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MECHANISMS OF INTRACELLULAR CALCIUM RELEASE DURING HORMONE AND NEUROTRANSMITTER ACTION INVESTIGATED WITH FLASH PHOTOLYSIS

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Summary

To understand the complex time course of cytosolic Ca^{2+} signalling evoked by hormones and neurotransmitters, it is necessary to know the kinetics of steps in the second-messenger cascade, particularly cooperative and inhibitory interactions between components that might give rise to periodic fluctuations. In the case of inositol trisphosphate (InsP_3)-evoked Ca^{2+} release, fast perfusion studies with subcellular fractions or permeabilised cells can be made if sufficient homogeneous tissue is available. Single-cell studies can be made by combining whole-cell patch-clamp techniques and microspectrofluorimetry with flash photolytic release of InsP_3 to give quantitative, time-resolved data of Ca^{2+} release from stores. A technical description is given here of flash photolysis of caged InsP_3, and the results of fast perfusion and flash photolytic experiments are reviewed. Studies of kinetics of Ca^{2+} release have shown that the InsP_3 receptor/channel is regulated first by positive and then by negative feedback by free cytosolic Ca^{2+} concentration, producing a pulse of Ca^{2+} release having properties that may be important in the spatial propagation of Ca^{2+} signals within and between cells. The properties of InsP_3-evoked Ca^{2+} release in single cells differ between peripheral tissues, such as the liver, and Purkinje neurones of the cerebellum. Purkinje neurones need 20–50 times higher InsP_3 concentrations and release Ca^{2+} to change the free cytosolic concentration 30 times faster and to higher peak concentrations than in liver. The InsP_3 receptors in the two cell types appear to differ in apparent affinity, and the greater Ca^{2+} efflux from stores in Purkinje cells is probably due to a high receptor density.

Introduction

There are many tissues in which hormone or neurotransmitter action on certain G-protein-coupled surface receptors results in the release of Ca^{2+} from intracellular stores by InsP_3. The rise in free [Ca^{2+}] activates physiological processes, such as ion channel

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opening, contraction or secretion, or metabolic processes, such as glycogenolysis. This process has been best described in non-excitable tissues, such as liver or exocrine cells. In general, it has a characteristic time course, showing delays that can be many seconds after receptor activation and periodic fluctuations of the intracellular Ca^{2+} concentration when hormonal stimulation is maintained. The complex kinetics suggests the presence of both regenerative and inhibitory interactions in the second-messenger reactions that regulate the rise and fall of free Ca^{2+} concentration. Kinetic methods, including flash photolysis and fast mixing, have been used to identify important interactions in the sequence of reactions. These will be reviewed here.

The existence of a similar system in neuronal tissue is supported by the presence of G-protein-coupled 'metabotropic' receptors, the presence of high densities of InsP_3 receptors and the demonstration of polyphosphoinositide metabolism and Ca^{2+} mobilisation in tissue derived from central nervous system (CNS). However, kinetic experiments on uniform neuronal cell populations or single cells are more difficult. As a result, the physiological role, means of activation and properties of InsP_3-evoked Ca^{2+} signalling in neurones are unclear. A comparison will be made here between data from neuronal and non-neuronal tissue.

Flash photolysis of photolabile 'caged' precursors is a technique now quite widely used in cell physiology to make rapid, precise changes of substrate or ligand concentration at sites within the cytosol or at cell surface receptors. The information obtained from experiments of this kind can be simply qualitative, for instance gaining evidence of the participation of a ligand such as calcium ions in a cellular process, or can be more quantitative, providing data on mechanism such as equilibrium constants and reaction rates. In the latter case, the time course of the re-equilibration following a concentration jump can give the rates of biochemical steps – ligand binding, conformation changes or ion translocation rates – that constitute the physiological response and can show the site of regenerative or inhibitory steps in the overall process. The technique is most usefully applied at sites within unstirred regions of the extracellular or intracellular space only slowly accessible by diffusion, where perfusion methods would be too slow to study kinetics.

Diffusional delays distort both the time course and steady-state concentration of physiological ligands applied by conventional perfusion methods, because of metabolism, uptake and binding. With flash photolysis, a physiologically inert, photolabile 'caged' derivative is allowed to reach diffusional equilibrium before photolysis by a brief light pulse releases a known concentration of free ligand at the site of action. Diffusional delays and metabolic breakdown are minimised and the rate of application and concentration of the ligand are determined by the speed and efficiency of photolysis.

The problem can be illustrated by comparing the action of intracellular InsP_3 when perfused from a whole-cell patch pipette with that seen with flash photolysis of caged InsP_3. Diffusional equilibration of small ions from the pipette is known to take tens of seconds (Fenwick *et al.* 1982) and for larger molecules may require many minutes (Pusch and Neher, 1988). The time course of loading the Ca^{2+} dye Fura-2 into a parotid gland acinar cell by diffusion from a patch pipette is shown in Fig. 1A by the rise of the fluorescence traces at 350 and 390nm excitation. Even in these small cells, several

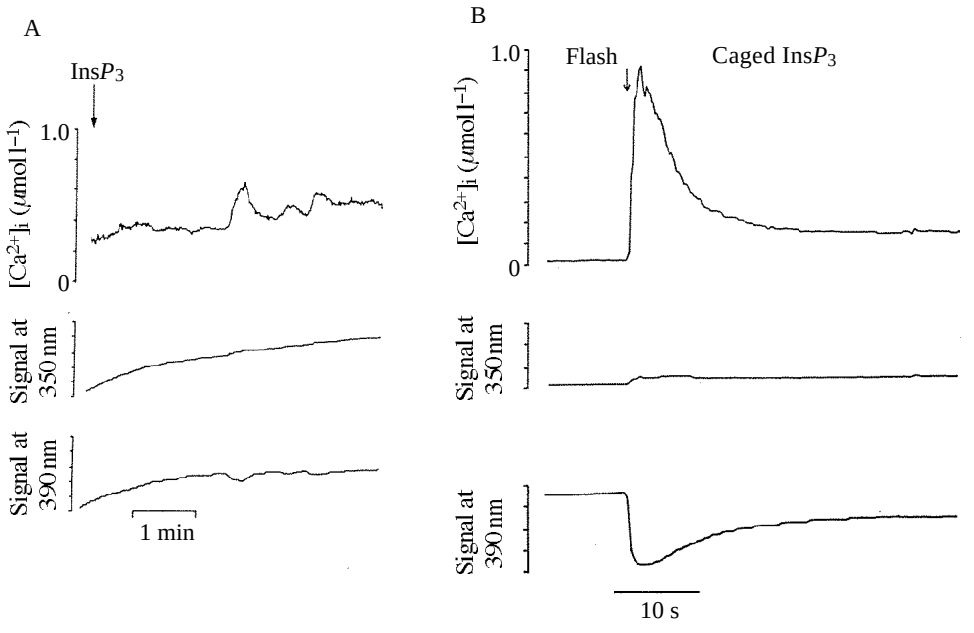

Fig. 1. InsP_3-evoked Ca^{2+} release in single parotid acinar cell – a comparison of pipette loading InsP_3 and flash photolysis of caged InsP_3 (see Gray *et al.* 1988). (A) The fluorescence signals (long-pass filter 490nm) produced by excitation at 350nm and 390nm in the lower two traces and the free Ca^{2+} concentration calculated from the ratio of fluorescence in the upper trace. On rupturing the membrane under a patch pipette containing 200 μmol l^{-1} Fura-2 and 10 μmol l^{-1} InsP_3 at the time indicated by the arrow, the rise of fluorescence signals excited at 350 and 390nm shows the time course of dye diffusion into the cell from the pipette. The free $[Ca^{2+}]$ calculated from the ratio of fluorescence is independent of dye concentration. In this cell, a fluctuating small rise of $[Ca^{2+}]$ was evoked by the presence of InsP_3, although in most cells tested in this way no obvious change of $[Ca^{2+}]$ occurred that could be attributed to InsP_3 in the pipette. (B) Recordings of the same variables from a cell loaded for several minutes with 200 μmol l^{-1} Fura-2 and 1 μmol l^{-1} caged InsP_3. At the time indicated by the arrow, a 1ms pulse of near-ultraviolet light was applied to release 0.5 μmol l^{-1} InsP_3, resulting in a fast Ca^{2+} transient.

