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# Differential regulation of calmodulin-dependent and -independent cyclic AMP phosphodiesterases from oviduct by fatty acids

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**SUMMARY** : Regulation of calmodulin-independent and -dependent cAMP phosphodiesterases from quail oviduct by various fatty acids was studied. The calmodulin-independent form was slightly activated by low concentrations (20 μM) of oleic, linoleic and arachidonic acid, higher concentrations were inhibitory. The basal activity of the calmodulin-dependent form was activated by linoleic acid and to a lesser extent by arachidonic acid at low concentrations and inhibited by higher concentrations of the two fatty acids. In contrast, arachidonic acid was a potent reversible inhibitor of calmodulin in the activation of this enzyme ($IC_{50}$ : 20 μM) whereas linoleic acid was inactive from 10 to 150 μM. The present results strongly suggest that the differential regulation of cAMP phosphodiesterases by these fatty acids could profoundly influence the level of cAMP in the oviduct and thus its subsequent effects. 

It is now firmly established that the activity of cyclic nucleotide phosphodiesterase (PDE) is largely involved in the control of the intracellular level of cyclic nucleotides. Multiple forms of PDE with differences in substrate specificity, kinetic behavior, and regulatory properties have been described from a variety of tissues [1-2]. The calmodulin (CaM) -dependent form of cyclic nucleotide PDE from brain can be activated by phospholipids and unsaturated fatty acids, in a $Ca^{2+}$-independent fashion [3-6]. In contrast, the cytosolic calcium-independent forms of PDE from lung or liver are not affected by phospholipids whereas the $Ca^{2+}$-independent form from bovine heart is non-competitively inhibited by some phospholipids or by D,L-palmitoyl-carnitine [7]. The cGMP-stimulated cAMP PDEs from calf (8) or rat liver (9) could be selectively inhibited by arachidonic acid.

In the quail oviduct, we have separated three forms of PDE, two were responsible for cAMP and cGMP hydrolysis and one of the two was CaM-sensitive [10]. The mechanism by which CaM-dependent and -independent cAMP PDEs are regulated in the oviduct is of peculiar interest because they are submitted to hormonal regulation and involved in both estrogen-induced [10,11] and antiestrogen-inhibited growth [12], through the control of the intracellular level of cAMP. On the other hand, estradiol and progesterone have been implicated as regulatory agents of the acylation-deacylation cycles involving various phospholipids,

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triacylglycerols and arachidonic acid in female reproductive organs of several species [13-15]. These results prompted us to investigate the influence of oleic acid, the major monounsaturated fatty acid (80 %) of oviduct phospholipids and arachidonic and linoleic acids, which account for more than 90 % of the n-6 polyunsaturated fatty acids of oviduct phospholipids, on the CaM-dependent and -independent cAMP PDE activities from quail oviduct.

## MATERIALS AND METHODS

**Chemicals :** 8-[$^3$H] cAMP (28 Ci/mmol), [U-$^{14}$C] adenosine (515 mCi/mmol) were from Amersham. Bovine brain CaM, essentially fatty acid-free bovine serum albumin, snake (Ophiophagus hannah) venom, unlabelled cAMP and fatty acids were purchased from Sigma Chemical Co (St. Louis, MO). QAE-Sephadex A-25 was obtained from Pharmacia (Uppsala, Sweden) and the resin AG 1-x 2 (200-400 mesh) was supplied by Bio-Rad Laboratories (Richmond, CA).

**Enzyme preparation :** Immature female quails (Coturnix coturnix japonica) received a daily injection of estradiol benzoate (0.1 mg/kg,im) for 3 consecutive days and were killed by decapitation 24 hours after the last injection. Oviducts were immediately removed, cleaned from connective tissue and homogenized at 4°C in an all glass dual homogenizer (Kontes) in 10 mM Tris-HCl buffer (pH 7.5) with 2 mM EDTA, 10 mM 2ß-mercaptoethanol, 0.21 M sucrose, and 75 µg/ml phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 105,000 x g for 1h. By ion exchange chromatography on QAE-Sephadex A-25, the crude PDE preparation (cytosol) was deprived from endogenous CaM and separated into two fractions containing the CaM-dependent ($F_1$) and -independent forms ($F_2$) of cAMP PDE, as described previously [12].

