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THE INFLUENCE OF INTRACELLULAR CALCIUM CONCENTRATION ON DEGRANULATION OF DIALYSED MAST CELLS FROM RAT PERITONEUM

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SUMMARY

1. Mast cells, isolated from rat peritoneum, were studied under tight-seal, whole-cell recording conditions. Membrane conductance, membrane capacitance and the concentration of free intracellular Ca^{2+}, $[Ca^{2+}]_i$, were measured simultaneously.

2. $[Ca^{2+}]_i$ could be accurately buffered to values between 0 and 1·5 μM only if relatively high concentrations of calcium buffers (in the millimolar range) were added to the pipette filling solution against which the cytoplasm was dialysed. At lower buffer concentrations $[Ca^{2+}]_i$ was markedly increased by hyperpolarizing the membrane.

3. When added to the pipette, guanosine-3-thio-triphosphate (GTP-γ-S), a non-hydrolysable analogue of guanosine triphosphate, stimulated a 3·3-fold increase in membrane capacitance, which is indicative of mast cell degranulation (Fernandez, Neher & Gomperts, 1984).

4. In weakly buffered cells, GTP-γ-S also induced a transient increase in $[Ca^{2+}]_i$ which, usually, preceded degranulation. Calcium buffers at 1–5 mM concentration suppressed this transient.

5. High $[Ca^{2+}]_i$ alone did not induce degranulation. However, it markedly accelerated GTP-γ-S-induced degranulation. When $[Ca^{2+}]_i$ was buffered to zero, an appreciable fraction of cells degranulated in response to GTP-γ-S, but very slowly, and only after a long lag phase.

6. Transient increases in $[Ca^{2+}]_i$, evoked either by GTP-γ-S, or by voltage changes, did not elicit capacitance changes during the lag phase, but accelerated the GTP-γ-S-induced degranulation response at later times.

7. Internally applied inositol 1,4,5-trisphosphate (IP_3) also induced transient increases in $[Ca^{2+}]_i$ which did not lead to secretion in the absence of GTP-γ-S.

8. It is concluded that an increase in $[Ca^{2+}]_i$ is neither necessary nor sufficient for secretion from dialysed mast cells. $[Ca^{2+}]_i$, however, acts synergistically with other stimuli to promote secretion. It is the more efficient the more time the other stimulus had been allowed for priming the cell.

INTRODUCTION

An increase in the concentration of free intracellular calcium ($[Ca^{2+}]_i$) is widely held to be a necessary and sufficient stimulus for histamine secretion in mast cells (Cochrane & Douglas, 1974; Foreman, Hallet & Mongar, 1977; Ishizaka, Hirata, Ishizaka & Axelrod, 1980; Gomperts, 1984). Studies with fluorescent indicator dyes such as quin2 and fura2 confirmed that such an increase actually occurs concomitant with secretion (White, Ishizaka, Ishizaka & Sha'afi, 1984; Beavan, Rogers, Moore, Hesketh, Smith & Metcalfe, 1984*b*; Neher & Almers, 1986).

However, tight-seal patch-clamp studies on single cells (whole-cell recording configuration) have shown that an increase in $[Ca^{2+}]_i$ alone is not sufficient to initiate secretion within the time span of a typical experiment (3–10 min). Dialysis of the cell interior with solutions buffered to $[Ca^{2+}]$ values between 0·2 and 10 μM-free calcium failed to induce the increase in membrane capacitance that is indicative of secretion (Fernandez, Neher & Gomperts, 1984). In this regard, mast cells differ markedly from chromaffin cells, where elevated $[Ca^{2+}]_i$ causes vigorous secretion as judged by an up to 2-fold increase in capacitance (Neher & Marty, 1982; Neher, 1986*a*; Penner, Neher & Dreyer, 1986).

Mast cells are known to lose their responsiveness to physiological stimulation by antigen within seconds to minutes after the beginning of the dialysis (Fernandez *et al.* 1984), probably due to the loss of some regulatory component(s) from the cytoplasm by diffusive dilution (or wash-out) across the cell–pipette boundary. Thereafter, a degranulation resembling the physiological response (Fernandez & Lindau, 1987) can still be elicited by intracellular application of GTP-γ-S, a non-hydrolysable analogue to GTP (Fernandez *et al.* 1984). The question arises whether lack of Ca^{2+} sensitivity is due to the loss of some regulatory component, or whether the mast cells are genuinely insensitive towards increased $[Ca^{2+}]_i$ in the absence of other stimuli. This paper describes some evidence in favour of the latter possibility. Secretion was assayed by electrical measurements, and $[Ca^{2+}]_i$ by the fluorescence of the indicator dye fura2 (Grynkiewicz, Poenie & Tsien, 1985). The results show that GTP-γ-S slowly sensitizes the exocytotic mechanism for Ca^{2+}, such that some 20–60 s after application of GTP-γ-S the rate of secretion can be markedly accelerated by increased $[Ca^{2+}]_i$.

The data from the combined electrical and optical measurements will also be used to outline the Ca^{2+}-buffering effects of various internal solutions under different experimental conditions. Some aspects of this investigation have been published in abstract form (Neher, 1986*b*; Neher, 1987).

METHODS

Rat peritoneal mast cells were obtained by peritoneal lavage after light ether anaesthesia and decapitation. They were plated onto cover-slips placed inside 35 mm culture dishes, and then stored in an incubator at 37 °C for 1–6 h until used. The medium for this incubation at 5% CO_2 contained (in mM): NaCl, 140; KCl, 2·8; $MgCl_2$, 2; $CaCl_2$, 1; HEPES–NaOH, 10; glucose, 5; $NaHCO_3$, 45; KH_2PO_4, 0·4; streptomycin (0·12 mg/ml); penicillin (64 μg/ml); pH 7·2. Experiments were performed at 23–26 °C in a Mg^{2+}-rich saline of the following composition (in mM): NaCl, 140; KCl, 2·5; $CaCl_2$, 2; $MgCl_2$, 5; glucose, 5; HEPES–NaOH, 10; pH 7·2. High $[Mg^{2+}]$ was found to be

helpful during seal formation as it prevented ATP-induced leaks (Bennett, Cockcroft & Gomperts, 1981).

Patch-clamp measurements were performed with Sylgard-coated pipettes as described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). Pipette resistance ranged between 2 and 3·5 MΩ when filled with the standard intracellular solution containing (in mM): potassium glutamate, 135; NaCl, 20; $MgCl_2$, 1; HEPES–NaOH, 10; Na_2ATP, 0·2; fura2 pentapotassium salt, 0·1; and various additions of Ca–EGTA buffer as indicated. GTP-γ-S was added at a 40 μM concentration. In twenty-one out of ninety-one recordings, however, only 20 μM-GTP-γ-S was used. Three experiments had 0·5 mM-ATP instead of 0·2 mM and four experiments were performed in a high-calcium (5 mM) Ringer solution. With these pipette filling solutions a liquid junction potential of −8 mV (potassium glutamate-side negative) developed at the tip of the pipette while the pipette was immersed in external saline. The potential values given have been corrected for this liquid junction potential, i.e. for a holding potential of 17 mV, the reading on the patch-clamp amplifier was set to +25 mV.

For preparing Ca–EGTA buffers a procedure was adopted that allows for possible impurities in EGTA (Miller & Smith, 1984): EGTA was neutralized with KOH for a final concentration of 200 mM at pH 6·5. A portion of this solution was diluted twofold, titrated to pH 7·2, and stored as 100 mM-stock solution of free EGTA at −20 °C. The other portion received small additions of $CaCl_2$. Between additions the solution was titrated back to pH 6·5 by KOH. Initially, the restoration of pH 6·5 required two moles of KOH for each mole $CaCl_2$ added. When calcium additions came close to saturation of EGTA the amount of KOH required dropped rapidly. The point where this amount reached one mole of KOH per mole of $CaCl_2$ was taken to indicate saturation of EGTA by Ca^{2+} (this was at 96 equivalents of EGTA for 100 equivalents of calcium). Subsequently, the solution was diluted for 100 mM final EGTA content and titrated to pH 7·2. It was frozen and stored at −20 °C as 100 mM stock of EGTA fully saturated with Ca^{2+}. A control measurement with a Ca^{2+}-sensitive electrode indicated that the concentration of free Ca^{2+} in that solution was ~ 20 μM. This assures that total calcium is equal to total EGTA within 1 % accuracy. Ca–EGTA buffers were made by adding various amounts of the two solutions to the pipette filling solution given above. $[Ca^{2+}]$ was calculated assuming an apparent dissociation constant (K_D) of the Ca–EGTA complex of 0·15 μM (Grynkiewicz *et al.* 1985). Besides improved accuracy the procedure described has the advantage that solutions do not acidify when mixing, as occurs when $CaCl_2$ is added to EGTA-containing solutions.

Capacitance measurements were performed with a two-phase lock-in amplifier in combination with a PDP 11 laboratory computer. Contrary to the procedure given by Neher & Marty (1982) the method employed now allowed tracking of large changes in capacitance as occur during mast cell degranulation. Details are given by Lindau & Neher (1987). Briefly, a 16 mV r.m.s., 800 Hz sine wave was added to the command potential of the patch clamp. The resulting sinusoidal current was measured by the lock-in amplifier at two mutually orthogonal phase angles. These signals were fed to the AD inputs of the computer together with the applied potential and the DC current (after filtering out the sinusoidal component). The computer used the three measured quantities to calculate the parameters of the equivalent circuit which are series resistance, R_S, membrane capacitance, C_M, and the parallel combination of leak and membrane conductance, G_M. The contributions of the pipette to the lock-in signals were cancelled during the cell-attached configuration before patch rupture.

