An extracellular role for calmodulin-like activity in cell proliferation

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1. Addition of extracellular pure pig brain calmodulin was found to modulate DNA synthesis and cell proliferation in K562 human leukaemic lymphocytes. At lower cell densities calmodulin significantly stimulated [³H]thymidine uptake; at higher densities it decreased it. 2. A protein biochemically indistinguishable from calmodulin was detected in the cell-conditioned media of rapidly dividing K562 cells. The concentration of calmodulin-like activity found in the conditioned media of these and a range of other normal and neoplastic cells (250–1636 ng/ml) was of the same order as would stimulate DNA synthesis in subconfluent cells. 3. Amounts of extracellular calmodulin-like activity and immunoreactivity varied during cell growth from low to high density, a peak of extracellular calmodulin preceding DNA synthesis in synchronized K562 cells. Extracellular calmodulin concentrations did not correlate with the presence of lactate dehydrogenase in the medium. 4. Inhibition of extracellular calmodulin activity by calmodulin antagonist immobilized on agarose beads, or by antibody to calmodulin, significantly decreased DNA synthesis. 5. These data strongly suggest that calmodulin or a very closely related protein can influence mitosis through an extracellular mechanism.

INTRODUCTION

A transient increase in cytosolic free Ca²⁺ is considered to be a universal requirement for cell proliferation (Durham & Walton, 1982). The Ca²⁺ is thought to complex with the low-M_r protein calmodulin, which then activates several key intracellular processes leading to cell division (Tomlinson *et al.*, 1984). Evidence for this includes reports of a specific peak of calmodulin synthesis preceding entry into S phase (Chafouleas *et al.*, 1982) and of a decrease of cell-cycle time achieved by introducing multiple copies of calmodulin mRNA into the cell (Rasmussen & Means, 1987). In addition, calmodulin antagonists arrest cell division at the late G₁/S phase of the cell cycle, which is just after the point of calmodulin synthesis (Chafouleas *et al.*, 1982).

Investigations of intracellular calmodulin, however, have failed to define a simple relationship between calmodulin concentration and the degree of proliferation in either normal or transformed cells (Veigl *et al.*, 1984; Mac Neil *et al.*, 1985b). In addition, a puzzling decrease in intracellular calmodulin a few hours before DNA synthesis has been noted (Chafouleas *et al.*, 1984).

The ability of exogenous calmodulin to stimulate DNA synthesis in cultured cells was reported several years ago by our (Mac Neil *et al.*, 1984a) and other laboratories (Boynton *et al.*, 1980; Gorbacherskaya *et al.*, 1983), but until now little consideration has actually been given to an extracellular role for calmodulin.

We present evidence that a protein biochemically indistinguishable from calmodulin is present in the conditioned media of normal and neoplastic cells and that addition and inhibition of extracellular calmodulin affects DNA synthesis. We also report that a peak of

extracellular calmodulin directly precedes DNA synthesis. This is the first report to suggest and provide evidence for an extracellular role for calmodulin.

MATERIALS AND METHODS

Chemicals

Calmodulin radioimmunoassay kits, cyclic [8-³H]-AMP(20–30 Ci/mmol) and [³H]thymidine were obtained from Amersham International, Amersham, Bucks., U.K. Serum-free medium HB101 and supplement were obtained from Du Pont NEN, Stevenage, Herts., U.K. Glutamine, penicillin, streptomycin and fungizone were obtained from Gibco Europe, Paisley, Scotland, U.K. Phosphate-buffered saline (Dulbecco's) was obtained from Flow Laboratories, Rickmansworth, Herts., U.K. All tissue-culture plastics were purchased from Sterilin, Teddington, Middx., U.K. W7-Agarose and horseradish-peroxidase-conjugated anti-sheep IgG were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Anti-calmodulin IgG was kindly donated by Dr. J. G. Chafouleas for initial studies and then obtained from Peninsula Laboratories (Europe), St. Helens, Merseyside, U.K. Calmodulin-deficient ox heart phosphodiesterase, king-cobra (*Ophiophagus hannah*) venom, Dowex 1 (X8; 200–400 mesh) anion-exchange resin and lactate dehydrogenase assay kits were obtained from Boehringer-Mannheim. Oncomodulin was kindly given by Dr. P. J. MacManus.

