Proliferative effect of ammodytin L from the venom of *Vipera ammodytes* on 208F rat fibroblasts in culture

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Ammodytin L, purified from the venom of *Vipera ammodytes*, triggers a rapid and dramatic lytic process in myotubes *in vitro*, as well as in differentiated muscle cells *in vivo*, through a mechanism that is not well understood. Despite its great sequence similarity to phospholipase A₂, it is devoid of any enzyme activity. Data on artificial membranes demonstrating a direct interaction between this toxin and the hydrophobic core of the lipid bilayer suggest that the toxin also acts on the lipid microenvironment in cell membranes. Recent experiments on living cells do not confirm this hypothesis, and a more intricate mechanism is proposed. *In vitro*, ammodytin L has necrotic effects only in well-differentiated myogenic cells, whereas other cell types such as platelets, red blood cells and lymphocytes show neither morphological nor functional alterations. In this work we

demonstrate that rat 208F fibroblasts in culture after ammodytin L challenge increase [³H]thymidine incorporation, indicating that this toxin has a myogenic effect. Moreover, ammodytin L increases intracellular Ca²⁺ by acting on intracellular stores probably by activating a phosphatidylinositol-specific phospholipase C. Preincubation of the cells with ammodytin L did not prevent the massive Ca²⁺ release evoked by bradykinin, a phenomenon observed when fibroblasts were incubated with both thapsigargin and ionomycin. Heparin, an agent that inhibits the necrotic effect of the myotoxin in myotubes, also reduces the effect of ammodytin L on DNA synthesis. Heparin inhibits only the late sustained increase in intracellular Ca²⁺ induced by the toxin.

INTRODUCTION

Ammodytin L (AMDL) is a component of *Vipera ammodytes* snake venom, which lacks intrinsic hydrolytic activity despite its great sequence similarity to secretory phospholipase A₂ (PLA₂) of class II [1,2]. We have demonstrated that AMDL interacts with the phospholipid bilayer in a completely different way from other active phospholipases [3,4]. We proposed that the ability of AMDL to penetrate the lipophilic core of artificial membranes is related to its ability to bind non-esterified fatty acids [5]. AMDL has been classified as a myotoxin because of its ability to cause necrosis in the skeletal muscle of *Xenopus laevis* larvae *in vivo* [6] and to destroy both primary and established myogenic cell lines in culture [1,7]. Over a wide range of concentrations (< 15 µg/ml), AMDL appears to be cytolytic only for established myotubes, whereas it prevents the fusion of undifferentiated cultured myoblasts. Furthermore, over the range of concentrations that destroy skeletal muscle, AMDL has no lytic effect on other cell types, such as platelets, erythrocytes and lymphocytes [7].

In all myogenic cell lines tested, AMDL was able to elevate cytoplasmic intracellular Ca²⁺ ([Ca²⁺]_i), by causing Ca²⁺ to be released from intracellular stores ([7]; S. Ruffini, unpublished work). An attractive hypothesis for this phenomenon is that the increase in [Ca²⁺]_i represents the first step in the cytotoxic action of AMDL; a role for Ca²⁺ has been in fact proposed in both necrotic and apoptotic events during cell death [8,9].

The fact that AMDL is tissue-specific suggests that there is a specific receptor on the target cells that binds the toxin. Recent studies have identified new physiological functions of secretory PLA₂s, such as stimulation of DNA synthesis [10,11], contraction

[12] and chemokinetic cell migration [13], which are not caused by their enzymic activity. However, further evidence suggests that secretory PLA₂s bind high-affinity receptors on the membrane of mammalian cells [10,14,15]. These findings may have great clinical relevance; patients with arthritis, endotoxic shock, psoriasis and acute pancreatitis have elevated levels of PLA₂ in synovial fluid, serum and bronchial lavage fluid [16].

Our aim was to understand the mechanism by which AMDL increases [Ca²⁺]_i in cultured cells. However, the study of AMDL-evoked [Ca²⁺]_i increase in myogenic cells is complicated by the fact that the lytic process begins a few minutes after the toxin challenge. Damage to the plasma membrane produces artificial results such as leakage of fluorescent probe, unspecific ion fluxes, etc. In order to find a suitable cellular model to clarify the nature of the toxin-induced [Ca²⁺]_i increase we screened many cell lines that are insensitive to the necrotic effect of AMDL. We found several that responded with a [Ca²⁺]_i increase but did not undergo lysis. We selected the fibroblast cell line 208F, which demonstrated high reproducibility in response to the toxin. Surprisingly, these cells greatly increase [³H]thymidine incorporation after stimulation with toxin.

