the term bistable-switch. Thus, there are at least two different but entirely compatible ways of thinking about the switches in fate produced by mutations such as sevenless and cut, one with strong evolutionary implications and the other with an emphasis on specific gene interactions.

We have considered two genes whose products act at different points in the long and still unknown chain of events that stretches from the determination of a cell in a specific location to become different from its neighbors, to the final differentiation of that cell or its progeny. The sevenless gene encodes a membrane-associated protein that probably plays a critical role in cell–cell communication and the initial determination process; the cut gene encodes a nuclear, probably DNA-binding protein which seems to be essential in normal differentiation. The switches in cell fate seen when these genes mutate to a loss of function can be interpreted on two levels, one mechanistic and the other evolutionary. As the catalogue of well-analysed genes grows, increasingly substantive interpretations of their role both in neural development and in the evolutionary history of developmental mechanisms can be expected.

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Intracellular Ca\(^{2+}\) activates phospholipase C

David A. Eberhard and Ronald W. Holz

It is well established that a receptor-mediated mechanism, perhaps involving a guanine nucleotide binding protein, directly activates polyphosphoinositide-specific phospholipase C. Recent evidence indicates that in excitable tissues a rise in cytosolic Ca\(^{2+}\) can also activate the phospholipase C. The activation of phospholipase C by Ca\(^{2+}\) can be a direct effect rather than a result of the Ca\(^{2+}\)-dependent release of neurotransmitters which activate phospholipase C through a receptor-mediated mechanism. Ca\(^{2+}\)-activated phospholipase C may represent a positive feedback system for Ca\(^{2+}\): small increases in cytosolic Ca\(^{2+}\) induced by Ca\(^{2+}\) influx across the plasma membrane may result in higher cytosolic Ca\(^{2+}\) concentrations due to IP\(_3\)-induced release of Ca\(^{2+}\) from intracellular stores. The activation of phospholipase C by Ca\(^{2+}\) may also provide a mechanism for diacylglycerol generation and protein kinase C activation following Ca\(^{2+}\) influx. Thus, the regulation of phospholipase C activity by Ca\(^{2+}\) may be physiologically important in regulating cytosolic Ca\(^{2+}\) and protein kinase C in excitable tissues.

Since the seminal review by Michell in 1975\(^{1}\) of the relationship between the metabolism of inositol phospholipids and cell receptor activation, considerable effort has been made to break down the breakdown of these lipids to specific cell functions. One of the central issues was whether a rise in intracellular Ca\(^{2+}\) is a result of or the cause of the hydrolysis of the phosphoinositides. Indeed, one of the suggestions from the Michell review was that phosphoinositide turnover results in the liberation of Ca\(^{2+}\) from intracellular stores. Subsequent work has confirmed this idea and we now understand that in many cell types, inositol trisphosphate (IP\(_3\)), which is a product of receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) by the enzyme phospholipase C (PLC)\(^{2-3}\), releases Ca\(^{2+}\) from intracellular, non-mitochondrial stores\(^{4}\).

A rise in intracellular Ca\(^{2+}\) can also occur secondary to influx of Ca\(^{2+}\) through ligand-gated channels or, in electrically excitable tissue, through voltage-gated ion channels. Micromolar Ca\(^{2+}\) activates several different phosphoinositide-specific PLC activities from brain\(^{5-8}\) and seminal vesicles\(^{9}\). In addition, micromolar Ca\(^{2+}\) causes production of diglyceride or inositol phosphates from permeabilized platelets\(^{8}\), prolactin tumor (GH3) cells\(^{10}\), chromaffin cells\(^{10}\) and insulinoma (RINm5F) cells\(^{11}\). The following discussion presents recent experiments showing that PLC activation can accompany Ca\(^{2+}\) influx, and suggests that Ca\(^{2+}\) itself is an important physiological activator of PLC in some tissues.

