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Low doses of cyclic AMP-phosphodiesterase inhibitors rapidly evoke opioid receptor-mediated thermal hyperalgesia in naïve mice which is converted to prominent analgesia by cotreatment with ultra-low-dose naltrexone

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ABSTRACT

Systemic (s.c.) injection in naïve mice of cyclic AMP-phosphodiesterase (cAMP-PDE) inhibitors, e.g. 3-isobutyl-1-methylxanthine [(IBMX) or caffeine, 10 mg/kg] or the more specific cAMP-PDE inhibitor, rolipram (1 μg/kg), rapidly evokes thermal hyperalgesia (lasting >5 h). These effects appear to be mediated by enhanced excitatory opioid receptor signaling, as occurs during withdrawal in opioid-dependent mice. Cotreatment of these mice with ultra-low-dose naltrexone (NTX, 0.1 ng/kg–1 pg/kg, s.c.) results in prominent opioid *analgesia* (lasting >4 h) even when the dose of rolipram is reduced to 1 pg/kg. Cotreatment of these cAMP-PDE inhibitors in naïve mice with an ultra-low-dose (0.1 ng/kg) of the kappa-opioid receptor antagonist, nor-binaltorphimine (nor-BNI) or the mu-opioid receptor antagonist, β-funaltrexamine (β-FNA) also results in opioid analgesia. These excitatory effects of cAMP-PDE inhibitors in naïve mice may be mediated by enhanced release of small amounts of endogenous bimodally-acting (excitatory/inhibitory) opioid agonists by neurons in nociceptive networks. Ultra-low-dose NTX, nor-BNI or β-FNA selectively antagonizes high-efficacy excitatory (hyperalgesic) Gs-coupled opioid receptor-mediated signaling in naïve mice and results in rapid conversion to inhibitory (analgesic) Gi/Go-coupled opioid receptor-mediated signaling which normally requires activation by much higher doses of opioid agonists. Cotreatment with a low subanalgesic dose of kelatorphan, an inhibitor of multiple endogenous opioid peptide-degrading enzymes, stabilizes endogenous opioid agonists released by cAMP-PDE inhibitors, resulting in conversion of the hyperalgesia to analgesia without requiring selective blockade of excitatory opioid receptor signaling. The present study provides a novel pharmacologic paradigm that may facilitate development of valuable non-narcotic clinical analgesics utilizing cotreatment with ultra-low-dose rolipram plus ultra-low-dose NTX or related agents.

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Abbreviations: AC, adenylyl cyclase; β-FNA, β-funaltrexamine; cAMP, cyclic AMP; CTX-B, cholera toxin-B subunit; GM1, monosialoganglioside; IBMX, 3-isobutyl-1-methylxanthine; NLX, naloxone; NTX, naltrexone; nor-BNI, nor-binaltorphimine; PDE, phosphodiesterase; PKA, protein kinase A

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1. Introduction

Collier et al. (1974, 1981) carried out pioneering studies demonstrating that acute systemic injection of *naïve* rodents with a cyclic AMP-phosphodiesterase (cAMP-PDE) inhibitor, e.g. IBMX, theophylline or caffeine, rapidly evokes a "*quasi*-morphine withdrawal syndrome" (including characteristic hyperalgesia) that is remarkably similar to naloxone (NLX)-precipitated excitatory withdrawal effects in chronic morphine-dependent animals. Collier et al. (1981) postulated that the opioid dependence-like effects of administration of these cAMP-PDE inhibitors to naïve mice may be mediated by "hypertrophy of a neuronal cAMP system…[that] releases endogenous opioid". The present study confirms and extends this research by demonstrating that systemic (s.c.) injection in *naïve* mice of IBMX or caffeine (10 mg/kg), as well as a much lower dose (1 μg/kg) of a more specific cAMP-PDE inhibitor, rolipram (e.g. Teixeira et al., 1997), rapidly evokes thermal hyperalgesic effects (lasting >5 h) that appear to be mediated by enhanced excitatory *opioid* receptor signaling, as occurs during withdrawal in opioid-dependent mice (e.g. Crain and Shen, 2007). Furthermore, cotreatment of mice with any one of these cAMP-PDE inhibitors plus ultra-low-dose naltrexone (NTX) (0.1 ng/kg–1 pg/kg, s.c.) results in prominent opioid *analgesia* (lasting >4 h)—even when the dose of rolipram is reduced to as low as 1 pg/kg. Cotreatment with rolipram plus an ultra-low dose (0.1 ng/kg) of the kappa-opioid receptor antagonist, nor-binaltorphimine (nor-BNI) or the mu-opioid receptor antagonist, β-funaltrexamine (β-FNA) also results in prominent opioid analgesia.

