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SPECIAL REPORT

Adrenomedullin inhibits spontaneous and bradykinin-induced but not oxytocin- or prostaglandin $F_{2\alpha}$-induced periodic contraction of rat uterus

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In isolated rat uterine strips, adrenomedullin (AM) inhibited the spontaneous periodic contraction in a concentration-dependent manner ($IC_{50} = 22.3 \pm 0.7$ nM). The inhibitory effect of AM was prevented by either AM_{22-52}, a putative antagonist for AM receptors, or calcitonin gene-related peptide $(CGRP)_{8-37}$, a putative antagonist for CGRP receptors. AM also attenuated bradykinin (BK)-induced periodic uterine contraction, which was blocked by AM_{22-52} or $CGRP_{8-37}$, whereas AM had no effect on the periodic contraction caused by oxytocin or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). RT–PCR analysis showed that mRNAs for calcitonin receptor-like receptor (CRLR), receptor-activity-modifying protein (RAMP)1, RAMP2 and RAMP3 were expressed in the rat uterus. These results demonstrate that AM selectively inhibits spontaneous and BK-induced periodic contraction *via* activating receptors for AM and CGRP.
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Abbreviations: AM, adrenomedullin; BK, bradykinin; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PG, prostaglandin; RAMP, receptor-activity-modifying protein; RT–PCR, reverse transcription-polymerase chain reaction

Introduction Adrenomedullin (AM), originally isolated from human pheochromocytoma as a hypotensive peptide (Kitamura *et al*., 1993), is widely distributed in various tissues, and plays multiple roles in the regulation of a variety of physiological functions (Kitamura & Eto, 1997; Cameron & Fleming, 1998). AM, consisting of 52 amino acids, shares the amino terminal ring structure and the carboxyl terminal amide structure with calcitonin gene-related peptide (CGRP), a vasodilating peptide (Kitamura *et al*., 1993). Receptors for AM and CGRP have been cloned in human neuroblastoma; combination of calcitonin receptor-like receptor (CRLR) with receptor-activity-modifying protein (RAMP)2 or RAMP3 produces receptor for AM, while combination of CRLR with RAMP1 produces that for CGRP (McLatchie *et al*., 1998; Foord & Marshall, 1999). AM has been shown to stimulate not only AM receptors but also CGRP receptors (Fraser *et al*., 1999).

AM and AM mRNA are highly expressed in the female reproductive system, such as posterior pituitary gland, uterus (Upton *et al*., 1997; Cameron & Fleming, 1998; Makino *et al*., 1999), and various foetoplacental tissues (Macri *et al*., 1996; Marinoni *et al*., 1998; Yotsumoto *et al*., 1998; Makino *et al*., 1999). Upton *et al*. (1997) reported that high concentration of AM (5 μM) inhibited tonic uterine contraction caused by galanin *via* activation of CGRP receptors, while the biological role of AM in the control of periodic uterine contraction remains elusive.

In the present study, to clarify the role of AM in the female reproductive system, we examined the effects of AM on periodic uterine contraction, and the expression of receptor for AM in rat uterus.

Methods *Isolation of uterine strips* Nonpregnant Sprague Dawley rats (8–12 weeks old, 230–280 g body weight) were given food and water *ad libitum*, and housed at 22°C with 50% humidity and a 12 h light/dark cycle. The rats were injected subcutaneously 1 μg 17β-oestradiol (Sigma, St. Louis, MO, U.S.A.) dissolved in 0.2 ml of 30% ethanol; oestradiol was administered to synchronize the menstrual cycle and to obtain a spontaneous periodic contraction of myometrium (Bek *et al*., 1988). After 24 h, uterine horns were isolated under pentobarbital anaesthesia (60 mg kg^{-1}, i.p. injection), and divided by a transverse cut into four segments of an equal length in modified Krebs-Ringer bicarbonate (KRB) solution (mM): NaCl 122, KCl 5, $CaCl_2$ 2.4, $MgSO_4$ 1, $NaHCO_3$ 26, EDTA 0.03, and dextrose 11, pH 7.4. Each segment was cut along the mesosalpinx insertion, and placed in a tissue chamber filled with 30 ml modified KRB solution under bubbled of 95% O_2/5% CO_2 at 37°C.

