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Spatial and Temporal Aspects of Cell Signalling [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1988 **320**, 325-343
doi: 10.1098/rstb.1988.0080

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Phil. Trans. R. Soc. Lond. B **320**, 325–343 (1988)
Printed in Great Britain

Spatial and temporal aspects of cell signalling

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As new techniques are developed to measure intracellular messengers it becomes increasingly apparent that there is a remarkable spatial and temporal organization of cell signalling. Cells possess a small discrete hormone-sensitive pool of inositol lipid. In some cells such as *Xenopus* oocytes and *Limulus* photoreceptors this phosphoinositide signalling system is highly concentrated in one region of the cell, so establishing localized calcium gradients. Another example is the hydrolysis of inositol lipids in eggs at the point of sperm entry resulting in a localized increase in Ins(1,4,5)P_3 and calcium which spreads like a wave throughout the egg. In hamster eggs this burst of calcium at fertilization recurs at 1–3 min intervals for over 100 min, a particularly dramatic example of spontaneous activity.

Spontaneous oscillations in intracellular calcium exist in many different cell types and are often induced by agonists that hydrolyse inositol lipids. We have made a distinction between oscillations that are approximately sinusoidal and occur at a higher frequency where free calcium is probably continuously involved in the oscillatory cycle and those where calcium falls to resting levels for many seconds between transients. In the former case, the oscillations are thought to be induced through a cytoplasmic oscillator based on the phenomenon of calcium-induced calcium release. Such oscillations can be induced in *Xenopus* oocytes after injection with Ins(1,4,5)P_3. A receptor-controlled oscillator based on the periodic formation of Ins(1,4,5)P_3 is probably responsible for the generation of the widely spaced calcium transients.

The function of such calcium oscillations is currently unknown. They may be a reflection of the feedback interactions that operate to control intracellular calcium. Another possibility emerged from observations that in some cells the frequency of calcium oscillations varied with agonist concentration, suggesting that cells might employ these oscillations as a way of encoding information. One advantage of using such a frequency-dependent mechanism may lie in an increase in fidelity, especially at low agonist concentrations. Whatever these functions might be, it is clear that uncovering the mechanisms responsible for such oscillatory activity will greatly enhance our understanding of the relation between the phosphoinositides and calcium signalling.

Introduction

As the biochemical details of the inositol lipid signalling pathway are unravelled, both spatial and temporal aspects are becoming increasingly important. Spatial aspects concern the way in which components of the signalling system are distributed within the cell to give localized second messenger domains. As more and more examples of oscillations in intracellular calcium become apparent, there is a growing interest in temporal aspects, particularly in trying to understand the basis and possible function of these regular calcium fluctuations. The fact that

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frequency of these oscillations can change in response to calcium-mobilizing agonists has raised the possibility that second messengers might be frequency-encoded. Such dynamic aspects add a new dimension to the central role of the phosphoinositides in cell signalling.

Spatial aspects

The receptors and enzymes responsible for inositol lipid hydrolysis may be localized within restricted regions of the cell. An example of this spatial organization is that the inositol lipids exist in separate pools (Berridge 1987). A small hormone-sensitive pool was found to be separate from the larger hormone-insensitive pool. Even the small phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) fraction appears to exist as two separate pools. The localization of receptors and G-proteins within the plasma membrane implies that lipids are hydrolysed at this site. The finding that PtdIns can be synthesized at the plasma membrane (Imai & Gershengorn 1987) makes it likely that the hormone-sensitive pool is mainly confined to the cell surface. However, lipid turnover associated with signalling may occur elsewhere in the cell. Of particular interest is that the nuclear membrane contains inositol lipid kinases, particularly the one that phosphorylates PtdIns to PtdIns4*P* (Smith & Wells 1983). Interest in the possible control of these kinases is likely to grow following the suggestion that PtdIns4*P* is a potent activator of DNA polymerase (Sylvia *et al.* 1987). There are indications that not all the polyphosphoinositide is in the nuclear envelope but some of it might be associated with chromatin (Cocco *et al.* 1987). A possible role in the control of growth is suggested by the marked increase in this non-membrane nuclear lipid at the time of differentiation. Although most attention has focused on the plasma membrane as the site of second messenger formation, it is clear that the turnover of lipids in other parts of the cell may also play a role in cell signalling.

The receptors that stimulate inositol lipid hydrolysis may not be distributed uniformly over the surface of the cell but may set up local second messenger gradients by being clustered within discrete areas. A classic example of such localization is found in *Limulus* photoreceptors where the R-lobe is very much more sensitive to light than the A-lobe (Payne & Fein 1987). Both rhodopsin and the Ins(1,4,5)P_3-sensitive endoplasmic reticulum is localized in the R-lobe. Another example is the *Xenopus* oocyte which has a high density of muscarinic receptors concentrated around the animal pole (Kusano *et al.* 1982). As these receptors are known to generate Ins(1,4,5)P_3 (Oron *et al.* 1985), this messenger and its associated calcium signal would be concentrated in the animal pole and could thus be responsible for setting up the orientated electrical field that surrounds such oocytes (Robinson 1979). The function for such a field is still unknown but it could play a role in establishing the anterior–posterior axis by segregating molecules such as the maternal mRNA which is uniformly distributed in stage I and II oocytes, but then migrates to form a crescent at the vegetable pole at later stages (Melton 1987). The fact that the teratogenic action of lithium can be blocked by injecting *Xenopus* embryos with *myo*-inositol provides further evidence that inositol lipids play a central role in the spatial organization of early development (Busa, this symposium).

At the time of fertilization, there is rapid and dramatic hydrolysis of PtdIns(4,5)P_2 resulting in the formation of Ins(1,4,5)P_3 which triggers a local increase in calcium at the point of sperm entry. There is also a suggestion that a calcium-induced formation of Ins(1,4,5)P_3 might be responsible for the wave of calcium that spreads from the point of sperm entry towards the

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opposite pole (Whitaker & Irvine 1984). In hamster eggs there is a similar spatially organized burst of calcium at fertilization. Calcium transients recur at 1–3 min intervals for over 100 min. The spatial organization can persist up to the third transient, thus providing a particularly dramatic example of both spatial and temporal aspects of cell signalling (Miyazaki *et al.* 1986). These temporal aspects will be considered in greater detail in the following section.

