Regulation of granule size in human and horse eosinophils by number of fusion events among unit granules

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1. We have investigated the granule size distributions in human and horse eosinophils by time-resolved patch-clamp capacitance measurements.

2. During exocytosis of single granules the electrical capacitance of the plasma membrane increases in discrete steps. The steps in horse cells are about six times larger than those in human cells in accordance with the difference in granule size.

3. In both species a multimodal capacitance step size distribution is observed with a first peak at 6–7 fF corresponding to granules with a diameter of about 450–500 nm and a surface area of about 0·7 μm^2, which we call the unit granule. The other peaks in the distributions correspond to multiples of the surface area of these units.

4. These results show that the larger granules are formed by fusion of several unit granules and the final size of mature granules is determined by the number of units allowed to fuse with each other. Whereas in human eosinophils most granules consist of one or two units, most granules of horse eosinophils are formed by fusion of seven to fifteen units.

5. The intracellular fusion events associated with vesicular traffic are believed to occur constitutively. In contrast, our results indicate that a cellular mechanism exists which regulates the size of the mature granules by determining the number of units allowed to fuse with each other. In view of our recent report that granule–granule fusion can be activated by GTPγS, this regulation may possibly involve GTP-binding proteins.

The primary physiological function of eosinophils is the defence against parasites. Killing of parasites is performed by secretion of cytotoxic proteins. These proteins are stored in cytoplasmic granules (Archer & Hirsch, 1963) which upon stimulation fuse with the plasma membrane releasing their contents by exocytosis (Henderson, Chi, Jörg & Klebanoff, 1983; Henderson & Chi, 1985; Nüße, Lindau, Cromwell, Kay & Gomperts, 1990; Lindau, Nüße, Bennett & Cromwell, 1993). After their synthesis the proteins are packed into vesicles in the Golgi apparatus and the secretory granules may be released into the cytoplasm from the *trans* Golgi network (TGN) (Bainton & Farquhar, 1970). However, granules which bud from the TGN are much smaller than most of the mature granules (Zucker-Franklin, 1980). The growth of granules could either be explained by continued fusion of small vesicles containing newly synthesized protein with the cytoplasmic granules, but association of such vesicles with granules is rare (Zucker-Franklin, 1980). Alternatively, as recently proposed for rat peritoneal mast cells (Alvarez de Toledo & Fernandez, 1990) and for endocrine cells (Tooze, 1991; Tooze, Flatmark, Tooze & Huttner, 1991) several granules derived from the TGN may fuse among themselves to form larger units. The final size of the granules will eventually be determined by the size and number of the vesicles which fuse to form the mature granules.

The size distribution of exocytotic granules is usually derived from morphometric measurements of electron micrographs. However, these methods are rather indirect since the granule profiles seen in thin sections are usually not equatorial and the conversion into a granule size distribution requires simplifying assumptions. In particular approximating the granules as spheres is not a very accurate approximation for the crystalloid-containing eosinophil granules (Henderson & Chi, 1985; Lindau *et al.* 1993).

Fusion of exocytotic granules with the plasma membrane leads to an increase of the plasma membrane area by the area of the granule membranes. The increase in plasma membrane area is associated with a proportional increase of plasma membrane capacitance which can be measured with very high resolution using the whole-cell mode of the patch-clamp technique (Neher & Marty, 1982; Lindau & Neher, 1988). The surface area of individual granules can thus be determined accurately irrespective of what the shape of the granule might be. The size distribution of the capacitance steps thus provides an exact measure of the

granule membrane area. With this method it was recently found that the membrane area distribution of rat peritoneal mast cell granules shows several equally spaced peaks (Alvarez de Toledo & Fernandez, 1990), suggesting that large granules may be formed by fusion of small granules with each other.

We have done capacitance measurements to determine the fine structure of the granule size distributions in eosinophils from human and horse blood. In these two species the size of the mature granules is very different and the capacitance steps are about 5–6 times larger in horse cells than in human cells. However, in both species the step size distributions are composed of distinct peaks separated by 6–7 fF, indicating that the mature granules in both species are formed from unit granules with a mean diameter of 450–500 nm. The dramatically different size of mature human and horse eosinophil granules develops due to the fact that an average human eosinophil granule consists of one to three unit granules whereas seven to fifteen units must fuse to form an average horse eosinophil granule. These results indicate that the fusion events associated with granule maturation are regulated by cellular mechanisms.

METHODS

Cell preparation

Horse eosinophils were isolated from 50 ml fresh blood obtained from the jugular vein of horses which was supplemented with EDTA. The leukocyte-containing plasma was continuously removed during sedimentation (20–40 min) and was then washed twice in Hanks' solution containing 30 mg ml^{-1} DNase at pH 7·2, centrifuging at 4 °C for 10 min at 300 *g*. Eosinophils were then purified on a discontinuous Percoll gradient in 15 ml conical tubes (Falcon). Four millilitres of 1·099 g ml^{-1} Percoll (Biochrom, Berlin, Germany) with physiological values of osmolarity and pH was overlaid with 3 ml of 1·089 g ml^{-1} Percoll and 4 ml of the leukocyte suspension. After centrifugation for 30 min at 25 °C and 400 *g* the pellet was washed twice and resuspended in Medium 199 (Biochrom) containing 4 mM glutamine (Biochrom), 0·6 mg ml^{-1} penicillin and streptomycin (Biochrom) and 4·2 mM $NaHCO_3$ at pH 7·2. The resulting preparation of eosinophils was 95% pure, with neutrophils as the main contaminating cell type, with a yield of 50%. The cells were used during the next 2 days after isolation.

