MOLECULAR AND CELLULAR BIOLOGY, July 1992, p. 3305–3312
0270-7306/92/073305-08$02.00/0

Vol. 12, No. 7

Role of Protein Kinase C in T-Cell Antigen Receptor Regulation of p21^{ras}: Evidence that Two p21^{ras} Regulatory Pathways Coexist in T Cells

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Received 30 January 1992/Accepted 4 April 1992

T-lymphocyte activation via the antigen receptor complex (TCR) results in accumulation of p21^{ras} in the active GTP-bound state. Stimulation of protein kinase C (PKC) can also activate p21^{ras}, and it has been proposed that the TCR effect on p21^{ras} occurs as a consequence of TCR regulation of PKC. To test the role of PKC in TCR regulation of p21^{ras}, a permeabilized cell system was used to examine TCR regulation of p21^{ras} under conditions in which TCR activation of PKC was blocked, first by using a PKC pseudosubstrate peptide inhibitor and second by using ionic conditions that prevent phosphatidyl inositol hydrolysis and hence diacylglycerol production and PKC stimulation. The data show that TCR-induced p21^{ras} activation is not mediated exclusively by PKC. Thus, in the absence of PKC stimulation, the TCR was still able to induce accumulation of p21^{ras}-GTP complexes, and this stimulation correlated with an inactivation of p21^{ras} GTPase-activating proteins. The protein tyrosine kinase inhibitor herbimycin could prevent the non-PKC-mediated, TCR-induced stimulation of p21^{ras}. These data indicate that two mechanisms for p21^{ras} regulation coexist in T cells: one PKC mediated and one not. The TCR can apparently couple to p21^{ras} via a non-PKC-controlled route that may involve tyrosine kinases.

The three *ras* proto-oncogenes, Ha-, Ki-, and N-*ras*, encode 21,000-molecular-weight guanine nucleotide-binding proteins that cause cell transformation when constitutively activated by point mutation (3). The activity of p21^{ras} is normally regulated by a cycle of binding GTP to give the biologically active form of the protein followed by hydrolysis of bound GTP to GDP (22). The GDP-bound form of p21^{ras} is inactive and is reactivated by exchange of bound GDP for free cytosolic GTP. Recent studies have shown that in T lymphocytes, triggering of the T-cell antigen receptor (TCR), the adhesion molecule CD2, or the interleukin 2 (IL-2) receptor caused a very rapid stimulation of p21^{ras}, as measured by its conversion from the GDP- to the GTP-bound state in activated cells (9, 11, 12). Ras proteins can also be regulated in fibroblasts by signals generated by the receptors for platelet-derived growth factor, epidermal growth factor, and insulin (5, 29, 30). In mast cells, p21^{ras} is activated by the cytokines IL-3 and granulocyte macrophage colony-stimulating factor (31).

In T lymphocytes, phorbol esters that activate protein kinase C (PKC) can mimic TCR triggering and induce accumulation of p21^{ras}-GTP complexes (9). The TCR is known to stimulate PKC by a mechanism involving the generation of diacylglycerols (DAGs; the endogenous PKC activators) via phospholipase C (PLC)-mediated hydrolysis of inositol phospholipids (PtdIns) (17, 27). TCR-stimulated PKC could thus mediate the p21*ras* activation seen upon triggering of this receptor. The proposal that PKC functions as an upstream regulator of p21^{ras} in T cells is in contrast to the previously described link between p21^{ras} and PKC in fibroblasts, where, based on observations that "active" p21^{ras} proteins are able to stimulate PKC (8, 25), PKC is thought to operate downstream of p21^{ras}. In this context, it is notable that signal transduction mechanisms involving PKC apparently do not explain how growth factors activate p21^{ras} in fibroblasts and myeloid cells, because in these cells, stimulation of PKC with phorbol esters does not induce the accumulation of p21^{ras}-GTP complexes, indicating that a PKC-mediated pathway for regulation of p21^{ras} is absent (18, 23). Furthermore, receptors such as the insulin receptor in fibroblasts and the IL-2 receptor in T cells can stimulate p21^{ras} even though signal transduction by these receptors is not considered to be mediated via PKC (11, 23). There must therefore be an additional, non-PKC-mediated route for controlling p21^{ras} activation. There are indications that regulation of protein tyrosine kinases (PTKs) may be important in the non-PKC pathway for activating p21^{ras} in fibroblasts (23). Similarly, in T cells, PTKs may be involved in TCR and IL-2 receptor regulation of p21^{ras}, since it is known that these receptors interact with nonreceptor PTKs (14, 15, 20).

It appears that in T cells, at least two mechanisms for p21^{ras} regulation coexist, one of which is PKC mediated and one of which is not. The molecular details of the non-PKC-mediated p21^{ras} control mechanism are not yet known, but the IL-2 receptor, which apparently does not activate PKC (24), must act on p21^{ras} proteins entirely through this second pathway. As discussed above, the TCR could couple to p21^{ras} via the PKC-dependent mechanism. This hypothesis is compatible with previous observations that PKC mediates TCR regulation of a number of secondary signal transduction responses, including regulation of c-*raf* and MAP-2 kinase (26, 32). However, the TCR can also couple to signal transduction pathways involving stimulation of PTKs (20), indicating that not all of the regulatory effects of the TCR are necessarily attributable to PKC activation. The object of the present study, therefore, was to explore the role of PKC in

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coupling the TCR to p21^{ras} in T lymphocytes. Our experiments were based on examining the activation of p21^{ras} by the TCR in a permeabilized cell system in which TCR activation of PKC was prevented by two strategies. First, receptor-stimulated PKC activity and function were blocked by a PKC pseudosubstrate peptide inhibitor (PS). Second, the activity of PtdIns-specific PLC is strictly dependent on calcium (28), and in permeabilized T cells, it is possible to prevent TCR stimulation of PLC and hence PKC stimulation by using intracellular buffers with zero calcium levels (13). Our experiments reveal that it is possible to fully block TCR activation of PtdIns metabolism and PKC activation without preventing the stimulation of p21^{ras} by this receptor. These data suggest that the TCR can use a PKC-"independent" mechanism to couple to p21^{ras}. Studies using herbimycin, an inhibitor of PTKs in T cells (19), suggest that the non-PKC mechanism for activating p21^{ras} in T cells may involve tyrosine phosphorylation.

