Patch and Whole Cell Calcium Currents Recorded Simultaneously in Snail Neurons

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ABSTRACT The flow of Ca ions through single Ca channels has been examined. The gigaseal method was used on identifiable snail neurons that were voltage clamped using a two-microelectrode voltage clamp method. Average Ca patch currents and whole cell currents have similar time courses. They are affected similarly by changes in temperature. The differences in amplitude and inactivation between Ba and Ca whole cell currents were present in the patch records. The stationary noise spectra recorded from ensembles of multichannel patches have two components with fast and slow time constants equivalent to two components in the whole cell tail current relaxations. Elementary current amplitudes measured from the variance-mean relationship and from noise spectra gave values comparable to measurements from single channels. The single channel I-V relationship was curvilinear and the maximum slope conductance in 40 mM Ca was 7 pS. The amplitude of unitary currents was unchanged at long times when inactivation had occurred; hence depletion is not involved in this process. Channel density was ~3 μm⁻² and was the same for Ba and Ca currents. The whole cell asymmetry currents gave very large values for the gating charge per channel. Changes in temperature from 29 to 9°C had only a slight effect on the two Ca tail current τ's at potentials where turn-on of patch and whole cell currents was markedly slowed and the peak amplitudes were reduced by one-third. Single channel recordings were obtained at these two temperatures, and the mean open time and the fast component of the closed times were scarcely affected. Unit amplitudes were reduced by 30% and the slow closed time component was doubled. Therefore, peak currents were reduced partly as a result of the reduction in unit amplitude, but mainly as a result of a reduction in opening probability, the latter arising from an increase of the long closed times. It is concluded that the behavior of single Ca channels in membrane patches is the same as it is in whole cells. Cooling from 29 to 9°C acts primarily on transitions among closed states and has little effect on the open to closed transition.

INTRODUCTION

Conduction of Ba ions in single Ca channels has been reported recently (Fenwick

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Ba was used in isotonic concentrations because the unitary currents could not otherwise be resolved. A description of conduction of the physiological ion Ca is necessary since there may be reasons to expect differences in the behavior of single Ca channels when Ca rather than Ba is the conducting ion. There may be channel binding sites with different affinities for the two ions (Hess et al., 1983); activation gating, independent of effects on surface charge, may differ (Fenwick et al., 1982; Saimi and Kung, 1982); and inactivation is clearly different (Brehm et al., 1978; Tillotson, 1979; Brown et al., 1981). While there have been preliminary reports of unitary Ca currents (Lux and Nagy, 1981; Brown et al., 1982; Cavalié et al., 1983), a detailed account has not yet appeared. In the present paper, we describe the properties of single Ca channels using Ca as the conducting ion. In addition, the possibility that the properties of the patch of membrane within the patch pipette may be altered (Fenwick et al., 1982b) has been examined by comparing patch currents with whole cell currents measured simultaneously. Since an intermediate metabolic step has been suggested for Ca currents (Reuter et al., 1982), the effects of temperature changes on channel conductance and kinetics were also investigated.

METHODS

The experiments were done on neuronal cell bodies of Helix pomatia (80–120 μm in diameter) located in the right parietal ganglion of the subesophageal mass. The cells form the dorso-lateral or D cluster (Sakharov and Salanki, 1969) and are characterized by Ca spikes and prominent inward Ca currents during depolarizing voltage steps (Figs. 1 and 2 of Lux and Hofmeier, 1982). After removal of connective tissue sheets using pronase E (0.5 mg/ml saline for 10 min), the cell cluster was dissected from the brain and exposed for 1–1.5 h to trypsin (10 mg/ml saline) at 35–37°C. The preparation was then rinsed intensively and maintained for 30 min in a saline containing (mM): 80 NaCl, 10 CaCl₂, 5 MgCl₂, 4 KCl, and 10 glucose, at pH 7.8 with 5 mM HEPES. Electron micrographs showed that the enzyme-treated neurons removed most but not all of the glial covering. This is in agreement with the reports of Kostyuk et al. (1974) and Neher (1982). In these experiments the axon was severed ~500 μm from the soma using a microtool. The axon then retracts into the soma (Lux and Hofmeier, 1982).

During experiments a Na-free solution containing (mM) 40 CaCl₂, 5 MgCl₂, 35 tetraethylammonium (TEA)-Cl, 5 4-aminopyridine (4-AP), and 20 Tris at the same pH was used. This was also the filling solution of the patch pipettes (see below). For Ba studies, Ba ion was substituted isosmotically for Ca ion. 2.5 μM tetrodotoxin (TTX) was added in the first half of the experimental series (see also Lux and Nagy, 1981) and omitted in the latter half without affecting the results.

The membrane potential of the cells was measured differentially between an intracellular microelectrode filled with 3 M KCl and a reference capillary electrode in the bath, located near the preparation (Fig. 1A). A current to voltage circuit, which provided a virtual ground, was used to collect whole cell current. The whole cell clamp current was delivered by a microelectrode of 2–5 MΩ resistance filled with either 2 M KCl or TEA-Cl. To minimize interaction with the patch and voltage-recording electrodes, the current electrode was shielded to the tip by an electrically grounded silver coat. The silver was applied by immersing the capillary tip into an organic silver chelate (Lapidol II; Doduko Co., Rockford, IL) and by subsequent annealing with heat as described by Lux and...
Hofmeier (1982) adapted from Llinas et al. (1981). The plated electrodes were then repeatedly dipped into a varnish (Humiseal; Columbia Chase Corp., Woodfield, NY) and thereby insulated from the bath. The conventional whole cell clamp circuit used an amplifier having ±140 V output with an amplification of 10^4, increasing to 10^5 below frequencies corresponding to the membrane time constant. With these precautions the voltage command had a rise time of 50 μs and the capacitative current transient had time constants of ≤60 μs. For the patch recordings the clamp gain was lowered to reduce high-frequency contributions to current noise. Under these conditions, voltage command levels were achieved within 60–150 μs and current transients settled with time constants of ~80–300 μs. Unless especially noted, the bath temperature was held at 18 ± 0.1°C with a thermoelectric device having the thermistor in a feedback configuration.

