Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 10917–10921, September 1999
Physiology

Inhibition of T cell proliferation by selective block of Ca^{2+}-activated K^+ channels

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Communicated by Hans H. Ussing, University of Copenhagen, Copenhagen, Denmark, July 19, 1999 (received for review March 26, 1999)

ABSTRACT **T lymphocytes express a plethora of distinct ion channels that participate in the control of calcium homeostasis and signal transduction. Potassium channels play a critical role in the modulation of T cell calcium signaling, and the significance of the voltage-dependent K channel, Kv1.3, is well established. The recent cloning of the Ca^{2+}-activated, intermediate-conductance K^+ channel (IK channel) has enabled a detailed investigation of the role of this highly Ca^{2+}-sensitive K^+ channel in the calcium signaling and subsequent regulation of T cell proliferation. The role IK channels play in T cell activation and proliferation has been investigated by using various blockers of IK channels. The Ca^{2+}-activated K^+ current in human T cells is shown by the whole-cell voltage-clamp technique to be highly sensitive to clotrimazole, charybdotoxin, and nitrendipine, but not to ketoconazole. Clotrimazole, nitrendipine, and charybdotoxin block T cell activation induced by signals that elicit a rise in intracellular Ca^{2+}—e.g., phytohemagglutinin, Con A, and antigens such as *Candida albicans* and tetanus toxin in a dose-dependent manner. The release of IFN-γ from activated T cells is also inhibited after block of IK channels by clotrimazole. Clotrimazole and cyclosporin A act synergistically to inhibit T cell proliferation, which confirms that block of IK channels affects the process downstream from T cell receptor activation. We suggest that IK channels constitute another target for immune suppression.**

All mammalian cells express potassium (K^+) channels in their cell membranes, and the channels play a dominant role in the regulation of the membrane potential. The intermediate-conductance, Ca^{2+}-activated K^+ channels (IK) are present in various blood cells, endothelial cells, and epithelial cells, but not in excitable tissue. Expression of IK channel mRNA has been established in a number of tissues—e.g., spleen, thymus, peripheral blood leukocytes, and T cells (1–3).

Physiologically, IK channels are important for Cl^- secretory epithelia, and for activation of T cells. Release of intracellularly stored Ca^{2+}, induced by agonists such as ATP, results in pronounced activation of IK channels. The activation of IK channels is followed by long-lasting or oscillatory hyperpolarizations of the cell membrane. The hyperpolarizations closely reflect the activity of intracellular Ca^{2+}. The IK channel (*i*) is highly K-selective; (*ii*) is activated by submicromolar concentrations of Ca^{2+}; (*iii*) has an inwardly rectifying conductance; and (*iv*) has as the calcium sensor calmodulin, which is constitutively bound to the channels (4). In T lymphocytes, the opening of the IK channels after the initial antigen stimulation of the T cell receptor hyperpolarizes the membrane and increases the influx of calcium. Furthermore, both the amount of mRNA and the number of expressed IK channels increases by 15- 20-fold upon activation of the T lymphocytes, indicating an important role for IK channels during lymphocyte proliferation (3). Previous studies have indicated the importance of Kv1.3 channels in hyperpolarizing the membrane potential to maintain an inwardly directed driving force for the secondary influx of calcium from the extracellular medium (5). Here we provide data suggesting that IK channels dominate the K^+ conductance of the activated T cell, and thus are responsible for providing the electrochemical driving force for Ca^{2+} entry into T cells. This idea, that inhibition of IK channel activity could decrease Ca^{2+} influx and subsequent T cell proliferation, has been evaluated in the present study.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells (PMBC) were obtained from healthy volunteers as described (6). In six experiments, T cells were depleted of plastic-adherent cells and purified by using nylon wool separation as described (7). All antigen $CD4^+$ T cell lines have been characterized previously (8).

Electrophysiology. The patch-clamp experiments were performed as described previously (2).

Salt solutions. (*i*) Extracellular. High-K^+ solution: 150 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% BSA, and 10 mM Hepes (pH 7.4). (*ii*) Intracellular solution: 150 mM KCl, 1 mM EGTA, 9 mM nitrilotriacetic acid, and 10 mM Hepes (pH 7.2). Solutions of 0.955 mM $CaCl_2$ and 5.585 mM $MgCl_2$ were added to obtain final free concentrations of 1 μM and 1 mM, respectively, as calculated with EQCAL software (Biosoft, Cambridge, U.K.). Clotrimazole, nitrendipine, and ketoconazole were purchased from Sigma. Charybdotoxin was from Alomone Labs, Jerusalem, Israel. Stock solutions were prepared in DMSO (organic compounds) or in extracellular solution (charybdotoxin) and added directly to the relevant experimental solution. All chemicals were of the purest grade commercially available.

