Volume 327, number 1, 35–40 FEBS 12717 July 1993

Effect of okadaic acid on protein phosphorylation patterns of chicken myogenic cells with special reference to creatine kinase

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Received 17 May 1993

Okadaic acid and other agents affecting cellular phosphorylation and dephosphorylation processes profoundly changed the phosphoprotein pattern of $^{32}P_i$-labelled chicken embryonic skeletal muscle cells. The phosphorylation states of proteins in the lower molecular weight range were especially increased. Immunoprecipitation of cellular extracts with anti-creatine kinase antibodies enabled us to identify creatine kinase (CK) phosphoproteins. B-CK was phosphorylated after treating the cultures with 1-oleoyl-2-acetyl-*sn*-glycerol, dibutyryl-cAMP, okadaic acid and combinations thereof, but not with 1,2-dioleoyl-*sn*-glycerol M-CK was also shown to be phosphorylated. The results indicated that in vivo, CK isoforms in muscle are subjected to control mediated by phosphorylation and dephosphorylation processes.

Phosphorylation of creatine kinase; Okadaic acid, Chicken; Myogenic cell

1. INTRODUCTION

Creatine kinase (CK) is a key enzyme of eukaryotic energy metabolism. During muscle contraction, CK catalyzes the regeneration of ATP from phosphorylcreatine (PCr) and ADP (reviewed in [1]). In differentiating chicken embryonic skeletal muscle cells, a CK isoenzyme transition from brain-type CK (B-CK) to muscle-specific CK (M-CK) takes place during the period of myotube formation; B-CK is expressed at a high level, until after M-CK expression has started. This isoenzyme switch is part of the biochemical differentiation which accompanies the morphological development of myoblasts. Cultured embryonic skeletal muscle cells undergo a very similar development and also show the B- to M-CK transition [2]. After leaving the cell cycle, postmitotic cultured myoblasts align themselves into arrays and subsequently fuse to form multinucleated myotubes (see references in [3]). The myotubes show sarcomeric striations and are able to contract in culture, suggesting that their cellular metabolism resembles the in vivo situation. Essential steps in the process of myoblast fusion depend on the action of various protein kinases, such as the phospholipid- and Ca^{2+}-dependent protein kinase (PKC) and the Ca^{2+}/calmodulin-dependent protein (CaM-) kinase [3]. Cyclic AMP-dependent protein kinase (PKA) activity is also present during the time of fusion [4].

Several recent reports indicated that rat, chicken and mouse B-CKs are phosphoproteins [5–7]. Rabbit M-CK was shown to autophosphorylate in vitro [8]. Mouse and human M-CK were phosphorylated in vitro by PKA and CaM-kinase [9], and mouse B-CK by PKC [10]. Phosphorylated forms of chicken and mouse B-CK have been shown to display an increased affinity for PCr [6,10]. It was therefore of interest to determine whether chicken CK is phosphorylated in vivo. The extracts of ^{32}P-labelled cultures of chicken myogenic skeletal muscle cells were analysed by SDS-PAGE, immunoprecipitation and 2D-gel electrophoresis. Cells were treated with okadaic acid (OA), a potent inhibitor of the catalytic subunits of type 1- (PP1) and type 2A phosphatases (PP2A) [11,12], and with other membrane permeable substances which influence cellular de-/phosphorylation processes. The use of these substances enabled us to show that, in living skeletal muscle cells, chicken B-CK as well as M-CK are indeed subject to phosphorylation and dephosphorylation.

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Abbreviations: B- and M-CK refer to brain- and muscle-type creatine kinase isoenzymes, respectively; CaM-kinase, Ca^{2+}/calmodulin-dependent protein kinase; DAG, diacylglycerol; dbcAMP, di-butyryl-3′-5′-cAMP; OA, okadaic acid; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PCr, phosphocreatine; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PP1 and PP2A, type 1 and type 2A phosphatase, respectively.

