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Cyclic AMP-like effects of polyamines on phosphatidylcholine synthesis and protein phosphorylation in human promyelocytic leukemia HL60 cells

Comparison with the effects of phorbol ester

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Spermine or putrescine increased cAMP levels through a catalase-sensitive mechanism, resulting in, most notably, a dephosphorylation of protein A (M_r 45000, p*I* 5.15) and protein B (M_r 45000, p*I* 4.9) and slightly increased phosphatidylcholine (PC) synthesis in HL60 cells. Exogenous dibutyryl cAMP mimicked the polyamine effects. 12-*O*-Tetradecanoyl phorbol-13-acetate (TPA) also promoted the protein dephosphorylation and PC synthesis, the effects augmented by R59022 and mimicked by exogenous 1-oleoyl-2-acetylglycerol. The effects of spermine (or dibutyryl cAMP) and TPA on PC synthesis were synergistic. It was suggested that cAMP-dependent protein kinase and protein kinase C might mediate, in an independent but interrelated manner, the effects of polyamines and TPA.

Polyamine; Phosphatidylcholine; cyclic AMP; Phosphorylation; Phorbol ester; (HL60 cell)

1. INTRODUCTION

Increases in ornithine decarboxylase activity and polyamine biosynthesis often accompany the onset of cell proliferation and differentiation (reviews [1-3]). Numerous polyamine effects on various cellular activities have been described [1-3], however none demonstrated a close relationship with the cAMP or Ca^{2+}/phospholipid-dependent PKC systems.

Human promyelocytic leukemia cell line HL60 is a good model to study cell differentiation since it can be induced to differentiate toward the monocytic pathway when treated with phorbol esters [4,5] or 1,25-dihydroxycholecalciferol [6,7] or along the granulocytic pathway when treated with dimethyl sulfoxide [8], butyric acid [8], hypoxanthine [8] and retinoic acid [9]. In addition to these agents, cAMP also promotes differentiation of HL60 cells [10,11].

During a study of the possible role of cAMP and polyamines in the phorbol ester-induced differentiation of HL60 cells, we observed significant elevation of cellular cAMP levels in the presence of spermine or putrescine, which was accompanied by

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; OAG, *sn*-1-oleoyl-2-acetylglycerol; db cAMP, dibutyryl cAMP; CAT, catalase; PC, phosphatidylcholine; A-PK, cAMP-dependent protein kinase

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apparently cAMP-mediated potentiation of the phorbol ester effect on PC synthesis as well as dephosphorylation of specific proteins. This paper is an account of these hitherto unrecognized, cAMP-related effects of polyamines which may be relevant to certain aspects of differentiation of HL60 cells.

2. MATERIALS AND METHODS

2.1. *Materials*

TPA and mezerein were purchased from Sigma; OAG from Avanti Polar Lipids; acrylamide from Kodak; compound R59022 from Janssen Life Sciences Product; ampholites (pH ranges 3.5–10 and 5–7) from LKB; [^{32}P]orthophosphate (carrier-free, in water) from ICN Radiochemicals; [*methyl*-^{14}C]choline chloride (50 mCi/nmol) and cAMP assay kit (code TRK 432) from Amersham.

2.2. *Cell culture*

The human promyelocytic leukemia cell line HL60 [12] was continuously cultured in RPMI 1640 medium (Gibco) supplemented with 20% heat-inactivated fetal calf serum, penicillin-streptomycin (50 U/ml and 50 μg/ml, respectively) and glutamine (2 mM). Cells were harvested for experiments at a density of 0.8–1.0 $\times$ 10^6/ml.

2.3. *Radiolabeling of cellular proteins and preparation of cell extracts*

Cells were first washed then incubated in fresh RPMI 1640 medium with $^{32}P_i$ (0.4 mCi/ml) at a density of 1 $\times$ 10^6/ml for 7 h. Aliquots (1 ml) of cells were transferred to small Eppendorf tubes and incubated for an additional 2 h with either of the following compounds; putrescine (0.5 mM), spermine (0.5 mM), db cAMP (0.5 mM) or 8-Br cAMP (0.5 mM). In other experiments cells were also treated for the last 45 min with TPA (20–500 nM), mezerein (100 nM) or OAG (25 μg/ml). Incorporations of $^{32}P_i$ were terminated by centrifuging the samples for 5 s at 10000 $\times$ g, aspirating supernatants and lysing cells with 50 μl gel buffer [13]. Stock solutions (10 mM) of TPA and mezerein were made up in dimethyl sulfoxide; the final concentration of this solvent never exceeded 0.1%, which had no detectable effect on cellular protein phosphorylation.