Patch clamp currents were recorded with the gigaseal method in the cell-attached configuration using pipettes of ~5 MΩ resistance (Hamill et al., 1981). The pipettes were made from Duran (Schott Optical Glass Inc., Duryea, PA) glass and were coated with Sylgard (Sigworth and Neher, 1980). They were directly connected to an I-V converter similar to that of Hamill et al. (1981) with the op amp specially selected for low-output voltage noise from a large batch of commercial Burr-Brown (Tucson, AZ) 5523K op amps (Fig. 1B). A 10-GΩ feedback resistor was used, and the background rms noise at both

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**Figure 1.** Experimental arrangement for recording patch and whole cell clamp currents (A) and action potentials (B). In this and subsequent figures, unless indicated otherwise, records are at 18°C. (A) A two-microelectrode system was used to produce the whole cell voltage clamp. Whole cell clamp current was recorded from an I-V converter connected to an electrode in the bath. For tail current measurements the axon was severed. The reference for the patch clamp was the same as that for the voltage clamp. However, when command pulses were applied to the patch pipette, the patch clamp headstage shielding was driven. (B) Action potentials from an identifiable *Helix* neurone during current steps of 1 nA. The identification of these neurons is given by Lux and Hofmeier (1982). Ordinarily, the action potentials of these cells are broad; following TEA injection, they become even broader. The resulting potential was ~60 mV; the peak of the action potential was +67 mV.
optimum high-frequency compensation (see below) and 1-kHz bandwidth was 0.05–0.10 pA. The pipette current was carefully nullified before formation of a seal. Recordings were made at a bandwidth between DC and 5 kHz using a high-frequency compensation circuit that was tuned by squaring the response to a ramp input of voltage applied to a capacitor at the input of the amplifier. Voltage and current data were recorded on analog tape at 5 kHz bandwidth and subsequently filtered to 1.0 or 1.5 kHz by two- or four-pole Bessel filters. The data were digitized using a PDP 11/34 minicomputer (Digital Equipment Corp., Marlboro, MA) at rates that ranged from 0.05 to 0.25 ms per point, depending upon the particular requirements. Control and test records had lengths of 5 ms to 2 s and intervals of 6–20 s were used between trials to avoid cumulative inactivation as checked by the whole cell currents. Linear components of leakage and capacitance were first subtracted from the test records using the average current produced by 10–20 hyperpolarizing pulses.

For noise experiments the pipettes had resistances of ~0.7–1 MΩ, which indicates that they were about three times larger than those used in single channel recording. The noise records, filtered to 1.2 kHz, were sampled mainly at 3 kHz. Sampling was begun after 50–100 ms, when the first component of inactivation of Ca currents was completed. A fast Fourier transform (FFT) algorithm was used for computing spectra. The individual spectra from 20–30 records of equal length produced at one potential were averaged. Slow trends were removed by subtracting pairs of records and doing the spectra on the resulting sums (Conti et al., 1980). The contributions from background spectra were removed by subtracting the spectrum obtained from the average of an equal number of individual spectra produced at either hyperpolarized or resting potentials. The resulting spectra were fitted to single or double Lorentzian functions with or without additional terms for constant levels and 1/f noise. For estimation of the parameters of the models we used a nonlinear least-squares algorithm (Marquardt, 1963, as it appears in Bevington, 1969). A nonstationary analysis of the mean and variance of the current was done with the larger patches also, using ensembles of 20–40 pairs of records (Conti et al., 1980; Sigworth, 1980).

Unitary events were analyzed as follows. In most cases the analog data were filtered prior to digitization using a four-pole low-pass Bessel filter with a 3-dB corner frequency (f0) of 1.0 kHz. Digitizing rates of 50–100 μs per point were used and the records were stored in buffers of 1,024–4,096 points. An interactive single channel analysis computer routine was used to obtain amplitude and kinetic histograms from the data in the following manner. The standard deviation of the background current noise (noise rms) before depolarization and in channel-free intervals was measured and used to set up two threshold detectors, one for openings with level φ and the other for incomplete closings. The latter was set above the baseline between 1.5 and 2 times the background rms noise and φ was set at 5–5 times rms noise. This was usually about half to two-thirds the amplitude of the openings studied presently. The activation potentials were <0 mV in order to satisfy these requirements. An opening transition was identified when the higher level was crossed and a closing transition was identified when the lower level was crossed. Once an event was detected, the program computed the average amplitude and measured the open time at the width corresponding to half-maximum amplitude. Idealized events were constructed by setting the points below the lower level to zero and taking the amplitude from the average of the values between the opening and closing transitions. These idealized values were used to construct the histograms. Events of <300 μs duration were rejected from the histogram analysis since they fell below the resolution of the recording system (Sachs et al., 1982). The average frequency of false positive events, λφ, is given by k f0 exp(−φ2/2σφ2) (Colquhoun and Sigworth, 1983), where φ is the threshold level for
detecting openings, \( \sigma \) is the level of background rms noise, and \( k \) is \( \sim 1.0 \). With an opening probability of 0.1 and a \( \phi/\sigma \) of 4, false events would have an average frequency of \(< 1/s\).

