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Neuropharmacology 50 (2006) 458–467
NEURO PHARMACOLOGY
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The human Ca^{2+}-activated K^+ channel, IK, can be blocked by the tricyclic antihistamine promethazine

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Received 13 July 2005; received in revised form 4 October 2005; accepted 10 October 2005

Abstract

Phenothiazines can be used as psychopharmaceutical agents and are known to cause many side effects during treatment since they interfere with many different cellular systems. Recently, phenothiazines were reported to block Ca^{2+}-activated potassium channels of the SK type. Therefore we investigated their effect on the functionally related class of Ca^{2+}-activated potassium channels of the IK type. The representative phenothiazine derivative promethazine (PTZ) blocked IK channels almost independently from the extracellular pH_o with an IC_{50} of 49 ± 0.2 μM (pH_o 7.4, $n = 5$) and 32 ± 0.2 μM (pH_o 6.2, $n = 5$) in whole cell experiments. The extracellularly applied membrane impermeable PTZ analogue methyl-promethazine (M-PTZ) had a strongly reduced blocking potency compared to PTZ. In contrast, intracellularly applied PTZ and M-PTZ had the same blocking potency on IK channels in excised inside out patch clamp experiments ($K_d = 9.3 \pm 0.5$ μM for PTZ, $n = 7$ and 6.7 ± 0.4 μM for M-PTZ, $n = 5$). The voltage dependency of the PTZ and M-PTZ block was investigated in excised inside out patch clamp experiments at a concentration of 100 μM. For both compounds the block was more pronounced at positive membrane potentials. The steepness of the voltage dependency was found to be 70 ± 10 mV (for PTZ) and 61 ± 6 mV (for M-PTZ) indicating that both compounds sensed approximately 40% of the entire membrane spanning electrical field from the inside. We conclude that PTZ and M-PTZ bind to a side in IK channels, which is located within the electrical field and is accessible from the intracellular side.

Keywords: Potassium channel; IK channel; Neuroleptic; Promethazine

1. Introduction

Tricyclic psychopharmaceutics share a tricyclic core structure. In the case of neuroleptics the tricyclic core structure has a planar orientation, which allows a conjugated ring system, whereas the phenyl rings have an angled orientation in the case of tricyclic antidepressants. Both classes of compounds are known to affect a huge variety of different cellular systems including ionic transport mechanisms (Delpon et al., 1992; Galeotti et al., 1999; Isenberg and Tamargo, 1985; Ogata and Narahashi, 1989; Ogata et al., 1989; Ogata and Tatebayashi, 1993). They also modulate the function of lipid bilayers through hydrophobic and electrostatic interaction (Carfagna and Muhoberac, 1993; Forrest and Mattai, 1983). These complex interactions may cause many side effects during therapeutical treatment with these compounds. Chlorpromazine treatment was reported to induce QT prolongation and torsade de pointes (Ochiai et al., 1990; Warner et al., 1996). In contrast to these findings, chlorpromazine as well as trifluoperazine had a proarrhythmic effect on isolated guinea-pig Purkinje fibers (Studenik et al., 1999). Derivatives of phenothiazine, like chlorpromazine, were also reported to cause pulmonary side effects like oedema (Li and Gefter, 1992). All these side effects could be explained by assuming that these compounds modulate ionic currents flowing through the plasma

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doi:10.1016/j.neuropharm.2005.10.005

membrane and therefore it was not surprising that these compounds were found to modulate different types of potassium currents (Isenberg and Tamargo, 1985; Ogata and Tatebayashi, 1993; Ogata et al., 1989). More recent reports showed that tricyclic psychopharmaceutics directly interacted with different types of K^+ channels like small conductance calcium-activated K^+ channels, SK channels (Carignani and Corsi, 2002; Dreixler et al., 2000; Terstappen et al., 2001), $K_v1.3$ channels of T-lymphocytes (Teisseyre and Michalak, 2003), rapidly inactivating K_A channels (Kuo, 1998), ether-a-go-go-related gene (HERG) channels (Jo et al., 2000; Teschemacher et al., 1999; Thomas et al., 2003) as well as with inwardly rectifying channels (Kobayashi et al., 2004). However, the blocking mechanism of K^+ channels by tricyclic antidepressants is still controversial. Extracellular binding sites were hypothesized for Ca^{2+}-activated SK channels and inwardly rectifying potassium channels (Carignani and Corsi, 2002; Kobayashi et al., 2004; Terstappen et al., 2001). In the case of rapidly inactivating K_A channels a block of the activated state was suggested by binding of uncharged imipramine to a binding site close to the extracellular pore region (Kuo, 1998). However, HERG potassium channels (Jo et al., 2000; Teschemacher et al., 1999) and $K_v1.3$ channels of T-lymphocytes (Teisseyre and Michalak, 2003) were shown to be blocked by tricyclic antidepressants in a voltage dependent manner, which requires the binding of a charged molecule.

In this report we describe the blocking of intermediate conductance calcium-activated potassium channels (IK channels) by PTZ, a representative phenothiazine derivative, as well as its membrane impermeable analogue M-PTZ. When both compounds were applied extracellularly, PTZ blocks IK channels with a much higher potency than M-PTZ. However, IK channels were found to be blocked by PTZ and M-PTZ with the same potency and the same voltage dependency when the compounds were applied to the intracellular side of excised inside out membrane patches. We further showed that the blocking potency of intracellularly applied M-PTZ was decreased, when the extracellular K^+ concentration was increased. This gives evidence that a binding site for PTZ and M-PTZ within IK channels is located within the membrane spanning electrical field of IK channels and that this binding site is only accessible from the intracellular side.

2. Materials and methods

2.1. Cell culture

HEK 293 cells stably expressing the human IK channel a generous gift from Prof. Daniel Devor (Pittsburgh, US (Jones et al., 2004) were grown in MEM medium with EARLE's salts and L-glutamax (Gibco, Germany) containing 10% FCS (PAA, Germany) and 300 μg/ml geneticine (Gibco, Germany) at 37 °C and 10% CO_2.

2.2. Methylation of promethazine

To a solution of 1 mmol promethazine hydrochloride in water (20 ml), a 6-M solution of sodium hydroxide was added and extracted with ether. The organic layer was dried with sodium sulfate and evaporated. The residue was dissolved in ether and an excess of dimethyl sulfate was added dropwise. Within the first hour after the completion of the addition of dimethyl sulfate the crude product starts to crystallize and the reaction mixture was stirred for an additional period of 12 h at room temperature. Then the mixture was filtered, the product was isolated and finally recrystallized from ethanol. Yield: 87%. ^{1}H NMR (DMSO-d_6, 300 MHz): δ/ppm (TMS) = 1.38 (d, 3H, $^3J = 6.5$ Hz, −CH−CH_3); 3.15 (s, 9H, $N(CH_3)_3$); 3.38 (s, 3H, O_3SO-CH_3); 3.71−3.78 (m, 1H, −CH_2−CH−CH_3); 4.06 (dd, 1H, $^3J = 8.6$ Hz, $^2J = 14.5$ Hz, $N-CH_2$−CH−); 4.63 (dd, 1H, $^3J = 2.8$ Hz, $^2J = 14.4$ Hz, $N-CH_2$−CH−); 7.06 (t, 2H, $^3J = 7.0$ Hz, Ar-H); 7.22−7.33 (m, 6H, Ar-H).

