virus-free females by infected males was demonstrated by the finding of LAC antigen in the bursa of one female and a disseminated infection in another mated to the same male.

By means of the FA technique, LAC viral antigen was detected in large amounts in the accessory sex glands of males, accessory gland fluids extruded from the ejaculatory duct, bursal contents, and mating plugs from recently mated females from the two previously LAC virus-free colonies, and in organs other than the lower reproductive tract (including heart, nerve ganglia, ovaries, gut, salivary glands, or fat bodies) of some of these females.

In infected males, antigen was also observed by FA in the testes and seminal vesicles, but in amounts much lower than those found in accessory sex gland fluids. Antigen was not observed within sperm. It would seem that LAC virus in *A. triseriatus* is venereally transmitted by male accessory sex gland fluid, which agrees with findings of other investigations with other vector-pathogen systems (*10*).

Additional knowledge of the natural cycle of LAC arbovirus is essential because of its growing public health importance and the difficulty of controlling the vector species (which survives in protected tree-hole sites) by conventional methods utilizing chemical insecticides. The frequency and importance of venereal transmission in nature are yet to be ascertained. However, such transmission may be an important supplement to transovarial and viremic mammalian blood virus sources for the natural endemic maintenance of this arbovirus.

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Internal Calcium Changes in a Bursting Pacemaker Neuron Measured with Arsenazo III

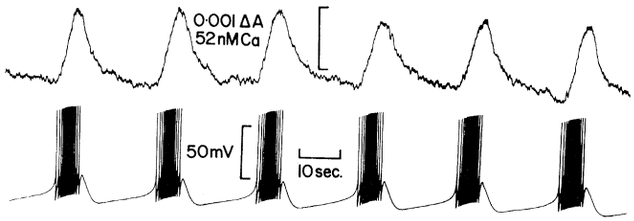
Abstract. *Arsenazo III was used to measure changes in the free intracellular calcium ion concentration during spontaneous bursting pacemaker activity in the* Aplysia *R15 neuron. Intracellular calcium increased during the burst, and this increase was sufficient to cause the hyperpolarization that followed. The results suggest that the interval between bursts is determined by the rate of subsequent decline of free intracellular calcium.*

One of the characteristic features of excitable cells is the ability to generate rhythmic activity. Certain neurons share with heart and smooth muscle cells the capability of spontaneously generating rhythmic patterns of action potentials, which are used to control or drive other cells. The origin of this behavior is still not fully understood. The neuron R15 (*1*) in the abdominal ganglion of the mollusk *Aplysia californica* is of this type (*2*) and has been extensively studied. Particular attention has been paid to the mechanism responsible for the hyperpolarization that occurs after each spontaneous burst of action potentials (*3–6*). Action potentials in *Aplysia* neurons are abolished by Ca^{2+}- and Na^+-free saline but not by Na^+-free saline alone (*7*), which suggests that a significant proportion of the inward current is carried by Ca^{2+} ions. A Ca^{2+} influx during the action potential has been directly shown with the Ca^{2+}-sensitive photoprotein aequorin (*8*). Injection of Ca^{2+} into *Aplysia* neurons causes a hyperpolarization, which results from an increase in K^+ conductance (*9*). It has been shown that the hyperpolarization that follows each spontaneously generated burst of action potentials is accompanied by an increase in K^+ conductance (*4, 6*). The possibility that the Ca^{2+} influx during a burst of action potentials may be sufficient to increase the K^+ conductance is strengthened by the demonstration of a posttetanic hyperpolarization in R15, following a train of action potentials induced by membrane depolarization. The effect is abolished by Ca^{2+}-free saline or injection of [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA), which suggests that it is mediated by an increase in free intracellular Ca^{2+} (*5*). It is conceivable, therefore, that free intracellular Ca^{2+} may increase during each train of action potentials and be an important determinant of the bursting behavior by causing, or assisting in causing, the hyperpolarization that follows.

To test this theory, it is necessary to determine directly whether free intracellular Ca^{2+} changes and to measure the size and time course of these changes throughout the burst cycle. The Ca^{2+}-sensitive dye arsenazo III, which undergoes an absorbance change on forming a complex with Ca^{2+} (*10*), has been used successfully to detect changes in free intracellular Ca^{2+} (*11, 12*). We used this dye to follow such changes during the burst cycle in R15, and we report here the results of these experiments.

