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Effects of sevoflurane on the cAMP-induced short-circuit current in mouse tracheal epithelium and recombinant Cl^- (CFTR) and K^+ (KCNQ1) channels†

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Background. An optimal level of airway surface liquid is essential for mucociliary clearance in lungs. The cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) and KCNQ1 channels in tracheal epithelium play key roles in luminal and basolateral membranes, respectively. The aim of this study was to examine the effects of sevoflurane on cAMP-induced chloride secretion by the mouse tracheal epithelium and the modulation of recombinant CFTR and KCNQ1 channels.

Methods. The equivalent short-circuit current (I_{sc}) of the mouse tracheal epithelium was measured using a flow-type Ussing chamber technique. Inhibition of Na^+ absorption was achieved through the luminal application of amiloride. cAMP-dependent Cl^- secretion was evoked by forskolin and isobutylmethylxanthine (Fsk/IBMX) applied to the basolateral side. The effect of sevoflurane on CFTR and KCNQ1 channels was assessed using a whole-cell patch clamp in human embryonic kidney 293T cells expressing CFTR and KCNQ1 channels.

Results. Fsk/IBMX induced a sustained I_{sc} that was suppressed by the application of sevoflurane [decreased by 49 (4.5)% at 190 μM]. The Fsk/IBMX-induced I_{sc} was also blocked by basolateral application of chromanol 293B, a blocker of the KCNQ1 K^+ channel. In KCNQ1-expressing cells, sevoflurane 190 μM reduced the outward currents to 59 (4.9)% at 80 mV. The CFTR current was not affected by sevoflurane (~360 μM).

Conclusions. These results suggest that the inhibition of KCNQ1 underlies sevoflurane-induced decrease in airway secretion.

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Electrolyte/water secretions from the airway epithelium form the airway surface liquid (ASL), which is vital for mucociliary clearance.[1 2] It is widely accepted that volatile anaesthesia suppresses airway clearance.[3] In secretory epithelia, the cAMP-dependent activation of luminal Cl^- channels [cystic fibrosis transmembrane conductance regulator (CFTR)] and basolateral K^+ channels (KCNQ1/KCNE3) play critical roles in electrogenic Cl^- secretion.[1–4] As the actions of inhaled anaesthesia are mediated via various ion channels,[5 6] it is likely that its effect on the respiratory tract could be the result of

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changes in ion channel activities. Indeed, it has been reported that halothane and isoflurane inhibit the short-circuit current (I_{sc}) and transepithelial potential differences in canine and rabbit tracheal epithelia.[7,8] Sevoflurane is associated with a relatively rapid induction of anaesthesia and is increasingly used.[9] Although sevoflurane causes less airway irritation and complications compared with other anaesthetics,[10–13] it is not known whether it affects epithelial secretion. In the present study, we examined the effects of sevoflurane on epithelial transport in mouse trachea using the Ussing chamber. To investigate the mechanisms further, a whole-cell patch clamp was used in mammalian cells transiently expressing CFTR and KCNQ1 channels.

Methods

Ussing chamber experiments

Trachea was harvested from mice of either gender (body weight, 25–35 g). The trachea was then split along the anterior side, and the pars membranacea was mounted into a tissue holder for the Ussing chamber (circular exposed area, 0.64 mm^2) with the aid of a dissecting microscope. The chamber (2 ml) was maintained at 37°C and continuously perfused with normal Tyrode's solution containing (in mM): 145 NaCl, 0.4 KH_2PO_4, 1.6 K_2HPO_4, 5 HEPES, 5 D-glucose, 1 $MgCl_2$, 1.3 $CaCl_2$ (pH 7.4) on both sides at a flow rate of 10–15 ml min^{-1}. Indomethacin (1 μM) was included in all experimental solutions to inhibit the endogenous formation of prostaglandins. The tissue was allowed to equilibrate for at least 30 min before the experimentation. Trans-epithelial resistance (R_{te}) was determined from the voltage deflection (ΔV_{te}) caused by the injection of short-current pulses (0.7 Hz, 1.4 s duration, 0.6 μA). The resistance of the empty chamber was subtracted. The equivalent I'_{sc} was calculated from the trans-epithelial voltage (V_{te}) and R_{te} according to Ohm's law. The electrical sign of the I_{sc} and V_{te} refers to the luminal side. Amiloride, indomethacin, forskolin (Fsk) and 293B were initially dissolved in dimethylsulfoxide (DMSO) and diluted with Tyrode's solution. The final concentrations of DMSO were $<0.1\%$.

