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Characterization of Ion Channels from *Acetabularia* Plasma Membrane in Planar Lipid Bilayers

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Summary. Plasma membrane from *Acetabularia acetabulum* was prepared by aqueous-polymer two-phase partitioning and incorporated into planar 1-palmitoyl-2-oleoyl phosphatidylethanolamine bilayers by stirring in the presence of a (*cis : trans*) 325 : 100 mM KCl gradient. Under these conditions five distinct K^+-selective channels were observed which had unitary chord-conductances (determined between 30 mV either side of the reversal potential) and frequencies of incorporation (in parentheses) of 1,600 pS (26%), 485 pS (21%), 259 pS (53%), 140 pS (37%) and 27 pS (37%). Two Cl^--selective channels were also observed, which had unitary chord-conductances of 8 and 48 pS and were present in 21 and 16% of bilayers, respectively.

The voltage dependencies of channel open probability (P_o), open-state time constant (τ_o) and closed-state time constant (τ_c) were determined for the 259, 140 and 27 pS K^+ channels. The P_o of all three channels increased with increasingly positive membrane potentials. Thus, since these channels were oriented with their extracellular face adjacent to the *cis* chamber, which was grounded, all would exhibit outward rectification in vivo. Changes in P_o were effected by modulation of τ_c in all channels, which shortened as membrane potentials became more positive, and also of τ_o in the 140 and 27pS channels, which increased as membrane potentials became more positive.

Extracellular (*cis*) KCl concentration (and/or the KCl gradient across the bilayer) affected the P_o of all three K^+ channels, shifting the P_o/membrane potential relationship in the direction of the change in the potassium reversal potential. In all channels this was achieved largely by changes in τ_c.

Key Words *Acetabularia* · K^+ channels · kinetics · planar lipid bilayers · voltage dependence

Introduction

Passive ion fluxes across the plasma membrane of the giant, unicellular, marine green alga *Acetabularia* have been studied extensively using both radioactive tracer and electrophysiological techniques (*reviewed by* Gradmann, 1984). The most quantitatively significant passive ion fluxes are those of Cl^- and K^+. Specifically, passive channel-mediated Cl^- efflux contributes to the depolarizing current observed during action-potentials (Gradmann, Wagner & Gläsel, 1973; Gradmann & Mummert, 1980; Wendler, Zimmerman & Bentrup, 1983; Mummert & Gradmann, 1991), although we note parenthetically that during the action-potential, large amounts of Cl^- may also be released in a mode which does not affect the electrical potential of the cytoplasm, possibly via a "vesicular shuttle" (Mummert & Gradmann, 1991), and passive K^+ fluxes dominate the ionic current across the plasma membrane under conditions when primary, electrogenic ion transport is eliminated (Gradmann, 1975; Gradmann & Mummert, 1980). At the molecular level more is known about the K^+ channels than about the Cl^- channels of the *Acetabularia* plasma membrane.

Potassium fluxes, currents and channels at the plasma membrane of *Acetabularia* have been studied using radioactive tracer (Mummert & Gradmann, 1976), impaling electrode (Gradmann, 1970, 1975; Gradmann & Bentrup, 1970) and membrane-patch voltage-clamping (patch-clamping; Bertl & Gradmann, 1987; Bertl, Klieber & Gradmann, 1988) techniques. Potassium fluxes and currents exhibit a characteristic outward rectification about the equilibrium (Nernst) potential for K^+ (E_K; Gradmann, 1970, 1975; Gradmann & Bentrup, 1970; Mummert & Gradmann, 1976) and patch-clamp studies on protoplasmic droplets have indicated the presence of at least two outwardly rectifying K^+ channels, which underlie this K^+ current (Bertl & Gradmann, 1987; Bertl et al., 1988). Both channels exhibit sigmoidal unitary current-voltage relationships over a voltage range between about −100 and 100 mV and have

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maximal unitary chord-conductances of approximately 120 and 70 pS at E_K in asymmetrical (pipette: estimated cytoplasmic concentration) 130 : 400 mM K^+ (Bertl & Gradmann, 1987; Bertl et al., 1988). The dominant, 120 pS K^+ channel is present at an abundance of one per μm^2 plasma membrane and its properties qualitatively account for the monovalent cation selectivity of the *Acetabularia* plasma membrane (Bertl & Gradmann, 1987). Furthermore, the analysis of single channel recordings of the 120 pS K^+ channel has provided a kinetic model of channel activity which accounts for the steady-state outward rectification of K^+ current. The model proposes one voltage-independent open-state time constant and two closed-state time constants, of which at least the shorter one decreases with more positive membrane potentials (Bertl et al., 1988).

In this paper we have studied the properties of single ion channels from *Acetabularia* plasma membrane using an alternative electrophysiological technique based on the incorporation of purified plasma membrane vesicles into planar lipid bilayers (PLB; White & Tester, 1992*a,b*). The PLB technique offers several advantages over the patch-clamp technique (White & Tester, 1992*b*). In the long-term, the ability to control closely both the lipid and the aqueous environment in this artificial and highly reduced system, and to incorporate purified, genetically altered or synthetic channel proteins or peptides, will not only allow the biochemical study of ion channels following their isolation and reconstitution but also provide a functional assay during ion channel purification (White & Tester, 1992*b*). However, to extrapolate from PLB studies on ion channels, it is important to establish initially whether the electrical properties of ion channels in PLB, such as conductivity, selectivity and kinetics, reflect those observed in the more physiological impaling-electrode and patch-clamp studies.

Since plasma membrane vesicles prepared by aqueous-polymer two-phase partitioning are predominantly of a single orientation and incorporate into PLB in a defined manner (*see* White & Tester, 1992*b*), the orientation of plasma membrane K^+ channels in PLB can be deduced. Thus, the studies described here can be compared with previous K^+ flux (Mummert & Gradmann, 1976), impaling electrode (Gradmann, 1970, 1975; Gradmann & Bentrup, 1970) and patch-clamp (Bertl & Gradmann, 1987; Bertl et al., 1988) studies of the *Acetabularia* plasma membrane. Consistent with previous studies, the K^+ channels most frequently observed in PLB were outwardly rectifying and, although not identical to those described in patch-clamp experiments, their unitary conductances (259, 140 and 27 pS) and gating kinetics closely resembled those of the patch-clamped channels.

Materials and Methods

Biological Material

Acetabularia acetabulum (formerly *A. mediterranea*) was cultured according to Hammerling (1963) in an artificial seawater medium described by Schweiger, Dehm and Berger (1977). For mass production, 8–16 10^6 young cells were grown under a 12 : 12 hr light : dark cycle for about three weeks in rolling cultures of 5–6 liter medium (Schmid & Gieseke, 1985) to an average length of 5 mm and a total chlorophyll quantity of 20–45 mg. Before the isolation of plasma membrane, the cells were collected in a nylon mesh and washed once in fresh culture medium.

Plasma Membrane Isolation

Plasma membrane was isolated by aqueous-polymer two-phase partitioning of a microsomal fraction obtained by differential centrifugation by the method of Smahel, Klieber and Gradmann (1992). Washed cells were resuspended in 100 ml of a homogenization medium containing (mM) 250 sucrose, 50 NaCl, 1 $MgCl_2$, 2 dithiothreitol (DTT), 0.5 phenylmethylsulfonylfluoride (PMSF) and 50 N-morpholinopropanesulfonic acid (MOPS) titrated to pH 7.2 with NaOH. The cells were disrupted by N_2-cavitation according to Goldfarb and Gradmann (1983) but the method was repeated twice at a pressure of 10 MPa and in the absence of EDTA. Cell debris and intact organelles were removed from the homogenate by centrifugation at 450 × *g* for 5 min and at 4,500 × *g* for 10 min, respectively. Microsomal membranes were then pelleted by centrifugation at 80,000 × *g* for 30 min and resuspended in less than 5 ml of a buffer containing (mM) 250 sucrose, 2 KCl and 5 potassium phosphate, pH 7.8, using a hand-held glass/glass homogenizer. Microsomal membranes were applied to an aqueous-polymer two-phase system to yield a 24 g system with a final concentration of 6.3% (w/w) polyethylene glycol 3350 (PEG), 6.3% (w/w) dextran T500, 2 mM KCl, 250 mM sucrose and 5 mM potassium phosphate buffer, pH 7.8. To enrich the plasma membrane vesicles, a batch procedure was adopted and membranes were partitioned three times, with successive replacement of the lower, dextran phase. The final PEG phase (U_3) was diluted about sixfold with a buffer containing (mM) 500 KCl, 5 Na_2EDTA, 2 DTT and 10 MOPS titrated to pH 7.2 with KOH, and membranes were pelleted by centrifugation at 110,000 × *g* for 90 min. The final plasma membrane fraction was resuspended at a concentration of 1 mg membrane protein ml^{-1} in a medium containing 5 mM N-tris-[hydroxymethyl]-methyl-2-aminoethane sulfonic acid (Tes), titrated to pH 7.5 using N-methyl-D-glucamine (NMDG). All preparative steps were performed at 0 to 4°C using precooled solutions. Plasma membrane preparations were stored at −70°C without loss of activity.

