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Published in:
Proceedings of the National Academy of Sciences

DOI:
10.1073/pnas.84.14.4905

Publication date:
1987

Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4905–4909, July 1987
Cell Biology

Aberrant transmembrane signal transduction in *Dictyostelium* cells expressing a mutated *ras* gene

(receptors/cAMP/cGMP/desensitization/chemotaxis)

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Communicated by J. T. Bonner, March 2, 1987 (received for review November 17, 1986)

ABSTRACT **_Dictyostelium discoideum_ cells contain a single _ras_ gene (_Dd-ras_) that is highly homologous to mammalian _ras_ genes. Cell transformation with a vector carrying a _ras_ gene with a (glycine → threonine) missense mutation at position 12 causes an altered morphogenesis. Extracellular cAMP signals regulate morphogenesis and induce chemotaxis and the activation and subsequent desensitization of adenylate and guanylate cyclase. cAMP signal transduction was investigated in _Dd-ras_-transformed cells. Transformants that overexpress the mutated _Dd-ras_-Thr^{12} gene show normal activation and desensitization of adenylate cyclase and normal activation of guanylate cyclase. However, cAMP induces a stronger desensitization of guanylate cyclase stimulation in the _Dd-ras_-Thr^{12} transformant than in transformants overexpressing the _Dd-ras_-Gly^{12} wild-type gene or in untransformed cells. This effect was correlated with a reduced chemotactic sensitivity of the transformant expressing the mutated _Dd-ras_-Thr^{12} gene.**

Ras genes code for GTP-binding proteins (1–7), which show some homology (4, 8–10) with the α-subunit of a group of guanine nucleotide-binding proteins (G proteins). Certain missense mutations in *ras* such as (glycine → threonine) at position 12 result in proteins with reduced GTPase activity and can induce neoplastic transformation (11–14). The cellular slime mold *Dictyostelium discoideum* carries a single *Dd-ras* gene that is highly homologous to the human *ras* genes (15, 16). *Dd-ras* expression appears to be essential for cell growth (17). Transformants that express a vector carrying a (Gly^{12} → Thr^{12}) missense mutation have aberrant morphogenesis, whereas cells that overexpress the endogenous *Dd-ras*-Gly^{12} gene show normal development (18). In the transformants the total *ras* gene product concentration is 2- to 4-fold higher than in untransformed cells.

Extracellular cAMP is a signal molecule for *Dictyostelium* cells and induces chemotaxis (19), morphogenesis (20), and cell differentiation (21). cAMP is detected by cell surface receptors, resulting in the activation of adenylate and guanylate cyclase (22). The produced cAMP is then secreted, by which the cAMP signal is relayed (23), whereas the produced cGMP remains largely intracellular and is probably involved in the transduction of the chemotactic signal (24). Guanine nucleotides alter the binding of cAMP to isolated membranes, suggesting that the receptor interacts with one or more G proteins (25–28). This hypothesis is substantially supported by the recent observation that GTP stimulates adenylate cyclase *in vitro* (29, 30).

The stimulation of adenylate and guanylate cyclase *in vivo* is transient, even when the cAMP concentration remains constant (31, 32), indicating desensitization. These desensitization processes show different kinetics: desensitization of guanylate cyclase stimulation has a $t_{1/2}$ of about 4 sec (32), whereas desensitization of adenylate cyclase stimulation occurs more slowly with a $t_{1/2}$ of 2–3 min (33). Desensitization of guanylate and adenylate cyclase is probably important for efficient chemotaxis, cell aggregation, and morphogenesis.

Chemotaxis and the activation and desensitization of adenylate and guanylate cyclase were investigated in cells expressing a mutant *Dd-ras* gene (*Dd-ras*-Thr^{12}) and in cells overexpressing the endogenous *ras* gene (*Dd-ras*-Gly^{12}) (18). It was found that signal transduction was not altered in the *Dd-ras*-Gly^{12} transformant. Cell transformation with *Dd-ras*-Thr^{12} did not affect the activation and desensitization of the adenylate cyclase response, which confirms and extends the observations of Reymond *et al.* (18). Neither was the activation of guanylate cyclase altered, but the cAMP-induced desensitization of this response was strongly promoted in the *Dd-ras*-Thr^{12} transformants. In addition, it was observed that transformation with *Dd-ras*-Thr^{12} reduced chemotactic sensitivity. Biochemical data suggest that these effects are not correlated with just an altered activity of adenylate cyclase, guanylate cyclase, surface receptors, surface phosphodiesterase (PDEase), intracellular cGMP PDEase, or GTP-mediated inhibition of cAMP-binding to isolated membranes.

