J. Physiol. (1980), **308**, pp. 259–285
With 12 text-figures
Printed in Great Britain

INTRACELLULAR CALCIUM ACCUMULATION DURING DEPOLARIZATION IN A MOLLUSCAN NEURONE

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(*Received 26 November 1979*)

SUMMARY

1. The bursting pacemaker neurone R-15 of *Aplysia* was injected with the Ca^{2+} sensitive dye arsenzo III. Changes in absorbance were measured with a differential spectrophotometer to monitor changes in free intracellular Ca^{2+} during membrane depolarization under voltage clamp conditions.

2. Dye absorbance increased linearly for depolarizing pulse durations up to 100 msec and approximately linearly between 100 and 300 msec, but for longer durations the absorbance change decreased.

3. The absorbance change *vs.* voltage relation increased steeply between -20 and 0 mV (e-fold per 8·5 mV), peaked at $+36$ mV and declined non-linearly to an estimated null or suppression potential of about $+139$ mV.

4. TTX (5×10^{-5} M) had no effect on the change in dye absorbance produced by brief or long duration stimuli whereas Ca^{2+} free ASW abolished all changes in dye absorbance.

5. The absorbance change saturated with increasing external Ca^{2+} concentrations. The relation between dye absorbance and external Ca^{2+} concentration was hyperbolic and for a small range of external Ca^{2+} concentration and membrane potentials could be fitted by a Michaelis–Menten expression where the dissociation constant and the maximum absorbance change are voltage dependent.

6. The absorbance change was reduced by external divalent ions which block the Ca^{2+} channel (e.g. Cd^{2+} and Ni^{2+}). The suppression of dye absorbance was increased by membrane depolarization and suggests that there is a voltage dependent site within the Ca^{2+} channel which binds divalent ions.

7. The decline of the absorbance–voltage relation from its peak to the suppression potential showed a greater nonlinearity when longer duration voltage clamp pulses were used. The non-linearity can be explained if the accumulation of Ca^{2+} ions next to the inner surface of the membrane during depolarization reduces the driving force on Ca^{2+} ions and thus decreases Ca^{2+} ion influx.

8. The suppression potential estimated from the absorbance–voltage relation increased 29 mV per tenfold change in the external Ca^{2+} concentration and thus can be used to estimate the Ca^{2+} equilibrium potential.

9. The change in dye absorbance produced by brief depolarizing voltage clamp

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0022-3751/80/2380-0623 $07.50 © 1980 The Physiological Society

steps was inactivated at positive holding potentials (50% inactivation at about −14 mV). Our results suggest that the slow decrease in dye absorbance during prolonged depolarization is caused by inactivation of the Ca^{2+} channel.

INTRODUCTION

In many excitable cells, Ca^{2+} ions enter during membrane depolarization and transiently accumulate in the cytoplasm. This process of Ca^{2+} accumulation is of general interest because changes in the free ionized internal Ca^{2+} concentration play a critical role in the activation of contractile proteins in muscle, the release of synaptic transmitters from nerve terminals and in the control of the membrane permeability to K^+ ions. Where Ca^{2+} ions enter the cell only through membrane channels during depolarization, then Ca^{2+} accumulation might be expected to be predictable directly from measurements of the membrane ionic current for Ca^{2+} ions and of the cell surface area and volume. Under normal conditions, however, the free intracellular ionized Ca^{2+} concentration is maintained at a very low level by internal buffering systems and by transport systems in the membrane (Baker, 1972; Brinley, 1978) and it is likely that only a small fraction of the *total* Ca^{2+} ion influx accumulates as *free* intracellular Ca^{2+}. For this reason, it is of interest to measure directly small changes in the free internal Ca^{2+} concentration, and the Ca^{2+} sensitive dye arsenazo III has been used successfully to measure such changes in nerve cells by several groups of investigators (Brown, Cohen, De Weer, Pinto, Ross & Salzberg, 1975; DiPolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976; Brown, Brown & Pinto, 1977; Thomas & Gorman, 1977; Ahmed & Connor, 1979). Arsenazo III has the additional virtue that changes in its absorbance are linear for Ca^{2+} concentration changes well below the dissociation constant of the Ca-arsenzao complex (DiPolo *et al.* 1976; Gorman & Thomas, 1978; Thomas, 1979), which makes it possible to obtain a reasonable estimate for the average change in the free intracellular Ca^{2+} concentration throughout the cell following membrane depolarization. In this paper we determine the relationship between transient changes in membrane potential and the Ca^{2+} accumulation in a molluscan pacemaker neurone. In the succeeding paper the relation between Ca^{2+} accumulation and changes in potassium conductance at different membrane potentials is determined. Aspects of this present study have been presented previously in a preliminary form (Thomas & Gorman, 1978; Gorman & Thomas, 1979).

METHODS

Experimental procedure

All experiments were done on the neurone R-15 (Frazier, Kandel, Kupfermann, Waziri & Coggeshall, 1967) in the abdominal ganglion of *Aplysia californica*. The ganglion was removed from the animal, the overlying sheath was dissected away to expose the cell and the ganglion was pinned to the base of a chamber through which artificial sea water (ASW) flowed at a constant rate and temperature (16 °C). The ASW contained 480 mM-Na^+, 10 mM-K^+, 10 mM-Ca^{2+}, 55 mM-Mg^{2+}, 620 mM-Cl^- and 15 mM-Tris-HCl at pH 7·8. Any variation in Ca^{2+} concentration or the addition of other divalent cations (e.g. Cd^{2+} or Ni^{2+}) to the ASW was compensated for by a corresponding change in Mg^{2+} concentration so that the total divalent cation concentration

remained constant. Control experiments suggested that these relatively small changes in Mg^{2+} concentration did not by themselves affect the results.

Many of the experimental procedures have been described previously (Thomas & Gorman, 1977; Gorman & Thomas, 1978). In brief, a pair of fibre optic probes was positioned on either side of the exposed R-15 soma so that a beam of light from one of the probes could be collected by the second probe after passage through the cell (path length 0·3 mm). Micro-electrodes used for recording and stimulation were filled with 3 M-KCl. Dye injection electrodes were filled with solutions of purified arsenazo III (98% pure, Sigma Chemical Co., St Louis, Missouri). Membrane potential was measured differentially with respect to an extracellular electrode which was independent from bath ground. The circuit used for these measurements has been given previously (Thomas, 1977). The remainder of the recording, stimulating and voltage clamp circuitry was conventional. Signals were displayed on an oscilloscope and on a three channel rectilinear pen recorder.

Spectrophotometer

Several changes were made in the pulsed wave-length, differential spectrophotometer (see Gorman & Thomas, 1978) which was used to monitor the changes in dye absorbance. The most significant change involved the use of a smaller size (9·4 mm diameter) of interference filter (Ditric Optics, Marlboro, Massachusetts) in the spinning rotor wheel, which in turn allowed the use of a smaller wheel and a higher rotation rate of 300 rev/sec. The six narrow band interference wave-lengths were 690, 660, 630, 600, 570 and 540 nm. Dye absorbance changes were measured differentially between 660 nm and 690 nm and the absorbance at the 570 nm isobestic wave-length was used to estimate the intracellular dye concentration. The absorbances at the other three wave-lengths gave useful information concerning the dye selectivity (see Gorman & Thomas, 1978, Fig. 6) which is important since the dye absorbance is also affected by changes in Mg^{2+} concentration and in pH. Further details of the spectrophotometer will be published separately.

Dye–Ca^{2+} interaction

Injections of arsenazo III into the neurones were made using pressure (5–30 lb/sq.in.) to give an intracellular concentration of about 0·3 mM. The use of 98% pure arsenazo III in place of the previous impure dye stock (Thomas & Gorman, 1977; Gorman & Thomas, 1978) necessitated a recalibration of the dye absorbance change in response to changes in internal Ca^{2+} and a re-evaluation of the interference by intracellular Mg^{2+}. The results of these experiments are reported in detail elsewhere (Thomas, 1979). They confirmed, however, that the composition of the dye–Ca^{2+} complex was 2 dye:1 Ca^{2+} (Gorman & Thomas, 1978) whereas the dye–Mg^{2+} complex was 1:1. The effective dissociation constants at pH 7·3 and 16 °C were $1{\cdot}8 \times 10^{-9}$ M^2 and 7×10^{-3} M respectively.

The increase in absorbance at 600 and 630 nM resulting from dye injection was greater than expected from the increase at the 570 nm isobestic wave-length, and the spectral dependence suggested that a reasonable proportion of the dye was present as the Mg complex. Comparison with the results of Mg^{2+} calibration experiments (Thomas, 1979) suggested that the effect was consistent with an intracellular free Mg^{2+} concentration of 2–3 mM which is in reasonable agreement with estimates in squid axon (Brinley & Scarpa, 1975). A value of 2·5 mM was adopted for the calibration of absorbance changes in response to changes in internal Ca^{2+}. The calibration experiments indicated that 2·5 mM-Mg^{2+} reduced the absorbance change in response to a step change in [Ca^{2+}] by a factor of three, which is large enough to place some uncertainty on calibration of the absorbance changes in response to changes in free intracellular Ca^{2+}. Although a precise comparison is impossible (different measurement wave-lengths, different dye stocks, etc.) the calibrations in the present experiments seem, nevertheless, to agree quite well with our previous estimates (Thomas & Gorman, 1977; Gorman & Thomas, 1978), but all such estimates must remain somewhat tentative.

To facilitate comparison of our results with those of other workers, a modified calibration procedure has been used in this study. In this procedure, the calibration is related directly to the dissociation constant of the Ca–EGTA complex, rather than to that of the Ca–arsenazo complex, although in practice there is little difference between the two methods. In a previous

study (Thomas, 1979), the apparent dissociation constant of the Ca–arsenazo complex was determined using a Ca^{2+} sensitive electrode, and the result was then used to estimate the apparent dissociation constant of the Ca–EGTA complex, by measuring the arsenazo absorbance change when Ca^{2+} ions were added to a solution containing EGTA. The procedure used here is the reverse one, namely to use an independent estimate for the apparent dissociation constant of the Ca–EGTA complex to calibrate the arsenazo absorbance change when Ca^{2+} ions are added to an EGTA solution. The absorbance changes obtained in experiments can then be related to this value by making the appropriate corrections for arsenazo concentration and optical path length, bearing in mind the 2 dye:1 Ca stoichiometry of the complex. A correction for interference by Mg^{2+} must still be made, however, so uncertainties from this source are, unfortunately, not removed. The calibrations were based on the estimate for the true stability constant for the Ca–EGTA complex of $10^{11.00}$, obtained originally by Bjerrum, Schwarzenbach & Sillen (1957), and more recently by Owen (1976). This compares with the value of $10^{11.16}$ which Thomas (1979) calculated from his estimate of the Ca–arsenazo dissociation constant, so the calibrations are quite similar regardless of which calibration technique is used.

