Control Mechanisms Governing the Infectivity of *Chlamydia trachomatis* for HeLa Cells: Modulation by Cyclic Nucleotides, Prostaglandins and Calcium

By MICHAEL E. WARD* AND HASSAN SALARI

Department of Microbiology, Southampton University Medical School, Southampton General Hospital, Tremona Road, Southampton SO9 4XY, U.K.

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Chlamydia trachomatis causes common infections of the eyes and genital tract in man. The mechanism by which this obligate intracellular bacterium is taken into epithelial cells is unclear. The results described here support the concept that chlamydial infection of HeLa cells is under bidirectional cyclic nucleotide control, with guanosine 3':5'-cyclic monophosphate (cGMP) acting as a stimulator, and adenosine 3':5'-cyclic monophosphate (cAMP) as an inhibitor. Treatment of the HeLa cells with the divalent cation ionophore A23187, with carbamoylcholine, or with prostaglandins known to increase the concentration of endogenous cGMP, also increased host cell susceptibility to chlamydial infection. Cyclic GMP was only effective if added at or before chlamydial inoculation, suggesting that its main effect was on chlamydial uptake. The stimulatory effect of cGMP, but not antagonism by cAMP, was abolished if the cells were first treated with any of four different inhibitors of prostaglandin synthesis, suggesting a critical role for endogenous prostaglandin synthesis. Centrifugation of chlamydiae on to host cells was followed by a rapid increase in the mobility of Ca^{2+} across the cell membrane. The interrelationships of these observations and the possibility that chlamydiae and other intracellular pathogens might evoke alterations in host cell prostaglandin and cyclic nucleotide concentrations to aid their own uptake are discussed.

INTRODUCTION

Chlamydiae are specialized bacteria which have adapted to obligate intracellular life in non-phagocytic cells by the development of a specialized growth cycle (Moulder, 1974). The rigid 0.3 μm diameter elementary body enters the host cell and enlarges within a cytoplasmic vacuole to form the reticulate body: the latter divides by binary fission producing the characteristic inclusion. Elementary bodies differentiate from the reticulate bodies; their release some 48–72 h after infection completes the growth cycle. Relatively little is known concerning the mechanism by which intracellular pathogens enter non-phagocytic host cells. In the case of chlamydiae special mechanisms must exist as they are taken up by HeLa or L cells 10–100 times faster than polystyrene latex particles or *Escherichia coli* (Byrne & Moulder, 1978). Chlamydial uptake requires energy from glycolysis (Kuo & Grayston, 1976) and electron microscope studies suggest that chlamydiae enter host cells by phagocytosis (Stirling & Richmond, 1977). Thus both the parasite and the host cell participate in chlamydial uptake and the stimulus for uptake must be generated at the host cell surface.

The cyclic nucleotides adenosine 3':5'-cyclic monophosphate (cAMP) and guanosine 3':5'-cyclic monophosphate (cGMP) are ubiquitous in eukaryotic cells. The intracellular concentrations of these nucleotides can be modulated by appropriate stimuli at the cell surface and control many critical cell functions, including phagocytosis and lysosomal degranulation (Cox & Karnovsky, 1973; Zurier *et al.*, 1974). Preliminary findings from this

laboratory suggested that the susceptibility of HeLa 229 cells to infection with *C. trachomatis* was under bidirectional cyclic nucleotide control, with cGMP stimulating and cAMP depressing chlamydial infectivity (Ward & Salari, 1980). The present paper confirms these observations and describes experiments suggesting that host cell prostaglandin biosynthesis and the concentration of intracellular calcium are also important in determining HeLa cell susceptibility to chlamydial infection.

METHODS

Materials. *Chlamydia trachomatis* strains A/SA-1/OT, E/DK-20/ON, H/UW-4/GCx and L3/404/LN were kindly provided by Drs S. Darougar and J. Trehan, Institute of Ophthalmology, London, U.K. After receipt, the strains were passaged once in hens' egg yolk sacs and frozen at -70°C . Reagents were obtained from the following sources: $N^6, O^{2'}$ -dibutyryl adenosine 3':5'-cyclic monophosphate (dibutyryl cAMP), $N^2, O^{2'}$ -dibutyryl guanosine 3':5'-cyclic monophosphate (dibutyryl cGMP), acetylsalicylic acid, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindol-3-ylacetic acid (indomethacin), carbamoylcholine chloride and emetine hydrochloride from Sigma; purified *N*-2,3-xylylanthranilic acid (mefenamic acid) from Parke Davis Co., Pontypool, Gwent, U.K.; 2-(2-fluoro-4-biphenyl)propionic acid (Froben) from Boots Ltd, Nottingham, U.K. Ionophore A23187 was a generous gift from Dr R. Hamill, Lilly Research Laboratories, Indianapolis, Ind. 46206, U.S.A., and prostaglandins E_1 , E_2 and $F_{2\alpha}$ promethamine salt were kindly donated by Upjohn Limited, Crawley, Sussex, U.K. ^3H -labelled amino acid mixture TRK.440 and $^{45}\text{CaCl}_2$ CES.3 were purchased from Amersham, and Hoechst 33258 from Hoechst (U.K.), Hounslow, Middlesex, U.K. Stock solutions of reagents were dissolved in MEM (see next section), with the exception of A23187 which was dissolved in ethanol.

