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Investigation of guanine-nucleotide-binding protein involvement and regulation of cyclic AMP metabolism in interleukin 1 signal transduction

Keith RAY,* Nicola THOMPSON, Nicola KENNARD, Philippa ROLLINS, Stephanie GRENFELL, Sarah WITHAM, Nicholas SMITHERS and Roberto SOLARI†

Department of Cellular Science, Glaxo Group Research Limited, Greenford Road, Greenford, Middx. UB6 0HE, U.K.

The involvement of guanine-nucleotide-binding proteins (G-proteins) and regulation of cyclic AMP (cAMP) in interleukin 1 (IL1) signal transduction has been investigated in EL4 and 7OZ/3 cells expressing Type 1 and Type 2 IL1 receptors respectively. Results show that in both cell types IL1 alone failed to induce changes in cellular cAMP levels, and in membrane preparations the cytokine had no significant effect on adenylate cyclase activity. In contrast, forskolin stimulated cAMP levels in cells and membranes. IL1 did not significantly alter GTPase activity or rate of guanosine 5′-[γ-[^{35}S]thio]triphosphate binding measured in membrane preparations from the EL4 and 7OZ/3 cells. In EL4-cell membrane preparations the kinetics of ^{125}I-IL1 binding were altered in the presence of guanosine 5′-[βγ-imido]triphosphate, resulting in the formation of a higher-affinity state for IL1 binding. Adenosine 5′-[βγ-imido]triphosphate at the same concentration was without effect. These results suggest that IL1 receptor function may be regulated by guanine nucleotides; however, the mechanism appears to differ from that exhibited by conventional G-protein-linked receptors. The lack of significant effects of IL1 on cAMP metabolism in these cells suggests that alternative pathways must exist to mediate the intracellular responses to stimulation via both types of the IL1 receptor.

INTRODUCTION

Interleukin 1 (IL1) describes a family of polypeptide cytokines produced primarily by cells of the monocyte/macrophage lineage, which elicit a wide range of biological actions, including the regulation of immune and inflammatory responses. Genes encoding two forms of IL1, IL1α and IL1β, have been cloned and sequenced (Auron *et al.*, 1984; March *et al.*, 1985; Oppenheim *et al.*, 1986) in addition to a natural IL1 receptor antagonist (Hannum *et al.*, 1990). Despite only limited primary sequence similarity between these gene products, they all bind to the same cell-surface receptors with approximately the same affinity (Kilian *et al.*, 1986; Bird & Saklatvala, 1986; Dower *et al.*, 1986). The biological effects of IL1 are considered to be elicited in response to high-affinity binding of the cytokine to specific receptors present on target cells. Studies of ^{125}I-IL1 affinity cross-linking to cell-surface binding sites (Solari, 1990) and the development of antibodies recognizing specifically an 80 kDa form of the IL1 receptor found on T cells have demonstrated that distinct forms of the IL1 receptor exist on different cell lineages (Bomsztyk *et al.*, 1989; Chizzonite *et al.*, 1989). Molecular cloning of cDNAs corresponding to the murine and human T-cell forms of the IL1 receptor have confirmed that the 80 kDa IL1 receptor found on T cells and fibroblasts and the 60 kDa IL1 receptor found on B cells represent two distinct gene products (Bomsztyk *et al.*, 1989; Chizzonite *et al.*, 1989; Sims *et al.*, 1989), which we shall term the type I and type II receptors respectively.

Transfection of the cloned type I IL1-receptor gene into CHO cells has been shown to be sufficient to confer biological responsiveness to IL1, suggesting that this single receptor chain is capable of transducing the IL1 signal (Curtis *et al.*, 1989). Despite these studies, the primary sequence of the receptor provides few clues to suggest possible mechanisms by which the signal-transduction process might occur. Furthermore, a number of features of IL1 receptor biology imply that the mechanisms involved in IL1 signalling may be novel. Firstly, the concentration of IL1 required to achieve half-maximal binding to its receptor (K_d = 50 pM) is known to exceed greatly the levels of cytokine which are required to produce biological responses in target cells. Secondly, the number of IL1 receptors per cell is relatively low, typically 1000 or less; therefore biological responses to IL1 appear to be initiated by the occupancy of a low number of receptors, perhaps as few as 1–10 per cell. Consequently, one would expect a highly efficient coupling and amplification system to transduce the IL1 signal and generate an intracellular response.

The ability of IL1 to affect various well-established signal-transduction pathways and second messengers has been investigated in some detail; however, no conclusive picture of receptor signalling events has yet emerged (Mizel, 1990; O'Neill *et al.*, 1990*a*). In T-cells, IL1 has been reported to increase phosphatidylserine biosynthesis (Didier *et al.*, 1988) and to increase diacylglycerol production from hydrolysis of phosphatidylcholine (Rosoff *et al.*, 1988), or possibly from hydrolysis of a novel phosphatidylinositol-glycan moiety (Dobson *et al.*, 1990). IL1-induced phosphatidylinositide hydrolysis was reported in macrophages (Wijelath *et al.*, 1988), but has not been seen in other cell types and the available evidence indicates that IL1 does not change intracellular Ca^{2+} levels (Abraham *et al.*, 1987; Didier *et al.*, 1988; Rosoff *et al.*, 1988). Despite the finding that IL1 stimulation induces production of diacylglycerol, evidence for a concomitant IL1-induced activation of protein kinase C is controversial (Abraham *et al.*, 1987; Ostrowski *et al.*, 1988; Munoz *et al.*, 1990). The question of involvement of cyclic AMP (cAMP) in the actions of IL1 is equally unresolved. IL1 stimu-

Abbreviations used: IL1, interleukin 1; IL2, interleukin 2; DTT, dithiothreitol; cAMP, cyclic AMP; GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; p[NH]ppA, adenosine 5′-[βγ-imido]triphosphate; LPS, lipopolysaccharide.

* To whom correspondence should be addressed.

† Present address: Yamanouchi Research Institute (U.K.), Littlemore Hospital, Oxford OX4 4XN, U.K.

lation has been reported to increase intracellular cAMP acutely in several cell types (Shirakawa *et al.*, 1988; Zhang *et al.*, 1988; Bomsztyk *et al.*, 1990; Munoz *et al.*, 1990) and to stimulate adenylate cyclase activity in membrane preparations (Shirakawa *et al.*, 1988; Chedid *et al.*, 1989). Others, however, have failed to observe cAMP responses to IL1 (Didier *et al.*, 1988; Shiroo & Matsushima, 1990; Fagarasan *et al.*, 1990; Kasahara *et al.*, 1990; Rodan *et al.*, 1990), and in T-cells elevation of cAMP concentration by pharmacological agents was found to inhibit the ability of IL1 to induce interleukin 2 (IL2) secretion (Rollins *et al.*, 1991).

