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Mechanism of Phospholipase C Activation through the T Cell Receptor/CD3 Complex

— Evidence for the Activation without Involvement of a Guanine Nucleotide-binding Protein —

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SUMMARY

The binding of monoclonal antibodies such as OKT3 to the T cell receptor (TcR)/CD3 complex elicites the hydrolysis of phosphatidylinositol 4, 5-bisphosphate, which yields two intracellular second messengers, diacylglycerol and inositol 1, 4, 5-trisphosphate. Possible involvement of a guanine nucleotide-binding protein (G-protein) in the activation of a polyphosphoinositide phospholipase C (PLC) through the TcR/CD3 complex has been studied in a human T cell leukemia line, Jurkat, made permeable to nucleotides by treatment with *Pseudomonas aeruginosa* cytotoxin. The OKT3-stimulated production of inositol phosphates was not enhanced by guanosine 5′-O-(3-thiotriphosphate) (GTPγS), a nonhydrolyzable GTP analog, at any concentration from 10 nM to 100 μM but was reduced by GTPγS at and above 10 μM. In the presence of 100 μM GTPγS, OKT3-stimulated production was reduced to 27% of the level found in the absence of GTPγS. Only GTPγS and no other nucleoside triphosphates including adenosine 5′-O-(3-thiotriphosphate) and GTP suppress the OKT3-stimulated production of inositol phosphates. NaF also suppressed the OKT3-stimulated production of inositol phosphates and $AlCl_3$ potentiated the NaF effect, which is consistent with the view that the active principle is AlF_4^-, an activator of G-proteins. NaF (10 mM) plus $AlCl_3$ (40 μM) reduced the OKT3-stimulated production to 18% of the control level. Therefore, the activation of G-proteins in

Abbreviations

TcR, T cell receptor; G-protein, guanine nucleotide-binding regulatory protein; PtdIns(4, 5)P_2, phosphatidylinositol 4, 5-bisphosphate; $[Ca^{2+}]i$, cytoplasmic free Ca^{2+}; PLC, polyphosphoinositide phospholipase C; PDGF, platelet-derived growth factor; GTPγS, guanosine 5′-O-(3-thiotriphosphate); ATPγS, adenosine 5′-O-(3-thiotriphosphate); GDPβS, guanosine 5′-O-(2-thiodiphosphate); ADPβS, adenosine 5′-O-(2-thiodiphosphate); $InsP_1$, $InsP_2$, $InsP_3$, and $InsP_4$, inositol mono-, bis-, tris-, and tetrakis-phosphates, respectively; $InsP_{2+3}$, $InsP_2$ plus $InsP_3$.

Jurkat cells by GTPγS and AlF_4^- results in the inhibition of PLC activation elicited with OKT3. Moreover, the inhibition of G-proteins by guanosine 5′-O-(2-thiodiphosphate) does not suppress the PLC activation elicited with OKT3. These results suggest that the coupling of the TcR/CD3 complex to a PLC is not mediated through a G-protein. These results also suggest the presence in Jurkat cells of an inhibitory G-protein involved in the control of PLC activity. The effect of treatment of Jurkat cells with cholera toxin on the PLC activation mediated through a stimulatory G-protein has been studied in a cell-free membrane system. Exposure of Jurkat cells to cholera toxin markedly suppressed the inositol polyphosphate formation in membranes evoked by GTPγS and NaF plus $AlCl_3$. Suppression by cholera toxin of the PLC activation mediated both through the TcR/CD3 complex and through a stimulatory G-protein can be explained by the activation of an inhibitory G-protein constituting a PLC system in Jurkat cells.

Key Words: T cell line, OKT3 monoclonal antibody, Inositol phosphate, Guanine nucleotide, Fluoroaluminate, Cholera toxin

INTRODUCTION

Monoclonal antibodies directed against the T cell receptor (TcR) and its associated CD3 complex elicite the hydrolysis of phosphatidylinositol 4, 5-bisphosphate [PtdIns(4, 5)P_2] and an increase in cytoplasmic free Ca^{2+}([Ca^{2+}]i) in human T cells such as Jurkat cells(24, 25, 36, 43, 46, 53). The initial event in this signal transduction pathway is the hydrolysis of PtdIns(4, 5)P_2, which yields two intracellular second messengers, diacylglycerol and inositol trisphosphate(24, 42, 43). It has been assumed that the CD3 complex plays an important role in signal transduction through the TcR/CD3 complex.

In Jurkat membranes, the hydrolysis of PtdIns(4, 5)P_2 is stimulated by guanosine 5′-O-(3-thiotriphosphate) (GTPγS), a nonhydrolyzable GTP analog, and fluoroaluminate(44), which indicates the presence in Jurkat cells of a guanine nucleotide-binding regulatory protein (G-protein) coupled to a polyphosphoinositide phospholipase C (PLC). However, it remains to be determined whether the G-protein is involved in PLC activation through the TcR/CD3 complex.