In vitro *contractility measurements* Four strips prepared from the same uterus were used to examine the effects of test compounds on myometrial contractility in parallel. The contractility of uterine strip was monitored by an isotonic transducer (Nihon Kohden, TD-112S, Japan) with 1 g tension, and recorded on a polygraph (Rikadenki, R-62, Japan). After a 40 min equilibrium of the spontaneous periodic contraction, the strip was preincubated with or without 1 μM AM_{22-52}, a putative antagonist for AM receptors (Eguchi *et al*., 1994), or 1 μM $CGRP_{8-37}$, a putative antagonist for CGRP receptors (Chiba *et al*., 1989), for 15 min, and exposed to 1–100 nM AM in a cumulative manner. In a separate experiment, uterine

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contraction was caused by 1 nM oxytocin, 1 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), or 10 nM bradykinin (BK), and the effects of 100 nM AM on the uterine contraction were examined in the absence or presence of AM_{22-52} or $CGRP_{8-37}$. Uterine strips were finally contracted by 45 mM KCl to confirm the uterine responsiveness. Peptides were obtained from Peptide Institute (Osaka, Japan).

Reverse transcription-polymerase chain reaction (RT–PCR) of AM and CGRP receptors Total RNA was extracted from rat uterus using TRIzol™ (Life Technologies, Tokyo, Japan) and RT–PCR was performed with a thermal cycler (Perkin Elmer Corp., Norwalk, CT, U.S.A.) using a RT–PCR kit (Toyobo, Osaka, Japan). According to the published sequences appeared in GenBank, we constructed primers specific for rat CRLR and RAMP1-3 as follows; CRLR (L27487), 5′-CCAAACA-GACTTGGGAGTCACTAGG-3′ (forward) and 5′-GCTGT-CTTCTCTTTCTCATGCGTGC-3′ (reverse), RAMP1 (AB02-8933), 5′-CACTCACTGCACCAAACTCGTG-3′ (forward) and 5′-CAGTCATGAGCAGTGTGACCGTAA-3′ (reverse), RAMP2 (AF162778) 5′-AGGTATTACAGCAACCTGC-GGT-3′ (forward) and 5′-ACATCCTCTGGGGGATCGGA-GA-3′ (reverse), RAMP3 (AB028935) 5′-ACCTGTCGGA-GTTCATCGTG-3′ (forward) and 5′-ACTTCATCCGGGG-GGTCTTC-3′ (reverse). The predicted sizes of PCR products using each primer pair are shown in Figure 3. PCR was performed using the following conditions: 30 cycles of 94°C 30 s, 60°C 30 s, and 72°C 90 s. At the end of PCR, samples were kept at 72°C for 10 min for final extension. The amplification products were separated by electrophoresis (2% agarose gel) and visualized by ethidium bromide staining. The PCR products were cut from agarose gel, purified by QIA quick gel extraction kit (Qiagen Gmbh, Hilden, Germany). The nucleotide sequence was determined by ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.).

Statistics Data (mean ± s.e.mean) were evaluated statistically of two-way ANOVA or one-way ANOVA with multiple comparisons using the Dunnett's test.

Results Uterine strips isolated from oestrogenized rats spontaneously contracted in a periodic manner (Figure 1a). Bath application of AM, in a cumulative fashion, inhibited the spontaneous periodic contraction in a concentration-dependent manner ($IC_{50} = 22.3 \pm 0.7$ nM) (Figure 1b,c,f), whereas the addition of vehicle alone had no effect (Figure 1a). The inhibitory effect of AM was reversible upon washout, even when the myometrium was completely relaxed by 100 nM AM (Figure 1c). Preincubation with either 1 μM AM_{22-52} or 1 μM $CGRP_{8-37}$ had no effect *per se*, but prevented the relaxing effects of 1–100 nM AM (Figure 1d–f) AM had no effect on high K^+-induced tonic contraction (Figure 1b).

