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Effect of dibutyryl cyclic AMP and isoproterenol on 7β-hydroxycholesterol cytotoxicity and esterification in spontaneous transformed cell lines derived from astrocyte primary cultures

Patrick Behr[a], Alexandre Kupferberg[a], Claude Leray[a], Laurence Schellenbaum[a], Paul-Francis Urban[b], Guy Vincendon[a] and Marcel Mersel[a]

[a]*Laboratoire de Neurobiologie Moléculaire des Interactions Cellulaires, Centre de Neurochimie du CNRS* and [b]*Unité 338 de l'INSERM, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France*

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Incubation of spontaneous transformed cells derived from astrocyte primary cultures with 30 μM 7β-hydroxycholesterol (7β-OH-CH) which is lethal to the cells or with 150 μM isoproterenol reduces the intracellular level of cAMP (4- and 2-fold respectively). Treatment of the cultures with 0.5 mM dibutyryl (db)-cAMP and 7β-OH-CH increases 3-fold the intracellular level of cAMP and both, db-cAMP and isoproterenol, raise the lethal effect of 7β-OH-CH and its esterification on C-3-OH by naturally occurring fatty acids (metabolite). Kinetic studies of net steryl-3-esters hydrolysis revealed that db-cAMP and isoproterenol lower that of cholesteryl-3-esters (2-fold) whereas the opposite is found for the metabolite. These data demonstrate that (i) high cAMP intracellular levels modulate differently the net hydrolysis of cholesteryl-3-esters and metabolite, (ii) isoproterenol acts otherwise than cAMP on 7β-OH-CH esterification, (iii) the cytotoxicity of 7β-OH-CH is linked to its own esterification. The accumulation of metabolite subsequent to db-cAMP or isoproterenol treatment as a result of acyl-CoA:cholesterol acyl transferase activation is discussed.

Oxysterol; cAMP; Isoproterenol, Transformed cell; Cytotoxicity

1. INTRODUCTION

Cyclic adenosine monophosphate (cAMP), a second messenger discovered by Sutherland et al. [1], is generated from ATP by the membrane-bound adenylate cyclase [2] which is activated when GTP binds to G proteins [3]. A key mechanism mediated by cAMP is the activation of protein kinases which in turn catalyse the phosphorylation of seryl or threonyl groups of some proteins in the target cell; this process leads to the modulation of activity of important polypeptides such as acetyl coenzyme A carboxylase, 3-hydroxy-3-methylglutaryl-CoA reductase [4] and kinase kinase [5]. During the last decade, the regulation of the cytosolic neutral cholesteryl ester hydrolase (NCHE; EC 3.1.1.13) by cAMP is well documented [4]. For example, Hajjar [4] and Middleton [6] have demonstrated that prostacyclin and forskolin which increase the intracellular cAMP level in cultured smooth-muscle cells from bovine thoracic arteries and in human vascular smooth-muscle cells activate also NCHE, probably via NCHE phosphorylation. In this context, Hajjar [2] and Onali et al. [7] have shown that short period incubation of arterial smooth muscle cultured cells with isoproterenol increases the intracellular cAMP level and NCHE activity whereas the opposite was found for long time incubation. The oxygenated derivatives of cholesterol, known commonly as oxysterols, have been demonstrated to have inhibitory effects on cholesterol biosynthesis, to modify some immunological responses and to exert toxic effects on high proliferative or tumor cells in vitro and in vivo [8]. Recently, we have demonstrated that 7β-hydroxycholesterol (7β-OH-CH) which is not present in cultured nerve cells, is lethal to spontaneously transformed cell lines (transformed cells) derived from neonatal rat astrocyte primary cultures (normal cells) [9]. Further investigations indicated that 7β-OH-CH is esterified via acyl-CoA:cholesterol acyltransferase (ACAT) on C-3-OH by fatty acids; we have also demonstrated that this metabolite (met) is implicated in 7β-OH-CH cytotoxicity [9,10]. These findings prompt us to elucidate whether the accumulation of met is a consequence of NCHE inaptitude to hydrolyse met. Dibutyryl-cAMP (db-cAMP) or isoproterenol were used in order to modulate NCHE activity via the variation of intracellular cAMP. We take also advantage of this model to demonstrate that the cytotoxic effect of 7β-OH-CH is linked to met formation.

