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Effects of Cyclosporine A on T Cell Development and Clonal Deletion

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Cyclosporine A (CsA) is an important immunosuppressive drug that is widely used in transplantation medicine. Many of its suppressive effects on T cells appear to be related to the inhibition of T cell receptor (TCR)–mediated activation events. Paradoxically, in certain situations CsA is responsible for the induction of a T cell–mediated autoimmunity. The effects of CsA on T cell development in the thymus were investigated to elucidate the physiologic events underlying this phenomenon. Two major effects were revealed: (i) CsA inhibits the development of mature single positive ($CD4^+8^-$ or $CD4^-8^+$) TCR-$\alpha\beta^+$ thymocytes without discernibly affecting $CD4^-8^-$ TCR-$\gamma\delta^+$ thymocytes and (ii) CsA interferes with the deletion of cells bearing self-reactive TCRs in the population of single positive thymocytes that do develop. This suggests a direct mechanism for CsA-induced autoimmunity and may have implications for the relative contribution of TCR-mediated signaling events in the development of the various T cell lineages.

CYCLOSPORINE A (CsA) IS AN IMMUNOSUPPRESSIVE drug that inhibits lymphokine production by helper T cells in vitro and has been widely used to alleviate tissue allograft rejection in vivo (*1*). It is also effective in preventing graft-versus-host disease (GVHD) secondary to allogeneic bone marrow transplantation (*2*). Surprisingly, irradiated hosts transplanted with syngeneic bone marrow and then treated with, and withdrawn from, CsA actually develop autoimmunity (*3*). This phenomenon, observed in rats (*3, 4*), mice (*5, 6*), and humans (*7*), is characterized by a GVHD-like syndrome that is transferable to naïve recipients by T cells. Removal of the thymus from transplanted animals prevents the induction of autoimmunity (*4*), thus, the thymus is required for the development of the autoreactive T cells. Several mechanisms have been postulated to explain these results, including repertoire alterations secondary to reduced class II major histocompatibility complex (MHC) molecule expression in the thymic medulla (*5*) or effects on the development of suppressor cells that normally regulate autoreactive T cell clones (*4, 6*). However, the direct effects of CsA on thymocyte development have not been fully explored.

Advances in the delineation of the molecular and cellular events involved in T cell differentiation enabled us to directly examine the effect of CsA on specific developmental stages. A critical early event in thymocyte differentiation is the progressive rearrangement of T cell receptor (TCR) gene segments leading to surface expression of either a TCR-$\gamma\delta$ or a TCR-$\alpha\beta$ (*8–10*). Thymocytes that productively rearrange and express TCR-$\gamma\delta$ maintain a $CD4^-8^-$ phenotype and appear to represent a stable distinct lineage from TCR-$\alpha\beta$–bearing thymocytes (*10–12*). Cells that fail to productively rearrange their γ or δ genes probably continue to rearrange their α and β genes and also initiate expression of the accessory molecules CD4 and CD8, resulting in a $CD4^+8^+$ TCR-$\alpha\beta^{lo}$ intermediate stage thymocyte (*10, 11, 13*). Deletion of potentially self-reactive T cells occurs at this stage (*14–16*). A small subset of $CD4^+8^+$ thymocytes ("double positive") undergoes a subsequent differentiation step characterized by an increase in surface TCR density and down-regulation of either CD4 or CD8 resulting in the "single positive" TCR-$\alpha\beta^{hi}$ phenotype of mature T cells (*11, 13*). There is an additional "positive selection" step resulting in the skewing of the T cell repertoire toward self-MHC restriction (*17*). The developmental step at which this occurs is unknown.

