Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4825–4829, May 1994
Medical Sciences

γ interferon activates a previously undescribed Ca^{2+} influx in T lymphocytes from patients with multiple sclerosis

(cytokines/Ca^{2+} homeostasis/protein kinase C)

GIANVITO MARTINO*†, EMILIO CLEMENTI‡§, ELENA BRAMBILLA*†, LUCIA MOIOLA*†, GIANCARLO COMI†, JACOPO MELDOLESI§, AND LUIGI M. E. GRIMALDI*†¶

*Neuroimmunology Unit, †Department of Neurology, and §Receptor Byochemistry Unit and Department of Pharmacology, Dipartimento di Ricerca Biologica e Tecnologica (DIBIT), Consiglio Nazionale delle Ricerche Cytopharmacology and B. Ceccarelli Centers, University of Milan, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy; and ‡Chair of Pharmacology, Faculty of Pharmacy, University of Reggio Calabria, 88021 Catanzaro, Italy

Communicated by Rita Levi-Montalcini, January 26, 1994

ABSTRACT **Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system. The etiology of the disease is still unknown. Activated T lymphocytes are considered essential in mediating the inflammatory process leading to demyelination in MS. They operate through a complex network of cytokines among which γ interferon (γ-IFN) plays a key role. Here we report that exposure to γ-IFN of T lymphocytes from patients with MS activates, by a protein kinase C-mediated pathway, a previously undescribed γ-IFN-activated Ca^{2+} influx, functionally coupled to the γ-IFN receptor. The influx, mainly expressed by $CD4^+$ T lymphocytes, was found in 12 of 15 (80%) patients with clinically active MS and in 14 of 30 (46%) patients with stable MS. The influx was found in only 3 of 24 (12%) control patients and in none of the 15 healthy subjects studied. Our results document the appearance in MS lymphocytes of a γ-IFN-activated, protein kinase C-dependent, Ca^{2+} influx that might be due to the expression of a new cation-specific plasmalemma channel. This finding suggests that at least part of γ-IFN's contribution to the pathogenesis of MS is exerted through a Ca^{2+}-dependent regulation of T lymphocyte activity.**

Multiple sclerosis (MS) is a clinically heterogeneous demyelinating disease of unknown etiology of the central nervous system. Combined clinical/pathological/epidemiological data suggest a genetic predisposition to the disease triggered by viral or autoimmune (or possibly both in concert) phenomena (1).

Immunological abnormalities leading to central nervous system demyelination in MS involve primarily T lymphocytes and are modulated by an intersecting network of cytokines in which T-lymphocyte-secreted γ interferon (γ-IFN) is considered essential. γ-IFN increases major histocompatibility complex class II expression on central nervous system resident cells (2), stimulates macrophages to release cytokines able to damage oligodendrocytes (i.e., tumor necrosis factor α) (3), and triggers the production by microglial cells of molecules potentially noxious to the myelin sheath, such as nitric oxide metabolites (4). The central role of γ-IFN in MS is also confirmed by the increase in exacerbation rate seen in MS patients undergoing systemic γ-IFN treatment (5) and by the protective effects of systemic administration of β-IFN (6). This latter effect has been attributed, at least in part, to inhibition of γ-IFN synthesis (7). However, the relationships between γ-IFN secretion, T-lymphocyte activation, and the γ-IFN-evoked intracellular signals occurring during different phases of MS are still elusive.

In the present study we have investigated the effects of γ-IFN on Ca^{2+} release from intracellular stores and influx across the plasmalemma in peripheral blood lymphocytes (PBLs) from patients with definite MS. Patients affected by other diseases (including active autoimmune diseases and other inflammatory and noninflammatory neurological diseases) and healthy donors were studied as controls.

We found that exposure of T lymphocytes from patients with MS to γ-IFN activates a previously undescribed Ca^{2+} influx functionally coupled to the γ-IFN-receptor (γ-IFN-R) and distinct from the influx processes described so far (8). This Ca^{2+} influx, which appears to be activated by a protein kinase C (PKC)-dependent intracellular pathway, is overexpressed in MS during the phases of clinical activity and tends to disappear during recovery from clinical exacerbation.

