Specific blockade of slowly activating I_{sK} channels by chromanols – impact on the role of I_{sK} channels in epithelia

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Abstract Chromanols, which were recently shown to inhibit cAMP-mediated Cl^- secretion in colon crypts via a blockade of a cAMP-activated K^+ conductance, were analyzed for their effects on distinct cloned K^+ channels expressed in *Xenopus* oocytes. The lead chromanol 293B specifically inhibited I_{sK} channels with an IC_{50} of 7 µmol/l without affecting the delayed rectifier Kv1.1 or the inward rectifier Kir2.1. Moreover, several other chromanols displayed the same rank order of potency for I_{sK} inhibition as demonstrated in colon crypts. Finally, we tested the effects of the previously described I_{sK} blocker azimilide on cAMP mediated Cl^- secretion in rat colon crypts. Similar to 293B azimilide inhibited the forskolin induced Cl^- secretion. These data suggest that I_{sK} protein induced K^+ conductances are the targets for the chromanol 293B and its analogues, and azimilide.

Key words: K^+ channel; cAMP; I_{sK}; Chromanol; Cl^- secretion

1. Introduction

Expression of the I_{sK} protein in *Xenopus* oocytes induces slowly activating, voltage-dependent potassium I_{sK} channels of an unknown subunit composition [1]. The gene encoding the I_{sK} protein was shown to be expressed in uterus [2], heart [3] and a number of epithelial cell types [1]. In cardiac ventricular myocytes, I_{sK} channels contribute to the repolarizing currents during action potentials and may be targets for novel class III antiarrhythmics [4]. The physiological role of I_{sK} channels in epithelial cells is unclear. The activity of I_{sK} channels is regulated by a number of second messengers including diacylglycerol, Ca^{2+}, nitric oxide, peroxides and cAMP [5]. Activation of K^+ channels by Ca^{2+} and cAMP has been shown to provide the electrical driving force for Cl^- secretion in tracheal and colon epithelial cells [6–10]. Recently, Lohrmann et al. [11] demonstrated that the inhibition of cAMP-activated K^+ channels by several chromanol derivatives completely abolishes Cl^- secretion in colon crypts. In whole-cell patch clamp experiments the inhibitory effects of these chromanols were confirmed on the basolateral, cAMP-activated K^+ conductance in rat colon crypts [9,12]. The aim of this study was therefore to identify the molecular targets for these

chromanols and to test the putative involvement of I_{sK} channels in Cl^- secretion of colon epithelial cells.

2. Materials and methods

2.1. Electrophysiological experiments in Xenopus oocytes

Handling and injection of *Xenopus* oocytes and synthesis of cRNA has been described previously in detail [13]. The two-microelectrode voltage clamp configuration was used to record currents from *Xenopus laevis* oocytes. In several sets of experiments oocytes were individually injected with cRNA encoding each of the K^+ channels rat I_{sK} [1], rat Kv1.1 [14] or rat Kir2.1 [15]. Recordings were performed at 22°C using a Geneclamp amplifier (Axon Instruments, Foster City, CA, USA) and MacLab D/A converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). Outward currents through I_{sK} or Kv1.1 channels were evoked with 15 s or 0.5 s depolarizing pulses to −10 mV from a holding potential of −80 mV, and filtered at 10 Hz and 1 kHz, respectively. K^+ inward currents through inwardly rectifying Kir2.1 channels were evoked by hyperpolarizing the cells for 0.5 s to −120 mV from a holding potential of −40 mV (filtered at 1 kHz). The amplitudes of the recorded currents were measured at the end of the test voltage steps. The control solution contained (mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (titrated with NaOH to pH 7.5 for the control solution). The microelectrodes were filled with 3 M KCl solution and had resistances of 0.5–0.9 MΩ. All chromanols used are listed in Table 1. Azimilide was a generous gift of Procter and Gamble Pharm. (Norwich, USA). Forskolin, IBMX and remaining reagents were purchased from Sigma (St. Louis, MO, USA). Data are presented as means with standard errors (S.E.M.), where n represents the number of experiments performed. A paired Student's t-test was used to test for statistical significance, which was obtained for $P < 0.05$.

