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Fusion pore expansion in horse eosinophils is modulated by Ca^{2+} and protein kinase C via distinct mechanisms

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Using the patch-clamp technique, we studied the role of protein phosphorylation and dephosphorylation on the exocytotic fusion of secretory granules with the plasma membrane in horse eosinophils. Phorbol 12-myristate 13-acetate (PMA) had no effect on the amplitude and dynamics of degranulation, indicating that the formation of fusion pores is insensitive to activation of protein kinase C (PKC). Fusion pore expansion, however, was accelerated ~2-fold by PMA, and this effect was abolished by staurosporine. Elevating intracellular Ca^{2+} to 1.5 μM also resulted in a 2-fold acceleration of pore expansion; this effect was not prevented by staurosporine, indicating that intracellular Ca^{2+} and activation of PKC accelerate fusion pore expansion via distinct mechanisms. However, fusion pores can expand fully even when PKC is inhibited. In contrast, the phosphatase inhibitor α-naphthylphosphate inhibits exocytotic fusion and slows fusion pore expansion. These results demonstrate that, subsequent to its formation, fusion pore expansion is under control of proteins subject to functional changes based on their phosphorylation states.

Keywords: capacitance/exocytosis/membrane fusion/patch-clamp/staurosporine

Introduction

During exocytosis, secretory organelles containing preformed material fuse with the plasma membrane releasing their contents through a resulting connection between the lumen of the organelle and the extracellular space, the fusion pore. Upon its formation, the small fusion pore appears to be a universal structure having a mean conductance of ~200 pS in beige mouse mast cells, human neutrophils and horse eosinophils. Within milliseconds, the pore then enlarges its conductance up to several nS, allowing rapid release of vesicle or granule contents (Lindau and Almers, 1995).

The fusion pore expansion rate is sensitive to a concentration of intracellular free calcium ($[Ca^{2+}]_i$) in the micromolar range (Fernández-Chacón and Alvarez de Toledo, 1995; Hartmann and Lindau, 1995). As the binding of Ca^{2+} ions to free phospholipid headgroups is negligible below 10 μM free Ca^{2+}, it has been suggested that pore expansion may involve more than lipidic interactions within membranes. Thus, although it is generally accepted that fusion pore expansion involves lipid incorporation (Monck *et al*., 1990; Nanavati *et al*., 1992; Lindau and Almers, 1995), even this lipidic state of pore expansion may be regulated by proteins (Fernández-Chacón and Alvarez de Toledo, 1995; Hartmann and Lindau, 1995). However, it has also been shown that significant Ca^{2+} binding, at even lower free concentrations, does occur between phosphatidylserine headgroups of closely apposed lamellae (Feigenson, 1986). As comparable domains might be present around early fusion pores during their initial expansion, it cannot be ruled out that the effect of Ca^{2+} in the physiological range might also involve this type of Ca^{2+} binding between anionic lipids. Whereas the possible contributions of such domains are difficult to test for, the involvement of proteins can be examined readily using a variety of agents known to modify protein function in specific ways.

Activation of kinases and phosphorylation of proteins have been shown to affect exocytosis in a variety of cell types (Howell *et al*., 1989; Coorssen *et al*., 1990; Ämmälä *et al*., 1994; Brewer and Roth, 1995; Hay *et al*., 1995; Wiedemann *et al*., 1996). At frog neuromuscular junctions, a kinase inhibitor, staurosporine, was shown to block destaining of vesicles preloaded with a fluorescent dye, FM1-43, reflecting the exocytotic loss of the dye, but not to block synaptic transmission. It was suggested that in the presence of staurosporine, a small fusion pore of 1–2 nm radius may open transiently for ~1 s, without post-exocytotic collapse of the vesicle into the plasma membrane (Henkel and Betz, 1995). Staurosporine is an inhibitor of protein kinase C (PKC). Although it may have additional effects at the concentrations used (2–5 μM), these results are a further indication of a role for proteins in fusion pore expansion.

