Evidence against Functional Heteromultimerization of the $K_{\text{ATP}}$ Channel Subunits Kir6.1 and Kir6.2*

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$K_{\text{ATP}}$ channels consist of pore-forming potassium inward rectifier (Kir6.x) subunits and sulfonylurea receptors (SURs). Although Kir6.1 or Kir6.2 coassemble with different SUR isoforms to form heteromultimeric functional $K_{\text{ATP}}$ channels, it is not known whether Kir6.1 and Kir6.2 coassemble with each other. To define the molecular identity of $K_{\text{ATP}}$ channels, we used adenoviral gene transfer to express wild-type and dominant-negative constructs of Kir6.1 and Kir6.2 in a heterologous expression system (A549 cells) and in native cells (rabbit ventricular myocytes). Dominant-negative (DN) Kir6.2 gene transfer suppressed current through heterologously expressed SUR2A + Kir6.2 channels. Conversely, DN Kir6.1 suppressed SUR2B + Kir6.1 current but had no effect on coexpressed SUR2A + Kir6.2. We next probed the ability of Kir6.1 and Kir6.2 to affect endogenous $K_{\text{ATP}}$ channels in adult rabbit ventricular myocytes, using adenoviral vectors to achieve efficient gene transfer. Infection with the DN Kir6.2 virus for 72 h suppressed pinacidil-inducible $K_{\text{ATP}}$ current density measured by whole-cell patch clamp. However, there was no effect of infection with the DN Kir6.1 on the $K_{\text{ATP}}$ current. Based on these functional assays, we conclude that Kir6.1 and Kir6.2 do not heteromultimerize with each other and that Kir6.2 is the sole $K_{\text{ATP}}$ pore-forming subunit in the surface membrane of heart cells.

Whereas the gating of classical ion channels is regulated by membrane potential, $K_{\text{ATP}}$ channels respond instead to changes in cellular energy metabolism. $K_{\text{ATP}}$ channels are present in the surface membrane of various excitable cells, including cardiac myocytes (1, 2) and pancreatic β cells (3). These channels play an important role in several cellular responses, notably insulin secretion and hypoxic vasodilation, by linking the metabolic status of the cell to its membrane potential (4).

Given the important physiological and pathophysiological roles of these channels, there is good reason to elucidate their molecular composition. There are two types of $K_{\text{ATP}}$ channels in cardiac muscle (1, 2, 5), the sarcolemmal channel and the mitochondrial channel (mito$K_{\text{ATP}}$) (6–10). Although the molecular identity of the mito$K_{\text{ATP}}$ channel is not known, the sarcolemmal $K_{\text{ATP}}$ channel consists of pore-forming potassium inward rectifier (Kir6.x) subunits and sulfonylurea receptors (SURs). Heterologous expression studies suggest that cardiac sarcolemmal channels are heteromultimers of pore-forming Kir6.2 subunits and SUR2A subunits (5, 11–14), a prediction confirmed by Kir6.2 knockout studies in mice (15). However, a possible contribution from products of the Kir6.1 gene, which is richly expressed in the normal heart and elevated after ischemia (16, 17), has not been excluded. Previous work has shown that smooth muscle $K_{\text{ATP}}$ channels consist of Kir6.1 subunits and SUR2B subunits. In addition, immunogold staining has localized Kir6.1 to the inner mitochondrial membrane in skeletal muscle (18). Recently, Baron et al. (19) have used pharmacological and molecular studies to argue that $K_{\text{ATP}}$ channels in neonatal atrial myocytes are formed by the heteromultimeric association of several SUR and/or Kir subtypes. Whereas both Kir6.1 and Kir6.2 were found to be expressed in these cells, their relative contribution to the formation of functional $K_{\text{ATP}}$ channels is not known. Different SUR isoforms can clearly coassemble with either Kir6.1 or Kir6.2; however, it is not known whether Kir6.1 and Kir6.2 coassemble with each other.

To investigate the assembly of this channel, we used viral vectors designed to overexpress wild-type or dominant-negative Kir6.1 or Kir6.2 constructs driven by inducible ec dysone promoters in a heterologous expression system (A549 cells) and in adult rabbit ventricular myocytes. The electrical properties of $K_{\text{ATP}}$ channels in these cells lead us to conclude that Kir6.1 and Kir6.2 do not form functional heteromultimers.

