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38. Cells reacted with bispecific anti-DNP/anti-DNS IgE (0.3 μg/ml) (*10*) (a fivefold to sixfold excess of IgE per receptor) for ≈16 hours were washed and reacted with the alternative ligands at 28°C. After 30 min, release of hexosaminidase was measured. Cells in separate wells were stimulated identically and analyzed for phosphotyrosine after 3 min. The doses of ligand (adjusted to induce ample phosphorylation of the receptors) were 10, 100, and 2500 ng/ml for the DNP-, oDNCP-, and 2NP-protein conjugates, respectively. Separate experiments confirmed that higher doses of the oDNCP and 2NP conjugates gave no additional release. The experiments were conducted at a lower than optimal temperature (*34*) because of the unusual difference in the temperature response of the cells to the two ligands: Whereas the DNP conjugate stimulated increased phosphorylation with increasing temperature between 0° and 37°C, the 2NP conjugate yielded decreased phosphorylation above 15°C, although the ratio of modification in γ compared with β was always around 2:1 (*35*).
39. We thank D. Holowka and B. Baird for gifts of purified DNS-BSA and anti-DNS IgE, for quadroma cells, and for advice on preparing the bispecific antibody.

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Fidelity of T Cell Activation Through Multistep T Cell Receptor ζ Phosphorylation

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The T cell receptor (TCR) αβ heterodimer interacts with its ligands with high specificity, but surprisingly low affinity. The role of the ζ component of the murine TCR in contributing to the fidelity of antigen recognition was examined. With sequence-specific phosphotyrosine antibodies, it was found that ζ undergoes a series of ordered phosphorylation events upon TCR engagement. Completion of phosphorylation steps is dependent on the nature of the TCR ligand. Thus, the phosphorylation steps establish thresholds for T cell activation. This study documents the sophisticated molecular events that follow the engagement of a low-affinity receptor.

The αβ TCR is part of a large protein complex composed of the CD3 γ, δ, ε, and ζ chains. These chains contain signaling motifs called ITAMs (immune receptor tyrosine-based activation motifs) with the consensus sequence $YXX(L/I)X_{6-8}YXX(L/I)$ (*1, 2*). Upon phosphorylation, this motif is sufficient to transduce signals from the TCR (*3*). The γ, δ, and ε chains each contain one ITAM, and ζ contains three ITAMs. The multiple ITAMs are thought to amplify signals from the TCR; however, it is not immediately clear why such a complicated receptor system arose solely for the purpose of signal amplification. Another function of the TCR complex could be to qualitatively evaluate ligands of the αβ TCR. To examine this possibility, we studied the effect of different physiologic TCR ligands on phosphorylation of individual tyrosines of the TCR ζ chain. Although ζ is not absolutely required for T cell development (*4, 5*), ζ is critical for the selection of the TCR repertoire and for the prevention of autoimmunity (*6*). With six potential phosphorylation sites, herein referred to as A1, A2, B1, B2, C1, and C2 (Fig. 1A), ζ could yield more than 60 different phospho-species and amplify initial signals. Thus, molecularly, ζ is well suited for processing information received by the αβ TCR. The mechanism of signal initiation through phosphorylation of ζ, however, has not been ascertained. Discreet phosphoforms of ζ exist in resting and in activated T cells, with apparent molecular sizes of 21 and 23 kD, respectively. Specific phospho-species giving rise to these discreet forms have not been identified because of the complexity of the molecule (*7–9*). The ratio of p21 and p23 can be altered after stimulation of T cells with suboptimal ligands, further suggesting a discriminatory role of ζ phosphorylation in T cell activation (*10, 11*).