A detailed analysis of the ATPase activities associated with the *Acetabularia* plasma membrane fraction prepared by the method used in this paper will be described elsewhere (Smahel *et al.*, 1992). However, it is important to note here that the membrane fraction used for the present studies was substantially enriched in vanadate-sensitive ATPase activity (approximately 10-fold above the microsomal fraction), which accounted for about 70% of the total ATPase activity of the fraction. In addition, there was little inhibition of ATPase activity by NaN_3 suggesting the absence of appreciable mitochondrial contamination.

Incorporation of Plasma Membrane Vesicles into Planar Lipid Bilayers

Plasma membrane vesicles were incorporated into planar lipid bilayers (PLB) as described previously (White & Tester, 1992*a*). Briefly, PLB were painted across a 0.2 mm diameter hole in the wall of a styrene copolymer cup from a decane dispersion of 30 mM synthetic 1-palmitoyl-2-oleoyl phosphatidylethanolamine (PE: Avanti Polar Lipids). The bilayer separated solution volumes of 800 μl in the styrene copolymer cup (*cis* side) and 5 ml in the outer perspex chamber (*trans* side). All solutions were Millipore filtered (pore diameter 0.2 μm). Aqueous solutions were buffered with 5 mM Tes, titrated to pH 7.5 using NMDG. Experiments were performed at room temperature (about 20 to 23°C). Plasma membrane vesicles from *Acetabularia* were added to the *cis* chamber (final concentration 25 to 75 μg membrane protein ml^{-1}) and incorporated into PLB by stirring in the presence of a (*cis : trans*) 325 : 100 mM KCl gradient. Once channels were detected, unfused vesicles were thoroughly perfused out within one minute, using 30 chamber volumes of 100 mM KCl.

Electrical Recordings

Currents were measured under voltage-clamp conditions using a low noise operational amplifier with frequency compensation connected to the bilayer chambers by calomel electrodes and 3 M KCl salt bridges (White & Tester, 1992*a*). To conform with traditional conventions, membrane voltage has been referenced *trans* with respect to *cis*. Thus, since plasma membrane vesicles prepared by aqueous-polymer two-phase partitioning are incorporated into PLB with their cytoplasmic face adjacent to the *trans* chamber (White & Tester, 1992*a,b*), the cited membrane voltage equals the cytoplasmic potential minus the noncytoplasmic potential. Movement of K^+ from the *trans* (cytoplasmic) to the *cis* (extracellular) chamber is indicated by a positive current and appears as an upward deflection in current traces. Data were stored on video tape after digitizing by a Sony audio to digital converter (PCM-701ES, 22 kHz per channel; Sony, Japan) and simultaneously displayed on an oscilloscope (Gould 1602, Gould Electronics, Hainault, Essex, UK).

Data Analysis

Data were analyzed using a microcomputer-based system after appropriate digitization (Cambridge Electronic Design, Cambridge, UK). In general, channel records were replayed, filtered at a corner frequency (−3 db) between 100 Hz and 5 kHz using a 5-pole low-pass Butterworth filter (LPF30; World Precision Instruments, New Haven, CT). For numerical analysis of channel open- and closed-duration time constants, records were sampled at between 500 Hz and 25 kHz. The amplitude of single channel currents was determined either directly from channel recordings, or from composite current-frequency distributions. Typically, channel open probabilities and open- and closed-duration time constants were determined from single channel recordings of between 1 and 5 min duration by the 50% threshold crossing method (Colquhoun, 1987). The transition between open- and closed-channel conductance states was assumed to occur at a value 50% of the unitary current and the duration of the opening or closure was calculated as the time between two such events. The open probability (P_o) was calculated either by summation of open durations, as the total time above the 50% current threshold divided by the total period of the recording, or, at membrane potentials close to the current reversal potential, from peak areas of composite current-frequency distributions. Thus, P_o was determined independently of open- and closed-duration time constants. The relationship between P_o and membrane potential was fitted to a Boltzmann equation of the form:

$$P_o = (1 + \exp(zF/RT)\,(V_o - V))^{-1}$$

where z is the hypothetical gating charge of the channel, F is Faraday's constant, R is the gas constant, T is the absolute temperature, V_o is the voltage at which P_o is half-maximal and V is the membrane potential. Open- and closed-duration time constants were determined by single (or multiple if necessary) exponential regressions of the frequency distributions of channel open- and closed-durations (*cf.* White & Tester, 1992*a*). Ionic activities were calculated using the coefficients supplied by Robinson and Stokes (1959) or Tamamushi and Goto (1970). All the experiments described in this paper were performed at least twice.

Abbreviations

P_o = probability of the channel being open; τ_c = apparent mean lifetime of the channel closed state; τ_o = apparent mean lifetime of the channel open state.

Results

Unitary Conductance and Selectivity of Ion Channels

When plasma membrane vesicles from *Acetabularia* were incorporated into PE bilayers in the presence of asymmetrical (*cis : trans*) 325 : 100 mM KCl, several distinct current transitions, differing in amplitude, were detected at the same holding potential. Current transitions typifying the most common ion channel activities are illustrated in Fig. 1. Unitary current/voltage curves were constructed from the amplitude of single (open channel) transitions (Figs. 2–4). The most frequently observed channel activities were more permeable to K^+ than to Cl^-, as indicated by their current reversal potentials (Figs. 2–4, Table). In the absence of data on the cationic specificity of these channels we refer to them here as "K^+-selective," but note that they may have a greater permeability to another (untested) cation. The three dominant K^+-selective channels had mean unitary chord-conductances (determined between 30 mV either side of the reversal potential) of 259, 140 and 27 pS (Table). The 259 pS channel was observed in 53% and both the 140 and 27 pS channels in 37% of a total of 19 bilayers. Two further K^+-selective channels were observed, which had extremely high mean unitary conductances of 1,600 and 485 pS and

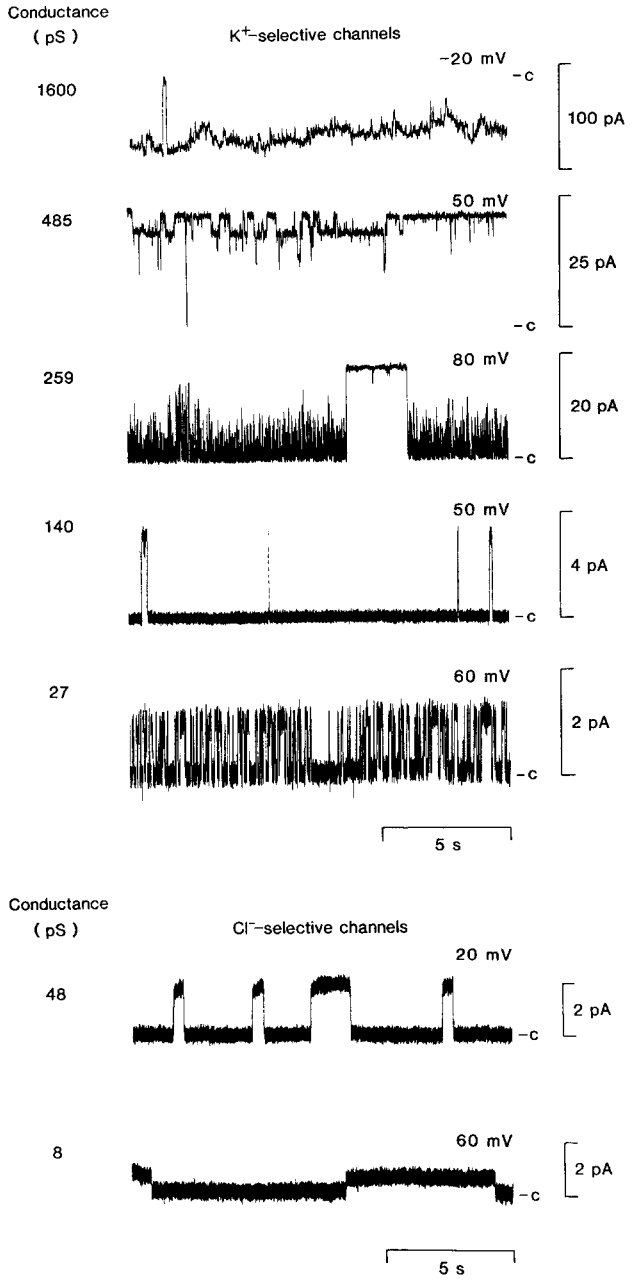

Fig. 1. Examples of characteristic single channel records of seven different channel types obtained when plasma membrane vesicles from *Acetabularia* were incorporated into PE bilayers. Recordings, which were filtered at 100 Hz, were made at the membrane potentials indicated in separate bilayer experiments in the presence of asymmetrical (*cis : trans*) 325 : 100 mM KCl. Conductances represent mean values determined between 30 mV either side of the reversal potential.

were present in 26 and 21% of bilayers, respectively. The 485 pS channel exhibited several distinct substates (Fig. 1).

The 1,600 and 485 pS K^+ channels (Fig. 2) exhibited almost linear unitary current/voltage relationships in asymmetrical (*cis : trans*) 325 : 100 mM KCl. However, the 259, 140 and 27 pS K^+ channels had nonlinear unitary current/voltage relationships, their unitary conductances increasing with more positive membrane potentials (Fig. 3). This phenomenon was not a consequence of electrodiffusion limitations since it was also observed in symmetrical 100 mM KCl.