Our study on the mechanism of the growth factor-stimulated activation of a PLC in a rat fibroblast line, WFB, indicates that a G-protein couples vasopressin- and bombesin-receptors to PLC activation but the coupling of the receptor for platelet-derived growth factor (PDGF) to a PLC is not mediated through a G-protein(20). It is interesting to find a comparable lag period of 10 to 15 s before

an increase in $[Ca^{2+}]i$ after the stimulation of Jurkat cells by monoclonal antibodies against the TcR/CD3 complex and after the stimulation of WFB and Swiss mouse 3T3 cells by PDGF(18, 20, 43, 46). A nonmeasurable lag period was observed in $[Ca^{2+}]i$ responses induced by vasopressin, bombesin, and prostaglandin $F_{2\alpha}$ in WFB and Swiss mouse 3T3 cells(20, 41). Two pathways are known through which a PLC is activated following binding of a ligand to a cell surface receptor(20, 38). A heterotrimeric G-protein is involved in one pathway as a signal transducer from a cell surface receptor to a PLC(8). In the other pathway, a receptor tyrosine kinase such as PDGF-receptor and EGF-receptor activates a PLC, PLCγ, by phosphorylating tyrosine residues of the PLC and by binding the PLC to the receptor(10, 33, 34, 52, 54). PDGF-receptor has a single membrane-spanning segment(55). All molecules constituting the TcR/CD3 complex have single membrane-spanning segments (for reviews, see Refs. 2 and 3). Participation of G-proteins in signal transduction has been proved in receptors with seven membrane-spanning segments(16, 30). The mechanism by which the TcR/CD3 complex activates a PLC remains unknown. Since the TcR/CD3 complex has at least seven components each with one membrane spanning segment(2, 3), it is possible in principle that the TcR/CD3 complex transmits the signal to an unknown G-protein. However, it is also equally possible that the TcR/CD3 complex activates a PLC by tyrosine phosphorylation. Evidence has been presented which indicates the association of a nonreceptor protein tyrosine kinase, $p59^{fyn}$, with the TcR/CD3 complex(40).

It has not been possible so far to reproduce the PLC activation initiated with monoclonal antibodies against CD3 in a cell-free membrane system of Jurkat cells(44). In this paper, we examined a possible involvement of a G-protein in the PLC activation through the TcR/CD3 complex in Jurkat cells made permeable to nucleotides by treatment with *Pseudomonas aeruginosa* cytotoxin. The findings obtained in this study suggest that the activation of a PLC through the TcR/CD3 complex is not mediated through a G-protein.

MATERIALS AND METHODS

Materials

Jurkat-FHCRC, a human T cell leukemia line, was obtained from Dr. James Watson. OKT3 monoclonal antibody was prepared from a culture supernatant of OKT3 hybridoma, obtained from the American Type Culture Collection (Rockville, MD), by protein A-Sepharose CL-4B (Pharmacia) chromatography. Cytotoxin from *Pseudomonas aeruginosa* was prepared from an autolysate of *P. aeruginosa* strain 158 as described previously(31). Commercial sources of chemi-

cals were as follows: creatine kinase, GTPγS, guanosine 5′-O-(2-thiodiphosphate) (GDPβS), adenosine 5′-O-(2-thiodiphosphate) (ADPβS), XTP, ITP, and CTP, Sigma Chemical Co. (St. Louis, MO); adenosine 5′-O-(3-thiotriphosphate) (ATPγS) and GTP, Boehringer Mannheim Yamanouchi(Tokyo, Japan); cholera toxin, List Biological Laboratories, Inc. (Campbell, CA); AG1-X8 (200-400 mesh, formate form), Bio-Rad (Tokyo, Japan).

Cell culture and preparation of permeabilized cells

Jurkat cells were maintained in Iscove's modified Dulbecco's medium supplemented with 5% heat-inactivated (56℃ for 30 min) FCS(Filtron Pty. Ltd., Altona, Victoria, Australia), 2 mM glutamine, 1 mM sodium pyruvate, 50 U/m*l* penicillin, and 50 μg/m*l* streptomycin(13). In each experiment, Jurkat cells were collected from cultures and suspended at 5×10^5 cells/m*l* in the growth medium. To this cell suspension, *myo*-[2-^{3}H]inositol, 15 Ci/mmol (American Radiolabeled Chemicals Inc., St. Louis, MO), was added at 2.5 μCi/m*l* unless otherwise stated. After labeling for 2 days, the cells were washed once with HEPES-buffered saline without Ca^{2+} and Mg^{2+}. The cells were then suspended at 5×10^6/m*l* in a permeabilized cell medium (pH 7.0), which contained 20 mM HEPES, 110 mM KCl, 10 mM LiCl, indicated concentrations of $MgCl_2$ and EGTA, 5 mM potassium succinate, 5 mM potassium pyruvate, 2.5 mM ATP, 5 mM phosphocreatine, 10 units/m*l* creatine kinase, 1 mg/m*l* bovine serum albumin, and $CaCl_2$. $CaCl_2$ was added to the medium to make the free Ca^{2+}-concentration 100 to 250 nM as indicated, which was determined fluorimetrically by the use of quin-2(50). The cells were treated with 2 to 8 μg protein of the pseudomonal cytotoxin/10^6 cells for 5 min at 37℃. The cytotoxin-treated cells were washed once with the permeabilized cell medium by centrifugation at 200× *g* for 5 min at 4℃ and then suspended at 4 to 7×10^6 cells/m*l* in the same medium.

Entrapment of GTPγS and GDPβS in Jurkat cells by electroporation

The *myo*-[2-^{3}H]inositol-labelled Jurkat was suspended at 10^7/0.4 m*l* in the permeabilized cell medium (4 mM $MgCl_2$, 2.5 mM EGTA, and the free Ca^{2+}-concentration of 180 nM) containing GTPγS or GDPβS. The suspension was transferred to an electroporation chamber kept in ice-water bath and an electric pulse was applied to the electrodes of the chamber at 1800 V/0.4 cm according to the method of Potter *et al.*(37). The cell suspension was then allowed to sit for 10 min at room temperature. The cells were spun down at 200×*g* for 5 min at 4℃ and then suspended at 10^7/m*l* in the permeabilized cell medium.

