STOICHIOMETRY AND APPARENT DISSOCIATION CONSTANT OF THE CALCIUM-ARSENAZO III REACTION UNDER PHYSIOLOGICAL CONDITIONS

ZAHUR AHMED, LAURA KRAGIE, AND JOHN A. CONNOR, *Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801 U.S.A.*

ABSTRACT In vitro and in situ tests have been run to characterize the reaction of the metallochromic indicator, arsenazo III, with calcium. Job plots as well as plots of indicator absorbance vs. $[Ca^{2+}]$ at different indicator concentrations show a 1:1 reaction stoichiometry. Equilibrium analysis and analysis using Adair's equation are also consistent with 1:1 complexes being formed and give estimates of 34 to 45 μM for the apparent dissociation constant. In situ tests were carried out using giant neurons from *Archidoris monteryensis*, a marine gastropod mollusc. Dye absorbance changes were measured during voltage clamp pulses which produced a fixed calcium influx. The dependence of absorbance change on total dye concentration is consistent with the formation of a 1:1 complex of Ca with ArIII if measurements are made during the initial period of the loading pulse, < 300 ms, although the apparent dependency changes with longer delay in measurements from the onset of the pulse.

INTRODUCTION

The measurement of intracellular free calcium concentration, $[Ca_i^{2+}]$, and the dynamics of its changes is a significant problem in cell biophysics because of the many processes Ca^{2+} is thought to regulate or influence (c.f. reviews by Carafoli and Crompton, 1978; Kretsinger, 1976). These measurements, however, have proved to be technically difficult because of the very low levels of Ca^{2+} encountered in cytoplasm, and because the time-course of $[Ca_i^{2+}]$ fluctuations is fairly rapid. As most standard analytical techniques are unsuitable because of speed, resolution, or inability to discriminate free from bound Ca, metallochromic indicator dyes (murexide, arsenazo III, antipyralazo III) and Ca^{2+} selective resin electrodes have come into increasing use. Each of these techniques has its own set of shortcomings and peculiarities, but at present the indicator method is clearly preferable for the measurement of small, rapid $[Ca_i^{2+}]$ fluctuations. Arsenazo III (ArIII) has been used to determine changes in $[Ca_i^{2+}]$ in several recent studies involving both nerve and muscle cells (c.f. Brown et al., 1975; Dipolo et al., 1976; Brown et al., 1977; Brinley et al., 1977; Miledi et al., 1977; Gorman and Thomas, 1978; Ahmed and Connor, 1979), and its favorable attributes, well covered in the above references, make it an extremely useful indicator of cytoplasmic calcium in functioning cells.

One of the difficulties encountered in using the dye for quantitative measurements of Ca^{2+} is that there is disagreement as to the stoichiometry of the Ca-ArIII reaction. For some types of in vitro, steady-state measurements this is not a serious problem, as appropriate standardizations can be run (c.f., Ohnishi, 1979). For transient measurements, however, where nonuniform concentrations of Ca^{2+} exist, as in active cells, one must combine simulation

studies with absorbance change measurements, requiring a knowledge of both stoichiometry and apparent dissociation constant. The early analyses of Budesinsky (1969) and Michaylova and Ilkova (1971) indicated a 1:1 stoichiometry. Dipolo et al. (1976) reported 1:1 stoichiometry with a K_D (apparent) ~ 30×10^{-6} M, as have Scarpa et al. (1979). However, a recent study by Thomas (1979) presented evidence for 2:1 dye to Ca stoichiometry with dissociation constants of $>3.0 \times 10^{-4}$ M and 1.8×10^{-9} M^2 for CaAr and $CaAr_2$, respectively. In the present study we ran several standard in vitro analyses for reaction stoichiometry, an analysis similar to Thomas' (1979), and also performed an in situ analysis of ArIII-Ca complexing. The results of all these tests are consistent with 1:1 complexing with an apparent dissociation constant of 30 to 40 μM at physiological salt concentrations.

MATERIALS AND METHODS

The in vitro and in situ experiments were done using 98% purified arsenazo III (lot #38C-7660 and #119C-7120; Sigma Chemical Co., St. Louis, Mo.) of effective molecular weight 1,135. In vitro absorbance or differential absorbance was measured at appropriate wavelengths in either a Cary 118 or a Cary 219 spectrophotometer (Varian Corp., Palo Alto, Calif.). The absorbance spectra of 200 μM dye (lot #119C-7120) in internal saline solution (Table I) in the absence of any added calcium and in the presence of 2 mM calcium is shown in Fig. 1. Differential absorbance was measured with 0 Ca^{2+} as the reference. Absorbance at 580 nm (an isosbestic point of ArIII) was measured to ascertain dye concentration. Optical measurements were made in cuvettes of 3 different path lengths, 0.1, 0.5, or 1 cm, depending on the concentration of the dye. Absorbances were normalized to a path length of 1 cm before analysis. Arsenazo III and calcium solutions were prepared in an internal solution mix (Table I). To avoid systematic dilution, as would occur during titration with calcium into a stirring cuvette, all experimental solutions for absorbance measurements were premixed in separate test tubes. Each test tube was rinsed with the designated solution of ArIII before the experimental solution was put in the test tube. Spectrophotometer cuvettes were rinsed with the experimental solution in question before the solution was transferred for absorbance measurements. The pH of the test tube solutions was measured to confirm that all mixtures were at pH 7.2. Where necessary, contaminant calcium in this mixture was measured by atomic absorption spectroscopy as described below, and the calcium concentration of the solutions was corrected accordingly. Data for Job, log-log, reciprocal, Scatchard, and Hill plots were obtained using the Cary 118 spectrophotometer. The Job plot was prepared using two separate stock solutions of calcium and ArIII, 300 and 500 μm each. The log-log plot, double reciprocal plot, and Hill plot were measured using four separate stock solutions of ArIII and $CaCl_2$ to give the data statistical

