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A Patch-Clamp Investigation of the *Streptococcus faecalis* Cell Membrane

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Summary. The patch-clamp technique was used to study the membrane of giant protoplasts from the gram-positive bacterium *Streptococcus faecalis*, demonstrating the presence of ion-conducting pores in the cytoplasmic membrane of procaryotes. The single channel recordings were characterized by a variety of conductances, ranging up to a few nanoSiemens, arising from stretch-activated, voltage-modulated, cooperative channels. Activation by stretch and voltage took place via both a decrease of the mean closed time and an increase of the mean open time of the channels, which are strictly controlled in intact cells, where they might constitute parts of a membrane apparatus or transport system.

Key Words stretch-activated channels · patch clamp · substates · cooperativity · gram-positive bacteria · *Streptococcus faecalis*

ABBREVIATIONS
SA: stretch-activated; SI: suction-independent; G: conductance; ΔP: pressure difference; TMA: tetramethylammonium; TEA: tetraethylammonium; MOPS: 4-morpholine-propanesulfonic acid; HEPES: 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Introduction

Despite the application of the patch-clamp technique (Hamill et al., 1981) to an ever-increasing number of biological systems, the energy-transducing membranes of mitochondria, bacteria and chloroplasts had, until recently, received scarce attention by the electrophysiologists, for technical reasons and possibly also out of the belief that channels would be incompatible with the maintenance of an electrochemical proton gradient. The patch-clamp work carried out so far on mitochondria (review: Kinnally, Antonenko & Zorov, 1992) has shown that chemiosmotic energy transduction does not imply the absence of channels, which merely need to be appropriately regulated. We report here the results of a patch-clamp investigation on the membrane of protoplasts obtained from *Streptococcus faecalis*, a gram-positive bacterium: it harbors a striking channel system which gives rise to multiple stretch-activated conductances.

A priori, there was reason to suspect that channels might be observed. The bacterial plasma membranes are rich in transport systems, since the cells must derive their livelihood from the environment. Among those whose mechanism has not yet been completely investigated are, e.g., the uptake of large molecules (iron complexes, vitamins, colicins) (Kadner, 1990; Postle, 1990; Webster, 1991), protein excretion (Holland, Blight & Kenny, 1990) and the rapid loss of solutes upon osmotic downshock (Tsapis & Kepes, 1977; Meury, Robin & Monnier-Champeix, 1985). Pore-forming proteins are suspected to play a role in these processes, at least as part of the carrier structure. Channels are also suspected to play a role in the uptake of genetic material in some transformation experiments (Hanahan, 1987) and in the entry of antibiotics into bacterial cells (Davis, 1987). A knowledge of the endogenous bacterial channels is mandatory if bacteria are to be used as vehicles for the expression and mutagenesis of channel proteins, as a convenient alternative to the current approaches based on oocytes. The presence, or lack, of channels in the plasma membrane of bacteria was also a question of some relevance from an evolutionary point of view.

With the exception of preliminary communications (Zoratti & Petronilli, 1988; Delcour et al., 1989*a*; Zoratti, Petronilli & Szabó, 1990), previous electrophysiological studies on bacterial membranes have all dealt with *Escherichia coli*, which presents the disadvantage of possessing two membranes. The Madison group first discovered a stretch-activated channel in *E. coli* (Martinac et al., 1987), and later presented evidence favoring its localization in the outer membrane (Buechner et al., 1990). Other studies have suggested instead that stretch-activated (and not) channels may be localized in the inner membrane (Berrier et al., 1989; Berrier et al., 1992). A large body of literature deals with the outer membrane porins of gram-negative organisms (Benz, 1988; Jap & Walian, 1990). *Streptococcus faecalis*,

like all gram-positive bacteria, has only one membrane, so that no ambiguity is possible. This work, therefore, establishes the existence of components of the plasma membrane of procaryotes which may act as *bona fide* channels.

Materials and Methods

Protoplast and Proteoliposome Preparation

Streptococcus faecalis ATCC9790 cells were grown to OD 0.3 in a defined-composition medium (CDM) (Roth, Shockman & Daneo-Moore, 1971). The cells (20 ml) were harvested and subjected to digestion of the cell wall with lysozyme (1.5 mg/ml, Sigma grade VI). After about 50 min of incubation at 37°C the protoplasts were diluted 10-fold in CDM + 0.25 M sucrose, and allowed to grow for about 3.5 hr, to a diameter of 2–4 μm. The protoplast suspension could be utilized for up to one week. Giant proteoliposomes were prepared from sonicated protoplasts and purified azolectin liposomes by the freeze-thaw method (Correa & Agnew, 1988). The azolectin : protein ratio (w : w) was 10–20. High-resistance seals were established on monolamellar vesicles or blebs. For electron microscopy work, one-day-old giant protoplasts were centrifuged and the pellet was fixed in 2.5% glutaraldehyde (0.1 M cacodylate buffer), post-fixed with 1% osmic acid in the same buffer, dehydrated and included in Epon 812. Thin sections were observed using a Philips EM 301.

Patch-Clamp Experiments

Pipettes were drawn from Hilgenberg 11411 glass to a tip diameter not exceeding 0.5 μm, and polished, but not coated. Pipette resistance in the typical medium was 3–5 MΩ. The success rate of seal formation was about 50%, but dropped to near zero, without an apparent reason, for a few preparations. In these cases we found that some improvement could be obtained by coating the pipette tip with hydrophobic residues by exposure to dimethyldichlorosilane or hexamethyldisilazan (Aldrich; 1% in benzene) vapors, followed by annealing at 150°C.

The protoplast suspension (20–30 μl) was deposited on the 0.17 mm-thick glass slide bottom of a homemade patch-clamp chamber fitting in the holder of a "patch-clamp tower" (List). The chamber (capacity: about 0.9 ml) was filled with the desired medium, and the protoplasts were allowed to settle to the bottom. The chamber was then extensively perfused with fresh medium. The protoplasts appeared as dark round objects. Unless otherwise specified, the medium used was symmetrical (in mM): 350 KCl, 10 $CaCl_2$, 1 $MgCl_2$, 5 HEPES/K^+, pH 7.2. Seal resistances were in the range of 1 to 10 GΩ. Protoplasts at times disintegrated spontaneously during seal formation, forming excised inside-out patches (*see also* Results). There was no noticeable difference in channel behavior between excised and cell-attached patches. A majority of the seals did not last long enough for meaningful experiments to be performed: in about 50% of cases the seal was lost upon application of stretch, in the initial phases of the experiment. Collapse of the gigohm resistance was likely to occur if voltages higher than ±60 mV were applied, so that most of our data are limited to this voltage range.

Suction was applied via a side arm of the pipette holder and measured by a mercury manometer. Pressure differences are given in units of mercury centimeters (cm Hg; 1 cm Hg = 1333.2 Pascal). The patch chamber was grounded through an agar bridge. The voltage at the pipette Ag/AgCl electrode was controlled via a List EPC-7. The output from the EPC-7 current-voltage converter was filtered by a Frequency Devices 902LPF 8-pole Bessel active filter, with corner frequencies of 3–10 KHz, and recorded on tape. Data analysis was carried out off-line using either an Indec L-11/73-70 data system with software developed in the laboratory of Dr. P. Hess, or Axon's TL-1-125 interface and pCLAMP 5.5.1 program set. Probability density histograms were fitted by a sum of exponentials using the least-squares method.

Voltages quoted in this paper are those of the bath, zero being conventionally assigned to the pipette electrode. Inward currents, i.e., cations flowing out from the pipette or anions flowing into it, are assigned a negative sign and plotted downwards.

In selectivity experiments, an I/V curve was first determined under symmetrical conditions. The chamber contents were then changed by withdrawing 800 μl of medium and adding an equal volume of the desired new bath solution, three consecutive times. The new bath medium consisted of either KCl at a different concentration (to determine P_K/P_{Cl}) or either M^+Cl^- or K^+X^- at the same concentration of the common ion as in the pipette (to determine P_K/P_M or P_{Cl}/P_X). I/V curves were determined again, and the reversal potential determined by linear regression of the data points, which always fell on a straight line in the restricted voltage range used. The permeability ratios could be calculated from the reversal potential by straightforward elaborations of the Goldman-Hodgkin-Katz equation (Hille, 1992).

Results

Protoplasts

Figure 1 shows the giant *S. faecalis* protoplasts used in this study. The outline of the protoplasts is well defined, with only occasional fuzzy segments. All the electron microscope images we obtained conformed to this example, indicating that the protoplasts conserved, at most, only isolated patches of cell wall residues.

