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Cholera toxin partially inhibits the T-cell response to phytohaemagglutinin through the ADP-ribosylation of a 45 kDa membrane protein

André E. NEL,*‖ Michel VANDENPLAS,† Marie M. WOOTEN,§ Rosemary COOPER,‡ Santie VANDENPLAS,† Anli RHEEDER† and Jahmil DANIELS‡

Departments of *Internal Medicine, †Biochemistry and ‡Microbiology, Stellenbosch Medical School, P.O. Box 63, Tygerberg 7505, Republic of South Africa, and §Department of Hematology–Oncology, Alabama Medical School, Birmingham, AL, U.S.A.

This study examines the influence of cholera toxin (CT) on T lymphocyte activation by the mitogenic lectin phytohaemagglutinin (PHA). CT suppressed lectin-induced [^{3}H]thymidine uptake in a dose-dependent fashion and acted synergistically with PHA in the generation of intracellular cyclic AMP. The toxin was assumed to act on G_s, because it also stimulated ADP-ribosylation of a 45 kDa membrane protein *in vitro*; no additional substrates were seen. The inhibitory effect of the adenylate cyclase/cyclic AMP pathway was shown to be directed at a concomitant stimulatory pathway, namely inositol phospholipid turnover. Lectin-stimulated ^{32}P incorporation into both phosphatidylinositol as well as its 4,5-biphosphate derivative was depressed in the presence of CT or exogenous dibutyryl cyclic AMP. This, in turn, was associated with reduced activation of C-kinase as determined by decreased lectin-induced translocation from the cytosol to the surface membrane. These results indicate that G_s probably acts as a transducer between the PHA receptor and adenylate cyclase and may give rise to an exaggerated adenylate cyclase response in the presence of CT. It would seem as if reduction in inositol phospholipid turnover is related to the elevation of cyclic AMP rather than a CT effect on a putative transducer which acts directly on phospholipase C. Our study does not exclude the existence of non-CT-sensitive transducers in this capacity.

INTRODUCTION

T-cell activation by antigens or mitogenic lectins requires several intracellular second messengers for signal transduction (Nel *et al.*, 1987). Phospholipase C (PL-C) mediated hydrolysis of inositol phospholipids yields diacylglycerol (DAG) and inositol trisphosphate (IP_3), which in turn lead to release of intracellular Ca^{2+} and generation of Ca^{2+}/phospholipid-dependent kinase (C-kinase) activity (Nishizuka, 1984; Berridge & Irvine, 1984; Tsien *et al.*, 1982; Imboden & Stobo, 1985). While these events play a stimulatory role in the autocrine growth cycle of T-cells (Acuto *et al.*, 1985; Manger *et al.*, 1985; Nel *et al.*, 1986), the exact role of an early concomitant peak of cyclic AMP activity remains uncertain; on the balance of evidence it would seem to exert an inhibitory influence (Strom *et al.*, 1977; Watson, 1976; Kaever & Resch, 1985; Wedner & Parker, 1976).

It is possible that lectin receptors may be coupled to adenylate cyclase via guanine nucleotide regulatory protein(s) such as heteromeric G_s or G_i (reviewed by Gill, 1982). Because cholera toxin (CT) or pertussis toxin (PT) are able to ADP-ribosylate and modify the function of G_s or G_i, respectively, they are useful tools for studying this system in lymphocytes (Hart & Finkelstein, 1975). This approach requires careful interpretation of the data, however, because it is now known that G-binding proteins which relay signals between receptors and alternative second messengers, such as PL-C and PL-A_2, may also be influenced by these toxins (Ohta *et al.*, 1985; Nakamura & Ui, 1985; Cockcroft & Gomperts, 1985; Buch *et al.*, 1986). Recent evidence obtained from Jurkat T-cells would seem to indicate that CT may inhibit antigen-receptor-induced responses through the ADP-ribosylation of a 43 kDa substrate that is distinct from $G_s\alpha$ (Imboden *et al.*, 1986).

We noticed a substrate of almost similar molecular mass in normal human T-cell membranes during ADP-ribosylation studies and found that CT acted inhibitory to PHA-induced [^{3}H]thymidine uptake. Measurement of cyclic AMP levels during PHA stimulation of CT-pretreated normal human and Jurkat T-cells revealed a synergistic increase of this second messenger. Our data would indicate that G_s is directly or indirectly involved in signalling via the PHA receptor. We also provide some evidence for its inhibitory effect on the C-kinase stimulatory pathway.

Abbreviations used: Ca^{2+}_i, intracellular free calcium concentration; C-kinase, Ca^{2+}/phospholipid-dependent kinase (protein kinase C); CT, cholera toxin; DAG, diacylglycerol; FCS, fetal calf serum; IP_3, inositol trisphosphate; IL-2, interleukin 2; G_s and G_i, guanine-nucleotide-binding proteins responsible for activation or inhibition of adenylate cyclase activity, respectively; PHA, phytohaemagglutinin (purified grade); PL-C, phospholipase C; PL-A_2, phospholipase A_2; PMSF, phenylmethanesulphonyl fluoride; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PT, pertussis toxin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate; GTPγS, guanosine 5'-[γ-thio]triphosphate.

‖ To whom correspondence and reprint requests should be addressed at present address: Division of Immunology, Department of Internal Medicine, UCLA School of Medicine Center for the Health Sciences, CHS 52/175, 10833 Le Conte Avenue, Los Angeles, CA 90024, U.S.A.