Our results suggest that these excitatory effects of cAMP-PDE inhibitors in naïve mice can best be accounted for by cAMP-enhanced release of small amounts of endogenous bimodally-acting (excitatory/inhibitory) opioid agonists by neurons in nociceptive networks in the spinal cord (seeSection 3.1). Cotreatment with ultra-low-dose NTX, nor-BNI or β-FNA selectively antagonizes high-efficacy excitatory (hyperalgesic) Gs-coupled opioid receptor-mediated signaling (Crain and Shen, 1995, 1998a, 2000) activated by the putative cAMP-induced release of endogenous opioid agonists. Cotreatment with ultra-low-dose NTX, nor-BNI or β-FNA results in rapid conversion to inhibitory (analgesic) Gi/Go-coupled opioid receptor-mediated signaling which normally requires activation by much higher doses of opioid agonists (e.g. Shen and Crain, 1997).

Low doses of *exogenous* morphine, dynorphin or other bimodally-acting opioid agonists (0.1–1 μg/kg, s.c.) rapidly evoke prominent opioid receptor-mediated thermal hyperalgesia in naïve mice, lasting for several hours (Crain and Shen, 2001; Shen and Crain, 2001). Cotreatment of these mice with ultra-low-dose NTX (0.1 ng/kg) blocks this hyperalgesia and results in rapid conversion to potent analgesia lasting >3 h (Crain and Shen, 2001). These correlative data provide a pharmacologic bioassay which may help to estimate the amount of *endogenous* opioid-agonist release that could account for the hyperalgesia observed in the present study of cAMP-PDE inhibitors in *naive* mice (see also Crain and Shen, 2007).

Opioid excitatory effects are also selectively blocked by cholera toxin-B subunit (CTX-B), which binds specifically to a putative allosteric GM1 ganglioside-regulatory site on excitatory Gs-coupled opioid receptors (Crain and Shen, 1998a, b; Shen and Crain, 1990b, 2001; see also Wu et al., 1997, 1998). Cotreatment of cAMP-PDE inhibitors with CTX-B also

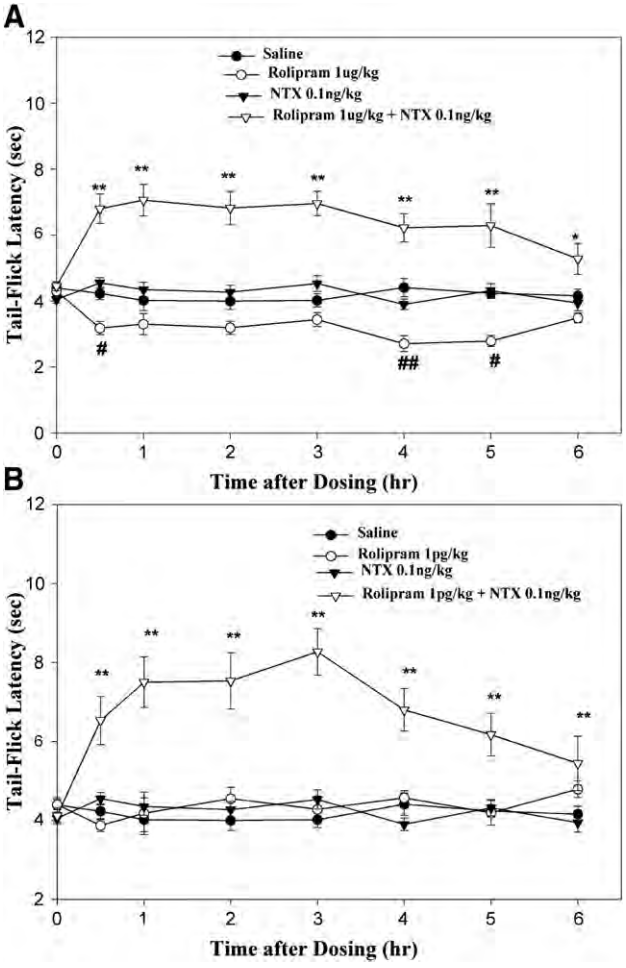

Fig. 1 – Presented are time-effect curves of warm-water-immersion (52 °C) tail-flick tests. A shows cotreatment of naïve mice with ultra-low-dose NTX blocks the acute hyperalgesic effects of a low (1 μg/kg) dose of a specific Type IV cAMP-PDE inhibitor, rolipram (A, curve ○) and results in prominent opioid analgesia (A, curve ▽). Control tests with saline (A, curve ●) or ultra-low-dose NTX (0.1 ng/kg) alone (A, curve ▼) show no detectable effect in all of the tail-flick assays in the present and previous studies (see data in Crain and Shen, 1995, 2001, 2007). Asterisks in A indicate statistically significant differences between time points on curve ● vs. curve ▽ (P<0.01; *P<0.05). Symbol # indicates statistically significant differences between time points on A, curve ● vs. curve ○ (## P<0.01, # P<0.05). (B): A remarkably similar degree of analgesia was elicited by cotreatment with ultra-low-dose NTX (0.1 ng/kg) plus a *million*-fold lower dose of rolipram (1 pg/kg) (B, curve ▽). By contrast, saline (B, curve ●) or NTX 0.1 ng/kg (B, curve ▼), or 1 pg/kg rolipram (B, curve ○) had no significant hyperalgesic effect when administered alone. Asterisks indicate statistically significant differences between time points on curve ● vs. curve ▽ (* P<0.05, **P<0.01). Note: all drugs were injected subcutaneously. In all figures, *n*=8 for each curve, error bars indicate S.E.M.**