Preparation of calmodulin

Pig brain calmodulin was prepared by the method of Kakiuchi *et al.* (1981). Preparations were over 95% pure

Abbreviation used: EGF, epidermal growth factor.

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as determined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Culture of cells

K562 human leukaemic lymphocytes were cultured in HB101 serum-free medium plus supplement. Supplement removal for 24 h resulted in cell synchronization.

In addition, media were also collected from a further range of normal, foetal and transformed cells in culture; the normal cells studied were human dermal fibroblasts, human epidermal keratinocytes, human non-toxic-goitre thyrocytes, human umbilical-vein endothelial cells, rat hepatocytes; the foetal cells studied were human foetal fibroblasts (A182), rat cloned myoblasts (L6) and mouse embryo Swiss fibroblasts (3T3); transformed cells were mouse B16 melanoma cells, human Chang liver cells, human Burkitt lymphoma (RAJ1) cells and rat kidney fibroblasts (NRK). These were cultured in a range of media, some with and some without sera present. In each case the corresponding cell-free medium was compared with the cell-conditioned medium.

Cell proliferation

Cell proliferation was determined both by counting cell number and by measuring [^3H]thymidine incorporation into DNA as previously described (Mac Neil *et al.*, 1984a). Cell number reflected [^3H]thymidine-uptake data for K562 cells.

Measurement of intracellular and medium calmodulin concentrations

Cell-conditioned media were collected from cells of high viability ($> 90\%$, as determined by their ability to exclude Trypan Blue), and centrifuged at 1000 *g* for 10 min to remove cells, and then conditioned and control cell-free media were heated to 95 °C for 5 min before cooling rapidly in ice. Media were then centrifuged at 11600 *g* for 10 min to remove denatured protein and stored at -20 °C until assay. Heat-treated extracts of K562 cells were prepared by removing tissue-culture medium by centrifugation at 1000 *g* for 10 min, washing once in phosphate-buffered saline and then resuspending in distilled water (which helped to burst the cells) and homogenizing with a glass Dounce homogenizer. Samples were then heated to 95 °C for 5 min, and cellular debris was removed as for media samples. All heat-treated supernatant samples were stored at -20 °C until assay. Samples were assayed for calmodulin-like biological activity by using a calmodulin-dependent cyclic nucleotide phosphodiesterase as previously described (Mac Neil *et al.*, 1984a) and for calmodulin immunoreactivity by using a calmodulin radioimmunoassay. The inter-assay coefficient of variation for this former assay is 17.7% (Mac Neil *et al.*, 1985a). Calmodulin values are expressed as either ng/ml or $\mu\text{g}/10^6$ cells.

Lactate dehydrogenase activity

Release of the intracellular enzyme lactate dehydrogenase was determined as an index of cell damage. Media were collected, centrifuged at 1000 *g* for 10 min to remove cells, and stored at 4 °C until assayed within 5 days with lactate dehydrogenase kits.

Western blotting of calmodulin in conditioned media

Conditioned medium (170 ml) was collected from K562 cells at exponential growth phase and centrifuged

for 10 min at 1000 *g* to remove cells. Calmodulin was extracted from the supernatant by the method of Kakiuchi *et al.* (1981) (this extraction greatly increased the amount of calmodulin-like activity present). Conditioned-medium-derived calmodulin and pig brain calmodulin (prepared by the same method) were analysed by SDS/polyacrylamide-gel electrophoresis on 20%-acrylamide slab gels, as described by Laemmli (1970), and transferred to 0.2 μm -pore-size nitrocellulose for 90 min at 150 mA, 4 °C. Samples were fixed with 0.1% glutaraldehyde for 30 min, and then blocked with 2% bovine serum albumin, 0.5% ovalbumin and 0.1% gelatin for 1 h. Incubation with sheep anti-calmodulin IgG (Peninsula Laboratories) at 5 $\mu\text{g}/\text{ml}$ was for 90 min at 20 °C. The second antibody used was horseradish-peroxidase-conjugated anti-sheep IgG, and the substrate was 0.05% (w/v) diaminobenzidine with 0.03% (v/v) H_2O_2 .

