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Review

Role of the mitochondrial ATP-sensitive K^+ channels in cardioprotection★

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The mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel was discovered more than a decade ago. Since then, several pharmacological studies have identified agents that target this channel some of which selectively target mitoK_{ATP}. These and other studies have also suggested that mitoK_{ATP} plays a key role in the process of ischemic preconditioning (IPC) and prevention of apoptosis. The mechanism by which mitoK_{ATP} exerts its protective effects is unclear, however, changes in mitochondrial Ca^{2+} uptake and levels of reactive oxygen species, and mitochondrial matrix swelling are believed to be involved. Despite major advances, several important issues regarding mitoK_{ATP} remain unanswered. These questions include, but are not limited to: the molecular structure of mitoK_{ATP}, the downstream and upstream mechanisms that leads to IPC and cell death, and the pharmacological profile of the channel. This review attempts to provide an up-to-date overview of the role of mitoK_{ATP} in cardioprotection.

The process of ischemic preconditioning (IPC) was first described by Murry and coworkers in 1986 (Murry *et al.*, 1986). IPC refers to a phenomenon in which brief episodes

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Abbreviations: APD, action potential duration; BLM, bilayer lipid membranes; 5-HD, 5-hydroxydecanoate; IPC, ischemic preconditioning; K_{ATP}, ATP-sensitive K^+ channel; mitoK_{ATP}, mitochondrial ATP-sensitive K^+ channel; mPTP, mitochondrial permeability transition pore; 3-NPA, 3-nitroproprionic acid; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SUR, sulfonylurea receptor.

of hypoxia provide protection against a subsequent more prolonged period of ischemia (Murry *et al.*, 1986; Kloner *et al.*, 1998; Cohen *et al.*, 2000). This protection includes a reduction in both infarct size and the incidence of cardiac arrhythmias. Several mediators have been proposed to play a role in IPC, including protein kinase C, various G-protein coupled receptors, and the generation of reactive oxygen species (ROS) and nitric oxide (Sato *et al.*, 1998; Sasaki *et al.*, 2000; Zhang *et al.*, 2001). The adenosine triphosphate sensitive K^+ (K_{ATP}) channels are believed to be the common effectors in the process of IPC. There are two K_{ATP} channels that have been identified in the heart, brain, liver, skeletal muscle and the kidneys: the cell surface (surface K_{ATP}) and the mitochondrial (mitoK_{ATP}) channels (Gross & Fryer, 1999; O'Rourke, 2000; Oldenburg *et al.*, 2002; Debska *et al.*, 2002; McCully & Levitsky, 2003).

Early studies suggested that opening of surface K_{ATP} channels may mediate the cardioprotective effects of IPC. The opening of the surface K_{ATP} channel leads to shortening of phase 3 of the action potential and hyperpolarizing cell membrane (Noma, 1983). This, in turn, would result in a reduction in the intracellular Ca^{2+} levels, which initially was thought to produce the cardioprotective effects. However, studies published as early as 1994 suggested that surface K_{ATP} channels did not play an important role in IPC. Yao and Gross (1994) showed that a low dose of bimakalin produced cardioprotective effects, even though it did not shorten action potential duration (ADP) at that low dose. Subsequently, Grover *et al.* (1996) studied the effect of the class III antiarrhythmic agent dofetilide on preconditioning at a dose that prevented APD shortening during ischemia. Their results showed that dofetilide abolished the APD shortening of ischemia, but did not alter the protective effects of preconditioning.

In 1991, Inoue *et al.* (1991) reported the identification of a K_{ATP} channel in the mitochondria. They patch clamped mitoplasts (mitochondria stripped of their outer membranes) from rat liver and identified K^+ selective channels, sensitive to inhibition by ATP, 4-aminopyridine and glibenclamide. The conductance of these channels was lower than that of surface K_{ATP} channels (about 10 pS in 100 mM matrix K^+ and 33 mM cytosolic K^+). Subsequent studies by several groups have demonstrated that mitoK_{ATP} may be the key player in IPC. This review will present and discuss the evidence implicating mitoK_{ATP} in cardioprotection.

