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Intracellular calcium regulation and the measurement of free calcium in 2H3 cells and synaptosomes

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Introduction

It is almost precisely 100 years since experiments on the contraction of frog heart muscle provided the first unequivocal demonstration of a requirement for calcium in a biological process. Since that time much evidence has accumulated indicating that cations play important roles within the cell in controlling metabolism. In particular, it is now established that changes in the intracellular concentration of calcium regulate not only contraction in muscle, but conduction in nerve, coupling of secretion to stimulus in cells such as the platelet and mast cell, the initiation of development in fertilized eggs and possibly, though more controversially, the activation of growth in normal cells.

Intracellular calcium regulation

The free calcium concentration within eukaryotic cells, ($[Ca]_i$), is the result of the combined actions of various pump-leak systems at both the plasma membrane and at internal membranes, such as the inner membrane of the mitochondria. Calcium influx across the plasma membrane may occur in at least two separate ways: (1) down the steep electrochemical gradient by facilitated diffusion: the process is relatively independent of membrane potential but the carriers may be affected by sodium and the channel may be hormone-modulated (Borle, 1982); (2) through a potential-dependent channel (Reuter, 1979) which probably only exists in excitable cells (see Carafoli, 1982). Following depolarisation of, for example, the squid giant axon, some 'early' calcium entry may occur through the Na^+ channels which gate the action potential. Tetrodotoxin specifically blocks the sodium channels and hence any 'early' calcium influx due to the imperfect selectivity of the sodium channels (Baker *et al.*, 1973a). The slow phase of calcium entry through potential-dependent, calcium-specific channels can be blocked by verapamil and its more active derivative D600 (Baker *et al.*, 1973b). In addition to the action of these drugs in specifically blocking calcium channels, heavy metal ions, in particular lanthanum, inhibit calcium conductance in both heart muscle fibre and smooth muscle membranes (Bulbring & Tomita 1969; Anderson *et al.*, 1971). In the squid giant axon the depolarisation-induced rise in $[Ca]_i$ is rapidly rebuffered with a half-time of about 15 s and, as this effect is ATP and CN^- (cyanide ion) dependent, it is presumed to reflect the action of the mitochondria. These organelles can show exquisite sensitivity in regulating their external calcium at a pre-determined low level (Nicholls, 1978). Isolated rat liver mitochondria at 30°C and pH 7.0 maintain extra-mitochondrial free calcium at 0.8 μM and the precision of this control is due to the mitochondria cycling calcium through independent influx and efflux pathways (Puskin *et al.*, 1976; Carafoli & Crompton, 1976). The efflux pathway in these mitochondria exchanges the cation for protons, whereas in heart, brain or brown fat mitochondria the exchange is of Ca^{2+} for Na^+.

In addition to the avid buffering capacity of the mitochondria, calcium may be actively transported into the smooth endoplasmic reticulum (from which it may be passively released) by a Mg^{2+}-dependent, Ca^{2+}-sensitive ATPase. A similar enzyme, located in the plasma membrane, also functions as one of the two major mechanisms by which calcium may be pumped out of the cell. In the erythrocyte, at least, this

pump is also regulated by calmodulin and the efflux rate it can achieve has been estimated from isotopic flux measurements as between 10 and 24 attomol $cell^{-1}$ min^{-1} (atto = 10^{-18}) (Ferreira & Lew, 1977). There is considerable evidence for the existence of a similar, if not identical, calmodulin-dependent (Ca^{2+} + Mg^{2+})-ATPase in a variety of cell membranes, including the smooth muscle of pig stomach (Wuytack *et al.*, 1981). A second plasma membrane extrusion mechanism exists by which the Na^+ chemical gradient drives calcium efflux against its electrochemical potential gradient. The activity of this Na^+/Ca^{2+} exchange system is dependent on the trans-membrane Na^+ gradient maintained by the ubiquitous Na^+-K^+-activated ATPase. Current evidence suggests a stoichiometry of $3Na^+:1Ca^{2+}$ and that the exchange protein functions independently of ATP, although the ionic affinity of the system is increased in the presence of ATP (Jundt & Reuter, 1977; Blaustein, 1977). The electrogenic Na^+/K^+ exchange catalysed by the Na^+-K^+ ATPase, may in turn be coupled to an electrically silent Na^+/H^+ exchange protein. In some cells this latter protein appears to regulate intracellular pH, whereas in others Cl^-/HCO_3^- exchange is the predominant mechanism.

