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Cell Cycle-Dependent Activity Change Of Ca^{2+}/Calmodulin-Dependent Protein Kinase II In NIH 3T3 Cells

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Although the blockage of a cell cycle by specific inhibitors of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) is well known, the activity profile of CaMK-II during the cell cycle in the absence of any direct effectors of the enzyme is unclear. The activity of native CaMK-II in NIH 3T3 cells was examined by the use of cell cycle-specific arresting and synchronizing methods. The total catalytic activity of CaMK-II in arrested cells was decreased about 30% in the M phase, whereas the Ca^{2+}-independent autonomous activity increased about 1.5-fold in the M phase and decreased about 50% at the G1/S transition. The *in vivo* phosphorylation level of CaMK-II was lowest at G1/S and highest in M. The CaMK-II protein level was unchanged during the cell cycle. When the cells were synchronized, the autonomous activity was increased only in M. These results indicate that the physiologically relevant portion of CaMK-II is activated only in M, and that the net activation of CaMK-II is required in mitosis.

Keywords: Ca^{2+}, Calmodulin, CaMK-II, Cell cycle, Phosphorylation

Introduction

CaMK-II is a ubiquitous multifunctional enzyme. It requires Ca^{2+}-bound calmodulin (CaM) for its activation, and its activity is involved in many cellular events (for a review, see Braun and Schulman, 1995). The kinase is in a large multimeric form of its subunits *in vivo* and shows unique autoregulatory functions (Hanson and Schulman, 1992). autophosphorylation of the CaMK-II subunit displaces the autoinhibitory domain in a subunit, which is very important (MacNicol *et al.*, 1990). autophosphorylation increases by several hundred-fold the affinity of the kinase for the CaM. When the intracellular Ca^{2+} level is reduced to basal levels, CaM is still trapped on the kinase for a certain period of time. Therefore, the kinase is independent on the Ca^{2+} level, and can exert full activity as long as CaM is trapped (Meyer *et al.*, 1992). The phosphorylated kinase itself especially maintains partial activity (20-80%), even after CaM dissociates from the kinase (Mukherji and Soderling, 1994). This kind of activity is known as the autonomous activity of CaMK-II, which is physiologically relevant for it is the fraction of the kinase that has been activated by the stimulation of cells.

In order to reproduce and multiply, every cell must execute the cell cycle during its life span. The cell cycle of eukaryotic cells is conventionally divided into four phases: G1, S, G2, and M (for a review, see Lundberg and Weinberg, 1999; Shapiro and Harper, 1999). Physiologically the G1 phase is the decision phase, in which it either commits to undergo another round of DNA synthesis (S phase) and continue the cell division (M phase), or to exit the cell cycle to enter a quiescent state (referred to as G0.) For the initiation and progression of the cycle, specific enzymes should be activated or expressed at the corresponding phase, otherwise cells stop growing and are arrested at a specific point on the cell cycle (Sielecki *et al.*, 2000).

It is well known that the intracellular level of Ca^{2+} transiently increased at the exit of G0, G1/S, G2/M and mid M; CaM also oscillates in a similar pattern during the cell cycle (for a review, see Lu and Means, 1993; Santella, 1998). Therefore, it can be easily assumed that the activity of native CaMK-II is also cell cycle-dependently changing. However, there is no direct evidence to confirm this assumption. There are several reports regarding the *in vivo* phosphorylation of various cellular proteins by CaMK-II at specific points of the cell cycle (Houglum *et al.* ,1997; Patel *et al.*, 1999). These also show the arrest of the cell cycle at G1/S by KN-series inhibitors of CaMK-II (Minami *et al.*, 1994; Tombes *et al.*, 1995; Morris *et al.*, 1998). No systematic study has been done to determine how the activity of native CaMK-II is changing as the cell cycle progresses.

