Proc. Natl. Acad. Sci. USA
Vol. 74, No. 4, pp. 1748–1752, April 1977
Neurobiology

Voltage-dependent facilitation of Ca^{2+} entry in voltage-clamped, aequorin-injected molluscan neurons

(calcium current/membrane conductance)

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Communicated by Paul D. Boyer, January 3, 1977

ABSTRACT **Voltage-clamp experiments were performed on giant neurons of the nudibranch *Anisodoris nobilis* injected with the Ca-sensitive photoprotein, aequorin. Depolarization beyond −10 to +5 mV produced an aequorin signal, the amplitude of which depended on the extracellular Ca^{2+} concentration, the amplitude of the depolarization, and its duration. In paired pulse experiments, the amplitude of the aequorin signal produced in response to the second of two identical depolarizing pulses was larger than that produced during the first, resulting from an increased entry of Ca^{2+} during the second pulse. The increment in Ca conductance inferred from the augmented signal during the second pulse was independent of Ca^{2+} influx during the first pulse but, instead, was related to the amplitude and duration of the first pulse.**

Calcium entry activated by membrane depolarization has been demonstrated electrophysiologically in nerve cell bodies, axons, presynaptic terminals, and, more recently, dendrites (1–8). Some of the Ca systems described in these membranes are distinguished by having much slower inactivation kinetics during maintained depolarization than the Na systems studied in the squid axon and the node of Ranvier (9, 10). Several lines of evidence have recently appeared that indicate that the Ca systems may differ from the Na systems in another respect—namely, with repetitive depolarization, the Ca systems may exhibit enhanced activation rather than inactivation.

Stinnakre and Tauc (11) recorded Ca transients associated with single action potentials in a giant neuron of *Aplysia* by measuring light emission after injection with the Ca-sensitive photoprotein, aequorin (12–14). These light signals grew larger with successive impulses during short trains. During repetitive firing, many molluscan neurons exhibit a broadening plateau of the soma spike (11, 15), an effect that is pronounced in the presence of the K-blocking agent, tetraethylammonium. The plateau and its duration depend on the extracellular Ca^{2+} concentration, $[Ca]_o$, and the plateau is abolished by Ca-blocking agents (16). A Ca-dependent spike broadening with repetitive firing has also been inferred from extracellular recordings near the terminals of crustacean motor axons (17). In paired pulse experiments, the second depolarizing pulse exhibits a deficit in outward current relative to K^+ efflux. This deficit has been ascribed to a facilitating inward current (18) that appears to be carried by Ca^{2+} since it disappears with application of Ca-blocking agents (19).

We have performed experiments, on voltage-clamped, aequorin-injected giant molluscan neurons, designed to examine further the facilitated activation of Ca conductance. The light emitted by the aequorin, termed "the aequorin signal," is a function of the intracellular concentration of free ionized calcium $[Ca]_i$ (12, 14). The signal produced in response to a depolarization was found to be augmented by a prior depolarization up to about +60 mV. We then had to determine if the augmentation of the aequorin signal results from: (*i*) a facilitated entry of extracellular Ca^{2+} into the cell through the surface membrane, (*ii*) an augmented release of Ca^{2+} from intracellular stores, (*iii*) loading of cytoplasmic Ca-binding sites during a depolarization, leading to less binding capacity and more free Ca^{2+} during a subsequent depolarization, or (*iv*) priming of the aequorin so that the same amount of Ca entering during a subsequent pulse produces more light. The last of these is of little physiological interest, whereas the first three possibilities might have relevance to neural function.

Our findings indicate that depolarization leaves the membrane in a state such that the entry of Ca^{2+} during a subsequent closely spaced depolarization is facilitated.

MATERIALS AND METHODS

Specimens of the dorid nudibranch *Anisodoris nobilis* were collected during the summer months at Friday Harbor Laboratories and kept in fresh running sea water. The cerebral mass was isolated and treated for 3 min at 15° with Pronase to permit removal of connective tissue without trauma to the neurons. The neuron somata of the cerebral ganglia were exposed, and cells having diameters of at least 250 μm were selected for large resting (about −40 mV) and action potentials (about 100 mV overall). The preparation was bathed in an artificial seawater containing 470 mM NaCl, 10 mM $CaCl_2$, 10 mM KCl, 50 mM $MgCl_2$, 10 mM glucose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes; Sigma). The pH was adjusted to 7.7 ± 0.1 with NaOH. For most of the experiments the calcium concentration of experimental solutions was increased to 20 mM, with Mg decreased to 40 mM. This produced no qualitative change in the behavior of the membrane, and it improved the signal:noise ratio of the aequorin response. Solutions were changed by flushing the bath with 15 volumes. The bath temperature was held at 15° ± 1° with a Peltier cooling device.