2.2. Rat distal colon, Ussing chamber experiments

Rats of either sex (body weight 100–200 g) were used. The animals were killed by decapitation. Ussing chamber experiments were performed as described recently [16]. The chambers were modified for rat tissue. The solutions on the two sides of the epithelia were circulated by a bubble lift system. The reservoirs were bubbled with carbogen (5% CO_2 and 95% O_2) and kept at 37°C by water jackets. The total volume on each side was approximately 15 ml. The solutions used on the two sides had the following composition (in mmol/l): NaCl 120, $NaHCO_3$ 25, K_2PHO_4 1.6, KH_2PO_4 0.4, Ca-gluconate 1.3, $MgCl_2$ 1, D-glucose 5. To this solution indomethacin (1 µmol/l) was added to inhibit endogenous prostaglandin formation. The measurements were performed in 'open circuit mode'. Transepithelial voltage (V_{te}) was referenced to the serosal side. Transepithelial resistance (R_{te}) was obtained by applying calibrated short current pulses (I) every 6 s. The voltage deflection (ΔV_{te}) caused by these current pulses with the mucosa-submucosa preparation present was corrected by that obtained for the empty chamber ($\Delta V_{te}'$). According to Ohm's law: $R_{te} = (\Delta V_{te} - \Delta V_{te}')/I$, the equivalent short circuit current (I_{sc}) was determined from V_{te} and R_{te}: $I_{sc} = V_{te}/R_{te}$. The usual protocol consisted of an equilibration period, usually 2–10 min. Then amiloride (10 µmol/l) was added to the mucosal perfusate. This abolished the lumen negative voltage caused by electrogenic Na^+ reabsorption. Thereafter Cl^- secretion was stimulated by adding forskolin (10 µmol/l) to the

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Table 1: A list of the chromanols used, their IC_{50} value for I_{sK}, and their chemical names. (n = number of experiments)

Code	Structure	IC_{50} (μmol/l), chemical name	Code	Structure	IC_{50} (μmol/l), chemical name
293B Racemate		6.89 ± 0.38, (n = 8) trans-6-Cyano-4-(N-ethylsulphonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane	439B Racemate		48.2 ± 1.74, (n = 4) trans-6-Cyano-3-hydroxy-2,2-dimethyl-4-(1,1-dioxo-2-isothiazolidinyl)-chromane
434B		5.05 ± 0.21, (n = 4) (+)-(3S,4R)-6-Cyano-4-(N-ethylsulphonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane	360B Racemate		58.8 ± 1.81, (n = 4) trans-6-Cyano-4-[N-(1-butylsulphonyl)-N-methylamino]-3-hydroxy-2,2-dimethyl-chromane
407B		30.7 ± 1.24, (n = 4) (-)-(3R,4S)-6-Cyano-4-(N-ethylsulphonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane	369B		48.2 ± 3.46, (n = 4) (+)-(3S,4R)-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-6-phenylsulphonyl-chromane
374B Racemate		19.2 ± 1.20, (n = 4) trans-6-Cyano-4-(N-methylsulphonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane	368B		> 300, (n = 4) (-)-(3R,4S)-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-6-phenylsulphonyl-chromane

serosal perfusate. When a stable secretory I_{sc} was obtained the putative blockers were added to the serosal perfusate.

3. Results

As previously described, expression of I_{sK} and Kv1.1 channels in *Xenopus* oocytes resulted in the induction of slowly and fast activating voltage-dependent K^+ currents, respectively [1,14], while Kir2.1 expression induced an inward-rectifier K^+ channel [15]. 293B (at 30 μmol/l; for chemical name see Table 1), the most potent cAMP-regulated K^+ channel inhibitor in rabbit and rat colon crypts [9,11,12], had no inhibitory effects on Kv1.1 and Kir2.1 (Fig. 1; $n = 6$ and 6 for Kv1.1 and Kir2.1, respectively). However, 293B (at 30 μmol/l) inhibited I_{sK} channels almost completely (Fig. 1; 91.9 ± 0.9%; $n = 8$). This effect was completely and rapidly reversible upon washout.

A concentration-blockade relation for 293B on I_{sK} yielded an IC_{50} of 6.9 μmol/l (Fig. 2; IC_{50} means ± S.E.M. for all chromanols are listed in Table 1), a value which corresponds to its effects on cAMP-regulated K^+ channels in colon crypts [11]. As previously mentioned, I_{sK} channels are positively regulated by activation of protein kinase A (PKA) [17]. Effects of 293B on I_{sK} were therefore also tested after PKA-mediated regulation. Superfusion of oocytes expressing I_{sK} with forskolin (10 μmol/l) and IBMX (1 mM) increased I_{sK} by 83.1 ± 16.5% ($n = 4$), similar to that described by Blumenthal and Kaczmarek [17]. The inhibitory potency of 293B on regulated I_{sK} was not significantly altered; following PKA-mediated increase of I_{sK} the IC_{50} for 293B was 5.18 ± 0.27 μmol/l ($n = 4$).

