

# Involvement of Chloride Channels in Progesterone Production During Meiotic Maturation of Follicle-Enclosed Oocytes of *Rana temporaria* and *Xenopus laevis*

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**ABSTRACT** The chloride channel blockers SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulphonic acid) and DIDS (4,4'-diisothiocyano-stilbene-2,2'-disulphonic acid) markedly suppressed progesterone production in the *Rana temporaria* and *Xenopus laevis* follicle-enclosed oocytes and oocyte maturation stimulated by the homologous pituitary suspension and hCG, respectively. Inhibition was dose-dependent and decreased with the increase of the hormone concentration. SITS did not affect progesterone production in the *R. temporaria* follicle-enclosed oocytes stimulated by dbcAMP. Substitution of sodium chloride for equimolar concentrations of sodium gluconate, methanesulfonate, glutamate, or formate significantly potentiated the gonadotropin-stimulated progesterone production and oocyte maturation in the both species. Possible involvement of chloride channels in progesterone production by the gonadotropin-stimulated amphibian follicle-enclosed oocytes is discussed. *J. Exp. Zool.* 278:422–428, 1997. © 1997 Wiley-Liss, Inc.

The study of action of the gonadotropic hormones on maturation of the amphibian follicle-enclosed oocytes (FEO) (Skoblina et al., '95a,b) led us to search for the role of interactions of follicle and medium in FEO maturation especially at low gonadotropin concentrations. It was shown that increased NaHCO<sub>3</sub> concentration decreases the minimal concentration of gonadotropic hormones that stimulated oocyte maturation in sturgeon (Goncharov, '78) and *Xenopus laevis* (Skoblina, unpublished results). Ca<sup>2+</sup>-dependent (Supplission et al., '91) and cAMP-dependent (Arellano and Miledi, '93, '94) chloride channels have been described in the amphibian FEOs. It was proposed that chloride channels or chloride ions are involved in luteinizing hormone (LH)-stimulated testosterone production in the rat Leydig cells (Choi and Cooke, '90) and progesterone production in the chicken granulosa cells (Morley et al., '91). Nothing is known about their involvement in the regulation of progesterone production in the follicle-enclosed oocytes of low vertebrates. We studied the influence of chloride channel blockers and chloride-deficient media on the progesterone production in FEOs of *R. temporaria* and *X. laevis* and oocyte maturation stimulated by the homologous pituitary suspension (PS) and hCG, respec-

tively. Progesterone, a physiological inducer of amphibian oocyte maturation (Masui and Clarke, '79), was assayed by radioimmunoassay (RIA).

## MATERIALS AND METHODS

Experiments were carried out on FEOs of 20 *R. temporaria* and 21 *X. laevis* females. *R. temporaria* females were collected during late autumn and housed in a cold room in darkness at 4°C. *X. laevis* females were bred in the animal rooms of the Institute of Developmental Biology, Russian Academy of Science. One or two months before the experiments, *X. laevis* females were injected with human chorionic gonadotropin (hCG) (Profasi, Italy) (350–500 IU/female) and 3 to 5 days before the experiments with PMSG (Sigma, St. Louis, MO; 100–350 IU/female). They were anesthetized by immersion into 0.2% ethyl m-aminobenzoate (MS-222; Sigma) and operated. *R. temporaria* females were killed by decapitation. The ovaries were removed, immersed in Ringer solution, divided with forceps into fragments containing one to ten FEOs each, and carefully mixed. Individual

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FEOs were isolated with forceps for every experimental variant. Progesterone production in *R. temporaria* FEOs was stimulated by the homologous PS (0.00125–0.01 pit/ml) and in *X. laevis* FEOs by hCG (5–40 IU/ml). These concentrations, except the highest, induce complete or near 100% oocyte maturation without overdosage of hormones (Skoblina and Kondratieva, '92).

