Events leading to the opening and closing of the exocytotic fusion pore have markedly different temperature dependencies

Kinetic analysis of single fusion events in patch-clamped mouse mast cells

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ABSTRACT The earliest event in exocytosis is the formation of a fusion pore, an aqueous channel that connects the lumen of a secretory granule with the extracellular space. We can observe the formation of individual fusion pores and their subsequent dilation or closure by measuring the changes in the admittance of patch-clamped mast cells during GTPγS-stimulated exocytotic fusion. To investigate the molecular structure of the fusion pore, we have studied the temperature dependency of the rate constants for fusion pore formation and closure. An Arrhenius plot of the rate of fusion pore formation shows a simple linear relationship with an apparent activation energy of 23 kcal/mol. In contrast, the Arrhenius plot of the rate of closure of the fusion pore is discontinuous, with the break at ~13°C. Above the break point, the rate of closure has a weak temperature dependence (7 kcal/mol), whereas below 13°C the rate of closure is temperature independent. This type of temperature dependency is characteristic of events that depend on diffusion in a lipid phase that undergoes a fluid–solid phase transition. We propose that the formation of the fusion pore is regulated by the conformational change of a molecular structure with a high activation energy, whereas the closure of the fusion pore is regulated by lipids that become phase separated at 13°C.

INTRODUCTION

Ultrastructural techniques have shown that an early event in the exocytotic fusion of a secretory granule is the formation of a narrow, water-filled pore spanning both the granule and plasma membranes and connecting the lumen of the secretory granule to the extracellular environment (Chandler and Heuser, 1980). After formation, the fusion pore rapidly enlarges to a size that is sufficiently large to allow the hydrated proteoglycan matrix of the granule to be expelled into the extracellular space. In the extreme case of exocytotic fusion pores in beige mouse mast cells, the fusion pores must enlarge to a diameter of several micrometers so that the abnormally large matrix can be released (Breckenridge and Almers, 1987). It seems obvious that, in the later stages of expansion, when the size of the fusion pore is several orders of magnitude bigger than any known macromolecule, the fusion pore must be made predominantly of lipids. However, the structure of the smaller precursors is unknown.

Measurements of changes in the admittance of a cell caused by the fusion of a secretory granule make it possible to obtain time-resolved measurements of the conductance of a single fusion pore from the instant of its formation and throughout its growth (Breckenridge and Almers, 1987; Alvarez de Toledo and Fernandez, 1988; Spruce et al., 1990). These measurements have shown that fusion pores begin abruptly with an initial conductance that varies between 30 and 3,000 pS; they subsequently expand rapidly in a fluctuating manner to an immeasurably large conductance (Spruce et al., 1990). Similar experiments showed also that fusion pores, after expanding to a large conductance value, could collapse, leaving an intact vesicle inside the cell (Fernandez et al., 1984; Spruce et al., 1990). Because these observations are similar to the changes in conductance caused by the conformational changes of ion channels, it has been proposed that the fusion pore begins as an ion channel that spans the vesicular and plasma membranes and that the initial opening, fluctuating conductance, and occasional closure correspond to conformational changes in the channel protein (Almers, 1990). In this model, irreversible expansion occurs when lipid molecules become interspersed between the channel subunits and the structure of the fusion pore has become predominantly lipidic (Almers, 1990). We have recently shown that a massive lipid flux of $\sim 10^6$ lipid molecules/s occurs through the fusion pore as a consequence of an increased surface tension in the secretory granule membrane (Monck et al., 1990). These experiments showed that while the fusion pore conductance fluctuates, large amounts of lipids are already part of the fusion pore structure. Furthermore, these experiments showed that lipidic fusion pores can close. These findings suggest that fusion pore closure may be dominated by the physical properties of the fusion pore lipids. In this work we explore this question by manipulating the physical properties of the plasma membrane and studying the effect on fusion pore kinetics.

Temperature is a convenient means of manipulating the physical properties of membranes (Lee and Chap-

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man, 1987). Because ion channels and other membrane transport mechanisms are affected by temperature in different and characteristic ways (Krasne et al., 1971; Boheim et al., 1980), we have designed experiments to attempt to identify the nature of the early stages of the fusion pore structure by probing its response to changes in temperature. In this work we have characterized the temperature dependence of the rate of formation of fusion pores and the rate at which they close. Unexpectedly, both events have markedly different temperature dependencies. Arrhenius plots of the rate of fusion pore formation reveal a strong temperature dependency with a large activation energy (23 kcal/mol) and no breaks. In contrast to the rate of formation, the rate of closure has a weak temperature dependency between 32 and 14°C (7 kcal/mol). However, between 14 and 13°C, there is a threefold change in the rate constant for closure; below 13°C closure is temperature independent. The temperature dependency of the rate of formation of the fusion pore is characteristic of a process with a high activation energy like the conformational change of a protein. In contrast, the closure of the fusion pore resembles the temperature dependency of processes that depend on diffusion in a pure lipidic phase that undergoes phase transition. We speculate that closure of the fusion pore is regulated by a lipid molecule that undergoes phase separation at 13°C and becomes excluded from the fusion pore structure delaying its closure.