Cell culture and ion channel transfection

KCNQ1 and CFTR channels were transiently transfected into human embryonic kidney (HEK-293) cells (ATCC, Manassas, VA, USA). HEK-293 cells were seeded in 12-well plates. On the next day, 1.5–2 μg per well of pcDNA vector containing the cDNA for KCNQ1 (donated by Dr K. Kunzelmann, Regensburg, Germany) or for CFTR (donated by Dr Min-Goo Lee, Yonsei University, Seoul, Republic of Korea) was mixed with 50–100 ng per well of pEGFP-C1 (Clontech) to identify transfected cells. The mixture was transfected into the cells using the transfection reagent, FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's protocol. After 30–40 h, the cells were trypsinized and used for whole-cell recording. To record KCNQ1 current, only those HEK-293 cells showing EGFP fluorescence were used.

Whole-cell patch clamp

Isolated cells were transferred into a small chamber (0.2 ml) on the stage of an inverted microscope equipped with a fluorescence system (IX-70, Olympus) and perfused continuously with normal Tyrode's solution at a rate of 10 ml min^{-1}. A glass microelectrode with a resistance of 2–2.5 MΩ was used to obtain a gigaohm seal. The conventional whole-cell patch clamp technique was used to hold the membrane potential at −60 mV with the aid of an Axopatch 200B patch-clamp amplifier (Axon Instruments). pCLAMP software Version 9.02 (Axon Instruments) was used for data acquisition and the application of the command pulses. The data were filtered at 5 kHz and displayed on a computer monitor. The data were analysed using Microcal Origin software, Version 6.0. The pipette solution for recording CFTR current contained 140 mM *N*-methyl-D-glucamate (NMDG)-Cl, 10 mM HEPES, 1 mM $MgCl_2$ and 5 mM ethyleneglycol-*bis*-(oxonitrilo)-tetraacetate (EGTA) at pH 7.2 (titrated with HCl). For recording K^+ current, we used KCl-rich pipette solution containing 140 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$ and 5 mM EGTA at pH 7.2 (titrated with HCl).

Chemicals

All chemicals were of the highest grade available and were obtained from Sigma-Korea (Seoul, Republic of Korea) with the exception of chromanol 293B (Tocris, UK) and sevoflurane (Abbott Korea, Seoul, Republic of Korea). Perfusate in the reservoir was vigorously bubbled with air containing various levels of sevoflurane using a vaporizer (Dräger Vapor® 2000, Drägermedical). The volume of the half chamber for the I_{sc} measurement was ~0.5 ml, and the volume of the bath for the patch clamp study was 0.2 ml. In both cases, complete exchange of the solution was achieved within 10 s.

The sevoflurane concentrations in the samples of the superfusing external solution were determined using the headspace analysis method on a Hewlett Packard 6890 GC/MSD flame ionization detection gas chromatograph (Hewlett Packard, Avondale, PA, USA). Aliquots of the bath solution were sampled directly from the recording chamber. Partial pressure values for sevoflurane used in this study (1, 3 and 5%) were within the clinical range. The bath solution contained sevoflurane 30, 190 and 360 μM when the solution in the reservoir bottle was equilibrated with sevoflurane 1, 3 and 5%, respectively. The aqueous concentration with sevoflurane 1% (30 μM) was below the limit of detection of the measurement system.

Therefore, the sublinearly smaller value of 1% solution (30 μM) than the higher values (190 and 360 μM corresponding to 3 and 5%, respectively) might have an underestimation because of the intrinsic limit of the measurement.