MATERIALS AND METHODS

Material and cells. Purified reduced streptolysin-O (SLO) and phytohemagglutinin were obtained from Wellcome Diagnostics (Dartford, Kent, United Kingdom). The peptide GS has the sequence Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, and its properties as a selective PKC substrate in T cells have been previously reported (1). The PKC pseudosubstrate peptide inhibitor PS has the sequence Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Glu-Lys-Asn-Val and is a potent inhibitor of the enzyme in vitro (16) and in permeabilized T cells (1). Human recombinant IL-2 was obtained from Cetus. Human T lymphoblasts were prepared as described elsewhere (6) by stimulating peripheral blood mononuclear cells (10^6/ml) in RPMI 1640–10% (vol/vol) fetal calf serum with 5 μg of phytohemagglutinin ml for 72 h. After being washed, cells were maintained in exponential growth phase in RPMI 1640–10% fetal calf serum supplemented with 20 ng of recombinant IL-2 per ml.

Cell permeabilization. T lymphoblasts were permeabilized with SLO as previously described (13). Cells were washed twice in phosphate-buffered saline and suspended at 5×10^7 cells per ml in permeabilization medium containing 0.4 IU of SLO per ml, 120 mM KCl, 30 mM NaCl, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.2), 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 5 mM $MgCl_2$. The total concentration of $CaCl_2$ required to give a free-Ca^{2+} concentration in the range of nominal 0 to 1 μM at pH 7.2 was predicted by the computer program CHELATE by using appropriate dissociation constants for Ca^{2+}, Mg^{2+}, and H^+ (13).

p21^{ras} activation. p21^{ras} proteins were immunoprecipitated with antibody Y13-259 from T lymphoblasts in which guanine nucleotides were labeled with ^{32}P. Two methods of guanine nucleotide labeling were adopted: (i) cells were labeled biosynthetically with $^{32}P_i$ as described elsewhere (9), and (ii) cells were permeabilized in a cytosolic buffer containing [α-^{32}P]GTP and 1 mM ATP. For the first method, a modification of the protocol previously described (9) was used. Cell lysis was performed in 50 mM HEPES (pH 7.4)–100 mM NaCl–1% Triton X-114–5 mM $MgCl_2$, 1 mg of bovine serum albumin, 10 μM benzamidine–10 μg of aprotinin per ml (lysis buffer A). Nuclei were removed by centrifugation at 15,000 × *g* for 4 min, and 0.5 M NaCl was added to the lysate. Lysates were incubated at 37°C for 2 min to induce precipitation of the detergent. Phase split was performed by centrifugation at 15,000 × *g* for 2 min. The detergent-containing pellet was suspended in ice-cold lysis buffer A containing 0.5 M NaCl, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate (SDS), and 1% Triton X-100 instead of Triton X-114. For the second method, cell lysis was in lysis buffer A containing 1% Triton X-100 instead of Triton X-114. After the nuclei were removed, 0.5 M NaCl, 0.5% deoxycholate, and 0.05% SDS were added to the lysate. For both protocols, immunoprecipitation was for 40 min with antibody Y-259 precoupled to protein A agarose via rabbit anti-rat immunoglobulin. Washing of immunoprecipitates and elution of guanine nucleotides bound to p21^{ras} was performed as previously described (9). Labeled nucleotides, separated by thin-layer chromatography (polyethyleniminecellulose), were quantitated by direct scanning for β radiation with an Ambis β Scanner. Results are expressed as proportion of p21^{ras} bound to GTP (i.e., ratio between GTP- and GTP-GDP-labeled nucleotides on p21^{ras}).

Peptide GS phosphorylation in permeabilized T cells. Peptide GS phosphorylation assays in cells permeabilized with SLO were performed as described before (1). Briefly, 5×10^6 cells were permeabilized with or without stimuli in 250 μl of permeabilization medium containing the indicated Ca^{2+} concentration, 100 μM [γ-^{32}P]ATP, and 125 μM peptide GS substrate in the presence or absence of different concentrations of PKC pseudosubstrate inhibitor (PS). Separation and evaluation of the peptide GS phosphorylation was performed as previously detailed (1). Results are expressed as picomoles of phosphate incorporated into the peptide during the assay time per number of cells used in the assay.

PA production in permeabilized T cells. T lymphoblasts (5×10^6) were permeabilized with or without stimuli in 250 μl of permeabilization medium containing different Ca^{2+} concentrations and 400 μM [γ-^{32}P]ATP. In some experiments, 50 μg of synthetic DAGs (stearoyl arachidonoyl glycerol [SAG], dioctanoylglycerol [DOG]) per ml was added to control the activity of endogenous DAG kinase. After 5 min of stimulation, lipids were extracted by the addition of 0.6 ml of chloroform-methanol-concentrated HCl (in a ratio of 1:2:0.01). After vortexing, a phase split was achieved by the addition of 150 μl of 10 mM HCl and 150 μl of chloroform followed by centrifugation at 8,000 × *g* for 5 min. Aliquots of the chloroform (lower) phase were loaded onto silica gel 60A thin-layer chromatography plates (Whatman, Kent, United Kingdom) that had been previously sprayed with a freshly prepared solution of 1% (wt/vol) potassium oxalate and allowed to dry for at least an hour. The chromatography plate was run in chloroform-methanol-acetone-acetic acid-water (80:26:30:24:16). Phospholipid species and phosphatidic acid (PA) were identified by comparison with unlabeled standards that were visualized by exposure to iodine vapor. In the conditions of the assay, any DAG produced is phosphorylated to form PA by endogenous DAG kinase activities.