METHODS

Experimental procedures

Mast cells were prepared from adult C57BL/6J mice of either sex (Jackson Laboratories, Bar Harbor, ME) after a procedure described in detail elsewhere (Alvarez de Toledo and Fernandez, 1990*a*). Briefly, mouse peritoneal mast cells were prepared by peritoneal lavage, plated onto glass-bottom culture chambers, and stored at 37°C under a 5% CO_2 atmosphere until use. The medium for this incubation contained (mM) 136 NaCl, 10 Hepes, 0.8 NaOH, 0.9 $MgCl_2$, 1.8 $CaCl_2$, 45 $NaHCO_3$, 0.8 K_2HPO_4, 2.5 glucose, 0.12 mg/ml streptomycin, and 0.64 mg/ml penicillin (pH 7.2). Typically, ~5% of the cells were mast cells. Recordings were usually made between 0.5 and 7 h of plating the cells in incubation Ringer's solution. The standard extracellular medium used for the experiments was a modified Ringer's solution containing (mM) 150 NaCl, 10 Hepes, 2.8 KOH, 1.5 NaOH, 1 $MgCl_2$, 2 $CaCl_2$, and 25 glucose (310 mmol/kg, pH 7.25).

Measurement of the cell membrane capacitance

The cell membrane capacitance was measured using the whole-cell mode of the patch-clamp technique. The pipette solution contained (mM) 125 K-glutamate, 10 Hepes, 7 $MgCl_2$, 3 KOH, 0.2 ATP, 1 $CaCl_2$, 10 EGTA, and 10 μM GTPγS (290 mmol/kg, pH 7.2). The Ca-EGTA buffer was prepared as described by Neher (1988). The free Ca^{2+} concentration in the pipette solution was 30 nM. The cell membrane capacitance was determined using a digital phase detector (Joshi and Fernandez, 1988), implemented on a system comprising a microcomputer (Compaq 386/25) and a data acquisition interface (IDA 15125; Indec, Sunnyvale, CA). Fire-polished pipettes were coated with silicone elastomer (Sylgard) resin and had resistances of 1–3 MΩ when immersed in the external solution. After achieving a high resistance whole-cell recording, the capacitive transient was canceled with the C-slow and G-series potentiometers. A computer-generated sine wave (833 Hz, 54 mV peak to peak) was filtered at a corner frequency of 830 Hz with a low pass filter (Bessel 902, Frequency Devices Inc., Haverhill, MA) and then fed into the stimulus input of the patch-clamp amplifier (EPC-7; List Electronics, Darmstadt, FRG). The current was filtered with an antialiasing filter at a corner frequency of 2 kHz (Butterworth, LP-120, Unigon Industries, Inc.) and sampled with a 15-bit analog-to-digital converter. The current was measured at two different angles from the stimulus, ϕ and $\phi - 90$. The phase detector was aligned so that one output (at $\phi - 90$) reflected the real part of the changes in the cell admittance (Re [ΔY]) and the second output reflected the imaginary part (Im [ΔY], proportional to changes in the cell membrane capacitance). Every capacitance determination was obtained by performing the phase detection over a period of eight sinusoidal cycles (one capacitance point every 14 ms). The phase was periodically adjusted by using the phase tracking technique (Fidler and Fernandez, 1989), which readily allows correct alignment of the phase detector. A calibration for the capacitance trace was obtained by unbalancing the C-slow potentiometer of the compensation circuitry of the patch clamp amplifier by 100 fF.

Temperature control

The temperature of the bathing solution was regulated by a homemade circuit, which applied current to two Peltier devices that were in thermal contact with the recording chamber. During the experiment, the temperature was sensed in the vicinity (1–4 mm) of the recorded cell with a small thermocouple (YSI-511, diameter 0.5 mm; Yellow Springs Instrument Co., Yellow Springs, OH). Under these conditions there was a small temperature gradient of ~0.3°C/mm between the edge and the center of the chamber. Therefore, the temperature was measured at the cell location immediately after each experiment. In this manner, the temperature of the cell was known to within ±0.5°C.

Changing the temperature had a small effect on the pH of the internal and external solutions. Cooling from 23 to 6°C increased the pH by ~0.2 U in both cases. The affinity of EGTA for Ca^{2+} is somewhat lower at 6°C (Harrison and Bers, 1987), but an increase of pipette solution pH to 7.4 (due to lowering the temperature) would result in a net decrease of free $[Ca^{2+}]$ from 30 nM at 22°C to 17 nM at 6°C. The kinetic parameters studied (mean duration between fusion events and incidence and duration of transient fusion events) are not sensitive to changes in $[Ca^{2+}]_i$ below 500 nM (Alvarez de Toledo and Fernandez, 1990*c*).

Data analysis

The Q_{10}s of certain parameters within a given temperature range were calculated using

$$Q_{10} = (P_1/P_2)^{10/(T_1-T_2)},$$

where P_1 and P_2 are values of the parameters at temperature T_1 and T_2, respectively. The energy of activation (E_a) was determined from the equation describing the slope of the Arrhenius plot,

$$E_a = R(\ln P_1 - \ln P_2)/(1/T_1 - 1/T_2),$$

where R = 1.987 cal/Kelvin-mol.

Data are expressed as the mean ± SE unless indicated otherwise. Paired or unpaired *t* tests were used where appropriate to determine differences between data sets.

Probability distribution functions were fitted to one exponential by using an iterative method based on the Marquardt-Levenberg algorithm. Although a higher number of exponentials could be used in these fits, we sought to use the simplest description of the data that gave reasonably good fits. In the majority of the cases a two-exponential fit was not warranted.