MATERIALS AND METHODS

Materials

PHA, histone IIIS, phosphatidylserine, diolein, cholera toxin, dibutyryl cyclic AMP, isobutylmethylxanthine and 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) were all purchased from Sigma. [^{32}P]P_i (40 mCi/ml) and a radioimmunoassay kit for measuring cyclic AMP levels were bought from Amersham (Arlington Heights, IL, U.S.A.), while Gamma Prep A was obtained from Promega Biotec (Madison, WI, U.S.A.). [α-^{32}P]NAD$^+$ and [^{3}H]phorbol 12,13-dibutyrate were from New England Nuclear. LK6D t.l.c. plates were purchased from Whatman. A pertussis toxin extract enriched for the islet-activating protein subunit was purchased from List Biological Laboratories (Campbell, CA, U.S.A.). Culture medium components were from Gibco. All other reagents were of the highest analytical grade available.

Cellular separation and culture

T-cell-enriched populations were obtained from the peripheral blood of normal adult volunteers. Mononuclear cells were separated by density centrifugation over Ficoll-Isopaque. After depletion of adherent cells and double cycle rosetting with AET-treated sheep erythrocytes (Pellegrino *et al.*, 1975), an enriched T-cell population exhibiting > 90% positive fluorescence for OKT-3 were obtained. This population contained 2–3% non-specific esterase positive cells. The cell viability was > 98% as judged by Trypan Blue exclusion and no toxicity was seen with any of the agents that were used for modifying cellular responses at doses used. Cells were cultured in RPMI medium, supplemented with 10% FCS and antibiotics, at 10^5 cells/well in flat-bottomed microtitre plates. Control cells received no further additions while stimulated cultures were incubated in the presence of 10 μg of PHA/ml for 54 h in a a 5% CO_2 environment at 37 °C. [^{3}H]Thymidine was added for 18 h before harvesting and scintillation counting. The effect of adding different doses and combinations of intact CT, PT, TPA and dibutyryl cyclic AMP were examined in this system as indicated.

A CD-2/CD-3-positive subclone of Jurkat cells was held in continuous culture and was grown in complete RPMI medium under similar conditions.

Assessment of inositol phospholipid turnover

Enriched T-cells or Jurkat cells were resuspended at 5×10^6 cells/ml and preincubated in phosphate-free Eagle's minimal essential medium. Cells were subsequently resuspended to similar density in the same medium containing 5% dialysed FCS in 1 ml culture tubes, to which various combinations of the following agents were added at different time periods: PHA, 10 μg/ml; CT, 100 ng/ml; dibutyryl cyclic AMP, 100 μM and forskolin, 10 μM. CT was introduced 30–120 min before addition of PHA to allow adequate cellular uptake and processing. Each tube was pulsed with 100 μCi of [^{32}P]P_i for 3–15 min prior to commencing cellular stimulation with PHA and/or dibutyryl cyclic AMP (Akeson *et al.*, 1984; Hui & Harmony, 1980). Reactions were terminated by extraction in 1.5 ml of chloroform/methanol (1:2, v/v), followed by addition of 0.5 ml of 2.4 M-HCl/0.1 M-KCl and 0.5 ml of chloroform (Schacht, 1981). After thorough mixing of their contents, the tubes were centrifuged and the lower phases were collected, washed and dried under N_2 and the dried lipids kept at −20 °C until used (Schacht, 1981). Lipids were dissolved in chloroform/methanol (2:1, v/v), and small aliquots were applied to Merck LK6D TLC plates. The developing solvent was chloroform/methanol/acetone/acetic acid/water (40:13:15:12:8, by vol.). After drying of the plates, autoradiograms were obtained and spots corresponding to all three inositol phospholipid species and a reference phospholipid (e.g. the spot for phosphatidylethanolamine) were scraped off and counted. Each sample was processed in duplicate and from this we calculated the stimulated uptake of ^{32}P into PI and PE. Phospholipid standards added to the extracted lipids were visualized by developing the plates in an iodine tank for 10 min.

ADP-ribosylation of lymphocyte membrane

Washed T-cells (10^8 cells/experiment) were homogenized with a Dounce homogenizer (10 strokes) in ice-cold hypo-osmotic medium containing 25 mM-Tris, pH 8.5, 5 mM-$MgCl_2$, 1 mM-EGTA, 1 mM-dithiothreitol and 50 k.i.u. of aprotinin/ml (Ribeiro-Niro *et al.*, 1985). Sucrose was added to a final concentration of 0.25 M and after the nuclei were removed, a crude membrane pellet was obtained by centrifugation at 12000 *g* for 10 min. The pellet was subsequently resuspended in an ice-cold potassium phosphate buffer, pH 7.5, containing 10 mM-thymidine, 1 mM-ATP and 50 k.i.u. of aprotinin/ml. Some tubes additionally received 0.1 mM-GTP or 0.1–10 μM-GTPγS (Owens *et al.*, 1985). CT-containing reaction vials also received 2.5 mM-Mg^{2+} but this cation was omitted from PT-containing vials. The homogenous membrane suspension (100 ng) was incubated with 10 μM-[α-^{32}P]NAD$^+$ (10^6 c.p.m./vial) in the presence of preactivated CT or PT, 0.1–2 μg and 0.02–0.4 μg/tube, respectively. Reactions were run at 30 °C for 30 min (Ribeiro-Neto *et al.*, 1985) and terminated by sedimenting the membranes at 12000 *g* and boiling the pellets in Laemmli (1970) sample buffer. Samples were submitted to polyacrylamide slab gel electrophoresis as previously described (Laemmli, 1970) and the stained gels were autoradiographed for 48 h at −80 °C with Kodak XAR film.