We initially investigated the effects of CsA on the various developing thymocyte subsets as defined by CD4, CD8, and TCR expression (Table 1 and Fig. 1). C57BR mice were lethally irradiated, reconstituted with autologous bone marrow cells, and injected intraperitoneally with CsA at 20 mg/kg daily for 25 days; at this time full reconstitution of the predominant thymocyte subsets with donor-derived cells is complete (*18*). Thymocytes from control mice analyzed by two-color flow cytometry (FC) had the normal subset distribution: 4% $CD4^-8^-$, 78% $CD4^+8^+$, 11% $CD4^+8^-$, and 7% $CD4^-8^+$ (Fig. 1C). In contrast, thymocytes from CsA-treated mice were depleted of single positive cells ($CD4^+8^-$ and

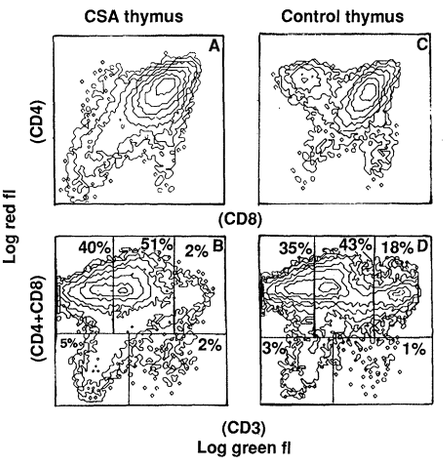

Fig. 1. Effect of CsA on developing thymocyte subsets. C57BR mice (Jackson Laboratory) were irradiated with 850R from a Gammacell 40 irradiator, reconstituted with 10^7 autologous bone marrow cells, and maintained in autoclaved cages on antibiotic water. Beginning 3 days after reconstitution and continuing for the next 23 days, mice received daily intraperitoneal injections of 20 mg of CsA per kilogram dissolved in olive oil (20 mice per experiment, referred to as CsA-treated) or an equal volume of olive oil alone (10 mice per experiment, referred to as controls). Age- and sex-matched experimental and control mice were treated and analyzed simultaneously within each experiment. Unfractionated thymocytes, pooled from all mice from CsA-treated (panel A) or control (panel C) groups were stained with fluorescein isothiocyanate (FITC)–labeled antibody to CD8 (Becton Dickinson) followed by biotinylated antibody to CD4 (H129.19) (*27*) plus allophycocyanin-avidin. Alternatively, unfractionated thymocytes from CsA-treated (panel B) or control (panel D) mice were stained with MAb to CD3-ϵ (500-A2) (*28*) plus FITC-labeled goat antibody to hamster immunoglobulin (Ig) (Kirkegard and Perry) and then with biotinylated anti-CD4 and biotinylated MAb to CD8 (Becton Dickinson) plus allophycocyanin-avidin. Samples (2×10^5 cells) were analyzed by FC on a FACS 440 (Becton Dickinson). Plots shown are representative of three separate experiments. Standard errors of the mean were less than 10%.

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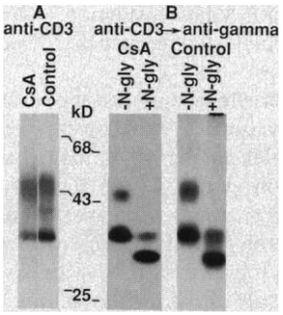

Fig. 2. TCR expression on $CD3^+$ $CD4^-8^-$ thymocytes from the CsA-treated thymus. Thymocytes were isolated from CsA-treated C57BR thymuses 25 days after irradiation and syngeneic bone marrow reconstitution as described in Fig. 1 or from normal adult C57BR mice. $CD4^-8^-$ thymocytes were then prepared by cytotoxic elimination with MAb to CD4 (RL-172.4) (*29*) and to CD8 (3-155) (*30*) plus complement. Cell surfaces were radioiodinated, cells were lysed in digitonin buffer and immunoprecipitates formed with (**A**) anti-CD3-ε (MAb 145-2C11) (*31*) or (**B**) anti-CD3, followed by SDS-elution and re-immunoprecipitation with an antiserum to Cγ1/2 peptide (with or without *N*-glycanase treatment). Immunoprecipitated proteins were separated by SDS–10% PAGE. Cytotoxic eliminations, radioiodination, immunoprecipitations and *N*-glycanase treatments were done as described (*12*).

