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Secretory responses of rat peritoneal mast cells to high intracellular calcium

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The patch-clamp technique was used to investigate the secretory responses of rat peritoneal mast cells at various intracellular calcium concentrations ($[Ca^{2+}]_i$). When Calcium was introduced into the cell with pipette-loaded dibromo-BAPTA, elevation of $[Ca^{2+}]_i$ into the range 1–10 μM induced membrane capacitance increases indicative of exocytosis in a concentration-dependent manner. At higher concentrations a decrease of the response was observed. Cells that were exposed to micromolar $[Ca^{2+}]_i$ underwent morphological alterations resulting in swelling, which is indicative of cytoskeletal alterations. The presence of dibromo-BAPTA (4 mM) strongly inhibited secretion induced by GTP-γ-S, thus hampering the contribution of G-protein-mediated stimulation. Application of the Ca^{2+} ionophore ionomycin resulted in transient increases in $[Ca^{2+}]_i$ which were parallelled by Ca^{2+}-dependent secretion. Effective buffering of the cytosolic calcium level below 1 μM abolished the secretory response. Our results show that an increase in $[Ca^{2+}]_i$ can trigger secretion, but only if it is high and sustained. During physiological stimulation, however, secretion proceeds at $[Ca^{2+}]_i$ below 1 μM. It is, therefore, concluded that mast cell degranulation under physiological conditions is not simply a result of an increase in $[Ca^{2+}]_i$, but that other second messenger systems in conjunction with calcium act synergistically in order to ensure fast and efficient secretion.

Mast cell; Secretion; Ca^{2+}; Ionomycin; GTP

1. INTRODUCTION

Elevation of cytosolic calcium concentration is known to accompany the exocytotic event in a number of different secretory cells. Varying with cell types two main mechanisms of raising intracellular calcium $[Ca^{2+}]_i$ have become evident. Excitable cells such as neurones, that need to release their vesicular contents very rapidly, achieve this mainly through the influx of extracellular Ca^{2+} through voltage-dependent channels [1,2]. Evidently, the resulting increase in $[Ca^{2+}]_i$ is sufficient to induce secretion in these cells [3,4]. Non-excitable cell types appear to be largely devoid of voltage-dependent ion channels [5,6]. When they are stimulated they release calcium from internal stores through the actions of second messengers. However, for exocytosis in some of these cells, particularly neutrophils, platelets and mast cells, this rise of $[Ca^{2+}]_i$ does not seem to be compulsory [7–9]. Their secretory mechanism rather seems to be under enzymatic control involving polyphosphoinositide metabolism. Apparently, both the enzymatic machinery as well as Ca^{2+}-controlled mechanisms are present in non-excitable and excitable cells [10,11]. However, their relative importance appears to be different in these cells.

We have attempted to investigate the role of intracellular calcium concentration on exocytosis of rat peritoneal mast cells. We have used the patch-clamp technique to study secretion at the single-cell

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level by monitoring cell membrane capacitance which registers changes in the membrane surface area as vesicles fuse with the plasma membrane [12]. In addition, this technique allows one to control the intracellular milieu, so that various calcium buffers could be introduced while measuring $[Ca^{2+}]_i$ with the Ca^{2+}-indicator dye fura-2 [13]. We have also studied the effects of the Ca^{2+} ionophore ionomycin which translocates extracellular Ca^{2+} across the plasma membrane into the cytosol.

Our results suggest that calcium at high concentrations for prolonged periods can enforce secretion. However, under physiological conditions the rise in $[Ca^{2+}]_i$ by itself is not sufficient to elicit the exocytotic process in mast cells.

