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Biochemical, cellular and pharmacological activities of a human neuropeptide FF-related peptide

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Abstract

We report on the biochemical, cellular and pharmacological activities of SQA-neuropeptide FF (Ser-Gln-Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe–NH_2), a peptide sequence contained in the human neuropeptide FF (neuropeptide FF, Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe–NH_2) precursor. Quantitative autoradiography revealed that, in the superficial layers of the rat spinal cord, SQA-neuropeptide FF displayed the same high affinity for [^{125}I]1DMe ([^{125}I]D-Tyr-Leu-(NMe)Phe-Gln-Pro-Gln-Arg-Phe–NH_2) binding sites (K_i = 0.33 nM) as did neuropeptide FF (K_i = 0.38 nM). In acutely dissociated mouse dorsal root ganglion neurones, SQA-neuropeptide FF reduced by 40% the depolarisation-induced rise in intracellular Ca^{2+} as measured with the Ca^{2+} indicator, Fluo-3. In mice, 1DMe and SQA-neuropeptide FF dose-dependently inhibited the antinociceptive effect of intracerebroventricular (i.c.v.) injections of morphine, but SQA-neuropeptide FF was less potent than 1DMe. Furthermore, SQA-neuropeptide FF, as well as 1DMe, produced marked hypothermia following third ventricle injections in mice. These data demonstrate that the human peptide, SQA-neuropeptide FF, exhibits biochemical and pharmacological properties similar to those of neuropeptide FF or neuropeptide FF analogues, and belongs to the neuropeptide FF family.

Keywords: Neuropeptide FF; Receptor, binding; Ca^{2+}, intracellular; Opioid; Analgesia; Hypothermia

1. Introduction

Neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe–NH_2) originally isolated from bovine brain (Yang et al., 1985) was identified in the central nervous system of several mammals including humans (Panula et al., 1987; Kivipelto et al., 1989). Neuropeptide FF is believed to act as a neurotransmitter since neuropeptide FF immunoreactivity is restricted to vesicles in axonal profiles in the spinal cord (Allard et al., 1991) and could be released from nerve terminals by depolarisation in a Ca^{2+}-dependent process (Allard et al., 1989; Zhu et al., 1992; Devillers et al., 1994). Neuropeptide FF generates its pharmacological effects by interacting with specific binding sites in the central nervous system (Allard et al., 1989). High densities of binding sites are found in areas implicated in the control of nociception, such as the thalamus, brainstem and spinal cord (Allard et al., 1992; Dupouy and Zajac, 1996). Pharmacological results indicate that neuropeptide FF decreases opioid antinociception (Yang et al., 1985; Gicquel et al., 1992) and can modify the development of opioid tolerance and dependence (Tang et al., 1984; Malin et al., 1990; Stinus et al., 1995). Neuropeptide FF antagonizes opioid antinociception following intracerebroventricular (i.c.v.) injection in rats (Yang et al., 1985; Millon et al., 1993) and mice (Kavaliers and Yang, 1989; Gicquel et al., 1992). Third ventricle injection of neuropeptide FF in non-dependent rats induces a morphine withdrawal-like behavioural syndrome (Malin et al., 1990). Furthermore, a highly selective antiserum directed against neuropeptide FF attenuates morphine tolerance (Lake et al., 1991). However, neuropeptide FF exerts complex pharmacological actions (Desprat and Zajac, 1994) since intrathecal infusion of neuropeptide FF produces antinociceptive effects in rats (Gouardères et al., 1996).

