AEQUORIN RESPONSE FACILITATION AND INTRACELLULAR CALCIUM ACCUMULATION IN MOLLUSCAN NEURONES

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SUMMARY

1. When molluscan neural somata are filled with the calcium-indicating photo-protein aequorin and subjected to a 1 Hz train of depolarizing pulses (0·3 sec duration to +15 mV) under voltage clamp, the successive photo-emissions due to calcium influx facilitate. The origin of this phenomenon was investigated in identified neurones from the abdominal ganglion of *Aplysia californica*.

2. Since outward currents inactivate cumulatively in successive pulses, the effective depolarization increases due to a series resistance error. Elimination of this error by electronic compensation or pharmacological block of outward current reduced aequorin response facilitation by only about 30 %, on the average.

3. When voltage-dependent sodium and potassium currents are blocked in tetra-ethylammonium (TEA)-substituted zero-sodium sea water, the remaining inward calcium currents display no facilitation. On the contrary, a slow decline during a pulse and a slight progressive depression in successive pulses are observed. Barium-substitution for calcium in the same medium eliminates a small residual potassium current insensitive to external TEA. The remaining inward barium currents also display depression instead of facilitation.

4. A non-pharmacological separation of calcium current was accomplished by measuring tail currents at the potassium equilibrium potential following depolarizing pulses. Calcium tail currents activate rapidly and then decline gradually and incompletely as depolarizing pulse duration is lengthened. Tail currents also show no evidence of facilitation; there is instead a slight depression of currents after successive pulses.

5. Increments of optical absorbance in neurones filled with the calcium-sensitive dye arsenazo III show a depression rather than facilitation to successive depolarizations in a train. The time course of these absorbance signals is consistent with the time-dependent depression of calcium current.

6. Calibration of arsenazo III response amplitude indicates that the dye reports only about 1 % of the calcium concentration increment expected from knowledge of cell volume and the charge carried by calcium current during a depolarizing pulse. This suggests that cytoplasmic buffering of free calcium must occur rapidly, on a time scale comparable to the response time of arsenazo III (about 1 msec) or more rapidly.

7. The slow potassium tail current following a depolarizing pulse is calcium-dependent and probably provides an approximate index of the internal sub-

membrane calcium concentration. Increments in this current after repetitive pulses display a slight progressive depression rather than facilitation.

8. Since neither calcium currents nor the concentration transients show facilitation, we conclude that aequorin response facilitation is due to the non-linear dependence of aequorin photo-emissions on calcium concentration. This conclusion is supported by a finding that the very different kinetics of arsenazo III responses and aequorin responses can be reconciled by a simple model representing calcium accumulation and known response properties of the two indicator substances.

9. In a train of impulses evoked by injecting depolarizing current into a neurone, the successive action potentials grow in duration. Nevertheless, a nearly constant calcium influx signalled by arsenazo III accompanies broadening action potentials.

INTRODUCTION

In 1973, Stinnakre & Tauc reported that molluscan central neurones filled with the calcium-sensitive photoprotein aequorin emitted increasingly intense flashes of light to successive action potentials in a train. More recently, Eckert, Tillotson & Ridgway (1977), Lux & Heyer (1977), and Stinnakre (1977) reported that the aequorin emissions to successive identical voltage-clamp depolarizations also facilitate. Since cytoplasmic calcium ions elicit aequorin photo-emissions and control a variety of normal cellular functions, the processes involved in aequorin response facilitation could be of wide importance. For example, similar processes may play a role in facilitation of transmitter release from nerve terminals (Katz & Miledi, 1968).

Originally, aequorin response facilitation was interpreted as evidence for a voltage-dependent facilitation of a membrane calcium influx. In contrast to this interpretation, calcium currents recorded from voltage-clamped molluscan somata show no suggestion of facilitation (Akaike, Lee & Brown, 1978; Tillotson & Horn, 1978; Connor, 1979). While voltage-clamp measurement of calcium current is subject to errors due to imperfect current separation, responses of the calcium-indicating dye arsenazo III also fail to facilitate (Ahmed & Connor, 1979).

We have explored the origin of aequorin response facilitation in *Aplysia* neurones by employing several different measures of calcium transients under conditions observed to optimize facilitation. We find no evidence for facilitation of inward calcium currents under voltage clamp, or of arsenazo III absorbance changes, or of calcium-dependent potassium currents. We can account quantitatively for the facilitation of aequorin photo-emissions by considering the nature of the calcium–aequorin reaction and the intracellular distribution of calcium in cytoplasm during and following a depolarization-induced influx. Moreover, the comparison of aequorin and arsenazo III measurements of calcium activity allow certain deductions about the fate of calcium that has entered the cell. We find evidence for powerful and rapid cytoplasmic binding of calcium (cf. Brinley, Tiffert, Scarpa & Mullins, 1977; Baker & Schlaepfer, 1978; Brinley, Tiffert & Scarpa, 1978), for a greatly slowed diffusion of free calcium in cytoplasm compared to water (cf. Hodgkin & Keynes, 1957; Blaustein & Hodgkin, 1969) and for a very slow extrusion or absorption of calcium by a membrane pump or intracellular organelles (cf. Blaustein, 1976; DiPolo, 1976; Blaustein, Ratzlaff & Schweitzer, 1978; Brinley et al. 1978).

METHODS

Experimental preparation

All results reported here were obtained in identified neurones from the abdominal ganglion of 200–400 g *Aplysia californica*. Many of the voltage-clamp and arsenazo III experiments have also been performed using neurones from the parietal ganglion of *Helix aspersa*, with similar results. Voltage-clamp and arsenazo III experiments were usually performed on clumps of axotomized cells, following the procedure of Connor (1977) to improve the isopotentiality of cells. For arsenazo III experiments, clumps of cells were pinned onto a thin Sylgard sheet bonded to the glass bottom of the recording chamber. Aequorin experiments were rarely successful with axotomized cells and were therefore usually performed on intact cells in a desheathed ganglion pinned to a similar Sylgard layer. A brief exposure to a few grains of pronase usually facilitated desheathing but a few experiments in which dissection was accomplished without pronase yielded similar results.

All experiments were performed in a controlled-temperature normal artificial sea water consisting of (mM): 495 Na^+, 10 K^+, 10 Ca^{2+}, 50 Mg^{2+}, 620 Cl^-, and 10 HEPES buffer pH 7·5 (half the HEPES is in anionic form). In barium sea water, cobalt sea water, and magnesium sea water, 10 mM-Ba^{2+}, Co^{2+} or Mg^{2+} was substituted for all the Ca^{2+}. In tris-(hydroxymethyl)-aminomethane sea water and tetraethylammonium sea water, $tris^+$ or TEA^+ was substituted isosmotically for all the Na^+. Detailed formulations are given in Zucker & Smith (1979).

Voltage clamp

In our voltage-clamp arrangement, we measure the transmembrane potential differentially between intracellular and extracellular voltage-recording electrodes, and apply wide-band negative feed-back between a second intracellular micro-electrode and extracellular grounding electrode. Our clamp includes a circuit for electronic series resistance compensation, following the method of Hodgkin, Huxley & Katz (1952). Series resistance was estimated from the instantaneous jump in potential following a step in current injected into the cell (Binstock, Adelman, Senft & Lecar, 1975). In practice, a 2 kHz square wave voltage was applied to the current-passing micro-electrode. A signal proportional to the current was sampled with a calibrated potentiometer and this voltage was subtracted from the measured membrane potential. The potentiometer was adjusted to nullify the series resistance potential jump, at which point its voltage was presumed equal to the series resistance error potential. In voltage clamp, the error potential could be added to the command potential to compensate for the portion lost to the series resistance.

The feed-back amplifier incorporated the modification described by Dionne & Stevens (1975) to boost the low frequency gain. The speed of the voltage clamp was maximized by using a wide-band negative capacity input electrometer to record from the voltage micro-electrode, by using a wide-band feed-back amplifier in the bath virtual ground circuit to maintain low input impedance at high frequencies, and by paying careful attention to circuit layout. When used in conjunction with well shielded glass micro-electrodes filled with 3 M-KCl and pulled to a resistance of 2 MΩ, and a low fluid level (200 μm above the neurone surface) to reduce stray capacitance between the voltage-sensing electrode and ground, we achieved capacitative current settling to 0·1 % of its maximum value within approximately 1 msec of command steps. A feed-back gain of up to 2×10^5 at 1 kHz could be used and still maintain stability. The rate of current settling is probably limited mainly by imperfect isopotentiality within the cell during a voltage step.

Aequorin procedures

Neurones were micro-injected with about 0·2–1·0 nl. of a solution of 0·2 mM-aequorin in 0·25 M-K_2SO_4, 2 μM-EDTA and 10 mM-phosphate buffer at pH 7·5. Light emitted as a consequence of the reaction of calcium with aequorin was collected and transmitted by a fibre-optic light guide to a photomultiplier tube (EMI 9789A) run at an anode voltage of 870 V. The anode current of the photomultiplier tube was measured using a current-to-voltage converter with a time constant of 30 msec. The anode current is proportional to the intensity of the light emitted by aequorin. For further details of our methods for the preparation, storage, assay and micro-injection of aequorin, and of detecting light, see Steinhardt, Zucker, & Schatten (1977).

Arsenazo III procedures

Cells were filled with arsenazo III to an estimated final concentration of 0·25 mM by inter-electrode ionophoresis from a micropipette filled with 75 mM-arsenazo III (Sigma Grade I). Our batch of arsenazo III contained less than 2 μM-calcium per m-mole dye. Light from a 100 W quartz-iodide lamp was projected through a 10 nm band-width interference filter (Ditric Optics, Marlboro, MA.) onto a field-stop diaphragm. That image was focussed by a camera lens (Switar f/1·4 25 mm) to form a 100 μm spot of monochromatic light on a single cell. The transmitted light
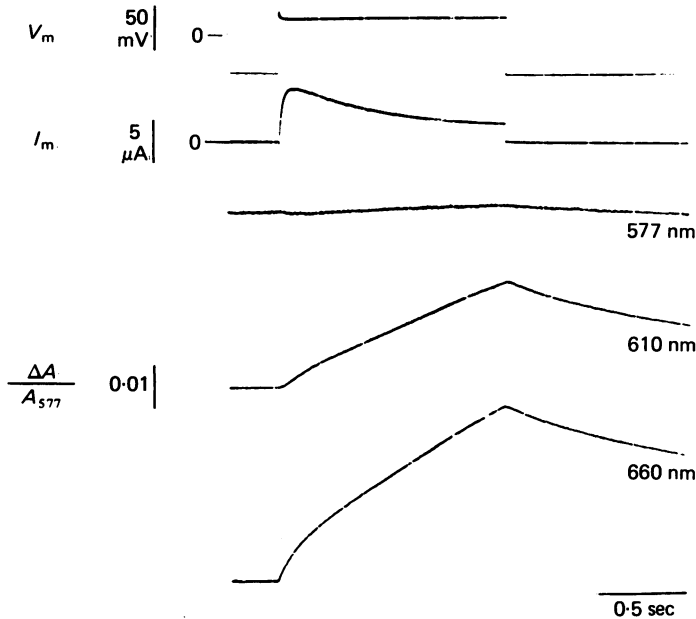

Fig. 1. Membrane current (I_m) and normalized absorbance changes ($\Delta A/A_{577}$) for a neurone filled with 0·25 mM-arsenazo III. The membrane potential (V_m) was de-polarized to $+15$ mV for one sec, and the absorbance change was recorded in three separate runs for incident light of 577, 610, and 660 nm. The response at the isosbestic point (577 nm) is less than 5 % that at 660 nm, and the ratio of responses at 660 and 610 nm (2:1) is that expected for a change only in intracellular calcium. Temperature 18 °C. Holding potential: -50 mV. In all Figures, outward current is up, unless indicated otherwise.

was collected by manipulating a 1/16 in. clad glass rod (American Optical) coupled to a low-noise photo-diode (UV 100, E.G. and G., Salem, MA.) to within 1 mm of the arsenazo III-filled neurone. The output of the diode was amplified to provide a signal proportional to the transmitted light intensity. The signal was filtered with a 3 msec time constant.

