A substance P-opioid chimeric peptide as a unique nontolerance-forming analgesic

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To elucidate mechanisms of acute and chronic pain, it is important to understand how spinal excitatory systems influence opioid analgesia. The tachykinin substance P (SP) represents the prototypic spinal excitatory peptide neurotransmitter/neuromodulator, acting in concert with endogenous opioid systems to regulate analgesic responses to nociceptive stimuli. We have synthesized and pharmacologically characterized a chimeric peptide containing overlapping NH_2- and COOH-terminal functional domains of the endogenous opioid endomorphin-2 (EM-2) and the tachykinin SP, respectively. Repeated administration of the chimeric molecule YPFFGLM-NH_2, designated ESP7, into the rat spinal cord produces opioid-dependent analgesia without loss of potency over 5 days. In contrast, repeated administration of ESP7 with concurrent SP receptor (SPR) blockade results in a progressive loss of analgesic potency, consistent with the development of tolerance. Furthermore, tolerant animals completely regain opioid sensitivity after post hoc administration of ESP7 alone, suggesting that coactivation of SPRs is essential to maintaining opioid responsiveness. Radioligand binding and signaling assays, using recombinant receptors, confirm that ESP7 can coactivate μ-opioid receptors (MOR) and SPRs *in vitro*. We hypothesize that coincidental activation of the MOR- and SPR-expressing systems in the spinal cord mimics an ongoing state of reciprocal excitation and inhibition, which is normally encountered in nociceptive processing. Due to the ability of ESP7 to interact with both MOR and SPRs, it represents a unique prototypic, anti-tolerance-forming analgesic with future therapeutic potential.

The neuropeptide substance P (SP) and endogenous opioids are intimately involved in the regulation of acute and chronic pain transmission (1–6). Overlapping distributions of SP- and opioid-containing neurons, as well as their corresponding G protein-coupled receptors, within the superficial dorsal horn of the spinal cord suggest major SP/opioid functional interactions (7–9). The superficial dorsal horn is an important site of functional integration and transmission of nociceptive input. Here neuronal signaling is mediated by both SP and excitatory amino acids released from primary afferent terminals with further modulation by opioid peptides originating from second-order spinal cord neurons. In light of the established literature indicating that SP- and opioid-expressing neurons presumably mediate opposite physiological effects at the spinal level, investigators have tended to overlook the role of SP and SP receptors (SPRs) in the regulation of endogenous opioid systems (10–14), particularly in the area of analgesic responsiveness. Previous pharmacological data from our group strongly suggest that SP released in the dorsal horn plays an important role in antinociception by regulating analgesic activity of the postsynaptic opioid systems (15, 16). In particular, we demonstrated that low doses of SP, when coadministered with marginally effective doses of morphine sulfate (MS) into the rat subarachnoid space, produce a markedly enhanced analgesic response. The pharmacological effect was blocked by previous treatment with naloxone, indicating that the potentiated analgesic response is mediated exclusively through activation of opioid-expressing neurons. Importantly, we have described a unique inhibitory effect of coadministered SP on the development of MS tolerance at the spinal level, thereby providing a theoretical and practical basis for the studies described in the present report.

Recently, two new endogenous opioids with high affinity and selectivity for the μ-opioid receptor (MOR) have been characterized (17). The endomorphin tetrapeptides, EM-1 (Tyr-Pro-Trp-Phe-NH_2) and EM-2 (Tyr-Pro-Phe-Phe-NH_2), isolated from human cerebral cortex and from rat medulla and spinal cord, are potent inducers of naloxone-reversible analgesia in rodents when administered by either the intracerebroventricular or the intrathecal route (18, 19). As described for a wide variety of MOR-preferring opioids (e.g., MS) administration of endomorphins over the course of a few days leads to rapid development of tolerance. Notably, EM-2 displays a similar pattern of distribution as described for SP with peptide localization in the dorsal root ganglion, as well as in the spinal and medullary dorsal horn, thereby suggesting a sensory origin for both ligands (20, 21). Comparison of the EM-2 and SP amino acid sequences revealed two phenylalanine residues that were shared by both peptides. This overlapping region was used in conjunction with the NH_2-terminal amino acids unique to EM-2 and COOH-terminal amino acids unique to SP to create a chimeric peptide capable of coincident activation of MOR and SPRs within the superficial dorsal horn. We now report that repeated administration of the chimeric molecule YPFFGLM-NH_2, designated ESP7, into the rat spinal cord produces opioid-dependent analgesia without loss of potency over 5 days. Additionally, tolerant animals completely regain opioid sensitivity after post hoc administration of ESP7, supporting the hypothesis that SPR-expressing spinal neurons are intimately involved in the regulation of opioid analgesia and maintenance of opioid responsiveness.