2.3. Whole cell patch clamp experiments

Cells were resuspended and placed onto poly-lysine-coated glass coverslips, which were positioned into a perfusion chamber directly before measurements. Cells were superfused with N-sol, pH 7.4 (in mM: NaCl 160, KCl 4.5, HEPES 5, $MgCl_2$ 1 and $CaCl_2$ 2, pH 7.4 adjusted with NaOH) and pH 6.2 (in mM: NaCl 160, KCl 4.5, MES 5, $MgCl_2$ 1 and $CaCl_2$ 2, pH 6.2 adjusted with NaOH) with and without 3, 10, 30, 100, and 300 μM promethazine hydrochloride (Sigma, Germany). M-PTZ was added to the N-sol pH 7.4 at a concentration of 100 μM. Pipettes were pulled in three stages with a pipette resistance ranging from 1.5 to 2.5 MΩ when filled with K-Asp solution containing 1 μM free Ca^{2+} (in mM: K^+ aspartate 135, EGTA 10, HEPES 10, $MgCl_2$ 2, $CaCl_2$ 8.422 corresponding to 1 μM of free Ca^{2+}, pH 7.2 adjusted with KOH). After whole cell perfusion with K-Asp solution containing 1 μM free Ca^{2+}, the membrane potential was clamped to −120 mV for 50 ms followed by a 400-ms voltage ramp from −120 mV to +60 mV using a HEKA EPC9 amplifier with Pulse and PulseFit (HEKA elektroniks, Germany) as data acquisition and analysis software. The membrane potential was kept at −80 mV for 5 s between ramps. Whole cell currents were measured at 0 mV. Since a strong over expression of functional IK channels was observed, no leak currents were subtracted. Relative currents were calculated according to I/I_{max} with I_{max} as the current measured in N-sol without PTZ. Relative currents were plotted against the PTZ concentration as mean values ± SEM.

2.4. Excised inside out patch clamp experiments

HEK 293 cells stably expressing human IK channels were placed onto poly-lysine-coated coverslips. Coverslips were placed into a perfusion chamber directly before measurements. Pipettes were pulled from borosilicate capillaries in three stages with a resistance of 0.3−0.7 MΩ when filled with K-sol (in mM: KCl 164.5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5, pH 7.4 adjusted with KOH). Chambers were permanently perfused with K-Asp with 1 μM free Ca^{2+} and low Ca^{2+} (in mM: K^+ aspartate 135, HEPES 10, EGTA 10, $MgCl_2$ 2.78, pH 7.2 adjusted with KOH).

For concentration response curves, membrane patches were excised from cells and membrane potentials were clamped to −80 mV for 100 ms followed by a 120 ms voltage step to +80 mV for 120 ms. The membrane potential was kept at −50 mV in-between voltage steps for 5 s. Compounds (PTZ and M-PTZ) were added to K-Asp with 1 μM free Ca^{2+} at concentrations of 3, 10, 30 and 100 μM.

For investigating the voltage dependency of the block, the membrane potential was stepped to −80 mV for 100 ms followed by 120 ms voltage steps to voltages between −60 mV and +80 mV in 20 mV increments. The membrane voltage was kept at −50 mV for 5 s in-between voltage steps.

For all experiments, steady state currents were measured as mean currents during the final 20 ms of the voltage step. Steady state currents measured in K-Asp with low Ca^{2+} as bath solution were assumed as leak currents and therefore subtracted from those measured in K-Asp with 1 μM Ca^{2+} with and without compound. Relative currents were calculated after subtraction of leak currents according to I/I_{max} with I_{max} as currents measured with 1 μM Ca^{2+} in the bath solution. Relative currents for membrane potentials of 80 mV were plotted as mean values ± SEM.

2.5. Curve fitting

Curves were fitted using the software package Igor Pro 3.12 (Wave Metrics, Oregon, USA). Calculated values were given as mean ± standard

deviation. The standard deviation obtained by the fitting routine gives the uncertainty of the fit.

3. Results

3.1. Whole cell patch clamp experiments

Whole cell patch clamp experiments were performed in order to investigate if PTZ (PTZ, Fig. 1A) is able to inhibit human IK channels.

IK channels were activated by whole cell perfusion with K-Asp containing 1 μM free Ca^{2+}. The slope of the whole cell ramp currents in N-sol (Fig. 1B) was linear with a reversal potential close to −80 mV. The application of PTZ in increasing concentration of up to 300 μM led to a reduction of whole cell currents, which was more pronounced at positive membrane potentials indicating a voltage dependent block. Whole cell currents measured at 0 mV were found to be 8.2 ± 1.4 nA at a pH_o 7.4 with N-sol as external bath solution. In the presence of 300 μM PTZ the remaining whole cell current at 0 mV was found to be 2 ± 0.6 nA, which corresponds to a relative current of 0.24 ± 0.08 ($n = 5$).

Since human IK channels contain a histidine at position 236 within their outer pore region we wanted to test if the inhibition of IK channels depends on the extracellular pH_o. Therefore, we tested the PTZ block at a pH_o of 6.2 (Fig. 1C). Again, the slope of the whole cell current ramps was found to be linear with N-sol (pH_o 6.2) as extracellular solution and the reversal potential was found to be close to −80 mV. Whole cell currents measured at 0 mV were found to be 6.8 ± 2.1 nA with N-sol (pH_o 6.2) as extracellular solution. The application of PTZ in increasing concentrations up to 300 μM to the extracellular bath solution led to a concentration dependent decrease of whole cell currents. Also at a pH_o of 6.2 the blockage was strongest at positive membrane potentials. In the presence of 300 μM PTZ the remaining whole cell currents measured at 0 mV were found to be 1.7 ± 0.4 nA, which corresponds to a relative current of 0.26 ± 0.03 ($n = 5$).

The relative currents at 0 mV were plotted against the PTZ concentration (Fig. 1D). The concentration dependence observed for PTZ at both pH_o values of 7.4 and 6.2 was similar. Best fits through the data points for concentration response curves could be calculated according to $I/I_{max} = 1/(1 + (C_{PTZ}/IC_{50})^h)$, with C_{PTZ} as the PTZ concentration in the bath solution and h as the Hill coefficient. The IC_{50}-values obtained by this fit were 49 ± 0.2 μM (Hill coefficient of 0.67 ± 0.01, pH_o 7.4) and 32 ± 0.15 μM (Hill coefficient of 0.49 ± 0.01, pH_o 6.2).

Because of its hydrophobic properties, PTZ can permeate the plasma membrane. In order to test, if the permeation of the plasma membrane is essential for the PTZ block of IK channels we tested the effect of the membrane impermeable PTZ analogue M-PTZ (M-PTZ, Fig. 2A) on currents through IK channels in whole cell patch clamp experiments.

