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Adipocyte plasma membranes contain two G_i subtypes but are devoid of G_o

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Antisera generated against synthetic peptides were used to identify G-protein α-subunits in plasma membranes from rat adipocytes. Applying the immunoblot technique, we detected two G_s α-subunits of 42 and 43 kDa, corresponding to the two cholera toxin substrates, and two G_i α-subunits of 40 and 41 kDa, corresponding to the two pertussis toxin substrates present in these membranes. The 40 kDa protein was tentatively identified as the G_{i2} α-subunit. A serum specific for the G_o α-subunit failed to detect any immunoreactive protein. Thus plasma membranes of adipocytes possess two forms of G_i but not G_o.

Signal transduction; G-protein; Synthetic peptide; Immunoblotting

1. INTRODUCTION

Guanine nucleotide-binding proteins (G-proteins) are involved in signal transduction across plasma membranes [1]. They are composed of three different subunits referred to as α-, β- and γ-subunits [2]. The α-subunits are substrates for ADP-ribosylating bacterial toxins, i.e. for cholera toxin, pertussis toxin or both, and differ by structural criteria including relative molecular masses on SDS gels (39–54 kDa).

Multiple cDNA clones encoding for G-protein α-subunits with molecular masses between 39 and 41 kDa have been identified (e.g. [3]). Among those are three G_i α-subunits termed G_{i1}, G_{i2} and G_{i3} with apparent molecular masses on SDS gels of 41, 41 and 40 kDa, respectively [4]. The G_o α-subunit which is highly abundant in brain [5] and in a pituitary cell line [6] migrates as a 39 kDa protein.

In adipose tissue, G-proteins mediate hormonal stimulation and inhibition of adenylate cyclase [7] and glucose transport [8–10] and stimulation of phosphoinositide hydrolysis [11]. G-proteins have also been proposed to mediate effects of insulin [12,13].

Membranes of adipocytes contain two substrates for each cholera and pertussis toxin [14]. The cholera toxin substrates may represent G_s α-subunits [15]. Based on the use of polyclonal antisera raised against purified G-proteins [16], the pertussis toxin substrates have been reported to represent α-subunits of a G_i-type G-protein and G_o.

Here, antisera raised against peptides corresponding to defined regions of G-protein α-subunits were used to identify G-protein α-subunits in plasma membranes of rat adipocytes. In contrast to previously reported data obtained

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Abbreviation: SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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with antisera raised against purified G-proteins, the present data indicate the absence of G_o and the presence of two G_i-type G-proteins, one of which is likely to represent G_{i2}.

2. MATERIALS AND METHODS

The α_{common} and the α_o peptide (see below) were a gift from Dr A. Herz (München). Pertussis toxin was a gift from Dr M. Yajima (Kyoto).

Highly insulin-responsive adipocytes were prepared from epididymal fat pads of male rats (170–200 g, Wistar strain, fed ad libitum) by collagenase treatment according to Rodbell [17] with modifications [18]. Plasma membranes and low-density microsomes were prepared by differential centrifugation as in [19].

3T3-L1 cells obtained from the American Type Culture Collection (Rockville, MD) were grown as described [20]. Undifferentiated cells were homogenized in Tris-HCl, EDTA, sucrose buffer (10, 1 and 250 mM, respectively; pH 7.4) with a Potter-Elvehjem homogenizer, and membranes were prepared by differential centrifugation [20].

G-proteins from human erythrocytes (G_s and G_i) were purified by the method of Codina et al. [21]. G-proteins from porcine brain (G_i and G_o) were isolated as described [22].

Peptides were obtained by Fmoc solid-phase peptide synthesis under continuous flow conditions, using BOP (Castro's reagent) for activation [23]. All peptides contained a cysteine residue at the amino terminus in order to facilitate cross-linking to keyhole limpet hemocyanin. Coupling of peptides was performed as described [24] with modifications [25]. New Zealand white rabbits were immunized, and antisera were obtained according to Mumby et al. [26].

SDS-PAGE was performed according to Laemmli [27] with modifications. Proteins were acetone-precipitated, dissolved in sample buffer and loaded onto a slab gel, composed of 8% (w/v) acrylamide, 0.21% (w/v) bisacrylamide and 4 M urea. Electrophoresis was performed at a constant current of 20 mA for 3.5 h. The inclusion of urea in the SDS gel effectively improved the resolution and caused a slight increase in the mobility of G-protein α-subunits. Immunoblotting (using ^{125}I-protein A for the detection of filter-bound antibodies) and autoradiography of filters were performed as described [22].

Pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of membrane proteins was performed as in [22] with two modifications. The toxin was preactivated in the presence of ATP (1 mM), and the assay was performed in the presence of Lubrol PX (0.1%, w/v). Cholera toxin-catalyzed [^{32}P]ADP-ribosylation was performed essentially as described [28]. The assay mixture contained 1 μM [^{32}P]NAD (37–74 kBq/assay tube) and (in mM) 10 $MgCl_2$, 1 EDTA, 10 thymidine, 0.1 GTP, 1 ATP, 10 DTT, 20 isonicotinic acid hydrazide, 3 dimyristoyl-L-α-phosphatidylcholine, and 250 potassium phosphate (pH 7.5).

Urea (Bio-Rad, München) was deionized by passage over an ion-exchange resin (AG 501-X8). ^{125}I-protein A was synthesized using Iodo-Gen as oxidizing agent [29]. [α-^{32}P]ATP was synthesized according to Johnson and Walseth [30] and [^{32}P]NAD according to Cassel and Pfeuffer [31]. Protein was determined according to Lowry et al. [32] with BSA as standard.

3. RESULTS AND DISCUSSION

Four different peptides derived from published sequences of cDNA encoding G-protein α-subunits were used for antiserum production (table 1). The α_{common} peptide, identical to that employed by Mumby et al. [26], occurs in all G-protein α-subunits except that of G_s and G_z, from which it differs in one and three amino acids, respectively [33]. The α_o peptide, also identical with that used by Mumby et al. [26], corresponds to a region specific for the G_o α-subunit. Two different peptides were designed for generation of antisera against G_i α-subunits. The $\alpha_{icommon}$ peptide is found in the α-subunits of G_{i1} and G_{i2}. It differs from the corresponding sequence in the α-subunit of G_{i3} in only one amino acid, and from the α-subunits of G_o, G_s and G_z in five or more amino acids. The α_{i2} peptide is specific for the G_{i2} α-subunit; it differs from the corresponding sequences of the α-subunits of G_{i1}, G_{i3}, G_o or G_z in 4 or more amino acids.

Immunization of rabbits with the peptides listed in table 1 yielded antisera that recognized G-protein α-subunits. Specificity of the antisera was tested with subunits of purified G-proteins, which were separated by SDS-PAGE in the presence of urea and blotted onto nitrocellulose filters (fig.1). The α_{common} peptide antiserum, AS 9, reacted with α-subunits of G-proteins purified from erythrocytes, i.e. the 42 kDa α-subunit of G_s and the 40–41 kDa α-subunits of G_i-type G-proteins. It also reacted with the α-subunit of G_o (39 kDa) purified from porcine brain (panel A). The $\alpha_{icommon}$ peptide antiserum, AS 19, recognized the 40–41 kDa G_i α-subunits of erythrocytes (panel B)

Table 1

Sequences of peptides used for generation of antisera

α_{common} peptide	(C)GAGESGKSTIVKQMK	antisera AS 8, AS 9
$\alpha_{icommon}$ peptide	(C)NLREDGEKAAREV	antiserum AS 19
α_{i2} peptide	(C)TGANKYDEAAS	antiserum AS 64
α_o peptide	(C)NLKEDGISAAKDVK	antiserum AS 6

Amino acid sequences are given in the one-letter code. Each peptide contained an additional cysteine residue (in parentheses) at the amino terminus in order to facilitate cross-linking to keyhole limpet hemocyanine. Code numbers of antisera used in the present study are given