Measurement of intracellular cyclic AMP production

Normal human E-rosette positive lymphocytes and Jurkat cells, resuspended in complete medium, were incubated for 30–180 min in the presence of absence of CT (100 ng/ml) and/or 10 μM-indomethacin. At the indicated time periods some of these cultures were stimulated with PHA (10 μg/ml) for a further 10–180 min. All cultures were made 0.1 mM with respect to isobutylmethylxanthine. The incubations were terminated and the cellular AMP extracted by treating duplicate washed cell pellets with 3 ml of ice-cold 67% ethanol in phosphate-buffered saline (Dobson & Strange, 1985). These tubes were kept at −20 °C overnight. Supernatants, after 2000 *g* sedimentation, were removed and saved, while pellets were washed once in 1 ml of 67% ethanol. The supernatants were evaporated under N_2 and the residue resuspended in 0.5 ml of water. Small aliquots of these were used for analysis of cyclic AMP by a commercially available radioimmunoassay kit (Amersham). Triplicate measurements were done on every cell pellet and results were expressed as pmol of cyclic AMP/10^7 cells.

Determination of [^{3}H]PDBu binding to T-cell membranes

Jurkat cells, resuspended in complete RPMI medium, were portioned into tubes at 5×10^6 cells/ml and preloaded with leupeptin (10 μg/ml) and CT (100 ng/ml) or 100 μM-dibutyryl cyclic AMP for 60 or 15 min, respectively. Leupeptin was used to prevent Ca^{2+}-dependent proteolysis of C-kinase (Melloni *et al.*, 1986). Cells were subsequently stimulated with PHA (10 μg/ml) or TPA (100 ng/ml) for 10 min. The cell pellets were lysed in ice-cold buffer A containing 20 mM-Tris, pH 7.5, 50 mM-2-mercaptoethanol, 1 mM-EGTA, 1 mM-PMSF, leupeptin (10 μg/ml) and aprotinin (100 k.i.u./ml) (Nel *et al.*, 1986*b*, 1987; Farrar & Anderson, 1985). Sucrose was added to a final concentration of 0.25 M and, after removal of the nuclei by low speed centrifugation, the cytosol was separated from residual particulate material by ultracentrifugation at 100000 *g*. The cytosolic fractions were used for partial purification of C-kinase across small DEAE columns (Farrar & Anderson, 1985) and C-kinase activity was determined as previously described (Nel *et al.*, 1986*a*,*b*). The particulate material was homogenized in 20 mM-Tris/HCl, pH 7.5, containing 10 mM-2-mercaptoethanol and used for determining [^{3}H]PDBu binding as previously described (Tanaka *et al.*, 1986). Briefly, 20 μl of homogenized particulate suspension was added to reaction vials containing 200 μg of phosphatidylserine, 5 mM-$MgCl_2$, 100 μM-Ca^{2+}, 10 μg of bovine serum albumin, and 50 nM-[^{3}H]PDBu (150×10^6 d.p.m.), in the absence or presence of unlabelled excess PDBu. After 20 min at 23 °C, 2 ml of ice-cold 0.5% dimethyl sulphoxide was added. The mixture was poured onto a glass-fibre filter which had been soaked in fresh 0.3% polyethylenimine solution for 1 h before use.

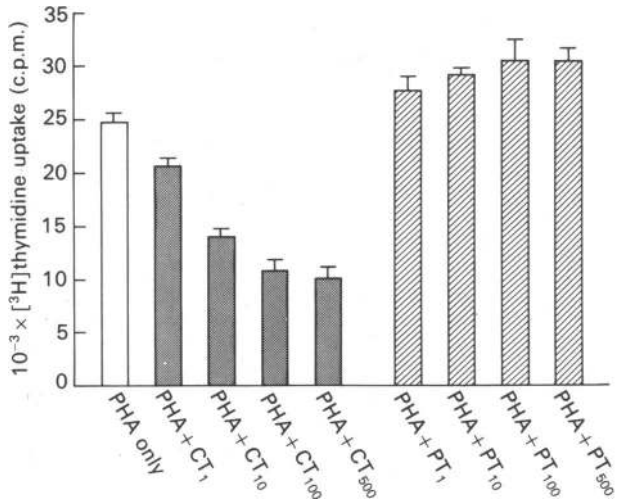

Fig. 1. Influence of CT and PT on PHA-stimulated [^{3}H]-thymidine uptake

Purified normal T-cells were treated in the absence or presence of 10 μg of PHA/ml for 54 h in triplicate wells of microtitre plates. [^{3}H]Thymidine (0.5 μCi/well) was added for a further 18 h and the cells harvested onto glass-fibre filters. Different doses of CT or PT (in ng/ml) were preincubated (1 h) with selective culture wells before stimulation with PHA (10 μg/ml). Data given are means ± S.E.M. of triplicate determinations from one representative experiment out of four; each of these were performed with a different donor's lymphocytes. [^{3}H]Thymidine uptake in unstimulated wells was 167 ± 39 c.p.m.

