Structure−Activity Relationship Studies of Spinorphin as a Potent and Selective Human $P2X_3$ Receptor Antagonist

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Spinorphin, an endogenous antinociceptive peptide (LVVYPWT), showed potent and non-competitive antagonism at the ATP-activated human $P2X_3$ receptor (IC_{50} = 8.3 pM) in a two-electrode voltage clamp assay with recombinant human $P2X_3$ receptors expressed in *Xenopus* oocytes. Single alanine substitutions from 1st to 4th amino acids and the cyclic form of LVVYPWT sustained antagonistic properties at the human $P2X_3$ receptors, whereas the threonine to alanine substitution resulted in an enhancing effect of the agonistic activity.

Introduction

$P2X_3$ receptors, members of the P2 purinergic receptor family, are ligand-gated ion channels activated by extracellular ATP[a] as an endogenous ligand. The expression of $P2X_3$ receptors is highly localized in the peripheral and central processes of sensory afferent neurons.[1−3] Extracellular ATP has been studied as a neurotransmitter at synapses in sympathetic ganglia[4] and in the brain.[5] The activation of $P2X_3$ receptors by ATP based on a pronociceptive effect has been shown to initiate the pain signaling involved in chronic inflammatory nociception and neuropathic pain due to nerve injury, implicating a possibility of a new drug target for pain control.[6,7] The reported $P2X_3$ receptor antagonists **1**−**3** are listed in Figure 1. The ATP analogue **1** (TNP-ATP)[8] and the pyridoxal 5′phosphate derivative **2** (MRS 2273)[9] were developed as potent $P2X_3$ receptor antagonists. Recently, **3** (A317491) was identified as a potent and selective non-nucleotide antagonist of the human $P2X_3$ receptor, with an IC_{50} of 99 nM in patch clamp assays and painkilling effects in several animal models.[6] However, most of the known antagonists for $P2X_3$ receptors, including **1**−**3**, contain anionic functional groups such as carboxylic acids[6] or phosphates,[9] which generally limit the bioavailability of these compounds as potential drug candidates.

Spinorphin, **5**, has been isolated from bovine spinal cords as an endogenous peptide (LVVYPWT)[10] with the inhibitory effect of enkephalin-degrading enzymes, resulting in analgesic activity in the tail pinch assay with intracerebroventricular administration.[11] Spinorphin has been reported to inhibit the effect of

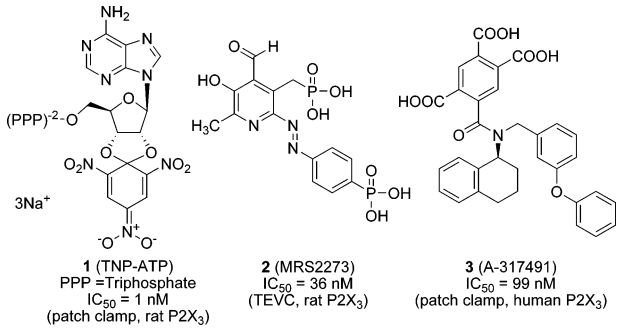

Figure 1. Structures and biological activities of the $P2X_3$ receptor antagonists.

2-MeS-ATP, a P2X receptor agonist, and induced nociception, which was not blocked by morphine, an inhibitor of bradykinin-induced nociceptive response.[12] Thus, a detailed structure−activity relationship study of spinorphin as the first potent peptide antagonist of $P2X_3$ receptors was undertaken. We report on the electrophysiological evaluation of seven alanine scanned spinorphin peptide derivatives, truncated peptide analogues, cyclic peptides, and a retroinverso peptide at the recombinant human $P2X_3$ receptors expressed in *Xenopus* oocytes to determine the channel blocking activity and active fragments of spinorphin.

