Biochemical and Biophysical Research Communications **284,** 126–132 (2001)
doi:10.1006/bbrc.2001.4937, available online at http://www.idealibrary.com on IDEAL®

Rac and p38 Kinase Mediate 5-Lipoxygenase Translocation and Cell Death

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Received April 30, 2001

5-Lipoxygenase (5-LO) is a key enzyme involved in the synthesis of leukotrienes from arachidonic acid, and its activation is usually followed by translocation to the nuclear envelope. The details of mechanisms involved in the translocation of 5-LO are not well understood, though Ca^{2+} is known to be essential. Here we show that ionomycin, a Ca^{2+} ionophore, induces 5-LO translocation and necrotic cell death in Rat-2 fibroblasts, suggesting a potential relationship between activation of 5-LO and cell death. These effects were markedly attenuated in Rat2-Rac^{N17} cells expressing a dominant negative Rac1 mutant. Pretreatment with SB203580, a specific inhibitor of p38 MAP kinase, or EGTA, a Ca^{2+} chelator, likewise diminished ionomycin-induced 5-LO translocation and cell death, but PD98059, a MEK inhibitor, did not. Thus, Rac and p38 MAP kinase appear to be components in a Ca^{2+}-dependent pathway leading to 5-LO translocation and necrotic cell death in Rat-2 fibroblasts.

Key Words: **Rac; p38 MAP kinase; 5-lipoxygenase; ionomycin; necrosis.**

The enzyme 5-lipoxygenase (5-LO) acts in concert with 5-LO-activating protein (FLAP) to catalyze the synthesis of leukotrienes (LTs) from arachidonic acid (AA) (1–5). Activation of soluble 5-LO is typically characterized by Ca^{2+}-dependent translocation to the nuclear membrane (6–8), which positions the enzyme close to its substrate and activating protein. However, recent studies have shown that 5-LO can be found in the cytosol of some resting cells, including polymorphonuclear neutrophils (PMNs) and peritoneal macrophages, but in the nucleus of others including mast cells and alveolar macrophages (2). Furthermore, the compartmentation of 5-LO is not static: *in vivo*, the enzyme rapidly moves from the cytosol into the nucleus when PMNs migrate from the blood into sites of inflammation and, *in vitro*, when they adhere to various substrates. Interestingly, upon stimulation, PMNs recruited into inflammatory sites secrete more leukotriene B4 (LTB_4) than those in peripheral blood. The movement of 5-LO from the cytosol into the nucleus thus correlates with increased LT synthesis in the affected cells. Similarly, alveolar macrophages, which also possess intranuclear 5-LO, produce much more LTB_4 than their progenitors, peripheral blood monocytes, in which the 5-LO is cytosolic (2, 9). Despite the critical role of 5-LO translocation in the synthesis of LTs, details of the mechanism by which 5-LO translocation is regulated are not clearly understood.

Rac belongs to the Rho family of GTPases, which mediate a wide variety of cellular responses, including the reorganization of the actin cytoskeleton, cell cycle progression, adhesion, metastasis, and gene transcription (reviewed in 10). Some members of the Rho GTPase family, including Rac, are also components of apoptotic and anti-apoptotic pathways (11–15). We recently found that activated Rac stimulates the synthesis of LTs in Rat-2 fibroblasts (16, 17), suggesting a possible signaling link between Rac and 5-LO activation. In the present study, therefore, we examined the effects of the Ca^{2+} ionophore, ionomycin, on 5-LO translocation in Rat-2 cells and characterized, in part, the signaling pathway involved.

MATERIALS AND METHODS

Chemicals and plasmids. Ionomycin, EGTA, paraformaldehyde, and Hoechst 33258 were obtained from Sigma Chemical Co. (St. Louis, MO). SB203580 and PD 098059 were from Research Biochemical International (Natick, MA). For construction of a mammalian expression vector encoding a green fluorescent protein (GFP)-5-LO

