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Cyclic GMP may serve as a second messenger in peptide-induced muscle degeneration in an insect

(cell death/*Antheraea polyphemus*/development/hormone action)

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ABSTRACT **At the end of metamorphosis, the intersegmental muscles of the moth *Antheraea polyphemus* undergo rapid degeneration in response to the peptide eclosion hormone (EH). Muscle death was preceded by a 22-fold increase in muscle guanosine-3′,5′-cyclic monophosphate (cGMP) titers, which peaked 60 min after peptide exposure; adenosine-3′5′-cyclic monophosphate (cAMP) titers remained unchanged. EH induced a dose-dependent increase in muscle cGMP content with a threshold dose similar to that needed to induce cell death. Exogenous cGMP, but not cAMP, mimicked the action of EH. Sodium nitroprusside, a potent stimulator of guanylate cyclase, and methylated xanthines, a class of 3′,5′-cyclic-nucleotide phosphodiesterase inhibitors, also induced the selective death of these muscles. It is concluded that an elevation of cGMP level is involved in EH-induced muscle degeneration. The intersegmental muscles become sensitive to EH at the end of adult development in response to the declining titers of the steroid molting hormones, the ecdysteroids. At earlier times, treatment with EH, exogenous cGMP, sodium nitroprusside, or methylated xanthines was ineffective in causing cell death. Nevertheless, treatment with EH at this time resulted in a marked increase in intersegmental-muscle cGMP. Thus, the onset of physiological responsiveness to the peptide hormone presumably results from biochemical changes distal to the EH receptors and guanylate cyclase.**

The actions of most peptide hormones are mediated by second messengers within cells (1). The two most widely studied second messengers are calcium and adenosine 3′,5′-cyclic monophosphate (cAMP). The role of another cyclic nucleotide, guanosine 3′,5′-cyclic monophosphate (cGMP), has proved to be more elusive and controversial (2–4). The best evidence for a biological role for cGMP comes from examinations of smooth muscle and rod photoreceptors. In both vascular and nonvascular smooth muscle, an increase in cGMP is thought to be involved in muscle relaxation (5–8). In the retina, cGMP appears to play a central role linking photon capture in rods with the subsequent reduction of the dark current (9–11). Pathological changes in cyclic nucleotide levels may also play a role in tissue dysfunction and disease (see ref. 12). For example, several studies have suggested that an abnormal increase in cGMP is responsible for spontaneously occurring retinal degeneration in both dogs and mice (13–15).

In order to better understand the potential role of cyclic nucleotides in cell death, we have studied the hormone-induced degeneration of the intersegmental muscles (ISM) of a moth. These muscles are a group of large embryonically derived muscles located in the third through the sixth abdominal segments in developing adult Lepidoptera. The ISM are last used by the animal during the process of adult emergence (eclosion), after which these muscles undergo rapid cell death (16–18). In the giant silkmoth *Antheraea polyphemus*, the death of these muscles requires first a decrease in the levels of ecdysteroids, the steroid molting hormones that promote adult differentiation, and then the appearance of the peptide, eclosion hormone (EH) (19, 20). In this study, we present evidence that the action of EH in triggering muscle death may involve cGMP as a second messenger.

MATERIALS AND METHODS

Animals. Diapausing male pupae of the giant silkmoth *A. polyphemus* were purchased from suppliers and stored at 4°C. Individuals were incubated at 25°C with a photoperiod of 17 hr of light:7 hr of darkness in order to break diapause and initiate adult development. Adult development in this species requires 17 days under these conditions. The progression of development was monitored by external markers as described by Walters (21).

Assays. In all experiments, animals were severed between the thorax and abdomen with a hemostat, and the head and thorax were discarded. Isolated abdomens prepared in this manner were viable and survived for several weeks. Typically, abdomens were isolated early on the last day (day 17) of development, which is prior to EH release from brain neurohemal sites. Thus, these abdomens are denied exposure to EH.

In some experiments, isolated abdomens were denervated after CO_2 anesthesia (22). A small patch of ventral cuticle was removed from over the ganglia and the abdominal central nervous system was removed through the incision. A crystal of phenylthiourea was placed in the wound to prevent blood blackening (23). The wound was then sealed with Tackiwax (Cenco, Chicago).