Temporal aspects

Latency

A characteristic feature of receptors that act through phosphoinositide hydrolysis is that there is a long latency between arrival of the agonist and the subsequent cellular response. The example shown in figure 1*b* is the transepithelial potential response of an insect salivary gland after addition of different concentrations of 5-hydroxytryptamine (5-HT). This neurohormone stimulates the hydrolysis of PtdIns(4,5)P_2 leading to the formation of Ins(1,4,5)P_3 which releases internal calcium to open chloride channels giving the potential responses shown in figure 1*b*. At low 5-HT concentrations, there is latency of approximately 20 s which is progressively reduced as the dose of 5-HT is increased (figure 1*a*, *b*). Similar long latencies have been described in smooth muscle (Bolton 1976) and *Xenopus* oocytes (Kusano *et al.* 1982; Miledi *et al.* 1982) responding to acetylcholine, in hepatocytes responding to vasopressin (Cooper *et al.* 1985) and in *N*-formylmethionyl-leucyl-phenylalanine (fMLP)-stimulated

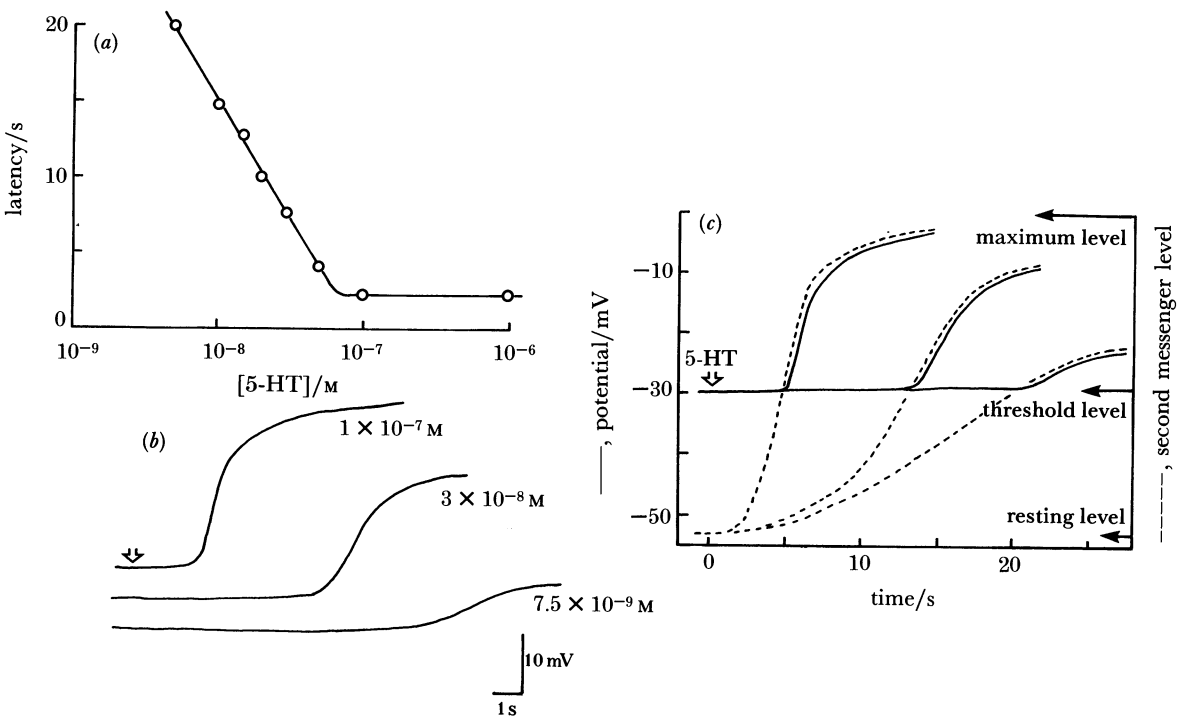

FIGURE 1. Relation between 5-hydroxytryptamine (5-HT) concentration and the latency of the transepithelial potential response of a blowfly salivary gland. Typical responses to varying concentrations of 5-HT added at the large arrow are shown in (*b*) and data obtained from such experiments was used to obtain the relationship shown in (*a*). The model outlined in (*c*) attempts to explain how latency may depend upon the time required to raise some second messenger (e.g. Ins(1,4,5)P_3 or calcium) from a resting level towards some threshold value necessary to trigger the change in membrane potential.

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neutrophils (von Tscharner *et al.* 1986). As in the insect salivary gland, the latency of the response in neutrophils (von Tscharner *et al.* 1986) and in the parotid (Merritt & Rink 1987) was found to decline as the concentration of agonist increased. This latency seems to depend upon some rate-limiting step associated with the receptor transduction process responsible for generating the appropriate messenger(s). Another explanation (see figure 1*c*) is that the resting level of the messenger(s), such as calcium, might lie somewhat below the threshold necessary to trigger the physiological response. Latency will then depend on how long it takes to raise the concentration of the messenger to this threshold level. The most likely rate-limiting step is the agonist-dependent formation of Ins(1,4,5)P_3 (Merritt & Rink 1987). A prediction from such a mechanism is that latency should be reduced by using low agonist concentrations to elevate second messenger levels close to threshold. Such an experiment has been done with *Xenopus* oocytes where a threshold concentration of 5-HT, which by itself had no effect, markedly reduced the latency of the response to low doses of acetylcholine (Parker *et al.* 1987). More attention must be paid to the temporal sequence of events that intervene between the arrival of an agonist and the initiation of its cellular response.

Oscillations

Second messenger systems are highly dynamic in that they not only respond to external signals but are subject to a great variety of internal feedback controls. In the case of calcium, an expression of this dynamism is that its level is frequently found to oscillate (figures 2–4). Such calcium oscillations are widespread (table 1), occurring in both undifferentiated (oocytes and eggs) and fully differentiated cells, indicating that they are not a property of any one cellular process but appear to be a basic feature of intracellular calcium regulation. These calcium oscillations have been measured either directly or have been inferred on the basis of

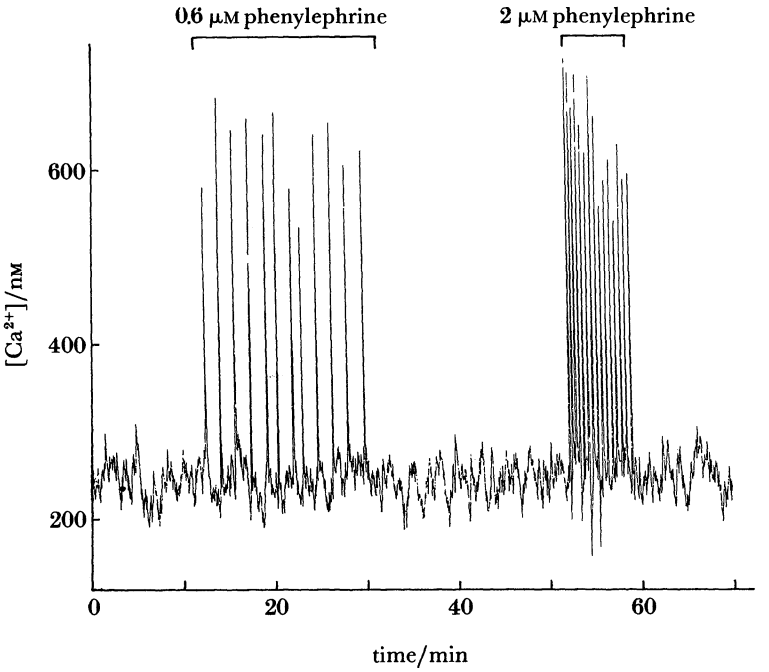

FIGURE 2. Effect of varying the concentration of phenylephrine on the frequency of calcium oscillations measured in a single hepatocyte loaded with aequorin.

