Glucose oxidase prevents programmed cell death of the silkworm anterior silk gland through hydrogen peroxide production

Ca^{2+}-PKC-caspase 3-like protease pathway mediates DNA and nuclear fragmentation in ecdysteroid-induced programmed cell death

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Abstract

20-hydroxyecdysone (20E) induces programmed cell death in the anterior silk gland of the silkworm. Here, we report the involvement of a Ca^{2+}-protein kinase C (PKC)-caspase 3-like protease in the 20E signaling pathway. The calcium ionophore can mimic 20E effects in inducing DNA and nuclear fragmentation, but such mimicry is only possible in glands precultured for 18 h with 20E. The simultaneous presence of cycloheximide with 20E in the preculture showed that *de novo* gene expression was needed to mimic 20E effects by the calcium ionophore. Both a PKC inhibitor and a caspase 3 inhibitor inhibited the mimicking effects. After substitution of the calcium ionophore for 20E, caspase 3-like protease was fully activated 12 h later and DNA and nuclear fragmentation occurred faster than continuous 20E stimuli. The results show the presence of a Ca^{2+}-PKC-caspase 3-like protease pathway in 20E signaling, and possible involvement of the pathway up to the mobilization of Ca^{2+} in regulating the speed of cell death *in vivo*.

Keywords: calcium ionophore, caspase 3, ecdysone, programmed cell death, protein kinase C, nuclear fragmentation, DNA fragmentation.

Introduction

Programmed cell death is an important event in animal development. The steroid hormones estrogens, androgens, progesterone, and glucocorticoids regulate cell death in mammalian and invertebrate tissue cells, as well as in cultured cells (Herold et al., 2006). In insects, 20-hydroxyecdysone (20E), an insect steroid hormone, triggers degeneration of larval-specific tissues through programmed cell death. In holometaborous insects, the tissues are partly or entirely eliminated from pupal bodies at larval–pupal transformation (Chinzei, 1975; Truman et al., 1992; Terashima et al., 2000; Fahrbach et al., 2005; Yin and Thummel, 2005; Mane-Padros et al., 2008). In *D. melanogaster*, salivary glands degenerate and disappear from pupal bodies, for which the genomic action of 20E is indispensable for activation of the death activator genes, *rpr* and *grim*, which trigger the death sequence (Yin and Thummel, 2005). In addition to the genomic action of 20E, the death sequence is completed through activation of caspase family proteases that are recognized as a factor in the non-genomic pathway in mammalian cell death, triggered by activation of membrane-bound ligand receptors. Although caspases and other factors, such as DIAP1 and Dronk, have been extensively studied in *Drosophila* (Leulier et al., 2006), little information is available on the entrance to activation of the caspase cascade. Because the fundamental mode to activate the caspase cascade in *Drosophila* could be very similar to that in mammalian cells (Twomey and McCarthy, 2005), it is probable that cell death execution involves a non-genomic action of 20E in programmed cell death in *Drosophila* salivary glands.

The silk gland of the silkworm, *Bombyx mori*, is a larval-specific tissue and degenerates completely 2 days after pupation, through programmed cell death triggered by the metamorphic peak of ecdysteroids in last-larval hemolymph (Chinzei, 1975; Terashima et al., 2000). The

anterior silk gland, the most anterior part of the silk gland, is composed of a single type of squamous cell with highly branched nuclei (Akai, 1983). Cell death induced by 20E *in vitro* begins with *de novo* gene expression (Terashima et al., 2000; Sekimoto et al., 2006, 2007), followed by nuclear condensation, cell shrinkage, DNA fragmentation, and nuclear fragmentation, in that order, and is completed with apoptotic body formation about 120 h after 20E stimulation (Terashima et al., 2000; Iga et al., 2007). Although 20E-induced cell death begins with regulation of gene expression (Sekimoto et al., 2006, 2007), the genomic action of 20E is not sufficient for completion of cell death in anterior silk glands (Terashima et al., 2000), and non-genomic action of 20E is indispensable for completion (Iga et al., 2007). The non-genomic pathway may begin by binding of 20E to a putative membrane ecdysone receptor (mEcR; Elmogy et al., 2004; Manaboon et al., 2009), and its activation results in nuclear and DNA fragmentation through activation of protein kinase C (PKC) and caspase 3-like protease (Iga et al., 2007).