Tissue culture. HeLa 229 cells (Flow Laboratories, Irvine, U.K.) were grown at 35.5°C in an atmosphere of 5% (v/v) CO_2 in air in Eagle's Minimal Essential Medium supplemented with 10% (v/v) foetal calf serum, non-essential amino acids, L-glutamine and sodium bicarbonate (MEM). The absence of mycoplasma contamination from the cell cultures was confirmed weekly by fluorescence microscope examination of fluorochrome-stained (Hoechst 33258) preparations of the cells (Russell *et al.*, 1975).

Chlamydial amino acid utilization. The effect of cyclic nucleotides and other host-cell perturbing agents on the ability of the cells to support chlamydial growth was assessed by measuring the chlamydia-dependent utilization of radioactive amino acids. HeLa 229 cells were grown overnight to confluent monolayers in 1.5 ml volumes of MEM in 24-well tissue culture trays (Costar, Cambridge, Mass. 02139, U.S.A.). Each well contained approximately 5×10^5 cells. After washing in Hanks' balanced salt solution (HBSS) the monolayers were incubated for 2 h at 35.5°C in MEM containing $1 \mu\text{g}$ emetine hydrochloride ml^{-1} with or without (control) the agent under test. Emetine hydrochloride blocks host cell (80S ribosome-dependent) protein synthesis without impairing the 70S ribosome-dependent protein synthesis of chlamydiae (Becker & Asher, 1972; Salari & Ward, 1981). After a further wash in HBSS, 500 μl volumes of either MEM (uninfected control) or of a dilution of yolk sac-derived chlamydiae in MEM, with or without the test agent, were inoculated into each well as appropriate. The chlamydial inoculum was standardized so that inclusions developed in approximately 30% of the control, emetine-treated, HeLa cells. The trays were centrifuged for 1 h at 2000 *g* then incubated for a further 2 h at 35.5°C in 5% (v/v) CO_2 in air to permit chlamydial adsorption. After washing in HBSS to remove unadsorbed chlamydiae, 1.5 ml MEM containing $1.5 \mu\text{g}$ emetine hydrochloride plus $1.5 \mu\text{Ci}$ tritiated amino acids with or without test agent was added to each well and the trays were incubated at 35.5°C . Uptake of radioactive amino acids into the infected cells was determined by first washing the cells thoroughly in HBSS at room temperature then solubilizing the cells in 1 ml 1% (w/v) sodium dodecyl sulphate (SDS) at 35.5°C overnight. The radioactivity in 800 μl samples of the resulting solution was quantified by liquid scintillation counting. The controls routinely included were uninfected cells plus and minus emetine to ensure that the host cell protein synthesis was blocked, and emetine-treated uninfected cells plus the test agent to ensure that the agent was not cytotoxic. Cytotoxicity was also monitored by direct microscope counting of the number of HeLa cells adherent to the cover slips after incubation with the test agent and prior to digestion in SDS. Preliminary experiments established that the bulk (>90%) of radioactivity taken up by emetine-treated, chlamydia-infected cells was incorporated into protein and could be recovered by precipitation with 5% (w/v) trichloroacetic acid. This incorporation was due to chlamydial protein synthesis as it was almost entirely prevented by the addition of the antichlamydial agent rifampicin at $1 \mu\text{g ml}^{-1}$. Comparison of the chlamydia-dependent radioactive amino acid incorporation in 10 replicate wells showed the incorporation to be highly reproducible with a range of counts within $\pm 4\%$ of the mean of 16410 c.p.m. Points plotting cumulative chlamydial amino acid incorporation versus time bore a simple linear relationship to each other (e.g. Fig. 3) up to peak incorporation. It was, therefore, considered unnecessary to use replicate wells for each sample in experiments plotting cumulative amino acid incorporation versus time. In experiments in which amino acid incorporation at a fixed period of time was measured, the c.p.m. value plotted is

the mean of three replicate wells. Minor variations in the magnitude of individual responses (e.g. to cGMP) were observed from experiment to experiment, for unknown reasons, but the trends were always consistent. Each experiment was repeated at least once to establish its reproducibility.

