Inhibition of $Ca_v3.1$ Channel by Silver Ions

P. Gromová*, M. Kurejová* and Ľ. Lacinová

Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia

Abstract. We have investigated the effects of AgCl and $AgNO_3$ on the $Ca_v3.1$ calcium channels stably expressed in the HEK 293 cells. Ca^{2+} was used as a charge carrier. Both forms of Ag^+ blocked the $Ca_v3.1$ channel and negatively shifted the I-V relations in a concentration-dependent manner. The inhibition of current amplitude by AgCl was voltage-dependent and increased with increasing amplitude of the depolarizing pulse. Furthermore, AgCl but not $AgNO_3$ accelerated the kinetics of current activation. No effect on current inactivation or steady-state inactivation of the channel was observed for AgCl or $AgNO_3$.

Key words: T-type calcium channel — $Ca_v3.1$ — Silver — AgCl — $AgNO_3$

Introduction

It is known that systemic exposure to heavy metals may affect physiological functions. One of the relatively common environmental contaminants is silver, which could be found in groundwater due to its common use in photoprocessing industry and in pesticides used for treatment of commercially grown flowers. Human toxicity of Ag^+ is low, yet they affect both the surface and intracellular ion channels. It has been shown that silver ions selectively influence Ca^{2+} release channels. The main interest has been focused on Ca^{2+} release channels in the sarcoplasmic reticulum (SR). Ag^+ induces Ca^{2+} release by binding to one or more sulfhydryls sites in the skeletal SR (Abramson et al. 1983; Salama and Abramson 1984) and cardiac SR (Prabhu and Salama 1990). The same effect of silver ions has been observed also on the HL-60 cells (Taguchi at al. 1991). This specific effect is referred to as Ag^+-induced calcium release.

Ag^+ induces transient contraction of intact skeletal muscle fibers followed by inhibition of excitation-contraction coupling (Oba and Hotta 1985). The authors suggested that development of Ag^+-induced tension is not associated with depolarization of the surface membrane but is more likely caused by specific action of

*Contributed equally to this work.

Correspondence to: Dr. Ľubica Lacinová, Institute of Molecular Physiology and Genetics Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava 37, Slovakia
E-mail: Lubica.Lacinova@savba.sk

Ag^+ on membrane proteins in the T-tubules. This mechanism was confirmed by the finding that Ag^+ binds to critical SH groups on the dihydropyridine receptor Ca^{2+} channel, thereby modifying the voltage sensor of the channel (Oba et al. 1992).

Ag^+ may also act as an effective inhibitor of potassium channel. Externally applied Ag^+ irreversibly blocked the strong inwardly rectifying K^+ channel by binding to the cysteine at position 149 in the H5 region (Dart et al. 1998).

It may be of interest whether Ag^+ also acts on other surface membrane ion channels. Interaction of Ag^+ with voltage-dependent calcium channels has not been studied yet. For our study, we have chosen the $Ca_v3.1$ low voltage-activated calcium channel. A wide range of responses of this channel to divalent (Lee et al. 1999; Lacinova et al. 2000; Harasztosi et al. 2001) and trivalent (Beedle et al. 2002) inorganic ions has been described. However, no information about interaction with monovalent metal ions has been reported so far. Investigation of possible effects of silver ions may be interesting also for methodological reasons. The ground electrode in patch clamp installations is often made of silver wire or silver pellet. Unless it is separated from the experimental chamber with an agar-bridge, Ag^+ could contaminate bath solution during experiments and cause unwanted effects.

We studied the modulation of $Ca_v3.1$ current by different concentrations of two forms of silver, $AgNO_3$ and AgCl, using $Ca_v3.1$ channels expressed in the HEK 293 cell line (Klugbauer et al. 1999). Our results show a complex yet reversible modulation of calcium current by silver ions at concentrations that may result from random contamination by a silver bath electrode.

Materials and Methods

All experiments were carried out on HEK 293 cells stably expressing the $Ca_v3.1$ subunit of T-type Ca^{2+} channel (CACNA1G). This gene has access number AJ012569 in the EMBL database. Construction of the expression vector has been described previously (Klugbauer et al. 1999). The cells were grown in minimal Eagle's medium with Earle's salts, containing 10% (v/v) fetal calf serum, 100 U/ml penicillin – streptomycin, and 0.04% (w/v) G418 (PAA Laboratories GmbH, Austria) at 37°C in a humidified atmosphere of air/CO_2 (95 : 5). The cells were harvested from their culture flasks by trypsinization and plated out 24–48 h before use in electrophysiological experiments.