Results and Discussion

Spinorphin **5** and other peptide derivatives were synthesized using solid-phase peptide synthesis on a Wang resin as depicted in Scheme 1. The synthesized peptides were tested in a functional ion channel assay[13] to measure the ATP-induced current at the recombinant mouse $P2X_1$ and human $P2X_3$ receptors, expressed in *Xenopus* oocytes, using the two-electrode voltage clamping (TEVC) technique. Since purinergic P1 and P2 receptors are present on the follicle cell layer of *Xenopus* oocytes,[14] the TEVC experiment was carried out on defolliculated oocytes. Control experiments were repeated with 2 μM ATP to confirm the reproducible magnitude of peak currents, and the antagonistic effect of each peptide was measured on the basis of the difference of the currents. The effects of spinorphin derivatives at the human $P2X_7$ receptors stably expressed in the HEK293 cells were determined with an ethidium accumulation assay[15] using BzATP as a selective agonist.

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[a] Abbreviations: ATP, adenosine 5′-triphosphate; BzATP, 3′-*O*-(4-benzoyl)benzoyladenosine 5′-triphosphate; DMEM, Dulbecco's modified Eagle medium; EDAC, 1-ethyl-3-(3-dimethlyaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; Fmoc, fluoren-9-ylmethoxycarbonyl; HEK, human embryonic kidney; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid); HOAT, 1-hydroxy-7-azabenzotriazole; HOBT, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; HRMS, high-resolution mass spectrometry; iso-PPADS, pyridoxal-α^5-phosphate-6-zaophenyl-2′,5′-disulfonic acid; KN62, 1-[*N*,*O*-bis(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperizine; NMP, *N*-methyl-2-pyrrolidinone; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; SAR, structure−activity relationship; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid.

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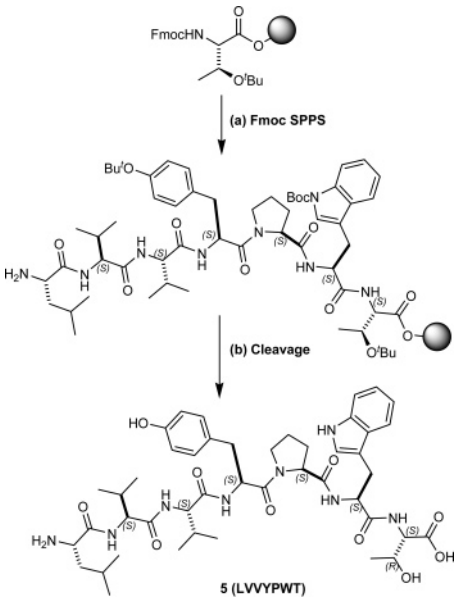
Scheme 1. Solid-Phase Synthesis of Spinorphin[a]



[a] (a) (i) 20% piperidine in NMP; (ii) EDCI, HOBt, Fmoc-amino acids, NMP, 2 h, 25 °C (repeated); (b) TFA/H_2O (95:5 v/v), 3 h, 25 °C.

Table 1. Antagonistic Effects of Peptide Analogues at Mouse $P2X_1$, Human $P2X_3$, and Human $P2X_7$ Receptors

		% inhibition or normalized current I/I_{max}		
compd	sequence	m$P2X_1$[a]	h$P2X_3$[a]	h$P2X_7$[b]
4 (iso-PPADS)		64.2 ± 3.1%[c]	48.3 ± 5.6%	97.6 ± 4.7%
5	LVVYPWT	7.4 ± 1.3%	67.2 ± 3.1%	8.3 ± 0.9%
6	AVVYPWT	inactive	82.5 ± 7.5%	inactive
7	LAVYPWT	8.1 ± 0.9%	48.4 ± 6.3%	7.6 ± 0.3%
8	LVAYPWT	inactive	53.4 ± 1.2%	inactive
9	LVVAPWT	inactive	52.3 ± 8.9%	inactive
10	LVVYAWT	inactive	12.9 ± 1.0%	inactive
11	LVVYPAT	4.2 ± 1.2%	17.0 ± 6.2%	16.7 ± 1.4%
12	LVVYPWA	9.4 ± 1.5%	2.5 ± 0.1[d]	6.7 ± 1.2%
13	cyclic LVVYPWT	11.5 ± 1.7%	36.3 ± 1.6%	8.8 ± 1.0%
14	cyclic AVVYPWT	6.4 ± 0.8%	11.4 ± 2.3%	inactive
15	(D)-TWPYVVL	15.5 ± 2.4%	inactive	inactive
16	VYP	nd	16.2 ± 2.9%[e]	nd
17	VYPW	nd	inactive[e]	nd
18	VYPWT	nd	inactive[e]	nd
19	YPW	nd	15.6 ± 5.4%[e]	nd
20	YPWT	nd	13.3 ± 3.5%[e]	nd
21	PWT	nd	32.7 ± 6.8%[e]	nd
22	VVYPWT	nd	29.3 ± 1.8%[e]	nd