Horse eosinophils exocytose large specific granules. Using the whole-cell patch-clamp technique, the correspondingly large capacitance of these granule membranes (C_V) makes it possible to record electrically the formation and expansion of the fusion pore between single granules and the plasma membrane, employing the fusion pore conductance (G_P) as an equivalent of the fusion pore size (Breckenridge and Almers, 1987; Spruce *et al*., 1990). We studied the influence of treatments favouring phosphorylation or dephosphorylation conditions on the exocytotic fusion pores formed during degranulation stimulated by intracellular application of GTPγS.

Results

PMA has no effect on amplitude and time course of degranulation stimulated by GTPγS

In single patch-clamped horse eosinophils, degranulation was stimulated by intracellular perfusion with solutions containing 20 μM GTPγS via the patch pipette in whole-cell configuration. Exocytosis was recorded by time-resolved measurements of cell membrane capacitance (C_m). The extent of degranulation was quantified by measuring the total increase in C_m. The dynamics of the degranulation process were characterized by: (i) the delay time between initiation of intracellular perfusion and the first C_m step indicating exocytosis of an eosinophil-specific granule; (ii) the frequency distribution of time intervals between individual fusion events (C_m steps); and (iii) the C_m step size distributions.

When cells were perfused with a control solution containing 20 μM GTPγS and <10 nM free calcium, C_m increased by 3.8 ± 0.2 pF (n = 34). The initial C_m of these cells was 2.8 ± 0.1 pF, comparable with previous reports (Scepek and Lindau, 1993; Hartmann and Lindau, 1995). Overall, the capacitance of individual cells increased by a factor of 2.4 ± 0.1. A typical degranulation trace is shown in Figure 1A. The delay between patch rupture and the first C_m step was 170 ± 20 s (n = 33). When the pipette solution also contained 100 nM of phorbol 12-myristate 13-acetate (PMA) (Figure 1B), the total increase in C_m was 4.0 ± 0.2 pF, representing a factor of 2.3 ± 0.1 (n = 22), and the delay to the first C_m step was 180 ± 20 s (SEM, n = 23). The time intervals between subsequent C_m steps were measured to characterize the rate of the exocytotic events during degranulation. The frequency distributions of these time intervals (data not shown) decay exponentially (Scepek and Lindau, 1993; Lindau *et al*., 1994). The time constants obtained from single exponential fits were 8 ± 1 s under control conditions (20 μM GTPγS and <10 nM $[Ca^{2+}]_i$) and 7 ± 1 s in the presence of 100 nM PMA. Thus, in spite of the addition of PMA, all these parameters were indistinguishable from control values. The C_m step size distributions were also unchanged (data not shown). Therefore, the rate at which stable fusion events occurred was not affected by addition of 100 nM PMA, nor was the total extent of degranulation or the types (sizes) of the granules fusing. When GTPγS was omitted and PMA applied alone, only very small C_m changes were observed in eight of 12 cells (67%). In these cells, the average increase was 0.3 ± 0.1 pF (n = 12), <10% of the increase observed in control cells. The delay to the first C_m step was 870 ± 90 s (SEM, n = 8). In the absence of both PMA and GTPγS, cells showed neither a significant capacitance increase nor any capacitance steps.

PMA and Ca^{2+} accelerate fusion pore expansion in different ways

To test if the extent or time course of fusion pore expansion is modulated by PKC activation, the time course of electrical conductance for individual fusion pores connecting the intragranular lumen to the extracellular space was calculated from the C_m trace. The resulting increase in pore conductance was approximated by fitting a straight line as previously described (Hartmann and Lindau, 1995). The slope of this line indicates the rate, r, of the conductance increase in

