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ESTIMATION BY AN ELECTROPHYSIOLOGICAL METHOD OF THE EXPRESSION OF OXYTOCIN RECEPTOR mRNA IN HUMAN MYOMETRIUM DURING PREGNANCY

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Summary—In order to evaluate the changes in uterine oxytocin receptor-specific mRNA during pregnancy, receptor expression in *Xenopus* oocytes are examined electrophysiologically following microinjection of mRNA from human uterus. In voltage-clamped oocytes injected with term myometrial mRNA, oxytocin elicited an inward current response. The amplitude of the oxytocin-induced current increased with increasing dose of oxytocin, but no current was elicited following stimulation with vasopressin. The oxytocin-induced current was completely eliminated as a result of pretreatment with a specific oxytocin antagonist. 21 of 27 oocytes injected with term myometrial mRNA showed a large amplitude (77.0 ± 16.1 nA) reaction to oxytocin. In comparison, only 3 of 13 oocytes injected with early gestational myometrial mRNA exhibited a small amplitude (4.6 ± 1.4 nA) reaction to oxytocin. No oxytocin response was observed in oocytes injected with non-pregnant myometrial mRNA. These results indicate that the striking increment in oxytocin sensitivity in term uterus depends on the increase in mRNA encoding oxytocin receptors.

INTRODUCTION

Parturition, the final and most dramatic event of human pregnancy, can be divided into three distinctive but overlapping phases. The first phase is the time of uterine preparedness for labor. The second phase is the time of forceful uterine contractions of active labor and delivery. The last phase is the time of puerperal contraction and involution of the uterus [1]. It has been reported that several compounds cause the contraction of uterine smooth muscle cells [2]. Among them, oxytocin and prostaglandins are the most potent compounds for causing uterine contraction. As compared with prostaglandins and its derivatives which have relatively high contractive effectiveness not only to pregnant uterus but also to non-pregnant uterus [3], oxytocin can effectively induce the contraction only to the uterus after the beginning of preparedness for labor. Although oxytocin is widely used for the induction of labor by obstetricians, we have often experienced the ineffectiveness of oxytocin for patients in term. Therefore, many investigators are interested in the mechanism by which the uterus reacts to oxytocin just before the appropriate time for labor. Analysis of this mechanism is expected to provide clues for the answer to the question of how delivery can occur spontaneously at term in most cases.

Since Soloff *et al.* [4] first reported that oxytocin specific bindings to rat uterine myometrium increased remarkably just before the beginning of labor [4], several investigators have observed the same phenomena in human uterus [5–7]. These studies on oxytocin receptors were performed with a binding assay. However, this method can not reveal the effect of gene control of oxytocin receptors on the myometrium during pregnancy.

Recent developments in molecular biological technology have made it possible to express various kinds of membrane receptors and ion channels on the surface membrane of *Xenopus*

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oocytes. It has been reported that the expression of several membrane receptors on the oocytes can be detected using an electrophysiological method when these receptors are coupled to the inositol phospholipid–protein kinase C (IP_3–PKC) system [8]. As oxytocin causes inositol phospholipid hydrolysis [9–12], the oxytocin receptor is also thought to couple to the IP_3–PKC system in *Xenopus* oocytes. Actually, Morley *et al.* [13] reported that oocytes injected with mRNA extracted from bovine endometrium reacted to oxytocin [13].

In order to investigate whether the increase in oxytocin receptors on the uterine myometrium after the preparedness of labor is regulated on the level of oxytocin receptor mRNA expression or on another level, we compared the expression of the oxytocin receptor mRNA in uterine myometrium among non-pregnant women, women in an early stage of pregnancy and term pregnant women with the aid of membrane current measurements under voltage-clamp conditions in *Xenopus* oocytes injected with poly $(A)^+$RNA from uterine myometrium.

