Temporal Activation of Ca\textsuperscript{2+}-Calmodulin-sensitive Protein Kinase Type II Is Obligate for Adipogenesis\textsuperscript{*}

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\textsuperscript{¶}The abbreviations used are: IBMX, isobutylmethylxanthine; CaM kinase II, Ca\textsuperscript{2+}-calmodulin-sensitive protein kinase, type II; FACS, fatty acyl-CoA synthase; LPL, lipoprotein lipase.

Differentiation of 3T3-L1 embryonic fibroblasts to adipocytes in response to induction by dexamethasone and isobutylmethylxanthine is blocked by inhibitors of Ca\textsuperscript{2+}-calmodulin-sensitive protein kinase type II, but not by inhibitors of protein kinase A or protein kinase C. CaM kinase II displays a biphasic increase in autonomous activity, rising after an initial transient peak from 1 to 15 h, declining at 24 h, followed by a sustained rise from 24 to 48 h, which is 2.5-fold greater than basal values at induction of adipogenesis. Adipogenesis was blocked effectively by CaM kinase II inhibitors, either KN-62 or KN-93, if the inhibitors are introduced at 6 h and maintained until 12 h of induction of adipogenesis. Equally effective, however, is inhibition of CaM kinase II activity at 24–48 h after induction, during the later phase of autonomous CaM kinase activity. Inhibition of cultures with KN-62 or KN-93 either for 0 to 6 h or for 12 to 24 h failed to influence adipogenesis. Two temporally-distinct phases of CaM kinase II activation, either 6 to 12 h or 24 to 48 h, if inhibited with either KN-62 or KN-93, blocked the conversion to adipocytes. Thus, a biphasic activation of CaM kinase II is obligatory for the progression of the embryonic fibroblasts to adipocytes. Inhibition of either phase of CaM kinase activity blocks adipogenesis and expression of several intermediate early gene products.

3T3-L1 embryonic fibroblasts provide a useful model for the study of cellular differentiation. In response to several inducers (e.g. high concentrations of insulin or dexamethasone and isobutylmethylxanthine (dexamethasone+IBMX)), in combination), cultures of 3T3-L1 fibroblasts progress to adipocytes, accumulating lipid and displaying activation of the number of genes. This paper is available on line at http://www-jbc.stanford.edu/jbc/
chopper. Emitted light was channeled into the photomultiplier via an ultraviolet dichroic mirror and interference filter of 510 nm (Corion, Holiston, MA). Measurements of fluorescence intensity were performed at a rate of 20 points/s, and the ratio of absorbance 345/380 nm was computed using the PTI software.

RESULTS AND DISCUSSION

The ability of several well-known selective inhibitors of protein kinase activities (19–22) were investigated for their influence on 3T3-L1 cell growth and differentiation in response to the inducers dexamethasone + IBMX (Fig. 1). Dexamethasone + IBMX-induced differentiation is >90% of 3T3-L1 embryonic fibroblasts within 6–7 days, as evidenced by staining of lipid accumulation by oil-red O (23). At concentrations an order of magnitude greater than their $K_i$ inhibitors for protein kinases were added to the cell culture media for 48 h, as indicated. The response was quantified by scoring of the percentage of cells progressing to differentiation (Fig. 1B). The bisindolylmaleimide inhibitor of protein kinase C (20) failed to alter either cell growth or the ability of the cells to progress to adipocytes in response to dexamethasone + IBMX. More than 90% of the cells displayed

FIG. 1. CaM kinase II inhibitors (KN-62 and KN-93), but neither protein kinase C inhibitor (bisindolylmaleimide) nor protein kinase A inhibitor (KT5720) inhibit dexamethasone + IBMX-induced 3T3-L1 cell differentiation. 3T3-L1 cells grown to confluence on coverslips in six-well plates (day 0) were either untreated (−DM) or induced to differentiation with dexamethasone + IBMX (+DM). Bisindolylmaleimide (1.4 μM), KT5720 (1 μM), KN-62 or KN-93 (10 μM) were added to the differentiation media for the first 48 h, as indicated. Panel A, at day 8, cells were fixed, stained with oil-red O, and photographed using a Zeiss Axiophot system. Panel B, the number of adipocytes relative to that of total cells on the coverslip represents the ratio of differentiation. The data are mean values ± S.E. from at least three separate experiments.