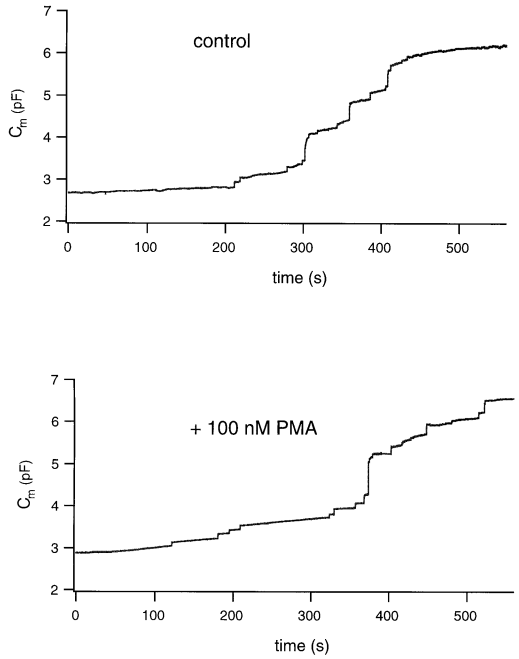

Fig. 1. Traces showing the increase in plasma membrane capacitance during degranulation of horse eosinophils in the absence (control) and presence of 100 nM PMA in the intracellular solution. In both experiments, the pipette solution contained 20 μM GTPγS and 7 mM EGTA ($[Ca^{2+}]_i$ <10 nM). The different delays for the first capacitance step represent the usual variability under control conditions (Scepek and Lindau, 1993).

nS/s. For C_m steps <50 fF, the time course of fusion pore conductance was not well resolved. Therefore, only C_m steps >50 fF were used for the fusion pore analysis. For steps >50 fF, the measured pore expansion rates were not correlated with the step sizes (data not shown; Hartmann and Lindau, 1995).

The measured expansion rates were grouped into bins, and frequency distributions were constructed. As shown previously (Hartmann and Lindau, 1995), the expansion rates are exponentially distributed (Figure 2). The characteristic expansion rates ρ were obtained from single exponential fits to the distributions obtained under the different experimental conditions. In the absence of PMA, the characteristic fusion pore expansion rate in cells stimulated at low $[Ca^{2+}]_i$ (Figure 2A) was 28 ± 2 nS/s (n = 354 fusion pores from 38 cells).

In the presence of 100 nM PMA, the pore expansion rate at low $[Ca^{2+}]_i$ (Figure 2B) was accelerated ~2-fold to 59 ± 7 nS/s (n = 206, 26 cells). When, instead of PMA, the inactive analogue 4α-phorbol-didecanoate (αPDD) was used at the same concentration (Figure 2C), the pore expansion rate was 27 ± 1 nS/s (n = 254, 23 cells), not significantly different from the control value. To provide further evidence that the effect of PMA is mediated by activation of PKC, staurosporine, an inhibitor particularly effective on membrane-bound PKC (Budworth and Gescher, 1995), was added together with PMA. Under these conditions (Figure 2D), the pore expansion rate was 26 ± 1 nS/s (n = 189, 16 cells), not significantly different from the value in the absence of PMA. Staurosporine thus completely abolished the accelerating effect of PMA on pore expansion.

In the absence of PMA (Figure 2E), 1.5 μM $[Ca^{2+}]_i$ increased the rate of fusion pore expansion to 47 ± 8 nS/s

