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Calcium and Barium Permeable Channels from *Aplysia* Nervous System Reconstituted in Lipid Bilayers

Mary D. Coyne*, Daniel Dagan**, and Irwin B. Levitan
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Summary. Ion channels permeable to barium and calcium were reconstituted from the *Aplysia* nervous system into phospholipid bilayers formed on the tips of patch electrodes. With asymmetrical concentrations of barium or calcium on the two sides of the bilayer, the single-channel currents reversed at the calculated barium or calcium reversal potentials, indicating that the channels were cation selective. Channels with conductances of 10, 25 and 36 pS were routinely observed. Calcium and barium were equally effective as charge carriers for the 36-pS channel, whereas magnesium was at least fifteenfold less effective. The gating of all three channels was independent of the voltage across the bilayer, but was affected by the dihydropyridine calcium channel agonist Bay K 8644 (Bay K). In the presence of Bay K but not in its absence, long discrete gating events were routinely observed, suggesting that the dihydropyridine increased the probability of long open states as it does for calcium channels in other systems.

Bilayers invariably contained more than a single channel (or conductance state). This was observed even when the *Aplysia* nervous system membranes were prepared in the presence of cytoskeleton disrupting agents, or when the membrane proteins were diluted extensively with exogenous phospholipid. Furthermore, transitions between conductance levels were observed with high frequency. These findings, together with the fact that all of the conductance states share certain properties including voltage-independence and sensitivity to Bay K, suggest that the apparent multiple channel types may in fact represent subconductance states of a single ion channel.

Key words calcium channels · *Aplysia* nervous system · channel reconstitution · dihydropyridines · conductance substates · single-channel recording

Introduction

Channels permeable to calcium ions are involved in controlling many cellular processes in both excitable and nonexcitable tissues (Reuter, 1983; Tsien, 1983). There appear to be several different kinds of calcium channels in neurons and cardiac cells (Nowycky, Fox & Tsien, 1985; Miller, 1985; Nilius et al., 1985), and perhaps in other cell types. To fully understand the control mechanisms in which these channels participate, a necessary step is the identification and characterization of the various species of calcium channels in a given cell or tissue. One particularly appropriate system in which to study calcium channel regulation is the central ganglia of the marine mollusc, *Aplysia*. A great deal of information exists about calcium currents and calcium-dependent processes in *Aplysia* neurons (*see*, for example, Eckert & Chad, 1984; Ewald & Levitan, 1987), and thus information at the single-channel level can be related directly to known physiological processes.

One way to examine a wide spectrum of ion channels from a given tissue is to reconstitute the channels from membrane vesicles into artificial lipid bilayers (Miller, 1983). A recent variant of this method which has been particularly useful is the formation of bilayers on the tips of patch electrodes ("tipdipping"—Wilmsen et al., 1983). Such bilayers are sufficiently stable to enable transfer of the electrode into a series of different solutions, and thus ion changes and pharmacological manipulations can be carried out with ease. We report here that a variety of channels permeant to calcium and barium ions, derived from membranes of the *Aplysia* nervous system, can be reconstituted in "tipdip" bilayers. Of the various channels seen, several were observed at sufficiently high frequency to allow a preliminary kinetic and pharmacological analysis.

Materials and Methods

Membrane Vesicle Preparation

Pleural, pedal, buccal and cerebral ganglia were dissected from adult *Aplysia* and were rinsed with ice-cold Tris-EGTA (10 mM,

* *Permanent address:* Department of Biological Sciences, Wellesley College, Wellesley, MA 02181.

** *Permanent address:* Rappaport Family Institute, Technion Faculty of Medicine, Department of Physiology and Biophysics, Haifa, Israel.

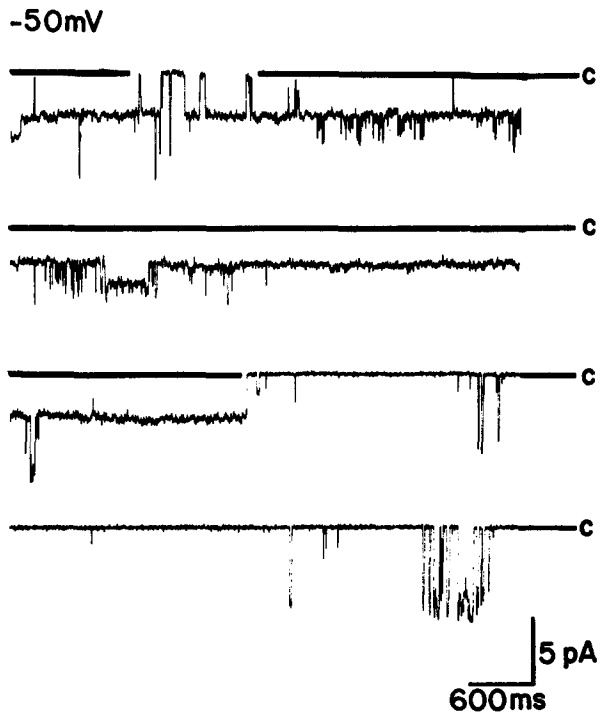

Fig. 1. Different kinds of channel activity in a bilayer formed at the tip of a patch electrode. The four current records were continuous. The zero current is marked by a solid line (*C*) and downward deflections indicate inward current in "wellular" convention. Leak current was subtracted in this and subsequent figures. At a membrane potential of −50 mV with symmetrical 100 mM $BaCl_2$, single-channel currents of four different sizes can be discerned with prolonged open states, rapid transitions, and silent or null mode gating