These considerations of the factors which control the plasma membrane ion pumps indicate that, in general, ionic changes within the cytosol are likely to be tightly coupled. For example, if the result of a ligand binding to its receptor on the cell surface is an increase in $[Ca]_i$, an increase in the K^+ permeability of the plasma membrane will follow, which in turn would modulate membrane potential: decrease in the K^+ gradient activates the Na^+-K^+ ATPase, hence the level of intracellular Na^+ falls and Ca^{2+} efflux via Na^+-Ca^{2+} exchange increases. Such a sequence may therefore provide a negative feedback loop controlling $[Ca]_i$. Hence, although an ionic signal across the plasma membrane may be the primary action of, for example, a growth-stimulating ligand, this may result in extensive cation flux changes through coupling, which *may* also alter their internal concentrations. In stimulus-secretion activation of, for example, the mast cell, it seems clear that the critical ionic event is an increase in $[Ca]_i$ (Pearce, 1982), but in the stimulation of normal cell growth each major cation (Ca^{2+}, Mg^{2+}, K^+, Na^+ and H^+) has had its supporters as being the primary determinant of mitogenesis and the coupling phenomenon has been largely ignored in attempts by authors to correlate the specific ionic changes which they can measure with the physiological response of the cell. This problem is compounded because one cannot infer that the primary signal is different in different types of cell simply because the pleiotropic pattern of ionic responses is different: coupling is a function of which of the transport systems are present in the plasma membrane and therefore of the differentiated phenotype of the cell. Finally, although the properties of the endoplasmic reticulum and mitochondria discussed above indicate that these organelles may be important in regulating transient, intracellular ionic responses, it should be borne in mind that it is the activity of the plasma membrane pumps, in combination with leakage across that membrane, that will determine the steady-state concentrations of free ions within the cytosol.

The mediation of cellular responses by calcium is presumably brought about by the interaction of the ion with specific calcium-binding proteins, thereby altering enzymatic activity and leading to the cellular changes observed. It is therefore the free cytoplasmic concentration or chemical potential of the ion which is crucial in regulating any response. It is now clear from measurements on a variety of different cell types that although the average, total calcium concentration within cells is about 1.5 mM (Lichtman *et al.*, 1979), less than 0.01% of this exists in the free, ionised form. Of the rest, 40–60% is sequestered in the mitochondria, either ionised, bound or precipitated as the carbonate or phosphate salts, about 20% occurs in the endoplasmic reticulum and the remainder is either accumulated in secretory granules or the nucleus, or bound to macromolecules. For the other major intracellular divalent cation, magnesium, recent estimates suggest total concentrations of about 12 mM with a free concentration of about 1 mM (Rink *et al.*, 1982). Therefore, as was pointed out by Heilbrun (1956), of the major intracellular cations, it is the very low level of ionised calcium that bestows its unique potential as a sensitive triggering agent.

Measurement of free, intracellular cation concentrations

Conventional methods for measuring the level of ionised calcium within cells, using either ion-selective electrodes or microinjection of calcium-sensitive molecules, such as the luminescence protein aequorin (Gilkey *et al.*, 1978), are difficult if not impossible to apply to small cells such as those of the circulatory system. This problem has prompted the development by Tsien (1980) of a prototype fluorescent calcium indicator ('quin 2'), derived from EGTA. Quin 2

may be introduced into cells as the non-polar acetoxymethyl ester (Figure 1) which, after crossing the plasma membrane, is hydrolysed by endogenous esterase activity to the tetra-anionic form, which is then trapped inside the cell. The fluorescence of quin 2 increases six-fold on binding calcium and, knowing the affinity for calcium (log K_{Ca} = 7.05 at 37°C), resting levels of $[Ca]_i$ may be determined by the procedure shown in Figure 2 (Hesketh *et al.*, 1983).

More recently, Smith and colleagues have synthesised a fluorescent pH indicator ('quene 1', Rogers *et al.*, 1983), as well as a series of non-fluorescent, fluorine-labelled molecules which show large ^{19}F-NMR chemical shifts on chelating divalent cations (Smith *et al.*, 1983). The ^{19}F chemical shifts and linewidths enable the identity and concentration of the chelated ion to be determined. In the acetoxymethyl ester form each of these indicators may be loaded into cells by the method described above. As the NMR indicators may be used in tissues or opaque suspensions of cells which are unsuitable for fluorescence measurements, these developments substantially extend the range of usefulness of intracellular chelators as M^{n+} indicators.

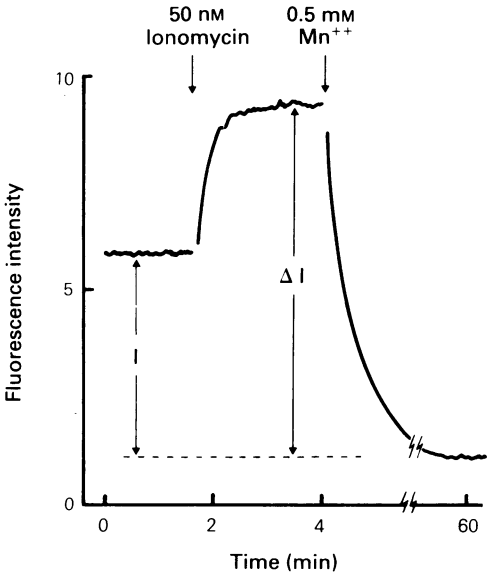

Figure 2 Measurement of $[Ca]_i$ using quin 2 fluorescence. Cells are loaded with intracellular quin 2, washed and suspended at an appropriate concentration in a continuously stirred fluorimeter cuvette. I represents the stable fluorescence intensity from the cells. The addition of either 50 nM ionomycin or 0.1% Triton X-100 yields the maximum fluorescence signal from quin 2 fully saturated with Ca^{2+} (Δ I). The background signal from the cells (autofluorescence and scattering) is obtained by the addition of 0.5 mM $MnCl_2$ (which quenches quin 2 fluorescence by > 99% in the presence of 1 mM Ca^{2+}). The fluorescence intensity of free quin 2 or the Mg^{2+}-quin 2 complex at pH 7.1 is 0.16 Δ I (Hesketh *et al.*, 1983). The per cent Ca^{2+}-quin 2 is then calculated as (I − 0.16 Δ I)/0.84 Δ I × 100.

