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12. Six phosphopeptides derived from the murine ζ ITAMs (*33*) were synthesized in our laboratory [AP1 = CLQDPNQLY(PO_4)NELNLGR, AP2 = CNLGRREEY(PO_4)DVLEKKR, BP1 = CEGVY(PO_4)NALQKDKMAEAY(PO_4)SEIG, BP2 = CKDKMAEAY(PO_4)SEIGTKG, CP1 = CGKGHDGLY(PO_4)QGLSTAT, CP2 = CSTATKDTY(PO_4)DALHMQT]. Peptides were purified with high-performance liquid chromatography, analyzed by mass spectrometry, quantitated by amino acid analysis (Beckman Model 6300), and coupled to keyhole limpet hemocyanin (KLH) using bromoacetylsuccinimide at a mass ratio of 1:4 peptide:KLH. Two New Zealand White rabbits were immunized with each peptide (750 μg/rabbit) 5 to 10 times with the first immunization emulsified in complete Freund's adjuvant and all subsequent ones in incomplete Freund's adjuvant. For blocking of antisera, peptides were added at 10^{-9} M to the protein immunoblotting solution.
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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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-

tors for which serotonin [5-hydroxytryptamine (5-HT)] is the endogenous agonist. Serotonin modulates many processes in mammalian peripheral and central nervous systems through its interactions with at least 14 receptor subtypes, all but one of which are G protein (heterotrimeric GTP–binding protein)–coupled (*2*). The 5-HT_3 subtype is the exception because it is a ligand-gated ion channel that shares functional and structural similarities with nicotinic acetylcholine receptors (*3*, *4*). Functional cDNA clones encoding defined serotonin receptor subtypes were used to selectively screen for and purify bioactive toxins through electrophysiological assays. The venom of marine snails was used for this search, because these organisms produce a vast array of small structurally constrained peptides that rapidly immobilize prey by targeting G protein–coupled receptors and ligand- or voltage-gated ion channels (*1*, *5*).

Crude venom extracts from several *Conus* species were tested for their ability to block serotonin-activated currents in *Xenopus* oocytes expressing recombinant 5-HT_3 receptors (*6*). Venom from *Conus geographus* produced potent and specific 5-HT_3 channel inhibition at 0.25 mg/ml (Fig. 1A). This effect was observed in three independent *C. geographus* venom preparations, one of which was fractionated by reverse-phase high-performance liquid chromatography (HPLC) to effect purification of the active component (Fig. 1B, left) (*7*). Chemical sequencing (*8*) and mass spectrometry (*9*) were used to characterize the isolated intact toxin. The 5-HT_3 receptor–inactivating peptide was 41 amino acids long, making it the largest *Conus* peptide thus far characterized (Fig. 1C). The peptide had an amidated COOH-terminus and contained 10 cysteine residues that, based on the observed intact mass, formed five disulfide bonds. Conotoxin peptides are grouped into families according to their disulfide-bonding pattern and their receptor target. Because the 5-HT_3 receptor–inactivating peptide has a unique molecular target and contains five disulfide bonds, it defines a novel family of conotoxins and was thus named GVIIIA σ-conotoxin. The amino acid composition of the peptide is notable for the abundance of glycines and threonines and the absence of any acidic residues (having a predicted isoelectric point of 11.8). Chemical sequence analysis did not reveal a standard or commonly modified amino acid at position 34. Mass spectrometry identified this residue as a bromotryptophan, a highly unusual posttranslational modification found in only three other naturally occurring peptides, all from *Conus* snails (*10*). These peptides, and most bromotryptophan-containing small organic molecules of marine origin, are brominated at the 6′ position on the tryptophan moiety. It is probable that the σ-conotoxin peptide is also brominated at this position, and co-chromatography data supported this notion. Epimerization between L and D isomers of tryptophan has been observed in one conotoxin peptide (*11*), and assignment of L-6- Br-Trp in position 34 was therefore determined directly by coelution of native and synthetic [L-6-Br-Trp^{34}Cys(PyE)^{36,38,40}] σ-$conotoxin_{(27–41)}$ fragments under conditions where synthetic σ-$conotoxin_{(27–41)}$ peptides containing L-6-Br-Trp^{34} and D-6-Br-Trp^{34} could be resolved (Fig. 1B, right) (*12*). Sequence analysis of a cloned cDNA encoding σ-conotoxin was consistent with the amino acid sequence for the purified toxin peptide, including a tryptophan at position 34 and a glycine at position 42, from which the COOH-terminal amide is presumably derived (*13*).

