Role of mitogen-induced calcium influx in the control of the cell cycle in Balb-c 3T3 fibroblasts

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Abstract — The role of mitogen-activated calcium influx from the extracellular medium in the control of cell proliferation was studied in Balb-c 3T3 fibroblasts. Stimulation of serumdeprived, quiescent cells with 10% foetal calf serum (FCS) induced a long-lasting (up to 70 min) elevation of intracellular free calcium concentration ([Ca²⁺]_i). Both the sustained [Ca²⁺]_i increase and the related inward current, described in a previous paper [Lovisolo D. Munaron L. Baccino FM. Bonelli G. (1992) Potassium and calcium currents activated by foetal calf serum in Balb-c 3T3 fibroblasts. Biochim. Biophys. Acta, 1104, 73-82], could be abolished either by chelation of extracellular calcium with EGTA or by SK&F 96365, an imidazole derivative that can block receptor-activated calcium channels. The effect of the abolition of these ionic signals on FCS-induced proliferation was investigated by adding either EGTA or SK&F 96365 to the culture medium during the first hours of stimulation of quiescent cells with 10% FCS. As measured after 24 h, a 22% inhibition of growth was observed when SK&F 96365 was added for the first hour, and stronger inhibitions, up to 56%, were obtained by adding the blocker for the first 2 or 4 h. Similar effects were observed with addition of 3 mM EGTA, though the inhibition was less marked for the 4 h treatment. By contrast, incubation with either substance in the next 4 h of serum stimulation did not influence cell growth, except for a slight inhibition observed when SK&F 96365 was applied from the 4th to the 8th hour. The reduction in growth resulting from the abolition of the early calcium influx was paralleled by an accumulation of cells in the G₂/M phase. Both growth inhibition and G₂/M accumulation were reversible, since after further 24 h in 10% FCS cells had fully recovered the exponential growth. These data indicate that the early calcium influx seen in response to mitogen stimulation develops on a timescale long enough to play a significant role in cell cycle progression, and that its block in the early G1 phase can lead to a reduction of proliferation by arresting cells in later stages of the cycle.

In many cell types, recruitment into and progression through the proliferative cycle are under the strict control of mitogens and growth factors [1]. It has been recognized that one of the parameters controlling the complex cascade of events triggered by mitogen stimulation is the cytosolic free calcium concentration, $[Ca^{2+}]_i$ [2–4]; one of the critical points at which [Ca²⁺]_i changes are involved is immediately at the G_0/G_1 transition [5]. Among the early $[Ca^{2+}]_i$ changes triggered by mitogens, much attention has been devoted to the transient increases due to release from intracellular stores [2,6,7], mainly via the phosphoinositide pathway [8]. Only recently, evidence for a long-lasting increase, due to calcium influx through the plasma membrane, has been obtained in various cell types [3,4,9]. This pathway has been reported to be activated not only by mitogens but also by other agonists in several preparations [10-13] either directly, or as a secondary response following the emptying of the intracellular calcium stores (store dependent calcium influx, SDCI, [13]; calcium release activated current, ICRAC, [14,15]).

A long-lasting calcium influx may play a key role in the control of the proliferative process, since many cells require the mitogen to be present in the extracellular medium for several hours in order to progress into the cell cycle and eventually proliferate [2], and mitogenic responses in non tumoral cells depend on the presence of extracellular calcium for the same time span [4,16]. Moreover, several authors have reported that the imidazole derivatives SC38249 and SK&F 96365, which reversibly block agonist-induced calcium influx [3,17], have an antiproliferative effect on fibroblasts [3] and neoplastic cell lines [18,19], even if the dependence of the antimitogenic action of SK&F 96365 on the calcium channel blocking effect has been questioned [18].

We have previously reported that in Balb-c 3T3 fibroblasts, made quiescent by deprivation in 1% foetal calf serum (FCS) for 48 h, stimulation with 10% FCS elicits an inward current, carried at least partially by Ca²⁺ ions, that can last for tens of minutes [20]. We have further shown that a current with similar characteristics, and a long-lasting $[Ca^{2+}]_i$ increase, are induced in the same preparation by basic fibroblast growth factor [21]. In the present study electrophysiological and cytofluorometrical techniques were employed in order to investigate the role of this current and of the ensuing $[Ca^{2+}]_i$ elevation in cytosolic calcium concentration, in the control of cell proliferation. We compared the effects of application of SK&F 96365 with those obtained by

chelating calcium ions in the extracellular medium in the first 4 h of mitogen stimulation. In both cases, we found a significant reduction of Balb-c 3T3 cell growth, even if the magnitude of the effect was somewhat different in the two experimental conditions. This inhibition was accompanied by an accumulation of cells in the G₂/M phase. Both agents had either significantly smaller effects, in the case of SK&F 96365, or no effect at all, for EGTA, if applied at a later stage of the progression into the cell cycle.

Materials and methods

Cell culture and incubation protocols

Balb/c 3T3 mouse fibroblasts (clone A31, American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Biochrom-Seromed, Berlin, Germany) supplemented with 10% foetal calf serum (FCS, Biochrom-Seromed) and 50 µg/ml gentamycin (Sigma, St Louis, MO, USA) at 37°C, in a humidified atmosphere of 5% CO₂ in air. Exponentially growing cultures were seeded at a density of 12.000 cells/cm² in DMEM containing 1% FCS 48 h before the experiments. For the determination of the effects of EGTA and SK&F 96365 on the proliferation rate and on the DNA content, after the serum deprivation period, cells were transferred to DMEM + 10% FCS containing either SK&F 96365 at the desired concentration or 3 mM EGTA. After 1, 2, or 4 h, SK&F 96365 or EGTA were removed by substitution with DMEM supplemented with 10% FCS. In controls, DMEM + 1% FCS was replaced directly with DMEM + 10% FCS. 24, 28 or 48 h after the reintroduction of 10% FCS, cells from the monolayer, harvested by trypsinization, were resuspended in phosphate buffered saline (PBS) and used for the experiments.

