

Extracellular K⁺ and Opening of Voltage-gated Potassium Channels Activate T Cell Integrin Function: Physical and Functional Association between Kv1.3 Channels and β 1 Integrins

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Abstract

Elevated extracellular K⁺ ([K⁺]_o), in the absence of “classical” immunological stimulatory signals, was found to itself be a sufficient stimulus to activate T cell β 1 integrin moieties, and to induce integrin-mediated adhesion and migration. Gating of T cell voltage-gated K⁺ channels (Kv1.3) appears to be the crucial “decision-making” step, through which various physiological factors, including elevated [K⁺]_o levels, affect the T cell β 1 integrin function: opening of the channel leads to function, whereas its blockage prevents it. In support of this notion, we found that the proadhesive effects of the chemokine macrophage-inflammatory protein 1 β , the neuropeptide calcitonin gene-related peptide (CGRP), as well as elevated [K⁺]_o levels, are blocked by specific Kv1.3 channel blockers, and that the unique physiological ability of substance P to inhibit T cell adhesion correlates with Kv1.3 inhibition. Interestingly, the Kv1.3 channels and the β 1 integrins coimmunoprecipitate, suggesting that their physical association underlies their functional cooperation on the T cell surface. This study shows that T cells can be activated and driven to integrin function by a pathway that does not involve any of its specific receptors (i.e., by elevated [K⁺]_o). In addition, our results suggest that undesired T cell integrin function in a series of pathological conditions can be arrested by molecules that block the Kv1.3 channels.

Key words: T cells • extracellular K⁺ • potassium channels • integrins • neuroimmunomodulation

Introduction

It is generally accepted that T cells are physiologically activated to function upon stimulation by specific antigens, superantigens, and immunocyte-secreted factors such as cytokines and chemokines. These specific stimuli convey their messages to the T cells through binding to specific surface-expressed receptors, such as the TCR, and the receptors to cytokines, chemokines, and growth factors.

Here, we investigated whether the activation of human resting T cells, resulting in rapid integrin-mediated functions, can occur without any such “classical” immunological triggering stimuli, and not necessarily through specific receptors. Specifically, we questioned whether modifying the ionic composition of the extracellular milieu, which

takes place under conditions of injury, stress, and even normal neurotransmitter stimulation, and which is typically characterized by elevated levels of extracellular K⁺ ions (1–10), can drive T cells into two integrin-mediated T cell functions: adhesion to an extracellular matrix (ECM)¹ component, and migration.

These integrin-mediated T cell activities are carried out only by activated T cells, and are required in a broad spectrum of normal and diseased conditions in general, and in inflammation and injury in particular. The activation of

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¹Abbreviations used in this paper: 4AP, 4-aminopyridine; CGRP, calcitonin gene-related peptide; ChTX, charybdotoxin; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DiBAC₄(3), bis-(1,3-dibutylbarbituric acid) trimethine oxonol; ECM, extracellular matrix; FN, fibronectin; [K⁺]_o, extracellular K⁺; KaTx, kaliotoxin; MgTx, margatoxin; MIP, macrophage-inflammatory protein; NoTx, noxioustoxin; Sub P, substance P; TEA, tetraethylammonium; TTX, tetrodotoxin; VLA, very late antigen.

the integrin moieties is a dynamic, rapid, and often transient process, and is the primary factor that allows T cells to attach to their ligands on cell surfaces and matrices (11, 12).

To date, the exact cellular mechanisms by which different physiological stimuli activate integrins on lymphocyte membranes have not been fully understood (13). Moreover, finding ways to selectively arrest integrin-mediated adhesion and migration of T cells remains a major challenge in various pathophysiological conditions, such as those involving T cell-mediated inflammation, graft rejection, autoimmunity, and malignancy.

In view of the above, we also investigated the possible existence of a pivotal T cell membranal component that operates as an on/off switch of $\beta 1$ integrin activation. We postulated that depolarization that leads to the opening of the T cell voltage-gated K^+ channels may serve as a common merging step through which various integrin-activating molecules on one hand, and elevated extracellular K^+ ($[K^+]_o$) levels on the other, induce $\beta 1$ integrin function of T cells.

Materials and Methods

T Cells. Human T cells were purified from the peripheral blood of healthy donors as follows: the leukocytes were isolated on a Ficoll gradient, washed, and incubated on Petri dishes (37°C , 10% CO_2 humidified atmosphere). After 2 h, the nonadherent T cells were removed and incubated on nylon wool columns (Novamed Ltd.). Nonadherent T cells were eluted, washed, and passed through human $\text{CD}3^+$ cell purification columns (Cedarlane Laboratories Ltd.). The resulting cell population was $>92\%$ T cells, as evaluated by $\text{CD}3$ staining shown in our previous studies. Myelin basic protein (amino acid sequence 87–99)-specific $\text{CD}4^+$ T cell lines, of the Th2 phenotype, were obtained from SJL/J mice as described (14).

Membrane Potential Measurements by Flow Cytometry. Purified normal human T cells were suspended ($\sim 3 \times 10^6/\text{ml}$) in serum-free medium for 1 h (37°C , 10% CO_2) to assure steady-state conditions. The cells were then distributed into individual tubes ($\sim 3 \times 10^6/\text{tube}$), washed, and resuspended either in normal RPMI or in rich K^+ RPMI solutions. After an additional 30-min incubation (37°C , 10% CO_2), 300 nM bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄[3]) dye (Molecular Probes) was added to the cells, and 5–10 min later the cells were applied to the FACSsort™ (Becton Dickinson); fluorescence was read at 488 nm.

Adhesion Assay. Adhesion of purified normal human T cells, isolated from a fresh human blood sample, was assayed in fibronectin (FN)-coated microtiter flat-bottomed wells, as described previously (15). In brief, normal human T cells, purified from a fresh blood sample, were labeled with $\text{Na}_2^{51}\text{CrO}_4$, washed, and resuspended in adhesion medium (RPMI 1640 supplemented with 0.1% BSA, 1 mM Ca^{2+} , 1 mM Mg^{2+} , and 1% HEPES buffer). The cells were then pretreated (30 min, 37°C) with rich K^+ solutions ranging from 10 to 50 mM K^+ , as indicated in the specific experiment, or with the desired proadhesive molecule, and added to FN-coated (1 $\mu\text{g}/\text{well}$; Sigma Chemical Co.) microtiter flat-bottomed 96-well plates (10^5 cells/well). The plates were placed in a humidified incubator (37°C , 30 min, 10%

CO_2), and then washed thoroughly several times with PBS to remove nonadherent T cells. The adherent T cells were lysed with 1% Tween 20 in 1 N NaOH, and the radioactivity in the resulting supernatants was determined in a γ -counter. For each experimental group, results were expressed as the mean cpm \pm SD of bound T cells from quadruplicate wells. Adhesion of treated T cells to BSA-coated (rather than FN) wells, and adhesion of untreated T cells to FN-coated wells were the standard negative controls.