$[Ca]_i$ and histamine release in 2H3 cells

The 2H3 rat basophil leukaemic cell line has many of the properties of the mast cell and many agents stimulate histamine release in both types of cell (McGivney *et al.*, 1981). In mast cells, $^{45}Ca^{2+}$ uptake experiments indicate that antigenic stimulation causes a large calcium influx

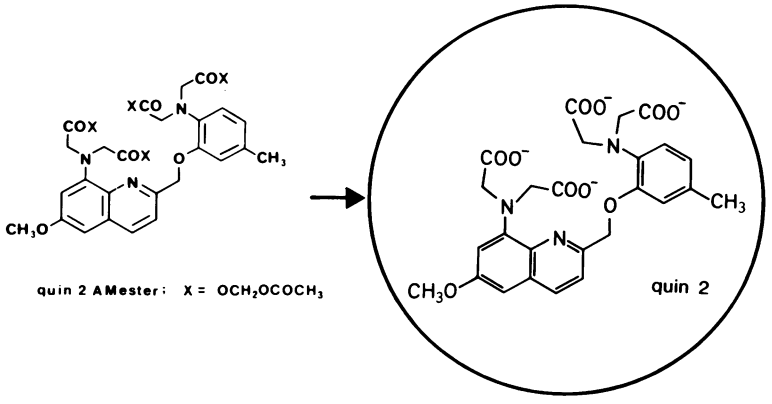

Figure 1 Intracellular accumulation of quin 2.

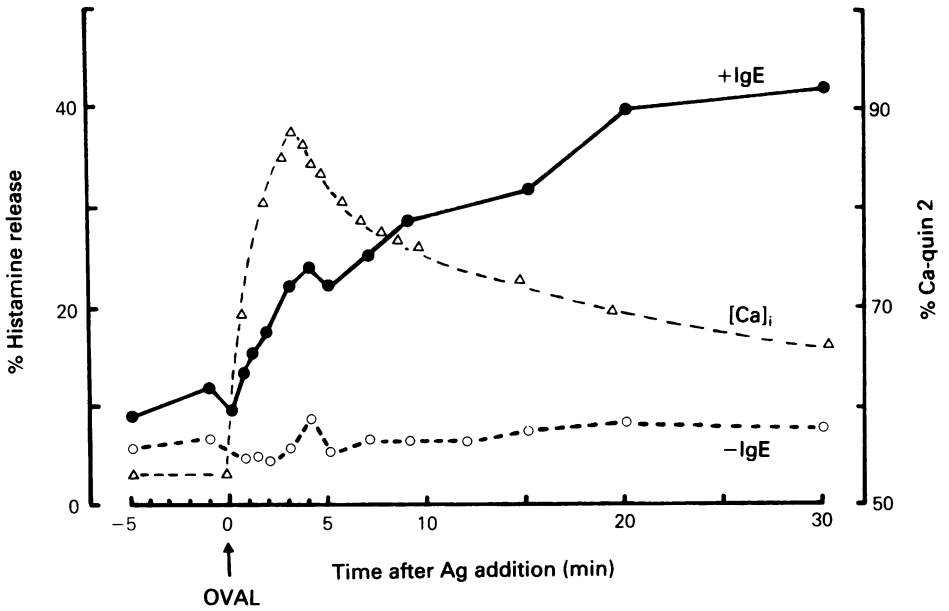

Figure 3 The fluorescence response and histamine release following antigenic challenge (ovalbumin, OVAL) in quin 2-loaded 2H3 cells. Samples of 2H3 cells grown in suspension culture (Beaven *et al.*, 1984a) were loaded in medium A (120 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.1% bovine serum albumin) at 10^6 cells/ml with 10 μM [^{3}H]-quin 2 acetoxymethyl ester for 60 min at 37°C. The cells were washed and suspended in 2 ml of medium A at 10^6/ml in a quartz cuvette with continuous magnetic stirring at 37°C in a Perkin Elmer 44B spectrofluorimeter. At time zero 2.5 μg/ml aggregated ovalbumin was added. At the times indicated 50 μl samples were removed for assay of histamine released from the cells by a single-step radioenzymatic method (Beaven *et al.*, 1983). ---△--- % Ca-quin 2, —●— % histamine release from cells sensitised with ovalbumin-specific IgE, ---○--- % histamine release from control (non-IgE sensitised) cells.