The abdominal ganglion was dissected to expose R15, and a fiber-optic probe was positioned on each side of the neuron. Light from a tungsten halide source was passed through a wheel driven at 12,000 rev/min by compressed air; the wheel contained several narrow-band interference filters of different wavelengths, which sequentially interrupted the light beam. The composite light signal that resulted was directed to R15 through one of the fiber-optic probes, and the fraction that passed through the cell was directed to a photodiode through the second probe. The output from the photodiode was electronically separated into the components that corresponded to each filter wavelength for subsequent manipulation. The dye was injected under pressure through an intracellular microelectrode to give an internal concentration (as judged by the absorbance increase) of approximately 0.3 m*M*. This concentration did not affect the electrical activity of the cell. One or more additional electrodes were inserted for injection of ions and electrical recording. Changes in dye absorbance were measured by differential recording between a wavelength at which Ca^{2+} caused no absorbance change (570 nm) and one at which the absorbance increase was maximal (650 nm). The experiments were performed in artificial seawater (*13*) at 16°C.

A typical result, shown in Fig. 1, provides strong evidence for an increase in the free internal Ca^{2+} concentration during the burst. The absorbance changes as-



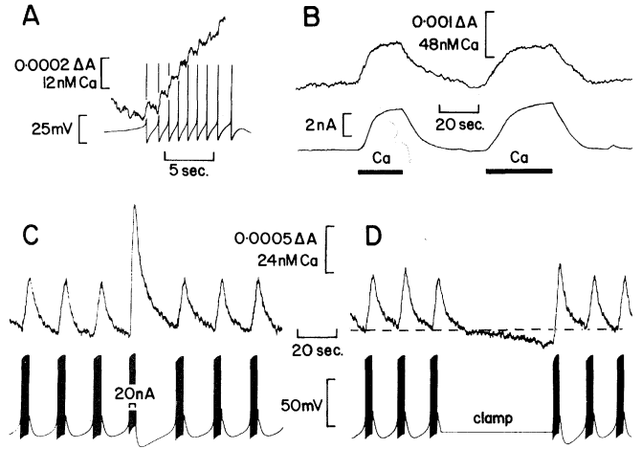


Fig. 1 (left). Changes in free internal Ca^{2+} concentration and membrane potential during spontaneous bursting pacemaker activity. Changes in dye absorbance (upper trace) accompany the spontaneous bursts of action potentials (lower trace) in the *Aplysia* R15 neuron. The equivalent change in intracellular Ca^{2+} (upper calibration bar) was estimated from in vitro dye absorbance data. Fig. 2 (right). Changes in free internal Ca^{2+} concentration under normal and experimentally manipulated conditions. (A) Absorbance increase during the burst shown at higher resolution than in Fig. 1. This result suggests that internal Ca^{2+} (diagonal trace) increases primarily in steps coincident with each action potential (bottom trace). (B) Absorbance increase (upper trace) and outward membrane current seen when Ca^{2+} is injected under pressure into a voltage-clamped cell. (C) Effect of a 20-na depolarizing current, passed during a single burst, on the dye absorbance (upper trace) and membrane potential (lower trace). There is a larger membrane hyperpolarization, and the next burst is delayed until the absorbance has almost declined to its normal baseline value. Records (C) and (D) are from the same cell and share the same calibration. (D) Effect of voltage-clamping R15 to the average value of the resting potential. The dye absorbance (upper trace) declines below its normal baseline value (dashed line) at the time when the next burst is expected. The next burst occurs immediately on release from the clamp, and the frequency of action potentials is somewhat higher than normal.

sociated with each burst were greatly reduced by lowering the external Ca^{2+} concentration from 10 m*M* to 1 m*M* and by saline solutions with normal Ca^{2+} (10 m*M*) containing 10 m*M* Mn^{2+} or Co^{2+} (*14*). These results suggest that much or most of the increase in the free internal Ca^{2+} concentration occurs as a result of influx of Ca^{2+} rather than release from an intracellular store. From an examination of recordings made at higher gain and speed (Fig. 2A), it is clear that Ca^{2+} entry occurs primarily in steps coincident with each action potential (the steps appear slightly rounded in the record, as a 1-hertz low-pass filter was used to reduce the contribution of low-frequency optical noise generated by the light source). The absorbance changes that occurred when the depolarizing waves did not give rise to action potentials were considerably smaller, whereas tetraethylammonium ions (which greatly prolonged each action potential) caused a much larger absorbance increase with each action potential.