2. MATERIALS AND METHODS

2.1. *Cell culture and $^{32}P_i$-labelling*

Primary cell cultures were prepared from thigh muscle of 11-day-old

white Leghorn chick embryo as described [13] The cells were plated at 4×10^5 cells per 6-cm plate in 3 ml standard medium (199 with Earle's salts (Amimed) supplemented with 1 mM glutamine, 10% horse serum, 2% chick embryo extract and standard concentrations of penicillin and streptomycin. A complete medium change was carried out 24 h after plating. At different developmental stages (myoblasts at 42 h and myotubes at 96 to 120 h after plating), the cells were starved for 3 h in phosphate-free medium (MEM with EBS, Amimed), followed by labelling for 150 min with 0 5 mCi $^{32}P_i$ (Amersham, PBS13) per dish and treatment with several agents which influence cellular phosphorylation/dephosphorylation processes For methionine labelling, 42 h cultures were starved for 1 h in serum free 199 medium, lacking methionine but containing antibiotics and glutamine as described above. Then cells were labelled for 2 h with standard medium supplemented with 100 μCi [^{35}S]methionine (Amersham) per dish

2.2 *Drug additions*

All agents were prepared as stock solutions and diluted with phosphate-free medium prior to use. The final ethanol concentration introduced by dissolved agents to the cell cultures was less than 0.1% Controls were dosed with corresponding amounts of ethanol The diacylglycerol (DAG) analogue 1-oleoyl-2-acetyl-*sn*-glycerol (here referred to as OAG) and 1,2-dioleoyl-*sn*-glycerol (DAG analogue which does not cross membranes), both from Sigma, were dissolved at 10^{-2} M in ethanol and diluted to a final concentration of 15 μM. Dibutyryl-3′-5′-cAMP (abbreviated as dbcAMP, Sigma) was prepared as 10^{-2} M stock solution in phosphate free medium and used at a final concentration of 10^{-4} M Okadaic acid (OA; kind gift from Dr. Y. Tsukitani, Fujisawa Chemical Co , Tokyo, Japan) was prepared as 5 mM ethanolic stock solution OA was added only for the last 45 min of labelling, whereas the other agents were present during the complete labelling period. Drug treated cells were controlled under the light microscope; no morphological changes were detectable using this method, even in myoblasts treated for 1 h with the highest OA concentration (1 μM) Additional evidence that the drug treatments did not severely damage the cells is given by the fact that myoblasts still differentiated to myotubes after OA treatment, provided that the drug-containing medium was replaced with conditioned medium, taken from untreated parallel cultures.

2 3. *Immunoprecipitation and gel analysis*

After washing in ice-cold PBS (phosphate-buffered saline· 100 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1 4 mM KH_2PO_4, pH 7.4), cells were harvested in 100 μl of buffer A (1% Triton X-100, 1% sodium deoxycholate, 0 1% SDS, 0.15 M NaCl, 20 mM Tris [pH 7 4]) and lysed by sonicating (Branson). The lysates were analysed either directly by standard 12% SDS-PAGE or on 2D-gels [14], or immunoprecipitated as described [15]. Briefly, extracts were preincubated with protein A-coated Sepharose beads (Sigma) for 1 h on ice. Sepharose beads were removed by centrifugation. Affinity purified anti-chicken-B-CK IgG [6,14] was added to the supernatants and incubated for 1 h on ice, followed by precipitation of the immune complex with protein A-coated Sepharose beads. After extensive washing with buffer A the immunoprecipitates were analysed by 2D-gel electrophoresis, blotting and autoradiography, as described [14].

3. RESULTS AND DISCUSSION

3.1. *Direct analysis of cellular extracts by SDS-PAGE and 2D-gel electrophoresis*

To examine the total population of phosphoproteins in myoblasts, cell cultures derived from thigh muscle of chicken embryos were labelled with $^{32}P_i$. Cell extracts were directly analysed by SDS-PAGE followed by autoradiography (Fig. 1). Treatment of the cell cultures