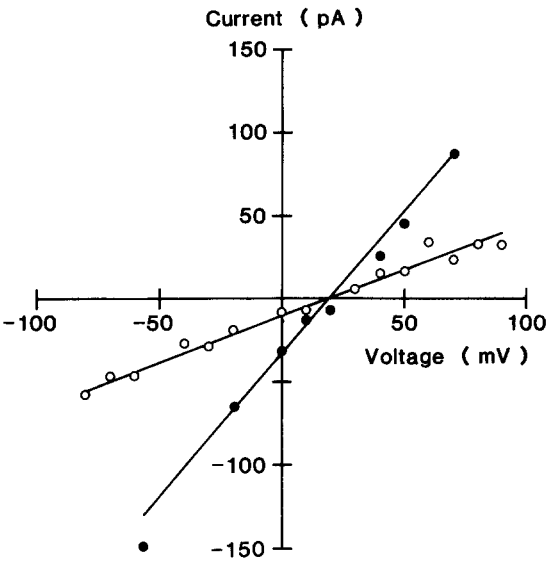

Fig. 2. The effect of membrane potential on the unitary current of two high-conductance K^+-selective channel types from *Acetabularia* plasma membrane incorporated into planar PE bilayers. Solutions contained asymmetrical (*cis : trans*) 325 : 100 mM KCl. Experiments were performed at 21°C and the K^+ reversal potential (E_K) for ideal selectivity was 27 mV. Data are from a single representative experiment for each channel. Mean unitary chord-conductances (determined between 50 and −10 mV) for these channels were 1,600 pS (●) and 485 pS (○).

Two Cl^--selective channels were observed (Fig. 4, Table). These had mean unitary conductances of 8 and 48 pS in asymmetrical 325 : 100 mM KCl and occurred in 21 and 16% of bilayers, respectively. Both these channels had linear unitary current/voltage relationships.

All channels (both K^+ and Cl^-) of a similar conductance grouping behaved identically in PE bilayers: They had similar selectivity, rectification and ion channel gating kinetics (e.g., Fig. 8). This implies that each channel type incorporated into PE bilayers in a defined orientation. Presumably, since plasma membrane vesicles prepared by aqueous-polymer two-phase partitioning have their cytoplasmic face predominantly internalized (Larsson, Widell & Kjellbom, 1987) and vesicles fuse with a bilayer such that the inside of the vesicle becomes exposed to the *trans* chamber, the ion channels of the plasma membrane are orientated with their cytoplasmic face exposed to the *trans* chamber in PLB (White & Tes-

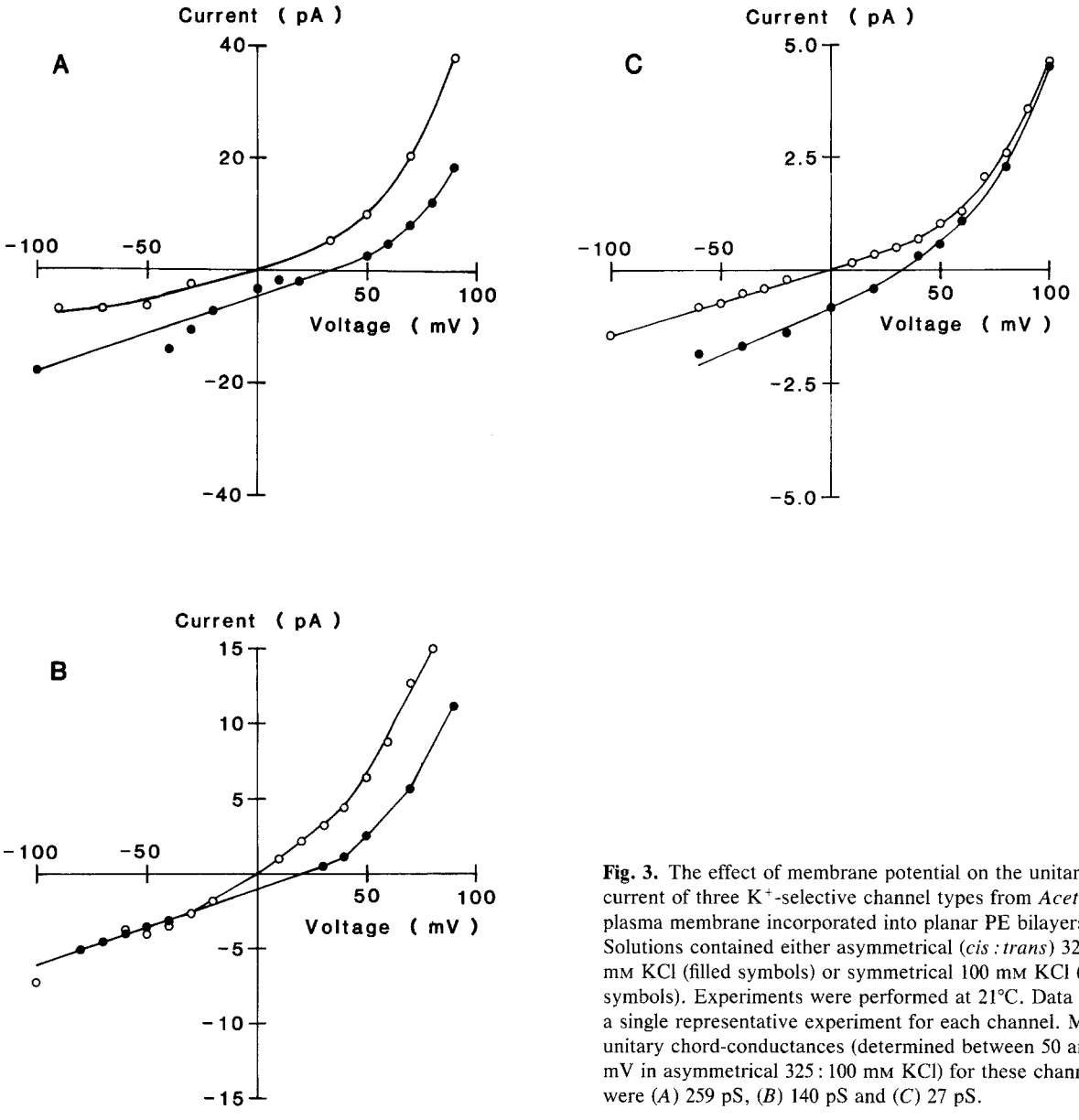

Fig. 3. The effect of membrane potential on the unitary current of three K^+-selective channel types from *Acetabularia* plasma membrane incorporated into planar PE bilayers. Solutions contained either asymmetrical (*cis : trans*) 325 : 100 mM KCl (filled symbols) or symmetrical 100 mM KCl (open symbols). Experiments were performed at 21°C. Data are from a single representative experiment for each channel. Mean unitary chord-conductances (determined between 50 and −10 mV in asymmetrical 325 : 100 mM KCl) for these channels were (*A*) 259 pS, (*B*) 140 pS and (*C*) 27 pS.

ter, 1992*b*). Since membrane potentials were recorded with reference to the *cis* chamber, we can surmise that the 259, 140 and 27 pS K^+ channels would all exhibit outwardly rectified unitary currents in vivo.

Gating Kinetics of K^+ Channels

Single channel recordings were regularly obtained for the most frequently observed K^+-selective channels (e.g., Figs. 1, 5–8). Thus, it was possible to determine the gating kinetics of the 259, 140 and 27 pS K^+ channels.

Gating Kinetics of the 259 pS K^+ Channel

The 259 pS channel showed bursting kinetics, often having long periods of closure interspersed with periods of rapid opening and closing (Fig. 5). No obvious subconductance states were observed for this channel. No analysis was made of the closed durations between bursts of channel activity, but the probability of the channel being in an open state (P_o) during an opening burst was voltage dependent (Fig. 6*D*). In asymmetrical 325 : 100 mM KCl, P_o decreased approximately as a Nernstian exponential function (a factor 0.1 per 58 mV) as more negative membrane potentials were applied from a maximal

Table. The unitary conductance and permeability of ion channels from *Acetabularia* plasma membrane and their frequency of incorporation into PE bilayers[a]

KCl (*cis* : *trans*) 325 : 100 mM				100 : 100 mM	
Conductance (pS)	E_{rev} (mV)		P_{Cl^-}/P_{K^+}	Conductance (pS)	Frequency (%)
	Mean	Range			
1,600 ± 50 (4)	19.0	(2)	0.15	ND	26
485 ± 23 (4)	19.3,	14 to 25 (3)	0.15	ND	21
259 ± 19 (9)	31.0	(2)	0.00	149 (2)	53
140 ± 17 (5)	24.0,	22 to 34 (3)	0.05	102 (2)	37
27 ± 1 (7)	27.3,	22 to 33 (4)	0.00	15 (2)	37
48 ± 2 (3)	−20.0	(2)	7.63	ND	21
8 ± 2 (4)	−13.3,	−5 to −25 (3)	3.21	ND	16

[a] Unitary conductance was determined as a chord-conductance between 30 mV either side of the reversal potential. The permeability ratio P_{Cl^-}/P_{K^+} was calculated using the Goldman-Hodgkin-Katz equation from the zero-current potential (E_{rev}) in asymmetrical 325 : 100 mM KCl. The theoretical E_{rev} for ideal K^+ selectivity was 27 mV and for ideal Cl^- selectivity −27 mV in asymmetrical 325 : 100 mM KCl. Results are expressed as means ± SE (for conductances) and means with ranges for (E_{rev}) from the number of determinations in parentheses.
ND: not determined.

