adsorbed state as an intermediate and with insertion as the rate-limiting step. The ability to resolve adsorption from insertion will permit investigation of the effects of pH, ionic strength, surface potential, and lipid compositions on these events independently. (ii) Colicin E1 adsorbs to and inserts into neutral membranes at acidic pH, and the basis of the interaction is thus likely hydrophobic. (iii) The absence of spectral change at residues 483 and 505 during adsorption suggests that the structure of the adsorbed state is similar to that in solution. (iv) The data presented are consistent with the insertion of helix 8 into the bilayer interior and the complete passage of the 8-9 interhelical loop across the membrane, although they cannot eliminate another recently proposed model in which the 8-9 interhelical loop remains in the solution on the same side to which the colicin was added (*23*).

Although the rates of the processes investigated here are slow, rapid-mixing EPR instrumentation presently available permits investigation of processes on the order of milliseconds (*13, 24*). Thus, site-directed spin-labeling methods of the type illustrated here can be used to investigate other, more rapid time-dependent molecular events such as protein folding.

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27. This work is dedicated to the memory of Professor Cyrus Levinthal.

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The Secretory Granule Matrix: A Fast-Acting Smart Polymer

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The secretory granule matrix is a miniature biopolymer that consists of a charged polymer network that traps peptides and transmitters when it condenses and releases them on exocytotic decondensation. Models of exocytotic fusion have treated this matrix as a short circuit and have neglected its electrical contributions. This matrix responded to negative voltages by swelling, which was accompanied by a large increase in conductance, and to positive voltages by condensing. Thus, the matrix resembled a diode. The swollen matrix exerted large pressures on the order of 12 bar. The responses took place within milliseconds of the application of the electric field. These findings suggest that matrix decondensation, and therefore product release, is controlled by potential gradients.

Most granule matrices are too small to be studied, but the relatively large (5-μm-diameter) matrices in the secretory granule of the mast cells of the beige (bg^J/bg^J) mouse are an exception. They contain a negatively charged heparin sulfate proteoglycan network that condenses in the presence of divalent cations, such as calcium or histamine (at acid pH), and decondenses in the presence of monovalent cations, such as sodium (*1, 2*). We studied the electrical properties of the secretory granule matrices of beige mice by placing the matrices in the tip of a glass pipette (*3*).

Several properties of the granule matrix changed dramatically in response to a change in the applied voltage. At zero or positive pipette potentials, the granule matrix was refractile and condensed and conducted poorly (Fig. 1, A and B). In contrast, at negative potentials, the matrix was transparent and swollen and conducted a large inward current. The matrix had swollen to half of its final size within one video frame of the application of a potential of −2.5 V. The instantaneous swelling was followed by a slower exponential phase (Fig. 1B) (*4*). At the same time, we observed an instantaneous (≤2.5 ms) inward current, which was half of the final value. As with the swelling, this instantaneous current was followed by a slower exponential phase (Fig. 1B) (*4*). When the voltage was turned off, both the current and the swelling returned to control levels within 300 ms. If we assume that the voltage dropped across the diameter of the matrix, the 2.5 V across the 5-μm matrix equaled a field strength of 5000 V cm^{-1}. For comparison, the 50-mV transmembrane potential that is necessary to open a sodium channel corresponds to an electric field of 50,000 V cm^{-1}. Therefore, the electric field needed to trigger matrix swelling is smaller than that required to open an ion channel.

In exocytosis, the secretory granule matrix expands against the cellular cytoskeleton (*5*). To see if a granule matrix subjected to an electric field could perform mechanical work, we used a miniature stress transducer to measure the force exerted by a swollen matrix (Fig. 1C) (*6*). We obtained a value of 131 ± 38 μg (mean ± SD; n = 5) (*7*); if 1 μm^2 of the granule is in contact with the stress transducer, this force translates to a pressure of ~12 bar.

