Differential pH sensitivity of Kir4.1 and Kir4.2 potassium channels and their modulation by heteropolymerisation with Kir5.1

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1. The inwardly rectifying potassium channel Kir5.1 appears to form functional channels only by coexpression with either Kir4.1 or Kir4.2. Kir4.1–Kir5.1 heteromeric channels have been shown to exist *in vivo* in renal tubular epithelia. However, Kir5.1 is expressed in many other tissues where Kir4.1 is not found. Using Kir5.1-specific antibodies we have localised Kir5.1 expression in the pancreas, a tissue where Kir4.2 is also highly expressed.

2. Heteromeric Kir5.1–Kir4.1 channels are significantly more sensitive to intracellular acidification than Kir4.1 currents. We demonstrate that this increased sensitivity is primarily due to modulation of the intrinsic Kir4.1 pH sensitivity by Kir5.1.

3. Kir4.2 was found to be significantly more pH sensitive ($pK_a = 7.1$) than Kir4.1 ($pK_a = 5.99$) due to an additional pH-sensing mechanism involving the C-terminus. As a result, coexpression with Kir5.1 does not cause a major shift in the pH sensitivity of the heteromeric Kir4.2–Kir5.1 channel.

4. Cell-attached single channel analysis of Kir4.2 revealed a channel with a high open probability ($P_o > 0.9$) and single channel conductance of ~25 pS, whilst coexpression with Kir5.1 produced novel bursting channels ($P_o < 0.3$) and a principal conductance of ~54 pS with several subconductance states.

5. These results indicate that Kir5.1 may form heteromeric channels with Kir4.2 in tissues where Kir4.1 is not expressed (e.g. pancreas) and that these novel channels are likely to be regulated by changes in intracellular pH. In addition, the extreme pH sensitivity of Kir4.2 has implications for the role of this subunit as a homotetrameric channel.

Inwardly rectifying potassium (Kir) channels are found in almost every cell type where they play key roles in controlling membrane potential, cellular excitability and K^+ fluxes (Nichols & Lopatin, 1997; Reimann & Ashcroft, 1999). A large number of clones have now been identified which encode Kir channels and these can be divided into seven major subfamilies (Kir1.0–Kir7.0). Until recently very little was known about the only member of the Kir5.0 subfamily, Kir5.1 (Bond *et al.* 1994). This subunit is unusual in that it only appears to form functional K^+ channels when assembled as a heteromeric channel with members of the Kir4.0 subfamily, Kir4.1 and Kir4.2 (Pessia *et al.* 1996; Pearson *et al.* 1999).

The functional role of these heteromeric Kir4.0–Kir5.1 channels remains unclear. However, recent studies have shown that Kir4.1–Kir5.1 heteromeric channels exist *in vivo* in renal tubular epithelia. In addition these heteromeric channels have been shown to be extremely sensitive to inhibition by protons within the physiological pH range (Tanemoto *et al.* 2000; Tucker *et al.* 2000; Xu *et al.* 2000; Yang *et al.* 2000). There is therefore an emerging role for these channels in the pH-dependent regulation of K^+ fluxes and acid–base homeostasis.

Extensive studies with Kir1.1, another pH-sensitive channel, have shown that the primary pH sensor is a lysine residue found within a highly conserved region of the proximal N-terminus (Fakler *et al.* 1996; Schulte *et al.* 1999). Lysine is normally fully protonated under physiological conditions. However, this lysine residue in Kir1.1 (K80) exhibits anomalous titration due to its close association with two highly conserved arginine residues, one in the intracellular N-terminus and one in

intracellular C-terminus (Schulte *et al.* 1999). This implies that the two intracellular domains of this channel are in close physical proximity to form this 'Arg-Lys-Arg' triad. This is supported by recent evidence which demonstrates a direct physical and functional interaction between the N- and C-terminal domains (Schulte *et al.* 1998; Tucker & Ashcroft, 1999).

Several different studies have also revealed that there is a correlation between the intrinsic sensitivity of a Kir channel to intracellular pH and the presence of a lysine residue at the equivalent position to K80 in Kir1.1. One such example is Kir4.1, which also forms pH-sensitive homomeric channels (Schulte *et al.* 1999; Yang & Jiang, 1999). Like Kir1.1, the pH sensitivity of Kir4.1 channels is primarily determined by a similar titratable lysine residue in the proximal N-terminus (K67), which is equivalent to K80 in Kir1.1. However the intrinsic pH sensitivity of Kir4.1 channels ($pK = 6.0$) is significantly lower than that of Kir1.1 ($pK_a = 6.9$) and regulation of Kir4.1 channels by pH_i is only likely to be significant in cases of extreme intracellular acidosis.

We and others have recently shown that the intrinsic pH sensitivity of Kir4.1 can be modified by hetero-polymerisation with Kir5.1 (Tanemoto *et al.* 2000; Tucker *et al.* 2000; Xu *et al.* 2000; Yang *et al.* 2000). Coexpression of Kir4.1 with Kir5.1 produces novel K^+ channels with a significantly 'enhanced' pH sensitivity ($pK_a = 7.35$). Inhibition of these channels by protons is direct and does not involve a reduction in the single-channel conductance (Tucker *et al.* 2000; Yang *et al.* 2000). Interestingly, the pH sensitivity of these heteromeric channels still appears to be governed by the Kir4.1 subunit. Mutation of the titratable lysine residue (K67M) in Kir4.1 almost completely abolishes the pH sensitivity of the heteromeric Kir4.1–Kir5.1 channels (Xu *et al.* 2000; Yang *et al.* 2000). However, the mechanism by which Kir5.1 alters the intrinsic pH sensitivity of Kir4.1 remains to be determined.

Although Kir4.1 and Kir5.1 have been shown to form heteromeric channels *in vivo* their tissue specific patterns of expression do not completely overlap. RT-PCR and Northern blot analysis have shown Kir5.1 to be expressed in several tissues where Kir4.1 is not, and vice versa (Bond *et al.* 1994; Shuck *et al.* 1997). It is therefore highly likely that other subunits permit functional expression of Kir5.1 in tissues where Kir4.1 is not found. We have therefore investigated the properties of the related subunit Kir4.2 which is also pH sensitive and which forms heteromeric channels with Kir5.1 (Pearson *et al.* 1999).