Inclusion counting. The effect of dibutyryl cAMP and dibutyryl cGMP treatment of HeLa cells on the number of inclusions developing from the standard inoculum of *C. trachomatis* was determined by direct counting of fluorochrome-stained inclusions. Confluent monolayers of HeLa 229 cells grown on 13 mm diameter coverslips in disposable 'bijou' bottles were treated for 2 h at 35.5 °C with various concentrations of dibutyryl cAMP and dibutyryl cGMP in the ranges of 10^{-3} – 10^{-6} M and 10^{-4} – 10^{-11} M, respectively, in MEM in the absence of emetine. The monolayers were then inoculated with chlamydiae and incubated for 60 h at 35.5 °C to permit inclusion development. The coverslips were fixed in methanol, and the inclusions were stained with Hoechst 33258 (Salari & Ward, 1979), then counted at a magnification of 625 $\times$ using a Leitz Ortholux II incident-light fluorescence microscope fitted with an ocular counting graticule. Quadruplicate coverslips were prepared for each variable and coded and the number of infected and uninfected cells in 32 randomly selected microscope fields was counted. The total number of cells per microscope field was approximately 90.

RESULTS

Effect of treatment with different concentrations of cyclic nucleotides on HeLa cell susceptibility to chlamydial infection. HeLa 229 cells were treated for 2 h before chlamydial inoculation and during subsequent incubation with different concentrations of dibutyryl cAMP or dibutyryl cGMP. [Dibutyryl analogues were used because they resist degradation by cellular phosphodiesterases and can penetrate intact HeLa cells (Kankel & Hilz, 1972).] The effects of these treatments on the number of chlamydial inclusions developing from a standard inoculum of *C. trachomatis* UW4 are shown in Fig. 1. In the absence of cyclic nucleotide treatment (control) a mean of 28 inclusions was observed per microscope field. Treatment with 10^{-3} M dibutyryl cAMP reduced the number of inclusions developing by approximately two-thirds; this effect was dependent on concentration and at concentrations $\leq 5 \times 10^{-4}$ M the number of inclusions was not significantly different from the controls. Treatment with dibutyryl cGMP at 10^{-4} – 10^{-5} M did not markedly affect the number of inclusions, but at concentrations in the range 10^{-6} – 10^{-8} M the number of inclusions was almost tripled. The cyclic nucleotides had no effect on the appearance of uninfected HeLa cell monolayers observed by phase-contrast microscopy.

These findings were confirmed using chlamydia-dependent uptake of radioactive amino acids as the parameter of growth (Fig. 2). At high concentrations dibutyryl cAMP decreased the susceptibility of HeLa 229 cells to chlamydial infection, whereas dibutyryl cGMP increased their susceptibility at concentrations in the range 10^{-6} – 10^{-11} M but not at higher, non-physiological concentrations.

Action of cyclic nucleotides on the susceptibility of HeLa cells to infection with different serotypes of *C. trachomatis*. Different serotypes of *C. trachomatis* differ markedly in the type of disease they produce. Therefore the effect of treatment with optimal concentrations of dibutyryl cAMP and dibutyryl cGMP on the susceptibility of HeLa cells to other strains of *C. trachomatis* was determined.

Strains SA1 (serotype A, trachoma isolate) and DK20 (serotype E, ophthalmia neonatorum) like UW4 (serotype H, genital tract infection) require centrifuge-assisted infection in tissue culture systems and have a 'slow' developmental cycle. By contrast, *C. trachomatis* 404 (serotype L3, lymphogranuloma venereum) produces a more invasive genital tract infection in man, has a 'fast' developmental cycle and infects HeLa or McCoy cells without the need for centrifugation. Despite these differences in the serotype and in the infections produced, growth of all the strains was enhanced by 10^{-6} M-dibutyryl cGMP and suppressed by 10^{-3} M-dibutyryl cAMP (Table 1).

Effect of compounds perturbing host cell cyclic nucleotide synthesis on chlamydia-dependent uptake of radio-labelled amino acids. A variety of compounds, including prostaglandins and carbamoylcholine, are known to affect the concentrations of intracellular

Fig. 1. Effect of different concentrations of dibutyryl cAMP (●) or dibutyryl cGMP (○) on the growth of *C. trachomatis* UW4 in HeLa 229 cells. HeLa 229 cells were treated for 2 h with different concentrations of cyclic nucleotide before a standardized inoculum of chlamydiae was centrifuged on to the cells. Inclusion counts were made after incubation for 60 h in the presence of the nucleotide. The control (□) shows the number of inclusions per field in the absence of cyclic nucleotide. The bars represent standard deviations.