2. MATERIALS AND METHODS

Rat peritoneal mast cells were purified on a percoll gradient as described [14] and suspended in culture medium M199 supplemented with fetal calf serum (10%), $NaHCO_3$ (45 mM), glucose (2.5 mM), streptomycin (0.12 mg/ml) and penicillin (0.64 mg/ml), pH 7.2. Cells were plated onto cover-slips placed inside 35-mm culture dishes and stored in an incubator at 37°C and 10% CO_2 for 1–6 h until use. Experiments were performed at 23–26°C in an Mg^{2+}-rich saline of the following composition (in mM): NaCl, 140; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 5; glucose, 11; Hepes-NaOH, 10; pH 7.2.

Patch-clamp measurements were performed with Sylgard-coated pipettes in the tight-seal whole-cell configuration. Pipette resistance, after filling with the solution given below, ranged between 3 and 5 MΩ. The solution for filling pipettes (intracellular solution) contained (in mM): K^+-glutamate, 135; NaCl, 20; $MgCl_2$, 1; Hepes-NaOH, 10; Na_2ATP, 0.2; fura-2 pentapotassium salt, 0.1 (Molecular Probes). In most experiments GTP (300 μM) was added to the intracellular solution in order to sustain the cell's ability to degranulate. Different ratios of Ca-dibromo-BAPTA and dibromo-BAPTA (Molecular Probes), a calcium chelator with a dissociation constant of 1.58 μM, were used to buffer calcium in the internal solution as indicated in section 3. In some experiments with ionomycin EGTA was used as chelator. Ionomycin (Calbiochem) was dissolved in extracellular solution (5 μg/ml) from a stock solution (5 mg/ml in DMSO) and applied directly onto the cell under investigation via pressure ejection from a second pipette. Compound 48/80 (Sigma) was applied the same way at a final concentration of 5 μg/ml. GTP-γ-S (kindly provided by Dr F. Eckstein, Göttingen) was used at 100 μM.

Capacitance measurements were performed with a 2-phase lock-in amplifier as in [15]. Briefly, a 16 mV r.m.s. 800 Hz sine wave was added to the command potential of the patch clamp (usually 17 mV). The resulting sinusoidal current was measured by the lock-in amplifier at two mutually orthogonal phase angles. The two signals together with the applied potential and the d.c. current were used to calculate access resistance R_A, 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.

The concentration of intracellular calcium $[Ca^{2+}]_i$ was measured by use of the fluorescent indicator dye fura-2. Cells were loaded with fura-2 pentapotassium salt by diffusion from the recording pipette. Fluorescence of fura-2 was excited alternatingly by light at 360 and 390 nm by means of a rotating filter wheel fitted to a slot in the excitation pathway. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio [13]. The calibration procedure and details of the measurement are described elsewhere [15].

A total of six signals (two fluorescence intensities; two lock-in signals; d.c. current and voltage) were fed into the computer and sampled every 0.5–2 s. Subsets of these and $[Ca^{2+}]_i$ were displayed on-line on a vector display. Later they were plotted on an HP 7470A (Hewlett Packard) plotter.

3. RESULTS

Three examples of the secretory responses to three different intracellular calcium concentrations are given in fig.1. In addition to the normal internal saline that was buffered with dibromo-BAPTA/Ca-dibromo-BAPTA ratios (3:1, 1:2 and 1:7), GTP (300 μM) was present in the pipette. While $[Ca^{2+}]_i$ buffered to values below

