Dependence of urokinase-type-plasminogen-activator induction on cyclic AMP-dependent protein kinase activation in LLC-PK₁ cells

David A. JANS,* Therese J. RESINK† and Brian A. HEMMINGS*

*Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, and †Department of Research, University Hospital, CH-4054 Basel, Switzerland

The activation of cyclic AMP-dependent protein kinase (cAMP-PK) *in vivo* was studied in LLC-PK₁ pig kidney cells and the mutant cell lines M18 and FIB5, which have total levels of cAMP-PK catalytic-subunit and regulatory-subunit activities comparable with those of parental cells. The extent of cAMP-PK activation (release of active catalytic subunit from the holoenzyme) was directly correlated with the cellular cyclic AMP concentration in LLC-PK₁ cells. In LLC-PK₁ cells, as well as in the mutants M18 and FIB5, the extent of the induction of urokinase-type plasminogen activator (uPA) by the cyclic AMP-mediated effectors calcitonin, vasopressin and forskolin was directly correlated with the levels of activated catalytic subunit. The 'receptorless' mutant M18, which is impaired in calcitonin- and vasopressin-receptor function, did not show any activation of cAMP-PK or uPA production in response to either hormone, whereas cAMP-PK and uPA responses to forskolin were about 35% higher than in parental cells. Analysis of the FIB5-cell line revealed a lesion affecting the regulation of adenylate cyclase activity, whereby basal and stimulated (both receptor- and non-receptor-mediated) adenylate cyclase levels were less than 36% of those in parental cells. The activation of cAMP-PK in response to cyclic AMP effectors was similarly reduced, and uPA induction was concomitantly lower than that in parental cells. The results demonstrate the dependence of uPA induction by cyclic AMP effectors on dissociation of the cAMP-PK holoenzyme, implying the importance of activated free cAMP-PK catalytic subunit in this process. Thus it is concluded that the mutations in the cellular cyclic AMP-generating apparatus of the M18 and FIB5 cell lines impair uPA induction by preventing cAMP-PK activation.

INTRODUCTION

Hormone-stimulated elevation of cyclic AMP is a key element in control of cellular processes (Steinberg, 1983; Walter *et al.*, 1979). Both long- and short-term effects of cyclic AMP appear to be exerted directly through cyclic AMP-dependent protein kinase (cAMP-PK) (Krebs & Beavo, 1979; Carlson *et al.*, 1979). The cAMP-PK exists as an inactive holoenzyme of two regulatory (R) and two catalytic (C) subunits (Carlson *et al.*, 1979). Upon cyclic AMP binding by the R subunits, the R₂C₂ complex dissociates to yield catalytically active C subunit (Bechtel *et al.*, 1977; Flockhart & Corbin, 1982). The profound effects of cyclic AMP on cellular processes are mediated, at least in part, via switches in transcription of specific genes, including those coding for ornithine decarboxylase (Bachrach, 1975), tyrosine aminotransferase (Ernest & Feigelson, 1978), alkaline phosphatase (Firestone & Heath, 1981) and urokinase-type plasminogen activator (uPA) (Dayer *et al.*, 1981; Nagamine *et al.*, 1983). The integral role of cAMP-PK in some of these processes has been demonstrated (Trevillyan & Byus, 1983; Jans & Hemmings, 1986), and surmised upon (Nagamine & Reich, 1985).

The precise role of cAMP-PK in cellular metabolism can be dissected by using mutant cells affected in cyclic AMP regulation and responsiveness (Gottesman *et al.*, 1980; Steinberg, 1983; Trevillyan & Byus, 1983; Jans & Hemmings, 1986). One approach is to study mutant cell lines that possess essentially the same total cAMP-PK