Two chloride channel blockers were used: SITS (4-acetamido-4'-isothiocyano-stibene-2,2'-disulphonic acid) and DIDS (4,4'-diisothiocyano-stibene-2,2'-disulphonic acid) at 0.01, 0.1, 1 and 10  $\mu$ M. FEOs of both species were incubated in the amphibian Ringer solution or in a Ringer solution with an inhibitor for 1 hour. Some FEOs were then left in either of these solutions and the rest of them were treated by the hormones with or without SITS or DIDS until the end of the experiment. The effect of SITS on the progesterone production in *R. temporaria* FEOs stimulated by dbcAMP (Sigma; 2 and 3 mM) was also studied. Besides the Ringer solution, we used a saline with chloride salts substituted for equimolar concentrations of the corresponding gluconates or with sodium chloride substituted for sodium methanesulfonate, glutamate, or formate. All solutions had pH 7.8 and osmolarity 217 to 228 mOsm.

Progesterone content was assayed in the FEOs of *R. temporaria* and in the incubation medium of the FEOs of *X. laevis*. Good correlation of the progesterone content between the FEOs and the medium was shown earlier (Schuetz and Lessman, '82; Fortune, '83; Patino and Purkiss, '93). In the *R. temporaria* FEOs, whose maturation was induced by low PS concentrations, progesterone production sharply increased 18 h after the beginning of PS treatment and remained at the high level thereafter. In our conditions, the highest concentration of progesterone in the medium was attained 6 h after the beginning of hCG treatment (Skoblina et al., '95b). Samples for progesterone RIA in *R. temporaria* were prepared as follows: progesterone was extracted 20–24 h after the beginning of experiment. FEOs from all experimental variants (three repetitions,  $n = 20$  in each repetition) were placed in 5-ml glass test tubes. The Ringer solution transferred with the FEOs was removed by filter paper. Steroids were extracted as described by Lessman and Schuetz ('82). One milliliter of methanol (Merck, Darmstadt, Germany, analytical grade) was added to each tube. Extraction was carried out in a shaker (100 oscillations/min) at the room temperature for 15 min. Methanol was carefully transferred into another set of

tubes and evaporated under nitrogen flow. Samples for progesterone RIA in *X. laevis* were prepared as follows: progesterone was extracted within 6 hours after the beginning of the hormonal treatment. The medium in the well was carefully mixed and 1 ml of it was removed and extracted by 2 ml of freshly distilled diethyl ether. All samples were stored at  $-20^{\circ}\text{C}$  until RIA. The efficiency of steroid extraction was higher than 95% (Skoblina et al., '95a). The progesterone content was measured as described elsewhere (Vourento et al., '89).

The rate of oocyte maturation (two repetitions,  $n = 30$ ) was determined within 48 hours in *R. temporaria* ( $16\text{--}17^{\circ}\text{C}$ ) and within 24 hours in *X. laevis* ( $20\text{--}22^{\circ}\text{C}$ ). The FEOs were boiled and cut under a dissection microscope. The absence of germinal vesicle served a criterion of FEO maturation. In the *X. laevis* FEOs, the presence of a white spot at the animal pole was an additional criterion. The percentage of maturation determined using these two criteria was similar in all cases.

Mean  $\pm$  S.E.M. were calculated for different experimental groups. The data were compared using Student's *t*-test.

## RESULTS

### *The influence of SITS and DIDS on progesterone production in FEOs of R. temporaria stimulated by PS or cAMP and in FEOs of X. laevis stimulated by hCG*

Since the initial progesterone level and its production stimulated in FEOs of different females by the same PS or hCG concentration can vary significantly, the results presented in each figure were obtained for FEOs of one female. The influence of the chloride channel blockers was studied on the FEOs of 11 *R. temporaria* and six *X. laevis* females. The both inhibitors (1 and 10  $\mu$ M) significantly decreased the progesterone production stimulated by PS (0.00125–0.005 pit/ml) or hCG (5–20 IU/ml) in FEOs of all females (Figs. 1A, 2A, 3A, and 4A). They did not affect the progesterone production stimulated by the highest gonadotropin concentration in both species (data not provided). SITS and DIDS (0.1  $\mu$ M) significantly decreased the progesterone production in FEOs of eight *R. temporaria* females at all PS concentrations, unlike in FEOs of three other females. The inhibitory effects of SITS and DIDS at 0.1  $\mu$ M on the hCG-stimulated progesterone production in *X. laevis* FEOs were insignificant (data not provided). The influence of SITS and DIDS at 0.01  $\mu$ M was studied on the FEOs of two *R. temporaria* females only: progesterone production was significantly decreased (Fig.

