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Influence of Specific Immunotherapy on the Activity of Human T Lymphocyte Kv1.3 Voltage-Gated Potassium Channels in Insect Venom Allergic Patients

Marita Nittner-Marszalska · Andrzej Teisseyre · Bożena Jaźwiec · Aneta Kowal · Magdalena Wujczyk · Antonina Gawlik · Maryla Krasnowska

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Abstract Kv1.3 channels play an important role in T lymphocytes function. $CD4^+$ and $CD4^+CD25^+$ T cells are two broad categories of T cells that are critically involved in the immunoresponse to allergens and that are also a major target for allergen immunotherapy. The aim of the study was to evaluate the effects of venom immunotherapy (VIT) on the activity of Kv1.3. channels on noncultured subsets: $CD4^+$ and $CD4^+CD25^+$ T cells of insect venom allergic patients. Eleven patients with allergic reactions to bee or wasp venoms participated in the study. The patients were provided VIT according to the ultrarush protocol. $CD4^+$ and $CD4^+CD25^+$ T cells were isolated from peripheral blood mononuclear cells of VIT-treated patients by an immunomagnetic method. We used the whole-cell patch clamp technique to investigate the whole potassium chord conductance (gK) of Kv1.3. channels in $CD4^+$ and $CD4^+CD25^+$ T cells of venom-sensitive patients before and during the course of VIT. The conductance of Kv1.3. channels on $CD4^+CD25^+$ T cells decreased during the course of VIT. On day 0 it was 0.054 ± 0.07 [nS], and on day 70 it was 0.008 ± 0.09 [nS] ($P = 0.03$). The observed decrease of the gK of the Kv1.3 channels in the subpopulation of activated T cells may contribute to T cell tolerance and functional unresponsiveness of these cells to allergen in the early stages of VIT.

Keywords Potassium channels · Kv1.3 · Voltage-gated ion channels · Insect allergy · Specific immunotherapy · T cells

M. Nittner-Marszalska (✉) · A. Kowal · M. Wujczyk · A. Gawlik · M. Krasnowska
Department of Internal Disease, Geriatrics and Allergology, Medical University, Wrocław, Poland
e-mail: marmarsz@gmail.com

A. Teisseyre
Department of Biophysics, Medical University, Wrocław, Poland

B. Jaźwiec
Department of Haematology, Medical University, Wrocław, Poland

Introduction

Specific allergen immunotherapy is a highly effective method in the treatment of immunoglobulin (Ig) E-mediated allergic diseases such as allergic rhinitis, asthma, and stinging insect allergy (Bousquet et al. 1998). This method is especially addressed to bee and wasp venom–sensitive patients who, after stinging, can develop acute systemic life-threatening allergic reactions. Insect venom immunotherapy (VIT) represents the only causal therapy in the treatment of insect allergy. VIT results in full protection, as indicated by the complete absence of allergic symptoms after subsequent stings in 80–90% of bee venom- and 95–100% of wasp venom-sensitive patients (Bonifazi et al. 2005). VIT is also recommended as a model for investigating the mechanisms of specific immunotherapy and the induction of tolerance to different allergens.

Although detailed immunological mechanisms of VIT have not been satisfactorily elucidated so far, the currently adopted concept postulates that T lymphocytes play a fundamental role in these processes and thus are a major target of immunotherapy. It seems that induction of tolerance in peripheral T cells constitutes an important step in VIT. The achieved peripheral T cell tolerance is mainly evidenced by suppression of proliferative and cytokine responses to major venom allergens and their T cell recognition sites. VIT exerts a suppressive effect on many

lymphocyte T functions: it decreases allergen-induced T cell proliferation, suppresses the subset of allergen-specific $CD4^+$ Th2 cells and the production of their cytokines, and decreases $CD4^+$ T cell numbers in late allergic response (Akdis and Akdis 2007). The $CD4^+CD25^+$ T cells constitute 5–10% of peripheral $CD4^+$ T cells that express the interleukin-2 receptor alpha chain (CD25). This subpopulation of CD4 T cells represents activated T cells.