Very recently, Perry et al. (1997) have cloned a human neuropeptide FF-related peptide gene, revealing two novel

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neuropeptides with amino acid sequences differing from those already known in bovines. The longer peptide differs from the peptide isolated from bovines by the substitution of two amino acids (AGEGLNSQFWSLAAPQRF–NH_2) and the sequence of human SQA-neuropeptide FF (SQAFLFQPQRF–NH_2) is identical to that of neuropeptide FF (FLFQPQRF–NH_2) except for a three-amino acid N-terminal extension in the human peptide. Surprisingly SQA-neuropeptide FF, in contrast to neuropeptide FF, has no effect on the amplitude of the monosynaptic component of the ventral root reflex response (Perry et al., 1997) and appears to be wholly inactive. In order to examine the precise physiological role of this peptide, we have investigated biochemical, cellular and pharmacological activities of SQA-neuropeptide FF in rodents, using tests in which neuropeptide FF and neuropeptide FF analogues are known to be active.

2. Materials and methods

2.1. Chemicals

Morphine hydrochloride was obtained from Francopia (Paris, France).

Neuropeptide FF, SQA-neuropeptide FF (Ser-Gln-Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2) and 1DMe (D-Tyr-Leu-(NMe)Phe-Gln-Pro-Gln-Arg-Phe–NH_2) were prepared by solid-phase synthesis on an automated peptide synthesizer (Applied Biosystem 430A) with *N*-terbutyl-oxycarbonyl amino acids and symmetric anhydride or hydroxybenzotriazole ester as activation chemistry (Gicquel et al., 1992). A *p*-methyl-benzhydrylamine resin (MHBA) was used as the solid support and *N,N′*-dicyclohexyl-carbodiimide was used for the coupling step.

The products were removed from the resin and simultaneously deprotected by reaction with liquid anhydrous hydrofluoric acid in the presence of anisol (5%) and *m*-cresol (5%) at −5°C. After evaporation, precipitation and washings with anhydrous diethyl ether and solubilization with aqueous acetic acid (30%), the crude peptides were lyophilized. Peptides were purified by preparative high-performance liquid chromatography on an Aquapore column (C8, 10 × 100 mm, 20 mm, Brownlee Labs) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The purity of the final products was assessed by analytical reverse phase liquid chromatography and fast atom bombardment mass spectrometry (Finningan Mat model TSQ700).

2.2. Binding assay and autoradiographic procedures

As previously described (Gouardères et al., 1997), slide-mounted rat spinal cord sections were preincubated at 25°C for 20 min in 50 mM Tris–HCl pH 7.4 containing 140 mM NaCl and 0.1% bovine serum albumin and washed twice for 3 min in 50 mM Tris–HCl at 25°C. Sections were then incubated at 25°C for 150 min with 0.1 nM [^{125}I]1DMe in 50 mM Tris–HCl buffer pH 7.4 containing 120 mM NaCl, 0.1% bovine serum albumin and 0.1 mM bestatin and various concentrations of displacer. Non-specific binding of [^{125}I]1DMe was determined in adjacent sections in the presence of 1 μM 1DMe. Slides were washed (4 × 3 min) in ice-cold Tris–HCl 50 mM buffer pH 7.4 and exposed for 4 days on Hyperfilm (Amersham) at room temperature.

The quantification of autoradiograms was calculated as nanoCurie per milligram (nCi/mg) of tissue equivalent by reference to commercially available iodinated standards (Amersham) co-exposed with the tissue sections.

2.3. Measurement of intracellular Ca^{2+}

The methods for isolating dorsal root ganglion neurones and recording the variations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) have been described previously (Roumy and Zajac, 1996). Briefly, male Swiss mice (Dépré, France, 20–25 g) were decapitated and six to eight dorsal root ganglia were dissected out. Ganglia were digested with collagenase (Sigma type II, 3 mg/ml, 40 min at 37°C) and trypsin (1 mg/ml, 10 min, 37°C). After a 3-min incubation with trypsin inhibitor (10 mg/ml, 25°C), the ganglia were rinsed three times with 1 ml buffer, and neurones were isolated by mechanical trituration through Pasteur pipettes. The cell suspension was incubated for 1 h with 2 μM Fluo-3-acetoxymethyl ester (Sigma) at room temperature. The cells were resuspended in fresh buffer and further incubated for 30 min to allow complete hydrolysis of Fluo3-acetoxymethyl ester. An aliquot (50 μl) was deposited on a microscope slide and 5 min were allowed for cell attachment before the start of perifusion (2 ml/min). The buffer (pH 7.3) contained (in mM): NaCl: 150, KCl: 5, $CaCl_2$: 2, $MgCl_2$: 1, glucose: 10, HEPES: 10 and 0.1% bovine serum albumin.