Electrophysiological analysis of σ-conotoxin activity demonstrated that it is a potent and reversible inhibitor of 5-HT_3 receptor channels. At 1 μM, σ-conotoxin completely inhibited 5-HT_3 responses to bath-applied serotonin (10 μM) (Fig. 2A). Washout of the toxin led to full recovery of 5-HT_3 channel

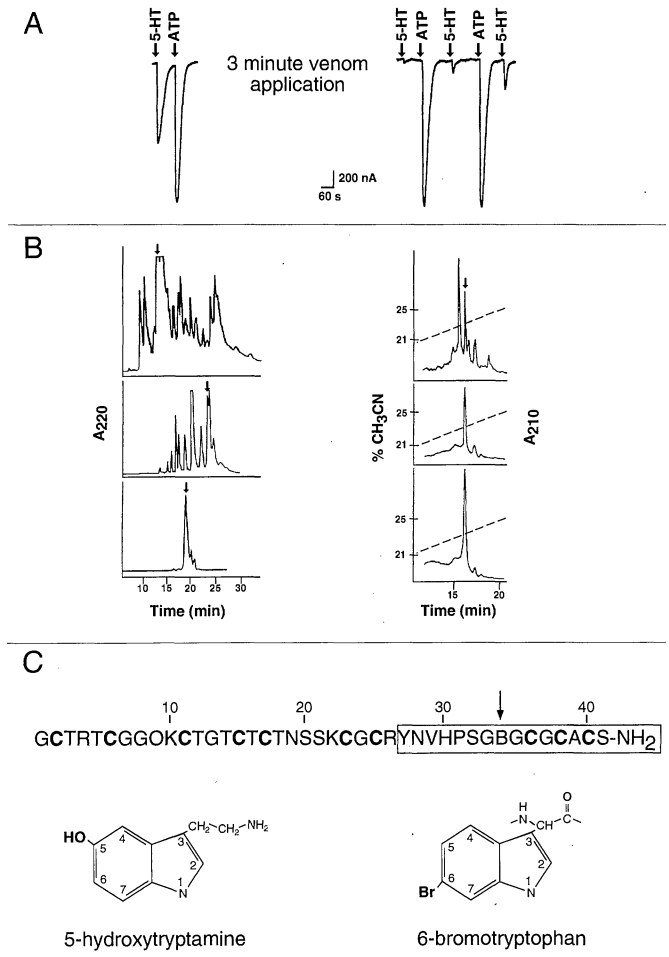

Fig. 1. Identification of σ-conotoxin as an inhibitor of 5-HT_3 receptor activity. (**A**) *Xenopus* oocytes were coinjected with cRNAs encoding 5-HT_3 and $P2X_2$ receptors and analyzed for serotonin-evoked (10 μM) or ATP-evoked (100 μM) currents. After determination of initial agonist responses, oocytes were removed from the recording chamber to minimize the volume of toxin used and incubated for 3 min in 5 μl of crude *C. geographus* venom extract (protein concentration, 0.25 mg/ml) in Ringers solution. Subsequent responses showed selective and complete elimination of serotonin-evoked currents, which eventually recovered after a prolonged washout period. (**B**) (**Left**) Purification of GVIIIA σ-conotoxin from *C. geographus* venom. Venom extract was purified with three sequential (upper, middle, and lower panels) reverse-phase HPLC steps. The fraction containing 5-HT_3 receptor antagonist activity is indicated with an arrow. Pure bioactive peptide (arrow, lower panel) was subjected to chemical sequencing and mass spectrometry. (**Right**) Coelution of the native and synthetic [L-6-Br-Trp^{34}Cys(PyE)^{36,38,40}] σ-$conotoxin_{(27–41)}$. HPLC chromatograms of (upper panel) native σ-$conotoxin_{(27–41)}$ (arrow), which was collected for reinjection; (middle panel) synthetic [L-6-Br-Trp^{34}Cys(PyE)^{36,38,40}] σ-$conotoxin_{(27–41)}$; (lower panel) coelution of native and synthetic [L-6-Br-Trp^{34}Cys(PyE)^{36,38,40}] σ-$conotoxin_{(27–41)}$ under conditions where the D-6-Br-Trp^{34}–containing synthetic σ-conotoxin fragment eluted at a distinct position from the L-6-Br-Trp^{34} and native σ-$conotoxin_{(27–41)}$ fragments. (**C**) Amino acid sequence of purified σ-conotoxin GVIIIA. O, hydroxyproline; B, 6-L-bromotryptophan; NH_2, COOH-terminal amidation. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; G, Gly; H, His; K, Lys; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr. The synthetic peptide used for coelution studies corresponds to the boxed sequence. The chemical structure for 6-bromotryptophan is compared with that of serotonin (5-hydroxytryptamine).

