Biochimica et Biophysica Acta, 716 (1982) 72–78
Elsevier Biomedical Press

BBA 21102

THE INHIBITION OF THE SERUM-STIMULATED INCREASE OF ORNITHINE DECARBOXYLASE BY IONOPHORES AND LOCAL ANESTHETICS

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(Received August 18th, 1981)
(Revised manuscript received December 10th, 1981)

Key words: Ornithine decarboxylase; Ionophore; Anesthetic; Serum stimulation; (Mouse leukemia cell)

The addition of fresh serum-containing growth medium to L1210 mouse leukemic cells in culture resulted in a 5-fold increase in ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) activity. The presence of microtubule disrupting agents (colchicine, vinblastine) or cations (5–10 mM K^+, Na^+ or Mg^{2+}) abolishes this increase of ornithine decarboxylase activity (Chen, K.Y., Heller, J.S. and Canellakis, E.S. (1976) Biochem. Biophys. Res. Commun. 70, 212–219). Based on these observations we proposed that fluctuation in cellular cation concentrations may act as a link between the membrane structure and ornithine decarboxylase. To test this proposal, we studied the effects of selective membrane perturbing agents such as ionophores and local anesthetics, on the serum-stimulated increase of ornithine decarboxylase activity in L1210 cells. Among the six ionophores tested, valinomycin was the most potent one, with I_{50} value (concentration that gives 50% inhibition of ornithine decarboxylase activity) of $6 \cdot 10^{-9}$ M. Dibucaine and tetracaine were also effective inhibitors at 10^{-4}–10^{-5} M. The I_{50} values of valinomycin on the protein synthesis and RNA synthesis, however, were greater than $1 \cdot 10^{-6}$ M. These results substantiate the notion that ornithine decarboxylase activity can be regulated at plasma membrane level and such regulation is related to the perturbation of cellular cation pools.

Introduction

The polyamines appear to be replaceable by other cations in some but not all of their functions [1–3]. Such functional replacement of polyamines by cations is also indicated by the inhibition of the serum-stimulated enhancement of ornithine decarboxylase activity in P388 and L1210 mouse leukemic cell by 5–20 mM Na^+, K^+ or Mg^{2+} [4]. Furthermore, we have recently found that Na^+ was essential for the stimulation of ornithine decarboxylase activity by asparagine in mouse neuroblastoma cells maintained in isotonic salts-glucose solution (Viceps-Madore, Chen, Tsou and Canellakis, unpublished data). The relationship between polyamines and cations is also suggested by the temporal changes in K^+ and Na^+ fluxes as well as the changes in the intracellular concentrations of K^+ and Na^+ that have been shown to occur during cell cycling [5,6]. In fact, when we correlated the results obtained from various laboratories, we noted that the loss of intracellular K^+ followed by a recovery of the K^+ that occurs during the G1/S phase [5,6], coincides temporally with the increase in ornithine decarboxylase activity during G1/S phase [7,8]. The causal relation-

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ship of these two phenomena remained to be established.

In addition to the relationship between polyamines and inorganic cations, we have previously presented evidence that the control of ornithine decarboxylase activity may occur through the plasma membrane [9,10]. For instance, it has been shown that the enhancement of ornithine decarboxylase activity is inhibited by low concentrations of colchicine, vinblastine and cytochalasin B that disrupt the microtubule-microfilament cytoskeleton [9] without affecting the transport of metabolites [11]. The specificity of these reaction upon microtubule-microfilament cytoskeleton is emphasized by showing that much higher concentrations of colchicine analogues, such as lumicolchicine and colchiceine, which do not affect the structure of the cytoskeleton also do not affect ornithine decarboxylase activity [9].

Experimental evidence from other laboratories also indicates the association of polyamines with discrete sites of cell membrane [12,13] as well as the modification of the activity of plasma-membrane-bound enzymes by low concentration of polyamines [14,15].

Additional data in favor of the control of ornithine decarboxylase activity through the plasma membrane is that the addition of extremely low concentrations of polyamines and α,ω-diamines (approx. 10^{-6} M) to the growth medium inhibits ornithine decarboxylase activity, despite the fact that the intracellular content of polyamines is greater by several orders of magnitude [10,16].

