A Phenylalanine in Peptide Substrates Provides for Selectivity between cGMP- and cAMP-dependent Protein Kinases*

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Bovine lung cGMP-binding cGMP-specific phosphodiesterase (cG-BPDE) is a potent and relatively specific substrate for cGMP-dependent protein kinase (cGK) as compared to cAMP-dependent protein kinase (cAK) (Thomas, M. K., Francis, S. H., and Corbin, J. D. (1990) J. Biol. Chem. 265, 14971-14978). A synthetic peptide, RKISASEFDRPLR (BPDEtide), was synthesized corresponding to the sequence surrounding the phosphorylation site in cG-BPDE. BPDEtide retained the cGk/cAK kinase specificity demonstrated by native cG-BPDE: the apparent Km of BPDEtide for cGK was 5-fold lower than that for cAK (Km = 68 and 320 μM, respectively). Vmax values were 11 μmol/min/mg for cGK and 3.2 μmol/min/mg for cAK. The peptide was not phosphorylated to a measurable extent by protein kinase C or by calcium/calmodulin-dependent protein kinase II. Thus, the primary amino acid sequence of the peptide substrate was sufficient to confer kinase specificity. Studies in crude tissue extracts indicated that BPDEtide was the most selective peptide substrate documented for measuring cGK activity. Peptide analogs of BPDEtide were synthesized to determine the contribution of specific residues to cGK or cAK substrate specificity. Substitution of a Lys for the amino-terminal Arg did not reduce cGK/cAK specificity; neither did the exchange of an Ala for the non-phosphorylated Ser nor the removal of the 3 carboxy-terminal residues. A truncated BPDEtide (RKISASE) served equally well as substrate (Km ~ 90 μM) for both kinases. However, restoration of the Phe, to yield RKISASEF, reproduced the original cGK/cAK specificity for BPDEtide (Km = 120 and 480 μM, respectively), primarily by decreasing the affinity of cGK. Addition of a carboxy-terminal Phe to the peptide RKRSRAE (derived from the sequence of the cGK phosphorylation site in histone H2B) or to the peptide LRRASLG (derived from the sequence of the cAK phosphorylation site in pyruvate kinase) also improved the cGK/cAK specificity by decreasing the affinity of cAK. These data suggested that the Phe in each substrate tested is a negative determinant for cAK.

Serine/threonine protein kinases target specific protein substrates in part through recognition of the primary amino acid sequence (4–10 residues in length) surrounding the phosphorylated residue (1–5). In most instances, features of these recognition motifs have been elucidated by comparison of the amino acids surrounding phosphorylation sites in known physiological substrates, thus allowing for definition of a substrate consensus sequence. Kinetic analyses of the phosphorylation of synthetic peptide analogs of these sequences have been essential for determination of the individual amino acids that are kinase specificity determinants. Frequently these simple peptides have also served as specific and efficient phosphate acceptors in vitro (2, 6–9).

Investigation of protein substrates of cGMP-dependent protein kinase (cGK) has been conducted using purified proteins and peptides, tissue extracts, and intact cells, but very few substrates specific for cGK have been documented (for reviews, see Refs. 10 and 11). Initial studies of cGK substrate specificity by this laboratory analyzed both protein and peptide substrates that were phosphorylated in vitro by homogeneous preparations of cGK and cAMP-dependent protein kinase (cAK) (12). These studies indicated that the same primary amino acid sequence (Arg-Arg-X-Ser(P)/Thr(P)-X) was sufficient for substrate recognition by both cAK and cGK, consistent with the close evolutionary relationship between these two kinases (10, 13). Detailed studies of peptide substrates by Glass and Krebs (6, 14) focused on distinctive sites in histone H2B which were phosphorylated preferentially, but not exclusively, by either cAK or cGK. cGK selectively phosphorylated a serine in the sequence RKRSRK in histone H2B. Peptide analogs of this sequence were synthesized as substrates in order to analyze the contribution of individual amino acids to kinase specificity (14). Subtle differences in cAK and cGK substrate specificities and the importance of certain residues for cGK phosphorylation became apparent.

