

# Hypercapnic Acidosis Activates $K_{ATP}$ Channels in Vascular Smooth Muscles

Xueren Wang, Jianping Wu, Li Li, Fuxue Chen, Runping Wang, Chun Jiang

**Abstract**—ATP-sensitive  $K^+$  channels ( $K_{ATP}$ ) couple intermediary metabolism to cellular activity, and may play a role in the autoregulation of vascular tones. Such a regulation requires cellular mechanisms for sensing  $O_2$ ,  $CO_2$ , and pH. Our recent studies have shown that the pancreatic  $K_{ATP}$  isoform (Kir6.2/SUR1) is regulated by  $CO_2$ /pH. To identify the vascular  $K_{ATP}$  isoform(s) and elucidate its response to hypercapnic acidosis, we performed these studies on vascular smooth myocytes (VSMs). Whole-cell and single-channel currents were studied on VSMs acutely dissociated from mesenteric arteries and HEK293 cells expressing Kir6.1/SUR2B. Hypercapnic acidosis activated an inward rectifier current that was  $K^+$ -selective and sensitive to levcromakalim and glibenclamide with unitary conductance of  $\approx 35$  pS. The maximal activation occurred at pH 6.5 to 6.8, and the current was inhibited at pH 6.2 to 5.9. The cloned Kir6.1/SUR2B channel responded to hypercapnia and intracellular acidification in an almost identical pattern to the VSM current. In situ hybridization histochemistry revealed expression of Kir6.1/SUR2B mRNAs in mesenteric arteries. Hypercapnia produced vasodilation of the isolated and perfused mesenteric arteries. Pharmacological interference of the  $K_{ATP}$  channels greatly eliminated the hypercapnic vasodilation. These results thus indicate that the Kir6.1/SUR2B channel is a critical player in the regulation of vascular tones during hypercapnic acidosis. (*Circ Res.* 2003;92:1225-1232.)

**Key Words:**  $K_{ATP}$  ■ Kir6.1 ■ hypercapnia ■ ATP ■ vasodilation

$K_{ATP}$  channels are expressed in various tissues including pancreatic  $\beta$ -cells, skeletal muscles, myocardium, vascular smooth myocytes, and central neurons where they couple the intermediary metabolism to cellular excitability.<sup>1,2</sup> Although these  $K^+$  channels are sensitive to ATP, studies have shown that they are also modulated by other nucleotides and phospholipids.<sup>3-9</sup> Previous studies have shown that  $K_{ATP}$  channels in myocardium and insulin-secreting cell line are activated by intracellular acidification.<sup>10,11</sup> Similar activation was observed in the cloned pancreatic  $K_{ATP}$  isoform (Kir6.2/SUR1) during hypercapnic acidosis.<sup>12-15</sup> The regulation of  $K_{ATP}$  by protons is particularly significant, because pH alterations occur in a large variety of physiological and pathophysiological conditions and are more frequently seen than sole energy depletion.

The pH sensitivity may allow the  $K_{ATP}$  channels to play a role in autoregulation of vascular tones. Experimental evidence suggests that the  $K_{ATP}$  channels may be involved in reactive hyperemia, as sulfonylurea blocks hyperemic vasodilation.<sup>16,17</sup> The autoregulation occurs in most tissues including the heart and brain in which it underlies the cardioprotective effect of ischemic preconditioning and the activity-dependent regulation of cerebral circulation.<sup>18-21</sup> The reactive hyperemia is produced by hypoxia, hypercapnia, acidosis, and accumulation of other metabolic products in local tissues.

Because under most physiological and pathophysiological conditions, ATP levels may not readily drop to submillimolar concentrations in cells to activate  $K_{ATP}$  channels,<sup>1,2</sup> demonstration of the  $CO_2$ /pH sensitivity of the  $K_{ATP}$  channels becomes critical for understanding the molecular basis of the autoregulation of vascular tones.

The vascular  $K_{ATP}$  channels are likely to be made of Kir6.1 and SUR2B. The heteromeric Kir6.1/SUR2B channel has several biophysical properties similar to the  $K_{ATP}$  channels described in mesenteric artery and portal vein.<sup>22-24</sup> Among these properties is the stimulation of channel activity by internal nucleoside diphosphates (NDP), which gave them the name  $K_{NDP}$  channel.<sup>22,23</sup> The Kir6.1 is indeed involved in vascular functions, as mice with the Kir6.1-gene defect have a high rate of sudden death associated with spontaneous electrocardiographic ST elevation followed by atrioventricular block.<sup>25</sup> To test the hypotheses that (1) the vascular  $K_{ATP}$  channel(s) is sensitive to  $CO_2$ /pH, and (2) its  $CO_2$ /pH sensitivity plays a crucial role in the vascular autoregulation, we performed these studies.

## Materials and Methods

### Cell Dissociation

Vascular smooth myocytes were freshly dissociated from rats (Sprague-Dawley, 200 to 400 g; Charles River Laboratories, Wil-

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ington, Mass). All animal experiments comply with the IACUC approval of the Georgia State University. The rats were anesthetized by inhaling saturated halothane vapor and decapitated. Under a microscope, mesenteric arteries were dissected free, cut into small pieces (1 to 2 mm), and placed in a 5 mL vial containing (in mmol/L) 120 NaCl, 5.4 KCl, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 0.3 Na<sub>2</sub>HPO<sub>4</sub>, 10 D-glucose, and 24 NaHCO<sub>3</sub>, with 0.5 mg/mL collagenase (Type F, Sigma). The solution was bubbled with 5% CO<sub>2</sub>-95% O<sub>2</sub> (pH 6.9). The incubation vial was maintained at 37°C and continually stirred for 45 minutes. The digested tissues will then be washed and triturated with a fire-polished glass Pasteur pipette to yield single smooth muscle cells.<sup>26,27</sup>

### Expression Kir6.1 and SUR2B in Mammalian Cell Line

Rat Kir6.1 (GenBank No. D42145) and rat SUR2B cDNAs (GenBank No. D86038) were cloned into a eukaryotic expression vector (pcDNA3.1, Invitrogen Inc). The Kir6.1/SUR2B channel was expressed in the human embryonic kidney (HEK293) cells as we detailed previously.<sup>15,28</sup> The cells were transfected using Lipofectamine 2000 (Invitrogen) with a 5- $\mu$ g mixture of Kir6.1 and SUR2B cDNAs in a 1:2 ratio and split twice weekly. To facilitate the identification of positively transfected cells, 0.5  $\mu$ g of green fluorescent protein (GFP) cDNA (pEGFP-N2, Clontech) were added to the cDNA mixture. Cells were disassociated from the monolayer using 0.01% trypsin 24 to 48 hours after transfection. A few drops of the cell suspension were added to a Petri dish, and then incubated at 37°C for at least 2 hours before experiments.