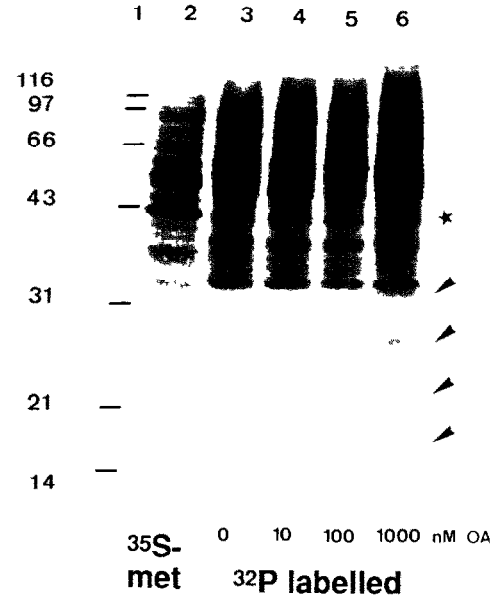

Fig 1 Effect of OA at different concentrations on protein phosphorylation $^{32}P_i$-labelled myoblasts were treated with different concentrations of OA, harvested and analysed by 12% SDS-PAGE followed by autoradiography. Cells analysed in lane 2 were metabolically labelled with [^{35}S]methionine, but not with $^{32}P_i$ Note the OA-dependent detection of phosphoproteins in lanes 5 and 6, indicated by arrowheads. The position of CK is indicated by an asterisk. Lane 1 corresponds to low molecular weight marker; the apparent molecular masses are indicated in kDa

with increasing concentrations of the phosphatase inhibitor okadaic acid (OA) specifically increased the phosphorylation states of a variety of proteins, especially in the low molecular weight range (Fig. 1, arrowheads). Intense phosphorylation of low molecular weight proteins after OA treatment has also been reported when extracts from OA-treated myoblasts were incubated with ^{32}P-γ-ATP [16]. Likely candidates for some of these proteins are myosin light chain or ribosomal protein S6 [12,17].

It was not possible to identify CK isoenzymes as phosphoproteins by SDS-PAGE analysis, since the region where CK migrated (Fig. 1, asterisk) was masked by many phosphorylated proteins. Therefore, 2D-gel electrophoresis was carried out with extracts of $^{32}P_i$-labelled cells (Fig. 2). By comparison of 2D-patterns of untreated cells with cells given 1 μM OA, many proteins which became heavily phosphorylated only after OA addition could be identified (Fig. 2b and d, triangles). Several other phosphoproteins showed increased phosphorylation states after OA addition (Fig. 2b and d, long arrows pointing down). The proteins that become phosphorylated after OA treatment are likely to be substrates of type-1 and type-2A phosphatases, dominant cellular phosphatases [17] which are targets of OA inhibition. Although OA has an almost 100 fold higher affinity to type-2A as compared to type-1 phosphatases, one could expect effects on both types at the OA con-

