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Research Article

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Ca^{2+} Responses to Interleukin 1 and Tumor Necrosis Factor in Cultured Human Skin Fibroblasts

Possible Implications for Reye Syndrome

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Abstract

Elevated concentrations of cytokines were found in the plasma of patients acutely ill with Reye syndrome (RS) but not in control subjects or recovered RS patients. To determine whether this disorder involves a genetically determined abnormal response to cytokines, the effects of tumor necrosis factor (TNF) and IL-1 on intracellular free Ca^{2+} were compared in cultured skin fibroblasts from control subjects and patients with RS. IL-1 and TNF caused rapid, transient, and concentration-dependent increases in cytosolic free Ca^{2+}. The peak cytosolic free Ca^{2+} was greater and occurred at higher concentrations of IL-1 and TNF in patient cells than in cells from age-matched controls. In control cells, the Ca^{2+} transient diminished sharply with increasing amounts of IL-1 or TNF above the maximum stimulatory concentration. In contrast, in patient fibroblasts this bell-shaped curve of concentration dependency was much less apparent. Bradykinin-stimulated Ca^{2+} transients were similar in the two groups and did not exhibit the bell-shaped concentration dependency. Thus, plasma cytokine levels are elevated in RS patients and the Ca^{2+} response to cytokines is increased in cells derived from these patients. We propose that the increased response reflects a genetic defect in cytokine receptor–modulated signal transduction. (*J. Clin. Invest.* 1991. 87:778–786.) Key words: cytokines • bradykinin • phorbol esters • signal transduction • cyclic AMP

Introduction

For more than 25 years, researchers have been seeking the cause of Reye syndrome (RS),[1] an often fatal hepatic encephalopathy that strikes children treated with aspirin during a viral illness (1–5). Our previous studies were focused on the liver (6), where disturbances in lipid metabolism appeared to play a role in the pathology of the disease. Based on finding large amounts of hepatic acyl CoA metabolites of lipid oxidation known to inhibit many enzymes (6–13), we suggested that mitochondrial β-oxidation is blocked at multiple acyl CoA dehydrogenases. Although these observations can explain the hyperammonemia, hypoglycemia, and hepatic pathology seen in RS, they do not identify the cause of the disorder.

A genetic defect, viral infection, salicylate toxicity, endotoxin, and these factors in combination have been put forth as possible causes of RS (2, 3, 14–20). It has also been suggested that cytokines such as IL-1 or tumor necrosis factor (TNF) may play a key role in the pathogenesis of this disease. TNF and IL-1 production follow viral infection or endotoxin stimulation (16, 19–25). A more recently identified cytokine (26), IL-6, produced by fibroblasts (27) and certain other cell types, shares many actions with TNF and IL-1 but with apparently greater hepatic specificity (26). These cytokines are believed to be the endogenous mediators of the toxic effects of endotoxin in vivo (23–31). Increased plasma levels of endotoxin have been found in RS (32). Moreover, aspirin amplifies TNF production in vitro (16) and thus indirectly may stimulate IL-1 production (21). Larrick and Kunkel proposed that RS is caused by augmented production of TNF, which they showed was exacerbated by aspirin (16). Kilpatrick et al. have extended this proposal and suggested that cytokines inhibit mitochondrial β-oxidation as part of their hepatic action (18). In support of this concept we have demonstrated an accumulation of inhibitory acyl CoA esters in livers of rats treated with low doses of endotoxin together with aspirin (18), a pattern similar to that observed in patients with RS (6).

IL-1 and TNF mediate pleiotropic pro-inflammatory effects on many cell types by an as yet unidentified signal transduction pathway (21, 22). We have measured the cytokines IL-1, TNF, and IL-6 and found them greatly elevated during the acute phase of RS. The present study also compares the early actions and interactions of IL-1 and TNF with that of bradykinin (BK) in human fibroblasts. We examined fibroblasts because they were available from several patients who had had RS and they express many defects characteristic of Reye-like syndromes. We focused on cytosolic free Ca^{2+} as a second messenger of stimulus-response coupling. Mitogenic stimulation by a variety of growth factors including BK transiently increases cytosolic free Ca^{2+}, independently of external Ca^{2+} (33), presumably by activation of phosphatidylinositol 4,5 bisphosphate (PIP_2)-specific phospholipase C (34). We found that IL-1 and TNF resembled BK in causing rapid, transient, concentration-dependent rises in cytosolic free Ca^{2+}, through mobilization of intracellular stores. Fibroblasts from patients with Reye syndrome exhibited enhanced maximal responsiveness to both IL-1 and TNF. Furthermore, whereas the

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1. *Abbreviations used in this paper:* BK, bradykinin; G-protein, guanine nucleotide-binding protein; PIP_2, phosphatidylinositol 4,5 diphosphate; RS, Reye syndrome; TNF, tumor necrosis factor.

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Ca^{2+} response in control cells was greatly decreased at concentrations of the cytokines above the optimal, the response of patient cells remained elevated at high concentrations of TNF and IL-1. These observations suggest that Reye syndrome may involve genetic defects in IL-1 and TNF receptor–modulated signaling.

Methods

Patients. Skin fibroblasts from four patients and three controls were obtained from the Reye Syndrome Tissue Culture Core Facility (Children's Hospital of Philadelphia, Philadelphia, PA; Dr Paul M. Coates, Director). Patients with Reye syndrome were identified by criteria set forth by the Communicable Disease Center, Atlanta, GA (2). These cells had been obtained from patients by informed consent at Children's Hospital of Philadelphia. Cells from one additional patient (also treated at Children's Hospital of Philadelphia) and two control cell lines were obtained from the Coriell Institute, Camden, NJ. The clinical characteristics of the patients from whom fibroblasts were obtained are shown in Table I. Control cells were from age-matched subjects without known metabolic disease.

