Detection and preliminary characterization of two enzymes involved in biosynthesis of platelet-activating factor in mouse oocytes, zygotes and preimplantation embryos: dithiothreitol-insensitive cytidinediphosphocholine: 1-*O*-alkyl-2-acetyl-*sn*-glycerol cholinephosphotransferase and acetyl-coenzyme A:1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine acetyltransferase

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The aim of this study was to determine whether the final enzymes in the two biosynthetic pathways for platelet-activating factor (PAF) (the 'de novo' and the 'membrane remodelling' pathways) are present in mouse embryos, zygotes and oocytes. The enzymes are dithiothreitol-insensitive cytidinediphosphocholine: 1-*O*-alkyl-2-acetyl-*sn*-glycerol cholinephosphotransferase (cholinephosphotransferase) in the *de novo* pathway and acetyl-coenzyme A:1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine acetyltransferase (acetyltransferase) in the membrane remodelling pathway. Activity of both enzymes was detected in the unfertilized oocyte, the zygote and also in the preimplantation embryo (48, 72 and 96 h after the ovulatory injection of hCG). In both cases the activity was destroyed by boiling and increased linearly with incubation time and the concentration of embryo homogenate present, indicating that the reactions were catalysed by enzymes. The product of the reactions was confirmed as PAF using HPLC and structural analyses by enzymatic digestion. Cholinephosphotransferase required Mg^{2+} and was inhibited by Ca^{2+} , while acetyltransferase required the presence of NaF (a phosphatase inhibitor). The activity of cholinephosphotransferase was similar in unfertilized oocytes and zygotes, and did not change significantly with advancing developmental stage in preimplantation embryos. Acetyltransferase had a significantly lower specific activity (0.078 ± 0.044 fmol PAF per oocyte per min, mean $\pm$ SEM) in unfertilized oocytes than in zygotes of corresponding age (0.358 ± 0.097 fmol PAF per zygote per min) ($P < 0.03$). During the preimplantation period, acetyltransferase activity was higher at the two-cell stage (0.578 ± 0.245 fmol PAF per embryo per min) than for compacted embryos (0.097 ± 0.056), while blastocysts displayed an intermediate activity (0.201 ± 0.002). For both enzymes, activity was variable at all developmental stages studied. The results indicate that the enzymes that catalyse the final step in both biosynthetic pathways are present within the oocyte and early embryo, while the activation of acetyltransferase following fertilization indicates that this enzyme may be of regulatory significance in the initiation of biosynthesis of PAF by zygotes.

Introduction

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is released by the preimplantation embryos of a number of species (mice: O'Neill, 1985a; humans: Collier *et al.*, 1988; sheep: Battye *et al.*, 1991). It has been detected using several different methods including a whole-animal

bioassay (O'Neill *et al.*, 1987), *in vitro* bioassays (Collier *et al.*, 1988), radioimmunoassays (Ammit and O'Neill, 1991) and mass spectroscopy (Kodama *et al.*, 1989), and has been independently confirmed for the mouse (Adamson *et al.*, 1991) and human (Punjabi *et al.*, 1990) embryo. However, some workers (Amiel *et al.*, 1989; Smal *et al.*, 1990) have failed to demonstrate the release of PAF by embryos. This conflict and a consideration of its possible causes have been considered in detail in a recent review by O'Neill (1993).

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Fig. 1. The reactions catalysed by cholinephosphotransferase (I) and acetyltransferase (II) involved in biosynthesis of platelet-activating factor (PAF) in mouse embryos: CDP: cytidine diphosphate; CMP: cytidine monophosphate; PCho: phosphocholine; R: C16–C18.

Two-cell mouse embryos cultured *in vitro* were shown to convert radiolabelled precursors into radiolabelled embryo-derived PAF (Wells and O'Neill, 1992). The precursors included intermediates of known PAF biosynthetic pathways (long-chain alcohols, lysoPAF, hexadecylacetyl-glycerol). Studies in various cell types indicate that PAF synthesis may occur via one of two biosynthetic pathways: the '*de novo*' and 'membrane remodelling' pathways (for review see Snyder, 1990). The immediate precursors of PAF in the *de novo* pathway are 1-O-alkyl-2-acetyl-*sn*-glycerol (alkylacetyl-glycerol) and cytidinediphosphocholine. In the membrane remodelling pathway, the substrates are 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lysoPAF) and acetyl-coenzyme A (acetyl-CoA). These reactions are shown in Fig. 1 and are catalysed by dithiothreitol-insensitive cytidinediphosphocholine: 1-O-alkyl-2-acetyl-*sn*-glycerol cholinephosphotransferase (EC 2.7.8.16, cholinephosphotransferase), and by acetyl-coenzyme A: 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphocholine acetyltransferase (EC 2.3.1.67, acetyltransferase), respectively (Snyder, 1990).

