Poly-3-Hydroxybutyrate/Polyphosphate Complexes Form Voltage-Activated Ca^{2+} Channels in the Plasma Membranes of *Escherichia coli*

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ABSTRACT The lipidic polymer, poly-3-hydroxybutyrate (PHB), is found in the plasma membranes of *Escherichia coli* complexed to calcium polyphosphate (CaPPi). The composition, location, and putative structure of the polymer salt complexes led Reusch and Sadoff (1988) to propose that the complexes function as Ca^{2+} channels. Here we use bilayer patch-clamp techniques to demonstrate that voltage-activated Ca^{2+} channels composed of PHB and CaPPi are in the plasma membranes of *E. coli*. Single channel calcium currents were observed in vesicles of plasma membranes incorporated into planar bilayers of synthetic 1-palmitoyl,2-oleoyl phosphatidylcholine. The channels were extracted from cells and incorporated into bilayers, where they displayed many of the signal characteristics of protein Ca^{2+} channels: voltage-activated, selective for divalent over monovalent cations, permeant to Ca^{2+}, Sr^{2+}, and Ba^{2+}, and blocked in a concentration-dependent manner by La^{3+}, Co^{2+}, Cd^{2+}, and Mg^{2+}, in that order. The channel-active extract, purified by size exclusion chromatography, was found to contain only PHB and CaPPi. This composition was confirmed by the observation of comparable single channel currents with complexes reconstituted from synthetic CaPPi and PHB, isolated from *E. coli*. This is the first report of a biological non-proteinaceous calcium channel. We suggest that poly-3-hydroxybutyrate/calcium polyphosphate complexes are evolutionary antecedents of protein Ca^{2+} channels.

INTRODUCTION

The physiological role(s) of calcium in bacteria is not well defined, yet bacteria maintain low intracellular calcium concentrations (Gangola and Rosen, 1987), and there is increasing evidence pointing to calcium involvement in a number of important cellular functions such as chemotaxis (Matsushita et al., 1989; Tisa and Adler, 1992) and cell division (Chang et al., 1986; Casaregola et al., 1991). While secondary systems for calcium export have been identified in a number of bacteria (Lynn and Rosen, 1987), mechanisms for calcium entry are less well known, although a protein with L-type channel characteristics has been reported in *Bacillus subtilis* (Matsushita et al., 1989).

In *Escherichia coli*, two distinct secondary systems for calcium extrusion have been identified, a calcium/proton exchanger and a calcium phosphate cotransporter (Rosen and McClees, 1974; Ambudkar et al., 1984); and recently a gene designated *chaA* was postulated to encode a calcium proton antiporter (Ivey et al., 1993). No calcium import systems have been identified, but the existence of a calcium channel is suggested by the inhibition of calcium-related chemotaxis by the calcium channel blocker, ω-conotoxin (Tisa et al., 1993).

Reusch and Sadoff (1988) found complexes of poly-3-hydroxybutyrate (PHB) and inorganic calcium polyphosphate (CaPPi) in the plasma membranes of *E. coli* and other bacteria, and suggested that the complexes may serve as calcium channels. The PHB/CaPPi complexes were discovered during fluorometric studies of membrane differentiation at the time of development of genetic competence; the complexes give rise to a sharp thermotropic transition at ~56°C, attributed to an increase in membrane viscosity resulting from the dissociation of the complexes (Reusch and Sadoff, 1983; Reusch et al., 1986). The PHB/CaPPi complexes are present in the plasma membranes during log-phase growth, but at low concentrations. They increase considerably in number during stationary phase and even more so when cells are suspended in ice-cold calcium buffers to render them genetically competent (Reusch et al., 1986). At high concentrations, alterations of the plasma membrane structure are observable by freeze-fracture electron microscopy (Reusch et al., 1987). A model was proposed for the complexes (Reusch and Sadoff, 1988) on the basis of molecular modeling with consideration for the physical properties and the molar ratios of the two polymers, the low dielectric membrane environment, and the coordination preferences of calcium. In this model, the amphiphilic PHB forms a helical polar-lined pore that surrounds and solvates the membrane-crossing CaPPi. It was proposed that this structure could operate as an ion channel.

Both PHB and PPi are ubiquitous homopolymers whose biological roles are poorly understood. PHB, a head-to-tail polymer of (*R*)-3-hydroxybutyrate, has long been known in certain bacteria as a high molecular weight polymer (60,000–1,000,000) sequestered within cytosolic inclusion bodies (Dawes and Senior, 1973; Anderson and Dawes, 1990). PHB of low molecular weight (~12,000) has been discovered more recently, not only in bacterial membranes (Reusch and Sadoff, 1983; Reusch et al., 1986), but also in a wide variety of plant and animal tissues (Reusch, 1989, 1992; Seebach et al., 1994). PHB has the molecular char-

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acteristics common to a select group of polymers that form ion-conducting salt complexes, i.e., it is a linear flexible molecule bearing electron-donating carbonyl ester oxygens at intervals that allow multiple bonding between the polymer chain and cations (Armand, 1987; Gray, 1992; Reusch, 1989). The ability of PHB to "dissolve" salts has been demonstrated by Bürger and Seebach (1993), who have shown that oligomers of (*R*)-3-hydroxybutanoic acid transport alkali and alkaline earth salts across chloroform layers, and by Reusch and Reusch (1993) who have shown that PHB in the bulk phase forms conductive complexes with lithium perchlorate. PPi also has molecular characteristics that contribute importantly to ion selection and transport. This polymeric anion is composed of linear chains of phosphoryl residues linked by phospho-anhydride bonds that have a free energy of hydrolysis comparable to that of ATP (Dawes and Senior, 1973; Kulaev, 1979). In addition to providing a framework of sufficient length to cross the bilayer, PPi has a high density of monovalent anionic sites that form the basis for its large capacity for cation exchange and its marked preference for binding divalent over monovalent cations (Corbridge, 1985).

In this study, we use planar bilayer voltage-clamp techniques to demonstrate the existence of voltage-activated calcium channels in the plasma membranes of *E. coli* composed of PHB and CaPPi, and we show that the complexes possess many of the characteristic properties of protein channels, i.e., they are permeant to Ca^{2+}, Sr^{2+}, and Ba^{2+}; selective for divalent over monovalent cations; and blocked in a concentration-dependent manner by La^{3+}, Co^{2+}, Cd^{2+} and Mg^{2+}, in that order.

MATERIALS AND METHODS

Preparation of competent cells of *E. coli*

E. coli DH5α cells were made genetically competent by a variation of the method of Hanahan (1983) as previously described (Reusch et al., 1986). Briefly, cells were cultured in SOB medium (2% Bacto-tryptone (Difco, Detroit, MI), 0.5% yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl to an absorbance at 550 nm of ~0.4. The cells were pelleted at low centrifugal speed (800 × *g*) at 4°C, and gently resuspended in ⅓ volume 100 mM KCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$ in 4-morpholine ethanesulfonic acid (Sigma Chemical Co., St. Louis, MO) neutralized to pH 6.3 with KOH for 30–60 min at 4°C. The cells were then collected under the same conditions as above.

Preparation of membrane vesicles

A pellet of *E. coli* DH5α competent cells was suspended in 10 mM Tris (tris (hydroxymethylaminomethane) Hepes (4-(2-hydroxyethyl)-1-piperazine sulfonic acid), pH 7.3 containing 20 μg/ml DNase 1 and 20 μg/ml RNase A and sonicated at 30% power in a 20% cycle with a Vibra Cell ultrasonicator (Sonics and Materials Inc., Danbury, CT) (for a total sonication time of 3 min at 4°C). Intact cells and debris were pelleted by centrifugation at 10,000 × *g* for 10 min, and the envelope fraction was collected on a pillow of 70% sucrose in 10 mM Tris Hepes, pH 7.3 by centrifugation of the supernatant at 35,000 rpm in a Beckman SWTi 41 rotor (Arlington Heights, IL) for 90 min. The membranes were collected with a syringe fitted with a J-shaped needle, washed with 10 mM Tris Hepes, pH 7.3, suspended in 250 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM Tris Hepes, and sonicated as above for 1 min.

To prepare vesicles of plasma and outer membrane fractions, the envelope fraction above was separated on step gradients (2.5 ml each of 30, 42 and 53% sucrose in 10 mM Tris Hepes, pH 7.3) over a pillow of 70% sucrose. The gradients were centrifuged at 25,000 rpm for 24 h at 4°C in the same rotor. Plasma and outer membrane fractions were collected and suspended in buffer as above. NADH oxidase was used as a marker for plasma membrane and ketodeoxyoctonate as a chemical marker for the outer membrane (Osborn et al., 1972; Weissbach and Hurwitz, 1959). Protein was determined with the Bio-Rad (Richmond, CA) Protein Assay Kit.