MATERIALS AND METHODS

The preparation of the *ras* transformants has been described by Reymond *et al.* (18). Cells were grown in HL-5 medium supplemented with 8 μg of the antibiotic G418 (Geneticin) per ml to select for cells that overexpress the transformation vector. Cells were grown to a density of 2–3 × 10^6 cells per ml, harvested in 10 mM KH_2PO_4/Na_2HPO_4, pH 6.5 (buffer A), and washed three times with buffer A. Chemotaxis and cell aggregation were investigated with the small population assay (19).

All assays were done as described in the references: chemotaxis and cell aggregation (19), the cAMP-induced cGMP response (32), the 2′-deoxyadenosine 3′,5′-monophosphate (dcAMP)-induced cAMP response (34), cAMP-binding to cells (Scatchard analysis) (35), developmental regulation of cAMP-binding to cells until 8 hr after starvation (36), inhibition of cAMP-binding to membranes by 30 μM guanosine 5′[γ-thio]triphosphate (GTP[γS]) and guanosine 5′[β-thio]diphosphate (GDP[βS]) (25), cell surface PDEase activity at 0.1 μM cAMP (32), intracellular cGMP-stimulated cGMP-PDEase isolated and activity measured at 10 nM

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Abbreviations: *Dd-ras*, *ras* gene of *Dictyostelium discoideum*; G proteins, the guanine nucleotide-binding regulatory proteins; *Dd-ras*-Gly^{12}, wild-type *ras* gene; *Dd-ras*-Thr^{12}, mutant gene carrying threonine substitution for glycine at position 12; PDEase, cyclic nucleotide phosphodiesterase.

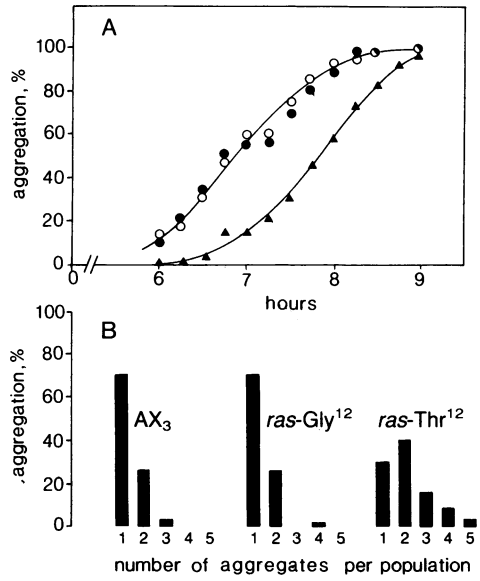

FIG. 1. Cell aggregation. Small 0.1-μl droplets containing about 500 cells were deposited on a hydrophobic agar surface (19). The number of droplets containing a cell aggregate (*A*) and the number of aggregates per droplet (*B*) were determined at different times after deposition of cells on the agar surface. *B* represents the situation when about 90% of the droplets contained at least one aggregate. About 60 populations of each cell type were observed. The results shown are representative for three experiments. ●, Untransformed cells; ○, *Dd-ras*-Gly^{12} transformants; and ▲, *Dd-ras*-Thr^{12} transformants.

cGMP in the absence and presence of the activator 8-bromoguanosine 3′,5′-monophosphate (8-Br-cGMP) (37), basal adenylate cyclase activity (30), and guanylate cyclase activity (38). Downregulation of surface cAMP receptors was induced by (S_P)-cAMP[S] or cAMP (39) and was done as described in the figure legend. Adenosine 3′,5′-monophosphorothioate, S_P-isomer {(S_P)-cAMP[S]} was a gift of J. Baraniak, W. J. Stec, and B. Jastorff (40).