Membrane Stretch-Sensitive Channel Activity

The mere application of a voltage difference across the membrane of a protoplast resulted in the observation of single-step, gated currents only in approximately 10% of the experiments. These are referred to below as "suction-independent" (SI) events (activity). In nearly all other cases, channel activity could be elicited by applying suction to the inside of the pipette, i.e., by stretching the membrane. Figure 2 illustrates this behavior: the application ("on") of a pressure difference of 15 cm Hg induced gating by a 2.2-nS channel, which stopped when suction was released ("off"). Activity of this type was the most frequently observed: about 60% of

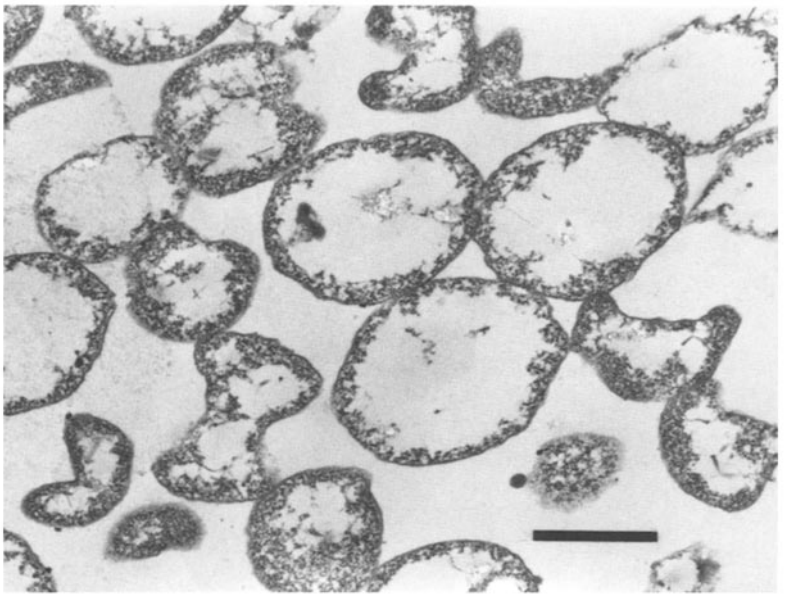

Fig. 1. EM image of *S. faecalis* giant protoplasts. Bar: 1 μm. *See* Materials and Methods for the experimental procedure followed.

patches exhibited similar SA conductances, in the range 2–3 nS. However, as we reported (Zoratti & Petronilli, 1988), many other SA conductances were also observed, and often the same membrane patch exhibited more than one. The most common other conductances measured 4–6 nS (twice the most common ones), 0.9–1.3 nS (depending on the substate occupied) and 0.30–0.35 nS. Figure 3 shows current records illustrating this behavior, from six separate experiments (*see also* Fig. 1 of Zoratti & Petronilli, 1988). Single-step conductance variations ranging from approximately 10 pS to at least 8.5 nS were observed. Since the amplifier gain was usually kept at low values, we have not gathered information on the conductances below 100 pS.

The "Initial Catastrophe"

In about 60% of cases, the stretch-induced activity was inaugurated by a sudden, fast and nearly always saturating burst of current, which we refer to as the "initial catastrophe." Examples are shown in Fig. 4*A–C*. In most (>90%) cases these events comprised some square current steps, with sizes in the range of the largest stretch-activated channels. Except for these steps, in all cases in which a catastrophe was recorded no channel openings were observed before it, even though in many cases suction was being repeatedly applied and released.

In synchrony with the electrical event, the protoplast attached to the pipette was observed to change suddenly from a dark to a lighter shade of gray or to become nearly transparent, suggesting that an equilibration of its contents with the medium had taken place. The capacitance of the patched membrane did not change appreciably, indicating that the catastrophe had not caused a transition to the whole-protoplast configuration. In about 40% of cases the initial catastrophe coincided with the destruction of the protoplast. In these cases also,the capacitance of the patched membrane did not change, suggesting that an excised inside-out patch had formed. We tentatively take these observations to indicate that suction caused a large, short-lived pathway to appear, connecting the protoplast inner compartment and the pipette interior. The considerable mechanical stress sustained by the protoplast and/or the flux of solution from the outside at times caused the protoplast to disintegrate. Given the presence of square current steps, the catastrophic event might involve the channels themselves. While the observation of channel activity after the catastrophe generally required a further increase in applied stretch, in several cases channel activity started immediately after the catastrophic event, and continued as long as suction was applied (Fig. 4*C*), or, in a few cases, also after suction was released. We have recorded three instances of a catastrophe occurring without any application of membrane stretch after seal formation. In two of these cases channel activity started with the catastrophic event and continued spontaneously afterwards. Figure 4*D* presents one of these events, distinguished by the additional unusual features of a relatively low peak conductance and long duration of the current burst. A large fraction of the protoplasts attached to the chamber bottom always turned from dark to semi-transparent in

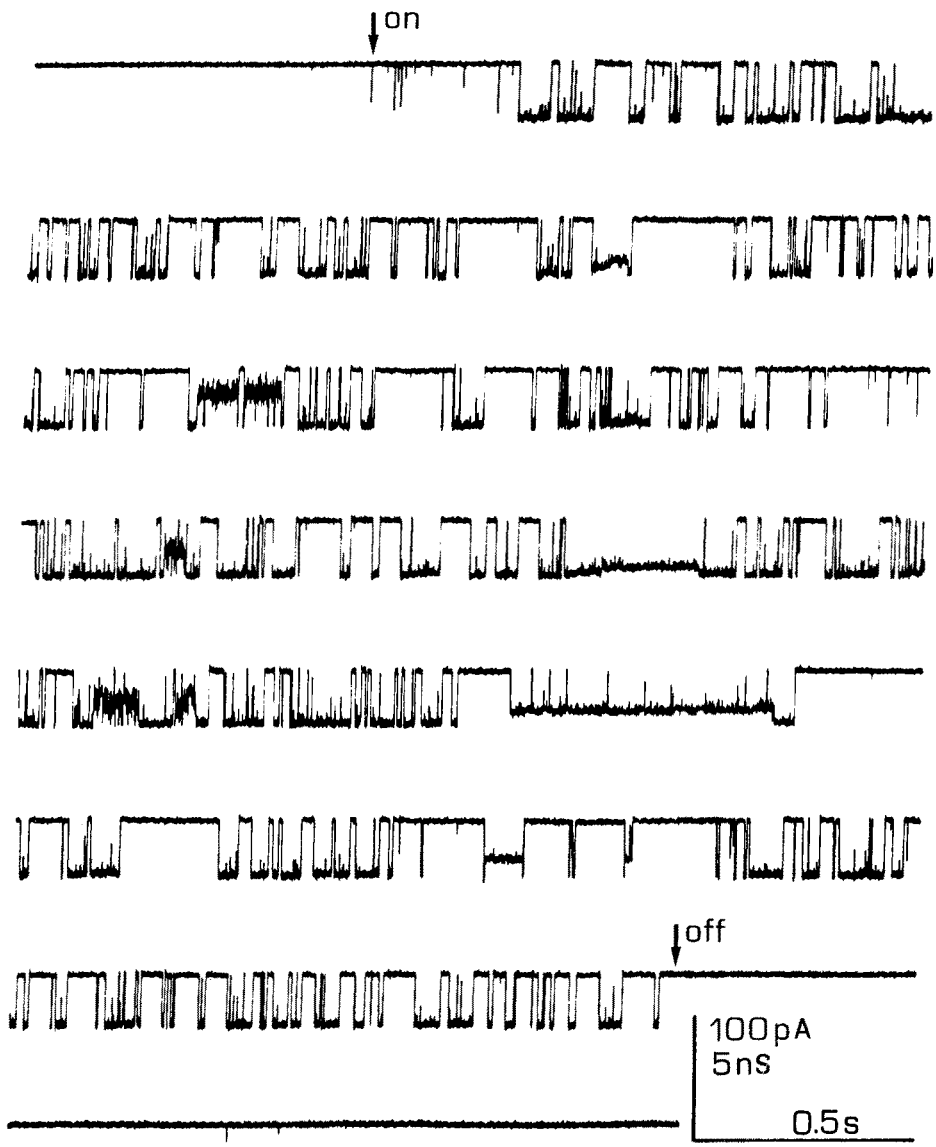

Fig. 2. An example of stretch-elicited activity. The current was recorded at −20 mV. At the moment marked by the arrows, the patch pipette was put into communication with a 3-liter glass jar held at −15 cm Hg ("on"), or with the atmosphere ("off"). Cell-attached patch. Main conductance level (*G*): 2.2 nS (*I*: 44 pA).

the course of the experiments, presumably because of spontaneous permeabilization events. High-resistance seals could not be established on them. In a few cases, we observed the dark-to-transparent transition while prodding the protoplast with the pipette in the attempt to establish a seal.

