ADENYLATE CYCLASE AND CYCLIC AMP-PHOSPHODIESTERASE ACTIVITIES OF FETAL MEMBRANES: EFFECT OF INSULIN

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Summary: Basal activity and response to insulin of adenylate cyclase and cyclic AMP-phosphodiesterase were measured in the fetal membranes before and after labor.

Basal activity of adenylate cyclase showed only slight variations after delivery when compared to that observed before labor. Enzyme activity was significantly although weakly decreased by insulin both in the amnion and in chorion before labor, while after delivery a weaker non-significant inhibiton was observed.

Basal activity of cyclic AMP-phosphodiesterase was lower after delivery with respect to that observed before labor. Insulin inhibited enzyme activity in all conditions.

Cyclic AMP levels in intact tissue were not substantially modified by insulin.

INTRODUCTION

Current knowledge of the physiology of the fetal membranes deals mainly with their possible role in the events that cause the onset of labor. This process involves the cyclic AMP (cAMP), but its regulation is not completely understood. In particular little is known about the adenylate cyclase activity of either the amnion or chorion ([1, 2]), whereas, as far as we know, their phosphodiesterase (PDE) activity has never been investigated. The amniotic fluid has been shown to contain substances, such as insulin, that might affect both enzymes. It has been shown that in several tissues insulin inhibits adenylate cyclase ([3]) and stimulates PDE ([4]) activities. It has also been reported that insulin concentration significantly increases in amniotic fluid during pregnancy ([5]), thus representing a potential regulator of amniotic membrane functions.

The aim of the present study was to understand the behavior of adenylate cyclase and cAMP-PDE of the fetal membranes by assaying their basal activity and their response to insulin both before and after labor.

METHODS

Preparation of membrane fractions

The fetal membranes were obtained from term physiological pregnancies either after spontaneous vaginal delivery or after cesarean section before the onset of labor. All the cesarean sections were electively performed either for breech presentation or in women who had previously been delivered by cesarean section.

The amnion and chorion were carefully separated. The maternal surface of the chorionic membrane was subsequently gently scraped by means of a knife. This procedure was proved to be effective in removing residual decidua from the membrane by histological examination. Both membranes were washed several times with cold sodium chloride (0.15 M) then stored at −20 °C for about one week before being processed. After thawing homogenates of single tissue samples were prepared in 6 vol (v/w) of iced buffer containing 250 mM sucrose, 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetracetic acid (EGTA) and 10 mM Tris-hydrochloride (Tris-HCl), pH 7.2 and centrifuged at 3,000×g for 10 min. The supernatant was centrifuged at

40,000×g for 20 min. This particulate fraction was resuspended by gentle homogenization with the same buffer to obtain a final membrane protein content of approximately 200 μg/100 μl.

Adenylate cyclase assay

Adenylate cyclase activity was measured in an assay medium containing, in a final volume of 0.4 ml, 100 mM Tris-HCl (pH 7.4), 4.5 mM magnesium sulfat-heptahydrat, 0.1 mM EGTA, 0.5 mM guanosine-5'-triphosphate, tetralithium salt-monohydrate (GTP), 1 mM 3-isobutyl-1-methyl-xanthine, 0.5 mM adenosine-5'-triphosphate, disodium salt-trihydrate (ATP), approximately 200 μg of membrane proteins plus insulin as indicated. The reaction was started by addition of ATP and was carried out for 5 min at 30 °C. The reaction was stopped by boiling. The samples were frozen and then thawed after at least one day. After centrifugation the cAMP was measured on the clear supernatant according to the method of Brown *et al.* (6).

Cyclic AMP-phosphodiesterase assay

cAMP-PDE activity was measured by a slight modification of the two step procedure of Thompson and Appleman (7). 200 μg of homogenate proteins were preincubated at 30 °C for 8 min in a medium containing, in a final volume of 200 μl, 40 mM Tris-HCl (pH 8.0), 5 mM magnesium chloride, 0.5 mM 2-mercaptoethanol, approximately 200,000 cpm of cyclic [^{3}H]AMP and insulin as indicated. In the range of substrate concentrations from 4.0×10^{-7} M to 1.0×10^{-4} M, Km and Vmax values were calculated by Lineweaver and Burk plots.

