PHOSPHORYLATION OF SKELETAL MUSCLE MYOSIN LIGHT CHAIN KINASE BY THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE

Arthur M. EDELMAN and Edwin G. KREBS

Howard Hughes Medical Institute Laboratory, Department of Pharmacology, University of Washington School of Medicine, Seattle, WA 98195, USA

Received 4 January 1982

1. Introduction

The phosphorylation of myosin, initially observed in skeletal muscle [1], has now been studied in other muscle types as well as in a variety of non-muscle tissues [2,3]. The clearest understanding of the function of myosin phosphorylation is in smooth muscle where it appears to be obligatory for actin activation of myosin ATPase activity and correlates with contraction (review [2]). In skeletal muscle, myosin phosphorylation appears to play more of a modulatory role, for example participating in post-tetanic twitch potentiation [4].

The phosphorylation of myosin occurs on one of the light chain subunits (LC_2 M_r 18 500 in skeletal muscle) catalyzed by a cyclic nucleotide-independent enzyme, myosin light chain kinase (MLCK). The enzyme has been purified from a variety of sources. From all sources it is completely dependent for activity upon the presence of calcium and the calcium-dependent regulator, calmodulin [5,6], and is highly specific for LC_2 of myosin. There are, however, some reported differences in the enzyme as isolated from different sources. Although highly specific for myosin as compared with other possible protein substrates, MLCK from a given muscle type preferentially phosphorylates the light chain from the same muscle type [7,8]. By SDS gel electrophoresis, the apparent M_r-values of the purified enzymes were reported to be 77 000–90 000 in skeletal muscle [9–13], 94 000 in cardiac muscle [14], but 130 000 in smooth muscle [8]. Several reports have suggested that the enzyme is the same size in all muscle types (M_r 130 000–160 000) [15,16]. However, since MLCKs of this size have not yet been purified to homogeneity from skeletal and cardiac muscle, the issue has remained unsettled.

Another apparent difference between MLCKs from the different muscle types lies in their ability to be regulated by cAMP-dependent phosphorylation. Cyclic AMP-dependent protein kinase was shown to phosphorylate smooth muscle MLCK causing a decrease in MLCK activity due to a drop in the enzyme's affinity for calmodulin [17,18]. Cardiac muscle MLCK was also reported to be phosphorylated, although either without effect on activity [14] or with a relatively small effect [15] (compared with smooth muscle MLCK).

To date, phosphorylation of skeletal muscle MLCK by cAMP-dependent protein kinase has not been demonstrated. Since cyclic nucleotide-dependent phosphorylation regulates a broad spectrum of enzymatic activities [19], the smooth muscle MLCK being one of them, the possibility of phosphorylation was investigated for skeletal muscle MLCK.

Here, we demonstrate that purified skeletal muscle MLCK can be phosphorylated at a significant rate by cAMP-dependent protein kinase. Unlike the smooth muscle MLCK, however, this phosphorylation does not affect the affinity of the enzyme for its activator, calmodulin.

Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl) ether-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; CaM, calmodulin; MES, 2[*N*-morpholino]ethanesulfonic acid; DTT, dithiothreitol; PIPES, piperazine-*N,N'*-bis-(2-ethane sulfonic acid); cAMP, adenosine 3':5'-cyclic monophosphate; LC_2, 18 500 M_r phosphorylatable myosin light chain; MLCK, myosin light chain kinase

2. Materials and methods

2.1. *Protein purifications*

Unless otherwise indicated all protein purification procedures were at 0–4°C.

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

Myosin and mixed myosin light chains were prepared from rabbit skeletal muscle as in [20,21] with minor modifications.