Correspondence address. M. Mersel, Laboratoire de Neurobiologie Moléculaire des Interactions Cellulaires, Centre de Neurochimie du CNRS, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France.

2. MATERIALS AND METHODS

2.1. *Synthesis of [^{3}H]7β-OH-CH*

Microsynthesis of [^{3}H]7β-OH-CH using [^{3}H]CH (40–60 Ci/mmol, Amersham, UK), was carried out according to Rong [11] except that (i) 7β-hydroxycholesterol-3-acetate was obtained by the reduction of 7-keto-cholesterol-3-acetate with $NaBH_4$ (Aldrich, USA) dissolved in H_2O (pH 8.0) and $CeCl_3$ $7H_2O$ (Jansen, Belgium) in methanol/tetrahydrofuran (1:1, v/v), and (ii) the hydrolysis of 7β-hydroxycholesterol-3-acetate was performed in 1 N NaOH methanolic solution. The final radioactive product was extracted in $CHCl_3$, purified and recovered by silica gel thin-layer chromatography (TLC) as previously described [9]. The structure and purity of the synthesized intermediary and final products were checked by gas–liquid chromatography (GLC) coupled to mass spectrometry (MS) as described previously [12]. Mass spectra revealed the typical molecular peaks of 7-keto-cholesteryl-3-acetate (M = 442) and 7β-OH-CH (M = 402).

2.2 *Cell cultures*

Spontaneously transformed cell lines were obtained from neonatal rat brain astrocyte primary cultures by repetitive subculturing as described previously [13]. Briefly, passages were obtained with 0.05% trypsin (EC 3.4.21.4; from bovine pancreas, Sigma, USA) in 0.04% Tyrode KCl. The harvested cells were seeded at a density of 200×10^3 cells in 35 mm diameter culture dishes (Falcon, 3001, USA). Culture medium (2 ml, Dulbecco's modified Eagle's medium, DMEM, Gibco, NY, USA) was supplemented with 10% foetal calf serum (FCS, Gibco) and cells were grown in a 95% air/5% CO_2 atmosphere at 37°C. To each subculture, a passage number (Pn) was attributed. The cells were observed with a Nikon phase contrast microscope (Japan) and the cell viability tested by the method of exclusion of Trypan blue dye at a final concentration of 0.05% in 9‰ NaCl. The cell number was determined as described previously [14].

2.3. *Treatment of cells with 7β-OH-CH and db-cAMP or isoproterenol*

The cells, 1 day in culture (DIC), were incubated with 0.5 mM db-cAMP (Sigma, USA) for 24 h and then treated either with 30 μM 7β-OH-CH (Sigma, USA) in 8 μl ethanol solution and 2.5 μM [^{14}C]stearate ([^{14}C]18:0) (60 mCi/mmol, Amersham, UK) or with [^{3}H]7β-OH-CH mixed with 30 μM non-radioactive 7β-OH-CH. Alternately, after incubation of 2 DIC-transformed cells with 7β-OH-CH and [^{14}C]18:0 for 4 h, 150 μM of isoproterenol (Sigma, USA) in 20 μl ethanolic solution was added. [^{14}C]18:0 was added in order to label 7β-hydroxycholesteryl-3-esters.

2.4. *Determination of db-cAMP*

The cultures were rinsed twice with 9‰ NaCl, and the cells harvested by means of a rubber policeman, in 500 μl 1 N perchloric acid. After homogenization and centrifugation (2,000 × *g* for 5 min), the supernatant was neutralized with K_2CO_3 and centrifuged again. cAMP contained in the final supernatant was determined with a cAMP kit assay (TRK 432, Amersham, UK).

2.5. *Lipid extraction and radioactive steroid analysis*

The cells were harvested in 9‰ NaCl and lipids extracted according to Folch et al. [15]. Subsequent to TLC chromatography [9] and exposure of the TLC plates to iodine vapors, areas corresponding to met or cholesterol esters (CHE) were scraped in 500 μl H_2O, dissolved in 5 ml scintillator (Biofluor, NEN, USA) and counted in a scintillation counter (United Technicologies Packard TriCarb 4000 series, USA). In some experiments the radioactive compounds were detected by autoradiography as previously reported [10,12].