Time course of Ca^{2+} accumulation

The time course of Ca^{2+} accumulation immediately adjacent to the internal surface of the membrane during depolarization may be quite different from the average change in Ca^{2+} concentration throughout the cell measured with arsenazo III. The total Ca^{2+} content of the cell is expected to increase linearly with time if the rate of Ca^{2+} influx is constant and is much greater than the rate of sequestration and extrusion. Since arzenazo is likely to be evenly distributed within the soma at the time measurements were made (see Brinley, 1978) and its absorbance change is proportional to the Ca^{2+} concentration as long as only a small proportion of the dye is complexed, the dye absorbance is expected to represent the change in free, ionized Ca^{2+} averaged throughout the soma.

The change in Ca^{2+} concentration with time at the inner membrane surface can be approximated if certain simplifying assumptions are made about cell geometry, Ca^{2+} influx and the diffusion coefficient for Ca^{2+} ions in the cytoplasm. The R-15 soma is approximately spherical and for the cells used in the present study has a diameter of about 300 μm. The Ca^{2+} diffusion coefficient in aqueous solution is close to $6{\cdot}4 \times 10^{-6}$ cm^2/sec (Hodgkin & Keynes, 1957; Kushmerick & Podolsky, 1969), but most of the Ca^{2+} in the soma is buffered and the results of Ca^{2+} injection experiments (Gorman & Thomas, 1980) suggest that at early times only about 1/50 of the Ca^{2+} ions that enter the soma are free. If it is assumed that the intracellular Ca^{2+} buffering is rapid, reversible and non-saturating and that the buffering sites are relatively immobile, a standard solution for diffusion into a sphere can be used in which the Ca^{2+} diffusion coefficient is given by the diffusion coefficient for Ca^{2+} ions multiplied by the proportion of the total Ca^{2+} which is free (see Crank, 1975, p. 327). The Ca^{2+} diffusion coefficient would then be about 1×10^{-7} cm^2/sec. Previous estimates for the Ca^{2+} diffusion coefficient in cytoplasm range from $1{\cdot}4 \times 10^{-7}$ to about 6×10^{-7} cm^2/sec (Hodgkin & Keynes, 1957; Blaustein & Hodgkin, 1969; Kushmerick & Podolsky, 1969) and therefore it would seem reasonable to explore solutions to the diffusion equation for Ca^{2+} diffusion coefficients between 10^{-7} and 10^{-6} cm^2/sec. It also seems reasonable to assume that, at least for short times, the Ca^{2+} influx is constant.

If a cell of radius a is initially at a uniform concentration C_0 and the diffusing substance enters at a constant rate F_0 per unit area surface, that is

$$D\partial C/\partial r = F_0, \quad r = a$$

then the concentration C^* at any time t and radial distance r can be determined from the equation

$$C^* - C_0 = \frac{F_0 a}{D}\left\{\frac{3Dt}{a^2} + \frac{1}{2}\frac{r^2}{a^2} - \frac{3}{10} - 2\frac{a}{r}\sum_{n=1}^{\infty}\frac{\sin(\alpha_n r)}{\alpha_n^2 a^2 \sin(\alpha_n a)}\exp(-D\alpha_n^2 t)\right\} \quad (1)$$

(Carslaw & Jaeger, 1959, p. 242; Crank, 1975, p. 96) where $a\alpha_n$s are the positive roots of

$$a\alpha_n \cot a\alpha_n = 1 \quad (1a)$$

The solution of eqn. (1) can be expressed in terms of the dimensionless variables Dt/a^2, $CD/F_0 a$ and ${}_0\int^{a} CD\mathrm{d}r/F_0 a^2$, where $C = C^* - C_0$.

The theoretical rise in internal Ca^{2+} concentration (CD/F_0a) at the inner membrane surface ($r = a$) at various times of interest between 0·01 and 1 sec for a cell with a radius of 0·015 cm is shown in Fig. 1. For $D = 10^{-7}$ cm²/sec, Dt/a^2 will range from 5×10^{-6} to 5×10^{-4} with a corresponding increase when a higher value of D is used. The closed circles plot the Ca^{2+} concentration integrated over the whole volume (${}_0\int^a CD\mathrm{d}r/F_0a^2$) at various times. The rise in Ca^{2+} concentration next to the membrane is initially fast, but slows appreciably with time whereas the integral of this change increases linearly.

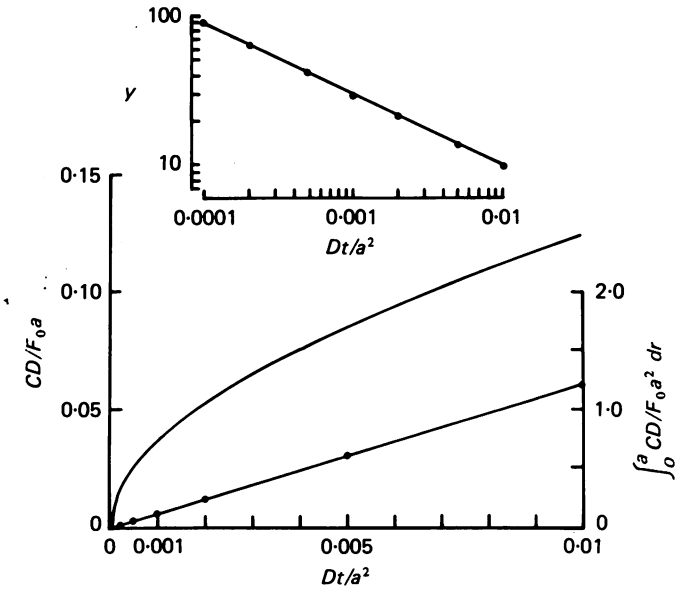

Fig. 1. Theoretical plot of the time course of the change in Ca^{2+} concentration next to the inner membrane surface (continuous curve) and the integral of this change over the entire volume (filled circles). Note that the integral values are multiplied by 100. The inset graph is a log–log plot of the ratio (y) of the Ca^{2+} concentration change at the inner membrane surface to the integral of this change at various values of Dt/a^2 (see text for further details).

For small values of Dt/a^2 (0·01 or less), the solution for diffusion into a sphere is very similar to that for an infinite medium, for which case the Ca^{2+} concentration at the inner membrane surface would increase with the square root of time. Since the integral of this change over the entire volume increses linearly with time, the ratio, y, of the change in Ca^{2+} concentration at the inner membrane surface to the integral of this change (where $y = (CD/F_0a)/({}_0\int^a CD\mathrm{d}r/F_0a^2)$ will decrease with the square root of time. The insert in Fig. 1 shows a plot of log y *vs.* log Dt/a^2 for values of Dt/a^2 between 0·0001 and 0·01.

The assumption that the buffer system is immobile and does not saturate may be questionable especially at the cell periphery, but it is difficult to make any specific prediction about buffer saturation until the internal Ca^{2+} sequestering system is better understood. Baker, Hodgkin & Ridgway (1971) assumed that Ca^{2+} was taken up by mitochondria at a rate of 0·1/sec, but more recent experiments (see Brinley, 1978) have suggested that mitochondrial uptake may be very slow and ineffective for Ca^{2+} sequestration in the physiological range. There is, however, a non-mitochondrial or X buffer system which is apparently rapid and reversible and may supply much of the buffering power of the cell under physiological conditions (Brinley, 1978).

Although eqn. (1) is a useful approximation, neither this nor the equation for diffusion into an infinite medium take into account the effects of invaginations in the cell membrane which occur in molluscan neurones (see Mirolli & Talbott, 1972; Graubard, 1975). A quantitative treatment of the effects of such invaginations on Ca^{2+} accumulation near the membrane surface is not feasible,

on account of the complex and variable geometry, but some qualitative predictions can be made. Where the invaginations are in sufficiently close proximity that the intracellular region between them is in the form of a relatively narrow cleft, the accumulation of Ca^{2+} ions at the inner surfaces of the membranes forming such a cleft will be relatively greater than for a sphere. This is because the value of Dt/a^2 (where a now represents the half-width of the cleft) will be much greater because of the reduction in a. Although the diffusion equation will of course depend on the cleft geometry, it will *always* tend towards a linear rise in Ca^{2+} concentration at the inner membrane surfaces of the cleft, for values of Dt/a^2 of around unity and above, if the rate of Ca^{2+} influx is constant (see Carslaw & Jaeger, 1959, pp. 113, 203 and 242).

Thus we have the extreme solutions of the Ca^{2+} concentration at the inner surface of the membrane varying with the square root of time over our range of interest (up to about 1 sec) when the membrane invaginations are neglected, and varying linearly with time (at later times within 1 sec range) for narrow cytoplasmic clefts of less than *about* 1 μm half-width. It is probable that both extremes apply in different regions of the R-15 neurone, but that for the cell as a whole and for early times, the square-root relation will be the better approximation.

RESULTS

The effect of stimulus duration

Fig. 2 shows the change in differential dye absorbance in voltage clamped R-15 neurones produced by a 100 msec depolarization of 80 mV from a holding potential of -50 mV. Typically, in these cells a voltage step to $+30$ mV produced a near maximum change in absorbance in normal (10 mM-Ca^{2+}) ASW and therefore this value was used in most test conditions. The response (Fig. 2*A*) started almost immediately and rose more or less linearly to a peak about 15 msec after termination of the voltage step. The decay of the absorbance change, however, always occurred in at least two phases and took 25 to 30 sec to return to baseline levels (Fig. 2*B*). This time increased for longer stimulus durations which produced a greater change in absorbance.