IK channels were activated by whole cell perfusion with K-Asp containing 1 μM free Ca^{2+}. N-sol was used as extracellular solution. Again, the measured whole cell ramp currents

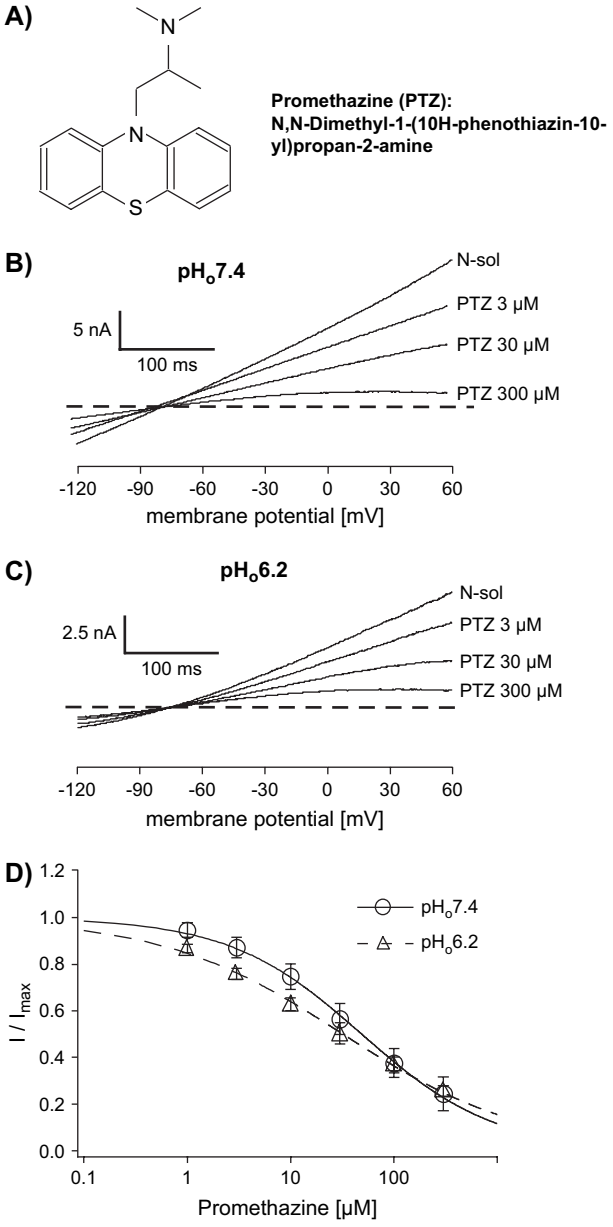

Fig. 1. Promethazine (PTZ) block of whole cell IK currents using voltage ramps. IK channels were activated by whole cell perfusion with K-Asp containing 1 μM of free Ca^{2+}. Membrane potential was clamped to −120 mV for 50 ms followed by a 400 ms voltage ramp from −120 mV to +60 mV. N-sol was used as extracellular bath solution. PTZ was added to the bath solution in increasing concentrations of 1, 3, 10, 30, 100, and 300 μM. (A) Chemical structure of PTZ. (B) and (C) Ramp currents of representative experiments at a pH_o of 7.4 (B) and 6.2 (C) without and with PTZ at the given concentrations. (D) Currents were measured at membrane potentials of 0 mV and relative currents were calculated as ratios of I/I_{max} with I as the current measured in N-sol containing M-PTZ and I_{max} as the current measured in N-sol without M-PTZ with a pH_o of 7.4 or 6.2. Relative currents were plotted against the M-PTZ concentration. Concentration response curves were calculated as best fits through the data points according to the Hill equation $I/I_{max} = 1/(1 + (C_{drug}/K_d)^h$, with IC_{50} as the half maximal blocking concentration and h as the Hill coefficient. Values were calculated to be $pH_o = 7.4$, $IC_{50} = 49 \pm 0.15$ μM and $h = 0.67 \pm 0.01$; $pH_o = 6.2$, $IC_{50} = 32 \pm 0.15$ μM and $h = 0.49 \pm 0.01$.

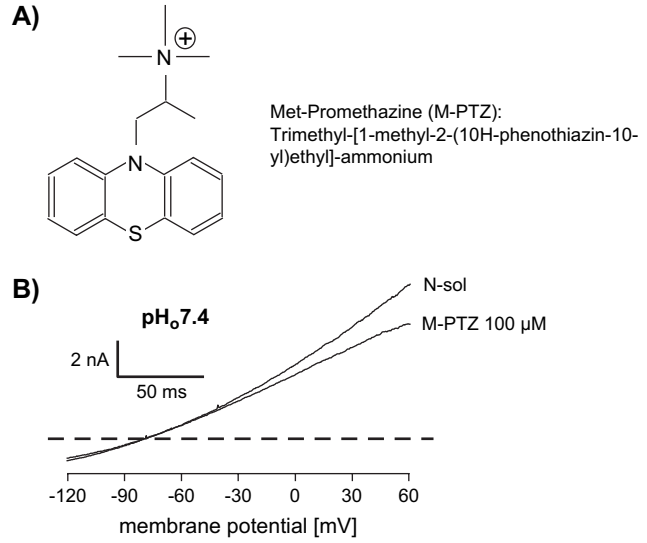

Fig. 2. Blockage of IK channels by the membrane impermeable M-PTZ. (A) Chemical structure and systematic name of methyl-promethazine (M-PTZ). (B) Representative ramp currents elicited similar as described in legend of Fig. 1. IK channels were activated by whole cell perfusion with K-Asp containing 1 µM free Ca^{2+}. N-sol was used as extracellular bath solution, to which M-PTZ was added at a concentration of 100 µM.

were found to be almost linear with a reversal potential at approximately −80 mV (Fig. 2B). Whole cell currents were measured at 0 mV and were found to be 6.9 ± 1.6 nA with N-sol (pH_o 7.4). The blocking effect of M-PTZ was tested for an extracellular concentration of 100 µM. Whole cell currents at 0 mV were 5.5 ± 1.3 nA (N-sol 100 µM M-PTZ), which correspond to a remaining relative current of 0.81 ± 0.03 ($n = 10$). Therefore, M-PTZ applied to the extracellular side blocked IK currents with a strongly reduced potency, when compared to PTZ (remaining relative current in the presence of 100 µM PTZ, 0.38 ± 0.07, $n = 5$).

In all whole cell patch clamp experiments the blockage of IK currents by PTZ as well as by M-PTZ were found to be more pronounced at positive than that at negative membrane potentials. This gives evidence for a voltage dependent blocking mechanism of IK channels from the intracellular side of the membrane.

