Tumor necrosis factor α (TNF-α) is a potent inhibitor of proliferation in several cell types, including thyroid FRTL-5 cells. As intracellular free calcium ([Ca²⁺]ᵢ) is a major signal in activating proliferation, we investigated the effect of TNF-α on calcium fluxes in FRTL-5 cells. TNF-α per se did not modulate resting [Ca²⁺]ᵢ. However, preincubation (10 min) of the cells with 1–100 ng/ml TNF-α decreased the thapsigargin (Tg)-evoked store-operated calcium entry in a concentration-dependent manner. TNF-α did not inhibit the mobilization of sequestered calcium. To investigate whether the effect of TNF-α on calcium entry was mediated via the sphingomyelinase pathway, the cells were pretreated with sphingomyelinase (SMase) prior to stimulation with Tg. SMase inhibited the Tg-evoked calcium entry in a concentration-dependent manner. Furthermore, an inhibition of calcium entry was obtained after preincubation of the cells with the membrane-permeable C₂-ceramide and C₆-ceramide analogues. The inactive ceramides dihydro-C₂ and dihydro-C₆ showed only marginal effects. Neither SMase, C₂-ceramide, nor C₆-ceramide affected the release of sequestered calcium. C₂- and C₆-ceramide also decreased the ATP-evoked calcium entry, without affecting the release of sequestered calcium. The effect of TNF-α and SMase was inhibited by the kinase inhibitor staurosporin and by the protein kinase C (PKC) inhibitor calphostin C (PKC) but not by down-regulation of PKC. However, we were unable to measure a significant activation of PKC using TNF-α or C₆-ceramide. The effect of TNF-α was not mediated via activation of either c-Jun N-terminal kinase or p38 kinase. We were unable to detect an increase in the ceramides (or sphingosines) content of the cells after stimulation with TNF-α for up to 30 min. Thus, one mechanism of action of TNF-α, SMase, and ceramide on thyroid FRTL-5 cells is to inhibit calcium entry.

An abundance of reports has shown that the cytokine tumor necrosis factor-α (TNF-α) has diverse effects upon several cell

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types (21). These effects of SP and SPP have also been observed in thyroid FRTL-5 cells (22–24). Recent reports also show that SP attenuated store-operated calcium entry (25, 26). Sphingosines and ceramides seem to have mostly opposite effects on cellular proliferation. As both SP and SPP mobilize sequestered calcium and stimulate calcium entry in FRTL-5 cells, two important events in the initiation of proliferation, we thought that it would be of interest to investigate whether ceramides could have any effect on the regulation of calcium fluxes in these cells. Our results showed that, in FRTL-5 cells, TNF-α, SMase, and ceramides potently attenuated calcium entry. Thus, one mechanism of action of TNF-α on thyroid cells is an inhibition of calcium entry.