293B is a racemate, and its stereoisomers (diastomers) 434B and 407B differed in their effects in colon crypts approximately 10-fold. Similarly, 434B and 407B had distinct inhibi-
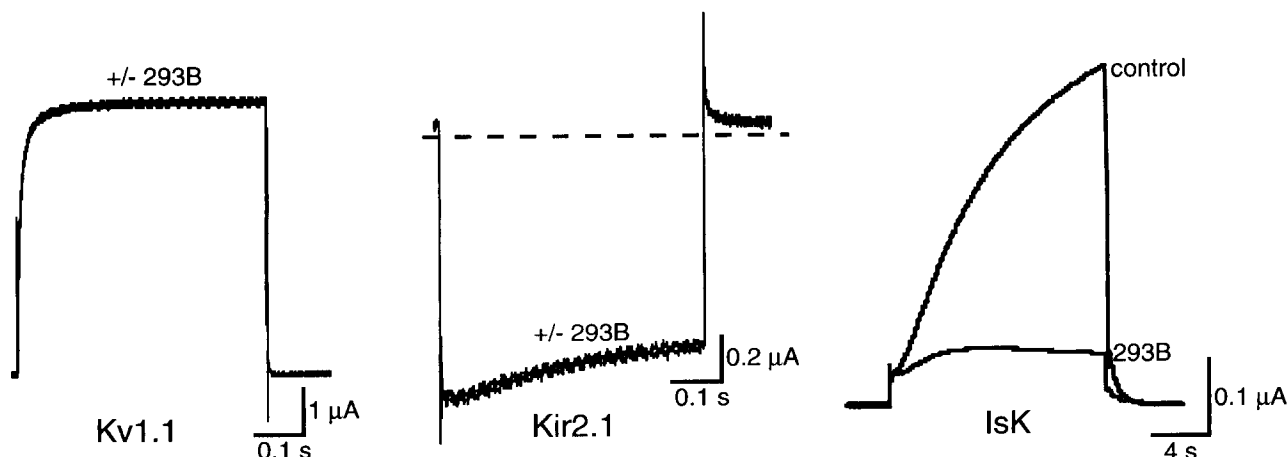

Fig. 1. Effects of 293B (30 μmol/l) on the K^+ channels Kv1.1, Kir2.1 and I_{sK}. The voltage clamp protocol for the K^+ channels is described in Section 2.