### Electrophysiology

Patch clamp experiments were performed at room temperature as described previously.<sup>12,15,26,27</sup> In brief, fire-polished patch pipettes were made from 1.2 mm borosilicate capillary glass (Sutter Instruments). Giant inside-out patches were used to study macroscopic currents in a cell-free condition using recording pipettes of 0.5 to 1.0 M $\Omega$ . Current records were low-pass filtered (2 kHz, Bessel, 4-pole filter, -3 dB), digitized (20 kHz, 16-bit resolution), and stored on computer disk for later analysis (pCLAMP8, Axon Instruments). The bath solution contained (in mmol/L) 140 potassium gluconate, 10 KCl, 1 MgCl<sub>2</sub>, 5 EGTA, 5 UDP, 10 D-glucose, and 10 HEPES (pH 7.4). The pipette was filled with the same solution. In ATP-containing solutions, pH was titrated to 7.4 after ATP (K<sup>+</sup> salt) was added. All ATP-containing solutions were prepared immediately before experiments and used for less than 4 hours. For whole-cell studies, the bath solution contained (in mmol/L) 125 KCl, 24 KHCO<sub>3</sub>, 5 NaCl, 1 MgCl<sub>2</sub>, and 5 EGTA (pH 7.4 after bubbled with 5% CO<sub>2</sub>). For isohydric hypercapnia, HCO<sub>3</sub><sup>-</sup> was increased to 38.4 and 48 mmol/L for 8% and 10% CO<sub>2</sub> studies, respectively, by reducing KCl concentration accordingly (pH 7.4). The pipette solution contained (in mmol/L) 145 KCl, 1 MgCl<sub>2</sub>, 5 UDP (Na<sup>+</sup> salt), and 5 EGTA (pH 7.4).

### In Situ Hybridization Histochemistry

The Kir6.1 and SUR2B cDNAs were produced by in vitro transcription. The antisense and sense riboprobes were labeled with digoxigenin using a riboprobe labeling kit (Roche Diagnostics). The length of the hydrolytic products was confirmed to be 400 to 500 bp with electrophoresis. Transverse mesenteric sections of 10  $\mu$ m were made using a Cryostat (Leica) at -20°C, and thaw-mounted onto 3-amino-propyltriethoxysilane coated slides. Tissue-endogenous phosphatases were inactivated with a treatment of 0.2 mol/L HCl for 8 minutes. The tissues were permeabilized with 1  $\mu$ g/mL RNase-free proteinase K in the TE buffer (100 mmol/L Tris-HCl, 50 mmol/L EDTA, pH 8.0) at 37°C for 30 minutes. After draining the prehybridization buffer, sections were overlaid with 50  $\mu$ L hybridization buffer containing 200 ng/mL cRNA probe that has been denatured at 80°C. Then the sections were incubated at 50°C in a humidified chamber for 16 hours. In a shaker water bath of 45°C, sections were washed twice with 2 $\times$ SSC (15 minutes each) and then twice with 1 $\times$ SSC (15 minutes each). Sections were incubated with 1:4000

alkaline phosphatase-conjugated sheep anti-DIG antibodies for 2 hours. Reactions were stopped at optimal color development by dipping the sections briefly in distilled water. Sections were then covered with an aqueous mounting solution (Crystal/Mount, Biomed).

### Isolated and Perfused Mesenteric Arteries

The mesenteric vascular bed was dissected from the intestinal wall. The third-order branches (100 to 200  $\mu$ m) of the superior mesenteric artery were isolated with a short secondary branch that was then cannulated with polyethylene-polyvinyl tubing (200  $\mu$ m/L in diameter). The tissue was then maintained at 37°C, and superfused with a modified Krebs-bicarbonate buffer containing (in mmol/L): 120 NaCl, 5.0 KCl, 1.2 MgSO<sub>4</sub>, 2.4 CaCl<sub>2</sub>, 10 D-glucose, 24 NaHCO<sub>3</sub>, and 0.1 EGTA. The tissue was allowed to equilibrate for  $\approx$ 30 minutes in this solution aerated with 5% CO<sub>2</sub>-95% O<sub>2</sub>. Baseline recordings were taken when the arteries were perfused and superfused (2 mL/min) with the modified Krebs-bicarbonate buffer bubbled with 5% CO<sub>2</sub>-95% O<sub>2</sub> (pH 7.4). Hypercapnia was produced by switching the superfusate to a solution containing (in mmol/L) 96 NaCl, 5.0 KCl, 1.2 MgSO<sub>4</sub>, 2.4 CaCl<sub>2</sub>, 10 D-glucose, 48 NaHCO<sub>3</sub>, and 0.1 EGTA. This solution had been bubbled with 10% CO<sub>2</sub>-90% O<sub>2</sub> for at least 30 minutes before use (pH 7.4). The same gas mixture flew over the tissue during the hypercapnic exposure. When 8% CO<sub>2</sub> was studied, NaHCO<sub>3</sub> concentration was reduced to 38.4 mmol/L with an increase in NaCl to 105.6 mmol/L.