For every experimental protocol, the plasma membrane integrity was checked by the propidium iodide (PI) exclusion test [22]. Cells suspended in PBS, as described above, were incubated with PI (10 μ g/ml) and analyzed by flow cytometry (*see* below).

[Ca²⁺]_i measurements

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with the fluorescent probe Fura-2 as described by Grynkievicz et al. [23]. Quiescent cells on glass coverslips were loaded with Fura-2 pentacetoxymethylester (Fura-2/AM) by a 30 min incubation period at 37°C with 3 µM Fura-2. The medium was then replaced with standard Tyrode solution (see below) and the coverslips were placed on an inverted Zeiss microscope with a fluorescence objective (Nikon 100×). Diaphragms were used to observe single cells. Fluorescence records were taken at excitation wavelengths of 350 nm and 380

Table

mark). Unless otherwise indicated, a standard Tyrode solution of the following composition (in mM): NaCl, 154; KCl, 4; CaCl₂, 2; MgCl₂, 1; NaHEPES, 5; glucose, 5.5; NaOH to pH 7.4 was superfused at a flow rate of 3 ml/min. When 20 mM BaCl₂ was used, NaCl was 127 mM. A microperfusion system Protocol G_0/G_1 S G_2/M n A. 3 mM EGTA added in the first hours of 10% FCS reintroduction. Cell cycle distribution evaluated after 24 h 10% FCS 48 h 1% FCS (quiescent) 90 ± 2 4 ± 1 5 6±1 48 h 1% FCS + 24 h 10% FCS (exponentially growing controls) 54 ± 3 37 ± 4 5 9 ± 2 48 h 1% FCS + 2 h 10% FCS and EGTA +22 h 10% FCS 46 ± 5 35 ± 3 19 ± 2* 5 48 h 1% FCS + 4 h 10% FCS and EGTA + 20 h 10% FCS 44 ± 2 35 ± 2 $21 \pm 2*$ 5 B. 40 µM SK&F 96365 added in the first hours of 10% FCS reintroduction. Cell cycle distribution evaluated after 24 h 10% FCS 48 h 1% FCS (quiescent) 90 ± 4 5 ± 2 5 ± 3 13 48 h 1% FCS + 24 h 10% FCS (exponentially growing controls) 48 ± 5 43 ± 5 8 ± 2 13 48 h 1% FCS + 2 h 10% FCS and SK&F 96365 + 22 h 10% FCS 37 ± 5 27 ± 4 37 ± 7* 13 48 h 1% FCS + 4 h 10% FCS and SK&F 96365 + 20 h 10% FCS 22 ± 3 24 ± 3 54 ± 5* 13

40 JM SK & F 96365 added between the 4th and 8th hour of 10% ECS reintroduction

U.	40 µm Sk&F 90505 audet between the 4 and 8 hour of 10% FCS femiliouccion	1.			
	Cell cycle distribution evaluated after 24 h 10% FCS				
	48 h 1% FCS (quiescent)	93 ± 2	3 ± 1	4 ± 2	6
	48 h 1% FCS + 24 h 10% FCS (exponentially growing controls)	45 ± 3	44 ± 5	11 ± 2	6
	48 h 1% FCS + 4 h 10% FCS + 4 h 10% FCS and SK&F 96365 + 16 h 10% FCS	46 ± 2	43 ± 3	11 ± 3	6
D.	40 μ M SK&F 96365 added in the first hours of 10% FCS reintroduction.				
	Cell cycle distribution evaluated after 28 h 10% FCS				
	48 h 1% FCS (quiescent)	90 ± 2	5 ± 2	5 ± 2	6
	48 h 1% FCS + 28 h 10% FCS (exponentially growing controls)	57 ± 5	31 ± 4	12 ± 2	6
	48 h 1% FCS + 4 h 10% FCS and SK&F 96365 + 24 h 10% FCS	21 ± 3	34 ± 2	45 ± 5*	6
E.	40 μ M SK&F 96365 in the first hours of 10% FCS reintroduction.				
	Cell cycle distribution evaluated after 48 h 10% FCS				
	48 h 1% FCS (quiescent)	94 ± 2	4 ± 1	2 ± 1	3
	48 h 1% FCS + 48 h 10% FCS (exponentially growing controls)	67 ± 3	20 ± 1	13 ± 2	3
	48 h 1% FCS + 4 h 10% FCS and SK&F 96365 + 44 h 10% FCS	70 ± 3	16 ± 1	14 ± 3	3

All experiments were performed in triplicate. Values are given as percentages.

nm and emission of 520 nm using a spectrophotometer from Cairn Ltd, UK. Temperature was 22-24°C. Calibration was done according to [24].

Electrophysiology

Cells used in the experiments were flat and firmly attached to the dish (35 mm dishes, Nunc, Denallowed to switch from this solution to the test ones. The changes took effect in about 3-5 s.