Human granulocytes were isolated from heparinized fresh blood by dextran sedimentation and centrifugation through a Ficoll–Paque (Pharmacia Fine Chemicals, Piscataway, NJ, USA) layer (1000 *g*, 20 min, 20 °C) (Bøyum, 1968). The pellet was resuspended in distilled water to lyse contaminating red cells. After 20 s, the same volume of 1·8% NaCl solution was added. The granulocytes were pelleted (100 *g*, 20 °C, 5 min) and the pellet was resuspended in Medium 199 supplemented as described above. The suspension of granulocytes contained typically 2–8% eosinophils as determined by Kimura stain. Under the microscope they could be clearly distinguished from the neutrophils by their granular appearance. In most experiments eosinophils were purified by negative selection using magnetic beads. Beads coated with sheep anti-mouse IgG (Dynabeads M-450; Dynal, Oslo, Norway) were incubated overnight with mouse anti-human CD16 IgG in a rotary mixer. Eighty microlitres of anti-CD16 IgG (0·2 mg ml^{-1} in 0·02% sodium azide) was added to 10^8 beads contained in a volume of 250 μl. To remove unbound antibody, the beads were washed 4 times in Dulbecco's phosphate-buffered saline containing 0·5% BSA. After 5 min rotation the beads were immobilized by the magnet and the fluid was gently aspirated and replaced. Then 10^8 beads were incubated with 10^7 cells in the rotating shaker for 30 min. During this period the beads bind to the neutrophils which were then removed using the magnet. With this method the final cell suspension contained > 90% eosinophils with a yield of 40–60%. The cells were stored in the described medium at room temperature for up to 12 h. The pH of all solutions was adjusted with KOH and HCl.

Patch-clamp experiments

The internal (pipette) solutions contained (mM): 125 potassium L-glutamate, 10 NaCl, 7 $MgCl_2$, 5 EGTA, 4·5 $CaCl_2$ and 1 Na_2ATP; and 20 μM guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS); pH 7·2–7·3. For experiments with human cells the pipette solution contained 1 mM EGTA and $CaCl_2$ was omitted. The external solution contained (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes–NaOH; pH 7·2–7·3. The osmolality of the external solution was adjusted with D(+)glucose (Merck) such that it exceeded that of the internal solution by a few milliosmoles per kilogram. About 50–100 μl of cell suspension was transferred into small Petri dishes with a coverslip as the bottom. When the cells had settled on the glass, the dish was perfused with external saline. Eosinophils could be clearly distinguished from the neutrophils by their granular morphology. When identified cells were occasionally treated with Kimura stain the green stain of the granules was always observed. All experiments were done at room temperature.

Capacitance measurements

For high-resolution capacitance measurements an 800 Hz sine wave was used as the command signal of the patch-clamp amplifier (EPC-7 or EPC-9, List Electronic) operating in the voltage-clamp mode. Sine wave amplitude was 20 mV r.m.s. In some experiments on human cells the amplitude was increased to 50 mV r.m.s. to improve the signal-to-noise ratio. The resulting current was analysed by a 2-phase lock-in amplifier (home built lock-in or model 5210, EG&G PAR, Princeton, NJ, USA) (Lindau & Neher, 1988) and sampled by a computer every 20–25 ms. After attainment of the whole-cell configuration the bulk capacitance of the cell was compensated. During the experiment the phase of the lock-in was determined using the phase tracking technique (Fidler & Fernandez, 1989; Lindau, 1991).

When the phase of the lock-in is set to the correct phase angle where changes in membrane capacitance (C_M) and access resistance (R_A) are well separated, one of the lock-in channels (Y_2) directly indicates the capacitance changes (ΔC_M) (Neher & Marty, 1982; Lindau & Neher, 1988). Capacitance steps were only used for quantitative analysis when the noise level allowed determination of the step size within ±2 fF. Steps with a corresponding change in the conductance channel (Y_1) exceeding 20% of the Y_2 change were also discarded (error of $\Delta C_M > 2\%$) and the capacitance calibration was repeatedly

determined by transiently unbalancing the capacitance compensation by 100 or 200 fF. The error in determining the size of individual capacitance steps was ±2 fF plus 4% of the actual step size.