Stimulation of permeabilized cells by OKT3, GTPγS, and AlF_4^- and determination of [^3H]inositol phosphates

Portions (0.1 m*l*) of the cytotoxin-treated cells were transferred to test tubes containing 0.1 m*l* of the permeabilized cell medium, 2 to 5 μl of OKT3 and, where indicated, 2 μl of nucleotides and their analogs, or 2 μl of a mixed solution of NaF and $AlCl_3$. The mixtures were incubated at 37℃ for 5 min. The reaction was terminated by adding 0.75 m*l* of chloroform-methanol (1 : 2, v/v). The mixture was mixed thoroughly and then partitioned into two phases by adding 0.25 m*l* each of chloroform and water. The upper phase containing various [^{3}H]inositol phosphates was removed and diluted with 4 m*l* of water. Total inositol phosphates in this solution were determined by the method of Berridge *et al.*(6) as described previously(19, 41).

RESULTS

Stimulation of inositol phosphate production by an anti-CD3 monoclonal antibody, OKT3, in Jurkat cells treated with pseudomonal cytotoxin

It has been shown that pseudomonal cytotoxin makes Jurkat cells and other animal cells permeable to ions and small molecules(32, 45, 49). Previously, we have shown that the treatment of Jurkat cells with the cytotoxin makes the cells responsive to NaF plus $AlCl_3$ by an increase in inositol phosphates(21). The results in Fig. 1*a* show that about 70% of inositol phosphates present in unstimulated Jurkat cells is released by treatment of 10^6 cells with 4-8 μg protein of the cytotoxin. The result again indicates that cytotoxin treatment makes Jurkat cells permeable to small molecules. The cytotoxin-treated Jurkat cells produced a much larger amount of inositol phosphates than intact Jurkat cells during a 5-min incubation at 37℃ in the absence of stimulus (Fig. 1*b*). GTPγS stimulated inositol phosphate production to a small extent in cytotoxin-treated Jurkat cells but not in intact cells (Fig. 1*b*). Inositol phosphate production in response to a monoclonal antibody against CD3, OKT3, was markedly augmented by cytotoxin treatment when the response was assayed by a 5-min incubation at 37℃ (Fig. 1*b*). It was previously shown that in cytotoxin-treated Jurkat cells the binding of monoclonal antibodies to the CD3 complex results in decreases in phosphatidylinositol, phosphatidylinositol 4-phosphate, and PtdIns(4, 5)P_2 and increases in inositol bisphosphate ($InsP_2$), inositol trisphosphate ($InsP_3$), and, to a lesser extent, inositol monophosphate ($InsP_1$)(21). Inositol phosphate production in response to various doses of OKT3 was compared between cytotoxin-treated Jurkat cells and intact Jurkat cells (Fig. 2). In this experiment, the cytotoxin-treated cells were incubated with OKT3 at 37℃ for 5 min and intact cells were incubated with OKT3 at 37℃ for 15 min in order to compare the

magnitude of the OKT3-stimulated production(21, 46). It was found that the magnitude of the response to OKT3 was larger in cytotoxin-treated cells than in intact cells (Fig. 2). Inositol phosphate production in the cytotoxin-treated cells

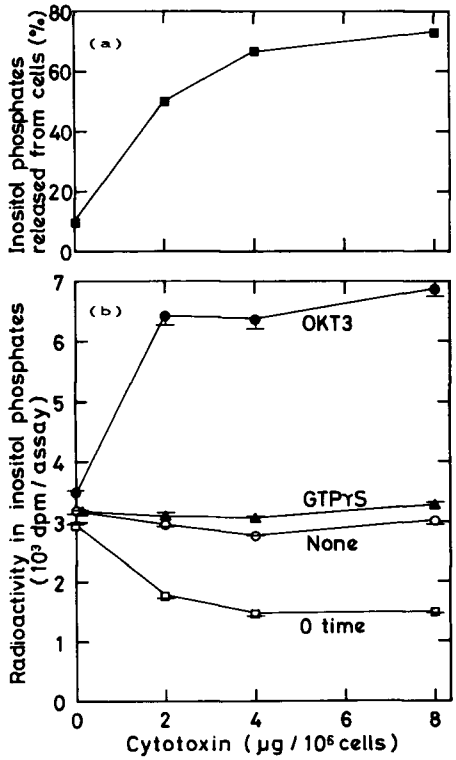

Fig. 1 Release of inositol phosphates from unstimulated Jurkat cells by treatment with various amounts of pseudomonal cytotoxin (*a*) and GTPγS- and OKT3-stimulated production of inositol phosphates by cytotoxin-treated Jurkat cells (*b*)

The *myo*-[2-^{3}H]inositol-labelled Jurkat cells were treated at 37℃ for 5 min with the indicated amount of the cytotoxin per 10^6 cells, after which the cells were subjected to a centrifugation at $200\times g$ for 5 min at 4℃. *Panel a* shows inositol phosphates released from the cells by cytotoxin treatment; inositol phosphates found both in the supernatant and in the cell pellet on centrifugation were taken as 100%. *Panel b* shows inositol phosphate production stimulated by GTPγS and OKT3. The cytotoxin-treated cells (6.3×10^5/assay) were stimulated at 37℃ for 5 min either by 100 ng protein of OKT3 (●) or by 100 μM GTPγS (▲) in the permeabilized cell medium, which was prepared in such a way as to contain 5 mM $MgCl_2$, 1 mM EGTA, and the free Ca^{2+}-concentration of 107 nM. The results are expressed as the mean±S. E. of three determinations. ○, unstimulated control cells incubated at 37℃ for 5 min; □, inositol phosphates found in the cytotoxin-treated cells before incubation.

