tubes orient themselves longitudinally rather than circumferentially. The effect of curvature may be important, especially in small vessels, where the effect on individual cells is the greatest.

Adaptive alterations in endothelial cell stress fibers seem likely in disease processes such as hypertension and atherosclerosis. An increase in the concentration of endothelial actin in hypertension has been suggested by staining with fluorescent antibodies (*6*), so a reevaluation with our techniques might be informative.

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Intracellular Calcium Measurements with Arsenazo III During Cyclic AMP Injections into Molluscan Neurons

Abstract. *Injections of cyclic adenosine monophosphate into molluscan neurons often produce a transient membrane depolarization. By using the calcium indicator dye arsenazo III, it was found that cyclic nucleotide injections into neurons of* Archidoris montereyensis *resulted in elevation of internal calcium concentrations. However, this was demonstrated to be a secondary consequence of an induced increase in membrane sodium permeability, and not due to any direct effect of cyclic adenosine monophosphate on cellular calcium influx or internal calcium regulating processes.*

Many different types of cells utilize intracellular second messengers, such as cyclic adenosine monophosphate (AMP), cyclic guanosine monophosphate (GMP), and Ca^{2+}, to transduce membrane signals into a wide range of cellular responses (*1*). Evidence has been accumulating that the concentration of these messengers is regulated not only by extracellular effectors—that is, first messengers such as hormones and neurotransmitters—but also through feedback relations with each other (*2, 3*). For example, there is considerable indirect evidence that elevation of the internal cyclic AMP concentration leads to an increase in the intracellular calcium concentration, $[Ca^{2+}]$, in cells exhibiting stimulus-secretion coupling, such as nerve terminals and endocrine cells (*3*). However, there are no direct measurements of such increases, nor clear indications of the mechanism or mechanisms that bring them about.

Intracellular injections of cyclic AMP or its derivatives into mammalian (*4*) and molluscan neurons (*5, 6*) have been reported to produce membrane depolarizations or increases in net inward current. In one reported instance this effect was shown to be the direct result of an induced calcium influx (*6*). However, in most instances the mechanism of action of cyclic AMP has not yet been deduced. We report here measurements on a set of reidentifiable molluscan neurons which show that increased intracellular levels of cyclic AMP do indeed lead to increased cellular $[Ca^{2+}]$, but that the coupling mechanism is indirect.

Giant neurons (~ 300 to 500 μm in diameter) within isolated ganglia from the circumoesophageal ring ganglia of *Archidoris montereyensis* were maintained in vitro at 12°C in a physiological saline of the following composition: 490 m*M* NaCl, 8 m*M* KCl, 10 m*M* $CaCl_2$, 20 m*M* $MgCl_2$, 30 m*M* $MgSO_4$, 10 m*M* MOPS, 5 m*M* glucose, and *p*H adjusted to 7.6. Recording electrodes were filled with 3*M* KCl and had resistances of 1 to 2 megohms. Standard two-electrode voltage clamping techniques were employed. Iontophoresis electrodes were filled with 0.1*M* HCl, 0.2*M* $CaCl_2$, or 0.2*M* cyclic AMP (grade I, Sigma). Pressure injection electrodes were filled with a buffered cyclic AMP solution (0.2*M*) at *p*H 7.4. We constructed *p*H microelectrodes according to procedures described by Thomas (*7*). The *p*H microelectrodes had tip diameters of 1 to 2 μm and response times—that is, 90 percent completion following a step change in *p*H—of approximately 1.5 minutes. We used *p*H electrodes with slopes of 54 to 58 mV per unit of *p*H change.

The indicator dye arsenazo III (grade I, Sigma) was used to examine changes in intracellular free Ca^{2+}. This dye has been used extensively for monitoring changes in cellular $[Ca^{2+}]$ (*8–14*). Neurons were iontophoretically injected with dye (10 mg/ml) under voltage clamp, using pulses of 200 to 400 nA for 0.8 second repeated at 1 Hz. Injections were quantified as described elsewhere (*12–14*). The dye is nontoxic when loaded into cells at concentrations up to 1 m*M*, and at this concentration it can detect submicromolar changes in cellular $[Ca^{2+}]$ (*15*). Changes in internal Ca^{2+} were detected by monitoring the absorbance of the dye at the differential wavelength pair 660 − 700 nm with a rotating wheel spectrophotometer (*16*).