Materials and Methods

Peptide Synthesis. ESP7 was synthesized in our laboratory by the solution method published previously and the final crude peptide was purified by gel filtration on Sephadex LH20 followed by preparative HPLC (22). ESP7 was prepared as a peptide-cyclodextrin (β-CD) complex in a molar of ratio 1:2 (23). In *in*

Abbreviations: AUC, area under the curve; β-CD, β-cyclodextrin; CRE, cAMP-responsive element; EM-1 and -2, endomorphin-1 and -2; IP, inositol phosphate; MPE, maximum possible effect; MS, morphine sulfate; MOR, μ opioid receptor; NTX, naltrexone; SP, substance P; SPR, NK-1 or substance P receptor; DAMGO, [D-Ala2,*N*-MePhe4,Gly5-ol]enkephalin; CI, confidence interval.

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vivo tests, the β-CD vehicle was found to be without analgesic effects when administered alone.

Intrathecal Catheterization. All experimental procedures used in the present study were approved by the Tufts University Animal Research Committee, protocol no. 05–97. Adult male Sprague–Dawley rats (200–250 g) were implanted with chronic indwelling intrathecal catheters by using a modified protocol of Yaksh and Rudy (24). Catheters were made of silastic tubing (ID = 0.30 mm; OD = 0.64 mm) and possessed a dead volume of 10 μl. Catheters measured a total of 11.5 cm with 7.5 cm inserted into the intrathecal space to the level of T13-L1. Rats were anesthetized with 5.0% isoflurane. The catheter was inserted through the alanto-occipital membrane and into the intrathecal space using a guide wire. Sutures were used to secure the placement of the catheter. The rats were allowed to recover from the surgery for 3–4 days. Rats exhibiting any sign of neurological or motor impairment, as evidenced by paralysis, abnormal gait, weight loss, or negligent grooming, were excluded from the study. Rats were housed separately to ensure catheter patency in a temperature- and light-controlled environment, with free access to food and water. After completion of drug testing, the catheter position was verified in each animal by postmortem examination of the spinal cord. The ESP7–β-CD complex was dissolved in sterile saline and injected in a volume of 10 μl, followed by 10 μl of saline to flush the catheter.

Tail-Flick Assay. During recovery from surgery, the rats were habituated to the laboratory environment and to the analgesic-testing apparatus. For measurement of the thermal antinociceptive properties of ESP7, a custom-made tail-flick apparatus (Department of Medical Engineering, New England Medical Center), consisting of a variable-intensity 300-W quartz projector bulb and a photodetector-automatic timer sensitive to 0.01-sec intervals, was used. During testing, rats were placed in the tail-flick chamber; a light source was directed at the underside of their tail, and the latency to remove the tail was recorded. The baseline latency was ≈3.5 sec, and the cutoff latency was set to 10 sec to avoid tissue damage. Three measurements were made at each pre- and post-treatment time point, and the results were averaged. Responses were expressed as percentage of maximum possible effect (%MPE): {%MPE = [(posttreatment latency − baseline latency)/(cutoff latency − baseline latency)] × 100}. The area under the curve (AUC) was calculated for each dose on each day using the trapezoidal method (25). The data were evaluated with one-way repeated measures ANOVA followed by Bonferroni-corrected pairwise comparisons. The Dunnett test was used for all pairwise multiple comparisons versus control. Significance was defined as a $P < 0.05$. It was expected that six animals would permit seven Bonferroni-corrected contrasts to be made at an overall power of at least 90%.

Receptor Binding Assays. The affinity of ESP7 for the MOR and SPRs was determined by using rat brain membrane preparations according to a modified version of the referenced protocols (26, 27). In brief, for the μ receptor, fresh frozen rat brains were homogenized in 40 vol of standard buffer (50 mM Tris·HCl, pH 7.4/0.2 mg/ml BSA/2.5 mM EDTA/40 μg/ml bacitracin/30 μg/ml bestatin/5 mM $MgCl_2$). After centrifugation at 15,000 × *g* for 20 min, the pellet was washed with standard buffer (+100 mM NaCl), followed by standard buffer alone. The membrane preparation was resuspended in 10 vol of incubation buffer (standard buffer with 4 μg/ml leupeptin and 2 μg/ml chymostatin). The same procedure was followed for the SPR except the wash with NaCl was eliminated and 5 mM $MgCl_2$ was replaced with 3 mM $MnCl_2$. Binding assays for the μ receptor were performed at 4 μC for 90 min. Each reaction contained incubation buffer (described above), brain membrane, 1.85 nM [^{3}H][D-Ala2,*N*-MePhe4,Gly5-ol]enkephalin (DAMGO) (DuPont/NEN), and increasing concentrations of cold competitor (ESP7). All assays were done in triplicate and repeated twice. Nonspecific binding was determined with 10 μM DAMGO (gift from National Institute on Drug Abuse) for the μ receptor. After incubation, the samples were filtered on a Brandell-Harvester apparatus using a GF/B filter presoaked in 50 mM Tris·HCl (pH 7.4) and 0.5% polyethyleneimine. A procedure that paralleled MOR binding assay was followed for the SPR except assays were performed at room temperature for 75 min using 0.1 nM [^{125}I]Bolton-Hunter-SP (DuPont/NEN). Nonspecific binding was assessed in the presence of 10 μM SP. All binding data were analyzed by using GraphPad (San Diego) PRISM and fit to a sigmoidal curve using nonlinear regression. K_is for ESP7 at each receptor were calculated.

