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CALMODULIN STIMULATES DNA SYNTHESIS BY RAT LIVER CELLS

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SUMMARY

Incubation in low (0.02 mM)-calcium medium prevented T51B rat liver cells from initiating DNA synthesis. Raising the calcium concentration in the medium from 0.02 to 1.25 mM caused these arrested cells to initiate DNA synthesis 1-2 hours later. The possibility of this rapid DNA-synthetic response to calcium addition being mediated through Ca-calmodulin complexes was suggested by the following observations: It was blocked by the putative Ca-calmodulin blockers chlorpromazine and trifluoperazine; the trifluoperazine-inhibited cells were stimulated by purified rat calmodulin; and purified rat calmodulin itself (10^{-7} to 10^{-6} moles/l) mimicked calcium action, unless the already low ionic calcium concentration in the calcium-deficient medium was reduced further by adding the specific calcium chelator EGTA.

INTRODUCTION

Calcium ions are needed briefly in later G1 phase of the growth-division cycle for the completion of prereplicative development and the initiation of DNA synthesis by a wide variety of non-neoplastic cells *in vitro* and *in vivo* (see reviews by Whitfield *et al.* (1,2) for lists of original references). Consequently, lowering the extracellular calcium concentration *in vitro* does not stop on-going DNA synthesis, but it does stop proliferative development in later G1 phase of the growth-division cycle (1,2). These blocked cells may then revert sooner or later to an earlier prereplicative state unless rescued by a timely elevation of the extracellular calcium level, which causes them promptly to initiate DNA synthesis and finish the cycle (1,2).

Since many of calcium's actions in the cell are now being attributed to the formation of Ca-calmodulin complexes, which

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stimulate many different enzymes (3,4), it would be reasonable to begin examining the role of these complexes in prereplicative development as suggested by Whitfield et al. (2). In this communication we present some preliminary evidence which indicates that Ca-calmodulin complexes might indeed be active in the calcium-dependent process or processes leading to the initiation of DNA synthesis.

MATERIALS AND METHODS

Non-neoplastic T51B epithelioid rat liver cells were isolated by Swierenga et al. (5). Proliferation of these cells, like that of their counterparts in the regenerating rat liver, can be inhibited by lowering the extracellular calcium concentration (1,2). To do this, the cells were first planted at a density of $0.7 \times 10^4/cm^2$ on 25 mm round plastic coverslips (in 35 mm plastic Petri dishes) in a high (1.8 mM)-calcium medium consisting of 90% (v/v) BME (Eagle's basal medium from Flow Laboratories, Rockville MD), 10% (v/v) FBS (fetal bovine serum also from Flow Laboratories), and the antibiotic gentamicin (from Microbiological Associates, Bethesda MD). They were then incubated for 24 hours at 37°C (in an atmosphere consisting of 95% and 5% CO_2) to ensure maximum attachment and spreading, after which the high-calcium medium was replaced by low (0.02 mM)-calcium medium consisting of 90% (v/v) commercially prepared (by Grand Island Biological Co., Grand Island NY) low (0.02 mM)-calcium BME and 10% (v/v) FBS, the ionic calcium concentration of which had been reduced to 0.02 mM with the specific calcium chelator EGTA (ethylene-bis-(oxyethylenenitrilo) tetraacetic acid) (6). Forty-eight hours later (i.e., 72 hours after the initial plating), some experiments began with the addition of calcium chloride to cultures in the low-calcium BME-FBS medium. However, in experiments with calmodulin, the calcium-deficient BME-FBS medium was discarded and replaced with equally calcium-deficient serum-free BME medium 10 minutes before addition of calcium and/or other agents. Serum-free medium was used simply to avoid any possible interference with calmodulin action by the EGTA required to lower the ionic calcium concentration in FBS.

Rat testicular calmodulin was purified to homogeneity according to MacManus (7). Chlorpromazine was obtained from Sigma Chemical Co. (St. Louis MO), and trifluoperazine was a gift from Smith, Kline and French (Montréal, Canada).