In all, except the kinetic studies, 10^{-6} M cyclic AMP for the low Km PDE was used. Reactions were initiated by the addition of substrate. After incubation at 30 °C for 5 min, the reaction was terminated by immersing the tubes for 1 min in boiling water. 50 μg of 5'-nucleotidase from Ophiophagus hanna venom were added and the incubation was carried out for 10 min at 30 °C and then stopped by adding 0.5 ml of a slurry of Dowex anion-exchange resin. After a stabilization period of 15 min at room temperature, the tubes were centrifuged and 0.2 ml of the clear supernatant was counted in a liquid scintillation spectrometer.

Assay for cyclic AMP levels

Fresh individual discs of amnion and chorion tissue (1.8 cm diameter) were preincubated for 45 min at 37 °C in a physiological solution (0.15 M NaCl) in a shaking bath flushed with a mixture of O_2/CO_2 (95%-5%). After preincubation, 0.5 mM GTP and when present 5 mM aminophylline were added and the tubes were incubated for 15 min. 0.1 mM EGTA and, when present, 0.1 mM insulin were added then the tubes were incubated for 10 min (final volume 2.0 ml). At the end of the incubation period tissues were transferred to cold 6% trichloroacetic acid (1 ml) then, after hard shaking, allowed to rest at 4 °C for one day. Subsequently the tubes were centrifuged for 10 min at 2,000×g and the supernatants were extracted 5 times with aqueous ethyl-ether. Neutralized supernatants were assayed for cyclic AMP as above described. Proteins were determined on the trichloroacetic acid precipitates, dissolved in 1 N NaOH. The data are expressed as pmol cAMP/mg protein.

Determination of protein

Protein concentrations were determined according to the method of Lowry *et al.* (8) using bovine serum albumin as standard.

Statistics

Statistical analysis was performed by Wilcoxon's test for paired observations.

RESULTS

Adenylate cyclase

Fig. 1 shows the dose response curves for inhibition of adenylate cyclase by insulin of the amnion and chorion from cesarean section. The hormone at various concentrations used, slightly inhibits enzyme activity with a maximum effect at 10^{-4} M. On the base of data from dose response curves, 10^{-4} M concentration of insulin was chosen. Subsequently adenylate cyclase activity was measured in 13 cases of spontaneous vaginal delivery (i.e. after labor) and in 8 cases of cesarean section (i.e. before labor). In Table 1 are reported the means (pmol cAMP/mg protein/5 min ± S.E.M.) of enzyme activity in basal condition and in the presence of 10^{-4} M insulin. As shown, the hormone exerts a slight but significant inhibition of the enzyme activity in both amnion and chorion before labor. After delivery the inhibitory action results negligible.

Table 1. – *Effect of insulin on adenylate cyclase activity of amnion and chorion from cesarean section and from spontaneous vaginal delivery.*

	No. of cases	Basal (Pmol cAMP/mg protein/5 min±S.E.M.)	Insulin (Pmol cAMP/mg protein/5 min±S.E.M.)
Cesarean section	8		
Amnion		25.19± 4.86	19.80±5.53 *
Chorion		38.06±10.65	32.35±9.63 *
Spontaneous vaginal delivery	13		
Amnion		28.43±3.38	27.59±3.64
Chorion		39.67±7.49	36.41±6.64

* Difference statistically significant at the 0.05 level. Insulin was 10^{-4} M.

Cyclic AMP-phosphodiesterase

The rate of cAMP breakdown during a 5 min incubation period showed a linear increase with homogenate protein concentrations up to 200 μg both in the amnion and in the chorion before (fig. 2) and after (fig. 3) labor. This enzyme behavior is the same in the presence of two different substrate concentrations (1 and 100 μM cAMP). Furthermore, in the presence of either low or high substrate concentrations, the chorion shows an enzyme activity higher than the amnion, both before and after labor (fig. 2 and 3).