Ionic currents were recorded in whole cell configuration of the patch clamp method using the EPC-10 patch clamp amplifier (HEKA Electronic, Lambrecht, Germany). The extracellular solution contained (mmol/l): N-methyl-D-glucamine 160; $CaCl_2$ 2; $MgCl_2$ 1; HEPES 10; and glucose 5; pH 7.4 (HCl). The intracellular solution contained (mmol/l): CsCl 130; EGTA 1; $MgCl_2$ 1; TEA-Cl 10; HEPES 10; and Na-ATP 5; pH 7.4 (CsOH). All drugs and chemicals were obtained from Sigma. Osmolarity of the internal solution was approximately 300 mOsm. Osmolarity of the external solution was adjusted by glucose to values 2–3 mOsm below that of the internal solution. A 100 mmol/l $AgNO_3$ stock solution prepared from deionized water was stored at 4°C and diluted in bath solution prior to the experiment.

Deionized water was made by Millipore Elix® Water Purification Systems. AgCl was diluted freshly in bath solution. Extracellular solutions were exchanged by a gravity-driven flow system with manually controlled valves.

Patch-clamp pipettes were manufactured from borosilicate glass with input resistance ranging from 1.6 to 2.5 MΩ. The capacitance of individual cells were ranged between 10 and 22 pF. Series resistance were ranged from 2.5 to 6 MΩ. The bath was grounded using an AgCl pellet connected to the experimental chamber through an agar bridge.

The holding potential (HP) in all experiments was −100 mV. The effect of the salts was investigated using 40 ms long depolarizing pulses applied from the HP to −30 mV with frequency of 0.2 Hz. Current-voltage relations were measured by a series of 40 ms long depolarizing pulses applied from the HP to membrane potentials between −70 and +70 mV.

Data were recorded using a HEKA Pulse 8.5 and analyzed with HEKA Pulsefit 8.5 software. The data were sampled at 20 kHz. Capacity transients and series resistance were compensated by procedures built in the EPC-10 amplifier. Leak current and capacity transients were subtracted using the P/4 procedure. Significance of the observed effects of Ag^+ was assessed by non-paired Student's *t*-test. Values of $p < 0.05$ were considered significant. All experimental values are expressed as mean ± SEM.

Results and Discussion

The aim of our study was to investigate the effects of Ag^+ on currents carried by Ca^{2+} through the $Ca_v3.1$ channel permanently expressed in the HEK 293 cells. A nearly physiological calcium concentration of 2 mmol/l was used. Two different silver salts were tested: AgCl and $AgNO_3$. AgCl dissolves relatively poorly in water, with maximum soluble concentration of 15 μmol/l. This form of silver may arise spontaneously in experimental chamber due to interaction between silver ground electrode and experimental solution. $AgNO_3$ solves readily in water, but when used in higher concentrations, Ag^+ may precipitate in the form of AgCl if Cl^- are present in the solution. In our experiments, we used a Cl^--containing bath solution to mimic the most usual experimental conditions.

Block of $Ca_v3.1$ channel by AgCl

Figure 1 shows effects of AgCl on the $Ca_v3.1$ channel. Inward calcium current was activated by a series of 40 ms long pulses from the HP of −100 mV to −30 mV (peak of I-V). The inhibition of current amplitude had a rapid onset and reversed readily upon washout (Figure 1A). In addition to the current inhibition, I-V relation was shifted in the presence of AgCl in hyperpolarizing direction (Figure 1B). To quantify the shifts, voltage dependence of channel conductance was calculated, normalized to the maximum, and fitted by the Boltzmann equation (Figure 1C). Under the control conditions, the half-maximum inactivation voltage was -42.6 ± 0.6 mV ($n = 16$) with the slope of 3.99 ± 0.14 mV. In the presence of 1 μmol/l AgCl, these

values were changed to -44.8 ± 0.5 mV ($n = 6; p < 0.05$ *vs.* control) and 3.67 ± 0.25, and in the presence of 10 μmol/l AgCl to -49.2 ± 0.6 mV ($n = 13; p < 0.001$ *vs.* control) and 4.73 ± 0.12 ($p < 0.001$ *vs.* control).