Fluorescence measurements on single cells were performed as described by Almers & Neher (1985). Briefly, a Zeiss IM 35 inverted microscope was equipped with a Xenon lamp, epifluorescence and a photomultiplier (photometer SF, Zeiss). Cells were loaded with fura2 pentapotassium salt by diffusion from the recording pipette. Fluorescence of fura2 was excited alternately by light at 360 and 390 nm by means of a rotating filter wheel fitted to a slot in the excitation pathway of the microscope. Light was collected from a circular area of 16 μm diameter in the centre of the field of view where the cell under investigation had been placed. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio as described by Grynkiewicz *et al.* (1985). Calibration constants were determined within single mast cells as described by Almers & Neher (1985). As the work progressed over a time period of about 9 months, the calibration constants seemed to change slowly, probably as a consequence of ageing of the lamp. For example, initially a limiting fluorescence ratio of 0·135 was determined when a cell was loaded with 10 mM-EGTA. A few months later experiments under similar conditions gave a ratio of 0·12; the change corresponding to −34 nM-Ca^{2+}. Calibration

measurements at zero Ca^{2+} were therefore repeated occasionally. If the result differed significantly from the previous one, both high and low limiting ratios were scaled by the same factor such that the low ratio agreed with the new measurement. It is estimated that values of $[Ca^{2+}]_i$ given may be in error of up to 50 nM due to these changes in calibration constants.

Unlike in earlier work (Almers & Neher, 1985), a hard-wired instrument based on sample-and-hold circuits was used to track the fluorescence signals at the two excitation wavelengths. Also, some of the experiments were performed on a set-up equipped with a Neofluar 1·3 NA, 100×, oil immersion objective (instead of 63×, 1·25 NA), and excitation filters made from Schott glasses. A combination of UG1 and GG385 (3 mm) was used for the long wavelength and UG11 (3 mm) for the short wavelength. The latter, together with the filter action of glass lenses of the microscope, resulted in a short-wave bandpass centred at wavelengths slightly larger than the isosbestic wavelength of fura2.

A total of six signals (two fluorescence intensities; two lock-in signals; DC current and voltage) were fed to the computer and sampled every 0·5–2 s. Subsets of these and $[Ca^{2+}]_i$ were displayed on-line on a vector display. Permanent copies were made later on a HP 7470A (Hewlett–Packard) plotter.

Materials. Salts were obtained from Merck; EGTA and HEPES were from Sigma; GTP-γ-S was purchased from Boehringer and subsequently purified on HPLC (high-pressure liquid chromatography) by Dr F. Eckstein at the Max-Planck-Institut für Experimentelle Medizin, Göttingen. IP_3 (D-myo-inositol 1,4,5-trisphosphate) was obtained from Amersham. Fura2 pentapotassium salt was from Molecular Probes.

RESULTS

Responses to a GTP-γ-S stimulus with weakly buffered cytosolic calcium

The experiment of Fig. 1 shows how electrical and fluorescence measurements may be used to simultaneously observe secretion and changes in cytosolic $[Ca^{2+}]_i$. The pipette sealed against the cell membrane contained standard solution (see Methods) which includes 40 μM-GTP-γ-S plus 0·15 mM-free EGTA and 0·15 mM-Ca–EGTA. The pipette potential was +17 mV. A positive potential was selected because under these conditons $[Ca^{2+}]_i$ was more stable and less dependent on leakage effects (see below). When the patch beneath the pipette tip was ruptured (arrow), a small outward current appeared (trace *A*) as an approximately 300 nS electrical connection was made with the cytosol (*B*). The somewhat irregular, but nearly ohmic current probably flowed through a leak conductance (0·3 nS in this experiment). At the time of patch rupture, the capacitance trace reported an increase of roughly 6 pF, the membrane capacitance of this particular cell, and fluorescence began to rise as fura2 entered the cell, approaching a maximum value with a half-time of about 20 s. As soon as fluorescence intensity reached a level allowing the fluorescence ratio at the two excitation wavelengths to be measured accurately, $[Ca^{2+}]_i$ reached a level of about 0·1 μM (trace *D*).

As the GTP-γ-S diffusing into the cell took effect, $[Ca^{2+}]_i$ slowly rose until a prominent Ca^{2+} transient developed, very similar to those described by Almers & Neher (1985), and Neher & Almers (1986). Following the transient, $[Ca^{2+}]_i$ stabilized at a level close to 0·25 μM. Such Ca^{2+} transients were observed regularly when GTP-γ-S was included in the pipette, but not when the nucleotide was absent. Capacitance started to increase immediately following the Ca^{2+} transient as the cell degranulated in response to GTP-γ-S (see Fernandez *et al.* 1984). The concomitant decline in series conductance (trace *B*) was typically, though not always, observed.

During the period marked by the bar, the holding potential was changed to −58 mV. This caused an inward current, as well as a rise in $[Ca^{2+}]_i$ to about 0·4 μM.

While $[Ca^{2+}]_i$ was increased, the time course of degranulation showed signs of being accelerated; this point will be taken up later. When the holding voltage was returned to 17 mV, $[Ca^{2+}]_i$ returned to about 0·25 μM. Similar effects of holding voltage on $[Ca^{2+}]_i$ were observed regularly in mast cells. They were more prominent when the input conductance of the cell was higher (see below), and smaller when it was lower (0·1–0·2 nS in some experiments). The $[Ca^{2+}]_i$ changes probably reflect changes in Ca^{2+} influx due to changes in electrical driving force across leaks or channels. No other conspicuous voltage-dependent phenomena were observed in mast cells.

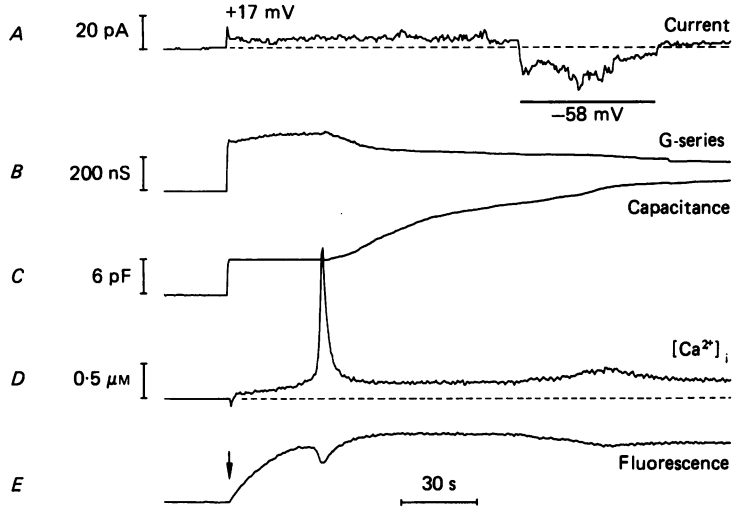

Fig. 1. GTP-γ-S-induced changes at loosely buffered calcium. The pipette contained 0·15 mM-Ca–EGTA and 0·15 mM-free EGTA in addition to the standard salts. The arrow marks the time of patch rupture. Holding voltage was +17 mV except for some period towards the end (marked by horizontal bar) when it was changed to −58 mV. *A*, current; *B*, series conductance; *C*, capacitance; *D*, free intracellular calcium; *E*, fluorescence at approximately 365 nm excitation wavelength in arbitrary units. Dashed lines indicate zero.

The zero-current potential of the cells was not investigated systematically. Infrequent tests gave values close to 0 mV (see also Lindau & Fernandez, 1986*a*). The majority of experiments was performed at a holding potential of +17 mV.

The effect of EGTA buffers on $[Ca^{2+}]_i$

Following Ca^{2+} transients as in Fig. 1, $[Ca^{2+}]_i$ approached a steady-state value that depended on the Ca^{2+}/EGTA ratio of the buffer, on the buffer strength, on the membrane voltage and on membrane conductance. It was suggested above that the influence of voltage and membrane conductance is due to voltage-driven Ca^{2+} inflow through leaks. Therefore, the influence of buffer composition on $[Ca^{2+}]_i$ will be considered first at positive membrane potentials (usually 17 mV) where the influence of leaks is small. Cells with membrane conductance smaller than 2 nS were selected, to further limit leakage effects.

Figure 2*A* plots the deviation $\Delta[Ca^{2+}]$ of the measured steady-state $[Ca^{2+}]_i$ from

the equilibrium calcium concentration of the buffer mixture, $[Ca^{2+}]_E$, *versus* the latter quantity. Only experiments with low buffer capacity were included in this plot. It is seen that for small values of $[Ca^{2+}]_E$ the measured $[Ca^{2+}]_i$ is always higher whereas for large $[Ca^{2+}]_E$ values $[Ca^{2+}]_i$ tends to be smaller than $[Ca^{2+}]_E$. This most likely reflects the action of endogenous calcium buffers tending to maintain $[Ca^{2+}]_i$ at its natural value. The best-fit line to all the data points intersects the abscissa at 0·28 μM which may be taken as an estimate for $[Ca^{2+}]_i$ in the absence of exogenous chelators under GTP-γ-S stimulation.