To depolarize the neurones, KCl was raised to 30 or 50 mM and NaCl reduced accordingly to maintain osmotic pressure. SQA-neuropeptide FF was added to the normal buffer at 10 nM final concentration and perifused for 20 min. The response to K^+ depolarization was measured every 5 min.

The cells were viewed with a 40/0.65 objective and a single neurone was illuminated using the field diaphragm. Fluo-3 fluorescence was excited at 488 nm and emitted light measured at 530 nm (10-nm bandwidth interference filters) with a photomultiplier detection system (Model 7070, Oriel, France). The output of the detection system was digitized (at 10 Hz and 10 successive values were averaged) and stored in a microcomputer. The intensity of fluorescence was expressed as the photomultiplier current.

2.4. Analgesia

Animals were tested in accordance with the ethical guidelines of the International Association for Study of Pain. This study was approved by the local committee of biomedical ethic (Comité Régional d'Ethique Biomédicale).

Male Swiss mice (Dépré, France, 20–25 g) were maintained at 21 ± 1°C on a 12:12-h dark/light cycle with free access to food and water. The nociceptive responses were assessed by the radiant heat tail-flick test with a cut-off time set to 8 s. Baseline tail withdrawal latencies were measured 15, 10 and 5 min before injection. Groups of 10 mice were injected and the tail flick responses were measured 20, 30, 40, 60 and 120 min after i.c.v. administration.

For i.c.v. injections, the mouse was hand-held and gently restrained, the skull was punctured perpendicular to the dorsal surface and 5 μl of drug were injected into the lateral ventricle over a period of about 5 s. The injection point was 3 mm caudal to the right orbit and 2.5 mm lateral to the midline of the head to a depth of 3 mm.

Changes in tail-flick latency responses were converted to maximum percentage effect (% MPE) calculated as: $100 \times [(\text{post-injection latency} - \text{baseline latency}/\text{cut-off value} - \text{baseline latency})]$, where the cut-off value was 8 s.

Analgesia measured as tail-flick latencies was compared with control data in a one-way analysis of variance (ANOVA) followed by post-hoc Dunnett or Scheffé's tests (Staview, Macintosh). The level of significance was set at $P < 0.05$.