Experimental procedure. Coverslips containing resting or activated T cells were placed in a perfusion chamber and continuously superfused with extracellular salt solution. In the whole-cell voltage-clamp experiments the series resistance as well as the cell capacitance were followed on-line and compensated by 70%. Initial series resistances were below 5 MΩ and usually remained constant throughout the experiments. Experiments were invariably discharged if series resistance increased above 10 MΩ or if slow capacitance cancellation failed. All experiments were performed at room temperature (21–25°C).

Proliferation Assays. Assays were performed in culture medium (RPMI medium 1640; GIBCO) supplemented with

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Abbreviations: CRAC channel, calcium-release activated Ca^{2+} channel; IK channel, Ca^{2+}-activated intermediate-conductance K^+ channel; PPD, purified protein derivative; PHA, phytohemagglutinin.

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10% fetal calf serum, 2 mM L-glutamine, 100 mg/ml penicillin, and 100 mg/ml streptomycin (Novo, Copenhagen) in 96-well round-bottom tissue culture plates (Nunc) in a final volume of 200 μl. PBMC or T cells plus irradiated plastic-adherent cells (APC) were cultured at 5×10^4 cells per well for 5 days with or without reagents as indicated. Twelve hours before harvest, [^{3}H]thymidine (1 mCi per well; 1 Ci = 37 GBq) was added. The cells were harvested onto glass fiber filters, [^{3}H]thymidine incorporation was measured in a scintillation counter, and the results were expressed as median cpm from triplicate cultures (6–8).

Cytokine Release Assay. Cells were incubated for 24 or 48 hr with or without antigens in a humidified atmosphere as above. Harvest of the supernatants was carried out after pelleting the cells. IFN-γ was measured by using IFN-γ-specific ELISA as described elsewhere (9).

RESULTS

Electrophysiology. Whole-cell recordings of Con A-activated T cells revealed the expression of IK channels in activated T cells. A typical whole-cell experiment exhibited a slightly inwardly rectifying current (150–1000 pA) in activated T cells (not shown). In contrast, only a linear current of maximally 10 pA was observed when a voltage ramp (−100 to +100 mV, 200 ms in duration) was applied to a resting T cell. The sensitivity of the endogenous IK channel in activated T cells to clotrimazole, ketoconazole, nitrendipine, and charybdotoxin is shown in Fig. 1. Ramp currents were elicited every 5 s, and the time course of the current at −80 mV is shown (Fig. 1). Clotrimazole (1 μM), a blocker of both endogenous and cloned IK channels (1–3), blocked the current fully (n = 4). Charybdotoxin (200 nM), a peptide blocker of both Kv1.3 and IK channels, fully inhibited the IK current in a potent and reversible way (n = 4), as did nitrendipine (1 μM, n = 3). Ketoconazole (1 μM) had no effect on the IK current in T cells (Fig. 1, n = 3). These results correspond closely to those obtained on the cloned IK channel (2).

T Cell Proliferation. Using the three most potent blockers of the cloned IK channel, clotrimazole, charybdotoxin, and nitrendipine, we investigated the effect of IK channel block on T cell proliferation. Activation of T cells upon antigen stimulation is strongly inhibited when clotrimazole is used as IK channel blocker (Fig. 2). Clotrimazole is not a selective compound, and in addition to its effects on IK channels it also blocks cytochrome P450 and calcium-release-activated Ca^{2+} (CRAC) channels. Therefore, we also studied the closely related compound ketoconazole, which in contrast to clotrimazole is a more potent blocker of CRAC channels than of IK channels.