Calmodulin-free light chains were prepared from the mixed light chain fraction as follows: light chains (85 mg) were applied to DEAE-cellulose (at 22°C) (25 × 1.5 cm) equilibrated with 25 mM Tris, 10 mM NaCl, 1 mM DTT, 7 M urea (pH 7.5) and eluted with a linear gradient (total vol. 300 ml) of 10–180 mM NaCl in the buffer. The position of the M_r 18 500 phosphorylatable light chain (LC_2) was detected by SDS gel electrophoresis. Fractions containing this subunit were pooled, dialyzed exhaustively against 0.5 mM Tris–HCl, 0.1 M NaCl, 0.1 mM DTT (pH 7.9) concentrated by vacuum dialysis and stored at –20°C. By densitometric scanning of the Coomassie blue-stained SDS gel, this preparation contained 69% LC_2 and 31% of the M_r 25 000 light chain. In this preparation and in the mixed light chain preparation, LC_2 was the only subunit of myosin subject to phosphorylation by MLCK.

Myosin light chain kinase was purified from back and hind limb skeletal muscle of freshly killed rabbits. The procedure employed ammonium sulfate fractionation followed by sequential DEAE-cellulose, Sephacryl S-200 and calmodulin–Sepharose chromatography. Additional chromatography on calmodulin–Sepharose and Sephacryl S-200 was utilized to remove minor contaminants. The purification was similar to one utilized for the purification of platelet MLCK [22]. The enzyme was essentially homogeneous (see fig.1) on sodium dodecyl sulphate (SDS) gel electrophoresis. By this technique, using the standards indicated (see section 2.4) an M_r of 91 000 was obtained. Our report for this preparation indicated M_r 95 000–97 000 [23]; however, when the M_r of one of the gel standards (β-galactosidase) was based on its amino acid sequence [24] rather than ultracentrifugation methods, M_r 91 000 for MLCK is obtained. The specific activity of the purified enzyme was 23 μmol ^{32}P incorp. min^{-1}. mg $MLCK^{-1}$.

Catalytic subunit of cAMP-dependent protein kinase (type II) was purified from bovine heart essentially as in [25]. By SDS–polyacrylamide gel electrophoresis the enzyme was apparently homogeneous. The specific activity was 0.86 μmol ^{32}P transferred to mixed histones . min^{-1}. mg^{-1} for the preparation used in determining the rate of phosphorylation of MLCK. The phrase cAMP-dependent protein kinase as used here refers to the isolated catalytic subunit.

Calmodulin was purified from bovine brain as in [26].

2.2. *^{32}P Incorporation into MLCK*

Unless otherwise indicated, 2.5 μM MLCK was incubated at 25°C with or without 0.1 μM cAMP-dependent protein kinase, 0.5 mM DTT, 2 mM EGTA, 0.1 M NaCl, 0.5 mg bovine serum albumin/ml, 0.1 mM [γ-^{32}P]ATP (0.3–0.5 Ci/mmol), 12.5 mM $MgCl_2$, 50 mM MES (pH 6.6) and traces of buffer components from the protein storage buffers for varying times. ^{32}P Incorporation was determined [27] by spotting an aliquot of the reaction mixture onto a 2 × 2 cm square of ET-cellulose paper (Whatman), which was then immersed in trichloroacetic acid containing 2% sodium pyrophosphate (0°C). After washing in this solution for 30 min the paper was washed twice for 15 min each time with 5% trichloroacetic acid (22°C), briefly rinsed in 95% ethanol, dried, and subjected to liquid scintillation spectrometry. Blank values (MLCK omitted or MLCK and protein kinase omitted) were subtracted.

2.3. *MLCK assay*

All enzyme assays were carried out at 25°C. Unless otherwise indicated, MLCK was assayed in a solution (pH 6.5) containing 0.1 M Pipes, 0.6 mM DTT, 0.6 mg bovine serum albumin/ml, 1 mM [γ-^{32}P]ATP (0.06 Ci/mmol), 12.5 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.2 mM EGTA, 0.03 M NaCl, 0.01 M KCl, 5.38 mg calmodulin-free myosin light chains/ml (LC_2 final conc. 200 μM), 1.3 nM MLCK, varying concentrations of calmodulin and traces of buffer components from the various protein storage buffers. After incubation for 12 min (during which time the reaction rate was linear) an aliquot was spotted on cellulose paper and processed as in section 2.2. Blank values (determined by the omission of substrate from the reaction) were subtracted. The free calmodulin concentrations in the assay were calculated as follows:

$$[CaM]_{free} = [CaM]_{total} - [CaM]_{bound}$$

$$[CaM]_{bound} = [MLCK]_{bound} = [MLCK]_{total} \times$$

(Fractional occupancy)

