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Visualization of calcium dynamics at fertilization of the sea urchin egg using fluorescent probes and digital imaging

MATHIAS HAFNER

Department of Applied Cell Biology,
Technical University of Mannheim, FRG

Intracellular calcium is the most dynamic second messenger known. The list of biological processes requiring calcium is so disparate that there can hardly be any cellular function that is not influenced by this ion. It serves for the control of a variety of cell functions, including fertilization, mitosis and differentiation. Enormous progress has been made in the understanding of the molecular mechanisms which mobilize calcium from internal stores and how this signal is detected by calcium sensitive proteins such as calmodulin. Recent advances in calcium measurement, including the imaging of intracellular free calcium are beginning to reveal how surprisingly heterogeneous, pulsatile or even oscillatory calcium is at the single cell level. In this review specific attention is paid to using fluorescent probes and digital imaging to detect and map the intracellular distribution of free calcium. Possible release mechanisms of calcium at fertilization of the sea urchin egg are discussed.

Key words: *calcium, digital imaging, fertilization, Fura-2.*

INTRODUCTION

Over a century ago, Sidney Ringer (1883) demonstrated that a solution of sodium chloride plus tap water was more efficacious in maintaining the normal functioning of the isolated frog heart than a corresponding one constituted from distilled water. He concluded that the minute amount of calcium present in London tap water antagonized the "injurious" effects of sodium. Testimony to the complex and multifaceted roles of calcium in biological systems was first provided by Heilbrunn (see *e.g.* Heilbrunn, 1952). He and subsequently his co-worker Daniel Mazia perceived that a basic property of all living cells is to utilize calcium. Their theory of "cell stimulation" proclaimed that when a cell was stimulated by various means, the concentration of ionized calcium within the cell dramatically increased.

Since the pioneering experiments of Mazia (1937), who observed an increase in calcium in the ultrafiltrates of homogenates of *Arbacia* eggs which had just been fertilized, enormous progress has been made in the understanding of the molecular mechanisms which release calcium into the cytoplasm, such as voltage operated calcium channels or inositol polyphosphates acting on internal calcium stores (for review see Berridge, 1993). Comparable advances are occurring in the understanding of calcium sensitive proteins such as calmodulin or cyclins (rev. by Whitaker and Patel, 1990). However, the critical link between calcium fluxes and calcium sensitive proteins is the concentration of free calcium in the cytosol. Normally there is a very steep, inwardly directed electrochemical gradient for calcium because the extracellular free calcium is in the millimolar range. This gradient is even

Correspondence to: Prof Dr M. Hafner, Technical University of Mannheim, Speyererstr. 4, D-6800 Mannheim 1, FRG. Fax: (49-621) 292-6420

higher in marine invertebrates (Blaustein, 1974). This occurs because resting intracellular free calcium is normally extremely well buffered: basal cytosolic calcium activity in virtually all animal cells is of the order of 70 - 100 nM. Due to this low background, small changes in the absolute amount of calcium in the cytosol can provide a very large signal-to-background ratio. The source of this calcium may be the extracellular fluid or intracellular stores. All you need to do then is to make a conformational change in some sort of protein molecule in the membrane which allows calcium to flow down at the electrochemical gradient into the cytoplasm producing first a change in the free calcium and then second one of these many effects, which involves sensing or decoding of the calcium signal by appropriate calcium sensitive proteins like calmodulin.

In most cell types calcium may have several second messenger functions; in neurons, *e. g.*, it is involved in neurotransmitter secretion, control of potassium conductance or axoplasmic transport. Since each of these activities must be regulated independently, and since they may occur simultaneously in different parts of the same cell, calcium signals must exhibit spatial as well as temporal resolution. This occurs due to the fact that calcium is strongly buffered and sequestered, and cannot readily diffuse through the cytoplasm (Hodgkin and Keynes, 1957; Rose and Loewenstein, 1976; Allbritton *et al*, 1992). However, this situation presents simultaneously an opportunity for the cell in that meaningful signals can be generated by means of using the natural chemical energy stored in form of the calcium gradient and releasing or admitting very small amounts of additional calcium, and a challenge for the experimenter in measuring such low activities without perturbation.

Recent technical advances have enabled calcium to be visualized dynamically with spatial resolution down to micrometers and temporal resolution to fractions of a second (*e.g.* Tsien, 1989a; Bright *et al*, 1989, Tsien and Harrotunian, 1990). Such images of calcium are beginning to show how surprisingly heterogenous and pulsatile, even oscillatory calcium is at the single cell level (rev. by Berridge and Irvine, 1989; Berridge, 1993). By "calcium imaging" we mean producing a picture of a cell in which the brightness at any location within the cell relates not to its superficial structure, but to the local value of calcium. Ideally these images should appear continuously in video form or real time. This sets the requirements for such images: i) a chemical indicator whose optical properties change measurable with calcium, ii) a microscope and suitable light source, iii) a sensitive video camera, iv) a computer controlled image processor which digitizes and processes the video information to produce the calcium image. This new approach was primarily initiated by R. Y. Tsien and co-workers, who first developed fluorescent indicators of free calcium ion concentration which could be loaded into cells in a non-disruptive manner (Grynkiewicz *et al*, 1985, Tsien, 1989b).