FIG. 2. Blockade of adipogenesis by 3T3-L1 cells treated with inhibitors of CaM kinase II: dose response. 3T3-L1 cells grown to confluence on coverslips in six-well plates (day 0) were either untreated (−DM) or induced to differentiation with dexamethasone + IBMX (+DM). KN-62 or KN-93 were added to the differentiation media at the final concentrations indicated. At day 8, cells were fixed and stained with oil-red O. The number of adipocytes relative to that of total cells on the coverslip represents the ratio of differentiation. The data are mean values ± S.E. from at least three separate experiments.
frank adipogenic conversion in the presence of the protein kinase C inhibitor. Similarly, treating cultures of 3T3-L1 cells with phorbol 13-myristate 12-acetate (10 μM, from −2 to +24 h of induction in response to dexamethasone + IBMX) in order to down-regulate protein kinase C did not alter adipogenesis (data not shown). Short-term activation of protein kinase C with phorbol 13-myristate 12-acetate (250 nM) for 10 min prior to the induction with dexamethasone + IBMX also was without effect on either the rate or the extent of adipogenic conversion (data not shown).

Treatment of cells with KT5720, a selective inhibitor of protein kinase A (19), did not block adipogenesis, more than 75% of the cells progressing to adipocytes within 8 days (Fig. 1). By 10 days nearly 100% of the KT5720-treated cells had undergone adipogenesis (not shown). These data are consistent with earlier data demonstrating the adipogenic conversion was unaffected by agents like forskolin or pertussis toxin treatment that elevate intracellular cAMP levels (5). In addition, suppressing intracellular cAMP levels with 2',5'-dideoxyadenosine (10 μM) was also without effect on the ability of the cultures to differentiate to adipocytes in response to dexamethasone + IBMX (not shown). Thus inhibition of either protein kinase C or protein kinase A activities fails to influence adipogenic conversion in 3T3-L1 cells.

In contrast to the lack of effect of inhibitors for either protein kinase A or C on adipogenic conversion in response to dexamethasone + IBMX, treating the cells with KN-62, an inhibitor of CaM kinase II, effectively blocks adipogenesis (Fig. 1, A and B). KN-62 is highly selective for CaM kinase II, displaying a Ki for CaM kinase II more than 2 orders of magnitude lower than those for protein kinase C, protein kinase A, and

**FIG. 3.** Autonomous CaM kinase II activity increases in response to induction of adipogenesis. Confluent 3T3-L1 cells were induced to differentiation by treatment with dexamethasone + IBMX. Cells were harvested at 0–120 min (panel A) and 2–48 h (panel B) post-induction and homogenized. Autonomous CaM kinase II activity was assayed immediately in cell homogenates, as described under “Experimental Procedures.” The data are mean values ± S.E. from at least three separate experiments.

**FIG. 4.** Measurements of intracellular free Ca^{2+} fail to identify an increase in response to induction of adipogenesis. Intracellular free Ca^{2+} measurements were performed as described under “Experimental Procedures.” Confluent 3T3-L1 cells on 25-mm circular coverslips were loaded with 2 μl of 1 mM fura-2 acetoxymethyl ester for 30 min at 37°C in the dark. After de-esterification in fresh Krebs-Henseleit buffer at 20°C for 15 min, 10 μl of 1 mM ionomycin (A) or 25 μM dexamethasone and 5 mM isobutylmethylxanthine, in combination, were added. Measurements of the ratio of fluorescence absorption (nm) 340/380 was monitored in real time.
myosin light chain kinase (21). KN-93, a second inhibitor of CaM kinase II with higher water solubility and equal selectivity for CaM kinase II as compared to protein kinase C, protein kinase A, and myosin light chain kinase (22), was evaluated also for its ability to affect adipogenesis. At 10 \( \mu M \) concentrations, both KN-62 and KN-93 effectively abolish the adipogenic response of 3T3-L1 cells to induction by dexamethasone+IBMX. The dose-response relationship between adipogenic conversion of 3T3-L1 cultures and the inhibitors of CaM kinase II was explored (Fig. 2). KN-62 and KN-93 both display the ability to block fully the adipogenic response, the more water-soluble KN-93 compound being less potent in the suppression of adipogenesis.

