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SERUM RAPIDLY MOBILIZES CALCIUM FROM AN INTRACELLULAR POOL IN QUIESCENT FIBROBLASTIC CELLS

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SUMMARY: Addition of dialysed fetal bovine serum to quiescent cultures of Swiss 3T3 cells loaded with $^{45}Ca^{2+}$ causes a very rapid increase in the rate of $^{45}Ca^{2+}$ efflux from an intracellular pool. Exposure to serum for 2 min leads to a fall of 0.59 nmol Ca^{2+}/mg protein in the intracellular Ca^{2+} content of the cells. Inhibitors of mitochondrial function prevent the stimulation of $^{45}Ca^{2+}$ efflux by serum. The stimulation of $^{45}Ca^{2+}$ efflux by serum is also observed in quiescent cultures of Rat-1, Swiss 3T6 and BHK cells and in secondary cultures of whole mouse embryo fibroblasts.

Quiescent cells can be stimulated to reinitiate DNA synthesis and cell division by addition of serum or growth-promoting factors (1,2). Understanding of the mechanism of action of proliferative stimuli requires the identification of the intracellular signals capable of initiating or modulating the proliferative response. Ion fluxes and redistributions are thought to play a role in mediating the action of mitogenic agents (2,3). An early increase in Na^+ influx is increasingly recognised as one of the earliest events elicited by mitogenic stimulation (2-4). Although divalent cations have been also implicated in the control of cell proliferation (5-14), little attention has been given to the early changes in Ca^{2+} flux in quiescent fibroblastic cells stimulated to reinitiate DNA synthesis and cell division. In a previous study, Tupper et al. (15) concluded that serum causes a rapid decrease in extracellular Ca^{2+} from a pool presumably bound to the cell surface and/or to secreted macromolecules. In contrast we report here that addition of serum causes a rapid increase in the rate of Ca^{2+} efflux from an intracellular pool of quiescent Swiss 3T3 cells which leads to a net decrease of the level of this cation in the cells.

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These, and similar findings with other cell types, raise the possibility that Ca^{2+} movements may mediate some of the actions of serum in quiescent cells.

MATERIALS AND METHODS

Cell culture: Stock cultures of Swiss 3T3 cells (16) propagated as previously described (17) were subcultured into 30 mm Nunc petri dishes (10^5 cells per dish) with Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum. The cells became confluent and quiescent in G_1/G_o (17) 6-9 days after plating. Whole mouse embryo, BHK and Rat-1 cells were subcultured in DME medium containing 0.5% fetal bovine serum and became quiescent 6 to 10 days later.

$^{45}Ca^{2+}$ efflux: Quiescent cultures of the various cell types were loaded with $^{45}CaCl_2$ (4μCi/ml) by incubating the cultures for 24 h in conditioned medium. $^{45}Ca^{2+}$ efflux was carried out at 37°C in an atmosphere of 10% CO_2:90% air in 2 ml of DME medium after removing the labelling medium and washing the cultures as indicated in the individual experiments. At the times indicated, samples of 0.2 ml were taken for counting and were replaced by 0.2 ml of fresh medium. The radioactivity left in the cells at the end of the efflux was extracted with 1 ml of solution containing 0.1% SDS, 2% Na_2CO_3 and 0.1 N NaOH. The sum of all the radioactivity in each sample plus the radioactivity left in the cells at the end of the efflux period was taken as the total radioactivity present in the cells at the beginning of the experiment.

Analysis of the various Ca^{2+} compartments: The radioactivity remaining in the cells at each time point was obtained by sequentially subtracting the radioactivity of each sample. It was plotted on semilogarithmic paper as per cent of the total initial radioactivity and the half-lives of exchange of the different pools were obtained by graphical analysis (18).

Materials: The serum in all experiments was fetal bovine obtained from Flow Laboratories. $^{45}CaCl_2$ was obtained from the Radiochemical Centre (Amersham, England). All other chemicals were of the purest grade commercially available.