P_o of about 0.076 at 90 mV. Fitting a Boltzmann equation to these data yielded values for the hypothetical gating charge of this channel in asymmetrical 325 : 100 mM KCl of one and an estimate of 170 mV for the voltage at which P_o was half-maximal. This channel exhibited extremely rapid transitions between open and closed states, which could not be resolved temporally even when filtered at 5 kHz. Thus, for the sake of simplicity, in the analysis of this channel actual series of short openings and closures were forced to appear as continuous closures by low-pass filtering (100 Hz). This approach was also taken in previous analyses of K^+ channels of the *Acetabularia* plasma membrane recorded using patch-clamp techniques, with which the results presented here will be compared (Bertl et al., 1988). Although this simplification provides an apparently consistent approach, for a more exact treatment of the kinetics of this channel the fast events should not simply be ignored (Bertl et al., 1988). Under the filtering constraint of our analysis, the decrease in P_o with increasingly negative membrane potentials could be attributed to an exponential increase in the mean lifetime of the apparent channel closed state (τ_c) as the membrane potential became more negative, rather than to an effect of membrane potential on the mean lifetime of the most frequently observed apparent channel open state (τ_{o1}), which was about 3.9 msec when filtered at 100 Hz and apparently independent of voltage (Fig. 6*E*). But the apparent voltage insensitivity of τ_{o1} might be an artifact imposed by the filtering constraint. Furthermore, at membrane potentials more positive than about 60 mV, a second, rarely observed open state with a mean lifetime (τ_{o2}) between 50 and 200 msec was also observed, but could not be determined accurately due to a lack of sufficiently detailed data. This slower τ_{o2} accounts for the deviation between the observed and calculated τ_{o1}, which is most evident at extreme positive membrane potentials (Fig. 6*E*).

The gating kinetics of the 259 K^+ channel was sensitive to the KCl concentration in the *cis* chamber (Fig. 6). In symmetrical 100 mM KCl, P_o was not only greater at any given voltage but also less voltage dependent than in asymmetrical 325 : 100 mM KCl. Fitting a Boltzmann equation to data obtained in symmetrical 100 mM KCl yielded estimates of 0.42 for the hypothetical gating charge and 237 mV for the voltage at which P_o was half-maximal. The greater P_o could be attributed to: (i) a higher apparent τ_{o1} in symmetrical 100 mM KCl, which was apparently voltage independent and about 7.2 msec when recordings were filtered at 100 Hz (and also greater than that observed in 325 : 100 mM KCl when recordings were filtered at 5 kHz, *data not shown*); and (ii) an apparent τ_c value which was an order of magnitude lower than that observed in asymmetrical conditions (Fig. 6*E*). The lower voltage dependency of P_o in symmetrical 100 mM KCl was a consequence of a reduced voltage dependency of τ_c.

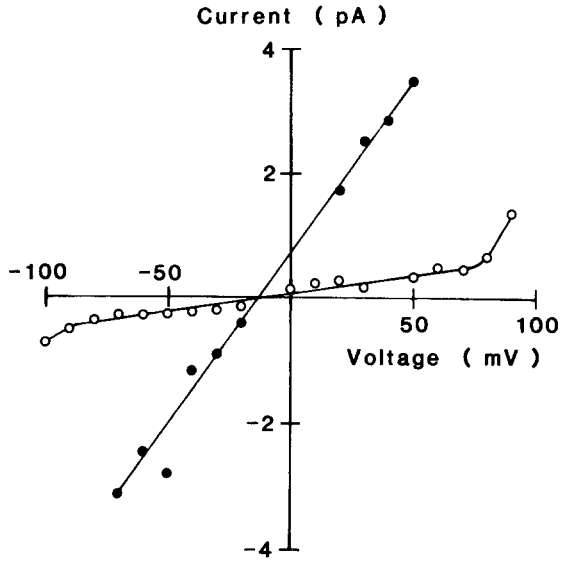

Fig. 4. The effect of membrane potential on the unitary current of two Cl^--selective channel types from *Acetabularia* plasma membrane incorporated into planar PE bilayers. Solutions contained asymmetrical (*cis : trans*) 325 : 100 mM KCl and experiments were performed at 21°C. The Cl^- reversal potential (E_{Cl}) for ideal selectivity was −27 mV. Data are from a single representative experiment for each channel. Mean unitary chord-conductances (determined between 10 and −50 mV) for these channels were 8 pS (○) and 48 pS (●).

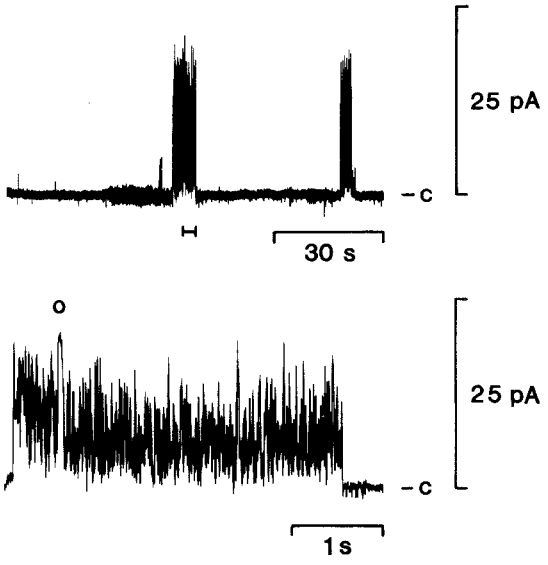

Fig. 5. Single channel recordings of the 259 pS K^+ channel from *Acetabularia* plasma membrane in asymmetrical 325 : 100 mM KCl at a membrane potential of 90 mV. The top recording illustrates the bursting kinetics of this channel. The bottom record, corresponding to the period indicated by the bar in the top recording, illustrates the discrete square jumps occurring within a burst (○) which are observed at a higher temporal resolution. Recordings were filtered at 100 Hz.

Gating Kinetics of the 140 pS K^+ Channel

The gating kinetics of the 140 pS K^+ channel were determined in detail only in symmetrical 100 mM KCl (Fig. 7). The channel P_o exhibited saturation approaching $P_o = 1$ at high positive membrane potentials (greater than about 100 mV; Fig. 7*D*). At membrane potentials more negative than 50 mV, P_o decreased exponentially with a greater than Nernstian dependence. The relationship between P_o and membrane potential was fitted by a Boltzmann equation with a hypothetical gating charge (z) of 1.97 ± 0.08 and a voltage at which P_o was half-maximal of 53.2 ± 0.5 mV (both mean ± SE, $n = 18$). The fit was not significantly different for $z = 2$. The frequency distribution of both channel open- and closed-durations could be fitted by a single exponential function at all membrane potentials (Figs. 7*A,B*), indicating a simple two-state model (one open state and one closed state) for channel kinetics (Sanders, 1990). The decline in P_o was attributed to a complex interaction of the dependence of both τ_o and τ_c on membrane potential (Fig. 7*E*). With increasingly negative membrane potentials, channel τ_c increased exponentially, whereas τ_o exhibited a general decrease, although its exact relationship with membrane potential was unclear.

The channel P_o was dependent upon KCl concentration in the *cis* chamber. When determined in (*cis : trans*) 325 : 100 mM KCl the 140 pS channel had a 10- to 30-fold lower P_o than in symmetrical 100 mM KCl at the membrane potentials tested (Fig. 7, *compare* Fig. 1). This was a consequence of an increase in τ_c, since τ_o was similar to that determined in symmetrical 100 mM KCl (*data not shown*).