Cytokine assays. Plasma samples were obtained from eight healthy control subjects and eight children (7–15 yr) during their acute illness who had been diagnosed with RS between 1982 and 1989. Plasma was stored at −70°C before assay. Analyses were performed using IL-1β and TNF-α ELISA kits (Cistron Biotechnologies, Pine Brook, NJ). Before IL-1β and TNF-α measurements, plasma samples were extracted twice with two volumes of chloroform according to the method of Cannon et al. (35). The IL-6 ELISA kit was obtained from Genzyme Corp. (Boston, MA). Duplicate determinations were done for each plasma sample. IL-1β, TNF-α, and IL-6 were not detectable in any of the parallel control samples or recovered RS patients. The limits of detection were 20 pg/ml for TNF-α, 4 pg/ml for IL-1β, and 0.3 ng/ml for IL-6.

Fibroblast growth and preparation. Cells were grown in L-15 medium containing 5% Serumax (Sigma Chemical Co., St. Louis, MO). Cells were removed from culture flasks by incubating for 8–10 min in 0.02% EDTA in Dulbecco's PBS or for 2–5 min in 0.05% trypsin. The Ca^{2+} responses of cells removed by either technique were similar, indicating that trypsin did not impair the cytokine receptor.

Ca^{2+} measurement. Cytosolic free Ca^{2+} was determined from changes in the excitation signals of the fluorescent indicator fura 2 at 340 and 380 nm, measuring emission at 510 nm (36), using a time-sharing fluorometer built and designed by the Bio Instrumentation Group (University of Pennsylvania) (37).

Cells were loaded with fura 2 using L-15 tissue culture medium with 15 mM Hepes, pH 7.4, containing 0.5% fatty acid free, dialyzed BSA filtered through a 0.45-μm Millipore filter. Cells were suspended in modified Krebs-Hepes buffer containing 120 mM NaCl, 5 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, and 0.5% BSA, pH 7.4. The cytosolic free Ca^{2+} was calculated as described previously (36, 38–40). The maximum Ca^{2+}/fura 2 and minimum free fura 2 signals were determined after addition of Triton X-100 to equilibrate Ca^{2+} across the plasma membrane. The minimum Ca^{2+}/fura 2 and maximum free fura 2 signals were determined after addition of EGTA (plus Tris buffer to maintain pH). The cytosolic free Ca^{2+} was calculated by measuring the two fura 2 signals as a percentage of the maxima using the equation: [free Ca^{2+}] = $K_d \cdot$ [percent fura 2 free/percent Ca^{2+}-fura 2 complex] where the K_d was 200 nM (36). Ca^{2+} transients were measured in suspensions of confluent fibroblasts between passages 10 and 24. No consistent variation in the responsiveness of the cells between passages 10 and 24 was observed (data not shown); however, one previously responsive patient cell line failed to respond at passage 25. Cells used for phorbol ester experiments were treated with 1 μM phorbol-12-myristate-13-acetate (PMA) either for 15 min or 16 h before addition of agonist (41).

Cytokine preparations. Purified human IL-1 and TNF were obtained from Endogen, Inc. (Boston, MA). Purified IL-1 is the naturally occurring form containing 75% IL-1β and 25% IL-1α. It has a molecular mass of 17 kD, a specific activity of 10^8 U/mg, and is supplied at a concentration of 200 U/ml. TNF-α, the only purified natural form of this cytokine, has a molecular mass of 17 kD, contains 10^8 U/mg, and is supplied at a concentration of 4,000 U/ml. Recombinant human IL-1β was from Cistron Biotechnologies, 500 U/ml and 10^6 U/mg, 95% pure with < 50 pg/μg of endotoxin.

Table I. Clinical Description of Patients with Reye Syndrome

Patient	1	2	3	4	5
Age (yr, mo)	15, 7	15, 10	7, 10	11, 4	14, 0
Sex	M	F	M	F	M
Prodromal illness*	URI	VIS	VAR	VIS	VIS
Aspirin used	Yes	Yes	Yes	Yes	Yes
Ammonia (μg/dl)	955	485	248	175	382
SGOT (IU)	254	236	550	400	889
SGPT (IU)	424	465	695	377	751
Outcome	died	survived	survived	survived	died

* URI, upper respiratory infection; VIS, viral syndrome; VAR, varicella.

Results

The relevance of cytokine effects on signal transduction depends on elevations of these compounds in patients during the course of their illness. The plasma levels of cytokines in patients with RS are shown in Table II. TNF, IL-1, and IL-6 levels were frequently elevated in acutely ill patients but not in control subjects or recovered patients (data not shown, since all values were below the level of detection of the assays). From the small number of patients with samples available on sequential

Table II. Cytokine Levels in Patients with Reye Syndrome

Patient	Stage	TNF (ng/ml)	IL-1 (pg/ml)	IL-6 (ng/ml)
1*	IV (Day 1)	84.4	<4	<0.3
	IV (Day 4)	2.6	11.6	>30
3	II‡	4.6	ND	0.34
4	II (Day 1)	0.19	<4	<0.3
	II (Day 2)	3.2	<4	<0.3
5*	IV (Day 1)	0.88	<4	<0.3
	IV (Day 2)	1.7	8.1	1.8
	IV (Day 3)	<0.02	50.2	>30
6*	II	1.5	15.2	<0.3
	III	11.5	11.6	ND
7	II	ND	16.5	<0.3
8	II	3.1	108	ND
9	II	1.6	<4	<0.3

Cytokine levels were measured using ELISA kits. Plasma samples from eight control subjects and two patients after recovery were below the level of detection. Patient designations 1–5 are the same as in Table I. Plasma was not available from patient 2 during the illness. The staging of Reye syndrome was according to criteria set forth by the Communicable Disease Center (2).