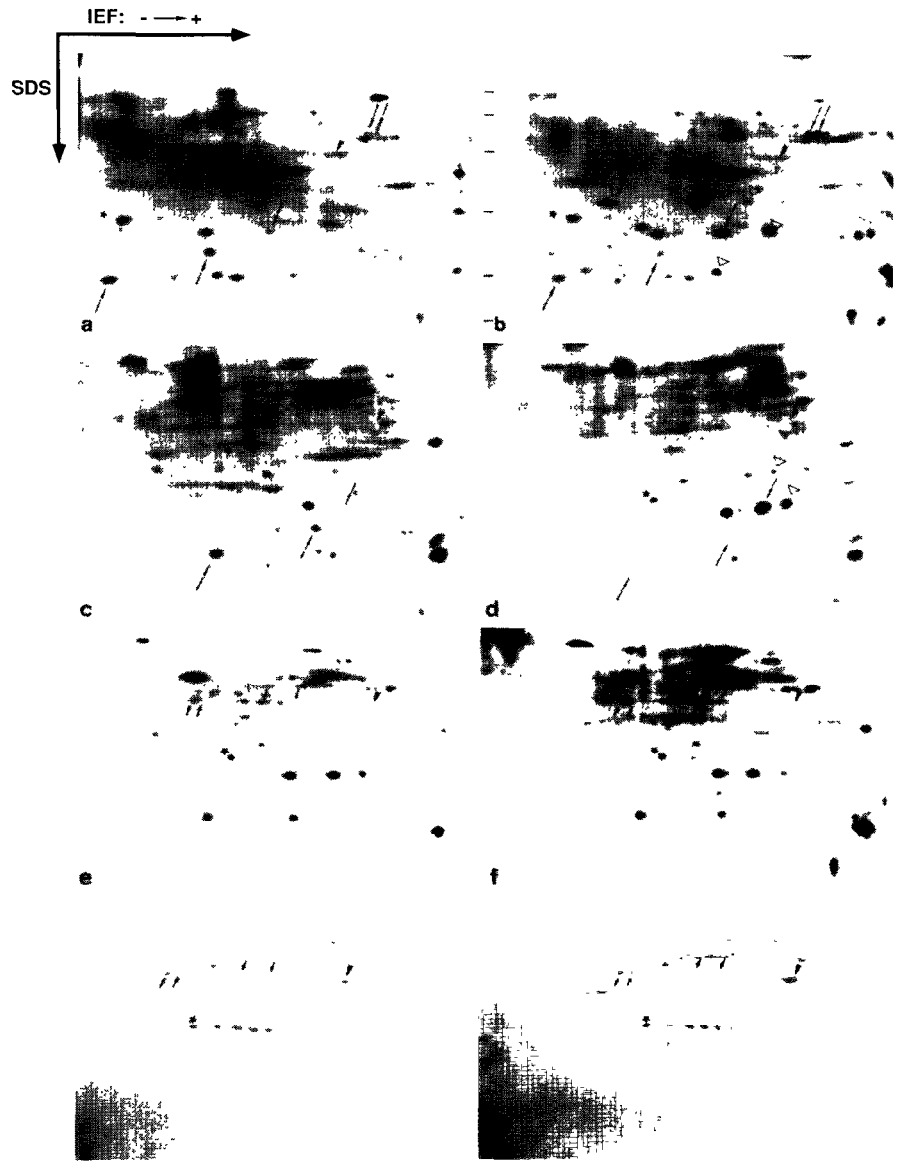

Fig. 2. 2D gel analysis of the protein phosphorylation patterns of $^{32}P_i$-labelled myogenic cells treated with OA and dbcAMP. The protein phosphorylation patterns of $^{32}P_i$-labelled cells treated with various agents were analysed by 2D-gel electrophoresis in the pH ranges 5–8 (a and b) and 3–10 (c–h) Autoradiograms from extracts of OA (1 μM) treated myoblasts or myotubes are shown in (b) and (d), respectively; the corresponding untreated controls are shown in (a) and (c). Triangles in panels (b) and (d) indicate protein spots unlabelled in controls but heavily phosphorylated upon OA addition. Long arrows pointing downwards in all panels indicate proteins markedly less labelled in controls versus OA treated cells, whereas long arrows pointing upwards show proteins with increased labelling in controls compared to OA treated cells. Autoradiograms (e) and (f) represent the phosphoprotein patterns induced by treatment of myotubes with dbcAMP (e) or with dbcAMP and 1 μM OA (f); (g) and (h) represent the protein pattern of the corresponding blots, stained with Ponceau red S Asterisks indicate the position of the most basic spot of a 2D-carbamylate marker (carbonic anhydrase, 30 kDa, Pharmacia). The two B-CK main subunit spots were located to positions above the third and fifth of the carbamylate marker spots (from left to right, Fig. 2g and h, small arrows pointing downwards), whereas M-CK subunits (indicated by short arrows pointing upwards) are more basic and migrate at slightly lower position. The position of actin is indicated by arrowheads in each panel The positions of molecular weight markers (97, 66, 43, 31 and 21 kDa) are indicated in panel (b)

centration used (1 μM). Unexpectedly, the phosphorylation status of some proteins was reduced by OA treatment (Fig. 2b and d, long arrows pointing upward). This finding could result from the fact that phosphatases themselves can be regulated by phosphorylation [12,18]. The phosphoprotein patterns of myoblasts and myotubes were similar but not identical (Fig. 2, compare b and d). Myotubes were also treated with di-butyryl-3′-5′-cAMP (dbcAMP), which activates PKA. Addition of dbcAMP to myotubes increased the phosphorylation states of a large number of proteins, similar to what was seen with OA treatment (compare Fig. 2d and e). Cells treated with both dbcAMP and OA (Fig. 2f) did not show large numbers of additional pro-