2.4. *Two-dimensional polyacrylamide gel electrophoresis*

The 2-dimensional gel electrophoresis procedure described by O'Farrell [14] was used, with modifications described by Steinberg and Coffino [13]. The first-dimension isoelectric focusing gels contained 9.2 M urea and 6% ampholites (1.5% ampholite, pH 3.5–10, and 4.5% ampholite, pH 5–7). Aliquots, corresponding to about 35 μg protein and containing 1.5 $\times$ 10^5 cpm (fig.1) or 2.0 $\times$ 10^5 cpm (fig.2) of acid-precipitable radioactivity, were loaded for gel electrophoresis. Radioactivity in various samples of the same experiment differed from each other by less than 10%. The first-dimension gels were run for 7000 V·h. The second-dimensional separation was accomplished using SDS-polyacrylamide gel electrophoresis. Proteins were stained with Coomassie blue. The ^{32}P-labelled proteins were detected by autoradiography of dried gels for 4 days at −20°C using Kodak XAR-5 film between Kodak Omatic intensifying screens. Gel patterns are shown with the acidic proteins to the right. Each experiment was performed at least three times to ensure the reproducibility of the findings.

2.5. *Measurement of radioactivity in specific proteins*

To quantitate the changes in ^{32}P content, after autoradiography of gels for 4 days, the respective proteins, as well as several reference proteins, were excised and counted for ^{32}P in a liquid scintillation spectrometer. Pieces of gel of identical sizes, corresponding to the appropriate proteins, were excised from radioactivity-free areas in the gels and served as a blank. The data given in the text are the mean values of three experiments with a maximal error of 15%.

2.6. *Measurement of PC synthesis in intact cells*

Cells were suspended in fresh RPMI 1640 medium and incubated with [*methyl*-^{14}C]choline chloride (0.75 μCi/ml) at a density of 2 $\times$ 10^6/ml for 3 h in the absence or presence of the agents listed in table 1. At the end of incubation, cells were pelleted and then phospholipids were extracted with a mixture of chloroform/methanol (2:1, v/v). PC was separated from other phospholipids by silica gel thin-layer chromatography and its ^{14}C content determined as in [15,16].

Table 1

Effects of spermine, db cAMP, TPA and R59022 on PC synthesis in HL60 cells

Addition	^{14}C-labeled PC (cpm)/10^6 cells per 3 h	
	None	TPA (200 nM)
None	2157 ± 28 (100)	3651 ± 49[a] (169)
db cAMP (0.5 mM)	2594 ± 85 (120)	5187 ± 129[b] (240)
Spermine (0.5 mM)	2462 ± 117 (114)	4944 ± 64[b] (229)
R59022 (25 μM)	2216 ± 46 (103)	4672 ± 80[b] (217)

[a] Significantly different from the control ($p < 0.05$)
[b] Significantly different from the value obtained with TPA alone ($p < 0.01$)

Cells (3×10^6/ml) were incubated with ^{14}C-labeled choline for 3 h as described in section 2. All agents were present during the entire incubation period. Data are means ± SE of three determinations. Similar results were obtained in two other separate experiments. The numbers in parentheses are percentages of the values compared to the control (no addition, no TPA), which was taken as 100%

2.7. *Determination of cAMP content in intact cells*

Cells were treated with spermine (0.5 mM), putrescine (0.5 mM), db cAMP (0.5 mM), 8-Br cAMP (0.5 mM) and TPA (100 nM) for 2 h or with OAG (25 μg/ml) for 45 min. CAT (50 μg/ml) was added 30 min before polyamines. Samples were deproteinized with ethanol and cAMP content was determined in triplicates using an Amersham cAMP assay kit according to Gilman [17].

