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Effects of intrathecal amylin on formalin-induced nociception and on cAMP accumulation in the rat embryonic spinal cells

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ABSTRACT

Amylin (AMY) is a member of calcitonin family of peptides. In this study, the effects of intrathecal (i.t) injection of AMY on the inflammatory pain and on the cAMP accumulation in the rat spinal cells were investigated. By using AMY receptor antagonists, we also studied the pharmacology of AMY receptors in the spinal cells. Formalin model of inflammatory pain was induced by intraplantar injection of formalin. AMY (0.06250–2500 pmol/rat) was administrated i.t 15 min before the injection of formalin. Antagonists were injected i.t 10 min before the injection of AMY and/or morphine. AMY reduced formalin-induced pain in a dose dependent mode. This effect was inhibited by the potent AMY antagonist, AC187 but not $CGRP_{8-37}$. $rAMY_{8-37}$, most commonly reported as a weak AMY antagonist, showed to be equally or more potent than AC187 in antagonizing the above effects. The opioid antagonist, naloxone, had no significant effects on AMY antinociceptive effects. Primary dissociated cell culture was used to investigate the effect of AMY on cAMP production and to characterize AMY receptors in the spinal cells. AMY moderately increases cAMP accumulation in the spinal cells with an EC_{50} value of 74.62 nM. This effect was not affected by $CGRP_{8-37}$ but was inhibited by AC187 and $rAMY_{8-37}$ with pA_2 values of 7.94 and 7.87 respectively.

In conclusion, effects of AMY in reducing formalin induced pain and on the cAMP accumulation by spinal cells are mediated through undefined receptors.

1. Introduction

AMY or islet amyloid polypeptide bears high structural similarities with calcitonin gene related peptide (CGRP) and other members of calcitonin family of peptides that include calcitonin (CT), adrenomedullin, intermedin, and three calcitonin receptor stimulating peptides (Hong et al., 2012; Katafuchi et al., 2009; Watkins et al., 2013). These peptides are expressed in peripheral tissues (Hong et al., 2012; Katafuchi et al., 2009; Watkins et al., 2013) and in various parts of brain and spinal cord (Owji et al., 1996; Takahashi et al., 2011; Yu et al., 2009). AMY is shown to play a role in feeding behavior (Hayes et al., 2014; Lutz, 2013), cognition and memory (Qiu et al., 2014) as well as neuroprotection (Adler et al., 2014). The presence of AMY immunoreactivity in the DRG (dorsal root ganglion) neurons and in the cell processes in the spinal dorsal horn (Huang et al., 2010; Mulder et al., 1997) suggests that this peptide may participate in the transmission of nociceptive signals. However, contradicting results have been reported regarding the involvement of AMY in nociception. Although knocking down of AMY in mice resulted in a reduced response to painful stimuli (Gebre-Medhin et al., 1998), but intracerebroventricular administration of AMY failed to reduce nociception in the tail immersion test (Bouali et al., 1995). Additionally, i.t administration of AMY showed to reduce the pain related behaviors in mice subjected to acetic acid-induced writhing test (Huang et al., 2010).

AMY is structurally more similar to CGRP and salmon calcitonin (sCT) than it is to other members of the calcitonin family (van Rossum et al., 1997). Thus early reports suggested that AMY acts via interaction with receptors for CGRP and sCT (Hay et al., 2004). Later findings revealed that receptors for AMY can be generated by the heterodimerization of CT receptors (CTR) with one of the receptor activity modifying proteins 1-3 (PAMP1-3), thus yielding AMY1, AMY2 and AMY3 receptors (Hay et al., 2004; Bailey et al., 2012; Walker and Hay, 2013). While CTR does not require RAMPs to bind and respond to CT, CGRP and adrenomedullin receptors are formed from combinations of a calcitonin receptor like receptor (CLR) and RAMPs (Woolley and Conner, 2013). CTR and RAMPs are highly expressed in the rat brain at both levels of mRNA (Baisley et al., 2014) and protein (Csati et al., 2012). mRNAs encoding three RAMPs and CTR exist in the spinal cord of mice (Huang et al., 2010). In the rats, although binding sites for [^{125}I]-eel-calcitonin (Guidobono et al., 1986) and the presence of RAMPs (Oliver et al., 2001; Marvizon et al., 2007) have been shown but the expression of CTR remains to be demonstrated.