Both the magnitude of the current through the matrix and the amount of swelling depended on voltage and displayed

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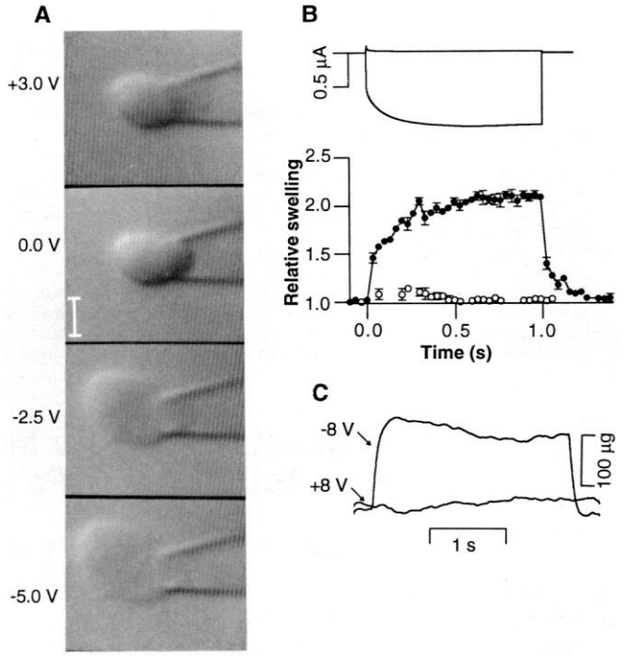

Fig. 1. Voltage-dependent changes of the physical properties of a secretory granule matrix that has been inserted into the tip of a glass pipette. (**A**) Changes in volume and refractive index in response to a voltage difference applied between the pipette interior and the bath. Bar equals 3 μm. (**B**) Time course of the changes in the current across (top) and the swelling (bottom) of a matrix in response to voltage pulses of +3 V (no current, open symbols) and −2.5 V (large inward current, closed symbols). (**C**) Time course of the force generated by a matrix that has swollen in response to a voltage pulse of −8 V. A control pulse of +8 V is also shown.

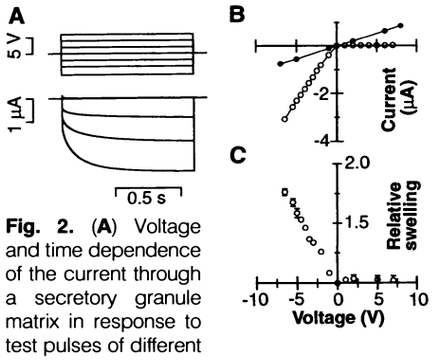

Fig. 2. (**A**) Voltage and time dependence of the current through a secretory granule matrix in response to test pulses of different amplitudes. (**B**) Steady-state current versus voltage [same axis as (C)] for the pipette alone (closed circles) and for a granule matrix (open circles). (**C**) Voltage dependence of matrix swelling (*22*).

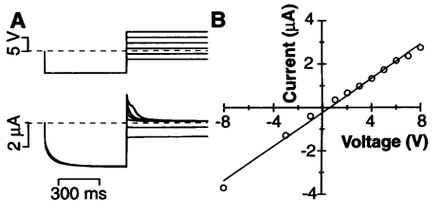

Fig. 3. Instantaneous current-voltage relation for a granule matrix. (**A**) Voltage pulse protocol (top) and current (bottom). (**B**) Instantaneous current-voltage relation (circles) for the same matrix. The straight line is a least squares fit to the data (slope = 390 nS).

strong rectification near 0 V (Fig. 2). Within the range of the negative voltages that we tested, the swelling increased with the magnitude of the voltage without saturation (Fig. 2C). However, the range of testable voltages was limited because the granule matrices, after swelling by severalfold, sometimes were pushed out of the pipette (*8*). Electrically, the matrix behaved like a diode whose conductance was 100-fold larger at negative potentials than at positive potentials (Fig. 2) (*9*). The rectification was not due to the asymmetrical placement of the granule matrix in the pipette tip because it was observed even when the granule matrix was placed symmetrically. The rectification must be characteristic of the matrix itself because, in the absence of a matrix, the pipette current was linear and ohmic at all voltages (Fig. 2B). The conductance of the pipette with a swollen granule in its tip was as much as six times that of the pipette tip alone (Fig. 2B) (*10*).

To test whether the rectification was a property of the polymer matrix or of the swelling, we decondensed the granule with the application of a negative voltage pulse of −8 V. We then changed the potential to a predetermined value and measured the instantaneous current after 2.5 ms—that is, before the granule had condensed (Fig. 3A). The conductance determined from the instantaneous slope was 390 nS, in contrast to a steady-state conductance of 510 nS for this matrix at negative potentials (Fig. 3B) (*11*). The approximate linearity of the instantaneous current-voltage relation suggested that the polymer itself did not behave as a diode but that the rectification was a consequence of the voltage-dependent swelling of the matrix.

