Ca^{2+} Redistribution from Bound to Free Form Is Required for Tumor Necrosis Factor Actions in 30A5 Preadipocytes

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Tumor necrosis factor/cachectin (TNF) inhibits differentiation of 30A5 preadipocytes into adipocytes. In this process, TNF inhibits the expression of the gene for acetyl-coenzyme-A carboxylase, the rate-limiting enzyme for biogenesis of long chain fatty acids. One of the early reactions caused by TNF is the Ca^{2+} redistribution of Ca^{2+} from the bound form to the free form. This Ca^{2+} redistribution results in a transient Ca^{2+} efflux. High concentrations of Mg^{2+} inhibit Ca^{2+} redistribution and efflux. This inhibition reverses the repression of acetyl-coenzyme-A carboxylase and reverses the TNF inhibition of the differentiation of 30A5 preadipocytes into adipocytes. This indicates that Ca^{2+} redistribution between the bound and the free form is an obligatory event in the sequence of actions caused by TNF in 30A5 cells. (Molecular Endocrinology 4: 1671–1678, 1990)

INTRODUCTION

Tumor necrosis factor/cachectin (TNF), a protein with a mol wt of 17,000, is secreted by macrophages in response to noxious stimuli, such as bacterial toxins, viruses, and cancer cells (1–3). TNF exhibits cytolytic and cytostatic effects on certain tumor cells as well as a large number of seemingly unrelated physiological effects in normal cells. For example, TNF induces macrophage-induced angiogenesis (4), bone resorption by osteoclasts (5), and secretion of collagenase in synovial cells (6). In adipocytes, TNF inhibits the synthesis of lipogenic enzymes (7, 8) by decreasing the rate of transcription of specific genes (9–11). In 30A5 preadipocytes, TNF completely blocks the differentiation of cells into adipocytes (8). During this inhibitory process, the synthesis of lipogenic enzymes, including acetyl-coenzyme-A carboxylase (ACC), is blocked at the transcriptional level (8, 9). In addition, TNF stimulates inorganic phosphate uptake and ATP synthesis in 30A5 cells (12).

Just how TNF exerts such diverse physiological and biochemical effects in different types of cells is not known. However, all of the affected cells contain specific high affinity receptors for TNF in their plasma membranes (13). The biochemical reactions triggered by the interaction between TNF and its receptors are essentially unknown. The affinity of TNF receptors and the cytolytic activity of TNF are down-regulated by activators of protein kinase-C, such as phorbol 12-myristate 13-acetate, suggesting that protein kinase-C may be involved somewhere in the chain of biochemical reactions of TNF action (13).

In the present studies we attempted to answer some questions about the early biochemical reactions that make TNF action possible. Our experimental results indicate that TNF causes the redistribution of Ca^{2+} from the bound to the free form, and that this Ca^{2+} metabolism is required for TNF action in the repression of ACC and in the inhibition of the differentiation of 30A5 preadipocytes into adipocytes.

RESULTS

Effects of Ca^{2+} Ionophore and TNF on ACC Synthesis and 30A5 Differentiation

The 30A5 preadipocytes (Fig. 1A) differentiate into adipocytes in the presence of insulin and dexamethasone (Fig. 1B). Differentiated adipocytes are loaded with lipid droplets (Fig. 1B). This differentiation is completely inhibited in the presence of TNF or Ca^{2+} ionophore A23187 (Fig. 1, C and D).

During differentiation the amount of ACC increases about 3- to 4-fold (8). This increase in the amount of enzyme is accompanied by an increase in the amount of carboxylase mRNA as a result of an increase in the