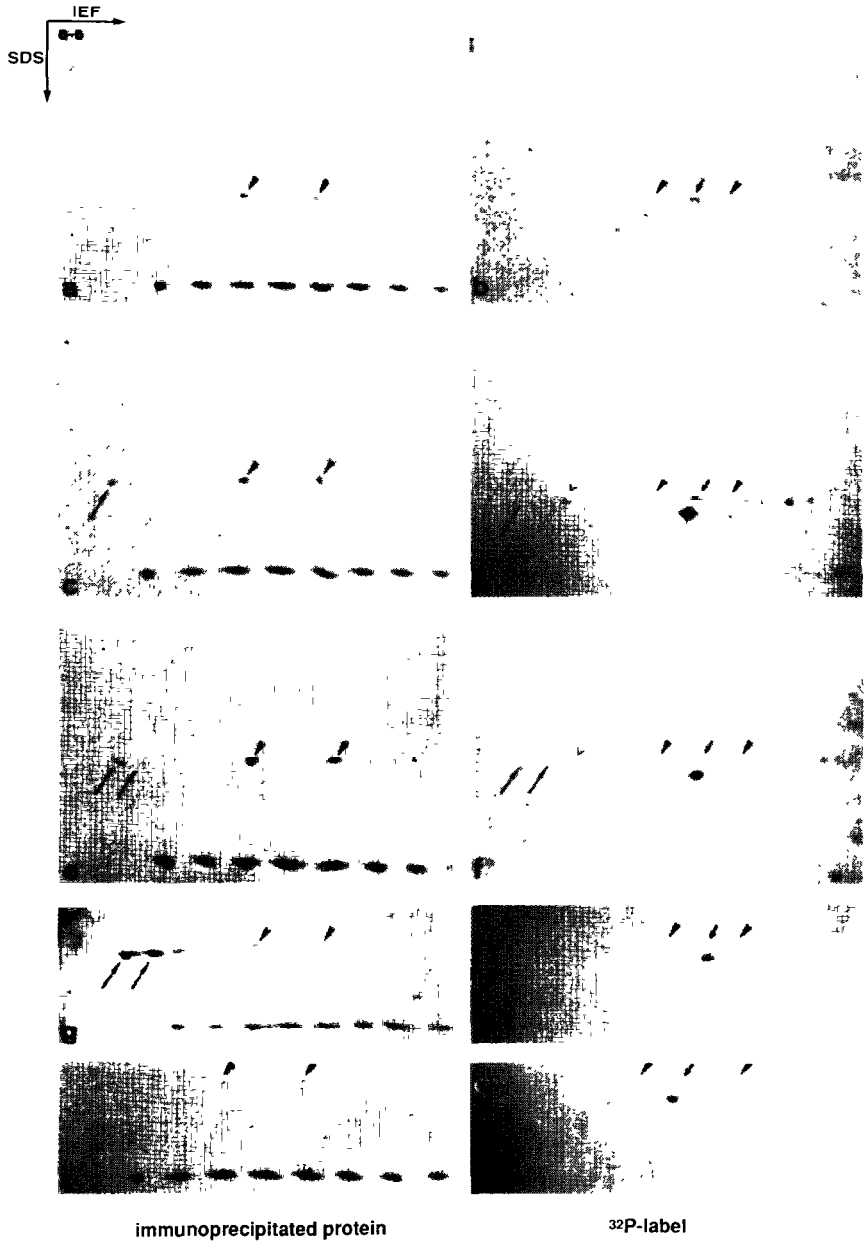

Fig. 3 Analysis of CK phosphorylation in myogenic cell cultures by immunoprecipitation with affinity purified anti-CK antibody. Lysates of drug-treated, $^{32}P_i$-labelled cells were immunoprecipitated with affinity purified anti-CK antibody, followed by 2D-gel analysis of the immunoprecipitates (pH range 5–8), blotting and autoradiography The cells have been treated with dbcAMP (a,b), a combination of OA and OAG (c,d), OA (e,f), OAG (g,h) or a combination of OA and dbcAMP (i,k). Staining for protein with Amido black (a,c,e,g and i) revealed the specific precipitation of CK isozymes and the positions of 2D-carbamylate marker spots (carbonic anhydrase, 30 kDa, Pharmacia). B-CK protein spots and their respective positions in the autoradiograms (b,d,f,h and k) are indicated by arrowheads. The position of M-CK is indicated by long arrows, pointing upwards. In (g) 2 μg of purified M-CK were added to confirm the position of M-CK on the gels. In all the treatments shown, a $^{32}P_i$-labelled spot (indicated by a short arrow) was observed at the acidic side of the main B-CK subunit spot; this is likely to arise from phosphorylation of B-CK (see text). A slight radioactive signal was also observed at the positions of the main B-CK subunit spots. At a position slightly more acidic than the main M-CK subunit, another phosphoprotein spot, derived from phosphorylation of M-CK, is marked in (d) and (f) by open triangles.

tein spots compared to those treated with dbcAMP alone (Fig. 2e), indicating that the group of proteins affected by these two treatments (OA and dbcAMP) is largely the same. It cannot be excluded that increased PKA activity is directly responsible for the increased phosphorylation of all these proteins; however, the fact that a similar group of proteins is phosphorylated in dbcAMP and OA treatments could also be due to an indirect consequence of PKA activation, namely the inhibition of type-1 phosphatases. PKA has been shown to phosphorylate the heat stable inhibitor-1, which in turn inhibits type-1 phosphatases [12]. Since these

