RAPID CHANGES OF INTRACELLULAR FREE CALCIUM CONCENTRATION

DETECTION BY METALLOCHROMIC INDICATOR DYES IN SQUID GIANT AXON

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ABSTRACT The metallochromic indicator dyes, arsenazo III and chlorophosphonazo III, were used in squid giant axons to detect rapidly the very small influxes of calcium that occur as a result of changes of membrane potential.

Changes of intracellular free calcium concentration have been implicated in a wide variety of physiological events such as excitation-contraction and excitation-secretion coupling, control of enzymatic reactions, cell-cell communication, modulation of specific ionic permeabilities in neurons, intracellular transmission of excitation in vertebrate photoreceptors and mediation of light adaption in invertebrate photoreceptors. Because intracellular free calcium concentration, Ca_i, is usually very low (10^{-8} M to 10^{-5} M) and changes of Ca_i may be rapid, direct detection of changes of Ca_i has been difficult. One useful technique involves the intracellular injection of the calcium-sensitive photoprotein, aequorin (1, 2). The luminescent reaction between aequorin and calcium ions has an appropriate sensitivity and quantum yield to allow detection of a change of Ca_i from a variety of single cells, such as squid giant axons (3, 4) and giant synapses (5), muscle cells (6, 7), neuron cell bodies (8), eggs (9), gland cells (10), and photoreceptors (11). However, the aequorin technique has several difficulties: (*a*) Aequorin is not readily available. (*b*) The kinetics of the luminescent reaction (12) are slower than those of some physiological events. (*c*) The stoichiometry of the Ca-aequorin reaction is in dispute (13, 14) so that it is difficult to quantitate the change of Ca_i that can be detected. (*d*) The high molecular weight (15) of aequorin may prevent rapid diffusion throughout the cytoplasm.

We wish to report an alternative technique for the detection of changes of Ca_i based

on the use of water-soluble metallochromic indicator dyes. The dyes are *bis* arylazo derivatives of chromotropic acid (16), specifically arsenazo III (mol wt 757; Sigma Chemical Co., St. Louis, Mo.; Aldrich Chemical Co., Milwaukee, Wisc.) and chlorophosphonazo III (mol wt 775; K&K Labs., Plainview, N.Y.). When calcium is added to a solution of either dye its absorption peak shifts towards longer wavelengths, resulting in an increase in absorption at long wavelengths and a decrease at shorter wavelengths. For arsenazo III, the long wavelength maximum of the difference spectrum (with and without calcium) is at 660 nm; chlorophosphonazo III has two long wavelength maxima, one at 620 nm and one at 680 nm. The maximum molar absorptivities of the difference spectra exceed 10^4 (16). Both dyes form 1:1 complexes with Ca at physiological pH (16, 17) and either dye can be used to detect small changes of Ca in the presence of high concentrations of Mg. Apparent stability constants for the calcium complexes of either dye determined with a dye concentration of 5×10^{-5} M in a medium containing 400 mM KCl, 3 mM $MgCl_2$, and 5 mM Tris-HEPES at pH 7.3 were about 10^4 M^{-1}.

In order to evaluate the applicability of the metallochromic dye technique to physiological problems, we have reexamined some properties of the entry of Ca associated with the action potential in the squid giant axon, previously described by Baker et al. (3) and Hallett and Carbone (4). Cleaned giant axons of the squid, *Loligo pealei,* were injected (18) with a dye solution previously freed of Ca by passage through a column of Chelex 100 (Bio-Rad Laboratories, Richmond, Cal.) in the potassium form. Some axons were injected with both a dye and tetraethylammonium chloride. An injected axon was voltage clamped using platinized-platinum electrodes as described by Cohen et al. (19). Changes of dye absorption (20) were measured from a 1.5 mm length of axon centered on the voltage-sensing internal electrode; incident light was filtered through a 10 nm bandpass interference filter, centered on the λ_{max} of the difference spectrum.

At an internal concentration of 0.3 mM, arsenazo III was relatively nontoxic to the axon. The potential-dependent conductance changes did not seem to be altered appreciably. Also, the strophanthidin-sensitive ^{22}Na efflux (21) was reduced by less than 10%.