**PDE assay :** The cAMP PDE activities of cytosol, $F_1$ and $F_2$ were measured by a two-step radioisotopic method [16] using [$^3$H] cAMP as the substrate [12]. The standard assay mixture (final volume 400 µl ; pH 8.0) contained 40 mM Tris-HCl, 5 mM $MgCl_2$, 3.75 mM 2ß-mercaptoethanol, 100 µg BSA, 0.1 µCi [$^3$H] cAMP, 0.25 µM cAMP, and 100 µl of the enzymatic preparation. After incubation at 30°C, the reaction was terminated by heating at 100°C for 1 min. Snake venom was added to the assay mixture to convert 5'-[$^3$H] AMP formed to [$^3$H] adenosine. Tritiated adenosine was isolated by anion-exchange chromatography and quantified with a liquid scintillation counter. Recovery of adenosine was monitored for each sample with [$^{14}$C] adenosine. Phosphodiesterase activity at 30°C was found to be linear with time and protein concentration as long as less than 30 % of the substrate was hydrolysed. Routinely, a 15 min incubation period was used, the enzyme was diluted to give about 20 % hydrolysis of substrate. The basal activity of each fraction was determined by adding 1 mM EGTA. For CaM-stimulated activity, the assays were supplemented with 0.5 mM $CaCl_2$ and 25 U/ml of purified CaM. When fatty acids were included in the assay, they were dissolved in a small volume of ethanol, diluted with the incubation buffer, sonicated and transferred to the assay mixture just before starting the reaction. The vehicle (ethanol diluted with buffer) was added to control samples. At the concentration used (less than 0.25 %) ethanol did not change the PDE activity. Fatty acids had no effect on the venom nucleotidase reaction used to convert [$^3$H] AMP into [$^3$H] adenosine in the second step of the method.

## RESULTS AND DISCUSSION

In quail oviduct homogenates, cAMP PDE activity determined at low substrate level (0.25 µM) is almost completely restricted to the cytosolic fraction [10]. As demonstrated previously [10,12] and summarized in table 1, a CaM-sensitive ($F_1$) and a CaM-insensitive ($F_2$) form of the crude enzyme were clearly separated by QAE-Sephadex chromatography. The activity of the first peak ($F_1$) was stimulated five-fold by the addition of exogenous $Ca^{2+}$ and CaM while only marginal stimulation of the activity of the second peak ($F_2$) was observed.

**Table 1** . Crude, basal, and CaM-stimulated activity of the cytosolic cAMP PDE and the isoenzymatic forms ($F_1$ and $F_2$) separated by ion exchange chromatography

| | PDE activity (pmol cAMP hydrolyzed/min/mg protein) | | |
|---|---|---|---|
| | Crude | Basal (EGTA 1 mM) | CaM (25 U/ml)-stimulated |
| S 105.000 | 262.8 ± 9.4 | 221.5 ± 6.4 | 457.6 ± 20.6 |
| $F_1$ | — | 74.4 ± 6.5 | 373.9 ± 34.4 |
| $F_2$ | — | 329.6 ± 25.8 | 400 ± 36.1 |

S 105.000, $F_1$ and $F_2$ represent the 105.000g supernatant of oviduct homogenate, the first and the second fractions collected after ion exchange chromatography, respectively. Results are the mean ± SEM of five different homogenates.