3. RESULTS

It has been reported previously that TPA stimulates PC synthesis in HL60 cells [18,19]. We observed now that db cAMP (table 1), or the cAMP-elevating agent prostaglandin E_2 (not shown), potentiated the stimulatory effect of TPA on PC synthesis in these cells. Polyamines such as spermine (table 1) and putrescine (not shown) and the diacylglycerol kinase inhibitor R59022 [20] also potentiated the TPA effect (table 1). Neither of these agents had any significant effect on PC synthesis when present alone.

Table 2

Effects of polyamines, TPA and OAG on cAMP levels in HL60 cells incubated with or without CAT

Addition	cAMP (pmol)/10^7 cells	
	None	+ CAT (50 μg/ml)
Expt 1		
None	33.3 ± 1.9 (100)	33.9 ± 0.8 (102)
Spermine (0.5 mM)	88.4 ± 7.1[a] (265)	44.9 ± 4.9[b] (135)
Putrescine (0.5 mM)	93.7 ± 5.3[a] (281)	46.4 ± 2.8[b] (139)
TPA (200 nM)	40.8 ± 3.6 (123)	38.4 ± 2.1 (115)
Expt 2		
None	40.7 ± 3.4 (100)	ND
OAG (25 μg/ml)	51.3 ± 4.2 (100)	47.9 ± 53 (118)

[a] Significantly different from the control ($p < 0.05$–0.01)
[b] Significantly different from the respective values obtained in the absence of CAT ($p < 0.05$–0.01)

In both experiments, cells (3×10^6/ml) were preincubated for 30 min with or without CAT followed by incubation for 2 h (expt 1) or 45 min (expt 2) in the absence or presence of the agents, as indicated. Data are means ± SE of three determinations. Similar results were obtained in another experiment. The numbers in parentheses are the percentages of the values compared to the control (no addition, no CAT), which was taken as 100%. ND, not determined