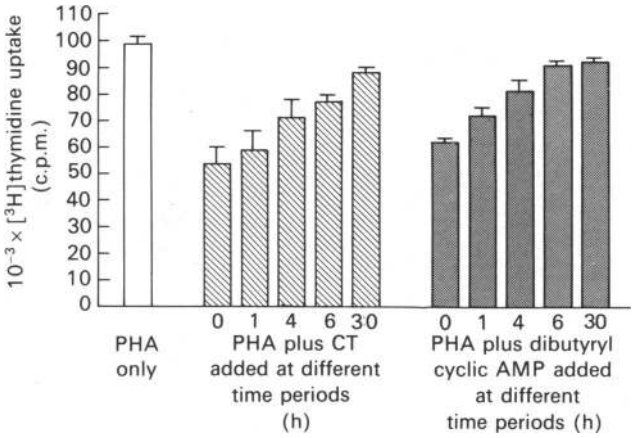

Fig. 2. Influence of CT (100 ng/ml) and dibutyryl cyclic AMP (100 μM) on PHA-stimulated [^{3}H]thymidine uptake in normal T-cells

Stimulation with PHA (10 μg/ml) was started at $t = 0$ but instead of preincubation with CT or dibutyryl cyclic AMP, these agents were introduced at time points corresponding to $t = 0$, 0, 1, 4, 6 or 30 h. Unstimulated cultures incorporated 1261 ± 121 c.p.m. Data given are means ± S.E.M. of triplicate determinations from one experiment; a second experiment yielded similar results.

Statistical analyses

Where appropriate, the Wilcoxon paired rank sum test was used.

RESULTS

Different effects of CT and PT on PHA-stimulated [^{3}H]thymidine uptake

Partial but significant ($P \leqslant 0.05$) inhibition of [^{3}H]-thymidine uptake responses was seen in the presence of CT at doses of 10–100 ng/ml (Fig. 1). Total response abrogation could not be achieved, even with larger doses (1000 ng/ml) or prolongation of the pre-exposure time period for up to 4 h. These results are in agreement with previous studies looking at the effect of CT on lectin-treated lymphocytes (Hart & Finkelstein, 1975). Maximum inhibition never exceeded 60% of the optimal PHA response. PT, on the other hand, not only failed to inhibit, but resulted in a small but measurable augmentation of the PHA response (Fig. 1).

Second messenger generation and signal transduction are carefully regulated events which should be delivered at the correct stage and for an appropriate time period during the cellular response. Since it has previously been shown that CT was ineffective if added 24 h or more after commencing mitogenic stimulation (Hart & Finkelstein, 1975), we were interested to see if we could narrow the time down further. Statistically significant ($P \leqslant 0.05$) inhibition could be obtained when CT was added within the first 6 h but not beyond this point (Fig. 2). A similar time-related profile of inhibition was obtained with exogenous dibutyryl cyclic AMP (Fig. 2).

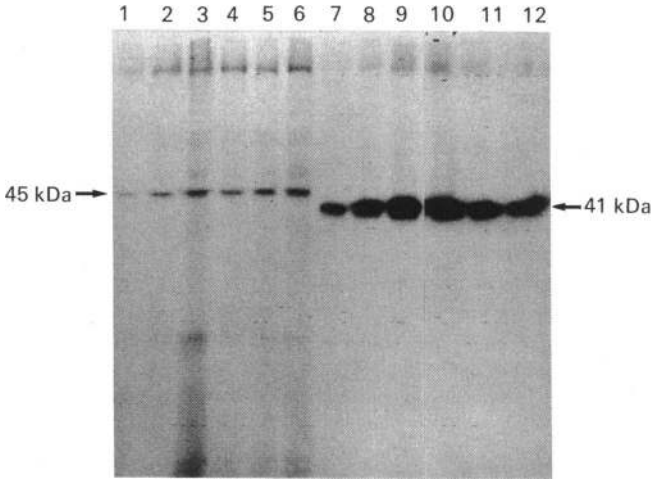

Fig. 3. Autoradiogram of a 10% SDS/polyacrylamide gel on which T-cell membrane extracts were analysed after ADP-ribosylation in the presence of CT (lanes 1–6) or PT (lanes 7–12)

Crude membrane extracts (100 μg of protein/100 μl reaction vial) were incubated with [α-^{32}P]NAD$^+$ for 30 min in the presence of 0.1 μg (lanes 1), 1 μg (lanes 2, 4, 5 and 6) and 2 μg (lane 3) of CT, or 0.02 μg (lane 7), 0.2 μg (lanes 8, 10, 11 and 12) and 0.4 μg (lane 9) of PT, respectively. In addition, some reaction vials received GTPγS to the amount of 0.1 (lanes 4, 10), 1 (lanes 5, 11) and 10 μM (lanes 6, 12). Reactions were terminated by boiling in SDS sample buffer. The molecular masses of labelled substrates are indicated. A duplicate experiment gave similar results.

[α-^{32}P]NAD$^+$-dependent ADP-ribosylation of membrane proteins in the presence of CT and PT

Fig. 3 shows the respective ADP-ribosylation profiles of membrane substrates for CT (lanes 1–6) and islet activitating protein-enriched PT (lanes 7–12). Labelling of the 45 kDa CT-sensitive substrate was dependent on the dose of toxin used (lanes 1–3), and was enhanced by the inclusion of GTPγS (lanes 4–6). In contrast, PT covalently modified different substrate(s) of 39–41 kDa; these incorporated proportionally more label than the 45 kDa sensitive substrate.

Synergy of CT and PHA in the generation of cyclic AMP and the influence of indomethacin

Although CT was an effective stimulus for increasing cyclic AMP production, the intracellular generation of this second messenger was synergistically enhanced in the presence of PHA (Fig. 4). As this suggested that the PHA receptor itself could regulate adenylate cyclase activity, we also measured cyclic AMP production during PHA treatment to confirm previous work which showed small but significant ($P \leqslant 0.025$) elevation of intracellular cyclic AMP levels (Kaever & Resch, 1985).

