LLC-PK$_1$ cell mutants in cAMP metabolism respond normally to phorbol esters

David A. Jans and Brian A. Hemmings

Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland

Received 17 June 1986

Mutants of the pig kidney cell line, LLC-PK$_1$, affected in cAMP metabolism, were examined for cAMP-dependent protein kinase (cAMP-PK) activity and for cAMP-mediated induction of urokinase-type plasminogen activator (uPA). The FIB4 and FIB6 mutant cell lines possessed about 10% parental levels of cAMP-PK activity and concomitantly reduced uPA production (10–20% parental) in response to calcitonin, forskolin and 8-bromo cAMP. The FIB1, FIB2 and FIB5 mutant cell lines had about 70% parental levels of cAMP-PK and the synthesis of uPA was 40–60% parental. Thus, cAMP-mediated induction of uPA showed a dependence on the absolute levels of cAMP-PK. However, uPA synthesis in response to phorbol-12-myristate-13-acetate by all of the mutants was similar to parental, which indicates that enzyme induction mediated by phorbol esters does not involve cAMP or cAMP-PK.

cyclic AMP dependence *Urokinase-type plasminogen activator* *Enzyme induction* *Protein kinase* *Somatic cell mutant*

1. INTRODUCTION

All available evidence [1–3] suggests that the profound effects of adenosine 3′,5′-monophosphate (cAMP) on cellular processes are exerted through phosphorylation of a variety of specific proteins, mediated by cAMP-dependent protien kinase (cAMP-PK) [4]. Among these effects include specific induction of a number of enzymes, including ornithine decarboxylase in glioma and neuroblastoma cells [5], hepatic tyrosine aminotransferase [6], cAMP phosphodiesterase in C-6 glioma cells [7], alkaline phosphatase in mouse L-cells [8], hepatic phospho*enol*pyruvate carboxykinase [9], and urokinase-type plasminogen activator (uPA) in LLC-PK$_1$ cells [10,11].

Here, the isolation of LLC-PK$_1$ mutant cell lines defective in cAMP metabolism is described. These were characterized in terms of cAMP-PK activity, and used to address the question of the role of the cAMP-PK in uPA induction. A direct correlation between cAMP-PK levels and uPA inducibility by cAMP effectors was observed. Significantly, the induction of uPA by phorbol esters [12,13] was unaffected in all of the mutants, indicating that cAMP and cAMP-PK are not involved in the induction of uPA by phorbol esters.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Pig epithelial cells, LLC-PK$_1$ [14] and mutants were cultured in plastic petri dishes in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum at 37°C. For hormonal treatments or addition of drugs, monolayer cultures were washed with serum-free DMEM and then incubated in serum-free DMEM containing the appropriate hormone or drug. Forskolin (Calbiochem) and isobutylmethylxanthine (IBMX, Sigma) were stored at −20°C as stocks of 10 and 25 mM solutions, respectively, in absolute ethanol, and appropriately diluted into media.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2.2. *Enzyme assays*

The catalytic activity of cAMP-PK was assayed using the synthetic peptide Leu-Arg-Arg-Ala-Ser-Ala-Gly (Kemptide, Bachem), essentially as in [15,16]. Protein kinase activity is expressed as units per mg protein (1 unit kinase activity is defined as the amount of enzyme that catalyzes the transfer of 1 nmol phosphate from ATP to the peptide per min). cAMP-PK regulatory subunit activity was quantitated using the Millipore filtration cAMP binding assay [17]. Plasminogen activator activity was determined by measuring the plasminogen-dependent hydrolysis of the synthetic peptide S-2251 (D-Val-Leu-Lys-*p*-nitroanilide, Bachem) and the activities are expressed in Ploug Units per mg cellular protein using purified urokinase (EC 3.4.4.a, Calbiochem) as standard. Media to be assayed were serially diluted to constant specific activity. Human plasminogen was purified as in [18,19]. Protein concentrations were measured according to Bradford [20] with bovine serum albumin as standard. Materials and chemicals were from standard sources as described [21].

Cell extracts were prepared in 20 mM 2-(*N*-morpholino)ethanesulphonate-NaOH, pH 6.8, containing 150 mM NaCl, 1 mM EDTA, and 0.2% (v/v) Triton X-100. For the cAMP binding assay the Triton X-100 was omitted.

