XE991 Reveals Differences in K⁺ Channels Regulating Chloride Secretion in Murine Airway and Colonic Epithelium

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ABSTRACT

The cognitive enhancer XE991 interacts with K⁺ channels consisting of KCNQ2 and KCNQ3 heteromultimers to block the M-current. XE991 can also block KCNQ1 K⁺ channels expressed in oocytes, but sensitivity is reduced when the channels are coexpressed with minK (KCNE1). The purpose of the study was to examine the interaction of XE991 with other types of K⁺ channel, especially those in the basolateral membranes of murine epithelia. K⁺ channel blockade was measured by the inhibition of chloride secretion resulting from depolarization. XE991 inhibited the chloride secretory current in colonic epithelia by an interaction with basolateral K⁺ channels when forskolin was used as the stimulus. However, when 1-ethyl-2-benzimidazolinone (EBIO) was used to stimulate chloride secretion, XE991 was ineffective unless charybdotoxin was also present. Because EBIO also activates Ca²⁺-sensitive K⁺ chan-nels, whereas forskolin activates only cAMP-sensitive K⁺ channels, it is concluded that the latter are the targets for XE991. XE991 had effects similar to those of 293B on epithelial chloride transport, for which the target is known to be KCNQ1/KCNE3 multimers. mRNA for both these components of the cAMP-sensitive K⁺ channels were found in high abundance in the colon, whereas KCNE1 was barely detectable. Furthermore, both XE991 and 293B were active in colonic epithelia from KCNE1 knockout mice. By contrast, in nasal epithelium, the forskolin sensitive chloride secretory current was barely sensitive to XE991 but was sensitive to clofilium. *Xenopus laevis* oocytes in which both KCNQ1 and KCNE3 had been expressed were significantly more sensitive to XE991 than oocytes expressing only KCNQ1.

KCNQ potassium channels occur in the heart, nervous tissue, and many epithelia (Busch and Suessbrich, 1997) and have important physiological functions. KCNQ1 (K$_V$LQT$_1$) can form heteropolymeric channels with an accessory sub-unit, KCNE1 (minK) to form I$_{Ks}$ channels, responsible for the slow component of the delayed rectifier potassium current that contributes to the repolarization of the cardiac action potential (Sanguinetti et al., 1996). We know that the sensitivity of KCNQ1 channels to both activators and inhibitors, such as mefanamic acid and 293B, respectively, is enhanced by complex formation with minK (Busch et al., 1997). A recent study can be interpreted to mean that chromanols, such as 293B, bind to the subunit KCNQ1, with the minK unit acting as an allosteric activator (Lerche et al., 2000). The cognitive enhancer XE991 increases stimulus evoked transmitter release in the central nervous system (Zaczek et al. 1998), probably by blockade of the M-channel (Aiken et al., 1996), a heteromultimer of KCNQ2 and KCNQ3 channel subunits (Wang et al., 1998a). XE991 also blocks KCNQ1 channels expressed in oocytes, but its efficacy is reduced

when the channel is expressed with KCNE1 (Wang et al., 2000). Presumably, if the activity of XE991 had been enhanced when KCNQ1 channels were expressed with KCNE1, the potential to cause long QT syndrome would have increased. Thus, the selectivity of the cognitive enhancer is dependent on the type of subunits colocalized with particular K⁺ channels.

Activation or inhibition of K⁺ channels in epithelia, particularly those secreting chloride, has potentially useful therapeutic applications in diseases as diverse as cystic fibrosis, where chloride secretion is deficient, and secretory diarrhea, where it is in excess. We have shown previously that the chromanol 293B, although very active on intestinal epithelia, is virtually inactive in airway epithelia, whereas for clofilium, the situation is reversed (MacVinish et al., 1998; Cuthbert et al., 1999b). In this report, we examine the effect of XE991 on K⁺ channels in chloride secretory epithelia from the intestine and the airways of mice. Our objective was to examine if the differences seen with 293B also applied to XE991 and, if so, whether this could be correlated with differences in the type of K⁺ channels present in the two epithelia. Again we found that the effects of XE991 are very

ABBREVIATIONS: PCR, polymerase chain reaction; KHS, Krebs Henseleit Solution; SCC, short circuit current; EBIO, 1-ethyl-2-benzimidazolin-one; ChTX, charybdotoxin; bp, base pair(s); minK, accessory subunit KCNE1.

different in these two types of epithelia. Sequences from various murine KCNQ-type K^+ channels and minK-like peptides were amplified by PCR to allow correlation of function with channel type. Although the colonic tissue showed the presence of both KCNQ1 and KCNE3 mRNA, the airway epithelium also showed, uniquely, the presence of mRNA for KCNQ2.

Materials and Methods

All the SCC experiments were performed on the isolated colonic mucosae or nasal epithelium of balbC mice. Mice were killed by CO_2 narcosis and the colons were placed in cold Krebs Henseleit solution (KHS) of the composition: 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11.2 mM glucose, pH 7.6 when bubbled with 95% O_2/5% CO_2 at 37°C. The muscle layers were dissected away and small pieces of mucosae were mounted in Ussing chambers with a window area of 20 mm^2. Up to four pieces of mucosae were used from a single mouse. Tissues were bathed on both sides with 20 ml of KHS at 37°C that was continually circulated with the gas lift using 95% O_2/5% CO_2. The tissues were voltage-clamped at 0 mV to measure the short circuit current (SCC) using a WPI Dual Voltage Clamp (Stevenage, Herts, UK) connected via an ADI Instruments Maclab 8e (Hastings, Sussex, UK) to a computer. The arrangement of voltage sensing and current passing electrodes was as described elsewhere (Cuthbert et al., 1999a) and current compensation for the current $\times$ time drop between the voltage electrodes was used. When colonic epithelium from IsK knockout mice were used, comparisons were made with tissues from their wild-type littermates. Nasal epithelia were mounted in Ussing chambers and voltage clamped as for the colonic epithelia and bathed in KHS solution as for the colon. However, the window area in the chambers was only 1.8 mm^2. The technique for the removal of the nasal epithelium from the nasal cavity was exactly as described elsewhere (MacVinish et al., 1997)

