Differential expression of cGMP-inhibited cyclic nucleotide phosphodiesterases in human hepatoma cell lines

Taku Murata, Masato Taira, Vincent C. Manganiello*

Pulmonary/Critical Care Medicine Branch, NHLBI, NIH, Bethesda, MD 20892, USA

Received 27 February 1996; revised version received 9 April 1996

Abstract PDE3 or cGMP-inhibited cyclic nucleotide phosphodiesterase (cGI PDE) activity was detected in homogenates of HepG2, Hep3B and HuH7, but not SK-Hep-1, human hepatoma cells. In HepG2 and Hep3B cells PDE3 activity was found predominantly in particulate fractions; in HuH7, in both particulate and supernatant fractions. cDNAs encoding two human PDE3s (an 'adipocyte' type, HcGIP1, and a 'cardiovascular' type, HcGIP2) have been cloned. HcGIP1 cDNA hybridized strongly with poly(A)$^+$ RNA species from HepG2 and Hep3B. Both HcGIP1 and HcGIP2 mRNAs were expressed in Hep3B and HuH7 cells. The nucleotide sequence of an ~300-bp cDNA fragment, isolated after RT-PCR cloning from HepG2 RNA, was identical to a sequence within the conserved domain of HcGIP1 cDNA, consistent with the presence of HcGIP1 mRNA in HepG2 cells.

Key words: Human hepatoma cell line; PDE3 or cGMP-inhibited cyclic nucleotide phosphodiesterase; PDE4 or cAMP-specific phosphodiesterase

1. Introduction

Seven cyclic nucleotide phosphodiesterase (PDE) gene families have been identified [1–5]. The PDE3 or cGMP-inhibited (cGI) PDE family is characterized by a high affinity for cAMP and cGMP (with V_{max} greater for cAMP than for cGMP) and competitive inhibition of its cAMP hydrolytic activity by cGMP, cilostamide (OPC 3689) and certain positive inotropic agents, including milrinone and enoximone [6]. PDE3s have been purified from adipose and cardiac tissues, rat liver, bovine aortic smooth muscle and human platelets [6–13]. Studies with specific PDE3 inhibitors suggest that PDE3s regulate cAMP pools important in myocardial contractility, platelet aggregation, vascular smooth muscle relaxation, lipolysis and vascular smooth muscle and T-lymphocyte proliferation [1–6,14–19]. cDNAs encoding two distinct but related PDE3 isoforms (cGIP1 and cGIP2) have been cloned from rat (R) and human (H) adipose and human (H) cardiac cDNA and genomic libraries ([20,21]; unpublished observations). cGIP1 and cGIP2 isoforms are products of two different genes, located on human chromosomes 11 and 12, respectively (unpublished observations). Their deduced primary amino acid sequences indicate that the C-terminal regions of PDE3s contain the catalytic domain conserved among all mammalian PDEs and that rat and human cGIP1 (or cGIP2) are more closely related than cGIP1 and cGIP2 from the same (rat or human) species [19–21]. Within the conserved domain of the PDE3s is an insertion of 44 amino acids which is not present in the conserved domains of other PDE families [19–21]. The N-terminal regulatory domain contains several consensus sites for cAMP-dependent protein kinase phosphorylation and hydrophobic putative membrane association domains [19–21].

Although there is considerable information regarding PDE3 in normal tissue, little is known of its presence in tumors. Recent in situ hybridization studies have indicated that rat cGIP1 mRNA is expressed in liver parenchymal cells, whereas rat cGIP2 mRNA is found in vascular elements [22]. In this study we observed differential expression of the two PDE3 mRNAs in several human hepatoma cell lines. Whether these findings reflect the ontological lineage of these tumor cell lines and/or a role for PDE3 in carcinogenesis remains to be established.

2. Materials and methods

2.1. Cell lines

Human hepatoma HepG2 cells, established from a human hepatoblastoma [23], were maintained in RPMI-1640 containing 10% fetal bovine serum. Human hepatoma cell lines Hep3B and HuH7, established from well-differentiated hepatocellular carcinomas, and SK-Hep-1 from hepatic adenocarcinoma, were maintained in Eagle's minimal essential medium containing 10% fetal bovine serum and 1% nonessential amino acids [23–26]. Media were changed every 2 days.