Measurement of Inositol Phosphate (IP) Formation. The NK-1 or SPR is coupled to a stimulatory G_q protein. Activation of the SPR increases the levels of phospholipase C, which subsequently cleaves phosphatidyl inositol into inositol triphosphate (IP_3) and diacylgycerol. For this reason, the potency of ESP7 at the SPR was assessed by quantifying its ability to stimulate IP production. The protocol of Blaker *et al.* (28) was followed. Then 10^6 COS-7 cells/10-cm plate were transfected with 5 μg of rat NK-1 receptor cDNA (29) or pcDNA1.1 (Invitrogen). After transfection, cells were split into 12-well plates (2×10^5 cells/well) and then labeled overnight with 3 μCi/ml of the IP precursor, myo-[^{3}H]inositol (DuPont/NEN). The next day, cells were stimulated for 60 min with ESP7 (0.01–38,000 nM) or SP (1,000 nM) in the presence of 10 mM LiCl (duplicate samples/reaction). LiCl was necessary to inhibit the degradation of IPs. Inositol metabolites were extracted with methanol/chloroform. Then, IPs, in the aqueous phase, were separated from other tritiated products by strong anion exchange chromatography. IPs were eluted with 2 M ammonium formate; noting that total IP production parallels IP_3 signaling. IP production was expressed as a fraction of the total cellular tritium content (sampled before fractionation of IPs) that was incorporated during overnight exposure to myo-[^{3}H]inositol (tritiated IPs/total tritium incorporated), to minimize intraassay variability. The EC_{50} for ESP7 was calculated by nonlinear regression analysis using GraphPad PRISM.

Measurement of Inhibition of cAMP-Mediated Signaling. HEK293 cells in 24-well (5×10^4 cells/well) or 96-well (1×10^4 cells/well) plates were transiently transfected with 200 ng/ml of wild-type rMOR and 1,000 ng/ml of a cAMP-responsive reporter gene construct by using a lipofection method (30). The reporter gene encoded firefly luciferase under the control of a cAMP-responsive element (CRE), and was a generous gift of M. R. Montminy, The Salk Institute, La Jolla, CA (31). Control cells were transfected with 200 ng/ml of pcDNA1.1 and 1,000 ng/ml of the CRE-luciferase construct to assess MOR-independent reporter gene activity.

To assess MOR-mediated inhibition of cAMP-dependent luciferase activity, experiments were performed in the presence of 10 μM forskolin, which activates adenylate cyclase. To define a concentration-response curve for DAMGO, EM-2 and ESP7-induced inhibition of adenylyl cyclase/cAMP activity, HEK293 cells were concomitantly treated a range of concentrations of these peptides (0.01–10,000 nM). Because of the lag time in the detection of cAMP-induced transcription of luciferase and subsequent protein formation, longer stimulation periods were used compared to assays that directly measure cAMP production. The optimal time course of ligand-induced inhibition of cAMP was explored. Based on these experiments, a 6-h time point at 37°C in serum-free media was used for further study (triplicate samples/reaction). For experiments completed in 24-well plates,

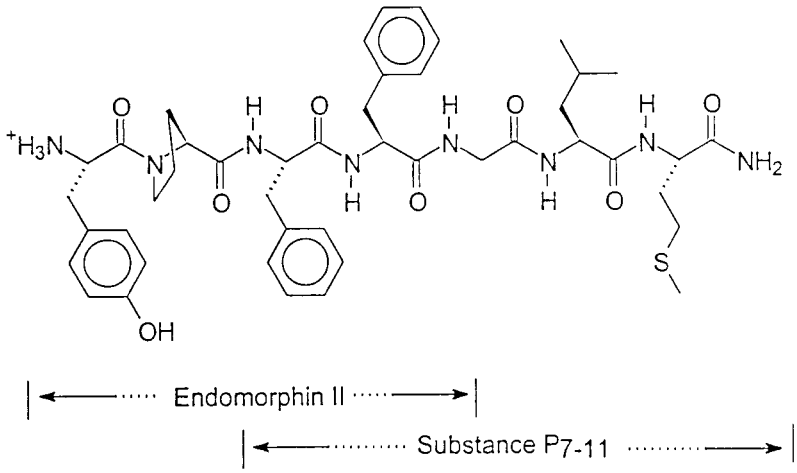

Fig. 1. Sequence and two-dimensional structure of ESP7. The amino acid sequence of ESP7 at the N terminus corresponds to that of EM-2 (Tyr-Pro-Phe-Phe-NH_2), and the overlapping sequence of ESP7 at the C terminus corresponds to that of SP (7–11) (SP = H-Arg-Pro-Lys-Pro-Gln-Gln-*Phe-Phe-Gly-Leu-Met-*NH_2). The drawing illustrates ESP7 (Tyr-Pro-Phe-Phe-Gly-Leu-Met-NH_2) with its overlapping opioid and SP moieties.