We first wished to identify the molecular composition of p21. We raised six antisera, each specific for one of the six phosphotyrosines in ζ (*12*). We demonstrated their specificity by using phosphorylated and unphosphorylated ζ proteins, as well as ζ proteins with substitutions of individual tyrosines to phenylalanine, where the tyrosine of interest cannot be phosphorylated (Fig. 1B) (*13*). The antibodies were used to examine the phosphorylation status of TCR ζ in the resting Th1 clone 3.L2, which is specific for Hb(64–76)/I-E^k (Fig. 2, leftmost lane of each panel) (*14*). Two of the six specific phospho-ITAM sera (against the B1 and C2 sites) recognized p21 in resting T cells (Fig. 2, D and G) (*15*). A2 was recognized variably (Fig. 2C) (*16*). This pattern was observed in at least seven independent experiments for each antisera. The B1 and C2 phosphotyrosines were located within the same ζ homodimer, because immunoprecipitation with anti-pC2 and subsequent protein immunoblotting with anti-pB1 revealed p21 (*17*). Thus, in resting T cells, the p21 form of ζ has two prominent phosphotyrosines, B1 and C2.

We also examined the pattern of ζ phosphorylation observed in resting mature T cells directly isolated from mice. We made use of TCR transgenic mice harboring the Hb(64–76)/I-E^k–specific TCR 2.102 (*14*) bred onto a RAG-1 deficient background (*18*). Spleens from such mice are greatly enriched in resting, mature $CD4^+$ T cells. Lysates from freshly isolated spleen cells were studied for their ζ phosphorylation (*19*). Phosphorylation of the p21 form of ζ, consisting of prominent B1 and weak C2 phosphorylation, was found (*17*), thus extending our findings to resting ex vivo T cells.

We next stimulated the T cell clone 3.L2 with antigen presenting cells (APCs) pulsed with the antigenic peptide Hb(64–76) and examined the phosphorylation of ζ (*15*). Such stimulation fully activates the 3.L2 T cell and causes cell proliferation (*14*). The phosphorylation of p21 rapidly increased, and a 23-kD phospho-form of ζ appeared (Fig. 2A). The increase in p21 phosphorylation was due

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to phosphorylation of additional ζ molecules at A2, B1, and C2, the three prominent phosphotyrosines of p21 in activated T cells (Fig. 2, B through G) (*20*). All six antisera to the phospho-ITAMs recognized p23. Therefore, upon full T cell activation, all six tyrosines in ζ became phosphorylated.

The phosphorylation steps leading from basal to full phosphorylation appeared to be ordered, because the phosphorylation of four sites was interdependent. When A2 was mutated into phenylalanine, anti-pB2 could not recognize the mutant (Fig. 1B; part f), indicating that phosphorylation of A2 was required for B2 phosphorylation. Anti-pB2 was not cross-reactive with position A2, because AP2, a phosphopeptide derived from A2, could not compete for recognition by anti-pB2, when BP2 could (Fig. 1C) (*12*). A similar interdependence existed between positions A1 and C1. When A1 was mutated, C1 was not phosphorylated (Fig. 1B; part g), showing that C1 phosphorylation required A1 phosphorylation. Again, anti-pC1 did not cross-react with position A1 (Fig. 1C). The interdependence of phosphorylation was not reciprocal, because A1 and A2 phosphorylation did not require C1 or B2 phosphorylation, respectively (Fig. 1B; parts c and d). Therefore, ITAM A phosphorylation preceded B2 and C1 phosphorylation (Fig. 1D). We could not observe an actual time difference for sequential phosphorylation in 3.L2 T cells, because all antibodies recognized p21 and p23 with similar kinetics (Fig. 2).

Ligand interactions with the TCR are of low affinity, and differences in affinity between immunogenic and self-derived, nonstimulatory ligands are small (*21*). Multiple ordered steps of ζ phosphorylation may help the TCR to distinguish a high background from faint antigenic signals while still providing specificity. To test this hypothesis, we stimulated our T cell clone with altered peptide ligands (APLs) that had single amino acid changes in their sequence when compared to immunogenic peptide (*22*). These peptides bind to I-E^k with similar affinities, but have different potencies to induce T cell functions: G72 and I72 do not cause 3.L2 T cell proliferation at any concentration, and D73 causes minimal proliferation only at the highest concentrations; all these peptides, however, can act as antagonists (*23*). The APLs caused altered TCR ζ phosphorylation: p21 phosphorylation was enhanced when p23 was only slightly phosphorylated (Fig. 3A) (*10, 11*). No ZAP-70 activation was observed with APL stimulation (Fig. 3B) (*10, 11, 24*), indicating a lack of downstream signal transduction. We used our antisera to analyze these alterations in ζ phosphorylation. We found that p21 phosphorylation was due to A2, B1, and C2 phosphorylation (Fig. 3C). Induction of p23 was not completely abrogated after G72, I72, and D73 stimulation. Namely, A1 was phosphorylated significantly, albeit reduced when compared with the stimulatory peptide (Fig. 3C; part a). Phosphorylation of B2 and C1, the two last tyrosines to become phosphorylated, was directly related to the strength of the TCR ligand (Fig. 3C; parts d and e). After stimulation with the weakest ligand, G72, B2 and C1 phosphorylation were completely absent. This indicates that p23 can contain incompletely phosphorylated ζ species, that is, spe-