The present study provides additional evidence on the control of ornithine decarboxylase activity through the plasma membrane, as evidenced by the effects of ionophores and cationic local anesthetics on the enhancement of ornithine decarboxylase activity.

A preliminary report of this work was presented at the 61st Annual Meeting of Federation of American Societies for Experimental Biology, Chicago, IL, April 1–8, 1977.

Materials and Methods

Chemicals

The following chemicals were purchased: DL-[1-^{14}C]ornithine (32.2 Ci/mol), DL-[4,5-H]leucine (50 μCi/mol), [2-^{14}C]uridine (50 mCi/mol) and Aquasol from New England Nuclear, Boston, MA; horse serum, fetal calf serum, Fischer's medium, Dulbecco's modified Eagle medium, and phosphate-buffered saline and Earle's balanced salt solution (salts/glucose solution) from Gibco, Grand Island, NY; valinoymcin, pyridoxal 5-phosphate from Sigma, St. Louis, MO; dicyclohexyl-18-crown-6 from Aldrich, Milwaukee, WI, dibucaine and tetracaine HCl from Crookes-Barnes Lab., Inc., Wayne, NJ; while nigericin, ionophores A204 and A23187 and monesin were kindly provided by Dr. R. Hamill of Eli Lilly Co.

Cell culture

L1210 mouse leukemic cells were grown in Fischer's medium supplemented with 10% horse serum at 37°C in a water-jacketed CO_2 incubator (95% air/5% Co_2) as previously described [9]. To stimulate the increase of ornithine decarboxylase activity in L1210 cells, they were grown to the early stationary phase ($8 \cdot 10^5$–$1.2 \cdot 10^6$ cells/ml) and then diluted 2- to 3-fold with fresh Fischer's medium containing 10% horse serum. N-18 mouse neuroblastoma cells were grown as monolayer cultures in Dulbecco's modified Eagle medium (with 4500 mg glucose per liter) supplemented with 10% fetal calf serum as previously described [17]. Cells at their early stationary phase of growth were washed with two 5 ml portions of the salts/glucose solution at 37°C and placed in 5 ml of the test medium which was either salts/glucose solution of fresh Dulbecco's medium. Various experimental additives including ionophores were added to L1210 leukemic cells immediately after dilution or to N-18 cells after reincubation for the experiments described as follows.

For the time-course study, portions of the cell suspension were removed at desginated times for enzyme assay. For the dose-response study, cells in the absence or presence of ionophores at various concentrations were incubated for 90 or 120 min and harvested for enzyme assay. For the studies of protein and RNA synthesis, 2 h after dilution, the cells were divided into 10 ml portions, valinomycin and either [^{2}H]leucine (0.2 μCi/ml) or [^{14}C]uridine (0.2 μCi/ml) were added. The cells were then incubated for one additional hour. The cells were

centrifuged, washed twice with phosphate-buffered saline, and suspended in 1 ml of assay buffer for enzyme assay. Leucine and uridine incorporations were measured according to the standard filter paper assay procedure [18].

Assay of ornithine decarboxylase activity

(a) L1210 cells. The cell suspension (10 ml) was centrifuged, cells were washed twice with phosphate-buffered saline and suspended in 1 ml of assay buffer which contains 0.1 mM EDTA, 50 μM pyridoxal phosphate and 5 mM dithiothreitol in 50 mM Tris-HCl (pH 7.4 at 22°C). The cells were broken by two cycles of freeze-thawing and centrifuged at $10000 \times g$ for 10 min. The supernatant fluid was used as crude enzyme extract for the assay or ornithine decarboxylase activity. The specific activity of ornithine decarboxylase was determined by measuring the release of $^{14}CO_2$ form DL-[1-^{14}C]ornithine as described previously at saturating substrate concentrations (0.56 mM ornithine) [9].

(b) N-18 neuroblastoma cells. At the designated time after incubation, the test medium was decanted and the cells were washed with phosphate-buffered saline. They were scraped off the dish with a rubber policeman, suspended in 0.8 ml of assay buffer, and processed for ornithine decarboxylase assay as described above.

Results

Six ionophores were tested for their effects on the stimulation of ornithine decarboxylase activity that occurred following dilution of cells into fresh Fischer's growth medium. Among those ionophores, A23187 is a divalent cation ionophore; the other five are monovalent cation ionophores. These five monovalent cation ionophores belong to two different subclasses [19]: valinomycin and dicyclohexyl-18-crown-6 are neutral cyclic closed antibiotics; nigericin, monesin and ionophore A204 are open-chain monocarboxylic acid antibiotics.