cells were subsequently lysed in extraction buffer containing 1% Triton X-100. Light emission was measured immediately by using a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego). Cell lysate from transfected cells (50 ml) was mixed with 250 ml of ATP buffer (43 mM glycylglycine, pH 7.8/22 mM $MgSO_4$/0.4 mg/ml BSA/0.6 mM EDTA, pH 8.0/1 M DTT/5 mM adenosine 5′-triphosphate). The solution was placed in the luminometer, and the bioluminescent reaction was initiated by injecting 100 ml of D-luciferin substrate. Light emission was measured during a 20-sec period. For experiments in 96-well plates, luciferase activity was measured by using the LucLite assay kit (Packard) and a Packard microplate scintillation counter. The IC_{50} values of DAMGO, EM-2, and ESP7 (inhibition of forskolin-induced luciferase activity) were calculated by nonlinear regression using the GraphPad PRISM computer program.

Results

The pharmacological and biochemical properties of the EM-2/SP chimera, designated ESP7 (Fig. 1), were evaluated. Intrathecal administration of 0.05, 0.2, or 1 μg of ESP7 produced significant, long-lasting analgesia over the course of 1–2 h, as monitored by the tail-flick test (Fig. 2*a*). At three administered dosages, analgesic responses were observed to reach a plateau at 20–40% of MPE; most likely reflecting the coincident and competing effects of EM-2 and SP on spinal MOR and SPRs. Importantly, 0.2 and 1 μg of ESP7, given once daily, effected equivalent analgesic responses over the 5-day course of administration. At the lower dose (0.05 μg), the pattern was comparable for 4 days, with tolerance developing only to the 0.05-μg dose on day 5 (Fig. 2*b*). The vehicle, β-CD, alone had no effect on analgesia.

The inhibition of the ESP7-mediated analgesia by naltrexone (NTX) illustrated that the chimeric peptide induced an opioid response (Fig. 3). Animals treated on day 1 with 1 μg of ESP7, and evincing a strong analgesic response, were treated on day 2 with 1 μg of NTX before ESP7 administration. The predicted analgesic effects of ESP7 were blocked by NTX. Additionally, slight hyperalgesia (not statistically different from baseline) was seen on day 2, most likely due to the activation of SPRs in the absence of opioid inhibition. On day 3, NTX was not given before ESP7 administration and a strong analgesic response resulted. NTX alone did not produce a response significantly different from baseline.

The ability of ESP7 to act as a nontolerance-forming opioid analgesic appeared to be mediated by SPRs, based on reversibility by the selective SPR antagonist RP67580 (Fig. 4). RP67580 (250 pmol) (K_i = 4 nM) was previously shown to selectively antagonize the SPR, with minimal activity at the NK-2 or NK-3 receptors (15). ESP7 administered in the presence of 250 pmol of RP67580 over the course of 4 days behaved as a typical MOR-preferring opioid analgesic. The peptide displayed a $T_{1/2}$ of approximately 1 day for the decay of opioid efficacy as a function of time, similar to that observed for MS administered in the absence of SP or MS administered in the presence of SP and RP67580 (15). RP67580 alone produced no analgesic effects.

The return of analgesia on day 5 after removal of the SPR antagonist suggested a unique role for simultaneous SPR receptor activation in MS tolerance reversal. The effect of ESP7 on pain responsiveness in tolerant animals was further examined by rendering rats tolerant to MS through repeated daily intrathecal injections. Then, ESP7 was administered to MS tolerant, partially withdrawn, animals. Notably, a restoration of opioid responsiveness was seen for 5–8 days after initial MS exposure (Fig. 5). All doses of ESP7 produced 40–60% of maximum analgesia for 4 days with no tolerance development.

The hypothesized ability of ESP7 to interact with both the MOR and SPRs *in vivo* was confirmed by *in vitro*-binding assays using rat brain membranes. In these experiments (not shown), ESP7 displayed a K_i of 218 nM [95% confidence interval (CI) = 94–505 nM, n = 2] for inhibition of binding of radio-labeled DAMGO, a prototypic μ-opioid agonist, to the MOR. For the SPR, ESP7 had a K_i of 289 nM (95% CI = 120–698 nM, n = 2) as determined by inhibition of binding of radio-labeled SP. The comparable affinity displayed by ESP7 for the MOR and SPRs further enhances the likelihood that coincident activation of equivalent subpopulations of spinal cord receptors underlies the observed pharmacological effect of opioid analgesia without tolerance development.