In this study we have used Kir5.1 specific antibodies to reveal abundant expression of Kir5.1 in the pancreas, a tissue where Kir4.2 (but not Kir4.1) is expressed (Shuck *et al.* 1997; Gosset *et al.* 1997). We have also further investigated the contribution of Kir5.1 to the pH sensitivity of Kir4.0–Kir5.1 heteromeric channels and have determined some of the primary biophysical properties of both Kir4.2 and Kir4.2–Kir5.1 channels. We demonstrate that in addition to a titratable lysine residue in the N-terminus, Kir4.2 has an additional pH-sensing mechanism involving the intracellular C-terminal domain. This mechanism renders homomeric Kir4.2 channels significantly more sensitive to intracellular acidification than Kir4.1 and is unaffected by hetero-polymerisation with Kir5.1. These results have important implications for the role of Kir4.2 in tissues where Kir5.1 is both present and absent.

METHODS

Immunohistochemistry

Procedures involving animals and their care have been conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, 1987). In accordance with these institutional guidelines, 60-day-old male Sprague-Dawley rats were deeply anaesthetised by intraperitoneal injection with Nembutal (40 mg kg^{-1}); tissues were washed and fixed by intracardiac perfusion with sodium phosphate buffer (PBS 0.1 M, pH 7.4) and with 10% buffered formalin. The production and specificity of the anti-Kir5.1 antibody has previously been described in detail (Salvatore *et al.* 1999). Sections of pancreas were prepared, fixed and stained as described before (Salvatore *et al.* 1999). Antibody staining was visualised using 3,5′-diaminobenzidine (DAB) and tissues were counterstained with methylene blue. Control sections were stained with the anti-Kir5.1 antibody previously absorbed to an excess of antigenic peptide (Salvatore *et al.* 1999).

Molecular biology

All channel subunits were subcloned into the oocyte expression vector pBF, which provides 5′ and 3′ untranslated regions from the *Xenopus* β-globin gene flanking a polylinker containing multiple restriction sites. *In vitro* mRNAs were generated using the SP6 polymerase. Kir subunits were joined in tandem as previously described (Pessia *et al.* 1996). This method provides control for the stoichiometry of the heteromeric channels and does not affect channel function (Pessia *et al.* 1996; Tucker *et al.* 2000). Kir4.1–Kir4.2 chimeras were constructed by extension overlap PCR. Splicing of the C-terminus was between residues 171 of Kir4.1 and 170 of Kir4.2.

Electrophysiology

Two-electrode voltage-clamp recording (TEVC). The care and handling of *Xenopus laevis* were in accordance with the highest standards of institutional guidelines in compliance with both national and international laws and policies (see above). Frogs underwent no more than two surgeries, separated by at least 3 weeks. Frogs were anaesthetised with an aerated solution of 3-aminobenzoic acid ethyl ester. Standard recording solution contained 90 mM KCl, 3 mM $MgCl_2$, 10 mM Hepes (pH 7.4) unless otherwise stated. Intracellular acidification was achieved using a potassium acetate buffering system (Choe *et al.* 1997). Only one pH/inhibition value per cell was determined in TEVC experiments. Microelectrodes were filled with 3 M KCl and had resistances of 0.1–0.5 MΩ. Recordings were performed at 22 °C, 18–48 h after injection with a GeneClamp 500 amplifier (Axon Instruments) interfaced to a Power Macintosh 7200/90 computer with an ITC-16 computer interface (Instrutech Corp., NY, USA). Currents were evoked by voltage commands from a holding potential of −10 mV, delivered in −10 mV increments from +50 to −120 mV, unless otherwise stated.

Patch-clamp recording

Patch-clamp recordings were performed at room temperature (22 °C) using an Axopatch 200B amplifier (Axon Instruments). Oocytes were bathed in a cytoplasmic solution containing 120 mM KCl, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM Hepes, 0.1 mM dithiothreitol (pH 7.2). Recording electrodes were pulled from borosilicate glass, dipped in sticky wax (Kerr, Emoryville, CA, USA) prior to polishing, and had resistances of 3–15 MΩ. The pipette solution contained 120 mM KCl, 10 mM Hepes, 200 μM $CaCl_2$ (pH 7.2). Patch records were obtained in the cell-attached and inside-out configuration by stepping the holding potential to various test potentials for 20–60 s. Junction potentials between bath and pipette solutions were properly nullified. Current traces at each holding potential were filtered at 1 kHz with a 4-pole low-pass Bessel filter and acquired at 5–10 kHz with a Pulse+PulseFit program (HEKA Elektronik GmbH, Germany). Channel activity was analysed with a TAC-TACfit program (Bruxton Co., Seattle, WA, USA) using the 50% threshold technique to determine the event amplitude. Channel openings were visually inspected before being accepted (event-by-event mode).

Inside-out giant patches were obtained by using borosilicate pipettes that had resistances of 0.4–1 MΩ. The composition of the intracellular and extracellular solutions was as described above. The cytoplasmic solutions of different pHs were applied through a fast-flow system by using multibarrel pipettes. Macroscopic currents were recorded by applying voltage ramps from −100 to 100 mV (filtered at 1 kHz and digitised at 5 kHz). For each giant patch all pH/inhibition data points were sequentially determined. The pHi–inhibition relationships were constructed by including in the analysis only giant-patch macroscopic currents showing minimal run-down assessed as complete return of the current amplitudes to control levels.