2.6. *Fatty acid analysis*

After TLC chromatography, areas corresponding to met were scraped and extracted with chloroform/methanol (2:1, v/v) and the organic phase centrifuged and evaporated under a nitrogen stream. The residue was submitted to BF_3-catalyzed methanolysis according to Morisson and Smith [16] and the fatty acid methyl esters analyzed by GLC as described previously [9]. Analysis of radioactive fatty methyl esters was carried out on a packed GLC-column (18% DEGS on chromosorb 80/100 WAW, Supelco, USA) using a DELSI 120 FL gas chromatograph (France) equipped with a 1/7 splitt. The column temperature and those of the injector and detector were 250°C.

2.7. *Determination of cholesteryl-3-esters and 7β-hydroxycholesteryl-3-esters hydrolysis*

[^{14}C]18:0 was added to 2 DIC cultures for 24 h. Cultures were rinsed twice with DMEM and then incubated either with db-cAMP or isoproterenol in DMEM which did not contain FCS according to Slotte et al. [17]. Cells were harvested at appropriate times, lipids extracted, chromatographed and radioactivity associated with CHE counted. Since treatment of the cells with db-cAMP or isoproterenol enhance drastically 7β-OH-CH cytotoxicity, 2 DIC cultures were incubated with 10 μM 7β-OH-CH and [^{14}C]18:0, rinsed with DMEM after 24 h and incubated with db-cAMP or isoproterenol in DMEM containing 5% FCS.

3. RESULTS AND DISCUSSION

We have shown that the cytotoxic effect of 7β-OH-CH to the transformed cells is proportional to intracellular met concentrations [10]. Levels of met were measured by addition of [^{14}C]18:0 and 7β-OH-CH to the cells and control experiments indicated that (i) 18:0 does not modify the 7β-OH-CH uptake, (ii) almost all of de novo synthesized met is 7β-OH-CH-3-[^{14}C]18:0 [10]. Since the aim of this work was to study the effect of cAMP on met hydrolysis, it was essential again to check that cAMP does not change the uptake of 7β-OH-CH and the fatty acyl profile of met.

The fatty acyl pattern of met formed in db-cAMP-treated cells (P88, P93 and P100) was similar to that found in the non-treated one (i.e. 6% for 14:0, 38% for 16:0, 6% for 16:1, 31% for 18:0, 14% for 18:1 and 3% for 18:2). Discrepancies in 18:0 and 18:1 content between this set of experiments and previous ones (14% for 18:0 and 27% for 18:1) [9] may be attributed to differences in culture age (3 DIC versus 8 DIC) and in the surface of the culture substratum (35 mm diameter culture dishes versus 80 mm). Changes in fatty acid profile from one batch of FCS to another may also explain this divergence. Treatment of the cells with [^{3}H]7β-OH-CH, autoradiography and counting the labelled sterols indicate that neither db-cAMP nor isoproterenol modified the uptake of [^{14}C]18:0 and the label distribution among the main lipid classes. The data indicate also that 7β-OH-CH was essentially esterified by [^{14}C]18:0 and that the uptake of 7β-OH-CH is not modulated by db-cAMP or isoproterenol.

We further checked the influence of db-cAMP, isoproterenol and 7β-OH-CH on intracellular cAMP levels; Table I shows that subsequent to dbcAMP or isoproterenol incubation for 24 h, intracellular levels of cAMP raised 3-fold and diminished 2-fold respectively. With respect to isoproterenol, the intracellular cAMP level increased 3-fold after 15 min, thus our observations are in agreement with those of Hajjar [2] and Onali et al. [7] which demonstrate a fast activation of adenyl-

ate cyclase, followed by a high hydrolysis of cAMP. Addition of 7β-OH-CH (30 μM) to the transformed cells reduced 4-fold the cAMP content and, as expected, isoproterenol could not modulate the level of cAMP (Table I). We have demonstrated that 30 μM 7β-OH-CH modifies the plasma membrane fluidity as well as the cholesterol/phospholipids ratio [18]. Since adenylate cyclase is sensitive to the compositional and dynamic microenvironment, we may assume that 7β-OH-CH affects this enzymatic activity. In contrast, 7β-OH-CH does not affect the amount of cAMP when the cells were preincubated with db-cAMP; as with control cells, the level increased markedly (Table I). In comparison to only 7β-OH-CH-treated cells, met biosynthesis and cellular death increased significantly (2.5- and 2.0-fold, respectively) when cultures were also incubated with db-cAMP or isoproterenol (Table I).

