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Short communication

THE INFLUENCE OF 8-PRENYLNARINGENIN ON THE ACTIVITY OF VOLTAGE-GATED Kv1.3 POTASSIUM CHANNELS IN HUMAN JURKAT T CELLS

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Abstract: Using the whole-cell patch-clamp technique, we investigated the influence of 8-prenylnaringenin on the activity of the voltage-gated Kv1.3 potassium channels in the human leukemic T lymphocyte cell line Jurkat. 8-prenylnaringenin is a potent plant-derived phytoestrogen that has been found to inhibit cancer cell proliferation. The results show that it inhibited the Kv1.3 channels in a concentration-dependent manner. Complete inhibition occurred at concentrations higher than 10 μM. The inhibitory effect of 8-prenylnaringenin was reversible. It was accompanied by a significant acceleration of channel inactivation without any pronounced change in the activation rate. Of the naringenin derivatives tested to date, 8-prenylnaringenin is the most potent inhibitor of the Kv1.3 channels. The potency of the inhibition may be due to the presence of a prenyl group in the molecule of this flavonoid. The inhibition of the Kv1.3 channels might be involved in the antiproliferative and pro-apoptotic effects of 8-prenylnaringenin that have been observed in cancer cell lines expressing these channels.

Key words: 8-prenylnaringenin, Kv1.3 channel, Jurkat T lymphocytes, Patch-clamp, Cancer cell proliferation, Cancer cell apoptosis

INTRODUCTION

8-prenylnaringenin (8-isopentenylnaringenin) is a prenylated flavonoid that is isolated from common hop (*Humulus lupulus*) [1]. The major source of this

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Abbreviations used: I_{prel} – relative peak current; Kv – voltage-gated potassium channels; T_{max} – time needed for the recorded currents to reach their maximal value

compound in the human diet is beer, in which the female flowers are used as a preservative and flavouring agent [2]. 8-prenylnaringenin was identified as the most potent phytoestrogen [3, 4], since it showed similar binding characteristics to α- and β-estrogen receptors [5]. It may also modulate the process of inflammation [6] and angiogenesis [7]. It was shown that 8-prenylnaringenin inhibits the activity of P-glycoprotein and MRP1 [8]. It could also inhibit cancer cell proliferation [9, 10] and induce apoptosis in the breast cancer cell line MCF-7 [11].

It remains unknown whether 8-prenylnaringenin influences the activity of ion channels, in particular the activity of voltage-gated potassium channels (Kv). However, it is known that the activity of some Kv channels is involved in cancer cell proliferation and apoptosis [12]. It was shown that inhibiting some Kv channels also inhibited the proliferation of cancer cells in various phases of this process [13]. Therefore, some Kv channels may be considered new and potentially important molecular targets in cancer therapy [12, 13].

The Kv1.3 channels belong to the group of Kv channels involved in the proliferation and apoptosis of some cancer tissues [13]. They are members of the *Shaker*-related Kv channel family and were discovered in 1984 in human T lymphocytes [14]. The biophysical properties of Kv1.3 channels were studied in more detail by Cahalan *et al.* [15]. Kv1.3 channels are expressed not only in human, murine and rat T lymphocytes, but also in many other tissues, both healthy and cancerous [13, 16]. The activity of Kv1.3 channels plays an important role in T lymphocyte cell function, particularly in setting the resting membrane potential, cell proliferation, apoptosis and volume regulation [17, 18]. Studies of Kv1.3 channels expressed in human T lymphocytes showed that blocking them inhibited cell proliferation in the G_1 phase [17]. Specific blockers of Kv1.3 channels may be applied in selective immunosuppression [17].

Several studies demonstrated that Kv1.3 channels have altered expression levels in some types of cancer, including breast, colon, pancreas and prostate cancer [13, 19-21]. A significantly increased expression of Kv1.3 channels was observed in the case of breast, colon and lymph node cancers [13, 19]. However, a markedly reduced expression of Kv1.3 channels was detected in kidney, pancreas and prostate cancer [19]. Importantly, it was shown that inhibition of Kv1.3 channels both by specific (MgTX) and non-specific (TEA) inhibitors also inhibits the proliferation of breast cancer cells [20] and lung cancer cells *in vitro* and *in vivo* [21]. Therefore, Kv1.3 channel inhibitors could be applied in therapy of some types of cancer.