Data are presented as mean  $\pm$  SE. ANOVA or Student's *t* test was used. Differences were considered to be statistically significant if *P*  $\leq$  0.05.

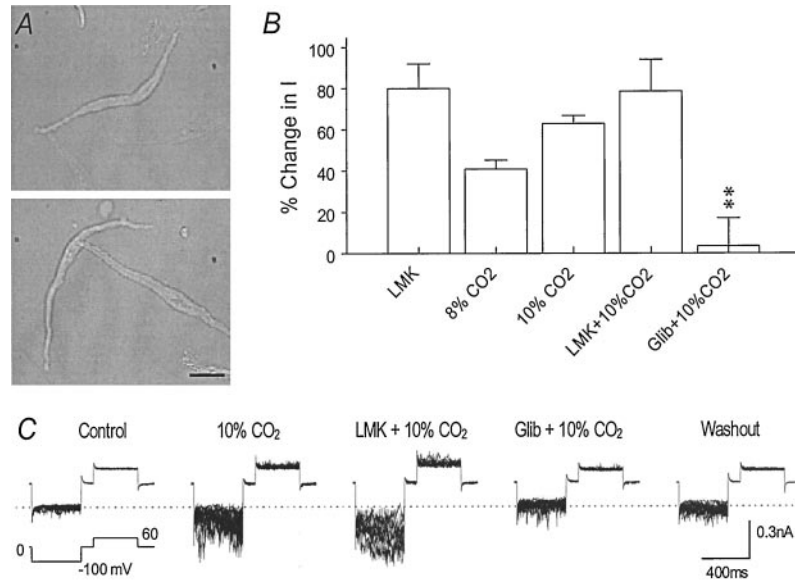
## Results

### Hypercapnia Activates K<sub>ATP</sub> Currents in Acutely Dissociated Vascular Smooth Myocytes

To understand how the vascular K<sub>ATP</sub> channels respond to P<sub>CO2</sub>/pH, we performed patch clamp experiments on VSMs freshly dissociated from rat mesenteric arteries. These dissociated cells showed clear 3-dimensional morphology with smooth surface. Cells were rejected for further studies, if they (1) became flat and lost 3-dimensional structure, (2) had membrane blebs or rough surface, (3) showed the sign of swelling or shrinkage, and (4) failed to attach to the surface of the recording chamber. Figure 1A shows a few examples of the dissociated VSMs.

Whole-cell currents were studied in these freshly dissociated VSMs in the voltage clamp mode. These cells expressed K<sup>+</sup> currents with weak inward rectification. These currents were activated by 10  $\mu$ mol/L levcromakalim, a K<sub>ATP</sub> channel activator, by 80.1  $\pm$  12.1% (n=4).

The inward rectifying currents were rather small at baseline. The current amplitude increased markedly when cells were exposed to 10% CO<sub>2</sub> (62.9  $\pm$  3.8%, n=15; Figure 1B). The current activation is reversible and dependent on CO<sub>2</sub> levels (Figure 1B). Complete recovery was seen in cells with a period (3 to 5 minutes) of washout. This effect was seen in most VSMs, whereas a small number of cells showed no response or was slightly inhibited during the CO<sub>2</sub> exposure. We assumed that they constituted distinct populations of VSMs, on which further studies were not attempted. Selective decrease in extracellular pH to 6.6 did not produce any evident increase in the current amplitude, suggesting that the current activation was mediated by intra- but not extracellular acidification. The current activation was almost completely abolished when there was 10  $\mu$ mol/L glibenclamide, a K<sub>ATP</sub>



**Figure 1.** Activation of  $K^+$  channels by  $CO_2$  in acutely dissociated mesenteric VSMs. **A**, Cells were freshly dissociated from the rat mesenteric artery and photographed with phase-contrast optics and a CCD camera at the resolution of  $1280 \times 860$  dpi. One VSM is shown in the top panel and two in the bottom panel. Calibration =  $50 \mu m$ . **B**, Inward rectifying  $K^+$  currents were studied in the VSMs. Currents were strongly activated by  $10 \mu mol/L$  levcromakalim (LMK). These currents were also activated by exposing the cells to  $8\% CO_2$ . Higher concentration of  $CO_2$  ( $10\%$ ) produced large current activation. In the presence of  $10 \mu mol/L$  levcromakalim,  $10\% CO_2$  enhanced only modestly the inward rectifying currents, reaching a level that was almost the same as with levcromakalim alone. Current activation was blocked by  $10 \mu mol/L$  glibenclamide (Glib). I, current;  $**P < 0.01$  in comparison with the  $10\% CO_2$ . **C**, Whole-cell currents were recorded from a VSM acutely dissociated from the mesenteric artery using a bath solution containing  $145 mmol/L K^+$  and  $25 mmol/L$  bicarbonate. This solution was constantly bubbled with  $5\% CO_2$  (pH 7.4). Pulse command potentials (bottom panel in Control) were applied to the patch from a holding potential of  $0 mV$ . Under this condition, small inward rectifying currents were seen at baseline (Control). These currents were strongly activated when the cell was exposed to a solution that contained  $48 mmol/L$  bicarbonate and was bubbled with  $10\% CO_2$  (pH 7.4). Exposure to  $10 \mu mol/L$  levcromakalim (LMK) with the hypercapnia further activated the currents by  $\approx 30\%$ . Glibenclamide (Glib,  $10 \mu mol/L$ ) completely suppressed the channel activation. Dotted line indicates the baseline level. Note that outward currents do not show marked changes, and 10 superimposed traces are shown in each panel.

channel blocker, in the perfusate bubbled with  $10\% CO_2$  ( $3.6 \pm 13.5\%$ ,  $n=7$ ;  $P < 0.01$  in comparison with  $10\% CO_2$ ) (Figures 1B and 1C). In the presence of levcromakalim ( $10 \mu mol/L$ ), the current amplitude was modestly augmented by  $10\% CO_2$  reaching a level ( $78.5 \pm 15.5\%$ ,  $n=7$ ) that was not different from the effect of levcromakalim alone ( $P > 0.05$ ; Figures 1B and 1C). The currents activated by  $10\% CO_2$  were almost 80% of the total currents activated by  $10 \mu mol/L$  levcromakalim, a concentration known to open most  $K_{ATP}$  channels (Figure 1B). The currents activated by  $CO_2$  showed weak inward rectification.