pH 7.5), followed by three 5-ml rinses with 5 mM HEPES-Tris, pH 7.5, containing 100 μM each of dithiothreitol, phenylmethylsulfonyl fluoride, N-*p*-tosyl-L-lysine chloromethyl ketone, and leupeptin (Sigma, St. Louis, MO) (buffer A). Ganglia were homogenized in a motorized Teflon-glass homogenizer (1 ml buffer A/5 ganglia) with 5-10 strokes at low speed. After the connective tissue sheath was removed, the material was further homogenized in a ground glass homogenizer, followed by centrifugation at 4°C for 15 min at 10,000 × *g*. The 10,000 × *g* supernatant was then centrifuged at 100,000 × *g* for 60 min, and the resulting pellet was resuspended by homogenization in 1.2 ml of buffer A.

Membrane Reconstitution

Phosphatidylserine and phosphatidylethanolamine (Avanti, Birmingham, AL) in a 1 : 3 wt/wt mixture were dried under nitrogen and sonicated in buffer A at a concentration of 80 μg/ml until a clear solution was obtained. *Aplysia* nervous system membrane vesicles were added to give a lipid/protein ratio of 1000 : 1 (wt/wt). The mixture was vortexed thoroughly, then frozen and thawed twice. Before freezing a third time at −70°C the preparation was aliquotted into a series of tubes. Single aliquots were thawed immediately prior to use in bilayer experiments.

Patch Electrodes

Patch electrodes were pulled from Jencons H15/10 hard borosilicate glass (Jencons Scientific, Leighton Buzzard, England), fire-polished, and filled with 5 mM HEPES-Tris buffer (pH 7.5) containing concentrations of divalent cations as described for individual experiments.

Monolayer/Bilayer Formation

Monolayers were formed by placing 20-50 μl of the reconstituted membranes into one well of a 96-well microwell plate (Falcon 3910), and underlaying 300 μl of buffer A. Bilayers were formed on patch electrode tips by dipping the electrode through the monolayer with positive pressure, releasing the pressure, pulling out of solution, and redipping (Wilmsen et al., 1983). With barium ions (100 mM) in both the electrode and the microwell, this manipulation resulted in bilayer formation in 85% of the attempts. Seal resistances ranged between 20-100 GΩ.

Bilayer Manipulations

Solutions were changed by transferring the electrode with bilayer into adjacent wells of the 96-microwell plate with the aid of a micromanipulator, or by adding drugs and mixing directly in the original well. A fresh sterile plate was used for each experiment, and all solutions were filtered through 0.45 μm filters. The microwell plate and manipulator were enclosed in an inverted Precision Thelco oven to minimize electrical and vibrational noise.

Single-Channel Recording

A Yale (New Haven) patch-clamp amplifier with a 10 GΩ feedback resistor was used. Activity was recorded on a beta video cassette recorder (Sony) after 16-bit digitization at 44 kHz with a pulse code modulator (Sony PCM-701-ES). After filtration at 1 kHz with an 8-pole Bessel filter (Frequency Devices), data were analyzed with an Indec (11/23) laboratory computer system. All voltages are given as if the inside of the electrode is the extracellular side of the membrane and the microwell is the intracellular side ('wellular' convention).

Results

From the earliest studies of single calcium channels (Reuter et al., 1982), investigators have utilized barium as the charge carrier, because barium usually carries current through these channels at least as well as calcium; in addition this helps to circumvent problems associated with Ca^{2+}-dependent inactivation. To optimize the conditions for detection and identification of channels permeant to divalent cations, barium acetate (or barium chloride) were used in asymmetrical concentrations on the two sides of the bilayer. Barium was identified as the charge car-

rier by shifting its reversal potential from that of the anion. The following asymmetrical concentrations of barium acetate in buffer A were used; (i) 200 mM pipette/100 mM well, (ii) 100 mM pipette/10 mM well, (iii) 500 mM pipette/1 mM well. The reversal potentials of barium and acetate are sufficiently separated under these conditions to allow for unequivocal identification of barium as the charge carrier. The calculated reversal potentials for these combinations of ion concentrations are respectively: (i) $E_{Ba} = +8.7$ mV, $E_{Ac} = -17$ mV: (ii) $E_{Ba} = +29$ mV, $E_{Ac} = -58$ mV; (iii) $E_{Ba} = +78$ mV, $E_{Ac} = -156$ mV. The channels in the bilayers were classified as cation selective if the single-channel currents were observed at the anionic reversal potential and changed sign at the barium reversal potential.

