performance liquid chromatograph with a Beckman ultraviolet detector operated at 214 nm with a zinc lamp. Samples were introduced with a 20-μl Rheodyne loop or a 50-μl Hamilton syringe. The mobile phase consisted of a 65:35 solution of methanol and 0.25 percent (weight to volume) aqueous ammonium dihydrogen phosphate buffer with a flow rate of 0.8 ml/min. The column (length, 25 cm; inner diameter, 4.5 mm) was an Apex model commercially packed with octadecylsilane reversed-phase material (particle size, 5 μm; Jones Chromatography). Experiments were performed at ambient temperature. A calibration curve was determined by plotting the auranofin peak height versus the gold concentration (in parts per million) of stock auranofin solutions. All solutions were filtered through 0.45- or 0.22-μm membrane filters before use.

6. Although the chromatographic peak at 4.5 minutes could have resulted from multiple components with similar retention times, the fact that the shape of the peak did not change when the synthetic single material 1-thio-β-D-glucopyranosato(triethylphosphine)gold(I) was added indicates that this material was the only component contributing to the peak.
7. Auranofin incubated in 0.01*M* HCl for 7 hours is largely unchanged; thus the bulk of auranofin ingested may be expected to reach the intestine intact [I. C. P. Smith, A. Joyce, H. Jarrell, B. M. Sutton, D. T. Hill, in *Bioinorganic Chemistry of Gold Coordination Compounds*, B. M. Sutton and R. G. Franz, Eds. (Smith Kline & French Laboratories, Philadelphia, 1983), pp. 47–57]. Apparently, contact with the mucosal cell surface initiates the deacetylation reactions since auranofin in the incubation medium outside the sac shows significant deacetylation as well.
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12. We thank J. Fondacaro for discussions of the everted gut preparation. Supported by NSF grant PCM 8023743 (R.C.E.), the Kroc Foundation (R.C.E. and K.T.E.), and Smith Kline & French Laboratories (R.C.E.).

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16 February 1984; accepted 22 May 1984

Single Channel Studies on Inactivation of Calcium Currents

Abstract. *Inactivation of calcium channels has been attributed to a direct reaction of calcium ions entering the cell with the calcium channel. For a single channel this hypothesis predicts a correlation between the amount of calcium entering during an opening or a burst of openings and the subsequent closed times. No such correlation was found, and the possibility that, upon entry, calcium ions produce inactivation is excluded.*

Activation of calcium channels in excitable membranes is voltage-dependent (*1, 2*), but inactivation has been described as voltage-dependent or current-dependent or both (*3, 4*). The current-dependent hypothesis attributes inactivation to a direct reaction between calcium ions that have entered the channel and the channel itself (*4, 5*). The opening of a single calcium channel provides an instantaneous and quantitative calcium injection, and the effect on inactivation can be investigated by observing subsequent single channel behavior.

Our experiments were performed with neuronal cell bodies in the right parietal ganglion of *Helix pomatia* with the use of the scheme shown in Fig. 1A, in which whole-cell and gigaseal ($>10^9\Omega$) patch clamp currents were recorded simultaneously (*6*). A sodium-free solution containing 40 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, 35 m*M* tetraethylammonium chloride, 5m*M* 4-aminopyridine, and 20 m*M* tris (*p*H 7.4) was used in the bath and patch pipettes. For measurements of barium current, barium ion was substituted isosmotically for calcium ion.

Unitary currents (Fig. 1B) were caused by the flow of calcium ions from the pipette into the cell. The rise and fall of averaged single-channel and whole-cell currents were similar. The time course of activation and inactivation increased with potential over the range of −25 to +5 mV. Changing the holding potential from −50 to −90 mV had no effect on single channel behavior during steps to these same potentials. Barium currents have also been studied and compared to calcium currents (*6*). The averaged single-channel and whole-cell barium currents also overlaid each other when scaled. The results showed that the average behavior of a single calcium or barium channel in a patch of membrane was the same as the behavior averaged from all the activated calcium or barium channels in the cell.