Since it is known that arachidonic acid is often released concomitantly to the induction of PI turnover (Nishizuka, 1984) and also that certain prostaglandins may influence intracellular cyclic AMP levels (Chouaib *et al.*, 1985; Butcher & Baird, 1968; Vercammen & Ceuppens, 1987), we were interested to see what the influence of a cyclo-oxygenase inhibitor would be on PHA-induced

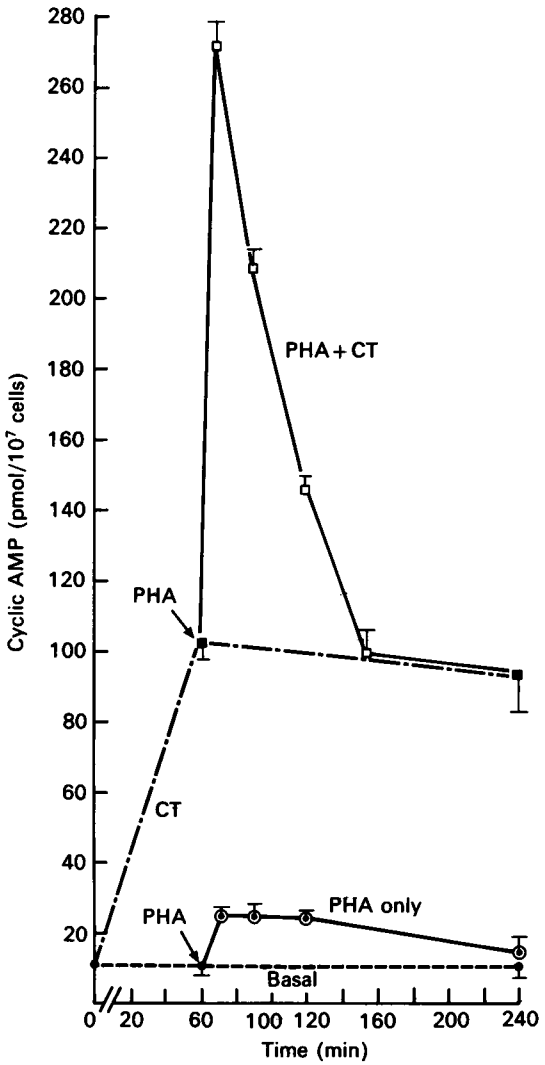

Fig. 4. Assessment of intracellular cyclic AMP generation in normal T-cells treated with PHA (10 μg/ml) and CT (100 ng/ml) in various combinations over a 4 h time period

Enriched T-cells (5×10^6 cells/tube) were resuspended in duplicate in complete medium before addition of CT. PHA was added to some of these cultures 60 min later (arrow) and reactions were terminated by treating the washed cell pellets in ice-cold 67% methanol in phosphate-buffered saline (Dobson & Strange, 1985). After extraction, cyclic AMP levels were measured by a standardized radioimmunoassay kit and results expresed as shown. Duplicate measurements were made for each tube. The number of experiments performed on normal human T-cells was three, and a similar profile was obtained with one experiment on Jurkat cells. The results were statistically significant at $P \leqslant 0.05$.

increases of this cyclic nucleotide. Results obtained with normal and Jurkat T-cells are shown in Table 1. Indomethacin at a concentration of 10 μM blocks cyclo-oxygenase quite effectively but does not interfere with PL-A_2 activity (Franson *et al.*, 1980). No inhibition of cyclic AMP generation was seen in Jurkat cells and a small but insignificant reduction was seen in normal PHA-treated lymphocytes (Table 1). Previous studies

have indicated that stimulated human peripheral blood lymphocytes could act as a source of arachidonic acid, but they were deficient in prostaglandin production (Goldyne & Stobo, 1982).

CT reduces PHA-induced inositol phospholipid turnover

Because sensitivity towards CT was an early phenomenon, we were interested to see whether toxin and/or dibutyryl cyclic AMP was, in fact, interfering with a concomitant stimulatory event such as inositol phospholipid turnover. T-cells were pulse labelled with [^{32}P]P_i for 3 min and stimulated with PHA (10 μg/ml) for time periods ranging between 5 and 180 min prior to extraction of the phospholipid phase and analysis by t.l.c. A 5–10-fold increase of ^{32}P incorporation into PI was seen in stimulated as compared with control cells (Fig. 5). The autoradiogram in Fig. 5(*b*) is representative of the 30 min stimulation point depicted in Fig. 5(*a*). A significant ($P \leqslant 0.05$) reduction of approx. 40% in the PHA-stimulated PI response was seen at every time interval under study (Fig. 5*a*). Two further experiments yielded similar results. Dibutyryl cyclic AMP also reduced the stimulated response by 36–40% (not shown). Although no clear differences could be detected by this method at the 5 min stimulation point, it was possible to perform measurements within this time period by prolonging pulse labelling to 15 min. The latter method also facilitated the detection of ^{32}P incorporation into phosphatidylinositol 4,5-bisphosphate. For instance, in a representative experiment control cell material incorporated 816±174 c.p.m. of ^{32}P; this increased to

Table 1. Effect of indomethacin on cyclic AMP levels

Culture conditions, extraction and measurements of cyclic AMP levels were the same as described in Fig. 4. CT and indomethacin were pre-incubated for 1 h prior to stimulation with PHA. The stimulation time period was 20 min. Number of experiments, 2.

