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Effects of neuropeptide FF on intracellular Ca^{2+} in mouse spinal ganglion neurons

Michel Roumy *, Jean-Marie Zajac

Laboratoire de Pharmacologie et Toxicologie Fondamentales, CNRS, 205 Route de Narbonne, 31077 Toulouse Cedex, France

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Abstract

Intracellular Ca^{2+} was measured in freshly dissociated mouse dorsal root ganglion neurons by using Fluo3 as fluorescent Ca^{2+} probe. Short perifusions (5–10 s) with 30 mM K^+ induced a sharp rise in fluorescence due to the entry of Ca^{2+} ions, in particular through L and N voltage sensitive Ca^{2+} channels opened by the action potentials that were triggered by depolarization. Perifusions with 1 or 10 nM (1DMe)Y8Fa (DYL(NMe)FQPQRFamide), a neuropeptide FF analog, suppressed the rise in fluorescence induced by short (5–10 s) K^+ perifusions within 30 min. However, when K^+ perifusions of longer duration were applied, Fluo3 fluorescence rose after an increased latency. Two other analogs, (2DMe)Y8Fa (DYDL(NMe)FQPQRFamide) and (3D)Y8Fa (DYDLDFQPQRFamide), had the same effect; similarly neuropeptide FF (FLFQPQRFamide, 1 nM, 30 min) reduced intracellular Ca^{2+} rise during depolarization. These features indicate that neuropeptide FF and its analogs exert their pharmacological effects by reducing the $[Ca^{2+}]_i$ transient induced by short depolarizations.

Keywords: Ca^{2+}, intracellular; Dorsal root ganglion neuron; Neuropeptide FF; (Mouse)

1. Introduction

Neuropeptide FF (FLFQPQRFamide), a FMRFamide-related peptide isolated from bovine brain (Majane and Yang, 1987; Yang et al., 1985) modulates opioid-mediated functions. Neuropeptide FF acts through specific receptors, not recognized by opiates (Allard et al., 1992), that are localized in specific brain and spinal cord areas implicated in the control of nociception (Allard et al., 1989, 1992). It antagonizes morphine analgesia after intracerebroventricular injection in rat (Yang et al., 1985) and mouse (Gicquel et al., 1992; Kavaliers and Yang, 1989) but induces long-lasting analgesia after intrathecal injection in rat (Gouardères et al., 1993b).

Opiates slow cell firing (North, 1986; North et al., 1987) and inhibit transmitter release (Cherubini and North, 1985; Jessel and Iversen, 1977; Kojima et al., 1994). These inhibitory effects are mediated by either an increase in K^+ conductances, that contribute to the neuron resting potential (Mihara and North, 1986; North and Williams, 1985) and/or to the repolarizing phase of the action potential (Werz and Mcdonald, 1983) or a decrease in Ca^{2+} conductances (Mcdonald and Werz, 1986; Rhim and Miller, 1994; Schroeder et al., 1991). In mouse dorsal root ganglion neurons, activation of μ- and δ-opioid receptors increases a K^+ conductance that results in the shortening of the action potential (Werz and Mcdonald, 1985) whereas activation of κ-opioid receptor decreases the conductance of a voltage sensitive Ca^{2+} channel of the N-type (Gross and Mcdonald, 1987). Similarly in rat dorsal root ganglion neurons, activation of μ-opioid receptor reduces the conductance of an N Ca^{2+} channel (Schroeder et al., 1991). All these effects decrease Ca^{2+} influx into neurons and possibly into presynaptic nerve endings resulting in a reduced transmitter release.

We hypothesized, therefore, that the rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) triggering transmitter release could also be regulated by neuropeptide FF and thus accounting for its modulation of opiate functions. There are high densities of neuropeptide FF receptors (Allard et al., 1992) as well as of μ- and δ-opioid binding sites (Gouardères et al., 1991, Gouardères et al., 1993c) in the superficial layers of the spinal dorsal horn. Similarly to μ- and δ-opioid receptors (Zajac et al., 1989) part of neuropeptide FF binding sites

* Corresponding author. Tel.: +33-61 17 59 07; fax: +33-61 17 59 94.

