both trypan blue exclusion and hydrolysis of fluorescein diacetate: 76 ± 5 percent of the cell fluoresced and 81 ± 6 percent excluded trypan blue. We then plated $4.4 \pm 0.3 \times 10^5$ viable cells by trypan blue exclusion (4.1×10^5 cells by hydrolysis of fluorescein diacetate). After 24 hours there were $4.2 \pm 0.1 \times 10^5$ attached cells. Therefore, these three assays gave closely correlated measures of the viability of cultured hepatocytes. In addition, the cultured hepatocytes were treated with A23187 (10 μg/ml) in the presence or absence of Ca^{2+} (Table 1). The cells were assayed for viability after 1 hour by trypan blue exclusion and fluorescein diacetate hydrolysis. In the presence of Ca^{2+}, 20 ± 3 and 21 ± 2 percent of the cells were found to be viable by the two methods, respectively. In the absence of Ca^{2+} the respective viabilities were 101 ± 8 and 101 ± 6 percent.

Our data give new insight into the mechanisms of toxic cell death. We show that the killing of rat liver cells in culture by the ten toxins studied involves at least two clearly definable steps. The first step represents a disruption of the integrity of the plasma membrane and is independent of extracellular calcium. Despite the widely differing mechanisms by which this injury is produced, there is a common functional consequence in what follows. The second step is dependent on extracellular calcium and most likely represents an influx of Ca^{2+} across the damaged plasma membrane and down a steep electrochemical gradient. This step represents, or at least initiates, a final common pathway by which the cells are killed. Recognition of this pathway has several important implications. First, it indicates that toxic cell death is ultimately a specific consequence of a disturbance in intracellular Ca^{2+} homeostasis and not some nonspecific slowing down of cellular metabolism or generalized disruption of membrane function. Second, attempts to specifically interrupt Ca^{2+} fluxes could have significant therapeutic consequences. Finally, our model should serve to focus future studies, both in vivo and in vitro, on the membrane consequences of toxin-cell interactions and how these membrane alterations specifically affect Ca^{2+} permeability.

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Rapid Killing of Single Neurons by Irradiation of Intracellularly Injected Dye

Abstract. *A simple technique for rapidly killing all or part of single neurons consists of filling the cell with Lucifer Yellow CH and irradiating all or part of it with intense blue light. Such treatment kills the irradiated part of the cell within a few minutes. Adjacent cells are not affected.*

Many neurobiological and developmental questions could be answered by selectively destroying single cells within their surrounding tissue and determining the effects of that destruction. Various methods of damaging or killing single cells have been used to identify neuronal function and connectivity and to investigate the dependence of cell function on developmental lineage (*1*). A promising method for destruction of single neurons by intracellular injection of proteolytic enzymes has recently been reported (*2*). We report an alternative technique that has several important advantages over injection of proteolytic enzymes. We killed single cells by filling them with a photoabsorptive dye and irradiating the surrounding tissue with high-intensity light. This method (i) avoids contaminating the tissue with inherently toxic or lytic substances, (ii) kills in minutes rather than hours, and (iii) can kill only a portion of a cell, if desired, and permits identification (during the experiment) of that portion.

One of two preparations used was the stomatogastric ganglion of the California

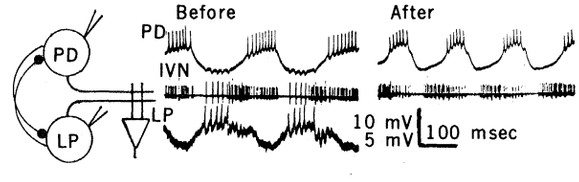

Fig. 1. The results of killing an LP neuron from the lobster stomatogastric ganglion. The diagram shows the reciprocal inhibitory connections between the LP and PD neurons as well as the axonal pathways that can be monitored extracellularly in the LVN. Recordings from a filled cell prior to irradiation show the spikes from the LP neuron on the LVN trace as well as discrete unitary postsynaptic potentials on the trace. After irradiation, the LP trace had depolarized off the screen; the extracellular LP spikes and the inhibitory postsynaptic potentials have disappeared, an indication that the LP cell has died.