RESULTS

Immunolocalisation of Kir5.1 in the pancreas

Due to the incomplete overlap in the tissue-specific distribution of Kir4.1 and Kir5.1 we chose to examine Kir5.1 expression in tissues where Kir4.2 is expressed but Kir4.1 is not. Using Northern blot analysis Shuck *et al.* (1997) have previously shown Kir4.2 mRNA to be abundantly expressed in the pancreas but found no evidence of Kir4.1 expression. To determine whether Kir5.1 is also expressed in pancreatic tissues we utilised highly specific anti-Kir5.1 polyclonal antibodies. These antibodies have been used previously to detect Kir5.1 expression in the kidney and testes (Salvatore *et al.* 1999; Tucker *et al.* 2000). Immunohistochemical analysis of 60-day-old rat pancreatic tissues revealed Kir 5.1 to be localised in both the exocrine (acini) and the endocrine (islets of Langerhans) regions (Fig. 1*A*). No detectable immunoreactivity was observed in the control staining (Fig. 1*B*). Interestingly, a particularly intense ABC-immunoperoxidase staining was detected in the pancreatic arteriolae (Fig. 1*C*). The endothelial cells of these vessels appear positively stained, although further work is required to determine a more detailed localisation of Kir5.1 within this structure.

Kir5.1 enhances the intrinsic pH sensitivity of Kir4.1

We and others have recently shown that hetero-polymerisation of Kir5.1 with Kir4.1 produces novel channels which have an enhanced sensitivity to inhibition by intracellular acidification (Tanemoto *et al.* 2000; Tucker *et al.* 2000; Xu *et al.* 2000; Yang *et al.* 2000). However, in addition to inhibition by acidification, Kir4.1–Kir5.1 channels also exhibit activation by alkalinisation. Figure 2*A* shows currents recorded from an excised inside-out membrane patch from *Xenopus* oocytes expressing Kir4.1–Kir5.1 channels. These channels exhibit clear activation upon exposure to recording

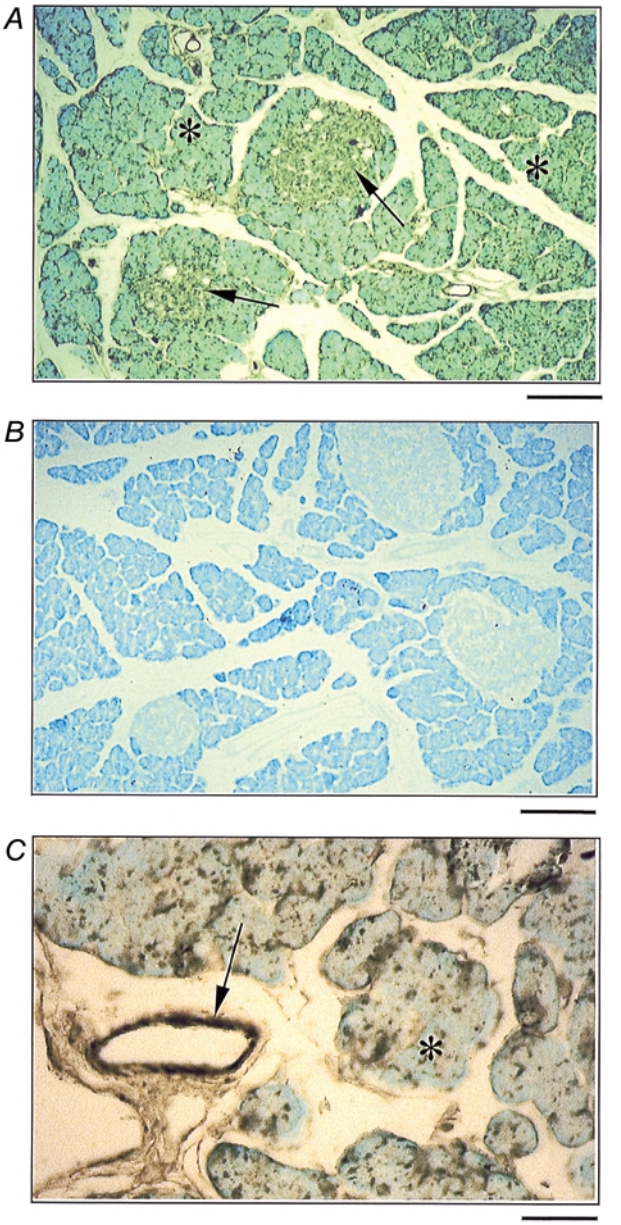

Figure 1. Immunohistochemical localization of Kir 5.1 subunits in the rat pancreas

A, Kir 5.1 immunoreactivity was detected in the islets of Langerhans (arrows) and also on the acinar cells (*). *B*, control slices show total absence of brown staining. *C*, an intense immunoreactivity is detectable in pancreatic arteriolae (arrow) and acinar cells (*). Scale bars: 25 μm in *A* and *B*; 10 μm in *C*.

solution at pH 8.0, which is readily reversible upon return to control solution at pH 7.0.

The primary determinant of the pH sensitivity of Kir4.1 is a lysine residue (K67) in the N-terminus (Schulte *et al.* 1999). In order to monitor the response of these channels to intracellular acidification we utilised a well-established potassium acetate buffering system previously used to study these channels (Choe *et al.* 1997). Whole-cell currents were then recorded by two-electrode voltage clamp from oocytes expressing different combinations of Kir subunits. Measurement of pH sensitivity using this method is less accurate than measurement in excised patches due to the activity of these channels in the pH 7.5–8.0 range and the inability to alkalinise the intracellular pH. However, the values recorded provide clear indication of the relative differences between channels and more accurate estimations of the pK_a were determined in giant inside-out excised patches and are shown in Fig. 5 and Table 1.