PATIENTS AND METHODS

Patient Selection. Forty-five patients (27 women, 18 men) with a mean age of 35.5 yr (range, 23–57 yr) were diagnosed as affected by definite MS (disease duration, 7.9 yr; range, 2–30 yr) according to the clinical classification of McDonald and Halliday (9). The clinical course of the disease was relapsing-remitting in 32 patients and relapsing-progressive in 13 patients. We subdivided patients as follows: MS patients experiencing a clinically active phase (13 relapsing-remitting and 2 relapsing-progressive patients) and MS patients clinically stable (6 relapsing-remitting and 24 relapsing-progressive patients). Activity of the disease was defined as a clinical status of exacerbation of previous symptoms and signs or appearance of new symptoms and signs, lasting >24 hr. In the group of patients with clinically active MS, PBLs were obtained within 10 days after the onset of neurological signs and symptoms. The control group included 24 patients (14 women, 10 men) with a mean age of 45.6 yr (range, 20–68 yr). Eleven patients were affected by other neurological diseases, including cerebrovascular disorders (5 patients), viral encephalomyelitis (3 patients), meningioma (1 patient), paraneoplastic neuropathy (1 patient), and neurosyphilis (1 patient). Thirteen patients were studied during an active phase of other autoimmune disorders, including rheumatoid arthritis (3 patients), type 1 insulin-dependent diabetes mellitus (2 patients), myasthenia gravis (2 patients), lupus erythematosus sistemicus (2 patients), autoimmune thyroiditis (1 patient), sarcoidosis (1 patient), chronic idiopathic polineuropathy (1 patient), and connective tissue disease of unknown origin (1 patient). Fifteen healthy donors (6 women, 9 men) with a mean age of 27.5 yr (range, 24–36 yr) were also

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Ab, monoclonal antibody; $[Ca^{2+}]_i$, cytosolic Ca^{2+}; γ-IFN, γ interferon; γ-IFN-R, γ-interferon receptor; MS, multiple sclerosis; PBL, peripheral blood lymphocyte; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; PKC, protein kinase C; FACS, fluorescence-activated cell sorting.

¶To whom reprint requests should be addressed.

included in the study. At the time of sample collection all patients had not received any steroid or other immunosuppressive treatments for at least the previous 3 months. Statistical analysis was performed by the Student's *t* test for unpaired data.

Purification of Lymphocyte Subsets. Human PBLs were isolated by conventional Ficoll/Hypaque density gradient centrifugation. Adherent cells were discarded to eliminate macrophages before fluorimetric analysis. Subsets of lymphocytes (B cells, $CD4^+$, $CD8^+$) were purified from macrophage-depleted PBLs by negative selection with anti-$CD4^+$- and anti-$CD8^+$-antibody coupled Dynabeads (Dynal, Oslo). The purity (>90%) of our preparations was confirmed by fluorescence-activated cell sorting (FACS) analysis.

γ-IFN-R-Enriched T Lymphocytes. γ-IFN-R-enriched T lymphocytes and the fluorescein isothiocyanate-conjugated anti-γ-IFN-R monoclonal antibody (Ab) γR99 (10) were provided by F. Novelli (University of Turin, Turin, Italy). γ-IFN-R-enriched T lymphocytes were obtained from two healthy subjects by culturing PBLs for 72 hr in complete medium (RPMI 1640 plus 10% fetal calf serum) supplemented with phytohemagglutinin (PHA) (10 μg/ml) or by alloactivation of PBLs (from three healthy donors) for 5 days with the Epstein–Barr virus-transformed lymphoblastoid cell line SL1 (10). To assess the purity and the levels of γ-IFN-R expression of our preparations, PHA-stimulated or alloactivated T cells were harvested after culture and tested by double-fluorescence FACS analysis with a phycoerythrin-conjugate anti-CD3 Ab (Becton Dickinson); the presence of γ-IFN-R was assessed by using the anti-γ-IFN-R Ab γR99.

Measurements of Cytosolic Ca^{2+}. Fluorimetric analyses were performed on PBLs, lymphocyte subsets (B cells, $CD4^+$, and $CD8^+$), and PHA-stimulated and alloactivated T cells as follows: Cells were resuspended in Krebs/Ringer/Hepes (KRH) medium containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, and 25 mM Hepes·NaOH (pH 7.4). Cells were washed three times by centrifugation, resuspended, and loaded for 30 min at 25°C with the Ca^{2+}-sensitive dye fura-2 (Calbiochem), administered in the same KRH medium as acetoxymethylester at the final concentration of 2.5 μM. Cells were then diluted in KRH medium to a final concentration of 3–4 × 10^6 cells per ml and kept at 37°C until use. Cell aliquots (4 × 10^6 cells) were centrifuged, resuspended by gentle swirling in 1.5 ml of KRH medium supplemented with 250 μM sulfinpyrazone (Sigma) (to prevent dye leakage), and finally transferred, under continuous stirring, to a thermostatted cuvette (37°C) in a Perkin–Elmer LS-5B fluorimeter (11). The experiments were carried out in plain KRH or according to the Ca^{2+}-free/Ca^{2+} reintroduction protocol (12). In this latter case, the samples were supplemented with excess EGTA (3 mM), and 1 pg of γ-IFN per ml (corresponding to an antiviral titer of 2.5 × 10^7 units/mg) (Genzyme) was added 1 min later. After 3–4 min of continuous recording, Ca^{2+} (3 mM) was reintroduced into the medium and the ensuing peak (Ca^{2+} influx) was recorded. In parallel experiments, the small cytosolic Ca^{2+} ($[Ca^{2+}]_i$) changes occurring in nonstimulated cells, as a consequence of medium Ca^{2+} chelation and reintroduction, were carefully evaluated and the corresponding values were subtracted from the experimental traces as described (12).

