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SYNTHETIC PEPTIDES BASED ON THE CALMODULIN-BINDING DOMAIN OF MYOSIN LIGHT CHAIN KINASE INHIBIT ACTIVATION OF OTHER CALMODULIN-DEPENDENT ENZYMES

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Nanomolar concentrations of synthetic peptides corresponding to the calmodulin-binding domain of skeletal muscle myosin light chain kinase were found to inhibit calmodulin activation of seven well-characterized calmodulin-dependent enzymes: brain 61 kDa cyclic nucleotide phosphodiesterase, brain adenylate cyclase, Bordetella pertussis adenylate cyclase, red blood cell membrane Ca^{++}-pump ATPase, brain calmodulin-dependent protein phosphatase (calcineurin), skeletal muscle phosphorylase b kinase, and brain multifunctional Ca^{++} (calmodulin)-dependent protein kinase. Inhibition could be entirely overcome by the addition of excess calmodulin. Thus, the myosin light chain kinase peptides used in this study may be useful antagonists for studying calmodulin-dependent enzymes and processes.

Synthetic peptides corresponding to the sequence of the calmodulin(CaM)-binding domain of skeletal muscle myosin light kinase (MLCK) are useful tools for investigating CaM-target enzyme interactions at the molecular level. These MLCK CaM-binding peptides are facilitating biochemical and spectral studies of CaM-MLCK interactions because they: a) are capable of mimicking many aspects of the interaction of CaM with native MLCK; b) are relatively small (Mr < 3000); and c) can be prepared in relatively large quantities using automated solid-phase chemical synthesis procedures [1-4]. The MLCK CaM-binding peptides differ from small hydrophobic molecules such as trifluoperazine in that the peptides bind CaM with high-affinity to form a unique, 1:1 complex in which the structures of both CaM and peptide are significantly altered [3,4]. These peptides, therefore, appear to be excellent probes for identifying residues on CaM that are responsible for interactions with native MLCK. In addition, these peptides are specific and potent inhibitors of the activation of MLCK by CaM [1,2].

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Abbreviations. CaM, calmodulin; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(B-aminoethyl ether)-N,N,N'N'-tetraacetic acid; LC20, gizzard 20kDa myosin light chain; MLCK, myosin light chain kinase; MOPS, 4-morpholinepropanesulfonic acid; QAE, quaternary triethylammonium; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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It is not known at present whether the synthetic MLCK CaM-binding peptides are capable of inhibiting the activation of other CaM-dependent enzymes. In fact, recent evidence from several laboratories showing that the activation of different target enzymes involves specific determinants on CaM for each enzyme [5-9] raises the question of whether the MLCK peptides are capable of inhibiting activation of all CaM target enzymes. Thus, to establish their potential efficacy as universal or selective CaM antagonists, the effects of the MLCK peptides on other CaM-dependent systems must be examined. More importantly, because the MLCK peptides and their derivatives are being used to characterize the structural features of the MLCK-binding site on CaM, the extent to which the MLCK peptides inhibit activation of other target enzymes will provide data as to the similarities between the MLCK-binding site and other target enzyme-binding sites on CaM. To address these two issues, a study of the effects of MLCK CaM-binding peptides on the activation of seven well-characterized CaM-dependent enzymes was undertaken and the results reported here.

Methods

Peptide Preparation. MLCK peptides were synthesized by solid phase methods using a Beckman 990B automated peptide synthesizer and were purified and characterized as described by Blumenthal and Krebs [1]. Two different MLCK peptides, referred to as MLCK I and MLCK IV in the text, were used. The sequence of MLCK I is: Lys-Arg-Arg-Trp-Lys-Lys-Asn-Phe-Ile-Ala-Val-Ser-Ala-Ala-Asn-Arg-Phe-Lys-Lys-Ile-Ser-Ser-Ser-Gly-Ala-Leu; that of MLCK IV is: Lys-Arg-Arg-Trp-Lys-Lys-Ala-Phe-Ile-Ala-Val-Ser-Ala-Ala-Ala-Arg-Phe-Lys-Lys-Ile. Both peptides inhibited skeletal muscle MLCK activity with similar Ki values (approx. 1 nM) [1].