Fig. 2*A* shows the antigen-induced T cell proliferation in the presence of vehicle (control), 10 μM ketoconazole, and 10 μM clotrimazole. The T cell proliferation was estimated as the [^{3}H]thymidine incorporation 6 days after antigen stimulation. T cell activation in the presence of ketoconazole was only slightly inhibited compared with controls (23% ± 3%). In contrast, nitrendipine (10 μM), clotrimazole (10 μM), and

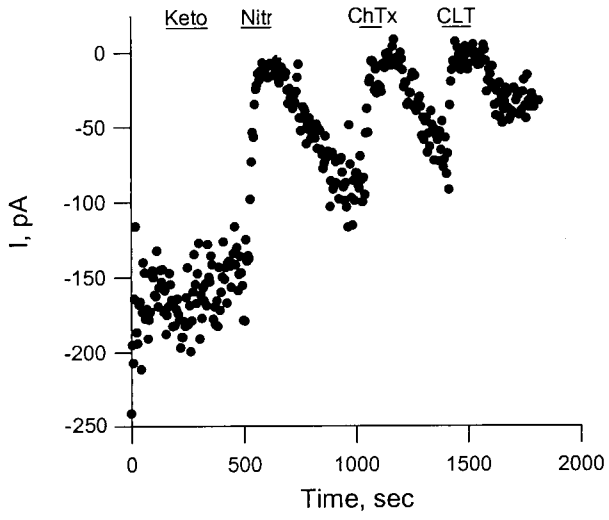

FIG. 1. Time course of whole-cell currents from a T cell activated by purified protein derivative (PPD) at −80 mV measured from voltage ramps (±100 mV every 5 s, 200-ms duration) under control conditions (extracellular K^+ solution in the bath) and during the application of the indicated compounds. The concentrations used were ketoconazole (Keto), 1 μM; nitrendipine (Nitr), 1 μM; charybdotoxin (ChTx), 200 nM; and clotrimazole (CLT), 1 μM. Free Ca^{2+} in the pipette solutions was 1 μM.

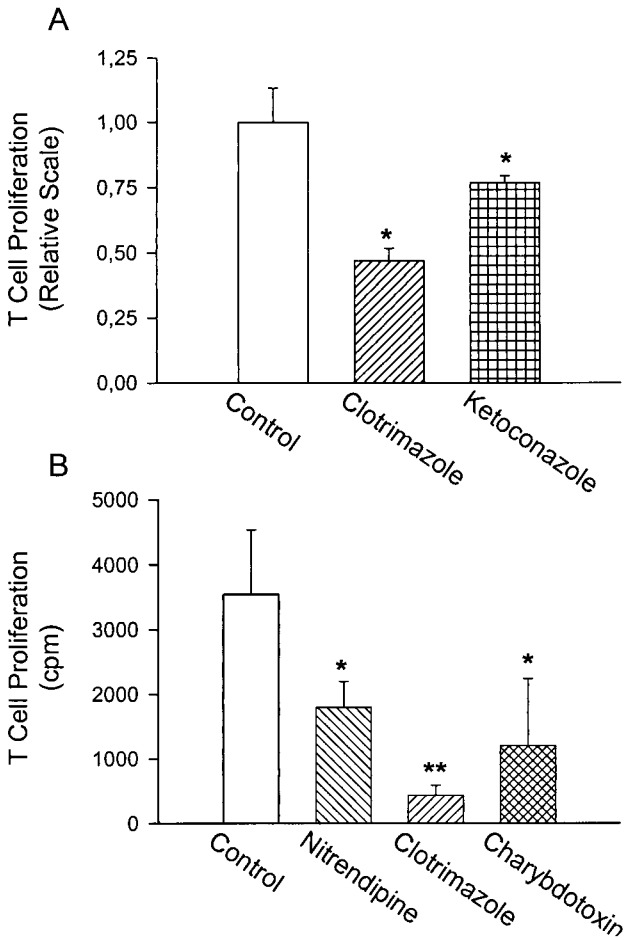

FIG. 2. (*A*) Effect of clotrimazole and ketoconazole on T cell proliferation. Cells were incubated for 5 days in culture medium with PPD and the IK channel blockers (10 μM clotrimazole or 10 μM ketoconazole), which were added 30 min before the addition of PPD. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents 12 independent experiments ± SE; control = $26 \pm 12 \times 10^3$ cpm per well; clotrimazole = $12 \pm 6 \times 10^3$ cpm per well; ketoconazole = $20 \pm 10 \times 10^3$ cpm per well. *, $P \leq 0.008$ vs. control. (*B*) Effect of nitrendipine, clotrimazole, and charybdotoxin on T cell proliferation. Cells were incubated for 5 days in culture medium with *Candida albicans* and the IK channel blockers (10 μM nitrendipine, 100 nM charybdotoxin, or 10 μM clotrimazole), which were added 30 min before the addition of *Candida albicans.* [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents 9 independent experiments ± SE for control, and 3 independent experiments in the presence of nitrendipine, clotrimazole, and charybdotoxin, respectively. *, $P \leq 0.05$ vs. control; **, $P \leq 0.01$ vs. control.

charybdotoxin (100 nM), all IK channel blockers, decreased T cell proliferation significantly (Fig. 2*B*). In the experiments shown, charybdotoxin inhibited T cell proliferation by 67%, nitrendipine by 50%, and clotrimazole by 89%.

