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Biochemical Pharmacology 67 (2004) 1513–1521
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Calpain as a multi-site regulator of cell cycle

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Received 22 August 2003; accepted 15 December 2003

Abstract

Calpain has long been implicated in the regulation of cell cycle, mostly based on studies with inhibitors that lack strict specificity toward the enzyme. Further, previous work has primarily focused on one particular point, the G_1 checkpoint, and made no attempt at dissecting the full cycle in terms of calpain action. To extend and complement these findings, we tested the effect of a specific inhibitor, PD 150606, on granulocyte-macrophage-colony stimulating factor (GM-CSF)-stimulated human TF-1 cells by flow cytometry following single- and double labelling by propidium iodide and bromodeoxyuridine. Using a new algorithm of analysis, we determined the time-dependence of the absolute number of cells leaving G_1, S and G_2M phases following the application of the inhibitor. Our results point to the simultaneous involvement of calpain activity in promoting the cycle at the G_1 checkpoint and somewhere in the G_2M compartment. Furthermore, the inhibitor significantly impedes the progress of cells through the S phase, indicating calpain activity in S phase checkpoint signalling. Overall, our analysis suggests that calpain regulates the cell cycle at more points than previously thought.

Keywords: Calpain inhibition; PD 150606; G_1 phase; S phase; G_2M phase; Mitosis

1. Introduction

Calpains are intracellular, Ca^{2+}-regulated cysteine proteases, which occur in all animals and also in lower eukaryotes and plants [1–3]. The calpain superfamily consists of more than a dozen genes; some gene-products have been amply studied whereas others have only been recently described and still await detailed characterisation. Most of our concepts with respect to calpain function are based on studies with the ubiquitous forms, μ- and m-calpain, which are found in all animal cells and are essential for life, as demonstrated by the lethality of disrupting the gene of their common small subunit [4]. In short, calpains are generally believed to regulate key cellular processes, such as differentiation, apoptosis, cell motility and cell cycle via signal-dependent limited cleavage of substrate proteins [1,2,5]. The evidence for their involvement in these physiological processes, however, is often not conclusive due to the use of inhibitors which display activity against other enzymes as well [6,7].

It appears well established, nevertheless, that calpain is part of the machinery controlling the cell cycle. Early studies have indicated its involvement in mitosis [8,9], but later the attention has shifted to its contribution to G_1 checkpoint signalling [10,11]. It has been shown by a range of small peptidyl inhibitors [10–15] and its specific, endogenous inhibitor protein, calpastatin [8,15–18] that calpain activity is necessary for the cell to pass through the restriction point. Although the disruption of the small subunit was initially reported to have no effect on fibroblast proliferation [4], subsequent studies have shown that it does affect the cycle at low plating density [18], in accord with earlier data on calpain's involvement in the clonal expansion phase of preadipocyte differentiation [15]. As for the possible targets of calpain action, most of the

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Abbreviations: BrdU, bromodeoxyuridine; Cdk, cyclin-dependent kinase; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; GM-CSF, granulocyte-macrophage-colony stimulating factor; NGS, normal goat serum; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; pRb, retinoblastoma gene product.

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doi:10.1016/j.bcp.2003.12.021

evidence points to the tumor-suppressor protein p53 [11,13,14,17,19], but cyclin D_1 [16], the Cdk inhibitor p27 [15] and the retinoblastoma-family member p107 [20] have also been implicated.

The inhibitors used in most of these studies, however, are not strictly specific to calpain, as they have a similar effect on cathepsins, papain and the proteasome. Further, almost all these studies have concentrated on a single phase of the cell cycle, whereas the sole flow-cytometric analysis of the whole cycle with non-specific thiol protease inhibitors has shown a double block affecting both the G_1S and G_2M compartments [12]. These data imply the possible involvement of calpain at multiple points of the cycle; based on this inference, we used flow cytometry following single- and double labelling and a specific calpain inhibitor, PD 150606 [21], to dissect calpain action in regulating the cell. PD 150606 is targeted against the calmodulin-like domain of calpain and, thus, it does not interfere with the action of other proteases [21]. It has been widely used to ascertain calpain action in various cell-biological phenomena [22–24] and its only side-effect reported so far is its inhibition of Ca^{2+}-permeable AMPA receptors [25]. Here we analysed its effect on the cell cycle of human TF-1 cells. These CD34(+) cells have not been reported to contain AMPA receptors and are good models of myelo-erythroid progenitor cells for which hormone deprivation and the ensuing synchronization control clonal maturation in the bone marrow [26,27]. Our data corroborate a significant contribution of calpain at the G_1/S boundary, and also suggest its simultaneous involvement in the G_2M compartment. An unexpected feature is the contribution to S phase progression, as inhibited cells spend significantly more time in S phase than control cells. In the aggregate, our results suggest the existence of three distinct sites where calpain is involved in cycle-specific signalling and call for further work at identifying its substrates.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise specified, all chemical reagents were purchased from Sigma. The tissue culture media were from Gibco, GM-CSF from New England Biolabs and PD 150606 from Calbiochem. NGS was obtained from Dakopatt, anti-BrdU monoclonal antibody from Becton Dickinson and FITC-conjugated affinity pure $F(ab')_2$ fragment of goat antimouse IgG from Jackson PD 150606 was used as follows. Typically, 5 mg was dissolved in 500 μL methanol, dried down by a vacuum concentrator in 10 μL aliquots and stored at −20°. The portions were dissolved in 20 μL DMSO on the day of use and diluted into the given medium to reach the desired final concentration.

