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CELLULAR UPTAKE OF CYCLIC AMP CAPTURED WITHIN PHOSPHOLIPID VESICLES AND EFFECT ON CELL-GROWTH BEHAVIOUR

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SUMMARY

Cyclic 3′,5′-adenosine monophosphate (cyclic AMP) was successfully incorporated within unilamellar phospholipid vesicles. Treatment of 3T3 and SV40-transformed 3T3 cells (SV3T3 cells) with vesicles containing cyclic AMP resulted in significant intracellular incorporation of vesicle-associated cyclic AMP and alteration of cell-growth behavior. Vesicles containing cyclic AMP caused a significant reduction in the growth rate of 3T3 and SV3T3 cells and effectively inhibited growth stimulation in stationary 3T3 cell cultures treated with insulin, serum and proteolytic enzymes. These alterations in cell-growth behaviour were produced by vesicle cyclic AMP at concentrations as low as 10^{-7} M. Similar modification of cell growth by addition of cyclic AMP or dibutyryl-cyclic AMP to the culture medium was achieved only at concentrations as high as 10^{-4} M plus 10^{-3} M theophylline. The ability of cyclic AMP trapped within vesicles to modify cell behaviour was influenced by the lipid composition of the vesicles. Cyclic AMP-containing vesicles composed of lipids that were in a "fluid" state at 37 °C produced marked growth inhibition while similar concentrations of cyclic AMP within vesicles prepared from "solid" lipids had no effect on cell proliferation. The influence of vesicle lipid composition on the mechanisms by which vesicles may be incorporated in to cells was discussed.

INTRODUCTION

Experimental studies on the interaction of biologically active molecules with mammalian cells cultured in vitro are often hindered by the failure of cells to incorporate molecules added to the culture medium. Even when incorporation does occur the molecules may not always be distributed to the required intracellular sites. These problems could be reduced if appropriate molecules could be trapped within a carrier vehicle which would not only protect the contents from the external environment but which would also be incorporated intracellularly by different mechanisms so that the contents would be delivered to specific intracellular compartments. For example, a single-walled carrier vehicle which could fuse with the cellular plasma

Abbreviation: TES, *N*-tris-(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid.

membrane would introduce its contents into the general cytoplasm, while the contents of a carrier vehicle incorporated by endocytosis would be concentrated instead in the lysosomal digestive apparatus of the cell. Certain of these requirements may be fulfilled by unilamellar phospholipid vesicles. These are small (approx. 250–300 Å diameter) spherical structures composed of a single bilayer of phospholipid molecules enclosing a single aqueous space within which a variety of water-soluble substances can be trapped [1, 2]. In this communication we describe the use of similar lipid vesicles containing cyclic adenosine 3′,5′-monophosphate (cyclic AMP) to modify the growth behaviour of cultured cells under conditions where cyclic AMP and dibutyryl-cyclic AMP added to the culture medium produced no detectable effect.

Several lines of evidence suggest that the intracellular concentration cyclic AMP is an important factor in cell-growth regulation. Intracellular cyclic AMP levels in slowly growing or non-dividing cells are significantly higher than in dividing cells [3–5] and the intracellular concentration of cyclic AMP varies throughout the cell cycle reaching a minimum during mitosis [6]. Tumor cells and virus-transformed cells have significantly lower levels of cyclic AMP than untransformed cells [4, 7], even when in the non-dividing state [8]. Several studies have also demonstrated that addition of exogenous cyclic AMP or, more commonly, dibutyryl-cyclic AMP, to the culture medium reduces the growth rate of both normal and transformed cells [9–15] and causes phenotypic reversion of certain of the altered morphological [14] and surface properties [15, 16] of transformed cells, so that they more closely resemble untransformed cells. However, these changes are produced only by high exogenous cyclic nucleotide concentrations (10^{-3} or 10^{-4} M) and little or no effect is obtained at concentrations below 10^{-5} M [10, 12, 17]. The results described here using phospholipid vesicles as carriers to enhance intracellular cyclic AMP levels show that similar changes in cell behaviour can be achieved with initial cyclic AMP concentrations as low as 10^{-7} M.

MATERIALS AND METHODS

Cells

BALB/c mouse 3T3 cells and 3T3 cells transformed by Simian virus 40 (SV3T3 cells) were cultured in 60-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) at 37 °C in a humidified 5 % CO_2 atmosphere in Dulbecco's modification of Eagles medium supplemented with 10 % calf serum as described previously [18]. Mouse L929 cells were grown in culture tubes in RPMI 1640 medium supplemented with 10 % calf serum on a roller incubator rotating at 12 rev./h. Cell culture media and sera were obtained from the Grand Island Biological Co., Grand Island, N. Y. To measure the number of cells present in cultures, cells were first detached from the surface of the culture vessel using 0.2 % EDTA in Ca^{2+}, Mg^{2+}-free saline for 3T3 and SV3T3 cultures or 0.2 % trypsin in Ca^{2+}, Mg^{2+}-free saline for L929 cultures an the number of cells measured by haemocytometer counts for 3T3 and SV3T3 [18]. L929 Cells were counted using a Coulter Model B electronic particle counter calibrated to provide equivalent counts to the mean of six haemocytometer counts of the same cell type. Between two and eight replicate cultures were counted to provide mean values of cell numbers at each time interval or treatment in any given experiment. Cell viability was determined as described elsewhere [19].

Preparation and properties of lipid vesicles

Unilamellar lipid vesicles were made as described previously [20] by sonication of multilamellar lipid vesicles (liposomes). The lipid composition of the vesicles used in the present experiments was selected on the following criteria. Egg phosphatidylcholine and dipalmitoylphosphatidylcholine were selected as the major components for "fluid" and "solid" vesicles, respectively, since at the experimental temperatures they are respectively above and below their gel–liquid crystalline transition temperature (T_c). The inclusion of positively charged stearylamine molecules in both "solid" and "fluid" vesicles was designed to facilitate capture of negatively charged cyclic AMP molecules [21], while cholesterol was included to inhibit the ability of various proteins (such as those present in serum added to the culture medium) to increase vesicle permeability [22].