selectively blocks excitatory opioid receptor-mediated signaling and results in rapid conversion to inhibitory opioid analgesia. Furthermore, cotreatment with a low subanalgesic dose of kelatorphan (Fournie-Zaluski et al., 1984), an inhibitor of multiple endogenous opioid peptide-degrading enzymes, appears to stabilize endogenous excitatory amounts of opioid agonists released by low doses of cAMP-PDE inhibitors, resulting in conversion of the usual hyperalgesia to prominent analgesia without requiring selective blockade of excitatory opioid receptor signaling.

The present results provide a novel pharmacologic paradigm that may facilitate development of valuable non-narcotic clinical analgesics utilizing, for example, cotreatment with ultra-low-dose rolipram plus ultra-low-dose NTX.

2. Results

2.1. *Cotreatment of naive mice with a low, or even subthreshold, hyperalgesic dose of a cAMP-PDE inhibitor plus ultra-low-dose NTX results in rapid conversion to prominent opioid analgesia*

Administration of a low (1 μg/kg) dose of a specific cAMP-PDE inhibitor, rolipram, to naïve mice resulted in long-lasting hyperalgesia (Fig. 1A: $P<0.01$ for curve ○ vs. ●). Cotreatment with ultra-low-dose NTX blocked rolipram-evoked hyperalgesia and resulted in rapid conversion to prominent opioid analgesia (Fig. 1A: $P<0.01$ for curve ▽ vs. ●). A remarkably similar degree of analgesia was elicited by cotreatment with ultra-low-dose NTX plus a *million*-fold lower dose of rolipram (1 pg/kg) (Fig. 1B: $P<0.01$ for curve ▽ vs. ●). By contrast, the same ultra-low dose of rolipram did not significantly alter baseline tail-flick latencies when administered alone (Fig. 1B: curve ○). Administration of a second dose of 0.1 ng/kg NTX *alone* to the same group of mice that were used in the experiment shown in Fig. 1A, at 24 h after the initial cotreatment, evoked significant analgesia, indicating that the single low dose of rolipram (1 μg/kg) was still effective for more than 1 day (data not shown).

Preliminary nociceptive tail-flick assays were carried out on naïve mice with less specific cAMP-PDE inhibitors: IBMX and caffeine (Collier et al., 1974, 1981; Arnold et al., 1982). Each of these agents (tested at 10 mg/kg, s.c.) elicited similar, but weaker, hyperalgesia in comparison to treatment with 1 μg/kg rolipram. Nevertheless, cotreatment with 0.1 ng/kg NTX resulted in rapid conversion to remarkably prominent analgesia as observed in the present tests with rolipram (data not shown).

2.2. *Cotreatment of naïve mice with an ultra-low dose of a selective kappa- or mu-opioid receptor antagonist plus a hyperalgesic dose of rolipram results in prominent opioid analgesia*

In order to determine the specific types of receptors that may mediate the opioid analgesia elicited by cotreatment with rolipram plus ultra-low-dose NTX (Figs. 1A, B), comparative tests were carried out with rolipram (1 μg/kg) plus either an ultra-low dose (0.1 ng/kg) of the kappa-opioid receptor antagonist, nor-BNI or the mu-opioid receptor antagonist, β-FNA. Both antagonists were quite effective, although the endogenous opioid analgesia unmasked by nor-BNI was much larger in magnitude and lasted >6 h (Fig. 2: $P<0.01$, for curves ▼ and ▽ vs. curve ● for saline).

