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Roles for the phosphatidylinositol cycle in early development

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Founded on the seminal studies and writings of Hokin, Michell and Berridge, a vast body of data now exists documenting the central importance of phosphatidylinositol (PtdIns) cycle activation in transducing information of many types across the plasma membrane. The great majority of these data derive from studies of terminally differentiated somatic cells. Nevertheless, the fact that many crucial events in animal development also involve transduction of information across the plasma membrane has recently led developmental biologists to search for regulatory roles for PtdIns cycle activity in such developmental processes as oocyte maturation, fertilization, and embryogenesis, with encouraging results. In this paper I briefly review the progress of such studies, beginning with the event in which the PtdIns cycle's role is best understood (fertilization), then progressing both backwards and forwards in developmental time to explore more speculative roles for the PtdIns cycle in oocyte maturation and pattern formation during embryogenesis.

1. Fertilization and the activation of development

The unfertilized eggs of many species are quiescent cells, bearing some resemblance to growth-arrested somatic cells in culture in that their cell cycle is arrested (in sea urchins after completion of meiosis, and in many vertebrates at metaphase of meiosis II), DNA synthesis is absent, mRNA and protein are synthesized only at low rates if at all, and, in general, the major catabolic and anabolic processes of the cell are at a low ebb. Within moments following sperm–egg contact, however, this situation is dramatically reversed, initiating a sequence of events collectively termed *egg activation*. Depending on the species considered, activation may involve such early events as plasma membrane depolarization (due to the opening of ion channels), cortical granule exocytosis, cytoskeletal events such as cortical contraction and modification of microvilli, and increases in respiration and NADP content. Subsequent events, coming some minutes later, typically include an increase in intracellular pH (pH_i) of about 0.4 units, elevation of protein synthesis rate, and the onset of cell division and DNA synthesis (see Epel (1975) for review). The ensuing sequence of 'cleavage divisions' can proceed at an astonishing rate; in the frog *Xenopus*, the interval between cleavages is about 35 min at room temperature, with no detectable G_1 or G_2 phases. In essence, fertilization transforms the quiescent, almost dormant egg into an active and rapidly proliferating cell system over the course of only a few minutes.

Egg activation and its regulation have long fascinated cell physiologists, and the study of this response by many workers provided what may have been the first clear evidence of an important role for intracellular free calcium ($[Ca^{2+}]_i$) changes in the regulation of proliferation. As first demonstrated with fish eggs microinjected with the Ca^{2+}-sensitive photoprotein aequorin (Ridgway *et al.* 1977; Gilkey *et al.* 1978), and subsequently for eggs of sea urchins

(Steinhardt *et al.* 1977; Eisen *et al.* 1984), frogs (Busa & Nuccitelli 1985) and mammals (Cuthbertson *et al.* 1981) via a variety of techniques, $[Ca^{2+}]_i$ transiently increases (typically from about 0.1 μM to roughly 1 μM) within seconds following fertilization, and two major lines of evidence (derived largely from studies of sea urchin and frog eggs) indicate that this transient $[Ca^{2+}]_i$ increase plays a fundamental role in regulating egg activation (see Whitaker & Steinhardt (1982) for review). First, the unfertilized eggs of many species can be artificially activated by treatment with the Ca^{2+} ionophore A23187, eliciting many of the responses seen at fertilization (Steinhardt *et al.* 1974). Secondly, when eggs are microinjected with Ca^{2+} buffers such as EGTA or BAPTA to prevent the $[Ca^{2+}]_i$ increase at fertilization, insemination no longer activates the egg (Kline 1988). Even the pH_i increase after fertilization, which is thought to regulate the 'late' events of egg activation (see Busa & Nuccitelli 1984), seems to depend upon the prior events which give rise to the $[Ca^{2+}]_i$ increase.