2.2. Cell line, culture conditions and stimulation

The factor-dependent human myelo-erythroid leukaemia cell line TF-1 [28] was kindly provided by Dr. C. Braun, Heinrich Pette Institute for Experimental Virology and Immunology. Cells were grown in RPMI medium without nucleosides, supplemented with 10% (v/v) fetal calf serum and 2.5 ng/mL GM-CSF. For synchronization, cells were deprived of GM-CSF overnight and stimulated by the hormone added at the above concentration; its addition was considered as $t = 0$ of the experiments. Cell viability and cell growth were determined by a trypan blue exclusion assay [29]. Western blot was carried out according to ref. [30] by using specific primary monoclonal antibodies purchased from Santa Cruz Biotechnology and secondary antibodies from Jackson ImmunoResearch.

2.3. One-parameter flow-cytometric analysis of cellular DNA content

Cell cycle analysis by propidium-iodide staining was performed according to ref. [31]. Briefly, 10^6 cells, treated with or without PD 150606, were washed with PBS, fixed overnight in 70% ethanol, washed again and treated with 100 μg/mL RNAse and 10 μg/mL propidium iodide in PBS for 30 min at 37°. Cells were analysed for DNA content by a FACS Calibur (Becton Dickinson) flow cytometer at excitation and emission wavelengths of 488 and 585 nm, respectively. Data were analysed by the ModFit software.

2.4. Two-parameter flow-cytometric analysis of cellular DNA content and BrdU incorporation

Cultured cells without synchronization were treated with 30 μg/mL BrdU for 20 min, washed and placed in a fresh medium plus GM-CSF with or without PD 150606 at the concentrations indicated. Starting from the addition of the inhibitor, three parallel samples were collected every 3 hr and fixed as described above. 2×10^6 fixed cells were washed with PBS and then resuspended and incubated in 3 N HCl for 30 min. After washing with 0.1 M $Na_2B_4O_7$, pH 8.5, to stop acid denaturation, the cells were resuspended in 180 μL PBS with 0.5% Tween 20, 1% NGS and labelled with 20 μL anti-BrdU monoclonal antibody for 1 hr at room temperature. The cells were then washed with PBS and incubated for 1 hr with FITC-conjugated $F(ab')_2$ fragment of goat antimouse IgG diluted 1:50 in PBS with 0.5% Tween 20 and 1% NGS. Following incubation with the secondary antibody, the cells were collected by centrifugation, resuspended in PBS with 2 μg/mL propidium iodide and 100 μg/mL RNAse, incubated overnight and analysed by flow cytometry by setting the excitation at 488 nm and measuring emission in the range 515–545 nm (FITC) and 600–660 nm (PI).

2.5. Evaluation of the absolute number of cells leaving G_1, S and G_2M

We obtained the percentage of BrdU+ cells ($\%Br^+(t)$) and of undivided BrdU+ cells ($\%ud^+(t)$) at time "t" by setting the appropriate regions of interest in the biparametric DNA-BrdU histogram. Obviously, $\%Br^-(t) = 100 - \%Br^+(t)$. Then, the DNA distributions of the whole population and of the BrdU+ subpopulation were separately fitted, obtaining cell cycle percentages $\%G_1(t)$, $\%S(t)$ and $\%G_2M(t)$ and $\%G_1^+(t)$, $\%S^+(t)$ and $\%G_2M^+(t)$. Percentages of BrdU− cells in the cell cycle phases were obtained using the formulae:

$$\%G_1{}^-(t) = \frac{100 \times \%G_1(t) - \%Br^+(t) \times \%G_1{}^+(t)}{\%Br^-(t)}$$

$$\%S^-(t) = \frac{100 \times \%S(t) - \%Br^+(t) \times \%S^+(t)}{\%Br^-(t)}$$

$$\%G_2M^-(t) = \frac{100 \times \%G_2M^-(t) - \%Br^+(t) \times \%G_2M^+(t)}{\%Br^-(t)}$$

Using these data, the time course of the number of BrdU+ and BrdU− cells in G_1, S and G_2M phases, relative to the number of cells at $t = 0$ hr, was calculated with the following algorithm.