These data, which demonstrate the relationship between 7β-OH-CH cytotoxicity and met biosynthesis, incited us to examine whether the increase in met accumulation subsequent to cAMP or isoproterenol addition is due to ACAT activation or to NCHE inhibition.

Fig. 1A shows that db-cAMP or isoproterenol slows the rate of apparent CHE hydrolysis in P111 and P112 (5 pmol/h, 3 pmol/h and 2 pmol/h for control, db-cAMP and isoproterenol-treated cells, respectively). Incubation of sister cells with 10^6 cpm TLC-purified tritiated CH (40–60 Ci/mmol, Amersham, UK) in 20 μl ethanolic solution and subsequent incubation either with db-cAMP or isoproterenol revealed a similar picture; thus, we assume that the decrease in [^{14}C]18:0-labelled CHE is caused by the hydrolysis of C-3 ester function. In contrast, when cells (P111 and P112) were also treated with 7β-OH-CH, db-cAMP or isoproterenol accelerated radioactive met degradation (Fig. 1B) (0.6 pmol/h and 0.3 pmol/h for cells treated with 7β-OH-CH or with 7β-OH-CH and db-cAMP or isoproterenol, respectively). In comparison to the kinetics of CHE hydrolysis in control cells (Fig. 1A), 7β-OH-CH-treated cells revealed (Fig. 1B, insert) a lower extent of radioactive CHE biosynthesis (3-fold) and hydrolysis (2-fold) as well as the inaptitude of db-cAMP or isoproterenol to significantly reduce the rate of apparent CHE hydrolysis. These observations may be explained by the fact that LDL contained in FCS activate ACAT and exogenous CH uptake, on the one hand, and that met competes with NCHE activity, on the other hand. We should keep in mind that studies dealing with the relationship between cAMP levels and NCHE activity were essentially carried out on homogenates whereas a few reports relate the same relationship in entire cultured cells [19]. Therefore, as proposed by other authors, we may assume that in our biological model cAMP stimulates both NCHE and ACAT activities and therefore our results express the net CHE hydrolysis.

Table I

Effect of dibutyryl-cAMP and isoproterenol on intracellular cAMP level and on 7β-OH-CH esterification[a], and cytotoxicity, in spontaneous transformed glial cell lines

Mode of treatment	Determinations		
	cAMP[b] (pmol/mg protein)	7β-OH-CH-3-ester[c] (epm/mg protein)	Percent of cytotoxicity[d]
Control	39 ± 6	–	–
db-cAMP[e]	110 ± 10	–	–
Ipr[f]	24 ± 4	–	–
7β-OH-CH[g]	10 ± 3	$70 \times 10^3 \pm 15 \times 10^3$	56
7β-OH-CH+db-cAMP	143 ± 10	$180 \times 10^3 \pm 25 \times 10^3$	93
7β-OH-CH+Ipr	11 ± 4	$175 \times 10^3 \pm 25 \times 10^3$	89

The cells were treated as described in Section 2 [a]7β-hydroxycholesterol-3-ester; [b]cyclic adenosine monophosphate; [c]10^3 epm represent 10 pmol of 7β-OH-CH-3-ester; [d]expressed as percent decrease of protein and DNA which were determined according to Lowry et al. [22] and Burton [23], respectively, [e]db-cAMP: dibutyryl cyclic AMP, [f]Ipr: isoproterenol; [g]7β-OH-CH: 7β-hydroxycholesterol. These values are means of duplicates for 3 independent determinations.