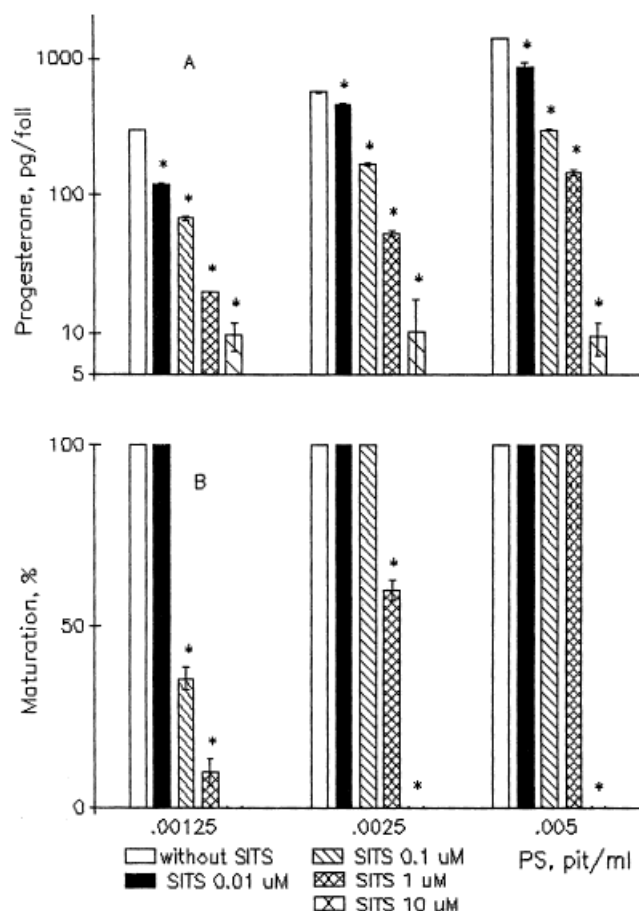


Fig. 1. The influence of SITS on the progesterone production (A) and maturation (B) of the follicle-enclosed oocytes of *Rana temporaria* stimulated by pituitary suspension (PS). Each point is the mean  $\pm$  S.E.M. of three measurements. The asterisks indicate significant differences from the preceding time-point of the same treatment ( $P < 0.05$ ).

1A). SITS (1 and 10  $\mu$ M) did not affect the progesterone production stimulated in FEOs of three females by dbcAMP (2 and 3 mM; Fig. 5).

#### **The influence of SITS and DIDS on oocyte maturation**

Some oocytes of six (out of 11) *R. temporaria* females matured spontaneously. We will not consider here the influence of the chloride channel blockers on oocyte maturation in these females. SITS and DIDS (10  $\mu$ M) inhibited oocyte maturation almost completely in five other females stimulated by all PS concentrations. The inhibitors at 1  $\mu$ M markedly suppressed oocyte maturation of all females, stimulated by one or two PS concentrations only and at 0.1  $\mu$ M oocyte maturation of three females stimulated by the lowest PS concentration (Figs. 1B, 2B). The oocytes of both fe-