Extraction of membrane complexes

The pellet of *E. coli* competent cells was washed with methanol (2×), methanol:acetone 1:1 (2×), and acetone (2×), and the dry residue was extracted 24 h or longer at 4°C with chloroform (10^{10} cells/ml $CHCl_3$). Because the complexes are sensitive to temperature and moisture, all solvents were ice-cold and dried with molecular sieves, 3 Å for alcohol and acetone, and 4 Å for chloroform (Aldrich Chemical Co., Milwaukee, WI).

Size exclusion chromatography of membrane complexes

The chloroform extract was filtered, using a 0.2 μm polytetrafluoroethylene syringe filter (Whatman Inc., Hillsboro, OR), and chromatographed at 4°C on a high performance lipid chromatography (HPLC) non-aqueous size exclusion column (Shodex K803; 8 mm × 300 mm, Waters, Millipore Division, Milford, MA) using chloroform as an eluent at a flow rate of 0.5 ml/min. Molecular weight standards were synthetic PHBs (courtesy of D. Seebach, Eidgenössischen Technischen Hochschule (ETH), Zürich, Switzerland) and polyisoprenes (Polysciences, Inc., Warrington, PA). Eluent fractions (250 μl each) were tested for channel activity in symmetrical solutions of 250 mM $BaCl_2$, 5 mM $MgCl_2$, and 10 mM Tris Hepes, pH 7.3.

Determination of PHB, PPi, and Ca

The chloroform extract of competent cells was washed with 10 mM Tris EDTA (TE buffer), pH 7.5. The chloroform layer was used for determination of PHB, and the aqueous wash for determination of PPi and Ca. PHB was measured by chemical assay by a variation of the method of Karr et al. (1983) as previously described (Reusch, 1989). Briefly, the dry sample was hydrolyzed in concentrated sulfuric acid at 92°C for 45 min, and the resulting crotonic acid was extracted, separated by HPLC chromatography using a Spectra-Physics SP8700 Chromatography System (Santa Clara, CA) with a Bio-Rad Organic Acids Column HPX87H, and quantitated by comparison of peak area measured on a Shimadzu (Kyoto, Japan) C-R3A Chromatopac Integrator with that of crotonic acid standards. PPi was determined by chemical assay as described by Hess and Derr (1975) or by enzymatic assay using polyphosphate kinase as described by Akiyama et al. (1992). The average molecular weight of PHB was determined by comparison of its elution time on a non-aqueous size exclusion chromatography with that of PHB standards and polyisoprene standards as above. The average molecular weight of PPi was determined by polyacrylamide gel electrophoresis using PPi standards prepared as described by Clark and Wood (1987). Calcium was determined by graphite furnace atomic absorption chromatography using a Varian (Palo Alto, CA) SpectrAA-300/400 Zeeman spectrometer.

Incorporation of channel extracts in planar bilayers

The chloroform solution containing PHB/Ca/PPi complexes was filtered and added to a solution of synthetic 1-palmitoyl,2-oleoyl phosphatidylcho-

line (POPC) (Avanti Polar Lipids, Birmingham, AL) in decane (40 mg/ml). The weight ratio of PHB to phospholipid was <1:1000. The chloroform was evaporated with a stream of dry nitrogen gas and the lipid solution was used to form a bilayer across a 250 μm aperture in a Delrin cup (Warner Instruments, Hamden, CT) separating two aqueous bathing solutions. All salts used in the bathing solutions were ultrapure (>99%) (Aldrich Chemical Co.).

Purification of PHB

The chloroform extract containing PHB/Ca/PPi was washed four times with dilute NaCl to remove PPi and other water-soluble contaminants. The chloroform was evaporated and the PHB was suspended in TE buffer, pH 7.5 and treated with 200 μg/ml proteinase K at 37°C overnight. The PHB was then extracted into chloroform, and the chloroform solution was washed three times with distilled water. The concentration of PHB was determined by chemical assay as described above.

Preparation of CaPPi

Calcium chloride (1 M) was added to a solution of sodium polyphosphate glass (average chain length 65) (Sigma Chemical Co.). The precipitate of CaPPi was collected by centrifugation and washed two times with 0.1 M $CaCl_2$ and one time with distilled water, and then dried by lyophilization.

Reconstitution of PHB/Ca^{2+}/PPi

A few mg of CaPPi were dried in a microwave oven (2 min, full power), ground to a fine powder in an agate mortar, and dried in the microwave oven again for 1 min. A chloroform solution of PHB (1 μg) was added to the powdered CaPPi, and the chloroform evaporated with a stream of dry nitrogen gas, leaving the CaPPi coated with a film of PHB. This mixture was then heated for 2 × 2 min in the microwave oven. PHB/CaPPi complexes were formed from portions of the dry mixture and incorporated into bilayers by each of the following methods. 1) A chloroform solution of POPC (10 μg) was added to the dry blend of PHB and CaPPi, and sonicated at 30% power in a 20% cycle with a VibraCell ultrasonicator for 2 min at room temperature, and then again for 2 min at 4°C. The supernatant was filtered with a Teflon syringe filter (0.2 μm) and added to a decane solution of POPC at a final concentration of 10 ng PHB/100 μg phospholipid. The chloroform was evaporated, and the lipid mixture was used to form a bilayer as above. 2) A chloroform solution of POPC was added to the dry blend of PHB and CaPPi (10 ng PHB/100 μg POPC), and the chloroform was evaporated to form a lipid film. Liposomes were formed by adding aqueous buffer (10 mM Tris Hepes, pH 7.3 containing 100 mM $CaCl_2$, 1 mM $MgCl_2$) and sonicating the mixture gently (Sonicor, Inst. Corp. bath sonicator; Copiaque, NY) for 30 min at room temperature. After centrifugation (1200 × g for 5 min), a portion of the liposomes (1–5%) was added to the *cis* aqueous bathing solutions and allowed to incorporate spontaneously into a bilayer of POPC. 3) PHB was added to phospholipid (10 ng PHB/100 μg POPC), and the mixture was used to form a bilayer. CaPPi (~1 mg) was then added to the aqueous bathing solution and a holding potential of 60–80 mV was applied *cis* to induce the formation of PHB/CaPPi complexes in the bilayer.

Voltage clamp and recording techniques

The *cis* solution (voltage command side) was connected via an Ag/AgCl electrode to the CV 201A head-stage input of an Axopatch 200A integrating patch clamp (Axon Instruments, Foster City, CA). The *trans* solution was held at virtual ground. Channel currents were filtered at 1 kHz and recorded on videotapes after pulse code modulation (PCM-200, A. R. Vetter Co., Rebersburg, PA). Currents were analyzed by replaying the tape