Determination of step sizes

The size of capacitance steps was initially measured by eye using horizontal cursor lines on the computer screen. To avoid any subjective bias in the analysis an automatic procedure for step size determination was developed. The steps were selected by visual inspection of the Y_2 and Y_1 trace on the computer screen and segments of thirty data points containing the step were stored separately. These segments were analysed by the computer using the following method. A straight line was fitted through the first seven data points and the r.m.s. noise level was determined from the scatter of these points around the line. If the next (8th) data point deviated from the straight line by more than three or (in recordings with very low noise) five standard deviations the computer suggested the beginning of a step. If the point was within the noise level, the fit started at the second point and so on. If the beginning of a step was found it was shown on the computer screen. In most cases the step was well identified by the computer. If an error was made due to a glitch in the trace, the experimenter could force the computer to continue the search. Once the step was found, a straight line was fitted through seven data points after step. This fit started after a gap of five data points which we allowed for opening of the fusion pore. The step size was taken as the distance between the two straight lines in the middle of the gap. All step sizes used for the analysis presented here were determined with this procedure.

Analysis of step size distributions

To reduce the noise in the step size distributions, the moving bin technique was applied. The number of steps within a certain range (the 'bin size') was calculated for bin positions separated by the 'bin increment', which is smaller than or equal to the bin size (Rahamimoff & Yaari, 1973).

To confirm that the observed peaks in the distribution are not due to statistical noise the measured distribution was smoothed by a 5- or 7-bin rolling average and the χ^2 test was applied to compare the measured with the smoothed distribution using standard methods. Although the smoothed distribution has effectively several free parameters we used the number of bins minus one as the number of degrees of freedom (Liao, Jones & Malinow, 1992).

As an additional test for the occurrence of peaks in the histograms and for the regularity of peak spacing we calculated the autocorrelation function (ACF) of the amplitude histograms (Aseltine, 1958):

$$\mathrm{ACF}(j) = \{\Sigma_i H(i)H(i+j)\}/\{\Sigma_i H(i)H(i)\},$$

where $H(i)$ is the number of events within the ith bin. The value on the abscissa is $j \times$ bin width (in fF). If the distribution contains equally spaced peaks, then the ACF should display a first maximum at the mean distance between neighbouring peaks in the original distribution, and maxima with decreasing amplitudes at multiples of the peak spacing. In contrast, peaks in the original distribution which occur randomly at uncorrelated positions average out generating a smooth ACF.

RESULTS

Capacitance steps in human and horse eosinophils

Intracellular application of 20 μM GTPγS to eosinophils isolated from human or horse blood via a patch pipette in the whole-cell configuration stimulates complete degranulation as indicated by a 2- to 3-fold capacitance increase. Typical examples of the measured capacitance traces during degranulation are shown in Fig. 1. The capacitance increase is partly continuous and partly due to discrete steps corresponding to the fusion of individual eosinophil granules as previously shown for guinea-pig eosinophils (Lindau *et al.* 1993). Most of the continuous increase occurs before the first large step. The steps observed in human cells (Fig. 1*A*) are about 5 times smaller than those in horse cells (Fig. 1*B*).

In human cells the mean step size was 12·7 ± 0·3 fF (mean ± S.E.M., $n = 557$ steps from 15 cells). A typical human eosinophil contains 300–400 granules and the capacitance increases from 2·9 ± 0·3 pF (mean ± S.D., $n = 30$) to a final value of 8·6 ± 1·4 pF (mean ± S.D., $n = 30$). The total increase was 5·7 ± 1·1 pF ($n = 30$); 0·48 ± 0·06 pF was apparently continuous or due to steps of less than 3 fF. The increase due to steps exceeding 3 fF is thus about 5·2 pF. Dividing this value by the mean step size gives about 400 steps per cell. The late parts of recordings were usually too noisy to determine the size of capacitance steps within ± 2 fF.

The continuous increase is likely to be generated by small secretory vesicles (Calafat, Kuijpers, Janssen, Borregaard, Verhoeven & Roos, 1993). It mainly preceded the large steps and started without delay after the cell was internally perfused with the pipette solution.

In horse eosinophils the mean step size was 73·7 ± 2·0 fF (mean ± S.E.M., $n = 1505$ steps from 74 cells). Complete degranulation of a horse eosinophil usually occurs in thirty to sixty capacitance steps giving a total capacitance increase from 2·94 ± 0·46 (mean ± S.D., $n = 109$) to 7·94 ± 1·18 pF (S.D., $n = 109$) corresponding to an increase of plasma membrane area by a factor of 2·74 ± 0·51 (mean ± S.D., $n = 109$). Of the total capacitance increase, 30 ± 10% is due to an apparently continuous rise which could not be resolved into individual steps. The stepwise increase reflecting exocytosis of the large specific eosinophil granules thus generates a mean capacitance increase of 3·5 pF. This is somewhat less than the corresponding value of human eosinophils, reflecting the fact that a larger surface area is contributed by many small granules than by a few large granules. The volume of an average human granule is about 8% of that of a horse granule. However, since the human cell has about 10 times more granules than a horse cell, the granules in both species store a similar total volume of secretory material.