The potency of the IK channel blockers was investigated further. A dose–response relation of IK channel block on T cell proliferation after antigen stimulation is shown in Fig. 3 *Upper*. T cell proliferation is increasingly inhibited by clotrimazole, and even at 10 μM, maximal inhibition is not yet accomplished. Both nitrendipine and charybdotoxin appear to be less potent and exert their maximal effect at a concentration of 5 μM and 10 nM, respectively (Fig. 3 *Upper*). This indicates that clotrimazole inhibits T cell proliferation by additional means other than IK channel block, maybe by interference with the intracellular Ca^{2+} store system as suggested previously for human melanoma cells (10). Taken together, the data for the IK channel blockers suggest that maximal inhibition of IK channels accounts for a 40–50% inhibition of the T cell proliferation.

During the course of the present study, a significant donor and stimulus variation was observed. For the T cell proliferation induced by mitogens the inhibition by 10 μM clotrimazole varied from 10% to 84% in the 14 donors studied, whereas the antigen-induced T cell proliferation was inhibited by 20–100% in the presence of 10 μM clotrimazole. An example of the varied suppression by the T cell response to different donors and proliferative compounds is shown in Fig. 3 *Lower*. T cells from three different donors respond differently; however the inhibition of the T cell proliferation is significant in T cells from all three donors, as clotrimazole at 10 μM inhibits approximately 40–80% of the proliferation response. Clotrimazole at 1 μM is ineffective except for donor 3. Clotrimazole inhibits antigen-mediated T cell proliferation by 50–90% when antigens such *Candida albicans*, PPD, and tetanus toxin are used.

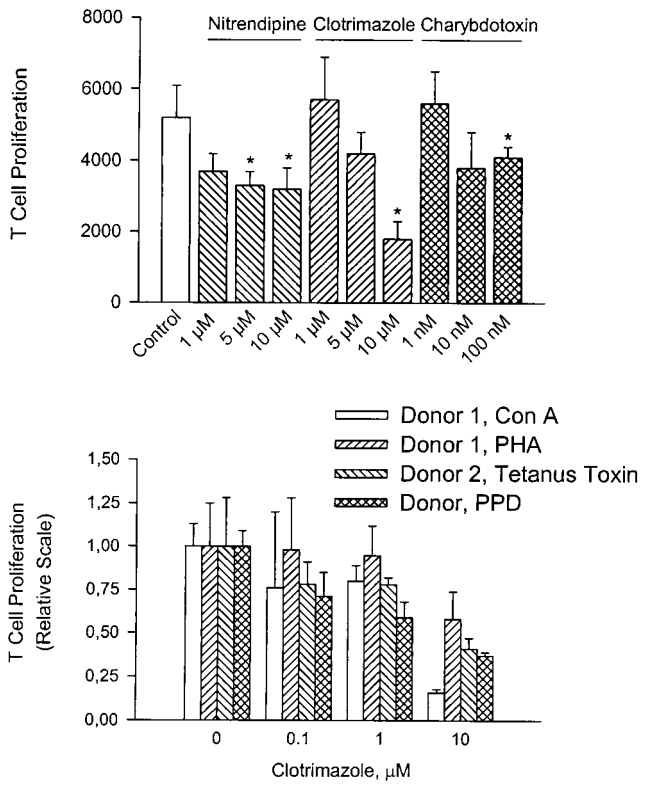

FIG. 3. (*Upper*) Dose–response relations of nitrendipine, clotrimazole, and charybdotoxin on T cell proliferation. Cells were incubated for 5 days in culture medium with PPD. The IK channel blockers, in the concentrations indicated, were added 30 min before the addition of antigen. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents nine independent experiments ± SE for control, and three independent experiments in the presence of nitrendipine, clotrimazole, or charybdotoxin, respectively. *, $P \leq 0.05$ vs. control. Six other experiments using *Candida albicans* or tetanus toxin as the antigen challenge gave similar results. (*Lower*) Donor variability of the clotrimazole effect on T cell proliferation. PHA, phytohemagglutinin. Cells from three different donors were incubated for 5 days in culture medium with mitogen (ConA or PHA) or antigen (tetanus toxin or PPD) and 10 μM clotrimazole, which was added 30 min before the addition of *Candida albicans*. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents three independent experiments ± SE.