EXPERIMENTAL PROCEDURES

Viral Vectors—The coding sequence for mouse Kir6.1 (or rabbit Kir6.2) was amplified using a polymerase chain reaction and cloned into pFPV-IRE5 (20) vector to generate polycistronic expression constructs.

Site-directed mutagenesis was performed using a QuickChange™ kit (Stratagene, La Jolla, CA) to modify codons 142 and 144 of wild-type mouse Kir6.1 (Kir6.1WT) to code for alanines instead of glycines to generate Kir6.1AFA (Fig. 1A). Similarly, wild-type rabbit Kir6.2 (Kir6.2WT) codons 131 and 133 were modified to code for alanines instead of glycines to generate mutant Kir6.2AFA. The wild-type and mutant Kir genes were cloned into a polycistronic adenovirus shuttle vector with ec dysone-inducible promoter coexpressing enhanced green fluorescent protein (pAdEGI) or human CD8 protein (pAdECDSI) to generate the modified vectors (21). Recombinant adenoviruses containing the various Kir6.1 and Kir6.2 constructs were generated by Cre-lox

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1 The abbreviations used are: mito$K_{\text{ATP}}$, mitochondrial ATP-sensitive potassium channel; Kir6.x, pore-forming potassium inward rectifier subunits; SUR(s) sulfonylurea receptor(s); WT, wild-type; AFA, glycine to alanine dominant-negative mutant.
Fig. 1. Schematic diagram showing the arrangement of the $K_{ATP}$ channel and the ecdysone-inducible adenovirus vector system. A, location of the AFA mutations in the Kir gene and the presumed subunit arrangement showing one example of a $K_{ATP}$ channel crippled by a dominant-negative Kir subunit (GFG → AFA). B, generation of the ecdysone-inducible adenovirus vector system. $\Psi$, packaging signal; Ecd Promoter, ecdysone-inducible promoter; IRES, internal ribosome entry site; ITR, inverted terminal repeat; MCS, multiple cloning site; pA, SV40 polyadenylation signal; RSV, Rous sarcoma virus;loxP, packaging site; RXR, retinoid X receptor; VgEGR, modified ecdysone receptor; EGFP, enhanced green fluorescent protein; CMV, cytomegalovirus.

recombination as described previously (21, 22). Fig. 1B summarizes the various adenovirus constructs used in this study.

Cell Isolation—Rabbit ventricular myocytes were isolated by enzymatic dissociation of adult rabbit hearts as described previously (9, 10, 23) and washed several times with calcium-free solution. Calcium concentration was gradually brought back to 1 mM. Cells were cultured on laminin-coated coverslips in M199 culture medium (CellGro; Mediatech, Herndon, VA) with 2% fetal bovine serum and 1% penicillin/ streptomycin at 37 °C.

Transfection and Infection—A human epithelial cell line A549 (CCL-185, American Type Culture Collection, Manassas, VA) was cotransfected with 1 µg of rabbit Kir6.2 and rat SUR2A (a gift of Dr. S. Seino, Chiba University School of Medicine, Chiba, Japan) using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) before infecting with various virus constructs. The virus constructs were coinfected with either the Kir or reporter-only virus and the receptor virus AdVgRXR at a ratio of about 10:1 as described previously (21). After about 1 h the cells were washed with virus-free culture medium. Expression was induced by the addition of 1 µM ponasterone A (Invitrogen, San Diego, CA) for up to 72 h before electrophysiological studies were conducted. We also transfected A549 cells using 6 µg of mouse Kir6.1 and mouse SUR2B (both gifts from Dr. Y. Kurachi, University of Osaka, Osaka, Japan) and LipofectAMINE before infecting as described above. Myocytes were cultured in 2% fetal bovine serum-containing M199 media and infected with various reporter and Kir constructs and induced for 72 h after infection before measuring the currents.