Intracellular PLC activation by Ca\(^{2+}\)

Support for direct receptor-mediated activation of PLC comes from demonstrations that, after stimulation of Ca\(^{2+}\)-mobilizing receptors, the generation of inositol phosphates precedes or coincides with a rise in intracellular Ca\(^{2+}\), and that IP\(_3\) releases Ca\(^{2+}\) from intracellular stores. Subsequent work has confirmed this idea and we now understand that in many cell types, inositol trisphosphate (IP\(_3\)), which is a product of receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) by the enzyme phospholipase C (PLC)\(^{2-3}\), releases Ca\(^{2+}\) from intracellular, non-mitochondrial stores\(^{4}\).

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from intracellular stores. However, investigators using preparations derived from excitable tissues have repeatedly described PLC activity that seems to result from an increase in cytosolic Ca²⁺. Akhtar and Abdel-Latif found that the Ca²⁺ ionophore A23187 caused the breakdown ofPIP₂ in iris smooth muscle. Griffin and Hawthorne found that A23187 caused the hydrolysis of PIP₂ and PIP in guinea-pig synaptosomes. Kendall and Nahorski observed that the production of inositol phosphates in rat cerebral cortical slices is increased by A23187 (Ref. 15), or by depolarization with elevated K⁺ (Ref. 16). The effect of the latter involves dihydropyridine-sensitive Ca²⁺ channels. We found that nicotinic receptor-channel stimulation and elevated K⁺ increased the formation of inositol phosphates in bovine adrenal chromaffin cells, an effect that could be inhibited by organic Ca²⁺ channel antagonists. All of these effects were either dependent upon medium Ca²⁺, or were strongly inhibited by EGTA.

Are Ca²⁺ effects direct or indirect?

A major question in the interpretation of these findings is whether PLC activation is a direct consequence of Ca²⁺ influx or is secondary to the Ca²⁺-dependent release of neurotransmitters, which in turn activates PLC through a receptor-mediated mechanism. For example, in an extension of their study cited above, Akhtar and Abdel-Latif found that the α₁-adrenergic receptor antagonist prazosin blocked the ionophore-induced hydrolysis of PIP₂, indicating that the effect was mediated by catecholamine release from nerve endings in the smooth muscle preparation. Unfortunately, the task of showing conclusively that PLC activation by Ca²⁺ in neural or secretory tissues is not caused by a released substance is not so straightforward. In many studies it seems unlikely that the stimulated formation of inositol phosphates results from the release of an endogenous substance, since the activation of PLC requires far less extracellular Ca²⁺ than is generally necessary to support transmitter release. As little as 10 μM Ca²⁺ (the estimated concentration of Ca²⁺ in 'Ca²⁺-free' media without EGTA) is sufficient for the ionophore-stimulated breakdown of polyphosphoinositides in synaptosomes and the ionophore- and depolarization-stimulated formation of inositol phosphates in brain slices.

In primary cultures of purified bovine chromaffin cells, there is strong evidence that a rise in cytosolic Ca²⁺ directly stimulates PLC. Exocytosis of catecholamine and PLC activation induced by nicotinic receptor-channel stimulation or by elevated K⁺ both require extracellular Ca²⁺ and are inhibited by organic Ca²⁺ channel antagonists. Thus, both processes are triggered by an influx of Ca²⁺. The following indicates that the activation of PLC is not caused by a secreted substance: (1) PLC activation by either agent requires less than one-tenth the extracellular Ca²⁺ concentration than does exocytosis; (2) addition of a nicotinic receptor antagonist after nicotinic agonist-induced secretion is essentially complete (in medium containing 2.2 mM Ca²⁺) totally blocks subsequent PLC activity; (3) medium from chromaffin cells that had been stimulated to secrete does not increase PLC activity in naive cells; and (4) increasing the incubation volume without increasing cell number does not alter the nicotinic stimulation of PLC. Thus, PLC activation is likely to be a direct result of Ca²⁺ influx rather than an indirect, receptor-mediated effect of a secreted substance. Muscarinic stimulation of bovine chromaffin cells (which does not cause secretion) activates PLC through a receptor-mediated mechanism that does not require Ca²⁺ influx. Hence, in the same cells, the mechanism by which PLC is activated depends upon the type of signal at the plasma membrane.