Use of W7-Agarose

Before incubation of cells with W7-agarose to decrease extracellular calmodulin activity, beads were washed three times in a large excess of phosphate-buffered saline at 4 °C, with centrifugation at 1000 *g* for 10 min to remove any free W7. Control agarose beads were handled similarly.

RESULTS

Effect of addition of pure calmodulin

Addition of exogenous calmodulin will stimulate DNA synthesis in a suspension culture of K562 human leukaemic lymphocytes (Fig. 1a). Cells were taken at exponential growth phase and resuspended in HB101 serum-free medium, used because of the high content of calmodulin in foetal-calf serum (Mac Neil *et al.*, 1984b). Purified pig brain calmodulin was added at concentrations of 0.1–10 $\mu\text{g}/\text{ml}$, and the effect on DNA synthesis was studied.

A significant increase in DNA synthesis occurred between 16 and 20 h, reaching a maximum at 24 h (Fig. 1a), and was barely detectable at 48 h (results not shown). At 24 h, maximal stimulation of DNA synthesis [cells at $(1-2) \times 10^5/\text{ml}$] was achieved at $2.2 \pm 0.6 \mu\text{g}/\text{ml}$ (130 nM; mean $\pm$ S.E.M. for nine expts.), with half-maximal stimulation at $0.8 \pm 0.2 \mu\text{g}/\text{ml}$ (50 nM; mean $\pm$ S.E.M. for nine expts.). Higher concentrations of calmodulin were inhibitory.

It was noted that exogenous calmodulin would stimulate proliferation in cells at low density, but would inhibit proliferation in the same cells at high density (Fig. 1b), suggesting that cell-derived calmodulin may be present in the medium.

Occurrence of calmodulin-like protein in medium

Conditioned media were collected at exponential phase from a range of normal, foetal and transformed cell lines and assayed for calmodulin-like activity by the ability to activate calmodulin-dependent bovine heart phosphodiesterase. The results of the study are summarized in Table 1. In all cases (except one) calmodulin activity in conditioned media (which ranged from 250 to 1636 ng/ml) was significantly greater than in the corresponding unconditioned media (which ranged from 1 to 700 ng/ml; $n = 13$ samples) and was within the range which we describe to stimulate DNA synthesis in K562 cells (Fig. 1). The one case where calmodulin activity was

Fig. 1. Effect of exogenous calmodulin on DNA synthesis in human K562 leukaemic lymphocytes grown in HB101 serum-free medium

In (a) cells at $1.5 \times 10^5/\text{ml}$ were incubated with calmodulin for a total of 12 h (●), 16 h (○), 20 h (Δ) or 24 h (▲) of incubation, receiving a pulse of [^3H]thymidine ($1 \mu\text{Ci}/0.2 \mu\text{l}$ well) during the final 4 h of incubation. In (b) cells at $2.7 \times 10^5/\text{ml}$ (●) and $2.4 \times 10^6/\text{ml}$ (○) were incubated for 24 h with calmodulin, receiving a final 4 h pulse of [^3H]thymidine. For both (a) and (b) points are means $\pm$ S.E.M. for triplicate cultures of a single representative experiment.

the same in the conditioned media as in the unconditioned was human foetal fibroblasts, where amounts in the unconditioned media were already fairly high (533 ng/ml).

Calmodulin was further identified in the conditioned medium of K562 cells by radioimmunoassay (Fig. 4b) and by immunoblotting. Fig. 2 shows that the species to which the anti-calmodulin antibody bound in conditioned medium showed a mobility similar to, although not identical with, that of authentic calmodulin and a characteristic change in mobility with Ca^{2+} (Molla *et al.*, 1981), having an apparent M_r of 22000–26000 in the presence of EGTA and of 20000–25000 in the presence of Ca^{2+} , as opposed to 20000 and 17000–20000 respectively for the purified calmodulin.