PtdIns *cycle involvement in sea urchin egg activation*

As the role of Ca^{2+} in egg activation was becoming firmly established in the late 1970s and early 1980s, advances in *somatic* cell physiology were uncovering the roles of the PtdIns cycle in regulating $[Ca^{2+}]_i$ changes in response to Ca^{2+}-mobilizing hormones. The first link between these fields was forged in 1984 with the demonstration that PtdIns cycle activity increases dramatically within seconds after fertilization of sea urchin eggs, with a 40% increase in phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) level *preceding* the exocytosis of cortical granules (an event known to be regulated by the $[Ca^{2+}]_i$ transient) (Turner *et al.* 1984). That same year, Whitaker & Irvine (1984) demonstrated that microinjection of as little as two attomoles of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) can trigger cortical granule exocytosis in the eggs of another sea urchin species, consistent with the concept that sperm–egg interaction might be coupled to the $[Ca^{2+}]_i$ transient via Ins(1,4,5)P_3-mediated Ca^{2+} release from internal stores. These same eggs possess a microsomal fraction, co-sedimenting with endoplasmic reticulum markers, which sequesters Ca^{2+} in an ATP-dependent fashion and releases Ca^{2+} in response to submicromolar concentrations of Ins(1,4,5)P_3 (Clapper & Lee 1985). More recent studies, employing the Ca^{2+}-sensitive dye fura = 2, confirmed that Ins(1,4,5)P_3 microinjection into these eggs can trigger a $[Ca^{2+}]_i$ transient similar to that observed at fertilization (Swann & Whitaker 1986). Because inositol trisphosphates rapidly accumulate within seconds following fertilization of these eggs (Ciapa & Whitaker 1986), it now seems reasonable to conclude that Ins(1,4,5)P_3, produced via increased turnover of PtdIns(4,5)P_2, mediates the $[Ca^{2+}]_i$ increase at fertilization in the urchin egg.

Frog egg activation

In the study of egg activation, some discoveries prove to be remarkably generalizable (such as the involvement of $[Ca^{2+}]_i$), whereas others prove unique to one or a few genera of organisms. For example, among deuterostomes such as urchins and vertebrates, the $[Ca^{2+}]_i$ increase at fertilization appears to depend largely on Ca^{2+} mobilization from internal stores, whereas protostome eggs are thought to rely on Ca^{2+} influx from the extracellular medium (Jaffe 1983) (the involvement, if any, of the PtdIns cycle in protostome egg activation remains to be explored). Thus, it is important to ask whether the Ins(1,4,5)P_3-mediated $[Ca^{2+}]_i$ increase at fertilization is unique to sea urchin eggs or whether, instead, it constitutes a general strategy for deuterostome egg activation.

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The frog (*Xenopus laevis*) egg, like that of the sea urchin, displays a marked, transient $[Ca^{2+}]_i$ increase at fertilization. As determined with a Ca^{2+}-selective microelectrode, $[Ca^{2+}]_i$ increases, on average, from a resting value of 0.4 μM to 1.2 μM over the course of about 2 min, recovering over the next 10 min back to its initial level (Busa & Nuccitelli 1985). By using either multiple Ca^{2+} electrodes (Busa & Nuccitelli 1985), aequorin (Kubota *et al.* 1987), or fura-2 (W. B. Busa, unpublished observations) it can be shown that this transient traverses the egg as a *wave* of propagated $[Ca^{2+}]_i$ increase and subsequent recovery, commencing at the sperm entry site and terminating at the antipode of the egg. Similar *propagated* $[Ca^{2+}]_i$ transients are also observed in the eggs of fish (Gilkey *et al.* 1978) and sea urchins (Eisen *et al.* 1984; Tsien & Poenie 1986), although in the latter (comparatively small) cells they are less readily resolved than in the eggs of fish and frogs (over 1 mm diameter). Iontophoretic injection of Ins(1,4,5)P_3 at sub-femtomole doses triggers a propagated $[Ca^{2+}]_i$ increase in the *Xenopus* egg which is essentially indistinguishable (with regard to its form, magnitude, and timing) from that seen at fertilization; this response is independent of extracellular Ca^{2+} (Busa *et al.* 1985). Owing (presumably) to its ability to mobilize Ca^{2+}, Ins(1,4,5)P_3 injection also triggers several other events of frog egg activation, including plasma membrane depolarization, cortical granule exocytosis, cortical contraction, and the abortive reinitiation of the cell cycle (Busa *et al.* 1985; Picard *et al.* 1985). Ins(1,4,5)P_3 is by far the most potent activator of frog eggs of all the inositol polyphosphates tested to date; in comparison, Ins(2,4,5)P_3 is about 8-fold less potent, Ins(1,4)P_2 is 100-fold less potent, and HPLC-purified inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) is about three orders of magnitude less potent than Ins(1,4,5)P_3 (W. B. Busa, unpublished observations). Finally, even enormous injections of such unrelated polyphosphates as fructose 1,6-bisphosphate consistently fail to activate eggs (Busa *et al.* 1985).