In agreement with the findings of Xu *et al.* (2000) the Kir4.1(K67M) mutation has profound effects on the pH sensitivity of both Kir4.1 and Kir4.1–Kir5.1 channels (Fig. 2*C*; Table 1). Interestingly, we found that either subunit is capable of acting as the pH sensor. Wild-type Kir5.1 does not possess a lysine residue at the equivalent position within the N-terminus. However, the pH sensitivity of these Kir4.1(K67M)–Kir5.1 channels could be restored by replacing the equivalent residue in Kir5.1 with a lysine residue, Kir4.1(K67M)–Kir5.1(M73K) (Fig. 2*B* and *C*; Table 1). This provides further evidence for the role of a titratable lysine residue at this position in

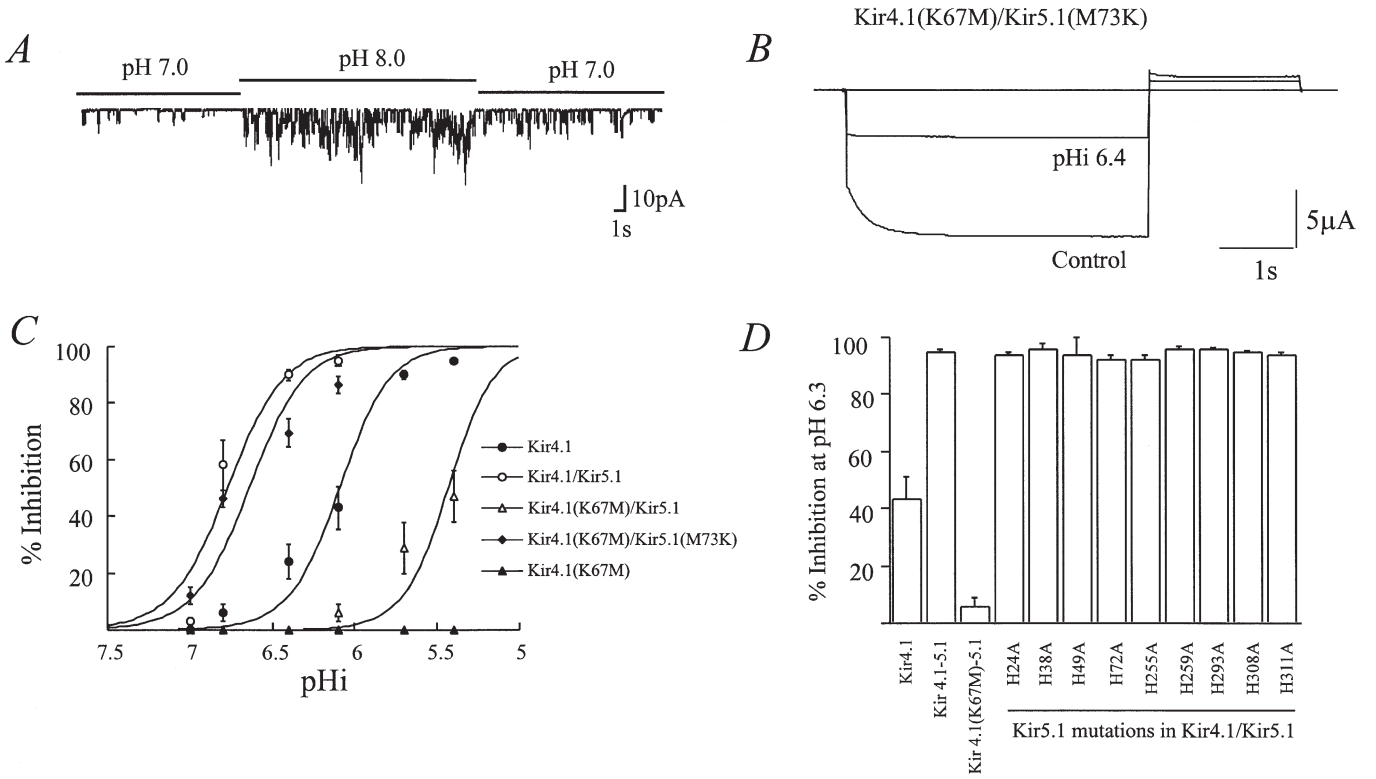

Figure 2. Modulation of Kir4.1–Kir5.1 channel activity by acidification and alkalinisation

A, inside-out patch recordings from oocytes expressing Kir4.1–Kir5.1 channels. Channel openings were evoked by hyperpolarising pulses to −100 mV, from a holding potential of 0 mV. The representative trace shows the effect on channel activity by the application of a cytoplasmic solution at pH 8, through a multibarrel fast-flow pipette. *B*, whole-cell representative current traces recorded from a *Xenopus* oocyte expressing Kir4.1(K67M)–Kir5.1(M73K) channels. Currents were evoked by hyperpolarisation to −100 mV for 4 s from a holding potential of −10 mV followed by a 2 s pulse to +40 mV. Current traces are shown before (control) and after intracellular acidification to the indicated value (pH_i 6.4). *C*, intracellular pH *vs.* current inhibition for Kir4.1–Kir5.1 (○), Kir4.1 (●), Kir4.1(K67M)–Kir5.1 (△), Kir4.1(K67M)–Kir5.1(M73K) (◆) and Kir4.1(K67M) (▲). Data points were obtained from currents recorded at −100 mV in the TEVC configuration in control conditions and during the perfusion of a membrane-permeant potassium acetate buffer that reduces the oocyte intracellular pH to the indicated value (mean ± S.E.M. of 6–8 oocytes). The continuous line shows the fit with the equation $1/[1 + ([H^+]_i - K)^n]$ from which the apparent pK_a values were calculated. *D*, relative current inhibition (as recorded in *B*) that was induced by intracellular acidification to pH 6.3 for wild-type and mutant channels.

determining the primary pH sensitivity of Kir channels (Fakler *et al.* 1996) and also suggests that Kir subunits utilise a common structural gating mechanism which is adapted for modulation by a variety of physiological parameters.

The mechanism by which Kir5.1 enhances the pH sensitivity of the heteromeric channel remains unclear. We therefore attempted to determine the contribution of Kir5.1 to the enhanced pH sensitivity of the heteromeric channel. Figure 2*D* shows the effect of individually mutating all the intracellular histidines in the Kir5.1 subunit and coexpressing them with Kir4.1 as a tandemly linked dimer. These results clearly show that none of the intracellular histidines in Kir5.1 contribute significantly to the enhanced pH sensitivity of the heteromeric channel.

