increase of lamella type granules in PTZ-incubated neurons.

Figure 2 shows the results of the calcium analysis carried out with the energy-dispersive type electron probe microanalyzer. The left parts of Fig. 2, A and B, show the electron micrographs of freeze-dried thin sections of normal and PTZ-treated cells, respectively. Because of freeze-drying and the absence of staining, the electron microscopic image was not good, but the granules were recognizable. The PTZ-treated granules were less dense than normal granules (Fig. 2B). The accelerating voltage was 60 kV, and the analysis was performed on several granules in each specimen. At the same time, several surrounding spots where there were no visible intracellular organelles were analyzed in the same manner, and no detectable calcium was found. The normal granules have a considerable amount of calcium (right part of Fig. 2A), and the PTZ-treated granule no longer had detectable calcium (right part of Fig. 2B); these results suggest that PTZ released the calcium previously bound to the granules. Henkart (*9*) and Brown *et al.* (*10*) demonstrated that the pigmented granules of the *Aplysia* neuron release calcium and are converted from dense granules into lamella type granules after illumination. We also compared the ganglia illuminated to those prepared under dark conditions. The preparation was performed in daylight under a fluorescent lamp (Toshiba FLR-40 SW tube) positioned about 0.5 m from the ganglia. This light supplied about 1000 lux over a wavelength range from 400 to 750 nm. The temperature of the solution bathing the ganglia was about 22°C. The results showed that the $L/L + G$ ratio of the illuminated ganglia is increased but far less than after PTZ treatment (middle column of Fig. 1C).

These results demonstrate that PTZ releases the intracellular calcium bound to the dense lysosome-like granules and converts them into lamella type granules. Our PTZ-induced ultrastructural change and release of calcium bear a remarkable resemblance to the light-induced change in the *Aplysia* neuron (*9*, *10*).

The bursting neurons of *Helix pomatia* and *Aplysia* show a voltage-dependent, slow inward current in the steady-state voltage clamp current-voltage curve. This is called the negative slope region or negative resistance characteristics (*3-6*). Electrophysiological analysis of this current has been carried out (*4-6*). Smith *et al.* (*4*) demonstrated that this current is carried by sodium ions, whereas Eckert and Lux (*5*) proposed that it is carried by calcium ions. Johnson (*6*) proposed the possibility of a responsive carrier of both sodium and calcium ions. We carried out additional experiments on the preparation of a contour map of sodium ions in normal and PTZ-treated neurons by the method described elsewhere (*7*). No clear change in the sodium distribution pattern was found between the normal and PTZ-treated neurons. Several additional experiments with a nondispersive type electron probe microanalyzer in the area where there were no organelles showed no definitive change in sodium. As previously demonstrated (*7*), the intracellular calcium scattered in the cytoplasm is concentrated in the cell membrane by PTZ treatment. This newly concentrated calcium in the cell membrane showed a different type of chemical shift of the x-ray wavelength, which means a new binding state of calcium occurs within the membrane (*11*). On the basis of the present findings together with earlier data (*7*), we believe that the bursting activity caused by PTZ can be explained as follows: PTZ acts on the intracellular granules and releases the bound calcium; the released calcium binds to the cell membrane structure and modifies the ionic channels and induces the bursting activity. The determination of the identity of the carrier ions responsible for the negative resistance characteristics of bursting activity will require further research. In any case, the calcium release from intracellular granules and the movement of the released calcium toward the cell membrane is the essential step required to induce the bursting activity of neurons.

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Ion Shower Milling: Its Application to Cell Membrane Removal

Abstract. *A thickness of about 100 angstroms of the cell membrane of an isolated single freeze-dried neuron of the snail can be etched off with an ion shower milling machine. The calcium content of the cell membrane area was more than one-fifth of the whole cell. The calcium content of the cell membrane area increased during pentylenetetrazole-induced bursting activity.*

In the nerve cell, the combination of ions and the cell membrane structure plays an important role. The ions bound to the membrane, however, cannot be measured separately, and it is desirable to measure various substances in the cell membrane and cytoplasm separately. For this purpose, only fractionation by the density gradient method has been used.

The ion shower milling machine has been used to etch thicknesses of several hundreds of angstroms off the surfaces of metals, glass, and organic polymers, and this machine has been of great value in the etching of transistors and integrated circuits. The ion shower milling machine was first applied to biological specimens by Lewis *et al.*, and they found a submembranous structural difference between normal erythrocytes and those that are characteristic of sickle cell anemia (*1*).

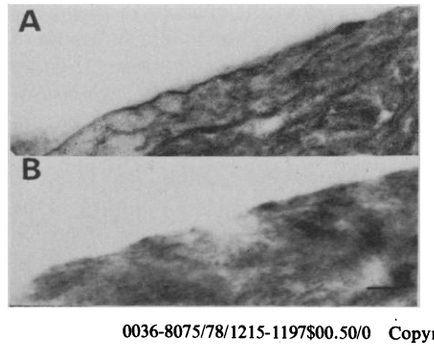

Fig. 1. Electron micrograph of a freeze-dried cell surface of the snail neuron (A) before etching of the cell surface and (B) after removal of the cell membrane area by the ion shower milling machine. Scale, 0.1 μm.

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We describe here the application of the ion shower milling machine to remove the cell membrane of the single isolated neuron. We also measured the content of calcium ions in the normal neuron and that during bursting activity induced by pentylenetetrazole (PTZ) both for the whole cell and for the cell with the membrane removed.

