Some mitogens cause rapid increases in free calcium in fibroblasts

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Received 10 January 1984; revised version received 1 March 1984

Quiescent 3T3 fibroblasts grown on microcarrier beads and loaded with the $[Ca^{2+}]$ indicator quin2 had a cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) of 154 ± 11 nM (SE; $n = 32$). Stimulation with the mitogens vasopressin, epidermal growth factor (EGF) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) caused a very rapid increase in $[Ca^{2+}]_i$ to a maximum of 200–500 nM after 60–90 s. $[Ca^{2+}]_i$ declined thereafter to a level above that in quiescent cells which was maintained for at least 15 min. In contrast no immediate effects on $[Ca^{2+}]_i$ were detected after the addition of the mitogens insulin or 12-*O*-tetradecanoylphorbol 13-acetate (TPA). These studies indicate that early changes in $[Ca^{2+}]_i$ may be involved in the action on fibroblasts of some, but not all, mitogens.

Free Ca^{2+} *Quin2* *3T3 fibroblast* *Growth factor* *Mitogenic stimulation*

1. INTRODUCTION

There is extensive evidence that the activation of quiescent eukaryotic cells into the cell cycle is accompanied by changes in both ion fluxes across the plasma membrane and intracellular ion concentrations. For example, it is well established that two early ionic signals mediate the fertilisation of sea urchin eggs, in which a rapid and transient increase in $[Ca^{2+}]_i$ from about 0.1 to 3 μM [1] triggers a persistent increase in intracellular pH (pH_i) by an unknown coupling mechanism [2]. Both the Ca signal, which is completed within 3 min, and the consequent in pH_i appear to be obligatory for the normal activation pathway leading to the initiation of DNA synthesis (review [3]).

These observations raise the question of whether all eukaryotic cells have evolved with common activation pathways from G_0 into the cell cycle, and in particular whether there are common ionic signals necessary for the stimulation of RNA and protein synthesis which normally precede DNA synthesis. The evidence concerning ionic signals in mitogenically stimulated mammalian cells is incomplete, but it has been shown that there is a rapid increase in $[Ca^{2+}]_i$ in lymphocytes within 1 min of the addition of mitogens [4,5], with no detectable increase in pH_i within 60 min [6]. In contrast, in fibroblasts there is an early increase in pH_i in response to mitogens that appears to be driven by Na^+/H^+ exchange [7,8] but no corresponding measurements of $[Ca^{2+}]_i$ over the same period have been reported. Here we demonstrate that normal responses to mitogens occur in 3T3 fibroblasts grown on microcarrier beads, and using the fluorescent indicator quin2 [9] we show that some but not all classes of mitogens cause a very early increase in $[Ca^{2+}]_i$.

2. MATERIALS AND METHODS

2.1. Cell culture

Swiss 3T3 fibroblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 44 mM $NaHCO_3$, 20 mg/l penicillin, 100 mg/l streptomycin, 5% foetal calf serum (Gibco) and 5% newborn calf serum (Gibco) in a humidified atmosphere of 10% CO_2/90% air at

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00145793/84/$3.00

37°C. 3T3 fibroblasts were seeded at a density of 5×10^4 cells/ml onto Cytodex 1 microcarrier beads (Pharmacia) at 1.5 mg/ml and grown to confluence in medium stirred continuously at 20 rpm. The medium was renewed on days 2 and 4 after seeding and experiments carried out on days 8–9. Alternatively, cells were grown directly attached to 30 mm petri dishes after seeding at 5×10^4 cells/ml; the medium was renewed on day 2 and the cells used on day 7. The cells were free from mycoplasma contamination.