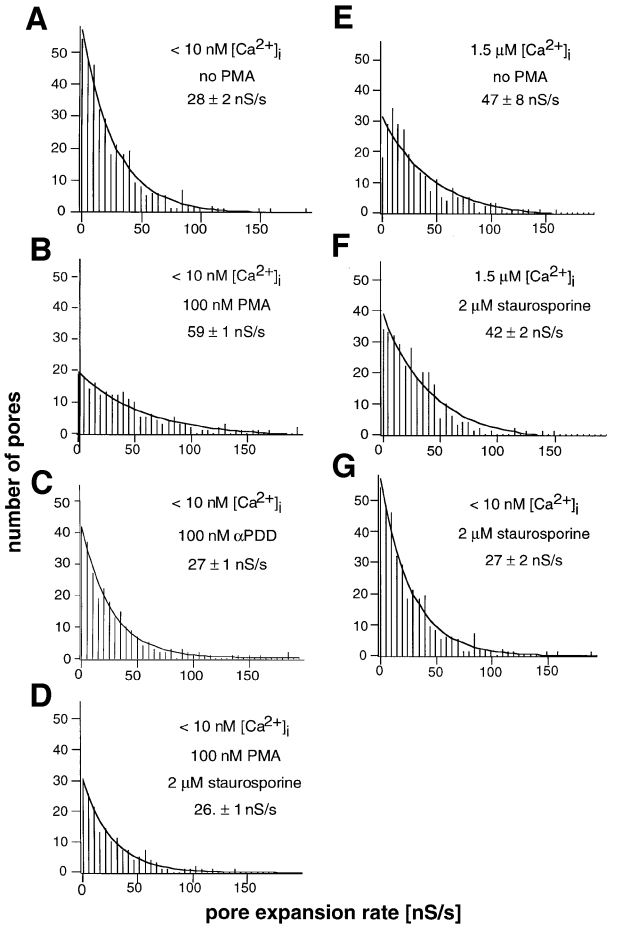

Fig. 2. Frequency distributions of fusion pore expansion rates under different conditions. In each case the characteristic rate was determined by a single exponential fit (smooth lines).

(n = 264, 21 cells). This is an almost 2-fold increase compared with the rate at low $[Ca^{2+}]_i$, in excellent agreement with a previous report (Hartmann and Lindau, 1995). The pore expansion rate can thus be accelerated either by PMA or by elevated $[Ca^{2+}]_i$. Measurements of $[Ca^{2+}]_i$ using the fluorescent indicator Fura-2 (200 μM; as in Coorssen *et al*., 1996) showed that with 7 mM EGTA in the pipette, $[Ca^{2+}]_i$ stayed constantly below 10 nM during degranulation stimulated by 20 μM GTPγS, with or without 100 nM PMA (data not shown). Thus, both GTPγS-induced fusion and the acceleration of pore expansion by PMA are not due to an elevation of $[Ca^{2+}]_i$. We used staurosporine in the absence of PMA to test whether the increase in pore expansion rate at high $[Ca^{2+}]_i$ was mediated by calcium-dependent activation of PKC. When 2 μM staurosporine was included in the pipette solution, the pore expansion rate at 1.5 μM $[Ca^{2+}]_i$ was not affected (Figure 2F; 42 ± 4 nS/s, n = 292, 16 cells). Staurosporine also had no effect on the pore expansion rate at low $[Ca^{2+}]_i$ (Figure 2G, 27 ± 2 nS/s, n = 231, 15 cells).

α-Naphthylphosphate inhibits degranulation stimulated by GTPγS

Addition of 5 or 10 mM α-naphthylphosphate (α-np), a broad spectrum phosphatase inhibitor, inhibited exocytosis

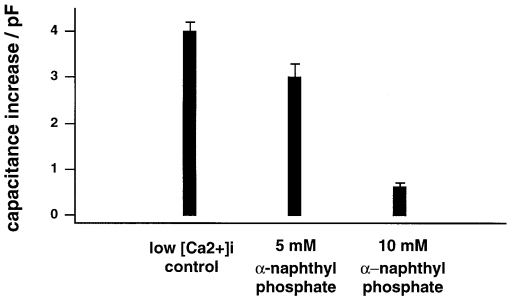

Fig. 3. Amplitudes of the total capacitance increase during degranulation in the absence (n = 34) and presence of 5 (n = 20) or 10 mM (n = 32) α-np.