**EXPERIMENTAL PROCEDURES**

**Materials—**Culture medium, serum, and hormones needed for the cell culture was purchased from Life Technologies, Inc., Biological Industries (Beth Haemek, Israel), and Sigma. Culture dishes were obtained from Falcon Plastics (Oxnard, CA) or from Greiner (Germany). GP109203X, N-acylsphingosine (C2-ceramide), and N-hexanoyl sphingosine (C6-ceramide) and the inactive forms N-acylsphinganine (di-hydro-C2) and N-hexanoylsphinganine (di-hydro-C6) were purchased from Calbiochem. Fura 2-AM and bisoxonol were purchased from Research Products International Corp. (IL). Rat thyroid FRTL-5 cells were a generous gift of Dr. A. F. Parlow (NHPP, NIDDK, National Institutes of Health). The localization of immunoreactive proteins was compared with those of prestained molecular weight markers (Life Technologies, Inc.). The signals were visualized by enhanced chemiluminescence (ECL, Amersham Corp., UK). The mixtures also contained leupeptin (12.5 μg/ml), phosphatidylinerse (PS, 40 μg/ml), and diacylglycerol (DAG, 8 μg/ml). PKC activity was calculated as the difference in the activity in the presence and the absence of DAG. The activity in the presence of DAG was calculated as the sum of the activities of the different subtypes of PKC (37). A sample of the extract was mixed with an excess of a phosphocellulose paper (1.5 cm in diameter). The plates were then washed twice with PBS (in millimolar concentrations: NaCl, 118; KCl, 4.6; glucose, 10; CaCl2, 1.0; HEPES, 20; pH 7.2) lacking 0.2% trypsin in 24-well tissue culture dishes. The medium was aspirated, and the cells were washed twice with HBSS buffer and incubated for at least 10 min at room temperature and washed once again. Fluorescence was measured with a Hitachi F2000 fluorometer. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM CaCl2 and the membranes from the cells to 10 mM CaCl2 and Trion X-100 to obtain maximal fluorescence. Chelating extracellular Ca2+ with 5 mM EGTA and the addition of Tris-base was used to elevate pHi above 8.3 to obtain minimal fluorescence. [Ca2+]i was calculated as described by Gryenwickicz et al. (28), using a computer program designed for the fluorimeter with a Kd value of 224 nm for Fura 2.

**Measurement of [Ca2+]i—**The medium was aspirated, and the cells were washed twice with HBSS buffer and incubated for at least 10 min at room temperature and washed once again. Fluorescence was measured with a Hitachi F2000 fluorometer. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM CaCl2 and Trion X-100 to obtain maximal fluorescence. Chelating extracellular Ca2+ with 5 mM EGTA and the addition of Tris-base was used to elevate pHi above 8.3 to obtain minimal fluorescence. [Ca2+]i was calculated as described by Gryenwickicz et al. (28), using a computer program designed for the fluorimeter with a Kd value of 224 nm for Fura 2.

**Activation of Protein Kinase C—**Immunoblots were run using a minigel apparatus (Midget Electrophoresis Unit, Pharmacia, Sweden). Proteins (1 and 3 μg per well for soluble and particulate proteins, respectively) were loaded onto 7.5% polyacrylamide gels and separated according to molecular weight. The proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were incubated three times for 15 min at 45 °C in Tween/TBS (TTBS, containing in millimolar concentrations: NaCl, 500; Tris-base, 20; pH 7.5, and 0.1% Tween 20) containing 5% fat-free dry milk and 15 min in TTBS. Then the membranes were incubated for 2 h with 1:4000–1:80,000 dilution of rabbit polyclonal anti-PKC antibodies that recognize α, β1, β2, γ, δ, ε, and δ subtypes of PKC (37). A horseradish peroxidase-labeled goat anti-rabbit antibody (Bio-Rad) was used as the secondary antibody, and the immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Corp., UK). The localization of immunoreactive proteins was compared with those of prestained molecular weight markers (Life Technologies, Inc.).

**Immunoblotting of PKC Isoenzymes—**SDS-polyacrylamide gel electrophoresis was run using a minigel apparatus (Midget Electrophoresis Unit, Pharmacia, Sweden). Proteins (1 and 3 μg per well for soluble and particulate proteins, respectively) were loaded onto 7.5% polyacrylamide gels and separated according to molecular weight. The proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were incubated three times for 15 min at 45 °C in Tween/TBS (TTBS, containing in millimolar concentrations: NaCl, 500; Tris-base, 20; pH 7.5, and 0.1% Tween 20) containing 5% fat-free dry milk and 15 min in TTBS. Then the membranes were incubated for 2 h with 1:4000–1:80,000 dilution of rabbit polyclonal anti-PKC antibodies that recognize α, β1, β2, γ, δ, ε, and δ subtypes of PKC (37). A horseradish peroxidase-labeled goat anti-rabbit antibody (Bio-Rad) was used as the secondary antibody, and the immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Corp., UK). The localization of immunoreactive proteins was compared with those of prestained molecular weight markers (Life Technologies, Inc.).