TABLE I
SOLUTIONS

Ion type	In vitro	In situ	
	Internal saline	Normal saline	TEA saline
	(mM)	*(mM)*	*(mM)*
Na^+	50	490	390
K^+	350	8	8
Mg^{2+} (Cl^-)	3	20	20
Mg^{2+} (SO_4^{2-})	0	30	30
Ca^{2+}	0	15	15
MOPS (H^+-buffer)	20	10	10
TEA	0	0	100
pH	7.2	7.4	7.4

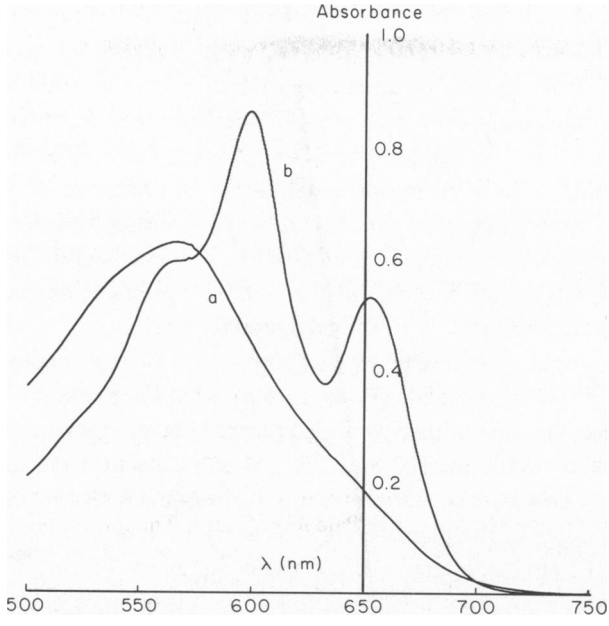

FIGURE 1 Absorbance spectra of ArIII in internal saline mix (Table I). Dye concentration 200 μM, pathlength 0.1 cm. (*a*) No Ca added to mixture. (*b*) 2 mM Ca.

appraisal. Data for Fig. 3 were obtained with Sigma dye lot #119C-7120 and in Cary 219 spectrophotometer (See Results section for solution mix).

The contaminant calcium in internal solution was estimated using atomic absorption spectrophotometry (Perkin-Elmer Corp., Norwalk, Conn.; model 305). Measurements were done at λ = 4227 Å using an air-acetylene, slightly yellow flame and Ca-Mg light source. Both the standard solutions and sample solutions had 5 mM EGTA to avoid phosphate (air-acetylene) interference. A standard calibration curve was obtained using $CaCO_3$ at 10, 25, and 50 μM, after zeroing the instrument with glass distilled water. In situ: studies were performed on neurons from the nudibranch mollusc, *Archidoris monteryensis,* obtained from the waters around Friday Harbor, Washington. The procedures involved in maintenance and dissection of the animals, a diagram of reidentifiable neurons, voltage-current clamp circuits, and the optical system have been described in previous communications (Connor, 1979; Ahmed and Connor, 1979, 1980). The behavior of intracellular ArIII absorbance changes were examined at different dye concentrations. In each case the neuron soma was held under a two-microelectrode voltage clamp at resting membrane potential and ArIII was injected iontophoretically.

RESULTS

In Vitro Characteristics of Dye

Five in vitro methods have been used to explore Ar-Ca interaction. The first two presented are Job's method and a "normalized slopes" method (c.f. Thomas, 1979) which demonstrate the stoichiometry of the reaction. Following these, data from other tests are given in the form of log-log plots, double reciprocal plots, and Hill plots. These methods have been used primarily for determination of the apparent dissociation constant for the reaction, K_D, but the results also suggest the possible stoichiometric ratio.

The most direct method of determining indicator-metal reaction stoichiometry is Job's

method (c.f. Bishop, 1972). Fig. 2 shows absorbance data plotted against dye-Ca mixture ratio, the standard form of a Job plot. Mixtures were prepared using the internal saline solution as a blank (Table 1) with contaminant calcium in the internal saline measured by atomic absorption spectroscopy as outlined in the Methods section. In two separate sets of determinations, each using two samples, the contaminant Ca ranged from 10 to 23 μM. Abscissa values in Fig. 2 have been corrected for a 10-μM level of Ca contamination. Correction for higher levels of contaminant Ca would have shifted the plot slightly to the left. Stoichiometry of the Ca-Ar reaction is indicated by the intersection point of asymptotes drawn through the right and left branches of the plot. This intersection occurs approximately at the 50% division on the abscissa, indicating 1:1 stoichiometry.

Thomas (1979) reported experiments which showed that two molecules of ArIII bind to a calcium ion in vitro. These experiments employed somewhat lower levels of $[Ca^{2+}]$ than those used for Job's method. His conclusion was drawn partially from experiments in which plots of $\Delta A_{660\text{-}690}$ vs. $[Ca^{2+}]$ were made for 100 and 200 μM dye concentration. Both 1- and 0.5-cm optical path lengths were used to normalize total absorbance to the same value (Fig. 1, Thomas, 1979). The slopes of the plots were different by a factor of 2. The ratio of the slopes of such plots yields the stoichiometric ratio of the complex as follows. Applying the law of additive absorbances to a solution of Ca and ArIII, the absorbance of ArIII and 660 nm is given by:

$$A_{660} = l \cdot \epsilon_{Ar} \cdot [Ar] + l \cdot \epsilon_{CaAr_n} \cdot [CaAr_n], \quad (1)$$

where $\epsilon_{Ar_{660}} = A_{min}/([Ar]_T \cdot l)$ and $\epsilon_{CaAr_{660}} = A_{max}/([Ar]_T \cdot n^{-1} \cdot l)$. A_{min} and A_{max} are the absorbances of the dye at 0 and at saturating calcium concentrations, respectively. $\epsilon_{Ar_{660}}$ and $\epsilon_{CaAr_{660}}$ are the molar extinction coefficients measured at 660 nm for the uncomplexed and fully complexed dye, respectively. n is the number of ArIII molecules bound per calcium ion. At a total dye concentration of $[Ar]_T$, optical path length l, and $[Ca^{2+}] = [Ca^{2+}]_1$, the differential