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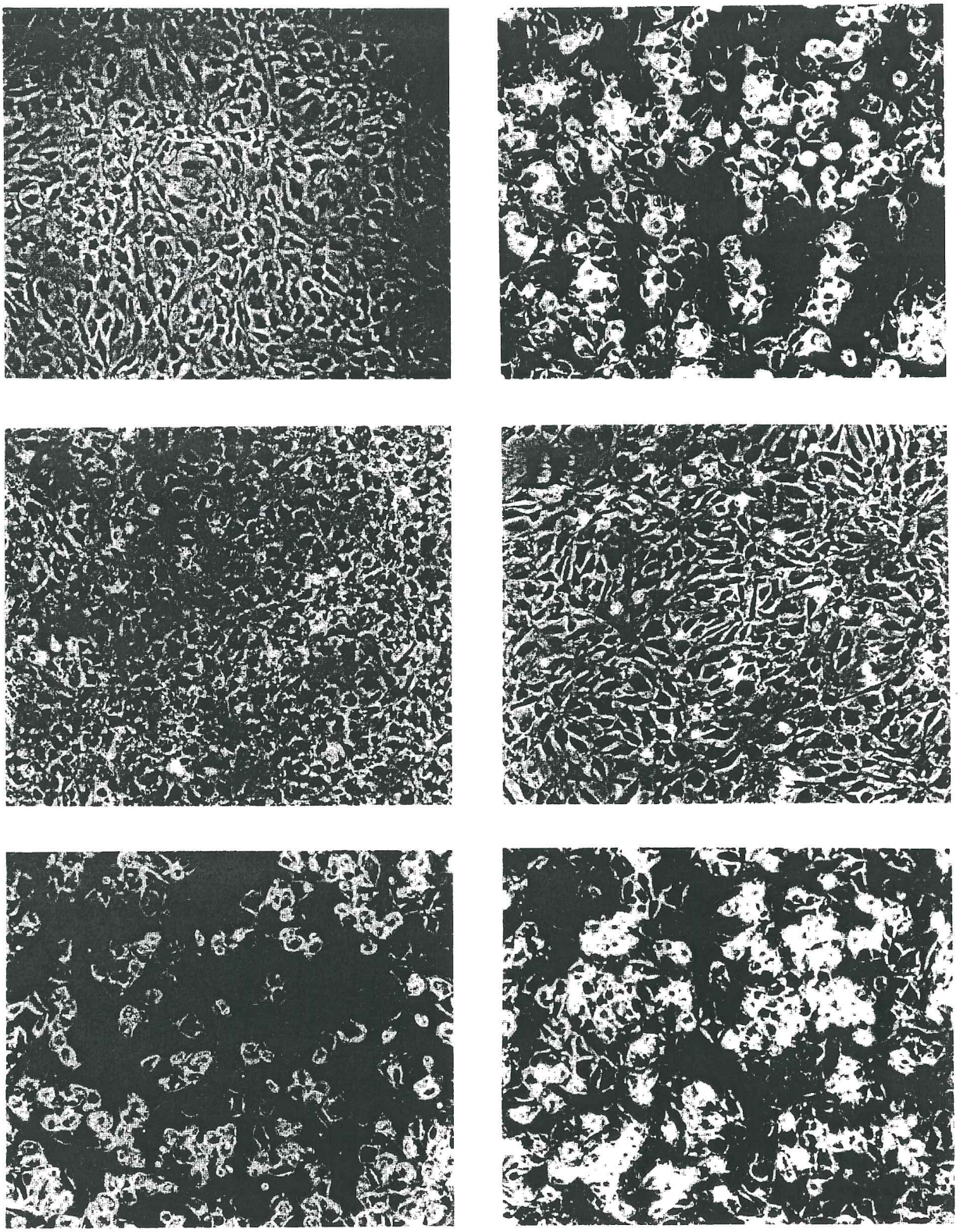

Fig. 1. Effect of Ca^{2+} Ionophore and TNF on Conversion of Preadipocytes to Adipocytes

Each panel represents 30A5 cells at ×100 magnification of the cell monolayer under phase contrast microscopy. A, Confluent cells; B, 9 days after confluence in the presence of hormone(s); C, 9 days after confluence, hormone(s) and TNF present throughout the 9 days; D, 9 days after confluence, hormone(s) and Ca^{2+} ionophore present throughout the 9 days; E, 9 days after confluence, hormones and 30 mM Mg^{2+} present throughout the 9 days; F, the same as E, except 200 U TNF were present in addition to the agents present in E.

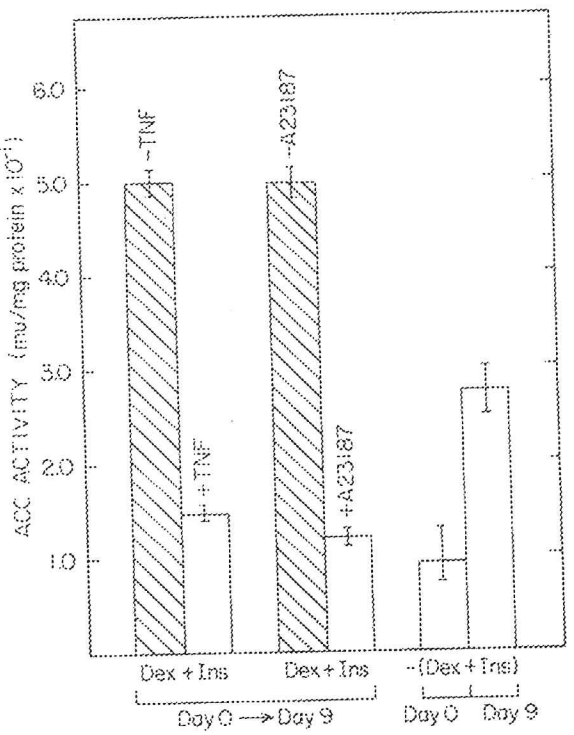

Fig. 2. Effect of Ca^{2+} ionophore and TNF on ACC Synthesis

Confluent cells on 60-mm dishes were incubated in Eagle's Basal Medium supplemented with 10% fetal bovine serum and appropriate hormone(s) in the presence and absence of TNF (400 U/ml) or 4-bromo-A23187 (1 mM) for 9 days at 37 C. On the ninth day, cells were washed twice with cold PBS, pH 7.0, and extracted with buffer containing digitonin. ACC activity was measured as previously described (see *Materials and Methods*). The ACC activity of day 0 cells and day 9 cells, which were cultured in the absence of hormones, served as controls. Dex, Dexamethasone; Ins, insulin. Each value represents the average value obtained from three plates.