Fig. 2. Effect of different concentrations of dibutyryl cAMP or dibutyryl cGMP on the growth of *C. trachomatis* UW4 in HeLa 229 cells. Growth after 30 h was monitored by measuring chlamydia-dependent incorporation of ^3H -labelled amino acids in the presence of $1 \mu\text{g}$ emetine ml^{-1} : uninfected cells alone (controls) (■), with dibutyryl cAMP (△) or with dibutyryl cGMP (▲). Infected cells alone (□), with dibutyryl cGMP (○) or with dibutyryl cAMP (●).

Table 1. *Effect of 2 h treatment with dibutyryl cAMP or dibutyryl cGMP on the susceptibility of HeLa 229 cells to subsequent challenge with C. trachomatis*

Means $\pm$ 95 % confidence interval for the population mean are shown.

Host cell treatment	Mean no. of inclusions per field 60 h after challenge with strain:		
	SA1	DK20	LGV404
None	12.8 $\pm$ 0.98	18.5 $\pm$ 0.51	42.5 $\pm$ 1.13
Dibutyryl cGMP (10^{-6} M)	22.9 $\pm$ 1.2	35.5 $\pm$ 1.34	61.0 $\pm$ 1.10
Dibutyryl cAMP (10^{-3} M)	4.6 $\pm$ 0.68	9.1 $\pm$ 0.5	17.4 $\pm$ 1.22

Fig. 3. Effect of cyclic nucleotides, carbamoylcholine and prostaglandins on the growth of *C. trachomatis* UW4. HeLa 229 cells in MEM containing $1 \mu\text{g}$ emetine ml^{-1} were treated for 2 h with 10^{-3} M-dibutyryl cAMP ($\square$), 10^{-6} M-dibutyryl cGMP ($\bullet$), 2×10^{-5} M prostaglandins E_1 ($\blacktriangledown$), E_2 ($\blacktriangle$) or $F_{2\alpha}$ ($\blacksquare$), 10^{-4} M-carbamoylcholine ($\triangle$) or MEM alone (control) ($\circ$). After chlamydial challenge fresh medium containing test agent and $1 \mu\text{Ci}$ ^3H -labelled amino acids ml^{-1} was added and chlamydia-dependent amino acid uptake determined. The dashed line indicates amino acid incorporation by emetine-treated uninfected cells (controls) alone or in the presence of each test agent; the bars indicate the ranges of results.

Table 2. Effect of 2 h treatment with prostaglandin E_1 or E_2 , dibutyryl cAMP or dibutyryl cGMP on the susceptibility of HeLa 229 cells to subsequent challenge with *C. trachomatis* strain UW4

Host cell treatment	No. of inclusions per field 60 h after challenge
	(Sample mean $\pm$ 95% confidence interval for population mean)
None	20.6 ± 0.8
Prostaglandin E_1 (5×10^{-7} M in MEM)	11.4 ± 0.76
Prostaglandin E_2 (5×10^{-7} M in MEM)	11.8 ± 0.54
Dibutyryl cAMP (10^{-3} M)	6.75 ± 0.56
Dibutyryl cGMP (10^{-6} M)	33.8 ± 0.83

cAMP or cGMP in a wide range of eukaryotic cells. Therefore such compounds would be expected to influence HeLa cell susceptibility to chlamydial infection. Figure 3 shows the effect on chlamydial amino acid uptake of treating HeLa 229 cells for 2 h before chlamydial challenge with dibutyryl cAMP (10^{-3} M), dibutyryl cGMP (10^{-6} M), prostaglandins E_1 , E_2 and $F_{2\alpha}$ (2×10^{-5} M) or carbamoylcholine (10^{-4} M).

Emetine-treated uninfected HeLa cells (controls) showed an insignificant amount of amino acid incorporation, which was not affected by any of the agents under test. A slight, but consistent, loss of incorporated radioactivity in these metabolically inhibited cells was due to gradual death and exfoliation from the monolayer. Maximal chlamydial amino acid incorporation was noted at 54 h post-inoculation. Beyond this time, loss of radiolabelled cells from the monolayer due to the combined effects of metabolic inhibition and chlamydia-dependent host cell lysis exceeded the cumulative incorporation of isotope resulting from continuing chlamydial development. Treatment with dibutyryl cGMP, carbamoylcholine and