The presence of calmodulin in this conditioned medium was not attributable to cell damage, as cell viability was high ($> 95\%$), as determined by Trypan Blue exclusion, and release of the intracellular enzyme lactate dehydrogenase was low (less than 200 munits/ml). Anti-calmodulin antibody failed to react with control cell-free media similarly extracted or with oncomodulin.

Effect of inhibition of extracellular calmodulin-like activity

The effect of decreasing extracellular calmodulin activity in the culture medium was studied in K562 cells by using the calmodulin antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7) bound to agarose beads, and anti-calmodulin IgG. The addition of either well-washed W7-agarose or anti-calmodulin IgG to the culture medium significantly decreased [^3H]-

thymidine incorporation and cell division (Fig. 3). Neither agarose beads alone nor normal IgG affected cell proliferation.

W7 inhibits cell division in K562 cells equally well when it is bound to agarose beads or in the unbound form (Barton *et al.*, 1987), when it is free to enter the cell (Hidaka *et al.*, 1981). The concentration of W7 required for 50% inhibition of DNA synthesis is $24 \pm 2 \mu\text{M}$ (Barton *et al.*, 1987). For W7-agarose half-maximal inhibition was obtained between 15 and 250 μM (see Fig. 3 and Table 2). As with W7-agarose, a similar variability in the inhibitory response to anti-calmodulin IgG was also found. This was examined further by taking K562 cells growing at four different original densities and resuspending them in fresh HB101 medium to a density of 5×10^4 cells/ml. The concentrations of W7-agarose and of anti-calmodulin antibody necessary to inhibit [^3H]thymidine incorporation by 50% were measured over a 16 h incubation (Table 2). The higher the original cell density, the greater was the inhibitory effect of W7-agarose and anti-calmodulin antibody. This suggests that for cells at high density there is a decrease in the amount of extracellular calmodulin available for inhibition by W7-agarose or calmodulin antibody.

Finally, the relationship of extracellular and intracellular calmodulin to cell proliferation was examined in non-synchronized and synchronized cell cultures.

Relationship of extracellular and intracellular calmodulin to proliferation

When K562 cells were taken from high-density slow-proliferating cultures and diluted into fresh medium, an

Table 1. Calmodulin-like activity in cell-conditioned media

Cell-conditioned media were collected from cells of high viability (> 90%) in exponential growth phase and prepared as described in the Materials and methods section. Samples were assayed for calmodulin-like biological activity by determining their ability to activate calmodulin-dependent cyclic nucleotide phosphodiesterase, as described in the Materials and methods section. Values shown are derived from heat-treated media extracts assayed over a minimum of three dilutions. All media extracts diluted in parallel to a standard curve of pure pig brain calmodulin. The inter-assay coefficient of variation for this assay is 17.7%.

Cells	Calmodulin activity (ng/ml)	
	Medium alone	Cell-conditioned medium
Human dermal fibroblasts	180	383
Human epidermal keratinocytes	170	500
Human non-toxic goitre	95	1000
Human umbilical-vein endothelial cells	95	700
Rat hepatocytes	38	444
Human foetal fibroblasts (A182)	533	533
Rat cloned myoblasts (L6)	4*	250
Mouse embryo Swiss fibroblasts (3T3)	4*	325
Mouse B16 melanoma cells	40	1636
Human K562 leukaemic lymphocytes	1*	250
Human Chang liver cells	135	1438
Human Burkitt lymphoma (RAJI) cells	700	1243
Rat kidney fibroblasts (NRK)	4*	555

* Serum-free medium.