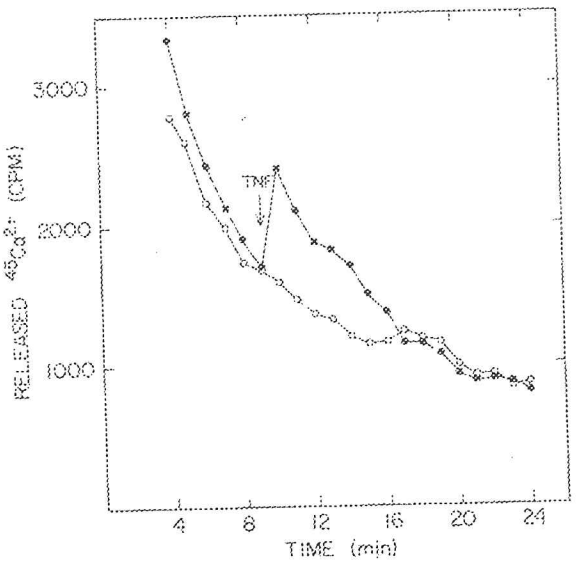

Fig. 3. Effect of TNF on $^{45}Ca^{2+}$ Efflux from 30A5 Preadipocytes in Serum-Free Eagle's Basal Medium

Confluent cells on 60-mm dishes were washed five times with prewarmed Hanks' Balanced Salt Solution, pH 7.0. Cells were equilibrated with $^{45}Ca^{2+}$ (15 μCi/ml) in 1.5 ml serum-free Eagle's Basal Medium for 3 h at 37 C. After incubation, cells were quickly washed twice with prewarmed serum-free Eagle's Basal Medium and then incubated in 1.3 ml serum-free Eagle's Basal Medium or Ca^{2+}-free serum-free Eagle's Basal Medium containing 2 mM EGTA, which was changed every minute for 24 min. The $^{45}Ca^{2+}$ released into the medium was assayed by counting in a Packard Scintillation Counter. TNF (400 U/ml) was added at 9 min of the efflux period.

rate of transcription (8). TNF decreases the rate of transcription and, thus, represses gene expression (16).

Inhibition of 30A5 cell differentiation by Ca^{2+} ionophore A23187 is also reflected in the repression of the expression of the ACC gene, as in the case with TNF (Fig. 2). In this experiment fully differentiated 30A5 cells in the presence of insulin and dexamethasone show about 3-fold higher ACC activity than that in 30A5 preadipocytes (Fig. 2). Since 30A5 preadipocytes undergo partial differentiation in the absence of hormones, small increases in ACC in the absence of hormones were also observed. TNF almost completely abolishes the increased synthesis of the enzyme (Fig. 2, lanes ± TNF), as did Ca^{2+} ionophore A23187 (Fig. 2, lanes ± A23187). This effect of Ca^{2+} ionophore on the induction of ACC during the differentiation of preadipocytes led us to examine the possible involvement of Ca^{2+} in TNF action in 30A5 preadipocytes.

Effect of TNF on $^{45}Ca^{2+}$ Efflux and Influx

We have examined the effects of TNF on both Ca^{2+} efflux and influx kinetics using $^{45}Ca^{2+}$. In examining Ca^{2+} efflux, 30A5 preadipocytes were preloaded with $^{45}Ca^{2+}$, and the release of free $^{45}Ca^{2+}$ into the medium was examined (Fig. 3). Following the establishment of steady state kinetics for $^{45}Ca^{2+}$ release, TNF or buffer solution was added, and $^{45}Ca^{2+}$ release was continuously monitored. As shown in Fig. 3, the addition of TNF produced an immediate $^{45}Ca^{2+}$ efflux pulse, which was followed by a reduced, but still stimulated, rate of efflux lasting several minutes. The same kinetics of $^{45}Ca^{2+}$ efflux were observed regardless of whether Ca^{2+} was present in the medium, suggesting that the stimulated $^{45}Ca^{2+}$ efflux occurs independently of Ca^{2+} influx (data not shown). These experiments suggest that one of the early effects of TNF is related to Ca^{2+} efflux, because TNF had no effect on the kinetics of $^{45}Ca^{2+}$ uptake (data not shown).