[a] The ion current was induced by 2 μM ATP at the recombinant P2X receptors expressed in *Xenopus* oocytes, and % inhibition of the ion current by 10 μM and 100 nM compounds was measured for mouse $P2X_1$ and human $P2X_3$ receptors, respectively (mean ± SEM, $n = 4$). [b] The accumulation of ethidium$^+$ was induced by 6 μM BzATP at the human $P2X_7$ receptors expressed in HEK293 cells, and % inhibition of the accumulation by 10 μM compounds was measured (mean ± SEM, $n = 3$). [c] 100 nM compound **4** (isoPPADS) was used. [d] Relative fold-increase of the inward current induced by 2 μM ATP in the presence of 100 nM compound **12** compared with 2 μM ATP alone. [e] 10 μM compounds were tested.

The antagonistic activity of spinorphin, **5**, and its derivatives at various P2X receptor subtypes is listed in Table 1. A P2X receptor antagonist, iso-PPADS, **4**, was used as a positive control, which displayed 64%, 48%, and 97% inhibition at 100 nM at the mouse $P2X_1$ and human $P2X_3$ and at 10 μM at the human $P2X_7$ receptor, respectively. Spinorphin and its derivatives with single alanine substitutions from the first to the fourth amino acid from the N-terminus of spinorphin showed higher (**5** and **6**) or equal (**7**−**9**) antagonistic potency compared with iso-PPADS at the human $P2X_3$ receptor.