**Phosphatidylinerse (PS), and DAG was the same as that obtained when only PS and DAG were omitted. The reaction was started by adding protein (0.7–1.5 μg). The samples were incubated for 5 min at 30 °C, and the reaction was stopped by spotting 25 μl of each reaction mixture onto Whatman P81 phosphocellulose paper (1.5 × 1.5 cm). The papers were washed three times in 75 mM phosphoric acid. After air-drying, the radioactivity measurements were determined. The activity in the presence of 2 μM NEM was expressed as the ratio of organic phosphate incorporated to substrate peptide/mg of protein/min.

**Measurement of [Ca2+]i—**The medium was aspirated, and the cells were harvested with HEPES-buffered saline solution (HBSS, in millimolar concentrations: NaCl, 118; KCl, 4.6; glucose, 10; CaCl2, 1.0; HEPES, 20; pH 7.2) lacking Ca2+ but containing 0.02% EDTA and 0.1 mM 32P-ATP. The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM CaCl2 and Trion X-100 to obtain maximal fluorescence. Chelating extracellular Ca2+ with 5 mM EGTA and the addition of Tris-base was used to elevate pHi above 8.3 to obtain minimal fluorescence. [Ca2+]i was calculated as described by Gryenwickicz et al. (28), using a computer program designed for the fluorimeter with a Kd value of 224 nm for Fura 2.

**Measurement of Calcium Entry**—The excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM CaCl2 and Trion X-100 to obtain maximal fluorescence. Chelating extracellular Ca2+ with 5 mM EGTA and the addition of Tris-base was used to elevate pHi above 8.3 to obtain minimal fluorescence. [Ca2+]i was calculated as described by Gryenwickicz et al. (28), using a computer program designed for the fluorimeter with a Kd value of 224 nm for Fura 2.
10%; SDS, 4%; bromphenol blue, 0.02%; Tris-base, 0.125 mm, pH 6.8). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. The proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membrane was incubated with 5% nonfat dry milk for 1 h at room temperature in Tris-buffered saline (TBS, in mM concentrations: NaCl, 500; Tris-base, 20, pH 7.5) to block the remaining binding sites. The blots were incubated with anti-active JNK antibody (1:5000) diluted in TBS containing 0.2% bovine serum albumin for 2 days. The medium was then changed to 0H/bovine serum albumin containing the appropriate concentrations of the test compounds and [3H]thymidine (0.4 μCi/ml), and the cells were incubated for 24 h. The cells were washed twice with cold PBS solution and once with cold 5% trichloroacetic acid. The trichloroacetic acid-insoluble precipitate was dissolved in 0.1N NaOH, and the radioactivity was measured by scintillation counting.

**Statistics**—The results are expressed as the means ± S.E. Statistical analysis was made using Student’s t test for paired observations. When three or more means were tested, analysis of variance was used.

**RESULTS**

**TNF-α, SMase, and Cell-permeable Ceramides Inhibit DNA Synthesis**—Previous studies have shown that TNF-α and C2- and C6-ceramides potently inhibit both the TSH- and the insulin- evoked incorporation of [3H]thymidine in DNA, i.e. DNA synthesis (6–8, 20). We confirmed these results and further showed that SMase also inhibited the incorporation of [3H]thymidine in response to TSH and insulin (data not shown). The inactive ceramides dihydro-C2 and dihydro-C6 had only marginal effects (data not shown).

