0026-895X/99/061000-06$3.00/0

MOLECULAR PHARMACOLOGY, **55:**1000–1005 (1999).

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

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Received August 18, 1998; accepted March 7, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

A variety of direct and indirect techniques have revealed the existence of ATP-sensitive potassium (K_{ATP}) channels in the inner membranes of mitochondria. The molecular identity of these mitochondrial K_{ATP} (mitoK_{ATP}) channels remains unclear. We used a pharmacological approach to distinguish mitoK_{ATP} channels from classical, molecularly defined cardiac sarcolemmal K_{ATP} (surfaceK_{ATP}) channels encoded by the sulfonylurea receptor SUR2A and the pore-forming subunit $K_{ir}6.2$. SUR2A and $K_{ir}6.2$ were expressed in human embryonic kidney (HEK)293 cells, and their activities were measured by patch-clamp recordings of membrane current. SurfaceK_{ATP} channels are activated potently by 100 μM pinacidil but only weakly by 100 μM diazoxide; in addition, they are blocked by 10 μM glibenclamide, but are insensitive to 500 μM 5-hydroxydecanoate. This pharmacology, which was confirmed with patch-clamp recordings in intact rabbit ventricular myocytes, contrasts with that of mitoK_{ATP} channels as indexed by flavoprotein oxidation. MitoK_{ATP} channels in myocytes are activated equally by 100 μM diazoxide and 100 μM pinacidil. In contrast to its lack of effect on surfaceK_{ATP} channels, 5-hydroxydecanoate is an effective blocker of mitoK_{ATP} channels. Glibenclamide's effects on mitoK_{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 $K_{ir}6.2$ in HEK cells and in myocytes revealed no targeting of mitochondrial membranes. The differences in drug sensitivity and subcellular localization indicate that mitoK_{ATP} channels are distinct from surface K_{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_{ATP}) channels as the end effectors. Such a key role was first attributed to the classical sarcolemmal K_{ATP} (surfaceK_{ATP}) channels (Gross and Auchampach, 1992; Speechly-Dick et al., 1995; Liang, 1997; Kloner et al., 1998), but recent evidence implicates the more nebulous mitochondrial K_{ATP} (mitoK_{ATP}) channels (Inoue et al., 1991; Garlid et al., 1997; Liu et al., 1998; Sato et al., 1998).

This work was supported by National Insitutes of Health Grants R01 HL52768 and R01 HL44065 (to E.M.), and T32 HL07227 (to H.H.), an American Heart Association-Maryland Fellowship (to H.H.), and a Banyu Fellowship in Lipid Metabolism and Atherosclerosis (to T.S.).

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Native cardiac mitoK_{ATP} channels share some pharmacological properties with their classical, sarcolemmal counterparts. Both channels are activated by pinacidil and inhibited by glibenclamide (Paucek et al., 1992; Garlid et al., 1997). Thus, specific inhibitors and activators are in demand for the study of cardiac mitoK_{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 surfaceK_{ATP} channels (Notsu et al., 1992; Garlid et al., 1997; Liu et al., 1998). Likewise, the extent to which the activator diazoxide opens surfaceK_{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 mitoK_{ATP} channels remains unclear, although cardiac surfaceK_{ATP} channels have been molecularly defined as an octameric complex of four pore-forming $K_{ir}6.2$ 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; K_{ir}, inwardly rectifying potassium channels; K_{ATP} channels, ATP-sensitive potassium channels; mitoK_{ATP}, inner mitochondrial membrane K_{ATP}; SUR, Sulfonylurea receptor; surfaceK_{ATP}, sarcolemmal (surface membrane) K_{ATP}.

distinguish unambiguously between surfaceK_{ATP} and mitoK_{ATP} channels, we compared native cardiac mitoK_{ATP} channels to molecularly defined and native surfaceK_{ATP} channels. Differences in pharmacology and subcellular distribution reveal clear distinctions between cardiac mitoK_{ATP} and surfaceK_{ATP} channels.

