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Role of G-proteins in T cell activation: non-hydrolysable GTP analogues induce early ornithine decarboxylase activity in human T lymphocytes

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Rapid activation of ornithine decarboxylase is one of the earliest recognized events during induction of a mitogenic response in human T lymphocytes. Here we show that the non-hydrolysable GTP analogues guanine-5-(γ-thio)trisphosphate and guanylyl-5-imidodiphosphate, introduced into human T cells by means of a transient membrane permeabilization technique, can replace an external mitogenic ligand, such as concanavalin A, in inducing early ornithine decarboxylase activity. Neomycin inhibits this rapid activation at concentrations known to bind to phosphoinositides. One of the two compounds formed in polyphosphoinositide breakdown, inositol-1,4,5-trisphosphate, also induces ornithine decarboxylase activity. The other, diacylglycerol, apparently does not, since the phorbol ester, tetradecanoyl phorbol acetate, which is thought to mimic the action of diacylglycerols, does not alter basal ornithine decarboxylase activity in T cells until several hours after administration. These findings indicate that guanine nucleotide-binding regulatory (G-) protein(s) participates in the transduction of the mitogenic signal. The intracellular target system for this G-protein may include phosphoinositide breakdown and generation of inositoltrisphosphate, which might be involved in the early activation of ornithine decarboxylase.

Key words: G-proteins/inositol metabolism/T cell activation/ornithine decarboxylase

Introduction

How the mitogenic signals from the surface membrane receptors are transferred to the cell nucleus is still largely unknown.

The G-proteins are a family of membrane-associated, guanine nucleotide-binding proteins, involved in signal transduction (Gilman, 1984; Limbird, 1981; De Wit and Snaar-Jagalska, 1985). Members of the family have been shown to couple several receptors to their various intracellular effectors (Gomperts, 1983; Bokoch and Gilman, 1984; Backlund *et al.*, 1985; Pfaffinger *et al.*, 1985; Breitweiser and Szabo, 1985; Barrowman *et al.*, 1986; Virgilio *et al.*, 1986), including phospholipase C activity producing inositol phosphates and diacylglycerol from phosphoinositides (Cockcroft and Gomperts, 1985; Blackmore *et al.*, 1985; Joseph, 1985; Uhing *et al.*, 1986). Most studied are the two G-proteins coupling stimulating and inhibiting receptors to adenylate cyclase (Gilman, 1984; Limbird, 1981). These two proteins consist of three subunits (α, β, γ) and differ only with respect to their α-subunits. More members of the G-protein family are continuously found.

The G-proteins are closely related to the products of the *ras* and *rho* (Madaule and Axel, 1985) genes, which presumably also regulate cellular functions in a GTP-dependent manner.

Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the synthesis of polyamines (Jänne *et al.*, 1978). Cell growth is invariably associated with high ODC activities and stimulated polyamine metabolism (Kay and Lindsay, 1973; Korpela *et al.*, 1981; Erwin *et al.*, 1983; Persson *et al.*, 1984; Kallio *et al.*, 1977, Haddox *et al.*, 1980). Mitogenic ligands (but not non-mitogenic) induce a 4- to 5-fold activation of ODC in T cells within 5–10 min (Scott *et al.*, 1984, 1985). This rapid rise in ODC activity is insensitive to cycloheximide and dependent on extracellular calcium. The early activation is also seen as a rapid rise in putrescine concentration (our unpublished data). The activity of the enzyme remains at this elevated level 2–3 h and then begins to rise in a cycloheximide-sensitive manner.

We have earlier shown that an intact inositol metabolism is obligatory for the early activation of ODC (Mustelin *et al.*, 1986). Here we show that the rapid ODC activation is inducible by non-hydrolysable GTP analogues, suggesting that a G-protein is an important link in the transduction of mitogenic signals.

Results

Transient membrane permeabilization

Incubation of blood T lymphocytes at 37°C in the presence of ATP (3 μM) and EDTA (0.5 mM) permits the entry of otherwise impermeable compounds. The permeability was measured by uptake of the fluorescent dye ethidium bromide, which binds to DNA and can be visualized under u.v. light (data not shown). The cells are not damaged by a brief permeabilization (3 μM ATP for 5 min); the viability, measured as trypan blue exclusion, is unchanged after a cycle of permeabilization and resealing with 1 mM Mg^{2+}. After sealing of the cell membranes the lymphocytes respond normally to concanavalin A (ConA) by rapid ODC activation, and to PHA by DNA synthesis and prolifera-

Table I. Influence of transient membrane permeabilization (3 μM ATP, 5 min) on cell responsiveness