RESULTS

Starvation of *D. discoideum* cells initiates cell aggregation that is mediated by chemotaxis to cAMP. The time course of cell aggregation was identical in control and *Dd-ras*-Gly^{12}-transformed cells but was delayed about 1 hr in the *Dd-ras*-Thr^{12} transformants (Fig. 1*A*). In these experiments the number of aggregates per droplet was also observed to be significantly higher in the *Dd-ras*-Thr^{12} transformant than in the other cell types (Fig. 1*B*), confirming previous observations (18). The signal for cell aggregation in *D. discoideum* is extracellular cAMP. The aggregation center autonomously secretes cAMP that is detected by surrounding cells, which respond to cAMP by a chemotactic movement toward the aggregation center and by the secretion of cAMP (cAMP relay). The kinetics of cell aggregation and the territory size of an aggregate are probably determined by multiple components—including the number of autonomous cAMP-oscillators, the efficiency to detect and relay cAMP, and by the efficiency of the chemotactic response to cAMP.

The chemotactic response to different cAMP concentrations was measured at different times after the addition of cAMP; no differences were observed between untransformed and *Dd-ras*-Gly^{12}-transformed cells. However, *Dd-ras*-Thr^{12}-transformed cells showed a reduced chemotactic sensitivity to cAMP (Fig. 2*A*), which is, at least partly, due to their slow chemotactic response (Fig. 2*B*). These cells were also less sensitive to folic acid, which is a chemoattractant for vegetative cells (Fig. 2*C*).

Fig. 3 compares the cAMP-induced accumulation of cGMP and cAMP in control cells and in *Dd-ras*-Gly^{12} and *Dd-ras*-Thr^{12} transformants. The cAMP response was essentially the same for the three cell types, suggesting that the activation and desensitization of adenylate cyclase are not altered in the *Dd-ras* transformants (Fig. 3*A*), confirming and extending previous observations by Reymond *et al.* (18). The initial rate of cGMP accumulation was identical in the three cell types (Fig. 3*B*), suggesting that also the activation of guanylate cyclase is unaltered in *Dd-ras* transformants. However, the cGMP accumulation was terminated precociously in the *Dd-ras*-Thr^{12} transformant. Dose–response curves of the cGMP accumulation at 10 sec after stimulation with different cAMP concentrations revealed that the maximal cGMP accumulation by saturating cAMP stimuli was reduced in the *Dd-ras*-Thr^{12} transformant, whereas the cAMP concentration that induced a half-maximal response was identical for the three cell types (data not shown). These results suggest that the *Dd-ras*-Thr^{12} transformant has neither a reduced initial cGMP accumulation nor a reduced sensitivity for cAMP, but shows a more rapid termination of cGMP accumulation. To confirm that the cGMP response in these cells was a function of the *Dd-ras*-Thr^{12} gene expression, this transformant was grown in the absence of G418. These cells had lost the transformation vector with the *Dd-ras*-Thr^{12} gene and had a wild-type developmental phenotype and cGMP response (data not shown).

The regulation of cGMP levels after cAMP stimulation has been previously shown to be a complex process involving the activation of guanylate cyclase (41), the termination of this activation by a rapid desensitization process (32), and the degradation of cGMP by a specific PDEase that is activated

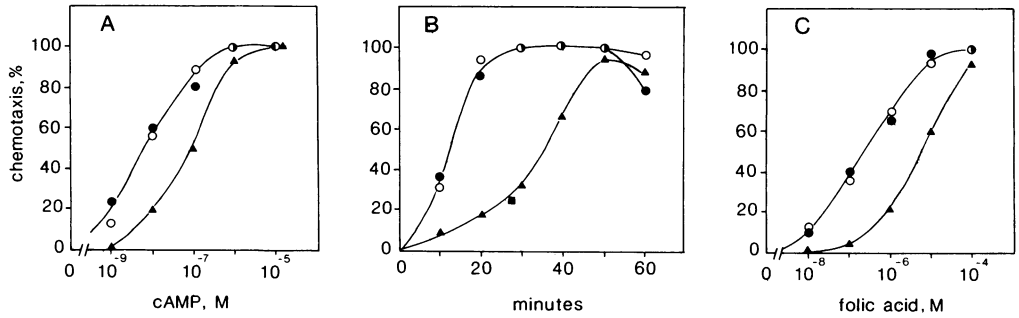

FIG. 2. Cells were deposited on hydrophobic agar as described in the legend for Fig. 1. Chemotaxis was tested by deposition of 0.1-μl droplets containing different concentrations of folic acid or cAMP close to the amoebal population and observing the chemotactic reaction at different times thereafter. (*A*) Chemotaxis of 6-hr-starved cells to cAMP. (*B*) Time course of the chemotactic reaction of 6-hr-starved cells to 1 μM cAMP. (*C*) Chemotaxis of 1-hr-starved cells to folic acid. The maximal responses are given in *A* and *C*. Results shown are the means of two independent experiments with 20 populations for each cell type. ●, Untransformed cells; ○, *Dd-ras*-Gly^{12} transformants; and ▲, *Dd-ras*-Thr^{12} transformants.