This paper reports the modification of assays for cholinephosphotransferase and acetyltransferase to allow detection of their activity in mouse embryo extracts. Preliminary characterization of the activity of the enzyme is performed and their activity in unfertilized oocytes, zygotes and preimplantation embryos is examined.

Materials and Methods

Materials

1-O-[Hexadecyl-1'2'- $^3\text{H(N)}$]-2-acetyl-*sn*-glycero-3-phosphocholine (^3H]PAF; specific activity, 2220.0 GBq mmol $^{-1}$), 1-O-[alkyl-1'2'- ^3H]-*sn*-glyceryl-3-phosphocholine (^3H]lysoPAF; specific activity, 1924.0 GBq mmol $^{-1}$), [acetyl- ^3H]CoA (specific activity, 173.9 GBq mmol $^{-1}$) and [methyl- ^{14}C]cytidinediphosphocholine (specific activity, 2.1 GBq mmol $^{-1}$) were supplied by NEN (Wilmington, DE). Sigma Chemical Co (St Louis, MO) supplied L- α -lysophosphatidylcholine, 1-O-alkyl (lysoPAF) and acetyl-CoA, Na salt. BSA was supplied by Pentex, Miles Inc (Kankakee, IL) and 1-O-hexadecyl-2-acetyl-*sn*-glycerol was purchased from Nova Biochem AG (Laufelfingen). All organic solvents were HPLC grade (BDH, Poole). All other chemicals used were the best grade for the purpose.

Embryo collection

Random-bred Swiss albino mice of the Quackenbush strain (Gore Hill Research Laboratories, NSW), 6–8 weeks old, were superovulated i.p. with 10 iu pregnant mares' serum gonadotrophin (Folligon, Intervet International, Boxmeer) followed 48 h later by an i.p. injection of 10 iu hCG (Chorulon, Intervet). They were either left unmated or paired with males of proven fertility overnight; the presence of a copulation plug indicated day 1 of pregnancy.

Unfertilized oocytes and zygotes were collected 24 h after hCG administration. The cumulus masses were released from the oviduct into Hepes-buffered human tubal fluid medium (Quinn *et al.*, 1985) supplemented with 3 mg BSA ml $^{-1}$ (Hepes-HTF/BSA). Oocytes or zygotes were freed from their cumulus masses by brief exposure to 300 iu hyaluronidase (Sigma Chemical Co) in Hepes-HTFM/BSA. They were then thoroughly washed in five changes of Hepes-HTFM/BSA. Embryos at the two-cell stage were recovered by flushing the oviducts of mated animals 48 h after hCG injection (day 2). Compacted embryos were collected 72 h after hCG administration and blastocysts were collected from the uterus approximately 96 h after hCG administration. In every case embryos were collected in Hepes-HTF/BSA. Embryos were washed twice in bicarbonate-buffered human tubal fluid medium (Quinn *et al.*, 1985) containing 3 mg BSA ml $^{-1}$ (HTFM/BSA). Embryos were transferred to the appropriate buffer on ice and then prepared for assay.

Preparation of embryo extracts

All operations were performed at 4°C and all buffers contained 3 mg BSA ml $^{-1}$. When performing dithiothreitol-insensitive cholinephosphotransferase assays, embryo extracts were prepared in 0.1 mol Tris l $^{-1}$ containing 0.5 mmol EGTA l $^{-1}$, 10 mmol MgCl $_2$ l $^{-1}$ and 5 mmol dithiothreitol (DTT) l $^{-1}$ (all from Sigma Chemical Co), pH 8.0 (Alonso *et al.*, 1982). Embryo extracts in which acetyltransferase activity was measured were prepared in 20 mmol Tris l $^{-1}$ containing 50 mmol NaF l $^{-1}$ (Sigma Chemical Co) and 5 mmol DTT l $^{-1}$, pH 7.4 (Lenihan and Lee, 1984; Lee, 1985).