2.2. *Incorporation of [^{3}H]uridine or [^{3}H]thymidine into nucleic acid*

For measurements of RNA synthesis, 3T3 cells attached to beads were transferred to 30 mm petri dishes in 1 ml of simplified medium (137 mM NaCl, 20 mM Hepes, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, 4.4 mM $NaHCO_3$, gassed with 10% CO_2 at pH 7.1). 3T3 cells which were grown directly attached to the dishes were resuspended in 1 ml of simplified medium. One μCi [^{3}H]uridine was added to each dish with or without mitogens. The cells (5×10^5/ml) were incubated for 3 h in a humidified atmosphere (10% CO_2/90% air) at 37°C before harvesting to measure [^{3}H]uridine incorporation into material insoluble in trichloroacetic acid. For measurements of DNA synthesis 3T3 cells attached to beads were transferred in 1 ml serum-free DMEM to 30 mm petri dishes and 3T3 cells directly attached to dishes were suspended in 1 ml serum-free DMEM. The cells (5×10^5/ml) were incubated with 0.5 μCi [^{3}H]thymidine with or without mitogens and harvested after 28 h to measure [^{3}H]thymidine incorporation into material insoluble in trichloroacetic acid. [5,6-^{3}H]Uridine (43 Ci/mmol) and [6-^{3}H]thymidine (5 Ci/mmol) were obtained from Amersham International. Arginine vasopressin, $PGF_{2\alpha}$, insulin (bovine pancreas) and TPA were obtained from Sigma and EGF ('culture grade') from Collaborative Research Inc.

2.3. *Quin2 loading and fluorescence measurements*

Cells attached to beads in the culture medium were washed by centrifugation (1 min, $100 \times g$) into serum-free DMEM supplemented with 20 mM Hepes, 20 mM $NaHCO_3$ and gassed with 10% CO_2 at pH 7.1. Cells (5×10^5/ml) were incubated with [^{3}H]quin2 acetoxymethyl ester (15 μM; 5.4 Ci/mol; prepared as [5]) for 45 min at 37°C in a humidified atmosphere of 10% CO_2/90% air. The cells were washed by centrifugation (1 min, $100 \times g$) into simplified medium and the intracellular quin2 concentration estimated as 2.5–3.0 mM by measuring the ^{3}H content of the cells. Intracellular quin2 fluorescence (excitation 339 nm, emission 492 nm) was measured in a Perkin Elmer 44B spectrofluorimeter using 1.5 ml of cell suspension (3×10^5 cells) in 1 cm quartz cuvettes with constant stirring at 37°C.

3. RESULTS

3.1. *Mitogenesis in 3T3 fibroblasts grown on microcarrier beads and on petri dishes*

Cells grown on microcarrier beads were quiescent by day 8 of the procedure described in section 2. The combination of EGF (12 ng/ml) with insulin (1 μg/ml) stimulated [^{3}H]thymidine incorporation into DNA in cells on microcarrier beads at least as effectively as in cells grown on petri dishes (159 000 and 166 000 cpm/dish respectively; fig.1). Incorporation of [^{3}H]thymidine into DNA was also stimulated in cells attached to dishes by the combination of 1 μg/ml insulin with 10 ng/ml vasopressin (152 000 cpm/dish), or with 100 ng/ml $PGF_{2\alpha}$ (125 000 cpm/dish), or with 10 ng/ml TPA (143 000 cpm/dish). The stimulation of [^{3}H]uridine incorporation into RNA during the first 3 h of mitogenic activation was very similar in cells attached to beads and in those grown on petri dishes (not shown).

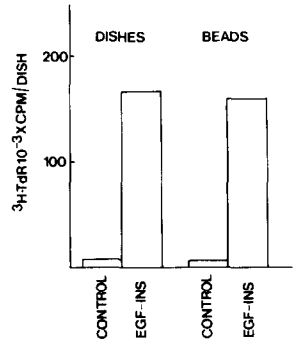

Fig.1. The stimulation of [^{3}H]thymidine incorporation into DNA by EFG (12 ng/ml) with insulin (1 μg/ml) in cells (5×10^5) grown on microcarrier beads and on petri dishes.

3.2. *The effect of growth factors on* $[Ca^{2+}]_i$

The fluorescence from quiescent 3T3 fibroblasts loaded with quin2 corresponded to a $[Ca^{2+}]_i$ of 154 ± 11 nM (SE; $n = 32$) calibrated as described in the legend to fig.2. The addition of vasopressin (10 ng/ml) to these cells caused a rapid increase in the fluorescence signal (fig.2). It is important to determine whether any changes in quin2 fluorescence are due to $[Ca^{2+}]_i$ rather than to changes in intracellular $[Na^+]$ or pH_i, since it is known that mitogens stimulate $^{22}Na^+$ influx into quiescent fibroblasts [10–12] and it has been shown that EGF and platelet-derived growth factor increase pH_i in quiescent human foreskin fibroblasts [7]. However, neither increasing the intacellular $[Na^+]$ with gramicidin D [13] nor changing pH_i with bicarbonate [13] or NH_4Cl had an effect on the fluorescence of cells loaded with quin2.