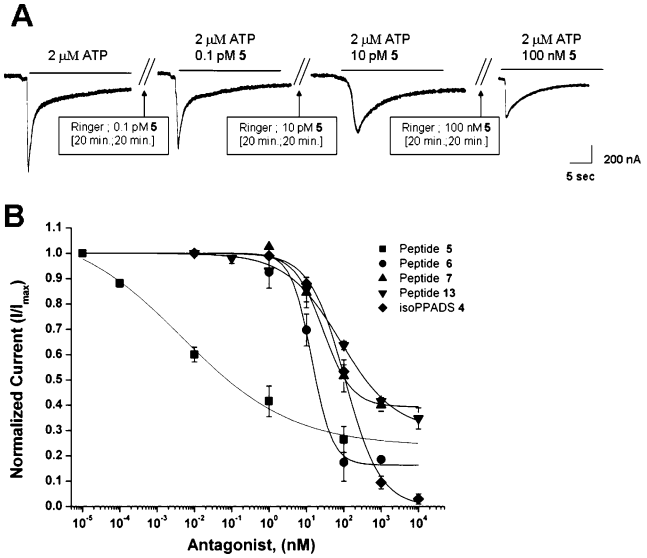


Figure 2. (A) Inhibition of human $P2X_3$ receptor-mediated currents by spinorphin. Representative inward current is elicited by 2 μM ATP in the control and in the presence of three concentrations of spinorphin, **5,** in *Xenopus* oocytes expressing human $P2X_3$ receptor subtypes. (B) Concentration−inhibitory curves of peptides **5** (■), **6** (●), **7** (▲), **13** (▼), and iso-PPADS **4** (◆) for human $P2X_3$ receptors. The continuous line for ATP is fit to the data using the equation $I = I_{max}/(1 + IC_{50}/L)^{nH})$, where I is the actual current for a ligand concentration (L), nH is the Hill coefficient, and I_{max} is the maximal current. The IC_{50} values were 8.3 ± 2.2 pM (**5**), 14.3 ± 5.1 nM (**6**), 32.4 ± 8.7 nM (**7**), 82.4 ± 17.0 nM (**13**), and 108 ± 11 nM (**4**). The Hill coefficients were −0.38 ± 0.10 (**5**), −1.08 ± 0.13 (**6**), −1.27 ± 0.15 (**7**), −0.60 ± 0.05 (**13**), and −0.92 ± 0.08 (**4**). Each value is the mean ± SEM of four observations. The recordings were carried out at a holding potential of −90 mV with 2 μM ATP.

The peptide analogues displaying antagonistic effects at the $P2X_3$ receptor are all highly selective, showing very weak or inactive activity at $P2X_{1,7}$ receptors. The alanine substitutions of the fifth and sixth amino acids of spinorphin (**10** and **11**) significantly reduced the antagonistic activity, displaying only 10−20% inhibition at 100 nM dose (Table 1). Interestingly, the substitution of threonine of spinorphin to alanine (peptide **12**) resulted in a 2.5-fold increase of ATP-induced ion current, showing a dramatic reverse of the antagonistic character of spinorphin into a strong potentiator of the ATP effect at the human $P2X_3$ receptor. Thus, the fifth to seventh amino acids in the sequence of spinorphin seem to be important in recognizing the human $P2X_3$ receptor as an antagonist. Peptides **13** and **14**, as the cyclic forms of peptides **5** and **6**, at 100 nM were found to have relatively weak antagonism, showing 36% and 11% inhibition against the ATP-induced ion current at the human $P2X_3$ receptor, respectively. A retroinverso peptide, **15**, an isomer with reverse sequence and inverted chirality, was inactive in 10 μM, suggesting that terminal functions such as primary amine and carboxylic acid units may need to be appropriately arranged.

Figure 2A shows the dose-dependent inhibition of **5** against the ATP-induced ion current at the human $P2X_3$ receptor with a complete reverse of the antagonistic effects after wash-out. The full dose-response curves of iso-PPADS **4**, spinorphin **5**, alanine substituted peptides **6** and **7**, and a cyclic form of spinorphin **13** are depicted in Figure 2B. Spinorphin showed the most potent antagonistic effect against the inward current elicited by ATP, with an IC_{50} of 8.3 pM and a Hill coefficient of −0.38, suggesting the possibility of a binding mode with negative cooperative antagonism. The alanine substituted peptides **6** and **7** displayed decreased antagonistic activity, with

IC_{50} of 14.3 and 32.4 nM and Hill coefficients of −1.08 and −1.27, respectively, which implies a changed binding mode from negative to positive cooperative antagonism due to the alanine substitutions. The cyclic form of the spinorphin sequence also has an appreciable antagonistic effect with an IC_{50} of 82.4 nM and Hill coefficient of −0.60.

In an effort to search the active fragments of spinorphin, which maintain antagonistic properties at the human $P2X_3$ receptor, a number of truncated peptide analogues were evaluated. However, removal of certain amino acid sequences from the original peptide resulted in a significant loss of antagonistic activity, showing 10−30% inhibition even at 10 μM, as presented in Table 1. In addition, the leucine-deleted peptide **22** was also less active than spinorphin, showing only 30% inhibition at 10 μM. The results suggest that most of the amino acids of spinorphin are important in conferring the antagonistic activity against the human $P2X_3$ receptor subtype.