Involvement of Specific Integrins in Elevated $[K^+]_o$ -induced T Cell Adhesion to FN. ^{51}Cr -labeled T cells were treated (30 min) with mAbs (2 $\mu\text{g}/\text{ml}$) specific to human integrin moieties: the CD29 molecule (the common $\beta 1$ integrin chain) or the $\alpha 2$ and $\alpha 5$ chains of the very late antigen (VLA) integrins (Serotec). The T cells were then suspended in rich K^+ solutions and seeded in FN-coated microtiter plates. The plates were returned to the incubator for an additional 30-min incubation, and T cell adhesion was determined as described previously.

Integrin-mediated Migration Assay. Cell migration was measured as described previously (16) by using a modification of the Boyden chamber assay. Cells were resuspended at $5 \times 10^6/\text{ml}$ either in normal RPMI 1640 or in rich K^+ RPMI solutions, both supplemented with 1% BSA. The cells were then placed in the upper wells of a 48-well chamber (50 $\mu\text{l}/\text{well}$). The lower well of the migration chamber contained either normal RPMI (control) or a chemoattractant (macrophage-inflammatory protein [MIP]-1 β , 0.1 ng/ml). The upper and lower wells were separated by a 8- μm -pore polycarbonate filter (Poretics), which was precoated (2 h, 37°C in a 10% CO_2 incubator) with bovine FN (Sigma Chemical Co.) solution (0.1%). The chambers were incubated at 37°C in a 10% CO_2 incubator for 2 h.

The filters were fixed, stained with solutions from a Diff-Quik staining kit (Dade Diagnostika GmbH), and mounted on slides using histological methods. Cell migration was measured by counting the number of cells migrating through the filter that adhered to the lower part of the filter (facing the bottom chamber). The cells that did not migrate remained either in the upper chamber or adhered to the upper part of the filter, and were not counted. Counting the migrating cells was performed using a $10\times$ microscope magnification in six replicate fields. The numbers represent the mean \pm SD of migrating cells per field.

Immunofluorescence Staining for Activated Integrins. Freshly purified normal human T cells were incubated (18 h, 37°C , 7.5% CO_2 humidified atmosphere) in normal culture medium. The cells were washed, counted, and 5×10^5 cells were incubated in elevated K^+ -RPMI medium (CO_2 incubator, 30 min). When needed, samples were preincubated with a Kv1.3 blocker for 20 min before being exposed to elevated K^+ depolarizing medium. The blockers remained in the cell suspension throughout the experiment. The cells were then incubated with the HUTS 21 mAb (5 $\mu\text{l}/\text{sample}$; a gift from F. Sanchez-Madrid, Hospital de la Princesa, Madrid, Spain), returned to the 37°C incubator for 10 min, and transferred to 4°C for 45 min. The cells were then washed and resuspended in PBS, to which FITC-conjugated goat anti-mouse antibody (1 $\mu\text{g}/\text{sample}$, 45 min, 4°C ; Jackson ImmunoResearch Laboratories) was added. After a final wash, the cells were resuspended in cold staining buffer (PBS, 1% FCS, 0.01% sodium azide) and analyzed by FACSsort™ (Becton Dickinson).

Electrophysiology. Purified human T cells, plated on poly-L-lysine-coated glass coverslips, were placed in a 1-ml recording chamber mounted on the stage of an Axiovert 35 inverted microscope (Carl Zeiss, Inc.). The whole-cell configuration of the

patch-clamp technique (17) was used to record the potassium macroscopic currents at room temperature ($22 \pm 1^\circ\text{C}$). Signals were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments), filtered <1 kHz through a 4-pole Bessel low-pass filter. Data were sampled at 2.5 kHz and analyzed using pClamp 6.0.2 software (Axon Instruments) and an IBM-compatible 486 computer in conjunction with a DigiData 1200 interface (Axon Instruments). The patch pipettes were pulled from borosilicate glass (fiber filled) with a resistance of 4–8 M Ω and were filled (in mM) with 120 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 10 Hepes, and 11 glucose at pH 7.4. The external solution contained (in mM) 140 NaCl, 1 MgCl₂, 2 CaCl₂, 5 KCl, 11 glucose, and 10 Hepes, at pH 7.4. All drugs used here were externally perfused, and the whole-cell recordings were performed after the effect reached saturation. Series resistances were in the range of 8 to 15 M Ω and were compensated by 90–95%. Before the peak amplitude measurements, traces were leak-subtracted by the Clampfit program (pClamp 6.0.2 software) and further analyzed using Axograph 3.0 software (Axon Instruments). The I/V curve was fitted by the Boltzmann distribution: $I/I_{\text{max}} = a/\{1 + \exp[(V_{50} - V_K)/s]\}$, where V_{50} is the half-maximal activation and s is the slope of the curve. All of the data are expressed as mean \pm SEM.

Immunoprecipitation and Immunoblotting. Membrane fractions were prepared as described (18) from fresh purified normal human T cells, incubated overnight with IL-2 (50 U/ml of human recombinant IL-2; Chiron Corp.). Immunoprecipitation and immunoblotting were carried out as described (18). Polyclonal anti-

Kv1.3 was purchased from Alomone Labs. For immunoprecipitation of the $\beta 1$ integrins, we used a mouse anti-human CD29 (clone 3S3; Serotec), and for immunoblotting the $\beta 1$ integrins we used either the B44 (Chemicon) or the JB1B (Serotec) anti-CD29 antibodies, according to the manufacturer's instructions. Control anti-human IL-2 receptor antibody was purchased from Serotec.

Results

T Cells Are Depolarized by Elevated Levels of Extracellular Potassium Ions. To test our hypothesis that T cells can be activated and driven to integrin-mediated function by depolarization and the subsequent opening of voltage-gated potassium channels, we first established conditions for producing net depolarization, without any additional factors. Thus, we prepared a series of RPMI solutions containing increasing concentrations of K⁺ ions (on the expense of decreasing concentrations of Na⁺ ions), exposed purified normal human peripheral T lymphocytes to these solutions, and measured the membrane potential by flow cytometry using the voltage-sensitive oxonol dye, DiBAC₄(3). Fig. 1 A confirms the correlation between the dye fluorescence intensity and the membrane potential (19, 20), and shows that human T cells are indeed depolarized (right shift of the fluorescence curve) when exposed to solutions containing elevated levels of K⁺ ions.