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function, with half-maximal activity returning within 8 to 23 min (average time 12.5 min; $n = 6$). This presumably reflects a slow dissociation rate of the toxin, because 5-HT_3 channel activity fully recovered from agonist stimulation within 2 min in the absence of toxin. To assess the specificity of σ-conotoxin for 5-HT_3 receptors, we compared its actions at several other neurotransmitter receptors that either bind the same agonist or share structural similarities (*14*). Whereas 5-HT_3 receptor activity was fully blocked by σ-conotoxin, no significant inhibitory effect was seen with the other receptors or channel complexes (Fig. 2B).

To determine whether σ-conotoxin is a competitive antagonist, we tested the ability of purified toxin to displace the competitive antagonist [^{3}H]-zacopride from HEK293 cells stably expressing 5-HT_3 receptors (*15*). These data show that σ-conotoxin potently displaced [^{3}H]-zacopride, with a median inhibitory concentration (IC_{50}) of 53 ± 3 nM, from which an inhibition constant (K_i) of 4.8 ± 0.3 nM was derived (Fig. 2C). The interaction of σ-conotoxin with the 5-HT_3 receptor rivals the highly specific and potent synthetic small molecule antagonists, such as zacopride, ondansetron, and MDL 72222, which have reported K_i's of 0.1 to 1.9 nM, 0.9 to 6.0 nM, and 5.3 to 55 nM, respectively (*16*). σ-Conotoxin has a Hill coefficient of 1.0, which suggests that it interacts with a single site or with multiple noncooperative sites. We also asked whether σ-conotoxin could interact with the 5-HT_4 subtype, a metabotropic receptor that is activated by some antagonists of the 5-HT_3 receptor. Competitive radioligand binding studies with the 5-HT_4 antagonist [^{3}H]-GR-113808 did not reveal any interaction between σ-conotoxin and the cloned 5-HT_4 receptor (*13*). We also asked whether σ-conotoxin could inactivate a chimeric ion channel in which the putative extracellular ligand binding domain of the 5-HT_3 receptor is replaced with the cognate region of the α7 nicotinic acetylcholine receptor (nAChR) (*17*). The failure of purified σ-conotoxin to block the α7/5-HT_3 chimera (Fig. 2B) is consistent with our findings that the toxin inactivates the 5-HT_3 receptor primarily through competitive antagonism, which is presumably mediated through interaction of the toxin with the extracellular domain of the receptor.

