IN VITRO OVULATION OF TROUT OOCYTES : EFFECT OF PROSTAGLANDINS ON

SMOOTH MUSCLE-LIKE CELLS OF THE THECA

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ABSTRACT

Ovulation (active expulsion of oocyte from the mature follicle) of trout follicles matured in vivo can be induced in vitro by adding $\text{PGF}_{2\alpha}$ at doses of 1 and 5 $\mu\text{g/ml}$. PGE_2 is ineffective.

The in vitro induction of ovulation by $\text{PGF}_{2\alpha}$ is inhibited in a calcium free medium or by inhibitors of calcium influx, particularly by Mn^{++} and La^{+++} , suggesting that ovulation process implies active contraction of the smooth muscle cells of the theca.

A significant but partial inhibition is also observed with cytochalasin B (1 and 5 $\mu\text{g/ml}$) demonstrating that contraction of other cell types than muscle, containing actin-like filaments, may also participate in the process.

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PROSTAGLANDINS

INTRODUCTION

Induction of meiotic maturation of trout follicle oocytes in vitro has been reported under the influence of gonadotropin hormones and certain steroids (1, 2, 3). Under incubation conditions (isotonic, balanced salt solution) used thus far, ovulation has never followed maturation, even though these processes are connected in vivo.

It may be hypothesized that ovulation is due to follicular envelope (follicular cells and theca) activities, composed of at least two distinct, consecutive phases :

1) The first phase is preparatory and occurs simultaneously with oocyte maturation ; follicle cells detach from the oocyte with which they have been associated within the long pores traversing the zona radiata (zona pellucida) (4, 5). At the same time, collagen fibrils, abundant between smooth muscle-like cells of the theca (6), seem to be attacked by enzymes similar to the proteases existing in other vertebrates (7, 8, 9).

2) In the second phase, there may be active contraction of the follicle, leading to expulsion of the oocyte.

The first phase is slow in the trout (72-96 hours at 10°C) and may not take place in vitro due to absence of necessary mediators for the required enzyme systems, or simply because precursors or cofactors are missing. On the other hand, the second phase is short, and an appropriate stimulus should be able to induce it.

Because the first phase introduced undue complexities, we chose ovaries in which germinal vesicle breakdown was complete and follicle cell detachment initiated in vivo. We attempted to answer the following questions :

1. Is ovulation per se (expulsion of the oocyte from the follicle) under direct pituitary control and, more precisely, under neuropituitary control ;

2. Would prostaglandins induce ovulation in fishes, as in mammals where direct or indirect local involvement of prostaglandins has been demonstrated to be absolutely necessary for initiation of ovulation (10, 11, 12, 13, 14, 15, 16, 17, 18).

3. As the preceding question can be answered positively, is in vitro ovulation in the trout due to active contraction of the smooth muscle-like cells of the theca or rather to disorganization and disruption of the tissues under the influence of enzymatic factors alone or both of these ?

MATERIAL AND METHODS

Ovaries were removed from 3-4 year-old rainbow trout (Salmo gairdnerii) during the normal spawning season (November-December). Knowing that the entire oocyte population matures and ovulates synchronously, females were chosen according to the state of in vivo maturation of a few oocytes squeezed out by abdominal stripping. The maturation stage selected for the experiments (separation of the oocyte from the follicular tissue) is reached approximately 12-24 hours subsequent to germinal vesicle breakdown and precedes natural ovulation by several hours. At that time, the follicle can be easily opened with a watchmaker forceps and the oocyte removed.

Incubation was carried out at 15°C in the same balanced salt solution used for studying egg maturation (2) with some changes in pH and buffer mentioned in the text and tables.

Prostaglandins were from The Upjohn Co., Kalamazoo, Michigan.