Detection of K^+ Channel Messenger RNA. Heart, lung, nasal epithelium, colon and jejunal tissues were dissected from balbC mice, and quick frozen in isopentane. Total RNA was isolated from each sample using the TRIzol extraction method (Invitrogen, Paisley, UK) (Chomczynski and Sacchi, 1986). For nasal epithelium tissues from ten mice were pooled; all other tissues were sampled from single animals. RNA samples were run on ethidium bromide agarose gels and gave the appropriate 28S to 18S bands, confirming the lack of degradation. Samples were double DNase digested before reverse transcription (Superscript II; Invitrogen, Paisley, UK) and the resulting cDNA used for PCR. Sense primers 5′ and antisense primers 3′ were designed for each K^+ channel or minK-like protein using Primer 3 software (*http://www.genome.wi.mit.edu/genome_software/other/primer3.html*) using the GenBank accession numbers given in Table 1. After reverse transcription using 1 μg total RNA, specific PCR products were obtained, but not in the absence of reverse transcription. The nature of the PCR products was confirmed by sequencing the amplified products on an ABI 310 sequencer (Applied Biosystems, Warrington, UK).

Expression in *Xenopus laevis* Oocytes. Full-length clones of hKCNQ1 and murine KCNE3 were obtained. The former was a kind gift from Dr. J. Vandenberg, and the latter was prepared by the usual methods. Standard PCR procedures were used to construct a Kozak consensus sequence GCCACC, immediately upstream of the ATG initiation codon in cDNA clones for KCNE3. These constructs were then subcloned into the *Hin*dIII-*Bam*HI site of the pBG7.2 vector, which provides the 5′- and 3′-untranslated regions of the *X. laevis* β-globin gene. In vitro transcription was achieved using capped cRNA made from *Nde*I-linearized cDNA using the T7 polymerase mMESSAGE mMACHINE Kit (Ambion, Austin, TX) according to the manufacturer's instructions.

X. laevis oocytes were prepared using collagenase (0.3%) (Sigma) in Ca^{2+}-free buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6). Isolated oocytes were microinjected, in a ratio of 10:1 with KCNQ1 and KCNE3 cRNAs or with KCNQ1 or KCNE3 cRNAs alone. Water-injected oocytes were used as controls. Each oocyte received two 50-nl injections. Oocytes were incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM MgCl2, and 5 mM HEPES at pH 7.6) at 18°C for several days before use.

Two-electrode voltage-clamp recordings were performed on cRNA-injected oocytes using an OC-725C amplifier (Warner Instruments Corp. Hamden, CT), using a Power Macintosh 8100/100 computer with an ITC-16 A/D board (Instrutech Corp., Greatneck, NY). Current traces were recorded with pulse-acquisition software (version 8.11; HEKA electronics, Lambrecht, Germany). Microelectrodes (3 M KCl) were used with resistances of approximately 1 MΩ.

Drugs used in the study, amiloride, charybdotoxin, clofilium, 1-ethyl-2-benzimidazolone (EBIO), and furosemide, were obtained from Sigma (Poole, Dorset, UK). XE991 was a gift from the DuPont Pharmaceutical Company and 293B was a gift from Dr. R. Greger. Stock solutions of drugs were made in water, alcohol, or dimethyl sulfoxide. The concentration of solvents added to tissues had no effect.

Results

Effects of XE991 on Colonic Epithelia. The effects of XE991 on the chloride secretory responses to two agonists, forskolin and EBIO, were investigated by short circuit current procedures. In all experiments, 100 μM amiloride was present in the apical bathing solution to eliminate electrogenic sodium absorption. Addition of 30 μM XE991 to colonic

TABLE 1
Potassium channel primers

K^+ Channel	Accession Number	Forward Primer	Bases	Reverse Primer	Bases
KCNQ1	U70068	GTTCCTCTCA GGCCCCTATC	2141–2160	GTTCCCTGAT GGTCTCTGGA	2340–2321
KCNQ2	NM010611	GTGCACCCAC AAAACACAAG	1488–1507	CAGGGACTAA GGCTCACCTG	1684–1665
KCNQ4	AF249747	ATTCAGCAGG ACACCTGGAC	486–505	ATGTTCGATG GACTGCACCT	685–666
KCNQ5	AF263836	ACAGTTTTCA GGCAGCGAGT	2371–2390	ATGACCGTGA CCTTCCAGTC	2570–2551
KCNE1	NM008424	CCCAATTCCA CGACTGTTCT	88–107	CAGCTTCTTG GATCGGATGT	291–272
KCNE3	AF076532	GGCTCTGAAC ACAACCCTTC	297–316	TTTGTCCACT TTGCGTGAAC	501–482
KCNE4	AF076533	AAGAGGCGGG AGAAGAAGTC	255–274	GAGCTCACAC TGTCCCCTTC	454–435
KCNN4	AF072884	AAGCACACTC GAAGGAAGGA	973–992	CCGTCGATTC TCTTCTCCAG	1187–1168

epithelia without prior stimulation caused a significant reduction in the basal SCC. In 11 preparations, the basal current was 54.5 ± 6.9 μA cm^{-2}, which was reduced to 29.2 ± 4.1 μA cm^{-2} ($P < 0.005$) in 10 min after addition of XE991, representing a 46.4% reduction in basal SCC. Because the basal current is known to result in part from the secretion of chloride ions (Cuthbert et al., 1999a) XE991 seems to inhibit this activity in the absence of a secretory stimulus.

The adenylate cyclase activator forskolin was used to promote chloride secretion in murine colonic epithelia. Addition of the K$^+$-channel blocker XE991 to the basolateral face of the tissues rapidly reduced the chloride secretory response by circa 75%. Epithelia pre-exposed to XE991 gave correspondingly reduced responses to forskolin. Subsequent addition of furosemide increased the SCC because of inhibition of remaining potassium secretion (Cuthbert et al., 1999a) (Fig. 1). The inset (Fig. 1c) shows partial concentration-response curves to forskolin and the inhibition caused by XE991. A noncompetitive interaction between forskolin and XE991 is indicated for the generation of electrogenic chloride secretion. A second inset in Fig. 1d shows the inhibition of SCC stimulated by forskolin by the cumulative addition of XE991. Inhibition was evident at 3 μM XE991 and reached 53% at 10 μM XE991. We have used 30 μM XE991 as a standard

concentration, which caused 73% inhibition, whereas 100 μM XE991 increased inhibition to only 82%. It seems unlikely that complete inhibition of the secretory response could be achieved with XE991.