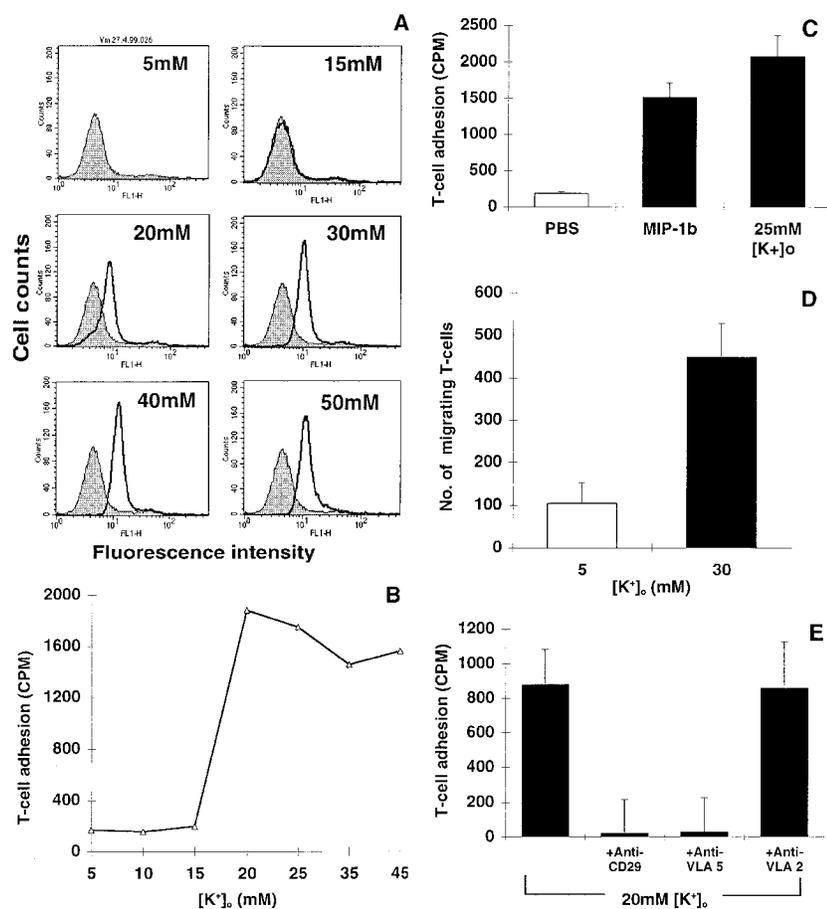


Figure 1. Elevated [K⁺]_o levels depolarize human T cells and induce $\beta 1$ integrin-mediated adhesion to FN and motility. (A) Depolarization of human T cells by elevated [K⁺]_o levels. Human peripheral blood T cells were incubated in either normal RPMI, or in RPMI solutions containing elevated concentrations (10–50 mM) of K⁺, and supplemented with the voltage-sensitive oxonol dye DiBAC₄(3) (300 nM). The fluorescence intensity (x-axis), proportional to the membrane potential (references 19, 20), was measured by FACS[®]. (B) T cell adhesion to FN induced by elevated [K⁺]_o levels. Purified human T cells were labeled with ⁵¹Cr, washed, and resuspended in either normal RPMI adhesion medium or in elevated [K⁺]_o RPMI solutions, and then added to FN-coated wells. The results represent the mean cpm of four to six independent adhesion microtiter wells. One experiment representative of six is depicted. (C) T cell adhesion induced by MIP-1 β (20 ng/ml) or 25 mM [K⁺]_o. (D) Elevated [K⁺]_o increases the chemotactic migration of resting human T cells towards MIP-1 β (0.1 ng/ml). The y-axis corresponds to the number of cells that migrated to the lower chamber and adhered to the bottom part of an FN-coated filter separating the two chambers. The cells were counted after fixation and staining. The results represent mean \pm SD of four replicates of one of two experiments performed. (E) Purified human T cells were pretreated with mAbs to the human integrins CD29 (the common $\beta 1$ chain), VLA-5 ($\alpha 5$), and VLA-2 ($\alpha 2$), and then exposed to an RPMI solution containing 20 mM [K⁺]_o. T cell adhesion to FN was then determined. The results represent the mean cpm \pm SD of four to six independent adhesion microtiter wells. One experiment representative of two.

Elevated Levels of Extracellular Potassium Ions Trigger Integrin-mediated T Cell Adhesion and Migration. We then tested whether elevated $[K^+]_o$ can trigger the adhesion of T cells to FN, a major component of the ECM. The adhesion of T cells to FN is known to be mediated by the $\beta 1$ integrins, and to require their activation (15). Thus, we incubated a similar number of $Na_2[^{51}Cr]O_4$ -labeled human T cells in solutions containing increasing concentrations of K^+ , and monitored their adhesion to FN-coated microtiter well plates.

Fig. 1 B shows that exposure of the human T cells to elevated levels of $[K^+]_o$ was sufficient to trigger their adhesion to FN. The dose-response relationship shows that T cell adhesion was induced by $[K^+]_o > 15$ mM, and that the activation is an "all or none" phenomenon. Thus, once reaching or exceeding the threshold, activation occurs, and stronger depolarizations caused by higher $[K^+]_o$ have no further augmenting effect. These results suggest that it is sufficient to depolarize the T cells to an extent that leads to the opening of only a fraction of the voltage-gated channels in order to trigger integrin-mediated adhesion.

The levels of T cell adhesion induced by elevated $[K^+]_o$ were found to be comparable to those evoked by the potent proadhesive and proinflammatory chemokines MIP-1 β (21; Fig. 1 C) and regulated upon activation, normal T cell expressed and secreted (RANTES; data not shown).

We next investigated whether elevated levels of $[K^+]_o$, in addition to causing T cells to adhere, could also augment their migration. We tested both migration towards chemoattractants such as MIP-1 β and IL-2 (16) and random migration (motility). T cells were thus allowed to migrate through a filter, from an upper chamber to a lower chamber containing (or not) a chemoattractant. At the end point, only the cells that migrated to the lower chamber (16) were counted. Fig. 1 D shows that a much higher number of T cells migrated towards MIP-1 β after preincubation (90 min) in an increased $[K^+]_o$ (30 mM) medium compared with normal (5 mM $[K^+]_o$) medium. Similar results were obtained when IL-2 was used as the chemoattractant (data not shown). Interestingly, elevated $[K^+]_o$ levels substantially enhanced the migration of T cells, even under conditions where no chemoattractant was present in the lower chamber (data not shown), showing that the overall random migration was increased. Together, these results suggest that elevated $[K^+]_o$ levels augment the basic motility of T cells.