Recently, it has been reported (Chedid *et al.*, 1989) that a novel pertussis-toxin-sensitive GTP-binding protein might be responsible for the transduction of stimulatory signals from the IL1 receptor to adenylate cyclase. Evidence that IL1 can stimulate GTP binding and GTPase activity in membrane preparations from IL1-responsive cells also suggests that a GTP-binding protein may be involved in IL1 signal transduction (O'Neill *et al.*, 1990*a*,*b*). Hormonal regulation of adenylate cyclase is generally considered to be mediated via the hetero-trimeric class of GTP-binding proteins. These have been shown to couple with a distinct family of receptors which possess common structural features, most notably seven putative membrane-spanning domains. Since the predicted form of the IL1 receptor does not possess these characteristics, a novel signal-transduction mechanism may be expected to account for its ability to activate adenylate cyclase in a GTP-dependent fashion. In order to investigate aspects of this signalling pathway in more detail, we have therefore re-examined the ability of IL1 to affect cAMP formation and G-protein function, using two different IL1-responsive cells lines expressing either the 80 kDa or the 60 kDa forms of IL1 receptor.

MATERIALS AND METHODS

Reagents

Human recombinant IL1α and IL1β were obtained from the Glaxo Institute of Molecular Biology, Geneva, Switzerland. [α-^{32}P]ATP ($\sim$ 30 Ci/mmol) and [8-^{3}H] cAMP (30 Ci/mmol) were from Amersham International, Amersham, Bucks., U.K. [γ-^{32}P]GTP ($\sim$ 30 Ci/mmol), guanosine 5'-[γ-[^{35}S]thio]triphosphate (GTP[^{35}S]), [^{32}P]NAD (1000 Ci/mmol) and cAMP radioimmunoassay kits were purchased from NEN Research Products, Dupont (U.K.), Stevenage, Herts., U.K. Nucleotides including adenosine 5'-[$\beta\gamma$-imido]triphosphate (p[NH]ppA), guanosine 5'-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) and GTP[S] were from Boehringer Mannheim, Lewes, East Sussex, U.K. Pertussis toxin was from Porton International, Salisbury, Wilts, U.K. Rat anti-mouse κ light chain conjugated to fluorescein isothiocyanate was obtained from Seralab, Horsham, Sussex, U.K. Electrophoresis reagents were obtained from Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K., and all other biochemical reagents were obtained from Sigma, Poole, Dorset, U.K.

Cell lines

The murine T-cell lymphoma EL 4.6.1.d10 (EL4) was kindly provided by Dr. Robson MacDonald of the Ludwig Institute, Epalinges, Switzerland, and was maintained as previously described (Rollins *et al.*, 1991). The murine pre-B-cell line 7OZ/3 was obtained from the American Tissue Culture Collection and was maintained according to the suppliers' instructions.

Determination of intracellular cAMP

The ability of IL1 to alter cellular cAMP concentration was measured by the following procedure. Briefly, cells (EL4 or 7OZ/3) were washed and resuspended in serum-free culture medium, divided into wells of a tissue-culture plate [4 × 6 wells at (3–4) × 10^6 cells per well in 1.5 ml] and incubated for 2 h at 37 °C. Experiments were initiated by addition of 50 μl of IL1 or forskolin diluted in culture medium to give the final concentrations shown. At the times stated, cells from individual wells were harvested and centrifuged for 30 s at 13000 rev./min in an Eppendorf Minifuge at room temperature. The supernatant was rapidly aspirated and the cell pellet was resuspended in 0.5 ml of ice-cold 0.2 M-HCl, and immediately frozen. For the cAMP assay, samples were neutralized and the cAMP content was measured after acetylation by using a radioimmunoassay kit (NEN) as described by the manufacturers.

Preparation of membranes for enzyme assays and binding studies

Cells ($\sim 5 \times 10^8$) in exponential growth phase were harvested, washed in phosphate-buffered saline (146 mM-NaCl/4.8 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4) and stored as a frozen pellet at −80 °C. To prepare membranes, the cell pellet was rapidly thawed and resuspended in buffer containing 50 mM-Tris/HCl, pH 7.6 (at 4 °C), 5 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride and 10 μg of soybean trypsin inhibitor/ml, and homogenized on ice with a Polytron (Kinematica G.m.b.H., Littau, Switzerland) at mark 7 for a 10 s burst. The homogenate was centrifuged at 500 *g* for 5 min at 4 °C. The supernatant was re-centrifuged at 30000 $g_{av.}$ for 30 min at 4 °C in a Sorvall SS34 rotor. The resulting pellet was resuspended in homogenization buffer at a protein concentration of approx. 5 mg/ml and stored at −80 °C.

Enzyme assays

Adenylate cyclase assays were conducted by the method of Salomon *et al.* (1974). The volume of each assay was 100 μl and contained the following reagents: 50 mM-Tris/HCl, pH 7.5, 5 mM-$MgCl_2$, 20 mM-phosphocreatine, creatine kinase (1000 units/ml), 1 mM-cAMP, 0.1 mM-isobutylmethylxanthine, 10 μM-GTP and 1 mM-[α-^{32}P]ATP (20–40 d.p.m./pmol). Cytokines and drugs were added to the assays at the concentrations indicated. Incubations were performed at 30 °C and initiated by addition of the membrane preparation. Linear rates of [^{32}P]cAMP formation were observed over at least the first 20 min period at membrane concentrations of up to 40 μg per assay point.

GTPase assays were carried out by a modification of the method of Cassel & Selinger (1976). Incubations in a final volume of 100 μl contained 25 mM-Tris/HCl, pH 7.4, 5 mM-$MgCl_2$, 1 mM-EGTA, 10 mM-phosphocreatine, 2 mM-dithiothreitol (DTT), 1 mM-ATP, 0.5 mM-p[NH]ppA, 1 mM-ouabain, 2 mg BSA/ml, 50 units of creatine kinase/ml and [γ-^{32}P]GTP (sp. radioactivity 2000 d.p.m./pmol, final concn. 0.5 μM). Drugs, cytokines or solvent vehicle as a control were added at the indicated concentrations. Incubations at 37 °C were initiated by additions of membranes (10–40 μg of protein per assay tube) and terminated at the times shown by addition of 1.0 ml of ice-cold charcoal suspension containing 50 mg of charcoal/ml, 10 mg of BSA/ml, 2 mg of dextran/ml and 10 mM-H_3PO_4. Tubes were centrifuged to separate charcoal-bound radioactivity, and 0.5 ml samples of the supernatants taken to measure ^{32}P release by liquid-scintillation spectrometry.