Defn.: Fractional occupancy = MLCK activity/ MLCK activity at saturating calmodulin

This calculation utilizes the facts that MLCK is com-

pletely dependent upon added calmodulin and that calmodulin and skeletal muscle MLCK interact with a 1:1 stoichiometry [12,13,23].

2.4. *Gel electrophoresis*

MLCK was analyzed in a 7.5% acrylamide SDS gel [28] with the following M_r standards: β-galactosidase (116 300); phosphorylase *b* (97 400); bovine serum albumin (66 200); IGG-heavy chain (51 600); ovalbumin (44 000); aldolase (40 000); deoxyribonuclease I (31 000).

2.5. *Phosphoamino acid analysis*

MLCK (3.5 μg) phosphorylated after incubation for 200 min under the conditions in section 2.2 was partially acid hydrolyzed in 1.25 ml 5.7 N HCl at 110°C for 2 h in the presence of 2 mg phenol and 0.3 mg each of unlabeled phosphoserine and phosphothreonine standards. After evaporation under vacuum, the residue was redissolved in 17 μl H_2O; 5 μl was then applied to 3 MM paper (Whatman) and electrophoresed at 3000 V for 100 min at pH 1.9 (2.5% formic acid, 7.5% acetic acid). Phosphoamino acids were detected with ninhydrin and autoradiography.

2.6. *Other methods*

Autoradiography was done using Cronex X-ray film (Dupont) with an intensifying screen at 22°C (fig.1) or −70°C (fig.2). Protein concentrations were determined spectrophotometrically for calmodulin ($E^{0.1\%}_{280} = 0.21$) and cAMP-dependent protein kinase ($E^{0.1\%}_{280} = 1.23$) and using a combined biuret–Lowry procedure [29] for MLCK and myosin light chains.

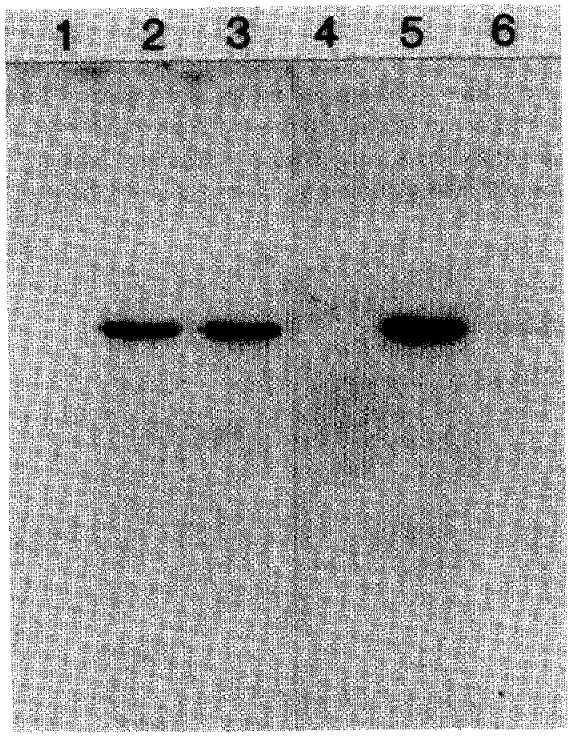

Fig.1. SDS–polyacrylamide gel electrophoresis of phosphorylated skeletal muscle myosin light chain kinase. MLCK was incubated for 90 min with or without catalytic subunit of cAMP-dependent protein kinase as in section 2 with the exception that albumin was omitted. Boiling for 3 min in the presence of 1% SDS and 2% β-mercaptoethanol served to stop the reaction. Samples were subjected to SDS gel electrophoresis as in section 2: Coomassie blue staining pattern; (4–6) autoradiograph of the gel; (1,4) complete reaction mix minus MLCK, including protein kinase; (2,5) complete reaction mix including both MLCK and protein kinase; (3,6) complete reaction mix including MLCK, minus protein kinase.

