Two phases of inositol polyphosphate and diacylglycerol production at fertilisation

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[^{3}H]Inositol and [^{3}H]arachidonic acid were used to label polyphosphoinositide phospholipids in sea urchin eggs. Both [^{3}H]inositol polyphosphate ($InsP_3$) and [^{3}H]diacylglycerol (DAG) increase at fertilisation. An early increase in $InsP_3$ occurs as the sperm-induced calcium transient crosses the egg and exocytosis occurs; a later increase in $InsP_3$ as calcium declines and the protein kinase C-dependent Na/H antiporter causes the cytoplasmic pH to increase. These results support suggestions that a calcium-induced hydrolysis of phosphatidylinositol bisphosphate occurs at fertilisation, that the production of diacylglycerol may be essential for exocytosis and that diacylglycerol production at fertilisation stimulates the Na/H antiporter. The increase in [^{3}H]inositol polyphosphate as calcium declines indicates that this second messenger may have some function later in the cell cycle.

Inositol trisphosphate *Diacylglycerol* *Phosphoinositide* *Ca^{2+} release* *Protein kinase C* *Fertilization*

1. INTRODUCTION

It has been suggested that inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG), 2 second messengers produced by the hydrolysis of polyphosphoinositide phospholipids [1,2], play a part in the activation of sea urchin eggs at fertilisation [3,4]. Microinjecting $InsP_3$ activates sea urchin eggs by releasing calcium from an intracellular store [3] and $InsP_3$ will release calcium from the same store in vitro [5]. The protein kinase C activator phorbol myristate acetate causes the intracellular alkalinisation essential to development [6] by stimulating the egg plasma membrane Na^+/H^+ antiporter [4]. Synthetic diacylglycerol has similar effects (Swann and Whitaker, unpublished). Sea urchin egg plasma membrane has a calcium-sensitive phospholipase C, which suggests that the production of $InsP_3$ and DAG may be stimulated by the calcium transient at fertilisation [7]. We report here measurements of $InsP_3$ and DAG production consistent with these ideas. We also find a second increase in $InsP_3$ when the calcium transient is subsiding which plays no part in the above scheme. This raises the possibility that $InsP_3$ has other functions as a second messenger during the cell cycle.

2. MATERIALS AND METHODS

Eggs of *Lytechinus pictus* (Pacific Biomarine, Venice, CA) were incubated in artificial seawater [8] in the presence of *myo*-2-[^{3}H]inositol (10–20 Ci/mmol; 100 μCi for 18 h) or 5,6,8,9, 11,12,14,15-[^{3}H]arachidonic acid (80–135 Ci/mmol; 10 μCi for 3 h) in a 50% (v/v) egg suspension. The egg jelly coat was subsequently removed by passage through Nitex mesh as the eggs were washed free of external radioactivity. Eggs retained 5% of [^{3}H]inositol, 35% of this in polyphosphoinositide phospholipids (PPI), and 20% of [^{3}H]arachidonic acid, 5% in PPI and 75% in

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other lipids. Eggs were fertilised in a 5% (v/v) suspension with a sperm concentration of 10^8/ml. Samples were taken at intervals into ice-cold trichloroacetic acid. The trichloroacetic acid pellets were washed with distilled water and phospholipids extracted for chromatography [7,9]. The aqueous phase was applied to Dowex anion-exchange columns after neutralisation and eluted to separate inositol phosphates [10]. This procedure separates inositol phosphate and bisphosphate from trisphosphates and higher polyphosphates. It is possible that the fraction we call $InsP_3$ contains tetrakis- and higher polyphosphates [11]; these inositol polyphosphates are considered to have calcium-releasing properties similar to $InsP_3$. Protein content of samples was determined using a Bradford assay [12].

[^{3}H]Arachidonic acid labelled PPI to constant activity during the 3 h incubation. 18 h incubation with [^{3}H]inositol labelled phosphatidylinositol (PtdIns) to constant activity. Phosphatidylinositol phosphate (PtdInsP) and phosphatidylinositol bisphosphate ($PtdInsP_2$) showed increasing activity up to 24 h after addition of [^{3}H]inositol.

3. RESULTS

There is an increase in [^{3}H]inositol-labelled $InsP_3$ within 10 s of adding sperm to egg suspensions (fig.1a). [^{3}H]$InsP_3$ has a peak at 26 ± 6.3 s (mean ± SE, n = 5) and a trough at 61 ± 12.5 s (n = 5). There follows a second sustained increase. [^{3}H]$InsP_3$ remains elevated for at least 30 min after fertilisation. At the peak, [^{3}H]$InsP_3$ is 66 ± 11.7% (n = 5) greater than that in unfertilised eggs. At the trough, [^{3}H]$InsP_3$ is comparable to that in unfertilised eggs. The later increase is 53% greater. Since the time to peak varies from experiment to experiment, the peak is broadened and reduced in height by combining the data from 4 experiments (fig.1b).