GTP-[^{35}S]binding assay

The effects of IL1 on binding of GTP[^{35}S] to cell membrane preparations were investigated by a method based on that described by Huff & Neer (1986). Binding assays were performed at 30 °C in buffer containing 27.5 mM-Tris/HCl, pH 7.4, 1.1 mM-ATP, 1.1 mM-$MgCl_2$, 1.1 mM-DTT, 1.1 mg of BSA/ml, 1.1 mM-ouabain, 1.1 mM-EDTA and GTP[^{35}S] at the concen-

trations shown. Incubations in a final volume of 100 μl were performed in a 96-well filter plate (Millipore) and were initiated by addition of membranes (10–40 μg/well). Reactions were terminated by rapid vacuum filtration of the samples, followed by washing of the filters with 3×0.2 ml of ice-cold wash buffer (25 mM-Tris/HCl, pH 7.4, 50 μM-EDTA, 5 mM-$MgCl_2$ and 1 mM-DTT). Radioactivity bound to the filter discs was determined by liquid-scintillation spectrometry. In assays to determine the effect of IL1 on GTP binding to membranes, a fixed final concentration of 50 nM-GTP[^{35}S] was used at a specific radioactivity of $\sim$ 10000 d.p.m/pmol (0.025 μCi per well). For saturation-binding studies the concentration of GTP[^{35}S] was varied from 4 to 500 nM (sp. radioactivity 20000 d.p.m./pmol), and incubations were performed for 60 min with 10 μg of membrane protein to achieve equilibrium binding. Control incubations containing a 100-fold molar excess of unlabelled GTP[S] were included to determine non-specific binding, which was usually less that 5% of the total bound radioactivity, and the binding data were analysed by linear transformations using the LIGAND program in accordance with the method of Scatchard (1949).

^{125}I-IL1-binding assays

The binding of ^{125}I-IL1α to crude membrane preparations was investigated as follows. Saturation-binding studies were carried out using 5 μg of membrane protein in RPMI 1640 culture medium containing 5% (v/v) foetal-calf serum and 0.01% (w/v) bacitracin. Incubations were performed in 96-well microtitre filtration plates (Millipore SV) with a final volume of 0.15 ml, at room temperature for 4 h to reach equilibrium. ^{125}I-IL1 (sp. radioactivity $\sim 4 \times 10^6$ d.p.m./pmol) was added in the concentration range 0–300 pM, and parallel incubations containing a 100-fold molar excess of unlabelled IL1 were used to determine non-specific binding. Binding was terminated by rapid vacuum filtration and repeated washing of the filters with ice-cold binding buffer, and the radioactivity associated with each filter disc was determined by γ-radiation spectrometry. The equilibrium binding studies were performed on EL4 membranes either with no additions or in the presence of 10 nM-p[NH]ppG or 10 nM-p[NH]ppA. The binding data were analysed by linear transformations using the LIGAND program in accordance with the method of Scatchard (1949).

The effect of p[NH]ppG on the kinetics of ^{125}I-IL1 association and dissociation from its receptor were also determined. For determination of the ligand/receptor association rate constants, 300 pM-^{125}I-IL1α was incubated with 50 μg of membrane protein in binding buffer, as described above, in a final volume of 0.2 ml, with or without addition of 10 nM-p[NH]ppG. Incubations were initiated by the addition of radiolabelled IL1α and terminated by vacuum filtration on to glass-fibre filters pre-treated with phosphate-buffered saline containing 1% (w/v) BSA. Non-specific binding to the EL4 membranes was determined with a 100-fold molar excess of unlabelled IL1. To measure the dissociation kinetics, 50 μg of membranes was incubated to equilibrium (4 h at room temperature) with 300 pM-^{125}I-IL1α. The membranes were extensively washed, and dissociation of receptor/ligand complexes was initiated by resuspending the membranes in binding medium containing 430 nM unlabelled IL1 with or without 10 nM-p[NH]ppG. At timed intervals the incubations were terminated by vacuum filtration as described above.

ADP-ribosylation of membrane preparation

EL4 cells were incubated overnight (18 h) in the absence or presence of 100 pM-IL1α or 100 pM-IL1β, and membranes were prepared as described above. ADP-ribosylation of pertussis-toxin-specific substrates present in each membrane preparation was carried out by published methods (Ribeiro-Neto *et al.*, 1985). The ADP-ribosylation reaction was performed in the absence or presence of pertussis toxin in order to demonstrate the specificity of the substrates, and also in the absence or presence of 100 pM-IL1α to determine any effect the cytokine may have on the pattern of ADP-ribosylation. Incubations were performed in a total volume of 120 μl and contained the following; 25 mM-Tris/HCl, pH 7.5, 10 mM-thymidine, 1 mM-EDTA, 1 mM-ATP, 8 mM-DTT, 100 μM-GTP, with or without 100 pM-IL1α, with or without 10 μg of pertussis toxin (pre-activated)/ml, 0.2 mg of BSA/ml, 10 μM-[^{32}P]NAD^+ ($\sim$ 2.5 μCi/sample) and the membrane suspension (60 μg/sample). After incubation for 45 min at 30 °C, reactions were terminated by addition of 1.0 ml of ice-cold 20% (w/v) trichloroacetic acid. The samples were subjected to SDS/PAGE (Maizel, 1971) and analysed by autoradiography.

Analytical procedures

Flow-cytometry analysis was performed to determine surface expression of Ig κ-light chains in the murine pre-B-cell line 7OZ/3. Cells, at a concentration of 5×10^5/ml, were incubated for 24 h either with no additions or with additions of 20 pM-IL1 or 1 μg of lipopolysacharide (LPS)/ml. The cells were subsequently analysed for surface κ-chain expression by using a Beckton–Dickinson FACS analyser as described previously (Quint *et al.*, 1989).

RESULTS

Biological responsiveness of target cells

The studies reported here concern the signal-transduction pathways activated by the IL1 receptor following binding of its ligand. Since there are two forms of IL1 receptor which represent distinct gene products, we have chosen to study two cell lines as models for each receptor type. The murine thymoma cell line, EL4, expresses the $\sim$ 80 kDa form of the receptor, which we shall refer to as the type I receptor, whereas the murine pre-B cell line 7OZ/3 expresses the $\sim$ 60 kDa form of the receptor, which we shall refer to as type II. Before embarking on studies of signalling pathways, it was essential to demonstrate that both of these cell lines expressed IL1 receptors and that they had a demonstrable biological response to stimulation with physiologically appropriate doses of the cytokine. The expression of cell-surface receptors for IL1 on both EL4 and 7OZ/3 was previously demonstrated by affinity cross-linking studies using radiolabelled IL1α and IL1β (Solari, 1990). Stimulation of EL4 cells with IL1 results in the induction of IL2 secretion, and the particular sub-clone of EL4 used in this study showed a half-maximal response at an IL1 concentration of $\sim$ 250 fM (Rollins *et al.*, 1991). The 7OZ/3 cells have been reported to up-regulate surface Ig κ-light-chain expression in response to IL1 or LPS stimulation (Sakaguchi *et al.*, 1980), and we consequently wished to confirm this response.

To establish that our 7OZ/3 cells were also responsive, cells were treated for 24 h with either IL1 (20 pM) or LPS (1 μg/ml). Flow-cytometry analysis was then performed to assess the levels of surface κ-chain expression. Results confirmed that both stimuli induced elevation of κ-chain expression, with LPS producing the slightly greater response (results not shown).