Phosphorylase Kinase Assay. The activity of phosphorylase b kinase was determined by measuring rates of ^{32}P incorporation into phosphorylase b using a filter paper assay method [10]. The assay reaction mixture (final volume of 50 µl) contained 50 mM magnesium acetate, 200 µM CaCl2, 5 mg/ml phosphorylase b, 0.9 µg/ml skeletal muscle phosphorylase kinase, and the indicated concentrations of CaM, MLCK peptide, or EGTA. The assay reaction was initiated (after a 5 min pre-incubation at 30°) by the addition of [γ-^{32}P] ATP (150-200 cpm/pmol, 0.4 mM final concentration). CaM was purified from bovine testis as previously described [11]. Recrystallized phosphorylase b from rabbit skeletal muscle was a generous gift from Dr. E.H. Fischer (University of Washington). Dr. C.P. Chan (University of Washington) graciously provided phosphorylase kinase that was purified from rabbit skeletal muscle essentially as described by Cohen [12].

CaM-dependent Protein Phosphatase (Calcineurin) Assay. Phosphatase activity was assayed as described by Blumenthal et al. [13] by determining rates of ^{32}Pi release from a synthetic phosphopeptide corresponding to residues 81-99 of bovine cardiac cAMP-dependent protein kinase regulatory subunit (RII). The assay reaction (50 µl final volume) contained 50 mM MOPS, pH 7.0, 15 mM 2-mercaptoethanol, 2 mM magnesium acetate, 2 mM MnCl2, 0.3 µg/ml bovine brain CaM-dependent phosphatase (prepared by the method of Sharma et al. [14]), and the indicated concentrations of bovine testis CaM, MLCK peptide, and EGTA. Following a 5 min preincubation at 30°, the reaction was started by the addition of ^{32}P-labeled synthetic RII peptide (100 µM final, 150 cpm/pmol).

Cyclic Nucleotide Phosphodiesterase Assay. The 61 kDa isozyme of CaM-dependent phosphodiesterase was purified from bovine brain using immobilized conformation-specific monoclonal antibodies [7] and CaM-Sepharose chromatography. Phosphodiesterase activity was assayed as described [7] at 30° in 20 mM Tris-HCl, 20 mM imidazole, pH 8, 3 mM MgCl2, 0.2 mg/ml bovine serum albumin, 1 mM [^3H]cAMP, and the indicated concentrations of bovine testis CaM, MLCK peptide, and EGTA. The reaction was initiated by the addition of [^3H]cAMP and terminated after 10 min.

Multifunctional Ca^{++}(CaM)-dependent Protein Kinase Assay. Multifunctional Ca^{++}(CaM)-dependent protein kinase (also termed CaM-dependent protein kinase II) was purified from rat brain essentially as described [15] and was a generous gift from Dr. H. Schulman (Stanford University). Protein kinase activity was measured by determining rates of ^{32}P incorporation into chicken gizzard muscle myosin light chain (LC20; prepared as previously described [16]) using a filter paper method [10]. The assay reaction (38 µl final volume) contained 50 mM Tris, pH 7.6, 0.6 mM dithiothreitol, 0.6 mg/ml bovine serum albumin, 80 mM NaCl, 0.5 mM CaCl2, 1.0 µg/ml multifunctional Ca^{++}(CaM)-dependent kinase, and the indicated concentrations of CaM and MLCK peptide. The reaction was performed at 25° and was started by the addition of Mg-[γ-^{32}P]ATP (1900 cpm/pmol, 0.2mM ATP final; 6mM MgCl2 final) and LC20 (40 µM final).