Embryos were transferred in a minimal volume using a drawn Pasteur pipette to the appropriate buffer in a micro-centrifuge tube. Generally, embryos were added at the rates of 2 embryos μl^{-1} (acetyltransferase assay) and 1 embryo μl^{-1}

(cholinephosphotransferase). The mixture was immediately sonicated on ice with a microtip probe of a Heat Systems Ultrasonics W-385 sonicator (New York) on setting 1 for 10 s. Aliquots of the mixture were used immediately in the appropriate enzyme assay.

Enzyme assays

All assays were performed in microcentrifuge tubes and incubated at 37°C. The assays were initiated by the addition of embryo homogenate and stopped by freezing. Assays were generally performed at least in duplicate. Blank reactions contained aliquots of the preparation buffer without embryos. All assay buffers contained 3 mg BSA ml⁻¹.

Cholinephosphotransferase. This assay is based on the method described by Alonso *et al.* (1982) with modifications. In the standard assay, 20 µl of embryo extract was brought to a final volume of 27 µl while being supplemented with hexadecylacetylgllycerol (added in 1 µl ethanol) and [methyl-¹⁴C]cytidinediphosphocholine (3.7 kBq) to give final concentrations of 52 µmol l⁻¹ and 66 µmol l⁻¹, respectively.

Acetyltransferase. This assay is based on procedures described by Lenihan and Lee (1984) and Lee (1985) with modifications. A 15 µl aliquot of embryo extract was brought to a final volume of 28 µl with 0.1 mol Tris l⁻¹, pH 6.9, containing lysoPAF (added in 1 µl ethanol) and [acetyl-³H]CoA (3.7 kBq), to give final concentrations of 24 µmol l⁻¹ and 215 µmol l⁻¹, respectively.

Extraction and HPLC of radiolabelled PAF from assay mixtures

Each assay sample was thawed by the addition of 0.43 ml 0.1 mol Tris l⁻¹, pH 7.4, containing 3 mg BSA ml⁻¹ and allowed to stand at room temperature for approximately 10 min. The samples were then mixed and 0.4 ml was extracted as described by Wells and O'Neill (1992) by a modified Bligh–Dyer technique (Bligh and Dyer, 1959).

The resultant lipid extracts were resuspended in 120 µl methanol and chromatographed on a Partisil strong cation exchange HPLC column, 10 µm, 250 mm × 4.6 mm (Whatman, Clifton, NJ). Phospholipids were eluted isocratically with a mobile phase of acetonitrile:methanol:water (300:150:35 v:v:v) at a flow of 1.5 ml min⁻¹ (Wells and O'Neill, 1992). Eluant was collected (1.5 ml fractions) and the amount of radioactivity in each fraction was determined by liquid scintillation counting. For each sample, the distribution of radiolabel throughout the elution profile was compared with the known distribution of [³H]PAF. PAF production was identified as the amount of radioactivity eluted in the same region as [³H]PAF after subtraction of the blank. The elution profiles of other phospholipids (phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, sphingomyelin and [³H]lysoPAF) were also examined to ensure that they did not co-migrate with PAF, as has been described by Wells and O'Neill (1992). These phospholipids plus [³H]PAF were routinely chromatographed before and after a series of unknown samples to determine the retention time of PAF. Unlabelled and labelled standards were detected by UV absorbance at 203 nm and liquid scintillation counting, respectively.

[³H]PAF could not be used as an internal standard; the efficiency of the extraction and HPLC were therefore monitored by routinely incubating trace amounts of [³H]PAF (approximately 1500 d.p.m. in 1 µl ethanol) in the appropriate buffer and processing in parallel. Aliquots of these [³H]PAF-containing samples were taken before extraction, at various stages of the procedure, and the amount of radioactivity present was determined. The percentage recovery of [³H]PAF was used to correct for the loss of activity during extraction and chromatography. The mean (± SEM) recovery of a [³H]PAF tracer from assay mixtures was 58.6% (± 2.2%) after extraction and HPLC. This value is similar to the percentage recovery of [³H]PAF tracer from HTFM/BSA previously reported (Wells and O'Neill, 1992).