We then examined the effects of AM on periodic uterine contraction caused by oxytocin, $PGF_{2\alpha}$ or BK. In uterine strips of non-oestrogenized rats, they contracted spontaneously with variable intervals and amplitudes (Figure 2a–e). Either 1 nM oxytocin (Figure 2a), 1 μM $PGF_{2\alpha}$ (Figure 2b), or 10 nM BK (Figure 2c) significantly stimulated the contractility. AM, at 100 nM, completely blocked the BK-induced contraction (Figure 2c) whereas it had no effect on the oxytocin- or $PGF_{2\alpha}$-induced periodic contraction even at 100 nM (Figure 2a,b). The inhibitory effect of AM on BK-induced periodic contraction was substantially prevented by preincubation of AM_{22-52} or $CGRP_{8-37}$ (Figure 2d,e). The inhibitory effect of AM on BK-induced periodic uterine contraction was also observed in uterine strips of oestrogenized rats (data not shown).

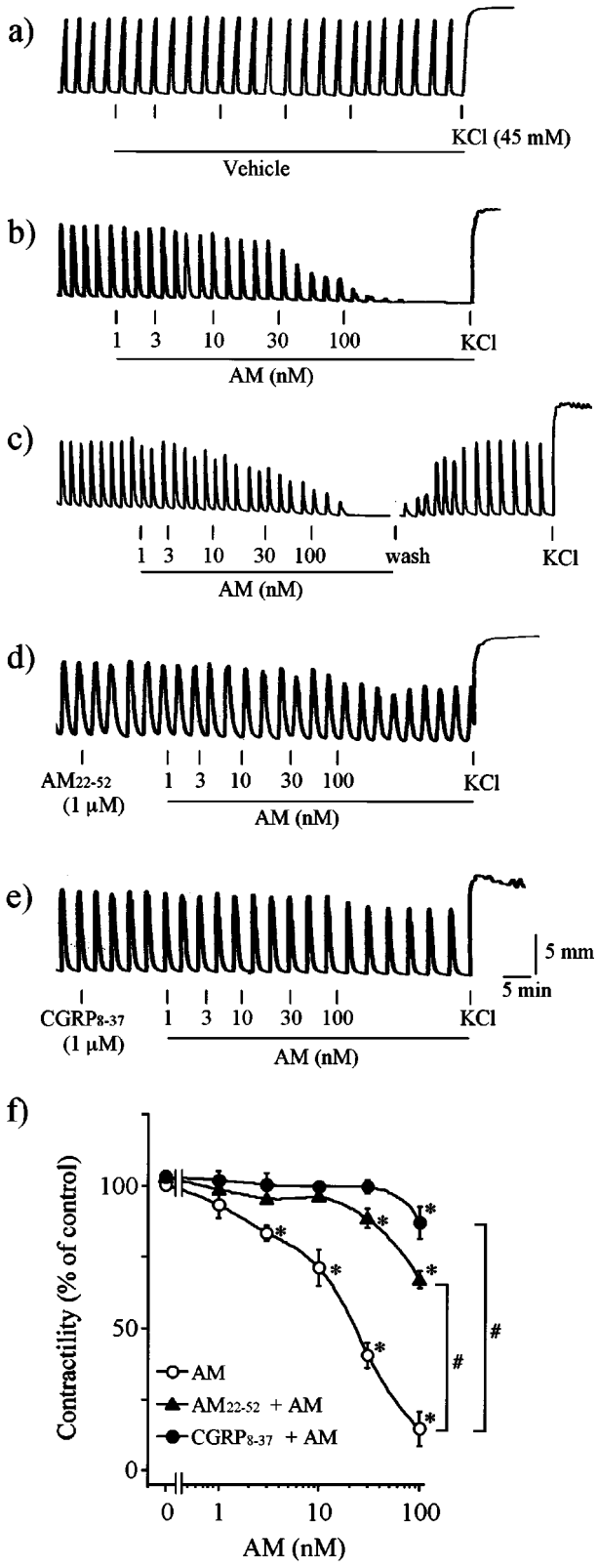

Figure 1 Effects of AM on spontaneous periodic contraction. Uterine strips of oestrogenized rats were treated with (a) vehicle; (b,c) AM; (d) AM_{22-52} + AM; (e) $CGRP_{8-37}$ + AM. Typical recordings from five separate experiments with similar results were shown. (f) effect of AM_{22-52} or $CGRP_{8-37}$ on the AM-induced inhibition of the contraction. $^*P < 0.05$, compared with the response without any peptide (one-way ANOVA); $\#P < 0.05$, compared with AM alone (two-way ANOVA).