Kir4.2 forms pH-sensitive channels with and without Kir5.1

The absence of Kir4.1 expression in certain tissues, for example pancreas (Shuck *et al.* 1996), where Kir5.1 is expressed, means that other subunits are likely to permit functional expression of Kir5.1. Kir4.2 shares 65% identity to Kir4.1 and is highly expressed in the pancreas (Shuck *et al.* 1997). Pearson *et al.* (1999) have previously shown that Kir4.2 also forms novel heteromeric channels with Kir5.1 and that Kir4.2 homomeric channels possess an intrinsic sensitivity to intracellular acidification. However the precise pH sensitivity of these channels has yet to be determined. In order to assess the pH sensitivity of Kir4.2 and Kir4.2–Kir5.1 channels both TEVC and excised giant patch recordings of channel activity were carried out (Fig. 3). In particular, the response of both

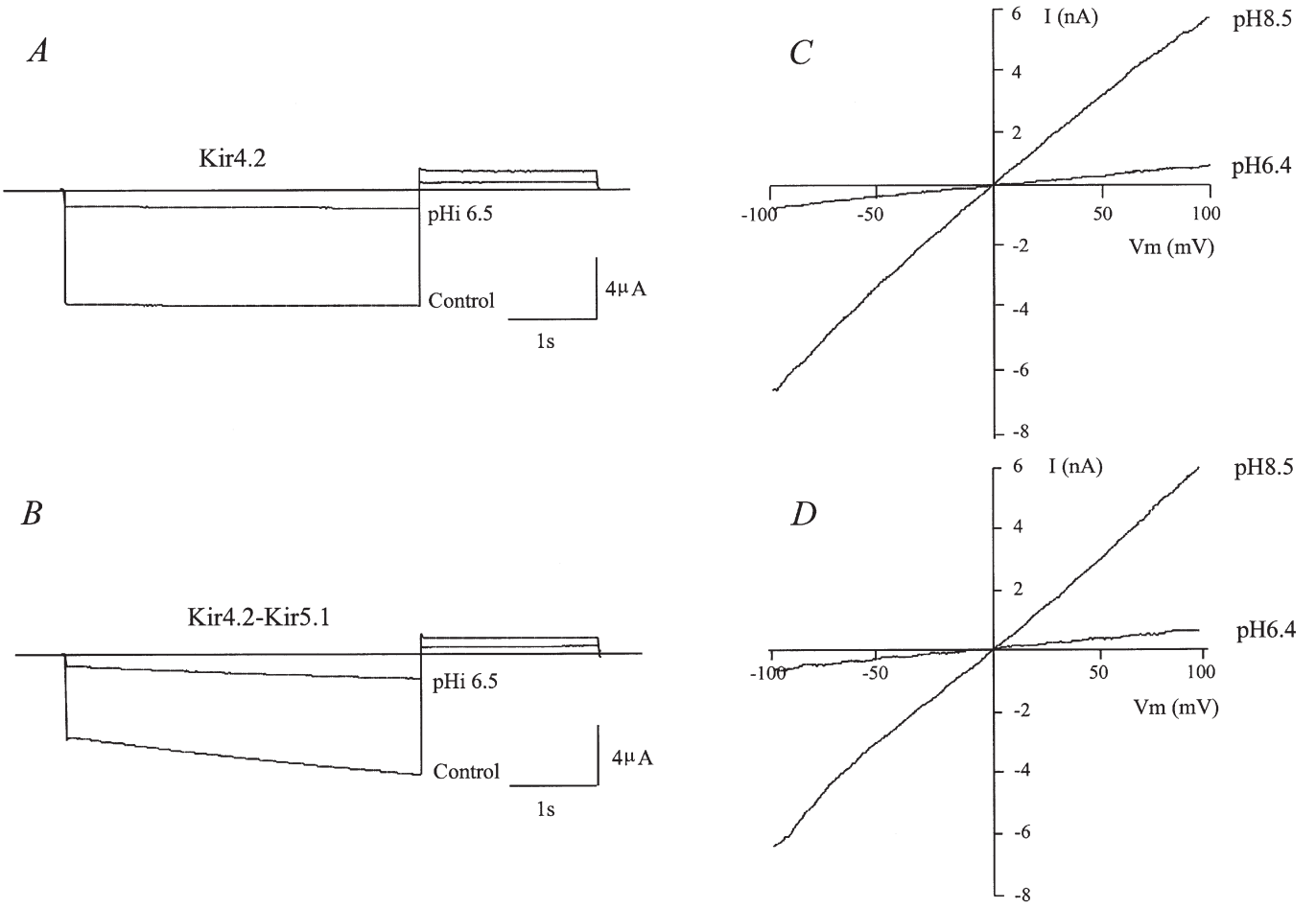

Figure 3. Kir4.2 and Kir4.2–Kir5.1 currents are inhibited by intracellular acidification

A, representative whole-cell current traces recorded in the TEVC configuration from *Xenopus* oocytes expressing the indicated channels. The recordings were performed as detailed in Fig. 2*B*. *C* and *D*, *I–V* curves that were evoked by 1 s voltage ramps from −100 to +100 mV and were recorded using inside-out giant patches excised from oocytes expressing Kir4.2 (*C*) and Kir4.2–Kir5.1 (*D*) channels. The currents shown exhibit little rectification as they were were recorded in the absence of Mg^{2+} and polyamines and during the cytoplasmic application of solutions at the indicated pHs through a multibarrel fast-flow system. Strong rectification was observed in the cell-attached configuration before patch excision into Mg^{2+}- and polyamine-free solution.