Our studies in recent years showed that some natural plant-derived compounds that exert an antiproliferative effect on cancer tissues (such as the isoflavone genistein and the substituted stilbene resveratrol) are inhibitors of Kv1.3 channels in human T lymphocytes [22, 23]. It is important that the antiproliferative effect on cancer cells and the inhibition of Kv1.3 channels occurred at comparable concentrations of the two named compounds. We concluded that the antiproliferative effect of genistein and resveratrol could be at least partially related to the inhibition of Kv1.3 channels.

Taking into account the inhibition of cancer cell proliferation by 8-prenylnaringenin and the possible involvement of Kv1.3 channels in this process, it was of interest to study the influence of 8-prenylnaringenin on the activity of Kv1.3 channels. Since Kv1.3 channels are expressed abundantly and endogenously in the human leukemic T cell line Jurkat [24, 25], these cells were used in our study as a model system. The results provide evidence that 8-prenylnaringenin effectively inhibits the Kv1.3 channels expressed in Jurkat T cells.

MATERIALS AND METHODS

Cell culture and solutions

The human leukemia T cell line Jurkat (clone E6-1) was purchased from the American Type Culture Collection (Manassas, VA). The Jurkat T cell line was grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% heat-inactivated FBS, 10 mM HEPES, and 2 mM glutamate. Cells were grown in culture plates at 37ºC in a 5% CO_2-humidified incubator.

During the experiments, cells were placed in an external solution containing 150 mM NaCl, 4.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.35), adjusted with NaOH (300 mOsm). The pipette solution contained 150 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES and 10 mM EGTA (pH 7.2), adjusted with KOH (280 mOsm). The concentration of free calcium ions in the internal solution was below 100 nM, assuming a dissociation constant for EGTA at pH 7.2 of 10^{-7} M [26]. Such a low calcium concentration was applied in order to prevent the activation of the calcium-activated K^+ channels $K_{Ca}2.2$, which are abundantly expressed in Jurkat T cells [27]. The chemicals were purchased from the Polish Chemical Company (POCH, Gliwice, Poland), except HEPES and EGTA, which were purchased from SIGMA. 8-prenylnaringenin was purchased from Alexis Biochemicals (Lausen, Switzerland).

Patch-clamp recordings

Dishes with cells were placed under an inverted Olympus IMT-2 microscope. Solutions containing the tested compounds were applied using a perfusion system developed in our laboratory. The pipettes were made from borosilicate glass (Hilgenberg, Germany) and fire-polished before the experiment and have a resistance in the range of 3-5 MΩ.

Whole-cell potassium currents in T lymphocytes were recorded using the patch-clamp technique [28]. The currents were recorded using an EPC-7 Amplifier (HEKA, Germany), low-pass filtered at 3 kHz, and digitized using a CED Micro 1401 analogue-to-digital converter (Cambridge, UK) with a sampling rate of 10 kHz. Two protocols of depolarizing voltage stimuli were used during the experiments. The influence of 8-prenylnaringenin on the activity of the channels was preliminarily studied with the voltage ramp protocol. Voltage ramps depolarizing the cell membrane from –100 mV up to +40 mV were applied every 20 s; the ramp duration was 340 ms and the holding potential was –90 mV.