Membrane potential was recorded and clamped with the circuit shown in Fig. 1*A*. The holding potential was set to −40 mV, and voltage steps were imposed with a time constant of 1–2 msec. The pulse program was repeated every 30 sec to allow adequate time for recovery processes. The reference potential was checked for drift after the experiment.

A purified ammonium sulfate precipitate of aequorin, prepared as described previously (12), was dissolved in 100 mM KCl and 4 mM *N*-tris(hydroxymethyl)methy-2-aminoethanesulfonic acid (Sigma), pH 6.8. The aequorin was assayed by exhaustive reaction of 0.5-μl samples with calcium acetate while the total number of quanta emitted was measured (20). The average value obtained in this assay was 1.3×10^{13} photons/μl of the injection solution. Assuming a quantum yield of 0.29 (21),

Abbreviations: $[Ca]_o$, Ca^{2+} concentration outside cell; $[Ca]_i$, Ca^{2+} concentration inside cell.

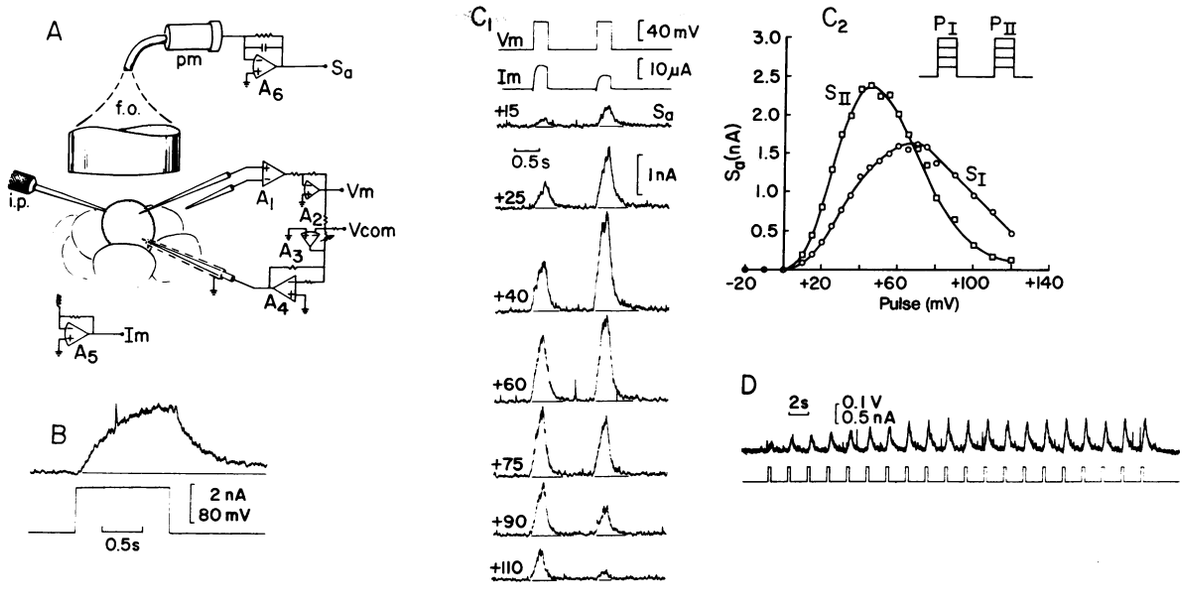

FIG. 1. (*A*) Experimental setup. Cells were voltage-clamped with a 2- to 5-MΩ voltage electrode and a 0.5- to 0.8-MΩ current electrode, both filled with 3 M KCl. The latter was shielded to within 100 μm of its tip with grounded silver (conducting) paint insulated with Carbowax. The bath was held at virtual ground, and current was measured through a 1.0 M KCl–agar bridge leading to the summing junction of A_5. The photomultiplier tube, *pm*, was operated at 800 V. Anode current was converted to voltage by A_6 with a time constant of 10 or 25 msec to give the aequorin signal *Sa*. *f.o.*, fiber optic wave guide; *Vm*, intracellular minus extracellular voltage; *Vcom*, command voltage; *i.p.* injector pipette: A_1, Burr Brown 3622; A_4, Datel 302A.