Multimodal granule size distribution in human cells

Capacitance steps measured in a human cell are shown together with horizontal grid lines in Fig. 2*A*. The spacing of the dotted lines is 7 fF. The observed steps are apparently multiples of this value. The size of 557 steps was determined using an automatic procedure (see Methods). The resulting frequency distribution is shown in Fig. 2*B* (histogram bars). It shows a prominent peak around 12 fF. There are apparently two minima, one between 9 and 10 fF and one around 17 fF, suggesting that the distribution may have multiple peaks. Figure 2*C* shows the same distribution smoothed with the moving bin technique. With this method distinct peaks are seen at 7, 12, 19 and possibly around 24 fF. The position of the peaks did not depend on the choice of the bin size and bin increment. The distance between the peaks is thus similar to the position of the first peak. A possible explanation would be that two subsequent capacitance steps follow each other so rapidly that they are not separated and are erroneously taken as one step of twice the true size and the third peak may correspond to unresolved triple events. To determine the probability of such events we have measured the time intervals between subsequent fusion events. The frequency distribution of these time intervals is shown in Fig. 2*D*. It can be fitted by a single exponential (continuous line) with a time constant of 2·5 s. In our recordings steps separated by more than 150 ms could always be clearly separated. The probability that two fusion events occur within 150 ms and are taken as one is thus less than 6%. In contrast, 60% of the steps in Fig. 2*B* are contained in the second and higher peaks (> 11 fF). The observed multimodal distribution is thus not a consequence of insufficient resolution of the measurement.

Comparison with morphometric data

The question arises whether the granules consisting of two or more unit granules are present in resting cells or whether they are formed by granule–granule fusion which is stimulated by GTPγS (Scepek & Lindau, 1993). The size distribution of granules in unstimulated human eosinophils has previously been estimated by measuring the granule profile areas in thin sections using electron microscopy (Henderson & Chi, 1985) and this data is shown as the histogram bars in Fig. 2*E*. For comparison we have converted the capacitance step size distribution of Fig. 2*A* into a distribution of section profile areas approximating the granules as spheres (Bach, 1967; Lindau *et al.* 1993) and assuming a specific membrane capacitance of 0·9 μF cm^{-2}. Both distributions agree very well, indicating that the measured capacitance step size distribution reflects the size distribution of specific eosinophil granules and is not significantly shifted due to granule–granule fusion which may occur during the degranulation process. Although the multimodal distribution is smeared due to the sectioning, the second peak is also detectable in the morphometric data, but had not been recognized previously. The multimodal distribution of capacitance steps thus reflects a corresponding multimodal size distribution of specific eosinophil granules which is a property of resting human eosinophils.

Significance of multiple peaks

To exclude the possibility that the distinct peaks in the distribution are due to statistical variations, a smoothed distribution was generated by calculating the rolling average over five adjacent bins (Fig. 2*B*, continuous line). The possibility that the measured distribution results from the smooth distribution by statistical scatter must be rejected ($P < 0{\cdot}001$, χ^2 test). To account for the sharp peak we have replaced the values at 11 and 12 fF in the smoothed distribution by the original values (Fig. 2*B*, dashed line). However, even with this distribution the deviations are still significant ($P < 0{\cdot}01$). The distinct peaks in the capacitance step size distribution are thus real and not due to statistical scatter.

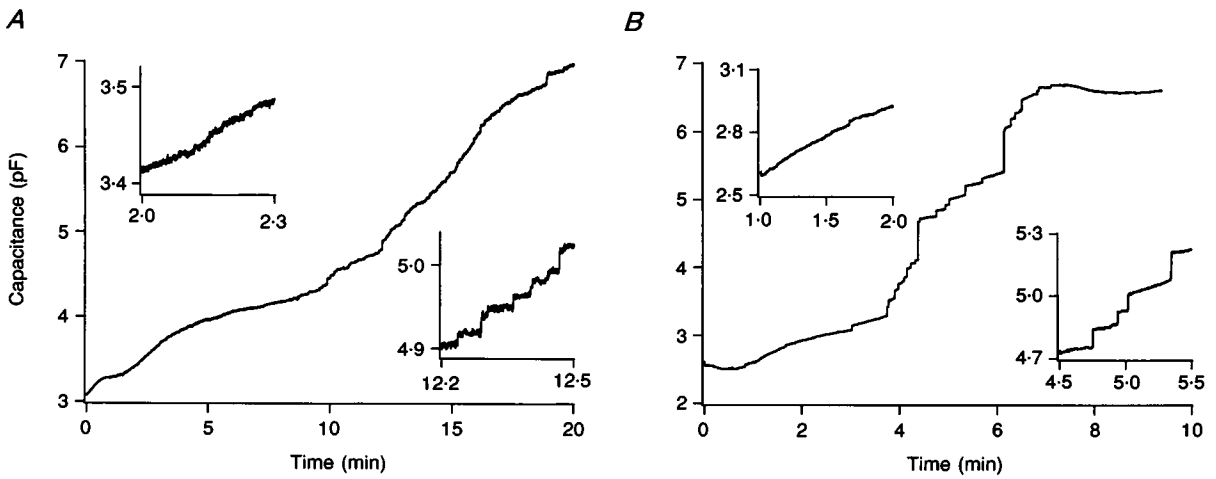

Figure 1. Typical records of the membrane capacitance increase during degranulation of a human (*A*) and horse (*B*) eosinophil

The pipette contained 20 μM GTPγS. The insets show parts of the early continuous increase and of the delayed stepwise increase on an expanded scale.