Gating Kinetics of the 27 pS K^+ Channel

At extreme positive membrane potentials the P_o of the 27 pS K^+ channel approached unity (Fig. 8*D*). At membrane potentials more negative than about 40 mV P_o decreased with a higher than Nernstian dependence. The relationship between P_o and membrane potential was fitted by a Boltzmann equation with a hypothetical gating charge (z) of 1.91 ± 0.17 and a voltage at which P_o was half-maximal of 65.7 ± 1.1 mV (both mean ± SE, $n = 41$). The fit was not significantly different for $z = 2$. Both τ_c and τ_o could be obtained by a single exponential fitting of frequency distributions of channel closed and open durations (Fig. 8*A,B*), suggesting a simple two-state model (one open state and one closed state) for channel kinetics (Sanders, 1990). The decline in P_o could be attributed to both: (i) an exponential increase in

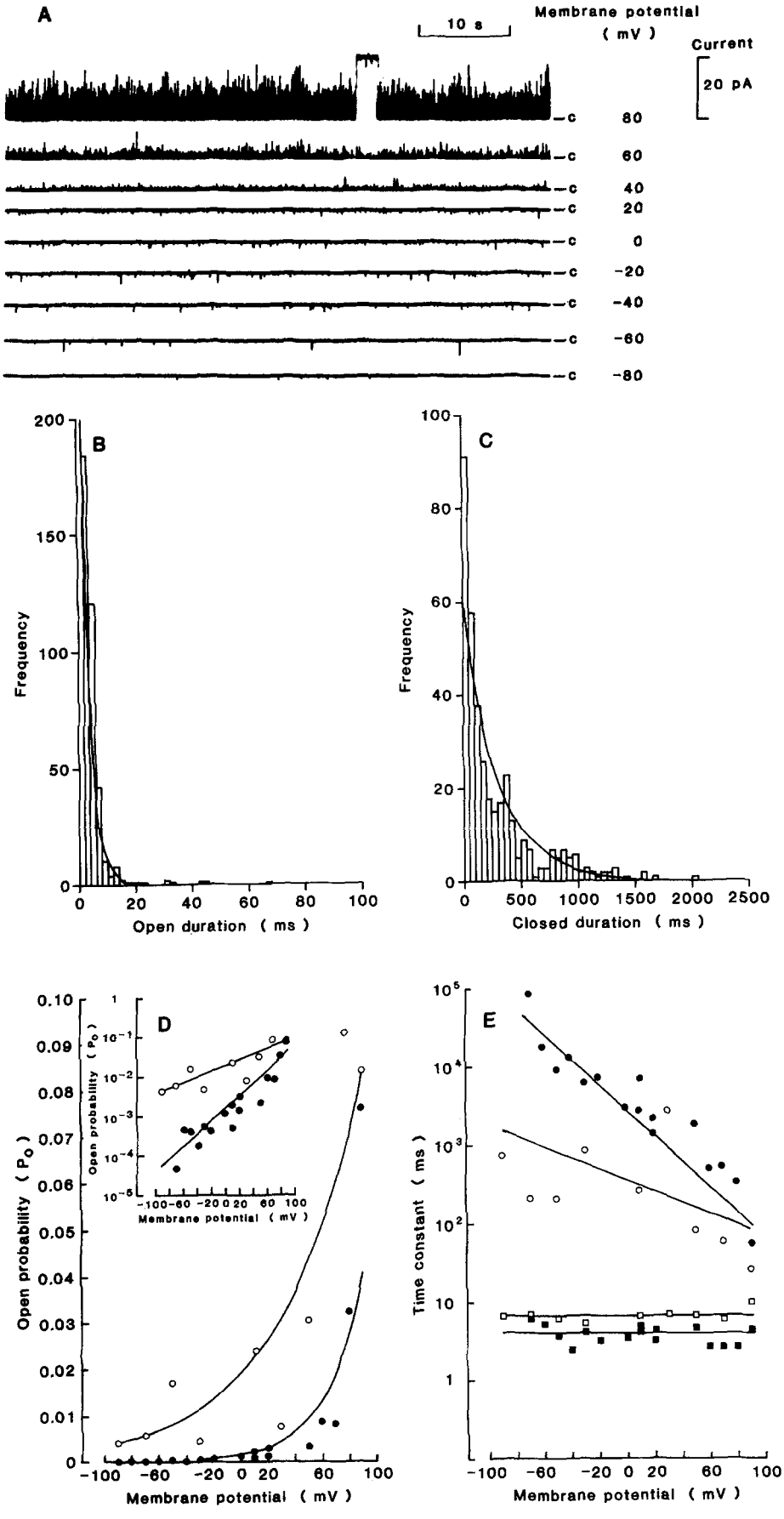
A
10 s
Membrane potential
(mV)
Current
20 pA
c 80
c 60
c 40
c 20
c 0
c -20
c -40
c -60
c -80
B
Frequency
Open duration (ms)
C
Frequency
Closed duration (ms)
D
Open probability (P_o)
Membrane potential (mV)
E
Time constant (ms)
Membrane potential (mV)

Fig. 6. (*A*) Current records of a bilayer containing a single 259 pS K^+ channel from *Acetabularia* plasma membrane determined at the membrane potentials indicated in the presence of (*cis : trans*) 325 : 100 mM KCl. The record was filtered at 100 Hz and sampled at 500 Hz. (*B*) Frequency-distribution histograms (discrete levels) and fitted probability density function (smooth curve, fitted to a single exponential function with τ_o = 2.7 msec and 429.4 initial events) of channel open-state durations for the 259 pS channel. Data were collected from a record lasting 2 min (380 events) of channels assayed in asymmetrical 325 : 100 mM KCl at 80 mV, filtered at 100 Hz and sampled at 500 Hz. (*C*) Frequency-distribution histograms (discrete levels) and fitted probability density function (smooth curve, fitted to a single exponential function with τ_c = 309.2 msec and 61.3 initial events) of channel closed-state durations for the 259 pS channel. Data were collected from a record lasting 2 min (380 events) of channels assayed in asymmetrical 325 : 100 mM KCl at 80 mV, filtered at 100 Hz and sampled at 500 Hz. (*D*) Open probability (P_o) for the 259 pS channel, assayed either in asymmetrical 325 : 100 mM KCl (●) or symmetrical 100 mM KCl (○), was calculated from single channel recordings lasting 2 min either using current frequency-distribution histograms or by summation of open and closed durations. *Inset:* log-linear plot of the relationship between channel open probability and membrane potential. The hypothetical gating charge and voltage at which half-maximal P_o was obtained were 0.91 and 170 mV in the presence of asymmetrical 325 : 100 mM KCl, and 0.42 and 237 mV in symmetrical 100 mM KCl. (*E*) Apparent open- (squares) and closed-state (circles) time constants for the 259 pS channel assayed either in asymmetrical 325 : 100 mM KCl (closed symbols) or symmetrical 100 mM KCl (open symbols), were calculated from single exponential regressions of either apparent open- or apparent closed-duration frequency distributions determined from current recordings filtered at 100 Hz and sampled at 500 Hz. Data for P_o and apparent τ_o were fitted by linear regression, while data for apparent τ_c were calculated assuming a simple kinetic model for the channel involving single open and closed states.

τ_c and (ii) an exponential decrease in τ_o with more negative membrane potentials (Fig. 8*E*).

At any membrane potential the absolute value of P_o was dependent upon KCl concentration in the *cis* chamber (Fig. 9). The dependence of P_o on membrane potential appeared to shift by a factor approximating the difference in E_K under contrasting conditions, with half-maximal P_o occurring about 40 mV more positive than E_K. However, the hypothetical gating charge for the channel was relatively unaffected by KCl concentration. Fitting a Boltzmann equation to data obtained in symmetrical 100 mM KCl yielded estimates of 1.81 ± 0.03 for the hypothetical gating charge of the channel and 47.1 ± 0.27 mV for the voltage at which P_o was half-maximal (both mean ± SE, n = 22).

Discussion

In our experiments five channels with a greater permeability to K^+ than Cl^-, differing in conductance, selectivity and kinetics, were observed when *Acetabularia* plasma membrane fractions were incorporated into PLB (Table). Although the cation selectivities of these channels were not determined, and it is possible that these K^+-selective channels may have a greater permeability to other ions, the presence of multiple K^+ channel types coexisting in the plasma membrane is not unusual and has been observed frequently; for example in the plasma membrane of leaf mesophyll cells (Moran *et al.*, 1984), guard cells (MacRobbie, 1988; Hedrich & Schroeder, 1989; Blatt, 1991), pulvinar cells (Moran, 1990), suspension cultured cells (Fairley, Laver & Walker, 1991) and root cells (White & Tester, 1992*a*). Indeed, two K^+ channels have previously been shown to be present in droplets of the plasma membrane derived from the *Acetabularia* cap using the patch-clamp technique (Bertl & Gradmann, 1987; Bertl et al., 1988).

Physiological Relevance of Channels Observed in PLB

To speculate on the physiological relevance of the K^+ channels incorporated into the PLB, it is important to compare the electrical properties of the single channels with the electrical characteristics of intact cells derived under voltage clamp. This is feasible since the plasma membrane vesicles we used were of a known orientation and incorporated into PLB in a defined manner; but we note that the standard KCl concentration gradient applied to channels in PLB (extracellular : cytoplasmic) 325 : 100 mM, is opposite to the common physiological conditions. In intact *Acetabularia* cells the dominant K^+ current observed under voltage clamp is an outwardly rectified current (Gradmann, 1975; Mummert & Gradmann, 1976). Such an outward rectification of the macroscopic K^+ current could be attributable to both or either of two, single channel characteristics: (i) the outward rectification of unitary current and/or (ii) the voltage dependency of P_o. Thus, since all three K^+ channels most commonly incorporated into PLB (259, 140 and 27 pS) exhibited outward rectification in both unitary conductance *and* P_o, all are candidates for the channel activity which underlies the outwardly rectified K^+ current observed in intact cells.