We never observed more than one initial catastrophe per protoplast. We presume that when the catastrophe was not observed, it took place before or during the formation of the high-resistance seal, before recording began. No catastrophic events, and no channel-like events, were observed in 15 control experiments on giant purified azolectin liposomes.

While the observations leave little doubt that the occurrence of the initial catastrophe and channel activity were intimately related, it is not clear whether the channels could be induced to function because some membrane structure came apart during the catastrophe, because of the loss or dilution of some cytoplasmic component, or for both reasons.

Rundown of the Stretch-Activated Channels

The stretch-induced channel activity often evolved in an instructive manner during an experiment. Figures 2, 3 and 5*A* present traces recorded from freshly

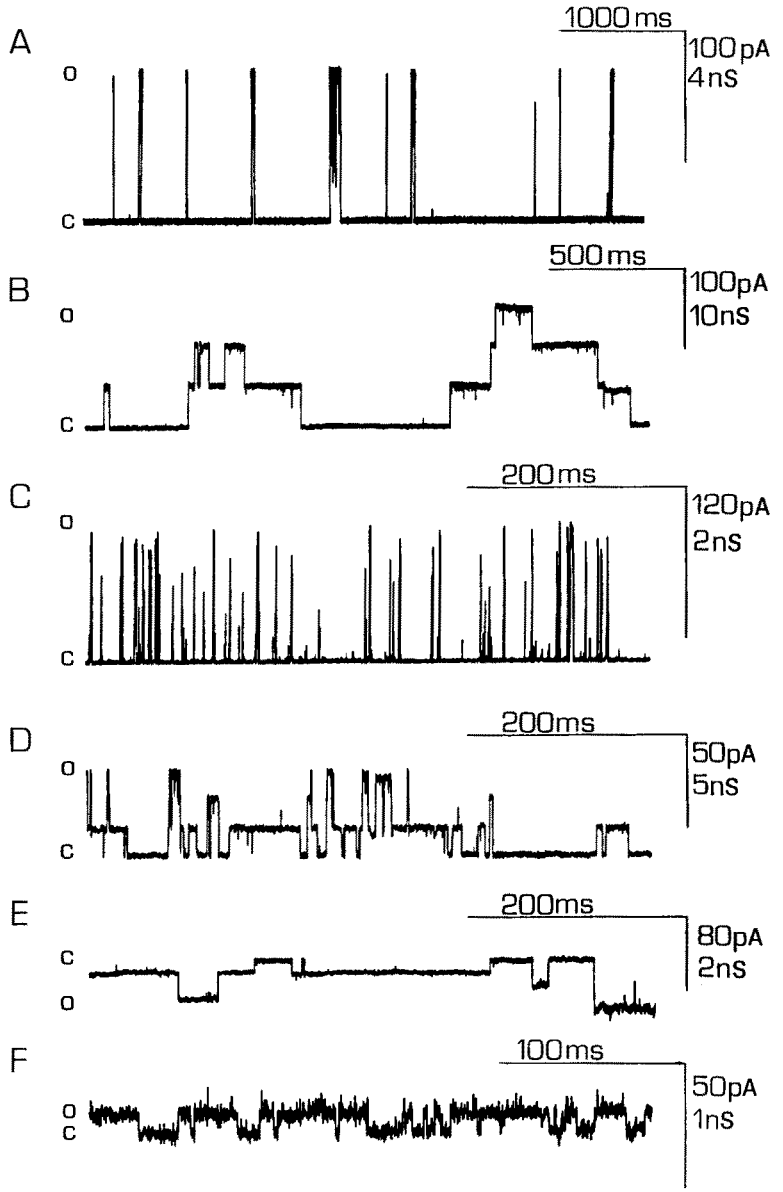

Fig. 3. Membrane stretch elicits various types of channel activity. The recordings were obtained under suction, from cell-attached (*A,B,E,F*) or excised (*C,D*) patches. The activity was strictly stretch-dependent: release of suction resulted in the closure of the channels. (*A*) ΔP: 20.5 cm Hg. *V:* 25 mV. *G:* 4.6 nS. (*B*) ΔP: 13 cm Hg. *V:* 10mV. *G:* 5 nS. (*C*) ΔP: 18 cm Hg. *V:* 60 mV. *G:* 1.7 nS. (*D*) ΔP: 10 cm Hg. *V:* 10 mV. Main conductances: 1.4, 2.9, 4.3 nS (Note the approximate 1.4 nS periodicity) (*E*) ΔP: 14 cm Hg. *V:* −40 mV. Main conductances: 0.35, 0.7 and 1.3 nS. (*F*) ΔP: 7 cm Hg. *V:* 50 mV. Conductance steps: 0.2 nS.

established patches. In "fresh" patches, activity was strictly stretch-dependent, in the sense that if suction was released, all activity ceased immediately. The continuous application of membrane stretch, the repetition of stretch/release cycles, or simply aging, induced changes in the characteristics of the stretch-elicited currents. Figure 5 shows an example of such a process. The suction-dependent high-conductance events (Fig. 5*A*) transformed themselves, one channel at a time, into lower-conductance channels (generally with different kinetic properties) until only the latter remained (Fig. 5*B*). The process often eventually led to a noisy activity of relatively low, heterogenous conductances (Fig. 5*C*), which continued to gate even if suction was released (Fig. 5*B–D*). In the example shown, the activity of these low conductances also gradually withered away (Fig. 5*D*), but could be recalled by reapplying suction (Fig. 5*E*). The original higher-conductance channels were not, however, to be seen again. In most cases, the suction-independent activity by the "daughter" channels continued indefinitely, and its characteristics could not be altered by suction. In some cases the loss of the requirement for suction was not accompanied by alterations of the conductance size on a timescale of a few minutes. This phenomenon may well reflect the persistence of membrane tension after suction was released.

The "rundown" closely parallels the phenomenon reported for *Bacillus subtilis* protoplasts (Zoratti et al., 1990). As in that case, a possible interpretation is that the high-conductance channels decay, if sub-

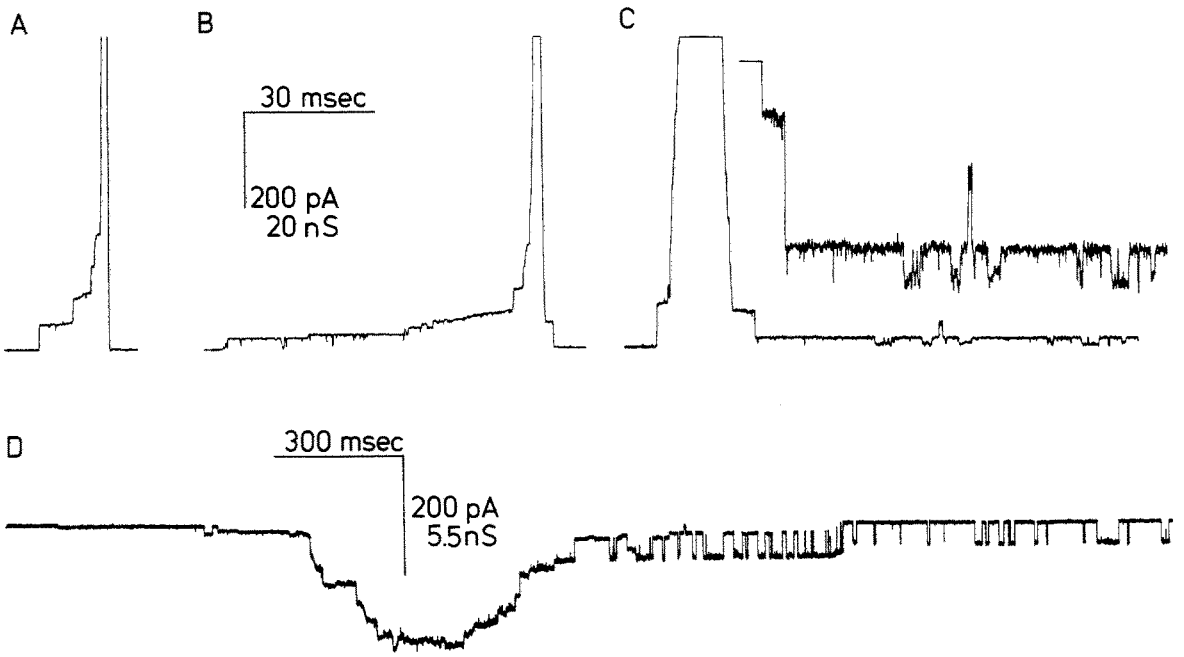

Fig. 4. Examples of "initial catastrophes." (*A*) ΔP: 10 cm Hg. *V:* 10 mV. Approx. sizes of the four recognizable conductance steps (in sequence): 5.5, 5.2, 5.8, 6.5 nS. (*B*) ΔP: 6 cm Hg. *V:* 10 mV. Conductance steps: 2.2, 0.7 and 5.4 nS. (*C*) ΔP: 15 cm Hg. *V:* 10 mV. Conductance steps: 9.1, 5.2, 1.8, 3.4 nS. Inset: amplified five-fold (*D*) *V:* −36 mV. Maximum conductance of the event: 5.6 nS. Conductance sizes: 1.6 and 1.0 nS.