**RESULTS**

In our experiments, the whole cell was voltage clamped using two microelectrodes (Lux and Hofmeier, 1982; Lux and Nagy, 1981), which made it possible to record macroscopic and patch currents simultaneously (Figs. 4 and 12). Exposure to trypsin at 35–37°C did not appear to alter the whole cell Ca currents as compared with untreated controls. The preparation remained in good condition for up to 30 h, as judged by measurements of cellular membrane potential, action potential, and action currents. The resting potential of the cells ranged from \(-40 \) to \(-110 \) mV and input resistances ranged from 6 to 50 MΩ. The action potentials had overshoots of \(~65 \) mV (Fig. 1B) and durations at 0 mV of 20–40 ms. The TEA-containing current microelectrode was inserted and TEA was injected for several minutes using currents of 5–10 nA, which gave a total charge of \( \sim 2 \times 10^{-6} \) C and an estimated concentration of 20–30 mM. The injection was discontinued after its effects on voltage clamp currents became steady. During this time, the action potential lengthened to a final value of \(~0.2–2 \) s at 0 mV without change in the amplitude of the overshoot. The injection of TEA increased peak inward currents and reduced the later outward currents produced by voltage clamp steps to +20 mV for 200 ms. The former effect was small and occurred shortly after the onset of the injection; the latter effect was large and became constant at the end of the injection. Injection of cesium ions using current strengths similar to those used for injecting TEA had much smaller effects on the outward currents. The outward currents after TEA were slower than those present prior to injection and were probably nonspecific in character (Brown et al., 1981). Above +30 to +50 mV, the nonspecific outward currents persisted.

Generally, the cells were held at their resting potentials for 30–60 min before measurements were taken. Depolarizing steps of 20–115 mV produced net inward currents (Fig. 2) of the sort that have been described previously (Eckert and Lux, 1976; Brown et al., 1981). Current was inward at +65 mV even without leakage correction. At these potentials, outward Ca currents were observed in heart cells by Reuter and Scholz (1977) and Lee and Tsien (1982), and in chromaffin cells by Fenwick et al. (1982a). However, the Ca⁺'s were lower in these cases, being 1.8, 1.0, and 5 mM, respectively. The relationship between peak current and voltage is shown in Fig. 2. The nadir of the relationship occurred at +30 mV. In 10 mM Ca, this point is at \( \approx 15–20 \) mV and the shift toward more positive potentials is to be expected from the effects of 40 mM Ca on surface potential (Wilson et al., 1983). Changing the holding potential to more negative values had no effect on the curve.

Ca currents are noteworthy because the currents revealed by deactivation, the tail currents, are fit by a sum of two exponentials giving rise to two time constants or \( \tau \)'s at potentials of \(-50 \) or \(-60 \) mV, where activation goes to zero. This is inconsistent with a Hodgkin-Huxley \( m^2 \) model, which predicts only a single-exponential decay (Brown et al., 1983). A three-state model of activation has
been proposed (Fenwick et al., 1982a). However, the two tail $\tau$'s do not predict the delay in the turn-on currents, and to be described adequately the activation process requires at least three time constants (Brown et al., 1983). Fig. 3 seems to support this point; turn-on at +20 mV is slowed greatly by cooling, whereas turn-off at −15 mV is not greatly affected, although the $\tau$'s changed a small amount, as indicated in the figure legend. The ratios of the two tail current $\tau$'s were between 7 and 10 and were not changed at the three temperatures. At potentials more comparable to the turn-off potential of −15 mV, turn-on is still greatly slowed by cooling (see Figs. 2 and 3 of the next paper [Brown et al., 1984] for turn-on at −5 mV and see Fig. 17C of Brown et al. [1983] for turn-

**FIGURE 2.** Whole cell clamp Ca currents. Na ions were omitted from the extracellular solution, TEA 30 mM and 4-AP were added, and TEA had been injected into the neuron to an estimated concentration of ~20 mM (see Methods). The extracellular Ca concentration was 40 mM. The axon was severed. A shows transient inward currents without leakage correction recorded at potentials up to +65 mV. A very delayed outward current appeared at the more positive potentials. Holding potential ($V_\text{H}$) was −50 mV in this and the subsequent figures. The peak $I$-$V$ relationship is shown in B. Currents were corrected for linear components of leakage and capacitance.

on at −27 mV). Thus, the absence of significant change in the relaxation rates of the tail currents at lower temperatures, despite the substantial changes in the turn-on of the currents at similar potentials, argues against the three-state sequential models of Ca current activation that have been proposed (Fenwick et al., 1982a; Brown et al., 1983).

The tail currents are due to closure of Ca channels for the following reasons. The two components identified here are blocked by Co substitution for Ca or addition of Cd (1.0 mM) to the solution. They are increased when Ba is substituted for Ca, as are the macroscopic peak $I$-$V$'s. The relaxation rates are controlled by the return potential and not the test potential, whereas the amplitudes are a function of the test potentials at any return potential. The tail
currents are very similar to those measured in Helix aspersa with the combined suction pipette-microelectrode method (Brown et al., 1983). Using an activation curve and an instantaneous I-V curve, both of which were obtained from tail current measurements, an isochronal I-V can be constructed that closely resembles a measured one (Brown et al., 1983).

The features of whole cell Ca currents have been presented because we wished to compare them with the patch Ca currents. At present no such comparison has been made. This is of importance because, as Fenwick et al. (1982b) have pointed out, the properties of the membrane within the patch pipette can be altered. Multichannel results are presented first since they gave larger currents that could be more readily compared with whole cell currents.

Patch Ca Currents

The patch pipette was applied and a satisfactory background noise was obtained with input resistances of 10–100 GΩ. The patch pipette was maintained at the bath potential and clamp steps were applied to the cell interior with the two-microelectrode voltage clamp; with this arrangement, the time constant of the...
capacitive transient in the patch circuit was usually 0.1–0.5 ms, although it could be as brief as 80 μs. The current-time integral of the transient per applied voltage change had the size required to charge a patch membrane of 2–20 μm² assuming a specific capacitance of 1 μF/cm² (Sigworth and Neher, 1980). The variable sizes were related to the different sizes of electrodes used, as well as to differences in the amount of membrane that was aspirated into the pipettes. Additional and smaller components of the artifact showed similarities in time course with that of the whole cell current and may reflect transient changes in the extracellular potential field surrounding the patch pipette. The potential in the patch pipette could also be changed independently from the potential in the rest of the cell. However, in this situation the charging time was always longer by a factor of ~2, despite reduction of the pipette capacitance by the Sylgard coat.

Patch currents were recorded repeatedly from different parts of the same neuron. This was performed without any apparent damage, although small inward currents of ~0.1 nA peak amplitudes usually appeared transiently upon removal of the patch electrode. The patch currents recorded from different parts of the cell soma were identical.