We found that the toxin causes a sustained [Ca²⁺]_i increase in 208F cells probably by activating a phosphatidylinositol-specific phospholipase C. Experiments with other Ca²⁺-mobilizing agents, such as thapsigargin (TPHS) and bradykinin BK, demonstrate that AMDL fails to deplete the *myo*-inositol trisphosphate (IP₃)-sensitive Ca²⁺ stores totally. In 208F cells heparin reduces both the AMDL-evoked increase in [Ca²⁺]_i and stimulation of proliferation, demonstrating that a link exists between these events.

Abbreviations used: AMDL, ammodytin L; BK, bradykinin; PLA₂, phospholipase A₂; TPHS, thapsigargin; [Ca²⁺]_i, intracellular calcium ion concentration; IP₃, *myo*-inositol trisphosphate.

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MATERIALS AND METHODS

Materials

AMDL was kindly provided by Dr. F. Gubensek (Jozef Stefan Institute, Ljubljana, Slovenia), and rat fibroblast cell line 208F by Dr. A. H. Wyllie (Department of Pathology, The University of Edinburgh Medical School, Edinburgh, Scotland, U.K.). Tissue culture media, fetal calf serum and sterile materials were supplied by Flow Laboratories; ionomycin and fura 2 acetoxymethyl ester were from Calbiochem (San Diego, CA, U.S.A.); BK and heparin came from Sigma (St. Louis, MO, U.S.A.); [^3H]thymidine and IP_3 radioreceptor assay were supplied by Amersham Corp. (Amersham, Bucks., U.K.). All other chemicals were of the highest grade commercially available.

Cell cultures

Fibroblasts were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin, at 37 °C under air/ CO_2 (19:1, v/v). Experiments were performed on cells after not more than 20 passages in culture. Cells were removed from flasks by trypsin treatment and seeded (1×10^6 cells) into 35 mm culture dishes.

IP_3 mass determination

Cells grown to confluency on 35 mm plates (10^6 cells/plate) were incubated for 4 h in RPMI 1640 and stimulated by a rapid shift to 1.0 ml of HEPES-buffered saline containing BK or AMDL. The reaction was terminated by the addition of 1.0 ml of cold trichloroacetic acid (20%); cells were then scraped off, transferred to test tubes and, after being left on ice for 30 min, centrifuged. The acid-soluble extract was washed three times with 3 vol. of water-saturated diethyl ether, neutralized with NaHCO_3 and IP_3 mass estimated by a commercially available specific radioreceptor assay (Amersham Corp.).

Measurement of $[\text{Ca}^{2+}]_i$

Cells grown in 25 cm^2 flasks were incubated for 15 min at room temperature in RPMI 1640 medium containing 1 μM fura 2/acetoxymethyl ester. After a wash with HEPES-buffered saline, cells were incubated for an additional 15 min in the same buffer, gently scraped off, centrifuged for 3 min at 900 g and finally resuspended in HEPES-buffered saline (0.3×10^6 cells/ml). The variation in fura 2 fluorescence after the addition of the substance to be tested was monitored at 37 °C with continuous stirring in a Perkin-Elmer LS-5 spectrofluorimeter at excitation and emission wavelengths of 340 nm and 500 nm respectively. Maximum and minimum fluorescence were determined after the addition of 0.1% Triton X-100 and 3 mM EGTA (after the pH had been shifted to 8.5) respectively. $[\text{Ca}^{2+}]_i$ was calculated as described by Grynkiewicz et al. [17].

Analysis of DNA synthesis

[^3H]Thymidine incorporation was used to measure the mitogenic response. DNA-synthesis experiments were performed by incubating the cell monolayers in Dulbecco's modified Eagle's medium in the presence of AMDL. After incubation, the monolayer was pulsed with 1 μCi of [^3H]thymidine/ml and

incubated for an additional 3 h. After trypsin treatment for 3 min, cells were harvested by centrifugation and treated with 5% trichloroacetic acid at 4 °C for 30 min. The acid-insoluble fraction was resuspended in 0.1% SDS/200 mM NaOH and samples were processed for liquid-scintillation counting in an LKB 1500 spectrometer. Each experiment was performed in triplicate.