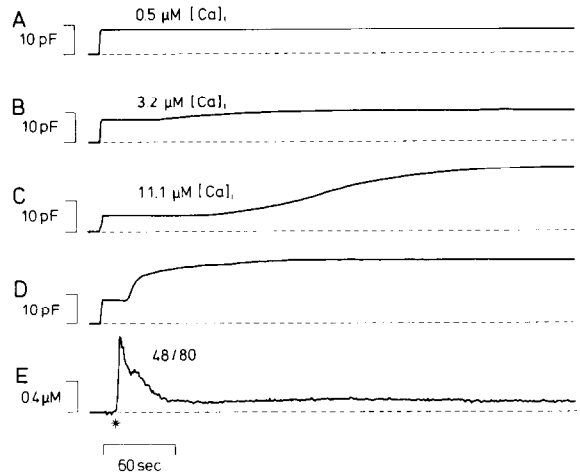

Fig.1. Secretory responses of mast cells stimulated by different intracellular calcium concentrations. Changes in cell membrane capacitance were measured, reflecting membrane area increases as a result of exocytosis. (A) The cell was dialyzed with standard intracellular solution which additionally contained GTP (300 μM) and a combination of dibromo-BAPTA/Ca-dibromo-BAPTA at a ratio of 3:1 mM, yielding a free $[Ca^{2+}]_i$ of ~500 nM. (B) Same as A except that $[Ca^{2+}]_i$ was buffered to ~3 μM (ratio = 1.33:2.66 mM). (C) Same as A with $[Ca^{2+}]_i$ buffered to ~11 μM (ratio = 0.5:3.5 mM). (D,E) Secretory response of a cell stimulated externally by compound 48/80 (5 μg/ml). Ca^{2+} buffers were omitted and changes in $[Ca^{2+}]_i$ were monitored by fura-2 (100 μM) included in the pipette filling solution. Application of compound 48/80 is indicated by the asterisk.

1 μM (fig.1A) remained ineffective in eliciting any significant secretion, higher Ca^{2+} levels of 3 μM (fig.1B) or 11 μM (fig.1C) progressively increased the amplitude of the secretory response. With even higher concentrations (30 μM, ratio 1:19) the relative degranulation amplitude (defined as the ratio of final capacitance to initial capacitance) again became smaller. It should be noted that $[Ca^{2+}]_i$ typically reached a high value within 10–20 s, but that the secretory response occurred only after prolonged exposure to high calcium. For comparison a typical more 'physiological' response of a mast cell stimulated with compound 48/80 is shown in fig.1. In the latter case $[Ca^{2+}]_i$ was left to vary freely by omitting Ca^{2+} buffers in the pipette filling solution. It can be seen that after stimulation with compound 48/80 most of the exocytotic activity (fig.1D) proceeds at levels below 1 μM (fig.1E).

The secretory response of cells stimulated with compound 48/80 is strongly dependent on the presence of GTP added to the pipette filling solution [14]. A requirement for GTP was also evident when stimulating cells with low $[Ca^{2+}]_i$, whereas in the presence of very high $[Ca^{2+}]_i$ the response became more and more independent of internally provided GTP. The relationship between $[Ca^{2+}]_i$ and the relative degranulation amplitude is shown in fig.2. The data ± SE were fitted using the Michaelis-Menten formalism, excluding the data points corresponding to 30 μM $[Ca^{2+}]_i$ and GTP-free internal solutions. The maximal degranulation amplitude was assumed to be 3.6 (mean maximal response attainable with GTP-γ-S [15]). The fitted dose-response curve yielded an apparent K_d of 4.7 μM with a Hill coefficient of 1.4. It should be pointed out that the fit did not describe the data for $[Ca^{2+}]_i$ values above 10 μM, where calcium seemed to be inhibitory.