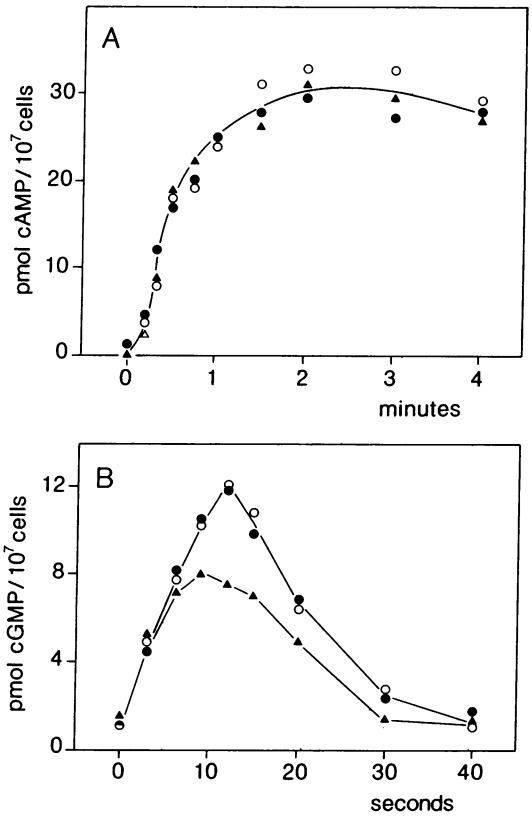

FIG. 3. cGMP and cAMP response. Cells were starved on nonnutrient agar at a density of 2.5×10^6 cells/cm^2 for 16 hr at 6°C, harvested, resuspended in buffer A, and aerated for at least 10 min. (*A*) Cells were stimulated with 5 μM 2'deoxyadenosine 3',5'-monophosphate (dcAMP) and 10 mM dithiothreitol at t = 0, and cAMP production was measured by isotope dilution assay (20). (*B*) Cells were stimulated with 0.1 μM cAMP at t = 0 and lysed at the indicated times with perchloric acid (21). The cGMP content was measured in the neutralized lysates with a RIA. Results shown are the means of triplicate determinations from three independent experiments. ●, Untransformed cells; ○, *Dd-ras*-Gly^{12} transformants; and ▲, *Dd-ras*-Thr^{12} transformants.

by cGMP (37). The three cell types studied were found to have essentially the same levels of basal guanylate cyclase and cGMP-stimulated PDEase activity (see Table 2).

Desensitization of guanylate cyclase was determined by using the analogue (S_P)-cAMP[S]. This analogue is not effectively hydrolyzed by cell surface PDEase and binds to surface receptors with lower affinity by a factor of 50–100 than cAMP. Cells were stimulated with a subsaturating concentration (0.2 μM) of (S_P)-cAMP[S]. This constant stimulus induced a transient cGMP response that was ≈ 44% of the response induced by a saturating cAMP concentration; this response was identical in the three cell types (Table 1). The constant (S_P)-cAMP[S] stimulus also induced partial desensitization of guanylate cyclase. The degree of desensitization was measured by restimulating the cells with a saturating cAMP concentration. Desensitization is defined as the decrease of responsiveness to cAMP that is induced by the pretreatment with (S_P)-cAMP[S]. In control cells and in *Dd-ras*-Gly^{12}-transformed cells the level of desensitization was about 40%, thereby being equal to the response that was induced by 0.2 μM (S_P)-cAMP[S]. This is the normal property of the cGMP response (32). In *Dd-ras*-Thr^{12} transformants, however, the response to the saturating cAMP stimulus after pretreatment with (S_P)-cAMP[S] was significantly lower than 60%, indicating that desensitization of the cGMP response was enhanced in these cells. This suggests that the precocious termination of the cGMP response as observed in Fig. 3*B* is probably due to enhanced desensitization of guanylate cyclase. Desensitization of adenylate cyclase was determined by a similar procedure. The results (Table 1) indicate that 0.2 μM (S_P)-cAMP[S] induced the same degree of desensitization of the cAMP response in the three cell types. Another form of desensitization — i.e., down-regulation of surface cAMP receptors (39) — was also not affected by overexpression of the mutated *Dd-ras* gene (Fig. 4).