Statsitics

Data are presented as original recordings and bar graphs of the mean (SEM) (for the number of tested mucosa or cells). Paired or unpaired Student's *t*-test was used for statistical analysis where appropriate. $P<0.05$ was considered significant.

Results

Table 1 summarizes the resting transepithelial voltages (V_{te}), tissue resistance (R_{te}) and equivalent I_{sc} calculated using Ohm's law. After equilibration in a normal Tyrode's solution on both sides of the chamber, a stable V_{te} plateau was confirmed. The initial spontaneous V_{te} was reduced by the luminal application of amiloride (10 μM), which blocks epithelial Na^+ channels. Mean changes in V_{te} and I_{sc} induced by amiloride were 2.6 (0.44) mV and 32 (5.0) μA cm^{-2}, respectively (n=13).

cAMP-dependent Cl^- secretion was stimulated by applying Fsk (2 μM) and isobutylmethylxanthine (IBMX, 100 μM) basolaterally in the presence of luminal amiloride. The addition of Fsk/IBMX induced a negative shift in V_{te} [ΔV_{te}=−6.1 (0.54) mV] and enhanced the lumen negative I_{sc} by −57.5 μA cm^{-2} (n=13). Such changes are typical of electrogenic Cl^- secretion in epithelial tissues. It is well known that luminal Cl^- channels (CFTR) and basolateral K^+ channels (KCNQ1) are activated subsequent to stimulation of cAMP-dependent pathways.[4 14] Consistent with this model, the application of the KCNQ1 channel blocker (chromanol 293B 100 μM) to the basolateral side completely suppressed the cAMP-induced I_{sc}, highlighting the significance of basolateral K^+ channels in Cl^- secretion (Fig. 1A and B). The effect of 293B was completely reversed by a washout with a Fsk/IBMX-containing solution (Fig. 1A).

Next, the effects of sevoflurane on the I_{sc} of the airway epithelia were tested. I_{sc} induced by Fsk/IBMX was reduced when perfused with a sevoflurane-bubbled solution (Fig. 1C). The inhibition was completely antagonized by washout. Efficacy of different concentrations of sevoflurane was tested. Figure 1D shows that sevoflurane 190 μM inhibited approximately half of the Fsk/IBMX-induced I'_{sc}

The observation that the inhibition of cAMP-induced I'_{sc} suggests that sevoflurane affects either CFTR or KCNQ1 in the tracheal epithelium. Whole-cell patch clamp experiments were performed to verify this hypothesis. In CFTR-expressing HEK293T cells, the application of Fsk/IBMX to the bath induced a large increase in membrane conductivities. The Fsk/IBMX-induced Cl^- currents were not affected by up to sevoflurane 360 μM (n=8, Fig. 2). No increase in Cl^- conductance was observed in non-expressing control cells when treated with Fsk/IBMX (n=6, data not shown).

Consequently, the effect of sevoflurane on the KCNQ1 current was examined in single cells. In the KCNQ1-expressing HEK293T cells, the membrane voltage was held at −60 mV and step-like depolarizing pulses (500 ms) were applied ranging from −40 to 80 mV with 20 mV intervals (see pulse protocol in Figs 3 and 4). The incremental step pulses induced slowly activating outward currents from −40 mV. Upon repolarization to −60 mV, which is still above the equilibrium potential for K^+, deactivation with slow kinetics was observed as an outward tail current. The slowly activating and tail outward currents were commonly suppressed by 293B (Fig. 3A). However, the blocking effect of 293B on KCNQ1 currents was less potent than the results acquired from Ussing chamber studies. It is possible that such differences are because of the coexpression of beta subunit (KCNE3 protein) in the native epithelial tissues, which modulates the sensitivity of KCNQ1 to 293B and other properties.[15] In control cells (HEK293T cells expressing only the GFP vector), the amplitudes of outward currents were generally small and the application of 293B had a negligible effect (Fig. 3B).