3. RESULTS

3.1. *Isolation of 'cAMP resistant' cell lines*

It was decided to generate mutant cell lines affected in cAMP metabolism using the phosphodiesterase (PDE) inhibitor IBMX [22] together with the adenylate cyclase (AC) activator forskolin [23]. Each had been shown in preliminary experiments to be cytotoxic. The combined use of these two reagents, elevating intracellular cAMP levels via stimulation of AC and by inhibition of PDE, was expected to bias the resistant cell population in favour of mutants affected at a point in cAMP metabolism distal to cAMP synthesis and degradation, such as cAMP-PK. Towards this end, LLC-PK_1 cells were seeded at 10^6/10 cm dish in DMEM containing 0.5 mM IBMX and 50 μM forskolin. Cells were grown for 3 weeks, passaged, grown for a further 3 weeks, and passaged and seeded into the same medium, except that the IBMX concentration was raised to 1 mM on the final seeding. Colonies began to form after a further 3 weeks and clonal cell lines (the FIB cell lines) derived.

The clonal cell lines were removed from selective media and maintained in DMEM. After 8 weeks, the FIB cell lines were returned to selective media to ascertain whether the resistance phenotype had reverted in the absence of selection. No significant reduction in cell viability was observed, and the basis for resistance of all cell lines was assumed to be genetic.

3.2. *cAMP-PK of mutants*

The cAMP-PK levels of cell extracts from five of the isolated mutant cell lines were assayed (table 1). Two distinct classes of mutants appeared. FIB1, FIB2 and FIB5 exhibited approx. 70% parental cAMP-PK activity, whereas FIB4 and FIB6 had very low cAMP-PK activity (<0.4 U/mg). In contrast to having altered cAMP-PK catalytic activities, all the variant cell lines tested appeared to be largely comparable to parental in terms of cAMP-binding (regulatory subunit) activities (table 1).

3.3. *uPA induction in mutants*

As the five mutant cell lines represented differing phenotypes with respect to cAMP-PK, it was possible to use them to investigate the link between cAMP-PK and uPA induction. Salmon calcitonin, forskolin and the cAMP analogue 8-bromo cAMP (BrcAMP) can all be used to stimulate uPA induction in LLC-PK_1 cells [10,11], as can phorbol-12-myristate-13-acetate (PMA) [24], which is believed to induce uPA via a different but convergent pathway utilising the C-kinase ([24] and see below). Cells were induced for 8 h with hormonal effectors in DMEM lacking serum, prior to assay of uPA levels in the culture media (table 2).

Mutant cell lines FIB1, FIB2 and FIB5 were similar in that each produced levels of uPA in response to calcitonin 40–60% of parental. Identical results were obtained with the polypeptide hormones vasopressin and human calcitonin (not shown). FIB1 responded similarly to forskolin (60% parental response) whereas FIB2 and FIB5 responded to a lesser extent (about 30% parental). The FIB4 and FIB6 lines appeared similar in that

Table 1

cAMP-PK levels and cAMP-binding activities in extracts from LLC-PK$_1$ mutant cell lines

Cell line	cAMP-PK activity (units/mg)	cAMP-binding activity ([^{3}H]cAMP/mg)
LLC-PK$_1$	3.03 ± 0.35	8.00 ± 0.28
FIB1	2.11 ± 0.11	ND
FIB2	2.00 ± 0.20	7.07 ± 0.08
FIB4	0.41 ± 0.05	5.86 ± 0.05
FIB5	2.10 ± 0.19	5.62 ± 0.10
FIB6	0.39 ± 0.07	7.26 ± 0.23

ND, not determined; activities are expressed ± SE of the mean

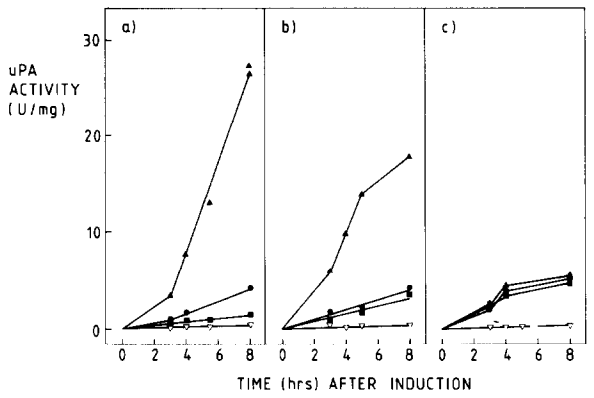

Fig.1. Time course of uPA production in response to the inducers 30 nM salmon calcitonin (a), 1 mM BrcAMP (b) and 160 nM PMA (c) in LLC-PK1 cells and mutants. (▲—▲) LLC-PK1, (●—●) FIB4, (■—■) FIB6, (▽—▽) control cells with no addition. Measurements were performed as in the legend to table 2, with duplicate plates sampled at the time intervals indicated.