For kinetic analysis the enzyme activity was measured in four tissue samples from spontaneous vaginal delivery and in four tissue samples from cesarean section under the standard conditions with different cyclic AMP concentrations ranging from 4.0×10^{-7} M to 1.0×10^{-4} M. The Km values extrapolated by double reciprocal plot according to Lineweaver-Burk imply the existence of two phosphodiesterase activities: a first with higher affinity (Km $\simeq 10^{-6}$ M) and a second with lower affinity for cyclic AMP (Km $\simeq 10^{-4}$ M). The Km values of both phosphodiesterase activities are quite similar in cesarean section and in spontaneous vaginal delivery while the Vmax values for both

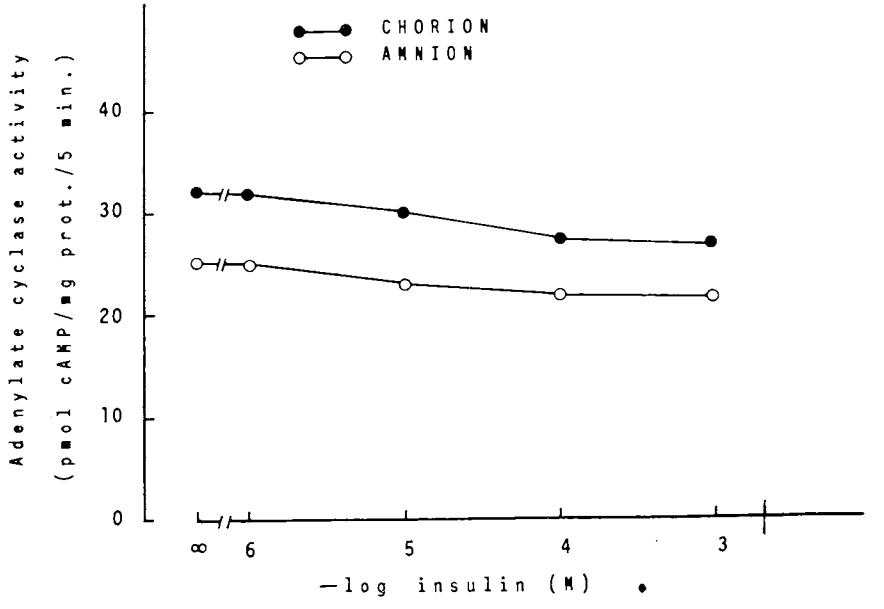

Fig. 1. – Effect of increasing concentrations of insulin on adenylate cyclase activity of the amnion and chorion from cesarean section.

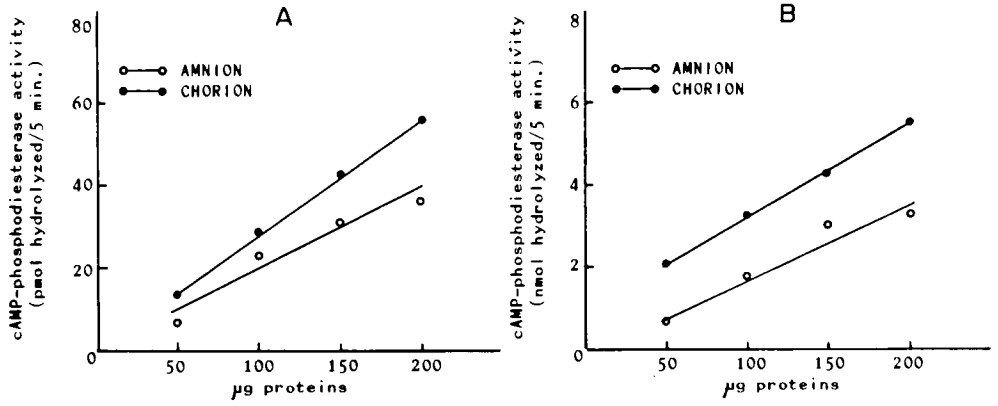

Fig. 2. – Linearity of basal cyclic AMP-phosphodiesterase activity of the amnion and chorion from cesarean section as a function of protein content. The cyclic AMP concentration was 1 μM in A and 100 μM in B.

enzyme activities seem to be higher in the cesarean section. The Km and Vmax values are summarized in Table 2.