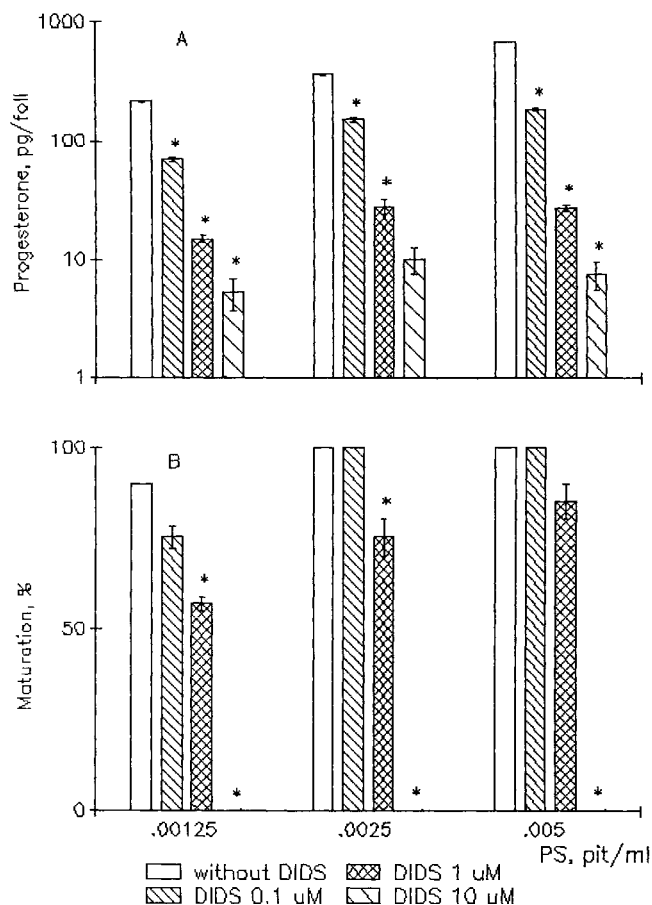


Fig. 2. The influence of DIDS on the progesterone production (A) and maturation (B) of the follicle-enclosed oocytes of *Rana temporaria* stimulated by pituitary suspension (PS). For details see Figure 1.

males treated with the inhibitors at 0.01  $\mu$ M matured in all PS concentrations.

None of the oocytes of 11 *X. laevis* females used in experiments did mature spontaneously. Both inhibitors at 10  $\mu$ M significantly suppressed oocyte maturation of all females stimulated by all hCG concentrations, at 1  $\mu$ M suppressed oocyte maturation stimulated only by lower hormone concentrations (Figs. 3B, 4B), and at 0.1  $\mu$ M did not affect oocyte maturation (data not provided).

#### **The influence of chloride ions substitution on progesterone production in FEOs of *R. temporaria* and *X. laevis* stimulated by PS or hCG, respectively**

The influence of chloride ions substitution on the progesterone production was studied in FEOs of 12 females of *R. temporaria* and six females of *X. laevis*. The absence of extracellular chloride ions