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0006-291X/01 $35.00

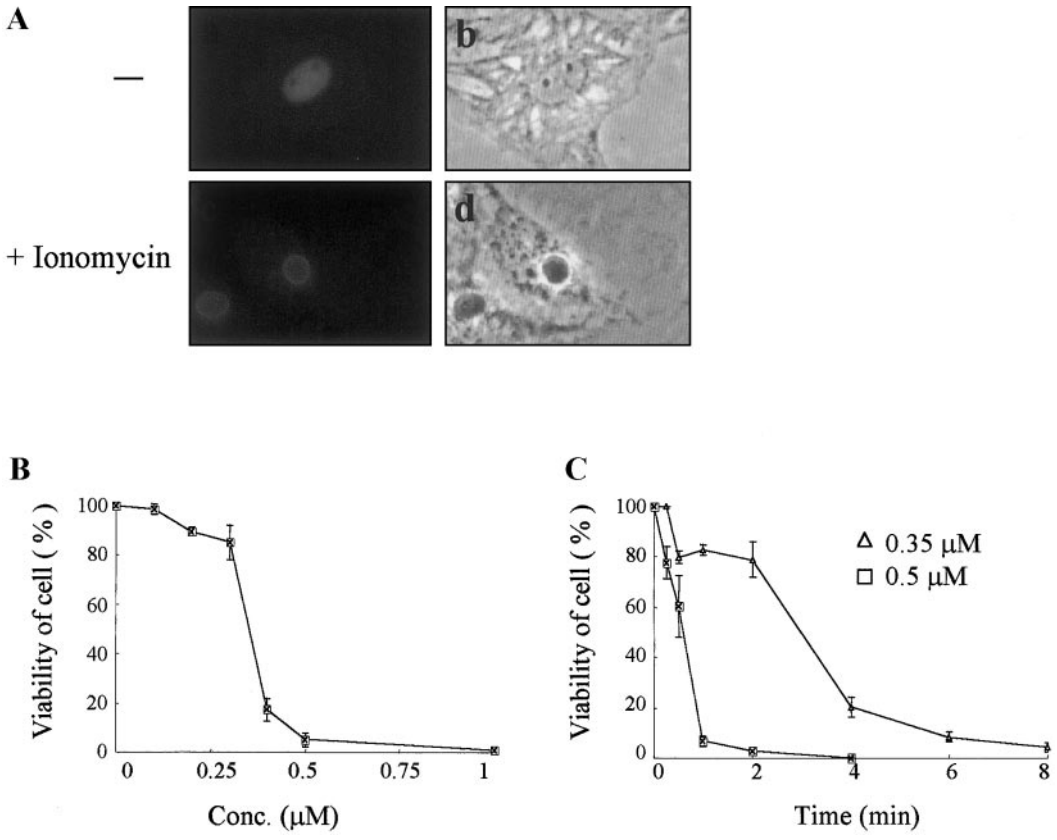

FIG. 1. Ionomycin concentration-dependently induces translocation of 5-LO and cell death. (A) Fluorescence (a and c) and corresponding bright-field (b and d) photomicrographs showing Rat-2 cells transiently transfected with GFP-5-LO fusion protein. Cell viability was evaluated followed exposure to the indicated concentrations of ionomycin (B) for the indicated times (C). At least 300 cells were counted from six different fields, and the percentages of dead cells were determined. Results are expressed as means ± SE of three independent experiments.

fusion protein, 5-LO cDNA was subcloned into the *Eco*RI site of pEGFP-N3 vector (Clontech Laboratories, Inc.). All other chemicals were from standard sources and were molecular biology grade or higher.

Cell culture and DNA transfection. Rat-2 fibroblasts were obtained from the American Type Culture Collection (ATCC, CRL 1764) and grown in DMEM supplemented with 0.1 mM nonessential amino acids (Gibco-BRL), 10% fetal bovine serum (FBS), and penicillin (50 units/ml)–streptomycin (50 mg/ml) (Gibco-BRL) at 37°C under a humidified atmosphere of 95% air/5% CO_2 (v/v). The stable Rat2-Rac^{N17} clone expressing RacN17 was described previously (18). Transient transfection was carried out by plating approximately 5×10^5 cells in 100 mm dishes for 24 h, after which calcium phosphate: DNA precipitates prepared with a total of 20 μg DNA were added to each dish (17). The total quantity of DNA in each transfection was kept constant at 20 μg by adding appropriate quantities of sonicated calf thymus DNA (Sigma Chemical Co.). For introducing the GFP-5-LO into Rat-2 or Rat2-Rac^{N17}, 4 μg of GFP-5-LO DNA was transfected using calcium phosphate methods. After 6 h of incubation with calcium phosphate:DNA precipitates, the cells were rinsed twice with phosphate-buffered saline (PBS) and then incubated serum-free DMEM for an additional 24 h.

