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ARTICLE

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The influence of zinc on the modulatory effect of sphingosylphosphorylcholine on Kv1.3 channels in human T lymphocytes

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Abstract In the present study, the whole-cell patch-clamp technique was applied to investigate the influence of co-application of zinc ions and sphingosylphosphorylcholine (SPC) on the SPC-induced shift of the activation midpoint and slowing of activation kinetics of Kv1.3 channels in human T lymphocytes. The results obtained provided evidence that the effects exerted by SPC and Zn were not additive. The shift was significantly diminished in a concentration-dependent manner upon co-application of 10 μM SPC and Zn in the concentration range 10–300 μM. However, the shift was not abolished in the presence of 100 and 300 μM of Zn co-applied with SPC. It was shown that the extent of the shift upon SPC and Zn co-application was similar to the shift observed for Zn applied without SPC. The slowing of the activation kinetics was also diminished upon SPC and Zn co-application; however, no clear dependence on concentration was observed. Moreover, the slowing was not abolished in the presence of 100 and 300 μM of Zn. It was shown that the slowing of the activation kinetics upon Zn and SPC co-application was primarily due to the effect exerted by SPC. The steepness of the voltage dependence of steady-state activation of the channels was not changed upon SPC and Zn co-application. Possible mechanisms underlying the observed phenomena and their possible physiological significance are discussed.

Keywords Patch clamp · Potassium channel · Sphingosylphosphoryl-choline · T lymphocyte · Zinc

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Abbreviations 4-AP: 4-aminopyridine · SPC: sphingosylphosphorylcholine · TL: human T lymphocyte

Introduction

Human T lymphocytes (TL) are relatively small (6–8 μm in diameter) and electrically non-excitable cells. Nevertheless, many different ion channel types are expressed in human TL cell membranes (Gallin 1991). Among them are potassium channels: voltage gated Kv1.3 channels, voltage-gated channels of unknown type, calcium-activated IKCa1 channels (expressed abundantly only in activated TL), SKCa2 channels (only in T cell line Jurkat) and voltage-independent channels gated by intracellular cAMP and probably by the membrane stretch (Teisseyre 2001).

Potassium channels play an important role in setting the cell resting membrane potential, cell mitogenesis, apoptosis and volume regulation (Cahalan et al. 2001). Because blockade of potassium channels can inhibit mitogenesis in its early stage, several synthetic blockers of potassium channels are being clinically tested as immunosuppressants (Chandy et al. 2001).

The predominant potassium channels in resting (not activated) TL are Kv1.3 channels (Cahalan et al. 2001; Chandy et al. 2001; Teisseyre et al. 2002). The channel activity is affected by a number of chemically unrelated compounds (Cahalan et al. 2001; Chandy et al. 2001). One of them is zinc ions (Zn), which are a well-known endogenous modulator of many types of ion channels (Harrison and Gibbons 1994). Results obtained recently in our laboratory showed that application of Zn ions at micromolar concentrations caused a concentration-dependent shift of the channel activation and inactivation midpoints by about 30 mV towards positive membrane potentials (Teisseyre and Mozrzymas 2002). This was accompanied by a pronounced decrease of the current activation rate, whereas the inactivation and deactivation rates remained unchanged. Moreover, the

current amplitude at +60 mV was reduced upon Zn application in a concentration-dependent manner. It is suggested that Zn binds to two distinct binding sites: binding to one site shifts the channel activation and inactivation midpoints and decreases the current activation rate, whereas binding to the other site reduces the current amplitude (Teisseyre and Mozrzymas 2002). The effect exerted by Zn on the channels may be related both to the stimulation of TL proliferation in vitro upon application of 200 μM Zn and inhibition of the proliferation at 800 μM concentration (Reardon and Lukas 1987). In other sets of experiments, possible modulatory effects of membrane lipid metabolites on the Kv1.3 channel activity were studied (Teisseyre et al. 2002). During these experiments it was shown that application of sphingosylphosphorylcholine (SPC) caused a modulatory effect that was similar to the effect exerted by Zn. There was a concentration-dependent shift of activation and inactivation midpoints by about 20 mV towards positive membrane potentials and a pronounced decrease of the current activation rate without alteration of the inactivation and deactivation rates. In contrast, application of SPC did not reduce the current amplitude. It was shown that the effect exerted by SPC was specific (Teisseyre et al. 2002). SPC is a potent and versatile lipid mediator in a variety of cell types, which also activates inward rectifier potassium channels coupled to muscarine acetylcholine receptors in guinea-pig atrial myocytes (Meyer zu Heringsdorf et al. 2002). The effect exerted by SPC on the channels might be related to the inhibition of TL proliferation in vitro at 10 μM SPC concentration in the TL cell line Jurkat (Xu et al. 1995).