Fifty-microliter Hamilton syringes were used to inject EH or drugs that affect cyclic nucleotide metabolism into abdomens. Experiments designed to examine changes in muscle cyclic nucleotide content were conducted at 22°C; studies of muscle degeneration were done at 25°C.

At the completion of an experiment, the isolated abdomens were opened mid-dorsally, pinned under saline (24), and eviscerated, and the lateral ISM were removed from segments 4–6. To determine the extent of muscle degeneration, the ISM were placed on preweighed foil squares, dried for 24 hr at 60°C, and weighed to the nearest 5 μg on a Mettler M5 balance. Cyclic nucleotide determinations were made on tissue that had been immediately frozen on dry ice and stored at −80°C.

Frozen muscles were homogenized in 2.25 ml of ice-cold acidic ethanol (1 ml of 1 M HCl/100 ml of 100% ethanol) to

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Abbreviations: EH, eclosion hormone; *i*-BuMeXan, 3-isobutyl-1-methylxanthine; ISM, intersegmental muscles; PDEase, 3′,5′-cyclic-nucleotide phosphodiesterase.

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which a trace of ^{3}H-labeled cyclic nucleotide had been added to monitor recovery. After centrifugation at 750 × *g* for 20 min to remove precipitated protein, the pellet was washed with 750 μl of acid ethanol and centrifuged. The supernatants were pooled and dried under a stream of filtered air, and their cyclic nucleotide contents were measured by the method of Steiner *et al.* (25) using a radioimmunoassay kit (New England Nuclear). Recoveries of labeled cyclic nucleotides were 75–90%. All assays were done in duplicate. The tissue pellet was digested in 1 N NaOH and its protein content was determined by the procedure of Lowry *et al.* (26) using an ovalbumin standard.

EH Preparation. EH used in this study was extracted from the corpora cardiaca of developing adult *Manduca sexta* moths according to the protocol of Reynolds and Truman (27). The corpora cardiaca, the storage and release sites for the peptide, were homogenized in saline, heated to 80°C for 5 min, and centrifuged to remove precipitated protein. The supernatant was subjected to gel filtration through a Sephadex G-50 column with 0.1 N acetic acid as the eluant. The EH-containing fractions were lyophilized and further purified by narrow-gradient isoelectric focusing on polyacrylamide gels. Hormone was eluted from gel slices with 0.1 N acetic acid (containing 0.1% bacitracin to prevent adsorption of the hormone to the walls of containers). A unit of EH activity is defined as the amount of hormonal activity found in one pair of corpora cardiaca (27) from pre-emergent *Manduca sexta*.

RESULTS

Inhibition of 3',5'-Cyclic-Nucleotide Phosphodiesterases (PDEases). Inhibition of the cyclic-nucleotide-degrading enzymes, the PDEases, often mimics the effects of hormones whose actions depend on an increase in cyclic nucleotide levels (28). To determine whether inhibition of PDEases could mimic EH by inducing ISM breakdown, day-17-isolated abdomens were injected with either theophylline or 3-isobutyl-1-methylxanthine (*i*-BuMeXan) at various concentrations, and the state of the ISM was determined 24 hr later. Both drugs have been shown to inhibit PDEases from numerous sources (28, 29), with *i*-BuMeXan exhibiting about 15 times the potency of theophylline. Treatment with both theophylline and *i*-BuMeXan resulted in a dose-dependent breakdown of the ISM (Fig. 1). At the highest dosages used, the state of the muscles after 24 hr was the same as that after treatment with the peptide. *i*-BuMeXan evoked cell death at a concentration of ≈3 nmol per abdomen (≈3 μM in the hemolymph, assuming a hemolymph volume of 1 ml), while 0.2 μmol of theophylline was needed for the same effect (≈0.2 mM in the hemolymph).