The likelihood of obtaining patches with low background noise and prominent inward unitary Ca channels even during large depolarizations was far greater in cells having the electrophysiological properties described in the first paragraph of the Results. Activity from nonspecific cation channels of the type reported by Colquhoun et al. (1981) and Yellen (1982) was not observed in these healthy cells. Cells with low input resistances, low resting potentials, and small action potentials were sources of unsatisfactory patches which showed larger background noise and bursts of activity that were random in amplitude and appeared to be several times noisier than the background noise. This sort of behavior may have been due to transient breakdown of the seal. There was also considerable activity from nonspecific cation channels.

The command pulses were varied from 5 ms to 1.0 s in duration, with intervals of 3 s between consecutive pulses of 5 ms duration and 30 s for pulses 1–2 s in duration. Longer intervals for larger pulses were necessary because recovery from inactivation is very slow (Eckert and Lux, 1976; Tillotson and Horn, 1978; Plant and Standen, 1981; Brown et al., 1981; Yatani et al., 1983). This restriction limited the number of trials that could be made on any patch since the patches rarely lasted longer than 1 h.

We compared the waveform of the patch currents with the simultaneously measured waveform of the whole cell currents, using larger cell-attached patches that contained several active channels. As noted, this allowed us to average fewer records than would have been required using single channel events. The latter course was not practical because of the long interval between pulses and the limited lifetime of the cell-attached patches. Fig. 4 shows that the averaged patch currents closely resemble the whole cell current. They rise to a peak and then decline slowly with time. The half-times to peak for the patch currents were identical to the half-times to peak for the whole cell current over potential ranging from ~20 to ~50 mV (Fig. 6C). When Ba replaces Ca, both the averaged patch current and the whole cell current are larger and inactivate less. This is consistent
with earlier observations (Brown et al., 1981). Both the current fluctuations from channel openings ($i_{Ca}$ and $i_{Ba}$) and the mean patch currents were relatively large at $\sim 0$ mV and this facilitated a nonstationary investigation of the variance-mean relationship and evaluation of the elementary Ca and Ba current amplitudes.

**Figure 4.** Fluctuations of Ca currents (left-hand panel, a–d) and Ba currents (right-hand panel, a–d) in micropatch recordings. Temperature, 18°C. (a) Five sequential traces of multiechannel patch currents before and during a step change of membrane potential from $-50$ to $-5$ mV. (b) The averaged currents from 29 samples for Ca and 32 samples for Ba are shown in the middle of b (thick continuous line). The corresponding plots of variance (from zero levels indicated by the thin line) are shown at the top in b. The baseline variance of 0.011 (pA)$^2$ has been subtracted. At the bottom in b are the whole cell Ca and Ba currents with amplitudes scaled to match the averaged multiechannel currents. The peak values were 12 and 15.5 nA, respectively. The rate of rise is faster for $i_{Ba}$ at this potential. Analog data were filtered at 1 kHz using a four-pole Bessel filter and sampled at 0.1 ms per point. Note the small initial outward currents in the averaged and whole cell currents. These may be asymmetry currents. (c) Plots of variances vs. means of fluctuation currents. Bin width, 0.05 pA; each point gives average and SD of samples within bins. The continuous line is fit with Eq. 2 using $N = 3$ for Ca and Ba and $i_{Ca, Ba} = 0.43$ and 0.52 pA, respectively.
The relationship is given by

\[ \sigma^2 = i \langle I \rangle - \frac{1}{N} \langle I \rangle^2 \]  

(1)

or equivalently

\[ \sigma^2 = Np_o[1 - p_o]^2 \]  

(2)

where \( p_o \) is the opening probability and is a function of time, \( \langle I \rangle \) is the mean current, \( N \) is the number of channels, and the background variance from other noise sources has been subtracted. As seen in Eq. 1, the initial slope (at small \( \langle I \rangle \)) of the variance-mean curve is a measure of the unitary amplitude; it gave 0.43 pA for \( i_{ca} \) and 0.52 pA for \( i_{ba} \), which gives a ratio of 1.21. Similar results were calculated from spectral curves of Ba and Ca current fluctuations at times when the mean current was nearly steady state. The variance-mean estimates from patches containing low numbers of channels \( (N \leq 3) \) were compared with current amplitudes of single openings. When major distributions were fitted by a Gaussian function the variance-mean values were within a standard deviation \((0.05-0.08 \ pA)\) of the major peak of the amplitude distribution.

The amplitude distributions from single Ca and Ba channels measured from data such as that shown in Fig. 10 had mean values similar to those obtained from the mean-variance curves. For recordings between -25 and 0 mV, the ratio of \( i_{ba}/i_{ca} \) has a mean of \( 1.24 \pm 0.15 \) (SD, \( n = 7 \), where \( n \) is the number of patches). The value of whole cell peak \( I_{ca} \) at -5 mV can be used to compute the number of channels per neuron. The probability of opening is \( \sim 0.2 \) at this potential (also see Fig. 4 of the accompanying paper) and a value of 139,500 channels per neuron is obtained. For a neuron 120 \( \mu m \) in diameter, this gives an average density of three channels per \( \mu m^2 \). The smaller patches we used usually showed activity from only one channel. When larger patch pipettes were chosen, as shown for the experiments of Figs. 4 and 5, there were three or four channels per patch.