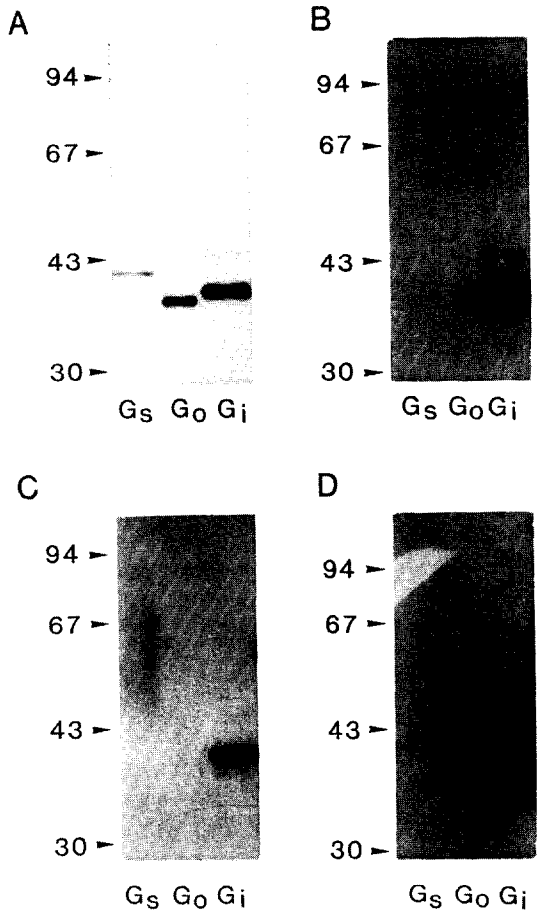

Fig.1. Characterization of peptide antisera with purified G-proteins. Autoradiographs of immunoblots performed with purified G-proteins are shown. G_s and G_i were purified from human erythrocyte, G_o from porcine brain. The amount of protein applied per lane of the SDS gel amounted to approx. 1 μg with respect to G-protein α-subunits. (A) α_{common} peptide antiserum (AS 9, 1:300). (B) $\alpha_{icommon}$ peptide antiserum (AS 19, 1:300). (C) α_{i2} peptide antiserum (AS 64, 1:300). (D) α_o peptide antiserum (AS 6, 1:300).

and 40–41 kDa proteins of brain preparations (see below); the α_{i2} peptide antiserum, AS 64, reacted with an 40 kDa α-subunit of G_i-type G-proteins purified from erythrocytes (panel C) and brain preparations (see below). The α_o-peptide antiserum, AS 6, detected the 39 kDa α-subunit of G_o purified from porcine brain (panel D). AS 19 did not cross-react with the G_o α-subunit, and AS 6 did not detect α-subunits in the various G_i preparations. The reactions of antisera with proteins of 39–43 kDa were blocked by preincubation with the respective synthetic peptide employed as hapten (not shown); this suggests that 39–43 kDa proteins were specifically recognized by antibodies against the employed hapten.

To characterize further the antisera, a cholate extract of brain membranes which contains G_s, G_{i1}, G_{i2} and G_o [34] was used. As shown in fig.2, AS 9 detected a narrow band at 43 kDa (presumably representing a G_s α-subunit), and a broad band in the 39–41 kDa region (fig.2A). With the various peptide antisera, this broad immunoreactive band was attributed to three different protein species: two G_i α-subunits (40, 41 kDa; fig.2B), one of which appeared to be the G_{i2} α-subunit (40 kDa; fig.2C), and to the G_o α-subunit (39 kDa; fig.2E). When AS 19 was added to AS 64, the second G_i α-subunit emerged as an additional band (fig.2D). The interaction of a protein of molecular mass $\geqslant$41 kDa with AS 19 (fig.2B,D) was unspecific, since it was not suppressed by preincubation of the serum with the $\alpha_{icommon}$ peptide (not shown). The resolution of G_i and G_o α-subunits (particularly that of the G_{i2} and G_o α-subunits) crucially depended on the presence of urea in the SDS gels. Only under these conditions was a 40 kDa protein, detected by AS 6 in addition to the 39 kDa G_o α-subunit, observed (fig.2E). This immunoreactive protein was also detectable in some but not all preparations of purified G_o.

In adipocyte plasma membranes, AS 9 detected four proteins corresponding to [^{32}P]ADP-ribosylated G-protein α-subunits (fig.3). Cholera toxin catalyzed the ADP-ribosylation of two proteins (42, 43 kDa; panel B) which were immunoprecipitated with the serum (not shown). These proteins representing the two forms of the G_s α-subunit corresponded to two similarly migrating immunoreactive proteins in the immunoblot (panel A). Pertussis toxin-catalyzed ADP-ribosylation revealed two substrates in the 40–41 kDa region (panel C); two corresponding proteins were detected by immunoblotting (panel A).