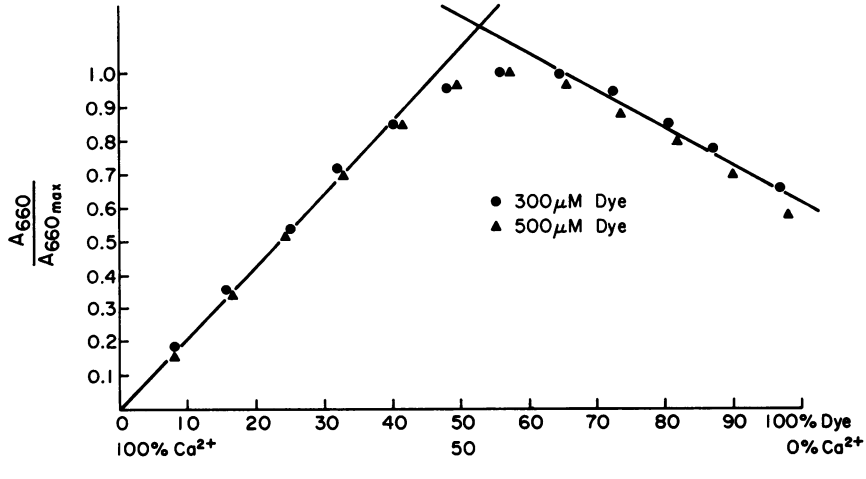

FIGURE 2 Job plot: Plot of absorbance vs. Ca-ArIII molar mixture ratio. Plots were done for two different stock solutions, 300 μM (•) and 500 μM (▲). Asymptotes to right and left hand branches of the plot intersect at approximately 50%, indicating 1:1 stoichiometry.

absorption at 660 nm with and without Ca^{2+} is, from Eq. 1:

$$\Delta A_{660_1} = \epsilon_{CaAr_n} \cdot l \cdot [CaAr_n]_1 + \epsilon_{Ar} \cdot ([Ar]_1 - [Ar]_T).$$

Differential absorption can be taken either at a single wavelength or a pair of wavelengths with no change in the form of the expressions. A similar expression will give ΔA_{660_2} i.e., at $[Ca^{2+}] = [Ca^{2+}]_2$. Therefore, the slope of ΔA_{660} vs. $[Ca^{2+}]$ plot is:

$$\frac{\Delta A_{660_2} - \Delta A_{660_1}}{[Ca^{2+}]_2 - [Ca^{2+}]_1} = \frac{1}{\Delta[Ca^{2+}]} \cdot (\epsilon_{CaAr_n} \cdot l \cdot \Delta[CaAr_n] + \epsilon_{Ar} \cdot l \cdot \Delta[Ar]).$$

At a given dye concentration a change in $[Ca^{2+}]$ will cause an equal and opposite change in complex and free dye concentrations, i.e.,

$$\Delta[CaAr_n] \cdot n = \Delta[Ar].$$

Therefore, the expression for slope may be rewritten as:

$$\frac{\Delta[CaAr_n]}{\Delta[Ca^{2+}]} \cdot l \cdot (\epsilon_{CaAr_n} + n \cdot \epsilon_{Ar}].$$

Similarly, at dye concentrations of $[Ar]'_T$ and path length l', the slope is

$$\text{slope}' = \frac{\Delta[CaAr_n]'}{\Delta[Ca^{2+}]'} \cdot l \cdot (\epsilon_{CaAr_n} + n \cdot \epsilon_{Ar}).$$

Therefore, the ratio of the slopes is:

$$\frac{\text{slope}'}{\text{slope}} = \frac{\Delta[CaAr_n]' \cdot l'}{\Delta[Ca^{2+}]'} \div \frac{\Delta[CaAr] \cdot l}{\Delta[Ca^{2+}]}.$$

For $[Ar] \gg [CaAr_n]$ the equilibrium dissociation relation gives

$$\frac{\Delta[CaAr_n]}{\Delta[Ca^{2+}]} \simeq \frac{[Ar]_T^n}{K_{D_{app}}},$$

where $K_{D_{app}}$ is the apparent dissociation constant for the CaArIII reaction. Combining the last two relationships reduces the expression for slope ratio to:

$$\frac{l'}{l} \cdot \frac{[Ar]_T'^n}{[Ar]_T^n}.$$

With:

$$l = xl' \text{ and } [Ar_T] = \frac{[Ar]'_T}{X},$$

the ratio of slopes is then equal to $X^{(n-1)}$. Since X is known, the stoichiometric ratio can be obtained.

We have carried out experiments with $X = 2$, 5, and 10, and dye concentrations of 1 mM, 200 μM, and 100 μM in a solution of 350 mM KCl, 150 mM MOPS, 5 mM EGTA at pH 7.3. The K_D of Ca-EGTA reaction was assumed to be 1.5×10^{-7} M (Dipolo et al., 1976). The data