Dose-response curves of the ionophores on the stimulation of ornithine decarboxylase activity of L1210 cells

In order to compare the effectiveness of these various ionophores, the dose-response curve of the effect of each ionophore on the stimulation of ornithine decarboxylase activity in L1210 cells was determined (Fig. 1). The I_{50} values, defined as concentrations of ionophores that give 50% inhibition of ornithine decarboxylase activity, and cation specificities of these ionophores are listed in Table I. From Fig. 1 and Table I, the following conclusions can be drawn:

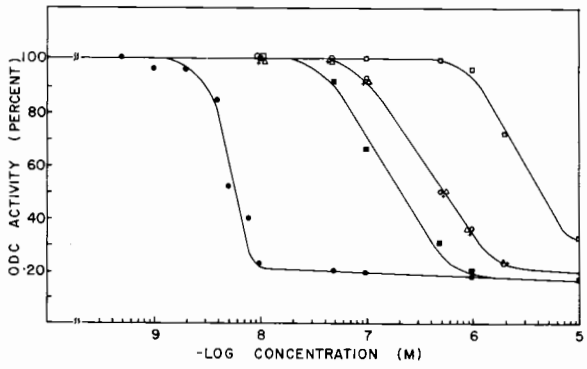

Fig. 1. Dose-response curve of the effect of various ionophores on the stimulation of ornithine decarboxylase (ODC) activity. L1210 cells at early stationary phase were diluted with fresh Fisher's medium supplemented with 10% horse serum in the presence of various concentrations of each individual ionophores. Cells were harvested 120 min after dilution for enzyme assay. Valinomycin (●——●), ionophore A23187 (■——■), ionophore A204 (+——+), monesin (△——△), nigericin (○——○), dicyclohexyl-18-crown-6 (DC-18) (□——□). None of these ionophores affected ornithine decarboxylase activity in the in vitro assay.

TABLE I

SPECIFICITIES OF VARIOUS IONOPHORES ON POTASSIUM SELECTIVITY AND INHIBITION OF ORNITHINE DECARBOXYLASE ACTIVITY

K_D represents the aqueous K^+ concentration necessary to half-saturate the ionophore in organic phase. Values were obtained from Ref. 27. I_{50} value is concentration of ionophores which gives 50% inhibition of enzyme activity as compared to control. Values were obtained from Fig. 1.

Ionophores	Cation specificity	K_D(mM)	I_{50}(M)($\times 10^9$)
Valinomycin	K^+	49	6
DC-18	K^+	200	4000
A23187	Ca^{2+}	–	500
Monensin	K^+, Na^+	38	200
Nigericin	K^+, Na^+	0.09	200
A204	K^+, Na^+	–	200

(i) Although valinomycin and dicyclohexyl-18-crown-6 belong to the same subclass of monovalent ionophores and both are K^+ specific ionophores with affinity constants that differ only about 5-fold [20], the I_{50} value of valinomycin was smaller than that of dicyclohexyl-18-crown-6 by three orders of magnitude.

(ii) The monovalent ionophores, nigericin, monesin and ionophore A204, gave identical I_{50} values, even though nigericin is 400-times more effective than monesin in its ability to bind K^+ [20]. These results are in line with the findings illustrated in (i) and support the notion that translocation of K^+ alone does not account for the inhibiting effects of these ionophores.

(iii) The calcium ionophore A23187 also inhibited the increase of ornithine decarboxylase activity, despite the fact that A23187 has been reported to be mitogenic in lymphocytes [21].

(iv) The shapes of dose-response curves of these ionophores were similar and were characterized by steep inflection points.

