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Washout Phenomena in Dialyzed Mast Cells Allow Discrimination of Different Steps in Stimulus-Secretion Coupling

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Transient increases of intracellular calcium and exocytotic activity of rat peritoneal mast cells following stimulation with compound 48/80 were monitored using the Ca-indicator dye fura-2 and the capacitance measurement technique. It is known that mast cells very rapidly lose their secretory response towards antigenic or compound 48/80-induced stimulation in the whole-cell recording configuration of the patch-clamp technique due to "washout" of signal mediators. In contrast, we found that calcium transients remained unaffected by intracellular dialysis for as long as 10 min.

The fast "washout" phenomenon of exocytosis could be overcome by supplementing the pipette filling solution with guanosinetriphosphate (GTP) indicating a major role for GTP-binding proteins in secretion. The restoration of exocytosis was transient and decayed within three minutes, suggesting diffusional escape of one or several other cytoplasmic substances involved in stimulus-secretion coupling. Quantitative aspects of this process and the implications of its differential effects on Ca-transients versus secretion are discussed.

INTRODUCTION

Histamine release from mast cells has widely been used as a model system for the study of exocytosis. It is believed that one pathway involved in signal transduction, starting from receptor occupancy until eventual exocytotic fusion of mast cell granules, comprises activation of polyphosphoinositide phosphodiesterase (phospholipase C)

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with a GTP-binding protein as a mediator between receptor and the lipase (Kennerly *et al.*, 1979; Beaven *et al.*, 1984; Nakamura and Ui, 1985; Cockcroft and Gomperts, 1985). As a result of phospholipid breakdown two second messengers are released: inositol trisphosphate (IP_3) and diacylglycerol (DAG). While IP_3 mediates the release of Ca^{2+} from intracellular stores, DAG activates protein kinase C (for review see Berridge and Irvine, 1984), which in turn is thought to bring about the secretory response by an as yet unknown mechanism. The crucial role of intracellular Ca^{2+}-concentration in triggering release, undisputed in many secretory systems (Katz, 1969; Baker and Knight, 1984) is still a matter of debate in mast cells (Gomperts, 1986).

Transient increases in intracellular calcium reminiscent of those obtained by introduction of IP_3 can be measured by the use of fluorescent calcium indicator dyes following stimulation of mast cells with a variety of secretagogues including antigen, compound 48/80 and intracellularly injected GTP-γ-S (Neher and Almers, 1986; Neher, 1987). Using the latter stimulus has proven valuable in monitoring the kinetic aspects of the secretory response by measuring the capacitance increase of the cell membrane as exocytotic fusion of granules proceeds (Neher, 1987). In other cases, where secretagogues are added exogenously, the establishment of the whole-cell recording configuration of the patch-clamp technique prevents the cells from undergoing degranulation, presumably due to fast washout of cytoplasmic constituents indispensable in the chain of events leading to exocytosis (Fernandez *et al.*, 1984).

The use of the recently introduced "slow whole-cell" configuration (Lindau and Fernandez, 1986) circumvents this apparent disadvantage of the conventional tight-seal whole-cell mode. However, it also prevents a fast and effective dialysis of the intracellular space which is often desired in order to selectively introduce agents into cells which normally have no access to the cytoplasm when added exogenously.

We have tried to re-establish the normal secretory response of mast cells in the conventional whole-cell configuration by adding GTP into the pipette filling-solution and report here that this procedure indeed enables mast cells to undergo degranulation following stimulation with compound 48/80. However, the restorative action of GTP was only transient and decayed within a few minutes, such that delayed application of 48/80 was again ineffective in eliciting secretion. Our data suggest that the fast washout of secretion found in whole-cell recordings from these cells is due to the loss of a considerable fraction of endogenous GTP. This appears to be paralleled by a slower washout of yet another readily dilutable cytoplasmic component required in the sequence of events resulting in exocytosis. In contrast, washout of neither substance seems to inflict the stimulus-induced increase of intracellular calcium concentration.

