have been reported to induce ATP hydrolysis caused by reverse-mode F$_1$F$_0$-ATPase activity (6, 17). To determine whether mitochondrial ATP hydrolysis was involved in the DNP-induced rapid activation of $I_{KATP}$, the effect of the F$_1$F$_0$ inhibitor oligomycin (cf. Fig. 2) was examined (4, 6, 11, 17). These experiments were performed under external K$^+$-free conditions to eliminate the potential for indirect inhibitory effects of oligomycin on the Na$^+$-K$^+$ pump (21); however, similar results were obtained in the presence of external K$^+$. Figure 4A shows that the increase in $I_{KATP}$ by DNP in the presence of pinacidil was inhibited by additional exposure to oligomycin. In addition, pretreatment with oligomycin abolished the DNP-induced activation of $I_{KATP}$ (data not shown). These findings indicate that DNP-induced activation of $I_{KATP}$ is mediated by F$_1$F$_0$-ATPase, probably because mitochondrial uncoupling not only interrupts ATP synthesis but also accelerates ATP hydrolysis, thus rapidly depleting subsarcolemmal ATP.

The first step of the interaction between mitochondrial matrix ATPase activity and the cytosol is the translocation of ATP and ADP across the inner membrane by ANT (cf. Fig. 2) (6). We tested the effects of the ANT inhibitor atractyloside (6, 15) on the DNP-induced acute activation of $I_{KATP}$. Figure 4B demonstrates that the application of atractyloside alone induced a small transient increase of $I_{KATP}$ but significantly attenuated the fast rising phase of DNP-induced activation. Although 300 $\mu$M atractyloside might not be able to inhibit ANT completely because of its poor cell membrane permeability, these data indicate that, in the presence of pinacidil, small perturbations of intracellular ATP can be detected by measuring $I_{KATP}$, revealing the existence of cross-talk between the mitochondrial inner membrane and the subsarcolemma.  

To define the contribution of the CK system (cf. Fig. 2) to the DNP-induced activation of $I_{KATP}$, the effects of DNFB were examined (5). DNFB was reported to inhibit both cytoplasmic and mitochondrial CK equally in rabbit isolated mitochondria (30) (cf. Fig. 2). DNFB did not activate $I_{KATP}$ within 10 min in the absence of pinacidil. In contrast, in the presence of pinacidil, subsequent exposure to DNFB modestly but consistently increased $I_{KATP}$ ($n = 6$). Additional exposure to DNP induced rapid activation of $I_{KATP}$, followed by a gradual decrease (Fig. 4C). Although the effect of DNFB may have been complicated by local ATP production by glycolytic enzymes or by incomplete inhibition of CK, it is important to note that the rising and the declining phases of $I_{KATP}$ were blunted by coapplication of...
DNFB. This finding suggests that the CK system contributes to energy transport between mitochondria and sarcolemma during mitochondrial uncoupling. Interestingly, second exposures to DNP in the presence of pinacidil also revealed slowing of the rapid rise of DNP-induced $I_{\text{K,ATP}}$ activation in a manner reminiscent of atractyloside (Fig. 4D).

To evaluate quantitatively the kinetics of activation of K$_{\text{ATP}}$ channels induced by DNP in the presence of pinacidil, the time courses of $I_{\text{K,ATP}}$ during the rapid rise and subsequent partial recovery were fitted by single exponential curves. Figure 5A shows representative data for the rising phase of DNP-induced $I_{\text{K,ATP}}$, whereas Fig. 5B shows the declining phase during the first exposure to DNP with (from Fig. 4C) or without (from Fig. 4D) concomitant DNFB. In the other groups, e.g., second exposure to DNP or first exposure to DNP with atractyloside, the early decline in $I_{\text{K,ATP}}$ was not observed. Figure 5C summarizes the data for the kinetics of the rising phase, indicating that the rate of rise is considerably blunted during second exposures to DNP ($P < 0.01$), with DNP + atractyloside ($P < 0.01$), and in the DNFB-treated group ($P < 0.05$). In addition, the declining phase of $I_{\text{K,ATP}}$ in the DNFB group was also slowed, as shown in Fig. 5D ($P < 0.05$). These results indicate that mitochondrial uncoupling induced-ATP hydrolysis by F$_{1}$F$_{0}$-ATPase is partially inactivated after a rapid activation and that CK inhibition slows the kinetics of DNP-induced $I_{\text{K,ATP}}$ activation, probably because under such conditions interactions between mitochondria and sarcolemma depend solely on bulk diffusion through the cytoplasm (cf. Fig. 2). Furthermore, there is a difference in the time course of DNP-induced $I_{\text{K,ATP}}$ activation between the first and second application, indicating cellular memory similar to that of preconditioning phenomena (24, 25). This effect of multiple exposures and the ANT inhibitor may reflect endogenous inactivation of the F$_{1}$F$_{0}$-ATPase activity as a result of mitochondrial ADP accumulation (8).

**DNP-induced activation of single K$_{\text{ATP}}$ channels in presence of pinacidil.** To eliminate the influence of intracellular dialysis by the whole cell patch electrode, we measured single K$_{\text{ATP}}$ channel currents in the cell-attached mode during application of DNP in the presence of pinacidil. Figure 6 shows that the $P_{o}$ of K$_{\text{ATP}}$ channels was not affected by exposure to pinacidil alone ($n = 18$), but additional application of DNP dramatically increased $P_{o}$. This DNP-induced activation of single-channel current was inhibited by the additional application of oligomycin. In only two cells, exposure to pinacidil alone induced activation of K$_{\text{ATP}}$ channels in the cell-attached mode. In the majority of cells ($n = 15$), DNP activated K$_{\text{ATP}}$ channels within 3 min after exposure to DNP in the presence of pinacidil, whereas in only three cells did channel activation by DNP take more than 3 min. In contrast, application of DNP alone did not activate any single-channel current in the cell-attached mode within 5 min ($n = 6$).