RESULTS

Exocytosis induced by guanine nucleotides is strongly dependent on temperature

The increase in cell surface area that occurs when secretory granules fuse with the plasma membrane can be observed as an increase in membrane capacitance using the patch-clamp technique. Fig. 1 shows the time courses of the membrane capacitance changes caused by the compound fusion of several hundred secretory granules (Alvarez de Toledo and Fernandez, 1990*a*) in mouse peritoneal mast cells at four different temperatures. The cells were stimulated by 10 μM GTPγS contained in the pipette solution. This stimulus bypasses most of the steps in the stimulus–secretion coupling pathway so that only the kinetics of those steps immediately before fusion pore opening are examined. The recordings show the cell membrane capacitance as a function of time starting when cell perfusion was initiated (i.e., the beginning of whole-cell recording mode). In each case the membrane area starts to expand after a lag period (Fernandez et al., 1984, 1987) that is significantly longer at low temperatures, increasing from ~1 min at 20°C to ~14 min at 6°C. The rate of degranulation was also strongly dependent on temperature. The rise time, defined as the time between 20 and 80% of the final capacitance change (see Fig. 1), increased fivefold when the temperature was decreased from 20 to 6°C. The Q_{10}s for the mean lag period and mean rise times, calculated between 10 and 20°C, were 7.3 and 4.5, respectively. The respective mean lag periods and mean

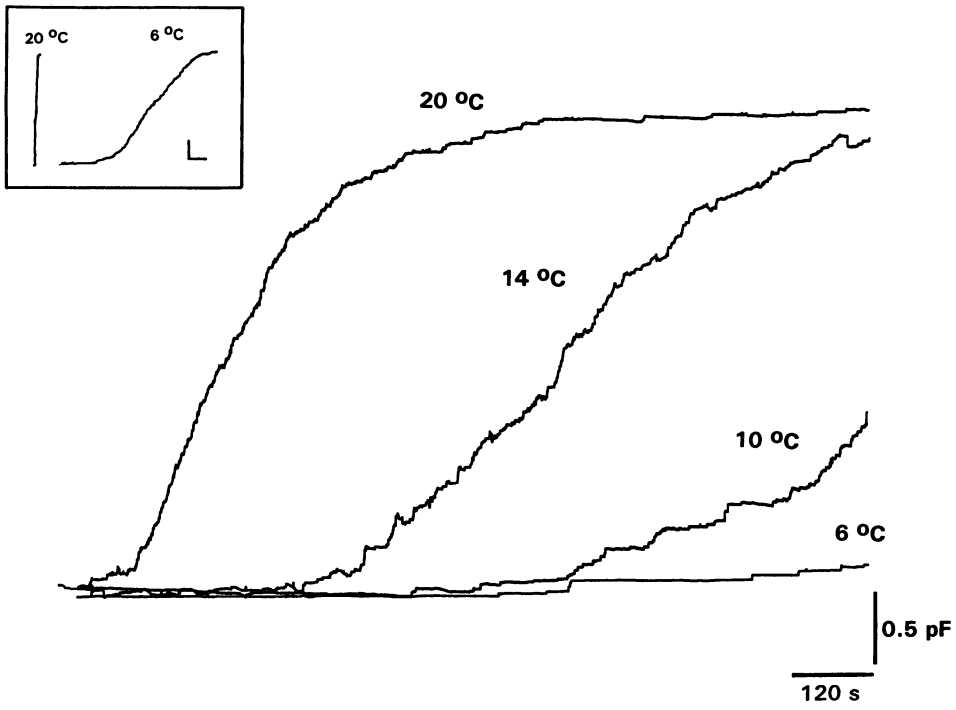

FIGURE 1 Effect of temperature on the rate and extent of exocytosis in mouse peritoneal mast cells. Exocytosis is measured as an increase in the cell membrane capacitance caused by the addition of the secretory granule membrane. The capacitance increases shown in this figure each consist of the fusion of several hundred secretory granules. The initial and final capacitances had the following values: 3.3–11.5 pF (6°C), 4.2–12.3 pF (10°C), 4.0–12.1 pF (14°C), and 3.9–12.0 pF (20°C). The respective lag periods and rise times were: 11.2 and 17.5 min (6°C), 8.1 and 12.3 min (10°C), 2.8 and 6.5 min (14°C), and 0.9 and 3.2 min (20°C). (*Inset*) Capacitance traces at 20 and 6°C plotted with a compressed time scale. Calibration bars, 2 pF, 5 min. The patch pipette contained the standard internal solution plus 10 μM GTPγS and $[Ca]_i$ in the nanomolar range (30 nM).

rise times were 13.8 ± 4.1 and 19.6 ± 2.8 min (6°C, n = 3), 9.5 ± 2.8 and 15.3 ± 4.1 min (10°C, n = 3), 3.2 ± 0.9 and 6.2 ± 2.6 min (14°C, n = 3), and 1.3 ± 0.5 and 3.5 ± 0.8 min (20°C, n = 6). The inset in Fig. 1 shows that a degranulation at 6°C proceeds to a similar extent and with a shape similar to that of a degranulation at higher temperatures. The extents of degranulation, expressed as the ratio of the final and initial capacitance (C_f/C_i), were 3.48, 2.93, 3.03, and 3.08 at 6, 10, 14, and 20°C, respectively (mean 3.11 ± 0.44, n = 31). Thus, only the lag period and rate of degranulation are affected by temperature; the total number of secretory granules that are released is not altered.

Kinetic analysis of the fusion of secretory granules

An advantage of monitoring the cell membrane capacitance during exocytosis is that it is possible to detect the fusion of individual secretory granules with the plasma membrane as step increases in capacitance. The ability to resolve the fusion of individual secretory granules makes possible a kinetic analysis of the single fusion events, analogous to the kinetic analysis of single ion channels. Fig. 2 shows a capacitance record like those shown in Fig. 1, but with an expanded amplitude scale to demonstrate the stepwise changes in capacitance caused by the exocytotic fusion of individual secretory granules. Two types of fusion events can be seen: irreversible step increases in capacitance (for example, the event marked with the *dot*) and transient fusion events (marked with *asterisks*). The latter occur when a secretory granule fuses with the plasma membrane and the narrow fusion pore subsequently closes, leaving an intact secretory granule inside the cell (Fernandez et al., 1984; Breckenridge and Almers, 1987; Alvarez de Toledo and Fernandez, 1988; Monck et al., 1990). We simply assume that these two types of fusion events correspond to fusion pores that are structurally identical.