Translocation of 5-LO. Serum-starved Rat-2 and Rat2-Rac^{N17} cells were transiently transfected with GFP-5-LO, exposed to selected concentrations of ionomycin and then fixed with 4% paraformaldehyde in PBS for 15 min. The fixed cells were stained with either 10 μg/ml Hoechst 33258 for 10 min or with an Annexin-V-FLUOS Staining Kit (Boehringer Mannheim) using the procedure recommended by the manufacturer. Changes in nuclear morphology and 5-LO translocation were observed microscopically using an Axiovert 25 inverted microscope (Carl Zeiss, Inc.); analysis of the acquired images was performed using SPOT software V2.2 (Diagnostic Instruments, Inc.).

SDS–PAGE and immunoblot analysis. Protein samples were heated at 95°C for 5 min and then subjected to SDS-PAGE on 8% acrylamide gels. The resolved proteins were then transferred to polyvinylidine difluoride membranes using a Novex wet transfer unit (2 h, 100 V). Membranes were then blocked overnight in Tris-buffered saline containing 0.01% (v/v) Tween 20 and 5% (w/v) nonfat dried milk, after which they were incubated for 2 h with the primary antibody (1:2000 dilutions anti-p38 or anti-phospho-p38) in Tris-buffered saline, and then for 1 h with horseradish peroxidase-conjugated secondary antibody. The blots were developed using enhanced chemiluminescence kits (ECL, Amersham Pharmacia Biotech).

RESULTS

Ionomycin-induced translocation of 5-LO and necrotic cell death. The intracellular distribution of 5-LO was initially analyzed in resting and ionomycin-treated Rat-2 fibroblasts. 5-LO was found to be uniformly dispersed within the nuclei of Rat-2 cells tran-