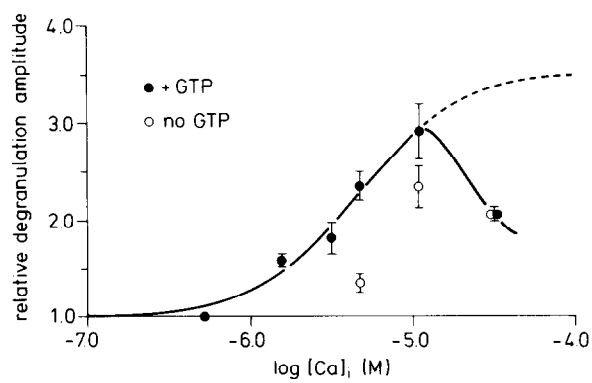

Fig.2. Dependence of the secretory response of mast cells on intracellular calcium concentration. The ordinate denotes the relative degranulation amplitude defined as the ratio of final capacitance to initial capacitance. The abscissa gives the $[Ca^{2+}]_i$ as calculated from the ratio of Ca^{2+}-free to Ca^{2+}-bound dibromo-BAPTA. Data points reflect means ± SE from 3–21 determinations. Experiments were conducted under identical conditions in the presence (●) and absence (○) of GTP (300 μM). The concentration-response curve was fitted to the data excluding values obtained in the absence of GTP and $[Ca^{2+}]_i$ of 32 μM, where secretion was inhibited. While the theoretical course of the fit is indicated by the dashed line, the data points for $[Ca^{2+}]_i$ of 11 and 32 μM were connected by eye. See section 3 for further details.

The values of $[Ca^{2+}]_i$ used in the data of fig.2 were calculated by assuming a dibromo-BAPTA dissocation constant of 1.58 μM. These values were in quite good agreement with those measured with fura-2 within the first 20–40 s after establishment of the whole-cell configuration. Thereafter, fura-2-fluorescence tended to indicate a progressive increase in calcium. We doubt that these changes reflect genuine concentration changes, since fura-2-fluorescence is inherently an inaccurate measure of such high calcium concentrations. Another possible reason for the apparent increase in $[Ca^{2+}]_i$ are morphological changes which were regularly observed after the prolonged presence of high intracellular Ca^{2+} levels. As demonstrated in fig.3A, mast cells, when challenged with high $[Ca^{2+}]_i$, swelled and acquired a balloon-like appearance with most of the cytoplasm and granules adhering to the pipette. These changes could only be observed when very slight positive hydrostatic pressure was applied through the patch pipette as is usually inadvertently exerted through the pipette filling solution. They disappeared as the hydrostatic pressure was relieved or made negative. The swelling usually occurred 20–40 s after patch rupture. It was not accompanied by significant capacitance changes but was paralleled by an increase in the fluorescence intensity as a result of the increase in total cell volume. For comparison, fig.3B illustrates the appearances of an intact mast cell and a cell kept in the whole-cell configuration (with slight positive hydrostatic pressure applied). Both cells were stimulated with compound 48/80.

In all experiments carried out with dibromo-BAPTA the total concentration of the chelator was kept at 4 mM. Only the ratios of the Ca^{2+}-bound and the Ca^{2+}-free forms of the chelator were varied. In order to test for possible side-effects of dibromo-BAPTA we have used a 1:3 ratio (Ca^{2+}-bound/Ca^{2+}-free dibromo-BAPTA $\approx$ 530 nM $[Ca^{2+}]_i$) while challenging the cell with 100 μM GTP-γ-S. To our surprise, the secretory response to this strong stimulus was almost completely inhibited, yielding a relative degranulation amplitude of 1.1 $\pm$ 0.04 (mean $\pm$ SE, n = 11).