Additional *in vitro* assays with recombinant receptors were performed to demonstrate the ability of ESP7 to act as a strong agonist at either the SPR or the MOR. To measure the potency at the SPR, COS-7 cells were transiently transfected with the rat SPR and the capacity of ESP7 to stimulate IP production was quantified as a function of peptide concentration. ESP7 induced IP production with an EC_{50} of 27 nM, approximately 1–2 orders of magnitude higher than the reported value for SP (32) (Fig. 6*a*). At saturating concentrations, ESP7 (1 and 10 μM) and SP

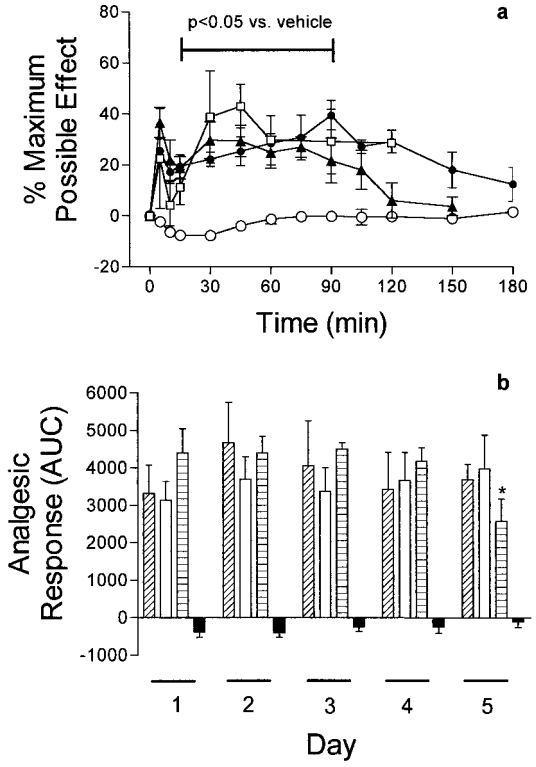

Fig. 2. (*a*) Time-dependent analgesic responses after intrathecal administration of 0.05 μg of ESP7 (●), 0.2 μg ESP7 (▲), 1 μg of ESP7 (□), or 1 μg of β-CD (○). Ordinal values represent tail-flick latency measurements normalized as %MPE (means ± SEM; $n = 6$ for 0.05 μg ESP7; $n = 8$ for 0.2 μg ESP7; $n = 5$ for both 1 μg of ESP7 and 1 μg of β-CD). All doses of ESP7 produce a modest analgesic response, which plateaus at ≈20–40% and remains effective for ≈1.5–2 h. The peptide vehicle, β-CD, alone has no analgesic efficacy. As indicated by the bar, all doses of ESP7 produced a significantly higher effect than the vehicle between 15 and 90 min of treatment ($P < 0.05$). (*b*) Repeated daily intrathecal administration of 0.05 μg of ESP7 (horizontally lined bars), 0.2 μg ESP7 (open bars), 1 μg of ESP7 (rising right-lined bars), or 1 μg of β-CD (filled bars). Ordinal values represent the daily analgesic response, as expressed by the AUC, calculated from experiments with a parallel design to that shown in Fig. 2*a* (means ± SEM; $n = 6$ for 0.05 μg ESP7; $n = 8$ for 0.2 μg ESP7; $n = 5$ for both 1 μg of ESP7 and 1 μg of β-CD). No tolerance develops to the analgesia produced by 0.2 or 1 μg of ESP7 in the tail-flick test for 5 days ($P > 0.05$); however, tolerance does develop to the 0.05-μg dose on day 5 (*, $P < 0.05$). One microgram of β-CD vehicle has no analgesic efficacy, and the effect of ESP7 is statistically different from baseline at all doses and on all days of treatment.

(1 μM) had comparable efficacy. Neither ESP7- nor SP-evoked IP production was observed in control cells transfected with the empty expression vector pcDNA1.1 (data not shown).

The potency of ESP7 at the rat MOR was compared to the high affinity MOR ligands, DAMGO and EM-2. HEK293 cells were simultaneously transfected with the rat MOR and a CRE-luciferase construct, as an indicator of cAMP-dependent transcription. DAMGO, EM-2, and ESP7 inhibited forskolin-stimulated activity (Fig. 6*b*) with IC_{50}s of 8.0 nM, 4.8 nM, and 94.8 nM; respectively. ESP7 at maximal concentrations inhibited forskolin-induced activity to a similar degree as the prototypic μ-opioid ligands, DAMGO and EM-2. None of the ligands decreased forskolin-induced activity in cells transfected with the empty expression vector pcDNA1.1 and CRE-luciferase construct alone (data not shown).