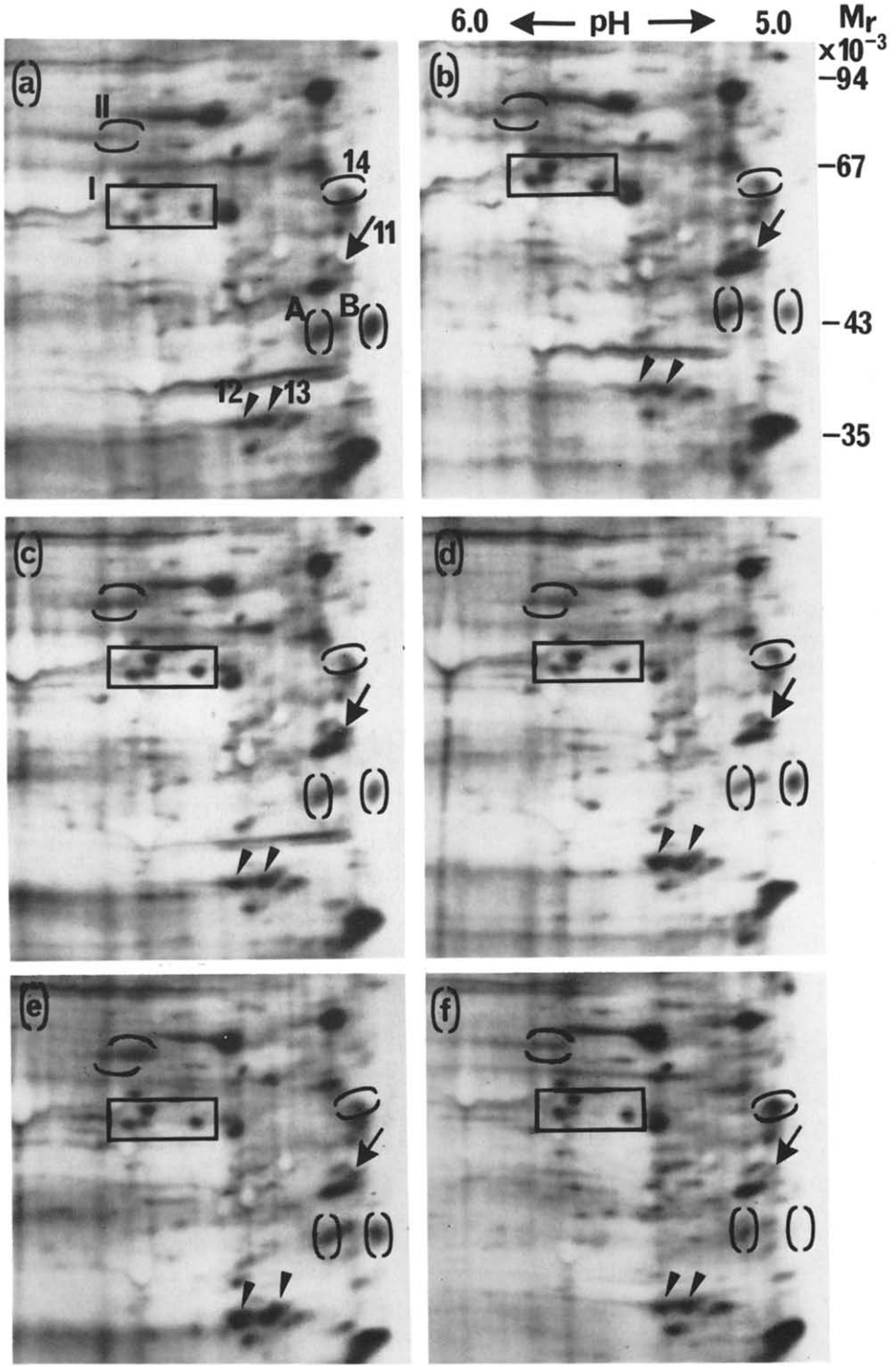


Fig.1. Effect of PKC activators on protein phosphorylation in HL60 cells. Cells were labeled with $^{32}P_i$ for a total period of 9 h without (a) or with 20 nM TPA (b), 100 nM TPA (c), 500 nM TPA (d), 100 nM mezerein (e) or 25 μg/ml OAG (f) present during the last 45 min of labeling period. About 1.5×10^5 cpm acid-precipitable radioactivity (about 35 μg protein) was loaded onto gels. Autoradiographs were exposed for 4 days as described in section 2.

Spermine or putrescine increased nearly 3-fold the cAMP levels in HL60 cells and this increase was effectively attenuated by CAT (table 2). TPA or OAG, in the presence or absence of CAT, in comparison, had no effect on the cAMP levels. The data from tables 1 and 2, when taken together, suggested that the potentiating effect of polyamines on the TPA-stimulated PC synthesis might be mediated by cAMP whose levels were in turn modulated by a catalase-sensitive (or H_2O_2-dependent) pathway.

Since most, if not all, of the cellular effects of TPA seem to be mediated by PKC activation [21,22], we investigated the possible interactions of cAMP, polyamines and TPA on PC synthesis at the level of protein phosphorylation. Compared to the control (fig.1a), 20 nM TPA (fig.1b) caused marked phosphorylation of several proteins in blocks I and II as well as that of protein 11. At a higher concentration (100 nM), TPA did not further increase phosphorylation of these proteins (fig.1c), but at 500 nM TPA further phosphorylation of proteins 12 and 13 was noted (fig.1d). In contrast, TPA at all concentrations tested inhibited phosphorylation of proteins A and B by 35 and 60%, respectively (fig.1, comparing a–d). The extents of inhibition reported in these and subsequent experiments were determined by measuring actual radioactivity in the individual proteins excised from the gels after the autoradiographs were developed.

