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Inflammation enhances peripheral μ-opioid receptor-mediated analgesia, but not μ-opioid receptor transcription in dorsal root ganglia

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Abstract

μ-Opioid receptor agonist [D-Ala^2,NMe-Phe^4,Gly^5-ol]enkephalin (DAMGO)-induced peripheral analgesic effects occur early in hindpaws inoculated with Freund's complete adjuvant and increase in parallel to the development of inflammatory signs. Antagonism of these effects by β-funaltrexamine, an irreversible μ-opioid receptor antagonist, suggests that the effective number of peripheral opioid receptors does not increase during early stages, but does so at later stages of the inflammation. As determined by a ribonuclease protection assay, μ-opioid receptor mRNA in dorsal root ganglia is abundant in untreated animals, but does not significantly increase following inflammation. Thus, peripheral analgesic efficacy of DAMGO is not correlated with transcription or number of μ-opioid receptors at early inflammatory stages. At later stages, however, the number of peripheral μ-opioid receptors appears to increase and may enhance opioid efficacy.

Keywords: Antinociception; μ-Opioid receptor; Dorsal root ganglion; Primary afferent neuron; Ribonuclease protection assay; β-Funaltrexamine

1. Introduction

Experimental and clinical studies demonstrate that local administration of low doses of opioid receptor agonists elicits potent analgesic effects in inflamed, but not in non-inflamed tissue (reviewed in Stein, 1993). These effects are opioid receptor-specific, because they have been shown to be dose-dependent, stereospecific and reversible by opioid receptor antagonists. Opioid binding studies show evidence for opioid receptors in dorsal root ganglia and on central terminals of primary sensory neurons (LaMotte et al., 1976; Fields et al., 1980). In addition, we have found opioid receptors on peripheral terminals of small-diameter cutaneous nerves (Stein et al., 1990; Hassan et al., 1993).

This study examined the analgesic efficacy of a locally applied μ-opioid receptor agonist [D-Ala^2,NMe-Phe^4,Gly^5-ol]enkephalin (DAMGO) in relation to (i) changes in paw volume as a parameter for the development of inflammation, (ii) the effective number of peripheral μ-opioid receptors in vivo and (iii) μ-opioid receptor mRNA levels in dorsal root ganglia.

2. Materials and methods

2.1. Animals

The NIH guidelines for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80-23) were followed. Male Wistar rats (180–225 g) were housed individually in cages lined with ground corn cob bedding, maintained on a 12/12 h (7 a.m./7p.m.) light-dark cycle and allowed free access to tap water and standard rodent chow. Room temperature and relative humidity were maintained at 22 ± 0.5° C and 60%, respectively. Inflammation was induced by an injection of 0.15 ml Freund's complete adjuvant into the right hindpaw of rats. Control ani-

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mals were injected with isotonic saline. After brief anesthesia with halothane six groups ($n = 5$) per each time interval were killed by decapitation at 0, 6, 24, 48 and 96 h after inoculation with Freund's complete adjuvant. Dorsal root ganglia were separately removed on each side after dorsal laminectomy, quickly frozen on dry ice and stored at −80° C until further use. For RNA extraction three dorsal root ganglia (L3-L5), which process the major part of sensory information from the rat's hindpaw (Swett and Woolf, 1985), were pooled from five animals.

2.2. Parameter of inflammation

As a parameter of inflammation, paw volume was determined by submerging each hindpaw to the tibiotarsal joint into a water-filled Perspex cell of a plethysmometer (Ugo Basile, Comerio, Italy). The volume of displacement, which is equal to the paw volume, was then read on a digital display. For each animal ($n = 12$), measurements were done twice (at each of the above time intervals) and the average calculated.