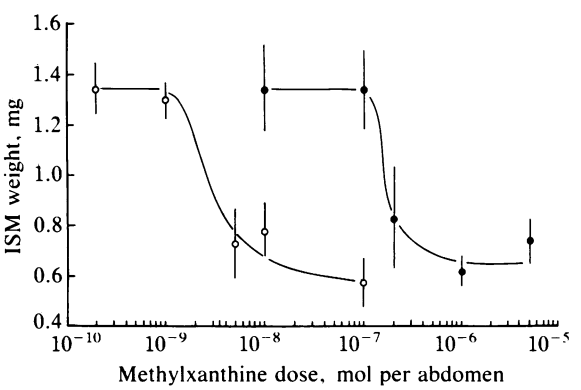

FIG. 1. Effects of the PDEase inhibitors theophylline (●) and *i*-BuMeXan (○) on the degeneration of the ISM of abdomens isolated from *A. polyphemus* on the 17th day of adult development. Muscle dry weights were determined 24 hr after drug injection. Muscles from untreated abdomens had a dry weight of 1.20 ± 0.10 mg (n = 4), while those from EH-treated abdomens weighed 0.46 ± 0.04 mg (n = 7). Values plotted are means ± SEM for five to seven abdomens per point.

Cyclic Nucleotide Induction of ISM Degeneration. The possible involvement of cyclic nucleotides in ISM degeneration was further examined by injecting isolated abdomens with various concentrations of either cAMP or cGMP and quantifying the extent of muscle degeneration after 24 hr (Fig. 2). Injection of cAMP, even at doses of 50 μmol per abdomen (corresponding to a hemolymph concentration of ≈50 mM), did not cause any loss of muscle mass. In contrast, injections of cGMP resulted in dose-dependent degeneration of the ISM, the threshold dose being about 0.5 μmol per abdomen (≈0.5 mM in the hemolymph). Maximal degeneration was induced by doses >5 μmol per abdomen. Thus, exogenous cGMP, but not cAMP, induced ISM degeneration in isolated abdomens.

EH-Induced Changes in Muscle Cyclic Nucleotide Concentrations. Two units of EH was injected into isolated abdomens and the ISM was removed for cyclic nucleotide determination at various times thereafter. EH exposure evoked only minor fluctuations in the cAMP content of the muscles (Fig. 3*A*). This lack of change was consistent with the inability of exogenous cAMP to evoke muscle degeneration.

In contrast to the meager changes in the cAMP levels in EH-treated muscles, there was a dramatic increase in the cGMP content of muscle after hormone administration. The concentration of cGMP within the muscles began to increase within 5 min of EH injection (Fig. 3*B*). It peaked after 60 min at a concentration 22 times the basal level and then slowly decreased, so that 3.5 hr after injection the concentration was still 10-fold greater than the basal concentration.

The magnitude of the cGMP increase after EH treatment was dependent on the amount of peptide administered. As seen in Fig. 4, 0.1 unit or less of EH resulted in only small increases in muscle cGMP by 60 min after EH injection, whereas doses >0.1 unit evoked marked increases in the cGMP levels. We have found that 0.1 unit of EH is also the threshold dosage of peptide required to trigger muscle death (20).

Effects of Sodium Nitroprusside. Sodium nitroprusside is a potent stimulator of guanylate cyclase and, therefore, is useful in examining the potential roles of cGMP in biological processes (5, 6, 30). Injection of 0.25 μmol of this drug per abdomen resulted in extensive ISM degeneration. Saline-injected controls had muscles that weighed 1.20 ± 0.10 mg (n = 4) 24 hr after injection, while sodium nitroprusside-injected abdomens had an ISM mass of only 0.51 ± 0.10 mg (n

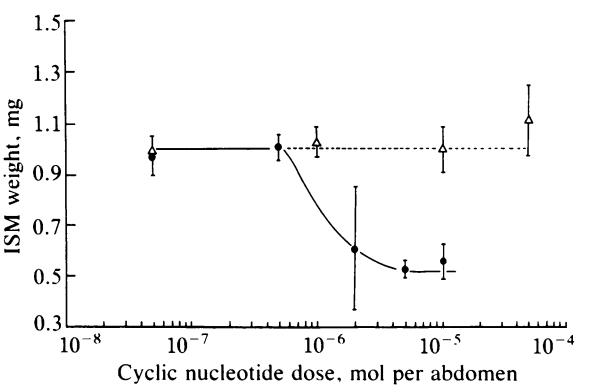

FIG. 2. Effects of exogenous cAMP (△) and cGMP (●) on the degeneration of the ISM of day-17-isolated abdomens. Muscle dry weights were determined 24 hr after injection and are plotted as the mean ± SEM for five or six abdomens per point.