Since we must detect very small changes in dye absorbance ($\Delta A = 0·001$), fluctuations in incident light pose an important source of noise. These were minimized by a reference beam correction procedure. A beam splitter directed a fraction of the incident light to a reference photo-diode. The gains of separate preamplifiers for the transmission and reference photo-diodes were set to give outputs equal to 5 V with the resting, dye-injected cell in place. The preamplified reference signal was subtracted from the corresponding transmission signal by a differential amplifier with a gain of 20, yielding an over-all sensitivity equivalent to 1 V per 1 % change in transmitted light intensity. This method results in cancellation of incident beam noise fluctuations as long as these fluctuations and changes in cell absorbance are small. For the conditions of our experiments, this reference beam correction gave nearly an order of magnitude reduction of base-line drift and low-frequency noise.

Changes in path length due to cell movement, or changes in dye concentration due to osmotic

variation, could produce fluctuations in transmitted light. These changes ought to be relatively broad-band, and should affect equally the absorbance at 577 nm, the isobestic point of the arsenazo III difference spectrum for calcium changes, and the absorbance at 660 nm, the peak of this difference spectrum. However, we found that the changes at 577 nm were 5 % or less of those at 660 nm (see Fig. 1), so no correction for such changes was necessary.

For small absorbance changes, the change in absorbance is linearly related to the change in transmitted light intensity (ΔI) by the formula $\Delta A = \log_{10} e \cdot \Delta I$. Thus the amplified photodiode output gave a direct measure of absorption changes, when scaled appropriately.

Arsenazo III calibration

At low calcium concentrations, the absorbance changes of arsenazo III vary linearly with calcium concentration and with the square of arsenazo III concentration (Gorman & Thomas, 1978; Thomas, 1979). Thus the reaction is first order with respect to calcium, and second order with respect to arsenazo III: $Ca + 2X \rightleftharpoons CaX_2$, where X denotes arsenazo III. Let K_D represent the dissociation constant for CaX_2, $\epsilon_{660}^{CaX_2}$ and ϵ_{660}^{X} represent the absorption coefficients at 660 nm for CaX_2 and X, ϵ_{577}^{X} represent the absorption coefficient for both X and CaX_2 at 577 nm (the isobestic point), and let the total concentration of arsenazo III (X) be in excess of calcium, and therefore be constant. Then by a derivation similar to that of Brinley *et al.* (1978), the absorbance change at 660 nm, expressed as a fraction of the absorbance at 577 nm, can be shown to be directly proportional to changes in low levels of calcium activity

$$\frac{\Delta A_{660}}{A_{577}} = \frac{\epsilon_{660}^{CaX_2} - 2\epsilon_{660}^{X}}{\epsilon_{577}^{X}} \cdot \frac{X}{K_D} \cdot \Delta Ca. \tag{1}$$

This fractional absorbance change is independent of path length, so the same relation holds for a cell of any diameter. However, the fractional absorbance change does depend on dye concentration. Moreover, since arsenazo III forms a complex with magnesium, and magnesium is present in molluscan axoplasm at a concentration of about 3 mM (Brinley & Scarpa, 1975), X will actually be a magnesium complex of arsenazo III. Finally, arsenazo III also reacts with hydrogen, so that the effective binding constant K_D could be a function of pH (Portzehl, Caldwell & Ruegg, 1964). Thus, any attempt to calibrate the arsenazo III signal must be done in solutions with the same dye, pH and magnesium concentrations as cytoplasm, and the same dye concentration as that used in the experiments.

Arsenazo III binds calcium tightly, with an effective K_D of about 15–60 μM (Scarpa, Brinley, Tiffert & Dubyak, 1978). For a second order reaction, the value of K_D will depend on the arsenazo III concentration. We have confirmed this approximate value of K_D by using calcium-selective electrodes (Růžička, Hansen & Tjell, 1973) to measure the free calcium concentration in solutions containing 0·25 mM-arsenazo III, 0·1 M-KCl, 10 mM-HEPES pH 7·3, and various amounts of added calcium. Measurements at higher ionic strength and in the presence of magnesium were impossible, due to the low selectivity of the calcium electrode. Since calcium is bound tightly by arsenazo III, it might seem that a buffering action of the dye could seriously perturb the calcium concentration to be measured. Apparently this is not the case, however, since physiological events controlled by calcium seem to be little affected by arsenazo III concentrations below 0·5 mM (Brown, Brown & Pinto, 1977; Gorman & Thomas, 1978; Ahmed & Connor, 1979). This is presumably due to the fact that cytoplasm already contains calcium buffers much more powerful than the added arsenazo III (Brinley *et al.* 1977, 1978; Baker & Schlaepfer, 1978), so that the dye at a concentration of 0·25 mM perturbs the total cytoplasmic buffering of calcium only negligibly.

In order to fill cells with a known constant concentration of dye, we adopted a procedure of injecting every neurone with arsenazo III while observing its absorbance at 577 nm. We measured the molar extinction coefficient (ϵ_{577}^{X}) of arsenazo III in 0·5 M-KCl, 10 mM-HEPES pH 7·3, 3 mM-MgCl$_2$, and 10 mM-EGTA as $2 \cdot 98 \times 10^4$ cm^{-1} mole^{-1}. We injected arsenazo III until the absorption changed the appropriate amount for the cell diameter (D), using $A = \epsilon \cdot X \cdot D$, indicating a final arsenazo III concentration of 0·25 mM. For these large absorbance changes, absorbance was measured as $A = \log_{10} (I_1/I_2)$, where I_1 is the transmitted light intensity before dye injection, and I_2 is the intensity after dye injection.

To calibrate a batch of arsenazo III, we measured the ratio of $\Delta A_{660}/A_{577}$ for solutions of 0·25 mM-arsenazo III, 0·5 M-KCl, 10 mM-HEPES pH 7·3, 3 mM-MgCl$_2$, and 10 mM-EGTA with

3–8 mM CaCl$_2$ added. The pH value of 7·3 was similar to several measurements of intracellular pH (average of four cells at 15 °C, 7·17) which we made using pH-sensitive micro-electrodes (Thomas, 1976). The free calcium concentration in our EGTA solutions was calculated according to the method of Portzehl *et al.* (1964), except that a value of pK = 10·7 was used for the major Ca–EGTA binding constant, based on our measurements with calcium-sensitive electrodes (Zucker & Steinhardt, 1979), after correction for the effect of high ionic strength (DiPolo,
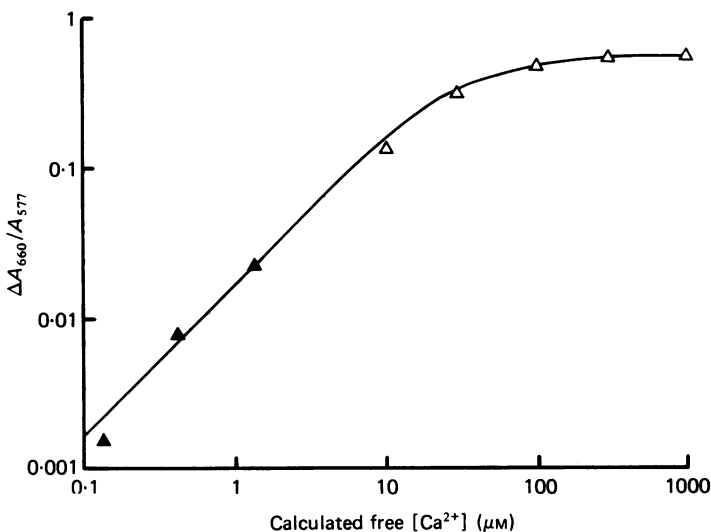

Fig. 2. Arsenazo III calibration curve for the relation between $\Delta A_{660}/A_{577}$ and free calcium concentration. Measurements were made using 1 mm cuvettes in a Cary 14 spectrophotometer on a solution of 0·25 mM-arsenazo III in 0·5 M-KCl, 10 mM-HEPES pH 7·3, 3 mM-MgCl$_2$ and 10 mM-Ca-EGTA buffer (filled triangles) or a solution of 0·25 mM-arsenazo III in 0·4 M-KCl, 50 mM-HEPES pH 7.3, 3 mM-free magnesium, and 50 mM-Ca-DPTA buffer (open triangles). The reference solution was identical to the test solution, but contained no dye. ΔA_{660} is measured with respect to a solution containing no added calcium. Measurements at 660 nm were corrected for variations at 577 nm. The calculations of calcium concentration used apparent dissociation constants of 0·35 μM for Ca-EGTA, 103 μM for Ca-DPTA, and 1·71 mM for Mg-DPTA. The curve is drawn to fit the points by eye, with an initial slope of 1·0.

Requena, Brinley, Mullins, Scarpa & Tiffert, 1976). This gave us buffers for free calcium levels between 0·1 and 1·0 μM. To extend this range, instead of EGTA we used a 50 mM solution of the weaker calcium chelator DPTA (1,3-diaminopropane-2-ol-*N*-*N*'-tetraacetic acid) in 0·4 M-KCl and 50 mM-HEPES pH 7·3. We added appropriate amounts of magnesium and calcium to get a final free magnesium concentration of 3 mM and calcium concentrations of 0·01–1·0 mM, using the procedure of Steinhardt *et al.* (1977) and the DPTA binding constants of Grimes, Huggard & Wilford (1963). The results of these calibration measurements are shown in Fig. 2.

In most experiments, measurement of dye absorbance was complicated somewhat by scattered light passing around the cell. If no correction were made for such light scattering, the concentration of arsenazo III injected into the cell would be underestimated. We estimated the amount of scattered light by occasionally filling with very large amounts of arsenazo III so that virtually no incident light would be transmitted through the cell. Such data allows correction of our measurement of dye absorption at 660 and 577 nm. From the latter, the probable actual dye concentration in our cells was calculated (typical values were 0·3–0·4 mM), and the conversion factor from $\Delta A_{660}/A_{577}$ to ΔCa (see Fig. 2) was corrected following eqn. (1).

In most experiments, voltage, current and optical signals were recorded simultaneously on magnetic tape and a chart recorder. However, neither instrument had a frequency response fast enough to record the fastest tail currents following voltage-clamp pulses. These were photographed directly from the oscilloscope screen using an automatic oscillograph camera.