Kv1.3 channels are voltage-gated potassium channels discovered in 1984 in human T lymphocytes via the patch clamp technique (Matteson and Deutsch 1984; DeCoursey et al. 1984). The biophysical properties of these channels were characterized in detail by Cahalan et al. (1985). Kv1.3 channels belong to the family of *Shaker*-related voltage-gated potassium channels (Gutman et al. 2005). Kv1.3 channels are expressed abundantly in human, mouse, and rat T lymphocytes, but also in many other tissues (Gutman et al. 2005). Activity of Kv1.3 channels plays an important role in T cell function, particularly in setting the resting membrane potential, cell proliferation, apoptosis, and volume regulation (Cahalan et al. 1985, 2001; Shieh et al. 2000; Cahalan and Chandy 2009). Kv.1.3 are localized in the immunological synapse formed between T cells and antigen-presenting cells, and they participate in the activation of human T cells. It is well known that inhibition of Kv1.3 channels also inhibits the cell proliferation in the G_1 phase (Cahalan et al. 2001; Panyi et al. 2006; Cahalan and Chandy 2009; Gulbins et al. 2010). Therefore, specific blockers of Kv1.3 channels may be applied in a selective immunosuppression (Panyi et al. 2006). The role of Kv1.3 in allergy and allergen immunotherapy is not yet known.

On the basis of these findings, we decided to examine the activity of Kv1.3 channels in freshly isolated, noncultured subsets of T cells, $CD4^+$ and $CD4^+CD25^+$, of venom-sensitive patients during VIT to asses the influence of VIT on the activity of these channels.

Materials and Methods

Patients

Eleven adult patients aged 16–57 years with a history of serious allergic reactions to bee or wasp venom (grade III or IV according the Mueller I–IV scale) were involved in the study. All patients had positive intracutaneous test results to venom at a concentration of 10^{-6} g/l. The patients were provided VIT with respective venom according to ultrarush protocol, starting at a dose of 0.1 μg venom and increasing during 3.5 h to the cumulative dose of 101.1 μg (day 0). The next vaccine doses were provided on day 7 (100 μg) and day 30 (100 μg). Blood samples were taken in all patients before VIT (on day 0), on day 7 before the maintenance dose of vaccine, and on days 30 and 70 (in five patients). During therapy, the patients did not report any side effects. Demographic data and the characteristics of the study group are listed in Table 1.

The study protocol was approved by the ethical committee of Wrocław Medical University.

Isolation of Human T Lymphocytes

The cells were isolated at the Department of Haematology, Wrocław Medical University, from the peripheral blood of insect (bee or wasp)-allergic patients. The procedure of blood sample extraction is in accordance with good medical practice and was approved by the bioethics committee at Wrocław Medical University.

$CD4^+CD25^+$ T cells were isolated from peripheral blood mononuclear cells (PBMC) by immunomagnetic method with the ready-to-use Dynabead Regulatory $CD4^+CD25^+$ T cell kit (Dynal/Invitrogen, Alab, Warszawa) according to the manufacturer's instruction. In brief, PBMCs were obtained from anticoagulated blood by standard centrifugation over 1077 g/cm^3 density medium (Gradisol L, AquaMedica, Łódź). Platelets were discarded with the supernatant by slow centrifugation (12 min at $80\times g$). PBMCs were stained with a cocktail of antibodies directed to non-CD4 antigens (mouse IgG antibodies to CD14, CD16, CD56, CDw123, CD36, CD8, CD19, and glycophorin A), washed, and incubated with Depletion MyOne Dynabeads, which formed magnetic complexes with cells coupled to antibodies from the cocktail. Non-CD4 cells were depleted by the magnet and $CD4^+$ fraction incubated with CD25 Dynabeads. $CD4^+CD25^+$ cells were next separated on the magnet, non-CD25 fraction collected to separate tube and magnetic beads were removed from CD25-positive cells by incubation with DETACHaBEAD and captured from the cell suspension by the magnet.