activities but are affected elsewhere in cyclic AMP metabolism. In the present study the LLC-PK₁ pig kidney cell line and its mutant derivatives, M18 (Jans *et al.*, 1986) and FIB5 (Jans & Hemmings, 1986), were examined. These three cell lines possess similar cAMP-PK regulatory and catalytic activities, but exhibit qualitative and quantitative differences in uPA production in response to various cyclic AMP agonists (Jans & Hemmings, 1986; Jans *et al.*, 1986; see also below). The experimental data presented here support the hypothesis that activation of cAMP-PK, and more specifically perhaps, the intracellular level of free cAMP-PK catalytic subunit, is the prime determinant of induction of uPA.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was from GIBCO (Grand Island, NY, U.S.A.) and fetal-calf serum from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Synthetic salmon calcitonin and [Arg⁸]vasopressin were from Peninsular (Palo Alto, CA, U.S.A.). ATP, cyclic AMP, the synthetic peptide Kemptide (Leu-Arg-Arg-Ala-Ser-Ala-Gly), bovine serum albumin (fatty-acid-free, fraction V) and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5'-[γ-³²P]ATP was obtained from Amersham International (Amersham, Bucks., U.K.),

Abbreviations used: cAMP-PK, cyclic AMP-dependent protein kinase; uPA, urokinase-type plasminogen activator; IBMX, isobutylmethylxanthine; p[NH]ppG, guanosine 5'-[βγ-imido]triphosphate.

phosphocellulose paper (P-81) from Whatman (Maidstone, Kent, U.K.), and the colorimetric substrate S-2251 (D-Val-Leu-L-Lys *p*-nitroanilide) from Bachem AG (Bubendorf, Switzerland). All other reagents were of the highest quality commercially available.

Cell culture

The parental LLC-PK₁ pig kidney epithelial cell line (Hull *et al.*, 1976) and mutant cell lines were cultured in DMEM supplemented with 10% (v/v) fetal-calf serum, streptomycin (0.2 mg/ml) and penicillin (50 units/ml) in a humidified incubator at 37 °C under an air/CO₂ (19:1) atmosphere.

Enzyme assays

Extracts for the assay of cAMP-PK catalytic activity were prepared and assayed as described previously (Hemmings, 1985; 1986; Jans & Hemmings, 1986; Jans *et al.*, 1986) with Kemptide as substrate. The assay is a modification of that described by Glass *et al.* (1978). Protein kinase activity is expressed as units/mg of protein (1 unit of kinase activity is defined as the amount of enzyme that catalyses the transfer of 1 nmol of phosphate from ATP to the peptide/min at 30 °C). Phosphotransferase activity in the absence of the substrate Kemptide was found to be negligible in LLC-PK₁ cells.

The cAMP-PK activity ratio expresses the catalytic subunit activity present in cell extracts (assayed in the absence of cyclic AMP) relative to the total stimutable activity (assayed in the presence of cyclic AMP) (Devis *et al.*, 1985; Hemmings, 1985, 1986; Jans *et al.*, 1986). The ratio gives a direct measure of the extent of cAMP-PK activation produced by treatment with different cyclic AMP agonists and has been shown to reflect the level of cyclic AMP in a number of tissues (Keely *et al.*, 1975). The use of Kemptide as substrate enables very small changes in the activity ratio to be determined, since the cAMP-PK from LLC-PK₁ cells is about 15 times more active with this substrate than with histone (B. A. Hemmings, unpublished work). Unlike histone (Corbin *et al.*, 1975), Kemptide promotes no dissociation of the holoenzyme. Stabilization of the partially dissociated cAMP-PK in cell extracts is ensured by the presence of 0.15 M-NaCl in the extract buffer and by maintaining cell extracts at 4 °C (Corbin *et al.*, 1975). Though all ratio measurements were made within 60 min of preparation of cell extracts, activity ratios were observed to remain constant up to 4 h after extract preparation, with a slight increase in the ratio detectable after overnight incubation at 4 °C. The inclusion of charcoal (10 mg/ml) in the extraction buffer, as recommended by Corbin *et al.* (1975) and Corbin (1983), was not observed to alter the activity ratio significantly and hence was not routinely used. Hormone- or agonist-induced cAMP-PK activity could be completely inhibited by the inclusion of 100 nM protein kinase inhibitor peptide 5-24 (Scott *et al.*, 1986) in the assay mix. Extracts for the measurement of adenylate cyclase (EC 4.6.1.1) activity were prepared and assayed as described previously (Jans *et al.*, 1986; Resink *et al.*, 1986), using a modification of the method of Salomon (1979).