3.2. Excised inside out patch clamp experiments

Excised inside out patch clamp experiments were performed in order to obtain a better accessibility to the intracellular side of IK channels. The effect of intracellular applied PTZ and M-PTZ on IK currents was investigated for a membrane potential of +80 mV. In these experiments K-sol was used as the pipette solution and K-Asp solutions with low Ca^{2+} and with 1 µM free Ca^{2+} were used as bath solutions to which the intracellular side of the excised membrane patch was exposed (Fig. 3A). Currents measured (Fig. 3B, C) in the presence of K-Asp solution with low Ca^{2+} in the bath solution were assumed to be background currents ($I_{low\ Ca}$). After exchanging the bath solution against a K-Asp solution with

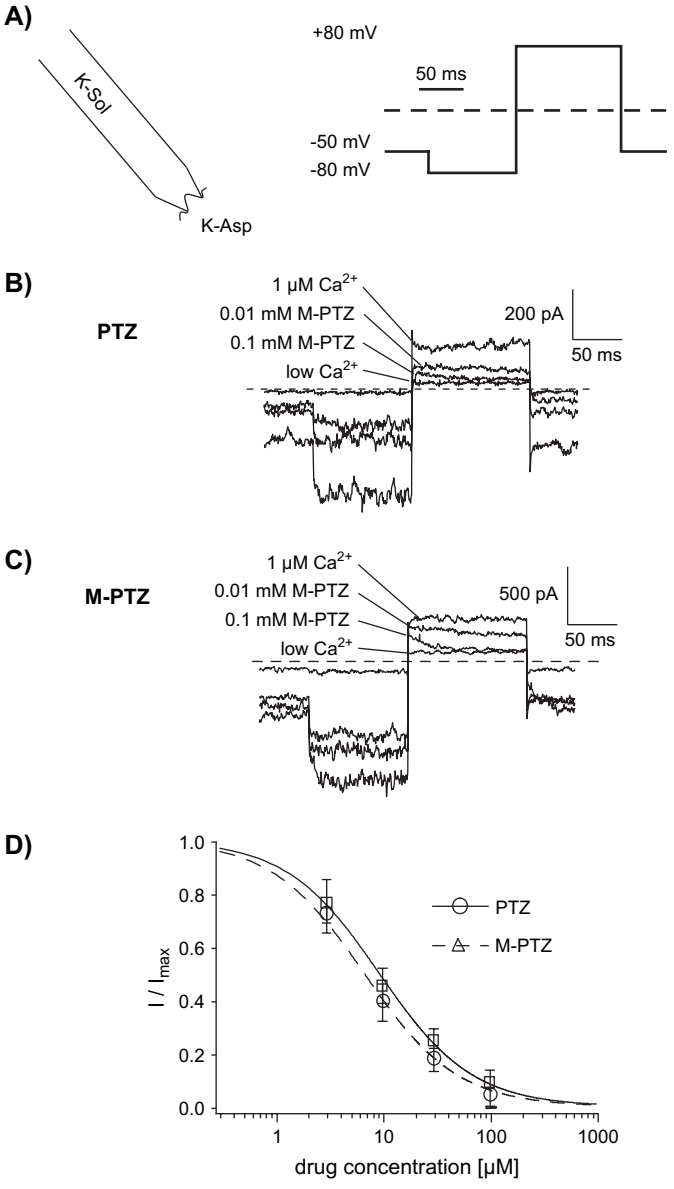

Fig. 3. Concentration dependent block of IK channels by promethazine (PTZ) and methyl-promethazine (M-PTZ) in excised inside out patch clamp experiments. (A) K-sol was used as the pipette solution. K-Asp was used as the bath solution, to which the intracellular side of the excised membrane patch was exposed. The membrane potential was clamped as shown. Representative currents are shown in (B) for experiments with PTZ and in (C) with M-PTZ. Background currents were estimated in K-Asp with low concentrations of free Ca^{2+} (low Ca^{2+}). IK currents were activated by exchanging the bath solution against a K-Asp solution containing 1 µM of free Ca^{2+} (1 µM Ca^{2+}). PTZ and M-PTZ were added to the K-Asp solution containing 1 µM free Ca^{2+} in increasing concentrations. For better clarity currents are shown for M-PTZ concentrations of 0.1 mM and 0.01 mM (B, 0.1 mM PTZ and 0.01 mM PTZ) and for M-PTZ for concentrations of 0.1 mM and 0.01 mM (C, 0.1 mM M-PTZ and 0.01 mM M-PTZ). (D) Currents were measured as steady state currents at the end of the depolarizing step. Background currents were subtracted from currents measured in K-Asp with 1 µM free Ca^{2+} with and without compound. Relative currents were calculated according to I/I_{max} with I as currents measured in K-Asp with 1 µM free Ca^{2+} containing M-PTZ or M-PTZ and I_{max} as currents measured in K-Asp with 1 µM free Ca^{2+} without any compound. Relative currents were plotted against the compound concentration and concentration response curves were calculated through the data points as best fits according to the Hill equation $I/I_{max} = 1/(1 + (C_{drug}/K_d)$ with an Hill coefficient of 1. The half maximal blocking concentration was calculated to be $K_d = 9.3 \pm 0.5$ µM for PTZ and $K_d = 6.7 \pm 0.4$ µM for M-PTZ.

1 μM free Ca^{2+} a strong increase in outward currents through the membrane patch was observed, indicating the activation of Ca^{2+} dependent currents through IK channels. The increase in currents through the membrane patch was instantaneous with the voltage step, indicating that there appears no time or voltage dependent opening of IK channels. Furthermore, no time or voltage dependent inactivation of IK channels could be observed. Compounds were added in increasing concentrations to the K-Asp solution with 1 μM free Ca^{2+}. The addition of PTZ (Fig. 3B) or M-PTZ (Fig. 3C) to the bath solution led to a reduction of currents through the membrane patch. When the membrane potential was stepped from −80 mV to +80 mV, a time dependent reduction of currents through the membrane was observed for both compounds, suggesting a time dependent PTZ and M-PTZ block of IK channels. Steady state currents at the end of the depolarizing pulse were found to be 20.6 ± 3.4 pA ($n = 10$) in K-Asp with low Ca^{2+} ($I_{low\ Ca}$), 96 ± 22.6 pA ($n = 10$) in K-Asp with 1 μM free Ca^{2+} ($I_{1\ \mu M\ Ca}$) and 21 ± 6.7 pA ($n = 7$) after application of 100 μM PTZ for experiments in which the effect of PTZ was tested. For experiments, in which the effect of M-PTZ was investigated, steady state currents were found to be 17.9 ± 6.2 pA ($n = 7$) in K-Asp with low Ca^{2+} ($I_{low\ Ca}$), 338.7 ± 164.2 pA ($n = 7$) in K-Asp with 1 μM free Ca^{2+} ($I_{1\ \mu M\ Ca}$) and 13.7 ± 2.6 pA ($n = 5$) after application of 100 μM M-PTZ. Relative steady state currents in the presence of PTZ and M-PTZ were calculated according to $I/I_{max} = (I_{drug} - I_{low\ Ca})/(I_{1\ \mu M\ Ca} - I_{low\ Ca})$ and were plotted against the drug concentration. Concentration response curves were calculated as best fits through the data points according to the Hill equation $I/I_{max} = 1/(1 + (C_{drug}/K_d))$. The K_d values were found to be almost identical for both compounds ($K_d = 9.3 \pm 0.5$ μM for PTZ and 6.7 ± 0.4 μM for M-PTZ).