2.3. Algesiometry

Nociceptive thresholds were evaluated by use of a modified Randall-Selitto paw pressure test with the observer blind to the experimental condition employed. Animals ($n = 6–7$ per group) were gently restrained and incremental pressure (maximum 250 g) was applied onto the hindpaw. The pressure required to elicit paw withdrawal, the paw pressure threshold, was determined. After baseline measurements, rats received an intraplantar injection of DAMGO (1–32 μg) (RBI), a highly selective μ-opioid receptor agonist. 5 min post injection, when paw pressure thresholds were maximal (Stein et al., 1989), paw pressure thresholds were reevaluated for different concentrations of DAMGO in order to construct dose-response curves. This procedure was carried out in different groups at each stage (2, 6, 12, 24, 96 h) of inflammation. In separate experiments β-funaltrexamine hydrochloride (β-funaltrexamine, 0.1–75 μg) (RBI), an irreversible μ-opioid receptor antagonist, was injected intraplantar alone or 2 h before DAMGO (8 μg) administration. Paw pressure thresholds were tested 5 min after DAMGO injection and dose-response curves were constructed for β-funaltrexamine at 6, 12, 24 and 96 h of inflammation. Paw pressure thresholds are given as percentage of maximum possible effect (% MPE) according to the formula: (paw pressure $\text{threshold}_{\text{postinjection}}$ − paw pressure $\text{threshold}_{\text{basal}}$)/(250 − paw pressure $\text{threshold}_{\text{basal}}$).

2.4. Ribonuclease protection assay

Total RNA was extracted from dorsal root ganglia L3-L5 for each side by applying the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). A 400-bp cDNA encoding rat μ-opioid receptor sequences corresponding to the 4th transmembrane domain to *C*-terminal region (Wang et al., 1993) and an Fnu4HI digested human γ-actin cDNA were used to generate antisense cRNA probes with [^{32}P]CTP as a label (specific activity 3×10^4, 10^3 Ci/mmol, respectively), using a Riboprobe kit (Promega). 10 μg of each total RNA sample were hybridized in solution to a μ-opioid receptor/human γ-actin probe mixture for 16 h at 42° C. Then the reaction mixture was subjected to digestion by RNase A and RNase T1 for 30 min in 37° C. Protected fragments were precipitated with a RNase Inactivation/Precipitation mixture (RPA II kit, Ambion), then dissolved, denatured and electrophoresed on 8 M urea/5% polyacrylamide gels. The gels were dried and densitometrically analysed on PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Each μ-opioid receptor mRNA value was normalized to the value of γ-actin mRNA from the same lane. The value obtained at each time interval of inflammation represents measurements of six different RNA samples (corresponding to the six groups), the mean calculated of one to three blots per RNA sample. Data are expressed as % of control (saline treated animals) from the same gel to exclude differences in gel conditions.

Table 1
Assessment of paw volume and baseline paw pressure thresholds after inoculation of the right paw with Freund's complete adjuvant

	Time post inoculation (h)					
	0	2	6	12	24	96
Volume (ml)						
Right paw	1.40 ± 0.02	2.09 ± 0.07 [b]	2.75 ± 0.11 [b]	2.78 + 0.10 [b]	2.67 ± 0.09 [b]	2.60 ± 0.08 [b]
Left paw	1.33 ± 0.04	1.45 ± 0.06	1.44 ± 0.06	1.32 + 0.08	1.29 ± 0.07	1.45 ± 0.08
Paw pressure threshold (g)						
Right	68.1 ± 4.4	62.1 ± 3.9	40.1 ± 3.9 [a]	40.4 ± 3.8 [a]	40.8 ± 3.7 [a]	40.3 ± 3.4 [a]
Left	71.0 ± 3.9	71.9 ± 3.1	66.3 ± 2.6	67.2 ± 2.7	71.5 ± 2.9	72.8 ± 2.5

Data are expressed as means + S.E.M.; [a] $P < 0.01$, [b] $P < 0.001$ indicate significant differences between right and left paws (Wilcoxon test).

3. Results

3.1. Parameters of inflammation

After inoculation with Freund's complete adjuvant the volume of inoculated paws increased continuously up to 12 h (Friedman test, $P < 0.05$) and did not significantly change thereafter (Friedman test, $P > 0.05$) (Table 1). The volume of non-inflamed paws did not significantly change during the same period of time (Friedman test, $P > 0.05$). The volume of inoculated paws was always significantly higher than that of contralateral non-inflamed paws after inoculation (Wilcoxon test, $P < 0.001$) (Table 1). Baseline paw pressure thresholds of the inflamed paws were significantly decreased at 6 h (Friedman test, $P < 0.05$) and did not significantly change thereafter (Friedman test, $P > 0.05$) (Table 1). Baseline values of the non-inflamed paws did not significantly change during the same observation period (Friedman test, $P > 0.05$). At 6, 12, 24 and 96 h, the pressure threshold of inoculated paws was significantly lower than that of contralateral non-inflamed paws (Wilcoxon test, $P < 0.01$) (Table 1).