The effect of insulin on enzyme activity was tested under high affinity conditions (in the presence of 10^{-6} M cyclic AMP) in five cases of cesarean section and in six cases of vaginal delivery. As shown in fig. 4, 10^{-4} M insulin caused a small but significant inhibition of enzyme activity in all the cases.

Cyclic AMP levels

Fig. 5 shows the cAMP content in the absence and in the presence of aminophylline, a cAMP-PDE inhibitor, in the amnion and chorion discs from six spontaneous vaginal delivery. In the absence of amino-

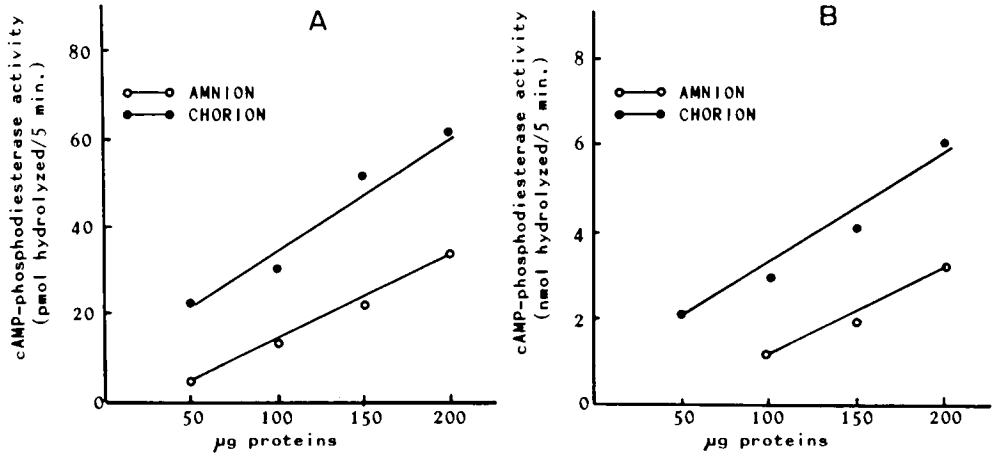

Fig. 3. – Linearity of basal cyclic AMP-phosphodiesterase activity of the amnion and chorion from spontaneous vaginal delivery as a function of protein content. The cyclic AMP concentration was 1 μM in A and 100 μM in B.

Table 2. – *Kinetic parameters of cyclic AMP-phosphodiesterase in the amnion and chorion from cesarean section and from spontaneuos vaginal delivery.*

	Km (μM)		Vmax (nmol/mg prot./5 min)	
	high affinity	low affinity	high affinity	low affinity
Cesarean section				
Amnion	5.99	258	2.52	92.10
Chorion	5.42	195	2.52	72.10
Spontaneous vaginal delivery				
Amnion	6.00	320	1.56	65.70
Chorion	5.36	142	1.19	46.30

Km and Vmax values were extrapolated from Lineweaver-Burk plots in the range of substrate concentrations from 4.0×10^{-7}M to 1.0×10^{-4}M.

phylline cAMP concentration is reduced by 30% with respect to values obtained in the presence of 5 mM of cAMP-PDE inhibitor in both the amnion and chorion. As shown in the same figure, 10^{-4} M insulin did not procedure any significant variation in cAMP content in either amnion or chorion nor in the absence or in the presence of aminophylline.

DISCUSSION

The cyclic AMP of the amniotic membrane seems to activate a protein kinase which is responsible for the phosphorylation of a phospholipase or a phospholipase proteic co-factor: this favours the release of arachidonic acid ([9]).