Efforts to document PtdIns cycle activation following fertilization of frog eggs have proved more challenging than for sea urchin eggs, owing to technical difficulties such as sluggish labelling with exogenous radiolabelled precursors and the notable asynchrony of fertilization in clutches of *Xenopus* eggs. Nevertheless, by using a mass assay, Le Peuch and coworkers (1985) observed roughly a ten-fold decrease in PtdIns(4,5)P_2 content after artificial activation of *Xenopus* eggs. In contrast, preliminary studies with radiolabelled precursors demonstrate a two-fold *increase* in PtdIns(4,5)P_2 during activation (J. Ferguson & R. Nuccitelli, personal communication). Resolution of this apparent discrepancy must await further studies, but it seems clear that frog egg activation involves changes in PtdIns cycle activity, and the previously cited studies clearly demonstrate the existence of an Ins(1,4,5)P_3-mobilized Ca^{2+} pool in the *Xenopus* egg, suggesting that here, as in the sea urchin egg, Ins(1,4,5)P_3 mediates the fertilization-induced $[Ca^{2+}]$ transient.

Evidence for G protein involvement

All of the evidence discussed above leads to the (admittedly simplistic) analogy of the fertilizing spermatozoan as a sort of giant Ca^{2+}-mobilizing growth factor. A question thus naturally arises concerning the identity of the mechanism coupling sperm–egg interaction with phospholipase C activation. In keeping with the growth factor analogy, several lines of evidence now suggest that guanine nucleotide-binding proteins (G-proteins) may be involved.

Sea urchin egg cortical preparations contain 47 and 40 kDa proteins which are specifically ADP-ribosylated by cholera and pertussis toxins, respectively (Turner *et al.* 1987). In their molecular masses and ability to serve as substrates for these toxins, these proteins are similar,

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and perhaps identical, to the α subunits of G_s and G_i, the guanine nucleotide-binding proteins of the cAMP signalling system. Microinjection of the non-hydrolysable GTP analogue GTPγS triggers cortical granule exocytosis in these eggs. This response is blocked by prior injection of EGTA, indicating that exocytosis in response to GTPγS reflects Ca^{2+} mobilization (Turner *et al.* 1986). Similarly, microinjection of cholera toxin also triggers cortical granule exocytosis in the absence, but not presence, of intracellular EGTA (Turner *et al.* 1987). These responses are in keeping with a role for G_s or a similar G-protein in activation of the urchin egg's phospholipase C, since both GTPγS and cholera toxin are potent activators of this protein. The most direct evidence for the involvement of a G-protein in coupling sperm–egg interaction with Ca^{2+} mobilization is the observation that microinjection of the non-hydrolysable GDP analogue, GDPβS (an inactivator of G proteins), prevents sea urchin egg activation upon subsequent insemination (Turner *et al.* 1986). An as yet unexplained observation from this same report is that eggs injected with GDPβS and inseminated subsequently fail to activate in response to microinjection of Ins(1,4,5)P_3, even though *uninseminated* eggs injected with GDPβS activate normally in response to Ins(1,4,5)P_3.

Xenopus eggs, too, are activated by microinjection of GTPγS (D. Kline & L. A. Jaffe, personal communication). In a particularly novel approach to testing for a role for G-proteins in frog egg activation, Kline and colleagues (1988) injected rat brain mRNA into immature *Xenopus* oocytes and were able to demonstrate, via electrophysiological techniques, the expression of functional serotonin receptors in these oocytes. On subsequent maturation *in vitro* (see below), these eggs could be activated by serotonin, displaying the membrane depolarization, cortical contraction, and cortical granule exocytosis characteristic of fertilized eggs (Kline *et al.* 1988; D. Kline & L. A. Jaffe, personal communication). As this receptor is known to stimulate PtdIns cycle activity via a G-protein, these results lend further credence to the hypothesis that a G-protein mediates egg activation. Very recent experiments employing the *purified* mRNA for the muscarinic acetylcholine receptor (also known to activate the PtdIns cycle via a G-protein) have yielded identical results (L. A. Jaffe, personal communication), providing compelling evidence of G-protein involvement in egg activation.