In order to demonstrate that active contraction of theca smooth-muscle cells induced by $\text{PGF}_{2\alpha}$ play a role in the ovulation process, experiments were carried out either in Ca^{++} free media (with and without EDTA) or in media containing one of the following inhibitors of Ca^{++} influx (19) :

- a) chlorpromazin (10^{-3}M)
- b) manganese ions ($5 \times 10^{-3}\text{M}$) in form of MnCl_2
- c) lanthanum ions ($5 \times 10^{-3}\text{M}$) in form of LaCl_3

The effect of Cytochalasin B on in vitro ovulations was tested to resolve if follicle contraction is due more precisely to actin-like filaments (20) observed in various cell lines or to muscle cells (21) (22) (23). It was added to the incubation medium in concentrations of 1 and 5 $\mu\text{g/ml}$ by adding adequate quantities from a stock solution of 2 mg/ml in dimethyl sulfoxide (DMSO). The effect of soybean trypsin

PROSTAGLANDINS

inhibitor (Sigma) was tested at 1 per cent to resolve if a trypsin-like enzyme could play a part in preparing the follicle for ovulation.

The action of crude pituitary and neuropituitary extracts of trout was investigated by using homogenates of fresh tissue in the incubation medium (5 mg tissue per ml of medium). The cell debris was removed by centrifugation. Furthermore the possible action of oxytocine (Sandoz-400 I.U/ml) and L8-vasopressin (Sandoz-10 pressor unit/ml) was evaluated.

The statistic significance of the difference in ovulation rate between experimental lots and controls is shown in Tables with the following symbols : N.S. : Not significant, * - ** - *** : significant difference respectively at the 5 %, 1 %, 0,1 % levels.

RESULTS

Effects of pituitary extracts and neuropituitary hormones on ovulation in vitro

Neither fresh pituitary extracts (up to 150 µg/ml) nor neuropituitary extracts (up to 100 µg/ml) of trout, oxytocine (10 I.U/ml) nor vasopressin (0.5 I.U/ml) induces ovulation in vitro of trout follicular eggs matured in vivo.

Prostaglandin action (Table I, figures 1 and 2)

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) can induce up to 100 per cent ovulation (range was 30-100 per cent) at a dose level of 5 µg/ml and up to 84 per cent ovulation (range 10-84 per cent) at a dose of 1 µg/ml (Table I). The variability of response in different females appears to express the state of maturation at which the follicles were selected at the beginning of incubation.

When the sensitivity range of the follicles of a given female is investigated, a sigmoid response curve is found as function of the dose. For instance as shown in Figure 1 for female n° 4, 20 per cent ovulation is triggered by 0.3 µg/ml, 50 per cent by 0.6 µg/ml, and a plateau higher than 80 per cent by more than 2.5 µg/ml.

Some follicles ovulate rapidly after 3 hours of incubation, accounting for 30-45 per cent of the ovulations. The remaining follicles react more slowly, and maximum ovulation is reached only after 18-20 hours of incubation.

PROSTAGLANDINS

FIGURE 1 : Percentage of ovulation of 8 different trout as a function of the dose of $\text{PGF}_{2\alpha}$ added 18 hours earlier

FIGURE 2 : Percentage of ovulation of 2 different trout as a function of time after addition of $5 \mu\text{g/ml}$ of $\text{PGF}_{2\alpha}$

PROSTAGLANDINS

At first the follicular envelope tears open, followed by active expulsion of the oocyte. The follicle subsequently appears extremely contracted. Those follicles, judged by morphological criteria as advanced, sometimes ovulate spontaneously without $\text{PGF}_{2\alpha}$ (Table I, females 2, 7, 9), but it seems in those cases that the eggs escape because of injury to the follicular tissue during dissection.

On the other hand, prostaglandin E_2 (PGE_2) is completely ineffective in ovulation in vitro (Table I). When administered simultaneously with $\text{PGF}_{2\alpha}$, it has neither a stimulatory nor an inhibitory effect.