Elevated Levels of Extracellular Potassium Activate T Cell $\beta 1$ Integrin Moieties. T cell recognition and adhesion to FN is mediated primarily by the $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins (13, 15, 22). To demonstrate that the adhesion to FN induced by elevated $[K^+]_o$ was mediated by these integrins, we used mAbs specific for the $\beta 1$ and $\alpha 5$ moieties, and a control mAb directed against a nonrelevant $\alpha 2$ moiety of the VLA-2 integrin. Fig. 1 E shows that adhesion to FN of resting T cells induced by 25 mM $[K^+]_o$ was specifically and significantly inhibited by the presence of an mAb against CD29 (the common $\beta 1$ integrin chain), and by the anti-VLA-5 mAb specific to the $\alpha 5$ integrin chain.

The adhesion was not influenced by the anti-VLA-2 ($\alpha 2$) mAb.

To obtain further direct evidence that elevated levels of $[K^+]_o$ activate the T cell $\beta 1$ integrin moieties and thereby promote T cell adhesion to ECM components, we used the HUTS 21 mAb (23), which recognizes only the activated form of the $\beta 1$ integrin, and is therefore termed an "activation reporter" antibody, whereas the second is an anti-CD29 mAb that inhibits the $\beta 1$ integrin ligation. Fig. 2 A shows the immunofluorescence of T cells incubated either in an elevated $[K^+]_o$ medium or in normal medium, and then stained with the HUTS 21 mAb followed by an FITC-conjugated secondary anti-mouse antibody. The clear shift in the fluorescence curve after T cell incubation with high concentration of $[K^+]_o$ compared with normal $[K^+]_o$ levels indicates that the $\beta 1$ integrins were activated, since only as such could they be recognized by the HUTS 21 mAb. Such activation was completely inhibited by a specific Kv1.3 blocker, margatoxin (MgTx; at 0.1 μ M), as shown in Fig. 2 B. These results suggest that elevated levels of $[K^+]_o$ activate the T cell $\beta 1$ integrin moieties and that this activation is mediated by opening the voltage-gated K^+ channels.

Taken together, these results confirmed that the T cell adhesion to FN induced by elevated levels of $[K^+]_o$ (like T cell adhesion induced by chemokines, cytokines, neuropeptides, and phorbol esters [15, 16, 21, 23]) is mediated by the $\beta 1$ integrins.

Various Physiological Molecules Trigger T Cell $\beta 1$ Integrin Function by Opening Voltage-gated K^+ Channels. Can depolarization and the subsequent opening the voltage-gated K^+ channels be a common mechanism by which different physiological stimuli (other than elevated levels of $[K^+]_o$) activate the T cell $\beta 1$ integrin-mediated functions? To answer this question, we tested whether the proadhesive effects of the chemokine MIP-1 β and the neuropeptide calcitonin gene-related peptide (CGRP [15]) are blocked by various voltage-gated K^+ channel blockers. Fig. 2 C shows that three highly specific Kv1.3 blockers, MgTx, kaliotoxin (KaTx), and noxiustoxin (NoTx), profoundly blocked MIP-1 β -induced T cell adhesion. Moreover, and in a comparable manner, all three blockers fully inhibited CGRP-induced T cell adhesion to FN (data not shown). The concentrations at which these highly specific Kv1.3 channel blockers inhibited the integrin-mediated T cell adhesion fall well within the concentration range (1–100 nM) previously reported to be effective for blockage of other T cell functions (19, 24–26).

To further support the involvement of the Kv1.3 channel in the $\beta 1$ integrin-mediated T cell function, and to investigate the possible contribution of other ion channels, we tested a series of additional channel blockers regarding their effect on MIP-1 β and CGRP-driven T cell adhesion to FN. The relevant blockers included charybdotoxin (ChTX) and tetraethylammonium (TEA), which block both voltage-gated and large conductance Ca^{2+} -dependent K^+ channels, and 4-aminopyridine (4-AP) and quinine, which block only voltage-gated K^+ channels. The nonrele-