Clotrimazole (10 μM) inhibits the T cell proliferation responses to alloantigens in different T cell subsets such as $CD4^+$, $CD45RA^+$, and CD45RO T cells, whereas no effect is observed in an Epstein–Barr virus-transformed B cell line (results not shown). Activation of T cells by bacterial superantigens, such staphylococcal enterotoxins (SEA and SEE) is inhibited by 10 μM clotrimazole. Clotrimazole inhibits expression of CD25 and of the MHC II complex, but has no effect on expression of CD45 (results not shown). The reason for the different sensitivity of antigen- and mitogen-induced T cell proliferation to IK channel block is not obvious. It might indicate different pathways of T cell activation, the antigen-induced response involving the inositol trisphosphate/calcium signaling pathway, and the mitogen-induced pathway being more broad in its mechanism of T cell activation, including both Ca^{2+}-dependent and Ca^{2+}-independent activation pathways.

Fig. 4 shows the release of IFN-γ in unstimulated T cells and in T cells stimulated by PPD in the presence of vehicle or clotrimazole (10 μM). Clotrimazole inhibits the release of

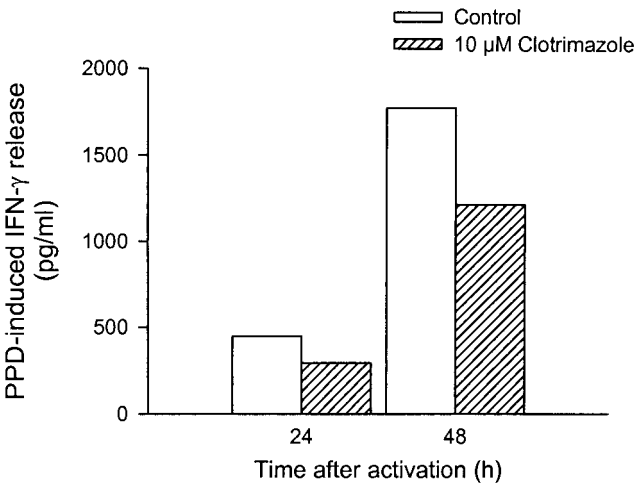

FIG. 4. Effect of clotrimazole on release of IFN-γ from activated T cells. Cells were incubated with or without PPD for the indicated periods before measurement of IFN-γ in supernatants. Similar results were obtained in two additional experiments using activation of T cells with tetanus toxin and *Candida albicans*.

IFN-γ from activated T cells by 34% in the experiment presented in Fig. 4, suggesting an inhibition of the orchestrated T cell response to antigens by the channel blocker. The average effect was an inhibition by 29% ± 5% ($n = 3$).

The activation of T cells via the T cell receptor is associated with activation of phospholipase C and subsequent elevation of intracellular Ca^{2+} released from intracellular stores and mobilized from the extracellular medium. The rise in $Ca^{2+}{}_i$ leads to the activation of the Ca^{2+}/calmodulin-dependent protein phosphatase 2B, also known as calcineurin, which is the target for immunosuppressive compounds such as FK506 and cyclosporin A. Concomitant inhibition of IK channels and calcineurin is expected to be additive at submaximal concentrations. Fig. 5 shows the effect of IK channel block on cyclosporin A inhibited T cell proliferation after antigen-induced T cell activation. T cells were stimulated with antigen in the presence of cyclosporin A or both cyclosporin A and clotrimazole. T cell proliferation was assayed by [^{3}H]thymidine incorporation 6 days after stimulation. The cyclosporin A-mediated inhibition of T cell proliferation is shifted leftwards by 10 μM clotrimazole, from a 50% inhibition of proliferation at approximately 25 nM cyclosporin A to half-maximal inhibition at 2.5 nM cyclosporin A. This result suggests that the antigen-induced T cell proliferation is highly sensitive to both IK channel block and inhibition of calcineurin (Fig. 5).