(Foreman *et al.*, 1977) and the increase in $[Ca]_i$ that this has been taken to imply, is considered as a Ca^{2+} signal which triggers degranulation (Gomperts *et al.*, 1983). 2H3 cells may be grown in suspension cultures and sensitised with mouse IgE to respond to antigen (Beaven *et al.*, 1984a). The histamine release thus stimulated occurs over 20 to 30 min, rather than the 1 to 2 min in normal peritoneal mast cells and this slow release, together with the use of quin 2, has enabled the time course of the Ca^{2+} signal and of secretion to be compared.

Figure 3 shows the change in quin 2 fluorescence, representing changes in $[Ca]_i$, due to the addition of aggregated ovalbumin to quin 2-loaded 2H3 cells, together with the extent of histamine release from the same cells. The onset of the calcium increase occurred within 10 s of antigen addition and the maximum was reached 2 to 3 min later. Similar fluorescence increases were induced by concanavalin A (20 μg/ml), A23187 (200 nM) or ionomycin (50 nM), although the maximal $[Ca]_i$ level was attained more rapidly (after 80 s) with the ionophores. Changes in

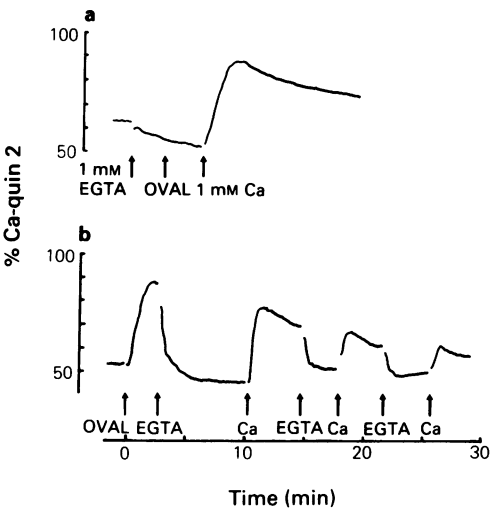

Figure 4 Effect of extracellular Ca^{2+} on the Ca^{2+} signal in quin 2-loaded 2H3 cells. (a) 1 mM EGTA was added to the cells followed by 2.5 μg/ml aggregated ovalbumin (OVAL) and 1 mM $CaCl_2$ at the times indicated. (b) Sequential additions of EGTA and $CaCl_2$ to stimulated cells, varying the free calcium concentration in the medium from < 5 μM to 1 mM respectively.

$[Ca]_i$ did not occur in cells unsensitised with IgE, except in response to the calcium ionophores.

The antigen-induced $[Ca]_i$ increase was from a level of 104 nM in resting cells to a maximum of 1.2 μM. Following this initial rise, the fluorescence signal declined slowly over 30 min, during which time $[Ca]_i$ was always above the level in resting cells, although the rate of histamine release was maximal when $[Ca]_i$ was increasing rapidly and declined during the period when $[Ca]_i$ was decreasing. Although A23187 induces both an increase in $[Ca]_i$ and histamine release in 2H3 cells, compound 48/80 elicits neither response in sensitised or unsensitised cells, in contrast to its effect on rat peritoneal mast cells (data not shown).

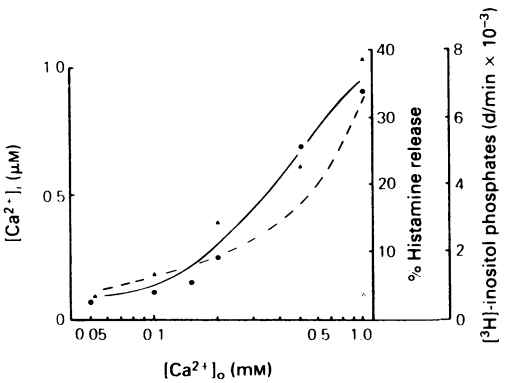

Figure 5 The Ca^{2+}-dependence of the maximum Ca^{2+} signal, histamine release and PI breakdown in 2H3 cells. The experimental protocol was as described in the legend to Figures 3 and 7. ---- Maximum $[Ca]_i$ in antigen-stimulated cells; (▲, △) total $[^3H]$-inositol phosphate production and (●, ○) histamine release after 20 min incubation in the presence (closed symbols) or absence (open symbols) of 2.5 μg/ml antigen.

Effect of external Ca^{2+} on the Ca^{2+} signal

Addition of 1 mM EGTA to the medium reduced the free calcium concentration to < 5 μM and caused an immediate drop in fluorescence due to the removal of Ca^{2+} from quin 2 which had leaked from the cells (Figure 4a). Aggregated ovalbumin failed to elicit a Ca^{2+} signal under these circumstances but this response was restored on addition of $CaCl_2$ to return the free calcium concentration in the medium to 1 mM. Once the response to antigen had been initiated it could immediately be aborted by the addition of EGTA and then restored by adding back $CaCl_2$ (Figure 4b). The fluorescence changes thus induced were more abrupt than the initial response to antigen, being complete in about 30 s. When the fluorescence signal is restored to its maximum by adding back calcium to the medium (Figure 4b) the time course of decay is the same as when the extracellular cation concentration is invariant (Figure 3). These data indicate that the antigen-induced Ca^{2+} signal is directly dependent on the level of extracellular Ca^{2+} ($[Ca]_o$). In fact the Ca^{2+} signal is induced to half-maximal levels in the presence of 0.4 mM $[Ca]_o$ and is only maximal when $[Ca]_o$ is > 1 mM (Figure 5). The histamine release profile exhibits a closely similar $[Ca]_o$ dependence (Figure 5).