Cells to be assayed for uPA induction were treated as described previously (Jans & Hemmings, 1986; Jans *et al.*, 1986). uPA activity was measured as outlined previously (Jans & Hemmings, 1986; Jans *et al.*, 1986)

with substrate S-2251 in a coupled assay with human plasminogen (prepared by affinity chromatography on lysine-Sepharose; Deutsch & Mertz, 1970; Goldberg, 1974). Human urokinase (EC 3.4.21.31, Calbiochem) was used as standard, with uPA activities expressed as Ploug units/mg of cellular protein. Protein was measured by the dye-binding assay of Bradford (1976), with bovine serum albumin (fatty-acid-free) as standard.

Cyclic AMP estimation

Monolayers of cells for cyclic AMP determination (90–95% confluency) were washed four times with serum-free DMEM, before a 5 min treatment with hormones or other agents. Cells were then washed five times with phosphate-buffered saline (140 mM-NaCl/1.5 mM-KH₂PO₄/2.8 mM-KCl/8 mM-Na₂HPO₄, pH 7.2) followed by lysis with boiling 0.5 mM-sodium acetate, pH 6.2, containing 0.2 mM-IBMX (Steinberg *et al.*, 1979). Samples were boiled for 3 min and maintained on ice until assayed for cyclic AMP. Cyclic AMP levels were measured by using the competitive-protein-binding assay kit from Amersham International, as described by Tovey *et al.* (1974).

RESULTS

Cyclic AMP production and cAMP-PK activation in LLC-PK₁ cells

The cell line LLC-PK₁ and its mutant derivatives M18 and FIB5 have already been characterized with respect to cAMP-PK levels, with the finding that all three possess essentially similar catalytic- and regulatory-subunit activities (Jans & Hemmings, 1986; Jans *et al.*, 1986). Preliminary observations on uPA induction in response to cyclic AMP effectors (Jans & Hemmings, 1986; Jans *et al.*, 1986) indicated that, despite similar levels of cAMP-PK activity, these cell lines exhibited rather different patterns of uPA induction by different cyclic AMP agonists. To investigate the role of cAMP-PK activity in the uPA-induction process (Jans & Hemmings, 1986), cAMP-PK activation was examined in the mutant cell lines in more detail. The cAMP-PK activity ratio (determined as described in the Materials and methods section) gives an index of the extent of agonist-induced cAMP-PK activation, i.e. release of C subunit from the holoenzyme (Devis *et al.*, 1985; Hemmings, 1985, 1986), and has been shown in other studies to reflect the level of cyclic AMP in a variety of tissues (Keely *et al.*, 1975). Though possibly not a strictly quantitative measure of cellular events, as discussed by Soderling *et al.* (1974) and Corbin (1983), it is assumed that the activity ratio measures events intracellularly rather than during the preparation of cell extracts (Corbin *et al.*, 1975; see also below).

To confirm that changes of the cAMP-PK activity ratio did indeed reflect elevation of intracellular cyclic AMP levels, concurrent series of cultures of LLC-PK₁ cells were assayed for both cyclic AMP concentration and cAMP-PK activation after treatment with cyclic AMP agonists. The hormones vasopressin and calcitonin, the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor IBMX were used to elevate intracellular cyclic AMP levels (Table 1). Measurements were performed after 5 min treatment with agonists, since cyclic AMP levels peak at this time (results not shown; Steinberg *et al.*, 1979). IBMX alone produced

Table 1. Cyclic AMP production and cAMP-PK activation in LLC-PK₁ cells and mutant derivatives

(a) Cells were grown to 90–95% confluency, washed four times with serum-free DMEM, and treated with effectors in serum-free DMEM for 5 min before cyclic AMP determination (see the Materials and methods section). Data are averages of four separate determinations, for which the S.E.M. was no greater than 16% of the value of the mean. (b) Cells were grown to 60–80% confluency, washed four times with serum-free DMEM, and then treated for 5 (ratios in parentheses) or 30 min in serum-free DMEM before determination of cAMP-PK activities of cell extracts in the presence or absence of 10 μ M-cyclic AMP (see the Materials and methods section). Data are the averages of four separate determinations.

