Previous research on urn cell complexes (*3, 4*) suggests that the mucus they release contributes to the host's protection against heterologous agents, including bacteria and other foreign cells. This report shows that one of the possible controlling mechanisms of mucus release could involve LTA and RCA_1-sensitive fucosyl and galactosyl residues of secretory receptors in the basal cell membrane of urn cell complexes. Internalization of these receptors (*12*) may trigger a cytoplasmic response leading to hypersecretion of mucus. This response may be intracellular acidification, although the possibility (*13*) that an altered *p*H may also induce the release of mucus-stimulating substances by other coelomic cells ("amebocytes") cannot be ruled out. Interestingly, increases in membrane fluidity and ionic permeability are among the earliest molecular events associated with lectin binding (*8*), and theoretically this phenomenon may influence the intracellular *p*H. Connections between intracellular *p*H and the turning on of cellular activities have been described in other systems (*12*). Some of the mucus-stimulating factors described in physiologic and pathologic serums, including poorly characterized macromolecular components (*4, 5*), antigen-antibody complexes, and cationic polypeptides (*6*), could interact with membrane receptors of mucous cells in a manner similar to that of lectins. Thus, lectins used as probes should be helpful in assessing the specificity of mucus-stimulating substances that operate under physiologic and pathologic circumstances.

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10. Fluorescein isothiocyanate-conjugated LTA and RCA_1 were obtained from Sigma Chemical Co. and used at a concentration of 200 μg/ml in FSW adjusted to *p*H 7.8. Formalin-fixed urn cell complexes were incubated for 10 minutes, rinsed three times in FSW, and observed under a Leitz Ortholux microscope, with the appropriate filters being used for fluorescein fluorescence. Controls contained $10^{-1}M$ L-fucose or D-galactose in staining and washing solutions. Photomicrographs were taken with Kodak Tri-X (ASA 400).
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Calcium Dependence of Toxic Cell Death: A Final Common Pathway

Abstract. *Primary cultures of adult rat hepatocytes were treated in the presence or absence of extracellular calcium with ten different membrane-active toxins. In all cases more than half the cells were killed in 1 to 6 hours in the presence but not in the absence of extracellular calcium. An effect of calcium on the primary mechanism of membrane injury by any of the agents cannot be implicated. Viability, as determined by trypan blue exclusion correlated well with other indices of viability such as plating efficiency and the hydrolysis of fluorescein diacetate. It is concluded that the cells are killed by processes that involve at least two steps. In each type of injury, disruption of the integrity of the plasma membrane by widely differing mechanisms is followed by a common functional consequence involving extracellular calcium, and most likely representing an influx of calcium across the damaged plasma membrane and down a steep concentration gradient. This later step represents, or at least initiates, a final common pathway for the toxic death of these cells.*

All cells in the body are bathed in a fluid very rich in Ca^{2+} ($10^{-3}M$), while intracellular Ca^{2+} concentrations are much lower, on the order of $10^{-6}M$ (*1*). The electrical potential across the plasma membrane of these cells tends to drive Ca^{2+} into them. Such a large electrochemical gradient is maintained by the relative impermeability of the plasma membrane to Ca^{2+} and by active extrusion. Damage to the plasma membrane by any one of a number of different mechanisms would disrupt this permeability barrier with a consequent influx of Ca^{2+}. Calcium ions are biologically very active, being capable of considerable disruption of metabolic order. Liver cells lethally injured by several quite different chemical toxins exhibit marked alterations in intracellular Ca^{2+} homeostasis with large accumulations of Ca^{2+} (*2*). Although consistent with an active role of Ca^{2+} in toxic liver cell death, such accumulations of Ca^{2+} could also be explained as simply the passive equilibration of Ca^{2+} concentrations in cells lethally injured by different and as yet unexplained mechanisms.

To explore more directly the role of Ca^{2+} in toxic cell death, we evaluated the dependence on extracellular Ca^{2+} of the toxic cell death of primary cultures of adult rat hepatocytes. The results (Table 1) show an absolute requirement for extracellular Ca^{2+} in the killing of these cells by ten different toxins which, although varying widely in chemical composition, share two characteristics. First, none of the agents seems to need metabolic activation to a proximate or ultimate toxin. Second, all of the ten compounds are capable of interacting with cell membranes; indeed, many are known hemolysins. The compound A23187 is a Ca^{2+} ionophore whose specific biological activity is to create Ca^{2+} channels and thereby overcome the permeability barrier represented by plasma membranes (*3*). Lysolecithin is known to react strongly with membranes (*4*). It is a hemolysin (*5*) whose mechanism of action is related to its ability to solubilize the lipid components and thus induce a change in the molecular organization of membranes (*6*). Amphotericin B is a polyene macrolide antibiotic that interacts specifically with sterols in cell membranes (*7*). Complexes of amphotericin B and sterol molecules are believed to form hydrophilic pores in the membranes, allowing increased ion permeability. Melittin is the main cytolytic component of bee venom (*8*). The first 20 amino acids of this peptide are mostly apolar, whereas the C-terminal hexapeptide is polar and highly basic (*9*). Melittin interacts with phospholipids both in liposomes and in biomembranes (*10*), causing a decrease in the mobility of the fatty acid chains and a rearrangement of the phospholipid head groups. Phalloidin is a bicyclic heptapeptide isolated from the toxic mushroom *Amanita phalloides* (*11*). It is a very specific hepatotoxin

