

*Review Letter***Cyclic AMP, calcium and control of cell growth****R.K. Ralph***Department of Cell Biology, University of Auckland, Auckland, New Zealand*

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The role of cyclic AMP and calcium in the control of normal and tumour cell growth is considered in relation to the question whether cyclic AMP is a true mitogen or co-mitogen. It is proposed that cyclic AMP normally controls the cell cycle at a point in G1 phase only by virtue of its ability to exclude calcium required by cells to progress past this point into S phase. Therefore increased influx of calcium by other routes induced by various factors can bypass the inhibitory effect of cyclic AMP and stimulate growth. In these circumstances cyclic AMP or calcium may or may not facilitate further progress into S phase according to the metabolic requirements of individual cells. The relevance to cancer cells is considered.

Cyclic AMP Calcium Mitogen Growth control

1. INTRODUCTION

The following short review and critique considers the role of cyclic AMP and Ca^{2+} in the control of normal and tumour cell growth and particularly the question whether cyclic AMP is a mitogen or co-mitogen. It is proposed that cyclic AMP is the normal inhibitor of the cell cycle only by virtue of its ability to exclude Ca^{2+} from cells in G1 phase and that increasing Ca^{2+} influx sufficiently in G1 can override the effect of cyclic AMP and stimulate growth. No attempt has been made to present all of the references relevant to the topic since most have been summarized in past and recent reviews referred to in the text.

2. GROWTH INHIBITION BY CYCLIC AMP

Numerous reviews and articles have summarized a multitude of facts suggesting that adenosine-3',5'-cyclic monophosphate (cyclic AMP) controls mammalian cell growth [1-12]. Some of the more pertinent observations are that growth-inducing agents such as serum usually decrease cyclic AMP in cells, that cyclic AMP will reverse the action of serum on growth, that agents

that increase cyclic AMP in cells often reduce growth, that there is an inverse relationship between intracellular cyclic AMP concentration and growth rate, that cyclic AMP or its analogues can inhibit growth and cause regression of some tumours, and that several tumour cell lines that do not respond to cyclic AMP contain mutant cyclic AMP-dependent protein kinases, illustrating the need for cyclic AMP-dependent protein phosphorylation to control their growth. Additional support for possible control of the cell cycle by cyclic AMP comes from observations that cyclic AMP is a normal cellular metabolite and that high intracellular concentrations usually stop growth in G1 phase of the cell cycle, often restoring a morphology more like that attained during normal differentiation. Furthermore, it has been demonstrated [13] that cyclic AMP is involved in growth arrest rather than progress through the cell cycle, supporting a negative or inhibitory role for cyclic AMP in growth control (for discussion see [12]). Also, the action of cyclic AMP requires transmission of the cyclic AMP signal via protein phosphorylation [14] and failure of cyclic AMP to inhibit growth has often been traced to alterations or defects in the transmission pathway [10,15-18].

Thus in toto there is considerable support for cyclic AMP as a pleiotypic mediator [19] controlling the normal mammalian cell cycle.

3. GROWTH STIMULATION BY CYCLIC AMP

Recently the idea that cyclic AMP inhibits growth has been challenged, largely on the basis that cyclic AMP analogues or cyclic AMP-elevating agents such as cholera toxin or prostaglandin E1 appear to act synergistically with various mitogens such as insulin or epidermal growth factor to stimulate DNA synthesis in some cells. These observations have led to proposals that cyclic AMP provides a mitogenic signal for 3T3 cells [20,21] and that it stimulates DNA synthesis in other cells [22–25]. Evidence for the positive control of proliferation of rat liver cells and thymic lymphocytes by Ca^{2+} , cyclic AMP or the interplay of Ca^{2+} and cyclic nucleotides has also been presented [22–26] together with indications that calcium is necessary or possibly sufficient, for the initiation of cell growth in some systems [26–30]. What then is the truth? Do different mechanisms control the growth of different cells, or is it possible to reconcile these apparently contradictory observations and provide a universal model for the control of mammalian cell growth?