$CD4^-8^+$) relative to $CD4^-8^-$ and $CD4^+8^+$ thymocytes (Fig. 1A).

The TCR expression within these subsets was assessed by two-color staining with a monoclonal antibody (MAb) specific for the murine CD3-ε chain (green) versus staining with MAb to CD4 plus CD8 (red). In the control thymus (Fig. 1D), cells expressing CD4, CD8, or both, can be divided into three groups on the basis of their level of CD3 expression: $CD3^{hi}$ thymocytes (18% of the total cells), which represent the $CD4^+8^-$ and $CD4^-8^+$ subsets (*11, 19*); $CD3^{lo}$ cells (43%), which represent about half of the $CD4^+8^+$ subset (*11*); and $CD3^-$ cells (35%), which are composed predominantly of the remaining $CD4^+8^+$ cells. The $CD3^-4^-8^-$ cells (3%) contain the thymic stem cell precursors (*18*), whereas the $CD3^+4^-8^-$ cells (1%) are, at this time point, mainly T cells that express TCR-γδ (see below).

Cyclosporine A decreased the absolute number of thymocytes (~50%) and had differential effects on thymocyte subsets. First, as was expected from the depletion of single positive cells described above, the population which displayed the greatest reduction in absolute cell number was the $CD3^{hi}$ subset (Table 1, 5% and 8% of control for the $CD4^+8^-$ and $CD4^-8^+$ cells, respectively). Within the $CD4^+8^+$ compartment, the relative proportion of $CD3^-$ and $CD3^+$ cells was essentially unaffected in the CsA-treated thymus. The absolute number of $CD4^+8^+$ cells was, however, slightly decreased.

We further analyzed the TCR expression of the $CD3^+4^-8^-$ population which, in contrast to the single positive subsets, was not decreased in the CsA-treated thymuses. The adult thymus contains two $CD3^+4^-8^-$ populations. One expresses TCR-γδ and represents a distinct T cell lineage (*12*). The other expresses TCR-αβ, high levels of Lyt 1, and appears late in ontogeny (*20*). To determine the relative representation of these subpopulations in the total $CD3^+$ $CD4^-8^-$ CsA-resistant population, TCR complexes from $CD4^-8^-$ thymocytes from bone marrow–reconstituted CsA-treated mice were immunoprecipitated with either MAb to CD3-ε or antibodies to a carboxyl-terminal TCR-γ peptide (anti–TCR-γ) (Fig. 2). The CD3 MAb primarily immunoprecipitated species of 35 and 46 kD, characteristic of the TCR γ and δ chains (*12*) (Fig. 2A). Virtually no TCR-αβ, which have a mobility on SDS–polyacrylamide gel electrophoresis (PAGE) between 37 and 43 kD, were detected from the CsA-treated group. In contrast, $CD4^-8^-$ thymocytes from normal adult mice expressed detectable amounts of TCR-αβ, although the predominant species was TCR-γδ. This TCR-αβ expression in the thymuses of normal adult mice is attributable to the $CD4^-8^-$ TCR-$\alpha\beta^+$ cells, a population that has not yet appeared 25 days after irradiation and reconstitution (*21*). The CD3-associated 35- and 46-kD material from the CsA-treated mice was TCR-γδ, as it could be re-immunoprecipitated by anti–TCR-γ (Fig. 2B). In addition, both the γ and δ chains had the same glycosylation pattern as $\gamma\delta^+$ thymocytes from untreated normal adult mice. Thus, in contrast to the marked inhibition of αβ-T cell development (Fig. 1 and Table 1), there is no discernible effect of CsA on γδ-T cell development.