Ca^{2+} wave propagation

A feature of Ins(1,4,5)P_3-mediated Ca^{2+} mobilization which thus far appears unique to activating eggs is the *propagation* of $[Ca^{2+}]_i$ transients across these giant cells, commencing at the sperm–egg interaction site and terminating at its antipode. Although no evidence exists to rule out the potential for propagated Ca^{2+} release in somatic cells, the rather different geometric constraints on somatic cell and gamete activation suggest that propagation may be especially important in the latter case. In the hormonally stimulated somatic cell the surface:volume ratio is generally relatively large, receptors are present across the cell, and agonist molecules may bind in large numbers over large expanses of the cell surface, thus potentially giving rise to the simultaneous activation of the PtdIns cycle (and $[Ca^{2+}]_i$ increase) across the cell. In contrast, eggs are unusually large cells (the *Xenopus* egg is *ca.* 1.3 mm in diameter!) and in many organisms (such as sea urchins and frogs) only a *single* sperm cell, a few micrometres in diameter, interacts with the egg at a single site on the plasma membrane, yet this spatially restricted event of sperm–egg interaction must be communicated to metabolic processes and structures throughout the vast volume of the egg cytoplasm. Propagation of Ca^{2+} release seems a particularly effective way to achieve this end.

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What mechanisms are involved in the propagation of $[Ca^{2+}]_i$ transients through the cytoplasm? Although a completely satisfying answer remains to be achieved, testable hypotheses have been advanced and explored in a preliminary fashion. In the sea urchin egg injected with Ins(1,4,5)P_3, the Ca^{2+} wave's propagation rate (as reflected by the propagation of cortical granule exocytosis) is constant over a fivefold range of Ins(1,4,5)P_3 concentration, ruling out the possibility that propagation results from simple diffusion of Ins(1,4,5)P_3 from the injection site (Whitaker & Irvine 1984). Further, the failure of the Ca^{2+} waveform to broaden as it traverses a millimetre of cytoplasm rules out passive diffusion of Ca^{2+} itself (Busa *et al.* 1985). Noting that PtdIns(4,5)P_2 hydrolysis could be triggered in sea urchin egg cortical preparations by an increase in $[Ca^{2+}]$ to 3 μM (equalling the peak $[Ca^{2+}]_i$ during activation), Whitaker & Irvine (1984) suggested an *autocatalytic* mechanism for wave propagation, in which Ins(1,4,5)P_3-stimulated Ca^{2+} release and Ca^{2+}-stimulated Ins(1,4,5)P_3 production cooperate to propagate Ca^{2+} mobilization in a regenerative fashion. In keeping with this model, neomycin (which inhibits PtdIns(4,5)P_2 hydrolysis (Whitaker & Aitchison 1985)) substantially blocks wave propagation in the sea urchin egg without preventing Ins(1,4,5)P_3-triggered Ca^{2+} mobilization *at the injection site* (Swann & Whitaker 1986). This observation falls short of providing compelling evidence for the autocatalytic model, however, because neomycin (like other polycations) is notoriously non-specific in its cellular effects: aside from inhibiting PtdIns(4,5)P_2 hydrolysis, it also chelates Ins(4,5)P_3 and inhibits ATP-dependent Ca^{2+} uptake (Prentki *et al.* 1986), blocks Ca^{2+}-dependent exocytosis (Crabb & Jackson 1985), and inhibits Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum (Palade 1987). More critical tests (perhaps employing antibodies to phospholipase C?) remain to be reported; however, the responses of neomycin-injected eggs are certainly in keeping with the autocatalytic model of wave propagation.