phosphatases are also inhibited by OA [11,12], it may be not surprising to see similar results with the two treatments. This explanation is further supported by the observation (not shown), that the phosphorylation states of only a few proteins were specifically increased when cells were treated with 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a DAG analogue which activates protein kinase C [19].

On blots stained for proteins, it was possible to identify the CK isoenzymes (Fig. 2g,h). Chicken B-CK consists of two subunits, which differ at the N-terminus and arise by differential splicing, having different pIs but a similar molecular weight of about 43 kDa [14, 20]. The molecular basis of the two subunit spots of M-CK, which have identical N-termini [21], is still unknown. Although there were radioactively labelled spots seen in the area where CKs are located (Fig. 2e,f), it was not possible by 2D-gels alone to unambiguously assign these spots to any of the CK isozymes.

3.2. *Analysis of cellular extracts by immunoprecipitation with anti-CK antibodies*

Due to the high complexity of the cellular phosphoprotein pattern, the phosphorylation of CK isoenzymes was investigated by immunoprecipitation of extracts of $^{32}P_i$-labelled myotubes using a specific affinity purified anti-CK antibody (Fig. 3). The antibody specifically reacts with B-CK, illustrated by comparing the protein patterns of myotube proteins (Fig. 2g and h) with those of precipitated CK proteins (Fig. 3a,c,e,g and i). Given in excess, the antibody also precipitates M-CK. Phosphoprotein spots derived from phosphorylation of B-CK (Fig. 3, small arrows) were detected after treatment of cells with dbcAMP, OA, OAG or combinations of these substances. Furthermore, when cells were treated with OA (or OA plus OAG), M-CK phosphoprotein spots, showing a slightly more acidic pI than the main M-CK subunit spot, were precipitated (indicated by open triangles). The pI shifts of phosphorylated B- and M-CK relative to the unphosphorylated subunits were very similar, amounting to approximately 0.1 pH units, as calculated from 2D-gels. Neither B-CK nor M-CK phosphoprotein spots were detected in untreated cultures or in cells treated with 1,2-dioleoyl-*sn*-glycerol (a DAG analogue that does not cross membranes); the spots were also absent when preimmune serum was used for immunoprecipitation (not shown).