* Patient died.

‡ Serum sample.

days, it appears that rather dramatic changes in the three cytokines develop with time. Elevated TNF concentrations occurred in all of the patients tested. Cytokine levels were generally highest in the patients who died (indicated with an asterisk in Table II). Patients 1 and 5, from whom blood samples were obtained on their last day of life, exhibited extremely high IL-6 concentrations at a time when TNF had diminished. To study IL-1 and TNF effects on signal transduction in human tissue, fibroblasts from patients 1 to 5 and five age-matched control subjects were obtained and used for the subsequent studies. Plasma from patient 2 was not available during the acute illness but fibroblasts and plasma were obtained several months after recovery.

Fibroblasts exhibit increases in cytosolic free Ca^{2+} in response to the peptide growth factor BK (33, 34, 42). Fig. 1, *A* and *B*, illustrates that purified IL-1 also causes a rapid but transient rise in cytosolic free Ca^{2+} lasting ~ 2 min. The IL-1–mediated Ca^{2+} transient rose with increasing IL-1 up to a maximum effective concentration. In the experiments illustrated in Fig. 1, a maximal effect was observed at 375 mU/ml (trace B), which is equal to 3.75 pg/ml or 220 fM. A maximal Ca^{2+}-elevating concentration of BK, which was similar in shape and duration to the transients induced by IL-1, is illustrated for comparison in trace C. Similar Ca^{2+} responses were also observed in human fibroblasts stimulated with TNF (traces E and F). The peak Ca^{2+} response occurred at 6.25 U/ml (trace F), which is equivalent to 62.5 pg/ml or 3.75 pM. In comparison to IL-1, higher concentrations of TNF were required to elicit Ca^{2+} transients in fibroblasts. We performed additional control experiments to verify these observations since other investigators have failed to document IL-1–mediated Ca^{2+} transients (43–45). First, we demonstrated that the vehicle in which the IL-1 is dissolved, 0.25% HSA (provided by Endogen), did not affect cytosolic free Ca^{2+} (data not shown). Next, we evaluated the effect of the β-isomer of recombinant IL-1 (rIL-1) on Ca^{2+} transients. As shown in trace D, rIL-1 also stimulated an increase in

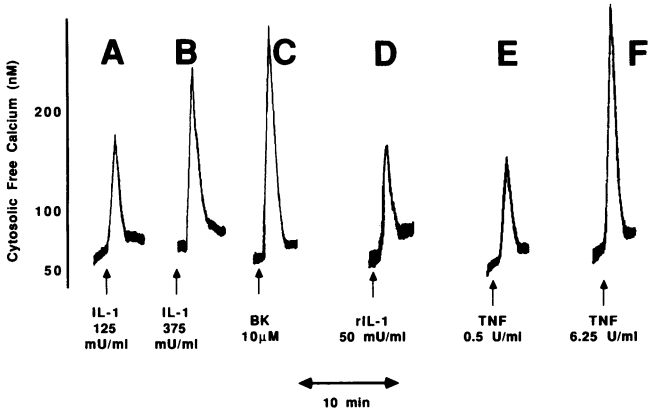

Figure 1. Illustration of the effect of IL-1 (*A* and *B*) BK (*C*), recombinant IL-1β (rIL-1; *D*) and TNF (*E* and *F*) on cytosolic free Ca^{2+} in human fibroblasts. Fura 2–loaded control fibroblasts (0.5 mg protein/ml) were suspended in modified Krebs-Hepes buffer containing 120 mM NaCl, 5 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, and 0.5% dialyzed fatty acid free BSA, pH 7.4. At the indicated times IL-1, BK, rIL-1, or TNF was added at the concentrations shown. rIL-1 β was used rather than purified IL-1 only in the trace shown in *D*. These traces illustrate experiments that were performed at least three times.

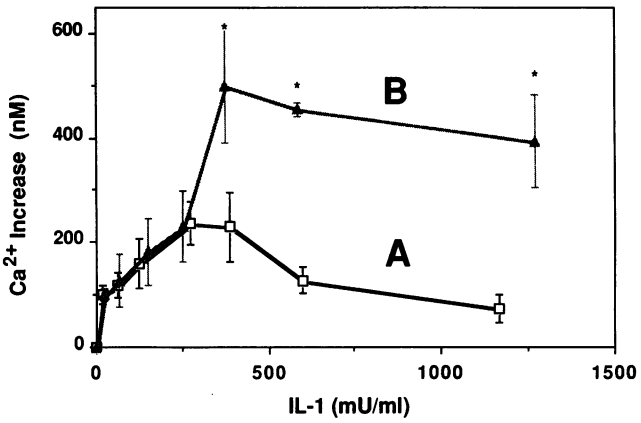

Figure 2. Comparison of the concentration dependence of IL-1 responses in fibroblasts from control subjects (*A*) and patients with Reye syndrome (*B*). Fura 2–loaded human fibroblasts from five control subjects and four patients were incubated as described in Fig. 1. Values represent means ±SE of 3–12 analyses for controls and 3–9 analyses for patients. Asterisks indicate a value that differs significantly from the control value according to Student's *t*-test.