	Cyclic AMP (pmol/10^7 cells)	
	Jurkat cells	Human E^+-lymphocytes
Unstimulated (control)	< 1	1.17±0.33
CT (100 ng/ml)	237.0±7.14	106.4±9.02
PHA (10 μg/ml)	1.58±0.21	3.8±0.33
PHA+indomethacin	1.91±0.58	2.5±0.22
PHA+CT	650.7±62.6	304.5±21.6
PHA+CT+indomethacin	664.3±10.3	243.4±5.1

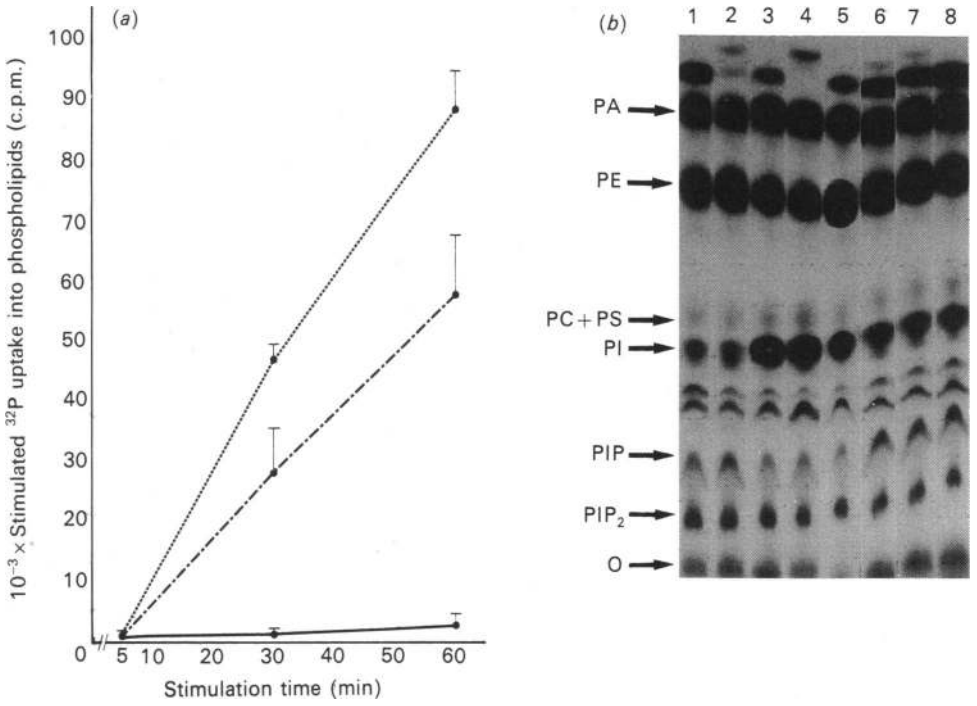

Fig. 5. ^{32}P incorporation into PI as assessed by t.l.c.

Jurkat cell suspensions were preloaded with CT (100 ng/ml) for 1 h or 100 μM-dibutyryl cyclic AMP for 15 min. Duplicate suspensions of phosphate-depleted cells were pulsed with 100 μCi of [^{32}]P_i for 3 min prior to stimulation with PHA for the indicated time periods. After extraction of phospholipids, equal amounts of radioactivity were loaded onto Merck LK 6D plates. These were developed as described together with phospholipid standards. After drying, the standards were visualized by iodine staining and radiolabelled phospholipid spots were detected by autoradiography. The number of experiments was four; two each for Jurkat cells and normal T-cells. (*a*) Plot to describe stimulated ^{32}P incorporation at various time periods in the presence (–·–·) or absence (------) of CT. Stimulated ^{32}P-incorporation into PE (——) is shown for comparison. (*b*) Autoradiograph of the 30 min time point in (*a*). Tracks: 1. 2, control cells, unstimulated; 3, 4, PHA stimulation for 30 min; 5, 6, PHA stimulation of CT-loaded cells; 7, 8, PHA stimulation after loading with dibutyryl cyclic AMP. Stimulated ^{32}P incorporation was calculated as follows: [^{32}P]PI in PHA-treated samples minus [^{32}P]PI in control samples = (59211±3938) − (12373±975) = (46838±1378) c.p.m. Further abbreviations: PA, phosphatidic acid; PIP, PIP$_2$, PI 4-phosphate and 4,5-bisphosphate; PC, phosphatidylcholine.

Table 2. Influence of CT and dibutyryl cyclic AMP on intracellular translocation of C-kinase in PHA-treated Jurkat cells

Jurkat cells were resuspended in complete medium and duplicate aliquots were preincubated with CT (100 ng/ml) or 100 μM-dibutyryl cyclic AMP for 60 and 15 min, respectively. Control cultures received no further additions (unstimulated control) or were treated with TPA (100 ng/ml) (positive control) for 10 min. CT and dibutyryl cyclic AMP pre-treated cultures were stimulated with PHA (10 μg/ml) for 10 min. The cell pellets were homogenized in 20 mM-Tris/HCl, pH 7.5, containing 10 mM-2-mercaptoethanol, 1 mM-EGTA together with proteolytic inhibitors and made 0.25 M with respect to sucrose. After removal of the nuclei, cytosolic and particulate materials were separated by ultracentrifugation. The particulates, which were assumed to include most of the surface membrane compartment, were homogenized in 20 mM-Tris/HCl, pH 7.5, and used for determination of [^{3}H]-PDBu binding in triplicate. The background d.p.m. (in the presence of a 500-fold excess of PDBu) never exceeded 15% of the maximal d.p.m. (absence of unlabelled PDBu) and was subtracted from the latter to yield specific binding in d.p.m. These values were used to calculate pmol of [^{3}H]-PDBu/mg of sample protein ($n = 2$).

