Evaluation of the Electrostatic Field Strength at the Site of Exocytosis in Adrenal Chromaffin Cells

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ABSTRACT Exocytosis in secretory cells consists of release from intracellular storage granules directly into the extracellular space via fusion of the granule membrane with the plasma membrane of the cell. It is considered here as comprising two distinct processes. One is the close apposition of granule and plasma membranes. The other arises from interactions between the two membranes during the process of apposition, leading to the formation of a fusion pore. In the following it is shown for the case of the adrenal medullary chromaffin cell that the fusion pore can be ascribed to electroporation of the granule membrane, triggered by the strong electric field existing at the site of exocytosis. Based on an electric surface charge model of the cytoplasmic side of the plasma membrane, resulting from the negatively charged phosphatidylserine groups, it is found that the electrostatic field strength at the site of exocytosis reaches values on the order of 10^8 V/m at small intermembrane distances of 3 nm and lower. The field strength increases with the size of the disc-shaped plasma membrane region generating the electric field, reaching an approximate limit for a radius of 10 nm, at a surface charge density of 5.4×10^{-2} C/m^2. According to previous experimental evaluations of threshold field strength, this field is sufficiently strong to cause membrane electroporation. This step is a precondition for the subsequent membrane fusion during the ongoing process of apposition, leading to secretion.

INTRODUCTION

There has been significant progress in clarifying the mechanism of exocytosis in secretory cells, mainly as a result of the identification of a number of proteins similar to those known to play a role in the fusion of intracellular membranes during constitutive exocytosis (Sollner et al., 1993; Rothman, 1994). The interactions between these proteins, as well as their dependence on calcium ions and other intracellular modulators, have now been characterized in broad outline, but a detailed charting of the sequence of events leading to exocytotic secretion is still missing (Augustine et al., 1996). In particular, whereas the steps preceding it, such as vesicle docking (Rothman, 1994), seem well accounted for, the mechanism of fusion between vesicle and plasma membrane, leading to pore formation, remains to be explained. The possibility that the latter event is a consequence of interactions in the lipid bilayers of the fusing membranes has been raised (Nanavati et al., 1992; Oberhauser and Fernandez, 1993; Chernomordik et al., 1995). One potentially relevant aspect that has not been considered in detail so far is the influence of the intracellular electrostatic forces on the interaction between components of the vesicle and plasma membranes, just before the fusion event. An analysis of the electrostatic interactions between charged phospholipid bilayer vesicles and their influence on calcium-induced membrane fusion has been carried out by Gingell and Ginsberg (1978). In the following we estimate the intensity of the local electrostatic field, arising from the electric charges of the membrane lipids located on the cytoplasmic side of the plasma membrane, and the role it may play in the intiation or facilitation of membrane fusion. It is found that when the two membranes approach each other during the stage of apposition (Rand and Parsegian, 1986), the electrostatic field reaches values known from previous studies to cause electrical breakdown of biological membranes, i.e., electroporation. The latter process, first demonstrated by measuring the release of stored catecholamines from adrenal chromaffin granules under the influence of short, high-intensity electric field pulses (Neumann and Rosenheck, 1972; Lindner et al., 1977), is now widely applied to introduce active molecules and macromolecules into the cell cytoplasm (Neumann et al., 1982, 1989; Tsong, 1987; Mir et al., 1991). It is also a well-known fact that electroporation is a precondition for the subsequent fusion between cell membranes (Teissie et al., 1982; Zimmermann, 1982; Neumann et al., 1989; Nickoloff, 1995).