In the present study, the activity of CaMK-II in NIH 3T3 cells was measured along the phases of the cell cycle without using direct effectors of the enzyme. We report here for the

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first time how the native activity of CaMK-II in mammalian somatic cells changed at specific phases during one turn of the cell cycle. It was observed that the physiologically relevant Ca^{2+}-independent activity of CaMK-II in NIH 3T3 cells was increased only in M, and was kept below the basal level during the interphase.

Materials and Methods

Materials Hydroxyurea (HU), bisbenzimide Hoechst 33342 fluorochrome (H33342), and nocodazole (Noco) were purchased from Sigma (St. Louis, USA). All media and reagents for cell culture were from Gibco BRL (Rockville, USA). The polyclonal anti CaMK-II antibody was purchased from Upstate Biotechnology (Lake Placid, USA), and a synthetic peptide substrate specific to CaMK-II, Syntide 2, was obtained from Calbiochem (La Jolla, USA). All radioactive materials were from Amersham (Piscataway, USA).

Cell culture and treatments NIH 3T3 cells were grown as a monolayer culture in a Dulbeccos modified Eagle medium (DMEM) that was supplemented with 10% (v/v) bovine calf serum (BCS) and antibiotics (penicillin at 100 units/ml and streptomycine at 50 μg/ml) at 37°C in an atmosphere of 5% CO_2. In an attempt to arrest the cells at a specific phase of the cell cycle, cells were treated with cell cycle-specific drugs, or different types of a culture medium as modified from the previous report (Suh, 2000). (a) When cells grew to about 70% confluency, they were incubated in serum-deficient (0.5% BCS) DMEM for 2 days. (b) The medium of exponentially growing cells was replaced by an isoleucine-deficient Eagle's minimum essential medium (EMEM-Ile) containing low BCS (5%), and the cells were cultured for 2 days. (c) The normally grown 70% confluent cells were incubated in 1 mM HU for 14 h. (d) An inhibitor of DNA-topoisomerase II, H33342 (13.3 μM), was applied to the normal medium of exponentially growing cells and incubated for 17 h. (e) Normally growing cells were treated with 1 μM nocodazole for 16 h. At the end of each designated culture period, the cell population was analyzed by flow cytometry. For the release of cells from their arresting points, the cells were washed by Hank's balanced salt solution (HBSS) three times and then incubated in fresh DMEM supplemented with 10% BCS. The viability was determined by a dye exclusion assay for each treatment of the cells.

Protein extraction Cells harvested from different treatments were washed with an ice-cold Tris-buffered saline (TBS) and resuspended in 4 volumes of an extraction buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02 mM leupeptin). After sonication on ice for 30 sec, the homogenate was centrifuged at 100 K × g for 30 min to obtain the soluble proteins. The aliquots of the soluble proteins were analyzed for the activity of CaMK-II and were not pre-treated to block other protein kinases using CaMK-II specific synthetic substrate.

Protein kinase assay CaMK-II activity was measured in a reaction mixture (final volume 100 μl) containing 30 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM dithiothreitol, 20 μg/ml CaM, either 2 mM Ca^{2+} (plus Ca^{2+}), or 1 mM EGTA (minus Ca^{2+}), 5 μg of a soluble protein extract, and 2 μg of Syntide 2. Syntide 2 is a synthetic peptide that is a highly specific substrate of CaMK-II (White *et al.* 1998). Therefore, it was unnecessary to pre-treat the soluble proteins of cells in order to block the other protein kinase activities. The mixture was incubated at 30°C for 1 min, and the reaction was started by the addition of 20 μM [γ-^{32}P]-ATP (100 cpm/pmol). The phosphorylation reaction was terminated after a 1 min incubation by the addition of a stop solution containing 100 mM EDTA, 25 mM Tris-HCl, pH 7.4, 33 mM EGTA, 10 mM sodiumpyrophosphate, and 10 mM β-glycerophosphate. An aliquot (60 μl) of each reaction mixture was applied onto the phosphocellulose membrane (Whatman p81), and rinsed 4 times with 1% phosphoric acid and once with ethanol. Radioactivity retained on the membrane was analyzed by liquid scintillation spectroscopy.