minutes were required to reach a steady level of fluorescence, indicating equilibration of dye concentration between pipette and cytosol, after establishing access to the cytosol (at the beginning of the trace). Applying 10 μmol l^{-1} InsP_3 simultaneously with Fura-2 produced an erratic and small increase of free $[Ca^{2+}]$, as monitored by the ratio of Fura-2 fluorescence signals and shown in the top trace. Loading 1 μmol l^{-1} 'caged' InsP_3 with the Fura-2 for 3min (Fig. 1B) followed by release of 0.5 μmol l^{-1} InsP_3 in the cytosol by a 1ms flash, given at the time indicated by the arrow, produced an immediate fast rise of free $[Ca^{2+}]$, followed by a slower decline to baseline levels. The time course of this response is more readily interpreted in terms of the kinetics of InsP_3-evoked Ca^{2+} release from stores and subsequent Ca^{2+} metabolism than that of Fig. 1A. In this system, InsP_3 released by photolysis produced Ca^{2+} release at concentrations as low as 200nmol l^{-1},

compared with concentrations of 10 μmol l^{-1} required with perfusion from the pipette (Evans and Marty, 1986; Capiod *et al.* 1987; Wakui *et al.* 1989). This suggests that metabolism of InsP_3 reduces considerably the concentration reaching the stores from the patch pipette. Similar considerations apply also to the second messengers inositol tetrakisphosphate (InsP_4), cyclic AMP and cyclic GMP.

Although the principle of flash photolysis is straightforward, there are a number of technical problems which will be reviewed briefly with reference to caged InsP_3.

Chemistry of photoreactive groups and photolysis mechanisms

Given that the photolytic yield and rate of release of the ligand are good enough to be useful, it is essential to know the extent to which the caged compound itself and by-products formed during photolytic decay interfere with the physiological system under investigation.

Caged compounds developed as physiological probes since 1978 have 2-nitrobenzyl or 2-nitrophenyl moieties (or close derivatives) as photolabile protecting groups attached covalently to the ligand (see Kaplan *et al.* 1978; McCray and Trentham, 1989). The reduction in activity of the caged ligand is presumably due to lowered binding affinity, produced either by changing the charge near an important binding group or by some steric effect impeding access to the binding site. Because of the remaining structural similarity, the caged ligand may act as a weak agonist or antagonist at the receptors for the free ligand.

The photochemical properties of caged compounds, particularly the mechanism of photolysis as exemplified by caged ATP, have been reviewed in detail recently by McCray and Trentham (1989). The photolysis reaction proceeds essentially in two steps, absorption of a photon of sufficient energy to generate an activated intermediate (or intermediates), followed by one or more slower dark reactions in which the intermediates decay to release products. On absorbing a photon, the activated intermediate is formed very quickly, on a nanosecond time scale. However, the decay to release products during the dark part of the reaction is relatively slow, in some cases in microseconds, but with some reactions very much slower, for example in 5–10ms with caged InsP_3. Whether these compounds are useful depends on the rates of the physiological process being investigated. The efficiency and rate of photolysis depend on the nature of the bond cleaved and the substituent groups on adjacent atoms. Changing these substituents can be used to eliminate residual biological activity and to modify the rate and efficiency of photolysis. Descriptions of the effects of chemical substitutions can be found in reviews by Wootton and Trentham (1989), McCray and Trentham (1989), Nerbonne (1986) and Lester and Nerbonne (1982). The importance of the experimental conditions in photolysis is discussed by McCray and Trentham (1989).

Synthesis and properties of caged InsP_3

The synthesis and purification of 2-nitrobenzyl derivatives, the effects of substituents on their properties and problems such as the presence of stereoisomers with differing biological activity are discussed in a methodological review by Walker (1991). In particular, he gives recipes for the synthesis and purification of several compounds,

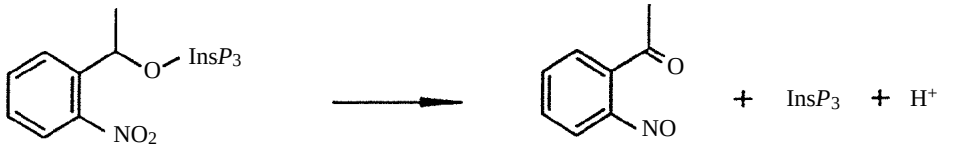

Fig. 2. Schematic photolysis reaction for caged InsP_3. The reaction releases a proton and a by-product, 2-nitrosoacetophenone.

including caged InsP_3. The approach used to 'cage' ATP, with a 2-nitrophenylethyl ester to protect the terminal phosphate (Walker *et al.* 1988), was applied by Walker *et al.* (1989) to generate the three photolabile isomeric 2-nitrophenylethyl phosphate esters of InsP_3, substituted on the 1, 4 or 5 phosphates. The isomers were separated by anion-exchange HPLC, characterised with nuclear magnetic resonance and their photochemical and physiological properties evaluated. The rate of the dark reaction was determined from the decay of aci-nitro and the formation of nitrosoacetophenone. The scheme of the photolysis reaction is shown in Fig. 2. At physiological ionic strength and pH (0.2mol l^{-1} and pH7.1), the P4 and P5 isomers photolysed with rates of 225 s^{-1} and 280 s^{-1}, respectively (half-times close to 3ms), and with quantum yields Q_p of 0.65, similar to the value of 0.63 determined for caged ATP in the same conditions. The interactions of the isomers with the InsP_3-evoked Ca^{2+} release process in smooth muscle and with enzymes involved with InsP_3 metabolism were tested. At 10 μmol l^{-1}, the P1 isomer produced maximal tension in permeabilised smooth muscle, indicating Ca^{2+} release from stores. The P4 and P5 caged isomers were inactive at 50 μmol l^{-1} in releasing Ca^{2+} and were ineffective in blocking Ca^{2+} release evoked by InsP_3. Similar results have been obtained for Ca^{2+} release from stores in isolated guinea pig hepatocytes, in which the caged InsP_3 was applied from whole-cell patch-clamp pipettes. The potency of InsP_3 released photochemically with low levels of photolysis, so that a high caged InsP_3 concentration remained, is similar to that obtained with high levels of photolysis and a low cage concentration. The potency in single hepatocytes with whole-cell perfusion and flash photolysis is similar to that obtained by applying InsP_3 to permeabilised hepatocyte suspensions (Burgess *et al.* 1984). A similar concentration range, 0.2–10 μmol l^{-1}, is required for activation of Ca^{2+} release in both cases, suggesting that inhibition by the unphotolysed cage was small.

The three isomers were tested by Walker *et al.* (1989) as substrates and inhibitors of 5-phosphatase and 3-kinase. The P1 isomer was hydrolysed to caged Ins(1,4)P_2 by phosphatase and the P4 and P5 isomers were neither substrates nor inhibitors of this enzyme. The P5 isomer inhibited 3-kinase, which phosphorylates InsP_3 to InsP_4, by about twofold at 10 μmol l^{-1}, and the P4 isomer was inactive on this enzyme. Neither was phosphorylated to InsP_4. Thus, the commercially available mixture of P4 and P5 isomers may inhibit 3-kinase. Further, the P1 caged Ins(1,4)P_2 has been used as a control for the effects of photolysis, release of the inactive bisphosphate in smooth muscle and liver showing no effect on the hormonal response of the system (Walker *et al.* 1987; Ogden *et al.* 1990).

Toxicity of photoproducts

The aromatic nitroso ketones released as by-products are reactive particularly with –SH groups of proteins and are potentially toxic. It is necessary to make control experiments in which the by-products of photolysis are released into the system without the ligand of primary interest. In some instances, custom-made analogues such as caged InsP_2 may be available, but compounds such as caged ATP or caged phosphate may be suitable. As a guide, it has been found that 50 μmol l^{-1} or less of nitrosoacetophenone released into cells appears to have no effect, but higher concentrations approaching 1 mmol l^{-1} are toxic. The toxicity can be alleviated by including thiols – mercaptoethanol, dithiothreitol (DTT) or glutathione – in the solution to react with nitrosoketones released.