4. MITOGENS AND CALCIUM

Current research has demonstrated that mitogens such as serum, insulin, epidermal growth factor, platelet-derived growth factor or lectins all affect Ca^{2+} availability or mobilize cations in cells [29–40]. In particular it appears likely that an amiloride-sensitive Na^+/H^+ antiport is activated by many mitogens causing Na^+ to enter cells in exchange for H^+ ions and provoking the subsequent exchange of intracellular Na^+ for Ca^{2+} ions as occurs in other systems [33,36,41–46]. As a consequence, numerous intracellular Ca^{2+} -activated processes including carbohydrate metabolism phospholipid turnover, protein phosphorylation, microtubule depolymerisation, protease activation, cyclic nucleotide phosphodiesterase activation and altered mitochondrial metabolism could occur as part of a programme leading eventually to DNA synthesis as cells are propelled into cycle

[19,47–55]. Depending upon its concentration and/or the availability of calmodulin or other factors, elevated intracellular Ca^{2+} could also decrease cyclic AMP availability by inhibiting adenylate cyclase or stimulating cyclic nucleotide phosphodiesterase which would further reduce the restraints exerted by cyclic AMP on growth [56,57]. These and other observations suggest that the intracellular (cytoplasmic?) Ca^{2+} concentration is critical for the initiation of cell growth and DNA synthesis, a conclusion supported by evidence that Ca^{2+} induces some cells to cycle [9,11,12], that removal of Ca^{2+} with EGTA usually negates the growth response [58–61] and that cyclic AMP levels rise in some cells when growth is inhibited by EGTA (J. Smart, unpublished).

5. CYCLIC AMP AND Ca^{2+} AVAILABILITY

The cyclic AMP analogue N^6, O' -dibutyryl-adenosine 3',5'-cyclic monophosphate (DB cyclic AMP) blocks growth initiation by serum and other mitogens and Ca^{2+} influx into cells [8,29,30,61,62]. This raises the question whether the inhibition of growth by cyclic AMP is due solely to its ability to prevent excess calcium from entering cells at a critical growth restriction point [12] in G1 phase of the cell cycle. Some evidence that this could be true exists. Thus concanavalin A activation of small lymphocytes requires Ca^{2+} and the influx of Ca^{2+} into concanavalin A-treated cells is inhibited by DB cyclic AMP, as is DNA synthesis [29]. Furthermore either DB cyclic AMP or EGTA prevents PY815 mouse mastocytoma cells from receiving a flux of Ca^{2+} required for growth [59] and either reagent inhibits the influx of Ca^{2+} and subsequent DNA synthesis upon restoration of serum to serum-deprived 3T3 cells [30]. Cyclic AMP also reduces Ca^{2+} availability in rat thymocytes and PC_{12} cells by stimulating Ca^{2+} extrusion and the Ca^{2+} ionophore A23187 can bypass the effect of cyclic AMP and stimulate thymocyte growth and PC_{12} cell adhesion [28,63,64]. In addition, A23187 short-circuits cyclic AMP-dependent mechanisms that decrease Ca^{2+} concentrations in platelets [6]. These and other observations suggest that normally cyclic AMP acts to decrease intracellular Ca^{2+} and that swamping the capacity of cyclic AMP to limit intracellular Ca^{2+} concentrations by opening new Ca^{2+} channels with mitogens, hormones or

ionophores permits intracellular Ca^{2+} concentrations to rise sufficiently to initiate those Ca^{2+} -dependent processes required to activate cells (cf. [61,62]) allowing them to proceed into cycle while possibly, although not necessarily, simultaneously lowering cyclic AMP levels [56]. Having progressed beyond the critical Ca^{2+} -dependent restriction point in G1 phase cells could then respond to increased cyclic AMP production induced by agents such as cholera toxin by activating various metabolic processes [48], and thereby enhancing cell cyclic progression.

It is also possible that after G1 phase growth has initiated, later cyclic AMP-dependent events may be essential in some cells before DNA synthesis can eventually occur, explaining the apparent requirement for cyclic AMP-dependent protein phosphorylation for initiation of DNA synthesis in Ca^{2+} -deprived rat liver or other cells blocked late in G1 phase or at the G1/S phase boundary [11,22,25,27]. However, there appears to be no necessity for cyclic AMP-dependent events for S49 lymphoma mutants to progress through the cell cycle [12, 13,66] so that either protein phosphorylation is not universally needed or the requirement for such cyclic AMP-dependent events is overcome in S49 lymphoma and possibly other transformed or tumour cells [27].

After an initial Ca^{2+} influx initiates growth, further Ca^{2+} -dependent events could also be necessary for some cells to progress into S phase since subsequent removal of Ca^{2+} can prevent the onset of DNA synthesis [11]. However, addition of DB cyclic AMP to cells that have progressed into S phase does not always prevent cell division, although it may delay or inhibit cells in G2 phase [5,8,59,67], suggesting either that the need for Ca^{2+} is past once cells enter S phase, or that the Ca^{2+} taken up during the initial influx is available or sufficient to sustain at least one cell division. Again the requirements for Ca^{2+} appear to be different for normal and tumour cells (see below).