In this study, we present evidence for Ca^{2+} acting as a second messenger for 20E signaling, which activates PKC and caspase 3-like protease, and activation of this pathway occurs only after the genomic action of 20E has been completed. We also discuss the possible role of cytosolic Ca^{2+} elevation in the regulation of the timing of death execution in the anterior silk glands.

Materials and Methods

Animals

Larvae of *B. mori* were reared and staged as previously described (Sakurai et al., 1998). Last (fifth) instar larvae exhibited a gut purge, a behavior to purge their gut content, in the

scotophase of day 6 or 7. We used only larvae in the photophase following the gut purge.

Hormones and Chemicals

20E (Sigma, St. Louis, MO) was dissolved in water (1 mg/ml) and stored at -20°C. The protein synthesis inhibitor, cycloheximide (CHX), and the calcium ionophore (calcimycin A23187) were purchased from Sigma, and myristoylated protein kinase C (PKC) peptide inhibitor solution was from Promega (Madison, WI). CHX and the calcium ionophore were dissolved individually in dimethylsulfoxide (DMSO). When added to the culture medium, each chemical concentration in DMSO was adjusted so that the final DMSO concentration was less than 5%, at which DMSO did not exert any effect on the response of anterior silk glands to 20E.

Tissue Culture and Observations

Anterior silk glands were rinsed in Grace's insect culture medium (Gibco BRL, Rockville, MD), and cultured individually in 0.3 ml medium using 24-well plates (Greiner Bio-One, Frickenhausen) at 25°C. Anterior silk glands cultured with 1 μM 20E exhibit serial changes in cell and nuclear morphology (Terashima et al., 2000). The glands become round in shape, and detach from each other, caused by cell shrinkage. Highly branched nuclei of the glands become thicker through nuclear condensation, followed by DNA and nuclear fragmentation. Cell death ends with the formation of numerous apoptotic bodies. When cultured with 1 μM 20E and 0.2 mM CHX, a translation inhibitor, anterior silk glands exhibit little change in morphology, but DNA and nuclear fragmentation are induced. This type of cell death has been designated as type C cell death (Iga et al., 2007). Because the calcium ionophore induced a cell death similar to type C cell death (Iga et al., 2007), we use the term ‘type C cell death’ in the present study to

indicate cell death induced by the calcium ionophore. In assays for the effects of chemicals and inhibitors, one of a pair of glands was used as a control and the contralateral gland as the experimental material.

DAPI staining

Anterior silk glands were fixed with 4% formaldehyde for 30 min, followed by washing in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), and then incubated in PBS containing 0.1 μg/ml DAPI (4',6-diamidino-2-phenylindole) at 25°C in the dark for 10 min. The glands were washed with PBS and observed under a fluorescence microscope using an UV excitation filter (BX-50, Olympus, Tokyo). Photographs were processed with Photoshop® (Adobe Systems Inc., San Jose, CA).

DNA Isolation and Agarose Gel Electrophoresis

Anterior silk glands were homogenized and mixed in DNA extraction buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA-NaOH, 0.1% sodium dodecylsulfate, pH 8.0) on ice. After treatment of the homogenate with RNase (20 μg/ml, 37°C, 30 min) and proteinase K (100 μg/ml, 50°C, 60 min), DNA was extracted using a standard phenol-chloroform and chloroform extraction method. After extraction, 1 μg of DNA was electrophoresed on 2% agarose gels and stained with ethidium bromide.

Caspase 3-like Protease Activity Assay

We measured caspase 3-like protease activity using a colorimetric substrate, Ac-DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline (BIOMOL, Plymouth Meeting,

PA) as described in Iga et al., 2007). Briefly, extracts of anterior silk glands were subjected to the assay in reaction buffer (50 mM HEPES, pH7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT, 10% glycerol) in the presence or absence of the caspase 3 inhibitor, Ac-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-CHO, BIOMOL). The solution was incubated at 37°C and absorbance was read at 405 nm every 15 min for 3 h using a Benchmark microplate reader (Bio-Rad, Hercules, CA).