	Membrane-associated C-kinase	
	(pmol of [^{3}H]PDBu/mg of protein)	(% of control)
Unstimulated control	928±135	100
CT (100 ng/ml)	920±4	99
TPA (100 ng/ml)	1350±91	146
PHA (10 μg/ml)	1230±3	133
PHA+CT	984±116	106
PHA+dibutryl cyclic AMP (100 μM)	917±16	99

3270±306 c.p.m. after 3 min of stimulation. It was reduced to 2190±42 c.p.m. (35%) and 2598±258 c.p.m. (21%) in the presence of CT and dibutyryl cyclic AMP, respectively. Measurement of ^{32}P incorporation into phosphatidic acid in the presence of CT and dibutyryl cyclic AMP yielded an identical trend.

CT prevents the intracellular translocation of C-kinase in PHA-treated cells

Since the hydrolysis of inositol phospholipid species generates DAG, which in turns leads to activation of C-kinase, we were interested to see if the activation of this kinase may be influenced by the presence of CT. There is as yet no direct way of showing the activation of C-kinase intracellularly, but it is known that the enzyme may be translocated from the cytosol to the surface membrane by certain receptors which induce inositol phospholipid turnover (Nishizuka, 1984; Nel *et al.*, 1986*b*; Nel *et al.*, 1987). Approx. 44% of cytosolic activity disappeared from PHA-treated cells, but this phenomenon was apparently much reduced (to only 3% of cytosolic activity) if stimulation was performed in the presence of CT. CT itself had no effect on cytosolic activity (not shown). Parallel examination of Tritonized particulate extracts revealed an opposite trend, but interpretation was complicated by high basal Ca^{2+}/PS-independent activity. For this reason we chose to perform [^{3}H]PDBu binding studies to the particulates, assuming that most surface membrane components will be included in this fraction. Nuclei were removed because these are potential targets for C-kinase displacement by intracellular cyclic AMP-dependent processes (Cambier *et al.*, 1987). Results are depicted in Table 2 and show a 30–40% increase in [^{3}H]PDBu binding in PHA-treated material; TPA was used as positive control and resulted in a 40–50% increase in membrane bound enzyme (Table 2). In the presence of CT or exogenous dibutyryl cyclic AMP, the PHA-induced rise in [^{3}H]PDBu receptor number was abrogated (Table 2).

Table 3. Comparison of the influence of CT and dibutyryl cyclic AMP on PHA- or TPA-induced [^{3}H]thymidine uptake

Culture conditions and methods were as for Fig. 1. Triplicate measurements were made of each experimental point and means±S.D. are given. A second experiment yielded similar data.

	[^{3}H]Thymidine uptake	
	(c.p.m.)	(% of maximum)
Unstimulated control	134±14	–
PHA (10 μg/ml)	67211±201*	100
PHA+CT (10 ng/ml)	44803±487	66.7
PHA+CT (100 ng/ml)	34621±244	51.5
TPA (10 ng/ml)	23273±301†	100
TPA+CT (10 ng/ml)	22043±384	94.7
TPA+CT (100 ng/ml)	19223±114	82.6
TPA+dibutyryl cyclic AMP (10 μM)	18932±97	81.3
TPA+dibutyryl cyclic AMP (100 μM)	20043±403	86.1

*, † 100% reference values

Effect of CT and dibutyryl cyclic AMP on TPA-induced responses

The phorbol ester TPA is a weak mitogen for human peripheral blood T-cells (Ashman, 1984) and a known direct agonist for C-kinase (Castagna *et al.*, 1982). The inhibitory doses of CT and dibutyryl cyclic AMP shown in Figs. 1 and 2 were ineffective at reducing [^{3}H]thymidine uptake in response to TPA treatment (Table 3). It would therefore seem as if events at and distal to C-kinase activation were minimally affected while pathways proximal to generation of C-kinase activity were susceptible.

DISCUSSION

In the present study we demonstrated that CT could act inhibitory to PHA-stimulated T-cell proliferation by enhancing the role of cyclic AMP, a second messenger which under physiological conditions seems to act as a modulator of the stimulatory PI pathway.

The mechanism by which CT could result in inhibition in this system may conceptually be approached from two different viewpoints: (i) direct interference, through

ADP-ribosylation, of a transducing protein which controls PL-C activity; or (ii) G_s-mediated generation of intracellular cyclic AMP which acts as modulator of events leading to C-kinase activation. We favour the latter possibility for the following reasons. Firstly, inhibition of the [^{3}H]thymidine uptake response was incomplete, indicating a modulation rather than severing of an activation pathway (Figs. 1 and 2). If a CT-sensitive G-protein was responsible for initiating the inositol phospholipid pathway, we would have expected complete response abrogation. Secondly, CT acted synergistically with PHA to generate adenylate cyclase activity (Fig. 4), which would seem to indicate that an existing response was enhanced. Further, elevated intracellular cyclic AMP was shown to interfere with stimulatory events (see below). Thirdly, the CT-sensitive substrate has a mass of 45 kDa, which is similar in size to $G_s\alpha$.

The exact mechanism of coupling between the lectin receptor and G_s is unknown. It may involve a direct physical interaction, but it is also possible that prostaglandins could be involved since it is known that members of the E-group are potent stimulators of adenylate cyclase by way of cellular receptors (Chouaib *et al.*, 1985; Vercammen & Ceuppens, 1987). We have, however, been unable to show that cyclic AMP generation was affected by introduction of the indomethacin to PHA-treated normal or Jurkat cells (Table 1). This is in agreement with previous studies which demonstrated that mitogenic stimulation of lymphocytes may induce arachidonic acid release but does not stimulate prostaglandin production simultaneously (Goldyne & Stobo, 1982). Taken together, it would seem as if the PHA receptor may be linked to adenylate cyclase by way of CT-sensitive G_s.