Mammalian Adenylate Cyclase Assay. CaM-sensitive adenylate cyclase was partially-purified from bovine brain using CaM-Sepharose affinity chromatography as described by Yeager et al. [17]. Adenylate cyclase activity was assayed at 30° for 20 min by the method of Salomon et al. [18]. Assays contained CaM-sensitive adenylate cyclase (24 µg/ml), 20 mM Tris-HCl, pH 7.5, 0.25 mM [α-^{32}P]ATP (100 cpm/pmol), 5 mM theophylline, 0.22 mM EGTA, 0.25 mM CaCl2, 0.1% bovine serum albumin and the indicated concentrations of CaM and MLCK peptide in a final volume of 0.25 ml. [^3H]cAMP (60,000 cpm/ml) was also included to monitor product recovery.

Bordetella pertussis Adenylate Cyclase Assay. Adenylate cyclase from Bordetella pertussis was purified as described by Shattuck et al. [19]. The activity eluting from QAE-Sepharose in peak 1 was pooled and used in these experiments. This enzyme activity was assayed as described above for the mammalian cyclase with the following changes: the enzyme (1.12 µg/ml) was assayed for 10 min at 30°; the [α- ^{32}P]ATP concentration was increased to 1.0 mM; 5 mM $MgCl_2$ was substituted for $MnCl_2$; and theophylline was omitted.

Ca^{++}-pump ATPase Assay. CaM-deficient red blood cell membranes were prepared from outdated blood by the procedure of Raess and Vincenzi [20]. ATPase activity was assayed by monitoring the rate of absorbance change at 366 nm (reference at 550 nm) over a 5 min period using the coupled enzyme method described by Foder and Scharff [21]. The assay mixture contained 18 mM histidine, 18 mM imidazole, pH 7.1, 80 mM NaCl, 15 mM KCl, 3.0 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM $CaCl_2$, 0.1 mM ouabain, 2.3 mM phosphoenol pyruvate, 0.45 mM NADH, 50 units each of pyruvate kinase (Sigma) and lactate dehydrogenase (Sigma), and CaM and MLCK peptide at the indicated concentrations. The red cell membranes (360 µg/ml) were preincubated with this mixture for 5-10 min at 37° and the ATPase reaction started by addition of ATP (1.5 mM final). CaM was purified from human red blood cells [22].

Results and Discussion

The enzymes employed in the present study represent a variety of well-characterized CaM-dependent activities that differ markedly in a number of respects including tissue of origin, physico-chemical and catalytic properties, and CaM-dependence. To facilitate direct comparison wherever possible, the enzymes were grouped into three categories on the basis of similarities in their CaM-dependent and divalent cation-dependent activities.

The first category of enzymes, consisting of phosphorylase kinase and CaM-dependent phosphatase (calcineurin), is comprised of those that show significant divalent cation-dependent/CaM-independent activity. In addition, both of the enzymes in this category contain integral divalent-metal cation-binding subunits. In phosphorylase kinase this subunit is identical to CaM and is termed the δ-subunit [23], while in calcineurin the metal-binding subunit is called the B-subunit and is homologous to CaM [24]. These enzymes were assayed with varying concentrations of CaM using a fixed concentration of peptide (Fig. 1). The concentration of peptide used was 2- to 3-fold greater than the concentration of exogenously added CaM required for 50% activation of that enzyme in the absence of peptide. In the case of phosphorylase kinase, the peptide did not inhibit Ca^{++}-activation in the absence of exogenous CaM, indicating that the peptide does not interfere with the function of the integral CaM (δ) subunit(s) of phosphorylase kinase. However, activation by exogenous CaM (Fig. 1) or skeletal muscle troponin-C (data not shown) was completely inhibited by the peptide. The effects of peptide on the inhibition of