Table 1. pK_a values for different combinations of Kir4.0 and Kir5.1

	pK_a ($n = 6$)	Hill coefficient
A. Whole-cell recordings, intracellular acidification		
Kir4.1	6.1 ± 0.1	1.8
Kir4.1–Kir5.1	6.8 ± 0.1	2.0
Kir4.1(K67M)–Kir5.1	5.4 ± 0.1	1.4
Kir4.1(K67M)–Kir5.1(M73K)	6.6 ± 0.1	1.3
Kir4.1(K67M)	Not sensitive	
Kir4.2	6.7 ± 0.1	1.8
Kir4.2(K66M)	5.4 ± 0.1	2.2
Chimera 31B	6.6 ± 0.1	2.6
Chimera 31C	5.9 ± 0.1	3.5
B. Excised inside-out patches		
Kir4.1	5.99 ± 0.05	0.8
Kir4.2	7.07 ± 0.06	0.9
Kir4.1–Kir5.1	7.35 ± 0.05	1.0
Kir4.2–Kir5.1	7.64 ± 0.09	1.2

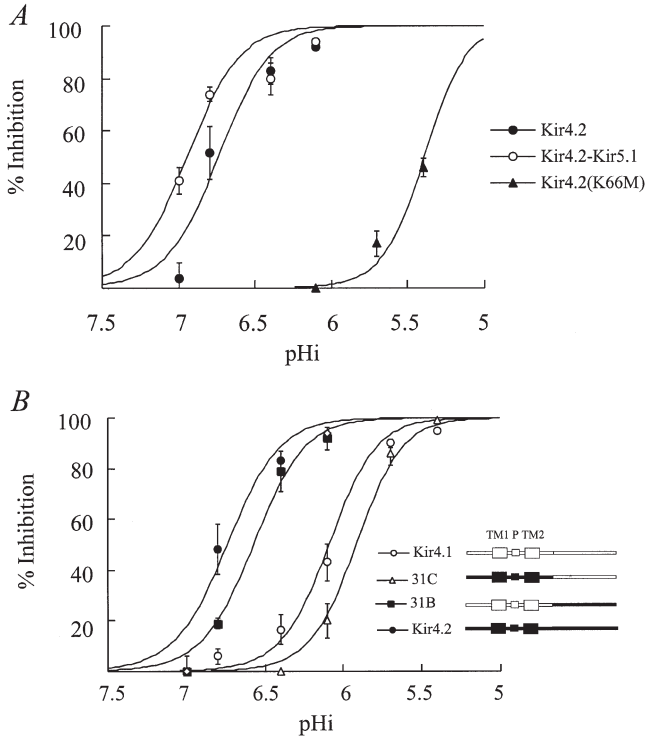

Figure 4

A, pH sensitivity of Kir4.2 and Kir4.2–Kir5.1 channels to intracellular pH. Intracellular pH *vs.* current inhibition determined in TEVC configuration for Kir4.2 (●), Kir4.2–Kir5.1 (○) and Kir4.2(K66M) (▲). *B*, increased sensitivity of Kir4.2 correlates with the C-terminal region. Intracellular pH *vs.* current inhibition determined as in *A* for Kir4.1 (○) and Kir4.2 (●) compared to chimeras 31B (■) and 31C (△) which exchange the intracellular C-terminus (see inset). The difference in sensitivity between the two subunits correlates with the C-terminus.

channels to intracellular acidification is shown in Fig. 4*A*. As expected Kir4.2–Kir5.1 channels were also responsive to changes in pH_i in the physiological range (Table 1). However, Figs 4*A* and 5 show that the response of these heteromeric Kir4.2–Kir5.1 channels to pH_i was only slightly different from that of Kir4.2 itself. Thus, despite their similarity, Kir4.2 is significantly more sensitive to changes in pH_i than Kir4.1 and in contrast to Kir4.1 this enhanced sensitivity is not markedly altered by heteropolymerisation with Kir5.1.

Differential pH sensitivity of Kir4.1 and Kir4.2

We therefore attempted to identify the structural elements which define the pH sensitivity of Kir4.2. This subunit also possesses a lysine residue at the equivalent position to K67 in Kir4.1 and K80 in Kir1.1. As expected mutation of this residue almost completely abolished the pH sensitivity of both homomeric Kir4.2 and heteromeric Kir4.2–Kir5.1 channels (Fig. 4*A*). This provides further evidence for the primary role of the Kir4.0 subunit in determining the pH sensitivity of the heteromeric Kir4.0–Kir5.1 channel and also further evidence for the role of a titratable lysine residue in determining this characteristic. However, this did not explain the increased pH sensitivity of Kir4.2 compared to Kir4.1 and the differential modulation of their pH sensitivity by Kir5.1 (Figs 4*A* and 5; Table 1). We therefore made chimeras between Kir4.1 and Kir4.2 and determined their response to changes in pHi. Figure 4*B* shows that the sensitivity of Kir4.2 to pH_i in the physiological range appears to correlate with the intracellular C-terminus.

Single channel properties of Kir4.2 and Kir4.2–Kir5.1

We have also investigated the principal single channel properties of both homomeric Kir4.2 and heteromeric Kir4.2–Kir5.1 channels. Using cell-attached membrane

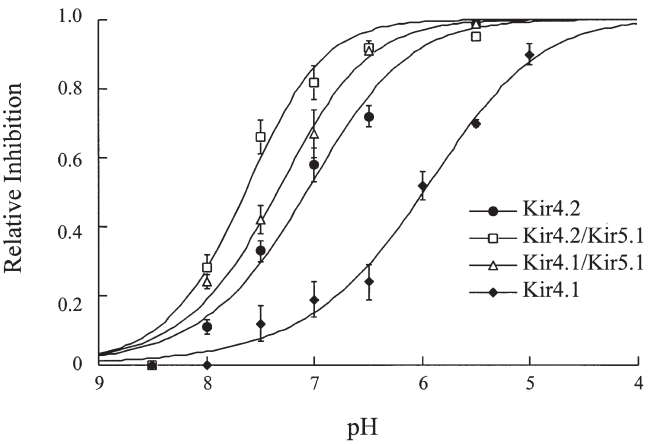

Figure 5. pH sensitivity of Kir4.2 and Kir4.2–Kir5.1 channels recorded in excised patches

pH *vs.* relative inhibition as determined by exposure of excised, inside-out giant membrane patches to different pH solutions.

patches from oocytes expressing either Kir4.2 alone, or expressing tandemly linked Kir4.2–Kir5.2, we have determined the single channel conductance of Kir4.2 and Kir4.2–Kir5.1. Representative single channel recordings for Kir4.2 are shown in Fig. 6*A*. This shows that Kir4.2 forms channels similar to Kir4.1 with an extremely high open probability ($P_o > 0.9$) and conductance of 25.2 pS. Figure 7 shows that heteropolymerisation of Kir5.1 with Kir4.2 profoundly alters the biophysical properties. These novel channels have a principal single channel conductance of 54.2 pS with additional subconductance levels. The peak values of the Gaussian distribution obtained from the recording shown in Fig. 7 are 5.1, 3.3 and 2.4 pA, indicating the presence of at least two subconductance levels of ~0.65 and ~0.45 of the full open state current. Moreover, these channels display a bursting pattern similar to Kir4.1–Kir5.1 (Pessia *et al.* 1996) with very long closed times (> 10 s) between bursts.