Two types of experimental observations might argue for an approach emphasizing the electrostatic interactions at the exocytotic site. One is that the dimensions of secretory fusion pores in their incipient stage, at ~1 nm diameter (Monck and Fernandez, 1992), as determined from patch-clamp experiments, are similar to those of electropores in lipid bilayers and biological membranes estimated from theory (Saulis and Venslauscas, 1993) as well as from experiments with model systems (Hibino et al., 1991; Tsong, 1991). The other concerns the kinetics of fusion pore evolution. It is now possible to follow stimulated catecholamine release from chromaffin and other secretory cells at the level of single storage granules with the aid of carbon-fiber electrode amperometry (Wightman et al., 1991), as well as patch-clamp capacitance (Neher and Marty, 1982;

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Alvarez de Toledo et al., 1993) and conductance (Breckenridge and Almers, 1987; Spruce et al., 1990; Hibino et al., 1991) measurements. These single-vesicle transients are characterized by half-widths in the millisecond range, except for occasional flickering in signal intensity that may end in pore closure before release is completed (Chow et al., 1992; Alvarez de Toledo et al., 1993). The rise times, in particular, should be related to the kinetics of formation of the fusion pore. The data, when compared to the kinetics of electric field-induced membrane permeabilization in suspensions of isolated chromaffin granules (Rosenheck et al., 1975), show a similar time course, suggesting that the electroporative model system might reflect, in this regard, the biological in situ event.

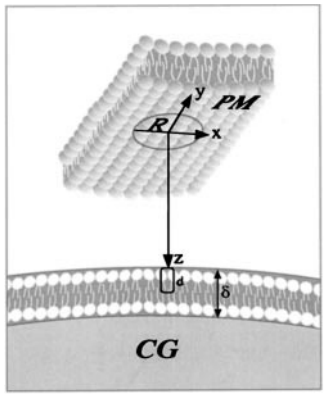

FIGURE 1 Schematic diagram of the electrostatic field calculation at the site of exocytosis. PM, Plasma membrane; CG, chromaffin granule. The dimensions are not to scale.

THE MODEL

When the calcium signal reaches a secretory vesicle docked near an exocytotic site, there is an additional closing in and alignment of short segments of the granule and plasma membranes, possibly driven by a scaffold of intermembrane proteins (Monck and Fernandez, 1992), until they are in close apposition. Electron micrographs show the two interacting regions lying flat against each other over a short range that may extend for several nanometers (Chandler, 1988; Vitale et al., 1995). This leads to the basic assumption of a planar geometry, used in these calculations. In contrast to this situation, it is also frequently observed that the plasma membrane caves inward, forming a dome-shaped extension with its apex directed toward the vesicle (Chandler and Heuser, 1980; Schmidt et al., 1983; Chandler, 1988). The implications of this nonplanar geometry will be discussed briefly later on.

Long-range Coulombic interactions

We consider a disc-shaped region of uniform net surface charge density on the cytoplasmic side of the chromaffin cell plasma membrane. The surface charge density is derived from the known lipid composition of this membrane (Wilson and Kirshner, 1976; Azila and Hawthorne, 1982), and the assumption, validated by data from many cell types, that phosphatidylserine (PS), the only membrane lipid in these cells that bears a net electric charge, is located on the monolayer facing the cytoplasm (Allan and Kallen, 1993). For a PS content of 11% of total phospholipids, there is one elementary charge per 2.95 nm^2, taking the mean area per lipid molecule as 0.65 nm^2. The radius of the disc-shaped region on the plasma membrane varies within a set of values in the nanometer range, chosen according to electron micrographs of chromaffin cells at a stage of incipient exocytosis. The planar geometry used in deriving the spatial variation of the field vector as a function of the local charge density (Wangsness 1979), yields the field strength, $E(z)$, in the direction normal to the plane of the membrane, whereas the in-plane components vanish (Fig. 1). The electric field strength along the direction connecting the disc to the apposed region of the chromaffin granule membrane is computed as a function of intermembrane distance z for a series of points located at a given depth d, within the granule lipid bilayer, assuming the bilayer-cytoplasm interface to be infinitely thin. The electrostatic potential associated with a disc-shaped uniform surface charge distribution of radius R (Wangsness, 1979) is

$$\Psi(z) = (\sigma_p/2\epsilon_c\epsilon_o)[(R^2 + z^2)^{1/2} - z], \quad (1)$$

where σ_p is the net surface charge density on the cytoplasmic side of the plasma membrane, ϵ_c is the dielectric constant of the cytoplasm, and ϵ_o is the permittivity of free space. $\sigma_p = 5.4 \times 10^{-2}$ C/m^2, as calculated from the PS content; $\epsilon_c = 80$; and $\epsilon_o = 8.85 \times 10^{-12}$ F/m.