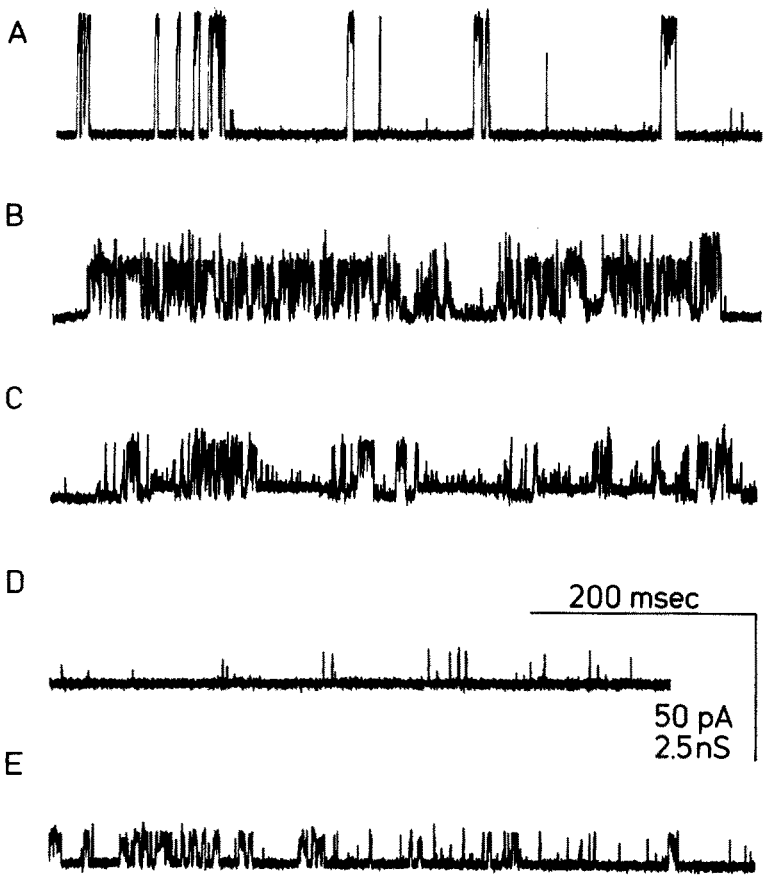

Fig. 5. An example of megachannel rundown. *V*: 20 mV. Traces recorded from the same protoplast-attached patch. (*A*) Fresh patch. Conductance: 2.5 nS. ΔP: 14 cm Hg. (*B–D*) Progressive rundown. Main conductances: 1.2, 1.6, 0.2, 0.6 nS. No suction applied. (*E*) Suction reapplied at a later time. Conductance: 0.6 nS. The activity in *E* was suction-dependent.

jected to stress, into lesser conductances with different properties. The phenomenon of decay, along with the short average duration of the high-resistance seals, made it difficult to carry out lengthy experiments appropriately. Thus, for example, it was impossible to obtain a set of curves relating open probability to applied pressure difference at various potentials.

Variability

The persistence of membrane tension after suction release may well provide the explanation for the observation of SI activity in some 10% of seals. We qualitatively observed that SI activity was more likely if the seal had been established with difficulty. Relatively low (<2 nS) conductances were more frequently observed in the case of SI activity than in suction-dependent activity, suggesting that in some cases rundown had occurred during seal establishment. The idea that SI activity was just a manifestation of the stretch-activated channels is supported by the fact that it also involved a variety of conductances, ranging up to a few nS, having the same voltage dependence as the SA activity (*see below*). Furthermore, in many cases the application of suction increased the open probability of "spontaneously" gating channels (*see below*, Fig. 11).

The channels of *S. faecalis* were not well behaved, even if the occurrence of rundown phenomena is neglected and allowance is made for the difficulty in applying quantitatively comparable membrane-stretch levels in different experiments. The channels sometimes switched between different kinetic modes, characterized by different mean open and closed times, in the absence of any changes in the operator-controlled experimental parameters. In other instances, a channel gradually increased or decreased its open probability, eventually remaining permanently open or closed. While activation by suction and positive voltages was always observed (*see below*), the amount of suction needed to elicit a given level of activity varied considerably from experiment to experiment. Much of this variability can be ascribed to differences in the patch shape and curvature from experiment to experiment (Guharay & Sachs, 1984; Milton & Caldwell, 1990; Morris, 1990; Sokabe & Sachs, 1990; Ruknudin, Song & Sachs, 1991). Other factors may be involved: the role of interactions between the channels and cell wall remnants, if any, remains to be explored; variations in the strength of the interaction may have been reflected in different activation thresholds. We also observed many instances of hysteresis in the dependence on both suction and voltage.

Despite this variability, the response to the variations of the experimental parameters studied was at least qualitatively reproducible and general. The various channels responded to stretch and to increasing voltage with an increase in their open probability, as exemplified below. In some 20% of the experiments, the channels maintained the same kinetic behavior long enough to allow meaningful quantitative data to be obtained. The experiments reported in the following sections were among these.

Modulation of Kinetic Parameters by Membrane Stretch

The determination of correlations between channel behavior and the pressure difference was complicated by the fact that the majority of patches contained more than one channel, often with different conductances. However, an increase in open probability with the applied transmembrane pressure difference was always observed, and the form of the relationship was always as described below. The small diameter of our pipette mouths (and membrane patches) may account at least in part for the high-pressure gradients (in comparison with other stretch-activated channels) needed to elicit activity. Often (but not always, *see* Fig. 2) the stretch-activated channels exhibited an apparent desensitization when the pressure difference was applied suddenly. An abrupt suction (stretch) increase elicited an immediate response (not to be confused with the initial catastrophe) after which the activity declined to a steady state (higher than before the pressure jump), in an approximately exponential process with a time constant of 0.5–0.6 sec (*not shown*). A similar phenomenon has been reported in the case of an *E. coli* channel (Buechner et al., 1990). Since the shape of the patch may sometimes be altered by a suction jump, and the time constant of the desensitization process was similar to that found by Sokabe and Sachs (1990) for the response of the curvature radius of patches to pressure difference steps, we presume that desensitization reflected the variations of geometrical parameters of the membrane patch. All the data and recordings presented, except Fig. 2, were obtained under steady-state conditions, following a slow (seconds) transition from one suction level to the other.

Pressure difference variations affected the channel open probability by altering both the mean open and the mean closed times of the channels. One representative experiment illustrating this observation is presented in Fig. 6. In that particular experiment, the patch exhibited two conductances, of 350 pS and 2.5 nS (the most commonly encountered conductance). The applied pressure difference was increased from 11 to 16 cm Hg, in steps of 1 cm Hg.