The result shown in Fig. 2 contrasts with the results obtained using aequorin under similar conditions (Eckert, Tillotson & Ridgway, 1977; Eckert & Tillotson, 1979). The aequorin signal begins after a delay of 20–25 msec, but thereafter rises sigmoidally and does not reach its peak until after the end of the depolarizing step. The decay of the signal is, however, much more rapid than that of the arsenazo absorbance change, being more than 90% complete within 1 sec of the end of the depolarizing step. Although this difference might suggest that one or the other technique distorts the time course of the intracellular free Ca^{2+} change, it is more probable that the discrepancy arises from the different way in which the two measurement techniques respond to an identical Ca^{2+} change.

The change in arsenazo absorbance is linear with the change in free Ca^{2+} concentration (DiPolo *et al.* 1976; Gorman & Thomas, 1978; Thomas, 1979), whereas the rate of light emission by aequorin does not bear a constant relation to free Ca^{2+} (Allen, Blinks & Prendergast, 1977). At very low Ca^{2+} concentrations (about 10^{-7} M-Ca^{2+} in the presence of physiological concentrations of intracellular K^+ and Mg^{2+}) the aequorin light emission is almost independent of Ca^{2+}, but as the Ca^{2+} concentration is increased, the rate of light emission increases markedly, approximating to a square or cubic relation, but declining again at very high Ca^{2+} levels, as it approaches saturation. This being the case, the aequorin signal would vary not only with the total amount of free Ca^{2+} in the cell, but also with its distribution (being higher if the distribution is less uniform), whereas the arsenazo absorbance change would vary with the Ca^{2+} distribution *only* if the dye anywhere approached saturation (in which case the effect would be to reduce the signal). When the nonlinearity and Ca^{2+} distribution effect of the aequorin technique are taken into account, the non-linear rise and the more rapid fall of the aequorin signal compared with the arsenazo absorbance change are as expected. When Ca^{2+} enters the cell during a depolarizing pulse, most of the increase in its concentration will occur in the vicinity of the cell membrane.

At the end of the pulse, the Ca^{2+} distribution is expected to become more uniform as the Ca^{2+} ions diffuse further into the cell, so the aequorin signal is expected to diminish even if the *total* internal Ca^{2+} concentration remains unchanged. In the real situation, the effect will be superimposed on the reduction due to sequestration and extrusion of Ca^{2+} ions, so even if (as is often done) the internal Ca^{2+} is estimated from the square root of the aequorin light emission in an attempt to overcome the problems of aequorin nonlinearity, it will still fall more rapidly than the arsenazo signal.

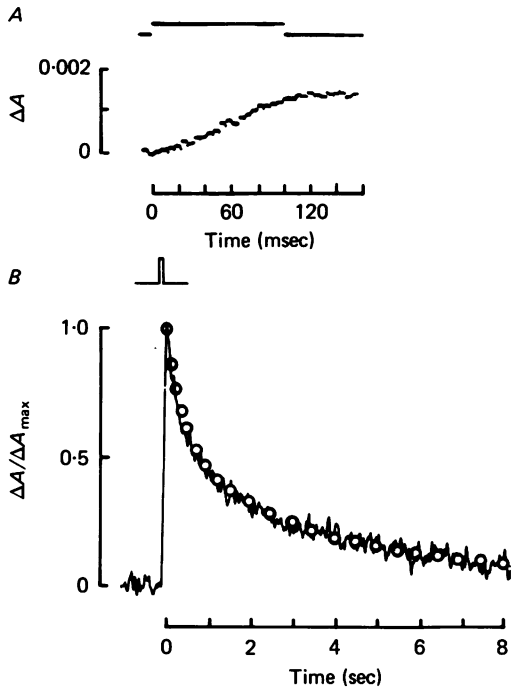

Fig. 2. Differential absorbance changes at 660–690 nm in response to step depolarizations from −50 to +30 mV in voltage clamped R-15 neurones. *A*, oscilloscope record (lower trace) of the absorbance change in response to a 100 msec depolarization (upper trace). A change in absorbance of 0·002 ΔA corresponds to an average increase in intracellular Ca^{2+} concentration of about 140 nM. *B*, absorbance change from a different cell in response to a similar stimulus (pen recorder record) to show the slow return of absorbance to the base line. The open circles superimposed on the falling phase represent an exponential decay curve composed of 3 time constants.

The relation between the change in peak absorbance and the duration of a constant amplitude membrane depolarization is shown in Fig. 3. The absorbance change is a linear function of pulse duration up to 100 msec (10–100 msec) and approximately linear between 100 and 300 msec (Fig. 3*A*); at longer durations (Fig. 3*B*) the absorbance change approaches an asymptote and for very long durations (> 5 sec) the absorbance change decreases (see Fig. 12). The initial linearity is compatible with the expected change in the integral of a step change in Ca^{2+} inward current under conditions where inactivation is minimal. Inactivation of the Ca^{2+} current in these cells has been shown to be slow and often incomplete (Eckert & Lux, 1976; Magura,

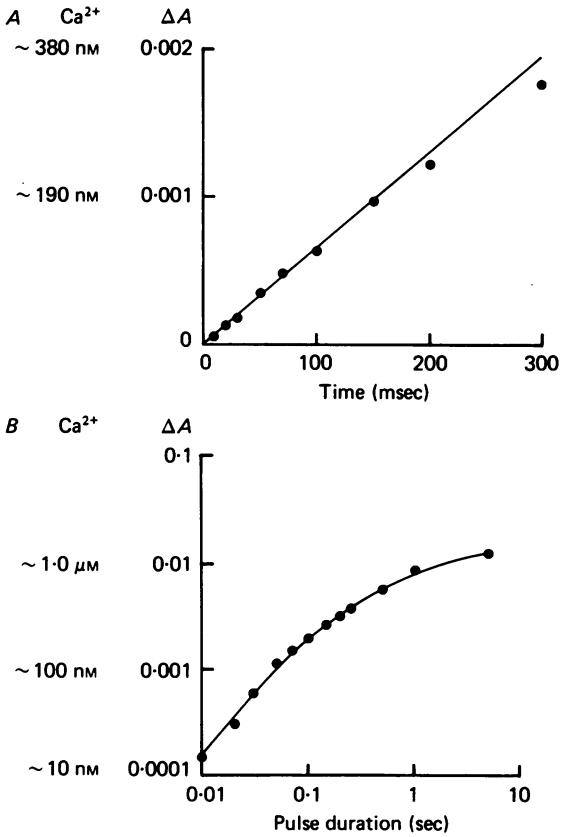

Fig. 3. Plots of the peak differential absorbance changes in two voltage clamped cells in response to step depolarizations of different durations from −50 to +30 mV. *A*, plot of absorbance change *vs.* pulse duration at short times to show that the change in internal Ca^{2+} is linear with pulse duration up to about 200–300 msec. *B*, plot of absorbance change versus pulse duration for a different cell to show that the increase in internal Ca^{2+} approaches saturation at longer pulse durations.

1977). In most of our experiments we used pulse lengths of 100–300 msec, but under some conditions the effects of pulse durations as short as 10 msec and as long as 90 sec were also investigated.

The effect of stimulus amplitude

Fig. 4*A* shows the change in absorbance in response to 300 msec step depolarizations from a holding potential of −50 mV to positive membrane potentials up to +130 mV. At stimulus durations of 100–300 msec the change in absorbance became apparent at about −20 mV, reached a maximum at +30 to +40 mV and thereafter declined to a minimum at potentials greater than +130 mV. In five cells it was found that between −20 and 0 mV the average absorbance change increased e-fold in 8·5 mV which is close to the increase in Ca^{2+} conductance (e-fold in 6 mV) reported

by Adams & Gage (1979*b*) for the same cell and to the increase in Ca^{2+} entry in squid axon (e-fold in 6·3 mV) measured with aequorin (Baker *et al.* 1971). In most experiments there was no true suppression or null potential, i.e. a clearly demarked positive membrane potential at which no change in absorbance was recorded. Usually there was a small residual absorbance change at potentials greater than +150 mV. In

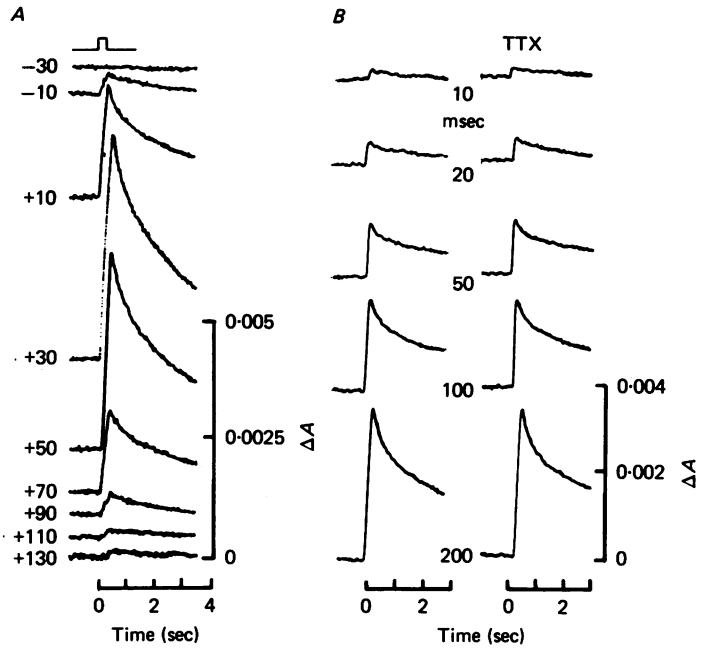

Fig. 4. Differential absorbance changes in two cells in response to step depolarizations to different potentials and to a constant potential at different durations. *A*, absorbance change in response to step depolarizations of 300 msec duration from −50 mV to the indicated values. A change in absorbance of 0·005 ΔA corresponds to an average increase in intracellular Ca^{2+} concentration of about 580 nM. *B*, absorbance changes in response to step depolarizations from −50 to +30 mV at the indicated pulse durations before (left side) and in the presence of 5×10^{-5} M-TTX. A change in absorbance of 0·004 ΔA corresponds to an average increase in intracellular Ca^{2+} concentration of about 420 nM.

some cells this absorbance change was clearly associated with a partial membrane repolarization during the pulse as a result of voltage clamp failure at very positive membrane potentials so some Ca^{2+} could still have entered, but this is unlikely to be the full explanation because similar changes in absorbance occurred at potentials greater than about +140 mV in Ca^{2+} free ASW containing 2 mM-EGTA. These changes were accentuated by voltage steps in excess of 500 msec. The basis for this residual change in absorbance has not been investigated, but it is unlikely to be mediated by Ca^{2+}.