2.2. PDE activity in cell extracts

Cultures were initiated with 1×10^6 HepG2 cells/10 cm diameter plate (Falcon Plastics) and Hep3B, HuH7 and SK-Hep-1 cells, at 0.5×10^6 cells/10 cm plate. After 5 days, cells were washed twice with phosphate-buffered saline (PBS), harvested with a rubber policeman, homogenized in 2 ml of ice-cold STS buffer [10 mM TES (pH 7.0), 250 mM sucrose, 10 μg/ml (each) of pepstatin and leupeptin, 5 μg/ml aprotinin, 3 mM benzamidine, 0.2 mM PMSF, 13 mM β-mercaptoethanol, 0.2 mM EDTA, 1 mM $MgCl_2$] and centrifuged ($100\,000\times g$, 45 min, 4°C) to obtain crude particulate and supernatant fractions. Particulate fractions were suspended in 1 ml of STS buffer.

2.3. cAMP PDE assay

cAMP-PDE activity was assayed by a modification of a published procedure [13]. Samples were incubated at 30°C for 10 min in a total volume of 0.3 ml containing 50 mM HEPES (pH 7.4), 0.1 mM EDTA, 8.3 mM $MgCl_2$, 0.1 μM [^{3}H]cAMP (18 000 cpm). PDE3 activity (pmol cAMP hydrolyzed per min) was measured as cAMP hy-

*Corresponding author. National Institutes of Health, Building 10, Room 5N-307, Bethesda, MD 20892, USA. Fax: (1) (301) 402-1610. E-mail: MANGANIV@FIDO.NHLBI.GOV

Abbreviations: cGI PDE, cyclic GMP-inhibited cyclic nucleotide phosphodiesterase; RT-PCR, reverse transcription-polymerase chain reaction; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate

The HcGIP1 (or HSPDE3B) cDNA sequence has been submitted to Genbank, Accession 438178.

PII S0014-5793(96)00410-3

drolysis that was inhibited by 0.5 μM OPC 3689, a specific inhibitor of PDE3s [1].

2.4. RNA isolation

Total RNA was isolated from human adipose tissue and hepatoma cells [37] and poly(A)$^+$ RNA was prepared using the Promega mRNA isolation system. Poly(A)$^+$ RNA from human heart and liver was purchased from Clontech.

2.5. Northern blots

To amplify the HcGIP1 cDNA probe (corresponding to nt 883–3339; Taira et al., unpublished data), oligonucleotide sense (5′-(TCC AGC TGC GTG TCG TTA GGA, corresponding to nt 883–903) and antisense (5′-CCG CTC GAG TCT AGA CTA TTC CTC TTC ATC TGC CT, corresponding to nt 3320–3339 with *Xho*I and *Xba*I sites underlined, respectively) PCR primers based on the nucleic acid sequence of HcGIP1 were synthesized on an Applied Biosystems 380B DNA synthesizer. To amplify the HcGIP2 cDNA probe (corresponding to nt 1837–3426 [20]), oligonucleotide sense (5′-CGA TGG GGA TCC AGT CGA ACA GAT GAC ACT GC, corresponding to nt 1837–1856 with *Bam*HI site) and antisense (5′-CAT GGA ATT CGA TCA CTG GTC TGG CTT TTG GGT, corresponding to nt 3406–3426 with *Eco*RI site) PCR primers were synthesized.

PCR amplification was carried out in a total volume of 50 μl (HcGIP1) or 100 μl (HcGIP2) containing 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM $(NH_4)S0_4$, 2 mM $MgCl_2$, 200 μM dNTPs, 0.1% Triton X-100, 10 μg/ml nuclease-free BSA and 2.5 units (HcGIP1) or 1 unit (HcGIP2) Pfu DNA polymerase (Stratagene) with 500 ng (HcGIP1) or 20 ng (HcGIP2) of template and 0.6 μM (HcGIP1) or 0.1 μM (HcGIP2) sense and antisense primers. PCR was performed under conditions of denaturing at 94°C for 15 s, annealing at 66°C for 30 s (two cycles), 64°C for 30 s (two cycles), 62°C for 30 s (two cycles), 60°C for 30 s (two cycles) for the first eight cycles and at 58°C for 30 s for the next 28 cycles and extension for each cycle at 72°C for 2 min 30 s, and with final extension at 72°C for 10 min. PCR products were purified using QIAEX (Qiagen) after electrophoresis in 1% agarose gel (50 V, 90 min).