**TNF-α Inhibits Calcium Entry**—In FRTL-5 cells, as well as in other cell types, changes in [Ca2+]i are probably important events in the initialization of cell proliferation (39, 40). We thus investigated the effect of TNF-α on [Ca2+]i, and on the entry of calcium in FRTL-5 cells. TNF-α (100 ng/ml, the highest dose tested) did not per se affect [Ca2+]i, in these cells (data not shown). To avoid any possible effects of TNF-α on receptor-mediated events, we activated calcium entry by stimulating the cells with the Ca2+ ATPase inhibitor thapsigargin (Tg) (41). Tg activates a rapid store-operated calcium entry in FRTL-5 cells (42). Pretreatment of the cells with TNF-α for 10–30 min potentely attenuated the Tg-evoked calcium entry in both a calcium-containing buffer and in a calcium-free buffer in a concentration-dependent manner (Fig. 1). We also observed that TNF-α did not inhibit the Tg-evoked mobilization of sequestered calcium. In cells pretreated with 100 ng/ml TNF-α for 10 min, the Tg-evoked release of intracellular calcium was 178 ± 15 nM, compared with 155 ± 10 nM in control cells. These experiments were performed in a calcium-free buffer to avoid any interference of Tg-evoked calcium entry.

To investigate whether the observed effect of TNF-α was due to activation of a sphingomyelinase, we reincubated the cells with the phosphatidylcholine-phospholipase C inhibitor D609. Previous investigations have shown that D609 effectively inhibits TNF-α-evoked events (16). However, we observed that D609 was a very potent modulator of calcium entry in FRTL-5 cells (data not shown). Furthermore, D609 also mobilized sequestered calcium in our cells (data not shown). Thus, D609 is apparently not a suitable compound for studies using intact cells, as its effects on calcium fluxes probably will affect a multitude of cellular events.

Activation of protein kinases, including PKC, is an important part of the signaling cascade evoked by TNF-α (15). We prein-
incubated the cells with different concentrations of exogenous SMase for 30 min. As shown in Fig. 3, SMase inhibited calcium entry in a concentration-dependent manner similar to that of TNF-α. Furthermore, SMase did not affect the amount of sequestered calcium. In cells treated with SMase (1 units/ml) for 30 min, the increase in \([\text{Ca}^{2+}]\), evoked by Tg in a calcium-free buffer was 130 ± 23 nM, compared with 155 ± 10 nM in control cells.

In the next series of experiments, the cells were incubated with 200 nM staurosporin for 10 min prior to addition of SMase (1 units/ml for 30 min). In these experiments, pretreatment with staurosporin totally abolished the effect of SMase (Fig. 4), in a manner similar to what was observed in cells treated with both staurosporin and TNF-α. To investigate whether the effect of SMase was mediated via activation of PKC, we pretreated the cells with 100 nM calphostin C. In these experiments calphostin C also abolished the effect of SMase (Fig. 4).}

\[\Delta [\text{Ca}^{2+}] = [\text{Ca}^{2+}]_{\text{peak}} - [\text{Ca}^{2+}]_{\text{plateau}}\]

\[\text{S.E. of 4–7 separate experiments.} \]

\[\text{S.E. of 6–7 separate determinations.} \]

\[\text{S.E. of 4–7 separate experiments.} \]

\[\text{S.E. of 6–7 separate determinations.} \]
The cells were harvested and loaded with Fura 2 as described under "Experimental Procedures." A, the cells were preincubated with staurosporin (200 nM for 10 min) prior to addition of vehicle or SMase (1 unit/ml). Control cells (trace a) or cells pretreated with SMase (1 unit/ml for 30 min; trace b) were stimulated with 2 μM thapsigargin (small arrowhead) in a calcium-free buffer, and then 

\[ \text{Ca}^{2+} \] (final concentration 1 mM) was added to the cells (large arrowhead). B, the cells were preincubated with calphostin C (100 nM for 10 min) prior to addition of vehicle or SMase. Control cells (trace a) or cells pretreated with SMase (1 units/ml for 30 min; trace b) were stimulated with 2 μM thapsigargin (small arrowhead) and then 

\[ \text{Ca}^{2+} \] (final concentration 1 mM) was added to the cells (large arrowhead). C, the cells were preincubated with PMA (2 μM for 24 h) prior to addition of vehicle or SMase (1 unit/ml). Control cells (trace a) or cells pretreated with SMase (1 unit/ml for 30 min; trace b) were stimulated with 2 μM thapsigargin (small arrowhead) in a calcium-free buffer, and then 

\[ \text{Ca}^{2+} \] (final concentration 1 mM) was added to the cells (large arrowhead). The traces shown are representative of 3–4 separate experiments.