Effect of TNF on Ca^{2+} Metabolism

TNF-mediated Ca^{2+} efflux in the absence of extracellular Ca^{2+} suggested that the efflux might be due to the increased free Ca^{2+} concentration in the cells as a result of the redistribution of the bound Ca^{2+} to free Ca^{2+}. To demonstrate that the Ca^{2+} efflux occurring after TNF addition is the result of Ca^{2+} redistribution between bound Ca^{2+} and free Ca^{2+} in the cell, the intracellular free Ca^{2+} was measured using a flow cytometer (Fig.

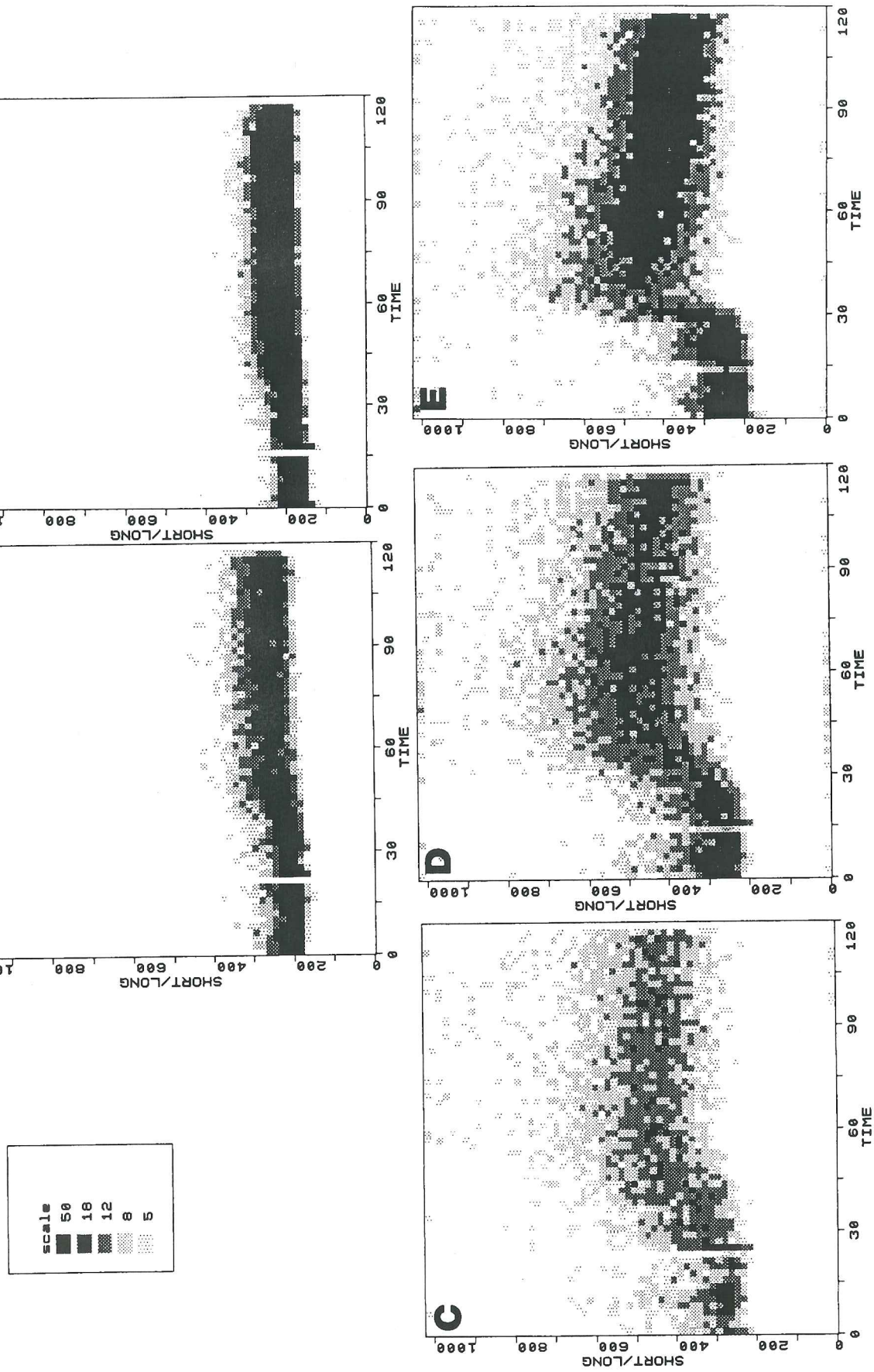

Fig. 4. Effect of TNF in the Generation of Free Ca^{2+}

Cells (5×10^5 cells/ml) were loaded with 1 mM Indo-1 for 5 min at 37 C. Loading kinetics were monitored to ensure that the cells were fully loaded. One hundred and fifty milliliters of cell suspension were placed into the sampler and introduced into the cytometer. Immediately after the first events were recorded to determine the ratio, 15 ml of the appropriate concentration of TNF were added via the injection system into the sample and mixed. Immediately, sample was continued into the cytometer flow cell, and continuous recordings were made for up to 120 sec/sample. The system was flushed with calcium-free buffer solutions between each run. A, Contained no TNF; B, 50 U TNF/ml; C, 100 U TNF/ml; D, 200 U TNF/ml; E, 400 U TNF/ml. Scale notes the number of 30A5 cells with a particular ratio of wavelength at a given time.