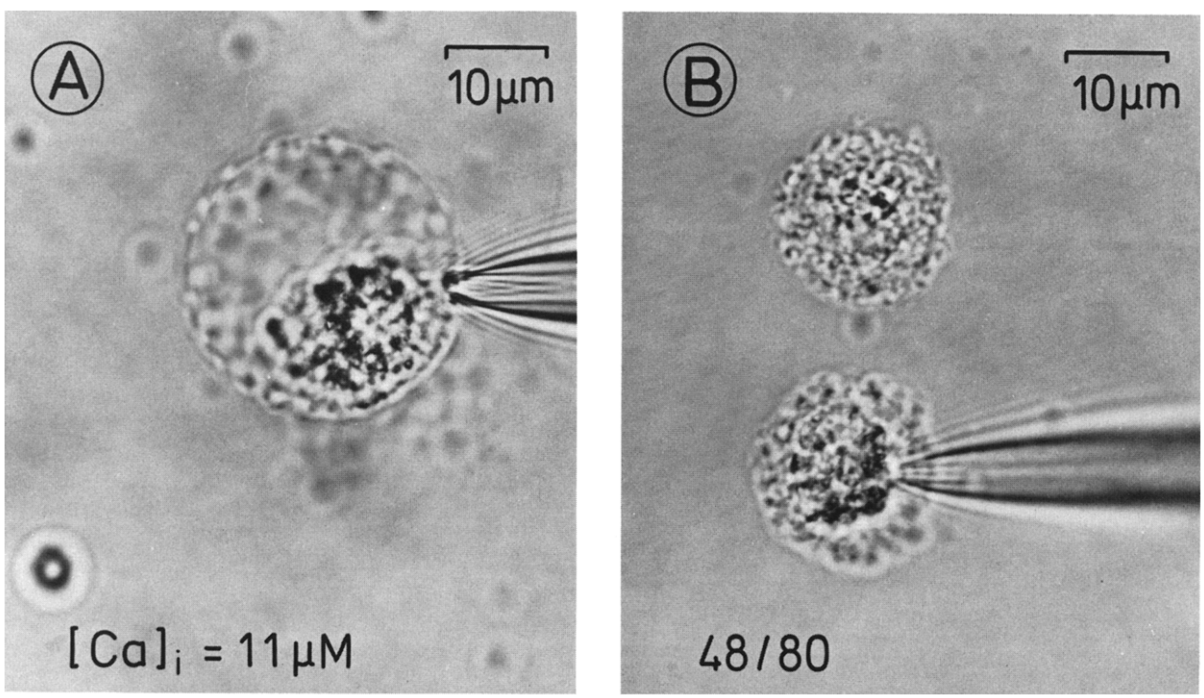

Fig.3. Morphological appearance of mast cells stimulated by high $[Ca^{2+}]_i$ (A) and compound 48/80 (B). In both cases the slight hydrostatic pressure exerted by the pipette solution was present. For comparison an unperturbed degranulated mast cell is shown in B.

Another way of increasing $[Ca^{2+}]_i$ is the application of the Ca^{2+} ionophore ionomycin which translocates extracellular Ca^{2+} into the cytosol [16]. For a controlled application we have applied ionomycin (5 μg/ml) for only brief periods by puffing it onto the cell under investigation by pressure ejection from a micropipette. This resulted in transient increases in intracellular calcium concentration as monitored by the fluorescent dye (fig.4B). As $[Ca^{2+}]_i$ increased to levels higher than 1 μM it was paralleled by a capacitance increase which ceased as $[Ca^{2+}]_i$ returned to resting levels (fig.4A). Repeated applications of ionomycin exerted similar secretory responses until eventually the cell became completely degranulated. When buffering the intracellular calcium concentration to near resting values, such that the translocated Ca^{2+} is effectively captured by the chelator, no secretory response could be elicited by repetitive application of the ionophore (fig.4C,D).