The voltage dependence of the PTZ as well as the M-PTZ block was investigated for both compounds at a concentration of 100 μM (Fig. 4). Background currents were measured in K-Asp solution with low Ca^{2+}. Exchanging the solution against a K-Asp solution with 1 μM free Ca^{2+} led to the activation of IK currents through the membrane patch. However, inward currents increased stronger than outward currents, which reflect a slight inward rectification of potassium currents through IK channels. Again, no time or voltage dependent activation or inactivation of IK currents could be observed. The addition of PTZ or M-PTZ at a concentration of 100 μM to the bath solution led to a time dependent reduction of currents through the membrane patch at membrane potentials more positive than 0 mV. Steady state currents in experiments with PTZ ($n = 7$, Fig. 4B) were found to range from −12.1 ± 3.1 pA to 19.2 ± 4.2 pA (K-Asp with low Ca^{2+} at −60 mV and +80 mV, respectively), from −167.5 ± 59.8 pA to 92.7 ± 28.1 pA (K-Asp with 1 μM free Ca^{2+} at −60 mV and +80 mV, respectively) and from −77.4 ± 26.9 pA to 23.6 ± 7.3 pA (K-Asp with 100 μM PTZ at −60 mV and +80 mV, respectively). In experiments with M-PTZ ($n = 5$, Fig. 4C) steady state currents were found to range from −4.7 ± 1.8 pA to 12.2 ± 4.6 pA (K-Asp with low Ca^{2+} at −60 mV and +80 mV, respectively), from −119.4 ± 40.3 pA to 89.1 ± 30.7 pA (K-Asp with low Ca^{2+} at −60 mV and +80 mV, respectively), from −49.2 ± 16.8 pA to 13.7 ± 2.6 pA (K-Asp with 100 μM M-PTZ at −60 mV and +80 mV, respectively).

Plotting the steady state currents through the membrane patch against the membrane potential in the presence and absence of PTZ and M-PTZ shows that the current is more reduced at positive membrane potentials (Fig. 4D). In order to quantify this voltage dependency of the PTZ and M-PTZ block, relative steady state currents in K-Asp containing 1 μM free Ca^{2+} with 100 μM PTZ and M-PTZ were plotted against the membrane potential (Fig. 5A, B). Again, the smaller remaining relative currents observed at positive membrane potentials compared to those at negative membrane potentials, result from the voltage dependency of the block of IK channels. Curves were calculated through the data points according to the Boltzmann equation $I/I_{max} = 1/(1 + \exp((E - E_{50})/k))$ with E_{50} as the membrane potential at half maximal block and k as a steepness factor. The E_{50} and k-values obtained from the best fits were $E_{50} = -89.2 \pm 10$ mV and $k = 69.9 \pm 9$ mV in the presence of 100 μM PTZ ($n = 7$) and $E_{50} = -103.5 \pm 8.4$ mV and $k = 60.7 \pm 6.4$ mV in the presence of 100 μM M-PTZ ($n = 5$). The steepness factor k is directly proportional to the relative electrical distance δ of the electrical field across the plasma membrane, which has to be sensed by PTZ and M-PTZ at their binding sites. Therefore, δ can be calculated according to the Woodhull model (Woodhull, 1973) $\delta = kRT/zF$ with R as the gas constant, T as the absolute temperature, z as the number of charges of the blocking molecule and F as the Faraday's constant. The electrical distance was calculated to be about $\delta = 0.4$ for both PTZ and M-PTZ from the inside.

3.3. Intracellularly applied M-PTZ

The effect of intracellularly applied M-PTZ was investigated for 100 μM M-PTZ in the pipette solution with N-sol as well as K-sol as extracellular solutions (Fig. 6). The membrane potential was clamped from −60 mV to +80 mV in 200 ms voltage steps with a 20 mV increment. Again, a time dependent block of IK currents was observed. The time constants measured at +80 mV were faster in N-sol ($\tau = 20.4 \pm 2.2$ ms, $n = 6$) compared to K-sol as bath solution ($\tau = 29.5 \pm 5$ ms, $n = 5$). The peak currents followed an almost linear current voltage relationship but steady state currents measured at the end of the 200 ms voltage pulse showed a slightly inward rectification. The ratio of steady state currents through peak currents was smaller at more positive than at negative membrane potentials, which again indicate a voltage dependent block. The voltage dependency was similar for experiments with N-sol ($k = 88.5 \pm 9.4$ mV) and K-sol ($k = 67 \pm 16$ mV) as well as to that one, obtained from the excised inside out patch clamp experiments. The E_{50} value in K-sol ($E_{50} = 102 \pm 18$ mV) was found to be shifted towards more positive membrane potentials compared to the one in N-sol ($E_{50} = 88 \pm 9$ mV) as extracellular solution. The strongly