From recordings such as those shown in Fig. 2, we can measure three parameters that define the formation and closure of fusion pores: (*a*) the interval between irreversible fusion events (interstep interval, t_i); (*b*) the duration or dwell-time of transient fusion events (t_t); and (*c*) the fraction of the total fusion events that are transient. Transient fusion events can be faster than the time resolution of the recordings (< 14 ms) or can last up to several seconds (see *asterisks* in Fig. 2). Similarly, the interstep interval covers a wide range of values. A practical representation of these parameters can be obtained by plotting the data as probability distributions. The probability distribution functions for the interstep interval, as well as that of the duration of transient fusion events, have been shown to be well represented by single exponentials (Alvarez de Toledo and Fernandez, 1990*a–c*).

The mean interstep interval can be obtained by measuring the interstep interval in the region where the rate of degranulation is linear, i.e., between 20 and 80% of the degranulation (see Fig. 1 and also Alvarez de Toledo and Fernandez, 1990*c*). In most experiments, this region includes ≥ 100 well-resolved fusion events and provides a probability distribution function that is well described by a single exponential, as shown in Fig. 3. In all cells analyzed, the time constant of this exponential is similar to the mean interstep interval, as expected from events that conform to a Poisson distribution. We used the time constant of the single exponentials fitted to each distribution as a measure of the mean value of these parameters. Fig. 3 shows that temperature strongly influences the interstep interval. A change in the temperature from 22 to 6°C increases the mean interstep interval by ~ eightfold. The mean interstep interval can be used to determine the rate constant for the rate-limiting step leading to fusion pore formation.

In mast cells, exocytosis occurs in the compound mode (Rohlich et al., 1971; Alvarez de Toledo and Fernandez, 1990*a*). Secretory granules fuse with the plasma membrane or with the membrane of previously fused granules. Thus, when a granule fuses with the plasma membrane, it provides a path for other granules, deeper in the cell, to fuse and release their secretory products. In this manner, granules fuse sequentially in a chain (Rohlich et al., 1971). In the linear part of the degranulation, the number of paths and the rate of fusion must be constant. Therefore, in this region the granules probably fuse in a linear chain. Thus, we can consider the interstep interval to be a measure of the rate-limiting step in the sequence of events that occurs between creation of the path when the first granule fuses and the next fusion event. Consequently, the rate of formation of the fusion pore is defined as the inverse of the mean interstep interval. It should be noted, however, that the exponential distributions shown in Fig. 3, *A* and *B*, do not necessarily represent the probability of forming a single fusion pore because it is likely that during a degranulation several paths are developing simultaneously. Thus, the exponential probability distribution that is measured is the product of the probability distribution function for the fusion of granules in each path and should, therefore, be an overestimate of the probability of any single granule fusing once the path has been created.

Fig. 3 also shows the probability distribution function for the length of time that the fusion pore exists before