The objective of this review is to discuss principles of use and required instrumentation and to demonstrate the usefulness of this approach in the study of the sperm-induced calcium wave at fertilization of the sea urchin egg. Clearly, with hundreds of papers already published I can only point out the major features, advantageous and problematic, of the technique and hope to provide a guide to the literature that details the important points.

OPTICAL INDICATORS FOR INTRACELLULAR CALCIUM

To design and interpret experimental investigations of calcium dependent processes and to make the study relevant to the living organism it is essential to know the free concentration of calcium inside intact cells and how that changes upon cell activation. Until recently such quantitative measurements were technically so hard that only few researchers dared to try and even then their efforts concentrated mainly on giant invertebrate cells like the squid axon. The situation changed dramatically with the availability of a new generation of fluorescent calcium indicators (*e. g.* Quin-2, Indo-1, Fura-2, Fluo-3) of which Fura-2 is

currently the most widely used (Fig 1). These are based on the tetra-carboxylic acid calcium chelator EGTA to which are added side-chains whose fluorescence is altered by the electron withdrawing effect of a bound calcium ion. Fura-2 has several properties that are advantageous compared with those of Quin-2 (Grynkiewicz *et al*, 1985). Its better absorption coefficient and quantum yield make it about 30 times brighter, so that loadings of 10 - 20 μM can give signals well above autofluorescence. To give an idea of the brightness, sea urchin eggs loaded with approx. 20 μM Fura-2 have a bluish-green glow easily visible when looked at under epifluorescence microscopy.

For optimal calcium measurements, the binding affinity of the indicator should be in the same range as the free calcium concentration to be measured. Fura-2 has a binding affinity such it can give useful indications of calcium from about 60 nM to 3 μM. As EGTA, Fura-2 also possess a selectivity for calcium over magnesium of 5 to 6 orders of magnitude. Such specificity is essential to detect the low resting calcium in the cytoplasm in the presence of 10^{-3} - 10^{-1} M Mg^{2+}, Na^+ and K^+. The excitation spectrum of Fura-2 is favorable in two ways: the binding of calcium shifts the fluorescence excitation spectrum of Fura-2 to shorter wavelengths so that increasing calcium increases the excitation efficiency at 340-350 nm and depresses that at 380 nm (Fig 2). At 360 nm Fura-2 has an isosbestic point were fluorescence intensity is independent of calcium. The emission spectrum peaks at 510 nm with negligible shift on calcium binding. This allows Fura-2 to be utilized as a dual excitation indicator, where the ratio of brightness obtained at 340 or 350 nm excitation to that at 380 nm excitation is used as a measure of calcium. Ratios are converted to calcium values using a calibration curve obtained from dye in buffers of known calcium. Instead of releasing the indicator to the incubation

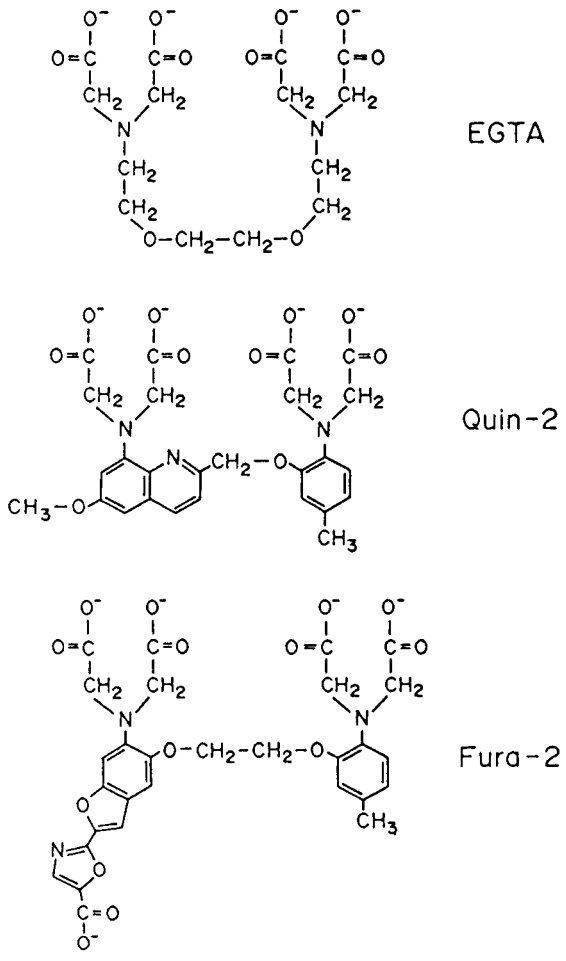

Fig 1: Comparison of the structures of EGTA and the fluorescent indicators of free calcium, Quin-2 and Fura-2.