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All known receptors for CT, CGRP and adrenomedullin induce the accumulation of cAMP in response to their agonists (Owji et al., 2008; Takhshid et al., 2006; Watkins et al., 2013). Nevertheless the second messenger system(s) linked to the AMY receptors are not very well defined. Although AMY is reported to stimulate production of cAMP in the soleus muscle (Pittner et al., 1996) and osteoclasts of rats (Granholm et al., 2011), but the peptide has also shown to decrease forskolin-stimulated insulin release from Rinm5F rat insulinoma cells with a concomitant decrease in cAMP levels (Suzuki et al., 1992). Likewise, the inhibitory effect of AMY on stimulated insulin release has shown to be suppressed by pertusis toxin (Silvestre et al., 1994), suggesting the coupling of Gi to AMY receptors in mediating this effect.

In view of the above mentioned contraindications, we examined the effects of i.t injection of AMY on the formalin model of inflammatory pain in rats. Owing to the uncertainty on the expression of CTR in the rat spinal cells, we used an embryonic primary culture model to investigate the effects of AMY on the cAMP accumulation in the spinal cord cells.

2. Material and methods

2.1. Chemicals

Rat AMY, $rAMY_{8-37}$, acetyl-(Asn30, Tyr32)-$calcitonin_{8-32}$ (AC187) and rat α-$CGRP_{8-37}$ were obtained from Bachem Americas, Inc. (Torrance, CA). All chemicals and cell culture materials used in this study were obtained from Sigma-Aldrich and Gibco BRL, respectively.

2.2. Animal treatments and surgery

Male Sprague–Dawley rats weighing 250 ± 20 g were anesthetized with ketamine (50 mg/kg)–xylazine (5 mg/kg) and intrathecal catheterization was performed as described by Yaksh and Rudy (1976). Briefly, a slit was made on atlanto-occipital membrane and 7.5 cm length of a polyethylene catheter (PE-10, Betcton Dickenson, San Jose, CA) was inserted into the subarachnoid space. The rostral part was sutured to the adjacent muscles to immobilize the catheter and the wound was closed in two layers with 4-0 silk. The position of the caudal tip was always confirmed after the animals were sacrificed. Rats showing neurological deficits during the recovery period of 7 days were excluded from the study. Final doses of morphine (15.0 nmol/rat) and peptides (0.0625–2500 pmol/rat) were prepared in 10 μl of sterile saline for i.t injection. Antagonists were administrated 10 min before the injection of AMY followed by 10 μl flushes of normal saline to clear the catheter. Formalin (2.5%, 50 μl) or saline was injected into the hind paw of rats 15 min after they received i.t injection of the drug or saline. Control rats were treated i.t with 10 μl of saline prior to an injection of 50 μl of saline into one hind paw. All experimental protocols were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Medical and Research Ethics Committee of the Shiraz University of Medical Sciences.

2.3. Formalin pain model and behavioral testing

Formalin-induced pain was used to study the effect of AMY on spinal nociception. According to Dubuisson and Dennis proposed model [1977], 50 μl of formalin solution (2.5%) was injected into the subcutaneous plantar surface of the right hind paw of rats. Animals were then placed in the testing chamber and numbers of flinches were recorded every 15 s. The cumulative numbers of flinches recording during 0–5 min after and 20–60 min after the injection were considered as acute pain (phase I) and late phase (phase II), respectively.

2.4. Preparation of dispersed rat embryonic spinal cells

Spinal cords from rat fetuses (embryonic day 17–18) were removed under aseptic conditions and were chopped into small pieces. Tissues were treated with 0.25% trypsin in Hanks' balanced salt solution (HBSS) at 37 °C for 10 min. Digested spinal tissues were then rinsed with culture media (DMEM containing 1% HEPES buffer solution, penicillin/streptomycin, 5% heat-inactivated fetal bovine serum and 5% normal horse serum) and triturated using flamed polished Pasteur pipettes. Dispersed spinal cells were then collected using centrifugation and were filtered through a 75 μM cell strainer, after being suspended in the culture media. 1×10^5 spinal cells were seeded in poly-D-lysine coated culture plates, and were cultured in a humid incubator at 37 °C with 5% CO_2 and 95% air.