METHODS

Anaesthesized male Wistar rats weighing 300–400 g were decapitated and exsanguinated. Peritoneal mast cells were obtained by injecting into the abdominal cavity 10 ml of a saline comprising (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, pH 7.2. The fluid was recollected and centrifuged for 1 min at $200 \times g$. The pellet was resuspended in the same

medium of which 2 ml were layered over a percoll gradient that contained 9 parts (v/v) percoll and 1 part (v/v) of 10 × concentrated saline. After centrifugation (35,000 × *g* for 20 min at 4°C) the band which contained the mast cell fraction (near the bottom of the tube) was aspirated, centrifuged (200 × *g* for 1 min) 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), penicillin (0.64 mg/ml), pH 7.2. Cells were plated onto washed cover slips placed inside 35 mm culture dishes and stored in an incubator at 37°C and 10% CO_2 for 1 to 6 hours until use.

Experiments were performed at 23–26°C in a Mg-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 at preventing ATP-induced leaks (Bennett *et al.*, 1981) during patch formation.

Patch-clamp measurements were performed with Sylgard-coated pipettes in the tight-seal whole-cell configuration as described by Hamill *et al.* (1981). Pipette resistance, after filling with the solution given below, ranged between 2 MΩ and 3.5 MΩ. The solution for filling pipettes (intracellular solution) contained (in mM): K-glutamate 135, NaCl 20, $MgCl_2$ 1, HEPES-NaOH 10, Na_2-ATP 0.2, fura-2 pentapotassium salt 0.1 (Molecular Probes). In experiments in which GTP was added to the intracellular solution it was used at a concentration of 300 μM. The mast cell secretagogue compound 48/80 (Sigma) was dissolved in extracellular solution (5 μg/ml) and applied directly onto the cell under investigation *via* pressure ejection from a second pipette.

Capacitance measurements were performed with a 2-phase Lock-In amplifier as detailed by M. Lindau and E. Neher (manuscript in preparation). Briefly, a 16 mV rms 800 Hz sine wave was added to the command potential of the patch clamp (usually + 25 mV). The resulting sinusoidal current was measured by the Lock-In amplifier at two mutually orthogonal phase angles. One of these phase angles coincided with a purely conductive signal, i.e. it was very close to zero, but allowed for small phase shifts of the measuring apparatus. This is different from previous uses of the Lock-In amplifier (Neher and Marty, 1982; Almers and Neher, 1987) where the phase was adjusted to also allow for phase shifts due to series resistance in the pipette-cell assembly. Contrary to previous uses also the membrane capacitance was not compensated. The two signals from the Lock-In amplifier were fed to the AD-inputs of a PDP 11–23 laboratory computer together with the applied potential and the DC-current (after filtering out the sinusoidal component). These were used 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 and 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 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. Light was collected from a circular area of 20 μM diameter in the center of the field of view where

the cell under investigation had been placed. $[Ca]_1$ was calculated from the fluorescent ratio as described by Grynkiewicz *et al.* (1985). Calibration constants were determined *in vivo* as described by Almers and Neher (1985).

A total of six signals (two fluorescent intensities; two Lock-In signals; DC-current and -voltage) were fed to the computer and sampled every 0.5 to 2 seconds. Subsets of these and $[Ca]_1$ were displayed online on a vector display. Later they were plotted on a HP 7470A (Hewlett Packard) plotter.

RESULTS

When mast cells were investigated using the whole-cell configuration they rapidly lost their ability to degranulate following stimulation with compound 48/80. Cells consistently failed to degranulate even when the stimulus was given shortly after establishment of the whole-cell mode. This "washout" phenomenon is exemplified in Fig. 1 where the secretagogue was administered 5 sec after patch rupture (the possibly fastest application under our experimental conditions). It can be seen that the cell capacitance (Fig. 1A, top trace) remains unaltered indicative of no major exocytotic (or endocytotic) activity.

The ability of compound 48/80 to elicit a transient rise in intracellular Ca^{2+}, however, was not impaired as illustrated in Fig. 1A (lower trace). Calcium transients manifested even when the drug was delivered as late as 10 min after establishment of whole-cell configuration during which time the cell was effectively dialyzed (Fig. 1B). Amplitude and shape of Ca-transients at various stimulation delays showed some variability (cf. Fig. 2) and were not noted to bear consistent differences. From these findings it appears that the loss of exocytotic activity caused by the diffusional exchange between cytoplasm and the effectively infinite volume of the patch pipette is not due to a disruption of the sequence of events that mediate the elevation of intracellular Ca^{2+}.