The results with a second agent, EBIO, which also stimulates chloride secretion were, by contrast, strikingly different from those with forskolin. XE991 caused only a minor (4%) inhibition of the EBIO-induced current. The discrepancy between the ability of XE991 to inhibit the responses to forskolin, while leaving those to EBIO unimpaired, even though both agents induce electrogenic chloride secretion, was resolved when tissues were pre-exposed to another channel blocker, charybdotoxin. Under these conditions, XE991 was able to reduce the response to EBIO by approximately 80%, as with forskolin (Fig. 2). Data from sets of experiments identical to those described in Figs. 1 and 2 are summarized in Fig. 3 and show that the differences between the effects of XE991 on the responses to forskolin and EBIO are statistically significantly different.

When colonic epithelia were pretreated with ChTX and XE991, subsequent addition of EBIO produced a chloride secretory current that faded rapidly, suggesting that one or both of the blocking agents required channels to be open before they were available for blockade. A comparison of the effects of EBIO in the presence and absence of both blocking agents is given in Fig. 4. As noted above, in the data shown in Fig. 1, when furosemide was added after XE991, it produced a current increase. Figure 4, c and d, contrasts the responses to furosemide when inhibiting a chloride secretory current and after the chloride secretory current was inhibited by a combination of K$^+$-channel blockers.

To gain more insight into why XE991 was unable to block chloride secretion after EBIO, we measured its direct effects on potassium currents. Nystatin was used to permeabilize the apical membrane to small cations in the presence of an
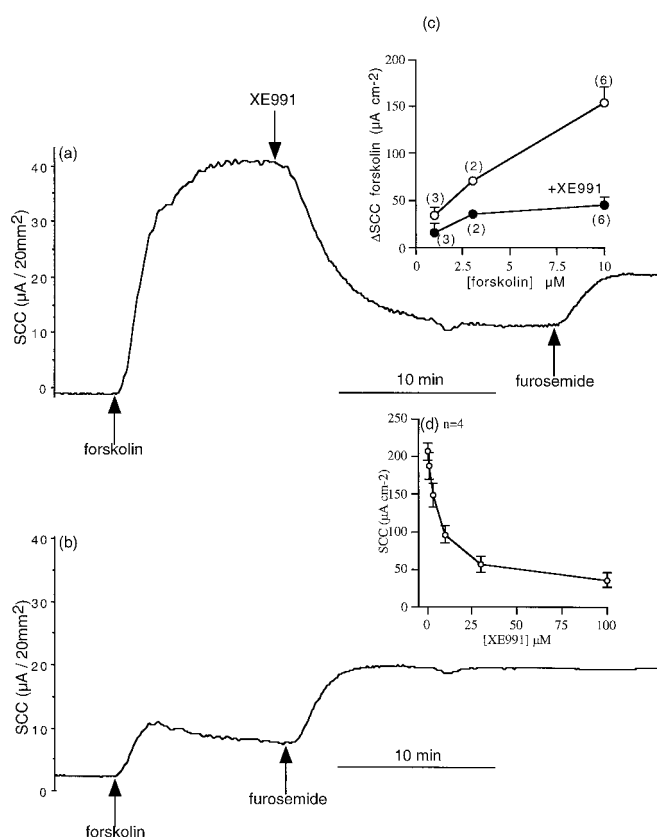
Fig. 1. Effects of XE991 on the responses to forskolin in colonic epithelia. SCC records from a pair of colonic epithelial preparations. a, the tissue was exposed to 10 μM forskolin, and after the current had stabilized, 30 μM XE991 was added. b, the tissue was exposed to 30 μM XE991 for 20 min before exposure to forskolin. Note that 1 mM furosemide produces an increase in SCC after exposure to XE991. c, a partial concentration response curve to forskolin is shown in the presence and absence of XE991 (30 μM). The inhibition of 10 μM forskolin responses by XE991 is shown in d, giving an EC$_{50}$ value of 10 μM. In c and d, the number of observations is given in parentheses.
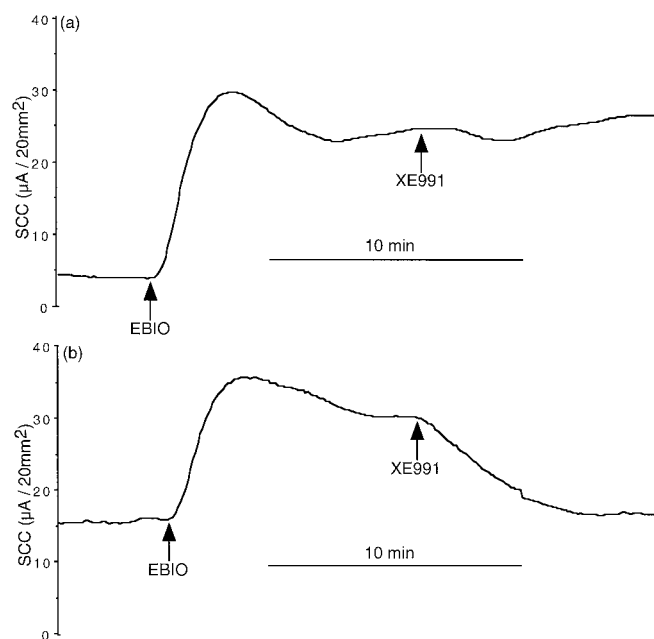
Fig. 2. Contrasts effects of XE991 on the responses to EBIO in the presence and absence of ChTX. a, XE991 (30 μM) was added basolaterally to a colonic epithelium after the response to 600 μM EBIO had reached steady state. b, the same procedure was used except the tissue had been preincubated with 50 nM ChTX for 20 min before EBIO was added.

apical-to-basolateral K^+ gradient (Cuthbert et al., 1999b). The presence of the potassium ion gradient increased the basal SCC, and nystatin increased this further to high levels because of an inward K^+ current. Addition of XE991 reduced this current by 87% (Fig. 5a). Addition of EBIO after nystatin further increased the current, which was inhibited by XE991, but only by an average of 30% (Fig. 5b). Thus, an inhibitory effect of XE991 on K^+ channels is demonstrated, following EBIO and in the absence of charybdotoxin, but the effect is less than in the absence of EBIO. Thus, in the absence of EBIO in nystatin-treated epithelia, the current is carried predominantly through cAMP-sensitive K^+-channels, which are constitutively open and sensitive to XE991. When further Ca^{2+}-sensitive K^+-channels are opened by EBIO, the effect of XE991 is weakened.

