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UPREGULATION OF PRONOCICEPTIVE MEDIATORS AND DOWNREGULATION OF OPIOID PEPTIDE BY ADRENOMEDULLIN FOLLOWING CHRONIC EXPOSURE TO MORPHINE IN RATS

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Abstract—Adrenomedullin (AM) belongs to a calcitonin gene-related peptide (CGRP) family and has been demonstrated to recruit CGRP following chronic use of morphine and neuronal nitric oxide synthase (nNOS) in inflammation. The present study investigated the possibility that AM initiates the changes of other molecules contributing to the development of morphine tolerance in its chronic use. Intrathecal (i.t.) co-administration of the AM receptor antagonist $AM_{22–52}$ (35.8 μg) inhibited tolerance to morphine-induced analgesia while a daily injection of the AM receptor agonist $AM_{1–50}$ (8 μg, i.t., bolus) for 9 days induced a decrease in the potency of morphine analgesia and thermal hyperalgesia. Persistent exposure of cultured dorsal root ganglion (DRG) explants to morphine (3.3 μM) for 4 days resulted in an increase in AM and CGRP mRNA levels. However, morphine failed to produce these effects in the presence of $AM_{22–52}$ (2 μM). The i.t. administration of morphine for 6 days increased the expression of nNOS in the spinal dorsal horn and DRG neurons but decreased expression of the endogenous opioid peptide bovine adrenal medulla 22 (BAM22) in small- and medium-sized neurons in DRG. Particularly, the co-administration of $AM_{22–52}$ (35.8 μg) inhibited the morphine-induced alterations in nNOS and BAM22. These results indicated that the increase in nNOS and CGRP expressions and the decrease in BAM22 were attributed to the increased AM receptor signaling induced by chronic morphine. The present study supports the hypothesis that the enhancement of AM bioactivity triggered upregulation of pronociceptive mediators and downregulation of pain-inhibiting molecule in a cascade contributing to the development of morphine tolerance.

Key words: adrenomedullin (AM), bovine adrenal medulla 22 (BAM22), calcitonin gene-related peptide (CGRP), morphine tolerance, neuronal nitric oxide synthase (nNOS).

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Abbreviations: AM, adrenomedullin; BAM22, bovine adrenal medulla 22; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; DRG, dorsal root ganglion; i.t., intrathecal; nNOS, nitric oxide synthase; PB, phosphate buffer; PBS, phosphate-buffered saline; RAMP2 or RAMP3, receptor activity-modifying protein 2 or 3; TFL, tail flick latency.

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INTRODUCTION

Adrenomedullin (AM) is a member of the calcitonin gene-related peptide (CGRP) family which also includes calcitonin, CGRP, intermedin/AM2 and amylin. AM was discovered in 1993 in human pheochromocytoma tissue extracts (Kitamura et al., 1993). It is a 52-amino acid peptide and induces biological activity by binding to the receptor complex which is calcitonin receptor-like receptor (CLR) associated with receptor activity-modifying protein 2 or 3 (RAMP2 or RAMP3) (Poyner et al., 2002). This peptide is abundantly expressed in the vascular endothelium and smooth muscle cells (Kato et al., 1995) and exerts a potent hypotensive effect as a vasodilator (Brain and Grant, 2004). AM is broadly distributed in the central nervous system (Shan and Krukoff, 2001) and acts as an important neuromodulator involved in the maintenance of homeostasis (Brain and Grant, 2004). AM and its receptor components are also expressed in superficial layers of the spinal cord and dorsal root ganglia (DRG) (Ma et al., 2006; Hong et al., 2009). AM is an important mediator for pathological pain as its expression in these key structures for pain processing is enhanced in acute (Ma et al., 2006) and chronic (Hong et al., 2009) inflammation and inhibition of AM receptor signaling by intrathecal (i.t.) administration of the selective AM receptor antagonist $AM_{22–52}$ (Hay et al., 2003) relieves inflammatory pain. AM has also been shown to be involved in the development of opioid tolerance (Hong et al., 2010; Wang et al., 2011a).

Opioids remain the most effective analgesics for the treatment of severe pain in the clinic (Arner et al., 1988). However, repeated administration of opiates results in analgesic tolerance and paradoxical pain sensitization (Chang et al., 2007). Hence, it is wise to inhibit opioid tolerance to improve pain management. We have observed that AM expression is greatly increased in DRG and the spinal dorsal horn following chronic exposure to morphine and i.t. $AM_{22–52}$ prevents (Hong et al., 2010) and reverses (Wang et al., 2011a) tolerance to morphine analgesia as well as its associated hyperalgesia. The mechanism may involve the enhancement of AM-evoked nociceptive activity in DRG and the spinal cord (Ma et al., 2006; Hong et al., 2009) which is presumed to counteract the antinociceptive effect of morphine, similar to other pronociceptive mediators (King et al., 2005). Interestingly, a single injection of morphine at a moderate dose also evokes enhanced AM activity reducing morphine-induced analgesia (Wang et al.,