3. Results

Fig.1 illustrates the phosphorylation of purified skeletal muscle myosin light chain kinase (M_r 91 000) by catalytic subunit of cyclic AMP-dependent protein kinase. Also illustrated is a slight apparent autophosphorylation of MLCK*.

The phosphorylation of MLCK by cAMP-dependent protein kinase occurred at serine although an extremely slight incorporation into threonine could also be detected (fig.2).

The time course of phosphorylation of MLCK is illustrated in fig.3. Under these conditions, multiple determinations yielded slightly >1 mol ^{32}P/mol MLCK (1.25 ± 0.12, SD). Adding fresh cAMP-dependent protein kinase after complete incorporation had been achieved led to only a very slight additional incorporation (0.08 mol ^{32}P/mol MLCK after an additional 100 min incubation). This indicates that the plateau in the time course is not due to inactivation of the protein kinase. The initial rate of phosphorylation of MLCK by protein kinase was 15.3 nmol $^{32}P \cdot min^{-1} \cdot mg$ cAMP-dependent protein kinase^{-1} when assayed at

* We have found, in addition, a calcium and calmodulin-dependent autophosphorylation of MLCK, which has a much greater rate than the apparent autophosphorylation in the presence of EGTA (unpublished)

1 μM MLCK. The slightly greater than stoichiometric phosphorylation of MLCK could be due, at least in part, to the slight apparent autophosphorylation (fig.3) (if this occurs at a different site than phosphorylation by cAMP-dependent protein kinase). The apparent autophosphorylation is not due to any contaminants

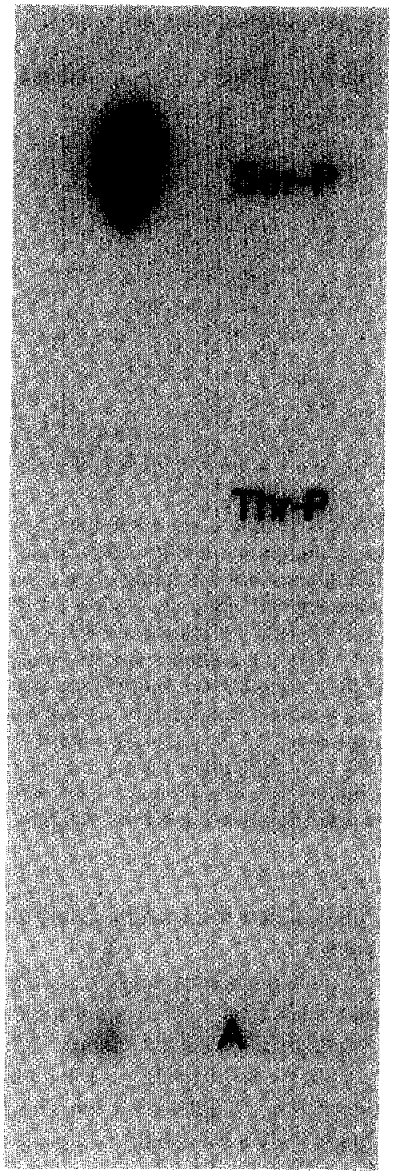

Fig.2. Identity of amino acid in myosin light chain kinase phosphorylated by catalytic subunit of cAMP-dependent protein kinase. Phosphoamino acid analysis was performed as in section 2. A = incompletely hydrolyzed material. The section of the paper containing the $^{32}P_i$ was removed prior to autoradiography.