Results

Calcium ionophore partly mimics the 20E action

Our preliminary results demonstrated that the calcium ionophore partly mimicked 20E's effects, inducing nuclear and DNA fragmentation, only in glands precultured for 18 h with 20E (Iga et al., 2007). To determine the optimal concentration of the calcium ionophore, we precultured anterior silk glands for 18 h with 1 μM 20E, and then cultured them in new medium containing the calcium ionophore, but not 20E, for another 120 h. At the end of the culture period, the glands were observed for the appearance of type C cell death (Fig. 1A). A concentration of 10 μM calcium ionophore effectively induced type C cell death in 90% of the glands, while a higher concentration was less effective.

To determine the time at which the 20E signaling pathway can be replaced with the calcium ionophore, we cultured anterior silk glands for various periods with 1 μM 20E, followed by culture in medium containing only 10 μM calcium ionophore for another 120 h, and then examined the occurrence of nuclear (Fig. 1B) and DNA fragmentation (Fig. 1C). In glands precultured for 6 h, the calcium ionophore induced neither nuclear fragmentation nor DNA fragmentation. Nuclei of glands precultured for 12 h were fragmented in 10% of glands,

while those with 18-h preculture exhibited nuclear fragmentation in 90% of glands (Fig. 1B). Similarly, DNA extracted from the latter glands displayed a clear ladder pattern, in contrast to that from the former glands (Fig. 1C). The occurrence of nuclear fragmentation and the DNA ladder pattern in glands with a 24-h preculture was the same as in those with 18-h preculture. Thus, anterior silk glands of gut-purged larvae must be exposed for 18 h to 20E before the calcium ionophore substitution for 20E, indicating that the components involved in 20E signal transduction from cytosolic Ca^{2+} elevation to induction of DNA and nuclear fragmentation become ready to act after between 12 and 18 h of exposure to 20E.

Time-course of type C cell death induction by calcium ionophore

We followed the time-course of the type C morphology after replacement with the calcium ionophore (Fig. 2A). Type C cell death was induced in 70% of glands 24 h after the replacement. When the glands were examined every 3 h, they exhibited no morphological changes until 12 h, and little at 15 h, after which cells underwent rapid type C cell death (Fig. 2B).

Gene expression is necessary to mimic 20E effects by the calcium ionophore

The required preculture period to mimic 20E effects by calcium ionophore (i.e., 18 h) is very similar to that for protein synthesis required for completion of 20E-induced cell death (Terashima et al., 2000). To examine if the calcium ionophore was capable of eliciting effects only after gene expression, we added a translation inhibitor, CHX, to the preculture medium simultaneously with 20E. Although CHX alone induced cell death at 2 mM, 0.2 mM CHX did not induce cell death, but retained the same protein synthesis inhibitory activity as 2 mM CHX (Iga et al., 2007). Accordingly, we precultured anterior silk glands with 0.2 mM CHX and 1 μM

20E for 18 h, then replaced them with new medium containing only the calcium ionophore (Fig. 3). In this preculture condition, neither type C cell death (Fig. 3A) nor DNA fragmentation (Fig. 3B) was induced, indicating that *de novo* gene expression by 20E was necessary for driving the signaling pathway by cytosolic Ca^{2+} elevation.

Involvement of PKC activation on calcium signaling

We examined the requirement for PKC activation in calcium ionophore-induced cell death. After preculture of anterior silk glands for 18 h with 20E, the glands were cultured in the presence of the calcium ionophore with or without the PKC inhibitor for another 120 h (Fig. 4). Nuclear condensation occurred in glands exposed to 20E for 18 h and then cultured further in 20E-free medium (Fig. 4a). In glands exposed to the calcium ionophore, the PKC inhibitor did not inhibit nuclear condensation (Fig. 4c), while completely inhibiting induction of type C cell death (Fig. 4C, D), nuclear fragmentation (Fig. 4b vs. c), and DNA fragmentation (Fig. 4E), indicating PKC activation was required to induce type C cell death and is located downstream of cytosolic Ca^{2+} elevation.

Caspase 3-like protease activation by calcium ionophore

Because caspase 3-like protease activation is necessary to induce nuclear and DNA fragmentation in 20E-induced cell death (Iga et al., 2007), we examined the effects of a caspase 3 inhibitor on the induction of type C cell death by the calcium ionophore (Fig. 5). Effects of caspase 3 inhibitor on cellular and nuclear morphology were the same as those of the PKC inhibitor (see Fig. 4): the caspase 3 inhibitor completely inhibited induction of type C cell death (Fig. 5A-D), nuclear fragmentation (Fig. 5b vs. c), and DNA fragmentation (Fig. 5E).