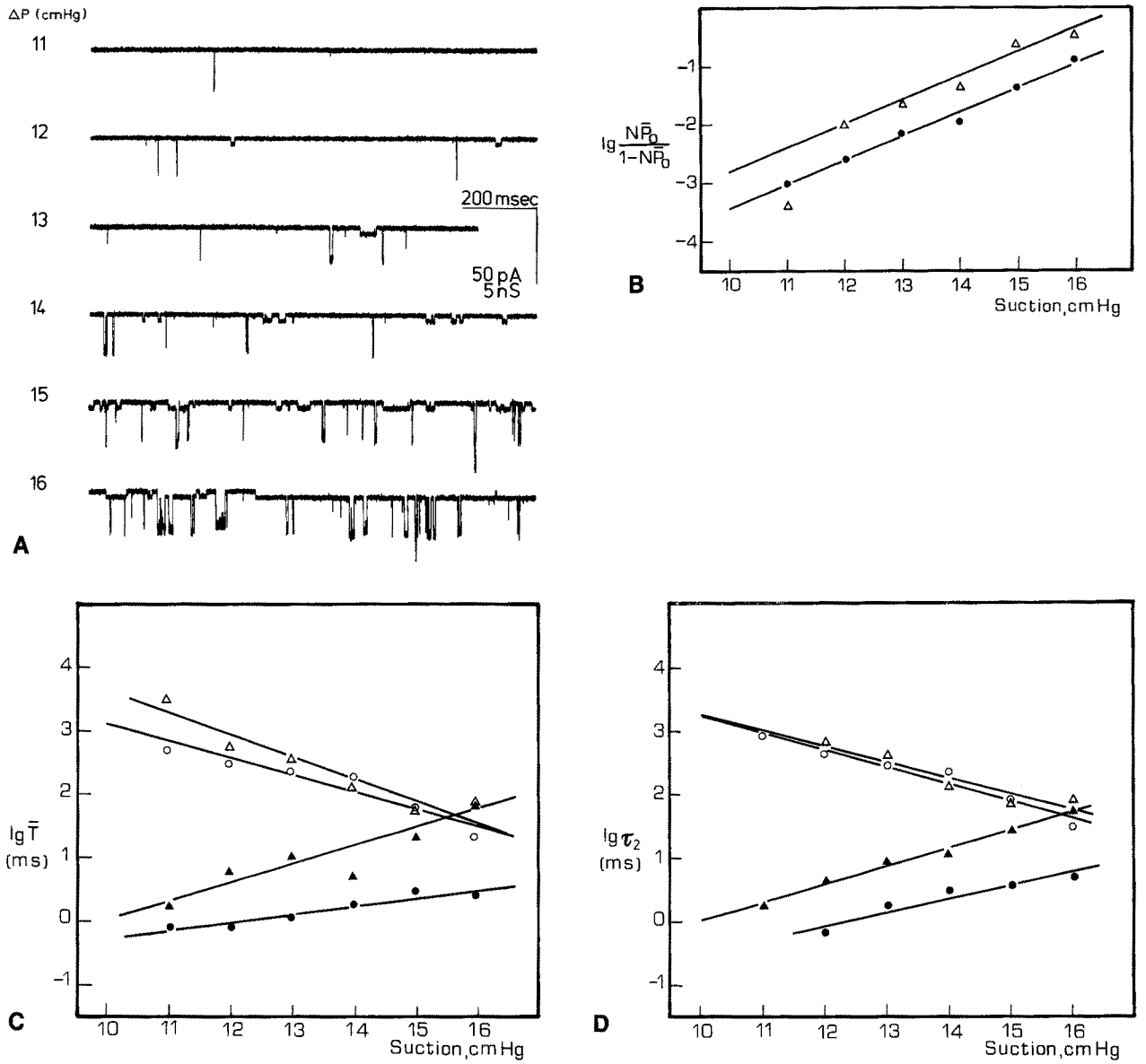

Fig. 6. Stretch affects both open and closed times. Data from one representative experiment on a protoplast-attached patch. (*A*) Exemplificative traces recorded at 11–16 cm Hg of applied suction. *V*: −10 mV. Filter 3 KHz, Dig. freq.: 10 KHz. Conductances: 2.5 and 0.35 nS. (*B*) Plot of the logarithm of the quantity $N\bar{P}o/(1\text{-}N\bar{P}o)$ for the low (△) and high (●) conductances *vs.* applied suction. A 10-fold variation occurs every 2.4 cm Hg for both conductances. (*C*) Plot of the logarithm of the open (▲, ●) and closed (△, ○) mean open times (msec) for the high (●, ○) and low (▲, △) conductances. (*D*) Plot of the logarithm of the longer time constants (msec) from the fitting of the probability density histograms. Symbols as in parts *B* and *C*.

Figure 6*B* shows the open probabilities of the two channels as a function of the applied pressure difference. The open probability increased 10-fold every 2.4 cm Hg (*e*-fold every 1.0 cm Hg) of suction for both conductances. Figure 6*C* shows the relationship between the mean open and closed (or rather "silent") times and applied transmembrane pressure difference. The probability density distributions for both conductances were adequately fitted by the sum of two exponentials. The lower time constant was below 1 msec at all pressure differences, and did not vary systematically. Its presence was due to a component of fast openings and to rapid intraburst closures. The higher time constants described the distribution of the majority of events, and varied with applied suction as shown in Fig. 6*D*. Since the patch contained an unknown number (*N*) of channels, the quantities referring to the closed ("silent") intervals merely provide a convenient way to evaluate the effect of suction. We have performed 17

determinations (13 separate experiments) of the $N\bar{P}o$ *vs.* ΔP relationship, for conductances ranging from 0.2 to 5 nS. The form of the relationships *vs.* applied suction was always as shown in Fig. 6. The change in applied suction causing a 10-fold variation of the channel activity ranged between 0.9 and 3.5 cm Hg (*e*-fold: 0.4–1.5 cm Hg), with 11 values between 1.5 and 3 cm Hg. There was no correlation with the conductance value. By comparison, the activity of the approx. 1 nS SA *E. coli* channel increased 10-fold every 1.8 cm Hg (Martinac et al., 1987).

Modulation by Voltage

Channel activity was voltage dependent: all other conditions being equal, increasing positive voltages were associated with increasing channel open probability. Figure 7 presents an experiment illustrating this behavior. The applied voltage was varied from 20 to 60 mV in 10-mV steps. The channel activity, $N\bar{P}o/(1\text{-}N\bar{P}o)$, varied 10-fold every 20 mV (*e*-fold every 8.5 mV). In 22 determinations (13 experiments) on conductances ranging from 0.2 to 3.4 nS the channel behavior always conformed to the pattern exemplified in Fig. 7. The voltage changes needed to induce a 10-fold variation in channel activity fell in the range 10–35 mV (*e*-fold: 4.3–15 mV), with 18 values in the 12–24 mV interval. Again, we did not observe any correlation with conductance size. By comparison, Martinac et al. (1987) reported, for the approx. 1 nS SA channel of *E. coli*, a 10-fold activity variation every 35 mV. As for modulation by stretch, modulation by voltage affected the mean duration of both the open and closed intervals, and, more specifically, the longer of the time constants of the two exponentials needed to fit the probability density histograms (Fig. 7).

There was no desensitization of the channels to voltage: in voltage jump experiments the open probability reached a new steady value immediately after the jump (*not shown*).

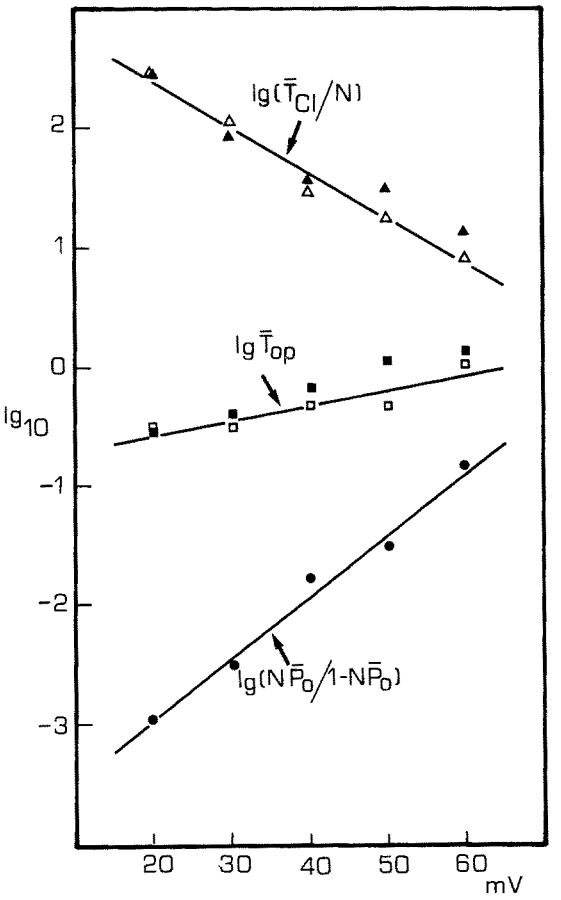

Fig. 7. Modulation of kinetic parameters by voltage. Data obtained from a protoplast-attached patch held at 21 cm Hg of pressure difference, containing an undetermined number (*N*; assumed to be 1 for calculation purposes) of 2-nS channels. The voltage was varied from 20 to 60 mV in 10-mV steps. The lines drawn represent the linear regression fit to the plots *vs.* voltage of the $\log_{10}$ of the following quantities: channel activity ($N\bar{P}o/(1 - N\bar{P}o)$) (●); mean open time expressed in milliseconds (□); mean silent interval ($\bar{T}cl/N$, in milliseconds) (△). The voltage changes needed for a 10-fold variation of these parameters were 20, 79 and 26 mV, respectively. The probability density distribution histograms for both the open and the closed (silent) times were fitted by two exponentials. The values of the longer time constants are also plotted (■: open times; ▲: silent intervals).

Substates and Cooperativity

For our purposes, we define "substates" as the different current levels of a multi-level event which could not be considered to arise from the operation of independent channels, on statistical grounds. "Cooperativity" refers to a possible explanation for the presence of substates, namely that the events in question may reflect the coordinated operation of a group of channels or channel components, and that the presence of substates may be due to the operation of subsets of these components.