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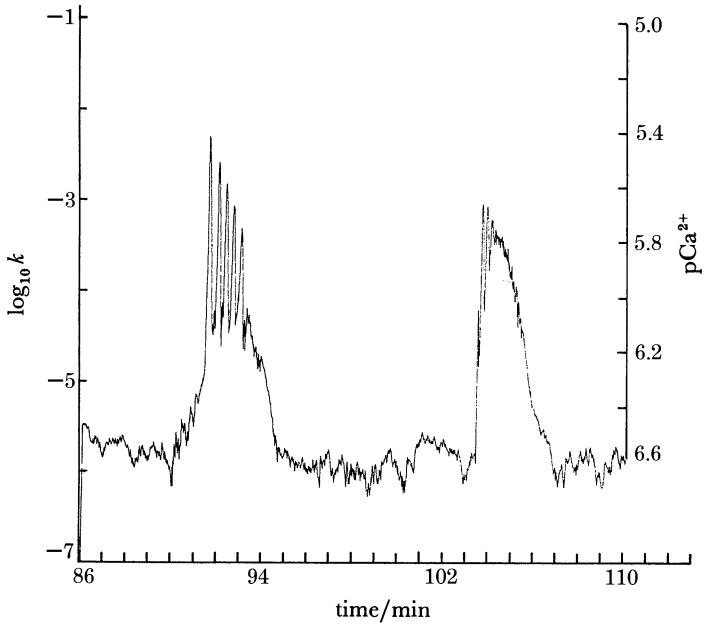

FIGURE 3. Oscillation in intracellular calcium recorded from a fertilized mouse egg (see Cuthbertson & Cobbold 1985).

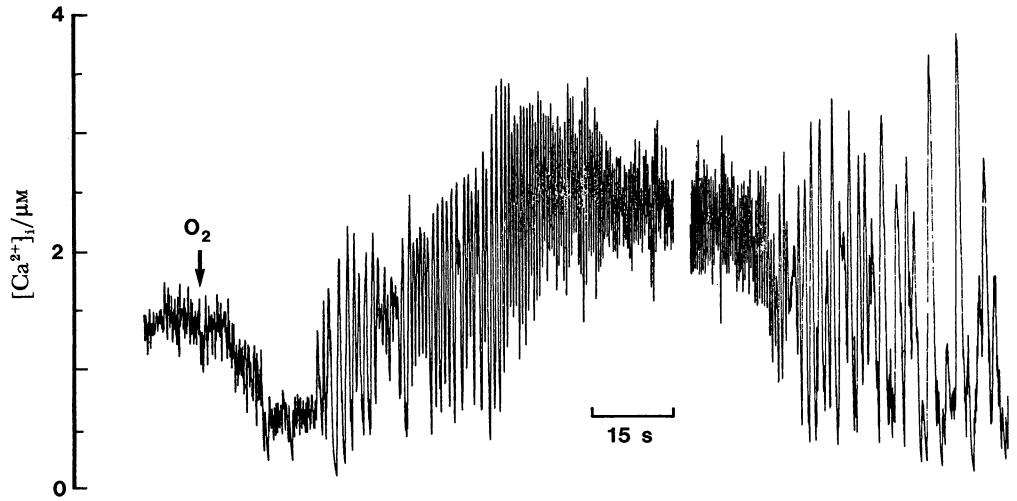

FIGURE 4. Intracellular calcium oscillations triggered by reoxygenation of an anoxic myocyte from adult rat ventricle. (See Allshire *et al.* 1987 for details.)

fluctuations in calcium-dependent plasma membrane conductances. That the latter is a valid approach has been confirmed in cultured epithelial cells (Yada *et al.* 1986), L-cells (Ueda *et al.* 1986) and in hamster eggs (Igusa & Miyazaki 1986), where membrane potential (due to the opening of a potassium channel) and intracellular calcium were found to oscillate in phase with each other. Such membrane oscillations are a secondary consequence of the internal oscillator in that they can be blocked with quinine in L-cells without interfering with intracellular calcium oscillations (Ueda *et al.* 1986). Likewise, oscillations in intracellular calcium in *Xenopus* oocytes have been recorded when the potential is clamped at a constant voltage (Parker & Miledi 1986).

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TABLE 1. OSCILLATORY PHENOMENA IN CELLS

(The examples are thought to result from oscillations in intracellular calcium measured either directly using calcium-sensitive indicators or indirectly due to the action of calcium on ion channels.)

cell & oscillatory phenomenon	stimulus	period/s	reference
cardiac muscle (intracellular Ca^{2+})	none	0.25	Allen *et al.* (1984)
rat myocytes (contraction)	caffeine	0.3–3	Kort *et al.* (1985)
astrocytes (membrane potential)	TPA	0.3–10	MacVicar *et al.* (1987)
anterior pituitary (membrane potential)	none	1–3	Poulsen & Williams (1976)
adrenocortical cells (membrane potential)	ACTH	2–10	Matthews & Saffran (1973)
lacrimal gland (potassium current)	acetylcholine	5–10	Evans & Marty (1986)
neutrophils (shape change)	f-Met-Leu, Phe	7–9	Wyman *et al.* (1987)
intestinal 407 cells (intracellular calcium)	5-HT or ACh	7–10	Yada *et al.* (1986)
parotid (intracellular calcium)	acetylcholine	8.0	Gray (1987)
hippocampal neurone (intracellular calcium)	none	8.7	Ogura *et al.* (1987)
L-cells (intracellular calcium)	none	12–27	Okada *et al.* (1986); Ueda *et al.* 1986
mouse oocytes (intracellular calcium)	TPA	17–35	Cuthbertson & Cobbold (1985)
rat hepatocytes (intracellular Ca^{2+})	NE, vasopressin, angiotensin II	18–240	Woods *et al.* (1987*a*, *b*)
macrophages (intracellular Ca^{2+})	none	19–69	Kruskal & Maxfield (1987)
Xenopus oocytes (chloride current)	acetylcholine	20	Miledi *et al.* (1982)
HeLa cell, (potassium current)	histamine	20–33	Sauvé *et al.* (1987)
mast cells, (intracellular calcium)	phosphatidylserine	30–60	Neher & Almers (1986)
B-lymphocyte (intracellular Ca^{2+})	antigen	50–75	Wilson *et al.* (1987)
hamster eggs (membrane potential)	fertilization	55	Igusa & Miyazaki (1983)
rat hepatocytes, (intracellular Ca^{2+})	ADP, ATP	*ca.* 60	Cobbold *et al.* (1988*b*)
rat liver, (Ca^{2+} efflux)	NE, vasopressin	90–240	Graf *et al.* (1987)
sympathetic ganglion (membrane potential)	caffeine	*ca.* 120	Kuba & Nishi (1976)
mouse embryo 3T3 (membrane potential)	none	160	Hülser & Lauterwasser (1982)
rabbit egg (membrane potential)	fertilization	300	McCulloh *et al.* (1983)
mouse oocytes (intracellular calcium)	fertilization	600–1800	Cuthbertson *et al.* (1981); Cuthbertson & Cobbold (1985)