Whole-cell patch-clamp recordings were performed using electrodes of 3–8 M Ω resistance connected to a RK 300 amplifier (Bio-Logic, France). The pipette solution contained (in mM): KCl, 133; MgCl₂, 3; EGTA, 0.1; HEPES, 5; NaGTP, 0.4; Na₂ATP, 5; Na₂PC, 5; KOH to pH 7.3.

Data were digitized and stored on a modified PCM video recorder system (Sony, Japan). Off-line analysis and generation of the voltage ramps used to record the I/V relationships (speed: 0.4 V/s) were performed with pCLAMP software (Axon Instr., USA). Capacitance measurements were performed by means of analog compensation. Temperature was 30–32°C. Some experiments were performed at 22– 24°C, with no detectable change in the response.

Cell growth

Cell number was determined with an electronic cell counter (ZM, Coulter Electronics, Hialeah, FL, USA). All experiments were performed in triplicate.

Flow cytometry of DNA distribution

Cell suspensions in PBS were centrifuged at 0-4°C, the pellet was washed with PBS (at 0-4°C), resuspended in ice-cold 70% ethanol and maintained at 0-4°C for at least 30 min. After centrifugation cells were incubated in the presence of DNase-free ribonuclease (Type 1-A) and PI at final concentrations of 0.4 and 0.18 mg/ml PBS, respectively. Fluorescence was measured using a FACScan flow cytometer (Becton & Dickinson, Mountain View, CA, USA) equipped with a 488 nm light source (argon laser). The flow rate was set at about 200 cells/s and at least 10^4 cells were analyzed for each sample. Two filters were used to collect the red fluorescence due to PI staining the DNA, one transmitting at 585 nm with a bandwidth of 42 nm (FL2), the other transmitting above 620 nm (FL3). FL2 and FL3 were registered on a linear and on a logarithmic scale, respectively. Forward (FSC) and side (SSC) light scatter were simultaneously measured. Debris were excluded from analysis by appropriately raising FSC and FL3 thresholds to values selected experimentally [25]. Data were recorded with a Hewlett Packard computer (HP 9000, model 300), using CellFit software (Becton & Dickinson). All experiments were performed in triplicate.

Statistical analysis

All values are given as means \pm SD.

In the cell proliferation and cycle distribution experiments, groups were compared using one-way analysis of variance, together with the Student-Newman-Keuls multiple range test, to determine where significant (P < 0.05) differences among the groups were located. Samples differing significantly from controls are marked with an (*) in figures and the Table.

In single-cell fluorometric $[Ca^{2+}]_i$ experiments, Student t test was used.

If not otherwise specified all chemicals and drugs were purchased from Sigma Chemical Co. St Louis, MO, USA. SK&F 96356 was kindly provided by Dr S. Pathak, Smith Kline Beecham Pharmaceuticals, UK.

Results

The long lasting increase in $[Ca^{2+}]_i$ induced by FCS and its dependence on calcium influx

The [Ca²⁺]_i changes induced by stimulation with 10% FCS in serum-deprived quiescent Balb-c 3T3 fibroblasts were analyzed by means of single-cell cytofluorometric measurements using the dye Fura-2. In 15 cells bathed in normal Tyrode solution, $[Ca^{2+}]_i$ was 104.0 ± 13.3 nM. In 11 of these cells, addition of 10% FCS to the solution induced a biphasic response, consisting of a transient increase in [Ca²⁺]_i followed by a long-lasting elevation to 130.9 \pm 12.7 nM (P < 0.001); a typical response is shown in Figure 1A. In the longest recording, the plateau was still present after 70 min. In 3 cells, 10% FCS induced only a transient [Ca²⁺]i increase (not shown), while in one cell no response could be observed. Chelation of extracellular calcium by addition of 3 mM EGTA to the bathing solution had no detectable effect on the basal [Ca²⁺]_i values, while it completely abolished the FCS-induced plateau (Fig. 1A), showing that this phase of the response is due



Fig. 1 Stimulation of quiescent Balb-c 3T3 fibroblasts with 10% FCS induces a long-lasting increase in $[Ca^{2+}]_i$ that is sensitive to inhibitors of calcium influx. (A) A typical biphasic response. The sustained plateau could be abolished by addition of 3 mM EGTA to the external medium; the same manipulation did not have any effect on basal $[Ca^{2+}]_i$ values. (B) Reversible abolition of the $[Ca^{2+}]_i$ plateau by 40 μ M SK&F 96365.

to an influx of calcium from the extracellular medium. Similar results were obtained on 5 other cells.

We then studied the effects of the imidazole derivative SK&F 96365, a blocker of agonist-activated calcium channels [17], on this sustained $[Ca^{2+}]_i$ increase. Figure 1B shows that 40 μ M SK&F 96365, when applied during the plateau phase of the response, abolished it completely and reversibly, in a way that closely resembled its suppression by elimination of Ca²⁺ ions from the external medium. Three other experiments gave similar results. The effects of 40 µM SK&F 96365 on the basal intracellular calcium concentration were tested in 6 cells. In 4 cases, no change was detected: in 2 experiments, on the other hand, a transient increase was observed (not shown), probably due to release from internal stores, in analogy with the observations of Magni et al. [3] in EGFR-T17 fibroblasts and of Nordström et al. [18] in Jurkat cells. When FCS was applied to the bath in the presence of SK&F 96365, only a transient increase could be observed (3 cells, data not shown).