In squid axons, there is evidence that a portion of the Ca^{2+} influx is blocked by TTX (Baker, Hodgkin & Ridgway, 1971) and therefore may represent Ca^{2+} movement through the fast Na^{+} channel. We have looked for, but find no evidence for, a similar

phenomenon in cell R-15. TTX at a concentration of 5×10^{-5} M had no measurable effect on the change in absorbance produced by depolarizing steps from -50 to $+30$ mV at short as well as at long durations (Fig. 4*B*). If there is any Ca^{2+} movement through the fast Na^+ channel it must be extremely small compared with its movement through TTX-insensitive channels.

The effect of Ca^{2+} free ASW and Ca^{2+} channel inhibitors

The relationship between absorbance changes and membrane potential is bell-shaped (Fig. 5). A similar relation holds for the aequorin light responses in squid axon

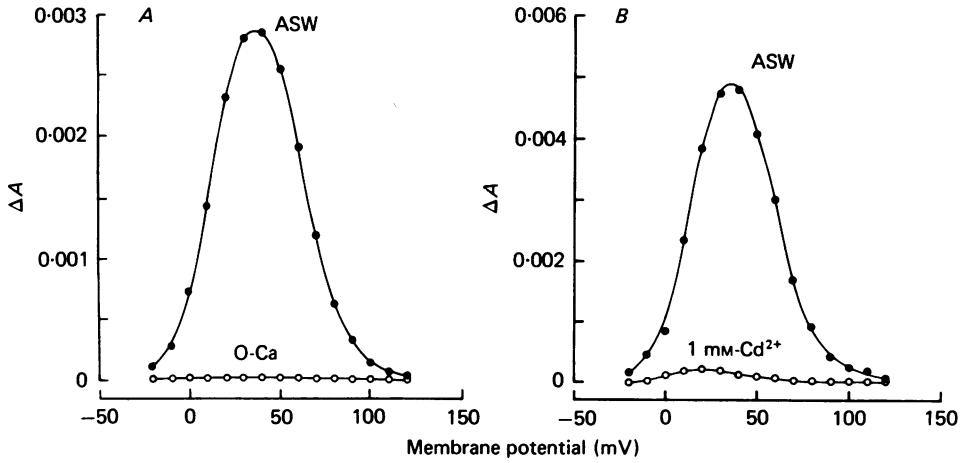

Fig. 5. Suppression of differential absorbance changes by Ca^{2+} removal and by the addition of Cd^{2+} ions. *A*, plot of the absorbance change in response to 300 msec step depolarizations from a holding potential of -50 mV to the indicated membrane potentials before and after removal of external Ca^{2+}. A change in 0·003 absorbance units corresponds to an average increase in intracellular Ca^{2+} of about 290 nM. *B*, plot of the absorbance change from a different cell in response to 300 msec step depolarizations from a holding potential of -50 mV before and after the addition of 1 mM-Cd^{2+} to normal ASW. A change in 0·006 absorbance units corresponds to an average increase in intracellular Ca^{2+} of about 700 nM.

(Baker *et al.* 1971) and in molluscan neurones (Eckert & Tillotson, 1978) *vs.* membrane potential as well as for the Ca^{2+} current *vs.* membrane potential in molluscan neurones (Akaike, Lee & Brown, 1978*b*). Removal of extracellular Ca^{2+} abolished the absorbance changes (Fig. 5*A*) indicating that any change in intracellular Ca^{2+} concentration produced by membrane depolarization is likely to represent an influx of Ca^{2+} from the external medium rather than from release from internal stores unless Ca^{2+} influx can trigger release. For short voltage clamp steps (< 1 sec) it is possible to detect changes in absorbance in ASW solutions containing 0·5 mM-Ca^{2+} and with longer steps (> 1 sec) in solutions containing 0·1 mM-Ca^{2+}, but not in Ca^{2+} free solutions with or without 2 mM-EGTA.

We showed elsewhere (Gorman & Thomas, 1978) that external La^{3+} was an effective

inhibitor of the absorbance change in normal ASW. In the present series of experiments the effects of the Ca^{2+} channel inhibitors Cd^{2+} and Ni^{2+} were tested. Both inhibit absorbance changes at all potentials when added to normal Ca^{2+} (10 mM) ASW. Cd^{2+} is as effective as La^{3+} whereas Ni^{2+} is slightly less effective. Fig. 5*B* shows the effect of 1 mM-Cd^{2+} on the absorbance versus potential relation. In normal ASW, 1 mM-Cd^{2+} reduced substantially the absorbance change at all potentials.

Effects of different Ca^{2+} concentrations

The preceding results suggest that the absorbance change produced by membrane depolarization can provide information about Ca^{2+} entry through membrane channels as well as about the subsequent increase in $[Ca]_i$. In barnacle muscle (Hagiwara & Takahashi, 1967) and in other molluscan neurones (Akaike *et al.* 1978*b*) evidence has been presented that the Ca^{2+} current is proportional to the number of sites occupied by Ca^{2+} at or near the surface membrane rather than to the external Ca^{2+} concentration. To determine whether a similar relation exists between ΔA and external Ca^{2+}, absorbance versus membrane potential curves were obtained from five cells in normal ASW, in Ca^{2+} free or Ca^{2+} free ASW with 2 mM-EGTA to remove residual external Ca^{2+}, and in at least five different ASW solutions containing Ca^{2+} concentrations between 0·5 and 20 mM. Each experiment took about 2 hr to complete, on account of the time required for the absorbance change in response to step depolarizations to equilibrate following each change in external Ca^{2+} concentration, and in all five cells the absorbance change in normal ASW declined during this time. This was associated with a reduction in absorbance at the 570 nm isosbestic wave-length, suggesting that it arose from a reduction in internal dye concentration (possibly as a result of diffusion of dye out of the soma into the axon). In three of the five experiments, the decline was, however, fairly small, and these results were analysed in detail.

Fig. 6*A* shows the relation between the absorbance change and membrane potential at different external Ca^{2+} concentrations for one of these cells. As the external Ca^{2+} concentration was increased the maximum absorbance change and the estimated suppression potential occurred at more positive membrane potentials. The absorbance data at a number of membrane potentials are replotted in Fig. 6*B* *vs.* external Ca^{2+} concentration and show that the change in absorbance produced by any constant depolarizing step increases asymptotically with the external Ca^{2+} concentration. A similar relationship between the Ca^{2+} current and external Ca^{2+} concentration was found by Hagiwara & Takahashi (1968) for barnacle muscle. The assumption was made that the Ca^{2+} channel has a site X which is either in the free state X or the bound state XCa when Ca^{2+} penetrates the channel (Hagiwara, 1975). When the electrochemical gradient of Ca^{2+} across the membrane is high, the reverse (outward) movement of Ca^{2+} ions through the channel can be neglected, so the passage of Ca^{2+} through the channel can be represented by

$$X + [Ca]_0 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X[Ca] \xrightarrow{k_2} X + [Ca]_i,$$

where k_n and k_{-n} are the forward and reverse rate constants.

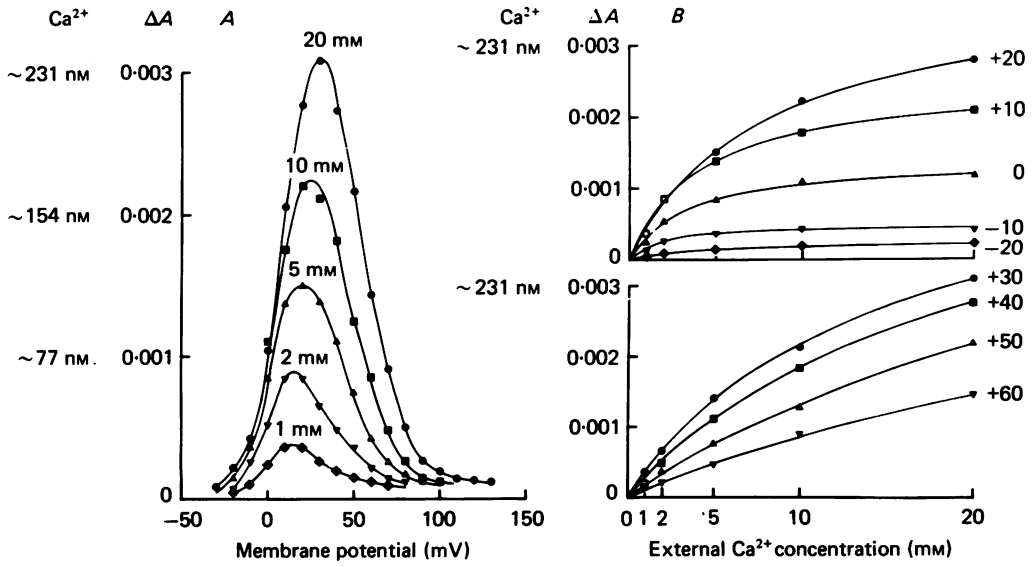

Fig. 6. Effects of external Ca^{2+} concentration on differential absorbance changes. *A*, plot of the absorbance change in response to 300 msec step depolarizations from a holding potential of −50 mV to various membrane potentials at the indicated external Ca^{2+} concentrations. *B*, plot of the absorbance changes *vs.* external Ca^{2+} concentration at the indicated membrane potentials for the same cell. The lines drawn through the experimental points represent the best fitting theoretical lines from eqn. (2).