Debye-Hueckel screening is accounted for by numerically solving the Poisson-Boltzmann equation for varying intermembrane distances (Overbeek, 1952), yielding $\Psi_s(z)$. The electrostatic field in the lipid bilayer, E_b, is calculated (Wangsness, 1979) as the negative gradient of the screened surface potential across the boundary between the two different dielectric media, cytoplasm and lipid bilayer:

$$E_b = (\sigma(z)/2\epsilon_b\epsilon_o)[1 - \mathcal{Z}/(R^2 + \mathcal{Z}^2)^{1/2}], \quad (2)$$

where $\mathcal{Z} = z + d$; the dielectric constant of the lipid bilayer $\epsilon_b = 2$; and $\sigma(z)$, the surface charge density corresponding to $\Psi_s(z)$, is obtained from

$$\sigma(z)/\epsilon_c\epsilon_o = \Psi_s(z)/\lambda \quad (3)$$

by modeling the cytoplasm as a symmetrical univalent electrolyte of a concentration of 0.15 M, corresponding to a Debye length $\lambda = 0.785$ nm. Contributions from higher valency ions are neglected, because they are in the micromolar to millimolar range. Calcium ions, even though they may accumulate in the region of the exocytotic site as a consequence of stimulation, do not reach concentrations higher than several tens of micromolarity during limited time periods of milliseconds (Heinemann et al., 1994). Similarly, from analytical data on granule membrane com-

position, as well as electrophoretic measurements, quoted by Phillips (1987) and by Neumann and Rosenheck (1972), respectively, the net surface charge density of the granule membrane is, at most, one-fifth that of the plasma membrane, and therefore the corresponding electric field, being of much shorter range, is not taken into account in the calculations.

Fig. 2 displays the calculated values of E_b as a function of d for a disc radius $R = 10$ nm, at three different values of the intermembrane distance, z, expressed in units of the Debye length, λ. Fig. 3 shows the variation in E_b as a function of z, at $d = 2.5$ nm. The local field strength within the lipid bilayer is seen to reach values in the range of 10^8 V/m and more as the intermembrane separation decreases. Are these field strengths sufficient to cause electroporation? The previously mentioned measurements of cell membrane permeabilization by high electric field pulses, using catecholamine release from chromaffin granules as a model for the neurosecretory process, suggest that they are.

From the dependence of the degree of catecholamine release on intensity and duration of externally applied electric field pulses (Neumann and Rosenheck, 1972; Lindner et al., 1977), the value of the induced transmembrane potential can be calculated from its relation to external field strength, E_e, and vesicle radius, r (Teissie and Tsong, 1981):

$$\phi = 1.5 r E_e \cos \alpha, \tag{4}$$

where α denotes the angle between field direction and the direction of the vesicle radius at any given point on its surface. The values of ϕ obtained in this way cover a range of ~350–450 mV, well within that of other biological membranes investigated (Neumann et al., 1989). If, as an approximation, it is assumed that the electric field strength due to the transmembrane potential is constant across the bilayer width of 5 nm, this yields a critical field strength of 7–9 × 10^7 V/m. This threshold zone, within and above