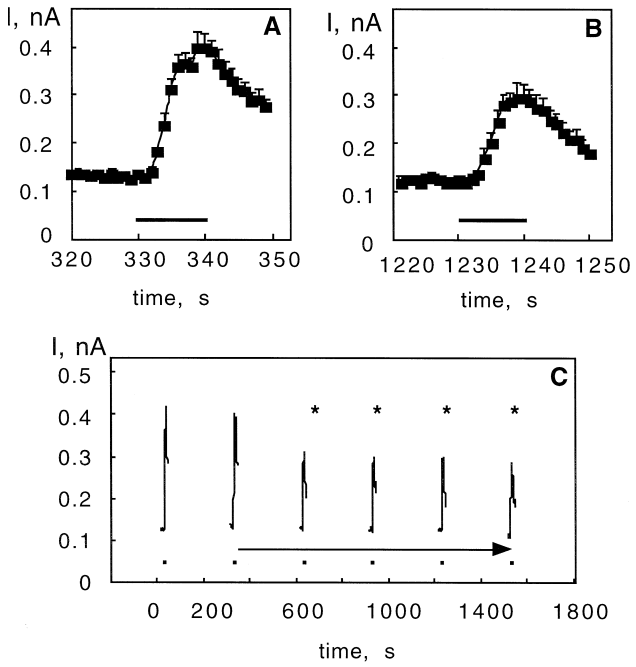

Fig. 2. Effects of SQA-neuropeptide FF on depolarization-induced rise in $[Ca^{2+}]_i$. The intensity of fluorescence was measured once per second and the mean intensity, for the five out of seven neurones affected by SQA-neuropeptide FF, was computed every second together with S.E.M., as shown in (A) (control) and (B) (after 15 min SQA-neuropeptide FF 10 nM). The whole experiment is illustrated in (C) (for the sake of clarity, S.E.M. was omitted). Horizontal lines in (A), (B), (C): indicate 30 mM K^+ superfusion. Arrow in (C): SQA-neuropeptide FF 10 nM. The ordinate is the photomultiplier current. (*) Indicates a significant difference from the controls ($P < 0.05$, unilateral Student's *t*-test).

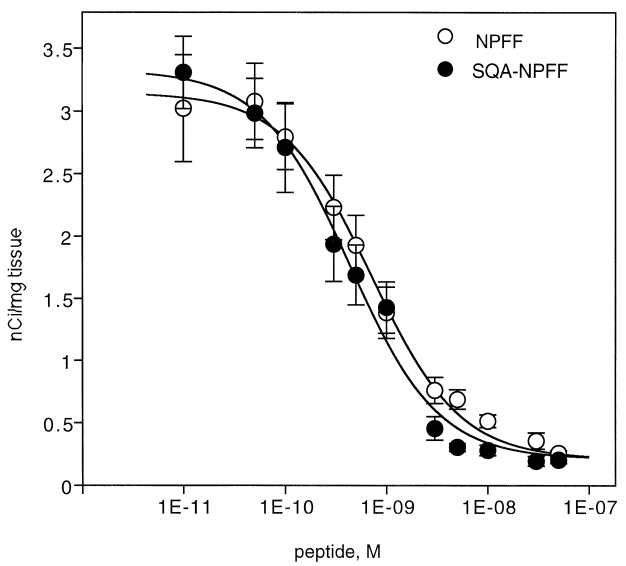

Fig. 1. Inhibition of [^{125}I]1DMe specific binding to slide-mounted rat spinal cord sections by neuropeptide FF and SQA-neuropeptide FF. The points are data from one representative experiment. Each point is the mean (±S.D.) of 12–18 measurements per concentration in laminae (I)–(II) of the dorsal horn. Curves were obtained by using non-linear regression analysis (Ultrafit, Macintosh) using a K_d value of 0.08 nM for [^{125}I]1DMe.

2.5. Hypothermia

Mice were injected in the third ventricle: 2 mm anterior to the interaural line, on the interhemispheric line to a depth of 2.5 mm. The animals were restrained in an aluminum device with their tails loosely taped to horizontal posts. The animals could not huddle together but the restraint did not otherwise affect convective heat exchange or thermoregulatory behaviour.

The ambient temperature was regulated at 21 ± 1°C. Colon temperature was measured with a thermistor probe (Ellab Instrument, Copenhagen) inserted to a depth of 2.5 cm into the rectum. Body temperature was recorded immediately before injection and at 10-min intervals for 60 min. Each animal was used only once.

The differences in mean body temperature between mice treated with 1DMe or SQA-neuropeptide FF and NaCl were subjected to ANOVA. Dunnett and Scheffé's tests (Staview, Macintosh) were used for the post-hoc pairwise comparison between means.

3. Results

3.1. Binding of SQA-neuropeptide FF to [^{125}I]1DMe binding sites in the rat spinal cord

The SQA-neuropeptide FF and neuropeptide FF were compared for their abilities to inhibit the specific binding of [^{125}I]1DMe in the superficial layers of the dorsal horn by quantitative autoradiography. In the presence of bestatin, an inhibitor of aminopeptidases, neuropeptide FF displayed a K_i value nearly identical to that previously determined (Gouardères et al., 1997) in this tissue ($K_i = 0.38 \pm 0.06$ nM, mean ± S.D., from three independent experiments). Under the same experimental conditions minimizing peptide degradation (prewash of sections and presence of bestatin), SQA-neuropeptide FF inhibited the specific binding of [^{125}I]1DMe with a calculated K_i value (0.33 ± 0.09 nM) very close to that of neuropeptide FF (Fig. 1).