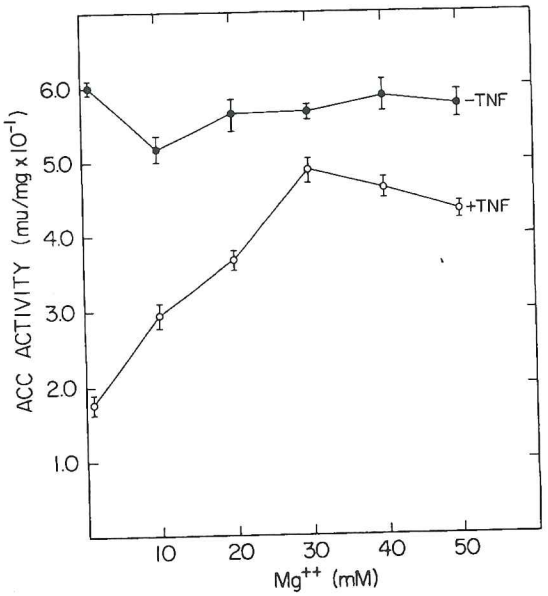

Fig. 5. Reversal of TNF Repression of ACC by Mg^{2+}

The 30A5 cells were subjected to the differentiation scheme described in *Materials and Methods* in the presence (200 U/ml) or absence of TNF. Different concentrations of magnesium acetate were included in the culture medium, as indicated in the figure. ACC activity was measured as described in *Materials and Methods*.

4). It is clear that a sudden surge of free Ca^{2+} occurs in the absence of external Ca^{2+} within 30 sec after the addition of TNF, and this is followed by a slow decrease in the free Ca^{2+}. The maximum free Ca^{2+} surge was observed at 200 U TNF. This experiment indicates that TNF causes Ca^{2+} redistribution from the intracellular bound form to the free form in a transient fashion in accordance with the $^{45}Ca^{2+}$ efflux observed.

Effects of Mg^{2+} on TNF Action on ACC Expression and 30A5 Preadipocyte Differentiation

Several intracellular components are involved in the regulation of Ca^{2+} in the cell (17); the endoplasmic reticulum-bound form of Ca^{2+}, mitochondrial uptake of Ca^{2+} at high cytosolic concentrations, and other Ca^{2+}-binding proteins, such as calcium-binding proteins. Ca^{2+} metabolism in some of these components is affected by Mg^{2+}. For example, Mg^{2+} inhibits Ca^{2+} uptake by mitochondria (18), and Mg^{2+} affects the formation of inositol 1,4,5-triphosphate, which is the intracellular agent involved in the release of the endoplasmic reticulum-bound form of Ca^{2+} by affecting the conformation of a specific G-protein (19).

To establish a causative relationship between TNF action and Ca^{2+} metabolism on ACC expression and 30A5 cell differentiation, the effects of Mg^{2+} on TNF action and Ca^{2+} metabolism were examined.

When 30A5 cells were incubated in the presence of different concentrations of Mg^{2+}, Mg^{2+} was very effective in reversing TNF action on ACC repression (Fig. 5). At 30 mM, Mg^{2+} almost completely reversed TNF repression. At these concentrations of Mg^{2+}, cells contained a relatively constant amount of ACC (Fig. 5). This striking effect of Mg^{2+} on TNF action is also observed in the differentiation of 30A5 cells. In the presence of 30 mM Mg^{2+}, the cells underwent normal differentiation in spite of the presence of TNF (Fig. 1F). This concentration of Mg^{2+} had no effect on the differentiation (Fig. 1E).

Under the same experimental conditions, Ca^{2+} redistribution caused by TNF (Fig. 6C) is also inhibited by Mg^{2+} (Fig. 6D). This establishes that under conditions that inhibit the generation of free Ca^{2+} TNF cannot repress ACC or inhibit cell differentiation.

Effect of Pertussis Toxin on $^{45}Ca^{2+}$ Efflux Mediated by TNF

If the $^{45}Ca^{2+}$ efflux pulse occurs in 30A5 cells as a result of TNF activation of G-protein, which leads to the formation of inositol 1,4,5-triphosphate, ADP-ribosylation of G-protein by the use of pertussis toxin should affect $^{45}Ca^{2+}$ efflux. The results of a test of this hypothesis are shown in Fig. 7. TNF-stimulated $^{45}Ca^{2+}$ efflux was examined in 30A5 cells that were incubated with different concentrations of pertussis toxin for 16 h. Pretreatment of the cells with the toxin (10 ng/ml) almost completely abolished the effect of TNF, indicating that the TNF-mediated $^{45}Ca^{2+}$ efflux pulse may be the result of the activation of G-protein (Fig. 7). However, further detailed experimental analysis is required to establish the relationship between phosphoinositide metabolism and TNF action.