they displayed about 10% parental uPA production in response to calcitonin and forskolin. These cells were studied in more detail, and the time course of uPA production was examined in response to calcitonin and to BrcAMP (which stimulates cAMP-PK directly). The results (fig.1) supported the above findings of severely reduced uPA induction. The responses of all FIB mutants to PMA were similar and comparable to parental (table 2, fig.1).

4. DISCUSSION

Our results support the concept of the direct relationship between cAMP-PK levels and induction of the enzyme uPA in LLC-PK$_1$ cells. cAMP-PK levels, and their response to cAMP-mediated hormonal stimulation in terms of uPA production is about 40–60% parental. The pivotal role of cAMP-PK in uPA induction by agents stimulating the cAMP cascade is clearly demonstrated. As the mutant cell lines possessed levels of R subunit activity comparable to parental (table 1), it can be concluded that it is the cAMP-PK catalytic activity (C subunit) that directly mediates uPA induction.

Table 2

uPA induction in mutant cell lines of LLC-PK1

Cell line	uPA activity (U/mg)			
	–	SCT	FSK	PMA
LLC-PK1	0.26 ± 0.04	30.8 ± 2.3	22.3 ± 1.2	4.9 ± 0.6
FIB1	0.19 ± 0.02	13.4 ± 1.1	14.2 ± 3.9	5.2 ± 2.2
FIB2	0.10 ± 0.05	16.4 ± 5.2	6.2 ± 0.7	4.4 ± 0.8
FIB4	0.30 ± 0.17	3.5 ± 0.5	2.4 ± 0.5	5.1 ± 0.4
FIB5	0.31 ± 0.12	15.5 ± 4.5	6.2 ± 0.3	4.9 ± 0.7
FIB6	0.46 ± 0.19	0.55 ± 0.1	2.0 ± 0.3	4.4 ± 0.1

Cells were grown to confluency in DMEM, washed four times, treated with reagents for 8 h in serum-free DMEM (0.5 mg/ml bovine serum albumin) and the conditioned medium sampled and assayed. SCT, salmon calcitonin (30 nM); FSK, forskolin (100 μM); PMA (160 nM)

Through the examination of mutants affected in cAMP-PK activity, it appears that the relative amount of cAMP-PK activity determines the extent of enzyme induction. The mutants FIB4 and FIB6 provide the clearest evidence. They possess about 10% of parental levels of cAMP-PK catalytic-subunit (C-subunit) activity and also are concomitantly severely reduced in uPA production in response to cAMP-mediated stimuli (calcitonin, forskolin, and BrcAMP). Similarly, FIB1, FIB2 and FIB5 appear to have about 70% of normal

The mutants FIB4 and FIB6 appear to be affected specifically in C-subunit activity. they possess an apparent structural mutation in the C-subunit protein are present. The residual kinase present in FIB4 and FIB6 exhibits parental levels of activation by cAMP and normal affinities for substrates ATP and Kemptide, but has about 10% parental V_{max}. In contrast, the 'cAMP resistant' mutants FIB1, FIB2 and FIB5 have only somewhat reduced C activity, and other data (Jans, D., Resink, T. and Hemmings, B., in preparation) show that they are affected in another component of cAMP metabolism, most probably adenylate cyclase.

The role of cAMP, and in particular cAMP-PK, in the induction of enzymes by phorbol esters, has been the subject of much experimentation (e.g. ornithine decarboxylase production in CHO cells [25–28]). The results here support those of others [28], in that PMA stimulated uPA induction appeared to be normal in all mutant cell lines affected in the cAMP cascade. The most conclusive demonstration of this is provided by the cell lines FIB4 and FIB6, which lack cAMP-PK catalytic activity and yet produce normal amounts of uPA in response to phorbol esters (table 2, fig.1). It appears that cAMP and cAMP-PK play no direct role in PMA-mediated (C-kinase) uPA induction.