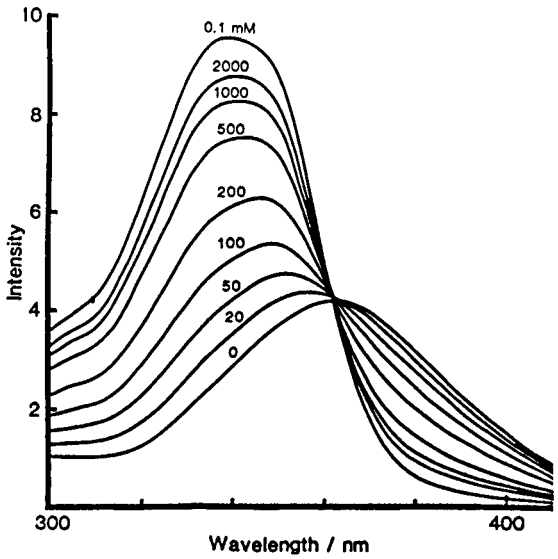

Fig 2: Family of excitation spectra of Fura-2 in a medium appropriate for measurements in cytoplasm as Fura-2 is titrated through a series of increasing calcium concentrations. Emission is measured at 505 nm throughout. The excitation maxima shifts toward 340 nm as calcium increases, the isosbestic point at 360 nm remains unchanged whereas an excitation minimum occurs at 380 nm.

medium and manipulating calcium there we alternatively can manipulate intracellular calcium without releasing the dye by permeabilizing Fura-2 loaded cells in the presence of such buffers. The latter approach gives calcium values under conditions that most closely mimic the conditions we measure during an experiment.

The dual wavelength method exploits the spectral changes to provide automatic compensation for variations in UV intensity, optical path-length through the cell, by simply measuring the proportion of available dye which is bound to calcium. Overall, the objective of calibration is to determine the fluorescence levels under conditions of maximum and minimum calcium binding. The obtained values are then related to the free calcium activity, through the known dissociation constant K_D (224 nM) and according to the mass action equation: calcium values (Ca^{2+}) are calculated from Fura-2 ratios (R) by the equation:

$$K\,(R - R_{min}) / (R_{max} - R) = (Ca^{2+})$$

where R_{min} and R_{max} are the ratios (340 nm/380 nm or 340 nm/360 nm) obtained in zero or saturating calcium concentrations, K is the product $K_d(F_o/F_s)$, where K_d is the effective dissociation constant, F_o is the 380 nm excitation signal in the absence of calcium and F_s is the 380 nm excitation signal at saturating calcium concentrations (Poenie *et al*, 1985). In practice one feels normally much more confident if calcium can be raised to saturating values with ionomycin at the end of the experiment and if the autofluorescence is then obtained by adding Mn^{2+} or EGTA. An additional advantage of this indicator is its availability as a membrane-permeable acetoxymethyl ester (Fura-2AM).

The above approach requires that the cells to be studied have esterases to produce the free acid which is hydrophilic and not membrane-permeable and therefore trapped inside the cell. Whereas most mammalian cells seem to have these non-specific esterases we never succeeded in getting Fura-2AM into sea urchin eggs, where we always had to microinject the free acid. However, the latter method circumvents the often occurring incomplete hydrolysis of the acetoxymethyl ester which can lead to compartmentalization in subcellular organelles. In addition, the final concentration of the indicator inside the microinjected cell can precisely be calculated. More details on the underlying assumptions and derivation of the calibration procedures can be found in Williams and Fay (1990) and Poenie (1990).

FLUORESCENCE DIGITAL IMAGING

The development of the new calcium indicators with above described potentials enabled us to examine the spatial organization of calcium changes at the single cell level with high accuracy, sensitivity and resolution under fluorescence microscopy and digital imaging. The major advantage of using this approach over conventional spectrofluorimetry of cell-suspensions clearly is that important changes (*e.g.* after the application of a stimulus) throughout a population of cells are not obscured due to the asynchronous nature of most calcium responses.

I shall now describe the important hardware components of a digital image processing system, which is summarized in Figure 3.

a) Microscope and illumination system: the central piece for imaging fluorescence emission from single cells is a epi-fluorescence microscope. For most applications, an inverted microscope is preferred, since cells are accessible from above for experimental manipulations while they are imaged from below with high numerical aperture and short working distance objectives. Objectives with high numerical aperture (> 1.25) are highly recommended because the light collecting efficiency varies with the square of the numerical aperture. It is crucial that all optical components are optimized for high UV-transmission (down to 340 nm), low autofluorescence, high light-gathering efficiency and low chromatic and spatial aberrations (*e.g.* 40 x 1.3 UV-CF Nikon). A