In an axon injected with arsenazo III and bathed in artificial seawater containing a high concentration (112 mM) of calcium, we detected, by averaging over many trials, a decrease in light intensity at 660 nm falling on the photodiode in response to individual depolarizing voltage-clamp steps (Fig. 1 *C*). This signal was reversibly attenuated by replacing most of the external calcium, Ca_o, with Mg (Fig. 1 *D*). The difference between the signals recorded in high and low Ca_o is not attributable to a change in light-scattering or other optical property of the axon itself. No difference was detectable at $\lambda = 750$ nm, where the absorption of the dye is negligible. Although light-scattering signals (19) were detected in unstained axons, these signals were small compared with the measured absorption changes and were affected only slightly by altering Ca_o. Furthermore, at a wavelength (530 nm) shorter than the isosbestic point for Ca-dye complexation, an absorption change of opposite sign was

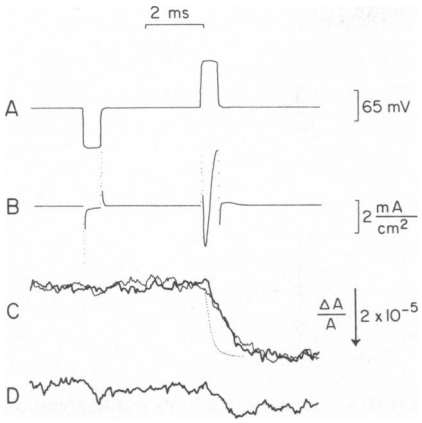

FIGURE 1

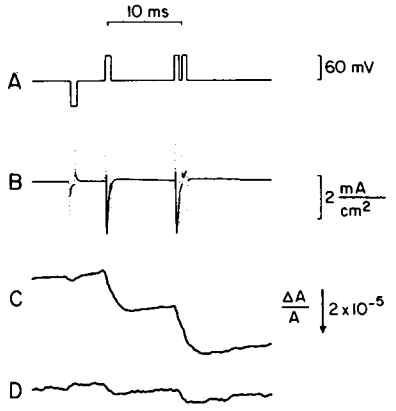

FIGURE 2

FIGURE 1 Changes in absorption during hyperpolarizing and depolarizing voltage-clamp steps of a dye-injected axon in high calcium (*C*) and low calcium (*D*) artificial seawaters. The two superimposed records in *C* were recorded before and after that shown in *D*. Potential steps are shown in *A* and current density recorded in low calcium seawater is shown in *B*. When the external calcium was reduced the absorption change resulting from the depolarizing step was also reduced. The dotted curve in trace *C* is explained in the text. The axoplasmic concentration of arsenazo III was 0.3 mM. All of the experiments were carried out at 21°C. The high calcium artificial seawater contained 112 mM $CaCl_2$, 350 mM NaCl, 10 mM KCl, and 5 mM Tris-HEPES, pH 7.8. The low calcium artificial seawater contained 10 mM $CaCl_2$, 350 mM NaCl, 10 mM KCl, 102 mM $MgCl_2$, and 5 mM Tris-HEPES, pH 7.8. In all of the figures the direction of the vertical arrow represents an increase in absorption and the size of the arrow represents the stated value of a change in absorption divided by the resting absorption due to the dye; the low frequency time constant was 120 ms; 512 sweeps were averaged; hyperpolarizing voltage is drawn downward; inward current is drawn downward; and the peak transmission of the interference filter was 660 nm.

FIGURE 2 Absorption changes in a dye-injected axon during a series of voltage-clamp steps, in high calcium seawater (*C*) and in high calcium seawater plus 100 nM tetrodotoxin (*D*). The potential steps are shown in *A*. The current densities in trace *B* were determined in seawater without tetrodotoxin. When two depolarizing steps were closely spaced, the inward current for the second step was greatly reduced and the absorption change for the two steps was only slightly larger than that from the single step. When tetrodotoxin was added, the calcium influx during these brief steps was greatly reduced. When the axon was returned to tetrodotoxin free seawater there was a recovery of inward current and a recovery of calcium influx. The axoplasmic concentration of arsenazo III was 0.3 mM and that of tetraethylammonium chloride was 8 mM; the latter was added to block outward potassium current that might obscure the degree of inactivation of the inward current. The light measuring system had a high frequency time constant of 540 μs.

elicited by depolarizing voltage-clamp steps. Also, there was no potential-dependent change in absorption or fluorescence (20, 22) if the axon was stained with dye applied extracellularly. From these findings, we conclude that the difference between signals in Fig. 1 *C* and *D* arises from an increase in absorption of the intracellular dye due to a stimulus-induced rise of intracellular free calcium. Similar results were obtained using chlorophosphonazo III.