Electrophysiology—To quantify $K_{ATP}$ channel activity, we measured agonist-induced membrane current using the whole-cell patch clamp technique (24). Cells were superfused with external solution containing (in mM) NaCl, 140; KCl, 5; CaCl$_2$, 1; MgCl$_2$, 1; and HEPES, 10 (pH 7.4) with NaOH at room temperature (−22 °C). The internal pipette solution contained (in mM) potassium glutamate, 120; KCl, 25; MgCl$_2$, 0.5; EGTA, 10; HEPES, 10; and MgATP, 1 (pH 7.2) with KOH. Currents were elicited by ramp pulses in A549 cells (between −110 and 150 mV from a holding potential of −80 mV). The current amplitude at 0 mV was measured at steady state after the application of the $K_{ATP}$ channel agonist P1075 (pinacidil analog, 30 µM). In rabbit ventricular myocytes, currents were elicited every 6 s by square pulses (in consecutive steps from a holding potential of −80 mV to −40 mV for 100 ms and 0 mV for 380 ms), and $I_{K_{ATP}}$ was quantified 15 and 30 min after exposure to 100 µM pinacidil. To be sure that the currents measured reflected agonist-induced $K_{ATP}$ channels, we excluded experiments in which pinacidil-induced currents were irreversible after washout of drugs (8 cells), although including those data would not have changed the conclusions.

Preliminary experiments revealed that 48–72 h were required to obtain robust dominant-negative effects; therefore, electrophysiological recordings were made 72 h after infection in both A549 cells and rabbit ventricular myocytes.

Statistical Analysis—All data are presented as mean ± S.E. Statistical analysis was performed by analysis of variance combined with the Bonferroni pairwise comparison test when needed. $p$ values < 0.05 were considered significant.

RESULTS AND DISCUSSION

We have previously shown that dominant-negative Kir6.2 constructs suppress $K_{ATP}$ current in heterologous expression systems and in rat neonatal cardiomyocytes (11). Thus, we predicted that infection with AdKir6.2AFA would suppress the cardiac sarcolemmal $K_{ATP}$ current (Fig. 1A). To address the question of heteromultimerization of the Kir6.1 and Kir6.2 subunits, we examined the effects of infection with various Kir and control virus constructs on $I_{K_{ATP}}$ A549 cells cotransfected with 6.2WT and SUR2A subunits. $I_{K_{ATP}}$ was elicited pharmacologically by addition of the pinacidil derivative P1075, which potently activates these channels (25). Fig. 2, A–D shows typical membrane currents recorded in A549 cells 72 h post-trans-
fection, in the absence (control) and presence of P1075. Panel A shows the wild-type current expressed by Kir6.2 and SUR2A. Fig. 2, B–D shows currents after cotransfection and infection with 6.1AFA (B), CD8 (C), or 6.2AFA (D) viral constructs. Only the 6.2AFA cell (D) shows notably suppressed drug-activated currents. Fig. 2E summarizes the data for P1075-induced current in the various groups of A549 cells. The uninfected cells had current densities similar to those infected with CD8 reporter virus, indicating that infection itself does not alter the membrane currents. Significant dominant-negative suppression of IKATP through Kir6.2-SUR2A channels was achieved by infection with the Kir6.2AFA virus (p < 0.001 versus control) but not with Kir6.1AFA.

To verify that Kir6.1AFA can function as a dominant-negative construct in Kir6.1 homomultimers, we heterologously expressed Kir6.1 + SUR2B channels. Kir6.1 and SUR2B have been reported to form smooth muscle-type KATP channels (26, 27). As with Kir6.2 and SUR2A, we measured membrane currents elicited by the KATP channel opener P1075. Fig. 3, A–D shows IKATP recorded in A549 cells 72 h after transfection, before and during exposure to P1075. Panel A shows the current produced by simple cotransfection with Kir6.1 and SUR2B DNA. Fig. 3, B–D shows the currents after cotransfection and infection with 6.1AFA (B), CD8 (C), or 6.2AFA (D) viral constructs. Here, the only permutation with suppressed P1075-inducible current was 6.1AFA (B). The pooled data in Fig. 3E reveal that infection with 6.1AFA significantly inhibited IKATP through Kir6.1 + SUR2B channels (p < 0.005 versus either no virus or CD8), but there was no effect of Kir6.2AFA.

The whole-cell currents produced by heterologously expressed Kir6.1 + SUR2B are about half as large as those produced by Kir6.2 + SUR2A channels (0.75 ± 0.2 versus 1.7 ± 0.3 nA at 0 mV). However, the number of functional channels is likely to be comparable, considering the known difference in single-channel conductance of the two channels (35 versus 80 picosiemens; Refs. 5 and 27) and assuming similar open probabilities. Thus, the lack of suppression by the 6.1AFA construct on 6.2WT + SUR2A coexpressed current (and, conversely, the lack of effect of 6.2AFA on 6.1WT + SUR2B current) is most likely due to the absence of coassembly.