	Without ATP treatment	With ATP treatment
ODC response		
Control	23.2 ± 4.2 (n = 20)	21.9 ± 4.3 (n = 9)
ConA (10 μg/ml)	92.4 ± 6.7 (n = 20)	91.6 ± 11.5 (n = 9)
GTPγS (100 μM)	25.7 ± 2.7 (n = 3)	83.8 ± 5.9 (n = 5)
[^{3}H]Thymidine incorporation		
Control	2508 ± 745 (n = 4)	2392 ± 522 (n = 3)
PHA (3 μg/ml)	100 356 ± 2824 (n = 4)	79 057 ± 964 (n = 3)

Phytohaemagglutinin (PHA)-induced [^{3}H]thymidine incorporation is measured after 72 h of culture and expressed as c.p.m./10^6 plated cells, ODC activity as pmoles of liberated $^{14}CO_2$/60 min/10^6 cells. Cell viability exceeded 95% in all experiments. SD and number of experiments are indicated.

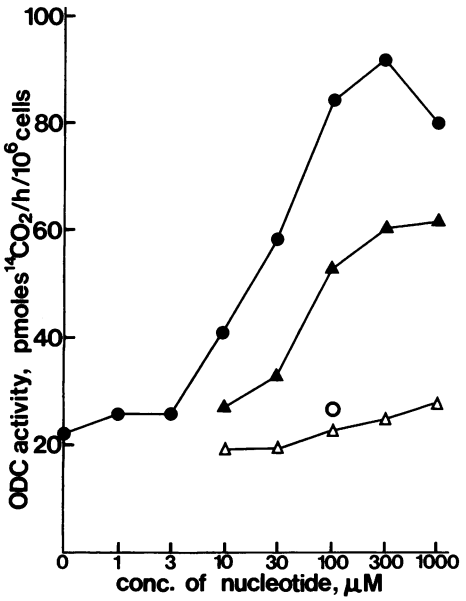

Fig. 1. Effects of GTPγS (●), GppNHp (▲) and GTP (△) on ODC activity 10 min after permeabilization (5 min). ODC activity after a similar treatment (GTPγS, 100 μM) without ATP permeabilization is also indicated (○). The activity is given as pmoles of liberated $^{14}CO_2$/60 min/10^6 cells.

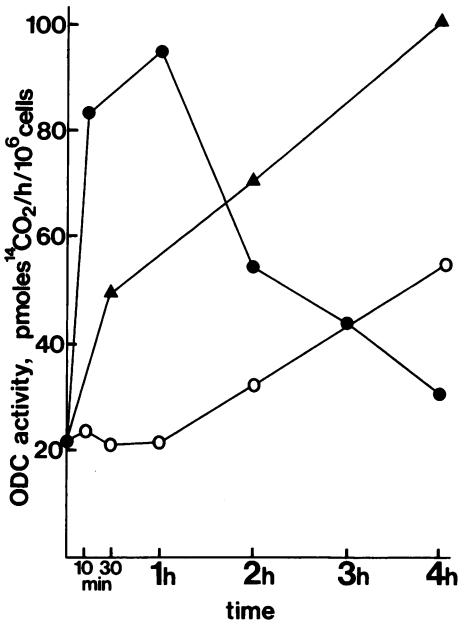

Fig. 3. The long-time effects of GTPγS 100 μM (●), TPA 100 nM (○) or both together (▲) on ODC activity measured at indicated times after permeabilization.

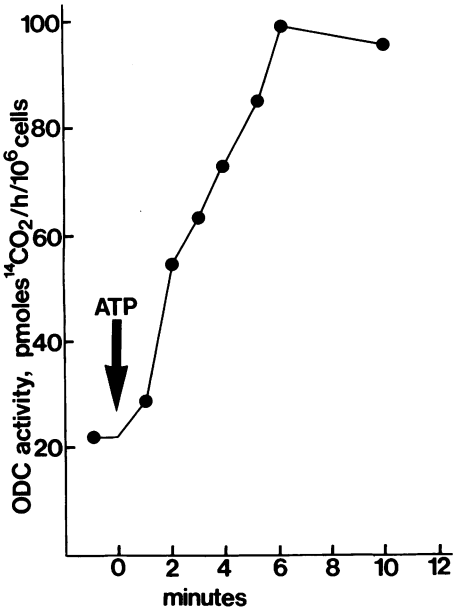

Fig. 2. ODC activity measured 10 min after the addition of 100 μM GTPγS to cells permeabilized for various times. The duration of the permeabilization is expressed as minutes from the addition of 3 μM ATP to the addition of 1 mM Mg^{2+}.