Influence of IL1 stimulation on cAMP levels in intact cells

In view of the recent reports (Mizel, 1989) that the acute effects of IL1 on target cells may be mediated via changes in intracellular cAMP levels, we investigated whether IL1 could affect cAMP concentrations in either EL4 or 7OZ/3 cells. The responses

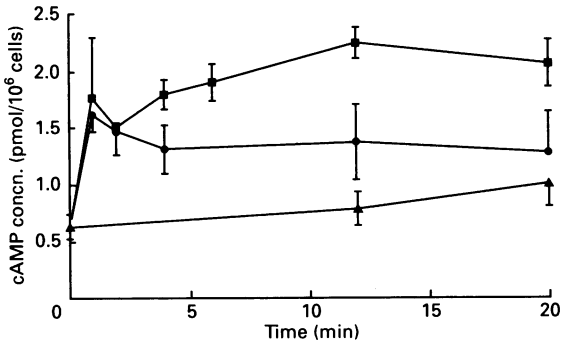

Fig 1. Stimulation of EL4 cells with a combination of IL1 and forskolin

EL4 cells were either incubated without receiving a stimulus (▲) or were stimulated with 1 μM-forskolin (●) or with a combination of 1 μM-forskolin with 1 nM-IL1 (■). At the indicated times after administration of the stimulus, cells were harvested and intracellular cAMP levels determined as described in the Materials and methods section. The Figure is a representative example of an individual experiment, and each point is the mean ± S.E.M. of triplicate determinations.

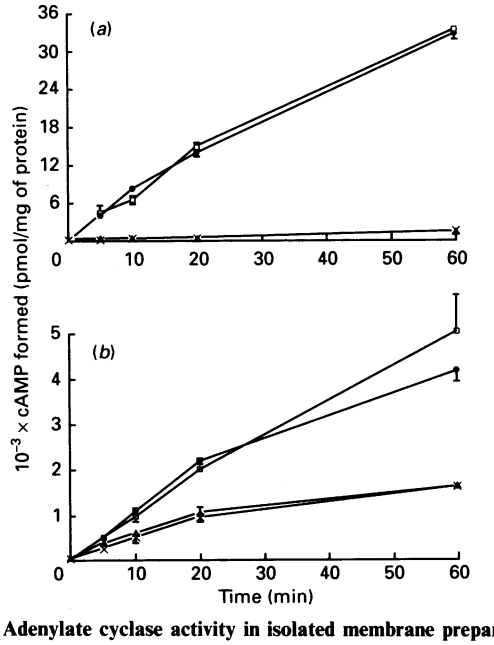

Fig. 2. Adenylate cyclase activity in isolated membrane preparations

Membranes were prepared from EL4 cells (*a*) or 7OZ/3 cells (*b*) by cell-fractionation procedures described in the Materials and methods section. The activity of adenylate cyclase in these membrane preparations was determined either under unstimulated conditions (×) or after stimulation with 1 nM-IL1 (▲), 10 μM-forskolin (●) or IL1 and forskolin in combination (□). Adenylate cyclase activity was measured by the formation of cAMP (in pmol/mg of membrane protein) over the indicated time course.

of these cells to stimulation with 24 pM-IL1 were tested, since this dose of cytokine is close to the K_d for receptor/ligand binding and sufficient to generate maximal biological responses. Over the period 2–120 min following addition of IL1 there was no significant effect on basal cAMP levels in either of the cell lines tested. As a positive control in both cases, adenylate cyclase was stimulated directly with 10 μM-forskolin. This resulted in a rapid elevation of cellular cAMP levels (within 2 min) in both cell types, with maximal concentrations reached between 5 and 20 min. Although considerable variation was found between experiments, resting levels of cAMP were similar in the EL4 and 7OZ/3 cells, that is between 0.1 and 0.5 pmol/10^6 cells. However, the maximum cellular response to forskolin stimulation of the 7OZ/3 cells (approx. 2-fold over basal) was consistently less than that observed with the EL4 cells (6-fold over basal). Despite this apparent inability of the IL1 receptor to activate cyclase directly after binding of ligand, when EL4 cells were stimulated with IL1 and forskolin together the intracellular accumulation of cAMP was prolonged and enhanced as compared with the response to forskolin alone (Fig. 1).

Influence of IL1 stimulation on adenylate cyclase activity in cell membrane preparations

In order to investigate further the possible involvement of adenylate cyclase in IL1 signal transduction, we examined the effect of IL1 stimulation on cyclase activity in membrane preparations from EL4 and 7OZ/3 cells. Figs. 2(*a*) and 2(*b*) show time courses for the formation of cAMP by membrane preparations from EL4 and 7OZ/3 cells respectively. In both cases, IL1 (1 nM) stimulation did not increase cyclase activity in the membranes as compared with control incubations with no additions, whereas forskolin stimulation (10 μM) resulted in a marked activation of cyclase. Doses of IL1 ranging from 10 pM to 10 nM were tested, and under no conditions could we detect an IL1-induced activation of cyclase activity (results not shown). However, when forskolin and IL1 were used in combination, the net effect on cyclase activity was variable. The results shown in Fig. 2 represent the most typical situation where IL1 did not influence forskolin activation of cyclase, but on occasions a significant inhibition of the forskolin activation of cyclase by co-stimulation with IL1 was observed (results not shown). The reasons for this variability have not been established. A significant observation to be made from the quantification of cyclase activity in EL4 and 7OZ/3 membranes is that in both cell lines the basal level of activity was similar (~ 50 pmol of cAMP formed/min per mg); however, in 7OZ/3 membranes cyclase activity was only poorly activated by forskolin stimulation as compared with EL4 membranes (Fig. 2).

To summarize data derived from experiments with different EL4 membrane preparations, basal cyclase activity of 63 ± 11 pmol of cAMP/min per mg ($n = 4$; mean ± S.E.M.) was not affected by 10 nM-IL1 (53 ± 7 pmol/min per mg; $n = 3$), whereas 10 μM-forskolin produced a 21-fold stimulation to 1313 ± 189 pmol/min per mg ($n = 4$).

G-protein involvement of IL1 signal transduction

The ability of bacterial toxins to modulate cellular responses to IL1 (Chedid *et al.*, 1989; Rollins *et al.*, 1991), together with reports that IL1 can activate membrane-associated GTPase activity and stimulate GTP binding (O'Neill *et al.*, 1990*b*) have suggested that IL1-receptor signal transduction might be mediated via a guanine-nucleotide-binding protein. We therefore sought to confirm such a mechanism by investigating the ability of IL1 to modulate various biochemical aspects of G-protein function in membrane preparations. Membrane preparations from EL4 cells were incubated in the presence or absence of 1 nM-IL1, and both the rate of GTP[^{35}S] binding (Fig. 3*a*) and the rate of [γ-^{32}P]GTP hydrolysis (Fig. 3*b*) were quantified. The results consistently failed to demonstrate IL1-induced increases in the rates of GTP binding or GTP hydrolysis. Comparable experiments on the rate of GTP hydrolysis in 7OZ/3 membranes (basal GTPase activity of 12 pmol/min per mg) also failed to indicate any IL1-induced stimulation. In order to establish that