Incubation in a calcium-deficient medium and in media containing calcium influx inhibitors (Table II)

Incubation in media lacking Ca^{++} and Mg^{++} does not appear to inhibit $\text{PGF}_{2\alpha}$ action. However, addition of EDTA (10^{-3}M) to the medium partially inhibits $\text{PGF}_{2\alpha}$ effect (especially in female 7). Chlorpromazine truly inhibits the ovulatory action of $\text{PGF}_{2\alpha}$ on the follicles of female 7, but surprisingly appears to initiate spontaneous ovulation of follicles of female 8, even in absence of $\text{PGF}_{2\alpha}$ at a significantly higher level than control.

The most clear inhibitory action in preventing ovulation is observed with La^{+++} and Mn^{++} ($5 \times 10^{-3}\text{M}$).

Effect of cytochalasin B (Table III)

Cytochalasin B at 1 or 5 $\mu\text{g/ml}$ significantly inhibits the ovulation response initiated by either 1 $\mu\text{g/ml}$ or 5 $\mu\text{g/ml}$ of $\text{PGF}_{2\alpha}$ in female 12. In females 11 and 13, inhibition occurs only with the higher doses of cytochalasin B (5 $\mu\text{g/ml}$) and $\text{PGF}_{2\alpha}$ (5 $\mu\text{g/ml}$). Inhibition is never complete in any of these cases at the concentrations employed.

Effect of trypsin-inhibitor (Table IV)

Trypsin-inhibitor appears to inhibit ovulation inducing action of $\text{PGF}_{2\alpha}$ significantly only in females 5 and 10; it has no effect on females 1, 2, 3 and 9 and the experiment gives somewhat paradoxal effects for female 3: it seems to stimulate spontaneous ovulation in absence of $\text{PGF}_{2\alpha}$. Nevertheless, with the exception of female 3, the genitive effect of trypsin-inhibitor seems to be manifested when "preparation" (maturation) of follicles appears to be incomplete, accompanied by a

PROSTAGLANDINS

TABLE I : OVULATORY RESPONSE OF IN VIVO-MATURED FOLLICLES OF DIFFERENT FEMALE TROUT TO IN VITRO ACTION OF $\text{PGF}_{2\alpha}$, PGE_2 OR $\text{PGF}_{2\alpha} + \text{PGE}_2$

Female n°	Treatment		Number of follicles incubated	Oocytes ovulated after 18 hours		Significance of the difference between data	
	nature	dose $\mu\text{g/ml}$		number	p.100		
1	control	-	25	0	0	(1)	(1) (2) ***
	$\text{PGF}_{2\alpha}$	1	25	21	84.0	(2)	
	$\text{PGF}_{2\alpha}$	5	25	22	88.0	(3)	(2) (3) N.S
	PGE_2	1	25	0	0	(4)	
	PGE_2	5	25	0	0	(5)	
	$\text{PGF}_{2\alpha} + \text{PGE}_2$	5 + 5	25	21	84	(6)	(3) (6) N.S
2	control	-	24	1	4.2	(1)	(1) (2) ***
	$\text{PGF}_{2\alpha}$	1	24	13	54.2	(2)	(2) (3) *
	$\text{PGF}_{2\alpha}$	5	21	18	85.7	(3)	
	PGE_2	1	24	0	0	(4)	
	PGE_2	5	24	0	0	(5)	
	$\text{PGF}_{2\alpha} + \text{PGE}_2$	5 + 5	25	21	84.0	(6)	(3) (6) N.S
3	control	-	20	0	0	(1)	
	$\text{PGF}_{2\alpha}$	1	21	2	9.5	(2)	
	$\text{PGF}_{2\alpha}$	5	21	8	38.1	(3)	(2) (3) N.S
	PGE_2	1	22	0	0	(4)	
	PGE_2	5	25	0	0	(5)	
	$\text{PGF}_{2\alpha} + \text{PGE}_2$	5 + 5	24	10	41.7	(6)	(3) (6) N.S
4	control	-	26	0	0	(1)	
	$\text{PGF}_{2\alpha}$	1	24	17	70.8	(2)	(1) (2) ***
	$\text{PGF}_{2\alpha}$	5	24	20	83.3	(3)	(2) (3) N.S
5	control	-	25	0	0	(1)	
	$\text{PGF}_{2\alpha}$	1	20	8	40.0	(2)	(1) (2) **
	$\text{PGF}_{2\alpha}$	5	20	15	75.0	(3)	(2) (3) *
6	control	-	25	0	0	(1)	(1) (2) N.S
	$\text{PGF}_{2\alpha}$	1	23	4	17.4	(2)	
	$\text{PGF}_{2\alpha}$	5	25	16	64.0	(3)	(2) (3) **
7	control	-	26	1	3.8	(1)	
	$\text{PGF}_{2\alpha}$	1	25	14	56.0	(2)	(1) (2) **
	$\text{PGF}_{2\alpha}$	5	24	20	83.3	(3)	(2) (3) N.S
8	control	-	25	0	0	(1)	
	$\text{PGF}_{2\alpha}$	1	25	16	64.0	(2)	(1) (2) ***
	$\text{PGF}_{2\alpha}$	5	27	25	92.6	(3)	(2) (3) *
9	control	-	25	3	12.0	(1)	
	$\text{PGF}_{2\alpha}$	5	25	25	100	(2)	**
10	control	-	25	0	0	(1)	
	$\text{PGF}_{2\alpha}$	5	25	8	32.0	(2)	*