For Northern blots, samples (~2.5 μg) of poly(A)$^+$ RNA in formaldehyde/formamide/MOPS were incubated at 65°C for 15 min and then subjected to electrophoresis in 1% agarose/formaldehyde gels (25 V, 19 h). Gels were stained with ethidium bromide, and mRNA was transferred to nylon membranes (Schleicher and Schuell) and fixed using a UV crosslinker (Stratalinker, Stratagene). Blots were prehybridized at 65°C in 5×SSC, 0.5% SDS, 2.5×Denhardt's solution, 200 μg/ml salmon sperm DNA, 20 mM Tris and hybridized at 65°C overnight with fresh solution containing ^{32}P-labeled HcGIP1 (883–3339) and HcGIP2 (1837–3426) probes (random priming kit, Stratagene). Following a wash at room temperature in 2×SSC/0.1% SDS, blots were washed at 65°C for 20 min with 1.0×SSC/0.1% SDS, for 20 min with 0.5×SSC/0.1% SSC, and then for 20 min with 0.2×SSC/0.1% SSC. Films were exposed for 5 days at −70°C with an intensifying screen. The blots shown in Fig. 3 were reprobed with ^{32}P-labeled human 6-actin (Clontech). Labeling, hybridization, and washing conditions were as described above.

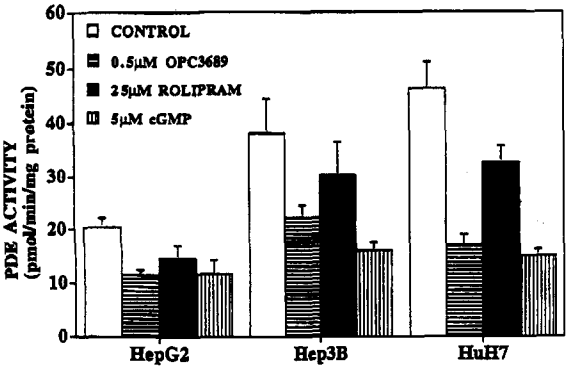

Fig. 1. Effect of inhibitors on PDE activity in hepatoma cells. Homogenates were prepared from hepatoma cell lines and assayed for PDE activity with or without inhibitors as described in Section 2. Data are mean ± S.E.M. of three experiments.

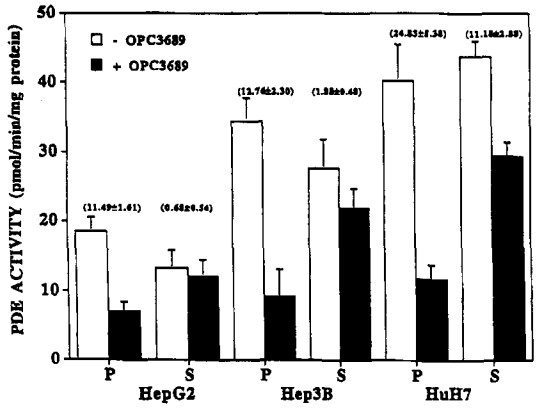

Fig. 2. Distribution of specific cGI PDE activity in hepatoma cells. Particulate (P) and supernatant (S) fractions were prepared from hepatoma cells and assayed for PDE3 activity with or without 0.5 μM OPC 3689 as described in Section 2. Numbers in parentheses represent total activity in particulate and supernatant fractions (pmol/min per dish). Data are mean ± S.E.M. of three experiments.

