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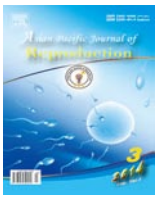

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Intermedin inhibits norepinephrine-induced contraction of rat seminal vesicle

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ABSTRACT

Objective: To study the effect of inter medin(IMD) on smooth muscle of rat seminal vesicles including the specific receptors and the signal pathways involved.**Methods:** The contraction of the seminal vesicle in response to norepinephrine (NE) and ADM2/IMD was studied by the organ bath method. The effects of antagonists for calcitonin gene related peptide (CGRP), adrenomedullin (ADM) and IMD receptors, and inhibitors of nitric oxide synthase, [L-NG-Nitroarginine Methyl Ester, L-NAME) and cAMP-dependent protein kinase (PKA), KT5720] were also investigated. The first overshoot, amplitude, frequency and basal tone were measured. **Results:** There is no significant effect of IMD on the initial overshoot, frequency and the basal tone in the seminal vesicle contraction. Only the amplitude of the contraction induced by NE was inhibited by IMD. The IMD inhibitory actions on amplitude were completely blocked by hADM22-52 and L-NAME, but not by hCGRP8-37 or KT5720. Furthermore, the action was diminished by IMD17-47. **Conclusion:** The results demonstrated that the inhibitory action of IMD on NE-induced seminal vesicle contraction was mediated via the ADM receptor(s) and the nitric oxide production pathway, partially by the IMD receptor, but not by the CGRP receptor and the cAMP-PKA pathway.

1. Introduction

Intermedin (IMD)/adrenomedulin 2(ADM-2), a novel peptide discovered in 2004[1,2], belongs to the calcitonin (CT)/ calcitonin gene-related peptide (CGRP) superfamily. The peptide was named intermedin because it was first discovered in the intermediate lobe of the pituitary[2]. Prepro-IMD was found to be a 146-150-amino-acid peptide and the C-terminal of its prepro-peptide showed about 30% and <20% sequence identity with adrenomedullin (ADM) and CGRP respectively. Three biologically active forms of IMD peptides can be produced from prepro-IMD after post-translational modification. They are a 47-amino-acid IMD (IMD1-47), a shorter 40-amino-acid IMD (IMD8-47) and the longest 53-amino-acid IMD (IMD1-53). They are generated by proteolytic cleavage at their different respective N-terminal sites[3].

Since IMD shares about 30% and 20% sequence homology with ADM and CGRP respectively, it shows vasodilatory effects with the action mediated by CGRP and ADM receptors[2-4]. ADM and CGRP preferentially bind to the calcitonin receptor-like receptor (CRLR)/specific receptor activity modifying proteins (RAMP) 2&3 and CRLR/RAMP1 receptor complexes respectively[5,6] while IMD shows similar affinity to all the 3 types of CRLR receptor complexes[2]. However, IMD may preferentially activate the ADM2 receptor[7]. Much work has been done recently in our laboratory on the roles of ADM in reproduction [8-12]. Wong *et al.* has also reported

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the expression of IMD mRNA, its peptide, and the receptor component in female reproductive organs including the ovary, oviduct and the uterus[13]. They also studied the inhibitory effects of IMD on uterine contraction and the signal pathways involved[13]. The inhibitory effects of ADM on norepinehrine-induced contraction of rat seminal vesicle have also been documented[14], which suggested that ADM plays a possible role in the control of seminal vesicle muscle contraction during ejaculation. All the evidence supports the hypothesis that IMD, may also play a role in the regulation of seminal vesicle contraction during ejaculation. The effects of IMD on NE-induced contraction of rat seminal vesicle was therefore investigated, together with the receptors and some signal pathways involved.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rat of 9-10 weeks was obtained from the Laboratory Animal Unit, Univeristy of Hong Kong Faculty of Medicine. The rats were killed by an overdose of sodium pentobarbitone. All the procedures were approved by the Committee on the Use of Live Animals for Teaching and Research (University of Hong Kong).

2.2. Immunohistochemistry

Paraffine sections of seminal vesicles were used for immunohistochemical (IHC) staining for IMD. The streptavidin-biotin-peroxidase complex method was used which involves the incubation of the sections with primary antibody specific to IMD (cat. no. H-010-52, Phoenix Pharmaceuticals, California, USA), a biotylinated secondary antibody that reacts with primary antibody, enzyme labeled streptavidin, and DAB substrate chromogen. The Rabbit ABC staining system kit (SC-2018, Santa Cruz Biotechnology, Inc. Santa Cruz, California, USA) was used.