Fig. 2. Western-blot analysis of calmodulin extracted from conditioned medium from K562 leukaemic lymphocytes

Details are given in the Materials and methods section. Lanes (a) and (b) show calmodulin from conditioned medium, and lanes (c) and (d) pig brain calmodulin. In (a) and (c) samples contained 10 mM-EGTA, in (b) and (d) samples contained 10 mM-Ca²⁺.

increase in both intracellular and then extracellular calmodulin preceded or accompanied their entry into exponential growth phase (Fig. 4). A similar picture was obtained whether biologically active (Fig. 4a) or immunoreactive (Fig. 4b) calmodulin was determined, although the numerical agreement between estimates of biologically active and immunoreactive calmodulin was

Table 2. Effects of W7-agarose and anti-calmodulin antibody on [³H]thymidine incorporation in K562 cells

K562 human leukaemic lymphocytes were taken from cultures at four different densities as indicated, centrifuged at 1000 *g* for 5 min and then resuspended in fresh HB101 medium to 5 × 10⁴ cells/ml. Cultures were incubated for 16 h with [³H]thymidine and a range of concentrations of well-washed (see the Materials and methods section) W7-agarose or anti-calmodulin IgG. The concentration of each required to produce 50% inhibition of [³H]thymidine incorporation is given for each cell density. Results shown are from a single representative experiment.

10 ⁻⁵ × Original culture density (cells/ml)	Original doubling time (h)	Concn. for 50% inhibition of proliferation	
		W7-agarose (μM)	Anti-calmodulin IgG (μg/ml)
0.5	16	240	25
1.1	28	195	16
2.8	32	75	10
7.2	> 72	15	5

poor. The increase in extracellular calmodulin was accompanied by a decrease in intracellular calmodulin. As cells entered their plateau phase of growth, extracellular calmodulin decreased and intracellular values returned to those found in the exponential phase. Lactate dehydrogenase activity in the medium remained relatively low (beneath 200 munits/ml) throughout the first 3 days of culture, rising to 400–500 munits/ml by day 6. Cell viability similarly was usually > 95% throughout

Fig. 3. Effect of inhibiting extracellular calmodulin activity

Human K562 leukaemic lymphocytes at 10^6 cells/ml were incubated for 24 h with anti-calmodulin IgG (a) or W7-agarose (b), and proliferation was determined by cell number (a) or [^3H]thymidine incorporation over 24 h (b) respectively. In (a) cells were incubated with either anti-calmodulin IgG (●) or normal human IgG (○) at equivalent concentrations. In (b) cells were incubated with either W7-agarose (addition of 100 μl of W7-agarose suspension was equivalent to 750 μM -W7-agarose in the incubation) (●) or non-coupled agarose (○) at equivalent concentrations. Values shown are means $\pm$ S.E.M. for five cultures of single representative experiments.

Fig. 4. Intracellular and extracellular calmodulin concentrations during the growth of K562 human leukaemic lymphocytes from low to high cell density

Lymphocytes were taken from high-density cultures, washed and plated at 10^6 cells/ml in fresh HB101 medium and sampled every 24 h for intracellular (○) and extracellular (●) calmodulin and cell number (■). Calmodulin was determined by activity assay (a) and radioimmunoassay (b). Results shown are of a single representative experiment. Cell viability remained high (95 %) at day 3 and thereafter fell to 80 % by day 6. Similarly, lactate dehydrogenase activity was less than 200 munits/ml throughout days 1–3, but rose to 900 munits/ml by day 6.

Fig. 5. Intracellular and extracellular calmodulin concentrations during synchronized growth of K562 human leukaemic cells

Cells were synchronized by supplement deprivation for 24 h and then resuspended in complete HB101 medium at zero time. Samples of the cultures were taken every 12 h for measurement of intracellular (○) and extracellular (●) calmodulin activity (details as given in the Materials and methods section) and every 4 h throughout the first 24 h for measurement of [³H]thymidine incorporation (▲). Cell number (■) was determined every 12 h. Cell viability was greater than 95 % throughout the experiment, and lactate dehydrogenase in the medium (results not shown) remained low (133 munits/ml at 36 h and 199 munits/ml at 72 h) and did not relate to extracellular calmodulin. Results shown are of a single representative experiment. A similar pattern was obtained whether calmodulin was measured by activity assay or radioimmunoassay (results not shown).

the first 3 days and then began to fall, to 70–80 % by day 6.

