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An Amiloride-Sensitive and Voltage-Dependent Na⁺ Channel in an HLA-DR-Restricted Human T Cell Clone¹

Zhong-Fang Lai,^{2,3*} Yu-Zhen Chen,^{3†} Yasuharu Nishimura,[†] and Katsuhide Nishi*

We investigated changes in voltage-gated Na⁺ currents and effects of extracellular Na⁺ on proliferation in HLA-DR-restricted human CD4⁺ αβ T cells after stimulation with a non-self antigenic peptide, M12p54–68. In the absence of antigenic peptide, neither single (*n* = 80) nor APC-contacted (*n* = 71) T cells showed voltage-gated inward currents recording with whole-cell patch-clamp techniques, even with Ca²⁺ and Na⁺ ions present in the perfusion solution. However, with the same recording conditions, 31% (26 of 84) of APC-contacted T cells stimulated with the antigenic peptide showed voltage-dependent inward currents that were elicited from –60 mV. The inward currents were not inhibited in extracellular Ca²⁺-free conditions or in the presence of 1 mM NiCl₂. However, they were completely inhibited in extracellular Na⁺-free conditions, which were made by replacing Na⁺ with iso-osmotic *N*-methyl-D-glucamine or choline. The Na⁺ currents were insensitive to tetrodotoxin, a classical blocker of Na⁺ channels, but were dose-dependently inhibited by amiloride, a potassium-sparing pyrazine diuretic. Furthermore, the Ag-specific proliferative response of T cells was completely inhibited in Na⁺-free Tyrode's solution and was suppressed by amiloride in a dose-dependent manner. Our findings suggest that activation of amiloride-sensitive and voltage-gated Na⁺ channels would be an important step to allow an adequate influx of Na⁺ and maintain a sustained high Ca²⁺ level during T cell activation. *The Journal of Immunology*, 2000, 165: 83–90.

T cell activation is initiated upon engagement of TCRs with an antigenic peptide in the context of MHC molecules and a costimulatory signal from APC. The signaling during T cell activation involves phosphorylation of multiple subunits of TCR/CD3 complexes, which are internalized after engagement of TCR with its ligands (1, 2). After ligating TCR with an MHC peptide, a rapid increase in intracellular protein-tyrosine phosphorylation occurred, and the intracellular calcium concentration ([Ca²⁺]_i)⁴ increased, an essential step in the pathway toward T cell activation, ultimately leading to proliferation, differentiation, or cell death (3).

Activation of several ionic channels, including Ca²⁺, K⁺, and Cl[–] channels, is important in the early phase of T cell activation. For example, the sustained elevation of [Ca²⁺]_i plays a crucial role during T cell activation and proliferation (4–6). A high level of [Ca²⁺]_i is required for activation and translocation of nuclear transcription factors, including NF-AT, NF-κB, and JNK, all of which are involved in *trans*-activation of several genes, including the IL-2 gene (7, 8). Although earlier electrophysiologic examinations suggest that human T lymphocytes do not express voltage-gated

Ca²⁺ channels (reviewed in Ref. 9), to maintain the TCR-activation-induced sustained elevation of [Ca²⁺]_i, a voltage-independent Ca²⁺ conductance known as calcium release-activated calcium channels is subsequently activated in response to TCR activation-induced depletion of intracellular Ca²⁺ stores (10–12).

On the other hand, transmembrane movement of sodium ions via Na⁺ channels is important for the maintenance of cellular physiological functions in a wide variety of cell types. Activation of voltage-gated Na⁺ channels induces a rapid Na⁺ influx and evokes action potentials in many cells, including neurons and cardiac and smooth muscle cells (13–17). For lymphocytes, although it was reported that an increase in the intracellular Na⁺ concentration ([Na⁺]_i) was observed in mouse splenocytes stimulated by Con A (18), and that Na⁺ was essential for entry into the proliferation cycle in pig lymphocytes (19), the precise role of Na⁺ channels in activation of T cells remains to be elucidated. Earlier studies showed that a small voltage-dependent Na⁺ inward current was recorded in a small percentage of human PBL, and that the current was sensitive to 100 nM tetrodotoxin (TTX) or was blocked in an external Na⁺-free environment (20). However, because TTX had no inhibitory effect on PHA-induced mitogenesis, previous studies suggested that if Na⁺ channels were present in T cells, they were not necessary for PHA-induced mitogenesis.

In the present study we have identified amiloride-sensitive and voltage-gated Na⁺ currents in the Ag-stimulated human T cells, but not exerted in nonstimulated T cells. We analyzed the effects of extracellular Na⁺ ions on Ag-specific T cell proliferation during stimulation with a physiological TCR ligand, MHC, plus an antigenic peptide. Our findings suggest that the activation of voltage-gated Na⁺ channels would play an important role during T cell activation.

Materials and Methods

Peptides and T cell clone

The peptide M12p54–68 (NRDLEQAYNELSGEA) and a T cell clone, YN5–32, were prepared as previously described (21–23). In brief, the CD4⁺ αβ T cell clone YN5–32 restricted by HLA-DR4 (DRA*0101 +

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⁴ Abbreviations used in this paper: [Ca²⁺]_i, intracellular free calcium concentration; [Na⁺]_i, intracellular sodium concentration; TTX, tetrodotoxin; NMDG, *N*-methyl-D-glucamine; fura-2/AM, fura-2/acetoxymethyl-ester; IS, interpeptide solution; ES, external solution; 4-AP, 4-aminopyridine; I_{Na}, Na⁺ current.

DRB1*0406) was established by stimulating PBMC with soluble M12p54–68 peptide as previously described (21). The peptide M12p54–68, which corresponds to aa residues 54–68 of the streptococcal M protein derived from group A β hemolytic streptococcal strain 12 (24) was used and was synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu, Tokyo, Japan), based on the F-moc strategy used in our laboratory. The peptide was purified by reverse-phase HPLC (Millipore, Bedford, MA).