6. IS CYCLIC AMP A MITOGEN?

Conjecture that cyclic AMP alone directly assists cells to enter cycle or that it is a mitogen or co-mitogen is based partly on studies of the stimulatory effects of cyclic AMP or cyclic AMP-elevating agents (e.g., glucagon, cholera toxin)

together with other mitogens (e.g., insulin) on the entry of quiescent 3T3 cells into S phase [20,21,24]. Unfortunately these studies did not measure intracellular Ca^{2+} concentrations or follow Ca^{2+} fluxes during growth stimulation so that no conclusions about effects on intracellular Ca^{2+} movement are possible. In these circumstances it is dangerous to ignore the possibility that the reagents used (e.g., insulin, glucagon, prostaglandins) could have affected Ca^{2+} availability in the cells and that the true mitogenic effects resulted from Ca^{2+} action. Insulin [9,36,68], glucagon [69], and prostaglandins [70] are all known to affect cell membranes, ion transport and Ca^{2+} availability in cells [48]. Furthermore cyclic AMP-elevating agents other than cholera toxin are not always effective in assisting growth [71]. Therefore the results of such experiments may depend upon whether the action of cyclic AMP or cyclic AMP-elevating agents on Ca^{2+} availability is overcome by the true mitogens.

Similar conclusions, about cyclic AMP normally stimulating growth drawn from experiments in which cyclic AMP, protein kinases or protein kinase inhibitors initiate DNA synthesis when added to cells whose growth is inhibited close to the G1/S phase boundary, should also be viewed with caution since these observations may not reflect events at the normal G1 phase growth restriction point [22,23,25,27]. Furthermore, cyclic AMP is rapidly degraded by serum and by cells and it is doubtful whether the protein kinases or protein kinase inhibitors could enter cells. Therefore, it seems possible that these reagents may alter cell surfaces affecting membrane permeability and ion transport. The fact that isoproterenol raised cyclic AMP levels but did not initiate DNA synthesis in parotid glands in hypocalcemic thyroparathyroidectomized rats illustrates the need for Ca^{2+} and not just cyclic AMP production for DNA synthesis [26]. Effects on Ca^{2+} availability in some of these systems could also depend upon the source of the cells studied, since male and female rat liver cells respond differently to hormones [72]. Consequently information regarding Ca^{2+} movement and availability is essential before intracellular cyclic AMP can be seriously considered as a true mitogen.

In view of the above considerations it appears to be too early to conclude that cyclic AMP is a true

mitogen or to reject the conclusion that mammalian cell growth is normally inhibited by cyclic AMP at a point in G1 phase of the cell cycle by depriving cells of sufficient Ca^{2+} for an event or events necessary for further cell cycle progression and that this is the main effect of cyclic AMP on the cell cycle, other later stimulatory effects of cyclic AMP merely arising from the widespread action of cyclic AMP and protein phosphorylation on metabolism [9,48]. In these circumstances increasing Ca^{2+} influx into the cytoplasm to levels exceeding the capacity of cyclic AMP to negate the influx would inevitably bypass the action of cyclic AMP and allow growth to proceed. Progression through the cell cycle may or may not require further cyclic AMP or Ca^{2+} action in different cells according to the nature of the cells and their individual requirements for further cyclic AMP- or Ca^{2+} -dependent metabolic events, while the necessary Ca^{2+} could be derived from intracellular sources replenished during the initial influx. It is possible that the cyclic AMP controlled restriction point in the cell cycle could correspond to G_D , the differentiation point in G1 described in [73] since various aspects of differentiation frequently accompany inhibition of growth by cyclic AMP or its analogues [2,10,12,74].