RESULTS

Aequorin response facilitation

When neurones are filled with aequorin and depolarized repetitively, the photo-emissions to successive constant pulses often facilitate. We find that this phenomenon is most pronounced with pulses 0·2–0·3 sec in duration, to a potential of +10 to +20 mV, repeated at about 1 Hz. Typical records of facilitating aequorin photo-emissions are presented in Fig. 3*A*, *C*. Aequorin response facilitation could arise from a facilitating calcium influx to successive pulses, from changes in buffering of a given calcium load after prior influx, or from changes in the calcium-sensitivity of aequorin. Below, we consider each of these possibilities in turn.

Calcium current during pulse trains

Series resistance effects

The facilitation of aequorin photo-emissions is accompanied by a progressive reduction in outward current, due mainly to cumulative inactivation of potassium channels (Heyer & Lux, 1976*b*; Eckert & Lux, 1977; Aldrich, Getting & Thompson, 1979*b*). Outward current inactivation, with the consequent reduction in series resistance error, will result in increasing proportions of the command potential being imposed across the cell membrane. Due to the high voltage-sensitivity of calcium influx and consequently of aequorin photo-emissions in the range of pulses to about +20 mV (Eckert *et al.* 1977; Lux & Heyer, 1977; Stinnakre, 1977; Zucker & Smith, 1979; see Fig. 10*A*), this reduction in series resistance error could result in facilitation of the calcium currents and consequently of aequorin responses.

In an earlier publication (Zucker & Smith, 1979) we employed a pharmacological method of reducing series resistance error. In four cells we isosmotically replaced Na^+ with TEA^+ to block the outward potassium current and inward sodium current and reveal a nearly constant small net inward current (Fig. 3*B*, *D*). Under these conditions, the series resistance error is constant and minimal. In some preparations facilitation was hardly affected, while in others, it was nearly abolished. This suggests a variable contribution of series resistance error to the facilitation process, but other factors must also play a role. The situation is complicated by a separate pharmacological effect of TEA (Zucker & Smith, 1979). TEA enhances the aequorin emissions, beyond any effect of sodium replacement or the reduction of the series resistance error. Thus the interpretation of TEA action on aequorin responses solely in terms of series resistance effects is impossible.

We sought to avoid these difficulties by compensating the series resistance error electronically, as discussed in the Methods. Fig. 4*A*, *B* show responses of the membrane potential to an approximately square-wave trajectory of membrane current, with and without series resistance compensation. The effect of such series resistance

compensation on aequorin photo-emissions is shown in Fig. 4C, D. Compensation boosts the voltage commands so that the membrane potential now reaches the full desired level in each pulse. Compensation enhances all photo-emissions, as expected for larger depolarizations, and facilitation is reduced from a 117 % increase by the
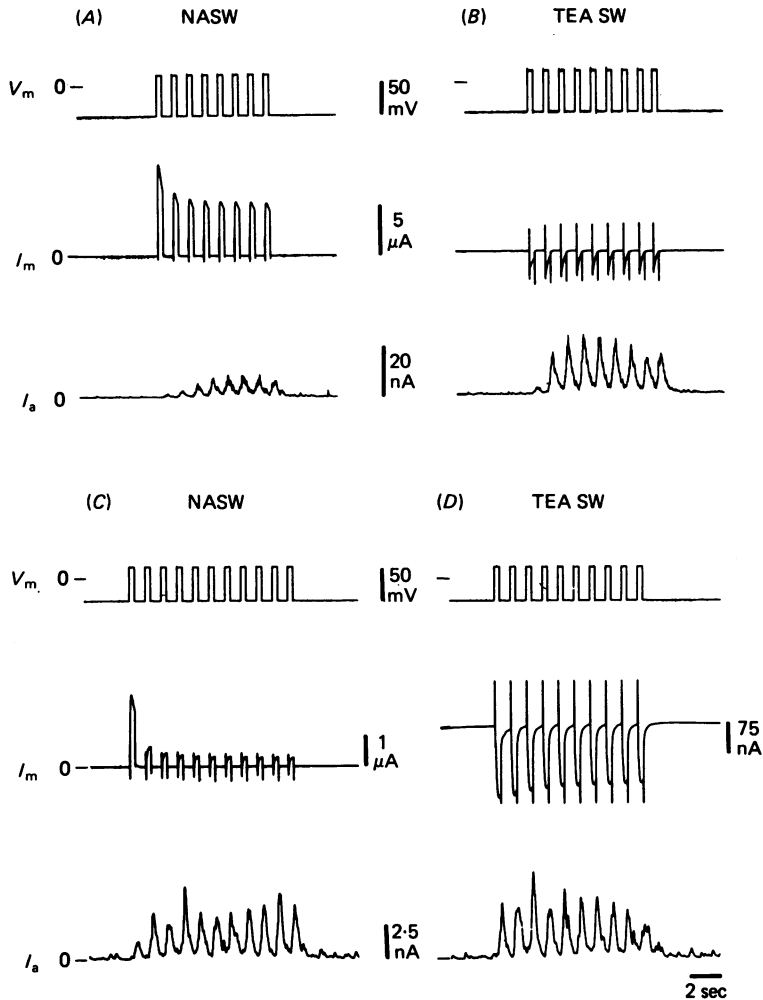

Fig. 3. Membrane currents (I_m) and aequorin photo-emissions measured as photo-multiplier anode current (I_a) recorded from cell R_2 (A and B) and R_{15} (C and D) in normal artificial sea water (NASW; A and C) or TEA sea water (TEA SW; B and D). The cells were stimulated with 0·3 sec pulses to +15 mV at 1 Hz. The holding potential was −50 mV for R_2 (A and B) and −40 mV for R_{15} (C and D). Temperature 18 °C. Aequorin signal filtered with 30 msec time constant in all Figures, except where stated otherwise.

fourth pulse compared to the first without compensation to a 55 % increase with compensation.

Since facilitation of aequorin emissions is maximal for uncompensated pulses to about +15 mV (Eckert *et al.* 1977; Lux & Heyer, 1977), the increased effective

depolarization in all compensated pulses might be responsible for the observed reduction in facilitation. However, even when the depolarizations were reduced so that the first compensated pulse was as effective as the first uncompensated pulse had been, facilitation was reduced with series resistance compensation (Fig. 4E).
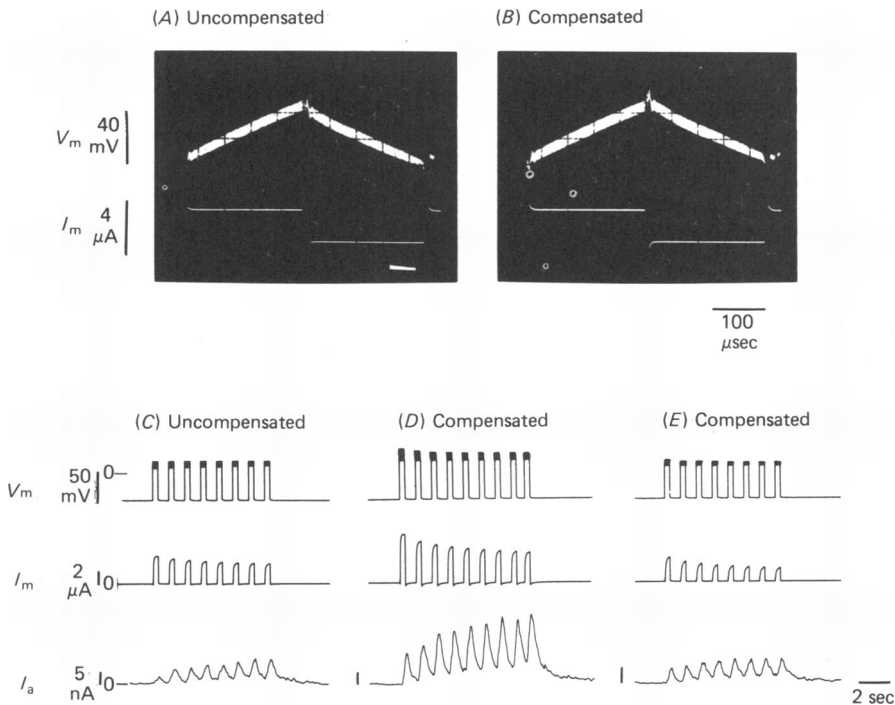

Fig. 4. Effect of series resistance compensation on aequorin photo-emissions. A, the membrane potential is recorded while injecting a 2 kHz square wave of current, 2·4 μA peak-to-peak amplitude. The sudden 4 mV jump in membrane potential at each current step corresponds to a 1·7 kΩ series resistance. This is electronically compensated by subtracting a signal proportional to the membrane current from the membrane potential until the series resistance error is nulled out (B). C–E, membrane currents and aequorin photo-emissions in response to a train of voltage-clamp pulses before (C) and after (D and E) series resistance compensation. In this cell, the series resistance was 2·4 kΩ. The unshaded part of the voltage pulse in C shows the expected level of the membrane depolarization in the presence of a series resistance error (shaded part) which subtracts from the constant command potential of +15 mV. In D, the command potential is automatically boosted by the shaded potential amount to keep the actual membrane depolarization at the desired constant level of +15 mV for each pulse. The fourth aequorin response was 117% greater than the first in C, and 55% greater in D. E, reducing the pulse amplitude to +5 mV with compensation did not restore the facilitation; it was now only 37%. Temperature 18 °C. A and B, cell L_4. C–E, cell L_2. Aequorin signal filtered with 80 msec time constant. Holding potential: −45 mV.

Similarly, the reduction of facilitation when the series resistance error was eliminated with TEA was not restored by changing the pulse amplitude (Zucker & Smith, 1979).

In six experiments like that illustrated in Fig. 4, we found that series resistance compensation reduced the facilitation of aequorin photo-emissions to between 28% and 100% of its level without compensation (average compensated facilitation was

69 % of uncompensated facilitation). We conclude that series resistance errors play a variable role in facilitation but are never entirely responsible for the phenomenon and frequently contribute a minor or even negligible effect.

Unfortunately, our procedure for series resistance compensation is subject to several sources of error. For example, precise measurements of rapid voltage steps are confounded by the response time of the micro-electrode recording system. Moreover, in some cells we found evidence of imperfect isopotentiality in the form of an axon spike, which comprised about 25 % of the peak transient inward current. This problem was particularly common in experiments in which no attempt was made to axotomize the cells. In such cells, part of the membrane current enters the axon and does not cross the resistance in series with the soma membrane. Hence, our procedure probably over-compensates the series resistance. From Graubard's (1975) anatomical and electrical measurements of the neurones we study, we estimate that for an axon and soma uniformly depolarized to + 15 mV, with a consequent 100-fold increase in membrane conductance due to potassium activation (Connor & Stevens, 1971c), about 71 % of the clamp current would enter the axon. The attenuation of axonal depolarization with distance from the soma will result in only part of the axon being activated, so less current will enter the axon. Nevertheless, soma series resistance will be over-compensated, suggesting that more than 70 % of the aequorin photo-emission facilitation is really due to factors other than series resistance error. Because of the small contribution of series resistance error, uncertainties in its compensation, and the de-stabilization of the voltage clamp introduced by series resistance compensation, most of the remaining experiments were conducted without compensation. However, each class of experiment described below (calcium currents, barium currents, deactivation tail currents, arsenazo III responses, and potassium tail currents) was performed at least twice using series resistance compensation, with little quantitative difference in the results.