2.3. Organ bath experiment

The coagulating glands and blood vessels connecting to the seminal vesicles were carefully removed before the seminal vesicles were dissected out. The seminal vesicles were washed free of fluid by Kreb's solution injected from the syringe and were placed into the oxygenated Kreb's solution containing 118 mM NaCl, 4.8 mM KCl, 1 mM $MgSO_4$, 1.15 mM NaH_2PO_4, 15 mM $NaHCO_3$, 10.5 mM glucose, and 2.5 mM $CaCl_2$. They were then tied with silk threads to holder electrodes in a 10 mL organ bath attached to a force transducer (Model 7 Polygraph, Grass, Massachusetts, USA). The tissues were perfused with Kreb's solution equilibrated with 95% O_2-5% CO_2 at 37 ℃. A tension of 0.1 g was applied and the tissues were allowed to stabilize for 50 minutes before the addition of drugs. To examine the effect of IMD on the NE induced contraction of seminal vesicles, the tissues were treated with 10 μM NE plus/minus 100 nM human IMD1-53 (cat no. 010-08, Pheonix Pharmaceuticals , California, USA) after equilibration for 50 minutes and the amplitudes of contractions were recorded for 20 minutes. For studying the receptors, the tissues were pre-incubated with the receptor antagonists for 30 minutes after 20 minutes equilibration and before the addition of NE and IMD. Receptor antagonists such as hADM22-52, hCGRP8-37 and IMD17-47 (cat. no. 010-04, 015-06 and 010-57, Phoenix Pharmaceuticals, California, USA), and enzyme inhibitors including KT5720 (cat. no. K3761, Sigma, St Louis, Missouri, USA) and L-NAME, (cat. no. 483125, Calbiochem, EMD Biosciences, San Diego, California, USA) were used in this study. Each treatment had its control from the same rat treated with 10 μM NE only. The basal tone, frequency, the first overshoot and contraction amplitude were measured from the period 2 to 12 mins after addition of NE and expressed as the percentage change compared to the control sample.

2.4. Statistics

The percentage changes were expressed as mean ± SEM and the statistical significance was assessed by One-way ANOVA followed by the Least Significant Difference (LSD) post-hoc test. The homogeneity of variances was also tested and Dunnett's T3 tests were used instead of LSD when $P<0.05$. All statistical analyses were performed using IBM SPSS Statistics 21 (Chicago, USA) and statistic significance was indicated when P was equal to or less than 0.05.

3. Results

3.1. Immunohistochemistry

IMD immunoreactivity was localized to the epithelial cells and some fibroblasts inside the epithelium (Figure 1).

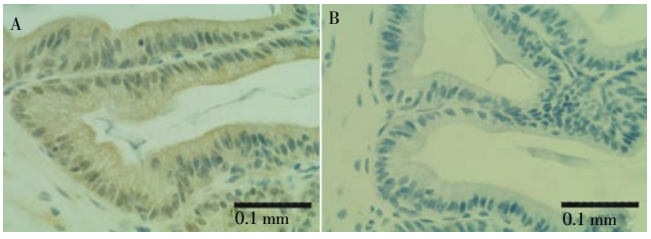

Figure 1. Immunocytochemical study of IMD in the rat seminal vesicles.
Positive IMD immunoreactivity localized to the epithelial cells and fibrobalsts (A) as compared to the negative control section (B).

3.2. Organ bath experiment

The addition of NE (10 μM) caused an increase in the contraction (first overshoot) of seminal vesicle (Figure 2A). When IMD was added at the same time, there are no significant differences in basal tone, frequency and first overshoot in both 10 nM IMD and 100 nM IMD groups relative to control group treated with NE alone (the control group, Table 1). However, there was a significant decrease in contraction amplitude in both 10 nM IMD (P<0.01) and 100 nM IMD (P<0.001) groups compared with the control and also a significant decrease in the 100 nM IMD group versus the 10 nM group (Table 1). These suggest that there was a dose response of IMD in inhibiting NE-induced contraction. The decrease in contraction amplitude in response to 100 nM IMD was not decreased by hCGRP8-37 or KT5720 pre-treatment. On the other hand, the inhibitory action of IMD was completely abrogated by hADM22-52 and greatly reduced by L-NAME (Figure 3). A significant reduction in % decrease in amplitude (P<0.001) was observed. There was still a significant decrease (P<0.01) in contraction amplitude in IMD17-47 group relative to the control group in spite of a significant reduction (P<0.01 compared with 100 nM IMD group (Figure 3). This suggests that the action of IMD was only partially blocked by IMD17-47. In all these studies, the receptor antagonists and enzyme inhibitors alone had no effect on the NE-stimulated contraction.