Treatment	Cell line . . .	(a) Cyclic AMP (pmol/mg of protein)	(b) cAMP-PK activity ratio ($\frac{\text{free C}}{\text{total C}}$)		
		LLC-PK ₁	LLC-PK ₁	M18	FIB5
No addition		8.1	0.03 (0.03)	0.03	0.03
30 nM-Salmon calcitonin		41.7	0.45 (0.22)	0.02	0.31
90 nM-[Arg ⁸]vasopressin		29.9	0.12 (0.20)	0.04	0.08
100 μ M-Forskolin		12.6	0.17	0.23	0.07
500 μ M-IBMX		8.8	0.11	0.11	0.09
+ Calcitonin		404	0.99	0.19	0.83
+ Vasopressin		390	0.94	0.18	0.79
+ Forskolin		158	0.63	0.53	0.50

Fig. 1. Linear regression between cyclic AMP concentration (5 min measurements) and cAMP-PK activity ratio (30 min measurements) in LLC-PK₁ cells

Data ($n = 8$) are taken from columns 1 and 2 of Table 1. Slope = 2.13; y -intercept = 0.149; x -intercept = -69.9; and regression = 0.952.

only a very minor increase above basal cyclic AMP levels (from 8.1 to 8.8 pmol/mg), but, when used in conjunction with the other effectors, greatly amplified their effects (about 10-fold). Salmon calcitonin was found to be the most potent inducer of cyclic AMP synthesis (Table 1).

Increases in cAMP-PK activity ratio in the LLC-PK₁ cells (measured after 30 min treatment with agonists) closely correlated with the elevation of cyclic AMP. Under basal conditions the cAMP-PK activity ratio was 0.03–0.05 and rose to the highest extent in response to calcitonin (0.45). The use of IBMX in conjunction with either calcitonin or vasopressin almost completely activated cAMP-PK in LLC-PK₁ cells (activity ratio of about 1.0). IBMX alone elevated cyclic AMP only marginally (see above), but increased the cAMP-PK activity ratio from 0.03 to 0.11 (3-fold). The cAMP-PK thus appears to be acutely sensitive, in terms of release

of the C subunit (i.e. increase in activity ratio), to small changes in cellular cyclic AMP levels. Values for cAMP-PK activity ratio after 5 min hormonal treatments are presented (see Table 1) because the vasopressin response peaks at 5 min (Hemmings, 1985). The forskolin response peaks after 1 h (results not shown). Although the system is a dynamic one, the observed direct correlation ($r = 0.95$) between elevation of intracellular cyclic AMP concentration (measured at 5 min) with cAMP-PK activity ratio (measured at 30 min) (Fig. 1) supports the use of the cAMP-PK activity ratio as a valid indicator of changes in cyclic AMP levels.

cAMP-PK activity ratio in mutants

cAMP-PK activation in response to vasopressin, calcitonin and forskolin was measured in the mutant cell lines and compared with parental-cell responses (Table 1). Line M18 did not respond to either vasopressin or calcitonin, but responded to forskolin to a somewhat greater extent than did LLC-PK₁. cAMP-PK activation in line M18 in response to IBMX plus vasopressin or calcitonin was not markedly higher than activation in response to IBMX alone (Table 1). Measurement of intracellular cyclic AMP levels in line M18 after hormonal treatment showed a similar response (results not shown).

In contrast with M18, the FIB5 cell line responded to vasopressin and calcitonin, but to a somewhat lesser extent than did LLC-PK₁ (Table 1). Its responsiveness to forskolin, as well as to forskolin, calcitonin, or vasopressin used in conjunction with IBMX (Table 1), was also reduced in comparison with LLC-PK₁ cells.

The cAMP-PK catalytic activities of lines LLC-PK₁, M18 and FIB5 (measured in the presence of cyclic AMP) were 2.32, 1.87 and 1.53 units/mg respectively, with cyclic AMP-binding (regulatory subunit) activities also very similar to one another (results not shown). Clearly

Table 2. Adenylate cyclase activities of homogenates of LLC-PK₁ cells and the mutant FIB5

Adenylate cyclase activity was measured as described in the Materials and methods section. Data represent mean values $\pm$ S.E.M. from five separate experiments.