Calcium channel behaviour

Pharmacological analysis of current during pulses. A net inward current at positive potentials in TEA sea water must be carried by calcium ions. The decreased inward current with successive pulses (Fig. 3) suggests that the calcium current progressively declines, rather than facilitates. However, the decline of net inward current (both during one pulse and between successive pulses) could reflect a slow activation of outward potassium current not fully blocked by TEA. Potassium current activation could mask a facilitating calcium current. Both transient outward current (Connor & Stevens, 1971b; Neher, 1971) and calcium-activated potassium current (Meech, 1972; Heyer & Lux, 1976b; Gorman & Thomas, 1978; Eckert & Tillotson, 1978) are relatively insensitive to TEA (Heyer & Lux, 1976b; Thompson, 1977).

The transient outward current may be inactivated in most cells by holding at a potential positive to − 45 mV (Connor & Stevens, 1971b; Neher, 1971). Unfortunately, there is at present no known agent or procedure for specific and complete elimination of calcium-activated potassium current. By substituting barium for calcium in the sea water, currents through the calcium channels can be viewed in isolation. Barium permeates the calcium channels (Eckert & Lux, 1976; Gola, Ducreux & Chagneux, 1977; Magura, 1977; Akaike et al. 1978; Connor, 1979) while failing to activate the calcium-dependent potassium current (Connor, 1979). Fig. 5 shows a typical record of the net inward currents observed in Ba sea water during a 1 Hz train of 0·2 sec pulses to + 20 mV. A nearly constant outward leak current, about 25 % the absolute magnitude of the net inward current, is observed in Mg sea water (Fig. 5). Thus the actual barium inward current is probably about 25 % larger than the net inward current. Barium currents also fail to facilitate, but rather

undergo a slow reduction in amplitude during each pulse and a slight cumulative reduction in successive pulses. Similar results were obtained from all four cells studied in this way.

Identification of calcium tail current. The above experiments relied on pharmacological intervention to isolate currents through calcium channels. One might suspect that behaviour of calcium channels could be altered by TEA or barium. To estimate
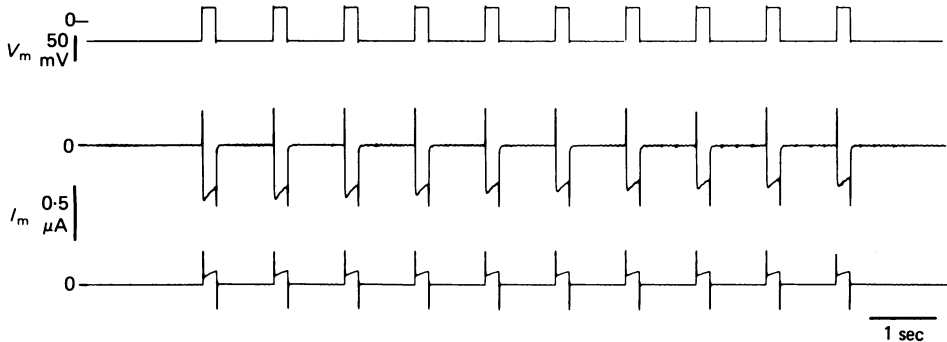

Fig. 5. Membrane currents in barium-substituted (middle trace) and magnesium-substituted (bottom trace) zero-calcium sea water. In both cases, sodium has been replaced with TEA. The top trace shows the membrane potential recorded in barium-substituted sea water. The record for magnesium-substituted sea water is identical, and is omitted from the figure. Cell R_2. Temperature 10 °C. Holding potential: -40 mV.

calcium currents in cells bathed in normal artificial sea water, we observed ionic tail currents following depolarizing pulses. Since sodium channels evidently inactivate nearly completely with depolarizations positive to 0 mV (Geduldig & Gruener, 1970; Connor & Stevens, 1971 c; Standen, 1975; Adams & Gage, 1976; Kostyuk & Krishtal, 1977 a; Connor, 1979), no sodium conductance should remain at the end of positive pulses lasting more than about 10 msec. By this time potassium current activation has begun but potassium tail currents may be minimized by stepping to near the potassium equilibrium potential following the depolarizing pulse. The potassium de-activation tail currents last over 100 msec at large negative potentials, and reverse at a potential near -60 mV (Connor & Stevens, 1971 c; Meech & Standen, 1975). Potassium tail currents at various voltages are shown in Fig. 6A. At the potassium equilibrium potential what remains are very rapid inward tail currents, which behave as expected for calcium current (Connor, 1977, 1979; Adams & Gage, 1979). The calcium tail current decays so rapidly that it is sometimes difficult to distinguish from the capacitance tail current. All experiments described in this section were performed on closely axotomized cells in order to obtain rapid capacitance current settling and therefore maximize the temporal separation of calcium tail current.

Two techniques were employed to further separate calcium tail currents from capacitance currents. First, we compared currents following depolarizing pulses of very different durations. A 1 msec pulse appears long enough to charge the membrane capacitance but too brief to activate appreciable ionic current in most cells, especially at low temperature (Connor & Stevens, 1971 a, c). As pulse duration is increased from 1 msec, the amplitude and duration of the inward tail also increase. On the basis of

experiments discussed below, it appears that the excess inward current following longer pulses is carried by calcium through channels which close with a time constant of about 1·5 msec at − 50 mV and 10 °C. At higher temperatures, the time course of the ionic tail current becomes more rapid and increasingly difficult to discriminate from the capacitance tail. Examples of tail currents following 1 msec and 300 msec pulses to + 20 mV are shown in Fig. 6B, C.
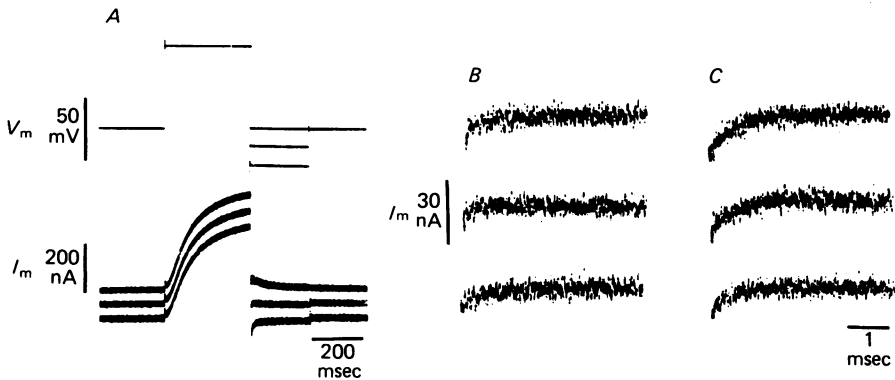

Fig. 6. Capacitance and inward current tail currents following depolarizing pulses. In A, a 0·3 sec pulse to + 25 mV is followed by 0·2 sec post-pulse potentials of − 40 mV, − 55 mV and − 70 mV (voltage records superimposed). The current records are shown displaced for the three post-pulse potentials of − 40 to − 70 mV from top to bottom. The post-pulse potential at which the slow potassium tail vanishes is used for measuring the fast inward tail currents after pulses to + 15 mV in B and C. B, capacitance currents following 1 msec pulses. C, capacitance plus inward calcium tail currents following 0·3 sec pulses. The first, fourth and eighth responses at 1 Hz are shown from top to bottom. Temperature 10 °C. L_B group cell. Series resistance compensated.

The second method used to distinguish capacitance and ionic tail currents depends on the approximately linear nature of the capacitance current. Thus, equal and opposite steps to the potassium equilibrium potential will generate equal and opposite capacitance transients. The asymmetry of capacitance attributed to gating current (Adams & Gage, 1976) is much less than the inward ionic tail current and may be neglected. Examples of tail currents following equal and opposite 75 mV steps to a − 55 mV post-pulse potential (i.e. pulses to + 20 and − 130 mV) are shown in Fig. 7. In normal artificial sea water the inward tail after a sufficient depolarizing pulse is always larger and slower than the outward tail after a balanced negative pulse. This asymmetry has the pharmacological and voltage sensitivities of calcium current, as discussed below. The large negative pulses used to obtain a capacitance tail sometimes damaged the cell membrane. To prevent this, we often used a negative pulse half the magnitude of the positive pulse, and recorded the tail current at twice the gain.

The pharmacological properties of inward tail currents were studied in twenty cells. Fig. 7 illustrates the most common results. In fifteen of these cells, the ionic tail currents clearly had the properties of a calcium current. Inward ionic tail currents were completely blocked in Co sea water or Mg sea water with no calcium. They were unaffected by tris or TEA substitution for sodium (except after very short pulses, see below). They were larger when barium replaced calcium. In the remaining five

cells, tris or TEA substitution for sodium reduced the tails to various extents, or substitution of Mg^{2+} or Co^{2+} failed to block them entirely. These observations may reflect an imperfect change in solution at the cell membrane or an incomplete sodium current inactivation (Smith, Barker & Gainer, 1975; Partridge, Thompson, Smith & Connor, 1979) and were not studied further.
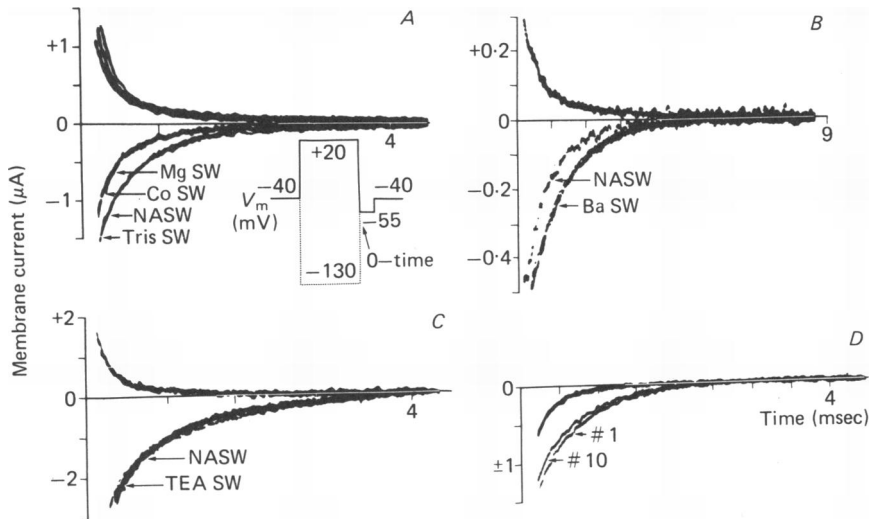

Fig. 7. Capacitance and inward tail currents in various solutions. *A*, positive capacitance currents following 30 msec pulses to -130 mV and inward capacitance plus ionic tail currents following pulses to $+20$ mV, measured at a post-pulse potential of -55 mV (see insert). Superimposed records after exposure to solutions in the following order: normal artificial sea water (NASW), Co sea water, tris SW, Mg SW. *B*, outward capacitance and inward capacitance plus ionic tail currents following 20 msec pulses to -100 mV and $+20$ mV, measured at the holding potential of -40 mV, in NASW and Ba SW. Following such relatively brief pulses, the potassium tail current was negligible. *C*, superimposed outward capacitance and inward capacitance plus ionic tail currents following 40 msec pulses to -100 mV and $+20$ mV, measured at the holding potential of -40 mV, in NASW and TEA SW. *D*, outward capacitance and inward capacitance plus ionic tail currents following 100 msec pulses to -130 mV and $+20$ mV repeated at 1 Hz and measured at a post-pulse potential of -55 mV. The capacitance tail currents have been inverted to facilitate comparison with the ionic tail currents. Currents following the first and tenth pulses are superimposed. Temperature 10 °C. *A* and *D*, cell R_2. *B*, cell R_{15}. *C*, a different cell R_2.