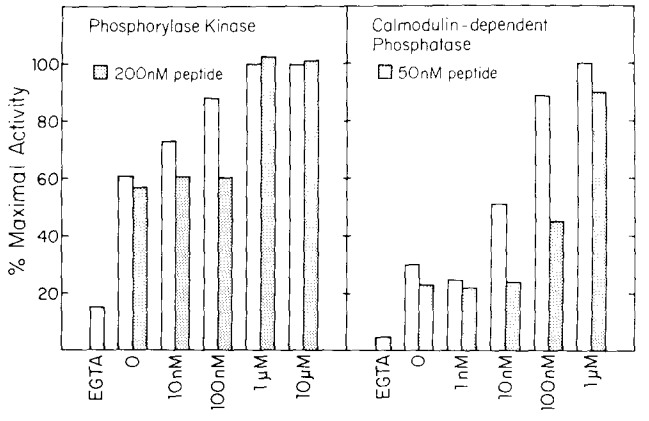

Figure 1. Effects of MLCK Peptide on the Activities of Phosphorylase Kinase and CaM-Dependent Phosphatase (Calcineurin). Phosphorylase kinase and calcineurin were assayed as described in Materials and Methods with the concentrations of CaM indicated on the x-axis. MLCK peptide (MLCK-IV; see Materials and Methods for amino acid sequence) was added at the concentrations indicated to those reaction mixtures represented by stippled bars and omitted from the reaction mixtures depicted by open bars. The bars denoted as "EGTA" contained 3 mM EGTA with no added CaM or peptide.

calcineurin phosphatase activity were similar to those seen with phosphorylase kinase (Fig. 1). The peptide did not inhibit Mn^{++}-stimulated activation of the phosphatase, but did inhibit activation by CaM. With either phosphorylase kinase or calcineurin, adding a molar excess of CaM relative to peptide relieved the peptide's inhibitory effects. The fact that the peptide did not inhibit the CaM-independent/divalent ion-stimulated activity of either enzyme suggests that the peptide has no direct effect on the catalytic domains of these enzymes, nor any effect on their respective integral divalent ion-binding subunits.

The second category of enzymes consists of those that exhibit more classical Ca^{++}/CaM-dependent behavior (i.e.- CaM-free preparations exhibit enzyme activities that are largely dependent on the presence of both Ca^{++} and CaM). Enzymes in this category included bovine brain 61 kDa cyclic nucleotide phosphodiesterase, multifunctional Ca^{++}(CaM)-dependent protein kinase, brain adenylate cyclase, and red cell Ca^{++}-pump ATPase. These enzymes were assayed with a fixed concentration of CaM and increasing concentrations of peptide (Fig. 2). In each case, sufficient CaM was added to achieve partial (50-80% of maximal) activation of the respective enzyme and this initial level of activity is indicated as 100% on the vertical axis of the graph. Because the concentration of CaM required for activation may be markedly different for any two enzymes shown in Fig. 2, the concentration of peptide required for inhibition will not always be equivalent for two particular enzymes. The preparations of phosphodiesterase and multifunctional kinase used had negligible (<1%) basal (CaM-independent) acitivities, whereas the adenylate cyclase and Ca^{++}-ATPase preparations exhibited significant levels of CaM-independent activity (approx. 20% of CaM-stimulated); none of the data shown in Fig. 2 were not corrected for CaM-independent contributions to total activity.

The first panel of fig. 2 shows the inhibition of cyclic nucleotide phosphodiesterase by increasing concentrations of MLCK peptide. Nanomolar concentrations of peptide effectively inhibited phosphodiesterase activity. Since this enzyme has an affinity for CaM comparable to that of the peptide (half-maximal activation, Kact, of this preparation of phosphodiesterase occurred at 1.2 nM CaM), an apparent Ki value could be calculated using an expression described previously [1,11]. The calculated Ki value (3.3 nM) is in good agreement with that obtained using skeletal muscle MLCK (0.9 nM [11]). An excess of CaM (120 nM) completely relieved the inhibition due to 32 nM peptide (data not shown).