Upon application of the voltage ramp protocol, the potassium currents in the Jurkat leukemia T lymphocytes were stably recorded for at least 20 min after "break-in" to the whole-cell configuration. During the offline analysis, the maximal value of Kv1.3 current at the end of a voltage ramp (+40 mV) was calculated. The leak current estimated at +40 mV was subtracted from the ramp current recorded at this voltage. The estimation of the leak current was performed by extrapolating the function fitting the linear component, which was supposed to be the leak current, to the potential value of +40 mV. In order to study the influence of 8-prenylnaringenin on the channels' activation and inactivation kinetics in more detail, another protocol of depolarizing voltage stimuli was applied. This protocol contained 7 depolarizing voltage steps in the range from –60 mV to +60 mV (20 mV increment, 100 ms step duration) applied every 20 s, with a holding potential of – 90 mV. All of the experiments were carried out at room temperature (22-24ºC) and the data are presented as means ± standard error.

Data analysis

Since the number of active channels varied significantly among the cells, the current amplitudes were presented in terms of a relative peak current (I_{prel}) defined as:

$$I_{prel} = I/I_{contr}$$

where I is the current amplitude and I_{contr} is the current amplitude recorded on the same cell under control conditions. Inactivation kinetics were fitted by the single exponential function and described by the value of inactivation time constant. Activation kinetics were described by the time-to-peak parameter (T_{max}), which is determined as the time needed to reach the peak value of Kv1.3 current upon the channels' activation during the application of a depolarizing voltage step. Statistical analysis was performed applying the one-way analysis of variance (ANOVA) or Student's unpaired t test. The results were considered statistically significant when $p < 0.05$.

RESULTS

Fig. 1 presents an example of the whole-cell currents recorded in Jurkat T lymphocytes under control conditions and in the presence of 3 μM 8-prenylnaringenin. This figure depicts the raw currents without leak subtraction. The currents were recorded applying the voltage ramp protocol (see the Materials and Methods section). The evoked currents contained a linear and a non-linear component. The linear current was presumed to be the leak current, whereas the non-linear component was due to the activation of Kv1.3 channels [27].

Applying 8-prenylnaringenin apparently reduced the amplitude of the Kv1.3 current to less than half of its control value. Moreover, in contrast to what was recorded under control conditions, the ramp currents recorded upon application of 8-prenylnaringenin decayed over time. This might indicate that inhibition of

8-prenylnaringenin was accompanied by an acceleration of channel inactivation. A more detailed study demonstrated that the acceleration of channel inactivation was actually the case (see below). The Kv1.3 currents recovered completely after the 8-prenylnaringenin was washed out (record "c"). This indicates that the inhibitory effect of 8-prenylnaringenin was reversible.

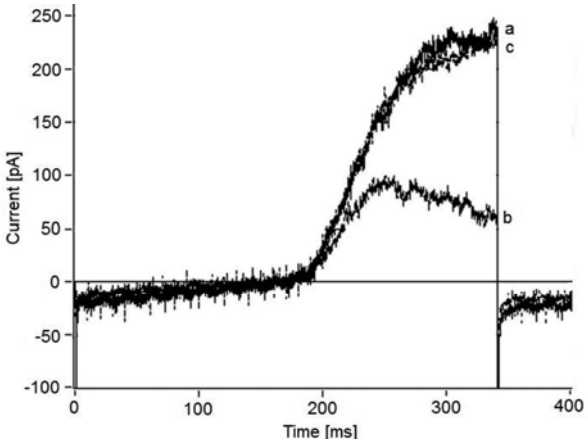

Fig. 1. 8-prenylnaringenin reduces the intensity of the whole-cell potassium currents recorded in Jurkat T lymphocytes using the voltage ramp protocol. The currents are shown under control conditions (a), with exposure to 3 μM 8-prenylnaringenin (b), and with the flavonoid having been washed out (c).

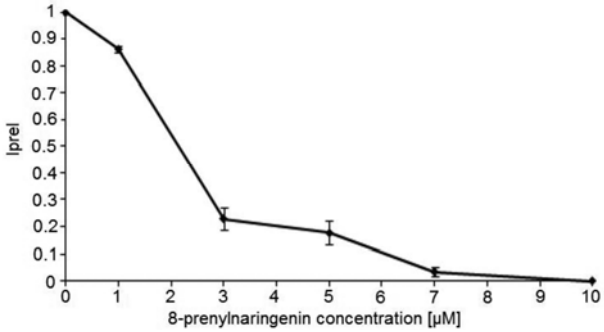

Fig. 2. The relative peak Kv1.3 current (defined above) plotted vs. 8-prenylnaringenin concentration.