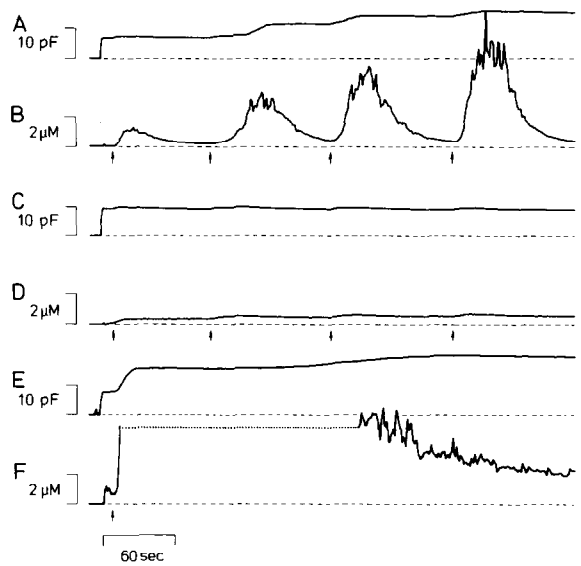

Fig.4. Effects of ionomycin on $[Ca^{2+}]_i$ and the resulting secretory response. (A,B) The cell was briefly flushed by external solution containing ionomycin (5 μg/ml) at the times indicated by arrows. The resulting increases in $[Ca^{2+}]_i$ and the concomitant increase in cell membrane capacitance are shown. (C,D) Same as A,B except that the internal solution was supplemented with a combination of EGTA/Ca-EGTA (5:5 mM) which effectively clamped $[Ca^{2+}]_i$ to near resting levels. (E,F) Ionomycin was applied once for a somewhat longer period (several seconds) at the time indicated by the arrow. The transient rise in $[Ca^{2+}]_i$ exceeded the measuring range of fura-2 and is therefore truncated (dotted line).

The level of intracellular calcium concentration could be varied by extending the application periods of ionomycin. Then $[Ca^{2+}]_i$ exceeded the accurate measuring range of fura-2. In these cases we frequently observed capacitance increases while $[Ca^{2+}]_i$ remained below 10 μM but as it increased further to unmeasurable values we observed a halt in secretion. As the $[Ca^{2+}]_i$ returned to the resting levels secretion resumed until $[Ca^{2+}]_i$ dropped below 1 μM (see fig.4E,F). In some experiments where $[Ca^{2+}]_i$ stayed at high levels for several minutes the same morphological changes were observed as described for dibromo-BAPTA experiments.

In a few experiments ionomycin was perfused into the recording chamber. This did not lead to steep rises of $[Ca^{2+}]_i$ as dramatic as those elicited by fast pressure ejection onto the cell. Typically, we found a slightly delayed slow transient increase in $[Ca^{2+}]_i$ to values in the range 0.5–2 μM which decayed to near resting values. In most cases this was not accompanied by degranulation of patch-clamped cells, whereas neighbouring cells did so to the full extent. It should be noted that ionomycin was able to induce small (0.3–0.8 μM) and transient (20–50 s) rises in $[Ca^{2+}]_i$ in nominally Ca^{2+}-free extracellular solution. This suggests that the ionophore may be able to release Ca^{2+} from internal stores.

4. DISCUSSION

The present study shows that $[Ca^{2+}]_i$ can bring about secretion provided the concentration is high enough and is sustained for long periods. From the dose-response curve derived from our data it becomes apparent that only Ca^{2+} levels well above 1 μM will be able to induce sizeable secretion with an apparent K_d of 4.7 μM. One possible reason for the high calcium requirement could be that we used as the chelating agent dibromo-BAPTA which we found to be a strong inhibitor of the exocytosis induced by GTP-γ-S. Evidence against such an explanation is provided by the fact that ionomycin-induced secretion in the absence of dibromo-BAPTA also required $[Ca^{2+}]_i$ of several micromolar. It is not clear at this point what may

be the cause of dibromo-BAPTA inhibition while even higher concentrations of EGTA have not been found to impede GTP-γ-S actions [15].

Since there is good reason to assume that a G-protein is involved in a late step of the secretory chain [14,17] this pathway may contribute to the responses we observed in the presence of GTP. Furthermore, the signal transduction cascade in mast cells is likely to comprise a number of Ca^{2+}-dependent enzymes [18] which may be subject to direct activation by high $[Ca^{2+}]_i$. It is well conceivable that under conditions where the G-protein-mediated signal is absent one may observe a shift of the dose-response curve to the right. This is in fact what was found in experiments where GTP was omitted from the pipette filling solution in agreement with studies on permeabilized cells [19]. However, at very high $[Ca^{2+}]_i$ secretion was less dependent on GTP.