With respect to 7β-OH-CH which is converted to met in the endoplasmic reticulum, we suppose that the association between met, NCHE and cAMP is not the same as for CHE which is formed as lipid droplets; thus, it is possible that, at the endoplasmic reticulum interface, minute quantities of cAMP only interact with NCHE and hence activate NCHE as expected. Indeed Stephens and Schroepfer [20] have observed that neutral 15-ketosterol oleate hydrolase activity was essentially present in the microsomal fraction of liver homogenate, whereas neutral cholesteryl oleate activity was present in both cytosolic and microsomal fraction, and thus only the level of cAMP in the endoplasmic reticulum modulates met hydrolysis. The concept of intracellular segregation of cAMP was also elaborated by Hollenga et al. [21]. The mechanism of action of β-adrenergic compounds on NCHE activity seems actually intricate, since Bernard et al. [19] have observed that the increase of cAMP subsequently to epinephrine treatment failed to stimulate NCHE. In this context we suggest that isoproterenol acts in a different manner to cAMP; isoproterenol constituted of a bulk of carboncycles substituted with hydroxyl functions may by itself modulate NCHE activity.

In summary, this is a first report demonstrating that db-cAMP and isoproterenol affect the net cholesteryl esters and 7β-hydroxycholesteryl-3-ester hydrolysis in different ways in our biological model. In spite of the enhanced hydrolysis of met in the transformed cells by db-cAMP or by isoproterenol, these compounds increase markedly the intracellular level of met. These observations suggest that either cAMP, and isoproterenol, or 7β-OH-CH activate(s) ACAT activity. This work demonstrates also the proportionality between 7β-OH-CH cytotoxicity and the level of intracellular 7β-hydroxy-cholesteryl-3-ester.