RESULTS

Quiescent cultures of Swiss 3T3 loaded with $^{45}Ca^{2+}$ for 24 h, were rapidly (4-5 sec) washed 3 times and transferred to DME medium containing Ca^{2+} at 1.8 mM. The efflux of radioactivity into the medium was monitored as a function of time. Graphical analysis of such a $^{45}Ca^{2+}$-efflux curve, performed as described under Materials and Methods, indicates the presence of at least three different Ca^{2+} pools with half-lives of exchange of 15 sec, 9 min and 64 min for the "very fast", "fast" and "slow" compartments respectively, as previously reported in other cell lines (13,18). The "very fast" pool is generally accepted to represent extracellular bound Ca^{2+}; in Swiss 3T3 cells, the size of this pool is decreased from 40% to

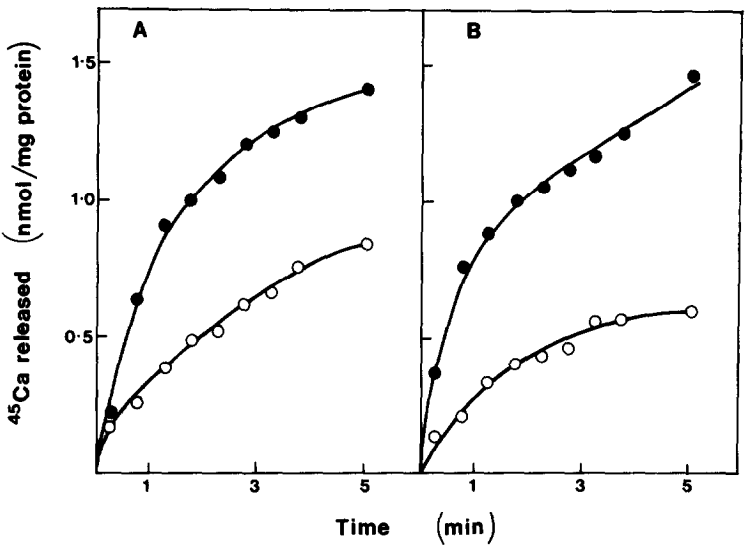

Fig.1: $^{45}Ca^{2+}$ efflux from quiescent cultures of Swiss 3T3 cells in the absence or presence of fetal bovine serum. Quiescent cultures of 3T3 cells were loaded with 4μCi/ml of $^{45}Ca^{2+}$ for 24 h. Prior to the efflux experiment the medium was removed and the cultures were washed A. seven times with 2 ml of DME medium at 37° or B. 5 times with 2 ml of 3 mM EGTA containing DME medium and 3 times with 2 ml of DME medium, at 37°. The efflux of $^{45}Ca^{2+}$ was carried out at 37°C in 2 ml of DME medium without (○) or with (●) 10% fetal bovine serum, as described in Materials and Methods.

less than 5% of total cell $^{45}Ca^{2+}$ by washing the cultures with DME medium (1.8 mm Ca^{2+}) containing 3 mM EGTA (results not shown).

Fig.1A shows that addition of serum causes a very rapid increase in the rate of $^{45}Ca^{2+}$ efflux; the effect can be detected as early as 15 sec after the addition of serum (the earliest time point determined). To assess whether the stimulation of $^{45}Ca^{2+}$ efflux by serum represents an increased mobilization of Ca^{2+} from an intracellular pool or only enhanced release of extracellularly bound Ca^{2+} (15), cultures of $^{45}Ca^{2+}$-loaded 3T3 cells were washed with EGTA-containing medium and then incubated in the absence or presence of 10% serum. As shown in Fig.1B the elimination of the "very fast" compartment by EGTA does not prevent the stimulation of $^{45}Ca^{2+}$-efflux induced by serum. This finding suggests that serum stimulates the mobilization of Ca^{2+} from an intracellular pool. In other experiments in which efflux of $^{45}Ca^{2+}$ was monitored for 90 min, we observed that serum decreases the half-life of exchange of the fast pool from 9 to 2