Fig. 4. Effect of adding dibutyryl cGMP (10^{-6} M) at different stages of the chlamydial growth cycle on its ability to stimulate chlamydial growth. A dibutyryl cGMP concentrate was added to emetine-treated HeLa 229 cells 2 h before inoculation with *C. trachomatis* UW4 (-2 h) at inoculation (0 h) or 3, 18, 24 and 30 h post-inoculation to achieve a final concentration of 10^{-6} M. Treatment with dibutyryl cGMP was continued until chlamydia-dependent ^3H -labelled amino acid incorporation was determined 40 h after inoculation. As controls, measurements were made of amino acid incorporation in uninfected, emetine-treated cells in the presence (a) or absence (b) of dibutyryl cGMP and of incorporation in infected cells in the absence of cGMP (c).

prostaglandins E_2 and $\text{F}_{2\alpha}$ caused a consistent increase in chlamydia-dependent amino acid uptake compared with control (untreated) cells. By contrast, dibutyryl cAMP and prostaglandin E_1 depressed chlamydial amino acid uptake. This opposed effect of the two E series prostaglandins was dependent on concentration; when retested at the lower, more physiological concentration of 5×10^{-7} M, prostaglandins E_1 and E_2 both consistently depressed the susceptibility of the HeLa cells to chlamydial infection (Table 2).

Action of cyclic nucleotides at different stages of the chlamydial growth cycle. Cyclic nucleotides might act early in the chlamydial growth cycle to affect the uptake and survival of the elementary bodies, or they might act later in the development cycle, perhaps influencing the differentiation of reticulate bodies to elementary bodies. To discriminate between these possibilities, the effect of adding dibutyryl cGMP (10^{-6} M) at different stages in the chlamydial growth cycle was examined. Addition of dibutyryl cGMP 2 h before challenge with *C. trachomatis* UW4 almost doubled chlamydia-dependent amino acid uptake when compared with the infected, untreated control cells (Fig. 4). Addition of dibutyryl cGMP with the challenge inoculum or 3–30 h after challenge had no significant effect. To ensure that this observation was representative of all *C. trachomatis* strains the experiment was repeated with further strains, the effect on chlamydial growth being assessed by inclusion counting. Results for strains DK-20 (Ward & Salari, 1980) SA1 and LGV404 (data not shown) again confirmed that both cAMP and cGMP were maximally effective when added 2 h before chlamydial challenge and had little effect if added 3 h after inoculation.

Role of endogenous prostaglandin. The possibility that endogenous prostaglandin synthesis might be important in the action of cyclic nucleotides on chlamydial growth was investigated. HeLa cells were grown overnight in the presence of the prostaglandin biosynthesis inhibitors acetylsalicylic acid (10^{-4} M), Froben, mefenamate or indomethacin (2×10^{-5} M) in order to deplete the cells of endogenous prostaglandin and block *de novo* synthesis. The susceptibility

Fig. 5. Role of host cell prostaglandins in the dibutyryl cGMP mediated stimulation of HeLa cell susceptibility to chlamydial infection. HeLa cell monolayers were incubated overnight with 10^{-4} M-acetylsalicylic acid (A), 10^{-5} M-Froben (F), 10^{-5} M-mefenamate (M) or 10^{-5} M-indomethacin (I) in MEM to inhibit prostaglandin synthesis and lower the concentrations of endogenous prostaglandins. The following day the medium was replaced with MEM containing cyclic nucleotide and/or prostaglandin synthesis inhibitor (Pg inhibitor) as appropriate. After 1 h the cells were challenged with *C. trachomatis* UW4 and then incubated with the appropriate cyclic nucleotide and/or inhibitor in medium containing $1 \mu\text{g}$ emetine ml^{-1} and $1 \mu\text{Ci}$ ^3H -labelled amino acids ml^{-1} . Cumulative chlamydia-dependent amino acid incorporation was determined 48 h after inoculation. As controls (numbered columns), amino acid incorporation by emetine-treated cells was also determined for uninfected cells in the absence (1) or presence (2) of test agent, for untreated infected cells (3), and for infected cells treated with either dibutyryl cAMP (4) or dibutyryl cGMP (5).

of normal and prostaglandin-depleted cells to infection with *C. trachomatis* UW4 after treatment with either dibutyryl cAMP or cGMP was compared using chlamydia-dependent radio-labelled amino acid incorporation (Fig. 5).