3. Results and discussion

PDE3s are very sensitive to inhibition by a number of inotropic and antithrombotic agents, including cilostamide and other OPC (Otsuka Pharmaceutical Co.) derivatives, imazodan, milrinone, enoximone, LY195115 [1,3,6,14,19]. cGMP is a competitive inhibitor of cAMP hydrolysis by PDE3 [6–14]. Inhibition of cAMP hydrolysis by cGMP also distinguishes PDE3s (cGI PDE) from PDE4s (cAMP-specific PDE) which exhibit a 'low K_m' for cAMP and are inhibited by rolipram but not by cGMP [1]. As shown in Fig. 1, in extracts of HepG2, Hep3B and HuH7 cells, PDE activity was inhibited by OPC 3689, cGMP and rolipram, indicating the presence of both PDE3 and PDE4 enzymes. In SK-Hep-1, the PDE activity was only slightly inhibited by OPC 3689 and rolipram, but was strongly inhibited by 5 μM cGMP (Table 1). It is very likely that this cGMP-inhibited activity reflects a PDE1 (calmodulin-sensitive PDE) since cAMP hydrolysis in SK-Hep-1 homogenates was inhibited by 0.1 mM EGTA plus 2 mM EDTA, activated by Ca and calmodulin, and the calmodulin-stimulated activity inhibited by cGMP (Table 1). Thus, in constrast to HepG2, Hep3B and HuH7 cells (Fig. 1) in which PDE3 and PDE4 are major contributors to hydrolysis of cAMP, these two PDEs hydrolyze little or no cAMP in extracts from SK-Hep1 adenocarcinomas in which a calmodulin-sensitive PDE is apparently a major PDE isoform (Table 1).

The apparent subcellular localization of PDE3s differs in different cells [6]. Most of the platelet PDE3 is cytosolic [12,13,27,28], whereas in adipocytes and hepatocytes, PDE3 activity is predominantly particulate [2,6,7,9,10,29,30] and in heart, both particulate and cytosolic [31–34]. In HepG2 and Hep3B most of the PDE3 activity was in the particulate fraction, but in HuH7 30% of PDE3 activity was in the supernatant fractions (Fig. 2).

cGIP1 mRNAs are relatively highly expressed in rat and 3T3-L1 adipocytes and human adipose tissue and cGIP2 mRNAs are expressed in rat heart, rat aorta, rat and human adipose tissue, human heart and human aorta [19–22]. It is