Crude cAMP hydrolyzing activity of the 105,000 x g supernatant (Fig. 1) was insensitive to stearic acid. Increasing concentrations of oleic acid from 10 to 200 μM increased hydrolysis of [$^3$H] cAMP with maximal activation (40 % $p < 0.01$) at 20 μM. In contrast, arachidonic acid (over 20 μM) and linoleic acid (over 50 μM) were inhibitory. At low concentration (20 μM) linoleic acid increased hydrolysis slightly (+ 16 %, $p < 0.05$). Potassium salts of fatty acids were also employed and the same results were obtained (not shown).

Effects of fatty acids on CaM-insensitive cAMP PDE (Fig. 2). The activity of fraction $F_2$ was not significantly affected by stearic acid. Increasing concentrations of oleic, linoleic or

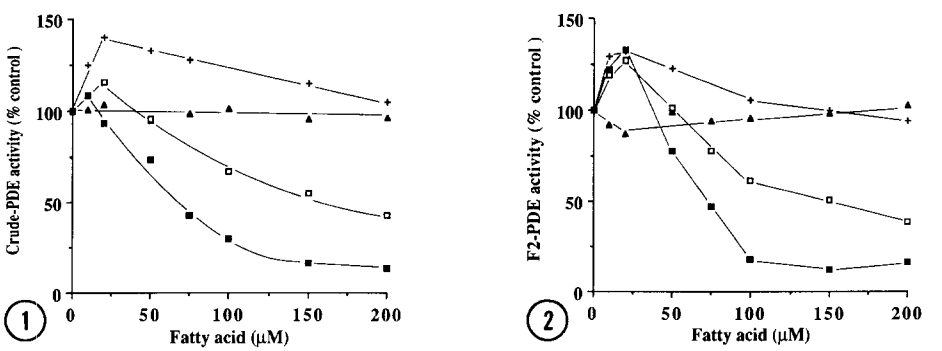


Fig. 1 . Effects of stearic, oleic, linoleic or arachidonic acid on crude cytosolic cAMP PDE.
cAMP PDE activity was measured without or with the indicated concentrations of stearic (▲), oleic ( + ), linoleic ( □ ) or arachidonic ( ■ ) acid. The control value (absence of fatty acid) was 262.8 ± 9.4 pmol cAMP hydrolyzed/min/mg protein. All results are averages of 3-5 determinations.

Fig. 2 . Effects of stearic, oleic, linoleic or arachidonic acid on CaM-independent cAMP PDE ($F_2$).
CaM-independent PDE activity ($F_2$) was measured without or with the indicated concentrations of stearic ( ▲ ), oleic ( + ), linoleic ( □ ) or arachidonic ( ■ ) acid in the presence of 1 mM EGTA in the incubation medium. The control value (absence of fatty acid) was 329.6 ± 25.8 pmol cAMP hydrolyzed/min/mg protein. All results are averages of 3-5 determinations.

arachidonic acids from 10 to 200 μM first increased hydrolysis of [$^3$H] cAMP, with maximal activation (linoleic : + 27.5 %, $p < 0.01$ ; oleic : +32 %, $p < 0.01$ ; arachidonic : + 32.5 %, $p < 0.01$) at 20 μM and then reduced the enzyme activity. Arachidonic acid was more effective than linoleic acid in inhibiting cAMP hydrolysis, $IC_{50}$ values (concentration of fatty acid required to inhibit phosphodiesterase activity by 50 %) were 65 and 130 μM, respectively. At concentrations over 20 μM, the stimulatory effect of oleic acid was gradually reduced but no significative inhibition was seen even at 200 μM.

It should be noted that the extent of inhibition of the CaM-independent oviducal enzyme by unsaturated fatty acids correlated with the number of double bonds. Similar inhibitory effects of unsaturated fatty acids have been described on the cGMP-stimulated cAMP PDE from calf (8) and rat (9) liver. Oleic acid had little effect while arachidonic acid was the most inhibitory ($IC_{50}$ = 70 μM for the calf enzyme) and its effect was increased in the presence of stimulatory concentrations of cGMP. However, no stimulation of cAMP hydrolysis by the purified bovine or rat liver enzyme with low concentrations of these unsaturated fatty acids was observed.