PHARMACOLOGY OF mitoK_{ATP} CHANNELS

The initial identification of mitoK_{ATP} in 1991 prompted several groups to further characterize the pharmacological profile of the channel. Table 1 lists some of the drugs that have been identified to modulate the activity of mitoK_{ATP} and surface K_{ATP} channels. Garlid's group has used two different approaches to study the channel. In their first approach, a protein fraction was purified on an DEAE-cellulose columns (Paucek *et al.*, 1992), and an ATP-binding affinity column (Bajgar *et al.*, 2001). The fraction was reported to contain 2 major proteins of 55 and 63 kDa in size; the larger protein was reported to bind to fluorescently labeled glibeclamide (Bajgar *et al.*, 2001). The protein fraction was then incorporated into liposomes containing a K^+-sensitive fluorescent marker (PBFI) (Jezek *et al.*, 1990; Paucek *et al.*, 1992). The emission intensity of PBFI increases when K^+ binds to it. Thus, the rate of fluorescence increase can be used to quantify K^+ transport into the proteoliposomes (Jezek *et al.*, 1990). A pH gradient or a permeable anion was used to compensate for the ion gradient generated by K^+ transport. Using this technique, Garlid's group showed that diazoxide opens mitoK_{ATP} about 2000 fold more potently than it opens surface

K_{ATP} channels (Garlid *et al.*, 1997). The agonist effect of diazoxide could be abolished by simultaneous treatment of proteoliposomes with 5-hydroxydecanoate (5-HD) or glibenclamide, with inhibitory constants (K_is) of 45–85 μM and 1–6 μM, respectively. The channel was also K^+ selective and sensitive to inhibition by ATP (K_i of 40 μM) and ADP (K_i of 280 μM) (Paucek *et al.*, 1992).

In their second approach, Garlid's group assessed steady state matrix volume by measuring light scattering at 520 nm in the intact isolated mitochondria (Jaburek *et al.*, 1998). The opening of mitoK$_{ATP}$ and influx of K^+ ions result in an increase in the osmotic pressure and water movement into the mitochondrial matrix, which would lead to an increase in the mitochondrial matrix volume (Brierley *et al.*, 1977; Szewczyk *et al.*, 1993; Garlid & Paucek, 2001). Thus, changes in the matrix volume can be used as an indirect measure of the mitoK$_{ATP}$ activity. Although there are limitations associated with this approach (such as hypoosmotic conditions of the experiments, use of supraphysiological ion gradients, etc.), these studies supported the pharmacological results obtained from the proteoliposome studies (Garlid & Beavis, 1986; Jaburek *et al.*, 1998).

Marban's group has taken a different approach to assay the function of mitoK$_{ATP}$ channels (Liu *et al.*, 1998; Hu *et al.*, 1999; Sato *et al.*, 2000a; 2000b; Seharaseyon *et al.*, 2000a; Sasaki *et al.*, 2000; 2003). This approach is based on the autofluorescence of mitochondrial flavoproteins and NADH, which increases with partial uncoupling generated by mitoK$_{ATP}$ opening (Hassinen & Chance, 1968; Chance, 1972; Chance *et al.*, 1972). The advantage of this technique is that it can be used in intact cells. However, there are also several limitations with this technique. Since the mitochondrial redox potential is a balance between NADH production and oxidation (i.e., NAD^+ production), it does not represent the overall rate of oxidative phosphorylation of the cell. Furthermore, mitochondrial membrane potential does not change significantly by partial oxidation of the redox pool due to enhanced proton pumping (O'Rourke, 2004). Nevertheless, these studies support results obtained by Garlid's group and, for the first

Table 1. Modulators of K_{ATP} channels and their selectivity toward mitochondrial and surface channels

	mitoK$_{ATP}$	mitoK$_{ATP}$ and surface K$_{ATP}$	Surface K$_{ATP}$
Openers	Diazoxide Nicorandil BMS-180448 BMS-191095	Cromakalim Pinacidil P-1060 Sildenafil Isoflurane EMD60480 Aprikalim	P-1075[1] MCC-134[2]
Blockers	5-HD MCC-134	Glibenclamide	HMR1098 (1833) Glimepiride[3]