Electrophysiology

Ionic currents were recorded in the whole-cell patch-clamp configurations as described previously (25). T cells were perfused with Tyrode's solution (external solution (ES)) containing 140 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl₂, 2.5 mM CaCl₂, 5.5 mM glucose, and 10 mM HEPES, and the pH was adjusted to 7.4 by adding NaOH. Pipettes were pulled from a vertical two-stage puller (PB-7, Narishige, Tokyo, Japan) and filled with an inter-pipette solution (IS) containing 140 mM KCl, 2.0 mM MgCl₂, 1.0 mM EGTA, and 10 mM HEPES, adjusted with KOH to pH 7.2. The resistance of pipettes was 4–6 M Ω . Membrane currents were acquired with an Axopatch-1B and were analyzed using the pCLAMP software program 6.04 (Axon Instruments, Foster City, CA). Once pulsed with 100 nM peptide for 5 h, the L cell transfectants expressing HLA-DR4 (1×10^5 cells) were mixed with 1×10^5 T cells and immediately placed on poly-L-lysine-coated glass coverslips 10 min before experiments. The cell size was ranged from 8 to 10 μ m in both peptide-stimulated and nonstimulated T cells. The contacted T cells could be distinguished from L cells under a microscope by their small size. The membrane capacitance was measured using a method described by Huynh and colleagues (26) and showed no difference between control and peptide-stimulated groups (membrane capacitance, 0.81 ± 0.03 , and 0.89 ± 0.05 μ F/cm², respectively). To record the outward currents, IS contained a K⁺-rich solution as described above. To record inward currents, IS was replaced with a K⁺-free solution containing 140 mM CsCl, 2.0 mM MgCl₂, 1.0 mM EGTA, and 10 mM HEPES, and the pH was adjusted to 7.2 by adding NaOH. T cells were perfused with a normal Tyrode's solution containing 1 mM quinidine (in some cases with 2 mM 4-aminopyridine (4-AP)), and then the ionic currents were recorded. To observe the effects of extracellular Na⁺, Na⁺-free solutions were made by replacement of Na⁺ with iso-osmotic *N*-methyl-D-glucamine (NMDG) or choline chloride. The pH of the Na⁺-free solution made by replacement of Na⁺ with NMDG was adjusted to 7.4 with HCl, whereas the pH of the choline-replaced Na⁺-free solution was adjusted to 7.4 with Tris-(hydroxymethyl)amino methane.

Cell proliferation

T cells (3×10^4 cells/well) were cultured in triplicate in 96-well micro-culture plates together with irradiated PBMC (1.5×10^5 cells/well) prepulsed with or without M12p54–68 peptide. Incorporation of [³H]thymidine was measured after 72 h in culture. During the last 18 h of culture, cells were pulsed with 1 μ Ci of [³H]TdR (sp. act., 6.7 μ Ci/mM), and cells were harvested. Radioactivity was measured using a scintillation counter. In some experiments T cells were incubated in a low Na⁺ Tyrode's solution (40 mM Na⁺ and 100 mM NMDG were added), and then [³H]TdR incorporation was investigated as described above.

Measurements of [Ca²⁺]_i

Measurements of [Ca²⁺]_i with fura-2/AM (Dojindo Laboratories, Kumamoto, Japan) were performed using a video-imaging system (ARGUS-50/CA, Hamamatsu Photonics, Hamamatsu, Japan) as described in our recent studies (27). The ratio of 340-nm/380-nm images was collected from the T cells stimulated with the antigenic peptide presented by HLA-DR4 molecules in normal Tyrode's solution, Ca²⁺-free or Na⁺-free solution, respectively, and then stored on a magneto optical disk every 4 s for later analysis.

Reagents and statistics

All salts used to prepare solutions and poly-L-lysine or other pharmacological agents were purchased from Sigma (St. Louis, MO). Data were analyzed by basic statistical methods, including two-tailed Student's *t* test (unpaired), and were expressed as the arithmetic mean \pm SD. *p* < 0.05 was considered statistically significant.

Results

Voltage-dependent Na⁺ currents in a human CD4⁺ T cell clone stimulated with HLA-DR-peptide complexes

Ionic currents were recorded using whole-cell patch-clamp techniques from the human T cell clone stimulated with or without an antigenic peptide, M12p54–68 in the context of HLA-DR4 (DRA*0101 + RB1*0406). In control resting T cells not stimulated with the peptide, 80 cells in a single state and 80 cells in a state of contact with L cells expressing HLA DR4 molecules, as APC, revealed the same pattern of outward K⁺ currents. Fig. 1A shows a representative recording of the outward currents activated from –50 mV in response to a series of depolarizing step pulses from –80 to +60 mV. A voltage-dependence upon depolarization pulses was evident. After T cells contacted APC prepulsed with a fully agonistic peptide, M12p54–68, the ionic currents observed in Ag-stimulated T cells could be divided into two types. In one, the amplitude of voltage-gated outward K⁺ currents (without inward currents) in the contacted T cells (64 cells) increased over that seen in resting T cells (Fig. 1, B and C) as reported by other investigators (3, 9, 28). These outward K⁺ currents were blocked by application of either quinidine (1 mM; *n* = 6) or 4-AP (2 mM; *n* = 3). In 23 contacted T cells, besides the enhanced outward currents, voltage-gated inward currents were also observed.

To observe possible inward currents in T cells contacting with APC prepulsed with the fully agonistic peptide, M12p54–68, the patch-pipette solution was changed to a K⁺-free IS containing 140 mM CsCl, 2.0 mM MgCl₂, 1.0 mM EGTA, and 10 mM HEPES, pH 7.2, and ionic currents were investigated in both single and contacted T cells perfused with ES containing 1 mM quinidine (in some cases with 2 mM 4-AP). The observation was began at 10 min after T cells and APC were placed on coated glass coverslips. In the absence of antigenic peptide, neither single T cells (*n* = 80; data not shown) nor APC-contacted T cells (*n* = 71) showed voltage-gated inward currents, even with Ca²⁺ and Na⁺ ions present in the perfusion solution (Fig. 1D). However, with the same recording conditions, ~31% (26 of 84) of the contacted T cells stimulated with the antigenic peptide showed voltage-dependent inward currents. The inward currents were activated from –60 mV and increased in amplitude with a voltage-dependent manner; the peak amplitude was obtained at about 0 mV. The inward currents appeared about 10–30 min after antigenic stimulation, and there was no appearance after Ag stimulation was longer than 30 min. Fig. 1, E and F, showed representative inward currents evoked by depolarization step pulses from –80 to +70 mV at a holding potential of –80 mV (Fig. 1E) and their I-V curves (Fig. 1F), respectively.