Identification of radiolabelled PAF

The ¹⁴C-labelled product resulting from the phosphocholine-transferase reaction was subject to enzymatic hydrolysis by phospholipase A₂ (bee venom; EC 3.1.1.4., Sigma Chemical Co), as described by Wykle *et al.* (1988). If the substrate had been incorporated into PAF, the product of phospholipase A₂ digestion would be [¹⁴C]lysoPAF. After hydrolysis, re-extraction and chromatography on the HPLC system described above was performed. The distribution of label in the elution profile was determined by liquid scintillation counting and compared with that of authentic lysoPAF.

Hydrolysis by phospholipase C (*Bacillus cerus*, EC. 3.1.4.3., grade I, Boehringer Mannheim, Castle Hill, NSW) of the radiolabelled product of the acetyltransferase reaction was performed as previously described (Wells and O'Neill, 1992). Zones of the TLC plates were scraped and radioactivity was eluted with hexane:diethylether (1:1, v:v). The distribution of the label was compared with that of [³H]alkylacetylgllycerol.

For both treatments, controls containing the appropriate labelled product but no phospholipase A₂ or phospholipase C were set up and incubated to ensure that no non-enzymatic hydrolysis occurred.

Statistical analyses

Data were tested for homogeneity by the Shapiro–Wilk test. The effect of changes to incubation conditions on enzyme activity was assessed where necessary by *t* tests between the treatment and its respective control value. The results are presented as percentage relative changes in activity. For the direct comparison of activity in oocytes and zygotes 24 h after hCG administration, *t* tests were also performed. The activity at different development stages was assessed by analysis of variance and, where applicable, multiple comparisons of means were made by the least significant difference test. All analyses were performed using the SPIDA statistics package, Statistical Computing Laboratory Pty Ltd, Eastwood, NSW.

Results

Two-cell mouse embryos (48 h after hCG administration) were used for the development of enzyme assays and preliminary

Fig. 2. The influence of incubation time (at 37°C) on the activity of (□) acetyltransferase and (Δ) cholinephosphotransferase in mouse embryos. Cholinephosphotransferase assays were incubated for a maximum of 60 min and acetyltransferase assays for up to 180 min. Each point represents the mean of two experiments, with each experiment performed in duplicate. PAF: platelet-activating factor.

characterization of activity. Enzyme activity was detected by conversion of substrate into radiolabelled PAF as detected by HPLC. The results are expressed as the fmol of [^3H]PAF or [^{14}C]PAF produced in the acetyltransferase or cholinephosphotransferase assays, respectively.

In preliminary experiments it was found that when embryos were prepared in buffer without exogenous BSA, a significant proportion of the incorporated label co-migrated with lyso-PAF. Furthermore, the embryo extracts could convert approximately 15% of exogenous [^3H]PAF into lysoPAF, suggesting the presence of an acetylhydrolase activity. Addition of 3 mg BSA ml^{-1} to the reaction mixture consistently caused the complete inhibition of this hydrolysis. Thus, in all experiments reported here, exogenous BSA was present in enzyme extracts to allow total enzyme activity to be assessed.

The cholinephosphotransferase activity that produced PAF from hexadecylacetyl glycerol and [^{14}C]cytidinediphosphocholine increased with incubation time, reaching an apparent maximum by 30 min (Fig. 2), and with the concentration of embryo homogenate present (Fig. 3). Its activity was destroyed by boiling and required the presence of exogenous alkylacetyl glycerol (Table 1). It was not affected ($P > 0.05$) by the absence of either EGTA or DTT from the buffers (Table 1). However, the absence of Mg^{2+} significantly reduced activity ($P < 0.01$) while the presence of $100 \mu\text{mol Ca}^{2+} \text{ l}^{-1}$ in the absence of EGTA also significantly reduced ($P < 0.01$) activity to 6% of control activity (Table 1). Standard conditions for assessing the specific activity of cholinephosphotransferase were defined as the activity in the extracts of 20 embryos or oocytes after incubation for 30 min in the reaction mixture described. The results represent the fmol [^{14}C]choline incorporated into PAF per embryo per min of incubation time.