Typical increases in $[Ca^{2+}]_i$ in response to additions of vasopressin (10 ng/ml), EGF (12 ng/ml) and $PGF_{2\alpha}$ (100 ng/ml) are shown in fig.3. The maximal increase in $[Ca^{2+}]_i$ above the resting $[Ca^{2+}]_i$ was 112 ± 25% (SE; $n = 6$) with vasopressin and 134 ± 46% (SE; $n = 9$) with EGF. $PGF_{2\alpha}$ caused similar but more variable increases in $[Ca]_i$. The response was very rapid with no detectable delay (less than 10 s) and was maximal within 60–90 s. $[Ca^{2+}]_i$ declined over the next 5 min to a level above basal which was sustained for at least 15 min after the addition of mitogen. This elevated level represented a 38 ± 9% (SE; $n = 7$) increase with vasopressin above the basal $[Ca^{2+}]_i$ and a 29 ± 10% (SE; $n = 8$) increase with EFG. In contrast no increase in $[Ca^{2+}]_i$ occurred over a period of 15 min after the addition of insulin (1 μg/ml) or TPA (10 ng/ml). Thus, although a large increase in

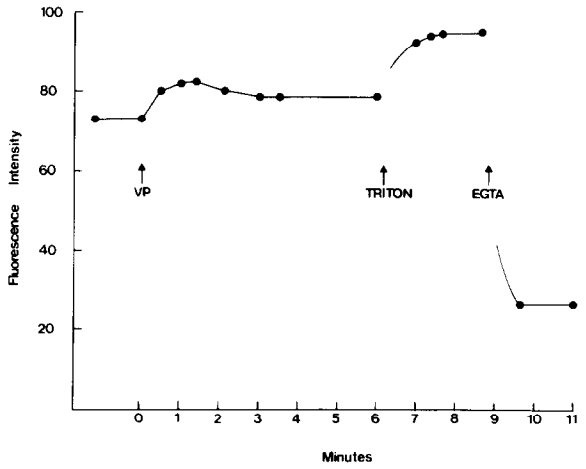

Fig.2. The quin2 fluorescence signal (arbitrary units) before and after the addition of vasopressin (Vp) (10 ng/ml). The signal was calibrated by releasing the quin2 from the cells by the addition of 0.02% Triton X-100 and then chelating the Ca^{2+} in the medium with 10 mM EGTA [4]. In calculating $[Ca^{2+}]_i$ a correction was made for the fluorescence from quin2 outside the cells which could be quenched immediately by the addition of 0.1 mM Mn^{2+} [5]. $[Ca^{2+}]_i$ was calculated using a dissociation constant of 89 nM [5] and the following expression: $[Ca^{2+}]_i$ (nM) = $89(F-F_{min})/(F_{max}-F)$ where F is the observed fluorescence from the cell suspension, F_{max} is the fluorescence after Triton X-100 addition, F_{min} fluorescence after EGTA addition and each fluorescence value is corrected for external quin2 as described above.

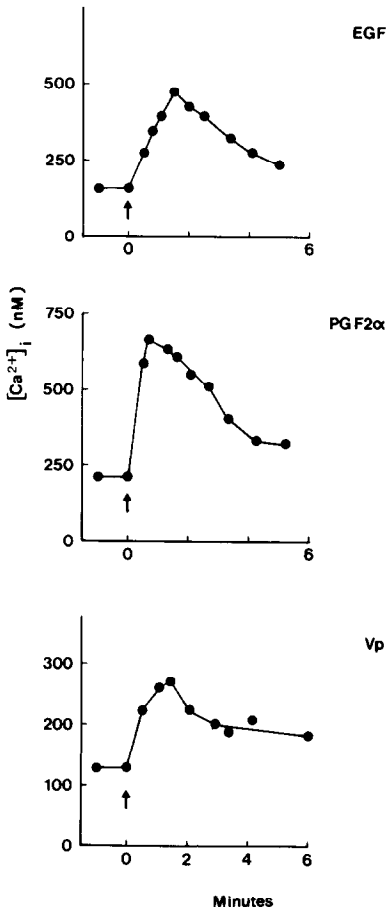

Fig.3. The increase in $[Ca^{2+}]_i$ after the addition of EGF (12 ng/ml), $PGF_{2\alpha}$ (100 ng/ml) and vasopressin (Vp) (10 ng/ml).