Pharmacological characteristics of the inward currents in T cells stimulated with Ags

To identify which cation involves TCR activation-induced inward currents, a Ca²⁺-free or Na⁺-free ES was used, respectively. Fig. 2 shows representative recordings of inward currents obtained from different T cells contacted with APC and prepulsed by peptides under five conditions: 1) Ca²⁺-free ES plus 1 mM EDTA, 2) normal ES plus 1 mM NiCl₂, 3) normal Ca²⁺ ES plus 0.1 mM TTX, 4) normal Ca²⁺ with Na⁺-free ES in which Na⁺ was replaced by iso-osmotic NMDG, and 5) Na⁺-free ES in which Na⁺ was replaced by choline. To block outward K⁺ currents, the IS containing 140 mM CsCl and 1.0 mM EGTA was used, and the T cells were perfused with a normal Tyrode's solution containing 1 mM quinidine or 2 mM 4-AP (see *Materials and Methods*). Then, whole-cell patch was performed at a holding potential of –80 mV,

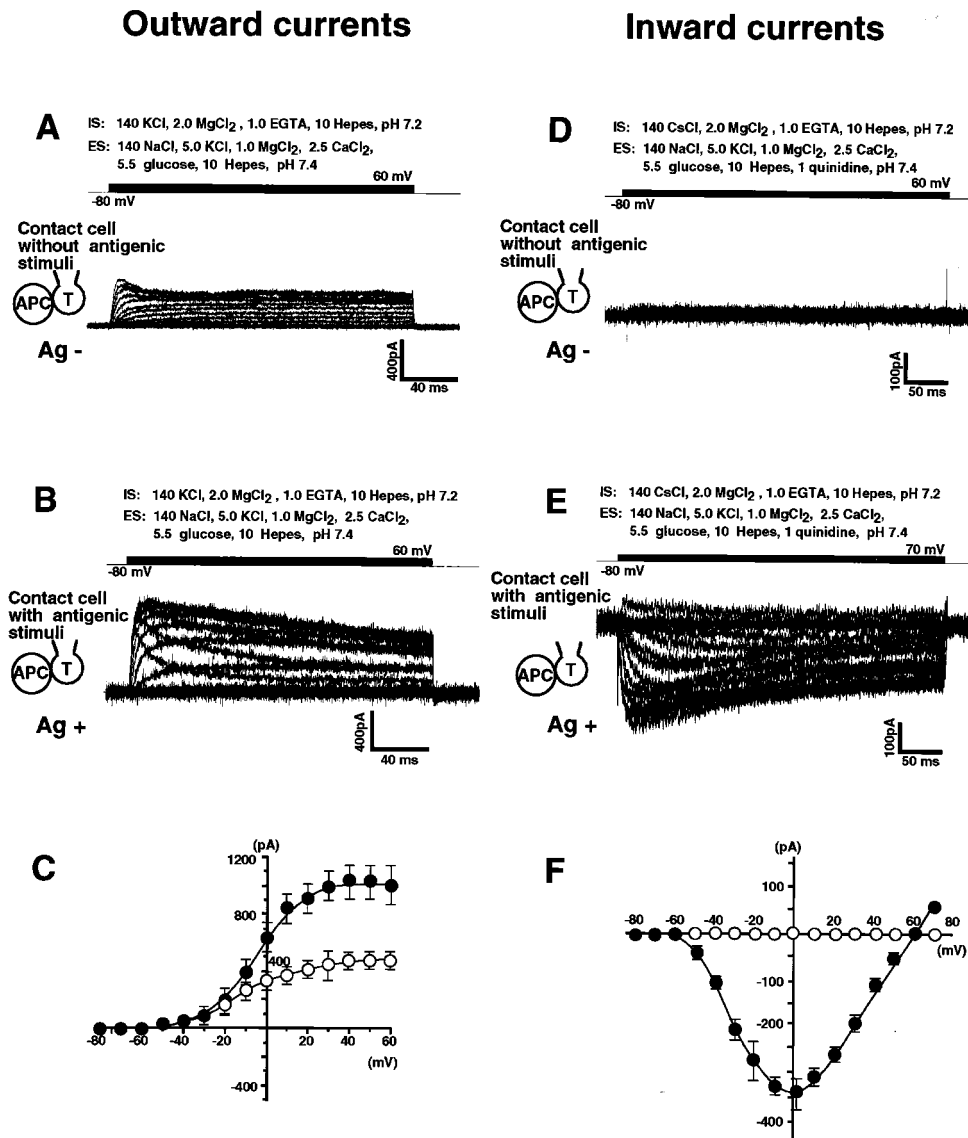
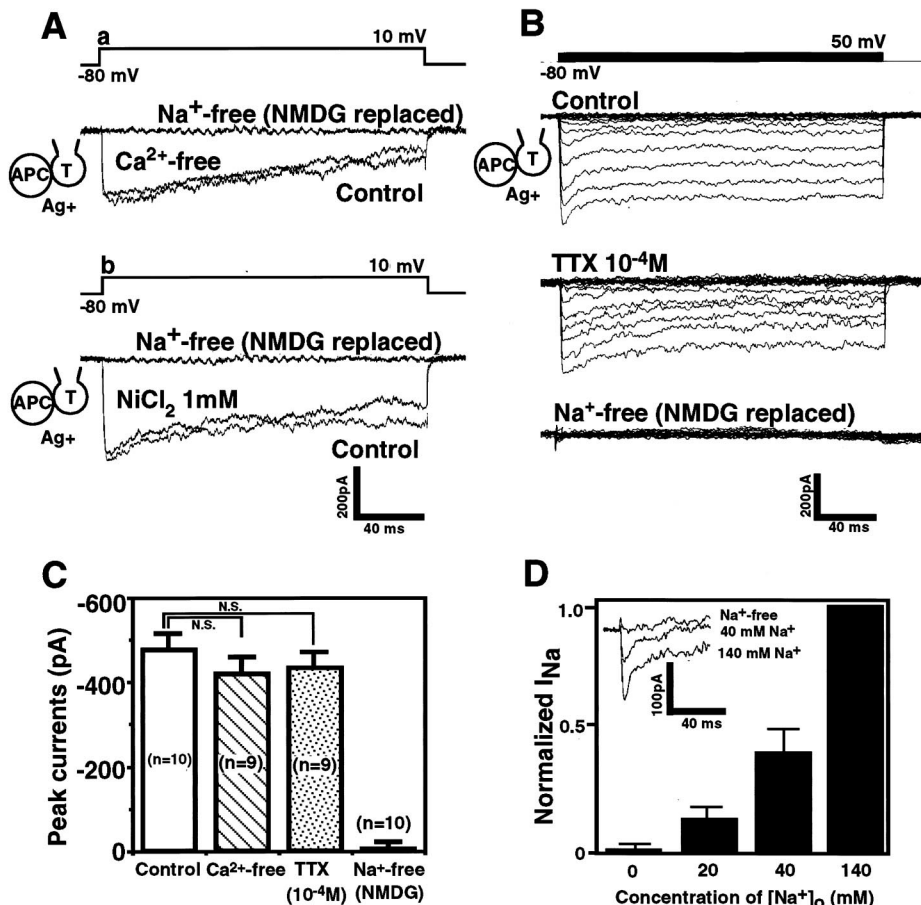