The larger conductance of the swollen matrix, relative to that of the pipette tip alone, may result from an increased concentration of mobile cations in the matrix relative to the bulk solution. It could also be explained by a model in which ionic conduction through the granule occurs by the hopping of mobile ions from site to site within the network. Simple electrodiffusion, which is responsible for the current through the pipette alone, conforms to Ohm's law, for which the current density is proportional to the electric field. In contrast, a mechanism in which ions hop can produce a current density that is larger than that predicted by Ohm's law (*12*). Furthermore, the hopping rate increases with the free volume available at a site (*12*), which is in agreement with our observation that the conductance of the matrix is larger at negative voltages.

The response of the secretory granule matrix can be compared with the analogous response of a synthetic polymer gel. An electric field applied across a gel, in addition to generating an electrochemical cationic current, pulls the (negatively charged) polymer gel network toward the positively charged electrode (*13*). This movement of the gel appears in a granule matrix that is constrained by a pipette as swelling when the pipette potential is negative and as condensation when it is positive. For uncharged spherical gels, the time constant τ for swelling is related to the diffusion coefficient of the gel, D, and the gel radius, a, by $\tau = a^2/D$, where D is, for example, 3×10^{-7} cm^2 s^{-1} for a polyacrylamide gel in water (*14*). If we assume that the diffusion coefficient for a secretory granule matrix is similar, the matrix that we used in Fig. 1B would have a time constant of 167 ms for swelling. This is similar to the 212-ms time constant for the slow phase of swelling. However, the instantaneous swelling phase (<30 ms, Fig. 1B) is much faster than that predicted by diffusion and remains unexplained.

In studies of single exocytotic fusion events, investigators have used the patch-clamp technique to monitor the conductance of the fusion pore that is formed during exocytosis. The fact that the granule matrix behaves like a diode raises the interesting possibility that some of the observed changes in the conductance of the fusion pore (*15, 16*) reflect changes in the conducting state of the secretory granule matrix as it hydrates and goes from a condensed nonconducting state to a highly conducting state. Because decondensation is thought to be a prerequisite for content release (*1, 17*), the data suggest that release is modulated by potential gradients. These gradients could arise at the interface of the granule matrix and the fusion pore during the discharge of the granule membrane potential (*18*) and as a consequence of Donnan equilibrium (*19*).

In their response to electric fields, secretory granule matrices behave like smart synthetic polymers, which have been used as drug carriers, sensors, valves, engines, and actuators (*20*). However, the speed, size, and electrical properties of granule matrices distinguish them from other gels and make them ideal for use in the design of fast chemoelectromechanical devices.