Two effects were anticipated upon changing from Ca to Ba solutions; first, an increase in the single channel currents (as was shown above), and second, an increase in the opening probability at potentials below a saturating voltage due to a change in surface potential that shifts the activation curve in a hyperpolarizing direction (Wilson et al., 1983). Thus, the ratio of whole cell peak \( I_{ba} \) and \( I_{ca} \) should be given by the ratio of the product of the ratio of the single currents and the ratio of the peak opening probabilities from the patches. The experiment in Fig. 4 yielded similar values for the opening probabilities, but this depends on the number of channels calculated for the two patches. In this case each patch was determined to have three channels. However, there is some uncertainty in calculating the number of channels in the patch by applying Eq. 1 to the data in Fig. 4C. The uncertainty arises from the scatter in the variance values and the fact that the mean-variance curves do not pass through a peak at this potential. In this experiment the ratio of the whole cell currents \( (1.3) \) was approximately the same as the ratio of the unitary currents \( (1.2) \). In one other case \( P_o \) was 0.2 for patch \( I_{ba} \) and 0.15 for patch \( I_{ca} \); the single channel currents were as above, and the ratio of the whole cell currents was 1.6, which agrees quite favorably
with the value of 1.65 predicted as described previously. Thus we find the calculated number of channels per cell is similar for Ba and Ca currents.

The relationship between the mean current and its variance is more accurately determined when the membrane is depolarized to an extent sufficient to activate at least 50% of the channels. Based on normalized tail current measurements, this would be expected at potentials greater than +15 mV (Brown et al., 1983). Under these circumstances Eq. 2 predicts that the mean-variance curve will go through a maximum. Results from an experiment of this sort are shown in Fig. 5. The variance as a function of time has two peaks as the opening probability rises above and falls below 0.5. There is considerable scatter in the variance values, and the minimum is only roughly near the current maximum. At +25 mV, the variance as a function of time also had clear peaks, and the first peak was reached later than at +50 mV. In Fig. 6 are variance-mean current plots; note that at +50 mV the variance reaches its maximum at a lower mean current than at +25 mV (compare Figs. 6A and B). The elementary currents, calculated from the initial slope of the mean-variance curve, have fallen to 0.22 and 0.15 pA at +25 and +50 mV, respectively (Fig. 6).

**Noise Spectra from Multichannel Patches**

The larger patch pipettes were used in noise experiments. The increase in patch current noise that resulted from activation of multiple channels was considerably greater than the background noise present at the holding potential (Figs. 7A and 8, filled circles). Spectra were assembled from sets of 20–40 patch clamp currents produced by voltage steps of 1–2 s duration after allowing 50–100 ms for the fast inactivation process to subside. Slow trends remaining in the mean currents were removed by pairwise subtraction. As a check on the steadiness of the fluctuations, we compared spectra during the first 200 ms after the peak with spectra at 200–400 ms and found no difference between the two sets of measurements. A similar result using this cross-validation technique was reported by
Krishtal et al. (1981) for noise measurements on whole cell Ca currents. These results, plus the fact that the mean current changed very slowly at these times, allowed us to interpret the spectra as arising from stationary rather than nonstationary channel activity (Sigworth, 1981). Some typical results are shown in Fig. 7B. In the majority of 60 analyses at potentials between −20 to +30 mV, the spectra had two clear corner frequencies. They were fitted with models of single Lorentzian functions or sums of two with and without a 1/f component. The best fit was selected as the model giving the smallest chi-squared error value (Bevington, 1969). In most cases only the double Lorentzian function produced a reasonable fit to the data. The double Lorentzian spectra were present at potentials between −20 and +50 mV, which was the range examined in these experiments. The high-frequency corner was reduced at +30 to +50 mV and with the exception of the value at −10 mV decreased as potential was increased. The lower-frequency corner appeared to have a minimum between 0 and +10 mV.

The time constants associated with the high-frequency corner \( (\tau_{\text{hif}}) \) and the low-frequency corner \( (\tau_{\text{lf}}) \) are given in Table I. At similar potentials these values were equivalent to those associated with tail current values (Fig. 3 and Table II). The two corners had similar values at comparable potentials when the currents were carried by Ba ions rather than Ca ions, but the number of Ba experiments was too small to permit a definite conclusion concerning this point. A spectrum for Ba currents at 0 mV is shown in Fig. 8.

The single channel current \( i \) can be estimated from the relationship between

\[
\sigma^2 = \frac{2i}{\tau}
\]

where \( \sigma^2 \) is the variance, \( i \) is the current, and \( \tau \) is the time constant. This relationship is illustrated in Fig. 6.
the mean current \( \langle I \rangle \) and the variance \( \sigma^2 \) using Eq. 1 and the relation

\[
\sigma^2 = \frac{\pi^2}{4} \sum_n S_n(0) f_n.
\]

**FIGURE 7.** Patch Ca current noise and power spectra. The Ca currents from a patch containing several active channels are shown on the left. Records were taken at 2.5 kHz bandwidth. Steps were to the indicated potentials. The voltage-dependent noise is clear. Hyperpolarizing potentials produced slight or no change from the background current noise present before the depolarizing step. The spectra were filtered at 2.0 kHz and digitized at 5.0 kHz. Pairwise subtraction of 10–24 records of the sort shown in the left part of the figure was done to remove the small amount of mean current drift or inactivation. Spectra from such corrected records were obtained by applying an FFT algorithm to 2,048 samples and averaging. The average background spectra were subtracted to produce the final spectra (right). The open squares were averaged condensations of the points contained in 1/20 of the linear distance within frequency decades (Conti et al., 1976). The solid lines were the best fits obtained by applying a sum of two Lorentzian functions to the data. The ordinate is divided in decades. The corner frequencies (−3 dB) are indicated by the arrows. The sums of the zero-frequency asymptotes for the spectra from −10 to +30 mV were 3.1, 7.0, 21.4, and \( 27.8 \times 10^{-9} \) A²s, the ratios of the slow and fast components of the spectra being 11.5, 3.4, 5.0, and 4.1, respectively.
Thus,

\[ i = \frac{\pi^2}{4} \sum \frac{S_n(0)f_n}{(1 - P_0)} \]

where \( S_n(0) \) is the zero-frequency asymptotes of the one-sided spectral components and \( f_n \) is the corner frequency. The background variance was removed by subtraction. For the recording of Fig. 7 at -10 and +10 mV, \( i \) was estimated to be 0.57 and 0.27 pA, respectively, and \( P_0 \), estimated from Fig. 4 of Brown et al. (1984), was 0.08 and 0.3. However, it should be noted that a reliable estimate is hampered by the scatter of calculated \( f_n \) values (see Table 1). Nevertheless, the \( i \) values calculated this way were of the order of values obtained from the variance-mean plots and values obtained directly from single channel measurements.