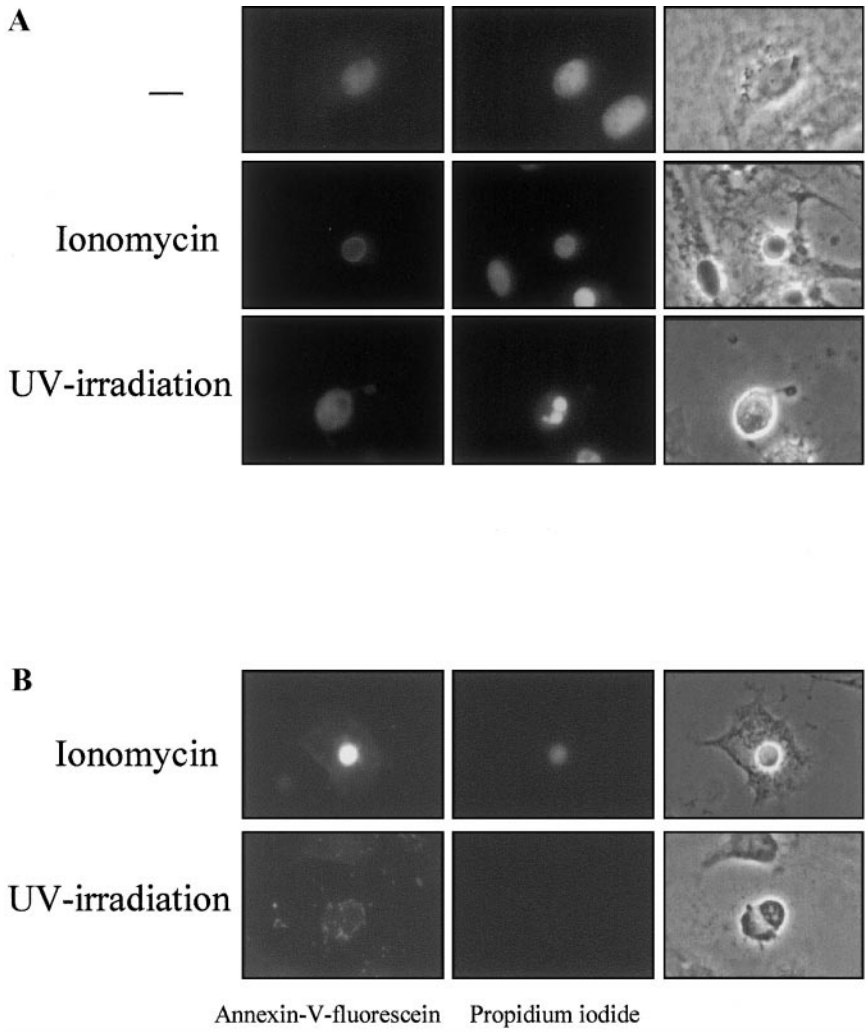

FIG. 2. Ionomycin induces necrotic cell death. (A) Rat-2 cells transiently transfected with GFP-5-LO fusion protein were exposed to 0.5 μM ionomycin for 2 min at 37°C or irradiated with UV (80 J). The photomicrographs show fluorescence (left panel) and corresponding bright-field (right panel) images, as well as images of cells stained with 10 μg/ml Hoechst 33258 (middle panel). (B) Rat-2 cells treated with ionomycin (0.5 μM, 2 min) or irradiated with UV (80 J) were stained with annexin-V-fluorecein or propidium iodide. Data are representative of three independent experiments.

siently transfected with 4 μg of GFP-5-LO plasmid and then serum-starved for 24 h (Fig. 1A-a). Upon addition of 0.5 μM ionomycin, however, GFP-5-LO rapidly translocated to the nuclear envelope (Fig. 1A-c). Bright-field microscopy showed that application of ionomycin caused the cells to rapidly round up and their nuclei to contract (Figs. 1A-b and 1A-d). More prolonged incubation in the presence of ionomycin caused cells to completely detach from the plates, indicating cell death. The effect of ionomycin was concentration-dependent, with significant cell death being observed at concentrations of 0.35 μM or above (Fig. 1B). With 0.35 μM ionomycin, for instance, ~80% of cells were dead within 4 min of exposure; more rapid cell death was observed at a concentration of 0.5 μM (Fig. 1C).

To resolve whether ionomycin-induced cell death was due to apoptosis or necrosis, cells were stained with Hoechst 33258. Consistent with induction of necrotic cell death, ionomycin (0.5 μM, 2 min) did not elicit nuclear fragmentation (Fig. 2A). By contrast, irradiation with UV (80J, 6 h) elicited pronounced nuclear fragmentation without translocation of 5-LO to the nuclear envelope. To further confirm that ionomycin induces necrotic rather than apoptotic cell death, ionomycin-treated cells were stained with annexin-V-fluorecein and propidium iodide, which are useful for distinguishing the two forms of cell death. Whereas UV-irradiated cells were stained weakly with only annexin-V-fluorecein, ionomycin-treated cells were strongly stained with both annexin-V-fluorecein and propidium iodide (Fig. 2B). Taken together, these re-