Materials and Methods

Functional Expression of K_{ir}6.2 + 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 μg/dish of equimolar plasmid DNAs of the rabbit cardiac K_{ir}6.2 subunit (Hu et al., 1999) and the rat SUR2A sulfonylurea receptor (Inagaki et al., 1996), supplemented with 0.2 μg/dish of mitochondrially 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_{ir}6.2 + SUR2A Channels. For functionally expressed surfaceK_{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_{ATP} channel activity. The holding current at −80 mV (near E_K) was also measured to verify the gigaseal stability. In the study of the effect of 5HD on membrane current, 1 min of K_{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_{ir}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, Palto 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 geneticin sulfate at 500 μg/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 infection of rabbit ventricular cells (isolated and cultured as described below). We first generated an isogeneic Kir6.2GFP 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 pRabKir6.2GFP. The expression cassette was removed and cloned into the multiple cloning site of modified adenovirus shuttle vector pAdECd8I (Johns et al., 1999) to generate pAdECd8Kir6.2GFP. The recombinant adenovirus containing the RabKir6.2GFP fusion gene product was generated by Cre-lox recombination as described previously (Johns et al., 1999). Isolated rabbit ventricular myocytes were coinfected with AdRabKir6.2GFP 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 μM 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- to 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 mitoK_{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.

Results

5HD Has Little Effect on Expressed Cardiac SurfaceK_{ATP} Channels. Figure 1 shows the effect of 5HD on currents elicited by 100 μM pinacidil in a HEK293 cell expressing $K_{ir}6.2$ + SUR2A, which forms cardiac-type surfaceK_{ATP} 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 ($3 \pm 3\%$, $n = 5$).

Diazoxide Weakly Activates Expressed Cardiac SurfaceK_{ATP} Currents. Diazoxide weakly activated expressed K_{ATP} 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 $4 \pm 1\%$ ($n = 26$, $P < .03$) 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 mitoK_{ATP} but not surfaceK_{ATP} 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 sarcolemmal 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 $K_{ir}6.2$ + 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 surfaceK_{ATP} channels encoded by $K_{ir}6.2$ and SUR2A.

Surface Membrane K_{ATP} Currents in Heart Cells. The pattern of pharmacologic responsiveness observed for heterologously expressed channels encoded by $K_{ir}6.2$ 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 surfaceK_{ATP} channels mimic the behavior of native cardiac surfaceK_{ATP} channels.

$K_{ir}6.2$ Does Not Target Mitochondrial Membranes. To address whether expressed $K_{ir}6.2$ subunits are targeted to mitochondrial membranes, we imaged HEK293 cells stably expressing the fusion construct GFP-$K_{ir}6.2$. Figure 4A–C shows the distribution of expressed $K_{ir}6.2$ 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

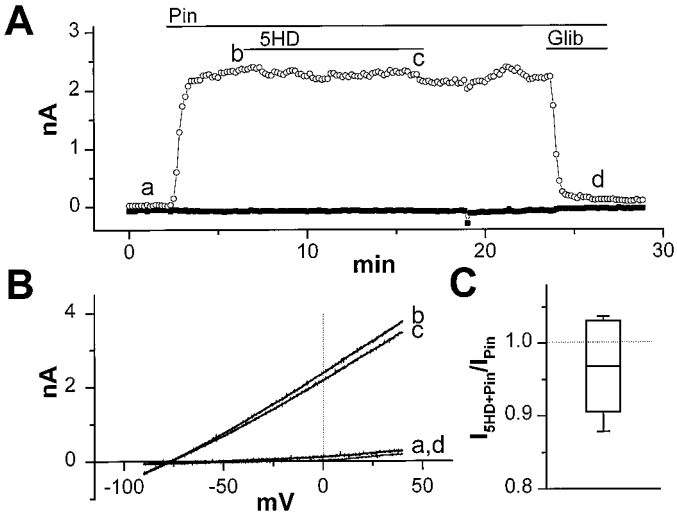

Fig. 1. Effects of 5HD on pinacidil-induced surfaceK_{ATP} channels expressed in HEK293 cells. A, time course of the membrane current measured at 0 mV (○) in the presence or absence of 100 μM pinacidil (Pin), 500 μM 5HD, or 10 μM glibenclamide (Glib). The holding current at −80 mV is also plotted (■). B, representative ramp currents recorded under drug-free conditions (a), when pinacidil was applied (b), when 5HD was added (c), and when glibenclamide was added (d), at the times indicated in A. Pinacidil was present (b–d). C, pooled data from five experiments of the relative current $I_{5HD+Pin}$ versus I_{Pin}, i.e., the current at time c versus that at time b as indicated in A.