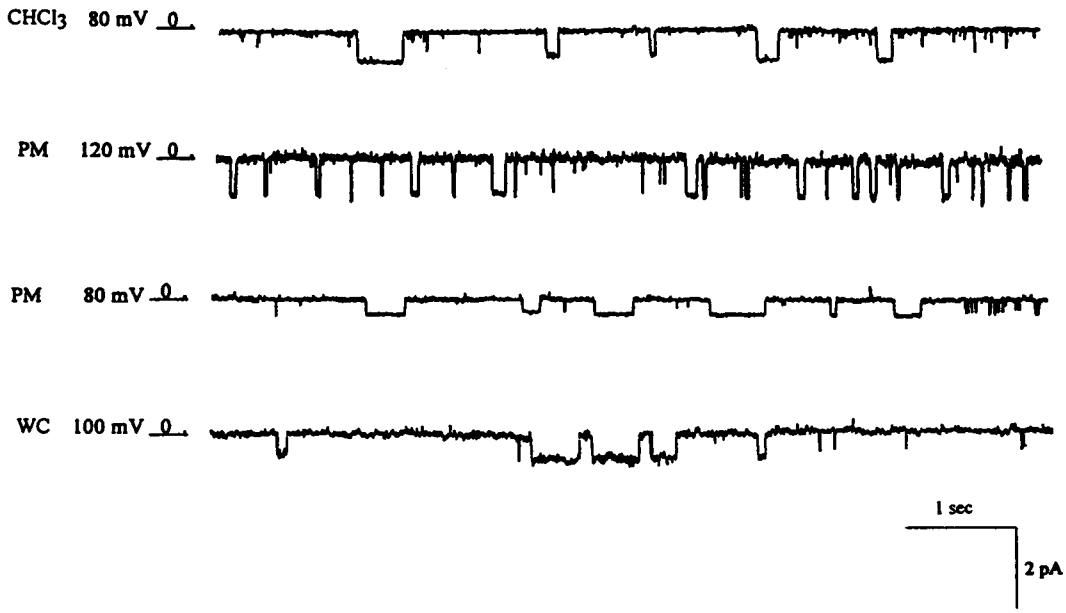


FIGURE 1 Calcium channels from *E. coli*. Single channel currents observed in membrane vesicles and extracts of *E. coli* competent cells when incorporated into planar bilayers of synthetic POPC between symmetric aqueous bathing solutions of 250 mM $CaCl_2$, 5 mM $MgCl_2$ in 10 mM Tris Hepes, pH 7.3. (*Line 1*) Membrane vesicles of whole cells (WC) were added to the *cis* bathing solution and the solution was gently stirred to allow spontaneous incorporation of the vesicles into the bilayer. Single channel currents were activated by a voltage step ≥60 mV. A representative current record at 100 mV is shown. (*Lines 2 and 3*) Plasma membrane vesicles (PM) were incorporated into the bilayer and single channel currents were activated as above. Representative records at 80 mV and 120 mV are shown. (*Line 4*) A chloroform extract of genetically competent cells was added to a decane solution of POPC. The chloroform was removed by evaporation, and the remaining lipid mixture was used to form the bilayer. Single channel currents were activated as above. A representative current record at 80 mV is shown.

through an eight-pole Bessel filter (200 Hz; Frequency Devices, Haverhill, MA) connected to a microcomputer via a Labmaster TI-1 DMA interface (Axon Instruments). Single channel events were digitized at 2 kHz with FETCHEX, and channel currents were measured and analyzed with FETCHAN and PSTAT (subroutines of pCLAMP 6 software; Axon Instruments).

RESULTS

Calcium channels in the plasma membranes of *E. coli*

Although PHB/CaPPi complexes are found in many bacteria, *E. coli* were used for this study because they do not synthesize PHBs of high molecular weight which coextract with and are difficult to separate from the low molecular weight polymer. *E. coli* DH5α cells were made genetically competent to increase the concentration of PHB/CaPPi complexes in the membranes (Reusch et al., 1986). The cells were broken by sonication, and vesicles of the cell envelopes were added to the *cis* side of symmetric bathing solutions of 250 mM $CaCl_2$, 5 mM $MgCl_2$, in 10 mM Tris Hepes, pH 7.3 on sides of a planar bilayer of POPC. The bathing solution was stirred gently to allow spontaneous insertion of the vesicles into the bilayer. No activity was observed in the absence of an applied voltage or at holding potentials <60 mV *cis* (*trans* virtual ground), but when a potential ≥60 mV was held for several minutes, single channel currents were observed signifying the presence of calcium-permeant channels in the envelope vesicles (Fig. 1, *line 1*).

The cell envelopes were separated into plasma and outer membrane fractions on density gradients, and vesicles of the plasma and outer membranes were separately added to the *cis* side of the above system. Single channel currents were observed with the plasma membrane vesicles when a voltage step of +60 mV or greater was maintained for several min (Fig. 1, *lines 2, 3*), but comparable voltage steps did not result in single channel activity with outer membrane vesicles.

Extracted and purified calcium channels contain PHB/PPi complexes

We then extracted the PHB/PPi complexes from *E. coli* DH5α competent cells with cold dry chloroform (Reusch and Sadoff, 1988). The extract was added to POPC in decane and, after removal of the chloroform, the mixture was used to form a planar lipid bilayer across symmetric bathing solutions of 250 mM $BaCl_2$ (or $CaCl_2$), 5 mM $MgCl_2$, in 10 mM Tris Hepes, pH 7.3. The channel activity of the extracts varied from sample to sample, and in most cases it was necessary to reduce the concentration of complexes by serial dilution of the mixture with a decane solution of POPC to obtain a stable bilayer. With increasing dilution, we observed burst activity, then multiple channels and finally single channels. Single channel currents were observed only with voltage steps of +60 mV or greater. This indicated that the extracts contained calcium-permeant ion channels with characteristics similar to those of the plasma membrane channels (Fig. 1, *line 4*).

The channel-active extract contained PHB (immunoassay and chemical assay; Reusch et al., 1992; Reusch, 1989) and PPi (enzymatic assay (Akiyama et al., 1992), but also protein, lipopolysaccharides, and other macromolecules. To further purify the channel-active material, the extract was chromatographed on a size exclusion column (Fig. 2), and eluent fractions (0.25 ml) were tested for single channel activity as above. Most protein did not enter the column and was recovered in the backwash. Peak channel activity was found in a fraction eluting in the molecular weight range 17,000 ± 4,000, as measured by standards of synthetic PHBs and polyisoprenes. This fraction contained PHB (chemical assay), PPi (enzymatic assay) and Ca^{2+} (graphite furnace atomic absorption spectroscopy); the ratio of PHB: PPi was 2:1 and the ratio of PHB to Ca^{2+} was 1:4 to 1:5. An aqueous extract of this fraction had no absorbance at 280 nm, and no protein was detected with silver stain. We found the activity in the purified extracts to be more labile than that in the plasma membrane vesicles or crude extracts; single channels could be obtained for up to 2 weeks when the extract was stored desiccated at 4°C, but activity was lost in a few minutes when the extract was exposed to humid room air. The stability of this extract was enhanced by the addition of phospholipid.

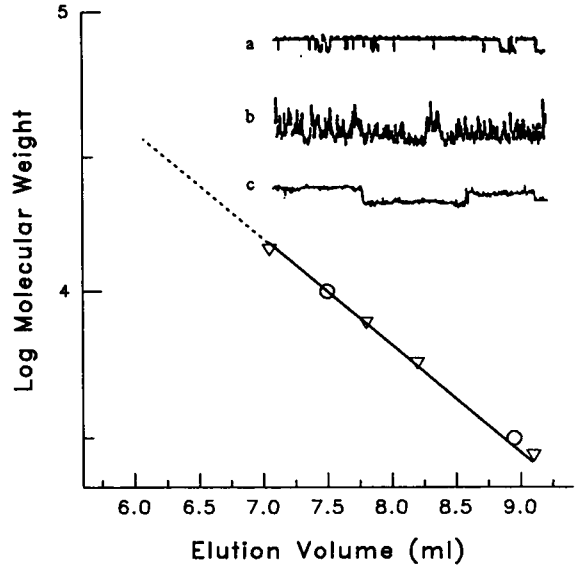


FIGURE 2 Purification of PHB/CaPPi channel complexes by HPLC size exclusion chromatography. Chloroform extracts of *E. coli* competent cells were chromatographed on a non-aqueous size exclusion column using chloroform as an eluent at a flow rate of 0.5 ml/min. Fractions (0.25 ml) were tested for single channel activity by incorporating them into a bilayer of POPC between symmetric solutions of 250 mM $BaCl_2$, 5 mM $MgCl_2$ in 10 mM Tris Hepes, pH 7.3 and applying a voltage step to +80 mV. Fractions showing activity were (*a*) 6.50–6.75 ml; (*b*) 6.75–7.00 ml; and (*c*) 7.00–7.25 ml. MW standards were 79 synthetic PHBs and 209 polyisoprenes.