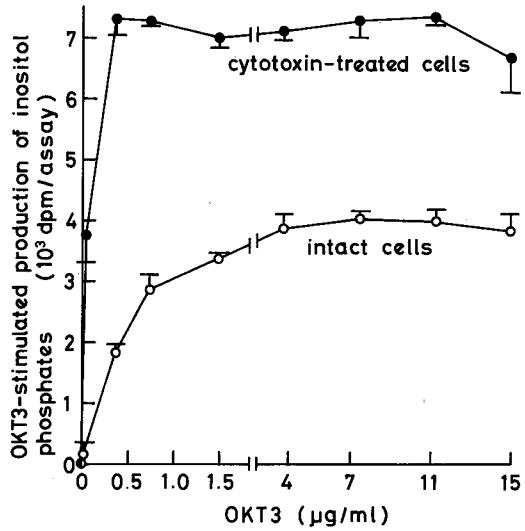

Fig. 2 Effect of various amounts of OKT3 on inositol phosphate production in intact Jurkat cells and in cytotoxin-treated Jurkat cells

A culture of the *myo*-[2-^{3}H]inositol-labelled Jurkat cells were divided into two equal portions. One portion was used as intact cells: the cells (6×10^5/assay) were incubated at 37℃ for 15 min in the presence of the indicated amount of OKT3 in 0.2 m*l* of HEPES-buffered saline in which a portion (10 mM) of NaCl had been replaced with 10 mM LiCl. The other portion was permeabilized by treatment with 7 μg protein of pseudomonal cytotoxin per 10^6 cells. The cytotoxin-treated cells (6×10^5/assay) were incubated at 37℃ for 5 min in the presence of the indicated amount of OKT3 in 0.2 m*l* of the permeabilized cell medium, which was prepared in such a way as to contain 6 mM $MgCl_2$, 1 mM EGTA, and the free Ca^{2+}-concentration of 254 nM. Formation of total inositol phosphates was determined. The results are expressed as the mean±S. E. (three determinations) of increases in inositol phosphates caused by OKT3 above control values obtained in the absence of OKT3. Intact cells (6×10^5 cells) contained 4,334±235 d. p. m. of inositol phosphates before incubation and 6,380±148 d. p. m. of inositol phosphates after 15 min-incubation in the absence of OKT3. The cytotoxin-treated cells (6×10^5 cells) contained 2,888±42 d. p. m. of inositol phosphates before incubation and 10,026±194 d. p. m. of instiol phosphates after 5 min-incubation in the absence of OKT3.

was maximally stimulated at 375 ng protein of OKT3 per m*l* (Fig. 2). This dose-response curve obtained in the cytotoxin-treated Jurkat cells is different from that obtained in intact Jurkat cells, in which the stimulation of inositol phosphate production by OKT3 increased up to 4 μg protein per m*l*. The response to OKT3 in the cytotoxin-treated cells was found to be more sensitive to low concentrations of OKT3. These results indicate that the cytotoxin-treated Jurkat cells retain the ability to respond to OKT3 with inositol lipid hydrolysis, though

quantitative differences are found between treated cells and intact cells in the OKT3-stimulated production of inositol phosphates.

Effects of GTPγS, fluoroaluminate, and GDPβS on OKT3-stimulated formation of inositol phosphates in cytotoxin-treated Jurkat cells

The effect of G-protein activation on the OKT3-stimulated hydrolysis of inositol lipids was studied by measuring the OKT3-stimulated release of inositol phosphates from the cytotoxin-treated Jurkat cells in the presence of various concentrations of GTPγS, an agonist of G-proteins (Fig. 3). In the absence of OKT3, GTPγS at 10 μM and 100 μM but not at or below 1 μM stimulated the release of inositol phosphates to a small extent (Fig. 3). The OKT3-stimulated production of inositol phosphates was not enhanced by GTPγS at any concentration from 10 nM to 100 μM but was reduced by GTPγS at and above 10 μM. In the presence of 100 μM GTPγS, the OKT3-stimulated production of inositol

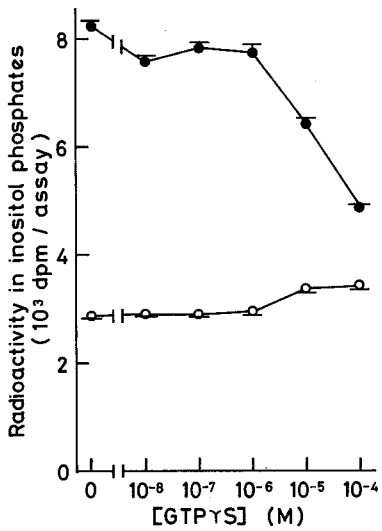

Fig. 3 Effect of GTPγS on OKT3-stimulated formation of inositol phosphates in cytotoxin-treated Jurkat cells

The *myo*-[2-^{3}H]inositol-labelled Jurkat cells were permeabilized by treatment with 4 μg protein of pseudomonal cytotoxin per 10^6 cells. The cytotoxin-treated cells (6×10^5/assay) were incubated at 37℃ for 5 min in the absence (○) and presence of 100 ng protein of OKT3 (●) in the permeabilized cell medium containing the indicated concentration of GTPγS. The permeabilized cell medium was prepared to contain 4 mM $MgCl_2$, 3 mM EGTA, and the free Ca^{2+}-concentration of 238 nM. Formation of total inositol phosphates was determined. The results are expressed as the mean±S. E. of three determinations.

phosphates, which can be calculated from the data in Fig. 3 as the increase in inositol phosphates caused by OKT3 above the values obtained in the absence of OKT3 stimulation, was reduced to 27% of the level found in the absence of GTPγS (Fig. 3).

