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Mu and delta opioid-stimulated [^{35}S]GTPγS binding in brain and spinal cord of polyarthritic rats

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Abstract

Polyarthritis induced by inoculation with complete Freund's adjuvant alters opioid peptides, but does not affect opioid receptor binding. This study was conducted to measure mu and delta opioid receptor-stimulated G-protein activity in brain and spinal cord of rats 19 days after injection of complete Freund's adjuvant or vehicle. Mu and delta opioid-stimulated [^{35}S]GTPγS binding measured autoradiographically in caudate–putamen, medial thalamus and periaqueductal gray was unchanged in polyarthritic rats. Delta opioid-stimulated [^{35}S]GTPγS binding was significantly decreased in the spinal cord of polyarthritic rats, whereas mu opioid-stimulated activity was unchanged. These data reveal that the functional activity of delta opioid receptors in the spinal cord is altered in polyarthritis.

Keywords: G-protein; Inflammation; Arthritis; Complete Freund's adjuvant

1. Introduction

Opioid drugs are among the most effective analgesics, but have limited effectiveness in treating chronic pain. One rat model of chronic inflammatory pain utilizes injection of complete Freund's adjuvant into the base of the tail, which produces a polyarthritic state characterized by inflammation, edema and hyperalgesia (Millan et al., 1986; Nagakura et al., 2003). The levels of the endogenous opioid peptides dynorphin and met-enkephalin are elevated in the spinal cord of polyarthritic rats (Millan et al., 1986; Pohl et al., 1997; Spetea et al., 2002), suggesting that endogenous opioid systems might play a role in modulating responses to chronic inflammation. Studies have generally found no changes in opioid receptor levels in the central nervous system (CNS) of polyarthritic animals (Cesselin et al., 1980; Millan et al., 1986; Delay-Goyet et al., 1989; Spetea et al., 2002), but the effect of chronic inflammation on opioid receptor-mediated G-protein activity has not been investigated in this model. Zollner et al. (2003) reported that mu opioid receptor-mediated G-protein activity was increased in the dorsal root ganglion and unchanged in the spinal cord of monoarthritic rats 4 days after complete Freund's adjuvant injection into the hindpaw. However, mu and delta opioid receptor-mediated G-protein activity has not been evaluated in the polyarthritic model after prolonged inflammation.

Opioids mediate antinociception via circuitry that includes the periaqueductal gray, rostral ventral medial medulla and spinal cord dorsal horn (Fields and Basbaum, 1978; Wang and Wessendorf, 2002). Both mu and delta opioid receptors are distributed throughout the forebrain, whereas mu opioid receptors predominate in the brainstem (Mansour et al., 1988). Opioid receptors are located on

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primary afferent terminals in the superficial laminae of the spinal cord, as well as in lamina V and surrounding the central canal at all spinal levels (Lamotte et al., 1976; Stevens et al., 1991b). Therefore, opioid receptors at a number of CNS levels could be affected by polyarthritis and serve as therapeutic targets.

Opioid receptors belong to the superfamily of G-protein-coupled receptors, containing seven transmembrane domains and transducing an agonist-stimulated signal through the G_i/G_o subfamily of G proteins (Fedynyshyn and Lee, 1989; Evans et al., 1992; Kieffer et al., 1992). Opioid receptor-activated G-proteins can be assessed using agonist-stimulated [^{35}S]GTPγS binding in both membrane homogenates and brain sections (Sim et al., 1995; Traynor and Nahorski, 1995). Previous studies have demonstrated region-specific decreases in mu opioid receptor-stimulated G-protein activity after chronic opioid administration (Sim et al., 1996; Sim-Selley et al., 2000), but the effect of chronic inflammation on opioid receptor function is not known. The goal of this study was to determine whether opioid receptor-mediated G protein activity is altered in polyarthritic rats using opioid-stimulated [^{35}S]GTPγS binding in rat brain and spinal cord. Regions of interest included the caudate–putamen because of its role in motor function, as well as the medial thalamus, periaqueductal gray and spinal cord dorsal horn because these areas mediate opioid-induced analgesia.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (350–375 g) were obtained from Harlan Laboratories (Indianapolis, IN). Rats were housed two per cage in an animal facility maintained at 22±2 °C on a 12-h light/dark cycle. Food and water were available ad libitum. All procedures were conducted in accordance with the regulations of the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2. Induction of arthritis