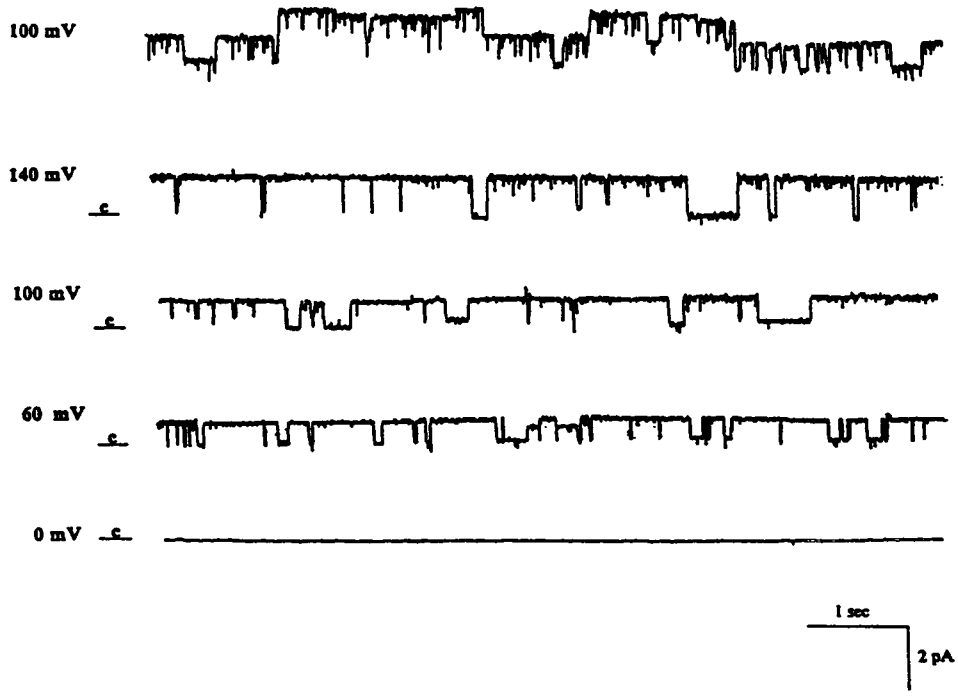


FIGURE 3 Representative unitary current fluctuations with Ca^{2+} in PHB/PPi channel complexes as a function of voltage. PHB/CaPPi complexes from *E. coli*, purified by size exclusion chromatography, were incorporated into a bilayer of POPC (10 ng PHB/100 μg POPC) between symmetric solutions of 250 mM $CaCl_2$, 5 mM $MgCl_2$ in 10 mM Tris Hepes, pH 7.3. Channels were activated by a voltage step to +60 mV, and the potential was then stepped to the value specified. (*Line 1*) Representative record at 0 mV. (*Lines 2–4*) Representative records for single channels at 60, 100, and 140 mV. (*Line 5*) Representative record for a bilayer containing at least two active channels at 100 mV.

PHB/PPi complexes form channels permeant to Ca^{2+}, Sr^{2+}, and Ba^{2+}

The activity of the chromatography-purified extract was examined by incorporating it into a bilayer of synthetic POPC between symmetric solutions composed of 10 mM Tris Hepes, pH 7.3, 5 mM $MgCl_2$, and 250 mM of either $CaCl_2$, $SrCl_2$, or $BaCl_2$. When holding potentials $\geq$60 mV were maintained for periods of several minutes at room temperatures (20–23°C), stepwise current fluctuations were observed (Fig. 3). The preparation was diluted with POPC as above to the point at which multiple channels (Fig. 3, *top line*) or single channels (Fig. 3, *lines 2–4*) could be observed. The conductances were not significantly altered when Ca^{2+} was reduced from 250 to 100 mM, indicating that binding sites in the channels were saturated with respect to the carrier cation. The higher concentrations were preferred because the channels were more stable at high salt concentrations. We surmise that the PPi is less likely to dissociate from the complex and dissolve in the aqueous medium when the bathing solutions are of high ionic strength. Mg^{2+} was not essential for channel activity, but it appeared to increase the stability of the channel complexes and acted as a mild blocker (see below), which made it easier to observe channel openings and closures. Consequently, Mg^{2+} was added to all solutions at an Mg^{2+}:M^{2+} ratio of 1:50; at this concentration, the conductance of the carrier ion was not significantly affected (see below).

The current records in Fig. 3 show movement of divalent cations from *cis* to *trans* at several potentials. Single channel current magnitudes increased with increasing potential, as did the probability the channel was in the open state (pO). At 60 mV, the probability was 0.45. Increasing the potential to 100 mV and then to 140 mV increased the probability to 0.57 and 0.72, respectively. Activity customarily lasted as long as the bilayer was intact, usually 20 min to an hour. Typically there were long periods of single channel activity

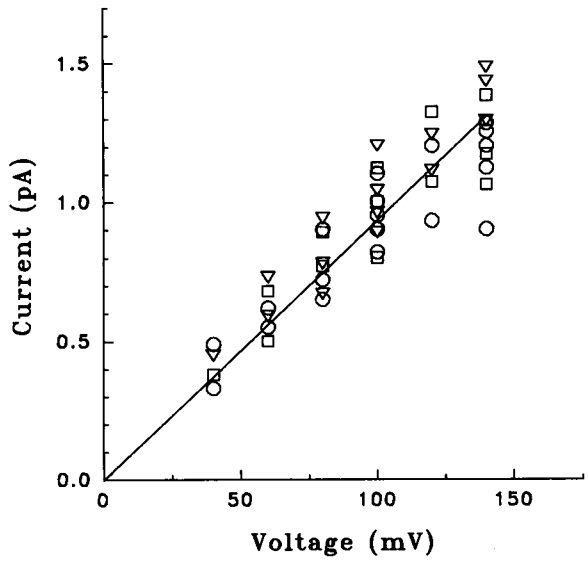


FIGURE 4 Current-voltage relations for PHB/PPi complexes bathed in Ca^{2+}, Sr^{2+}, and Ba^{2+}. PHB/PPi channel complexes from *E. coli*, purified by size exclusion chromatography, were incorporated into planar bilayers of POPC (10 ng/100 μg POPC) between symmetric solutions of 10 mM Tris Hepes, pH 7.3, 5 mM $MgCl_2$ and 250 mM of either $CaCl_2$, $SrCl_2$, or $BaCl_2$. Channels were activated by a voltage step to +60 mV and the potential was then stepped to the value specified. ○ Ca^{2+}; □ Sr^{2+}; ▽ Ba^{2+}. Each data point is the current magnitude of representative single channels from one trial. The slope of the first order regression line was ~9 pS.

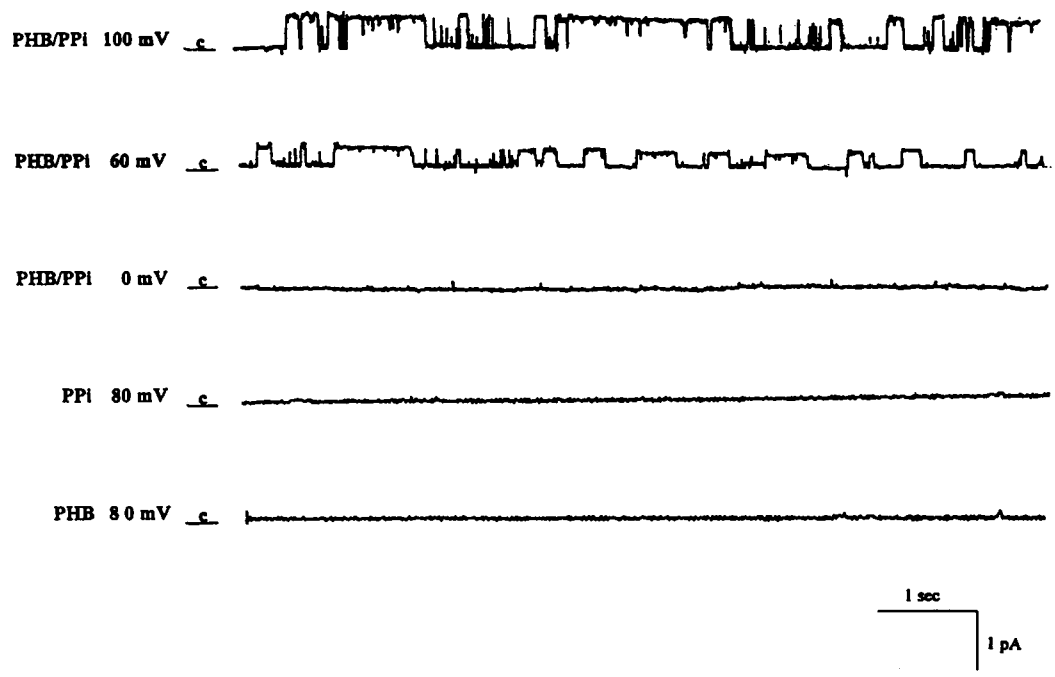


FIGURE 5 Reconstituted channel complexes. (*Line 1*) PHB was isolated from *E. coli* competent cells and purified (see Materials and Methods), and then incorporated into a bilayer of synthetic POPC (10 ng PHB/100 μg POPC) between symmetric solutions of 250 mM $CaCl_2$, 5 mM $MgCl_2$ in 10 mM Tris Hepes, pH 7.3. Holding potentials of 60–120 mV *cis* yielded no channel activity. (*Line 2*) CaPPi was prepared synthetically and added in small amounts (<1 mg) to the aqueous bathing solution on the *cis* side. The bilayer contained only synthetic POPC. Holding potentials of 60–120 mV *cis* yielded no channel activity. (*Lines 3–5*) A dry mixture of PHB and CaPPi was incorporated into liposomes of POPC (100 ng PHB/100 μg POPC with CaPPi in excess). A portion of the liposomes (~1%) was added to the *cis* aqueous bathing solution and allowed to incorporate spontaneously into a bilayer of POPC. Single channel currents were activated by a voltage step to +60 mV, and the potential was then stepped to the value specified. Representative current records at 0, 60, and 100 mV are shown.