$[Ca^{2+}]_i$ is an almost immediate response to the addition of some mitogens, others do not have this effect.

The auto-fluorescence from quiescent 3T3 fibroblasts that were not loaded with quin2 was not affected by growth factors. This contrasts with changes in the auto-fluorescence from hepatocytes after the addition of epinephrine and phenylephrine which has been attributed to an increase in the concentration of reduced pyridine nucleotides in response to the hormones [14].

4. DISCUSSION

Increases in quin2 fluorescence, attributable to an increase in $[Ca^{2+}]_i$, are initiated with no detectable delay after the exposure of 3T3 fibroblasts to EGF, vasopressin, or $PGF_{2\alpha}$ each of which has a well-defined mitogenic action on these cells. The $[Ca^{2+}]_i$ increased from 154 ± 11 nM (SE; $n = 32$) in quiescent cells to a maximum of 200–500 nM after 60–90 s and subsequently declined to a steady level (~200 nM) slightly above that in unstimulated cells.

Vessopressin has recently been shown to cause an increase in $[Ca^{2+}]_i$ by 3-fold to 600 nM within 15 s of addition to quin2-loaded hepatocytes [14]. In these cells the α_1-adrenergic agonists epinephrine and phenylephrine induced comparable increases in $[Ca^{2+}]_i$ which were transient and $[Ca^{2+}]_i$ returned almost to the level in resting cells over a period of 5–10 min [14]. Similar transient increases in $[Ca^{2+}]_i$ have also been observed in 2H3 rat basophil leukaemic cells stimulated with antigen [15] and in B lymphocytes (at low intracellular quin2 concentrations) in response to anti-Ig antibody [16]. The new steady-state $[Ca^{2+}]_i$ levels reached in these systems and in 3T3 fibroblasts reflect a new balance between influx and efflux pathways across the plasma membrane of stimulated cells, but the mechanism by which $[Ca]_i$ is increased is not established. Neither insulin nor TPA caused any rapid changes in $[Ca^{2+}]_i$ in 3T3 fibroblasts, although both have mitogenic activity on these cells. This contrasts with the effect of TPA on murine thymocytes, in which it gradually lowers $[Ca^{2+}]_i$ in the absence of mitogens and reverses the $[Ca^{2+}]_i$ increase in cells stimulated with concanavalin A [4].

Previous studies investigating Ca^{2+} in stimulated fibroblasts have analysed $^{45}Ca^{2+}$ fluxes. Most of these have examined the effects of the addition of serum to quiescent cells and are subject to uncertainties in that serum, contains a variety of growth factors and can also cause a transient depolarisation of the cells [18] which is not observed with EGF [17]. If the cells become transiently leaky on exposure to serum the ionic fluxes observed under these conditions may not be related to the mitogenic action of serum. The only $^{45}Ca^{2+}$ study with pure mitogens on 3T3 cells showed that varopressin stimulated $^{45}Ca^{2+}$ efflux whereas EGF and insulin had no effect on $^{45}Ca^{2+}$ efflux [19]. In human foreskin fibroblasts, EGF and vasopressin stimulated $^{45}Ca^{2+}$ efflux [20], and in A431 human carcinoma cells EGF can stimulate both influx and efflux of $^{45}Ca^{2+}$ [21,22]. Although the $^{45}Ca^{2+}$ and quin2 data are generally consistent in their indications of changes in Ca^{2+} metabolism, it is very difficult to make quantitative correlations between $^{45}Ca^{2+}$ flux data and $[Ca]_i$ measurements by quin2. The interpretation of $^{45}Ca^{2+}$ data is complex and cannot provide any direct information about changes in $[Ca^{2+}]_i$, whereas quin2 may not always act as a non-perturbing indicator of $[Ca^{2+}]_i$ [5].

The increase in $[Ca^{2+}]_i$ reported here in 3T3 cells in response to some mitogens are much faster than the corresponding increases in pH_i recently reported in human fibroblasts using an intracellular indicator derived from fluorescein [7]. However, the latter studies were performed at 30–32°C, rather than 37°C, and this may slow the pH response observed. A comparison of the time course of $[Ca^{2+}]_i$ and pH_i changes in 3T3 fibroblasts under the same experimental conditions will be reported shortly.

ACKNOWLEDGEMENTS

This work was supported by a grant from the SERC to J.C.M. and M.V.T. was a recipient of an MRC studentship. Technical assistance was provided by Mr R.W. Plumb.

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