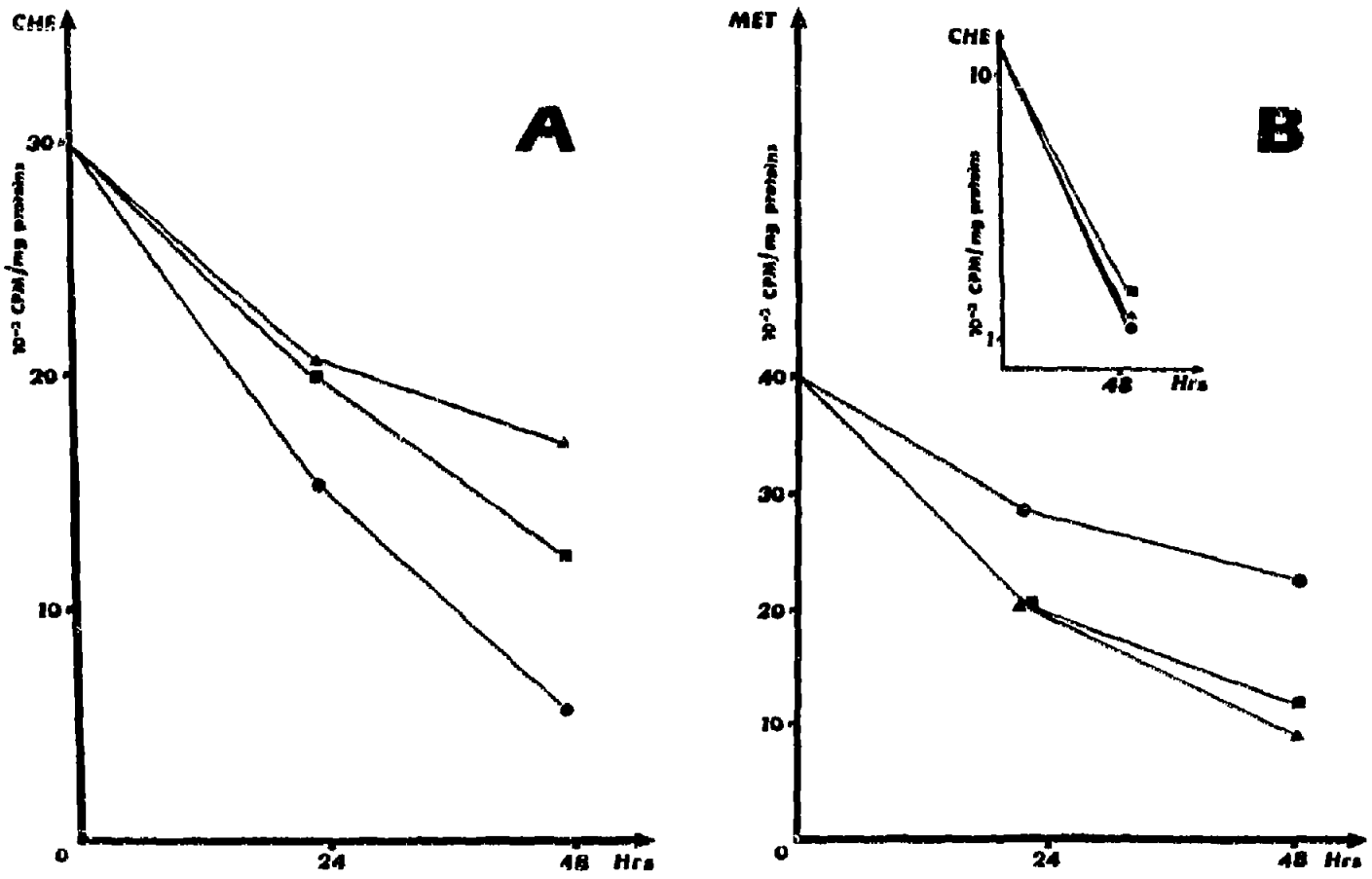

Fig. 1 Effect of db-cAMP and isoproterenol on cholesterol ester (CHE; panel A) and 7β-hydroxycholesteryl-3-ester (MET; panel B) hydrolysis; insert B: Rate of CHE hydrolysis. The cells were treated as described in Section 2. (●) Control; (■) db-cAMP, (▲) isoproterenol. These values are means of triplicates for 2 independent determinations.

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REFERENCES

[1] Sutherland, E.W., Rall, T.W. and Menon, T. (1962) J. Biol. Chem. 237, 1220–1227.
[2] Hajjar, D.P. (1986) Arch. Biochem. Biophys. 247, 49–56.
[3] May, D.C., Ross, E.M., Gilman, A.G. and Smigel, M.D. (1985) J. Biol. Chem. 260, 15829–15833.
[4] Hajjar, D.P. (1985) Biochem. Pharmacol. 34, 295–300.
[5] Hardie, D.G., Carling, D. and Sim, A.T.R. (1989) Trends Biochem. Sci. 14, 20–23.
[6] Middleton, A. and Middleton, B. (1990) Biochem. J. 267, 607–614.
[7] Onali, M., Schwartz, J.P. Hanbauer, I. and Costa, E. (1981) Biochim. Biophys. Acta 675, 285–292.
[8] Smith, L.L. and Johnson, B.H. (1989) Free Radical Biol. Med. 7, 285–332.
[9] Kupferberg, A., Teller, G., Behr, P., Leray, C., Urban, P.F., Vincendon, G. and Mersel, M. (1989) Biochim. Biophys. Acta 1013, 231–238.
[10] Kupferberg, A., Behr, P. and Mersel, M. (1990) BBA Report 1046, 106–109.
[11] Rong, S (1983) Activité antitumorale des dérivés hydrosolubles des stéroides polyoxygénés. Thesis, Université Louis Pasteur, Strasbourg, France.
[12] Behr, P., Kupferberg, A., Leray, C., Urban, P.F. and Mersel, M. (1991) FEBS Lett. 280, 202–206.
[13] Mersel, M., Tholey, G., Delaunoy, J.P., Rebel, G., Flory, E. and Mandel, P. (1985) C.R. Acad. Sci. Paris, Série III, 301, 811–815.
[14] Mersel, M., Benenson, A., Delaunoy, J.P., Devilliers, G. and Mandel, P. (1983) Neurochem. Res. 8, 449–463.
[15] Folch, J., Lee, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–499.
[16] Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600–608.
[17] Slotte, J.P., Hedström, G. and Bierman, E L. (1989) Biochim. Biophys. Acta 1005, 303–309.
[18] Kupferberg, A., Cremel, G., Behr, P., Van Dorsselaer, A., Luu, B. and Mersel, M. (1991) Mol. Cell. Biochem. 101, 11–22.
[19] Bernard, D.W., Rodriguez, A., Rothblat, G.H. and Glick, J.M. (1991) J. Biol. Chem. 266, 710–716.
[20] Stephens, T.J. and Schroepfer, G.J. (1989) Biochim. Biophys. Acta 1001, 127–133.
[21] Hollenga, C , Brower, F. and Zaagma, J. (1991) Eur. J. Pharmacol. 200, 325–330.
[22] Lowry, O.H., Rosebrough. N.J., Farr, A.K. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[23] Burton, K. (1956) Biochem. J. 62, 315–323.