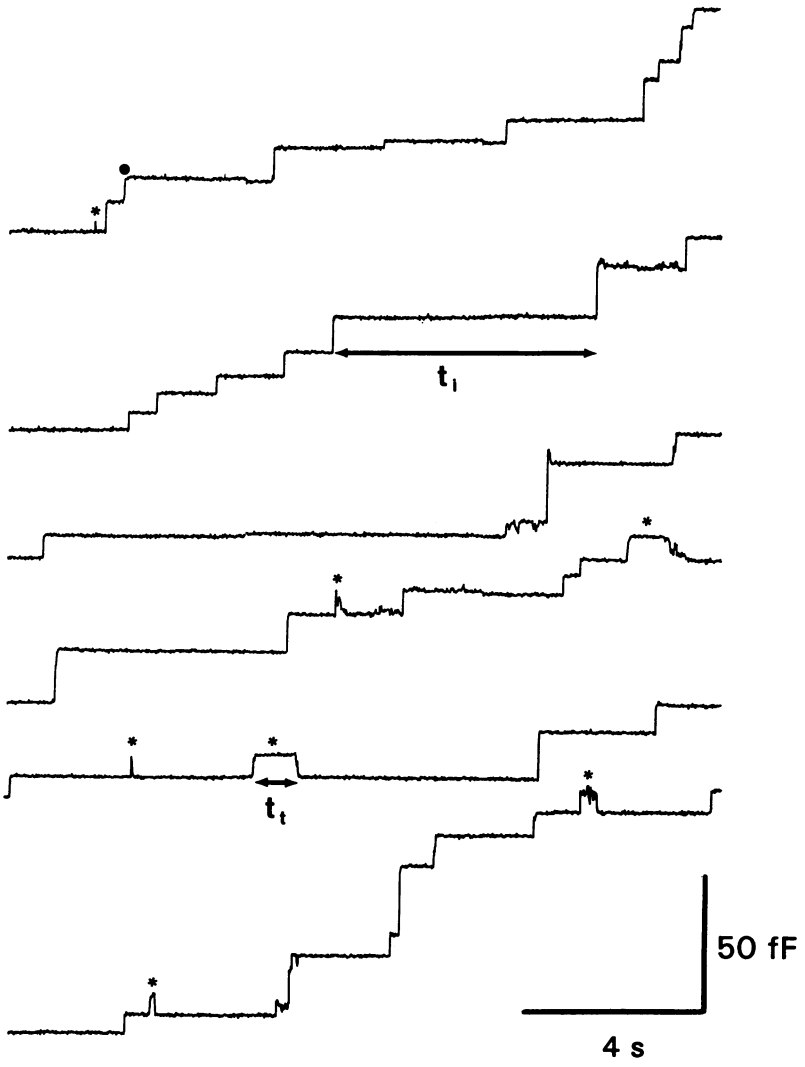

FIGURE 2 Step changes in cell membrane capacitance that occur during the course of mast cell degranulation induced by intracellular GTPγS. All of these events correspond to the fusion of single granules with the plasma membrane. The traces are from a continuous record (total of 90 s, from *bottom* to *top*) that occurred after 18 min of cell perfusion with pipette solutions containing 10 μM GTPγS. This record shows two types of events: (*a*) irreversible fusion events (*dot*) and (*b*) transient fusion events (*asterisk*). From these data we can measure the time between irreversible fusion events (interstep interval, t_i) and the duration or dwell time of the transient fusions events (t_t). Temperature: 13°C.

closure. As for the interstep interval, the probability distribution function is well fitted by a single exponential. The dwell time is strongly temperature dependent. A decrease in temperature from 22 to 6°C increases the mean duration of the transient fusion events by about four times. The transient fusion event mean dwell time and the fraction of transient fusion events can be used to determine a rate constant for the closure of the fusion pore. The inverse of the mean dwell time is a measure of the rate at which a fusion pore is leaving the open configuration. The fraction of reversible fusion events represents the probability that a fusion pore will close. Therefore, we can calculate the rate of closure of the fusion pore as the fraction of transient fusion events multiplied by the inverse of the mean transient fusion dwell time.

Effect of temperature on the rate of formation and closure of the fusion pore

The temperature dependence of the mean interval between irreversible fusion events, the mean interstep interval, is shown in Fig. 4*A*. The mean interstep interval is greatly increased as the temperature is decreased. The relationship is not linear: the interval decreases

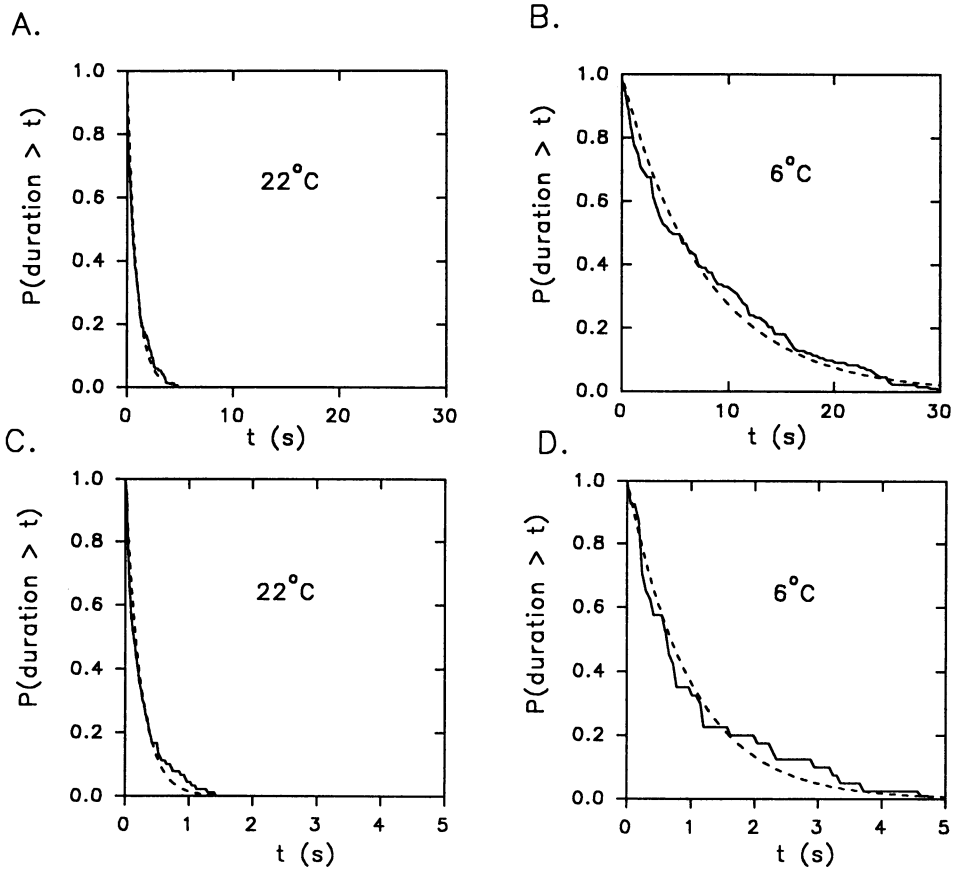

FIGURE 3 The probability distribution functions of the interstep interval and the duration of transient fusion events are well described by single exponentials. The characteristic time of the exponential distribution is strongly temperature dependent. The data were obtained by measuring time between fusion events (interstep interval, t_i; *A* and *B*) or by measuring the time between the initial increase in capacitance and the return to the basal level, indicative of complete closure of the fusion pore (duration of transient fusion events, t_t; *C* and *D*), as indicated in Fig. 2. The probability distribution function, $P(t)$, is the probability of finding events separated by a time $\geq t$ or the probability of a transient fusion event having a lifetime $\geq t$. Nonlinear single exponential fits of the data are shown by the dashed lines. (*A*) $P(t)$ of the interval between steps at 22°C. 161 events measured in the region where the rate of fusion is constant (between 20 and 80% of the degranulation). The time constant was 941 ± 15 ms and the mean value was 969 ± 75 ms. (*B*) $P(t)$ of the interstep interval at 6°C. The time constant was 7,756 ± 145 ms and the mean value was 7,872 ± 703 ms (132 events measured in the linear region of the degranulation). (*C*) $P(t)$ of the duration of transient fusion events at 22°C. Data obtained from the same cell as in *A*. The time constant was 230 ± 7 ms and the mean value was 279 ± 32 ms (90 events). (*D*) $P(t)$ of the duration of transient fusion events at 6°C. Data obtained from the same cell as in *B*. The time constant was 980 ± 27 ms and the mean value was 1,105 ± 191 ms (28 events).