2014). This finding implies that the development of morphine tolerance can be attributed to the accumulation of increased AM that is released following each administration of morphine. Given that it can recruit CGRP following chronic morphine (Hong et al., 2010) and initialize a nNOS/CGRP cascade in cultured DRG explants (Wang et al., 2013), AM must be one of the key molecules contributing to morphine tolerance. The present study was designed to examine the hypothesis that the peptide AM is one of the upstreams in a cascade that occurs in chronic morphine. As the opioid peptide bovine adrenal medulla 22 (BAM22) is involved in the development of morphine tolerance (Jiang et al., 2006; Chen et al., 2008), effect of inhibition of AM receptor signaling on morphine-induced change of BAM22 was also determined.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (250–320 g; Animal Center of Fujian Medical University, Fuzhou, China) were housed individually at 22 °C with 50 % humidity under a 12-h light/dark cycle and were given free access to food and water. The experimental procedures described in the present study were approved by the animal care committee at the Fujian Normal University and the guidelines for the treatment of animals were in accordance with the International Association for the Study of Pain. All efforts were made to minimize animal suffering and the number of animals.

I.t. catheter implantation

For the spinal administration of drugs to the animal, an i.t. catheter was implanted in the subarachnoid space as described before (Yaksh and Rudy, 1976). Under phenobarbital (50-mg/kg, i.p.) anesthesia, a polyethylene catheter (PE-10 tubing, Stoelting, Wood Dale, IL, USA) was inserted through a small hole made in the atlanto-occipital membrane and threaded 8–8.5 cm down the i.t. space to the lumbar enlargement level of the spinal cord. The rostral part was sutured to the muscle to immobilize the catheter and the wound was closed in two layers with 4-0 silk suture thread. After surgery, the rats were housed individually, had free access to food and water and were allowed to recover for at least 7 days before habituation and behavioral testing. Only animals with no evidence of neurological deficits after catheter placement were used in this study. The proper location of the catheter was confirmed by assessing sensory and motor blockade after i.t. injection of 10 μl of 2% lidocaine (Shengong, Shanghai, China). At the end of the study, location of the catheter was examined by postmortem dissection.

The chronic morphine protocol consisted of administration of morphine hydrochloride (Northeast Pharmaceutical Group, Shenyang, China) at a dose of 20 μg (i.t.) per day for 6 days to each rat. This protocol has been proven to generate tolerance to morphine antinociception (Mao et al., 1994; Cai et al., 2007b). I.t. injection of $AM_{22–52}$ (AM receptor antagonist) or $AM_{1–50}$ (AM receptor agonist, Huadatianyuan Biological Co., Shanghai, China) was given at various times. All the drugs and vehicle were administered under conscious condition.

Assessment of nociceptive behavior

Tail flick latency (TFL) was determined by the radiant heat tail flick assay using a Tail Flick Meter (IITC Life Science Inc., CA, USA). Radiant heat was focused on the underside of the tail 3 cm from its distal end and tail flick latency automated by equipment with 0.01-s precision. Radiant heat intensity was adjusted to produce on average a baseline of 2–3 s (high intensity) or 7.0–9.0 s (low intensity) in naive rats. The cutoff latency was established at 10 or 20 s to prevent possible tissue damage. TFL at any test time point was measured three times at 2-min intervals and the mean value of these measurements was taken. The investigator was blind to the test drug conditions.

TFL in the pain test was converted to a percentage of maximum possible effect (%MPE) using the following formula:

$$\%\text{MPE} = ([\text{post-drug latency} - \text{baseline latency}]/[\text{cut-off time} - \text{baseline latency}]) \times 100\%$$

For the assessment of the efficacy of acute morphine, cumulative morphine dose–response curve was constructed using the tail flick assay. Animals were given i.t. ascending doses of morphine every 30 min until a maximal level of antinociception was reached. TFL was determined 20 min after morphine administration to get the peak effect of morphine as shown previously (Cai et al., 2007b). The morphine dose–response curves were constructed and the ED_{50} values of the agonist were determined from these curves.

Immunohistochemistry

Rats received 6-day treatment of saline or drugs. On day 7, one hour following i.t. administration of saline or drugs, the animals were anesthetized with pentobarbital (i.p.) and perfused intracardially with cold 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The L4–6 segments of the spinal cord and DRG were removed and postfixed in the same fixative overnight. The tissues were then transferred to 30% sucrose in PB for cryoprotection. The spinal cord segments (40 μm) and DRG (10 μm) were cut on a cryostat and every 4th section was collected on the slides. Immunohistochemistry was performed at room temperature on free-floating sections (spinal cord) or slides (DRG) using an avidin–biotin complex technique as described previously (Cai et al., 2007a). To permit comparisons across various groups, sections from different treatments were processed simultaneously. After being pre-treated with 0.3% H_2O_2 and 5% normal goat/rabbit/horse serum, sections were incubated with rabbit polyclonal anti-neuronal nitric oxide synthase (nNOS, 1:200, BD Transduction Laboratories, San Diego, CA, USA) antisera for 24 h at 4 °C. The tissues were then transferred to biotinylated secondary IgG complex