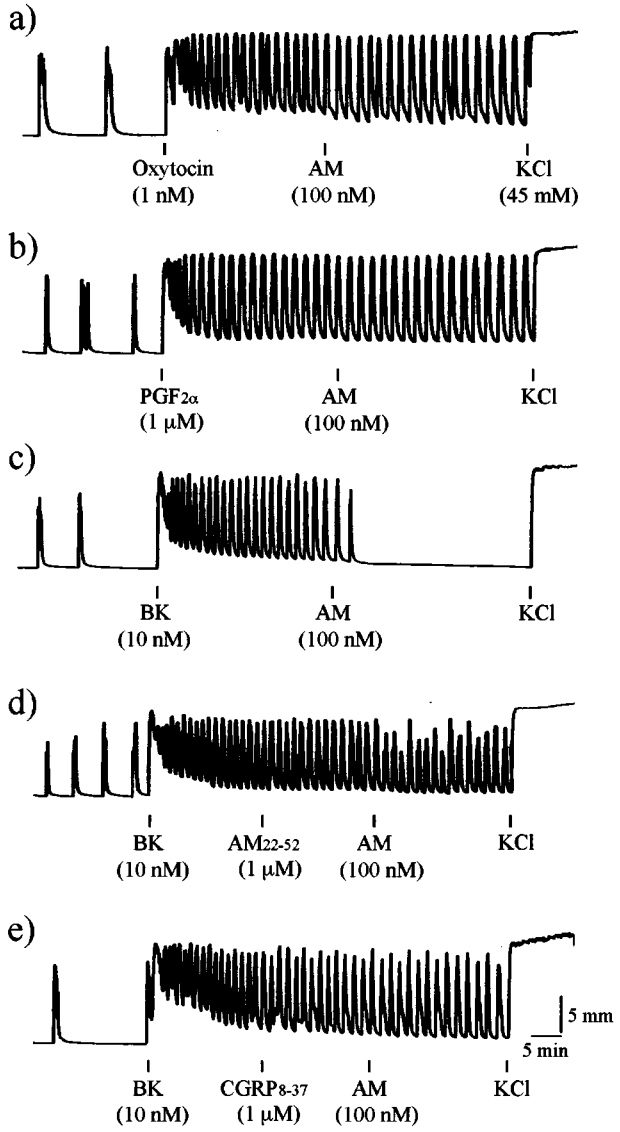

Figure 2 Effects of AM on periodic contraction caused by oxytocin, $PGF_{2\alpha}$ or BK. Uterine strips of non-oestrogenized rats were pretreated with (a) oxytocin; (b) $PGF_{2\alpha}$; (c–e) BK and further treated with (a–c) AM; (d) AM_{22-52}+AM; (e) $CGRP_{8-37}$+AM. Typical recordings from five separate experiments with similar results were shown.

RT–PCR analysis showed that rat uterus expressed mRNAs for CRLR, RAMP1, RAMP2, and RAMP3. The PCR products were sequenced and found to be identical to the reported sequences of the rat CRLR and RAMPs (Figure 3).

Discussion Our main finding is that AM reversibly inhibits spontaneous periodic uterine contraction in a concentration-dependent manner. Thus our finding demonstrates that AM has a regulatory function in female reproductive system as a modulator of uterine contractility.

We also observed that AM inhibited BK-induced periodic uterine contraction, while it did not affect oxytocin- or $PGF_{2\alpha}$-induced periodic and high K^+-induced tonic contraction. These results suggest that AM does not have an active dilatory effect on myometrium. The inhibitory effect of AM could be operated by the prevention of mechanism(s) for spontaneous and/or BK-induced periodic contractions.