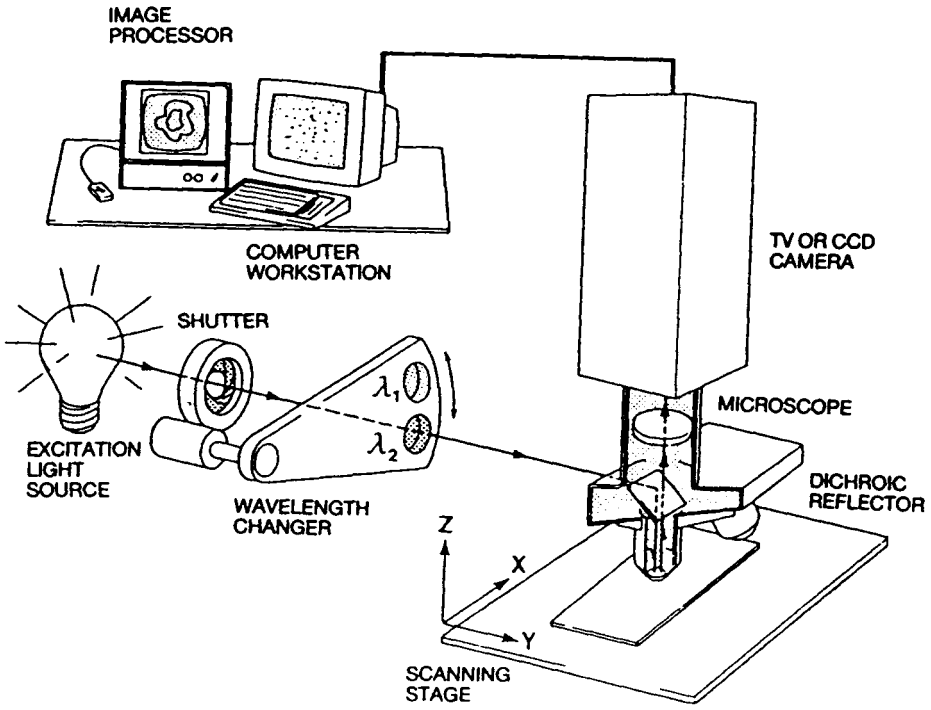

Fig 3: Schematic diagram of the basic elements of a epifluorescent ratio imaging microscope system. The elements include a light source, a computer controlled shutter and excitation wavelength selector, microscope with dichroic beam splitter, low-light-level imaging detector, control and data acquisition computer.

good transmission of the relevant UV wavelength of each individual optical element is particularly important. If *e. g.* the transmission of each element is 50%, then with n elements the total transmission would be 0.5^n. A 6-lens system would therefore give only 1.5% transmission! The microscope will also provide transmission optics for direct observation and manipulation of the cells. Since Fura-2 is currently the indicator of choice a xenon arc lamp is generally superior to mercury to deliver the excitation light because xenon provides a much more even spectrum of illumination. However, xenon arc lamp produces a large amount of energy in the infrared region. Thus, it is advisable to include a heat filter (*e.g.* Corning 754) early in the light path. The microscope is further equipped with some mechanics for frequently alternating between at least two excitation wavelengths. For their selection multilayer interference filters usually centered around 340 nm and 380 nm with about 10 nm bandwidth should be used. Due to higher light transmission at 380 nm, it is worthwhile to use neutral density filters in order to achieve similar illumination intensities. Interference filters are typically assembled in an automated device (mechanical chopper or rotating wheel) capable of frequently switching between filters. Furthermore, the filter changer must provide computerized control modules to allow synchronization with image frame acquisition and a shutter to prevent cellular photodamage and photobleaching when data is not being collected.

b) Imaging devices and image processing: the availability of specialized video cameras, capable of subdividing the photon flux according to the points of origin and with sensitivities greater than the human eye, has quantitative fluorescence imaging made possible. Two main types of two dimensional detectors are currently in use: the silicon intensified target (SIT) camera, and the intensified charge-coupled device (ICCD) camera. A SIT camera uses analogue technology and provides an image with good spatial resolution and low noise, but is rather

slow to respond to changes in image intensity (lag times up to 1 sec). Thus after switching wavelengths the time which is needed for the camera to stabilize limits the time resolution in dual-wavelength imaging. A CCD camera uses a solid-state charge coupled device to collect electrons from a photocathode. This semiconductor layer is divided into discrete, electrically isolated electronic "wells" which are defined by gates in the surface of the sensor. Thus unlike a SIT-tube there is a separate detector for each pixel. The electron-hole pairs created by arriving photons cause a potential which is proportional to the photonflux at each discrete pixel. Subsequently the signal is read out by sequentially shifting the electron charge in the potential well. Thus, the obtained image is free from shading and geometrical distortion and responds immediately to changes in illumination. Major disadvantages of CCD's are that spatial resolution is limited by the number of elements and noise from thermal generation of electrons (dark current) which can be minimized by cooling. The characteristics which should be considered when selecting an appropriate imaging detector are: i) linearity of the output signal with respect to the input signal (this depends on the so called gamma factor of the camera), ii) spatial resolution (which is limited by the number of TV-scan lines in the case of tube cameras or the number of pixels for solid-state detectors), iii) temporal resolution (limited by the degree of image persistence, which can be expressed as a percentage of the signal left on the phosphor output after a complete video frame (33 msec), iv) sensitivity (defined by the lowest light level that can be detected with an acceptable signal-to-noise ratio) and v) quantum efficiency (= the ratio of photons arriving over photons detected). More details on these parameters can be found in Bright *et al* (1989) and Tsien and Harrotunian (1990).

