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AMYLIN SUPPRESSES ACETIC ACID-INDUCED VISCERAL PAIN AND SPINAL *C-FOS* EXPRESSION IN THE MOUSE

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Abstract—Amylin is a member of calcitonin or calcitonin gene-related peptide (CGRP) family. Immunohistochemical study revealed a dense network of amylin-immunoreactive (irAMY) cell processes in the superficial dorsal horn of the mice. Numerous dorsal root ganglion (DRG) and trigeminal ganglion cells expressed moderate to strong irAMY. Reverse transcriptase-polymerase chain reaction (RT-PCR) revealed amylin receptor mRNA in the mouse spinal cord, brain stem, cortex, hypothalamus and hippocampus. The nociceptive or antinociceptive effects of amylin were evaluated in the acetic acid-induced writhing test. Amylin (0.1, 0.5 and 1 mg/kg, intraperitoneally (i.p.) or 1–10 μg, intrathecally (i.t.)) reduced the number of writhes in a dose-dependent manner. Pretreatment of the mice with the amylin receptor antagonist salmon calcitonin (8–32), either by i.p. or i.t., antagonized the effect of amylin on acetic acid-induced writhing test. Locomotor activity was not significantly modified by amylin injected either i.p. (0.01–1 mg/kg) or i.t. (1–10 μg). Measurement of *c-fos* mRNA by RT-PCR or proteins by Western blot showed that the levels were upregulated in the spinal cord of mice injected with acetic acid and the increase was attenuated by pretreatment with amylin (10 μg, i.t.). Collectively, our result demonstrates that irAMY is expressed in DRG neurons with their cell processes projecting to the superficial layers of the dorsal horn, and that the peptide by interacting with amylin receptors in the spinal cord may be antinociceptive.

Key words: antinociception, dorsal root ganglia, intrathecal, *c-fos*, amylin receptor.

Amylin, a 37 amino acid peptide secreted from pancreatic β-cells upon stimulation by meal or glucose (Cooper et al., 1987; Westermark et al., 1987), is a member of the calcitonin family which includes calcitonin (CT), calcitonin gene-related peptide (CGRP) and adrenomedullin (AM) (Muff et al., 2004). Amylin-like immunoreactivity (irAMY) has been reported in the gut (Mulder et al., 1994), osteoblast (Gilbey et al., 1991) and CNS of humans and rats (Skofitsch et al., 1995; D'Este et al., 2000). Amylin is abundantly expressed in rat dorsal root ganglion (DRG) cells, some of which contain substance P and pituitary adenylate cyclase-activating polypeptide (PACAP) (Mulder et al., 1995). Further, irAMY and CGRP-immunoreactivity overlaps in the motor nuclei of the hindbrain and spinal cord (Skofitsch et al., 1995; Gebre-Medhin et al., 1998).

Amylin is structurally similar to both salmon CT (sCT, about 30% homology) and CGRP (about 50% homology) (van Rossum et al., 1997). Similar to CGRP receptors, amylin receptors are heterodimeric complex of calcitonin receptor (CTR) and receptor activity modifying proteins (RAMP) (Christopoulos et al., 1999). High affinity amylin binding sites are located in many regions of the rat and monkey brains; including the hypothalamus, nucleus accumbens, dorsal raphe and area postrema (Stridsberg et al., 1993; Sexton et al., 1994; Paxinos et al., 2004). Little is known relative to the function of amylin in the mammalian nervous system. Amylin has been proposed as an anorexigen affecting the gastrointestinal system and ingestive behavior (Riediger et al., 2001; Young, 2005; Edelman et al., 2008). The peptide has also been shown to exert anti-inflammatory activity (Clementi et al., 1995) and protect gastric mucosa in various ulcer models (Samonina et al., 2004). Variable results have been reported relative to its involvement in nociception. For example, central administration of amylin failed to induce antinociception (Bouali et al., 1995; Sibilia et al., 2000), while peripheral administration (s.c. or i.p.) produced antinociception (Young, 1997).