Other PKC activators, mezerein at 100 nM (fig.1e) and OAG at 25 μg/ml (fig.1f), stimulated or inhibited the above proteins in a manner quite similar to that observed for TPA, with the following notable exceptions: OAG appeared to be most active in stimulating phosphorylation of protein 14 and inhibiting that of protein A (70%) and protein B (>90%). db cAMP (0.5 mM) did not stimulate phosphorylation of proteins in block I or any other proteins, but inhibited phosphorylation of proteins A and B by 30 and 90%, respectively (fig.2d). The effects of 8-Br cAMP (0.5 mM) were similar to but less pronounced than those of db cAMP (fig.2e). The cAMP-elevating prostaglandin E_2 mimicked the effects of the cAMP analogs (not shown), further supporting the role of cAMP or A-PK in protein dephosphorylation. R59022, without effects when present alone (not shown), potentiated the inhibitory effects of TPA on phosphorylation of proteins A and B (fig.2, comparing a, f and g), also suggesting a possible involvement of PKC in dephosphorylation of certain cellular proteins.

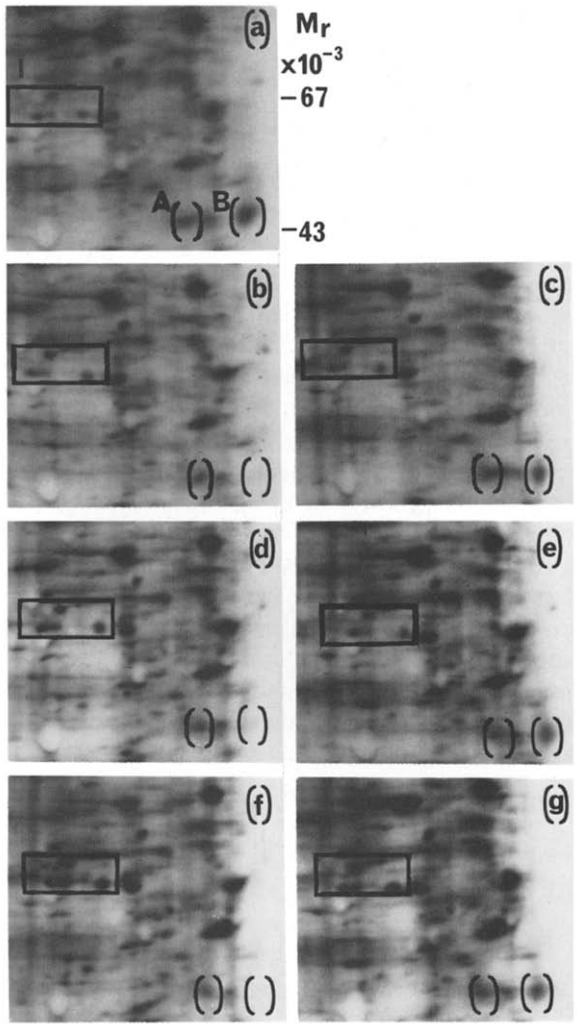
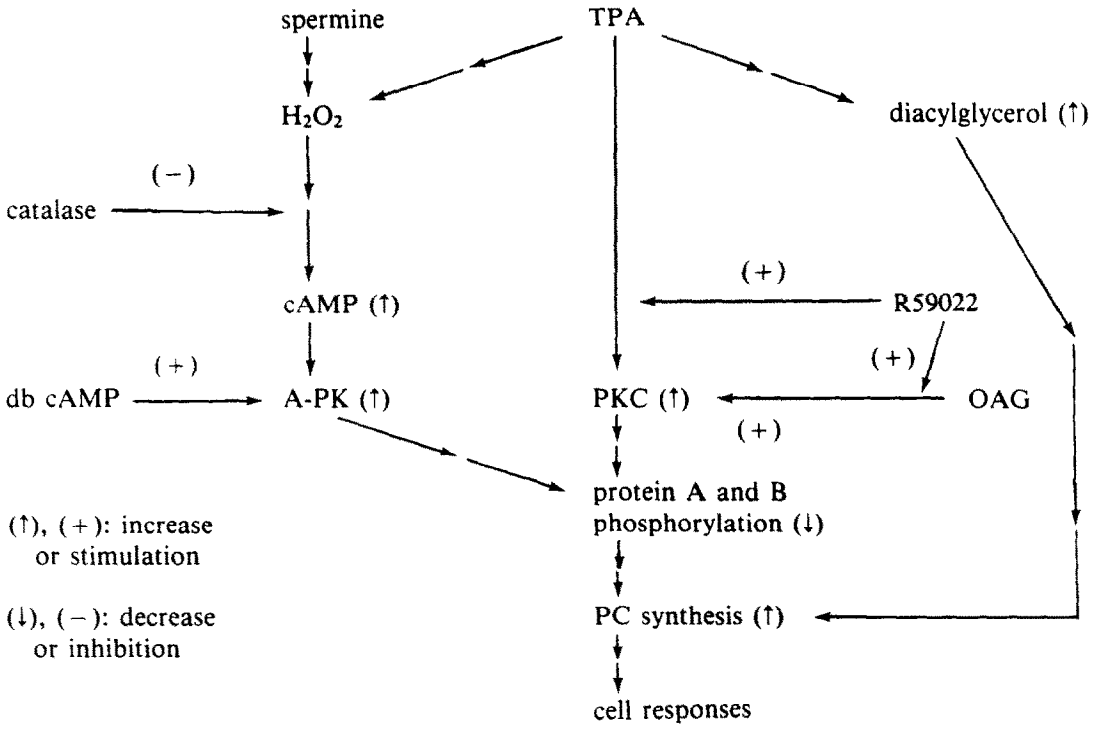