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are located on the primary afferent endings (Gouardères et al., 1993a). We may expect therefore, that the cell bodies express neuropeptide FF receptors in their membranes. In this study, we report the effects of neuropeptide FF and analogs resistant to degradation by peptidases (Gicquel et al., 1994), on $[Ca^{2+}]_i$, in freshly dissociated mouse dorsal root ganglion neurons.

2. Materials and methods

2.1. Cell isolation

Male CDF1 mice were decapitated and the lumbar vertebral column was cut out, placed in ice cold buffer, opened and 6–8 dorsal root ganglia were dissected out. After 1 h of incubation at 37°C with collagenase (Sigma type II, 3 mg/ml) ganglia were rinsed four times with 1 ml buffer containing 0.1% bovine serum albumin at room temperature and were mechanically dispersed by passage through glass pipettes of decreasing tip diameter. The cell suspension (0.3 ml) was incubated for 1 h with 2 μM Fluo3-AM (Sigma) at room temperature. After a 10 s centrifugation (9500 × *g*) the cells were resuspended in fresh buffer (0.3 ml) and incubated for 30 min to allow a complete hydrolysis of Fluo3-AM. An aliquot (50–100 μl) was deposited on a microscope slide and 5 min were allowed for cell attachment before the start of perifusion

2.2. Measurements of $[Ca^{2+}]_i$

The cells were viewed with a 40/0.65 objective and a single cell was illuminated using the field diaphragm. The selected neurons had their cell body diameters at the lower end of the size distribution. Fluo3 fluorescence was excited at 488 nm and emitted light measured at 530 nm (10 nm bandwidth interference filters). Emitted light was measured with a photomultiplier detection system (Model 7070, Oriel, France). The output of the detection system was digitized (at 10 Hz and ten successive values were averaged) and stored in a microcomputer. The fluorescence signal was expressed as the photomultiplier current (nA).

2.3. Solutions and drugs

The cells were perifused (1.8 ml/min, room temperature) with a solution containing (in mM): NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; Hepes, 10; glucose, 10. The pH was adjusted to 7.30 at room temperature and bovine serum albumin (0.1%, Fraction V, Euromedex, France) was added. Cells were depolarized by a short (5–10 s) perifusion with the same buffer in which KCl was raised to 30 mM and NaCl reduced accordingly to maintain osmotic pressure. Solution change was made possible by means of an electrically operated valve.

Fluo3-AM (1 mM, Sigma) and nifedipine (100 mM, Sigma) were dissolved in dry dimethylsulfoxide and stored at −20°C. The toxin ω-conotoxin GVIA (Sigma) was dissolved (200 μM) in sterile distilled water and stored at −20°C. The peptides, neuropeptide FF (FLFQPQRF-amide), (1DMe)Y8Fa (DYL(NMe)FQPQRFamide), (2DMe)Y8Fa (DYDL(NMe)FQPQRFamide) and (3D)Y8Fa (DYDLDFQPQRFamide), synthesized as previously described (Gicquel et al., 1994), were dissolved (1 mM) in distilled water and stored at −20°C as 100 μl aliquots. On the day of experiment they were further diluted (1, 10 μM) with the perifusion buffer and stored on ice. Lidocaine (0.2%) was dissolved in the perifusion buffer. Cells were incubated for 4 min with 1 μM ω-conotoxin GVIA. The other drugs were applied by perifusion.

2.4. Statistical analysis

Values are expressed as mean ± S.E.M. Means were compared with Student's *t* test.

3. Results

3.1. Depolarization-induced rise in $[Ca^{2+}]_i$

Short exposures (5–10 s) of dorsal root ganglion neurons to 30 mM K^+ caused a fast increase in Fluo3 fluorescence (Fig. 1A, trace 1) which was multiplied by 3.10 ± 0.14 ($n = 48$ neurons). During 90 s perifusions with 30 mM K^+, $[Ca^{2+}]_i$ reached a value that remained stable (Fig. 1A, trace 2) or decayed very slowly: in six cells, fluorescence after 90 s K^+ was 80 ± 8% of peak fluorescence. Lidocaine (0.2%, 4 min), a local anesthetic that blocks Na^+ channels, suppressed the K^+-induced $[Ca^{2+}]_i$ rise in all five neurons tested (not shown). Nifedipine (10 μM, 10 min), an antagonist of L-type Ca^{2+} channels, reduced (Fig. 1B) the fluorescence rise by 52 ±