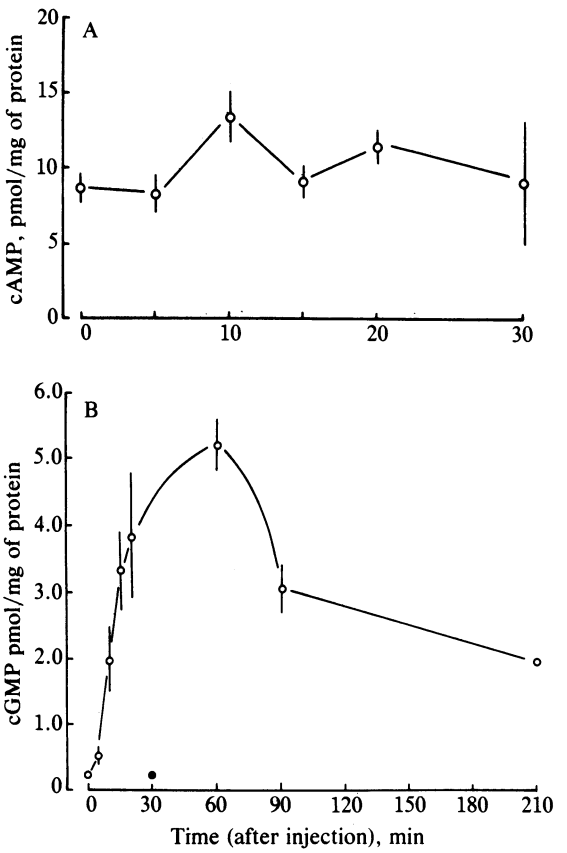

FIG. 3. Cyclic nucleotide levels in the ISM at various times after injection of 2 units of EH into day-17-isolated abdomens. (*A*) cAMP. (*B*) cGMP. Mean ± SEM for five abdomens per point after injection with EH (○) or saline (●).

= 8) and no contractile ISM. It should be emphasized that the alary, external, and cardiac muscles, all of which normally survive in the adult, were present and contractile in the treated abdomens. Thus, the effect of the drug was restricted to the ISM. It should be noted that doses of sodium nitroprusside >0.5 μmol per abdomen were toxic.

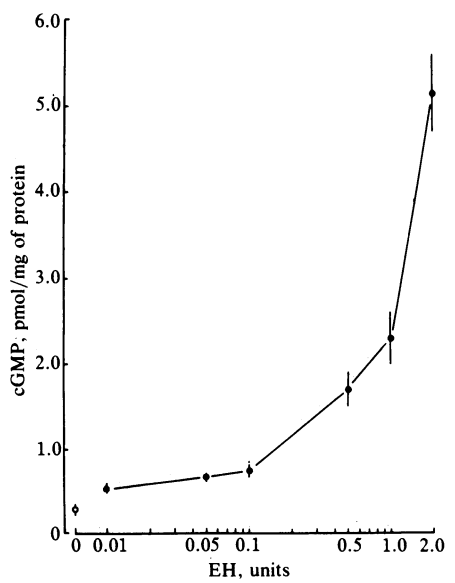

FIG. 4. Relationship between the dosage of EH injected into day-17-isolated abdomens and the titer of cGMP in their ISM. Muscles were removed 60 min after injection of EH. Mean ± SEM (n = 5) for EH-treated (●) and saline-treated (○) abdomens.

Relationship of the Cyclic Nucleotide Response to the Onset of Physiological Sensitivity. The ISM of *A. polyphemus* become competent to degenerate in response to EH only on the last day of adult development (day 17) (19, 20). Injection of EH on day 16, the day preceding eclosion, had no effect on the state of the ISM (Table 1). Doses of *i*-BuMeXan, cGMP, or sodium nitroprusside that induced ISM degeneration on day 17 had no effect on the muscles of day-16-isolated abdomens (Table 1). Thus, not only the peptide but also its putative second messenger were ineffective on day 16. This phenomenon was further examined by injecting 1 unit of EH into day-16-isolated abdomens and subsequently determining the levels of cGMP in their muscles. Muscles from day-16-isolated abdomens had basal levels of 196 ± 46 fmol of cGMP/mg of protein (n = 5). In response to EH treatment, the levels of cGMP increased 15-fold within 60 min to an average value of 3044 ± 361 fmol/mg of protein (n = 5). Thus on the 16th day of development, EH caused the normal increase in muscle cGMP even though it was incapable of evoking cell death. These data suggest that by day 16 the cellular receptors for EH are present and capable of effecting an increase in cGMP but that some subsequent biochemical step is inoperable.