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whose mechanism of action is related to its ability to interact with actin microfilaments intimately associated with liver cell plasma membranes (*12*). Methylmethanesulfonate (MMS) and ethylmethanesulfonate (EMS) are methylating and ethylating agents, respectively. Their reactivity results in the alkylation of many tissue macromolecules including membrane constituents. Both MMS and EMS are widely used mutagens. *N*-Acetoxyacetylaminofluorene (*N*-acetoxy AAF) is a reactive derivative of the arylamine hepatocarcinogen acetylaminofluorene (AAF) capable of arylamidating a variety of cellular constituents. It is both mutagenic (*13*) and carcinogenic (*14*). Lastly, silica and asbestos are mineral dusts which are potent hemolysins and cytotoxic to macrophages (*15, 16*). Silica is thought to form hydrophobic bonds with membrane phospholipids; asbestos interacts with surface sialic acid residues.

The data in Table 1 indicate that in a Ca^{2+}-containing medium each of these agents kills, in virtually all cases, at least half of the cells, as measured by the failure of trypan blue exclusion. In the absence of added Ca^{2+} in the culture medium (in a total Ca^{2+} concentration of less than 20 μM by atomic absorption spectrophotometry, including Ca^{2+} bound to medium constituents so that the free-Ca^{2+} concentration is actually much lower) the same compounds are not toxic. This absence of cell death in Ca^{2+}-free medium does not seem to represent simply a slowing down of the expression of the toxic response. Although the hepatocytes cannot be maintained in Ca^{2+}-free medium for much longer than 12 to 18 hours, there is no loss of viability in the presence of the ten toxins for at least this length of time. Trypan blue uptake is not dependent on Ca^{2+}, as shown by the staining of 10 to 15 percent of the freshly isolated hepatocytes suspended in Ca^{2+}-free medium. A Ca^{2+} gradient across the plasma membrane is therefore necessary for expression of the toxicity of these widely differing agents. The dependence of the toxicity of the ionophore A23187 on this gradient is a direct demonstration of the lethal consequences of an influx of Ca^{2+} across the plasma membrane. Furthermore, the Ca^{2+} dependence of A23187 also argues that Ca^{2+} is directly related to cell death and not merely facilitative, because the only known action of A23187 is to move Ca^{2+} across membranes. However, it is not known whether Ca^{2+} is solely responsible for the cell death or if it simply initiates other changes that kill the cells. The simplest interpretation of the Ca^{2+} dependence of the toxicity of the other nine agents is that in each case the toxin causes disruption of the permeability-barrier function of the plasma membrane, allowing a similar, lethal influx of Ca^{2+} down the steep electrochemical gradient between the outside and inside of the cell.

An alternative interpretation is that each agent requires extracellular Ca^{2+} to produce membrane injury. It seems very unlikely, however, that the diverse toxin-membrane interactions produced by the ten compounds studied would all be dependent on extracellular Ca^{2+}. Furthermore, the lysis of red blood cells by lysolecithin (*5*), melittin (*10*), silica, and asbestos (*15*) occurs in Ca^{2+}-free medium (*7*). We have shown that the interaction of phalloidin with liver cell plasma membranes does not require extracellular Ca^{2+} (*17*). Finally, in this study hepatocytes treated with ^{14}C-labeled MMS in the presence or absence of Ca^{2+} in the culture medium showed no difference in the extent of methylation of the cells, as measured by trichloroacetic acid–insoluble radioactivity (data not shown).