Activity of cellular p21^{ras} GAPs. T lymphoblasts (5×10^7) were suspended in 1 ml of permeabilization buffer (see above) at 37°C (with or without calcium) for each treatment. Cells were unstimulated (control) or stimulated with phorbol ester or anti-TCR antibody (UCHT1) at the same time they were permeabilized with SLO (0.4 IU/ml). The cells were incubated for 5 min at 37°C, collected by centrifugation (15,000 × *g*, 30 S), and lysed in 1 ml of lysis buffer (50 mM HEPES [pH 7.5], 1% Triton X-100, 5 mM $MgCl_2$, 20 mM NaCl, 10 mM benzamidine, 10 μg of trypsin inhibitor per ml, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM *para*-nitrophenyl phosphate, 1 mM sodium orthovanadate,

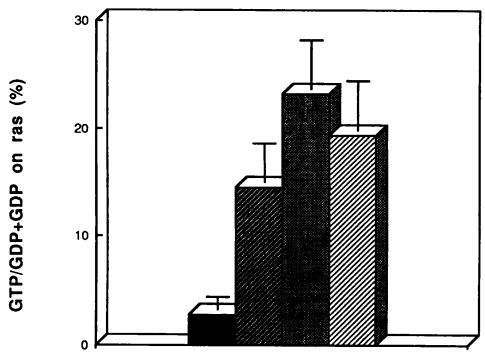


FIG. 1. Effects of different stimuli on the activation state of p21^{ras} in T lymphoblasts. β-scan data corresponding to thin-layer chromatography of the guanine nucleotides eluted from immunoprecipitates of p21^{ras} from T lymphoblasts permeabilized with SLO in the presence of 100 nM Ca^{2+}, 5 mM Mg^{2+}, and [α-^{32}P]GTP. Cells were either unstimulated (■) or stimulated with 100 ng of PDBu (▧), 50 μg of DOG (▨), or 10 μg of the monoclonal antibody UCHT1 (▩) per ml for 5 min. After cell lysis, immunoprecipitation was performed with p21^{ras} monoclonal antibody Y13-259 as indicated in Materials and Methods. Data are expressed as the ratio of [α-^{32}P]GTP to total α-^{32}P-labeled nucleotide on p21^{ras} expressed as percentages and are averages of the results obtained from five experiments. No nucleotides were visualized when the immunoprecipitation was performed with an irrelevant antibody.

20 mM NaF). This lysate was clarified by centrifugation at 350,000 × *g* for 10 min and normalized for protein concentration. Various concentrations of the lysate were assayed for GTPase-activating protein (GAP) activity (15 min at 37°C) in duplicate against bacterially expressed wild-type p21^{ras} by using the method previously described (9). Lysate was diluted as appropriate with lysis buffer.

RESULTS

Activation of p21^{ras} in SLO-permeabilized cells. In SLO-permeabilized T cells, the activation of p21^{ras} can be monitored by examining the kinetics of exchange and hydrolysis of [α-^{32}P]GTP on p21^{ras} proteins in stimulated or quiescent T cells. In T cells permeabilized in a cytosolic buffer that maintains calcium levels at 100 nM free Ca^{2+} (the intracellular calcium concentration of quiescent T cells), the PKC activators phorbol dibutyrate (PDBu) and DOG were able to induce an increase in the proportion of p21^{ras}-GTP complexes (Fig. 1). In the absence of stimulation, approximately 1% of the p21^{ras} was in the GTP-bound state, whereas in PDBu- and DOG-stimulated cells, approximately 20% of p21^{ras} bound GTP. Similarly, T-cell activation with the TCR agonist UCHT1 also results in activation of p21^{ras}. We have shown previously that the accumulation of p21^{ras}-GTP complexes as a result of PKC stimulation and TCR triggering in permeabilized T cells results from a decrease in the hydrolysis of [α-^{32}P]GTP on p21^{ras}: no differences in nucleotide exchange kinetics have been detected (9).

Effect of a PKC pseudosubstrate peptide inhibitor on p21^{ras} activation. To clarify the role of PKC in TCR regulation of p21^{ras}, the effect of a specific PKC pseudosubstrate peptide inhibitor (PS) (2, 16) on p21^{ras} activation in permeabilized cells was examined. In initial experiments, the conditions required for the PS to block PKC activation by PDBu and TCR triggering were determined. To measure PKC activation in permeabilized cells, a rapid and sensitive assay for all known PKC isozymes was used. This assay is based on the phosphorylation of a PKC substrate peptide (peptide GS), and its specificity and sensitivity in permeabilized T cells have been previously described (1). The kinetics of peptide GS phosphorylation in PDBu- and TCR-activated SLO-permeabilized T cells demonstrate that 100 μM of the PS peptide efficiently blocked GS phosphorylation induced by PDBu and the TCR agonist UCHT1 (Fig. 2A). These data support previous observations that the PKC PS is an inhibitor of the phosphorylation of cellular substrates for PKC in T cells and is able to equally block PKC activation induced by phorbol esters or TCR agonists (1, 2).

Figure 2B shows the effect of the PKC PS on p21^{ras} regulation in SLO-permeabilized T cells. The PS at 100 μM was able to efficiently block the PDBu-induced accumulation of p21^{ras}-GTP complexes in T lymphocytes, which correlated with its effects on PDBu-induced PKC activation (Fig. 2A versus Fig. 2B). The PKC PS did not inhibit exchange of guanine nucleotide onto p21^{ras} in unstimulated or PDBu-activated cells. In TCR-stimulated, SLO-permeabilized T cells, [α-^{32}P]GTP was also able to exchange rapidly on the p21^{ras} proteins irrespective of the presence of the PKC PS (Fig. 2B). However, in contrast to the inhibiting effect of the PS on PDBu regulation of p21^{ras}, TCR-induced accumulation of p21^{ras}-GTP complexes was apparently not blocked by the PS (Fig. 2B). The data in Fig. 3 are the means of three experiments with the PKC PS inhibitor. The data confirm that this inhibitor can induce >80% inhibition of PDBu-induced p21^{ras} activation while having no significant effect on UCHT1-induced accumulation of p21^{ras}-GTP complexes.