In KCNQ1-expressing cells, sevoflurane application decreased the amplitude of the outward current in a concentration-dependent manner (Fig. 4). Interestingly, the inhibitory effects also showed a time dependency. A larger decrease in the outward current was observed towards the end of the depolarizing pulse (Fig. 4A). To evaluate the inhibitory effects, the amplitudes of the currents in the presence of sevoflurane were measured at depolarizing pulses with a duration of 500 ms and were plotted against the voltage ($I-V$ curve, Fig. 4B, left panel). In control cells, sevoflurane application produced no significant changes (Fig. 4B, right panel). Figure 4C shows the amplitudes of the remaining currents normalized to the control as a function of test voltage. In summary, the steady-state amplitudes of the outward currents (500 ms of depolarization to 80 mV) were reduced to 67 (4.5), 59 (4.9) and 50 (5.4)% by sevoflurane 30, 190 and 360 μM, respectively (n=7, Fig. 4C).

Table 1 Summary of the V_{te}, R_{te} and equivalent I_{sc} in mouse trachea. Agonists and inhibitors were added to the luminal (lu) or basolateral (bl) side as indicated. Means (SE); n=30. Fsk, forskolin; IBMX, isobutylmethylxanthine

	V_{te} (mV)	R_{te} (Ωcm^2)	I_{sc} (μA cm^{-2})
Control	−5.4 (0.67)	85 (9.2)	−70 (6.4)
Amiloride (lu)	−2.6 (0.44)	92 (10.2)	−32 (5.0)
FSK/IBMX (bl)	−7.4 (0.98)	86 (8.4)	−88 (7.8)
Chromanol 293B (bl)	−2.5 (0.52)	92 (10.7)	−38 (7.2)

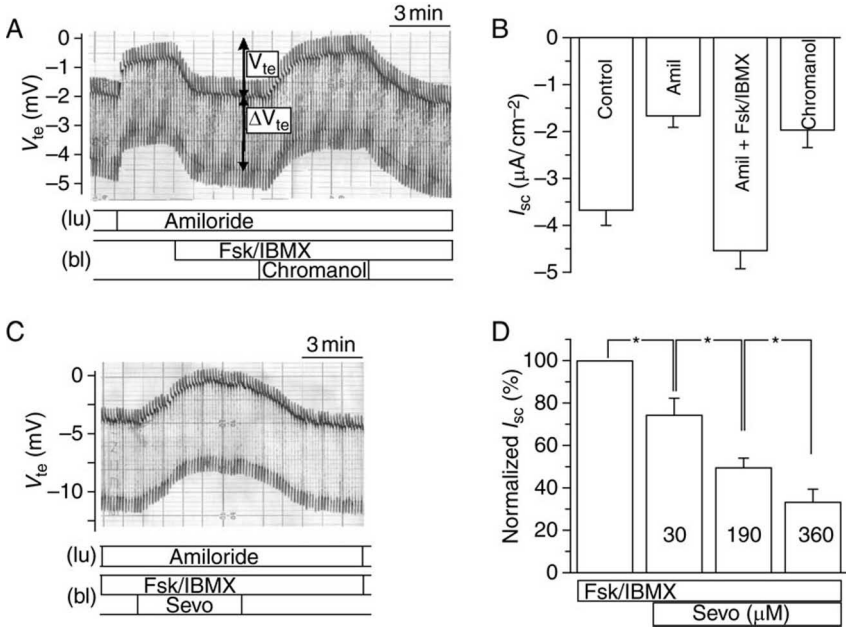

Fig 1 Ussing chamber experiments in mouse trachea. (A, C) Original recordings of the V_{te}. The upper border of the trace is V_{te}, the downward deflection (amplitude ΔV_{te}) is the response to current injections from which R_{te} and the equivalent I_{sc} were calculated. The bars below indicate the luminal (lu) or basolateral (bl) application of amiloride (10 μM), Fsk (2 μM)/IBMX (100 μM), chromanol 293B (chromanol, 10 μM) and sevoflurane (sevo, 360 μM). (B) Summary of the I_{sc} measured during the initial control and each phase of drug application. (D) Representation of the I_{sc} measured at the steady state of the response to different sevoflurane concentrations as demonstrated in C. Asterisks indicate statistical significance ($P<0.05$, paired *t*-test).