The second panel of Fig. 2 shows the effect of increasing MLCK peptide concentration on the activity of the multifunctional Ca^{++}(CaM)-dependent protein kinase. The higher concentrations of peptide required for inhibition compared to the phosphodiesterase (first panel, Fig. 2) reflect the higher Kact of this enzyme for CaM. The addition of 5 μM CaM completely relieved the inhibition due to 320 nM peptide (data not shown). The observed inhibition of the kinase by nanomolar concentrations of peptide indicates the high specificity of the peptide for CaM, since these assays were performed in the presence of 40 μM gizzard myosin light chain, a Ca^{++}-binding protein homologous to CaM.

The inhibition of mammalian CaM-dependent adenylate cyclase obtained by increasing concentrations of MLCK peptide is shown in the third panel of Fig. 2. This enzyme preparation exhibited a significant amount of activity in the presence of excess peptide; this residual activity was not inhibited by the addition of a molar excess of EGTA relative to Ca^{++} (data not shown). This is consistent with the peptide's acting on CaM rather than through a direct inhibition of the catalytic domain of the enzyme.

The effects of increasing MLCK peptide concentration on the activity of red cell membrane Ca^{++}-pump ATPase is shown in the fourth panel of Fig. 2. Similar to the mammalian adenylate cyclase (third panel), this enzyme has a relatively high basal activity (activity in the absence of any added CaM) that was not inhibited by

excess peptide. This again suggests the peptide is acting solely via CaM and not through a direct effect on catalytic activity. If the peptide is incubated at 37° for prolonged periods (1 hr) with this enzyme preparation, little or no inhibition of ATPase activity is observed (data not shown). This is most likely due to the presence of proteases and/or peptidases in the red cell membrane preparation that degrade the peptide to an inactive form during the long incubation.

The final category is composed of a single enzyme, Bordetella pertussis adenylate cyclase, which displays CaM-dependent activity in the presence and absence of Ca^{++} [25]. Data obtained with this enzyme are shown in Figure 3. This enzyme was assayed in the presence or absence of Ca^{++}, CaM, or both, and the effect of increasing MLCK peptide concentration on each activity determined. The peptide had no effect on the activity of the Bordetella adenylate cyclase in the absence of Ca^{++}, whereas the peptide caused nearly total inhibition of CaM-dependent activity in the presence of Ca^{++}. These data are consistent with the interaction of the peptide and CaM being Ca^{++}-dependent and with the peptide's inhibitory effects being mediated through its binding to CaM.

The data presented here indicate that a synthetic peptide corresponding to the CaM-binding domain of rabbit skeletal muscle MLCK antagonizes the interaction of CaM with a number of other CaM-dependent enzymes. Evidence that the MLCK peptide acts by binding to CaM was provided by the following observations: 1) The peptide was effective at nanomolar concentrations, consistent with its high affinity for CaM; 2) Peptide inhibition was overcome by adding a molar excess of CaM; 3) Several of the enzymes studied exhibited