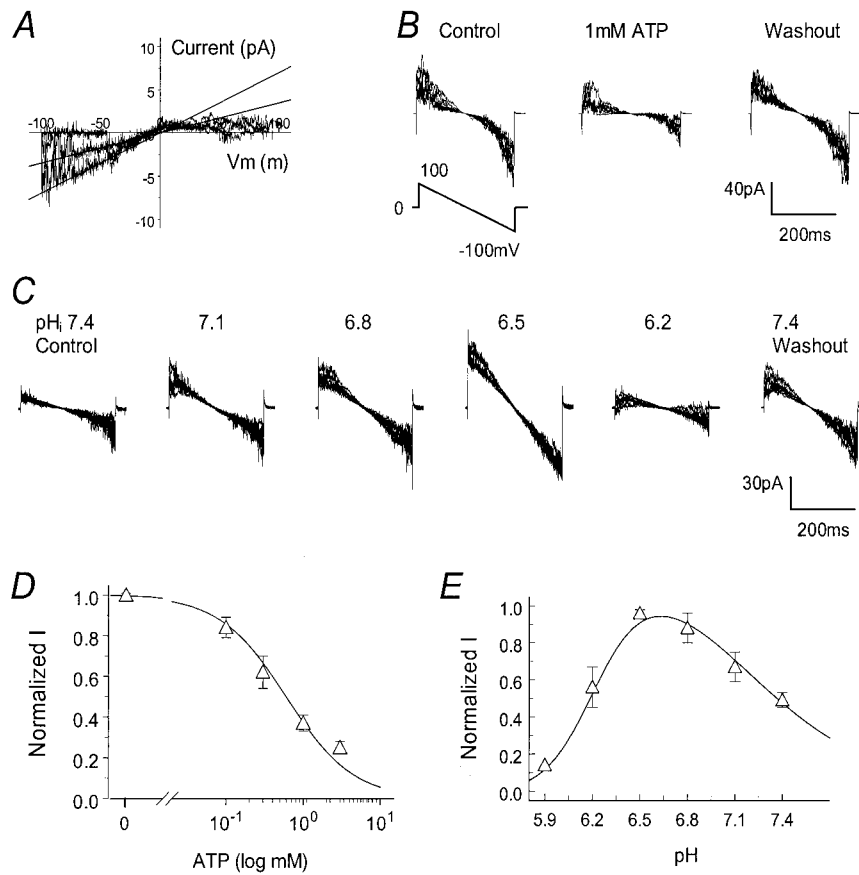
Single-channel currents were studied in inside-out patches obtained from the dissociated VSMs. With equal concentrations of  $K^+$  ( $145 mmol/L$ ) on both sides of patch membranes, inward rectifying currents were observed. These currents were  $K^+$  selective, and had unitary conductance of  $35.0 \pm 1.2 pS$  ( $n=9$ ) (Figure 2A). These currents showed rapid rundown after patch excision. Such rundown was alleviated in the presence of  $5 mmol/L$  UDP and  $1 mmol/L$   $Mg^{2+}$  in the internal solution. Under such a condition, the currents were inhibited by ATP (Figure 2B) with  $IC_{50}$   $0.6 mmol/L$  (Figure 2D). The current amplitude increased when the internal surface of patch membranes was exposed to acidic pH. The maximal activation occurred at pH 6.5 to 6.8, at which the inward rectifying currents increased by  $103.0 \pm 17.7\%$  ( $n=5$ ). Further acidification caused inhibition of channel activity (Figures 2C and 2E), which was only partially reversible or

even irreversible in some cells. Exposure of the intracellular patch membranes to isohydric hypercapnia ( $10\% CO_2$ , pH 7.4) did not have any significant effect on the currents ( $1.7 \pm 4.4\%$ ,  $n=4$ ), suggesting that the effect is produced by pH rather than molecular  $CO_2$ .

In addition to these acid-activated currents, we observed currents that were inhibited by acidic pH in a small number of patches. The acid-inhibited currents that were also  $K^+$ -selective with inward rectification were insensitive to ATP and glibenclamide, suggesting that they are not recorded from  $K_{ATP}$  channels.

### Kir6.1 and SUR2B Are Expressed in Vascular Smooth Muscles

The currents activated during hypercapnic acidosis showed several properties similar to the cloned Kir6.1 channel.<sup>22,23</sup> To demonstrate the expression of the Kir6.1 and SUR2B in mesenteric arteries, we performed in situ hybridization experiments using antisense riboprobes labeled with digoxigenin. Distributions of the Kir6.1 and SUR2B mRNAs were revealed under bright-field microscope. Coexpression of Kir6.1/SUR2B mRNAs was observed in mesenteric arteries (Figure 3A). Control experiments were done in the renal tissue in which arteries but not veins nor the tubular epithelium were stained, indicating that the labeling with these riboprobes is specific (Figure 3B). Consistent results were obtained from 4 rats for each of these tissues. The sense riboprobes did not show any positive labeling in these tissues.