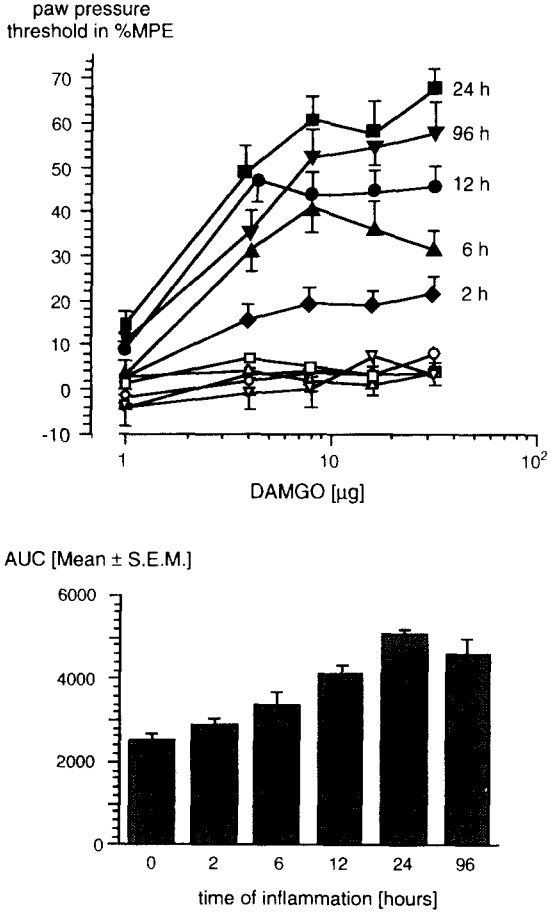

Fig. 1. *Top panel*: effects of intraplantar DAMGO (1–32 µg) on nociceptive thresholds in untreated paws (open symbols) and in paws inoculated with Freund's complete adjuvant (closed symbols) at 2 (♦), 6 (▲), 12 (●), 24 (■) and 96 h (▼) after inoculation. DAMGO effects are dose-dependent ($P < 0.05$ ANOVA, linear regression). *Bottom panel*: efficacy of DAMGO, determined as area under the dose-response curve (AUC), at different time intervals of inflammation. AUC increases linearly until 24 h ($P < 0.001$ ANOVA, linear regression), but is not different at 24 and 96 h ($P > 0.05$, unpaired *t*-test).

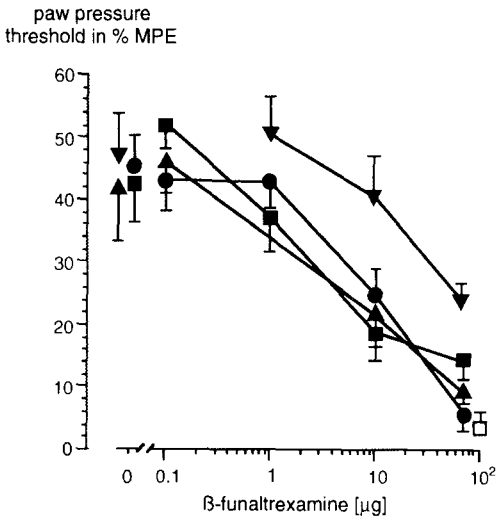

Fig. 2. Antagonism of DAMGO (8 µg) effects by intraplantar β-funaltrexamine. Dose-response curves were not significantly different at 6 (▲), 12 (●) and 24 h (■) of inflammation ($P > 0.05$, two-way ANOVA), but at 96 h (▼) ($P < 0.001$, two-way ANOVA). 70 µg of β-FNA (□) alone did not change nociceptive thresholds.