with occasional intervals of inactivation. A voltage step of at least +60 mV was required to activate the channels, but once activated, single channels could be observed below this voltage (Fig. 4). At <40 mV, the single channel currents were too small and brief to measure, and at potentials of 120 mV and above, lipid activity and open current noise increased appreciably. There was a variation of 20–25% in the magnitude of the single channel currents, and there were frequent simultaneous double openings and closings suggesting cooperative activity. Subconductance current levels were also frequently observed. We did not perceive significant differences in conductance between Ca^{2+}, Sr^{2+}, and Ba^{2+}, but small differences would not be noticed because of the large experimental variations. The single channel slope conductance, estimated from the slope of the first order regression line, was ~9 pS.

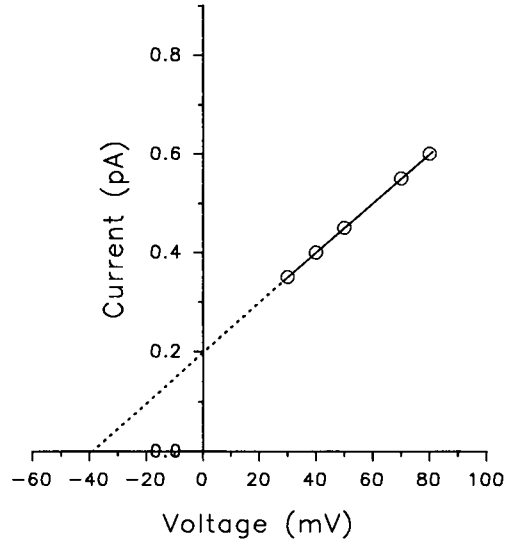


FIGURE 6 Selectivity of PHB/PPi channel complexes for divalent over monovalent cations. Selectivity for Sr^{2+} over Na^+. Single channel current voltage relations for PHB/PPi complexes, extracted from *E. coli* and purified by size exclusion chromatography, and incorporated into bilayers of synthetic POPC with 200 mM $SrCl_2$, 10 mM Tris Hepes, pH 7.3 *cis* and an isotonic solution of 8 mM $SrCl_2$, 288 mM NaCl, 10 mM Tris Hepes, pH 7.3 *trans*. The equilibrium potentials (calculated from concentration) were $E_{Sr} = -40$ mV, $E_{Cl} = +7$ mV, and E_{Na} is nominally plus infinity.

Reconstituted calcium channels of PHB/Ca/PPi

Although no protein was detected in the channel-active fractions, no assay is sufficiently sensitive to rule out the presence of a few protein molecules. To establish the composition of the channels unequivocally, the PHB/CaPPi complexes were reconstituted from PHB, extracted from *E. coli*, and purified of contaminating proteins; and CaPPi,

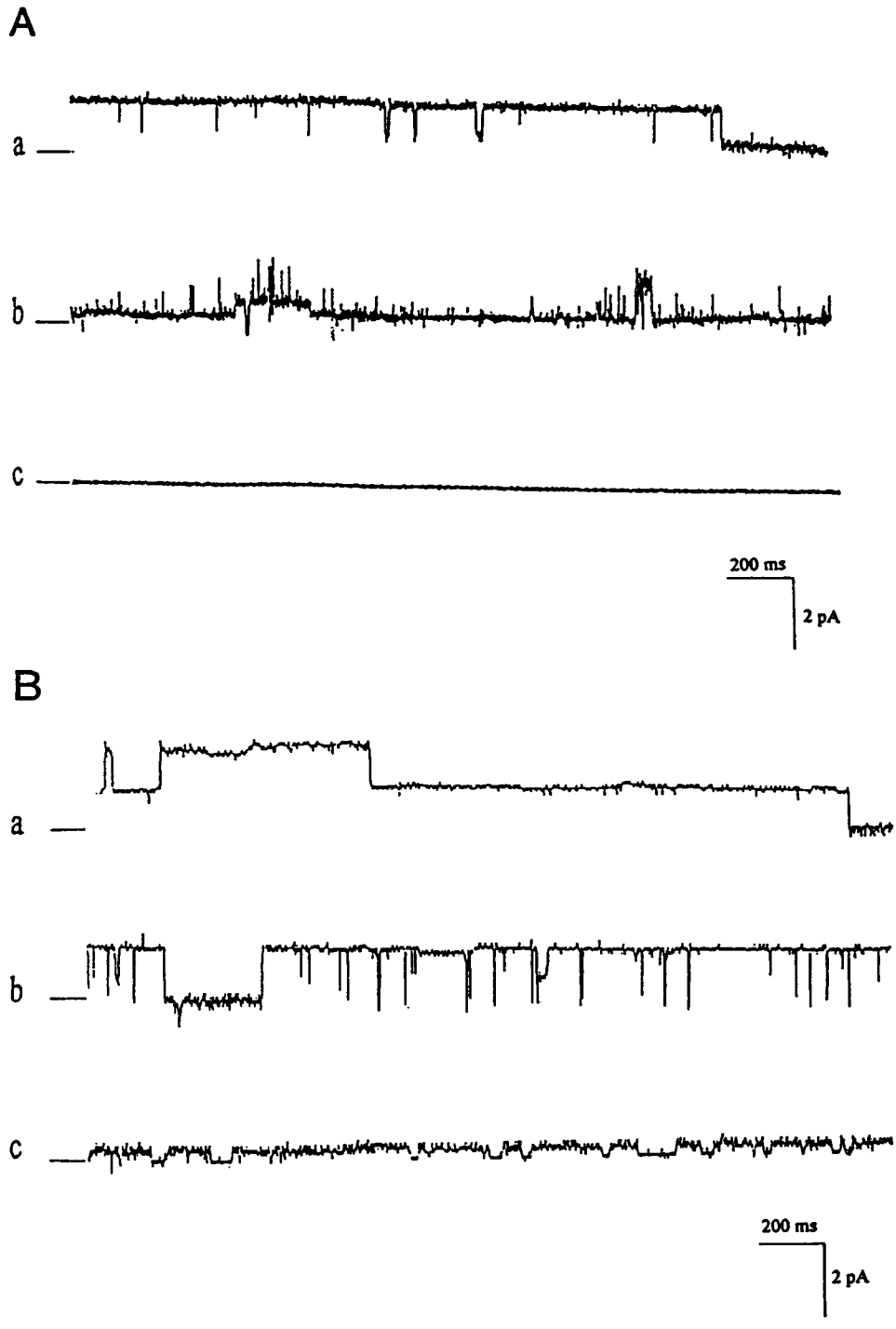


FIGURE 7 Block of PHB/PPi ion channels. (*A*) Block by La^{3+}. Representative single channel current steps at 120 mV for PHB/PPi complexes, extracted from *E. coli*, purified by size exclusion chromatography, and incorporated into POPC bilayers between symmetric solutions of 100 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Tris Hepes, pH 7.3, and with $LaCl_3$ added to the *cis* side as stated. (*a*) No $LaCl_3$; (*b*) 0.20 mM $LaCl_3$; (*c*) 0.55 mM $LaCl_3$. (*B*) Block by Mg^{2+}. Representative single channel current steps at 120 mV for PHB/PPi complexes, prepared as above and incorporated into POPC bilayers between symmetric solutions of 100 mM $CaCl_2$, 10 mM Tris Hepes, pH 7.3, and with $MgCl_2$ added to the *cis* side as stated. (*a*) No $MgCl_2$; (*b*) 2 mM $MgCl_2$; (*c*) 20 mM $MgCl_2$.

prepared from commercial sodium polyphosphate, average chain length 65, and calcium chloride. Three procedures were used (see Materials and Methods). 1) Complexes of PHB/CaPPi were formed in chloroform solution, and the complexes were incorporated into the bilayer in the same manner as the extracted complexes. 2) PHB/CaPPi was incorporated into liposomes, and the liposomes were added to the aqueous bathing solutions for spontaneous insertion into the bilayer. 3) PHB was incorporated into the bilayer, CaPPi was added to the aqueous bathing solutions, and a potential of 60 mV was applied to induce the formation of the complexes within the bilayer.