[^{3}H]Arachidonate-labelled DAG shows a similar pattern of early increase (fig.2a): a rise within 10 s, a peak increase of 44 ± 7.7% at 21 ± 5.5 s (n = 7). No pronounced second peak of DAG was detected. Again, combining the data from 7 experiments leads to a reduction in the apparent value of the peak and a broadening of the time course (fig.2b).

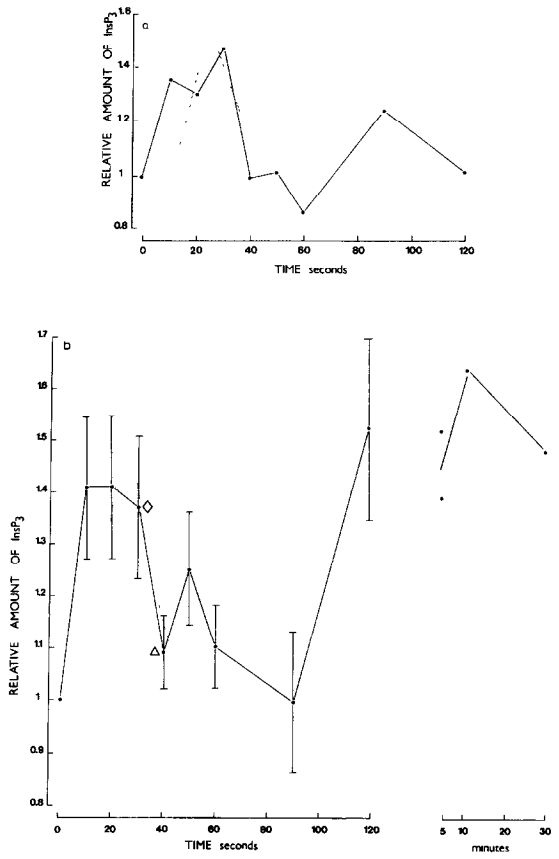

Fig.1. Changes in [^{3}H]inositol polyphosphate with time after insemination. (a) Data from a single experiment. (b) Combined data from 5 experiments. Mean and SE are shown. (◇) n = 4, (△) n = 3. The trough is significantly smaller than the peak ($p < 0.05$, Student's t-test). The dashed curve indicates the population time course of an event associated with the fertilisation wave (see legend to fig.3).

Fertilisation is not synchronous, even at the sperm densities we have used, as fig.3 shows. Fertilisation is 95% complete at 10 s. Fig.3 also indicates how the proportion of eggs undergoing the transient increase in intracellular calcium varies with time after insemination. The calcium transient begins at the earliest 7 s after fertilisation, but may be delayed substantially in individual eggs: at times greater than 7 s the population follows a constant transition probability [13]. We cannot therefore interpret the data of figs 1 and 2 as reproducing the time-dependent changes in each egg.

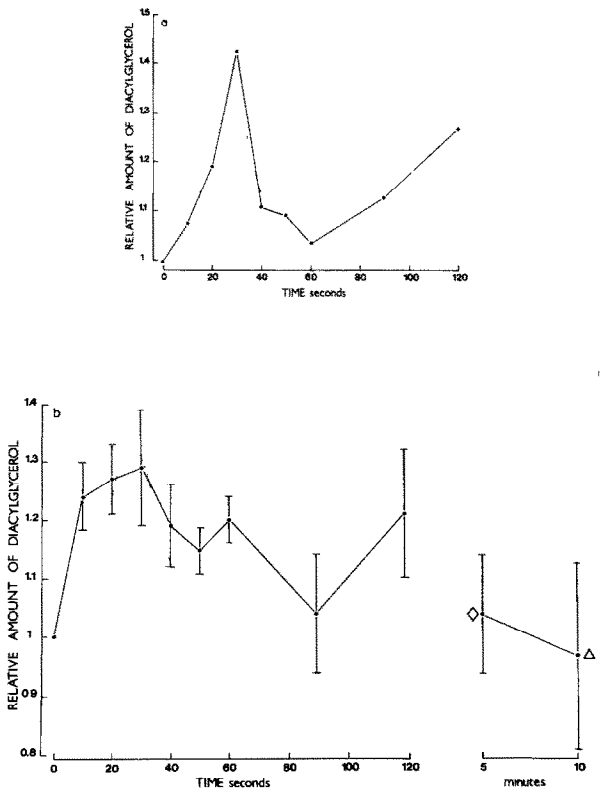

Fig.2. Changes in [^{3}H]arachidonate-labelled DAG with time after insemination. (a) Data from a single experiment. (b) Combined data from 7 experiments. Mean and SE are shown. (◇) $n = 5$, (△) $n = 3$. The dashed curve indicates the population time course of an event associated with the fertilisation wave (see legend to fig.3).