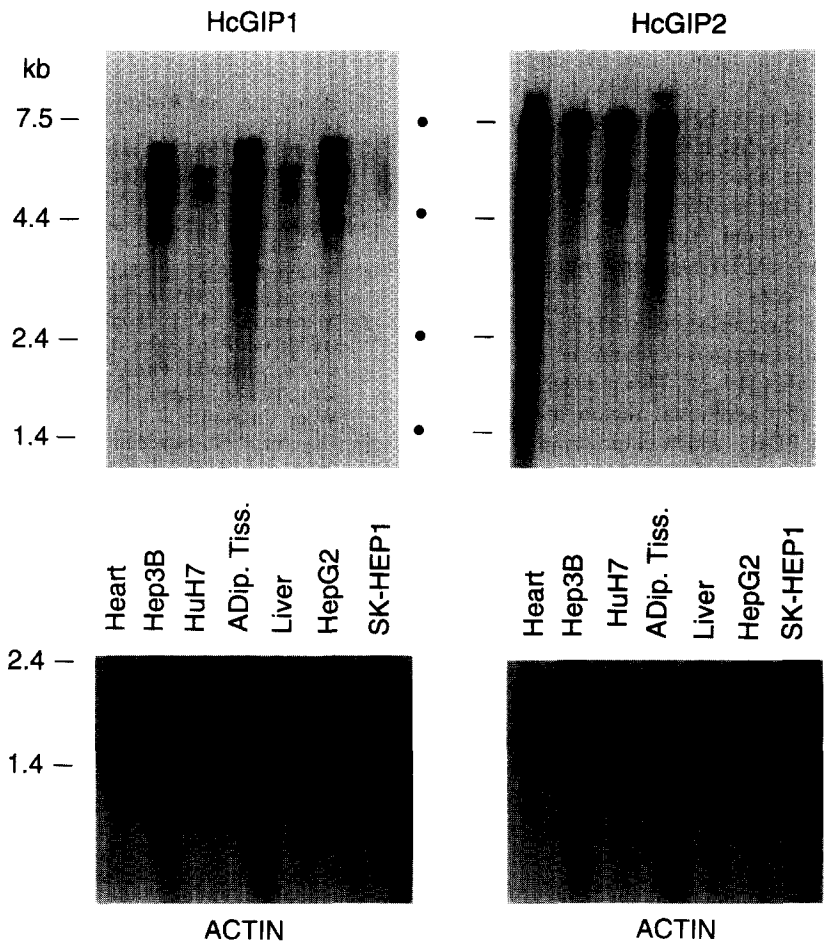

Fig. 3. Northern blots hybridized with (A) HcGIP1 or (B) HcGIP2 cDNA probes. Total RNA was prepared from the indicated tissues [37]; poly(A)$^+$ RNA (2.5 μg) was purchased or prepared, applied to each lane, subjected to electrophoresis in a 1% agarose-formaldehyde gel, and transferred to a nylon membrane as described in Section 2. Blots were prehybridized at 65°C, hybridized at 65°C overnight with ^{32}P-labeled cDNA (the HcGIP1 probe, nt 883–3339, or HcGIP2, nt 1837–3426), and washed as described in Section 2. Films were exposed for 5 days at −70°C with an intensifying screen. The same blots were reprobed with ^{32}P-labeled human 6-actin cDNA (Clontech).

likely that the RcGIP2 mRNA in adipose tissue reflects the presence of vascular elements, since RcGIP2 cDNA hybridized weakly, if at all, to mRNA from isolated rat fat cells or differentiated murine 3T3-L1 adipocytes [21] and in situ hybridization experiments also indicated that RcGIP1 mRNA is abundant in fat cells and RcGIP2 mRNA in vascular smooth muscle [22].

HcGIP1 cDNA hybridized much more strongly with

Table 1
Type I Ca^{2+}/calmodulin sensitive PDE activity in SK-Hep-1 cells

Additions	cAMP hydrolysis (pmol/min per mg protein)	
	−cGMP	+cGMP (5 μM)
None	208 ± 10.9	22 ± 5.6
OPC 3689 (0.5 μM)	205 ± 8.2	
Rolipram (25 μM)	200 ± 28.9	
EGTA (0.1 mM), EDTA (2 mM)	151 ± 15.9	19 ± 3.5
EGTA, EDTA, Ca^{2+} (2 mM)	203 ± 17.4	56 ± 9.7
EGTA, EDTA, Ca^{2+}, calmodulin (0.1 μg)	430 ± 26.8	44 ± 3.5

Homogenates from SK-Hep-1 cells were assayed in duplicate for cAMP PDE activity with and without cGMP and the indicated additions as described in Section 2. Values represent mean ± S.E.M. (n=3).

poly(A)$^+$ RNA species of 6.4 and 5.4 kb from Hep3B, HepG2 and adipose tissue than from HuH7 and liver (Fig. 3). HcGIP1 hybridized weakly with mRNA from HuH7, and little, if at all, with mRNA from heart or SK-Hep-1 (Fig. 3). On the other hand, HcGIP2 cDNA hybridized with poly(A)$^+$ RNA species of 8.4 and 7.2 kb from heart and adipose tissue. HcGIP2 cDNA hybridized with ~7.2-kb mRNA from Hep3B and HuH7. HcGIP2 cDNA did not apparently hybridize strongly with mRNA from HepG2 and SK-Hep-1 (Fig. 3). Thus, neither PDE3 mRNA, i.e. HcGIP1, nor HcGIP2 mRNA was apparently detected in SK-Hep-1 cells, consistent with the lack of inhibition of SK-Hep-1 PDE activity with cilostamide (Table 1). There have been no other reports of cGIP1 and cGIP2 mRNAs expressed in single cell populations as they seem to be in Hep3B and HuH7 cells. It will be of interest to determine which PDE4 isoforms are present in HepG2, Hep3B and HuH7 cells.

The additional region in the conserved catalytic domain of PDE3 does not correspond to sequences within the conserved domains of other PDE families [19–21]. Using degenerate primers from both sides of this additional region in the conserved catalytic domain of PDE3, which could amplify either HcGIP1 or HcGIP2 cDNAs, a ~300-bp fragment was isolated by RT-PCR cloning from HepG2 RNA (data not shown); its nucleotide sequence was identical to the corresponding region in the conserved domain of HcGIP1, consistent with the finding that the HcGIP1 cDNA probe hybridized strongly and the HcGIP2 cDNA probe weakly, if at all, to HepG2 mRNA (Fig. 3).