Some possible causes of inactivation can be excluded. The absence of outward unitary currents established that inactivation was a property of single calcium channels. Mean open times and amplitudes of openings were compared between the rising and falling phases (Fig. 2). These were unchanged as were the open time distributions, which were fitted by the same single-exponential function during the two phases. Therefore, neither a change in unitary conductance nor in open state caused inactivation. The closed times were distributed in at least a double-exponential manner (*7*), and the average values of the closed times during the first 50 to 75 msec which encompassed the peak of the current were compared with the average values during the last 200 msec, when the current was inactivating and had become almost steady. The average value of the closed time increased approximately threefold between these times, which compares well with the reduction of the frequency of opening in the averaged currents (Fig. 1B).

We deduced that if inactivation were due to a cytoplasmic process produced

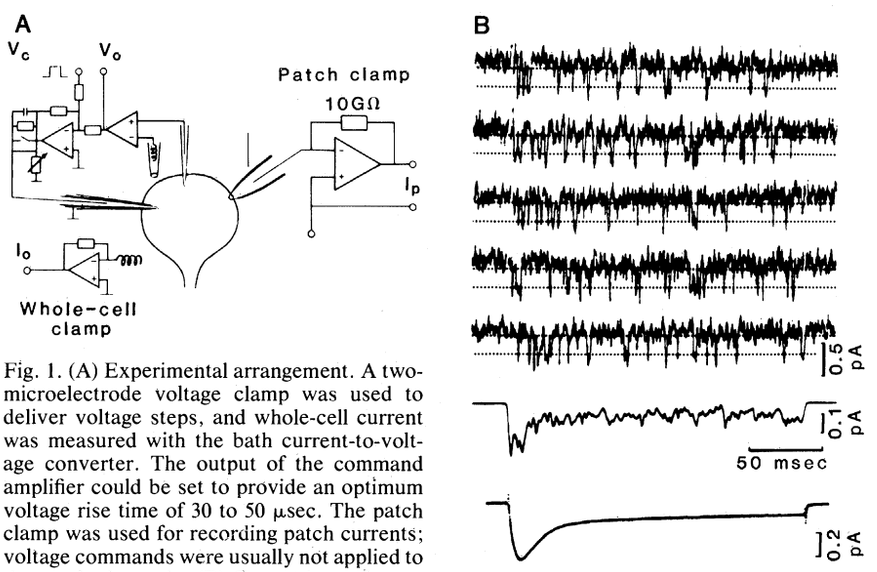

Fig. 1. (A) Experimental arrangement. A two-microelectrode voltage clamp was used to deliver voltage steps, and whole-cell current was measured with the bath current-to-voltage converter. The output of the command amplifier could be set to provide an optimum voltage rise time of 30 to 50 μsec. The patch clamp was used for recording patch currents; voltage commands were usually not applied to the patch electrode. (B) Comparison of averaged single-channel and whole-cell currents. Five samples of single-channel events are shown. Immediately below is the averaged current obtained from 42 single-channel records, in which one failure occurred. The bottom record shows a whole-cell current that was recorded simultaneously, showing a similar inactivation time course. Holding potential (V_H) was −50 mV; test potential was to a membrane potential (V_M) of 0 mV; pulse duration was 200 msec; intervals between pulses were 10 seconds. Temperature was 29°C to increase rates of activation and inactivation. The effects were also present at 20°C. Bandwidth was 1.0 kHz and sampling rate was 5 kHz. Threshold for openings was about three times the root mean square of the background noise and for closing about 1.5 times the root mean square of the noise.

by calcium entry, the process must be localized to the vicinity of single channels. Evidence against a role for generalized calcium entry through the cell body was obtained by experiments in which calcium was omitted from the bath solution. Calcium was replaced by magnesium and 10 m*M* nickel was added so that the whole-cell calcium current was completely blocked. Single-channel patch currents similar to those shown in Fig. 1B were obtained, confirming that for a calcium-entry model only currents through single channels need to be considered. Another way to examine this possibility was to record from a depolarized patch while holding the rest of the cell at −50 mV. The averaged patch currents obtained in this manner were similar to whole-cell currents recorded subsequently.