***in vivo* phosphorylation of cellular proteins** Arrested cells at designated points of the cell cycle in 60 mm culture dishes were washed three times with HBSS, 4 h before the end of the designated culture period. Further incubation of the cells in a phosphate-free medium followed for 1 h. The *in vivo* phosphorylation of the cellular proteins was carried out by the application of a carrier-free $^{32}P_i$ (0.5 mCi/ml) to the medium and incubation for 3 h. CaMK-II was identified from the soluble proteins by an anti CaMK-II polyclonal antibody, and its phosphorylation level was analyzed by autoradiography.

[^{3}H]-thymidine incorporation NIH 3T3 cells were treated with EMEM-Ile for 2 days, washed three times with HBSS, and cultured in fresh DMEM supplemented with 10% BCS. At this point, [^{3}H]-thymidine (0.2 μCi/ml) was added to label the cellular DNA. The cells on each culture dish were washed three times with ice cold TBS every 1 or 2 h. The washed cells were lysed on the dish by 0.8 ml of TBS containing 0.1% Triton X-100 for 15 min. To measure the incorporation of [^{3}H]-thymidine into DNA, 50 μl of the total cell lysate was applied to the scintillation cocktail and the radioactivity was counted.

Results

Effect of the cell cycle-specific treatment In order to study the changes in the activity and phosphorylation level of CaMK-II during the cell cycle, NIH3T3 cells were arrested at specific phases of the cell cycle by specific media, or drugs with known action mechanisms or targets. The drugs and treatments used in this study exerted no direct effect on the catalytic activity and autophosphorylation level of CaMK-II, when examined in the *in vitro* assay system (data not shown). At the end of each treatment, cells were analyzed by flow cytometry to determine the phase where the majority of the cells were arrested.

As shown in Fig. 1, and summarized in Table 1, each treatment worked effectively to arrest cells at specific phases of the cell cycle. Two days of serum-deprivation arrested most of the NIH 3T3 cells in the G0 phase. Cultivation of the cells

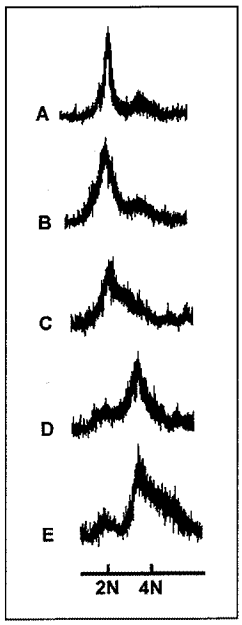

Fig. 1. A flow cytometry analysis of the DNA content of NIH 3T3 cells after treatments to arrest the cells at the designated phase of the cell cycle. (A) Untreated control cells. (B) Cells treated with isoleucine-deficient EMEM for 2 days. (C) Cells treated with 1 mM hydroxyurea for 14 h. (D) Cells treated with 13.3 μM Hoechst 33342 for 17 h. (E) Cells treated with 1 μM nocodazole for 16 h.

in EMEM-Ile for 2 days, which limited the availability of a nitrogen source, arrested cells in the early G1 phase (Tobey, 1973). This method was non-toxic because most of cells were viable after the designated incubation period. Cells cultivated for 14 h in a HU containing medium, an inhibitor of ribonucleotide reductase regulating the level of deoxyribonucleotides (Tobey *et al.*, 1988), were arrested at the G1/S transition with relatively low efficiency. Since HU was cytotoxic, as reported earlier (Lerza *et al.*, 1998), some of the cells were detached from the dish and showed a positive response to the dye exclusion assay. Cells growing in the medium supplemented with H33342 stopped growing at the G2/M transition after a 17 h cultivation. This drug was known as an inhibitor of DNA-topoisomerase II (Tobey *et al.*, 1990). A well-known anti-microtubule drug (Brizuela *et al.*, 1988), nocodazole, arrested cells at M after a 16 h incubation. Its substantial arresting efficiency is higher because cells undergoing mitosis were detached from their mother cells and eventually lost during the cell preparation.