Many aspects of transmembrane signal transduction were not altered in *D. discoideum* cells that express the mutated *ras* gene (Table 2). We have analyzed equilibrium binding of cAMP to cell surface receptors, the developmental regulation of cAMP binding to cells until 8 hr after starvation, equilibrium binding of cAMP to membranes and its inhibition by GTP[γS] and GDP[βS], basal adenylate cyclase activity, basal guanylate cyclase activity, cell surface PDEase activity, and intracellular cGMP-PDEase activity in the absence and presence of the activator 8-bromoguanosine 3',5'-monophosphate. None of these activities were significantly different in the three cell types that we have investigated (Table 2).

These results demonstrate that a glycine to threonine mutation at position 12 in the *Dictyostelium ras* protein has a specific effect on chemotaxis and on desensitization of the cGMP response. It does not affect other signal transduction pathways investigated thus far.

DISCUSSION

Expression of a Gly^{12} → Thr^{12} *ras* missense mutation in *D. discoideum* transformants at levels 2- to 4-fold higher than the endogenous *Dd-ras* gene results in aberrant morphogenesis (18). This is not observed in cells that overexpress the normal *Dd-ras* gene (*Dd-ras*-Gly^{12}). Cell aggregation of the *Dd-ras*-Thr^{12} transformants was somewhat delayed and resulted in

Table 1. Response to cAMP after desensitization by (S_P)-cAMP[S]

Pretreatment	Stimulus	Cell response, %		
		AX_3	*Dd-ras*-Gly^{12}	*Dd-ras*-Thr^{12}
cGMP response				
—	0.1 μM cAMP	100 (9.3)	100 (9.2)	100 (6.8)
—	0.2 μM (S_P)-cAMP[S]	43 ± 2	44 ± 3	44 ± 6
0.2 μM (S_P)-cAMP[S]	0.1 μM cAMP	61 ± 5	58 ± 6	26 ± 6
cAMP response				
—	5 μM dcAMP	100 (22)	100 (24)	100 (21)
0.2 μM (S_P)-cAMP[S]	5 μM dcAMP	65 ± 4	63 ± 6	62 ± 4

Cells were prepared as described in the legend for Fig. 3. The cGMP response was determined at 10 s after addition of the stimulus. The responses to 0.1 μM cAMP were set at 100% for each cell type, and the number in parenthesis represents pmol cGMP formed per 10^7 cells. The pretreatment with 0.2 μM (S_P)-cAMP[S] was for 30 s. Desensitization is defined as the decrease of responsiveness to 0.1 μM cAMP after pretreatment with 0.2 μM (S_P)-cAMP[S]. The cAMP response was determined at 5 min after addition of the stimulus (5 μM dcAMP and 10 mM dithiothreitol). The pretreatment with 0.2 μM (S_P)-cAMP[S] was for 5 min.

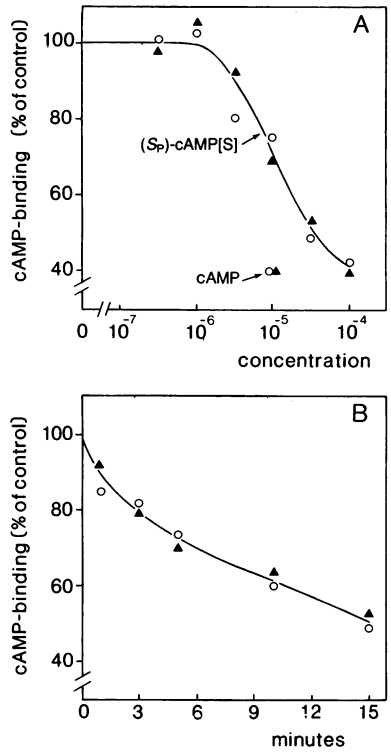

FIG. 4. Downregulation of surface cAMP receptors. Cells were starved for 5 hr in buffer A, harvested and resuspended in buffer A at a density of 10^8 cells/ml. (*A*) Cells were incubated with different concentrations of (S_P)-cAMP[S] or 10 μM cAMP plus 10 mM dithiothreitol for 15 min. (*B*) Cells were incubated for the indicated period with 30 μM (S_P)-cAMP[S]. Then cells were washed twice with 15 ml of ice-cold phosphate buffer, and the binding of 1 μM [^{3}H]cAMP was measured. ○, *Dd-ras*-Gly^{12}; ▲, *Dd-ras*-Thr^{12}.

smaller aggregates (Fig. 1). This could be related to the reduced chemotactic sensitivity of these cells (Fig. 2). The appearance of multiple tips after cell aggregation (18) may be caused by the same physiological defect because chemotaxis to cAMP appears to be involved in late morphogenesis (42).