Diazoxide and nicorandil can activate surface K_{ATP} channel at high concentrations. [1]P-1075 is selective for surface K_{ATP} channel in rabbit myocytes. In isolated rat mitochondria, it activated mitoK$_{ATP}$. [2]MCC-134 selectively activates surface K_{ATP} and inhibits mitoK$_{ATP}$. [3]Glimepiride inhibits surface K_{ATP} without any effect on cardioprotection.

time, demonstrated greater selectivity of diazoxide for mitoK$_{ATP}$ over the surface K$_{ATP}$ channel in intact cells.

mitoK$_{ATP}$ has also been studied in bilayer lipid membranes (BLM). In their initial report on mitoK$_{ATP}$, Garlid's group demonstrated K^+ channel activity when their purified mitochondrial fraction was incorporated into BLMs (Paucek *et al.*, 1992). However, they used 1 M concentrations of K^+ in their studies, concentrations significantly higher than mitoK$_{ATP}$ K_m for K^+. Mironova's group has reported the isolation of a 55 kDa protein from the mitochondria by an ethanol/water extraction of inner mitochondrial membrane (Grigoriev *et al.*, 1999; Mironova *et al.*, 1999; Marinov *et al.*, 2001), however, the identity of this protein remains unclear. When incorporated into LBM, it displayed mitoK$_{ATP}$ activity. The channel activity was increased and its selectivity was reduced by sulfur-reducing agent, dithiotheritol. In a recent report by Zhang *et al.* (2001), K^+ channel activity with a conductance of 56 pS was demonstrated when an enriched mitochondrial membrane fraction was incorporated into BLMs. Consistent with earlier observations, diazoxide activated the channel, while 5-HD and glibenclamide inhibited the channel.

It has been demonstrated in C2C12 skeletal myoblasts that the opening of mitoK$_{ATP}$ by diazoxide stimulates cellular O_2 consumption, a reduction in ATP levels in the cell and a small decrease in $\Delta\Psi_m$ (Minners *et al.*, 2000). Similar results were found using isolated rat skeletal muscle mitochondria and L6 myoblasts (Debska *et al.*, 2002). These changes in respiration and membrane potential have been used to study mitoK$_{ATP}$ channel, and these results also support the pharmacological profile of mitoK$_{ATP}$.

MECHANISM OF PROTECTION

It is unclear how the opening of a K^+ channel in the mitochondria would lead to cardioprotection. However three hypotheses have emerged to explain the link between mitoK$_{ATP}$ channel opening and cardioprotection: (1) a decrease in the mitochondrial Ca^{2+} uptake, (2) swelling of the mitochondrial matrix and changes in ATP synthesis, and (3) changes in the levels of reactive oxygen species (ROS).

Regarding the first hypothesis, diazoxide and pinacidil have been shown to reduce the magnitude of mitochondrial Ca^{2+} uptake, and the effect of both can be reversed by 5-HD (Holmuhamedov *et al.*, 1999). This change in mitochondrial Ca^{2+} uptake is thought to be mediated by a partial depolarization of $\Delta\Psi_m$ in response to mitoK$_{ATP}$ opening. Murata *et al.* (2001) showed that opening of mitoK$_{ATP}$ resulted in a reduction of Ca^{2+} accumulation in the mitochondria during simulated ischemia reperfusion. Inhibitors of the mitochondrial permeability transition pore (cyclosporine A and bongkrekic acid) markedly suppressed mitochondria Ca^{2+} concentrations only during reperfusion. Similar results were obtained by Wang and coworkers who showed that mitoK$_{ATP}$ opening attenuates the mitochondrial Ca^{2+} accumulation during ischemia in intact hearts (Wang *et al.*, 2001).

It has been known for some time that the opening of mitoK$_{ATP}$ causes mitochondrial matrix swelling, and that this in turn activates the respiratory chain providing more ATP to support the recovering myocardium (Halestrap, 1989; Grover & Garlid, 2000; O'Rourke, 2000). Halestrap's group took a different approach to study this phenomenon. Since it is difficult to observe changes in mitochondrial matrix volume by electron microscopy (a 25% increase in volume causes only a 3% change in the mitochondrial diameter) they used ^3H_2O and [^{14}C]sucrose to measure the mitochondrial volume (Lim *et al.*, 2002). Their studies showed that two 5-minute cycles of preconditioning or 50 μM of diazoxide both increased matrix volume by 58% and 88%, respectively. Diazoxide was also shown to in-

hibit succinate and 2-oxyglutarate oxidation. However, 5-HD not only did not reverse the effect of diazoxide and preconditioning it actually increased matrix volume when it was added by itself. The authors concluded that cardioprotection may be due to inhibition of respiration rather than mitochondrial matrix swelling by the opening of mitoK$_{ATP}$. This is in stark contrast to earlier reports, which suggested that mitoK$_{ATP}$ opening and mitochondrial matrix swelling underlie the process of IPC (discussed in more detail later).

