The effect of cyclic cytidine 3′,5′-monophosphate (cCMP) on the in vitro development, hatching and attachment of the mouse blastocyst

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Summary. The in vitro development and attachment of hatched mouse blastocysts on the untreated substratum was enhanced by 10 μM dibutyryl cCMP (dbcCMP). The result suggests that cCMP has an effect on embryonic development and on the blastocyst attachment process.
Key words. cCMP; cyclic nucleotides; blastocyst; mouse; hatching.

Cyclic CMP is a pyrimidine compound[1], in contrast to cAMP[2–4] and cGMP[5] which are purine compounds. The role of cCMP as an intracellular messenger is unknown. Recent studies indicate that cCMP is a mediator for pigeon crop-sac epithelial cell growth[6], activates protein kinases[7], initiates hemoglobin synthesis[8] and is found at high intracellular levels in rapidly-growing hepatoma cells[9]. In addition, the cCMP phosphodiesterase (PDE) is different from cAMP-specific PDE[10]. The objective of the present study was to study the possible effects of dbcCMP on the development of the blastocyst in vitro.

Superovulation was induced in 6-week female Swiss-Webster mice with a single 5 IU pregnant mares serum gonadotropin (PMSG) i.p. injection followed by 5 IU i.p. human chorionic gonadotropin (hCG) injection 48 h later. The animals were mated and the presence of a vaginal plug the following morning indicated successful copulation. Embryos (early 8-cell stage) were flushed out of the oviducts 64-65 h after the hCG injection and were placed in Falcon No. 3037 petri dishes containing either 1 ml culture medium as control or 1 ml culture medium containing 10 μM dbcCMP. The dbcCMP compound was water-soluble and was dissolved in culture medium which served as the vehicle medium. The concentration of dbcCMP (10 μM) chosen was based on the approximate concentration used in a previously reported study on cultured calf liver cells[8]. The cultures were incubated at 37 °C with a 5% CO_2 in air moist atmosphere.

The culture medium consisted of Ham's F-10 (with L-glutamine, 5.6 mM D-glucose; GIBCO, Grand Island, NY) supplemented with 2.1 g/l sodium bicarbonate, 245.2 mg/l calcium lactate, 75 mg/l penicillin G and 75 mg/l streptomycin[11]. In addition, the medium contained 4 mg/ml human serum albumin fraction V (HSA-V). The pH and osmolarity were adjusted to 7.2 and 290 mOsm respectively. All culture media were equilibrated in a 5% CO_2 in air mixture at 37 °C overnight in the incubator prior to use on the following day. Embryos were examined by phase contrast and light microscopy at 24, 48 and 72 h after the start of incubation for indications of development to the blastocyst stage, hatching, attachment and outgrowth. The significance of the data was analyzed using the chi-square test statistic and a difference with $p \leq 0.05$ was considered significant.

The results are presented in the table. The percentage of embryos developing from the 8-cell stage to the blastocyst stage was significantly higher in the presence of dbcCMP in comparison with the control. The observation suggests that the mouse embryo at the 8-cell to blastocyst stage possesses the competence to respond to the dbcCMP stimulus. The observed enhancement effect of dbcCMP on the development and proliferation of the 8-cell mouse embryonic cells is supported by evidence in other studies that indicate a role of cCMP in promoting the proliferation of cells[6,9].

The percentage of hatched blastocysts attaching to the substratum was significantly higher in the dbcCMP treatment group in comparison with the control. However, there was no difference in the percentage of embryo hatching in the 2 groups although numerically the dbcCMP treatment resulted in more hatched blastocysts. Previous reports[12] indicated that the hatched mouse blastocyst had a low attachment rate (0–6%) on petri dishes and that the presence of bovine serum albumin (BSA) did not facilitate attachment. In this study, the HSA-V appeared to overcome the limitations of BSA in promoting attachment (25% in controls). The observation of a 2-fold higher percentage of blastocysts attaching in the presence of dbcCMP provides evidence of an effect of dbcCMP in the attachment process. The mechanisms by which cyclic nucleotides direct the attachment of the polar trophoblastic cells to the substratum in the implantation process is unknown. Studies with fibroblast cells indicate that cAMP is involved in enhancing the adhesiveness of the cells to the substratum[13] and it is possible that the same mechanism in response to cCMP may be functioning to attach and implant the embryos. The precise mechanism of action of cCMP in embryonic development will require further exploration.

All hatched blastocysts from both groups that attached to the petri dish substratum developed trophoblastic outgrowths with intact inner cell mass. Differentiation of the cells to the primary ectoderm and endoderm layers was not studied as the cultures were terminated early after blastocyst attachment and outgrowth. A few attached blastocysts in a separate study were allowed to continue in culture in the presence of dbcCMP for 5 days. There was no visible evidence of a cytotoxic effect of dbcCMP and the blastocysts developed to the egg cylinder stage (stage 9)[14,15]. The effect of dbcCMP on the development of the egg cylinder stage embryos will be examined in a future study.

In the present study, we demonstrated an effect of dbcCMP in promoting the development of the 8-cell mouse embryo to the blastocyst stage and in promoting the attachment of the hatched blastocyst. The result suggests that the 8-cell mouse embryo is responsive to the cCMP analog stimulus.