Since both Zn and SPC are physiologically significant modulators of Kv1.3 channels, and both exert similar effects on the channel activity, it was of interest to elucidate the effects of SPC and Zn co-application. For that reason, experiments were performed with a saturating concentration of SPC (10 μM) co-applied with various zinc concentrations. The obtained data provide evidence that the effects exerted by SPC and Zn are not additive. It may be also suggested that the mechanism of interactions of SPC and Zn with the potassium channels is similar.

Preliminary results of this study were published as an abstract (Teisseyre and Michalak 2003).

Materials and methods

Cell separation, solutions and pipettes

Human TL were separated from peripheral blood samples from six healthy donors using a standard method described elsewhere (Hirano et al. 1977). After separation, cells were cultured for at least 24 h in the standard RPMI-1640 medium (Sigma) supplemented with 5% vol/vol horse serum (Sigma).

Upon experiment, the cells were placed in the external solution containing (in mM): 150 NaCl, 4.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES at pH 7.35 and 300 mosm. The pipette solution contained (in mM): 150 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 EGTA at pH 7.2 and −300 mosm. The concentration of free calcium in the internal solution was below 100 nM, assuming a dissociation constant for EGTA at pH 7.2 of 10^{-7} M (Grissmer et al. 1993). Such a low calcium concentration was applied in order to prevent the activation of calcium-activated IKCa1 channels (Grissmer et al. 1993). The reagents were provided by the Polish Chemical Company (POCH, Gliwice, Poland), except for HEPES and EGTA, which were purchased from Sigma. SPC and $ZnCl_2$ were also from Sigma. Dishes with cells were placed under an inverted Olympus IMT-2 microscope. External solutions containing Zn and Zn co-applied with SPC were applied using a fast perfusion system, RSC 200 (Bio-Logic, Grenoble, France). Pipettes were pulled from a borosilicate glass (Hilgenberg, Germany) and fire-polished before the experiment. The pipette resistance was in the range 3–5 MΩ.

Electrophysiological recordings

Whole-cell potassium currents in TL were recorded applying the patch-clamp technique (Hamill et al. 1981). The currents were recorded using an EPC-7 amplifier (List Electronics, Darmstadt, Germany), low-pass filtered at 3 kHz, digitized using the CED Micro 1401 (Cambridge, UK) analogue-to-digital converter with a sampling rate of 10 kHz. A standard protocol of depolarizing voltage stimuli containing seven pulses in the range from −60 mV to +60 mV (20 mV increment) was applied every 10 s; the pulse duration was 40 ms and the holding potential −90 mV. The linear (ohmic) component of the current was subtracted off-line from the final record. The data were analysed using the WCP J. Dempster program.

The data are given as the mean ± standard error. All experiments were carried out at room temperature (22–24 °C).

Data analysis

Since the number of active channels varied significantly among the cell population, the steady-state activation of the channels was presented in terms of a relative chord conductance (gK_{rel}) defined by the equation: $gK_{rel} = gK/gK_{60}$, where gK = chord conductance and gK_{60} = chord conductance at the membrane potential of +60 mV. The chord conductance was calculated according to the definition: $gK = I_p/(V - V_{rev})$, where I_p is the amplitude of the current, V is the membrane potential and V_{rev} is the reversal potential of the current. The voltage dependence of steady-state activation was fitted by a Boltzmann function given by the equation: $gK_{rel}(V) = 1/[1 + \exp\{-(V - V_n)/k_n\}]$, where V_n is the activation midpoint and k_n is the steepness of the voltage dependence. The activation kinetics was fitted by applying a power function that was successfully used for analysis of our previously published results (Teisseyre et al. 2002), given by the equation: $I(t) = I_p[1 - \exp(-t/\tau_n)]^2$, where τ_n is the activation time constant.

