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Reduction of adenylyl cyclase activity by cholera toxin in myeloid cells

Long-term down-regulation of Gsα subunits by cholera toxin treatment

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In IPC-81 cells, the adenylyl-cyclase activation by cholera toxin produces an elevation of cAMP that causes a rapid cytolysis. A resistant clone with deficient cholera toxin-induced cyclase activity (yet sensitive to cAMP) showed a rapid decrease in the amount of membrane-bound Gsα (42–47 kDa) detectable soon after ADP-ribosylation of these proteins; pertussis toxin-sensitive G proteins (41 kDa) were not affected. Resistant cells showed a rapid decrease of Gsα that is consistent with the finding that cAMP did not accumulate in these cells. Cholera toxin treatment of resistant cells had long-lasting effects (several weeks) on the level of Gsα in the cell membrane. The duration of Gsα decrease does not correspond to the probable life of catalytically active cholera toxin in the cells, and suggests a regulated process more complex than a proteolytic degradation targeted on ADP-ribosylated molecules.

Adenylyl-cyclase; GTP-binding protein; Down-regulation

1. INTRODUCTION

G proteins are essential for coupling many intracellular signals generated by activated membrane receptors to intracellular effector enzymes and ion channels [1–5]. Numerous signals transduced through the AC-cAMP-protein kinase A pathway play key roles in regulating hematopoiesis. In myeloid cells, cAMP as a result of cholera toxin (CT)-induced AC activation, provides a potent intracellular signal for differentiation and negative growth regulation [6–13].

It has been observed that some lineages of normal or leukemic cells, despite normal sensitivity to cAMP, are refractory to CT-induced proliferation inhibition. This implies some alteration in the response to CT of the G protein-cyclase complex. CT stabilizes the GTP-bound conformation of Gsα and decreases its GTPase activity, mimicking receptor-GSα coupled intracellular signalling and increasing AC activity [4]. Yet, the mechanisms by which cells, after CT exposure, switch AC from the active to the inactive state, remain unclear.

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Abbreviations: CT, cholera toxin; PT, pertussis toxin; CTs cells, cholera toxin-sensitive cells; CTr cells, cholera toxin-resistant cells; AC, adenylyl-cyclase; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Previously, we isolated a myeloid leukemia (IPC-81) for which CT treatment generated a sustained AC activation that blocked cell proliferation and induced a rapid cAMP-dependent cytolysis [14,15]. Interestingly, the rapid eradication of sensitive cells by cytolysis promoted the emergence of a few CT resistant clones. Though they were cultured for months in presence of CT, these cells were sensitive to cAMP analogs. The functional defect was located at the level of the AC enzymic complex [16].

In this paper, we show that CT resistant cells respond to CT exposure with a rapid decrease of Gsα membrane proteins; it was noticed that the long-lasting effects of CT exposure on Gsα did not correspond to the probable intracellular life of CTα chain.

2. MATERIALS AND METHODS

2.1. *Cells*

An in vitro adapted cell line, IPC-81 [14], was isolated from the rat myeloid leukemia (BNML), and cultured in RPMI medium supplemented with 10% horse serum. Two clones are grown in our laboratory: CTs and CTr cells which are CT-sensitive and CT-resistant, respectively [16]. The S49 cyc-mutant of the S49 rat lymphoma, a Gsα deficient cell line [17] was provided by Dr J. Bockaert (Montpellier, France).

2.2. *Membrane preparation*

10^8 cells were washed 3 times in Tris-HCl (10 mM, pH 7.4) with 0.25 M sucrose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM KCl and 0.10 mg/ml leupeptin (buffer A), then frozen at −80°C and thawed twice, and homogenized with a Kontes potter. The homogenate was centrifuged (2500 × *g*, 4°C, 20 min) to remove the nuclei. The supernatant was collected and centrifuged at 21000 × *g*, at 4°C, for 30 min. The pellet (semi-purified membranes) was resuspended in Tris-HCl

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(10 mM, pH 7.4) with 0.25 M sucrose, and leupeptin (0.10 mg/ml) (buffer B). Proteins were determined by the method of Lowry [18].