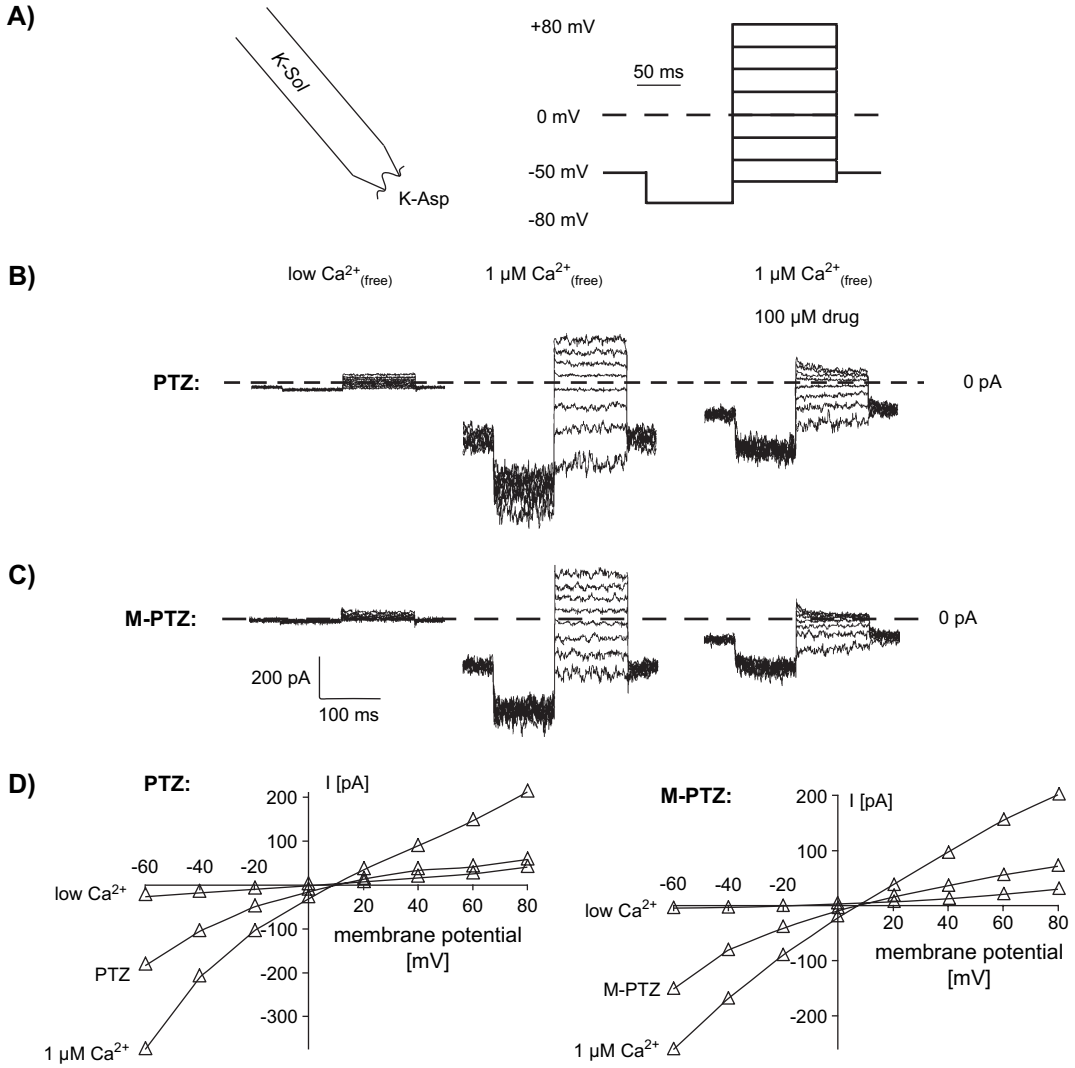

Fig. 4. Voltage dependent block of promethazine (PTZ) and methyl-promethazine (M-PTZ) block of IK currents in excised inside out patch clamp experiments using voltage steps. (A) Pipettes were filled with K-sol. The intracellular side of the excised patch was exposed to K-Asp solution. The excised inside out configuration and the voltage clamp protocol are shown. Representative experiments for PTZ (B) and M-PTZ (C) are shown. Currents with K-Asp containing low concentrations of free Ca^{2+} (low Ca^{2+}) were assumed as background currents. IK currents were activated by exchanging the K-Asp solution with 1 µM free Ca^{2+} (1 µM Ca^{2+}). Currents in the presence of K-Asp containing 1 µM free Ca^{2+} and 100 µM of PTZ (B) and with 100 µM M-PTZ (C) are shown. (D) Steady state currents for the experiments shown in (B) and (C) were measured at the end of the voltage step and were plotted against the membrane potential for experiments with PTZ (on the left hand side) and for experiments with M-PTZ (on the right hand side) with currents in the presence of K-Asp containing low concentrations of free Ca^{2+} assigned as low Ca^{2+}, currents measured in the presence of K-Asp with 1 µM free Ca^{2+} as 1 µM free Ca^{2+} and those with K-Asp 1 µM Ca^{2+} containing 100 µM PTZ (assigned as PTZ) and those containing 100 µM M-PTZ (assigned as M-PTZ).

positive E_{50} values obtained in the whole cell experiments showed that the potency to block IK channels of intracellularly applied M-PTZ is strongly reduced to that one observed in the excised inside out patch clamp experiments.

4. Discussion

Tricyclic antidepressants and neuroleptics are known to affect ionic transport mechanisms across the cell membrane through many mechanisms. Recent data showed that these compounds directly interacted with potassium channels and were therefore able to modulate different kinds of potassium currents. Phenothiazines such like PTZ were reported to block Ca^{2+}-activated potassium channels so called SK3 channels with IC_{50}s ranging from 31 µM for PTZ to 48 µM for trifluoperazine (Terstappen et al., 2001). Furthermore, phenothiazines have been shown to be able to displace apamin from its binding site in SK3 channels (Terstappen et al., 2001). Since apamin is known as a peptidic SK channel blocker which binds to the extracellular pore region, Terstappen et al. (2001) suggested a competitive mechanism for the apamin displacement by phenothiazines and hypothesized an extracellular phenothiazine binding site in SK channels.

Here we present that intermediate conductance Ca^{2+}-activated potassium channels are also blocked by PTZ. The IC_{50}-values, which could be calculated from the whole cell patch clamp experiments were similar to those IC_{50}-values

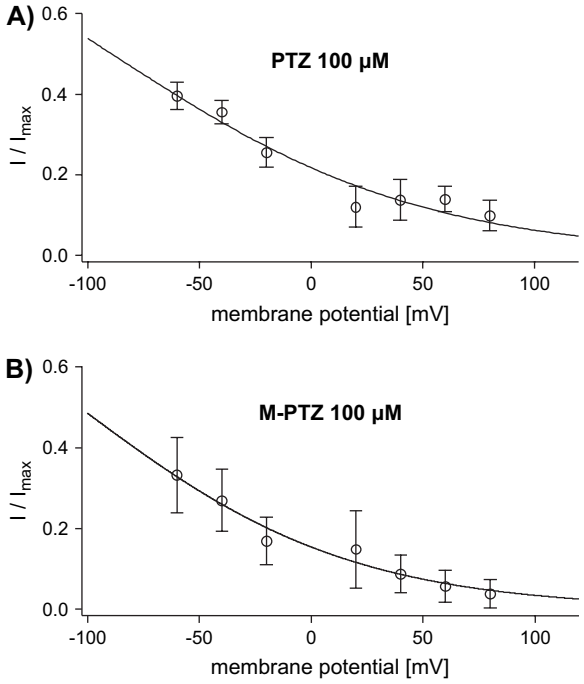

Fig. 5. Voltage dependence of promethazine (PTZ) and methyl-promethazine (M-PTZ) block of currents through IK channels. Currents measured in excised inside out patch clamp experiments are shown in Fig. 4. Background steady state currents measured with K-Asp with low Ca^{2+} were subtracted from steady state currents measured with K-Asp with 1 µM free Ca^{2+} with and without PTZ or M-PTZ. Relative steady state currents were calculated according to I/I_{max}, with I_{max} as steady state currents measured in the presence of 1 µM free Ca^{2+} without compound after background subtraction and I as steady state currents measured in the presence of compound after background subtraction. Data points were plotted as mean ± SEM ($n = 4$) against the membrane potential in (A) for experiments with 100 µM of PTZ and (B) for experiments with 100 µM of M-PTZ. Curves through the data points were calculated according to Boltzmann equation $I/I_{max} = 1/(1 + \exp((E - E_{50})/k))$ with E as the membrane potential, E_{50} as the potential at half maximal block and k as steepness factors of the curves.

obtained for the human SK3 channel of 31 µM (Terstappen et al., 2001). However, the fact that lowering the outer pH_o from 7.4 to 6.2 had nearly no effect on the IC_{50}-value for the PTZ blockage of IK channels made it likely that bound PTZ did not interfere with positive charges located within the outer pore region at H236 in human IK channels. There are two simple explanations for this observation. One might be that PTZ binds as an uncharged molecule and therefore it does not interfere electrostatically with charges located in the vicinity of its binding site. The other possibility might be that PTZ binds far away from H236 and therefore cannot sense any electrostatical repellence caused by a protonation of H236.