Recently, a cGMP-specific cGMP-binding phosphodiesterase (cG-BPDE) was described that was phosphorylated rapidly and relatively specifically by physiological concentrations of purified cGK (15, 16). Although cG-BPDE also served as substrate for cAK, cGK was a more efficient catalyst than an equimolar concentration of cAK (16). Furthermore, phosphorylation of cG-BPDE by either cAK or cGK occurred only when cGMP was bound to the allosteric site of the phospho-

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1 The abbreviations used are: cGK, cGMP-dependent protein kinase; cG-BPDE, cGMP-binding cGMP-specific phosphodiesterase; BPDEtide, a peptide (RKISASEFDRPLR) derived from the sequence of the phosphorylation site in cG-BPDE; H2B peptide, a peptide (RKRSRAE) derived from the site in histone H2B (RKRSRK) preferentially phosphorylated by cGK; cAK, cAMP-dependent protein kinase; CaMK II, calcium/calmodulin-dependent protein kinase II.
di esterase, supporting the possibility that cG-BPDE is a physiological substrate for cGK. In the present study, synthetic peptide analogs derived from the sequence surrounding the phosphorylation site in cG-BPDE (16) were analyzed to determine residues which contributed to differences in cGK and cAK substrate specificities. Availability of more potent and specific peptide substrates for cGK could prove beneficial in studies of partially purified enzyme and could provide insight into the functional roles of this kinase in vivo.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Purification**—Peptides were synthesized from N-Boc-D-amino acid derivatives on a Beckman 990 System; cleavage of peptide from the resin with hydrofluoric acid was performed by Multiple Peptide Systems, San Diego, CA. Cleaved peptides were purified by reverse phase chromatography on a high performance liquid chromatography C18 column, then subjected to several cycles of Speed-Vac evaporation and resuspension in deionized water to remove solvents. Peptides were resuspended in a minimum volume of water, brought to neutrality with NH₄HCO₃, and stored at -20 °C. Peptide concentrations and purities were determined by amino acid analyses using a Waters PicoTag reverse phase high performance liquid chromatography system.

**Purification of Kinases**—The catalytic subunit of cAK was purified from calf thymus following the method of Flockhart and Corbin (17); in the text, cAK indicates the catalytic subunit of the catalytic kinase. Type Ia cGK was purified from bovine lung according to the method of Corbin and Döskeland (18).

**Protein Kinase Assays**—In standard assays, the activities of cGK and cAK were assayed in the presence of CAMP. Kinase activity was adjusted to reflect total activity in a particular fraction.

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**Results and Discussion**

**Characterization of BPDEtide Phosphorylation**—Purified cG-BPDE was phosphorylated in vitro by either cGK or cAK at a single site to a stoichiometry of 0.6-1.6 mol of 32P/mol of dimer (16). Under the conditions employed, phosphorylation of the intact phosphodiesterase by cGK proceeded at an apparent 10-fold higher rate than by an equimolar amount of cAK. A specificity index was derived as a means to compare the ability of cGK and cAK to catalyze the phosphorylation of each peptide. The index is the ratio of the mean of the apparent Vₘₐₓ/Kₐₙₐₜ values for the particular kinase. A larger ratio implies a better peptide substrate for the kinase of interest.

**Characterization of BPDEtide Phosphorylation**—Purified cG-BPDE was phosphorylated in vitro by either cGK or cAK at a single site to a stoichiometry of 0.6-1.6 mol of 32P/mol of dimer (16). Under the conditions employed, phosphorylation of the intact phosphodiesterase by cGK proceeded at an apparent 10-fold higher rate than by an equimolar amount of cAK; cG-BPDE was not a substrate for either protein kinase C or for CaMK II. The phosphorylation site sequence, RKISAFDRPLR, was determined initially by analysis of tryptic digests (16) and a peptide, RKISAFDRPLR (BPDEtide), corresponding to the phosphorylation site in cG-BPDE, was synthesized and tested as a protein kinase substrate. BPDEtide retained the kinase specificity demonstrated by the native phosphodiesterase. The apparent Kₐₙₐₜ of BPDEtide for phosphorylation by cGK was 68 μM, 5-fold lower than that of cAK (320 μM); the Vₘₐₓ value of cGK was 3-fold higher than that of cAK (Table I). The BPDEtide specificity index for cGK as compared to cAK was 16 (Table I), which was consistent with the more efficient phosphorylation of the native cG-BPDE by cGK. In addition, neither CaMK II nor protein kinase C catalyzed phosphorylation of BPDEtide to a