To investigate whether the interaction of peptide **5** with human $P2X_3$ receptors is competitive, the dose-response curves of ATP-induced currents in the presence of three concentrations of peptide **5** were compared (Figure 3A). Each peak current was normalized to the current value obtained from the treatment with 200 μM ATP, a concentration that showed the maximum ATP-evoked current; the EC_{50} from the dose-response curve of ATP in the absence of antagonist (control) was determined to be 1.66 μM. Increasing concentrations of peptide **5** shifted the dose-response curve of ATP to the right with reduced the maximal current, resulting in EC_{50} values of 8.45, 27.7, and 54.3 μM for 100 nM, 500 nM, and 1 μM of peptide **5**, respectively. The Hill coefficient, nH, of each dose-response curve was also changed (0.84, 0.73, 0.68, and 0.57 for control and 100 nM, 500 nM, and 1 μM peptide **5**, respectively), indicating that the mode of antagonism of peptide **5** is noncompetitive. An alanine-substituted peptide of the first amino acid, **6**, was also noncompetitive at the human $P2X_3$ receptor, showing increasing EC_{50} values and decreasing slopes (Figure 3B).

A competition study between the positive allosteric modulator (peptide **12**) and the negative allosteric modulator (spinorphin **5**) was performed at the recombinant human $P2X_3$ receptors to determine whether both ligands bind to the same allosteric site or not (Figure 4). The dose-dependent antagonism of spinorphin **5** against 2 μM ATP in the presence of 20 nM (fixed concentration) peptide **12** was evaluated. The inhibition curve of the mixed peptides was shifted to the right side, showing decreased IC_{50} (48.1 ± 37.4 pM) and a similar value of slope (−0.32 ± 0.05) compared with those values of spinorphin (IC_{50} = 8.3 ± 2.2 pM, slope = −0.38 ± 0.10). These data suggest that the two oppositely acting allosteric modulators bind to the same binding site in human $P2X_3$ receptors.

In summary, we established potent antagonism and structure−activity relationships of a series of spinorphin derivatives by electrophysiological evaluation with $P2X_{1,3,and7}$ receptors expressed in *Xenopus* oocyte or mammalian cell systems. Although the mode of antagonism was noncompetitive, the peptide derivatives showed great potency and specificity for the human $P2X_3$ receptors in comparison to the mouse $P2X_1$ and human $P2X_7$ receptors. The peptide antagonists discovered in this study will be valuable for future study regarding the structure and function of P2X receptors, and the information collected on the peptide sequences for antagonism can be used for further design of non-peptide antagonists.