No activity was detected with the individual constituent polymers; however, single channel currents were observed with PHB/CaPPi complexes formed by all three procedures. The presence of PHB in the bilayer, at concentrations of 10 ng PHB/40 μg POPC between symmetric bathing solutions of 250 mM $CaCl_2$, 5 mM $MgCl_2$, in 10 mM Tris Hepes, pH 7.3, did not produce ion currents at holding potentials of 60–120 mV (Fig. 5, *line 1*). This rules out the possibility

that protein or peptide contaminants in the PHB were responsible for the channel activity. There was also no activity when the bilayer contained only POPC and synthetic CaPPi was added to the bathing solutions (Fig. 5, *line 2*). However, when PHB was in the bilayer and CaPPi was added to the aqueous bathing solutions, voltage steps ≥60 mV for periods of 15 min to an h resulted in single channel activity (not shown). Single channel currents were most easily observed when liposomes containing PHB/CaPPi were added to the bathing solutions and allowed to fuse with the bilayer spontaneously. Representative records are shown in Fig. 5, lines 3–5. These results demonstrate clearly that PHB and CaPPi are both necessary and sufficient for channel activity.

Selectivity of PHB/PPi complexes for divalent cations

In the absence of Ca^{2+}, the PHB/PPi channel complexes were permeant to monovalent cations; however, Ca^{2+} (or Sr^{2+}) competed strongly against monovalent cations. The selectivity of the channel for divalent over monovalent cations and for cations over anions is illustrated in Fig. 6, which shows the single channel current-voltage relations with 200 mM $SrCl_2$, 10 mM Tris Hepes, pH 7.3 *cis*, and an isotonic solution of 8 mM $SrCl_2$, 288 mM NaCl, 10 mM Tris Hepes, pH 7.3 *trans*. The reversal potential was close to the equilibrium potential for Sr^{2+} of −40 mV calculated from the Nernst equation, whereas the chloride equilibrium potential was +7 mV and the sodium equilibrium potential was nominally plus infinity.

Concentration-dependent blocking by transition metal cations and Mg^{2+}

In many types of cells, Ca^{2+} currents are blocked by a variety of transition metals (Hagiwara and Byerly, 1981; Tsien, 1983), presumably because they compete with Ca^{2+} for binding sites. We found La^{3+}, Co^{2+}, and Cd^{2+} to be impermeant in the channel complexes, and to increase closed times in a concentration-dependent manner with the order of effectiveness $La^{3+} > Co^{2+} > Cd^{2+}$ (Fig. 7 *A*). With symmetric solutions of 100 mM $CaCl_2$, 1 mM $Mg Cl_2$ in 10 mM Tris Hepes, pH 7.3, the single channel current was completely inhibited by 0.6 ± 0.1 mM (4) La^{3+}, 1.5 ± 0.5 mM (3) Co^{2+}, or 8 ± 2 mM (3) Cd^{2+}.

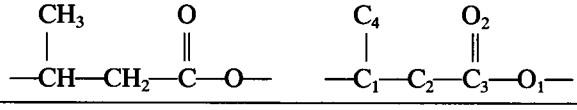
Mg^{2+}, although permeant, also blocked the Ca^{2+} current, but less effectively (Fig. 7 *B*). With symmetric solutions of 100 mM $CaCl_2$ in 10 mM Tris Hepes, pH 7.3, the main effect of adding 2 mM $Mg Cl_2$ or less to the *cis* side was to increase the frequency of channel closings, but raising Mg^{2+} concentrations further significantly diminished single current magnitudes. Complete blocking of Ca^{2+} currents required >20 mM Mg^{2+}.

DISCUSSION

In this study, we show that the plasma membranes of *E. coli* contain voltage-activated calcium channels. We further demonstrate that the channels display many of the characteristics associated with protein calcium channels in higher organisms, and that they are composed of two linear homopolymers with exceptional physical properties, PHB and PPi.

PHB is a flexible, linear molecule which in the solid state forms left-handed 2_1-helices (Cornibert and Marchessault, 1972) and in solution retains a flexible helical form (Marchessault et al., 1970). If the bond angles remain constant, then the shape of the molecule can be described by four backbone dihedral angles (Table 1). Computer modeling studies suggest that the amphiphilic PHB molecule may assume a large number of exolipophilic endopolarophilic helical forms with ester groups in the preferred antiperiplanar orientation, and also indicate that the conformation of the molecule is highly sensitive to small changes in one or more of the three less constrained backbone dihedrals (Fig. 8, Table 1). A change of only 1° in a single dihedral angle can effect a change in helical rise of as much as 2.5 Å. This elasticity implies that PHB may wrap around other macromolecules and conform to their contours. The highly charged polymeric anion, PPi, is more rigid, but the P-O-P

TABLE 1 Backbone dihedral angles and helix parameters for some exolipophilic-endopolarophilic conformations of PHB



PHB_{140} Structures	A	Conformational variants of A (change in dihedral angle)						B	C
Residues/turn	14							12.5	9
C_2–C_3–O_1–C_1	−176							−174	176
C_3–O_1–C_1–C_2	85.8	−1.0	1.0					91.7	73.9
O_1–C_1–C_2–C_3	−155.5			−1.0	1.0			−153.7	−142.5
C_1–C_2–C_3–O_1	−104.1					−1.0	1.0	−112.7	−142.5
Rise (Å)	4.5	2.4	7.0	5.5	3.0	6.7	2.7	5.0	5.0
Internal diameter (Å)	16.4	16.1	16.8	15.9	17.0	16.8	16.1	14.8	11.0

Bond angles are the same in all conformers: O2–C3–O1 = 125°; O2–C3–C2 = 125°; C2–C3–O1 = 110°.