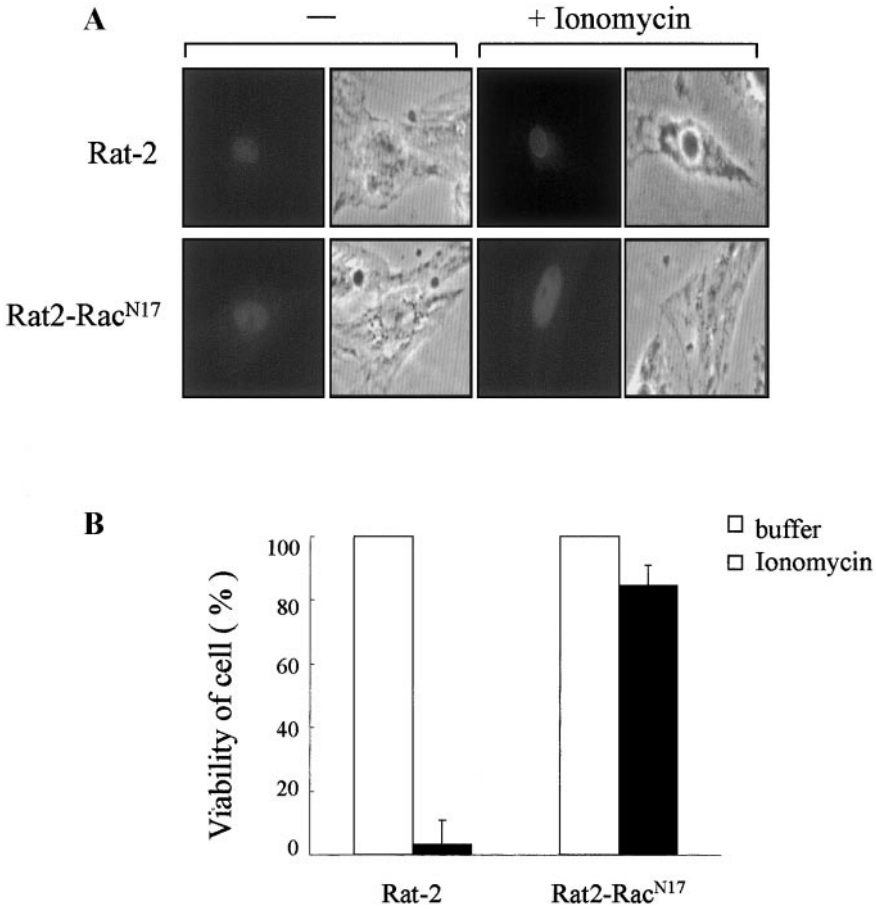

FIG. 3. Ionomycin induces 5-LO translocation and cell death via Rac1. (A) Fluorescence and corresponding bright-field photomicrographs of Rat-2 and Rat2-Rac^{N17} cells transiently transfected GFP-5-LO fusion protein and exposed to 0.5 μM ionomycin (2 min, 37°C) (+) or to buffer (−). (B) Rat-2 and Rat2-Rac^{N17} cells were exposed to 0.5 μM ionomycin (2 min, 37°C) or to buffer, after which at least 300 cells from six different fields were examined, and the percentages of dead cells determined. Results are expressed as means ± SE of three independent experiments.

sults confirm that ionomycin induces a necrotic cell death in Rat-2 fibroblasts.

Involvement of Rac in the translocation of 5-LO and cell death. On the basis of our recent finding that Rac1 enhances the synthesis of leukotrienes (16, 17), we examined the extent to which Rac1 is involved in ionomycin-induced translocation of 5-LO and the resultant necrotic cell death. It was found that whereas ionomycin (0.5 μM)-induced 5-LO translocation and cell death were clearly detected within a few seconds in Rat-2 cells, the response was markedly attenuated in Rat2-Rac^{N17} cells stably expressing dominant negative mutant form of Rac1 (Figs. 3A and 3B).

Role of p38 MAP kinase in ionomycin-induced cell death. Environmental stresses as well as proinflammatory cytokines, such as TNF-α or interleukin-1, are known to activate p38 MAP kinase via Rho family GTPases (22). To determine whether p38 MAP kinase is involved in the response to ionomycin, we pretreated cells with 15 μM SB203580, a specific p38 MAP kinase inhibitor, which dramatically diminished ionomycin-induced translocation of 5-LO (Fig. 4A) and cell death (Fig. 4B), suggesting a role of p38 kinase in the affected signaling cascade. Similar effects were elicited by chelation of extracellular Ca^{2+} using EGTA (2 mM), which is consistent with the Ca^{2+} dependency of 5-LO translocation. In addition, 0.25 μM ionomycin elicited a dramatic increase in the level of phosphorylated p38 MAP kinase within 2 min (Fig. 5). That this effect was substantially diminished in Rat2-Rac^{N17} cells suggests Rac is situated upstream of p38 kinase in the signaling pathway activated by ionomycin. In contrast, pretreatment with 10 μM of PD98059, a MEK inhibitor, had no effect on the response to ionomycin (Figs. 4A and 4B).