To prepare "fluid" vesicles containing cyclic AMP, 4 μmoles of egg phosphatidylcholine, 1 μmole stearylamine and 3 μmoles cholesterol were mixed in chloroform, evaporated to dryness in vacuum and suspended under nitrogen in 0.5 ml of the cyclic AMP-containing buffer solution. The latter contained cyclic AMP (90 mM), NaCl (10 mM), L-histidine (2 mM), *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) (2 mM), and EDTA (0.1 mM) adjusted to pH 7.4 with 1 M NaOH, and was stored at −10 °C until use. The suspension of lipids in cyclic AMP-containing buffer was then mechanically shaken for 10 min at 37 °C and finally sonicated in a bath-type sonicator for 1 h at 37 °C under nitrogen [20]. The suspension was then diluted with 1.5 ml of buffer containing 100 mM NaCl, pH 7.4, and left at room temperature (21 °C) for 1 h. It was then passed through a Sephadex G-50 column, which effectively separated the vesicles (together with approx. 20 % of the original cyclic AMP) from the free cyclic AMP. The lipid peak was eluted with the void volume and contained 1 μmole of phosphate per ml. This preparation was then used to treat cells either undiluted or diluted as described previously [19] except that the cells were incubated continuously in medium containing vesicles. The intracellular incorporation of vesicles containing ^{3}H-labeled cyclic AMP or radiolabeled lipid molecules was measured after incubation of cells with vesicles under the conditions outlined in Results, followed by detachment of cells from the surface of the culture vessel after which they were washed three times with ice-cold PBS, suspended in Brays solution (L929 cells) or "Aquasol" (New England Nuclear liquid scintillation fluid (3T3 and SV3T3 cells) and the amount of cell-associated radioactivity determined in a Beckman LS230 or Packard Tricarb liquid scintillation counter.

The lipid mixture used to prepare "solid" vesicles plus entrapped cyclic AMP contained 4 μmoles dipalmitoylphosphatidylcholine, 1 μmole of stearylamine and 4 μmoles cholesterol and this was suspended in cyclic AMP-containing buffer and treated identically to the "fluid" vesicles described above.

For vesicles containing ^{3}H-labeled cyclic AMP or [^{3}H]dipalmitoylphosphatidylcholine or [^{14}C]cholesterol, the appropriate isotopes were added to the organic solvent before evaporation of the lipid mixture to dryness and suspension in aqueous medium. Both "fluid" and "solid" vesicles were relatively impermeable to the cyclic AMP captured within the vesicles. Dialysis of vesicles indicated an 0.8 % per hour "leak" of captured cyclic AMP from vesicles at 37 °C and 0.5 % per hour at 0 °C. Overnight dialysis at 4 °C revealed a 4 % total "leak". In contrast, 80 % of free

cyclic AMP diffused through the dialysis bags within 1 h at 37 °C.

As shown previously [21, 23, 24] our method of sonication converts more than 95 % of the phospholipid into unilamellar vesicles with an external diameter of approx. 250 Å and a captured volume of 0.2–1.0 μl per μmole phosphate. Since vesicles of this size contain approx. 3000 lipid molecules [23, 25] it can be calculated that there are $2 \cdot 10^{14}$ vesicles per μmole of phospholipid. The amount of captured cyclic AMP (measured as ^{3}H-labeled cyclic AMP) under the conditions used here was 0.2 μmole cyclic AMP per μmole of phospholipid which is equivalent to a captured cyclic AMP concentration (vesicle cyclic AMP) in the undiluted vesicle dispersion of $2 \cdot 10^{-4}$ M or $1 \cdot 10^{-9}$ pmole of cyclic AMP per vesicle.

The transition temperature (T_c) of individual lipids and the vesicles used in experiments was determined by differential scanning calorimetry as described before [24]. Egg phosphatidylcholine, with or without stearylamine, became "solid" only at temperatures below 0 °C. Dipalmitoylphosphatidylcholine preparations used in the present experiments showed an endothermic peak at 42 °C which was broadened by addition of cholesterol and completely abolished at a molar ratio of 1 : 1. Previous studies have shown that dipalmitoylphosphatidylcholine–cholesterol membranes are rigid (motion inhibited), although not crystalline [26–28]. The presence of high concentrations of cyclic AMP (90 mM) was found to have no effect on the thermotropic properties of the lipids used here.

Lipids and other chemicals

Cholesterol (U.S.P. crystalline) and palmitic acid (puriss) were obtained from Fluka A.G., Switzerland. Cholesterol was recrystallized twice from methanol before use. Stearylamine (cetylamine) was obtained from K and K laboratories, Plainview, N. Y. Phosphatidylcholine was extracted from egg yolk as described [29]. Dipalmitoylphosphatidylcholine was synthesized by the method of Robles and Van den Berg [30] and contained more than 99 % palmitic acid. Both phospholipids were purified on silicic acid and found to be pure on thin-layer chromatography. [8-^{3}H]Adenosine-3′,5′-cyclic phosphate (^{3}H-labelled cyclic AMP) as ammonium salt in 50 % aqueous (spec. act. 30 Ci/mmole) and [4-^{14}C]cholesterol (spec. act. 61 Ci/mole) were obtained from Amersham–Searle, Arlington Heights, Ill. [^{3}H]-Dipalmitoylphosphatidylcholine was prepared by catalytic hydrogenation of dipalmitolcylphosphatidylcholine by New England Nuclear Corp., Boston, Mass. This lipid was synthesized and purified by methods identical to those used for dipalmitoleylphosphatidylcholine. After tritiation, the final product was repurified on silicic acid and the final preparation had a specific activity of 4 Ci/μmole. Adenosine-3′,5′-cyclic monophosphate, sodium salt (cyclic AMP); N^6,$O^{2'}$-dibutyryl adenosine-3′,5′-cyclic monophosphate, monosodium salt; theophylline; crystalline bovine insulin; L-histidine were obtained from the Sigma Chemical Co., St. Louis, Mo. and pronase (grade B) from Calbiochem, La Jolla, Calif. All other chemicals were reagent grade. Water was twice distilled, the second time in an all-glass apparatus.