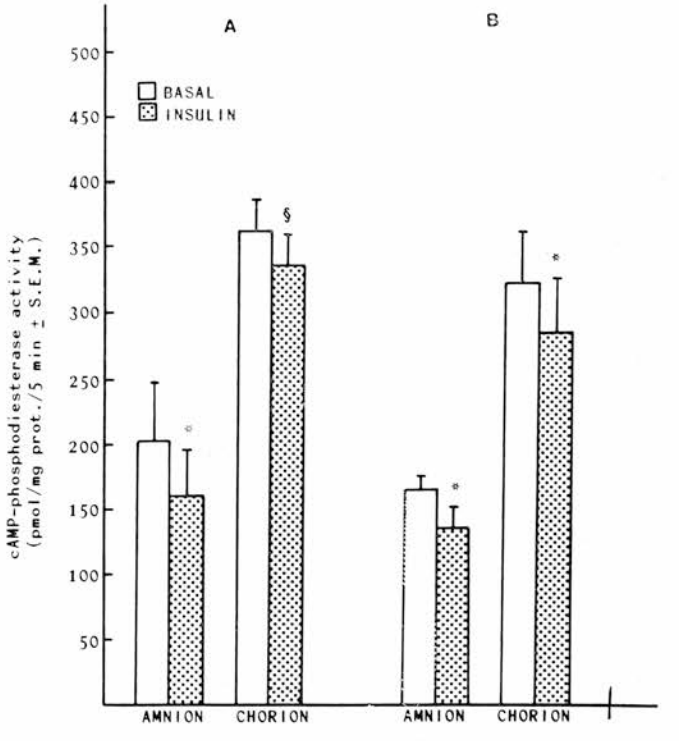

Fig. 4. – Effect of 10^{-4}M insulin on the low Km phosphodiesterase of the amnion and chorion from cesarean section (A) and from spontaneous vaginal delivery (B).
* Difference statistically significant at the 0.01 level.
§ Difference statistically significant at the 0.05 level.

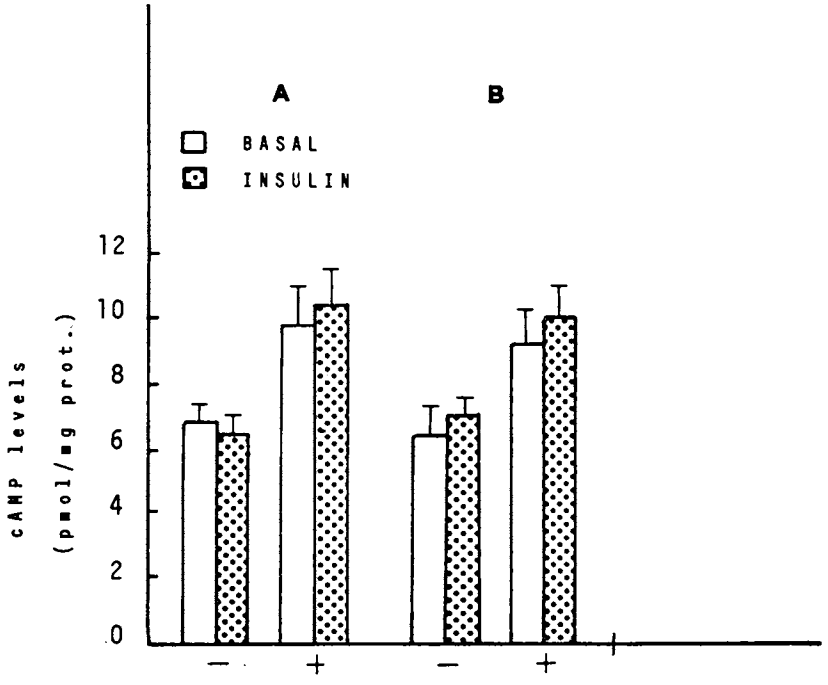

Fig. 5.– Effect of 10^{-4} M insulin on the cyclic AMP levels of amnion (A) and chorion (B) from spontaneous vaginal delivery, in the absence (−) and in the presence (+) of 5 mM aminophylline.