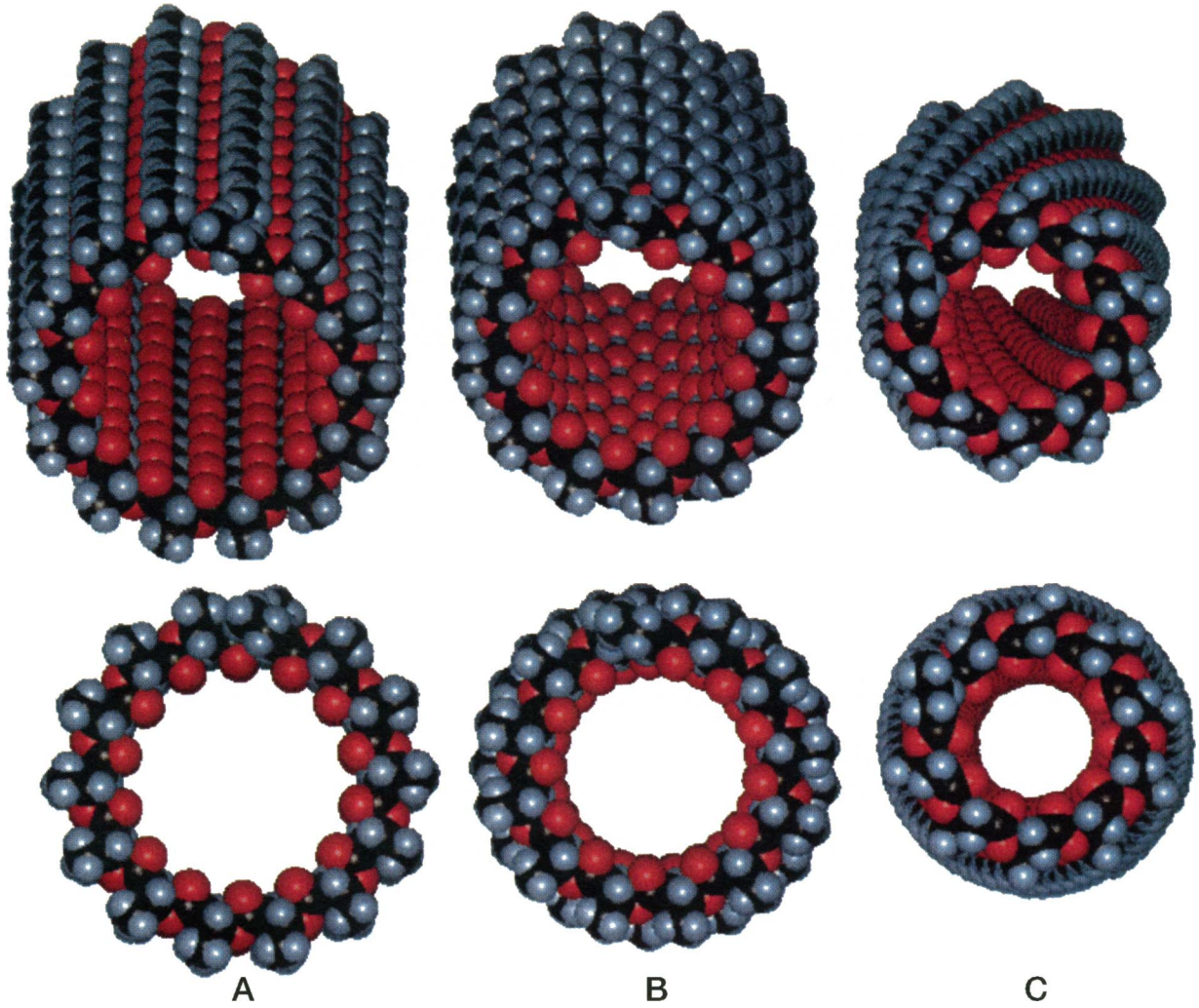


FIGURE 8 (*A*) Some exolipophilic-endopolarophilic helical conformations of PHB. Computer-generated models (Insight II, Biosyms Technologies, Inc., San Diego, CA) of the amphiphilic PHB molecule in helical conformations with the methyl groups facing outward and the ester groups facing inward. All three conformers are 140 residues in length and have the ester groups in the preferred antiperiplanar arrangement (see Table 1). (*A*) Ester groups are aligned at each turn of the helix; (*B*) ester groups alternate; (*C*) ester groups are helically skewed.

linkages that join the phosphoryl tetrahedra have great rotational flexibility so that adjacent residues can twist into a number of different conformations (Fig. 9). As a result, the inorganic polyphosphates are polymorphic (Corbridge, 1985; Majling and Hanic, 1980), and their conformations are sensitive to cation charge and coordination geometry. Consequently, we surmise that the PPi chains in the channel continually adjust their conformations in response to the charge and coordination requirements of transient cations.

Present estimates of the lengths of the polymers are 130–150 residues for PHB (MW ~12,000), as measured by size exclusion chromatography (Seebach et al., 1994), and 55–70 residues for PPi (MW ~5000), as determined by acrylamide gel electrophoresis (Castuma et al., 1995). The molecular size of the channel complex, estimated here as 17,000 ± 4,000, suggests a 1:1 relationship between the two polymers. In a hydrophobic environment such as the membrane, it is assumed that the PHB will position its more lipophilic methyl moieties to face the fatty acyl chains of the phospholipids and surround the polar PPi framework helix with its hydrophilic ester carbonyl oxygens (Reusch and Sadoff (1988); Figs. 8 and 10, *A* and *B*). In such a structure, a multi-lane channel is formed between the two polymers, with the outer wall lined with solvating carbonyl oxygens and the inner wall girdled by ladders of paired monovalent phosphoryl anions. Cations may move through the channel in parallel single file lanes in response to concentration or voltage gradients. In the idealized model, each Ca^{2+} is held in an ionophoretic cage solvated by eight oxygens–four phosphoryl oxygens from two adjoining units of PPi (total charge-2), and four ester carbonyl oxygens (two neighboring PHB units from one turn and two contiguous units from the turn directly above or below) (Fig. 10 *D*). All binding sites are identical and spaced at frequent intervals along the channel wall, hence there is no net potential energy cost to ion movement within the channel (Fig. 10 *C*). Recently,

Seebach et al. (1994) put forward an alternate model for the membrane complexes in which the PHB molecules fold to form square-shaped sheets of ~50 × 50 Å. In this model, ellipsoid arrangements of PHB segments from neighboring sheets cooperatively solvate CaPPi.

The response of the complexes to voltage gradients and their permeability to monovalent cations, when Ca^{2+} is absent, is in accordance with the behavior of many protein Ca^{2+} channels, as is the blocking by La^{3+}, Co^{2+}, and Cd^{2+} (McCleskey and Almers, 1985; Tsien et al., 1987). For PHB/PPi complexes, these behaviors derive from the individual and combined molecular properties of the two polymers, which may be exploited to discriminate among cations by ion size, binding energy, hydration energy, and coordination geometry. The polymeric anion, PPi, with its string of negative charges bridging the bilayer can act as a sensor of the membrane potential, and may react to a change in this potential by stretching or sliding within the pore, thus initiating an ion flow. At the interfaces, PPi attracts cations to the mouth of the channel where its paired monovalent sites preferentially bind divalent cations (Eisenman and Horn, 1983) (Fig. 10*A*). Thus the molecular properties of PPi alone may account for the blocking of Na^+ currents by Ca^{2+} or Sr^{2+} (Fig. 6). PPi, however, does not discriminate well among divalent cations nor against trivalent cations. It is PHB that must serve as a second selectivity filter, by preferentially solvating those cations whose coordination geometries best conform to the spatial arrangement of its ester carbonyl oxygens, and by defining together with PPi the size and shape of the cation equilibrium cavity (Fig. 10 *C*). Mg^{2+} can enter the channel but it competes poorly with Ca^{2+} for binding sites because of its smaller size, smaller coordination number, regular octahedral coordination geometry, preference for nitrogen ligands, and slow rate of water exchange (Martin, 1990). The transition metals should also bind tightly to the anionic PPi at the channel face, but they are inhibited from entering because of their size, charge, or coordination geometry. By occupying the binding sites at the channel opening, they block the entry of permeant cations.

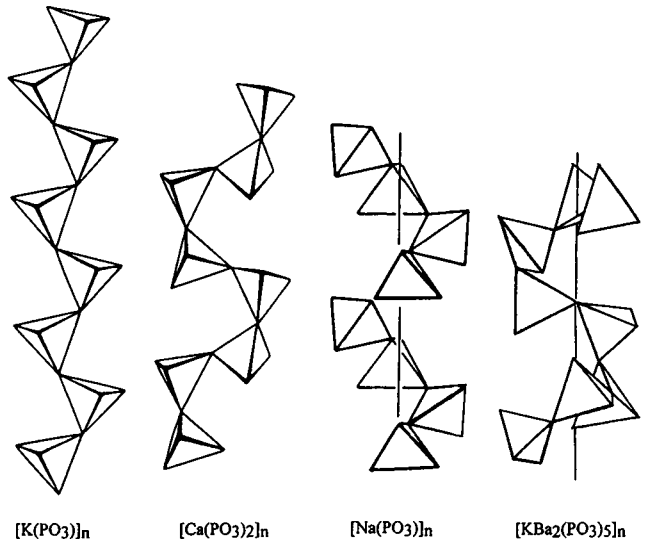


FIGURE 9 Some chain configurations of PPi salts. Drawings show a few of many possible configurations of PPi salts taken from crystal structures (Corbridge, 1985; Matheja and Degens, 1971). Structures illustrated all contain eight phosphoryl residues (PO_3^-), with each residue represented by a tetrahedron. The P-O-P bonds joining the tetrahedra have considerable rotational flexibility, thus allowing the chains to twist into a large variety of conformations.

The mechanism described above is consistent with the observed cation permeance and single channel conductance of the channel. As expected, Ca^{2+} is particularly well suited to bind to the channel sites. It has a preference for forming seven or eight coordinate bonds to oxygen ligands and is well known for its ability to cross-link molecules with irregular geometries (Lehn, 1973; Simon et al., 1973). Sr^{2+} and Ba^{2+}, although increasingly larger in diameter, have coordination geometries similar to that of Ca^{2+} and may also be expected to be permeant. Ba^{2+} did not carry the current with significantly greater efficiency as it does in many, but not all, calcium channels (Dubas et al., 1988). This suggests that channel diameter is a limiting factor so that the lower binding affinity associated with increasing ion size is countered by increased steric hindrance. Deformation of one lane of the channel by the larger Ba^{2+} may constrict neighboring lanes and reduce total current.