Discussion

Clinically, opioids are widely used to alleviate both acute and chronic pain symptoms (34). Opioid administration, however, is

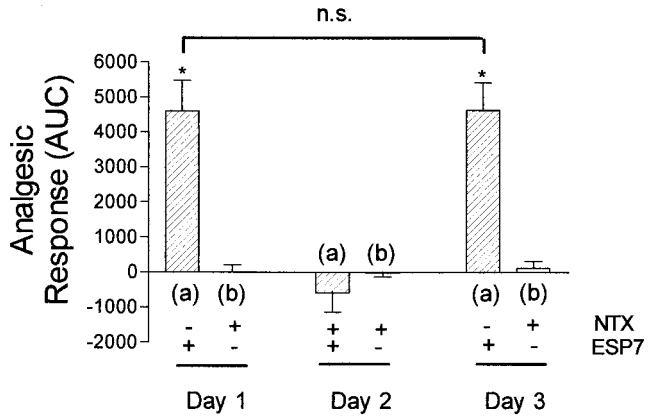

Fig. 3. Effect of intrathecal NTX administration. ESP7 (1 μg) was injected daily for 3 days. On day 2, 1 μg of NTX was injected 10 min before ESP7 (lane a). On days 1, 2, and 3, 1 μg of NTX was given alone as a control (lane b). Ordinal values represent the analgesic response as expressed by the AUC (means ± SEM; $n = 3$ for 1 μg of ESP7; $n = 5$ for 1 μg of NTX control) for each day of analgesic testing. ESP7 alone triggered significant analgesic effects vs. the baseline defined in untreated animals (*, $P < 0.05$). NTX blocks the analgesic effects of ESP7 on day 2 with slight hyperalgesia (not statistically significant, $P > 0.05$). A level of ESP7-induced analgesia similar to day 1 is recovered on day 3, when no antagonist is present (n.s.; $P > 0.05$). The effects of NTX alone are not significantly different from baseline.

fraught with undesirable sequelae including tolerance and dependence. Consequently, many patients must receive escalating doses of opioid to maintain adequate analgesia. When the efficacy or potency of a pharmacological agent decreases with repeated administration, tolerance has occurred. The mechanisms underlying the development of tolerance have received much attention and appear to be linked both to neuronal

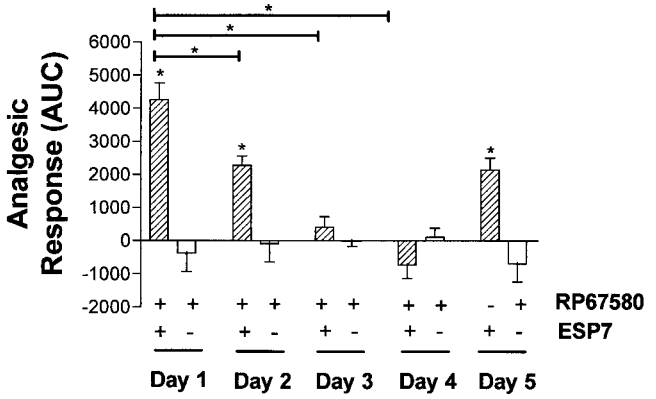

Fig. 4. Effect of intrathecal RP67580 administration. RP67580 (250 pmol), a high affinity SP antagonist, was injected intrathecally 10 min before 1 μg of ESP7 for 4 days (rising right-lined bars). On day 5, no RP67580 was given. RP67580 (250 pmol) was administered intrathecally alone for 5 days as a control (open bars). Ordinal values represent the daily analgesic response as expressed by the AUC (means ± SEM; $n = 6$ for treated group; $n = 5$ for control). After preinjection of RP67580, the analgesic effect of ESP7 is only significantly different from baseline on days 1 and 2 (*, $P < 0.05$), suggesting tolerance development. Tolerance begins to develop to the analgesic effects of ESP7 on day 2, as indicated by the bars, (*, $P < 0.05$ vs. effect of ESP7 on day 1) with complete tolerance or a return to baseline on day 3. Slight hyperalgesia is present on day 4, suggesting a withdrawn state, although the effect is not statistically significant from baseline. On day 5, RP67580 was not delivered and significant analgesia was regained (*, $P < 0.05$). RP67580 alone has no effect on analgesia and is not statistically different from baseline ($P > 0.05$).