(*B*) Aequorin signal (upper trace) in response to a 1200-msec pulse (lower trace) to +55 mV. Holding potential (Vh) = −40 mv. $[Ca]_o$ = 20 mM.

(*C*) Paired-pulse experiment. (C_1) Top three traces are voltage (*Vm*), membrane current (*Im*), and aequorin signal (*Sa*) recorded simultaneously during depolarization to +15 mV with two equal pulses, P_I and P_{II}, separated by a 1-sec interval. Remaining traces show the aequorin signals recorded in response to pairs of equal pulses to the absolute voltages indicated. Vh = −40 mV throughout. $[Ca]_o$ = 20 mM. (C_2) Amplitudes of aequorin signals (S_I and S_{II}) plotted against pulse voltages. The negative shift of the peak of the S_{II} curve relative to the peak of the S_I curve occurred in most, but not all, preparations. The S_{II} maximum was always higher than the S_I maximum.

(*D*) Aequorin responses (upper trace) to a train of 300-msec pulses (lower trace) to +30 mV. Vh = −40 mV. $[Ca]_o$ = 10 mM.

we estimate the aequorin concentration in the injection solution to be about 8×10^{-5} M.

The aequorin solution was pressure-injected with brief pulses of about 0.7 kg/cm^2 through a double-bevelled (22) glass capillary. To prevent Ca contamination of the aequorin solution, the preparation was briefly bathed in calcium-free artificial seawater during the insertion of the injection pipette (23). A volume of aequorin solution was injected corresponding to about 20% of the original cell volume, while the membrane potential was continuously monitored with the voltage electrode. The injection was terminated upon any significant loss in the overshoot or undershoot of evoked action potentials; if the loss exceeded 5 mV, the preparation was discarded. The aequorin emission was converted to a current with an EMI 9635A photomultiplier tube. Membrane voltage, membrane current, and aequorin signal were stored on magnetic tape with a band pass of dc to 2.5 kHz and were subsequently transcribed with a strip chart recorder at reduced speed or, in some cases, were digitally processed with an averaging computer (Nicolet 1074, Madison, WI). Preparations typically continued to produce normal membrane currents and aequorin signals for several hours. During the early period there sometimes was a time-dependent moderate increase (10–20%) in the aequorin signal, but beyond that the signal strength stabilized, with little or no indication of aequorin depletion.

RESULTS

Aequorin signals were recorded from about half the cells that appeared to have been successfully injected. During a prolonged voltage clamp pulse, the signal increased at a progressively slowing rate to a maximal intensity. In Fig. 1*B*, an aequorin signal during a 1200-msec pulse to +55 mV is shown. The rising phase began after a delay of 10–25 msec, and the signal attained a voltage-dependent maximum within about 1 sec after onset of the pulse. The experiments that follow were done with pulses lasting 300 msec or less, during which the rise in signal was often nearly linear. The signal persisted briefly after the end of the pulse and then decayed. In most cells, decay of the aequorin signal was more than 95% complete within 1 sec after the end of the pulse. Significant slowing of the decay of the light signal was associated sometimes with high (i.e., > +100 mV) or prolonged pulses. A reduced rate of signal decay was also associated with a lowering of the bath temperature.

The amplitude of the aequorin signal was a function of voltage (Fig. 1*C*). The signal first became detectable at about −10 to +5 mV and exhibited a maximum between +30 and +70 mV. Further increase in positive potential was accompanied by a progressive drop in the amplitude of the aequorin signal. The falling limb of the curve generated in some preparations intersected the voltage axis at potentials between +100 and +160 mV. The curve more commonly flattened at high potentials, the pulse of high voltage producing small signals that failed to exhibit full suppression. A slowly developing aequorin signal with an abnormally slow recovery was often seen after large (> +100 mV) potentials, but true "off" responses with kinetics like those of normal responses were not seen. This suggests that the Ca channels close rapidly after repolarization to holding voltage, so that the amount of Ca^{2+} entering after repolarization is too small for detection.