Results and discussion

Figure 1A depicts whole-cell currents in a TL recorded by applying a standard protocol of depolarizing stimuli as described in Materials and methods. The currents are activated upon membrane depolarization to potentials more positive than −40 mV. Application of 5 mM 4-aminopyridine (4-AP), which is a selective blocker of Kv1.3 channels in TL (Zegarra-Moran et al. 1999), inhibited the currents to less than 5% of the control value (Fig. 1B). The blocking effect is reversible (not shown). This indicates that the recorded currents are predominantly due to activation of Kv1.3 channels.

The effect of application of zinc ions at 100 μM concentration on the currents is presented in Fig. 2A.

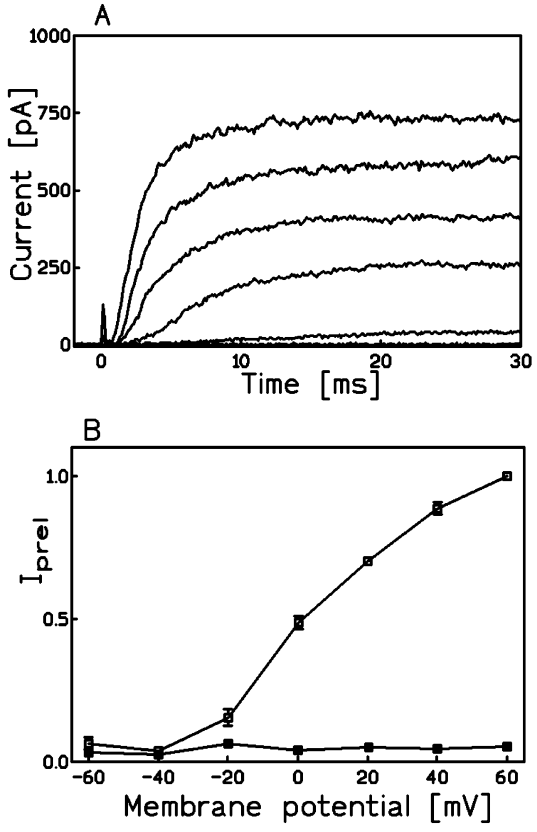

Fig. 1 **A** Example of the whole-cell currents in a TL recorded by applying a standard protocol of depolarizing stimuli (see Materials and methods). For clarity, only the first 30 ms of the records are shown. **B** Relative peak current to voltage relationship obtained under control conditions (*open squares*, $n = 6$) and upon application of 5 mM 4-AP (*filled squares*, $n = 6$). Data points in **B** were connected by a point-to-point line

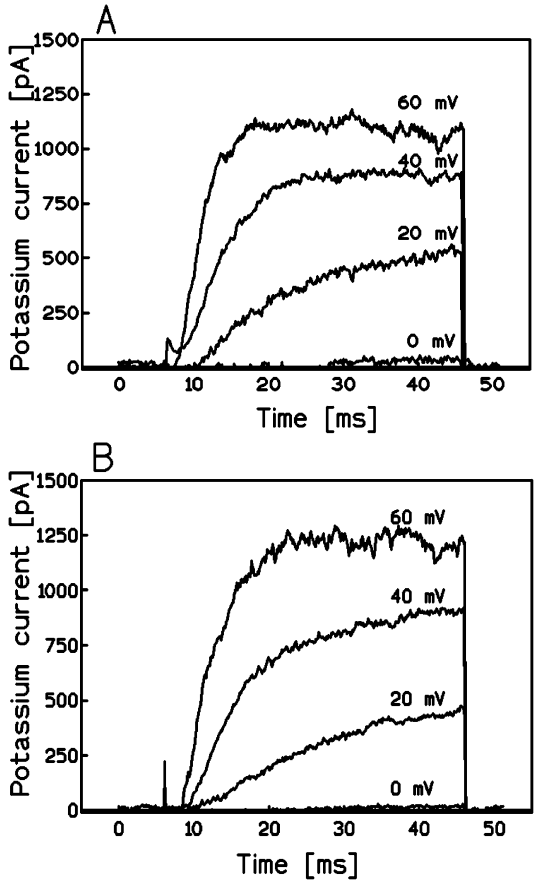

Fig. 2 Whole-cell potassium currents recorded in a TL in the presence of 100 μM Zn (**A**) and 10 μM SPC co-applied with 100 μM Zn (**B**)

According to our results obtained previously (Teisseyre and Mozrzymas 2002), at such a concentration the effect exerted by zinc ions on the channel gating is saturated.