FIGURE 1. Changes in ionic currents in the T cell clone stimulated with an antigenic peptide. T cells were cultured and then plated in poly-L-lysine-coated glass coverslips 10 min before the experiment. To observe outward currents, a K⁺-containing solution was chosen as an IS, as described in *Materials and Methods*. *A*, In resting T cells not stimulated by the antigenic peptide, only voltage-gated outward currents were elicited (the holding potential was -80 mV; step pulses from -80 to $+60$ mV). *B*, When stimulated with the peptide presented by HLA-DRB1*0406 molecules, the amplitude of outward currents in 64 contacted T cells was larger than that in resting T cells. *C*, The outward currents showed a voltage dependence during depolarization with step pulses from -80 to $+60$ mV, and the current-voltage (*I-V*) curves are indicated. *D*, In resting T cells with a K⁺-free IS (K⁺ ions were replaced by Cs⁺ ions), no detectable inward currents were recorded in either single (0 of 80 cells) or APC-contacted (0 of 71 cells) T cells in the absence of the antigenic stimuli. *E*, On the other hand, using a K⁺-free IS, a voltage-sensitive inward current was elicited in 31% (26 of 84) of contacted T cells (but not in single cells) at a holding potential of -80 mV during depolarization with step pulses from -80 to $+70$ mV. *E*, Representative recording of inward currents in a whole-cell patch clamp configuration was shown (*A*). *F*, The inward currents were activated from -60 mV during step pulses, and the *I-V* curves of inward currents are shown. ○, the currents recorded from resting T cells; ●, the currents recorded from T cells contacted with APC prepulsed with the antigenic peptide, M12p54–68. Ag+, stimulated with the antigenic peptide; Ag–, without stimulation of the antigenic peptide.

depolarizing with 10 mV steps from -80 to $+60$ mV. After recording of inward currents in control conditions, the perfusion solution was switched to a Ca²⁺-free solution plus 1 mM EGTA or to an ES containing 1 mM NiCl₂. Unexpectedly, the inward currents were not suppressed in the Ca²⁺-free condition. Nickel, an effective inhibitor of TCR-activated Ca²⁺ influx during T cell activation, at a concentration of 1 mM also had no effects on the inward currents (Fig. 2*A*). Thus, transmembrane movement of Ca²⁺ is apparently not involved in the inward currents. On the contrary, as the currents were completely blocked in the case of NMDG replacing Na⁺-free ES (Fig. 2*B*), the inward currents were considered Na⁺ currents.

To examine the possible TTX sensitivity of the inward currents, T cells were perfused with ES containing TTX at a concentration ranging from 0.1 μ M to 0.1 mM, and then the inward currents were recorded. However, the inward current showed its TTX-resistant characteristics even though TTX was applied at a high concentration (0.1 mM). Fig. 2*B* shows a representative recording in which the TTX-insensitive currents were completely suppressed in a Na⁺-free solution. Similar results were obtained from nine other cells, and results are summarized in Fig. 2*C*. To test the extracellular Na⁺ dependence of the inward currents, the Na⁺ current (I_{Na}) was evoked at $+10$ mV with a holding potential of -80 mV in six cells, and then each cell was exposed to Na⁺ at 0, 20, 40, and 140

FIGURE 2. Extracellular Na⁺ dependence of TCR full agonism-induced inward currents. *A*, A Ca²⁺-free solution (*A-a*) or 1 mM nickel, a Ca²⁺-channel blocker (*A-b*), did not suppress the inward currents, whereas an external Na⁺-free (NMDG-replaced) environment completely blocked them. *B*, TTX, a classical Na⁺ channel blocker, at a high concentration (0.1 mM) did not block the inward currents. *C*, Similar data were reproducibly obtained from different experiments and summarized (numbers in parentheses indicate numbers of patched cells). *D*, Bar graphs show the effects of changes in extracellular Na⁺ ([Na⁺]_o) on the amplitude of the inward current recorded (*n* = 6). The inset illustrates suppression of the inward currents in Na⁺-free conditions and partial recovery in low Na⁺ solutions (40 mM Na⁺). The Na⁺ current (I_{Na}) was evoked at +10 mV from a holding potential of -80 mV.



mM, respectively (Fig. 2D). Suppression of the inward currents under Na⁺-free conditions and partial recovery at the same cell when the perfusion solution was switched to a low Na⁺ solution were evident. To determine whether the effect of NMDG on Na⁺ currents is related to a specific action on TCR, choline chloride was selected as another replacement for NaCl, and the same protocols were performed. As shown in Fig. 3, Na⁺ currents were completely inhibited in a choline-replaced Na⁺-free solution (with 140 mM choline chloride). On the other hand, when T cells were perfused with a low Na⁺ (40 mM) ES, the amplitude of inward Na⁺ currents was suppressed, but the reversal potential was the same as that seen in normal Na⁺ conditions (about +20 mV). Similar results were obtained and are summarized in Fig. 3B.

Since it was reported that TTX-resistant Na⁺ currents were inhibited by amiloride, a potential potassium-sparing diuretic, and its analogous in B lymphocytes (29), we investigated the effects of amiloride on Na⁺ currents in six T cells contacted with APC pre-pulsed with the peptide. As shown in Fig. 4, amiloride inhibited the voltage-gated Na⁺ currents in a dose-dependent manner (IC₅₀, ~15 μM).