We used the D neurons of the subesophageal ganglion of the Japanese land snail, *Euhadra peliomphala*, in the active season. Two groups of dissected ganglia were prepared, one of which was incubated in normal snail Ringer solution and the other in snail Ringer solution containing $5 \times 10^{-2} M$ PTZ. After 10 minutes of incubation, both groups were quickly frozen by being dipped in a liquid Freon 12 pool surrounded by solid Freon 12 cooled by liquid nitrogen and freeze-dried without passing through a liquid phase at a temperature below −35°C. We also made some freeze-dried specimens at a temperature below −80°C, but the results were the same. The D neurons were dissected from the freeze-dried ganglion with fine forceps under a binocular microscope. Each freeze-dried neuron was cut into two hemispherical shapes under the microscope, and half of each group was placed on the ion shower stage. The other half was not etched.

We used the Kaufman type ion shower milling machine (Elionix ISM-S) and carried out ion etching for 3 minutes in argon at a pressure of 6×10^{-5} torr. The ion-accelerating voltage was 500 V, and the ion current was 200 μA. In order that the surface of the neuron be etched evenly, the sample stage was inclined to 45° and rotated both around the vertical axis and the stage axis during ion etching. The etched and nonetched D neurons were weighed with a fish-pole balance placed in a transparent plastic box with manipulating windows which was located in a room kept at a constant temperature (20°C) and constant humidity (40 percent) according to the microtechnique of Lowry and Passonneau (*2*). The mean weight of a freeze-dried D neuron was 93.6 ± 9.8 ng (mean ± standard deviation, $N = 31$). The weight of an ion-etched neuron was almost the same as that of a nonetched neuron.

Figure 1 shows the electron micrograph of the cell surface of a freeze-dried normal neuron (A) and an ion-etched neuron (B) prepared by the conventional Epon embedding method. The ultrastructure of the ion-etched neuron was not as well preserved as that of the normal neuron because of freeze-drying but the cell membrane was completely removed. Figure 2 shows the calcium content of normal cells and ion-etched cells with the membranes removed measured by a flameless atomic analyzer (JEOL JAA-7000; sensitivity, 0.5 part per trillion) for both normal and PTZ-treated cells. The difference between the whole cell calcium content of a neuron dipped into normal snail Ringer and that of a neuron dipped into calcium-free Ringer was negligible. This demonstrates that more than one-fifth of the whole cell calcium is distributed within the membrane area. In the case of PTZ-treated cells, the calcium content of the cell membrane area was considerably increased compared with normal cells, as shown in Fig. 2 (*3*).

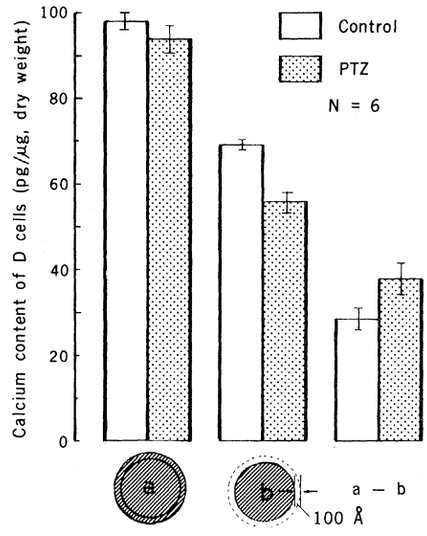

Fig. 2. Calcium content of normal and PTZ-treated neurons. The pair of bars at the left shows the whole cell calcium content, the middle bars show the calcium content after removal of the cell membrane area (about 100 Å), and the bars at the right are calculated values of the calcium content of the cell membrane area.

These findings demonstrate that the calcium accumulates in the cell membrane area during bursting activity induced by PTZ. The ion shower milling machine provides a promising technique for cell membrane analysis.

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3. A question may arise about the possibility of the redistribution of ions or molecules during the ion shower milling procedure. We examined this possibility by carrying out the following experiment. Two groups of several cells each were prepared: one was used as a control, and the other was ion-showered under the same conditions as we used in this experiment. The distribution pattern of calcium on the inner surface of the hemispherically cut cells of both groups was analyzed by computer-controlled ion distribution mapping (*4*). No change in the distribution pattern was found. One may question whether a change in the calcium distribution may occur during the freeze-drying. If the ion distribution change came from freeze-drying, the D cells and I cells would show the same change tendency under the same procedure. However, after the same freeze-drying procedure the calcium distribution map of the PTZ-treated D and I cells showed completely different distribution patterns, as described in (*4*).
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5. We thank M. Hotta for help in manipulating the ion shower milling machine.

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The Pineal Gland: A Biological Clock in vitro

Abstract. *Circadian rhythmicity was studied by following the course of* N-*acetyltransferase activity in the pineal glands of chickens in vitro. The results indicate (i) a daily change during day 1 of organ culture in constant dark that was dependent on the time the chickens were killed, (ii) equivocal persistence of the daily change in constant dark during 6 to 7 days of organ culture, (iii) an effect of light, (iv) inhibition by adrenergic agents and cycloheximide, and (v) stimulation by dibutyryl adenosine 3′,5′-monophosphate and related compounds.*

Serotonin *N*-acetyltransferase in the pineal gland of the chicken takes part in the conversion of the indole serotonin to the pineal hormone melatonin (*1*). There are circadian rhythms in melatonin production, and these are believed to be regulated by a circadian rhythm in serotonin *N*-acetyltransferase activity (NAT) (*2*, *3*).

The daily rhythms seen in NAT in the pineal gland are thought to be of special importance for the following reasons. (i) The pineal gland has been established as the source of the circadian rhythm in sparrows by investigators using ablation, transplant, and hormone replacement techniques (*4*); (ii) pineal gland NAT has in vivo a circadian rhythm that is precisely timed and regulated by light and dark (*2*); and (iii) the NAT rhythm has been observed to reach a peak in the dark time in all species of mammals and birds so far studied, irrespective of whether the species was nocturnal or diurnal in behavior (*5*).

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