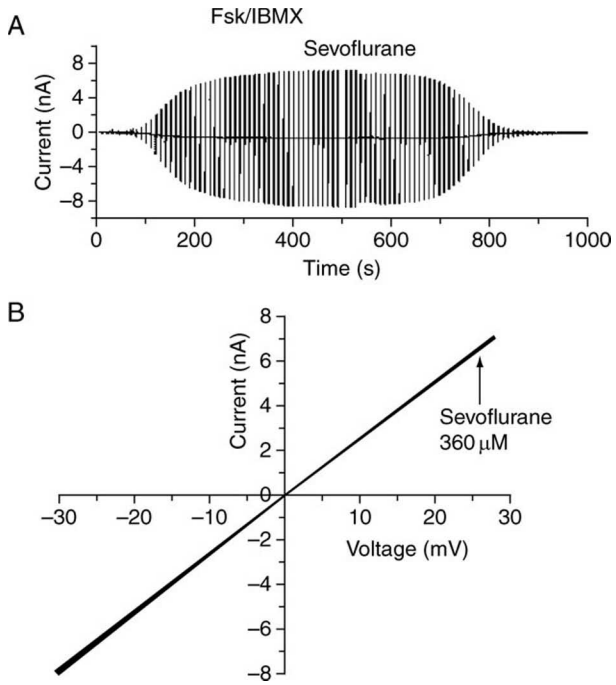

Fig 2 Effects of sevoflurane on CFTR Cl^- current in HEK293T cells. The Cl^- currents were measured in the whole-cell configuration. (A) After establishing the whole-cell configuration with NMDG–Cl^- solution in the patch pipette, the CFTR channels were activated using a cAMP cocktail, Fsk 2 μM and IBMX 100 μM. The recorded cells were held at 0 mV and ramp-like pulses from −30 to 30 mV (0.1 V s^{-1}) were applied every 10 s. (B) The current–voltage relationship was obtained from the response to the ramp pulses. The bath solution containing sevoflurane 360 μM had no effect on the CFTR current.

Discussion

This study examined the effects of sevoflurane on the transepithelial secretion of electrolytes in the murine tracheal epithelium. In addition, the effects of sevoflurane on cloned channels that are thought to be important in cAMP-induced secretion were also examined. Sevoflurane inhibited KCNQ1 in a reversible manner. This suggests that sevoflurane impairs Cl^- secretion indirectly by inhibiting the KCNQ1 channels in the basolateral membrane. The cAMP-induced I_{sc} is associated with the Cl^- secretion, which is accompanied by the paracellular transport of Na^+ and water. Therefore, the inhibitory effects on Cl^- secretion and the subsequent disturbance of the ASL formation might explain the suppression of mucociliary clearance by the volatile anaesthetics.[3]

Previous reports using halothane as a test agent have shown an inhibition of the I_{sc} in canine trachea in both the presence and absence of stimulation of cAMP signalling pathways.[7] Moreover, mechanical stimuli-induced transepithelial voltage was decreased by halothane and isoflurane in the rabbit trachea.[8] In the airway epithelium, both the beta-adrenergic pathway and the paracrine hormones such as prostaglandin E_2 activated cAMP-dependent intracellular signalling cascades. It should be noted that in these studies, indomethacin was not included in the experimental solutions.[7 8] Therefore, it is probable that the endogenous prostaglandins have already stimulated cAMP-signalling pathways. To prevent the influence of