EXPERIMENTAL

Samples

Non-pregnant uterine myometrium, pregnant uterine myometrium at an early gestational stage (13th week) and pregnant uterine myometrium at term (38th week) were obtained with informed consent from surgical specimens. Pregnant myometrium at term was from a patient with uterine rupture noticed just after vaginal delivery. These tissues were washed with ice cold PBS to remove blood, snap-frozen in liquid nitrogen and stored at −70°C until used for RNA extraction.

Preparation of Xenopus laevis oocytes. Adult *Xenopus laevis* females were obtained from Hamamatsu Seibutsu Kyozai (Shizuoka, Japan) and maintained at 18°C. Ovary fragments were excised from frogs aseptically under hypothermic conditions to provide adequate anesthesia. Oocytes were defolliculated by 3 h treatment with 2 mg/ml collagenase (Sigma, St Louis, MO, U.S.A.) in modified Barth's saline medium (MBS, see below) from which Ca^{2+} was omitted. Oocytes were cultured in MBS medium consisting of: NaCl, 88 mM; KCl, 1 mM; $Ca(NO_3)_2$, 0.33 mM; $CaCl_2$, 0.41 mM; $MgSO_4$, 0.82 mM; $NaHCO_3$, 2.4 mM; and Tris–HCl (pH 7.6), 7.5 mM supplemented with gentamycin (10 μg/ml) and mycostatin (5 U/ml) at 19°C before and after the microinjection. The medium was changed at least once a day at which time any dead or dying cells were removed.

Extraction of RNA and microinjection into oocytes. RNA was prepared with the guanidium–CsCl method [14] with slight modifications. 10 g of frozen tissue was crushed in liquid nitrogen and dissolved in 100 ml of 5.5 M guanidium isothiocyanate, 100 mM 2-mercaptoethanol, 0.5% Sarcosyl, 25 mM sodium citrate (pH 7.0), layered onto a 20 ml cushion at 5.7 M CsCl, 0.1 M EDTA (pH 7.0) and centrifuged for 24 h at 20°C in a SRP28 rotor (Hitachi Koki Co. Ltd., Ibaraki, Japan) at 25,000 rpm. The RNA pellet was washed with cold 75% ethanol, dissolved in TE buffer [10 mM Tris–HCl and 1 mM EDTA (pH 7.2)] and reprecipitated with ethanol. For isolation of mRNA, the total RNA was chromatographed over a 1.5 ml column of oligo-dT cellulose [15] (Type 3; Collaborative Research Inc., Bedford, MA, U.S.A.), precipitated with ethanol and dissolved in deionized distilled (dd)H_2O at 1.0 mg/ml. The quantity of mRNA was assessed by spectrophotometry at 260 nm and the quality monitored by means of the electrophoresis pattern in formaldehyde/formamide agarose gel and the results of Northern blotting with β-actin cDNA probe (Wako Pure Chemical Industries, Ltd, Osaka, Japan) [16].

After the selection of healthy Dumont stage V and VI oocytes [17], the cells were injected with 50 nl (= 50 ng) of mRNA. Control oocytes were not injected or injected with 50 nl of ddH_2O. Injected cells were cultured in MBS medium for 30–48 h at 19°C.

Electrophysiological measurements

Whole cell current measurements were performed with a conventional two-microelectrode voltage-clamp method [18]. Voltage recording and current injecting micropipettes were filled with 3.0 M KCl solution and had electric resistance between 1.0 and 4.0 MΩ. The oocyte was placed in a small bath which was continuously perfused with amphibian saline consisting of: NaCl, 115 mM; KCl, 1 mM; Tris–HCl (pH 7.2), 5 mM and $CaCl_2$, 1.8 mM. After insertion of the micropipettes, the membrane resting potential was between −30 and −70 mV. This potential was controlled with a voltage-clamp amplifier (Nihon Koden CEZ-1100, Tokyo, Japan). The membrane current signal was filtered through a low path filter (RC = 30 Hz) and recorded on a

pen recorder (Nihon Koden WT-625G). The assayed ligands were perfused at the indicated concentrations dissolved in amphibian saline. The measurements were made at room temperature.