Fig.4 illustrates the further identification of the 40–41 kDa proteins with sera specific for G_i and G_o α-subunits. Membranes from 3T3-L1 fibroblasts, which reportedly contain G_o [35], or a mixture of G_i and G_o, purified from porcine brain, were run as controls. Surprisingly, AS 6 failed to

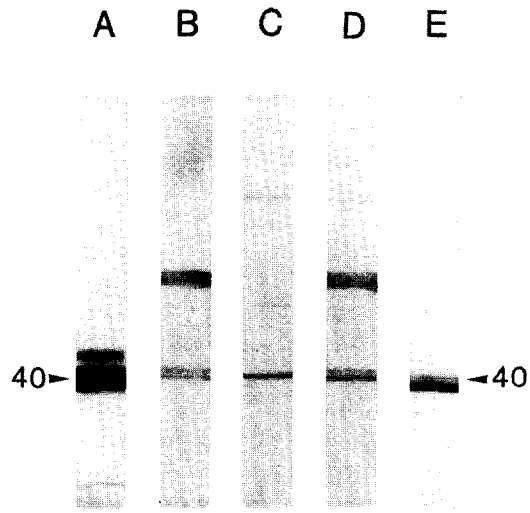

Fig.2. Detection of G-protein α-subunits with peptide antisera in cholate extracts of porcine brain membranes. Autoradiograph of an immunoblot is shown. Membranes from porcine brain cortex were prepared and extracted with 1% cholate as described [22]. 390 μg extract were applied to an SDS gel of 10 cm width. Following SDS-PAGE and transfer of proteins, nitrocellulose filters were cut into 1.5 cm wide strips and incubated with various antisera. (A) α_{common} peptide antiserum (AS 9, 1:300). (B) $\alpha_{icommon}$ peptide antiserum (AS 19, 1:400). (C) α_{i2} peptide antiserum (AS 64, 1:400). (D) $\alpha_{icommon}$ peptide antiserum (AS 19, 1:400) plus α_{i2} peptide antiserum (AS 64, 1:400). (E) α_o peptide antiserum (AS 6, 1:1500).

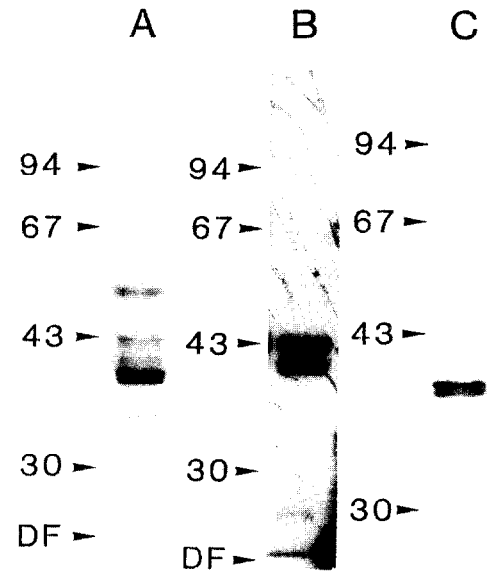

Fig.3. G-protein α-subunits in adipocyte plasma membranes as detected with an α_{common} peptide antiserum and with [^{32}P]ADP-ribosylation. Autoradiographs of an immunoblot and of SDS gels are shown. Immunoblotting was performed with adipocyte plasma membranes (100 μg of protein) and the α_{common} peptide antiserum, AS 8 (1:300) (A). For [^{32}P]ADP-ribosylations, plasma membranes (50 μg protein) were incubated with ^{32}P-NAD and cholera toxin (B) or pertussis toxin (C).