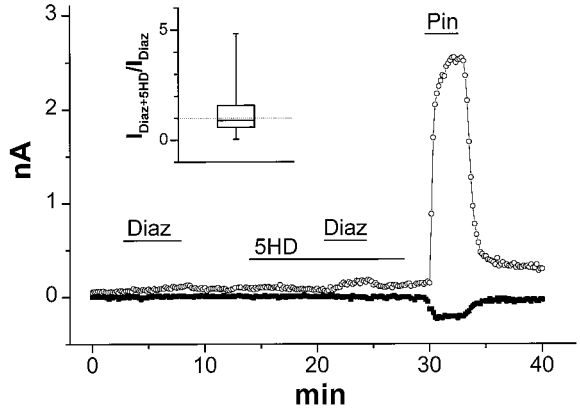

Fig. 2. Effects of diazoxide and 5HD on $K_{ir}6.2$ + SUR2A channels expressed in a HEK293 cell. The cell was first exposed to 100 μM diazoxide (Diaz) alone, then again in the presence of 500 μM 5HD, and finally to 100 μM pinacidil (Pin). The inset box graph shows the effect of 5HD from seven experiments expressed as the ratio of the second current elicited by diazoxide + 5HD versus the first current with diazoxide alone (P = N.S.).

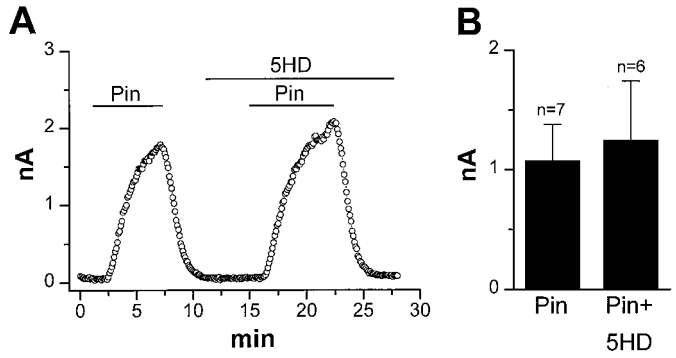

Fig. 3. Pharmacological sensitivity of native surfaceK_{ATP} channels in rabbit ventricular myocytes. A, time course of membrane current elicited by pinacidil (100 μM) in the absence and presence of 5HD (500 μM). B, pooled data for pinacidil-induced membrane current measured in the absence and presence of 5HD. P = N.S.

mitochondria (Fig. 5B). From these experiments, we conclude that $K_{ir}6.2$ subunits do not target mitochondrial membranes.

5HD Inhibits mitoK_{ATP} in Intact Rabbit Ventricular Myocytes. Having studied the effects of diazoxide and 5HD on expressed surfaceK_{ATP} channels, we then examined their effects on the native mitoK_{ATP} in rabbit cardiomyocytes. We previously established single-cell methodologies to assay surfaceK_{ATP} and mitoK_{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_{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 the