In conclusion, the data presented here as well as elsewhere [24,28] suggest that the cAMP-PK and C-kinase are able to induce transcription of the uPA gene using either independent pathways, or a pathway that converges at a 'post-kinase' step. That uPA induction by the C-kinase pathway is quantitatively and qualitatively different from that of the cAMP-PK ([24], and this paper) suggests that the two pathways may utilise different transacting elements (or are subject to different desensitization/downregulation mechanisms [24]). The basic mechanism is likely to be similar in both, however: activation of kinase leads to phosphorylation of a trans-acting element, which in the phosphorylated form can induce transcription of the uPA gene. Thus, the rate of transcription of the uPA gene would be determined by the relative kinase and phosphatase activities, which determine the phosphorylation state of the trans-acting element.

ACKNOWLEDGEMENTS

The authors thank Manuela Schwarz for invaluable technical assistance, Jon de la Harpe for setting up the assay for uPA, and Yoshikuni Nagamine, Brian R. Rudkin and Michael S. Altus for reading the manuscript and providing helpful suggestions.

REFERENCES

[1] Pastan, I.H., Johnson, G.S. and Anderson, W.B. (1975) Annu. Rev. Biochem. 44, 491–523.
[2] Steinberg, R.A. (1983) Biochemical Actions of Hormones (Litwack, G. ed.) vol. 11, pp. 25–65, Academic Press, New York.
[3] Lohmann, S.M. and Walter, U. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 18, 63–117.
[4] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–939.
[5] Bachrach, U. (1975) Proc. Natl. Acad. Sci. USA 72, 3087–3091.
[6] Ernest, M.J. and Feigelson, P. (1978) J. Biol. Chem. 253, 319–322.
[7] Schwartz, J.P. and Passoneau, J.V. (1974) Proc. Natl. Acad. Sci. USA 71, 3844–3848.
[8] Firestone, G.L. and Heath, E.C. (1981) J. Biol. Chem. 256, 1396–1403.
[9] Beale, E.G., Hartley, J.L. and Granner, D.K. (1982) J. Biol. Chem. 257, 2022–2028.
[10] Dayer, J.-M., Vassalli, J.-D., Bobbitt, J.L., Hull, R.N., Reich, E. and Krane, S.M. (1981) J. Cell Biol. 91, 195–200.
[11] Nagamine, Y., Sudol, M. and Reich, E. (1983) Cell 32, 1181–1190.
[12] Wigler, M. and Weinstein, I.B. (1976) Nature 259, 232–233.
[13] Miskin, R., Easton, T.G. and Reich, E. (1978) Cell 15, 1301–1312.
[14] Hull, R.N., Cherry, W.R. and Weaver, G.W. (1976) In Vitro, 12, 670–677.
[15] Roskoski, R. (1983) Methods Enzymol. 99, 3–6.

[16] Hemmings, B.A. (1985) Curr. Top. Cell. Regul. 27, 117-132.
[17] Gilman, A.G. (1970) Proc. Natl. Acad. Sci. USA 67, 305-312.
[18] Deutsch, D. and Mertz, E.T. (1970) Science 170, 1095-1096.
[19] Goldberg, A.R. (1974) Cell 2, 95-102.
[20] Bradford, W.M. (1968) Anal. Biochem. 72, 248-249.
[21] Jans, D.A., Resink, T.J., Wilson, L.E., Reich, E. and Hemmings, B.A. (1986) Eur. J. Biochem., submitted.
[22] Beavo, J.A., Rogers, N.L., Crofford, O.B., Hardman, J.G., Sutherland, E.W. and Newman, E.V. (1970) Mol. Pharmacol. 6, 597-603.
[23] Seamon, K.B. and Daly, J.W. (1981) J. Biol. Chem. 256, 9799-9801.
[24] Degen, J.D., Estensen, R.D., Nagamine, Y. and Reich, E. (1985) J. Biol. Chem. 260, 12426-12433.
[25] Mufson, R.A., Astrup, E.G., Simsiman, R.C. and Boutwell, R.K. (1977) Proc. Natl. Acad. Sci. USA 74, 657-661.
[26] Perchellet, J.-P. and Boutwell, R.K. (1980) Cancer Res. 40, 2653-2660.
[27] Perchellet, J.-P. and Boutwell, R.K. (1981) Cancer Res. 41, 3918-3926.
[28] Trevillyan, J.M. and Byus, C.V. (1983) Biochim. Biophys. Acta 762, 187-197.