Effects of extracellular Na⁺-free and amiloride on proliferation of T cells

We then investigated whether an Na⁺-free environment induced any changes in T cell activation and proliferation in the T cell clone. Fig. 5A shows the effects of a Na⁺-free solution, in which Na⁺ was replaced by NMDG, on the Ag-specific proliferative response in the T cell clone. Ag-stimulated cells proliferation was completely inhibited in an extracellular Na⁺-free solution (NMDG-replaced) as well as in a Ca²⁺-free solution. In a low Na⁺

Tyrod's solution (40 mM Na⁺ and 100 mM NMDG), T cell proliferation was only partially inhibited.

As shown in Fig. 5B, Ag-specific T cell proliferation was inhibited by amiloride in a dose-dependent manner. Amiloride at about 10 μM began to inhibit T cell proliferation and completely inhibited the response from ~100 μM (IC₅₀, ~15 μM). It is interesting to note that the inhibitory effect of amiloride on T cell proliferation showed the same concentration dependency as that on inward Na⁺ currents in Fig. 4.

Effects of absence of Na⁺ on TCR-activated intracellular Ca²⁺ response

After engagement of TCR with its ligand, it is known that an inositol triphosphate-evoked Ca²⁺-release from intracellular stores followed by a sustained elevation of [Ca²⁺]_i via Ca²⁺ influx through Ca²⁺ release activated Ca²⁺ channels. To determine how Na⁺-free conditions affect the Ca²⁺ response induced by the antigenic stimuli, we investigated changes in [Ca²⁺]_i in T cells stimulated with M12p54-68 peptide. As shown in Fig. 6A, antigenic stimulation induced a transient and small sinusoidal peak followed by a high and sustained Ca²⁺ increase (ratio ≥1.0 and duration >10 min; Fig. 6A, upper panels), similar to our previous observation (27). However, in a Ca²⁺-free solution, the Ca²⁺ response induced by the antigenic peptides was markedly suppressed (Fig. 6A, middle panels). Only a transient and small sinusoidal peak followed by a small and sustained increase without high responses were found. In the extracellular Na⁺-free condition (NMDG-replaced), the Ca²⁺ response was also partially suppressed, even though Ag stimulation and extracellular Ca²⁺ were

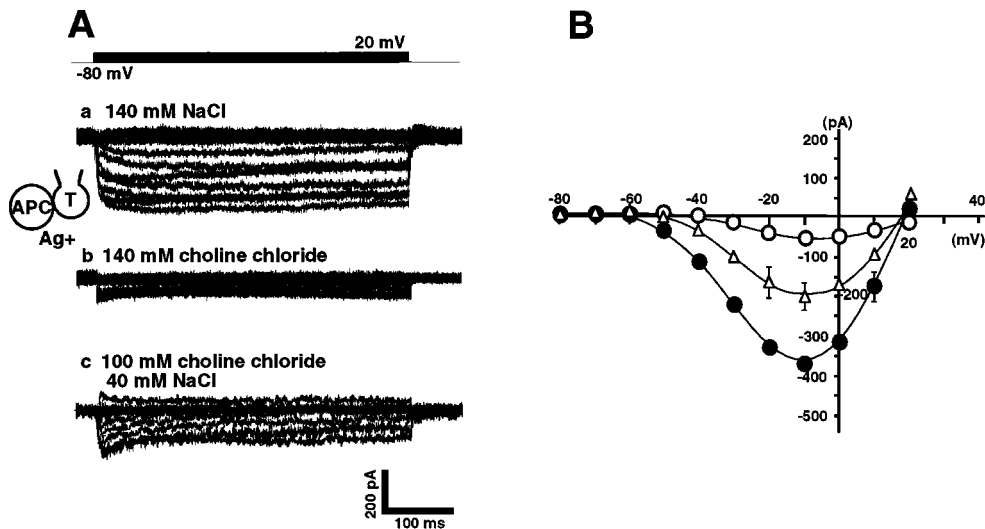


FIGURE 3. Effects of choline on the Na⁺ currents. *A*, Representative recordings of inward currents after application of choline, another replacement for Na⁺. *a*, The inward Na⁺ currents were elicited in normal Na⁺ conditions at a depolarized step range from -80 mV to +20 mV at a holding potential of -80 mV. The reversal potential was approximately +20 mV. *b*, In the same cell the Na⁺ currents were completely inhibited by a Na⁺-free ES in which Na⁺ was replaced with 140 mM choline. *c*, In another T cell perfused with ES containing 40 mM Na⁺ and 100 mM choline, the Na⁺ currents were partially suppressed by reduction of external Na⁺ concentration, but the reversal potential was the same as that shown in *a*. *B*, The current-voltage (*I*-*V*) curves of inward currents in peptide-stimulated T cells perfused with or without choline chloride. Data showed that the Na⁺ currents were also inhibited by choline chloride. ●, the currents recorded at a normal Na⁺ solution (*n* = 6); △, the current recorded at a low Na⁺ condition (100 mM choline chloride with 40 mM NaCl; *n* = 4); ○, the currents recorded from T cells treatment with Na⁺-free solution (choline replaced; *n* = 6).

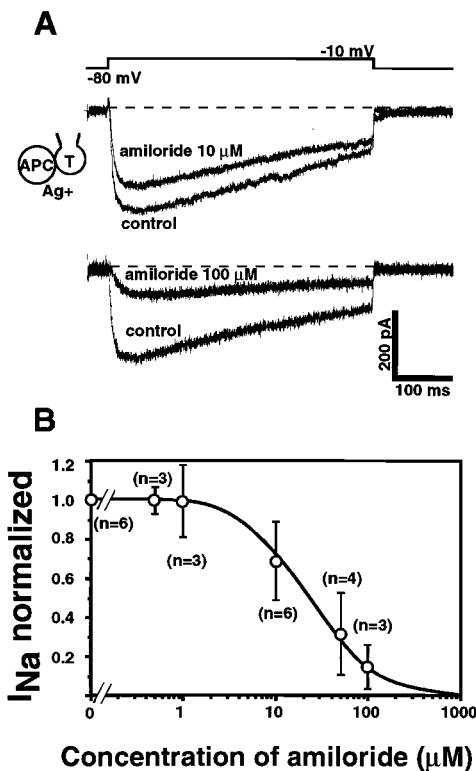


FIGURE 4. Effects of amiloride on Na⁺ currents. *A*, Representative recordings for TCR stimulation of activated Na⁺ currents in the presence or the absence of amiloride. The Na⁺ currents were elicited at a step stimulation from a holding potential (-80 mV) to -10 mV, and then amiloride at concentration of 10 or 100 μM was added for 2 min before recording. *B*, Concentration-dependent inhibition of amiloride on the amplitude of I_{Na}. Data for I_{Na} were normalized from three to six T cells. ○, normalized I_{Na} after application of amiloride at the indicated concentration.

present (Fig. 6A, lower panels). Fig. 6B summarized the Ca²⁺ responses after Ag stimulation with or without Ca²⁺ or Na⁺ ions in ES, respectively. These results indicated that the intracellular Ca²⁺ response was suppressed in the absence of extracellular Na⁺.