In summary, in tissues with ligand-gated channels that are permeable to Ca²⁺ or in excitable tissues, two distinct mechanisms for PLC activation exist: direct receptor coupling (probably through a GTP-binding protein) to PLC, or activation induced by a rise in cytosolic Ca²⁺. This conclusion is shown schematically in Fig. 1.

Ca²⁺ may modulate the response to receptor/G protein activation of PLC

The effects of intracellular Ca²⁺ and receptor/G protein activation on modulation of PLC activity may be interrelated. The ability of muscarinic agonists to stimulate polyphosphoinositide turnover is enhanced by Ca²⁺ ionophore in synaptosomes. The effects of the Ca²⁺ ionophore are probably distal to the muscarinic receptor, since the ionophore does not alter the binding characteristics of the receptor. An interaction between Ca²⁺- and G protein-dependent mechanisms of PLC regulation is demonstrated by the synergy between Ca²⁺ and the guanine nucleotides in activating PLC in permeabilized cells. An increase in cytosolic Ca²⁺ may, therefore, increase the sensitivity of PLC to receptor activation.

Influx of Na⁺ may act through Ca²⁺ to activate PLC

Na⁺ ions have also been implicated in PLC activation. Gusovsky et al. reported that a number of agents that cause Na⁺ influx through various mechanisms also increase the accumulation of inositol phosphates in guinea-pig synaptoneurosomes. The effect is blocked by EGTA but does not involve voltage-dependent Ca²⁺ channels. Sodium channel activators also increase inositol phosphate accumulation in chick heart cells. Increased intracellular Na⁺ may cause more Ca²⁺ to enter the cytoplasm by Na⁺/Ca²⁺ exchange across the inner mitochondrial membrane and across the plasma membrane. Indeed, large elevations of cytosolic Ca²⁺ occur in chick heart cells upon incubation with sodium channel activators.

Excitatory amino acid receptors linked to ionic channels may activate PLC through a rise in cytosolic Ca²⁺

Quisqualate (Q), and N-methyl-D-aspartate (NMDA) excitatory amino acid receptors have been linked to PLC activation in the striatum and in cul-
The ability of NMDA receptor stimulation to activate PLC is profoundly inhibited by physiological concentrations of extracellular Mg$^{2+}$ (Ref. 24); the inhibition by Mg$^{2+}$ is non-competitive for the NMDA binding site and can be overcome by depolarization. In contrast, Mg$^{2+}$ has little effect on the activation of PLC through Q receptors$^{24}$. The effect of Mg$^{2+}$ on the functional coupling of the NMDA receptor to PLC is remarkably similar to its effect on the NMDA receptor-associated channel$^{25}$, which is permeable to Ca$^{2+}$ and Na$^+$. Since Mg$^{2+}$ is thought to inhibit influx by directly blocking the channel, it is possible that PLC activation by NMDA receptor agonists occurs as a result of Ca$^{2+}$ entry. The Ca$^{2+}$ dependency of the NMDA activation of PLC has not been demonstrated and the possibility that the activation results from the release of a neurotransmitter that directly activates PLC through another membrane receptor has not been excluded.

**Physiological implications**

The direct receptor-mediated activation of PLC results in production of IP$_3$ and diacylglycerol (DAG), each of which are second messengers. IP$_3$ (or metabolites) releases Ca$^{2+}$ from intracellular stores and, thereby, increases cytosolic Ca$^{2+}$ (Fig. 1). DAG activates protein kinase C. When Ca$^{2+}$, either through depolarization- or ligand-gated channels, enters the cells, the same second messengers are generated. However, in this case the effect of IP$_3$ to increase cytosolic Ca$^{2+}$ represents a positive feedback system rather than the initial event that increases cytosolic Ca$^{2+}$ (Fig. 1). Because in intact cells PLC activation by Ca$^{2+}$ probably occurs upon small increases in cytosolic Ca$^{2+}$ (see above), IP$_3$ production may be an important amplification system to increase cytosolic Ca$^{2+}$ when Ca$^{2+}$ influx is small. Large increases in cytosolic Ca$^{2+}$ caused by Ca$^{2+}$ influx are less likely to be significantly increased by IP$_3$. A positive feedback system whereby cytosolic Ca$^{2+}$ induces increased cytosolic Ca$^{2+}$ through the action of IP$_3$ involves a number of biochemical steps. It is unlikely to be important where rapid Ca$^{2+}$ changes in response to depolarization are necessary, such as at the neuromuscular junction. However, the pathway may operate in generating slower or more prolonged synaptic or cellular responses. The involvement of PLC and IP$_3$ in regulating cytosolic Ca$^{2+}$ in the presynaptic terminal could therefore serve an integrating function for incoming signals.