RESULTS

Effect of vesicle cyclic AMP on cell proliferation: comparison with effects of free cyclic nucleotides

Treatment of 3T3 and SV3T3 cell cultures with "fluid" lipid vesicles con-

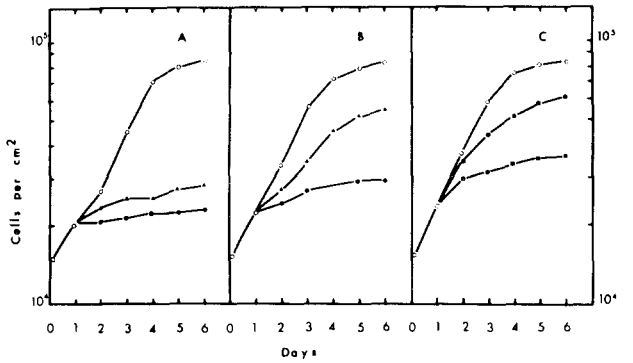

Fig. 1. Growth rate of mouse 3T3 cells in the presence of different concentrations of "fluid" lipid vesicles containing cyclic AMP (A), free dibutyryl-cyclic AMP plus 10^{-3} M theophylline (B) and free cyclic AMP plus 10^{-3} M theophylline (C). Control cell cultures (○–○) were grown in medium without cyclic nucleotides. Cyclic nucleotide concentrations ■–■, 10^{-3} M; ●–●, 10^{-4} M; and ▲–▲, 10^{-5} M. 60-mm plastic Petri dishes were seeded at day 0 with $3 \cdot 10^5$ 3T3 cells in normal medium. At day 1 the medium in the experimental cultures was replaced with medium containing differing concentrations of free cyclic AMP or dibutyryl-cyclic AMP or lipid vesicles (phosphatidylcholine, stearylamine, cholesterol) containing entrapped cyclic AMP as outlined in Methods. The number of cells per dish was measured at daily intervals in both the experimental and the untreated control cultures and the results expressed as the number of cells per cm^2 of the culture dish. Each point represents a mean value obtained from cell counts on 12 Petri dish cultures in three separate experiments.

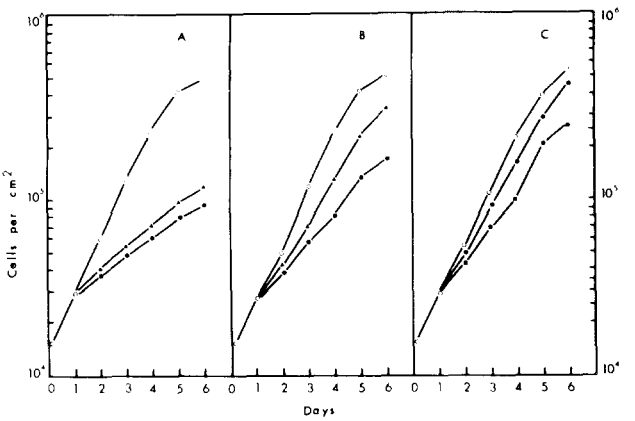

Fig. 2. Growth rate of SV3T3 cells in the presence of different concentrations of "fluid" lipid vesicles containing cyclic AMP (A), free dibutyryl-cyclic AMP plus 10^{-3} M theophylline (B) and free cyclic AMP plus 10^{-3} M theophylline (C). Control cell cultures (○–○) were grown in medium without cyclic nucleotides. Cyclic nucleotide concentrations: ■–■, 10^{-3} M; ●–●, 10^{-4} M; and ▲–▲, 10^{-5} M. The experimental conditions and techniques were identical to those described in the legend to Fig. 1.

taining cyclic AMP at an external concentration of 10^{-4} or 10^{-5} M caused a significant reduction in their growth rate and final density (Figs. 1A and 2A). The extent of growth inhibition in 3T3 and SV3T3 populations treated with vesicle cyclic AMP at these concentrations was comparable to that produced by addition of 10^{-4} M

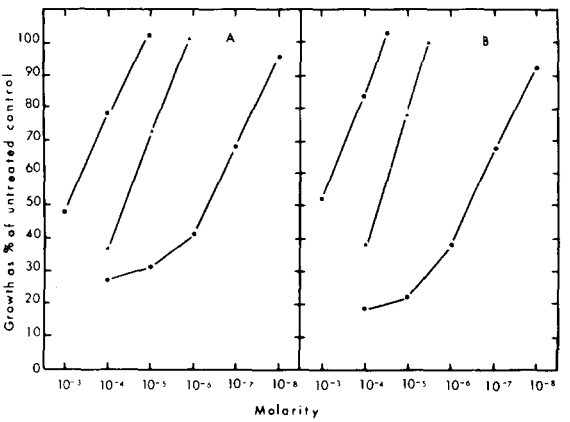

Fig. 3. Effect of increasing molar concentrations of cyclic AMP trapped within "fluid" lipid vesicles (●–●), free dibutyryl-cyclic AMP plus 10^{-3} M theophylline added to the culture medium (▲–▲), or cyclic AMP plus 10^{-3} M theophylline added to the culture medium (■–■) on the growth of mouse 3T3 (A) and SV3T3 cells (B). The number of cells in experimental cultures was measured after 5 days incubation in medium supplemented with the various cyclic nucleotide preparations and expressed as a percentage of the number in control cell cultures grown for the same period in medium without cyclic nucleotides. Each point represents a mean value obtained from 12 Petri dish cultures in three separate experiments.

dibutyryl-cyclic AMP and 10^{-3} M theophylline to the culture medium (Figs 1B and 2B). However, exogenous dibutyryl-cyclic AMP (10^{-5} M) and cyclic AMP (10^{-3} or 10^{-4} M) were less effective in inhibiting cell proliferation than vesicle cyclic AMP at comparable concentrations, even when supplemented with 10^{-3} M theo phylline (Figs 1B, 1C, 2B and 2C).