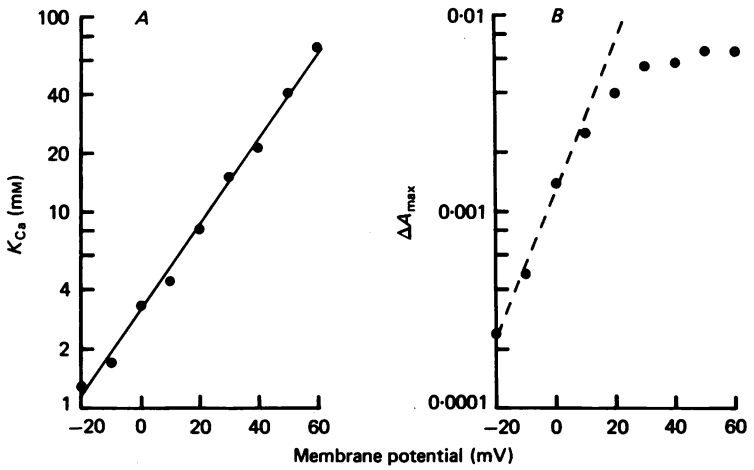

Fig. 7. Logarithmic variation of the apparent dissociation constant (K_{Ca}) and the maximum absorbance change (ΔA_{max}) with membrane potential. The filled circles in *A* and *B* represent values used to calculate the theoretical lines fitted to the experimental points in the plots shown in Fig. 6*B*.

If we assume that the absorbance change (ΔA) is proportional to the Ca^{2+} current (I_{Ca}), then

$$\Delta A(V) = \frac{\Delta A_{max}(V)}{1 + \frac{K^*_{Ca}(V)}{[Ca]_0}} \quad (2)$$

where ΔA is the peak absorbance change at a given membrane potential, V, $\Delta A_{max}(V)$ is ΔA when all the sites are occupied by Ca^{2+}, and $K^*_{Ca}(V)$ is the apparent dissociation constant of the Ca^{2+} binding site at V. The curves drawn through the experimental points in Fig. 6*B* were calculated using eqn. (2). The data are reasonably well fitted between the potential range of -20 to $+60$ mV, but not at more positive potentials. Values for K^*_{Ca} and ΔA_{max} used to construct these curves are plotted *vs.* membrane potential in Fig. 7. Both K^*_{Ca} (Fig. 7*A*) and ΔA_{max} (Fig. 7*B*) change as a function of membrane potential. For this cell, $K^*_{Ca} = 2{\cdot}9$ mM at 0 mV and increased e-fold per 18·8 mV change in potential between -20 and $+60$ mV; ΔA_{max} increased e-fold per 13·3 mV change in potential between -20 and $+10$ mV, but approached an asymptote at more positive potentials. Two other cells, examined over the Ca^{2+} concentration range of 0–10 mM, showed an identical pattern of results. For the three cells, the mean value for K^*_{Ca} at 0 mV was 2·1 mM and is similar to the value (5·4 mM) reported for the Ca^{2+} current in snail neurones at the same potential (Akaike *et al.* 1978*b*), but much smaller than the values (25–40 mM) reported for K^*_{Ca} in barnacle muscle (Hagiwara & Takahashi, 1967). On average, K^*_{Ca} increased e-fold per 24 mV change in potential between -20 and $+60$ mV and ΔA_{max} increased e-fold per 10·5 mV increase in potential between -20 and $+10$ mV.

In these experiments the concentration of external Mg^{2+} was always greater than the external Ca^{2+} concentration. Mg^{2+} does not pass through the Ca^{2+} channel, but does interact with the presumed binding site X with a dissociation constant K_{Mg} (Hagiwara & Takahashi, 1967). Under these conditions, the apparent dissociation constant K^*_{Ca} is related to the calcium dissociation constant K_{Ca} by $K^*_{Ca} = (1 + [Mg]_0/K_{Mg})K_{Ca}$. In barnacle muscle the ratio $K_{Ca}:K_{Mg}$ is in the range 1:3 to 1:4 (Hagiwara & Takahashi, 1967). If a similar ratio applies to our results then K^*_{Ca} is an over-estimate of K_{Ca}.

Divalent ion effects on absorbance

The results given above and elsewhere (Thomas & Gorman, 1977; Gorman & Thomas, 1978) indicate that a number of di- and trivalent cations suppress the absorbance change produced by membrane depolarization in normal (10 mM-Ca^{2+}) ASW when added to the external medium. The order of suppression effectiveness is La^{3+}, $Cd^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$ and is basically similar to the sequence found for suppression of the Ca^{2+} current by external cations in other tissues (Hagiwara, 1975) and in other molluscan neurones (Kostyuk & Krishtal, 1977; Akaike *et al.* 1978*b*). Fig. 8*A* shows a plot of the absorbance change *vs.* voltage in normal Ca^{2+} ASW before and after the addition of 5 mM-Ni^{2+}. At this concentration, Ni^{2+} produced a partial suppression of the absorbance and shifted the maximum absorbance to more negative potential values.

If we assume that a divalent ion, M^{2+}, produces its suppression effect by binding to a selectivity site X* but does not pass through the membrane and therefore is only on one side then the reaction can be written

$$[M]_0 + X^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X^*M,$$

where k_1 and k_{-1} are the forward and reverse rate constants. If the assumption that ΔA is proportional to I_{Ca} is also made, then the probability, p, that the sites are *not* occupied by M^{2+} at membrane potential, V, is given by

$$p = \frac{\Delta AM^{2+}(V)}{\Delta A(V)} = \frac{k_{-1}}{k_{-1} + k_1[M]_0}, \tag{3}$$

where ΔAM^{2+} and ΔA are the absorbance changes at V in the presence of and in the absence of M^{2+} in the external medium.

Eqn. (3) may be replaced (see Woodhull, 1973; Dubois & Bergman, 1977) by

$$p = \frac{1}{1 + [M]_0 B \exp(-z\delta FE/RT)}, \tag{4}$$

where δ is a position parameter that can vary between 0 and 1 and corresponds to the fraction of the membrane potential, E, acting on the site, $[M]_0$ is the concentration of the blocking ion, z is its valence, B is a constant that depends on concentration but not potential, and R, T and F have their usual meanings. Fig. 8*B* shows that the experimental values for p are reasonably well fitted with eqn. (4) using $\delta = 0{\cdot}28$. In a similar experiment, but with 0·2 mM-Cd^{2+}, instead of Ni^{2+}, experimental values for p were well fitted with eqn. (4) using $\delta = 0{\cdot}95$. Although they are based only on single experiments, these preliminary results suggest strongly that divalent ions from the external medium enter into the channel to reach a selectivity site or barrier. It is possible that Ni^{2+} and Cd^{2+} bind to different sites within the membrane, but this point needs to be examined further.

The absorbance–voltage relation

The free intracellular Ca^{2+} concentration of molluscan neurones has been estimated to lie between 10^{-8} and 10^{-7} M (Meech & Standen, 1975). An estimate of the Ca^{2+} equilibrium potential, E_{Ca}, and hence the intracellular Ca^{2+} concentration, can be obtained from the potential at which the absorbance change is suppressed. If the relation $I_{Ca} = G_{Ca}(V - E_{Ca})$ is obeyed, and if at positive potentials (e.g. above +50 mV; see Akaike *et al.* 1978*b*) G_{Ca} approaches a maximum and constant value, then the absorbance–voltage relation would be linear, intercepting the voltage axis at E_{Ca}.

Unfortunately, this is not the case, and the slope instead decreases as the suppression is approached (see Figs. 5, 6 and 8), which places some doubt on estimates of E_{Ca} by this method. Nevertheless, it is possible to make some tentative estimates, since the slope of the absorbance–voltage relation becomes more nearly linear at very positive potentials, i.e. above +100 mV. This approach to linearity is clearer in some experiments (e.g. Fig. 5*B*) than in others, but in most experiments an approximate estimate of the suppression potential could be made. In five cells, the average value of the suppression potential obtained for 300 msec pulses was $+139{\cdot}0 \pm 6{\cdot}1$ mV (S.E. of mean). If this is a reliable estimate of E_{Ca}, then the free intracellular Ca^{2+} concentration, $[Ca]_i$, can be found from the relation

$$[Ca]_i = \frac{[Ca]_0}{\exp(2FE_{Ca}/RT)} \tag{5}$$

which corresponds to $[Ca]_i = 1{\cdot}4 \times 10^{-7}$ M. The residual non-linearity in the absorbance–voltage relation at very positive potentials means that we may underestimate the true value of the suppression potential, so the above estimate of $[Ca]_i$ is likely to be on the high side.

One independent check which can be made on the approximate validity of this method is that the estimated suppression potential should change by 29 mV for a

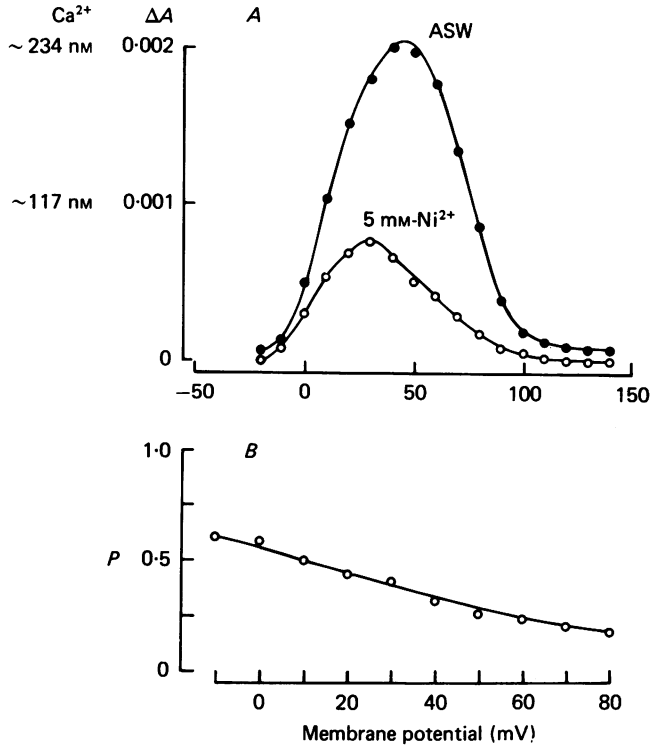

Fig. 8. Reduction of differential absorbance changes by external Ni^{2+}. *A*, plot of absorbance change in response to 300 msec step depolarizations from a holding potential of −50 mV to various membrane potentials before and after the addition of 5 mM-Ni^{2+} to normal ASW. *B*, plot of the ratio *p vs.* membrane potential. The line through the experimental points represents the best fitting theoretical lines from eqn. (3) (see text).

tenfold change in external Ca^{2+} concentration, as predicted by the Nernst equation. This was investigated in three cells, the results from one of which are shown in Fig. 6, and the average values for the suppression potential in different external Ca^{2+} concentrations are shown as open circles in Fig. 9. The straight line fitted to these points has a slope of 29 mV per tenfold change in external Ca^{2+} concentration, and this complete agreement with the Nernst equation is very much better than might have been expected from the problems inherent in making such estimates.