Next, we measured the change in caspase 3-like protease activity after replacement of 20E with the calcium ionophore (Fig. 5F). During the preculture period with 20E, caspase 3-like protease was not activated. After replacement of 20E with the calcium ionophore, enzyme activity increased slightly at 6 h, followed by a large increase, more than 100-fold, in the 6-h period between 6 and 12 h. The high activity was retained for 6 h, after which it gradually decreased to 70% of the peak level by 36 h.

Discussion

The present study showed the involvement of the Ca^{2+}-PKC-caspase 3 pathway in 20E-induced cell death in anterior silk glands. However, the calcium ionophore elicited death-inducing activity only in glands that had been exposed for 18 h to 20E. In the anterior silk glands, *de novo* gene expression is necessary for cell death induction, in which gene transcription and translation are estimated to be complete after 8 h and 18 h of exposure to 20E, respectively (Terashima et al., 2000; Iga et al., 2007). These results indicate that during the first 18 h of gland culture with 20E, preparation of component(s) involved either directly or indirectly in the non-genomic action of 20E involves Ca^{2+} signaling up to DNA and nuclear fragmentation, via PKC and caspase 3.

Calcium ionophore-induced type C cell death was accompanied by DNA and nuclear fragmentation, but neither cell shrinkage nor apoptotic body formation (Iga et al., 2007). Culture of glands with 20E and 0.2 mM CHX induced type C cell death. However, CHX alone at that concentration did not induce cell death, but did effectively inhibit translation (Iga et al., 2007). Accordingly, some of the component(s) in the signaling pathway connecting Ca^{2+} and caspase 3-like protease may not be present in gland cells at the time of gut purge. Alternatively,

CHX may inhibit the expression of factor(s) involved in the activation of the signaling pathway. In mammals and insects, several caspase inhibitors are involved in the regulation of apoptosis (Callus and Vaux, 2007). In *Drosophila*, DIAP1, a homolog of the inhibitor of apoptosis protein (IAP), suppresses apoptosis, and gene expression of DIAP1 is under the control of *hid*, the death activator gene that is up-regulated by 20E in the salivary glands (Wang et al., 1999; Yoo et al., 2002; Yin and Thummel, 2005). Thus, in type C cell death induced by the simultaneous presence of 20E and CHX, CHX could inhibit translation of such an inhibitor, and this inhibitor may act downstream of Ca^{2+} because *de novo* gene expression is needed for the action of the calcium ionophore to induce nuclear and DNA fragmentation. This indicates that the genomic action of 20E is to prepare factors downstream of Ca^{2+} or to liberate the pathway from inhibition. The details of the molecular aspects remain to be explored.

20E could also induce an inhibitor of inhibitors, such as in the vertical relationship of E74, bFTZ-F1, DIAP1, and DIAP2 in *Drosophila* salivary glands, or directly inhibit inhibitor expression (Jiang et al., 1997, 2000; Yin and Thummel., 2004), as in the late genes in Ashburner's model (Ashburner et al., 1974). Either way, the calcium ionophore may elicit its effects only after suppressive effects in the pathway are eliminated. Timed replacement of 20E with the calcium ionophore showed that a 12-h exposure to 20E was not sufficient but that an 18-h exposure was sufficient to mimic the 20E effects to induce type C cell death by the calcium ionophore. Since protein synthesis needed for programmed cell death is apparently completed by 18 h (Terashima et al., 2000), the signaling pathway downstream of Ca^{2+} may become functional as a result of genomic action by 18 h.