The inward ionic tail currents were further characterized by their kinetics and voltage-dependence. Fig. 8*A* shows data from one of twelve experiments in which the magnitude of the ionic inward tail current was studied as a function of the duration of the depolarizing pulse. The tail reaches a maximum about 50 msec and declines gradually and incompletely after longer pulses (note the logarithmic time scale). When sodium ions are replaced with either tris or TEA, the tail currents are similar, except for a reduction in magnitude after very brief ($\leqslant$ 10 msec) pulses. In this cell, there was a slight activation of sodium current even at 1 msec. These results confirm that sodium ions contribute little to fast tail currents after pulses longer than 10 msec, which should fully inactivate sodium channels (Geduldig & Gruener, 1970;

Connor & Stevens, 1971 c; Standen, 1975; Adams & Gage, 1976; Kostyuk and Krishtal, 1977 a; Connor, 1979). The time-dependence of the inward tail currents in sodium-free sea water presumably gives an indication of the time course of calcium current during the pulse.

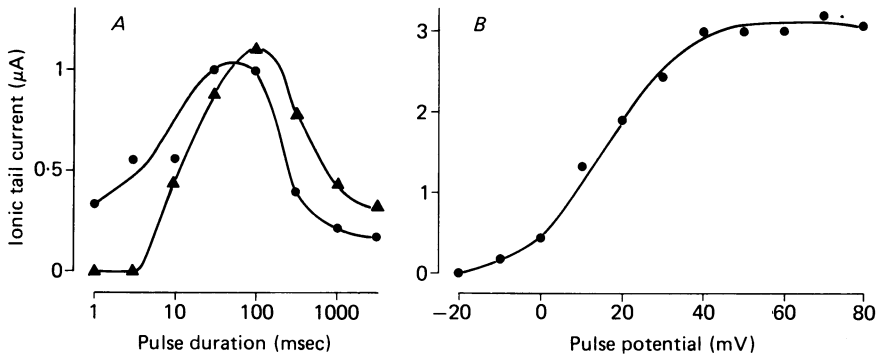Fig. 8B plots the magnitudes of inward ionic tail current following 40 msec

Fig. 8. A, magnitude of inward calcium deactivation tail currents after pulses to + 20 mV of various durations (shown on abscissa) in artificial sea water (ASW) (circles) and TEA SW (triangles). Twice the capacitance currents following equal duration pulses to − 77·5 mV were subtracted from the negative tail currents after positive pulses, and the currents were evaluated 0·5 msec after the voltage step to the post-pulse potential of − 45 mV. B, magnitude of inward calcium deactivation tail currents in ASW after 40 msec pulses to various potentials (shown on abscissa), measured 0·5 sec after the voltage step to − 40 mV and corrected by subtracting twice the capacitance currents following negative pulses half as large as the positive pulses. Curves fitted by eye. Temperature 10 °C. A and B, two different R_2 cells.

depolarizations of various amplitudes. Similar results were obtained from six neurones in artificial sea water, barium sea water, TEA sea water and Ba-TEA sea water. The activation voltage-dependence for ionic tail current is similar to that determined by different means in many other studies of molluscan calcium channels (Geduldig & Gruener, 1970; Standen, 1975; Eckert & Lux, 1976; Gola et al. 1977; Magura, 1977; Kostyuk & Krishtal, 1977a; Akaike et al. 1978; Tillotson & Horn, 1978; Connor, 1979). The kinetics and activation of tail currents were also similar to the time- and voltage-dependence of the inward currents in Ba-TEA sea water, after correction for the leak conductance (see Fig. 5).

One limitation in characterizing calcium tail current in Na sea water is the necessity of stepping exactly to the potassium equilibrium potential to completely eliminate potassium tail currents. Long pulses to very positive potentials appear to cause a shift in the potassium equilibrium potential, probably due to extracellular potassium accumulation. Potassium current then flows inward at the former reversal potential and sums with the calcium tail. Potassium tail current was recognized by its prolonged decay, and disappearance with small corrections of the post-pulse potential. The measurements reported here are confined to a region of pulse amplitude and duration where such potassium current interference was negligible.

Effect of repeated activation on calcium tail current. These results suggest that tail currents provide a relatively non-invasive measure of calcium channel behaviour, in that data can be obtained in normal sea water, and later ionic substitutions can

be used to confirm their identification as calcium currents. We now turn to the question of how these tail currents behave in trains of depolarizing pulses. Fig. 6*B*, *C* shows inward tail currents following the first, fourth and eighth pulses in 1 Hz trains of 1 and 300 msec depolarizations to +15 mV. Fig. 7*D* depicts tail currents following the first and tenth pulse in 1 Hz trains of 100 msec pulses to +20 and −130 mV, with a pulse potential of −55 mV. The capacitance currents are constant, while the calcium tail currents display depression of about 20–25 % in later pulses. Similar results were obtained with tail currents observed in tris-, TEA-, Ba-, and Ba-TEA sea water. No facilitation of calcium tail currents was observed in nine experiments, for pulses to between 0 and +30 mV, of durations between 20 and 300 msec, and at frequencies between 0·5 and 2 Hz. Calcium behaviour inferred from the calcium tail current is thus very similar to that inferred from barium current, and we conclude that both probably provide a fairly accurate indication of calcium current during pulse trains. Aequorin response facilitation in these neurones must therefore arise at some stage subsequent to membrane calcium entry.

Arsenazo III responses

Aequorin facilitation could arise from a property of cytoplasmic buffering of calcium loads. To investigate this possibility, we measured changes in arsenazo III absorbance during calcium influx. Since arsenazo III is a nearly linear indicator of free calcium concentration over a wide range (Fig. 2), its absorbance should be proportional to the average free calcium concentration in the cytoplasm sampled by the light beam.

Fig. 9 shows the effect of a train of depolarizing pulses on absorbance in a neurone filled with arsenazo III. The record is typical of the results of twelve experiments. The absorbance at 660 nm begins to increase abruptly at the beginning of each pulse, rises with a slightly declining slope during the pulse, and begins to fall abruptly at the end of each pulse. The successive increments in absorbance do not facilitate, rather they show a slight depression, so that by the eighth 0·3 sec pulse to −20 mV in a 1 Hz train, the increment is only 73 % of the response to the first pulse. This finding indicates that the calcium concentration change produced by successive pulses, like the calcium current itself, does not facilitate but instead declines during a train. After the last pulse, the absorbance signal recovers according to a complex time course, with an initial rapid phase of decay lasting a few seconds followed by a slower component of recovery lasting over a minute (cf. Ahmed & Connor, 1979).

Since the arsenazo III signal is proportional to the average free intracellular calcium concentration, its rate of change should be proportional to the difference between the rate of calcium influx through the membrane and the rate of calcium efflux and internal binding or uptake. Inflexions to the rate of change of arsenazo signals at the beginning or end of a pulse may be used as a measure of the voltage-dependent calcium flux into cytoplasm. In Fig. 9, the absolute magnitude of slope change at the end of the first arsenazo III response is 73 % of the change in slope at its beginning. The change in slope at the end of the last response is 80 % of the change in slope at its beginning. Finally, the change in slope at the beginning (or end) of the last response is 73 % (or 71 %) of the same measure for the first response. This gradual decline of calcium influx during individual pulses and between successive pulses is as would be

expected from the electrical estimates of voltage-dependent calcium current described above (see Figs. 3, 5 and 8A).

By reference to Fig. 2, the change in absorbance during the first pulse in the train shown in Fig. 9 should correspond to the appearance of approximately 220 nM-Ca^{2+} in cytoplasm. This figure can be compared to a concentration increment of 13 μM

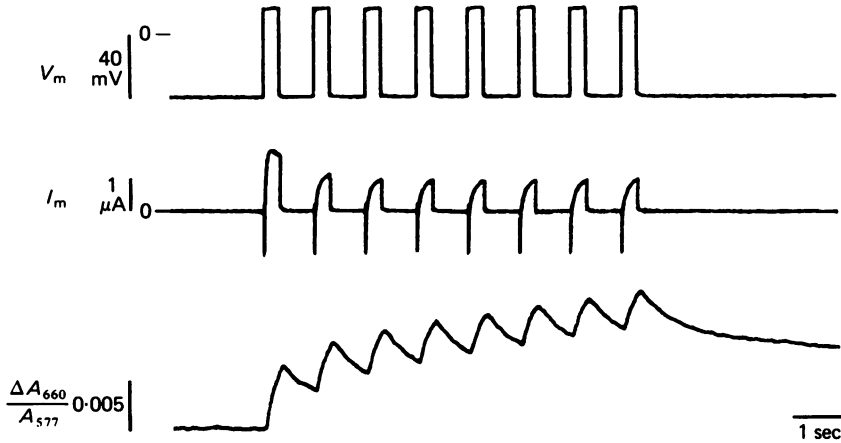Fig. 9. Membrane currents and absorbance changes at 660 nm in cell L_2 filled with 0·25 mM-arsenazo III, when depolarized to +15 mV for 0·3 sec at 1 Hz. The normalized absorbance change calibration bar corresponds to a change in average intracellular free calcium of about 175 nM.

expected from our estimates of the time integral of current during such a pulse and cell volume. The large difference between these values suggests that most of the calcium entering the cell is bound intracellularly (cf. Baker & Schlaepfer, 1978) and is not available to react with arsenazo III.

Calcium-activated potassium tail current during pulse trains

The above results with arsenazo III suggest that the increments in internal calcium concentration do not facilitate to successive depolarizations. An intrinsic index of intracellular calcium concentration is provided by the calcium-activated potassium current. The slow component of potassium current following depolarizing pulses evidently depends on the magnitude of calcium influx during the pulse (Meech & Standen, 1975; Thompson, 1977), and exhibits a time-dependence comparable to that of intracellular calcium accumulation (Gorman & Thomas, 1978). Although the point has not yet been conclusively established, the presently available evidence is consistent with the simple notion that potassium conductance is controlled directly by the calcium concentration adjacent to the inner membrane surface (Eckert & Tillotson, 1978).

