Protein kinase C and cyclic AMP-dependent protein kinase phosphorylate phospholemman, an insulin and adrenaline-regulated membrane phosphoprotein, at specific sites in the carboxy terminal domain

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Phospholemman, a transmembrane, 72 residue protein enriched in striated muscle and heart [Palmer, Scott and Jones (1991) *J. Biol. Chem.* **266**, 11126–11130], is phosphorylated in response to insulin [Walaas, Horn and Walaas (1991) *Biochim. Biophys. Acta* **1094**, 92–102]. The present study is aimed at identifying the phosphorylation sites of this protein. A synthetic peptide, GTFRSS⁶³IRRLS⁶⁸TRRR (in the single letter code) and consisting of phospholemman residues 58–72, is a substrate for both protein kinase C and cyclic AMP (cAMP)-dependent protein kinase, with K_m values of 6–7 μ M for both enzymes. Amino acid sequencing of the phosphopeptide shows that protein kinase C

phosphorylates both Ser-63 and Ser-68, while cAMP-dependent protein kinase phosphorylates Ser-68. Thermolytic phosphopeptide mapping of ³²P-labelled phospholemman from rat diaphragms shows that treatment with insulin results in labelling of phosphopeptides containing both Ser-63 and Ser-68, whereas treatment with adrenaline results in labelling of the phosphopeptide containing Ser-68. Hence, insulin and adrenaline regulate the phosphorylation of phospholemman, presumably through protein kinase C and cAMP-dependent protein kinase, respectively, on partly overlapping phosphorylation sites.

INTRODUCTION

Phosphorylation and dephosphorylation of proteins is of general importance among the insulin-regulated signalling systems which regulate cellular activities (Denton, 1986; Czech et al., 1988; Dent et al., 1990; White and Kahn, 1994). A transmembrane protein of apparent 15 kDa molecular mass when analysed by SDS/PAGE can be phosphorylated in skeletal muscle sarcolemma *in vitro* by an endogenous, insulin-sensitive protein kinase (Walaas et al., 1977, 1979). This protein, recently denoted phospholemman (Palmer et al., 1991), is a substrate for *in vitro* phosphorylation catalysed by both cyclic AMP (cAMP)-dependent protein kinase and protein kinase C (Presti et al., 1985; Walaas et al., 1988a,b). In intact skeletal muscle, addition of insulin can mimic tumour-promoting phorbol esters in increasing both protein kinase C activity (Walaas et al., 1987) and phospholemman phosphorylation (Walaas et al., 1991). Other studies have shown that phospholemman is phosphorylated following activation of α - or β -adrenergic receptors in myocardial sarcolemma and after activation of β -adrenergic or vasopressin receptors in smooth muscle (Jones et al. 1979, 1980, 1981; Manalan and Jones, 1982; Boulanger-Saunier et al., 1985; Presti et al., 1985; Boulanger-Saunier and Stoclet, 1987).

Following purification from canine myocardium, the primary structure of phospholemman has recently been deduced from the nucleotide sequence of the cloned cDNA (Palmer et al., 1991). The protein contains 72 amino acids with an intracellular, positively charged carboxy terminal domain. This part of the protein, comprising amino acids 58–72, contains at least four

residues, including Ser-62, -63 and -68, and Thr-69, which appear as possible consensus sequences for cAMP-dependent protein kinase and protein kinase C (Kemp and Pearson, 1990). It is presently not known if any of these phosphorylation sites are hormonally regulated in intact tissue. We have therefore subjected a synthetic phospholemman peptide, G⁵⁸TRFSS⁶³IRRLS⁶⁸TRRR⁷², to phosphorylation catalysed by both cAMP-dependent protein kinase and protein kinase C, and determined the sites of phosphorylation labelled by these kinases. Both cAMP-dependent protein kinase and protein kinase C phosphorylate Ser-68, while protein kinase C also phosphorylates Ser-63. Furthermore, we find that insulin regulates the phosphorylation of both Ser-63 and Ser-68, while adrenaline regulates phosphorylation of Ser-68 in skeletal muscle *in situ*.

MATERIALS AND METHODS

[γ -³²P]ATP and ³²P_i were from Amersham (Bucks, U.K.). Diolein, phosphatidylserine and thermolysin were from Sigma (St. Louis, MO, U.S.A.). Ion exchange resin AG 1X8 (acetate form) was from BioRad (Richmond, CA, U.S.A.). A synthetic peptide, comprising the extreme 15 carboxy terminal amino acids [residues 58–72 of the canine protein, GTFRSSIRRLSTRRR (in single letter code)] of phospholemman (Palmer et al., 1991) was prepared at The Peptide Synthesis Facility, Forskningsparken, University of Oslo. Male Wistar rats (125–150 g body wt.) were from Møllegaard, Denmark. Protein kinase C was purified from rat brain (Woodgett and Hunter, 1987), while the catalytic subunit of cAMP-dependent protein kinase, calmodulin and

Abbreviations used: cAMP, cyclic AMP; CaM kinase II, Ca²⁺/calmodulin-dependent protein kinase II.