Role of PLC-mediated PtdIns metabolism in p21^{ras} regulation. The major mechanism for TCR regulation of PKC involves TCR-induced, PLC-mediated hydrolysis of PtdIns, which generates inositol polyphosphates that modulate intracellular calcium levels and DAGs that stimulate PKC (17, 27). DAGs produced from the hydrolysis of PtdIns are phosphorylated by DAG kinases to form PA, which is then used for the cyclic resynthesis of PtdIns. Accordingly, an effective way to block TCR stimulation of PKC is to block TCR induction of PtdIns metabolism. PtdIns-specific PLC is a calcium-dependent enzyme (28), and when T cells are SLO permeabilized in a cytosolic buffer with calcium buffered to <1 nM, agonist-induced PtdIns hydrolysis and hence PKC activation are prevented (13). Accordingly, in T cells permeabilized in a 100 nM calcium buffer in the presence of [γ-^{32}P]ATP, TCR triggering induces a 20-fold increase in the levels of ^{32}P-labeled PA after TCR triggering (Fig. 4), whereas TCR-induced PA production is completely prevented in a 0 nM calcium buffer (Fig. 4). The failure to detect ^{32}P-labeled PA in the 0 nM Ca^{2+} buffer did not reflect the loss of DAG kinase function, since the phosphorylation of exogenous SAG and DOG was not significantly affected (<20% inhibition) by the absence of calcium in the SLO-permeabilized cells (Fig. 4). These data indicate that TCR-induced PLC activity and PA production are prevented in cells permeabilized in the absence of calcium. To confirm that TCR-induced PKC activation was also prevented, the ability of UCHT1 to induce phosphorylation of the PKC substrate peptide GS in either a 100 or a 0 nM Ca^{2+} buffer was monitored (Fig. 5). The data (Fig. 5) show that PDBu and DOG could efficiently stimulate PKC in the 0 nM Ca^{2+} buffer. In contrast, the TCR agonist UCHT1 did not induce PKC activation in the absence of calcium, as judged by peptide GS phosphorylation.

Since TCR-mediated PKC activation was blocked in the 0 nM Ca^{2+} buffer, the ability of the TCR to induce p21^{ras} activation in this buffer was examined. The data in Fig. 6

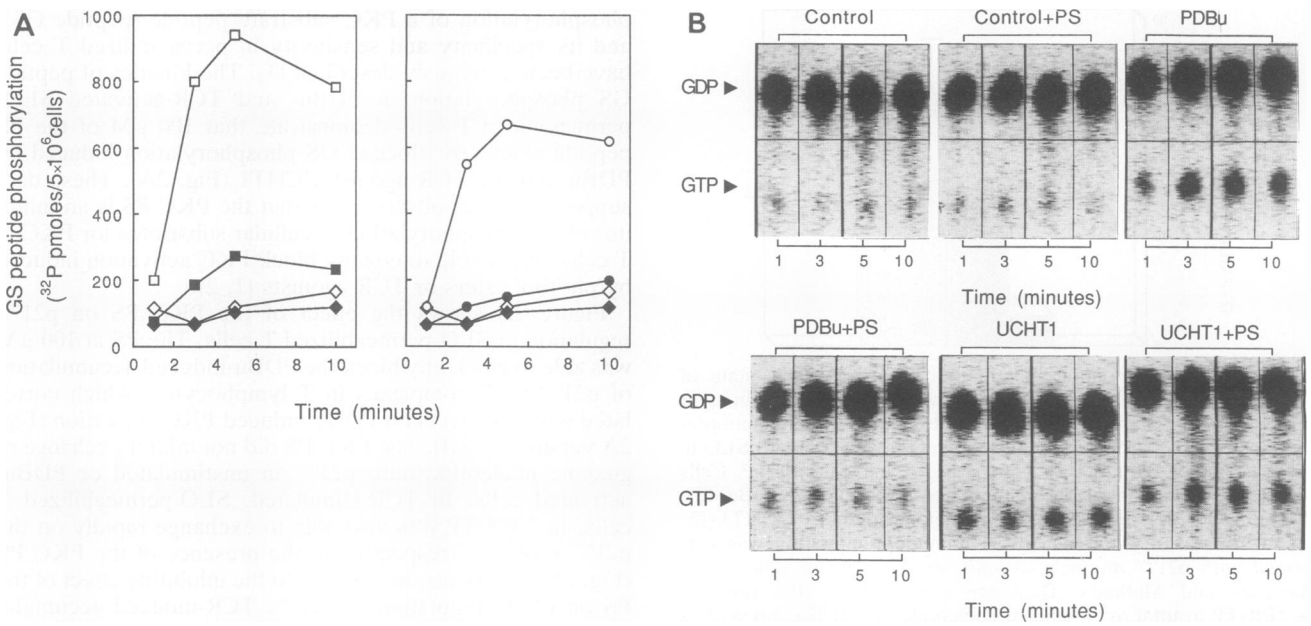


FIG. 2. Effect of a PKC pseudosubstrate peptide inhibitor on the stimulation of GS peptide phosphorylation and p21^{ras} activation in permeabilized T lymphoblasts. (A) T lymphoblasts were permeabilized in the presence of 100 nM Ca^{2+}, [γ-^{32}P]ATP, and 250 μM peptide GS. Cells were either unstimulated (◇, ◆) or stimulated with 100 ng of PDBu (□, ■) or 10 μg of UCHT1 (○, ●) per ml for the indicated times in the absence (open symbols) or presence (closed symbols) of 100 μM peptide PS. The peptide GS was recovered and washed, and ^{32}P incorporation in it was quantitated by scintillation counting. Data are means of duplicate values expressed as picomoles of phosphate incorporated into peptide during the assay time per number of cells used. (B) Thin-layer chromatogram of the guanine nucleotides eluted from Y13-259 immunoprecipitates of p21^{ras} that were permeabilized and stimulated as indicated in panel A. The positions at which GTP and GDP standards ran are indicated.

compare UCHT1, DOG, and PDBu activation of p21^{ras} in either a 100 or a 0 nM Ca^{2+} buffer. All three stimuli were able to regulate the accumulation of p21^{ras}-GTP complexes in both the presence and the absence of calcium in the permeabilization buffer. There was a partial inhibition of TCR activation of p21^{ras} (20 to 40% range in three experiments) in the absence of calcium compared with >95% inhibition of TCR-controlled PA production and PKC activation under the same conditions (Fig. 4 and 5).