Visual inspection of the current records provided ample evidence for an extensive substate structure of the giant channels of *S. faecalis*. One-step opening transitions were commonly followed by partial closures to a lower conductance (Fig. 8*A*). These visits were generally brief, but in many cases (*see* Fig. 8*C* for an example) channels were observed to function in different conductance states for seconds. Substates were ubiquitous and plethoric. Figure 8 presents a few representative recordings. Often a certain degree of regularity was evident in the spacing of the substate levels. Figure 8*B–C* presents a rapidly gating single channel which visited eight

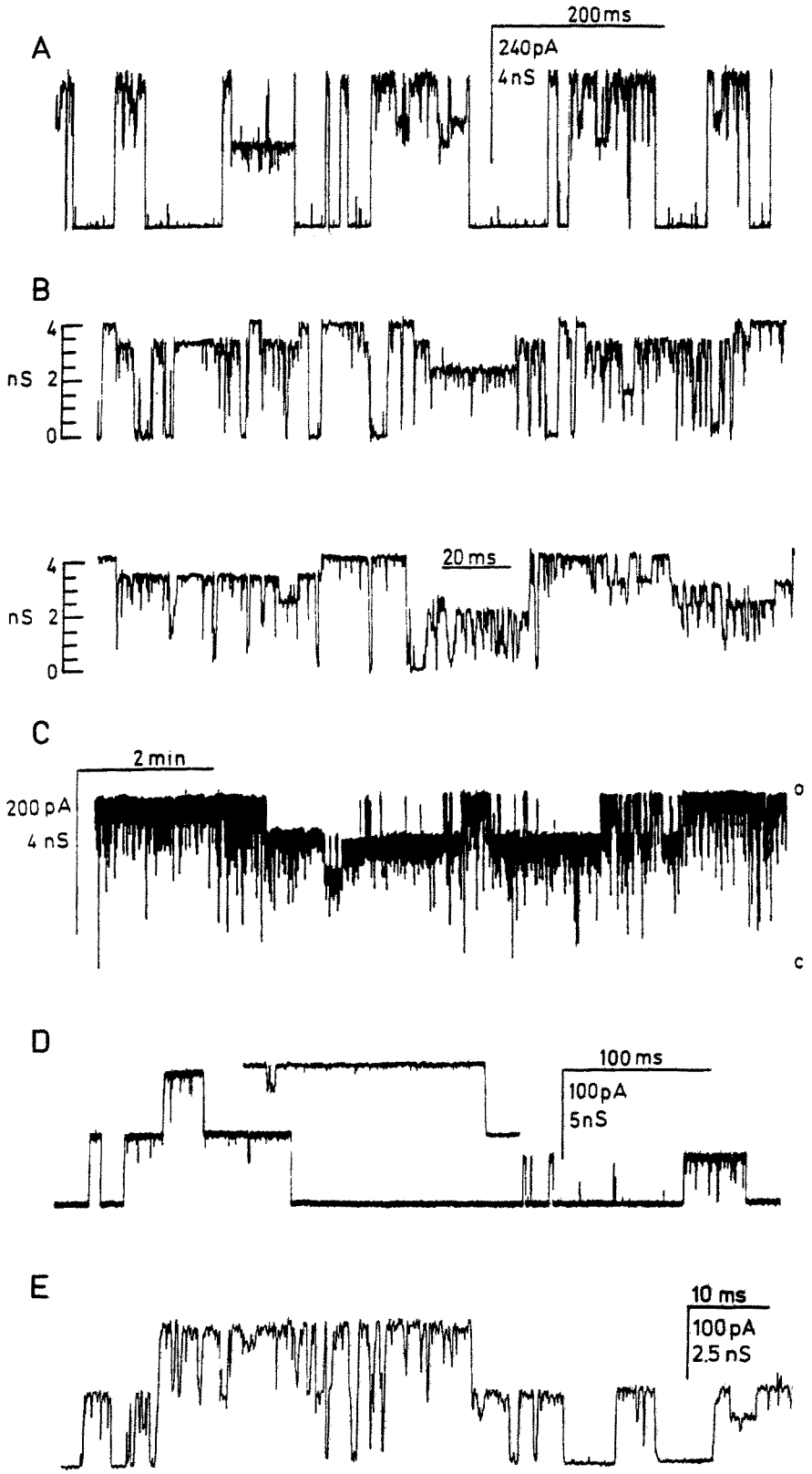

Fig. 8. Selected current traces showing evidence for a substate structure of the channels. (*A*) Excised patch. ΔP: 16 cm Hg. *V:* 60 mV. Full conductance: 4.4 nS. Major substate levels: 2.4 and 3 nS. (*B*) Composite trace illustrating the presence of at least eight regularly spaced substates in a 4-nS channel functioning "spontaneously" in a patch activated by the previous application of stretch. *V:* 50 mV. (*C*) A current plot from the same experiment as in *B*, on a compressed time scale. The major substate levels are clearly visible. (*D*) The conductance of substate levels often matched that of apparently independent, self-standing channels. In this example, a 4-nS channel (*I:* 78 pA) briefly closed to a 2.6-nS substate (*I:* 52 pA) (inset: 5× time scale). A few milliseconds later apparently independent 2.6-nS channels were observed. Protoplast-attached patch. ΔP: 18 cm Hg. *V:* 20 mV. (*E*) An example of partially coordinated gating of two channels. Several nearly simultaneous opening and closing events indicate a degree of interaction between two 2.5-nS channels (themselves endowed with substates). Protoplast-attached patch. ΔP: 12 cm Hg. *V:* 40 mV.

approximately regularly spaced levels. This suction-independent activity (in a patch previously subjected to stretch) began suddenly, in a manner not to be expected from a collection of independent channels. The activity was ascribed to what could be formally considered a single channel also because of the frequent full-amplitude nonresolvable gating events, which would not be expected if the activity were due to several channels gating independently.

Often the conductance level of these substates matched that of independent channels in the same patch, as illustrated in Fig. 8*D*. In this case, a channel briefly visited a substate (inset) at 2.6 nS, the size of events observed several milliseconds later. Records such as the one in Fig. 8*E* suggested to us that the channels may cooperate. The trace presents two conductances of equal size, possessing substates (*see, e.g.*, the right part of the figure), which gated simultaneously or nearly simultaneously in 9 out of 49 resolved gating events.

Behavior compatible with an interaction among the channels was also observed in some multi-channel patches. Figure 9 illustrates the point with reference to a specific experiment in which the patch contained at least twenty 1-nS channels operating spontaneously in an apparently independent manner. No suction was applied in this experiment after seal formation. The record on which Fig. 9 is based was taken starting 17 min after the establishment of the seal, so that presumably channel behavior was not influenced by variations in a residual membrane tension. The total current amplitude histogram (20 approximately evenly distributed peaks) can be viewed as a plot of the probability of finding a given number of channels open at any time during the recording. If the channels were truly independent, this probability would follow a binomial distribution. Vice versa, the observed distribution cannot be fitted by any value of the open probability for a single channel. The binomial distributions expected for two values of open channel probability are plotted to illustrate the point. A similar behavior has been reported for at least one other stretch-activated channel (Kirber, Walsh & Singer, 1988). This lack of conformity to the statistically predicted behavior may mean that the channels were not in fact behaving independently, but rather influenced each other. Alternatively, the patch may have contained two or more populations of independent "similar" (Mannivannan et al., 1992) channels, having the same properties except for different open probabilities. The nature of the possible interaction among channels is obscure at this point. However, an indication that the channels may be close to each other in the membrane comes from the observation illustrated in Fig. 10. When the patch exhibited activity by several channels, the average size of the current steps gradually decreased as the total current flowing through the patch increased: the more channels were already open, the lower was the apparent conductance of the ones gating. This behavior presumably reflected the overlap of the regions in which the ion concentra-

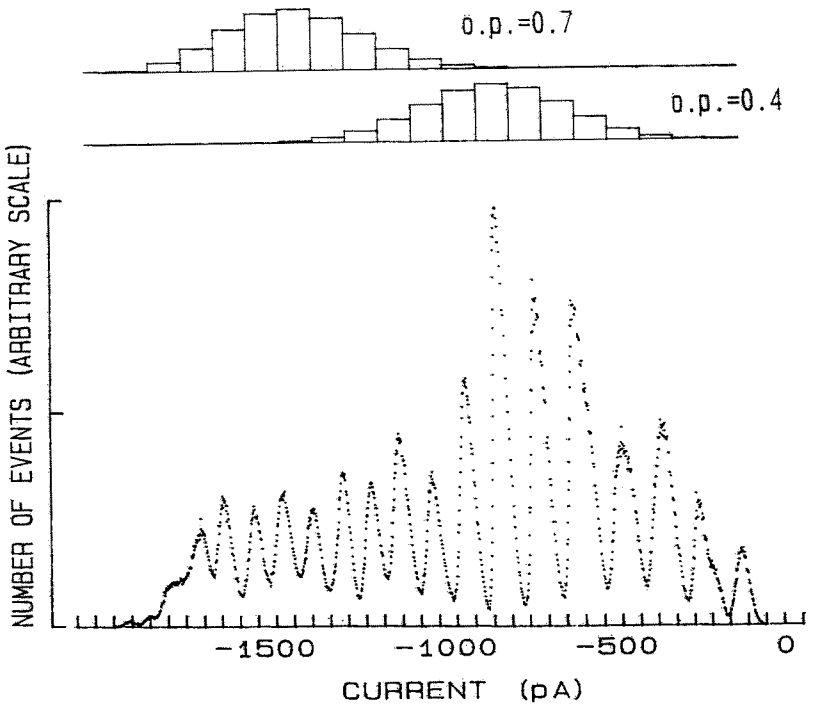

Fig. 9. Evidence for interaction between channels in a patch. Current amplitude histogram of a 2-min. stretch of spontaneous activity from a multi-channel patch. *V*: −40 mV. Inset: histograms predicted by the binomial distribution law for the indicated open probability values. *See* text for details.