The membrane impermeable PTZ analogue, M-PTZ, blocks IK channels with a strongly reduced potency compared to PTZ, when it is applied to the extracellular solution. Possible explanations for this observation might be that this particular chemical modification of PTZ led to its strongly reduced blocking potency. Another explanation might be that an intracellular binding site is not accessible for the membrane impermeable M-PTZ.

Excised inside out patch clamp experiments were performed in order to get a better accessibility to the intracellular side of IK channels. In these experiments we applied PTZ as well as M-PTZ to the intracellular side of IK channels. In contrast to whole cell experiments, we observed that both compounds block IK channels with the same potency. This indicated that the accessibility of the intracellular side of IK channels is essential for IK channel blockage by either PTZ and M-PTZ rather than an altered binding affinity of M-PTZ due to its structural difference compared with PTZ.

In order to investigate the voltage dependency of the PTZ block of IK channels we performed excised inside out patch clamp experiments. During these experiments, the pH of the bath solution was 7.2. PTZ and has a pK_a value of 9.4 and therefore most of the PTZ molecules are protonated. We observed a voltage dependent blockage of IK channels by PTZ, which is strongest at positive membrane potentials. From these observations we concluded that PTZ binds as a charged molecule to IK channels. Since PTZ is a positively charged molecule at a given pH of 7.2, it seems that PTZ enters the channel from the intracellular side of the membrane and when bound senses some of the membrane spanning electrical field. From the electrical distance we can conclude that PTZ senses approximately 30–40% of the electrical field, which is applied to the membrane. Such a voltage dependency of the block by phenothiazines has not been described for Ca^{2+}-activated SK channels (Terstappen et al., 2001; Carignani and Corsi, 2002). Contrary to this, the phenothiazine derivative trifluoperazin was reported to block $K_v1.3$ channels (Teisseyre and Michalak, 2003) in a voltage dependent manner. In this case the blockage was strongest at depolarizing membrane potentials. Taken together, the voltage dependency of the PTZ block of IK channels seems to be more similar to the block of K_v channels (Teisseyre and Michalak, 2003) by trifluoperazine than to that one described for SK channels (Terstappen et al., 2001).

PTZ was also able to block IK channels, even when it was added to the extracellular bath solution in whole cell experiments. This seems to contradict the conclusion that PTZ binds from the intracellular side. However, it was already reported that the phenothiazine derivative chlorpromazine is able to block potassium channels by binding from the intracellular side, even when it is applied to extracellular solution. This observation led to the conclusion that phenothiazines can permeate the cell membrane (Ito et al., 2002). Therefore, we presume that PTZ is able to permeate the cell membrane in whole cell patch clamp experiments and can bind to a binding site, which is accessible from the intracellular side of the channel. A similar blocking mechanism was already reported for the blockage of $K_v1.3$ channels by verapamil (Rauer and Grissmer, 1996). Such a mechanism would result in an effective cytosolic PTZ concentration, which can differ from the extracellular concentration and can be influenced by many different effects, like the membrane permeability or the ability of the cell to buffer PTZ. The concentration dependence of the PTZ block was estimated in whole cell experiments. However, a sufficient fit of the concentration response curves would only

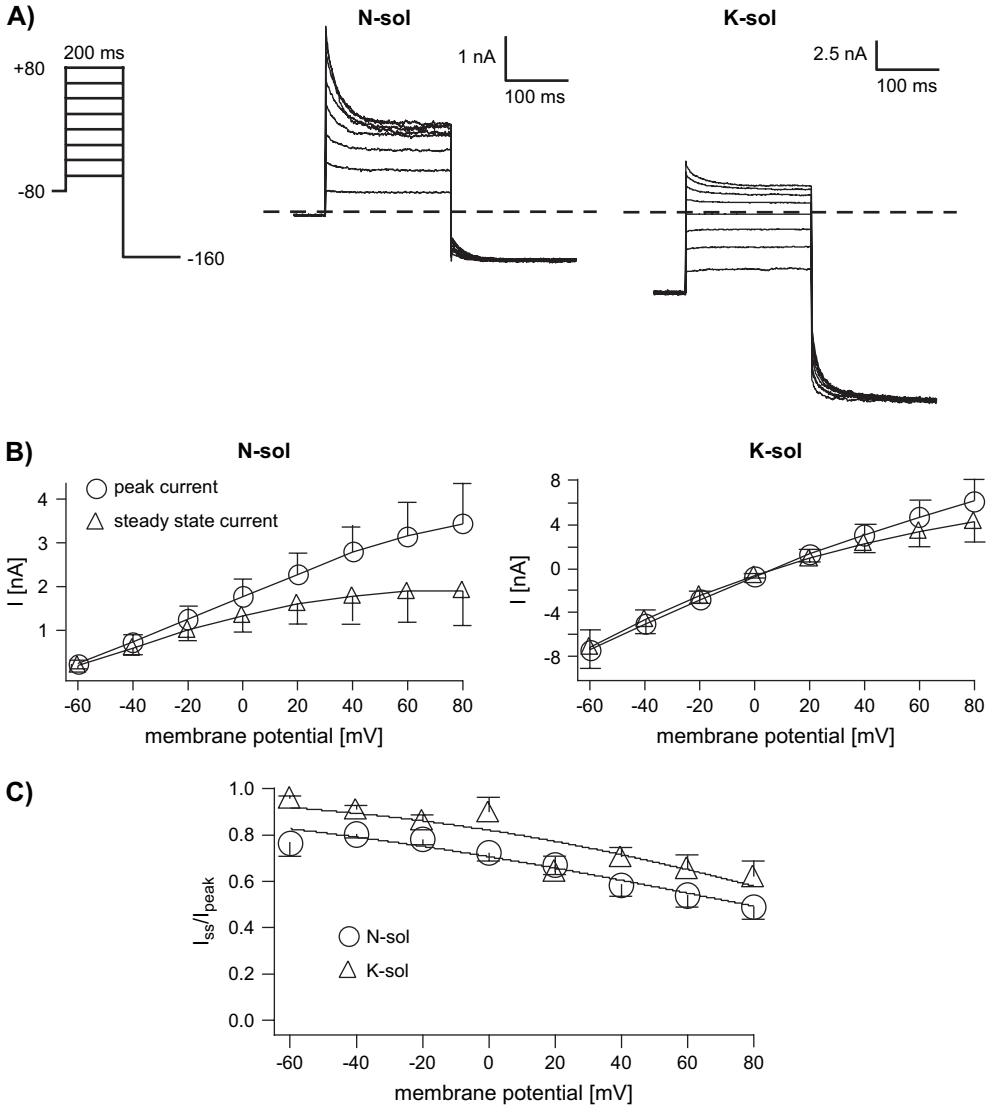

Fig. 6. Effect of intracellularly applied M-PTZ. M-PTZ (100 μM) was added to the pipette solution. (A) The membrane potential was stepped from −60 mV to +80 mV with a 20 mV increment for 200 ms followed by a 200 ms hyperpolarizing step to −160 mV. The potential was kept at −80 mV for 10 s in-between voltage steps. Representative whole cell currents with N-sol and K-sol as extracellular solutions are shown. (B) Peak currents and steady state currents measured in N-sol and K-sol were plotted against the membrane potential. (C) The ratio between peak currents (I_{peak}) and steady state currents ($I_{steady\ state}$) was calculated and plotted against the membrane potentials (data points are given as mean values ± SEM for at least five experiments). Curves represent best fits through data points according to $I_{steady\ state}/I_{peak} = 1/(1 + \exp((E - E_{50})/k))$ with E as the membrane potential, E_{50} as the potential at half maximal block and k as steepness factors of the curves.

be achieved with a Hill coefficient below one. Such a Hill coefficient cannot be explained by a certain stoichiometry of the blocking reaction. It is much more likely that this reduction of the curve's steepness results from the voltage dependence of PTZ block in addition to the effects of the extra and intracellular environments on the effective PTZ concentration.