2.3. *ADP-ribosylation assay*

ADP-ribosylation was carried out on semi-purified membranes, as described by Berthillier et al. [19] and Jacquemin et al. [20]. ^{32}P-labelled membrane proteins were analysed on a 10% SDS-PAGE [21], followed by autoradiography and microdensitometric scanning of films. For quantitative measurements, 50 μg of ADP-ribosylated membranes, resuspended in 50 μl of ice-cold buffer B, were deposited on 3MM Whatman filters, then precipitated in 10% TCA at 4°C overnight. Filters were washed twice with ice-cold TCA 5%, then with ethanol/ether (1:1), dried and counted. ADP-ribosylation by pertussis toxin was carried out with 1 μg toxin for 100 μg membrane following the same procedure [20], except that the GTP concentration was 0.1 mM and nicotinamide was omitted.

2.4. *Adenylyl cyclase activity assay*

Membranes (15–30 μg) were incubated in 100 μl of 25 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 20 mM creatine phosphate, 100 u/ml creatine phosphokinase, 1 mM [α-^{32}P]ATP (50 cpm/pmol), 10^{-6} M GTP for 20 min at 37°C. GTPγS (Sigma) was added as indicated. The reaction was stopped by addition of 100 μl of a solution containing 2% SDS, 40 mM ATP, 1.4 mM cAMP (pH 7.5). 20000 cpm of [^{3}H]cAMP and 800 μl H_2O were added to each sample and [^{32}P]cAMP was isolated by ion-exchange chromatography by the method of Salomon et al. [22]. ^{3}H-counts were used to evaluate cAMP recovery. Assays were carried out in triplicate.

2.5. *Immunoblotting with Gsα antiserum*

Gs antisera ('RM' and 'CS1') were obtained in rabbit after repeated immunization with a conjugate of Keyhole Limpets Hemocyanin and a synthetic peptide corresponding to the RMHLRQYELL C-terminal sequence of GSα [23,34]. 100 μg membrane proteins were separated on a 10% or 12.5% SDS-PAGE [21] and transferred to nitrocellulose membranes. The blots were washed twice in buffer C (10 mM Tris-HCl, pH 7.5, with 500 mM NaCl) with 5% fat-free dry milk (30 min each), then incubated overnight at 20°C with Gsα antiserum (dilution 1/300) in buffer C with 5% fat-free dry milk. The blots were then washed repeatedly with buffer C and incubated with [^{125}I]Protein A (100 000 cpm/ml) in 50 ml buffer C with 5% fat-free dry milk for 45 min at 20°C. The blots were then washed and dried before autoradiography.

2.6. *Gsα functional assay*

Cell membranes (5 mg/ml) were extracted with 1% Na-cholate and Gsα activity in the extract was evaluated by its ability to complement the Gsα deficiency of S49 cyc-membranes in vitro [17,22,23]. The Na-cholate extract from CTr cells (100 μl) was added to cyc-membranes (100 μg) and incubated at 30°C for 20 min with GTP (100 μM); CTs cell membranes were used as a control. Na-cholate extracts were centrifuged (100 000 × *g*, 10 min, 4°C) and the pellets resuspended in 100 μl of buffer B and tested for AC activity.

3. RESULTS

Our previous studies concerning the IPC-81 CT-resistant cells (CTr) have shown a deficient AC activity associated with CT treatment [16]. Cells were resistant to high doses of CT, while they were sensitive to prostaglandins ($PGE_{1,2}$) and cAMP analogs. This work implied that a specific AC anomaly was involved, and presumably concerned Gsα, Gsα ADP-ribosylation, or activation of the catalytic subunit by Gsα. Here, we studied the feature of Gsα in sensitive or resistant cells and the effects of prolonged exposure of these cells to CT.