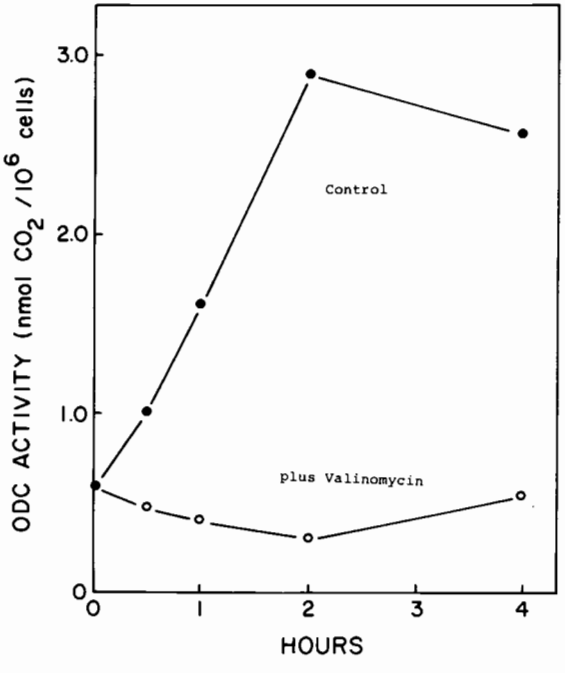

Fig. 2. Time course of the effect of valinomycin on the stimulation of ornithine decarboxylase (ODC) activity. L1210 cells were maintained in fresh Fisher's medium supplemented with 10% horse serum in the absence (●——●) or in the presence (○——○) of $2 \cdot 10^{-8}$ M valinomycin.

The effect of valinomycin on the time course of the stimulation of ornithine decarboxylase activity

Ornithine decarboxylase activity is greatly increased in L1210 cells when cells at the stationary phase of growth are diluted with fresh growth medium. The maximal ornithine decarboxylase activity occurs 2–4 h after addition of the growth medium [4]. Fig. 2 shows that valinomycin, at $2 \cdot 10^{-8}$ M, completely inhibited the enhancement of ornithine decarboxylase activity. On the other hand, when valinomycin was added to L1210 cells in which activity had already been enhanced, there occurred a decay in ornithine decarboxylase activity with a $T_{1/2}$ value of 40–45 min. The concurrent presence of 10 mM asparagine counteracted this inhibitory effect of valinomycin on ornithine decarboxylase activity and extended the $T_{1/2}$ value of ornithine decarboxylase activity to 90–95 min (Table II). Similarly, the presence of asparagine also prolonged the half-life of ornithine decarboxylase activity of N-18 cells when valinomycin was added to the cell cultures maintained in the salts/glucose solution (Table II). These findings are consistent

TABLE II

$t_{1/2}$ VALUES OF ORNITHINE DECARBOXYLASE ACTIVITY IN L1210 AND N-18 CELLS

Ornithine decarboxylase activity of L1210 cells was stimulated for 2 h, various drugs were added to the cultures. At 15 min intervals, cells were harvested for assay. $T_{1/2}$ values were estimated from the enzyme decay kinetics. Ornithine decarboxylase activity of N-18 cells was stimulated by adding 10 mM asparagine to the cultures maintained in the salts/glucose solution. 6 h after incubation, cells were then rinsed twice with fresh salts/glucose solution and reincubated in fresh salts/glucose solution in the presence of various drugs as indicated in the table. At 15 min intervals, cells were harvested for assay. $T_{1/2}$ values were estimated from the enzyme decay kinetics.

Additions	$T_{1/2}$ values (min)	
	L1210 cells	N-18 cells
Cycloheximide (50 μg/ml)	40–45	30
Valinomycin ($2 \cdot 10^{-8}$ M)	40–45	30
Cycloheximide (50 μg/ml) + asparagine (10 mM)	90–95	400–420
Valinomycin ($2 \cdot 10^{-8}$ M) + asparagine (10 mM)	90–95	400–420

with our previous observations that ornithine decarboxylase activity in cultured cells can be stabilized by the presence of its inducer, asparagine [17]. They also lend support to the notion that the decay of ornithine decarboxylase activity in the presence of valinomycin (or cycloheximide) is not a consequence of the inhibition of protein synthesis, since it can be prevented by the addition of the inducing agent.

Macromolecular syntheses and the stimulation of ornithine decarboxylase activity

Fig. 3 shows that $1 \cdot 10^{-8}$ M valinomycin inhibited ornithine decarboxylase activity by more than 70% whereas RNA and protein synthesis were only slightly inhibited (less than 20%). This result, however, cannot distinguish the following two possibilities:

(i) The inhibitory effect of valinomycin on the enhancement of ornithine decarboxylase activity is not related to the inhibition of macromolecular synthesis.