Replacement of 20E with the calcium ionophore after an 18-h preculture with 20E induced caspase 3-like protease activation much more rapidly and in higher amounts than in glands

cultured in the continuous presence of 20E (Fig. 6; see Iga et al., 2007). In 20E-induced cell death, caspase 3-like protease began to be active from 72 h and peaked at 96 h (Iga et al., 2007). Thus, after completion of transcription, it takes at least 54 h (18-72 h) to activate caspase 3-like protease. In culture conditions with replacement of 20E with the calcium ionophore after an 18-h preculture with 20E, caspase 3-like protease was activated 12 h after the replacement (Fig. 6F). This short period indicates that the signaling pathway between Ca^{2+} and caspase 3-like protease is ready for full operation 18 h after culture with 20E. Thus, a comparison of the two culture conditions, continuous presence of 20E and replacement of 20E with the Ca^{2+} ionophore, indicates that the pathway between activation of putative mEcR and Ca^{2+} mobilization (Manaboon et al., 2009) operated rather slowly, and the Ca^{2+}-PKC-caspase 3-like protease pathway may be driven rapidly once cytosolic Ca^{2+} exceeds a threshold level. Accordingly, the molecular mechanisms to establish the pathway for Ca^{2+} mobilization may be of importance in time-regulation of the cell death sequence, which takes as long as 144 h to complete *in vitro*. This may be partly involved in regulation of the *in vivo* death sequence, which takes 5-6 days from the onset of the prepupal period to day 2 of the pupal stage (Terashima et al., 2000).

Early gene expression may also be involved in regulation of the timing of death execution. In *Blattella germanica*, knockdown of *E75* using RNAi techniques accelerates the timing of cell death of prothoracic glands (Belles et al., 2007). In *Drosophila* salivary glands, E75 suppresses diap1, which inhibits cell death (Yin and Thummel, 2004). In *Drosophila* egg chambers, over-expression of E75A induces apoptosis of nurse cells, and over-expression of E75B suppresses apoptosis induced by nutritional shortage (Terashima and Bownes, 2006). In *Bombyx* anterior silk glands, *E75A* is up-regulated in response to an increase in the ecdysteroid

titer in the early to middle prepupal period (Sekimoto et al., 2006), during which death execution begins. *In vitro* exposure of the glands to 20E also up-regulated *E75A*, indicating that E75A in the early to middle prepupal period *in vivo* and for the first 18 h *in vitro* may serve to temporarily suppress death progression. Both substantially slower establishment of the Ca^{2+}-PKC-caspase 3-like protease pathway and hierarchical gene regulation of early genes, death activator genes, and suppressor genes could be one of the molecular mechanisms for the slow progression of cell death in *Bombyx* anterior silk glands.

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Figure legends

Figure 1. A calcium ionophore is capable of mimicking 20E effects only after exposure to 20E. (A) Optimal concentration of the calcium ionophore to induce type C cell death (see Iga et al., 2007 for type C cell death). Anterior silk glands were cultured for 18 h with 1 μM 20E (preculture), and then 20E was substituted with calcium ionophore. The ratio of type C cell death was evaluated 120 h after preculture (see Iga et al., 2007) and expressed as a percentage of the total number of glands (n = 8). (B) Minimum time-period of exposure to 20E for calcium ionophore to induce type C cell death, as indicated by nuclear fragmentation and (C) DNA fragmentation 120 h after preculture. The occurrence of nuclear fragmentation is expressed as a percentage of the total number of ASGs (n = 8). M, marker for 100 bp DNA ladder.

Figure 2. Time-course of type C cell death occurrence with the calcium ionophore. Anterior silk glands were precultured for 18 h with 1 mM 20E, followed by replacement with new medium containing 10 μM calcium ionophore, and the glands were observed (A) every 24 h or (B) every 3 h. Open circles, control glands cultured with DMSO; closed circles, glands cultured with calcium ionophore. Each datum point in (A) is a mean with SD (n = 8). In (B) the occurrence of type C cell death is expressed as a percentage of the total number of ASGs (n = 10-20).

Figure 3. Protein synthesis during preculture with 20E is indispensable to mimic the effects of 20E by the calcium ionophore on DNA and nuclear fragmentation. Anterior silk glands were precultured with 1 μM 20E or 20E + 0.2 mM CHX for 18 h, then 20E was substituted with 10 mM calcium ionophore. (A) Nuclear fragmentation (n = 5 for each condition) and (B) DNA

fragmentation. DNA was extracted from glands cultured for 120 h after preculture. M, marker for 100 bp DNA ladder.