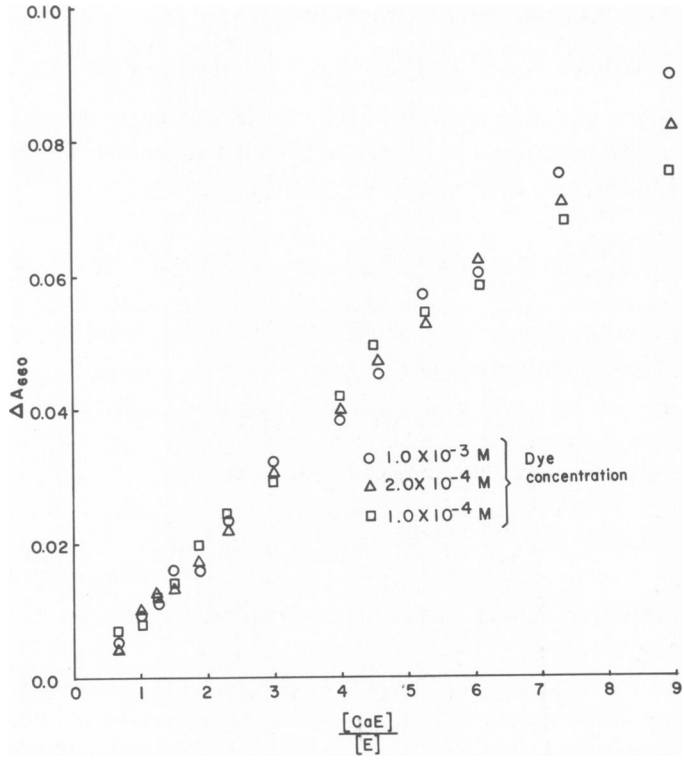

FIGURE 3 Plots of differential absorbances at 660 nm vs. $[Ca^{2+}]$ expressed as the ratio of Ca-EGTA complex (CaE) to uncomplexed EGTA (E). Three different concentrations of ArIII were used in cuvettes whose pathlength normalized the "zero" calcium dye absorbance to the same value: (□), 100 μM and 1 cm; (△), 200 μM and 0.5 cm; (○), 1,000 μM and 0.1 cm. Total EGTA concentration was 5 mM in all cases.

are presented in Fig. 3 and do not show significant slope differences. That is, the ratios of the slopes are around 1 and not 2, 5, and 10, as should have been the case if the dye to Ca stoichiometry were 2:1. By this test, then, the stoichiometry appears to be 1:1 even at lower $[Ca^{2+}]$.

Since neither of the above methods gives an accurate measure of K_D, and since the Job method has inherent limitations (c.f. Bishop, 1972), other less direct methods have also been employed. A brief outline of the theoretical framework involved in log-log, double reciprocal, and Hill plots as applied to indicator solutions is given along with the data. Using Eq. 1, the complex concentration, $[CaAr_n]$, and the free dye concentration, [Ar], at any calcium concentration are given by:

$$[CaAr_n] = \frac{A_x - A_{min}}{n(A_{max} - A_{min})} \cdot [Ar]_T \tag{2}$$

and

$$[Ar] = \frac{A_{max} - A_x}{A_{max} - A_{min}} \cdot [Ar]_T. \tag{3}$$

A_x is total absorbance at a particular calcium concentration. Ion combination indicators like ArIII participate in equilibria according to:

$$n \cdot \mathrm{Ar} + \mathrm{Ca}^{2+} \rightleftharpoons \mathrm{Ca\,Ar}_n$$

$$K_D = \frac{[\mathrm{Ca}^{2+}]\,[\mathrm{Ar}]^n}{[\mathrm{CaAr}_n]}. \tag{4}$$

Therefore, if the total amounts of calcium and ArIII in a solution are known, one can proceed to study the indicator properties. In the absence of the value of n, the analysis was carried out assuming both $n = 1$ and $n = 2$.

From four different stock solutions of purified ArIII and calcium in the internal solution mix, 28 test tubes were prepared with different Ca^{2+} and ArIII concentrations. A_{max} at each dye concentration was measured at a calcium concentration 10 times the concentration of the dye. A_{min} at each dye concentration was measured with no added calcium and 1 mM EGTA. A_x was measured for the different dye and calcium concentrations with a range of free dye concentrations between 5 and 477 μM, which was estimated from Eq. 3. $[CaAr_n]$ was obtained from Eq. 2 using $n = 1$ and $n = 2$. Free calcium concentration, $[Ca^{2+}]$, was obtained by subtracting $[CaAr_n]$ from the total calcium concentration. All measurements were made in the Cary 118 spectrophotometer (Varian Corp.) at 660 nm. According to Eq. 4, the slope of the plot of log $[CaAr]/[Ca^{2+}]$ vs. log [Ar] when $n = 1$, or of the plot of log $[CaAr_2]/Ca^{2+}]$ vs. log $[Ar]^2$ when $n = 2$, should be 1. Figs. 4 *A* and *B* are such plots, respectively. The plot of Fig. 4 *A* has a least square fit slope of 1.05 with a correlation coefficient of 0.95, and that of Fig. 4 *B* is 0.25 with a correlation coefficient of 0.92. Despite the relatively high correlation coefficient, the data of Fig. 4 *B* deviate from a straight line in a regular pattern. These results, therefore, also suggest that one ArIII binds per calcium ion. At log $[CaAr]/[Ca^{2+}] = 0$, the plot of Fig. 4 *A* yields a K_D of 37.6 μM.

A second method of analysis for K_D employs a simplification of Adair's equation (c.f. Edsall and Wyman, 1958), which assumes a sequence of binding steps, each assigned a macroscopic binding constant. This method has the advantage that it does not require free calcium concentration. The first assumption to be made is that the n binding sites on Ca^{2+} are equivalent and independent. This reduces Adair's equation to the form:

$$\theta = \frac{\bar{\nu}}{n} = \frac{K\,[\mathrm{Ar}]}{1 + K\,[\mathrm{Ar}]},$$

where K is the association constant and $\bar{\nu} = n\,[CaAr_n]/[Ca_{total}]$. The well-known plots of this equation are the double reciprocal plot and Scatchard plot. In Fig. 5 *A* we have plotted $1/\theta$ vs. $1/[Ar]$, i.e., the double reciprocal plot, assuming $n = 1$ and $n = 2$. The intercept of the plot should give the number of calciums complexed with the dye. When we assumed $n = 1$, the intercept was 1, and when we assumed $n = 2$, the intercept was ~ 2, indicating that two calciums bind to two molecules of the ArIII, suggesting a 1:1 complexing. The K_D obtained from the 1:1 plot is 45.0 μM. A similar conclusion has been arrived at from the Scatchard plot (not shown), which yields a K_D of 35.5 μM.