When the distribution of Fig. 2*B* was fitted with a sum of two Gaussians (not shown) the positions of the first two maxima were 7·3 ± 0·3 and 12·5 ± 0·2 fF, suggesting that two or more granules of about 6–7 fF capacitance fuse with each other forming a larger granule. This fusion already occurs in resting cells before stimulation.

Multimodal granule size distribution in horse eosinophils

The capacitance steps in horse cells are typically 5–6 times larger than those in human cells. When the frequency distribution is generated using a correspondingly larger bin width of 12 fF (Fig. 3*A*) a periodic multimodal distribution is not obtained. The distribution shows a first peak around 10 fF and a second, broad peak between 50 and 100 fF. To determine if the distribution shows a fine structure of peaks with a similar separation distance as the human cells, we plotted the part up to 100 fF using a bin width of 1 fF (Fig. 3*B*). The x-axis tick marks are at a distance of 6 fF. There is obviously a large number of peaks coinciding with these positions. The first peak is at 6–7 fF and the peak

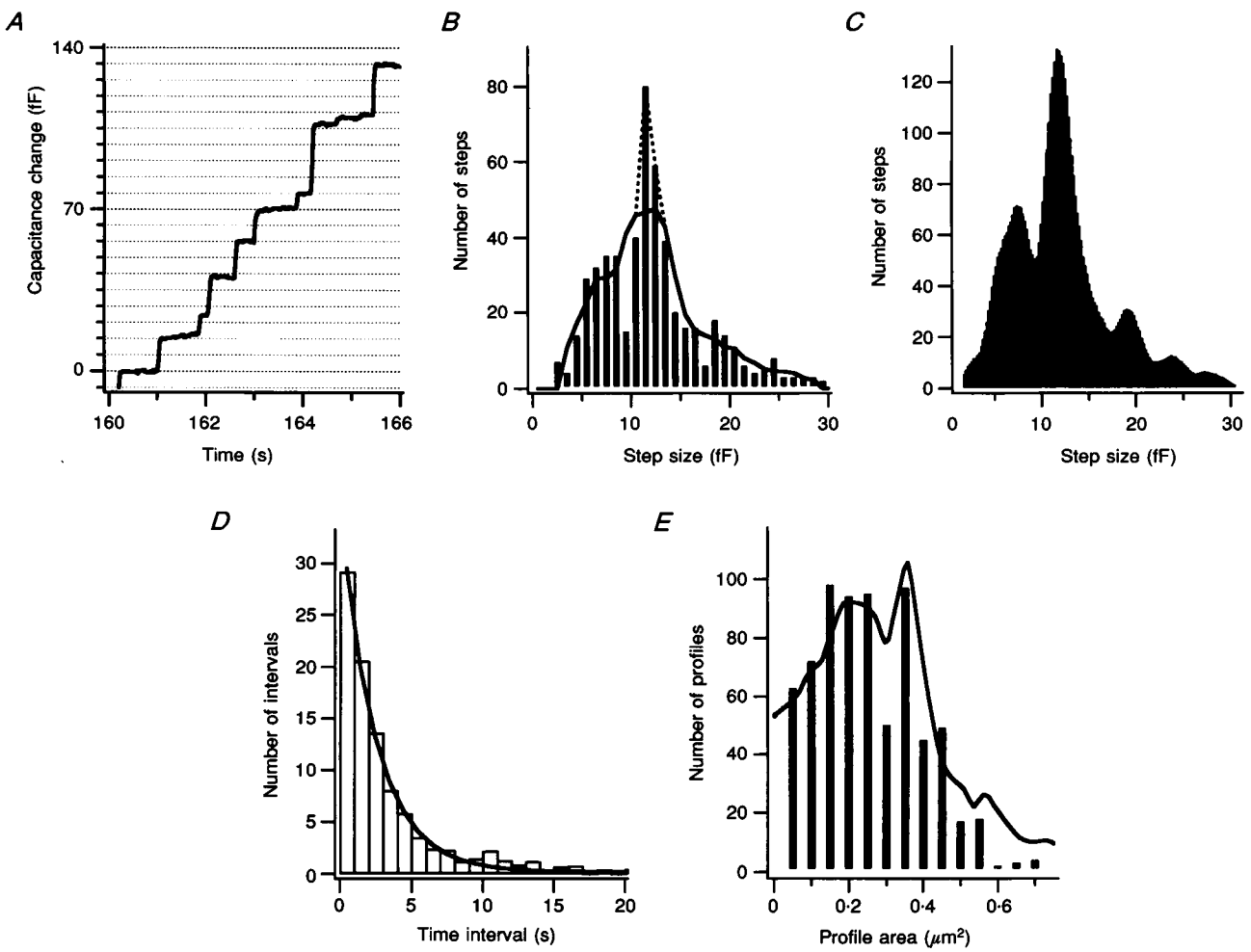

Figure 2. Capacitance steps in human eosinophils

A, capacitance steps in human eosinophils. The steps are multiples of 7 fF, as indicated by the dotted lines. *B*, frequency distribution of capacitance step sizes in human eosinophils stimulated with 20 μM GTPγS; bin size, 1 fF. The continuous line is the rolling average over 5 adjacent bins. The χ^2 value for the difference between the smoothed and the original distribution is 69·1. Assuming 25 degrees of freedom (number of bins minus one) this value indicates that the possibility that the measured distribution reflects only statistical deviations from the smooth distribution can be rejected ($P < 0{\cdot}001$). To account for the sharp peak, the values at 11 and 12 fF in the smoothed distribution were replaced by the original values reducing the degrees of freedom by 2. This distribution could also be rejected ($\chi^2 = 42{\cdot}5$, $P < 0{\cdot}01$). *C*, frequency distribution generated from the same data using the moving bin technique with