The amplitude of the aequorin signal for a given depolarization depended on the extracellular Ca^{2+} concentration. The

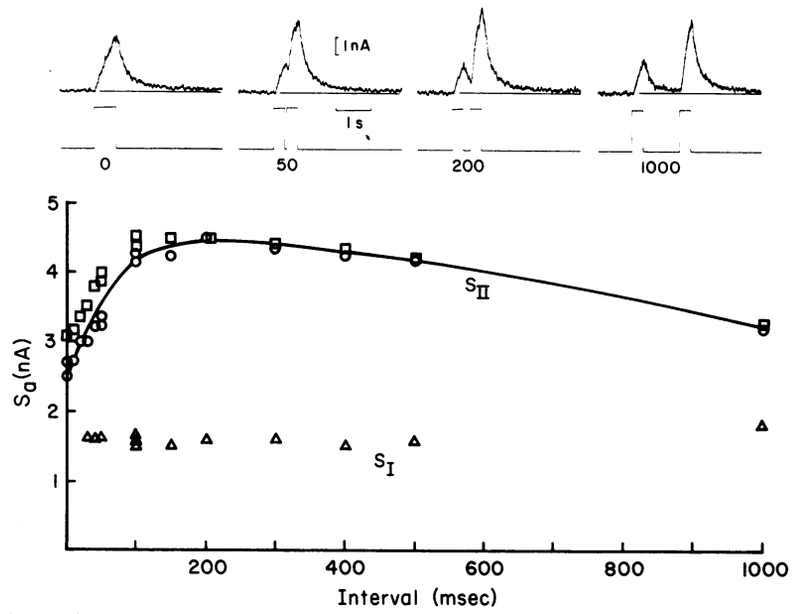

FIG. 2. Signal augmentation as function of signal interval. *Upper.* Representative records showing aequorin signals (upper trace) and membrane potential (lower trace). Numbers below indicate pulse interval in msec; 300-msec pulses to +50 mV; holding voltage = −40 mV. *Lower.* Amplitude of second-pulse signal (S_{II}) plotted against interval. □, measured from base line; ○, measured from projected trajectory of S_I; △, amplitudes of corresponding S_Is. $[Ca]_o$ = 20 mM.

signal amplitude typically increased about 4-fold in going from 10 to 20 mM $[Ca]_o$ and 6-fold in going from 10 to 100 mM $[Ca]_o$. In preparations bathed in artificial sea water without added Ca^{2+} (contaminant Ca^{2+} assumed to be $\leq 10^{-4}$ M), the aequorin signal was absent.

When depolarizing pulses were presented in a train of constant amplitude (Fig. 1*D*), the first few aequorin responses exhibited a progressive increase in amplitude similar to that observed during a train of spikes in *Aplysia* (11, 23). Augmentation (i.e., enhancement) of the signal by a prior depolarization was investigated systematically in twin-pulse experiments in which the pulses were equal in amplitude and duration and were separated by a 1-sec interval. For potentials up to about +60 mV, the aequorin signal associated with the second pulse exhibited a greater rate of rise and was significantly larger than the aequorin signal associated with the first pulse (Fig. 1*C*). The curve for the signal with the second pulse achieved a significantly higher maximum than did that with the first pulse in every case.

The relative depression of the second-pulse signal at the higher potentials and the negatively shifted second-pulse peak must be considered cautiously because of an uncorrected tissue resistance in series with the membrane capacitance and a depression of outward membrane current during the second pulse (Fig. 1C_1). These two factors could produce a compression of the second pulse voltage axis and a concomitant compression toward the left of the corresponding signal curve. To minimize this effect, the remaining experiments were done with pulses of smaller amplitudes.

Augmentation of the second-pulse signal was a function of the interval between the pulses (Fig. 2). The ratio of the second-pulse signal to the first was maximal for intervals of 100–500 msec and decreased gradually for greater intervals. At shorter intervals there was a steep decrease in augmentation, but the ratio remained larger than unity even when the projected trajectory of the first-pulse signal by itself was subtracted. These temporal relationships were compressed toward shorter times at higher temperatures (i.e., 25°) and were extended toward longer times at lower temperatures (i.e., 5°).