Transmembrane signal transduction reveals a specific effect of the expression of the *Dd-ras*-Thr^{12} gene. The *Dd-ras*-Thr^{12} transformant shows a normal cAMP-induced activation and desensitization of adenylate cyclase, which

Table 2. Biochemical characteristics of *ras* transformants

	AX_3	*Dd-ras*-Gly^{12}	*Dd-ras*-Thr^{12}
cAMP-binding to cells	100*	105/99	90/120
cAMP-binding to membranes	100*	92/83	80/128
% inhibition cAMP-binding by GTP[γS]	80/81	76/72	76/81
% inhibition cAMP-binding by GDP[βS]	52/58	58/55	62/57
Adenylate cyclase	100*	76/93	90/100
Guanylate cyclase	100*	138/71	116/86
Cell surface PDEase	100*	98/94	91/91
Intracellular cGMP PDEase	100*	87/100	110/99
-Fold stimulation cGMP PDEase by 8-Br-cGMP	2.1	2.0	2.1

Untransformed cells (AX_3) and the *ras*-transformed cells were analyzed for several biochemical properties as indicated in the methods. Results from two independent experiments are shown; the stimulation of cGMP-PDEase by 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP) was determined once with triplicate samples.

*Value of untransformed cells was set at 100%.

extends the previous results on the regulation of adenylate cyclase by GTP *in vitro* (18). The activation of guanylate cyclase by cAMP is probably not altered, but the increase of cGMP does not continue as long as in untransformed cells. This precocious termination of the cGMP response appears not to be due to a lower activity of guanylate cyclase or to a higher activity of cGMP-stimulated PDEase, but this termination is probably caused by the earlier termination of guanylate cyclase stimulation due to enhanced desensitization (Table 1). It has been suggested that cGMP is involved in the transduction of chemotactic signals (24) and that *D. discoideum* cells desensitize by the chemotactic signal (43). The present results showing that a single mutation in the *Dd-ras* gene alters both desensitization of guanylate cyclase stimulation and chemotaxis supports this hypothesis. The observation that the chemotactic response to both cAMP and folic acid is affected suggests that the alteration in *Dd-ras*-Thr^{12} transformants affects a common step in the transduction of these chemotactic signals.

Recently it has been shown that the addition of inositol 1,4,5-trisphosphate ($InsP_3$) or calcium to permeabilized *D. discoideum* cells results in the accumulation of cGMP levels (44, 45). In mammalian cells $InsP_3$ is derived from the receptor- and G-protein-mediated activation of phospholipase C, which hydrolyses phosphatydylinositol 4,5-bisphosphate (46). The second product of this reaction is diacylglycerol, which is the endogenous activator of protein kinase C. The latter enzyme can be activated artificially by phorbol diesters, such as phorbol 12-myristate 13-acetate (PMA) (47). In mammalian cells it has been shown that the activation of protein kinase C may function as a negative feedback loop in the receptor-mediated activation of phospholipase C (48–50).

The present results suggest that in *D. discoideum ras* does not affect the regulation of adenylate cyclase but potentiates the cAMP-induced desensitization of guanylate cyclase. It is tempting to suggest that this effect is related to the activation of phospholipase C or protein kinase C. However, although $InsP_3$ and PMA affect signal transduction in *D. discoideum* (44, 45, 51), neither the existence of phospholipase C nor that of protein kinase C has been directly demonstrated in this organism thus far. The hypothesis that *Dd-ras* is involved in the regulation of phospholipase C and protein kinase C activity is worth further investigation.

We gratefully acknowledge Theo Konijn for stimulating discussions and critical reading of the manuscript and C. Silan for technical assistance. This work was supported by a grant from the C. and C. Huygens Fund, which is subsidized by the Netherlands Organization for the Advancement of Pure Scientific Research (ZWO) and by Grant GM 37830 from the Public Health Service.

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