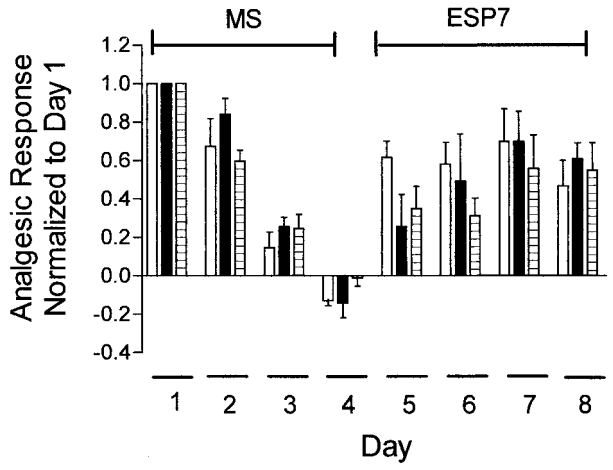

Fig. 5. MS tolerance reversal by ESP7. MS (5.0 μg) was given intrathecally to three groups of rats on days 1–4 to induce tolerance. On days 5–8, the rats were administered 0.05 (open bars), 0.2 (filled bars), or 1 μg of ESP7 (horizontally lined bars) intrathecally. Analgesia was measured each day for 60 min after drug injection using the tail-flick assay. Ordinal values represent daily analgesic responses measured as the AUC and normalized to respective values on day 1 [means ± SEM; $n = 6$ for MS (days 1–4); $n = 6$ for all doses of ESP7 (days 5–8)]. Tolerance begins to develop to MS by day 2 and no analgesia is present by day 4 to indicate complete tolerance or a return to baseline. Administration of any dose of ESP7 on days 5–8 recovers 40–60% of the analgesic response of MS on day 1, with all analgesic responses significantly different from in tolerant animals (day 4) ($P < 0.001$). All doses of ESP7 produce similar levels of analgesia. Furthermore, no tolerance develops to ESP7 analgesia during the 4-day treatment.

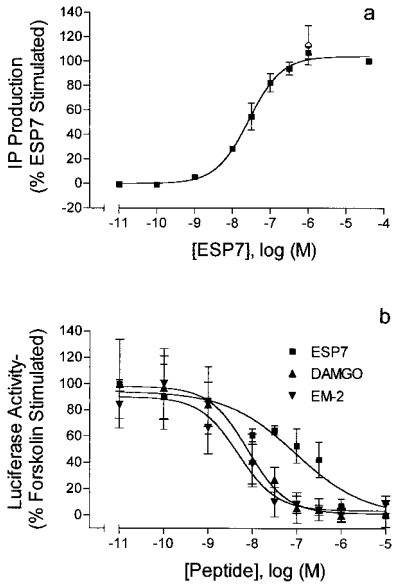

Fig. 6. (*a*) ESP7 and SP induced IP production in COS-7 cells expressing a recombinant rat substance P receptor. Data points represent means ± SEM of three independent experiments. SP (1 μM; ○), a saturating concentration, was used to define maximal stimulation by a full agonist. ESP7 (■) induces a concentration-dependent increase in IP production (EC_{50}, 27 nM; 95% CI = 19–38 nM). IP stimulation by the two highest concentrations of ESP7 is not significantly different from the stimulation by the full agonist, SP ($P > 0.05$). (*b*) Inhibition of forskolin-stimulated CRE luciferase activity (a marker of cAMP-mediated effect) by DAMGO, EM-2, and ESP7 in HEK293 cells transiently expressing the rat MOR. Data points represent means ± SEM of three experiments done in triplicate, each normalized to the effect of forskolin alone (10 μM = 100%) and unstimulated luciferase activity (= 0%). DAMGO (▲), EM-2 (▼), and ESP7 (■) produce concentration-dependent inhibition of forskolin-stimulated, cAMP-dependent luciferase activity. The IC_{50}s for DAMGO, EM-2, and ESP7 are 8.0 nM (95% CI = 2.1–31.1 nM), 4.8 nM (95% CI = 1.2–18.7 nM), and 94.8 nM (95% CI = 21.6–415 nM), respectively. ESP7 acts as a strong agonist at the rMOR with a 10-fold lower potency than DAMGO and EM-2.

adaptations involving alterations of receptor and/or transmitter expression, as well as to second messenger-mediated intracellular events after drug exposure. Interestingly, the development of tolerance to MS is significantly attenuated in rats receiving nociceptive stimulation (33), in concurrence with anecdotal data and results from controlled studies demonstrating markedly attenuated tolerance development in patients receiving opioids for postoperative pain (34). These preclinical and clinical observations suggest that spinal excitatory peptides, such as SP, which trigger nociception, may play a beneficial role in delaying tolerance development to opioids.

Results from previous experiments in our laboratory directly emphasize a pivotal role for SP in opioid analgesia (15). Low concentrations of SP, when coadministered with marginally effective doses of MS into the rat subarachnoid space, produce a markedly enhanced analgesic response as monitored by the tail-flick test. The pharmacological effect is blocked by previous treatment with naloxone, indicating that the potentiated analgesic response is mediated exclusively through activation of opioid-expressing neurons. The SP-mediated potentiation of opioid analgesia depends on the dose of coadministered SP. Thus, the SP dose–response curve appeared to be of a bell-shaped configuration, reflecting the opioid-potentiating analgesic properties of SP at low concentrations and the traditional hyperalgesic effects realized at significantly higher concentrations. A likely mechanism underlying the peptide-mediated enhancement of opioid analgesia is the ability of SP to release endogenous opioid peptides within the local spinal cord environment (35). Thus, the antinociceptive role of spinally released SP may be to initiate an amplification mechanism for opioid action subsequent to painful stimuli. These results provide evidence that spinal tachykinin and opioid systems have a direct functional interaction in the dual modulation of local nociceptive responses.