Effects of XE991 on Nasal Epithelia. In a previous report, we showed that the effects of K^+ channel blockers on chloride secretion depended on the particular epithelium investigated (MacVinish et al., 1998; Cuthbert et al., 1999). For example, the chromanol 293B was effective in the colon but had only a minor effect on murine nasal epithelium, indicating that different K^+ channels were involved in the two tissues. The same proved to be true for XE991, which caused less than 10% inhibition of the forskolin-activated chloride current in murine nasal epithelium. The ineffectiveness of

XE991 in nasal epithelium was not different when used alone or in the presence of ChTX (Fig. 6, a to c). By contrast, clofilium, a class III antiarrhythmic agent, was effective in blocking the chloride secretory effect, either alone or in the presence of ChTX plus XE991.

Effects of XE991 on Jejunal Epithelium. To test whether the profile described for murine colonic epithelium was unique, a limited study was made with murine jejunal epithelium. Phloridzin (100 μM) was added to the apical bathing solution to inhibit electrogenic sodium/glucose transport. The tracing illustrated in Fig. 7 shows that, like the colon, the forskolin-sensitive current is sensitive to XE991, whereas the residual current is insensitive to clofilium but sensitive to furosemide. Consequently, the jejunum is similar to the colon and unlike the nasal epithelium.

Which K Channels Are Present in Murine Colonic and Nasal Epithelium? To discover whether the difference in response to XE991 in nasal and colonic epithelia can be ascribed to different K^+ channels, RNA was extracted from various tissues and reverse transcribed. The resulting cDNAs were then used for PCR. Pairs of primers were selected for the α and β subunits of the KCNQ group of K^+ channels and for KCNN4, an intermediate-conductance, calcium-activated potassium channel. No primers for KCNQ3 and KCNE2 were made because the murine sequences for these are unknown. The primers were designed to give 200-bp
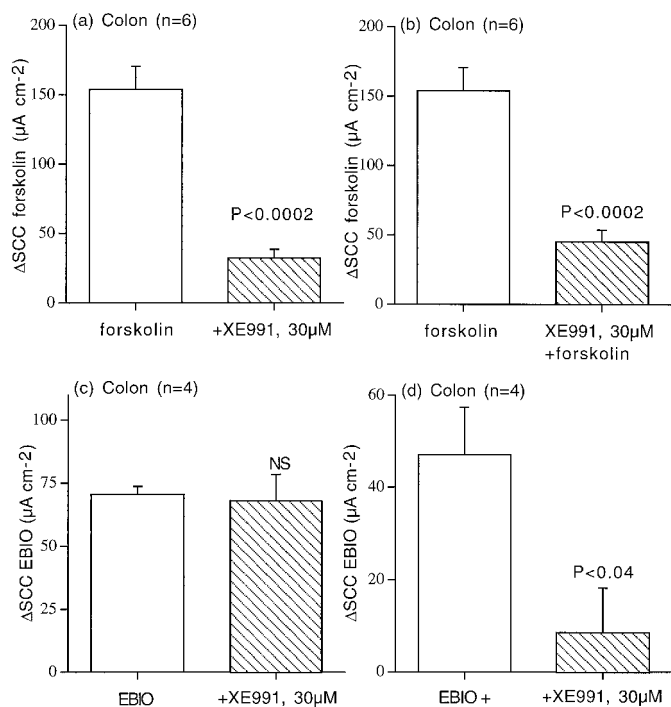
Fig. 3. Contrasts the inhibitory effects of XE991 on the colonic epithelial responses to forskolin and to EBIO. The inhibitory effects of 30 μM XE991 on the responses to forskolin are shown in a and b. a, XE991 was added to the basolateral side after the response to 10 μM forskolin had reached a steady state value. b, tissues were exposed to 30 μM XE991 for 20 min before forskolin responses were assessed and the results compared with those in the absence of XE991. Significant inhibitions of the responses to forskolin were found in a and b; mean inhibition corresponded to 79.2% in a ($P < 0.0002$) and 70.6% in b ($P < 0.0002$). In c and d, the secretory response was evoked with 600 μM EBIO. Addition of 30 μM XE991 in c had no significant effect on the current, reducing the response by only 3.9%. In d, 50 nM ChTX was added to the basolateral side for 20 min before EBIO was added. Addition of 30 μM XE991 after EBIO caused a significant reduction of the response of 81.8% ($P < 0.04$).
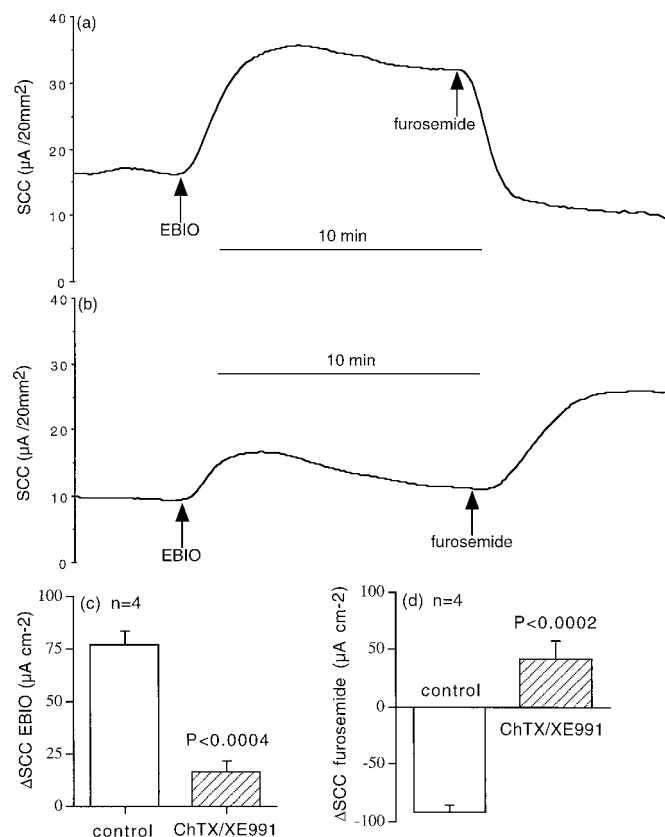
Fig. 4. Inhibition by ChTX together with XE991 on the responses to EBIO. The effect of 600 μM EBIO on SCC and its inhibition by 1 mM furosemide is shown in a. b, a paired preparation was subjected to the same protocol after incubation for 20 min with 50 nM ChTX and 30 μM XE991 applied basolaterally. Note the reversal of the effect of furosemide in b. Quantitative data from four pairs of colonic epithelial preparations, treated as in a and b, are presented in c and d.

products from samples extracted from heart, lung, nasal epithelium, colon and jejunum. A typical gel, showing the presence of 200-bp products using primers for KCNQ1, KCNQ2, KCNQ4, KCNQ5, KCNE1, KCNE3, KCNE4, and KCNN4, is shown in Fig. 8.