The identification of σ-conotoxin demonstrates that the serotonergic system is a target for venoms of predatory snails. The 5-HT_3 receptor is the first known molecular target of any Br-Trp–containing conotoxin, and perhaps this derivatized tryptophan residue is an important determinant of the pharmacological specificity of σ-conotoxin, because the endogenous agonist for 5-HT receptors is a hydroxylated tryptophan derivative (Fig. 1C). Indeed, the 6-Br-Trp moiety is located within the largest intercysteine segment of the toxin, a hypervariable region of conotoxin peptides that has been hypothesized to play a critical role in defining target specificity (*5*, *18*). Thus, perhaps the 6-Br-Trp moiety is situated within a constrained loop of the toxin in a configuration that favors interaction with the serotonin binding site. Tests of this hypothesis await the availability of functional synthetic toxin peptide containing substitutions at the Br-Trp position. A related question is whether other Br-Trp–containing conotoxins target 5-HT receptors. We tested the ability of two such peptides, bromocontryphan and bromoheptapeptide, to block the activity of 5-HT1A, 5-HT2A, 5-HT2C, and 5-HT_3 receptors. No inhibition was observed, although many other receptor and channel subtypes still remain as potential targets for brominated toxins.

Predatory strategies of *Conus* snails include multiple simultaneous mechanisms for immobilizing prey through neuromuscular and sensory blockade and excitotoxic shock (*5*). Inactivation of 5-HT_3 receptors could contribute to inhibition of neurotransmitter release at motor or sensory synapses. Irrespective of whether 5-HT receptors in fish are bona fide physiological targets for conotoxins, venom of the *Conus* snail can be viewed as a combinatorial peptide library that maintains a broad spectrum of neuroactive ligands capable of incapacitating prey through myriad molecular mechanisms. σ-Conotoxin is a potent reagent with which to probe the agonist binding site of one member of an important class of ligand-gated ion channels.

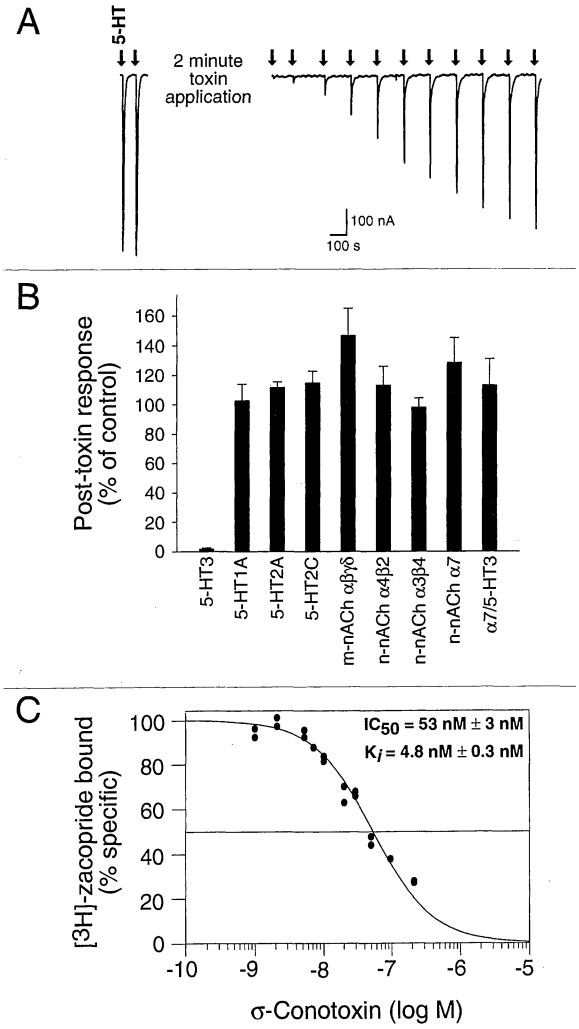

Fig. 2. Purified σ-conotoxin GVIIIA is a highly specific and reversible competitive antagonist of the 5-HT_3 receptor. (**A**) A *Xenopus* oocyte expressing the 5-HT_3 receptor was challenged with serotonin (10 μM, arrows) before and after incubation in purified σ-conotoxin GVIIIA (1 μM). Continuous washing of the oocyte resulted in recovery of half-maximal serotonin-evoked responses by ~12.5 min. Direct addition of purified toxin to the recording chamber did not elicit responses, demonstrating that the toxin has no measurable agonist activity. (**B**) *Xenopus* oocytes expressing a given serotonin receptor subtype (5-HT_3, 5-HT_{1A}, 5-HT_{2A}, or 5-HT_{2C}), an nAChR complex [muscle (m-) α1β1γδ, neuronal (n-) α4β2, n-α3β4, or n-α7], or chimeric receptor (α7/5-HT_3) were used to assess the specificity of σ-conotoxin action. Oocytes were examined for their response to serotonin or acetylcholine in the two-electrode voltage-clamp configuration. Data were normalized to the response of each oocyte before exposure to toxin. Error bars indicate average responses ± SEM. Uninjected control oocytes showed no response to serotonin or acetylcholine. (**C**) σ-Conotoxin and zacopride compete for binding to the 5-HT_3 receptor. Membrane preparations from stably transfected HEK293 cells expressing the mouse 5-HT_3R-A receptor were incubated with the radiolabeled competitive antagonist [^{3}H]-zacopride (1 nM) in the absence or presence of various concentrations of purified σ-conotoxin.