**Fig. 4.** Modulation of the effect of SMase on the thapsigargin-evoked calcium entry in FRTL-5 cells. The cells were harvested and loaded with Fura 2 as described under "Experimental Procedures." A, the cells were preincubated with staurosporin (200 nM for 10 min) prior to addition of vehicle or SMase (1 unit/ml). Control cells (trace a) or cells pretreated with SMase (1 unit/ml for 30 min; trace b) were stimulated with 2 μM thapsigargin (small arrowhead) in a calcium-free buffer, and then 

\[ \text{Ca}^{2+} \] (final concentration 1 mM) was added to the cells (large arrowhead). B, the cells were preincubated with calphostin C (100 nM for 10 min) prior to addition of vehicle or SMase. Control cells (trace a) or cells pretreated with SMase (1 units/ml for 30 min; trace b) were stimulated with 2 μM thapsigargin (small arrowhead) and then 

\[ \text{Ca}^{2+} \] (final concentration 1 mM) was added to the cells (large arrowhead). C, the cells were preincubated with PMA (2 μM for 24 h) prior to addition of vehicle or SMase (1 unit/ml). Control cells (trace a) or cells pretreated with SMase (1 unit/ml for 30 min; trace b) were stimulated with 2 μM thapsigargin (small arrowhead) in a calcium-free buffer, and then 

\[ \text{Ca}^{2+} \] (final concentration 1 mM) was added to the cells (large arrowhead). The traces shown are representative of 3–4 separate experiments.

30 μM dihydro-C2. Furthermore, in PKC down-regulated cells, C6-ceramide attenuated the Tg-evoked calcium entry (data not shown). We also tested C6-ceramide and obtained a decreased calcium entry in Tg-stimulated cells. However, the effect of C2 was smaller than that observed with C6-ceramide (data not shown). Dihydro-C2 had no effect on calcium entry (data not shown).

**Fig. 5.** SMase inhibits the thapsigargin-evoked barium entry in FRTL-5 cells. The cells were harvested and loaded with Fura 2 as described under "Experimental Procedures." Control cells (trace a) or cells pretreated with SMase (1 unit/ml for 30 min; trace b) were stimulated with 2 μM thapsigargin (small arrowhead) in a calcium-free buffer, and then 

\[ \text{Ca}^{2+} \] (final concentration 1 mM) was added to the cells (large arrowhead). The traces shown are representative of 3–4 separate experiments.

TNF-α, C6-ceramide, and DHC6-ceramide and the Activation of PKC—In addition to activating the sphingomyelinase pathway, TNF-α may activate PKC (15). Recent studies have shown that FRTL-5 cells express the α, δ, ε, and ζ isoforms of PKC (45), and our initial experiments confirmed these findings (data not shown). However, we were unable to show an activation of PKC in cells stimulated with neither TNF-α, C6-ceramide, nor DHC6-ceramide (Table I). In control experiments PMA significantly activated PKC (Table I).