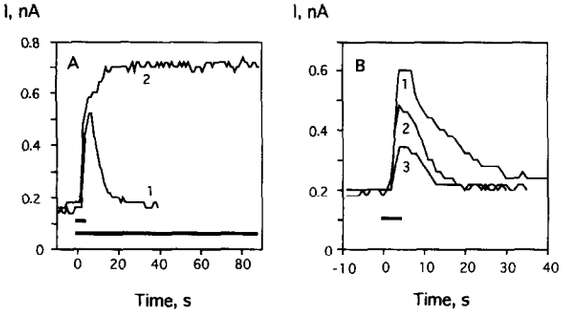

Fig. 1. Depolarization-induced rise in fluorescence in dorsal root ganglion neurons. A: Effects of 5 s (trace 1) and 90 s (trace 2) of perifusions with 30 mM K^+ in the same dorsal root ganglion neuron. Heavy black lines indicate the timing of K^+ perifusions. B: Fluorescence increases during 5 s of 30 mM K^+ perifusions (as indicated by heavy line) under control conditions (trace 1), after nifedipine at 10 μM, 10 min (trace 2) and after nifedipine plus ω-conotoxin GVIA at 1 μM, 4 min (trace 3). Recordings in A and B are from two different neurons.

7% ($n = 10$) whereas ω-conotoxin GVIA (1 μM, 4 min), an N-type Ca^{2+} channel antagonist, reduced it (Fig. 1B) by 45 ± 7% ($n = 6$). Other voltage sensitive Ca^{2+} channels may exist in dorsal root ganglion neurons since a rise in $[Ca^{2+}]_i$ persisted (Fig. 1B) in 3 out of 4 neurons after treatment with nifedipine plus ω-conotoxin GVIA.

These data suggest that during a 30 mM K^+-induced depolarization, dorsal root ganglion neurons fired action potentials and that the Ca^{2+} signal measured was the time integral of Ca^{2+} entering the cells through L, N and other voltage sensitive Ca^{2+} channels.

3.2. Effects of neuropeptide FF and analogs

3.2.1. Neuropeptide FF analogs

Three analogs were used: (1DMe)Y8Fa 10 nM ($n = 5$), 1 nM ($n = 4$), 0.1 nM ($n = 6$); (2DMe)Y8Fa 1 nM ($n = 4$) and (3D)Y8Fa 6 nM ($n = 2$). The effects of these analogs developed slowly and were only partially reversible even after 60 min washing in normal buffer. This precluded the determination of dose-response relationships.

At concentrations between 1 and 10 nM the three analogs had the same effects and the 15 neurons treated will be described as a single group. The analogs did not alter resting $[Ca^{2+}]_i$ but affected the $[Ca^{2+}]_i$ rise induced by depolarization (Table 1). Two effects were observed as illustrated in Fig. 2A.

(1) In this neuron, after a 10 min perifusion with 10 nM (1DMe)Y8Fa, the depolarization-induced rise in $[Ca^{2+}]_i$ had the same latency and magnitude as the control (compare traces 1 and 2) but declined more slowly when the neuron was returned to the normal K^+ buffer (the time constant of the exponential decrease rose from 15 s, trace 1, to 30 s, trace 2).

(2) After 20 min of (1DMe)Y8Fa perifusion the same K^+ stimulation caused no increase in $[Ca^{2+}]_i$ (Fig. 2A, trace 3). The slowing down of $[Ca^{2+}]_i$ recovery after K^+ depolarization was recorded in eight neurons (Table 1): the time constant of fluorescence decrease rose from 10.5 ± 1.4 s in the controls to 29.4 ± 3.4 s ($P < 0.05$) after neuropeptide FF analogs. The suppression of the $[Ca^{2+}]_i$ rise induced by short depolarizations was observed in 13 neurons (Table 1) and occurred after 10–20 min perifusions with neuropeptide FF analogs. In nine of these 13 neurons, we tested the $[Ca^{2+}]_i$ response to K^+ perifusions of longer