RESULTS

AMDL, in the concentration range 4–20 $\mu\text{g}/\text{ml}$, induced an increase in [^3H]thymidine incorporation into subconfluent rat fibroblast cells 208F (Figure 1) grown for 24 h in Dulbecco's modified Eagle's medium in the absence of serum. 208F fibroblasts continued to progress through their cycle even in the absence of serum. At higher AMDL concentrations, we observed a decrease in DNA synthesis. When cells were stimulated in the presence of heparin (2–20 $\mu\text{g}/\text{ml}$), the effect of the toxin on [^3H]thymidine incorporation progressively diminished (Table 1). Concentrations of heparin above 10 $\mu\text{g}/\text{ml}$ reduced DNA synthesis in serum-deprived 208F cells (Table 1).

AMDL increased $[\text{Ca}^{2+}]_i$ in 208F fibroblasts (Figures 2a and 2b). Over the range of concentrations tested (1–15 $\mu\text{g}/\text{ml}$), the kinetics of Ca^{2+} mobilization were independent of toxin con-

Figure 1 Effect of AMDL concentration on [^3H]thymidine incorporation in 208F fibroblasts

The control value was 5600 ± 500 c.p.m./mg of protein. Results are means $\pm$ S.D. for three to five independent experiments carried out in triplicate.

Table 1 Effect of heparin on AMDL-induced [^3H]thymidine incorporation in 208F cells

Cells were stimulated with AMDL (7 $\mu\text{g}/\text{ml}$) for 24 h in the presence or absence of different heparin concentrations. After 24 h [^3H]thymidine incorporation was estimated as described in the Materials and methods section. Values are means $\pm$ S.D. for at least three independent experiments.

Heparin ($\mu\text{g}/\text{ml}$)	AMDL	[^3H]Thymidine incorporation (c.p.m./mg of protein)
0	—	5457 $\pm$ 978
	+	9850 $\pm$ 1730
2	—	5567 $\pm$ 1450
	+	8210 $\pm$ 2400
10	—	4979 $\pm$ 980
	+	6323 $\pm$ 2113
20	—	4035 $\pm$ 1650
	+	4450 $\pm$ 2400

Figure 2 Time-dependent action of AMDL (7 µg/ml) on $[Ca^{2+}]_i$ in 208F fibroblasts in the presence (a) or absence (b) of extracellular Ca^{2+}

In (b) the Ca^{2+} value is indicated as relative fluorescence.

Table 2 Effect of AMDL (7 µg/ml) on $[Ca^{2+}]_i$ increase and $[^3H]$ thymidine incorporation in different cell lines

Experiments were carried out as described for 208F fibroblasts in the Materials and methods section. $[Ca^{2+}]_i$ increase is expressed as nM after 1 min of AMDL stimulation, and $[^3H]$ thymidine incorporation as percentage increase over the control. Determinations for each cell line are means $\pm$ S.D. for at least three independent experiments. ND, not determined. RASMC, rat aortic smooth muscle cells; L6, proliferative myoblast mouse cells; SK-N-BE, human neuroblastoma; B3.2, *Xenopus laevis* kidney cells, 208F, rat fibroblasts.

Cell line	$[Ca^{2+}]_i$ increase (nM)	$[^3H]$ Thymidine incorporation (% of control)
RASMC	270 $\pm$ 23	180 $\pm$ 15
L6	180 $\pm$ 48	110 $\pm$ 20
SK-N-BE	300 $\pm$ 79	200 $\pm$ 40
B3.2	ND	115 $\pm$ 15
208F	230 $\pm$ 30	190 $\pm$ 35

Figure 3 Time-dependence of IP_3 accumulation after stimulation of 208F fibroblasts with 1 mM BK (●) or 7 µg/ml AMDL (○)

Results are means $\pm$ S.D. for three independent experiments carried out in triplicate.