Treatment	Cell line . . .	Adenylate cyclase activity (pmol/min per mg)	
		LLC-PK ₁	FIB5
No addition		19 $\pm$ 2	6 $\pm$ 2
Vasopressin (90 nM)		106 $\pm$ 22	26 $\pm$ 4
Calcitonin (30 nM)		134 $\pm$ 15	26 $\pm$ 5
p[NH]ppG (10 μ M)		62 $\pm$ 16	17 $\pm$ 7
+ Vasopressin		86 $\pm$ 16	27 $\pm$ 2
+ Calcitonin		88 $\pm$ 12	22 $\pm$ 1
Forskolin (10 μ M)		58 $\pm$ 12	21 $\pm$ 5
NaF (10 mM)		78 $\pm$ 16	21 $\pm$ 2

the three cell lines were comparable in cAMP-PK, but exhibited differences in activation of the enzyme present.

Adenylate cyclase activities

Line FIB5 exhibited reduced cAMP-PK responsiveness *in vivo* to all agonists, and particularly to forskolin (adenylate cyclase stimulator) and IBMX. IBMX-mediated activation effectively gives a measure of basal adenylate cyclase activity, as IBMX elevates intracellular cyclic AMP levels by inhibiting phosphodiesterase-catalysed cyclic AMP catabolism. Line FIB5 accordingly appeared to have lower basal adenylate cyclase activity than the parental cell line, suggesting a possible mutation in adenylate cyclase. This might explain the phenotype of reduced responsiveness to agents stimulating adenylate cyclase. Accordingly, basal adenylate cyclase activity and adenylate cyclase activation in response to hormones and to the non-hydrolysable GTP analogue Gp[NH]pp were determined (Table 2). FIB5 exhibited 32% of basal adenylate cyclase activity

Fig. 2. Linear regression between uPA production (8 h measurements) and cAMP-PK activity (30 min measurements) in LLC-PK₁, M18 and FIB5 cell lines

Data ($n = 12$) are taken from Table 3. Slope = 41.38; y-intercept = 1.54; x-intercept = -0.037; and regression = 0.903.

compared to parental LLC-PK₁ cells and approximately 20–25% of the parental activity in response to vasopressin or calcitonin. Total adenylate cyclase activities stimulated by p[NH]ppG, forskolin or NaF were less than 36% that of line LLC-PK₁. Adenylate cyclase activity was stimulated about 4-fold by vasopressin or calcitonin in line FIB5 (Table 2), as opposed to parental-line responses of 6-fold or 7-fold stimulation of activity respectively. These data strongly suggest that FIB5 contains a mutation affecting adenylate cyclase.

Adenylate cyclase activation of line M18 has been reported elsewhere (Jans *et al.*, 1986). The data indicate that line M18 displays complete refractoriness of adenylate cyclase to hormonal stimulation.

cAMP-PK and uPA production

The M18 and FIB5 mutant cell lines appear to possess total cAMP-PK levels essentially comparable

Table 3. uPA production and cAMP-PK catalytic-subunit activity in LLC-PK₁ cells and mutants

(a) cAMP-PK activities (free catalytic subunit) in cell extracts (assayed in the absence of cyclic AMP from the assay) are calculated from Table 1 (30 min treatments). Total activities (assayed in the presence of cyclic AMP) were 2.32, 1.87 and 1.53 units/mg for LLC-PK₁, M18 and FIB5 respectively. (b) Cells were grown to 80–90% confluency, washed four times with serum-free DMEM, and then treated for 8 h in serum-free DMEM. Conditioned media were assayed for uPA activity as described in the Materials and methods section, where activity is expressed as Ploug units per mg of cellular protein. Data represent the averages of four separate determinations, for which the S.E.M. was less than 12% of the value of the mean.