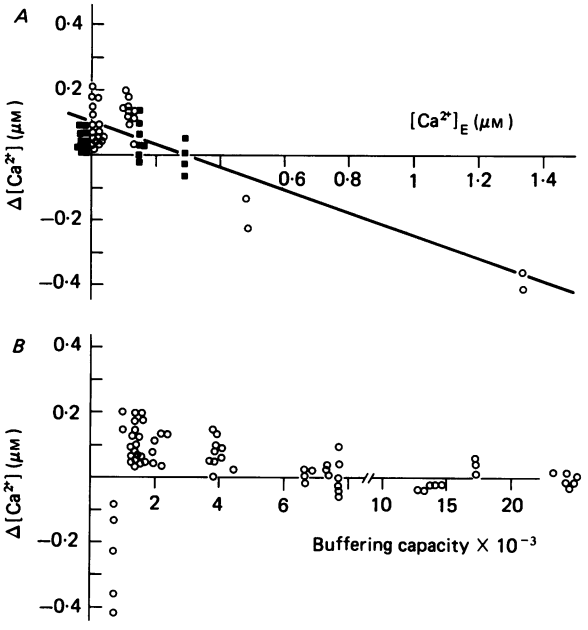

Fig. 2. The influence of calcium buffers on $[Ca^{2+}]_i$. The quantity plotted on the ordinate in both *A* and *B* is the difference ($\Delta[Ca^{2+}]$) between measured intracellular free calcium $[Ca^{2+}]_i$ and the equilibrium calcium concentration $[Ca^{2+}]_E$. The $[Ca^{2+}]_i$ was measured approximately 1–2 min after patch rupture. In *A*, $\Delta[Ca^{2+}]$ is plotted against $[Ca^{2+}]_E$ (see above); in *B* it is plotted against the equilibrium buffering capacity (κ) of the pipette filling solution. See Fig. 3 for an equivalent buffer concentration scale. Table 1 gives the definition of κ and exemplar values for $[Ca^{2+}]_E$ and κ. Each symbol represents one cell. In some instances the symbols are slightly shifted along the abscissa for clearer representation. Circles in *A* represent experiments with $\kappa < 3000$ whereas squares represent experiments with $3000 < \kappa < 10000$. In *B*, six measurements plotted at the very right end of the abscissa were obtained with $25000 < \kappa < 70000$.

The scatter in $[Ca^{2+}]_i$ values decreased when pipettes were filled with solutions of higher buffering capacity. Buffering capacity κ is defined as $d[Ca^{2+}]_{tot}/d[Ca^{2+}]_{free}$ where $[Ca^{2+}]_{tot}$ is the total calcium concentration in a given solution and $[Ca^{2+}]_{free}$ is the free calcium concentration. Table 1 given some examples of the buffer mixtures used, their equilibrium calcium concentration, $[Ca^{2+}]_E$, and the buffering capacity. Figure 2*B* plots the $\Delta[Ca^{2+}]$ values from Fig. 2*A* against the buffering capacity of the pipette filling solution. It also includes values from solutions with high buffering capacity ($\kappa > 10000$, which is, for instance, > 3 mM-EGTA–3 mM-Ca–EGTA). It is

seen that at low buffer capacity, the deviations varied between +0·2 and −0·42 μM with the extremes coming from experiments where $[Ca^{2+}]_i$ was weakly buffered to extremely low or high values, respectively (as shown in *A*). At $\kappa > 10000$, $[Ca^{2+}]_i$ values varied much less. For the latter values mean and standard deviations of $\Delta[Ca^{2+}]$ were found to be 1 ± 30 nM in thirteen cells. This scatter at high buffering capacity to a large part reflects errors in the calibration constants of the Ca^{2+}-measuring system (see Methods).

TABLE 1. Calcium buffers and buffer capacity of some of the solutions used assuming an apparent dissociation constant for EGTA of 0·15 μM at pH 7·2 (Grynkiewicz *et al.* 1985)

Ca^{2+} bound to EGTA (mM) ([Ca.E])	0	0	0	0·15	1
Free EGTA (mM) ([E])	0·1	0·5	10	0·15	1
Free calcium (μM)† ($[Ca^{2+}]_E$)	0	0	0	0·09	0·14
Buffering capacity*† (κ)	1320	3970	66880	1030	3800
Ca^{2+} bound to EGTA (mM) ([Ca.E])	5	7·5	1·6	1·8	9·1
Free EGTA (mM) ([E])	5	2·5	0·4	0·2	0·9
Free calcium (μM)† ($[Ca^{2+}]_E$)	0·15	0·44	0·48	0·91	1·37
Buffering capacity*† (κ)	17100	4430	790	280	658

* $\kappa = \frac{d[Ca^{2+}]_{tot}}{d[Ca^{2+}]_{free}} = [E]_{free}/(K_D + [Ca^{2+}])$. This definition differs from the more common definition of buffering capacity for pH buffers which is d[base]/dpH. It was preferred because the quantity measured here is $[Ca^{2+}]_i$ and not pCa.

† Fura2 pentapotassium salt was treated in this calculation as if it were EGTA. It was present at 0·1 mM in all solutions.

At positive potentials no marked effect of membrane conductance (G_M) on $\Delta[Ca^{2+}]$ could be observed even at the lowest buffering capacity as long as G_M was smaller than 2 nS. For larger membrane conductance and low κ ($300 < \kappa < 2000$) deviations as large as 0·5 μM were observed. These cells, however, were probably damaged, since these high values of G_M were usually associated with irregular, discrete changes in G_M. With moderately to strongly buffered solutions ($\kappa > 4000$) even these cells did not deviate more than 0·15 μM from the equilibrium buffer value as long as G_M remained smaller than 10 nS.

At negative potentials (−20 to −60 mV) the influence of G_M was much more dramatic. With low buffering capacity ($\kappa < 2000$) even cells as low in input conductance as 0·5 nS showed a $\Delta[Ca^{2+}]_i$ of 0·2 μM or more. Only highly buffered cells ($\kappa > 10000$) had $\Delta[Ca^{2+}]$ values smaller than 0·1 μM for conductance values up to 4 nS.

These results indicate that under conditions that more or less cover the range of electrophysiological experiments, $[Ca^{2+}]_i$ is under control if the internal solution has a buffering capacity of 10000 or more, as may be achieved with a mixture of 3 mM-Ca–EGTA and 3 mM-free EGTA. This conclusion will be reinforced later by considering Ca^{2+} transients.

Ca^{2+} transients

Ca^{2+} transients like those shown in Fig. 1*A* were observed in forty-four cells dialysed with weakly to moderately buffered solutions. In five cells a first transient was followed by a second one. In twenty-five moderately to highly buffered cells no

transient could be observed. The latency of the transients was 43 ± 23 s (mean $\pm$ S.D.) largely independent of buffering strength and $[Ca^{2+}]_E$. The amplitude, however, was severely attenuated at higher buffering capacity. Figure 3 plots the amplitude of transients against the instantaneous buffering capacity, κ', calculated from the total EGTA concentration, $[E]_{tot}$, and the measured $[Ca^{2+}]_i$ at the base of the transient according to

$$\kappa' = [E]_{tot} K_D / ([Ca^{2+}]_i + K_D)^2 .$$

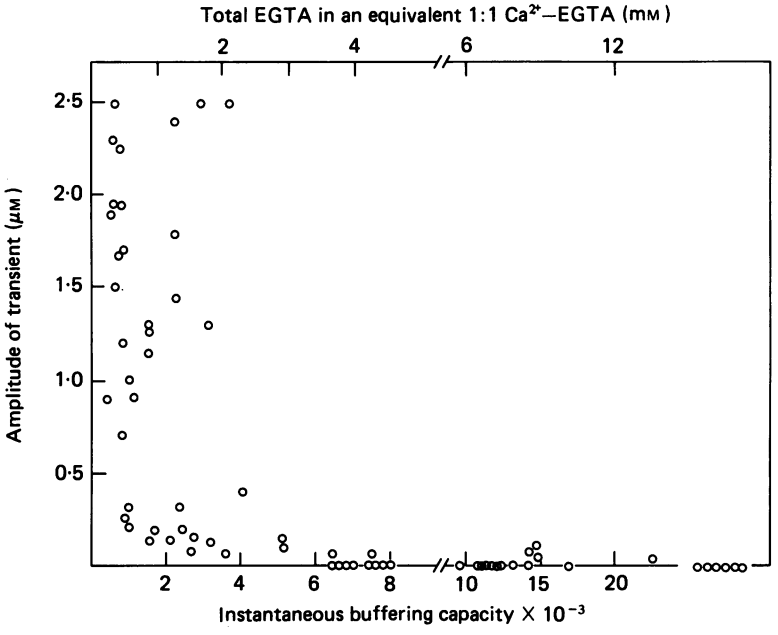

Fig. 3. Amplitude of Ca^{2+} transients as a function of instantaneous buffering capacity. Each cell is represented by one symbol. Amplitude was measured as the difference between peak $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ shortly before the transient. Amplitudes larger than 2·5 μM were plotted at 2·5 μM since the fura2 measurement is not very reliable at these high calcium concentrations. Cells which did not produce a transient were plotted at the baseline. For these cells the equilibrium buffer capacity κ (see text) was used for the abscissa, rather than κ'. The scale above the Figure gives the equivalent total EGTA in a 1:1 mixture of Ca^{2+}-bound and free EGTA, which has the same buffer capacity as given in the scale below. It should be noted, however, that pipettes did not always contain 1:1 mixtures, but solutions as given in Table 1. Values in the Figure are plotted against the lower scale, which is κ'.

This was preferred over κ in order to allow for the fact that in many cases $[Ca^{2+}]_i$ at the base of the transient deviated quite markedly from $[Ca^{2+}]_E$. It is seen that full-sized transients could occur up to $\kappa' = 4000$. However, in many cells transients were reduced in size already at $\kappa' \sim 1000$. Above $\kappa' = 6000$, transients were smaller than 0·05 μM. All of the cells where no transients were observed had κ' values larger than 6000. For the above calculation of κ' it was assumed that at the time of the transient, EGTA had equilibrated already between the pipette and the cell interior. This is likely to be the case in most experiments, as Fig. 1*E* shows that fura2

(molecular weight 831) had reached approximately 70% of its final concentration at the time of the transient.