3.1. *ADP-ribosylation of Gsα and Giα by cholera and pertussis toxins*

CT-resistant cells (CTr), selected by a long-term exposure to CT, were washed and grown for 7 days without CT. This allowed about 10 cell cycles and a 10^3 clonal cell expansion in the absence of CT. Membranes from both sensitive (CTs) and resistant (CTr) cells were prepared. ADP-ribosylation of G proteins in CTr and CTs cell membranes by CT was measured (Fig. 1A). The CTs ADP-ribosylated membranes revealed radioactive bands (47 kDa and 42 kDa) corresponding to the Gsα protein subunits. CTr membranes were significantly deficient in Gsα protein ADP-ribosylation (Fig. 1A). Both CTs and CTr cells, when preincubated for 4 h with CT 10^{-8} M prior to the ADP-ribosylation assay, showed no ADP-ribosylation (not shown). CTs cell Gsα protein ADP-ribosylation is about 8 times higher than CTr cell ADP-ribosylation (CTs = 1.30 pmol [^{32}P]NAD/mg protein; CTr = 0.18 pmol [^{32}P]NAD/mg protein). After subtraction of the non-specific radioactivity, densitometry measurements revealed an even greater difference in ADP-ribosylation (CTs/CTr = 9.7). The ADP-ribosylation by pertussis toxin (PT) showed (Fig. 1B) a prominent 41 kDa band in both CTr and CTs cells; labellings were approximately equal in sensitive and resistant cells. The minor 38 kDa band suggests a partial degradation of Giα during the procedure.

3.2. *Immunoblotting with Gs antiserum*

The expression of Gsα in CTs, CTr cells and the effects of chronic exposure of CTr cells to CT were studied. CTs and CTr cells did express roughly similar amounts of Gsα (47 kDa and 42 kDa); CT treatment of CTr over 7 days resulted in undetectable amounts of Gsα both in membrane and cytosol (Fig. 2A,B). The kinetics of Gsα decrease upon CT treatment was analysed (Fig. 2C). In CTs cells treated with CT, the M_w of Gsα (both subunits) increased after 30 min (43 kDa and 49 kDa, respectively) as a consequence of ADP-ribosylation. After 4 h of CT treatment, the amount of Gsα decreased mainly in the 43 kDa band. After 6 h, studies on Gsα in these cells were complicated by the process of cytolysis; studies on cell clones refractory to cytolysis indicated that the two Gsα subunits became totally undetectable after 12–15 h of treatment (not shown). In CTr cells, a shift to higher M_w of Gsα subunits corresponding to ADP-ribosylation was similarly observed. Gsα was rapidly lost after 1–2 h of CT treatment: it was nearly undetectable after 4–6 h (Fig. 2C). The phenomenon affected first the 47–49 kDa subunit and later the 42–43 kDa subunit. The time required for CT-treated CTr cells grown in CT-free medium to recover the normal Gsα amount was surprisingly long. After 7–15 days of culture in CT-free medium, Gsα were expressed again and had M_w corresponding to the non-ADP-ribosylated

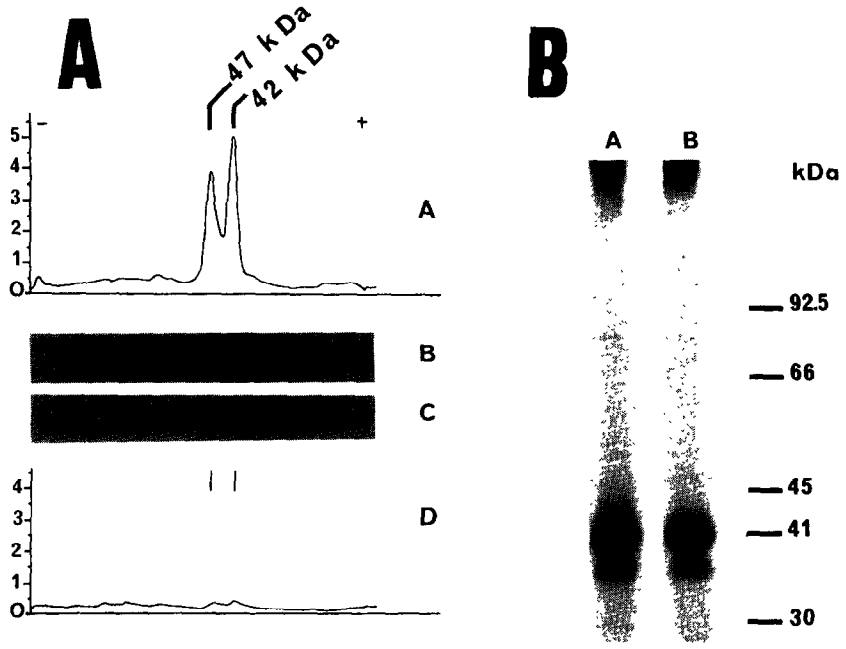

Fig. 1. Membrane Gsα and Giα protein ADP-ribosylation in CTs and CTr cells in presence of cholera or pertussis toxins. Cells were cultured without CT for two weeks before the ADP-ribosylation assays. (*a*) ADP-ribosylation by CT: (A) CTs cells, microdensitometer scanning; (B) CTs cells, autoradiography; (C) CTr cells, autoradiography; (D) CTr cells, microdensitometer scanning (ordinates indicate arbitrary units used for densitometric measurements). (*b*) ADP-ribosylation by PT: (A) CTs cells, autoradiography; (B) CTr cells, autoradiography.