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The specificity of cGK compared to cAK for BPDEtide was greater than that for the H2B peptide (RKRSRAE), the peptide substrate most commonly used for selective measurement of cGK activity (Fig. 1 and Table I). In the present study, the apparent $K_m$ of cGK for H2B peptide was 38 $\mu$M, as compared to 71 $\mu$M for cAK, in agreement with previously reported values (14). However, the $V_{max}$ for cGK phosphorylation of the H2B peptide (9.2 $\mu$mol/min/mg) was nearly 2-fold higher than that for cGK, resulting in a specificity index of 1.0 (Table I). Thus, the H2B peptide was a relatively specific substrate for cGK when assayed at concentrations near the $K_m$, but this selectivity was not apparent at higher concentrations. The pH optimum of cGK phosphorylation of H2B peptide was approximately 8.0; maximal phosphorylation of BPDEtide occurred at pH 7.2 (data not shown). Both the H2B peptide and BPDEtide were good substrates for cGK (compare $V_{max}/K_m$ values of 0.13 and 0.16, respectively), but the same peptides exhibited a 13-fold difference in $V_{max}/K_m$ values for cAK (Table I). Both the low affinity of cAK for BPDEtide and the low $V_{max}$ contributed to the 16-fold difference in the specificity indices between these peptides. In these studies, BPDEtide was a much more specific substrate for cGK than was the H2B peptide.

Modification of Full-length BPDEtide—An arginine located amino-terminal and proximal to the phosphorylated residue has been established as an important specificity determinant for several protein kinases, including cyclic nucleotide-dependent protein kinases (1, 2, 4, 6). An analog of BPDEtide in which the amino-terminal arginine was replaced by lysine, KKISASEFDRPLR, was synthesized to assess the importance of that arginine to kinase specificity. As shown in Table I, the apparent $K_m$ obtained for cGK increased from 68 to 160 $\mu$M, and the $V_{max}$ decreased approximately 4-fold. However, the effect of the lysine substitution on cAK kinetics was far more dramatic: $K_m$ and $V_{max}$ values for cAK could not be determined since saturating substrate concentrations were not achieved, even at peptide concentrations of 3.5 mM. The arginine appeared to be an important residue for cGK peptide affinity and was critical for the interaction of cAK with the peptide.

Amino acid analyses of tryptic digests of $^{32}$P-labeled cG-BPDE revealed that only the amino-terminal (first) serine in the native cG-BPDE sequence RKISASEFDRPLR was phosphorylated by either cGK or cAK (16). Substitution of an alanine for the second serine (RKISAAEFDRPLR) did not significantly affect the kinetic values of either kinase or the specificity index, as compared to BPDEtide (Table I). This result suggested that only the first serine in BPDEtide was phosphorylated by cGK or cAK, as in the native phosphodiesterase, and that the second serine in BPDEtide was not a substrate determinant for either kinase. Although further studies are required, this peptide could be a substrate of choice in routine cGK measurement since it retains the kinase specificity and kinetic values of BPDEtide and has only a single phosphorylatable residue.