Under the assumption of spatial homogeneity one can calculate the amount of calcium captured by EGTA (together with fura2) during the rising phase of the transient. The concentration of calcium bound to EGTA [Ca.E] is given by

$$[\mathrm{Ca.E}] = [\mathrm{E}]_{\mathrm{tot}} \frac{[\mathrm{Ca}^{2+}]_{\mathrm{i}}}{[\mathrm{Ca}^{2+}]_{\mathrm{i}} + K_{\mathrm{D}}}.$$

Thus, the amount of calcium taken up (expressed as a concentration effective throughout the accessible cell volume), is:

$$\Delta[\mathrm{Ca.E}] = ([\mathrm{E}]_{\mathrm{tot}} + [\mathrm{F}])\,\{[\mathrm{Ca}^{2+}]_{\mathrm{i,peak}}/([\mathrm{Ca}^{2+}]_{\mathrm{i,peak}} + K_{\mathrm{D}}) - [\mathrm{Ca}^{2+}]_{\mathrm{i,base}}/([\mathrm{Ca}^{2+}]_{\mathrm{i,base}} + K_{\mathrm{D}})\},$$

where $[\mathrm{E}]_{\mathrm{tot}}$ is the total concentration of EGTA and [F] is the total concentration of fura2. For simplicity, the chelating effect of fura2 was treated as if it were identical to that of EGTA.

Values for Δ[Ca.E] ranged between 70 and 950 μM with a mean $\pm$ S.D. of 285 ± 220 μM. There was a clear positive correlation between Δ[Ca.E] and buffering capacity. Such a correlation is expected because at high buffering capacity EGTA can compete better with the endogenous buffers for the calcium. Unfortunately, precise conclusions cannot be drawn from these data, since at high buffering capacity no transient could be observed at all in many cells. These are likely to be cells with small Δ[Ca.E] values, such that the remaining values are biased. It is clear, however, that more than 100 μM-calcium can be captured by high EGTA (2–5 mM) during a transient.

The half-width of the transient was in the range 2·5–6 s for full-sized transients (> 1 μM) and between 4 and 17 s for strongly attenuated transients ($< 0{\cdot}2$ μM).

Transients of very similar form were also observed when the pipette contained 0·2–1 μM-inositol 1,4,5-trisphosphate (IP_3) instead of GTP-γ-S. Figure 4 compares transients under IP_3 with those induced by GTP-γ-S. Their half-width was somewhat shorter (2–4 s). The main difference with respect to GTP-γ-S-induced transients was that their latency after patch rupture was only 1–10 s (twenty experiments). In fact, at higher IP_3 concentrations (2–10 μM), transients appeared so early that only the trailing edges could be observed. At concentrations $< 0{\cdot}2$ μM no transients were elicited at all. In contrast, GTP-γ-S-induced transients never occurred earlier than about 10 s, even at higher GTP-γ-S concentration (see also Fernandez & Lindau, 1987). The other difference between the two cases was that IP_3-induced transients were never followed by degranulation.

Capacitance changes

When $[\mathrm{Ca}^{2+}]_{\mathrm{i}}$ was only weakly buffered, degranulation usually started during the $[\mathrm{Ca}^{2+}]_{\mathrm{i}}$ transient or immediately afterwards, as judged by the capacitance increase (see also Fig. 1). In some cells a slight capacitance increase could be observed already before the transient. In ninety cells the mean value of capacitance before degranulation was $6{\cdot}44 \pm 1{\cdot}36$ pF (mean $\pm$ S.D.). In those cases where degranulation

was allowed to go to completion, capacitance increased by a factor of $3{\cdot}29 \pm 0{\cdot}58$ (forty-four cells). This degranulation amplitude did not depend on $[Ca^{2+}]_i$. The average, given above, excludes cells which did not degranulate, but it includes some cells which degranulated very slowly due to buffering to low $[Ca^{2+}]$ values (see below). If only cells that degranulated within 200 s were counted, the ratio was $3{\cdot}58 \pm 0{\cdot}57$ (fifteen cells).

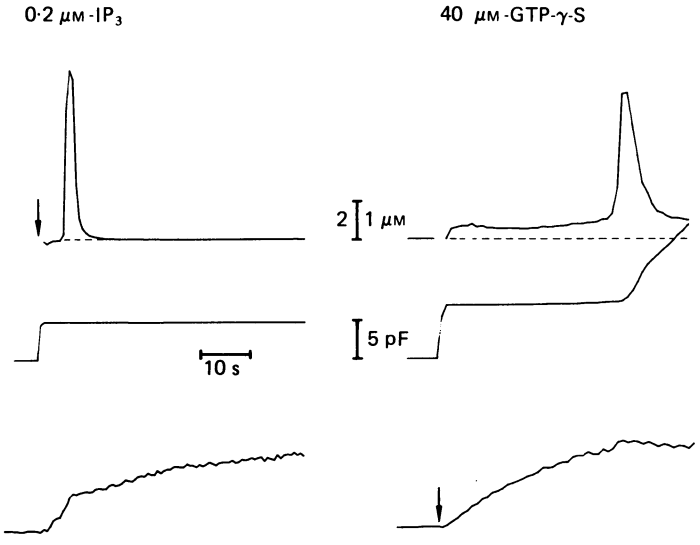

Fig. 4. Ca^{2+} transients induced by IP_3; comparison with GTP-γ-S-induced transients. The lowermost trace in each case shows the fluorescence intensity in arbitrary units at approximately 355 nm for an illustration of the dye-loading time course after patch rupture (arrow). D-myo-inositol (1,4,5)-trisphosphate (0·2 μM) was contained in the pipette during the recording on the left side. It gained access to the cytoplasm at the time of the patch rupture together with fura2. The right side shows an experiment similar to the one of Fig. 1. In both experiments the pipette contained 0·1 mM-EGTA in addition to the usual salts; ATP was missing in the experiment with IP_3; the GTP-γ-S experiment had 0·5 mM-ATP instead of the usual 0·2 mM.

Both the latency of the capacitance increase and the rise time were markedly influenced by the calcium concentration. Figure 5 gives two examples of cells strongly buffered at low (*A*) or high (*B*) $[Ca^{2+}]$ values. Figure 6 plots mean values against $[Ca^{2+}]_i$. Rise time was defined as the time difference between 20 and 80% of the degranulation amplitude. Latency was the time difference between patch rupture and the 20% point. The values plotted close to the ordinate are averages from seventeen cells buffered to values smaller than 60 nM. Eight cells buffered to this low $[Ca^{2+}]_i$ did not start to degranulate within the observation period of 7–8 min. They are excluded from this plot. In five additional cells, loaded with 10 mM-EGTA, external calcium was withdrawn and replaced by 0·1 mM-EGTA (four cells) or 1 mM-EGTA (one cell). Three of these, including the cell at 1 mM-external EGTA, degranulated within 5–10 min of patch rupture.

The mean delay of the degranulation response decreased from about 200 s at zero

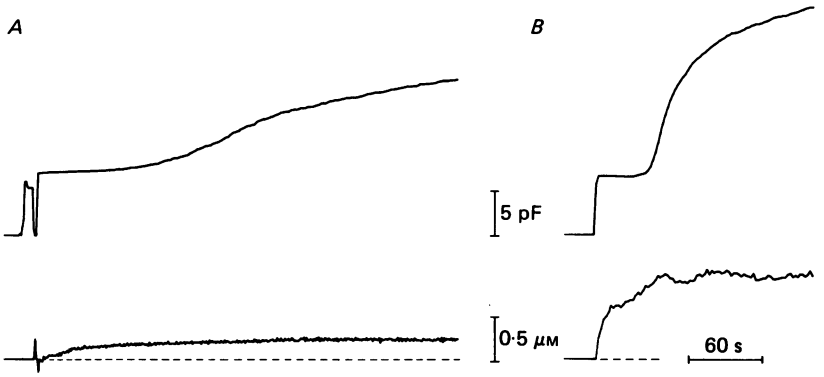

Fig. 5. The degranulation response under conditions of strong calcium buffering to low (*A*) and high (*B*) calcium concentrations. In *A* the pipette contained 5 mM-Ca–EGTA and 5 mM-free EGTA which brought $[Ca^{2+}]_i$ to values between 0·15 and 0·2 μM. In *B* the pipette contained 9·1 mM-Ca–EGTA and 0·9 mM-free EGTA which brought $[Ca^{2+}]_i$ close to 1 μM. Note that $[Ca^{2+}]_E$ of 1·5 μM was not reached in this cell since buffering capacity of this solution is only about 500. Both cells were obtained from the same animal.

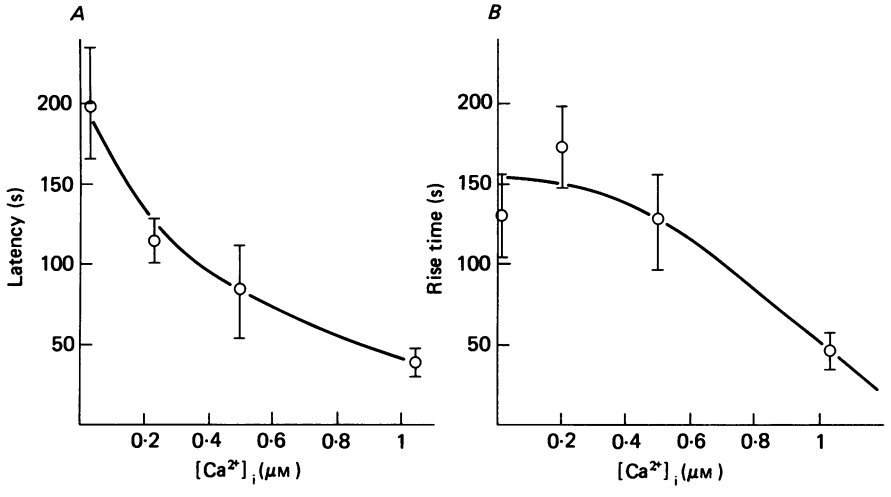

Fig. 6. Latency (*A*) and rise time (*B*) of degranulation responses as a function of free intracellular calcium. Mean values ± standard error of the mean are given. Values from forty-six experiments were grouped into four categories according to $[Ca^{2+}]_i$ values, measured at steady state. There were seventeen experiments at $0\ \mu M \leqslant [Ca^{2+}]_i \leqslant 0{\cdot}06\ \mu M$; sixteen experiments at $0{\cdot}15\ \mu M \leqslant [Ca^{2+}]_i \leqslant 0{\cdot}3\ \mu M$; eight experiments at $0{\cdot}35\ \mu M \leqslant [Ca^{2+}]_i \leqslant 0{\cdot}7\ \mu M$ and five experiments at $0{\cdot}8\ \mu M \leqslant [Ca^{2+}]_i \leqslant 1{\cdot}5\ \mu M$. Experiments at low $[Ca^{2+}]_i$ in which the cells did not degranulate were excluded from this plot. These are eight experiments for the lowest point and two experiments for the value at 0·25 μM. See text for further explanations.