bin size 2 fF and bin increment 0·2 fF. *D*, frequency distribution of the time intervals between subsequent capacitance steps. The smooth line is a single exponential fit giving a time constant of 2·5 s. *E*, the distribution of *B* was converted into the expected frequency distribution of granule profile areas in thin sections approximating the shape of the granules as spherical (continuous line). The bars are morphometric data from Henderson & Chi (1985).

spacing is also 6 fF. The peaks are thus separated by a similar distance as those in the human cells but in the horse cells much larger multiples of the unit occur.

Most granules in unstimulated horse eosinophils are larger than 1 μm (Henderson *et al.* 1983) and are thus much larger than the 6–7 fF unit granule. The granules consisting of many units are thus present in resting cells and not formed during the degranulation stimulated by GTPγS. It was previously found that intracellular GTPγS stimulates granule–granule fusion, but this becomes significant only at GTPγS concentrations higher than the 20 μM used here (Scepek & Lindau, 1993). Furthermore, it should be noted that in the previous paper (Scepek & Lindau, 1993) the relative contribution of steps with a certain size to the total capacitance increase was plotted instead of the plain number of steps with a certain size as in Fig. 3. Intracellular granule–granule fusion at high GTPγS concentrations leads to a reduction in the number of normal single granule steps and the occurrence of exceptionally large steps instead. The multigranular compounds are generally much larger than the range of step sizes associated with exocytosis of single granules. For comparison the number of steps as a function of size measured at 80 μM GTPγS is shown in Fig. 3*C*. In this range the shape of the distribution is similar to that obtained with 20 μM GTPγS and is not much affected by granule–granule fusion. This further confirms that the distributions described here are features

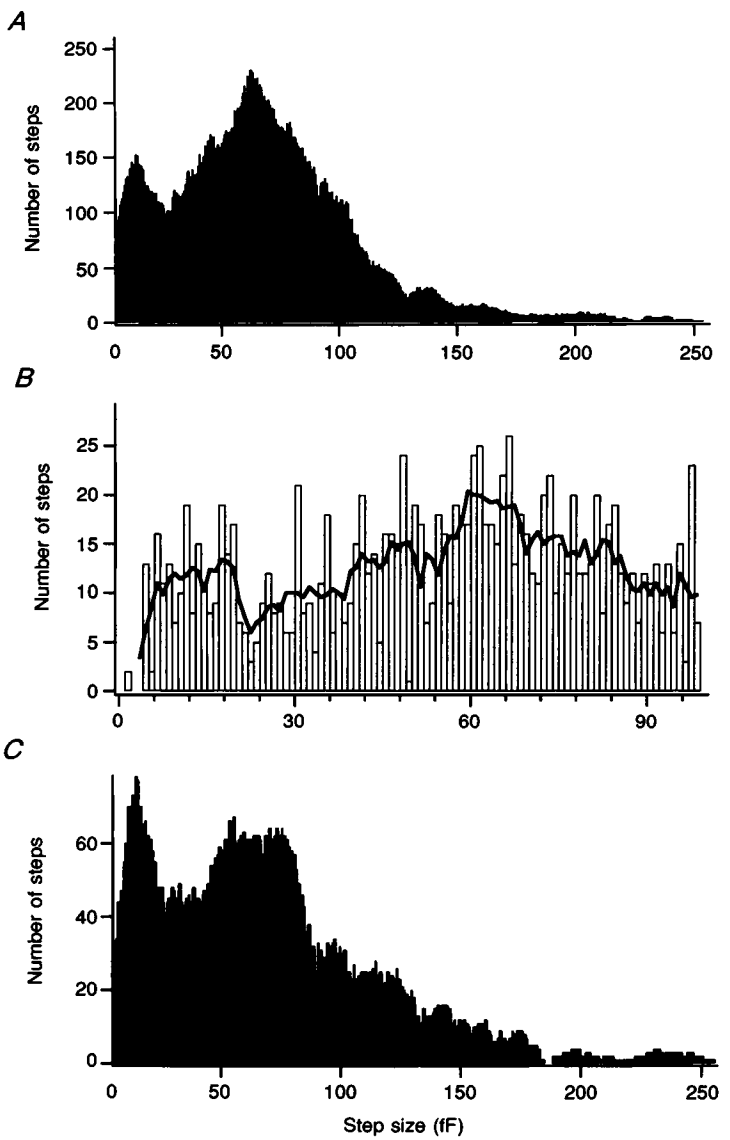

Figure 3. Frequency distribution of capacitance step sizes in horse eosinophils stimulated with 20 μM GTPγS