Light sources

The optimal wavelengths for photolysis of nitrophenyl derivatives are in the near ultraviolet, 300–360nm, longer than those that damage proteins or nucleic acids and in a region where cells are relatively transparent. Light flashes in the near ultraviolet with sufficient intensity for flash photolysis can be produced by lasers, flashlamps or shuttered arc lamps. The energy density required is about 50–500mJcm^{-2} at 300–360nm. The pulse should be short enough, 1ms or less, not to limit the rate of photolysis. Details of light sources and their utilisation have been reviewed by McCray and Trentham (1989).

Xenon arc flashlamps produce pulses of 0.5–1ms duration and energies up to 150mJ at 300–350nm (Schott UG11 or Hoya U 350 filter). Details of the design and performance of a lamp available commercially are given by Rapp and Guth (1988). The lamp arc can be focused directly to an image of about 4mm in diameter at a focal length of 3cm.

Calibration of photolysis

The quantum yield, Q_p, of a caged molecule is the proportion of molecules which, having absorbed a photon of light, then break down to release products. To determine Q_p requires measurement of the light absorbed as well as the extent of photolysis, and is useful for photochemical comparison. For routine use of flash photolysis, it is important to know simply the percentage conversion of cage to product under experimental conditions. How easy this is to determine depends on the complexity of the experimental arrangement. Two approaches have been used. In the most straightforward case, where the light is focused to a large spot from above, a 5–10 μl drop of solution can be exposed to a flash and the changes estimated chemically, for instance by HPLC for caged phosphates (Ogden *et al.* 1990; Walker *et al.* 1988). If photolysis is carried out in combination with microspectrofluorimetry, protons released stoichiometrically during photolysis can be measured directly with a pH-sensitive dye (Walker *et al.* 1988; Khodakhah and Ogden, 1993). As a guide to the extent of photolysis, caged ATP and caged InsP_3 (the 2-nitrophenylethyl esters) are photolysed by about 50% with a 500 mJcm^{-2} pulse of 300–360 nm light from a xenon arc flashlamp applied to a 10 μl drop.

Simultaneous flash photolysis and Ca^{2+} measurement

Experiments in which free [Ca^{2+}] is changed by flash photolysis are designed either to study the effects of [Ca^{2+}] on a physiological variable, such as contractile tension, membrane conductance or secretion, or to study the mobilisation and sequestration of free Ca^{2+} by the cell. In some instances, the Ca^{2+}-sensitivity of the tension or conductance may be known and provides a measure of the free [Ca^{2+}]. However, it is often necessary to make independent measurements of free [Ca^{2+}] with an optical technique, such as Ca^{2+}-binding dyes, and the use of both simultaneously may give greatly enhanced information about the system. The light pulse can produce direct optical interference in two ways, from saturation of the photomultipliers (or other photosensitive element) during the flash and by a slowly decaying visible phosphorescence induced by the flash in glass components, such as coverslips and objective lenses. The direct effect of the flash can be removed by a fast shutter or by electronically gating the dynodes of the photomultiplier during the flash. The phosphorescence is less easily dealt with and requires a near-ultraviolet filter between the preparation and the microscope optics and the use of quartz components for the chamber (Ogden *et al.* 1991).

A second form of interference arises from continuous photolysis of caged compounds by excitation light during [Ca^{2+}] measurement with fluorescent dyes. The most widely used Ca^{2+} probes are the fluorescent dyes Indo-1 and Fura-2, which are excited at near-ultraviolet wavelengths and can be used without appreciable photolysis only with weak excitation intensity. Indo-1 and Fura-2 have the advantage of spectral shifts on binding Ca^{2+}, permitting [Ca^{2+}] measurements independently of dye concentration and geometric factors by the use of the ratio of fluorescence at two wavelengths. However, a weak excitation intensity means, in practice, that time resolution is limited by the need to integrate the emitted light over 100ms or more to achieve an acceptable signal/noise ratio (as in Fig. 1), and the good time resolution possible with flash photolysis is lost. There are three commonly used dyes excited at longer wavelengths, Fluo-3 or Calcium Green at fluoroscein (480nm) and Rhod-2 at rhodamine (520nm) wavelengths, which can be used at high excitation intensity.

Time-resolved measurements of free [Ca^{2+}] require a detection system that is linear over the whole [Ca^{2+}] range of even local transients, which may reach 100 $\mu mol\,l^{-1}$ adjacent to Ca^{2+} channels for short times (e.g. Chad and Eckert, 1984). Distortions arise when the dye is locally approaching saturation and does not contribute proportionately to the spatial average. The fluorescent dyes commonly used (Fura-2, Indo-1, Fluo-3) have equilibrium dissociation constants near the resting [Ca^{2+}] and therefore give a good signal at low [Ca^{2+}] levels. However, their response characteristics approach saturation at relatively low [Ca^{2+}] and, as a result, transient detection and time resolution is poor. A second problem is that they are extensively bound to Ca^{2+} even at resting levels, buffering Ca^{2+} to a greater extent than the native buffers. As a result, they distort spatial aspects of Ca^{2+} signalling that arise from Ca^{2+} binding to fixed cytosolic buffers by acting as a mobile Ca^{2+} carrier and speeding Ca^{2+} diffusion. A fluorescent dye for which the problems of distortion and buffering are minimal is MagFura-2 (Furaptra) which, when

excited at 420nm, is quenched by Ca^{2+} to almost zero fluorescence, like Fura-2, and so is easily calibrated, but has the advantage of a relatively low Ca^{2+} affinity, around 50 $\mu mol\,l^{-1}$ (see Konishi *et al.* 1991).

Origin of the delay in hormone responses in guinea pig hepatocytes and other peripheral tissues

Calcium-mobilising hormones bind to cell surface receptors and set in motion a sequence of reactions comprising activation of a pertussis-insensitive G-protein, phospholipase C, and generation of InsP_3, which releases Ca^{2+} from a component of the endoplasmic reticulum. Hepatocytes are a good 'model' system for single-cell studies, partly because they have provided much information about the biochemistry of second-messenger action and also because Ca^{2+} release from stores is the only means of Ca^{2+} signalling. In hepatocytes, the Ca^{2+} has a primary function of activating phosphorylase kinase *via* calmodulin, producing glycogenolysis and glucose release. A secondary action (in guinea pig and rabbit) is to activate K^+-permeable and Cl^--permeable ion channels in the surface membrane, resulting in rapid KCl loss from the cell (Burgess *et al.* 1981). The potassium ion conductance is an apamin-sensitive Ca^{2+}-activated channel that can serve as a monitor for intracellular free Ca^{2+} concentration in the range 0.3–2.0 $\mu mol\,l^{-1}$ (Capiod and Ogden, 1989*b*). The whole-cell patch-clamp technique permits both the perfusion of the cell with caged InsP_3 and the measurement of the K^+ conductance to monitor Ca^{2+} release. Single-cell microelectrode or patch-clamp recordings have shown that, when noradrenaline acting through α-adrenoceptors is applied rapidly at high concentration to single hepatocytes, the K^+ conductance increase follows with a variable delay of minimum duration 2s and up to 90s or more (Field and Jenkinson, 1987; Capiod and Ogden, 1989*a*; Ogden *et al.* 1990). Flash photolysis of caged InsP_3 has been used in a qualitative fashion to indicate the point in the sequence of reactions where the delay may occur. A number of studies have compared the time courses of responses to Ca^{2+}-mobilising hormones and to InsP_3 released by photolysis from caged InsP_3. Fig. 3 shows, for comparison, in the same hepatocyte the Ca^{2+}-activated K^+ conductance following with 8s delay after application of noradrenaline (upper trace) and flash photolytic release of 0.5 $\mu mol\,l^{-1}$ InsP_3, with a delay of 0.3s, in the lower trace. A pulse of InsP_3 at moderate concentration, 0.5–1 $\mu mol\,l^{-1}$, initiates a rise of $[Ca^{2+}]$in 100–200ms in most tissues studied, compared with delays in the onset of the effect of Ca^{2+}-mobilising hormones of 1s or longer (smooth muscle: Walker *et al.* 1987; hepatocytes: Capiod *et al.* 1988; *Xenopus* oocytes: Miledi and Parker, 1989; vascular endothelia: Carter and Ogden, 1992). The delays in Ca^{2+} mobilisation by hormones are therefore at a step prior to, or during, the generation of InsP_3. The delays with hormone vary from one cell type to another, and in some cases have been shown to be shorter at high than at low hormone concentration, even though the ensuing response has a rise time and amplitude that are much the same. It should also be mentioned that responses of this kind have, in some cases, been shown to propagate across and between cells, and, in the case of primary cultures of hepatocytes, that the response originates at the same point each time in a particular cell (Rooney *et al.* 1990). In exocrine acinar cells, there is