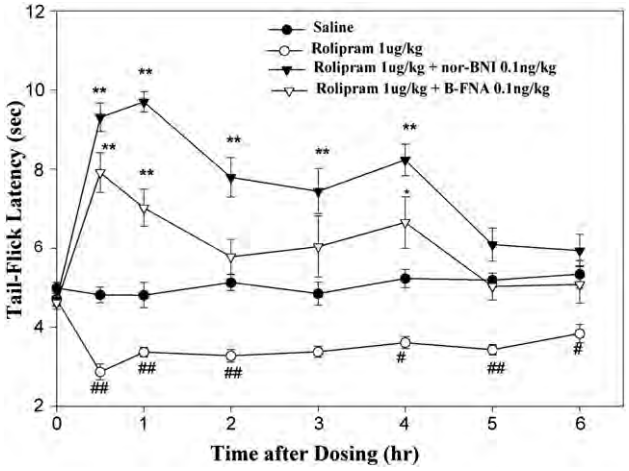

Fig. 2 – Cotreatment of mice with an ultra-low dose of specific mu- or kappa-opioid receptor antagonists also blocks the acute hyperalgesic effects of rolipram and unmasks prominent opioid analgesia. Cotreatment with the kappa-opioid receptor antagonist, nor-binaltorphimine (nor-BNI, 0.1 ng/kg) unmasks a larger magnitude of analgesia (curve ▼) than does a similar dose of the mu-opioid receptor antagonist, β-funaltrexamine (β-FNA) (curve ▽), although both antagonists produce analgesic effects lasting >4 h, comparable to cotreatment with ultra-low-dose NTX (cf. Fig. 1A). Control tests with 0.1 ng/kg nor-BNI or β-FNA alone do not significantly alter baseline tail-flick latencies (data not shown; as occurs with 0.1 ng/kg NTX, e.g. Fig. 1A: curve ▼). Asterisks indicate statistically significant difference between time points on curve ● vs. curve ▼ or curve ▽ ($P<0.01$; *$P<0.05$). Symbols # indicate statistically significant difference between time points on curve ● vs. curve ○ (## $P<0.01$, # $P<0.05$).**

2.3. *Cotreatment of naïve mice with a low dose of CTX-B plus ultra-low-dose rolipram also results in opioid analgesia that is blocked by delayed injection of high-dose NTX*

Cotreatment of mice with 1 pg/kg rolipram plus another selective blocker of excitatory opioid receptor signaling, CTX-B (10 μg/kg) (see Section 1) also resulted in prominent opioid analgesia (Fig. 3A: $P<0.01$ for curve ▽ vs. ●). Administration of CTX-B alone had no effect on baseline tail-flick latency (Fig. 3A, curve ○). In other mice cotreated with rolipram (1 μg/kg) plus CTX-B (10 μg/kg), the prominent unmasked analgesia (Fig. 3B: curve ▼) was rapidly blocked by delayed injection of high-dose NTX (1 mg/kg) at 3 h after initial drug treatment (arrow near curve ▼ in Fig. 3B). This result provides further evidence that the analgesia unmasked by CTX-B is mediated by endogenous inhibitory opioid receptor signaling.

2.4. *Cotreatment of naïve mice with a low dose of kelatorphan stabilizes endogenous opioid agonists released by ultra-low-dose rolipram and results in rapid onset of analgesia*

In order to provide a more direct test of our pharmacological evidence suggesting that ultra-low-dose rolipram can elicit release of endogenous opioid agonists, mice were cotreated with low doses of kelatorphan, a highly efficient inhibitor of multiple endogenous opioid peptide-degrading enzymes (Fournie-Zaluski et al., 1984). Previous studies showed that doses of kelatorphan as low as 2.5 mg/kg (i.v.) elicited potent NLX-reversible antinociceptive effects in normal rats, evaluated by vocalization responses to paw pressure (Kayser et al., 1989). In the present study, administration of much lower doses of kelatorphan, 0.1–1 mg/kg (s.c.) to mice did not alter the baseline tail-flick latency in our tail-flick assay (Fig. 4, curves ▼ and ▽). Ultra-low-dose rolipram alone (1 pg/kg, s.c.) was also ineffective (Fig. 4, curve ○; see also Fig. 1B: curve ○). By contrast, cotreatment of 0.1–1 mg/kg kelatorphan plus 1 pg/kg rolipram evoked prominent dose-dependent analgesia (Fig. 4: $P<0.01$ for curves ● and □ vs. rolipram-alone curve ○ or kelatorphan-alone curves ▼ and ▽).

3. Discussion

The present study in mice utilized subcutaneous injections of ultra-low-dose NTX and other agents with warm-water-immersion tail-flick assays (as in many of our previous studies, e.g. Crain and Shen, 1995, 2000, 2001, 2004, 2007). The reliability and the validity of this antinociceptive assay and its specificity for analyzing opioid receptor signaling functions have been confirmed by elegant studies utilizing intra-spinal infusion of ultra-low-dose NTX in morphine-treated rats (Powell et al., 2002; Abul-Husn et al., 2007) and correlative biochemical analyses of ultra-low-dose NLX in morphine-treated rats (Wang et al., 2005; Tsai et al., personal communication see also, Tsai et al., in press). The results of our assays have also guided clinical applications in treating chronic pain patients (Chindalore et al., 2005; Cruciani et al., 2003; see also reviews in Crain and Shen, 2000; Sloan and Hamann, 2006).