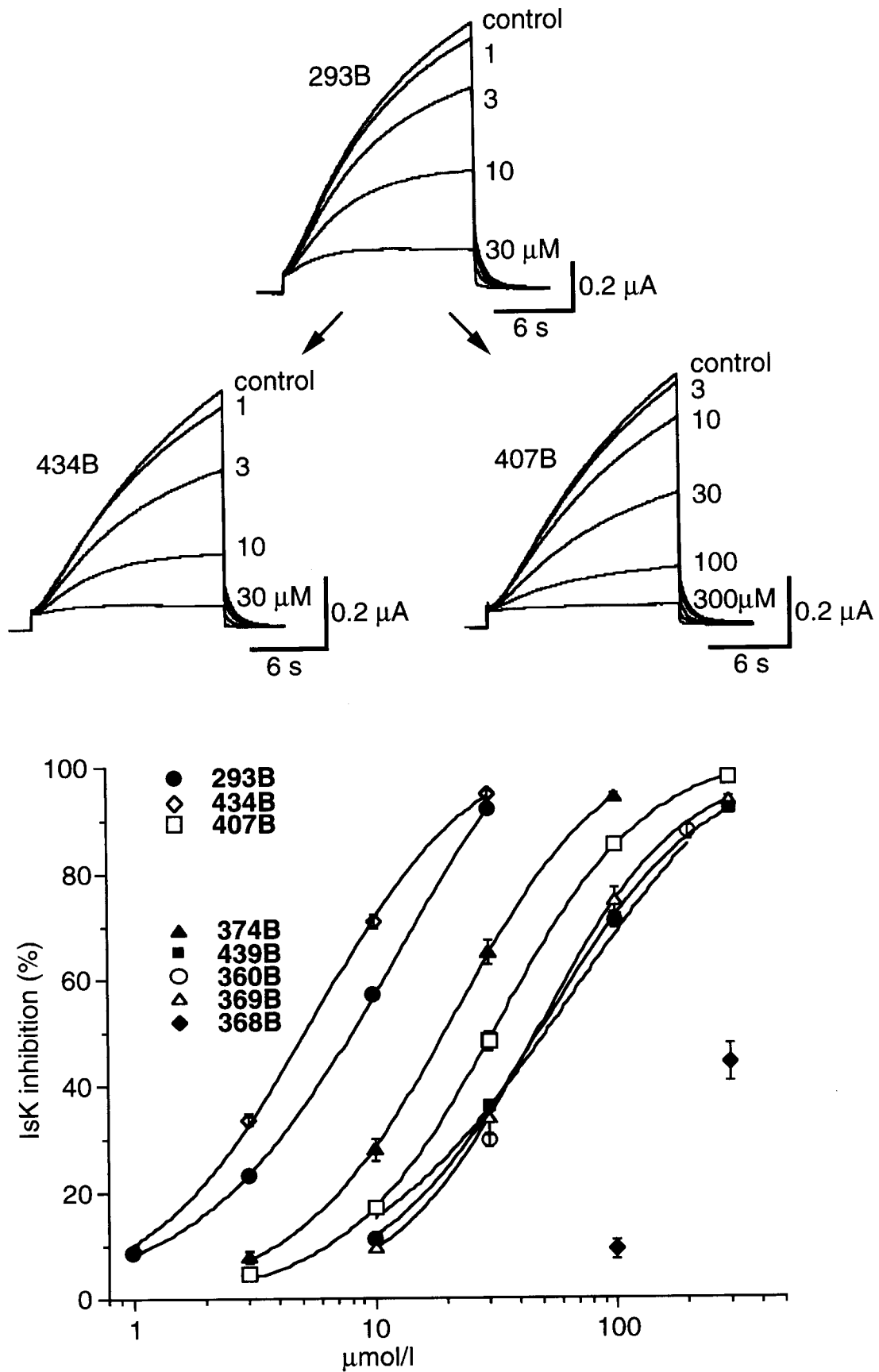Fig. 2. Effects of the chromanol 293B and its enantiomers 434B and 407B on I_{sK} channels expressed in *Xenopus* oocytes. I_{sK} was evoked with 15 s depolarizing steps from −80 to −10 mV. The graph illustrates the concentration-inhibition relation for all chromanols tested. The data present means ± S.E.M. The IC_{50} values are given in Table 1.
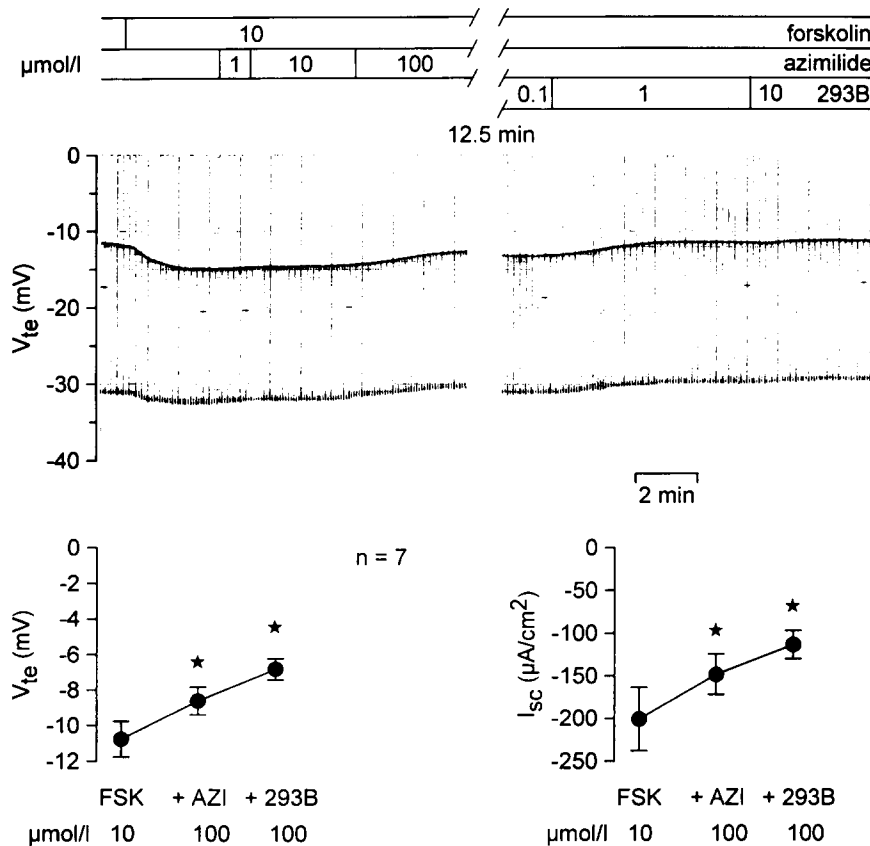
Fig. 3. Rat distal colon, Ussing chamber experiment. Upper trace: original record of transepithelial voltage (V_{te}, continuous line). Voltage deflections are caused by constant current injection to determine transepithelial resistance. The effect of forskolin is partially reversed by the addition of azimilide in increasing concentrations. 293B shows an additional effect on V_{te} in the presence of azimilide. Lower panels: Effect of azimilide (AZI) and additional effect of 293B on V_{te} and equivalent short circuit current (I_{sc}) in the presence of forskolin (FSK). *Significantly different from V_{te} and I_{sc} under FSK alone.