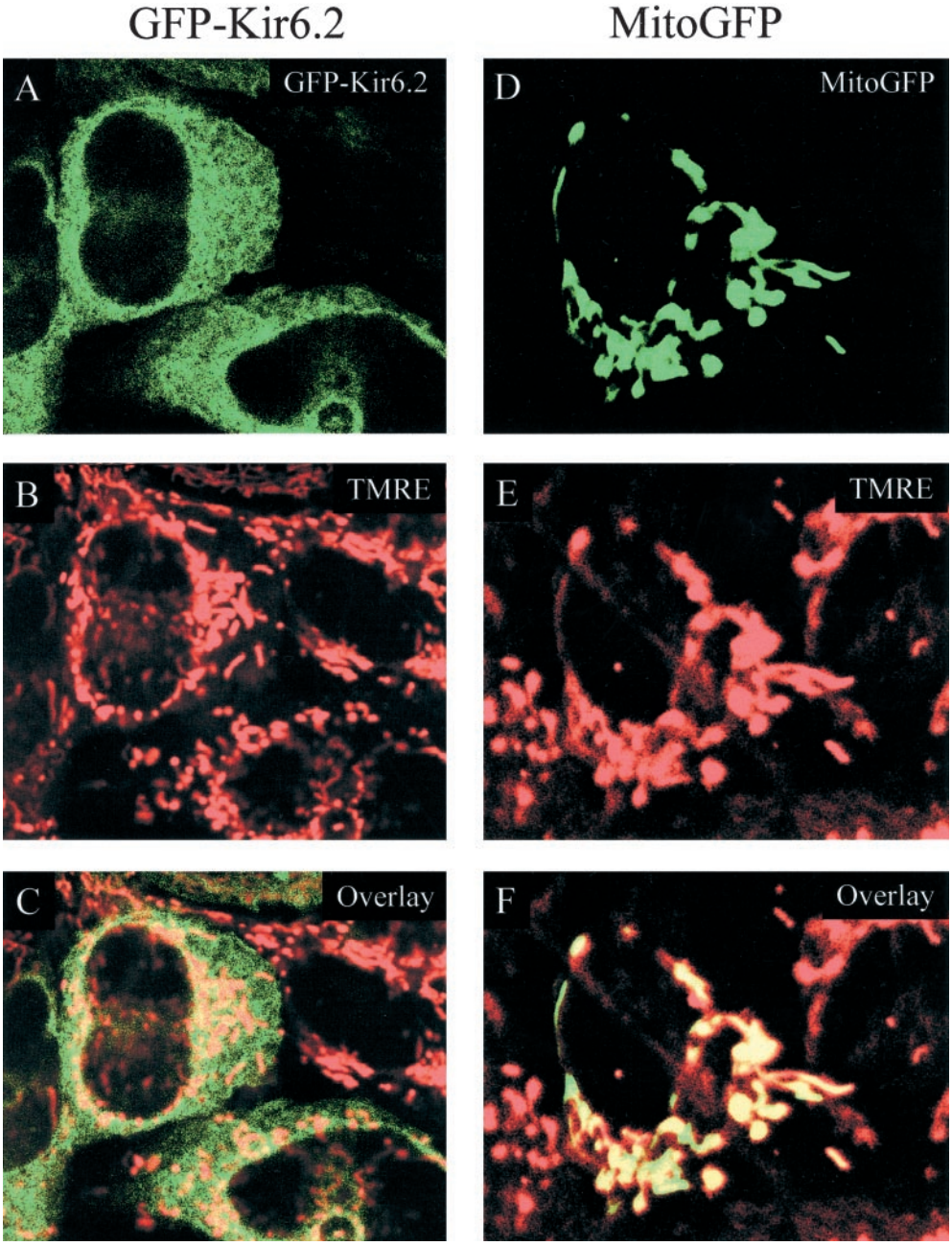

Fig. 4. Confocal fluorescent images of HEK293 cells stably expressing $K_{ir}6.2$ fused with GFP (left column, A–C), and HEK293 cells transiently expressing mitochondrial GFP (right column, D–F). Green signals indicate the locations of GFP-$K_{ir}6.2$ (A) and mitochondrially targeted GFP (D), and red 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 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 (Szewczyk 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 ($K_{ir}6.2$ + 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 $K_{ir}6.2$ 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 (Szewczyk 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