A comparison was also made between the time constants derived from the patch current spectra and the time constants calculated from ensemble averages of patch tail currents (Lux, 1983). A fast time constant was identified with values similar to the fast times shown in Table II.

We turn now from considerations of multichannel currents to the currents arising from single channels.

**Single Calcium Channel Calcium Currents**

Unitary currents were probabilistic: at a given potential their latencies, open times, and rates of opening appeared random. Taking into account the standard deviation of the background noise, amplitudes of single events appeared constant at any given potential. When the single channel current records were summed, the resulting average current was identical to the whole cell current, as shown in Figs. 2 and 12 of the accompanying paper (Brown et al., 1984). An interesting feature was the occurrence of openings in groups or bursts with much longer
T A B L E I

Characteristics of Patch Ca Current Noise Spectra

<table>
<thead>
<tr>
<th>Membrane potential</th>
<th>( n )</th>
<th>( f_{1/2}^{(1)} )</th>
<th>( f_{1/2}^{(2)} )</th>
<th>( S_0(0)/S_0(0) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>+30, +50 mV</td>
<td>7</td>
<td>33.4±16.3 (4.8 ms)</td>
<td>297±190 (0.54 ms)</td>
<td>4.6±2.5</td>
</tr>
<tr>
<td>+20 mV</td>
<td>5</td>
<td>34.7±11.1 (4.6 ms)</td>
<td>349±238 (0.46 ms)</td>
<td>5.0±2.9</td>
</tr>
<tr>
<td>+10 mV</td>
<td>6</td>
<td>29.9±7.5 (5.5 ms)</td>
<td>345±116 (0.46 ms)</td>
<td>5.7±3.2</td>
</tr>
<tr>
<td>0 mV</td>
<td>12</td>
<td>28.2±16.5 (5.66 ms)</td>
<td>429±55 (0.37 ms)</td>
<td>5.0±2.4</td>
</tr>
<tr>
<td>−10 mV</td>
<td>8</td>
<td>43.1±33.8 (3.7 ms)</td>
<td>520±223 (0.49 ms)</td>
<td>6.9±4.5</td>
</tr>
<tr>
<td>−20 mV</td>
<td>5</td>
<td>35.5±20.1 (4.15 ms)</td>
<td>546±365 (0.29 ms)</td>
<td>7.0±6.5</td>
</tr>
</tbody>
</table>

Pooled data (mean ± SD) are shown from 13 patches, each with determinations at two or more membrane potentials as indicated. \( n \) is the number of observations (see text). \( f_{1/2}^{(1)} \) and \( f_{1/2}^{(2)} \) are the corner frequencies of the slow and fast components, respectively. The values given in brackets are the corresponding mean time constants. \( S_0(0)/S_0(0) \) is the ratio of the amplitudes at zero frequency of the low- and high-frequency components.

intervals between the bursts (Figs. 9 and 12; Figs. 2 and 3, Brown et al., 1984). This aspect has been described before (Fenwick et al., 1982a; Brown et al., 1982; Reuter et al., 1982; Hagiwara and Ohmori, 1983). The closed intervals within bursts were irregular, too.

Patches were inferred to have a single channel present by the following type of argument. These patch records showed no overlapping openings at potentials where the probability of opening, \( P_o \), and the record length were sufficiently great that, assuming the openings to be independent, a significant number of multiple openings should have been observed. For example, at 0 mV, \( P_o \) was ~0.2 (Wilson et al., 1983; Fig. 4, Brown et al., 1984). If there were two active channels in the patch, the binomial theorem predicts that single independent openings should occupy 18% of the record. Openings of double amplitude should occur over 1% of the record. In records of 400 ms length with mean open times of ~1 ms, roughly 80 single openings and 4 openings of double amplitude should occur on the average. For 10 repetitions, 40 double openings should be observed were the active number of channels two. Given these

T A B L E I I

Comparison of Activation Time Constants

<table>
<thead>
<tr>
<th>Membrane potential</th>
<th>( n )</th>
<th>( \tau_{fast} )</th>
<th>( \tau_{slow} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noise spectra</td>
<td>15</td>
<td>0.40±0.16</td>
<td>3.85±2.10</td>
</tr>
<tr>
<td>Macroscopic tails</td>
<td>4</td>
<td>0.46±0.20</td>
<td>2.20±1.82</td>
</tr>
</tbody>
</table>

Mean values (± SD) of \( n \) observations with the different methods are given.
considerations, the failure to observe any openings of double amplitude means that only one single active channel was present.

Openings became more frequent as the membrane patch was depolarized. At positive potentials, unitary amplitudes were measurable (Fig. 9d) but discharge patterns became uncertain because of the lower signal to noise ratio.

![Figure 9](image-url)

**Figure 9.** Single calcium channels recorded from a cell-attached patch at 28°C. (a) Record at $V_M = -10$ mV, $V_H = -50$ mV. Data were recorded at 1.0 kHz bandwidth with a four-pole Bessel filter; the sampling rate was 100 μs per point. The lines are set at 1.5 and 4 times background noise rms. Note the two different time bases. b–d are records at the potentials indicated. The top solid line is the mean value of the background noise and the next line is 1.5 times rms. The threshold for opening was three times background rms, except in d, where only the lower level was used. In the subsequent five records the values between the two lines are set to zero. The remaining points represent the amplitudes of the open transitions. Mean values for these points are combined with the corresponding zeros to give the idealized openings used for histograms. Events <300 μs in duration were below the rise time of the recording system and were excluded from data analysis.