tory potencies on I_{sK} with respective IC_{50} of 5 μmol/l and 31 μmol/l (Fig. 2; Table 1). Moreover, 5 other chromanol derivatives were tested which had been shown to exert distinct inhibitory potencies on the cAMP-regulated K^+ conductance in colon crypts in the study of Lohrmann et al. [11]. These chromanols also exerted distinct inhibitory potencies on I_{sK} expressed in *Xenopus* oocytes with IC_{50} values of 20–500 μmol/l, in the same order of potency (for details see Table 1).

I_{sK} channels and the cardiac conductance I_{Ks} have recently been shown to be inhibited by the novel class III antiarrhythmic azimilide [4]. In Ussing chamber experiments ($n = 7$) the effects of azimilide on transepithelial transport were examined. Transepithelial voltage (V_{te}) and equivalent short circuit current (I_{sc}) were taken as a measure of electrogenic Cl^- transport similar to what was previously described [11].

Stimulation of cAMP-dependent Cl^- secretion by forskolin (FSK, 10 μmol/l) increased V_{te} from -7.7 ± 0.7 mV to -10.8 ± 1.0 mV and I_{sc} increased from -125 ± 21 μA/cm² to -201 ± 37 μA/cm². In the presence of FSK azimilide inhibited V_{te} and I_{sc} concentration-dependently by 3% (1 μmol/l), 15% (10 μmol/l) and 69% (100 μmol/l). At a concentration of 100 μmol V_{te} and I_{sc} were reduced by 2.2 ± 0.4 mV and by 52 ± 16 μA/cm² (Fig. 3), respectively, which was close to V_{te} and I_{sc} before FSK treatment. 293B (100 μmol/l) had an additional effects in the presence of azimilide (100 μmol/l) and blocked the remaining FSK stimulated secretion completely. In all experiments V_{te} and I_{sc} were reduced by additional superfu-

sion with 293B (100 μmol/l) to -6.8 ± 0.6 mV and 113 ± 17 μA/cm² (Fig. 3), respectively.

4. Discussion

In colon crypt cells luminal cAMP-mediated Cl^- secretion requires the activation of a basolateral K^+ conductance to provide the necessary electrical driving force for Cl^-. Ecke et al. [12] demonstrated recently in whole cell patch clamp experiments an inhibition of the basolateral K^+ conductance in rat colon crypts by novel chromanol derivatives, thereby completely inhibiting Cl^- secretion caused by several hormones. A number of observations of the present study indicate that I_{sK} channels are the source of this epithelial K^+ conductance: (a) I_{sK} inhibition mediated by chromanols occurred at similar concentrations and with a similar rank order of potency as observed for the colon cAMP-regulated K^+ conductance [11]; (b) the chromanols inhibited I_{sK} channels potently without affecting other cloned K^+ channels; (c) especially intriguing is the stereospecificity of the I_{sK} inhibition by chromanols. The stereoisomers of the racemate compound 293B inhibited I_{sK} channels with different potencies, again in perfect correlation with their effects in colon crypts [11]; (d) finally, the I_{sK} blocker azimilide also inhibits FSK mediated Cl^- secretion in colon crypts. However, azimilide is with an estimated IC_{50} of 48 μM for I_{sc} in rat colon about 10 times weaker than for I_{sK} channels expressed in *Xenopus* oocytes [4].