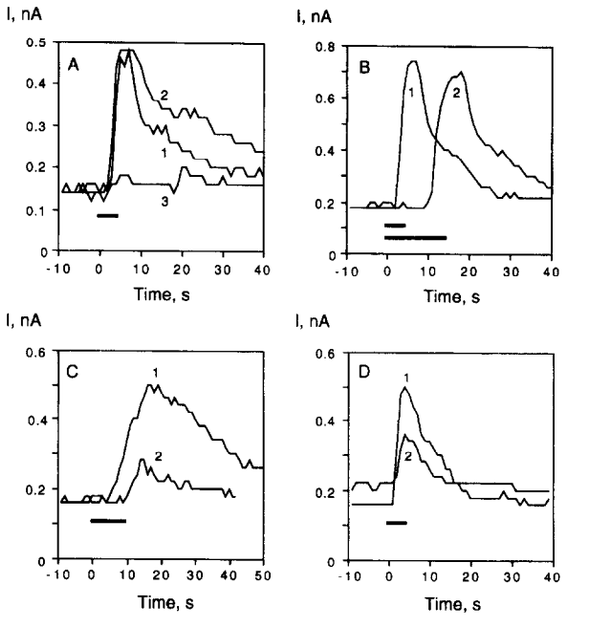

Fig. 2. Effects of neuropeptide FF and analogs. A: Ca^{2+} response of a dorsal root ganglion neuron to 5 s of 30 mM K^+ (heavy black line) under control conditions (trace 1), after 10 min (trace 2) and after 20 min (trace 3) of 10 nM (1DMe)Y8Fa. B: Trace 1, response of a dorsal root ganglion neuron to 5 s of 30 mM K^+ under control conditions; trace 2, response of the same neuron to 15 s of K^+ after 20 min of (1DMe)Y8Fa plus a 20 min washing in normal buffer. C: Responses of a dorsal root ganglion neuron to 10 s of 30 mM K^+ before (trace 1) and after (trace 2) 20 min of (1DMe)Y8Fa at 0.1 nM. D: Ca^{2+} response of a dorsal root ganglion neuron to 5 s of 30 mM K^+ before (trace 1) and after (trace 2) 20 min of neuropeptide FF at 1 nM. Recordings in A, B, C, D were obtained from four different dorsal root ganglion neurons.

duration when the response to control K^+ stimulation had been suppressed by neuropeptide FF analogs as illustrated in Fig. 2B. In this neuron, under control conditions, 5 s of 30 mM K^+ caused a rise in $[Ca^{2+}]_i$ after a 2 s latency (Fig. 2B, trace 1). After 20 min of (1DMe)Y8Fa at 1 nM, the neuron was insensitive to this K^+ stimulation and remained so for at least 20 min after washing in normal buffer (not illustrated). The neuron was then perifused for 15 s with 30 mM K^+ that caused a rise in $[Ca^{2+}]_i$ after 9 s (Fig. 2B, trace 2), i.e., 4.5 times the control latency. In the nine neurons the latency for the increase in $[Ca^{2+}]_i$ rose from 4.2 ± 0.6 s in controls to 12.5 ± 2.3 s after perifusion with neuropeptide FF analogs ($P < 0.05$). During these

Table 1
Ca^{2+} responses of dorsal root ganglion neurons to neuropeptide FF and analogs

	(1DME), (2DME), (3D) 1–10 nM; $n = 15$	(1DME) 0.1 nM; $n = 6$	NPFF 1 nM; $n = 9$
Slowing down of $[Ca^{2+}]_i$ recovery	8	1	1
Suppression of response to K^+	13	1	0
Reduction of response to K^+	0	4 (39 ± 8%)	3 (38 ± 11%)
No effect	2	1	5

The values are the number of neurons exhibiting a particular response. The values in brackets indicate the percentage reduction in the depolarization induced rise in fluorescence.

prolonged K^+ stimulations the peak fluorescence was 1.02 ± 0.1 times its control value ($P > 0.05$).