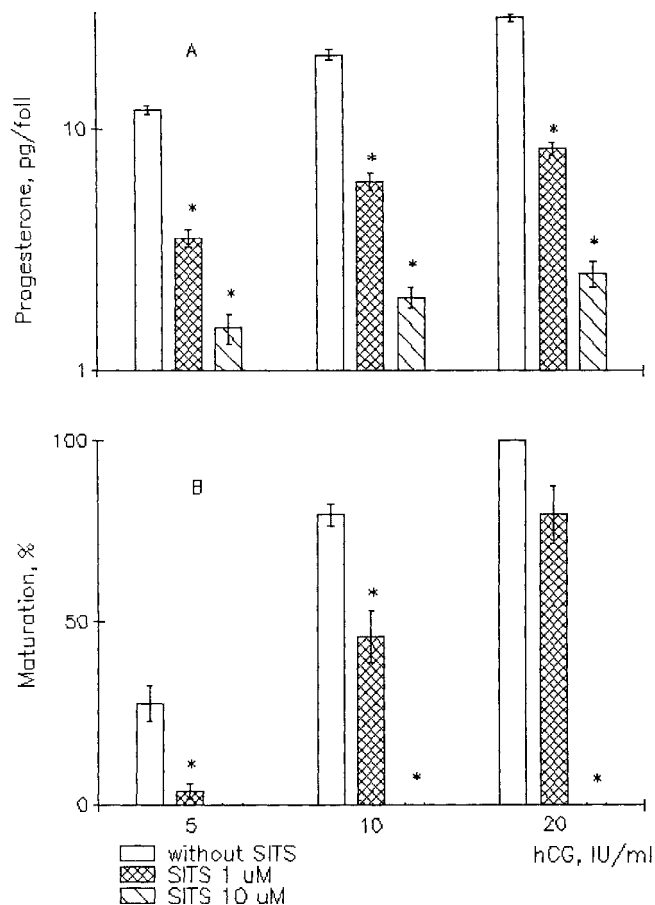


Fig. 3. The influence of SITS on the progesterone production (A) and maturation (B) of the follicle-enclosed oocytes of *Xenopus laevis* stimulated by hCG. For details see Figure 1.

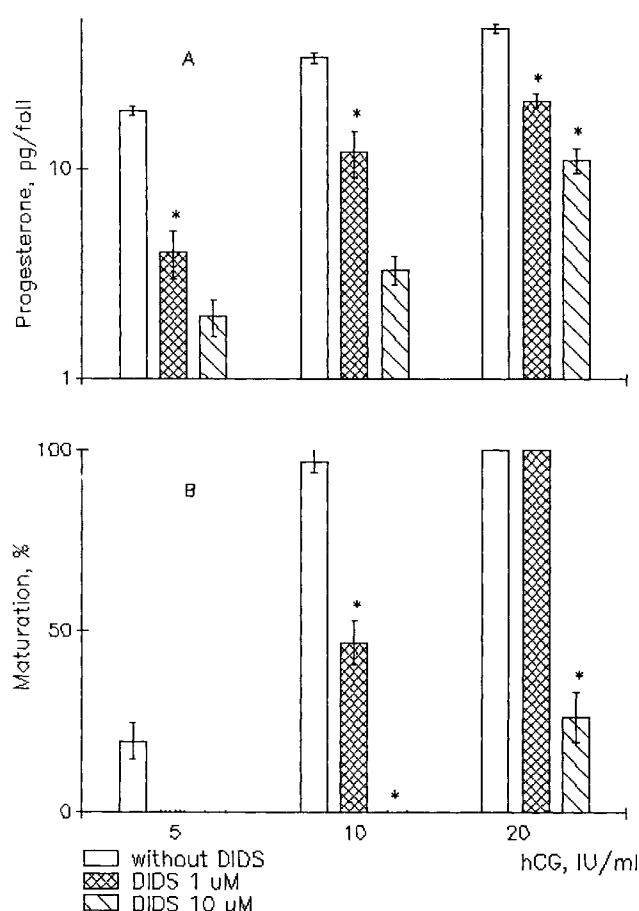


Fig. 4. The influence of DIDS on the progesterone production (A) and maturation (B) of the follicle-enclosed oocytes of *Xenopus laevis* stimulated by hCG. For details see Figure 1.

(it was substituted for equimolar concentrations of gluconates) or their decreased concentration (sodium chloride was substituted for sodium glutamate, methanesulfonate or formate) significantly increased the progesterone production in FEOs stimulated by lower PS or hCG concentrations (Figs. 6A, 7A). In chloride-deficient media, the progesterone production by unstimulated FEOs increased. In some cases the increase was significant (Fig. 6A). Progesterone production stimulated by the highest PS or hCG concentration in most cases decreased insignificantly (data not provided).

#### **The influence of chloride ions substitution on oocyte maturation**

The effect of chloride ions substitution on the gonadotropin-stimulated oocyte maturation was studied on FEOs of 12 *R. temporaria* and 10 *X. laevis* females. The absence of chloride ions or their lowered concentration increased the rate of oocyte maturation in both species. The effect of

chloride-deficient media was significant in all *X. laevis* females, especially at low hCG concentration (Fig. 7B). The high rate of *R. temporaria* oocyte maturation in the control made this effect insignificant in the most cases. The rate of "spontaneous" oocyte maturation in all *R. temporaria* females was significantly higher after substitution than in the Ringer solution (Fig. 6B).