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spiny lobster *Panulirus interruptus*. This ganglion contains approximately 30 neurons that interact via chemical and electrical synapses. Each cell is identifiable by its connectivity and function within the ganglionic circuit. The ganglion and its peripheral nerves were dissected out and pinned in a chamber filled with physiological saline solution. The individual neurons could be recorded from and stimulated intracellularly by using techniques reported in (*3*).

The cell to be killed was filled with the dye Lucifer Yellow CH by means of intracellular iontophoresis (*4*). The peak light absorption of this dye occurs at 426 nm (blue); it fluoresces brilliantly at 540 nm (yellow) (*5*). The dye itself had no measurable effects on the resting potential, input resistance, or shapes of action potentials and postsynaptic potentials in any of the cells we studied. To kill the dye-filled cell, the ganglion was irradiated with blue light of high intensity (*6*). Within 5 minutes, the membrane potential of the dye-filled cell depolarized to zero, all postsynaptic potentials to its follower cells disappeared, and after an initial period of high-frequency "damage discharge," action potentials were no longer measurable in the cell's peripheral axon.

The results of one such experiment are shown in Fig. 1. The lateral pyloric (LP) cell was filled with Lucifer Yellow. The activities of the LP cell and one of its follower cells, a pyloric dilator (PD) cell, were recorded by KCl intracellular microelectrodes. An extracellular electrode was placed on the lateral ventricular nerve (LVN), which contains their axons (along with several others). Before irradiation, the spontaneous activities of the LP and PD neurons were measured and the normal reciprocal inhibition was observed (*7*). The inhibitory postsynaptic potentials from the LP cell to the PD cell were particularly well defined. After the ganglion was irradiated for 3 minutes, the membrane potential of the LP cell had depolarized to zero (off the scale), the inhibitory postsynaptic potentials to the PD cell had disappeared, and action potentials from the LP were no longer measurable in the peripheral nerve. In such experiments, killing one cell disrupted the ganglion's output pattern by inactivating integral ganglionic circuit elements. However, neither the resting potentials, the input resistances, nor the spike and postsynaptic potential shapes were altered in other cells of the ganglion.

Lucifer Yellow is freely diffusible across gap junctions, which are the substrates of electronic synapses (*5*). The stomatogastric ganglion contains two strongly coupled PD cells (*7*). We performed several experiments in which one cell was injected with the dye and allowed to leak some into the electronically coupled cell for various lengths of time. When the ganglion was irradiated, the latter cell was damaged to an extent dependent on the amount of dye it had accumulated. But, when one PD cell was rapidly filled and immediately irradiated, no signs of damage were observable in its partner. Rather, an increase in the input impedance approximately equal to the coupling coefficient was measured, indicating that the cells uncoupled during the inactivation of the dye-filled cell.

Since Lucifer Yellow is not electron-dense, dye-filled processes could not be easily located with an electron microscope. Rather than filling the cells with an additional marker (which might have confused subsequent interpretations), we performed several experiments in which the lateral giant fiber (LGF) in the abdominal nerve cord of the crayfish *Procambarus clarkii* was used. Each fiber consists of a longitudinal chain of six axon segments coupled by gap junctions (*8*) and can easily be located in electron microscopy sections by its size and position in the cord. Lateral giant fibers that had been filled with Lucifer Yellow and irradiated until their resting potentials depolarized to zero showed complete dissolution of cytoplasmic structure (Fig. 2B). Control fibers that had either been irradiated or filled with Lucifer Yellow appeared no different from unfilled, nonirradiated fibers (Fig. 2A). Cells killed with this technique were monitored for as long as 18 hours after irra-