DNA-synthetic activity was assessed from the proportion of cells whose nuclei were labeled during a 1-hour exposure to [^{3}H]-thymidine (5 μCi/ml of medium; specific activity 20 Ci/mmole; from Amersham, Arlington Heights IL). The autoradiographic procedure was that of Whitfield et al. (8) in which the radioactivity is found only in the DNA (and not in the RNA or cold acid-soluble nucleotide fraction) and produces approximately 500 silver grains in the nuclear track emulsion overlying the nucleus of each DNA-synthesizing cell.

RESULTS AND DISCUSSION

Around 30 to 40% of the cells in T51B cultures were reversibly blocked in late G1 phase by end of 48 hours of incubation in calcium-deficient 90% (v/v) BME - 10% (v/v) FBS medium containing

only 0.02 mM calcium. Raising the calcium concentration from 0.02 to 1.25 mM caused these blocked cells to initiate DNA synthesis within 1-2 hours regardless of whether they were still in BME-FBS medium or had been covered with serum-free, calcium-deficient BME medium 10 minutes before calcium addition (Figs. 1 and 2).

If this rapid DNA-synthetic response to calcium addition were mediated through Ca-calmodulin complexes, it should have been suppressible by low concentrations of chlorpromazine and trifluoperazine, which are currently believed to bind specifically to, and hence specifically to block the various actions of, these complexes (3,4,9). A 15-minute preexposure of calcium-deprived cells in BME medium to a low concentration of trifluoperazine (2.5×10^{-6} moles/1) prevented their DNA-synthetic response to calcium addition (Figure 1). The drug did not block calcium action simply by non-specifically damaging or killing the cells, because addition of purified rat calmodulin (10^{-6}moles/1) either along with calcium, or 2 hours afterwards elicited a rapid DNA-synthetic response from the trifluoperazine-treated cells (Fig. 1). A 15-minute preexposure of calcium-deprived cultures in BME-FBS medium to chlorpromazine (at the lowest maximally effective concentration of 1.4×10^{-5} moles/1) also blocked the DNA-synthetic response to calcium addition (Fig. 2).

Purified rat calmodulin at a concentration of either 10^{-7} or 10^{-6} moles/1 also stimulated otherwise untreated cells in calcium-deficient, serum-free BME medium to initiate DNA synthesis as rapidly and as effectively as calcium addition (Fig. 3). However, the DNA-synthetic response of the calcium-deprived cultures to calmodulin was comparatively brief: It lasted for only 2 hours (Figs. 1 and 3) while the response to calcium addition lasted for at least 8 hours (Figs. 1 and 2).