The apparently normal development of γδ-T cells in the presence of CsA suggested the possibility that the activation of these cells was resistant to CsA. This was not the case, however, since anti-CD3 antibody stimulation of $CD3^+4^-8^-$ TCR-γδ thymocytes was completely inhibited by CsA in vitro (*21*). Thus, TCR-mediated activation of TCR-γδ thymocytes is CsA-sensitive even though these cells develop normally in the presence of CsA in vivo.

The relative insensitivity of γδ-T cells to the effects of CsA suggests that alterations in the αβ-T cell population may account for the induction of autoimmunity in CsA-treated animals. This may be because CsA not only reduces the numbers of developing single positive thymocytes, but also interferes with self-tolerance induction in those

Table 1. Effect of CsA on thymocyte subpopulations.

Population*	Treatment group (cells per thymus) × 10^6	
	Control	CsA
$CD3^-4^-8^-$	3.3	3.0 (91%)†
$CD3^{hi}4^-8^-$(TCR-γδ)	1.1	1.2 (109%)
$CD3^{hi}4^+8^-$(TCR-αβ)	12.1	0.6 (5%)
$CD3^{hi}4^-8^+$(TCR-αβ)‡	7.7	0.6 (8%)
$CD3^-4^+8^+$	38.5	24.0 (62%)
$CD3^{lo}4^+8^+$(TCR-αβ)	47.3	30.6 (65%)

*Thymocytes from CsA and control groups were prepared as in Fig. 1. The absolute cell numbers were calculated by multiplying the total number of cells per thymus (CsA group, 6×10^7; control group, 1.1×10^8) by the percentages of the indicated populations based on FC analyses of the type shown in Fig. 1. The results shown are from a single experiment representative of three others. Experimental design and statistical analysis was performed as in Fig. 1. †Values represent the percentage of the control cell yield. ‡Because of the existence of a small number of $CD3^-4^-8^+$ thymocytes, the value for $CD3^{hi}4^-8^+$ cells was derived from two-color FC analysis of CD3 versus CD8.

single positive cells that manage to mature in the presence of the drug. A major mechanism of intrathymic tolerance induction is the physical deletion of T cells expressing autoreactive TCR (*14, 15*). We used KJ23 MAb, which recognizes $V\beta17a^+$ TCR (*14*) (which are associated with a high degree of anti–I-E reactivity), to directly test whether CsA inhibits the deletion of potentially self-reactive thymocytes in the C57BR strain, which possesses a functional Vβ17a gene and is $I\text{-}E^+$. Since elimination of autoreactive thymocytes that express Vβ17a is most evident in the single positive subset (*14*), these cells were enriched by cytotoxic elimination of $CD4^+8^+$ and most $CD4^-8^-$ cells with the J11d MAb (*22*) and complement. Consistent with the decreased number of single positive T cells in the thymuses of CsA-treated mice (Table 1), the yield of $J11d^-$ thymocytes was approximately 10% that of control thymuses. Both control and CsA-treated $J11d^-$ thymocyte populations were primarily constituted of cells that expressed high levels of CD3 and CD4 or CD8 (but not both). The ratios of $CD4^+8^-$ to $CD4^-8^+$ cells were similar in both groups (Fig. 3, A and C). As previously described (*14*), Vβ17a-bearing cells were deleted from the control C57BR $J11d^-$ thymocyte population (Fig. 3D). In contrast, however, Vβ17a-bearing cells were readily detectable among $J11d^-$ thymocytes from CsA-treated C57BR mice in both the $CD4^+8^-$ and $CD4^-8^+$ populations. Thus, a small number of $J11d^-$ single positive thymocytes mature in the presence of CsA, and clonal deletion is inhibited in this population.

Our results demonstrate two major effects of CsA on T cell development. First, CsA

interferes with the development of mature TCR-$\alpha\beta^{hi}$ single positive cells without affecting the development of $\gamma\delta$-T cells. Second, CsA blocks the deletion of the potentially autoreactive T cell clones among the small number of $\alpha\beta$-T cells that do mature in the presence of CsA.