3.1. *Endogenous opioid analgesia elicited by cotreatment with a cAMP-PDE inhibitor plus ultra-low-dose NTX*

The prominent thermal hyperalgesia evoked in the present study by injection of naive mice (s.c.) with rolipram (1 μg/kg), as well as with IBMX or caffeine (10 mg/kg), is consonant with previous studies that demonstrated mechanical hyperalgesia in rats following peripheral administration of cAMP-PDE inhibitors which elevate cAMP in primary sensory neurons (Cunha et al., 1999; Ouseph et al., 1995) or by infusion of 8-br-cAMP directly into the dorsal spinal cord (Sluka, 1997; see also Taiwo and Levine, 1991; Taiwo et al., 1989). However, this is the first demonstration that *opioid* receptor-mediated *analgesia* can be elicited in *naïve* mice by cotreatment of low hyperalgesic or subthreshold excitatory doses of rolipram or other cAMP-PDE inhibitors with ultra-low-dose NTX.

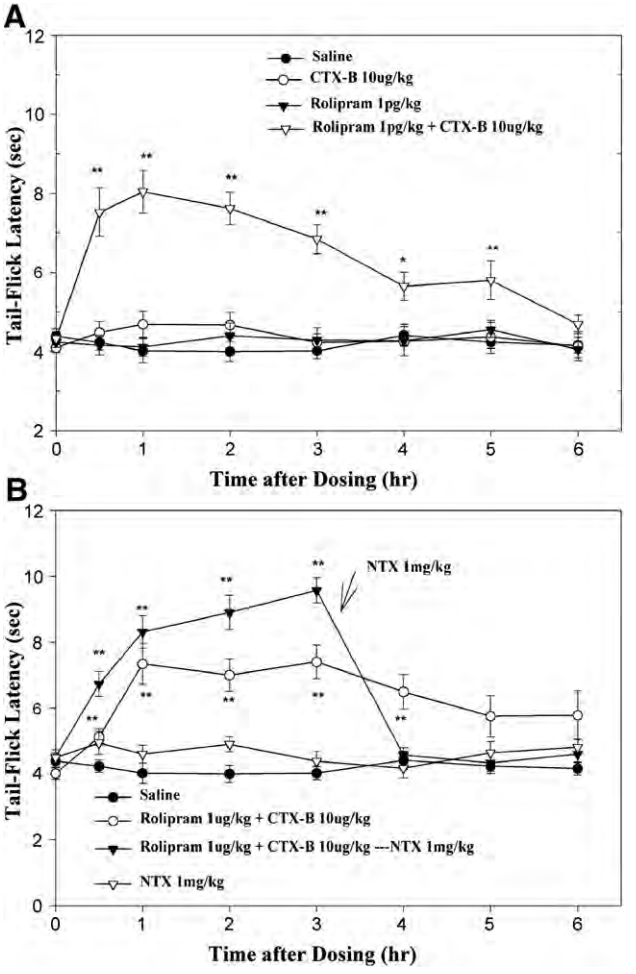

Fig. 3 – Cotreatment of mice with a low dose of rolipram plus CTX-B also results in prominent opioid analgesia which can be rapidly blocked by high-dose NTX. (A) Cotreatment with an ultra-low-dose of rolipram (1 pg/kg) plus a low dose of CTX-B (10 μg/kg) elicits prominent analgesia lasting >5 h (A, curve ▽), as occurs after cotreatment with ultra-low-dose NTX (Fig. 1A, B). Control test with saline (A, curve ●) or rolipram 1 pg/kg alone (A, curve ▼) does not alter baseline tail-flick latency, nor does CTX-B alone (A, curve ○). Asterisks indicate statistically significant difference between time points on curve • vs. curve ▽ ($P<0.01$; *$P<0.05$). (B): Analgesia unmasked by cotreatment with a hyperalgesic dose of rolipram (1 μg/kg) plus CTX-B is rapidly blocked by delayed injection of high-dose NTX (1 mg/kg) at 3 h after initial cotreatment (see B, arrow near curve ▼).**

As noted in Section 1, the excitatory *opioid* receptor-mediated hyperalgesic effects evoked by injection of cAMP-PDE inhibitors in *naïve* mice can best be accounted for by cAMP-enhanced release of small amounts of endogenous bimodally-acting opioid agonists by neurons in nociceptive networks in the spinal cord. This view is supported by our present evidence that kelatorphan, a specific inhibitor of endogenous opioid peptide-degrading enzymes (Fournie-Zaluski et al., 1984; Kayser et al., 1989) stabilizes putative endogenous opioid agonists released by cAMP-PDE inhibitors resulting in rapid onset of analgesia (see Section 3.4).