The potassium currents in the presence of zinc were activated only at potentials higher than 0 mV (Fig. 2A). This suggests that the channel activation midpoint (V_n) is shifted towards positive membrane potentials. Moreover, the kinetics of current activation is significantly slower in the presence of zinc than under control conditions (see Figs. 1A and 2A). This is in agreement with our previous results (Teisseyre and Mozrzymas 2002).

Figure 2B depicts the effect of co-application of 100 μM Zn and 10 μM SPC on the currents. In our previous experiments it was shown that the effects exerted by SPC on the channel gating were saturated at such concentrations (Teisseyre et al. 2002). The amplitudes of the currents upon co-application of SPC and Zn and upon application of Zn alone were comparable. However, the kinetics of current activation is markedly slower upon SPC and Zn co-application than when Zn is applied alone (compare Fig. 2A and Fig. 2B; see below).

In order to examine the joint effects of SPC and Zn more precisely, experiments were performed with 10 μM SPC co-applied with Zn at concentrations of 10–100 μM. Figure 3A depicts the shift of the steady-state activation curve upon SPC application without Zn, whereas the shift of the curve upon SPC and Zn co-application is presented in Fig. 3B–D. It can be observed that the SPC-induced shift is most pronounced in the absence of Zn. When 10 μM Zn is co-applied with SPC, the shift of the curve is remarkably diminished (compare Fig. 3A and Fig. 3B). Further increase of Zn concentration reduces the shift in a concentration-dependent manner (see Fig. 3B–D). However, the shift is not abolished completely when Zn is applied at a concentration of 100 μM (see Fig. 3D). Such a small shift occurs also when the Zn concentration is raised to 300 μM (data not shown).

The absolute value of V_n in the presence of 10 μM SPC and at the same concentration of Zn was 7.62 ± 0.77 mV ($n = 10$), which is close to the value obtained for SPC when applied without Zn [6.61 ± 1.43 mV ($n = 10$)]. On the other hand, in the presence of 10 μM SPC co-applied with 100 μM Zn, the value of V_n was 18.58 ± 0.93 mV ($n = 10$), similar to value obtained for 100 μM Zn applied alone [14.33 ± 1.61 mV ($n = 10$)]. The data demonstrate that the shift of the activation midpoint is significantly more

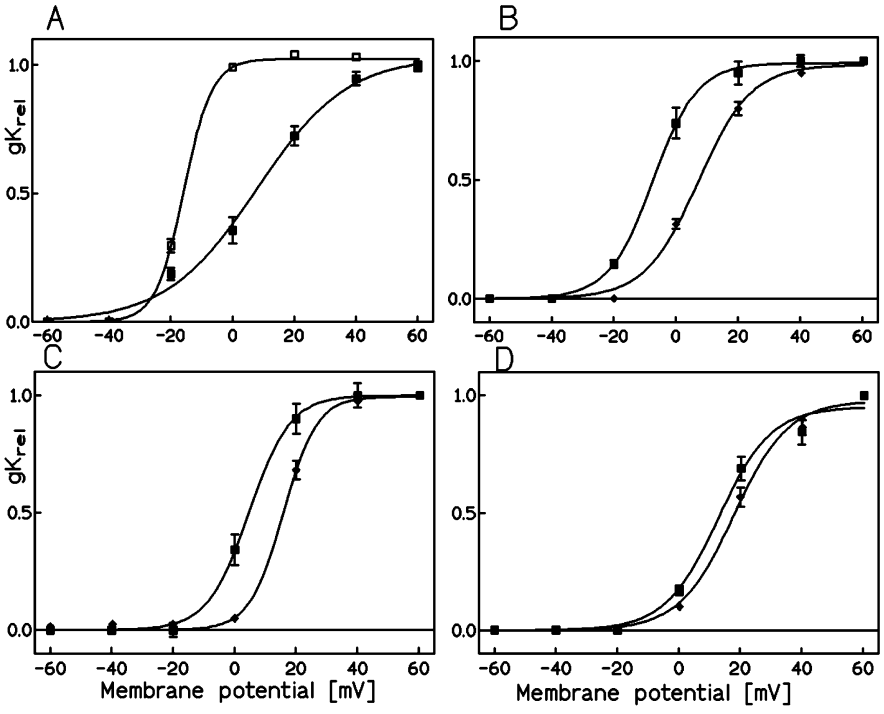

Fig. 3A–D Steady-state activation of the currents as a function of the membrane potential. *Left-hand curves* in **A–D** represent relative chord conductance calculated in the presence of Zn at concentrations of 0 (**A**) ($n=10$), 10 (**B**) ($n=10$), 50 (**C**) ($n=5$) and 100 μM (**D**) ($n=10$). *Right-hand curves* show the conductances for Zn at concentrations listed above, co-applied with 10 μM SPC. Data points were fitted by a Boltzmann function (see Materials and methods)

pronounced upon Zn application than when SPC is applied.