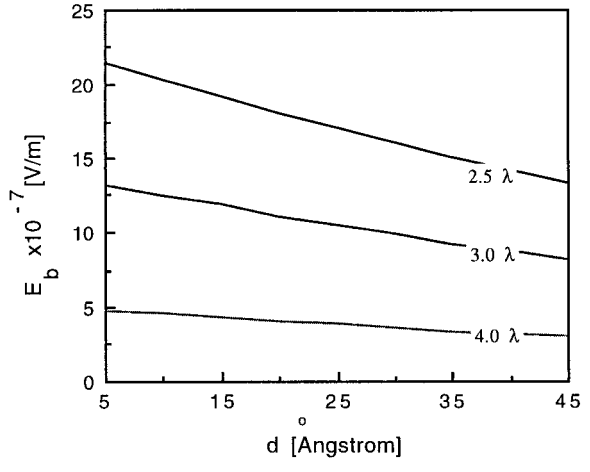

FIGURE 2 Electrostatic field strength within the granule lipid bilayer as a function of the distance, d, from the cytoplasm- membrane interface, calculated for intermembrane separations, z, of 2.5, 3.0, and 4.0 Debye lengths. $R = 10$ nm.

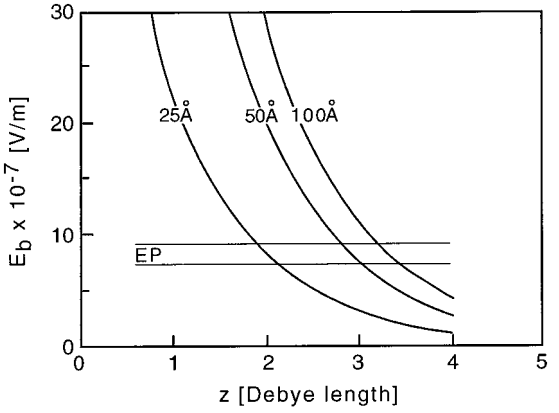

FIGURE 3 Field strength as a function of intermembrane separation for disc radii, R, of 10, 5, and 2.5 nm. The zone designated EP is the critical threshold for membrane electroporation, as estimated from previous work (Lindner et al., 1977).

which electroporation of the lipid bilayer could be expected to occur, is shown by the horizontal lines in Fig. 3, denoted EP. Although, for the R values shown, the dependence of E_b on disc radius is pronounced, this is no longer the case for much larger radii because of the screening in the double layer. Fig. 4 demonstrates this point. From Fig. 3 it is seen that permeabilization of the lipid bilayer may be expected to occur at intermembrane distances of 2.5–3.5 Debye lengths, i.e., z of ~2–3 nm.

Because at very short distances, higher order effects arising from inhomogeneities in the surface charge distribution (Cevc, 1990), as well as membrane hydration (Marsh, 1989; Rand and Parsegian, 1989) and steric repulsion (Helm et al., 1992), come into play, the closest distance of approach for which the E_b values are calculated is 1 Debye length. Still, some of these effects may be significant at this intermembrane separation, and therefore a comparison of the Coulombic and the repulsive steric-hydration pressure has been carried out. Fig. 5 indicates that steric interactions are comparable in magnitude to those of Cou-

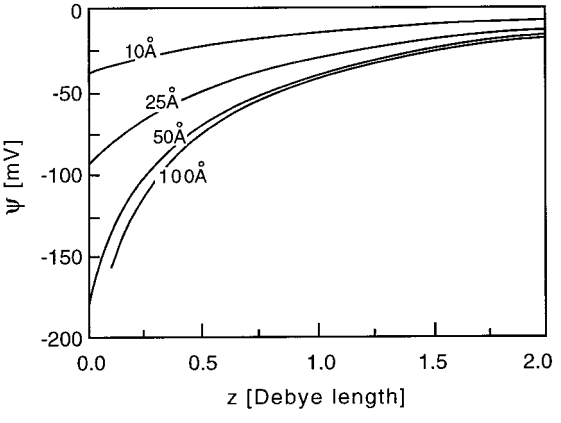

FIGURE 4 Screening of the plasma membrane surface potential, calculated from Eq. 1, as a function of distance in the double layer. The concentration of monovalent electrolyte in the cytoplasm is 0.15 M.