Cells from CD25-positive and -negative fractions were washed, counted, and controlled for CD4/CD25 expression by cytofluorimeter by means of mouse anti-CD25 and anti-CD4 monoclonal antibodies coupled to fluorescein isothiocyanate and phycoerythrin, respectively (Beckman Coulter, Comesa, Warszawa).

Patch Clamp Recording

During the experiments, the cells were suspended in the extracellular solution containing (in mM): 150 NaCl, 4.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH = 7.35 adjusted with NaOH, 300 mOsm. The pipette solution contained (in mM): 150 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 EGTA; pH = 7.2 adjusted with KOH, 300 mOsm. The reagents were provided by the Polish Chemical Company (Gliwice, Poland), except for HEPES and EGTA, which were

Table 1 Demographic data and clinical characteristics of patients

Patient no.	Age (years)	Sex	Insect responsible for allergic reaction	Intracutaneous skin test with venom at the concentration of 10^{-6} g/l (mm)	Venom-specific IgE (class)
1	57	M	Bee	15 × 20	2
2	44	F	Wasp	12 × 10	1
3	27	M	Bee	12 × 13	2
4	48	F	Wasp	18 × 18	2
5	33	M	Wasp	16 × 14	3
6	43	M	Bee	8 × 8	0
7	16	M	Bee	13 × 12	0
8	43	M	Bee	13 × 13	2
9	38	F	Bee	15 × 12	2
10	40	M	Wasp	10 × 9	3
11	19	F	Bee	7 × 9	6

purchased from Sigma (St. Louis, MO, USA). The concentration of free calcium in the internal solution was below 100 nM, assuming the dissociation constant for EGTA at pH = 7.2 of 10^{-7} M (Grissmer et al. 1993). Such a low calcium concentration was applied to prevent the activation of calcium-activated $K_{Ca}3.1$ channels (Grissmer et al. 1993). Dishes with cells were placed under an inverted Olympus IMT-2 microscope. Pipettes were pulled from a borosilicate glass (Hilgenberg, Germany) and fire-polished before the experiment. The pipette resistance was in the range of 3–5 MΩ.

Whole-cell potassium currents in T lymphocytes were recorded applying the patch clamp technique (Hamill et al. 1981). The currents were recorded with an EPC-7 amplifier (Heka, Germany), low-pass filtered at 3 kHz, and digitized with a CED Micro 1401 (Cambridge, UK) analog-to-digital converter with the sampling rate of 10 kHz. A standard depolarizing sequence contained 10 voltage ramps applied every 20 s depolarizing the cell membrane gradually from −100 mV up to +40 mV; ramp duration was 340 ms and holding potential −90 mV. Upon application, the voltage ramp protocol potassium currents in T lymphocytes were stably recorded for at least 20 min after break-in to the whole-cell configuration. To calculate the Kv1.3 current at +40 mV, the estimated leak current at +40 mV was subtracted from the ramp current recorded at this voltage. The estimation of the leak current at +40 mV was performed by extrapolation of the function fitting the linear component that was supposed to be the leak current, recorded at potentials below the activation threshold for Kv1.3 channels, to the potential of +40 mV. The reversal potential of recorded currents was estimated by the tail-current protocol described in detail elsewhere (Teisseyre et al. 1996; Teisseyre and Mozrzymas 2002). The whole-cell potassium chord conductance (gK) was calculated in nanosiemens [nS] applying the definition

$$\mathrm{gK} = I_p/\left(V_p - V_{rev}\right), \quad (1)$$

where I_p is the calculated peak Kv1.3 current at the end of the voltage ramp (after subtraction of the estimated leak current at the end of the voltage ramp), V_p is the membrane voltage at the end of the voltage ramp (+40 mV), and V_{rev} is the estimated reversal potential of the currents (−75 mV). All experiments were carried out at room temperature (22–24°C).