Let $N^-(t)$ and $N^-(0)$ be the (absolute) total numbers of BrdU− cells at time "t" and time zero (corresponding to the end of BrdU pulse and the beginning of inhibitor treatment). If cell loss is negligible (in comparison to the mitotic rate), the increase in cell number in the 0–t interval equals the number of mitotic events. In the BrdU− subpopulation, cells that initially divided were in G_2M at $t = 0$, while others (in G_1 at $t = 0$ hr) had to exit G_1, traverse S before entering G_2M and to undergo division. Thus, the cell number increment in the BrdU− subpopulation is equal to the decrease of the number of BrdU− cells in G_2M, up to the time new cells enter G_2M. This initial interval was assessed by visual inspection of the flux of the cloud of BrdU− cells exiting G_1, which reached G_2M at $t = 12$ hr in controls but were still in S phase at $t = 24$ hr in treated samples. Within that interval the following formula holds:

$$N^-(t) - N^-(0) = N_{G_2M}{}^-(0) - N_{G_2M}{}^-(t) \quad (1)$$

where $N_{G_2M}{}^-(t)$ is the absolute number of BrdU− cells in G_2M. On the other hand, by definition $N_{G_2M}{}^-(t) = N^-(t) \times \%G_2M^-(t)/100$. Taking formula (1) and the definitions into account, with simple algebra we obtained the formula:

$$N_{G_2M}{}^-(t) = \left(\frac{N(0) \times \%Br^-(t)}{100}\right) \times \left(\frac{1 + \%G_2M^-(0)}{100}\right) \times \left(\frac{\%G_2M^-(t)}{100 + \%G_2M^-(t)}\right) \quad (2)$$

Formula (2) gives the number of cells remaining in G_2M until time "t", i.e. in the initial interval defined above, providing the quantities in the right side are measured. These quantities all come from the DNA-BrdU histograms at time zero and "t", except for the initial absolute number of cells at time zero ($N(0)$), that was independently measured using an hemocytometer. Once $N_{G_2M}{}^-(t)$ and $N(0)$ are known, it is possible to calculate $N^-(t)$ using formula (1) and the identity $N^-(0) = N(0) \times \%Br^-(0)/100$.

For what concerns G_1 phase, the number of cells remaining there throughout the 0–t interval was calculated by the number of BrdU− cells in that phase at time "t" minus the number of cells, which have entered G_1 as a result of division of cells initially in G_2M:

$$\mathrm{Nres}_{G_1}{}^-(t) = \frac{N^-(t) \times \%G_1{}^-(t)}{100} - 2 \times (N_{G_2M}{}^-(0) - N_{G_2M}{}^-(t)) \quad (3)$$

This formula holds true, as long as the division of BrdU− cells is sustained only by cells initially in G_2M. In the case of TF-1 cells this period lasts for about 15 hr in controls and more than 24 hr in treated samples.

With regards to cells initially in S phase, their exit from S phase was calculated with reference to BrdU+ cells. First, the time course of the absolute number of BrdU+ undivided cells was calculated by the formula:

$$N_{ud}{}^+(t) = \frac{N(0) \times \%Br^+(t)}{100}\left(\frac{1 + \%ud^-(0)}{100}\right) \times \frac{\%ud^+(t)}{100 + \%ud^+(t)} \quad (4)$$

that can be obtained in a way similar to that of formula (2). Once $N_{ud}{}^+(t)$ and $N(0)$ are known, $N^+(t)$ can be calculated:

$$N^+(t) = \frac{N(0) \times \%Br^+(0)}{100} + N_{ud}{}^+(0) - N_{ud}{}^+(t) \quad (5)$$

Because undivided BrdU+ cells comprised both cells in S+ and G_2M+, the number of cells remaining in S phase at time "t" can be calculated by the formula:

$$N_S{}^+(t) = N_{ud}{}^+(t) - N_{G_2M}{}^+(t) = N_{ud}{}^+(t) - \frac{\%N^+(t) \times \%G_2M^+(t)}{100} \quad (6)$$

Formula (6) holds true as long as undivided cells can be unequivocally distinguished from divided BrdU+ cells. In the TF-1 cell line, a robust separation between undivided and divided BrdU+ cells was feasible up to $t = 18$ hr in controls and $t = 24$ hr in treated samples.

3. Results

3.1. Effect of calpain inhibition on cell proliferation and viability

TF-1 cells were synchronized by overnight GM-CSF deprivation. Thereafter the hormone was re-added and the