Arthritis was induced as previously described (Cox and Welch, 2004). A volume of 0.1 ml of mineral oil vehicle or complete Freund's adjuvant in mineral oil (heat-killed *Mycobacterium butyricum*, 5 mg/ml; Difco Laboratories, Detroit, MI) was injected intradermally into the base of the tail. Animals remained in their cages for 12 days. From days 12–19, the animals were acclimated to paw-pressure testing by holding the rat in the position for testing and turning the device "on" to let the rats become acclimated to the noise of the weight moving. No pressure was applied to the feet until day 19, when inflammation resulted in a generalized polyarthritis. The paw-pressure test was performed using the Analgesy-Meter (Ugo-Basile, Varese, Italy), designed to exert a force on the paw that increases at a constant rate. The paw pressure threshold was defined as the pressure in grams at which the rat began to withdraw the paw. The criteria for inclusion in the study was as follows: vehicle-treated rats having a baseline paw pressure threshold of greater than 100 g, and complete Freund's adjuvant-treated rats having a baseline paw pressure threshold of less than 100 g. An upper limit of 500 g was set for all testing.

2.3. Agonist-stimulated [^{35}S]GTPγS autoradiography

Rats that met the above criteria were killed by rapid decapitation 19 days after complete Freund's adjuvant inoculation. Brains were removed and immediately frozen in isopentane at −35 °C. Coronal sections (20 μm) were cut on a cryostat maintained at −20 °C and thaw-mounted onto gelatin-subbed slides. Brain sections were collected at the levels of the caudate–putamen, thalamus and periaqueductal gray. Slides were dried under a vacuum and stored in a humidified chamber at 4 °C overnight, then stored desiccated at −80 °C until use. Slides were incubated for 10 min in 50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA and 100 mM NaCl (assay buffer) at 25 °C. Slides were then transferred to assay buffer containing 2 mM GDP and 10 mU/ml adenosine deaminase and incubated for 20 min at 25 °C. Agonist-stimulated activity was determined by 2 h incubation at 25 °C in the presence of 10 μM [D-Ala^2, NMePhe^4, Glyol^5]enkephalin (DAMGO) or 10 μM [D-Pen^2, pCl-Phe^4, D-Pen^5]enkephalin (DPDPE) in assay buffer containing 2 mM GDP, 10 mU/ml adenosine deaminase and 0.04 nM [^{35}S]GTPγS. Basal activity was measured in the absence of agonist. Slides were rinsed twice in ice-cold Tris buffer (50 mM, pH 7.4) for 2 min and once in deionized water for 30 s. Slides were dried overnight and placed in cassettes with ^{14}C microscales and Kodak X-Omat MR film. Films were developed after 3–5 days and digitized with an XC-77 video camera (Sony, Tokyo, Japan). The NIH Image program for Macintosh was used for analysis. All data are reported as mean values±S.E.M. for triplicate sections from brains from 6–8 rats. Net stimulated [^{35}S]GTPγS binding was determined by subtracting basal [^{35}S]GTPγS binding from agonist-stimulated [^{35}S]GTPγS binding.

2.4. Agonist-stimulated [^{35}S]GTPγS binding in membrane homogenates

Whole spinal cords from vehicle- and complete Freund's adjuvant-treated rats were removed, frozen immediately on dry ice for 10 min and then stored at −80 °C until use. For each assay, spinal cords were thawed and homogenized in 10 ml assay buffer, followed by centrifugation at 50,000×*g* for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in cold assay buffer. Membranes were incubated with adenosine deaminase (4 mU/ml) for 10 min at

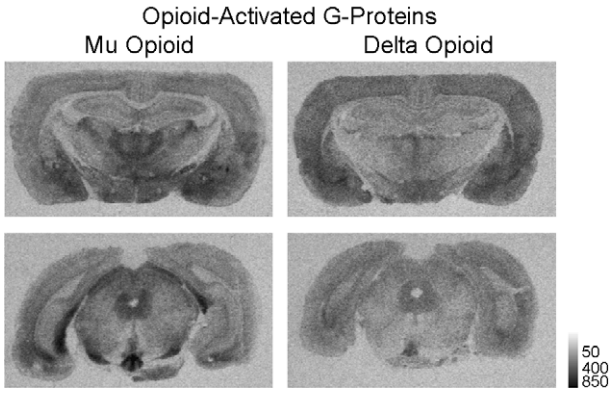

Fig. 1. Autoradiograms of brain sections comparing mu and delta opioid receptor-activated G-proteins in vehicle- treated rats. Autoradiography was performed as described in Materials and methods using 10 μM DAMGO or DPDPE for mu and delta opioid receptors, respectively. Representative autoradiograms depict sections at the levels of the thalamus (top row) and periaqueductal gray (bottom row).