centration: the onset of $[Ca^{2+}]_i$ mobilization was rapid and its intracellular concentration reached a plateau in less than 1 s (Figure 2a). In some experiments we observed a brief delay (1–3 s) between the first increase and a more sustained phase (results not shown). Subsequent administration of AMDL was unable to evoke further $[Ca^{2+}]_i$ increase. The AMDL concentration able to induce 50% of the maximal effect was between 1 and 2 µg/ml; higher toxin concentrations provoked a pro-

Figure 4 Time-dependent action of 1 mM BK on $[Ca^{2+}]_i$ in 208F fibroblasts in the absence (a) or presence (b) of 7 µg/ml AMDL

portional $[Ca^{2+}]_i$ increase up to 230 ± 30 nM (control value 95 ± 20 nM) at 10 µg/ml AMDL. At the same AMDL concentrations as those used in the previous experiments, the toxin did not induce leakage of cytoplasmic components (results not shown) assessed as lactate dehydrogenase release into the culture medium.

We tested different cell lines to investigate the specificity of the AMDL effect on both $[Ca^{2+}]_i$ increase and $[^3H]$ thymidine incorporation. As shown in Table 2, several of the cell lines tested showed an increase in $[Ca^{2+}]_i$ after AMDL challenge. Neither B3.21 *Xenopus* kidney cells nor myoblasts appeared to be stimulated to proliferate by the toxin.

The source of the Ca^{2+} involved in the AMDL-stimulated response was investigated by eliminating extracellular Ca^{2+} and by adding 2 mM EGTA to the extracellular medium (Figure 2b). Without extracellular Ca^{2+} , the magnitude of the transient rise in $[Ca^{2+}]_i$ was proportional to the amount of Ca^{2+} released from the stores. The addition of 7 µg/ml AMDL resulted in a marked increase in $[Ca^{2+}]_i$, followed by recovery to pre-exposure levels. Another experimental approach was used to define the contribution of external Ca^{2+} to AMDL activity, namely the use of mM Ni^{2+} , a non-selective Ca^{2+} -channel blocker. Ni^{2+} failed to inhibit AMDL-induced $[Ca^{2+}]_i$ increase, supporting the idea that the rapid phase of intracellular Ca^{2+} accumulation at least is dependent on an intracellular source (results not shown).

Experiments were conducted to investigate the role of the IP_3 -sensitive intracellular Ca^{2+} stores in the cell response to AMDL. Figure 3 shows intracellular IP_3 accumulation in fibroblasts after stimulation with AMDL (7 µg/ml) or 1 mM BK. Both agonists stimulated IP_3 accumulation, but the effect of AMDL was three times smaller than that of BK. The maximal response for both BK and AMDL was reached after 15–20 s from the cell challenge, and thereafter the IP_3 concentration gradually decreased, returning to control values within 10–15 min.

A set of experiments was designed to determine which stores act as the Ca^{2+} source after treatment with toxin by using different agents known to elevate $[Ca^{2+}]_i$ in fibroblasts. In medium containing 1.5 mM Ca^{2+} (Figure 4a), BK induced a $[Ca^{2+}]_i$ peak with a rapid and markedly transient ion mass elevation (1.1 ± 0.25 µM) followed by a lower and more sustained phase (300 ± 70 nM). The late phase did not correlate with IP_3 accumulation and was abolished by a brief preincubation with both EGTA and Ni^{2+} . This event can be ascribed to an influx of external ions through plasma-membrane channels. When cells were prestimulated with 7 µg/ml AMDL, no changes were observed in the BK-induced $[Ca^{2+}]_i$ peak (Figure 4b). Further experiments were conducted to determine the effect in 208F cells

Figure 5 Effect of BK (a,b) and TPMS (c,d) on AMDL (7 μ g/ml) stimulation of $[Ca^{2+}]_i$ in the absence (a,c) or presence (b,d) of extracellular Ca^{2+} .

208F cells were stimulated with TPMS (10 mM) or BK (1 mM).

of prestimulation with BK on AMDL-induced $[Ca^{2+}]_i$ increase. Under our experimental conditions we were unable to detect a significant change in the late phase of the BK-evoked $[Ca^{2+}]_i$ peak.