The effects of calcium entry were examined further with the use of selected records from ensembles of single-channel records. In any ensemble there are some records, such as the fifth record in Fig. 1B, in which opening frequency during the rise to the peak of the averaged current is relatively low. The currents averaged from such samples were compared with averages from those records in which the initial opening rate was particularly high. We found no example in which the larger initial calcium currents depressed the subsequent current average, as would be expected from calcium entry–dependent inactivation (Fig. 3A). The result also implied that channels can enter an inactivated state from the closed state and that the inactivated state is not absorbing. This test compared averaged currents over periods of 100 to 300 msec, and the question remained as to whether a short-term correlation between calcium entry and inactivation existed; that is, whether longer openings were followed by longer closings. We found that if each open period were plotted against the subsequent closed period, the relation was random. This was true for the first 75 msec, when the occupation probability for the open state was high, or for subsequent portions of the record that had low occupation probability. However, conclusions from event-by-event comparisons may be restricted by the possibility that openings or closings were briefer than the time required for the inactivation reaction proposed (*8*). Moreover, fast flickering to an activated closed state may not be related to inactivation.

To determine whether a correlation existed, we used bursts to compare averaged open-state occupation probabilities in segments of records beyond times of single events. On the basis of the calcium-entry hypothesis, we expected to find a negative correlation; that is, bursts with higher opening probabilities (P_o's) should have been followed by periods with lower P_o's. The single-channel data were scanned in segments by a time window (W_1) set at 6 to 40 msec, which encompassed the duration of most of the bursts. The average probability of opening within W_1 [$P_o(W_1)$] was computed; when $P_o(W_1)$ exceeded a preset value P_n, which was an integer multiple of the average P_o for the ensemble, a burst was found. The program continued to scan with W_1; when $P_o(W_1)$ fell below the threshold, the end of the previous setting of W_1 was marked as the end of the

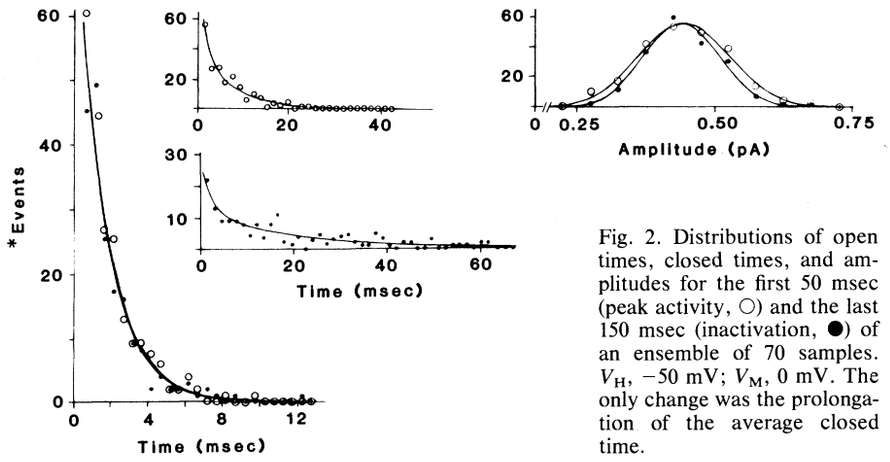

Fig. 2. Distributions of open times, closed times, and amplitudes for the first 50 msec (peak activity, ○) and the last 150 msec (inactivation, ●) of an ensemble of 70 samples. V_H, −50 mV; V_M, 0 mV. The only change was the prolongation of the average closed time.