The inhibitory effect of AM on the periodic uterine contraction was antagonized by AM_{22-52} or $CGRP_{8-37}$,

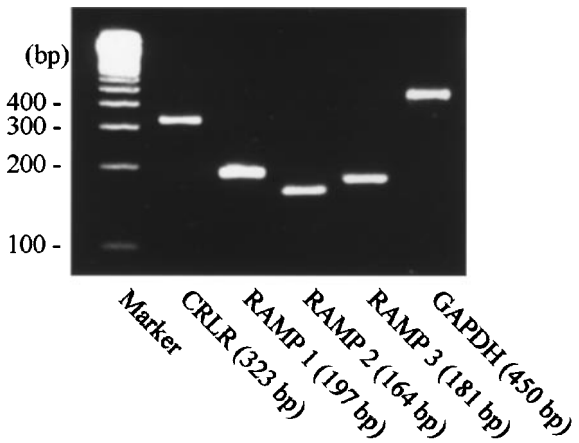

Figure 3 Expression of mRNA for CRLR, RAMPs in rat uterus. RT–PCR products of CRLR and RAMPs were separated by agarose gel electrophoresis. The DNA size marker was 100 bp ladder as shown on the left. The predicted sizes of RT–PCR products are shown at the bottom.

suggesting that AM reacted with receptors for AM and CGRP. In addition, our RT–PCR experiments showed that rat uterus expressed mRNAs for CRLR, RAMP1, RAMP2 and RAMP3, although we cannot exclude the possibility that mRNAs for CRLR or RAMPs may come from blood vessels in the uterus. Collectively, our results suggest that rat uterus possesses both AM receptors and CGRP receptors both of which are reactive to AM (Fraser *et al*., 1999). Our results are in agreement with those of previous report in which both ^{125}I-AM binding and ^{125}I-CGRP binding were entirely displaced by AM in membrane fraction prepared from rat uterus (Upton *et al*., 1997). Although AM_{22-52} is generally accepted as an antagonist for AM receptors (Eguchi *et al*., 1994; McLatchie *et al*., 1998), it is reported to suppress CGRP (but not AM)-induced vascular dilation in cat hindlimb (Champion *et al*., 1997). If AM_{22-52} is acting on CGRP receptors, AM might act *via* CGRP receptors in the rat uterus.

Levels of AM peptide and mRNA in rat uterus are as high as those in adrenal medulla (Upton *et al*., 1997; Cameron & Fleming, 1998). In human and rat uterus, AM is mainly located in the endometrium (Cameron & Fleming, 1998; Michishita *et al*., 1999). These findings suggest that AM may act on the myometrium in a paracrine manner.

It has become evident that levels of AM, AM receptors and CGRP receptors significantly increase during pregnancy in various maternal and foetal tissues. Abundance of AM mRNA in uterus increased 1.8 to 4.5 fold during pregnancy (Upton *et al*., 1997; Makino *et al*., 1999). Immunohistochemical analyses showed that trophoblast giant cells in placenta produced a large amount of AM, and secreted it into the surrounding maternal and foetal tissues (Yotsumoto *et al*., 1998). In pregnant rat uterus, AM binding sites and CGRP binding sites were increased by 10 and 4 fold, respectively (Upton *et al*., 1997; Dong *et al*., 1998), whereas the amount of CGRP peptide decreased to undetectable level (Upton *et al*., 1997). These findings suggest that the increased binding sites of AM and CGRP as well as the increased local production of AM, but not CGRP, in pregnant uterus play a crucial role in the maintenance of uterine quiescence.

In the present study, AM selectively inhibits BK induced, but not oxytocin- or $PGF_{2\alpha}$-induced periodic uterine contraction. The abnormal expression of BK, an autacoid causing inflammatory responses (De La Cadena *et al*., 1991), has been implicated in triggering infection-driven preterm labour

(Schrey *et al*., 1992), whereas oxytocin- or $PGF_{2\alpha}$-induced uterine contraction is generally thought to be involved in normal labour. These findings imply that AM selectively inhibits preterm contraction caused by BK, and it might contribute to the maintenance of pregnancy without interfering normal labour promoted by oxytocin or $PGF_{2\alpha}$.

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