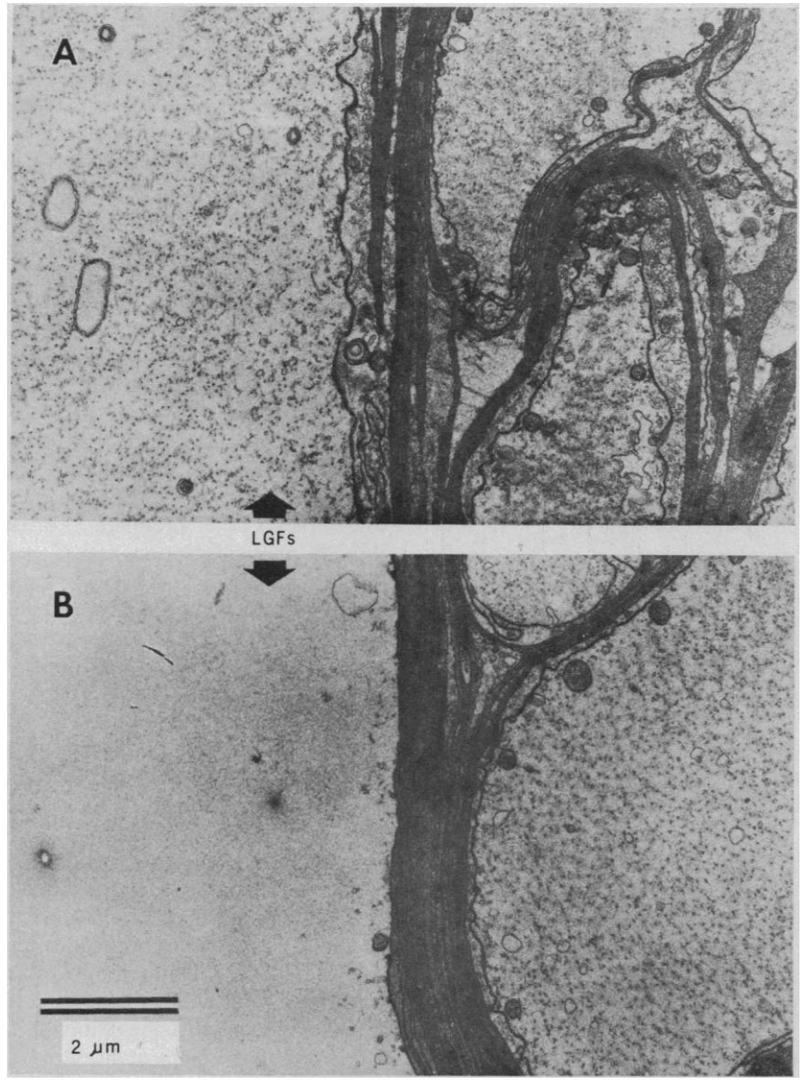

Fig. 2. Electron micrographs of crayfish LGF's. (A) An LGF filled with Lucifer Yellow but not irradiated. Note that it is identical in appearance to the small fibers to its right, which were neither filled nor irradiated. (B) An LGF filled, illuminated for 10 minutes at full-beam intensity, and fixed immediately. Note the dissolution of its cytoplasmic structure. To its right are smaller, unfilled fibers, which were irradiated but not filled; also compare with the control LGF in (A).

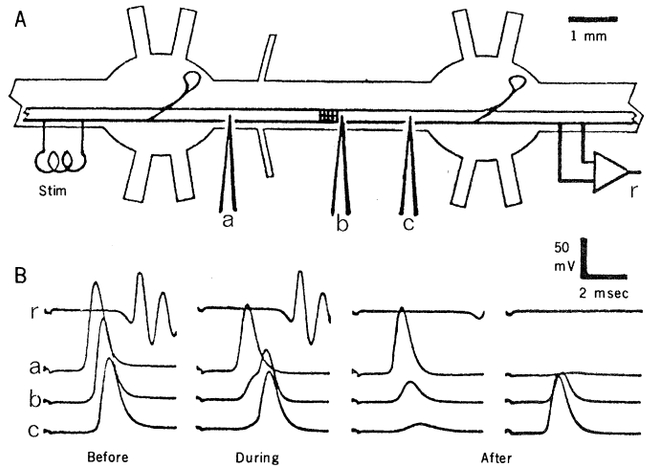

Fig. 3. The results of partial killing of a dye-filled lateral giant neuron from a crayfish. (A) Diagram of crayfish ventral nerve cord. Lateral dimensions are accurate. One segment of a lateral giant axon was recorded intracellularly at *a*, *b*, and *c*. Extracellular stimulating (*stim*) and recording (*r*) electrodes were placed on adjacent segments. The crosshatched area near electrode *b* was subsequently irradiated with blue light. (B) Extracellular (*r*) and intracellular (*a*, *b*, *c*) recordings from the axon immediately before irradiation, 5 minutes into the irradiation period, and immediately after it. For the last recording, we reversed the function of the extracellular electrodes and triggered spikes from the opposite direction.

diation; although the function of other cells in the same tissue remained perfectly normal during this period, no recovery of the inactivated cells was measured. Electron microscopy of such killed and monitored cells showed that cytoplasmic structure remained disintegrated. Moreover, breakdown of mitochondria and of the plasma membrane had begun. It should be noted, however, that a recovery time of 18 hours is developmentally brief. It remains to be tested whether or not such neurons could recover after days or weeks.