When cells were stimulated with AMDL after pretreatment with BK, in both the presence and absence of extracellular Ca^{2+} ions, we observed a reduction in the AMDL-induced $[Ca^{2+}]_i$ increase (Figures 5a and 5b). In order to evaluate the source of

the intracellular Ca^{2+} stores mobilized by AMDL, we also used TPMS. TPMS is a microsomal Ca^{2+} -ATPase inhibitor which induces the release of Ca^{2+} from IP_3 -sensitive intracellular stores [18,19]. In 208F fibroblasts, in both the presence and absence of Ca^{2+} in the medium, the complete depletion of IP_3 -sensitive Ca^{2+} stores by TPMS (10 mM) prevented the AMDL-induced $[Ca^{2+}]_i$ increase (Figures 5c and 5d).

Finally, the effect of heparin on toxin-dependent $[Ca^{2+}]_i$ increase was studied, in order to correlate the effect of AMDL on DNA synthesis with its action on intracellular Ca^{2+} homeostasis. Figure 6 shows that heparin at concentrations ranging between 1 and 10 μ g/ml did not significantly affect the early peak of the AMDL-induced $[Ca^{2+}]_i$ increase. We did, however, observe a progressive decrease in the late sustained phase of $[Ca^{2+}]_i$ elevation which disappeared at heparin concentrations of 10 μ g/ml.

DISCUSSION

From the results obtained we can conclude that: (1) AMDL, at nanomolar concentrations, stimulates DNA synthesis in 208F rat fibroblasts in culture and that this effect is negatively regulated by heparin; (2) AMDL induces an increase in $[Ca^{2+}]_i$ consisting of a very rapid transient ion mass elevation, followed by a lower and more sustained phase. The release of neither non-esterified fatty acids nor lactate dehydrogenase could be detected after toxin challenge, demonstrating that the pharmacological effects of AMDL were not mediated by cell damage or a non-specific release of arachidonic acid.

The mechanism of action by which snake venom myotoxins exert their toxic effect is still unknown. The *in vivo* tissue specificity of these toxins is significant for the presence of a receptor in the target cell membranes; however, AMDL at nanomolar concentrations affects physiological events in nearly all the cell lines tested including rat vascular smooth cells, human neuroblastoma SK-N-BE and proliferative myoblasts L6 (Table 2). In these cell lines, AMDL increases $[Ca^{2+}]_i$ and stimulates $[^3H]$ thymidine incorporation, but it shows necrotic activity only in differentiated myotubes [7].

Gutiérrez and co-workers [20], using phospholipase myotoxins from *Bothrops asper* venom in a micromolar range on different cell lines in culture, demonstrated that myotoxins have a broad cytolytic activity. Similarly, micromolar concentrations of different myotoxins from *Bothrops* venom show necrotic activity in cultured 3T3 fibroblasts [21]. These reports support our observation (Figure 1) that AMDL, at concentrations higher than 2 μ M (i.e. 25 μ g/ml), inhibits $[^3H]$ thymidine incorporation into 208F fibroblasts, indicating that a toxic process is involved.

On the basis of these observations, two main hypotheses on the mechanism of action of myotoxins can be proposed: (i) myotoxins exert a detergent-type action on the plasma membrane of a variety of cell types, but the injury at nanomolar concentrations is lethal only for muscle cells and differentiated myotubes; (ii) myotoxins bind to one class of receptors on myogenic cells and to another on non-myogenic cells.

Our data on the increase in $[Ca^{2+}]_i$ (Figure 2) strongly suggest that AMDL in fibroblasts does not act by damaging cell membranes. No additive effect of subsequent AMDL addition was observed on $[Ca^{2+}]_i$, and, after 208F cells were challenged with AMDL, the cells retained their ability to respond to BK (Figure 4). Finally, we can exclude the possibility that AMDL damages the plasma membrane through a non-selective mechanism, since, if this were so, we would observe a decrease and not an increase in fura 2 fluorescence when cells are stimulated in the presence of EGTA (Figure 2).

Figure 6 Effect of different heparin concentrations on AMDL (7 μ g/ml) stimulation of $[Ca^{2+}]_i$.

We conclude that the effect of AMDL on the increase in $[Ca^{2+}]_i$ in 208F fibroblasts is due to the mobilization of intracellular stores of Ca^{2+} , the depletion of which causes the opening of membrane channels to allow a refilling process, indicating that the receptors are occupied by AMDL.