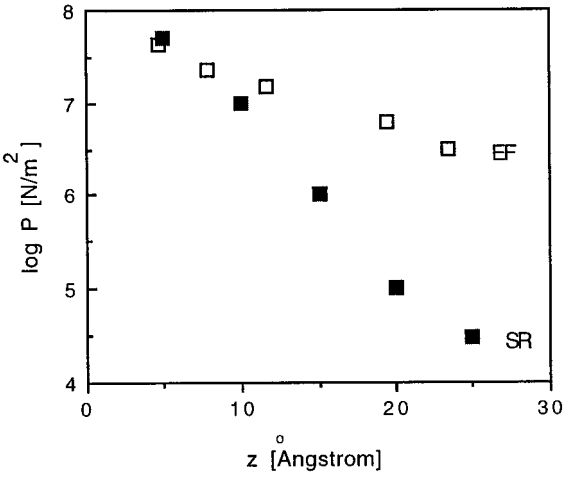

FIGURE 5 Comparison of the pressure exerted by the electric field (EF), calculated for $R = 10$ nm and surface per lipid headgroup = 0.65 nm^2 with the steric pressure (SR) between two lipid bilayers, as adapted from the paper of Helm et al. (1992).

lombic origin at z values of ~1λ and lower, suggesting that electroporation, for which the critical distance is ~2.5–3.5λ, may occur while repulsion between the lipid bilayers in the course of apposition is still small. Furthermore, when one takes the effect of membrane hydration on electrostatic field strength into account by assuming a spatially variable dielectric response at very short distances (Cevc, 1990), significantly higher E_b values are found in the region of the critical threshold, and thus membrane breakdown may occur at even somewhat longer intermembrane distances (not shown).

DISCUSSION

The essential feature of the model presented above is the idea that fusion pore formation starts as a perturbation in the structure of the lipid bilayer of the secretory vesicle membrane. This perturbation is a result of the forces exerted on charged as well as dipolar constituents of the lipid bilayer and, possibly, lipid-associated protein domains, by the high electrostatic field strength, generated by the fixed plasma membrane surface charges, at the site of exocytosis. When, at distances of separation of ~2–3 nm, lipid dislocation has risen to a sufficiently high degree, fusion between the apposed membranes is triggered. Electric breakdown in lipid bilayers has been amply documented (Teissie and Tsong, 1981; Lopez et al., 1988; Needham and Hochmuth, 1989), and models of the lipid character of the fusion pore in its initial stages are able to account satisfactorily for the patch-clamp results (Monck and Fernandez, 1992). It has also been shown that mechanically stressed lipid bilayers will fuse, owing to exposure of hydrophobic domains when brought into close apposition of ~2 nm (Helm et al., 1992). Furthermore, patch-clamp studies of secretory granules from beige mouse mast cells have demonstrated that pipette-induced lateral tension in the granule membrane and high-voltage transmembrane pulses can be used interchangeably to create fusion pore-like events (Oberhauser and Fernandez, 1993). Whereas satisfactory electromechanical models have been developed to account for the tension-voltage relations in lipid bilayer vesicles under defined conditions (Crowley, 1973; Needham and Hochmuth, 1989), none such exist, as yet, for biological membranes in the prefusion stage. One reason for this is the fact that, whereas the chemical identity, as well as interactions, of many of the proteins involved in exocytosis are now well known (Rothman and Soellner, 1997), the physics of the process still eludes us. Do mechanical tensions in the lipid bilayer develop during apposition, as a result of interactions with the protein scaffold? And if so, are they lateral or transversal with respect to the plane of the membrane? Do they affect the two bilayer leaflets to the same degree? Could it be that the ATP-dependent membrane "priming" (Augustine et al., 1996) is the stage at which tension is generated, as a preparatory step for subsequent electric field-induced fusion? Qualitatively, it may be expected that, if tension is present in the granule membrane, it will reduce the electric field strength required for electroporation, and therefore the latter will occur at a longer intermembrane distance (Fig. 3).