To exclude the possibility that DNP-induced activation of $I_{\text{K,ATP}}$ is caused by a direct effect of DNP on the channels, we recorded single-channel current in inside-out excised patches during application of DNP in the presence of pinacidil. Exposure to DNP did not enhance the activity of K$_{\text{ATP}}$ channels (Fig. 7, 1 mM intracellular [MgATP]). The single-channel currents were also not enhanced by DNP in bath solutions containing either 0 or 3 mM intracellular [MgATP] (data not shown). These results indicate that DNP-induced $I_{\text{K,ATP}}$ activation is not mediated by direct effects of DNP on K$_{\text{ATP}}$ channels. In addition, we confirmed that 30 μM oligomycin had minimal effects on K$_{\text{ATP}}$ in the excised inside-out patch (data not shown).
DISCUSSION

The present study demonstrates that mitochondrial uncoupling can induce rapid activation of $I_{K_{ATP}}$ in the presence of pinacidil. This activation is due to ATP hydrolysis by $F_1F_0$-ATPase, as confirmed by the sensitivity to oligomycin. In the absence of pinacidil, DNP does not have an acute effect on $I_{K_{ATP}}$, indicating that, although the mitochondria are inhibited, the ATP depletion is not severe enough to cross the threshold for activation of sarcolemmal $K_{ATP}$ channels. Other studies confirm that under normal conditions, mitochondrial inhibitors produce a slow effect on $I_{K_{ATP}}$. Leysens et al. (17) reported that in rat myocytes exposed to FCCP, there was a rapid decrease in intracellular ATP, as indicated by an increase in free $Mg^{2+}$, but no rapid activation of $I_{K_{ATP}}$. Regarding this point, Alekseev et al. (2) reported that DNP alone directly activated $I_{K_{ATP}}$ in guinea pig ventricular myocytes. However, the DNP-induced activation of $I_{K_{ATP}}$ observed in the present study differs with respect to the requirement for pinacidil and the very rapid time course of $I_{K_{ATP}}$ development. By decreasing the sensitivity of sarcolemmal $K_{ATP}$ channels to ATP inhibition using pinacidil, we were able to reveal dynamic early changes in energy balance within the cell during mitochondrial uncoupling.

Because sarcolemmal $K_{ATP}$ channels do not open immediately in response to mitochondrial uncouplers (1), systems must be present that are able to blunt the ATP hydrolytic effect of the mitochondria. One such system could be glycolysis. Weiss and Lamp (27, 28) proposed that glycolytic enzymes localized to the sarcolemma may preferentially produce ATP to inhibit $K_{ATP}$ channel opening. This could serve as a local damper on the effect of mitochondrial ATP consumption provided that glycolytic substrates (either exogenous or endogenous) are available. Another possibility is that sufficient stores of phosphocreatine are available to buffer the fall in cytosolic ATP, a mechanism relying on the presence of an active CK system. The role of the CK equilibrium as a transporter of energy equivalents between energy-producing and energy-consuming sites (ATP shuttle; see Refs. 3 and 14) should actually exacerbate subsarcolemmal ATP depletion. Maintenance of ATP transport to distant sites depends on a large positive ATP concentration gradient at the energy-consuming membrane, which would become negative if the mitochondria began to hydrolyze ATP. This, in fact, could be a crucial link between the mitochondria and the rapid activation of sarcolemmal $K_{ATP}$ channels.

A pronounced rapid transient of $I_{K_{ATP}}$ is evident at the onset of DNP exposure in the presence of pinacidil (e.g., Figs. 1C and 3). This finding is reminiscent of the results of Leysens et al. (17), who observed a similar transient in intracellular $[Mg^{2+}]$ that was blunted by oligomycin when myocytes were exposed to an uncoupler. The transient is likely to be due to an early burst of ATP breakdown by $F_1F_0$-ATPase that is somehow self-limiting. This is supported by blunting of this phase by atractyloside and by the attenuation of the transient phase when pinacidil is applied after DNP (Fig. 1E) or when DNP is applied for the second time (Fig. 4D). Although the mechanism of self-limitation...
remains to be determined, local depletion of the nucleotide pool or the binding of an endogenous ATPase inhibitor protein (11) are plausible explanations.

Clearly, the conversion of mitochondria from energy-producing to energy-consuming organelles will substantially impact the response of the myocyte to metabolic stress. This mechanism is likely to be active under conditions known to dissipate the mitochondrial inner membrane potential, such as during mitochondrial permeability transitions (6), ischemia (26), or spontaneous redox oscillations (22). Indeed, in the case of redox oscillations in cardiac cells, we have observed close coupling between mitochondrial redox transitions, mitochondrial membrane potential dissipation, and sarcolemmal $I_{K_{ATP}}$ activation (20). Further investigation into the contribution of mitochondrial energy consumption to the cellular pathophysiology of metabolic inhibition, apoptosis (7, 16), and ischemia will be facilitated by the approaches described in the present study.

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