A second case of Adair's equation involves the assumption that infinite positive cooperativ-

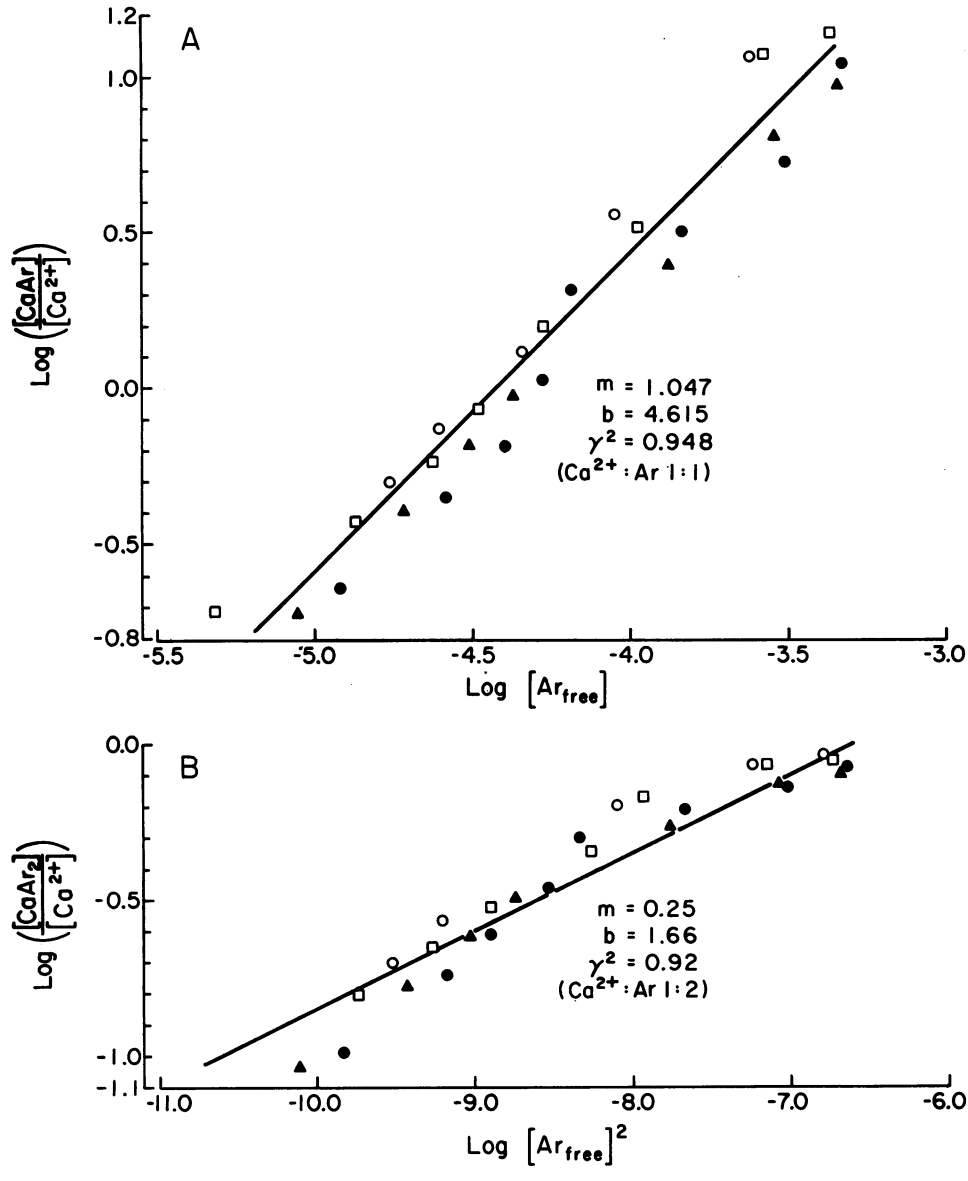

FIGURE 4 (*A*) Plot of log [CaAr]/[Ca^{2+}] vs. log [Ar]; (*B*) plot of log [$CaAr_2$]/[Ca^{2+}] vs. log [Ar]^2. Solid line in each case is the least square fit line (see text).

ity exists between the n sites on Ca^{2+}, which reduces the equation to:

$$\frac{\theta}{1-\theta} = K\,[\mathrm{Ar}]^N.$$

This is the equation for the well-known Hill plot where log ($\theta/1 - \theta$) vs. log [Ar] gives the value of N, the Hill coefficient. The Hill coefficient cannot be <1 for a physically realizable reaction. The value of $N = 1$ results from no cooperativity between sites or, as a special case,

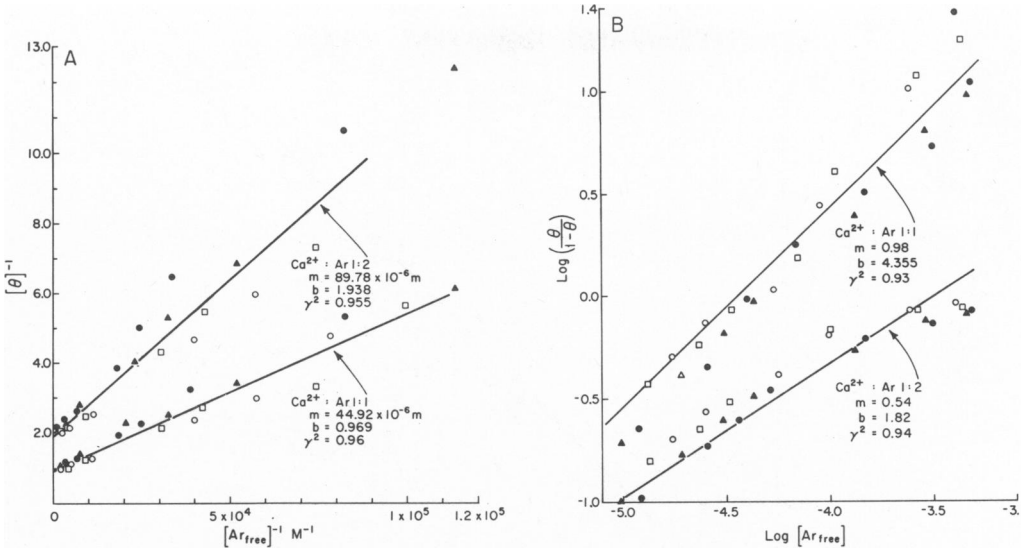

FIGURE 5 (*A*) Double reciprocal plot: plot of θ^{-1} vs. $[Ar]^{-1}$, assuming $n = 1$ and $n = 2$. (*B*) Hill plot: plot of $\log \theta/(1-\theta)$ vs. $\log [Ar]$, assuming $n = 1$ and $n = 2$. In both cases the solid line is the least square fit.

only one site, whereas if there is infinite positive cooperativity, N will equal n. In Fig. 5 *B* we have constructed Hill plots from the CaArIII data assuming both $n = 1$ and 2. The least square fit line assuming $n = 1$ has a slope of 0.98, while the line assuming $n = 2$ has a slope of 0.53, which is not in the possible range of Hill coefficients. These data would then also suggest that the stoichiometric ratio of the Ca-Ar complex is 1. The K_D value obtained from the intercept of 1:1 plot is ~44 μM.