Effect of fatty acids on CaM-sensitive cAMP PDE. Both basal activity and $Ca^{2+}$-CaM-stimulated activity of fraction $F_1$ were measured. The basal activity (Fig. 3), which was measured with 1 mM EGTA in the assay mixture, was stimulated by increasing concentrations of oleic acid, with maximal activation (+ 80 %, $p < 0.01$) at 150 μM. Linoleic and arachidonic acids at low concentrations also increased the hydrolysis of [$^3$H] cAMP. Activation was maximal at 20 μM but linoleic acid was about 3 times more potent than arachidonic acid (+ 66.3 %, $p < 0.01$ and + 23.1 %, $p < 0.05$, respectively). Higher concentrations of both fatty acids were inhibitory.

As shown in table 1, the cAMP hydrolyzing activity of fraction $F_1$ was increased by 5-fold in the presence of 0.5 mM $Ca^{2+}$ and 25 U/ml of CaM, which is the smallest concentration of CaM necessary for maximal stimulation of enzyme [12]. Under these conditions, arachidonic acid inhibited the activation of cAMP PDE by exogenous $Ca^{2+}$/CaM complex in a concentration-dependent manner (Fig. 4), with an $IC_{50}$ value of 20 μM. Linoleic acid was inactive from 10 to 150 μM and inhibitory at higher concentrations ($IC_{50}$ : 235 μM). Stearic acid was found to be inactive on both basal and $Ca^{2+}$-CaM-stimulated activity of fraction F1 at all the concentrations used. An increase of CaM concentration from 25 to 250 U/ml in the assay mixture did not significantly increase PDE activity in the absence of arachidonic acid, but completely suppressed the inhibitory effect of 20 μM arachidonic acid (Fig. 4, insert). This suggests that arachidonic acid is a reversible inhibitor of CaM in PDE activation. This activity could result from a direct effect on the conformation of CaM as suggested recently by Chiba et al. (17).

These results show that regulation of CaM-dependent PDE from quail oviduct by polyunsaturated fatty acids is quite different from that of the CaM-dependent PDE purified from bovine [4,6] or porcine [3] brain. Oleic, linoleic and arachidonic acid have been described as $Ca^{2+}$-independent activators of the brain enzyme, half-maximal stimulation occurring at 30-40 μM (6). The degree of stimulation of cAMP hydrolysis was almost comparable to that elicited by saturating concentrations of CaM (18). Activation of the brain enzyme by these substances

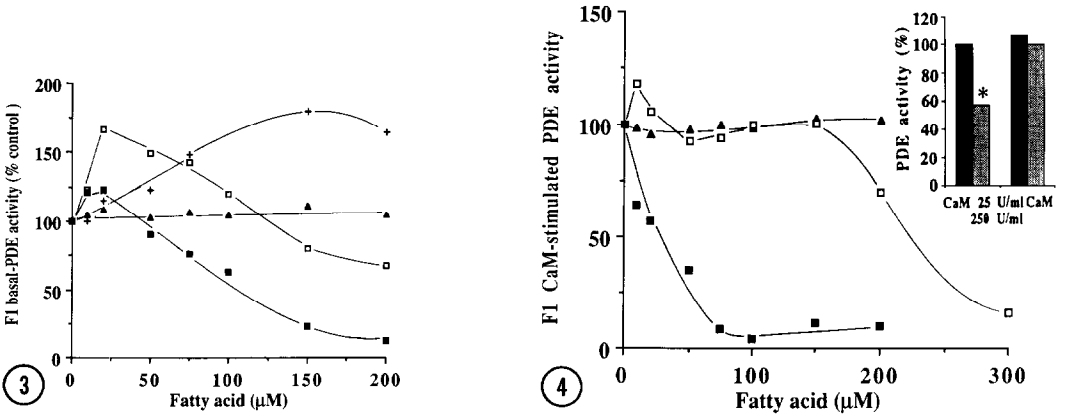


Fig. 3 . Effects of stearic, oleic, linoleic or arachidonic acid on basal CaM-dependent cAMP PDE ($F_1$).