$[Ca^{2+}]_i$ to about 50 s at ~ 1 μM (Fig. 5). The rise time also shortened when $[Ca^{2+}]_i$ was increased above 0·5 μM.

The experiments show that elevated $[Ca^{2+}]_i$ speeds degranulation. However, it should be pointed out that degranulation can occur at a $[Ca^{2+}]_i$ as small as 10 nM. About half of the cells examined at EGTA/Ca^{2+} ratios > nine degranulated (solutions

contained either 10 mM-EGTA alone, or 1 mM-Ca–EGTA/9 mM-EGTA, or 0·5 mM-Ca–EGTA/4·5 mM-EGTA, or 2 mM-EGTA alone). It should also be noted that while low $[Ca^{2+}]_i$ retards degranulation, it has no such effect on the latency of Ca^{2+} transients.

Development of Ca^{2+} sensitivity following stimulation

The experiments above have shown that when $[Ca^{2+}]_i$ is kept constant by strong buffering, the rate of degranulation is strongly influenced by $[Ca^{2+}]_i$. More information on the relationship between degranulation and $[Ca^{2+}]_i$ can be obtained by changing $[Ca^{2+}]_i$ during an experiment. A first clue can be obtained by observing the temporal relationship between the endogenous Ca^{2+} transient and the degranulation response. The latency of the transient displays considerable variability between cells. It is most noteworthy that there is no rigid coupling between the transient and the degranulation response. Among nineteen cells with Ca^{2+} transients larger than 1 μM, seven cells did not change their capacitance visibly during the transient. In a few cells degranulation started during the transient (see Fig. 4*B* for an example) and in five cells degranulation started already before the transient. In these cases the degranulation rate was markedly accelerated during the transient. Figure 7 compares two cases with extreme time relationships. In *A* the transient goes to completion before degranulation starts. In *B* the cell has gained already 1·6 pF at the time the transient rises; the rate of degranulation changes approximately 10-fold within 3–4 s while $[Ca^{2+}]_i$ is high, and diminishes again when $[Ca^{2+}]_i$ returns to basal levels.

These examples show that calcium is definitely not the most important parameter influencing degranulation in mast cells. It appears that another stimulus has to be acting, which in the experiments reported here is GTP-γ-S or some signal derived from it. This other signal has to prime the cell and start secretion. Only thereafter can calcium accelerate the secretory process. This was particularly obvious in a few examples when two transients appeared in sequence. Usually, the first transient did not induce degranulation, whereas the second one transiently increased the rate of secretion. Figure 8 shows an example.

Changes in $[Ca^{2+}]_i$ can also be brought about by changes in membrane voltage (see Fig. 1). This can be utilized to probe the cell's calcium sensitivity at various times after GTP-γ-S stimulation. Figure 9 shows an experiment where this effect was amplified by increased extracellular calcium (5 mM). The cell was repeatedly hyperpolarized to -108 mV. Initially, these hyperpolarizations were not very effective because membrane resistance was high. Membrane hyperpolarization was increased stepwise until at -108 mV $[Ca^{2+}]_i$ started to rise abruptly due to the sudden appearance of some leak conductance. Thereupon the holding potential was set back to the standard value of $+17$ mV and $[Ca^{2+}]_i$ started to return towards baseline. At this time the endogenous Ca^{2+} transient occurred (marked by *). So far, both transients in $[Ca^{2+}]_i$ did not influence capacitance. Some time later the cell was hyperpolarized again for a period of time sufficient to bring $[Ca^{2+}]_i$ close to 2 μM. This time capacitance increased by a small amount. Thereafter secretion slowed again as $[Ca^{2+}]_i$ returned to basal levels, but each subsequent voltage-induced Ca^{2+} transient markedly enhanced secretion. It should be noted that the 5th transient (marked

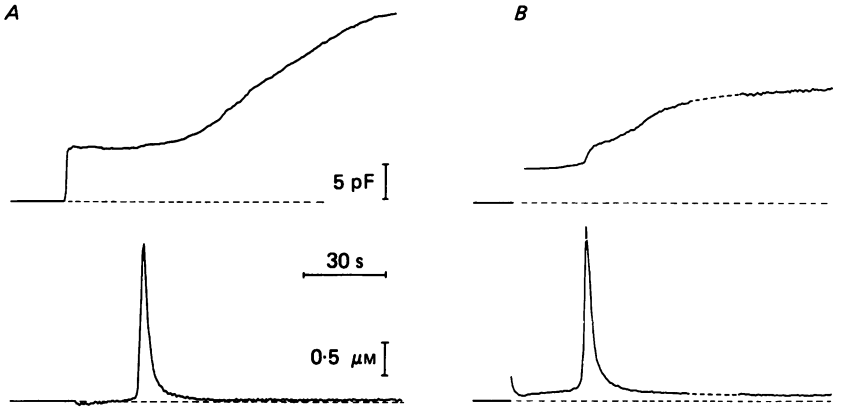

Fig. 7. Different time relationships between Ca^{2+} transient and degranulation. Standard conditions except that the cell in *B* was stimulated with only 20 μM-GTP-γ-S. *A*: 0·05 mM-Ca–EGTA; 0·44 mM-EGTA. *B*: 0·1 mM-Ca–EGTA; 0·9 mM-EGTA. In *B* the measurement was interrupted for about 20 s (dashed lines) in order to observe the cell.

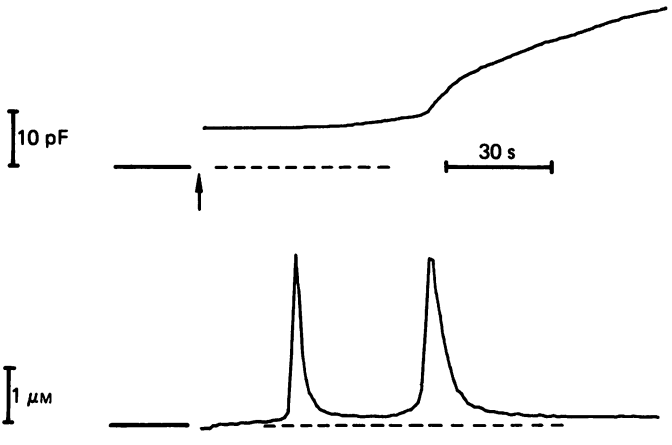

Fig. 8. Two endogenous transients. Capacitance (upper trace) is seen to stay almost constant during the first Ca^{2+} transient (lower trace) but to change markedly in its rate of increase during the second transient. Standard conditions: 0·05 mM-Ca–EGTA; 0·44 mM-EGTA. The two dashed lines indicate zero level and initial value of the capacitance trace, respectively; the arrow marks the time of patch rupture.

by **) produced a response much greater than the 3rd transient although it was much smaller. Obviously Ca^{2+} sensitivity had increased in the meantime. The 7th and 8th transients, finally, had only small effects because degranulation was nearly complete.

Better controlled methods of changing $[Ca^{2+}]_i$ will have to be developed for a quantitative study of the relationship between rate of secretion, $[Ca^{2+}]_i$ and time. A few qualitative features, however, are already apparent:

(1) Degranulation in response to abrupt increases in $[Ca^{2+}]_i$ was only rarely observed before degranulation at basal $[Ca^{2+}]_i$ levels had started. If this did occur, degranulation continued after return to basal $[Ca^{2+}]_i$ indicating that the lag phase had ended.

(2) No further capacitance increase could be elicited by raising $[Ca^{2+}]_i$ when capacitance had reached a plateau and degranulation had gone to completion.

(3) No capacitance increase was seen in response to increased $[Ca^{2+}]_i$ (induced either by IP_3 or by voltage changes) when GTP-γ-S was absent from the pipette filling solution.

All these features point towards a synergistic action of GTP-γ-S (or the signal it passes on) and calcium.

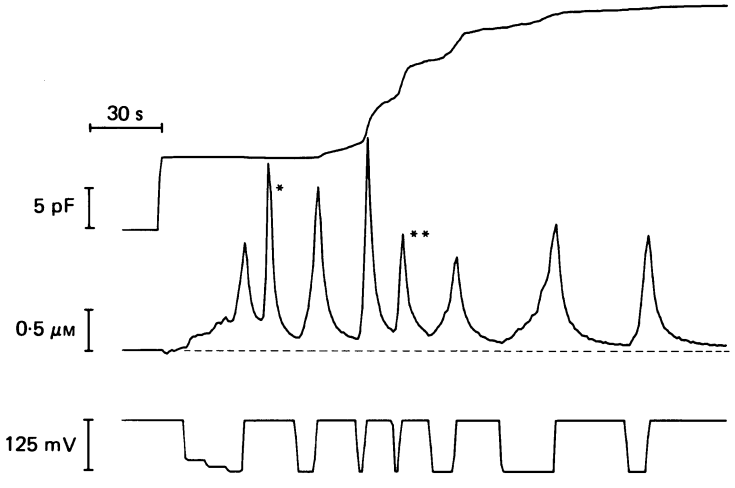

Fig. 9. Voltage-induced changes in $[Ca^{2+}]_i$. Upper trace is capacitance, centre trace is $[Ca^{2+}]_i$ and lower trace is holding voltage. Holding voltage was changed as explained in the text. * marks an endogenous Ca^{2+} transient. See text for meaning of **. The conductance was very low initially; it increased to about 1 nS during the first and second hyperpolarization. Changes in $[Ca^{2+}]_i$ always lag behind voltage changes since the voltage-induced Ca^{2+} inflow changes $[Ca^{2+}]_i$ very slowly. The pipette contained 0·2 mM-free EGTA; 4 mM-$CaCl_2$ was added to the standard external saline in this experiment to give a total external Ca^{2+} concentration of 5 mM.