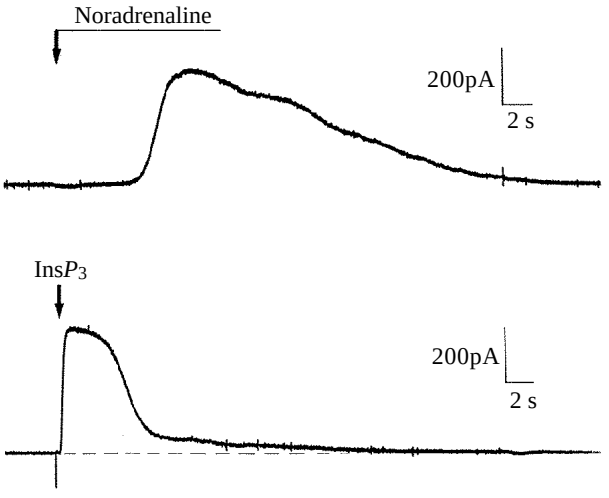

Fig. 3. Comparison of the time course of activation of outward K^+ current in a single guinea pig hepatocyte under whole-cell patch-clamp with 10 μmol l^{-1} external noradenaline (upper trace, delay 8s) and later with photolytic release of 0.5 μmol l^{-1} Ins*P*$_3$ from 1 μmol l^{-1} caged Ins*P*$_3$ (lower trace, delay 300ms). Membrane potential 0mV. Cl^--free solutions.

evidence that the site of Ins*P*$_3$ production is at the opposite pole of the cell from the region of Ca^{2+} release (Kasai and Augustine, 1990; see Marty, 1991). Thus, the spatial arrangement of the different elements of the signalling pathway and diffusion of intermediates may contribute to the delay.

It is possible that the delay is due to the time taken to accumulate sufficient local concentration of a mediator, Ins*P*$_3$ or Ca^{2+} or both, to set off a regenerative reaction sequence (Marty *et al.* 1989; Parker and Miledi, 1989; Crossley *et al.* 1991). This idea could be tested by release of Ins*P*$_3$ by photolysis during the delay in hormone action to see whether the response can be triggered. Attempts to do this have shown no 'triggering' action of Ins*P*$_3$ in hepatocytes (T. Capiod and D. Ogden, unpublished observations) but it must be remembered that Ins*P*$_3$ is released by this means throughout the cell and, if a spatial element is important, experiments with photolysis restricted to small areas of the cell would be needed.

The GDP/GTP exchange reaction and dissociation of subunits of the G-proteins coupling receptors to phospholipase C (PLC) are also thought to be slow (rate 0.03min^{-1}, Pang and Sternweiss, 1990) but catalysed by the activation of hormone receptors. Caged GTP-γ-S is potentially a useful agent to activate G-protein independently of hormonal stimulation, as has been done with G-protein interactions with Ca^{2+} channels (Dolphin *et al*. 1988). Experiments with a caged 2-nitrophenylethyl ester of GTP-γ-S show first a delay of several seconds in activation of Ca^{2+} release and, second, that this delay is not reduced by application of noradrenaline, which would accelerate GTP/GDP exchange. On the basis of this observation, the delay would appear to be after G-protein activation and before Ins*P*$_3$ action on stores, i.e. during production of Ins*P*$_3$ by PLC.

Mechanism of InsP_3-evoked Ca^{2+} release

Flash photolysis of caged InsP_3 introduced into single hepatocytes or other cells during whole-cell patch-clamp can give information on the concentration-dependence, kinetics and regulatory interactions during InsP_3 action that would otherwise only be obtained with fast mixing experiments in permeabilised cells or subcellular fractions. Microinjection of InsP_3 itself or caged InsP_3 followed by photolysis does not give information on the concentration applied because of the difficulty of estimating the quantities injected and the cell volume.

The simplest hypothesis of how the Ca^{2+} release might occur is gating by cytosolic InsP_3 of an ion channel in the store-cytoplasm membrane. In this case, the rate of Ca^{2+} flux into the cytosol will be proportional to the open probability of the channels. The *rate of change* of Ca^{2+} concentration in the cytosol, $d[Ca^{2+}]/dt$, is proportional to the net flux of Ca^{2+} and provides the best measure of open probability of the InsP_3 channels within each cell. However, it may be expected to vary from cell to cell, not least because of geometric factors such as the ratio of InsP_3 receptor density to cytosolic volume.

Initial delays

Experiments with caged InsP_3 and the Ca^{2+} dye Fluo-3 in permeabilised smooth muscle (Somlyo *et al.* 1992), in hepatocytes (Ogden *et al.* 1990, 1991) and in vascular endothelial cells (Carter and Ogden, 1992; see Fig. 4) have shown a delay in the activation of Ca^{2+} efflux of several hundred milliseconds at low InsP_3 concentration (0.1–0.5 $\mu mol\,l^{-1}$), which becomes much shorter, less than 20ms, at high concentration (10–100 $\mu mol\,l^{-1}$). The short delay at high concentration is not much longer than the time required for photolysis and suggests direct activation of the Ca^{2+} efflux channel by InsP_3. The concentration range of InsP_3 activation of receptors is given by the range over which the delays were observed to become shorter, from about 0.2 to 5 $\mu mol\,l^{-1}$. These data from photolytic release of InsP_3 in single hepatocytes and other tissues agree with data from rapid mixing experiments with permeabilised hepatocytes (Champeil *et al.* 1989). In permeabilised rat basophilic leukaemia cells, a similar reduction of the delay at high concentration was found with rapid mixing experiments, but in this case over a lower concentration range and with a longer delay (65ms) at high concentration (Meyer *et al.* 1990). In all tissues tested with both photolytic and fast mixing experiments, the speed of activation at high concentration is consistent with a directly gated channel, being much faster, for instance, than rates of activation of G-protein-coupled channels in the surface membrane (Hartzell *et al.* 1991).

The delay at low InsP_3 concentrations is not predicted for simple models of ligand gating, and two general explanations have been proposed. The first is a high (n=4) cooperativity in InsP_3 binding seen in permeabilised rat basophilic leukaemia cells (Meyer *et al.* 1990). In permeabilised smooth muscle, the delay is consistent with n=1 or n=2 (Somlyo *et al.* 1992). The second explanation is a cooperative or co-agonist effect between InsP_3 and Ca^{2+} at low free Ca^{2+} concentrations. This idea is supported by observations of the Ca^{2+}-dependence of InsP_3 action in smooth muscle (Iino, 1990; Iino and Endo, 1992), brain microsomes (Finch *et al.* 1991) and reconstituted InsP_3 receptor