### **DISCUSSION**

It was proposed that chloride channels can play the role of a second messenger at low gonadotropin concentrations (Choi and Cooke, '90). But it was shown that the chloride channels blockers (SITS and DIDS) reduced the accumulation of extracellular cAMP in the chicken granulosa cells (Morley et al., '91) and that only the cAMP-dependent chloride conductance is activated at the resting potential in the Leydig cells (Noulin and Joffe, '93). Nevertheless, in amphibians, as well

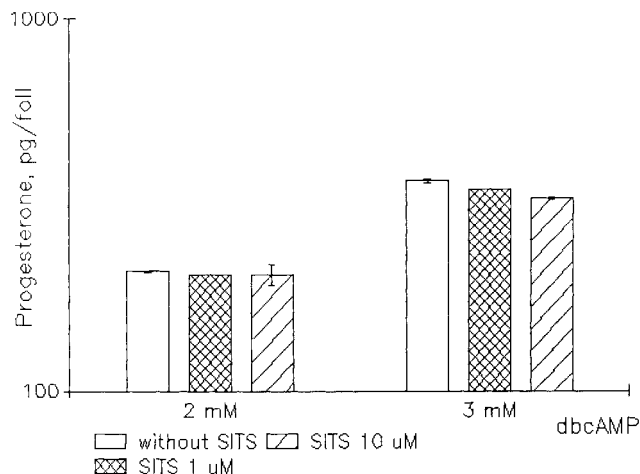


Fig. 5. The influence of SITS on the progesterone production of the follicle-enclosed oocytes of *Rana temporaria* stimulated by dibutyrylcyclic AMP (dbcAMP). For details see Figure 1.

as in mammals and birds, chloride channel blockers inhibited gonadotropin-stimulated steroidogenesis, especially at low PS or hCG concentration. The increased hormone concentration was followed by decreased inhibitory influence of SITS and DIDS on the progesterone production by FEOs. We did not observe any effect of SITS and DIDS on testosterone or estradiol production by the *R. temporaria* FEOs (Skoblina et al., '95b), so they seem not to enhance the progesterone metabolism. Note that SITS and DIDS can affect some other pathways of chloride transfer (Russel and Brodwick, '79). The data about the regulation of chloride transfer in amphibian FEOs are rather contradictory. In some cases, the permeability of FEOs for chloride ions is connected with chloride channels (Schlichter, '89; Katayama and Widdicombe, '90; Chao and Katayama, '91), while in others, with  $\text{Na}^+\text{-K}^+\text{-Cl}^-$ -cotransfer (Chipperfield and Fry, '82; Bartel et al., '89; Keicher and Meech, '94). In our preliminary experiments, bumetanide at 100  $\mu\text{M}$  did not inhibit the progesterone production in *X. laevis* FEOs stimulated by all hCG concentration, although at 10  $\mu\text{M}$  it strongly inhibited chloride reuptake by *Rana* oocytes (Keicher and Meech, '94). SITS and DIDS can also affect the intracellular pH, chloride activity (Vaughan-Jones, '79; Kaila et al., '92), and calcium level (Kimura et al., '88; Romero and Ortiz, '88). In our preliminary experiments, SITS did not affect the intracellular pH of *R. temporaria* and *X. laevis* follicle cells (Nikiforova et al., unpublished results). The inhibition by DIDS of  $\text{Ca}^{2+}$ -ATPase