An important problem remains, however, as to why the absorbance–voltage relation is so markedly non-linear at less positive potentials. The same phenomenon

has been observed by Ahmed & Connor (1979) in absorbance–voltage plots from other molluscan neurones, and can be seen in the light response *vs.* voltage relations of aequorin-injected squid axons (Baker *et al.* 1971), but is much less apparent in the Ca^{2+} current *vs.* voltage plots from internally perfused molluscan neurones (Akaike *et al.* 1978*b*). The most probable explanation is that at less positive potentials (i.e. those around +70 mV), the inward Ca^{2+} current is large enough to increase $[Ca]_i$ significantly, and that the consequent decrease in the driving force for Ca^{2+} entry determines the non-linearity.

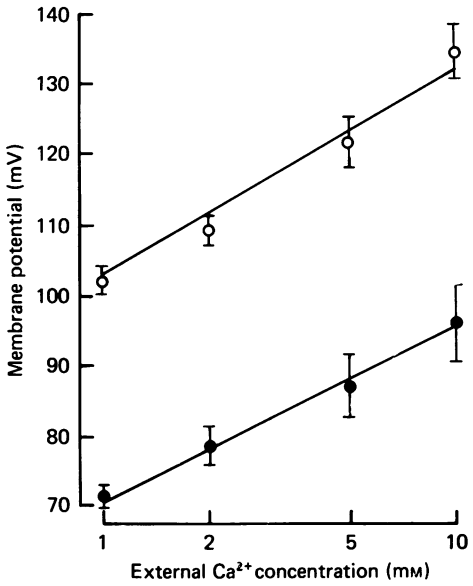

Fig. 9. Effects of external Ca^{2+} concentration on the suppression potential. Plot of the suppression potential for the differential absorbance change produced by 300 msec pulses *vs.* the external Ca^{2+} concentration (open circles). The filled circles represent the potential values estimated from the intercept of a tangent to the absorbance curve at +70 mV with the voltage axis at different external Ca^{2+} concentrations. Both plots are the means of triplicate determinations ± the standard error.

If this explanation is correct, then the absorbance–voltage relation should be more nearly linear for shorter duration pulses, since the increase in $[Ca]_i$ will be correspondingly less. The experiment shown in Fig. 10 suggests that the absorbance–voltage relation does indeed become more nearly linear as the pulse duration is reduced from 200 to 20 msec.

By an extension of this argument, it may be possible to use the non-linearity of the absorbance–voltage relation to estimate the extent of the Ca^{2+} accumulation near the inner surface of the cell membrane over this range of potentials. The simplest approach is to draw tangents to the absorbance–voltage curve, and to use the x-axis intercepts as estimates of an apparent Ca^{2+} equilibrium potential, E^*_{Ca} (see inset in Fig. 10), from which the Ca^{2+} accumulation can be assessed.

Unfortunately, these cannot be exact estimates, since the procedure assumes that E^*_{Ca}, and hence the Ca^{2+} accumulation, is constant, whereas it will vary with the Ca^{2+} current and hence also with the pulse potential. The slope of the current–voltage relation will actually be

$$\frac{dI_{Ca}}{dV} = G_{Ca}\left(1 - \frac{d}{dV}E^*_{Ca}\right) \tag{6}$$

which will represent the Ca^{2+} conductance only if $d/dV\,E^*_{Ca}$ is close to zero, and estimates of E^*_{Ca} from the x-axis intercept of the slope of the absorbance–voltage relation (a measure of dI_{Ca}/dV) will be valid only when this condition is met. $[Ca]_i$ will vary with I_{Ca}, and hence also with $G_{Ca}(V - E^*_{Ca})$, so as the pulse potential is decreased away from E^*_{Ca}, $[Ca]_i$ will increase, and

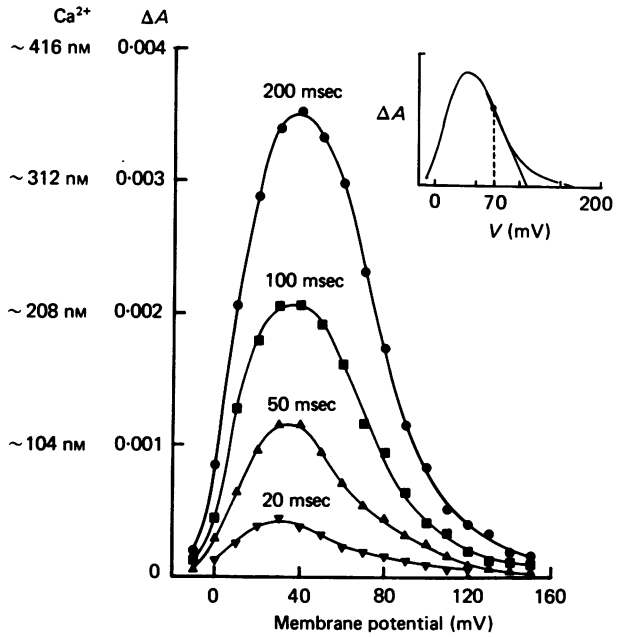

Fig. 10. Effects of different stimulus durations on the absorbance change. Plot of the differential absorbance changes produced by depolarizing pulses at the indicated durations from a holding potential of −50 mV to different membrane potentials. A change of 0·004 absorbance units corresponds to an increase in intracellular Ca^{2+} of about 420 nM. The inset shows the graphical procedure used to estimate the Ca^{2+} equilibrium potential at various stages of the absorbance–voltage relation (see text for further details).

thereby decrease E^*_{Ca}. From the Nernst equation, E^*_{Ca} will vary with $-\ln [Ca]_i$, so the variation of E^*_{Ca} with $[Ca]_i$ will become proportionately less as $[Ca]_i$ becomes larger, i.e. as the pulse potential is moved further below E^*_{Ca}. Accordingly, estimates of E^*_{Ca} by this method will become relatively more accurate as the pulse potential is reduced.

Although it is possible to quantify this argument by solution of the appropriate equations, some of the parameters involved are hard to assess. An alternative approach is to say that if the variation of E^*_{Ca} with pulse potential determines the nonlinearity of the absorbance–voltage relation, then for potentials over which the relation is reasonably linear, $d/dV\,E^*_{Ca}$ is likely to be small, and hence the x-axis intercept of the absorbance–voltage relation is likely to be a reasonable estimate of E^*_{Ca}. Our results suggest that this condition is most closely met at potentials around +70 mV (see inset in Fig. 10), and accordingly this potential has been used for the E^*_{Ca} estimates below.

For the same five cells as were used for the estimate of the suppression potential, the average value of the intercept of a tangent to the absorbance–voltage relation at +70 mV in normal ASW was $+101 \pm 4{\cdot}7$ mV (S.E. of mean). Just as for the suppression potential estimates, this intercept is expected to vary by 29 mV per tenfold change in external Ca^{2+} concentration. The filled circles in Fig. 9 show the results of such estimates for the same three cells as were used to obtain the suppression potential estimates (open circles in the same Figure). The slope of the line fitted to these points is 25·5 mV per tenfold change in external Ca^{2+} concentration, which is reasonably close to the 29 mV expected.

The value estimated for E^*_{Ca} at +70 mV can be used to estimate the extent of Ca^{2+} accumulation next to the inner surface of the membrane during a depolarizing pulse. From eqn. (5), for an external Ca^{2+} concentration of 10 mM and $E^*_{Ca} = +101$ mV, $[Ca]_i$ at the inner surface of the membrane would be $2{\cdot}9 \times 10^{-6}$ M. For the same five cells, the size of the arsenazo III absorbance change (which represents the average value of $[Ca]_i$ throughout the cell) was equivalent to $[Ca]_i = 0{\cdot}14 \times 10^{-6}$ M. This would make $[Ca]_i$ at the inner surface of the membrane about 21 times the cell average. If a similar ratio applies at the peak of the absorbance–voltage relation for 300 msec pulses (i.e. at about +35 mV), then the maximum value for $[Ca]_i$ at the inner surface of the membrane would be about 7×10^{-6} M in these experiments, based on a maximum absorbance change which was equivalent to $[Ca]_i$ (average) = $3{\cdot}4 \pm 0{\cdot}7 \times 10^{-7}$ M for the five cells.

Inactivation and facilitation

There is indirect evidence that the Ca^{2+} current can be facilitated from measurements of the K^+ current (Heyer & Lux, 1976) and from measurements of the aequorin light signal (Eckert *et al.* 1977; Lux & Heyer, 1977) during repetitive stimulation under voltage clamp conditions. Fig. 11 shows a comparison of the effects of brief, repetitive depolarizing pulses from −50 to +30 mV at several frequencies in normal ASW. The absorbance change to individual pulses remained reasonably constant during a repetitive train at all frequencies.We have not observed facilitation of the absorbance change in any experiments where repetitive stimuli were used. A similar conclusion has been reached by others based on results of the effects of repetitive stimulation on the Ca^{2+} current (Akaike *et al.* 1978*b*) and on changes in intracellular Ca^{2+} as measured by arsenazo III (Ahmed & Connor, 1979).