The effect of AgCl on the kinetics of current activation and inactivation was analyzed by fitting individual current traces measured during the I-V relation protocol by the m2h form of the Hodgkin-Huxley equation. AgCl had no significant effect on the time constant of current inactivation (results not shown), but significantly and reversibly accelerated current activation (see inset in Figure 1A).

To illustrate the voltage dependence of the AgCl block, inhibition of current amplitude was evaluated for each depolarizing potential, that activated a measurable inward current. At 1 μmol/l concentration of AgCl, the current block was essentially voltage independent. At 10 μmol/l, the inhibition increased with increasing amplitude of the depolarizing pulse (Figure 1D).

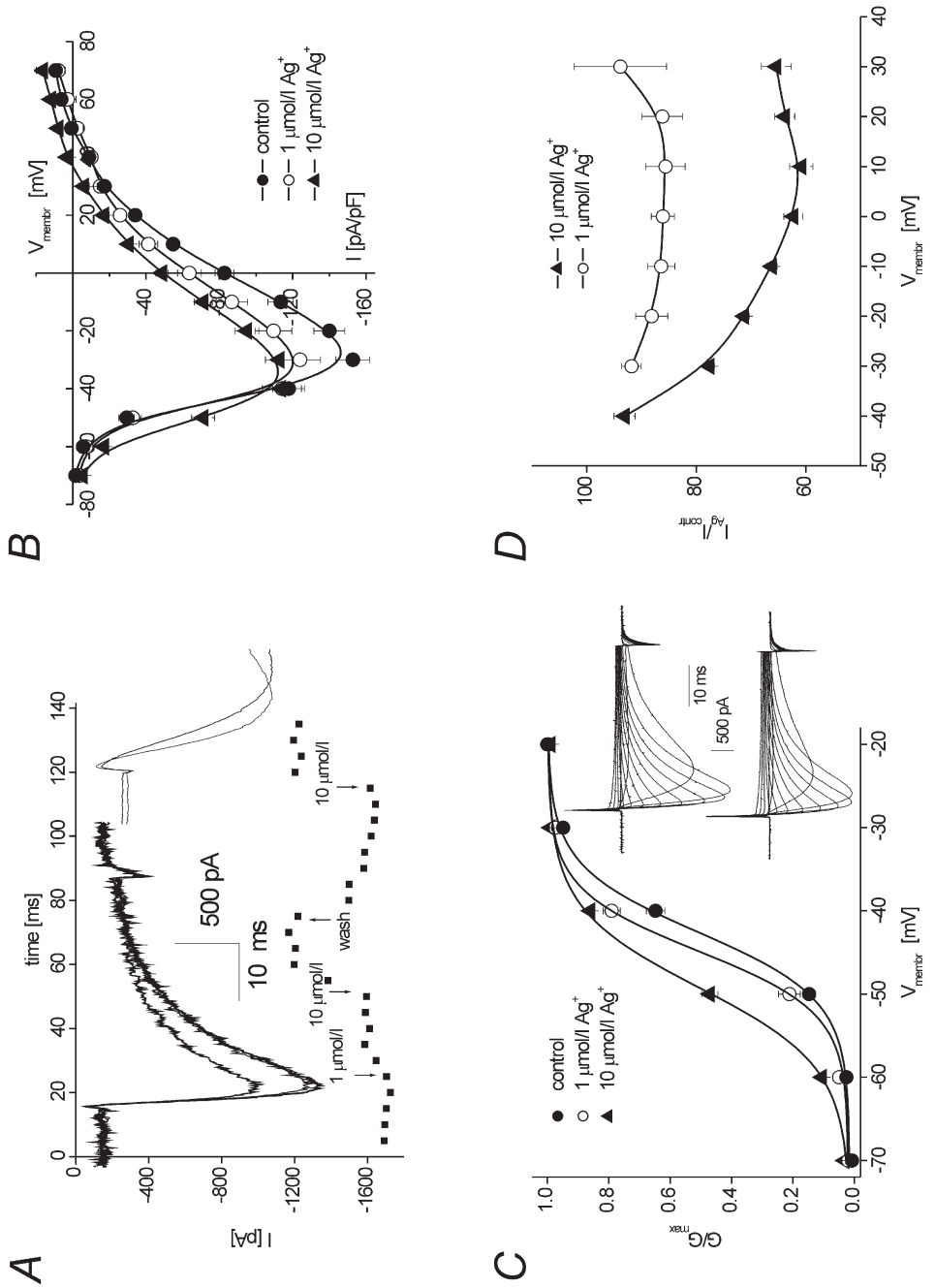
Steady-state inactivation of the $Ca_v3.1$ channel was not significantly affected by Ag^+. Half-maximum current inactivation was reached at -92.0 ± 1.2 mV ($n = 6$) and -93.6 ± 2.3 mV ($n = 4$), with slopes 4.74 ± 0.27 and 4.27 ± 0.46 under the control conditions and in the presence of 10 μmol/l AgCl, respectively (data not shown).