From the foregoing analysis we have concluded that one dye binds per calcium ion for the ionic medium used and, within the concentration ranges of dye and calcium used, that the average value of the apparent dissociation constant for the Ca-Ar complex is ~40 μM.

In Situ Characteristics of Dye

The preceding experiments have indicated that ArIII forms 1:1 complexes with Ca, in vitro. The next question to be addressed is whether this stoichiometry appears to hold in situ. To assess this we have measured the absorbance change of the dye during repeated, identical voltage clamp pulses applied at successively greater ArIII concentrations within the same neuron. The intracellular concentration of ArIII ($[Ar_T]$) was estimated from the absorption at 580 nm, cell diameter, and in vitro extinction coefficient, $\epsilon_{580} = 2.98 \times 10^4$ M^{-1} cm^{-1}. In six neurons, a mixture of ArIII and Na_2 $^{35}SO_4$ (New England Nuclear, Boston, Mass.; 100 μCi/20 μmol dye) was injected under pressure (Coles and Brown, 1976; Ahmed and Connor, 1980). Under these conditions dye concentration ($[Ar_T]$) was estimated both from optical absorbance and isotope content. Fig. 6 is a plot of the estimates of $[Ar_T]$ obtained from the isotope method and ϵ_{580} method. The data show good agreement, so in general only the ϵ_{580} method was used for the determination of $[Ar_T]$. Where dye concentration was increased in a neuron during an experiment, a 15-min diffusion interval was allowed after each incremental injection before measurements were taken.

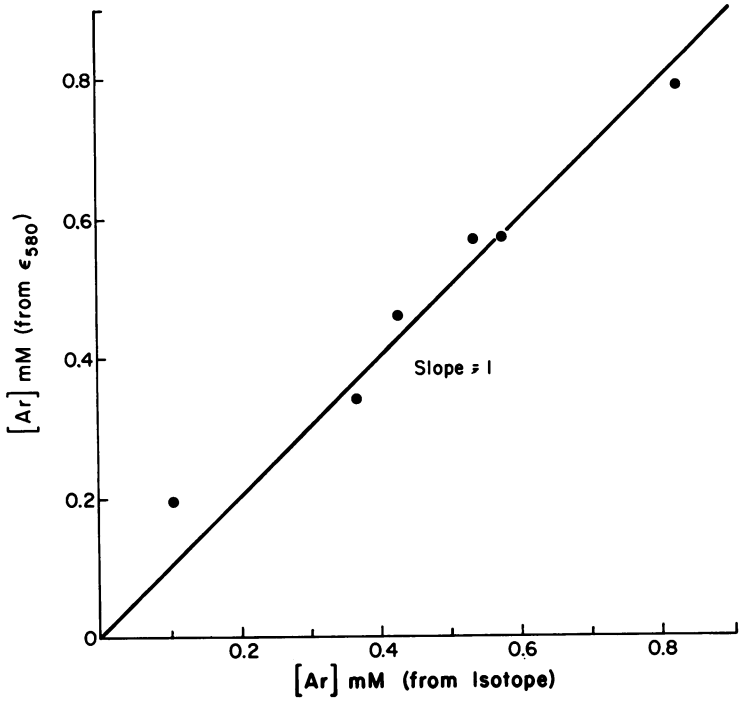

FIGURE 6 Plot of intracellular [ArIII] estimated by two methods. The ordinate is the [ArIII] estimated from the extinction coefficient (ϵ_{580}-method), and the abscissa is that estimated by isotope mixture method. The solid line is of unity slope.

A basic assumption underlying such experiments is that Ca influx is uniform from pulse to pulse and is not affected by the changing dye concentration (see also Gorman and Thomas, 1978). The data shown in Fig. 7 support this assumption. In this figure we have plotted the peak magnitude of the secondary inward current (Fig. 7 *A*) vs. [ArIII] for six neurons. This current is carried predominantly by Ca^{2+} and is the major Ca^{2+} influx during voltage clamp in marine gastropods (c.f. Geduldig and Gruener, 1969; Connor, 1977, 1979). It can be seen that there is very little dependence on dye concentration. Although an apparent rapid transient in records such as Fig. 7 *A* or in Fig. 8 *A*, it has been shown elsewhere that the Ca current fully activates in ~10 ms (T = 12°C) and undergoes only partial inactivation (<20%) in the first 200–300 ms of its time-course (Connor, 1979; Ahmed and Connor, 1979). The relaxation of total membrane current toward zero or to outward levels is due to activation of potassium conductance which superimposes an outward current on the calcium current (Connor, 1977, 1979). Neurons were bathed in TEA saline (Table I) for the records shown. Under these conditions most of the potassium conductance increase is due to a Ca-dependent mechanism (c.f. Meech, 1974, 1978; Connor, 1979).