(ii) The synthesis of ornithine decarboxylase is much more sensitive to the inhibitory effect of valinomycin than general protein synthesis.

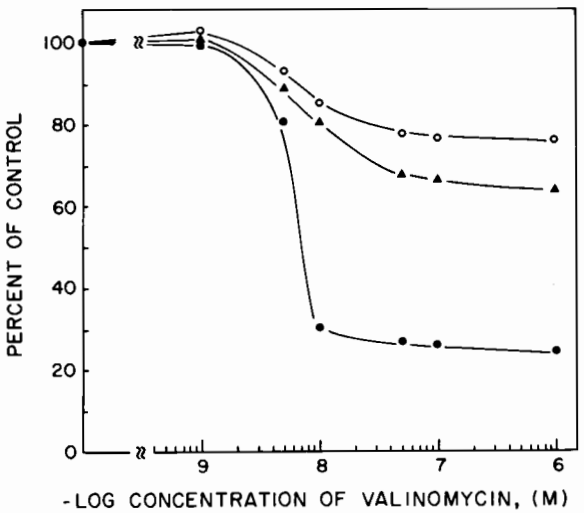

Fig. 3. Effect of valinomycin on the ornithine decarboxylase activity, RNA and protein syntheses of L1210 cells. Ornithine decarboxylase activity, (●——●); [^{14}C]uridine incorporation, (○——○): [^{3}H]leucine incorporation (▲——▲).

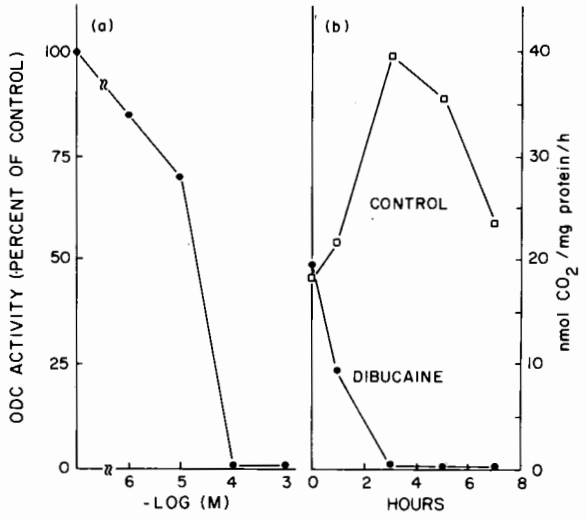

Fig. 4. Effect of dibucaine on the serum-stimulated increase of ornithine decarboxylase (ODC) activity. (a) Dose-response study, ornithine decarboxylase activity in L1210 cells was stimulated by dilution with fresh Fisher's medium plus 10% horse serum in the presence of the indicated concentrations of dibucaine (●——●). Enzyme activity was measured 4 h after incubation; (b) the time-course experiment was performed in the presence of $1 \cdot 10^{-4}$ M dibucaine.

Effect of local anesthetics on the enhanced ornithine decarboxylase activity

Additional evidence for the regulation of ornithine decarboxylase activity through membrane-associated sites comes from the membrane perturbing effect of dibucaine and tetracaine on ornithine decarboxylase activity. Fig. 4 shows that the stimulation of ornithine decarboxylase activity was inhibited 100% at $1 \cdot 10^{-4}$ M and 45% at $1 \cdot 10^{-5}$ M dibucaine, within 4 h. These concentrations of dibucaine have been reported to increase the fluidity of the cell membrane [22]. Similar results were obtained with tetracaine (data not shown).

Discussion

The observation that the increase of ornithine decarboxylase activity is inhibited by micromolar concentrations of microtubule-disrupting agents [9] suggests that the plasma membrane/cytoskeletal structure may play a role in the regulation of intracellular ornithine decarboxylase activity [9,10].

Additional evidence for such an association has been sought in the present study by relating membrane perturbations, caused by membrane-specific drugs, to the change of intracellular ornithine decarboxylase activity.

Possible sources of membrane perturbation are changes in surface charges, in membrane potential and in ion permeability. Indeed, Chen, et al. [4] have shown that small increases of Na^+, K^+ or Mg^{2+} (5–20 mM) in the growth medium, eliminated the anticipated increase of ornithine decarboxylase activity that normally occurred upon the dilution of cultured cells from the stationary phase.