cytosolic free Ca^{2+}. Similar data were obtained in two additional cell lines (data not shown).[2]

Basal cytosolic free Ca^{2+} levels obtained in patients and control cell lines did not differ significantly (83±9, n = 96, for controls and 78±7 nM, n = 80, for patients), nor did maximal peak responses to 1 μM BK (888±192 nM, n = 19, for controls and 976±159 nM, n = 16, for patients). Increasing BK concentrations above the maximal level did not alter the observed Ca^{2+} peak (data not shown). However, cells from patients with RS responded to IL-1 and TNF with higher maximum Ca^{2+} peaks and right-shifted concentration response curves compared with cells from control subjects. To characterize this difference detailed concentration dependencies were obtained. The mean peak Ca^{2+} transients at different concentrations of IL-1 are shown in Fig. 2. The concentration dependency curves below 300 mU/ml are similar for control and patient groups. However, higher peak increases were obtained in the patient group and hence both peak responses (240 nM Ca^{2+} at 250 mU/ml IL-1 for control vs. 500 nM Ca^{2+} at 400 mU/ml IL-1 for patients) and the half-maximal IL-1 concentrations (170 mU/ml for control vs. 350 mU/ml for patients) were higher in patient than in control cells. In addition the declining portion of the bell-shaped curve or inhibitory phase of the response occurred at lower concentrations of IL-1 in the control group (half-maximal Ca^{2+} response at 750 mU/ml) than in the patients (half-maximal at 2 U/ml). Although variations in the concentration dependency at high IL-1 levels were observed among patients, a decreased Ca^{2+} transient was observed above 5 U/ml IL-1 in cells from all patients.

The mean Ca^{2+} transients obtained from fibroblasts at different concentrations of TNF are shown in Fig. 3. The maximal Ca^{2+} peak in patient cells occurred at 8 U/ml TNF and in control cells at 4 U/ml TNF. In addition, the calculated TNF levels at which half-maximal inhibition of the Ca^{2+} peak oc-

2. Attempts to block the response to IL-1 with specific antibodies to IL-1 were unsuccessful since both immune and nonimmune sera induced Ca^{2+} transients.

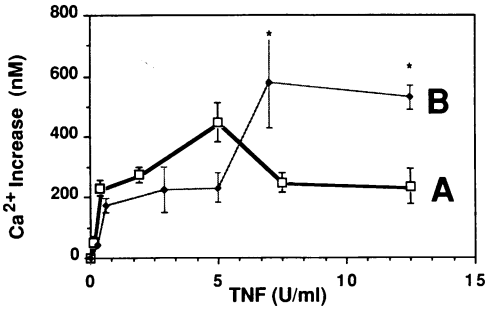

Figure 3. Comparison of the concentration dependence of TNF responses in fibroblasts from control subjects (*A*) and patients with Reye syndrome (*B*). Fura 2-loaded human fibroblasts from five control subjects and four patients were incubated as described in Fig. 1. Values represent means ±SE of 5–10 analyses for controls and 5–11 analyses for patients. Asterisks indicate a value that differs significantly from the control value according to Student's *t*-test.

curred was higher in cells from patients (40 U/ml) than from controls (15 U/ml).

Studies were next undertaken to determine the source of the elevated intracellular Ca^{2+}. Fig. 4 demonstrates that IL-1 and TNF released Ca^{2+} from internal stores. When external Ca^{2+} was removed by chelation with EGTA just before addition of the agonist, the Ca^{2+} transient was similar to that observed in the presence of Ca^{2+} (compare trace A with B and trace C with D). This indicates that IL-1– and TNF-induced Ca^{2+} transients were independent of extracellular Ca^{2+}. In contrast, when internal stores were depleted by pretreatment with low concentrations of the Ca^{2+} ionophore, ionomycin, the Ca^{2+} increases induced by IL-1 (compare traces E and F) and TNF (compare traces G and H) were markedly diminished, suggesting dependence on internal Ca^{2+} stores. Mitochondrial blockade with antimycin A did not influence the Ca^{2+} rise (data not shown) identifying a nonmitochondrial pool as the internal Ca^{2+} source. Similar dependence on intracellular Ca^{2+} was observed with BK (data not shown), consistent with previous reports in the literature (33, 45).

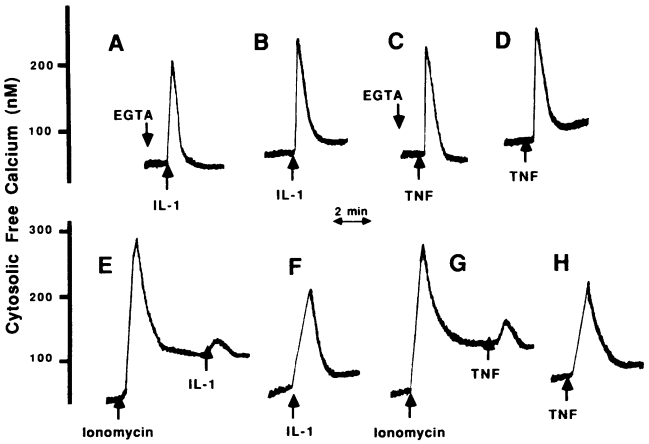

Figure 4. IL-1– and TNF-induced Ca^{2+} transients are dependent on intracellular Ca^{2+} stores. Fura 2-loaded fibroblasts were incubated as described in Fig. 1, except traces A and C contained no Ca^{2+} and 100 μM EGTA. IL-1 was added at 400 mU/ml, TNF at 2 U/ml, and ionomycin at 2 μM, as indicated. These traces illustrate experiments that were performed at least three times with control and patient cell lines.