Effect of cell cycle-specific treatments on the CaMK-II activity The activity of CaMK-II was measured in the cells arrested at different phases of the cell cycle by use of a highly specific synthetic peptide substrate. The total catalytic activity level of the kinase in G0, G1, G1/S, and G2/M, which was activated by the addition of 2 mM Ca^{2+} (plus Ca^{2+} activity), were similar to that of the exponentially growing asynchronized control cells (Fig. 2A). In contrast, the total activity of CaMK-II in the M phase was about 30% lower than that of the control cells. The total activity of CaMK-II reflected a summation of both the Ca^{2+}-dependent and independent activity. However, it is well known that the physiologically relevant activity of CaMK-II is its Ca^{2+}-independent activity (Mukherji and Soderling, 1994; Braun and Schulman, 1995). This kind of activity can last a certain amount of time, even after the Ca^{2+} level is lowered to the basal level. The Ca^{2+}-independent activity was measured directly from the assay mixture without Ca^{2+} (minus Ca^{2+} activity), and with EGTA to chelate any possible Ca^{2+} contamination. The Ca^{2+}-independent activity at each arresting point showed evident changes (Fig. 2B). The activities in G0, G1 and G1/S decreased about 20-50%, and those at G2/M and M increased about 20-45%. The Ca^{2+}-independent activity, especially at G1/S, showed the lowest activity, whereas the highest was at M.

Effect of cell cycle-specific treatments on the autonomous activity In order to understand how the physiologically relevant activity of CaMK-II changed at each arresting point, the proportion of Ca^{2+}-independent activity to the total activity in each phase of the cell cycle was determined (Fig. 3A). This proportional activity was designated as autonomous activity, which was the direct expression of the physiologically effective activity of the CaMK-II in cells. The cells arrested at G0, G1, and G2/M showed similar autonomy to that of the control. However, autonomy decreased at G1/S and increased at M. The percent of change of autonomous activity showed a small change around 15% at G0, G1 and G2/M (Fig. 3B).

Table 1. Cell population arrested at a specific phase of the cell cycle

Treatment	Condition or concentration	Treatment period	Arresting phase	Cell population (%)
Serum deficient DMEM	DMEM supplemented with 0.5% BCS	2 days	G0	90.0 ± 5.1
Nitrogen source-limited medium	EMEM minus Ile, 5% BCS	2 days	G1	86.4 ± 4.9
Hydroxyurea	1 mM	14 h	G1/S	72.1 ± 5.6
Hoechst 33342	13.3 μM	17 h	G2/M	76.5 ± 6.6
Nocodazole	1 μM	16 h	M	84.2 ± 2.7

Cell population was determined by flow cytometry and indicates the proportion of cells arrested at an expected phase of the cell cycle. All culture media were DMEM supplemented with 10% BCS otherwise indicated.