Skin Tests

Lyophilized bee and wasp venom (Pharmalgen, Alk-Abello, Denmark) was used for skin tests. Skin tests were performed by administering intracutaneous injections of 0.02 ml dilution of bee or wasp venom at the concentration of 10^{-6} g/l. The test was considered positive if a wheal at least 5 mm in diameter with surrounding erythema resulted after 20 min.

Statistical Analysis

For statistical evaluation, Friedman's repeated-measure analysis of variance for dependent variables followed by the Kendall post hoc test comparisons were used. Data are expressed as mean values (±SD) with confidence intervals of mean differences for the pre- and posttreatment comparisons. P values of <0.05 were considered statistically significant. Statistical evaluation was performed by Statistica software, version 8.0 for Windows (StatSoft, Tulsa, OK).

Results

For all study patients, immunotherapy was performed according to the predesigned scheme: on day 0, the

accumulated dose of venom was 101.1 μg, on day 7 it was 201.1 μg, and on day 30 it was 301.1 μg. Subsequent doses of venom were well tolerated by the patients. The course of immunotherapy was performed successfully and the immunotolerance of the venom was reached. No systemic adverse effects were observed.

An example of T lymphocyte whole-cell raw currents (without leak subtraction) is depicted in Fig. 1. The current was recorded in a T lymphocyte applying a voltage ramp (see "Materials and Methods" section). Application of the ramp evoked a current, which contained two components: a linear current supposed to be the leak current, followed by a nonlinear current activated upon membrane depolarization. Our previous experiments demonstrated that the nonlinear component was predominantly due to activation of Kv1.3 channels (Teisseyre et al. 1996, 2007; Teisseyre and Mozrzymas 2002, 2006). First of all, our studies demonstrated that the nonlinear currents recorded by either voltage steps (Teisseyre et al. 1996; Teisseyre and Mozrzymas 2002) or by voltage ramps (Teisseyre and Mozrzymas 2006) were clearly voltage dependent. The reversal potential of the currents estimated by the tail current protocol, described in detail elsewhere (Teisseyre et al. 1996; Teisseyre and Mozrzymas 2002), was equal to about −75 mV. This value is close to the Nernst equilibrium potential for potassium ions under physiological conditions (−87 mV). Raising the extracellular potassium concentration from the physiological level to 150 mM shifted the reversal potential value to about 0 mV, which is in accordance with the properties of potassium-selective channels (Teisseyre et al. 1996; Teisseyre and Mozrzymas 2002). Moreover, the nonlinear current was sensitive to block by 5 mM 4-amino-pyridine, which is a well-known blocker of voltage-gated potassium channels including the Kv1.3 type (Teisseyre and Mozrzymas 2002). Finally, the nonlinear current underwent the process of cumulative inactivation, which is the characteristic feature of Kv1.3 channels (Teisseyre et al. 2007).

Figure 2 shows examples of normalized whole-cell currents recorded in T lymphocytes from the same patient applying the same voltage ramp protocol on days 0, 30, and 70 of VIT. The only clear difference is a significantly increased relative contribution of the leak component in the current recorded on day 70. This is a consequence of the fact that the Kv1.3 current on day 70 was small, so the leak current recorded on this day had much higher relative contribution to the total current. However, normalized Kv1.3 currents recorded on all the days of VIT were not significantly different one from another. This may indicate that VIT did not change gating properties of Kv1.3 channels in human T lymphocytes.

The mean values of gK in the study group of venom-treated patients are shown in Table 2 and Figs. 3 and 4.