If the quin 2-loaded cells are maintained in medium of low free Ca^{2+} (< 5 μM, Figure 4a) the rate of the slow decrease in fluorescence provides a measure of the rate of Ca^{2+} efflux from the cells. This was > 60 attomol $cell^{-1}$ min^{-1}, equivalent to the removal of 0.05 mM Ca^{2+} $cell^{-1}$ min^{-1}, assuming an intracellular volume of 1,100 fl. On readdition of Ca^{2+} to the medium, Ca^{2+}-depleted cells take up Ca^{2+} at comparable rates, indicating that at physiological concentrations of Ca^{2+} in the medium $[Ca]_i$ homeostasis is maintained by the dynamic balance of large calcium fluxes across the plasma membrane. This finding is consistent with previous deductions concerning calcium homeostasis in lymphocytes based on $^{45}Ca^{2+}$ flux measurements (Hesketh *et al.*, 1977).

Effect of M^{n+} ions on the Ca^{2+} signal

On addition of 10 μM La^{3+} the quin 2 fluorescence from loaded 2H3 cells remained stable, although an immediate drop in fluorescence occurs, similar to that due to EGTA addition, caused by quenching of external quin 2. This concentration of La^{3+} completely blocks the Ca^{2+} signal due to antigen, however, and when added after the signal has reached its maximum, immediately decreases $[Ca]_i$ to levels close to those before stimulation (Figure 6a). Chelation of La^{3+} by 1 mM Mg^{2+}-EGTA restores the Ca^{2+} signal (Figure 6a).

On the other hand, 100 μM Zn^{2+} slowly permeated 2H3 cells, as shown by the slow decline in intracellular quin 2 fluorescence (Figure 6b). In the presence of Zn^{2+}, however, aggregated ovalbumin caused a large rise in $[Ca]_i$, comparable with that normally observed (*cf.* Figures 3 and 6b).

The observations with La^{3+} and Zn^{2+} indicate that the former does not rapidly permeate 2H3 cells, because the resting $[Ca]_i$ is unperturbed. The inhibition by this ion of the antigen-induced fluorescence increase demonstrates, however, that although the normal influx and efflux path-

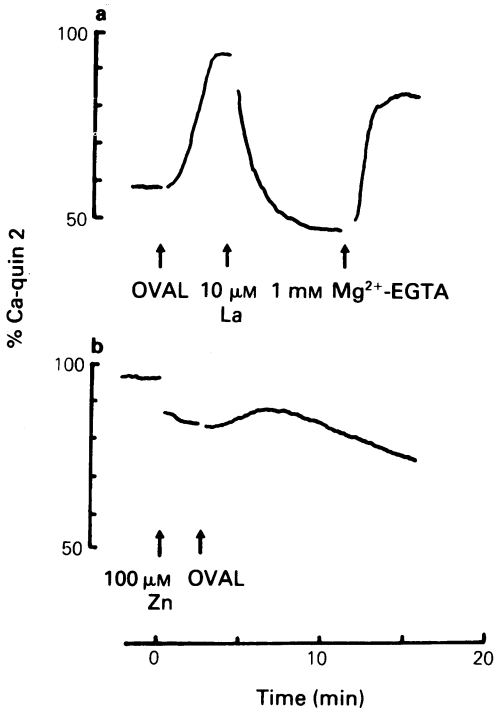

Figure 6 Effect of M^{n+} ions on the Ca^{2+} signal. (a) Addition of 10 μM La^{3+} after aggregated ovalbumin (OVAL), followed by 1 mM Mg^{2+}-EGTA. (b) Effect of 100 μM Zn^2 on the Ca^{2+} signal in response to aggregated ovalbumin.

ways of Ca^{2+} across the plasma membrane are unaffected, the Ca^{2+} signal pathway is inhibited. The direct correlation of the Ca^{2+} signal with histamine release described above was also observed when La^{3+} was used to inhibit the Ca^{2+} response. In the case of Zn^{2+}, however, histamine release was almost totally inhibited (< 5% of normal stimulated release), although the Ca^{2+} signal was essentially normal. Elevation of the intracellular concentration of this ion therefore appears to uncouple the Ca^{2+} signal from the physiological response.

The observations arising from the use of low concentrations of calcium in the medium or the addition of La^{3+} suggest that the signal influx pathway, activated in 2H3 cells by antigenic stimulation, is functionally independent of the normal influx pathway. This concept is supported by the fact that, although the Ca^{2+} signal is only half-maximal when $[Ca]_o = 0.4$ mM (Figure 5), $[Ca]_i$ in resting cells is constant at $[Ca]_o > 0.1$ mM. This suggests that external, low affinity Ca^{2+} site(s) (K_m = 0.4 mM) must be occupied when the signal pathway is activated. Furthermore, although depletion of cellular ATP to less than 5% of normal concentrations, for example by incubation with 10 mM sodium azide and 10 mM 2-deoxyglucose, totally inhibits the Ca^{2+} signal, it has little effect on $[Ca]_i$ in resting cells (data not shown).