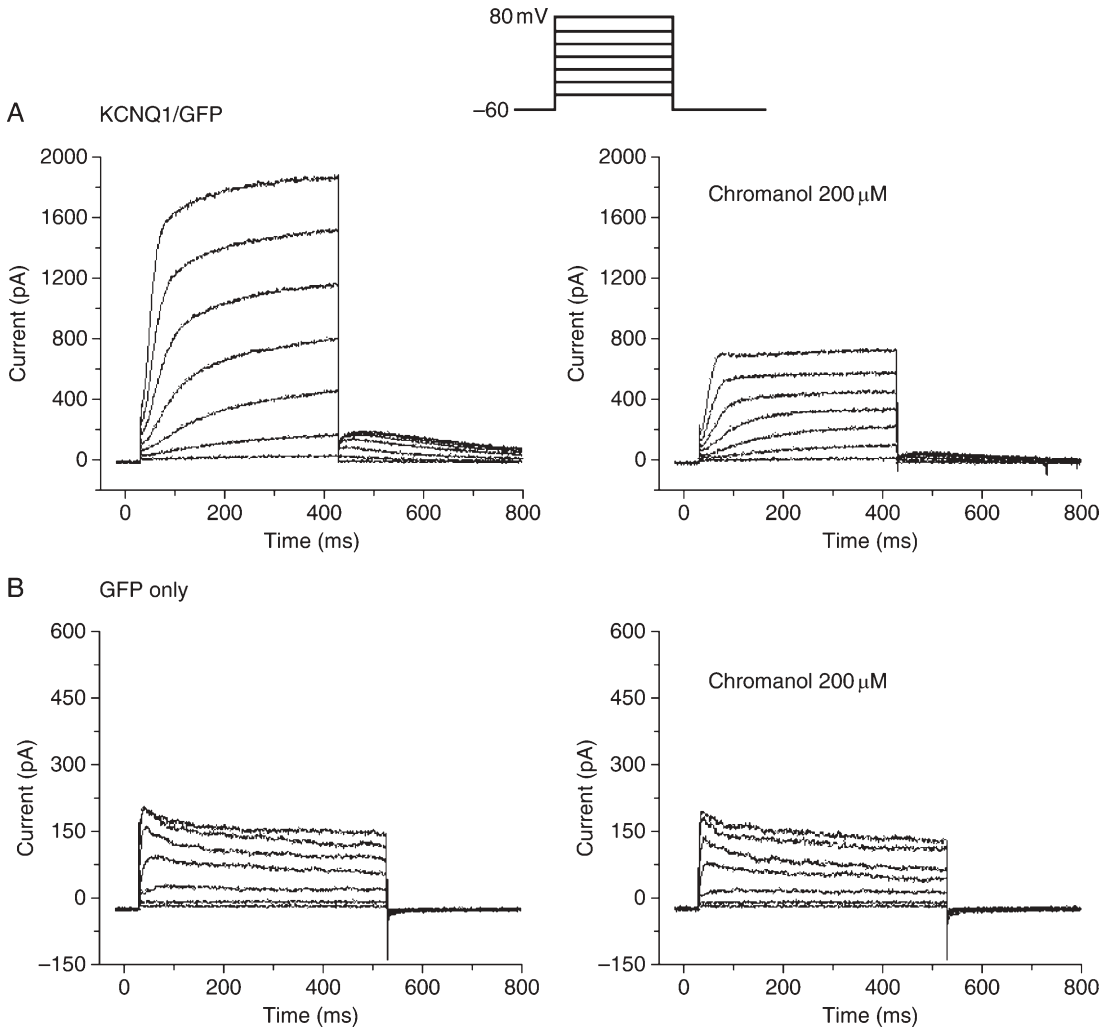

Fig 3 Effects of chromanol 293B on KCNQ1$^-$ mediated K^+ currents. Representative whole-cell currents in HEK293 cells expressing KCNQ1/GFP (A) or GFP alone (B) were measured. Step pulses from −40 to 80 mV (20 mV of interval, 400 ms of duration) were applied as shown in the inset. Slowly activating and deactivating outward currents were observed in the cells expressing KCNQ1, which were largely suppressed by chromanol 293B (200 μM). In contrast, control HEK293 cells showed relatively small outward currents with little sensitivity to chromanol 293B.

endogenous prostaglandins, we included indomethacin in our Ussing chamber experiments.