Fig. 10 shows a high-gain record of membrane current during a 1 Hz train of 0·3 sec pulses to +15 mV. The slow potassium tails are outward at the holding potential (−40 mV). Outward current accumulates with pulses in a train, and declines slowly afterwards (Fig. 10A) with kinetics generally reminiscent of arsenazo III calcium

responses. The increments in slow outward tail current produced by succeeding pulses in a train are also similar to arsenazo III responses, and to our calcium current measures, in never showing evidence of facilitation. Rather, they show a decline of about 25 %. Typical data are shown in Fig. 10 B.
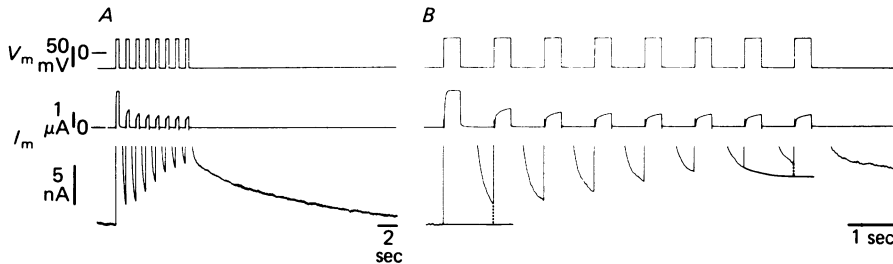

Fig. 10. Membrane current at two different amplifications during a 1 Hz train of 0·3 sec pulses to +30 mV, shown at two different time bases in *A* and *B*. The high gain current record displays slow outward potassium tail currents. The extrapolated tails were traced from currents recorded after a single pulse and a six pulse train. The dotted line represents an estimate of the increment in the slow tail contributed by the preceding pulse. Cell R_{15}. Temperature 17 °C. Holding potential: −40 mV.

Intracellular calcium–aequorin reaction kinetics

Sensitivity of aequorin to calcium. The arsenazo III and potassium tail results suggest that there is facilitation neither of calcium current nor of the calcium concentration transient. One might therefore suspect that aequorin response facilitation arises from the reaction kinetics of aequorin itself. It is known that aequorin signals measured in calcium buffer solutions are approximately proportional to the 2·5th power of the calcium ion concentration (Allen, Blinks, & Prendergast, 1977; Steinhardt *et al.* 1977). Earlier studies have noted that facilitation could be explained by such a non-linearity (Stinnakre & Tauc, 1973; Eckert *et al.* 1977; Lux & Heyer, 1977; Zucker & Smith, 1979). A given influx of calcium may produce a constant increment in calcium concentration, but if some free calcium remained from a previous influx, the new increment would occur at a higher position on the 2·5-power calcium sensitivity curve for aequorin. The result would be a larger response. This idea has been tested in a rough way by measuring the tail of the aequorin response immediately before a facilitated photo-emission and comparing the 2·5th root of the facilitated photo-emission to the sum of 2·5th roots of the tail and the unfacilitated photo-emission. Such calculations almost always predict even more facilitation than we observe (for example, Figs. 3 *A*, *B* and 4) except in cases where series resistance effects seem prominent (Fig. 3 *C* and *D*). Thus, it would seem that the 2·5th power-law calcium sensitivity could in fact be the major basis for observed facilitation of aequorin responses. Unfortunately, the reasoning above is strictly valid only for indicator responses from a single, well-stirred compartment, while there must be large gradients of calcium ions within cells during voltage-dependent calcium influx.

Numerous observations suggest that calcium concentration increases within cells can be highly localized near a source of net influx (Blaustein & Hodgkin, 1969; Andresen, Brown & Yasui, 1979; Baker, Hodgkin & Ridgway, 1971; Rose &

Loewenstein, 1975). The non-linear sensitivity of aequorin to calcium implies that the response will be sensitive to spatial distribution of a calcium concentration transient, as well as to total number of excess ions appearing in the cytoplasm. A localized region of high calcium concentration just inside the surface membrane would be expected to dominate the 2·5th power-law aequorin responses to inward
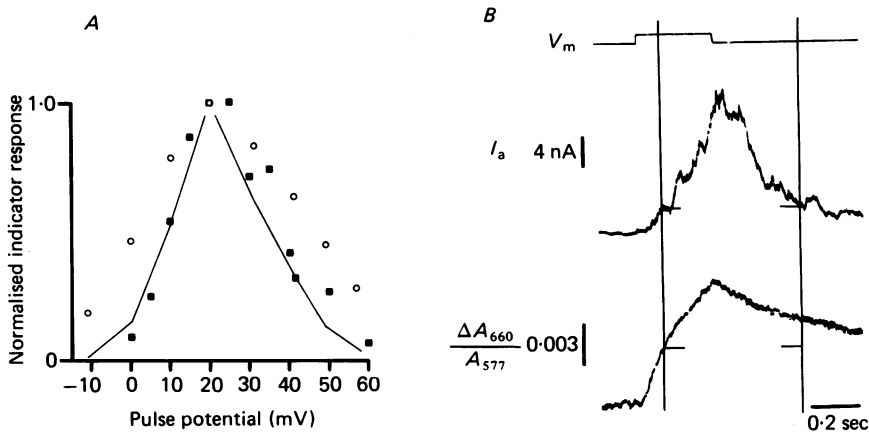

Fig. 11. *A*, arsenazo III (squares) and aequorin (circles) responses as a function of pulse amplitude, for 0·3 sec pulses. Both responses were normalized to the same peak. The line represents the arsenazo III responses raised to the 2·5th power and normalized. Aequorin data from cell R_2 in TEA SW; arsenazo III data from cell L_2 in ASW. *B*, aequorin photo-emission (I_a) and arsenazo III absorbance ($\Delta A_{660}/A_{577}$) signals from two different L_2 cells in ASW, in response to a $+20$ mV 0·3 sec pulse, from a holding potential of -45 mV. The vertical lines mark two times when the aequorin signals are equal. Temperature 18 °C. Series resistance compensated for aequorin response.

calcium current. The space and time profiles of calcium accumulation and diffusion therefore become central to quantitative interpretation of aequorin response facilitation.

Comparison of aequorin and arsenazo III responses. Distinctive differences between aequorin and arsenazo III responses seem to provide evidence for spatial localization of calcium transients. We have studied both aequorin (eleven cells) and arsenazo III (twenty-two cells) responses to carefully matched depolarizations in specific identified cells. It seems justified to assume that the intracellular calcium transients were generally similar regardless of which indicator was employed in a specific experiment. None the less, there are many striking differences in response kinetics which seem to be absolutely characteristic of the two different indicators. As noted above, successive aequorin responses facilitate while arsenazo III responses do not. Several other characteristic differences may help to explain the facilitation result.

Fig. 11*A* shows the voltage-dependence of arsenazo III and aequorin responses during 0·3 sec pulses. Responses of both indicators rise to a maximum near $+30$ mV, where calcium conductance reaches full activation (Fig. 8*B*), and become smaller with more positive pulses, presumably due to reduction of the electrochemical gradient driving calcium influx. The voltage-dependence seen with the two indicators differs in that the aequorin peak is narrower, with steeper voltage-dependence on both sides

of the maximum response. The continuous line in Fig. 11A is drawn through points representing the 2·5th power of the arsenazo III response amplitudes. The line seems to give a good prediction of aequorin response amplitude, as would be expected for calibrations in well-stirred buffers. This result does not, however, establish that the cytoplasm actually behaves as a well-stirred compartment. A power relation as apparent in Fig. 11A would be expected to hold approximately assuming only that the concentration transient at every point in the cell has a linear dependence on magnitude of calcium influx. This point can be justified by general consideration of indicator responses governed by power-law sensitivity curves.

The calcium sensitivities of both aequorin and arsenazo III can be approximated over some concentration interval by a relation of the form:

$$r = k[Ca^{2+}]^p \, dx^3 \tag{2}$$

where k is a constant sensitivity coefficient characteristic of the indicator and the apparatus, and $[Ca^{2+}]$ is the concentration of calcium ions. The response, r, is measured in a volume dx^3, which is assumed to be infinitesimal or well-stirred. Over most of the probable range of intracellular calcium ion concentrations the measured response is best fit by setting $p = 2·5$ for aequorin and by $p = 1·0$ for arsenazo III. The over-all response R to a spatially non-uniform distribution of calcium ions, designated $Ca_1(x)$, is simply eqn. (2) expressed as an integral over the entire cell volume monitored:

$$R = \int k \, Ca_1^p(\mathbf{x})dx^3. \tag{3}$$

If one supposes that the calcium transient arising due to depolarization, $Ca_1(\mathbf{x}, t)$, is governed by effectively linear processes, then a transient should depend on magnitude of calcium current in a linear way. Such dependence can be expressed by:

$$\triangle Ca_1(\mathbf{x}, t) = Q(V) \, U(\mathbf{x}, t), \tag{4}$$

where $Q(V)$ is a function representing the voltage-dependence of calcium influx, and $U(\mathbf{x}, t)$ is some function expressing the unvarying form after amplitude normalization of all calcium transients in space and time. From eqns. (3) and (4), the response to cell depolarization can be expressed by:

$$\triangle R(V, t) = \int k[Q(V)U(\mathbf{x}, t) + Ca_1(0)]^p dx^3 - \int k \, Ca_1^p(0)dx^3, \tag{5}$$

where $Ca_1(0)$ represents the resting free calcium level. In the case where $p = 1$, or in general if one assumes that the value of $Ca_1(0)$ is negligible in comparison to $Ca_1(\mathbf{x}, t)$, eqn. (5) becomes:

$$\triangle R(V, t) = Q^p(V)\int k \, U^p(\mathbf{x}, t)dx^3. \tag{6}$$

Eqn. (6) implies that $\sqrt[p]{(\triangle R)}$ should be directly proportional to $Q(V)$, provided that $\triangle R$ is sampled at a fixed time t in relation to the pulse. Since $Q(V)$ is presumed to be similar in aequorin and in arsenazo III experiments, the results shown in Fig. 11A confirm that both indicators function inside cells as expected from *in vitro* procedures.

These results would then further suggest that intracellular calcium transients have a fairly linear dependence on calcium current amplitude. Our voltage-dependence results, subject to the limitations of eqn. (6) are consistent with the existence of the expected spatial gradients of intracellular calcium. By the same token, however, these data cannot prove that such gradients exist or provide information about them.

The time-dependence of the two indicator signals should be fundamentally more sensitive to spatial gradients than the voltage-dependence. While the profile of intracellular calcium at fixed time may simply scale up and down with pulse amplitude, profiles sampled at different times during and after calcium current flow should have very different shapes. In keeping with such an expectation, the much faster recovery time course of aequorin responses as compared to arsenazo III responses (see Figs. 3 and 9, for example) cannot be reconciled even approximately by the $^p \sqrt{(\triangle R)}$ operation. While such disparities could in principle arise from time-dependence of indicator reactions, known properties of neither indicator would seem likely to account for the observed discrepancies unless there were in fact a non-uniform and time-varying distribution of intracellular calcium. In this case, variation of the integral term in eqn. (6) would account for the failure to reconcile responses at different values of t by $^p \sqrt{(\triangle R)}$.

Fig. 11B shows one example of a time-course difference suggestive of a varying intracellular calcium distribution. The responses of the two indicators to matched pulses shown in the Figure differ in a way that is typical of every one of our observations. The ruled vertical lines indicate times at which aequorin responses are equal during and after the pulse. If calcium distributions were the same at these times, arsenazo III signals should also be equal. This is never the case. Differences between the two indicator responses at the same time pairs, and other differences in time course, therefore seem to provide evidence for spatial redistribution of free calcium during and after depolarizing pulses.