A, bin size 12 fF, bin increment 0·6 fF; *B*, bin size 1 fF, bin increment 1 fF. The continuous line is a five-point rolling average. This smoothed distribution could be rejected as the parent distribution ($\chi^2 = 176$, 95 degrees of freedom, $P < 0{\cdot}001$). *C*, step size distribution from cells stimulated with 80 μM GTPγS (bin size 12 fF, bin increment 0·6 fF).

present in unstimulated cells and are not a consequence of the GTPγS stimulation.

Significance of the peaks

The probability that the peaks are only due to statistical scatter was estimated by comparison with a 5-bin smoothed histogram (Fig. 3*B*), and was found to be less than 0·1% and also less than 0·1% when only the range from 40 to 100 fF is considered (χ^2 test). The data of Fig. 3 were obtained from two horses. When only the steps obtained from each horse were plotted separately the peaks were unchanged and the distributions were not significantly different from the full data set (data not shown).

For a more quantitative assessment of the regularity of the peaks we calculated the autocorrelation function (ACF) of the frequency distribution. This method is a sensitive detector of periodic features in noisy data (Aseltine, 1958). To obtain a smooth curve a moving bin frequency distribution was created using a bin width of 2 fF and a bin increment of 0·2 fF (Fig. 4*A*). The ACF calculated for this distribution (Fig. 4*B*) shows an overall decline which is due to the effect of a finite data window which generates a triangular ACF for a constant parent function. However, when a part of it is shown on an expanded scale (Fig. 4*C*), an oscillating pattern is seen. To illustrate the regularity of peaks more clearly, the ACF was smoothed by a five-point rolling average (Fig. 4*C*, dashed line) (Edwards, Konnerth & Sakmann, 1990) and the smoothed ACF was subtracted from the original ACF. The difference (Fig. 4*D*) shows a characteristic periodic pattern. The maxima are equally spaced with a repeating interval of 6·25 fF as indicated by the vertical dotted lines.

DISCUSSION

Multimodal step size distributions

The data presented here show that the electrical capacitance and thus the surface area of secretory granules of both human and horse eosinophils are multimodally distributed. The smallest units have an electrical capacitance