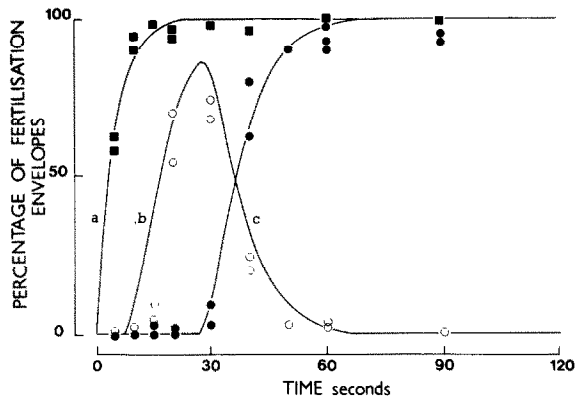

Fig.3. Time course of fertilisation in an egg population at the egg and sperm densities used to measure [^{3}H]$InsP_3$ and [^{3}H]DAG. (■) Fertilised eggs. (○) Eggs undergoing the transient increase in cytoplasmic free calcium. (●) Eggs in which the fertilisation wave is completed. (Two experiments are shown.) The fertilisation rate is constant and the number of unfertilised eggs falls exponentially with time [26]. The data indicate a fertilisation rate constant of 0.2 s^{-1} (curve a). Once fertilised, individual eggs undergo the calcium transient at a time $t_1 + t_2$ where $t_1 = 7$ s and t_2 is exponentially distributed with a transition probability of 0.15 s^{-1} [13]. Convolution of the 2 exponential functions gives the proportion of eggs in which the calcium transient has begun with time after insemination. The fertilisation wave propagates across the egg in 20 s [3,15], which gives the curve of the proportion of eggs completing the calcium transient (curve c). The proportion of eggs undergoing the calcium transient (curve b) is obtained by subtracting the proportion in which the calcium transient has begun from the proportion in which the fertilisation wave is completed. Thus far there are no ad hoc assumptions and no arbitrary constants. The curves in figs 1 and 2 are obtained by convolution from curve b by assuming that the fertilisation wave is a zone of constant breadth which travels across the egg with a constant velocity of 5 μm/s [3,27] and represents the time course of $InsP_3$ or DAG concentration if they are at high concentration in the active zone. Scaling the ordinate to our data suggests a 5–10-fold increase in $InsP_3$ concentration averaged over the cell volume or a 50–200-fold increase for an active zone of 5–10 μm wide.

However, we can fit the data of fig.3 without recourse to arbitrary constants. The curves in the figure use the fertilisation rate, which we measured, the 7 s delay and transition probability measured by Shen and Steinhardt [13] and the propagation time of the calcium transient which we measured previously [3]. If we then assume that a wave of $InsP_3$ and DAG production crosses each egg in 20 s [3], using the curves of fig.3 we can construct a population time course from which we can infer the peak increase in radioactivity in individual eggs. These are the dashed curves in figs 1 and 2. The curves indicate that increases of 5–10-fold occur in individual eggs and that, if indeed a wave of $InsP_3$ and DAG production crosses the egg, then the local increase in individual eggs may be 50–200-fold for both [^{3}H]$InsP_3$ and for [^{3}H]DAG.

4. DISCUSSION

Our data indicate a consistent biphasic increase in the production of $InsP_3$ and a peak of DAG production at fertilisation. It is of interest how faithfully these alterations in radiolabelled $InsP_3$ reflect changes in the amounts of $InsP_3$ and DAG.

A 3 h incubation with [^{3}H]arachidonate labels phosphoinositides to apparent equilibrium. The alterations in [^{3}H]DAG therefore reflect alterations in chemical amount of DAG. DAG increases by 44% at the early peak. The increase in [^{3}H]$InsP_3$ is 66%. Since the action of a phospholipase C will produce $InsP_3$ and DAG in equivalent stoichiometric proportions, the similarity in the percentage increase of $InsP_3$ suggests that alterations in [^{3}H]$InsP_3$ may also reflect alterations in the chemical amount of $InsP_3$. With this assumption and those of the legend to fig.3, the increase in $InsP_3$ amounts to a concentration of 0.5–5 μM. This is a concentration of $InsP_3$ which will activate eggs when microinjected [3]. It appears from a comparison of the distribution of radiolabelled inositol and arachidonate in PPI that incubation of eggs with [^{3}H]inositol for 18 h labels $PtdInsP_2$ to one quarter of its equilibrium activity. It seems likely that a proportion of the early increase in [^{3}H]$InsP_3$ reflects an increase in the rate of production of $InsP_3$ from $PtdInsP_2$.