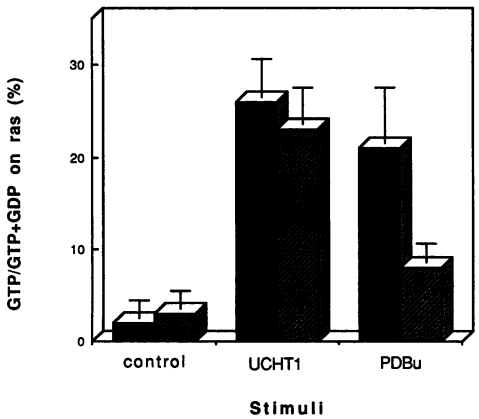


FIG. 3. Effect of pseudosubstrate inhibitor of PKC on the stimulation of p21^{ras} in permeabilized T lymphoblasts. T lymphoblasts were permeabilized as described in the legend to Fig. 1 in the absence (■) or presence (▨) of 75 μM PKC pseudosubstrate inhibitor (PS). Cells were either unstimulated (control) or stimulated with 10 μg of monoclonal antibody UCHT1 or 100 ng of PDBu per ml for 5 min prior to lysis, and immunoprecipitation of p21^{ras} was with monoclonal antibody Y13-259 as described in the text. Nucleotides were separated by thin-layer chromatography and quantitated by direct scanning for β radiation. The histogram shows the proportion of p21^{ras} bound to GTP, and the data are means of three experiments.

Previous studies have established that stimulation of PKC in T cells results in inhibition of the activity of a p21^{ras}-GAP protein (9). The data in Fig. 7 reveal that TCR stimulation of T cells permeabilized in either a 100 or a 0 nM Ca^{2+} buffer results in inhibition of p21^{ras}-GAP activity in cell lysates. Thus, the TCR can induce inactivation of p21^{ras}-GAP proteins in the absence of PKC stimulation.

PTK inhibitor herbimycin prevents TCR-induced p21^{ras} activation. One of the earliest consequences of TCR triggering is the tyrosine phosphorylation of a number of proteins (20). To determine whether protein tyrosine phosphorylation was important in p21^{ras} activation, the effect of the PTK inhibitor herbimycin was examined. It has been previously described that herbimycin treatment of T cells inhibits TCR-mediated substrate tyrosine phosphorylation, PtdIns hydrolysis, and calcium elevation. In contrast, phorbol ester-induced effects (i.e., PKC-mediated phosphorylation and activation of c-*raf* kinase) were resistant to herbimycin (7, 19). In preliminary experiments, it was established that, in agreement with previous reports (19), T lymphoblasts exposed to 1 μM herbimycin for 24 h were unable to induce tyrosine phosphorylation of cellular substrates in response to TCR triggering as detected by Western blotting (immunoblotting) analysis with antiphosphotyrosine antibody (data not shown). As predicted, TCR regulation of PtdIns hydrolysis was also inhibited by herbimycin pretreatment of cells, as judged by the inhibitory effect of this tyrosine kinase inhibitor on TCR-mediated generation of PA (Fig. 8A).

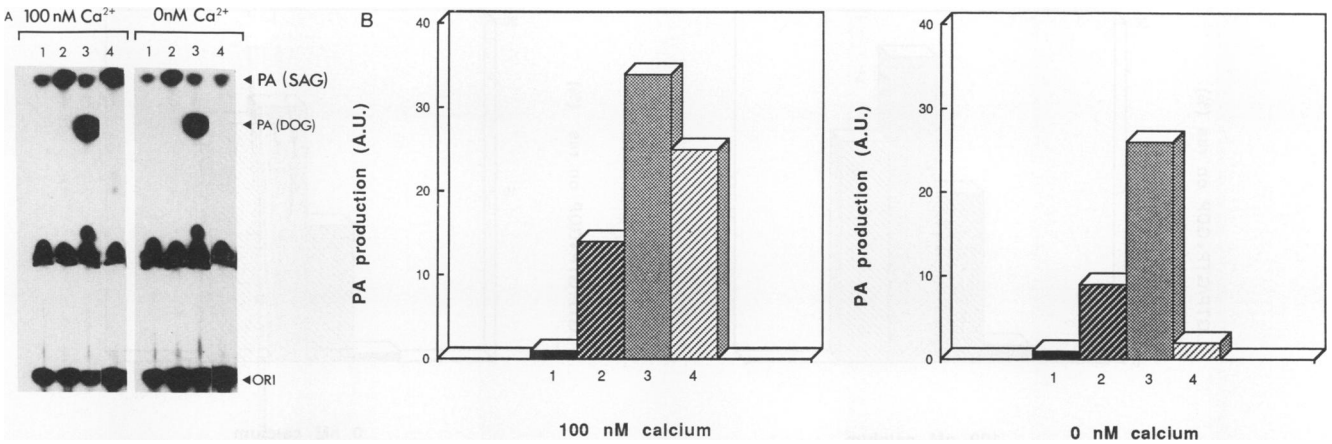


FIG. 4. PA production in response to UCHT1 in T lymphoblasts permeabilized with 0 nM Ca^{2+} buffer. T lymphoblasts were permeabilized with SLO in the presence of 5 mM Mg^{2+} and [γ-^{32}P]ATP with either 100 or 0 nM Ca^{2+} buffer. Cells were either unstimulated (lane and bar 1) or stimulated with 50 μg of SAG (lane and bar 2), 50 μg of DOG (lane and bar 3), or 10 μg of monoclonal antibody UCHT1 (lane and bar 4) per ml. The phospholipids extracted after 5 min by addition of an acidified chloroform-methanol mixture were separated by thin-layer chromatography in parallel with lipid standards and were subjected to autoradiography (A) and β-emission-scan quantification (B). The positions corresponding to PA SAG and PA DOG are shown.