Discussion

In the present study our results have clearly shown that an amiloride-sensitive Na⁺ channel was present in Ag-stimulated T cells and suggest that Na⁺ ions participate in T cell activation in human T lymphocytes. First, we have obtained evidence that the Na⁺-selective and voltage-gated inward currents were activated by physiological stimulation of TCR in a human T cell clone, YN5-32, which was specific to the nonself antigenic peptide M12p54-68 in the context of HLA-DR4 molecules. The activation of this voltage-gated Na⁺ channel was sensitive to amiloride, a potassium-sparing pyrazine diuretic, and was suppressed by removal of extracellular Na⁺. Second, extracellular Na⁺-free conditions and amiloride inhibited T cell proliferation. Our results suggest that an increase in [Na⁺]_i via activation of the amiloride-sensitive Na⁺ channel may participate in the early phase of intracellular signaling that leads to T cell activation and proliferation. If this is true, the inhibitory effects of Na⁺-free solutions on T cell proliferation in the present study may be explained as a consequence of its suppression of Na⁺ influx via voltage-gated Na⁺ channels.

Because depletion of extracellular Na⁺ would impair several Na⁺-dependent ion transport systems, including Na⁺-Ca²⁺ and Na⁺-H⁺ exchangers, the effects of Na⁺-free solutions on T cell activation in the T cell clone may be due to inhibition of these ion transports. Indeed, the importance of the Na⁺-Ca²⁺ exchanger in T cell activation was suggested by previous studies that showed that various amiloride derivatives were effective inhibitors of a sustained increase in [Ca²⁺]_i and cell proliferation stimulated by ligation of the CD3-TCR complex in Jurkat T or human peripheral T cells (30, 31). Thus, the effects of amiloride on T cell activation

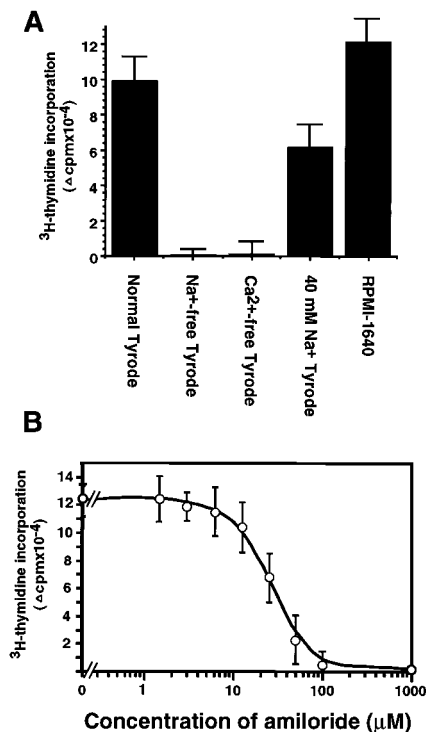


FIGURE 5. Inhibition by Na⁺-free solution of the proliferative response of T cells stimulated with the peptide M12p54–68. **A**, Effects of Na⁺-free and Ca²⁺-free solutions on T cell proliferation. T cells (3×10^4) were cultured with irradiated PBMC (1×10^5) prepulsed with the antigenic peptide in the indicated solution for 72 h, and the proliferative responses were quantitated. Normal Tyrode's solution, normal Tyrode's solution that contained 140 mM NaCl and 2.5 mM CaCl₂, Na⁺-free Tyrode's solution in which Na⁺ ions were replaced with equimolar NMDG, Ca²⁺-free Tyrode's solution, 40 mM Na⁺ Tyrode's solution, 40 mM Na⁺, and 100 mM NMDG, RPMI 1640, and RPMI 1640 medium supplemented with 10% heat-inactivated human plasma and antibiotics were added. Both Na⁺-free and Ca²⁺-free conditions inhibited the proliferative response. Data were obtained from three independent experiments. The Δcpm was calculated by subtracting the mean cpm of triplicate responses observed in antigenic stimulation with mean cpm observed in culture with peptide-unpulsed PBMC. The later value did not exceed 50 cpm in any solution. **B**, Effects of amiloride on [³H]TdR uptake by YN5–32 T cell clone stimulated with the irradiated PMBC prepulsed with peptide. [³H]TdR incorporation was observed in the presence of amiloride at the indicated concentrations (0, 1.5, 3, 6, 12, 25, 50, 100, or 1000 μM). The mean ± SD of triplicate responses are indicated for each concentration of amiloride. Amiloride began to inhibit [³H]TdR incorporation from about 10 μM and reached complete inhibition at 1000 μM. ○, [³H]TdR uptake by YN5–32 T cells after application of amiloride at the indicated concentration.

in the past and in the present study would result from its blockade of the Na⁺-Ca²⁺ exchanger.