The continuous requirement of metabolic energy and high $[Ca]_o$ for maintenance of the Ca^{2+} signal and the correlation of the latter with the extent of degranulation indicate that calcium influx due to the transient opening of a channel, allowing calcium to enter the cell down the concentration gradient, is insufficient to stimulate release. It appears that the signal is an actively maintained, dynamic balance of calcium influx and efflux. The rate of increase of quin 2 fluorescence following antigenic stimulation of sensitised 2H3 cells enables the net influx rate of the Ca^{2+} signal pathway to be estimated as > 1,000 attomol Ca^{2+} $cell^{-1}$ min^{-1}, equivalent to 0.91 mM Ca^{2+} $cell^{-1}$ min^{-1} and some 15-fold greater than the normal influx rate. At the maximum $[Ca]_i$ reached (> 1 μM) the efflux and influx rates must balance and it therefore appears that the rate of the normal efflux pathway may be increased by a similar factor in response to the rise in $[Ca]_i$ occurring in stimulated cells.

The Ca^{2+} signal in 2H3 cells and phosphatidylinositol breakdown

The above data indicate that elevation of $[Ca]_i$ is an early and obligatory event in the degranulation of 2H3 cells in response to antigen. In view of the hypotheses suggesting that the stimulation of phosphatidylinositol (PI) metabolism is connected with the increase in $[Ca]_i$ in the activation of cells of many types (Irvine *et al.*, 1982; Ishizuka *et al.*, 1983), we have compared the kinetics of the Ca^{2+} signal with those of the breakdown of pre-labelled PI, to determine whether the latter might be involved in Ca^{2+} signal generation in 2H3 cells.

The time course of production of [^{3}H]-inositol 1-phosphate, [^{3}H]-inositol (1,4)-bisphosphate and [^{3}H]-inositol (1,4,5)-trisphosphate in response to antigen in quin 2-loaded cells is compared with that of the change in $[Ca]_i$ in Figure 7. Increases in the levels of all three [^{3}H]-inositol phosphates were detectable within 10 to 40 s, coincident with the onset of the $[Ca]_i$ increase. The accumulation of [^{3}H]-inositol phosphates continued for at least 80 min, although histamine release was essentially complete within 30 min and the decrease in $[Ca]_i$ from the maximum

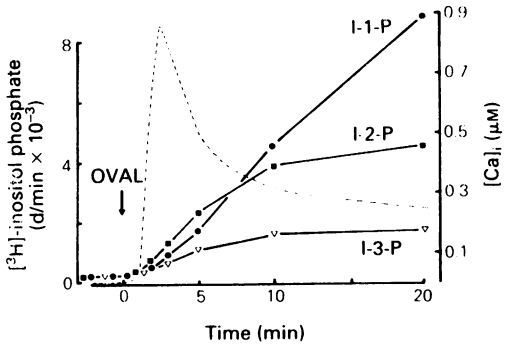

Figure 7 Relationship between $[Ca]_i$ and antigen-stimulated phosphatidylinositol breakdown in 2H3 cells. 2H3 cells were sensitised with IgE, labelled with [^{3}H]-inositol, loaded with quin 2 and suspended in medium A (Figure 3) containing 5 mM LiCl. 2.5 μg/ml aggregated ovalbumin (OVAL) was added at time zero and samples withdrawn and assayed for [^{3}H]-inositol phosphate by a method based on that of Berridge *et al.* (1982) and (Beaven *et al.*, 1984b). ---- $[Ca]_i$ (calculated from the % Ca^{2+}-quin in Figure 3 by the method of Hesketh *et al.*, 1983); ● [^{3}H]-inositol 1-phosphate (I-1-P); ■ [^{3}H]-inositol (1,4)-bisphosphate (I-2-P) and ▽ [^{3}H]-inositol (1,4,5)-trisphosphate (I-3-P).

began after about 3 min. The antigen dose-response curves for [^{3}H]-inositol phosphate production and histamine release were very similar, both being stimulated by 0.01 μg/ml aggregated ovalbumin and maximal at 1–10 μg/ml antigen (data not shown).

Effect of $[Ca]_o$ and M^{n+} ions on PI breakdown

Antigenic stimulation of PI breakdown showed a closely similar dependence on $[Ca]_o$ to that of the Ca^{2+} signal and histamine release (Figure 5). This observation suggests that PI breakdown might be a consequence of the rise in $[Ca]_i$ following stimulation of 2H3 cells. However, as described above, 100 μM Zn^{2+} or 10 μM La^{3+} both inhibit histamine release and the latter totally blocks the Ca^{2+} signal, but neither of these ions significantly inhibit stimulated PI breakdown (data not shown). Furthermore, A23187, which causes large increases in $[Ca]_i$ and release of histamine from 2H3 cells, has little effect on PI metabolism.