DISCUSSION

TNF exerts multiple physiological effects in a variety of cells. Although the mechanism underlying TNF action is not well understood, extensive biochemical studies reveal that, at least in the case of the lipogenic enzyme system, the effect of TNF is exerted at the level of specific genes (9–11). On the other hand, how the existence of interaction between TNF and the plasma membrane receptor is translated into the subsequent biochemical reactions is unknown. In the present studies we have established that TNF binding to the receptor causes a transient Ca^{2+} efflux peak.

Intracellular metabolism of Ca^{2+} is affected by Mg^{2+} at several points. Mg^{2+} inhibits Ca^{2+} uptake by mitochondria, and Mg^{2+} also affects inositol 1,4,5-triphosphate formation by changing the effective conformation of G-proteins that might be involved in the action of TNF. TNF activation of G-proteins in GL-60 and mouse L-909 cells has been reported (20). In our preliminary experiments, we have also observed TNF activation of G-protein in 30A5 cells. However, how TNF activation of G-proteins is related to the observed Ca^{2+} efflux requires further investigation. These experiments are being carried out. Since Mg^{2+} inhibits mitochondrial

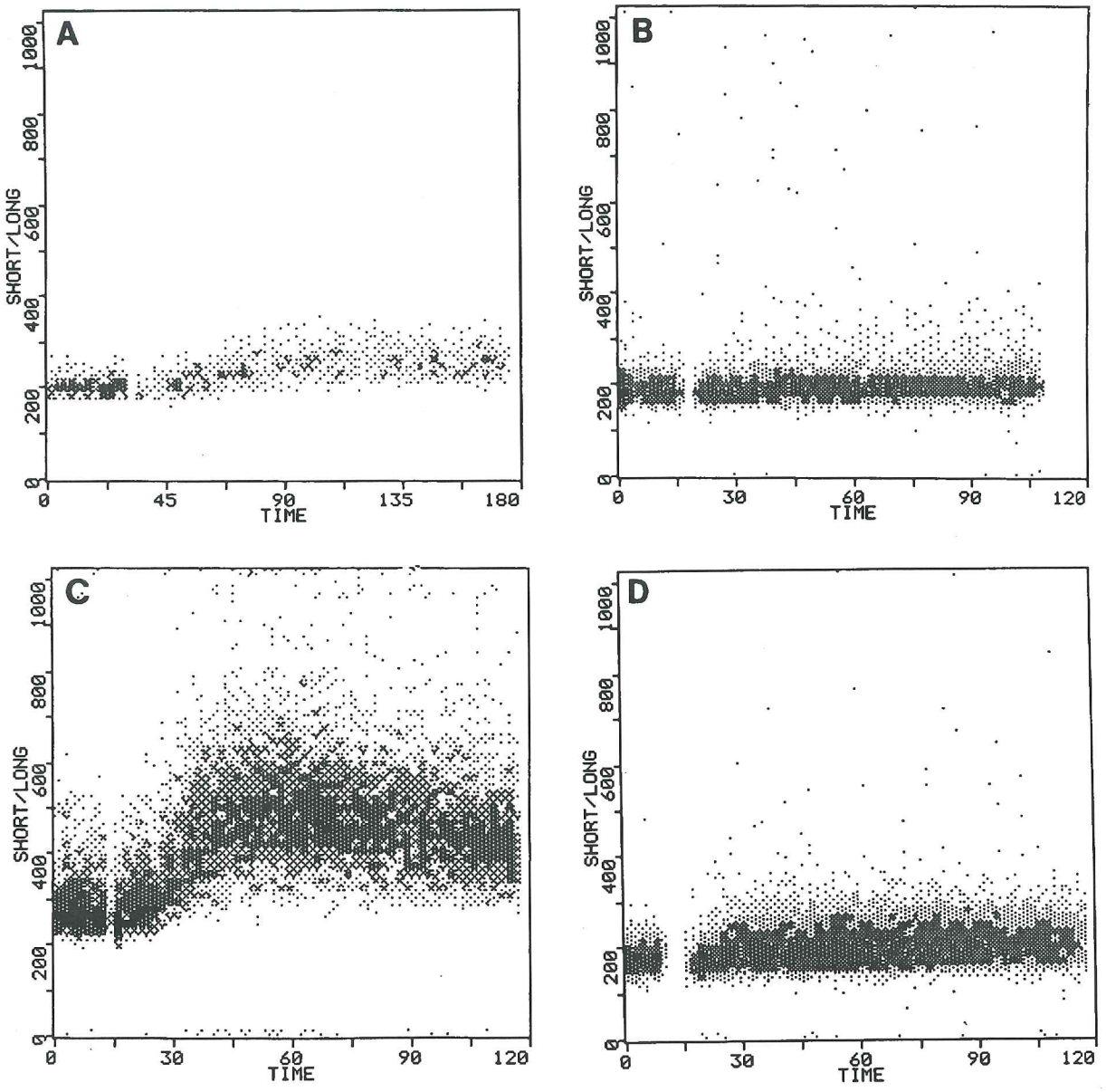

Fig. 6. Effect of Mg^{2+} on Free Ca^{2+} Generation by TNF

The experimental conditions were the same as described in Fig. 4, except that cells were preincubated as follows: A and C, no additions; B and D, 30 mM Mg^{2+} for 5 min before the experiment at 37 C.

uptake of Ca^{2+}, the Mg^{2+} reversal of several of the effects of TNF may not be related to Mg^{2+} action at this uptake step.