Block of Ca_v 3.1 channel by $AgNO_3$

To determine the effects of $AgNO_3$ on the T-type calcium channel, a concentration range of 0.1–300 μmol/l was used. Individual concentrations of $AgNO_3$ were applied sequentially. The calcium current was inhibited by Ag^+ in a concentration-dependent manner, with blocking efficiency reaching $38.5 \pm 3.5\,\%$ at 300 μmol/l (Figure 2A). The $AgNO_3$ block was fast and reversible. Representative traces of current records at concentrations of 1, 10, and 100 μmol/l are shown in the inset in order of decreasing current amplitudes.

I-V relations at the control conditions and at 1, 10, and 30 μmol/l of $AgNO_3$

Figure 1. Inhibition of T-type calcium channel current by AgCl. **A.** Time-course of the peak amplitude of the T-type whole cell current in response to a 40 ms long pulse from the HP to -30 mV. Arrows indicate the application of AgCl (1 and 10 μmol/l) followed by washout. Current traces in the absence and presence of 1 and 10 μmol/l AgCl in order of decreasing amplitudes are shown in the left inset. The right inset compares the kinetics of current activation under the control condition (black) and in the presence of 10 μmol/l AgCl (gray). The traces were scaled to the same amplitude to facilitate comparison. **B.** Voltage dependence of current densities (I-V) measured under the control conditions (●) and in the presence of 1 (○) and 10 (▲) μmol/l AgCl. **C.** Individual I-V relations were converted into conductances and normalized to the maximum conductance. Control conditions (●), 1 μmol/l AgCl (○), 10 μmol/l AgCl (▲). Solid lines represent fits of experimental data by the Boltzmann equation. The current traces recorded during the I-V protocol under the control conditions and in the presence of 10 μmol/l AgCl are shown in the inset. **D.** Voltage dependence of current inhibition by 1 (○) and 10 (▲) μmol/l AgCl evaluated from the I-V relations for depolarizations that activated a measurable inward current.


A
time [ms]
I [pA]
500 pA
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1 µmol/l
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wash
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B
V membr [mV]
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control
1 µmol/l Ag+
10 µmol/l Ag+
C
G/G max
control
1 µmol/l Ag+
10 µmol/l Ag+
10 ms
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1 µmol/l Ag+
V membr [mV]

in bath solution are shown in Figure 2B. $AgNO_3$ not only inhibited calcium current amplitude but also caused a shift of the I-V relations in hyperpolarizing direction. To quantify the shifts, the channel conductance was calculated, normalized to the maximum, and fitted by the Boltzmann equation (Figure 2C). Under the control conditions, the half-maximal inactivation voltage was -44.4 ± 2.1 mV ($n = 5$) with the slope of 4.08 ± 0.48 mV. In the presence of 1 μmol/l $AgNO_3$ these values changed to -45.8 ± 0.72 ($n = 4$) and 3.32 ± 0.38 mV, in the presence of 10 μmol/l to -49.1 ± 1.2 mV ($n = 4$) with the slope of 3.1 ± 0.27 and to -51.4 ± 0.6 mV ($n = 2$) with the slope of 3.79 ± 0.66 at 30 μmol/l concentration of $AgNO_3$. In contrast to AgCl, $AgNO_3$ did not affect significantly current kinetics (data not shown).