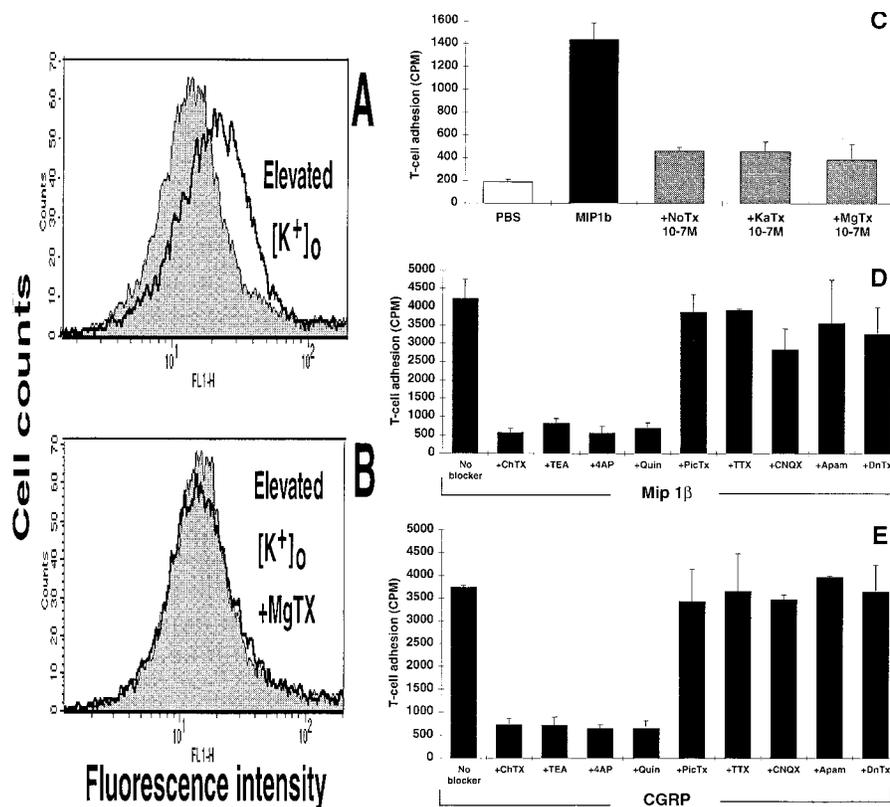


Figure 2. (A) Elevated $[K^+]_o$ levels activate $\beta 1$ integrin moieties. Human T cells were incubated either with a normal 5 mM RPMI solution (gray line, filled histograms) or with RPMI solutions containing elevated levels of K^+ (black line, open histograms) and stained with the anti-activated $\beta 1$ integrin (HUTS 21) mAb. (B) Human T cells were treated as in A, but in the presence of MgTx (10^{-7} M). (C) Inhibition of MIP-1 β -induced T cell adhesion by Kv1.3-specific blockers MgTx, KaTx, and NoTx. (D and E) Inhibition of MIP-1 β and CGRP-induced T cell adhesion by additional relevant and nonrelevant ion channel blockers ChTx (10^{-8} M), TEA (10^{-3} M), 4-AP (10^{-4} M), quinine (Quin, 10^{-5} M), picrotoxin (PicTx, 10^{-5} M), TTX (10^{-6} M), CNQX (10^{-7} M), apamin (Apam, 5×10^{-9} M), and dendrotoxin (DnTx, 10^{-7} M). The results represent the mean cpm \pm SD of four to six independent adhesion microtiter wells.

vant blockers (controls) included: apamin, a small conductance Ca^{2+} -dependent K^+ channel blocker; dendrotoxin, a blocker of the Kv1.1 and Kv1.2 voltage-gated K^+ channels, which are not expressed in human T lymphocytes; picrotoxin, a Cl^- channel blocker; tetrodotoxin (TTX), an Na^+ channel blocker; and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a glutamate/ α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-gated receptor channel blocker.

The results showed that T cell adhesion induced by MIP-1 β (Fig. 2 D) and by CGRP (Fig. 2 E) is specifically blocked by the four voltage-gated K^+ -channel blockers ChTx, TEA, 4-AP, and quinine and unaffected by all other nonrelevant blockers.

When the results of the usage of the above 13 different blockers are taken together, and considering the similar pharmacological profiles and order of potency of the Kv1.3 blockers, it appears that the mechanism by which MIP-1 β and CGRP induce T cell adhesion involves the opening of the voltage-dependent Kv1.3 channel.

Is the T cell adhesion induced by elevated levels of $[K^+]_o$, such as that induced by MIP-1 β and CGRP, also mediated by the Kv1.3 channel? If so, it should be blocked by the specific Kv1.3 blockers. Fig. 3, A–C, shows that 25 mM $[K^+]_o$ -induced T cell adhesion was inhibited, in a dose-dependent manner, by the three highly specific Kv1.3 blockers MgTx (A), KaTx (B), and NoTx (C). Complete inhibition was exerted also by ChTx, TEA, 4-AP, and quinine, whereas the other nonrelevant blockers had no effect (Fig. 3 D). Once again, the concentrations of the

blockers found effective to block the elevated $[K^+]_o$ -induced integrin-mediated T cell adhesion are in line with those used to block other T cell activities and functions (19, 24–26). These findings show that T cell adhesion induced by elevated levels of $[K^+]_o$ (as by MIP-1 β and CGRP) requires the opening of voltage-gated Kv1.3 channels.

The Unique Physiological Ability of Substance P to Block T Cell Adhesion Correlates with an Inhibitory Effect on Voltage-gated K^+ Currents. Can the T cell Kv1.3 channels be blocked by physiologically relevant molecules, rather than by toxins? This question arises because, although numerous factors promote integrin-mediated T cell adhesion to FN, to the best of our knowledge no physiological compounds known to block this function have so far been identified. However, in a previous study (15), we found that substance P (Sub P), through its T cell-expressed NK1 receptor, and at physiological concentrations, has a so far unique ability to block integrin-mediated adhesion of T cells to FN, induced by a variety of proadhesive factors. Accordingly, we tested whether Sub P can also inhibit T cell adhesion induced by elevated levels of $[K^+]_o$. Fig. 3, E and F, shows that Sub P completely blocks T cell adhesion induced by either CGRP or elevated levels of $[K^+]_o$. The dose-response curve for this inhibition resembles that observed for the inhibition by Sub P of other proadhesive molecules, including MIP-1 β (15).

Can the inhibitory effect of Sub P on T cell adhesion be explained, at least in part, by the blocking of Kv1.3 channels? To answer this question, we tested, by electrophysio-