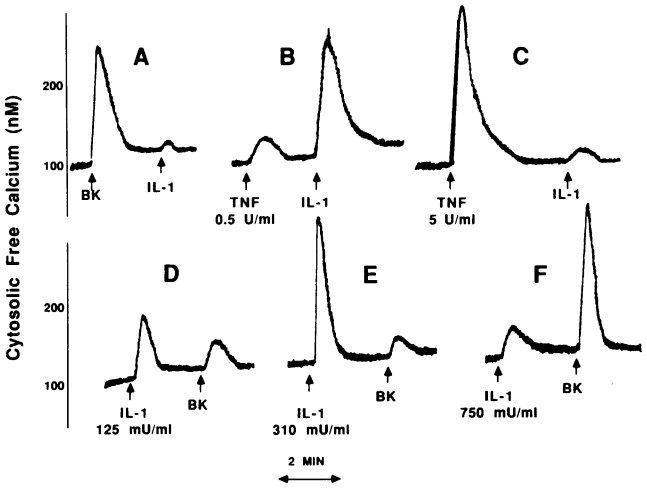

Figure 5. Interactions between cytokines and BK in human fibroblasts. Fura 2-loaded fibroblasts were incubated as described in Fig. 1. IL-1 was 400 mU/ml (traces *A–C*) or as indicated (traces *D–F*), TNF concentrations are indicated in parentheses; BK was 1 μM. These traces illustrate experiments that were performed at least three times with control and patient cell lines.

Because of the apparently similar mechanism of Ca^{2+} rise induced by the three agonists tested, the interactions among them were investigated. Fig. 5 *A* shows that prior addition of a maximal stimulatory concentration of BK blunts the response to IL-1 (compare traces A and E). The ability of TNF to diminish the response to IL-1 depended on the concentration of TNF (Fig. 5, *B* and *C*). Below the maximal concentration of TNF (Fig. 5 *B*), a large IL-1 response followed the small TNF response, whereas at a near maximal concentration of TNF (Fig. 5 *C*), the IL-1 response was largely inhibited. The ability of BK to increase Ca^{2+} was similarly influenced by prior addition of IL-1 and TNF (TNF data not shown).

It was not clear from the previous data whether the first agonist impaired the response to the second agonist through a membrane receptor event, competition for the same Ca^{2+} pools or generation of a second messenger such as diacylglycerol. Increasing IL-1 concentrations elicited the type of bell-shaped curve (Fig. 5, *D–F*) described in Fig. 2. However, the ensuing BK response appeared to depend on the magnitude of the Ca^{2+} transient rather than on the concentration of IL-1. Thus the response to BK was greatly diminished after a maximal IL-1–induced Ca^{2+} transient (Fig. 5 *E*), but increased after a higher but less effective dose of IL-1 (Fig. 5 *F*). These data suggest that the interaction among agonists is distal to receptor binding. The different agonists may compete for the same internal stores or the first agonist may activate protein kinase C.

Short-term exposure of cells to the phorbol ester PMA activates protein kinase C, which in some cell types appears to feedback-inhibit Ca^{2+} responses (for review see references 46 and 47), probably by protein kinase C–mediated block of receptor-induced PIP_2 breakdown (48). Longer exposure to phorbol esters appears to deplete cellular protein kinase C content, preventing this feedback mechanism (47). The Ca^{2+} transients caused by IL-1 and TNF were diminished in fibroblasts from control subjects and patients by short (15 min) exposure

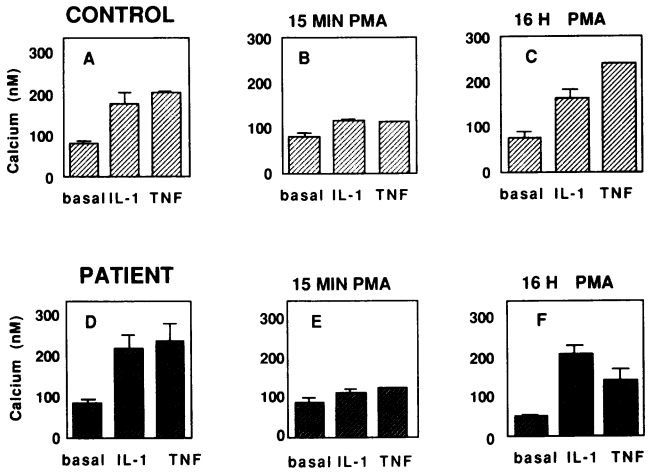

Figure 6. Effect of phorbol esters (PMA) on cytosolic free Ca^{2+} in cultured human fibroblasts from control subjects (*A–C*) and patients with Reye syndrome (*D–F*). Fura 2–loaded human fibroblasts were incubated as described in Fig. 1. IL-1 (125 mU/ml) and TNF (1 U/ml) were added in the absence of added PMA (*A* and *D*) or in cells treated for 15 min (*B* and *E*) or 16 h (*C* and *F*) with 1 μM PMA. Values represent means±SEM for 3–14 observations or duplicate observations where no standard errors are shown.

of the cells to PMA (Fig. 6, compare *A* with *B* and *D* with *E*). In contrast, prolonged exposure (16 h) of fibroblasts to PMA did not affect IL-1–or TNF-mediated Ca^{2+} transients in control cells (Fig. 6 *C*). The patient cells exhibited lower basal Ca^{2+} and diminished response to TNF (Fig. 6 *F*), although the significance of these findings is not understood. In addition, the inhibitions observed at high concentrations of IL-1 and TNF were retained in both patient (Fig. 7) and control (data not shown) cells after long-term exposure to phorbol esters, suggesting that the inhibitory phase of the concentration curve was not mediated by protein kinase C activation. Furthermore, the enhanced response to BK at higher, less effective, concentrations of IL-1 and TNF were also retained after long-term exposure to PMA (Fig. 7, compare *A–D* with *E–H*), indicating that the agonists compete for the same internal stores.