The first phase of PPI second messenger production appears broadly to coincide with the calcium transient which occurs at fertilisation [14,15]. The stimulation of $PtdInsP_2$ hydrolysis at this stage and the finding that increasing cytoplasmic calcium using the calcium ionophore A23187 results in hydrolysis of $PtdInsP_2$ (not shown) support the postulate that eggs possess a physiologically relevant calcium-activated phospholipase C [7]. The production of $InsP_3$ would result in an autocatalytic cycle of $InsP_3$-induced calcium release and calcium-induced $PtdInsP_2$ hydrolysis [3]. The substantial production of DAG at this stage is consistent with the idea that calcium-generated DAG production may promote exocytosis [7,16–21]. However, our data also suggest that production of $InsP_3$ and DAG has begun some seconds before cytoplasmic calcium concentration increases (fig.3). This is consistent with a sperm-induced phospholipase C activity which may be responsible for triggering the autocatalytic cycle responsible for the calcium wave [22].

The level of DAG returns to that of unfertilised eggs over a period of 5 min. This time course coincides with that of stimulation of the egg membrane Na/H antiporter which appears to be regulated by protein kinase C [4], DAG's known target as a second messenger [2]. The decline of DAG indicates that its rate of removal increases as the cytoplasmic pH rises. Preliminary experiments suggest PPI as the source of DAG, but we cannot rule out the possibility that other phospholipids act as substrates for the phospholipase C.

The second phase of $InsP_3$ production has no known function. Intracellular calcium concentrations are falling as $InsP_3$ rises; this does not rule out its being the 1,4,5 (calcium-releasing) isomer [1], however, as the sea urchin egg calcium store has been shown to become refractory to $InsP_3$ in vitro [5]. Ins 1,3,4-P_3 appears later in the PPI response in parotid gland [23] and may merely reflect the continued production of its pair, DAG. It is possible, however, that inositol polyphosphate may be important as a second messenger to later cell cycle events [24,25].

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REFERENCES

[1] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[2] Nishizuka, Y. (1983) Phil. Trans. Roy. Soc. B302, 101–112.
[3] Whitaker, M.J. and Irvine, R.F. (1984) Nature 312, 636–639.
[4] Swann, K. and Whitaker, M.J. (1985) Nature 314, 274–277.
[5] Clapper, D.L. and Lee, H.-C. (1985) J. Biol. Chem., in press.
[6] Whitaker, M.J. and Steinhardt, R.A. (1982) Q. Rev. Biophys. 15, 593–666.
[7] Whitaker, M.J. and Aitchison, M.J. (1985) FEBS Lett. 182, 119–224.
[8] Whitaker, M.J. and Baker, P.F. (1983) Proc. Roy. Soc. B218, 397–413.

[9] Homa, S.T., Conroy, D.M. and Smith, A.D. (1980) Biochem. Biophys. Res. Commun. 95, 1321–1327.
[10] Griffin, H.D., Sykes, M. and Hawthorne, J.N. (1979) Biochem. Soc. Trans. 7, 348–353.
[11] Batty, I.R., Nahorski, S.R. and Irvine, R.F. (1985) Biochem. J. 232, 211–215.
[12] Spector, T. (1978) Anal. Biochem. 86, 142–146.
[13] Shen, S.S. and Steinhardt, R.A. (1984) Proc. Natl. Acad. Sci. USA 81, 1436–1439.
[14] Poenie, M., Alderton, J., Tsien, R.Y. and Steinhardt, R.A. (1985) Nature 315, 147–150.
[15] Whitaker, M.J. (1985) J. Physiol. 365, 5P.
[16] Habernicht, A.J.R. et al. (1981) J. Biol. Chem. 256, 12329–12335.
[17] Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D. (1984) Biochem. J. 222, 195–201.
[18] Irvine, R.F., Brown, K.D. and Berridge, M.J. (1984) Biochem. J. 222, 269–272.
[19] Hawthorne, J.R. and Pickard, M.R. (1979) J. Neurochem. 32, 5–14.
[20] Allan, D. and Michell, R.H. (1975) Nature 258, 343–348.
[21] Turner, P.B., Sheetz, M. and Jaffe, L.A. (1983) Nature 310, 414–415.
[22] Turner, P.B. and Jaffe, L.A. (1985) J. Cell Biol., in press.
[23] Irvine, R.F., Letcher, A.J., Lander, D.J. and Downes, C.P. (1984) Biochem. J. 223, 237–243.
[24] Toomooka, Y., Imagawa, W., Nandi, S. and Bern, H.A. (1983) J. Cell. Physiol. 117, 290–296.
[25] Schneyer, C.A. (1974) in: Secretory Mechanisms of Exocrine Glands (Thorn, N.A. and Petersen, O.H. eds) Munksgaard, Copenhagen.
[26] Rothschild, Lord and Swann, M.M. (1951) J. Exp. Biol. 28, 403–416.
[27] Jaffe, L.F. (1983) Dev. Biol. 99, 265–276.