We were able to calculate the amount of dye injected by measuring the absorbance increase at 570 nm after injection, and, from in vitro dye calibration data obtained with Ca-EGTA buffers (*15*), to estimate the changes in intracellular Ca^{2+} to which the experimental absorbance changes correspond. The absorbance increase during the burst corresponds to an intracellular Ca^{2+} increase of $6 \times 10^{-8} M$ (Fig. 1). We observed that absorbance increases also occur when R15 is depolarized under voltage clamp (*16*), and preliminary experiments to find the reversal potential of this response suggested an internal Ca^{2+} concentration of less than $10^{-7} M$. The increase during the burst is, therefore, a significant fraction of the baseline Ca^{2+} concentration.

Arsenazo III is not absolutely specific for Ca^{2+}. However, of the other ions to which it is sensitive (*17*), only Mg^{2+} is present at a significant concentration in the cell, but the dye is considerably less sensitive to Mg^{2+} than to Ca^{2+} (*10*). The absorbance spectra of the Mg^{2+}- and Ca^{2+}-arsenazo complexes are sufficiently different (*11*) for it to be possible to discriminate between them. This was a useful feature of the dye, because it allowed us to demonstrate that there was no significant change in intracellular Mg^{2+} during the burst.

When the dye binds Ca^{2+}, the absorbance increases maximally at two wavelengths, 650 and 600 nm, with a minimum at 630 nm. At wavelengths shorter than the isosbestic wavelength of the dye at 570 nm, there is a decrease in absorbance. When the dye binds Mg^{2+}, there is also a decrease in absorbance at wavelengths shorter than 570 nm, but the increase at longer wavelengths shows only a single broad peak, centered at 610 nm (*11*). Thus, it is possible to discriminate between the formation of the Ca^{2+}- and Mg^{2+}-arsenazo complexes by measuring the absorbance change simultaneously at two wavelengths longer than 570 nm, since the absorbance increase at one wavelength relative to that at the other depends on which complex is formed. We chose wavelengths of 630 and 650 nm for this test (the absorbance change at each wavelength was measured differentially with respect to the 570-nm isosbestic wavelength). We made simultaneous absorbance measurements at these wavelengths during the burst, and compared the result with the effects of Ca^{2+} and Mg^{2+} injection. When Ca^{2+} was injected, the absorbance increase at 630 nm was only 72 percent as great as the increase at 650 nm. In contrast, the absorbance increase after Mg^{2+} injection was 22 percent greater at 630 nm than at 650 nm. During the burst, the absorbance increase at 630 nm was only 69 percent as great as the increase at 650 nm. The very good agreement of the absorbance change during the burst with that observed on Ca^{2+} as opposed to Mg^{2+} injection (within the error of measurement) suggests that it represents an essentially pure Ca^{2+} change.

Injections of Ca^{2+} that caused absorbance increases comparable to those observed during the burst were also sufficient to hyperpolarize the membrane, and the recovery of membrane potential had a time course similar to the recovery of the absorbance change. This correspondence is seen more clearly in a voltage-clamped cell, where the time course of the outward current induced by Ca^{2+} injection is very similar to the time course of the absorbance change (Fig. 2B). (In contrast, Mg^{2+} injections that caused absorbance increases comparable to those observed during the burst had no significant effect on the membrane potential, and the absorbance increases recovered five to six times more slowly than those observed for Ca^{2+} injection or during the burst.)

As R15 has a resting membrane resistance of several megohms, we calculate that the Ca^{2+} dependence of the outward current (Fig. 2B) is sufficient to account for the hyperpolarization following the burst (*18*). Further evidence for the in-

volvement of intracellular Ca^{2+} in the hyperpolarization is shown by passing a current during the burst (Fig. 2C). A depolarizing current of 20 na approximately doubled the number of action potentials during the burst and caused a comparable absorbance increase. The hyperpolarization that followed was larger and of longer duration, and the onset of the next burst was delayed until the absorbance had almost returned to its baseline value. This result suggests that the onset of the burst is determined by the fall of the free intracellular Ca^{2+} concentration below some threshold value. When the cell was voltage-clamped after a burst to the average value of its resting potential, the absorbance declined below its baseline value at the time when the next burst was expected (the interval between bursts in R15 is normally quite regular). The next burst occurred immediately after the cell was released from voltage clamp; the frequency of action potentials was somewhat higher than normal, an effect that can be attributed to the internal Ca^{2+} concentration being lower than normal at the start of the burst.