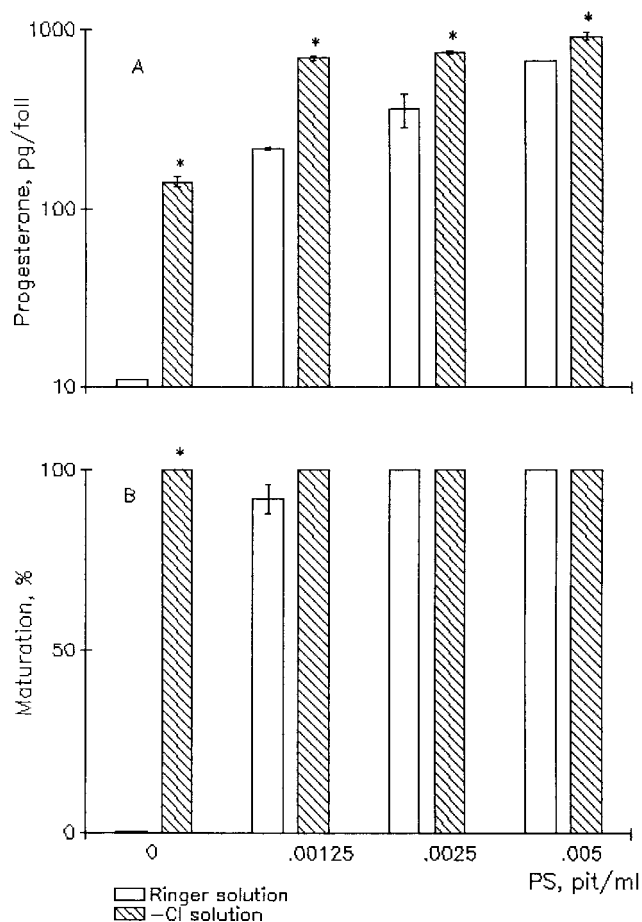


Fig. 6. The influence of substitution of chloride ions for equimolar concentrations of corresponding gluconate on the progesterone production (A) and maturation (B) of the follicle-enclosed oocytes of *Rana temporaria* stimulated by pituitary suspension (PS). For details see Figure 1.

(Kimura et al., '88; Romero and Ortiz, '88) could increase rather than decrease the intracellular  $\text{Ca}^{2+}$  (and steroid) level.

Chloride-deficient media exert different effects on the gonadotropin-induced steroidogenesis in various systems: they stimulate steroidogenesis in mammals (Choi and Cooke, '90) and amphibians (our results) and inhibit it in birds (Morley et al., '91). Enhanced steroidogenesis was observed at low hormone concentrations and also in unstimulated amphibian FEOs. It is known that some anions substituted for chloride stimulate chloride transfer, while others inhibit it (Russel and Brodwick, '79). Since all chloride substitutes used in our experiments enhanced the progesterone production in FEOs of the both species, the mechanism of their action appears to be the same. The stimulating influence of chloride-