Fig. 8 *A* shows the time-course of ArIII absorbance changes (λ = 660 nm) along with membrane current measured for three values of [ArIII]. The shape of the signal, as well as amplitude, changes with dye concentration. Figs. 8 *B* and *C* show a log-log plot of dA_{660}/dt vs. [ArIII] for five neurons. The rate of absorbance change in Fig. 8 *B* was measured during the first 200 ms of the voltage clamp pulse, while in Fig. 8 *C*, dA_{660}/dt was measured near the end

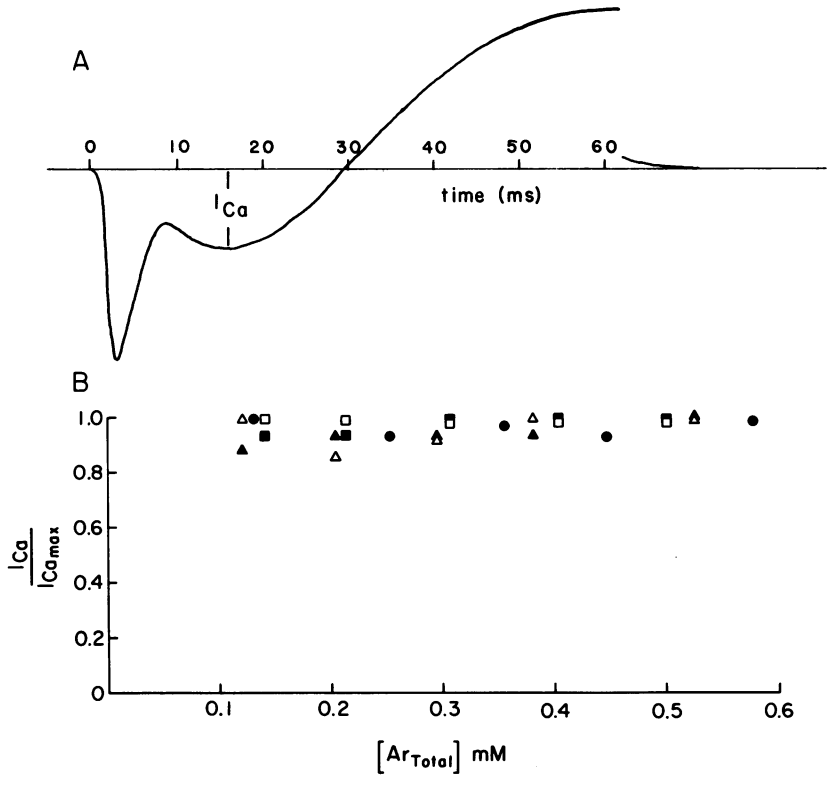

FIGURE 7 (*A*) Tracing of membrane current under voltage clamp (V_c = +20 mV) from neural soma of *Archidoris*. Magnitude of secondary inward current (I_{Ca}) is indicated on the trace. (*B*) Plots of I_{Ca} vs. internal ArIII concentration $[Ar]_T$ from four neurons. I_{Ca} was measured for identical voltage pulses to +20 mV and was normalized to the pre-injection I_{Ca} in each cell. $[Ar]_T$ was determined from ϵ_{580} and cell diameter (see Methods).

of the 1.3-s pulse for the same five neurons. Similar data were taken from 12 neurons using both purified and nonpurified dye. As pointed out by Gorman and Thomas (1978), the nonzero slope of the plots indicates that the dye complexes only a minor fraction of the incoming Ca^{2+} over the range of [ArIII] used, the rest being chelated by intrinsic buffers, sequestered, or pumped out. There is, however, a marked difference in the slope of the plots depending on when the measurements were taken. The data points of Fig. 8 *B* lie along a line with a slope of 1, except for the points corresponding to the smallest dye concentrations (<150 μM). These leftmost points consistently fell below the extrapolated unity slope fit to the right-hand data points. In contrast, the data of Fig. 8 *C* are well fit by a line of slope 2 over the range of dye concentrations tested, as were the data of Gorman and Thomas (1978), which were also obtained after long periods of current flow (10 s).

If one assumes a simplified model in which local equilibrium exists between Ca^{2+}, ArIII, and an endogenous calcium buffer, BB, having an apparent dissociation constant K_{D_B}, the following relation holds:

$$[CaAr_n] = \frac{[Ar]^n \cdot [Ca_T]}{(K_{D_A} + [Ar]^n)\, K_{D_B} + K_{D_A}\,[BB]} \cdot K_{D_B},$$