The blocking effect of 8-prenylnaringenin was concentration dependent. Upon application of 1, 3, 5 and 7 μM 8-prenylnaringenin, the relative peak current (I_{prel}) was respectively reduced to 0.86 ± 0.012 (n = 5), 0.38 ± 0.04 (n = 7), 0.25 ± 0.043 (n = 5) and 0.034 ± 0.015 (n = 5) of the control value. The decrease in the current amplitude was statistically significant ($p < 0.05$, Student's t test) for all concentrations applied. Further increase in the 8-prenylnaringenin concentration to 10, 15, 20 and 30 μM caused a complete inhibition of Kv1.3

currents in Jurkat T lymphocytes. The inhibitory effect of 8-prenylnaringenin was reversible at all concentrations (not shown). Fig. 2 shows the relative peak current (I_{prel}) as a function of 8-prenylnaringenin concentration.

As mentioned above, the inhibitory effect of 8-prenylnaringenin on Kv1.3 channels was probably accompanied by a significant increase in inactivation rate. A more detailed study performed using a protocol with depolarizing voltage steps provided evidence that such an increase actually took place. Fig. 3 shows examples of normalized whole-cell potassium currents recorded using a voltage step to +60 mV under control conditions and upon exposure to 7 μM 8-prenylnaringenin. The figure depicts the currents after leak subtraction performed during offline analysis. The inactivation was much more rapid in the presence of the flavonoid than under control conditions.

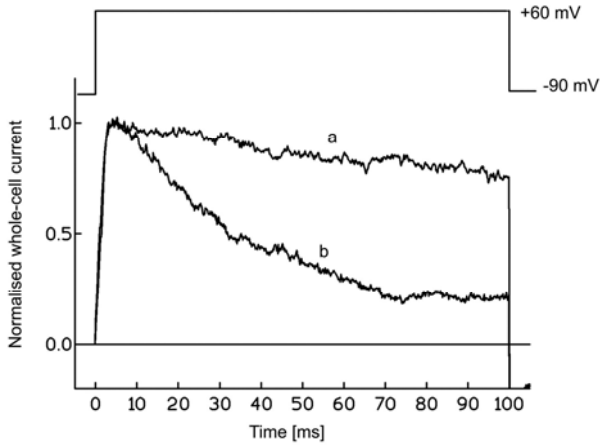

Fig. 3. Examples of normalized whole-cell potassium currents, after leak subtraction, recorded using a voltage step from the holding potential of –90 mV to +60 mV (shown schematically above the records) under control conditions (a) and upon exposure to 7 μM 8-prenylnaringenin (b).

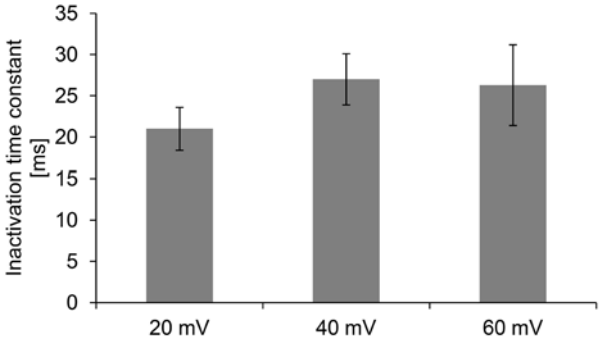

Fig. 4. The inactivation time constant in the presence of 7 μM 8-prenylnaringenin.

Fig. 4 illustrates the inactivation time constant calculated at different values of membrane potential in the presence of 7 μM 8-prenylnaringenin. Inactivation time constants were 20-25 ms upon 8-prenylnaringenin exposure. These values were significantly lower ($p < 0.05$, Student's t-test) than the inactivation time constant of ca. 200 ms estimated for Kv1.3 channels under control conditions [29]. The values of inactivation time constants obtained in the presence of 8-prenylnaringenin did not significantly differ for all membrane potentials in the range from +20 to +60 mV ($p > 0.05$, one-way ANOVA).