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6. 5-HT_3 receptors were coexpressed with ATP-gated ion channels of the $P2X_2$ subtype. Differential sensitivities to serotonin and ATP were analyzed to control for nonspecific toxicity. *Xenopus* oocytes and cRNA transcripts were prepared as described in (*3*) and in A. J. Brake, M. J. Wagenbach, D. Julius, *Nature* **371**, 519 (1994). All animals were housed and cared for by University of California, San Francisco (UCSF) veterinary staff under federal and UCSF guidelines. The cRNA transcripts were injected 24 hours after oocyte harvest and electrophysiological recordings were made 36 to 72 hours after injection. Oocytes were voltage-clamped with two electrodes (holding potential, −70 mV) using Geneclamp 500 (Axon Instruments), and data were digitized with MacLab MK II software. Microelectrodes (Narishige GD-1, Sea Cliff, NY) were filled with 3 M KCl and had resistances of 0.5 to 1.5 megohm. Oocytes were perfused by gravity at a rate of 1 ml/min with Ringers solution [90 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes (pH 7.6), 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$] in a 30-μl recording chamber at room temperature. Agonists were applied for 3 s.
7. Crude *C. geographus* venom was collected from dissected ducts, lyophilized immediately, and stored at −70°C. Lyophilized crude venom (360 mg) was resuspended in 30 ml of 40% acetonitrile with 0.5% trifluoroacetic acid (TFA), vortexed, and sonicated four times for 30 s, with brief cooling intervals. The extract was clarified by centrifugation before HPLC purification. Venom extract was diluted 20-fold with 0.1% TFA and fractionated on a Vydac C18 preparative column (2.5 cm by 25 cm), with a guard column (22 mm by 50.8 mm) using 1000 ml of 0.1% TFA in a linear gradient from 0% to approximately 90% acetonitrile (flow rate of 20 ml/min). The active fraction was further fractionated on a Vydac C18 semipreparative column (10 mm by 250 mm) using 150 ml of 0.1% TFA with a linear gradient of 4.5 to 31.0% acetonitrile (a 0.9% increase in acetonitrile per minute at a flow rate of 5 ml/min). The final purification step was done on a Vydac C18 analytical column (4.6 mm by 250 mm) using 50 ml of 0.1% TFA with a linear gradient of 13.5 to 27.0% acetonitrile (a 0.45% increase in acetonitrile per min at a flow rate of 1 ml/min). For functional studies, purified σ-conotoxin was stored in 50 mM tris-Cl (pH 7.6) with bovine gamma globulin (0.2 mg/ml), which had no effect on electrophysiological or radioligand binding assays.
8. Approximately 1 nmol of peptide in 70 μl of HPLC elution buffer was treated with dithiothreitol (DTT) (9 mM final concentration) after the pH was raised to 7.5 with 1.0 M tris-base. Argon was bubbled through the solution, and the reaction was incubated at 65°C for 15 min. After cooling to room temperature, 4 μl of 20% 4-vinyl pyridine in ethanol was added, and the reaction was incubated in the dark for 25 min at room temperature. The reaction was diluted fivefold with 0.1% TFA, and the modified peptide was isolated by HPLC using a Vydac C18 analytical column (4.6 mm by 250 mm) with a linear gradient of 2 to 42% acetonitrile in the presence of 0.1% TFA. The sequence was determined with Edman chemistry (Applied Biosystems model 477A Protein Sequencer, Protein/DNA Core Facility, University of Utah Cancer Center), with the exception of a blank cycle at position 34.