Figure 1A shows the recording arrangement, and Fig. 1, B and C, shows records of experiments during which separate injections of Ca^{2+} and H^+ were made into a neuron containing 0.3 m*M* arsenazo III. Dye absorbance was simultaneously monitored at two wavelength pairs, 660 − 700 nm and 610 − 700 nm. These dual-wavelength measurements were used in order to reduce light scattering or dilution artifacts by subtracting a relatively ion-insensitive wavelength (700 nm) from the recording. Calcium ion injection resulted in a greater increase in absorbance at 660 − 700 nm than at 610 − 700 nm, a result at odds with in vitro dye measurements (*9*) but consistent with in situ results (*10, 12*). The subsequent recoveries of the absorbance signals were analyzed and are thought to result from cellular calcium buffering systems, cellular extrusion of calcium,

and diffusion of the ion away from the recording probe (*10, 12, 17*). The faster recovery of the 610 − 700 nm signal is not completely understood, although a major factor is the local intracellular *p*H (pH_i) change that results from the Ca^{2+} injection (*14, 18*). The *p*H microelectrode (top trace) did not detect the small $[H^+]$ change in this experiment because of its distance from the recording site.

Iontophoretic injections of hydrogen ions had the reverse effect of the dual-wavelength absorbance measurements (Fig. 1). Both signals decreased with the 610 − 700 nm signal showing the greater decrease, a result that reflects the *p*H dependence of the differential absorbance spectrum of the magnesium-arsenazo complex (*9, 19*), since this divalent ion predominates in situ. A simultaneous measurement of pH_i with a microelectrode (top trace) confirmed the much larger increase in $[H^+]$. Here again the recoveries of the absorbance signals reflect cellular hydrogen buffering, extrusion, and diffusion of the ion away from the sampling region. Since the *p*H microelectrode had a response time of ~ 2 minutes, its reported measurement lags the much faster dye signal. Also, it was necessary for H^+ to diffuse to the *p*H electrode, which contributed to the lag, whereas the absorbance signals were monitored at the injection electrode tip.

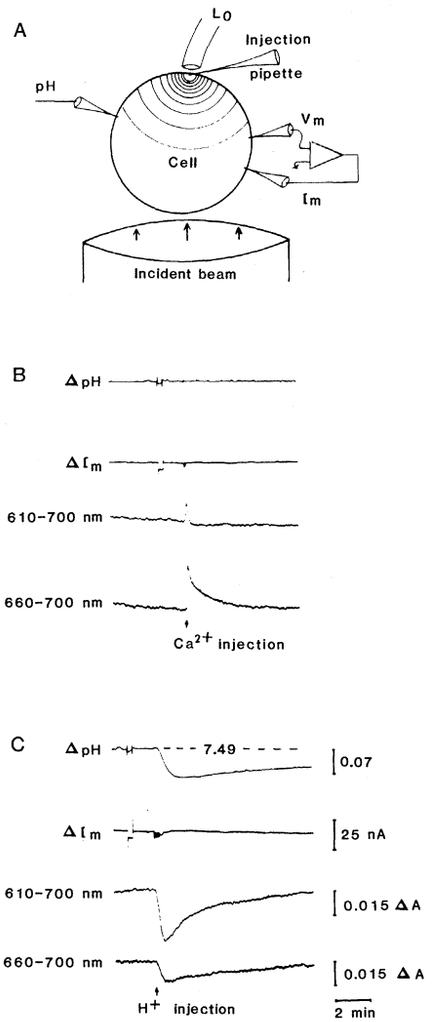

Fig. 1. (A) Diagrammatic representation of the recording arrangement, showing the relative size of the incident and receiving (L_0) optical fibers in relation to cell body diameter. Not illustrated are the additional neurons in the isolated ganglion. V_m and I_m refer to voltage and current microelectrodes. (B and C) Simultaneous measurements of pH_i (top traces), membrane holding current (second traces), and dual-wavelength absorbance signals (bottom two traces) of an arsenazo-filled neuron injected with Ca^{2+} and H^+ under voltage clamp. See text for details. The *p*H electrode and intracellular voltage signals were routed into a differential amplifier, whose output was used to measure pH_i. Thus, a negative voltage step prior to each injection demonstrated complete cellular penetration of the *p*H electrode since there was no net change in pH_i signal. Absorbance signal time constant (τ) was 1 second.