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Spontaneous activity is very sensitive to external conditions, particularly the presence of natural agonists or certain pharmacological agents (table 1). In most cases, such agonist-induced oscillations occur after a short latency, for example 15–20 s after the addition of acetylcholine to *Xenopus* oocytes (Miledi *et al.* 1982). There are some agonists, however, that induce oscillations after a much longer latency. For example, mammary epithelial cells begin to display hyperpolarizing responses 3 h after addition of EGF, or depolarizing responses 6–12 h after insulin (Enomoto *et al.* 1986). The mechanism underlying these slowly developing oscillations in response to growth factors is likely to differ from those that occur rapidly in response to the stimuli summarized in table 1. Many of the agonists that act quickly to induce oscillations are also known to stimulate the hydrolysis of inositol lipids, suggesting a link between the two processes. Further evidence for such an association is that oscillations can be induced by agents such as TPA (Cuthbertson & Cobbold 1985) or phosphatidylserine (Neher & Almers 1986), which activate protein kinase C, or by the injection of Ins(1,4,5)P_3 (Oron *et al.* 1985; Evans & Marty 1986; Parker & Miledi 1986; Capiod *et al.* 1987).

The frequency of calcium oscillations varies enormously between different cell types (table 1) ranging from the very rapid fluctuations seen in heart cells (Kort *et al.* 1985; Capogrossi *et al.* 1986) to the widely spaced transients occurring every 10–20 min as described in mouse oocytes after fertilization (Cuthbertson *et al.* 1981). Within a single cell type, oscillator frequency is very sensitive to agonist concentration, as will be described later. However, such agonist-dependent changes cannot explain the wide range of frequencies displayed in table 1, which seem to point to the existence of different oscillatory mechanisms. At the outset we should distinguish between calcium oscillations that are approximately sinusoidal where free calcium is probably continuously involved in the oscillatory cycle, as for example in the rapid calcium oscillations in heart cells (figure 4) (Allshire *et al.* 1987), and those oscillators where free calcium can fall to resting levels for many seconds between transients. In the latter instance, best typified by the repetitive free-calcium transients seen in hepatocytes (figure 2) or

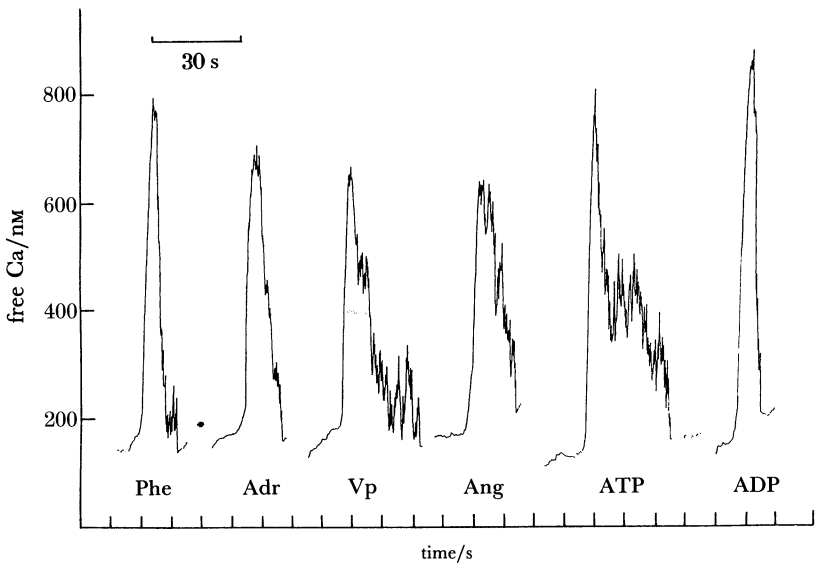

FIGURE 5. Representative free Ca transients showing the differences in transient duration for different agonists. Transients from several different hepatocytes are shown here, but differences in transient shape can be routinely recorded in the same individual cell. Time constant 1 s for the transients, 15 s for resting levels. Time scale in 10 s intervals. Phe, phenylephrine; Adr, adrenaline, Vp, arg^8 vasopressin; Ang, angiotensin II.

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mouse eggs (figure 3), free calcium seems to be the output of an independent oscillatory mechanism. Both oscillatory mechanisms could occur in the same cell, for instance the mouse oocyte calcium transients, besides repeating at 10–20 min, also oscillate at their peak with a period of about 10 s, reminiscent of the continuous oscillations that phorbol esters induce in these cells (Cuthbertson & Cobbold 1985). The individual calcium transients seen in hepatocytes also display complex oscillatory patterns which vary depending on the agonist (figure 5). When stimulated with norepinephrine or ADP the transients are reasonably symmetrical whereas the other agonists (vasopressin, angiotensin II and ATP) induce asymmetrical transients with the slower falling phase often displaying higher-frequency oscillations. Such complex transients may reflect the co-existence of two separate oscillatory mechanisms, as outlined in the following section.

The cellular basis of calcium oscillations

Based on the cellular origin of such oscillations, Berridge & Rapp (1979) recognized two types, one generated by ionic events at the plasma membrane and the other originating intracellularly. Examples of the former are pacemaker cells in the heart, insulin-secreting β-cells and certain pituitary cells or burster neurons in the CNS where oscillations are generated by an interplay of plasma membrane conductances. Membrane depolarization due to the opening of voltage-operated calcium channels alternates with membrane hyperpolarization resulting from the opening of calcium-dependent potassium channels (Wallen & Grillner 1987). In such plasmalemmal electrical oscillators, the periodic opening of the voltage-dependent calcium channels leads to fluctuations in internal calcium (Gorman & Thomas 1978; Schlegel *et al.* 1987). Because oscillations in L-cells were unaffected by agents such as caffeine, dantrolene and oxalate which influence intracellular stores but were blocked by the calcium channel antagonists Ca^{2+}, nifedipine and D600, it was concluded that the oscillator was based in the plasma membrane (Okada *et al.* 1982). It was proposed that external stimuli, acting either directly or indirectly via a receptor, opened channels in the plasma membrane causing calcium to flow into the cell while the resulting rise in the level of calcium was then lowered by a calcium pump. Although such a model that envisages periodic alterations in the balance between influx and efflux might produce oscillations in intracellular calcium (with some additional assumptions), it cannot easily account for the observed pulsatile patterns. The evidence for the assumption that the oscillations in L-cells involve extracellular calcium is far from convincing and is contradicted by the lack of electrophysiological signs of calcium currents, and the occasional appearance of 'normal' transients after prolonged incubation in calcium-free medium (Henkart & Nelson 1979). In most of the examples shown in table 1, the oscillator is relatively independent of external calcium, for example *Xenopus* oocytes (Parker & Miledi 1986), HeLa cells (Sauvé *et al.* 1987), peritoneal mast cells (Neher & Almers 1986), cultured intestinal cells (Yada *et al.* 1986) or macrophages (Kruskal & Maxfield 1987). Studies of rat peritoneal mast cells reveal that influx of external calcium cannot account for the rapid rise in the calcium transient, which must therefore originate from a spontaneous release of calcium from intracellular stores (Neher & Almers 1986).