PROSTAGLANDINS

TABLE II : EFFECT OF Ca^{++} FREE MEDIUM OR Ca^{++} INFLUX INHIBITORS ON OVULATION IN VITRO INDUCED BY $\text{PGF}_{2\alpha}$ IN TROUT FOLLICLES MATURED IN VIVO

N° of females	Treatment	Dose of $\text{PGF}_{2\alpha}$ $\mu\text{g/ml}$	Number of follicles incubated	Oocytes ovulated after 18 hours		Significance of the difference between data	
				number	p. 100		
7	control	0	26	I	3.8	(1)	
		1	25	14	56.0	(2)	(1) (2) ***
		5	26	20	83.3	(3)	(2) (3) NS
	medium without Ca^{++} & Mg^{++}	0	25	0	0	(4)	(1) (4) NS
		1	25	8	32.0	(5)	(2) (5) NS
		5	25	21	84.0	(6)	(3) (6) NS
	medium without Ca^{++} & Mg^{++} + EDTA 10^{-3}M	0	25	0	0	(7)	(1) (4) NS
		1	25	0	0	(8)	(2) (8) ***
		5	25	4	16	(9)	(3) (9) ***
	chlorpromazin 10^{-3}M	0	25	0	0	(10)	(1) (10) NS
		1	25	0	0	(11)	(2) (11) ***
		5	25	0	0	(12)	(3) (12) ***
	Mn^{++} 5.10^{-3}M	0	26	0	0	(13)	(1) (13) NS
		1	25	0	0	(14)	(2) (14) ***
		5	25	0	0	(15)	(3) (15) ***
	La^{+++} 5.10^{-3}M	0	25	0	0	(16)	(1) (16) NS
		1	25	0	0	(17)	(2) (17) ***
		5	25	0	0	(18)	(3) (18) ***
8	control	0	25	0	0	(19)	
		1	25	16	64.0	(20)	(19) (20) ***
		5	27	25	92.6	(21)	(20) (21) *
	medium without Ca^{++} & Mg^{++}	0	25	0	0	(22)	
		1	25	14	56.0	(23)	(20) (23) NS
		5	25	23	92.6	(24)	(21) (24) NS
	medium without Ca^{++} & Mg^{++} + EDTA 10^{-3}M	0	25	0	0	(25)	
		1	26	5	19.2	(26)	(20) (26) **
		5	25	21	84.0	(27)	(21) (27) NS
	chlorpromazin 10^{-3}M	0	25	9	36.0	(28)	(25) (28) **
		1	25	15	60.0	(29)	(20) (29) NS
		5	26	20	76.9	(30)	(21) (30) NS
	Mn^{++} 5.10^{-3}M	0	25	0	0	(31)	
		1	25	0	0	(32)	(20) (32) ***
		5	25	1	4	(33)	(21) (33) ***
	La^{+++} 5.10^{-3}M	0	26	0	0	(34)	
		1	26	0	0	(35)	(20) (35) ***
		5	26	0	0	(36)	(21) (36) ***