The close similarity between the profiles for PI breakdown and the Ca^{2+} signal when $[Ca]_o$ is varied suggests that a common, low affinity binding site(s) (K_m = 0.4 mM Ca^{2+}) activates both PI breakdown and the signal influx pathway. The fact that PI breakdown can be activated in the presence of low concentrations of La^{3+}, however, although the Ca^{2+} signal is blocked, leaves unresolved the central question of whether PI breakdown precedes the Ca^{2+} signal and is obligatory for its generation, or whether PI breakdown is stimulated independently of the signal influx pathway in 2H3 cells.

$[Ca]_i$ and pH changes in synaptosomes during transmitter release

Figure 8 represents changes in the free Ca^{2+} concentration $[Ca]_i$ in rat brain synaptosomes loaded with quin 2. Depolarisation of the synaptosomes by addition of KCl increases the % Ca^{2+}-quin 2 saturation in proportion to the K^+ added (Figure 8a) in an effect that is abolished if the Ca^{2+} in the medium is first completely chelated by EGTA. Equivalent additions of NaCl do not affect quin 2 fluorescence, indicating that the K^+-induced changes are not osmotic effects. The stable, free Ca^{2+} concentration within the synaptosomes at physiological concentrations of Ca^{2+} in the medium (1.2 mM) was estimated at 104 nM, using EGTA to quench any extra-synaptosomal Ca^{2+}-quin 2 fluorescence (as shown for 2H3 cells in Figure 4a). Neurotransmitter release by the synaptosome during potassium depolarisation was confirmed by a two-fold increase in α-amino [^{14}C] butyric acid release due to 22 mM K^+ which was undiminished in preparations loaded with quin 2 (Richards *et al.*, 1984).

The calcium channel blocker verapamil produced a small decrease in $[Ca]_i$ and depressed the increase therein due to K^+ depolarisation (Figure 8). The normal $[Ca]_i$ rise caused by K^+ depolarisation was antagonised by the subsequent addition of verapamil (Figure 8b). Similar results were obtained with 40 μM D600 (gallopamil).

These results indicate a direct correlation between $[Ca]_i$ and transmitter release in functional synaptosomes. Verapamil and D600 are known to act at micromolar concentrations as specific calcium channel blockers in excitable tissues (Reuter, 1983), and verapamil reduces both the release of putative amino-acid transmitters and the uptake of $^{45}Ca^{2+}$ by depolarised synaptosomes (Norris *et al.*, 1983). The present data using the intracellular Ca^{2+} indicator quin 2 are consistent with these effects, although they do not constitute conclusive evidence that verapamil specifically blocks calcium channels in synaptosomes.

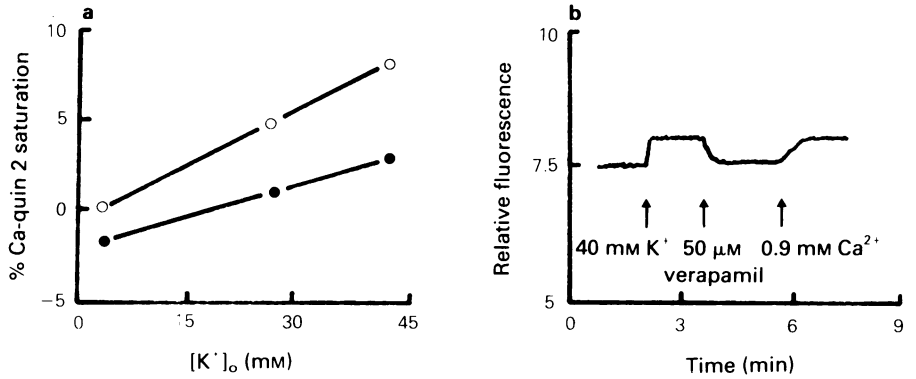

Figure 8 Change in % saturation of quin 2 due to K^+ depolarisation of synaptosomes. Synaptosomes prepared from rat brains were dispersed in artificial cerebrospinal fluid (ACSF) (2 mg protein/ml) and loaded with quin 2 acetoxymethyl ester (10 μM for 30 min at 37°C). The synaptosomes were washed twice, resuspended in ACSF (1.2 mM Ca^{2+}) and fluorescence determinations carried out as described in the legend to Figure 3. (a) % Ca^{2+}-quin 2 saturation as a function of the extrasynaptosomal K^+ concentration with (●) 50 μM verapamil and (○) without verapamil. (b) Time course of quin 2 fluorescence change due to 40 mM K^+ and the antagonism by 50 μM verapamil.

Intrasynaptosomal pH

Using the fluorescent indicator quene 1 the stable internal pH of unstimulated synaptosomes in medium containing 5 mM $NaHCO_3$ at pH 7.4 was 7.04. In the absence of $NaHCO_3$ the intrasynaptosomal pH decreased continuously, suggesting that the bicarbonate ion may regulate pH in nerve cells, as has been found in other systems (Thomas, 1976).