In order to characterize this shift more precisely, the differences of activation midpoint (δV_n) were calculated from values of V_n obtained in the presence and in the absence of 10 μM SPC for all Zn concentrations used. Figure 4 depicts δV_n values as a function of Zn concentration. As is shown in this figure, δV_n is decreased, but it is not abolished with increase of Zn concentration.

The value of k_n in the presence of 10 μM Zn was 7.26 ± 0.32 mV ($n=10$) and it was 8.85 ± 0.64 mV ($n=10$) for co-application of 10 μM Zn and 10 μM SPC. When the Zn concentration was raised to 100 μM the value of k_n was 9.90 ± 1.39 mV ($n=10$); it did not change significantly upon co-application of 100 μM Zn and 10 μM SPC (9.46 ± 0.88 mV; $n=10$). These data clearly demonstrate that the steepness of the voltage dependence of steady-state activation is not altered upon Zn and SPC co-application.

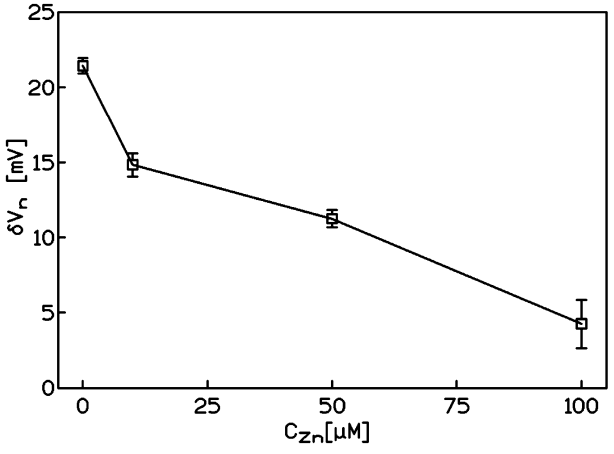

Fig. 4 Difference values of the activation midpoint defined in the text as a function of zinc concentration. The number of experiments is the same as in the case of Fig. 3. The data points were connected by a point-to-point line

In our previous studies it was revealed (Teisseyre et al. 2002) that application of SPC also slows the activation kinetics of the currents Since the SPC-induced steady-state activation shift is diminished in the presence of Zn, the SPC-induced slowing of the activation kinetics is also probably reduced. In order to demonstrate this effect, the difference value of the activation time constant ($\delta\tau_n$) between τ_n values obtained for the currents recorded in the presence and absence of SPC were calculated.

In Fig. 5 the $\delta\tau_n$ values as a function of membrane potential are presented. It can be observed that the SPC-induced decrease of the current activation rate is strongly voltage dependent, being the most potent at 0 mV and the least potent at +60 mV. As expected, the decrease is most pronounced in the absence of Zn (Fig. 5, upper curve) and is significantly reduced in the presence of Zn. However, the activation kinetics is still considerably slower upon SPC application even in the presence of 100 μM Zn (Fig. 5, lower curves). Moreover, the extent of SPC-induced slowing of the activation kinetics does not depend on Zn concentration (Fig. 5, lower curves).

Figure 6 depicts the absolute values of the activation time constants in the presence of SPC and Zn applied separately and upon co-application of SPC and Zn. It can be observed that activation time constants are significantly higher upon application of 10 μM SPC than

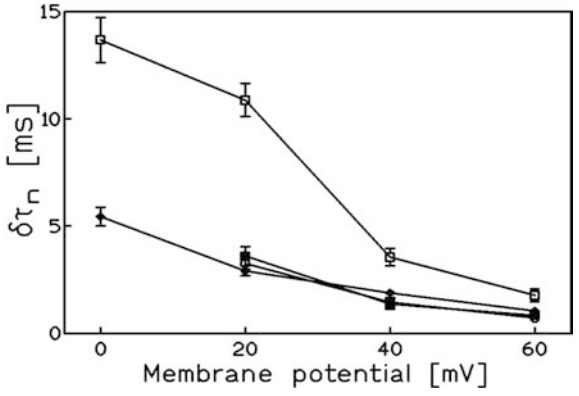

Fig. 5 Difference values of the activation time constants obtained in the presence of 10 μM SPC and zinc at concentrations of 0 (*open squares*, $n=10$), 10 (*open circles*, $n=10$), 50 (*filled diamonds*, $n=5$) and 100 (*filled squares*, $n=10$) in relation to control conditions, which are the absence of SPC and the presence of 0, 10, 50 and 100 μM of zinc, respectively. The data points were connected by a point-to-point line