cGMP Changes in Denervated Muscle. The ability of EH to induce ISM degeneration results from its direct action on the muscle, rather than an indirect effect mediated by the nervous system (20). Nevertheless, it was possible that the observed changes in muscle cGMP content in response to EH could have resulted from an indirect effect of the hormone on central nervous system activity. Indeed, it has recently been shown that glutamate, the insect neuromuscular transmitter, activates guanylate cyclase in insect muscle membranes (31). Consequently, we examined the effects of EH on denervated ISM. Isolated abdomens were denervated and injected with either saline or EH. The cGMP content of their muscles was measured after 60 min. Denervated ISM from the saline-injected group had endogenous cGMP levels of 455 ± 60 fmol/mg of protein (n = 7), whereas those from EH-treated abdomens (1 unit of EH per abdomen) showed cGMP levels of 3669 ± 498 fmol/mg of protein (n = 4). This 8-fold increase is of the same magnitude as that seen 60 min after undenervated isolated abdomens were treated with the same dose of EH (see Fig. 4). Therefore, the ability of EH to increase ISM cGMP content apparently results from a direct action on the muscle.

Table 1. Effects of various treatments on the degeneration of the ISM of abdomens isolated during the last 2 days of adult development

Drug	Dose	ISM weight, mg	% contractile
		Day 17	
None	—	1.20 ± 0.10 (4)	100
EH	1 unit	0.46 ± 0.04 (7)	0
i-BuMeXan	0.1 μmol	0.56 ± 0.06 (10)	10
cGMP	5 μmol	0.42 ± 0.06 (8)	0
NP	0.25 μmol	0.51 ± 0.10 (8)	0
		Day 16	
None	—	1.71 ± 0.14 (5)	100
EH	2 units	1.82 ± 0.20 (5)	100
i-BuMeXan	0.1 μmol	1.43 ± 0.13 (7)	100
cGMP	5 μmol	1.54 ± 0.07 (5)	100
NP	0.25 μmol	1.85 ± 0.14 (10)	100

Abdomens were isolated and injected with the indicated drugs on the 16th or 17th day of adult development. Dry weights of ISM 24 hr after injection are given as the mean ± SEM, followed in parentheses by the number of abdomens tested. NP, sodium nitroprusside.

DISCUSSION

Peptide hormones usually induce changes in intracellular concentrations of second messengers, which in turn initiate some changes in the physiological state of the stimulated cell. One of the major classes of intracellular second messengers is the cyclic nucleotides. More than a decade ago, four criteria were established for defining a system as being regulated by a cyclic nucleotide (32). Modified to include cGMP-mediated systems, they are (*i*) that the cell be capable of synthesizing the cyclic nucleotide in question, (*ii*) that inhibition of PDE either mimics or potentiates the action of the hormone, (*iii*) that exogenous cyclic nucleotide mimics the action of the hormone, and (*iv*) that an increase of the cyclic nucleotide concentration precedes the physiological response of the cell to the peptide.

We have examined the EH-induced degeneration of the ISM of *A. polyphemus* to determine whether cyclic nucleotides are involved in this phenomenon. The data presented in this report suggest that cyclic GMP, but not cAMP, is involved in this response. Examination of this system has shown that cGMP satisfies the four criteria above. First, although we have not directly assayed guanylate cyclase the presence of cGMP within the muscle and the ability of EH to increase the cGMP titer strongly indicate that the cyclase is present.

Second, injection of drugs that inhibit PDEases mimic EH by inducing ISM degeneration. The two methylated xanthines tested here, *i*-BuMeXan and theophylline, are potent inhibitors of PDEases in both vertebrate and invertebrate tissues (28, 29). *i*-BuMeXan has been shown to be a much more potent inhibitor of PDEases than theophylline (28); it was also much more potent in mimicking the actions of EH. Furthermore, crude preparations of PDEases from the ISM were inhibited by these agents (unpublished data).