30 °C, then protein was determined by the method of Bradford (1976). Membranes (8 μg) were incubated with 0.1 nM [^{35}S]GTPγS, 30 μM GDP and varying concentrations of DAMGO or DPDPE in a final volume of 500 μl. Basal binding was assessed in the absence of agonist, and non-specific binding was determined in the presence of 10 μM unlabeled GTPγS. Assay tubes were incubated for 2 h at 30 °C and the reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters. Bound radioactivity was measured by liquid scintillation spectrophotometry after extraction with ScintiSafe Econo 1 scintillation cocktail. Data are reported as mean±S.E.M. values of six experiments performed in triplicate. Nonlinear iterative regression analyses of agonist concentration–effect curves were determined using JMP 5.0.1 software (SAS, Cary, NC).

2.5. Drugs

[^{35}S]GTPγS (1250 Ci/mmol) was purchased from New England Nuclear (Boston, MA); GDP and adenosine deaminase were purchased from Sigma (St. Louis, MO). DAMGO and DPDPE were provided by the Drug Supply Program of the National Institute on Drug Abuse. All other reagent-grade chemicals were obtained from Sigma or Fisher Scientific (Pittsburgh, PA).

3. Results

3.1. Agonist-stimulated [^{35}S]GTPγS autoradiography

Mu and delta opioid receptor-mediated G-protein activity was assessed using DAMGO- or DPDPE-stimulated [^{35}S]GTPγS binding, respectively. Regions of interest included caudate–putamen, thalamus and PAG, as shown in Fig. 1. Visual inspection of brain sections from vehicle- and complete Freund's adjuvant-treated rats revealed similar levels of mu and delta opioid-stimulated [^{35}S]GTPγS binding. This observation was confirmed by densitometric analysis (Table 1), which showed no difference in agonist-stimulated [^{35}S]GTPγS binding between the two groups. Basal binding was also similar between vehicle- and complete Freund's adjuvant-treated brains in all regions examined (Table 1).

3.2. Agonist-stimulated [^{35}S]GTPγS binding in rat spinal cord membranes

DAMGO- and DPDPE-stimulated [^{35}S]GTPγS binding was examined in membranes from spinal cords of vehicle- or complete Freund's adjuvant-treated rats. Both agonists produced concentration-dependent stimulation of [^{35}S]GTPγS binding in spinal cord membranes from vehicle- and complete Freund's adjuvant-treated rats (Fig. 2). Although the magnitude of stimulation by DAMGO was similar in both groups (Fig. 2A), DPDPE-stimulated activity appeared to be decreased in complete Freund's adjuvant-treated rats (Fig. 2B). Nonlinear regression analysis of the concentration–effect curves revealed

Table 1
The effect of complete Freund's adjuvant-induced arthritis on basal and DAMGO- or DPDPE-stimulated [^{35}S]GTPγS autoradiography in the rat brain

Region	Basal [^{35}S]GTPγS binding		Net DAMGO-stimulated [^{35}S]GTPγS binding	
	Vehicle	CFA	Vehicle	CFA
Caudate–putamen	148±17	161±12	480±20	500±14
Thalamus	192±29	144±20	310±39	383±24
Periaqueductal gray	262±16	267±6	263±17	228±18
Region	Basal [^{35}S]GTPγS binding		Net DPDPE-stimulated [^{35}S]GTPγS binding	
	Vehicle	CFA	Vehicle	CFA
Caudate–putamen	248±20	239±8	271±21	268±22
Thalamus	227±11	201±13	91±9	105±12
Periaqueductal gray	379±9	378±14	70±11	65±12

Data are expressed as mean net agonist-stimulated [^{35}S]GTPγS binding (nCi/g)±S.E.M. of triplicate sections from 6–8 vehicle- or complete Freund's adjuvant (CFA)-treated animals per group.