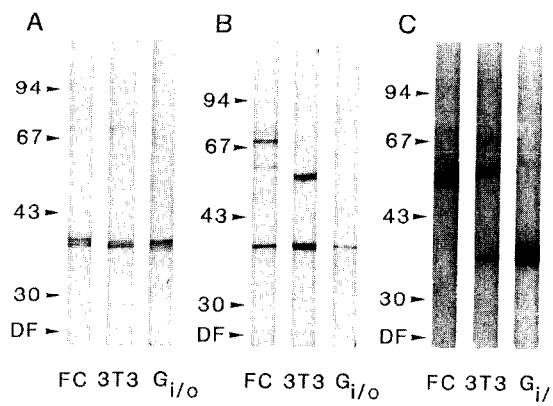

Fig.4. Identification of 40–41 kDa G-protein α-subunits in adipocyte plasma membranes with peptide antisera. Autoradiographs of immunoblots are shown. SDS gels were loaded with plasma membranes from fat cells (100 μg; FC) and, as positive controls, with membranes from 3T3-L1 cells (100 μg; 3T3) or a mixture of G_i/G_o purified from porcine brain (3 μg; $G_{i/o}$). Nitrocellulose filters were incubated with the $\alpha_{icommon}$ peptide antiserum (AS 19, 1:150; A), α_{i2} peptide antiserum (AS 64, 1:300; B) or α_o peptide antiserum (AS 6, 1:300; C).

detect any G_o α-subunits in the adipocyte plasma membranes (panel C). In contrast, the serum recognized a 39 kDa protein in membranes from 3T3-L1 fibroblasts and the G_i/G_o mixture. The blots obtained with AS 19 (panel A) revealed that the adipocyte plasma membranes contained two G_i α-subunits which fully account for the doublet detected with pertussis toxin-catalyzed [^{32}P]ADP-ribosylation or with AS 9 (see fig.3). The smaller species of the G_i α-subunits (40 kDa) may represent the G_{i2} α-subunit, since it was also detected with AS 64 (panel B).

The present results indicate that adipocyte plasma membranes contain two G_i subtypes but are devoid of detectable quantities of G_o. The latter conclusion is based on the failure of a specific serum to detect a G_o α-subunit, whereas the serum clearly detected this protein in positive controls. In addition, pertussis toxin-catalyzed ADP-ribosylation revealed only two substrates in

the 40 kDa region both of which were identified as G_i α-subunits. Therefore, neither method of G-protein detection provided any evidence for the presence of an additional G_o α-subunit in adipocyte plasma membranes.

In contrast to our findings, previous reports show the presence of G_o α-subunits in rat adipocyte plasma membranes [16,36]. The conclusions drawn in these reports were based on the finding that an antiserum raised against a purified 39 kDa G-protein α-subunit, assumed to represent the α-subunit of G_o, recognized a protein of about 39 kDa in adipocyte plasma membranes. Although the authors showed that their antiserum did not recognize a 41 kDa G_i α-subunit, the possibility cannot be excluded that the preparations used for immunization of rabbits were contaminated by the 40 kDa G_{i2} α-subunit which is not resolved from the 39 kDa G_o α-subunit by SDS-PAGE in the absence of urea. For this reason and because of the high degree of homology among G-protein α-subunits, a cross-reaction of sera used in these previous studies with the G_{i2} α-subunit appears possible. This may also apply to an antiserum raised against a 39 kDa G-protein α-subunit from bovine brain, which reacted with a 40 kDa protein in membranes of human adipocytes.

The present data obtained with highly specific antisera against synthetic peptides suggest that adipocyte plasma membranes contain two G_i-type G-proteins but no G_o. Thus, all receptor-mediated, pertussis toxin-sensitive responses of adipocytes may occur via G_i-type G-proteins, i.e. inhibition of adenylate cyclase [7], stimulation of glucose transport by adenosine [8,10], and stimulation of phosphoinositide hydrolysis [11].