3.2. Effects of SQA-neuropeptide FF on intracellular Ca^{2+} in mouse spinal ganglion neurones

It was shown earlier that neuropeptide FF and neuropeptide FF analogues, at concentrations between 1 and 10 nM, reduced and even suppressed responses to short depolarizations (Roumy and Zajac, 1996) in mouse spinal ganglion neurones. Dorsal root ganglion neurones were depolarized by a 10-s superfusion with 30 mM K^+ ($n = 7$ neurones). The rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured under control conditions and SQA-neuropeptide FF (10 nM) was then superfused for 20 min (Fig. 2C). SQA-neuropeptide FF had no effect on resting $[Ca^{2+}]_i$ in any of the neurones tested. In five of seven neurones (71%), the magnitude of the $[Ca^{2+}]_i$ rise was significantly reduced after SQA-neuropeptide FF. This decrease amounted to 40% after the 5-min SQA-neuropeptide FF superfusion (Fig. 2).

Four other neurones were depolarized with 50 mM K^+ (5 s). In three of these neurones, SQA-neuropeptide FF (10 nM, 20 min) caused a significant reduction of 45% in the $[Ca^{2+}]_i$ rise (data not shown).

3.3. Anti-opioid effects of SQA-neuropeptide FF

The anti-opioid effects of i.c.v.-injected SQA-neuropeptide FF and 1DMe are shown in Fig. 3. SQA-neuropeptide FF alone at 22 nmol did not modify significantly the threshold response in the tail-flick test. In contrast, 22 nmol SQA-neuropeptide FF significantly decreased the antinociceptive effect of coinjected morphine (5.5 nmol) in the lateral ventricle (Fig. 3A).

1DMe coinjected into the lateral ventricle reversed significantly the analgesia induced by 5.5 nmol morphine. Comparison of the dose–response curves of SQA-neuropeptide FF and 1DMe (Fig. 3B) revealed that doses of 20 nmol SQA-neuropeptide FF were needed to inhibit significantly morphine analgesia while 3 nmol 1DMe was active.

3.4. Hypothermia

SQA-neuropeptide FF produced a marked hypothermia following third ventricle administration to mice (Fig. 4A). Body temperature was minimal 10 min following injection of 10 or 22 nmol SQA-neuropeptide FF and returned to the saline level after 40 min. The SQA-neuropeptide FF-induced hypothermia was dose-dependent (Fig. 4B), with 10 nmol being the lowest effective dose.