3.2. Algesiometry

Early phase (0–24 h): After intraplantar injection of DAMGO (1–32 µg) paw pressure thresholds increased dose dependently and reached a maximum elevation at 8 µg in paws inoculated with Freund's complete adjuvant ($P < 0.05$, analysis of variance (ANOVA)), but not in contralateral saline treated paws ($P > 0.05$, ANOVA) (Fig. 1, upper panel). The plateau, but not the slope of DAMGO's dose-response curves increased in correlation to the duration of the inflammatory process. The efficacy of DAMGO, as determined by the area under the dose-response curve (Fig. 1, lower panel), increased linearly ($P < 0.001$, ANOVA; $P < 0.001$ linear regression). Based on these results, a test dose of 8 µg of DAMGO was chosen for the following experiments with β-funaltrexamine. β-Funaltrexamine (0.1–70 µg), injected into inflamed paws 2 h before DAMGO (8 µg), antagonized its effects dose dependently ($P < 0.001$, ANOVA; $P < 0.001$; linear regression ANOVA) (Fig. 2). At 6, 12 and 24 h after the onset of inflammation, dose-response curves of β-funaltrexamine were not significantly different ($P > 0.05$, two-way ANOVA). β-Funaltrexamine given alone did not change paw pressure thresholds until 120 min post injection ($P > 0.05$, ANOVA) (Fig. 2) demonstrat-

ing that, in contrast to other models (Jiang et al., 1990), agonist effects are not detectable in this situation.

Late phase (24–96 h): DAMGO's efficacy (area under the curve) did not change significantly between 24 and 96 h ($P > 0.05$, unpaired t-test) (Fig. 1). However, significantly higher doses of β-funaltrexamine were required to antagonize DAMGO effects at 96 h as compared to 6, 12 and 24 h ($P < 0.001$, two-way ANOVA) (Fig. 2).

3.3. Ribonuclease protection assay

In dorsal root ganglia L3-L5 of control (saline-treated) rats μ-opioid receptor mRNA levels were readily detectable at a hybridization density ratio of 4.23 ± 0.44% μ-opioid receptor/γ-actin (mean ± S.E.M.). μ-Opioid receptor mRNA levels were not significantly different between animals with inflammation and control animals within each gel at any time after inoculation with Freund's complete adjuvant ($P > 0.05$, Kruskal-Wallis test) (Fig. 3, upper panel). In addition, there were no significant differences between the ipsi- and contralateral side ($P > 0.05$, Wilcoxon test). At no time interval of inflammation did γ-actin mRNA levels significantly change within each gel ($P > 0.05$, Kruskal-Wallis test) (Fig. 3, lower panel).

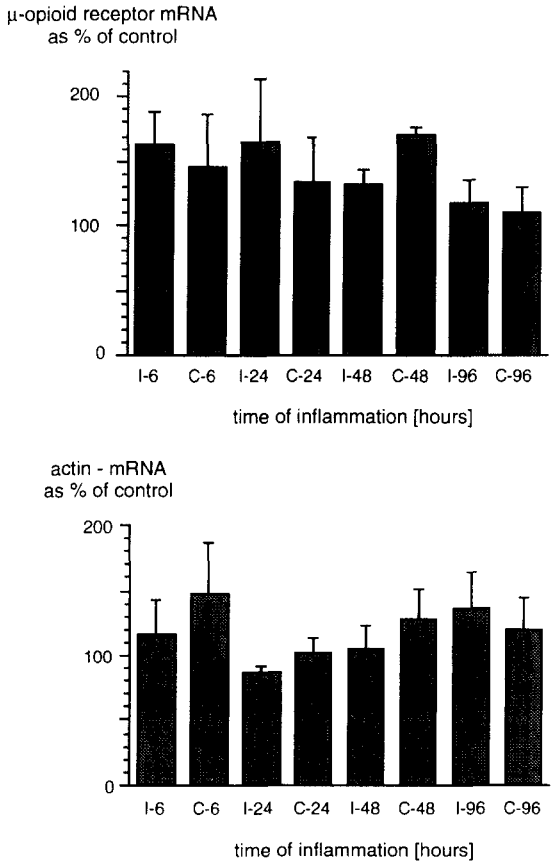

Fig. 3. *Top panel*: μ-opioid receptor mRNA levels in ipsi- (I) and contralateral (C) dorsal root ganglia (L3-L5) at different time intervals of inflammation. Data represent the mean + S.E.M. of six different RNA samples (one to three blots per sample) and are expressed as percentage of control. Differences between μ-opioid receptor mRNA levels at various time intervals of inflammation or control are not significant ($P > 0.05$, Kruskal-Wallis test). *Bottom panel*: γ-actin mRNA levels in ipsi- (I) and contralateral (C) dorsal root ganglia are shown as a control for the same time intervals as seen above. Differences are not significant ($P > 0.05$, Kruskal-Wallis test).