Acetyltransferase activity also increased with the concentration of embryo homogenate present (Fig. 3) and the incubation time, with a plateau occurring after 120 min (Fig. 2). No activity was detected in the absence of lysoPAF, or when extracts were boiled for 15 min prior to assay (Table 1). The absence of NaF (a phosphatase inhibitor) reduced activity

Fig. 3. The activity of (□) acetyltransferase and (Δ) cholinephosphotransferase in the presence of increasing concentrations of mouse embryo homogenates. The abscissa values represent the equivalent number of embryos in the homogenate added to the assay. Acetyltransferase assays were incubated for 180 min and cholinephosphotransferase assays for 30 min. Each point represents the mean of two experiments. PAF: platelet-activating factor.

($P < 0.01$) to 9.1% of the control activity (Table 1). Standard conditions for assessing the specific activity of acetyltransferase were defined as the activity in the extracts of 30 embryos or oocytes following a 180 min incubation period in the reaction mixture. The results represent fmol [^3H]acetate incorporated into PAF per embryo per min of incubation time.

The products of both reactions were confirmed to be PAF by structural analysis with enzyme digestion. The products of several reactions were pooled and, after hydrolysis by phospholipase A_2 , 87% of the total radioactivity produced by the cholinephosphotransferase reaction co-migrated with lysoPAF on HPLC. Treatment of the acetyltransferase reaction product with phospholipase C resulted in 89% of the total radioactivity detected in the profile co-migrating with alkylacetyl glycerol.

The specific activity of the two enzymes was compared in unfertilized oocytes and zygotes of the same age. The activity in embryos throughout the preimplantation period of pregnancy was also assessed. For acetyltransferase, the activity in unfertilized oocytes collected 24 h after hCG injection was significantly lower (0.078 ± 0.044 fmol PAF per oocyte per min^{-1} , mean $\pm$ SEM, $n = 4$) than that found in zygotes of equivalent age (0.358 ± 0.097 fmol PAF per zygote per min, $n = 6$) ($P < 0.03$, t test). By comparison, there was no significant difference ($P > 0.05$, t test) in the activity of cholinephosphotransferase between unfertilized oocytes (3.298 ± 1.080 fmol PAF per oocyte per min, $n = 3$) and zygotes (2.307 ± 0.400 , $n = 3$).

Table 1. The effect of various treatments of mouse embryo homogenates or modifications to the assay buffer on the relative activity of cholinephosphotransferase and acetyltransferase

Enzyme	Treatment	Activity (% of control (mean $\pm$ SEM))
Cholinephosphotransferase	Control	100
	Boiling (15 min)	0
	- DDT	108.3 $\pm$ 13.4
	- alkylacetyl glycerol	0
	- EGTA	82.8 $\pm$ 3.5
	- Mg ²⁺	8.6 $\pm$ 1.7
	- EGTA, + Ca ²⁺	6.2 $\pm$ 0.7
Acetyltransferase	Control	100
	Boiling (15 min)	0
	- lysoPAF	0
	- NaF	9.1 $\pm$ 6.7

DTT: dithiothreitol.

Cholinephosphotransferase activity was variable within and between developmental stages of the preimplantation period. There was a trend for the activity to be highest at the two-cell stage (48 h after hCG injection) with a reduction of activity with increasing time of development; however, this trend was not significant ($P > 0.05$) (Fig. 4a). Acetyltransferase activity showed considerable variability for all stages of development (Fig. 4b). The activity found in two-cell stage embryo homogenates was significantly higher than in compacted embryos (72 h after hCG administration) ($P < 0.01$), while zygotes and blastocysts had intermediate values ($P > 0.05$).

Discussion

This paper reports the first successful detection of the PAF biosynthetic enzymes, cholinephosphotransferase and acetyltransferase in extracts of oocytes, zygotes and preimplantation embryos. The results confirm the observation of feeding experiments *in vitro* (Wells and O'Neill, 1992), which showed that embryos can biosynthesize PAF. Together, the results of these two studies suggest that the release of PAF by embryos is probably due to its synthesis rather than solely due to the release of preformed stores.

For both the cholinephosphotransferase and acetyltransferase reactions, the activities were heat labile and dependent upon the time that the reaction was allowed to proceed, and was linear with increasing concentrations of embryo homogenates. Such results indicate that the reactions were enzymatically catalysed. The radioactive product of the reactions can be considered to be PAF since it migrated with authentic PAF during HPLC, while enzymatic digestion of the reaction products by phospholipase A₂ and phospholipase C resulted in the expected degradation products.