However, it is well known that activation of the Na⁺-Ca²⁺ exchanger may have two different models. One is that activation of this exchanger would induce a Na⁺ influx with a adequate Ca²⁺ efflux, which is present in many type cells, including myocardial cells and smooth muscle cells, and its function is to extrude Ca²⁺ and avoiding excessive elevation of [Ca²⁺]_i. Another model is a reversal model, which may operate in some pathological conditions, such as hypoxia and ischemia, and result in a abnormal increase in [Ca²⁺]_i. According to the first model, activation of the Na⁺-Ca²⁺ exchanger would induce a decrease in [Ca²⁺]_i via Na⁺ influx with Ca²⁺ efflux during T cell activation. And this Ca²⁺ efflux coupled with Na⁺ influx should be inhibited and induced an

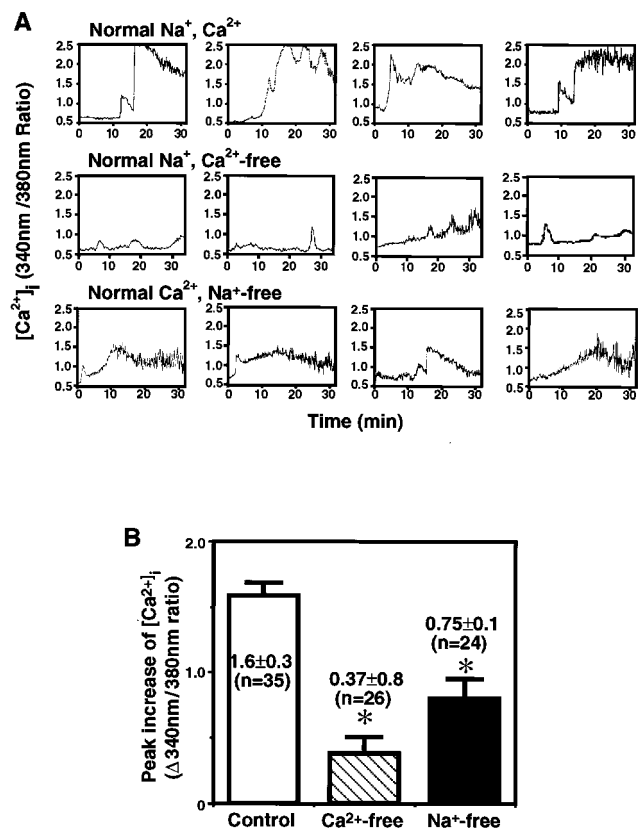


FIGURE 6. Effects of Na⁺-free solution on [Ca²⁺]_i elevation induced by the antigenic stimuli in the T cell clone. **A**, Sample recordings of changes in [Ca²⁺]_i in T cells after stimulation with the antigenic peptide M12p54–68 and changes in [Ca²⁺]_i in T cells perfused with normal Tyrode's solution (normal Na⁺ and normal Ca²⁺; upper panels), with a Ca²⁺-free solution (middle panels), or with a Na⁺-free solution (NMDG-replaced; lower panels). **B**, Similar results were obtained, and the peak increase in [Ca²⁺]_i (Δ340 nm/380 nm ratio) is summarized. □, antigenic stimulation induced Ca²⁺-response in YN5–32 T cell clone in a control condition with normal Ca²⁺ and normal Na⁺ solution (n = 35); ▨, the Ca²⁺ response in a Ca²⁺-free condition (n = 26); ■, the Ca²⁺ response in a Na⁺-free condition (n = 24). *, p < 0.05 compared with the control group (by unpaired t test).

increase in [Ca²⁺]_i during extracellular Na⁺-free environments. Obviously, this is not the case, because we found that the Ag-stimulated elevation in [Ca²⁺]_i in T cells was partially suppressed in the absence of extracellular Na⁺ (see Fig. 6).

Thus, one possibility, as described in Fig. 7, is that the Na⁺-Ca²⁺ exchanger would be activated via a reverse model with Na⁺ efflux/Ca²⁺ influx to maintain a sustained increase in [Ca²⁺]_i during T cell activation. According to this hypothesis, the [Na⁺]_i would be decreased during T cell activation, because activation of the Na⁺-Ca²⁺ exchanger may induce the Ca²⁺ influx as well as adequate Na⁺ effluxes. However, previous studies clearly showed that the [Na⁺]_i significantly increased during T cell activation stimulated by lectins (18, 19, 32, 33). This conflicted result may indicate that some mechanisms were operating during T cell activation to increase [Na⁺]_i to maintain necessary stimulation of the reverse Na⁺-Ca²⁺ exchange. In the present study one mechanism may be a TCR stimulation-induced activation of Na⁺ channels. Because our results obtained with patch-clamp techniques in the present study clearly showed that Na⁺ currents were activated in these activated T cells, they may explain why [Na⁺]_i does not decrease but, rather, increases during T cell activation. Activation