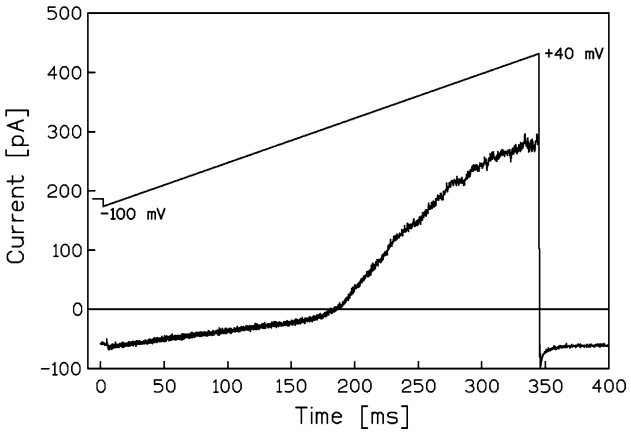

Fig. 1 Example of T lymphocyte whole-cell ion currents as a function of time recorded in a T lymphocyte using a voltage ramp depolarizing the cell membrane from −100 mV to +40 mV

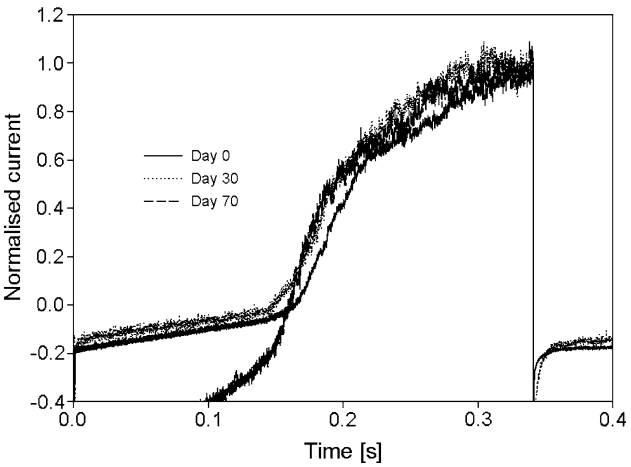

Fig. 2 Examples of normalized T lymphocyte whole-cell ion currents as a function of time recorded in T lymphocytes from the same patient using the same voltage ramp protocols on days 0, 30, and 70 of VIT

The gK value decreased during treatment, and the difference in gK on day 0 (before treatment) and on day 70 was statistically significant in the $CD4^+CD25^+$ lymphocyte T subpopulation (Fig. 3).

Discussion

In this study, we investigated the influence of venom-specific immunotherapy (VIT) in bee or wasp venom-sensitive patients on the whole-cell potassium conductance (gK) in two subsets of T cells, $CD4^+$ and $CD4^+CD25^+$. The results of the study provide conclusive evidence that in the early course of VIT, the whole-cell potassium conductance in the subpopulation $CD4^+CD25^+$ T cells is decreased.

The decrease of the whole-cell potassium conductance observed in the course VIT provided to our insect

Table 2 Levels of gK [nS] before and during venom-specific immunotherapy in bee and wasp venom-sensitive patients

Conductance	No. of study patients (no. of studied cells)	Mean gK [nS] (95% CI)	SD	Median
gK $CD4^+$				
Day 0	11 (336)	0.090 (0.04–0.138)	0.072	0.07
Day 7	11 (172)	0.113 (0.03–0.20)	0.126	0.08
Day 30	11 (249)	0.053 (0.02–0.08)	0.046	0.03
Day 70	5 (120)	0.027 (0.01–0.04)	0.01	0.027
gK $CD4^+25^+$				
Day 0	11 (160)	0.054 (0.004–0.1)	0.07	0.018
Day 7	11 (96)	0.031 (0–0.05)	0.04	0.008
Day 30	11 (103)	0.019 (0–0.05)	0.038	0.008
Day 70	5 (43)	0.008 (0–0.02)	0.009	0.008