The results in Tables 1 and 2 show the specificity of GTPγS effect on the OKT3-stimulated production of inositol phosphates in the cytotoxin-treated Jurkat cells. Only GTPγS and no other nucleoside triphosphates, including ATPγS, GTP, XTP, ITP, and CTP, suppress the OKT3-stimulated release of inositol phosphates.

Fluoroaluminate, AlF_4^-, is the other reagent which directly activates G-proteins by binding to the guanine nucleotide binding site of the α-subunits of these proteins in GDP form and by mimicking the role of the γ-phosphate of GTP(7,

Table 1. *Effects of GTPγS, ATPγS, GDPβS, ADPβS, NaF, and NaF plus $AlCl_3$ on OKT3-stimulated formation of inositol phosphates in cytotoxin-treated Jurkat cells*

Additions	OKT3-stimulated increase in inositol phosphates (d. p. m./assay)
None	3,188±136
100 μM GTPγS	1,334±138
100 μM ATPγS	3,038±156
100 μM GDPβS	2,988±102
1 mM GDPβS	3,136± 98
100 μM ADPβS	3,064±116
1 mM ADPβS	2,762± 60
10 mM NaF	778± 84
10 mM NaF plus 40 μM $AlCl_3$	564± 78

The *myo*-[2-^{3}H]inositol-labelled Jurkat cells were permeabilized by treatment with 7.5 μg protein of pseudomonal cytotoxin per 10^6 cells. The cytotoxin-treated cells (5.6×10^5/assay) were incubated at 37℃ for 5 min in the absence and presence of 100 ng protein of OKT3 in the permeabilized cell medium containing the indicated nucleotide analog, NaF, or NaF plus $AlCl_3$. The permeablilized cell medium was prepared to contain 6 mM $MgCl_2$, 1 mM EGTA, and the free Ca^{2+}-concentration of 254 nM. Formation of total inositol phosphates was determined. The results are representative of three separate experiments and are expressed as the mean±S. E. (three determinations) of increases in inositol phosphates elicited by OKT3 above control values (in the absence of OKT3). The control values were 3,100±36 d. p. m. in the absence of additions, 3,314±68 d. p. m. in the presence of 100 μM GTPγS, 3,142±46 d. p. m. in the presence of 100 μM ATPγS, 3,286±82 d. p. m. in the presence of 100 μM GDPβS, 1,972±24 d. p. m. in the presence of 1 mM GDPβS, 2,794±64 d. p. m. in the presence of 100 μM ADPβS, 1,758±52 d. p. m. in the presence of 1 mM ADPβS, 2,400±74 d. p. m. in the presence of 10 mM NaF, and 2,532±46 d. p. m. in the presence of 10 mM NaF plus 40 μM $AlCl_3$.

47). An addition of 10 mM NaF plus 40 μM $AlCl_3$ to the cytotoxin-treated Jurkat cells reduced the OKT3-stimulated production of inositol phosphates to 18% of the control level (Table 1). NaF also suppressed the OKT3-stimulated production (Table 1), and $AlCl_3$ potentiated the NaF effect, which is consistent with the view that active principle is AlF_4^-. Therefore, GTPγS and fluoroaluminate activate G-proteins in Jurkat cells and this activation results in the inhibition of inositol phosphate production elicited by OKT3.

GDPβS is a metabolically stable analog of GDP(12) and has been used extensively as a competitive inhibitor (an antagonist) of G-protein activation, inhibiting agonist-stimulated adenylate cyclase activity(12), PLC activity(9, 23, 51), and phospholipase A_2 activity(39). GDPβS at 100 M and 1 mM has no effect on the OKT3-stimulated production of inositol phosphates in the cytotoxin-treated Jurkat cells (Table 1). No difference was observed in the effects of GDPβS and ADPβS on the OKT3-stimulated production (Table 1). GDP and ADP at 0.5 mM also had no effect on the OKT3-stimulated production of inositol phosphates (Table 2). These results indicate that an inhibition of G-proteins by GDPβS does not suppress the PLC activation mediated through the TcR/CD3 complex.

Table 2. *Effects of various nucleoside di- and tri-phosphates on OKT3-stimulated formation of inositol phosphates in cytotoxin-treated Jurkat cells*

Additions	OKT3-stimulated increase in inositol phosphates (d. p. m./assay)
None	2,188± 28
100 μM GTPγS	634± 64
0.5 mM GTP	2,192± 56
0.5 mM XTP	2,198±172
0.5 mM ITP	2,134± 32
0.5 mM CTP	2,162± 42
0.5 mM GDP	2,144± 80
0.5 mM ADP	2,120± 52

The experiment was performed as described in the legend to Table 1 except that each incubation mixture contained the indicated nucleotides. Each assay contained 5.4×10^5 cells. The results are expressed as the mean±S. E. (three determinations) of increases in inositol phosphates evoked by OKT3 above control values (in the absence of OKT3). The control values were 2,798±60 d. p. m. in the absence of nucleotides, 2,964±68 d. p. m. in the presence of 100 μM GTPγS, 2,236±72 d. p. m. in the presence of 0.5 mM GTP, 2,488±16 d. p. m. in the presence of 0.5 mM XTP, 2,512±80 d. p. m. in the presence of 0.5 mM ITP, 2,490±74 d. p. m. in the presence of 0.5 mM CTP, 1,844±74 d. p. m. in the presence of 0.5 mM GDP, and 2,120±38 d. p. m. in the presence of 0.5 mM ADP.