In early experiments using lower lipid/protein ratios and 100 mM barium on both sides of the bilayers, gating was observed in more than 80% of the bilayers made from *Aplysia* membrane vesicles (Fig. 1). Invariably there appeared to be more than one active channel or conductance level in the bilayer, possibly due to aggregation of channels or to insufficient dilution by the added phospholipid. To determine whether aggregation of channels could result from cytoskeletal linkage of channels, membranes were prepared in the presence of the microtubule and microfilament disrupting drugs, colchicine (10 μM) and cytochalasin B (20 μM). No reduction in the number of conductance levels per bilayer was observed after these treatments. Further attempts to reduce the number of active channels per bilayer were made by adding more phospholipids during membrane reconstitution (*see* Materials and Methods). At the high dilutions finally used (1000:1 (wt/wt) phospholipid/protein), the number of bilayers with active channels decreased to about 30%, but the number of conductance levels was still always greater than one.

The pattern of gating of barium-permeable channels was episodic, with periods of activity interspersed with relatively long periods of inactivity (Fig. 1). The three sizes of channels observed most often are shown in Fig. 2*A*. With 100 mM Ba on both sides of the membrane, the unitary current amplitudes at −90 mV were approximately 1 pA (top), 2.2 pA (middle), and 3.9 pA (bottom), as indicated by amplitude histograms (Fig. 2*B*). A plot of open-channel current amplitude *vs.* open-channel duration (Fig. 3) demonstrates that the time resolution of the recording system is adequate to distinguish between these three conductance states even at open durations as low a 4 msec. The three channels had chord conductances of 11, 24, and 43 pS in 100 mM symmetrical barium acetate (Fig. 2*B*), and linear open-channel current-voltage relationships with slope conductances of 10, 25, and 36 pS (Fig. 4). The 10 and 25 pS conductance states were found consistently, while the 36 pS channels occurred in a small percentage of the membranes. While concerted gating of several channel sizes was the dominant feature during bouts of high activity, occasionally one of the channels could be observed independent of the others, and under these conditions could be examined for voltage dependence and ionic selectivity.

We chose to examine the ion selectivity of the 36 pS channel in more detail because its conductance was greater than that of reported calcium channels. Reversal potentials of single-channel currents were measured for this channel with 100 mM calcium acetate/10 mM barium acetate in the electrode and 10 mM calcium acetate/100 mM barium acetate in the well. The deviation of the observed reversal potential of the single-channel currents from 0 mV, which is a measure of the channel's selectivity for the different cations, was only 1 mV under these conditions (Fig. 5*A*). An estimate of P_{Ba}/P_{Ca} using the Goldman-Hodgkin-Katz equation gives a value close to one, indicating that this channel discriminates only poorly between Ba^{2+} and Ca^{2+} ions. However, when magnesium was substituted for Ca^{2+} in the same kind of experiment, the reversal potential in this case was −23 mV (Fig. 5*B*), indicating a much lower permeability for Mg^{2+} than for Ba^{2+} ($P_{Ba}/P_{Mg} = 16$).

The dihydropyridine calcium-channel agonist Bay K 8644 (Bay K) was able to increase the level of channel activity in the bilayers. After initial formation of the bilayers in the absence of Bay K, often only minimal single-channel activity was observed. Upon exposure to Bay K the level of activity increased dramatically within minutes. Figure 2*A* demonstrates the independent gating of the three main-size categories of channels in the presence of Bay K. In another experiment (Fig. 6), a progressive augmentation of channel activity was seen over a 3-min period of time after addition of Bay K. Initially there were few openings, and they were of brief duration. As the exposure to Bay K continued, the openings became longer and at least two sizes of channels were recognizable. Eventually one channel remained open nearly all the time. This pattern was routinely observed when the bilayer exhibited some channel activity prior to Bay K treatment. Bilayers which lacked *any* channel activity in the absence of Bay K remained inactive when transferred to a well containing the agonist.

Channel activity in the bilayers in the presence or absence of Bay K was voltage independent. In