molecules (42 kDa and 47 kDa). The intensity of these bands was, however, much lower than in CTs cells. Normal levels were found after 20–30 days, on average.

3.3. *Adenylyl cyclase activity*

AC in cell membranes was evaluated to find a correlation between this activity, the level of Gsα and the resistance to CT. We analysed: (i) CTr cells cultured in presence of CT (no Gsα detected by immunoblotting), (ii) CTr cells after several weeks of culture in CT-free medium (normal level of Gsα), and (iii) CTs cells as the control (normal level of Gsα). As shown in Table I, AC activity stimulated by GTPγS is significantly lower for CT-treated CTr cells; the GTPγS-dependent AC activity was recovered in CTr cells cultured in CT-free medium, as cells re-expressed Gsα (Fig. 2D).

3.4. *Reconstitution of Gsα function in S49 cyc- cell membranes*

Complementation of S49 cyc- cell membranes with membrane extracts free of AC activity was previously used to detect functional Gsα molecules [26]. Reconstitution of S49 cyc- membranes with extracts of CTr cells treated with CT, have no significant amount of functional Gsα (Table II). This result corroborates the absence of Gsα on immunoblots (Fig. 2), and the low AC activity (Table I). CTr cell membranes, in this experiment, showed a low level of functional Gsα (basal and GTPγS-dependent) compared to CTs cell membranes; this situation is typical of CTr cells during

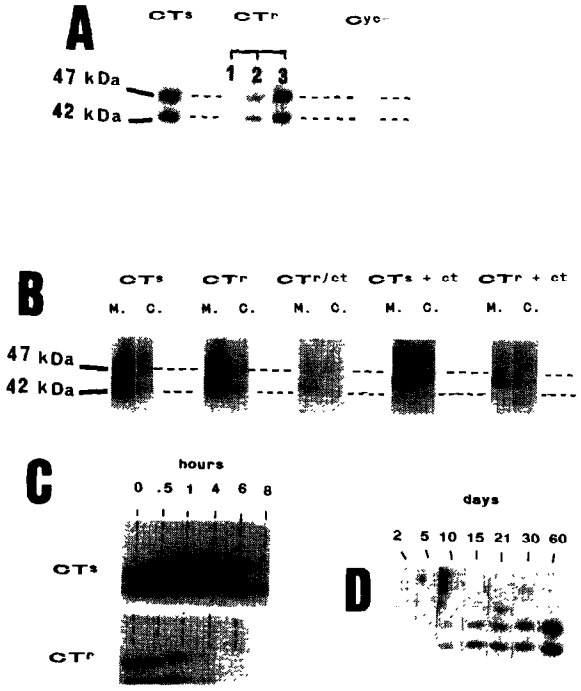

Fig. 2. Immunoblotting with anti-Gsα rabbit antiserum, after SDS-PAGE. (A) Gsα expression in CTs cells (CTs) and CTr cells (CTr): one week (1), two weeks (2), one month (3) after the cessation of CT treatment; S49 cyc- cells (Cyc-). (B) Gsα expression at the membrane (M.) and in cytosol (C.) CTr/CT: CTr cells cultured for 7 days with 10^{-8} M CT; CTs + ct and CTr + ct : CTs and CTr cells respectively, incubated for 5 h with 10^{-8} M CT. (C) Kinetics of Gsα decrease during CT treatment of CTs and CTr cells. (D) Re-expression of Gsα in CTr cells after the cessation of CT treatment.