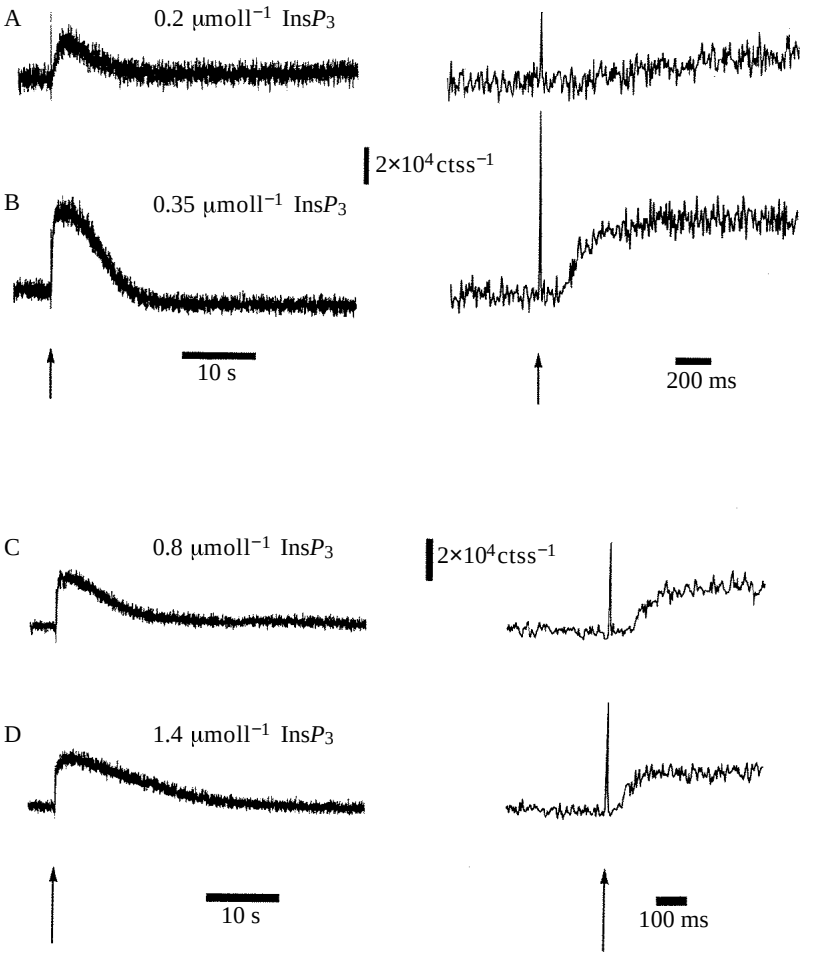

Fig. 4. Fluo-3 fluorescence detection of Ca^{2+} release from stores in porcine cultured aortic endothelial cells with whole-cell patch-clamp to show the delay after photolysis of caged InsP_3 and the rate of rise of fluorescence. (A,B) Release of 0.2 and 0.35 μmol l^{-1} InsP_3 at the time indicated by the arrow; sequential records from same cell. (C,D) Release of 0.8 and 1.4 μmol l^{-1} InsP_3 in the same cell. Membrane potential −44mV. The right-hand traces are expanded regions of the left-hand traces. From Carter and Ogden (1992).

(Bezprozvanny *et al.* 1991). Although a co-agonist action of Ca^{2+} and InsP_3 can explain the delay quite well, direct experimental tests are difficult. The experiments of Iino and Endo (1992) show that, at strongly buffered [Ca^{2+}] of 300nmol l^{-1}, the delay to photoreleased InsP_3 is removed and the sigmoid initial rise of [Ca^{2+}] is absent and is replaced by a single exponential rise, consistent with loss of the cooperative action of Ca^{2+}.

To summarise, the presence of a delay suggests cooperativity in InsP_3 action, as multiple InsP_3 binding and/or a co-agonist action of Ca^{2+}. The very short delays at high InsP_3 concentration are consistent with a direct ligand-gated mode of channel activation.

The concentration range of InsP_3 action, from longest to shortest delay, is 0.1–5 μmol l^{-1} in the peripheral tissues studied.

Rate of rise of $[Ca^{2+}]$

To study channel activation as a function of InsP_3 concentration or on changing other variables, the maximal rates of rise of free $[Ca^{2+}]$ should be measured in conditions where other Ca^{2+} fluxes into or out from the cytosol are small. In whole-cell recording from guinea pig liver cells, the Ca^{2+}-activated K^+ conductance of the surface membrane provides a good measure of the submembrane free $[Ca^{2+}]$, with a steep Ca^{2+}-dependence in the range 0.3–2 μmol l^{-1} Ca^{2+}. The rate of rise of the conductance increased with InsP_3 concentration between 0.5 and 10 μmol l^{-1} when compared within the same cell (Ogden *et al.* 1990). These results have been confirmed with the Ca^{2+} indicator dye Fluo-3 (Ogden *et al.* 1991). In endothelial cells, an increase in the rate of rise of the Fluo-3 signal was found in the range 0.2–5 μmol l^{-1} InsP_3 (Carter and Ogden, 1992). A dependence of the rate of change of the Fluo-3 fluorescence on InsP_3 concentration over a similar range has been found in permeabilised smooth muscle (Somlyo *et al.* 1992).

It is of interest to know the slope of the relationship between $d[Ca^{2+}]/dt$ and log [InsP_3]. The Hill coefficient, *n*, in principle, gives a *lower-limit* estimate of the number of InsP_3 molecules binding to produce activation. In single intact cells, data with Fluo-3 have proved quantitatively unreliable because of dye binding to proteins and because the high affinity for Ca^{2+} will underestimate the rate of change of free $[Ca^{2+}]$, especially if there is spatial localisation of the $[Ca^{2+}]$ rise to regions near the release sites. Data on the efflux/InsP_3 concentration relationship have come mainly from fast mixing experiments. A high potency of InsP_3, active at 4nmol l^{-1}, and a large Hill slope of 3–4 at low concentrations, suggesting high cooperativity, were found in permeabilised rat basophilic leukaemia cells (Meyer *et al.* 1988). In permeabilised liver cells (Champeil *et al.* 1989), the maximum rates of rise of $[Ca^{2+}]$ measured with Quin-2 increased with concentration in the range 0.1–10 μmol l^{-1} with a Hill coefficient of 1.6, indicating a small degree of cooperativity. The cooperativity seen in hepatocyte suspensions was reduced at high (10 μmol l^{-1}) $[Ca^{2+}]$ (Combettes *et al.* 1993). In an InsP_3-sensitive microsomal preparation from the CNS, the concentration range measured by $^{45}Ca^{2+}$ efflux was 30nmol l^{-1} to 10 μmol l^{-1} with a Hill coefficient of 1.0, under conditions of constant buffered free Ca^{2+} concentration (Finch *et al.* 1991). Similar results were found with single-channel recording from InsP_3 receptors isolated from cerebellum and incorporated into bilayers (Bezprozvanny *et al.* 1991). The maximum InsP_3-evoked open probability, of up to 15% at 2 μmol l^{-1}, occurred at a free $[Ca^{2+}]$ of 0.3 μmol l^{-1} and declined at higher $[Ca^{2+}]$. In smooth muscle, a Hill coefficient of 1–2 was reported by Somlyo *et al.* (1992) and of 2 by Iino and Endo (1992), both studies performed using flash photolysis of caged InsP_3 and with Fluo-3 as the Ca^{2+} indicator in permeabilised strips.

The strong dependence of InsP_3 potency on the free $[Ca^{2+}]$ discussed above suggests that the discrepancies between different studies, particularly in cell suspensions, may result from local Ca^{2+} accumulation. The data of Iino and Endo (1992), which gave n=2, were carried out with strongly buffered Ca^{2+} in order to avoid the influence of variable

[Ca^{2+}] on the InsP_3 cooperativity estimated, and were measured at the foot of the InsP_3 concentration range. It would appear that the value of the Hill coefficient is between 1 and 2, suggesting that at least two InsP_3 molecules need to bind to produce channel opening, but some degree of uncertainty remains about the value and significance of this number. An analogy may be made with studies of the nicotinic receptor, where good measurements of the Hill coefficient were difficult to make, even in this more accessible preparation, and in fact the firmest evidence for two bindings in activation was from the biochemical demonstration of two binding sites in each receptor oligomer.