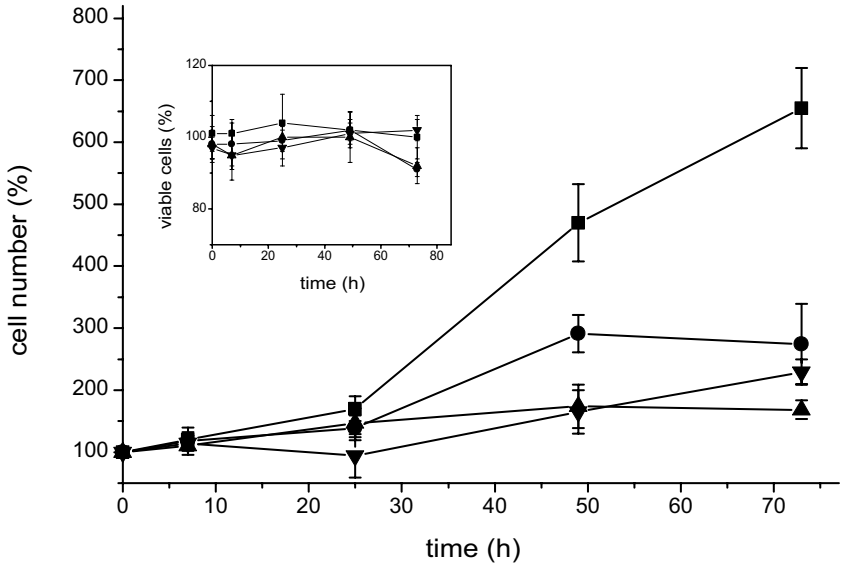

Fig. 1. Calpain inhibition retards proliferation of TF-1 cells. TF-1 cells were synchronized by overnight deprivation and subsequent re-addition of 2.5 ng/mL GM-CSF without (■) or with the calpain inhibitor PD 150606 at a concentration of 20 μM (●), 40 μM (▲) or 130 μM (▼). Cell number, determined at the times indicated, is given as the percentage of the initial count at the addition of hormone. The viability of cells was determined by trypan blue exclusion and is given in percentage of the initial value (inset); error bars in both cases correspond to SD of 5–8 separate experiments.

cells were treated with 20, 40, 130 μM or no (control) PD 150606, the specific calpain inhibitor ($t = 0$ hr). At 20 and 40 μM concentrations, the inhibitor was added every 24 hr to compensate for its loss due to degradation and to make sure the lack of an effect on viability is not due to its elimination from the system as a result of its degradation. Cell number and viability were determined by a trypan-blue exclusion assay at various times. As shown in Fig. 1, re-administration of the natural growth hormone resulted in the exponential growth of cells following a lag period. Growth was significantly hindered by the inhibitor, with a half-effective concentration between 20 and 40 μM. Upon treatment with 130 μM PD 150606, the cell number hardly changed by 24 hr and increased by about 50% by 48 hr, which indicates that the growth of cells slowed down significantly but was not fully blocked even at this inhibitor concentration. Application of the inhibitor caused no toxic side-effects: in all cases the viability of cells remained above 95% for at least 2 days (Fig. 1, inset).

3.2. *One-parametric flow-cytometric analysis of calpain inhibition*

To determine which phase of the cell cycle the inhibitor alters, a one-parametric FACS analysis was performed. Synchronized cells were treated with the inhibitor at a saturating concentration, 80 μM, and samples were collected regularly for a period of 30 hr. Our data point to the slowing down of all three phases of the cell cycle upon calpain inhibition. Figure 2A demonstrates that upon re-administration of the hormone, cells re-entered the cell cycle and reached the S phase following a 9-hr lag period, in accord with previous observations with other cell types [10,11]. PD 150606 much extended this delay, with cells starting to enter the S phase only 18 hr after hormone addition (Fig. 2B). For the proliferation of TF-1 cells, GM-CSF-stimulated activation of the ERK-MAPK pathway is essential [32]. To find out if calpain inhibition interferes with this pathway, we have tested ERK activation (phosphorylation), Elk-1 activation, p38 phosphorylation and the level of c-Fos by Western blot using specific antibodies. As all these were unaffected (Fig. 3), the point of attack must lay in late G_1, possibly at the restriction point. This conclusion is underscored by that if PD 150606 was added 6 hr after the re-administration of GM-CSF, the block was still effective and caused an even further delay in the cycle (data not shown).

Interestingly, the extent of delay was not fixed, as it depended on cell density. In one experiment, when the cells were plated out denser, the delay in entering the S phase became significantly shorter (data not shown), in accord with previous observations of the effect of plating density [18]. Furthermore, S phase itself apparently became longer in the presence of the inhibitor, as cells entering the S phase at 9 hr reach the G_2M phase about 6 hr later in the absence of the inhibitor (Fig. 2A), but fail to do so in its presence for up to 12 hr (i.e. they reach the S phase at 18 hr but not G_2M even by 30 hr); the duration of phases can be determined by the two-parameter analysis (see later). This observed extension of S phase and the absence of relative movement out and into G_2M suggest that calpain inhibition does not only arrest the cell cycle at the G_1 checkpoint but also impedes S and G_2M phase progression.