Herbimycin did not block GTPγS, i.e., G-protein-induced PA production, or the phosphorylation of exogenous SAG.

The data in Fig. 8B show that in intact $^{32}P_i$-labeled cells, herbimycin pretreatment could completely block TCR-induced accumulation of p21^{ras}-GTP complexes but did not prevent PDBu-induced stimulation of p21^{ras}. To explore the role of PTKs in the PKC-independent pathways of p21^{ras} activation, the effect of herbimycin on TCR coupling to p21^{ras} in T cells permeabilized by SLO in the 0 nM Ca^{2+} buffer was determined, since these are conditions under which the TCR is unable to stimulate PKC. As shown in Fig. 8C, pretreatment with herbimycin efficiently abolished the TCR-mediated activation of p21^{ras} in T cells permeabilized in the absence of calcium, while the PDBu and DOG stimulation of p21^{ras} were unchanged.

DISCUSSION

In T lymphocytes, phorbol esters and DAGs, which are activators of PKC, can induce activation of p21^{ras}. The stimulatory effects are blocked by a PKC pseudosubstrate peptide inhibitor, PS, which indicates the existence of a PKC-controlled route for p21^{ras} regulation. One immediate consequence of triggering the TCR is stimulation of PKC, and since the TCR also activates p21^{ras}, it was proposed that PKC is involved in TCR coupling to p21^{ras}. However, the present data demonstrate that when TCR stimulation of PKC is blocked, then TCR stimulation of p21^{ras} still occurs. First, the PKC inhibitor PS, which could efficiently prevent TCR stimulation of PKC, did not block TCR-induced accumulation of p21^{ras}-GTP complexes. Second, the TCR could

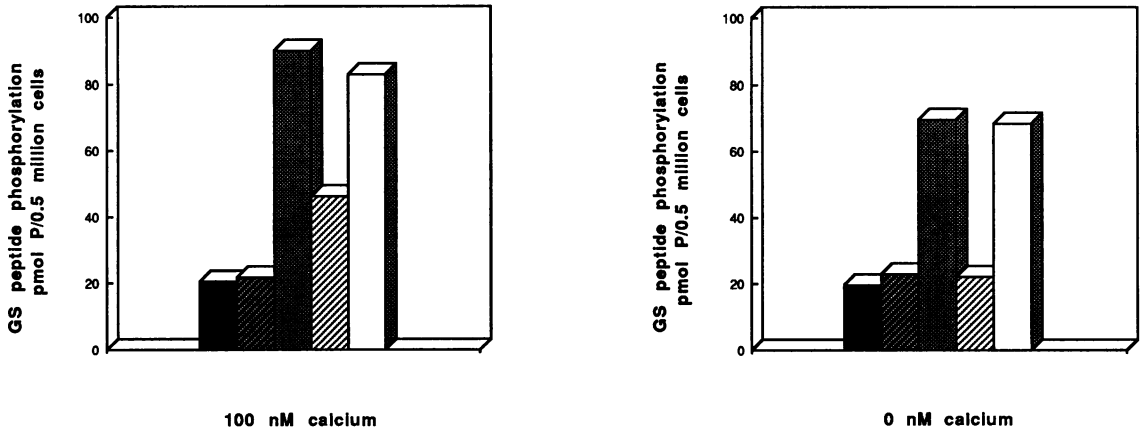


FIG. 5. Peptide GS phosphorylation induced by different stimuli in T lymphoblasts permeabilized with 0 nM Ca^{2+} buffer. T lymphoblasts were permeabilized with SLO in the presence of 5 mM Mg^{2+}, [γ-^{32}P]ATP, and 125 μM peptide GS with either 100 or 0 nM Ca^{2+} buffer. Cells were either unstimulated (control [■]) or stimulated with 50 μg of SAG (▨), 50 μg of DOG (▩), 10 μg of monoclonal antibody UCHT1 (▨), or 100 ng of PDBu (□) per ml for 5 min. The peptide was recovered and washed, and ^{32}P incorporation into it was quantified by using scintillation counting as indicated in Materials and Methods. Data are averages of duplicate values and are representative of several experiments.

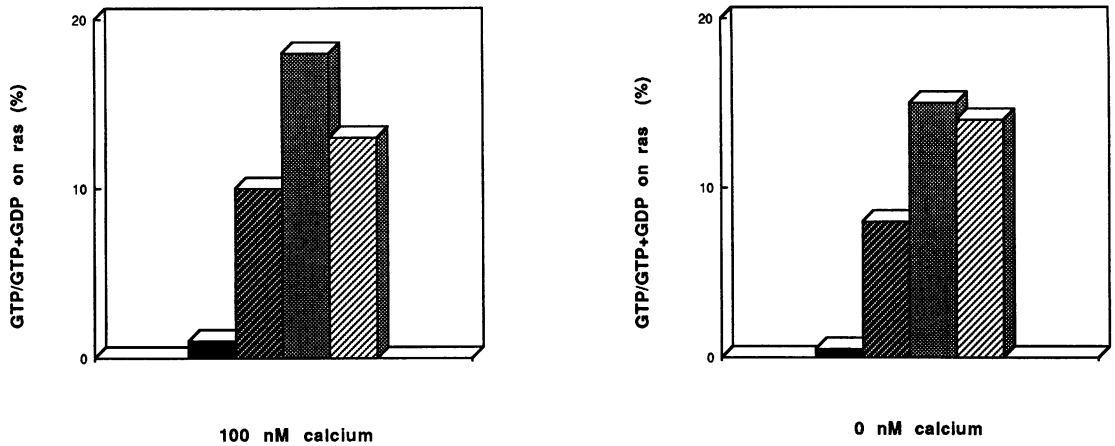


FIG. 6. Effects of different stimuli on the activation state of $p21^{ras}$ in T lymphoblasts permeabilized with 0 nM Ca^{2+} buffer. T lymphoblasts were permeabilized with SLO in the presence of 5 mM Mg^{2+} and [α-^{32}P]GTP with either 100 or 0 nM Ca^{2+} buffer. Cells were either unstimulated (control [■]) or stimulated with 50 μg of DOG (▨), 10 μg of monoclonal antibody UCHT1 (▩), or 100 ng of PDBu (▨) for 5 min. After cell lysis, the guanine nucleotides eluted from immunoprecipitates of $p21^{ras}$ were resolved by thin-layer chromatography, and the proportion of labeled nucleotides bound to $p21^{ras}$ was quantified by β-emission scan. The histogram shows the ratio of [α-^{32}P]GTP to total α-^{32}P-labeled nucleotide as a percentage, and the data are representative of several experiments.