Sustained levels of $[Ca^{2+}]_i$ above 1 μM caused severe morphological changes unrelated to a normal degranulation response, but similar to the alterations that have been observed in the presence of high Cl^- concentration [20]. The plasma membrane became responsive to slight changes of hydrostatic pressure through the patch pipette. These effects usually preceded the secretory response. It appears likely that the cytoskeleton is affected in some way by the presence of calcium making the cells more susceptible to external perturbations. If the cytoskeleton plays an important role in secretion, damage to it would impose a serious constraint on the secretory response.

The experiments with the Ca^{2+} ionophore ionomycin gave results qualitatively similar to those when introducing Ca^{2+} buffers, although a proper control of $[Ca^{2+}]_i$ was difficult to obtain. Calcium-dependent secretion was elicited by micromolar $[Ca^{2+}]_i$, while extremely high $[Ca^{2+}]_i$ impeded secretion. It is not clear what process is inhibited by high $[Ca^{2+}]_i$. However, it does not seem to involve an irreversibly toxic event, since the cell can resume secretion after returning to lower Ca^{2+} levels. The finding that buffering $[Ca^{2+}]_i$ to low values also abolishes the secretory response to ionomycin suggests that in mast cells a rise in $[Ca^{2+}]_i$ is a necessary requirement for ionophore action. This contrasts with the case of stimulation by compound 48/80 which will induce secretion under conditions of buffered $[Ca^{2+}]_i$ [21], presumably by activating the polyphosphoinositide metabolism, such that resting levels of $[Ca^{2+}]_i$ suffice to bring about degranulation.

A rise in intracellular calcium is among the early events that take place when mast cells are stimulated with a variety of agents. However, the role of calcium in the secretion process of mast cells remains controversial [22]. Relatively intact mast cells that have been briefly preloaded with fura-2 respond to antigenic or compound 48/80 stimulation with a transient increase in $[Ca^{2+}]_i$ up to several micromolar, but degranulation occurs after Ca^{2+} levels are within the range 0.2–1 μM [23]. When clamping $[Ca^{2+}]_i$ to these relatively low levels using EGTA buffers one also obtains a full secretory response following stimulation with compound 48/80, suggesting that the transient of high $[Ca^{2+}]_i$ is not required for secretion [21]. On the other hand, when mimicking the stimulation-induced changes in $[Ca^{2+}]_i$ by introducing inositol trisphosphate into the cell, there is no secretory response elicited despite the generation of very similar Ca^{2+} transients that probably result from the release of Ca^{2+} from intracellular stores [24]. This suggests that Ca^{2+} transients alone are not sufficient to cause degranulation.

The present finding of Ca^{2+}-enforced secretion at $[Ca^{2+}]_i$ above 1 μM reconciles previous patch-clamp measurements [9,15] with earlier studies employing calcium injection [25], ionophores and permeabilized cells [26] where calcium was found to be a sufficient stimulus. The effects of high calcium have probably gone unnoticed in patch-clamp investigations because they usually occur only with a delay and in a relatively narrow concentration range, in which $[Ca^{2+}]_i$ cannot be controlled accurately by EGTA at pH 7.2 (which were the conditions exclusively used in those studies). In addition, it is conceivable that other factors such as the composition of the pipette filling solution or the size of the tip of the pipette (which determines the speed of dialysis and 'washout' [27]) could determine the responsiveness of mast cells towards $[Ca^{2+}]_i$. Along this line lies the fact that in permeabilized mast cells one may obtain variable Ca^{2+}-concentration-response relationships depending on the additional presence or absence of various nucleotides [19,21]. It should be stressed, however, that high $[Ca^{2+}]_i$, which in our experiments appears to act as a rather unspecific

fusogen, does not prevail during physiological stimulation. The physiologically more important event in stimulus-secretion coupling seems to involve activation of other second messenger systems, while calcium may fulfill a supportive role. In this respect, mast cells are clearly distinct from excitable cells where under similar experimental conditions a 'calcium only' stimulus of 1 μM or less leads to dramatic exocytosis [28,29].

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