Embryonic cholinephosphotransferase activity was not affected by the presence of 5 mmol DTT l⁻¹. This property is similar to cholinephosphotransferase activities of the *de novo* pathway of PAF synthesis in various rat tissues (Renooij and Snyder, 1981; Woodard *et al.*, 1987). The finding that the activity was not significantly higher in the absence of DTT

Fig. 4. The specific activity of (a) cholinephosphotransferase and (b) acetyltransferase at various times throughout the preimplantation phase of pregnancy in mice. Each bar represents the mean $\pm$ SEM of the number of experiments shown in brackets above each bar. PAF: platelet-activating factor.

leads to the suggestion that DTT-insensitive cholinephosphotransferase was the only enzyme present in the embryo that could add phosphocholine to alkylacetyl glycerol. This study does not exclude the possibility that the enzyme activity present may also be capable of catalysing the conversion of 1-O-alkyl-2-long chain acyl-*sn*-glycerol to 1-O-alkyl-2-long

chain acyl-*sn*-glycero-3-phosphocholine, a precursor of PAF in the membrane remodelling pathway (see Wells and O'Neill, 1992).

The insensitivity of cholinephosphotransferase to DTT has been used as a means of distinguishing it from another cholinephosphotransferase activity that is implicated in the synthesis of phosphatidylcholine (Woodard *et al.*, 1987). The sensitivity of the embryonic cholinephosphotransferase to Ca^{2+} and its requirement for Mg^{2+} illustrates further similarities with the DTT-insensitive cholinephosphotransferase of other tissues (Woodard *et al.*, 1987).

The inhibition of acetyltransferase activity in the absence of NaF (a phosphatase inhibitor) from the reaction mixture suggests that the embryonic enzyme may be regulated by its phosphorylation state, and confirms its similarity in this regard to this type of acetyltransferase from other sources (Lenihan and Lee, 1984). This study does not exclude the possibility that the enzyme activity may also be capable of converting 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate to 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphate, a precursor of alkylacetyl glycerol in the *de novo* biosynthetic pathway. In rat spleen tissue, these two activities have been differentiated by studies of pH optima, sensitivity to preincubation temperatures and substrate inhibition studies (Lee *et al.*, 1986). Such detailed characterization was beyond the scope of this study.

Both enzymes required exogenous lipid substrate for activity, suggesting that under the conditions of the assay, there was no significant accumulation of endogenous precursors in the embryo homogenates. In the case of cholinephosphotransferase, this is in contrast to the situation in the inner medulla of the rat kidney (Woodard *et al.*, 1987).

It has been reported that the release of PAF (Ammit and O'Neill, 1991) and the conversion of radiolabelled substrates into radiolabelled PAF released by embryos (Wells and O'Neill, 1992) is highly variable. In this study, the activity of both enzymes studied was also highly variable. Despite this variability, it was possible to detect a significant increase in the activity of acetyltransferase in fertilized embryos. Cholinephosphotransferase by contrast was not different in oocytes and zygotes, with a consistently higher specific activity than acetyltransferase.

The activation of acetyltransferase in the zygote suggests that its activity may be of regulatory significance for the synthesis of embryo-derived PAF, as it corresponds to the time that PAF release is first observed (O'Neill, 1985b). It has been adequately demonstrated in a variety of cell types that acetyltransferase may be activated after increase of the intracellular Ca^{2+} concentration by ionophores and that activation also requires phosphorylation of the enzyme, possibly by protein kinase C (Lenihan and Lee, 1984). Pulsatile increases in intracellular Ca^{2+} concentration are known to occur in mouse zygotes (Swann, 1992), while the requirement for NaF to detect embryo acetyltransferase is consistent with a requirement for the enzyme to be phosphorylated. Further studies will indicate whether this is the mode of regulation of embryonic acetyltransferase.

Measurement of specific activity of enzymes in embryo homogenates does not necessarily reflect the activity of these enzymes *in situ*. It is not valid to extrapolate results from such studies to the biosynthetic activity of the embryo, and even

less valid to make suppositions about the relationship between the enzymatic activity studied here and the amount of PAF released by embryos *in vivo*.

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