2.5. Assay of cAMP

To study the effect of AMY on cAMP accumulation, the spinal cells were preincubated with serum-free DMEM containing 1 μM of the cAMP phosphodiesterase inhibitor RO-20-1724 (4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone dissolved in DMSO) for 30 min. AMY (10^{-6} to10^{-12} M) were then added to the culture media and incubated for an additional 10 min. Ice-cold ethanol (100%) was added to culture media to terminate the reaction. To characterize AMY receptors, AMY receptor antagonists ($rAMY_{8-37}$, AC187 and rat α-$CGRP_{8-37}$) were added to the medium for 15 min before the stimulation with agonist. The adenylyl cyclase activator, forskolin (1 μM), was used as a positive control to assess the maximal possible accumulation of cAMP by spinal cells. A sandwich Elisa method was used to measure cAMP (R& D System Inc).

2.6. Statistical analysis

SPSS software (SPSS, Chicago, IL, USA) was used for statistical analysis. Differences between groups were assessed by ANOVA followed by either Tukey's or Dennett's post-hoc analysis. Graph Pad Prism program (version 5) was applied to analyze the cAMP data. Concentration-response curves were fitted to a sigmoidal curve to obtain EC_{50}s and maximum responses. For each antagonist dose ratios were calculated and used to obtain pA_2 according to log (antagonist)–log (dose ratio − 1). The pA_2 is the negative logarithm of the concentration (M) of an antagonist which will cause a twofold increase in the EC_{50} of the agonist. Data are shown as mean ± SEM. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of i.t administration of AMY and morphine on the formalin-induced nociception

The numbers of flinches were scored after formalin intraplantar injection. Rats showed a biphasic pain response. The phase I (early phase) began immediately after injection and peaked at 5 min post-injection. After a 10–15 min quiescent period, the phase II (late phase) started and lasted 40 min. I.t pretreatment of formalin injected rats by morphine (15.0 nmol) or different doses of AMY resulted in dose-dependent decreases in the mean number of flinches in the phase I and II (Fig. 1). The inhibitory effects of AMY on formalin nociception tended to reverse at higher concentrations of the peptide in a hormetic-like manner. The increasing effects of AMY was observed at AMY concentrations of more than 250 pmol and 62.5 pmol respectively in phase I and phase II of formalin-induced nociception. Fig. 2 shows prototypical time courses of formalin-induced nociception in rats that were pretreated i.t by saline, AMY and/or morphine 10 min before formalin injection. Thus, during phase I, rats pretreated i.t by either AMY (62.5 pmol/10 μl) or morphine (15.0 nmol/10 μl) displayed mean number of flinches that were statistically lower than those of control animals ($P = 0.003$ and $P < 0.001$ respectively, ANOVA followed by

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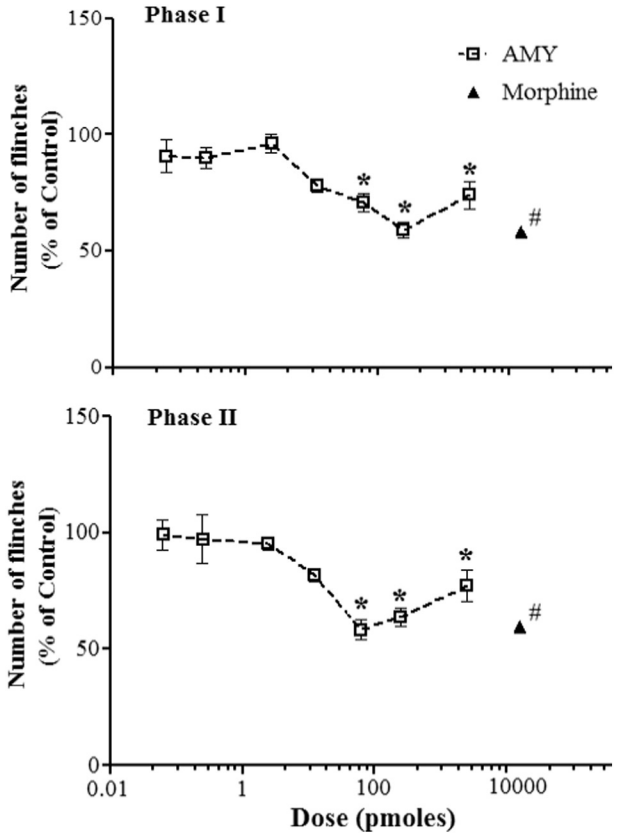