Table I

Adenylyl cyclase activation by GTPγS: Differences between cell types

	Adenylyl cyclase activity in IPC-81 cells (pmol [^{32}P]cAMP/min/mg protein)		
	CTs	CTr	CTr/CT
Basal	254 ± 41	149 ± 3	197 ± 7
GTPγS	1289 ± 171	1345 ± 48	477 ± 75

CTr/CT cells were cultured for a week with CT (10^{-8} M), then washed 3 times before membrane preparation (CTr/CT); control, CTr cells (CTr) were cultured for at least two weeks in CT-free medium. Adenylyl cyclase activity of 20 μg membrane, stimulated (or not = basal) with GTPγS was measured after a 20 min incubation (37°C).

Table II

Titration of Gsα activity by reconstitution of S49 cyc- cell membranes

	Adenylyl cyclase activity in S49 cyc- membrane reconstituted with IPC-81 membrane cholate extracts (pmol [^{32}P]cAMP/40 μg protein)			
	CTs	CTr	CTr/CT	S49 cyc-
Basal	106 ± 12	57 ± 7	72 ± 3	41 ± 31
GTPγS	1013 ± 9	436 ± 10	42 ± 4	48 ± 4

S49 cyc- cell membranes were prepared as described. Cholate extracts (100 μl; 100 μg protein, from 5 mg of membrane) were added to 100 μg S49 cyc- cell membrane and incubated as described, before titration of AC activity at 37°C for 20 min.

the first weeks that followed the cessation of CT treatment.

4. DISCUSSION

Previous work using the CT-sensitive IPC-81 cell line has shown that rare CT-resistant clones can be selected by prolonged exposure to CT [16]; CTr cells still respond by cytolysis to PGE mediated AC activation and cAMP analogs, hence suggesting a functional defect related to AC activation by CT.

In this paper, we demonstrate that, despite low or undetectable levels of ADP-ribosylated Gsα proteins found after prolonged exposure to CT, the AC defect cannot be explained by decreased ADP-ribosylation. Other results support this conclusion: CTr cells cultured for several weeks or a month in CT-free media showed CT mediated ADP-ribosylation of two protein bands (43 kDa and 49 kDa), subsequently immunocharacterized as Gsα by two distinct anti-GSα antisera. The shift of M_w related to ADP-ribosylation was clearly observed on Gsα Western-blots.

Immunoblotting experiments with anti-Gsα antisera (Fig. 2) revealed that the treatment of CTr cells with CT resulted in a rapid decrease of Gsα labelling after ADP-ribosylation, down to undetectable levels (5 h), while in CTs cells Gsα was still titrable, when the cAMP-induced cytolysis proceeded (8 h). The phenomenon was clearly restricted to Gsα; neither GiαADP-ribosylation by PT was affected (Fig. 1), nor Giα expression decreased - as indicated by anti-Giα antiserum titration (not shown). In conclusion, CT-mediated decrease of Gsα can explain the absence of ADP-ribosylation following CT treatment, and the low AC activity, a phenomenon sufficient to avoid CT-mediated cytolysis; it also explains why cAMP-dependent cytolysis remains.

Recently, Chang and Bourne described the progressive disappearance of Gsα with CT treatment in GH_3 cells [27]. However, in these cells the decrease of Gsα was not accompanied by a complete loss of CT-induced AC activity. The authors conclude that CT treatment increases the susceptibility of Gsα to proteolytic degradation. Milligan et al. [24] have demonstrated a cAMP-independent down-regulation of Gsα, but not of Giα by CT treatment of L6 skeletal myoblasts. This down-regulation occurred subsequent to CT catalysed ADP-ribosylation. MacLeod and Milligan recently demonstrated [28] that a biphasic regulation of AC activity by CT in neuroblastoma × glioma hybrid cells is due to the activation and subsequent loss of the α subunit of Gs.

In IPC-81 cells, CT induces Gsα decrease by mechanisms which are probably similar. In CTs cells, while Gsα degradation proceeds, the AC activity remains, high enough to irreversibly damage cells through intracellular cAMP elevation. On the contrary, in CTr cells Gsα degradation is enhanced to the point that AC generates inoperative cAMP levels. cAMP treatments of IPC-81 cells neither modify Gsα levels (Western blots) nor Gsα ADP-ribosylation (data not shown); this confirmed previous reports [27,28] that Gsα decrease by CT is a cAMP-independent process. An interesting result is that CTr cells retain the mark of CT treatment for a considerable time (up to 2 weeks, i.e. 20 cell cycles on average). These data argue against the presence of still active CT molecules in the cell at that time. ADP-ribosylation by CT, as proposed by others [24,27,28] may serve to target Gsα molecules for degradation by proteases, but the process is certainly more complex; differences in the kinetics of Gsα decrease, between the two cell clones analyzed herein and also between two Gsα isoforms, remain unexplained.