Figure 4. PKC inhibitor inhibits the effects of the calcium ionophore. Anterior silk glands were precultured for 18 h with 1 μM 20E, then cultured with (A) DMSO, (B) calcium ionophore, or (C) calcium ionophore and PKC inhibitor. (A–C) light micrographs; (a–c) DAPI-stained images showing nuclear morphology. (D) Inhibition of type C cell death, and (E) DNA fragmentation by PKC inhibitor. The occurrence of type C cell death is expressed as a percentage of the total number of ASGs (n = 10-12). M, marker for 100 bp DNA ladder. Scale bar = 100 μm (A–C), 20 μm (a–c).

Figure 5. The downstream calcium ionophore stimulus involves activation of caspase 3-like protease. Anterior silk glands were cultured for 18 h with 1 μM 20E (preculture), then cultured with (A) DMSO, (B) calcium ionophore, or (C) calcium ionophore and caspase 3 inhibitor for 120 h after preculture. (A–C) light micrographs; (a–c) DAPI-stained images showing nuclear morphology. (D) Inhibition of type C cell death induction, and (E) DNA fragmentation by caspase 3 inhibitor. The occurrence of type C cell death is expressed as a percentage of the total number of ASGs (n = 8). (F) Time-course of caspase 3-like protease activation by the calcium ionophore. Closed circles, caspase 3 activity in glands cultured with calcium ionophore; open circles, glands cultured with DMSO. Caspase 3-like protease activity is expressed relative to the value in freshly dissected glands (-18 h) as 1. M, marker for 100 bp DNA ladder. Scale bar = 100 μm (A–C), 20 μm (a–c).

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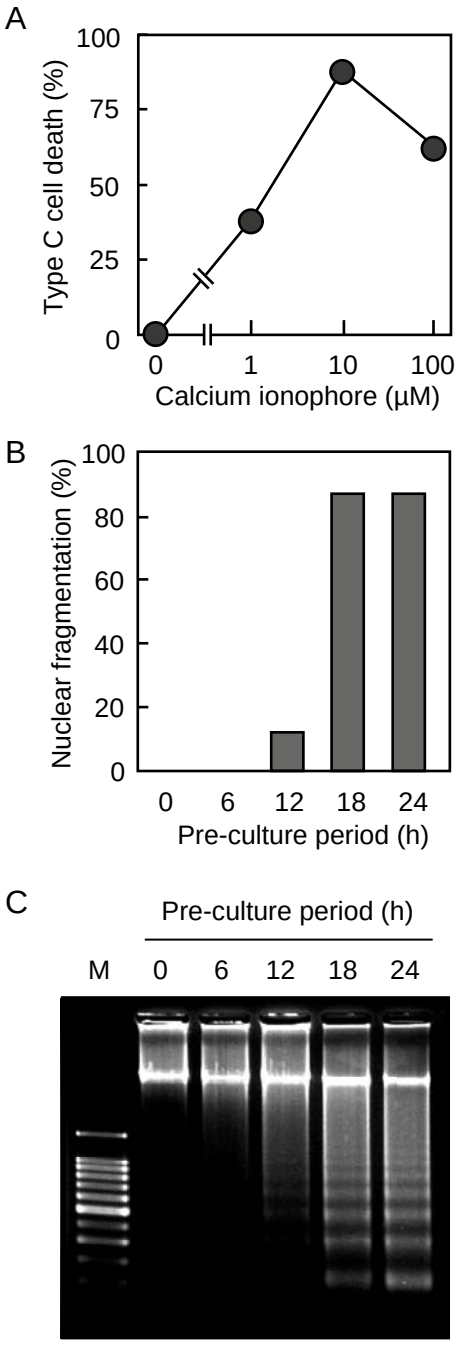
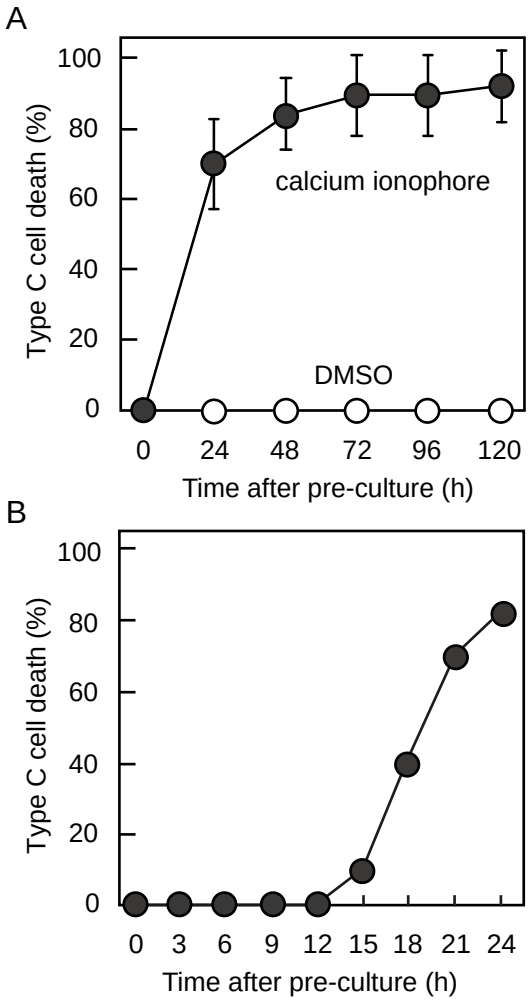
Iga et al. Figure 1