In the present report we have chosen to study the effects of two different classes of well-known membrane perturbing agents, ionophores and local anesthetics, on the regulation of ornithine decarboxylase activity.

Cationic local anesthetics display a complex interaction with phospholipid membranes [23], and at low concentrations, disrupt the coupling between surface membrane receptors and the cytoskeletal structure [22]. Other observations indicate that they can cause marked changes in both surface charges and ion permeability [24]. Their inhibitory effect on the increase of ornithine decarboxylase activity would be in keeping with the known dependence of ornithine decarboxylase activity on cation transport [4].

Ionophores are highly selective cation carriers, the presence of which in the plasma membrane causes specific cation fluctuations by altering ion permeability or cation exchange rates [19,20]. The present experimental evidence indicates that ionophores are potent inhibitors of the enhancement of intracellular ornithine decarboxylase activity, valinomycin being the most potent of those tested. None of the ionophores inhibited ornithine decarboxylase activity when tested in in vitro assays (data not shown); consequently, we are not examining a direct inhibition of ornithine decarboxylase by interaction with the ionophores. Uncoupling of oxidative phosphorylation by valinomycin, which occurs at micromolar concentrations [25], is not a likely cause of inhibition of ornithine decarboxylase activity, since the inhibition occurs at concentrations of valinomycin that are about three orders of magnitude lower. Other known functions of valinomycin on cultured cells involve its association with the plasma membranes [19,20], its inhibition of protein synthesis [26], and its specificity for K^+ binding [27]. Among these properties of valinomycin, its specificity for K^+ binding appears less likely to be a cause for the inhibition of ornithine decarboxylase activity for the following reasons:

Although both valinomycin and dicyclohexyl-18-crown-6 are K^+ specific ionophores with slight differences in binding constants (K_D for K^+ complex formation valinomycin is 49 mM, dicyclohexyl-18-crown-6 is 200 mM), valinomycin was more than 1000-fold as effective as an inhibitor of ornithine decarboxylase when compared to dicyclohexyl-18-crown-6 (Fig. 1). Furthermore, it should be noted that another class of monovalent cation ionophores, nigericin, monesin and ionophore A204, that are less selective for K^+ and differ in their binding affinity to various monovalent cations, gave almost identical dose-response curves and larger I_{50} values compared to valinomycin. Thus it appears that the cation specificity of ionophores may not be the primary reason for the inhibition of ornithine decarboxylase activity.

Our results with five different monovalent ionophores and one divalent ionophore also indicate that, although they differed in their potency on the inhibition of the enhancement of ornithine decarboxylase activity, all gave similar inverted S-shaped dose-response curves. This phenomenon suggests that all of the ionophores may exert their inhibitory effect by a similar mechanism.

Breitbart and Herzberg [26] have reported that $1 \cdot 10^{-6}$ M valinomycin has no effect on ^{42}K exchange, while it reversibly inhibits protein synthesis. On the other hand, $1 \cdot 10^{-5}$ M valinomycin inhibits ^{42}K exchange and irreversibly inhibits protein synthesis. The dose-response curve that we obtained when studying the effect of valinomycin on ornithine decarboxylase activity, on protein synthesis, and on RNA synthesis (Fig. 3), indicates that the inhibition of ornithine decarboxylase activity occurs at lower concentrations than the inhibition of protein synthesis. Such a disparity suggests that the inhibition of ornithine decarboxylase activity occurs before the general protein synthesis is turned off. This would imply

that either the enhancement of ornithine decarboxylase activity is independent of protein synthesis or alternatively, the synthesis of ornithine decarboxylase is more sensitive to the effect of valinomycin than is the general protein synthesis. In either case, the fluctuation of cations is probably involved.

We have previously proposed that fluctuation of intracellular cation pools may act as a linkage between plasma membrane/cytoskeletal structure and intracellular ornithine decarboxylase activity. The present studies using local anesthetics and ionophores further substantiate the notion that ornithine decarboxylase activity can be controlled at plasma membrane level and such control is related to the perturbation of intracellular cation pools. The detailed molecular mechanism, however, remains to be elucidated.

Acknowledgment

This work was supported by American Cancer Society grant BC and USPHS grant CA-04823 and a USPHS Research Careers Award to E.S.C.

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