A critical test of any wave propagation hypothesis is particularly important in light of the following unresolved issue. In the unactivated eggs of sea urchins and frogs, any treatment which *locally* elevates $[Ca^{2+}]_i$ (including injection of Ins(1,4,5)P_3 or of Ca^{2+} itself, application of Ca^{2+} ionophores or wounding the plasma membrane with a fine needle in Ca^{2+}-containing medium) will trigger a Ca^{2+} wave like that seen at fertilization. In contrast, no conditions have yet been identified under which propagated Ca^{2+} waves can be triggered in *previously activated* eggs: the propagation mechanism, whatever its basis, appears to be inactivated following the Ca^{2+} wave even though normal Ins(1,4,5)P_3-triggered Ca^{2+} release can still be demonstrated in the activated egg (Busa *et al.* 1985). This observation is seemingly inconsistent with the autocatalytic model, particularly in light of the fact that PtdIns(4,5)P_2 levels may be *higher* in activated eggs (Turner *et al.* 1984; J. Ferguson & R. Nuccitelli personal communication). These inconsistencies led us to propose an alternative wave propagation model for the frog egg (Busa *et al.* 1985). In it, *initiation* of the Ca^{2+} wave at the sperm entry site is proposed to be due to local Ins(1,4,5)P_3-mediated Ca^{2+} mobilization (similar to the autocatalytic model), but *propagation* of this $[Ca^{2+}]_i$ increase beyond the sperm entry site is due to Ca^{2+} mobilization from a *second* Ca^{2+} store, one which either breaks down or fails to recharge following the wave. To explain the observations discussed above, we tentatively proposed that this second pool might exhibit Ca^{2+}-induced Ca^{2+} release like that observed in both skeletal and cardiac muscle sarcoplasmic reticulum (SR). Two diagnostic features of such Ca^{2+}-induced Ca^{2+} release from SR are its stimulation by caffeine and inhibition by procaine. In preliminary studies, we have failed to observe either Ca^{2+} release or inhibition of the Ins(1,4,5)P_3-triggered Ca^{2+} wave when

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Xenopus eggs are injected with 10 mM (final concentration) caffeine or procaine, respectively (W. B. Busa, unpublished observations). Efforts are currently under way to test directly for Ca^{2+}-induced Ca^{2+} release in microsomal fractions from *Xenopus* eggs. Thus, at present, the preponderance of evidence is in favour of the autocatalytic model of Ca^{2+} wave propagation, but it remains to be seen how this model can account for the lack of wave propagation in *activated* eggs despite the continued presence of Ins(1,4,5)P_3-mobilizable Ca^{2+} stores and elevated PtdIns(4,5)P_2 levels. Perhaps the PtdIns(4,5)P_2 synthesized following activation resides in a 'metabolically inactive' pool, as discussed by other participants in this symposium, but this remains to be seen.

2. The PtdIns cycle in an alternate pathway of oocyte maturation

Before their release from the ovary, oocytes exist in an immature form in which (for many species) they are arrested in meiosis, are unreceptive to spermatozoa and cannot be activated. The hormonally induced events culminating in progression through meiosis and the acquisition of sperm receptivity and activatibility, collectively termed 'oocyte maturation', are particularly well understood in the *Xenopus* oocyte, where the responsible hormone (progesterone) triggers an inactivation of adenylate cyclase via a cholera toxin-sensitive G-protein, thus leading to depression of intracellular cAMP levels and inactivation of cAMP-dependent protein kinase, this latter response being sufficient to induce maturation (see Maller (1983) for review). Years of controversy concerning the possible involvement of $[Ca^{2+}]_i$ increase in progesterone-induced maturation have recently been resolved with the compelling demonstration that no detectable $[Ca^{2+}]_i$ changes accompany maturation (Cork *et al.* (1987) and references therein). Nevertheless, some evidence now exists documenting the *Xenopus* oocyte's possession of a hormone-activated PtdIns cycle, which may provide an alternative pathway for triggering oocyte maturation, although the physiological significance of this pathway remains to be demonstrated.