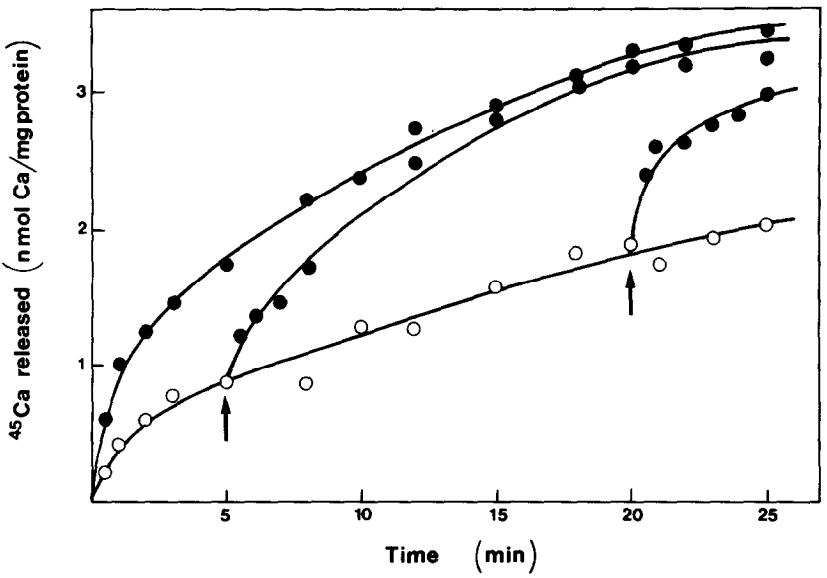

Fig.2: The stimulation of $^{45}Ca^{2+}$ efflux from Swiss 3T3 cells is also observed when the addition of serum is delayed for 5 or 20 min after the onset of $^{45}Ca^{2+}$ efflux. $^{45}Ca^{2+}$-loaded cultures of Swiss 3T3 cells were washed as described in Fig.1A and incubated with 2 ml of $^{45}Ca^{2+}$-free DME medium with (●) or without (○) 10% fetal bovine serum. At the times indicated by the arrows some of the control cultures received 10% serum.

min while that of the slow pool is only slightly affected (results not shown).

Further evidence supporting the conclusion that serum causes Ca^{2+} mobilization from an intracellular pool comes from the experiment shown in Fig.2 in which the addition of serum is delayed for 5 or 20 min after the cultures are washed and transferred to medium without radioactive Ca^{2+}. Regardless of the time at which serum is added, it causes a rapid increase in the rate of $^{45}Ca^{2+}$ efflux. Since at the times of serum addition (i.e. 5 and 20 min) the calculated amount of $^{45}Ca^{2+}$ remaining in the "very fast" pool represents less than 1% of the initial $^{45}Ca^{2+}$ in this pool, the results indicate that serum mobilizes Ca^{2+} from an intracellular pool rather than from a surface-localized compartment.

The stimulation of $^{45}Ca^{2+}$ efflux by fetal bovine serum could represent an increased $^{45}Ca^{2+}$-$^{40}Ca^{2+}$ exchange or a net depletion of a cellular pool of Ca^{2+}. Fig.3A shows that in the absence of any extracellular Ca^{2+}, serum produces a substantial increase in the rate of Ca^{2+} release from 3T3 cells,