In the pain signaling pathways, the immediate early gene *c-fos* is promptly expressed in neurons in response to a painful stimulus (Sagar et al., 1988). Several reports have shown that the expression of *c-fos* mRNA or immunoreactivity is upregulated in the spinal cord following noxious visceral stimulation (Rodella et al., 1998; de los Santos-Arteaga et al., 2003; Lee and Seo, 2008). In contrast, expression of *c-fos* in the spinal cord was attenuated by pretreatment of analgesia (Lee and Seo, 2008). Thus, expression of *c-fos* mRNA or protein can be used as a marker of neurons activated by pain sensation (Harris, 1998).

In this study, results from a multidisciplinary approach including immunohistochemistry, molecular biology and *in vivo* pharmacology, support the hypothesis that amylin is antinociceptive in an acetic acid-induced writhing mouse model.

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Abbreviations: AM, adrenomedullin; cc, central canal; CGRP, calcitonin gene-related peptide; CT, calcitonin; CTR, calcitonin receptor; DRG, dorsal root ganglion; i.p., intraperitoneally; irAMY, amylin-immunoreactive; i.t., intrathecally; PACAP, pituitary adenylate cyclase-activating polypeptide; RAMP, receptor activity modifying protein; RT-PCR, reverse transcriptase-polymerase chain reaction; s.c., subcutaneously; sCT [8–32], salmon calcitonin [8–32]; SEM, standard error of the mean; TACTV, total activity; TRG, trigeminal ganglion.

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EXPERIMENTAL PROCEDURES

Experimental animals

Adult male ICR mice (Ace Animal Inc, Boyertown, PA, USA) weighing 25–30 g were used in this study. Experimental protocols were reviewed and approved by the Temple University Institutional Animal Care and Use Committee, in accordance with the 1996 NIH Guide for the Care and Use of Laboratory Animals. Animals were housed under a 12 h light/dark cycle with free access to food and water. Animals were moved to the behavioral testing room one day prior to testing and were acclimated in the observation box at least 1 h before the experiments. Efforts were made to minimize the number of mice and animal suffering from pain-related studies.

Immunohistochemistry

Mice anesthetized with urethane (1.2 g/kg, i.p.) were intracardially perfused with cold 0.1 M phosphate buffered saline (PBS) followed by freshly prepared 4% paraformaldehyde/0.2% picric acid in PBS. Tissue were processed for irAMY by the avidin–biotin complex procedure (Dun et al., 2006). Sections were incubated in amylin antiserum (1:3000 dilution, a rabbit polyclonal against feline amylin, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) for 48 h. Sections were then incubated in biotinylated anti-rabbit IgG (1:300 dilution, Vector Laboratories, Burlingame, CA, USA) for 2 h, and rinsed with PBS and incubated in avidin–biotin complex solution for 1.5 h (1:100 dilution, Vector Laboratories). Following several washes in Tris-buffered saline, sections were developed in 0.05% diaminobenzidine/0.001% H_2O_2 solution and washed for at least 2 h with Tris-buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air-dried, dehydrated with absolute alcohol followed by xylene, and coverslipped with Permount (Fisher Scientific, Hanover Park, IL, USA).

In the control study, several sections of DRG or spinal cord from each animal were incubated in amylin antiserum pre-absorbed with amylin peptide (1 μg/ml, Phoenix Pharmaceuticals, Inc.) overnight. Quantification of irAMY neurons in DRG was conducted using the image analysis software (ImageJ 1.41o, Wayne Rasband, NIH). Images were captured at $\times$40 magnification. One hundred neuron profiles were measured from five DRG sections. Each irAMY neuron was sorted into six groups according to their diameters (1–20, 20–25, 25–30, 30–35, 35–40 or $>$40 μm) and expressed as a percentage of 100 neurons.

Acetic acid-induced writhing test

Procedures for acetic acid-induced writhing test are described in detail (Collier et al., 1968). Amylin (0.03, 0.1, 0.5 or 1 mg/kg) or vehicle was injected i.p. 15 min before an i.p. injection of acetic acid (0.6%, 0.3 ml/30 g). The number of writhes was counted between 5 and 15 min after the last injection. For the antagonist experiment, sCT [8–32] (0.5 mg/kg) or vehicle was injected i.p. 15 min before the administration of amylin (0.1 mg/kg). Acetic acid tests were conducted 15 min after the injection of amylin. Intrathecal administration of amylin (0.3, 1, 5 or 10 μg/mouse) or vehicle was given 10 min before an i.p. injection of acetic acid (0.6%, 0.3 ml/30 g). sCT [8–32] (5 μg/mouse, i.t.) was given 10 min before amylin (5 μg/mouse, i.t.) and acetic acid or acetic acid alone.