Whether all actions of TNF in different systems can be reversed should be investigated. The information obtained in such investigations would be valuable in formulating the axis of biochemical events in TNF actions.

MATERIALS AND METHODS

Materials

Commercial products were obtained from the following sources: Eagle's Basal Medium (M. A. Products, Walkersville, MD); fetal bovine serum (Gibco, Grand Island, NY); dexamethasone and insulin (Collaborative Research, Waltham, MA); [^{14}C]sodium bicarbonate (55.5 mCi/mmol; Research Product International, Mount Prospect, IL); [$^{45}Ca^{2+}$] $CaCl_2$ (25 Ci/g; ICN, Irvine, CA); AMP-PNP (Boehringer Biochemicals, St. Louis, MO); pertussis toxin (List Biological Laboratories, Inc, Campbell, CA); creatine phosphokinase (Sigma Chemical Co., St. Louis, MO); and 4-bromo-A23187 (Sigma Chemical Co.). Indo-1 was purchased from Molecular Probes (Eugene, OR). TNFα was a generous gift from Dr. Tatsro Nishihara of the Suntory Institute (Osaka, Japan). The specific activity of TNF was 2.02×10^6 U/mg protein based on the mouse L929 cytotocicity assay (20). Endotocin contamination was less than 0.49 ng/mg protein according to the manufacturer's information. The 30A5 cells, derived from 10T 1/2 mouse fibroblasts by 5-azacytidine treatment, were obtained

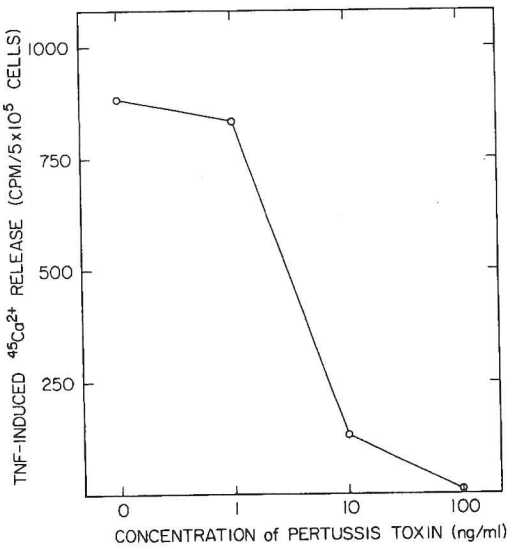

Fig. 7. Effect of Pertussis Toxin on $^{45}Ca^{2+}$ Efflux Mediated by TNF

Confluent cells on 60-mm plates were pretreated with various concentrations of activated pertussis toxin for 16 h at 37 C in Eagle's Basal Medium containing 10% fetal bovine serum. After washing, cells were equilibrated with $^{45}Ca^{2+}$ (15 mCi/ml) for 3 h at 37 C in Eagle's Basal Medium. $^{45}Ca^{2+}$ efflux was measured as previously described (14). TNF was added at 9 min. TNF-induced $^{45}Ca^{2+}$ release from pertussis toxin-pretreated cells was calculated from the difference between $^{45}Ca^{2+}$ released in the presence and absence of 200 U/ml TNF at 10 min of efflux.

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Cell Culture

Culture plates (60 mm) were initially seeded with approximately 3.5×10^4 cells. Confluent growth was reached 5 days later. Cells were grown in Eagle's Basal Medium supplemented with 10% heat-inactivated fetal bovine serum. At confluence, fresh medium containing 10^{-6} M dexamethasone and 5 mg/ml insulin was added. After 3 days the medium was changed to include only insulin, and the cells were maintained in this medium for the remainder of their differentiation. The culture medium was changed every 3 days with fresh medium supplemented with insulin.

ACC Assays

ACC was extracted from cells as previously described (8), except for the following modifications. Cells from a 60-mm culture dish were washed twice with cold PBS, pH 7.0. Two hundred milliliters of a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaF, 0.25 M sucrose, 0.4 mg/ml digitonin, and 1.5 mM phenylmethylsulfonylfluoride were added, and the samples were shaken for 2 min in an ice bath. The supernatant was adjusted to 80 mM phosphate, pH 7.0. The amount of protein in the supernatant was measured by the bicinchoninic acid method (14). BSA was used as the standard.