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100
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40
20
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Type C cell death (%)
calcium ionophore
DMSO
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24
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72
96
120
Time after pre-culture (h)
B
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80
60
40
20
0
Type C cell death (%)
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3
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9
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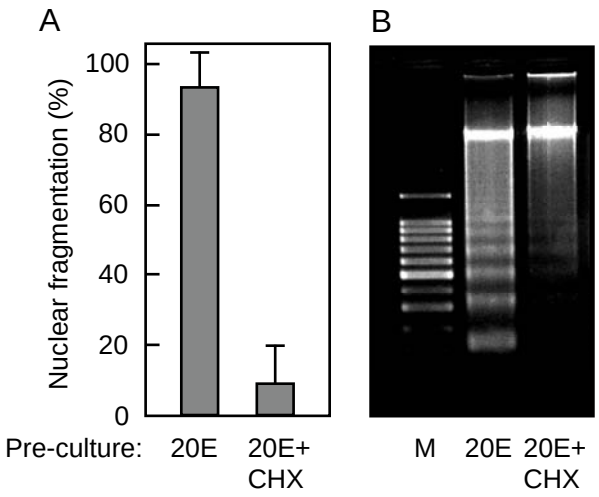
21
24
Time after pre-culture (h)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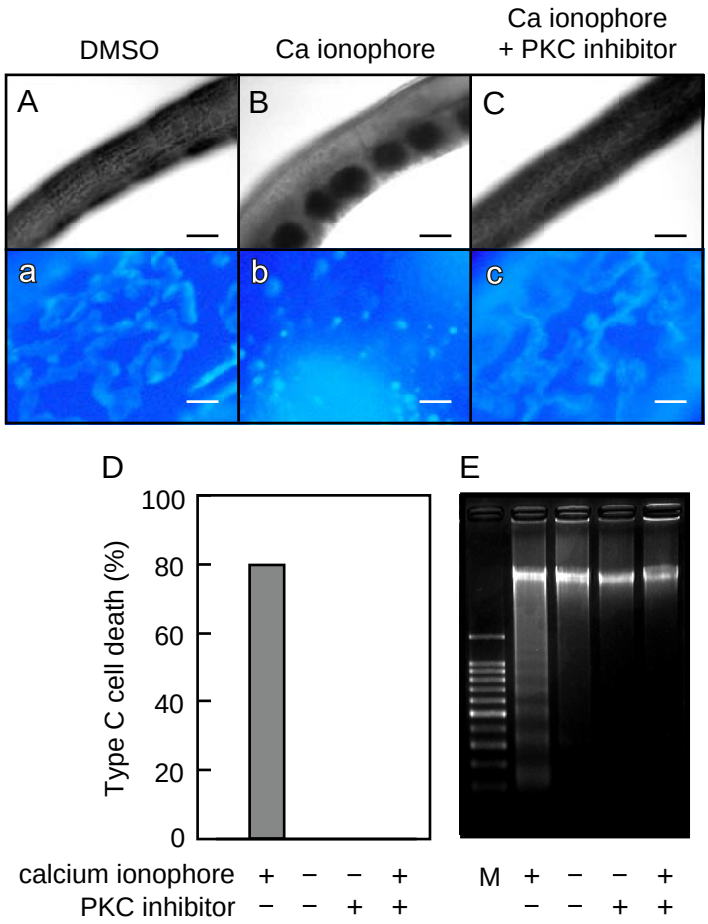
20E+ CHX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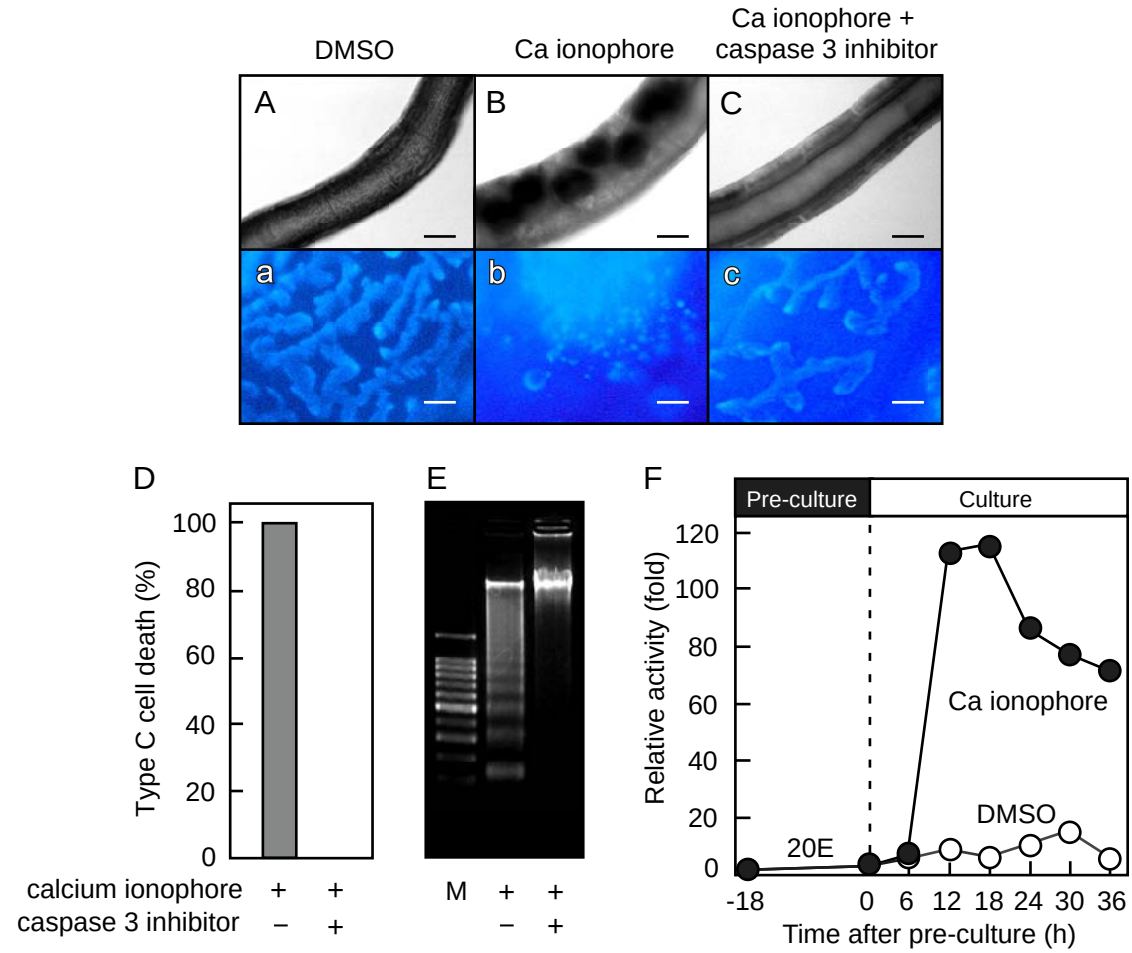
M + − − +
− − + +


DMSO
Ca ionophore
Ca ionophore +
caspase 3 inhibitor
A
B
C
a
b
c
D
Type C cell death (%)
100
80
60
40
20
0
calcium ionophore + +
caspase 3 inhibitor − +
E
M + +
− +
F
Pre-culture
Culture
Relative activity (fold)
120
100
80
60
40
20
0
Ca ionophore
DMSO
20E
-18 0 6 12 18 24 30 36
Time after pre-culture (h)