Ligands and chemicals

The synthetic oxytocin was from Sigma. [Lys^8]-vasopressin and [Arg^8]-vasopressin were from Peninsula Labs (Belmont, CA, U.S.A.). [d(CH_2)$_5$Tyr(OMe)^{2}Orn^8]-vasotocin, the antagonist of oxytocin [19] was obtained from Peninsula Lab. All other chemicals used in our experiments were of analytical grade.

Statistical analysis

Chi-square test and Mann-Whitney tests were performed with a StartFlex personal computer program (ViewFlex, Tokyo, Japan). The calculated results represent mean ± SEM.

RESULTS

To ascertain that the receptor expressed on mRNA injected oocytes is oxytocin specific, the electrical responses in the oocytes were analyzed by injecting into the oocyte about 50 ng of mRNA from pregnant uterine myometrium at term. Figure 1(A) shows the responses of a single oocyte to 3 sequential stimuli of 10^{-8}, 10^{-7} and 10^{-6} M of oxytocin. No response could be detected to 10^{-8} M of oxytocin. And as shown in Fig. 1(B), the amplitude of membrane current elicited by oxytocin increased in a dose dependent manner. To determine further whether acquired oxytocin response was mediated by the oxytocin-specific receptor and not by other receptors which crossreact with oxytocin, we compared the responses to vasopressin and to oxytocin. As seen in Fig. 2(A), no obvious responses were observed when the oocyte was stimulated by either 10^{-6} M of [Lys^8]-vasopressin or 10^{-6} M of [Arg^8]-vasopressin. We investigated the effect of one of several compounds that have been found to be oxytocin receptor antagonists [18, 19], [d(CH_2)$_5$Tyr(OMe)^2,Orn^8]-vasotocin. The oocyte that responded to 10^{-7} or 10^{-6} M of oxytocin was treated with 10^{-6} M of [d(CH_2)$_5$Tyr(OMe)^2,Orn^8]-vasotocin for 1 min after which 10^{-7} or 10^{-6} M of oxytocin was applied. The response to oxytocin was completely inhibited by the antagonist treatment [Fig. 2(B)]. These findings indicate that the electrophysiological response of the mRNA