Fig.2. Comparison of the inhibitory effects of spermine, cAMP analogs and TPA on protein phosphorylation in HL60 cells. Cells were labeled with $^{32}P_i$ for a total period of 9 h without (a) or with 0.5 mM spermine (b), 0.5 mM spermine + 50 μg/ml of CAT (c), 0.5 mM db cAMP (d) or 0.5 mM 8-Br cAMP (e) present during the last 2 h, or with 500 nM TPA + 25 μM R59022 (f) or 500 nM TPA alone (g) present during the last 45 min of the labeling period. CAT and R59022 were added 30 min before spermine or TPA. About 2×10^5 cpm acid-precipitable radioactivity was loaded onto gels and autoradiographs were exposed for 4 days.

4. DISCUSSION

This study showed the hitherto unrecognized interactions among polyamines, cAMP and PKC on both protein phosphorylation/dephosphorylation and PC synthesis in HL60 cells. The common denominator for the stimulated PC synthesis elicited by spermine is likely mediated through H_2O_2-dependent cAMP formation, because CAT markedly attenuated the spermine effects and furthermore, exogenous db cAMP effectively substituted for spermine. Although the mechanism by which H_2O_2 or other active oxygen species stimulated cAMP formation is still unclear, there are previous studies reporting H_2O_2-mediated spermine effects in other tissues or cell lines [23,24]. Endogenous cAMP or exogenous db cAMP activates A-PK, which could promote dephosphorylation of proteins A and B by phosphorylating and activating certain phosphoprotein phosphatases. Alternatively, the regulatory subunit of A-PK can directly activate phosphatases, leading to dephosphorylation. It has been reported that the regulatory subunit of the enzyme can directly modulate certain phosphatases [25,27]. Our recent work (unpublished) on PC synthesis in HL60 cells indicates that, similarly to other cell types [28–31], TPA alone stimulated the breakdown of PC which in turn supplies the synthetic pathway with the rate-limiting substrate diacylglycerol. TPA also stimulates the formation of CDP-choline [32] and in HL60 cells this pathway is potentiated by cAMP (unpublished). It appears, therefore, that only if enough diacylglycerol is available will the increased formation of CDP-choline be reflected in increased PC synthesis. This is probably the reason why spermine and cAMP alone had a relatively small effect on PC synthesis.

The possible interplays of TPA, spermine, CAT and db cAMP in HL60 cells are depicted below. Protein dephosphorylation and at least one step of PC synthesis were regulated by two independent but interrelated pathways involving A-PK or PKC. A synergistic or potentiating effect observed for the combination of spermine (or db cAMP) and TPA (or R59022) on PC synthesis (table 1) probably best support this hypothesis. Identification of the functional properties of proteins A and B would be required to elucidate the complex cellular events evoked by polyamines and/or TPA.