In this experiment, the incubation medium without bicarbonate was buffered with Tris-Hcl $8.3 \times 10^{-3}\text{M}$ at pH 7.5 to allow dissolution of La^{+++} , under an atmosphere of 50 % O_2 - 50 % N_2

PROSTAGLANDINS

TABLE III : EFFECT OF CYTOCHALASIN B ON OVULATION IN VITRO WITH PGF_{2α} OF TROUT FOLLICLES MATURED IN VIVO

Female n°	Treatment			Number of follicles incubated	Oocytes ovulated after 18 hours		Significance of the difference between pairs of data
	PGF _{2α} μg/ml	Cyto.B μg/ml	D.M.S.O. μg/ml		number	p.100	
II	0	0	0.5	25	I	4	N.S
		I	0.5	25	2	8	
		0	2.5	25	I	4	N.S
		5	2.5	25	0	0	
	1	0	0.5	25	8	32	N.S
		I	0.5	25	2	8	
		0	2.5	25	4	16	N.S.
		5	2.5	25	I	4	
	5	0	0.5	25	15	60	N.S
		I	0.5	24	15	62.5	
		0	2.5	25	18	72	*
		5	2.5	25	8	32	
12	0	0	0.5	50	3	6	N.S
		I	0.5	50	3	6	
		0	2.5	50	2	4	N.S
		5	2.5	50	2	4	
	1	0	0.5	49	32	65.3	***
		I	0.5	49	11	22.4	
		0	2.5	50	22	44	***
		5	2.5	50	4	8	
	5	0	0.5	49	45	91.8	***
		I	0.5	50	12	24	
		0	2.5	50	42	84	***
		5	2.5	50	6	12	
13	0	0	0.5	50	0	0	
		I	0.5	50	0	0	
		0	2.5	50	0	0	
		5	2.5	50	0	0	
	I	0	0.5	50	2	4	N.S
		I	0.5	49	I	2	
		0	2.5	50	0	0	N.S
		5	2.5	48	I	2.I	
	5	0	0.5	50	46	92	N.S
		I	0.5	50	49	98	
		0	2.5	49	34	68	***
		5	2.5	50	2	4	

PROSTAGLANDINS

TABLE IV : EFFECT OF TRYPSIN INHIBITOR (1 p. 100) ON IN VITRO OVULATION WITH PGF_{2α} OF TROUT FOLLICLES MATURED IN VIVO

Female n°	Treatment		Number of follicles incubated	Oocytes ovulated after 18 hours		Significance of the difference between data	
	PGF _{2α} μg/ml	trypsin-inhibit. μg/ml		number	p. 100		
1	-	-	25	0	0	(1)	(2) (3) N.S
	5	-	25	22	88	(2)	
	5	10	25	15	60	(3)	
	-	10	25	0	0	(4)	
2	-	-	24	1	4.2	(1)	(2) (3) N.S
	5	-	21	18	85.7	(2)	
	5	10	24	20	83.3	(3)	
	-	10	25	10	40.0	(4)	
3	-	-	20	0	0	(1)	(2) (3) N.S
	5	-	21	8	38.1	(2)	
	5	10	25	15	60.0	(3)	
	-	10	25	7	28.0	(4)	
5	-	-	25	0	0	(1)	(2) (3) *
	5	-	20	15	75	(2)	
	5	10	20	4	20	(3)	
9	-	-	25	3	12	(1)	(2) (3) N.S
	5	-	25	25	100	(2)	
	5	10	25	25	100	(3)	
	-	10	25	1	4	(4)	
10	-	-	25	0	0	(1)	(2) (3) *
	5	-	25	8	32	(2)	
	5	10	25	0	0	(3)	
	-	10	25	0	0	(4)	

relatively weak response to $\text{PGF}_{2\alpha}$ action (females 5, 10).