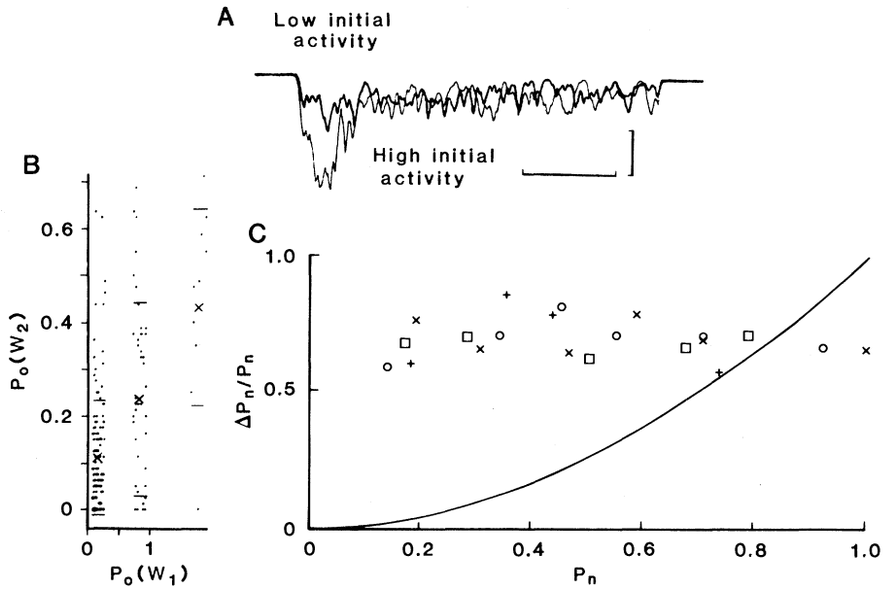

Fig. 3. (A) Comparison of the effects of high and low initial single-channel activity on the steady single channel activity. V_H, −50 mV; V_M, 0 mV. Bath solution contained Mg^{2+} and Ni^{2+} to block whole-cell calcium current. Seventeen samples with low initial activity ($P_a < 0.085$) and 21 samples with high initial activity ($P_a > 0.16$) were averaged. The complete ensemble contained 67 records. Despite considerable differences between initial activities, the steady levels were identical. Calibrated at 100 msec, 0.1 pA. (B) Examples of burstergrams. Steps of potential were 400 msec in duration and were from −50 to 0 mV. $P_o(W_1)$ were set at one and five times the average P_o of the ensemble. $P_o(W_2)$ means and standard deviations indicated. The larger points represent more than one value. $P_o(W_1)$'s that were greater than 1 arose from intense bursts of twice the window duration. The mean values in W_2 shifted towards higher P_o's, which was in contrast to the shift toward lower P_o's predicted by the hypothesis. (C) The fractional change in the difference between P_o's in W_1 and W_2 plotted against a range of P_o's given as P_n where n is a multiple of the average P_o of the ensemble. The curve $\Delta P_n/P_n = (P_n)^2$ follows from the last equation in (*8*) when the maximum value of 1 for $\Delta P_n/P_n$ was attained at $P_n = 1$. W_1 and W_2 durations were 6 and 20 msec, respectively. Results are from 21 burstergrams, each containing between 11 and 300 values at indicated levels; x is from a patch with bath solution identical to pipette solution; ○, □, and + are from three different patches having external Ni^{2+} and Mg^{2+} in the bath to prevent whole-cell calcium entry. The constancy of the measured results reflects the constancy of the spillover into W_2 of the falling phase of bursts of different intensity. This falling phase had a calculated time constant of about 15 msec.

burst. The probability of opening after the burst [$P_o(W_2)$] was computed within a time window (W_2) that could be set at several times the value of W_1. The expected negative correlation was not observed (Fig. 3B). The relationship between $P_o(W_1)$ and $P_o(W_2)$ (burstergram) seemed to show a positive correlation resulting from the carry-over of activity from the higher levels in W_1 to the lower levels in W_2.

The results of the burstergram may also be compared with predictions of the calcium-entry hypothesis for inactivation (*8*). The prediction is for a quadratic relation between the value of P_o during a burst and the subsequent change in P_o after the burst (Fig. 3C). The predictions were not proved by the results. Burstergrams for patches with two channels showed similar results.

We determined that inactivation occurred in averaged single calcium channel currents during a step change in potential and that it was due to a decrease in the rate of opening. Inactivation was not due to generalized calcium entry, and effects of localized calcium entry were excluded. The process appears to include a voltage-dependent component (*3*), although we have not extensively investigated this aspect at the single channel level. Nevertheless our results do not rule out an indirect, modulatory effect that calcium currents may have on transitions among closed states. In this case the channel, once activated, might be free to pass through the remaining states in the usual way. This implies that the effect of calcium entry is significant for a longer time than that encompassed by our experiments. This could explain the effect of changes in calcium current amplitude on recovery from steady-state inactivation (*9*).