It was of interest to determine whether just part of a cell could be damaged. To test this, a neuron was required that could be filled with the dye and recorded from intracellularly at several widely separated sites. Here again the crayfish LGF was used (each axonal segment is approximately 100 μm in diameter and 6 to 8 mm long, allowing multiple recordings along its length). One axonal segment was filled iontophoretically with Lucifer Yellow. Then, as shown in Fig. 3, bipolar extracellular electrodes were placed on the nerve cord to either side of the dye-filled segment. Three KCl-filled intracellular microelectrodes were inserted at positions along the filled axon segment. Then a small portion of the axon segment was irradiated through a mask that was placed under the nerve cord. The segment was 6.7 mm long; an area 0.4 mm long near its center was irradiated (the crosshatched area in Fig. 3). Within 5 minutes, the action potentials began to broaden as they passed through the irradiated region. Within 7 minutes, active propagation through the irradiated region was completely blocked, at which time the irradiation was terminated. Now only a small, passively propagated potential from the nonirradiated region was measured here. To determine the viability of the axon on the other side of the irradiated region, the axon was stimulated at the other end (see Fig. 3B). This portion of the axon also conducted an action potential which was blocked in the damaged region. No depolarization was measurable at any of the electrodes when only this small area was illuminated. The axon remained in this condition for at least 1 hour, after which the recordings were terminated.

The mechanism by which irradiation kills a dye-filled cell is not known. There are, however, two likely possibilities. The first is the production of heat through photoabsorption; the second is the production of a toxic substance through photodecomposition of the dye. Heating must occur to some extent [indeed, other researchers have used it to damage or kill cells (*9*)]. If it is the principal killing mechanism, then any photoabsorptive dye would be expected to kill the cell upon intense irradiation. To test this prediction, lobster stomatogastric cells were filled with Fast Green J or Procion Rubine (both of these are vital dyes totally unrelated in structure to Lucifer Yellow CH, and they would not be expected to yield similar breakdown products). Cells were filled iontophoretically until made very dark in color and were then irradiated with a conventional tungsten microscope lamp at its highest intensity. Cells filled with either dye were killed after less than 5 minutes of irradiation.

Although many optically dense dyes might allow cell destruction by irradiation, two properties of Lucifer Yellow make it the dye of choice. First, the broad-spectrum photodensity of dyes such as Fast Green J render cells filled with them susceptible to damage under normal experimental illumination. Lucifer Yellow only absorbs visible light in the blue region, which can easily be filtered out of normal light without impairing visualization of the preparation prior to killing the cell. Second, the visibility of cells filled with Lucifer Yellow, even during the procedure, is much better than most other dyes give after fixation and clearing of the tissue. This allows visualization of the cells in situ.

This technique promises to greatly simplify experiments that require quick, efficient elimination of one cell from a complex neuronal circuit. Elimination of a fraction of a single neuron's dendritic tree may now be possible through a combination of this technique and microbeam irradiation. Questions concerning the correlation between neuronal structure and function can thus be addressed with a degree of refinement previously unattainable.

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6. Tissue was illuminated with a Zeiss 100-W mercury lamp through a large working-distance dark-field condenser. The beam was filtered through a Zeiss BG38 red absorption filter, a Zeiss BG12 exciter filter, and a Pomfret 426-nm interference filter. The beam intensity at the tissue, as measured with a YSI-Kettering 65 radiometer, was 2.5×10^7 erg/cm²-sec. With our microscope (Wild Heerbrugg M5 at ×100), a beam intensity of 0.85×10^4 erg/cm²-sec was just sufficient for us to see fluorescence of cell bodies and did not noticeably damage cells during a 30-minute illumination period. However, a beam intensity of 2×10^4 erg/cm²-sec, which was just sufficient for us to see fluorescence of neuropil processes, killed the cells within 30 to 40 minutes.
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