In most systems used, data are stored sequentially until the memory is full, for later "off-line" processing, calcium calculation and display. However, a few such as the Imagine Image Processor (Synoptics Ltd, Cambridge, UK) have sufficient processing power to carry out real-time dual wavelength ratio imaging of calcium at video rate. The storage requirements are determined by the number of images to be stored, the image size (number of pixels) and the data precision. A digital image represents a large amount of computer information which must be manipulated all at once. Precision is defined by the number of distinct levels allowed for the intensity at each pixel. It is equal to 2^n where n is the number of bits used for digitization. Therefore, a 8-bit digitizer will allow 256 levels of precision (expressed in gray levels) for every sample point. That means a storage capacity for a standard 512 x 512 image at 8-bit resolution of more than 2 mega bits of information. This corresponds to 262.144 bytes which is more than most lab-computers have as memory. From this little calculation it becomes clear that a time series composed of several hundred images will fill the memory of most computers and other mass storage media like magnetic tape cartridges, hard disc drives or CD rom or optical disc drives are needed.

An image processing system consists of several components, each with its own unique function (Fig 4). The output from the video camera is an analog electrical signal, representing image brightness scanned on a raster of *e. g.* 484 lines every 1/30 sec (in the US). The task of the image processor is to convert this signal point by point into an image of calcium as a function of time. It does this by using an analog to digital converter (ADC) which produces coded numbers that are a measure of light intensity. The digitizer samples the signal at regular intervals along each video scan line, converting the electronic output into a string of discrete digital values that map the optical image into the computer. Each discrete value in the digital image represents the average light intensity measured over the sampling interval of a pixel. The next component is the image processor (compare with Fig 4), which is usually composed of three distinct parts: i) digital image memory, ii) array processor (for high speed manipulation like arithmetic or logical operations on data) and iii) host computer. Usually the array processor and the digital image memory are combined in one device along with additional memory

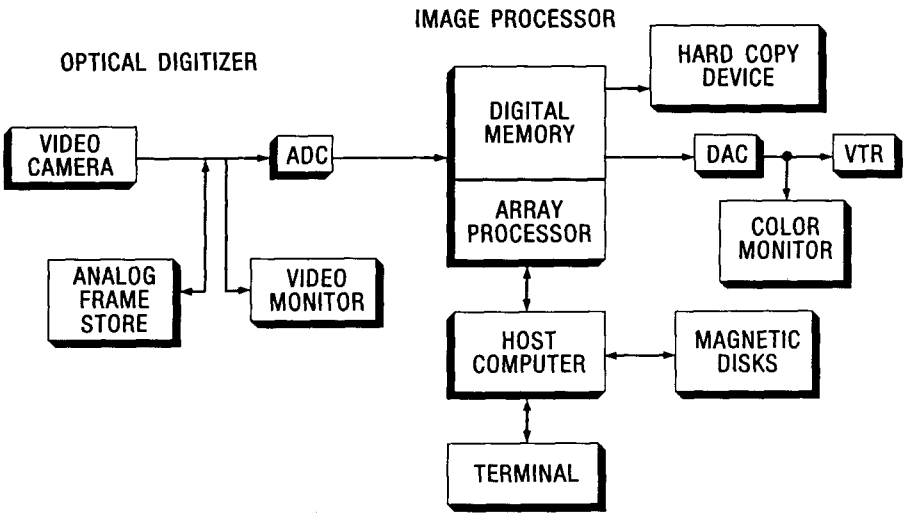

Fig 4: A block diagram of the imaging system computer hardware. For details see text.

areas that store look up tables (LUT). Here, input (gray-) values are matched directly to output values.

Once an image has been converted into digital form, it can be manipulated within the memory of the computer in ways that effect *e.g.* image contrast or overall brightness. These manipulations are performed using Intensity Transformation Functions (ITF) and effect only the gray values of individual pixels and do not alter the spatial information of the image. Most image processing systems take advantage of a specific form of computer memory called Intensity Transformation Table, or simply LUT. The hardware can be constructed that the digital image can be passed directly through a LUT resulting in a gray value of each pixel in the input image being converted to the output value specified by the LUT. Since these transformations are performed by hardware, they can be done very quickly, in real times at video rates. For calcium imaging ITF's loaded into LUT with implemented color transformations are frequently used. This allows a pseudocolor display of intracellular calcium. The digital image is routed through 3 different LUT, which in turn are directed to the red, green and blue color guns of the monitor. The result is an image with better contrast because the human eye distinguishes many more different colors than it does shades of gray.