DISCUSSION

This study demonstrates that other subunits such as Kir4.2 are likely candidates to facilitate functional expression of Kir5.1 in tissues where Kir4.1 is not expressed and suggests a role for these channels in the pH-dependent regulation of K^+ fluxes. As well as determining some of the basic properties of Kir4.2 and Kir4.2–Kir5.1 channels we also demonstrate that Kir5.1 has different effects on the intrinsic pH sensitivity of Kir4.1 and Kir4.2. This is due to the presence of an additional pH-sensing mechanism involving the C-terminus of Kir4.2.

Relative tissue distribution of Kir4.1, Kir4.2 and Kir5.1.

As several previous studies have shown, Kir5.1 and Kir4.1 do not share perfectly overlapping patterns of tissue distribution (Bond *et al.* 1994; Shuck *et al.* 1997; Salvatore *et al.* 1999; Tucker *et al.* 2000). The detection of

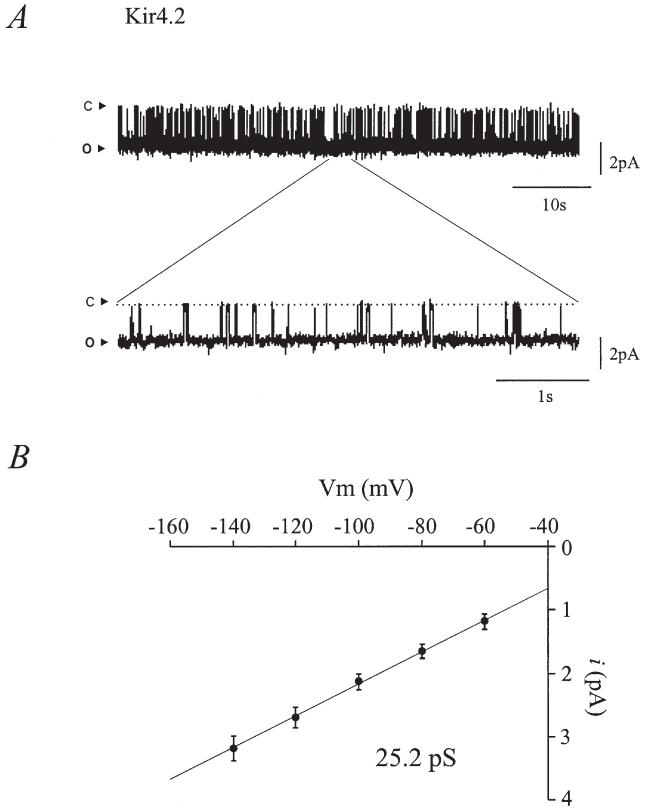

Figure 6. Single-channel properties of homomeric Kir4.2

A, representative cell-attached patch recording at −100 mV from an oocyte expressing Kir4.2 channels. The bottom record is shown on an expanded time scale. *B*, current–voltage relationship of Kir4.2. A slope conductance of 25.2 pS was calculated from the fit with a linear regression line. The extrapolated zero-current potential was approximately −14 mV. Each data point represents the average channel current calculated from amplitude histograms (mean ± S.D. of 6 patches pulled from different oocytes).

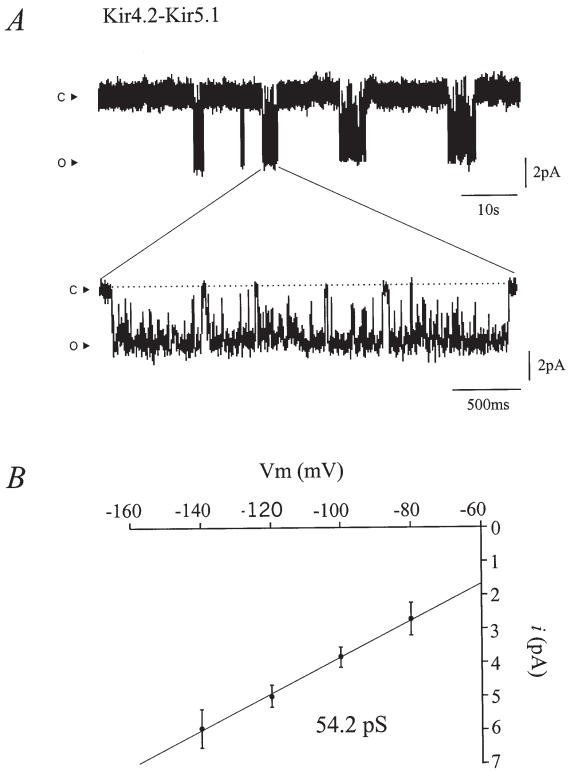

Figure 7. Single-channel properties of heteromeric Kir4.2–Kir5.1 channels

A, representative cell-attached patch recording at −100 mV from an oocyte expressing tandemly linked Kir4.2–Kir5.1 channels. The bottom record is shown on an expanded time scale. *B*, current–voltage relationship of Kir4.2–Kir5.1 channel. A slope conductance of 54.2 pS was calculated from the fit with a linear regression line. The extrapolated zero-current potential was approximately −30 mV. Each data point represent the average channel current calculated from amplitude histograms (mean ± S.D. of 6 patches pulled from different oocytes).