Previous work was concerned with the evaluation of the critical transmembrane potential for electroporation of spherical or planar membranes exposed to an external transmembrane electric field. In contrast to those studies, we look here at the gradient of the potential associated with the unilaterally situated source of evenly distributed electric charges on the cytoplasmic side of the plasma membrane. Furthermore, because from experimental studies there appears to be no more than one fusion pore per single vesicle exocytosis, we may consider the intramembrane field as the electrostatic force exerted on a point charge located on the z axis (Fig. 1), at a given depth in the lipid bilayer. It may be relevant in this respect that the dimensions of the fusion pore in its initial stage, ~1 nm diameter (Monck and Fernandez, 1992), correspond fairly well to that of a single lipid molecule with a headgroup surface of 0.65 nm^2. It is, thus, an intriguing question whether fusion pore formation starts as a dislocation of a single molecule from the lipid bilayer. For a field strength of 10^8V/m, the force exerted on a single charge is 1.6×10^{-11} N, and the energy required for displacing this charge for a distance of 2.5 nm, the half-width of the bilayer, is 4×10^{-20} J, a value in the range of those calculated for electropore formation (Saulis and Venslauskas, 1993).

The assumption that the membrane surface charge is evenly distributed usually works well (Cevc, 1990; McLaughlin, 1989; Peitzsch et al., 1995), especially for relatively high charge densities and ionic strengths, as in the present case. A recent theoretical evaluation of the electrostatic potential adjacent to PS-containing phospholipid bilayers, in which their detailed spatial structure was taken into account (Peitzsch et al., 1995), has given values that differ by a factor of ~2 from those of the present work.

Thus the 25-mV equipotential surface for a charge density of $1e/2.72\ nm^2$ (25% PS) is calculated to be at a distance of ~0.65 nm from the interface, whereas in our case of a charge density $1e/2.95\ nm^2$ (22% PS), the distance is ~1.1 nm for $R = 10$ nm, and 0.93 nm for $R = 2.5$ nm (Fig. 4), or, when adjusting for the difference in salt concentration (0.1 M versus 0.15 M), 1.4 nm and 1.1 nm, respectively.

Electric field energy in the lipid bilayer

The energy of pore formation in the presence of an imposed transmembrane potential can be written, according to Hui (1995), as

$$W_e = 2\pi r\gamma - \pi r^2\Gamma - \pi r^2[\epsilon_o(\epsilon_c - \epsilon_b)\phi^2/2\delta], \qquad (5)$$

where r is the pore radius, $\delta = 5$ nm is the bilayer width, $\gamma = 1 \times 10^{-11}$ N is the line tension, and $\Gamma = 2 \times 10^{-3}$ N/m is the surface tension of the membrane (Abidor et al., 1979; Sung and Park, 1997). Because, for an initial pore radius of 0.5 nm, the second term is more than one order of magnitude smaller than the first, i.e., $\sim 1.5 \times 10^{-21}$ J versus 3×10^{-20} J, an instability in the membrane lipid under the influence of the intramembrane electric field will occur when the first and third terms become equal in absolute value. When ϕ is expressed in terms of E_b, this happens when the field strength reaches 1.5×10^8 V/m, i.e., at $z =$ 2.5 Debye lengths, for a disc radius, R, of 10 nm.