An alternative model for protection induced by mitochondrial swelling has been proposed by Garlid's group (Kowaltowski *et al.*, 2001). According to this model, matrix swelling may lead to a more optimal contact between membrane proteins on the inner and outer membranes of the mitochondria, which may in turn lead to an increase in ADP transport and ATP synthesis.

Reactive oxygen species are a double-edge sword when it comes to cardioprotection. The ROS generated during the preconditioning period is thought to be protective (Vanden Hoek *et al.*, 1998; Pain *et al.*, 2000; Forbes *et al.*, 2001). However, the ROS that is produced during reperfusion, is detrimental and causes cell death (Zweier *et al.*, 1987; Vanden Hoek *et al.*, 2000; Ozcan *et al.*, 2002). It is thought that the opening of mitoK$_{ATP}$ results in an increase in the protective ROS produced during preconditioning phase and a decrease in the levels of ROS generated during reperfusion phase (O'Rourke, 2004).

MOLECULAR STRUCTURE OF mitoK$_{ATP}$

Despite efforts by several groups, the molecular structure of mitoK$_{ATP}$ remains unclear. The structure of the surface K$_{ATP}$ channel was solved in the mid 1990's and is thought to be composed of a tetramer of inward rectifier K^+ channels (Kir6.x) surrounded by four sulfonylurea receptor (SUR) subunits (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1995a; 1995b; 1996). It is thus proposed that mitoK$_{ATP}$ may also contain subunits similar to Kir and SUR proteins.

As mentioned before, Garlid's group has partially purified a mitochondrial fraction with mitoK$_{ATP}$ channel activity that contains, among several other proteins, 55 and 63 kDa components (Bajgar *et al.*, 2001). They have proposed that the 63 kDa protein in the fraction is the sulfonylurea binding subunit, and the 55 kDa is the pore forming unit of the channel. Preliminary reports also suggest that the 63 kDa protein binds to fluorescently labeled glibenclamide (Bajgar *et al.*, 2001). However, the identity of these proteins remains elusive.

Susuki *et al.* (1997) suggested the existence of a Kir6.1-like protein in the mitochondria. In their study, an antibody against Kir6.1 immunolocalized to the mitochondria, where it bound a 51 kDa protein. In two recent studies, antibodies against Kir6.1, Kir6.2 and SUR2A subunits detected proteins in the mitochondria of the heart and brain (Lacza *et al.*, 2003a; 2003b). However, results obtained by Seharaseyon *et al.* (2000b) do not support this notion. A dominant negative Kir6.1 construct with a mutation in the K^+-channel pore signature sequence was adenovirally delivered into adult myocytes. This construct did not have any effect on the diazoxide activated mitochondrial redox state, suggesting that mitochondria probably do not contain a Kir6.1. However, it is still possible that a Kir protein could exist in the mitochondria, which would not heterodimerize with the dominant negative from of Kir6.1. Genetic knockout of the Kir6.1 gene in mice results in sudden death associated with vasospasm and prinzmetal angina, but does not change mitoK$_{ATP}$ opening in response to diazoxide (Miki *et al.*, 2002).

Further fueling the fire of controversy over the molecular structure of mitoK$_{ATP}$ is data suggesting that the Krebs cycle enzyme, succinate dehydrogenase (SDH) may be a tar-

get of drugs that have been traditionally thought to be mitoK$_{ATP}$ modulators (Hanley *et al.*, 2002; Lim *et al.*, 2002). Furthermore, several pharmacological agents that target SDH also induce the process of IPC (Ockaili *et al.*, 2001; Ozcan *et al.*, 2002; Horiguchi *et al.*, 2003). For example, SDH inhibitors, malonate and 3-nitropropionic acid (3-NPA), have been shown to attenuate oxidant stress on the heart and have cardioprotective effects. Diazoxide also inhibits SDH, although the effects occur at concentrations about 10–100 fold higher than those that target mitoK$_{ATP}$. Thus, several investigators have questioned the very existence of mitoK$_{ATP}$ and have proposed that respiratory inhibition, rather than mitoK$_{ATP}$ opening, induces IPC (Hanley *et al.*, 2002; Lim *et al.*, 2002).