Treatment of control 3T3 and SV3T3 cell populations with similar numbers of lipid vesicles without cyclic AMP, or a mixture of vesicles suspended in medium containing free cyclic AMP (10^{-4} or 10^{-5} M) or incubation of cells in the buffer used to suspend the vesicles had no effect on cell growth rate, indicating that the reduced growth rates shown in Figs 1A and 2A for cells treated with vesicles containing cyclic AMP were due to the entrapped cyclic AMP.

Further experiments revealed that the growth of 3T3 and SV3T3 cell populations could be reduced by incubating cells with vesicles containing cyclic AMP at concentrations as low as 10^{-7} M (Fig. 3). This contrasts with the effect of free cyclic AMP and dibutyryl-cyclic AMP added to the culture medium which were unable to inhibit cell proliferation at concentrations below 10^{-4} and 10^{-5} M, respectively (Fig. 3). Cells treated with vesicles containing cyclic AMP showed no significant reduction in viability compared with untreated control cultures. In addition, observations on the plating efficiency of vesicle-treated SV3T3 cells after returning them to normal culture medium confirmed that their viability was similar to that of untreated control cells.

Measurement of the amount of ^{3}H-labeled cyclic AMP within the lipid vesicles incorporated by 3T3 and SV3T3 cells revealed significantly greater intracellular incorporation of ^{3}H-labeled cyclic AMP than in cell populations incubated with the same concentrations of ^{3}H-labeled cyclic AMP added to the culture medium (Table 1).

TABLE I

INCORPORATION OF ^{3}H-LABELED CYCLIC AMP ADDED TO THE CULTURE MEDIUM (FREE CYCLIC AMP) AND ^{3}H-LABELED CYCLIC AMP WITHIN PHOSPHOLIPID VESICLES BY 3T3 AND SV3T3 CELLS

Treatment[a]	Concentration (M)	Cell type	Percentage incorporation[b]	Cell growth as percentage of untreated control[c]	^{3}H-labeled cyclic AMP per 10^6 cells[d] (pmoles)	Ratio of incorporated vesicle cyclic AMP: free cyclic AMP	Projected number of vesicles per cell[e]
Vesicle cyclic AMP	10^{-4}	3T3	2.61	26	$5.21 \cdot 10^3$	6.98	$5.21 \cdot 10^6$
Free cyclic AMP	10^{-4}	3T3	0.37	75	$7.45 \cdot 10^2$		–
Vesicle cyclic AMP	10^{-4}	SV3T3	2.40	19	$4.79 \cdot 10^3$	8.32	$4.79 \cdot 10^6$
Free cyclic AMP	10^{-4}	SV3T3	0.29	79	$5.76 \cdot 10^2$		–
Vesicle cyclic AMP	10^{-5}	3T3	4.91	32	$9.82 \cdot 10^2$	12.08	$9.82 \cdot 10^5$
Free cyclic AMP	10^{-5}	3T3	0.41	103	$8.13 \cdot 10^1$		–
Vesicle cyclic AMP	10^{-5}	SV3T3	5.39	26	$1.07 \cdot 10^3$	13.72	$1.07 \cdot 10^6$
Free cyclic AMP	10^{-5}	SV3T3	0.39	94	$7.79 \cdot 10^1$		–

[a] 60-mm plastic Petri dishes were seeded with $3 \cdot 10^5$ 3T3 or SV3T3 cells in normal medium and incubated at 37 °C for 24 h. The culture medium was then replaced with medium containing different concentrations of "fluid" lipid vesicles containing ^{3}H-labeled cyclic AMP (vesicle cyclic AMP) or different concentrations of ^{3}H-labeled cyclic AMP plus 10^{-3} M theophylline (free cyclic AMP). Control cultures were incubated in normal culture medium without cyclic nucleotides. The results represent mean values derived from three separate experiments. 20 replicate 60-mm Petri dish cultures were used per experiment. Eight dishes were used to measure intracellular levels of ^{3}H-labeled cyclic AMP in cells incubated with free cyclic AMP (four dishes) or vesicle cyclic AMP (four dishes). The remaining 12 dishes were used for measurements of cell growth in untreated control cultures (four dishes) and in the experimental cultures incubated with free cyclic AMP (four dishes) or vesicle cyclic AMP (four dishes).

[b] Cell-associated ^{3}H-labeled cyclic AMP expressed as a percentage of the total ^{3}H-labeled cyclic AMP measured after 24 h incubation in medium containing free cyclic AMP or vesicle cyclic AMP.

[c] Calculated from cell counts on control and experimental cultures 6 days after inoculation.

[d] Calculated from the measured values for cell-associated cyclic AMP after 24 h incubation with free cyclic AMP or vesicle cyclic AMP and the measured values for the radioactivity per μmole ^{3}H-labeled cyclic AMP.

[e] Calculated from experimental values of the amount of ^{3}H-labeled cyclic AMP bound per μmole of lipid in the vesicle preparation. 1 vesicle contains $1 \cdot 10^{-9}$ pmole cyclic AMP (see Methods).