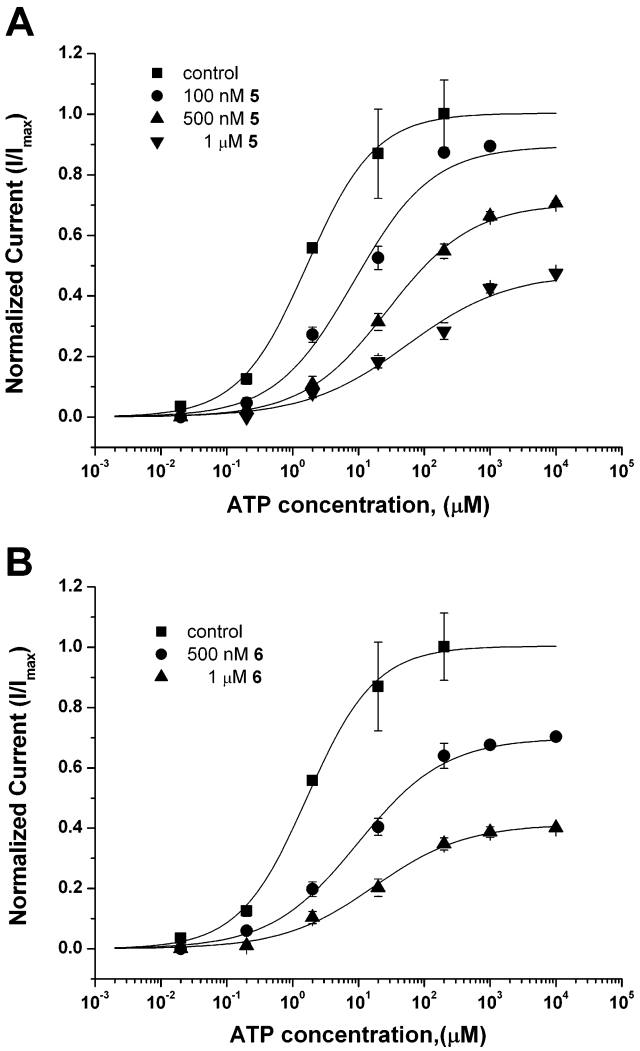


Figure 3. Concentration−response curves for ATP in the presence of increasing concentrations of spinorphin **5** and peptide **6** as indicated. The data correspond to oocytes expressing human $P2X_3$ subunits. In part A, the EC_{50} values and Hill coefficients for ATP (μM) are as follows: (■) for the control, 1.66 ± 0.23, 0.84 ± 0.09; (●) for 100 nM **5**, 8.45 ± 2.00, 0.73 ± 0.11; (▲) for 500 nM **5**, 27.7 ± 1.9, 0.68 ± 0.03; (▼) for 1 μM **5**, 54.3 ± 15.9, 0.57 ± 0.08. In part B, the values are as follows: (■) for the control, 1.66 ± 0.23, 0.84 ± 0.09; (●) for 500 nM **6**, 9.76 ± 1.26, 0.66 ± 0.05; (▲) for 1 μM **6**, 17.3 ± 4.2, 0.63 ± 0.07. Each value is the mean ± SEM of three to seven oocytes.

Materials and Methods

Synthesis, Materials, and Analytical Methods. Spinorphin **5** and other peptide derivatives were synthesized using solid-phase peptide synthesis on a Wang resin loaded with Fmoc-protected amino acids. Note that the representative procedure for the synthesis of spinorphin is depicted in Scheme 1.

Pyridoxal 5′-phosphate monohydrate and aniline 2,4-disulfonic acid used in the azo coupling reaction to prepare **4**[9] were purchased from Aldrich (St. Louis, MO) and TCI (Tokyo). All amino acids used the L-configuration unless otherwise indicated. Fmoc-protected amino acids were purchased from Novabiochem (Darmstadt, Germany). All other reagents and solvents were of analytical or peptide synthesis grade and purchased from Merck (Darmstadt, Germany) and B&J Bioscience.

Proton nuclear magnetic resonance spectroscopy was performed on a JEOL JNM-LA 300WB spectrometer, and spectra were taken in DMSO-d_6. Unless otherwise noted, chemical shifts are expressed as ppm downfield from internal tetramethylsilane or as relative ppm from DMSO (2.5 ppm). Data are reported as follows: chemical

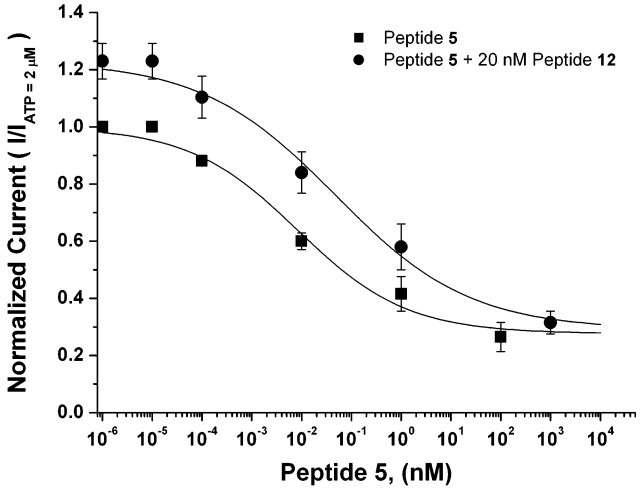


Figure 4. Dose-dependent antagonism of spinorphin, **5,** against 2 μM ATP in the presence of 20 nM (fixed concentration) peptide **12.** The inward current induced by activation by 2 μM ATP with peptide **5** only (squares) and with peptide **5** in the presence of 20 nM peptide **12** (circles) was measured at the recombinant human $P2X_3$ receptors, expressed in *Xenopus* oocytes, using the twin-electrode voltage-clamping technique. The IC_{50} and slope of peptide **5** in the presence of 20 nM peptide **12** were 48.1 ± 37.4 pM and −0.32 ± 0.05, respectively. All data points were each the mean ± SEM of four observations.

shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; b, broad; app, apparent), coupling constants, and integration. Mass spectrometry was carried out on MALDI-TOF and FAB instruments. High-resolution mass spectra (*m*/*z*) were recorded on a FAB instrument. [FAB source: JEOL FAB source and ion gun (Cs ion beam, 30 kV acceleration)]. High-resolution mass analysis was performed at the Seoul Branch Analytical Laboratory of the Korea Basic Science Institute.

The determination of purity was performed on a Shimadzu SCL-10A VP HPLC system using a Shimadzu Shim-pack C18 analytical column (250 mm × 4.6 mm, 5 μm, 100 Å) in linear gradient solvent systems. Solvent system A was (0.1% TFA in H_2O)/(0.1% TFA in CH_3CN) varying from 95:5 to 35:65 in 30 min with a flow rate of 1 mL/min. Solvent system B was (0.1% TFA in H_2O)/(0.1% TFA in MeOH), varying from 95:5 to 35:65 in 30 min with a flow rate of 1 mL/min. Peaks were detected by UV absorption using a diode array detector.

The cRNA of the human $P2X_3$ receptor was obtained by reverse transcription of the cDNA of the human $P2X_3$ receptor, which was generously provided by Dr. W. Stuhmer and Dr. F. Soto of the Max Planck Institute. The EST clones containing full-length cDNAs of mouse $P2X_1$ (clone no. 4189541) and human $P2X_7$ receptor (clone no. 5286944) were purchased (Invitrogen, CA), and their sequences were confirmed by DNA sequencing.

Peptide Synthesis. The peptides were synthesized by the stepwise fluoren-9-ylmethoxycarbonyl (Fmoc) solid-phase method. Fmoc amino acids were stored as 0.5 M *N*-methyl-2-pyrrolidinone (NMP) solutions. Coupling reagents were predissolved in NMP (0.5 M solutions), with the activators EDCI and HOBt as 2 M solutions. All cases utilized 10 equiv of amino acids and coupling reagents at a single 2 h coupling time. Fmoc deprotections were performed with 20% piperidine in NMP. Peptide cleavage from the resin of the amino acid side chains was carried out for 3 h with TFA/H_2O (95:5 v/v). The resins were washed with TFA and the filtrates partially evaporated. The crude products were precipitated with diethyl ether, collected by centrifugation, dissolved in H_2O, and lyophilized. For the synthesis of cyclic peptides, to a solution of the corresponding linear peptide, HOAT (3 equiv) and DMAP (catalytic amount) in DMF/DCM was added to a solution of PyBOP (3 equiv) and DIEA (6 equiv) in DCM. The reaction mixture was stirred for 48 h at room temperature and purified by prep-HPLC.