Electrophysiology

The sustained $[Ca^{2+}]_i$ plateau described above, dependent on calcium influx, can be related to the inward current that we have previously shown to be

activated by FCS [20] or basic fibroblast growth factor [21] in the same preparation. This current can last up to tens of minutes, has a reversal potential near 0 mV and is carried, at least partially, by calcium ions: in fact, it can be observed with external solutions containing 0 mM NaCl and with 2 mM CaCl₂ as the only permeant cation [20,21]. For these reasons, we examined the effects of application of 40 µM SK&F 96365 on the ionic currents recorded from Balb-c 3T3 fibroblasts, by means of patch clamp recordings in the whole cell configuration. Experiments were performed in the voltage clamp mode at a holding potential (V_h) of -50 mV. A typical recording of a FCS-activated current is shown in Fig. 2A. Since it has been reported [26,27] that SK&F 96365 may affect many other currents, in addition to the agonist-activated one, we first tested the effects of 40 µM SK&F 96365 on the basal electrophysiological properties of unstimulated, quiescent Balb-c 3T3 cells. In 3 cells, the compound induced no detectable change in the basal current recorded at $V_h = -50$ mV; accordingly, the currentvoltage relationships, obtained by applying voltage ramps from -100 to +50 mV, were unaffected (not shown). In 12 cells, however, 40 µM SK&F 96365 reduced the basal current flowing at $V_h = -50 \text{ mV}$ (Fig. 2B; mean reduction: $1.6 \pm 1.0 \text{ pA/pF}$). Comparison of I/V relationships in control conditions

and in the presence of the blocker (Fig. 2B) shows that the current component abolished by SK&F 96365 has a reversal potential of about 0 mV, and can be ascribed to a nonselective conductance. No evidence could be observed for a block of K⁺ currents, as reported by Schwartz et al. [26] in the range 10–200 μ M, and by Franzius et al. [27] at micromolar concentrations of SK&F 96365.

Α

0

-10 PA/PF

-20

С

0

0

-10

-20

0

pAVpF

In a total of 20 experiments, 10% FCS stimulation induced an inward current, whose mean peak density was 12.1 ± 8.5 pA/pF. Again, some vari-



Fig. 2 SK&F 96365 blocks the inward cationic current activated by FCS. (A) A typical inward current seen in response to stimulation with 10% FCS ($V_h = -50 \text{ mV}$). (B) The effects of 40 μ M SK&F 96365 on the electrophysiological properties of unstimulated cells. In most cells, a reduction in the basal current flowing at $V_h = -50 \text{ mV}$ was observed (inset); the I/V relationships, obtained by applying voltage ramps from --100 to +50 mV before (1) and during (2) SK&F 96365 application, show that this agent abolishes a current component with a reversal potential near 0 mV. (C) Reversible block of the FCS-induced inward current by 40 μ M SK&F 96365. (D) I/V curves obtained before (C) and during (SKF) application of 40 μ M SK&F 96365 on a cell bathed in an external solution containing 20 mM BaCl₂, showing inhibition of the voltage-dependent inward current.

ability was observed for the effects of 40 μ M SK&F 96365 on this current. In 5 cells, the drug completely and reversibly suppressed it (not shown). On the other hand, in 15 experiments the block was not complete (Fig. 2C) and amounted to 63.4 ± 18.4%.

It has been reported that SK&F 96365 can also block voltage-operated calcium channels [17], and we have previously reported that Balb-c 3T3 fibroblasts possess high threshold voltage-operated calcium channels that can be blocked by 2 µM nifedipine [28]. In the present experiments, however, performed on cells serum-deprived for 48 h, the voltage-activated calcium current (ICa.L) was usually barely detectable, or not detectable at all. Only when 2 mM CaCl₂ was substituted with 20 mM BaCl₂ in the external solution, could the voltage-dependent inward current still be observed and was substantially reduced by 40 µM SK&F 96365 (Fig. 2D), in agreement with the results of Merritt et al. [17]. Similar results were obtained in two other cells.

Block of calcium influx and inhibition of cell growth

The role of the sustained calcium signals in the control of cell proliferation, and the effects of their abolition either by application of the blocker SK&F 96365 or by chelation of calcium in the external medium were subsequently investigated. Since the longest recordings indicated that the $[Ca^{2+}]_i$ plateau evoked by mitogen stimulation could last 1 h or more, the approach was to suppress it in the first 1–4 h of stimulation of quiescent cells with 10% FCS, i.e. in correspondence of the Go/G₁ transition and of the early G₁ phase ('competence' phase, [1]).

Figure 3 shows a dose-response curve obtained for incubation with SK&F 96365 in the first 2 h of serum reintroduction. Growth inhibition was maximal at the highest dose tested, 80 μ M; at this concentration, however, toxic effects were also observed, as indicated by the PI exclusion test. Therefore, 40 μ M SK&F 96365 was used in all other experiments.

The effects of different incubation times were then examined. Figure 4B shows that, as compared to controls, a significant reduction of growth (22%) could already be observed when 40 μ M SK&F 96365 was applied during the first hour of FCS stimulation; with 2 h of exposure to SK&F 96365, the reduction amounted to about 34%; with 4 h, it reached 56%.

The same experimental protocols were repeated with 3 mM EGTA (and no SK&F 96365) added to the external medium (Fig. 4C). 1 h incubation with EGTA in the presence of FCS led to a significant (19%) inhibition of growth; with 2 and 4 h treatments, the reduction amounted, respectively, to 26% and 36%. With the 1 and 2 h treatments, the effects were not significantly different for the two agents, while with the 4 h protocol 3 mM EGTA caused a significantly lower reduction than 40 μ M SK&F 96365. For incubation times up to 4 h in 40 μ M SK&F 96365 no signs of toxicity were observed, as assessed by the PI exclusion test (*see* Materials and methods).