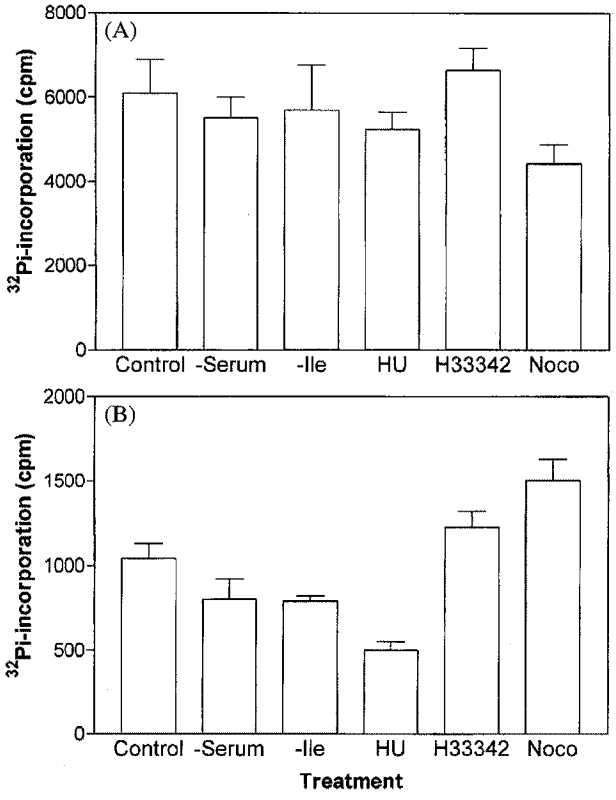

Fig. 2. The activity of CaMK-II in the cells arrested at specific phases of the cell cycle. (A) The total activity in the presence of 1 mM Ca^{2+} (plus Ca^{2+} activity). (B) Activity in the absence of Ca^{2+} and in the presence of 1 mM EGTA (minus Ca^{2+} activity). Control, asynchronously growing cells; -Serum, cultivation of cells in serum deficient DMEM for 2 days (G0); -Ile, in isoleucine deficient EMEM for 2 days (G1); HU, in DMEM containing 1 mM hydroxyurea for 14 h (G1/S); H33342, in DMEM containing 13.3 μM Hoechst 33342 for 17 h (G2/M); Noco, in DMEM containing 1 μM nocodazole for 16 h (M).