9. A solution containing 5 nmol of the peptide in 350 μl of elution buffer was concentrated sevenfold under vacuum, and 0.5 mg of Endoproteinase Arg-C [in 40 ml of 200 mM tris-Cl (pH 7.6) and 20 mM $CaCl_2$] was added. DTT and EDTA were added to final concentrations of 5 mM and 0.5 mM, respectively, and the total reaction was incubated at 37°C for 18 hours. The digest was diluted threefold with 0.1% TFA, and fragments were separated by HPLC using a Vydac C18 analytical column (4.6 mm by 250 mm) with a linear gradient of 0 to 54% acetonitrile in the presence of 0.1% TFA. The most abundant fragments were pyridylethylated and sequenced as above or analyzed by laser desorption mass spectrometry. Electrospray mass spectrometric analyses of (i) the intact toxin $[M + 4H]^{4+}$ species (M_{obs} = 4187.6 and M_{calc} = 4188.42 daltons, where M represents the monoisotopic mass) and (ii) the native [Cys-(PyE)^{36,38,40}] σ-conotoxin$_{(27–41)}$ $[M + 3H]^{3+}$ species (M_{obs} = 1932.0; M_{calc} = 1931.67 daltons) indicated the presence of Br-Trp and amidation of the COOH-terminus of the peptide. Mass spectrometry was performed with a Bruker Esquire (Bruker Daltonics, Billerica, MA) electrospray quadrupole ion trap mass spectrometer. Samples (in 0.055% aqueous TFA and approximately 25% acetonitrile) were diluted 1:1 with 1% acetic acid in methanol and infused at 1 ml/min. Spectra were measured in the maximum scan resolution mode with trap ion current set (and calibrated) at 5000 ions to ensure full resolution of the multiply charged species isotopic clusters. Mass accuracy was typically better than 200 parts per million under the conditions used.
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12. The [L-6-Br-Trp^{34}Cys(PyE)^{36,38,40}] σ-conotoxin$_{(27–41)}$ and [D-6-Br-Trp^{34}Cys(PyE)^{36,38,40}] σ-conotoxin$_{(27–41)}$ were synthesized using the fluorenylmethoxycarbonyl (Fmoc) strategy as previously described (*10*), with the three cysteine residues being protected during synthesis with acetamido methyl groups that were stable to both the TFA deprotection and hydrofluoric acid cleavage steps. After purification of the linear peptides, the acetamido methyl groups were removed with silver trifluoromethane sulfonic acid. The peptides were reduced with tris-(2-carboxyl ethyl)phosphine and pyridylethylated with 4-vinyl pyridine. The corresponding native fragment was generated by reduction and pyridylethylation of the intact peptide and Arg-C hydrolysis and identified by mass spectrometric analysis. The intense earlier eluting species corresponds with native [Cys-(PyE)^{6,11,15,17,23,25,36,38,40}] σ-conotoxin$_{(5–41)}$. Chromatography was carried out on a Vydac C18 reverse-phase column (2.1 mm by 150 mm) using a linear gradient of 0.45% CH_3CN/min (buffer A, 0.055% aqueous TFA; buffer B, 90% CH_3CN + 0.05% TFA; flow rate, 200 ml/min).