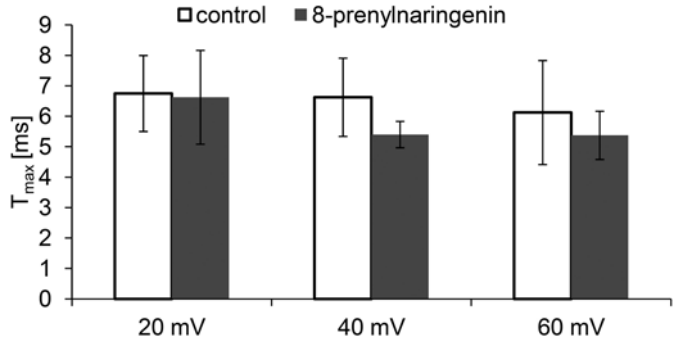

Fig. 5. T_{max} values for control conditions and upon exposure to 7 μM 8-prenylnaringenin.

Since the inactivation rate of the Kv1.3 currents was significantly higher after exposure to 8-prenylnaringenin, it was of interest to study the influence of the compound on the channels' activation kinetics. Fig. 5 illustrates the activation kinetics in terms of the T_{max} parameter estimated for the currents recorded under control conditions and upon application of 7 μM 8-prenylnaringenin. In contrast to the influence on the inactivation kinetics, exposure to the flavonoid did not change the activation kinetics significantly ($p > 0.05$, Student's t-test).

DISCUSSION

We studied the influence of 8-prenylnaringenin on the activity of Kv1.3 channels expressed in Jurkat T cells. Our results provide evidence that upon exposure to 8-prenylnaringenin the activity of the channels was inhibited in a concentration-dependent manner. Complete inhibition occurred at flavonoid concentrations higher than 10 μM and the blocking effect was reversible. The effect was accompanied by a significant acceleration of the inactivation rate, whereas the activation kinetics remained unchanged.

The inhibitory effect of 8-prenylnaringenin on Kv1.3 channels seems to be much more potent than the channel inhibition caused by other natural plant-derived compounds that exert an antiproliferative effect on cancer cells, such as genistein or resveratrol [22, 23]. The results of our previous studies showed that genistein did not completely inhibit the channels' activity when applied at

a concentration of 80 μM, whereas the inhibitory effect of resveratrol was not complete even at 200 μM [22, 23]. By contrast, these new results demonstrate that 8-prenylnaringenin completely inhibited Kv1.3 channels at concentrations higher than 10 μM.

In contrast to what was observed in this study for 8-prenylnaringenin, the inhibitory effects of genistein and resveratrol on Kv1.3 channels were not accompanied by an acceleration of the inactivation rate [22, 23]. Those effects were related to a significant slowing down of activation rate that was not observed in this study [22, 23]. Finally, the inhibitory effects of genistein and resveratrol were clearly time-dependent, whereas no such clear dependence on time was observed in this study. On the other hand, it must be taken into consideration that studies on the influence of genistein and resveratrol were not performed on Jurkat T cells, but on Kv1.3 channels in human T lymphocytes isolated from human peripheral blood [22, 23]. However, preliminary studies performed on Kv1.3 channels in human T lymphocytes isolated from peripheral blood demonstrated that these channels were inhibited by 8-prenylnaringenin and the magnitude of the inhibition did not significantly differ from the effect observed in Jurkat T cells [Gąsiorowska – unpublished results]. Therefore, the observed differences in the inhibitory effects of 8-prenylnaringenin, genistein and resveratrol were not due to the use of different model systems. They were probably due to the involvement of different mechanisms of channel inhibition by 8-prenylnaringenin, genistein and resveratrol.