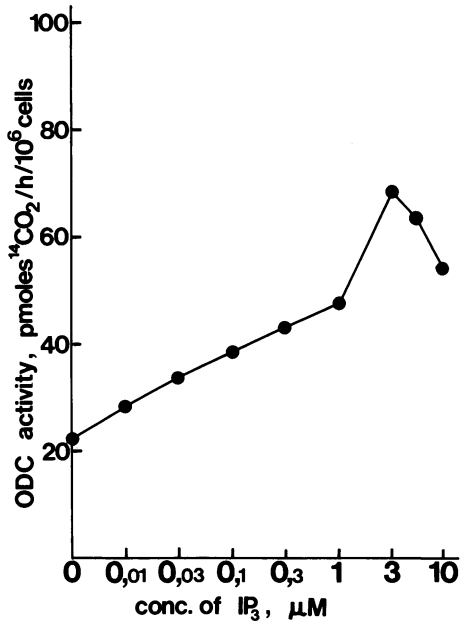

Fig. 4. Induction of ODC activity by IP_3 measured 10 min after permeabilization.

tion. This indicates that the ATP-induced transient membrane permeabilization does not permanently damage the cells (Table I).

Effects of nucleotides on ODC activity

Introduced by the ATP method (3 μM, 5 min), guanine-5-(γ-thio)triphosphate (GTPγS) and guanylyl-5-imidodiphosphate (GppNHp) induce a rapid elevation of ODC activity in 10 min. The response is dose-dependent (Figure 1), with half-maximal effects at 30 μM or GTPγS and 50 μM for GppNHp. At 300 μM GTPγS induces ODC activity that in some experiments was higher than that induced by ConA. The effects of different durations of the permeabilization are shown in Figure 2.

Other guanine nucleotides tested [guanosine-5-diphosphate guanosine-5-(β-thio)diphosphate, cyclic guanosine-3,5-monophosphate], and the non-hydrolysable ATP analogue ATPγS, induced marginal or no activation of ODC (≤20% of the elevation induced by ConA, results not shown).

The elevation in ODC activity induced by 100 μM GTPγS remained at the level it had reached in 10 min for 1 h and then declined to basal level in 4 h (Figure 3).

Effects of inositol triphosphate and phorbol ester on ODC activity

Inositol triphosphate (IP_3) induced about 70% of the ODC activity obtained with ConA (10 μg/ml). Maximal response was seen at ~3 μM and even 0.03 μM produced a significant ODC response (Figure 4). Tetradecanoyl phorbol acetate (TPA),

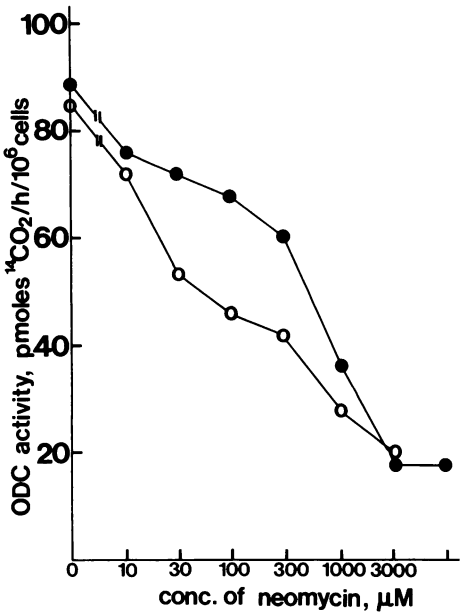

Fig. 5. Inhibition of ODC inducibility by neomycin, when ConA (●) or 100 μM GTPγS (○) were used as stimulants.

100 nM, caused no change in ODC activity during the first hour of incubation, later the activity began to rise slowly (Figure 3).

Neomycin blocks the activation of ODC

Neomycin blocked the induction of ODC activity both in permeabilized and in unpermeabilized cells. This inhibition of ODC activity was seen both after treatment with ConA and with GTPγS (but is stronger when GTPγS is used) (Figure 5). Neomycin also strongly blocked the elevation of ODC activity in lymphocytes measured 24 h after stimulation with ConA.

Discussion

Polar compounds do not readily penetrate an intact plasma membrane. At least some types of cells can be made permeable to small compounds, such as GTPγS, GppNHp or IP_3, by a recently described transient membrane permeabilization technique (Gomperts, 1983; Cockcroft and Gomperts, 1985; Gomperts and Fernandez, 1985). The mechanism is unknown. To our knowledge, this is the first demonstration of its application on lymphocytes.

In most systems studied, added GTP does not significantly activate G-proteins, probably due to its rapid hydrolysis. In contrast, non-hydrolysable GTP analogues activate G-proteins by blocking their inactivation. The G-protein thus remains in an actively transmitting mode (Gilman, 1984; Bockaert *et al.*, 1985; Barrowman *et al.*, 1986; Cockcroft and Gomperts, 1985).