Third, exogenous cyclic nucleotides can mimic the action of EH. In this system, however, it is cGMP, rather than the more conventional cAMP, that induces ISM degeneration. Injection of cGMP caused the death of these muscles in a dose-dependent manner, while cAMP, even at doses 100 times greater than cGMP, was without effect.

The final criterion, that hormone-induced increases in cyclic nucleotide precede the physiological response of the tissue, is also met in this system. Injection of EH into isolated abdomens had no effect on cAMP levels in the muscles, while the cGMP levels increased dramatically. This increase in cGMP levels is the earliest known change that occurs in the ISM after EH treatment and precedes by several hours the first electrophysiological change within the muscles, which is an apparent increase in the input resistance and a decrease in membrane capacitance (33). Further support for the involvement of cGMP in ISM degeneration is the observation that the threshold dose of EH necessary to cause the biological response of cell death is similar to that needed to increase cGMP concentration. Therefore, the observed increase in cGMP titers within the ISM was not due to unphysiological doses of the hormone but, rather, occurred with doses of hormone normally seen by the muscles at the time of eclosion.

EH acts on two other lepidopteran tissues besides the ISM: the central nervous system (34) and the wing epidermis (35). Injection of EH caused a dose-dependent increase in central nervous system titers of cGMP that preceded the onset of the eclosion behavior (36). Likewise, there was an accumulation of cGMP in isolated EH-treated wings (unpublished data). It therefore appears that all identified target tissues for EH exhibit an increase in cGMP that is associated with the action of the peptide.

Recently, the involvement of cGMP in the regulation of normal cell physiology has been questioned (2–4). One of the arguments used to suggest that changes in cGMP levels are not necessary for the physiological response with which they are associated is that pharmacological increases in cGMP with nitro-containing compounds do not mimic the actions of the hormones in question. Drugs such as sodium nitroprusside are potent stimulators of guanylate cyclase in cells (5, 6). This agent has been used to dissociate the rise in cGMP levels from the relaxation of vascular and nonvascular smooth muscle (3, 4, 37). We tested sodium nitroprusside and found that it could mimic EH and induce ISM degeneration. As indicated above, the ability of this drug to kill the muscles was restricted to only those muscles that normally die in response to the peptide. Although this observation strengthens the argument that cGMP is involved in ISM degeneration, certain cautions must be offered. First, doses >0.5 μmol of sodium nitroprusside were toxic to isolated *A. polyphemus* abdomens, and so the dose that was effective in causing selective ISM degeneration was almost enough to cause death. Second, Lincoln and Keely (38) have shown that, while both acetylcholine and sodium nitroprusside increase rat smooth muscle cGMP levels, only acetylcholine was able to increase cGMP-dependent protein kinase activity and thus bring about the anticipated muscle relaxation.

In several biological systems, activation of guanylate cyclase appears to require the entry of extracellular calcium (39). In one insect skeletal muscle in which the role of calcium has been examined, Robinson *et al.* (31) have shown that the neurotransmitter glutamate can stimulate guanylate cyclase in the absence of calcium in broken cell preparations. At present, we have no data concerning the calcium requirements of guanylate cyclase in EH-induced ISM degeneration.

The observation that cGMP is intimately associated with the death of the ISM provides a tool to study the development of hormone responsiveness in this system. During the last days of adult development, the endogenous titers of the ecdysteroids decrease (18–20). The withdrawal of this steroid renders the ISM competent to degenerate in response to EH on the last day of adult development. The onset of responsiveness does not appear to be due to the appearance of EH receptors on the muscles. Injection of EH into day-16 abdomens failed to induce ISM degeneration but nevertheless caused a dramatic increase in the cGMP titer. Furthermore, in experiments bypassing the EH receptors, injections of cGMP, *i*-BuMeXan, or sodium nitroprusside were ineffective at this stage of development. Taken together, these data suggest that some biochemical step, distal to both the receptor and the activation of guanylate cyclase, is subject to steroid regulation. Quite possibly, the decrease in steroid titer controls the appearance of either protein kinases or substrates. This developmental maturation of hormone responsiveness in the ISM may provide a powerful tool for delineating the biochemical pathway that ultimately leads to cell death.

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