In the experiments aimed at establishing the voltage dependence of the Ca^{2+} influx, parallel aliquots of cells were analyzed while suspended in the medium as above or modified by isosmotic substitution of 30, 60, or 120 mM NaCl with KCl.

Experiments aimed to assess the existence of an electroneutral cotransport were performed in samples of cells loaded with fura-2 as above, washed, and resuspended in a lightly buffered medium containing 0.3 M sucrose/5 mM Hepes/Tris, pH 7.4, and devoid of Cl^- and PO_4^{3-}.

La^{3+} measurements were performed as described (12). Briefly, cells (loaded with fura-2 as above) were resuspended in sulfate-free, phosphate-free KRH medium supplemented with 1 mg of gelatin per ml and tested. La^{3+} (50 μM) was added 3 min after administration of 1 pg of γ-IFN per ml and 1 min before Ca^{2+} reintroduction in the medium.

Characterization of γ-IFN-Induced Ca^{2+} Influx. We tried to interfere with the γ-IFN-induced Ca^{2+} influx with drugs known to act on all other Ca^{2+} channels described so far (8). The following drugs were used: SC 38249 (provided by H. Ruegg, Sandoz), a blocker of voltage-gated, second messenger-operated, and store-dependent Ca^{2+} channels (12–14); specific blockers of voltage-gated Ca^{2+} channels [1 μM diltiazem (Sigma) and 20 μM verapamil (Sigma) for the L type; 0.2 μM ω-conotoxin (provided by E. Sher, Consiglio Nazionale delle Ricerche Cytopharmacology Center, Milan) for the N type] (8, 15); and the adenylyl cyclase activator forskolin (10 μM) (Sigma). Overnight pretreatment with pertussis toxin (0.1 μM) (Sigma), a selective blocker of G_o/G_i-type GTP-binding proteins (16), was also tested.

Mn^{2+} permeability was measured by quenching of fura-2 fluorescence as described (12). Cells loaded with fura-2 were suspended in an incubation medium from which $CaCl_2$ was omitted, KH_2PO_4 was reduced to 0.6 mM, and $MgCl_2$ was substituted for $MgSO_4$. Fluorescence was excited at 360 nm, the Ca^{2+}/fura-2 isosbestic wavelength at which changes are caused by Mn^{2+} quenching (17). Emission was recorded at 500 nm. Mn^{2+} (100 μM) was added to the medium 2 min after thapsigargin (Calbiochem) (which induces depletion of intracellular Ca^{2+} stores), PHA, or exposure to γ-IFN. At the end of the recordings, Mn^{2+} quenching values were estimated in each preparation by cell permeabilization with Triton X-100 (0.1%).

In macrophages, transmembrane signaling at the γ-IFN-R is known to include activation of PKC (18, 19). The role of this kinase in the influx induced by γ-IFN in MS lymphocytes was therefore investigated with administration of staurosporin (0.1 μM) (Sigma), a PKC blocker (20), and phorbol 12-myristate 13-acetate (PMA; 0.1 μM) (Sigma), a PKC activator.

RESULTS

Results are summarized in Figs. 1–4. In no case was any $[Ca^{2+}]_i$ increase observed in PBL preparations exposed to γ-IFN while bathed in Ca^{2+}-free medium (see Figs. 1, 2, and 4). This excludes an effect of γ-IFN on intracellular Ca^{2+} release. In contrast, Ca^{2+} influx was documented by $[Ca^{2+}]_i$ increase in the cells bathed in Ca^{2+}-containing medium. Consistent increases of $[Ca^{2+}]_i$ after exposure to γ-IFN were observed in lymphocytes of 12/15 (80%) MS patients in a clinically active phase and of 14/30 (46%) of those in a clinically stable phase (Fig. 1*A*). The observed influx was slow, with an initial delay of ≈10 sec followed by a progressive 2- to 3-min rise of the $[Ca^{2+}]_i$ (Fig. 1*A*). Plateau values in acute and stable patients were, on the average, 46% (range, 23–82) and 44% (range, 30–68) higher than resting levels, respectively. In the continuous presence of γ-IFN, the plateau phase of the influx was maintained for prolonged periods of time (tens of minutes). When excess EGTA was added, $[Ca^{2+}]_i$ returned quickly to or below resting levels (Fig. 1*A*). In contrast, none of the patients with autoimmune disorders or healthy subjects exhibited $[Ca^{2+}]_i$ increase after exposure to γ-IFN. Among the other neurological disease patients, only 3 of 11 (27%) (affected by viral encephalomyelitis, ischemic cerebrovascular stroke, or meningioma) exhibited moderate $[Ca^{2+}]_i$ increases (average of 20% over basal values; range, 16–25; active MS vs. other neurological disease