The ability of CaM kinase II inhibitors to block the induction of adipogenesis in 3T3-L1 cells suggested some critical role of CaM kinase II activity in the differentiation process. Activation of CaM kinase II activity was investigated in the first 2 h of induction by dexamethasone+IBMX (Fig. 3A). The autonomous activity increased by 120 min, more than doubling. Analysis of the first minute of induction of adipogenesis by dexamethasone+IBMX revealed a sharp, transient peak of autonomous CaM kinase II activity (not shown). By 10 s of induction, CaM kinase II activity increased 5-fold (3.5% as compared to 0.75% at \( t = 0 \)), the activity declining to control levels with 30–60 s (not shown). Measured from 2 h to 2 days post-induction with dexamethasone+IBMX, autonomous CaM kinase II activity increased 4–8 fold (Fig. 3B). Autonomous kinase activity increased from 0.75 to 4.0% at 2 to 18 h (first phase) and then displayed a sharp decline at 24 h. Following the decline, autonomous CaM kinase II activity rebounded to 2.5% (second phase), where it remained up until 48 h post-induction. Thus, after a brief transient activation, CaM kinase II activity increases from 1 to 18 h of adipogenesis. This first phase declines by 24 h, followed by a second phase of autonomous CaM kinase II activity which remains 4–5-fold greater than that in control cells treated only with vehicle. Treating confluent cultures of 3T3-L1 cells for either 2 or 12 h with ionophore A23187 (1 \( \mu M \)) or ionomycin (3 \( \mu M \)) in either Dulbecco’s modified Eagle’s medium or Dulbecco’s modified Eagle’s medium supplemented with Ca\(^{2+}\) to 25 \( \mu M \) fails to induce adipogenic conversion of the embryonic fibroblasts (data not shown). Elevating the concentration of either ionophore by 10-fold results in cell death (data not shown). Even in the presence of inducers, CaM kinase II inhibitors do not alter the rate of adipogenesis (data not shown). These data suggest that activation of CaM kinase II activity itself is not sufficient a stimulus to induce the adipogenic progression.

The transient activation of CaM kinase in 3T3-L1 clones induced by addition of dexamethasone+IBMX was examined from a second perspective, measurements of intracellular free Ca\(^{2+}\). In cells pre-loaded with the calcium-sensitive probe fura-2, measurements of intracellular free Ca\(^{2+}\) were possible (Fig. 4A). As a control, the ability of the ionophore ionomycin to stimulate an increase in intracellular free Ca\(^{2+}\) was demonstrated. Ionomycin produced a rapid and robust increase in intracellular free Ca\(^{2+}\). Similar measurements performed in cells stimulated with dexamethasone+IBMX during the first 10 min of induction reveal no significant changes in intracellular free Ca\(^{2+}\) (Fig. 4B). Measurements at later time points stained with oil-red O, and photographed using a Zeiss Axiophot system. Panel B, the number of adipocytes relative to that of total cells on the coverslip represents the ratio of differentiation. The “+” denotes cultures progressing to >85% differentiation. The “-” denotes cultures that failed to progress to adipocytes (<5% differentiation). The data are mean values ± S.E. from at least three separate experiments.
likewise failed to identify a significant change in the concentration of intracellular free Ca$^{2+}$ (data not shown). Thus the temporal nature of a Ca$^{2+}$ transient, if stimulated by inducers of adipogenesis, may have precluded detection by this approach.