**Importance of c-Jun N-terminal Kinase and p38 Mitogen-activated Protein Kinase—**TNF-α may activate JNK (15) and p38 mitogen-activated protein kinase (46) in several cell types. Furthermore, at least in human thyroid cells, JNK may be activated by a PKC-mediated mechanism (47). When our cells were stimulated with TNF-α (100 ng/ml), a transient activation of JNK was observed after 1 and 3 min of stimulation (data not shown). This effect was absent in cells pretreated with PMA (1 μM for 24 h, data not shown). As the effect of TNF-α on Tg-
evoked calcium entry still occurred in cells pretreated with PMA, it is not likely that JNK is involved in the attenuating effect of TNF-α on store-operated calcium entry. Furthermore, in cells pretreated for 30 min with the p38 kinase inhibitor SB203580 (final concentration 10 μM), TNF-α still abrogated the Tg-evoked increase in calcium. In cells pretreated with SB203580 and then with TNF-α (100 ng/ml) for 10 min, the readout of calcium to cells stimulated with 2 μM Tg increased [Ca^{2+}], transiently by 459 ± 40 nm and stabilized at a plateau level 201 ± 12 nm above the prestimulatory [Ca^{2+}], level. In cells treated with SB203580, but not with TNF-α, the values were 587 ± 35 and 269 ± 18 nm, respectively (p < 0.05 for both values). SB203580 per se did not affect the Tg-evoked increase in [Ca^{2+}], compared with control cells (data not shown).

*Production of Ceramide and Sphingosine in FRTL-5 Cells in Response to TNF-α—* Stimulating the cells with TNF-α (final concentration 100 ng/ml) for up to 30 min did not result in a significant increase in ceramide production, although SMase (100 milliunits/ml) potently increased ceramide content of the cells (Table II). Furthermore, TNF-α did not decrease the amount of sphingomyelin in the cells, although this was clearly obtained with SMase (Table II). In addition, we could not see an increase in cellular sphingosine content in response to a 30-min incubation with TNF-α. Thus, the lack of an effect of TNF-α on ceramide production was not the result of a rapid conversion of ceramide to sphingosine (data not shown). A similar lack of an increase in sphingosine content was obtained when the cells were stimulated with SMase (data not shown).

*Ceramides Inhibit ATP-evoked Calcium Entry—* Previous studies have shown that ATP evokes calcium entry in FRTL-5 cells (48). It was of interest to investigate whether the tested ceramides also could attenuate ATP-evoked calcium entry. Neither C2-ceramide nor dihydro-C2 affected the transient increase in [Ca^{2+}], in response to 100 μM ATP (Fig. 7). The ATP-evoked increase in [Ca^{2+}], in control cells was 880 ± 71 nm, in cells treated with 30 μM C2-ceramide 796 ± 85 nm, and in cells treated with 30 μM dihydro-C2 733 ± 69 nm. However, C2-ceramide clearly attenuated the plateau phase of the ATP-evoked change in [Ca^{2+}], i.e. calcium entry. Dihydro-C2 was without an effect (Fig. 7). A similar lack of an effect of TNF-α and C2-ceramide on the ATP-evoked transient increase in [Ca^{2+}], has also been reported (12). Furthermore, in cells stimulated with ATP in a calcium-free buffer, the ATP-evoked increase in [Ca^{2+}], was 53 ± 11 nm in control cells, in cells treated with 30 μM C2-ceramide 55 ± 10 nm, and in cells treated with 30 μM dihydro-C2 the response to ATP was 65 ± 10 nm. Similar results were obtained using C5 and dihydro-C5 (data not shown). It is interesting to note that the ATP-evoked receptor-mediated, transient calcium entry (48) was not affected by the tested compounds.

**DISCUSSION**

In the present investigation we show that TNF-α inhibits store-operated calcium entry in FRTL-5 thyroid cells. The same effect was observed when the cells were treated with SMase and membrane-permeable ceramide derivatives. In a recent observation, Barger et al. (19) showed that TNF-α inhibits calcium entry in hippocampal neurons in response to glutamate. However, in that study the importance of SMase or ceramides was not evaluated. Although we were unable to observe an increase in ceramide production after incubating the cells with TNF-α for periods relevant for the inhibition of calcium entry, our observation is the first to suggest that TNF-α, SMase, and ceramides acutely inhibit calcium entry. Another mechanism of action has been shown in osteoblasts. In these cells, several cytokines, including TNF-α, inhibited a parathyroid hormone-evoked increase in [Ca^{2+}], by abrogating the parathyroid hormone-induced formation of inositol 1,4,5-trisphosphate (49). However, this effect required at least 8 h of incubation with TNF-α.