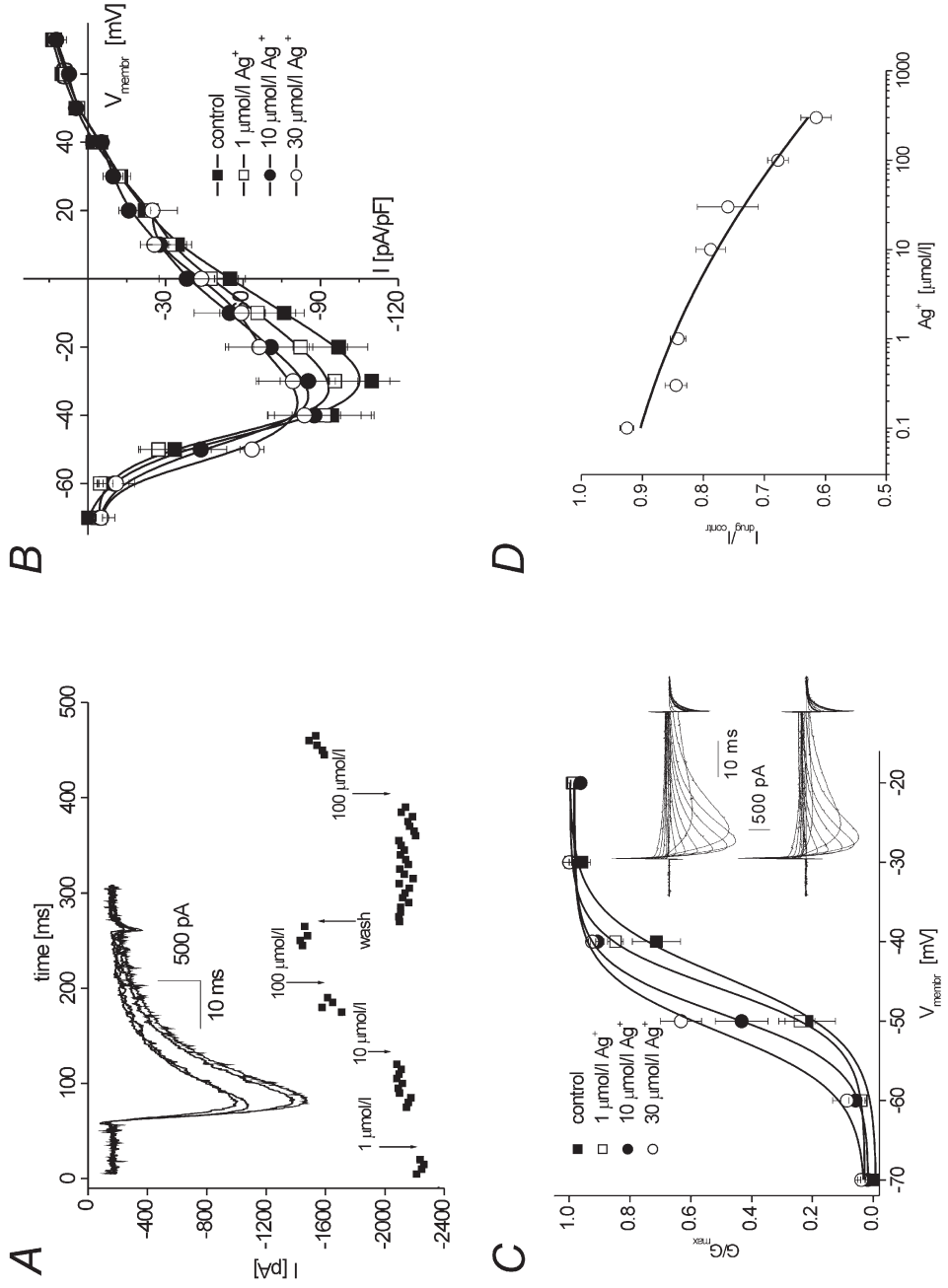
The block of current amplitude by $AgNO_3$ was moderately voltage-dependent. The voltage dependence started at the concentration of 1 μmol/l. At all concentrations, the current block increased monotonically with increasing depolarization (data not shown).

The concentration dependence of current amplitude inhibition (Figure 2D) was fitted by the Hill equation. The IC_{50} value obtained by extrapolation of experimental data was 3.5 mmol/l.

In conclusion, our data showed quite complex modulation of current through the $Ca_v3.1$ channel. The underlying mechanism differs from that described for the Kir2.1 channel (Dart et al. 1998). An interaction of Ag^+ with cysteine residues can be excluded, as there are no cysteines in the pore region of the $Ca_v3.1$ channel and the mechanism of the channel block differs principally in its kinetics from the effect described by Dart and coauthors for interaction of Ag^+ with the cysteine residues of the Kir2.1 channel.