Our earlier experiments provide evidence that the flavonoid naringenin, a precursor compound for 8-prenylnaringenin, does not inhibit Kv1.3 channels when applied at a concentration of 30 μM [30]. The same study showed that exposure to two synthetic derivatives of naringenin, 4',7-dimethylether and 7-methylether, both at 30 μM, respectively inhibited Kv1.3 channels to 4% and 29% of the control activity. On the other hand, the results of this study demonstrate that 8-prenylnaringenin completely inhibited Kv1.3 channels even at a concentration of 10 μM. Of all the naringenin derivatives tested to date, 8-prenylnaringenin seems to be the most potent inhibitor of Kv1.3 channels. It is possible that the potency of 8-prenylnaringenin to inhibit Kv1.3 channels is a consequence of the presence of a prenyl group in the molecule.

If the ability of 8-prenylnaringenin to inhibit Kv1.3 channels is due to the presence of such a prenyl group, it seems likely that other prenylated flavonoids or chalcones would be potent inhibitors of Kv1.3 channels. It is known that some compounds from these groups, such as 6-prenylnaringenin, xanthohumol and isoxanthohumol, are potent inhibitors of cancer cell proliferation, and xanthohumol and 6-prenylnaringenin are even more potent inhibitors of proliferation than 8-prenylnaringenin [10]. The influence of these compounds on the activity of Kv1.3 channels remains to be elucidated. However, the results of preliminary studies indicate that xanthohumol inhibits Kv1.3 channels in Jurkat T lymphocytes [Gąsiorowska – unpublished results].

The inhibition of Kv1.3 channels by 8-prenylnaringenin may be of physiological significance. It is known that inhibition of Kv1.3 channels could also inhibit proliferation of some cell types, including breast, colon and prostate cancer cells [13, 20, 21]. It is also known that 8-prenylnaringenin inhibits the proliferation of cells of the human prostate cancer cell lines PC-3 and DU-145 [10]. However, the half-blocking concentration of 8-prenylnaringenin for the inhibition of proliferation of PC-3 and DU-145 cells was 33.5 ± 1.0 μM and 43.1 ± 1.2 μM, respectively [10]. These values are significantly higher than the concentrations needed for the complete inhibition of Kv1.3 channels reported in this study. Therefore, further studies are needed to answer to the question whether the inhibition of Kv1.3 channels is involved in the inhibition of proliferation of the prostate cancer cell lines.

The inhibition of Kv1.3 channels by 8-prenylnaringenin might also be involved in the pro-apoptotic activity of this compound. Studies performed in recent years provide evidence that Kv1.3 channels are expressed not only in the plasma membrane but also in the inner mitochondrial membrane in human T lymphocyte and Jurkat T cells [18]. It is known that inhibition of Kv1.3 channels expressed in human T lymphocyte mitochondria by the pro-apoptotic protein Bax is the first crucial event in the mitochondrial pathway of T cell apoptosis [31]. In other studies, it was shown that inhibition of mitochondrial Kv1.3 channels in Jurkat T lymphocytes by specific inhibitors, such as margatoxin (MgTX), triggers the beginning of the apoptotic cascade in isolated mitochondria, similar to the events that occur after Bax-induced inhibition [31]. Recently obtained data provide evidence that Kv1.3 channels are expressed in the inner mitochondrial membrane in non-lymphocyte cancer cell lines, such as the human prostate cancer cell line PC-3 or breast cancer cell line MCF-7 [18]. It is possible that 8-prenylnaringenin is able to diffuse across the plasma membrane and reach intracellular compartments, so it may inhibit Kv1.3 channels in the mitochondria. Brunelli *et al.* showed that 8-prenylnaringenin induces apoptosis of MCF-7 cells when applied at 10 and 100 μM concentrations [11]. Our study showed that a 10 μM concentration of the flavonoid is high enough to completely inhibit Kv1.3 channels in the plasma membrane. Whether mitochondrial Kv1.3 channels are as sensitive to the inhibition by 8-prenylnaringenin as the channels in the plasma membrane and whether blocking of these channels is involved in 8-prenylnaringenin-induced apoptosis of MCF-7 cells remains to be elucidated.

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