Phosphorylation of B-CK was found at all developmental stages of myogenic cells investigated. Since the radioactive signals were rather faint, they were harder to detect in early cultures as compared to differentiated myotubes. The signals assigned as phosphorylated M-CK were only detected in late cultures, consistent with the CK isozyme expression in these cultures [2]. In OAG treated cultures, an unidentified phosphoprotein of about 40 kDa (Fig. 3h) was co-precipitated with different batches of anti-B-CK antibodies. This protein was not phosphorylated after treatment with dbcAMP (Fig. 3b) or in 1,2-dioleoyl-*sn*-glycerol treated or untreated controls (not shown), indicating that it was phosphorylated by PKC. OA markedly increased the phosphorylation status of this protein (Fig. 3d,f), indicating that it is under strong control of phosphatase PP1 or PP2A.

Mouse and rat B-CK were already proposed to be substrates of PKC in mouse keratinocytes [7] or in rat microtubule preparations [5], respectively. Since phosphorylated B-CK was detected in cultures treated with OAG, it seems likely that PKC is directly or indirectly involved in the phosphorylation of B-CK in chicken myogenic cells. On the other hand, phosphorylated B-CK was also found when myogenic cells were treated with dbcAMP. As discussed above, the stimulation of B-CK phosphorylation by dbcAMP may be explained by direct phosphorylation of CK by PKA. But indirect effects of cAMP-dependent PKA activation, such as inhibition of PP1 through phosphorylation of the heat stable inhibitor-1 [12], may also lead to increased phosphorylation of CK. The primary sequence of chicken B-CK [20] contains putative phosphorylation sites for PKC, CaM-kinase and PKA (Table I) which are all present in chicken myogenic cells [3,4]. However, no structural information is available yet whether these sequence stretches are on the surface of the CK proteins and thus accessible to kinases. Unfortunately, the amount of immunoprecipitated CK phosphoprotein was not sufficient for phosphopeptide analysis; thus, further pharmacological and biochemical data will be needed to unambiguously identify the protein kinase(s) involved in the phosphorylation of CK in chicken myogenic cells.

Table I

Putative phosphorylation sites of chicken B-CK

B-CK amino acid sequence	Kinase	Consensus
120 DLDPNYVLSS**R**VR**T**G**R**SIRGF 150	PKC	RxxS/TxR
230 NEEDHL**R**VI**S**MQKGGNMKEVF 250	CaM-kinase	RxxS/T
310 EVLKRLRLQK**R**G**T**GGVDTAAV 330	PKA	RRxS/T or RxS/T

Peptide stretches taken from different regions of the chicken B-CK amino acid sequence [22] which contain sequences (shown in bold face) corresponding to the consensus sites of protein kinase C (PKC), the multifunctional calcium/calmodulin-dependent protein kinase (CaM-kinase) and the cAMP-dependent protein kinase (PKA), which accepts RRxS/T but also RxS/T as recognition motif [23,24]. The phosphate acceptor residues, indicated by asterisks, may either be serine or threonine residues. The putative sites are fully conserved within the entire CK isoprotein family (for references see [25,26]). Amino acids are indicated in the single letter code; 'x' represents amino acid residues that are less essential for recognition [23]

In summary, we have shown that chicken B- and M-CK can be phosphorylated and dephosphorylated in vivo. This phosphorylation may play an important role in the cellular energy metabolism, since phosphorylated B-CK has an increased affinity for PCr [6]. The higher affinity of phosphorylated CK for PCr may increase the enzyme's ability to transfer the high-energy phosphate from PCr to ADP, thereby providing a higher ATP supply for ATP-requiring processes at low PCr levels. ATP is needed for a large number of energy-requiring processes, including the kinase activities of the various protein kinases acting in myoblast fusion and the intracellular processes they mediate. These increased local energy requirements may be provided in part by the phosphorylation of CK by the responsible kinase.