The effect of 0.1 nM (1DMe)Y8Fa was studied in six neurons (Table 1). In one neuron the increase in $[Ca^{2+}]_i$ in response to 10 s 30 mM K^+ perifusion was suppressed after 20 min perifusion with the analog whereas in four other neurons the increase in fluorescence intensity was reduced by $39 \pm 8\%$ (Fig. 2C, Table 1). In two of these neurons the latency of the $[Ca^{2+}]_i$ rise was increased by 3 and 5 s (Fig. 2C)

3.2.2. Neuropeptide FF

Resting $[Ca^{2+}]_i$ was not altered in the 9 neurons perifused with 1 nM neuropeptide FF. In five neurons neuropeptide FF had no effect (Table 1): the magnitude of the $[Ca^{2+}]_i$ rise after 20 to 30 min neuropeptide FF was 0.96 ± 0.015 times the control amplitude. In one cell the Ca^{2+} transient decayed more slowly (Table 1) while in 3 other neurons its magnitude was reduced by $38 \pm 11\%$ (Table 1 and Fig. 2D). In three additional neurons, neuropeptide FF 100 nM was perifused for 30 min; one neuron was not affected whereas in the other two the depolarization-induced rise in fluorescence was reduced by 37 and 50%, respectively.

4. Discussion

In this study, we report that neuropeptide FF and three of its analogs reduced the $[Ca^{2+}]_i$ rise induced by depolarization. The analogs, primarily designed for a better resistance to peptidase degradation (Gicquel et al., 1992), have more intense effects than neuropeptide FF since they are able to suppress the Ca^{2+} response to brief depolarizations. Considering the low cell concentration used in the preparation and thus the low peptidase activity, it is likely that the degradation of neuropeptide FF was negligible. In addition, in the three neurons perifused with 100 nM neuropeptide FF, although the $[Ca^{2+}]_i$ rise was reduced in magnitude it was not suppressed as with the analogs. It is likely, therefore, that (1DMe), (2DMe) and (3D)Y8Fa have greater intrinsic pharmacological activity than neuropeptide FF.

There are few experimental data on the action of neuropeptide FF on neurons. Guzman et al. (1989) reported no effect of neuropeptide FF on resting fetal mice dorsal root ganglion neurons maintained in culture. This is consistent with the present findings since resting $[Ca^{2+}]_i$ was not affected by neuropeptide FF and analogs; only the depolarization-induced $[Ca^{2+}]_i$ rise was reduced in our experiments. In about 10% of neurons of fetal mouse spinal cord maintained in culture, neuropeptide FF produced a transient hyperpolarization followed by a long-lasting depolarization during which the neurons fired action potentials (Guzman et al., 1989). This suggests that neuropeptide FF might produce different effects in various neurons, but this remained to be documented.

μ- and δ-opioid agonists were shown to reduce the depolarization-induced rise in $[Ca^{2+}]_i$ in neurons of the nucleus tractus solitarius (Rhim and Miller, 1994) and in NG 108-15 cells (Jin et al., 1992) through modulation of voltage sensitive Ca^{2+} channels. Such a reduction is expected in many neurons where opioid receptors are coupled to Ca^{2+} or K^+ channels. In the present study, neuropeptide FF and its analogs reduced and even suppressed the $[Ca^{2+}]_i$ rise induced by depolarization. Though the underlying ionic mechanisms are very likely to be different from those involved in the opioid-induced reduction in $[Ca^{2+}]_i$ rise, both opioid and neuropeptide FF would have functionally the same effect on intracellular Ca^{2+}. If the dorsal root ganglion neuron soma may be considered as a model of the primary afferent ending in the dorsal horn of the spinal cord, we may expect a pro-opioid effect of neuropeptide FF on these nerve endings, i.e., a reduction in transmitter release. Such an effect could account, at least partly, for the analgesia induced by intrathecal injection of neuropeptide FF (Gouardères et al., 1993b).

Neuropeptide FF analogs suppressed the $[Ca^{2+}]_i$ rise induced by short depolarizations in mouse dorsal root ganglion neurons by increasing the Ca^{2+} transient latency, which overcame the duration of K^+ perifusion. By contrast, opiates reduced the magnitude of the $[Ca^{2+}]_i$ rise without altering its time course (see, e.g., Rhim and Miller, 1994).

Our data revealed that neuropeptide FF acts at the cellular level by reducing the $[Ca^{2+}]_i$ rise during depolarization; the exact ionic mechanisms underlying this response remain, however, to be investigated.

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