To rule out any involvement of the voltage-activated calcium channels, that, even if present in low density, are not completely blocked by 40 μ M SK&F 96365, similar protocols were performed using 2 μ M nifedipine, which in these cells completely blocks the voltage-activated calcium chan-

28000

 \pm SD (n = 3).







Fig. 4 Block of calcium influx during the first 1–4 h of 10% FCS stimulation reduces cell proliferation. (A) Schematic representation of the incubation protocols. (B) Quiescent cells were incubated with 40 μ M SK&F 96365 in the first (1, 2 or 4) h of stimulation with 10% FCS, then harvested and counted 24 h later. Column EXP shows the cell density of control cultures, after 24 h of 10% FCS stimulation, without addition of either SK&F 96365 or EGTA or nifedipine. (C) The same experimental protocols as in (B) were repeated with incubation in 3 mM EGTA. (D) Incubation with 2 μ M nifedipine in the first 4 h of 10% FCS stimulation did not inhibit cell growth. In both (B), (C) and (D), the start of Y axis scale represents the density of serum-deprived quiescent cultures, as measured at the time of addition of 10% FCS (13319 ± 690 cells/cm² for experiments with SK&F 96365, n = 11; 12944 ± 1355 cells/cm² for experiments with nifedipine, n = 3). Numbers of experiments are shown inside the bars. Values are given as means ± SD.

nels [28]. No effect on cell growth could be observed with nifedipine added in the first 4 h of stimulation with 10% FCS (Fig. 4D).

Abolition of calcium influx blocks cells in the G_2 /M phase

The cell cycle distribution of Balb-c 3T3 cells, in control conditions and with different protocols of calcium influx inhibition, was studied by means of flow cytometry. The Table shows the distribution of cells deprived for 48 h in 1% FCS, indicating an

extensive arrest of cell proliferation (*see* also Fig. 5A). After 24 h in 10% FCS (Table and Fig. 5B), the distribution was typical of exponentially growing cultures. When 3 mM EGTA was added during the first 2 h of 10% FCS reintroduction, after 24 h in FCS a significant increase in the percentage of cells in G₂/M was observed (Table and Fig. 5C). Similar values were obtained with 4 h incubation times (Table and Fig. 5D). With the corresponding incubation protocols using SK&F 96365, even higher percentages of cells were found in G₂/M at the end of the 24 h period (Table and Fig. 5E,F).



DNA CONTENT

Fig. 5 Block of early calcium influx causes an accumulation in the G_2/M phase in FCS-stimulated cells. Panels show representative cell cycle (DNA) distributions. (A) Largely quiescent cultures, after 48 h of serum deprivation. (B–F) Cultures stimulated with 10% FCS for 24 h: (B) with no addition of calcium influx inhibitors, showing a pattern typical of exponentially growing cells; (C,D) with 3 mM EGTA added in the first 2 h and 4 h, respectively: (E,F) 40 μ M SK&F 96365 was added in the first 2 and 4 h, respectively.

In order to test whether the effects of the blocker and of calcium chelation were specifically related to the abolition of the calcium influx observed as an early response to FCS stimulation, the incubation protocols described above were repeated after 4 h of restimulation with FCS, i.e. between the 4th and 8th hour, and cells were counted, as usual, after 24 h in 10% FCS. Chelation of extracellular calcium during this time span had virtually no effect on cell growth (Fig. 6B), while 40 μ M SK&F 96365 significantly reduced growth by 38% only when applied for 4 h (Fig. 6C). Even in this case, the cell cycle distribution did not significantly differ from that of exponentially growing controls (*see* Table).

Additional experiments were performed to check if the effects of the abolition of mitogen-induced early calcium influx could be ascribed to a reversible block. The protocol of incubation with 40 μ M SK&F 96365 in the first 4 h of restimulation with FCS was used, but cells were counted, and DNA distribution examined, after 28 h of FCS stimulation, i.e. cells were left in the presence of 10% FCS for 4 additional hours. Significant reduction of growth (1 h: 24%; 2 h: 26%; 4 h: 43%) and accumulation in G₂/M phases (as observed with 4 h incubation protocol) could still be observed (Fig. 7B, D and Table).

On the other hand, when cells were incubated for 1-4 h with SK&F 96365, but cultures were analyzed after 48 h in 10% FCS, growth in cultures treated with the blocker had fully recovered (Fig. 8B); cell cycle distribution (as measured with the 4 h incubation protocol) was as in controls (Fig. 8C,D and Table).