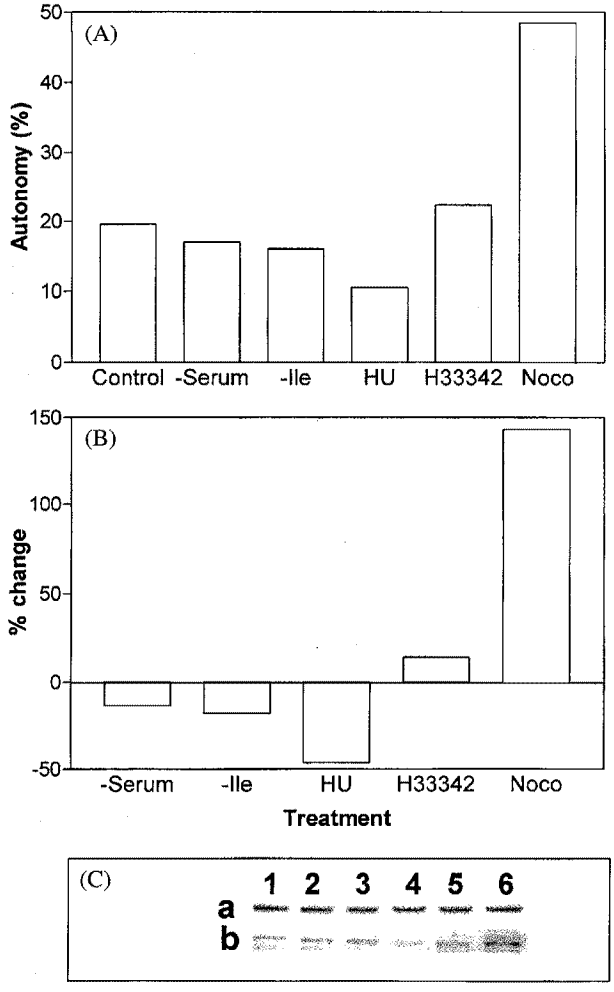

Fig. 3. Change of Ca^{2+}-independent autonomous activity of CaMK-II arrested at designated phases. (A) Effect of treatments on autonomous activity. Control, asynchronously growing cells; -Serum, G0 arrest; -Ile, G1 arrest; HU, G1/S arrest; H33342, G2/M arrest; Noco, M arrest. (B) Percent of change of autonomous activity. (C) a, Western blotting of CaMK-II from cytosolic fraction of cellular proteins. A protein band of 60 kDa was recognized by anti CaMK-II polyclonal antibody. (1, the soluble proteins from untreated control cells; 2, from cells arrested in G0; 3, from cells arrested in G1; 4, from cells arrested at G1/S; 5, from cells arrested at G2/M; 6, from cells arrested in M). b, Autoradiography of *in vivo* phosphorylated cytosolic CaMK-II. The protein band of phosphorylated CaMK-II was identified by an antibody and its phosphorylation level was determined by the exposure of the immuno blot membrane to X-ray film for 8 days at -70°C.

However, there was a large increase of autonomy at M (1.5-fold), and a decrease at G1/S (50%). However, the origin of this kind of change in autonomy was not identified. The changes of autonomy at G1/S and M might come from the change in the CaMK-II protein level, or modification of the kinase. Therefore, the protein and the *in vivo* phosphorylation levels of the kinase were examined (Fig. 3C). The western blot of the cellular protein extract from each arresting point with the polyclonal anti CaMK-II antibody showed no change in the immunostaining density (row *a*). This indicated that the CaMK-II protein level did not change during the cell cycle. Thus, the change in the autonomous activity was not from the change in the protein level. On the other hand, the autoradiography of the *in vivo* phosphorylated cellular protein extract showed a similar changing pattern to that of the relative change of autonomy (row *b*). There was almost no change at G0, G1, and G2/M, while there was a small decrease at G1/S and a large increase at M. It indicated that the autonomous activity of CaMK-II was related to the level of phosphorylation: more phosphorylation on the kinase showed a higher autonomy, and less phosphorylation showed a lower autonomy, or even an inhibition of the kinase activity.

Change of CaMK-II activity during the cell cycle Even though the treatments performed in the present study did not