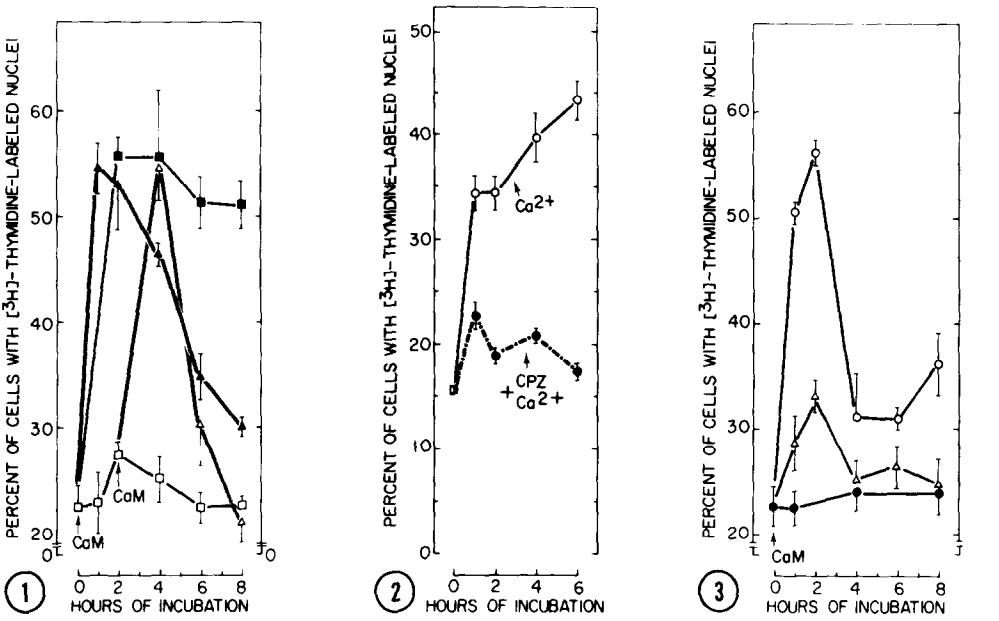

Fig. 1. The rapid DNA-synthetic response of calcium-deprived T51B rat liver cells to calcium addition, the prevention of this response by trifluoperazine, and the ability of purified rat calmodulin (CaM) to elicit a rapid DNA-synthetic response from the trifluoperazine-treated cells. (■), the calcium-deficient (0.02 mM) BME-FBS medium was discarded after 48 hours and replaced with calcium-deficient (0.02 mM) serum-free BME medium 10 minutes before the addition (at time "0") of enough calcium chloride to raise the extracellular calcium concentration to 1.25 mM; (□), trifluoperazine (2.5×10^{-6} moles/l) was added 15 minutes before calcium chloride; (▲), calmodulin (CaM; 10^{-6} moles/l) was added to the trifluoperazine-treated cultures at the same time as calcium; (△), calmodulin (CaM; 10^{-6} moles/l) was added to the trifluoperazine-treated cultures 2 hours after calcium. In these, and the experiments of Figs. 2 and 3, cells were exposed to [^{3}H]-thymidine (5 μCi/ml) for one hour without addition of calcium and/or other agents in order to obtain the initial (time "0") activity, and between 0 and 1, 1 and 2, 2 and 3, etc. hours after addition of calcium and/or other agents. Cells were fixed at the ends of the labeling periods which are the times recorded along the absissae. Each point is the mean ± S.E.M. of the values from 4 cultures.

Fig. 2. The ability of chlorpromazine (CPZ) to block the rapid DNA-synthetic response of calcium-deprived T51B rat liver cells to calcium addition. Cells in calcium-deficient BME-FBS medium were exposed to chlorpormazine (1.4×10^{-5} moles/l) 15 minutes before the addition of enough calcium chloride (at time "0") to raise the calcium concentration in the medium from 0.02 to 1.25 mM. Each point is the mean ± S.E.M. of the values from 4 cultures.

Fig. 3. The ability of rat testicular calmodulin to elicit a rapid DNA-synthetic response from calcium-deprived T51B rat liver cells. (●), the calcium-deficient (0.02 mM) BME-FBS medium was discarded after 48 hours and replaced with calcium-deficient (0.02 mM), serum-free BME, but nothing was added; (○), at time "0" (10 minutes after replacement of calcium-deficient BME-FBS medium with calcium-deficient, serum-free BME medium) rat calmodulin was added to a final concentration of 10^{-6} moles/l; (△), EGTA was added along with calmodulin to a final concentration of 10^{-5} moles/l in order to reduce further the already low (2×10^{-5} moles/l) calcium concentration in the calcium-deficient BME medium. Each point is the mean ± S.E.M. of the values from 4 cultures.

The DNA-synthetic response of the calcium-deprived T51B cells to calmodulin addition might have been due to the action of calmodulin itself or to Ca-calmodulin complexes formed by the interaction of the regulator with the calcium ions remaining in the calcium-deficient BME medium (i.e., 2×10^{-5} moles/l). Some support for Ca-calmodulin complexes being the active agents was provided by the observation that a further reduction of the extracellular ionic calcium concentration in the calcium-deficient BME medium with EGTA (10^{-5} moles/l) reduced the DNA-synthetic response to calmodulin addition by about 70% (Fig. 3).

Preliminary though they are, these observations strongly suggest that calcium stimulates the initiation of DNA synthesis in calcium-deprived T51B rat liver cells through the formation of Ca-calmodulin complexes. More importantly, they suggest the possibility that a brief redistribution of cellular Ca-calmodulin complexes might be one of the key events in later prereplicative development which triggers a process or processes leading to the initiation of DNA synthesis

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