Our results suggest a role for a G-protein(s) in the transduction of the activation signal in human T lymphocytes. This G-protein is unlikely to be identical with those involved in cyclic AMP (cAMP) metabolism, since the early activation of ODC is not a cAMP-mediated response.

G-proteins have recently been shown to functionally couple surface membrane receptors to phosphoinositide breakdown (Cockcroft and Gomperts, 1985; Blackmore *et al.*, 1985; Joseph, 1985; Uhing *et al.*, 1986). It has also been demonstrated that neomycin binds to phosphoinositides (Tadolini and Varani, 1986; Lodhi *et al.*, 1976) and thereby inhibits their breakdown (Cockcroft and Gomperts, 1976; Downes and Michell, 1981; Carney *et al.*, 1985). It is therefore possible that the G-protein involved in the early activation of ODC is identical with the one(s) inducing phosphoinositide breakdown.

Fleischman and co-workers (1986) recently reported that transformation of fibroblasts with any of the three different *ras* oncogenes leads to elevated production of inositol phosphates and diacylglycerol and loss of phosphatidylinositol-4,5-bisphosphate. Transfection of fibroblasts with human c-Ha-*ras* gives high and dose-dependent ODC activities (Sistonen *et al.*, 1986). Since the *ras*-encoded proteins are thought to participate in the regulation of growth and the ODC inducing G-protein transduces (at least a part of) the growth signal in T cells, it is tempting to speculate that this G-protein is a cellular *ras* product.

The induction of ODC activity by IP_3 could indicate that the generation of this compound is involved. It might also, however, reflect the fact that IP_3 elevates the intracellular calcium concentration, which is needed for the response (Scott *et al.*, 1984).

Our results indicate a dual control of ODC activity in proliferating T cells. A rapid rise in the activity of pre-existing enzyme is mediated by an inositol-requiring, calcium-dependent reaction, initiated by a G-protein. A later rise in ODC activity is mediated by *de novo* synthesis of ODC, involving a protein kinase C-mediated mechanism (Otani *et al.*, 1985; Sasakawa *et al.*, 1985; Jetten *et al.*, 1985). This stringent control of ODC activity during early mitogenesis further indicates an important role for the enzyme in the regulation of cell proliferation.

Materials and methods

Isolation of lymphocytes

Human lymphocytes were isolated by Ficoll-Isopaque gradient centrifugation from buffy coats kindly provided by the Finnish Red Cross Transfusion Service. In some experiments the T lymphocytes were further enriched by passage through a nylon wool column; this did not significantly influence any results. Cell viability was measured using trypan blue and always exceeded 95%.

Permeabilization procedure

In order to remove divalent cations the cells were washed several times in PBS supplemented with 0.02% EDTA (~0.5 mM) and suspended in this medium at 30–80 × 10^6/ml. ATP (3 μM) and the solutes to be internalized were added and the cells kept at 37°C for 5 min before 1 mM magnesium acetate was added to reseal the membrane lesions. The cells were then resuspended in RPMI 1640 medium and ConA (10 μg/ml) added to the positive control. The efficiency of the permeabilization was tested by ethidium bromide uptake by the cells in 3 μM ATP. The uptake was measured as percent of fluorescent nuclei under u.v. light from at least 200 cells.

ODC assay

The permeabilized or unpermeabilized cells were incubated at 37°C for 10 min immediately after the permeabilization procedure. A positive control (ConA) was always included. The incubation was terminated by a wash in ice-cold saline and the cells suspended in 25 mM Tris–HCl, 0.1 mM EDTA, 5 mM dithiothreitol, pH 7.5 and frozen in liquid nitrogen. The samples were thawed and sonicated and ODC activity was measured from the supernatants after a centrifugation at 42 000 *g* at 4°C for 45 min by the $^{14}CO_2$ liberation assay as described earlier (Seely *et al.*, 1982). The results are mostly given as percent of the positive control in the same experiment. In Table I ODC activity is given as pmoles $^{14}CO_{28}$/60 min/10^6 cells.

Measurement of [^{3}H]thymidine incorporation

Cells, permeabilized or unpermeabilized, were plated out at 5–25 × 10^5 cells/ml in RPMI 1640 supplemented with 10% human serum. The cells were incubated in a 5% CO_2 atmosphere at 37°C for 72 h, during the last 6 h in the presence of 1 μCi [^{3}H]thymidine/microwell (200 μl). The cells were harvested and radioactivity counted by liquid scintillation.

Materials

All nucleotides were purchased from Boehringer Mannheim (BRD). ConA and neomycin were from Sigma and IP_3 from Amersham (UK).

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