To probe the roles of Kir6.1 and Kir6.2 in native cells, we infected these viral constructs in adult ventricular myocytes in primary culture. Whole-cell patch recordings revealed that KATP current was greatly suppressed at 72 h in Kir6.2AFA-infected cells compared with green fluorescent protein-, 6.2WT-, 6.1WT-, or 6.1AFA-infected cells in primary culture. Fig. 4 summarizes the current densities measured 15 and 30 min after exposure to pinacidil in cells infected with all the viral constructs tested. Note that 6.2AFA-infected cells displayed pinacidil-induced current densities significantly lower than the others (at 30 min, 11.3 ± 2.7 picoampere/picofarad, n = 7 versus control, 36.8 ± 4.1 picoampere/picofarad, n = 6, p < 0.01). None of the other constructs had any effect on KATP current density, indicating that infection with 6.2AFA signifi-

Fig. 2. KATP currents induced by the agonist P1075 in A549 cells 72 h after transfection with Kir6.2 and SUR2A and, when indicated, infection with various adenovirus constructs. The current amplitude at 0 mV was measured at steady state after the application of 30 μM P1075. A, no virus; B, 6.1AFA; C, CD8; D, 6.2AFA; and E, summarized data for the currents. **, p < 0.001 versus no virus. Cont, control.
cantly suppressed the native sarcolemmal $K_{\text{ATP}}$ current in rabbit ventricular cells. Whereas the results are clear-cut at 72 h, no effect was evident at 24 h, and only a trend was apparent at 48 h (data not shown). This time course is consistent with that described for effective suppression of other K$^+$ channels by dominant-negative constructs in myocytes and neurons (20). The length of time required for effective suppression presumably reflects the time required for the assembly of the non-functional tetramer relative to the degradation of previously synthesized functional channels.

The results are notable in several respects. First of all, they confirm that Kir6.2 is crucial for cardiac surface $K_{\text{ATP}}$ channels. Second, the absence of an effect of exogenous wild-type Kir6.2 expression on the surface $K_{\text{ATP}}$ channel indicates that the availability of Kir6.2 is not a rate-limiting factor in the biogenesis of these channels (as might be the case if there were a reserve store of presynthesized but unpaired SUR2 proteins). Third, the lack of an effect of Kir6.1WT or Kir6.1AFA on the sarcolemmal $K_{\text{ATP}}$ channels, taken together with the lack of suppression of 6.1AFA on the heterologously expressed Kir6.2$^1$SUR2A channel, and of 6.2AFA on Kir6.1$^1$SUR2B channels, indicate that Kir6.1 and Kir6.2 do not form functional heteromultimers either in A549 cells or in native cardiac myocytes. The latter finding leaves open the question of what role, if any, Kir6.1 may play in heart cells. Specifically, the present experiments do not exclude a role for Kir6.1 in the formation of mito$K_{\text{ATP}}$ channels (18), which are crucial for the endogenous cardioprotective phenomenon known as ischemic preconditioning (25).

![Fig. 3. $K_{\text{ATP}}$ currents induced by P1075 in A549 cells 72 h after transfection with Kir6.1 and SUR2B and, when indicated, infection with various adenovirus constructs. The current amplitude at 0 mV was measured at steady state after the application of 30 μM P1075. A, no virus; B, 6.1AFA; C, CD8; D, 6.2AFA; and E, summarized data for the currents. *, $p < 0.005$ versus no virus. Cont, control.](image)

![Fig. 4. $K_{\text{ATP}}$ currents induced by pinacidil in rabbit ventricular myocytes infected with various adenovirus constructs 72 h earlier. The current amplitude at 0 mV was measured 15 and 30 min after application of 100 μM pinacidil. *, $p < 0.005$ versus control. **, $p < 0.001$ versus control.](image)
cence as the end point, may help to address possible contributions of Kir6.1 and/or 6.2 to mitoK\textsubscript{ATP} channels.