In thyroid cells, TNF-α inhibits an array of different functions. Some of these, like the inhibition of proliferation (20), the inhibition of type I 5-deiodinase (11), and the inhibition of TSH-evoked production of hydrogen peroxide (12), have clearly been shown to be mediated via the production of ceramides. Of these events, at least the activation of proliferation is crucially dependent on intracellular calcium, and especially on calcium entry (39, 40). Based on our findings in the present study it is.

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**Table I**

| Lipid          | Cytosol fraction | Particulate fraction |
|----------------|------------------|----------------------|
| Control        | 0.47 ± 0.15      | 0.68 ± 0.19          |
| C2-ceramide    | 0.52 ± 0.11      | 0.66 ± 0.11          |
| DHC6-ceramide  | 0.82 ± 0.11      | 0.66 ± 0.19          |
| TNF-α          | 0.60 ± 0.11      | 0.70 ± 0.17          |
| PMA            | 0.72 ± 0.19      | 1.89 ± 0.67          |

*The results are given as pmol of 32P/mg of protein/min.*

**Table II**

| Lipid          | Control | TNF-α | SMase |
|----------------|---------|-------|-------|
| Control        | 100 ng/ml | 100 ng/ml | 100 ng/ml |
| Lipid          | 102 ± 3 | 1283 ± 132 |
| Ceramide       | 87 ± 12 | 44 ± 6   |

*The results are given as the mean ± S.D. of triplicate determinations.*

**Fig. 7.** Effect of C2-ceramide on the ATP-evoked calcium entry in FRTL-5 cells. The cells were harvested and loaded with Fura 2 as described under “Experimental Procedures.” C2-ceramide (final concentration 30 μM; trace c) or DHC6-ceramide (final concentration 30 μM; trace b) was added to the cells (white arrowhead), and then the cells were stimulated with 100 μM ATP (black arrowhead). Control cells, trace a. The traces shown are representative of 5 separate experiments.
thus tempting to speculate that the TNF-α-evoked inhibition of calcium entry is one important mechanism in inhibition of proliferation. Clearly it cannot solely explain the effects of TNF-α on FRTL-5 cells, especially as TNF-α has cytotoxic effects that probably are mediated via calcium-independent signaling pathways. Furthermore, TNF-α inhibits the activation of thyroid peroxidase and the production of thyroid hormones (4, 6–8, 10, 11). Both the activation of thyroid peroxidase and the production of thyroid hormones involve calcium-dependent events in FRTL-5 cells (50–54). Thus, it is possible that the inhibitory effect of TNF-α on these processes also is, at least in part, mediated via inhibition of calcium entry. It is also interesting to note that TNF-α may be produced by thyroid epithelial cells (55), suggesting an autocrine function for TNF-α.

Our results suggest that the mechanism by which TNF-α attenuated calcium entry could involve activation of SMase and the production of ceramides. This signaling pathway is usually connected to the binding of TNF-α to the p55 TNF-α receptor (13, 14). Although binding of TNF-α to FRTL-5 cells has been shown (3), there presently exists no information on the type of TNF-α receptors present in these cells. Our results show that TNF-α did not induce a measurable increase in ceramide for up to 30 min of incubation. In a recent study in FRTL-5 cells an effect of TNF-α on ceramide production was observed, but the first measurements were made 2.5 h after stimulation (12). Thus, we cannot exclude the possibility that TNF-α induced a small or localized increase in ceramide production which we were unable to detect.