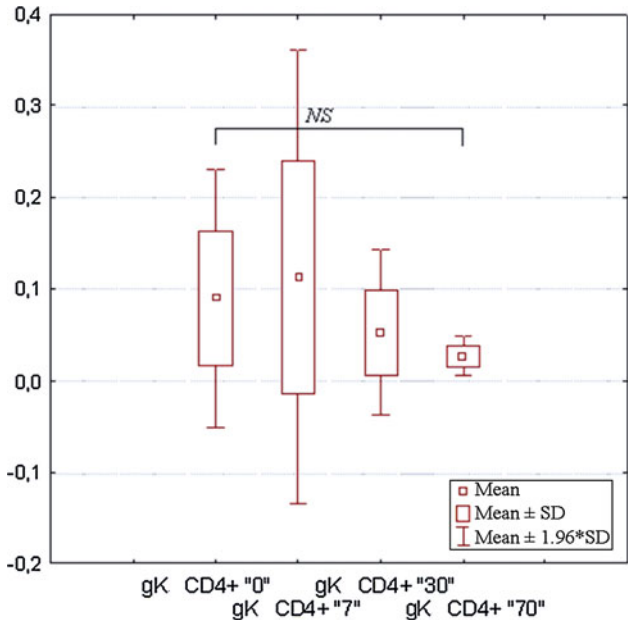

Fig. 3 Mean gK values [nS] in $CD4^+$ T cells

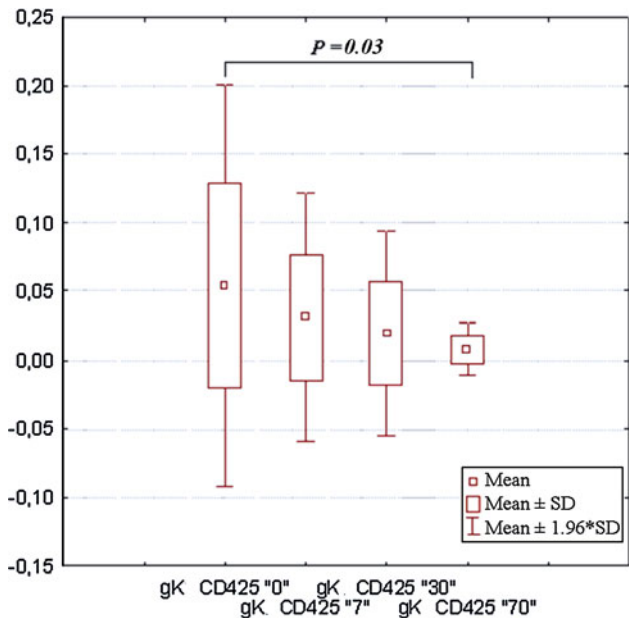

Fig. 4 Mean gK values [nS] in $CD4^+CD25^+$ T cells

venom-sensitive patients was due to the reduction of the amplitude of the whole-cell potassium currents. Reduction of the currents' amplitude may be a consequence of decrease of ion currents passing through the channels, decrease of number of active channels without changing the current passing through each channel in the cell membrane, or both. At present, it is known that changes in the whole-cell potassium conductance in T cell-mediated diseases such as sclerosis multiplex, type 1 diabetes mellitus, rheumatoid arthritis, systemic lupus erythematosus (Wulff et al. 2003; Beeton et al. 2006; Nicolaou et al. 2007), chronic renal failure (Teisseyre et al. 1996), and pollinosis (Krasnowska et al. 2005) are due to changes in channel expression rather than a decrease of the currents passing through the channels. On the basis of our results, we cannot definitively conclude which factor is responsible for the decrease of the whole-cell potassium conductance observed in our study.

It is well known that expression of Kv1.3 channels changes in the T cell cycle. Activated T cells always display more channels than resting cells; however, activity of each channel remains unaffected upon cell activation (Cahalan et al. 2001). This increase is most significant in case of the effector memory T cell (T_{EM}) subpopulation, in which the average number of active Kv1.3 channels per cell is raised from 200–300 in resting cells to 1500–1800 in activated cells (Wulff et al. 2003). In case of naive T cells and central memory T cell (T_{CM}) subpopulations, the

number of active Kv1.3 channels rises from 200–300 to about 400 channels per cell upon activation (Wulff et al. 2003). Thus, the increased number of active Kv1.3 channels per cell can be considered as a hallmark of T cell activation. Increase of the average T cell whole-cell potassium conductance caused by increased contribution of activated or preactivated cells to the entire cell population was observed in case of T cell-mediated autoimmune diseases such as sclerosis multiplex (Wulff et al. 2003), type 1 diabetes mellitus, and rheumatoid arthritis (Beeton et al. 2006) and in chronic renal failure after a long-term hemodialysis therapy (Teisseyre et al. 1996). In another autoimmune disease, systemic lupus erythematosus, the number of active Kv1.3 channels per cell was not changed significantly, but the channel compartmentalization into the immunological synapse formed between the T cell and the antigen-presenting cell was significantly accelerated, similar to what was observed in the case of preactivated healthy T cells (Nicolaou et al. 2007).