The intracellular levels of the cAMP of the amniotic membrane are regulated by several substances such as isoproterenol and prostaglandin E_1 ([10]) and E_2 ([9]), while the behavior of adenylate cyclase and cAMP-PDE necessitates the exploration both of amnion and the chorion.

In previous experiments we have demonstrated the presence in the fetal membranes of an adenylate cyclase activity which responds to prostaglandins E_1 and $F_{2\alpha}$ ([2]).

The present study demonstrates the following:

1) The basal adenylate cyclase activity of the fetal membranes is substantially unchanged after delivery.

2) The fetal membranes contain two forms of cAMP-PDE: high and low affinity. For both, the maximum velocity, both in the amnion and in the corion, is reduced following labor; for the Km smaller variations seem to occur after spontaneous delivery only for the low affinity form.

3) Before labor insulin, weakly although significantly, decreases the adenylate cyclase activity, while after delivery a negligible inhibition occurs.

4) Contrary to whate has been reported in other tissues, insulin significantly inhibits the PDE activity both before and after labor.

The inhibitory effect of insulin on adenylate cyclase was in agreement with the literature. The inhibitory effect of the hormone on PDE activity is difficult to interpret since it is known that insulin stimulates the enzyme in other tissues ([4, 7]). On the other side it is known that several molecular forms of the enzyme exist with different behavior toward either phy-

siological regulator agents, such as calmodulin or pharmacological agents in several tissues ([11]). Therefore a possible explanation for the inhibitory action of insulin on PDE could be the presence of an insulin-inhibitable PDE molecular form in the fetal membranes. In other tissues insulin is known to decrease the intracellular levels of cAMP by inhibiting adenylate cyclase, through a specific guanine nucleotide regulatory protein ([3]), as well as by stimulating cAMP-PDE activity ([4, 7]). However in our experiments the behavior of both enzymes under the insulin action would rather suggest that the hormone does not favor a decrease of cAMP concentration in the fetal membranes from physiological pregnancies. Accordingly cAMP levels, when measured in intact tissue, in the absence and in the presence of aminophylline, were not significantly affected by insulin addition. Taken together the findings suggest that in our system insulin acts mainly by inhibiting cAMP-PDE activity rather than through an inhibition of adenylate cyclase.

The clinical implications of our data deal with the mechanisms that control the onset of labor. It is known that cAMP increases in the amnion at the beginning of labor, and that it plays a key role in the events that lead to the synthesis of prostaglandins. Therefore a decrease of cAMP levels under the action of insulin would speak for a role of the hormone against the onset of labor, while an increase of the nucleotide could suggest an opposite role. Since in our experiments the hormone influenced the adenylate cyclase and cAMP-PDE activities in a way that seems not to affect the levels of the nucleotide significantly, it should be concluded either that it is not directly involved in the control of labor or that its action is mediated by a cAMP-independent process.

However our results might not be appropriate to the diabetic pregnancy.

Spontaneous preterm labor represents a harmful complication that takes place 8-10 times more frequently in the diabetic compared to non-diabetic population. Such high frequency could be explained in part by related pathology, i.e. congenital malformations and hydramnios, while it remains unexplained in more than two thirds of the cases. Preterm maturation of the combined different hormonal factors which take part in the beginning of parturition was suggested among the possible explanations. Amniotic levels of fetal insulin are known to be significantly increased in the diabetic pregnancy compared to normal. Furthermore high and rising amniotic insulin levels at an early stage of gestation may indicate a high risk of preterm onset of labor ([12]).

Although in our study insulin seems not to affect the levels of cAMP in the fetal membranes, higher amniotic concentrations of the hormone coupled to the different properties of the diabetic amniotic membranes might give different results that therefore remain to be investigated.

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