Effects of GTPγS and GDPβS on OKT3-stimulated formation of inositol phosphates in electropermeabilized Jurkat cells

The effects of GTPγS and GDPβS on the OKT3-stimulated production of inositol phosphates was also examined in Jurkat cells made permeable to these guanine nucleotide analogs by exposure to a high-voltage electric discharge (Table 3). Entrapment of 100 μM GTPγS in Jurkat cells reduced the OKT3-

Table 3. *Effects of GTPγS and GDPβS on OKT3-stimulated formation of inositol phosphates in electropermeabilized Jurkat cells*

Additions	Inositol phosphates formed (d. p. m./assay)	
	Control	OKT3 (Δ control)
None	820±42	1,490±62(670)
1 μM GTPγS	702±94	1,376±98(674)
100 μM GTPγS	918±96	1,306±82(388)
1 mM GDPβS	850±18	1,588±70(738)
2 mM GDPβS	782±12	1,778±48(996)

The *myo*-[2-^{3}H]inositol-labelled Jurkat cells were subjected to electroporation in a medium containing the indicated concentrations of GTPγS or GDPβS as described in the Materials and methods section. Portions (0.1 m*l*) of the electropermeabilized cells were transferred to test tubes containing 0.1 m*l* of the permeabilized cell medium, which contained 100 ng protein of OKT3 where indicated. The mixtures were incubated at 37℃ for 10 min and total inositol phosphates were determined. The results are expressed as the mean±S. E. (three determinations) of increases in inositol phosphates during 10 min of the incubations. "Δ control" indicates OKT3-dependent increases above control values.

stimulated formation of inositol phosphates to 58% of the control level in the electropermeabilized cells. In the absence of OKT3, GTPγS had no clear effect on inositol phosphate formation. GDPβS enhanced the OKT3-stimulated production of inositol phosphates to some extent in the electropermeabilized Jurkat cells.

Effect of exposure of Jurkat cells to cholera toxin on PLC activation in membranes by GTPγS and AlF_4^-

As was reported by Imboden *et al.*(26), the exposure of Jurkat cells to cholera toxin (0.1 μg protein/m*l* of culture) for 3 hr completely inhibited the OKT3-stimulated production of inositol phosphates (Table 4). The results in Table 4 indicate that basal increase in inositol phosphates during a 15-min incubation in the absence of OKT3 was also markedly reduced by the exposure to cholera

Table 4. *Effect of treatment of Jurkat cells with cholera toxin on OKT3-stimulated production of inositol phosphates*

Stimuli	Inositol phosphates produced (d. p. m./assay)	
	No cholera toxin	Cholera toxin
None	2,966±568	476±236
OKT3	9,976±690	396± 68

Jurkat cells were labelled with 5 μCi of *myo*-[2-^{3}H]inositol/m*l* of medium for 2 days. Three h before the end of labeling, the culture (7×10^5 cells/m*l*) was divided into two portions. Cholera toxin was added to one portion at 0.1 μg protein/m*l* and the culture was incubated at 37℃ for 3 h. The other portion was incubated without the addition. After being washed once, the cells (6.6×10^5/assay) were incubated at 37℃ for 15 min in the absence and presence of 1.9 μg protein of OKT3 in 0.2 m*l* of HEPES-buffered saline in which a portion (10 mM) of NaCl had been replaced with 10 mM LiCl. Total inositol phosphates was determined. The results are expressed as the mean±S. E. (three determinations) of increases in inositol phosphates during the 15-min incubation. Before the incubation, the cells without toxin treatment contained 11,484±502 d. p. m. of inositol phosphates and 259,706±1,526 d. p. m. of inositol lpids per 6.6×10^6 cells. Before the incubation, the cells treated with cholera toxin contained 10,858±474 d. p. m. of inositol phosphates and 269,242±22,352 d. p. m. of inositol lipids per 6.6×10^6 cells.

toxin. Before the incubation, the cholera toxin-treated and untreated Jurkat cells contained almost the same amounts of ^{3}H-label both in inositol phosphates and in inositol lipids.

The effect of exposure of Jurkat cells to cholera toxin on the PLC activation mediated through a G-protein was studied in a cell-free membrane system. GTPγS and NaF plus $AlCl_3$ stimulate the formation of $InsP_2$ and $InsP_3$ in crude membranes prepared from Jurkat cells by the method described previously(44) (Table 5). OKT3 does not stimulate inositol polyphosphate formation in the membranes under the conditions. Treatment of Jurkat cells with cholera toxin (0.1 μg protein/m*l* of culture) for 3 hr markedly reduced the GTPγS- and AlF_4^--stimulated formation of inositol polyphosphates in the membranes prepared from the treated cells (Table 5). These results indicate that the exposure of Jurkat cells to cholera toxin suppresses the PLC activation mediated both through the TcR/CD3 complex and through a stimulatory G-protein.