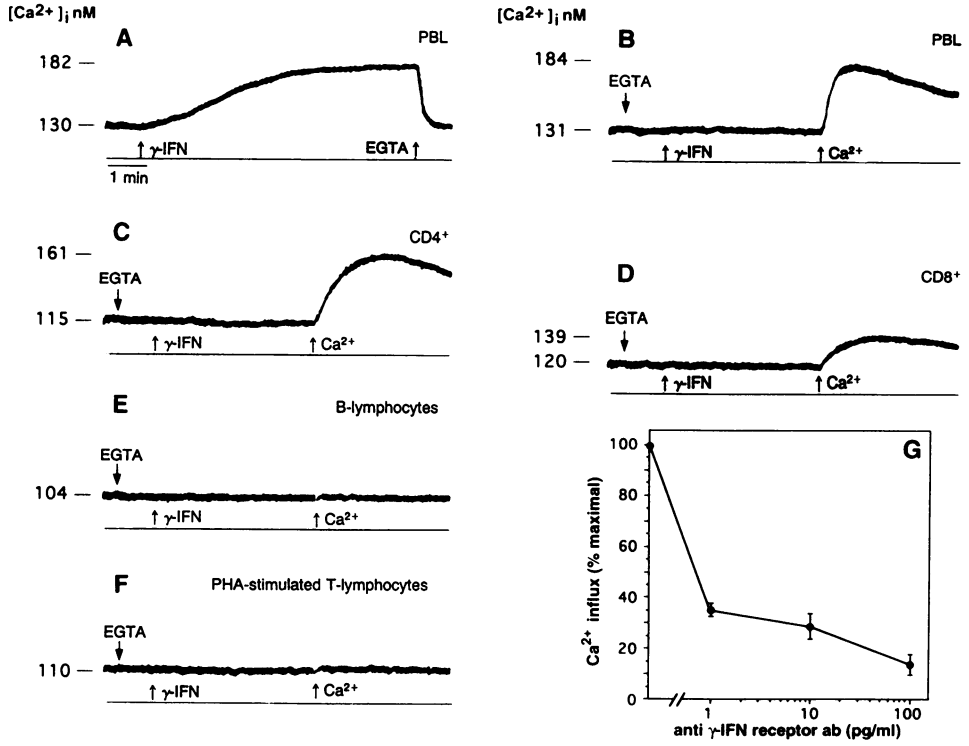

FIG. 1. Influx-sustained $[Ca^{2+}]_i$ responses induced in PBLs and in different lymphocyte subsets by γ-IFN. (*A–E*) Traces have been recorded in fura-2-loaded cells from a representative MS patient. PBLs were exposed to γ-IFN (1 pg/ml) in Ca^{2+}-containing (*A*) or Ca^{2+}-free (*B*) [obtained with the addition of excess (3 mM) EGTA where indicated] KRH medium. Under the latter conditions, a $[Ca^{2+}]_i$ rise (due to influx of the cation across the plasmalemma) was observed only when Ca^{2+} (3 mM) was reintroduced into the medium. Experiments as in *B*, but carried out with $CD4^+$, $CD8^+$, and B lymphocytes, are illustrated in *C*, *D*, and *E*, respectively. (*F*) PHA-activated T lymphocytes. (*G*) Experiments as in *B* (mean of three ± SD) in which the PBL preparations were preincubated for 30 min with the indicated concentrations of anti γ-IFN-R Ab GIR-301 (21) before exposure to γ-IFN. Traces obtained from total PBLs or lymphocyte subsets (B cells, $CD4^+$, $CD8^+$) not showing any γ-IFN-induced Ca^{2+} influx were superimposable to the trace in *E*.

patients, $P < 0.001$; stable MS vs. other neurological disease patients, $P < 0.05$). When lymphocytes were exposed to γ-IFN in the Ca^{2+}-free medium, the $[Ca^{2+}]_i$ rise observed after reintroduction of the cation was quicker and easier to measure than the slow rise observed in the Ca^{2+}-containing medium (see Fig. 1 *A* and *B*). Thus, although our experiments were carried out in both experimental conditions, only results obtained by the second approach are presented in the figures following Fig. 1*A*.

The $CD4^+$ lymphocyte subset exhibited the highest γ-IFN-induced influx, while $CD8^+$ lymphocytes responded weakly, and B lymphocytes were consistently negative (Fig. 1 *C–E*).