THE CALCIUM WAVE AT FERTILIZATION OF THE SEA URCHIN EGG

The advent of calcium-sensitive indicators, like Fura-2, and image analysis have made it possible to study the initiation of calcium release upon an external stimulus and the subsequent distribution of the calcium signal within a single cell. One of the most dramatic examples of such an event is fertilization. Calcium signals can either occur uniformly, or they can initiate at a discrete location of the cell from where they may propagate. At fertilization, the sperm (the external "signal") clearly dictates this initiation site: within seconds of sperm-egg attachment, the activation of a mature egg starts with a dramatic increase in the intracellular free calcium at the sperm entry point (Fig 5). This calcium explosion (Lionel Jaffe calls it even "calcium-tsunami") occurs in all deuterostome eggs investigated so far (review Jaffe, 1985; Jaffe, 1991), where it appears to be an important primary regulatory signal that triggers the initiation of development (Schatten and Hülser, 1983).

Imaging techniques using either the calcium-dependent luminescent protein, aequorin (Gilkey *et al*, 1978; Eisen *et al*, 1984; Miyazaki *et al*, 1986; reviewed by Jaffe, 1985 and 1991), or Fura-2 (Hafner *et al*, 1988) have revealed a conducting band of calcium release which travels towards the

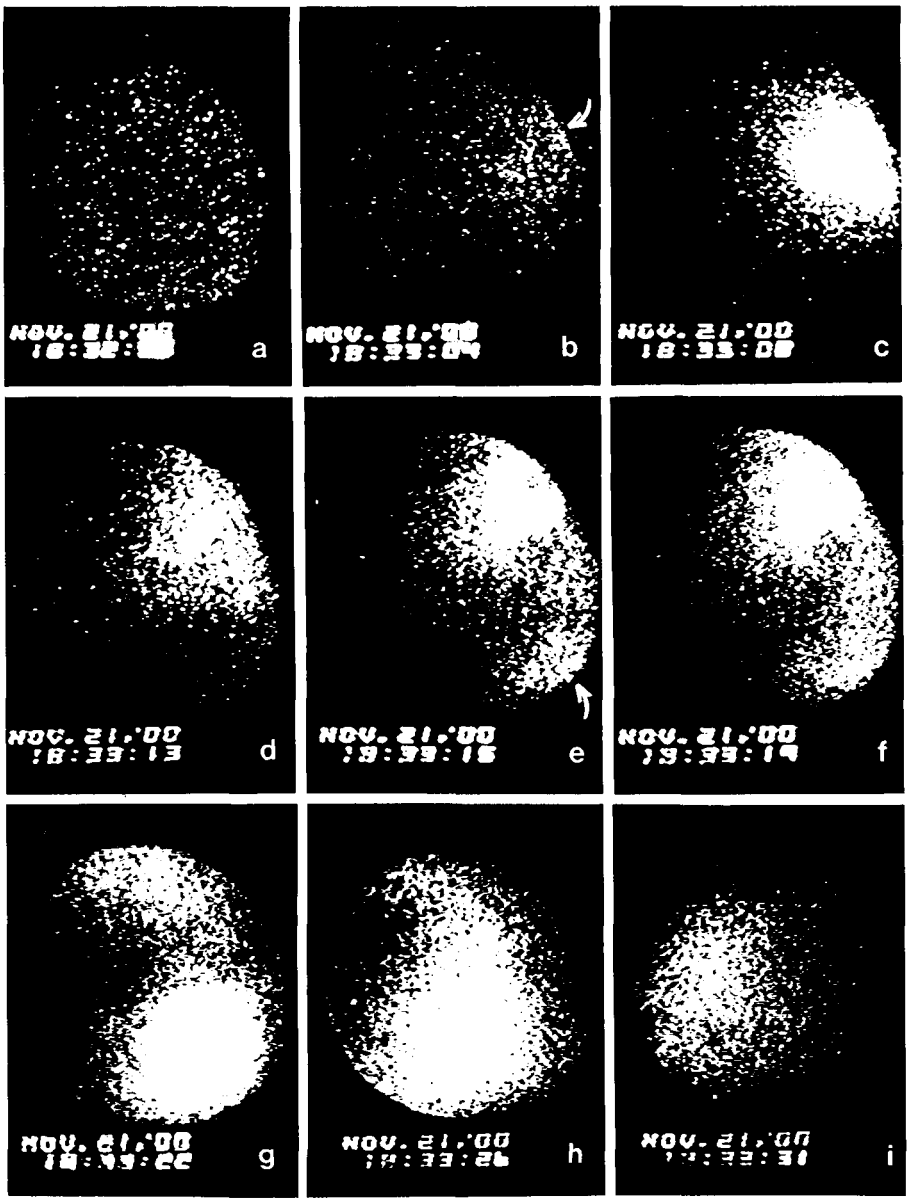

Fig 5: Calcium explosion at fertilization in a dispermic egg of the sea urchin *Lytechinus pictus* visualized with Fura-2 after image processing (a-i). Ten seconds after the entry of the first sperm (arrow, b) is visible by the increased calcium; a second focus of released calcium appears (e), indicating the successful hit of another sperm (arrow, e). The second wave increases as it spreads over the egg, whereas the first wave appears to be blocked in its propagation (adapted from Hafner *et al*, 1988).

opposite pole of the egg. As a first visible result of the high intracellular free calcium, a wave of cortical granule exocytosis follows, but Epel and coworkers were able to relate also other biochemical events, like increase in O_2 consumption, activation of NAD kinase or sustained increase in intracellular pH through the activation of the Na^+/H^+ exchanger (reviewed Epel, 1989; Whitaker and Patel, 1990). In fact, microinjection of calcium-chelators (*e.g.* BAPTA) inhibits initiation of that developmental sequence (Hamaguchi and Hiramoto, 1981; Swann and Whitaker, 1986; Turner *et al*, 1986; Mohri and Hamaguchi, 1991). We recently were able to demonstrate (Hafner *et al*, 1988) the

wave like behavior of that band in the egg of the sea urchin *Lytechinus pictus*. This steep positive calcium gradient is expanding simultaneously in all directions with the same velocity (11 μM/s). The quantitative analysis shows a peak free calcium of about 2.5 μM. Even before the sharp zone of elevated calcium reaches the opposite pole of the egg the calcium concentration at the sperm entry point already starts to fall (Fig 5), the beginning of bringing back calcium to the resting level. Kühtreiber and co-workers (1993) using a calcium specific vibrating probe clearly showed that this reduction is at least partially due to an actual loss of calcium through the action of a plasma membrane calcium pump, which can be as high as 36% of the eggs total calcium.