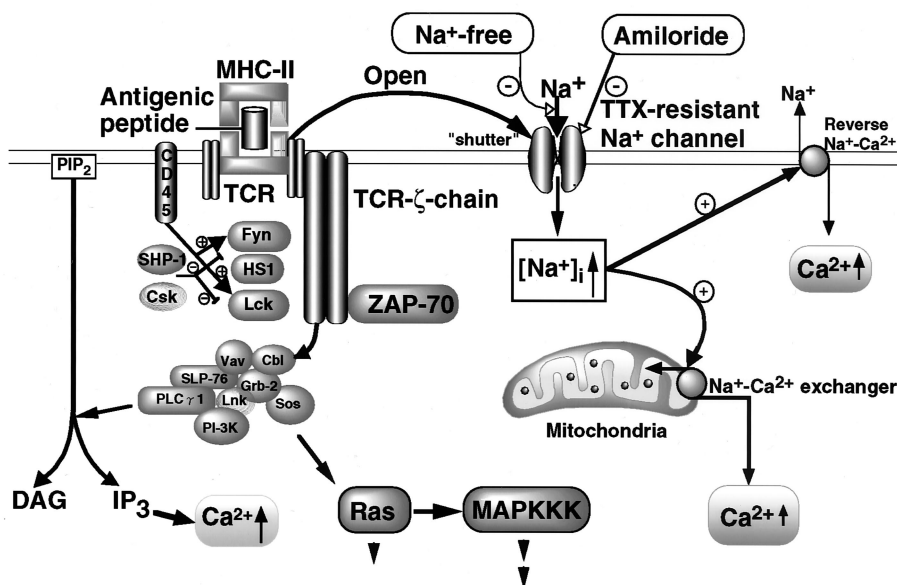


FIGURE 7. A putative model for the role of the amiloride-sensitive and TTX-resistant Na^+ channel in the T cell activation. Our results suggest that engagement of TCR with the MHC-peptide complex would directly activate or remove the “shutter” of a voltage-dependent, amiloride-blockable, but TTX-resistant, Na^+ channel presented in resting T cells. Activation of this Na^+ channel then induces influxes of Na^+ . The increased $[\text{Na}^+]_i$ may enhance the Na^+ - Ca^{2+} exchange in plasma membrane with a reverse model or stimulate the Na^+ - Ca^{2+} exchange in mitochondrial membrane to result in a sustained increase in $[\text{Ca}^{2+}]_i$. Therefore, the TCR stimulation-induced Ca^{2+} response may, at least, involve two important pathways: 1) TCR stimulation induces an increase in inositol trisphosphate and Ca^{2+} release from Ca^{2+} stores, then triggers activation of calcium release-activated calcium channels (I_{CRAC} ; see text); and 2) TCR stimulation removes the shutter of Na^+ channels and induces an increase in $[\text{Na}^+]_i$. The elevated $[\text{Na}^+]_i$ may enhance the operation of the reverse Na^+ - Ca^{2+} exchange in plasma membrane to increase Ca^{2+} influx or trigger the Na^+ - Ca^{2+} exchange in mitochondrial membrane to promote Ca^{2+} release from mitochondrial stores.

of a voltage-gated Na^+ channel at the T cell membrane may provide an influx of Na^+ and maintain $[\text{Na}^+]_i$ at a similarly high level. Therefore, this high level of $[\text{Na}^+]_i$ may allow activation of reversal of the Na^+ - Ca^{2+} exchanger and result in a sustained influx of Ca^{2+} and an adequate efflux of Na^+ . Recent studies in cultured human coronary myocytes also showed that an atypical Na^+ current can regulate Ca^{2+} homeostasis (34). On the other hand, another possibility is that a TCR stimulation-induced increase in $[\text{Na}^+]_i$ via the Na^+ channels may trigger the Na^+ - Ca^{2+} exchanger present in mitochondrial membrane to enhance Ca^{2+} release from mitochondria, as reported by Hoth and colleagues (35). Although we have no evidence to show which level of $[\text{Na}^+]_i$ concentrations is necessary for activation of Na^+ - Ca^{2+} exchange in mitochondrial membrane, it is possible that the opening of Na^+ channels would maintain a related high $[\text{Na}^+]_i$ that may ensure operation of the Na^+ - Ca^{2+} exchange to promote Ca^{2+} release from mitochondria (Fig. 7).

The amiloride-sensitive Na^+ - H^+ exchanger has been noted in human T cells and a leukemic T cell line stimulated with IL-2 or anti-CD3 Ab (36, 37) and has been reported in epithelial (38) and cardiac cells (39). Thus, the effects of amiloride on T cell proliferation may be explained by suppression on the Na^+ - H^+ exchanger. However, this is unlikely because previous reports showed that activation of the Na^+ - H^+ antiport was not required for lectin-induced proliferation of human T lymphocytes (40). The amiloride analogue, dimethylamiloride, a specific inhibitor of the Na^+ - H^+ exchanger, at 1 μM inhibited the Na^+ - H^+ exchanger by 90% without significant inhibition of DNA synthesis or Ig production in human PBMC (41). Recent research in PL.17 cell lines for studies of the altered TCR ligands during early T cell signaling also indicated that the acid release increased, not decreased, after application of antigenic peptides (42). Thus, the inhibitory effects of Na^+ -free solutions or amiloride on T cell activation and prolifer-

ation were probably not due to their suppression of the electrical silent Na^+ - H^+ exchanger.

Even though TTX, a potential blocker of Na^+ channels, was used at a high concentration, it did not inhibit the amiloride-sensitive Na^+ inward currents in the present study. This may be why TTX had no inhibitory effect on lectin-induced mitogenesis in previous studies. Recently, similar amiloride-sensitive and TTX-resistant Na^+ currents were also reported in human B lymphocytes (29) and some epithelial cells (43–45). However, the pharmacological characteristics of amiloride-sensitive and TTX-resistant Na^+ currents in our T cell clone were different from those in B lymphocytes (29) and some epithelial cells (44). The effective concentration used in the present study (IC_{50} , $\sim 15 \mu\text{M}$) was higher than that in B lymphocytes (IC_{50} , 2 μM) (29). Therefore, this difference may indicate that the Na^+ channel in our T cell clone was a different type from that in B lymphocytes. At this moment, we have no more evidence to confirm their molecular characteristics. However, the concentrations of amiloride used in the present study are similar to those used in several studies of amiloride on DNA synthesis and Ig production in human PBMC (41) and human peripheral T cells (46). Thus, the difference in sensitivity to amiloride may imply that the amiloride-sensitive Na^+ channels expressed in T cells are at least functionally different from those in B cells. It is interesting that the concentrations of amiloride used in the present study to inhibit T cell proliferation were similar to those used to suppress Na^+ currents. These results suggest that amiloride inhibited T cell proliferation at least in part by its inhibition of activation of Na^+ channels. The similar inhibitory effects of amiloride on proliferative response were also reported in murine splenocytes stimulated with Con A in previous investigations (18, 32).

It is clear that the voltage-dependent Na^+ currents were only observed in those T cells contacted with APC prepulsed with the antigenic peptide. This fact suggests that the activation of Na^+

currents would be related to T cell activation in the present study. However, the mechanisms involved are still unclear. As a possible explanation, the signaling triggered by TCR engagement may regulate membrane permeability to Na⁺ or activation of Na⁺ channels in Ag-stimulated T lymphocytes. Stimulation of TCR with its ligand may remove the "shutter" that presented in membrane of T cells to prevent the opening of Na⁺ channels under resting conditions or directly activated the Na⁺ channels (Fig. 7). However, to elucidate interaction between Na⁺ and T cell activation, further investigations at the molecular level, including single-channel studies and analysis and identification of structure amiloride-sensitive Na⁺ channels, are required.

In conclusion, our findings suggest that the fully agonistic peptide-induced physiological engagement of TCR would activate a voltage-dependent Na⁺ channel and result in influxes of Na⁺ to increase [Na⁺]_i in the HLA-DR-restricted human T cell clone. Thereby, the TCR activation induced rapid elevation in [Na⁺]_i via activation of Na⁺ channels may act to maintain a sustained elevation of [Ca²⁺]_i, resulting in T cell activation and proliferation. To know whether this Na⁺ channel is also present in other T lymphocytes, further investigations need to be undertaken.

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