**Truncated Peptides Derived from BPDEtide—**cGK was an efficient catalyst for phosphorylation of either cG-BPDE or BPDEtide. However, since the sequence immediately sur-

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**Table I**

**Summary of apparent kinetic constants and ratios for peptide substrates of cGK compared to cAK**

Synthetic peptides were assayed as described under "Experimental Procedures." Values are the mean ± S.E. of at least three separate determinations. The substrate specificity of cGK and cAK for each peptide was analyzed by computing the $V_{max}/K_m$ ratio for each kinase. A larger ratio indicates a better substrate for the kinase. The specificity index is the ratio of the $V_{max}/K_m$ for cGK divided by the $V_{max}/K_m$ for cAK. The larger the value, the better the peptide serves as substrate for cGK as compared to cAK.

| Substrate            | $K_m$ $\mu$M | $V_{max}$ $\mu$mol/min/mg | $V_{max}/K_m$ | Specificity index, cGK/cAK |
|----------------------|--------------|-----------------------------|---------------|---------------------------|
| RKRSRAE (H2B peptide) | 38 ± 4       | 5.1 ± 0.4                   | 1.0           |                           |
| RKISASEFDRPLR (BPDEtide) | 68 ± 3       | 1.1 ± 1.8                   | 16            |                           |
| KKISASEFDRPLR        | 160 ± 7      | 2.8 ± 0.1                   | —             | 0.017                     |
| RKISASEFDRPLR        | 50 ± 3       | 8.0 ± 0.4                   | 1.6           | 0.011                     |
| RKISASEFDR           | 80 ± 13      | 5.6 ± 0.4                   | 0.70          | 1.8                       |
| RKISASEF             | 120 ± 12     | 5.2 ± 0.4                   | 0.93          | 8.1                       |
| RKISASEFDR           | 140 ± 22     | 5.6 ± 0.6                   | 0.40          | 11                        |

*—, no phosphorylation of substrate was measurable.*

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rounding the phosphorylated serine in both substrates (RKISA) was similar to the established substrate consensus sequence of cAK, the low affinity of this kinase for both the protein and the peptide substrates was unexpected. Three truncated peptides were synthesized from the BPDEtide sequence by deletion of carboxyl residues to determine whether these amino acids contributed to cGK/cAK specificity.

Both cGK and cAK had approximately equal affinities for the shortest truncated BPDEtide RKISASE (Table I). The $V_{\text{max}}/K_m$ ratio for cGK decreased from a value of 0.15 for BPDEtide to 0.07 for this peptide, but the ratio for cAK was increased 4-fold, suggesting that the carboxyl-terminal portion of BPDEtide contained negative substrate determinants for cAK.

Restoration of the phenylalanine, to produce the octapeptide RKISASEF, re-established most of the kinase selectivity demonstrated by BPDEtide. The affinities of both kinases for the octapeptide were decreased as compared to BPDEtide, but the 4-fold difference in $K_m$ between cGK and cAK was maintained. $V_{\text{max}}$ values were also lower than those of BPDEtide (Table I). Addition of the phenylalanine slightly decreased the $V_{\text{max}}/K_m$ ratio for cGK as compared to that of the peptide RKISASE, but the ratio for cAK was decreased approximately 7-fold. Therefore, the specificity index was increased to 8.1 for the peptide RKISASEF (Table I). Addition of 2 more carboxyl residues (RKISASEFDR) did not significantly alter the kinetic values from those obtained for the octapeptide. These results suggested that the phenylalanine residue was a key negative substrate determinant for cAK in the BPDEtide sequence.

Since the negative effect of the phenylalanine on cAK peptide affinity could be a nonspecific effect due to increased peptide length, an alanine was added to the carboxyl terminus of the shortest truncated BPDEtide, RKISASE. This peptide (RKISASEA) was of equal length to that containing phenylalanine and was used as a control peptide for analysis of the effects of phenylalanine. Addition of the alanine did not significantly affect phosphorylation by cGK as compared to that of the peptide RKISASEF (Table II); however, the apparent $K_m$ for cAK was decreased by 30% and the $V_{\text{max}}$ was increased 3-fold, causing a decrease in the specificity index from a value of 8.1 to 2.0. The specificity index of RKISASEA was similar to that of RKISASE, which was a relatively nonspecific peptide substrate for cGK (Table II).