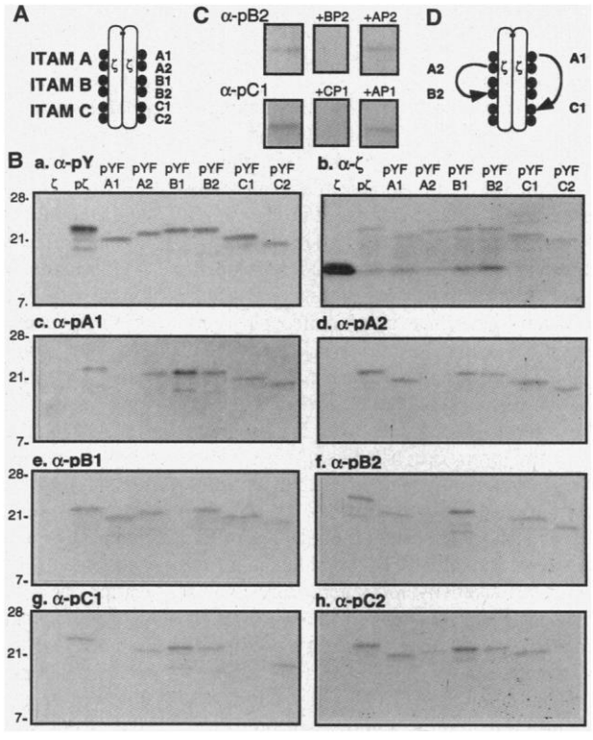

Fig. 1. Demonstration that anti-ITAM sera are sequence- and phosphorylation-specific. (**A**) Schematic representation of ζ. The three ζ ITAMs are referred to as ITAM A, B, and C. Tyrosines are referred to as A1, A2, B1, B2, C1, and C2. Six phosphopeptides spanning these tyrosines were used to immunize rabbits. (**B**) Protein immunoblot analysis of different ζ proteins to determine the specificity of antisera. ζ and ζ with mutations of tyrosines (Y) to phenylalanines (F) at A1, A2, B1, B2, C1, or C2 were expressed in HeLa cells alone or in the presence of constitutively active kinases to obtain unphosphorylated ζ (ζ), phosphorylated ζ (pζ), and pζ with Y → F mutations. Cells were lysed, ζ protein was precipitated using the mAb CO and separated on SDS-PAGE. Precipitates were titrated to contain similar amounts of ζ protein based on an anti-ζ protein immunoblot and were then kept constant for all the experiments shown. Molecular size markers (in kilodaltons) are to the left of the figure. (a and b) Protein immunoblots using mAb 4G10 to phosphotyrosine (α-pY) or polyclonal anti-ζ serum (α-ζ). All of the bands shown represent different migrations of ζ protein. (c through h) Protein immunoblots using anti–phospho-ITAM antibodies. Experiments were done five to nine independent times with similar results. (**C**) Anti-pB2 and anti-pC1 are not cross-reactive with A2 or A1, respectively. Protein immunoblotting with anti-pB2 or anti-pC1 was performed. Equal amounts of pζ were loaded on a gel and transferred to nitrocellulose. The nitrocellulose filter was cut and incubated with anti-pB2 alone and with BP2 or AP2 peptide, or with anti-pC1 alone and with CP1 or AP1 peptide. Experiments were done three independent times with identical results. (**D**) Phosphorylation of A1 and A2 precedes phosphorylation of position C1 and B2, respectively.