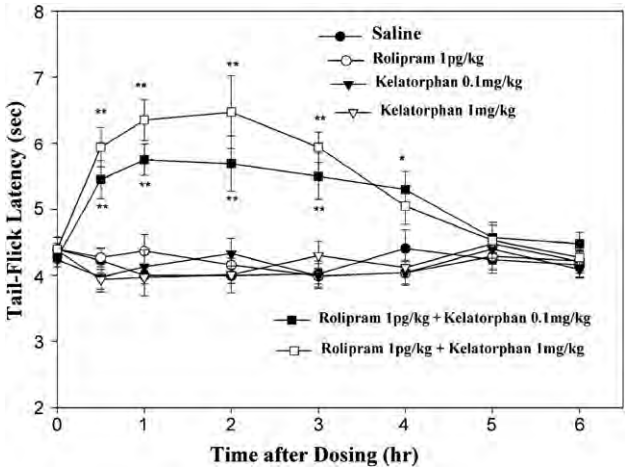

Fig. 4 – Cotreatment of mice with a low, subanalgesic dose of kelatorphan, an inhibitor of multiple endogenous opioid peptide-degrading enzymes, stabilizes putative endogenous opioid agonists released by ultra-low-dose of rolipram, resulting in rapid onset of analgesia. Control test with saline (curve ●) or low-dose rolipram (1 pg/kg) alone (curve ○) does not alter baseline tail-flick latency, nor does administration of kelatorphan (1 mg/kg) alone (curve ▽). By contrast, cotreatment with 1 pg/kg rolipram plus 1 mg/kg kelatorphan elicits prominent analgesia (curve □), even when the dose of kelatorphan is reduced to 0.1 mg/kg (curve ■). Asterisks indicate statistically significant difference between time points on curve ● vs. curve ■ or curve □ (*P<0.05, **P<0.01).

Furthermore, Arnold et al. (1982) demonstrated that intra-atrial infusion of caffeine (20 mg/kg) in naïve rats results in a rapid and sustained (2.5 h) increase in endogenous plasma β-endorphin levels. Similar biochemical assays are needed to confirm the putative release of endogenous opioid agonists by administration of rolipram to naïve mice.

Increased release of endogenous opioid agonists, as well as other excitatory transmitters, from primary afferent neurons may be mediated by activation of high-efficacy excitatory Gs-coupled opioid receptors linked to K^+ and Ca^{2+} channels via adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) transduction cascades that result in augmented Ca^{2+}-dependent neurotransmitter release (Crain and Shen, 1990, 1992, 1998a, 2000; Shen and Crain, 1989, 1990a, 1994; Suarez-Roca and Maixner, 1993, 1995). These cAMP-stimulated excitatory opioid receptor-mediated hyperalgesic effects can then be selectively blocked by ultra-low-dose NTX, resulting in conversion to inhibitory opioid analgesia. Furthermore, recent studies by Tsai et al. (in press, personal communication) have provided the *first direct biochemical evidence* that ultra-low-dose naloxone injection in morphine-treated rats not only rapidly blocks Gs-coupled opioid receptors, but also evokes an *acute* action on Gi mRNA expression that results in a rapid increase in Gi protein synthesis in the spinal cord. This dual Gs/Gi "switching" effect of ultra-low-dose naloxone provides a novel biochemical mechanism that elegantly confirms our present and previous pharmacologic tail-flick assays demonstrating rapid conversion of endogenous (as well as low-dose exogenous) opioid receptor-mediated hyperalgesia to analgesia by acute cotreatment with ultra-low-dose naltrexone (see also Tsai et al., in press). cAMP-evoked opioid excitatory effects may also account for previous evidence that application of cAMP-PDE inhibitors or forskolin to neurons in nociceptive networks counteracts inhibitory Gi-coupled opioid receptor-mediated signaling (Crain et al., 1986; Hosford and Haigler, 1981; Jurna, 1984; Shen and Crain, 1989; see also Ho et al., 1973).

3.2. *Endogenous kappa or mu-opioid analgesia following cotreatment with rolipram plus ultra-low-dose nor-BNI or β-FNA*

Our evidence that low-dose rolipram elicits thermal hyperalgesia in naive mice that can be converted to prominent analgesia by cotreatment with ultra-low-dose nor-BNI or β-FNA (Fig. 2) suggests that rolipram-induced hyperalgesia may be due, at least in part, to the release of endogenous *kappa*- and *mu*-opioid agonists that activate excitatory kappa and mu-opioid receptor-mediated signaling of neurons in nociceptive systems. There appears to be abundant releasable endogenous kappa-opioid agonists, e.g. dynorphin, in the dorsal roots as well as the perikarya of primary afferent neurons (Basbaum et al., 1986; Shen and Crain, 1994). Dynorphin has been shown to have complex pronociceptive (Wang et al., 2001), as well as antinociceptive, effects. However, recent studies provide strong evidence that *endogenous* kappa opioids play a major role in mediating spinal analgesia (Cheng et al., 2002; see also Fei et al., 1987; Han and Xie, 1984). Further studies are required to determine if delta or other types of endogenous opioid agonists, e.g. β-endorphin (Arnold et al., 1982; Loh et al., 1976; Tseng, 1995; Waldhoer et al., 2004), may also play roles in mediating hyperalgesia evoked by cAMP-PDE inhibitors in naïve mice.