4-fold between 20 and 32°C and >20-fold between 6 and 32°C. As would be expected, the mean interstep interval has a similar temperature dependence to the rise time of the macroscopic increase in membrane capacitance that results from the sequential fusion of all the secretory granules during a degranulation (Fig. 1), the Q_{10}s calculated from the values at 10 and 20°C being 4.1 and 4.5, respectively.

The effect of temperature on the mean dwell time of transient fusion events and the fraction of total fusion events that are reversible are shown in Fig. 4, *B* and *C*, respectively. The mean duration of the transient fusion events is not a simple function of temperature. Below 13°C there is no significant change in the dwell time (at $P < 0.05$ level, 2-tailed *t*-test), the average duration being ~1 s (1.08 ± 0.28 s, 226 transient fusion events, 9 cells). Between 13 and 14°C there is an abrupt decrease to ~600 ms (593 ± 97 ms, 165 events, three cells), and from 14 to 32°C there is a progressive shortening of the transient fusion event duration (Q_{10} = 1.8 between 14 and 24°C). The fraction of transient fusion events changes very little between 6 and 13°C or between 14 and 32°C (Fig. 4 *C*). However, between 13 and 14°C there is a sudden increase in the incidence of the transient fusion events, from 17 to ~28% (see Fig. 4). Thus, low temperature makes it less likely that a fusion pore will close.

Fig. 5 *A* shows an Arrhenius plot of the data shown in

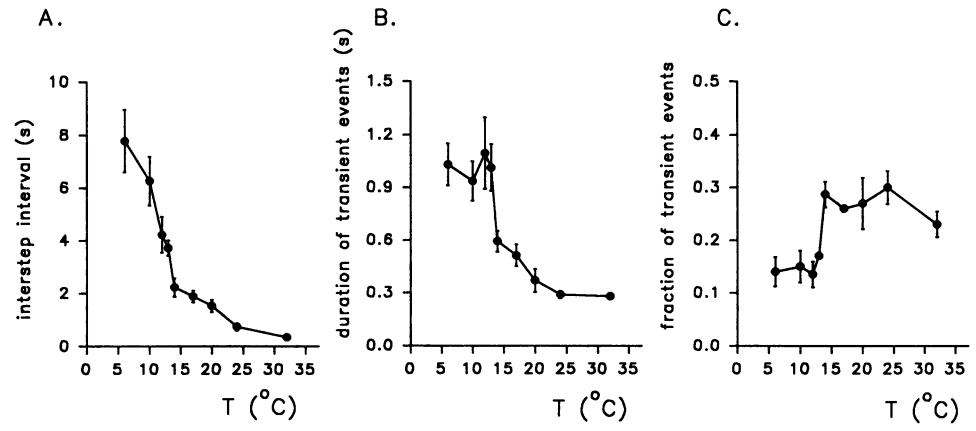

FIGURE 4 Mean interstep interval (*A*), mean duration of transient fusion events (*B*), and incidence of transient fusions (*C*) as a function of the temperature. The number of experiments at the different temperatures are as follows: 3 cells at 6°C (441 irreversible fusion events, 76 transient fusions), 3 cells at 10°C (265 irreversible events, 44 transient transient fusions), 2 cells at 12°C (220 irreversible events, 43 transient fusions), 1 cell at 13°C (306 irreversible events, 63 transient fusions), 3 cells at 14°C (416 irreversible events, 165 transient fusions), 1 cell at 17°C (32 irreversible events, 11 transient fusions), 6 cells at 20°C (592 irreversible events, 246 transient fusions), 10 cells at 24°C (1,390 irreversible events, 526 transient fusions), and 2 cells at 32°C (381 irreversible events, 128 transient fusions). The temperature values are accurate within ±0.5°C.

Fig. 4 *A*. The activation energy for the rate of formation of the fusion pore (calculated as the inverse of the average interstep interval) is 23 kcal/mol, suggesting that, unlike diffusion, the rate-limiting step in fusion pore formation has a large activation energy. The rate of closure of the fusion pore is plotted as an Arrhenius plot in Fig. 5 *B*. Below 13°C the rate of closure of the fusion pore is independent of temperature, and above 14°C the rate of closure is weakly temperature dependent with an activation energy of 7 kcal/mol. The rate of closure