Treatment	Activity (units/mg)					
	LLC-PK ₁		M18		FIB5	
	cAMP-PK	uPA	cAMP-PK	uPA	cAMP-PK	uPA
No addition	0.079	0.34	0.060	0.08	0.052	0.60
30 nM-Salmon calcitonin	1.05	36.0	0.037	0.09	0.480	25.0
90 nM-[Arg ⁸] vasopressin	0.281	20.7	0.065	0.25	0.116	6.6
100 μ M-Forskolin	0.393	22.3	0.424	30.7	0.113	6.2

with those of the parental LLC-PK₁ cell line, but, owing to mutations at other points in cyclic AMP metabolism, exhibit altered cAMP-PK activation profiles. It was therefore possible to investigate the relationship between the state of cAMP-PK activation and uPA induction. The three cell lines were assayed for uPA production in response to vasopressin, salmon calcitonin and forskolin (Table 3). Conditioned medium was collected and assayed after 8 h of treatment. uPA production is linear at this time (Jans & Hemmings, 1986; Jans *et al.*, 1986). The cAMP-PK activity in cell-free extracts, i.e. free C, resulting from 30 min hormonal treatment, are also presented in Table 3. A direct correlation ($r = 0.903$) was observed between cAMP-PK activity, and uPA production (at 8 h) for each cell line and for each treatment (Fig. 2). Treatment of LLC-PK₁ cells with calcitonin resulted in the highest increases in catalytic-subunit activity (1.05 units/mg) and uPA production (36.0 units/mg at 8 h). The responsiveness of line FIB5 to cyclic AMP-agonists was qualitatively similar to that of LLC-PK₁, but the stimulated cAMP-PK activity was reduced in comparison and uPA production was correspondingly lower. Whereas the M18 cell line did not respond to either calcitonin or vasopressin in terms of cAMP-PK or uPA induction, treatment of the cells with forskolin produced cAMP-PK activation and resulted in high levels of uPA production.

DISCUSSION

In the present study LLC-PK₁ cells and two of its mutants which possess similar overall cAMP-PK activities (of both catalytic and regulatory subunits) were compared with respect to the relationship between cAMP-PK activation and uPA induction. The results clearly indicate that cAMP-PK activation is a prerequisite for uPA production in response to agents elevating intracellular cyclic AMP concentration. Where cAMP-PK activation fails to occur, as for example in hormonal treatment of the mutant M18, which lacks both vasopressin and calcitonin receptors (Jans *et al.*, 1986), no uPA is induced. In addition, the magnitude of cAMP-PK activation correlated very well with the amount of uPA produced.

The FIB5 cell line (Jans & Hemmings, 1986) appears to possess a mutation at the level of adenylate cyclase. Basal adenylate cyclase activity was about 32% that of the parental line, and cyclase activities promoted by hormones, p[NH]ppG, forskolin and NaF were all 20–36% of the activities observed for LLC-PK₁ cells. Stimulation of adenylate cyclase activity relative to basal, induced by calcitonin or vasopressin, was also decreased compared with the parental line. The putative mutation results in a marked reduction of cAMP-PK activation (about 40–70% of that of the parental line) in response to hormones, forskolin and the phosphodiesterase inhibitor IBMX. The consequence of this possible adenylate cyclase mutation is also seen in the reduced uPA production in response to calcitonin (70%), vasopressin (32%) and forskolin (28%) (relative to that of the parental line).

The direct correlation between the extent of cAMP-PK activation and the magnitude of uPA production implies strongly that the process of uPA induction is dependent on cAMP-PK catalytic activity and implies a possible role for a phosphorylated intermediate in the process (Jans & Hemmings, 1986). It should not be overlooked,

however, that activation of cAMP-PK also liberates free regulatory-subunit dimers from the inactive holoenzyme, for which a role cannot be dismissed by this study. Mechanisms of uPA induction by cyclic AMP effectors have been proposed (Nagamine & Reich, 1985) postulating the possible role of the R_{II} subunit of cAMP-PK in this process. Although the experiments described here do not discount a possible role for R_{II} as delineated above, the proportionality of the relationship between cAMP-PK activation and uPA described above, and the fact that Type I, and particularly Type II, cAMP-PK are activated in the process (Ng *et al.*, 1983; Hemmings, 1985; B. A. Hemmings & D. A. Jans, unpublished work) suggest that it is most likely the cAMP-PK catalytic activity that is of primary importance.