Our results indicate that inactivation need not obligatorily be coupled to activation, as the calcium-entry hypothesis requires. These observations are not restricted to neuronal calcium channels. Similar results were obtained in rat and guinea pig ventricular myocytes and avian dorsal root ganglionic cells (*10*), and we suggest that they could apply generally to the behavior of single calcium channels.

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8. Calcium entering the cell reacts with a site S to form a complex CaS that inactivates the channel (*5*). The equilibrium, with dissociation constant K, is assumed to be instantaneous on the time scale of calcium channel activation. The probability P of a channel being open is expressed by
$$P = \frac{K}{[\mathrm{Ca}] + K}$$
where [Ca] is the intracellular calcium concentration. The change of [Ca] by entry through a channel relates to the probability that a channel, having been opened, will continue to be open; that is,
$$\frac{d[\mathrm{Ca}]}{dt} = D_P$$
where D is a constant relating to initial [Ca] and geometric factors. The solution to the differential equation is
$$[\mathrm{Ca}] = K \pm (2L)^{1/2}\{(t - t_0) + ([\mathrm{Ca}]_0 + K)^2(2L)^{-1}\}^{1/2}$$
where $L = DK$ and t_0 and $[\mathrm{Ca}]_0$ are integration constants. [Ca] is proportional to the current I and thus
$$\frac{I(t)}{2F^{-1}} = -0.5(2L)^{1/2}\{(t - t_0) + ([\mathrm{Ca}]_0 + K)^2(2L)^{-1}\}^{1/2} = \frac{i}{2F^{-1}} P_o(t)$$
for the probability of channel openings (F is Faraday's constant). After differentiating,
$$\frac{dP}{dt} = -RP^3$$
which relates opening probability with its change via calcium entry. The constant R ($A^{-2} \cdot sec^{-1}$) defines the initial conditions for each evaluation and applies, since sample periods with different occupation times were preceded by similar average opening activity.
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11. Supported in part by NIH grants NS11453 and HL25145. We thank D. L. Wilson and H. Zucker for their help.

29 December 1983; accepted 2 April 1984

Sedimentation Field Flow Fractionation of DNA's

Abstract. *Sedimentation field flow fractionation (SFFF) is a method for purifying and providing mass or size distribution information on samples containing particulates or soluble macromolecules. Since SFFF separations are based on simple physical phenomena related to first principles, molecular weight (or particle sizes) can be determined without calibration standards. SFFF is a gentle technique suited for fractionating biomolecules. Studies with the fragile λ DNA (molecular weight, 33×10^6) and smaller supercoiled plasmids have shown that these materials are not altered during SFFF separation; molecular weights and conformation remain unchanged, and biological activity is not reduced. Recoveries of nucleic acids approach 100 percent. Typically, components with about 20 percent difference in mass can be separated essentially to baseline if required. Fractionation time is usually independent of molecular weight, and separations often can be carried out within an hour.*

Sedimentation field flow fractionation (SFFF) is capable of the high-resolution separation of a variety of soluble macromolecules and organic and inorganic colloids with molecular weights ranging from 10^6 to 10^{13} (*1, 2*). Separations are performed in a thin open channel with a single, continuously flowing carrier liquid under the influence of an external centrifugal force field. The laminar flow profile generated within this thin channel causes mobile phase velocity to reach a maximum in the channel center and to approach zero velocity at the walls. Interaction of suspended or dissolved molecules with the external centrifugal force field that is applied perpendicular to the channel flow forces larger or more dense particles into slower flow streams nearer the wall so that they elute later than smaller or less dense particles. Thus, species elute in the order of increasing mass or particle density in the form of high-resolution "fractograms" similar to chromatograms.

The theoretical basis, equipment, and techniques of SFFF have been described (*1, 2*). The general application of this method to the particle-size distribution analyses of colloids and other suspended particulates also has been demonstrated (*3, 4*). Initial experiments have shown