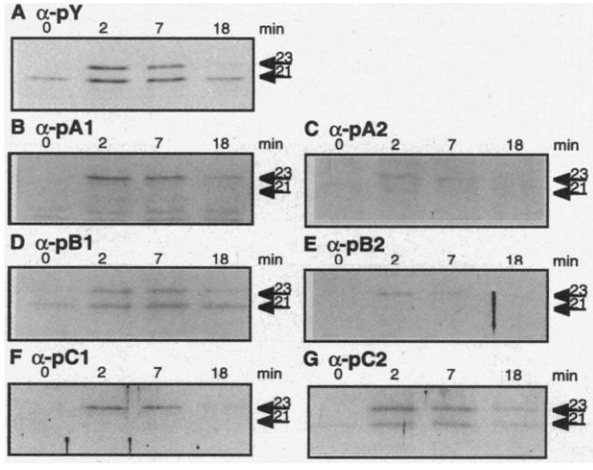

Fig. 2. Phosphorylation of individual ζ tyrosines in resting and activated T cells. Protein immunoblots were done using the mAb 4G10 to phosphotyrosine (α-pY) (**A**) or antibodies against the six ζ phosphotyrosines (**B** through **G**). The 3.L2 T cells (2×10^7) were activated by the addition of APCs alone (0) or Hb(64–76) peptide–pulsed APCs for 2, 7, or 18 min at 37°C. Cells were lysed, and the TCR complex was precipitated with the mAb 500.A2 to CD3ε. Samples were separated on 13% SDS-PAGE gels and transferred to nitrocellulose. Positions of p21 and p23 were determined by phosphotyrosine protein immunoblotting of controls on each nitrocellulose filter and are indicated. Independent experiments with similar results were performed seven to nine times for each antisera.

cies with A1 phosphorylation in the absence of B2 and C1 phosphorylation. For the slightly stronger ligand I72, C1 phosphorylation was absent, and B2 phosphorylation was still detected. Finally, for the strongest APL, D73, B2 phosphorylation was strong, and only C1 phosphorylation was absent. Thus, although ζ phosphorylation was initiated and even proceeded substantially for D73, ζ phosphorylation was not completed for any of the suboptimal ligands.

Our analysis shows that completion of ordered, successive ζ phosphorylation is dependent on the nature of the TCR ligand. When the αβ TCR recognized a ligand with only a subtle change compared to the immunogenic ligand, ζ phosphorylation was arrested at an intermediate stage, and subsequent signal transduction was abrogated. Thus, multistep ζ phosphorylation molecularly sets thresholds that determine whether a TCR-ligand interaction is adequate to result in T cell activation. Ordered ζ phosphorylation also could protect T cells from inappropriate activation by making it unlikely that nonspecific phosphorylation would result in the phosphorylation of all six tyrosines. Completion of ζ phosphorylation might be dependent on the interaction time of TCR and ligand, or on energy provided by this interaction. In this sense, the multiple steps of ζ phosphorylation can also be understood as a kinetic proofreading mechanism for T cell activation (*25*).

Once completed, ζ phosphorylation also provides an amplification mechanism of initial signals. Quantitative and redundant effects of ζ ITAMs on signal initiation have been established in many studies (*5, 6*). For example, functional T cells develop in $\zeta^{-/-}$ mice when reconstituted with ζ completely lacking or with a reduced number of ITAMs. Most likely, during development in such mice, ζ can be bypassed through the CD3 chains (*26, 27*). However, the threshold for selection of specific T cells is altered in these mice, a different T cell repertoire is selected, and the mice harbor autoreactive T cells. In normal mice, the discreet steps of ζ phosphorylation may determine thresholds for the positive or negative selection of any given αβ TCR in the thymus.

Inactive ZAP-70 has been reported to be constitutively associated with phospho-ζ in thymocytes and resting lymph node T cells (*8*). Similarly, in the resting 3.L2 T cell clone, kinase inactive ZAP-70 was associated with the TCR complex (*17*). ZAP-70 preferably associates with biphosphorylated ITAMs rather than monophosphorylated ITAMs (*28*). We did not identify a biphosphorylated ζ ITAM in resting T cell clones or in freshly isolated splenic T cells. It is therefore possible that in resting T cells, binding of inactive ZAP-70 occurs through a single SH2 domain to ζ pB1 or pC2, or to an undetected biphosphorylated ζ ITAM. Alternatively, the association might be mediated through TCR complex components other than ζ. Our analysis of individual ζ tyrosine phosphorylation also showed that processive phosphorylation of ζ does not necessarily lead to ZAP-70 activation. After stimulation with the APL D73, two ITAMs, ITAM A and B, were doubly phosphorylated, yet ZAP-70 was not activated (Fig. 3B), indicating that another signal for ZAP-70 activation was missing.