Our experiments demonstrate that changes in intracellular Ca^{2+} play a fundamental role in bursting pacemaker activity. They suggest that the interval between bursts depends on the rate at which free intracellular Ca^{2+} is sequestered or transported out of the cytoplasm. They also suggest, however, that at least one other factor is involved in the generation of the burst. If activation of an outward current by intracellular Ca^{2+} were the only factor, the membrane potential would hyperpolarize during the burst as a result of the increase in intracellular Ca^{2+} concentration, and this is clearly not the case (Fig. 1). The delay in onset of the hyperpolarization until the end of the burst could be due either to inhibition of the Ca^{2+}-activated outward current or to antagonism by a maintained inward current during the burst. Experiments on analogous cells in the mollusk *Helix* (*19*) suggest that the second explanation is more probable, as they provide evidence for a voltage-dependent inward current, showing slow activation and inactivation, which is carried primarily by Ca^{2+} ions.

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14. The magnitude of the membrane potential oscillation associated with each burst was also reduced by treatment with low external Ca^{2+} or by saline solutions containing Mn^{2+} or Co^{2+}. However, at the concentrations used, these treatments did not completely abolish the absorbance or membrane potential changes.
15. The dye was calibrated at 16°C in the presence of 500 m*M* KCl and 2.5 m*M* $MgCl_2$, *p*H 7.3, to match intracellular conditions. Di Polo *et al.* (*11*) give the effective Ca-EGTA/EGTA dissociation constant at this *p*H and ionic strength as $1.5 \times 10^{-7} M$; the additional presence of 2.5 m*M* $MgCl_2$ is not expected to increase it significantly. The calibration was linear over the range studied (0 to $4.5 \times 10^{-7} M$ free Ca^{2+}, based on the dissociation constant above). Although we believe that our calibration gives a resonable estimate of changes in free intracellular Ca^{2+}, it should be realized that there are several potential sources of error in attempting to match in vitro conditions with those in the cell, so the values given can be only approximate.
    We should stress that our dye calibration is based on changes in *free* intracellular Ca^{2+}. The corresponding changes in concentration of the Ca-dye complex (which can be calculated from the molar extinction coefficient of the complex) are somewhat higher, but the presence of the dye at the concentration used in these experiments is unlikely to increase significantly the total intracellular buffering power toward Ca^{2+}. The increase in *total* intracellular Ca^{2+} during the burst may be many times higher than the increase in free Ca^{2+}, but physiologically the free Ca^{2+} level is expected to be the more important parameter.
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Sesquiterpene Antitumor Agents: Inhibitors of Cellular Metabolism

Abstract. *Helenalin and tenulin injected into CF_1 male mice bearing Ehrlich ascites tumors inhibit DNA synthesis and DNA polymerase enzymatic activity in the tumor cells. Helenalin inhibited protein synthesis. Both drugs increased the concentration of adenosine 3',5'-monophosphate, and interfered with glycolytic and mitochondrial energy processes. Cholesterol synthesis was also inhibited, resulting in lower serum cholesterol levels in tumor-bearing animals. Data obtained in vitro indicate that the cyclopentenone-bearing sesquiterpene lactone and related compounds do not alkylate purine bases of nucleic acids but rather undergo a Michael-type addition reaction with the sulfhydryl groups of reduced glutathione and* L-*cysteine. Thus, the inhibition of cellular enzyme activities and metabolism that has been observed with these drugs might be explained by the occurrence of a Michael-type reaction.*

Helenalin (**1**), a sesquiterpene lactone, has been shown to be a potent antitumor agent in the Walker 256 ascites carcinosarcoma and Ehrlich ascites tumor, and marginally active in the P-388 lymphocytic leukemia screen (*1–3*). Its cytotoxic activity has been delineated in the HEp-2 cell line. Studies of the relation between the structure and activity of this compound have established that one of the structural requirements for significant cytotoxic antitumor activity is that the $O{=}C{-}C{=}CH_2$ moiety be part of an ester or part of a ketone or lactone (*4, 5*). Kupchan has demonstrated that the α-methylene-γ-lactone system can act as the alkylating center for cytotoxic antitumor lactones, for example, elephantopin, vernolepin, and euparotin acetate. A Michael-like reaction between biological nucleophiles, such as L-cysteine (*6*) or sulfhydryl-containing enzymes, for example, phosphofructokinase (*7*) and glycogen synthetase (*8*), was proposed. The $O{=}C{-}C{=}CH_2$ system in a ketone unit, for example, the β-unsubstituted cyclopentenone moiety, might act as an essential alkylating center, particularly since the α-methylene-γ-lactone was observed to be less important than the cyclopentenone