7. CANCER CELL GROWTH

There is a general belief that cancer cells have an altered or aberrant Ca^{2+} metabolism [11,75–77]. For example, unusual features of cancer cells are that they will continue to grow at Ca^{2+} concentrations well below those required for normal cell growth [78–83] and that they contain higher Ca^{2+} concentrations than their normal counterparts [84–86]. These observations could be explained if cancer cells have low 'effective' levels of cyclic AMP which allow intracellular Ca^{2+} concentrations to rise sufficiently to overcome G1 phase growth restriction (cf. [10,15,16]). However, other reasons for the higher Ca^{2+} in cancer cells are possible. These include altered cell membranes with additional or activated Ca^{2+} channels [36,76,85], altered mitochondrial function and Ca^{2+} metabolism [85,87–89], altered Ca^{2+} influx or efflux capacity [90–94], altered Ca^{2+} transport by membranes of the endoplasmic reticulum [94] and even the possible inability of some cancer cells

to exclude Ca^{2+} [91]. Because cyclic AMP is known to affect several of these processes it is also possible that either the multiple effects of low 'effective' cyclic AMP, or independent alterations in any one or more of these functions could cause elevated intracellular Ca^{2+} and provoke growth of different cancer cells.

Changes in the ratios, expression or distribution of different cyclic AMP-dependent and independent protein kinases or kinase subunits have been detected in proliferatively activated cells and tumour cells [95–103] which could alter protein phosphorylation and modify Ca^{2+} fluxes, possibly as an initial consequence of reduced cyclic AMP [104]. However, because of the interlocked nature of the various mechanisms that control intracellular Ca^{2+} it is extremely difficult to discern which is the most important process controlling Ca^{2+} availability or which processes are unusual in individual tumour cells. Cytoplasmic Ca^{2+} is normally maintained at such low concentrations ($\leq 10^{-6}$ M) that relatively small changes in Ca^{2+} transport could be sufficient to alter significantly intracellular Ca^{2+} concentrations and induce Ca^{2+} -dependent growth processes.

Studies with amiloride suggest that quiescent cells have a minimally active amiloride-sensitive Na^+ channel whereas mitogen-stimulated cells and tumour cells appear to have an active Na^+ channel [32,33,41–43,105–108]. Increased Na^+ influx via such channels could explain the higher Ca^{2+} in tumour cells since intracellular Na^+ is known to exchange for extracellular Ca^{2+} in cells [36,45,48,109]. Furthermore, exchange of intracellular Na^+ for protons could increase intracellular pH, further assisting cells into cycle [38,43,110–113]. High intracellular Na^+ is another common feature of rapidly proliferating cells or transformed cells that correlate with cell proliferation [105,114]. The rapid stimulation of the Na^+/K^+ ATPase of liver cells by agents such as glucagon or epinephrine may also reflect increased Na^+ influx since Na^+ ions are known to increase Na^+/K^+ ATPase activity in other cells [115–118]. Exchange of Na^+ with Ca^{2+} might then explain the ability of these hormones to induce DNA synthesis in liver cells blocked at the G1/S phase boundary by Ca^{2+} deprivation [27].

As well as understanding the mechanisms that control intracellular Ca^{2+} concentrations it is im-

portant to define the specific event(s) in cells which require Ca^{2+} in order to initiate growth. Ca^{2+} , like cyclic AMP, is a pleiotypic mediator that affects a wide variety of cellular processes [48,119]. Therefore co-ordinate control of growth by Ca^{2+} rather than control of one or two specific events or processes must be considered. Many effects of Ca^{2+} on cellular metabolism involve the Ca^{2+} -binding protein calmodulin [86,119]. However, the recent discovery that protein kinase C, a Ca^{2+} and phospholipid-dependent, diacylglycerol-activated tyrosine-specific protein kinase is activated in cells by phorbol esters and that other mitogens such as insulin, epidermal growth factor and platelet-derived growth factor all activate similar tyrosine-specific protein kinases in cells raises the possibility that C type protein kinases could respond to Ca^{2+} influx by phosphorylating many proteins in cells to coordinately induce cell growth [120–124]. Therefore, just as Ca^{2+} and cyclic AMP act as synarchic messengers to control cellular metabolism [48] it is also likely that they are complementary pleiotypic mediators co-ordinating growth or quiescence. How better to achieve this than through protein phosphorylation controlled by different protein kinases uniquely switched on by Ca^{2+} or cyclic AMP? How better to subvert such a system than as the oncogenic viruses have done by increasing the production in cells of protein kinases not subservient to cyclic AMP [125–127]? Perhaps these viruses also alter Ca^{2+} transport mechanisms to ensure that the kinases are fully activated?

In view of the above considerations it is now important to identify precisely those actions of cyclic AMP and Ca^{2+} that control cell growth and to determine their metabolic consequences. It may then be possible to correct, or bypass these effects and eventually control or eliminate cancer cells in a logical manner.

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