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Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) were gifts from A. Horiuchi and A. C. Nairn, The Rockefeller University. Casein kinase 2 was a gift from S. Laland, University of Oslo. Other reagents, of analytical grade or better, were from standard commercial suppliers.

Phosphorylation of synthetic peptide

The stoichiometry of phosphorylation of the synthetic peptide was analysed by incubating 0.1 mM peptide (dissolved in water) with 1 mM ATP (containing 750 c.p.m./pmol of ^{32}P) in a medium containing 50 mM Hepes, pH 7.4, and 10 mM MgCl_2 . Reactions were initiated by addition of the catalytic subunit of cAMP-dependent protein kinase (final concn. 12 nM), purified protein kinase C (final concn. 10 nM) in the presence of 0.5 mM CaCl_2 , phosphatidylserine (50 $\mu\text{g}/\text{ml}$) and diolein (2 $\mu\text{g}/\text{ml}$), purified CaM kinase II (final concn. 10 nM) in the presence of 0.5 mM CaCl_2 and 1.5 μM calmodulin, or purified casein kinase 2 as described (Walaas et al., 1991). Samples were incubated in a shaking water bath at 30 °C for varying periods of time. The reactions were terminated by addition of acetic acid (final concn. 10%, v/v), and phosphorylated peptide was quantitated by spotting aliquots onto P81 phosphocellulose paper (Millipore), washing and Cerenkov counting of the filter papers (Witt and Roskoski, 1975). When additivity of phosphorylation was examined, the peptide was incubated in medium containing phosphatidylserine, diolein, calcium and Mg-ATP as described. Phosphorylation was initiated by addition either of the catalytic subunit of cAMP-dependent protein kinase for 60 min followed by protein kinase C for an additional 60 min, or addition of protein kinase C for 60 min followed by the catalytic subunit of cAMP-dependent protein kinase for 60 min. The incubations were terminated and the results analysed as described.

Kinetics of peptide phosphorylation

To examine the efficiency of phosphorylation of the peptide, incubations were performed in a medium containing 50 mM Hepes, pH 7.4, 10 mM MgCl_2 , 0.2 mM ATP, 0.1 $\mu\text{g}/\mu\text{l}$ BSA and peptide concentrations ranging between 10 and 200 μM . Phosphorylations were initiated by addition either of the catalytic subunit of cAMP-dependent protein kinase (final concn. 5 nM) or protein kinase C (final concn. 2 nM) (added with Ca^{2+} , phosphatidylserine and diolein as described above). Incubations were carried out for 5 min at 30 °C. Initial experiments verified that these incubations led to initial rate conditions. Kinetic constants were assessed by examination of double reciprocal plots of $1/V$ versus $1/[S]$.

Phosphoamino acid analysis

Following phosphorylation incubations for 60 min, aliquots of ^{32}P -labelled phosphopeptide were purified by anion exchange chromatography on AG 1 $\times$ 8 (Kemp et al., 1976) and dried in a vacuum centrifuge. HCl (6 M) was added, and the samples were hydrolysed at 110 °C for 90 min under nitrogen. The samples were dried, phosphoamino acid standards were added, and the samples were subjected to electrophoresis on cellulose plates at pH 1.9 and pH 3.5 (Nairn and Greengard, 1987). ^{32}P -labelled components were detected by autoradiography, while phosphoamino acid standards were detected by ninhydrin staining.

Chemical determination of phosphorylated seryl residues

Samples which had been phosphorylated either by cAMP-dependent protein kinase or protein kinase C were subjected to

anion exchange chromatography, dried, resuspended in water and subjected to reverse-phase h.p.l.c. on a Vydac C_{18} column (4.6 mm $\times$ 25 cm). The column was eluted at a flow rate of 1 ml/min in solvent A [0.1% (v/v) trifluoroacetic acid], using a gradient of solvent B [0.085% (v/v) trifluoroacetic acid/70% (v/v) acetonitrile] as follows: 0–3 min 5% B, 3–8 min 5–21% B, 8–25 min 21–27% B, and 25–27 min 27–50% B. Eluting peptides were detected as absorbance at 214 nm, while phosphopeptides were detected by Cerenkov counting of fractions. Phosphorylated peptides were subjected to ethanethiol treatment to convert phosphoserine to *S*-ethylcysteinyl derivatives (Holmes, 1987), and the peptides were subjected to automated Edman degradation and amino acid sequencing. The presence of the phenylthiohydantoin derivative of *S*-ethylcysteine was taken as evidence of a phosphoseryl residue in the phosphorylated peptide.