Like progesterone, insulin and insulin-like growth factor are able to induce maturation of *Xenopus* oocytes (Maller & Koontz 1981), but several lines of evidence suggest that this induction involves an alternative set of 'second messengers' to those employed for progesterone-induced maturation. Injection of the oncogene product H-*ras* p21 (a mutated guanine nucleotide-binding protein with much-reduced GTPase activity) induces maturation in *Xenopus* oocytes *without* eliciting a detectable decrease in cellular cAMP levels (Birchmeier *et al.* 1985). This cAMP-independent induction of maturation may be mediated via PtdIns cycle activation, because microinjection of H-*ras* p21 rapidly stimulates diacylglycerol and inositol polyphosphate production in *Xenopus* oocytes (Lacal *et al.* 1987) and, in turn, the diacylglycerol analogue 12-*O*-tetradecanoylphorbol 13-acetate induces maturation of these oocytes (Stith & Maller 1987). That a cellular *ras* protein (or homologue) may be involved in transducing insulin's stimulation of maturation is suggested by the observation that two *ras* p21 monoclonal antibodies, 6B7 and Y13-259, specifically inhibit maturation in response to insulin (Korn *et al.* 1987; Deshpande & Kung 1987). Intriguingly, these same antibodies have no effect on progesterone-induced maturation, suggesting differing pathways for progesterone- and insulin-induced maturation. This seemingly straightforward result is confounded, however, by the observation that a different *ras* p21 monoclonal antibody, 238, actually *accelerates* progesterone-induced maturation, consistent with its ability to inhibit the adenylate cyclase activity of oocyte

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plasma membrane preparations (Sadler *et al.* 1986). In some batches of oocytes, antibody 238 even triggers maturation without progesterone. Thus, different antibodies to a single guanine nucleotide-binding protein seem able either to block insulin-induced, or promote progesterone-induced, maturation, but not both.

Although it must be emphasized that progesterone is the only known physiologically relevant inducer of *Xenopus* oocyte maturation (and thus cAMP may be the only physiologically relevant second messenger involved), the apparent ability to trigger maturation experimentally via either the PtdIns cycle or the cAMP system is certainly intriguing and deserves further study. In particular, the results of antibody studies suggesting that *ras*-like protein(s) mediate both insulin- and progesterone-induced maturation, perhaps via these two distinct signalling systems, warrants close attention. Does a single guanine nucleotide-binding protein serve as a transducer in both these systems, or do different '*ras* antibodies' target different proteins in the oocyte? Can activation of protein kinase C elicit the same cellular response (maturation) as inactivation of cAMP-dependent protein kinase, and if so, how? Perhaps the most important question of all (although certainly the least stimulating) concerns whether or not we are simply being led astray by data collected under widely varying conditions. Seasoned veterans of *Xenopus* oocyte maturation research will readily attest to the ease with which different laboratories, employing slightly different protocols, can arrive at opposing conclusions.

3. A role for the PtdIns cycle in pattern formation?

Although it has been the target of active study for a full century, the phenomenon of pattern formation (the spatial organization of cell differentiation during embryonic development) remains largely a mystery. Although many empirical data have been gathered concerning the events whereby the essentially homogeneous egg is converted into the highly anisotropic adult form, the cell physiological bases of these events are essentially unknown, and their genetics have only begun to be outlined. The study of pattern formation has been strongly influenced by the concept of 'positional information' (Wolpert 1969), i.e. the notion that cells have access to information concerning their positions in the embryo, which they use to guide their appropriate differentiation. Some elaborations of this theory have proposed the existence of *gradients* of diffusible 'morphogens', acting much like the axes of a Cartesian graph to specify cell position, but investigators have met with little success in identifying these putative morphogens.

A potential breakthrough in understanding the cell physiological basis of pattern formation was achieved recently by Kao and coworkers (1986), in their reinvestigation of the ancient observation that lithium ion can elicit severe developmental abnormalities in the embryos of many organisms. Rather than topically applying lithium salts, as all previous workers had done, they microinjected LiCl into single, defined blastomeres of the early (32-cell stage) *Xenopus* embryo, and observed a remarkable result: when Li^+ (but no other cation) was injected into a cell on the prospective ventral side of the embryo in the unpigmented (so-called 'vegetal') hemisphere, the tadpole resulting from further development was largely normal with one minor exception: it sported two well formed heads instead of the usual one. In brief, lithium microinjection into this single cell was able to respecify the formation of dorso-anterior structures, leading to head duplication and the formation of so-called 'Janus-twin' embryos.