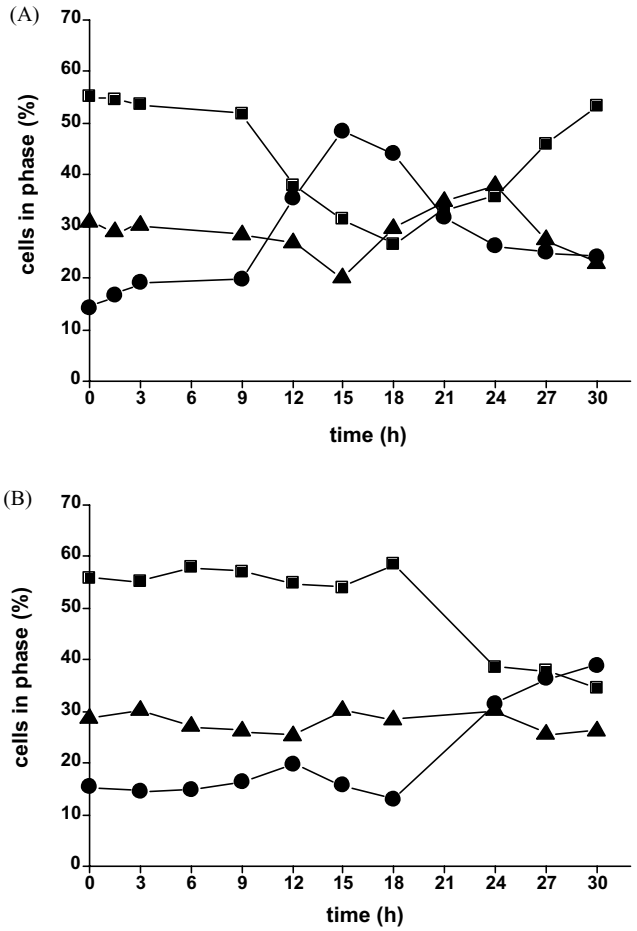

Fig. 2. Calpain inhibition delays all three phases of the cell cycle of TF-1 cells. TF-1 cells were synchronized by overnight deprivation and subsequent re-addition of 2.5 ng/mL GM-CSF in the absence (A) or presence (B) of 80 μM PD 150606. At the times indicated, cells were labelled with propidium iodide and their DNA content was determined by FACS analysis. The initial plots (not shown) were deconvoluted to determine the percentage of cells in the G_0G_1 (■), S (●) and G_2M (▲) phase of the cell cycle. A significant and reproducible delay in phase transitions was observed in three independent experiments. Due to a large variation in the initial distribution of cells within the three phases of the cell cycle, the results of one representative experiment are shown here.

3.3. Two-parametric flow-cytometric analysis of calpain inhibition

Our one-parametric FACS analysis implies that calpain is involved in controlling the cell cycle at multiple points, not only at the G_1 checkpoint as most results suggested previously. In order to obtain more certainty regarding the exact place of calpain action, two-parametric kinetic studies were performed following BrdU incorporation into non-synchronized cells. Treatment with 80 μM PD 150606 revealed that calpain inhibition blocks cells at three different points of the cell cycle. Figure 4 shows the time course of biparametric DNA-BrdU histograms in untreated (Fig. 4A) and treated (Fig. 4B) samples. Biparametric DNA-BrdU+ histograms allow to split the whole cell population into BrdU+ and BrdU− subpopulations. BrdU+ cells were in S phase at $t = 0$ hr (the time of the pulse labelling), while BrdU− cells occupied G_1 and G_2M at the outset. By visual inspection of the sequence of controls (Fig. 4A), one can catch the movement of BrdU+ cells, distributed through early, middle and late S phase at $t = 0$ hr, shifting to late S and G_2M with some cells divided and already in G_1 by $t = 7$ and 9 hr. Subsequently, at $t = 18$ hr, some of these cells have re-entered S phase. BrdU− cells, initially in G_1 and G_2M, were in part detected in early S at $t = 7$ hr, then moved through S, reaching the S/G_2M boundary by $t = 12$ hr. This kinetics differs significantly from that of treated cells (Fig. 4B), for which the cycle appeared largely frozen, i.e. the distribution of the clouds of BrdU+ and BrdU− cells showed only minor shifts for at least up to12 hr. However, by visual inspection of the time course of biparametric DNA-BrdU histograms, the fate of BrdU+ and BrdU− subpopulations can be followed only in a qualitative way. In order to quantify the exit of cells from the phase they occupied at the beginning of treatment, we devised a new procedure. For this purpose, we derived three formulae that permit to calculate the absolute numbers of G_1, S and G_2M cells remaining in the respective phases after the start of the treatment. The formulae used combinations of cell-cycle percentages, obtained by the flow cytometric analysis and a single independent measure of the total number of cells at time zero. The only condition of their validity was that cell loss was negligible, as verified by a trypan blue test of viability (>95%, in control and treated samples at all times). Each formula has a known interval of validity, which in our cell line and with the adopted experimental design, began at time zero (i.e. the time of the end of pulse BrdU labelling and the start of inhibitor treatment) and lasted not less than 12 hr in controls and 18 hr in the treated samples. The length of these intervals allowed to follow the outflow of cells from G_1, S and G_2M almost completely, both in treated and untreated samples.