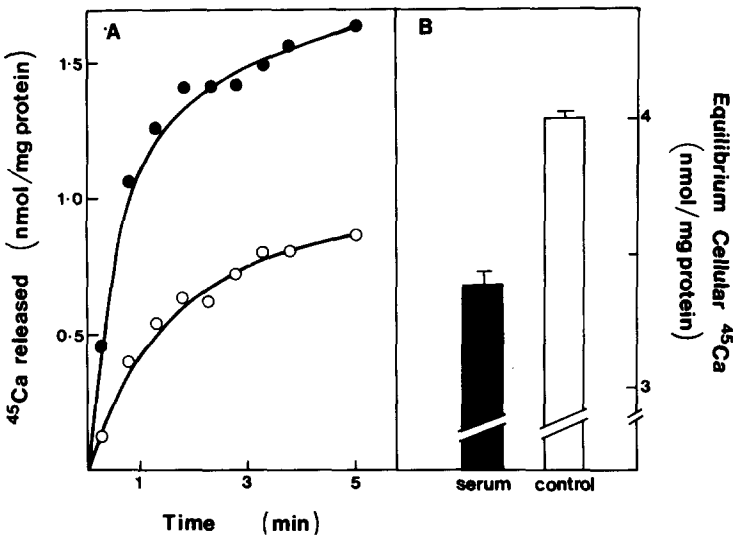

Fig.3: The stimulation of $^{45}Ca^{2+}$ efflux by serum does not represent an increased $^{45}Ca^{2+}$-$^{40}Ca^{2+}$ exchange. A. Quiescent cultures of Swiss 3T3 cells loaded with $^{45}Ca^{2+}$ (4μCi/ml) for 24 h were washed 7 times at 37°C with 2 ml of Ca^{2+}-free DME medium containing 50μM EGTA. The efflux experiment was carried out in 2 ml of the same medium without (○) or with (●) 10% of fetal bovine serum which was dialyzed against 2 mM EGTA/saline. B. Quiescent cultures of Swiss 3T3 cells were labelled with 4μCi/ml of $^{45}Ca^{2+}$ for 24 h. Then, either serum or saline (0.11 ml) were added directly to the medium (1 ml) in the presence of the isotope (i.e. under isotopic equilibrium conditions). After 2 min of incubation at 37°C, the cultures were washed 7 times with 2 ml of 3 mM EGTA-containing DME medium at 37°C. The radioactivity left in the cells was determined as described in Materials and Methods. The results represent the mean ±SEM of 5 measurements.

thereby excluding the possibility of an increased $^{45}Ca^{2+}$-$^{40}Ca^{2+}$ exchange as the sole cause of the stimulation of $Ca2^{+}$ efflux. Further, when $^{45}Ca^{2+}$-loaded cells are treated for 2 min with 10% serum under equilibrium $^{45}Ca^{2+}$ conditions (see legend to Fig. 3) there is a decrease of 0.59 ± 0.069 nmol Ca^{2+}/mg protein in the intracellular Ca^{2+} content of the cultures (Fig 3b). In agreement with these results, the initial rate of $^{45}Ca^{2+}$ uptake into the cells (i.e. uptake measured after washing the cells in the presence of EGTA) is not significantly enhanced by serum, even 5 min after its addition (results not shown).

Inhibitors of mitochondrial function are known to cause a release of Ca^{2+} from several cell types (18,19) including Swiss 3T3 cells (Table 1). Pretreatment for 10 min of $^{45}Ca^{2+}$-loaded cultures of Swiss 3T3 cells with either rotenone (at 1 μM) or rutamycin (at 2 μg/ml) inhibits by 74-80% the

Table 1: Effect of the pretreatment with mitochondrial inhibitors on serum-induced $^{45}Ca^{2+}$-efflux.

Preincubation	Cell Ca^{2+}	Serum-induced Ca^{2+}-efflux (nmol/mg protein)	% Inhibition
-	4.9	0.82	0
+ Rotenone (1 μM)	3.8	0.21	74
+ Rutamycin (2 μg/ml)	3.6	0.16	80

Quiescent of cultures Swiss 3T3 cells were loaded with $^{45}Ca^{2+}$ as described in Materials and Methods. Prior to the efflux experiment some of the cultures were treated for 10 min with either rotenone (1μM) or rutamycin (2μg/ml). The cultures were subsequently washed seven times with 2 ml of DME medium containing 3 mM EGTA and the amount of $^{45}Ca^{2+}$ released into DME medium with or without 10% serum was determined as described in Materials and Methods. "Cell Ca^{2+}" represents the Ca^{2+} present in cells at the end of the preincubation period. Serum-induced Ca^{2+}-efflux is expressed as the difference in the amount of ^{45}Ca released from serum-stimulated and control cultures after 2 min of efflux. Similar results were obtained in three independent experiments.

subsequent release of $^{45}Ca^{2+}$ induced by serum (Table 1), suggesting that the same intracellular Ca^{2+} pool is affected by treatment with both fetal bovine serum and the inhibitors of mitochondrial function.