The PHB/PPi channel complex may be viewed as a single molecule of an ion-conducting, solid polymer electrolyte (Armand, 1987) positioned across a membrane, with CaPPi forming a salt bridge between the cytoplasm and the extracellular medium. Prevailing theories of ion conduction by these substances indicate that the polymer must be in the elastomeric state to permit the polymer backbone to undergo the segmental motions that effect the transfer of cations from one coordinating site to the next (Fig. 11; Gray, 1992). It is probable from the above that there is a great diversity of PHB/PPi conformations and that the arrangement of the atoms is less rigid and uniform than is suggested by the diagrammatic models. Within each molecular complex there may be regions of atypical structure and liquidic character as the two polymers adjust in synchrony to the size, charge, and coordination requirements of individual cations passing through. Such plasticity of structure may account for the many subconductance states observed in the channel records.

The PHB/Ca^{2+}/PPi complex is a "minimalist" Ca^{2+} channel; every atom contributes and none can be spared. Both the polymeric salt and the solvating polymer are composed of rudimentary molecules available to the earliest cells; consequently, the association between CaPPi and PHB may date back to primordial times. Despite its deceptive simplicity, this polymer electrolyte complex exhibits many of the characteristics of proteinaceous Ca^{2+} channels, i.e., it is selective for divalent over monovalent cations, permeant to Ca^{2+}, Sr^{2+}, and Ba^{2+}, and blocked by La^{3+}, Co^{2+}, and Cd^{2+}. The many points of resemblance between the postulated structure of the

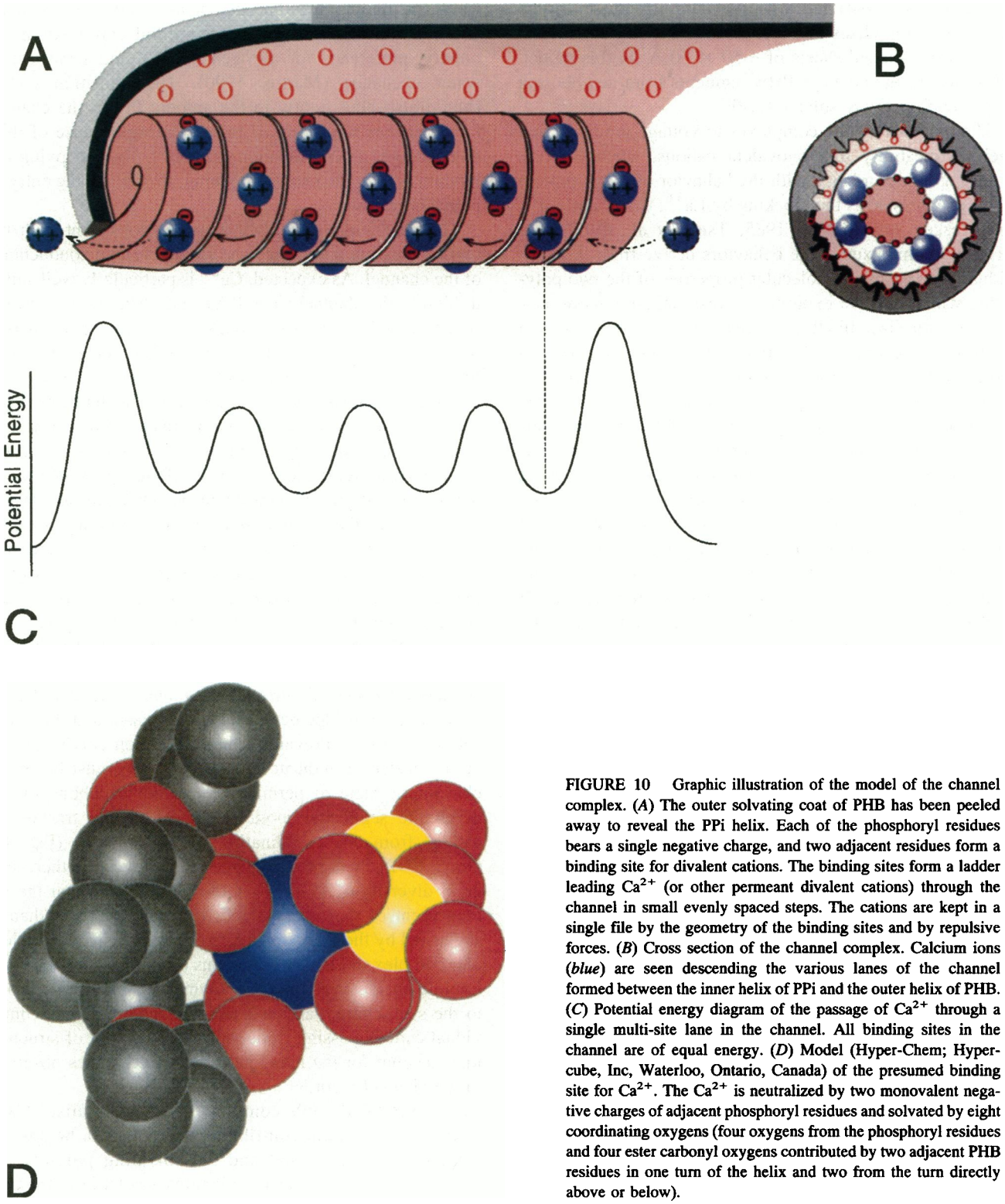


FIGURE 10 Graphic illustration of the model of the channel complex. (*A*) The outer solvating coat of PHB has been peeled away to reveal the PPi helix. Each of the phosphoryl residues bears a single negative charge, and two adjacent residues form a binding site for divalent cations. The binding sites form a ladder leading Ca^{2+} (or other permeant divalent cations) through the channel in small evenly spaced steps. The cations are kept in a single file by the geometry of the binding sites and by repulsive forces. (*B*) Cross section of the channel complex. Calcium ions (*blue*) are seen descending the various lanes of the channel formed between the inner helix of PPi and the outer helix of PHB. (*C*) Potential energy diagram of the passage of Ca^{2+} through a single multi-site lane in the channel. All binding sites in the channel are of equal energy. (*D*) Model (Hyper-Chem; Hypercube, Inc, Waterloo, Ontario, Canada) of the presumed binding site for Ca^{2+}. The Ca^{2+} is neutralized by two monovalent negative charges of adjacent phosphoryl residues and solvated by eight coordinating oxygens (four oxygens from the phosphoryl residues and four ester carbonyl oxygens contributed by two adjacent PHB residues in one turn of the helix and two from the turn directly above or below).

channel complex and multiple site, single file Ca^{2+} channel structures described by Almers and McCleskey (1984), Hess and Tsien (1984), and Tsien et al. (1987), suggest PHB/PPi complexes may serve to further our understanding of the fundamental structural features and molecular mechanisms underlying ion transport.

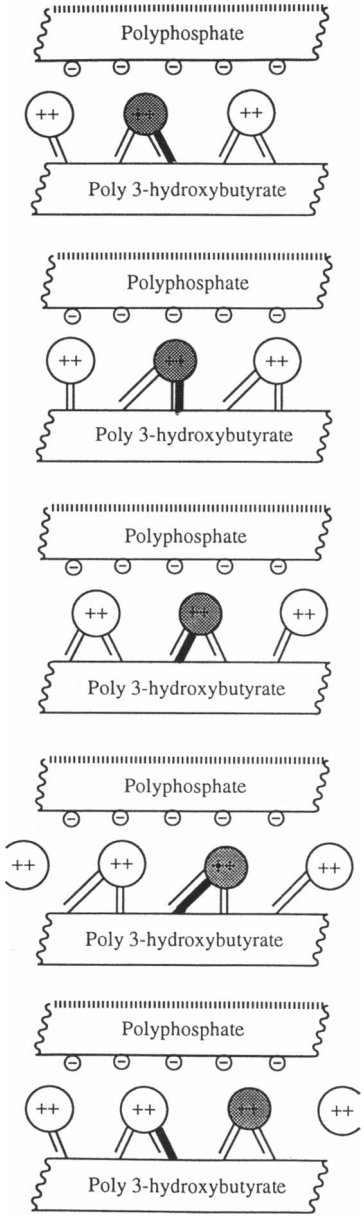


FIGURE 11 Schematic diagram of Ca^{2+} moving through a lane of a PHB/PPi channel. A single lane of the PHB/PPi channel in which the monovalent negative charges of PPi line the inner wall and the ester carbonyl oxygens of PHB (represented by lines joining PHB to Ca^{2+}) line the outer wall. Segmental motions of the PHB backbone transport the cations from one binding site to the next equivalent binding site. One Ca^{2+} is shaded and one ester carbonyl bond is bold to make it easier to follow their putative movements during ion conduction.

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