Addition of a Carboxyl-terminal Phenylalanine to Selected Peptides—A series of experiments was conducted to determine whether a phenylalanine placed 4 residues carboxyl-terminal to a phosphorylated serine, as in BPDEtide, would serve as a negative substrate determinant for cAK in other peptide sequences. The H2B peptide and Kemptide, peptide substrates relatively selective for cGK or cAK, respectively, were synthesized with a phenylalanine in this position. As shown in Table II, addition of a phenylalanine to the H2B peptide (RRKRSRAEF) increased the apparent $K_m$ for cAK 3-fold above that of the parent peptide, but did not significantly affect any other kinetic values for either kinase. Thus, the decrease in cAK affinity alone would account for the increase in the specificity index from a value of 1.0 for the H2B peptide to that of 5.3 for the peptide RRKRSRAEF (Table II). The presence of a phenylalanine 4 residues carboxyl-terminal to the serine produced a similar increase in cGK substrate specificity for both the H2B peptide and the peptide RKISASE. The phosphorylation kinetics of cGK were relatively unaffected by the presence of a phenylalanine, but the affinity of cAK for these peptides was diminished significantly, resulting in greater selectivity of each peptide for cGK as compared to cAK.

As a control, an analog of the H2B peptide was synthesized with an alanine added to the carboxyl terminus (RKRSRAEA). The affinities of both kinases for this peptide were increased significantly as compared to either the H2B peptide or to the H2B peptide containing phenylalanine (Table II); $V_{\text{max}}$ values were increased as well, albeit to a lesser extent. To our knowledge, the affinity of cGK for

**Table II**

*Effect of addition of phenylalanine to selected peptide substrates*

Synthetic peptides were assayed as described under “Experimental Procedures.” Values are expressed as the mean ± S.E. of at least three separate determinations. The relative substrate specificity of cGK as compared to cAK was analyzed by computing the $V_{\text{max}}/K_m$ ratio for each kinase, then dividing the cGK ratio by the cAK ratio (specificity index). A larger index indicates a better substrate for cGK as compared to cAK.

| Substrate | $K_m$ (μM) | $V_{\text{max}}$ (μmol/min/mg) | Specificity index, cGK/cAK |
|-----------|------------|-------------------------------|--------------------------|
| cGK       | cAK        |                               |                          |
| Truncated BPDEtide RKISASE | 80 ± 13 | 93 ± 14 | 5.6 ± 0.4 | 3.7 ± 0.4 | 1.8 |
| Truncated + Phe RKISASEF | 120 ± 12 | 480 ± 76 | 5.2 ± 0.4 | 2.6 ± 0.3 | 8.1 |
| Truncated + Ala RKISASEA | 140 ± 14 | 340 ± 12 | 6.4 ± 0.5 | 7.7 ± 1 | 2.0 |
| H2B peptide RKRSRAEF | 38 ± 4 | 71 ± 9 | 5.1 ± 0.4 | 9.2 ± 1 | 1.0 |
| H2B peptide + Phe RKRSRAEF | 36 ± 2 | 230 ± 39 | 6.6 ± 0.7 | 7.9 ± 1 | 5.3 |
| H2B peptide + Ala RKRSRAEA | 4 ± 0.3 | 16 ± 2 | 9.9 ± 0.8 | 19 ± 3 | 2.1 |
| Kemptide LRRASLG | ND* | 39 ± 4 | ND | 9.8 ± 1 | ND |
| Kemptide + Phe LRRASLAGF | ND | 33 ± 10 | ND | 8.0 ± 3 | ND |
| Kemptide + Ala LRRASLAGAA | ND | 11 ± 3 | ND | 20 ± 2 | ND |

*ND, not determined.*
Phenylalanine to both the H2B peptide and the shortest to Kemptide had no significant effect (Table I). However, the truncated BPDEtide (RKISASE), addition of phenylalanine peptide LRRASLGg of equal length (Table I). The affinity of phenylalanine had a pronounced negative effect on the decrease in cAK affinity produced by the addition of the phosphorylated serine and the phenylalanine was added (LRRASLGLF) to maintain the same distance between the phosphorylated serine and the phenylalanine: synapsin I and fructose-1,6-bisphosphatase from yeast (19, 20). It will be of interest to determine whether the phenylalanine is a negative determinant in these phosphorylation sequences. A phenylalanine is present, but at a different location, in ATP citrate-lyase in the phosphorylation sequence TASFSE (21), and in phenylalanine hydroxylase in the sequence SRKLSBFg (22; B = Asx).