DISCUSSION

Many questions about PLC activation through the TcR/CD3 complex remains to be answered. This is mainly due to a failure to reproduce the anti-CD3 antibody-stimulated hydrolysis of polyphosphoinositides in a cell-free membrane system. The results obtained in this study suggest that the coupling of the TcR/

CD3 complex to a PLC is not mediated through a G-protein or, at least, through a G-protein of ordinary properties ascribed to Gs, Gi, and Gt(14). This conclusion has been drawn from the effects of GTPγS and GDPβS on the OKT3-

Table 5. *Effect of treatment of Jurkat cells with cholera toxin on PLC activation in membranes by GTPγS, NaF plus $AlCl_3$, and OKT3*

Additions	$InsP_n$	Inositol phosphates formed (d. p. m./assay)	
		No cholera toxin	Cholera toxin
None	$InsP_1$	300±56	494±40
	$InsP_{2+3}$	524±13	128±10
	$InsP_4$	202±35	129±29
GTPγS	$InsP_1$	526±31	374±36
	$InsP_{2+3}$	1,976±24	338±27
	$InsP_4$	359±16	107±25
NaF plus $AlCl_3$	$InsP_1$	471±64	261±49
	$InsP_{2+3}$	2,730±33	410± 8
	$InsP_4$	267±30	55± 5
OKT3	$InsP_1$	253±36	457±33
	$InsP_{2+3}$	471±33	106±12
	$InsP_4$	175±20	93±18

The same culture of Jurkat cells labelled with *myo*-[2-^{3}H]inositol and treated with and without cholera toxin as described in the legend to Table 4 was used in this experiment. The labelled cells were washed once with HEPES-buffered saline without Ca^{2+} and Mg^{2+}, and disrupted at 4℃ by N_2 cavitation in a medium (pH 7.4) composed of 10 mM HEPES, 110 mM KCl, 1 mM EGTA, 10 mM LiCl, 1 mM ATP, and 5 mM $MgCl_2$ as described previously(44). A crude membrane fraction was obtained by centrifugation of $670\times g$, 10 min-supernatant at $2\times10^5\times g$ for 30 min at 4℃. The membranes (74 μg protein originated from about 1.3×10^6 cells/assay) were incubated at 37℃ for 5 min in the absence and presence of 1.9 μg protein of OKT3, 100 μM GTPγS, and 10 mM NaF plus 40 μM $AlCl_3$, as indicated, in 0.2 m*l* of a medium (pH 7.4 and the free Ca^{2+}-concentration of 131 nM) composed of 10 mM HEPES, 27 mM KCl, 1 mM EGTA, 10 mM LiCl, 2 mM ATP, 5 mM $MgCl_2$, 225 μM $CaCl_2$, and 2 mM 2, 5-diphosphoglycerate. Formation of $InsP_1$, $InsP_2$ plus $InsP_3$ ($InsP_{2+3}$), and inositol tetrakisphosphates ($InsP_4$) was determined. $InsP_1$, $InsP_2$, and $InsP_3$ were separated by chromatography on AG1-X8 (formate form) columns as described previously(5, 8, 41). $InsP_4$ was eluted from the column with 0.1 M formic acid/2 M ammonium formate(48). The results are expressed as the mean±S. E. (three determinations) of increases in inotitol phosphates during the 5-min incubation. Before the incubation, the membranes prepared from the cells without toxin treatment contained 595±20 d. p. m. of $InsP_1$, 130±0 d. p. m. of $InsP_2$ plus $InsP_3$, and 168±8 d. p. m. of $InsP_4$, and the membranes prepared from the cells treated with cholera toxin contained 841±63 d. p. m. of $InsP_1$, 121±11 d. p. m. of $InsP_2$ plus $InsP_3$, and 134±4 d. p. m. of $InsP_4$.

stimulated production of inositol phosphates in cytotoxin-treated Jurkat cells. We found that GTPγS did not enhance the OKT3-stimulated inositol phosphate production at any concentration from 10 nM to 100 μM (Fig. 3). This is an unexpected result when we postulate that the coupling of the TcR/CD3 complex to a PLC is mediated through a G-protein, because in receptors coupled to a G-protein, the rate of binding of GTPγS to the G-protein is enhanced greatly in the presence of an agonist. The result obtained by the use of GDPβS also indicates the coupling of the TcR/CD3 complex to a PLC without the participation of a G-protein. It has been shown that GDPβS at 1 mM inhibits the G-protein-dependent pathway of PLC activation in a rat fibroblast line, WFB, treated with pseudomonal cytotoxin(20). GDPβS has no effect on the OKT3-stimulated production of inositol phosphates in the cytotoxin-treated Jurkat cells (Table 1).

The OKT3-stimulated production of inositol phosphates was suppressed by the addition of GTPγS and AlF_4^- (Fig. 3 and Table 1). These results suggest the presence in Jurkat cells of an inhibitory G-protein involved in the control of PLC activity, which has been identified to be coupled to a D_2 dopaminergic receptor in rat anterior pituitary cells(27). Suppression by GTPγS was observed at 10 and 100 μM of the analog, the concentrations at which a small stimulation of inositol phosphate production by GTPγS alone was also observed (Fig. 3). Specificity of the GTPγS effect (Tables 1 and 2) and potentiation of the suppressive effect of NaF with $AlCl_3$ (Table 1) indicate that an activated G-protein is involved in the suppression of the OKT3-stimulated production of inositol phosphates.