Because the increase in the number of active Kv1.3 channels per cell can be a hallmark of T cell activation, a decrease of active channels' number may be a hallmark of the cell suppression. It is well known that a selective inhibition of T cell Kv1.3 channels leads to a selective immunosuppression that may be beneficial in medical treatment of T cell-mediated autoimmune diseases such as sclerosis multiplex, type 1 diabetes mellitus, and rheumatoid arthritis (Wulff et al. 2003; Beeton et al. 2006). The selective immunosuppression is possible because in autoantigen-specific T_{EM} cells, which are involved in the diseases' pathogenesis, only Kv1.3 channels are overexpressed (Wulff et al. 2003; Beeton et al. 2006). Therefore, these cells cannot escape from suppression when Kv1.3 channels are inhibited. On the other hand, activated naive and T_{CM} cells can easily escape from suppression because of a great number of calcium-activated potassium channels, $K_{Ca}3.1$ (Wulff et al. 2003; Beeton et al. 2006).

The reduction of the $CD4^+CD25^+$ T cell whole-cell potassium conductance observed in our study group occurred progressively in the course of VIT and was statistically significant after 70 days of VIT. An assumption can be made then that the reduction resulted from the immunosuppressive effect exerted by the VIT, and not from the character and the chronic nature of the disorder. The observation raises yet another question: is the reduction caused by toxic ingredients of the bee or wasp venom, or is it an effect of the immunomodulating mechanisms of immunotherapy? Many small-molecule modulators of Kv1.3 have been identified, the best known of them being scorpion toxins (charybdotoxin, margatoxin, and kalitoxin), but there is a lack of evidence that the peptide ingredients of bee or wasp venom should exert a similar effect. What supports the assumption that the reduction of the $CD4^+CD25^+$ T cell whole-cell potassium conductance is an effect of venom-specific immunological treatment rather than of an alleged influence of toxic venom ingredients is the fact that our patch clamp experiments were performed on day 70 of immunotherapy—that is, 33 days after administering the last dose of venom.

Specific VIT exerts a powerful influence on the immunological system. Successfully treated patients develop specific T cell hyporesponsiveness or even unresponsiveness to the entire allergen, which is manifested in vivo by withdrawal of allergic symptoms and in vitro by decrease in T cell proliferation and decrease in $CD4^+$ T cell production of proallergic cytokines (Th2 cytokines) (Akdis and Akdis 2007). In both processes, the activity of Kv1.3 channels plays an important role (particularly in T cell proliferation and apoptosis) (Shieh et al. 2000; Cahalan et al. 2001; Beeton et al. 2006). The induction of tolerance appears to be a result of functional inactivation of allergen-specific $CD4^+$ T cells. Over the course of VIT, we observed the reduction of the whole-cell potassium conductance in $CD4^+$ T cells, but especially in $CD4^+CD25^+$ T cells—which means in activated $CD4^+$ T cells because CD25 is an activation marker of effector T cells.

In conclusion, taking into account the fact that an effective immunoresponse requires an appropriate spatiotemporal activation, differentiation, and elimination of peripheral T cells, and that functional activity of the Kv1.3 channels plays an important role in these processes in T cell-mediated diseases, the observed decrease of the whole-cell potassium conductance may contribute to T cell tolerance and functional unresponsiveness of these cells to the allergen in the early stages of VIT. The pathophysiological relevance of these findings needs to be investigated in further studies.

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