Threshold and quantal effects

There are a number of reports indicating that regenerative phenomena occur during InsP_3 action. Parker and Ivorra (1990*a*, 1993) found that increasing InsP_3 concentration by incremental photolysis of caged InsP_3 showed a 'threshold' concentration for activation of Ca^{2+}-dependent Cl^- conductance and for the peak [Ca^{2+}] estimated by dye in *Xenopus* oocytes. In these experiments, [Ca^{2+}] was monitored with Rhod-2 fluorescence confocally within the oocyte from a spot of a few micrometres in diameter, to avoid spatial averaging of the signal from a large number of Ca^{2+} storage elements that may have had different sensitivities. InsP_3 concentrations were generated by increasing the duration or transmission of a steady ultraviolet irradiation of caged InsP_3 microinjected into the oocyte, but the absolute concentrations were not known. Above threshold, the amplitude of the Ca^{2+} fluorescence was constant with increasing InsP_3 concentration, indicating a constant 'quantal' Ca^{2+} release. The rate of rise of the Ca^{2+} fluorescence signal was greater as InsP_3 concentration was increased, suggesting that the rate of Ca^{2+} efflux from stores, and therefore receptor activation, increased with concentration even though the peak did not. The data therefore suggest that some process quickly terminated Ca^{2+} efflux so that the final concentration of Ca^{2+} did not increase much at high concentration (see discussion below). The advantage of Parker and Ivorra's approach is the confocal localisation of the observations to a small region of the oocyte's Ca^{2+} store, but observations of conserved 'quantal' Ca^{2+} release have been reported in other cells in response to thio-InsP_3 or hormonal stimulation (Muallem *et al.* 1989; Taylor and Potter, 1990). The reasons for the threshold and 'quantal' behaviour are not known, but they may be linked with Ca^{2+} regulation of InsP_3 sensitivity.

Cooperative and inhibitory interactions of Ca^{2+} and InsP_3

The complex kinetic behaviour of the response of many cell types to Ca^{2+}-mobilising hormones indicates the presence in the second-messenger system of steps that are regenerative or highly cooperative, and also of inhibitory mechanisms that restore the system. Some mechanisms of this kind have been shown to occur in the InsP_3-evoked Ca^{2+} release step. First, it is well documented that, immediately following InsP_3-evoked Ca^{2+} release, the release mechanism becomes refractory to further InsP_3 action and that recovery of sensitivity has a time course of tens of seconds. Fig. 5 shows data from experiments in which consecutive twin pulses of InsP_3 were released photolytically in a

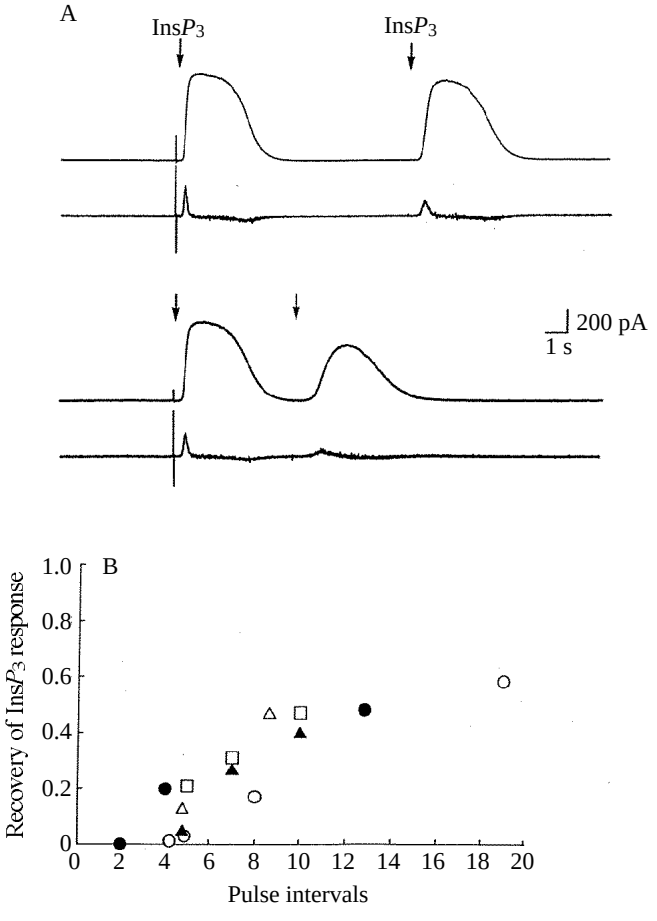

Fig. 5. Recovery of InsP_3 sensitivity in twin-pulse experiments in guinea pig hepatocytes. (A) Pulses of 1 μmol l^{-1} InsP_3 released at the arrows with an interval of 10s, a recovery period of 100s, then an interval of 5s, same cell. Top traces in each panel are Ca^{2+}-activated K^+ current; lower traces show the rate of rise of current, which measures $d[Ca^{2+}]/dt$. (B) Plot of the rate of rise of the second response relative to that of the first to show the time course of recovery of InsP_3 sensitivity. Different symbols show results from different hepatocytes. From Ogden *et al.* (1990).

hepatocyte. The sensitivity of the Ca^{2+} release mechanism to InsP_3, measured by the rate of rise of the Ca^{2+}-activated conductance, is suppressed to zero immediately after the first pulse and recovers with a half-time of 10–20s as the pulse interval is increased. It occurs in *Limulus* photoreceptors (Payne *et al.* 1988, 1990), guinea pig hepatocytes (Ogden *et al.* 1990), *Xenopus* oocytes (Parker and Ivorra, 1990*b*), brain synaptosomes (Finch *et al.* 1991), rat hepatocytes (Combettes *et al.* 1992, 1993) and in single-channel recordings from cerebellar InsP_3 receptors in bilayers (Bezprozvanny *et al.* 1991). It has been shown to be due to raised cytosolic $[Ca^{2+}]$ and is attributed to an action directly on the channel or with a closely associated protein. This mechanism terminates InsP_3-evoked Ca^{2+} release

in guinea pig hepatocytes (and most probably in other tissues). It has a rapid onset (Finch *et al.* 1991; Iino and Endo, 1992; Levitan *et al.* 1993) and may be important in the declining phase of hormonally induced Ca^{2+} spikes and in conserving stored Ca^{2+}. Estimates of the Ca^{2+} concentration needed to produce half-inhibition in the steady state are 0.5–1 $\mu mol\,l^{-1}$ in brain microsomes (Finch *et al.* 1991) and 3–15 $\mu mol\,l^{-1}$ in permeabilised hepatocytes (Combettes *et al.* 1993). The onset of inhibition has been reported to have a time constant of 580ms (brain microsomes, Finch *et al*. 1991). An immediate inhibition was reported by flash-released Ca^{2+} in smooth muscle (Iino and Endo, 1992) and complete inhibition within 200ms in *Limulus* photoreceptors (Levitan *et al.* 1993). The time course of recovery from inhibition is 10–50s. It is likely that the inhibition characterised by Ca^{2+} release measurements in cells has a counterpart in a Ca^{2+}-induced increase of InsP_3 binding affinity, coupled with a Ca^{2+}-induced desensitisation of the InsP_3 receptor, described in hepatocyte microsomes by Pietri *et al.* (1990).

An inhibitory effect of the decline in Ca^{2+} concentration within the store has been proposed as a regulatory mechanism (Irvine, 1990) but experimental tests do not support an important role for this mechanism (Combettes *et al*. 1992; Shuttleworth, 1992).

A positive interaction has been demonstrated between InsP_3 and cytosolic Ca^{2+} in preparations from CNS and smooth muscle. Finch *et al.* (1991) described a strong co-agonist action of Ca^{2+} and InsP_3 with an apparent equilibrium constant for Ca^{2+} of 0.66 $\mu mol\,l^{-1}$ and a Hill coefficient for InsP_3 action of 1 at constant $[Ca^{2+}]$. Bezprovzanny *et al.* (1991) described an increase in open probability of cerebellar InsP_3 channels at Ca^{2+} concentrations up to 0.5 $\mu mol\,l^{-1}$. In smooth muscle, Iino (1990) first described an increased rate of InsP_3-induced Ca^{2+} release at a free $[Ca^{2+}]$ up to 0.3 $\mu mol\,l^{-1}$ and showed subsequently that, if $[Ca^{2+}]$ was suddenly increased to about 0.3 $\mu mol\,l^{-1}$, the rate of InsP_3-induced efflux was immediately increased (Iino and Endo, 1992; see also Finch *et al*. 1991; Combette *et al.* 1993). Thus, InsP_3-evoked Ca^{2+} release has the elements – positive feedback by Ca^{2+} to increase Ca^{2+} efflux – that could produce a regenerative component to the response. There is, however, no direct evidence that this occurs in intact cells, particularly that Ca^{2+} applied in the presence of InsP_3 can initiate Ca^{2+} efflux from stores. This is a point that needs to be tested.