DISCUSSION

The results of the present study indicate that ionomycin induces a rapid translocation of 5-LO to the nuclear membrane, which is followed by necrotic cell

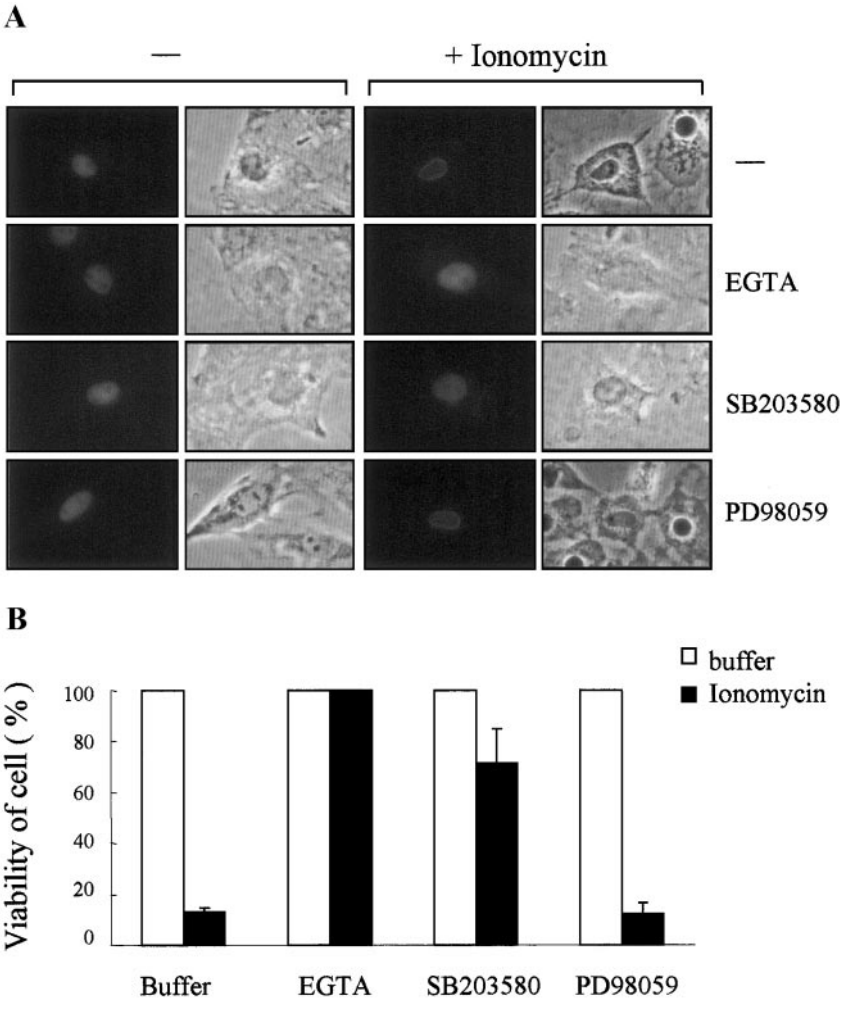

FIG. 4. Ionomycin-induced 5-LO translocation and cell death require activation of p38 MAP kinase. (A) Fluorescence and corresponding bright-field photomicrographs of Rat-2 cells transiently transfected with GFP-5-LO fusion protein, pretreated with SB203580 (15 μM) or PD98059 (10 μM) for 15 min or with EGTA (2 mM) for 30 min, and exposed to 0.35 μM ionomycin (+) or buffer (−) for 6 min at 37°C. (B) Viability was calculated by examining at least 300 cells from six different fields and determining the percentages of dead cells. Results are expressed as means ± SE of three independent experiments.

death. Comparison of ionomycin-induced 5-LO translocation in Rat-2 and Rat2-Rac^{N17} cells established a role for Rac played a role in those processes, while experiments with SB203580 established a role for p38 MAP kinase. Furthermore, activation of p38 kinase was also dramatically repressed in Rat2-Rac^{N17} cells, suggesting that p38 kinase is situated downstream of Rac in ionomycin-signaling. We do not yet know the p38 kinase target molecule(s) mediating 5-LO translocation and cell death, but one recent report suggests a possible role for p38 kinase-dependent MAP kinase-activated protein kinase 2 (MAPKAP kinase 2) in 5-LO phosphorylation in Mono Mac 6 cells and polymorphonuclear leukocytes (23).