when 100 μM Zn is applied. This indicates that, in contrast to the steady-state activation, SPC slows the activation kinetics more efficiently than Zn does. The same figure contains data from experiments with 10 μM SPC co-applied with 100 μM Zn. It can be observed that the time constants obtained in the case of SPC and Zn co-application are almost identical to the values obtained when SPC was applied alone. Thus, the decrease of the current activation rate observed upon SPC and Zn co-application was due to the slowing of the kinetics caused by SPC.

Our results may suggest that Zn and SPC interact with the potassium channels by a similar mechanism, which remains to be elucidated. The mechanism of zinc interactions with "delayed rectifier" potassium channels, which produce similar effects on the channel activity as described here for Kv1.3 channels, has been investigated in detail in other laboratories (Gilly and Armstrong 1982; Spires and Begenisich 1992, 1994; Kuo and Chen 1999). According to the proposed model, zinc binds to a binding site when the channel is closed and stabilizes its closed and non-inactivated state by a direct interaction with the gating charge. This may explain the shift of the steady-state activation and inactivation in the presence of zinc. Binding of zinc ions also prolongs the time period to the first opening during the channel activation. This causes the decrease of the activation rate of the whole-cell currents. Once the channel opens, zinc ions are removed from the binding site and they cannot be re-bound until the channel is closed (Gilly and Armstrong 1982). Whether such a mechanism underlies the modulatory effects exerted on TL Kv1.3 channels by Zn and SPC remains unknown as yet.

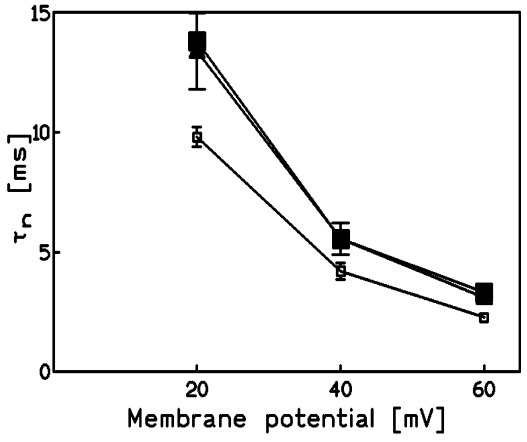

Fig. 6 Activation time constants as a function of membrane potential. *Open squares*, 100 μM Zn ($n=10$); *filled squares*, 10 μM SPC ($n=10$); *filled triangles*, 10 μM SPC co-applied with 100 μM Zn ($n=6$)

Our results clearly demonstrate that the effects exerted by Zn ions and SPC are not additive. One hypothesis that may explain this phenomenon is binding of SPC and Zn to the same or overlapping binding sites. However, some results may argue against such a mechanism. First, our results demonstrate that the SPC-induced shift of the activation midpoint and slowing of the activation kinetics is not abolished even in the presence of saturating Zn concentrations. Moreover, it must be taken into account that Zn and SPC molecules are chemically unrelated and they bind to the protein with apparently different affinities. Thus, it seems likely that both substances interact with different binding sites. Another possibility is that SPC and Zn bind to different sites that interact with each other. Finally, it might be possible that both substances bind in independent way to different sites but they interact with the same gating mechanism. Under such conditions the observed lack of additivity of the effects exerted by Zn and SPC may be due to inability of the channel gating mechanism to produce further conformational changes upon Zn and SPC co-application. The fact that the SPC-induced slowing of the activation kinetics is similar when 10 μM SPC is applied without Zn and when the same concentration of SPC is co-applied with 100 μM Zn might favour the later hypothesis. However, further studies are necessary to elucidate this problem.

The results of our experiments might have possible physiological significance. Since the effect exerted on Kv 1.3 channels by Zn and SPC applied separately might be physiologically significant for TL proliferation, it would be of interest to investigate how the co-application of both substances at various proportions would influence this process. This problem remains a subject for further studies.

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