The means by which sperm-egg interaction triggers intracellular calcium release and the mechanisms by which the calcium wave propagates through the egg are still not known in detail. Microinjection of the second messenger inositol 1,4,5-triphosphate ($InsP_3$) not only gives rise to a wave of cortical granule exocytosis but also stimulates partial resumption of the cell cycle indicating the involvement of sperm induced hydrolysis of a plasma membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2). Recently, considerable evidence has accumulated demonstrating that the egg utilizes this pathway endogenously. Microinjection of antibodies against PIP_2 considerably reduced the amount of calcium released in the egg cortex of *Xenopus* (Larabell and Nuccitelli, 1992). Moreover, using hamster eggs, Miyazaki and co-workers (1992) microinjected monoclonal antibodies to the $InsP_3$ receptor and where not only able to inhibit sperm-induced calcium waves but also calcium-oscillations. Although this evidence suggests a central role for $InsP_3$-induced calcium release there is still controversy concerning the way in which it contributes to the further propagation of the calcium wave and of calcium-oscillations which appear in murine, ascidian and *Xenopus* eggs (Cuthbertson and Cobbold, 1985; Miyazaki *et al*, 1992; Speksnijder *et al*, 1989). However, calcium oscillations and waves appear to be closely related. The mechanism by which this phenomenon occurs is unknown but one could imagine either a positive feedback effect on the phospholipase C, thereby generating periodic formation of $InsP_3$ or alternatively a release of calcium stimulated by calcium induced calcium release (for review see Berridge, 1993). In the latter model calcium elevated at a discrete initiation site like the sperm-attachment point diffuses to an adjacent calcium store to stimulate its own release and so on down to the opposite pole.

Figure 6 shows a simplified model illustrating the inositol phosphate/calcium signalling in sea urchin eggs. Under circumstances where there are more initiation sites like the dispermic egg in Figure 5 (a-i) separate waves may result. These waves can collide with each other (Fig 5 f, g) or even annihilate each other (Fig 5 g-i). This indicates that there is a refractory period following the wave, which probably depends on the time required to refill the calcium stores to the point at which they could once again respond to an inositolphosphate or calcium signal. The membrane stores from which calcium is released contain three major components: calcium pumps, –binding proteins and –channels, thus the intracellular target of $InsP_3$ appears to be the endoplasmic reticulum (ER) where all these components can be found (Girard *et al*, 1991; Oberdorf *et al*, 1988; Rakow and Shen, 1990; Gillot *et al*, 1991).

The interdependency between calcium signal propagation and the ER compartment were demonstrated by a reorganization of the structure of the tubular ER cisternae surrounding cortical granules. These changes also begin as a wave that passes across the egg just like the wave of calcium release (Terasaki and Jaffe, 1991). The approximate temporal correlation of the rise and fall in free calcium and the structural changes in the ER suggests that these events are functionally related.