The sequence of the first 5 residues in BPDEtide, RKISA, resembles that of physiological substrates of cAK (19, 20). The remaining residues in BPDEtide are present in similar, and often identical, positions in other cAK substrates. At least seven substrates contain a serine in a position analogous to that in RKISAS, and three have a glutamic acid in a position analogous to that in RKISASE (19, 20). The protein pp60 contains an aspartic acid in the same location in RKISASD, and two other substrates have an aspartate within 2 residues of the phosphorylated serine (19). Published phosphorylation sequences often do not extend as far beyond the phosphorylated residue as is necessary for comparison of carboxyl-terminal arginines, prolines, and leucines with those in BPDEtide, but several substrates of cAK have one or more of these 3 amino acids within 5 residues carboxyl-terminal to the phosphorylated serine (19, 20).

Although there are far fewer documented cGK-specific protein substrates (10, 11), the type 1 regulatory subunit of cAK has a tyrosine in a position analogous to the phenylalanine in cG-BPDE (23). To our knowledge, this is the only cGK substrate other than cG-BPDE containing an aromatic residue in this position (10, 19). Investigation of the importance of phenylalanine positioning relative to the phosphorylated residue, as well as the effect of other aromatic residues on kinase affinity for substrate, may provide further insight into the specificity determinants of cGK and cAK.

**Peptide Substrate Specificity in Crude Systems**

The effectiveness of BPDEtide as a selective substrate for cGK in a mixture of protein kinases was determined by assaying fractions from ion exchange chromatography of a crude cytosolic extract of rat lung (see "Experimental Procedures"). As shown in the column profile in Fig. 2A, Kemptide phosphorylation was evident across a broad peak; the contribution of the catalytic subunit of cAK to the profile was confirmed by addition of the cAK inhibitor peptide to the assay (Fig. 2B). The profile of Kemptide phosphorylation overlapped that of BPDEtide and H2B peptide (Fig. 2A), indicating that Kemptide was phosphorylated by cGK as well as by cAK. However, BPDEtide and H2B peptide were not phosphorylated to a significant extent by fractions containing high cAK activity (e.g. fractions 210–250). The H2B peptide was phosphorylated by the same fractions as was BPDEtide, but yielded lower activity (Fig. 2A). Two minor peaks were detected, indicating phosphorylation of H2B peptide by cAK (fractions 225–255) and by an unidentified kinase (fractions 50–75). Addition of the cAK inhibitor peptide to the BPDEtide assay decreased
phosphorylation along the trailing edge, in fractions containing significant cAK (Fig. 2C).

BPDEtide was recently used to measure specific cGK activation in crude cytosolic extracts of pig coronary artery following treatment with various drugs and hormones (24). In these studies, BPDEtide was a preferable peptide substrate compared to the H2B peptide: addition of the cAK inhibitor peptide decreased basal phosphorylation (−cGMP) of the H2B peptide by 40%, but had no effect on phosphorylation of BPDEtide. The activity ratio of cGK (−cGMP/+cGMP) measured using BPDEtide was unaffected by addition of cAK inhibitor peptide; however, addition of the inhibitor to the H2B peptide assays decreased the activity ratio by 25%. The truncated BPDEtide RKISASEF was also a selective peptide substrate in these studies and could prove useful for assaying cGK activity in crude systems.

Analyses of synthetic peptide analogs of the phosphorylation site in cG-BPDE (RKISASEFDRPLR) revealed that the amino acid sequence was sufficient to confer cGK/cAK peptide substrate specificity. BPDEtide was phosphorylated in a manner very similar to that of native cG-BPDE in that it was a better substrate for cGK than for cAK, was only phosphorylated on the first serine residue, and was not a substrate for either protein kinase C or for CaMK II. The phenylalanine in the BPDEtide sequence was shown to be a strong negative determinant for cAK, and may account for the specificity of native cG-BPDE for cGK as compared to cAK (16). Phenylalanine in a similar location was also a negative determinant for cAK in the H2B peptide and in Kemptide. Addition of an alanine to the truncated BPDEtide RKISASE, to the H2B peptide, and to Kemptide significantly improved these peptides as substrates for cAK. A lysine exchanged for the arginine at the amino terminus greatly diminished the ability of BPDEtide to serve as substrate for cGK, but eliminated the ability of cAK to phosphorylate this peptide. In summary, BPDEtide appears to be the most selective peptide substrate for cGK yet characterized and may be used to assay cGK activity in crude tissue extracts with virtually no interference from cAK.