KCNQ1 was expressed in all five tissues examined and therefore is a potential target for XE991, whereas KCNQ2 was found only in nasal epithelium, perhaps an indication of the differing responses to XE991 and clofilium in the colon and the nose. However, KCNQ2 is expressed in the olfactory bulb (Tinel et al., 1998), so its presence in the nasal epithelium may result from contamination with sensory olfactory neurons. KCNQ4 was found in all tissues, although the signal was weakest in the jejunum. KCNQ5 was apparently expressed only in the lung. Figure 7 shows that KCNE1 is most abundant in the heart, whereas KCNE3 seems to be present in all tissues except the heart. The apparent exclusivity, however, was not as absolute, as shown. In some gels, weakly staining bands for KCNE3 were present in heart and similarly weak bands for KCNE1 were seen with the other four tissues. Others have reported that KCNE1 is present in the heart but not in the colon and that the small intestine contains KCNE1 (Chouabe et al., 1997). Recently, Demolombe et al. (2001) described the differential expression of KvLQT1 (KCNQ1) and its regulator, IsK (KCNE1), in mouse tissues using in situ hybridization. In general, their findings are in agreement with those described here. None of the tissues examined showed evidence of KCNE4. All the tissues, with the exception of the heart, showed PCR products indicative of expression of KCNN4. The PCR products were subsequently cloned and sequenced. The sequences for KCNQ1, KCNQ2, KCNQ5, KCNE1, KCNE3, KCNE4, and KCNN4 were as predicted.

Observations with IsK Knockout Mice. Further evidence for the involvement or otherwise of KCNE1 subunits (minK) in the chloride secretory response was obtained by experiments with tissues from IsK knockout mice comparing these with wild-type controls. Inhibition of responses to forskolin by clofilium and 293B were measured in nasal and colonic epithelia, respectively, whereas XE991 was examined in only the mutant colonic tissue. No differences in the inhibitory responses were found between tissues without KCNE1 and those possessing the subunit, suggesting that the KCNE1 subunit is not involved in the inhibition of chloride secretion by K$^+$ channel blockers (Table 2).

Expression in *X. laevis* Oocytes. Indirect evidence above suggests that KCNQ1/KCNE3 multimers is the target for XE991. To test this directly, KCNQ1 was expressed in *X. laevis* oocytes, either alone or together with KCNE3, and examined by two-electrode voltage clamping. When KCNQ1 was expressed alone, a voltage-dependent current-voltage relationship was found, whereas in the presence of KCNE3, the relationship was linear (Fig. 9c). XE991 was more effective in inhibiting the clamp current, measured at 6 s after stepping the voltage from -80 mV to $+40$ mV, when both cRNAs were expressed than when KCNQ1 was expressed alone (Fig. 9d). The EC$_{50}$ value was approximately 20 μM in the presence of both KCNQ1 and KCNE3, but was >100 μM in the presence of KCNQ1 alone. Water- or KCNE3-injected oocytes showed virtually no current responses to changes in voltage.