The recently discovered endogenous opioid tetrapeptide agonist, EM-2, possesses high affinity and selectivity for the MOR. EM-2 includes two phenylalanine residues at the C terminus similar to the residues found in the middle region of the excitatory peptide, SP. These common residues suggested a means to design a chimeric peptide with both opioid and SP domains that could coactivate both types of receptors in the dorsal horn and allow the involvement of opioids and SP in pain and tolerance to be further explored. The peptide chimera was designated ESP7 (YPFFGLM-NH_2). Displaying apparent K_is for MOR and SPRs of 218 nM and 289 nM, respectively, an EC_{50} of 27 nM for SPR-mediated stimulation of IP production, and an IC_{50} of 94.8 nM for MOR-mediated inhibition of cAMP production, ESP7 represents a moderately high affinity agonist at both receptor sites.

Our pharmacological data suggest that in an integrated behavioral system, the *in vivo* spinal cord, coincident activation of MOR and SPRs by ESP7 enables maintenance of opioid-dependent analgesic response over time. ESP7, at three different doses, produces significant analgesia that does not diminish with repeated administration over 5 days. Tolerance develops only to the lowest dose of ESP7 (0.05 μg) on day 5. The level of ESP7 analgesia does not reach that of MS (i.e., 100% MPE), presumably because of the ability of ESP7 to activate simultaneously both stimulatory and inhibitory systems within the dorsal horn. Further experimentation to determine the pharmacological effects of ESP7 show that ESP7 analgesia is NTX-reversible. In fact, ESP7 in the presence of NTX produces slight hyperalgesia, most likely due to the unmasking of the SPR-mediated nociceptive effects. ESP7 behaves similar to a typical opioid ($T_{1/2} = 1$

day), when delivered with the SPR antagonist, RP67580, suggesting the importance of the SP moiety in delaying tolerance development. This behavioral evidence may explain why tolerance developed to the lowest dose of ESP7 on day 5 (Fig. 2*b*). ESP7 (0.05 μg), as opposed to 0.2 or 1 μg, may not activate SPR-expressing neurons potently enough.

ESP7 appears to exert its pharmacological effects by a novel, albeit ostensibly logical, mechanism: coincident activation of spinal receptors. Due to the sparse colocalization of SPR and MORs on individual neurons within the superficial layers of the dorsal horn, one molecule of ESP7 is probably not coactivating both receptors on the same neuron, but rather separate molecules of ESP7 are most likely acting at each receptor on closely spaced neurons (36). The small population of neurons coexpressing SPR and MORs also makes the formation of heterodimers between these two receptors less likely (37). Anatomical studies illustrate that SPRs are located primarily on the somato-dendritic surfaces of neurons (i.e., postsynaptically), whereas MORs are found both postsynaptically and on axonal components (i.e., presynaptically) (38). ESP7 may, by causing release of chemical mediators from SPR-expressing neurons, be indirectly activating secondary messengers systems within MOR-expressing neurons to alter the development of tolerance.

In addition to the ability of ESP7 to retard opioid tolerance, it can restore significant analgesia to MS tolerant rats (Fig. 4). The level of analgesia that occurs in this setting is 40–60% of the morphine-induced level on day 1 of treatment. This suboptimal effect is likely due to either the presence of the excitatory SP moiety or an insufficient dose of ESP7. SPR activation in MS tolerant animals may alter signaling pathways within dorsal horn neurons and thus reverse opioid tolerance. An alternative explanation is that MS and EM-2 may act at distinct MOR subtypes (μ_1 vs. μ_2) (39). The EM-2 moiety of ESP7 may bind to a different MOR. Because this receptor subtype would have not yet been desensitized, ESP7 would lead to an analgesic response.

Our present data using the chimeric opioid/tachykinin peptide ESP7 strongly suggest a novel role for SP in the inhibition of opioid tolerance at the spinal level. In addition, these data suggest a dynamic role for SP and related tachykinins, released from primary afferent or intrinsic neurons, in maintaining a viable and active opioidergic tone at the level of the spinal cord. ESP7 represents a valuable probe of the interactions between tachykinin and opioid systems due to its affinity and potency at both the MOR and SPRs. Theoretically, equivalent pharmacologic results to ESP7 could be obtained by coadministering a specific ratio of the peptide components, EM-2 and SP. However, combining the two moieties into one peptide creates a more kinetically favorable situation. Equivalent populations of spinal MORs and SPRs are targeted and activated by the chimeric peptide, thereby critically defining the anatomical locus of action to interactive SP and opioid cell groups. Additionally, potential differences in drug distribution, time course of action, and metabolism are obviated by combining the two agents into a chimeric compound. To use one medication instead of two is preferable in terms of side effects, simplicity and cost effectiveness of production and clinical distribution. Furthermore, the sequence of the opioid/SP chimera is amenable to chemical tailoring (40) to differentially target both receptors through altered affinity at each site, thereby optimizing analgesic endpoints with minimal tolerance development. This chimeric peptide is a promising future therapeutic candidate for use in both acute and chronic pain, as well as for exploring the relationship between tolerance and addiction in drug abuse complications.

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