The relationship between extracellular calmodulin and the initiation of DNA synthesis was examined in further detail in K562 cells synchronized by supplement deprivation for 24 h, then returned to complete medium at low cell density (5×10^4 cells/ml). DNA synthesis was measured for the first 24 h by incorporation of [³H]thymidine during 4 h periods. Cell number was determined every 12 h. Samples of the culture were recovered every 12 h, and calmodulin activity in both the medium and the cell lysate was determined. In a total of four experiments with K562 cells we found in each case a peak of intracellular and extracellular calmodulin activity preceding an increase in cell number.

Closer examination (Fig. 5) showed that this peak of intracellular and extracellular calmodulin activity preceded an increase in DNA synthesis, and was then followed by a doubling of cell number. At high cell density the rate of cell division was low, as were extracellular calmodulin concentrations.

DISCUSSION

Stimulation of DNA synthesis by extracellular calmodulin has previously been reported in rat liver cells (Boynton *et al.*, 1980), human peripheral blood lymphocytes (Gorbachskaya *et al.*, 1983) and B16 melanoma cells (Mac Neil *et al.*, 1984a). Until now, there has been little further investigation of this observation, largely because calmodulin has been considered to be exclusively intracellular; it does not possess a classical secretion sequence (Babu *et al.*, 1985) and it has only recently been reported in extracted fluids (Mac Neil *et al.*, 1988). However, interleukin 1, another low- M_r extracellular

protein, also lacks a secretion sequence (Auron *et al.*, 1984; Lomedico *et al.*, 1984), and we find concentrations of calmodulin in cell-conditioned medium which lie within a range of concentrations that will modulate the proliferation of K562 cells in culture.

The protein measured in conditioned medium is biochemically indistinguishable from calmodulin in that it activates a calmodulin-dependent phosphodiesterase and binds to calmodulin-specific antibodies in both radioimmunoassay (source Amersham) and Western-blot analysis (source Peninsula). In the latter the labelled bands have a mobility similar to, although not identical with, calmodulin and undergo a change in mobility in the presence of Ca^{2+} , which is characteristic of calmodulin (Molla *et al.*, 1981). The slight difference in mobility of the medium-extracted protein may be attributable to the greater amount of protein present or to the presence of other proteins in the conditioned medium which can alter the apparent M_r of calmodulin (Husain *et al.*, 1985). Alternatively, the protein is one which is immunologically and biologically very similar to calmodulin.

The relatively poor correlation between biologically active and immunoreactive measurements of calmodulin, as was apparent for both intracellular and extracellular calmodulin in this study (Fig. 4), has been noted by others and attributed to a different portion of the molecule being recognized in each assay (Veigl *et al.*, 1984). However, in agreement with Veigl *et al.* (1984), we find in the present and other studies (Mac Neil *et al.*, 1985a,b) that the pattern of response is similar for both estimates of calmodulin.

Another Ca^{2+} -binding protein, oncomodulin, is found in a wide variety of transformed cells (Gillen *et al.*, 1987). Like calmodulin, it has been reported to stimulate DNA synthesis in cultured cells when added to the medium

(Boynton *et al.*, 1982). This protein, however, has an M_r of 11500 and is reported to be immunologically dissimilar from calmodulin (Gillen *et al.*, 1987; Brewer *et al.*, 1984). We find that oncomodulin, kindly given by P. J. MacManus, does not bind the Peninsula antibody.

The importance of extracellular calmodulin to cell division is suggested by the degree of inhibition caused when exogenous antagonists are used to decrease calmodulin activity in K562 cell-culture medium and by previous unexplained reports relating to DNA synthesis. For example, in starfish oocytes re-initiation of meiosis was prevented only when calmodulin antagonists were present extracellularly, not when they were injected into the cell (Doree *et al.*, 1982). In Ca^{2+} -deprived rat liver cells anti-calmodulin antibody will block the DNA-synthesis response to the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate ('TPA') and to Ca^{2+} (Jones *et al.*, 1982), and in the same cells the inhibitory effects of the calmodulin antagonist trifluoperazine can be removed by addition of exogenous calmodulin (Boynton *et al.*, 1980).