that this difference was due to a decrease in maximal stimulation of [^{35}S]GTPγS binding by DPDPE in complete Freund's adjuvant-treated relative to vehicle-treated rats (Table 2). The E_{max} value of DPDPE-stimulated [^{35}S]GTPγS binding was 27% lower in complete Freund's adjuvant-treated than in vehicle-treated rat spinal cord. Although the EC_{50} value of DPDPE appeared to be increased by 1.6-fold in complete Freund's adjuvant-treated rats, this was not a significant difference. In contrast to the delta opioid agonist, the maximal stimulation and EC_{50} value for DAMGO did not significantly differ between groups. Basal [^{35}S]GTPγS binding was also not different in spinal cords of complete Freund's adjuvant- versus vehicle-treated rats (135±14 versus 155±9.4 fmol/mg, respectively). Thus, these results indicate that complete Freund's adjuvant-induced arthritis attenuated the maximal magnitude of delta opioid receptor-mediated G-protein activation in spinal cord membranes without affecting mu receptor-mediated or basal activity.

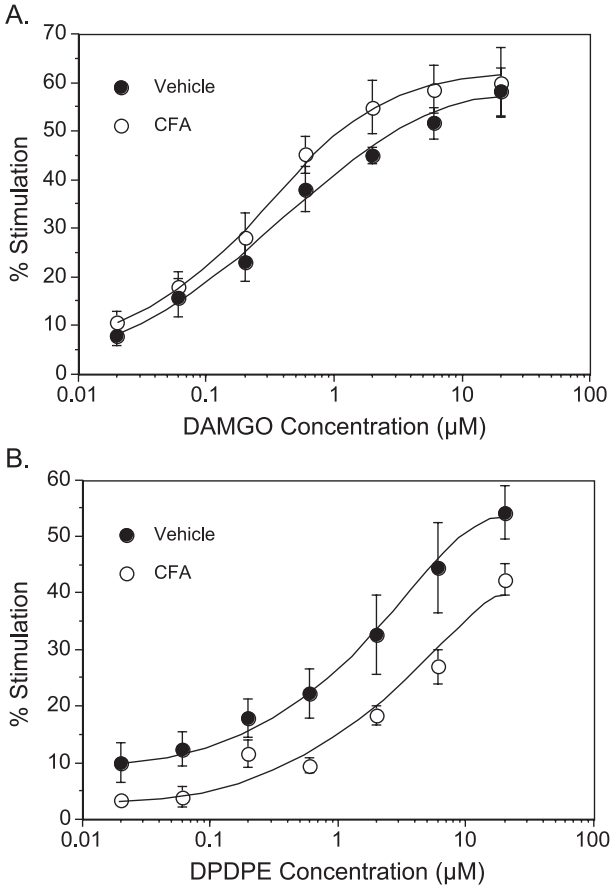

Fig. 2. Concentration–effect curves for (A) DAMGO-stimulated and (B) DPDPE-stimulated [^{35}S]GTPγS binding in spinal cords of vehicle- and complete Freund's adjuvant (CFA)-treated rats. Experiments were conducted using 0.02–20 μM DAMGO or DPDPE in spinal cord membranes. The E_{max} and EC_{50} values calculated from this data are presented in Table 2.

Table 2
E_{max} and EC_{50} values of mu and delta opioid receptor-mediated stimulation of [^{35}S]GTPγS binding in spinal cord membranes

Agonist	Vehicle		Complete Freund's adjuvant	
	E_{max} (% Stim)	EC_{50} (nM)	E_{max} (% Stim)	EC_{50} (nM)
DAMGO	55±3.8	372±161	62±6.5	248±113
DPDPE	53±4.2	1245±504	38±5.1*	1980±645

Values are mean±S.E.M. of 5–6 separate experiments, and were derived from nonlinear regression analyses of the concentration–effect curves shown in Fig. 2.

* $p<0.05$ different from vehicle-treated rats.