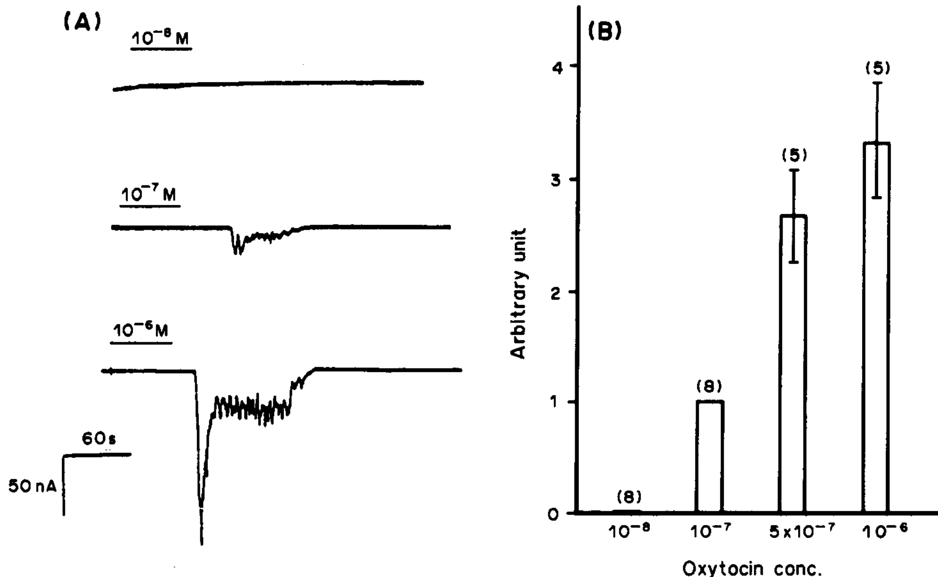

Fig. 1. Concentration dependence of oxytocin responsiveness in voltage-clamped oocytes injected with term myometrial mRNA. (A) Oxytocin-induced membrane currents of voltage-clamped oocyte injected with term myometrial mRNA. 10^{-8}–10^{-6} M of oxytocin was applied to the same oocyte sequentially as indicated by bars. Between the stimulations, amphibian saline was perfused for more than 15 min. (B) Eight oocytes were stimulated with 10^{-8} M of oxytocin but none responded. When 10^{-7} M of oxytocin was applied, the mean amplitude of the responses was defined as 1.0. The same oocyte was stimulated sequentially by 5×10^{-7} M or 10^{-6} M or both. When response amplitude was evaluated in terms of the ratio to the response of 10^{-7} M oxytocin, every oocyte showed larger responses to higher concentrations of oxytocin. Bars represent SEM. The numbers indicate the number of oocytes per data point.

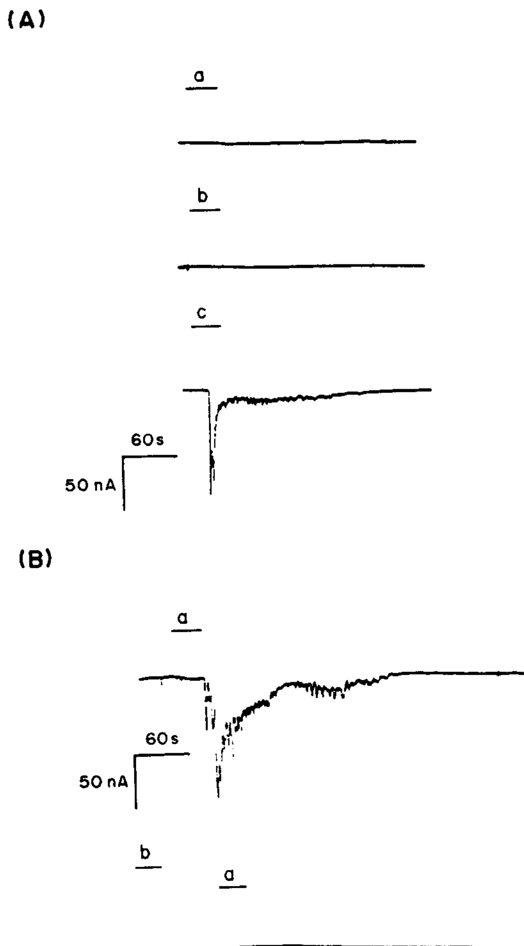

Fig. 2. (A) Changes in membrane currents of oocyte injected with term myometrial mRNA in response to [Lys^8]-vasopressin (a), [Arg^8]-vasopressin (b) and oxytocin (c). 10^{-6} M of each ligand was applied to the same oocyte sequentially as indicated by bars. Between the stimulations, amphibian saline was perfused for more than 15 min. The oocyte that responded to oxytocin showed no changes in membrane current when [Lys^8]- or [Arg^8]-vasopressin was applied. (B) Effect of oxytocin-specific antagonist [d(CH$_2$)$_5$Tyr(OMe)^5,Orn^8]-vasotocin on the oxytocin-induced membrane current. The oocyte which was injected with term myometrial mRNA and responded to 10^{-6} M of oxytocin (a) was perfused for 15 min with amphibian saline. 10^{-6} M of antagonist was then applied (b) just before the stimulation with 10^{-6} M of oxytocin (a). This antagonist completely inhibited any oxytocin responses. Results in (A) and (B) were reproducible in more than three oocytes.

injected oocyte to oxytocin is mediated by the oxytocin-specific receptor.