The dose-dependent inhibition observed (Fig. 1*G*) in PBLs preincubated with an Ab against the γ-IFN-R (Ab GIR-301; Genzyme), able to abrogate all binding of γ-IFN to γ-IFN-R (21), suggests that the Ca^{2+} influx is triggered by stimulation of the receptor. However, expression of the γ-IFN-R *per se* appears insufficient to induce Ca^{2+} influx since receptor-rich cells from healthy donors, such as resting B lymphocytes, or PHA-stimulated and alloactivated T-lymphocyte preparations (constituted by 85–95% of $CD3^+$ cells and by 50–70% of γ-IFN-R positive cells as assessed by FACS analyses; data not shown), failed to exhibit γ-IFN-induced $[Ca^{2+}]_i$ responses (Fig. 1*F*). Similar results were also observed with the γ-IFN-R-enriched lymphomonocytic cell line U937 (19).

Omission of Na^+ and Cl^- from the medium failed to modify the γ-IFN-induced influx (Fig. 2 *A* and *B*), thus excluding the involvement of neutral cotransporters and antiporters (8, 12). In contrast, influx was blocked by La^{3+} (Fig. 2*C*) and progressively inhibited by KCl-induced depolarization (Fig. 2*D*).

The γ-IFN-activated Ca^{2+} influx, revealed in MS lymphocytes, presents unique features compared to all other known Ca^{2+} influx processes (8, 12, 15). Fig. 3 shows that the γ-IFN-activated influx is impermeable to Mn^{2+} [as revealed

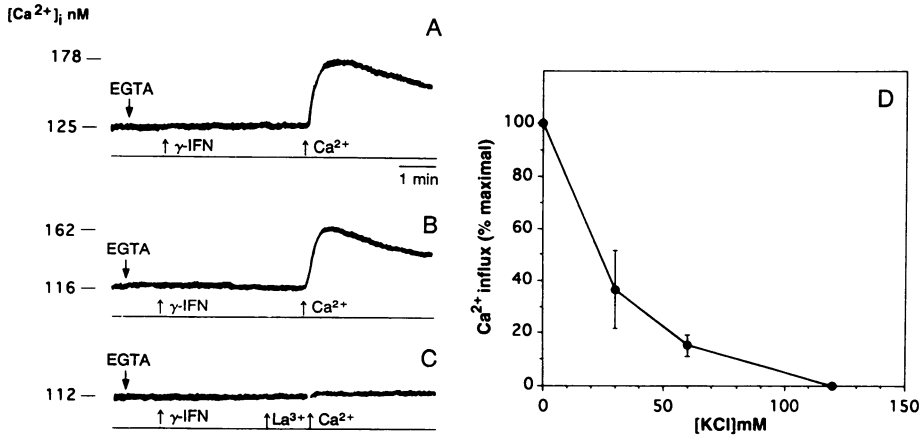

FIG. 2. γ-IFN-induced influx of Ca^{2+} in PBLs from patients with MS: Effects of ion substitutions and additions into the medium. All experiments shown were carried out according to the Ca^{2+}-free/Ca^{2+} reintroduction protocol, as described for Fig. 1*B*, using PBLs from a representative MS patient. (*A*) Standard KRH medium. (*B*) Medium containing 0.3 M sucrose and 5 mM Hepes/Tris (pH 7.4). (*C*) Sulfate- and phosphate-free KRH medium supplemented with 1 mg of gelatin per ml. La^{3+} (50 μM) was added where indicated. (*D*) Medium as described for Fig. 1*B*, but with the indicated concentrations of KCl replacing NaCl equimolarly. (*D*) Data are mean values (±SD) of three separate experiments.