![PLC Activation Diagram](image)

The ability of intracellular Ca$^{2+}$ to regulate PLC activity suggests that a rise in cytosolic Ca$^{2+}$ initially induced by direct receptor-mediated activation of PLC may in turn contribute to the prolonged activation of PLC. The rise in cytosolic Ca$^{2+}$ may be a link in a positive feedback system for PLC. For example, in rat parotid cells, continuous incubation with substance P or a muscarinic agonist causes a large, transient increase in cytosolic Ca$^{2+}$ followed by a lower, sustained increase (which is largely dependent upon extracellular Ca$^{2+}$)$^{26}$. The effects on Ca$^{2+}$ are paralleled by a large, transient and then a smaller, sustained increase in inositol (1,4,5)-trisphosphate and inositol tetrakisphosphate (IP$_4$). In addition to the inositol phosphates being responsible for the increased cytosolic Ca$^{2+}$, increased cytosolic Ca$^{2+}$ in the presence of extracellular agonist may contribute to the sustained level of activation of PLC. Thus, PLC once activated may have a tendency to maintain its own activity. A similar positive feedback effect may occur when PLC is activated by Ca$^{2+}$ influx and a small increase in cytosolic Ca$^{2+}$; a further increase in cytosolic Ca$^{2+}$ induced by inositol phosphates could contribute to sustained PLC activation.

There are a number of homeostatic mechanisms that would keep such a positive feedback mechanism in check. Following termination of the initial
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stimulus, cytosolic Ca\(^{2+}\) would return to resting levels through sequestration in organelles such as mitochondria, and through efflux across the plasma membrane. In addition, elevated intracellular Ca\(^{2+}\) may decrease the ability of IP\(_3\) to release sequestered Ca\(^{2+}\) (Ref. 27). Furthermore, protein kinase C (PKC) activation by DAG (see below) may inhibit PLC activity through a negative feedback loop\(^{28,29}\).

DAG and a rise in cytosolic Ca\(^{2+}\) will act synergistically to activate PKC\(^{30}\). Studies investigating the effects of exogenous activators of PKC indicate that PKC may be involved in increasing the sensitivity of the intracellular exocytotic machinery to Ca\(^{2+}\), in regulating the permeability of ion channels, in the desensitization of receptors, and in synaptic plasticity (see Ref. 31 for review). PKC is translocated and activated in bovine chromaffin cells during nicotinic stimulation\(^{32}\) or depolarization\(^{33}\) and in hippocampus following high-frequency electrical stimulation of the perforant pathway in vivo\(^{34}\). Although it is uncertain whether the activation of PKC in these systems requires activation of PLC, the biochemical machinery exists to link functionally these two enzymes.

Concluding remarks
Recent evidence indicates that a rise in cytosolic Ca\(^{2+}\) can be the cause of the activation of polyphosphoinositide-specific phospholipase C as well as the result of its activation. The proposition that Ca\(^{2+}\) influx can regulate phospholipase C activity in excitable cells should not be viewed as a challenge to the receptor-activation model of control of phospholipase C. Both modes of regulating phospholipase C occur, even in the same cell. Regulation of phospholipase C activity by Ca\(^{2+}\) influx is an extension of some of the known characteristics of phospholipase C and may fulfill the demands of specialized cell functions.

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