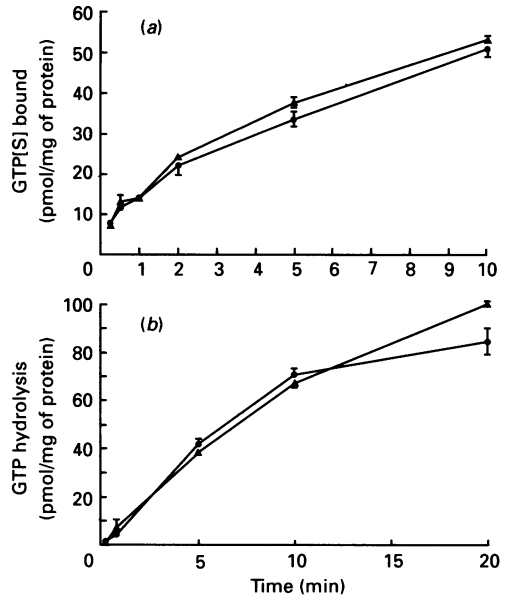

Fig. 3. Effect of IL1 on GTP binding and GTPase activity in EL4 membranes

Membranes were prepared from EL4 cells by cell-fractionation procedures. The membranes were incubated in the absence (●) or presence (▲) of 1 nM-IL1 for the time course indicated, and the binding of GTP[^{35}S] (*a*) or the hydrolysis of [γ-^{32}P]GTP (*b*) was determined as described in the Materials and methods section. Each point represents the mean ± S.E.M. of triplicate determinations.

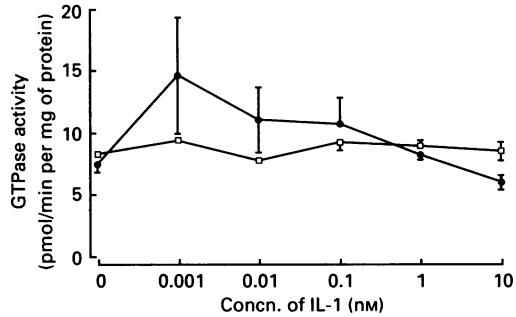

Fig. 4. Effect of increasing doses of IL1 on GTPase activity in EL4 and 7OZ/3 membranes

Membranes prepared from EL4 cells (●) or 7OZ/3 cells (□) were incubated with increasing concentrations of IL1 and the rate of [γ-^{32}P]GTP hydrolysis was determined over a 5 min period. Each point represents the mean ± S.E.M. of triplicate determinations.

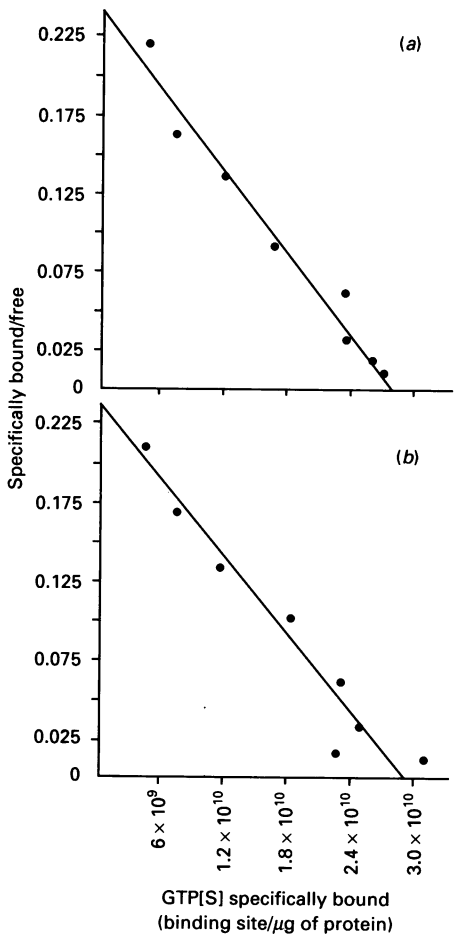

Fig. 5. Equilibrium binding of GTP[^{35}S] to EL4 membranes

This was performed in the absence (*a*) or presence (*b*) of 1 nM-IL1. The Figures represent Scatchard transformations of the equilibrium-binding data. Both gave linear plots with an identical $B_{max.}$ of 2.8×10^{10} binding sites per μg of membrane protein, with a K_d of 30 nM.

the lack of effect on GTPase activity was not simply a question of IL1-receptor occupancy with ligand, we quantified GTPase activity in EL4 and 7OZ/3 membranes at a range of IL1 concentrations from 1 pM to 10 nM (Fig. 4). In neither membrane preparation did IL1 cause a significant change in the net GTPase activity.

Analysis of the influence of IL1 on the interaction of GTP with EL4 membranes was also performed by equilibrium binding studies (Fig. 5). In these experiments, saturation binding of GTP[^{35}S] to EL4 membranes was performed to equilibrium in the presence or absence of IL1. Transformation of the binding data into Scatchard plots revealed that GTP bound to a single class of binding site on the membranes with an apparent equilibrium dissociation constant (K_d) of 30 nM and to 2.8×10^{10} binding sites per μg of membrane protein ($B_{max.}$). In the presence of IL1 neither the K_d nor the $B_{max.}$ of GTP[^{35}S] binding was altered.

Effects of nucleotide analogues on ^{125}I-IL1 binding to membrane preparations

A characteristic feature of many G-protein-coupled receptors is that in membrane preparations GTP or its non-hydrolysable analogues can cause a decrease in the affinity of agonist binding. The possibility that guanine nucleotides might alter binding of ^{125}I-IL1 to EL4 membranes was therefore investigated. Owing to the variability in the measurement of ^{125}I-IL1 binding to membrane preparations, all of the quoted values represent means from three independent determinations. The Scatchard plots in Fig. 6 show the data from one representative set of experiments, whereas the data presented in Table 1 are the means ± S.E.M. In equilibrium binding studies, Scatchard analysis of the ^{125}I-IL1 binding data revealed a single class of binding sites with a K_d of 53.6 pM and a $B_{max.}$ of 28.1×10^6 binding sites per μg of membrane protein (Table 1, Fig. 6*a*). When the binding studies were performed in the presence of 10 nM-p[NH]ppG, the data were converted from a one-site linear Scatchard plot to a curvilinear