These observations are of particular relevance to the study of the cell physiology of pattern

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formation because they present us with a tool we have previously lacked: a chemically defined agent, the biochemical effects of which are fairly well understood, with a profound yet readily interpretable effect on pattern formation. Thus, the known biochemical effects of Li^+ suggest where to look to discover the determinants or morphogens guiding this process.

Among the recognized effects of Li^+ on cells, one seems to stand out in terms of its potential significance in cell signalling and differentiation: Li^+ potently inhibits inositol 1-phosphatase (Hallcher & Sherman 1980), the last in a series of enzymes that dephosphorylate inositol trisphosphates to free inositol, thus disrupting the PtdIns cycle at this crucial 'choke point'. Hormonally stimulated cells treated with LiCl typically display elevated inositol phosphate levels (Berridge *et al.* 1982), as expected, and elevation of diacylglycerol levels has also been observed (Drummond & Raeburn 1984). Thus, my colleagues and I have recently begun to test whether the developmental effects of Li^+ on *Xenopus* embryos might be mediated via its effects on the PtdIns cycle, with the ultimate goal of testing whether PtdIns cycle products such as inositol polyphosphates or diacylglycerol (or the messengers they themselves regulate, such as $[Ca^{2+}]_i$ or intracellular pH) may play roles in pattern formation, either as 'diffusible morphogens' or by more subtle means.

In an initial approach to the questions outlined above, we have asked whether exogenous *myo*-inositol can rescue Li^+-injected embryos. We reasoned that, by counteracting the depletion of cellular *myo*-inositol levels (and consequent inhibition of polyphosphoinositide resynthesis) expected of Li^+-treated cells, we might, in a sense, 'short circuit' lithium's inhibition of the PtdIns cycle. A similar approach has previously been employed to rescue the induction of ornithine decarboxylase in Li^+-treated lymphocytes (Mustelin *et al.* 1986).

When 32-cell *Xenopus* embryos are injected in a ventral, vegetal blastomere with 8 nl of 0.25 M LiCl, more than 80% subsequently display duplication of dorso-anterior structures such as the anterior neural tube, cement gland and eye rudiments, and less than 10% of injected embryos are grossly normal (table 1). In contrast, similar injections of NaCl are without developmental consequences. However, when Li^+ is injected in a 1:2 molar ratio with *myo*-inositol, nearly 40% of injected embryos are subsequently found to be normal ($p < 0.01$ by Student's *t*-test). *Myo*-inositol alone (i.e. without Li^+) has no effect on development under these conditions. As a control, embryos were also injected with Li^+ plus *epi*-inositol, a non-biological positional isomer of inositol not employed in the PtdIns cycle. *Epi*-inositol, in sharp contrast with the *myo*-isomer, had no effect on Li^+-induced teratogenesis.

Interpretation of the above results is complicated by the high levels of embryo damage observed with combined Li^+ + *myo*-inositol injections (table 1). Nearly 50% of embryos so injected displayed detachment of noticeable numbers of cells from the embryo proper, with or without a spina bifida-like syndrome in which the neural tube failed to close completely. The predominance of damaged embryos, observed only with combined injections of Li^+ + *myo*-inositol, raises a potentially trivial explanation for the observed 'rescue': the abnormal demands on the PtdIns cycle imposed by the combination of these agents might so sicken the injected cell and its progeny that at best they are rendered incapable of participating in the respecification of dorso-anterior patterning, and at worst they are lost entirely from the embryo. That this trivial explanation probably cannot account for *myo*-inositol's rescuing effect is strongly indicated by recent observations by my colleague R. Gimlich (Busa & Gimlich 1988). Gimlich observed that a cell *adjacent to* the cell injected in the studies just described is a more sensitive target for Li^+, responding fully to doses of Li^+ only one quarter of those

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TABLE 1. DEVELOPMENTAL RESCUE OF Li^+-INJECTED *XENOPUS* EMBRYOS BY *MYO*-INOSITOL