Treatment of HeLa cells with each of the four inhibitors of prostaglandin synthesis slightly but consistently depressed chlamydia-dependent amino acid incorporation. In both normal and prostaglandin-inhibited HeLa cells treatment with dibutyryl cAMP caused a comparable decrease in susceptibility to chlamydial infection. The increased susceptibility of normal HeLa cells to chlamydial infection resulting from dibutyryl cGMP treatment was blocked in cells treated with any of the four prostaglandin synthesis inhibitors. Thus, the stimulatory effect of dibutyryl cGMP on chlamydial growth in HeLa cells, but not the inhibitory action of dibutyryl cAMP, is evidently mediated via the biosynthesis of prostaglandins or related compounds.

Action of intracellular calcium. The roles of intracellular calcium and of cyclic nucleotides are often interrelated. The effect on chlamydial development of treating HeLa cells with A23187, an ionophore which selectively elevates intracellular Ca^{2+} content, was investigated.

HeLa 229 cells were treated before chlamydial inoculation with cyclic nucleotide or with ionophore (Fig. 6). In some cases, cyclic nucleotide-treated cells were exposed to the ionophore before challenge. The effects of these treatments on chlamydial development were determined by measuring the chlamydia-dependent incorporation of radio-labelled amino acids. The results show that dibutyryl cGMP or A23187 treatment of HeLa cells increased their susceptibility to infection with *C. trachomatis* whereas dibutyryl cAMP treatment decreased it. This inhibitory effect of dibutyryl cAMP was abolished if the cells were subsequently treated with ionophore before chlamydial challenge. Thus A23187 treatment

Fig. 6. Effect of treatment with the calcium ionophore A23187 on the susceptibility of HeLa cells to infection with *C. trachomatis* UW4. HeLa 229 cells were incubated with 10^{-3} M-dibutyryl cAMP (●) or 10^{-6} M-dibutyryl cGMP (○) for 2 h, with $1 \mu\text{g}$ A23187 ml^{-1} (▲) for 1 h; or with dibutyryl cAMP (2 h) followed by A23187 for 1 h (△), in MEM. Control infected (□) or uninfected (▽) cells remained untreated. After washing, the cells were challenged and incubated in MEM containing $1 \mu\text{g}$ emetine ml^{-1} , $1 \mu\text{Ci}$ ^3H -labelled amino acids ml^{-1} and, as appropriate, cyclic nucleotides. Cumulative amino acid incorporation due to chlamydial growth was then determined.

stimulated chlamydial infection, showing it had no significant toxic effect on the host cells (since chlamydial growth requires active host cell metabolism), and modified the interactions of the host cells with cyclic nucleotides.

Effect of chlamydial interaction with the HeLa cell surface on host cell calcium mobility.

The experiment with A23187 suggested that if chlamydiae at the cell surface were capable of increasing calcium mobility in the host cell then they might trigger changes in the cell favourable to their own internalization. To investigate this possibility, the effect of chlamydiae on the influx and efflux of $^{45}\text{Ca}^{2+}$ in the host cell was determined.

To determine calcium influx, a purified tissue-culture derived inoculum of *C. trachomatis* UW4 (Salari & Ward, 1981) in MEM was centrifuged at 0°C on to a monolayer of HeLa 229 cells in order to deposit chlamydiae on the host cell surface. At this temperature the rate of enzyme-dependent reactions is low and membrane mobility greatly reduced. Thus it was considered unlikely that at 0°C any chlamydia-induced changes in cell physiology could occur. Following centrifugation, the medium on the cells was replaced at 0°C with a special medium, consisting of Hanks' balanced salts solution deficient in non-radioactive calcium, but containing 5 mM-Mg^{2+} and $2 \mu\text{Ci } ^{45}\text{Ca}^{2+} \text{ ml}^{-1}$ (modified HBSS). The cells were then rapidly warmed to 35.5°C to restore enzyme activity and membrane mobility. At this stage, chlamydial adhesion to the membrane can be strengthened due to membrane fluidity increasing the area in contact with the chlamydial particle, and uptake begins. Radioactivity due to the associated influx of $^{45}\text{Ca}^{2+}$ into chlamydial-inoculated or control (uninfected) cells was determined over a range of incubation times by washing the cell monolayer free of extracellular $^{45}\text{Ca}^{2+}$, followed by solubilization in 1% (w/v) SDS and scintillation counting. The results (Fig. 7) showed that chlamydial interaction with the host cell surface caused a rapid and marked increase in the uptake of calcium which was not observed in the uninfected controls. To distinguish whether this apparent uptake was due to increased calcium mobilization or to increased calcium binding sites at the host cell surface it was necessary to determine if there was a corresponding increased calcium efflux.