Reconstruction of indicator responses. To investigate the possibility of explaining differences between aequorin and arsenazo III responses by spatial calcium variations, we have reconstructed indicator responses mathematically from a model for intracellular calcium movements. The intracellular calcium model used was developed previously as part of a study of bursting pace-maker activity in neurones of the nudibranch mollusc *Tritonia diomedia* (Thompson, 1976; Smith, 1978). Indicator responses were calculated by substituting values for Ca_i (x, t) predicted from the calcium model into eqn. (5) above, assuming a resting calcium level $Ca_i(0)$ of 100 nM (DiPolo *et al.* 1976).

Description of model. Factors likely to influence the intracellular calcium transient induced by depolarization include: the dependence of calcium current on time and voltage, the diffusion and binding of calcium ions in cytoplasm, and the active efflux or uptake mechanism which must maintain the low resting concentration. The intracellular calcium model we used, developed in detail elsewhere (Smith, 1978), represents each of these factors in a simplified way. Calcium current is represented here by equations fit to our voltage-clamp data from *Aplysia*. The equations reproduce the slow partial inactivation of calcium current described in connexion with Figs. 3 and 5–9. Ionized calcium is assumed to diffuse with binding to a uniformly distributed population of fixed cytoplasmic binding sites. For a case where the binding reaction

is assumed to be well below saturation and occur very rapidly, such diffusion should follow the equation:

$$\frac{\partial\, Ca_1\,(\mathbf{x},\,t)}{\partial t} = \frac{D}{\beta+1}\,\nabla^2\, Ca_1\,(\mathbf{x},\,t), \tag{7}$$

where $D = 6\cdot4\times10^{-6}$ cm²/sec is a diffusion coefficient appropriate to free aqueous diffusion of calcium ions (see Blaustein & Hodgkin, 1969), and $\beta = 100$ is a ratio of bound to free calcium giving an approximation to binding observed in squid axoplasm (Brinley *et al.* 1977, 1978; Baker & Schlaepfer, 1978). Diffusion is assumed to occur within a simplified cell geometry represented as a membrane-bound sphere 300 μm in diameter. Finally, active calcium removal is represented as an efflux, J_e, given by:

$$J_e = k_e\, Ca_1\,(a,\,t), \tag{8}$$

where k_e is a rate constant and $Ca_1\,(a,\,t)$ represents the calcium concentration at the membrane inner surface. Experimental measurements of efflux from squid axons can be approximated at low calcium by eqn. (8) with k_e values on the order of 10^{-3} cm/sec (Blaustein, 1976; DiPolo, 1976).

Eqns. (5), (7) and (8) were solved for indicator responses to calcium current by numerical techniques implemented on a digital computer. Spherical symmetry was assumed, and space was represented discretely by a series of thin concentric shells.

Though the model employs a greatly simplified representation of each factor likely to affect intracellular calcium ion metabolism, it adheres closely to directly measured parameter values. The single parameter adjusted by preliminary computing runs was the active efflux rate constant, k_e, which was set by finding the value to achieve the best prediction of arsenazo III response time course. The value chosen, $k_e = 3\cdot2\times10^{-3}$ cm/sec, is within the range appropriate to measurements of calcium efflux from squid axons (Blaustein, 1976; DiPolo, 1976).

Predictions from model. Fig. 12A shows predicted time courses of intracellular free calcium and of indicator signals in response to a simulated voltage-clamp pulse. Fig. 12B represents predicted indicator responses to a simulated train of depolarizations equivalent to that imposed experimentally in obtaining the data shown in Fig. 12C.

The model supports the expectation that intracellular calcium accumulation should be highly localized near the surface membrane. Fig. 12A shows that the peak free calcium rise during a 300 msec pulse falls to approximately 2 % of its value adjacent to the surface membrane by a depth of 4·3 μm within the spherical model cell. At later times after a pulse however, and during trains of pulses (top panel, Fig. 12B), free calcium increases propagate somewhat more deeply into the cell. The spatial scale of diffusion evident in the model is greatly restricted compared to that expected for calcium diffusion in water. This restriction is a consequence in the model of cytoplasmic calcium binding (see eqn. (7)).

Generally good agreement between wave forms in Fig. 12B and 12C shows that kinetics of intracellular calcium diffusion could indeed provide a simple explanation for major differences in signals from the two indicators. For instance, the much more rapid return to base-line response predicted for an aequorin-like indicator, in comparison to an arsenazo III-like indicator, is seen to arise from spatial redistribution of

free calcium following pulse termination. The predicted aequorin response falls rapidly as the localized high calcium concentration near the membrane is reduced by diffusion, whereas the arsenazo III response is insensitive to localization, and recovers only as calcium is actually removed from cytoplasm.

Facilitation of predicted aequorin response. Fig. 12*B* shows that the model predicts
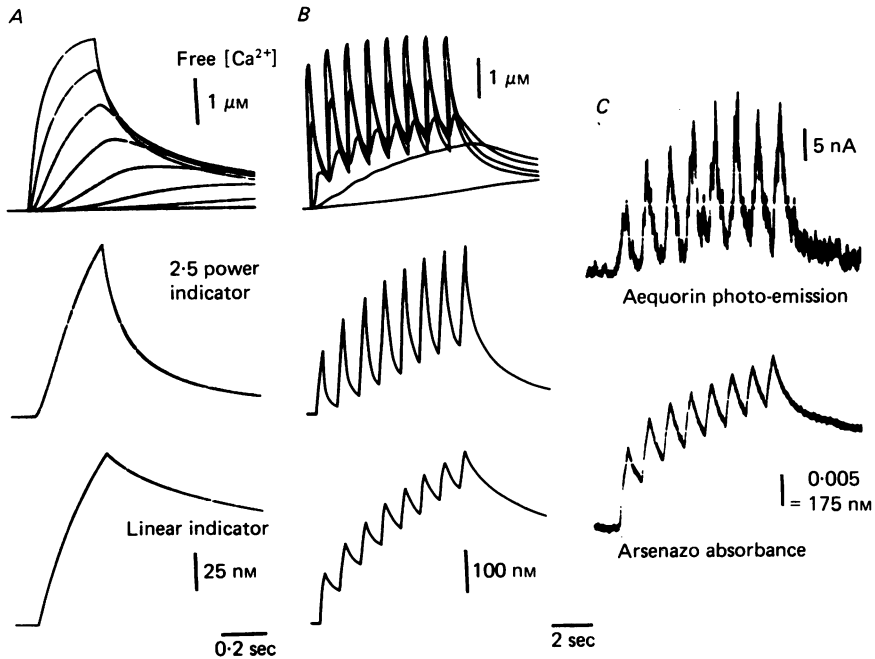

Fig. 12. *A*, theoretical prediction of calcium concentrations during and after 0·3 sec depolarizations to +15 mV, at the following depths (μm) below the plasma membrane: 0·2, 0·6, 1·2, 1·8, 2·8, 4·3, 6·5 and 10·8. Below these are shown the predicted responses of a cell filled with aequorin and a cell filled with 0·25 mM-arsenazo III. *B*, model predictions of calcium concentrations at depths of 0·2, 1·2, 2·8, 6·5, and 18·8 μm, and predicted aequorin and arsenazo III responses to a 1 Hz train of 0·3 sec pulses to +15 mV. *C*, typical aequorin photo-emissions and arsenazo III absorbance changes to such a train of pulses. Data from two different L₂ cells at 18 °C. Series resistance compensated for aequorin response.

facilitation of responses to an indicator having a 2·5-power calcium sensitivity. The trace in Fig. 12*C* is a typical experimental aequorin result, representing a time course and extent of facilitation in the middle range of our results. The model and experimental traces show a very similar pattern of growth and summation of responses, at least for the first five or six pulses in a train. The predicted response inappropriately continues to facilitate somewhat during later pulses, whereas most actual cells show a more pronounced levelling off or slight reversal of response growth, as in Fig. 12*C* This discrepancy seems minor, and could be due to a very small error in our measurements of calcium current depression.

We wish to emphasize here that the aequorin response calculated from the model is truly a prediction, in the sense that all parameters were set in advance on the basis

of independent experimental measurements. Considering that no parameters were adjusted to fit solutions to any aspect of aequorin responses, prediction of response facilitation seems remarkably accurate.

Calcium influx in nerve impulses

Although the evidence against facilitation of the voltage-dependent calcium channels now seems compelling, it is still possible that the facilitation of aequorin photo-emissions to a train of nerve impulses (Stinnakre & Tauc, 1973) reflects a real facilitation of calcium influx. This is especially true because successive impulses in a
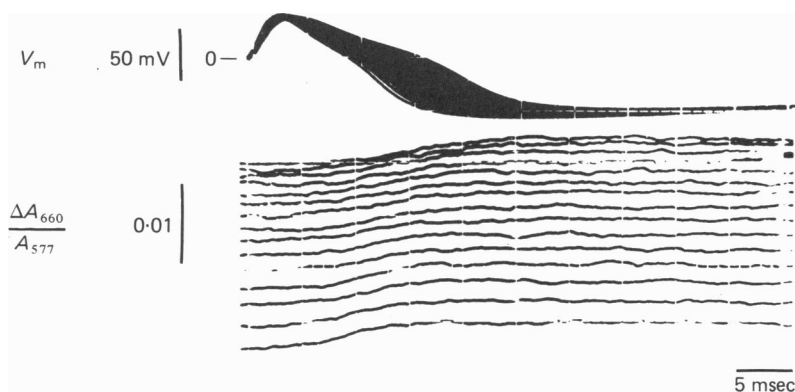

Fig. 13. Arsenazo III absorbance-changes accompanying a 10 Hz train of action potentials elicited by a 50 nA depolarizing current. The first impulse is the briefest, and the corresponding absorbance signal is the bottom-most one. The normalized absorbance calibration bar corresponds to an average change in internal calcium concentration of about 200 nM.

train in molluscan somata grow in duration, due to a cumulative depression or in-activation of potassium currents (Aldrich, Getting & Thompson, 1979a). However, we find that the arsenazo III absorbance-changes to successive broadening spikes in a train do not facilitate (Fig. 13). In six experiments, we found absorbance-changes to be constant (within a measurement uncertainty of about 10 %), while spikes broad-ened up to 50 %. Broadening of the action potentials occurs primarily at potential levels near zero, where there is little calcium influx, compared to that accompanying the peak of the action potential (Fig. 8B). During similar spike trains, the aequorin photo-emissions grow to more than double their initial size. Our model duplicates this behaviour, demonstrating that it also is due to the non-linearity of aequorin and the accumulation of submembrane calcium.

DISCUSSION

Mechanism of facilitation

Light output from aequorin-loaded neurones results from a process that can be considered as a sequence of four stages: (1) the surface membrane is depolarized by nerve activity or a voltage-clamp circuit, (2) depolarization opens calcium channels, causing a net influx of calcium ions, (3) the influx raises the concentration of calcium

ions in cytoplasm and (4) increased calcium elicits increased photo-emission by aequorin in the cytoplasm.