Extracellular calmodulin may also be relevant to other cellular processes. Wong *et al.* (1980) found that addition of calmodulin will stimulate thromboxane production in intact platelets, and Wong & Cheung (1979) have demonstrated that extracellular calmodulin will stimulate phospholipase A_2 activity in platelets. We have so far only examined the role of extracellular calmodulin in mitogenesis.

Our current study demonstrates that the response to the addition or inhibition of extracellular calmodulin is related to the density at which the cells were originally growing and not that to which they are resuspended. This implies a change in the cells themselves rather than a change in the extracellular environment during culture. The concentration of extracellular calmodulin decreases as the cell growth rate slows (as can be seen in Figs. 4 and 5). This could explain the variable responses to W7-agarose and anti-calmodulin antibody. If extracellular calmodulin release is relatively low in cells taken from higher densities, then such cultures could be more sensitive to W7-agarose and anti-calmodulin antibody. This does not explain, however, how the same concentration of exogenous calmodulin can stimulate DNA synthesis in cells taken from low original density and inhibit DNA synthesis in cells from high original density. The ability of extracellular calmodulin to inhibit cell proliferation may be as important as the ability to promote DNA synthesis.

Immediate attempts at explaining the role of calmodulin in mitogenesis can only be speculative. Reports (Colca *et al.*, 1987; Graves *et al.*, 1986; Lin *et al.*, 1986) suggest that calmodulin interacts with other mitogens such as insulin and epidermal growth factor (EGF). Insulin will stimulate the phosphorylation of calmodulin in both intact (Colca *et al.*, 1987) and broken (Graves *et al.*, 1986) adipocytes and, in the latter, calmodulin will enhance receptor kinase activity. Calmodulin-binding sites have been found on the EGF receptor of A431 cells, and calmodulin is said to be phosphorylated by the EGF receptor (Lin *et al.*, 1986). Also the calmodulin antagonist chlorpromazine is reported to decrease the binding of ^{125}I -labelled EGF to transformed human fibroblasts (Bodine & Tupper, 1984) and to mouse 3T3 cells (Selinfreund *et al.*, 1986). In our own laboratory

(Mac Neil *et al.*, 1988) we find that calmodulin concentrations in a range of human body fluids relate significantly with those of EGF (correlation coefficient 0.79, $P < 0.01$ for nine fluids), with approx. 80 mol of calmodulin/mol of EGF. Preliminary results indicate that addition of exogenous EGF to K562 cells decreases the extracellular calmodulin activity in a dose-dependent manner.

A transient increase in intracellular free Ca^{2+} appears to be intrinsic to the actions of EGF and other growth factors (Moolenaar *et al.*, 1986). In some cases, such as that of platelet-derived growth factor, the rise is achieved by mobilization of intracellular stores, whereas the binding of EGF to its receptor induces Ca^{2+} entry from outside the cell (Moolenaar *et al.*, 1986). No calmodulin-binding site has so far been reported on the platelet-derived-growth-factor receptor.

One could speculate, therefore, that extracellular calmodulin may mediate Ca^{2+} entry into the cell. In this respect it would be interesting to determine whether other Ca^{2+} -binding proteins behave similarly, although other authors have failed to find any such mitogenic response to rabbit skeletal-muscle parvalbumin (Boynton *et al.*, 1982).

Alternatively, calmodulin may influence mitogen-receptor complex internalisation, thought to be related to the mitogenic signal (for review, see Gregoriou & Rees, 1984), as it has been suggested that calmodulin facilitates the recruitment of clathrin to the plasma membrane for the assembly of coated pits (Mooibroek *et al.*, 1987).

To conclude, the ability of tumour cells to proliferate in abnormally low extracellular Ca^{2+} has never been adequately explained. The early hypothesis that transformed cells contain greater concentrations of intracellular calmodulin, thereby allowing them to proliferate almost independently of extracellular Ca^{2+} concentration, has not been substantiated for K562 or other cell types. We now suggest that investigation of the mechanism of extracellular calmodulin action may provide important information on the control of proliferation in both normal and transformed cells.

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