What is the mechanism by which the supraphysiological generation of cyclic AMP may interfere with T-lymphocyte responses? Is this by way of activating an inhibitory pathway or could it be an indirect form of inhibition aimed at the stimulatory inositol phospholipid/C-kinase pathway? (Kaibuchi *et al.*, 1982; Nel *et al.*, 1987). While this study is inadequate to address the former possibility, our results clearly demonstrated that high intracellular cyclic AMP may interfere with PHA-stimulated phosphoinositide turnover (Fig. 5), as well as the intracellular activation of C-kinase as measured by its intracellular translocation (Table 2). The former event is probably the more critical in terms of the inhibitory influence, because direct activation of cellular responses with the C-kinase agonist, TPA, were not subject to inhibition by CT or cyclic AMP (Table 3). Also, CT or dibutyryl cyclic AMP had to be present at an early stage of the cellular response to exert an inhibitory effect (Fig. 2). This is in agreement with previous findings (Strom *et al.*, 1977) and coincides with the time period during which the inositol phospholipid cycle is active. Whether the target step is the triggering event, namely phosphatidylinositol bisphosphate hydrolysis by PL-C, or subsequent stages of the turnover cycle, e.g. phospholipid kinase activity, is unknown at present, but it is noteworthy that Imboden *et al.* (1986) have recently shown that stimulation of adenylate cyclase activity does not interfere with inositol trisphosphate release during ligation of antigen receptors on Jurkat cells. We therefore favour the latter possibility. It has previously been reported that cyclic AMP may suppress PI turnover

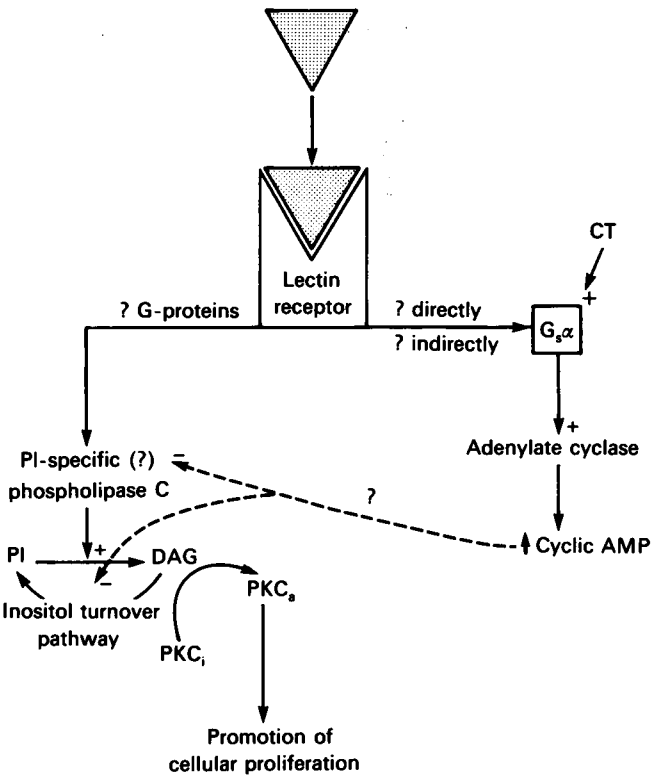

Fig. 6. Proposed scheme of events to explain how concomitant generation of two second messengers interacts to regulate the PHA response

According to this hypothesis adenylate cyclase activity may be directly or indirectly linked to the PHA receptor via $G_s\alpha$. Through ADP-ribosylation of G_s, CT interferes in this pathway and leads to a prolonged or excessive rise in intracellular cyclic AMP levels. The inhibitory role of this nucleotide on PI turnover and generation of C-kinase activity is exaggerated in the presence of CT. The net effect is therefore a diminished PHA response. PKC_i, inactive protein kinase C; PKC_a, activated enzyme.

(Kaibuchi *et al.*, 1982) as well as intracellular translocation of C-kinase (Anderson *et al.*, 1985; Chen *et al.*, 1986).

Our data do not provide a direct clue to the possible involvement and identity of G-binding protein transducers between the PHA receptor and PL-C, but Imboden *et al.* (1986) suggest that a CT-sensitive substrate may be involved. We are not convinced, however, that their 43 kDa substrate differs from our 45 kDa $G_s\alpha$ protein, because despite a rise in intracellular cyclic AMP they failed to provide evidence of a second membrane protein in their ADP-ribosylation studies. It is possible, of course, that ADP-ribosylation of $G_s\alpha$ may lead to the release of $\beta\gamma$-dimers which, in turn, may effect other G-proteins (Gill, 1982). Evidence has recently been provided which suggests the existence of guanine-nucleotide binding proteins that couple surface receptors to the breakdown of inositol-containing lipids during T-cell mitogenesis (Mire-Sluis *et al.*, 1987; Aussel *et al.*, 1988).

In considering all of the aforementioned we would like to propose that the generation of cyclic AMP during mitogenic stimulation plays a modulatory role (Fig. 6). If the balance of control is shifted in favour of the modu-

lator, e.g. through the use of CT, the magnitude of the stimulatory response diminishes. Adenylate cyclase activity may therefore act to fine-tune the PI response to lectins and communicates with the lectin receptor by way of G_s. Grove & Mastro (1987) have also suggested that lymphocyte mitogenesis depends on the dual activation of both kinase pathways.

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