13. L. J. England *et al.*, data not shown.
14. Each receptor or channel complex was expressed in oocytes, and agonist-evoked currents resulting from activation of second-messenger pathways (5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C}) or direct ion-channel gating (5-HT_3 and nAChRs) were measured. The plasmids encoding cDNAs for the mouse 5-HT_3 receptor (pMX205-1); human 5-HT_{1A} (pSP-G21); rat 5-HT_{2A} (pSR2); rat 5-HT_{2C} (pSR1c); mouse muscle AChR subunits α1, β1, γ, and δ (BMA407, pBMB49, BMG419, and BMD451, respectively); rat neuronal nicotinic receptor subunits α3, α4-1, β2, β4, and α7 [pPCA48E(3), pHYA23-1E(1), pPCX49, pZPC13, and pCDM6x1, respectively]; and the chimeric G protein $G\alpha_{q/o_5}$ were linearized and transcribed as described [B. K. Kobilka *et al. Nature* **329**, 75 (1987); D. Julius, K. N. Huang, T. J. Livelli, R. Axel, T. M. Jessell, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 928 (1990); D. Julius, A. B. MacDermott, R. Axel, T. M. Jessell, *Science* **241**, 558 (1988); J. Patrick, J. Boulter, D. Goldman, P. Gardner, S. Heinemann, *Ann. N.Y. Acad. Sci.* **505**, 194 (1987); J. Boulter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7763 (1987); R. M. Duvoisin, E. S. Deneris, J. Patrick, S. Heinemann, *Neuron* **3**, 487 (1989); B. Conklin, Z. Farfel, K. D. Lustig, D. Julius, H. R. Bourne, *Nature* **363**, 274 (1993)]. Fifty nanoliters of each cRNA transcript was injected 24 hours after oocyte harvest, and electrophysiological recordings were made 36 to 72 hours after injection. Agonist (5-HT_3, 10 μM 5-HT; 5-HT_{1A}, 0.5 μM 5-HT; 5-HT_{2A} and 5-HT_{2C}, 3 μM 5-HT; muscle nAChR, 0.5 μM ACh; neuronal α4β2 nAChR, 10 μM ACh; or neuronal α3β4 and α7 AChR, 500 μM ACh) was applied for 1 to 3 s using gravity perfusion (1 ml/min). Agonist concentrations exceeded median effective concentration values of the receptors examined by at least threefold. After establishment of reproducible baseline responses and a washout period of 5 to 20 min, each oocyte was removed from the recording chamber and soaked in 5 μl of purified σ-conotoxin (1 μM) diluted in Ringers solution. After a 2-min incubation, oocytes were returned to the chamber, and agonist-evoked responses were recorded. Data were collected as described (*6*), and values for all oocytes tested under the above conditions were plotted (5-HT_3, n = 25; 5-HT_{1A}, n = 10; 5-HT_{2A}, n = 6; 5-HT_{2C}, n = 6; muscle nACh α1β1γδ, n = 6; neuronal nACh α4β2, n = 7; neuronal nACh α3β4, n = 8; neuronal nACh α7, n = 10; and α7/5-HT_3 chimera, n = 16).
15. Crude membranes were prepared from human embryonic kidney HEK293 cells stably expressing mouse 5-HT_3R-A receptors (P3 cell line). Five micrograms of membrane protein were incubated with 1 nM *S*-[methoxy-^{3}H]-zacopride (84 Ci/mmol) (Amersham) plus various concentrations of purified σ-conotoxin in binding buffer [10 mM tris-Cl and 1 mM EDTA (pH 7.6)] for 40 min at room temperature. The binding reaction was terminated with 5 ml of ice-cold buffer, rapidly filtered through GF/B filters presoaked in 0.3% polyethyleneimine, then washed twice with 5 ml of ice-cold buffer. Nonspecific binding was determined in the presence of 10 μM LY-278,584 (Research Biochemicals International, Natick, MA). Filters were added to 5 ml of ScintiVerse II scintillation fluid (Fisher Scientific) and counted in a Beckman LS6500 Scintillation counter. The percent of [^{3}H]-zacopride bound = 100 × {[specific counts per minute in the presence of toxin]/[specific counts per minute in the absence of toxin]}. An experimentally determined IC_{50} value of 53 ± 3 nM was used to calculate the K_i value of 4.8 ± 0.3 nM as described [Y. C. Cheng and W. H. Prusoff, *Biochem. Pharmacol.* **22**, 3099 (1973)].
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