Peptide 5 (Spinorphin, LVVYPWT). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.74 (s, 1H, Trp), 9.15 (s, 1H, Tyr), 8.46 (d, J = 8.4 Hz, 1H, Tyr), 8.06 (d, J = 7.5 Hz, 1H, Trp), 7.95 (m, 2H, Leu), 7.86 (d, J = 9.6 Hz, 1H, Thr), 7.79 (d, J = 7.8 Hz, 1H, Val), 7.73 (d, J = 8.4 Hz, 1H, Val), 7.55 (d, J = 7.5 Hz, 1H, Trp), 7.28 (d, J = 7.8 Hz, 1H, Trp), 7.13 (s, 1H, Trp), 7.03 (d, J = 8.4 Hz, 2H, Tyr), 7.02−7.09 (m, 1H, Trp), 6.95 (dd, J = 8.4, 7.5 Hz, 1H, Trp), 6.60 (d, J = 8.4 Hz, 2H, Tyr), 4.64 (m, 1H, Tyr), 4.55−4.61 (m, 1H, Trp), 4.28−4.36 (m, 1H, Leu), 4.21−4.27 (m, 2H, 2H of each Val), 4.10−4.19 (m, 2H, each 1H of Thr), 3.84 (t, J = 6.9 Hz, 1H, Pro), 3.51−3.63 (m, 2H, Pro), 3.19−3.24 (m, 1H, Trp), 2.97−3.05 (m, 1H, Trp), 2.64−2.77 (tt, J = 10.2, 9.8 Hz, 2H, Tyr), 1.83−1.92 (m, 3H, 2H of each Val + 1H of Pro), 1.72 (m, 2H, Pro), 1.56 (m, 1H, Leu), 1.46 (dd, J = 6.3, 6.9 Hz, 2H, Leu), 1.03 (d, J = 6.3 Hz, 3H, Thr), 0.78−0.89 (m, 12H, 12H of each Val), 0.68−0.74 (d, J = 6.9 Hz, 6H, Leu). HRMS (FAB) Calcd for $C_{45}H_{65}N_8O_{10}$: 877.4824. Observed *m*/*z* 877.4829. HPLC (t_R, min): system A, 21.6; system B, 25.6.

Pharmacology: Antagonist Activity at Recombinant Mouse $P2X_1$ and Human $P2X_3$ Receptors. *Xenopus* oocytes were harvested and prepared as previously described.[22] Defolliculated oocytes were injected cytosolically with mouse $P2X_1$ and human $P2X_3$ receptor cRNA (40 nL, 1 μg/mL), respectively, and incubated for 24 h at 18 °C in Barth's solution and kept for up to 12 days at 4 °C until used in electrophysiological experiments.

ATP-activated membrane currents (V_h = −90 mV) were recorded from cRNA-injected oocytes using the two-electrode voltage-clamp technique (Axoclamp 2B amplifier). Voltage recording (1−2 MΩ tip resistance) and current-recording microelectrodes (5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were held in an electrophysiological chamber and superfused with Ringer's solution (5 mL/min, at 18 °C) containing the following (mM): NaCl, 110; KCl, 2.5; HEPES, 5; $BaCl_2$, 1.8, adjusted to pH 7.5.

ATP was superfused over the oocytes for 60−120 s and then washed out for 20 min. For inhibition curves, data were normalized to the current evoked by ATP, at pH 7.5. Test substances were added for 20 min prior to ATP exposure; all peptides were tested for reversibility of their effects. The concentration required to inhibit the ATP response by 50% (IC_{50}) was taken from Hill plots constructed using the formula $\log(I/I_{max} - I)$, where I is the current evoked by ATP in the presence of an antagonist. Data are presented as the mean ± SEM (n = 4) from different batches of oocytes.

Ethidium$^+$ Accumulation Assay at Human $P2X_7$ Receptors. Human $P2X_7$-expressing HEK293 cells, human embryonic kidney cells, were grown in DMEM supplemented with 10% fetal bovine serum as monolayer culture at 37 °C in a humidified atmosphere of 5% CO_2. Cells were harvested with treatment of trypsin/EDTA solution and collected by centrifugation (200*g* for 5 min). The cells were resuspended at 2.5 × 10^6 cells/mL in assay buffers consisting of the following (mM): HEPES, 10; KCl, 140; glucose, 5; EDTA, 1 (pH 7.4). Then ethidium bromide (100 μM) was added. Cell suspensions were added to 96-well plates containing the $P2X_7$ receptor agonist, ATP or BzATP, at 2 × 10^5 cells/well. Plates were incubated at 37 °C for 120 min, and cellular accumulation of ethidium$^+$ was determined by measuring fluorescence with a Bio-Tek instrument FL600 fluorescent plate reader (excitation wavelength of 530 nm and emission wavelength of 590 nm). When the effects of antagonists such as PPADS, KN62, and test peptides were studied, antagonists were treated together with agonist without preincubation.

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Supporting Information Available: ^{1}H NMR, HRMS, and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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