Less is known about this second type, which is the main concern of this review, where oscillations seem to arise from a periodic release of calcium from internal stores. Two main mechanisms have been invoked to account for this oscillatory release of internal calcium. In one

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mechanism, the oscillator is receptor-controlled in that the plasma membrane plays a direct role in generating the signal, most likely the periodic formation of Ins(1,4,5)P_3, which triggers the oscillatory release of calcium. In the other mechanism, the oscillator is cytoplasmic in that it resides within the organelle responsible for releasing calcium, perhaps through the phenomenon of calcium-induced calcium release.

Receptor-controlled oscillators

Models based on the periodic generation of second messengers

Various attempts have been made to explain calcium oscillations on the basis of the feedback interactions that exist between various second messenger pathways. These models all incorporate the same basic principle of having a positive loop whereby some process generates a calcium signal which is then counteracted by a negative feedback loop which inhibits the calcium release process. One such model proposed by Rapp & Berridge (1977) was based on feedback interactions operating between cyclic AMP and calcium. Agonists were thought to induce oscillations by first raising the intracellular level of cyclic AMP which then mobilized calcium. The feedback loop was closed by calcium acting back negatively to inhibit further production of cyclic AMP. The clear implication of such models is that the oscillation in calcium is driven by an oscillation in some other second messenger such as cyclic AMP.

With the discovery that Ins(1,4,5)P_3 functions as a calcium-mobilizing second messenger (Berridge & Irvine 1984; Berridge 1987) this ubiquitous signal pathway has been incorporated into more recent models (Cobbold *et al.* 1988*a*; Sauvé *et al.* 1987; Woods *et al.* 1987*a*). The most detailed and explicit model has been formulated by Cobbold *et al.* (1988*a*) to explain the calcium oscillations that occur in hepatocytes responding to various agonists. It is proposed that oscillations in Ins(1,4,5)P_3 are established through a negative feedback loop whereby protein kinase C regulates the membrane transduction step responsible for generating this messenger (figure 6). A key feature of the inositol lipid signalling system is that the hydrolysis of PtdIns(4,5)P_2 generates two initial messengers: (*a*) the Ins(1,4,5)P_3 that mobilizes calcium; and (*b*) diacylglycerol (DG) which, with calcium, activates protein kinase C (C-kinase; Nishizuka 1984). There are numerous reports that one function of the DG–C-kinase pathway is to inhibit further hydrolysis of PtdIns(4,5)P_2 (Berridge 1987), and this represents the negative feedback component responsible for reducing the formation of Ins(1,4,5)P_3 and the calcium signal. This reduction in calcium, together with the decline in DG formation, will relieve the inhibition, so allowing the transduction mechanism to generate another pulse of Ins(1,4,5)P_3, a process that may be facilitated by a co-operative step involving G-proteins.

A co-operative step, positive feedback or some other equivalent non-linearity is required for the model to account for the observed pulsatile calcium signals. The cytoplasmic concentration of Ins(1,4,5)P_3 is predicted to be under the second-by-second control of the receptor, or of receptor-specific elements such as G proteins, because of the key observation that the time-course of individual free calcium transients is different for different agonists (Woods *et al.* 1987*a*, *b*; Cobbold *et al.* 1988*a*, *b*) (see figure 5). Such agonist-specific transients have been recorded in a single cell and would be difficult to explain if the oscillatory mechanism were remote from the receptors. Thus oscillatory calcium release from the endoplasmic reticulum cannot be induced simply by Ins(1,4,5)P_3; such a mechanism would lack information as to which receptor species was active. The model explains the differences in the form of the

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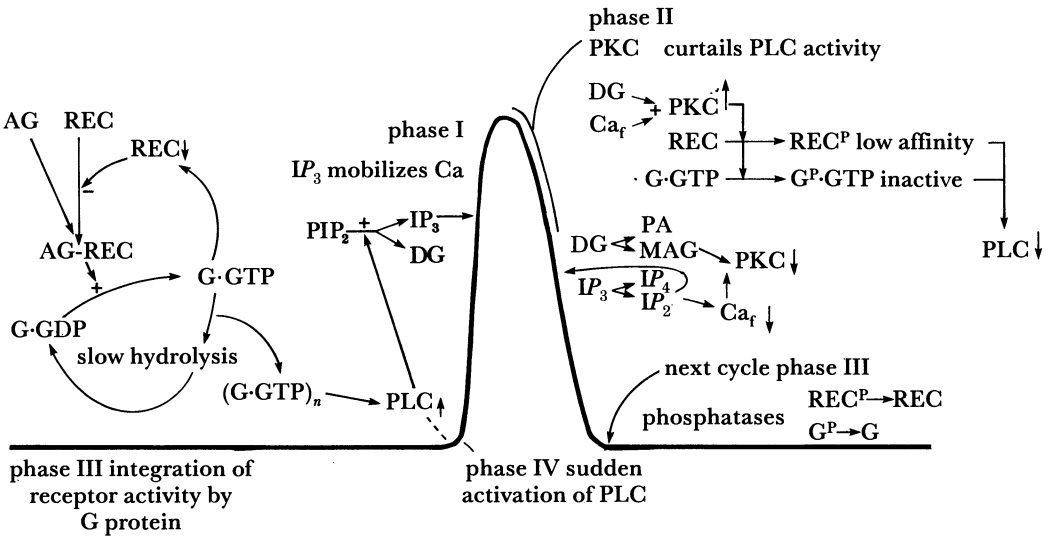

FIGURE 6. Diagram of the hypothetical scheme for generating a free Ca transient (heavy line). Phase I: Ins(1,4,5)P_3 mobilizes calcium. Phase II: the activation of protein kinase C (PKC) phosphorylates the receptor (REC) and the G-protein (G) resulting in a decrease in the activity of phospholipase C (PLC). The activity of PKC declines as the second messengers are removed. Diacylglycerol (DG) is converted to phosphatidic acid (PA) or monoacylglycerol (MAG) and Ins(1,4,5)P_3 is either phosphorylated to inositol Ins(1,3,4,5)P_4 or is dephosphorylated to inositol Ins(1,4)P_2. Phase III: phosphatases re-activate the REC and the G-protein allowing them to interact with an agonist (AG) to begin another cycle. Phase IV: the activated G-proteins $(G \cdot GTP)_n$ stimulate PLC to produce another burst of Ins(1,4,5)P_3 and DG.

transient, which occurs during the falling phase of free calcium, by differences in the speed with which negative feedback from protein kinase C inactivates the receptors or their G proteins. The model requires that the lifetime of Ins(1,4,5)P_3 is brief in relation to the time-course of the transients, allowing the instantaneous free Ca level to be a function of the instantaneous Ins(1,4,5)P_3 concentration. Obtaining direct evidence for such oscillations in the level of Ins(1,4,5)P_3 will be important for testing this hypothetical model, but will be difficult using current technology because continuous measurements are needed in one cell.