Fig. 1. Effects of i.t pretreatment of rats by morphine and different doses of AMY on the nociception during phase I and phase II following intraplantar formalin injection. Data are Mean ± SEM of number of flinches for five to six rats. Asterisks (*) in the AMY group and hash symbol (#) in the morphine-treated group show a significant difference ($P < 0.05$, ANOVA followed by Dunnett's test) compared to control group.

Dunnett's test). No significant differences were found between the average of number of flinches in rats pretreated by morphine and those pretreated by AMY ($P = 1.0$, ANOVA followed by Dunnett's test). The same pattern of results was obtained during the phase II of formalin test (Fig. 2). Intraperitoneal injection of AMY had no significant effects on the formalin-induced pain in both phases of I and II as compared with the saline-treated rats (data not shown).

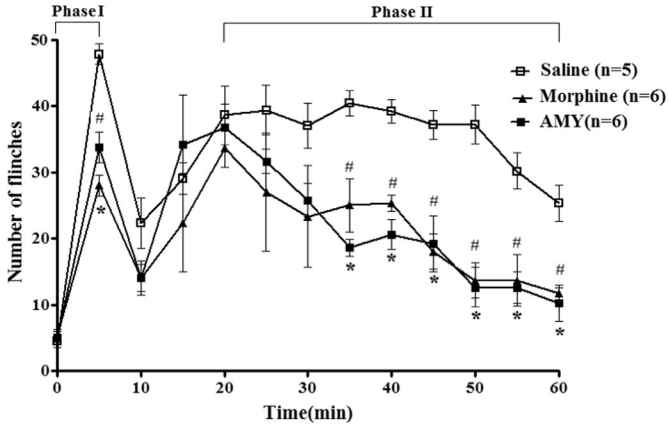

Fig. 2. Prototypical time courses of formalin-induced nociception in rats that were pretreated i.t by saline (control group), AMY (62.5 pmol) and/or morphine (15.0 nmol) 10 min before formalin injection. Drugs were administered 10 min before formalin injection. Data are presented as number of flinches versus time. Each point on the graph represents the Mean ± SEM of five to six animals. *$P < 0.01$ saline versus AMY-treated group, #$P < 0.01$ saline versus morphine-treated group. No significant differences found between AMY and morphine-treated groups (ANOVA followed by Dunnett's test).

3.2. Effects of i.t administration of AC187, rAMY$_{8\text{-}37}$, and CGRP$_{8\text{-}37}$ on the AMY-induced antinociception in the formalin test

The average of formalin-induced flinches obtained for different pretreatments during both phases of I (0–5 min) and II (20–60 min) of formalin test are summarized in the Table 1. In the phase I of formalin test, the mean number of flinches in the rats treated i.t by AMY (62.5 pmol) was significantly lower ($P = 0.01$) than those that had received i.t rAMY$_{8\text{-}37}$ (1.25 nmol/10 μl) prior to AMY administration. AC187, however, was not able to block the abovementioned effect of AMY at the i.t dose of 1.25 nmol/10 μl ($P = 0.98$) but it blocked the antinociceptive effect of AMY at the dose of 2.5 nmol/10 μl ($P < 0.001$). There was also no significant differences ($P = 1.00$) between the average of number of flinches of rats pretreated by AMY (62.5 pmol) and those that i.t received the CGRP antagonist, CGRP$_{8\text{-}37}$ (1.25 nmol/10 μl), before the agonist. In the phase II of the formalin-induced pain behavior, the analgesic effects of AMY (62.5 pmol/10 μl) were significantly reversed ($P < 0.001$) in rats pretreated by rAMY$_{8\text{-}37}$ (1.25 nmol/10 μl). The antinociceptive effects produced by i.t pretreatment of AMY was not significantly reduced by 1.25 nmol/10 μl of AC187 ($P = 0.98$) but was inverted when the i.t dose of the antagonist was elevated to 2.50 nmol/10 μl ($P < 0.001$). No significant changes were observed in the number of flinches of rats injected i.t by 1.25 nmol/10 μl of either rAMY$_{8\text{-}37}$ ($P = 0.99$) or AC187 ($P = 1.0$) in comparison to saline-treated animals. CGRP$_{8\text{-}37}$ however showed antinociceptive effects when injected at a doses of 1.25 nmol/10 μl ($P = 0.02$).