Kinetics

The time course of single-vesicle amperometric transients of catecholamine secretion from isolated chromaffin cells (Chow et al., 1992; Alvarez de Toledo et al., 1993; Jankowski et al., 1994), in the absence of pedestals, is similar to that of electric field pulse-induced membrane permeabilization in chromaffin granules (Rosenheck et al., 1975). The latter (Fig. 6) were recorded by the changes in light scattering of whole granule suspensions or by the fluorescence of diphenylhexatriene incorporated into the granule membrane. The former changes were interpreted according to Mie light scattering theory (van de Hulst, 1957) as being due to the change in membrane refractive index when the aqueous medium enters the membrane, and they therefore reflect the membrane permeabilization from its earliest stage on a microsecond time scale. The fluorescence monitors essentially the same process, because the entrance of water quenches the emission from diphenylhexatriene. The rise times are in the 100 μs range, and the decay is described in terms of two relaxation times, a fast one of ~2–3 ms and a slower one in the 30-ms range. Although no detailed comparison with the line shapes of the amperometric transients can be made, because additional factors, such as the speed of dissociation of the vesicular matrix, affect the amperometric signal (Jankowski et al., 1994), the essentially invariant rising phase may be assumed to reflect the evolution of an electric field-induced pore in

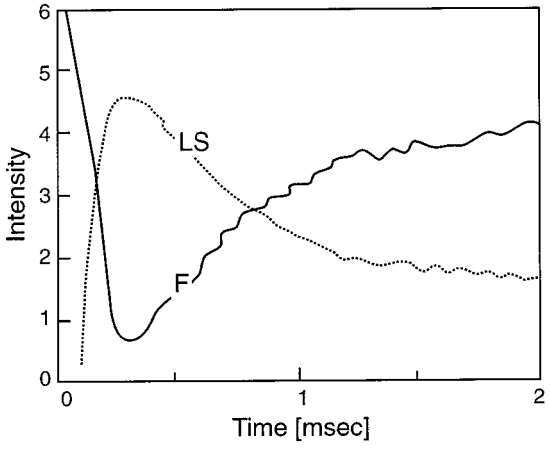

FIGURE 6 Fast transient changes in the optical signals recorded from chromaffin granules subjected to high-intensity electric field pulses with a decay half-time of 40 μs, as adapted from Rosenheck et al. (1975). The intensity scale is in arbitrary units. F, Fluorescence of chromaffin granule suspension in 0.3 M sucrose, doped with the fluorescent dye diphenylhexatriene, $E_e = 10$ kV/cm. LS, Light scattering from chromaffin granule membranes in 0.3 M sucrose. $E_e = 10$ kV/cm.

both the cellular catecholamine release and the model system. Further evidence favoring this assumption has become available very recently from the work of Marszalek et al. (1997). These authors concluded, on the basis of amperometric measurements of secretion from individual mast cell secretory granules that had been subjected to electroporation, that the rising phase of the signal reflected the time course of pore formation.

The frequent appearance of dome-shaped extensions of the plasma membrane, before exocytosis, has been mentioned earlier. The electrostatic field arising from an evenly distributed plasma membrane charge with this type of morphology is nonuniform (Pohl, 1978), and would give rise to a translational movement of both charged and dipolar lipid molecules in the direction of higher field strength, near the plasma membrane. The dislocations in the lipid bilayer resulting from these movements, whereby granule membrane lipids are propelled toward the plasma membrane, could act as precursors in the process of fusion pore formation, e.g., as an efficient way of exposing hydrophobic membrane domains. The geometry of this type of interaction resembles to a certain extent that between hemispherical tip and planar substrate in the atomic force microscope. The electrostatic force between lipid bilayers covering the tip and substrate in an atomic force microscope has been calculated on the basis of the Derjaguin approximation (Levadny et al., 1996). The force-distance dependences found by these authors in the region of small membrane separations differ from the present ones by somewhat less than an order of magnitude. This difference may be due, partly at least, to the fact that the lipid bilayer charge density chosen for their calculations is five times smaller. Exposure of hydrophobic domains might also be enhanced by mechanical stresses in a dome-shaped bilayer with a small

radius of curvature (Israelachvili, 1985). However, the net result for both planar and nonplanar interactions will depend primarily on the strength of the electrostatic field at the site of exocytosis.

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