4. Discussion

This study investigated the effect of complete Freund's adjuvant-induced arthritis on mu and delta opioid receptor-mediated G-protein activity in brain and spinal cord. Results revealed that delta opioid receptor-stimulated G-protein activity was decreased in the spinal cord of complete Freund's adjuvant-treated rats. No changes were found in mu or delta opioid receptor-mediated G-protein activity in brain or mu-stimulated activity in spinal cord, consistent with reports that opioid receptor levels are not altered in polyarthritic rats (Cesselin et al., 1980; Millan et al., 1986; Delay-Goyet et al., 1989; Spetea et al., 2002). Delta opioid receptor-mediated G-protein activity was decreased despite this lack of change in opioid receptor binding. However, a lack of correlation between receptor levels and activation of G-proteins is not uncommon (Sim-Selley et al., 2000), and might reflect receptor-G-protein uncoupling that can occur in the absence of receptor downregulation. The dynorphin system is upregulated in polyarthritic animals, as indicated by findings that levels and release of prodynorphin-derived peptide immunoreactivity (-ir), as well as prodynorphin gene expression, are increased in the spinal cord of polyarthritic rats (Millan et al., 1986; Hollt et al., 1987; Przewlocka et al., 1992; Pohl et al., 1997; Ballet et al., 2000; Spetea et al., 2002; Cox and Welch, 2004). Although these findings suggest that kappa opioid receptor activity might also be altered in polyarthritic animals, the levels of kappa receptors in rat CNS are very low (Mansour et al., 1988; Besse et al., 1991; Stevens et al., 1991b) and kappa opioid-stimulated [^{35}S]GTPγS binding can not be adequately assessed in the rat model (Childers et al., 1998).

Mu opioid receptor-mediated G-protein activity was not altered in the spinal cord of polyarthritic animals, as previously reported in monoarthritic rats (Zollner et al., 2003). It has therefore been suggested that peripheral opioid receptors might play a primary role in the response to inflammation (Stein et al., 1990). Increases in mu opioid receptor-ir, and mu opioid receptor-mediated G-protein activity have been detected in the dorsal root ganglia after intraplantar injection of complete Freund's

adjuvant or carageen (Ji et al., 1995; Zollner et al., 2003). Similarly, increased delta opioid receptor-ir and mRNA have been detected in the lumbar spinal cord 3 days after intraplantar injection of complete Freund's adjuvant (Cahill et al., 2003). However, these are highly localized models of inflammation in which opioid receptors are assessed within days of treatment. The temporal characteristics of the arthritic model are likely to influence in vitro results. Polyarthritis is characterized by four phases: preclinical (week 1), acute (weeks 2–4), postclinical (weeks 5–8) and recovery (weeks 9–11) (de Castro Costa et al., 1981; Calvino et al., 1987). Animals in the present study were assessed at 19 days, during the peak of the arthritic response. It is possible that different results would be obtained during different stages of the inflammatory process. In fact, delta opioid receptors and met-enkephalin synthesis and release exhibit a biphasic response to intraplantar injection of complete Freund's adjuvant or sciatic nerve constriction, with increases or no change during the early phase of injury and decreased receptor levels and peptide release in the late phase (days 10–14) (Stevens et al., 1991a; Przewlocka et al., 1992). In addition, the present study was conducted in membranes prepared from the whole spinal cord. Opioid receptor density varies in different spinal cord laminae, therefore localized alterations in receptor-mediated G-protein activity might not be detected in the whole spinal cord preparation. Moreover, opioid receptors at specific spinal cord levels could be preferentially affected by chronic inflammation.

The levels of met-enkephalin-ir and proenkephalin mRNA expression are significantly increased in spinal cord tissue from polyarthritic rats (Cesselin et al., 1980; Millan et al., 1986; Pohl et al., 1997; Spetea et al., 2002). In contrast, spinal outflow of met-enkephalin-ir peptides measured in the cerebrospinal fluid is decreased in polyarthritic animals (Bourgoin et al., 1988; Pohl et al., 1997; Ballet et al., 2000; Cox and Welch, 2004). The effect of these alterations on enkephalin levels locally at the delta opioid receptor and their possible contribution to changes in delta opioid receptor-mediated G-protein activity is not clear. Delta opioid receptor desensitization could occur in the spinal cord of polyarthritic rats in response to chronically increased enkephalin levels. Interestingly, delta opioid receptor-ir is colocalized with Met^5-enkephalin-ir in some axon terminals in the superficial layers of the spinal cord, suggesting that delta opioid receptors might act as autoreceptors in this region (Cheng et al., 1995). These data suggest that desensitization of delta opioid receptors might alter regulation of met-enkephalin in primary afferent terminals in polyarthritic animals. This possibility is supported by the finding that the delta agonist (D-Thr^2)-Leu-enkephalin-Thr (DTLET) decreased release of met-enkephalin-ir material in control rats, but enhanced release in polyarthritic rats (Ballet et al., 2000). These data, considered in the context of the present results, suggest that chronic inflammation during polyarthritis alters endogenous delta opioid peptides, leading to desensitization of delta opioid receptors in the spinal cord.

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