Discussion

In recent years, ample evidence has accumulated that stimulation with a wide range of agonists, and among them mitogens and growth factors, induces in many cell types a long-lasting increase in intracellular calcium, in addition to a fast transient. Moreover, it has been exhaustively demonstrated that such an increase is due to influx of calcium through the plasma membrane [3,4,9,10,13-15,26]. This influx has attracted the interest of many researchers, since in several preparations progress into



B



Fig. 6 The effect on Balb-c 3T3 cell growth of calcium influx inhibitors applied 4 h after the beginning of 10% FCS stimulation. (A) Schematic representation of the incubation protocols. (B) Cells were incubated with 3 mM EGTA for different times between the 4th and the 8th hour of 10% FCS stimulation; cell density of serum deprived quiescent cultures, as measured at the time of 10% FCS addition, (starting point of Y axis) was 12849 ± 1378 (n = 5). (C) The same experimental protocols as in (B) were repeated with incubation in 40 μ M SK&F 96365; density of quiescent cultures, measured as in (B): 12796 ± 1181 (n = 4). In both (B) and (C), column EXP shows the cell density of exponentially growing control cultures, after 24 h of 10% FCS stimulation, without addition of either SK&F 96365 or EGTA. Numbers of experiments are shown inside the bars. Values are given as means ± SD.

the cell cycle up to cell division is known to depend on the presence of extracellular calcium, at least at some critical points [4,5,16]. In the present paper, we show that stimulation of serum-deprived, guiescent Balb-c 3T3 fibroblasts with 10% FCS induces a sustained [Ca²⁺]_i increase, dependent on calcium influx, that can last for times up to 1 h or more. This finding is of great relevance, since it is commonly accepted that, for cells in which proliferation depends on the presence of external stimuli, the mitogen has to be applied for many hours in order to be effective [2]. In many cell types, the pathway of calcium influx has been shown to be activated indirectly by the depletion of intracellular calcium stores. Even if the present experiments did not specifically address this point, tests performed on a similar response activated by bFGF in the same preparation [21] ruled out this possibility, in agreement with the observations of Estacion and Mordan [4].

Two experimental approaches have been used to investigate the dependence of cell proliferation on



Fig. 7 SK&F 96365-induced block of cell proliferation is still evident after 28 h of 10% FCS stimulation. (A) Schematic representation of the incubation protocols. (B) Serum-deprived quiescent cells were incubated with 40 μ M SK&F 96365 for the first (from 1 to 4) h of stimulation with 10% FCS, then dissociated and counted 28 h after the beginning of FCS stimulation. Column EXP refers to controls, with no SK&F 96365 added. Numbers of experiments are shown inside the bars. Density of quiescent cells was 12364 ± 1418 (n = 5). Values are given as means ± SD. (C,D) Representative DNA distribution in controls (C) and in cultures treated with SK&F 96365 for the first 4 h, as measured after 28 h in 10% FCS (D).

calcium influx in our preparation: chelation of calcium in the external medium and use of potential blockers of calcium influx [3,9,17,18,26,27]. The second approach has some advantages, since it does not involve a drastic change in the environment; however, the known blockers of receptor-operated calcium influx, such as the imidazole derivatives SC38249 [3] and SK&F 96365 [17] are not very specific, and many side effects have been reported: block of voltage-operated calcium channels [17] as well as of K⁺ and Cl⁻ channels [26,27], activation of release from internal stores [3,18] and activation of calcium influx through other types of cation channels [26]. In Balb-c 3T3 cells, we have found that for concentrations up to 40 μ M, SK&F 96365 had only limited side effects: only in some cases it did induce a release from intracellular stores, as observed in microfluorometric experiments; it neither elicited any calcium influx, nor induced the activation of any inward current, when applied in the absence of the mitogen; finally, it had no detectable effect on K⁺ currents recorded in basal conditions.

Fig. 8 Reversibility of SK&F 96365-induced block of cell proliferation. (A) Schematic representation of the incubation protocols. (B) Serum-deprived quiescent cells were incubated with 40 μ M SK&F 96365 for the first (from 1 to 4) h of stimulation with 10% FCS, then dissociated and counted 48 h after the beginning of FCS stimulation. Column EXP refers to controls, with no SK&F 96365 added. Numbers of experiments are shown inside the bars. Density of quiescent cells was 13054 ± 674 (n = 3). Values are given as means ± SD. (C,D) Representative DNA distribution in controls (C) and in cultures treated with SK&F 96365 for the first 4 h, as measured after 48 h in 10% FCS (D).

In 80% of the cells tested, on the other hand, the blocker apparently abolished a current component having a V_{Rev} of about 0 mV, causing a small shift in the basal current recorded at $V_h = -50$ mV. From our experiments, it cannot be excluded that a fraction of the SK&F 96365-sensitive cationic conductance activated by FCS (and by bFGF [21]) was already present in unstimulated cells.

We have not tested the possibility that SK&F 96365 blocks a chloride conductance, as reported by Franzius et al. [27]. However, as we have shown previously [20,21], the current seen in response to mitogen stimulation in our preparation is not dependent on Cl⁻ ions; moreover, the amplitude of the shift in the basal current, when observed, is small as compared to the current densities of the responses to FCS. As for the reported block of voltage-activated calcium channels by SK&F 96365 [17], Balb-c 3T3 cells possess L-type channels [28], and their involvement in the influx of calcium could not be excluded in cytofluorometrical experiments, in which cells are not voltage-clamped at -50 mV. However, in the present experimental conditions the current carried by these channels is usually detectable only in the presence of high concentrations of barium ions. Moreover, 2 μ M nifedipine, that in our preparation completely blocks this current [28], had no effect on serum-induced proliferation. For all these reasons, a role of these channels in the events described in this paper is rather unlikely.