3.3. Effects of i.t administration of naloxone on the AMY, and morphine-induced antinociception in formalin test

To explore the possible role of opioid receptors in the AMY-induced antinociception, a well-known antagonist of opioid receptors, naloxone (28.0 nmol/10 μl), was injected i.t 10 min before the i.t injection of morphine (15.0 nmol/10 μl) and AMY (62.5 pmol). Data were analyzed by ANOVA followed by Tukey's test. As shown in the Fig. 3, naloxone, as expected, blocked the morphine antinociception during both phases I and II of formalin test. In the phases I and II of formalin-induced pain, naloxone failed to affect the antinociceptive effects of AMY. These data imply that naloxone had no significant effects on AMY antinociceptive effects.

3.4. Effects of AMY on cAMP accumulation in the rat embryonic spinal cells

An embryonic spinal cell primary culture model was used to evaluate the effects of AMY on the cAMP accumulation in the spinal cells. The basal level of cAMP in cells of our model was 3.95 ± 0.54 pmol/mg protein. As shown in the Fig. 4, AMY increased levels of cAMP in spinal cells dose

Table 1
Effects of i.t injection of AMY and its antagonists on the average number of flinches obtained for different i.t pretreatments during both phases of I and II of formalin test.

Intrathecal treatments	Number of flinches	
	Early phase (I)	Late phase(II)
Sham	10.0 ± 0.0^a	52.0 ± 20.5^a
Saline	47.8 ± 1.6^b	324.0 ± 12.6^b
AMY (62.5 pmol)	33.8 ± 2.3^c	189.0 ± 11.7^c
AMY(62.5 pmol) + AC187(1.25 nmol)	36.5 ± 6.5^c	247.0 ± 13.5^c
AMY (62.5 pmol) + AC187(2.5 nmol)	48.6 ± 1.1^b	387.0 ± 15.3^b
AMY(62.5 pmol) + rAMY$_{8\text{-}37}$ (1.25 nmol)	51.0 ± 4.0^b	392.0 ± 4.5^b
AMY(62.5 pmol) + CGRP$_{8\text{-}37}$(1.25 nmol)	27.0 ± 6.5^c	228.0 ± 23.5^c
AC187 (1.25 nmol)	43.3 ± 3.2^b	369.0 ± 12.2^b
rAMY$_{8\text{-}37}$ (1.25 nmol)	54.0 ± 1.0^b	446.0 ± 13.5^b
CGRP$_{8\text{-}37}$ (1.25 nmol)	30.6 ± 5.2^c	216.0 ± 14.8^c

Effects of i.t administration of AMY and its antagonists on the average number of flinches during both phases of I and II of formalin test. Values for each group represent mean ± S.E.M number of flinches of 5–6 rats subjected to the formalin test. Values with the same letters in each phase of formalin test are not significantly different at $P < 0.05$ (ANOVA followed by Tukey's test).