Some indication that calcium oscillations may occur without fluctuations in Ins(1,4,5)P_3 has emerged from studies showing that oscillatory activity can be induced when this messenger is perfused into cells from the lacrimal gland (Evans & Marty 1986) or liver (Capiod *et al.* 1987) via a patch electrode or when it is injected into *Xenopus* oocytes (figure 7) (Parker & Miledi 1986; Miledi *et al.* 1987). However, some of these Ins(1,4,5)P_3-induced oscillations differ significantly from those produced normally. The oscillations induced in hepatocytes after internal perfusion with Ins(1,4,5)P_3 were not sustained (Capiod *et al.* 1987) and were quasi-sinusoidal and 'damped' rather than appearing as recurring isolated pulses. Also, the Ins(1,4,5)P_3-induced oscillations in lacrimal glands occurred with a period of 1.7 s, which is very much shorter than the 5–10 s period observed with acetylcholine (Evans & Marty 1986). It seems much more likely that these Ins(1,4,5)P_3-induced calcium oscillations are generated by the cytoplasmic oscillatory mechanism described below.

Cytoplasmic oscillators

The second type of model considers that oscillations, particularly those showing sinusoidal and higher frequency characteristics, may be established through a mechanism associated with

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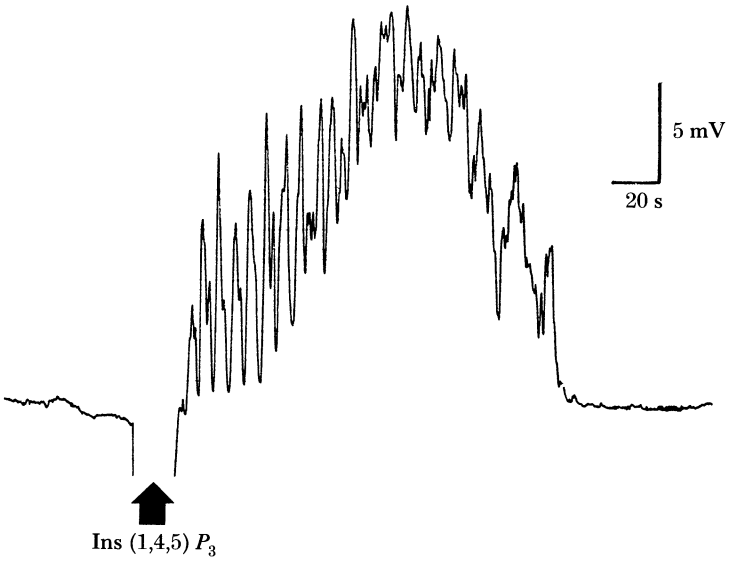

FIGURE 7. Oscillations in membrane potential of a *Xenopus* oocyte after iontophoretic injection of Ins(1,4,5)P_3. Membrane potential was recorded through the same electrode used to inject Ins(1,4,5)P_3. The downward deflection at the arrow represents the hyperpolarizing response induced during the period of current injection (M. J. Berridge, unpublished observation).

the organelle responsible for releasing calcium. As discussed earlier, calcium is an integral component of the oscillator where it is envisioned to act either as a negative feedback regulator of release (see article by Payne *et al.* (this symposium) for further details of this model) or as a trigger of calcium release.

Calcium induced calcium release model

The phenomenon of calcium-induced calcium release, originally described in muscle (Endo 1977), has an autocatalytic feature which led to the suggestion that it might form the basis of oscillatory activity (Kuba & Takeshita 1981; Yada *et al.* 1986; Igusa & Miyazaki 1983). Some support for such a mechanism has come from studies on skinned cardiac cells which contract in an oscillatory way due to a cyclic release and re-sequestration of calcium (Fabiato & Fabiato 1975). This oscillatory behaviour requires fairly precise conditions, particularly with regard to the level of free calcium and to the extent of calcium buffering. The latter must be sufficient to stabilize calcium levels but not high enough to compete with the re-sequestering of calcium that provides the signal for the next cycle. Fabiato & Fabiato (1975) came to the conclusion 'that the triggering of phasic contractions was related to the filling of the internal stores to a critical level at which a fraction of the Ca^{2+} content is released'. A similar phenomenon has been described in the sarcoplasmic reticulum (SR) of skeletal muscle where a certain level of preloading was required before spontaneous release occurred (Palade *et al.* 1983). It would seem that the calcium-sensitive process which triggers the spontaneous release of calcium is located within the SR. Another prerequisite for release was that the calcium concentration bathing the SR must be reduced below 0.3 μM. Therefore, not only must the internal store be charged up to some threshold level, but the external calcium concentration must also be low before release occurs. These observations may underlie the complex patterns of free calcium oscillations that can be recorded by using aequorin in anoxic rat cardiomyocytes upon

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reoxygenation (figure 4) (Allshire *et al.* 1987). In these cells free calcium oscillations start a few seconds after a reoxygenated cell has reduced free calcium from *ca.* 1.5 μM to *ca.* 0.5 μM. These oscillations are eliminated by caffeine, implicating the SR in the process.

Just which part of the endoplasmic reticulum (ER) of non-muscle cells contributes to such oscillatory activity is unclear. Available evidence suggests that only a proportion of the calcium accumulated by the ER can be released by Ins(1,4,5)P_3. This Ins(1,4,5)P_3-sensitive store may be a separate organelle (calciosome) which has many of the properties of the SR of muscle (Volpe *et al.* 1987). For these calciosomes to generate oscillations in the continued presence of Ins(1,4,5)P_3, it is necessary to assume that they must become desensitized to Ins(1,4,5)P_3 to allow for the periodic recharging of the pool, which is then released either by a resensitization process or through the calcium-induced calcium release mechanism described earlier. Because there is no evidence for desensitization of the Ins(1,4,5)P_3 receptor, it is more likely that oscillations are a property of the Ins(1,4,5)P_3-insensitive calcium pool and that this is induced to oscillate through the extra load imposed upon it by the calcium released from the Ins(1,4,5)P_3-sensitive pool. A build-up of calcium within the Ins(1,4,5)P_3-insensitive calcium store might be responsible for triggering the spontaneous release of calcium back into the cytoplasm (figure 8).