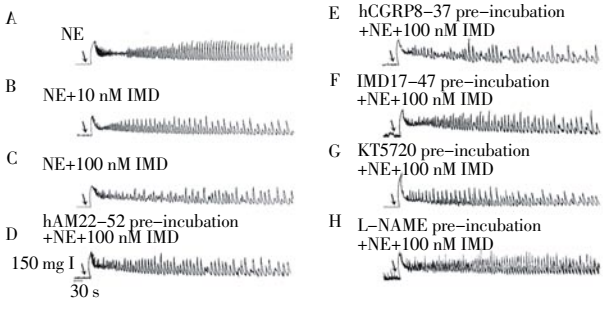

Figure 2. Tracings of seminal vesicle contraction induced by NE and the effects of receptor antagonists and KT5720 and L-NAME IMD dose-dependemtly inhibited muscle contracton induced by NE in seminal vesicles (A-C). The increase in contraction amplitude was inhibited by 100 nM IMD (C). hADM22-52 (D), but not hCGRP8-37 (E), completely blocked the inhibitory effect of IMD. IMD17-47 also partially blocked the inhibitory effect of IMD (F). KT5720, did not block the inhibitory effect of IMD (G), unlike L-NAME (H).

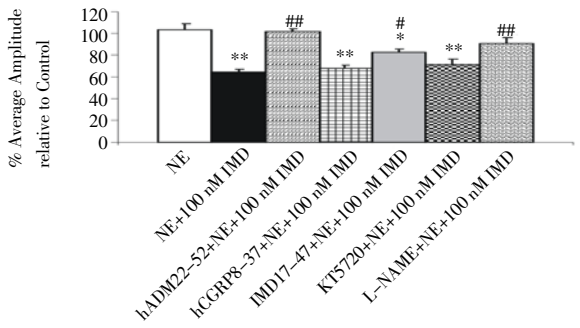

Figure 3. Inhibiton of amplitude response to NE by IMD and the effects of receptor antagonists and KT5720 and L-NAME.

Data presented as mean ± SEM. (n=5, except group hCGRP8-37 (n=4) and group IMD17-47 (n=7). $^{*}P$<0.01, $^{**}P$<0.001 *vs*. NE; $^{\#}P$<0.01, $^{\#\#}P$<0.001 *vs*. 100 nM IMD.

Table 1

Dose response to IMD in inhibiting NE-stimulated muscle contraction in the seminal vesicle.

% relative to NE-stimulated only	IMD dose		
	0 nM	10 nM	100 nM
Basal tone	93.17±14.17	77.45±18.63	67.80±13.42
Frequency	100.49±4.17	98.00±1.62	96.73±3.63
First overshoot	99.46±7.23	91.68±15.21	71.57±11.65
Average amplitute	103.12±5.81	82.06±7.73*	64.53±2.91$^{**\#}$

One-way analysis of variance followed by post-hoc LSD test. $^{*}P$<0.01, $^{**}P$<0.001 compared with NE and $^{\#}P$<0.01 compared with 10 nM IMD. Data presented as mean ± SEM (NE, NE+10nM IMD, n=5; NE+100nM IMD, n=6).

4. Discussion

An inhibitory effects of IMD on contractile response of smooth muscle has been reported for the uterus[13]. Together with, the positive staining for the peptide, the inhibitory effect of IMD on seminal vesicle contraction here suggests that IMD may be involved in the regulation of seminal vesicle contraction.

Our findings from studies using receptor antagonists and enzyme inhibitors suggests that IMD inhibits seminal vesicle contraction mostly by a specific ADM receptor and via NO pathway, and the CGRP receptor and cAMP-dependent protein kinase pathway are not involved. In the smooth muscles of the vasculature, the NO pathway was involved in the vasodilatory actions of IMD[4, 14-15]. cAMP has been reported to be involved in IMD vasorelaxant actions on the coronary artery[14] and aortic rings[16] but not on pulmonary artial rings[17]. The result also suggests that the specific IMD receptor antagonist may only partially block the IMD receptor. These findings are similar to those for IMD in uterine contraction in that in both organs the NO pathway was involved but the PKA pathway was not[13]. They differ from the finding in the uterus in the relative importance of the receptors involved. In the uterus, both CGRP and ADM receptor

antagonists partially blocks while the IMD receptor antagonist completely blocks the inhibitory actions of IMD[13].

These results are different from the previous study of inhibitory effects of ADM where the inhibition of smooth muscle contraction was prevented by hCGRP8-37 and was not mediated by the NO pathway[18]. These findings suggest that different pathways and receptors mediate the effect of different peptides such as ADM (via CGRP receptor) and IMD (via ADM1, ADM2 receptor and specific IMD receptor). Besides various inhibitors[19-21] and ADM[18], IMD may be a possible therapeutic agent for inhibiting seminal vesicle contraction to delay ejaculaton stimulated by the sympathetic system.

Conflict of interest statement

We declare that we have no conflict of interest.

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