Fig. 7

Fig. 8

Fig. 7. Effect of infection by chlamydiae on the uptake of $^{45}\text{Ca}^{2+}$ by HeLa 229 cells. A purified inoculum of *C. trachomatis* UW4 was centrifuged on to the surface of a monolayer of HeLa 229 cells at 0 °C. The tissue culture medium was replaced with MEM at 0 °C deficient in unlabelled Ca^{2+} but containing $^{45}\text{Ca}^{2+}$. After rapid warming to 35.5 °C, the uptake of $^{45}\text{Ca}^{2+}$ into infected (●) and control uninfected (○) cells was compared.

Fig. 8. Effect of chlamydial infection on the efflux of $^{45}\text{Ca}^{2+}$ from HeLa 229 cells. Confluent monolayers of HeLa 229 cells were labelled by incubation at 35.5 °C for 4 h with MEM deficient in Ca^{2+} but containing 2 μCi $^{45}\text{Ca}^{2+}$ ml^{-1} . After washing in normal MEM at 0 °C a purified inoculum of *C. trachomatis* UW4 in MEM was centrifuged on to the radioactive monolayer at 0 °C. After rapid warming to 35.5 °C, the efflux of $^{45}\text{Ca}^{2+}$ from the infected cells (●) into the supernatant medium was compared with the efflux from similarly processed control, uninfected cells (○).

To determine efflux, confluent monolayers of HeLa 229 cells were labelled with $^{45}\text{Ca}^{2+}$ for 4 h at 35.5 °C in modified HBSS containing 2 μCi $^{45}\text{Ca}^{2+}$ ml^{-1} . The radio-labelled cells were thoroughly washed with ice-cold phosphate-buffered saline to remove unincorporated $^{45}\text{Ca}^{2+}$, then inoculated by centrifugation at 0 °C with the purified tissue-culture derived inoculum of *C. trachomatis* UW4. After inoculation, the medium on the cells was replaced with normal HBSS at 0 °C, the cells were rapidly warmed to 35.5 °C, and the efflux of $^{45}\text{Ca}^{2+}$ from the labelled cells into the supernatant medium was determined at various time intervals. The results (Fig. 8) showed that chlamydial interaction markedly enhanced the efflux of calcium over that noted in uninfected (control) cells. Thus interaction between purified chlamydial elementary bodies and the host cell surface resulted in an increased mobilization of calcium which was reflected by an increase in both the influx and efflux of the cation.

DISCUSSION

It is now generally accepted that the cyclic nucleotides cAMP and cGMP, together with calcium, are major components of an internal signalling system regulating eukaryotic cell activities. Two types of cyclic nucleotide-based control systems have been identified (Goldberg *et al.*, 1974). In the monodirectional system either cAMP or cGMP mediates an effect in cooperation with calcium. In the bidirectional system the effect of cGMP or calcium antagonizes the effect of cAMP. The results described in this paper showed that exogenous dibutyryl cGMP or compounds, such as carbamoylcholine and prostaglandin F_{2a} , known to increase endogenous eukaryotic cell cGMP levels, increased the susceptibility of HeLa cells

to chlamydial infection. Treatment of HeLa cells with the ionophore A23187, which elevates intracellular Ca^{2+} (Reed & Lardy, 1972), also stimulated infection. By contrast, dibutyryl cAMP, or prostaglandin E_2 which stimulates endogenous eukaryotic cell cAMP synthesis (Samuelsson *et al.*, 1978), depressed the chlamydial infection. This effect of cAMP was abolished if the cyclic nucleotide-treated cells were treated with A23187 before chlamydial challenge. These findings, together with the fact that dibutyryl cGMP was only active at much lower concentrations (10^{-6} – 10^{-11} M) than dibutyryl cAMP (10^{-3} – 10^{-4} M) were consistent with the operation of a bidirectional control system (Goldberg & Haddox, 1977) in which cGMP or intracellular Ca^{2+} functions as agonist and cAMP as antagonist. The only apparently disparate finding in this respect was the opposed action of prostaglandins E_1 and E_2 at 2×10^{-5} M; in general prostaglandins of the E series act as cAMP inducers (Samuelsson *et al.*, 1978). However, as observed in other systems this opposed effect was dependent on the prostaglandin concentration (Salzman, 1972); when retested at the more physiological concentration of 5×10^{-7} M, prostaglandins E_1 and E_2 both depressed the susceptibility of HeLa cells to chlamydial infection as would be expected of cAMP inducers.