In summary, the present study demonstrates almost identical pharmacological properties of I_{sK} channels expressed in *Xenopus* oocytes and the cAMP-activated K^+ conductance in colon crypts. Moreover, parallel studies could also demonstrate a 293B mediated inhibition of the I_{sK} protein underlying cardiac conductance I_{Ks} in guinea pig cardiocytes [18] and I_{sK} channels in strial marginal cells in the inner ear [19]. The present study suggests therefore that I_{sK} channels are the molecular target for the chromanol 293B and its analogues.

References

[1] Takumi, T., Ohkubo, H. and Nakanishi, S. (1989) Science 242, 1042–1045.

[2] Folander, K., Smith, J.S., Antanavage, J., Bennett, C., Stein, R.B. and Swanson, R. (1990) Proc. Natl. Acad. Sci. USA 87, 2975–2979.

[3] Varnum, M.D., Busch, A.E., Bond, C.T., Maylie, J. and Adelman, J.P. (1993) Proc. Natl. Acad. Sci. USA 90, 11528–11532.

[4] Busch, A.E., Malloy, K.-J., Groh, W., Varnum, M.D., Adelman, J.P., North, R.A. and Maylie, J. (1994) Biochem. Biophys. Res. Commun. 202, 265–270.

[5] Busch, A.E., Waldegger, S. and Lang, F. (1995) Drugs News Perspect. 8, 278–282.

[6] Welsh, M.J., Smith, P.L. and Frissell, R.A. (1983) J. Membr. Biol. 71, 209–218.

[7] Loo, D.D.F. and Kaunitz, J.P. (1989) J. Membr. Biol. 110, 19–28.

[8] Lohrmann, E. and Greger, R. (1995) Pflüger's Arch. 429-494–502.

[9] Warth, R., Riedemann, N., Bleich, M., Van Driessche, W., Busch, A.E. and Greger, R. (1996) Pflüger's Arch. 432, 81–88.

[10] Bleich, M., Riedemann, N., Warth, R., Kerstan, D., Leipziger, J., Hör, M., Van Driessche, W. and Greger, R. (1996) Pflüger's Arch. 432, 1011–1022.

[11] Lohrmann, E., Burhoff, I., Nitschke, R.B., Lang, H.-J., Mania, D., Englert, H.C., Hropot, M., Warth, R., Rohm, W., Bleich, M. and Greger, R. (1995) Pflüger's Arch. 429, 517–530.

[12] Ecke, D., Bleich, M., Lohrmann, E., Hropot, M., Englert, H.C., Lang, H.-J., Warth, R., Rohm, W., Schwartz, B., Frase, G. and Greger, R. (1995) Cell. Physiol. Biochem. 5, 204–210.

[13] Busch, A.E., Kopp, H.-G., Waldegger, S., Samarzija, I., Süßbrich, H., Raber, G., Kunzelmann, K., Ruppersberg, J.P. and Lang, F. (1995) J. Physiol. 491, 735–741.

[14] Stühmer, W., Stocker, M., Sakmann, B., Seeburg, P., Baumann, A., Grupe, A. and Pongs, O. (1988) FEBS Lett. 242, 199–206.

[15] Fakler, B., Brändle, U., Glowatzki, E., Zenner, H.P. and Ruppersberg, J.P. (1994) Neuron 13, 1413–1420.

[16] Greger, R., Nitschke, R.B., Lohrmann, E., Burhoff, I., Hropot, M., Englert, H.C., Lang, H.-J. (1991) Pflüger's Arch. 419, 190–196.

[17] Blumenthal, E.M. and Kaczmarek, L.K. (1992) J. Neurosci. 12, 290–296.

[18] Busch, A.E., Suessbrich, H., Waldegger, S., Sailer, E., Greger, R., Lang, H.-J., Lang, F., Gibson, K.J. and Maylie, J.G. (1996) Pflüger's Arch 432, 1094–1096.

[19] Shen, Z., Marcus, D.C., Sunose, H., Chiba, H. and Wangemann, P. (1996) Audit. Neurosci. (in press).