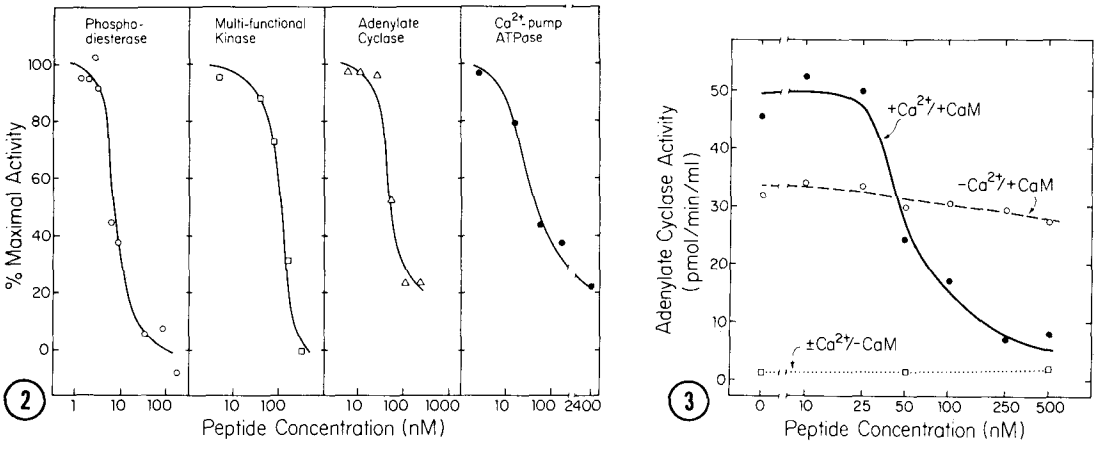

Figure 2. Effects of MLCK Peptide on the Activities of Four Mammalian CaM-Dependent Enzymes. Details of enzyme assays are described in Materials and Methods. Each enzyme was assayed at a fixed concentration of CaM and the concentration of MLCK peptide was varied as indicated. Maximal activity is defined as the activity of the enzyme in the absence of added peptide. The first panel depicts the activity of cyclic nucleotide phosphodiesterase (○) which was assayed with 3.3 nM CaM and increasing concentrations of MLCK peptide (MLCK-I; see Materials and Methods for amino acid sequence); in the absence of peptide this concentration of CaM gave 70% of the activity seen with saturating CaM concentrations. The second panel shows the activity of multifunctional Ca^{++} (CaM)-dependent protein kinase (□) as a function of MLCK peptide (MLCK-IV). The concentration of CaM present in the myosin light chain preparation used as substrate in this assay was sufficient to activate the kinase to 60% of that activity observed with saturating CaM concentrations; therefore no additional CaM was added to the assay mixture. The activity of brain adenylate cyclase (△) as a function of MLCK peptide (MLCK-IV) is shown in the third panel. The concentration of CaM in the assay (50 nM) was sufficient to activate the enzyme to 50% of that activity observed with saturating CaM. The fourth panel shows the effect of MLCK peptide (MLCK-IV) on the activity of red cell membrane Ca^{++} -pump ATPase (●). The final concentration of CaM in this assay was 133 nM.

Figure 3. Effects of MLCK Peptides on Bordetella pertussis Adenylate Cyclase Activity. Enzyme activity was assayed as described in Materials and Methods in the presence of the indicated concentrations of MLCK peptide (MLCK-IV). EGTA (0.22 mM) was included in all assays, whereas Ca^{++} (0.25 mM) and CaM (50 nM) were included (+) or not included (-), as indicated.

CaM-independent activities and these activities were not affected by concentrations of peptide that completely inhibited CaM-dependent activity; 4) Inhibition of the Bordetella adenylate cyclase was Ca^{++}-dependent, consistent with the interaction of peptide and CaM being Ca^{++}-dependent. Considering their high affinity and the broad spectrum of target enzymes they antagonize, the MLCK CaM-binding peptides should be generally useful tools in studying CaM-dependent enzymes and processes. The MLCK peptides may be particularly useful in identifying new CaM-dependent processes as recently demonstrated with the melanotropin-sensitive adenylate cyclase [26].

That a relatively small peptide derived from the CaM-binding domain of a target enzyme can antagonize the activation of seven different CaM-dependent enzymes indicates that a molecule of CaM only interacts with one target enzyme at a time. The simplest explanation for this is that the peptide and these different target enzymes compete for a common binding determinant or domain on CaM. Alternatively, a conformational change in CaM may be induced by peptide binding that distorts or obscures determinants required for target enzyme recognition at sites distinct from the peptide binding site, thus antagonizing enzyme activation. At present it is not possible to distinguish between these two alternatives; however, it is anticipated that structural studies of CaM interactions with synthetic peptides based on other target enzyme CaM-binding domains will provide the data necessary to answer this important question.