Discussion

We have shown that XE991 can reduce electrogenic chloride secretion in the murine colon when it is added basolaterally. This result supports the general thesis that hyperpolarization increases chloride efflux through the apical membrane by increasing the electrical gradient. Conversely, blockade of basolateral K$^+$-channels, by causing depolarization, will reduce chloride secretion. Because XE991 reduces the basal SCC without prior stimulation of SCC, this suggests that some K$^+$ channels are constitutively open, as has been argued by others (Schroeder et al., 2000).
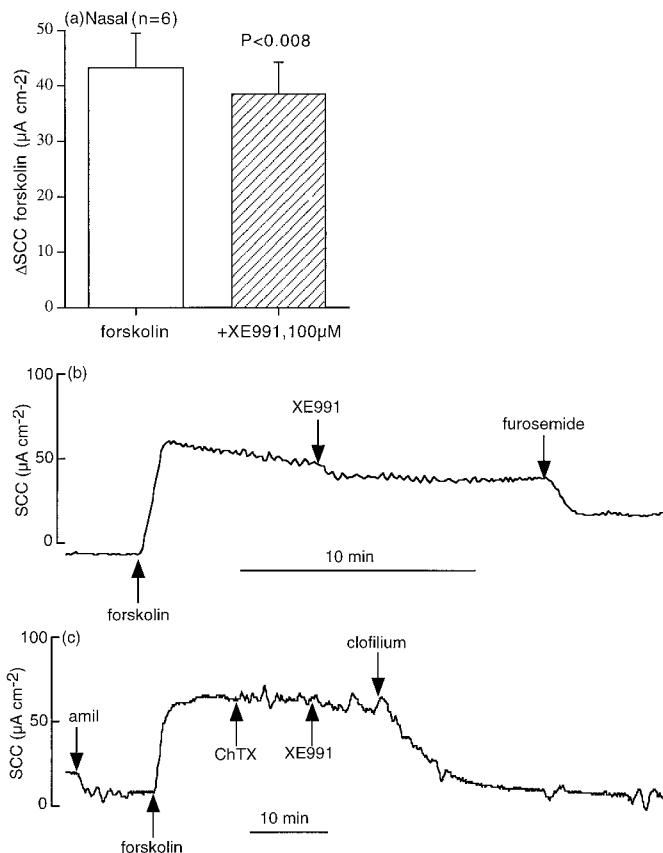
Both forskolin and EBIO have multiple actions on chloride
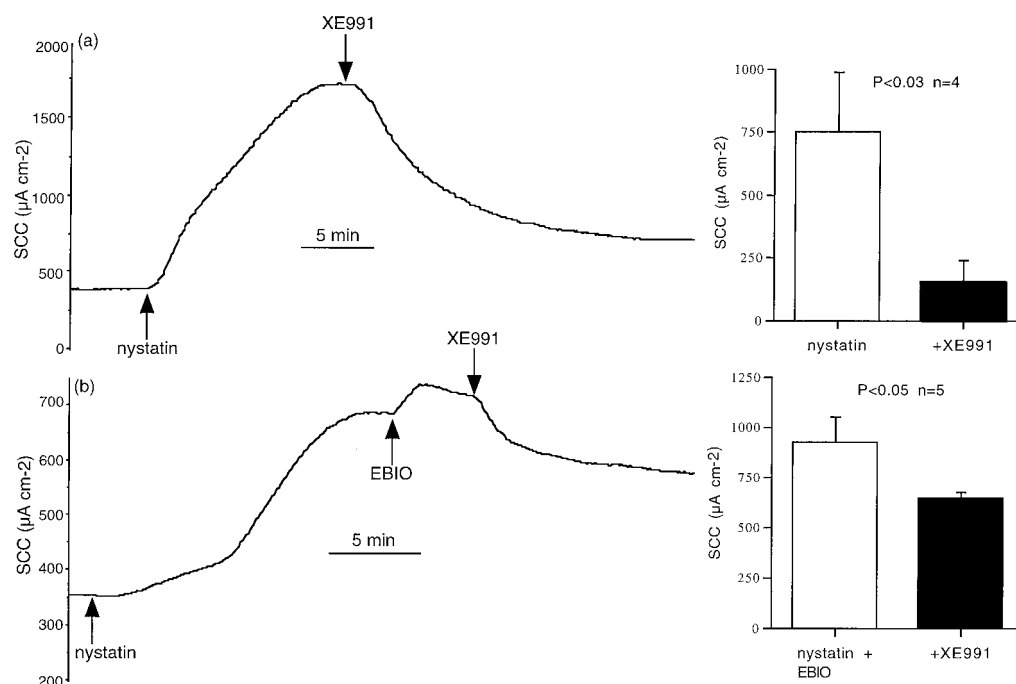
Fig. 5. Effect of XE991 on epithelial K$^+$ currents. Colonic epithelia were subjected to an apical to basolateral potassium ion gradient. Essentially, this consisted of replacing NaCl in the apical solution with potassium gluconate and in the basolateral solution with sodium gluconate (see Cuthbert et al., 1999b). Nystatin (180 μg/ml) was used to permeabilize the apical membranes, resulting in an inward K$^+$ current. Inhibition by 30 μM XE991 in the presence and absence of 600 μM EBIO is shown in a and b, respectively. The insets show the data from several experiments, the reduction in current being significant in both instances (paired t test). a, XE991 caused a reduction in current of 87.1 $\pm$ 10.3% and of 26.2 $\pm$ 6.8% in b; these values were significantly different ($P <$ 0.002)

secretory epithelia. Both activate apically located CFTR chloride channels, either by increasing cAMP (Cuthbert et al., 1999b) or by direct action (Devor et al.,1996a,b), and both activate basolateral K$^+$ channels, again either via cAMP or by a mixture of direct and indirect actions (Devor et al.,1996a,b; Cuthbert et al., 1999b; Syme et al., 2000). How-

Fig. 6. Relative lack of effect of XE991 on nasal epithelium. a, addition of 100 μM XE991 to the basolateral side of murine nasal epithelium, previously stimulated with 10 μM forskolin had a small but significant effect on the SCC using a paired t test. The reduction in the current had a mean value of 11.5%. b, record showing the effect of 100 μM XE991 and 1 mM furosemide on SCC after stimulation with 10 μM forskolin. c, as in b, showing that 50 nM ChTX does not increase the inhibitory effect of XE991. Clofilium (100 μM) rapidly reversed the effect of forskolin.
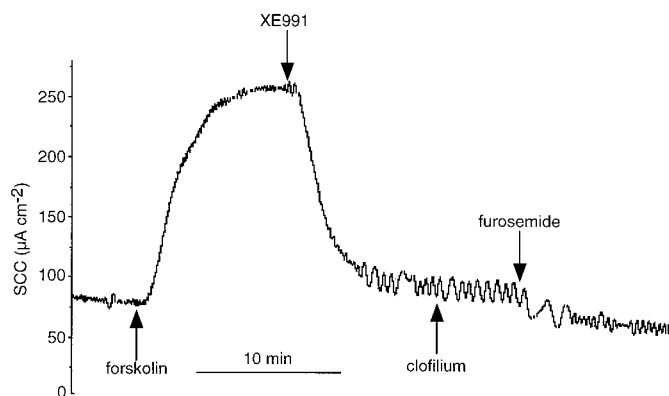
Fig. 7. Sensitivity of jejunal epithelium to XE991. Murine jejunal epithelium was treated with amiloride and phloridzin (both 100 μM) on the apical side to block electrogenic sodium absorption and electrogenic sodium-glucose transport. Forskolin (10 μM) increased SCC, and this was sensitive to 100 μM XE991, causing 85% inhibition. The residual current was insensitive to 100 μM clofilium, but sensitive to 1 mM furosemide.