ACC activity was assayed in a final volume of 100 ml containing 50 mM sodium phosphate (pH 7.0), 10 mM sodium citrate, 8 mM magnesium acetate, 1 mM dithiothreitol, 10 mg/ml BSA, 2.25 mM ATP, 0.5 mM acetyl-coenzyme-A, and 5 mM [^{14}C]sodium bicarbonate (55.5 mCi/mm), and 5 mg protein at 37 C for 10 min. A reaction mixture without ATP, acetyl-coenzyme-A, and sodium bicarbonate was preincubated for 30 min at 37 C, and the reaction was started by adding these reagents. To stop the reaction, 50 ml 6.0 N HCl were added, and samples were centrifuged for 8 sec in a Microfuge. ONe hundred milliliters of the supernatant were used to determine the amount of $^{14}CO_2$ fixed in malonyl coenzyme-A. One unit of ACC activity was defined as the micromoles of malonyl coenzyme-A formed per min at 37 C.

$^{45}Ca^{2+}$ Efflux

The measurement of $^{45}Ca^{2+}$ efflux was performed as described by Miasiro *et al.* (15). Confluent cells on 60-mm dishes were washed five times with prewarmed Hanks' Balanced Salt Solution, pH 7.0. Cells were equilibrated with $^{45}Ca^{2+}$ (15 mCi/ml) in 1.5 ml serum-free Eagle's Basal Medium for 3 h at 37 C. After incubation, cells were quickly washed twice with prewarmed serum-free Eagle's Basal Medium and then incubated in 1.3 ml serum-free Eagle's Basal Medium or Ca^{2+}-free, serum-free Eagle's Basal Medium containing 2 mM EGTA, which was changed every minute for 24 min. The $^{45}Ca^{2+}$ released into the medium was assayed by counting in a Packard Scintillation Counter (Downers Grove, IL). TNF (200 U/ml) was added 9 min into the efflux period. The radioactivity of $^{45}Ca^{2+}$ remaining in the cells was counted by dissolving the cells in 500 ml 0.1% sodium dodecyl sulfate.

$^{45}Ca^{2+}$ Influx

To measure $^{45}Ca^{2+}$ influx, cells were washed five times with prewarmed Hanks' Balanced Salt Solution, pH 7.0, and incubated for 15 min at 37 C in serum-free Eagle's Basal Medium. After preincubation, cells were incubated in 1.5 ml serum-free Eagle's Basal Medium containing 5 mCi/ml $^{45}Ca^{2+}$ in the presence and absence of TNF for 3 min at 37 C. The cells were then washed five times with ice-cold Ca^{2+}-free Hanks' Balanced Salt Solution containing 2 mM EGTA at 4 C. Cells were dissolved in 500 ml 0.1% sodium dodecyl sulfate, and radioactivity was measured.

Flow Cytometry

All cytometry was performed using an Elite cytometer (Coulter Cytometry, Hialeah, FL). The instrument was equipped with three laseres. These were a 15-milliwatt air-cooled argon laser (model 2201, Cyonics, San Jose, CA) operating at 488 nm, a 10-milliwatt air-cooled helium neon laser (model 106–1, Uniphase, Mantica, CA) operating at 632.5 nm, and a 5-watt water-cooled argon laser (Coherent Innova 90–5, Mountainview, CA) tunable from 528.7 to 351.2 nm. For the calcium studies the 5-watt water-cooled argon laser was used for all studies. Separate cross-cylindrical beam-shaping optics were installed for this laser, providing an elliptical beam profile for cell illumination of approximately 15×60 μm, using a 40 mm/80 mm confocal beam shaping optic. The water-cooled laser was operated with a continuous 160-milliwatt light output at a wavelength of 351–363 nm. The BioSense flow cell was used with a 250 mm^2 quartz channel with a variable restrictor to control velocity.

A special kinetic sampler designed for the Elite cytometer was used for these experiments. This sampler allowed for the rapid introduction of very small volumes of activating agent into the cell suspension. Because of the very short length of the sample introduction line, it is possible to routinely sample cells within 1 sec after introduction of the activating agent. The kinetic sampler maintained a constant temperature of 37 C during analysis.

Forward angle light scatter was measured using the standard split photodiode detector. Right angle light scatter and fluorescence were measured using four photomultiplier tubes. Dichroic splitting and bandpass filters (Omega Optical, Brattle-

boro, VT) were installed as follows. Ninety degree light scatter was diverted using a 380-nm dichroic. Short calcium fluorescence was diverted using a 400-nm dichroic long pass filter, followed by a 395-nm band pass filter. Long calcium fluorescence was collected using a 515-nm long pass filter, followed by a 525-nm band pass filter. Additionally, the ratio signal and time were collected as additional parameters on the Elite. All data were collected in the listmode to allow subsequent reanalysis.

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