The presence of a receptor for non-toxic soluble PLA_2 s has been described in different tissues [14,22–24], as well as in different cell lines [11,14,15] and, so far, several receptors from different tissue have been cloned [10,15,25]. Moreover, Arita and co-workers reported that pancreatic PLA_2 induces cell proliferation in several cell lines [11,14]. These authors postulated that PLA_2 exerts its action only by binding to a specific receptor and not through the release of biologically active non-esterified fatty acids.

A problem arising from the results reported here concerns the Ca^{2+} stores sensitive to the receptor–toxin complex. We observed a small but reproducible IP_3 mass elevation after stimulation of 208F cells with toxin (Figure 3), which suggests that the binding of AMDL to its receptor activates a phosphatidylinositol-specific phospholipase C. The increase in IP_3 induced by AMDL is probably related to the rapid phase of $[Ca^{2+}]_i$ increase observed within seconds of cell challenge.

BK increases IP_3 four times as much as does AMDL (Figure 3). A similar response is also observed for $[Ca^{2+}]_i$ (about 1100 nM compared with 250 nM) (Figure 4). In fibroblasts, whereas TPBS caused complete depletion of the intracellular hormone-sensitive Ca^{2+} pool and prevented the effect of BK on the increase in the cytosolic free Ca^{2+} , AMDL caused only partial depletion of the pool (Figure 5). The observation that AMDL was unable to deplete the hormone-sensitive Ca^{2+} stores completely was confirmed in a human neuroblastoma cell line well characterized for its response to charbacol [26]. In SK-N-BE cells, the toxin was unable to modify the increase in Ca^{2+} induced by 1 mM charbacol (A. Spinedi, personal communication). Other growth factors such as hepatocyte growth factor and epidermal growth factor have similar effects on the hormone-sensitive- Ca^{2+} pool [27]. We ruled out the possibility that AMDL acts by mobilizing non- IP_3 -sensitive Ca^{2+} pools, since (i) in Ca^{2+} -free medium, BK greatly reduced the AMDL-induced $[Ca^{2+}]_i$ increase (Figure 5) and (ii) cell pretreatment with TPBS totally inhibited the AMDL-induced $[Ca^{2+}]_i$ increase (Figure 5). Thus both AMDL and BK appear to act via the same mechanism, i.e. increasing intracellular IP_3 mass.

Heparin was used to discriminate between the different mechanisms through which AMDL exerts its pharmacological activity on different cell lines. Heparin has been reported to inhibit the necrotic activity of other PLA_2 -like myotoxins [28,29]. Lomonte and co-workers [29] reported that heparin binds myotoxin II in the region of residues 105–117 (115–129 in the numbering system of Renetseder et al. [30]), which is the cationic region thought to be responsible for the cytotoxic activity of the toxin. AMDL shows an extremely close similarity in this region to myotoxin II, and our findings (not shown) also indicate that heparin abolishes the toxic effects of AMDL on C2C12 myotubes.

As reported in the Results section, heparin was able to block the effect of AMDL on DNA synthesis (Table 1). Heparin has been reported to interfere with cell proliferation [31,32], and, in agreement with this, in experiments using concentrations of heparin greater than 20 μ g/ml, we observed that heparin blocked DNA synthesis in 208F cells.

Increased concentrations of heparin caused a slight decrease in AMDL-induced $[Ca^{2+}]_i$ increase during the early phase, whereas in the late phase the levels progressively decreased to control values. The late phase of the AMDL-induced $[Ca^{2+}]_i$ plateau is dependent on the presence of extracellular Ca^{2+} (Figure 3). The

Ca^{2+} flowing through the plasma membrane probably causes the Ca^{2+} oscillations that regulate the phosphorylation of proteins, which is a key event in the cellular response to growth hormones [33,34]. A role for extracellular Ca^{2+} influx in growth-factor-induced cell proliferation has been determined in several cell lines, including osteoblastic cells [35], hepatocytes [36] and pancreatic AR42J cells [37].

We can conclude that AMDL exerts a proliferative effect on 208F fibroblasts by binding to a receptor that also acts by mobilizing intracellular and extracellular Ca^{2+} . Heparin blocks the proliferative effect of AMDL by inhibiting the extracellular Ca^{2+} influx, which is the late sustained phase of Ca^{2+} increase essential for the progress of the cell cycle.

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