Variability among mast cells

Mast cells in this study showed considerable variability. For instance, both latency and rise time of capacitance responses at basal $[Ca^{2+}]_i$ values extended over a 5-to 10-fold range. Variability was also observed in the height and latency of the capacitive transient, the number of transients and membrane conductance. Most cells had conductances in the range 0·2–2 nS without any obvious Ca^{2+} dependence. However, a few cells had transient increases in conductance, as described by Lindau & Fernandez (1986*b*), concomitant with Ca^{2+} transients. There was much more variation among different animals than among cells from the same animal. Mean values given in this study reflect pooled data from many animals.

DISCUSSION

The basic finding of this investigation is that a rise in the intracellular concentration of free calcium ($[Ca^{2+}]_i$) can accelerate, but not initiate, the degranulation of mast cells. This result, which is surprising in view of previous mast

cell literature, can be discussed with respect to two questions. The first question is whether an increase in $[Ca^{2+}]_i$ is a necessary prerequisite of secretion. The answer given here is that this is not the case. Degranulation in a given mast cell goes to completion in about 3–5 min, if the cell is stimulated by GTP-γ-S and $[Ca^{2+}]_i$ is buffered to basal values around 0·2 μM. This is only a factor of three slower than at a $[Ca^{2+}]_i$ of around 1–1·5 μM, a concentration which is usually observed in a plateau phase following antigenic stimulation (Neher & Almers, 1986). Approximately half of the cells degranulated even when $[Ca^{2+}]_i$ was buffered to values below 50 nM. These cells did so to the full extent, although slower. It should be pointed out, however, that in this study degranulation was induced by GTP-γ-S. It is well conceivable that GTP-γ-S bypasses an earlier, more strongly Ca^{2+}-dependent step in stimulus–secretion coupling.

An absolute requirement of secretion in mast cells for Ca^{2+} has been inferred from ionophore and tracer flux studies on intact cells as reviewed by Gomperts (1984). More recent studies using permeabilized mast cells and Ca^{2+}-indicator dyes have come to more varied conclusions: when mast cells are permeabilized with ATP^{4-} they degranulate when calcium is present during permeabilization (Gomperts, 1984). Following reclosure they can be made to secrete by relatively high external calcium if they had been pre-loaded with GTP analogues (Gomperts, 1983). Similarly, cells permeabilized by streptolysin-O have a Ca^{2+} requirement for nucleotide-induced secretion (Howell & Gomperts, 1987). However, these cells have to be metabolically depleted before permeabilization. Otherwise they secrete during the permeabilization process in the absence of nucleotides.

$[Ca^{2+}]_i$ has been measured by quin2 in mast cells (White *et al.* 1984). It was found that $[Ca^{2+}]_i$ increases upon stimulation by antigen. Concomitantly histamine is secreted. However, when cells were exposed for 1 min to Ca^{2+}-free media prior to antigen stimulation the increase in $[Ca^{2+}]_i$ was only a fraction of that with Ca^{2+}-containing external media. Nevertheless, secretion was hardly changed (White *et al.* 1984, Fig. 1). This can be taken as evidence against an absolute Ca^{2+} requirement of exocytosis in mast cells. Another study employing quin2 found an increase in $[Ca^{2+}]_i$ only in the absence of manganese. Addition of manganese to the external solution prevented a rise in $[Ca^{2+}]_i$ although the cells degranulated in response to compound 48/80. It was concluded that the majority of the quin2 signal arises from quin2 bound to granules and is released during exocytosis (Bibb, Cochrane & Morel-Laurens, 1986; see also Almers & Neher, 1985, for the case of fura2). Thus, those experiments are not readily interpretable. From the results presented here it would be expected that only very small increases in $[Ca^{2+}]_i$ would occur when quin2 is applied at final concentrations of several millimolar, as is common in quin2 studies. Secretion at basal levels of $[Ca^{2+}]_i$ has been reported by Neher & Almers (1986) based on fura2 measurements.

Quin2 measurements have also been performed with rat basophilic leukaemia cells. In one study an absolute requirement of secretion on external calcium has been found (Beavan *et al.* 1984*a*). It seems, however, that calcium may be needed, at least in part, for binding of the specific antigen used (ovalbumin). Sagi-Eisenberg, Lieman & Pecht (1985) also used quin2 to measure $[Ca^{2+}]_i$ in the same preparation. They found that the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) at

intermediate concentrations blocked the antigen-induced increase in $[Ca^{2+}]_i$ but augmented secretion. In other cell types, such as permeabilized platelets (Haslam & Davidson, 1984) and neutrophils (Sha'afi, White, Molski, Shefcyk, Volpi, Naccache & Feinstein, 1983; DiVirgilio, Lew & Pozzan, 1984; Barrowman, Cockcroft & Gomperts, 1986), Ca^{2+}-independent secretion has been clearly established.

The next question relates to the problem of whether an increase in $[Ca^{2+}]_i$ is sufficient to induce secretion. The answer given by this study is that this is not the case. It has been reported by Fernandez *et al.* (1984) that buffering calcium concentration in the pipette to values between 0·2 and 10 μM generally failed to induce degranulation in the absence of GTP-γ-S. This statement was confirmed in the present investigation (data not shown). It can be extended by the observation that transient rises in intracellular $[Ca^{2+}]_i$ up to several micromolar induced by exogenously supplied inositol 1,4,5-trisphosphate (IP_3) did not cause any secretion. Furthermore, increases in $[Ca^{2+}]_i$ induced by calcium inflow through the plasma membrane or through leaks did not cause secretion in the first 20–30 s following membrane patch rupture. Only in synergy with a GTP-γ-S stimulus was there a slowly developing sensitivity towards calcium.

This, again, is surprising in view of earlier studies where calcium injected intracellularly by microelectrodes was found to degranulate mast cells (Kanno, Cochrane & Douglas, 1973), in view of studies on permeabilized mast cells (Howell & Gomperts, 1987) and in view of patch-clamp results on chromaffin cells (Neher & Marty, 1982). In all these cases an increase in $[Ca^{2+}]_i$ was sufficient to induce secretion.

This raises the question of which assay system most reliably reports the properties of the intact cell. Previously, we have postulated that the loss of cellular components through diffusional exchange between cytoplasm and pipette may be responsible for the lack of Ca^{2+} sensitivity in patch-clamped mast cells (Fernandez *et al.* 1984). In the light of the present investigation this proposal seems less likely. In the first few seconds after patch rupture, when the cell should be very little disturbed, it is found to be insensitive towards calcium. Even an IP_3-induced Ca^{2+} transient, which usually happens within 2–5 s after patch rupture and brings $[Ca^{2+}]_i$ to several micromolar, does not induce secretion. As time goes on the cell develops rather than loses Ca^{2+} sensitivity.

'Wash-out', on the other hand, is a well-known phenomenon in tight-seal recording (see Neher, 1986*a*). It should be stressed in this context that all data of this study were obtained in the first 5–10 min of tight-seal recording. The fact that within this observation period GTP-γ-S-induced secretion is very similar in time course and total extent to that obtained by antigenic stimulation under 'slow whole cell' conditions (Fernandez & Lindau, 1987) argues against severe wash-out problems. However, it cannot be excluded that prolonged exposure of cells to high $[Ca^{2+}]_i$ in the absence of wash-out effects might eventually induce secretion. This would reconcile the present results with studies on ATP^{4-}-permeabilized cells. It should also be pointed out that the range of calcium concentrations explored in this investigation was limited to values smaller than 1·5 μM due to the exclusive use of EGTA as a calcium buffer.

Possible molecular models of stimulus–secretion coupling in mast cells

The physiological stimulus for mast cell release is dimerization of immunoglobulin-E-receptors by antigen (Ishizaka & Ishizaka, 1984). Furthermore, it has been established that break-down of inositol phospholipids together with Ca^{2+} mobilization is one of the early events in mast cell secretion (Kennerly, Sullivan & Parker, 1979; Pearce, 1982; Beavan *et al.* 1984*a*, *b*). More recently, it has been suggested that a GTP-binding protein forms the link between receptor dimerization and phospholipid break-down (Nakamura & Ui, 1985; Cockcroft & Gomperts, 1985). The general scheme proposed by Berridge & Irvine (1984) marks a pathway whereby phospolipid break-down generates two second messengers, diacylglycerol and IP_3. The results reported here contain several pieces of evidence in support of such a scheme. The fact that GTP-γ-S is the most reliable stimulus for degranulation in internally dialysed cells may reflect permanent activation of the GTP-binding protein referred to above. Furthermore, the transient increase in $[Ca^{2+}]_i$ most probably reflects release of calcium from intracellular stores induced by IP_3 as discussed by Neher & Almers (1986). The fact that a very similar transient can be directly induced by IP_3 further strengthens that point. The delay regularly observed when this transient is induced by GTP-γ-S, as compared to direct IP_3 application, might represent the time required for activation of the GTP-binding protein and for phospholipid breakdown. Finally, the concept of the dual-signal pathway (Berridge & Irvine, 1984) includes the possibility that one signal alone (IP_3 or Ca^{2+}) is ineffective in the absence of the other one (diacylglycerol and C-kinase activation).