There is direct evidence that the Ca^{2+} current inactivates (Magura, 1977; Kostyuk & Krishtal, 1977; Akaike *et al.* 1978*b*; Tillotson, 1979). The decline in the absorbance signal with time has been noted previously (Gorman & Thomas, 1978) and could reflect a voltage dependent inactivation of Ca^{2+} influx. To determine whether the inactivation is voltage dependent a different type of experiment was used. The membrane was depolarized briefly with 100 msec pulses to +30 mV at 10 sec intervals from a holding potential of −50 mV. The procedure was repeated before, during and after an extended step change of the holding potential. Fig. 12*A* shows that during a maintained depolarization to +10 mV there was an initial large change in absorbance which was followed by a slow decline to a minimum. The change in absorbance to a brief repetitive pulse, however, was essentially abolished after

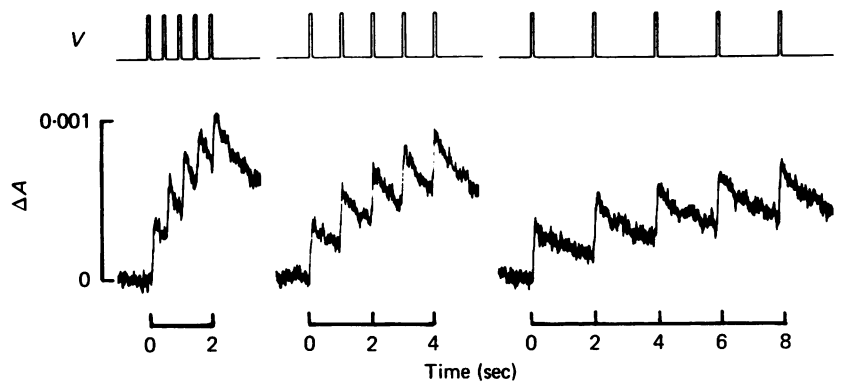

Fig. 11. Effects of repetitive stimulation on the differential absorbance change. The upper records show trains of repetitive 100 msec duration voltage pulses from −50 mV to +30 mV at frequencies of 2, 1 and 0·5 Hz (from left to right). The bottom records show superimposed absorbance changes in normal ASW.

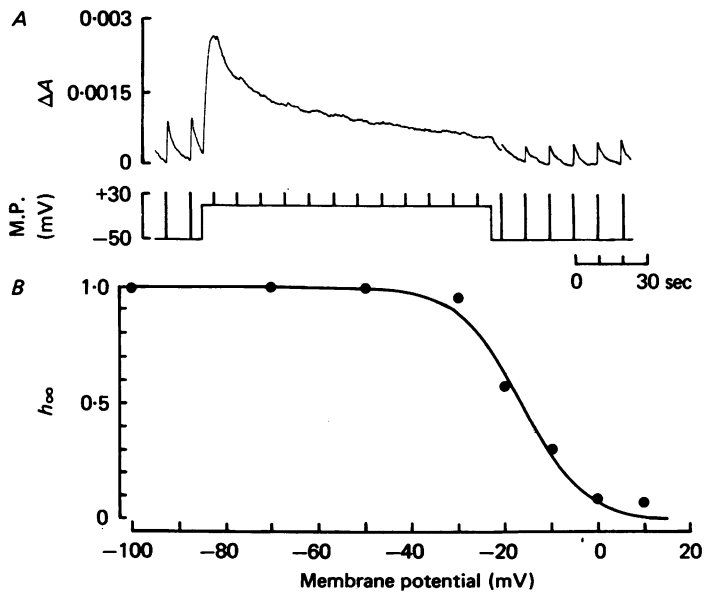

Fig. 12. Inactivation of the differential absorbance change. *A*, absorbance changes in response to 100 msec depolarizing voltage steps at 15 sec intervals from a holding potential of −50 to +30 mV and from a holding potential of +10 to +30 mV. A change of 0·003 absorbance units corresponds to an increase in intracellular Ca^{2+} of about 600 nM. *B*, plot of the steady-state absorbance change ($\Delta A/\Delta A_{max}$) *vs.* membrane holding potential. A theoretical curve from eqn. (6) (see text) is fitted to the experimental points.

about 30 sec during the maintained depolarization and recovered slowly after its termination. Recovery was still not complete by the time the absorbance base line had returned to its previous level after the end of maintained depolarization, and it continued for several minutes afterwards.

The time course of the inactivation and the subsequent recovery of the absorbance change depended on the membrane potential and was multiexponential. Fig. 12*B* shows the steady-state inactivation curve obtained in normal ASW. The continuous curve was calculated from the equation (Hodgkin & Huxley, 1952).

$$\frac{\Delta A}{\Delta A_{max}} = \left[1 + \exp \frac{V - V_h}{k}\right]^{-1} \quad (7)$$

where $\Delta A/\Delta A_{max}$ is the ratio of the peak absorbance change produced by a 100 msec pulse in the presence of a conditioning depolarization or hyperpolarization to the absorbance change in its absence, V is the value of the conditioning potential, V_h is the potential at which $\Delta A/\Delta A_{max} = 0{\cdot}5$ and k is the slope factor. In four cells the average value for V_h was $-14{\cdot}3$ mV and for k it was 7·2 mV for an e-fold change in $\Delta A/\Delta A_{max}$. It has been reported that the Ca^{2+} conductance 'inactivates' at hyperpolarized as well as at depolarized membrane potentials (Geduldig & Gruener, 1970). We find no evidence for inactivation at conditioning hyperpolarized potentials more negative than -50 mV.

DISCUSSION

Na^+, K^+ and Ca^{2+} ions are the primary charge carriers through voltage dependent channels in excitable cells. The factors which determine the movement of Ca^{2+} ions through these channels, however, differ from those for Na^+ and K^+ ions in several respects. For example, in cell R-15, with an extracellular Ca^{2+} concentration of 10 mM, a resting potential of about -50 mV and an intracellular Ca^{2+} concentration of about 10^{-7} M, the distribution of Ca^{2+} ions is approximately seven orders of magnitude away from equilibrium, thus the driving force acting on Ca^{2+} ions is many times greater than the driving force on either Na^+ or K^+ ions. Moreover, the relative value of the Ca^{2+} electrochemical gradient will vary considerably more than that for Na^+ or K^+ ions during excitation partly because of the double charge on the Ca^{2+} ion (the electrochemical gradient will change by an order of magnitude for a 29 mV rather than for the 59 mV change in membrane potential expected for a monovalent ion) and partly because the intracellular Ca^{2+} concentration is so low that a small Ca^{2+} influx has a high probability of accumulating near the membrane and thereby altering the electrochemical gradient. The results described in this paper provide information about the entry of Ca^{2+} ions through voltage dependent channels in the membrane, about the transient accumulation of Ca^{2+} ions within the cytoplasm during depolarization and about the interaction of Ca^{2+} influx and Ca^{2+} accumulation.

The influx of Ca^{2+} ions, measured from changes in the Ca^{2+} current at different membrane potentials, has been widely studied in *Aplysia* and in other molluscan neurones in recent years in intact cells (Geduldig & Gruener, 1970; Kostyuk, Krishtal & Doroshenko, 1974; Standen, 1975; Magura, 1977; Connor, 1979; Adams & Gage,

1979*a*, *b*), in perfused cells (Kostyuk & Krishtal, 1977; Akaike *et al.* 1978*b*) and in chemically altered cells (Tillotson & Horn, 1978; Tillotson, 1979) using a variety of procedures to eliminate or suppress other membrane currents. The behaviour of the absorbance change at different membrane voltages most closely resembles the behaviour of the Ca^{2+} current in perfused cells and chemically altered cells. Our results show that Ca^{2+} entry in the intact cell occurs primarily, if not exclusively, through a TTX-insensitive channel, in contrast to the squid axon membrane where Ca^{2+} enters through separate channels which are TTX sensitive and TTX insensitive (Baker *et al.* 1973). This channel is activated at about -45 to -40 mV (see Gorman & Thomas, 1978) and is only partially inactivated at all potentials more negative than 0 mV (50% inactivation at about -14 mV). We find no evidence that the channel is inactivated at negative membrane potentials (Geduldig & Gruener, 1970; Adams & Gage, 1979*b*). It is likely that the apparent inactivation of the Ca^{2+} current at hyperpolarized membrane potentials in cell R-15 (Adams & Gage, 1979*b*) is caused by incomplete suppression of an outward current (Neher, 1971; Standen, 1975).

The dye absorbance increases linearly when the soma is depolarized for times up to at least 100 msec (see Fig. 3). This behaviour is consistent with the expected average change in free ionized Ca^{2+} throughout the soma (see Fig. 1) measured by arsenazo III. The negative departure which is apparent at longer times almost certainly represents a reduction in the net rate of influx produced by inactivation rather than from saturation of the dye absorbance change because the estimated change in the Ca^{2+} concentration at the inner membrane surface is typically below the maximum possible dye absorbance change.

There is a variety of evidence to suggest that ions may move discontinuously through excitable membrane channels composed of discrete energy barriers and wells, and that their movement can be described by transition state or Eyring rate theory (see Hille, 1975). Movement of an ion through a system of this type is determined by its mobility, i.e. its ability to jump barriers, and its affinity for binding sites or wells within the channel as well as by its concentration gradient and the electric field across the membrane. The voltage dependence of the rate constants for ion movement between internal compartments, the saturation of binding sites at high ionic concentrations and the competitive inhibition of ionic movement by other ions which bind to internal sites within the channel are features of this type of system and, thus, can be accounted for. Our results show that the rise in internal Ca^{2+} saturates when external Ca^{2+} is elevated, but that saturation is reduced substantially by making the inside of the cell positive with respect to the outside. Since the maximum absorbance change (and, therefore, the maximum Ca^{2+} entry) increases and the binding constant for the reaction decreases (where the binding constant is the reciprocal of the apparent dissociation constant) when the inside of the membrane is made positive (see Fig. 7) it is possible that the mobility of Ca^{2+} in the channel increases whereas its affinity for a binding site within the channel decreases at positive membrane potentials. The Ca^{2+} current in perfused molluscan neurones shows similar behaviour (Akaike, Fishman, Lee, Moore & Brown, 1978*a*; Akaike *et al.* 1978*b*). Our results also show that small amounts of divalent cations (e.g. Ni^{2+} and Cd^{2+}) block Ca^{2+} entry when added to the external side of the membrane and that this inhibition is greatest at positive membrane potentials. A reasonable explanation of these results is that

there is a binding site within the channel and that those divalent ions which penetrate, but do not pass through the channel are bound more effectively at positive membrane potentials. The exact energy profile experienced by any ion, including Ca^{2+}, in traversing a membrane channel is not known, but it is possible that there are multiple barriers that determine which ions move through the channel and multiple binding sites that determine the order of affinity with which different divalent (or trivalent) ions block the channel.