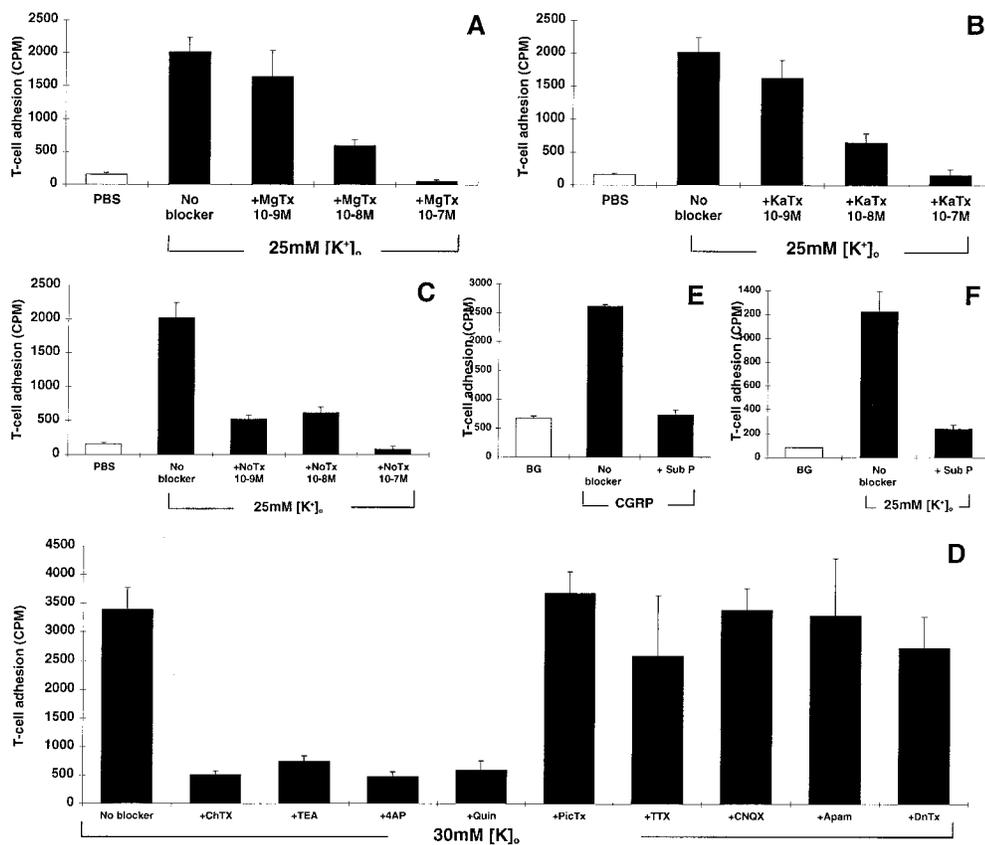


Figure 3. Elevated $[K^+]_o$ -induced T cell adhesion is blocked by Kv1.3 channel blockers and Sub P, and unaffected by non-relevant blockers. ^{51}Cr -labeled human T cells were incubated in elevated $[K^+]_o$ RPMI solutions and added with 10^{-9} to 10^{-7} M MgTx (A), KaTx (B), or NoTx (C), or with an additional series of relevant and nonrelevant ion channel blockers (D; as specified in the legend to Fig. 2, D and E). The adhesion of the T cells to FN-coated wells was then measured. Similarly, human T cells were exposed either to CGRP (10^{-8} M; E) or to an elevated (25 mM) $[K^+]_o$ -containing solution (F), in the presence or absence of Sub P (10^{-8} M), and then tested for their adhesion to FN. BG, background adhesion. Each figure represents one of three experiments performed; data are expressed as the mean \pm SD of four replicate microtiter wells.

logical techniques, whether Sub P, in a similar manner to its primary action on neurons (27), reduces the T cell membrane voltage-dependent $[K^+]_o$ conductance. Whole-cell membrane currents were recorded from normal human T cells, in the presence of Sub P, using the voltage-clamp

technique (17). Known blockers (MgTx, NoTx, ChTX, quinidine, and apamin) served as positive and negative controls. These experiments were performed while keeping in mind that one cannot expect a parallel potency relationship between the block of T cell adhesion and the block of

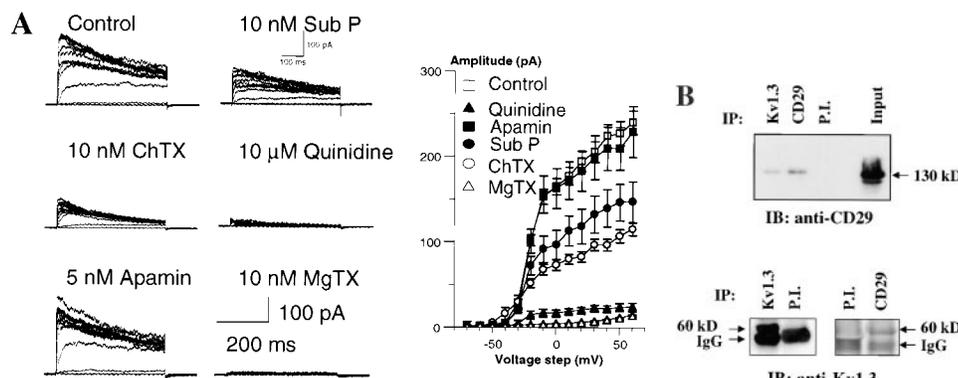


Figure 4. (A) Electrophysiological recordings of whole-cell K^+ currents from purified human T cells, in the presence of Sub P (10 nM) and various Kv1.3 channel blockers. Typical whole-cell K^+ currents were obtained by stepping the membrane from a holding potential of -80 mV to $+60$ mV (500 ms) in 10-mV increments. The effect saturated after ~ 8 min. Quinidine (10 μ M) and ChTX (10 nM) inhibited the maximal amplitude of the K^+ currents by 90 and 46%, respectively. Apamin, as a negative control, had no inhibitory effect on the K^+ currents. The peak amplitudes of

the control (\square , $n = 13$), apamin (5 nM; \blacksquare , $n = 8$), ChTX (10 nM; \circ , $n = 11$), Sub P (10 nM; \bullet , $n = 10$), MgTX (10 nM; \triangle , $n = 5$), and quinidine (10 μ M; \blacktriangle , $n = 9$) were plotted against the voltage step and fitted using the Boltzmann distribution. Statistical difference is expressed by SE. (B) Physical association between Kv1.3 channels and $\beta 1$ integrins in human T cells. Top, The extract of membrane fraction prepared from purified normal human T cells was subjected to parallel immunoprecipitations (IP) with either anti- $\beta 1$ integrin (CD29), anti-Kv1.3, or preimmune (P.I.) negative control antibodies. Immunoblotting of these precipitated immune complexes, as well as of a portion of the lysate (Input, 10%), was carried out using an anti- $\beta 1$ integrin antibody. The $\beta 1$ integrin is detected in membrane extracts precipitated with either the anti- $\beta 1$ integrin or the anti-Kv1.3 antibody, as well as in the lysate. Bottom, Similar immunoprecipitation as in the top panel, and blotting with the anti-Kv1.3 antibody. The Kv1.3 protein is detected in membrane extracts precipitated with either the anti-Kv1.3 or the anti- $\beta 1$ integrin antibody. Two different gels from two independent experiments are shown to illustrate the immunoprecipitation of Kv1.3 either by anti-Kv1.3 antibodies (left) or by anti-CD29 antibodies (right).

Kv1.3 channels measured in an electrophysiological setting, since the two experimental systems differ in almost every single parameter (e.g., composition of the extracellular medium, T cell condition [i.e., whether or not the T cell is intact or in suspension], temperature, incubation time, and the number of cells examined at a time).

Representative whole-cell currents are shown in Fig. 4 A. The membrane of untreated human T cells was stepped from a holding potential of -80 mV to $+60$ mV, in 10 -mV increments. After application of Sub P (10 nM), the maximal K^+ currents were reduced by 39% , from 241 ± 14 pA ($n = 10$) to 148 ± 24 pA ($n = 10$). A 100 -fold higher Sub P concentration (1 μ M) reduced the current by $\sim 45\%$, to 109 ± 11 pA ($n = 10$).