Based on inhibition of cAMP hydrolysis by cilostamide and cGMP and on Northern blot hybridizations, PDE3s were present in hepatoblastoma (HepG2)- and hepatocellular (Hep3B and HuH7)-derived cells and little, if at all, in cells derived from an adenocarcinoma (SK-Hep-1). HcGIP2 mRNA was detected in hepatocellular tumor cells (Hep3B and HuH7) but not (or in much lower amounts) in the hepatoblastoma-derived HepG2 cells, whereas HcGIP1 mRNA was found in all three hepatoma cell lines. Whether one or both PDE3 isoforms plays a role in the development or differentiation of hepatocellular carcinomas or hepatoblastoma (as opposed to hepatic adenocarcinomas) remains to be established.

Although our results are limited to a small number of cell lines, knowledge of specific PDE isoenzyme gene family members in specific tumor cells might be important in designing therapy to inhibit tumor cell growth. It is known that cAMP and cAMP analogs produce antiproliferative effects in various human cancer cell lines [35,36], in some cases perhaps by altering expression of RI and RII regulatory subunits of cAMP-dependent protein kinases [35]. Identification of the PDE types in tumor cells might allow use of specific inhibitors (e.g. compounds analogous to milrinone and cilostamide for PDE3 or to rolipram for PDE4) as adjunct therapeutic agents to potentiate the responses to cAMP analogs. This conceptual approach might be relevant, since it has been reported that low (μM) concentrations of 8-Cl-cAMP, which inhibit cell growth of a number of human cancer cell lines, were hydrolyzed by PDE3 at 30% of the efficiency of cAMP [36]. Several studies have in fact reported synergistic interactions between specific PDE3 inhibitors (cilostamide [18], CI-930 [15,18], SKF94836 [16] and milrinone [17]) and specific PDE4 inhibitors (Ro 20-1724, rolipram [16–18]) in attenuation of DNA synthesis and proliferation of rat [18] and porcine vascular smooth muscle cells [16] and rat [17] and human T-lymphocytes [15].

Acknowledgements: The authors wish to thank Dr. Martha Vaughan for reading this manuscript and Ms. Carol Kosh for excellent secretarial assistance.