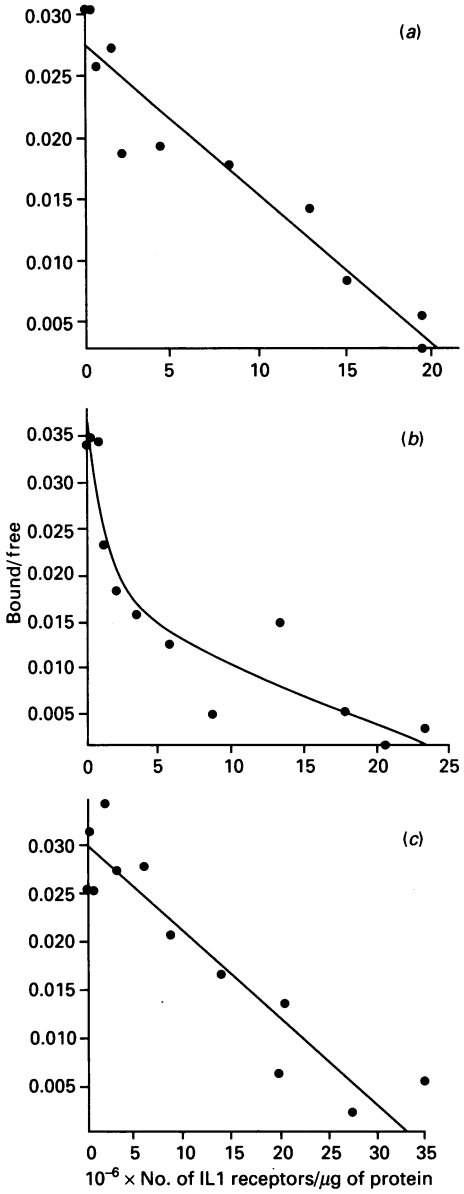

Fig. 6. Effect of nucleotide analogues on ^{125}I-IL1 binding to EL4 membranes

Equilibrium binding of ^{125}I-IL1 to EL4 membranes was performed with no additions (*a*), or in the presence of 10 nM-p[NH]ppG (*b*), or in the presence of 10 nM-p[NH]ppA (*c*). Scatchard transformations of the equilibrium-binding data revealed that under control conditions (*a*) and in the presence of p[NH]ppA (*c*) linear plots were obtained. In the presence of p[NH]ppG (*b*) a curvilinear plot was obtained. The Figures shown are a representative example of one of three independent experiments.

plot with a best fit to a two-site model (Table 1, Fig. 6*b*). Based on these transformations, ^{125}I-IL1 binding in the presence of GTP displays a high-affinity component with a K_d of 2.0 pM and a $B_{max.}$ of 0.8×10^6 binding sites per μg of membrane protein, and a low-affinity component with a K_d of 141.0 pM and a $B_{max.}$ of 43.5×10^6 binding sites per μg of membrane protein. As an additional control, binding studies were performed in the presence of the non-hydrolysable ATP analogue p[NH]ppA (Table 1, Fig. 6*c*). This nucleotide had no influence on ^{125}I-IL1 binding, and the Scatchard plots were for a single site with a K_d of 53.0 pM and a $B_{max.}$ of 22.6×10^6 binding sites per μg of membrane protein.

Table 1. Equilibrium binding of ^{125}I-IL1 to EL4 membrane preparations

Binding studies were performed with no nucleotide additions or in the presence of 10 nM-p[NH]ppA or 10 nM-p[NH]ppG. Binding data were transformed by the method of Scatchard (1949) and the K_d and number of binding sites/μg of membrane protein determined. Values represent means ± S.E.M. from three independent experiments.

Nucleotide	K_d (pM)	10^{-6} × Receptors (no./μg of protein)
None	53.6 ± 7.9	28.1 ± 4.3
p[NH]ppA	53.0 ± 17.3	22.6 ± 6.5
p[NH]ppG	141.0 ± 28.4	43.5 ± 12.0
	2.0 ± 0.15	0.76 ± 0.1

The GTP-induced alterations in the binding of IL1 to its receptor, as shown in Fig. 6(*b*), may be due to changes in the association rate or dissociation rate or both. To establish if p[NH]ppG modified the rate of association of IL1 with its receptor, EL4 membranes were incubated with ^{125}I-IL1 in the absence and presence of p[NH]ppG at 22 °C, and a time course for specific binding was determined (Fig. 7*a*). From these data we calculated a half-time ($t_{\frac{1}{2}}$) for receptor/ligand association of ~ 25 min, and semi-logarithmic transformation of the initial association rate (Fig. 7*a* inset) yielded a linear plot consistent with binding to a homogeneous class of binding site. Analysis of the gradient reveals that the experimentally observed association rate, $k_{obs.}$, for IL1 binding to its type I receptor was 25.3×10^3 min^{-1}. The addition of p[NH]ppG enhanced the rate of IL1 association with its receptor, and the $t_{\frac{1}{2}}$ for association was decreased from ~ 25 min to ~ 5 min. A semi-logarithmic transformation of the association rate in the presence of p[NH]ppG failed to produce a linear plot (Fig. 7*a* inset), suggesting a greater complexity of interaction than IL1 binding to a single population receptors with a single affinity. Having established the effect of GTP on the association rate of IL1 with its receptor, we proceeded to investigate the dissociation kinetics. For these studies EL4 membranes were incubated to equilibrium with ^{125}I-IL1 at 4 °C, washed thoroughly, then resuspended in medium, at 22 °C, containing 430 nM unlabelled IL1 in the presence or absence of p[NH]ppG. A time course for the dissociation of ^{125}I-IL1 from its receptor is shown in Fig. 7(*b*). In control membranes, 20 % of the ^{125}I-IL1 bound at zero time had dissociated from its receptor after 4 h, and a semi-logarithmic linear transformation of the data revealed that the dissociation rate constant, $k_{diss.}$, was 5.53×10^3 min^{-1}. The addition of p[NH]ppG slowed the dissociation rate considerably, and less than 10 % of the ^{125}I-IL1 bound at zero time had dissociated from its receptor after 4 h. Semi-logarithmic transformation of the dissociation data in the presence of p[NH]ppG failed to produce a linear plot, and consequently a $k_{diss.}$ could not be calculated. To confirm the validity of these kinetic data, we can compare the K_d calculated by Scatchard plots of equilibrium-binding studies (Fig. 6*a*) with a K_d calculated from these kinetic studies. The association rate constant ($k_{ass.}$) was calculated as 6.59×10^7 M^{-1}·min^{-1} from the $k_{obs.}$ and $k_{diss.}$ as follows (where [L] represents the ligand concentration), $k_{ass.} = (k_{obs} - k_{diss.})/[L]$. The K_d calculated from the ratio of $k_{diss.}/k_{ass.}$ was consequently 83.9 pM, which agreed closely with the K_d of 53.6 pM calculated by Scatchard analysis (Fig. 6).