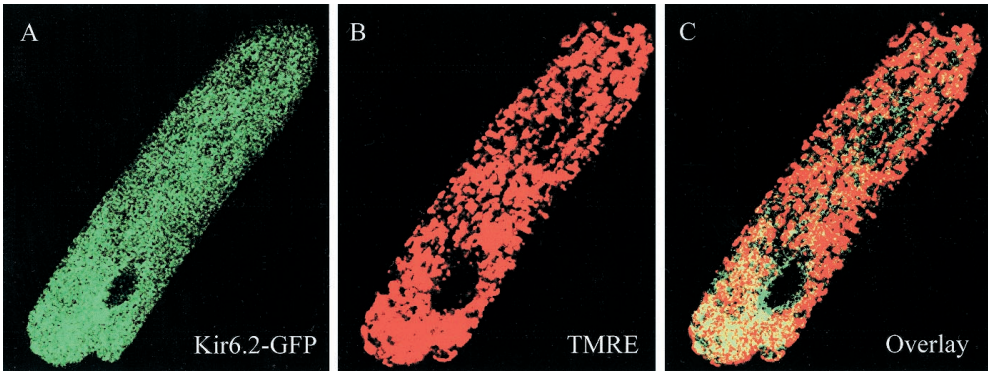

Fig. 5. Subcellular distributions of Kir6.2 and mitochondria in a cardiac myocyte. A, confocal image of Kir6.2 fused with enhanced GFP in a rabbit ventricular myocyte that had been infected with AdRabKir6.2GFP 72 h earlier (see *Materials and Methods*). B, pattern of TMRE staining in the same cell. C, overlay image of A and B.

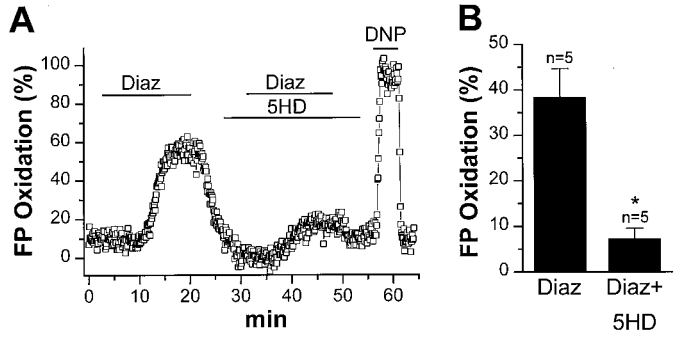

Fig. 6. Effects of diazoxide and 5HD on flavoprotein fluorescence in intact rabbit ventricular myocytes. A, diazoxide (Diaz, 100 μM) induced flavoprotein (FP) oxidation and its inhibition by 5HD (500 μM). B, summarized data for percentage of diazoxide-induced flavoprotein oxidation in the absence and presence of 5HD. The flavoprotein fluorescence was calibrated by exposing the cells to DNP (100 μM) at the end of experiments. *$P < .005$ versus Diaz alone group.

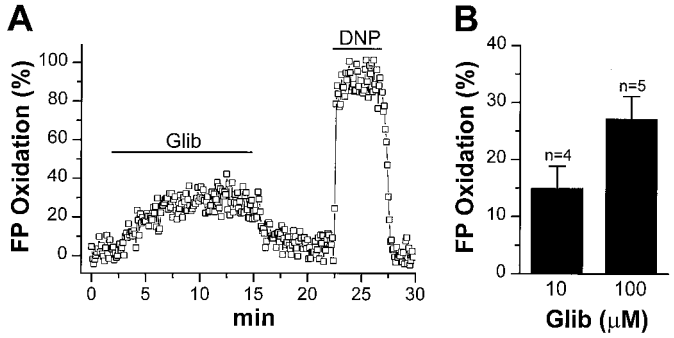

Fig. 7. Effect of glibenclamide on flavoprotein fluorescence in rabbit ventricular myocytes. A, glibenclamide (Glib, 100 μM) induced a reversible increase in flavoprotein (FP) oxidation, consistent with its reported uncoupling effect. B, concentration-dependent oxidative effect of glibenclamide.

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_{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_{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_{ATP} channel blocker is very important, especially given that mitoK_{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_{ATP} channels. At 500 μM, 5HD did not inhibit pinacidil-induced currents through expressed or native cardiac surfaceK_{ATP} channels, nor did it suppress diazoxide-induced surfaceK_{ATP} currents. In native cardiomyocytes, 5HD dramatically suppresses mitoK_{ATP} channel activity, yet it does not affect surfaceK_{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_{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_{ATP} channels as key players in the mechanism of ischemic preconditioning.

Acknowledgments

We thank Ms. Maria Janecki for technical assistance, Dr. Susumu Seino for providing SUR2A, and Dr. Yoshihisa Kurachi for providing mouse $K_{ir}6.2$.

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