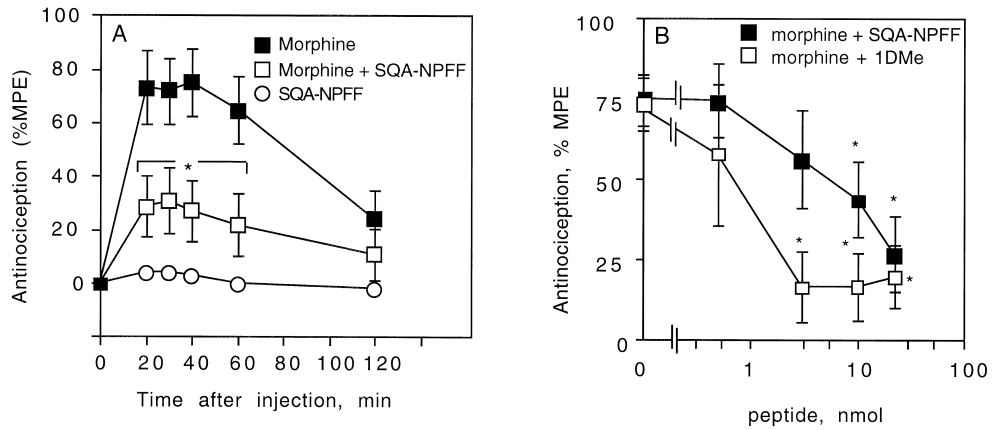

Fig. 3. Anti-opioid effects of SQA-neuropeptide FF. (A) Time course of 5.5 nmol morphine-induced antinociception was measured in the tail-flick test in mice with or without coinjected 22 nmol SQA-neuropeptide FF. Data are means (±S.E.M.) of changes in tail-flick latencies calculated as maximum percentage effect (% MPE) in 10 mice. (*) $P < 0.05$: significantly different from morphine-treated group. (B) Dose–response relationships for intracerebroventricular SQA-neuropeptide FF and 1DMe on coadministered morphine analgesia. The tail-withdrawal latency was measured 30 min after coinjection of SQA-neuropeptide FF or 1DMe with morphine (5.5 nmol). Antinociception is expressed as maximal percent effect (% MPE). Each point represents the mean (±S.E.M.) of 10 measurements. (*) $P < 0.05$ significantly different from morphine.

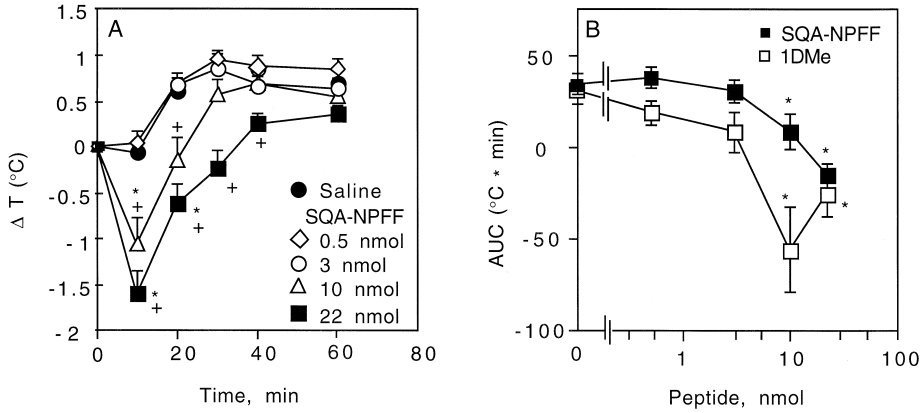

Fig. 4. Effects of SQA-neuropeptide FF on body temperature. (A) Time course of the change in body temperature after intracerebroventricular injection of SQA-neuropeptide FF. Each point represents the mean rectal temperature (±S.E.M.) of 10 animals. (+) $P < 0.05$ vs. saline. (*) $P < 0.05$ vs. baseline. (B) Dose–response relationships for SQA-neuropeptide FF and 1DMe on hypothermia following third ventricle administration. Each point is the mean (±S.E.M.) of 10 determinations, representing the time integral (AUC) of the temperature vs. time relationship following injection in the third ventricle over a 60-min period (the AUC was computed by trapezoidal approximation). (*) $P < 0.05$ vs. baseline.

1DMe produced a marked hypothermia after i.c.v. administration. The decrease in body temperature began 20 min after injection and was maximal at 20 or 40 min, depending on the dose. On a molar basis, SQA-neuropeptide FF induced quantitatively the same effects as 1DMe (Fig. 4B).

4. Discussion

The present study showed that the human peptide, SQA-neuropeptide FF, and neuropeptide FF have very similar biochemical, cellular and pharmacological properties.

(1) SQA-neuropeptide FF exhibits a high affinity for neuropeptide FF binding sites in the superficial layers of the dorsal horn of rat spinal cord. The experimental conditions used (prewashed slices in the presence of bestatin) minimize peptide degradation and increase the apparent affinity of neuropeptide FF (Gouardères et al., 1997).