To investigate the mechanism of aequorin response facilitation, we have attempted to characterize the behaviour at each of these stages during trains of identical voltage-clamp pulses. We find that only the aequorin response itself facilitates, suggesting that facilitation must arise entirely from the cytoplasmic calcium–aequorin reaction (stage 4). Increments of cytoplasmic free calcium (stage 3), inferred from arsenazo III absorbance and from calcium-activated potassium current, appear to decline slightly during pulse trains, although there is considerable temporal summation of calcium loads from individual stimuli. The stoichiometry of the calcium–aequorin reaction implies that linear summation of calcium loads should produce photo-emissions with the supra-additive summation characteristics referred to as facilitation. Calcium influx (stage 2), measured as calcium current, also appears to decline slightly with successive pulses in a train. Finally, series resistance errors in voltage-clamp control of membrane potential (stage 1) sometimes increase the amount of aequorin response facilitation observed. This effect is generally small, however, and calcium current does not actually facilitate, but simply declines less before compensation of the series resistance error.

Contrary to our own conclusions, earlier voltage-clamp data suggested a facilitating inward calcium current (Heyer & Lux, 1976a). Moreover, an increasing deficit between measurements of potassium efflux and net outward currents in pulse trains (Lux & Eckert, 1974; Heyer & Lux, 1976a) was attributed to a facilitating inward calcium current. Finally, earlier studies with aequorin (Eckert et al. 1977; Lux & Heyer, 1977; Stinnakre, 1977) suggested that at least part of the facilitation of aequorin responses was due to facilitation of calcium current. The non-linear calcium dependence of aequorin photo-emissions was believed to be quantitatively inadequate to explain the observed aequorin response facilitation.

All three points of evidence must be re-evaluated in light of more recent studies. First, the earlier voltage-clamp measurements suggesting inward current facilitation were subject to considerable ambiguity in separating calcium current from other components of ionic current. Having benefit of new data on calcium channel activation kinetics (Connor, 1977, 1979; Kostyuk & Krishtal, 1977a; Akaike et al. 1978), the facilitating inward tail currents described by Heyer & Lux (1977a) seem much too slow to represent calcium current, and may instead represent variations in potassium current due to external accumulation and conductance inactivation effects. With improved methods for measurement of calcium influx, there have been several reports of failure to observe calcium channel facilitation (Akaike et al. 1978; Tillotson & Horn, 1978; Ahmed & Connor, 1979). Secondly, the growing deficit in potassium efflux compared to outward current (Lux & Eckert, 1974; Heyer & Lux, 1976a) was due in part to inactivation of potassium channels. The deficit may also have been over-estimated by neglecting slow potassium tail currents in the integration of outward currents, and the effect of a potassium-activated potassium absorption pump (Heinemann & Lux, 1977) in the efflux calculations. Finally, recent studies of the calcium–aequorin reaction (Allen et al. 1977; Steinhardt et al. 1977) show that the photo-emission has a higher-order dependence on calcium than previously believed (approximately power-law with an exponent of 2·5 instead of 2·0). This non-linearity

could therefore make a greater contribution than was expected to the facilitation observed in the earlier studies.

Limitations of experimental measurements

None of our measures of calcium influx is perfect. The net inward currents in TEA sea water probably include some calcium-activated potassium current incompletely blocked by TEA (Heyer & Lux, 1976*b*; Thompson, 1977), whose slow activation will appear as an inward current inactivation. The calcium channel may not transport barium with the same kinetics as calcium (cf. Magura, 1977 to Akaike *et al.* 1978), which could invalidate conclusions drawn about calcium facilitation from barium currents. Calcium tail current is difficult to measure because its rapid decay challenges the temporal resolution of the voltage-clamp arrangement. The relationship between calcium-activated potassium current and intracellular free calcium has not yet been determined directly. Series resistance errors can interfere with measurements of calcium tail currents, calcium-activated potassium currents, arsenazo III responses, and aequorin responses, although they appear to significantly influence only the highly voltage-sensitive aequorin responses. Due to imperfect isopotentiality of cells series resistance compensation can only be approximate.

Interpretation of the optical indicator responses requires consideration of a number of additional complications:

(1) The absorbance of arsenazo III is sensitive not only to changes in calcium concentration, but also to changes in magnesium and hydrogen ion concentrations (DiPolo *et al.* 1976; Brown *et al.* 1977; Gorman & Thomas, 1978). The sensitivity to pH changes is similar to the sensitivity to p_{Ca} changes, and since intracellular calcium is known to affect intracellular pH (Meech & Thomas, 1977), the possibility of a contribution of pH changes to our results must be acknowledged. Fortunately, the difference spectra for pH and magnesium changes are quite distinct from the calcium difference spectrum. For low level calcium ion changes, the ratio of absorbance changes at 660 and 610 nm is about 2·2:1, while for magnesium and hydrogen ion changes this ratio is about 1:2·5 (Brown *et al.* 1977; Gorman & Thomas, 1978). In our experiments, this ratio had a value of about 2:1 (Fig. 1), so that at least 90 % of the signal is due to a calcium ion change.

(2) Despite the presence of powerful cytoplasmic calcium buffers (Brinley *et al.* 1977, 1978; Baker & Schlaepfer, 1978), arsenazo III might alter the normal level and distribution of calcium in cytoplasm. If this were true, we would expect to see an effect of increased dye concentration on the form of the arsenazo III responses. However, we have observed that the behaviour of the arsenazo III signal to a train of pulses is unaffected by increasing the dye concentration with repeated ionophoretic injections, up to a final concentration of 0·5 mM.

(3) Our estimated maximal local calcium concentration (about 5 μM in Fig. 11) is close to the apparent binding constant for arsenazo III with calcium (calculated at 7·5 μM from Fig. 2; see also Thomas, 1979). Thus the submembrane calcium might reach levels sufficiently high that arsenazo III will no longer respond linearly to the calcium concentration transients. However, even for calcium concentrations of 20 μM, the arsenazo III response only deviates from linearity by 20 %, so that it is unlikely that arsenazo III signals are seriously distorted by saturation of the indicator.

(4) The aequorin signal is highly specific to calcium ion changes, and is unaffected by pH changes in the physiological range (Blinks, 1978). Aequorin binds calcium less tightly than arsenazo III (Blinks, 1978; Scarpa *et al.* 1978), and is thus less likely to disturb intracellular calcium or be near saturation. However, the aequorin photo-emission is centred at 450 nm, which is an absorption maximum for the cytoplasm of some highly pigmented *Aplysia* neurones which we have used, such as R_2 (Arvanitaki & Chalazonitis, 1961). Thus, such cells may screen out some of the aequorin light, especially that arising from the centre of the cell. Nevertheless, we have observed similar aequorin responses from faintly pigmented cells, such as R_{15}, and our model calculations suggest that most of the aequorin light is generated just beneath the membrane, so it is unlikely that the aequorin signal is seriously distorted by cytoplasmic absorption.

(5) Neither aequorin nor arsenazo III responses to calcium concentration changes are instantaneous. The slow kinetics of their calcium reactions could distort the time course of the calcium changes they sense. However, the responses we observe are significantly slower than the reaction rates for either indicator (Blinks, 1978; Scarpa *et al.* 1978), so it is unlikely that this source of distortion is important in our experiments.

Despite all these limitations in the interpretation of each of our measures of calcium influx, the agreement between all the linear indices strengthens our conclusion that the calcium influx declines slightly in successive pulses, at the same time that aequorin photo-emissions facilitate.

Inferences from calcium indicator responses

We have shown that the observed time courses of calcium current, arsenazo III signals and aequorin signals can be reconciled quantitatively by a simple model taking into account the known stoichiometry of the two indicators and experimentally based estimates of factors governing calcium movements in cytoplasm. It is reasonable to conclude that certain aspects of the model have been confirmed by the results, because the alternatives would have produced very different results. For example, the close fit between the predicted and observed magnitudes of our calibrated arsenazo III responses makes it extremely likely that most of the calcium entering the cytoplasm is bound. If there were no such cytoplasmic binding, we should observe responses many times those recorded, and the decay of the aequorin signals, reflecting the dilution of internal calcium, would be even faster. It is also extremely likely that the cytoplasmic binding is very rapid, occurring within a few msec at most. Otherwise the arsenazo III signal would consist of a rapid spike followed by a decline whose time course would reflect binding (Blaustein & Hodgkin, 1969).

Other aspects of the model are less easily distinguished from the alternatives. For example, the slow decay of the arsenazo III signal could reflect extrusion of calcium by a membrane pump or absorption of calcium into intracellular organelles such as mitochondria. In our model the first process predominated, but the alternative may not be excluded by our results.

The model predicts significant changes in the submembrane calcium concentration, from a resting level of about 100 nM (DiPolo *et al.* 1976) to peaks of several μM during $+15$ mV pulses. Factors which have been neglected in the present computations, but

would seem likely to particularly affect the calcium accumulation very near the membrane, include infoldings of the surface membrane, possible non-uniform disposition of calcium binding or sequestering sites in cytoplasm, local saturation of binding sites, and membrane surface charge. Such limitations notwithstanding, a large increase in submembrane calcium such as we predict would reduce the calcium equilibrium potential (E_{Ca}) significantly.

It might be thought that this large reduction in E_{Ca} during a pulse and in successive pulses is responsible for the decline in calcium current during a pulse and its depression in successive pulses. If a constant conductance characteristic is assumed for calcium channels, this would be the case. However, at the holding and pulse potentials of our experiments we are very far from E_{Ca}, even after internal calcium accumulation. Under these circumstances, the net flux through a calcium channel ought to be very insensitive to intracellular calcium concentration, and there should be virtually no change in calcium current accompanying the expected changes in internal calcium. For example, using a Goldman model of the calcium channel (Hodgkin & Katz, 1949), we calculate that an increase in submembrane calcium from 100 nM to 10 μM would reduce the calcium current by only about 0·1 %. Thus the more substantial depression in calcium current we observe is not likely to be due simply to a reduction in driving force due to internal calcium accumulation. Rather, it may reflect a direct effect of internal calcium on calcium flux through membrane channels (Kostyuk & Krishtal, 1977b; Tillotson, 1979). Recently, Akaike *et al.* (1978) have reported that raising internal calcium concentration to 2 μM in perfused snail neurones reduces the calcium influx by about 12 %. This may be compared to the 25 % reduction in calcium current we observe when the calculated submembrance calcium concentration reaches several μM. Alternatively, the decline in calcium influx may reflect a voltage-dependent inactivation of the gating of calcium channels. Another possibility is that the reduction in calcium current reflects a depletion of the local extracellular calcium just outside the membrane. Calcium influx is nearly directly proportional to this local calcium concentration (Akaike *et al.* 1978).

Although there is no facilitation of calcium influx, there is a prolonged rise of submembrane calcium concentration in a train of pulses or action potentials, due to the residual calcium from previous activity. The non-linearity of aequorin then leads to a facilitation of transient photo-emissions responding to each influx. This is exactly the mechanism proposed in the residual calcium hypothesis of synaptic facilitation (Katz & Miledi, 1968; Rahamimoff, 1968; Miledi & Thies, 1971). The present evidence may be taken as supporting the plausibility of that hypothesis. Moreover, changes in the intracellular profile of calcium concentration with repeated activity may have important consequences for the activation of calcium-sensitive enzymes, just as they are important in the stimulation of aequorin photo-emissions.

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