In summary, it is proposed that a component of the ER has an inherent capacity to oscillate and is triggered by an overload of calcium which is then released and sequestered in a periodic manner. This hypothesis can account for many of the experimental manipulations known to induce oscillatory activity. Injection of Ins(1,4,5)P_3 will discharge calcium from the Ins(1,4,5)P_3-sensitive pool, which will then induce oscillations by overloading the Ins(1,4,5)P_3-insensitive pool. A similar explanation could account for the oscillations induced in sympathetic ganglion cells following addition of caffeine (Kuba & Nishi 1976) which, like Ins(1,4,5)P_3, is capable of mobilizing internal calcium. As discussed earlier, injection of enough calcium to induce a response similar to that of Ins(1,4,5)P_3 evoked no oscillations, but these begin to appear when the calcium injections are given repeatedly (Gillo *et al.* 1987). Such repeated injections may charge up the calcium store to a point where the free calcium reaches threshold for spontaneous release. One reason why oocytes may require rather severe treatment with calcium in order to induce oscillations is because in the absence of Ins(1,4,5)P_3 calcium can be taken up by both the Ins(1,4,5)P_3-insensitive and sensitive pool. After repeated injections of calcium into *Xenopus* oocytes, their responsiveness to acetylcholine is greatly enhanced (Nomura *et al.* 1987), suggesting that a large proportion of the calcium enters the Ins(1,4,5)P_3-sensitive pool. Any mechanism that leads to an overload of calcium within the ER seems to be responsible for setting up oscillatory activity. A similar explanation may also account for the onset of oscillatory activity that occurs when oocytes are injected with large amounts of calcium (Miledi & Parker 1984; Dascal *et al.* 1985; Gillo *et al.* 1987).

Any model of this oscillator must take account of the observation that the frequency of certain calcium oscillations are very sensitive to ambient calcium concentration (Fabiato & Fabiato 1975; Kuba & Nishi 1976; Allen *et al.* 1984; Kort *et al.* 1985; Benham & Bolton 1986). A key feature of the above model is that calcium is spontaneously released once its concentrations reaches some threshold level within the cisternae of the ER. This threshold will be reached quicker at higher external calcium concentrations because the pumps will be able to sequester calcium faster and could thus explain why the oscillator accelerates as the cytoplasmic level of calcium is elevated.

The model as described so far has assumed that oscillatory activity originates from a single

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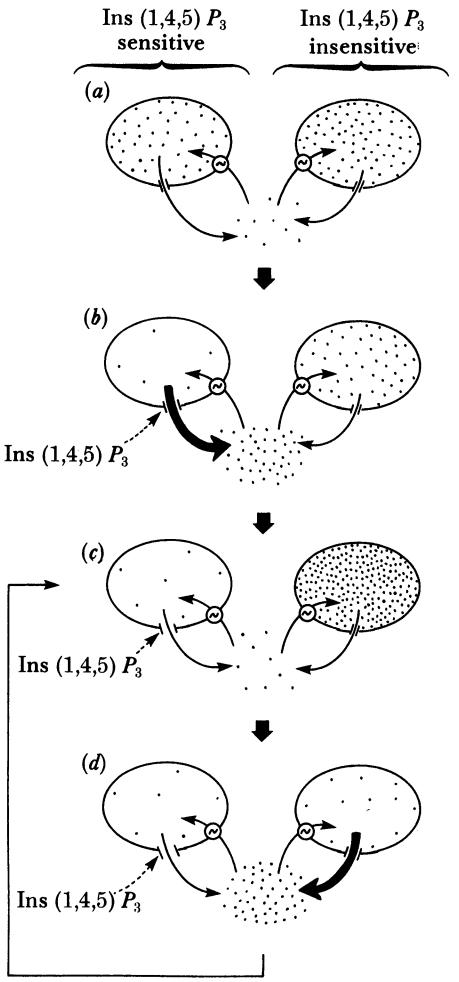

FIGURE 8. Cytoplasmic oscillator model based on calcium-induced calcium release. (*a*) Resting condition with calcium cycling between the cytoplasm and both pools. (*b*) An increase in Ins(1,4,5)P_3 triggers calcium release from the Ins(1,4,5)P_3-sensitive pool. (*c*) In the continued presence of Ins(1,4,5)P_3 calcium is taken up preferentially by the Ins(1,4,5)P_3-insensitive pool. (*d*) Overloading the Ins(1,4,5)P_3-insensitive pool with calcium triggers a spontaneous release of calcium back into the cytoplasm. As long as Ins(1,4,5)P_3 keeps the Ins(1,4,5)P_3-sensitive pool discharged, conditions (*c*) and (*d*) will follow each other, thus setting up oscillations in cytoplasmic calcium.

compartment, most likely the Ins(1,4,5)P_3-insensitive store. However, the possibility must be considered that these stores may communicate, at least temporarily, with the Ins(1,4,5)P_3-sensitive store. This possibility must be taken seriously in the light of recent evidence that GTP may function to facilitate communication between intracellular compartments (Chueh *et al.* 1987). In skeletal muscle, all of the SR can sequester calcium but the release site is concentrated in the terminal cisternae. If a similar arrangement exists in non-muscle cells, oscillatory activity may depend on the rate of transfer of calcium from Ins(1,4,5)(P_3-insensitive into the Ins(1,4,5)P_3-sensitive regions. Another possibility is that Ins(1,3,4,5)P_4 might play a role in facilitating this communication between intracellular calcium compartments (Irvine *et al.*, this symposium). This is of interest because this putative messenger does induce oscillations when injected into *Xenopus* oocytes (Parker & Miledi 1987). Further analysis of the dynamics and localization of oscillatory activity should lead to a better understanding of Ins(1,4,5)P_3-induced calcium mobilization.

Conceivably, these two oscillatory mechanisms (receptor-controlled and cytoplasmic) may

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co-exist to account for some of the complex oscillatory patterns described earlier. Brief Ins(1,4,5)P_3 pulses would produce single calcium transients, with the agonist-sensitive calcium release mechanism acting as a non-linear transducer between the Ins(1,4,5)P_3 and calcium signals. Longer Ins(1,4,5)P_3 pulses would invoke a larger mobilization of calcium which may trigger the cytoplasmic mechanism to induce a higher frequency oscillatory release of calcium. This concept fits some of the observed oscillatory patterns, for example the individual transients in response to ATP in hepatocytes (figure 5) and to fertilization in mouse oocytes (figure 3) where pulses with periods of the order of minutes contain oscillations with periods of the order of seconds.