The properties of K^+ channels previously documented in the *Acetabularia* plasma membrane by the patch-clamp method (Bertl & Gradmann, 1987; Bertl et al., 1988) apparently differ from those described here in several respects. First, the patch-clamp studies described two K^+-selective channels, with slope unitary conductances of 120 and 70 pS in (pipette : estimated cytoplasmic concentrations)

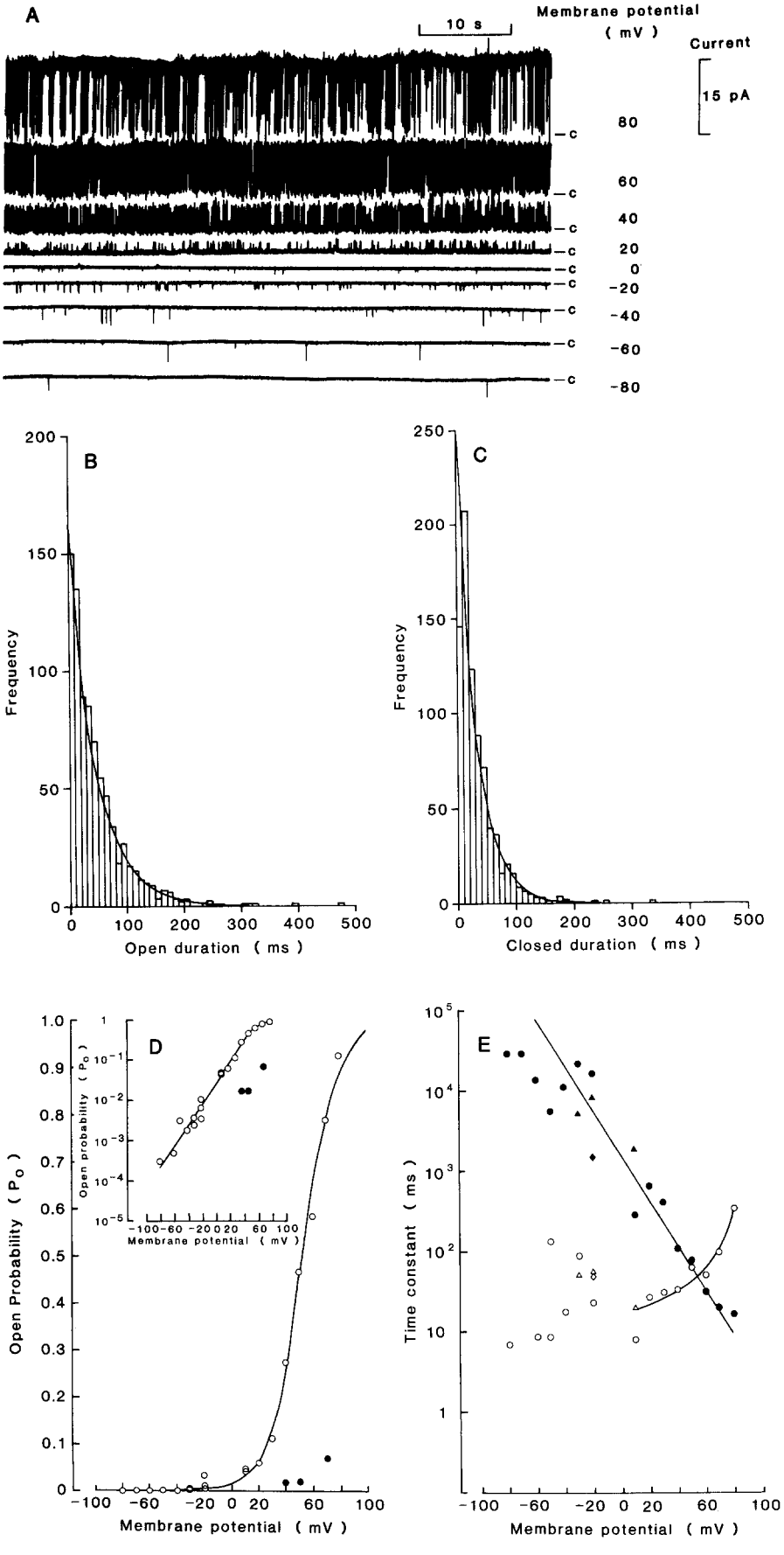
A
10 s
Membrane potential
(mV)
Current
15 pA
80
60
40
20
0
-20
-40
-60
-80
c
B
Frequency
Open duration (ms)
C
Frequency
Closed duration (ms)
D
Open probability (P_o)
Membrane potential (mV)
Open Probability (P_o)
Membrane potential (mV)
E
Time constant (ms)
Membrane potential (mV)

Fig. 7. (*A*) Current records of a bilayer containing a single 140 pS K^+ channel from *Acetabularia* plasma membrane determined at the membrane potentials indicated in the presence of symmetrical 100 mM KCl. The record was filtered at 100 Hz and sampled at 500 Hz. (*B*) Frequency-distribution histograms (discrete levels) and fitted probability density function (smooth curve, fitted to a single exponential function with τ_o = 46.6 msec and 163 initial events) of channel open-state durations for the 140 pS channel. Data were collected from a record lasting 1 min (813 events) of channels assayed in symmetrical 100 mM KCl at 60 mV, filtered at 100 Hz and samlped at 500 Hz. (*C*) Frequency-distribution histograms (discrete levels) and fitted probability density function (smooth curve, fitted to a single exponential function with τ_c = 31.3 msec and 252 initial events) of channel closed-state durations for the 140 pS channel. Data were collected from a record lasting 1 min (813 events) of channels assayed in symmetrical 100 mM KCl at 60 mV, filtered at 100 Hz and sampled at 500 Hz. (*D*) Open probability (P_o) for the 140 pS channel assayed in either asymmetrical 325 : 100 mM KCl (●) or symmetrical 100 mM KCl (○), calculated from single channel recordings lasting 1 min either using current frequency-distribution histograms or by summation of open and closed durations. Data obtained in symmetrical 100 mM KCl were fitted to a Boltzmann equation with a hypothetical gating charge (z) of 1.97 ± 0.08 and a voltage at which P_o was half-maximal of 53.2 ± 0.5 mV (both mean ± SE, n = 18). *Inset:* log-linear plot of the relationship between channel open probability and membrane potential. (*E*) Open- (open symbols) and closed-state (filled symbols) time constants for the 140 pS channel calculated from single exponential regressions of either open- or closed-duration frequency distributions. Time constants were determined from current recordings filtered at 100 Hz and sampled at 500 Hz. Different symbols represent repetitive observations on a single bilayer.

130 : 400 mM K^+. At first glance these conductances do not obviously match with the conductance of any of the K^+ channel activities observed in the PLB. In addition, both of the previously documented channels exhibited saturating currents at extreme clamp potentials, contrasting with the rectification of the unitary current observed for K^+ channels incorporated into the PLB. Such differences in the characteristics of single channel currents may result from the different methodologies used. In particular, patch clamping was performed in the cell-attached mode. It is possible, therefore, that cytoplasmic factors might influence the conductance properties of the channel. Indeed, under cell-attached conditions it is to be expected that the competition of ions for permeation is a major factor determining both the unitary conductance and current saturation of the channel. This is especially pertinent considering the voltage-dependent inhibition of outwardly rectifying K^+ channels by Na^+ (Thiel & Blatt, 1991) and the high cytoplasmic Na^+ concentration, which approximates 50 to 200 mM, in *Acetabularia* (Saddler, 1970; Amtmann, Klieber & Gradmann, 1992).

Despite differences in unitary current characteristics, many of the kinetic characteristics of the 259 pS channel studied in the PLB resemble those of the 120 pS channel observed in the patch-clamp records (*cf.* Bertl & Gradmann, 1987; Bertl et al., 1988). These include (i) bursting behavior, (ii) the presence of a long time constant (τ_{o2}) at extreme negative membrane potentials, (iii) a voltage-independent τ_{o1}, (iv) a voltage-dependent τ_c which increased with negative membrane potentials in a near Nernstian manner, and (v) a voltage-dependent P_o, which decreased in a Nernstian manner with negative membrane potentials and did not approach unity even at extreme membrane potentials. These characteristics of the 259 pS channel are unique among the channels incorporated into PLB. It is therefore possible that the patch-clamp 120 pS channel and the 259 pS channel observed in PLB are identical. Furthermore, the high frequency of incorporation into PLB of the 259 pS channel presumably reflects its abundance (and frequent detection) in the native plasma membrane in patch-clamp experiments.

One of the two K^+ channels observed in PLB, which had lower unitary conductances than the 259 pS, might correspond to the lower-conductance K^+ channel observed in patch-clamp studies (70 pS; Bertl & Gradmann, 1987). The ratio in conductances of the PLB and patch-clamped K^+ channel above, suggests that the 140 pS K^+ channel observed in PLB more closely resembles the 70 pS channel in patch-clamp experiments. The absence of kinetic data for the patch-clamped 70 pS channel does not allow any further comparison.