The inward current activated by 10% FCS, which we have shown to be carried at least partially by Ca^{2+} ions [20], was completely and reversibly suppressed by 40 µM SK&F 96365 in 5 cells, while the block was only partial in 15 cells. Yet in cytofluorometric experiments the abolition of the sustained [Ca²⁺]; increase was nearly complete in all cells tested. This discrepancy may be related to the fact that the inward current activated by FCS is carried not only by Ca^{2+} , but also by Na⁺ ions [20], and we cannot exclude that more than one channel type was involved, possibly with different sensitivities to the blocking effect of SK&F 96365. On the other hand, the FCS-induced growth was not completely inhibited by 40 µM SK&F 96365, when applied up to 4 h. This concentration was used throughout the experiments because it was the highest that did not cause detectable toxic effects, as assessed with PI exclusion tests, for the above incubation times; it thus represented a good compromise between the required action on the receptor-activated calcium influx and the absence of side effects that could hamper the interpretation of the results. For longer exposure times or at higher concentration (80 µM), a toxic effect of SK&F 96365 was observed (unpublished results). Other authors incubated the cells with imidazole compounds for longer times, respectively 48 h at 10 µM [3] and 24 h at 20 µM [18], without observing toxic effects. This may reflect a higher sensitivity of our preparation to SK&F 96365. On the other hand, with these long incubation times the observed effects on growth cannot be unequivocally ascribed to the suppression of the calcium influx that is activated in the initial period of the mitogen stimulation, and other explanations should not be excluded. In our experiments, on the contrary, it was possible to discriminate between different intervals in the early response of quiescent cells to the mitogen, showing that even 1 h of incubation in a calcium-free medium already produced a

significant reduction in growth, and that the effect was increased by longer incubation times. Removing extracellular calcium for 1-4 h immediately after this initial time span had no significant effect on cell growth. Similar protocols with 40 µM SK&F 96365 gave comparable results, except that, with the drug applied in the first 4 h, the reduction of growth was significantly more pronounced that with EGTA, and some inhibition was observed even when the blocker was applied for 4 h between the 4th and 8th hour of FCS reintroduction. Thus, it cannot be excluded that SK&F 96365, besides acting on the early calcium influx, may exert some other inhibitory effect on cell cycle progression. The latter possibility was already suggested by Nordström et al. [18]. In the present experiments, however, such an effect would only be additive to the block of the early calcium influx. It must be noted also that the data of Nordström et al. [18] were obtained in quite different experimental conditions, i.e. incubating transformed cells with SK&F 96365 for 24 h. Moreover, in our case the effects of 40 µM SK&F 96365 and of calcium-free solutions were not significantly different for incubation times up to 2 h, suggesting that the calcium influx-independent action developed only after the first 2 h of serum stimulation. Recently, it has been reported that SK&F 96365 blocks calcium influx in endothelial cells by inhibition of cytochrome P450 [29]; it is not clear if this inhibition can affect other targets, in addition to calcium-permeable channels.

Estacion and Mordan [30] have shown that in C3H 10T1/2 fibroblasts stimulated with PDGF, Ca²⁺ influx is necessary for competence induction (i.e. in the first part of G₁), but not for the subsequent progression into the cell cycle that depends on progression factors such as insulin. Consistently, our results indicate that proliferation of cultured fibroblasts seems to be dependent, by 40-50% at least, on the sustained calcium influx observed as an early response to mitogen stimulation. This signal conceivably concurs with others in a complex cascade that regulates cell cycle progression. An involvement of the transient component (presumably due to release from intracellular stores) can be ruled out, because in our experiments SK&F 96365 apparently has no effect on this component, in agreement with data from other authors [3, 18].

With all the protocols aimed to block the early calcium influx, we observed a marked accumulation of cells in the G₂/M phase after 24 h in the continued presence of FCS, i.e. from 20-23 h after the removal of the calcium influx inhibitors. A similar G2/M arrest was previously reported by Nordström et al. [18] on Jurkat cells, in different experimental conditions, as noted above. In our experiments, a comparable effect was also produced by chelation of extracellular calcium. Therefore, this arrest should be viewed, rather than as a 'toxic' effect of SK&F 96365, as an aftermath of the suppression of the early mitogen-induced calcium influx. On the other hand, since the G₂/M arrest was still evident at 28 h, after 4 additional hours of FCS stimulation, suppression of calcium influx clearly involved some more complex process than a simple cell cycle delay. The G₂/M arrest was fully reversible, however, since by 48 h, after 24 additional hours of FCS stimulation, cell growth had fully recovered and cell cycle distribution was as in exponentially-growing cultures.

Taken together, our findings point to the importance of the role played by the influx of calcium from the extracellular medium in the first hours of re-entry into the replicative cycle. This stage, and the G₁/S transition, seem to be the critical points at which calcium and calcium-binding proteins, such as calmodulin, are required [5]. Calcium and calmodulin have been shown to be involved in the regulation of transcription factors and in the expression of immediate early genes [31,32]. Due to the limitations of the techniques employed, we could not establish if the $[Ca^{2+}]_i$ elevation observed for times up to 1 h can actually last even longer. Apparently, suppressing calcium influx in the first 4 h after mitogen reintroduction did not prevent cells from progressing up to S and G₂ phases, so it remains to be understood how an ionic event that takes place in the first part of the G₁ phase can exert its action at later stages. Moreover, since our data indicate that block of early calcium influx does not completely inhibit the growth of Balb-c 3T3 fibroblasts, other events must concur in a complex pattern of regulatory signals. Cell proliferation involves an interdependent and regulated set of events that must be strictly coordinated for the replicative cycle to develop regularly and successfully. The role of the

early calcium influx in this pattern is now beginning to be understood, but additional studies will be necessary to clarify this fundamental issue.