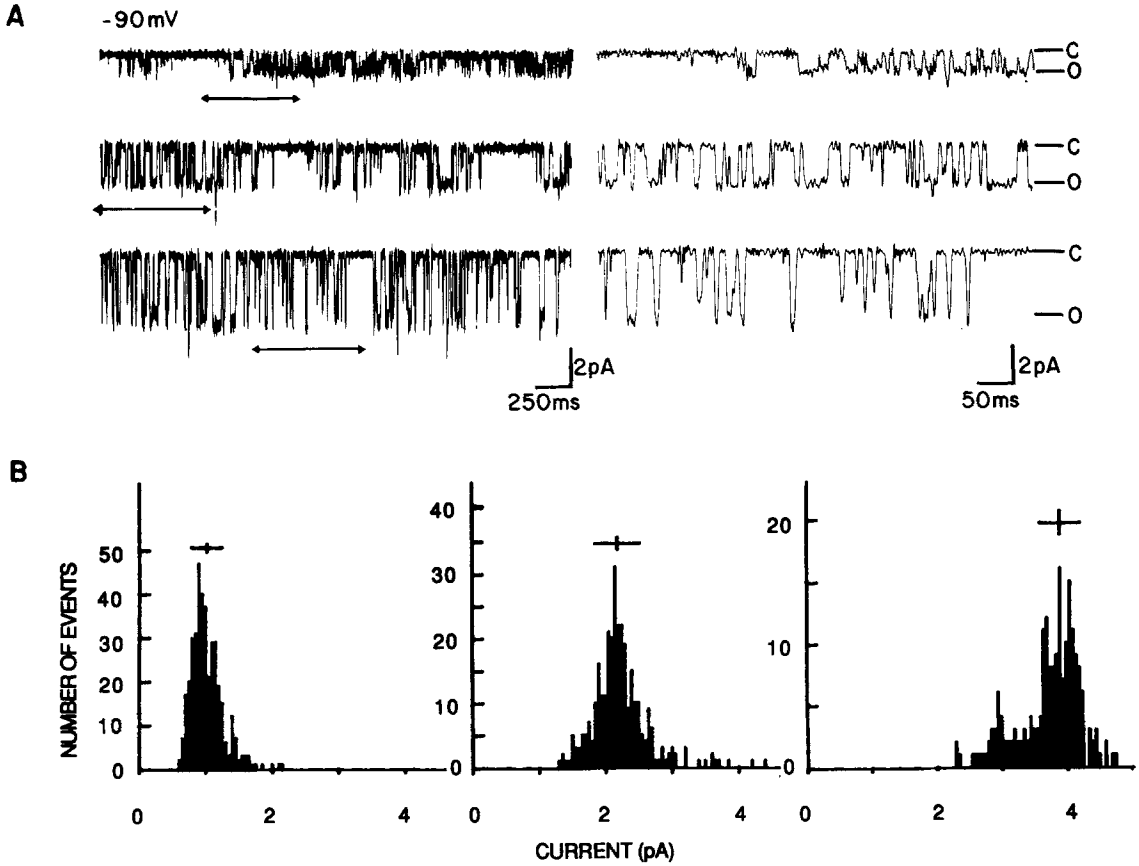

Fig. 2. Three different sizes of barium-permeable channels. (*A*) Activity of three different sizes of channels in a bilayer are shown after treatment with 20 μM Bay K. Membrane potential was −90 mV, with 100 mM symmetrical barium acetate. Horizontal bars between arrowheads under the current records on the left indicate time periods shown at an expanded time scale on the right. (*B*) Amplitude distribution histograms from the same data showing the three most frequently seen conductance states. The maximum values for the number of events for each conductance state were equalized for easier comparisons (note the different vertical scales). The total number of events analyzed was 389 for the smallest channel (left) (mean amplitude 1.02 ± 0.23 pA), 304 events for the middle sized channel (center) (2.18 ± 0.33 pA), and 169 events for the large channel (right) (3.9 ± 0.3 pA). The corresponding chord conductances are 11, 24 and 43 pS

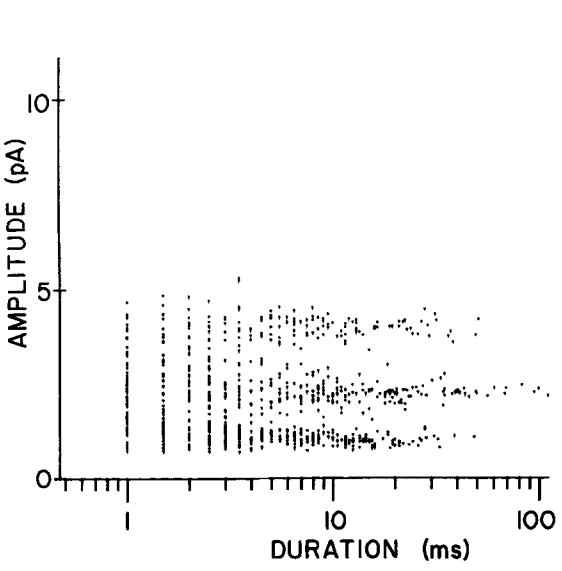

Fig. 3. Channel amplitude *vs.* open duration scatter plot from the same data shown in Fig. 2. The bilayer was held at −90 mV in symmetrical 100 mM barium acetate, in the presence of 20 μM Bay K. Note that the three sizes of channels shown are distinguishable down to about 4 msec duration openings

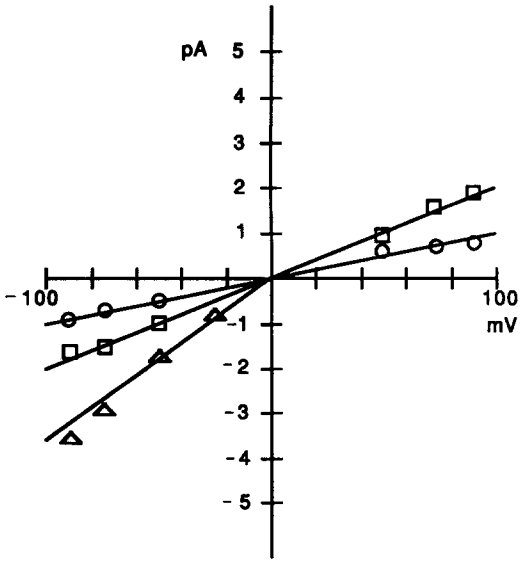

Fig. 4. Open-channel current voltage relationships with symmetrical barium acetate (100 mM) in the presence of 10 μM Bay K for the three conductance states observed most frequently. Slope conductances calculated with linear regression were 10 pS (circles), 25 pS (squares) and 36 pS (triangles)