Cyclosporine A exerts many of its effects on T cells via specific inhibition of TCR-mediated activation (*1, 23*). The data presented here suggest that TCR-generated signals may be important in T cell development. The precise point at which CsA blocks is unclear. However, the relative lack of effect of CsA on the development of $CD4^-8^-$ and $CD4^+8^+$ thymocytes (including both $CD3^+$ and $CD3^-$ subsets) suggests that the inhibition may occur at a late stage, perhaps at the transition of $CD4^+8^+$ TCR-$\alpha\beta^{lo}$ precursors to single positive TCR-$\alpha\beta^{hi}$ cells. This inhibition does not appear to be a general toxic effect, as the development of other thymocyte populations proceeds normally. Additional evidence for a late block in T cell development is that CsA does not affect TCR gene rearrangement, the critical early event in thymocyte differentiation (*21*). The developmental pattern we observed in the presence of CsA is provocatively similar to that reported in mice treated in vivo with MAb to class I or class II molecules, which blocked the development of $CD4^-8^+$ or $CD4^+8^-$ thymocytes, respectively, without affecting the $CD4^-8^-$ or $CD4^+8^+$ populations (*24*). Cyclosporine A and antibodies to MHC molecules may interfere with the mechanism by which developing T cells are positively selected for restriction to self-MHC molecules. Thus, CsA's effect on T cell development in the thymus is consistent with earlier studies demonstrating the involvement of TCR-$\alpha\beta$/MHC molecule interactions in positive selection (*17*). Whether this effect involves direct inhibition of TCR-mediated signaling, inhibition of the production of a lymphokine critical for T cell differentiation, or alterations in thymic MHC molecule expression is currently under investigation.

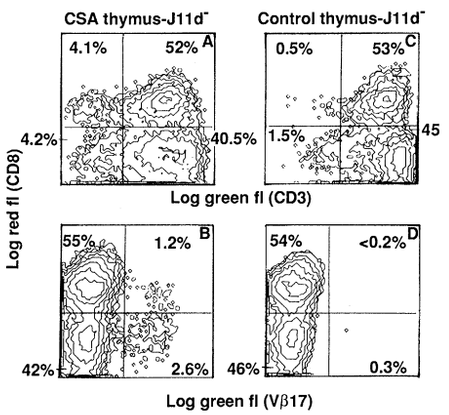

Fig. 3. CsA blocks clonal deletion of self-reactive thymocytes. $J11d^-$ thymocytes from CsA-treated and control C57BR mice (treated as in Fig. 1) were prepared with J11d MAb and rabbit complement (Cedarlane) to remove immature cells. Viable cells were recovered from the interface of Ficoll-Hypaque (Pharmacia) density gradients. Reproducibly, only 10% of the control number of $J11d^-$ thymocytes were recovered from the CsA-treated thymuses consistent with the 90% reduction in the number of single positive cells as described in Fig. 1. The cells were cultured for 3 hours to enhance TCR surface expression and were then stained with biotinylated MAb to CD8 (Becton Dickinson) plus allophycocyanin-avidin followed by anti-CD3 (MAb 500-A2) plus FITC-labeled goat antibody to hamster Ig (Kirkegard and Perry) (panels A and C) or by anti-Vβ17a (MAb KJ23) plus FITC-labeled goat antibody to mouse IgG2a (Southern Biotechnologies) (panels B and D). Samples (2×10^5 cells) were analyzed by FC as described in Fig. 1. The experiment shown is representative of three others.

In contrast to its marked effects on $\alpha\beta$-T cells, CsA did not appreciably affect the development of $\gamma\delta$-T cells. This does not reflect a general resistance of $\gamma\delta$-T cells to the effects of CsA since their in vitro proliferative response to anti-CD3 antibody stimulation was completely inhibited by the drug (*21*). Thus there are fundamental differences between the developmental physiology of $\gamma\delta$- and $\alpha\beta$-T cells. In contrast to $\alpha\beta$-T cells, the intrathymic development of $\gamma\delta$-T cells may be independent of any MHC interaction or TCR-mediated activation event.