The questions addressed by our results are whether or not some particular structure or conformation of Gsα confers an increased stability to CTs Gsα molecules (or confers unstability to CTr Gsα), or whether CT has the capacity to turn on some physiological process for post-translational down-regulation or turnover of Gsα. The studies of these cells should tell us more about these regulatory processes.

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REFERENCES

[1] Stryer, L. and Bourne, H.R. (1986) Annu. Rev. Cell Biol. 2, 391-419.
[2] Casey, P.J. and Gilman, A.G. (1988) J. Biol. Chem. 263, 2577-2580.
[3] Lochrie, M.A. and Simon, M.I. (1988) Biochemistry 27, 4957-4965.
[4] Spiegel, A.M., Carter, A., Brann, M., Collins, R., Goldsmith, P. et al. (1988) in: Recent Progress in Hormone Research, vol. 44, pp 337-375.
[5] Bourne, H.R. (1989) Nature 337, 504-505.
[6] Colon-Otero, G., Sando, J.J., Sims, J.L., McGrath, E., De Jensen and Quesenberry, P.J. (1987) Blood 70, 686-693.
[7] Wu, M.C., Zaun, M.R. and Wu, F.M. (1989) FEBS Lett. 244, 338-342.
[8] Lanotte, M., Gombaud-Saintonge, G. and Tertian, G. (1986) Exp. Hematol. 14, 724-731.
[9] Lanotte, M., Arock, M., Lacaze, N. and Guy-Grand, D. (1986) J. Cell. Physiol. 129, 199-206.
[10] Olsson, I. and Breitman, T.R. (1982) Cancer Res. 42, 3924-3933.
[11] Olsson, I., Breitman, T.R. and Gallo, R.G. (1982) Cancer Res. 42, 3928-3933.
[12] Long, M.W., Heffner, C.H. and Gragowski, L.L. (1988) Exp. Hematol. 16, 195-200.
[13] Tortora, G., Clair, T., Katsaros, D., Ally, S., Colamonici, O., Neckers, L., Tagliaferri, P. et al. (1989) Proc. Natl. Acad. Sci. USA 86, 2849-2852.
[14] Lacaze, N., Gombaud-Saintonge, G. and Lanotte, M. (1983) Leuk. Res. 7, 145-154.
[15] Lanotte, M., Hermouet, S., Gombaud-Saintonge, G. and Dobo, I. (1986) Leuk. Res. 10, 1319-1326.
[16] Hermouet, S. and Lanotte, M. (1989) Leukemia 3, 289-293.
[17] Bourne, H.R., Coffino, P. and Tomkins, G.M. (1975) Science 187, 750-752.
[18] Lowry, O.H. et al. (1971) J. Biol. Chem. 234, 465.
[19] Berthillier, G., D'Alayer, J. and Monneron, A. (1982) Biochem. Biophys. Res. Commun. 109, 297-304.
[20] Jacquemin, C., Thibout, M., Lambert, B. and Correze, C. (1986) Nature 323, 182-184.
[21] Laemmli, U.K. (1970) Nature 227, 680-685.
[22] Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541-548.
[23] Simonds, W.F., Goldsmith, P.K., Codina, J., Unson, C.G. and Spiegel, A.M. (1989) Proc. Natl. Acad. Sci. USA 86, 7809-7813.
[24] Milligan, G., Unson, C.G. and Wakeman, D.J.O. (1989) Biochem. J. 262, 643-649.
[25] Kaslow, H.R., Farel, Z., Johnson, G.L. and Bourne, H.R. (1979) Mol. Pharmacol. 15, 472-483.
[26] Sternweis, P.C. and Gilman, A.G. (1979) J. Biol. Chem. 254, 3333-3340.
[27] Chang, F.H. and Bourne, H.R. (1989) J. Biol. Chem. 264, 5352-5357.
[28] MacLeod, K.G. and Milligan, G. (1990) Cellular Signalling (in press).