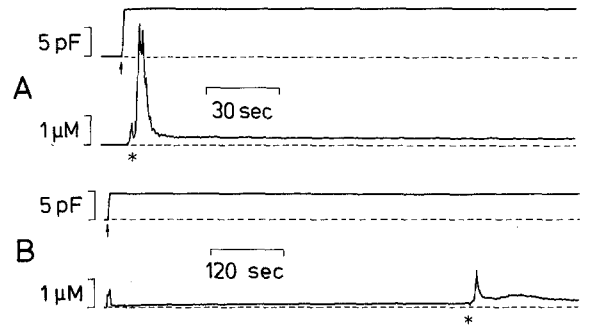

Fig. 1. Effect of compound 48/80 (5 µg/ml) on intracellular Ca^{2+}-concentration (lower traces) and cell membrane capacitance (upper traces) in the absence of GTP. Compound 48/80 was applied 5 sec (A) and 600 sec (B) after establishment of whole-cell configuration. Time of patch rupture (↑) and drug application (*) are indicated in the graphs. Amplitude and shape of Ca-transients at various stimulation delays showed some variability and were not noted to bear consistent differences. Note the different time scale in A and B.

GTP-binding proteins play an essential role in stimulus-secretion coupling of many cell types, and GTP-γ-S (a non-hydrolysable analogue of GTP) is an effective secretagogue in mast cells when allowed to gain intracellular access through patch pipettes in the whole-cell configuration (Fernandez *et al.*, 1984). Hence, it was conceivable that GTP was the missing link in our experiments and we therefore included 300 μM GTP in the pipette filling solution. Unlike its analogue GTP-γ-S, GTP by itself neither induced an intracellular calcium transient nor did it cause mast cell degranulation within the usual observation periods of 4–8 min. On rare occasions a slow and rather small capacitance increase was observed in cells which were investigated for longer periods (10–20 min).

The most striking result when supplementing the intracellular solution with GTP consisted in the restoration of the secretory response of mast cells following stimulation with 48/80. In addition to the generation of the Ca transient we consistently observed a full degranulation of the mast cell, i.e. the cell capacitance increased by a factor of about three above its initial value by the end of the secretory process. Figure 2A illustrates a typical response of a mast cell when challenged with 48/80 shortly after patch disruption. However, when the secretagogue was administered at later times, there was a gradual decrease in the attainable degranulation amplitude. Two examples of this are given in Figs 2B and 2C in which the drug was delivered at 90 and 180 sec (after whole-cell), respectively.

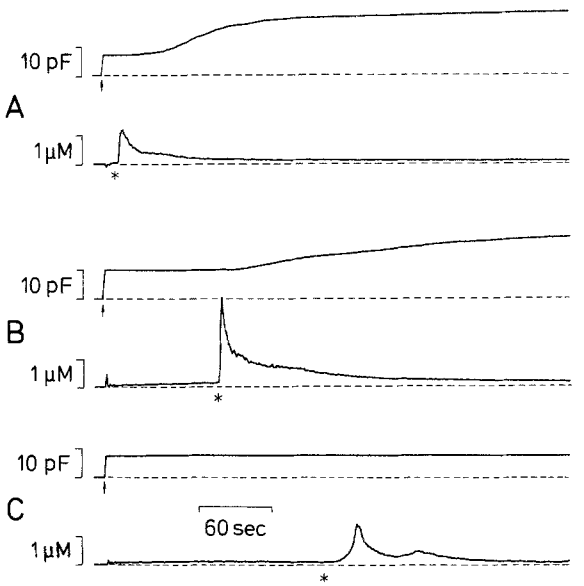

Fig. 2. Effect of compound 48/80 (5 μg/ml) on intracellular Ca^{2+}-concentration (lower traces) and cell membrane capacitance (upper traces) when GTP (300 μM) was present in the pipette filling solution. Compound 48/80 was applied 10 sec (A), 90 sec (B) and 180 sec (C) after establishment of whole-cell configuration. Patch rupture (↑) and drug application (*) are indicated in the graphs.