(2) SQA-neuropeptide FF inhibits the depolarization-induced rise in intracellular Ca^{2+} in mouse root dorsal ganglion neurones. Neuropeptide FF, as well as 1DMe, reduced by a maximum of 50% the depolarization-induced rise in Ca^{2+} (Roumy and Zajac, 1996). Thus, the ability of SQA-neuropeptide FF to mobilize intracellular Ca^{2+} seems very similar to that of neuropeptide FF.

(3) SQA-neuropeptide FF dose-dependently inhibits morphine analgesia following i.c.v. injection in mice.

1DMe has so far been the most potent neuropeptide FF analogue available since it is protected against aminopeptidases and exhibits a very high affinity towards neuropeptide FF binding sites (Gicquel et al., 1992). In the tail-flick test, the lowest anti-opioid dose of neuropeptide FF is 22 nmol (Gicquel et al., 1994). Thus, SQA-neuropeptide FF is as potent as neuropeptide FF to inhibit supraspinal opioid antinociception. In the presence of the peptidase inhibitor, bestatin, i.c.v. injection of neuropeptide FF in rats only caused a slight hyperalgesia (Yang et al., 1985), whereas neuropeptide FF alone had no significant effect on the basal nociceptive response in mice (Kavaliers, 1990). Similarly, SQA-neuropeptide FF alone, injected i.c.v., was ineffective in the tail-flick test.

(4) SQA-neuropeptide FF alone produces hypothermia following third ventricle administration.

Neuropeptide FF analogues injected in the third ventricle induced hypothermia unrelated to an interaction with the opioid system since this pharmacological action is not modified by naloxone or morphine (Desprat and Zajac, 1997).

Our data indicate that neuropeptide FF, as well as neuropeptide FF analogues, produce all the effects induced by SQA-neuropeptide FF at the same dose or over similar concentration ranges (Gicquel et al., 1992, 1994; Roumy and Zajac, 1996; Desprat and Zajac, 1997).

Because no neuropeptide FF antagonist has been characterized at this time, only the dose–effect relationships define the specificity of the pharmacological effects. On a molar basis, SQA-neuropeptide FF appears to be as potent as neuropeptide FF, but 1DMe remains more effective to reverse morphine analgesia and to induce hypothermia (Gicquel et al., 1992, 1994).

Despite their different N-terminal parts, SQA-neuropeptide FF and neuropeptide FF displayed the same affinity under experimental conditions that minimize enzymatic breakdown, and the same pharmacological activity. Previous data provide evidence that the C-terminal region of neuropeptide FF would be essential for the biological response while the N-terminal segment is responsible for high-affinity binding (Gicquel et al., 1994). Thus, shortening the neuropeptide FF sequence from the N-terminal produced only a moderate decrease in affinity until neuropeptide FF-(4–8) was reached. Similarly, N-terminal lengthening of neuropeptide FF does not modify neuropep-

tide FF receptor recognition since SQA-neuropeptide FF still exhibits high affinity.

Surprisingly, Perry et al. (1997) describe SQA-neuropeptide FF as an inactive peptide and in fact, SQA-neuropeptide FF did not modify the amplitude of the monosynaptic component of the ventral root reflex response as did neuropeptide FF (Perry et al., 1997). These results should indicate, in contradiction with our data, that SQA-neuropeptide FF exhibits a low affinity towards neuropeptide FF receptors. It is possible that, in this particular model, differences in degradation rates between neuropeptide FF and SQA-neuropeptide FF could explain such results. In the same manner, SQA-neuropeptide FF could possess the same efficacy as neuropeptide FF and could be seen as inactive in this test if the response is at the limit of detection.

Our data are the first direct evidence that the human peptide, SQA-neuropeptide FF, has biochemical and pharmacological properties very similar to those of neuropeptide FF analogues and, in all probability, belongs to the neuropeptide FF family.

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