The amplitude measurements were performed at the peak of the currents, in response to $+60$ mV. Application of MgTx (10 nM) and quinidine (10 μ M) inhibited almost completely (90%) the K^+ currents (Fig. 4 A), from 204 pA ± 14.3 ($n = 13$) to 21.3 ± 3.5 ($n = 9$), whereas ChTX (10 nM) inhibited the K^+ currents by 46% , from 204 pA ± 14.4 ($n = 13$) to 109.3 ± 7.5 ($n = 11$). In agreement with the T cell adhesion experiments, the application of apamin (5 nM) had no effect on the T cell K^+ currents (202.8 ± 25.7 , $n = 8$, vs. 204 ± 14.3 pA).

These results demonstrate that Sub P blocks the voltage-gated K^+ currents in T cells (like known Kv1.3 blockers), and suggest that its inhibitory effect on T cell adhesion is mediated, at least partially, by Kv1.3 channel blocking. These results also suggest that it is sufficient to close only a fraction of the Kv1.3 channels in order to block the T cells $\beta 1$ integrin function.

Naturally, Sub P may affect the integrins and the adhesion process by multiple mechanisms, including K^+ channel-dependent and K^+ channel-independent pathways. Nevertheless, in view of the recent suggestion that the Kv1.3 channel may be an attractive target for immune suppression (19), Sub P can now be considered as a relevant physiological effector.

A Physical Association between the T Cell Voltage-gated Kv1.3 Channels and $\beta 1$ Integrins. Regarding the mechanism by which the T cell voltage-gated K^+ channels "talk" to the $\beta 1$ integrins and consequently affect their activation state, we investigated whether there is a physical association among these membranal components. Such an association may allow direct communication between these two entities. Accordingly, we prepared membranes from purified normal human T cells and performed parallel immunoprecipitations with the anti- $\beta 1$ integrin mAb and the anti-Kv1.3 antibody. As controls, the T cell membrane extracts were immunoprecipitated with an irrelevant anti-IL-2 receptor antibody, and with IgG from nonimmunized (pre-immune) animals. Immunoblotting of these different immunoprecipitations with the anti-CD29 antibody (Fig. 4 B, top) showed the presence of the $\beta 1$ integrin in the membrane extracts immunoprecipitated with either the anti-CD29 or the anti-Kv1.3 antibody, but not in membrane extracts precipitated with the anti-IL-2 receptor antibody (data not shown) or with the control IgG from non-

immunized mice. The immunoblots showed an apparent molecular weight of ~ 130 kD for the $\beta 1$ integrin. This is consistent with previous studies (28, 29) showing that the apparent molecular weight of the $\beta 1$ subunit ranges from 115 to 140 kD, depending on the maturity of the proteins, i.e., mainly on posttranslational modifications such as glycosylation.

Reciprocally, when the same immunoprecipitation products were blotted with the anti-Kv1.3 channel antibody, the Kv1.3 protein (mol. wt. 60 kD) was recognized in membranes immunoprecipitated with either the anti-Kv1.3 or the anti-CD29 antibody, but not in those precipitated with the control antibodies (Fig. 4 B, bottom).

Taken together, these results show that the anti- $\beta 1$ integrin antibody precipitated the Kv1.3 protein (as well as the $\beta 1$ integrin itself), and that the anti-Kv1.3 antibody precipitated the $\beta 1$ integrin protein (as well as the Kv1.3 protein). The results suggest that the Kv1.3 channels and the $\beta 1$ integrins are physically associated in normal human T cells, and may therefore have the potential to communicate directly with one another.

Discussion

Extracellular elevated K^+ ion concentrations were reported in various pathophysiological situations, such as injury to the central and peripheral nervous systems, trauma, tissue ischemia (cerebral and myocardial), epilepsy, local inflammation, and metabolic poisoning (1–8), as well as after neurotransmitter stimulation (9, 10). Neurons, like many other cell types, can be depolarized by elevated levels of $[K^+]_o$.

Interestingly, a recent study (9) showed an elevation of the extracellular K^+ level (of up to 16 mM above the normal $[K^+]_o$) in the myoendothelial space, effluxing from endothelial cells in response to stimulation by acetylcholine and muscarinic agonists. This endothelium-derived elevated $[K^+]_o$ was shown to shift the membrane potential of adjacent smooth muscle cells, and to rapidly relax the arteries. These observations indicate that fluctuations in $[K^+]_o$ levels, originating within the blood vessel itself, are important in regulating mammalian blood pressure and flow.

Can such a simple modification of the ionic extracellular environment also be a trigger for T cell function? Taken together, the results presented here demonstrate that an elevated $[K^+]_o$ level is indeed a sufficient trigger for activation of T cell $\beta 1$ integrin moieties, and thereby for activation of integrin-mediated functions. Thus, we propose that such a mechanism, not realized thus far, does exist, whereby a mere excess of extracellular K^+ ions in the environment of a modified tissue initiates T cell integrin-mediated immune reactions. The latter reactions are absolutely essential for extravasation and the subsequent migration of T cells from the blood vessels into tissues, and for various other immune reactivities (22).

Our proposed scheme (Fig. 5), illustrating our findings and current thoughts and incorporating several relevant publications (1–10), suggests that an elevated concentration

of $[K^+]_o$ may occur in at least two immunologically relevant situations: (a) upon injury, elevated $[K^+]_o$ levels may result from a nonspecific efflux through a leaky membrane of injured, damaged, metabolically impaired, or stressed cells (other than T lymphocytes); and (b) under normal or inflamed conditions, local nerve endings may release neurotransmitters that may cause a channel-mediated efflux of K^+ ions from different non-T target cells. Recently reported examples are the effect of acetylcholine on endothelial cells (9) and the effect of *N*-methyl-d-aspartate (NMDA) on neurons (10).

We propose that the elevated $[K^+]_o$ from these two global sources may depolarize neighboring or patrolling T cells, leading to Kv1.3 gate opening and integrin activation.

In parallel, neurotransmitters may directly stimulate T cells through their respective receptors (shown here for CGRP and in our recent studies [14, 15] also for somatostatin and neuropeptide Y). Such stimulation may have the ability to depolarize the T cells, and may follow a similar integrin-activation path. In addition, neurotransmitters can directly induce other T cell functions, such as cytokine secretion, as shown previously in our recent study (14). Hence, the combined activity of locally secreted neurotransmitters may represent the rapid beneficial contribution of the nervous system to the undergoing function of the immune system in a troubled locus.