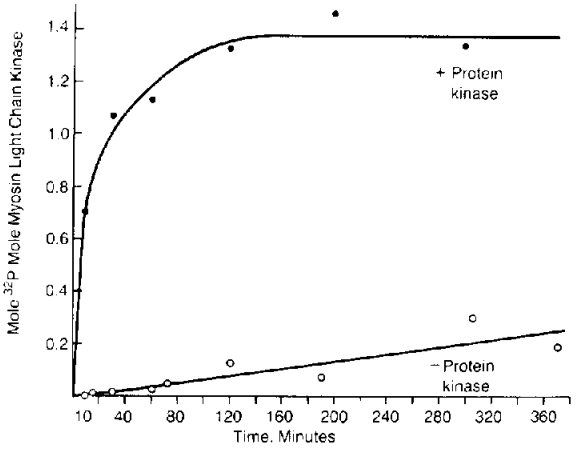

Fig.3. Time course of phosphorylation of skeletal muscle myosin light chain kinase in the presence (•——•) or absence (○——○) of cAMP-dependent protein kinase. MLCK (2.5 μM) was incubated for varying lengths of time with or without 0.1 μM cAMP-dependent protein kinase and ^{32}P incorporation into MLCK measured as in section 2.

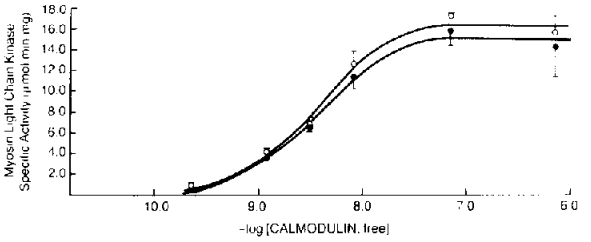

Fig.4. Activation of phosphorylated (•——•) or non-phosphorylated (○——○) myosin light chain kinase by calmodulin. MLCK was phosphorylated as in section 2 with the exception that 2 μM cAMP-dependent protein kinase was utilized to reduce the time needed for this incubation. The non-phosphorylated control was the complete incubation minus cAMP-dependent protein kinase, $n = 3$ separate incubations for each condition. After 10 min (during which time the levels of incorporation measured as in section 2 were 1.35 mol ^{32}P/mol MLCK for the phosphorylation condition and 0.02 mol ^{32}P/mol MLCK for the non-phosphorylation condition), all incubations were diluted 200-fold into ice cold buffer containing 50 mM MES (pH 6.6), 0.1 M NaCl, 2 mM EGTA, 0.5 mM DTT and 0.5 mg bovine serum albumin/ml. MLCK activity was then measured as in section 2. Values were corrected for a slight increase in the blank in the presence of cAMP-dependent protein kinase (perhaps due to a low level of LC_2 phosphorylation by cAMP-dependent protein kinase [30]). Each of the 6 incubations generated a separate calmodulin titration curve. Averaged curves are shown ± SD.

present in the albumin used to stabilize the enzymes during the kinetic experiments in that the extent is unaffected by the omission of albumin, but theoretically could be due to trace amounts of a contaminating kinase(s) in the MLCK preparation. Autophosphorylation in the presence of EGTA has also been observed for MLCK from smooth muscle [17]. In addition, a slow second site phosphorylation by cAMP-dependent protein kinase could also contribute to the greater than stoichiometric phosphorylation of MLCK. Upon prolonged incubation with high concentrations of enzyme (0.6 μM cAMP-dependent protein kinase, 3–5 h), 1.6–1.9 mol ^{32}P/mol MLCK was incorporated (not shown).

Purified skeletal muscle MLCK was completely dependent for activity upon the presence of calcium and calmodulin with K_a 1–4 nM for calmodulin. When the purified MLCK was phosphorylated by cAMP-dependent protein kinase, there was no change in the K_a of MLCK for calmodulin and only a slight, statistically non-significant, (at $P_{0.05}$ by t-test) change in the V_{max} (fig.4). In addition, when phosphorylated *vs* non-phosphorylated MLCK was assayed at subsaturating concentrations of light chain, ATP and calmodulin the progress curves were essentially identical (not shown).