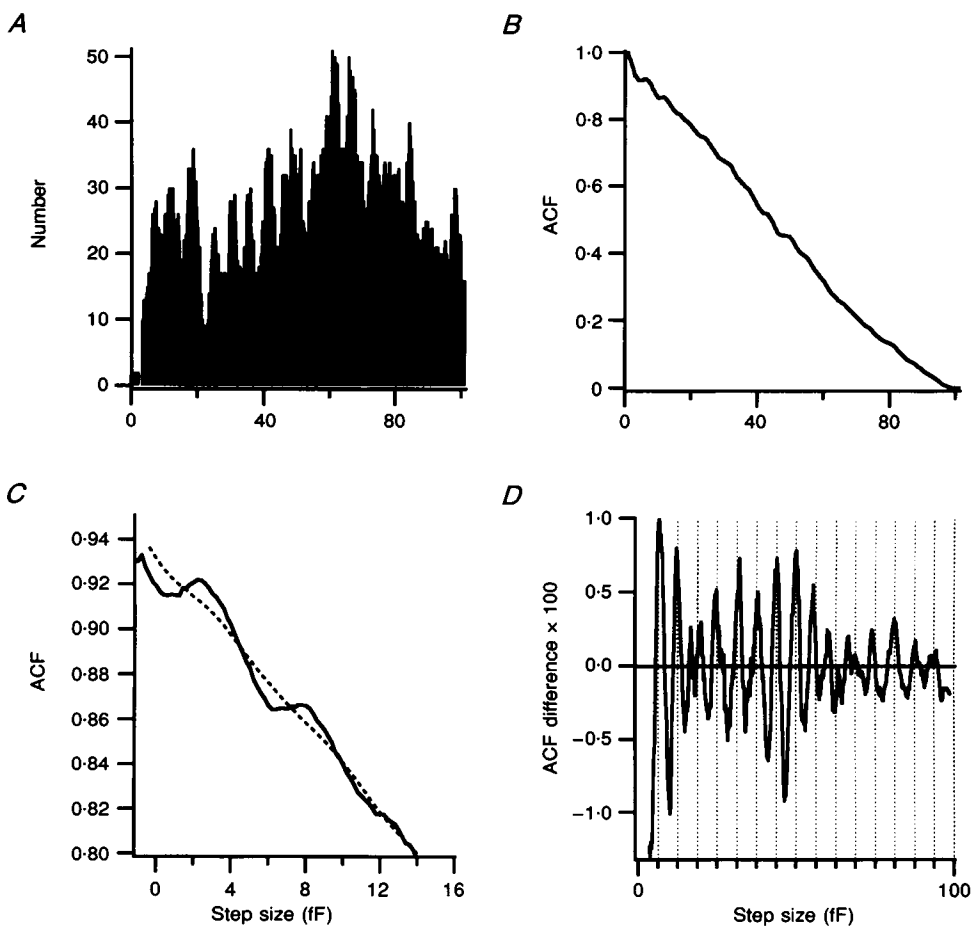

Figure 4. Capacitance steps in horse cells

A, moving bin distribution of the capacitance steps in horse cells using a bin width of 2 fF and a bin increment of 0·2 fF. *B*, autocorrelation function (ACF) of the distribution shown in *A*. *C*, the left-most part of the ACF on an expanded scale (continuous line) and its 25-point rolling average (dashed line). *D*, difference between the ACF and the smoothed ACF. The maxima have a periodicity of 6·25 fF as indicated by the dotted grid lines.

of 6–7 fF corresponding to vesicles with a diameter of about 450–500 nm. Most of the granules in human cells consist of one or two of these units, but larger ones were occasionally also observed. Comparison with morphometric data showed that this distribution is a property of the resting cells.

Horse eosinophil granules are generally much larger, but the step size distribution also shows a first peak at 6–7 fF and several peaks at multiples of this value. These results suggest that the granules are formed by fusion of several unit granules. The size of the unit granules is revealed by the location of the first peak and corresponds to the spacing between the peaks in the histogram.

Formation of large granules by fusion of small units

A multimodality in the distribution of granule sizes of exocytotic cells was reported previously for rat mast cells (Hammel, Lagunoff, Bauza & Chi, 1983; Hammel, Lagunoff & Krueger, 1988; Alvarez de Toledo & Fernandez, 1990) and human lung mast cells (Hammel *et al.* 1985) and was interpreted as the result of the fusion of unit granules with each other to form the mature granules.

Our results extend this observation to eosinophils. The membrane capacitance of the unit granule in human and horse eosinophils is 6–7 fF, corresponding to vesicle diameters of 450–500 nm. The size of the mature granules is controlled by the size and number of units which fuse to form them. We have shown here that the primary units in human and horse eosinophils have a similar size. The remarkable difference in the final size of mature granules is due to the fact that an average human granule consists of two units whereas a mature horse granule is formed by fusion of 7–15 units.

In eosinophilic myelocytes from rabbit bone marrow, granule formation was observed beginning with the appearance of dense material within the Golgi and subsequent budding of dense-cored vacuoles from the TGN. These vacuoles with a diameter between 0·3 and 0·5 μm merge into larger forms (Bainton & Farquhar, 1970). In human eosinophilic promyelocytes, granules which had just left the Golgi apparatus were much smaller than the granules seen in the periphery of the same cell. It would in principle be possible for enlargement of the granules to occur by vesicular traffic from the TGN to the secretory granules. However, in eosinophils small vesicles which could fuse with the immature granules to enlarge them were not seen in their proximity (Zucker-Franklin, 1980). Fusion of immature secretory granules with each other has also been discussed as a mechanism to explain the differences in size between immature and mature secretory granules of PC12 cells (Tooze *et al.* 1991). There is thus increasing evidence that immature granules bud from the TGN and mature secretory granules may be formed by fusion of several of these units. Both the immature and the mature granules have the ability of exocytotic fusion with the plasma membrane in response to stimulation (Tooze *et al.* 1991).

In contrast to regulated exocytosis, the intracellular fusion events associated with vesicular traffic are generally believed to occur constitutively. However, since the size of the unit granule is similar in horse and human eosinophils, cellular mechanisms must exist regulating the fusion events during granule development. It is not yet known what mechanisms determine the final size of the secretory granules. Why are most of the granules in human cells formed by two units and why is a typical horse granule formed by more than ten units? Small GTP-binding proteins play an essential role in vesicular traffic and intracellular fusion events (Balch, 1990). Although their effectors have not been clearly identified, they appear to be under control of guanine nucleotide releasing proteins (GNRPs) and GTPase activating proteins (GAPs) (Bourne, Sanders & McCormick, 1990). In addition, more recently evidence has been obtained that heterotrimeric GTP-binding proteins may also be involved in intracellular transport (Leyte, Barr, Kehlenbach & Huttner, 1992). Such mechanisms could possibly play an important role in regulating intracellular granule–granule fusion.

Very recently, it was found in this laboratory that granule–granule fusion can be activated by GTPγS (Scepek & Lindau, 1993). Although in eosinophils the formation of secretory granules by fusion of immature granules occurs during differentiation of the cells, it is possible that the same mechanisms of granule–granule fusion are activated in mature cells by high concentrations of GTPγS, suggesting that the fusion events associated with granule maturation may be under control of GTP-binding proteins. These should be distinct from those mediating exocytotic granule–plasma membrane fusion to retain specificity and differential regulation of these fusion events (Tooze, 1991).

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