Role of Ca^{2+} feedback

The calcium ions which accumulate in the cytosol adjacent to sites of Ca^{2+} efflux would initially increase InsP_3-evoked release and, at later times, inhibit release by the mechanism described above, which comes into play at higher Ca^{2+} concentrations. The result would be a pulse of Ca^{2+} released into the cytosol. Given that Ca^{2+} is heavily buffered by immobile binding sites to an extent of 50–100 bound/free (see, for example, Neher and Augustine, 1992), Ca^{2+} diffusion will be considerably slowed from that in free solution by about the same proportion. A pulse of Ca^{2+} released at the store will diffuse slowly, on a time scale of 50–100ms over 1 μm. Therefore, conditions exist in which the Ca^{2+} concentration adjacent to the receptor could rise to high levels as Ca^{2+} permeates through the channel. Conditions like this are thought to exist in the cytoplasm adjacent to Ca^{2+} channels in the surface membrane. Calculations based on the dimensions of a

channel with adjacent buffering of Ca^{2+} indicate that a moderate Ca^{2+} flux, corresponding to a channel current of about 0.5pA, will quickly, in 1–2ms, produce a large Ca^{2+} concentration of 10 μmol l^{-1} or higher within 0.1 μm of the channel (see, for example, Chad and Eckert, 1984), sufficient in this case for inhibition of the InsP_3-evoked efflux (50% at 3–15 μmol l^{-1}, Combettes *et al.* 1993). The brief, local high pulse of Ca^{2+} concentration will diffuse into the adjacent cytosol, becoming diluted over distances of about 1 μm in 100ms. On reaching adjacent receptor channels, the Ca^{2+} will contribute to their activation by the InsP_3 present. It is easy to envisage this cycle occurring at each receptor/channel and producing a spatially propagated wave, possibly locally restricted if channel open time were relatively brief and if the time course were dominated by the diffusion of Ca^{2+}. Control of the cycle could be, in some circumstances, by local Ca^{2+} buffering and by the channel density.

Mechanism of periodic fluctuations of $[Ca^{2+}]$

Hormonal stimulation of non-excitable cells often results in repetitive fluctuations of $[Ca^{2+}]$, which may be periodic spikes every 20s to 4–5min, as shown for a single guinea pig hepatocyte with 10 μmol l^{-1} noradrenaline in Fig. 6B, or disorganised fluctuations about a plateau level, as shown for a different hepatocyte in Fig. 6A (for reviews, see Berridge, 1990; Jacob, 1990; Petersen and Wakui, 1990; Cobbold *et al.* 1991; Marty, 1991). Models for systems with periodic fluctuations in the concentration of reactants require one or more autocatalytic or cooperative steps, to provide the rise in concentration, and an inhibitory step, or exhaustion of a reactant, to account for the fall. Two approaches can be made experimentally with flash photolysis, one to isolate

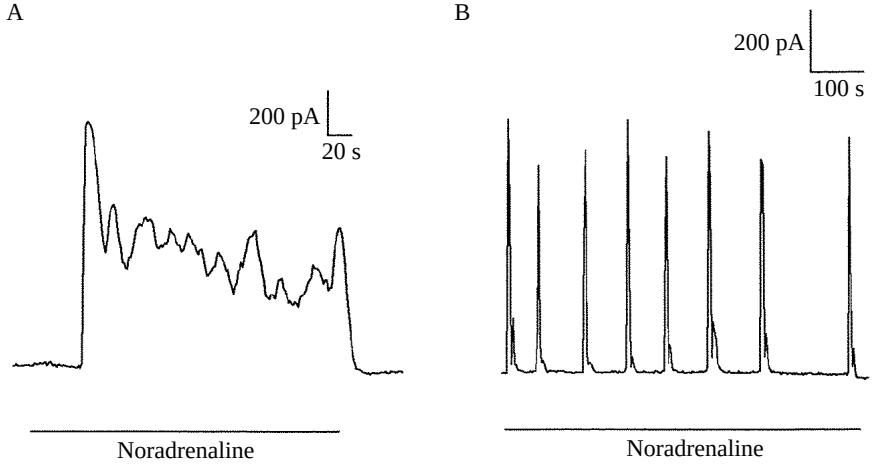

Fig. 6. Fluctuations of intracellular Ca^{2+} concentration in a guinea pig hepatocyte evoked by 10 μmol l^{-1} noradrenaline acting *via* α-adrenoceptors. Ca^{2+} concentration was monitored by Ca^{2+}-dependent K^+ conductance with whole-cell patch-clamp at 0mV, Cl^--free solutions. (A) Disorganised fluctuations of Ca^{2+} concentration; (B) regular spiking of Ca^{2+} concentration in a different cell.

regenerative and inhibitory steps under controlled, non-fluctuating conditions, the other to probe the sensitivity to intermediates such as InsP_3 during different parts of the cycle. The fluctuations of [Ca^{2+}] seen in the continuous presence of InsP_3 or its stable thio derivative in several cell types (Evans and Marty, 1986; Capiod *et al.* 1987; Wakui *et al.* 1989; Ogden *et al.* 1990; Payne and Potter, 1991) indicate that regenerative and inhibitory elements are present even in the simplest case of InsP_3 interaction with the Ca^{2+} store. Taking the first approach, the cooperative and inhibitory interactions of Ca^{2+} with InsP_3, described above for measurements under stable conditions, could form the basis of the simple type of InsP_3-evoked Ca^{2+} fluctuations with periods of 10–30s. However, it is clear that there are three or more different patterns of Ca^{2+} fluctuations or spiking that occur with hormonal stimulation and may involve additional steps, such as interactions between the receptor/G-protein/PLC and the diacylglycerol/protein kinase C/Ca^{2+} pathways (Cobbold *et al.* 1991; Llano and Marty, 1987; Sanchez-Bueno *et al.* 1990). In slowly spiking systems, the interval between spikes, of 1min or more, is too long to be accounted for by the Ca^{2+} inhibition of InsP_3 action discussed above, which has a half-time for recovery of 10–20s. Taking the second approach, photolytic release of InsP_3 during the interspike interval in slowly spiking REF52 fibroblastic cells produced a sharp rise in [Ca^{2+}], confirming in this case that the release process was not inhibited and that cytosolic InsP_3 was below the concentration required to initiate Ca^{2+} release (Harootunian *et al.* 1988). Although this would appear to be a good experimental approach, systematic studies with caged InsP_3 or with caged Ca^{2+} chelators to test the sensitivity to InsP_3 and [Ca^{2+}] during different parts of the cycle have yet to be made.

InsP_3-evoked Ca^{2+} release in neurones and glia

Glial cells in culture, both astrocytes and Schwann cells, respond to neurotransmitters with Ca^{2+} mobilisation from intracellular stores. The response may be evoked with excitatory amino acids, monoamines, peptides, ATP and cholinergic ligands. In more physiological conditions, propagated Ca^{2+} signalling has been observed among glia in organotypic cultures of hippocampal slices following stimulation of input pathways (Dani *et al.* 1992). Experiments with caged InsP_3 in cultured cerebellar astrocytes show Ca^{2+} mobilisation with characteristics similar to those seen in nonexcitable cells; the concentration range is 0.2–5 $\mu mol\,l^{-1}$ and responses are transient, lasting 5–20s, and show delays of up to 200ms at low concentrations (Khodakhah and Ogden, 1993). The role of Ca^{2+} mobilisation in glial physiology has not been demonstrated but, by analogy with other cells, it may play a part in the regulation of ion fluxes and volume, *via* Ca^{2+}-activated channels, and in the regulation of metabolic processes such as glycogenolysis. It is difficult to record from glia *in situ*, and the significance of data in culture is unclear since it is likely that glial characteristics, such as the sensitivity to neurotransmitters and the role of second-messenger systems, change in culture.