Thermolytic digestion of ^{32}P -labelled peptide

To compare the phosphorylated seryl residues in the synthetic peptide with the phosphorylated domains of phospholemman labelled *in situ*, the 15 amino acid peptide was phosphorylated with [γ - ^{32}P]ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase or protein kinase C for 60 min as described. Following purification by anion exchange chromatography, the samples were dried, resuspended in ammonium bicarbonate (50 mM) and subjected to proteolysis with thermolysin (100 $\mu\text{g}/\text{ml}$) for 24 h at 37 °C. The resulting thermolytic phosphopeptides were then divided into two sets of aliquots. One set was subjected to purification by reverse phase h.p.l.c. on a Pharmacia SuperPac Pep-S C_{18}/C_2 column (4.0 mm $\times$ 25 cm). The column was eluted for 90 min at a flow rate of 1 ml/min in solvent A [0.115% (v/v) trifluoroacetic acid] using a linear gradient of 5–25% solvent B [0.085% (v/v) trifluoroacetic acid/70% (v/v) acetonitrile]. Eluting peptides were detected as absorbance at 220 nm, while phosphopeptides were detected by Cerenkov counting of fractions. The radioactive peptides were collected and subjected to N-terminal amino acid sequence analysis, using automated Edman degradation on a 477A Protein Sequencer with an on-line 120A phenylthiohydantoin amino acid analyser (Applied Biosystems, Foster City, CA, U.S.A.). The thermolytic peptides were aligned within the 15 amino acid peptide by comparison of the experimentally derived amino acid sequences with the primary structure of the peptide.

The other set of aliquots was dried, resuspended and separated in two dimensions on thin layer silica plates, using electrophoresis at pH 3.5 in the first dimension and ascending chromatography in the second dimension, as described (Walaas et al., 1991). The thermolytic phosphopeptides were visualized by autoradiography.

Analysis of phospholemman in isolated diaphragms

Rat diaphragm preparations were prepared and incubated, in the absence or presence of insulin (100 nM), adrenaline (10 μM) or isoproterenol (10 μM) for 10 min, as described (Walaas et al., 1991). Phospholemman was then isolated from the prelabelled diaphragm preparations, using chloroform/methanol extraction and acetone precipitation, and subjected to thermolytic fingerprinting, as described (Walaas et al., 1991).

Miscellaneous methods

Protein was analysed by the method of Smith et al. (1985). SDS/PAGE was performed using the buffers of Laemmli (1970). Figures of autoradiograms were prepared using a Hewlett Packard ScanJet IIC/ADF scanner, and Desk Scan II (version

1.0, Hewlett Packard) together with Adobe Photoshop (version 2.5, Adobe Systems, Europe B.V.) software.

RESULTS

Phospholemman peptide phosphorylation

Incubation of the 58–72 amino acid peptide, derived from the carboxy terminal part of canine phospholemman, with the catalytic subunit of cAMP-dependent protein kinase or protein kinase C in the presence of [γ - 32 P]ATP led to a time-dependent incorporation of 32 P into the peptide, which reached maximal levels after 30–60 min (Figure 1). Calculation of stoichiometry indicated that cAMP-dependent protein kinase catalysed incorporation of approx. 0.8 mol of phosphate/mol of peptide, while protein kinase C catalysed incorporation of approx. 1.7 mol of phosphate/mol of peptide (mean of duplicate determinations, from two separate experiments). Moreover, when incubation with cAMP-dependent protein kinase for 60 min was followed with protein kinase C for a further 60 min, an approximate doubling of 32 P incorporation was observed, which was similar in extent to that obtained by protein kinase C alone (Figure 1). In contrast, when incubation with protein kinase C for 60 min was followed by cAMP-dependent protein kinase for 60 min, no further increase in the phosphorylation of the peptide was seen (Figure 1). Taken together, these results indicated that protein kinase C catalysed phosphorylation of at least two residues, while cAMP-dependent protein kinase catalysed phosphorylation of at least one residue in the phospholemman peptide. Moreover, at least one of these phosphorylation sites appeared to be shared by both kinases. In contrast, incubation with CaM kinase II or casein kinase 2 for 60 min led to a phosphorylation stoichiometry of less than 0.05 mol/mol (not shown).