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REFERENCES

[1] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[2] Hildebrandt, J.D., Codina, J., Risinger, R. and Birnbaumer, L. (1984) J. Biol. Chem. 259, 2039–2042.
[3] Jones, D.T. and Reed, R.R. (1987) J. Biol. Chem. 262, 14241–14249.
[4] Graziano, M.P. and Gilman, A.G. (1987) Trends Pharmacol. Sci. 8, 478–481.
[5] Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. 259, 13806–13813.
[6] Rosenthal, W., Hescheler, J., Hinsch, K.-D., Spicher, K., Trautwein, W. and Schulz, G. (1988) EMBO J. 7, 1627–1633.
[7] Londos, C., Cooper, D.M.F., Schlegel, W. and Rodbell, M. (1978) Proc. Natl. Acad. Sci. USA 75, 5362–5366.
[8] Joost, H.G. and Göke, R. (1984) FEBS Lett. 167, 5–9.
[9] Joost, H.G., Weber, T.W., Cushman, S.W. and Simpson, I.A. (1986) J. Biol. Chem. 261, 10033–10036.
[10] Kuroda, M., Honnor, R.C., Cushman, S.W., Londos, C. and Simpson, I.A. (1987) J. Biol. Chem. 262, 245–253.
[11] Moreno, F.J., Mills, I., García-Sáinz, J.A. and Fain, J.N. (1983) J. Biol. Chem. 258, 10938–10943.
[12] Houslay, M.D. (1985) in: Molecular Mechanisms of Transmembrane Signalling (Cohen, P. and Houslay, M.D. eds) pp.279–334, Elsevier, Amsterdam, New York.
[13] Londos, C., Honnor, R.C. and Dhillon, G.S. (1985) J. Biol. Chem. 260, 15139–15145.
[14] Malbon, C.C., Rapiejko, P.J. and Garciá-Sáinz, J.A. (1984) FEBS Lett. 176, 3010–3016.
[15] Robishaw, J.D., Smigel, M.D. and Gilman, A.G. (1987) J. Biol. Chem. 261, 9587–9590.
[16] Rapiejko, P.J., Northup, J.K., Evans, T., Brown, J.E. and Malbon, C.C. (1986) Biochem. J. 240, 35–40.
[17] Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
[18] Joost, H.G., Weber, T.W. and Cushman, S.W. (1988) Biochem. J. 249, 155–161.
[19] Simpson, I.A., Yver, D.R., Hissin, P.J., Wardzala, L.J., Karnieli, E., Salans, L.B. and Cushman, S.W. (1983) Biochim. Biophys. Acta 763, 393–407.
[20] Frost, S.C. and Lane, M.D. (1985) J. Biol. Chem. 260, 2646–2652.
[21] Codina, J., Rosenthal, W., Hildebrandt, J.D., Birnbaumer, L. and Sekura, R. (1985) Methods Enzymol. 109, 446–465.
[22] Rosenthal, W., Koesling, D., Rudolph, U., Kleuss, C., Pallast, M., Yajima, M. and Schultz, G. (1986) Eur. J. Biochem. 158, 255–263.
[23] Frank, R. and Gausepohl, H. (1988) in: Modern Methods in Protein Chemistry (Tscheche, H. ed.) vol.3, pp.41–60, De Gruyter, Berlin.
[24] Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T.M., Sutcliffe, J.G. and Lerner, R.A. (1982) Cell 28, 477–487.
[25] Hescheler, J., Rosenthal, W., Hinsch, K.-D., Wulfern, M., Trautwein, W. and Schultz, G. (1988) EMBO J. 7, 619–624.
[26] Mumby, S.M., Kahn, R.A., Manning, D.R. and Gilman, A.G. (1986) Proc. Natl. Acad. Sci. USA 83, 265–269.
[27] Laemmli, U.K. (1970) Nature 227, 680–685.
[28] Ribeiro-Neto, F.A.P., Mattera, R., Hildebrandt, J.D., Codina, J., Field, J.B., Birnbaumer, L. and Sekura, R.D. (1985) Methods Enzymol. 109, 566–572.
[29] Salacinski, P.R.P., McLean, C., Sykes, J.E.C., Clement-Jones, V.V. and Lowry, P.J. (1981) Anal. Biochem. 117, 136–146.

[30] Johnson, R. and Walseth, T. (1979) Adv. Cyclic Nucleotide Res. 10, 135–167.
[31] Cassel, D. and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. USA 75, 2669–2673.
[32] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[33] Fong, H.K.W., Yoshimoto, K.K., Eversole-Cire, P. and Simon, M.I. (1988) Proc. Natl. Acad. Sci. USA 85, 3066–3070.
[34] Mumby, S.M., Pang, I.-H., Gilman, A.G. and Sternweis, P.C. (1988) J. Biol. Chem. 263, 2020–2026.
[35] Gierschik, P., Morrow, B., Milligan, G., Rubin, C. and Spiegel, A. (1984) FEBS Lett. 199, 103–106.
[36] Ros, M., Northup, J.K. and Malbon, C.C. (1988) J. Biol. Chem. 263, 4362–4368.
[37] Homburger, V., Brabet, P., Audigier, Y., Pantaloni, C., Bockaert, J. and Rouot, B. (1987) Mol. Pharmacol. 31, 313–319.