Studies by the use of isolated membranes have demonstrated the presence in Jurkat cells of a G-protein(s) capable of activating a PLC(44). Recently, a serotonin receptor has been shown to activate a PLC in Jurkat cells probably by coupling to the stimulatory G-protein(4). However, serotonin did not induce a $[Ca^{2+}]i$ response in the Jurkat cells used in this experiment. This does not make it possible to compare peceptor-stimulated PLC activation through a G-protein-mediated pathway and that through a G-protein-independent pathway. In the case of a rat fibroblast line, WFB, vasopressin, bombesin, and PDGF initiate the activation of a PLC(s). We studied the effects of GTPγS, GDPβS, and AlF_4^- on the inositol phosphate production stimulated by vasopressin, bombesin, and PDGF in WFB cells made permeable to nucleotides by treatment with either saponin or pseudomonal cytotoxin(20). GTPγS markedly enhanced the vasopressin- and bombesin-stimulated production of inositol phosphates. In the same experiment, GTPγS did not enhance the PDGF-stimulated production of inositol phosphates at any concentration from 10 nM to 10 μM. The PDGF-stimulated production of inositol phosphates was suppressed in the presence of 10-100 μM

GTPγS and AlF_4^-. GDPβS markedly suppressed the vasopressin- and bombesin-stimulated production of inositol phosphates. The PDGF-stimulated production of inositol phosphates was not reduced in the presence of GDPβS. From these results, it was concluded that the coupling of vasopressin- and bombesin-receptors to a PLC is mediated through a G-protein but a G-protein is not involved in the coupling of PDGF-receptor to a PLC(20).

The GTPγS-stimulated release of inositol phosphates was significant when assayed in membrane (44 and Table 5) but was small when assayed in permeabilized cells (Figs. 1*b* and 3). Although the stimulatory effect of GTPγS on inositol phosphate release was small, GTPγS markedly inhibited the OKT3-stimulated release of inositol phosphates in the cytotoxin-treated cells. Essentially the same efect of GTPγS on the OKT3-stimulated release of inositol phosphates was found both in the cytotoxin-treated Jurkat cells and in Jurkat cells made permeable to GTPγS by electroporation (Table 3). Therefor, it seems possible to conclude that the low stimulatory effect of GTPγS on inositol phosphate release is not due to the low permeability of the cytotoxin-treated cells to GTPγS. It has been postulated that GTPγS activates both stimulatory and inhibitory G-proteins coupled to PLC in Jurkat cells. A difference in the relative activations by GTPγS of the stimulatory and inhibitory pathways may possibly explain the difference in GTPγS effect on inositol phosphate release found in membranes and in permeabilized cells.

It has been shown in a mouse B cell lymphoma line, WEHI-231, that a G-protein is involved in PLC activation initiated with the binding of an antibody to membrane IgM(15). This conclusion was drawn from results obtained by the use of WEHI-231 cells permeabilized to GTPγS and GDPβS by saponin treatment. The results obtained in WEHI-231 cells are strikingly different from those obtained in Jurkat cells and reported here. In the permeabilized WEHI-231 cells, PLC activation initiated with anti-IgM antibodies was greatly enhanced by GTPγS and was suppressed by high concentrations of GDPβS. Membrane immunoglobulins and T cell receptors are cell surface receptors of evolutionary relatedness(22). Therefore, it is a surprise to find a difference in the mechanisms of signal transduction through these receptors. However, it seems possible for these receptors to transduce signals by different mechanisms because these receptor molecules have single membrane-spanning segments and short cytoplasmic tails(2, 29, 39). Thus, transmembrane signaling through these receptors may be possible only with the help of associated molecules such as the CD3 complex, which probably mediates coupling to the effector, a PLC. In this respect, it is interesting to know that the receptor-mediated hydrolysis of inositol lipids was not enhanced by GTP addition and was not reduced by GDPβS when membranes

prepared from normal murine B cells were stimulated with an anti-immunoglobulin antibody(17).

Imboden *et al.*(26) found that cholera toxin inhibits a TcR/CD3 complex-mediated increase in inositol phosphates and that in $[Ca^{2+}]i$. Their results indicate that cholera toxin affects events that occur subsequent to the binding of monoclonal antibodies to the TcR/CD3 complex and prior to the activation of a PLC. A substrate for the cholera toxin ADP-ribosyltransferase distinct from Gsα, the stimulatory α-subunit of a G-protein in adenylate cyclase system, seems to regulate the signal transduction by the TcR/CD3 complex because the inhibition of PLC activation by cholera toxin occurs 1 to 2 h after the ADP-ribosylation of Gsα and after an increase in cellular cAMP content in Jurkat cells(26). Putative substrate for cholera toxin involved in the control of PLC activation through the TcR/CD3 complex was not identified(26). ADP-ribosylation by cholera toxin activates Gsα by inhibiting the receptor-stimulated activity of GTP hydrolysis(1, 11) and by decreasing the affinity of Gsα for G$\beta\gamma$(28). Therefore, cholera toxin, probably, does not inhibit but activates a G-protein involved in the control of PLC activation mediated through the TcR/CD3 complex. The results obtained by the use of permeabilized Jurkat cells suggest the presence of an inhibitory G-protein involved in the control of PLC activity in Jurkat cells. Moreover, the results shown in Table 5 indicate that exposure of Jurkat cells to cholera toxin markedly suppresses PLC activation mediated through a stimulatory G-protein. Therefor, it seems reasonable to postulate that cholera toxin stabilizes the putative inhibitory G-protein in an active form, which will block the couplings of both the TcR/CD3 complex and a stimulatory G-protein to a PLC.

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