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REFERENCES
1. Zetterqvist, Ö., Ragnarsson, U., Humble, E., Berglund, L., and Engström, L. (1976) Biochem. Biophys. Res. Commun. 70, 696–703
2. Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977) J. Biol. Chem. 252, 11391–11397
3. Pearson, R. B., Woodgett, J. R., Cohen, P., and Kemp, B. E. (1985) J. Biol. Chem. 260, 14471–14476
4. Soderling, T. R., Schwarzer, C. M., Payne, M. E., Jett, M. F., Porter, D. K., Atkins, J. L., and Richtand, N. M. (1986) in Hormones and Cell Regulation (Nunes, J., Dumont, J. E., and King, R. J. B., eds) Vol. 139, pp. 141–157, John Libbey Eurotext Ltd., Montrouge, France
5. Kennedy, P. J., and Krebs, E. G. (1991) J. Biol. Chem. 266, 15555–15568
6. Glass, D. B., and Krebs, E. G. (1979) J. Biol. Chem. 254, 9728–9738
7. Daile, P., Carnegie, P. R., and Young, J. D. (1975) Nature 257, 416–420
8. Masaracchia, R. A., Kemp, B. E., and Walsh, D. A. (1977) J. Biol. Chem. 252, 7109–7117
9. Hashimoto, Y., and Soderling, T. R. (1987) Arch. Biochem. Biophys. 252, 418–425
10. Glass, D. B. (1990) in Peptides and Protein Phosphorylation (Kemp, B. E., ed) pp. 209–233, CRC Press, Boca Raton, FL
11. Walter, U. (1989) Rev. Physiol. Biochem. Pharmacol. 113, 41–88
12. Lincoln, T. M., and Corbin, J. D. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3239–3243
13. Corbin, J. D., Thomas, M. K., Wolfe, L., Shabb, J. B., Woodford, T. A., and Francis, S. H. (1990) Adv. Second Messenger Phosphoprotein Res. 24, 411–418
14. Glass, D. B., and Krebs, E. G. (1982) J. Biol. Chem. 257, 1196–1200
15. Thomas, M. K., Francis, S. H., and Corbin, J. D. (1990) J. Biol. Chem. 265, 14964–14970
16. Thomas, M. K., Francis, S. H., and Corbin, J. D. (1990) J. Biol. Chem. 265, 14971–14978
17. Folkheart, D. A., and Corbin, J. D. (1984) in Brain Receptor Methodologies (Marangos, P. J., Marangos, P. J., Campbell, I. C., and Cohen, R. M., eds) Part A, pp. 209–215, Academic Press, New York
18. Corbin, J. D., and Deekeland, S. O. (1983) J. Biol. Chem. 258, 11381–11387
19. Pearson, R. B., and Kemp, B. E. (1991) Methods Enzymol. 200, 62–81
20. Zetterqvist, Ö., Ragnarsson, U., and Engström, L. (1990) in Peptides and Protein Phosphorylation (Kemp, B. E., ed) pp. 171–187, CRC Press, Boca Raton, FL
21. Pierce, M., Palmer, J., Keutmann, H., Hall, T., and Avruch, J. (1982) J. Biol. Chem. 257, 10681–10686
22. Wretborn, M., Humble, E., Ragnarsson, U., and Engström, L. (1980) Biochem. Biophys. Res. Commun. 93, 403–408
23. Grashien, R. L., and Krebs, E. G. (1980) J. Biol. Chem. 255, 1164–1169
24. Jiang, H., Colbran, J. L., Francis, S. H., and Corbin, J. D. (1992) J. Biol. Chem. 267, 1015–1019