A very straightforward explanation of the synergistic action of calcium and the GTP-γ-S stimulus is offered by the properties of protein kinase-C. The latter is a Ca^{2+}-dependent enzyme which in the absence of other stimuli requires 10–100 μM-calcium. In the presence of diacylglycerol or phorbol esters, it becomes much more sensitive towards calcium such that it is partially activated by basal $[Ca^{2+}]$ levels (Nishizuka, 1984). In this framework the increase in Ca^{2+} sensitivity observed after GTP-γ-S might be due to the build-up of diacylglycerol. However, preliminary attempts to mimic this process by simultaneous presentation of calcium and phorbol esters have failed, so far.

Protein kinase-C definitely is not the only place where modulation of sensitivity towards $[Ca^{2+}]_i$ can take place (see review by Rasmussen & Barrett, 1984). Another candidate is phospholipase-C which has been shown to increase its Ca^{2+} sensitivity after TRH stimulation in pituitary cells (Martin, Lucas, Bajjalieh & Kavalchyk, 1986) or following GTP-γ-S stimulation of polymorphonuclear lymphocytes (Smith, Cox & Snydermann, 1986). However, one aspect of the data argues against such an interpretation: the latency of the Ca^{2+} transient, which most probably reflects build-up of IP_3, did not markedly depend on $[Ca^{2+}]_i$, whereas the latency of degranulation did. In fact, there was quite a marked dissociation in time between the transient and the degranulation process. The onset of the two signals almost coincided under conditions of loose calcium buffering, whereas degranulation was very much delayed with respect to the Ca^{2+} transient, when $[Ca^{2+}]_i$ was buffered to low values. It thus seems that at least some of the Ca^{2+} sensitivity of degranulation must reside in a later

step. Also, Beavan, Moore, Smith, Hesketh & Metcalfe (1984*a*) find that phosphatidyl-inositol breakdown in rat basophil leukaemic cells does not depend on a rise in $[Ca^{2+}]_i$.

The discussion, so far, considers only the very first steps in the stimulus–secretion pathway. It is likely that further Ca^{2+}-dependent steps occur subsequently. Possible examples are a second GTP-dependent step (Barrowman *et al.* 1986) or activation of phospholipase A_2 (Nakamura & Ui, 1985).

The Ca^{2+} transient

Transient elevations of intracellular calcium have been observed in a number of non-excitable cells such as liver cells (Woods, Cuthbertson & Cobbold, 1986) and oocytes (Orchard, Eisner & Allen, 1983; Eusebi, Miledi & Takahashi, 1985). They are generally held to reflect release of calcium from intracellular stores by inositol 1,4,5-trisphosphate. For mast cells, it has been shown by Neher & Almers (1986) that stimulation by compound 48/80 or by antigen leads to such transients which do not depend on the presence of extracellular calcium. In this study it is shown that transients of very similar properties can be elicited both by GTP-γ-S and by IP_3. IP_3 was effective at concentrations as small as 0·2–1 μM which is exactly the range of concentrations where Ca^{2+} release from permeabilized pancreatic acinar cells can be observed (Streb, Irvine, Berridge & Schulz, 1983). The Ca^{2+} transient can be suppressed by solutions containing EGTA at buffer capacity of 4000 or greater (e.g. by a solution containing 0·6 mM-EGTA–0 Ca^{2+} or else by a solution containing 2·4 mM EGTA–1·2 mM-Ca^{2+}). The buffer can then capture an amount of calcium during the rising phase of the transient which corresponds to an equivalent cytoplasmic concentration of at least 100–500 μM. This is exactly the range of values for total calcium release found in pancreatic acinar cells (Streb *et al.* 1983; Streb & Schulz, 1983). If the mean cell volume is taken to be 1·4 pl (based on the mean value of capacitance of 6·44 pF, spherical geometry and specific capacitance of 1 μF cm^2) and the relative volume accessible to calcium is assumed to be 47% (Helander & Bloom, 1974; total volume excluding granules), a total amount of calcium of 0·07–0·35 fmol calcium per cell is obtained. This would correspond to approximately 1–5 nmol/mg dry weight, a number which compares to 450 pmol/mg dry weight of calcium released by IP_3 from 'permeabilized liver cells' (Joseph, Thomas, Williams, Irvine & Williamson, 1984*a*) and of up to 3 nmol/mg dry weight from insulin-secreting tumor cells (Joseph, Williams, Corkey, Matschinsky & Williamson, 1984*b*). The results discussed above imply that the mast cell must contain some 0·35 fmol endogenous calcium buffers (calcium binding proteins etc.) since only a small fraction of total calcium released appears as free calcium in the absence of added calcium buffer.

IP_3 diffusing from the pipette to the cell elicited Ca^{2+} transients in an all-or-none manner. At a 0·2 μM pipette concentration transients either appeared within 2–10 s after patch rupture or not at all. At these times IP_3 probably has not yet reached diffusional equilibrium. Thus, one would have to postulate that concentrations lower than 0·2 μM can elicit transients or else that it is sufficient to reach this value locally at the point of entry to elicit a generalized transient. Considering the first alternative the question would remain why lower pipette concentrations do not produce

transients after full equilibration. An answer to this question probably would also have to take into account degradation of IP_3 inside the cell and the possibility of desensitization of the intracellular IP_3 receptor mechanisms.

Desensitization would also help to explain the finding that the continued presence of IP_3 elicits not a permanent but a *transient* increase in $[Ca^{2+}]_i$. The transient nature of the calcium response in permeabilized liver cells was explained, at least in part, by Ca^{2+} reuptake after IP_3 degradation (Joseph *et al.* 1984*a*). This mechanism does not apply here, where IP_3 keeps diffusing from the pipette. Alternatively the downstroke of the transient could be explained by rate-limited binding of calcium to endogenous buffers after depletion of the intracellular stores.

Voltage dependence of $[Ca^{2+}]_i$ and intracellular calcium buffering

One of the more surprising findings of this study was that $[Ca^{2+}]_i$ depended quite strongly on the holding voltage, unless calcium was buffered by a high concentration (several millimolar) of EGTA or else the cell was very tight ($G_M < 0{\cdot}5$ nS). This reflects inflow of calcium through the plasma membrane or through leaks. Changes in membrane conductance in the range of 0·1 nS were observed by Lindau & Fernandez (1986*b*) to accompany degranulation. It was argued that these may be attributable to the additional membrane being externalized during degranulation. They were assumed not to have relevance for $[Ca^{2+}]_i$. The present results raise the possibility that small conductance changes can lead to significant changes in $[Ca^{2+}]_i$ even if they generate currents that are an order of magnitude smaller than calcium currents typically observed in excitable cells. A calcium current of only 1 pA (corresponding to a Ca^{2+}-selective conductance of approximately 80 pS at 0 mV) would be able to saturate 0·3 fmol endogenous calcium buffer (see above) within 1 min. This estimate is certainly not very accurate since it neglects pump and other transport processes, but it shows that very small currents may be sufficient to induce slow changes in $[Ca^{2+}]_i$.

Calcium homeostasis can be overcome by pipette-applied buffers only when these have buffering capacity of 10000 or more (e.g. by a solution containing 1·5 mM-EGTA or a solution containing 6 mM-EGTA and 3 mM-$CaCl_2$). Thus, the situation in these small cells is much better than that in perfused snail neurones where even 10 mM-EGTA was totally insufficient to set $[Ca^{2+}]_i$ to low values (Byerly & Moody, 1984). With weaker buffering, $[Ca^{2+}]_i$ tends towards a mean value of 0·28 μM (see Fig. 2*A*), regardless of the $[Ca^{2+}]$ in the buffer mixture. The GTP-γ-S-stimulated cell would probably adopt this value in the absence of exogenous buffers. It should be noted, however, that this is not the $[Ca^{2+}]_i$ of an unperturbed cell, as the cells at the time of the measurements had been exposed to a GTP-γ-S stimulus for a few minutes; very often they had partially degranulated by that time, and they had been held at +17 mV. The $[Ca^{2+}]_i$ of unstimulated, less perturbed cells was given as 0·155 μM by Almers & Neher (1986).

Cellular mechanisms for calcium homeostasis include endogenous buffers (calcium-binding protein), as well as Ca^{2+} pumps and exchange mechanisms both in the plasma membrane and in organelles (Baker & Umbach, 1987). If it is assumed that endogenous buffers have equilibrated by the time of the measurement, then the deviation $\Delta[Ca^{2+}]_i$ of $[Ca^{2+}]_i$ from the intrinsic Ca^{2+} concentration reflects a

competition between steady-state pumping rates and diffusional exchange of buffer between pipette and cell. An analysis analogous to that by Melzer, Rios & Schneider (1986) on Ca^{2+} movements in skeletal muscle should allow calculation of pumping rates from $[Ca^{2+}]_i$ data. This, however, would also have to take into account diffusion of buffer to and from the pipette, such that quantitative data on that diffusional exchange is a prerequisite for such an analysis.

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REFERENCES

ALMERS, W. & NEHER E. (1985). The Ca signal from fura-2 loaded mast cells depends strongly on the method of dye-loading. *FEBS Letters* **192**, 13–18.

BAKER, P. F. & UMBACH, J. A. (1987). Calcium buffering in axons and axoplasm of *Loligo*. *Journal of Physiology* **383**, 369–394.

BARROWMAN, M. M., COCKCROFT, S. & GOMPERTS, B. D. (1986). Two roles for guanine nucleotides in the stimulus–secretion sequence of neutrophils. *Nature* **319**, 504–507.