The effect of dbcCMP on the in vitro development, hatching and attachment of Swiss-Webster mouse embryos in vitro[a]

Treatment	No. developing to blastocyst (%)	No. of hatching blastocysts (%)	No. of attached embryos (%)
Control	29/50 (58.0)	14/29 (48.3)	6/24 (25.0)
10 μM dbcCMP	44/57 (77.2)[b]	28/44 (63.6)	17/34 (50.0)[b]

[a] Embryos were recovered at the 8-cell stage from mated superovulated mice. [b] Significantly different from the control ($p \leq 0.05$).

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0014-4754/87/080929-02$1.50 + 0.20/0

A mutation of *Euplotes vannus* causing induction of intraclonal conjugation (selfing) in low $[K^+]_o$

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8 August 1986

Summary. A newly discovered recessive mutation, *clk*, in the marine ciliate *Euplotes vannus* is responsible for induction of intraclonal *c*onjugation at *l*ow, i.e. < 2 instead of 10 mM $[K^+]_o$. Heredity was assured by crosses to standard clones and by backcrosses. Pairing in *clk* clones is prevented by K^+ at 2 mM, by Rb^+ and Cs^+ at 4 mM, not by Li^+, NH_4^+, and TEA^+ up to 10 mM. Clones with the *clk* phenotype were found in several natural populations.
Key words. Intraclonal pairing; low potassium concentration; *Euplotes vannus*.

Potassium ions apparently play crucial roles in conjugation processes in ciliates[2]. High extracellular concentrations affect pair formation in two opposite directions: induction of intraclonal conjugation in *Paramecium*[3], and inhibition of conjugation between opposite mating types in *Euplotes*[4]. In this report, an effect of reduced external potassium concentration on sexual behavior is described: a newly found mutant *(clk)* of the marine *E. vannus* is induced to intraclonal conjugation when transferred to medium with low (< 2 mM) $[K^+]$.

Materials and methods. All clones of various origins (table 2) are members of the *vannus* morphotype of the *Euplotes vannus-crassus-minuta* group[5-7]. Mutant clones are descendents of a stock from Naples/Italy; for designation see 'Heredity'. Cells were bred in artificial seawater (mM): NaCl 465.3; $NaHCO_3$ 2.4; KCl 10; $CaCl_2$ 10.4; $MgCl_2$ 24.8; $MgSO_4$ 28.1 in petri dishes[8] resulting in populations of 8–16000 cells in 8 ml medium. They were transferred to low K^+-medium by sieving. K^+ analogues were applied as chlorides. Ion compositions were altered by addition of appropriate adjusting media in 1:1 proportion. Changes in the nuclear apparatus were visualized by a modified orcein method[8], and Con A binding site fields on the cell membrane by means of horseradish peroxidase[9] (during the latter treatment, cells were partly damaged.) Experiments were performed at 24.5 °C in a constant temperature room.

Results and discussion. Description of the phenotype. Pairs appeared 2–3 h after transfer to 1 mM K^+, and in a still higher proportion at 0 mM (fig.). The percentage increased rapidly, but decreased after 5–6 h. All details were in accordance with normal conjugation, including susceptibility to 5 µM cycloheximide. Courtship behavior, adherence of two mates in angled position, and quick rotation[9] were observed. Only the pre-conjugation phase (lag phase) was longer. Also the typical field of Con A binding sites in the pairing region[9] occurred. Aceto-orcein preparations revealed performance of micronuclear divisions together with macronucleus destruction[8]. The most advanced cytological stage, however, was that of 8 micronuclei; the majority of pairs progressed only to the 2 or 4 micronuclei stage. Thereafter the partners separated without having exchanged pronuclei. Consequently, exconjugants with macronuclear anlagen were not found. Although the series of cytogenetic events was thus not completed to synkaryon formation, all traits indicate that conjugation was induced in the mutant cells, but continued only to a certain point. The inability to terminate conjugation could be due to the detrimental effects of lack of K^+ for so many hours.

Effects of K^+ analogues. Specificity of action of potassium ions on *clk* clones was tested by comparison with other monovalent cations (table 1). K^+ inhibited pair formation at 2 mM, the few exceptional pairs (fig.) being temporary, with-

Table 1. Effects of ions on intraclonal conjugation in a *clk*-clone (B26) of *Euplotes vannus*

Ion	Observations* at mM			
	1	2	4	10
K^+	+	−	−	−
Rb^+	+	+	−	−
Cs^+	+	+	−	−
Li^+	+	+	+	+
NH_4^+	+	+	+	+
TEA^+**				+

* + = conjugation; − = no pairs; ** tetraethylammonium.

Table 2. Reaction of clones of *Euplotes vannus* from different origins to medium without K^+

Origin	Clones tested	Observations*		
		−	(−)	+
Naples/Italy	31	17	7	7
Tropea/Italy	1	0	0	1
Brindisi/Italy	2	0	1	1
Vrsar/Jugoslavia	3	1	2	0
Barcarès/France	2	0	0	2
Quiberon/France	7	7	0	0
Patras/Greece	2	2	0	0
Nafplion/Greece	2	2	0	0
Mombasa/Kenya	18	18	0	0
Morehead/North Carolina	3	0	2	1

* − = no reaction; (−) = single pairs; + = conjugation, including induction of micronuclear divisions.