ever, as shown here for the colon, XE991 inhibits the response to forskolin without a significant effect on the response to EBIO. This suggests that the target for XE991 is the cAMP-sensitive K$^+$ channel, which has been shown to be a KCNQ1-KCNE3 heteropolymeric channel (Schroeder et al.,2000). Messenger RNAs for both the channel alpha subunit [KCNQ1(KvLQT1)] and the associated minK-related peptide (KCNE3) are present in the murine colon (Fig. 7). Furthermore, it is presumably these channels that are constitutively open in the absence of agonists.
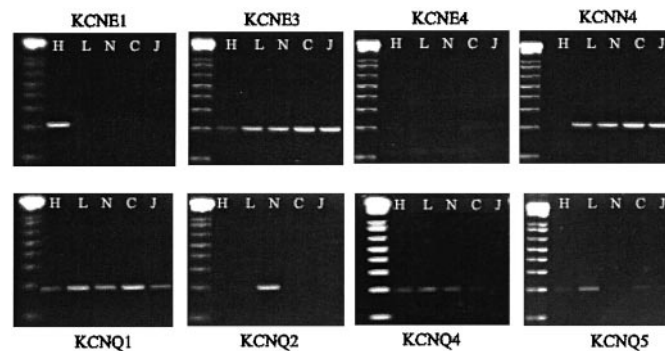
EBIO activates intermediate conductance Ca^{2+}-sensitive K$^+$ channels by increasing number $\times$ open probability (Syme et al., 2000). XE991 had no significant effect on the responses to EBIO unless the tissues were pretreated with ChTX, after which XE991 was effective. The presence of mRNA for the intermediate conductance Ca^{2+}-sensitive K$^+$ channel (KCNN4) was shown to be present in the epithelial tissues used in this study. Because, it is argued, the cAMP-sensitive K$^+$-channels are constitutively open, then in the presence of EBIO, both the Ca^{2+}- and the cAMP-sensitive K$^+$ channels will be activated and hyperpolarization is probably supramaximal; the rate of chloride secretion is rate-limited by the chloride permeability of the apical membrane. Under these conditions, blockade of either the cAMP- or Ca^{2+}-sensitive K$^+$ channels will have little effect and blockade of both types of channel is necessary to see inhibition. Most of the data presented with the IsK knockout mice tissues were obtained at a much earlier time, before the work on XE991 was commenced. At that time, the existence of multiple minK-related peptides was only postulated (Attali et al., 1993) and the failure to see any differences in responses in mice with and without minK was puzzling. Recently, we carried a few further studies using IsK knockout tissues in which XE991 was used instead of 293B. Again, no differences were found, confirming the earlier findings with 293B. Two conclusions from these findings might be as follows: first, the KCNQ1/KCNE1 heteropolymer is not the target for either XE991 or 293B; second, XE991 and 293B probably have the same target. Because the mRNA for KCNE1, the original minK, was found predominantly in the heart (Fig. 7), it is unlikely that this minK peptide is involved in functional responses in the colonic or nasal epithelium. However, it does not follow that XE991 and 293B do not interact with the heteropolymeric channel KCNQ1/KCNE1; indeed, both drugs have been shown to be active on cardiac tissues or oocytes expressing

Fig. 8. PCR products obtained using primers specific for murine K$^+$-channels. The expected size of the products was 200 bp in all instances. Ladders (1 kilo-bp) are shown between each group. H, L, N, C, and J correspond to cDNAs made from murine heart, lung, nasal epithelium, colon, and jejunum.

TABLE 2

Effect of clofilium, 293B and XE991 on forskolin responses in colonic and nasal epithelia from IsK knockout mice.

P values give the significance of the effects of the blocking agents.

|	ΔSCC (μA cm^{-2})				n	P	%I
	Forskolin 10μM	After clofilium 100μM	After 293B 10μM	After XE991 30μM			
Colon ($-/-$)	153.2 ± 12.8	—	36.4 ± 5.4	—	11	<0.0001	76.2
Colon (WT)	167.4 ± 11.2	—	45.9 ± 7.9	—	10	<0.0001	72.5
Colon ($-/-$)	143.6 ± 23.9	—	—	34.8 ± 6.5	8	<0.001	75.8
Colon (WT)	78.8 ± 9.3	—	—	12.3 ± 7.9	5	<0.001	84.4
Nasal ($-/-$)	50.8 ± 11.3	16.9 ± 6.7	—	—	23	<0.003	66.7
Nasal (WT)	63.9 ± 16.0	21.8 ± 10.1	—	—	8	<0.01	66.0

WT, wild type; ($-/-$), minK knockout; n, number of observations; %I, percentage inhibition.

both components (Bosch et al., 1998; Wang et al., 2000). It is clear that the target for 293B in the colon is the KCNQ1/KCNE3 K$^+$ channel (Schroeder et al., 2000; Kunzelmann et al., 2001), and because XE991 and 293B are equally effective in wild-type and IsK knockout mice, obviously KCNE1 is not essential for the activity of either agent. However, when KCNQ1 is expressed with KCNE1 in oocytes, the sensitivity to XE991 is reduced (Wang et al., 2000), whereas it is enhanced to 293B (Busch et al., 1997). Assuming KCNE3 also reduces the affinity of XE991 for the colonic K$^+$ channel, then the EC$_{50}$ value of 10 μM found in this study is impressive. It is possible that, in the colon, there is some KCNQ1 uncomplexed with KCNE3 and that XE991 interacts with this entity, whereas 293B reacts with the KCNQ1/KCNE3 complex.

The reversal of the responses to furosemide after XE991 is to be expected. The chloride secretory response in the colon is accompanied by modest K$^+$ secretion (Cuthbert et al., 1999a). When the former is inhibited by XE991, subsequent blockade of the Na-K-2Cl cotransporter will inhibit the latter, giving an increase in SCC.

PCR analysis of cDNAs derived from a variety of murine tissues, including epithelia, confirm that KCNN4, a Ca^{2+}-dependent K$^+$ channel, is present in both nasal and colonic epithelia. We and others have shown that EBIO activates this channel (Cuthbert et al., 1999b; Syme et al., 2000), although its effects on nasal epithelium are small and somewhat transient compared with the effects on the colon. In airway epithelia from other species (i.e., man), the effects of EBIO are more pronounced (Devor et al., 2000). The reasons for these differences are unknown, and we have no data on the expression levels of the Ca^{2+}-sensitive K$^+$ channel protein in the nasal epithelium of mice. The nasal epithelium of the mouse was unique in another way: it was the only tissue containing the mRNA for KCNQ2. Whether KCNQ2 can form heteropolymers with KCNE3, which are insensitive to XE991, is unknown and can only be determined by expression studies in cells lacking these entities. It is known that KCNQ2 is rather insensitive to clofilium (Yang et al., 1998), whereas it is sensitive to XE991. Thus the pharmacological profile of clofilium and XE991 in the nasal epithelium remains obscure. Possibly other β-subunits, still undiscovered, will provide the answer. KCNQ2 can form modulated K$^+$ channels with KCNE2 subunits (Tinel et al., 2000) but the murine homolog of KCNE2 has yet to be discovered. 293B, which seems to share with XE991 the same target in colonic epithelia, is similarly unable to inhibit the forskolin-generated current in nasal epithelium (MacVinish et al., 1998). XE991 is thought to act on KCNQ2/KCNQ3 multimers in the central nervous system (Wang et al., 1998a) when acting as a