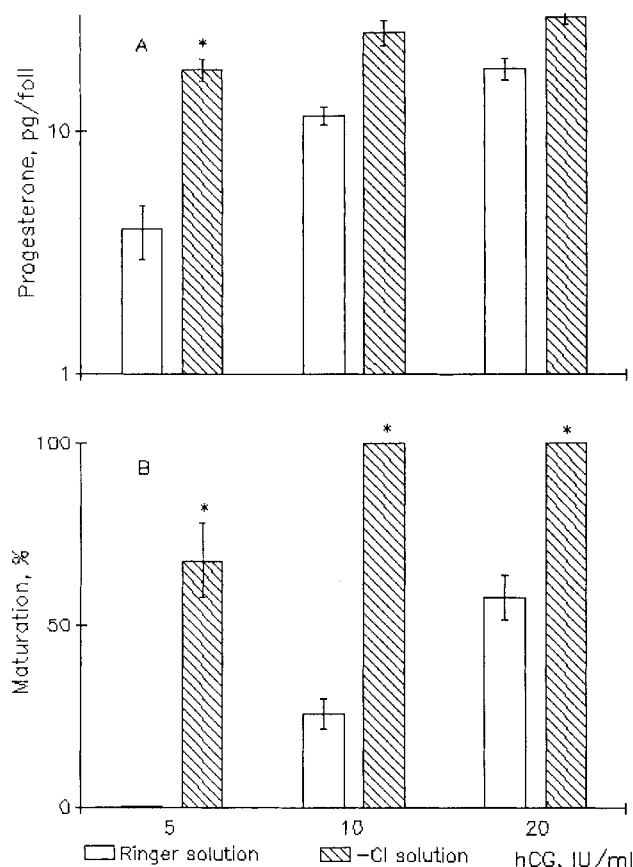


Fig. 7. The influence of substitution of sodium chloride for equimolar concentration of sodium methanesulfonate on the progesterone production (A) and maturation (B) of the follicle-enclosed oocytes of *Xenopus laevis* stimulated by hCG. For details see Figure 1.

deficient media can be explained by an increased intracellular  $\text{Ca}^{2+}$  level. Two facts favor this suggestion: (1) substitution of chloride ions for isothionate prevents the agonist-stimulated  $\text{Ca}^{2+}$  ion efflux from the rat neonatal pituitary (Acs et al., '93). If the intracellular  $\text{Ca}^{2+}$  level of amphibian follicle cells is also elevated in the chloride-deficient media, one could expect an increased progesterone production in the amphibian FEOs, since it is calcium-dependent (Kleis-San Francisco and Schuetz, '86, '87); and (2) addition of  $\text{Ca}^{2+}$  ions to gluconate solution leads to significant differences in the rate of spontaneous maturation of FEOs. If the rate of oocyte maturation in gluconate solution is low, addition of  $\text{Ca}^{2+}$  ions increases it; if it is high, decreases it (Skoblina et al., '96).

It was shown earlier that the increased extracellular calcium concentration stimulated progesterone production by the amphibian FEOs, but

still more elevated concentration inhibited it (Kleis-San Francisco and Schuetz, '86). It is possible that the different effects of chloride-deficient media on steroidogenesis in birds (Morley et al., '91) and mammals (Choi and Cooke, '90) are connected with different intracellular  $\text{Ca}^{2+}$  levels in their cells, especially because LH concentrations (0.01–0.1 ng/ml), whose effect was enhanced in chloride-deficient medium in mammals, were not used in birds. At LH concentration higher than 1 ng/ml, a decreased progesterone production was also observed in mammals. Insignificant decrease of progesterone production at the highest PS or hCG concentrations was also observed in our experiments. But one cannot also exclude different effects of chloride-deficient media on the cAMP level in steroid-producing cells of different species. Measurements of cAMP and intracellular calcium in the amphibian follicle cells will help to solve this problem.

Since progesterone is a physiological inducer of amphibian oocyte maturation (Masui and Clarke, '79), changes in its production should affect the rate of oocyte maturation. The inhibition of progesterone production by the chloride channel blockers did reduce the rate of oocyte maturation, while stimulation of the progesterone production after chloride ions substitution increased it. However, oocyte maturation proved to be less sensitive to the chloride channel blockers and more sensitive to chloride ion substitution. We propose that even the lowest PS concentration used stimulates production of more progesterone than is necessary for the induction of oocyte maturation. Hence, its partial reduction is not accompanied by the inhibition of oocyte maturation. The higher sensitivity of oocyte maturation to chloride-deficient media can be explained by their stimulating effect on  $\text{Ca}^{2+}$  level in the oocyte, in addition to its action on progesterone production. It is known that oocyte maturation can be stimulated by increased or decreased concentration of extracellular  $\text{Ca}^{2+}$  (Cicirelli and Smith, '87).

To answer the question whether the influence of chloride channel blockers and chloride-deficient media is connected with the intracellular  $\text{Ca}^{2+}$  level,  $\text{Ca}^{2+}$  should be assayed in the follicle cells of the FEOs stimulated with gonadotropins in different conditions. It can not also be excluded that SITS, DIDS and chloride-deficient media affected cAMP level differently in different systems. We could not find any published data on chloride measurements in the steroidogenic cells and we are going to carry out these studies further.

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