Measurement of cellular uptake of free ^{3}H-labeled cyclic AMP or ^{3}H-labeled cyclic AMP within vesicles at concentrations below 10^{-5} M was impossible because of the low number of counts. However, measurements of cellular uptake of lipid vesicles prepared from ^{14}C- or ^{3}H-labeled lipids which also contained cyclic AMP at similar concentrations to those used in the above experiments revealed that the proportion of the vesicle population incorporated by cells increased at lower dilutions (Table II). At all concentrations, a strikingly large number of lipid vesicles were incorporated by cells, but as shown above, cytotoxic effects were not observed. If both vesicles and cells are considered as spheres (an acknowledged simplification in the case of cells) with radii of 150 and 50 000 Å, respectively, then the incorporation of $5.86 \cdot 10^6$ vesicles per 3T3 cell as shown in Line 1 of Table II would increase the volume of the

TABLE II

INCORPORATION OF ^{3}H- AND ^{14}C-LABELED LIPID VESICLES BY MOUSE 3T3 AND SV3T3 CELLS

Cell type	Vesicle concentration (μmoles phosphate per 10^6 cells)	Vesicle cyclic AMP concentration (M)	Percentage incorporation[a]	Number of vesicles per cell[b]	Projected number of pmoles cyclic AMP per 10^6 cells[c]
3T3	0.5	10^{-4}	2.93	$5.86 \cdot 10^6$	$5.86 \cdot 10^3$
SV3T3	0.5	10^{-4}	2.82	$5.64 \cdot 10^6$	$5.64 \cdot 10^3$
3T3	$5 \cdot 10^{-2}$	10^{-5}	5.4	$1.18 \cdot 10^6$	$1.18 \cdot 10^3$
SV3T3	$5 \cdot 10^{-2}$	10^{-5}	6.5	$1.30 \cdot 10^6$	$1.30 \cdot 10^3$
3T3	$5 \cdot 10^{-3}$	10^{-6}	8.9	$1.78 \cdot 10^5$	$1.78 \cdot 10^2$
SV3T3	$5 \cdot 10^{-3}$	10^{-6}	8.2	$1.64 \cdot 10^5$	$1.64 \cdot 10^2$
3T3	$5 \cdot 10^{-4}$	10^{-7}	10.2	$2.04 \cdot 10^4$	20.4
SV3T3	$5 \cdot 10^{-4}$	10^{-7}	10.6	$2.13 \cdot 10^4$	21.3
3T3	$5 \cdot 10^{-5}$	10^{-8}	12.6	$2.51 \cdot 10^3$	2.51
SV3T3	$5 \cdot 10^{-5}$	10^{-8}	13.9	$2.78 \cdot 10^3$	2.78

[a] 60-mm plastic Petri dishes were seeded with $3 \cdot 10^5$ 3T3 or SV3T3 cells in normal medium and incubated at 37 °C for 24 h. The culture medium was then replaced with medium containing different concentrations of "fluid" lipid vesicles with trapped cyclic AMP and also containing trace amounts of [^{3}H]dipalmitoylphosphatidylcholine and [^{14}C]cholesterol and incubated for a further 24 h at 37 C. The amount of cell-associated radioactivity was then determined as described in Methods and expressed as a percentage of the total radioactivity in the original vesicle population. The results represent mean values derived from measurements on six separate cultures at each dilution.

[b] Calculated from the known radioactivity per μmole of lipid; 1 μmole lipid contains $2 \cdot 10^{14}$ vesicles (see Methods).

[c] 1 vesicle contains $1 \cdot 10^{-9}$ pmole cyclic AMP as outlined in the legend to Fig. 1. Compare these values with the measured values given in Table I.

cell by $8.30 \cdot 10^{13}$ Å^3 or 16 % of the total cell volume. More detailed information on the incorporation of lipid vesicles by mammalian cells, together with data on the kinetics of vesicle uptake, will be presented elsewhere (Papahadjopoulos, D., Mayhew, E. and Poste, G., unpublished).

Since each vesicle contains $1 \cdot 10^{-9}$ pmole cyclic AMP then it can also be calculated from the data in Table II that treatment of cells with vesicles at external concentrations of 10^{-6} and 10^{-7} M would result in intracellular incorporation of 178 and 20.4 pmoles of cyclic AMP, respectively, per $1 \cdot 10^6$ 3T3 cells and 164 and 21.3 pmoles of cyclic AMP per $1 \cdot 10^6$ SV3T3 cells (Table II). Increases in intracellular cyclic AMP levels of this order have been detected in previous studies using exogenous cyclic nucleotides at the higher concentrations of 10^{-4} and 10^{-5} M [8, 31]. It is of interest, therefore, to note that the extent of growth inhibition in 3T3 and SV3T3 cell populations produced by 10^{-6} and 10^{-7} M vesicle cyclic AMP is comparable to that obtained in cultures treated with 10^{-4} and 10^{-5} M exogenous dibutyryl-cyclic AMP plus theophylline (Fig. 3).

The projected values for the number of vesicles incorporated per cell listed in Table I which were calculated from the data on ^{3}H-labeled cyclic AMP incorporation agree well with the actual measured values for vesicle uptake given in Table II. The general similarity of these values suggests that little ^{3}H-labeled cyclic AMP

TABLE III

GROWTH OF MOUSE L929 CELLS AFTER TREATMENT WITH PHOSPHOLIPID VESICLES CONTAINING ^{3}H-LABELED CYCLIC AMP (VESICLE CYCLIC AMP) OR EXOGENOUS ^{3}H-LABELED CYCLIC AMP (FREE CYCLIC AMP)

Treatment[a]	Initial density (cells per $cm^2 \cdot 10^4$)	Cell growth as percentage of untreated control[b]	^{3}H-labeled cyclic AMP per 10^6 cells[c] (pmoles)	Ratio of incorporated vesicle cyclic AMP: free cyclic AMP
Vesicle cyclic AMP	1.2	114	$8.1 \cdot 10^3$	5.4
Free cyclic AMP		108	$1.5 \cdot 10^3$	
Vesicle cyclic AMP	3.2	89	$3.9 \cdot 10^3$	3.6
Free cyclic AMP		105	$1.1 \cdot 10^3$	
Vesicle cyclic AMP	9.3	81	$4.4 \cdot 10^3$	3.3
Free cyclic AMP		109	$1.3 \cdot 10^3$	
Vesicle cyclic AMP	15	103	$1.4 \cdot 10^3$	3.1
Free cyclic AMP		89	$4.3 \cdot 10^2$	
Vesicle cyclic AMP	18	98	$2.3 \cdot 10^2$	0.8
Free cyclic AMP		104	$2.8 \cdot 10^2$	

[a] Roller tube cultures at the stated densities were incubated in medium containing 10^{-4} M ^{3}H-labeled cyclic AMP or medium containing "fluid" lipid vesicles containing 10^{-4} M ^{3}H-labeled cyclic AMP as described in Methods.