Locomotor activity test

Locomotor activity of the mice was measured by a computerized monitoring system (Digiscan DMicro, Accuscan Inst, Columbus, OH, USA), which consists of a metal frame containing 16 parallel infrared photobeams and receivers into which a standard plastic cage (42$\times$20$\times$20 cm^3) was placed. Photobeam breaks were recorded as total activity (TACTV) and stored on a computer coupled to the activity monitors. Groups of mice were tested for 10 min before and 50 min post i.p. injection (vehicle, amylin 0.01, 0.1 or 1 mg/kg) or 45 min post i.t. injection (vehicle, amylin 1, 5 or 10 μg/mouse). TACTV was determined by the number of times the animal broke the light beams and used for statistical analysis.

CTR and RAMP mRNA analysis by reverse transcriptase-polymerase chain reaction (RT-PCR)

Mice anesthetized with urethane (1.2 g/kg, i.p.) were decapitated with guillotine and various brain regions were dissected out. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript II first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). PCR was performed with a 5′ primer (TGGTTGAGGTTGTGCCCAATGGA) and a 3′ primer (CTCGTGGGTTTGCCTCATC TTGGTC) for CTR (Wang et al., 1998), and a 5′ primer (TCGTACCACAGGCATTGTGATGGA) and a 3′ primer (ACTCCTGCTTGCTGATCCA CATCT) for β-actin under the following conditions: 95 °C for 2 min; 40 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min; and then 72 °C for 10 min (CTR); 94 °C for 2 min; 29 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min; and finally 72 °C for 10 min (β-actin). The primers of CTR generated a product of 503 bp from the CTRb mRNA, and a product of 392 bp from the CTRa mRNA (Wang et al., 1998). Similarly, primer sequences for RAMP1 with a 5′ primer (AGGACTTGAGAGTGGCTCTGCATT) and a 3′ primer (ATGCTGTCACTACTGTCC ATGGCT) and the conditions: 94 °C for 2 min; 38 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min; and finally 72 °C for 10 min. Primer sequences for RAMP3 with a 5′ primer (TCATCACTGGAATCCA CAGGCAGT) and a 3′ primer (GTGGCCAAAGCAAACCAG ACAGAA) and the conditions: 94 °C for 2 min; 37 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s; and finally 72 °C for 10 min. The amplified products were subjected to electrophoresis in a 1% agarose gel and stained with ethidium bromide. The image was acquired with a FujiFilm Las-1000 imaging system (Fujifilm Medical Systems, Stamford, CT, USA). The digitized images were quantified with the Image Gauge software (FujiFilm Medical Systems, Stamford, CT, USA).

c-fos mRNA analysis by RT-PCR

Mice anesthetized with urethane (1.2 g/kg, i.p.) were decapitated with guillotine and spinal cords were dissected out 30–40 min after writhing test. Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript II first-strand synthesis system for RT-PCR. PCR was performed with a 5′ primer (AAACCGCATGGAGTGTGTTGTTCC) and a 3′ primer (TCAGACCACCTCGACAATGCATGA) for *c-fos*, and a 5′ primer (TCGTACCACA GGCATT GTGATGGA) and a 3′ primer (ACTCCTGCTTGCTGATCCACATCT) for β-actin under the following conditions: 94 °C for 2 min; 30 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s; and finally 72 °C for 10 min (*c-fos*); 94 °C for 2 min; 29 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min; and finally 72 °C for 10 min (β-actin). Three groups of mice were analyzed as follows: vehicle, vehicle with acetic acid, and amylin (10 μg /mouse, i.t.) with acetic acid (n=6).

c-fos analysis by Western blot

Three groups of mice were analyzed in this series of experiments: vehicle, vehicle with acetic acid, and amylin (10 μg /mouse, i.t.) with acetic acid (n=6). Fresh-frozen spinal cord samples were obtained 2 h after writhing test and immediately stored at −80 °C. Samples of approximately 100 mg were lysed in ice cold lysis buffer [10 mM Tris pH 7.6, 1% Triton X-100, protease inhibitor cocktail (Roche Diagnostics, Manheim, Germany)], and protein