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REFERENCES

1. Noma, A. (1983) Nature 305, 147–148
2. Trube, G., and Hescheler, J. (1984) Pfluegers Arch. Eur. J. Physiol. 401, 178–184
3. Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P., IV, Boyd, A. E., III, Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D. A. (1995) Science 268, 423–426
4. Achari, F. M. (1988) Annu. Rev. Neurosci. 11, 97–118
5. Inagaki, N., Gonoi, T., Clement, J. P., IV, Wang, C. Z., Aguilar-Bryan, L., Bryan, J., and Seino, S. (1996) Neuron 16, 1011–1017
6. Inoue, I., Nagase, H., Kishi, K., and Higuti, T. (1991) Nature 352, 244–247
7. Paucek, P., Mironova, G., Mahdi, F., Beavis, A. D., Woldegiorgis, G., and Garlid, K. D. (1992) J. Biol. Chem. 267, 26062–26069
8. Garlid, K. D., Paucek, P., Yarov-Yarovoy, V., Murray, H. N., Darbenzio, R. B., D'Alonzo, A. J., Lodge, N. J., Smith, M. A., and Grover, G. J. (1997) Circ. Res. 81, 1072–1082
9. Liu, Y., Sato, T., O'Rourke, B., and Marbán, E. (1998) Circulation 97, 2463–2469
10. Sato, T., O'Rourke, B., and Marbán, E. (1998) Circ. Res. 83, 110–114
11. Lalli, M. J., Johns, D. C., Janecki, M., Liu, Y., O'Rourke, B., and Marbán, E. (1998) Pfluegers Arch. Eur. J. Physiol. 436, 957–961
12. Babenko, A. P., Gonzalez, G., Aguilar-Bryan, L., and Bryan, J. (1998) Circ. Res. 83, 1132–1143
13. Jovanovic, A., Jovanovic, S., Lorenz, E., and Terzic, A. (1998) Circulation 98, 1548–1555
14. Hu, H., Sato, T., Seharaseyon, J., Liu, Y., Johns, D. C., O'Rourke, B., and Marbán, E. (1999) Mol. Pharmacol. 55, 1000–1005
15. Miki, T., Tashiro, F., Iwanaga, T., Nagashima, K., Yoshitomi, H., Aihara, H., Nitta, Y., Gonoi, T., Inagaki, N., Miyazaki, J., and Seino, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11968–11973
16. Inagaki, N., Tsuura, Y., Namba, N., Masuda, K., Gonoi, T., Horie, M., Seino, Y., Mizuta, M., and Seino, S. (1995) J. Biol. Chem. 270, 5691–5694
17. Akao, M., Otani, H., Horie, M., Takano, M., Kuniyayu, A., Nakayama, H., Kouchi, I., Murakami, T., and Sasayama, S. (1997) J. Clin. Invest. 100, 3053–3059
18. Suzuki, M., Katake, K., Fujikura, K., Inagaki, N., Suzuki, T., Gonoi, T., Seino, S., and Takata, K. (1997) Biochem. Biophys. Res. Commun. 241, 693–697
19. Baron, A., van Beever, L., Monnier, D., Roatti, A., and Baertschi, A. J. (1999) Circ. Res. 85, 707–715
20. Johns, D. C., Nuss, H. B., and Marbán, E. (1997) J. Biol. Chem. 272, 31598–31603
21. Johns, D. C., Marx, R., Mains, R. E., O'Rourke, B., and Marbán, E. (1999) J. Neurosci. 19, 1691–1697
22. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., and Phipps, M. L. (1997) J. Virol. 71, 1842–1849
23. Liu, Y., Gao, W. D., O'Rourke, B., and Marbán, E. (1996) Basic Res. Cardiol. 91, 450–457
24. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Pfluegers Arch. Eur. J. Physiol. 391, 85–100
25. Sato, T., Sasaki, N., Seharaseyon, J., O'Rourke, B., and Marbán, E. (2000) Circulation, in press
26. Isomoto, S., Konno, C., Yamada, M., Matsumoto, S., Higashiguchi, O., Horie, Y., Matsuza, Y., and Kurachi, Y. (1996) J. Biol. Chem. 271, 24321–24324
27. Yamada, M., Isomoto, S., Matsumoto, S., Konno, C., Shindo, T., Horie, Y., and Kurachi, Y. (1997) J. Physiol. (Lond.) 499, 715–720