In Fig. 3 are the results when a 900-msec depolarization was presented as nine 100-msec pulses (*left*) and as a single 900-msec pulse (*right*). Since the amplitude of the aequorin signal is a positive function of the intracellular free Ca^{2+} concentration, the time integral of the signal must also be a positive function of the time integral of the increase in Ca concentration inside the cell, $[Ca]_i$. Assuming the intensity of the light signal to be a power function of $[Ca^{2+}]$ with an exponent greater than 1.0, a given number of Ca^{2+} entering over a greater span of time should produce both a smaller signal amplitude and a smaller time integral of the signal because there would be more time for dispersion of Ca^{2+} by diffusion or removal by sequestering and pumping activity and hence a lower concentration of free Ca^{2+}. This effect is seen at small pulse amplitudes (Fig. 3*A* and *A'*). When depolarization distributed in time as a series of pulses produces a larger signal integral, the amount of Ca^{2+} entering the cell must be greater than during a sustained pulse. Enhanced Ca entry during a distributed depolarization may result in part from time- and voltage-dependent inactivation kinetics of the Ca conductance during the sustained pulse. Evidence for inactivation can be seen in the time-dependent relaxation of the aequorin signal during sustained depolarizations in Fig. 3. The relaxation is greatest at the higher voltages (*C'* and *D'*). This suggests that the kinetics of inactivation of the Ca channel are voltage dependent, with inactivation beginning during a sustained depolarization and progressing more rapidly at higher potentials. Thus, the enhanced signal integral seen with intermittent depolarization may be due in part to removal, during the intervals of repolarization, of an inactivation of Ca channels that develops during periods of depolarization.

In addition to inactivation during sustained pulses and the reversal thereof by intermittent repolarization (Fig. 3), there is a treppe-like increase in amplitude seen in the successive signals. This signal augmentation, already demonstrated in Figs. 1 and 2, cannot be explained by inactivation.

We can ask, now, if the augmentation of the aequorin signal by a prior membrane depolarization results from (*i*) an actual increase in the entry of Ca^{2+} or (*ii*) a nonlinear increase of either

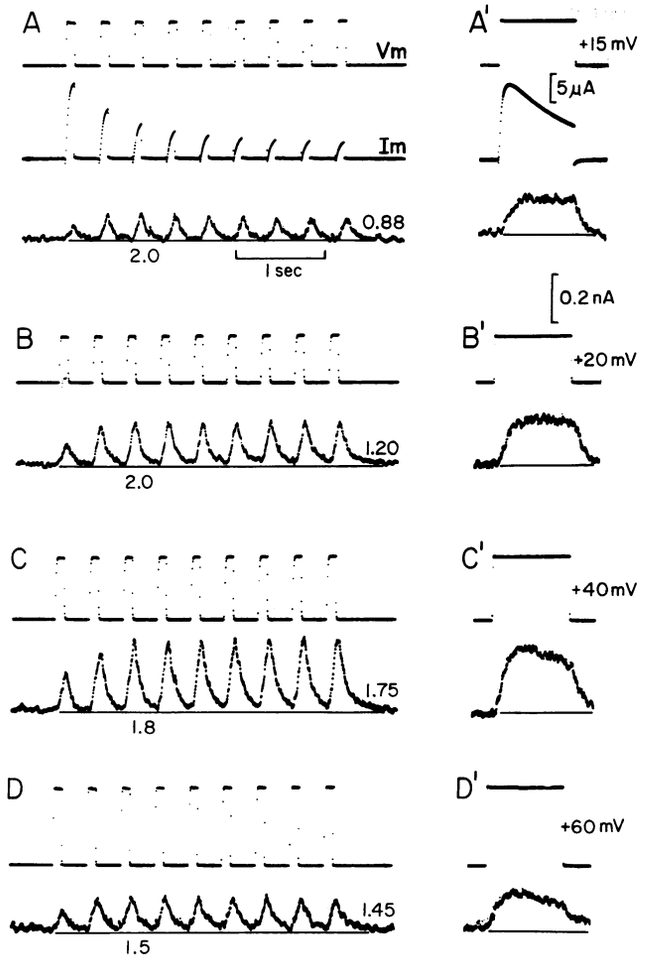

FIG. 3. Comparison of distributed (*Left*) and sustained (*Right*) 900-msec depolarizations. Pulse voltages are indicated next to each sustained voltage pulse. *Vm* = membrane voltage, *Im* = membrane current. The signal ratio of distributed-pulse:sustained-pulse integrals is indicated above the right end of each distributed aequorin signal. Relaxation of the aequorin signal during sustained pulses is independent of the signal amplitude and hence cannot be due to a decrease in aequorin activity. The number below the third-pulse signal of each train (*Left*) gives amplitude ratio of this signal to the first-pulse signal. Holding voltage = −40 mV. n = 8. $[Ca]_o$ = 20 mM.