In addition to the three most frequently observed K^+ channels in PLB, two other K^+-selective channels were detected. These had remarkably high unitary conductances (1,600 and 485 pS in 325 : 100 mM KCl). They are considerably greater than conductances of K^+ channels reported in patch-clamp surveys for plasma membrane K^+ channels in either algae or higher plants (Hedrich & Schroeder, 1989; Tester, 1990). At present, only porins (Benz, 1986) and the voltage-dependent anion channel of the mitochondrion (VDAC; Colombini, 1986) are known to exhibit nanoSiemen conductances in approximately 100 mM KCl. However, the 1.6 nS channel that we observed in PLB is unlikely to be a VDAC or porin since it is cation-selective (the VDAC is anion-selective) and it exhibits channel-like gating (porins rarely close). However, there is a report of a mechanosensitive cation channel at the plasma membrane of the bean rust fungus (*Uromyces appendiculatus*) having a unitary chord-conductance of 601 pS in 290 mM KCl (Zhou et al., 1991). Furthermore, when plasma membrane vesicles from rye (*Secale cereale*) roots

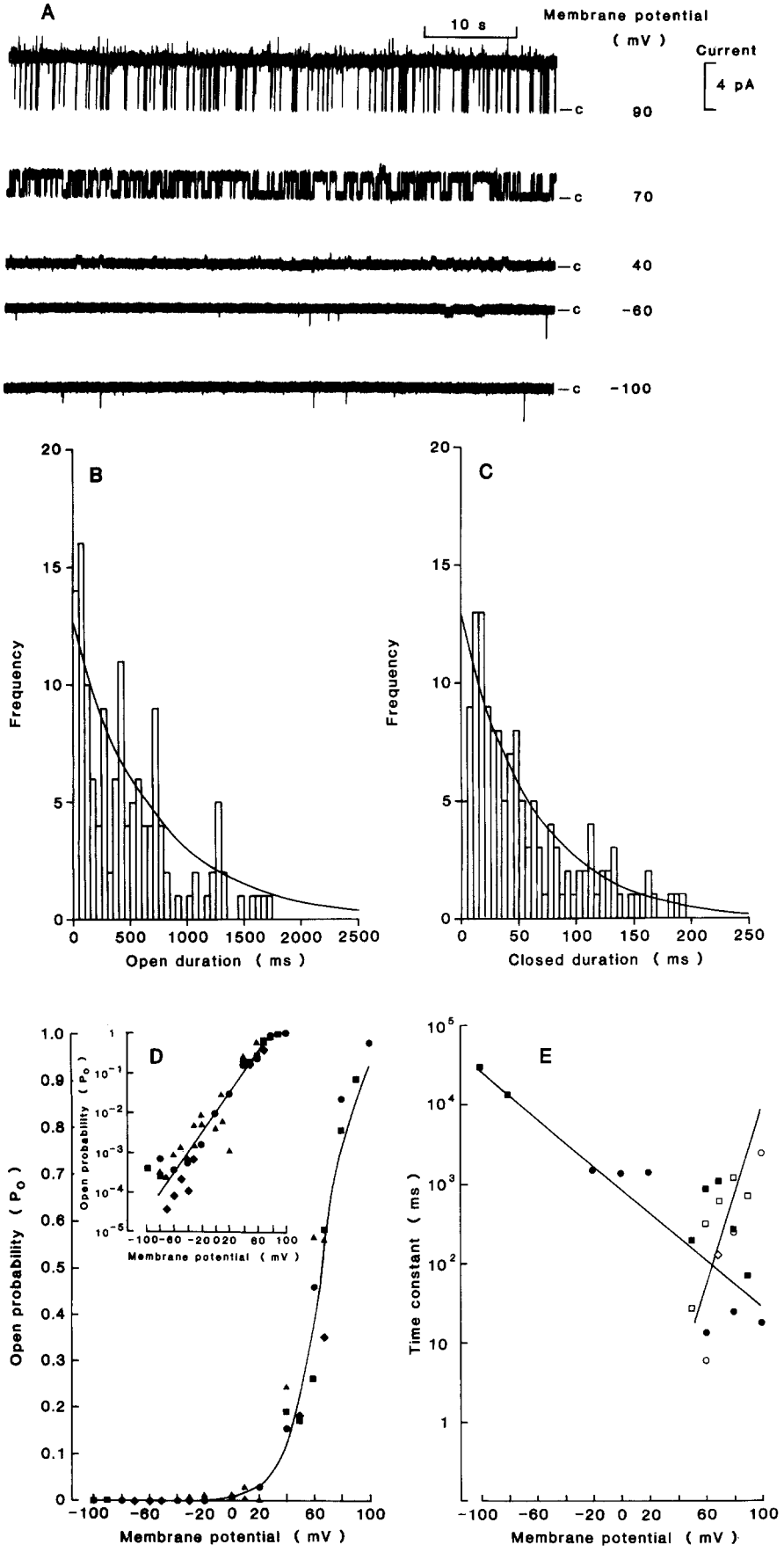
A
10 s
Membrane potential
(mV)
Current
4 pA
c
90
70
40
-60
-100
B
Frequency
Open duration (ms)
C
Closed duration (ms)
D
Open probability (P_o)
Membrane potential (mV)
E
Time constant (ms)
Membrane potential (mV)

Fig. 8. (*A*) Current records of a bilayer containing a single 27 pS K^+ channel, and a smaller channel, from *Acetabularia* plasma membrane determined at the membrane potentials indicated in the presence of (*cis : trans*) 325 : 100 mM KCl. The record was filtered at 100 Hz and sampled at 500 Hz. (*B*) Frequency-distribution histograms (discrete levels) and fitted probability density function (smooth curve, fitted to a single exponential function with τ_o = 712 msec and 12.5 initial events) of channel open-state durations for the 27 pS channel. Data were collected from a record lasting 1 min (139 events) of channels assayed in asymmetrical 325 : 100 mM KCl at 90 mV, filtered at 100 Hz and sampled at 500 Hz. (*C*) Frequency-distribution histograms (discrete levels) and fitted probability density function (smooth curve, fitted to a single exponential function with τ_c = 61.9 msec and 12.8 initial events) of channel closed-state durations for the 27 pS channel. Data were collected from a record lasting 1 min (138 events) of channels assayed in asymmetrical 325 : 100 mM KCl at 90 mV, filtered at 100 Hz and sampled at 500 Hz. (*D*) Open probability (P_o) for the 27 pS channel calculated from single channel recordings lasting 1 to 5 min either using current frequency distribution histograms or by summation of open and closed durations. Different symbols indicate observations on separate bilayers. Data were fitted to a Boltzmann equation with a hypothetical gating charge (z) of 1.91 ± 0.17 and a voltage at which P_o was half-maximal of 65.7 ± 1.1 mV (both mean ± SE, n = 41). *Inset:* log-linear plot of the relationship between channel open probability and membrane potential. (*E*) Apparent open- (open symbols) and closed-state (filled symbols) time constants for the 27 pS channel were calculated from single exponential regressions of either open- or closed-duration frequency distributions. Data for closed-state time constants at membrane potentials more positive than −40 mV are not shown, since these approached the corner frequency of the filtering and no slower closed-state time constants were detected. The presence of a smaller channel in some bilayers (*see A*) did not affect the analysis of time constants appreciably, since its conductance was much less than the 27 pS channel. Different symbols indicate observations on separate bilayers.

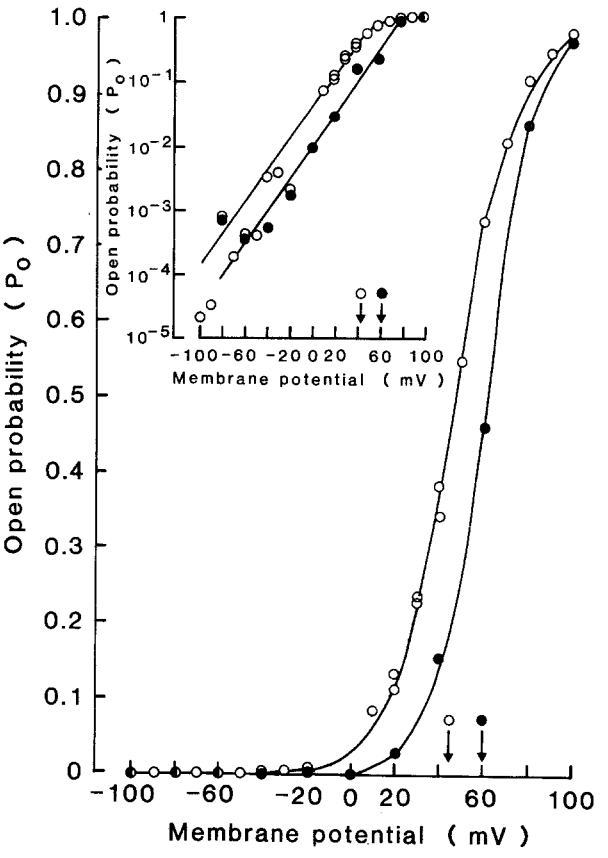

Fig. 9. The relationship between channel open probability (P_o) and membrane potential for the 27 pS K^+ channel from *Acetabularia* plasma membrane determined in (*cis : trans*) 325 : 100 mM KCl (●) or symmetrical 100 mM KCl (○). Data were fitted to Boltzmann equations. The hypothetical gating charges and voltages at which P_o was half-maximal were 2.22 ± 0.08 and 61.0 ± 0.5 mV and (mean ± SE, n = 10) in 325 : 100 mM KCl and 1.81 ± 0.03 and 47.1 ± 0.3 mV (mean ± SE, n = 22) in symmetrical 100 mM KCl. *Inset:* Log-linear transformation of the data presented in the main figure. Open probabilities were calculated from single channel recordings, filtered at 100 Hz and sampled at 500 Hz, lasting 1 to 2 min, either using current frequency-distribution histograms or by summation of open and closed durations. Observations were made in a single experiment, on the same bilayer. The membrane potential at which P_o is half-maximal is indicated for both asymmetrical and symmetrical conditions. The theoretical reversal potential for K^+ is 0 mV in symmetrical 100 mM KCl and 27 mV in asymmetrical 325 : 100 mM KCl.

were incorporated into PLB, high conductance channels (500 and 194 pS in 280 : 100 mM KCl) were observed at high frequencies (White & Tester, 1992*a,b*). At present it is not possible to make any definitive statement on the role(s) of such large conductance channels in the *Acetabularia* plasma membrane, since there are no obvious, complementary electrical measurements in intact cells.