**Figure 2.** A, Single-channel currents were recorded from a VSM acutely dissociated from the mesenteric artery. Equal concentrations of  $K^+$  (145 mmol/L, in the presence of 5 mmol/L UDP and 1 mmol/L  $Mg^{2+}$ ) were used on both sides of the inside-out patch membrane. Two active channels were recorded at  $pH_i$  6.5 in an inside-out patch. Both of them were closed at  $pH_i$  7.4. Acid-activated currents had a unitary slope conductance of 35 pS with inward rectification. B, With ramp command potentials from 100 to  $-100$  mV at a holding potential of 0 mV, inward rectifying currents were recorded at baseline ( $pH_i$  7.4). These macroscopic currents were reversibly inhibited by 1 mmol/L ATP. C,  $pH_i$  sensitivity was studied in another inside-out patch. Small currents were seen at  $pH_i$  7.4. The current amplitude increased when the internal surface of the patch membrane was exposed to acidic pH. Maximal current activation occurred at  $pH_i$  6.5. Further decrease in  $pH_i$  caused channel inhibition. D, Dose-dependent current inhibition by ATP. Data were expressed using the Hill equation (line):  $y = 1 / (1 + ([ATP]/IC_{50})^h)$ , where  $IC_{50}$  is the midpoint channel inhibition,  $h$  the Hill coefficient.  $IC_{50} = 0.6$  mmol/L;  $h = 1.0$ . E, Dose-dependent response was studied under the same condition as shown in C, and is described using a sum of two Hill equations (line):  $y = \{1 / [1 + (pH/pK_1)^{h_1}]\} + \{1 / [1 + (pK_2/pH)^{h_2}]\} - 1$ , where  $pK_1 = 7.20$  the midpoint channel activation,  $h_1 = 1.2$  the Hill coefficient for channel activation,  $pK_2 = 6.20$  the midpoint channel inhibition, and  $h_2 = 3.4$  the Hill coefficient for channel inhibition. Evident increase in the current amplitude was seen at  $pH_i$  7.1; maximal current activation occurred at  $pH_i$  6.5; and the currents showed inhibition at  $pH_i$  6.2 or lower. Note that 3 superimposed traces are shown in A, and 8 in each panel of B and C. Data are presented as mean  $\pm$  SE ( $n = 4$  to 5).

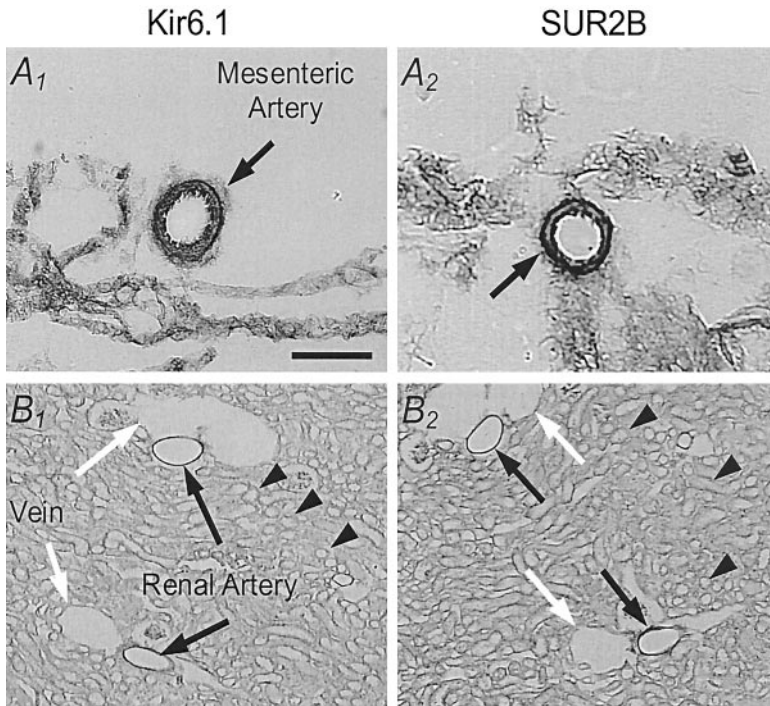
### Similar Activation Is Seen in the Kir6.1/SUR2B Channel During Hypercapnic Acidosis

The Kir6.1/SUR2B channel was expressed in HEK293 cells. Inward rectifying currents with single-channel conductance of  $35.7 \pm 0.3$  pS ( $n = 9$ ) were recorded from these cells in excised inside-out patches (Figure 4A). As reported previously, channel activity rapidly declined after excision. In the presence of 5 mmol/L UDP and 1 mmol/L  $Mg^{2+}$ , these channels remained active and were inhibited by ATP with  $IC_{50}$  0.5 mmol/L (Figure 4D). They were also stimulated by 10  $\mu$ mol/L levcromakalim and inhibited by 10  $\mu$ mol/L glibenclamide (not shown).

The  $pH_i$  sensitivity of the Kir6.1/SUR2B channel was studied in excised inside-out patches. Symmetrical concentrations of  $K^+$  (145 mmol/L) were applied to both sides of the patch membranes. Exposure of the internal surface of patch membranes to a perfusate with various pH levels produced concentration-

dependent channel activation (Figure 4C). The open-state probability ( $P_o$ ) reached maximal at  $pH_i$  6.8 to 6.5. Further acidification caused channel inhibition, which resembled the channel rundown as washout led to poor recovery. The pH- $P_o$  relationship for the Kir6.1/SUR2B channel was almost identical to that for the VSM  $K_{ATP}$  currents shown in Figure 2E with  $pK$  7.2 for the channel activation (Figure 4E), suggesting that these channels can be activated in the physiological pH range. In contrast to  $P_o$ , single-channel conductance ( $34.7 \pm 0.9$  pS,  $n = 6$ , at  $pH$  6.2) did not show any significant change ( $P > 0.05$ ; Figure 4B). Isohydric hypercapnia had no effect on the single-channel currents ( $1.7 \pm 5.8\%$ ,  $n = 5$ ). Consistent with these results, the whole-cell Kir6.1/SUR2B currents were activated by  $CO_2$  ( $101 \pm 20\%$  by 8%  $CO_2$ ,  $n = 4$ ;  $162 \pm 27\%$  by 10%  $CO_2$ ,  $n = 6$ ). Thus, all the baseline biophysical properties and channel responses to  $CO_2$ /pH are consistent with the acid-activated current recorded from the VSMs.