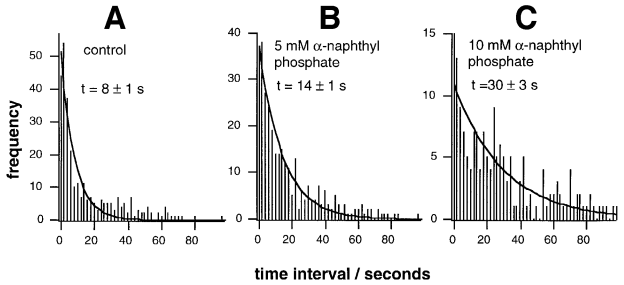

Fig. 4. Frequency distributions of time intervals between fusion events in the absence and presence of α-np. The time constants reflecting the exocytotic fusion rates were obtained by single exponential fits (smooth lines).

(Figure 3). At 10 mM, α-np blocked exocytosis completely in 20% of the cells (seven out of 39), as they did not show any C_m increase. In the remaining 80% of the cells, a few capacitance steps occurred. The average C_m increase in the degranulating cells was only 0.4 ± 0.1 pF (n = 32), ~10 times smaller than in control cells. At 5 mM, α-np caused only a small reduction of the stimulated C_m increase to 3.0 ± 0.3 pF (n = 20, P <0.05), and all cells showed C_m steps indicating exocytosis of specific granules. To characterize the dynamics of exocytosis, the time intervals between consecutive specific granule fusion events (C_m steps) were measured. The frequency distributions of these time intervals in the presence and absence of α-np are shown in Figure 4. The distributions decay exponentially, and the corresponding rates of exocytosis were determined by single exponential fits. The time constant under control conditions (20 μM GTPγS and low $[Ca^{2+}]_i$) was τ = 8 ± 1 s (exocytotic rate k = 0.125/s); with the addition of 5 mM α-np, the rate constant decreased almost 2-fold (k = 0.071/s) and with 10 mM α-np ~4-fold (k = 0.033/s). The exocytotic fusion rate during degranulation is thus decreased by the phosphatase inhibitor.

α-Naphthylphosphate decreases the fusion pore expansion rate

The frequency distributions of fusion pore expansion rates measured in the presence of α-np are shown in Figure 5. Characteristic expansion rates obtained from single exponential fits were 19 ± 1 nS/s at 5 mM α-np (n = 246, 20 cells) and 10 ± 1 nS/s at 10 mM α-np (n = 165, 39 cells). These rates are significantly lower than those measured in control cells (28 ± 2 nS/s). Thus, the

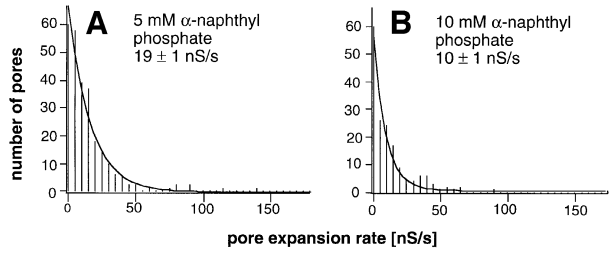

Fig. 5. Frequency distributions of fusion pore expansion rates at two different concentrations of α-np. The characteristic rates were obtained from single exponential fits (smooth lines).

phosphatase inhibitor also decreased the rate of fusion pore expansion.

Discussion

In this study, we used measurements of cell membrane capacitance to study the influence of protein phosphorylation and dephosphorylation on different parameters of both GTPγS- and Ca^{2+}-induced exocytosis in horse eosinophils. PMA had no effect on the total amplitude and time course of GTPγS-induced degranulation, suggesting that the process of fusion pore formation is insensitive to activation of PKC. However, PMA treatment accelerates expansion of exocytotic fusion pores once they have been formed. This effect is not observed when the inactive analogue αPDD is used or when PMA is applied together with the kinase inhibitor staurosporine. These results indicate that the acceleration of pore expansion by PMA is mediated by PKC rather than other targets of PMA such as Munc13 (Brose *et al*., 1995). Fusion pore expansion is also accelerated by elevated $[Ca^{2+}]_i$, but this effect is insensitive to staurosporine. Since with PMA and 7 mM EGTA in the pipette solution, $[Ca^{2+}]_i$ stayed below 10 nM throughout the degranulation, the accelerating effect of PMA is not due to an increase in $[Ca^{2+}]_i$. Fusion pore expansion is thus modulated by $[Ca^{2+}]_i$ and PKC via distinct mechanisms. Staurosporine also had no effect on expansion of GTPγS-induced fusion pores at <10 nM $[Ca^{2+}]_i$, indicating that a GTP-binding protein is directly involved at a late stage of exocytosis, rather than indirectly enhancing exocytosis via kinase activation (Coorssen *et al*., 1990; Coorssen and Haslam, 1993; Coorssen, 1996). These results are consistent with observations from several other cell types showing that the exocytotic event itself is insensitive to staurosporine (Koopman and Jackson, 1990; Terbush and Holz, 1990; Niessen and Verhoeven, 1994).