**Single Unit Amplitudes**

The amplitudes of the openings were averaged between the transitions to and from the open states to give the average amplitude of each opening. The transitions were identified at amplitudes that were half the maximum values. The openings were then idealized (Fig. 10) for the subsequent construction of amplitude, open time, and closed time histograms. At 18°C the amplitudes of single openings have mean values of 0.48 and 0.37 pA at -20 and 0 mV, respectively, and were similar to values obtained from mean-variance plots and noise spectra at this temperature. The amplitudes of the openings have a distribution that is slightly skewed toward the higher amplitudes (Fig. 10). The distribution is attributed to the background noise, and the skewing results from
the fact that smaller openings were eliminated by the threshold settings. There was no change in unit amplitude during long pulses in which inactivation was present in the averaged patch currents (Fig. 12). The mean unitary values were identical at 50 and at 400 ms.

**Conduction in Single Ca Channels**

The relationship between single channel current and potential was explored between $-30$ and $0$ mV, where the amplitudes could be satisfactorily resolved. Above 0 mV the elementary currents calculated from mean-variance relationships (Fig. 4) were used. The results are shown in Fig. 11. A maximum slope conductance of $7.0 \pm 1.3$ pS was calculated between $-20$ and 0 mV. As noted previously (Lux and Nagy, 1981), this value is higher than earlier values of Krishtal et al. (1981) and Akaike et al. (1978a). The values are comparable to those reported for pituitary clonal by Hagiwara and Ohmori (1983), although they used isotonic Ba solutions. They are less than the 25 pS reported for heart cells by Reuter et al. (1982). The $I-V$ curve between 0 and $+50$ mV is similar to the instantaneous $I-V$ curve calculated from whole cell tail currents (Brown et al., 1983).

**Effects of Changes in Temperature**

Changing the temperature from 29 to 9°C reduced the currents recorded from single channels and slowed the initial opening rate, as shown in Fig. 12 (see also Figs. 2 and 3 of Brown et al., 1984). The peak amplitude and the rate of inactivation of the averaged single channel currents are smaller at the lower temperature. Similar changes occur in the whole cell currents. The amplitude histograms (Fig. 13) have mean values of 0.49 and 0.33 pA at 29 and 9°C, respectively. These are in a ratio of 1.5 and give a $Q_{10}$ of 1.2. The peak of the averaged single channel currents changed by a ratio of three, which compares with the results from macroscopic currents. After correction for the effects of cooling on unit amplitude, the peak probability of opening changed by $\sim2$ and
FIGURE 11. Current-voltage relationship for Ca channels. Filled symbols are from two experiments and represent the mean values of amplitude histograms. The open symbols are calculated from mean-variance curves.

FIGURE 12. Single channel recordings at 29 (A) and 9°C (B). \(V_H\) was \(-50\) mV, \(V_M\) was 0 mV, pulse duration was 200 ms. Representative single channel records are shown for each case. The three traces below in each set of single channel records are, from top to bottom: the averaged current from the single unit patch records, the averaged current from the idealized single unit traces, and the current from the whole cell recorded simultaneously with the patch currents. Only a single sample of the latter is shown. Currents from the single unit patch recordings are averaged from 37 traces at 29°C and 49 traces at 9°C. Only one failure occurred and that was at the lower temperature.
had a $Q_{10}$ of 1.5. It appears that the change in size of the peak Ca current during cooling is due more to the change in the peak probability of opening rather than the change in unit amplitude. The open time histograms were virtually unchanged, the mean open times being 1.05 \( (29^\circ C) \) and 1.2 ms \( (9^\circ C) \). The open

![Amplitude histograms for the single Ca channel of Fig. 12.](image)

**FIGURE 13.** (A) Amplitude histograms for the single Ca channel of Fig. 12. The histograms are skewed toward layer values due to a limited signal to noise ratio. Values are 0.483 ± 0.052 pA at 29°C and 0.32 ± 0.058 pA at 9°C. (B) Open time distributions. In B and C the right-hand histograms are for 29°C. Under the same conditions mean open times were 1.05 ms at 29°C and 1.20 ms at 9°C. Average values in milliseconds at the same potential were, from 10 cells at 29°C, 1.48 ± 0.52; from 5 cells at 9°C, 1.52 ± 0.48; and from 20 cells at 18°C, 1.60 ± 0.56. At least 500 openings were measured for each cell. (C) Closed time distribution. Histograms of closed times were fit with a sum of two exponentials with “fast” and “slow” time constants. The fast time constant was 1.88 ms at 29°C and 1.82 ms at 9°C. The slow time constant had a mean value of 4.9 ms at 29°C and 11 ms at 9°C. The small number of events in C is due to the fact that the measurements were made for only the first 50 ms of the step at 28°C and the first 50 ms of the step at 9°C. The errors in our measured distributions can be estimated modifying the approach of Sachs et al. (1982). Under our filtering conditions, events of ≤300 µs in duration were not detected. This would lead to an underestimate of ~10% in the 300–600-µs bin of the open time distribution and ~15% for the fast closed time distribution.
time distributions between the first and second halves of the records were the same. The closed times were measured during the first 50 ms, when inactivation was not prominent, and were fit with two exponentials as described in Fig. 13. The fast time constant from the fit to the closed times was little affected by temperature; it was 1.88 and 1.82 ms at 29 and 9°C, respectively. However, the slower closed time \( \tau \) was increased from 4.9 to 11.0 ms, giving a ratio of \( \sim 2.2 \) with a \( Q_{10} \) of 1.5. This is the primary reason for the reduced probability of opening. Early in the records the probability of opening was also affected markedly by the effects of cooling on the latencies to first opening. The time to peak of the average patch current changed by a ratio of \( \sim 3 \), giving a \( Q_{10} \) of 1.8, and the whole cell current time to peak changed by a ratio of \( \sim 5 \) (similar results are found in Brown et al., 1983). Much of this effect is due to the effect on the latency to first opening, as discussed in the next paper.