The sequential phosphorylation of ζ could provide an explanation for earlier studies, in which mutation of both tyrosines in ITAM A was functionally more severe than in ITAM B or C (*29*), or more severe than single mutations introduced into any of the ITAMs (*30*). Also, when the natural arrangement of the three ζ ITAMs was changed, such as through internal truncations, interdependence of phosphorylation might have been abrogated (*27*). The mechanism of sequential TCR ζ phosphorylation is not clear. ITAM A phosphorylation did not inevitably induce B2 and C1 phosphorylation, suggesting additional regulation of these sites, such as from kinases or phosphatases (*31*). In addition, phosphorylation-driven conformational changes might occur in ζ, as suggested by our observed changes in electrophoretic mobility of ζ mutants. It is also feasible that the homodimeric state of ζ influences the availability of individual sites for phosphorylation.

Our study adds to the understanding of altered ligands of the TCR, whose specific biologic effects have puzzled T cell biologists. Stimulation with APLs causes an arrest in ζ phosphorylation. Subsequently, intermediate phospho-ζ species may accumulate and retain the ability to initiate some signaling pathways through single SH2 domain–containing proteins that would otherwise be displaced by the action of ZAP-70 (*32, 28*).

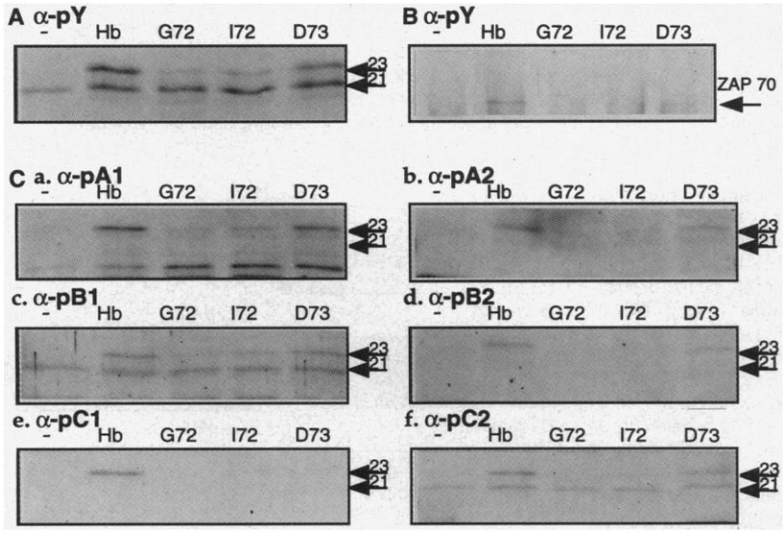

Fig. 3. Phosphorylation of individual ζ tyrosines in T cells after stimulation with APLs. We stimulated 3.L2 T cells (2×10^7) with APCs pulsed with the agonist peptide Hb, with the weak antagonist G72, with the strong antagonist I72, or with the weak agonist D73 for 3 min. Cells were lysed, and the TCR complex was precipitated with mAb 500.A2 to CD3ε (**A** and **C**) or with anti–ZAP-70 (**B**). Protein immunoblots were done with mAb 4G10 to phosphotyrosine (α-pY) [(A) and (B)] or antibodies to the six ζ phosphotyrosines (C). Experiments shown are representative of three to five independent experiments.