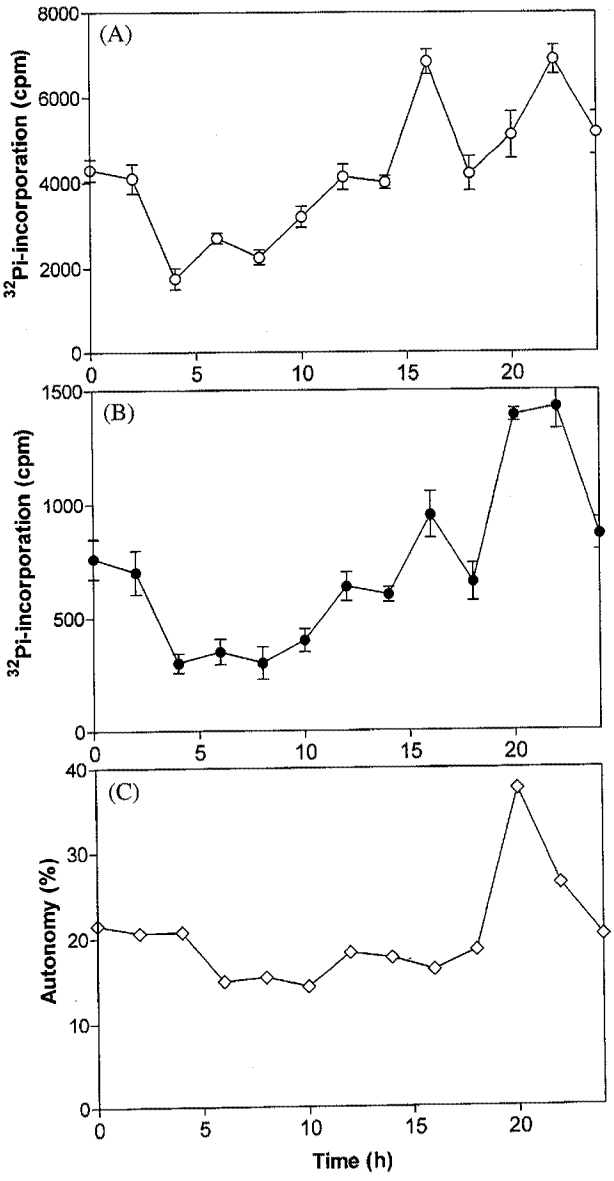

Fig. 4. CaMK-II activity change in NIH 3T3 cells released from G1 arrest. (A) The enzyme activity under 1 mM Ca^{2+} (plus Ca^{2+} activity) during the progression of the cell cycle. (B) The enzyme activity in the absence of Ca^{2+} (minus Ca^{2+} activity). (C) Change of Ca^{2+} -independent autonomous activity during the cell cycle.

exert any direct effect on the CaMK-II activity itself, it was still not a physiological event to arrest cells at specific phases. Therefore, the possibility could not be ruled out that the changes in autonomy at G1/S and M, as shown previously, might result from an unknown side effect of each treatment. In order to clarify this argument, it was necessary to examine whether a similar pattern of activity change occurred during one turn of the cell cycle. For this purpose, cells were released from their arrested phase, and the profile of the CaMK-II activity change was investigated. The cells arrested at G1 were released by a changing medium to a normal one, and

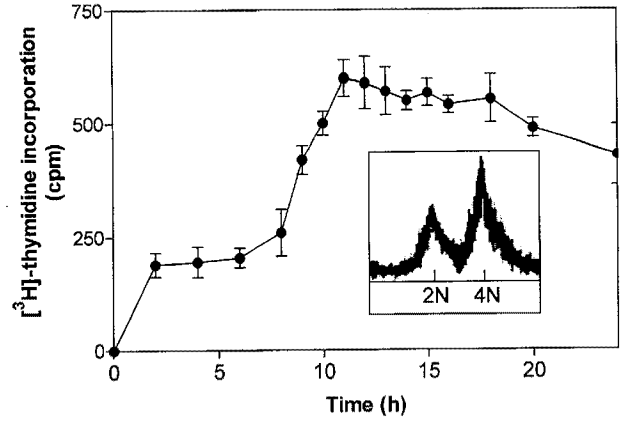

Fig. 5. Incorporation of [^{3}H]-thymidine into cellular DNA in NIH 3T3 cells synchronized from G1 phase. (Inset) Flow cytometry analysis of cells harvested 20 h after release, which showed the highest autonomous activity of CaMK-II.

synchronously proceeded the cell cycle. The cells at the designated time points from release were harvested and assayed for CaMK-II activity (Fig. 4). Generally, the total catalytic activity of CaMK-II in synchronized cells showed a slow increase following a decrease for a short period of time as cells proceeded the cycle. Two distinctive total catalytic activity peaks at 16 h and 22 h time points from the release were observed (Fig. 4A). The pattern of Ca^{2+}-independent activity was similar to that of total activity; decrease in an earlier period and increase in a later period (Fig. 4B). According to the calculation of duration of each phase of the cell cycle (Suh, 2000), the falls of total and Ca^{2+}-independent activity during the earlier period of release happened during the G1/S and S phases. When the change of autonomy during the cell cycle was analyzed, a single autonomy peak was observed at the 20 h point from the release, and it was about 40% higher than the control (Fig. 4C). It also demonstrated decreased autonomy during the early period of the release experiment.

As the cells proceeded the cell cycle, the autonomous activity of CaMK-II reached the highest point once during the cycle. In order to identify the phase in which the peak was shown, DNA labeling and flow cytometry was performed (Fig. 5). The synchronized cells from the G1 arrest started to synthesize DNA at 8 h after release from G1, and reached the maximum at 11 h after release. Therefore, it took at least 8 h for the cells to enter the S phase, which agreed with the result mentioned previously, as well as the previous report (Suh, 2000). Since the peak of autonomy was observed at the 20 h point, the phase of the cell cycle in which the peak existed was examined (Fig. 5, inset). The flow cytometry showed that the peak of autonomy was in the M phase. Although the histogram demonstrated a relatively large population of cells at G1, the majority of cells were still at the M phase. This result confirmed that the highest autonomous activity of CaMK-II was observed only at M.