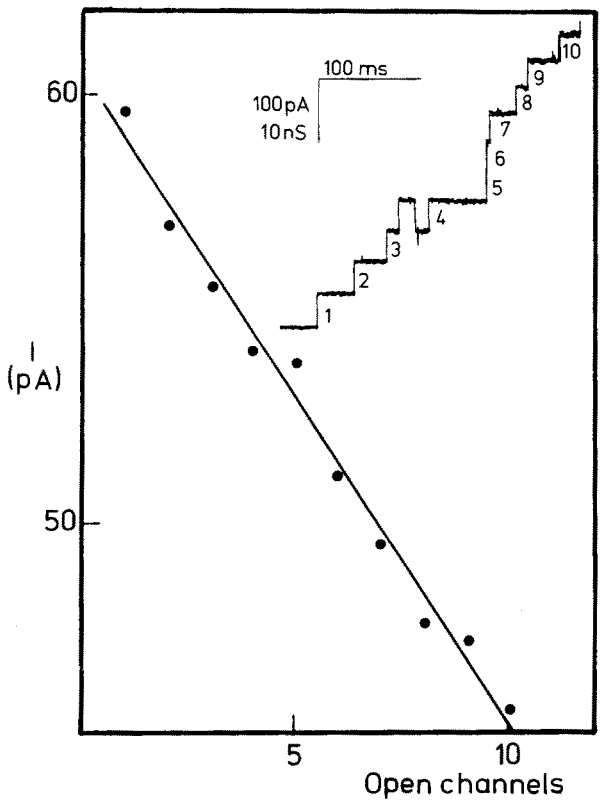

Fig. 10. Dependence of current step size on the number of open channels in a patch. The inset shows part of a stretch-elicited multi-channel event. The size of the current steps in 15 such events recorded from the same patch was determined, and averages were constructed separately for the first, second, etc., steps. The number of measurements contributing to the averages varied from 6 (10 open channels) to 29 (1 open channel). The plot of these average values *vs.* the number of open channels shows that the apparent conductance of a channel decreased with the number of channels already open in the patch. Suction (*not measured*) was repeatedly applied to elicit the multi-step events. *V:* 10 mV.

tion and voltage profiles were distorted by the presence of an ion flux through each channel. According to Laüger (1976) this distortion decreases with r_0/r, where r_0 might be identified with the radius of the pore (modeled as a cylinder) and r is the radial distance from the pore mouth. It can therefore be concluded that in the experiment presented the pores were separated, on average, by a distance not greater than several pore diameters. This behavior was not observed in all cases in which superimposed current steps were recorded. In the experiment of Fig. 9 a decrease in the apparent conductance of the channels (distance among the histogram peaks) was noticeable only when several channels were open, i.e., when high currents were flowing. The extent of the effect described above is expected to be a function of the channel open probability (as well as of the size of the cluster and of the distribution of the channels): at low $\bar{P}o$ values, any given open channel would have a low chance of being close enough to another open one to be affected by its operation (note that the values plotted in Fig. 10 are averages).

Selectivity

The large size of the conductances suggested a low selectivity. Indeed, the channels discriminated poorly among the cations and anions most commonly used in electrophysiological work. We have limited the investigation of this aspect of the channel properties to selected ionic couples for certain conductances (Table). We found evidence of discrimination only in one experiment involving a 0.4-nS conductance and the K^+/TEA ionic couple. K^+ was favored by a factor of 5 (reversal potential: 12 mV; pipette: 350 mM KCl, bath: 350 mM TEA · Cl). Unfortunately, TEA, as well as other, bulkier tetraalkylammonium cations, had a deleterious effect on seal stability, so that we were unable to gather other data using this class of compounds. In all other cases examined, the reversal potential did not exceed 2–3 mV, indicating a very low level of discrimination among the ions used.

Reincorporation into Proteoliposomes

We have performed five preliminary experiments on giant proteoliposomes. Seals were easily established on these vesicles. Gating channels were observed in four cases. The activity was practically undistinguishable from that observed in protoplasts, except for a higher occurrence of "spontaneous" activity (two out of four cases). Figure 11 shows an example of activation by stretch, resulting in an increased

Table. Lack of discimination among some common ions by several conductances

Ionic couple	Conductances (nS)	Permeability ratio
K^+/Na^+	0.26, 1.7, 2.5, 3.7	1
K^+/Cs^+	1.0, 1.7, 2.5	1
K^+/TMA	0.4, 1.6, 2.0, 2.7, 3.3	1
K^+/Ba^{2+}	1.1, 2.1	1
K^+/TEA	0.4	5
K^+/Cl^-	0.32, 0.85, 1.1, 1.7, 2.5, 2.7	1
Cl^-/AcO^-	1.0, 3.5	1

List of ionic couples compared and the conductances (measured under asymmetric conditions) for which the comparison was carried out. A permeability ratio of 1 means that the reversal potential did not exceed +/− 3 mV. *See* text for details.

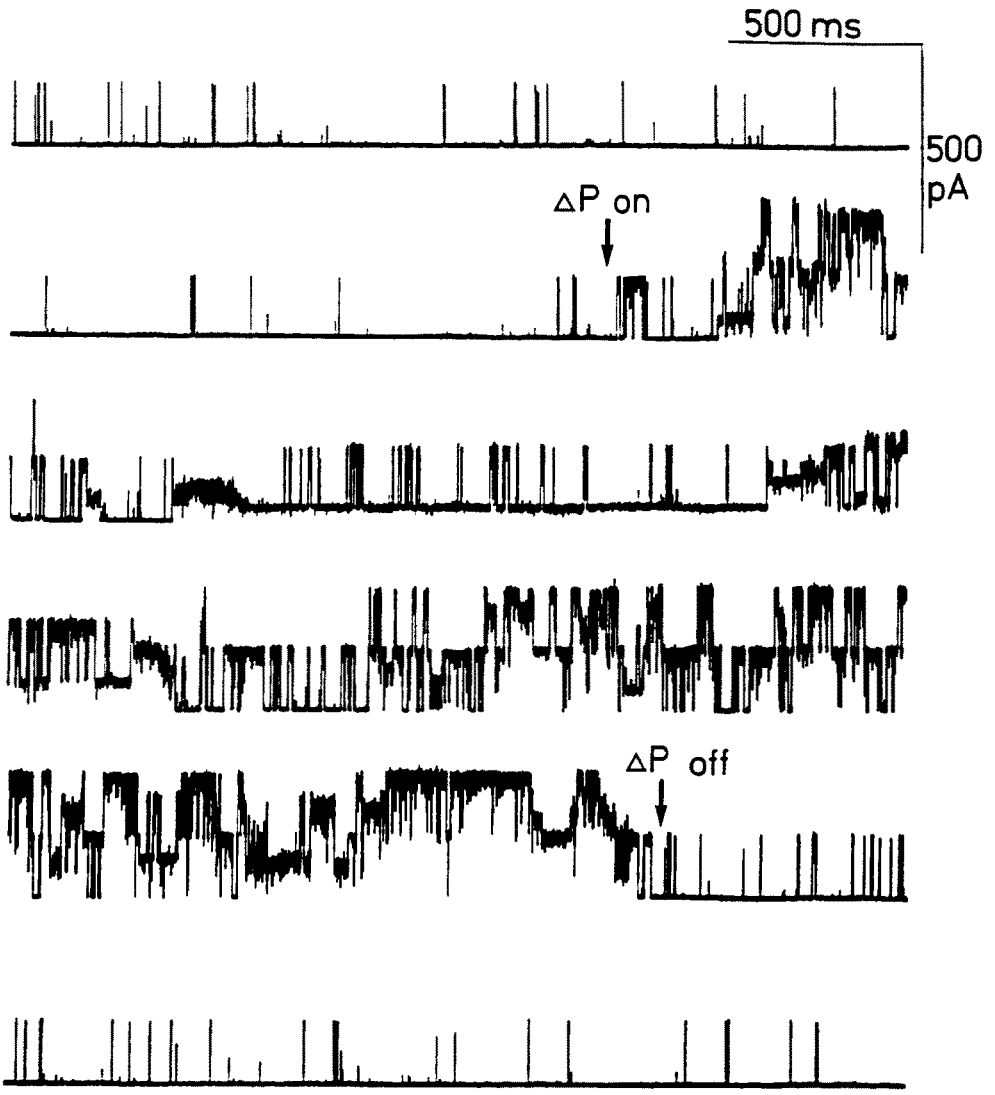

Fig. 11. Channels in proteoliposomes. Activation by stretch. Proteoliposome-attached patch. ΔP: 5 cm Hg. No suction had previously been applied to the patch (except for seal formation). *V:* 50 mV. Main conductance: 3 nS. Notice that the size of the major conductance is the same before and after the application of suction.

open probability for the same channels which were gating spontaneously before suction was applied. No channel-like events were observed in 15 control experiments on giant azolectin liposomes.