If it is assumed that all the calcium enters the axon uniformly during the first 300 μs of the depolarizing step, that there are no diffusion delays, that the complexation reaction has no delay, and given that the light measuring system had a time constant of

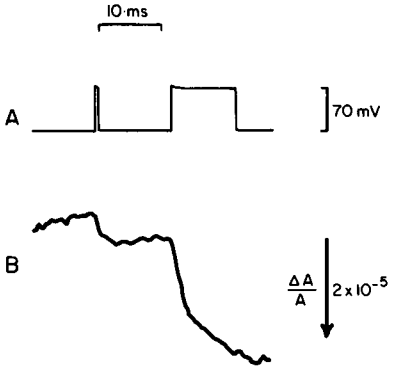

FIGURE 3 Changes in absorption (*B*) during a brief (0.6 ms) and a long (10 ms) depolarizing step (*A*). The long step resulted in an additional absorption change which we presume to represent the slow component of Ca entry found by Baker et al. (3). The peak inward currents were 1 mA/cm. The axoplasmic concentration of arsenazo III was 0.3 mM, the axoplasmic concentration of tetraethylammonium chloride was 8.5 mM. The light measuring system had a high frequency of 540 μs.

170 μs, then the absorption increase should have the time course shown as the dotted curve in Fig. 1 *C*. The measured absorption increase lags this calculated curve by 400 μs, suggesting that the Ca-dye complexation reaction must be faster than 400 μs.

The rapid kinetics of the technique allowed us to reexamine the relationship between the Ca entry and the increase of sodium conductance evoked by a depolarizing voltage-clamp step. When the second of a pair of closely spaced depolarizing steps elicited a mostly inactivated sodium conductance increase (Fig. 2 *B*), the Ca influx associated with the closely spaced pair of steps was only slightly larger than the Ca influx associated with a single depolarizing step (Fig. 2 *C*). The absorption change was not saturated at this value of Ca influx: in other axons, if the first 50 mV step was followed, 10 ms later, by a 100 mV depolarizing step, a larger calcium influx was recorded. Similarly, when the sodium conductance increase was blocked by tetrodotoxin (TTX: Fig. 2 *D*), the increase in dye absorption was also markedly reduced. These two findings show that much of the Ca entry during a brief depolarizing step was associated with the increase in sodium conductance. Also, a brief depolarizing pulse induced a smaller change in dye absorption than did a longer depolarizing pulse (Fig. 3); this finding is consistent with there being a late phase of Ca entry. Thus, our results obtained with the metallochromic dye technique confirm the results and interpretation of Baker et al. (3) using the aequorin technique.

A small brief hyperpolarizing voltage-clamp step elicits little or no change in absorption at 660 nm (Figs. 1 and 2). However, large hyperpolarizing steps lasting longer than 3 ms lead to substantial increases in absorption. This finding again agrees with the results obtained with the aequorin technique (4).

In summary, we used metallochromic indicator dyes in squid giant axons to detect the very small influxes of Ca that occur as a result of changes of membrane potential. These dyes have rapid response kinetics to increased Ca_i, and seem to be rela-

tively nontoxic to the cell membrane. Analogues of these dyes differ in their effective binding constant for Ca, their selectivity, net molar absorptivity, wavelength of maximum absorption, etc. We suggest that appropriately chosen metallochromic indicators ought to be useful in studying changes of ionized calcium in many cells and cell-free systems, particularly in those systems in which the changes of ionized calcium may be larger (11) than those occurring in squid giant axons (10^{-8} M per impulse [23]).

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