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3. Isolated secretory granules were prepared by sonification of purified beige (bg^J/bg^J) mouse mast cells that were obtained by peritoneal lavage (*21*). Some of these granules were devoid of membranes, and others lost their membranes within a few hours after exposure to the bathing solution. Granule matrices that were devoid of an intact granular membrane were recondensed by exposure to an acidic solution that contained histamine (*1, 2*). A condensed matrix was inserted into the tip of a glass pipette with the application of suction. The pipette voltage relative to the bath was controlled by a current-to-voltage converter. In one case, we cut a piece of a swollen matrix; this piece had the same electrical properties of a whole matrix, which indicates that the properties of the matrix do not depend on remnants of the granule membrane. Furthermore, granule matrices that were pretreated with Triton-X (30% in histamine saline) gave similar results. Glass pipettes had tip resistances of 5 to 15 megohms in the histamine solution. Unless otherwise specified, the solution in both the bath and the pipette contained 12 mM histamine dichloride and 0.5 mM citric acid (pH 3.5). The current-to-voltage converter had a gain of 1.2 mV nA^{-1} and was followed by a unity-gain differential amplifier. We used an IDA interface (INDEC Systems, Sunnyvale, CA) with a COMPAQ 386-25 computer and the CCLAMP software (INDEC Systems) to apply the voltages and to sample the currents at 2.5-ms intervals. To measure the swelling, we imaged isolated granule matrices and analyzed them as described (*1, 21*).
4. We observed similar voltage-dependent behavior in all 36 granule matrices. In all cases, the time course of the current was very similar (compare Figs. 1B, 2A, and 3A). In two cases we did a frame-by-frame analysis (~45 frames per experiment) of swelling in response to a voltage pulse. The time courses were similar with an instantaneous phase (≤30 ms) and a slower phase with a time constant of 212 ms for one matrix at −2.5 V (Fig. 1) and 422 ms for the second matrix at −1.8 V. Relative swelling was calculated as A_t/A_0, where A_t is the cross-sectional area at an arbitrary time t and A_0 is the cross-sectional area 165 ms before the onset of the voltage. The data correspond to the swelling of a single granule matrix in response to a single voltage pulse. Cross-sectional areas for each frame were measured seven times, and each data point represents the mean ± SD.
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7. In several instances, the force exerted when the granule was subjected to the first voltage pulse was larger than the force exerted on subsequent pulses because the granule was pushed up and cut by the glass pipette.
8. When the granule was pushed out, we observed an abrupt decrease in current to a level that corresponded to the current through the pipette alone. These changes were reversed when the granule was brought back into the glass pipette with suction and formed a tighter electrical seal.
9. The matrix conductance changed from 4.6 ± 5 nS (range, 0.72 to 15 nS; n = 8) at positive potentials to 480 ± 40 nS (range, 440 to 560 nS; n = 8) at negative potentials. The relative conductance was, however, underestimated because we did not correct for the leak current through the pipette-matrix seal. This leak current could account for most of the current at positive voltages.
10. The pipette conductance was 130 ± 50 nS (range, 70 to 200 nS; n = 5). At negative potentials, the matrix current exceeded the pipette current by a factor of 4.21 ± 1.55 (range, 2.50 to 6.14; n = 5).
11. The mean instantaneous and steady-state conductances at negative voltages were 352 ± 48 nS (range, 280 to 400 nS; n = 4) and 470 ± 35 nS (range, 430 to 510 nS; n = 4), respectively.
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22. Relative swelling is calculated as A_V/A_0, where A_V and A_0 are the cross-sectional areas at an arbitrary voltage and at 0 V, respectively. Each point is the mean ± SD for five measurements of cross-sectional area made on the same image. We used a long depth-of-field objective for three of eight experiments. For these three experiments, the swelling had a slope that was 0.35 to 0.77 relative-swelling unit per volt. For the remaining five experiments, we used a short depth-of-field objective. The swelling had a slope of 0.061 to 0.18 relative-swelling unit per volt. On swelling, the matrix was probably going out of the plane of focus of the shorter depth objective, which would result in an apparently smaller relative swelling. The experiment shown in Fig. 2C was conducted with a short depth-of-field objective.

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Structure of the Thiamine- and Flavin-Dependent Enzyme Pyruvate Oxidase

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Pyruvate oxidase from *Lactobacillus plantarum* is a tetrameric enzyme that decarboxylates pyruvate, producing hydrogen peroxide and the energy-storage metabolite acetylphosphate. Structure determination at 2.1 angstroms showed that the cofactors thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD) are bound at the carboxyl termini of six-stranded parallel β sheets. The pyrophosphate moiety of TPP is bound to a metal ion and to a βααβ unit corresponding to an established sequence fingerprint. The spatial arrangement of TPP and FAD suggests that the oxidation of the oxyethyl intermediate does not occur by hydride displacement but rather by a two-step transfer of two electrons.

Pyruvate oxidase (E.C. 1.2.3.3) is important in the aerobic growth of lactobacteria (*1*). The enzyme (E) catalyzes the oxidative decarboxylation of pyruvate in several steps

$$\text{Pyruvate} + \text{TPP-E-FAD}_{ox} \rightleftharpoons \text{Oxyethyl-TPP-E-FAD}_{ox} + CO_2 \quad (1)$$

$$\text{Oxyethyl-TPP-E-FAD}_{ox} \rightleftharpoons \text{Acetyl-TPP-E-FAD}_{red} \quad (2)$$

$$\text{Acetyl-TPP-E-FAD}_{red} + O_2 \rightleftharpoons \text{Acetyl-TPP-E-FAD}_{ox} + H_2O_2 \quad (3)$$

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$$\text{Acetyl-TPP-E-FAD}_{ox} + P_i \rightleftharpoons \text{Acetylphosphate} + \text{TPP-E-FAD}_{ox} \quad (4)$$

where P_i is inorganic phosphate. The released energy is partially stored in acetylphosphate, which can be used by acetate kinase to convert adenosine diphosphate to adenosine triphosphate (*1*). The enzyme requires TPP, FAD, and a divalent cation such as Mn^{2+} for catalytic activity. Sequence similarity in the TPP binding region suggests that pyruvate oxidase is related to the important enzyme pyruvate decarboxylase (*2*), which, however, does not oxidize the substrate and thus lacks FAD.

Recombinant pyruvate oxidase from