Discussion

Our observations, collected in more than 300 experiments on *S. faecalis* protoplasts, leave no doubt that, under the experimental conditions we employed, components of the membrane of this bacterium act as stretch-activated, voltage-modulated ion conducting channels. The activity is characterized by multiple, high conductances, and by the liability of the channels, whose properties often undergo drastic changes during the experiments. These pores share a common requirement for pre-activation in the "initial catastrophe." What physical event corresponds to the current burst of the catastrophe is not clear at present: it might signal the collapse of a membrane structure or apparatus to which the channels would belong. This hypothesis rests largely on the observation that the catastrophic event itself nearly always comprised a few discrete step-wise conductance changes, suggesting a relationship to the channels to be seen thereafter. One of several possible speculations may consider an interaction between the pores and remnants of the cell wall, which would restrain the channels until the interaction is destroyed.

The various conductances have important properties in common: activation by stretch and voltage, inhibition by lanthanides (Szabó et al., *in preparation*), and (within the limits of our observations) low selectivity among small ions. Our observations suggest the presence of a modular structure of the channel system, which we propose to consist of sets of interacting components, gating cooperatively (*see* Results). The abundance of conductances itself suggests that they arise from the simultaneous gating of components: it would be necessary otherwise to find a plausible reason for the presence of several distinct channels with such high conductances. The literature describes several channels formed by cooperating subunits. The multi-subunit K^+ channel studied by Geletyuk and Kazachenko (1989) is also subject to a rundown process reminiscent of our observations. Another appropriate model might be provided, e.g., by the pore-forming protein of *Entamoeba histolytica,* which has been reported to exhibit discrete substate levels as well as a smear of conductances below the maximal value (Keller et al., 1989). The pores are oligomers (Lynch, Rosenberg & Gitler, 1982), and probably possess a "barrel-stave" structure (Keller et al., 1989).

Observations similar to those concerning *S. faecalis* have been reported by us in communications on *B. subtilis* and *E. coli* (Szabó et al., 1990; Zoratti et al., 1990). The latter bacterium has also been the subject of several patch-clamp studies by the Madison (Martinac et al., 1987; Martinac, Adler & Kung, 1990; Delcour et al., 1989*b*, 1989*c*, 1991; Buechner et al., 1990) and the Orsay (Berrier et al., 1989; Berrier, 1992) groups. The approx. 1-nS channel described by Martinac et al. (1987) is stretch-activated and voltage-modulated in a manner analogous to the conductances of gram-positive bacteria. The work by the Orsay group (Berrier et al., 1989) and ours (Szabó et al., 1990), has demonstrated the presence in *E. coli* membranes of a whole array of stretch-sensitive conductances with a definite resemblance to the complex picture presented by *S. faecalis* protoplasts.

The large size of some of the conductances we observed is reminiscent of the characteristics of the porins of the outer membrane of gram-negative bacteria. The latter have been studied mainly after isolation and reconstitution in planar lipid bilayers (Benz et al., 1978; Schindler & Rosenbusch, 1978, 1981; Benz, 1988; Jap & Walian, 1990), and only now is the patch-clamp technique beginning to be applied, so far only on proteoliposomes (Delcour et al., 1989*b*, 1991; Berrier, 1992). OmpC and OmpF have been found not to be stretch-sensitive in proteoliposomes (B. Martinac and A. Ghazi, *personal communication*). Whether porins are voltage gated has been the object of debate (Jap & Walian, 1990). The emerging consensus seems to be that, in bilayers, the major *E. coli* porins tend to close at relatively high voltages of either sign (reviewed by Jap & Walian, 1990). In patch-clamp experiments on proteoliposomes, OmpC has been reported to close irreversibly at high positive voltages (Delcour et al., 1989*b*, 1991). Analogies can be found in the cooperativity exhibited by both the *S. faecalis* channels and the porins of gram-negative bacteria (Schindler & Rosenbusch, 1978, 1981; Delcour et al., 1989*b*, 1991; Berrier, 1992). Porin cooperativity is not limited to the concerted gating of the three units of the porin trimer, but may extend to clusters, which also exhibit hysteresis effects (Schindler & Rosenbusch, 1978, 1981). Other similarities include the presence of substates, and the dependence of the experimentally observed channel conductance on the size of the cluster (Schindler & Rosenbusch, 1981).

The properties of the *S. faecalis* channels are of some interest from a biophysical point of view. The activity ($N\bar{P}o/(1-N\bar{P}o)$) and the longer (interburst) time constants of the channels depend exponentially on the first power of the applied transmembrane pressure difference, i.e., of membrane tension (Guharay & Sachs, 1984; Gustin et al., 1988; Morris, 1990) (Fig. 5). A Boltzmann-type expression containing only first-power terms was found to adequately fit the $\bar{P}o$ *vs.* ΔP curves obtained from *E. coli* (Martinac et al., 1987, 1990). The literature on stretch-activated channels also contains reports of exponential dependence on the square of pressure (tension) (reviewed by Morris, 1990). Recently, Sachs and Lecar (1991) have presented a theoretical formulation including both first- and second-order terms.

We know of only two other cases in which an influence of the applied membrane stretch on both open and closed mean times has been shown (Kirber et al., 1988; Davidson, Tatakis & Averbach, 1990). The majority of the reports in the literature describe an effect of membrane tension on the interburst closed times only (reviewed by Morris, 1990). It is also relevant to note that some of the stretch-activated channels described in the literature share with the *S. faecalis* pores a behavioral instability, with apparently random variations of the gating pattern (Cooper et al., 1986; Christensen, 1987; Lansman, Hallam & Rink, 1987).

Our data do not provide information on the mechanism by which application of stretch to the membrane affects channel gating. In the case of eukaryotic cells, Sachs and coworkers favor a model envisioning an interaction with the cytoskeleton (Guharay & Sachs, 1984; Sachs & Lecar, 1991). In the case of bacteria, one would probably consider

bonds to the exoskeleton, i.e., the cell wall. In our case, the cell wall is digested away by lysozyme to transform the cells into protoplasts. The EM images show little evidence of important cell wall residues (Fig. 1), yet we cannot completely exclude that some remnants are left, or synthesized anew, and have a role in determining channel activity. The channels, however, often retain their stretch sensitivity even after fusion with (an excess of) liposomes.

The giant stretch-activated channels may well be responsible for the loss of osmolites from cells subjected to osmotic downshock, thus acting as a safety valve for the cells (Berrier et al., 1992). This may be their only task, or it might be merely a side benefit to be derived from their presence. Channel-like structures (besides porins) are suspected to exist in bacterial envelopes (*see* Introduction). A candidate might be provided, for example, by the SecY/E system (review: Wickner, Driessen & Hartl, 1991), responsible for protein traffic and localization in the bacterial envelopes, which is present in gram-positive bacteria as well (Suh et al., 1990; Koivula, Palva & Hemila, 1991). The work of Simon and Blobel (1991, 1992) has given strong support to the idea that protein translocation takes place via a channel-like structure in both eucaryotes and procaryotes.

Research on bacterial channels faces now an array of questions of relevance to the fields of microbiology and, possibly, pharmacology. How are these channels controlled in intact cells? Do they have physiological functions other than protection from osmotic downshock? Might they be normally involved in transmembrane traffic or the conduction of macromolecules? Do they provide a pathway for the entry of drugs? On a more basic level, more questions need to be answered on the properties, modulation and interactions of the pores. Interdisciplinary efforts ought to reap considerable rewards from this field.

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