Our group recently undertook studies to characterize the structure of mitoK$_{ATP}$ (Ardehali *et al.*, 2003). Based on their overlapping pharmacological profiles, we hypothesized that SDH and mitoK$_{ATP}$ interact both functionally and physically. We first decided to identify proteins that physically interact with SDH. Using the co-immunoprecipitation technique, we identified at least four SDH-associating proteins: mitochondrial ATP-binding cassette protein-1, adenine nucleotide translocator, ATP synthase, and inorganic phosphate carrier. A fraction containing this complex was then partially purified using a differential centrifugation technique. Incorporation of this fraction into liposomes or lipid bilayer yielded K^+-channel activity with sensitivity to the known mitoK$_{ATP}$ modulators. In order to better link this complex with the mitoK$_{ATP}$ activity, we studied the effect of SDH inhibitors. Addition of 3-NPA or malonate resulted in a significant increase in mitoK$_{ATP}$ activity, which was reversed by the addition of the mitoK$_{ATP}$ inhibitor, 5-HD. These results suggest that there is a complex of (at least) five proteins in the mitochondrial inner membrane, which is capable of transporting K^+ with characteristics similar to those of mitoK$_{ATP}$ (Fig. 1). We propose that SDH influences mitoK$_{ATP}$ activity through its physical and functional interaction with this complex. A complex of multiple unrelated proteins carrying a unique function is not an unfamiliar concept, as a similar structure has

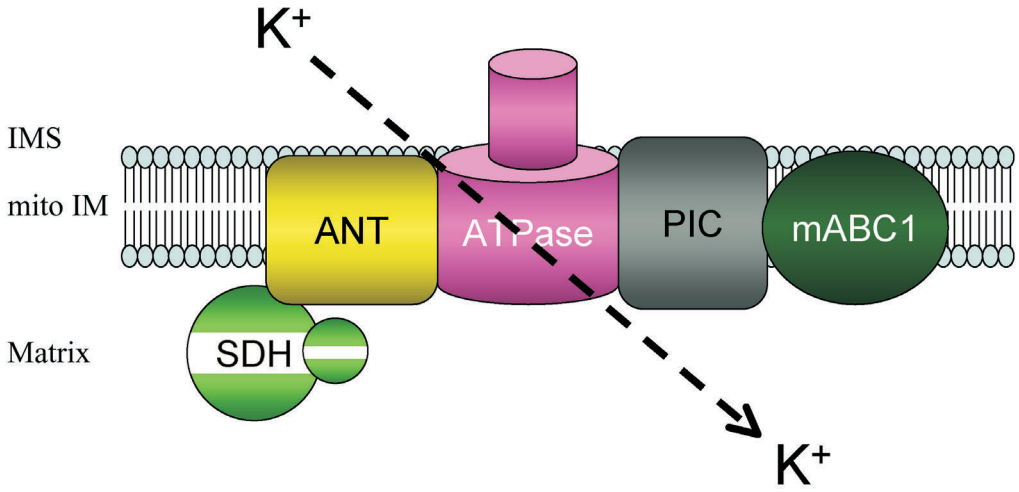

Figure 1. Schematic presentation of the proposed structure of a mitochondrial complex with mitoK$_{ATP}$ activity.