Modulation of current properties was dependent on form in which Ag^+ was delivered. Water-soluble $AgNO_3$ affects the $Ca_v3.1$ channel in a less complex manner. Inhibition of current amplitude was not voltage-dependent and current kinetics

Figure 2. Inhibition of T-type calcium channel current by $AgNO_3$. **A.** Time-course of the peak amplitude of the T-type whole cell current in response to a 40 ms long pulse from the HP to -30 mV. Arrows indicate the application of $AgNO_3$ (1, 10 and 30 μmol/l) followed by washout. Breaks between the applications of individual salt concentrations were used for measurements of I-V relations. Current traces in the absence and presence of 1, 10 and 30 μmol/l of $AgNO_3$ in order of decreasing amplitudes are shown in the inset. **B.** Averaged current density-voltage relations recorded under the control conditions (●) and in the presence of 1 (○), 10 (■) and 30 (□) μmol/l $AgNO_3$. **C.** Conductance-voltage relations calculated from individual I-V relations normalized to the maximum conductance at the control conditions (●) and in the presence of 1 (○), 10 (■) and 30 (□) μmol/l $AgNO_3$. Solid lines represent fits of experimental data by the Boltzmann equation. The current traces recorded during the I-V protocol under the control conditions and at the 10 μmol/l concentration of $AgNO_3$ are shown in the upper and lower inset, respectively. **D.** Dose-response relationship for the $AgNO_3$ block of calcium currents as seen in the $Ca_v3.1$ channels. Percentage of unblocked peak current is plotted against the salt concentration. Solid line represents the Hill equation with an IC_{50} of 3.5 mmol/l and Hill coefficient of 0.21.


A
time [ms]
I [pA]
500 pA
10 ms
100 µmol/l
wash
10 µmol/l
1 µmol/l
B
V_membr [mV]
I [pA/pF]
control
1 µmol/l Ag+
10 µmol/l Ag+
30 µmol/l Ag+
C
G/G_max
V_membr [mV]
control
1 µmol/l Ag+
10 µmol/l Ag+
30 µmol/l Ag+
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was not altered. Interaction between channel and silver ions had low affinity and milimolar concentrations would be necessary for inhibition of 50 % of current amplitude. Extent of current inhibition by less soluble AgCl was comparable at peak of I-V relation, but increased significantly with increasing depolarization and altered kinetics of individual current traces.

The source of difference between the effects of AgCl and $AgNO_3$ is unknown. We can exclude Cl^- as a possible agent causing the voltage dependence of channel inhibition and/or acceleration of current activation because its maximum concentration represents less than 0.1 % of the total Cl^- concentration in the bath solution. One could speculate that a part of Ag^+ released from $AgNO_3$ molecules precipitates in the form of AgCl in Cl^--based bath solution and, because of this decrease in the actual concentration, $AgNO_3$ is less efficient in the interaction with the channel than AgCl. If this were the case, the extent of amplitude inhibition should be significantly smaller, but no such effect was observed.

The leftward shift in voltage dependence of channel activation and the acceleration of activation kinetics suggest a direct interaction of Ag^+ with the voltage sensor of the $Ca_v3.1$ channel. However, no final conclusion in this respect can be drawn from our experiments. To prove such a mechanism, measurement of the effect of Ag^+ on gating current would be necessary.

The observed effects of AgCl appeared at concentrations close to the upper limit of it's solubility in water-based solution. Nevertheless, when an Ag/AgCl electrode is dipped in the bath solution for a long time, contaminations in this concentration range may occur. As a result, in the course of an electrophysiological experiment a moderate decrease in current amplitude and/or an acceleration of current activation may occur. This artifact may be superposed on other effects investigated in the experiment, and may influence their interpretation if the observed phenomena are minor to moderate in their extent. The onset of the effect of Ag^+ released from Ag/AgCl electrodes directly dipped in bath solution depends on several factors, including the composition of the bath solution, in particular the concentration of Cl^-, the geometry of experimental set-up, such as the distance of the measured cell from the grounding electrode and/or the presence or absence of continuous solution flow that could counteract diffusion of Ag^+ towards the measured cell. Due to the complexity of these factors, it is impossible to predict the onset of the effect of silver ions on Ca^{2+} current. Anyway, it is recommendable to avoid a direct contact of silver electrode with bath solution.

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