4. Discussion

These results indicate that skeletal muscle MLCK shares with the cardiac and smooth muscle enzymes the property of phosphorylation by cAMP-dependent protein kinase. For the skeletal muscle enzyme this phosphorylation appears not to be involved in the modulation of MLCK activity. This is similar to that reported for the purified cardiac muscle MLCK [14], although in [15], a 3-fold change was noted in the affinity of a partially purified cardiac MLCK for calmodulin after incubation with cAMP-dependent protein kinase. By contrast, smooth muscle MLCK shows a 10–20-fold drop in its affinity for calmodulin upon phosphorylation by cAMP-dependent protein kinase [18].

It is, at present, unclear whether the purified M_r 91 000 skeletal muscle MLCK is a tissue specific isozyme or is identical with the larger smooth muscle enzyme but is modified by proteolysis during isolation. If the skeletal and smooth muscle enzymes are indeed identical in the tissue, it should be possible to demonstrate an effect of cAMP-dependent phosphorylation upon skeletal muscle MLCK activity similar to the effect on smooth muscle MLCK when the skeletal muscle enzyme is utilized from a very early stage of purification, i.e., when presumably no or minimal proteolysis had occurred. We have tried to find a change in the affinity of MLCK for calmodulin in crude skeletal muscle supernatants after incubation with MgATP, cAMP, and cAMP-dependent protein kinase and have been unsuccessful (unpublished). This suggests either that skeletal muscle MLCK is degraded extremely rapidly in the extract, or is an isozyme whose phosphorylation does not alter its activity.

Smooth muscle MLCK is phosphorylated by cAMP-dependent protein kinase at 2 sites in the presence of EGTA (initial rate = 0.2 μmol . min^{-1}. mg cAMP-dependent protein kinase^{-1}) and 1 site when calmodulin is bound to MLCK (initial rate = 0.02 μmol . min^{-1}. mg cAMP-dependent protein kinase^{-1}) [18,31]. Our results were that skeletal muscle MLCK incubated with cAMP-dependent protein kinase in the presence of EGTA incorporated slightly >1 mol ^{32}P at an initial rate of 15.3 nmol . min^{-1}. mg^{-1}. This rate is ~25-fold slower than the phosphorylation by cAMP-dependent protein kinase of pyruvate kinase but is roughly equal to the rate of phosphorylation of ATP citrate lyase and cardiac troponin I (based on [32–34], correcting for differing assay conditions). Given 0.32 μmol cAMP-dependent protein kinase/kg [35], and correcting for temperature, it is theoretically possible for MLCK to be phosphorylated in skeletal muscle with a $t_{1/2}$ of ~1 min. One possible role for this phosphorylation consistent with such a rate is suggested by studies on the phosphorylation of myosin itself. In [36] it was shown that the phosphorylation of skeletal muscle myosin alters its conformation making it less susceptible to proteolysis. Such a process could operate in vivo to regulate myosin turnover and by analogy the phosphorylation of MLCK might serve a similar regulatory role.

Acknowledgements

We wish to thank Floyd Kennedy and Barbara Flug for the preparation of cAMP-dependent protein kinase, Warren Heideman for a portion of the calmodulin used, and Sally Hopkins for expert secretarial assistance. This work was partially supported by a postdoctoral fellowship to A. M. E. from the Muscular Dystrophy Association. E. G. K. is a Senior Investigator of the Howard Hughes Medical Institute.