stimulate $p21^{ras}$ in T cells permeabilized in the absence of calcium, which prevents TCR-induced PtdIns metabolism and PKC activation. There was a partial loss of TCR stimulation of $p21^{ras}$ in the zero-calcium buffer, which could mean that non-PKC $p21^{ras}$ control mechanisms are regulated by cytosolic calcium. These data indicate that the TCR can couple to $p21^{ras}$ via a non-PKC-mediated route and support the possibility that two intracellular pathways for $p21^{ras}$ regulation coexist in T cells: one PKC mediated and one not. PTKs are known to be important in TCR signal transduction mechanisms. The effects of the PTK inhibitor herbimycin on $p21^{ras}$ activation indicate that tyrosine kinases are also important in the non-PKC-mediated route for TCR regulation of $p21^{ras}$. The identities of the PTKs and substrates involved in $p21^{ras}$ regulation remain to be resolved.

The present series of experiments illustrate that the TCR is able to use a PTK-dependent non-PKC route to couple to $p21^{ras}$ when TCR activation of PKC is prevented. However, the experiments do not prove that PKC has no role in TCR regulation of $p21^{ras}$, because in a normal T-cell activation response, TCR stimulation of PKC would occur. Thus, both the PKC and PTK mechanism could simultaneously regulate $p21^{ras}$. If PKC is not involved in TCR activation of $p21^{ras}$, then it is necessary to explain why signals from phorbol ester- or DAG-stimulated PKC but not from TCR-stimulated PKC are transduced to $p21^{ras}$. One explanation could be that TCR triggering is not able to achieve the appropriate level of PKC stimulation necessary for $p21^{ras}$ stimulation. An alternative explanation could be that phorbol esters and DOG will activate the complete spectrum of both calcium-dependent and calcium-independent PKC isotypes expressed in T cells (PKC α, β, δ, ε, ζ, and θ) (21), whereas TCR triggering may activate a limited number of PKC isotypes and perhaps stimulate PKC isotypes that are not involved in $p21^{ras}$ activation.

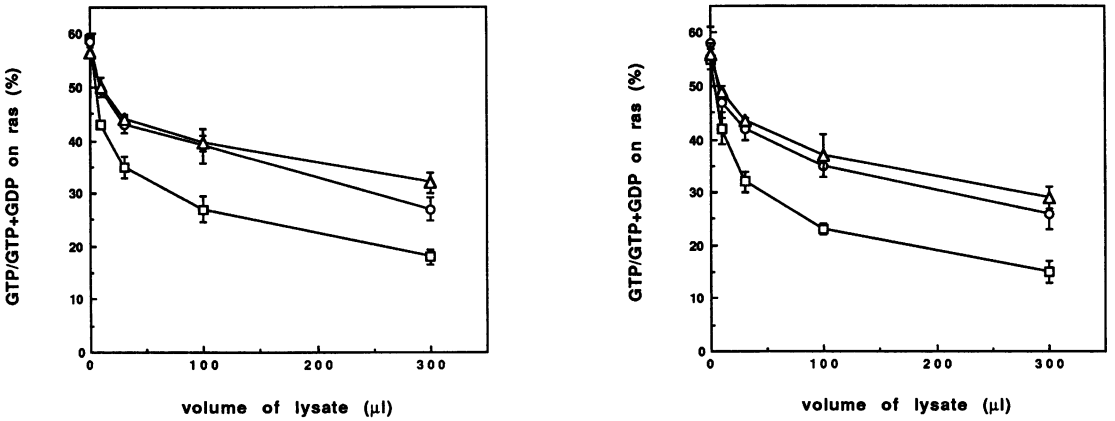
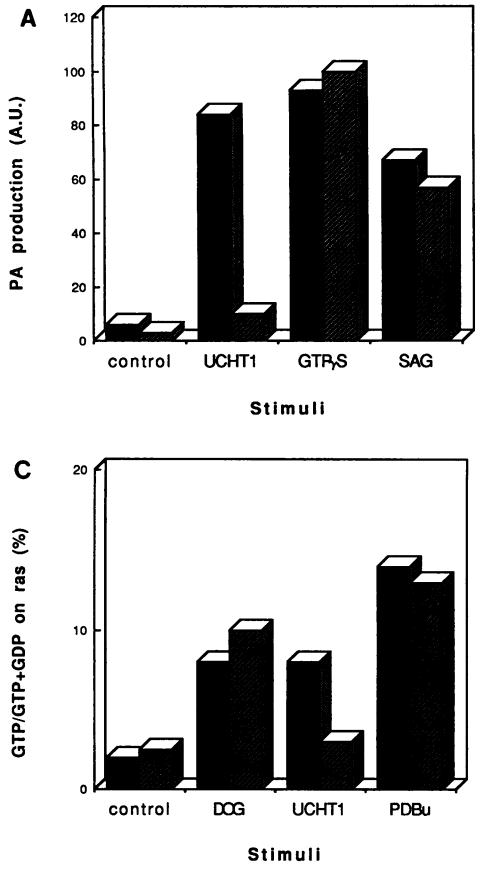


FIG. 7. Effect of PDBu and TCR triggering on GAP activity in T lymphoblasts permeabilized in the presence of 100 nM (B) or 0 nM (A) free Ca^{2+}. Cells were either unstimulated (□) or stimulated with 50 ng of PDBu (△) or 10 μg of anti-TCR monoclonal antibody UCHT1 (○) per ml. The relative amount of GTP remaining on added purified $p21^{ras}$ initially bound to [α-^{32}P]GTP is plotted against the volume of lysate added (see Materials and Methods for details). Data are averages of duplicate values and are representative of three experiments.