The ability of serum to stimulate $^{45}Ca^{2+}$ efflux is not confined to Swiss 3T3 cells, but can be also demonstrated in quiescent cultures of Rat-1 cells, secondary cultures of mouse embryo fibroblasts (Fig.4), mouse 3T6 cells and hamster kidney cells (BHK) (results not shown).

DISCUSSION

The present findings indicate that addition of dialysed fetal bovine serum to quiescent cultures of Swiss 3T3 cells and other fibroblast cell lines induces the mobilization of Ca^{2+} from an intracellular store(s). Our study demonstrates that this is one of the earliest events (15 sec) which takes place in quiescent fibroblasts after the stimulation with serum. Changes in Ca^{2+} distribution as a result of mitogenic stimulation are not restricted to serum-stimulated cells since we have recently observed that the neurohypophyseal nonapeptide vasopressin, which is a potent mitogen for Swiss 3T3 cells (20) stimulates the efflux of $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$-loaded cultures of quiescent Swiss 3T3 cells and causes a net decrease in the

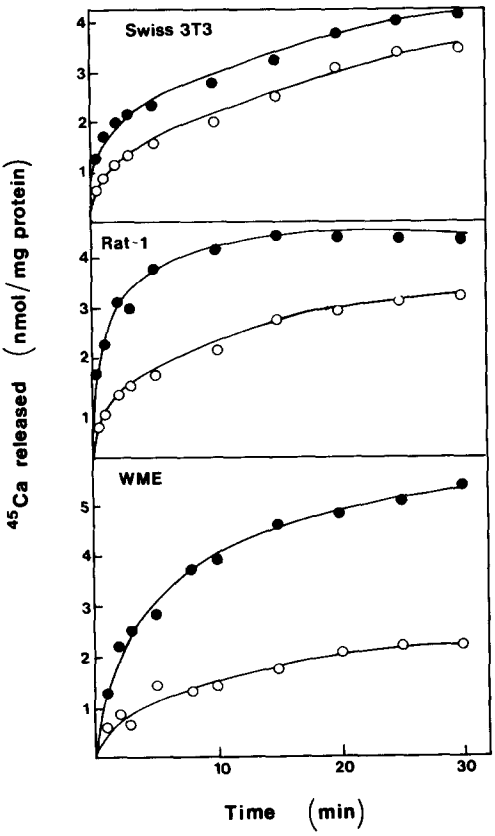

Fig.4: Effect of fetal bovine serum on $^{45}Ca^{2+}$ efflux from different cell types. All the cell cultures were pre-loaded with 4μCi/ml of $^{45}Ca^{2+}$ for 24 h, washed seven times with DME medium at 37°C, and the efflux experiments were carried out in this medium either in the absence (○) or presence (●) of 10% serum. The effect of serum on $^{45}Ca^{2+}$ efflux from 3T3 cells is shown for comparison.

cellular Ca^{2+} content (manuscript in preparation). In contrast, neither insulin (10μg/ml) nor epidermal growth factor (5 ng/ml) affected $^{45}Ca^{2+}$ efflux from 3T3 cells. In view of the inhibitory effect of rotenone or rutamycin on the stimulation of Ca^{2+} efflux by serum, it appears that mitogenic stimulation releases Ca^{2+} from a mitochondrial store. It is plausible that as a result of this Ca^{2+} flux there is an increase in the cytosolic concentration of this cation which, in turn, leads to the observed efflux of Ca^{2+} from the cell mediated by the plasma membrane Ca^{2+} ATPase pump. Cytosolic Ca^{2+} is known to modulate the activity of a wide variety of processes including metabolism, secretion, microtubule organization, motility and membrane transport (21-24). In view of the

rapid enhancement of Ca^{2+} fluxes upon growth stimulation shown in this paper, an analysis of the cause-effect relationship between Ca^{2+} movements, monovalent ion fluxes and other events associated with mitogenesis as well as further studies on the effect of pure growth-promoting agents on the cellular distribution of Ca^{2+} are warranted.

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