DISCUSSION

The data presented here demonstrate that the block of IK channels by nitrendipine, clotrimazole, and charybdotoxin leads to inhibition of the proliferative T cell response and inhibition of the release of IFN-γ from activated T cells. None of the compounds studied is selective for IK channels, but when the results are put together they support this conclusion. The inhibition of the proliferative T cell response is caused by specific interference with the pathways that induce a rise in intracellular Ca^{2+}, including those stimulated by tetanus toxin, anti-CD3, *Candida albicans*, PHA, PPD, or Con A. The increase in intracellular Ca^{2+} is recognized as an obligatory step in the cascade of signals that finally results in T cell proliferation. The early rise in Ca^{2+} is followed by a sustained increased level that results from the influx of extracellular

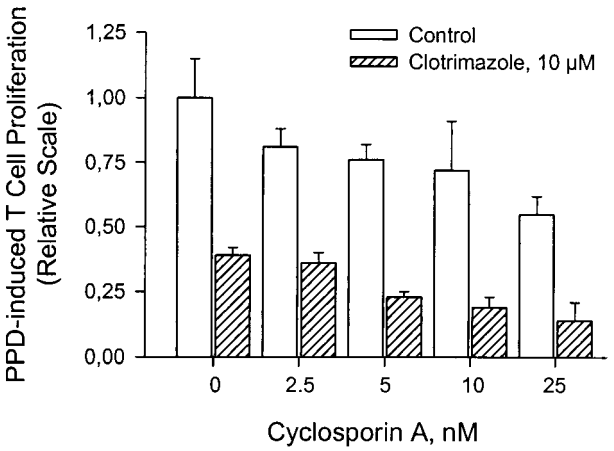

FIG. 5. Effect of clotrimazole on cyclosporin A-mediated inhibition of T cell proliferation. Cells were incubated for 5 days in culture medium with PPD in the presence of cyclosporin A alone as indicated or cyclosporin A plus clotrimazole. Clotrimazole (10 μM) was added 30 min before the addition of antigen. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents three independent experiments ± SE. *, $P \leq 0.05$ vs. control. Eleven other experiments using *Candida albicans,* tetanus toxin, Con A, or PHA as the antigen/mitogen challenge gave similar results.

Ca^{2+}. It was previously shown that T cell proliferation is inhibited in the absence of extracellular Ca^{2+}, and attention was focused on the role of Kv1.3 in the early cellular events following stimulation of the T cell receptor (5). Indirect results obtained by use of the selective Kv1.3 channel blockers margatoxin and noixiustoxin did lead to the conclusion that Kv1.3 dominates the K conductance of the T cell, and that inhibition of Kv1.3 is sufficient to mediate block of T cell proliferation (5). The possible involvement of IK channels in T cell activation has not been addressed previously, but there has been speculations about their playing a significant role in postactivation and secondary immune phenomena (12). Theoretically, IK channels are likely to be even more important for setting the membrane potential of T cells, since they do not close at negative membrane potentials, unlike Kv1.3, and the expression of IK channels is increased by 15- to 20-fold during T cell activation (3).

T cell activation is accompanied not only by a rise in intracellular Ca^{2+} concentration but also by membrane hyperpolarization when stimulated by antigens or mitogens. The long-lasting hyperpolarization is the consequence of the activation of IK channels by Ca^{2+} and closely reflects the activity of intracellular Ca^{2+}. The question raised in this paper is then: how does inhibition of IK channels influence the T cell proliferation? We suggest that depolarization of T cells mediated by block of IK channels is sufficient to inhibit T cell proliferation by elimination of the inward driving force for Ca^{2+} through CRAC channels. Preliminary results from Fanger and Cahalan[¶] suggest that Th1 helper cells are subject to an increased Ca^{2+} influx after calcium depletion as compared with Th2 cells. The difference can be accounted for by a difference in IK channel expression with Th1 cells expressing significantly larger numbers of IK channels than do Th2 cells. Therefore, an additional role of IK channels in T cells might be to allow memory T cells to respond more quickly to a secondary exposure to antigens by a rapid rise in intracellular Ca^{2+} concentration caused by influx from the extracellular medium. Whether the donor variability found in this study can be accounted for by a differential expression of IK channels in the different subsets of T cells has not been investigated.

In conclusion, the present investigation suggests an additional mechanism for immune suppression. IK channels are expressed solely in peripheral tissue, and the pharmacology of IK channels is unique. These characteristics of the IK channel make it an ideal target for immune suppression. Further, it will be interesting to determine the relevance of IK channels as a target for immune suppression *in vivo*.

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Vibeke Meyland-Smith is gratefully acknowledged for expert technical assistance. B.S.J. holds the Paul Bergsøe Research Fellowship from the Danish Natural Science Research Council. N.K.J. holds a fellowship from The Carlsberg Foundation. The work was supported by the Danish Health Research Council, The Beckett Foundation, and the Velux Foundation (to S.P.O.).

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