K^+ Channels Have Multiple Kinetic Mechanisms for Outward Rectification

There are several possible single channel kinetic mechanisms to effect outward rectification in K^+ currents. The outward rectification of all K^+ channels studied here in PLB always had a component resulting from an increase in τ_c with more negative membrane potentials. This is consistent with previous observations in *Acetabularia* (Bertl et al., 1988). However, the effect of membrane potential on τ_o differed between K^+ channels: The τ_o of the 259 pS K^+ channel was apparently voltage insensitive, whereas the τ_o of both the 140 and 27 pS was voltage dependent. Thus, in both the voltage dependence of τ_c and voltage independence of τ_o, the 259 pS channel appeared to follow the kinetics of the dominant K^+ channel observed in patch-clamp studies of *Acetabularia* (Bertl *et al.*, 1988) more closely than did either the 140 or 27 pS channel.

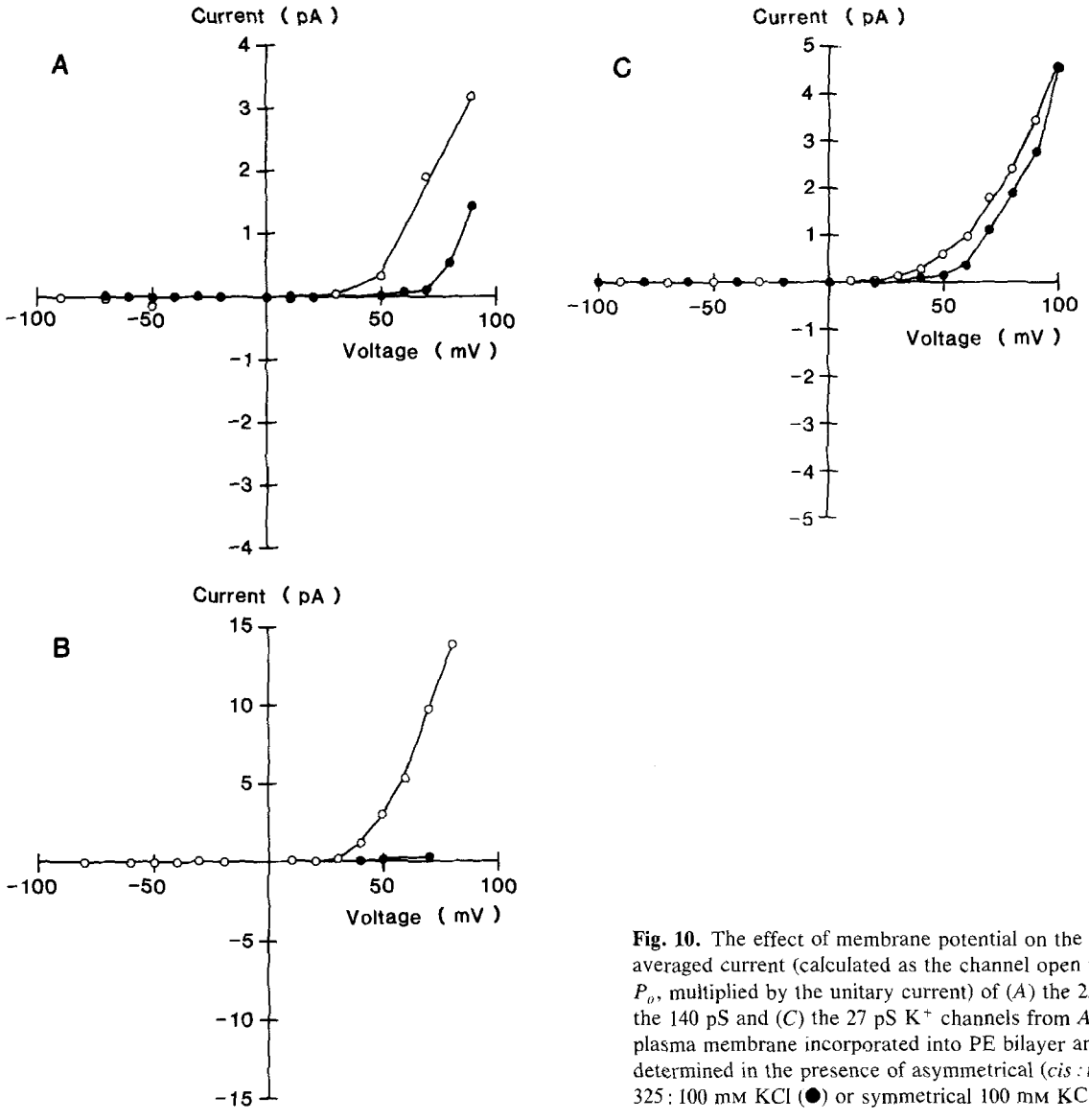

Fig. 10. The effect of membrane potential on the time-averaged current (calculated as the channel open probability, P_o, multiplied by the unitary current) of (*A*) the 259 pS, (*B*) the 140 pS and (*C*) the 27 pS K^+ channels from *Acetabularia* plasma membrane incorporated into PE bilayer and determined in the presence of asymmetrical (*cis* : *trans*) 325 : 100 mM KCl (●) or symmetrical 100 mM KCl (○).

For both the 140 and 27 pS K^+ channels in PLB, in addition to an increase in τ_c, τ_o decreased with more negative membrane potentials. This kinetic pattern is analogous to that observed for outwardly rectifying plasma membrane K^+ channels of *Chara* internodes and *Amaranthus* cotyledons, where both the τ_o and τ_c are voltage dependent, τ_o becoming shorter and τ_c longer as the membrane potential becomes more negative (Azimov, Geletyuk & Berestovskii, 1987; Terry, Tyerman & Findlay, 1991). The pattern observed in guard cells, in which the τ_o of the outwardly rectifying K^+ channel appears to increase with more positive membrane potentials, while τ_c remains constant (Schroeder, Hedrich & Fernandez, 1984; Hosoi, Iino & Shimazaki, 1988), was not observed for K^+ channels in PLB.

Dependence of P_o on External KCl Concentration

An interesting feature of all the outwardly rectifying K^+ channels incorporated into the PLB was their sensitivity of gating to extracellular (*cis*) KCl concentration and/or the KCl gradient across the bilayer (Figs. 6, 7 and 9). The modulation of K^+ channel activity by extracellular K^+ concentration is a property already documented for a number of outward rectifying K^+ channels of the plant plasma membrane (Iijima & Hagiwara, 1987; Ketchum, Shrier & Poole, 1989; Blatt, 1991; Lew, 1991). In the PLB, when the extracellular KCl concentration was changed, both the P_o/voltage relationship (Figs. 6, 7 and 9) and the time-averaged current/voltage rela-

tionship (Fig. 10) of all K^+ channels shifted along the voltage axis in the same direction as the shift in E_K. This gating feature has been interpreted as a mechanism that ensures K^+ efflux even at high external K^+ concentrations (Blatt, 1991). If these results can be extrapolated to the *trans* plasma membrane K^+ concentration gradients occuring in vivo, which remains to be tested, these channels would mediate a physiological K^+ efflux upon plasma membrane depolarization, such as would occur during repolarization of the plasma membrane following an action potential.

Conclusion

The present paper illustrates the difficulties in comparing single channel characteristics derived from the more physiological patch-clamping technique with the more reduced, and biochemically-based, PLB technique. First, although several similarities in the kinetic characteristics of an outwardly rectifying 259 pS K^+ channel incorporated into PLB were similar to those of an outwardly rectifying 120 pS K^+ channel observed in patch-clamp experiments of the *Acetabularia* plasma membrane, their unitary conductances and unitary current/voltage relationships appeared to differ. Thus, to judge whether these channel activities are truly identical it will be necessary to compare selectivity and pharmacological properties also. Second, many more channel types were observed in PLB than in patch-clamp studies. In this respect, the PLB method may be advantageous since it allows the detection and characterization of channels which are usually electrically silent under physiological conditions: For example, our PLB study demonstrates the presence of Cl^- channels at the plasma membrane, which are implied from voltage-clamp studies and tracer fluxes in intact *Acetabularia* (Mummert & Gradmann, 1991) but have not yet been detected in patch-clamp investigations (Bertl et al., 1988).

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