In the neuromuscular junction, staurosporine blocks release of FM1-43 from synaptic vesicles pre-loaded with this membrane-staining dye, but does not block release of the endogenous neurotransmitter acetylcholine (Henkel and Betz, 1995). This suggested that staurosporine interfered with the post-exocytotic collapse of synaptic vesicles into the surface membrane (Henkel and Betz, 1995). In agreement with this hypothesis, our data demonstrate that in eosinophils, staurosporine retards fusion pore expansion in PMA-treated cells. This indicates that the effect of staurosporine on the exocytotic fusion pore may indeed be mediated by kinase inhibition. Although fusion pore expansion can be affected by staurosporine treatment, we found that in eosinophils, pore expansion and irreversible fusion still occur in its presence.

Intracellular application of the phosphatase inhibitor α-np inhibits exocytosis induced by GTPγS; similar effects have been noted previously in platelets (Coorssen *et al*., 1992). This result is in accordance with several studies indicating a role for a dephosphorylation step in a variety of other cell types. Exocytosis in *Paramecium tetraurelia* involves the dephosphorylation of a 65 kDa phosphoprotein (Zieseniss and Plattner, 1985). In basophilic RBL-2H3 cells, protein tyrosine dephosphorylation was shown to be involved in exocytosis at a step distal to the mobilization of Ca^{2+} and activation of PKC (Santini and Beaven, 1993). Studies on permeabilized mast cells suggest that dephosphorylation of an unknown regulator protein is an enabling reaction for exocytotic membrane fusion to occur, because the provision of ATP retards the onset of exocytosis following provision of Ca^{2+} and GTPγS. In the absence of ATP, the protein appears to be dephosphorylated rapidly, and the onset of secretion is prompt (Churcher and Gomperts, 1990). A protein phosphatase could be the target enzyme for G_E, a putative G protein thought to regulate more terminal events in exocytosis (Gomperts, 1990). In preliminary experiments, the more specific inhibitors of protein phosphatase types 1 and 2A, cantharidin and okadaic acid, were found to be without effect (data not shown), suggesting that another type of phosphatase may be critical; type 2B seems less likely due to the low $[Ca^{2+}]_i$ used in many of these experiments. In basophilic RBL-2H3 cells, several inhibitors of tyrosine phosphatases block exocytosis (Santini and Beaven, 1993). In preliminary experiments using the tyrosine phosphatase inhibitor ortho-vanadate (1 mM), degranulation was also blocked in horse eosinophils (data not shown), suggesting that tyrosine phosphorylation may regulate exocytosis also in this cell type.

In addition to inhibiting exocytosis, α-np also caused a decrease of the fusion pore expansion rate. This result was in contrast to naive expectation since both PKC activation and phosphatase inhibition increase phosphorylation but have opposing effects on the fusion pore expansion rate. Taken together, however, the phenomena simply indicate that fusion pore expansion is modulated via more than one phosphorylation site, possibly on more than one protein. One or more PKC-mediated phosphorylations can lead to an increased pore expansion rate, whereas phosphorylation at different sites favours decreased pore expansion rates. All the data shown here strongly suggest that the expansion of the exocytotic fusion pore is not a purely lipidic process but is modulated by proteins.