The fact that cGMP was only effective when added before chlamydial inoculation (Fig. 4) shows that the nucleotide must affect an early stage in the process of infection e.g. chlamydial attachment or uptake. *Chlamydia trachomatis* UW4 in either the presence or absence of cGMP fails to grow in HeLa cells unless centrifuged on to the host cell surface, suggesting that it is defective in its ability to attach to this cell line irrespective of the presence of cGMP. Analysis of the binding of polyvalent ligands to cell membranes suggests that invagination of the host cell membrane following virus or chlamydial attachment may be the most thermodynamically stable configuration for the membrane to adopt (Patterson *et al.*, 1979). Uptake will be achieved either by fusion of the opposed plasma membrane of the invagination to form an intracellular vacuole or by sequential binding of the host cell membrane to the chlamydial surface resulting in uptake by a 'zipper mechanism' similar to that described by Griffin *et al.* (1975). In the case of the chlamydiae, close adhesion to the membrane and uptake are apparently part of the same process and cannot readily be distinguished. The finding that cGMP was relatively ineffective when added at chlamydial challenge, rather than 2 h before, suggests time is required to bring about an appropriate metabolic change. This change occurred irrespective of the presence of the protein synthesis inhibitor emetine. One possibility is that cGMP increases host cell membrane fluidity, mobility or ability to fuse, so favouring uptake.

Two different experiments point to a major role for prostaglandins in determining the susceptibility of HeLa cells to chlamydial infection. Firstly, addition of exogenous prostaglandins directly influenced chlamydial infectivity. Secondly, the stimulatory effect of cGMP was blocked by prior treatment of the cells with any of the four, structurally unrelated, inhibitors of prostaglandin biosynthesis tested. The stimulatory effect of A23187 treatment on chlamydial infection might also have been due to stimulation of prostaglandin biosynthesis, perhaps by calcium-dependent stimulation of phospholipase A_2 in the host cell membrane. Certainly, in macrophages, A23187 treatment leads to a burst of prostaglandin biosynthesis (Gemsma *et al.*, 1979). In this context the inhibitory action of cAMP on HeLa susceptibility to chlamydial infection might be explained by its ability to inhibit prostaglandin biosynthesis, either by blocking the release of precursor fatty acids from membrane lipids by phospholipase A_2 (Minkes *et al.*, 1977) or by inhibiting the formation of prostaglandin endoperoxides (Malmsten *et al.*, 1976). This would explain why the inhibitory action of cAMP on chlamydial infection was unaffected by prior treatment of the HeLa cells with prostaglandin biosynthesis inhibitors.

The results presented show that alterations in the concentrations of cyclic nucleotides and prostaglandins are capable of interacting to affect the susceptibility of HeLa cells to chlamydial infection. Further understanding of the mechanism involved and of the role of prostaglandin synthesis will necessitate direct assay of individual prostaglandin and cyclic

nucleotide responses and the use of more selective inhibitors of arachidonate metabolism. A critical question is whether chlamydiae themselves perturb the host cell membrane so as to bring about alterations in host cell control systems which favour their own uptake. The experiments with $^{45}\text{Ca}^{2+}$ suggest that interaction of chlamydiae with the host cell surface enhances Ca^{2+} efflux and influx through the cell membrane, raising the concentration of intracellular calcium, which, on the basis of the A23187 experiment, should directly or indirectly further chlamydial uptake. It is unlikely that this chlamydia-induced ion flux is selective for Ca^{2+} , as large inocula of live or inactivated *C. psittaci* are known to cause a rapid loss of various ions from mouse L cells following infection (Chang & Moulder, 1978). Increased Ca^{2+} mobility in the host cell membrane by activation of calcium-dependent membrane phospholipase A_2 should lead to enhanced prostaglandin biosynthesis. This, in turn, would stimulate cGMP production: in cell-free systems both Ca^{2+} (Goldberg & Haddox, 1977) and fatty acids related to prostaglandin biosynthesis (Goldberg *et al.*, 1978) activate guanylate cyclase, whilst in intact cells elevated intracellular Ca^{2+} is generally accompanied by a raised concentration of cGMP. Phospholipase A_2 activation in the membrane leads to the formation of lysolecithin, a potent membrane fusion agent (Weltzien, 1979) which might enhance chlamydial uptake by increasing membrane fluidity. The next step is to determine whether interaction of chlamydiae with the host cell membrane brings about short- or long-term alterations in the levels of host cyclic nucleotides or prostaglandins. Further study of the mechanisms discussed here may throw new light on the action of compounds known to potentiate the susceptibility of tissue culture cells to infection by chlamydiae present in clinical material. At present, studies on the immunochemistry of trachoma-inclusion conjunctivitis agents are limited by the problems of producing sufficient quantities of pure chlamydiae. It is to be hoped that an understanding of the factors controlling chlamydial interaction with host cells *in vitro* will lead to better systems for the large-scale growth of these important organisms.

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