The mechanism by which the elevated $[K^+]_o$ level triggers $\beta 1$ integrin-mediated T cell activity involves depolar-

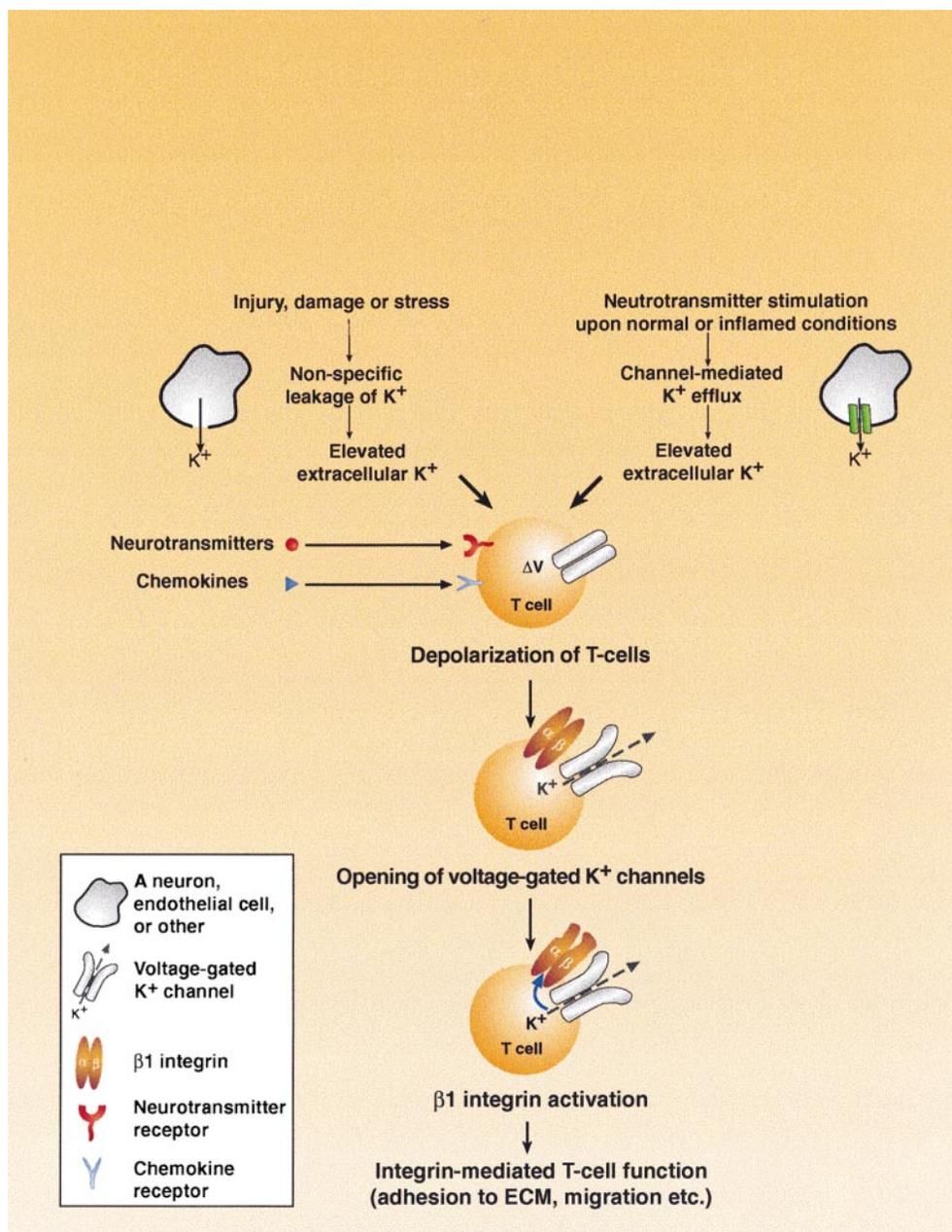


Figure 5. A speculated scheme for the activation of T cell $\beta 1$ integrin function by elevated extracellular K^+ , and the crucial role of the Kv1.3 channels. See Discussion for details.

ization and the subsequent opening of Kv1.3 voltage-gated K⁺ channels, which are suggested in this study to be physically associated with the β1 integrins. Based on our results, we hypothesize that this phenomenon reflects a “tyranny of the minority,” whereby it is sufficient to open only a fraction of the Kv1.3 channels in order to activate the T cell β1 integrin function, or to close only a fraction of the channels in order to block it. We further speculate that the opening of the Kv1.3 channels after depolarization is associated with a conformational change of the channel, which in turn imposes a conformational change of the neighboring β1 integrin moieties, leading to their activation. This, taken together with previous observations showing that T cell depolarization caused by voltage-gated K⁺ channels blockers does not allow activation of the cells (24), suggests that the above Kv1.3 conformational change and/or efflux of K⁺ ions through the newly opened channels, rather than the modulation of the membrane potential per se, may be the major factor contributing to the activation of the surface-expressed integrins and to the subsequent integrin-mediated function.

Although our study provides the first evidence of a physical and functional interaction between voltage-gated K⁺ channels and β1 integrin moieties in T lymphocytes, a functional interaction between integrins and the G protein-activated inward rectifier K⁺ (GIRK) channels was recently observed in the heart and brain (30). The GIRK channels, through their conserved amino acid sequence Arg-Gly-Asp (RGD) sequences (absent in Kv1.3 channels), may directly bind to integrins. Moreover, integrins have been previously shown to be linked to the activity of K⁺ channels (other than Kv1.3) in other cell types (31–34).

In T cells, the voltage-gated K⁺ channels (35, 36) are responsible for setting the membrane potential and for regulating the cellular volume after exposure to hypoosmotic solutions (37). The expression of the Kv1.3 (also termed *n* type) channel in murine T cells varies consistently with the cellular developmental state and functional class (37, 38). Numerous studies have shown that K⁺ channel blockers can inhibit T cell function, concluding that the voltage-gated K⁺ channels are required for T lymphocyte activation (19, 24, 25, 35–37, 39). The results of the present study demonstrate that the Kv1.3 channel plays a critical role in a sequence of events that leads from external stimuli (such as specific chemokines and neurotransmitters on the one hand, and elevated [K⁺]_o on the other) to the activation and function of the T cell β1 integrins.

In addition, this study identifies novel physiological modulators of the Kv1.3 channels, and thus of integrin-mediated T cell activity: the neuropeptide CGRP, the chemokine MIP-1β, and elevated extracellular K⁺ levels as activators, and in contrast, the neuropeptide Sub P as a blocker.

This study was supported by grants to M. Levite from the Volkswagen-Stiftung Foundation and the Rochlin Foundation.

Submitted: 20 September 1999

Revised: 16 December 1999

Accepted: 4 February 2000

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