References

[1] Perrie, W. T., Smillie, L. B. and Perry, S. V. (1973) Biochem. J. 135, 151–164.
[2] Stull, J. T., Blumenthal, D. K. and Cooke, R. (1980) Biochem. Pharmacol. 29, 2537–2543.
[3] Adelstein, R. S. and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921–956.
[4] Manning, D. R. and Stull, J. T. (1979) Biochem. Biophys. Res. Commun. 90, 164–170.
[5] Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M. and Uenishi, K. (1978) J. Biol. Chem. 253, 1338–1340.
[6] Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K. and Hartshorne, D. J. (1978) Biochemistry 17, 253–258.
[7] Stull, J. T., Blumenthal, D. K., DeLanerolle, P., High, C. W. and Manning, D. R. (1978) Adv. Pharmacol. Therap. 4, 171–180.
[8] Adelstein, R. S. and Klee, C. B. (1981) J. Biol. Chem. 256, 7501–7509.
[9] Pires, E. and Perry, S. V. (1977) Biochem. J. 167, 137–146.
[10] Yazawa, M. and Yagi, K. (1977) J. Biochem. (Tokyo) 82, 287–289.
[11] Nairn, A. C. and Perry, S. V. (1979) Biochem. J. 179, 89–97.
[12] Blumenthal, D. K. and Stull, J. T. (1980) Biochemistry 24, 5608–5614.
[13] Crouch, T. H., Holroyde, M. J., Collins, J. H., Solaro, R. J. and Potter, J. D. (1981) Biochemistry 20, 6318–6325.
[14] Wolf, H. and Hofmann, F. (1980) Proc. Natl. Acad. Sci. USA 77, 5852–5855.
[15] Walsh, M. P. and Guilleux, J. C. (1981) Adv. Cyclic Nucl. Res. 14, 375–390.
[16] Guerriero, V. and Means, A. R. (1981) Fed. Proc. FASEB 40, 1738.
[17] Adelstein, R. S., Conti, M. A., Hathaway, D. R. and Klee, C. B. (1978) J. Biol. Chem. 253, 8347–8350.
[18] Conti, M. A. and Adelstein, R. S. (1981) J. Biol. Chem. 256, 3178–3181.
[19] Krebs, E. G. and Beavo, J. A. (1979) Annu. Rev. Biochem. 48, 923–959.
[20] Kielley, W. W. and Bradley, L. B. (1956) J. Biol. Chem. 218, 653–659.
[21] Holt, J. C. and Lowey, S. (1975) Biochemistry 15, 4600–4609.
[22] Hathaway, D. R. and Adelstein, R. S. (1979) Proc. Natl. Acad. Sci. USA 76, 1653–1657.
[23] Andreason, T. J., Keller, C. H., LaPorte, D. C., Edelman, A. M. and Storm, D. R. (1981) Proc. Natl. Acad. Sci. USA 78, 2782–2785.
[24] Fowler, A. V. and Zabin, I. (1978) J. Biol. Chem. 253, 5521–5525.
[25] Bechtel, P. J., Beavo, J. A. and Krebs, E. G. (1977) J. Biol. Chem. 252, 2691.
[26] LaPorte, D. C., Toscano, W. A. jr and Storm, D. R. (1979) Biochemistry 181, 2820–2825.
[27] Corbin, J. D. and Reimann, E. M. (1975) Methods Enzymol. 38/C, 287–290.
[28] Laemmli, U. K. (1970) Nature 227, 680–685.
[29] Dorsey, T. E., McDonald, P. W. and Roels, O. A. (1977) Anal. Biochem. 78, 156–164.
[30] Noiman, E. S. (1980) J. Biol. Chem. 255, 11067–11070.
[31] Adelstein, R. S. (1981) personal communication.
[32] Berglund, L., Ljungstrom, D. and Engstrom, L. (1977) J. Biol. Chem. 252, 613–619.
[33] Guy, P. S., Cohen, P. and Hardie, D. G. (1980) FEBS Lett. 109, 205–208.
[34] Stull, J. T. and Buss, J. E. (1977) J. Biol. Chem. 252, 851–857.
[35] Hofmann, F., Bechtel, P. J. and Krebs, E. G. (1977) J. Biol. Chem. 252, 1441–1447.
[36] Ritz-Gold, C. J., Cooke, R., Blumenthal, D. K. and Stull, J. T. (1980) Biochem. Biophys. Res. Commun. 93, 209–214.