These in-depth analyses (Fig. 5) revealed that all phases were significantly prolonged. The block in G_1 is reflected in Fig. 5A: apparently, no G_1 cells leave this phase in the first 3 hr, not even in controls, which is probably due to a temporary block induced by cell manipulation. Then, a progressive outflow from G_1 follows and almost all untreated G_1 cells leave this phase by 12 hr. In contrast, no outflow from G_1 is observed in inhibitor-treated cells for up to $t = 12$ hr, and some cells remain in G_1 for as long as 24 hr. Similarly, treated S-phase cells move slowly and come out of S phase (Fig. 5B) with a significant delay compared to untreated cells, in perfect agreement with the results of the one-parametric analysis above. Control cells stay in S phase for 9–12 hr on the average; for treated cells, the average duration of S phase is about 21 hr. The absolute number of cells remaining in G_2M (Fig. 5C) indicated a G_2M delay in treated samples, that was activated roughly 3 hr after the start of treatment.

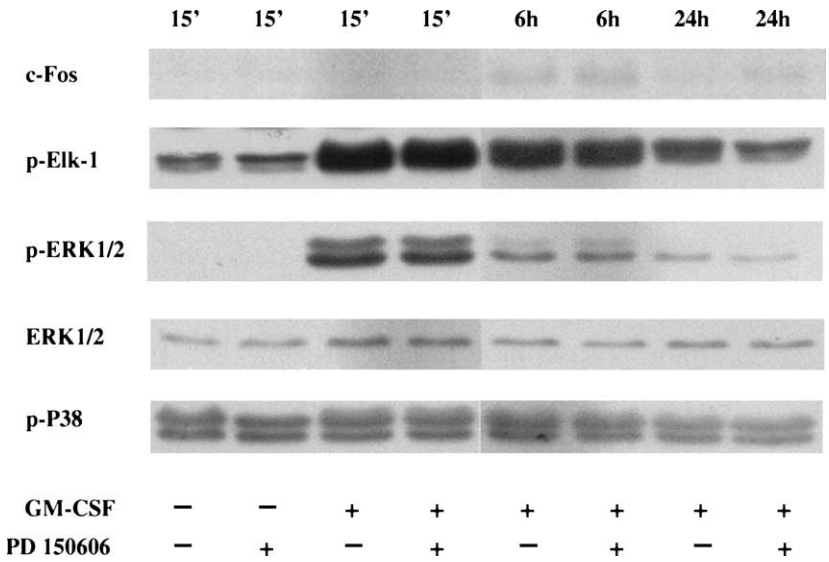

Fig. 3. Calpain inhibition does not affect the ERK-MAPK pathway. TF-1 cells were synchronised by overnight deprivation and subsequent re-addition of 2.5 ng/mL GM-CSF, either in the absence or the presence of 80 μM PD 150606 as in Fig. 2. At the times indicated, cells were collected and p38 phosphorylation (p-P38), ERK1/2 level and activation (p-ERK1/2), Elk-1 activation (p-Elk-1) and c-Fos level were checked by Western blotting as given in Section 2.

4. Discussion

Previous studies have established that calpain is involved in regulating the cell cycle at the G_1 checkpoint, and possibly also in the G_2M compartment. These studies, however, lack consistency due to the use of various normal and transformed cell-lines and a whole range of inhibitors of different specificity. Further, most studies have focused on a given point of the cycle only, and no attempt has been made to address the full cycle at the same time. The possibility of the involvement of calpain in S phase checkpoint signalling has not been raised at all. To straighten these things up, we tested the effect of a specific inhibitor, PD 150606, on TF-1 cells by flow cytometry following single and double labelling. Unlike with other cells, for which non-physiological serum-deprivation has been used for synchronization [7,11,12,16], TF-1 cells can be synchronized by physiological hormone deprivation which is absolutely essential for the maintenance of normal hematopoiesis [26,27]. Our results provide evidence that calpain regulates the cycle of these cells at three points, as follows.

The first point of control is the G_1 checkpoint or restriction point. We found that GM-CSF-deprived TF-1 cells return to G_1 and reach S phase in about 9 hr upon GM-CSF addition; in the presence of PD 150606, it takes at least 18 hr. These data corroborate all previous assertions that calpain has a role in making the decision to replicate chromosomal DNA. As the early phase of the ERK-MAPK pathway (ERK activation, Elk-1 activation and c-myc) was unaffected by calpain inhibition, our data are consistent with earlier suggestions that calpain may degrade p53 [11,13,14,17,19] that results in the down-regulation of the Cdk inhibitor p21 [11,13,19]; a direct effect on cyclin D_1 [16], the Cdk inhibitor p27 [15] and the retinoblastoma-family member p107 [20] is also possible. When calpain is inhibited, the ensuing inhibition of Cdk2 and/or Cdk4 may keep pRb in a hypophosphorylated state [13,15], which prevents the activation of downstream genes needed for the entry into the S phase.

The second point where calpain inhibition interferes with the cell cycle is within S phase, which has not been previously observed. Our kinetic analysis shows that it takes significantly longer for cells to proceed through S phase in the presence, than in the absence, of the inhibitor. This observation does warrant some speculation into the possible regulatory substrates involved. As stated above, calpain inhibition may prevent the activation of S-phase genes by keeping pRb in a hypophosphorylated state [13,15] and/or stabilizing its homologue, p107 [20]. We suggest here that this may also explain the observed effect of S phase retardation. One of the genes downstream of pRb, PCNA, is known to act as a DNA polymerase processivity factor and to promote S phase progression. In addition, PCNA interacts with the C-terminal domain of DNA polymerase ε, a calpain-substrate itself [33], which is also known to be involved in S phase checkpoint signalling [34]. Although further factors may come into the picture later on, these proteins are good candidates to start with.