Trifluoperazine, a phenothiazine derivative, is known to antagonize calmodulin by directly binding to it (Cook et al., 1994; Vandonselaar et al., 1994). Calmodulin has been shown to be an accessory subunit of IK (Fanger et al., 1999) and SK channels (Bruening-Wright et al., 2002; Keen et al., 1999; Schumacher et al., 2001). A commonly accepted model for IK/SK channel activation is that calmodulin acts as a Ca^{2+} sensor and mediates the activation of IK and SK channels (Bruening-Wright et al., 2002; Fanger et al., 1999; Keen et al., 1999; Schumacher et al., 2001). Therefore the question arises if phenothiazines can prevent the activation of IK and SK channels via antagonizing of calmodulin. Previous studies addressing this question came to contrary results. The blockage of IK whole cell currents by the calmodulin antagonizing phenothiazine derivative trifluoperazine was already been described (Khanna et al., 1999). Others (Del Carlo et al., 2002) were not able to reproduce these results. In their study IK currents were elicited by pulsing the membrane potentials to −80 mV. Under these conditions 10 μM trifluoperazine had no effect on IK channels. We observed that PTZ inhibits IK channels in a voltage dependent manner. From our excised inside out patch clamp experiments we observed at a PTZ concentration of 100 μM and a membrane potential of −60 mV a reduction of IK currents of roughly 55%. If a voltage

dependent trifluoperazine block would be presumed similar to that one we observed for the PTZ block, someone would expect that 10 μM trifluoperazine applied at a membrane potential of −80 mV would reduce IK currents to a much lower amount. Under these conditions, the trifluoperazine effect on IK currents might be that low that it would not be detected in the experimental approach, which was carried out by Del Carlo et al. (2002). This could explain the contrary results of Del Carlo et al. (2002) and Khanna et al. (1999). PTZ as well as other derivatives of phenothiazine were reported to block SK channels through binding to an extracellular binding site, because these compounds are reported to displace apamin from its binding site in SK channels. The apamin displacement is reported to be the consequence of a direct interaction of phenothiazines to SK channels. A blockage of SK channels by these tricyclic compounds via antagonization of calmodulin would not displace apamin from its binding site. In addition the voltage dependency of the PTZ block of IK channels observed in our experiments indicates that PTZ binds to a binding site which allows its intrusion into the membrane spanning electrical field from the inside. This assumption is underlined by mainly two observations. First, the time constant of the blocking reaction increases with the elevation of extracellular K^+ concentrations and second, the E_{50} value shifted towards more positive membrane potentials with increasing extracellular K^+ concentrations, whereas the voltage dependency remains unaffected by extracellular K^+. This indicates to us that M-PTZ bound to its binding site can sense the occupancy of K^+ at the extracellular part of the conducting path way, as it was described for the intracellular Ba^{2+} block of BK-channels (Neyton and Miller, 1988a,b). Such a mechanism makes a direct interaction between the blocker and the channel molecule necessary. Again, a blocking mechanism via calmodulin antagonization would presumably not be voltage dependent but independent from extracellular K^+ concentrations. Taken together, our results in combination with previously published results strongly suggest that PTZ blocks IK channels via a direct interaction with the channel molecule rather than via the antagonization of calmodulin.

It was recently reported that chlorpromazine, a derivative of PTZ, is able to prevent the short circuit current (I_{sc}) through Calu3 cell layers (Ito et al., 2002), which is partially forced by the activation of a charybdotoxin sensitive Ca^{2+}-activated potassium current. The IC_{50}-value for the I_{sc} inhibition by chlorpromazine was found to be dependent from the side of the Calu3 cell layer to which chlorpromazine was applied. The IC_{50}-value found for chlorpromazine applied to the basolateral side of the cell layer (Ito et al., 2002) was identical to that one, which was observed in the present study for the PTZ blockage of IK channels in whole cell experiments. A Ca^{2+}-activated and charybdotoxin sensitive current through the basolateral membrane of epithelial cells was shown to be carried by IK channels (Devor et al., 1999). Therefore, our results suggest that tricyclic antidepressants are able to block IK channels in epithelial cells by binding directly to a binding site within the conducting pathway of IK channels. This binding site is predominantly accessible from intracellular. Such a blocking mechanism of IK channels would affect transepithelial sodium and potassium chloride secretion, as it was already discussed (Ito et al., 2002). A single application of 50 mg promethazine results in a plasma concentration of up to 19.3 ng/ml (Strenkoski-Nix et al., 2000), which corresponds to roughly 1 μM. A similar plasma concentration is reported for the treatment with a medial dosage of 600 mg per day of chlorpromazine. This is shown to result in a plasma concentration of roughly 1–2 μM (Amaral et al., 2001). Therefore, it seems that drug concentrations close to the IC_{50} of 30 μM as it was observed in our whole cell experiments might never be reached during therapeutical treatment. However, it is reported that in lung tissues basic ambiphilic substances can reach up to 25-fold higher plasma concentration (Daniel, 2003; Jonkman et al., 1983; Karson et al., 1993; Muller, 1996). Such an accumulation, which can be presumed for other basic ambiphilic substances like phenothiazines, would lead to locally elevated concentrations close to K_d values of 30 μM and therefore, might cause toxic side effects.

In conclusion, we showed that human IK channels stably expressed in HEK 293 cells were blocked by PTZ in a voltage dependent manner. From the voltage dependency we concluded that PTZ interacts directly with the channel molecule at a binding site, which is accessible from intracellular and that PTZ senses approximately 40% of the membrane spanning electrical field. Furthermore, we showed that the M-PTZ block of IK channels interferes with extracellular K^+. This finding implies a binding site, which most likely enables PTZ and M-PTZ partially entering the conducting pathway of IK channels. Therefore, we excluded a blocking mechanism of IK channels by PTZ via the antagonization of calmodulin. Our experiments support the hypothesis that the reported dysfunction of NaCl and KCl transport, which is shown to be partially caused by the inhibition of basolateral located Ca^{2+}-activated potassium channels might be caused by a direct interaction of phenothiazines with IK channels.

Acknowledgements

We would like to thank Prof. Daniel Devor (Pittsburgh, US) for providing us with the IK-expressing cell line. This project was supported by the Faculty of Medicine, University of Ulm (Project P. 770) and by the DFG (Gr 848/8-2).

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