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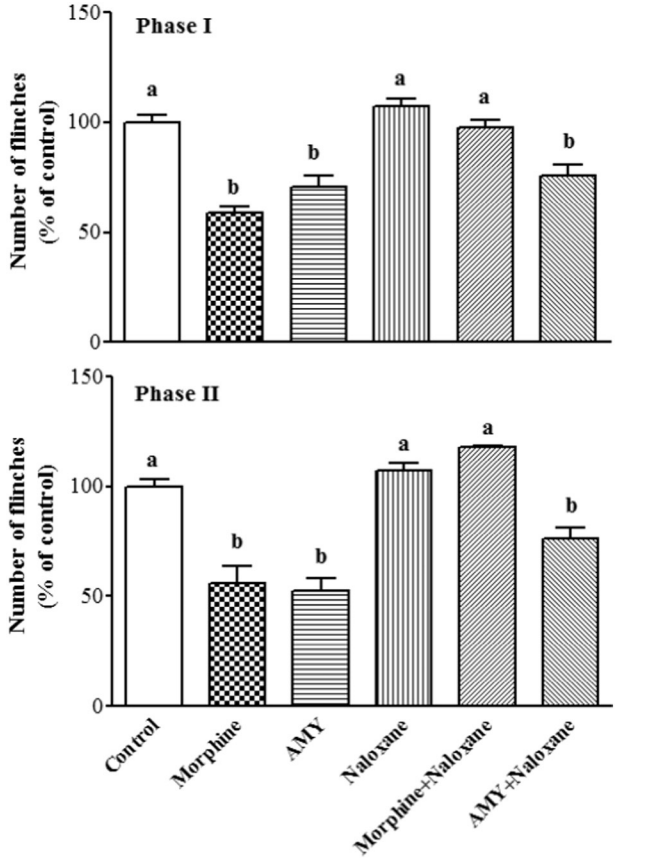

Fig. 3. Effects of naloxone on the antinociception induced by AMY and morphine in the phase I and phase II of formalin test. Naloxone (28.0 nmol, i.t) was injected 10 min before rats were treated i.t by morphine (15.0 nmol) and/or AMY (62.5 pmol). Each point on the graph represents the Mean ± SEM of five to six animals. The statistical differences between groups were evaluated by analysis of variance followed by Tukey's test. Values with the same letters are not significantly different at $P < 0.05$.

dependently with an EC_{50} value of 74.62 nM. A maximal response which was obtained at 1 μM of the peptide was approximately 6.99 ± 1.91 times of the basal level. Whereas forskolin (1 μM) increased levels of cAMP 54.76 ± 8.04 folds over the basal level.

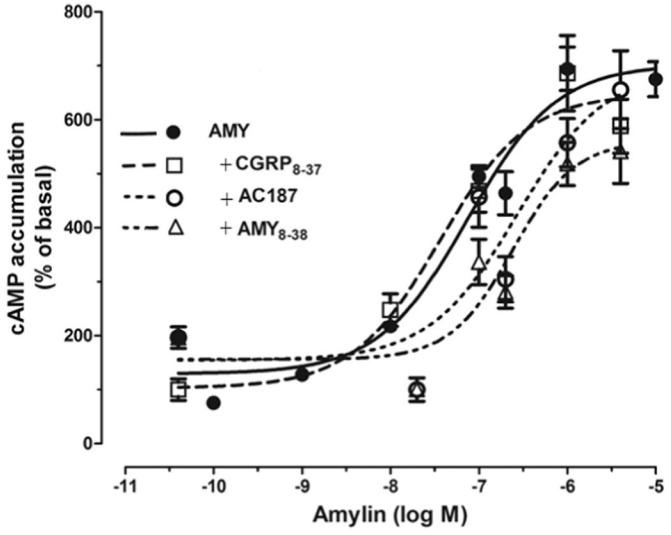

Fig. 4. Characterization of AMY-induced cAMP accumulation in the rat spinal cord cells. Data are Mean ± S.E.M from at least three independent experiments and are expressed as the percentage of basal cAMP level. The concentration response curves were estimated by fitting each line to a logistic Hill equation, as described in materials and methods.

3.5. Functional characterization of AMY receptors

To characterize the nature of AMY receptors in the embryonic spinal cells, we examined the effects of 0.3 μM concentrations of various AMY antagonists, AC187, $CGRP_{8-37}$ and $rAMY_{8-37}$ on cAMP accumulation induced by increasing concentrations of AMY (Fig. 4). Both AC187 and $rAMY_{8-37}$ shifted the concentration response curve of AMY to the right whereas $CGRP_{8-37}$ failed to inhibit stimulating effect of AMY up to the concentration of 1 μM (data not shown). From the sigmoidal concentration response curves, dose ratios (EC_{50} antagonist/EC_{50} agonist) were calculated. Thus, AC187 and $rAMY_{8-37}$ shifted the concentration-effect curve of AMY to the right with pA_2 values of 7.94 and 7.87, respectively (Table 2).