We demonstrated that CsA blocks the deletion of autoreactive thymocytes as evidenced by their increased expression of self-reactive TCRs. The effect may be due to interference with the TCR-mediated signals that are generated after high-affinity interactions of $CD4^+8^+$ precursors with self antigens. Therefore, TCR occupancy at an immature stage of T cell development may initiate the clonal deletion process. Alternatively, CsA reduces the expression of class II MHC molecules on cells in the medullary region of the thymus (*5*). Since Vβ17a-bearing TCR recognize I-E molecules, it is therefore possible that clonal deletion might be abrogated secondary to a reduction in Ia molecule expression on bone marrow–derived elements thought to be critical for tolerance induction (*25*). The block of clonal deletion in $CD4^-8^+$ thymocytes could also be explained by a CsA-induced decrease in Ia molecule expression, because deletion occurs at a stage when precursor thymocytes express not only CD8, but also CD4, the appropriate accessory molecule for class II MHC molecule recognition (*16*). This model and the signaling model detailed above can be distinguished by experiments in which medullary Ia molecule expression is restored in the presence of CsA, perhaps by the infusion of interferon-γ.

Our results provide a potential explanation for the paradox of CsA-induced autoimmunity (*3–7*). Cyclosporine A prevents the transition of 90% of cells to the single positive mature stage. In the small subset that does mature, clonal deletion is prevented by interference with TCR-mediated signal transduction or by reduction of Ia molecule expression on bone marrow–derived elements. The autoreactive T cells then seed the periphery. Their self-reactivity, however, is not manifested until the withdrawal of CsA, which relieves the block in lymphokine production and initiates the autoimmune process.

Note added in proof: Similar results on the blockage of clonal deletion by CsA have been obtained using a different MAb that recognizes anti–I-E reactive $VB11^+$ TCRs (*26*).

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Identification of an Intracellular Peptide Segment Involved in Sodium Channel Inactivation

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Antibodies directed against a conserved intracellular segment of the sodium channel α subunit slow the inactivation of sodium channels in rat muscle cells. Of four site-directed antibodies tested, only antibodies against the short intracellular segment between homologous transmembrane domains III and IV slowed inactivation, and their effects were blocked by the corresponding peptide antigen. No effects on the voltage dependence of sodium channel activation or of steady-state inactivation were observed, but the rate of onset of the antibody effect and the extent of slowing of inactivation were voltage-dependent. Antibody binding was more rapid at negative potentials, at which sodium channels are not inactivated; antibody-induced slowing of inactivation was greater during depolarizations to more positive membrane potentials. The peptide segment recognized by this antibody appears to participate directly in rapid sodium channel inactivation during large depolarizations and to undergo a conformational change that reduces its accessibility to antibodies as the channel inactivates.

VOLTAGE-SENSITIVE SODIUM CHANnels mediate the rapid increase in Na^+ permeability during the rising phase of the action potential in many excitable cells (*1*). Their ion conductance is regulated on the millisecond time scale by two experimentally separable processes: voltage-dependent activation, which controls the rate and voltage dependence of the Na^+ conductance increase upon membrane depolarization, and inactivation, which mediates the Na^+ conductance decrease during a maintained depolarization (*1*). The principal protein component of Na^+ channels is the α subunit, a glycoprotein of approximately 260 kD (*2*). It is expressed in association with β1 (36 kD) and β2 (33 kD) subunits in nerve and β1 subunits in muscle (*2*). Messenger RNA encoding the α subunit is sufficient to direct the synthesis of functional Na^+ channels in *Xenopus* oocytes (*3*), although their inactivation is slower than native Na^+ channels (*4*). The primary structures of Na^+ channel α subunits from rat brain and eel electroplax have been inferred from the nucleotide sequence of cDNA clones (*5*). However, the relations between molecular structure and the mechanisms of ion permeation and channel gating are not yet understood. Here we identify a highly conserved intracellular segment of the α subunit that is involved in channel inactivation by use of site-directed antibodies.