From the present experiments, we cannot distinguish whether the proteins involved are affected directly by Ca^{2+} and/or phosphorylation, or the effects are mediated by other regulatory proteins. The stimulation of exocytosis by GTPγS certainly indicates an involvement of G proteins, which are subject to modification by phosphorylation (Yamane and Fung, 1993). Recent evidence suggests that PKC activation enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules (Gillis *et al*., 1996). In contrast to endocrine cells stimulated by Ca^{2+}, which exocytose only a few percent of secretory granules, degranulation

stimulated by intracellular GTPγS is near maximal in granulocytes and a variety of other haematopoetic cells (Fernandez *et al*., 1984; Nüβe and Lindau, 1988; Nüße *et al*., 1990). In these cell types, degranulation of permeabilized cells in response to Ca^{2+} and GTPγS is an all-or-none event (Hide *et al*., 1993); at sub-optimal agonist concentrations, not all cells degranulate, but those which do degranulate completely. Exocytotic competence is thus not restricted to a subset of readily releasable granules.

Although the proteins forming the exocytotic fusion pore have not yet been identified, our results indicate that proteins control not only the formation but also the subsequent expansion of the fusion pore. At this stage, the fusion pore is modulated by PKC- and Ca^{2+}-binding proteins. Fusion pore control may be important specifically in neurosecretion where transmitter release is performed in a cycle of rapid exo-endocytosis (Smith and Neher, 1997) and where staurosporine also inhibits collapse of vesicles into the plasma membrane (Henkel and Betz, 1995). It was shown recently that synaptic vesicles retain their identity through the endocytic cycle (Murthy and Stephens, 1998), suggesting that the fusion pore may remain under tight control allowing for exo-endocytosis without complete incorporation of the vesicle into the plasma membrane.

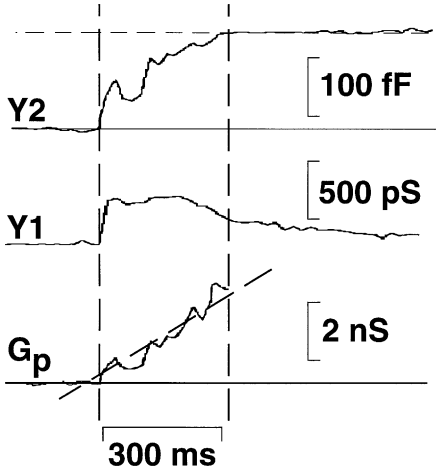

Fig. 6. Analysis of fusion pore expansion. When the fusion pore conductance is low (between vertical dashed lines), the Y2 trace is reduced and a transient increase appears in the Y1 trace. The pore conductance G_P was calculated from the Y2 trace and the vesicle capacitance C_V which is attained in the Y2 trace when the fusion pore expands (horizontal dashed line).

Materials and methods

Cell preparation

Fresh blood was drawn from the jugular veins of horses, eosinophils were isolated, and were purified over discontinuous Percoll gradients as previously described (Scepek and Lindau, 1993). Purified eosinophils were suspended in Medium 199 containing 4 mM glutamine, 4.2 mM $NaHCO_3$ and penicillin/streptomycin (pH 7.2–7.3), stored at room temperature and used within 2 days.