[b] Calculated from cell counts on untreated control and cyclic AMP-treated cultures after 3 days incubation.

[c] Calculated from measured values of cell-associated ^{3}H-labeled cyclic AMP after 24 h incubation in medium containing free cyclic AMP or vesicle cyclic AMP.

is lost from vesicles during their uptake by cells. Observations of the uptake by 3T3 and SV3T3 cells of different dilutions of vesicles containing a 2 : 1 ratio of ^{14}C- to ^{3}H-labeled lipids revealed a similar ratio of ^{14}C (cholesterol) to ^{3}H (dipalmitoylphosphatidylcholine) counts in material incorporated intracellularly, which further suggests that the vesicles were incorporated as intact structures, rather than cellular incorporation of individual radiolabeled lipid molecules from vesicles by molecular exchange between vesicles and the cellular plasma membrane.

In contrast to the marked growth inhibition found in 3T3 and SV3T3 cell populations treated with "fluid" lipid vesicles containing cyclic AMP, treatment of mouse L929 cells under the same conditions had no effect on the pattern of cell proliferation, despite significant intracellular incorporation of ^{3}H-labeled cyclic AMP within vesicles (Table III). The failure of vesicle-associated cyclic AMP to modify the growth behaviour of mouse L cells, despite substantial cellular incorporation of vesicles and cyclic AMP, contrasts with the marked inhibition found in 3T3 and SV-3T3 under the same conditions. However, Schroder and Plagemann (32) found that the proliferation of L cells was unaffected by exogenous cyclic AMP concentrations as high as 12 mM, and Weiss [33] found that cyclic AMP affected the adhesive properties of a number of mammalian cells though L cells were not affected. The effect of cell density on the incorporation of both vesicle cyclic AMP and free cyclic AMP by L cells is also difficult to explain. However, a recent study by Grimes and Schroeder [34] found that the effects of dibutyryl-cyclic AMP on polyoma-transformed

cells were more marked at lower cell densities but no data was given on intracellular cyclic AMP levels.

Effect of vesicle cyclic AMP on growth stimulation in stationary 3T3 cell cultures induced by serum, insulin or proteolytic enzymes

High concentrations of serum [35, 36] insulin [37, 38] and proteolytic enzymes [39] have been shown to stimulate cell division in stationary-phase cultures of normal fibroblasts. The stimulation of cell growth by these agents is accompanied by a fall in intracellular cyclic AMP levels [3, 4, 8, 31] and growth stimulation can be inhibited by addition of dibutyryl-cyclic AMP (10^{-3} or 10^{-4} M) to the culture medium [8, 12].

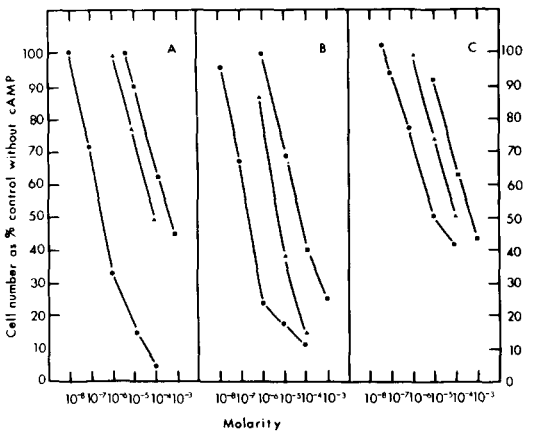

Fig. 4. The effect of different molar concentrations of cyclic AMP within "fluid" lipid vesicles (●–●), free dibutyryl-cyclic AMP plus 10^{-3} M theophylline (▲–▲), and free cyclic AMP plus theophylline (■–■) on the increase in cell number in 4-day-old stationary mouse 3T3 cell cultures following growth stimulation induced by insulin (A), pronase (B) and serum (C). The increase in cell number in experimental cultures treated with cyclic nucleotides is expressed as a percentage of the increase in cell number found in control cultures 56 h after growth stimulation by similar treatment with insulin, pronase or serum in the absence of cyclic AMP. Each point represents a mean value from cell counts on 12 separate Petri dish cultures in three separate experiments. (A) Inhibition of growth stimulation by insulin. The culture medium was removed from control stationary 3T3 cell cultures and the cells incubated in fresh medium containing 0.1 unit crystalline insulin. Experimental cultures were treated identically except that the various cyclic nucleotide preparations were included in the fresh culture medium in addition to insulin. After incubation for 18 h at 37 °C, the medium was removed from both control and experimental cultures, the cells washed twice in PBS, the original growth medium from the cultures replaced and the number of cells measured after further incubation for 38 h at 37 °C. (B) Inhibition of growth stimulation by proteolytic enzymes. Stationary 3T3 cell cultures were incubated with 10 μg/ml pronase (grade B) for 10 min at 37 °C in Ca^{2+}, Mg^{2+}-free saline. The cultures were then washed three times with Ca^{2+}, Mg^{2+}-free saline and new culture medium added. The enzyme-treated control cell cultures were grown in normal medium and experimental cultures in media supplemented with the various cyclic nucleotide preparations. After 56 h incubation at 37 °C the number of cells in experimental cultures was measured and compared with that in the protease-treated control cultures without cyclic nucleotides. (C) Inhibition of growth stimulation by serum. The medium was removed from stationary 3T3 cell cultures which were then washed twice with warm Ca^{2+}, Mg^{2+}-free saline before addition of fresh medium containing 50 % calf serum to the control cultures or a similar medium supplemented with the cyclic nucleotide preparations to the experimental cultures. The number of cells in both experimental and control cultures was measured after incubation for 56 h at 37 °C.

It was considered of interest, therefore, to investigate the effect of similar growth-stimulating agents on stationary 3T3 cell cultures that had been incubated with vesicles containing cyclic AMP.