We thank Ms. Manuela Schwarz for skilled technical assistance, and Dr. Michael S. Altus, Dr. Christoph Moroni and Dr. Yoshikuni Nagamine for reading the manuscript and providing helpful advice. Dr. John Scott (University of Washington, Seattle, WA, U.S.A.) is also thanked for the gift of the protein kinase inhibitor peptide 5-24.

REFERENCES

- Bachrach, U. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3087–3091
- Bechtel, P. J., Beavo, J. A. & Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 2691–2697
- Bradford, W. M. (1976) *Anal. Biochem.* **72**, 248–249
- Carlson, G. M., Bechtel, P. J. & Graves, D. J. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol.* **50**, 41–115
- Corbin, J. D. (1983) *Methods Enzymol.* **99**, 227–232
- Corbin, J. D., Keely, S. L., Soderling, T. R., & Park, C. R. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 265–279
- Dayer, J.-M., Vassalli, J.-D., Bobbitt, J. L., Hull, R. N., Reich, E. & Krane, S. M. (1981) *J. Cell Biol.* **91**, 195–200
- Deutsch, D. & Mertz, E. T. (1970) *Science* **170**, 1095–1096
- Devis, P. E., Grohol, S. H. & Taub, M. (1985) *J. Cell. Physiol.* **125**, 23–35
- Ernest, M. J. & Feigelson, P. (1978) *J. Biol. Chem.* **253**, 319–322
- Firestone, G. L. & Heath, E. C. (1981) *J. Biol. Chem.* **256**, 1396–1403
- Flockhart, D. A. & Corbin, J. D. (1982) *CRC Crit. Rev. Biochem.* **12**, 133–186
- Glass, D., Masaracchia, R., Feramisco, J. & Kemp, B. (1978) *Anal. Biochem.* **87**, 566–575
- Goldberg, A. R. (1974) *Cell* **2**, 95–102
- Gottesman, M. M., LeCam, A., Bukowski, M. & Pastan, I. (1980) *Somatic Cell Genet.* **6**, 45–61
- Hemmings, B. A. (1985) *Curr. Top. Cell. Regul.* **27**, 117–132
- Hemmings, B. A. (1986) *FEBS Lett.* **196**, 126–130
- Hull, R. N., Cherry, W. R. & Weaver, G. W. (1976) *In Vitro* **12**, 670–677
- Jans, D. A. & Hemmings, B. A. (1986) *FEBS Lett.* **205**, 127–131
- Jans, D. A., Resink, T. L., Wilson, L., Reich, E. & Hemmings, B. A. (1986) *Eur. J. Biochem.* **160**, 407–412
- Keely, S. L., Corbin, J. D., & Park, C. R. (1975) *J. Biol. Chem.* **250**, 4832–4840
- Krebs, E. G. & Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48**, 923–939
- Nagamine, Y. & Reich, E. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4606–4610
- Nagamine, Y., Sudol, M. & Reich, E. (1983) *Cell* **32**, 1181–1190
- Ng, K. W., Livesey, S. A., Larkins, R. G. & Martin, T. J. (1983) *Cancer Res.* **43**, 794–800
- Resink, T. J., Stucki, G., Grigorian, G. Y., Zschauer, A. & Buhler, F. R. (1986) *Eur. J. Biochem.* **154**, 451–456

- Salomon, Y. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 35–55
- Scott, J. D., Glaccum, M. B., Fischer, E. H. & Krebs, E. G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1613–1616
- Soderling, T. R., Corbin, J. D. & Park, C. R. (1974) *Methods Enzymol.* **38**, 358–367
- Steinberg, R. A. (1983) in *Biochemical Actions of Hormones II* (Litwack, G., ed.), pp. 25–65, Academic Press, New York
- Steinberg, R. A., Steinberg, M. G. & van Daalen Wetters, T. (1979) *J. Cell. Physiol.* **100**, 579–588
- Tovey, K. C., Oldham, K. G. & Whelan, J. A. M. (1974) *Clin. Chim. Acta* **56**, 221–234
- Trevillyan, J. M. & Byus, C. V. (1983) *Biochim. Biophys. Acta* **762**, 187–197
- Walter, U., Costa, M. R. C., Breakefield, X. O. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3251–3255

Received 1 July 1986/17 November 1986; accepted 19 December 1986