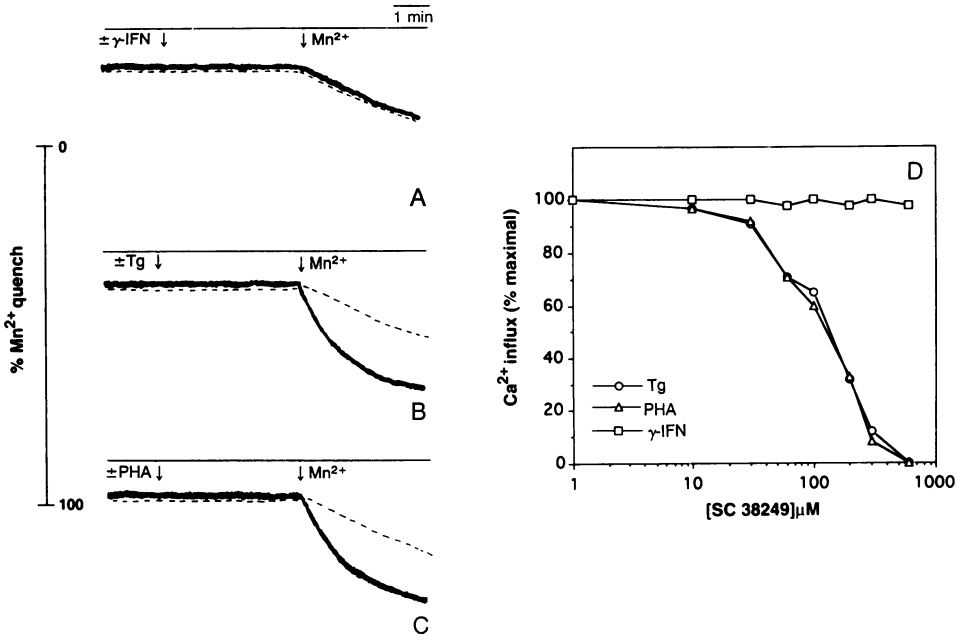

FIG. 3. Mn^{2+} and Ca^{2+} influx into PBLs from patients with MS. (*A–C*) Traces show fura-2 fluorescence recorded from suspensions of PBLs from a representative MS patient, bathed in a Ca^{2+}- and sulfate-free, phosphate-poor medium, excited at 360 nm, without (dashed lines) and with (continuous traces) the indicated treatments: γ-IFN (1 pg/ml), thapsigargin (Tg; 0.1 μM), PHA (20 μg/ml). Mn^{2+} (100 μM) was added where indicated. (*D*) PBLs suspended in the Ca^{2+}-containing KRH medium exposed first to the above indicated treatments and 3–4 min later to the indicated concentrations of the channel blocker SC 38249. (*D*) Data are from a single experiment representative of three consistent experiments.

by the fura-2 quenching technique (12)] (Fig. 3*A*) and insensitive to the imidazole drug SC 38249 (Fig. 3*D*). In contrast, the channels activated in the same PBL preparations by thapsigargin and by PHA were positive in the latter two tests (Fig. 3 *B–D*). Likewise, the γ-IFN-activated influx was unaffected by specific blockers of voltage-gated Ca^{2+} channels (diltiazem, verapamil, ω-conotoxin), by forskolin, and by overnight pretreatment with pertussis toxin.

The role of PKC in the influx induced by γ-IFN in MS lymphocytes was investigated (Fig. 4 *A–E*). When the PKC blocker staurosporin (20) was administered, a 40–60% decrease of the Ca^{2+} influx was observed (Fig. 4*A*). Conversely, administration of PMA induced a Ca^{2+} influx superimposable on that activated by γ-IFN (Fig. 4 *C* and *D*). In lymphocytes from healthy donors or patients unresponsive to γ-IFN, the phorbol ester did not affect $[Ca^{2+}]_i$ (Fig. 4*E*), even after preactivation with PHA. Finally, coadministration of optimal doses of PMA and γ-IFN did not increase the effect of either agent (Fig. 4*B*), confirming that both act on the same influx mechanism.

DISCUSSION

Our results demonstrate in T lymphocytes (primarily $CD4^+$) of MS patients the appearance of a γ-IFN-induced Ca^{2+} influx activated by PKC and functionally coupled to the γ-IFN-R. The nature of this Ca^{2+} influx cannot be established with certainty inasmuch as electrophysiological experiments have not been carried out yet. However, some of its properties (i.e., the blockade by La^{3+} and the voltage-dependence of its functioning) strongly support the influx to be sustained by a cationic channel. Compared to previously described channels, the putative one activated by γ-IFN in MS lymphocytes exhibits different features concerning pharmacology and ion permeability. The very slow activation rate, documented by the progressive $[Ca^{2+}]_i$ rise during the first 2–3 min, is consistent with a mechanism of coupling with the receptor by an enzyme (PKC) rather than a G protein. Once the $[Ca^{2+}]_i$ plateau is reached, however, it is maintained for many minutes and can therefore exert a long-standing effect on the target cells. The plateau levels of the influx we observed are moderate compared to $[Ca^{2+}]_i$ elevations de-