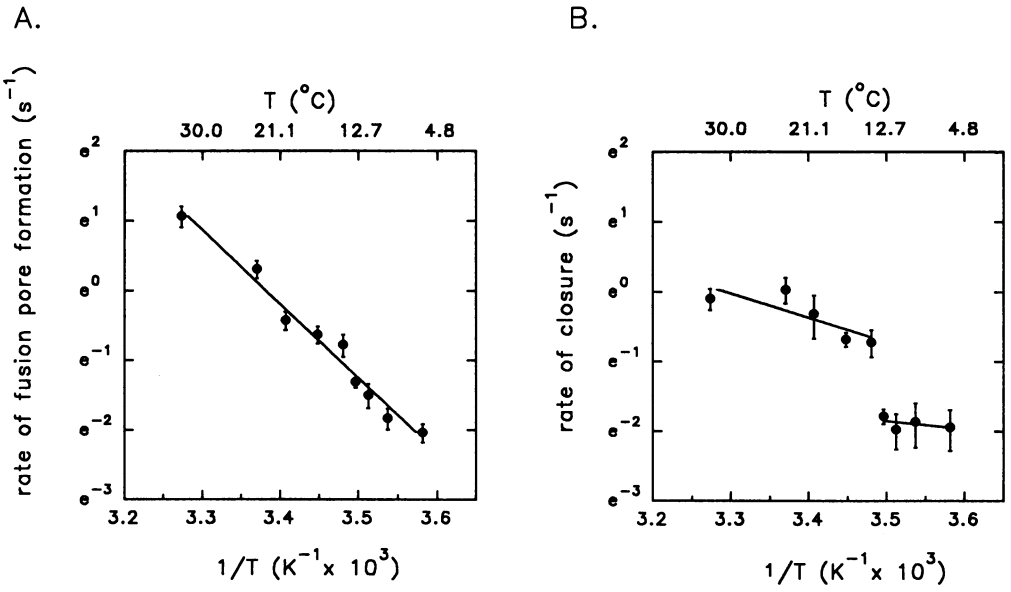

FIGURE 5 Arrhenius plots for the apparent rate of formation (*A*) and closure (*B*) of the fusion pore. (*A*) The rate of fusion pore formation was defined as the inverse of the mean interstep interval. The slope of the fitted line gives an activation energy of 23 kcal/mol. (*B*) The rate of closure of the fusion pore was defined as the inverse of the mean duration of transient fusion events multiplied by the fraction of transient events. The slope calculated above 13°C corresponds to an activation energy of 6.95 kcal/mol ($r = 0.82$). Below 13°C the rate of closure of the fusion pore is considered to be independent of temperature (slope = 2.1 kcal/mol, $r = 0.48$).

changes abruptly by a factor of three between 13 and 14°C.

DISCUSSION

The experiments presented in this paper aim to probe the molecular structure of the fusion pore by changing the physical properties of the cell membrane through changes in temperature. The molecular structure of the fusion pore and its regulatory mechanisms are currently unknown. One model proposes that the molecular structure of the fusion pore resembles that of an ion channel (Almers, 1990; Almers and Tse, 1990). This model is consistent with the experimental observation that fusion pores open abruptly with a well-defined conductance that subsequently can expand and close again. According to this model, the final irreversible expansion occurs when lipid molecules intersperse between the protein subunits that make up the pore. The ion channel model shares many characteristics with two well-studied systems: the gap-junction channel, which spans the extracellular gap between two membranes, and the alamethicin channel, which opens and closes through the aggregation and disaggregation of protein subunits that diffuse freely in the membrane. One might expect that the temperature dependency of the fusion pore formation and closing would share characteristics with one or both of these two systems. Our results show that the rate at which single secretory granules fuse with the cell surface membrane is strongly affected by the temperature (Q_{10} = 4.1, calculated from the values at 10 and 20°C; Fig. 4*A*). According to the Arrhenius Law, the logarithm of the rate constant of a reaction is inversely proportional to the temperature. As Fig. 5*A* shows, there is a good correlation (r = 0.980) between the natural logarithm of the rate of fusion pore formation and $1/T$. The activation energy for the rate of fusion pore formation (23 kcal/mol) represents a value that is higher than simple diffusion (5–10 kcal/mol; Bockris and Reddy, 1977) but is compatible with those found for conformational changes in peptides and proteins (10–90 kcal/mol; Hilton and Woodward, 1978; Laidler and Peterman, 1979). This suggests that the rate-limiting step between activation of the G-protein that initiates the stimulus for exocytosis and opening of the fusion pore may involve molecular conformation changes with a high activation energy. It should be noted, however, that the rate of formation, as calculated here, represents the rate-limiting step leading to the opening of the fusion pore. This step may not be associated directly with changes in the fusion pore structure, such as the opening of an ion channel, but, instead, could correspond to a more proximal cellular event involved in regulation of fusion pore opening.

The temperature dependency of the rate of closure of the fusion pore shows a pattern strikingly different (Fig. 5*B*) from those usually associated with conformational changes in proteins such as opening and closing of ion channels. The Arrhenius plot of the rate of closure of the fusion pore has a discontinuity at ~13°C. Above 13°C the rate of closure has a weak temperature dependence, with an activation energy of 7 kcal/mol, whereas below 13°C the rate of closure is temperature independent. A temperature dependency of this type resembles the temperature dependency of processes that depend on diffusion in a lipid phase that undergoes a phase transition. We cannot exclude the possibility that a discontinuity could arise from a highly cooperative interaction in a macromolecular structure such as the cytoskeleton. However, from other experiments, we know that, on formation of a fusion pore, a large flow of lipid molecules through the fusion pore is established (10^6 lipid molecules/s; Monck et al., 1990). Although the function of this flow of lipids is still unclear, the observation implies that the fusion pore is mostly lipid when it closes (Monck et al., 1990), making it reasonable to suppose that the temperature dependency observed is related to the physical properties of the lipid molecules that form the fusion pore.

Several studies have shown that freezing of the hydrocarbon interior of the phospholipid bilayer prevents free diffusion but has a characteristically different and smaller effect (possibly hindering conformational flexibility) on the activity of integral proteins like ion channels and pumps. The effect of a phase transition on a membrane process that is diffusion limited is evidenced by a sudden change in the measured parameter at the transition temperature, and this change can be demonstrated as an abrupt discontinuity in an Arrhenius plot. Examples of events showing this type of Arrhenius plot include those for the conductivity induced by mobile ion carriers, such as nonactin or valinomycin, incorporated into artificial lipid bilayers (Krasne et al., 1971) and the rotational motion of the Ca^{2+}-transporting ATPase from sarcoplasmic reticulum incorporated in synthetic lipid vesicles (Hoffmann et al., 1980). In contrast, membrane processes that depend on a conformational change of an integral protein often have an inflection in the Arrhenius plot at the phase transition temperature, but not a discontinuity. Examples include the conductance of ion channels (Krasne et al., 1971), the activity of membrane enzymes (Churchill et al., 1983), and the catalytic activity of ion pumps (Hoffmann et al., 1980).