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References

[1] Blumenthal, D.K. and Krebs, E.G. (1987) Methods Enzymol. 139, 115-126.
[2] Blumenthal, D.K. and Krebs, E.G. (1988) In Calmodulin (Cohen, P. and Klee, C.B., eds.) Molecul. Aspects Cell. Regul., vol. 5, pp. 341-356, Elsevier, Amsterdam.
[3] Klevit, R.E., Blumenthal, D.K., Wemmer, D.E., and Krebs, E.G. (1985) Biochemistry 24, 8152-8157.
[4] Klevit, R.E. and Blumenthal, D.K. (1987) In Proceedings of the 5th International Symposium on Calcium Binding Proteins in Health and Disease, pp. 333-347, Academic Press, Orlando, FL.
[5] Newton, D., Klee, C., Woodgett, J., and Cohen, P. (1985) Biochim. Biophys. Acta 845, 533-539.
[6] Craig, T.A., Watterson, D.M., Prendergast, F.G., Haiech, J., and Roberts, D.M. (1987) J. Biol. Chem. 262, 3278-3284.
[7] Hansen, R.S. and Beavo, J.A. (1986) J. Biol. Chem. 261, 14636-14645.
[8] Putkey, J.A., Draetta, G.F., Slaughter, G.R., Klee, C.B., Cohen, P., Stull, J.T., and Means, A.R. (1986) J. Biol. Chem. 261, 9896-9903.
[9] Zimmer, M. and Hofmann, F. (1987) Eur. J. Biochem. 164, 411-420.
[10] Roskoski, R. (1983) Methods Enzymol. 99, 3-6.
[11] Blumenthal, D.K., Takio, K., Edelman, A.M., Charbonneau, H., Titani, K., Walsh, K.A., and Krebs, E.G. (1985) Proc. Natl. Acad. Sci. USA 82, 3187-3191.
[12] Cohen, P. (1973) Eur. J. Biochem. 34, 1-14.
[13] Blumenthal, D.K., Takio, K., Hansen, R.S., and Krebs, E.G. (1986) J. Biol. Chem. 261, 8140-8145.
[14] Sharma, R.K., Taylor, W.A., and Wang, J.H. (1983) Methods Enzymol. 102, 210-219.
[15] Schulman, H. (1984) J. Cell Biol. 99, 11-19.
[16] Gallis, B., Edelman, A.M., Casnellie, J.E., and Krebs, E.G. (1983) J. Biol. Chem. 285, 13089-13093.
[17] Yeager, R.E., Heideman, W., Rosenberg, G.B., and Storm, D.R. (1985) Biochemistry 24, 3776-3783.
[18] Salomon, Y., Londos, D., and Rodbell, M. (1974) Anal. Biochem. 58, 541-548.
[19] Shattuck, R.L., Oldenburg, D.J., and Storm, D.R. (1985) Biochemistry 24, 6356-6362.
[20] Raess, B.U. and Vincenzi, F.F. (1980) J. Pharmacol. Methods 4, 273-283.
[21] Foder, B. and Scharff, O. (1981) Biochim. Biophys. Acta 649, 367-376.
[22] Raess, B.U. and Vincenzi, F.F. (1980) Molec. Pharmacol. 18, 253-258.
[23] Grand, R.J.A., Shenolikar, S., and Cohen, P. (1981) Eur. J. Biochem. 113, 359-367.
[24] Aitken, A., Klee, C.B., and Cohen, P. (1984) Eur. J. Biochem. 139, 663-671.
[25] Greenlee, D.V., Andreasen, T.J., and Storm, D.R. (1982) Biochemistry 21, 2759-2764.
[26] Gerst, J.E. and Salomon, Y. (1988) J. Biol. Chem. 263, 7073-7078.