A complex of at least 5 proteins, containing ATP-binding cassette protein-1 (mABC1), SDH, ATP synthase (ATPase), inorganic phosphate carrier (PIC) and adenine nucleotide translocator (ANT), is present in the inner membrane of the mitochondria. This complex is capable of transporting K^+ with characteristics similar to those of mitoK$_{ATP}$. The pore-forming unit of the channel has not been characterized yet. IMS, intermembrane space; mito IM, mitochondrial inner membrane.

been proposed for the mitochondrial permeability transition pore (mPTP) (Weiss *et al.*, 2003). The identity of the pore-forming unit of the channel is not clear at this point and is under investigation. These findings provide the first tangible clues to the structure of mitoK$_{ATP}$ channels.

mitoK$_{ATP}$ AND DELAYED CARDIOPROTECTION

The acute phase of IPC lasts about 1–2 h after the preconditioning phase. A second window of protection appearing about 24–72 h after the brief ischemic periods has also been described and termed delayed IPC (Kuzuya *et al.*, 1993; Marber *et al.*, 1993). The delayed protection is generally less effective and more cycles of brief ischemia are needed to exert protection. In addition to brief ischemic periods, several pharmacological agents, including diazoxide (Takashi *et al.*, 1999), opioids (Fryer *et al.*, 1999), adenosine (Sato *et al.*, 1998), and monophosphoryl lipid A (Mei *et al.*, 1996) have also been shown to induce delayed IPC. The role of mitoK$_{ATP}$ in delayed IPC was first reported by Mei and coworkers in 1996 (Mei *et al.*, 1996). They showed that monophosphoryl lipid A treatment in adult mongrel dogs resulted in a delayed reduction in infarct size, reversible by glibenclamide and 5-HD. Similar results were obtained by treatment of rat or rabbit hearts with adenosine, opioids, diazoxide, and these protective effects were reversed by 5-HD (Sato *et al.*, 1998; Fryer *et al.*, 1999; Takashi *et al.*, 1999).

mitoK$_{ATP}$ AND APOPTOSIS

It is believed that IPC may decrease both apoptosis and necrosis, and that apoptosis contributes to ischemia-mediated myocardial cell death. Takashi *et al.* (1999) showed that diazoxide decreased markers of apoptosis from ischemia/reperfusion injury, and that this effect was reversed by 5-HD. Akao and coworkers also studied the role of mitoK$_{ATP}$ in apoptosis. They showed that diazoxide inhibits H_2O_2-induced apoptosis in neonatal rat cardiac myocytes and that 5-HD blocked this effect (Akao *et al.*, 2001). In their study, they assessed apoptosis by measuring cytochrome *c* release, caspase activation, loss of mitochondrial membrane potential and cleavage of poly(ADP-ribose) polymerase. They later characterized three different stages in H_2O_2-induced cell death in neonatal rat cardiomyocytes. The first phase (also known as the priming phase) is characterized by calcium-dependent morphological changes with preserved membrane potential. In the second phase, which was termed the depolarization phase, mPTP opening leads to rapid depolarization of the membrane. The third phase is characterized by cell fragmentation. They showed that diazoxide decreased the likelihood of cells undergoing the priming phase while an mPTP opener (cyclosporine A) slowed membrane depolarization (i.e., phase 2). They concluded that apoptosis is composed of three phases and that mitoK$_{ATP}$ plays a role in protection against early stages of apoptosis by preserving mitochondrial integrity.

CONCLUSIONS

The mitoK$_{ATP}$ channel has been studied extensively since its discovery in 1991. Based on a large body of evidence, this channel is believed to play a major role in IPC and apoptosis. However, many questions remain to be answered. Although evidence strongly suggests a role for mitoK$_{ATP}$ in IPC, the selectivity of the drugs used to modulate mitoK$_{ATP}$ over surface K$_{ATP}$ remains controversial. The potential contribution of the surface K$_{ATP}$ channel to cardioprotection was re-

cently revisited in Kir6.2 knockout mice. IPC reduced the infarct size in the heart of wild type but not knockout mice.

Perhaps, the most challenging issue in this field is the identification of the structure of mitoK_{ATP} channel. The recent finding that a macromolecular supercomplex confers mitoK_{ATP} activity opens up new directions for future studies. The identity of the pore-forming unit of the channel, however, remains unclear. Furthermore, it is not clear how these proteins interact and whether there are other associating proteins in this complex.

Despite unanswered questions and remaining ambiguities, many studies in this field consistently support the existence of a K_{ATP} channel in the mitochondria, which when activated, leads to protection against cell death and apoptosis. The pharmacological and structural profile of this channel is of paramount importance, since targeting this channel may provide new therapeutic strategies for ischemic heart disease.

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