The results summarized in Fig. 4, show that the growth stimulation in stationary 3T3 cell populations following treatment with 0.1 unit of insulin (Fig. 4A) or brief treatment with proteolytic enzymes (Fig. 4B) was effectively inhibited by incubation of the cells with "fluid" lipid vesicles containing cyclic AMP at concentrations as low as 10^{-6} M. In contrast, dibutyryl-cyclic AMP or cyclic AMP, together with theophylline, added to the culture medium were able to inhibit growth stimulation only at very high concentrations (10^{-3} or 10^{-4} M) and were increasingly ineffective at lower concentrations (Figs 4A and 4B), a finding in agreement with previous observations on the effects of exogenous cyclic nucleotides [12].

Vesicles containing cyclic AMP were less effective, however, in inhibiting growth stimulation in 3T3 cell cultures treated with high concentrations of serum (Fig. 4C). Similarly, both free cyclic AMP and dibutyryl-cyclic AMP were also less effective (Fig. 4C) when compared with their effects at the same concentrations on growth stimulation induced by insulin (Fig. 4A) or pronase (Fig. 4B).

Effects of cyclic AMP incorporated into "solid" vesicles

The inhibition of growth in 3T3 and SV3T3 cell populations described in the previous sections was produced by cyclic AMP trapped within vesicles composed of lipids that were in a "liquid" or so called "fluid" condition at the experimental temperature. In contrast, identical concentrations of cyclic AMP incorporated within vesicles composed of lipids that were predominantly "solid" at 37 °C had no signi-

TABLE IV

INCORPORATION BY 3T3 AND SV3T3 CELLS OF FREE ^{3}H-LABELED CYCLIC AMP. ^{3}H-LABELED CYCLIC AMP WITHIN VESICLES (VESICLE CYCLIC AMP) COMPOSED OF "SOLID" LIPIDS AND CELLULAR INCORPORATION OF "SOLID" VESICLES CONTAINING ^{14}C-LABELED LIPIDS

Cell type	Treatment[a]	Cell growth as percentage of untreated control[a]	Uptake		
			^{3}H-labeled cyclic AMP percentage of total[a]	^{3}H-labeled cyclic AMP per 10^{6} cells[a] (pmoles)	Number of ^{14}C-labeled vesicles per cell[b]
3T3	10^{-4} M vesicle cyclic AMP	93	2.2	$4.40 \cdot 10^{3}$	$6.19 \cdot 10^{6}$
	10^{-4} M free cyclic AMP	72	0.27	$5.42 \cdot 10^{2}$	–
SV3T3	10^{-4} M vesicle cyclic AMP	98	2.6	$5.20 \cdot 10^{2}$	$6.40 \cdot 10^{6}$
	10^{-4} M free cyclic AMP	66	0.33	$6.61 \cdot 10^{2}$	–
3T3	10^{-5} M vesicle cyclic AMP	112	4.2	$8.41 \cdot 10^{2}$	$9.6 \cdot 10^{5}$
	10^{-5} M free cyclic AMP	94	0.3	$6.44 \cdot 10^{1}$	–
SV3T3	10^{-5} M vesicle cyclic AMP	114	5.2	$1.04 \cdot 10^{3}$	$1.1 \cdot 10^{6}$
	10^{-5} M free cyclic AMP	95	0.28	$5.59 \cdot 10^{1}$	–

[a] Experimental conditions are identical to those outlined in the legend to Fig. 1 except for the altered lipid composition of the vesicles.

[b] Experimental conditions are identical to those outlined in the legend to Table II except for the altered lipid composition of the vesicles.

ficant effect on the proliferation of 3T3 and SV3T3 cells, despite significant uptake of vesicles and cyclic AMP by the cells (Table IV). Cyclic AMP incorporated in vesicles prepared from "solid" lipids was also unable to inhibit stimulation of cell growth in stationary 3T3 cell cultures induced by insulin.

DISCUSSION

The present results demonstrate that unilamellar lipid vesicles can serve as effective carrier vehicles for introducing biologically active molecules into cultured cells under conditions where similar molecules added to the culture medium are ineffective. The experiments described here have shown that vesicles containing cyclic AMP can induce significant growth inhibition in 3T3 and SV3T3 cell populations at concentrations where exogenous cyclic nucleotides added to the culture medium produce no detectable effect on cell proliferation. The intracellular levels of ^{3}H-labeled cyclic AMP found in cells treated with vesicles containing ^{3}H-labeled cyclic AMP are substantially higher than most of the values reported in the literature for endogenous levels of intracellular cyclic AMP in 3T3 [3, 4, 7, 8] and other mammalian cells [5, 6, 8] and the levels found after exposure of cells to cyclic nucleotides [10^{-3}–10^{-5} M) added to the culture medium [8]. However, a recent study from Dulbecco's laboratory [40] has found that treatment of stationary mouse embryo cells with prostaglandin E_1, insulin and theophylline increased the endogenous cyclic AMP level from 20 to 4000 pmoles per mg protein within as little as 30 min. The latter values are very similar to the highest values found here. However, the very high levels of intracellular ^{3}H-labeled cyclic AMP found in vesicle-treated cells may not necessarily reflect the level of active cyclic AMP. For example, only a fraction of the intracellular cyclic AMP derived from vesicles may be released in an active form at the correct intracellular site and a substantial fraction may remain inactive if retained within intact vesicles.