(*Xenopus* embryos were injected at the 32-cell stage in a single ventral, vegetal blastomere with 8 nl of buffer composed of 0.2 mM HEPES (pH 7.6 with KOH), 0.05 mM EGTA, and the indicated agent(s) (0.25 M LiCl and/or 0.5 M *myo*- or *epi*-inositol), then incubated for 24 h at room temperature before visual scoring for developmental defects. Results are presented as mean ± s.e.m. percentage of embryos displaying each phenotype. Numbers in parentheses beside each injectate indicate the number of embryos injected and the number of females from which they were produced (1 female per experiment).)

	phenotype		
injectate	dorso-anterior duplication	normal	damaged
Li^+ (116/5)	82.1 ± 3.2	9.4 ± 4.8	8.4 ± 3.7
Li^+ + *myo*-Ins (159/6)	14.0 ± 3.8	37.1 ± 6.6	49.0 ± 6.6
Li^+ + *epi*-Ins (81/3)	86.6 ± 2.9	10.8 ± 3.9	2.5 ± 1.3
myo-Ins (90/3)	0.0	92.3 ± 2.2	7.7 ± 2.1

employed in the studies shown in table 1. Even lower doses of *myo*-inositol yield full rescue when this cell is injected (1:1 molar ratio with Li^+, or one eighth of the dose previously employed) and, most significantly, the incidence of damaged embryos is now reduced to near zero. Possibly, in our original studies, we were not injecting the cell that was the true target of Li^+-induced teratogenesis, but Li^+ and *myo*-inositol were diffusing into this cell via the embryo's extensive gap-junctional communication network, thus accounting for the higher (and apparently somewhat toxic) doses originally required. At the lower doses we are now able to employ there is no apparent toxicity (no cell loss or spina bifida and injected cells divide normally). Further, intracellular recordings with Li^+-selective microelectrodes demonstrate that *myo*-inositol does not depress the intracellular free Li^+ concentration of Li^+-injected embryos (neither by chelation nor by stimulating its removal from the cell). It thus seems clear that rescue of Li^+-injected embryos with *myo*- but not *epi*-inositol is fully in keeping with the hypothesis that lithium's teratogenic effect is due to its ability to inhibit the PtdIns cycle, raising the possibility (currently under investigation) that some PtdIns cycle product(s) also play a role in directing *normal* pattern formation.

4. CONCLUSION

As might be expected in view of its relative youth, the study of the PtdIns cycle's roles in development has thus far contributed little, if anything, in the way of new insights to its parent discipline, the study of the PtdIns cycle's roles in somatic cell physiology. The reverse cannot be said, however; concepts first elucidated in studies of the regulatory roles of Ins(1,4,5)P_3 and diacylglycerol in somatic cells have proved (and continue to prove) invaluable in shedding light on such long-standing mysteries of developmental biology as the means by which the sperm activates the egg, and hold some promise of enlightening the even greater mystery of the physiological basis of pattern formation. As we move beyond the initial phenomenological phase of research (largely characterized by developmental biologists borrowing interesting compounds from their biochemical brethren and injecting them into cells to 'see what happens') I expect that we shall begin to see more fundamental insights emerging, because developing systems often present particularly extreme examples of basic cell physiological problems and often constitute quite accessible systems with which to study basic cell biology.

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Discussion

(*Questioner not identified.*) What is the relation between GTP-binding proteins, sperm–egg fusion and egg activation?

W. B. Busa. This question has not been addressed in frog eggs, but Michael Whitaker may want to comment on the situation in sea urchin eggs.

M. J. Whitaker (*Department of Physiology, University College London, U.K.*). Both electrophysiology (McCulloh & Chambers 1986) and ultrastructural data (Longo *et al.* 1986) suggest that sea urchin egg activation occurs after fusion of egg and sperm. The idea that the sperm acts like a hormone in activating sea urchin (Turner *et al.* 1987) and frog (Kline & Jaffe 1987) eggs by receptor stimulation of a GTP-binding protein linked to phosphoinositidase C is attractive. However, there are several pieces of evidence that suggest (*a*) that fusion of egg and sperm is the first thing to occur at fertilization (McCulloh & Chambers 1986), and (*b*) that phosphoinositide metabolism is not necessary for sperm–egg fusion, as it is not inhibited by neomycin or GDPβS (Swann *et al.* 1987). This points back to the older idea (Jaffé 1980) that the sperm injects an activating messenger into the egg when it fuses.

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