ADP-ribosylation studies

It has been suggested that the stimulatory coupling of IL1

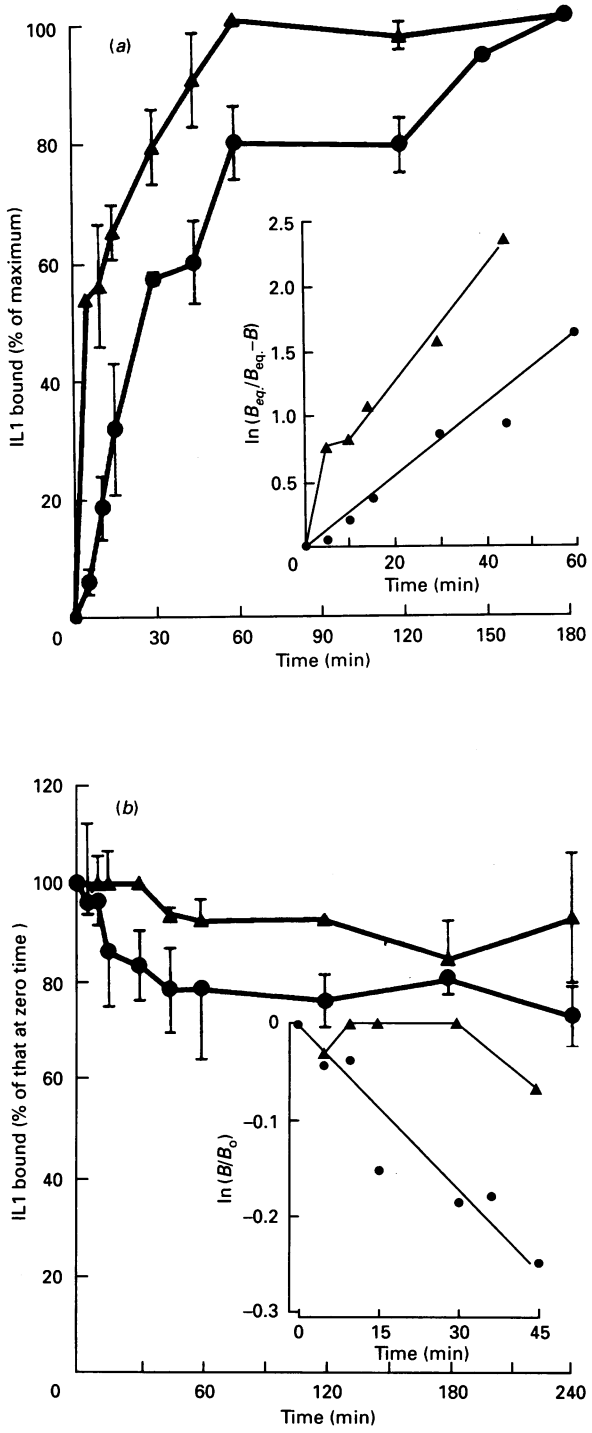

Fig. 7. Effect of p[NH]ppG on the kinetics of IL1 binding to its receptor

The association rate constant (*a*) and dissociation rate constant (*b*) for ^{125}I-IL1 binding to its receptor on EL4 membrane preparations were determined as described in the Materials and methods section. The rate of binding of ^{125}I-IL1 to the membranes, or the rate of dissociation of bound ^{125}I-IL1 from membranes, was measured in the absence (●) or presence (▲) of 10 nM-p[NH]ppG. Binding at each time point was normalized (*B*), and the amount of ^{125}I-IL1 bound at equilibrium (B_{eq}) was taken to be 100% (*a*). Dissociation was also normalized, and the amount of ^{125}I-IL1 bound at zero time (B_0) was taken to be 100% (*b*). Each point represents the mean ±S.E.M. from three independent experiments. The insets show semi-logarithmic transformations of the association and dissociation data in the absence (●) or presence (▲) of p[NH]ppG.

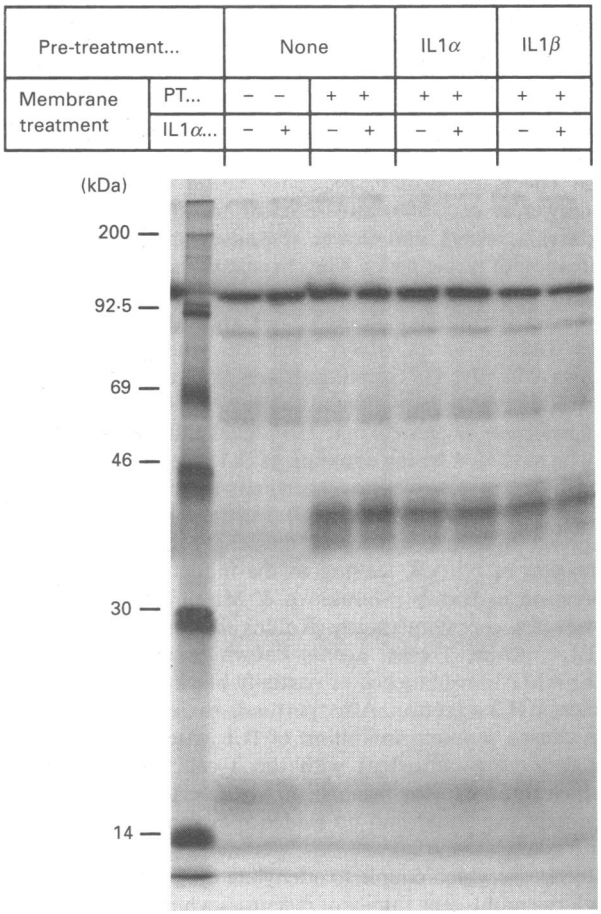

Fig. 8. EL4-cell-membrane ADP-ribosylation studies

EL4 cells were incubated for 24 h with or without the addition of IL1α or IL1β, and membrane preparations were made. The membranes were incubated with [^{32}P]NAD in the absence or presence of pertussis toxin (PT) alone or with a combination of PT and IL1. After the incubation, the membranes were analysed by SDS/PAGE and autoradiography. PT-specific substrates for ADP-ribosylation are only in the 39–41 kDa range.

receptors to adenylate cyclase might be mediated via a 46 kDa pertussis-toxin-sensitive G-protein (Chedid *et al.*, 1989). Since pertussis-toxin substrates are generally of lower molecular mass (39–41 kDa) and are considered to mediate inhibitory rather than stimulatory regulation of cyclase, this suggested that a novel G-protein might be involved in IL1-receptor coupling. In order to identify this component, membranes were prepared from EL4 cells and subsequently incubated with [^{32}P]NAD$^+$ in the presence of pertussis toxin either alone or plus IL1. Inclusion of IL1 during cell pretreatment or membrane incubations was undertaken to assess whether IL1-receptor occupation might influence ADP-ribosylation of a specific pertussis-toxin substrate, corresponding to the putative G-protein. After the incubation, the ADP-ribosylated proteins were analysed by SDS/PAGE and autoradiography (Fig. 8). The autoradiograph clearly shows that the major substrates for pertussis-toxin-specific ADP-ribosylation are in the range 39–41 kDa. Neither preincubation of the cells with IL1 nor performing the ADP-ribosylation reaction in the presence of IL1 altered the pattern of ADP-ribosylation. In parallel experiments, the major cholera-toxin-specific substrates for ADP-ribosylation displayed a mass of 46 kDa (results not shown).

DISCUSSION

The biological effects of IL1 appear to be mediated by at least two distinct types of cell-surface receptors, one of 80 kDa first identified on T-cells and fibroblasts, and a 60 kDa form found on B-cells. It is known that IL1 stimulation in these cells involves rapid effects on protein phosphorylation (Kaur *et al.*, 1989; Hulkower *et al.*, 1989) and receptor transmodulation (Bird & Saklatvala, 1989), and slower responses, including the altered expression of target genes. The chronic transcriptional responses to IL1 stimulation have been shown to be mediated via activation of the transcription factors NF-kB and AP-1 (Muegge *et al.*, 1989; Osborn *et al.*, 1989). However, the early biochemical pathways involved in coupling IL1-receptor activation with these intracellular responses remain unclear.