In order to examine the amount of oxytocin receptor-specific mRNA during pregnancy, we compared oxytocin-induced current among oocytes injected with mRNA from term, early gestational and non-pregnant myometrium. 21 out of 27 (78%) oocytes injected with mRNA from myometrium at term showed a response to 10^{-6} M of oxytocin. The amplitude of the inward current was 77.0 ± 16.1 nA (mean $\pm$ SEM) with a maximum response of 225 nA. On the other hand, 3 out of 13 (23%) oocytes injected with mRNA from early stage of gestational myometrium responded to 10^{-6} M of oxytocin. The mean amplitude of the inward current of the 3 oocytes was 4.6 ± 1.4 nA. Finally, 0 of 14 oocytes injected with non-pregnant myometrium mRNA showed any response to 10^{-6} M of oxytocin stimulation (Table 1).

Serially diluted mRNA from term myometrium was injected to compare the level of oxytocin receptor-specific mRNA in the myometrium at different gestational stages. Oocytes used in this experiment were from a different *Xenopus laevis*. The mean amplitude of responses to 10^{-6} M oxytocin in the oocytes injected with 50 nl of 1 mg/ml mRNA was 122.5 ± 28.6 nA. When the mRNA was diluted to 1/10, 1/50 and 1/100 and the same volume (50 nl) was injected into each oocyte, the mean amplitude of oxytocin responses to 10^{-6} M of oxytocin were 85.0 ± 34.0 nA, 10.0 and 6.2 ± 0.6 nA, respectively. These responses were observed in 5/5, 2/5 and 5/9 oocytes injected with the respective diluted mRNAs (Fig. 3). These results suggest that the amount of oxytocin receptor-specific mRNA is around 1/50–1/100 in early gestational myometrium and $< 1/100$ in non-pregnant myometrium as compared with that in term myometrium.

DISCUSSION

Recent advances in molecular biological methods have made it possible to express membrane receptors for various kinds of ligands such as serotonin [20], vasopressin [21], angiotensin II [21], thyrotropin-releasing hormone [22], substance P [23] and substance K [24] in *Xenopus* oocytes injected with mRNAs. By using this method for screening the cDNA library, cloning of several cDNAs encoding the membrane receptor has been achieved [25–27].

Table 1. Comparison of oxytocin responses in oocytes injected with mRNAs extracted from non-pregnant, early stage gestation and term uterine myometrium

RNA origin	Response ratio	Current amplitude (mean $\pm$ SEM)
Non-pregnant myometrium	0% (0/14)	—
Early gestational myometrium	23% (3/13)	4.6 ± 1.4 nA[a]
Term myometrium	78% (21/27)	77.0 ± 16.1 nA[a]

[a] $P < 0.02$ (Mann-Whitney test); $\chi^2 = 25.1$; and $P < 0.01$.

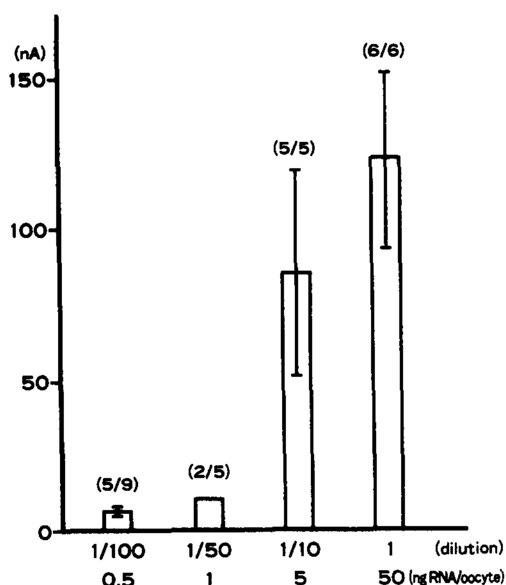

Fig. 3. Effect of dilution of term uterine myometrial poly(A)$^+$ RNA. Messenger RNA of 1 mg/ml was diluted to 1/10, 1/50 and 1/100 with ddH_2O and 50 nl of each sample was injected into oocytes. Each oocyte was exposed to 10^{-6} M of oxytocin and the amplitude of the membrane current was recorded. Bars represent SEM. In the numbers expressed as x/y, x denotes the number of responding oocytes and y the number of assayed oocytes. The mean value was calculated except for that of non-responding oocytes.