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13. Proteins were expressed in HeLa cells using recombinant T7 vaccinia virus and lipofectace (Gibco) (*34*). Six Y → F mutants were made using PCR mutagenesis. To phosphorylate ζ molecules, cells were cotransfected with equal amounts of cDNAs for $p56^{lckF505}$ and for $p58^{fynF528}$ encoding constitutively active kinases. After lysis of cells, ζ protein was precipitated with the monoclonal antibody (mAb) CO. For the generation of CO, CBA/J mice were immunized with the peptide CDGLY(PO_4)QGLSTATKDTY(PO_4)DALH derived from ITAM C, and spleen cells were fused with a B cell hybridoma. CO recognizes all ζ proteins independent of phosphorylation (*17*). Proteins were separated on 13% SDS PAGE gels, and molecular sizes were determined. Expression of each mutant without kinases gave rise to a 16-kD form of ζ. The band with the lowest mobility in pζ co-migrated with p23 from activated T cells. Mutagenesis of individual tyrosines affected the mobility of phospho-ζ differently, suggesting different effects of individual phosphotyrosines on ζ structure (*30*). Anti-pA1 serum was precipitated with ammonium sulfate and passed over an agarose column containing the engineered phosphopeptide CGKY(PO_4)GKLGKY(PO_4)GKY(PO_4)KGLGK or the unphosphorylated AP1 peptide. Anti-pA2 was purified similarly. Anti-pC2 was purified on the nonspecific phosphotyrosine column only. Anti-pB1, anti-pB2, and anti-pC2 were specific without purification; all sera were titrated to give the best background-to-signal ratio in protein immunoblotting. Antiserum to ζ for protein immunoblotting was raised as in (*35*), and the mAb to pY was 4G10 (UBI). Protein immunoblots were detected by ECL (Amersham), with techniques according to the manufacturer's instructions, except that blots were washed in 0.5% NP-40 containing phosphate-buffered saline for an additional 15 min after each antibody incubation.
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16. A2 phosphorylation in resting T cells was observed in four of seven experiments. When it was observed, it was consistently weaker than B1 and C2 phosphorylation when compared to maximal phosphorylation upon T cell activation. Thus, A2 phosphorylation may not be as prominent as B1 and C2 phosphorylation in resting T cells. Anti-pA1 did not recognize p21, but consistently reacted with minor migration forms of p-ζ of less than 21 kD. These minor forms were also present in the protein immunoblot to pY after longer exposure of the film. Anti-pC2 recognized p21; thus, p21 is not a form of η, an alternatively spliced form of ζ that does not contain tyrosine C2. p21 and p23 were not recognized by monoclonal or polyclonal antibodies to ζ, presumably because the amount of protein in p21 and p23 was too low to be detected (*17*).
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18. A. Grakoui and P. Allen, in preparation.
19. We washed 7.5×10^7 spleen cells from 4- to 6-week-old 2.102 RAG-$1^{-/-}$ mice twice with cold Hank's balanced salt solution and then lysed the cells. The TCR complex was precipitated with 10 μg of mAb 500.A2 to CD3ε and analyzed using protein immunoblotting as described for Fig. 2 and 3.
20. Anti-pB2 serum could also recognize p21 in activated T cells, although this was only apparent after long exposure of the film. p21 with pB2 may represent a minor fraction of pζ in the transition to full phosphorylation. Because B2 phosphorylation in p21 of resting T cells was not detected, p21 can probably contain multiple phospho-species that are not resolved electrophoretically. Similarly, p23 contained several species (Fig. 3 and text).
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Inactivation of a Serotonin-Gated Ion Channel by a Polypeptide Toxin from Marine Snails

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The venom of predatory marine snails is a rich source of natural products that act on specific receptors and ion channels within the mammalian nervous system. A 41–amino acid peptide, σ-conotoxin GVIIIA, was purified on the basis of its ability to inactivate the 5-HT_3 receptor, an excitatory serotonin-gated ion channel. σ-Conotoxin contains a brominated tryptophan residue, which may be important for peptide activity because the endogenous ligand for the 5-HT_3 receptor is a hydroxylated derivative of tryptophan. σ-Conotoxin inactivates the 5-HT_3 receptor through competitive antagonism and is a highly selective inhibitor of this receptor. Serotonin receptors can now be included among the molecular targets of natural polypeptide neurotoxins.

Molecular targets of natural polypeptide neurotoxins include neurotransmitter receptors and voltage-gated ion channels from many different families (*1*). An important group of neurotransmitter receptors that seems to have been excluded as a toxin target is the large family of recep-