DISCUSSION

The absence of ovulation in vitro as a response to the addition of fresh pituitary and neuropituitary extracts of trout seems to exclude a direct or immediate influence of the pituitary on ovulation in its strictest sense. The non-involvement of neuropituitary hormones may be confirmed by the inability of mammalian oxytocine and vasopressin to act, although these hormones are admittedly different from those of the trout (24).

The positive action of $\text{PGF}_{2\alpha}$ on ovulation in vitro is clear and repeatable, as demonstrated by the experiments. In contrast, PGE_2 did not have any effect. The necessary local involvement of prostaglandins in mammalian ovulation has been well demonstrated by blocking ovulation in vivo with indomethacine as an inhibitor of prostaglandin synthesis (10, 11, 12, 13, 18) and by the in situ quantitation of prostaglandins in pre- and postovulatory follicles (15, 17). It appears that $\text{PGF}_{2\alpha}$ is particularly effective in ovarian contraction, as was demonstrated in rabbit (16, 25) and in rhesus monkey (26). Our own observations in trout, presenting evidence for $\text{PGF}_{2\alpha}$ ovulatory activity, are thus in agreement with the results on mammals. The in vitro ovulation induced with $\text{PGF}_{2\alpha}$ is certainly accomplished by contractions of smooth muscle cells which are present in the theca (6). As Mn^{++} and La^{+++} are commonly used to block the influx of calcium necessary for muscle contraction (19), it may be hypothesized that inhibition of ovulation by them is achieved through these smooth muscle cells. The difference of response to chlorpromazine between females 7 and 8 is still difficult to explain simply considering only two animals ; but the powerful inhibition by La^{+++} and Mn^{++} appears to provide sufficient evidence. Moreover, the fact that the calcium free medium does not block ovulation is not surprising if one considers the calcium content of the incubated tissues, which may be sufficient to support contraction : but if EDTA was used to complex free Ca^{++} ions more fully, contractile activity was inhibited. On the other hand the significant inhibition of the ovulatory response to $\text{PGF}_{2\alpha}$ by cytochalasin B may imply that cells other than

PROSTAGLANDINS

the smooth muscle cells can contract and may be involved in ovulation. However, most of data presented thus far in the literature concerns the effect of cytochalasin B or D on striated or cardiac muscle and not on smooth muscle (21, 22, 23).

As concerns the necessary "preliminaries" to ovulation (dissociation of follicle cells from oocytes and opening of multiple layers of the follicle), they may not be fully completed in vivo when the follicles are taken for incubation. If a trypsin-like enzyme was involved, it probably would have been inhibited by the trypsin-inhibitor thus lowering the percentage of ovulation with $\text{PGF}_{2\alpha}$. In fact the results are not very clear, but it seems that trypsin-inhibitor manifests a negative effect when "preparation" of follicles appears to be incomplete (weak response to $\text{PGF}_{2\alpha}$) revealing a marginal effect ; thus it may be that a trypsin-like enzyme is marginally implicated in follicular preparation to ovulation, but other enzymes play probably the principal role. In mammals, an enzyme possessing some characteristics similar to collagenase seems to be essential in preparing the follicle (8, 9).

Various aspects of the experiments show that $\text{PGF}_{2\alpha}$ acts on smooth muscle cells of the theca, causing contractions, and that this action is probably preceded by the enzymatic preparation of the follicle. It remains to be shown that the observed effect is not only an artefact due to the extraphysiological or pharmacological effects of prostaglandins and resulting from an interference in the normal process of ovulation.

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