The third point where calpain is involved in cell-cycle decisions is in the G_2M compartment. This finding is consistent with the seminal observation of Schollmeyer that calpain activity is involved in the progression to metaphase and/or in the transition from metaphase to anaphase [8]. Since this early observation has not been followed up later, the information on the underlying molecular details is very scarce. The primary candidate is the c-mos protooncogene product, part of the cytostatic factor

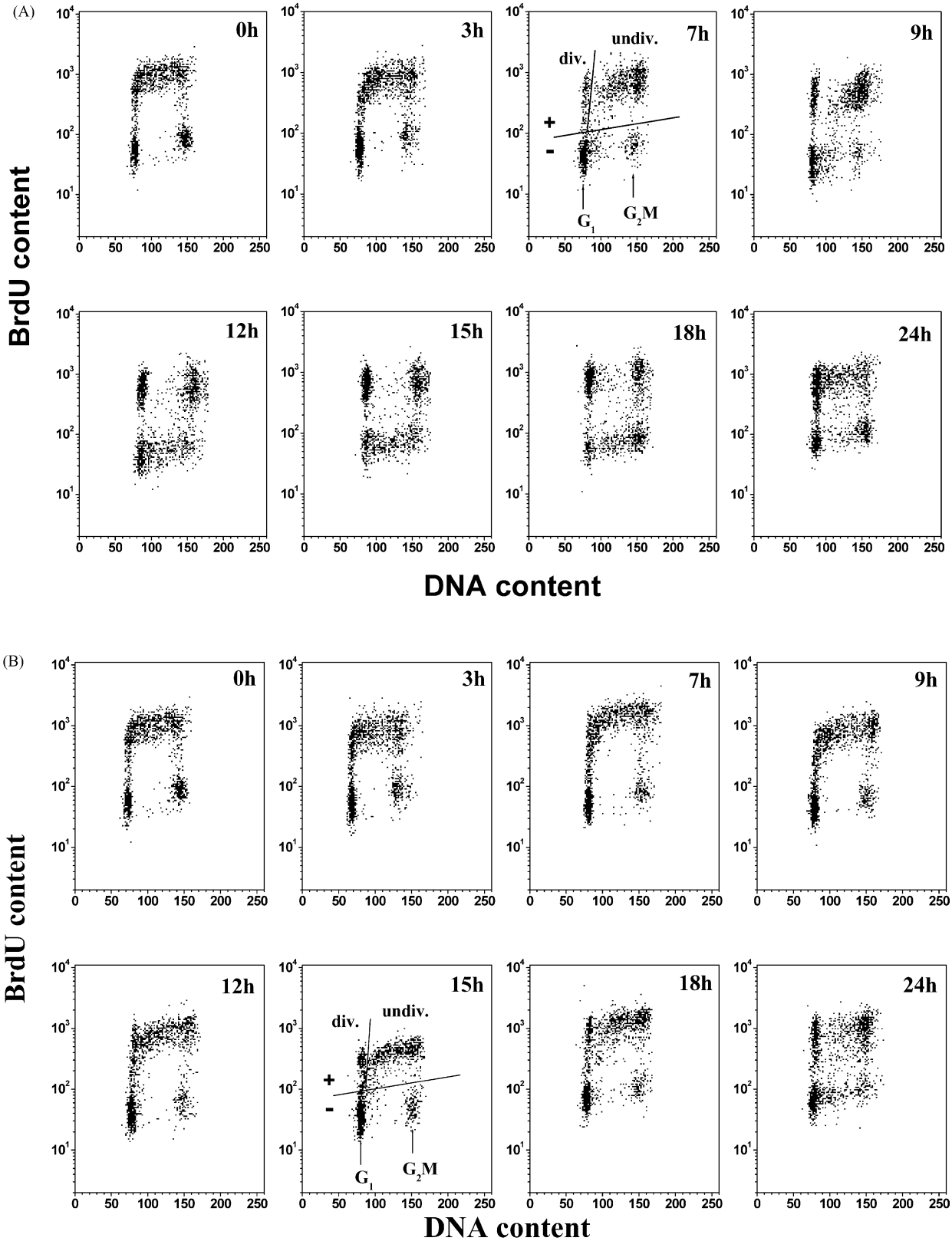

Fig. 4. Two-parametric flow-cytometric analysis of the effect of calpain inhibition on the cell cycle. Non-synchronized TF-1 cells were labelled with BrdU for 20 min and allowed to proceed further through the cell cycle in the absence or presence of 80 μM PD 150606. At the time indicated, aliquots were fixed and simultaneously analysed by flow cytometry for BrdU incorporation and total DNA content. The movement through the cell cycle for $t = 0$, 3, 7, 9, 12, 15, 18 and 24 hr for control (A) and inhibitor-treated (B) cells is shown. To help explain the evaluation of the absolute number of cells leaving G_1, S and G_2M, thin lines have been drawn on the plot $t = 7$ hr (A) and $t = 15$ hr (B) to separate BrdU-labelled (+) and non-labelled (−), as well as divided (div.) and undivided (undiv.) cells; cells in G_1 and G_2M phases are also marked (for details, see Section 2).