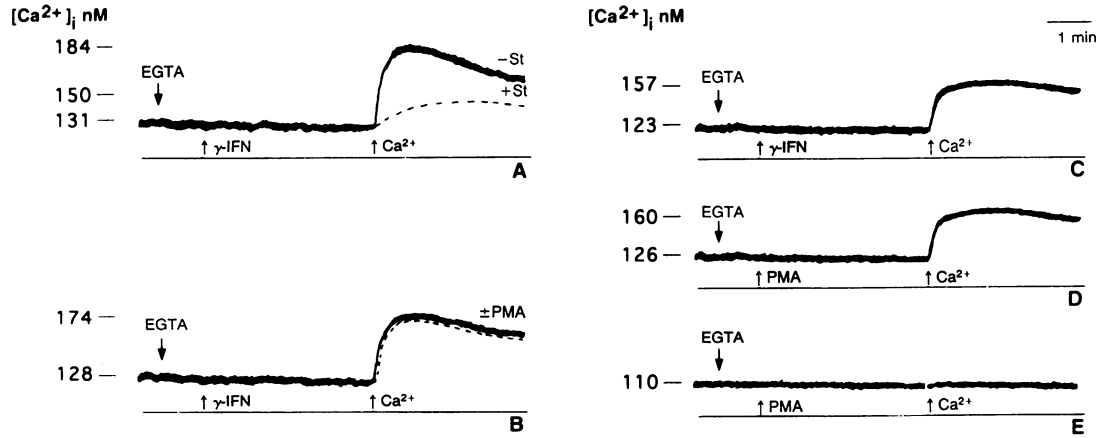

FIG. 4. Demonstration of the PKC-mediated role in the γ-IFN-induced Ca^{2+} influx. Traces (*A* and *B*, and *C* and *D*) were obtained with PBLs from two representative patients with MS. All experiments were carried out according to the Ca^{2+}-free/Ca^{2+} reintroduction protocol as described for Fig. 1*B*. (*A*) Comparison between the $[Ca^{2+}]_i$ responses induced by γ-IFN (1 pg/ml) in PBLs without (continuous trace) and with (dashed line) pretreatment with staurosporin (St; 0.1 μM, administered 15 min before γ-IFN). (*B*) Comparison of the responses induced by γ-IFN (1 pg/ml) administered without (continuous trace) and with (dashed line) PMA (0.1 μM). (*C* and *D*) Comparison of the responses induced by γ-IFN (1 pg/ml) and PMA (0.1 μM) administered to parallel aliquots of PBLs. (*E*) Lack of $[Ca^{2+}]_i$ effect of PMA (0.1 μM) administered to PBLs of a healthy control.

scribed in other cell systems using different stimuli (8, 12). It should be emphasized, however, that the analyzed cell preparations are far from homogeneous. In the responding subsets of lymphocytes the obtained values might therefore be much higher than herewith reported.

γ-IFN, mostly produced by activated T cells, is known to modulate the activity of macrophages by up-regulating surface expression of major histocompatibility complex class II molecules. The possibility that T lymphocytes are not only the producers but also the targets and effectors of γ-IFN is still open. In fact, although it is known that γ-IFN-R is up-regulated during human T-lymphocyte alloactivation (10), only limited evidence has been provided for an autocrine/paracrine control of T lymphocytes by γ-IFN (22). Our demonstration of a PKC-dependent Ca^{2+} influx in response to the interaction of γ-IFN with its receptor reveals an intracellular pathway by which such control can occur.

The cytotoxic and immune enhancing effects of γ-IFN on cell targets are considered crucial for T-lymphocyte-mediated central nervous system demyelination. This is confirmed by the increase in exacerbation rate seen in MS patients undergoing systemic γ-IFN treatment (5). Even the protective effects of systemic administration of β-IFN (6) have been attributed, at least in part, to its inhibition of γ-IFN synthesis (7). Our finding provides evidence for a cellular mechanism by which γ-IFN could trigger relapses in MS patients.

The γ-IFN-activated Ca^{2+} influx was not detected in 20% of MS patients studied within 10 days after the onset of an acute exacerbation. However, we cannot exclude that the expression of a relatively short-lived influx occurs in an early clinical, or even preclinical, phase (23). To preliminarily characterize the kinetic of the γ-IFN-activated Ca^{2+} influx, we have serially evaluated the presence of this influx in PBL samples collected every 4 days from a patient with relapsing-remitting MS experiencing an acute exacerbation. We demonstrated a close correlation between the continuous decline of the γ-IFN-activated Ca^{2+} influx and the disappearance of the symptoms (40% over resting value, first recorded at day 4; absence of detectable Ca^{2+} influx, finally recorded at day 20 when the patient had completely recovered). The recent evidence of transcriptional cytokine gene activity in circulating PBLs from MS patients observed 4–6 weeks prior to an acute attack, combined with the apparently short life-span of the γ-IFN-activated Ca^{2+} influx, might explain the absence of this influx in some of our MS patients.¶

Our observation that T lymphocytes from almost half of MS patients with clinically stable disease express the γ-IFN-activated Ca^{2+} influx is not in contrast with the possible role of this channel in the immunopathological processes occurring in MS. Magnetic resonance imaging studies have in fact clearly demonstrated ongoing disease activity in the absence of clinical signs and symptoms (24). When we analyzed PBLs from three relapsing-remitting MS patients with a particularly benign course of MS [>10 yr of disease, expanded disability status graded <3 according to Kurtzke (25), no evidence at the time of PBL collection of clinical activity or gadolinium-enhanced inflammatory lesions by magnetic resonance imaging of the brain], we did not detect any γ-IFN-activated Ca^{2+} influx.

Although this influx is a prominent feature of T lymphocytes from MS patients exposed to γ-IFN, we still need to define its involvement in other immune-mediated processes as well as its susceptibility to other extracellular stimuli. The detection of this signaling pathway of the γ-IFN-R in PBLs from three of our control patients points out that it can operate in other diseases beside MS. It is conceivable that the activation of a ubiquitous intracellular enzyme such as PKC could result from other extracellular signals; γ-IFN is probably only one, presumably the most important in MS.