It has been suggested that IL1 signal transduction in T-cells may be mediated by the coupling of IL1 receptors to stimulation of adenylate cyclase by a novel pertussis-toxin-sensitive G-protein (Mizel, 1989; Chedid *et al.*, 1989). Furthermore, cAMP has been proposed to act as a second messenger in the IL-1-induced activation of NF-kB, leading to the induction of κ-light-chain expression in B-cells (Shirakawa & Mizel, 1989; Mizel, 1990). However, in a previous study (Rollins *et al.*, 1991) we found that, in EL4 murine T-cells, agents known to elevate intracellular cyclic AMP levels blocked or partially blocked the ability of IL1 to induce IL2 secretion. Also, pertussis-toxin treatment of target cells caused a potent inhibition of IL1 actions. Thus, although our data were consistent with the T-cell IL1 receptor being coupled to an effector mechanism via a pertussis-toxin-sensitive G-protein, we could find no evidence in support of the effector being adenylate cyclase.

Receptors which couple to adenylate cyclase appear to belong to a large multi-gene family of receptors which utilize G-proteins to regulate transmembrane signal-transduction events. Members of this receptor family are predicted to possess common structural elements, which include seven hydrophobic transmembrane domains. There is some evidence that other types of receptor, including those for transforming growth factor-β, granulocyte/macrophage colony-stimulating factor and insulin-like growth factor II may also modulate membrane G-proteins (Corey & Rosoff, 1989; Howe & Leof, 1989; Okamoto *et al.*, 1990). However, the membrane topology of these receptors differs from that predicted, and the nature of the G-protein coupling has yet to be established. Several recent reports have presented evidence to suggest that IL1 receptors on T- and B-cells may be coupled directly to activation of a G-protein (Chedid *et al.*, 1989; O'Neill *et al.*, 1990*a*,*b*). Our studies reported here have failed to provide support for this model. Using cells which show typical biological responses to IL1, we have been unable to detect significant effects of the cytokine on rates of GTP hydrolysis or GTP[S] binding (Figs. 3–5 inclusive). Consideration of the number of IL1 receptors present in EL4 membranes (28×10^6 sites/μg) show that these are greatly exceeded by the number of GTP[S]-binding sites (2.8×10^{10} sites/μg). Since biological responses to IL1 occur at concentrations which would produce only low receptor occupancies (e.g. 1% of total), under such conditions the ratio of 'activated' receptors to G-proteins would be extremely low, i.e. 1:100000. Clearly, unless liganded IL1 receptors can couple G-proteins with exceptionally high efficiency or frequency, significant IL1-induced changes in the rates of GTP binding or hydrolysis in membrane preparations will be difficult to measure above the background.

It has been suggested that IL1 receptors may be coupled to stimulation of adenylate cyclase via a 46 kDa pertussis-toxin-sensitive membrane component present in EL4 and 7OZ3 cells (Chedid *et al.*, 1989). Generally, it has been found that pertussis toxin ADP-ribosylates G-protein α-subunits with a molecular mass in the range 39–41 kDa. Also, pertussis-toxin-sensitive G-proteins are typically implicated in negative rather than positive regulation of adenylate cyclase. Properties of the pertussis-toxin substrate described above therefore suggested that a novel G-protein species was involved in IL1 signal transduction. In the present study, however, we have been unable to confirm the existence of a 46 kDa pertussis-toxin substrate in EL4 cell membranes (or in fibroblasts; results not shown), although [^{32}P]ADP-ribosylation of other 39–41 kDa substrates was clearly evident. These observations therefore bring into question the general existence of a novel 46 kDa G-protein mediating IL1 signalling, but do not exclude the possibility that another pertussis-toxin-sensitive G-protein may be involved.

An important property of G-protein-coupled receptors is that their affinity for agonist ligands is decreased in the presence of guanine nucleotides. We have found that p[NH]ppG, but not p[NH]ppA, influenced ^{125}I-IL1 binding to EL4 cell membranes, suggesting that IL1-receptor function may also be modulated by guanine nucleotide binding, either directly or indirectly by a guanine-nucleotide regulatory component. However, since the effects of guanine nucleotide were complex and involved the generation of receptor populations with both increased and decreased affinities for IL1, the mechanism for guanine nucleotide regulation differs from that exhibited by other G-protein-coupled receptors. In this case, the results raise the possibility that guanine regulation, perhaps involving a novel G-protein, may contribute to the stabilization of a high-affinity form of the IL1 receptor which is important for signalling at low IL1 concentrations.

It has been proposed that activation of adenylate cyclase and stimulation of cellular cAMP levels are important steps in the IL1 signalling pathway in some cells (Mizel, 1989). However, in a number of ways the results presented here conflict with this model. Firstly, in studies using cell lines which express either the type I or type II IL1 receptor we have been unable to demonstrate an acute cAMP response to IL1 stimulation. Thus, although both the EL4 cells (Rollins *et al.*, 1991) and the 7OZ3 cells showed biological responses to IL1, indicating the presence of functionally coupled IL1 receptors, and gave a significant cAMP response to forskolin, indicating the presence of functional adenylate cyclase, neither cell type responded to IL1 with increased cAMP levels. Furthermore, IL1 did not significantly stimulate adenylate cyclase activity directly in membrane preparations from these cells, although again forskolin produced responses in both preparations.

Evidence that IL1 acutely stimulates cAMP levels in a variety of cell types, including lymphocytes (Shirakawa *et al.*, 1988), fibroblasts (Shirakawa *et al.*, 1988; Zhang *et al.*, 1988; Chedid *et al.*, 1989; Munoz *et al.*, 1990) and endothelial cells (Renkonen *et al.*, 1990) has been presented. However, in some of these reports changes were marginal, and in other reports no direct effects of IL1 on cAMP synthesis were observed in T-cells (Didier *et al.*, 1988), osteosarcoma cells (Rodan *et al.*, 1990), pituitary tumour cells (Fagarasan *et al.*, 1990) or astrocytoma cells (Kasahara *et al.*, 1990). Still further reports have shown that IL1 could elevate intracellular cyclic AMP levels, but that this effect was poorly reproducible (Bomsztyk *et al.*, 1990). Reasons for these discrepancies are not clear and may reflect variation in IL1 receptor coupling in different cell types. However, at present no distinct pattern of acute IL1 receptor coupling to cyclase has emerged from these studies. The ability of IL1 to induce cAMP formation during prolonged stimulation (hours) of some cells appears less controversial (Raz *et al.*, 1989; Burch *et al.*, 1989; Leighton & Pfeilschifter, 1990). However, this effect appears to be indirect and may be secondary to induction of prostaglandin biosynthesis.

Our results have shown that IL1 can potentiate the affects of forskolin on cAMP accumulation, which indicates that IL1 may indirectly regulate responses through the cyclase pathway. Other reports which support this possibility have shown that IL1 can potentiate (Rodan *et al.*, 1990) or inhibit (Fukuoka *et al.*, 1989) the effects of other hormones on adenylate cyclase. The mechanism by which the IL1 receptor cross-talks or modulates the activity of cyclase is not clear, but might involve phosphorylation of cyclase itself or of regulatory G-proteins. IL1 is known to stimulate the phosphorylation of a number of proteins, including the epidermal growth factor receptor (Bird & Saklatvala, 1989, 1990), but the nature of the kinase involved has yet to be established.

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