We found that ionomycin (above 0.35 μM) caused necrotic rather than apoptotic cell death. However, we could detect apoptotic cell death at lower concentration (e.g., 0.15 μM) of ionomycin with long incubation (>5 h) (data not shown), suggesting that patterns of Ca^{2+} ionophore-induced cell death are dosage-specific; thus the necrotic cell death induced by ionomycin is likely specific for higher concentration (above 3.5 μM in Rat-2 fibroblasts). Similar to our results, Ca^{2+} ionophores have been previously shown to concentration-dependently induce either apoptosis or necrosis (24). When cultured cortical neurons were exposed to 0.25 μM ionomycin or 0.1 μM A-23187, they underwent apoptosis characterized by early degeneration of neurites, cell body shrinkage, chromatin condensation and internucleosomal DNA fragmentation. By contrast when the ionomycin concentration was increased to 1–3 μM, the neurons underwent necrosis characterized by early cell body swelling without DNA laddering.

Maccarrone *et al.* (25) recently reported that selective inhibitors of 5-LO (i.e., MK886 and caffeic acid) protected CHP100 cells against HIV gp120-induced ne-

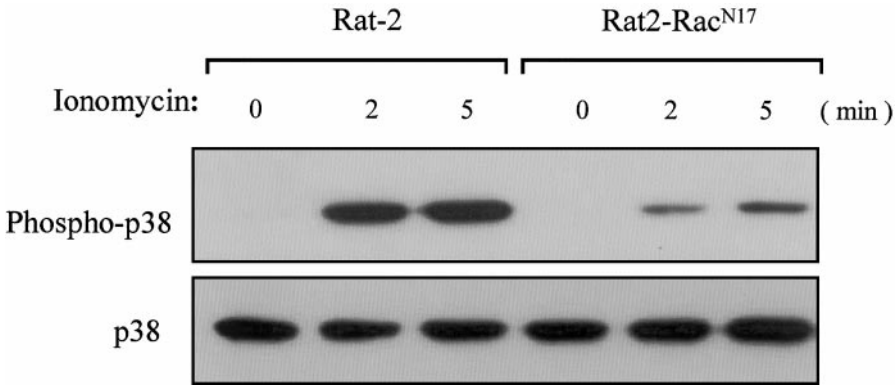

FIG. 5. Ionomycin evokes activation of p38 MAP kinase. Rat-2 and Rat2-Rac^{N17} cells were exposed to 0.35 μM ionomycin for the indicated times. Samples of their total protein were then extracted, resolved by SDS–PAGE and immunoblotted using anti-p38 MAP kinase (p38) and anti-phospho-p38 MAP kinase (Phospho-p38) antibodies as probes.

crosis, thereby linking activation of 5-LO and necrotic cell death. Nonetheless, details of the mechanism by which 5-LO mediates necrosis remain completely unknown. Indeed, to our knowledge, there has been virtually no information available on the role of Rac1 in the regulation of 5-LO translocation and subsequent necrotic cell death. While we found that ionomycin-induced Ca^{2+} influx led to necrotic cell death via Rac1, many death-inducing agents acting via Rho GTPases cause apoptotic cell death (5, 21, 26, 27). For example, Fas-induced apoptosis is mediated by activation of a Ras-Rac signaling pathway (21), and dominant-negative mutants of each Rho family member and *Clostridium difficile* toxin B, a nonselective inhibitor of Rho family members, strongly inhibited the susceptibility of cells to CTL- and Fas-induced apoptosis (26). Future studies elucidating the linkage between Rac and 5-LO translocation will be crucial for our complete understanding of the regulation of 5-LO signaling and necrotic cell death.

ACKNOWLEDGMENTS

We thank Dr. Abramovitz (Canada Merck, Canada) for the human 5-LO cDNA. This work was supported by grants from the National Research Laboratory (NRL), Molecular Medical Science Research (02-03-A-05), Life Phenomena and Function Research Group Program-2000 from the Ministry of Science & Technology, the Ministry of Health and Welfare (HMP 98-B-2-0007), Hallym Academy of Science, Hallym University, and the Brain Korea 21 (BK21) Program.

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