Whether, and to what extent, the selective activation of Ca^{2+} influx in T lymphocytes accounts for the deleterious effects of γ-IFN in patients with MS can only be inferred at present. A better definition of the relationship between clinical activity and the appearance of this influx might ultimately lead to more accurate diagnoses and to the development of new strategies for the treatment of MS.

We thank Nicola Canal, Piercarlo Marchisio, Antony Reder, Aaron Fox, and Debbie Nelson for helpful discussion. We also thank Emanuele Bosi, Bruno Colombo, Gianfranco Ciboddo, Ubaldo Del Carro, Massimo Filippi, Giuseppe Magnani, Silvia Mammi, Vittorio Martinelli, Rocco Pastore, Antonella Poggi, Mariaemma Rodegher, Marco Rovaris, Giulio Truci, and Antonietta Volontè for providing patients. This work was supported in part by grants from Telethon–Italy (G.M.), Italian Society for Multiple Sclerosis and Lions Club International Foundation (L.M.E.G.), and Consiglio Nazionale delle Ricerche - Target Project Biotechnology and Bioinstruments (J.M.).

1. Matthews, W. B., Compston, A., Allen, I. V. & Martyn, C. N. (1991) *McAlpine's Multiple Sclerosis* (Churchill Livingstone, Edinburgh).
2. Cannella, B. & Raine, C. S. (1989) *J. Neuroimmunol.* **24**, 239–248.
3. Selmaj, K. & Raine, C. S. (1988) *Ann. Neurol.* **23**, 339–346.
4. Hartung, H.-P., Jung, S., Stoll, G., Zielasek, J., Schimdt, B., Archelos, J. J. & Toyka, K. V. (1992) *J. Neuroimmunol.* **40**, 197–210.
5. Panitch, H. S., Hirsch, R. L., Haley, A. S. & Johnson, K. P. (1987) *Lancet* **i**, 893–895.
6. The IFNB Multiple Sclerosis Study Group (1993) *Neurology* **43**, 655–661.
7. Rudick, R. A., Carpenter, C. S., Cookfair, D. L., Tuohy, V. K. & Ransohoff, R. M. (1993) *Neurology* **43**, 2080–2087.
8. Tsien, R. W. & Tsien, R. Y. (1990) *Annu. Rev. Cell Biol.* **6**, 715–760.
9. McDonald, W. I. & Halliday, A. M. (1977) *Br. Med. Bull.* **33**, 4–9.
10. Novelli, F., Giovarelli, M., Gentz, R., Zucca, M., di Pierro, F., Garotta, G. & Forni, G. (1993) *Eur. J. Immunol.* **23**, 1226–1231.
11. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450.
12. Clementi, E., Scheer, H., Zacchetti, D., Fasolato, C., Pozzan, T. & Meldolesi, J. (1992) *J. Biol. Chem.* **267**, 2164–2172.
13. Rink, T. J. (1990) *FEBS Lett.* **268**, 381–385.
14. Ciardo, A. & Meldolesi, J. (1990) *Eur. J. Pharmacol.* **188**, 417–421.
15. Miller, R. J. (1992) *J. Biol. Chem.* **267**, 1403–1406.
16. Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M. & Satoh, T. (1991) *Annu. Rev. Biochem.* **60**, 349–400.
17. Sage, S. O., Merrit, J. E., Hallam, T. J. & Rink, T. J. (1989) *Biochem. J.* **258**, 923–926.
18. Hamilton, T. A., Becton, D. L., Somers, S. D., Gray, P. W. & Adams, D. O. (1985) *J. Biol. Chem.* **260**, 1378–1381.
19. Xue-dong, F., Goldberg, M. & Bloom, B. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5122–5125.
20. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402.
21. Sheehan, K. C. F., Calderon, J. & Schreiber, R. D. (1988) *J. Immunol.* **140**, 4231–4237.
22. Hardy, K. J. & Sawada, T. (1989) *J. Exp. Med.* **170**, 1021–1026.
23. Chofflon, M., Juillard, C., Juillard, P., Gauthier, G. & Grau, G. E. (1992) *Eur. Cytokine Netw.* **3**, 523–531.
24. Miller, D. H., Rudge, P., Johnson, G., Kendall, B. E., Macmanus, D. G., Moseley, I. F., Barnes, D. & McDonald, W. I. (1988) *Brain* **111**, 927–939.
25. Kurtzke, J. F. (1983) *Neurology* **33**, 1444–1452.

¶During the 9th Congress of the European Committee for Treatment and Research in Multiple Sclerosis held in Florence, Italy (Oct. 31–Nov. 2, 1993), P. Rieckmann, M. Albecht, U. Michel, S. Poser, and the MS Study Group reported in MS patients an increase of tumor necrosis factor α mRNA expression 4–6 weeks prior to a relapse.