BEAVAN, M. A., MOORE, J. P., SMITH, G. A., HESKETH, T. R. & METCALFE, J. C. (1984*a*). The calcium signal and phosphatidylinositol breakdown in 2H3 cells. *Journal of Biological Chemistry* **259**, 7137–7142.

BEAVAN, M. A., ROGERS, J., MOORE, J. P., HESKETH, T. R., SMITH, G. A. & METCALFE, J. C. (1984*b*). The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. *Journal of Biological Chemistry* **259**, 7129–7136.

BENNETT, J. P., COCKCROFT, S. & GOMPERTS, B. D. (1981). Rat mast cells permeabilized with ATP secrete histamine in response to calcium ions buffered in the micromolar range. *Journal of Physiology* **317**, 335–345.

BERRIDGE, M. J. & IRVINE, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315–321.

BIBB, P. C., COCHRANE, D. E. & MOREL-LAURENS, N. (1986). Loss of quin 2 accompanies degranulation of mast cells. *FEBS Letters* **209**, 169–174.

BYERLY, L. & MOODY, W. (1984). Intracellular calcium ions and calcium currents in perfused neurones of the snail, *Lymnea stagnalis*. *Journal of Physiology* **352**, 637–652.

COCHRANE, D. E. & DOUGLAS, W. W. (1974). Calcium-induced extrusion of secretory granules (exocytosis) in mast cells exposed to 48/80 or the ionophores A-23187 and X-537A. *Proceedings of the National Academy of Sciences of the U.S.A.* **71**, 408–412.

COCKCROFT, S. & GOMPERTS, B. D. (1985). Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature* **314**, 534–536.

DI VIRGILIO, F., LEW, D. P. & POZZAN, T. (1984). Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. *Nature* **310**, 691–693.

EUSEBI, F., MILEDI, R. & TAKAHASHI, T. (1985). Aequorin–calcium transients in mammalian fast and slow muscle fibers. *Biomedical Research* **6**, 129–138.

FERNANDEZ, J. M. & LINDAU, M. (1987). Intracellular stimulation of mast cells with guanine nucleotides mimic antigen stimulation. *Biophysical Journal* **51**, 398a.

FERNANDEZ, J. M., NEHER, E. & GOMPERTS, B. D. (1984). Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature* **312**, 453–455.

FOREMAN, J. C., HALLETT, M. B. & MONGAR, J. L. (1977). The relationship between histamine secretion and ^{45}calcium uptake by mast cells. *Journal of Physiology* **271**, 193–214.

GOMPERTS, B. D. (1983). Involvement of guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors. *Nature* **306**, 64–66.

GOMPERTS, B. D. (1984). Calcium and cellular activation. In *Biological Membranes*, vol. 5. ed. CHAPMAN. D., pp. 290–358. London: Academic Press.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HASLAM, R. J. & DAVIDSON, M. L. (1984). Guanine nucleotides decrease the free $[Ca^{2+}]$ required for secretion of serotonin from permeabilized blood platelets. Evidence of a role for a GTP-binding protein in platelet activation. *FEBS Letters* **174**, 90–95.

HELANDER, H. F. & BLOOM, G. D. (1974). Quantitative analysis of mast cell structure. *Journal of Microscopy* **100**, 315–321.

HOWELL, T. W. & GOMPERTS, B. D. (1987). Rat mast cells permeabilized with streptolysin-O secrete histamine in response to Ca^{++} at concentrations buffered in the micromolar range. *Biochimica et biophysica acta* **927**, 177–183.

ISHIZAKA, T., HIRATA, F., ISHIZAKA, K. & AXELROD, J. (1980). Stimulation of phospholipid methylation, Ca^{2+} influx, and histamine release by bridging of IgE receptors on rat mast cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **77**, 1903–1906.

ISHIZAKA, T. & ISHIZAKA, K. (1984). Activation of mast cells for mediator release through IgE receptors. *Progress in Allergy* **34**, 188–235.

JOSEPH, S. K., THOMAS, A. P., WILLIAMS, R. J., IRVINE, R. F. & WILLIAMSON, J. R. (1984*a*). *myo*-Inositol 1,4,5-trisphosphate. A second messenger for the hormonal mobilization of intracellular Ca^{2+} in liver. *Journal of Biological Chemistry* **259**, 3077–3081.

JOSEPH, S. K., WILLIAMS, R. J., CORKEY, B. E., MATSCHINSKY, F. M. & WILLIAMSON, J. R. (1984*b*). The effect of inositol trisphosphate on Ca^{2+} fluxes in insulin-secreting tumor cells. *Journal of Biological Chemistry* **259**, 12952–12955.

KANNO, T., COCHRANE, D. E. & DOUGLAS, W. W. (1973). Exocytosis (secretory granule extrusion) induced by injection of calcium into mast cells. *Canadian Journal of Physiology and Pharmacology* **51**, 1001–1004.

KENNERLY, D. A., SULLIVAN, T. J. & PARKER, C. W. (1979). Activation of phospholipid metabolism during mediator release from stimulated rat mast cells. *Journal of Immunology* **122**, 152–159.

LINDAU, M. & FERNANDEZ, J. M. (1986*a*). A patch-clamp study of histamine-secreting cells. *Journal of General Physiology* **88**, 349–368.

LINDAU, M. & FERNANDEZ, J. M. (1986*b*). IgE-mediated degranulation of mast cells does not require opening of ion channels. *Nature* **319**, 150–153.

LINDAU, M. & NEHER, E. (1987). Patch clamp techniques for time-resolved capacitance measurement in single cells. *Pflügers Archiv* (in the Press).

MARTIN, T. F. J., LUCAS, D. O., BAJJALIEH, S. A. & KOWALCHYK, J. A. (1986). Thyrotropin-releasing hormone activates a Ca^{2+}-dependent polyphosphoinositide phosphodiesterase in permeable GH_3 cells. *Journal of Biological Chemistry* **261**, 2918–2927.

MELZER, W., RIOS, E. & SCHNEIDER, M. F. (1986). The removal of myoplasmic free calcium following calcium release in frog skeletal muscle. *Journal of Physiology* **372**, 261–292.

MILLER, D. J. & SMITH, G. L. (1984). EGTA purity and the buffering of calcium ions in physiological solutions. *American Journal of Physiology* **246**, C160–166.

NAKAMURA, T. & UI, M. (1985). Simultaneous inhibitions of inositol phospholipid breakdown, arachidonic acid release, and histamine secretion in mast cells by islet-activating protein, pertussis toxin. *Journal of Biological Chemistry* **260**, 3584–3593.

NEHER, E. (1986*a*). Patch clamp studies on the role of calcium in secretion. In *Progress in Zoology*, vol. 33, ed. LÜTTGAU, H. CH., pp. 275–286. Stuttgart, New York: Gustav Fischer Verlag.

NEHER, E. (1986*b*). Inositol 1,4,5-triphosphate and GTP-γ-S induce calcium transients in isolated rat peritoneal mast cells. *Journal of Physiology* **381**, 71*P*.

NEHER, E. (1987). Stimulation by GTP-γ-S renders secretion in mast cells sensitive towards free intracellular calcium. *Biophysical Journal* **51**, 433a.

NEHER, E. & ALMERS, W. (1986). Fast calcium transients in rat peritoneal mast cells are not sufficient to trigger exocytosis. *EMBO Journal* **5**, 51–53.

NEHER, E. & MARTY, A. (1982). Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **79**, 6712–6716.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**, 693–698.

ORCHARD, C. H., EISNER, D. A. & ALLEN, D. G. (1983). Oscillations of intracellular Ca^{2+} in mammalian cardiac muscle. *Nature* **304**, 735–738.

PEARCE, F. L. (1982). Calcium and histamine secretion from mast cells. In *Progress in Medical Chemistry*, vol. 19, ed. ELLIS, G. S. & WEST, G. B. Amsterdam: Elsevier Medical Press.

PENNER, R., NEHER, E. & DREYER, F. (1986). Intracellularly injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells. *Nature* **324**, 76–78.

RASMUSSEN, H. & BARRETT, P. Q. (1984). Calcium messenger system: an integrated view. *Physiological Reviews* **64**, 938–984.

SAGA-EISENBERG, R., LIEMAN, H. & PECHT, I. (1985). Protein kinase C regulation of the receptor-coupled calcium signal in histamine-secreting rat basophilic leukaemia cells. *Nature* **313**, 59–60.

SHA'AFI, R. I., WHITE, J. R., MOLSKI, T. F. P., SHEFCYK, J., VOLPI, M., NACCACHE, P. H. & FEINSTEIN, M. B. (1983). Phorbol 12-myristate 13-acetate activates rabbit neutrophils without an apparent rise in the level of intracellular free calcium. *Biochemical and Biophysical Research Communication* **114**, 638–645.

SMITH, CH. D., COX, C. CH. & SNYDERMAN, R. (1986). Receptor-coupled activation of phosphoinositide-specific phospholipase C by an N protein. *Science* **232**, 97–100.

STREB, H., IRVINE, R. F., BERRIDGE, M. J. & SCHULZ, I. (1983). Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* **306**, 67–69.

STREB, H. & SCHULZ, I. (1983). Regulation of cytosolic free Ca^{2+} concentration in acinal cells of rat pancreas. *American Journal of Physiology* **245**, G347–357.

WHITE, J. R., ISHIZAKA, T., ISHIZAKA, K. & SHA'AFI, R. I. (1984). Direct demonstration of increased intracellular concentration of free calcium as measured by quin-2 in stimulated rat peritoneal mast cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **81**, 3978–3982.

WOODS, N. M., CUTHBERTSON, K. S. R. & COBBOLD, P. H. (1986). Repetitive transient rises in cytoplasmic free calcium in hormone-stimulated hepatocytes. *Nature* **319**, 600–602.