cognitive enhancer and will block KCNQ1 channels when expressed in oocytes (Wang et al., 2000), its activity being reduced when expressed with KCNE1. A drastic reduction in sensitivity to XE991 through a similar mechanism is unlikely in murine nasal epithelium, because little or no mRNA for KCNE1 is detectable. Although the concentration of XE991
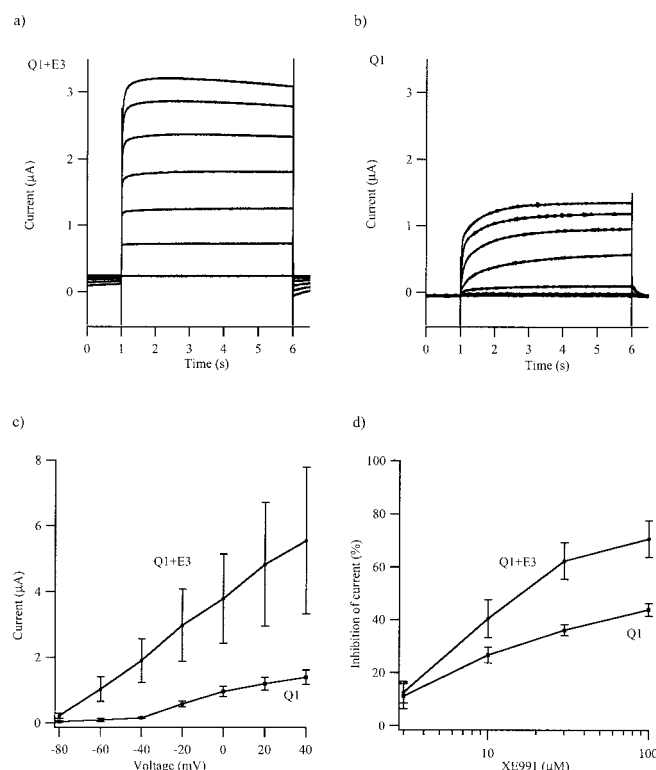
Fig. 9. Data obtained with *X. laevis* oocytes injected, 3 to 4 previously, with cRNA for KCNQ1, KCNE3, or both. a and b, families of current curves for voltage-clamped oocytes in response to stepwise changes in membrane potential from -80 mV to $+40$ mV, in steps of 20 mV. a, an oocyte was injected with cRNAs for both KCNQ1 and KCNE3, whereas in b, the oocyte was injected with the cRNA for KCNQ1 only. c, data for five oocytes injected with both messages and 12 oocytes injected with only the message for KCNQ1. Mean values and S.E. are shown. At all voltages, the current values for oocytes injected with both cRNAs was significantly greater than the currents obtained with oocytes injected with only KCNQ1 (by at least $P < 0.01$). The mean current generated in water injected oocytes voltage clamped at $+40$ mV was 0.18 μA ($n = 3$) and for oocytes injected with the cRNA for KCNE3 only and clamped at $+40$ mV was 0.16 μA ($n = 3$). d, the percentage inhibition of the voltage clamp current at 6 s and $+40$ mV by XE991, added externally to the bath. Mean values and S.E. are given for five oocytes injected with cRNAs for KCNQ1 and KCNE3 and for seven oocytes injected with cRNA for KCNQ1 only. The values at XE991 concentrations of 30 μM and 100 μM were significantly different ($P < 0.012$ and $P < 0.019$, respectively).

was raised to 100 μM, inhibition of the forskolin responses in nasal epithelium was only 10%. We have no evidence that this is caused by inhibition of uncomplexed KCNQ1. Forskolin-stimulated current in nasal epithelium was blocked by clofilium. The long QT syndrome is associated with mutations in the KCNQ1 gene that forms channels with the KCNE1 β-subunit. When expressed together in oocytes, currents were inhibited by clofilium (Yang et al., 1997), yet this agent is ineffective in the colon (MacVinish et al., 1998). Because the KCNQ1/min K family of channels can have multiple stoichiometries (Wang et el., 1998b) the differences between nasal and colonic epithelia and the actions of inhibitors such as XE991, clofilium, and 293B may be very subtle. The mRNA for KCNQ5 was found only in the lung and is therefore irrelevant to this discussion. KCNQ4, generally associated with sensory pathways in neuronal tissue (Kharkovets et al., 2000), may be expressed in both the nasal and colonic epithelium. Whether KCNQ4, either alone or complexed with a minK-like protein, may be a target for clofilium or XE991 requires expression studies.

From the limited study of the mouse jejunum, its profile with respect to the sensitivity to XE991 seems to be identical to that of the colon. mRNA for KCNQ1 and KCNE3 are present in this tissue and this together with the ability of XE991 to inhibit the chloride secretory current generated by forskolin supports this view.

Finally, to show that KCNQ1/KCNE3 multimers are the target for XE991, at least in colonic epithelium, we expressed both these proteins in *X. laevis* oocytes. We obtained current voltage relationships for KCNQ1 alone and combined with KCNE3 that were similar to those reported by others (Schroeder et al., 2000; Kunzelmann et al., 2001). Importantly, although the EC_{50} value for XE991 was 20 μM for oocytes expressing both KCNQ1 and KCNE3, the value was more than 100 μM for oocytes expressing KCNQ1 alone. This contrasts with the finding that expression of KCNQ1 with KCNE1 reduces the sensitivity to XE991 (Wang et al., 2000). The EC_{50} value for the KCNQ1/KCNE3 multimers expressed in oocytes (20 μM) is near the value found for the inhibition of the forskolin induced SCC by XE991. Thus, the results in oocytes support the deductions we have made for the murine colon.

In summary, XE991 is able to inhibit the chloride secretory current generated by activation of CFTR chloride channels and cAMP-sensitive K$^+$ channels by targeting the latter, which are made up of KCNQ1/KCNE3 multimers, while having no effect on Ca^{2+}-sensitive K$^+$ channels. The low sensitivity of airway epithelial K$^+$ channels to XE991 may result from subtle, as-yet-undiscovered differences in the assembly of α and β subunits of the channel.

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