Whether fertilization affects calcium levels only in the peripheral ooplasm by activation of specialized calcium stores or "calciosomes" (Krause 1991) restricted to the peripheral ooplasm is difficult to ascertain and definitely not possible by using

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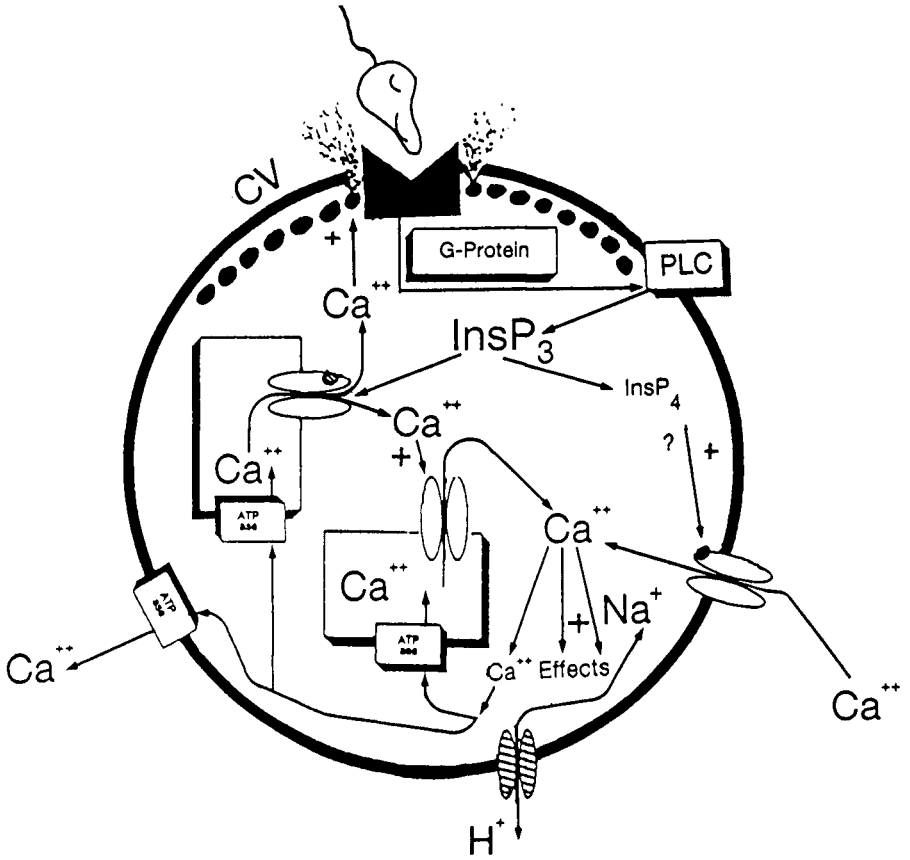

Fig 6: Simplified summary of inositol phosphate/calcium signalling in a sea urchin egg at fertilization (see text for details). Sperm and membrane receptor interaction ultimately produces inositol 1,4,5-triphosphate ($InsP_3$). $InsP_3$ releases stored intracellular calcium from the ER. If additional extracellular calcium influx facilitated by $InsP_4$ occurs has not clearly been shown. The increase in free calcium leads to exocytosis of cortical vesicles and stimulation of other biochemical events of which activation of the Na^+/H^+ exchanger is one example. Calcium probably also inhibits its own $InsP_3$-induced release before it is taken back into the stores or even pumped out of the cell by ATPases. Calcium-induced calcium release may lead to the propagation of the calcium wave throughout the entire egg.

isolated cortices. To address this question, calcium imaging with confocal laser scanning microscopy and time-lapse recordings of optical sections was applied by Stricker and co-workers (1992). However, their careful analysis of the fertilization-induced calcium wave indicated that there is rather a global elevation throughout the interior of the egg than just in the cortex. Otherwise, the study yielded another exciting finding, since the authors were able to clearly demonstrate a substantial rise in intracellular free calcium within the nucleus, suggesting the involvement of calcium release from specific stores in the nuclear envelope. Studies in other cell types (Burgoyne *et al*, 1989) indicate the existence of such a differentially regulated calcium pool.

Although the biological significance of the change in nuclear calcium remains unclear, calcium-mediated events have been discussed as triggers or modulators for a variety of important nuclear activities such, as nuclear envelope breakdown, modulation of chromatin structure or gene expression (*e.g.* Tombes *et al*, 1992; Patel *et al*, 1989; Whitaker and Patel, 1990) or appearance of the mitotic apparatus (rev. by Petzelt and Hafner, 1989). A great challenge for the future is to understand how cells are able to operate and interpret this fine language of calcium messages and how the signalling pathway that begins with the sperm-induced calcium release contributes to the sequence of events that culminate in DNA-synthesis, cell division and differentiation.

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