$[Ca]_i$ or of the aequorin signal relative to Ca^{2+} entry. In one approach we ionophoresed Ca^{2+} into the aequorin-injected cell to see if the signal showed augmentation with paired pulses of Ca^{2+}. The signals produced by two successive ionophoretic pulses summed if they overlapped in time. With increased interval, the second signal was equal to or slightly larger than the first (Fig. 4*A*). The slow kinetics of the signal suggest that delivering Ca^{2+} from a point source (the pipette) is of limited usefulness for investigating the behavior of Ca^{2+} entering the cell in a distributed manner through the surface membrane.

A more critical demonstration that augmentation of the aequorin response is not due to any physiological or artifactual consequences of Ca^{2+} entry during a preceding pulse can be seen in Fig. 4*B*. Voltage pulses of different combinations of amplitude and duration were presented, and signal augmentations (indicated by numbers below signals) were compared. Augmentation was found to be a function of pulse amplitude and duration and not a function of the preceding aequorin signal. Thus, augmentation was greater with brief (50 msec) pulses to +40 mV (B_3) than with longer (300 msec) pulses to +10 mV (B_2) even though the initial aequorin signals in both

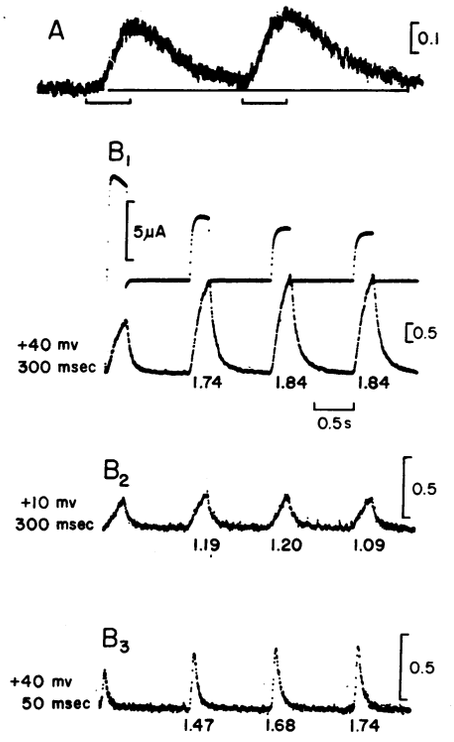

FIG. 4. (*A*) Aequorin responses to ionophoretically injected Ca^{2+}. A bevelled pipette filled with 0.1 M $CaCl_2$ with tip resistance of 20 MΩ was connected in series with a 1000-MΩ limiting resistor. Two equal 1-sec 100-nA ionophoretic current pulses were delivered while the cell was held at −40 mV under voltage clamp. Interval between pulses was 2.5 sec. n = 10.

(*B*) Relationship of signal augmentation to pulse amplitude and duration. Four pulses at 1/sec. Holding voltage = −40 mV. $[Ca]_o$ = 20 mM. Pulse voltages and durations are shown at left. (B_1) Membrane current (*Upper*) and aequorin signal (*Lower*). (B_2) and (B_3) Aequorin signals only. Numbers below signals give amplitudes relative to amplitudes of the initial signal of each train. Scale at right indicates anode current in nA. Augmentation is stronger for the +40 mV pulses than for the +10 mV pulses; at +40 mV the pulses lasting 300 msec produced greater augmentation than did the pulses lasting 50 msec.

pulse trains were of equal amplitude, and the time integral of the initial signal in B_2 was much greater. In other experiments (not shown), pulses of given amplitudes and durations produced larger aequorin signals when $[Ca]_o$ was increased; however, the increase in signal strength was not accompanied by an increase in the second-pulse signal:first-pulse signal ratios. Taken together, these findings indicate that the degree of signal augmentation is independent of the entry of Ca^{2+} during a previous depolarization and lead to the conclusion that, for a given interval between equal-sized pulses, the augmentation of the aequorin signal is a function of the voltage and duration of the depolarizing pulses and is not related to the entry of Ca^{2+} during the first pulse.