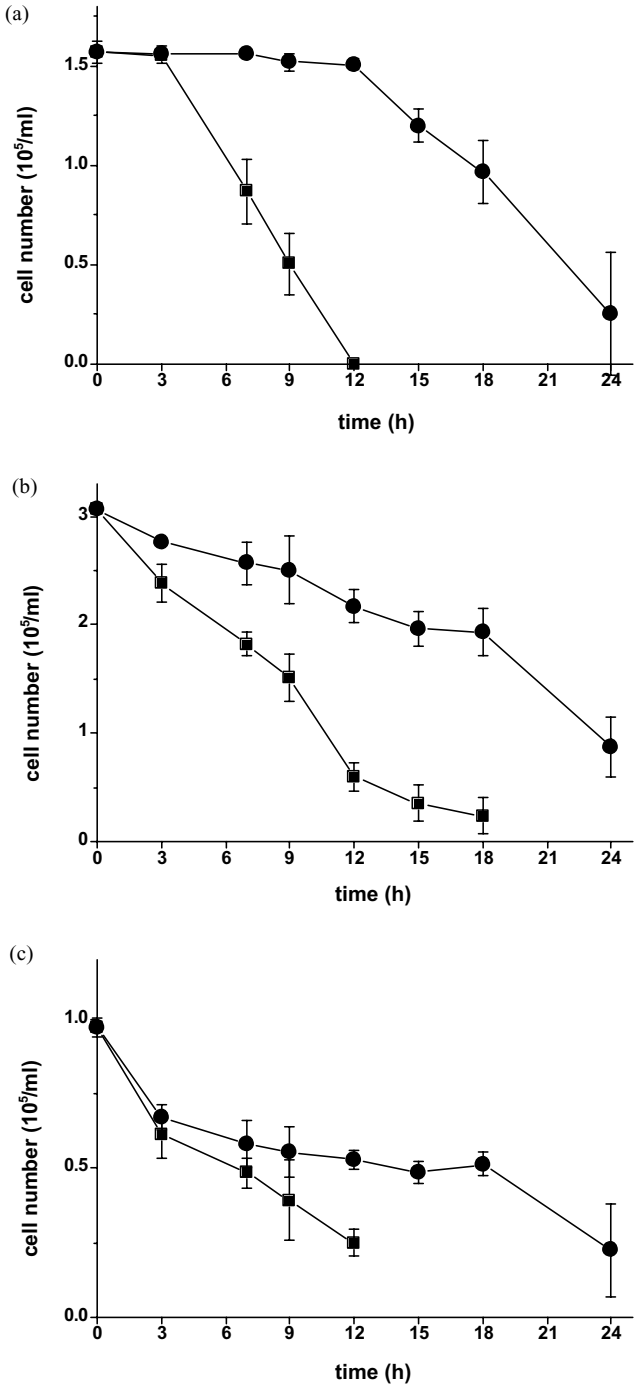

Fig. 5. Calpain inhibition interferes with the cell cycle at three separate points. All plots in Fig. 4 have been analysed and the absolute number of cells remaining in G_1, S and G_2M were calculated using formulae (3), (6) and (2), respectively (cf Section 2.5). The initial number of cells was 5.6×10^5 cells/mL. The kinetics of the outflow from G_1 (A), S (B) and G_2M (C) in the absence (■) and presence (●) of the inhibitor is shown. The results are mean and standard deviation of three replicates.

(CSF), which stimulates maturation-promoting factor (MPF) activity and promotes cell-cycle arrest at the metaphase/anaphase boundary [35]. c-Mos is degraded by calpain on fertilization of Xenopus eggs [9], an event probably crucial for the completion of meiosis. In light of our results, the generality of this regulatory step is worthy of further studies. Another possible link with calpain is related to p53 degradation, originally implicated in the G_1 restriction point. It has been shown recently that p53 directly [36] and/or via p21 [37] and PCNA [38], forms part of the G_2 checkpoint; calpain may thus affect cell cycle via this unexpected connection.

In the aggregate, our results suggest that calpain serves as a multi-site regulator of the cell cycle by acting at least three different control points. Whereas we have some good candidates for its target proteins at the G_1 checkpoint, we know much less about its involvement in mitosis and next to nothing about its role in promoting S phase. Unraveling its substrates in all these cellular settings, thus, will certainly be an intriguing future direction in calpain research.

Acknowledgments

This work was supported by grants T 32360, T 34255 and TS 040723 from OTKA and 1/A0020/2002 from the Ministry of Education and Ministry of Health grant ETT 206/2001.

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