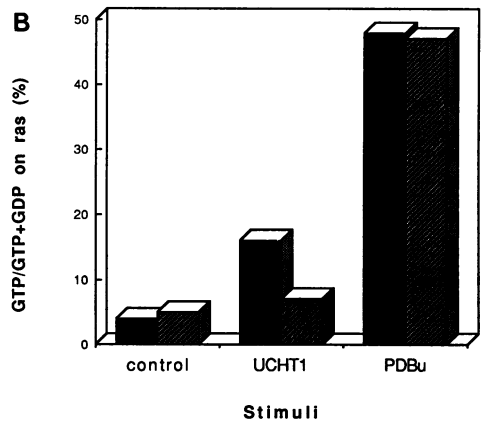


FIG. 8. Effect of herbimycin on the activation state of p21^{ras} in T lymphoblasts. Cells (5×10^6/ml) were preincubated with vehicle (0.05% dimethyl sulfoxide [■] or 1 μM herbimycin (▨) for 24 h prior to each experiment. (A) Effect of herbimycin on PA production directed by different stimuli in permeabilized T lymphoblasts. T lymphoblasts were permeabilized in the presence of [γ-^{32}P]ATP and 100 nM Ca^{2+} buffer. Cells were either unstimulated (control) or stimulated with 10 μg of monoclonal antibody UCHT1 per ml, 100 μM GTPγS, or 50 μg of SAG per ml for 5 min. Phospholipids were extracted and resolved by thin-layer chromatography, and the figure shows the β-emission-scan data corresponding to the PA position. (B) Effect of herbimycin on the activation state of p21^{ras} in intact cells. After preincubation, cells were labeled with $^{32}P_i$ as indicated in Materials and Methods. Intact cells were unstimulated (control) or stimulated for 30 min with 10 μg of UCHT1 or 100 ng of PDBu per ml. Immunoprecipitation of p21^{ras} was with monoclonal antibody Y13-259. Nucleotides were separated by thin-layer chromatography and quantitated by direct scanning for β radiation. (C) Effect of herbimycin on the p21^{ras} activation state in cells permeabilized with the 0 nM Ca^{2+} buffer. Cells were permeabilized in the presence of [α-^{32}P]GTP and 5 mM Mg^{2+}. Cells were either unstimulated (control) or stimulated with 50 μg of DOG, 10 μg of monoclonal antibody UCHT1, or 100 ng of PDBu per ml. Immunoprecipitation of p21^{ras} was with monoclonal antibody Y13-259, and the figure shows the quantitation of guanine nucleotides on p21^{ras} by direct scanning of β radiation. Data are representative of several experiments.

Increases in GTP levels on p21^{ras} proteins in TCR- or PKC-stimulated T cells appear to be mediated by a decrease in p21^{ras} GTPase activity, since guanine nucleotide exchange rates on p21^{ras} are constitutively high and unchanged by T-cell activation (9). The GTPase activity of p21^{ras} is controlled by GAPs. The best characterized of these are p120^{GAP} and the NF1 protein (33, 37). In T cells, the observed stimulation of p21^{ras} in TCR- or phorbol ester-stimulated cells correlated with an inhibition of p21^{ras}-GAPs (9) (Fig. 7). The present study demonstrates that there is also a TCR- but non-PKC-mediated mechanism for inactivation of p21^{ras}-GAPs, since TCR-induced GAP inhibition could occur in the zero-calcium buffer despite the uncoupling of TCR stimulation of PKC. It is unclear how the GAPs are inhibited, although PKC and TCR regulation of GAP activity does not appear to involve direct effects on p120^{GAP} or NF1, since these proteins undergo no significant changes in serine/threonine or tyrosine phosphorylation during T-cell activation (8a). One possibility is that T cells express regulatory proteins for p21^{ras}-GAPs and that these are the direct targets for PKC- or TCR-stimulated kinases. For p120^{GAP}, two associated proteins, p62 and p190, in fibroblasts have been described (10), but little is known about their regulation in T cells.

An alternative mechanism whereby GAP activity could be regulated involves the production of mitogenic lipids. Lipids and lipid metabolites such as PA, polyphosphoinositides, and arachidonic acid strongly inhibit the activity of the NF1 protein and, to a lesser extent, that of p120^{GAP} (4, 35). There are also reports of a GTPase inhibitory protein that blocks the action of GAPs on p21^{ras} and can be activated by DAGs and the lipids that inhibit GAPs (34). These lipids are unlikely to be important in PKC regulation of p21^{ras}, because there is no evidence in T lymphocytes that PKC induces increases in cellular levels of lipid metabolites such as arachidonic acid or PA. Furthermore, in fibroblasts, PKC regulation of lipid metabolism and PA production from both PLC- and PLD-mediated phosphatidylcholine hydrolysis is well documented, yet PKC-controlled pathways for p21^{ras} activation do not occur (23). It is less clear, however, whether lipid metabolites could play a role in TCR regulation of p21^{ras}. The present data show that in T cells permeabilized in a zero-calcium buffer, an absence of TCR induction of PA production resulted in only a partial inhibition (20 to 40%) of TCR induction of "active" p21^{ras}-GTP complexes. Thus, a major residual TCR-p21^{ras} response can occur in the absence of TCR-induced PtdIns hydrolysis, suggesting that PtdIns metabolism is not involved in the non-PKC p21^{ras} regulatory mechanism. However, we have not examined alternative pathways of lipid metabolism involving PLA_2 or PLD that might generate arachidonic acid or PA in T cells, and thus we would not exclude a role for these in TCR regulation of p21^{ras}-GAPs.

ACKNOWLEDGMENT

We thank E. Rozengurt for helpful comments on the manuscript.

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