Acknowledgements Dr. Y. Tsukitani is acknowledged for the kind provision of okadaic acid and Mrs. E. Zanolla for expert photography work. Special thanks go to Dr. E. Furter-Graves for carefully reading the manuscript and valuable discussion. This work was supported by an ETH graduate student scholarship to W H and by funding from the Swiss Society for Muscle Disease to T W

REFERENCES

[1] Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H.M. (1992) Biochem. J. 281, 21–40.
[2] Perriard, J.-C., Caravatti, M., Perriard, E.R. and Eppenberger, H M (1978) Arch. Biochem. Biophys 191, 90–100.
[3] David, J.D., Faser, C.R. and Perrot, G P (1990) Dev. Biol. 139, 89–99.
[4] Rogers, J.E., Narindrasorasak, S., Cates, G A. and Sanwal, B.D (1985) J. Biol. Chem 260, 8002–8007.
[5] Mahadevan, L.C., Whatley, S A., Leung, T.K.C. and Lim, L (1984) Biochem. J. 222, 139–144.
[6] Quest, A F.G., Soldati, T., Hemmer, W., Perriard, J.-C., Eppenberger, H.M. and Wallimann, T. (1990b) FEBS Lett. 269, 457–464.
[7] Chida, K., Kasahara, K., Tsunenaga, M., Kohno, Y., Yamada, S., Ohmi, S. and Kuroki, T (1990) Biochem Biophys Res Commun. 173, 351–357.
[8] Hemmer, W., Glaser, S J., Hartmann, G.R., Eppenberger, H.M. and Wallimann, T (1991) in: Cellular Regulation by Protein Phosphorylation (Heilmeyer, L.M.G. ed) Cellular Regulation by Protein Phosphorylation H 56, pp. 143–147, Springer-Verlag, Berlin.
[9] Friedman, D.L., Puelo, P R. and Perryman, M.B. (1990) FASEB J 4, A2232.
[10] Chida, K., Tsunenaga, M., Kasahara, K., Kohno, Y. and Kuroki, T. (1990) Biochem. Biophys. Res Commun. 173, 346–350.
[11] Bialojan, C and Takai, A (1988) Biochem. J. 256, 283–290.
[12] Cohen, P (1989) Annu. Rev. Biochem 58, 453–508.
[13] Entwistle, A., Curtis, D.H. and Zalin, R.J. (1986) J. Cell Biol 103, 857–866.
[14] Soldati, T., Schäfer, B W and Perriard, J.-C. (1990) J. Biol. Chem 265, 4498–4506
[15] Soldati, T and Perriard, J -C. (1991) Cell 66, 277–289.
[16] Kim, H S., Chung, C H., Kang, M -S. and Ha, D.B (1991) Biochem. Biophys. Res. Commun. 176, 1044–1050
[17] Haystead, T.A J., Sim, A.T.R., Carling, D., Honnor, R C., Tsukitani, Y., Cohen, P and Hardie, D.G. (1989) Nature 337, 78–81
[18] Hashimoto, Y., King, M.M. and Soderling, T.R (1988) Proc Natl. Acad. Sci USA 85, 7001–7005.
[19] McCaffrey, P.G., Friedman, B.A and Rosner, M.R. (1984) J Biol. Chem. 259, 12502–12507
[20] Wirz, T., Brändle, U., Soldati, T., Hossle, J.P. and Perriard, J -C. (1990) J. Biol. Chem 265, 11656–11666.
[21] Hemmer, W. (1993) Swiss Federal Institute of Technology, Zürich, Ph D. Thesis No. 10026.
[22] Hossle, J.P., Schlegel, J., Wegmann, G., Wyss, M., Böhlen, P., Eppenberger, H.M., Wallimann, T and Perriard, J.-C (1988) Biochem Biophys. Res Commun 151, 408–416.
[23] Kemp, B E and Pearson, R.B (1990) Trends Biochem. Sci 15, 342–346.
[24] Pearson, R.B. and Kemp, B.E. (1991) Methods Enzymol 200, 62–81.
[25] James, P., Wyss, M., Lutsenko, S., Wallimann, T. and Carafoli, E. (1990) FEBS Lett. 273, 139–143
[26] Wyss, M., Smeitink, J., Wevers, R.A. and Wallimann, T. (1992) Biochim. Biophys. Acta 1102, 119–166