Discussion

There have been many reports indicating the cell cycle-dependent change of intracellular levels of Ca^{2+} and CaM (for a review, see Santella, 1998). Generally, the transient increases in cytosolic Ca^{2+} were observed at G1/S, G2/M, and in M (Whitaker *et al.*, 1990; Means, 1994; Whitaker 1995; Whitefield, 1995). The physiological activity, or level of cellular CaM, also oscillated according to the phase of the cell cycle. It was especially high at G1/S (Chafouleas, 1984), G2/M (Rasmussen *et al.*, 1987), and M (Davis, 1992). Therefore, it is not difficult to assume that the activity of CaMK-II would also be cell cycle-dependent just as Ca^{2+} and CaM are. However, there has been no direct experimental evidence showing whether, or how the kinase activity changes during the cell cycle. There have only been a few reports showing that a few specific inhibitors of CaMK-II blocked the cell cycle (Minami *et al.*, 1994; Tombes *et al.*, 1995; Johnson *et al.*, 1998; Morris *et al.*, 1998; Patel *et al.*, 1999).

In the arresting experiment, the cells arrested respectively at the G0, G1, and G2/M phases showed a similar autonomy to that of the asynchronized control cells, which meant that there was no meaningful net activation of CaMK-II at those phases. However, the fraction of physiologically activated CaMK-II was increased at M and decreased at G1/S. It meant that the net physiological activation of CaMK-II was carried out in mitosis, whereas a low degree of net inhibition was exerted at G1/S. The present study, demonstrating an inhibition of CaMK-II by the arresting cells at G1/S, coincided with previous reports showing that the treatment with CaMK-II inhibitors specifically arrested cells at G1/S (Johnson *et al.*, 1998; Patel *et al.*, 1999). Therefore, this result indicated that keeping the CaMK-II activity below the basal level at G1/S might be necessary for the cell cycle to proceed. This was supported by activity changing the profile of CaMK-II in synchronized cells. The cells synchronously proceeding the cell cycle also showed an inhibition of the autonomy during interphase.

Even though it is unknown how the kinase activity changes during other phases of the cell cycle, the present study observed the activation of CaMK-II autonomy only in the M phase of the arrested/synchronized somatic cells. The cells arrested in the M phase showed almost a 1.5-fold increase of CaMK-II autonomy. The cells synchronously proceeding the M phase also showed about a two-fold activation. It has been suggested that activation of CaMK-II was necessary for relief from the metaphase II arrest in mammalian eggs (Dupont, 1998). Therefore, it is likely that activation of CaMK-II in mitosis in mammalian somatic cells is also required for cytokinetics during the cell division by phosphorylating many cellular proteins, including cytoskeletons. Pointing out the exact substrate protein of activated CaMK-II in mitosis is not a simple matter, because the enzyme has too many substrates, as can be imagined from its other name -"multifunctional enzyme" (Braun and Schulman, 1995).

The autonomy profile that has been presented resulted from a change in the phosphorylation state of CaMK-II, not from a change in the enzyme level. The enzyme level was unchanged during the cell cycle, while the *in vivo* phosphorylation state of CaMK-II was changed along the cell cycle. This profile correlated well with that of autonomy during the cell cycle. The *in vivo* phosphorylation of CaMK-II seemed to be an autophosphorylation event, according to substantial evidence previously reported (MacNicole *et al.*, 1990; Hanson and Schulman, 1992; Meyer *et al.*, 1992; Mukherji and Soderling, 1994). However, the involvement of other protein kinase(s) cannot be ruled out. This needs to be clarified.

In conclusion, the activity of CaMK-II was cell cycle specifically regulated by phosphorylation, which may have a role in the proceeding of the interphase and completion of mitosis.

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