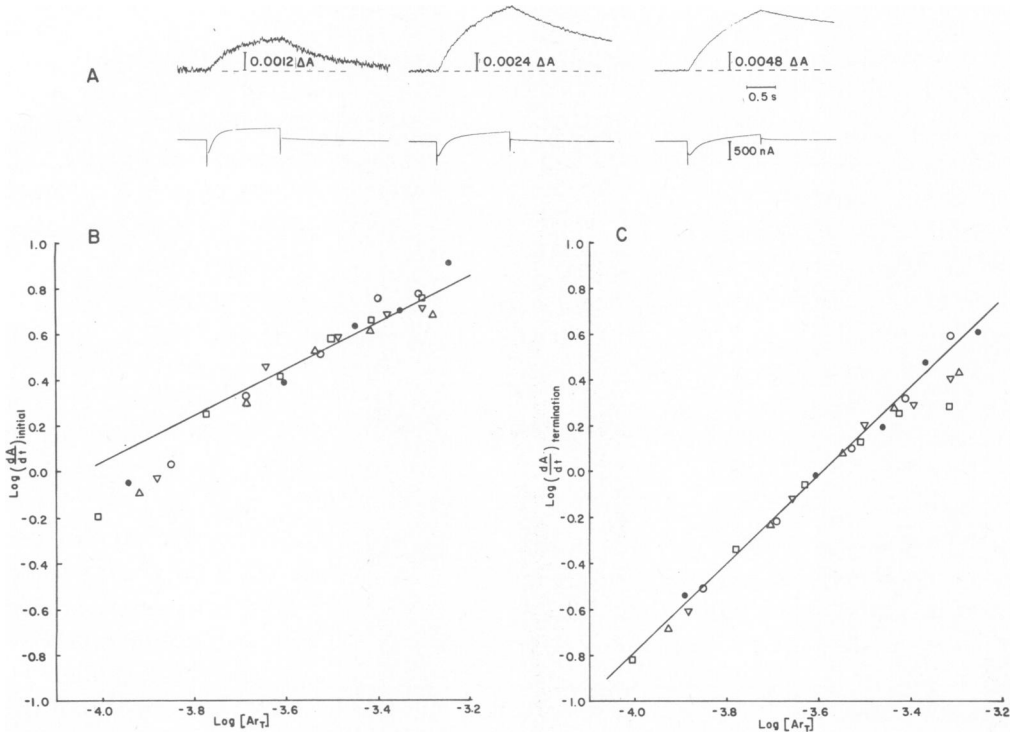

FIGURE 8 (*A*) Raw data showing absorbance changes (λ = 660 nm) and membrane current for three dye concentrations: 120 μM, left record; 290 μM, middle record; 520 μM, right record. (*B, C*) Plots of absorbance rate of change dA/dt vs. dye concentration ($[Ar]_T$) for five neurons. In *B*, dA/dt was determined from the initial slope of records such as shown in *A*, and in *C*, dA/dt was measured during the last 100 ms of the 1.3-s voltage clamp pulses.

where Ca_T is the total calcium added to the system. For Ca_T loads which produce small absorbance changes ($\Delta A \ll 1$) it is probably safe to approximate $[Ar]^n$ by $[Ar_T]^n$, where $[Ar_T]$ is the injected quantity of ArIII. (This assumption is somewhat problematical since intracellular diffusion barriers might give rise to local saturation of the dye.) Then, if ArIII is a minor buffer system compared with the intrinsic buffer, $K_{D_A} \cdot [BB] \gg K_{D_B} \cdot [Ar_T]^n$ and the expression for $[CaAr_n]$ reduces to:

$$[CaAr_n] \approx \frac{[Ar_T]^n \cdot [Ca_T]}{K_{D_A} \cdot [BB]} \cdot K_{D_B}.$$

If it is further assumed that the applied Ca load is small compared with [BB], then $[BB] \approx [BB_T]$, and the denominator is a constant independent of $[Ar_T]$ and log-log plots of ΔA or $[CaAr_n]$ (the absorbing species) vs. $[Ar_T]$ should have a slope equal to n. For purposes of illustration in Fig. 8, we have plotted rates of change; in Fig. 8 *B* it makes little difference whether rate or total change is plotted, because the initial rate is fairly constant, and in Fig. 8 *C* the rate at the 1.3-s pulse termination should give a better comparison with the data of Gorman and Thomas, which were obtained using total ΔA for very long pulses. A simple plot of ΔA at 1.3 s is heavily biased by the initial rate of absorbance change.

DISCUSSION

The results of the in vitro tests reported here demonstrate that ArIII forms 1:1 complexes with Ca. Although some of the tests are indirect and seemingly redundant, they all suggest the same conclusion and are necessary in a thorough analysis. The methods used for the determination of K_D require the stoichiometry of the reaction; therefore analysis was carried out assuming $n = 1$ and $n = 2$. The log-log plot is able to give a K_D value, but since there was no independent measure of free calcium, the subsequent analysis with the Adair's equation was necessary because it uses total calcium. The estimated value of K_D for the Ca-Ar reaction obtained from different analyses ranged between 35.5 and 45 μM with a mean value of ~40 μM. This agrees with the findings of Dipolo et al. (1976) and Scarpa et al. (1978).

The dye concentrations normally used in situ (100–500 μM) were used in the in vitro experiments. It is more difficult to compare levels of $[Ca^{2+}]$, because in situ figures are speculative at this point; however, the lower range of Ca^{2+} for Figs. 2 and 3 is probably not far different from $[Ca_i^{2+}]$ in active neurons. In Fig. 2 the lowest $[Ca^{2+}]$ was approximately 0.5 μM, assuming a K_D for the ArIII-Ca reaction of 40×10^{-6} M, while in Fig. 3 the lowest $[Ca^{2+}]$ was 0.12 μM, assuming a K_D for the EGTA-Ca reaction of 0.15×10^{-6} M. We have reported resting $[Ca_i^{2+}]$ of approximately 0.1 μM in *Archidoris* neurons (Ahmed and Connor, 1979), a value similar to the 40 nM measurement of Dipolo et al. (1976) in squid axon. Given the large Ca influx during voltage clamp of *Archidoris* and other gastropod central neurons, it is possible that $[Ca_i^{2+}]$ reaches micromolar concentrations locally near the membrane for transient periods. The in vitro studies would therefore give a useful representation of dye behavior in situ. At present we cannot offer an explanation for the differences between our Fig. 3 and Thomas' (1979) Fig. 1, except to note that the measurement systems were of very different design.

The in situ data of Fig. 8 *B* are consistent with a 1:1 stoichiometry, while those of Fig. 8 *C* would indicate a 2:1 complex of dye to Ca. We have greater confidence that the various assumptions necessary for the equilibrium analysis are more nearly met for brief periods of Ca influx than for long periods, and therefore regard the in situ data as being consistent with the formation of 1:1 complexes. For example, the approximation of uncomplexed [Ar] and [BB] by total amounts should better hold in the initial period of calcium influx than at later times. Also, the independence of Ca influx (hence Ca_T) from [ArIII] can be demonstrated from voltage clamp records only during the initial period (Fig. 7). At later times potassium current has activated and obscured the calcium current. It is possible that the phenomenon reported by Tillotson (1979), of increased internal calcium causing a blockage of calcium channels, is partially responsible for the slope differences of Figs. 8 *B* and *C*. With this mechanism operative, increasing the concentration of the Ca-complexing dye would lead to progressively larger calcium influx and the effect would be most noticeable at long, rather than short, periods of influx. Other factors, such as dye saturation or differing rates of cellular uptake, cannot be discounted. We would note, though, that the difference in slopes of Figs. 8 *B* and *C* is opposite to what would be predicted by a simple change in dye-Ca stoichiometry, from 2:1 at low $[Ca^{2+}]$ to 1:1 on going from lower to higher $[Ca^{2+}]/[Ar]$.

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