CaM-dependent PDE activity was measured without or with the indicated concentrations of stearic ( ▲ ), oleic ( + ), linoleic ( □ ) or arachidonic ( ■ ) acid in the presence of 1 mM EGTA in the incubation medium. Activities are expressed relative to the activity of the enzyme in the absence of fatty acid (74.4 ± 6.5 pmol cAMP hydrolyzed/min/mg protein). All results are averages of 3-5 determinations.

Fig. 4 . Effects of stearic linoleic or arachidonic acid on CaM-stimulated cAMP PDE ($F_1$).

CaM-dependent PDE activity was measured without or with the indicated concentrations or stearic ( ▲ ), linoleic ( □ ) or arachidonic ( ■ ) acid in the presence of 25 U/ml CaM and 0.5 mM $CaCl_2$ in the incubation medium. Activities are expressed as :

$$\frac{\text{(CaM-stimulated - basal activity) in the presence of fatty acid}}{\text{(CaM-stimulated - basal activity) in the absence of fatty acid}} \times 100.$$

All results are averages of 3 determinations.

Insert : CaM-dependent PDE (F1) was measured without (black columns) or with (dotted columns) 20 μM arachidonic acid in the presence of either 25 U/ml CaM and 0.5 mM $CaCl_2$ or 250 U/ml CaM and 0.5 mM $CaCl_2$. Phosphodiesterase activity is expressed relative to the activity of the enzyme stimulated by 25 U/ml CaM in the absence of arachidonic acid. Results are averages of 3 determinations.

* significantly different ($p < 0.01$) from the control activity.

was accompanied by simultaneous decrease of CaM stimulation, suggesting competitive interaction of these different effectors at the same site (6, 18). For the oviduct enzyme, only marginal stimulation of the basal activity was observed with low concentrations (10-20 μM) of arachidonic acid and higher concentrations were inhibitory. Moreover, reversible inhibition of $Ca^{2+}$/CaM-dependent cAMP hydrolysis by arachidonic acid at low concentrations ($IC_{50}$ = 20 μM) was also demonstrated. Linoleic acid was about 12-fold less potent ($IC_{50}$ = 235 μM). It should be noted that specific inhibition of another $Ca^{2+}$/CaM-dependent enzyme (CaM-kinase II) by arachidonic acid with a comparable $IC_{50}$ (24 μM) was demonstrated in nerve terminals (19).

In previous studies, we have shown that estradiol indirectly stimulates the proliferation of quail oviduct epithelial cells in vivo (20) and that this indirect mechanism involves the regulation of cAMP levels (10, 11, 21). Cumulative results show a significant correlation between growth inhibitory potency of triphenylethylene antiestrogens and their efficiency to inhibit CaM-dependent-cAMP PDE (12, 22, 23). Moreover, it has been reported by numerous laboratories that the amount and type of dietary fat can significantly influence the development of estrogen sensitive tumors (24). Thus, the differential regulation of CaM-dependent and -

independent cAMP PDEs from oviduct by unsaturated fatty acids described in this study and more particularly the inhibition of the CaM-dependent isoenzyme by arachidonic acid may be of physiological significance in the control of oviduct growth.

Concentration of free arachidonic acid in the immature oviduct is around 10 μM (unpublished result). But since phospholipase $A_2$ activity (25), arachidonate turnover (26), and eicosanoid production (27) in estradiol-target cells are stimulated by estradiol, it is likely that arachidonate, or else more active metabolites, could attain sufficient concentrations following estradiol injection to affect PDEs activity, cAMP levels, and cell cycle progression. In addition, the activities of fatty acids were observed in the presence of 0.025 % BSA which binds these molecules, suggesting that in the absence of BSA, the efficient doses will be lower.

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