4. Discussion

4.1. Effects of AMY on formalin-induced pain

The localization of AMY in small DRG neurons and in the major site of terminal nociceptors suggests that this peptide may participate in nociceptive transmission. Contradicting results, however, have been reported relative to AMY involvement in nociception. In the present study we considered the effects of i.t injection of AMY on the formalin-induced inflammatory pain model in rats. The formalin-induced nociceptive response has two phases that involve different mechanisms. The phase I (neurogenic pain) reflects acute pain and results from direct chemical stimulation of nociceptive C-fibers. The phase II (inflammatory pain) involves the action of inflammatory mediators in the peripheral tissues as well as the functional changes in the neurons of spinal dorsal horn (Franca et al., 2001). Opioids, when administered centrally, inhibit both phases of I and II equally (Dubuisson and Dennis, 1977), while peripherally acting drugs, such as cyclooxygenase inhibitors and corticosteroids only inhibit the late phase II (Yamamoto and Nozaki-Taguchi, 2002). In our current study, we demonstrated that i.t injection of AMY reduced formalin-induced pain in a concentration dependent manner in both phases of I and II. This result is in agreement with those of a recent study showing that i.t administration of AMY reduced the writhing reflex in mice with a maximal effective dose of 10 μg (Huang et al., 2010). This value is higher than the 62.5 pmol (250 ng) we presently report for the antinociceptive effects of AMY in the formalin test. The difference could be due to the animal model of pain (acetic acid-induced visceral pain model versus formalin-induced inflammatory pain) and/or the animal species (mice versus rats) used in the two studies. Like morphine, AMY showed antinociceptive effects in both phases of formalin-induced pain which confirms that the antinociceptive effect of AMY is mediated through a central mechanism. However, the opioid antagonist, naloxone, that blocked the analgesic effects of morphine, failed to reverse the inhibitory effects of AMY in formalin test, implying that the antinociceptive effect of AMY was mediated at least partly through an opioid independent mechanism. Our data also showed that AMY at a dose of 62.5 pmol/rat was able to induce an antinociceptive effect that was close to that produced by a higher dose (15.0 nmol/rat) of morphine. However, to establish the relative potency of AMY versus morphine, a full dose–response curve for morphine should be prepared and the derived EC_{50} be compared with the corresponding value for AMY.

Excitatory effects of the two other members of calcitonin family of peptides i.e. adrenomedullin and CGRP on nociception has already

Table 2
Effects of antagonists on cAMP accumulation induced by AMY in embryonic rat spinal cells.

Antagonist	Log EC_{50}	pA2
AC187	−6.56	7.94
AMY_{8-37}	−6.62	7.87
$CGRP_{8-37}$	−7.44	<4.0

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been reported (Takhshid et al., 2004; Ma et al., 2006). We have shown that AMY affected the formalin-induced pain behaviors in a dose response pattern reminiscent of a U-shaped curve. Therefore our results raise the possibility that AMY exerts its spinal pain related biological effects via two types of receptors. One of these receptors may be inhibitory with higher affinities for AMY and the other receptor is possibly excitatory with a lower affinity for the peptide. For example, AMY is conceivable to be pronociceptive if it interacts with CGRP and/or adrenomedullin receptors which have shown excitatory effects on nociception in the spinal cord (Ma et al., 2006). Another possibility is that the pronociceptive effects observed at high doses of AMY is mediated by the supraspinal regions.