The recent demonstration that IL-1 and TNF rapidly increase cAMP levels in thymocytes (49) and human fibroblasts (27) prompted evaluation of the role of cAMP in cytokine-mediated Ca^{2+} transients. We asked specifically whether exposure to forskolin, which activates adenylate cyclase and elevates cAMP, or the cAMP analogue, dibutyryl cAMP, modulated IL-1– and TNF-induced Ca^{2+} peaks. We found that both forskolin (data not shown) and dibutyryl cAMP (Fig. 8) increased cytosolic free Ca^{2+}. In contrast to the response previously observed in cultured clonal β-cells (39), the Ca^{2+} rise in the fibroblasts was independent of extracellular Ca^{2+} and was inhibited by 15 min pretreatment with PMA (data not shown). When IL-1 was added before dibutyryl cAMP, the response to dibutyryl cAMP was markedly diminished (Fig. 8 *A*). However, dibutyryl cAMP added before IL-1 exerted little effect on the IL-1–induced Ca^{2+} transient (Fig. 8 *B*), even at concentrations as high as 1 mM (Fig. 8 *C*). Thus our data indicate that a cytokine-mediated cAMP rise may partially explain the Ca^{2+} transients we have observed in our studies, although the ability of the cytokines to further elevate Ca^{2+} in the presence of elevated dibutyryl cAMP levels suggests involvement of additional components in their signal transduction pathway. The responses of patient and control cells to elevation of cAMP did not differ significantly (data not shown).

Several probes of Ca^{2+} channel activity yielded negative results. Depolarization with 30 mM K^+ did not elevate cytosolic free Ca^{2+} or alter the subsequent response of the depolar-

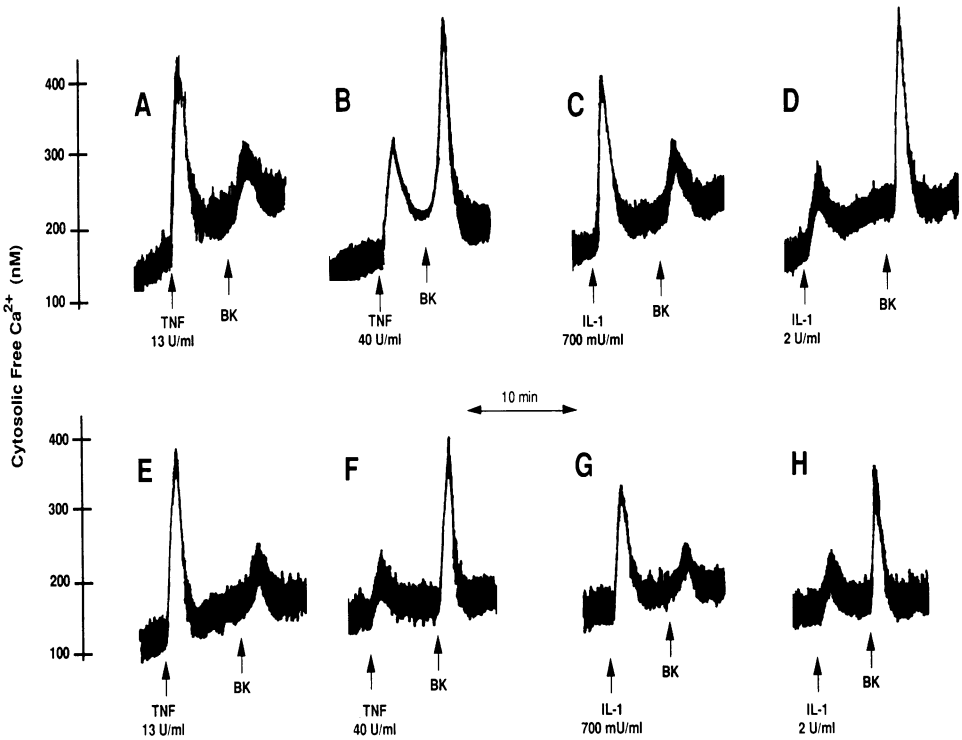

Figure 7. Effect of phorbol esters (PMA) on interactions between cytokines and BK in cultured human fibroblasts from patients with Reye syndrome. Fura 2–loaded fibroblasts were incubated as described in Fig. 1. TNF and IL-1, at concentrations indicated, and BK (1 μM) were added in the absence of added PMA (*A–D*) or in cells treated for 16 h (*E–H*) with 1 μM PMA. These traces illustrate experiments that were performed at least three times with control and patient cell lines.

