PHOSPHOLIPASE A ACTIVITY IS NOT ASSOCIATED WITH EARLY EFFECTS OF COLICIN El.

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SUMMARY

A comparison was made of the time course of phospholipid changes and other early biochemical changes caused by colicin El, conveniently monitored through changes in fluorescence intensity of the probe N-phenyl-1-naphthylamine. Under conditions where the fluorescence intensity attained half of its maximum value in 2 minutes, there was very little change in lysophosphatidyl ethanolamine level for 30 minutes. It is concluded that changes in phospholipase A activity are not associated with the early biochemical effects of colicin El. Phosphatidic acid levels decrease slightly after addition of colicin El, showing that an increase in phospholipase D activity also does not occur.

INTRODUCTION

The question of how very small amounts of exogenously added colicin can cause profound and specific intracellular effects is one which bears on the general problem of ligand-membrane and protein-membrane interactions. Two general mechanisms which have been proposed to explain the colicin effects are: (A) Structural or conformational changes in the cell envelope which extend over a large part of the cell surface (1,2). (B) Degradative enzymatic activity intrinsic to the colicins or induced by them; in degrading DNA (3,4) and ribosomal RNA (5-11) colicins E2 and E3 exert effects which appear to be the consequence of such enzymatic activity. It has been reported that effects of colicins El and K also are associated with degradative enzymatic activity, a phospholipase A activity which results in increased levels of lysophosphatidyl ethanolamine (12). The phospholipase activity was

documented more carefully for colicin K (12). The rate of change of lysophosphatidyl ethanolamine (LPE) level shown in ref. 12 at high multiplicities of colicin K can be compared with the rate at which this colicin exerts a biochemical effect, for example, release of intracellular potassium (13). The half-time for the increase in LPE obtained in ref. 12 is about 15 min. at 37° with 60 killing units of colicin K, compared with one min. for potassium efflux at comparable multiplicities (13). Based on this literature comparison, it would seem that the phospholipase activity associated with colicin K action may be a secondary effect. In this work we report simultaneous measurements of the rate of phospholipid changes caused by colicin El and the rate of fluorescence intensity change of a bound fluorescent probe N-phenyl-1-naphthylamine. The kinetics of the fluorescent probe response have been shown to be very similar to the kinetics of the colicin El- induced changes in ATP and potassium level, and the probe response itself may reflect the primary structural changes in the cell envelope caused by colicin El (14-16).

METHODS

(1) Growth conditions, media, colicin:

E. coli B/1,5, kindly sent to us by Dr. Simon Silver, was grown for 6 generations to about 10^9 cells/ml in 100 ml of the following medium: 0.1 M morpholinopropane sulfonic acid buffer, titrated to pH 7.0 with KOH, 5 mC of ^{32}P orthophosphate (Schwartz-Mann), and (in g/l) glucose, 2.0; NH_4Cl, 1.0; NaCl, 0.5; $MgSO_4$, 0.22; Na_2HPO_4, .08. The cells were harvested by centrifugation, washed once, concentrated 10-fold, and resuspended in the above medium without ^{32}P at a concentration of about 10^{10}/ml. Colicin was purified according to the procedure of Schwartz and Helinski (17).

(2) Fluorescence measurements: The fluorescence measurements were made in a Shimadzu spectrophotometer operated in the single beam mode as

described previously (14). The fluorescence probe used was N-phenyl-1-naphthylamine (Eastman) used without further purification at a concentration of 4 μM. The stirred sample (5.5 ml) in a one by two cm. cuvette was exposed to excitation light at 363 nm, with the emission defined by a 2 mm thick 2 M solution of sodium nitrite and a Corning 3-75 filter.

(3) Measurement of phospholipase activity: Aliquots for lipid analysis were taken from the cuvette during measurement of the fluorescence intensity change. The cuvette was masked so that the removal of the samples for analysis did not change the area of the sample exposed to the detector. 0.1 ml samples were taken for both protein and lipid analysis before the addition of dye and colicin El and at intervals thereafter. Samples were placed in 10 ml chloroform-methanol (1:1, v/v) and held for 6 hr. Insoluble material was removed by centrifugation and soluble nonlipid materials were removed by washing (18). Carrier E. coli lipids plus phosphatidic acid were added, extracts were concentrated, and separated by two-dimensional chromatography (19). All known phospholipids of E. coli (20) were separated cleanly from each other in the solvent systems used. Individual phospholipids were localized by brief exposure of plates to iodine vapors and outlined. After sublimation of iodine, areas of silica gel containing phospholipids were scraped into vials. Radioactivity was determined by liquid scintillation counting in a toluene-PPO-POPOP mixture which contained hydroxide of hyamine.