If Ca^{2+} is not necessary for the disruption of membrane structure by these agents, our results indicate that Ca^{2+} is necessary to couple this injury to cell killing as measured by trypan blue exclusion. Is trypan blue exclusion a valid assessment of liver cell viability? We examined this question by comparing trypan blue exclusion with two alternative assays of the viability of cells in culture: plating efficiency and the hydrolysis of fluorescein diacetate (*18*). Freshly isolated hepatocytes readily attach to plastic surfaces, spread out, and remain viable for several days. The number of attached cells is a measure of the number of viable cells originally present. This value can be compared with the number of viable cells in the same original suspension as determined by trypan blue or the hydrolysis of fluorescein diacetate (*18*). Nonviable cells hydrolyze fluorescein diacetate but fail to retain the free fluorescein and therefore do not fluoresce. We assayed freshly prepared hepatocytes for

Table 1. Calcium dependence of toxic liver cell death. Isolated hepatocytes were prepared from the livers of nonfasted female Wistar rats (150 to 200 g) by the method of collagenase perfusion (*19*). Collagenase (250 ml; 100 U/ml; type 1, Sigma) in Hanks balanced salt solution (BSS), *p*H 7.4 (Microbiological Associates), was recirculated through the liver for 15 minutes at 37°C at a flow rate of 32 ml/min. A yield of (2 to 4) × 10^8 cells per liver with 85 to 90 percent viability (trypan blue exclusion) was obtained. Hepatocytes were plated in plastic multiwells (Costar) 1.6 cm in diameter at a density of 5 × 10^4 per square centimeter in Williams E (Flow) containing 10 percent inactivated (56°C for 10 minutes) fetal calf serum (Flow), garamycin (50 μg/ml), and insulin (0.02 U/ml) (complete Williams). After incubation at 37°C in a humidified atmosphere of 5 percent CO_2 and 95 percent air for 90 minutes to allow the cells to attach, the cultures were rinsed three times with warm Hanks BSS and incubated for 15 to 10 hours in complete Williams E. Before treatment the cells were rinsed four times with warm Ca^{2+}-free Hanks BSS and placed in Williams E with or without 3.6mM $CaCl_2$ and without serum in both cases. The cells were treated with the agents indicated at the doses and for the times that gave no loss of viability in Ca^{2+}-free medium and maximum killing in the presence of Ca^{2+}. Cell viability was assayed by trypan blue exclusion. Trypan blue (0.4 percent in 0.15 percent NaCl; Gibco) was added directly to the cultures at a final concentration of 0.01 percent. Within 10 minutes the number of attached cells that excluded the dye was counted, using a 10-mm^2 eyepiece grid in an inverted microscope at ×200 magnification. Trypan blue exclusion is expressed as a percentage of the number of unstained cells in untreated cultures. All measurements were made by counting three to five fields each in triplicate cultures. The A23187 (gift from R. L. Hamill, Eli Lilly Co.), lysolecithin (Sigma), EMS (Sigma), and *N*-acetoxy AAF (gift of D. S. R. Sarma) were dissolved in ethanol. Melittin (Sigma) and phalloidin (Boehringer Mannheim) were dissolved in Ca^{2+}-free Hanks BSS. The MMS (Eastman) was dissolved in H_2O. Amphotericin B (Fungizone, Squibb) was added directly to the cultures. Silica and asbestos (chrysotile type B) were added directly to the cultures. Silica and asbestos (chrysotile type B) were particles 2 to 5 μm in diameter obtained from the Pneumoconiosis Research Unit, Johannesburg, South Africa. They were suspended at 1 mg/ml in Ca^{2+}-free phosphate-buffered saline.

Treatment	Viability (%)	
	Medium plus Ca^{2+}	Medium minus Ca^{2+}
None	100 ± 3	101 ± 3
A23187 (10μg/ml for 1 hour)	6 ± 1	98 ± 5
Lysolecithin (15 μg/ml for 3 hours)	19 ± 1	97 ± 9
Amphotericin B (200 μg/ml for 3 hours)	30 ± 3	98 ± 3
Melittin (0.05 μg/ml for 6 hours)	46 ± 1	103 ± 6
Phalloidin (50 μg/ml for 1 hour)	38 ± 3	103 ± 5
Methylmethanesulfonate (100 μg/ml for 6 hours)	48 ± 8	99 ± 2
Ethylmethanesulfonate (200 μg/ml for 6 hours)	36 ± 6	106 ± 2
N-Acetoxyacetylaminofluorene (10 μg/ml for 6 hours)	58 ± 3	101 ± 3
Silica (100 μg/ml for 3 hours)	26 ± 9	100 ± 6
Asbestos (100 μg/ml for 3 hours)	45 ± 6*	104 ± 11

*Ca^{2+} concentration was 1.8 mM.

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

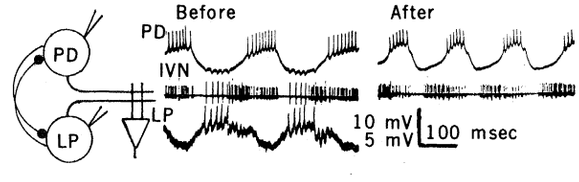

Fig. 1. The results of killing an LP neuron from the lobster stomatogastric ganglion. The diagram shows the reciprocal inhibitory connections between the LP and PD neurons as well as the axonal pathways that can be monitored extracellularly in the LVN. Recordings from a filled cell prior to irradiation 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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