Figure 2 shows recordings of electrical activity, internal $[Ca^{2+}]$, and pH_i of an arsenazo-filled neuron (0.5 m*M*) after pressure injections of a buffered cyclic AMP solution. In Fig. 2A the cell was not voltage clamped, and an injection induced a train of action potentials. The 660 − 700 nm absorbance increase occurred during the period of induced electrical activity, indicating a rise in internal $[Ca^{2+}]$. The absorbance recovered after the firing to a level below the original baseline. Monitoring of intracellular *p*H showed a decrease both during and after the induced activity, and thus the shift in 660 − 700 nm baseline was probably the result of this pH_i decrease (*14, 18*). As illustrated in the right-hand part of the record, both these effects were produced by simple current stimulation of action potentials. Comparable injections of 5′-AMP were without effect on resting potential.

Figure 2B shows records from the same neuron under a different condition—voltage clamped at its resting potential, −40 mV. In this case a much larger injection of cyclic AMP induced a reversible increase in membrane holding current (middle trace) lasting more than twice as long as the induced depolarization in Fig. 2A. Nevertheless, there was no increase in dye absorbance at 660 − 700 nm coincident with the current flow. After a pronounced lag, however, the dye signal decreased and the *p*H microelectrode demonstrated a delayed cellular acidification with a magnitude and duration that could account for the absorbance decrease. Subsequent studies showed that both the induced current and the subsequent decrease in pH_i are dependent on the dose of cyclic AMP; large doses were shown to lower pH_i by 0.1 to 0.2 *p*H unit.

The inward current evoked by cyclic AMP injection was examined with ion-substituted saline solutions and voltage clamping at various holding potentials. Replacing sodium in the superfusion solution with Trisma (base) or tetramethylammonium chloride blocked the nucleotide-induced response. Bathing the cells in $5 \times 10^{-4}M$ ouabain did not affect the response, nor did perfusing the cells in potassium-free or calcium-free saline. Voltage clamping neurons at potentials between −30 and −100 mV did not re-

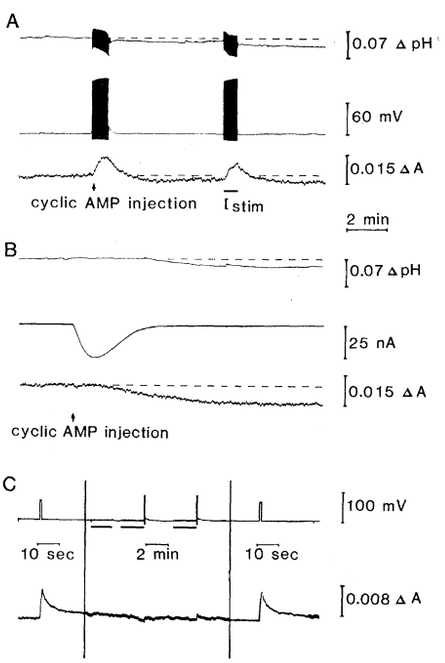

Fig. 2. Pressure injections of a buffered cyclic AMP solution (*p*H 7.5) into the same neuron (pH_i 7.46) with and without the membrane voltage clamped. (A) Without voltage clamp, the injection elicited a train of action potentials (middle trace) and a corresponding increase in the 660 − 700 nm signal (lower trace, τ = 1 second) indicating a rise in intracellular $[Ca^{2+}]$. Simultaneous monitoring of pH_i (top trace) showed an induced acidification during and after the train of spikes. Both changes were mimicked by current stimulation (I_{stim}) of a similar train of spikes. (B) With voltage clamped, a much larger injection elicited a large inward current (middle trace), a decreased 660 − 700 nm signal (lower trace, τ = 1 second), and a pH_i decrease. (C) Arsenazo III absorbance changes at 660 nm (lower trace) generated by identical voltage clamp pulses (upper trace) before (left) and after (right) several iontophoretic injections (middle) of cyclic AMP. The two voltage step records are nearly identical, indicating no change in Ca^{2+} influx or regulation characteristics. After each injection period (horizontal bars) voltage clamp was discontinued. The second and third injections were followed by a short train of spikes. Two additional injections were administered between the voltage steps, but are not shown. Absorbance τ = 50 msec.

verse the direction of the induced current. Taken together, these results indicate that cyclic AMP injections induce a reversible increase in the membrane sodium current of these neurons.