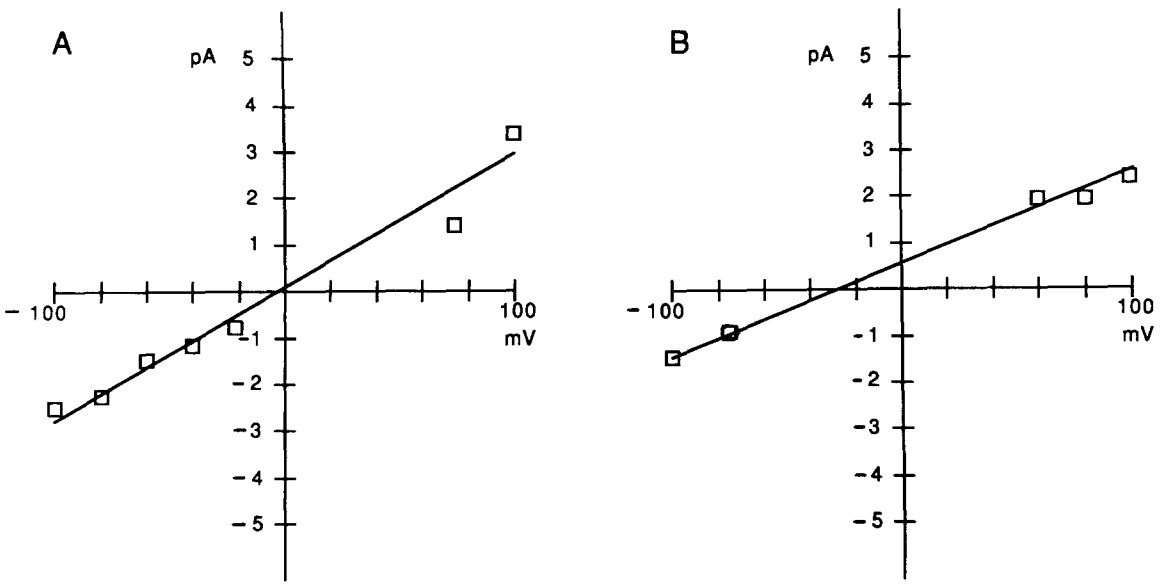

Fig. 5. Current voltage curves for the large 36 pS channel in the presence of 10 μM Bay K under biionic conditions to measure ion selectivity. (*A*) 100 mM $Ba(Ac)_2$/10 mM $Ca(Ac)_2$ in the electrode; 10 mM $Ba(Ac)_2$/100 mM $Ca(Ac)_2$ in the well. Reversal potential −1 mV. (*B*) 100 mM $Ba(Ac)_2$/10 mM $Mg(Ac)_2$ in the electrode; 10 mM $Ba(Ac)_2$/100 mM $Mg(Ac)_2$ in the well. Reversal potential −23 mV

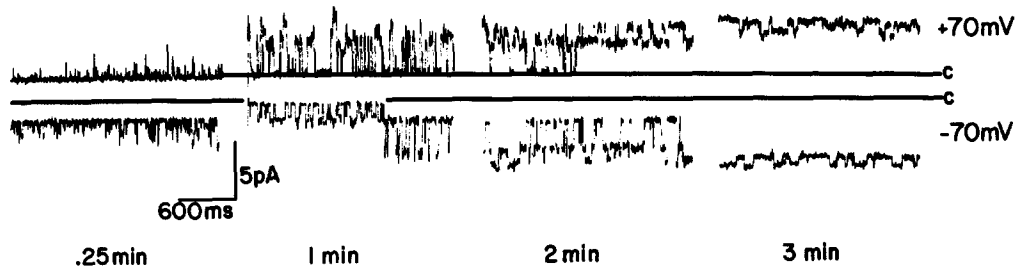

Fig. 6. Onset of the effect of Bay K on voltage-independent gating in symmetrical 100 mM $BaCl_2$. The holding voltage in each pair of sweeps is +70 mV for the top and −70 mV for the bottom trace. Zero current is marked with a solid line (*C*) and open states are downwards in the lower traces of each pair (inward current) and upwards in the upper traces (outward current). The distance between the traces in each pair does not reflect the leak current, which has been subtracted. Time after transfer into 10 μM Bay K is noted under each pair of traces. After transfer of the electrode with the bilayer into Bay K, the gating of several channels was augmented with a progressive change to longer open times. Notice the similarity in the number of channels open at both positive and negative voltages

Fig. 6 where the holding potential was alternated between +70 and −70 mV for 2-sec periods, two channel sizes can be seen gating at both positive and negative voltages. Although changes in open probability did occur with time, these shifts in gating characteristics did not result from the voltage changes and were observed at both voltage polarities. In Fig. 6, (in the 1-min record) a 1-pA channel appeared to gate at −70 mV but not at +70 mV; however, such apparent voltage-dependent gating was seen only rarely.