DISCUSSION

Because the aequorin signal is weak or absent at low $[Ca]_o$ ($<10^{-3}$ M) and increases monotonically with increasing $[Ca]_o$, the signal must result from a reaction of the aequorin with Ca^{2+} entering the cell through the surface membrane. Various relationships of emitted light intensity to free Ca^{2+} concentration have been reported for the Ca–aequorin reaction, ranging from linear to powers of nearly 3 (14, 21). We have noted a 4-fold increase in signal intensity after changing from 10 to 20 mM

$[Ca]_o$; this suggests a power of 2 relationship in the present experiments, if I_{Ca} is assumed to be linear with $[Ca]_o$ over that range of concentrations. If this relation were known to be consistent throughout our experiments, the actual ratio of Ca^{2+} entry during the second pulse relative to Ca^{2+} entry during the first pulse could be approximated by taking the square root of the ratio of the second to the first signals.

Of the four ways listed in the *Introduction* in which augmentation of the aequorin signal might arise, three have been ruled out in these experiments. The possibility of intracellular release was ruled out by the finding that the signal depends on the presence of extracellular Ca^{2+}. Cytoplasmic loading and priming of aequorin were ruled out by the demonstration that the amount of Ca^{2+} entering during a depolarization has no effect on the degree of signal augmentation seen during a subsequent depolarization. Augmentation of the aequorin signal appears, therefore, to represent a facilitated entry of Ca^{2+}. This in turn implies that Ca activation is facilitated by a prior depolarization.

The kinetics of Ca activation appear to be voltage dependent; the kinetics of inactivation are very slow at low potentials (refs. 2 and 3; Fig. 3*A'* and *B'*) and appear to be more rapid at high potentials (Fig. 3*C'* and *D'*). The current-voltage relationships of the Ca channels, studied in the squid presynaptic terminal, obtained in the presence of 3-aminopyridine suggest that opening of the channel involves about five Hodgkin-Huxley n-type gating units (7). Assuming the Ca channel consists of such multiple n-type units, all of which must be activated before the channel is "open," we can imagine that pulses of small and moderate voltages produce all possible states of partial activation in the population of channels during the depolarization, as well as full activation. Upon repolarization, those channels that were fully opened close completely and perhaps exhibit a temporary state of refractoriness. In contrast, those channels only partially activated by the depolarization may relax slowly and, because several particles are already primed, show an enhanced probability of opening during a subsequent depolarization, to produce a facilitated entry of Ca^{2+}. The relaxation of the aequorin signal seen with prolonged pulses at higher potentials (Fig. 3*C'* and *D'*) indicates that inactivation begins while the membrane is still depolarized.

In molluscan neurons, as in a number of other tissues, a component of the outward K current is activated by the entry of Ca^{2+} (24, 25). Because the outward membrane current recorded during voltage-clamp depolarizations exhibits a progressive depression during a train of pulses (Figs. 3 and 4), it might be conjectured that depression of outward current during a train of depolarizations results from reduced entry of Ca^{2+}. However, this cannot be the case since the aequorin signal increases rather than decreases while the outward current drops. Although the depression of net outward current seen in successive depolarizations roughly mirrored the increase in amplitude of the aequorin signal in some experiments (Figs. $1C_1$ and $4B_1$), the similarity was not apparent in other experiments (Fig. 3*A*). The depression in net outward current cannot, therefore, result entirely from charge subtraction by a facilitating inward partial current, although some charge cancellation by an inward current has been shown to take place (18, 19).

Our findings suggest that a state of partial activation of a large proportion of the population of Ca channels persists after repolarization with a decay time of several seconds. While those channels remain in this state, complete activation to the current-carrying state in response to a subsequent depolarization is facilitated. A similar persistent state of activation of Ca conductance may participate at presynaptic endings in the modulation of transmitter release, a process known to depend on Ca^{2+} entry (26). A facilitated Ca^{2+} influx may have special relevance in those synapses that exhibit a facilitated release of transmitter but in which release is related linearly to the entry of Ca^{2+} (7, 27).

Note Added in Proof. In an independent aequorin study on *Helix* neurons, H. D. Lux and C. Heyer (*Neuroscience*, in press) also found a facilitated entry of Ca^{2+} during repetitive depolarization.

We thank the Director of Friday Harbor Laboratories of the University of Washington for providing space and running seawater facilities, and we thank Drs. Johnson and Shimomura for the gift of purified aequorin. Dr. Robert Zucker, Douglas Junge, William New, and Peter Getting provided advice at various times, Dr. Paul O'Lague provided helpful comments on the manuscript, and Timothy Eckert collected specimens. Supported by U.S. Public Health Service Grants NS 8364, 5 SOF RR07009, NS 10919, and NSF BMS 74-19464.

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