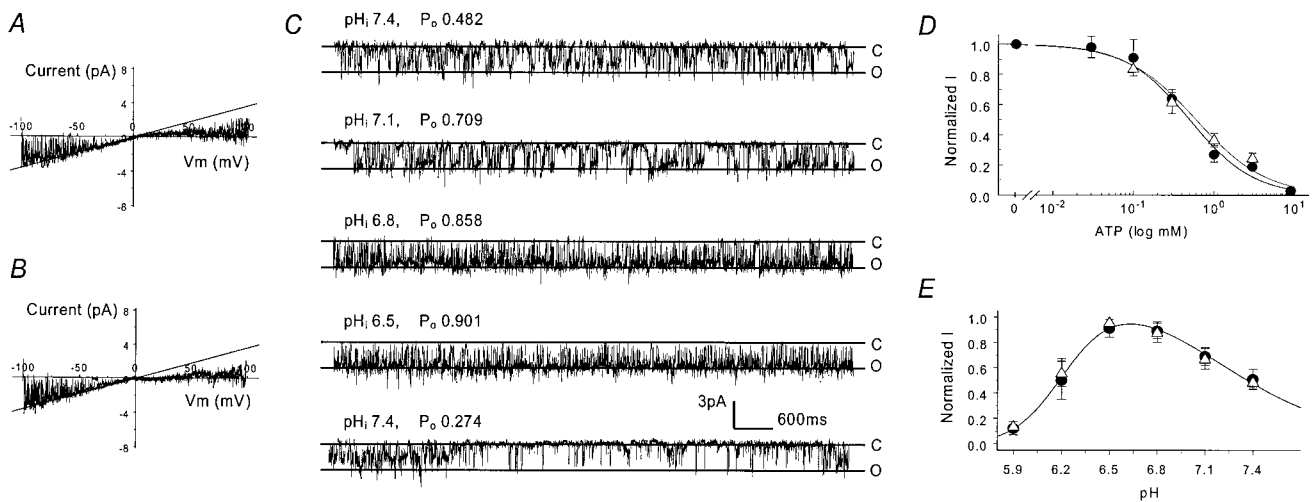


**Figure 3.** Expression of Kir6.1 and SUR2B in vascular smooth muscles. In situ hybridization histochemistry was performed in mesenteric and renal tissues using antisense riboprobes labeled with digoxigenin. A, Positive stains of both Kir6.1 and SUR2B were seen in the mesenteric arteries. B, In the kidney, arteries (black arrow) but not veins (white arrow) neither tubular epithelium (arrowhead) were positively labeled. Calibration=50  $\mu$ mol/L for A and B and 125  $\mu$ mol/L for C.

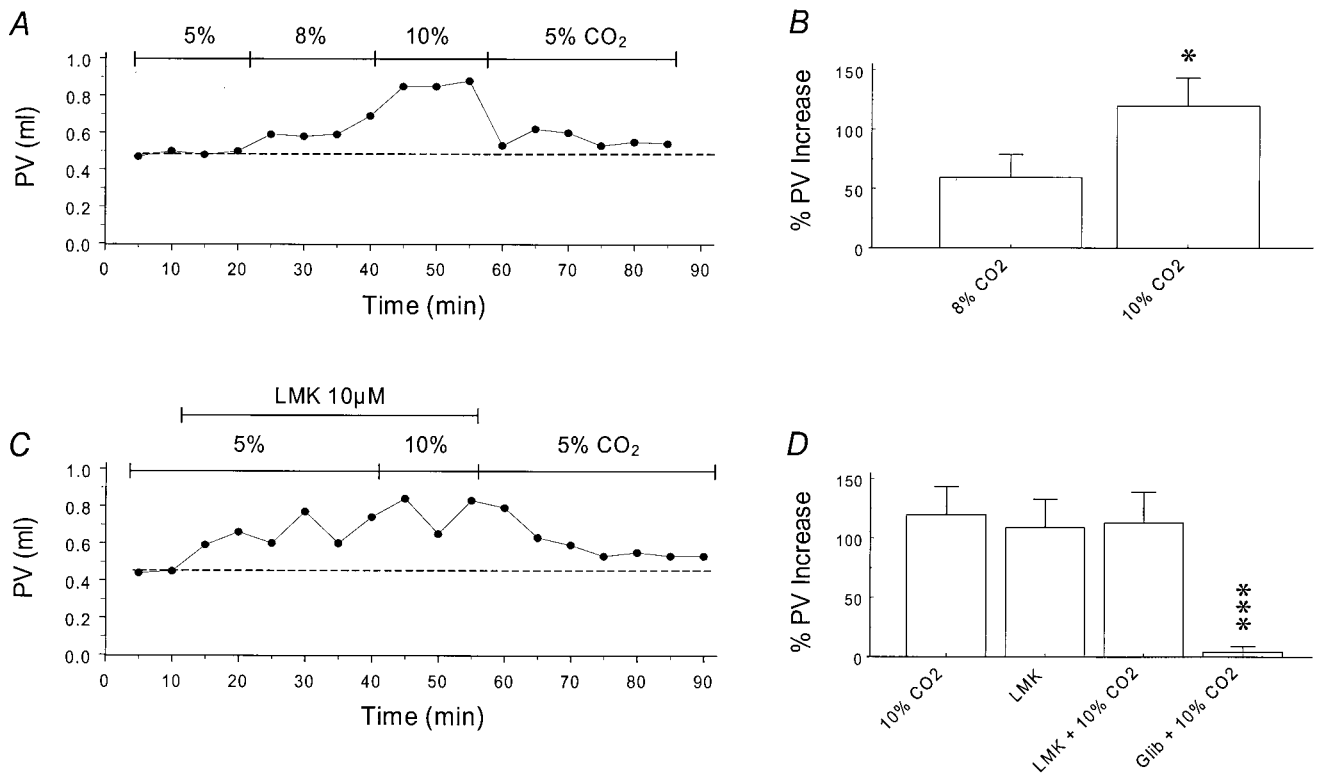
**Hypercapnia Produces  $K_{ATP}$  Channel-Dependent Vasodilation of the Isolated and Perfused Mesenteric Artery**