Adenylate cyclase in the tracheal epithelium was directly stimulated with a Fsk/IBMX cocktail in order to exclude the possibility that sevoflurane interferes with receptor-dependent signalling mechanisms. Thus, under the current conditions, the inhibitory effect of sevoflurane strongly indicates that a direct inhibition of ion channels/transporters is related to Cl^- secretion. According to the electrogenic Cl^- secretion model of epithelial tissues, activation of the luminal Cl^- and basolateral K^+ channels is the most significant event. In the case of cAMP-dependent secretion, both luminal CFTR and basolateral KCNQ1 channels are strong candidate targets for cAMP-dependent protein kinase A (PKA).

The most important clinical feature of cystic fibrosis, which is the genetic mutation of CFTR, is the impaired airway clearance and recurrent infections.[2] In this study, the CFTR current was unaffected by sevoflurane (up to 360 μM) whereas the KCNQ1 current was inhibited considerably from 30 μM. Although sevoflurane-induced inhibition of total outward current was incomplete in the KCNQ1-expressing HEK cells, the intrinsic outward currents in control (non-expressing HEK cells) were relatively insensitive to sevoflurane (Fig. 4B, right panel). The inhibitory effects on KCNQ1 channels *per se* could be more potent than our results suggest.

In the tracheal and intestinal epithelia, the heteromeric complex of KCNQ1 and its partner protein KCNE3 is suggested to be a functional unit of the basolateral K^+ channels regulated by PKA.[4 15] Although we did not examine the effects in a double expression system (KCNQ1/KCNE3), the inhibition by sevoflurane was clearly demonstrated. Therefore, it is likely that the alpha