The ability of cyclic AMP within vesicles to alter cell-growth behaviour when incorporated intracellularly suggests that at least a certain proportion of the vesicle associated cyclic AMP is released from vesicles into the cytoplasm. This presumed distribution of cyclic AMP in the cytoplasm also indicates that at least some vesicles fuse with the plasma membrane releasing their contents into the cytoplasm. The possibility that vesicles can fuse with the plasma membrane of cells has been suggested by the ability of vesicles of differing lipid composition to induce cell fusion [19], though more definitive evidence for this process has been provided by recent observations which have shown that lipid molecules from vesicles labeled with radioisotopes (Papahadjopoulos, D., Mayhew, E. and Poste, G., unpublished) or electron-histochemical markers (Pagano, R., personal communication) can be identified in the plasma membrane of cells immediately after treatment with vesicles. In addition, one interpretation of the present finding that cyclic AMP trapped within vesicles composed of "solid" phospholipids did not affect cell proliferation, despite being incorporated intracellularly, is that these vesicles are incorporated by a different mechanism than vesicles composed of "fluid" phospholipids. For example, vesicles composed of "solid" phospholipids are unable to fuse cells [19], a process that is considered to require fusion between the vesicles and the plasma membranes of adjacent cells [19, 41]. "Solid" vesicles also show a very limited capacity to fuse with

each other compared with vesicles of similar size composed of fluid phospholipids [42]. Consequently, solid vesicles containing cyclic AMP might not be able to fuse with the plasma membrane and may instead be incorporated intact by endocytosis. If this interpretation is correct then solid vesicles and their contents would be localized in the lysosomal digestive apparatus of the cell thereby limiting the liklehood of the cyclic AMP reaching the cytoplasm.

The results in Fig. 3 indicate that over a 1000-fold dilution the biological effects produced by vesicle cyclic AMP are surprisingly constant. This contrasts with the effects of free cyclic AMP and suggests that cyclic AMP within vesicles may be less susceptible than free cyclic AMP to degradation by phosphodiesterases in the cellular plasma membrane and the culture medium.

The use of lipid vesicles as carriers for introducing material into cells need not be restricted to vesicles of the size used here. Vesicles of appropriate lipid composition can be produced over a wide size range [2] and separation of different size classes is easily achieved by gel filtration or ultracentrifugation [25, 42]. Thus, larger (1–10 μm diameter) multilamellar lipid vesicles, so called "liposomes" [1] offer the advantage over the vesicles used here that larger material, including macromolecules, can be incoporated within their multiple aqueous compartments. Two recent studies have shown that multilamellar "liposomes" can be incorporated by mammalian cells and release their contents intracellularly [43, 44], though no data was given on the number of vesicles incorporated. However, these studies demonstrated that marker molecules within the liposomes were released intracellularly in the lysosomal digestive apparatus, which suggests that these larger vesicles are incorporated by endocytosis. Once within lysosomes the membranes of the vesicle are ruptured, presumably by lysosomal lipases, releasing the vesicle contents to act on intra-lysosomal substrates. In this respect, it would be of interest to investigate the value of liposomes as therapeutic vehicles for introducing enzymes into the lysosomes of cultured cells from patients with lysosomal storage disease that lack one or more specific lysosomal enzymes [45].

In addition to the intracellular release of molecules trapped within vesicles, the present finding that cells incorporate large numbers of vesicles without overt cytotoxicity suggests that vesicles could be used to modify the composition of cellular membrane systems as, for example, in the suggested fusion of unilamellar "fluid" vesicles with the cellular plasma membrane. In this respect, it is pertinent that a very recent study has shown that incubation of lymphocytes with lecithin–cholesterol unilamellar vesicles results in significant cellular incorporation of vesicle lipids and alteration in the "fluidity" of the plasma membrane as determined by fluorescence polarization [46]. Similarly, work in this laboratory has shown that incubation of cells with unilamellar vesicles can produce significant alterations in their susceptibility to agglutination by plant lectins (D. Papahadjopoulos, G. Poste and K. Jacobson, unpublished observations). Finally, since methods are available for introducing proteins and glycoproteins into the lipid membrane of vesicles [2] this offers the further experimental possibility of using lipid vesicles as carriers to introduce new protein molecules into the plasma membrane of cells cultured in vitro.

The possible routes by which lipid vesicles of differing size or composition and their contents might be incorporated into cells as outlined in the above discussion is summarized in Fig. 5.

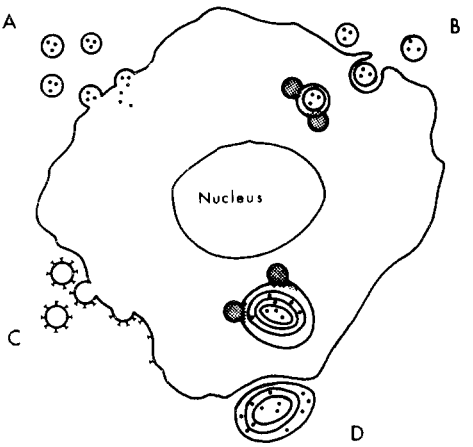

Fig. 5. Schematic representation of possible mechanisms for the incorporation of lipid vesicles by cultured cells. (A) Unilamellar vesicles composed of lipids that are "fluid" at 37 °C. The vesicles may fuse with the plasma membrane of the cell so that material trapped within the internal aqueous space of the vesicle (●) is released into the cytoplasm. (B) Unilamellar vesicles composed of lipids that are "solid" at 37 °C. Due to their "solid" membrane the vesicles may not fuse with the plasma membrane and are instead incorporated by endocytosis. Following endocytosis, the endocytotic vacuole containing the vesicle fuses with lysosomes (shown as stippled structures). The action of lysosomal lipases on the lipid vesicle would be expected to digest the vesicle membrane liberating its contents. (C) Unilamellar vesicles composed of lipids that are "fluid" at 37 °C and which contain a protein or glycoprotein bound (electrostatically or hydrophobically) in the vesicle membrane. The vesicles may fuse with the cellular plasma membrane as in A and the vesicle membrane, including the associated protein, would be incorporated into the plasma membrane. (D) Multilamellar lipid vesicles (liposomes). The available evidence indicates that the bulk of liposomes are incorporated into the cellular lysosomal digestive apparatus, presumably by endocytosis. Information is not available on the effect of the lipid composition of liposomes on their uptake by cells and the possibility remains that fusion might occur between the plasma membrane and the outer bilayer(s) of the liposomes even though the remainder of the liposome is endocytosed. Alternatively, liposomes may be endocytosed intact. In either case, material trapped within the multiple aqueous compartments of endocytosed liposomal structures would be released within the lysosomal digestive apparatus as in example B above.

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