We do not yet know how the attenuation of calcium entry occurs in response to stimulation with TNF-α, SMase, or ceramide. In the recent report by Barger et al. (19), it was suggested that NFκB transcription factor may be involved in the TNF-α-evoked attenuation of calcium entry. However, in our experiments the cells were treated with TNF-α for 24 h prior to testing for an inhibition of calcium entry. Our experiments show that TNF-α is effective within 10 min of application to the cells and the Cβ- and Cγ-ceramides within a few minutes. Such a rapid activation of NFκB has been reported in HL-60 cells (17, 56). As TNF-α has been reported to activate NFκB in human thyroid cells (18), we cannot exclude the possibility that NFκB mediates the TNF-α-evoked inhibition of calcium entry in FRTL-5 cells. In another recent report, a short (1–2 min) preincubation with Cβ-ceramide was also shown to attenuate calcium influx evoked with N-formyl-methionyl-leucyl-phenylalanine (57). The mechanism by which this inhibition was obtained was not established.

Our experiments performed in the presence of staurosporin suggest that a kinase apparently is of importance in mediating the effect of TNF-α. Some effects of TNF-α and sphingomyelinase have been shown to be mediated via activation of PKC (13, 58). In FRTL-5 cells, the α, δ, ε, and ζ isoforms of PKC have been detected (45). Of these isoforms, α, δ, and ε can be down-regulated by PMA, whereas the ζ isoform is insensitive to PMA (45). Previous studies have suggested that the PKC isoform activated by TNF-α is the ζ isoform (13, 58). This finding could explain why TNF-α and SMase were effective in PKC down-regulated cells but ineffective in cells treated with sphingosine C. This could also explain why the abrogating effect of PMA and TNF-α on calcium entry was additive. In other cell types, specific isozymes of PKC regulate calcium entry (59, 60). There is, however, a discrepancy between these observations and the fact that we could not measure an activation of PKC with either TNF-α or Cβ-ceramide (although PMA did so in control experiments). The PKC experiments might be hampered by the fact that about 60% of the PKC in our cells was already associated with the particulate fraction prior to stimulation, making a small effect of either TNF-α or Cβ-ceramide difficult to detect. We also observed that TNF-α evoked a transient activation of JNK, and this effect was absent in cells pretreated with PMA. However, as the effect of TNF-α on thapsigargin-evoked calcium entry still occurred in cells pretreated with PMA, it is not likely that JNK is involved in the TNF-α-evoked attenuation of store-operated calcium entry. Furthermore, as the p38 kinase inhibitor SB203580 did not inhibit the effect of TNF-α, we think it is unlikely that p38 kinase is mediating the effect of TNF-α on store-operated calcium entry.

The effect of ceramide was not due to conversion of ceramide to sphingosine, as we were unable to detect an increase in sphingosine content after stimulating the cells with either TNF-α or SMase. However, we have recently shown that the PMA-evoked activation of PKC depolarizes the membrane potential, resulting in decreased calcium entry due to a decreased electrochemical driving force for calcium (42). As the effect of TNF-α on store-operated calcium entry was abolished by inhibitors of PKC activity, an effect of TNF-α (and ceramide) on membrane potential cannot be excluded. Indeed, preliminary results suggest that TNF-α and ceramide evoke a depolarization of the membrane potential and that this effect is attenuated by calphostin C (21). These observations are consistent with a recent report showing that ceramide depolarizes the membrane potential in oligodendrocytes by inhibiting inwardly rectifying K+ channels (61). Furthermore, we cannot exclude the possibility that TNF-α also could modulate store-operated calcium channels, especially as a protein kinase has been shown to inhibit the calcium release-activated calcium current (I_{CRAC}) (62). This possibility appears unlikely as a recent report shows that ceramide does not modulate I_{CRAC} (26). The identification of the steps involved in the TNF-α/ceramide-evoked signaling pathway will be of crucial importance in understanding the mechanism(s) by which TNF-α inhibits calcium entry. Furthermore, this information may help in understanding the mechanisms regulating calcium entry in cells.

In conclusion, we have defined a novel mechanism of action for TNF-α, i.e., an inhibition of calcium entry. This observation will probably help in understanding the effects of this cytokine (and probably also of SMase and of ceramide) in thyroid cells and in other cell systems.

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