3.3. *Endogenous opioid analgesia elicited by cotreatment with rolipram plus CTX-B*

As noted in Section 1, opioid excitatory effects are selectively blocked by CTX-B which binds specifically to a putative allosteric GM1 ganglioside-regulatory site on excitatory Gs-coupled opioid receptors (Crain and Shen, 1998a,b; Shen and Crain, 1990b; see also Wu et al., 1997, 1998). The unmasking of prominent opioid analgesia in naïve mice cotreated with ultra-low-dose rolipram plus CTX-B (Fig. 3A) is therefore in good agreement with similar results obtained in mice cotreated with ultra-low-dose rolipram plus ultra-low-dose NTX, nor-BNI or β-FNA (Figs. 1A, B, Fig. 2). Furthermore, the rapid block of this CTX-B-unmasked analgesia by delayed injection of high-dose NTX (1 mg/kg) at 3 h after initial drug cotreatment (Fig. 3B) provides additional evidence that this analgesia is mediated by endogenous inhibitory opioid receptor signaling.

3.4. *Effects of kelatorphan cotreatment provide support for release of endogenous opioid agonists by cAMP-PDE inhibitors*

Our pharmacologic nociceptive bioassays suggest that ultra-low-dose rolipram elicits release of small amounts of endogenous bimodally-acting opioid agonists. These results are supported by the present evidence that cotreatment of mice with a low *sub*analgesic dose of kelatorphan, an inhibitor of

multiple endogenous opioid peptide-degrading enzymes (Fournie-Zaluski et al., 1984; Kayser et al., 1989), stabilizes excitatory amounts of endogenous opioid agonists released by an ultra-low dose of rolipram, resulting in rapid onset of analgesia (Fig. 4). In the presence of kelatorphan, sufficient quantities of these endogenous opioid agonists are prevented from rapid metabolic degradation so that analgesic responses can be evoked without requiring selective blockade of excitatory opioid receptor signaling.

3.5. *Remarkable potency of ultra-low-dose rolipram in activating excitatory opioid receptor signaling*

The potency of ultra-low doses of rolipram in activating excitatory opioid receptor signaling in naive mice that can be converted to endogenous opioid analgesia by cotreatment with ultra-low-dose NTX or CTX-B is surprising. The "pivotal role of cyclic AMP in the sensitization of the primary sensory neurons" (Cunha et al., 1999) has been demonstrated in many hyperalgesia studies (e.g. Ferreira and Nakamura, 1979; Taiwo and Levine, 1991; Taiwo et al., 1989; see also Collier et al., 1974, 1981). The type IV cAMP-PDE isoenzyme appears to be the most relevant enzyme in cAMP regulation in cells involved in hyperalgesic processes (Teixeira et al., 1997; see also Houslay et al., 2005). Behavioral studies have shown that administration of moderately low systemic doses of rolipram (ca. 1 μg/kg) in rodents and humans result in significant enhancement in memory, although aversive side-effects in the dose-range tested appear to have impeded clinical applications (Bach et al., 1999; Barad et al., 1998; see also Houslay et al., 2005). The present demonstration of prominent endogenous opioid analgesia elicited by a *million*-fold lower dose of rolipram (ca. 1 pg/kg) in mice cotreated with ultra-low-dose NTX may provide significant insights into cellular mechanisms underlying endogenous opioid analgesia and cAMP-mediated modulations of other CNS functions.

Maintenance of opioid receptor-mediated hyperalgesia for many hours after injection of cAMP-PDE inhibitors in the present study is interesting in view of the relatively rapid metabolic degradation of the small quantities of endogenous opioid peptide agonists (e.g. Roques, 2000; Roques and Noble, 1995; Roques et al., 1999) that appear to be released so as to activate excitatory opioid receptor signaling in these mice. Pharmacokinetic studies indicate that the half-life of rolipram in plasma after systemic administration may be at least several hours (Krause et al., 1990). Furthermore, rolipram-induced elevation of cAMP may increase the efficacy and duration of the release of endogenous opioid agonists as a result of a cAMP/PKA-dependent glycosyltransferase (Scheideler and Dawson, 1986), positive-feedback phosphorylation cycle (Crain and Shen, 1992) that prolongs activation of excitatory GM1-regulated, Gs-coupled opioid receptors (Crain and Shen, 1998a,b; Wu et al., 1997, 1998).

3.6. *Clinical application of endogenous opioid analgesic preparations*

Systemic injection with inhibitors of multiple endogenous opioid peptide-degrading enzymes, e.g. kelatorphan (Fournie-Zaluski et al., 1984), which attenuate rapid enzymatic degradation of opioid peptides released in the CNS, elicits potent NLX-reversible antinociceptive effects (Kayser et al., 1989; Le Guen et al., 2003; Roques and Noble, 1995). These results indicate that clinically significant analgesia may be produced by elevation of the extracellular levels of endogenous opioid agonists released either tonically or following stimulus-evoked depolarization of neurons in nociceptive systems (Noble and Roques, 2007; Roques, 2000; Roques and Noble, 1995; Roques et al., 1999).