Functional significance of calcium oscillations

There are numerous suggestions that oscillations may function in controlling a variety of physiological processes such as learning (Woollacott & Hoyle 1977), fertilization (McCulloh *et al.* 1983; Miyazaki *et al.* 1986), egg maturation (Schlichter 1983), glycogen metabolism (Woods *et al.* 1986), fluid secretion (Rapp & Berridge 1981), cell growth (Enomoto *et al.* 1986) and chemotaxis in neutrophils (Wyman *et al.* 1987). A role in cell growth is also suggested by the fact that antigen-induced oscillations in B-lymphocytes can last for at least 6 h and perhaps longer (Wilson *et al.* 1987). What might be the functional significance of having an oscillatory mechanism? In the case of calcium, there may be considerable advantages in using such an oscillatory mechanism based predominantly on the mobilization of internal calcium, which raises the whole question of the role of internal versus external calcium in agonist-dependent cell signalling. If cells draw upon the inexhaustible external calcium reservoir, they run the very serious risk of becoming swamped with calcium especially during prolonged stimulation when the calcium level would be elevated for prolonged periods. In the case of a typical plasmalemmal electrical oscillator, such as a pituitary cell, which relies upon external calcium, there is very precise control of calcium entry by means of voltage-operated channels. Opening of the calcium channel for just 10 ms generates a calcium transient that lasts for at least 2 s and is sufficient to stimulate secretion (Schlegel *et al.* 1987). In non-excitable cells where the control over calcium entry is likely to be much slower, there is an obvious danger of being swamped with calcium. Such overloading might be avoided by relying predominantly on internal calcium which is released periodically with each transient acting to maintain some slowly inactivating calcium-dependent process (Woods *et al.* 1987*a*).

Another reason for using free calcium transients may be to simplify the problem of regulating intermediate levels of free calcium in the face of fluxes, themselves dependent upon calcium-calmodulin-activated pumps, which tend to reduce free calcium. Sustaining an intermediate level of free calcium in the face of upwardly spiralling fluxes might not only present a difficult control problem but would also be energetically wasteful. By resorting to transients, however, free calcium may be regarded as being either raised or resting, simplifying the control pathway. Moreover, by restricting the generation of Ins(1,4,5)P_3, expenditure of energy sustaining the second messenger is minimized. It is conceivable that calcium sequestering was an early evolutionary development whereas use of calcium as a second messenger may have evolved later and was superimposable on the background of rigid cellular calcium homeostasis only by perturbing free calcium transiently. In summary, oscillatory signals can be sustained indefinitely without excessive energy cost in the presence of both fast activation kinetics (allowing immediate responses) and strong homeostatic controls (providing security for the maintenance of viability).

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On a more speculative note, it has been postulated that oscillations may represent a way of encoding information. The cell may employ a frequency-encoded messenger (Rapp & Berridge 1981; Rapp 1987; Schlegel *et al.* 1987). Instead of using an amplitude-dependent system, the internal signal is digitized in the form of constant-amplitude calcium transients and information is conveyed by varying frequency. The notion that intracellular messengers might be frequency encoded emerged from experiments on the blowfly salivary gland where oscillation frequency was found to vary with the dose of 5-HT over the same range of concentration which controlled the rate of fluid secretion (Rapp & Berridge 1981). A similar phenomenon has been described in hepatocytes (figure 2) (Woods *et al.* 1986, 1987*a*) and intact liver (Graf *et al.* 1987) where oscillation frequency was found to vary with the dose of glycogen-mobilizing hormones. This relation between frequency and agonist concentration was not found in the rat parotid (Gray 1987) which must raise some doubt concerning the existence of such a frequency-encoded system.

If certain cells do use a frequency-encoded system, how might variations in frequency be translated back into an analogue response to give a smooth change in some physiological response? One mechanism suggests itself based on the ability of calcium to stimulate the phosphorylation of proteins which will induce a cellular response until this activity is terminated by the appropriate phosphatase. The sequence is then repeated by the next calcium spike, the time spent in the active phosphorylated state will depend on the frequency of the calcium oscillations. For such a model, the physiological response of individual cells will also be periodic (in effect under 'proportional control') but because cells oscillate out of phase with each other their individual contributions will sum to give a smooth response. Studies on cultured cells have established that despite close ionic coupling through gap junctions, neighbouring cells oscillate out of phase with each other (Hülser & Lauterwasser 1982).

Why should cells generate repetitive free calcium transients using frequency-modulation to encode information on extracellular agonist levels? What is the advantage over the supposedly more simple amplitude-modulated systems? One reason may lie in an increase in fidelity, especially at low agonist concentrations. For an amplitude-dependent mechanism, small variations in hormone concentration have to be translated into small changes in second messenger level and such minor adjustments would be prone to serious distortion by random noise. However, if all effective agonist concentrations are translated into an unambiguous calcium spike with frequency rather than concentration being the variable parameter, the signalling system will be less corrupted by noise.

The two mechanisms of control may not necessarily be mutually exclusive in that cells might employ a frequency-encoded system at low agonist concentrations to regulate day-to-day responses, but may switch to an amplitude-dependent mechanism for regulating longer term adaptive changes such as cell proliferation. Variations in the patterning of the internal signal will greatly expand its second messenger repertoire and may help explain how single messengers can control more than one process within the cell.

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Discussion

C. P. Downes (*Smith Kline & French Research Ltd, Welwyn, U.K.*). In comparing the intracellular Ca^{2+} signals evoked in single cells by different agonists and attempting to relate these to data from cell populations for agonist-induced accumulation of inositol phosphates, it is important to know whether all the cellular effects of each agonist necessarily involves PtdIns(4,5)P_2 hydrolysis. To be more specific, α_1 receptors have, until recently, been regarded as a homogeneous population of receptors that utilize the PtdIns(4,5)P_2 hydrolysis effector system. However, recent data (Han *et al.* 1987) suggest there are at least two populations of α_1 (i.e. prazosin-blockable) receptors with only one population being coupled to PtdIns(4,5)P_2 hydrolysis, although both receptors seem able to use Ca^{2+} as an intracellular signal. Caution may therefore be needed when equating α_1 receptor-stimulated Ca^{2+} signals with inositol trisphosphate production.

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M. J. Berridge. This is a useful cautionary comment which has an important bearing on the models proposed here to account for oscillatory activity which both depend upon the

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generation of Ins(1,4,5)P_3. If an agonist acts solely at the cell surface to stimulate an influx of extracellular calcium, it is unlikely that it will induce oscillatory activity unless it overloads the intracellular pools sufficiently to activate the cytoplasmic oscillator.

M. R. Hanley (*MRC Molecular Neurobiology Unit, University of Cambridge Medical School, Cambridge, U.K.*). What are Dr Berridge's thoughts on how Ins(1,3,4,5)P_4 might fit into a mechanistic hypothesis for the control of oscillatory behaviour by intracellular and extracellular calcium? I am attracted to the fact that individual types of hepatocyte receptors have 'molecular signatures' in the shapes of their calcium spikes, which might suggest that there are comparable 'molecular signatures' in the patterns of inositol phosphate production.

M. J. Berridge. It is an intriguing idea that the 'molecular signatures' might reflect variable inositol phosphate production with Ins(1,3,4,5)P_4 being an important part of the pattern. This idea is supported by the observation that Ins(1,3,4,5)P_4 can induce oscillatory activity when injected into *Xenopus* oocytes (Parker & Miledi 1987). One interesting possibility is that Ins(1,3,4,5)P_4 might influence oscillatory activity by altering the lines of communication between intracellular calcium stores as has been suggested by Irvine *et al.* (this symposium).

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