The suppression potential for Ca^{2+} entry increases 29 mV per tenfold change in the external Ca^{2+} concentration and thus can be used to determine the Ca^{2+} equilibrium potential. The average intracellular Ca^{2+} concentration (140 nM) estimated from the suppression potential in normal (10 mM) external Ca^{2+} is 300–600 times smaller than the value estimated from the Ca^{2+} current suppression potential in intact molluscan neurones (Adams & Gage, 1979*a*; Connor, 1979), but these estimates are likely to be complicated by the presence of outward K^+ currents which are incompletely blocked at positive membrane potentials. The estimated free intracellular Ca^{2+} concentration is, however, about 2 times greater than indirect estimates from other molluscan neurones (Meech & Standen, 1975; Brown, Brodwick & Eaton, 1977) and 2–4 times greater than direct estimates from squid axon (Brinley, 1978), but there are reasons for suspecting that our estimate is on the high side. Our estimate is based on measurements of the suppression potential for the change in $[Ca]_i$ and not on the change in Ca^{2+} current. A small change in current through the membrane may go undetected because the Ca^{2+} influx is insufficient to produce an appreciable change in dye absorbance. Our preceding results (Gorman & Thomas, 1978) indicate that it is possible to detect a change in $[Ca]_i$ at about -40 mV when long depolarizing pulses (> 5 sec) are used, whereas changes in $[Ca]_i$ only become apparent at about -20 mV following short depolarizing pulses (< 1 sec). It is likely that a similar error occurs at very positive membrane potentials when the Ca^{2+} influx is brief and small. An error of 20 mV would increase the suppression potential to $+159$ mV and reduce the internal Ca^{2+} concentration to about 28 nM which is consistent with measurements from squid axons.

The non-linear decline of Ca^{2+} entry at positive potentials in R-15 is similar to the decline of the Ca^{2+} current (corrected for residual outward currents) in chemically altered molluscan neurones (Tillotson, 1979), but differs from the almost linear decline of the Ca^{2+} current in perfused neurones where the intracellular Ca^{2+} concentration is constant (Akaike *et al.* 1978*b*). Our results and those of Ahmed & Connor (1979) suggest that this difference can be explained by a rise in the Ca^{2+} concentration next to the inner membrane surface during depolarization which decreases the driving force on Ca^{2+} ions and, thus, reduces Ca^{2+} influx. A similar explanation was used by Beeler & Reuter (1970) to account for changes in the cardiac muscle Ca^{2+} current at different membrane potentials. We estimate an average increase next to the membrane of about 7 μM at the end of a 300 msec pulse to $+36$ mV, but this increase may be greater at the mouth of the Ca^{2+} channel if the current is localized there. The reduction of the Ca^{2+} current in *Aplysia* neurones which occurs when two closely spaced depolarizing pulses are used (Tillotson, 1979) might be explained by the change in driving force on Ca^{2+} ions, but this is unlikely to be the full explanation because a similar reduction does not occur when the current is carried by Ba^{2+} in

place of Ca^{2+} ions. It is possible that Ca^{2+} accumulation also has a direct effect of Ca^{2+} inactivation (Brehm & Eckert, 1978; Tillotson, 1979).

The accumulation of Ca^{2+} ions at the inner surface of the membrane obviously depends on the duration of the depolarizing pulse. Since a 200 or 300 msec depolarization to +70 mV is hardly a physiological stimulus, the conclusion that the accumulation near the membrane is about 21 times the average accumulation throughout the cell under these conditions might not appear to have much physiological relevance. As described previously, however, these are the only conditions under which the procedure for estimating the Ca^{2+} accumulation at the inner surface of the membrane (from tangents to the declining phase of the absorbance–voltage relation) is approximately valid, but it is possible to extrapolate the result to cover the physiological range. Under normal conditions the membrane is unlikely to depolarize to +70 mV, but the Ca^{2+} current at this potential, as estimated from the dye absorbance change, is roughly similar to that at +10 to +20 mV (see Fig. 10), which is of course within the physiological potential range, so the Ca^{2+} current at +70 mV is within the range of Ca^{2+} currents which are of physiological interest.

The duration of the current, as determined by the length of the pulse, is, however, about 10–20 times longer than might be expected during a single action potential, so the Ca^{2+} accumulation at the inner surface of the membrane will be greater than for a single action potential. On the other hand, during bursting pacemaker activity in the R-15 neurone, successive action potentials will result in a greater Ca^{2+} accumulation than for a single action potential (the extent of the increase will depend on the interspike interval and the rate of diffusion of Ca^{2+} away from the membrane during each interval). The findings for a 200 or 300 msec pulse can, however, be extrapolated to cover other pulse durations. For a constant Ca^{2+} current, the average increase in Ca^{2+} concentration throughout the cell will be proportional to the pulse duration, but the Ca^{2+} concentration at the inner surface of the membrane is expected to increase with the square root of the pulse duration (see Fig. 1). This means that as the pulse is shortened, the accumulation at the inner surface of the membrane *relative* to the average accumulation throughout the cell will increase (with the reciprocal of the square root of the pulse duration). Thus the accumulation of about 21 times the cell average for a 300 msec pulse would predict an accumulation of 115 times the average for a 10 msec pulse, whereas the actual accumulation at the membrane during a 10 msec pulse would be about 5·5 times less than for a 300 msec pulse.

If both the Ca^{2+} concentration at the inner surface of the membrane and its average concentration throughout the cell at the end of a period of Ca^{2+} entry are known, then it should be possible to calculate a value for the Ca^{2+} diffusion coefficient, D, in cytoplasm. The procedure is to estimate from Fig. 1 the value of Dt/a^2 for which the Ca^{2+} concentration at the inner surface of the membrane is 21 times higher than its value integrated over the whole cell, where $t = 0{\cdot}3$ sec and $a = 0{\cdot}015$ cm. This occurs for $Dt/a^2 = 0{\cdot}002$, i.e. $D = 1{\cdot}5 \times 10^{-6}$ cm^2/sec. The value of D obtained in this way is up to 10 times greater than expected from the estimates given in the Methods, but there is at least one major reason for the discrepancy. The model assumes the cell to be a perfect sphere, whereas in fact the cell membrane is invaginated. We argued in the Methods section that the invaginations were probably not sufficient to distort the time course of Ca^{2+} accumulation next to the membrane

by very much from the square root relation expected for short times; however, the increase in cell membrane *area* caused by the invaginations will invalidate the above calculation of D. If the cell membrane area is greater than that of a sphere of the same radius, then for a given value of Ca^{2+} at the inner surface of the membrane, the average value throughout the cell will be proportionately higher than for a true sphere. Accordingly, if Fig. 1 is to be used to estimate D in this way, then the actual ratio of 21 must be multiplied by the extent of the membrane invagination in order to obtain the 'true sphere equivalent' ratio.

Electrophysiological and anatomical estimates suggest that the total extent of membrane invagination is about 6–7 fold (Mirolli & Talbott, 1972; Graubard, 1975), but the regions of the cell where the invaginations are expected to be most extensive – especially the basal region in the vicinity of the axon hillock – are outside the light path between the fibre optics probes, and over the main part of the soma (from which we obtain our optical records), the cell membrane area is probably increased about threefold by the membrane invagination. This estimate gives a 'true sphere equivalent ratio' of about 21×3, i.e. about 63. Reference to Fig. 1 shows that the two relations differ by a factor of 63 when D is equal to about $1{\cdot}7 \times 10^{-7}$ cm^2/sec (for $t = 0{\cdot}3$ sec and $a = 0{\cdot}015$ cm).

This estimate is now well within the range expected, but it cannot be a very accurate one, since it is clearly very sensitive to the value of the 'surface/average' $[Ca]_i$ ratio which we use to obtain it. The theoretical basis for the sensitivity is that for a constant Ca^{2+} flux, the average $[Ca]_i$ is independent of D, whereas $[Ca]_i$ at the inner membrane surface varies with $(D)^{-\frac{1}{2}}$. Accordingly, the estimated value of D varies with the inverse *square* of the surface/average $[Ca]_i$ ratio. Thus we cannot attach very great confidence to our estimate of D, but conversely, we can argue that our estimate for the surface/average $[Ca]_i$ ratio must be a fairly good one. This is because it will vary with $(D)^{-\frac{1}{2}}$, and since D (from other estimates) is likely to be fairly close to our estimate of $1{\cdot}7 \times 10^{-7}$ cm^2/sec, the possible range of values for the surface/average $[Ca]_i$ ratio (depending on the value chosen for D and on the extent of membrane invagination) is relatively narrow, but our estimate is well within it.

There are important similarities between the intracellular accumulation of Ca^{2+} at different membrane potentials in cell R-15 and the change in post-synaptic potential at different pre-synaptic potentials. The membrane of the pre-synaptic terminal shows an increase in its permeability to Ca^{2+} ions upon the arrival of a pre-synaptic action potential, and a variety of evidence indicates that release of synaptic transmitter depends on the entry and subsequent accumulation of Ca^{2+} within the terminal (see Katz, 1969). If the release of synaptic transmitter is a monotonically increasing function of Ca^{2+} accumulation (see Llinas, Steinberg & Walton, 1976), the amplitude and relation of the change in post-synaptic potential to the change in pre-synaptic potential can be used to provide an estimate of Ca^{2+} ion accumulation in the terminal at different membrane potentials. The shape of the post-synaptic versus pre-synaptic voltage plot (Kusano, 1970) in normal external Ca^{2+} is similar qualitatively to the absorbance *vs.* voltage curve of cell R-15. The post-synaptic membrane potential increases rapidly with a positive shift in pre-synaptic membrane potential, but reaches a maximum and thereafter declines toward the suppression potential of the post-synaptic response. This decline is not linear and has a marked inflection at positive pre-synaptic membrane potentials. The rapid increase in post-synaptic potential presumably reflects the voltage dependence of the Ca^{2+} conductance and is

similar to the increase in Ca^{2+} entry in R-15 and in squid axon (Baker *et al.* 1971). The non-linear decline of the post-synaptic response at positive pre-synaptic potentials is also similar to the decline in Ca^{2+} entry in R-15 and may be a characteristic of systems where an interaction between Ca^{2+} influx and Ca^{2+} accumulation can occur.

We are indebted to Mr Mark Shafer for programming and for technical assistance. This work supported by U.S.P.H.S. grant NS11429.

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