Kir5.1 expression in the pancreas is of particular interest. Kir4.1 expression is either low or absent in this tissue, but Kir4.2 is abundantly expressed (Shuck *et al.* 1997). This suggests that in cases where Kir4.1 is absent, Kir4.2 may heteropolymerise with Kir5.1 to form novel pH-sensitive channels. Figure 1 shows Kir5.1 expression to be detectable in both the exocrine and endocrine regions of the pancreas and interestingly in the pancreatic arteriolae. The exocrine regions of the pancreas are responsible for the secretion of bicarbonate as well as digestive enzymes. This involves the production of bicarbonate by carbonic anhydrase and excess protons. These are removed via the Na^+–H^+ exchanger which is in turn driven by the Na^+–K^+-ATPase. Secretion of both H^+ and HCO_3^- therefore results in a build-up of intracellular K^+. It is therefore possible that heteromeric Kir4.2–Kir5.1 channels are involved in the pH-dependent secretion of K^+ produced by this route.

Mechanisms of pH sensing

Initially the dramatic effect of Kir5.1 on the intrinsic pH sensitivity of Kir4.1 suggested that Kir5.1 may possess additional pH-sensing mechanisms. However, as shown in Fig. 2, this is unlikely to be the case. Individual mutation of each intracellular histidine in Kir5.1 had no significant effect on the pH sensitivity of the heteromeric Kir4.1–Kir5.1 channels. By contrast mutation of lysine 67 (K67M) in Kir4.1 completely abolished the pH sensitivity of these channels. Some debate still continues as to whether this lysine residue is actually titrated at physiological pH. However, studies by Schulte *et al.* (1999) have provided good evidence for anomalous titration of this lysine residue and have shown that this is due to an interaction of this lysine with two highly conserved arginine residues, one in the N-terminus and one in the C-terminus. These arginine residues are thought to create a positively charged environment around the lysine residue and produce anomalous titration with a pK_a in the physiological range. These arginine residues are absolutely conserved in all known Kir channels.

Kir subunits which do not possess an appropriate lysine residue in the N-terminus are not generally sensitive to changes in intracellular pH within the physiological range. Further evidence to support the role of this titratable lysine residue comes from the double mutant Kir4.1(K67M)–Kir5.1(M73K) where the equivalent position in Kir5.1 is replaced with a lysine residue. Figure 2*B* and *C* shows that this mutation essentially reverses the effect of the Kir4.1(K67M) mutation and brings the pK_a back into the physiological range. It is therefore unlikely that the principal pH-sensing mechanisms are elsewhere on the Kir channel, and because only two subunits in the heteromeric channel possess 'titratable lysines' this also implies that closure of all four gates is not essential for channel closure. The results also suggest that the highly conserved region of the proximal N-terminus containing this lysine residue forms part of an evolutionarily conserved gating mechanism involving a physical association of both intracellular domains. Indeed, recent studies have indicated that conserved interactions between the N- and C-terminal domains of the Kir channel subunits may provide a common structural gating mechanism. In addition, the two arginine residues which produce anomalous titration of the pH-sensitive lysine residue both lie within conserved regions which contribute to the physical association of the N- and C-terminal domains of Kir channels (Schulte *et al.* 1998, 1999; Tucker & Ashcroft, 1999). Taken together, these results indicate that protonation of this lysine residue might influence the interaction between the N- and C-terminal domains and thereby provide a mechanism for modulating gating of the channel.

Differential modulation of Kir4.0 pH sensitivity by Kir5.1 and structural implications

In addition to modulating the gating and single channel properties of Kir4.1 channels, heteropolymerisation with Kir5.1 causes a significant shift in the pH sensitivity bringing the pK_a of Kir4.1–Kir5.1 channels into the physiological range. However, Kir5.1 has relatively little influence on the intrinsic pH sensitivity of Kir4.2. This subunit has previously been shown to be pH sensitive, although the pK_a value was not known. In this study we have obtained a pK_a value of approximately 7.1 for Kir4.2 in excised patches (see Table 1). This ranks Kir4.2 as one of the most pH sensitive of all the Kir channels. Although heteropolymerisation with Kir5.1 causes a small but significant shift in pH sensitivity, this shift is minor compared to the effect of Kir5.1 on Kir4.1 because it is masked by the additional pH sensitivity of Kir4.2. Chimeric analysis revealed that this enormous difference in the intrinsic pH sensitivity of Kir4.1 and Kir4.2 is due to differences in the C-terminus of these subunits. Figure 4*B* shows that transfer of the C-terminus of Kir4.2 into Kir4.1 can enhance the pH sensitivity to a value similar to Kir4.2. However, this effect is secondary to the primary pH sensor of Kir4.2 provided by a titratable lysine residue at an equivalent position to K67 in Kir4.1. Figure 4*A* shows that mutation of this residue (K66M) almost completely abolishes the pH response of these channels.

The ability of Kir5.1 to modulate the intrinsic pH sensor of Kir4.1 (K67) implies that the *inter*-subunit interactions produced by heteropolymerisation of Kir5.1 with Kir4.1 can influence the *intra*-subunit interactions which define the anomalous titration of the N-terminal lysine pH sensor. It is likely that even small adjustments in the steric arrangement of the 'Arg-Lys-Arg triad' could have a dramatic effect on the pK_a of the residue and thereby the pH-dependent regulation of channel activity.

Physiological consequences of differential heteropolymerisation and pH modulation

The ability of individual potassium channel subunits to heteropolymerise is of fundamental importance in generating diversity of function from a limited number of gene products. The ability of Kir5.1 to have a profound effect on the pH sensitivity of Kir4.1 may be important for the role of this channel in renal tubular epithelia where this heteromeric channel is thought to be involved in acid–base homeostasis. However, a slightly lower pH sensitivity may be important in those tissues where Kir4.1 forms homomeric channels and where Kir5.1 is not expressed. The fact that the distribution of Kir5.1, Kir4.1 and Kir4.2 does not appear to be completely overlapping suggests that a variety of both heteromeric and homomeric combinations of these channels are involved in the pH-dependent regulation of K^+ fluxes.

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