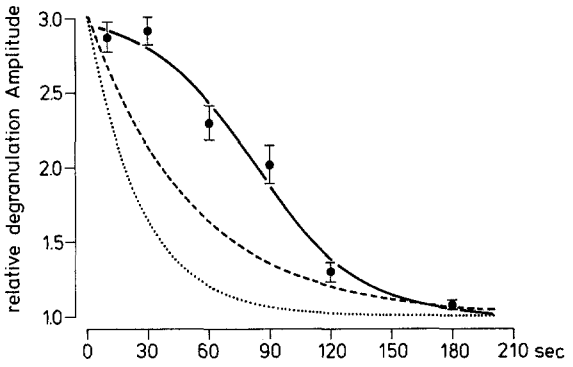

Fig. 3. Reduction of the relative degranulation amplitude as a function of the time-lagged application of compound 48/80. Data points correspond to the ratio of the cell capacitance after completion of the secretory response and the initial capacitance value. Degranulation was considered terminated when capacitance values remained steady for 30–120 sec. Each data point reflects mean values from 6–14 determinations ±SEM. Details concerning the fitting procedure and the meaning of the exponential curves included in the graph are provided in the text.

In order to quantify this "slow" washout, revealed after overcoming the "fast" washout due to GTP, we systematically applied 48/80 externally at different time-lags after establishment of the whole-cell configuration. In all these experiments GTP (300 μM) was present in the pipette filling solution. As a measure of the secretory response we used the "relative degranulation amplitude" defined as the ratio of the cell capacitance after completion of degranulation and the initial capacitance. From the values measured (provided in Fig. 3) it can be seen that after three minutes no significant exocytotic activity can be elicited by 48/80.

The time course of the decay does not seem to reflect a simple exponential function. The solid line in the graph was fitted to the data points by the least-squares method using an equation derived from Michaelis-Menten kinetics (see below). This is based on some reasonable assumptions: (a) the decrease of the degranulation amplitude is due to the loss of a cytoplasmic substance by diffusion into the pipette. This process follows an exponential time course $c = c_0 e^{-t/\tau}$, (b) the relative degranulation amplitude is strictly determined by the concentration of the substance at the time at which the stimulus (48/80) is delivered but is essentially independent of a further possible dilution of the substance as degranulation proceeds (see Discussion), (c) the substance is involved in some kind of chemical reaction during its action and the yield of this reaction is linearly related to the relative degranulation amplitude.

Based on these assumptions the following equation, relating the relative degranulation amplitude and the time of 48/80-application after patch rupture, can be derived:

$$R = \frac{M-1}{1+\left(\frac{K}{c}\right)^n} + 1 = \frac{M-1}{1+\left(\frac{K}{c_0}\right)^n e^{+tn/\tau}} + 1$$

where R is the relative degranulation amplitude, M the maximal relative degranulation amplitude, K the Michaelis-Menten constant, n the Hill coefficient and $c = c_0\, e^{-t/\tau}$ the concentration of the substance at the time t of 48/80-application. The solid line in Fig. 3 fitted to our data yields the following values: $M = 3.04$, $\tau = n \times 26$ sec, $(K/c_0)^n = 0.042$.

With these values one may obtain the presumed exponential decay of the concentration of the substance within the cytoplasm. The time course for an assumed Hill coefficient of 1 is represented in Fig. 3 by the dotted line. It can be seen that at the time at which a half-maximal relative degranulation amplitude can be obtained, the concentration of the substance would have decayed to about 4% of the initial concentration. In our opinion a coefficient of 2 gives a more reasonable exponential curve (Fig. 3, dotted line) in which $\tau = 52$ sec (broken line). Further implications are detailed in the Discussion.

DISCUSSION

The results presented provide evidence that the secretory response of mast cells to compound 48/80, observed in the whole-cell configuration of the patch-clamp technique, is inflicted by fast and slow washout phenomena. The rapid loss of the ability to degranulate (fast washout) is due to the diffusional escape of GTP into the patch pipette. This is strikingly evident from the restoration of the cell's ability to degranulate when GTP is provided within the pipette filling solution. This further stresses the idea that GTP-binding proteins play a crucial role in secretion from these cells. The slow washout which is seen in the presence of GTP presumably involves diffusion of one or several other cytoplasmic constituents required in stimulus-secretion coupling.