References

[1] Beavo, J.A. and Reifsnyder, D.H. (1990) Trends Pharmacol. 11, 150–155.
[2] Conti, M., Jin, S.-C., Monaco, L., Repaske, D.R. and Swinnen, J.V. (1991) Endocr. Rev. 12, 218–234.
[3] Thompson, W.J. (1991) Pharmacol. Ther. 57, 13–33.
[4] Beavo, J.A., Conti, M. and Heaslip, R.J. (1994) Mol. Pharmacol. 46, 399–405.
[5] Manganiello, V.C., Murata, T., Taira, M., Degerman, E. and Belfrage, P. (1995) Arch. Biochem. Biophys. 322, 1–13.
[6] Manganiello, V.C., Smith, C.J., Degerman, E. and Belfrage, P. (1990) GMP-inhibited cyclic nucleotide phosphodiesterases. In: Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action (Beavo, J.A. and Houslay, M.D. eds.) pp. 87–117, Wiley Chichester.
[7] Degerman, E., Newman, A., Rice, K., Belfrage, P. and Manganiello, V.C. (1987) J. Biol. Chem. 162, 5797–5807.
[8] Harrison, S.A., Reifsnyder, D.H., Gallis, B., Cadd, G.G. and Beavo, J.A. (1986) Mol. Pharmacol. 29, 506–514.
[9] Pyne, N.J., Cooper, M.E. and Houslay, M.D. (1987) Biochem. J. 242, 33–42.
[10] Boyes, S. and Loten, E.G. (1988) Eur. J. Biochem. 174, 303–309.
[11] Rascon, A., Belfrage, P., Lindgren, S., Andersson, K.-E., Stavenow, L., Rice, K., Manganiello, V. and Degerman, E. (1992) Biochim. Biophys. Acta 1134, 149–156.
[12] Grant, P.G. and Colman, R.W. (1984) Biochemistry 23, 1801–1807.
[13] Degerman, E., Moos, M. Jr., Rascon, A., Vasta, V., Meacci, E., Smith, C.J., Lindgren, S., Andersson, K.-E., Belfrage, P. and Manganiello, V.C. (1993) Biochim. Biophys. Acta 1205, 189–198.
[14] Weishaar, R.E., Cain, M.H. and Bristol, A. (1985) J. Med. Chem. 28, 537–545.
[15] Robicsek, S.A., Blanchard, D.K., Djeu, J.Y., Krazanowski, J.J., Szentivanyi, A. and Polson, J. (1991) Biochem. Pharmacol. 42, 869–877.
[16] Souness, J.E., Hassall, G.A. and Parrott, D. (1992) Biochem. Pharmacol. 44, 857–866.
[17] Marcoz, P., Prigent, A.F., Lagarde, M. and Nemoz, G. (1993) Mol. Pharmacol. 44, 1027–1035.
[18] Pan, N., Arauz, E., Krazanowski, J.J., Fitzpatrick, D.F. and Polson, J.B. (1994) Biochem. Pharmacol. 48, 827–835.
[19] Manganiello, V.C., Taira, M., Degerman, E. and Belfrage, P. (1995) Cell. Signalling, 7, 445–455.
[20] Meacci, E., Taira, M., Moos, M. Jr., Smith, C.J., Movsesian, M., Degerman, E., Belfrage, P. and Manganiello, V.C. (1992) Proc. Natl. Acad. Sci. USA 89, 3721–3725.
[21] Taira, M., Hockman, S., Calvo, J., Taira, M., Belfrage, P. and Manganiello, V.C. (1993) J. Biol. Chem. 268, 18573–18579.
[22] Reinhardt, R.R., Chin, E., Zhou, J., Taira, M., Murata, T., Manganiello, V.C. and Bondy, C.A. (1995) J. Clin. Invest. 95, 1528–1538.
[23] Knowles, B.B., Howe, C.C. and Aden, D.P. (1980) Science 209, 497–499.
[24] Fogh, J. and Trempe, G. (1975) Human tumor cell lines. In: Human Tumor Cells in Vitro (Fogh, J. ed.) pp. 115–159, Plenum Press, New York.
[25] Nakabayashi, H., Taketa, K., Yamane, T., Miyazaki, M., Miyano, K. and Sato, J. (1984) Gann 75, 151–158.
[26] Nishiyama, M. and Wands, J.R. (1992) Biochem. Biophys. Res. Commun. 183, 280–285.
[27] Alvarez, R., Banerjee, G.L., Bruno, J.J., Jones, G.L., Littschwager, K., Strossberg, A. and Venuti, M.C. (1986) Mol. Pharmacol. 29, 554–560.
[28] Macphee, C.H., Harrison, S.H. and Beavo, J.A. (1986) Proc. Natl. Acad. Sci. USA 83, 6660–6663.

[29] Kono, T., Robinson, F.W. and Sarver, J.A. (1975) J. Biol. Chem. 250, 7826–7835.
[30] Boyes, S. and Loten, E.G. (1989) Biochem. Biophys. Res. Commun. 162, 814–820.
[31] Kaufman, R.F., Crowe, G., Utterback, B. and Robertson, D.W. (1987) Mol. Pharmacol. 30, 609–616.
[32] Kithas, P.A., Artman, M., Thompson, W.J. and Strada, S. (1989) J. Mol. Cell. Cardiol. 21, 507–517.
[33] Lugnier, C., Muller, P., Lebec, A., Beaudry, C. and Rousseau, E. (1993) J. Pharmacol. Exp. Ther. 265, 1142–1151.
[34] Smith, C.J., Krall, J., Manganiello, V.C. and Movsesian, M.A. (1993) Biochem. Biophys. Res. Commun. 190, 516–521.
[35] Cho-Chung, Y.S. (1993) Int. J. Oncol. 3, 141–148.
[36] Yokozaki, H., Budillon, A., Clair, T., Kelley, K., Corvan, K., Rohlff, C., Glazer, R.I. and Cho-Chung, Y.S. (1993) Int. J. Oncol. 3, 424–430.
[37] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 152–159.