The ability of inhibitors of CaM kinase II to block adipogenesis and the time course of autonomous CaM kinase II activation during dexamethasone+IBMX-induced adipogenesis prompted us to investigate the time constraints on the exposure to the inhibitor with respect to its ability to block differentiation. As shown by oil-red O staining (Fig. 5A) and quantification of the extent of adipogenic conversion (Fig. 5B), blockade of adipogenesis by CaM kinase II inhibitor was not effective, if restricted to the first 6 h of the induction protocol. Whereas treatment with a CaM kinase II inhibitor from 0 to 12 h blocks adipogenesis, treatments from 0 to 6 h, 6 to 24 h, or 12 to 24 h fail to block adipogenesis. These data suggest that one period of CaM kinase II activation from 6 to 12 h of induction (Fig. 3A) is critical for adipogenic conversion to occur, a hypothesis supported by the ability of a 6–12 h treatment of KN-93 to be fully effective in blocking adipogenesis.

Interestingly, inhibition of CaM kinase II activity at 6–12 h was not the only window of opportunity for blockade of adipogenesis. Treating cells with KN-93 (or KN-62, not shown) from 0, 6, 12, or 24 h to the end of the 48-h induction period is equally effective in blocking adipogenesis as was the brief 6-h exposure to inhibitor from 6 to 12 h. The identification of two distinct phases for blockade of adipogenesis by CaM kinase II inhibitors may well reflect the two distinct phases of CaM kinase activation observed as the cell progress through adipogenesis (Fig. 3).

We explored the influence of CaM kinase II inhibition on expression of marker genes for adipogenic conversion (Fig. 6). Cells were induced to adipogenesis with dexamethasone+IBMX and KN-93 was added at either the 6–12 h or 24–48 h periods. aP2, fatty acyl-CoA synthetase (FACS), and lipoprotein lipase (LPL) are well known markers of adipogenesis, LPL being an early gene product, aP2 and FACS being intermediate early gene products (see Ref. 24). The mRNA levels of glyceraldehyde-phosphate dehydrogenase, which do not change during differentiation (24), were probed also. Inhibition of CaM kinase II activity from 24 to 48 h essentially blocked the expression of aP2, FACS, and LPL mRNAs. Treating the cells with inhibitor for a period of 6–12 h following induction of adipogenesis severely attenuated the activation of the marker genes. These data demonstrate an obligate role of CaM kinase II activation in the early program of adipogenic conversion, confirming the phenotypic displays of these cells stained for lipid accumulation with oil-red O at day 8 following treatment with CaM kinase inhibitors (Fig. 5A).

Activation of CaM kinase II is critical to several aspects of cell physiology and biology, particularly regulation of the cell cycle (25, 26), control of nuclear envelope breakdown (27), and promotion of neurite outgrowth and growth cone mobility (28). In the current work we report the activation of CaM kinase II during adipogenesis. Activation of CaM kinase II has been shown to lead to phosphorylation of C/EBPβ, a member of the βZip family of transcription factors (29), providing one possible paradigm linking CaM kinase activation to gene expression. In addition, CaM kinase II has been shown to phosphorylate a non-consensus site on ATF-1 and CREB, which inhibits activation of these two transcription factors (30), providing another possible role of CaM kinase II activation and gene expression. More importantly, blockade of the CaM kinase II activity, but not of either protein kinase A or protein kinase C, prevents the progression of 3T3-L1 embryonic fibroblasts to adipocytes. The activation of CaM kinase II is dynamic and has precise temporal boundaries for interruption by selective inhibitors like KN-62 or KN-93. Identification of substrates for CaM kinase II critical to adipogenic progression remains an important goal. In summary, activation of CaM kinase II is shown to be an obligate step toward induction and progression of adipogenesis.

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