Arsenazo III was further used to examine possible changes in the voltage-dependent calcium conductance or in cellular calcium regulation during cyclic AMP injections. Figure 2C (left) shows the characteristic increase and subsequent recovery of the signal at 660 nm during and after a voltage step to +20 mV for 1 second. During the following several minutes a total of five intracellular iontophoretic injections of cyclic AMP were administered under voltage clamp, of which three are represented in Fig. 2C (middle). When the voltage was unclamped after an injection, the cell exhibited the nucleotide-induced depolarization that usually elicited action potentials. Immediately after the fifth injection (not shown), the voltage was stepped to +20 mV in the same fashion as before. The absorbance, recorded in Fig. 2C (right), showed no observable change in either the characteristics of calcium influx or its subsequent regulation as reflected by the recovery of the dye signal.

In summary, we were unable to detect changes in resting Ca^{2+} levels of voltage clamped neurons after intracellular cyclic AMP elevation. In addition, no changes were found in the characteristics of calcium influx or its subsequent regulation during and after nucleotide injections. Although cells were studied from each of the six ring ganglia, only the large, identifiable neurons were examined. We have not ruled out the possibility that other neurons in *Archidoris* may exhibit direct cyclic AMP–induced effects on cellular calcium parameters, like those in certain *Aplysia* neurons (*6*). While the absence of cyclic nucleotide–induced calcium changes in the neurons used in this study indicates that there are no direct effects, it should be stressed that increased intracellular cyclic AMP levels will have a large indirect effect on internal $[Ca^{2+}]$ under physiological conditions. That is, the steady inward current induced by cyclic AMP will depolarize the membrane of unclamped neurons and thereby generate action potentials with corresponding calcium influx.

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Independent Pathways for Secretion of Cholesterol and Apolipoprotein E by Macrophages

Abstract. *Cholesterol-loaded macrophages secrete cholesterol and apolipoprotein E. The current studies show that this secretion occurs by two independent pathways. In the absence of serum, the cells secrete apolipoprotein E, but not cholesterol. In the presence of monensin (an inhibitor of protein secretion), the cells secrete cholesterol, but little apolipoprotein E. After secretion, apolipoprotein E and cholesterol associate with high-density lipoprotein to form a particle that can deliver cholesterol to the liver by receptor-mediated endocytosis. We conclude that apolipoprotein E does not function to remove cholesterol from macrophages but rather to participate in "reverse cholesterol transport."*

In humans or animals with hypercholesterolemia, cholesteryl esters accumulate as cytoplasmic lipid droplets in macrophages (*1*). Possible mechanisms for such accumulation have been disclosed by studies of macrophages in vitro. These studies have shown that mouse and human macrophages have surface receptors that allow them to take up and degrade large amounts of plasma lipoproteins that have been chemically altered. For example, when the low-density lipoprotein (LDL) of human plasma is modified by acetylation or treatment with malondialdehyde, the modified lipoprotein binds to receptors on cultured macrophages and is taken up by endocytosis and delivered to lysosomes (*2*). Within the lysosome the cholesteryl esters of acetyl-LDL are hydrolyzed, and the liberated cholesterol is transferred to the cytoplasm where it is reesterified and stored in cholesteryl ester droplets (*3*).

Under appropriate conditions, macrophages can rapidly secrete the large amounts of cholesterol that they have stored in the cytoplasm. Secretion requires the presence in the culture medium of an agent, such as high-density lipoprotein (HDL), that is capable of binding cholesterol (*4*). In the absence of a cholesterol-binding substance, the cytoplasmic cholesteryl esters are continuously hydrolyzed and reesterified in a sequence termed the cholesteryl ester cycle (*4*). When HDL is present, the hydrolysis of the stored cholesteryl esters continues, but reesterification no longer occurs and the liberated cholesterol is secreted from the cell. Macrophages can secrete huge amounts of cholesterol in this fashion—up to 200 μg per milligram of cell protein per day (*4*).

At the same time that macrophages secrete cholesterol, they also synthesize and secrete large amounts of apolipoprotein E (apo E), a normal protein component of plasma lipoproteins (*5*). First detected in mouse macrophages, the secretion of apo E has also been shown to take place in macrophages derived from circulating human monocytes (*5*). The