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REFERENCES

[1] Janne, J., Poso, H. and Raina, A. (1978) Biochim. Biophys. Acta 473, 241–293.
[2] Pegg, A.E. and McCann, P.P. (1982) Am. J. Physiol. 243, C212–C221.
[3] Tabor, C.W. and Tabor, H. (1984) Annu. Rev. Biochem. 53, 749–790.
[4] Rovera, G., Santoli, D. and Damsky, C. (1979) Proc. Natl. Acad. Sci. USA 76, 2779–2784.
[5] Huberman, E. and Callaham, M. (1979) Proc. Natl. Acad. Sci. USA 76, 1293–1297.
[6] Miyaura, C., Abe, E., Kuribyashi, T., Tanaka, H., Konno, K., Nishii, Y. and Suda, T. (1981) Biochem. Biophys. Res. Commun. 102, 937–943.
[7] Tanaka, H., Abe, E., Miyaura, C., Kuribayashi, T., Konno, K., Nishii, Y. and Suda, T. (1982) Biochem. J. 204, 713–719.
[8] Collins, S.J., Bodner, A., Ting, R. and Gallo, R.C. (1980) Int. J. Cancer 25, 213–288.
[9] Breitmen, T.R., Selonick, S.E. and Collins, S.J. (1980) Proc. Natl. Acad. Sci. USA 77, 2936–2940.
[10] Ollson, I.L., Breitman, T.R. and Gallo, R.C. (1982) Cancer Res. 42, 3928–3933.
[11] Chaplinski, T.J. and Niedel, J.E. (1982) J. Clin. Invest. 70, 953–964.
[12] Collins, S.J., Gallo, R.C. and Gallagher, R.E. (1977) Nature 270, 347–349.
[13] Steinberg, R.A. and Coffino, P. (1979) Cell 8, 719–733.
[14] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[15] Kiss, Z. and Farkas, T. (1975) Biochem. Pharmacol. 24, 999–1002.
[16] Kiss, Z. (1976) Eur. J. Biochem. 67, 557–561.
[17] Gilman, A.G. (1970) Proc. Natl. Acad. Sci. USA 67, 305–312.
[18] Cassileth, P.A., Suholet, D. and Cooper, R.A. (1981) Blood 58, 237–243.
[19] Cooper, R.A., Braunwald, A.D. and Kuo, A.L. (1982) Proc. Natl. Acad. Sci. USA 79, 2865–2869.
[20] Courcelles, B.C., Roevens, P. and Van Belle, H. (1985) J. Biol. Chem. 260, 15762–15770.
[21] Nishizuka, Y. (1986) Science 233, 305–312.
[22] Anderson, W.B. and Salomon, D.S. (1985) in: Phospholipids and Cellular Regulation (Kuo, J.F. ed.) vol.2, pp.127–170, CRC Press, Boca Raton.
[23] Livingston, J.N., Gurney, P.A. and Lockwood, A.H. (1977) J. Biol. Chem. 252, 560–562.
[24] Casciery, M.A., Mumford, R.A. and Katzen, H.M. (1979) Arch. Biochem. Biophys. 195, 30–34.
[25] Gergely, P. and Bot, G. (1977) FEBS Lett. 82, 269–272.
[26] Khatra, B., Printz, R., Cobb, C.E. and Corbin, J.D. (1985) Biochem. Biophys. Res. Commun. 130, 567–573.
[27] Jurgensen, S.R., Chock, P.B., Taylor, S., Vandenheede, J.R. and Merlevede, W. (1985) Proc. Natl. Acad. Sci. USA 82, 7565–7569.
[28] Mufson, R.A., Okin, E. and Weinstein, I.B. (1981) Carcinogenesis 2, 1095–1102.
[29] Guy, G.R. and Murray, A.W. (1982) Cancer Res. 42, 1980–1985.
[30] Grove, R.I. and Schimmel, S.D. (1982) Biochim. Biophys. Acta 711, 272–280.
[31] Daniel, L.W., Waite, M. and Wykle, R.L. (1986) J. Biol. Chem. 261, 9128–9132.
[32] Paddon, H.B. and Vance, D.E. (1980) Biochim. Biophys. Acta 620, 636–640.