**DISCUSSION**

Currents arising from single or multiple Ca channels contained in a patch of membrane should be similar to Ca currents arising from the whole cell. This was examined directly by simultaneous measurements of patch and whole cell currents and was demonstrated to be the case in a variety of circumstances by using a wide range of temperatures to change kinetics and amplitudes. The solutions used for the single channel measurements were decidedly more physiological than the isotonic Ba solutions used by other investigators. Nevertheless, 40 mM Ca, which we used to enhance the amplitude of the unitary currents, is still four times the usual concentration. However, whole cell Ca currents from snail neurons have been characterized at these concentrations and the behavior at 40 mM closely resembles the behavior at physiological concentrations after allowing for changes in gating and surface concentration due to surface charge effects (Wilson et al., 1983). As expected from whole cell current measurements, patch Ba currents are larger than patch Ca currents and inactivate more slowly. Isotonic Ba was not used here, but, as discussed in the next paragraph, it seems probable that single channel measurements in isotonic Ba may suitably reflect single channel behavior in more physiological solutions. Another advantage of using Ca as the extracellular ion is that any uncertainty arising from Ba conduction by Ca-activated nonspecific cation channels can be excluded since these channels do not conduct Ca ions (Colquhoun et al., 1981; Yellen, 1982). With our approach the voltage dependence of the unitary opening probabilities could be established with certainty since the membrane potential was measured directly. In the studies on smaller cells using cell-attached patches, the membrane potential had to be inferred.

In general, the single channel behavior observed—bursts of small amplitude currents—was similar to that described by other investigators and provides further confidence in the patch measurements. Given the difficulties in isolating whole cell Ca currents from nonspecific currents or from Ca-activated currents, the results from the patch studies may also be useful in evaluating whole cell current measurements. The question as to whether inactivation occurs at all in whole cell current recordings has some uncertainty, in many cases because of the
attendant problems of Ca-activated currents. The problem is readily settled at
the single channel level. As Figs. 4 and 9 hint and Fig. 12 shows, Ca channel
openings are less frequent at longer times following a depolarizing voltage clamp
step and show no change in amplitude. This is clear evidence that inactivation is
due to a reduction in opening probability. The fact that the unitary amplitudes
are unchanged at long times excludes depletion as a possibility. Intracellular
accumulation would have smaller, probably undetectable, effects. Note also that
another example of the usefulness of patch measurements is the high signal to
noise ratio of the patch current fluctuations. Noise measurements from whole
cell currents (Akaike et al., 1978b; Kostyuk et al., 1981) have not been consistent
with measurements of tail current kinetics. The earlier results showed only single
Lorentzian spectra with the corners related to either the $\tau_{\text{slow}}$ or the $\tau_{\text{fast}}$ of the
spectra measured presently. The patch data, probably because of the enhanced
amplitude of the current noise, resolved the differences nicely: the two frequency
components of activation were similar in both the spectra and the macroscopic
relaxations. This indicates a minimum of three states of activation and the
expression of this at the single channel level is the bursting behavior of the
transitions. However, the facts that the tail $\tau$'s do not predict the delay in turn-
on of current (Brown et al., 1983) and that cooling had a much larger effect on
turn-on compared with turn-off indicate the presence of a fourth state in the
activation diagram. This is not apparent in the spectra or the tails, probably
because the initial condition of the system for turn-on emphasizes different
kinetics. Thus, only two of the three time constants for a four-state model may
be resolved by these methods.

The slope conductance is similar to that reported by Hagiwara and Ohmori
(1983) but is $\sim 35\%$ of the value reported by Reuter et al. (1982). The latter two
groups used isotonic Ba and larger values relative to ours would be expected
from the constant field expression as applied to Ca current. However, differences
in surface charge between cell types could reconcile our results with those of
Hagiwara and Ohmori (1983). A difference in surface potential of 50–90 mV
could result in currents about twice the value of ours over a similar voltage
range, although the slope conductances would be equivalent.

The number of functional channels per cell was the same whether Ba or Ca
was the extracellular cation. The number of binding sites for the dihydropyridine
class of Ca channel blockers has been reported to be affected by the particular
divalent cation present in the extracellular solution (Gould et al., 1982). In brain,
the number of binding sites in Ba was one-third the number in Ca. Taken
together with our result, this indicates that the stoichiometry of the binding of
Ca channel blockers to functional Ca channels may be variable.

We have estimated $\sim 140,000$ channels per neuron. If one attributes the
asymmetry currents present in these neurons (Fig. 4; Figs. 5 and 12 of Brown et
al., 1983) to charge movement associated with Ca channels (Kostyuk et al.,
1981; Adams and Gage, 1979), the number of charges moving through the
membrane per channel can be calculated. We have calculated $\sim 5 \times 10^7$ esu
(electrostatic units) as the asymmetry charge per cell, giving $\sim 350$ esu per
channel. Similar estimates may be made using the gating currents reported by
Kostyuk et al. (1981) and the present calculations of channel density. However, the conductance-voltage relationship (Fig. 4, Brown et al., 1984; Fig. 12, Brown et al., 1983) shows a maximal e-fold charge for membrane potential changes of \( \sim 10 \text{ mV} \). This predicts \( \sim 2-3 \) esu per channel. Hence, it seems likely that most of the charge movement is not associated with channel opening, as has been suggested previously (Brown et al., 1979; Hagiwara and Byerly, 1981). The same may be said for gating currents ascribed to Ca channels in \textit{Aplysia} (Adams and Gage, 1979).

Cooling from 29 to 9°C reduced peak currents. It did so partly because the unit amplitudes were reduced but mainly because the opening probability fell. The explanation for this was simple. Mean open times were unchanged, as were the brief closed times within a burst, but the closed time between bursts was prolonged, producing a large reduction in the probability of opening. The closed time measurements are particularly complicated at the higher temperature because inactivation was prominent, and the contribution of this process to the closed time distributions has not been examined. We conclude that cooling had its major effect on transitions among closed states and had very little effect on the open to closed transition. The effect is likely to be on a closed state not shown in the three-state models of activation that have been proposed (Cachelin et al., 1983; Fenwick et al., 1982a; Brown et al., 1982) and this possibility is examined in the next paper (Brown et al., 1984).

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REFERENCES