4. Discussion

This study demonstrates that (1) upon intraplantar injection of Freund's complete adjuvant, paw volume and antinociceptive efficacy of DAMGO of inoculated paws increase linearly up to a maximum at 12 and 24 h, respectively, (2) doses of β-funaltrexamine required to abolish DAMGO effects are constant up to 24 h, (3) between 24 and 96 h, paw volume and DAMGO effects remain unchanged, whereas doses of β-funaltrexamine increase, (4) μ-opioid receptor mRNA levels are readily detectable in dorsal root ganglia of control animals and do not significantly change at any time interval up to 96 h of inflammation.

During the first 24 h after inoculation of the hindpaw with Freund's complete adjuvant the paw volume, a typical parameter of inflammation, and the antinociceptive efficacy of intraplantar DAMGO increases in parallel. Throughout this time, baseline paw pressure thresholds are decreased in inflamed paws but remain constant and, therefore, cannot account for these changes in DAMGO efficacy. The early appearance of DAMGO effects suggests that μ-opioid receptors are preexistent on peripheral sensory nerves. In fact, our assessment of μ-opioid receptor mRNA levels in dorsal root ganglia indicates a basal gene expression and probable synthesis of μ-opioid receptors in control animals. This is also supported by recent studies investigating the anatomical distribution of μ-opioid receptor mRNA (Thompson et al., 1993; Maekawa et al., 1994) by in situ hybridization.

To assess the effective number of peripheral μ-opioid receptors in vivo, we used β-funaltrexamine, which covalently binds and irreversibly inactivates μ-opioid receptors, thereby decreasing the number of available receptors (Jiang et al., 1990; Mjanger and Yaksh, 1991). At early stages of the inflammation (0–24 h) DAMGO effects were antagonized by constant concentrations of intraplantar β-funaltrexamine, suggesting that the effective number of peripheral μ-opioid receptors does not significantly change. Consistent with this observation, μ-opioid receptor mRNA levels of dorsal root ganglia do not significantly change over the same period of inflammation. This is in line with our previous finding that an increase in number of peripheral opioid receptors, produced by axonal transport to the periphery, requires several days (Hassan et al., 1993). Thus, the simultaneous development of inflam-

mation and increased efficacy of DAMGO suggests other mechanisms that act in a more direct and immediate manner. For instance, it has been described in membrane preparations that a low pH increases opioid efficacy to inhibit adenyl cyclase by decreasing the inactivation rate of receptor-coupled G proteins (Selley et al., 1993). A low pH as a consequence of inflammation could play an immediate role in our model of inflamed paw tissue. Another possible mechanism has recently been demonstrated (Antonijevic et al., 1995): inflammation produces a disruption of the perineural barrier around peripheral sensory nerves which is critical for the access of agonists to opioid receptors, particularly in the early phase.

At later stages (24–96 h) significantly higher doses of β-funaltrexamine are required to antagonize DAMGO effects, suggesting that the effective number of peripheral μ-opioid receptors increases. Alternatively, changes in opioid receptor affinity or receptor coupling to G-proteins may occur. However, our previous studies (Hassan et al., 1993) show that axonal transport and presence of opioid receptors in peripheral paw tissue increase after 48 and 96 h, respectively, thereby demonstrating an 'up-regulation' of peripheral opioid receptor proteins. In contrast, neither μ-opioid receptor mRNA nor DAMGO efficacy increase beyond 24 h. Neither of these findings, however, exclude an increased number of receptors at peripheral nerve terminals. Although mRNA levels remain unchanged, posttranscriptional and/or posttranslational changes may lead to altered receptor synthesis, but these are not detected by our methodology (Tsutsumi et al., 1993). Furthermore, DAMGO has an extremly high intrinsic activity which yields maximal effects at a small fraction of occupied μ-opioid receptors and, therefore, further increases in receptor number may not result in increased effects (Mjanger and Yaksh, 1991).

In summary, peripheral antinociceptive effects of DAMGO appear early during inflammation, which is consistent with preexistent μ-opioid receptors and a readily detectable basal level of μ-opioid receptor transcription in primary afferent nerves. At early stages of inflammation, DAMGO efficacy does not correlate with the number of peripheral μ-opioid receptors, but may be determined by other factors such as local pH and perineural permeability. At later stages, however, an increased number of peripheral opioid receptors may enhance the efficacy of opioid agonists.

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