There is ample evidence for the involvement of a GTP-binding protein in initiating polyphosphoinositide breakdown *via* activation of phospholipase C (for review, see Joseph, 1985). The liberated second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3) promote activation of protein kinase C and release of calcium from intracellular stores, respectively. This view has found some support by the findings that introduction of IP_3 into mast cells induces transient increases in $[Ca^{2+}]_i$ (Neher, 1986) and this is mimicked by GTP-γ-S when injected intracellularly as well as by 48/80 or antigen when added extracellularly (Neher, 1986; Neher and Almers, 1986). It was therefore conceivable that the fast washout of the secretory response was due to the lack of GTP, thus impairing phosphoinositide breakdown.

However, in experiments in which no GTP was present in the pipettes, the generation of Ca-transients by 48/80 was not subject to any obvious rundown even after prolonged periods in which most of the endogenous cytoplasmic GTP should have vanished. One possible explanation for this finding would be that the action of 48/80, which has been shown to involve polyphosphoinositide breakdown (Nakamura and Ui, 1985), occurs at a step subsequent to the GTP-binding protein, possibly involving direct activation of phospholipase C. Alternatively, there may be a different way of inducing Ca-transients, which does not require the presence of cytoplasmic GTP or only very small concentrations of it.

Providing GTP with the intracellular solution resulted in restoration of the

degranulation process. Since this effect was only transient one has to assume some kind of 'slow washout' caused by diffusional drift of one or several as yet unknown cytoplasmic substances. As already mentioned in the results section we made some basic assumptions in order to provide quantitative estimates on the washout process. One of these assumptions merits particular attention as it infers that the relative degranulation amplitude is critically determined by the concentration of the substance at the time at which the stimulus (48/80) is delivered, but is essentially independent of it as degranulation proceeds. Good support for this comes from a number of experiments in which the time course of degranulation extended over 5–15 minutes. In fact, the secretory response hardly ever went to completion before 3–6 minutes, whereas delayed stimulation with 48/80 at this time would usually not induce secretion at all. This would either suggest that the substance is merely required as an impulsive trigger of the secretory process or, if required during exocytosis, it be transferred from a freely diffusible cytoplasmic state into a non-diffusible, possibly membrane-bound state. A well-known example for the latter possibility is the translocation of protein kinase C from cytoplasm to plasma membrane following stimulation (Kraft and Anderson, 1984).

Certainly, it is tempting to infer protein kinase C as a potential candidate for causing the observed slow washout. However, a quantitative study (Pusch and Neher, in preparation) investigating the diffusional exchange between patch pipettes and small cells would imply that the diffusion time constant of 26 sec (Hill coefficient = 1) as measured with access resistance of 6 MΩ (mean value in this study) corresponds to a molecule with a relative molecular mass between 300 and 1500. Even when assuming a time constant of 52 sec (Hill coefficient = 2) this would yield a rather small molecular mass of 2400 to 12,000 compared to protein kinase C ($M_r = 82{,}000$).

It should be noted, however, that the model assumptions we made are to some extent arbitrary and cannot rule out protein kinase C as the substance responsible for the observed loss of the secretory response. Intriguingly, one would expect a diffusion time constant of about 150 sec for a substance of $M_r = 82{,}000$ which could reasonably well reflect the reduced exocytotic activity of mast cells stimulated that late. This possibility could be tested by providing the enzyme together with GTP in the pipette filling solution.

Taking the above given numbers seriously, one would expect for intracellular concentration of GTP ($M_r = \sim 500$) a decrease with a diffusion time constant of about 17–35 sec, in which case it appears surprising that even with very fast application of 48/80 no degranulation can be elicited. A possible explanation for this latter finding would infer that rather small decreases in intracellular GTP-levels may disable the cell to degranulate. More likely than this appears the possibility that the GTP-requiring step may not strictly coincide with the time of 48/80 application but presumably has some time-lag. Indeed, the delay between application of 48/80 and the initiation of exocytotic activity usually ranged between 15 and 30 seconds. This would be in good agreement with the notion that a GTP-binding protein may be involved in a late step of exocytosis as has been reported for neutrophils (Barrowman *et al.*, 1986).

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