If the vascular  $K_{ATP}$  channels play a role in regulation of vascular tones, hypercapnia should lead to vasodilation by activation of this channel. To test this hypothesis, we performed experiments on isolated arteries. The third-order branches of the mesenteric artery were perfused and super-

fused with modified Krebs solutions. Under a given perfusion pressure (70 mm Hg), the perfusion solution was collected at the other end of the arteries every 5 minutes. The perfusate volume (PV) was accounted as an index of vasodilation caused by isohydric hypercapnia (note that extracellular acidification had no effect as shown above). The PV increased by  $59.4 \pm 19.3\%$  ( $n=6$ ) when the arteries were exposed to a superfusate bubbled with 8%  $CO_2$  (with



**Figure 4.** Activation of Kir6.1/SUR2B currents by acidic pH. A, Single-channel recording was done in an inside-out patch obtained from an HEK cell transfected with Kir6.1/SUR2B. Symmetrical concentrations of  $K^+$  (145 mmol/L, 5 mmol/L UDP) were applied to both sides of the patch membrane. Unitary conductance was measured with a ramp command potential from  $-100$  to  $100$  mV at  $pH_i$  7.4. Straight line indicates a slope conductance of 35 pS;  $V_m$ , membrane potential. B, Conductance retained 35 pS at  $pH_i$  6.2. C, At membrane potential of  $-80$  mV, an active channel was recorded in an inside-out patch at  $pH_i$  7.4. Channel activity ( $P_o$ ) increased at  $pH_i$  7.1.  $P_o$  was almost doubled and plateaued at  $pH_i$  6.8 and 6.5. C indicates closure; O, opening. Note that  $P_o$  was measured from a 20 second stretch of data. D, Dose-dependent inhibition of currents by ATP.  $IC_{50}=0.6$  and  $0.5$  mmol/L, and  $h=1.0$  and  $1.2$ , for the VSM currents (triangle) and the Kir6.1/SUR2B (circle), respectively. E, Current- $pH_i$  relationship is expressed as shown in Figure 2D. The relationship of Kir6.1/SUR2B channel with  $pH_i$  (circle) can be described with the same equation used for the VSM channel (triangle) (ie,  $pK_1=7.20$ ,  $pK_2=6.20$ ). Data are presented as mean  $\pm$  SE ( $n=4$  to 5).



**Figure 5.** Involvement of  $K_{ATP}$  channels in hypercapnic vasodilation of mesenteric arteries. **A**, Consecutive records from an isolated mesenteric artery that was perfused and superposed with the Krebs solutions. Vascular tensions were indicated by perfusion volume (PV) collected at a constant pressure of 70 mm Hg each 5 minutes. Exposure of the artery to superfusates bubbled with 8% or 10%  $CO_2$  produced concentration-dependent increases in the perfusion volume. Such a vasodilation was reversible, as washout led to almost complete recovery. Broken line indicates the baseline level. **B**, Percentage PV increase with 8% and 10%  $CO_2$ . PV collected each 5 minutes and averaged from the last 2 to 3 collections. Data are presented as mean  $\pm$  SE (\* $P$ <0.05). **C**, In another mesenteric artery, the PV increased with the exposure to levcromakalim (LMK, 10  $\mu$ mol/L) but failed to show any significant further increase during hypercapnia in the presence of LMK. Washout led to evident recovery. **D**, Summary of effects of the  $K_{ATP}$  channel activator and blocker on the  $CO_2$ -induced vasodilation. Glibenclamide (Glib, 10  $\mu$ mol/L) almost completely abolished the increase in the PV during hypercapnia (\*\* $P$ <0.001 in comparison with the 10%  $CO_2$ ). After the arteries were pretilated with 10  $\mu$ mol/L LMK, 10%  $CO_2$  failed to produce further increase in the PV ( $P$ >0.05 in comparison with the LMK alone).

38.4 mmol/L  $HCO_3^-$ , pH7.4). This effect was reversible and dependent on  $PCO_2$  levels, as 10%  $CO_2$  (with 48 mmol/L  $HCO_3^-$ , pH7.4) raised the perfusate volume by  $119.7 \pm 23.7\%$  ( $n=9$ ) (Figures 5A and 5B). Such a hypercapnia-induced vasodilation was abolished almost completely in the presence of 10  $\mu$ mol/L glibenclamide in the perfusate and superfusate ( $4.3 \pm 4.8\%$ ,  $n=4$ ) (Figure 5D). Levcromakalim (10  $\mu$ mol/L) enhanced the perfusate flow by  $109.0 \pm 23.9\%$  ( $n=4$ ). After the levcromakalim-induced vasodilation, 10%  $CO_2$  did not produce any significant further increase in the PV ( $112.7 \pm 25.5\%$ ,  $n=4$ ;  $P$ >0.05 in comparison to levcromakalim exposure) (Figures 5C and 5D). Such a value was not different statistically from that with 10%  $CO_2$  exposure alone, suggesting that activation of  $K_{ATP}$  channels underscores the hypercapnic vasodilation in the mesenteric artery.

### Discussion

Several lines of experimental evidence have suggested that the vascular  $K_{ATP}$  channels are pH sensitive. The cerebral circulation is automatically regulated according to local neuronal activity,  $PO_2$  and  $PCO_2$  levels.<sup>29–31</sup> Such a regulation can be attenuated by  $K_{ATP}$  channel blockers.<sup>32,33</sup> Similar regulation of vascular tones has been reported in several

peripheral tissues such as the coronary, renal, pulmonary, and skeletal muscular circulations.<sup>34–38</sup> However, these previous studies on the  $K_{ATP}$  channels are suggestive but not conclusive, because they depended on pharmacological agents that are known to affect other ion channels as well.<sup>39,40</sup> The fact that there are a large number of ion channels expressed in the VSMs has greatly limited the further understanding of the molecular basis of the pH sensitivity with the pharmacological approach. Indeed, we observe that certain  $K^+$  channels are inhibited by acidic pH in the present study. Therefore, the information about the molecular identity of the acid-activated channels becomes crucial for any further investigations on the pH-dependent regulation of vascular tones.