The present study using an electrophysiological method showed the responsiveness to oxytocin of *Xenopus* oocytes injected with mRNA from term myometrium. The typical response induced by oxytocin consisted of a rapid, transient and large inward current followed by a slow, prolonged and fluctuating oscillatory inward current. The reversal potential of this current was about −28 mV, implying that the current was carried predominantly by chloride ions (data not shown). This current was not elicited by vasopressin and was antagonized by the oxytocin-specific antagonist. Therefore, the receptor expressed on mRNA-injected oocytes is coupled to the IP_3–PKC system [3] and must be the oxytocin-specific receptor.

Clinically, oxytocin has been widely used for the induction of labor. However, oxytocin is effective only in patients in whom the preparation of the uterus for active labor has already been completed [1]. This change in responsiveness to oxytocin was thought to be due to the increment in oxytocin receptor molecules expressed in the uterine myometrium. However, regulation of oxytocin receptor expression at the gene level has not been demonstrated since oxytocin receptor cDNA has not yet been cloned. In the serial dilution experiment shown in Fig. 3, the amplitude of the membrane current elicited by oxytocin depended on the amount of the oxytocin receptor-specific mRNA contained in the poly (A)$^+$ RNA pool. A comparison of the oocyte responses summarized in Table 1 leads us to speculate that about 1/50 to 1/100 of the oxytocin receptor mRNA and $< 1/100$ of the mRNA in term myometrium may be expressed in the myometrium of, respectively, early gestation and non-pregnant state. It has been reported that an amount of only 1/100 of oxytocin is required to induce uterine contraction in pregnant women at term as compared with non-pregnant women [28]. Several investigators have also reported that the number of binding sites for oxytocin on membrane fractions of human uterine myometrium at term is more than 100 times higher than that on non-pregnant myometrial membrane [4–6]. The increase in oxytocin receptor mRNA observed in our experiment agrees with these clinical and experimental observations. However, our experiment could not clarify whether this increment in mRNA depends on the control of gene transcription or on the stability of oxytocin receptor mRNA. The existence of a molecule that antagonizes the oxytocin receptor activity in non-pregnant or early pregnant myometrium is also hypothesized. To address these points, experiments such as Northern blotting or a nuclear ‘run-off’ using a cDNA probe of the oxytocin receptor should be carried out. The hormones and factors that can induce this drastic increase in oxytocin receptor mRNA are also of great interest. In animal experiments, administration of estrogen increased the number of binding sites of oxytocin in non-pregnant rat myometrium while progesterone inhibited this effect [12]. It has been reported that plasma estradiol 17-β is maximal on the day of parturition and that the plasma estradiol 17-β/progesterone ratio closely parallels the change in oxytocin bindings in pregnant rat uterus [4]. In human, however, no clear relationship has been revealed between the concentration of plasma sex steroid hormones and the level of oxytocin receptors on uterine myometrium or the day of parturition [29, 30].

To investigate the mechanism controlling the induction of oxytocin receptor mRNA, the structure of the 5′ flanking region of the oxytocin receptor gene is of great interest. We are now in the process of compiling a cDNA

library from the mRNA of human term myometrium to obtain oxytocin receptor cDNA. This should aid the investigation of the oxytocin receptor gene structure in order to understand the transcriptional control of the oxytocin receptor gene leading to preparedness of labor in the uterus.

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