A preliminary kinetic analysis of the activity of the three sizes of channels was carried out in the presence of Bay K. Measurements in the absence of Bay K were not made because openings were very infrequent and of such brief duration that they were not well resolved by the bilayer recording system. Open duration distributions could be fit by the sums of two exponential curves for the two smaller channels and a single exponential curve for the largest (Fig. 7*A*). The mean open times for the three sizes at −90 mV, calculated from the time constants of the fitted curves, ranged from 7 to 40 msec (Table). Closed duration distributions could be fitted as sums of two exponentials for the 25 and 36 pS conductance channels, and a single exponential for the 10 pS channel (Fig. 7*B*). The values of the closed time constants for the three channels ranged from 4 to 31 msec (Table). These values were obtained by analyzing long stretches of record containing many transitions. However, if small selected sections of the data were analyzed separately for closed dura-

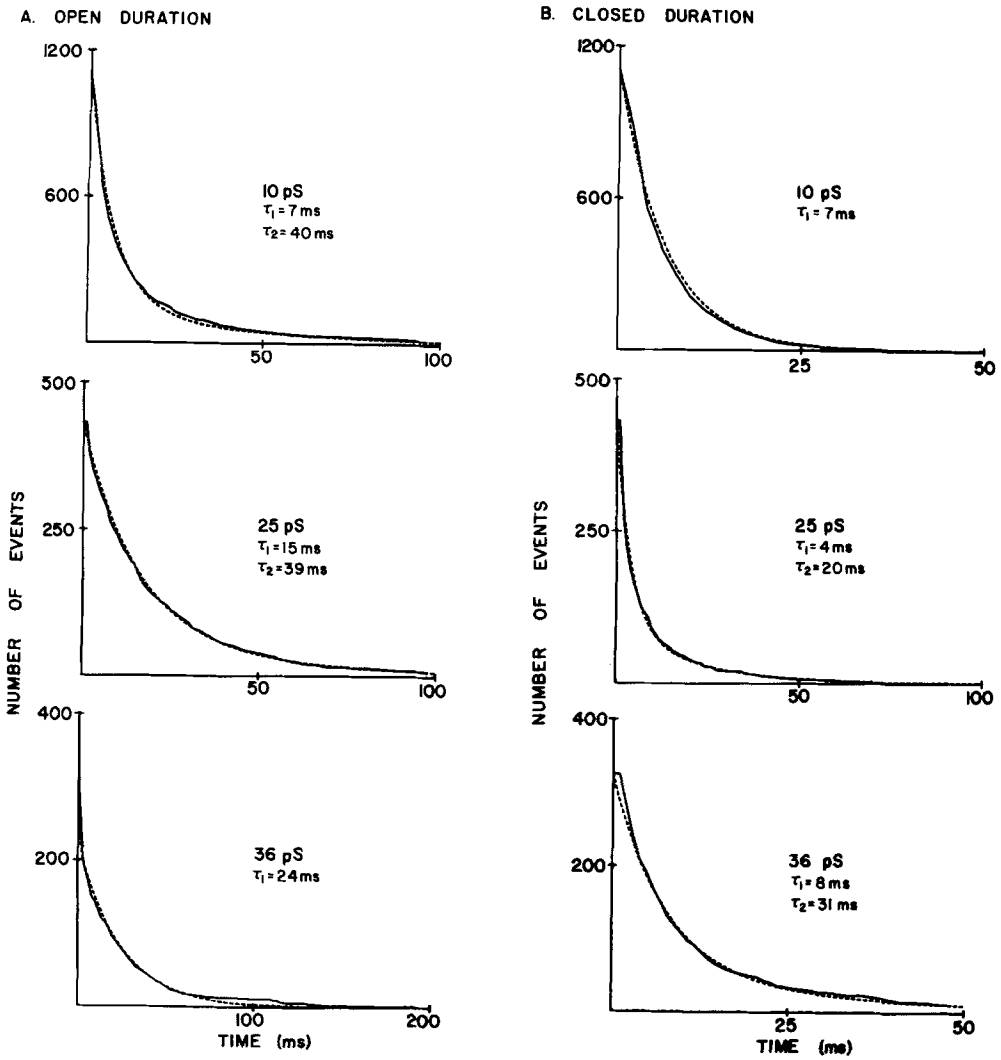

Fig. 7. Kinetic analysis. (*A*) Open-time distributions of the three conductance states shown in Fig. 2, in the presence of Bay K. The sum of two exponential time constants was used to fit the data for the 10 and 25 pS conductance states. A single exponential fit the data for the 36 pS state. (*B*) Closed time distributions of the three conductance states. Double exponential fits were used for the 25 and 36 pS conductance states, and a single exponential fit for the 10-pS state. The time constants for each conductance state appear on the plots and are summarized in the Table

Table. Summary of open and closed time constants for the three conductance states

Conductance state (pS)	Time constants msec			
	Open		Closed	
	τ_1	τ_2	τ_1	τ_2
10	7	40	7	
25	15	39	4	20
36	24		8	31

Data from the analysis shown in Fig. 7.

tions, the τ values varied considerably, suggesting a spontaneous shift between various gating modes (*see*, e.g. Fig. 1, 2 and 8).