The addition of 10 mM NH_4Cl induced a rapid transient increase in pH (Figure 9), similar to that observed in lymphocytes (Rogers *et al.*, 1983) and fibroblasts (Moolenaar *et al.*, 1983). When synaptosomes were depolarised by addition of 40 mM K^+ there was no significant change in internal pH (Figure 9) although under the same conditions there is an increase in $[Ca]_i$ (Figure 8). This observation using the pH indicator quene 1 suggests that potassium depolarisation does not activate Ca^{2+}/H^+ exchange across the synaptosomal membrane.

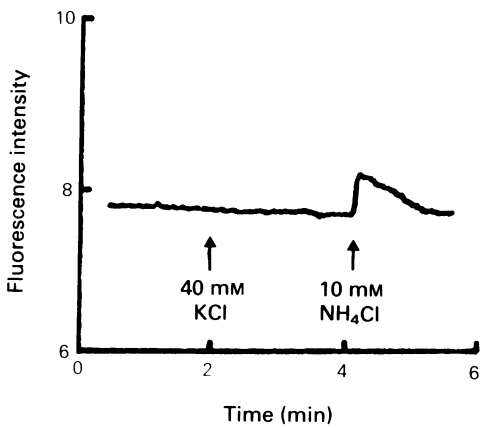

Figure 9 Changes in the internal pH of synaptosomes determined using the fluorescent indicator quene 1. Rat brain synaptosomes were loaded with indicator as described in the legend to Figure 8, but using 5 μM [^{3}H]-quene 1 acetoxymethyl ester. The fluorescence signals were quantitated by lysing the synaptosomes with 1.0% Triton X-100 in 0.5 mM EDTA/0.5 mM EGTA and making serial additions of 1 M Tris and 0.5 M HCl to calibrate the fluorescence of the released quene 1 as a function of pH (Rogers *et al.*, 1983).

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Discussion

Skidmore Both from experiments of others using less sensitive techniques and from this data, the rate of histamine release is maximal well before the peak calcium signal. Therefore it seems that very little of the calcium influx is responsible for histamine release.

Hesketh I take your point. The problem is that the *rate* of increase in the calcium signal is very rapid and the maximum rate of increase of intracellular calcium equates with the maximum rate of histamine release. It is probably incorrect to relate release of the mediator to the absolute *levels* of calcium.

Skidmore Have you done similar experiments mapping the calcium changes in rat pleural or peritoneal mast cells and does the response differ significantly from that seen in the 2H3 cells?

Hesketh No it does not differ significantly. The reasons why all data presented here refer to 2H3 cells is the high auto-fluorescence which is a major problem experienced with rat peritoneal cells.

Skidmore Are the kinetics of calcium signalling similar?

Hesketh Yes. It is quite surprising that the calcium signal is not faster in the peritoneal cell where histamine release is achieved more quickly. The quin-2 reaction is not rate-limiting since you can see much faster quin-2 calcium reactions in other cells.

Small When you added lanthanum after ovalbumin and the calcium signal went down, the new signal achieved was much lower than the original baseline, suggesting some effect on resting turnover.

Hesketh This is due to extracellular quenching by heavy metal ions of the indicator, rather than a physiological effect.

Church Is it possible to perform experiments combining quin-2 and perhaps ^{45}Ca to see whether the accumulation of calcium occurs as a result of movement from mitochondrial stores or from the extracellular environment?

Hesketh I have not specifically looked at this but it is possible to get, for example, the mitochondria to release the calcium by adding an inhibitor such as azide. This causes a massive transient increase in intracellular calcium levels, which is presumably reduced eventually by pumping out by the cell membrane. Alternatively, one could remove extracellular calcium and then look at the calcium signal in the presence of quin-2 which, if present, would suggest that the rise in calcium was derived from intracellular stores, of which mitochondria could be just one.

Skidmore Rat peritoneal mast cells do release histamine in the absence of extracellular calcium. Do these cells still produce a quin-2 fluorescence peak, under these conditions?

Hesketh In the 2H3 cells they do not; if you remove extracellular calcium, the quin-2 signal disappears but that is not a general rule.

Pearce If you activate rat peritoneal mast cells in the absence of extracellular calcium, you do see a rise in quin-2 fluorescence (see Pearce, 1985).

Barnes Have you worked with quin-2 in isolated smooth muscle cells? We have experienced high auto-fluorescence and were unable to distinguish a calcium signal on stimulation of contraction.

Hesketh No. We have collaborated with Taylor in San Diego examining a cultured muscle cell line in which quin-2 works well but this is clearly not the same. There are three problems; the first is that you cannot get the indicator into the cell, though we have only found this impossible so far in *E. coli*; the second problem is one of high intrinsic auto-fluorescence; the third is natural leakage of the indicator out of the cell.

Barnes Is the indicator specific for calcium; for example does magnesium interfere?

Hesketh Quin-2 is relatively insensitive to magnesium with about a 10^4 difference in affinity for binding calcium and magnesium. It is however exquisitely sensitive to heavy metal ions (e.g. lanthanum, zinc, cerium) which quench the substance.