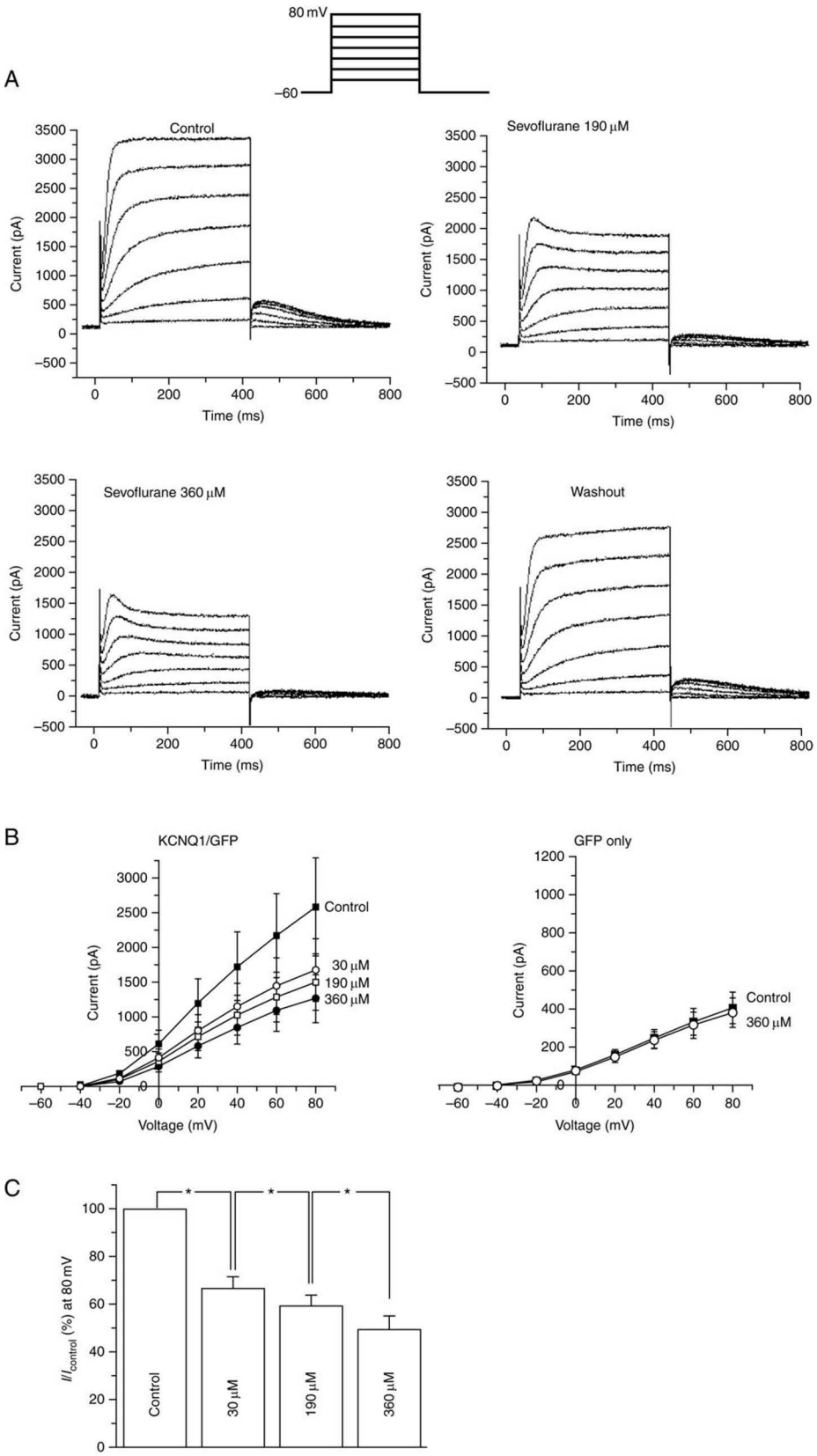

Fig 4 Inhibition of KCNQ1 current by sevoflurane in HEK293 cells. (A) Representative current traces showing the inhibitory effects of sevoflurane. (B) Summary of the $I-V$ curves under control and various sevoflurane concentrations ($n=7$). (C) Summary of normalized changes in the KCNQ1 current induced by different sevoflurane concentrations. The steady-state outward current amplitude at 80 mV was measured for each condition and normalized to the control value [$I/I_{control}$ (%) at 80 mV]. Asterisks indicate statistical significance between the two groups ($P<0.05$, paired t-test).

subunit (KCNQ1), not the beta subunit (KCNE3), may be the major pharmacological target for sevoflurane in the tracheal epithelium.

As the KCNQ1 protein is rarely expressed in neuronal cells, the inhibition of KCNQ1 by sevoflurane would not be the cause of its anaesthetic action. Instead, KCNQ1 is widely expressed in the secretory epithelial tissues such as the gastrointestinal tract, uterine endometrium, pancreas and vestibular epithelium of the inner ear. More importantly for anaesthesiologists, the heteromeric complex of KCNQ1 and KCNE (min-K protein) comprises the slowly activating voltage-dependent K^+ current (I_{Ks}) in cardiac ventricular myocytes. I_{Ks} is responsible for the late repolarization phase of the cardiac action potential, and mutation of KCNQ1 or min-K leads to hereditary long QT-syndrome.[16] Indeed, it has been reported that sevoflurane inhibits the I_{Ks} in guinea-pig ventricular myocytes and increases the duration of the action potential.[17] The increased duration of the ventricular action potential is reflected in the surface electrocardiogram as a prolonged QT-interval, which is arrhythmogenic.

In conclusion, sevoflurane inhibits the heterologously expressed KCNQ1 channels and decreases the cAMP-dependent I_{sc} in the murine trachea. In contrast, the exit pathway for Cl^- in the luminal membrane, CFTR, was not affected by sevoflurane. Considering the observation that KCNQ1 is the alpha subunit of cAMP-regulated K^+ channels in the basolateral membrane of tracheal epithelium, these results suggest that the basolateral K^+ channels in the tracheal epithelium may be one of the potential targets for the respiratory side effects evoked by volatile anaesthetics.

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