While the emphasis above has been on analysis of records in which independent gating of the three types of channels could be seen (e.g. Fig. 2*A*), usually more than one channel was active (Figs. 1 and 8). As shown in Fig. 8, on numerous occasions within even a short stretch of record there are transitions between several current amplitudes. The question arises whether this is due to the simultaneous gating of several channels, or represents substates of a single-channel entity. In the experiment shown in Fig. 8*A*, long transitions of approximately

4 pA dominated the first several seconds, after which there was an abrupt change (arrow), where the channel apparently shifted to an intermediate amplitude level of 1 pA for the next several seconds. Next, two brief high frequency "buzzes" of 1 pA amplitude occurred (middle trace in Fig. 8*A*). Finally, another mode of gating occurred with simultaneous shifts in the open-state probability and the current amplitude. While the four current amplitudes found in this record might be attributed to four different channels, the coincidence of transitions, and the closing of one channel simultaneously with the opening of another, might best be explained by gating between substates of a single channel. Further support for the concept of substates comes from the frequency with which apparently simultaneous openings of channels with different conductances occurred (filled arrows in Fig. 8*B*). In addition, partial closings to an apparent substate level (open arrows in Fig. 8*B*) were often observed. Such gating between substates (or, alternatively, a complex cooperative gating mode of several channels) was a consistent finding in a large proportion of our recordings and occurred at frequencies much higher than predicted by assuming simultaneous openings and closings of independent channels.

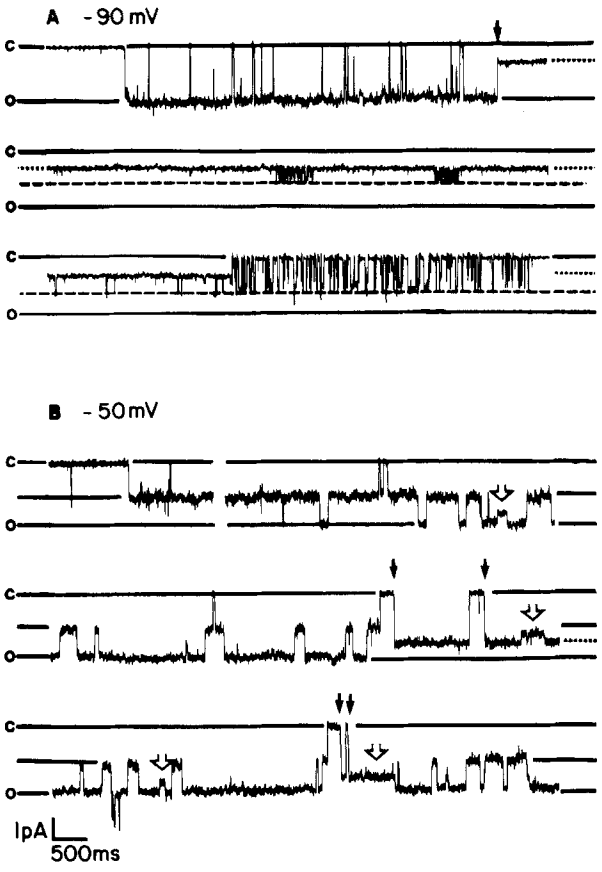

Fig. 8. Demonstration of different gating modes and intermediate current amplitudes in symmetrical 100 mM $Ba(Ac)_2$ in the presence of 10 μM Bay K. *A* and *B* each shows a continuous record from a different bilayer. Solid arrows indicate some of the possible substate transitions. Open arrows indicate some of the intermediate conductance states. (*A*) Both long duration open states and rapid transitions among three different conductances can be seen. (*B*) Long duration open states and transitions to intermediate conductance levels are evident

Discussion

Because of the wealth of physiological information available about calcium currents and their modulation in various *Aplysia* neurons, it is of great interest to characterize the calcium channels responsible for these macroscopic calcium currents. Among other things these studies demonstrate that *Aplysia* nervous system, like many other tissues (Miller, 1985; Nilius et al., 1985; Nowycky et al., 1985), contains more than a single kind of calcium channel.

Reconstitution of channels into artificial phospholipid bilayer membranes has been used widely in recent years to investigate the single-channel properties of a variety of different channel types (Miller, 1983; Miller, 1986). Reconstitution provides excellent experimental control, including ready access to both sides of the membrane, and indeed is the only way to study channels derived from intracellular membranes (e.g. Labarca, Coronado & Miller, 1980; Smith, Coronado & Meissner, 1985). Although in the past it has been difficult to study calcium channels in isolation from their cell of origin, several recent investigations have demonstrated that under appropriate conditions functional calcium channels can be observed reconstituted in artificial bilayer membranes (Ehrlich et al., 1984, 1986; Nelson, French & Krueger, 1984; Smith et al., 1985; Flockerzi et al., 1986; Nelson, 1986; Rosenberg et al., 1986). The present results extend these findings to channels derived from the *Aplysia* nervous system and demonstrate that a number of different conductances permeable to calcium and barium are present in membranes derived from this tissue.