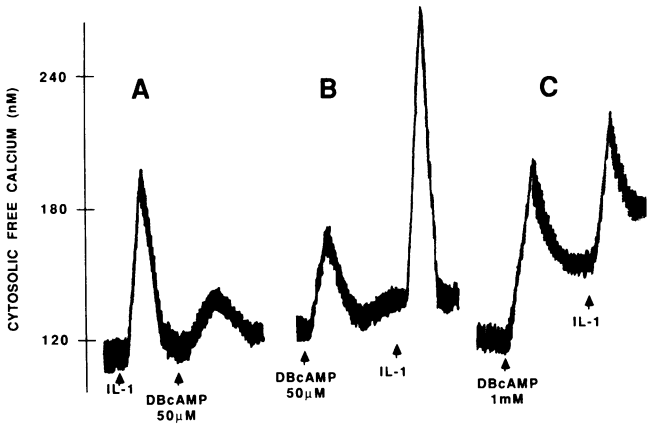

Figure 8. Illustration of the interaction between IL-1 and dibutyryl cAMP (DBcAMP) in cultured human fibroblasts. Fura 2-loaded human fibroblasts were incubated as described in Fig. 1. IL-1 was added at 400 mU/ml, DBcAMP at 50 μM or 1 mM, as indicated. These traces illustrate experiments that were performed two times with control and patient cell lines.

ized cells to IL-1 or TNF (data not shown). Another approach to evaluating Ca^{2+} channels in fibroblasts took advantage of the ability of Mn^{2+} to quench the fura 2 signal after its entry through Ca^{2+} channels (50, 51). In human fibroblasts Mn^{2+} itself elevated cytosolic free Ca^{2+} and diminished subsequent responses to IL-1, TNF, and BK (data not shown). Divalent cation-mediated Ca^{2+} peaks in fibroblasts have been previously shown to be independent of extracellular Ca^{2+} (52). The rate of quench of the fura 2 signal after Mn^{2+} addition was very slow. Neither depolarization nor addition of IL-1, TNF, forskolin, or BK influenced this rate in either patient or control cells (data not shown).

Discussion

Dermal fibroblasts from patients with RS exhibit exaggerated Ca^{2+} responses to high concentrations of IL-1 and TNF but not BK. In control cells the Ca^{2+} transients mediated by IL-1 and TNF increase with increasing cytokine concentrations to a maximal value and then exhibit a concentration-dependent decrease as cytokine levels are increased further. The cells from patients differed from controls in exhibiting higher maximal Ca^{2+} responses, higher half-maximal cytokine concentrations for observed Ca^{2+} effects, and blunted inhibition of the Ca^{2+} response at high cytokine levels.[3] This may be a genetic characteristic of patients with RS since it is retained through many passages in tissue culture and is observed in all five of the patients evaluated in this study, including one patient from whom fibroblasts were obtained several months after recovery. Additional proof of a genetic defect in cytokine signal transduction will require follow-up studies of surviving patients and their families.

The physiological relevance of the observed effects of high and low cytokine levels must be related to the actual concentrations that prevail in vivo. Others have reported values for IL-1 (from < 20 mU/ml to 5 U/ml, which is equivalent to 0.2–50 pg/ml) and TNF (10 pg/ml to 30 ng/ml, which is equivalent to 0.1–300 U/ml) in patients with various diseases such as septic shock and hepatic failure (53–55). The average values obtained from our patients (Table II) of ~ 20 pg/ml (2 U/ml) for IL-1 and 10 ng/ml (100 U/ml) for TNF are at the high end of this range. In addition, we found that TNF elevations preceded IL-1 and IL-6 increases in three of four patients from whom multiple samples were obtained, consistent with the suggestion by Fong and Lowry (56) of a feedback loop in which TNF stimulates IL-1 and IL-6 production and the latter cytokine in turn inhibits TNF production.

IL-1 and TNF concentrations in our patients were in the range of cytokine levels where effects on free Ca^{2+} are observed in patient but not control fibroblasts. Maximum Ca^{2+} transients in patient fibroblasts appear to occur at high concentrations between 0.5 and 1.5 U/ml (5 and 15 pg/ml) for IL-1 and between 5 and 15 U/ml (0.5 to 1.5 ng/ml) for TNF, concentrations where control fibroblasts are nonresponsive. Furthermore, our findings demonstrate that IL-1 and TNF increase cytosolic free Ca^{2+} at concentrations equal to or lower than those reported to cause other effects in human fibroblasts, including stimulation of mRNA production (57, 58), cyclooxygenase synthesis (59), protein phosphorylation (60), cAMP production (27, 49), and growth (22, 59, 61–63). The rapidity of the Ca^{2+} response (seconds), as early as any effect observed in fibroblasts, suggests that it may be an important component of signal transduction by IL-1 and TNF.

Previous studies of TNF or IL-1 have not reported changes in cytosolic free Ca^{2+} in human fibroblasts (43–45). There are several possible reasons why Ca^{2+} transients might not have been observed. First, as we have noted in this study, the response to both IL-1 and TNF is bell-shaped, with loss of the Ca^{2+} response above ~ 2 U/ml IL-1 and 20 U/ml TNF. Thus, experiments evaluating Ca^{2+} responses at 1–100 ng/ml (100–10,000 U/ml) (43), 100–500 pg/ml (10–50 U/ml) (45), or 50 U/ml (44) did not include sufficiently low concentrations of IL-1. Second, the Ca^{2+} response may be cell or species specific and only occur in human cells or skin fibroblasts. Third, TNF and IL-1 appear to bind to BSA, and therefore, the Ca^{2+} responses to submaximal concentrations of cytokines are diminished with increasing concentrations of BSA (data not shown). Fourth, prestimulation of cells with high concentrations of serum, which presumably contains various growth factors, blunts cytokine-mediated Ca^{2+} responses. The responses are only apparent in cells that have been grown in low serum concentrations or been serum-deprived for ~ 12 h.