Previous studies using RT-PCR and Northern blot have suggested that Kir6.1 and SUR2B may form  $K_{ATP}$  channels in blood vessels, based on the logical reasoning that they are expressed in all tissues.<sup>41,42</sup> Better evidence was found by Yamada et al,<sup>24</sup> who demonstrated a  $K_{ATP}$  channel by coexpression of Kir6.1 and SUR2B with all features of the vascular  $K_{NDP}$  channel described in mesenteric artery and portal veins.<sup>22,23</sup> This vascular  $K_{ATP}$  channel is observed in our current studies in the VSMs. We compare this channel with the Kir6.1/SUR2B channel expressed in mammalian cell line.

Our results support the idea that this vascular  $K_{ATP}$  channel is equivalent to the Kir6.1/SUR2B channel for the following reasons: (1) Kir6.1 and SUR2B are coexpressed in mesenteric arteries as shown in our *in situ* hybridization studies; (2) we have recorded functional  $K_{ATP}$  currents from the VSMs dissociated from these arteries; (3) the acid-activated currents in the VSMs can be blocked in the presence of  $K_{ATP}$  channel blocker, and largely eliminated with prior activation of  $K_{ATP}$  channels by the channel activator; (4) the acid-activated currents in VSMs show single-channel properties and nucleotide sensitivity similar to the Kir6.1/SUR2B channel; (5) the pH sensitivity of the vascular  $K_{ATP}$  channel is the same as the Kir6.1/SUR2B. The  $CO_2$  effect on cloned Kir6.1 channels is greater than on vascular  $K_{ATP}$ , because the percentage effect shown appears to be exaggerated by the high density of Kir6.1 channels over the low levels of background currents in the HEK293 expression system. Therefore, the  $K_{ATP}$  channel that we showed in mesenteric arteries appears to be the Kir6.1/SUR2B isoform.

Using cloned  $K_{ATP}$  channels, we have recently shown that the Kir6.2 channel can detect intracellular pH.<sup>12–15</sup> Similar to the Kir6.1/SUR2B shown in the present study, the pancreatic  $K_{ATP}$  isoform (Kir6.2/SUR1) is activated during hypercapnia.<sup>12–15</sup> Such channel activation is independent of ATP and relies on a histidine residue in the C terminus.<sup>12–14</sup> With pK 7.1, the pH sensitivity allows the channel to be activated in most physiological and pathophysiological conditions.<sup>12</sup> These studies as well as our current observations indicate that both Kir6.1 and Kir6.2 are activated by intracellular protons, making a clear contrast to all other Kir channels that are inhibited by intracellular protons.<sup>43</sup>

Our results show that the  $CO_2$ /pH sensitivity does allow the vascular  $K_{ATP}$  channel to regulate vascular tones. In the isolated and perfused mesenteric arteries,  $CO_2$  produces concentration-dependent vasodilation. The hypercapnia-induced vasodilation is abolished in the presence of 10  $\mu$ mol/L glibenclamide. Levromakalim enhances the perfusate flow and attenuates  $CO_2$ -induced further vasodilation. All these are consistent with our patch clamp studies in the mesenteric VSMs. In addition, we find that the maximal vasodilation is reached by 10%  $CO_2$  (Figure 5D) with  $\approx 80\%$  of the  $K_{ATP}$  channel activation (Figure 1B), suggesting that the vascular  $K_{ATP}$  channel is regulated by physiological levels of  $PCO_2$ .

The phenomenon that vascular tones are regulated according to metabolic state has been known for a long time, although the molecular basis was rather poorly understood.<sup>29</sup> Such an autoregulation depends on sensors in local tissues for the detection of metabolic products/substrates, effectors for the control of vascular smooth muscle contractility, and certain coupling mechanisms between the sensors and effectors. Our observations in the present study indicate that the  $K_{ATP}$  channel plays a key role in this process.  $K_{ATP}$  channels are widely known to control membrane potential and cellular excitability.<sup>1,2</sup> With the  $CO_2$ /pH sensitivity, this vascular  $K_{ATP}$  channel is therefore capable of coupling the  $PCO_2$ /pH levels to cellular excitability. Our results obtained from the isolated and perfused arteries show that such a property indeed enables the  $K_{ATP}$  channel to regulate vascular tones according

to the ambient  $PCO_2$  levels. Therefore, our studies on tissue expression, comparison of channel biophysical properties, and functional relevance to vascular tones have begun to shed insight into the molecular basis of vascular regulation during hypercapnia. We believe that the demonstration of  $K_{ATP}$  channel activation by hypercapnic acidosis is not only a remarkable finding but also has profound impact on the understanding of circulation physiology and the design of therapeutic modalities for several cardiovascular diseases, as the manipulation of this  $K_{ATP}$  channel by changing  $PCO_2$  or intracellular pH may provide an intervention to vascular tones in various tissues during metabolic stress. Thus, the identification of a specific membrane protein coupling hypercapnic acidosis to arterial tones contributes significantly to the understanding of the vascular regulation under physiology and pathophysiological conditions.

In conclusion, our studies have shown that hypercapnia and intracellular acidification activate a  $K^+$  current in the mesenteric VSMs that is sensitive to  $K_{ATP}$  channel blocker and activator. The channel is stimulated by millimolar concentrations of UDP and inhibited by ATP in the presence of UDP with unitary conductance of 35 pS. All these properties are also observed in the Kir6.1/SUR2B channel under the same experimental condition. In isolated and perfused mesenteric arteries, inhibition of the  $K_{ATP}$  channel eliminated vasodilation during hypercapnia. These as well as the coexpression of Kir6.1 and SUR2B mRNAs in the arteries indicate that the channel activated during hypercapnic acidosis in the mesenteric VSMs is likely to be made of the Kir6.1 and SUR2B and plays an important role in vascular regulation during hypercapnic acidosis.

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