AMY has shown high agonistic effects on rAMY1, rAMY3 and rCT receptors in transfected cells (Bailey et al., 2012). Hence, we used AMY receptor antagonists $rAMY_{8-37}$, AC187 and $CGRP_{8-37}$ to characterize the nature of receptors that mediate inhibitory effects of AMY on the formalin-induced hyperalgesia. AC187 is generally accepted as a good discriminator of amylinergic responses (Young, 2005). $rAMY_{8-37}$ is also generally accepted as a selective but weak antagonist at AMY receptors with an affinity profile of AMY1 > AMY3 ⋙ rCT (Hay et al., 2005). Salmon CT_{8-32} is another widely used AMY antagonist that is used to discriminate between AMY and CGRP receptors, but it is not able to discriminate between AMY and CT receptors effectively (Poyner et al., 2002). Herein, we used $CGRP_{8-37}$, the classic antagonist of CGRP 1 receptor (CLR/RAMP1) to discriminate between AMY and CGRP receptors. Thus, i.t pretreatment of rats with either AC187 or $rAMY_{8-37}$, reversed the analgesic effects of AMY. Interestingly, $rAMY_{8-37}$ was more potent than or at least equipotent to AC187 in inhibition of analgesic effects of AMY in formalin test. Moreover, $CGRP_{8-37}$ failed to reverse the analgesic effects of AMY when injected at the same concentrations as $rAMY_{8-37}$ and AC187. In a recent study, we have examined the effects of i.t administration of AMY and sCT on formalin-induced c-fos expression in the dorsal horn of rat spinal cord. Findings showed that sCT was more potent than AMY in inhibition of the c-fos expression (Khoshdel et al., 2014). Furthermore, a similar pattern of antagonistic effects to that of present study ($rAMY_{8-37}$ ≥ AC187 ≫ rα-$CGRP_{8-37}$) were obtained for the inhibitory effects of AMY and sCT on c-fos expression, except that $rAMY_{8-37}$ was a more potent antagonist for AMY than for sCT. The order of potencies of these antagonists at AMY1 and AMY3 receptors have been reported as AC187 > rα-$CGRP_{8-37}$ ≫ $rAMY_{8-37}$ in cell culture studies (Bailey et al., 2012). Findings of our previous (Khoshdel et al., 2014) and present study, however, imply that in the spinal cord AMY and sCT probably act through receptors that are equally if not more sensitive to antagonist effects of $rAMY_{8-37}$ than AC187 and insensitive to $CGRP_{8-37}$. Despite expression of RAMPs and also presence of specific binding sites for radiolabeled sCT which binds to both calcitonin and AMY receptors, there is no solid data on the expression of CTR in the rat spinal cord. Further studies are warranted to characterize the nature of AMY receptors in this tissue.

4.2. Effects of AMY on cAMP accumulation in rat embryonic spinal cells

We have previously used primary embryonic spinal cells of rats to characterize the effects of adrenomedullin and CGRP on cAMP accumulation (Takhshid et al., 2006). In this study we used the same model to functionally characterize AMY receptors in the rat spinal cells. Similar method was recently used by Walker and Hay (2013) for characterization of AMY and CGRP receptor in trigeminal ganglion neuron of rats. Our results showed that although AMY increases cAMP accumulation in the spinal cells but its potency (EC_{50} of 74.62 nM) is much lower than what we previously reported for adrenomedullin and CGRP (EC_{50}s of 0.79 nM and 5.01 nM respectively). The maximal effect of AMY on cAMP levels was approximately 12.76% of that of forskolin, an efficacy that is much smaller than those previously reported for adrenomedullin (100%) and CGRP (38.88%) (Takhshid et al., 2006). These data imply that cAMP system may not be a major transduction pathway linked to AMY receptors in spinal cells or alternatively AMY has a relatively low density of receptors in these cells. Our results also showed that $rAMY_{8-37}$ antagonized AMY stimulated cAMP accumulation ($pA_2 = 7.94$) with the same potency as AC187 ($pA_2 = 7.87$) in spinal cells. Several previous studies have indicated the antagonistic effects of $CGRP_{8-37}$ at rAMY1 receptors (Bailey et al., 2012). However $CGRP_{8-37}$ failed to antagonize AMY-induced cAMP accumulation under our experimental conditions ($pA_2 < 4$).

In summary, findings of the present study revealed that AMY reduced the inflammatory pain induced by subcutaneous injection of formalin and caused a small accumulation of cAMP in the rat spinal cells. The fact that $rAMY_{8-37}$ was equally or more potent than AC187 in antagonizing these effects and that $CGRP_{8-37}$ failed to antagonize these effects of AMY implies that AMY may act through undefined receptors in the spinal cord. Owing to the uncertainty on the presence of CTR in the rat spinal cord, further work is required to establish the nature of AMY receptors in this tissue.

Conflict of interest

None declared.

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