Na^+ channel α subunits consist of four homologous transmembrane domains that have approximately 50% amino acid sequence identity (Fig. 1A) (*5*). These are connected by hydrophilic segments that are predicted to be intracellular (Fig. 1A). Electrophysiological studies have shown that intracellular application of proteases and amino acid–specific reagents causes removal of Na^+ channel inactivation (*6*). These results indicate that regions of the Na^+ channel structure that are required for inactivation are located on the intracellular surface of the channel protein and are accessible to macromolecular reagents.

To identify functionally important regions on the intracellular surface of the Na^+ channel, antibodies were prepared (*7*) against synthetic peptides (SP1, SP11, SP19, and SP20) with amino acid sequences that correspond to both conserved and variable sequences (Table 1) of the intracellular segments between the four homologous domains of Type II rat brain Na^+ channel [(R_{II}) (*5*)] (Fig. 1A). These antibodies recognize the Na^+ channel purified from rat brain in native form, and their affinity for the native protein is comparable to their affinity for the peptide used as antigen (*7*). The functional effects of these antibodies, which were affinity-purified by adsorption to immobilized Na^+ channel α subunits (*8*), were analyzed by recording Na^+ currents of rat muscle cells in the whole-cell voltage clamp configuration (*9–11*). Rat skeletal muscle cells were dissociated from 20-day embryos, maintained in vitro for 4 days to allow fusion into multinucleated myotubes, treated with colchicine to obtain round "myoballs," and studied after a total of 8 to

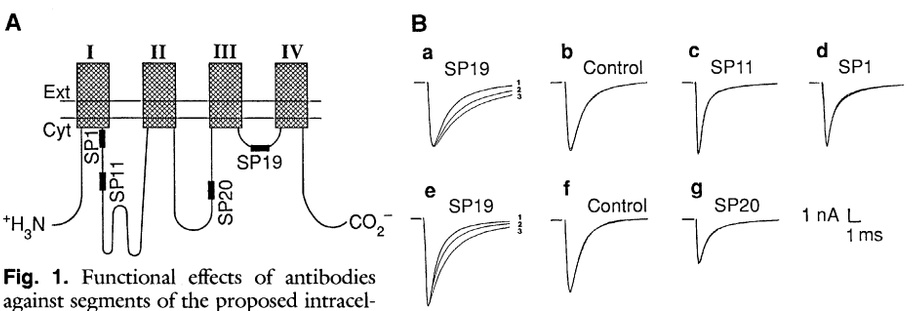

Fig. 1. Functional effects of antibodies against segments of the proposed intracellular domains of the Na^+ channel α subunit. (**A**) Schematic representation of the primary structure of Na^+ channel α subunit with designated peptide segments that are recognized by the four site-directed antibodies SP1, SP11, SP20, and SP19. (**B**) Na^+ currents during intracellular exposure to different sequence-directed antibodies. Na^+ currents were elicited from a holding potential of −70 or −110 mV by a 100-ms hyperpolarizing prepulse to −160 mV followed by a 9-ms test pulse to −5 mV. In each panel, two or three sequential Na^+ current traces are presented at increasing times of exposure to the antibody solution in the recording pipette. (a) Anti-SP19 for 6 (trace 1), 12 (trace 2), and 23 (trace 3) min at −110 mV; (b) control for 7 and 31 min at −110 mV; (c) anti-SP11 for 4 and 26 min at −110 mV; (d) anti-SP1 for 8 and 41 min at −110 mV; (e) anti-SP19 for 8 (trace 1), 25 (trace 2), and 61 (trace 3) min at −70 mV; (f) control for 8 and 68 min at −70 mV, and (g) anti-SP20 for 7 and 60 min at −70 mV. Na^+ current traces were normalized to allow direct comparison of time courses.

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