Although it cannot be stated with certainty that the various channels (or conductance states) described here are indeed calcium channels, several lines of evidence suggest they are. Certainly the channels exhibit a preference for cations over anions, although their selectivity for divalent as op-

posed to monovalent cations has not been investigated in detail. The 36 pS channel was equally permeable to barium and calcium, and much less permeable to magnesium, as would be expected for a calcium channel. Furthermore, the gating of all three of the conductance states described here was sensitive to the dihydropyridine calcium-channel agonist Bay K, suggesting that they might indeed represent true calcium channels.

Although the large size and convoluted surface membrane of *Aplysia* neurons makes many of them difficult to patch clamp, Chesnoy-Marchais (1985) recently described some properties of one type of calcium-permeable channel in membrane patches of unidentified neurons from *Aplysia* pleural and cerebral ganglia. There are several similarities between these latter channels and those described in this paper. For example, in both cases similar conductances were observed (approximately 10 and 25 pS), no inactivation occurred, open times were long and varied (1-100 msec), and patches with only one channel were rare. In a recent study it has been shown that the effects of phorbol esters and protein kinase C on calcium currents in *Aplysia* bag cell neurons (DeRiemer et al., 1985) can be attributed to the appearance of a new type of calcium channel in the phorbol ester-treated cells (Strong et al., 1987). Although the properties of the bag cell neuron calcium channels have not yet been described in detail, they are similar in conductance to several observed by us in the bilayers.

An interesting feature of the channels described here is that their gating was apparently independent of the voltage across the bilayer. Patch recording studies in *Aplysia* (Chesnoy-Marchais, 1985) and *Helix* (Lux & Nagy, 1981) neurons, as well as in many other cell types (for a summary *see* Tsien, 1987), have generally described voltage-dependent gating of calcium channels. This might suggest that these *Aplysia* channels in tipdip bilayers are cation channels rather than calcium-selective, since voltage-independent cation channels have been described from several cell types (Maruyama & Petersen, 1982; Yellen, 1982), and at least some *Aplysia* neurons exhibit a voltage-independent cation current (Kramer & Zucker, 1985). Another possibility is that these channels are voltage-dependent in their native membrane environment, but lose their voltage dependence at some stage of the reconstitution procedure. However, other calcium channels reconstituted into artificial bilayers (Nelson et al., 1984; Ehrlich et al., 1986; Rosenberg et al., 1986) have been shown to exhibit voltage-dependent gating. Finally, we cannot exclude the possibility that the channels described here are derived from intracellular membrane sources. No attempt was made to obtain a pure plasma membrane preparation for these experiments, and the crude membrane fraction will certainly contain endoplasmic reticulum vesicles. It is not clear whether calcium channels in intracellular membranes should be expected to be voltage dependent, although it is of interest in this regard that sarcoplasmic reticulum fractions from skeletal muscle contain a calcium channel that exhibits some limited voltage-dependence in artificial bilayers (Smith et al., 1985).

The effects of Bay K on the channel gating shown here are consistent with the previously described mode of action of this dihydropyridine calcium channel agonist (Hess, Lansman & Tsien, 1984; Kokubun & Reuter, 1984; Reuter et al., 1985). It was often difficult to observe discrete gating events in the absence of Bay K, and thus we were unable to carry out a quantitative kinetic analysis of gating under these conditions. In contrast, discrete gating with long and easily quantitated open events was readily apparent for all three channel sizes in the presence of Bay K. It has been proposed that Bay K increases the probability of long open events, and this would certainly have made more of the gating transitions fall into the range where the frequency response of the bilayer recording system allowed their detection.

Perhaps the most interesting and novel aspect of the present study has to do with channel clustering and the apparently nonindependent gating of the several different channels (or conductance states). We routinely observed more than a single conductance state per bilayer, even when the membrane proteins were diluted extensively by the addition of exogenous phospholipid. This does not appear to be due to clustering mediated by cytoskeletal elements, since the same pattern was observed when the membranes were prepared in the presence of the cytoskeleton-disrupting agents colchicine and cytochalasin B. Furthermore, the nonindependent gating suggests that the results cannot be explained simply by some stickiness of channel proteins leading to artifactual clustering. Rather it seems more reasonable to attribute the individual transitions through several different conductance levels to gating between subconductance states of a single ion channel. Gating between substates appeared to be much more common in the presence of Bay K, but this may simply have been due to prolongation of channel open times with consequent longer dwell times in each substate. Substates have now been observed for a variety of different ion channels (e.g. Labarca & Miller, 1981; Miller, 1982; Sachs, 1983; Kazachenko & Geletyuk, 1984; Krouse, Schneider & Gage, 1986), although to our knowledge they have not yet been described for calcium/barium-

permeable channels. Just how such substate transitions influence the lifestyle of the cells in which the channels reside remains to be determined.

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