RESULTS AND DISCUSSION

The time course of the colicin El-induced increase in fluorescence intensity of bound N-phenyl-1-naphthylamine and of lysophosphatidyl ethanolamine is shown in Fig. 1. There is very little change for 30 minutes in the LPE level compared to the control sample taken just before addition of colicin. The colicin-induced increase in probe fluorescence

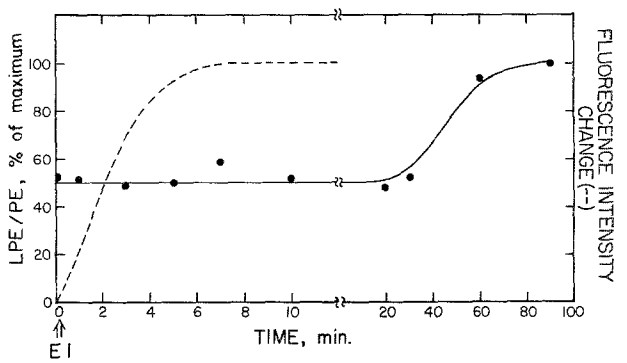

Figure 1. Time course of increase of fluorescence and LPE level caused by colicin El. Cell survival assayed 15 min. after colicin addition, 1%. Conditions otherwise as in METHODS.

intensity attains one-half of its maximum value in two minutes. The fluorescence change reaches a plateau value after four minutes at which time the initial fluorescence intensity is doubled. Colicin El does cause a substantial increase in LPE level, as reported by Cavard et al. (12), but under our experimental conditions where a much lower multiplicity ($m \simeq 5$) of colicin has been used, the changes in LPE level occur 30 min. or more after colicin addition.

The absolute levels of LPE:PE measured as a function of time after colicin addition are shown in Table 1. The levels shown are an average of two experiments. In each experiment there was no change ($\pm$ 20%) for 30 minutes. The uncertainty in absolute level is also about 20%. The levels are somewhat smaller than those reported by Cavard et al. (12). The oppositely directed changes in cardiolipin (increasing levels) and phosphatidyl glycerol (decreasing) shown in Table 1 are in approximate agreement with the effects of colicin K on these phospholipids (12), although the PG level measured here is larger. Cardiolipin and PG changes do occur within 3-5 minutes after adding colicin, although they plateau well after the fluorescence probe response. Cavard et al. (12) showed that a number of other agents besides colicins K and El which

Table 1

Phospholipid Levels as a Function of Time after Addition of Colicin E1.

Sample interval	LPE/PE	CAR/PE	PG/PE	CAR+PG/PE	PA/PE
min	x 10^3	x 10^2	x 10^1	x 10^1	x 10^3
0, Control	2.45	7.34	2.46	2.97	1.82
1	2.38	7.88	2.54	2.77	1.94
3	2.29	9.21	2.35	2.90	1.62
5	2.34	9.15	2.19	2.76	1.63
7	2.75	11.47	2.10	2.81	1.00
10	2.43	10.07	2.16	2.79	1.49
20	2.25	11.12	2.03	2.77	1.24
30	2.41			2.74	
60	4.37			3.13	
90	4.67			3.03	

Abbreviations: LPE, lysophosphatidyl ethanolamine; PE, phosphatidyl ethanolamine; CAR, cardiolipin; PG, phosphatidyl glycerol; PA, phosphatidic acid.

would decrease ATP level or disrupt cellular integrity or metabolism, such as dinitrophenol, chloramphenicol, penicillin, or deprivation of carbon sources, would cause similar changes in cardiolipin and PG. Continued formation of cardiolipin from phosphatidyl glycerol has also been observed when the culture was resuspended in saline solution in the absence of an energy source (21). The changes in cardiolipin and PG levels after colicin addition may then be secondary consequences of the change in ATP level. The existence of oppositely directed changes in cardiolipin and PG levels is qualitatively consistent with a mode of cardiolipin synthesis from phosphatidyl glycerol (22,23).

The comparison of the time course of the changes in probe

fluorescence and LPE levels caused by colicin E1 (Fig. 1) implies that any increase in phospholipase A activity caused by colicin E1 is a secondary effect occuring well after this colicin has caused changes in envelope structure and exerted its complete effect on intracellular ATP and potassium levels. That the LPE changes might be secondary was originally suggested by comparisons (16) of the rates of the LPE changes (12) for colicin K with the faster response for potassium (13) and ATP (24) changes. Scandella and Kornberg have purified a phospholipase A1 from E. coli which they have suggested might be responsible for changes in membrane integrity following phage infection and colicin addition (25). It has been shown that the changes in free fatty acid which occur after infection with phage T4 (26) or Salmonella phage P22 (27) are associated with lysis and not with primary penetration events. The increase in LPE 30 minutes after addition of fairly low multiplicities of colicin in Fig. 1 may be a consequence of a change in cellular integrity.

Additional evidence which argues against a role for phospholipase A activity in mediating the primary effects of colicin E1 comes from measurements of colicin E1-induced change in the polarization of fluorescence of the probe N-phenyl-1-naphthylamine bound to E. coli. Colicin E1 causes an increase in the fluorescence polarization and the lifetime of fluorescence, indicating that the colicin causes an increase in effective rigidity or viscosity of the cell envelope (manuscript in preparation). It would be expected that substitution of lysophospholipid in a membrane would decrease rather than increase the microviscosity and this has been documented by fluorescence polarization and lifetime measurements in the case of dispersions of egg lecithin and lysolecithin (28).

The other data shown in Table 1 demonstrate that the cardiolipin plus phosphatidylglycerol: PE ratio showed no net change during 90 minutes of incubation with colicin, indicating the absence of any extensive net degradation of these phospholipids. The last column shows that the trace

component detected in the lowest amounts, phosphatidic acid (PA) present at 1-2 parts per thousand of PE, shows a small decrease after about 10 minutes. The absence of any increase in phosphatidic acid levels would suggest that significant phospholipase D-mediated degradation of E. coli phospholipids can be ruled out as a factor in colicin El- induced lethality.

It is concluded from the above data that there is at present no evidence in favor of degradative enzymatic activity intrinsic to or induced by colicin El as part of its primary mode of action.

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