The signal transduction mechanism for cytokines has not been elucidated. Our data implicate an early role of cytosolic free Ca^{2+} in triggering the cascade of events involved in the pleiotropic cytokine pathway at low cytokine concentrations. The mechanism by which Ca^{2+} is elevated appears to involve primarily internal stores. The similarities between the BK and cytokine responses are consistent with a similar signal transduction pathway. BK activates the phosphoinositide pathway via a guanine nucleotide-binding protein (G-protein) coupled to phospholipase C (64). It is not known whether metabolites of this pathway are affected by cytokine concentrations that induce Ca^{2+} transients. However, it is necessary to postulate that IL-1 and TNF, which may share an initial Ca^{2+}-transient generating step with BK, have additional effects that are different, since some cytokine effects on human fibroblasts are observed

3. In preliminary studies, we also found that growth stimulation of fibroblasts by the cytokines was greater in patient cells at high cytokine concentrations.

within the range where we obtain Ca^{2+} responses (27, 49, 57–60, 65, 66), but others occur at higher concentrations where Ca^{2+} transients are not observed (58, 66, 67).

The bell-shaped concentration curve suggests that IL-1 and TNF may operate through two mutually incompatible signal transduction pathways: a Ca^{2+}-mediated pathway at low cytokine concentrations; and a second, as yet undefined, pathway that prevents cytokine-induced Ca^{2+} transients. Bell-shaped curves are also observed for a number of other responses to IL-1 and TNF (27, 57, 58, 68). Although the significance and mechanism of this phenomenon is not known, several possible explanations may pertain. First, the bell-shaped response curve could represent two independent actions of the cytokines mediated through two receptor subtypes each linked with a different effector system. Several groups have reported two classes of binding sites, although no correlation between binding and action has been demonstrated (22, 69, 70). A second possibility is that receptor-ligand internalization (69, 71) is an essential step in cytokine-mediated Ca^{2+} transients and is inhibited at high receptor occupancy. Third, the cytokines could interact with two different effector systems through the same receptor depending on ligand-receptor occupancy. Thus, a low level cytokine-receptor interaction could involve G-protein stimulation of a PIP_2-specific phospholipase C, whereas multiple cytokine-receptor units, which might exhibit a structurally altered complex, could promote interaction with different G-proteins and thus other effectors.

The relationship between Ca^{2+} hyperresponsiveness to high concentrations of IL-1 or TNF and patient morbidity remains to be determined. Although the two cytokines are structurally unrelated and have been shown to recognize separate receptors, they are quite similar in many of their actions (21, 22, 27, 57–63), including enhanced production of c-*fos* and c-*myc* protooncogene mRNA, collagenase, proteinases, prostaglandins, and IL-6, as well as stimulation of growth. The enhanced Ca^{2+} response could damage directly, or indirectly via stimulation of IL-6 output (26) which occurs during the terminal stage of the disease. One possibility is that hyperresponsitivity to Ca^{2+} blocks the action of other essential agonists either through depletion of Ca^{2+} stores or activation of protein kinase C enzymes. Both the competition for internal Ca^{2+} stores and the decreased Ca^{2+} transients after treatment with phorbol esters that we have observed make diminished responsiveness to other agonists probable at cytokine levels where large Ca^{2+} transients are observed. A second possibility is that exaggerated Ca^{2+} responses may reflect a receptor-coupling defect. This seems unlikely due to the similar responses to low concentrations of cytokines. A third possibility is that differences in receptor number (of a normal receptor) are responsible for the observed differences in Ca^{2+} response. Although receptor number may be hormonally or metabolically regulated (41, 65, 72–74), this seems improbable in the cultured fibroblasts because they are grown under identical conditions. Fourth, since IL-1 and TNF may need to be internalized to exert some of their effects (69–71), differences in internalization or receptor cycling may be involved in the hyperresponsiveness of patient cells. Finally, diminution of the Ca^{2+} response at high cytokine levels in control cells may be a protective mechanism mediated by a regulatory component that is lacking or abnormal in patient cells.

A hypothetical working model of the pathophysiology of RS involves the inability to turn off the Ca^{2+} signal at high cytokine concentrations. It is suggested that this defect leads to cell damage either directly, by blocking responses to other agonists, or indirectly, through production of IL-6. We propose that this destructive process involves inhibition of fatty acid oxidation. Stimulation of fatty acid synthesis, which has been noted to accompany cytokine elevation (28–30), presumably results from malonyl CoA–mediated inhibition of fatty acid oxidation (75–77) with resulting increases in cytosolic long chain acyl CoA levels (77). Because of the specific pattern of metabolic accumulation (6) it may be postulated that diminished energy production follows specific inhibition of amino and fatty acid oxidation at flavin-linked enzymes, resulting in accumulation of acyl CoA substrates for these enzymes. If the model is correct, inhibition of the Ca^{2+} transient, together with provision of substrate not requiring flavin-linked enzymes, such as glucose, might protect patients during the acute phase of the illness. The pathological consequences of alteration of the bell-shaped response curve would only appear, as in patients who develop RS, when three conditions co-exist: (*a*) A viral illness that stimulates local cytokine production by circulating monocytes or tissue-fixed macrophages; (*b*) the addition of aspirin to amplify cytokine production; and (*c*) a genetic or acquired loss of the protective inhibitory component of the response to high cytokine levels.

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