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References

- Aaronson SA. (1991) Growth factors and cancer. Science, 254, 1146-1153.
- 2. Soltoff SP. Cantley LC. (1988) Mitogens and ion fluxes. Annu. Rev. Physiol., 50, 207-223.
- Magni M. Meldolesi J. Pandiella A. (1991) Ionic events induced by epidermal growth factor. J. Biol. Chem., 266, 6329-6335.
- Estacion M. Mordan LJ. (1993) Competence induction by PDGF requires sustained calcium influx by a mechanism distinct from storage-dependent calcium influx. Cell Calcium, 14, 439-454.
- Means AR. (1994) Calcium, calmodulin and cell cycle regulation. FEBS Lett., 347, 1-4.
- Zagari M. Stephens M. Earp HS. Herman B. (1989) Relationship of cytosolic ion fluxes and protein kinase C activation to platelet-derived growth factor induced competence and growth in Balb-c 3T3 cells. J. Cell. Physiol., 139, 167-174.
- Villereal ML. Byron KL. (1992) Calcium signals in growth factor signal transduction. Rev. Physiol. Biochem. Pharmacol., 119, 67-121.
- Berridge MJ. (1993) Inositol trisphosphate and calcium signalling. Nature, 361, 315-325.
- Meldolesi J. Clementi E. Fasolato C. Zacchetti D. Pozzan T. (1991) Ca²⁺ influx following receptor activation. TIPS, 12, 289-292.
- Lewis RS. Cahalan MD. (1990) Ion channels and signal transduction in lymphocytes. Annu. Rev. Physiol., 52, 415-430.
- Gericke M. Droogmans G. Nilius B. (1993) Thapsigargin discharges intracellular calcium stores and induces transmembrane currents in human endothelial cells. Pflügers Arch., 422, 552-557.
- Penner R. Matthews G. Neher E. (1988) Regulation of calcium influx by second messengers in rat mast cells. Nature, 334, 499-504.
- 13. Clementi E. Scheer H. Zacchetti D. Fasolato C. Pozzan T.

Meldolesi J. (1992) Receptor-activated Ca²⁺ influx. J. Biol. Chem., 267, 2164-2172.

- Hoth M. Penner R. (1993) Calcium release-activated calcium current in rat mast cells. J. Physiol. (Lond.), 465, 359-386.
- Penner R. Fasolato C. Hoth M. (1993) Calcium influx and its control by calcium release. Curr. Opin. Neurobiol., 3, 368-374.
- Kojima I. Mogami H. Shibata H. Ogata E. (1993) Role of calcium entry and protein kinase C in the progression activity of insulin-like growth factor-I in Balb-c 3T3 cells. J. Biol. Chem., 268, 10003-10006.
- Merritt JE. Armstrong WP. Benham CD. et al. (1990) SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. Biochem. J., 271, 515-522.
- Nordström T. Nevanlinna HA. Andersson LC. (1992) Mitosis-arresting effect of the calcium channel inhibitor SK&F 96365 on human leukemia cells. Exp. Cell. Res., 202, 487-494.
- Soo Lee Y. Sayeed MM. Wurster RD. (1993) Inhibition of human brain tumor cell growth by a receptor-operated Ca²⁺ channel blocker. Cancer Lett., 72, 77-81.
- Lovisolo D. Munaron L. Baccino FM. Bonelli G. (1992) Potassium and calcium currents activated by foetal calf serum in Balb-c 3T3 fibroblasts. Biochim. Biophys. Acta, 1104, 73-82.
- Munaron L. Distasi C. Carabelli V. Baccino FM. Bonelli G. Lovisolo D. (1995) Sustained calcium influx induced by basic fibroblast growth factor in Balb-c 3T3 fibroblasts. J. Physiol. (Lond.), 484, 557-566.
- Del Bino G. Lassota P. Darzynkiewicz Z. (1991) The Sphase cytotoxicity of camptothecin. Exp. Cell. Res., 193, 27-35.
- Grynkievicz G. Poenie M. Tsien R. (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. J. Biol. Chem., 260, 3440-3450.
- 24. Almers W. Neher E. (1985) The Ca signal from Fura-2

loaded mast cells depends on the method of dye-loading. FEBS Lett., 192, 13-18.

- Barbiero G. Duranti F. Bonelli G. Amenta J.S. Baccino F.M. (1995) Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. Exp. Cell Res., In press.
- Schwartz G. Droogmans G. Nilius B. (1994) Multiple effects of SK&F 96365 on ionic currents and intracellular calcium in human endothelial cells. Cell Calcium, 15, 45-54.
- Franzius D. Hoth M. Penner R. (1994) Non specific effects of calcium entry antagonists in mast cells. Pflügers Arch., 428, 433-438.
- Lovisolo D. Alloatti G. Bonelli G. Tessitore L. Baccino FM. (1988) Potassium and calcium currents and action potentials in mouse Balb-c 3T3 fibroblasts. Pflügers Arch., 412, 530-534.
- Graier WF. Simecek S. Sturek M. (1995) Cytochrome P450 mono-oxygenase-regulated signalling of Ca²⁺ entry in human and bovine endothelial cells. J. Physiol. (Lond.), 482, 259-274.
- Estacion M. Mordan LJ. (1993) Expression of voltage-gated calcium channels correlates with PDGF-stimulated calcium influx and depends upon cell density in C3H 10T1/2 mouse fibroblasts. Cell Calcium, 14, 161-171.
- Sheng M. Thompson MA. Greenberg ME. (1991) CREB: a Ca²⁺-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science, 252, 1427-1430.
- Roche E. Prentki M. (1994) Calcium regulation of immediateearly response genes. Cell Calcium, 16, 331-338.

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