Patch-clamp experiments

About 50–100 µl of cell suspension were transferred into a Petri dish having a glass coverslip as a bottom. After a few minutes to allow the cells to settle on the glass, the dish was perfused with standard external saline (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES–NaOH, pH 7.2–7.3). The whole-cell configuration, with full contact between the cytosol and the solution inside the micropipette, was used to dialyse the cells with the pipette solution. Access resistance was ~5 MΩ. The pipette solution contained 125 mM potassium-L-glutamate, 10 mM NaCl, 7 mM $MgCl_2$, 7 mM EGTA, 1 mM Na_2ATP, 10 mM HEPES–NaOH, 20 µM GTPγS (unless noted otherwise), pH 7.2–7.3, and no added $CaCl_2$ ($[Ca^{2+}]_i$ <10 nM, as measured with a Ca^{2+}-sensitive electrode, with Fura-2, and as calculated using the computer program BAD v. 4.42; Brooks and Storey, 1992). PMA, α-np and staurosporine were added as described below. For high Ca^{2+} concentration ($[Ca^{2+}]_i$ = 1.5 µM, as measured with a Ca^{2+}-sensitive electrode), the solution contained 4.5 mM $CaCl_2$ and 5 mM EGTA instead of 7 mM EGTA. $[Mg^{2+}]_{free}$ was estimated to be 3.8 mM at low $[Ca^{2+}]_i$ and 5.8 mM at high $[Ca^{2+}]_i$. The osmolality of the external solution was adjusted with D(+)glucose (Merck) to exceed that of the pipette solution by a few mOsmol. All experiments were done at room temperature. All values given are means ± SEM of the indicated number of cells, unless noted otherwise.

Capacitance measurements

Changes in C_m were measured as the out-of-phase component (Y2) of the cell admittance change using an EPC-9 patch-clamp amplifier (List Electronics, Darmstadt, Germany). The command voltage signal was an 800 Hz, 20 mV (r.m.s.) sine wave and the current output signal was analysed by a two-phase lock-in amplifier (5210, EG&G PAR, Princeton, NJ) (Neher and Marty, 1982; Lindau and Neher, 1988) and sampled by computer every 22 ms. The bulk capacitance of the cell was compensated immediatedly after attaining the whole-cell configuration. During the subsequent continuous measurement of C_m, the phase error of the lock-in amplifier was determined using the phase tracking technique (Fidler and Fernandez, 1989) and automatically readjusted. In the presence of compounds found to inhibit degranulation, C_m was recorded for at least 30 min. In actively exocytosing cells, recording was discontinued after complete degranulation (apparent by visual inspection; Scepek and Lindau, 1993) or when C_m was stable for several minutes. The total C_m increase was taken as the difference between the initial and the final values. This increase includes both the stepwise increase due to the fusion of specific granules, and the smooth initial increase most probably due to the fusion of small vesicles (<3 fF) which cannot be resolved into individual steps. On average, these small vesicles contribute ~10–15% to the overall C_m increase under control conditions.

To determine the exocytotic rate during degranulation, the time intervals between consecutive capacitance steps were measured in all cells recorded under a given condition. These time intervals were grouped into bins and a frequency distribution was constructed. The frequency distribution was fitted with a single exponential providing the exocytotic rate, k. The time constant of degranulation is the reciprocal of this rate constant, $\tau = 1/k$.

During the fusion of a granule with capacitance C_V to the plasma membrane, a low fusion pore conductance G_P reduces the change of the Y2 signal which is then no longer equal to ωC_V but becomes $Y2 = \omega C_V/[1 + (\omega C_V/G_P)^2]$. Simultaneously, a correlated transient Y1 signal, $Y1 = [(\omega C_V)^2/G_P]/[1 + (\omega C_V/G_P)^2]$, appears ($\omega = 2\pi \cdot 800$ Hz) (Figure 6). Knowing C_V from the final step size, we calculated the time course of G_P from the capacitance trace and confirmed it by comparison of the predicted and measured conductance traces (Breckenridge and Almers, 1987; Lindau, 1991). The time course of fusion pore expansion during the first few hundred milliseconds after fusion was approximated by fitting a straight line through the increase in Gp, providing the expansion rate, r, in nS/s (Hartmann and Lindau, 1995) (Figure 6). All expansion rates determined under particular experimental conditions were grouped into bins and a frequency distribution was constructed. The distribution was fitted with a single exponential $n(r) = n(0) \cdot \exp(-r/\rho)$, thereby providing the characteristic expansion rate, ρ. All fits were performed on Macintosh computers using the program IGOR (Wavemetrics, Lake Oswego, OR). The results provide the best-fit parameters and standard errors.

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