In *neurones* there is evidence of Ca^{2+} mobilisation by neurotransmitters that is independent of extracellular Ca^{2+} (dorsal root ganglion cells, Murphy and Miller, 1988; cerebellar Purkinje cells, Llano *et al.* 1991). The kinetics of InsP_3-evoked Ca^{2+} release has been studied with experiments in Purkinje neurones in thin cerebellar slices. A large,

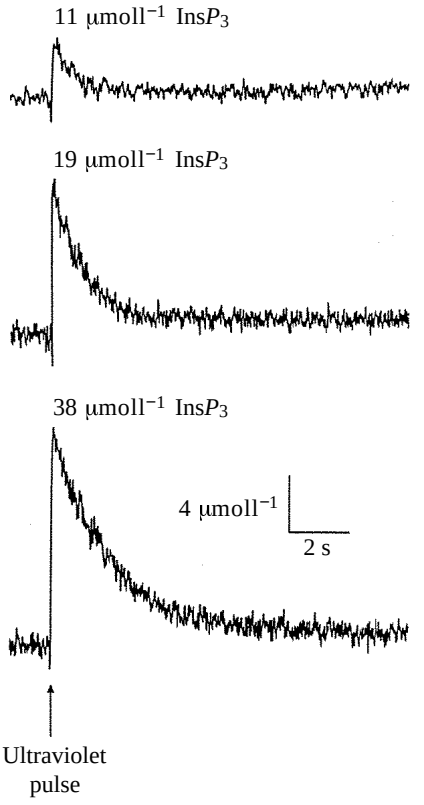

Fig. 7. Free Ca^{2+} concentration in a Purkinje cell soma in a rat cerebellar slice. Whole-cell patch-clamp, membrane potential −70mV, 500 $\mu mol\,l^{-1}$ Furaptra. Flash photolysis of caged InsP_3 to release 11 $\mu mol\,l^{-1}$, 19 $\mu mol\,l^{-1}$ and 38 $\mu mol\,l^{-1}$ InsP_3 into the soma. From Khodakhah and Ogden (1993).

rapid Ca^{2+} mobilisation from stores was found with photolytic release of InsP_3. The properties of Ca^{2+} release in Purkinje cells differ from those in peripheral tissues, described above. The InsP_3 concentrations needed are in the region of 10 $\mu mol\,l^{-1}$ or higher, 20 times higher than those needed in astrocytes and other nonexcitable cells, with maximal efflux still not attained at 80 $\mu mol\,l^{-1}$ InsP_3 (Khodakhah and Ogden, 1993). The overall characteristics are similar, but with shorter delays of up to 70ms at a low concentration, 10 $\mu mol\,l^{-1}$, of InsP_3, this being reduced to less than 20ms at concentrations above 33 $\mu mol\,l^{-1}$. The fluorescence increase was very large compared with the Ca^{2+}-induced fluorescence produced in the same cell either by depolarisation in voltage-clamp or with action potential discharge. The rate of rise of the Ca^{2+} concentration is much greater, up to 1mmol $l^{-1}\,s^{-1}$, more than 30 times the maximum seen in hepatocytes, and the peak levels of Ca^{2+} concentration are also much higher. The decline of the Ca^{2+} transient is quite fast in most cells, the response to a pulse of InsP_3 lasting a few seconds as in other tissues. Examples of the Ca^{2+} transients evoked by three

concentrations of InsP_3 in a Purkinje cell are shown in Fig. 7. It was essential to monitor Ca^{2+} concentration with the low-affinity dye Furaptra (Magfura-2) in whole-cell patch-clamp. The basis for the difference in properties between liver and Purkinje cell InsP_3 receptors may be in the isoform of receptor present, suggesting that the type 1 isoform present in Purkinje cells may have a lower InsP_3 affinity. The density of receptors per unit cytosolic volume, which is very high in Purkinje cells, would result in a high d[Ca^{2+}]/d*t* compared with peripheral tissues.

The physiological role of 'metabotropic' Ca^{2+} release from stores in neurones is difficult to demonstrate, but Ca^{2+} release by InsP_3 will result in short-term changes in excitability due to activation of Ca^{2+}-gated K^+ channels (Khodakhah and Ogden, 1993), and possibly in slower changes, such as long-term depression in cerebellar Purkinje cells. The reason for the lower apparent affinity of InsP_3 receptors and the much faster and larger Ca^{2+} mobilisation by InsP_3 in Purkinje cell may be related to a synaptic rather than hormonal function of the phosphoinositide system.

Ca^{2+} influx during Ca^{2+} mobilisation

The rise of cytosolic [Ca^{2+}] during hormonal Ca^{2+} mobilisation has been shown, in many cell types, to have an initial phase independent of the presence of external Ca^{2+}. This phase is therefore thought to be due entirely to release from Ca^{2+} stores. A later phase of the response, in which the [Ca^{2+}] often shows periodic fluctuations dependent on the presence of external Ca^{2+}, is thought to require Ca^{2+} influx (see reviews by Jacob, 1990; Marty, 1991). Current hypotheses concerning the regulation of this influx invoke InsP_3, InsP_4 or a combination of the two, possibly acting *via* Ca^{2+} stores either by means of a ligand-regulated connection of the store to the external solution or by depletion of the store itself, which then activates refilling from the external solution. Evidence has been obtained for InsP_3-induced Ca^{2+} influx, by both 1,4,5- and 2,4,5-isomers alone (e.g. Kuno and Gardner, 1987; Parker and Miledi, 1987; Matthews *et al.* 1989), for influx requiring both InsP_3 and InsP_4 (e.g. Morris *et al.* 1987) and for influx requiring InsP_4 alone (Luckhoff and Clapham, 1992). The problems of access and metabolism encountered with InsP_3 applied from the patch pipette also occur with InsP_4, but a 'caged' InsP_4 is not available to obtain evidence of its role or of the time course and concentrations required.

Ca^{2+} influx current *via* store depletion (Hoth and Penner, 1992) has been demonstrated in several tissues and appears to require stringent buffering of intracellular [Ca^{2+}] and drastic depletion of stores compared with that expected with physiological stimulation. Photolysis of caged InsP_3 has been used to show a Ca^{2+} influx in T-cells with milder conditions (McDonald *et al*. 1993). The time course of Ca^{2+} influx suggested that it may be *via* the stores, but similar experiments with caged InsP_3 in other systems have not been reported. The question of whether Ca^{2+} influx is *via* the stores or directly through the surface membrane in physiological conditions has not been satisfactorily resolved.

Caged cyclic AMP and cyclic GMP

The role of cyclic AMP as a second messenger in cytosolic Ca^{2+} regulation has been

investigated with flash photolysis in two systems: enhanced Ca^{2+} influx through non-inactivating high-threshold Ca^{2+} channels in cardiac muscle; and Ca^{2+} release from stores in hepatocytes. Two derivatives of cyclic AMP have been used as caged precursors, both as esters of the phosphate: the 4,5-dimethoxy-2-nitrobenzyl ester and the 2-nitrophenylethyl ester. The photochemistry of the caged cyclic nucleotides is discussed by Wootton and Trentham (1989). The 2-nitrophenylethyl esters photolyse slowly, with rates of about $5\,s^{-1}$ at pH7 and $100 mmol\,l^{-1}$ salt, and fairly efficiently, with a Q_p about 0.4. The 4,5-dimethoxy-2-nitrobenzyl esters photolyse more rapidly but with lower efficiencies. Caged cyclic nucleotides are lipophilic and may partition within cell membranes, where photolysis rates and efficiencies may differ from those in the cytosol.

Photolytic release of cyclic AMP increased the amplitude and the rate of decay of Ca^{2+} current in heart, probably through phosphorylation *via* protein kinase A (Nerbonne *et al.* 1984; Charnet *et al.* 1991). It may be noted that cyclic AMP released in the cytosol acts with little delay, unlike α-adrenoceptor stimulation by noradrenaline, which augments Ca^{2+} current after a delay of several seconds.

The Ca^{2+}-mobilising actions *via* adenylyl cyclase of β-receptor stimulation in hepatocytes are less well documented than those *via* InsP_3. This pathway can produce Ca^{2+} mobilisation alone in a smaller proportion of cells, often after very long delays of 1 min or more (Capiod *et al.* 1991). Cyclic AMP released from either cage mobilises Ca^{2+} with an average delay of 3s. The cyclic AMP mechanism is insensitive to heparin, providing evidence that InsP_3 is not involved by an indirect route (Noel and Capiod, 1991).

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