The present study introduces a related pharmacologic paradigm for development of valuable endogenous opioid analgesic preparations utilizing remarkably low doses of rolipram (Houslay et al., 2005) or other cAMP-PDE inhibitors. Systemic administration of these specific cAMP-PDE inhibitors in naïve mice appears to enhance the release of relatively small quantities of *endogenous* bimodally-acting *opioid agonists* that elicit excitatory opioid receptor-mediated signaling. These excitatory effects can then be converted to inhibitory opioid receptor-mediated analgesia simply by cotreatment of these naïve mice with innocuous doses of NTX or other selective blockers of excitatory opioid receptor signaling (Crain and Shen, 1995, 2000; Shen and Crain, 2001).

It should be emphasized that the nociceptive tail-flick assays utilized in the present study provide an animal model that focuses on spinal cord mechanisms, as in our previous studies demonstrating the "paradoxical" effects of ultra-low-dose NTX on morphine-treated mice (Crain and Shen, 1995; Shen and Crain, 1997). Further studies are required to evaluate the degree to which cotreatment with cAMP-PDE inhibitors plus ultra-low doses of NTX or other opioid receptor antagonists may evoke similar, as well as much more complex, endorphinergic-mediated effects on brainstem or other supraspinal centers (e.g. Schaible, 2007; see also Loh et al., 1976; Bloom et al., 1976; Tseng, 1995). Nevertheless, the mouse spinal cord model used in our present and previous studies may provide valuable clues to guide improved treatment of pain (Crain and Shen, 2000), as already observed in clinical trials of ultra-low-dose NTX cotreatment of chronic pain patients with various opioid agonists (Chindalore et al., 2005; Cruciani et al 2003; Sloan and Hamann, 2006). It will be of great interest to determine if clinical application of the present novel non-narcotic pharmacologic paradigm may result in maintenance of a significant degree of endogenous opioid analgesia during ultra-low-dose rolipram plus ultra-low-dose NTX cotreatment of pain patients with minimal tolerance/dependence liability and *extremely* low risk of aversive side-effects.

4. Experimental procedures

The protocols of this research project including the care and humane use of the mice have been approved by the Animal Institute Committee at the Albert Einstein College of Medicine, in accordance with the Guide adopted by the U.S. National Institutes of Health.

4.1. *Animal test groups*

Swiss–Webster male mice (20–25 g, Charles River, NY) were housed in groups of five, maintained on a 12 h light/dark

cycle, and provided water and food *ad libitum* for 1–3 days prior to antinociception tests. Comparative tests were generally carried out on the same day with two or more groups of 8 mice receiving a specific cotreatment plus an appropriate control group of 8 mice. All animal test groups were used for only one assay.

4.2. *Antinociception and hyperalgesia assays in mice*

Nociceptive and antinociceptive effects in mice were measured using a warm-water-immersion tail-flick assay similar to methods previously described (Crain and Shen 1995, 2000, 2001, 2007; Shen and Crain, 1997, 2001). Each mouse was permitted to enter a tapered plastic cylinder (with air holes). The cylinder was slightly larger than the body size, with the tail freely hanging outside the cylinder. The cylinder provided a secluded environment into which the animals voluntarily enter. During the tail-flick assay the cylinder was handled without direct contact with the animal. One-third of the tail from the tip was immersed into a water-bath maintained at 52 °C (±0.1°) with an electronic thermoregulator (Yellow Springs). The latency to a rapid tail-flick was recorded and the mouse was returned to its cage during the period between tests. Mice with control latencies >6 s were excluded from these tests and a 10 s cutoff was used to minimize tissue damage. Five sequential control tests were made, each with a 10 min interval. The latencies of the last 4 tests were averaged to provide a pre-drug value. Time-effect curves were plotted using tail-flick latencies as the ordinate. Experimentally induced increases in control tail-flick latency provide a measure of antinociceptive or analgesic effects, whereas decreases in tail-flick latency indicate hyperalgesic effects (Crain and Shen, 2001; Shen and Crain, 2001).

4.3. *Statistical analyses*

Differences between treatment groups were examined for statistical significance by means of ANOVA with Neuman–Keuls tests (Tallarida and Murray, 1987). *P* values of less than 0.05 were considered to be statistically significant.

4.4. *Materials*

The following drugs were used: 3-isobutyl-1-methylxanthine (IBMX), caffeine, rolipram, naltrexone (NTX), nor-binaltorphimine (nor-BNI), β-funaltrexamine (β-FNA) (Sigma, St. Louis, MO); cholera toxin B (List); and kelatorphan (a gift from Dr. Bernard Roques via Dr. Eric Simon).

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