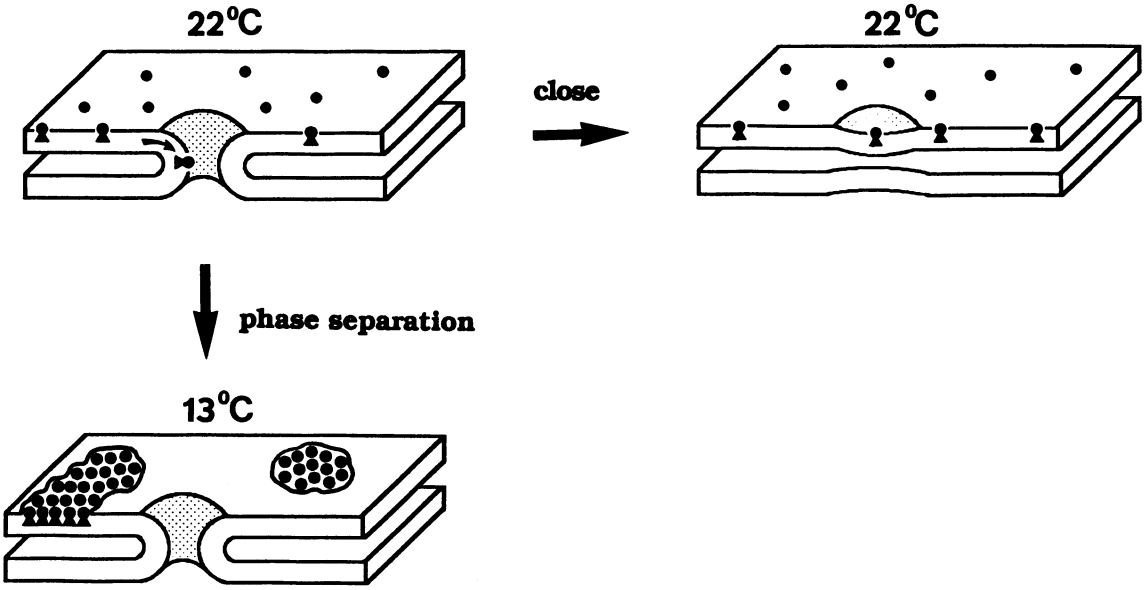

FIGURE 6 A simple model that accounts for the temperature dependence of the rate of closure of the fusion pore. If the equilibrium of the lipids that form the fusion pore is disturbed by incorporating new lipids into the pore bilayer, a new packing or elastic equilibrium could lead to membrane shape changes that favor spontaneous closure of the fusion pore. According to the model, closure of the fusion pore at room temperature is promoted by the diffusion of a single bilayer-perturbing type of lipid into the membrane in the neck of the fusion pore. Below 13°C, this lipid phase separates into rigid microdomains and is no longer able to diffuse into the fusion pore and cause its closure.

The remarkable analogy between the data of Fig. 5 *B* and the data from carrier diffusion (Krasne et al., 1971; Boheim et al., 1980) suggests that the mechanism that drives the closure of the fusion pore is a diffusional process that is strongly affected by the lipid phase of the membrane. The phase transition of artificial bilayers made from a single type of phospholipid always occurs over a narrow temperature range (usually 0.1–3°C; Albon and Sturtevant, 1978). In contrast to this, biological membranes contain complex mixtures of lipids that do not change from a fluid to a solid state in a single, highly cooperative phase transition. The transition breadth of biological membranes is usually 5–15°C (Crowe et al., 1989). Surprisingly, the sharp (within 1°C) decrease of the rate of closure of the fusion pore that occurs at 13°C resembles a diffusional process that is sensing the phase transition in a bilayer made of a homogeneous lipid.

It has been proposed that, rather than a homogenous phase transition, phase separation can occur in biological membranes at physiological temperatures; the result is the coexistence of distinct lipid microdomains of differing chemical composition (Karnovsky et al., 1982; Yechiel and Edidin, 1987; Tocanne et al., 1989; Rodgers and Glaser, 1991). Furthermore, phase separation and changes in the connectivity of the phase structure of lipid domains can occur within a narrow temperature range (Vaz et al., 1989). These observations suggest a simple model that can explain the temperature dependency of the closure of the fusion pore (Fig. 6). We propose that closure is promoted by a single type of lipid molecule that can enter the pore. Below the critical temperature this lipid separates into a solid microdomain in the plasma membrane so that this lipid is no longer available for the molecular events that cause the closure of the fusion pore. The entry of a specific type of lipid molecule into the fusion pore may increase its free energy by either increasing the packing energy of the fusion pore structure (Gruner, 1985) or by increasing the elastic energy of the pore through a change in the spontaneous radius of curvature (Israelchavilli, 1980; Markin et al., 1984). An increase in the free energy of the pore would then increase the probability of its closure. Elucidation of the molecular structure of the fusion pore and the mechanism by which it can close requires further experimentation. Toward this aim the simple model presented in Fig. 6 makes two predictions: (*a*) in the mast cell membrane there should be a class of lipids that undergoes phase separation at 13°C; and (*b*) the availability of these lipids should promote the closure of the fusion pore. These predictions can be tested with techniques used to study phase separation and with the techniques outlined in this work.

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