We also compared the extent of peptide phosphorylation by determination of retention times of the phosphorylated peptide on a reverse-phase h.p.l.c. column, from which the dephosphopeptide eluted at a retention time of approx. 20 min (Figure 2,

Figure 1 Time course of phosphorylation of phospholemman peptide

Peptide (0.1 mM) was incubated with [γ - 32 P]ATP (1 mM) in a medium containing 50 mM Hepes, pH 7.4, and 10 mM MgCl₂. Reactions were initiated either by addition of the catalytic subunit of cAMP-dependent protein kinase (PK-A, final concn. 12 nM, ●) or purified protein kinase C (PK-C, final concn. 10 nM, ○) in the presence of 0.5 mM CaCl₂, phosphatidylserine (50 μ g/ml) and diolein (2 μ g/ml) and allowed to continue for up to 60 min at 30 °C. When additivity of phosphorylation was examined, the catalytic subunit of cAMP-dependent protein kinase was followed after 60 min by addition of protein kinase C (▲), CaCl₂ and phosphatidylserine/diolein, while protein kinase C was followed after 60 min by addition of the catalytic subunit of cAMP-dependent protein kinase (△). The incubations were terminated at the times indicated and the results analysed as described in the text. The data represent mean of duplicate determinations from one representative experiment, which was repeated twice.

Figure 2 Purification by h.p.l.c. of phosphorylated phospholemman peptide

Phospholemman peptide was incubated without (trace A) or with (trace B) cAMP-dependent protein kinase for 60 min, (trace C) protein kinase C for 30 min, or (trace D) protein kinase C for 60 min as described in the legend to Figure 1. Following addition of acetic acid [10% (v/v)] and anion exchange chromatography, the samples were dried, and varying amounts were subjected to reverse-phase h.p.l.c. on a Vydac C₁₈ column as described in the text. Peptides were detected as absorbance at 214 nm (A_{214}). Data are representative of three experiments, where the elution times of the peptides vary within 1 min.

trace A). When the peptide was stoichiometrically phosphorylated for 60 min with the catalytic subunit of cAMP-dependent protein kinase, the dephosphopeptide eluting at 20 min disappeared and a 32 P-labelled peptide eluting at approx. 15 min retention time appeared instead (Figure 2, trace B). Phosphorylation of the peptide by protein kinase C to approx. 50% of maximal labelling gave one 32 P-labelled peptide eluting at approx. 15 min, and another eluting at approx. 14 min (Figure 2, trace C), while maximal phosphorylation with protein kinase C resulted in the peptide being completely converted to a 32 P-labelled peptide eluting at approx. 14 min (Figure 2, trace D).

Kinetic analysis of phospholemman peptide phosphorylation

Incubation of varying amounts of peptide with both the catalytic subunit of cAMP-dependent protein kinase and protein kinase C showed that both phosphorylation reactions obeyed Michaelis–Menten kinetics, with Lineweaver–Burk plots (not shown) indicating apparent K_m values of 7 μ M with cAMP-dependent protein kinase and 6 μ M with protein kinase C (mean of triplicate values from two independent experiments). The phospholemman peptide therefore appeared to be a good substrate for both cAMP-dependent protein kinase and protein kinase C.

Phosphoamino acid analysis

Since the phospholemman peptide contains both seryl and threonyl residues which could represent putative phosphorylation sites, we performed acid hydrolysis and phosphoamino acid analysis on peptide samples which had been phosphorylated by cAMP-dependent protein kinase or protein kinase C for

Figure 3 Analysis of phosphoamino acids in phospholemman peptide

Phospholemman peptide was incubated with [γ - 32 P]ATP in the presence of cAMP-dependent protein kinase or protein kinase C for 60 min as described in the legend to Figure 1. The samples were subjected to anion exchange chromatography, dried in a vacuum centrifuge, and hydrolysed at 110 °C for 90 min in 6 M HCl under nitrogen. 32 P-labelled amino acids were separated by electrophoresis on cellulose plates at pH 1.9 and pH 3.5. They were detected by autoradiography, while phosphoamino acid standards (P-SER, phosphoserine; P-THR, phosphothreonine, P-TYR, phosphotyrosine), run on the same plates, were detected by ninhydrin staining.

60 min. The results showed that the peptide was exclusively phosphorylated on serines by both kinases (Figure 3).

Identification of phosphoseryl residues

Since only seryl residues appeared to be phosphorylated in the phospholemman peptide, we used the method of Holmes (1987) to identify these phosphorylation sites. The peptide was phosphorylated for 60 min with either cAMP-dependent protein kinase or protein kinase C, subjected to h.p.l.c. (Figure 2, traces B and D), and the different peptides were treated with ethanethiol. Subsequent amino acid sequencing showed that cAMP-dependent protein kinase had catalysed stoichiometric phosphorylation of residue 11, which corresponded to phospholemman Ser-68, while protein kinase C had catalysed stoichiometric phosphorylation of residues 6 and 11, which corresponded to phospholemman Ser-63 and Ser-68. Moreover, analysis of incompletely phosphorylated peptide samples which had been incubated with protein kinase C for short periods of time indicated that this

enzyme initially catalysed phosphorylation of Ser-68, and only after extensive labelling of this residue would Ser-63 become phosphorylated (results not shown).

Characterization of thermolytic phosphopeptides

Identification of phosphorylation sites was also undertaken on the 32 P-labelled 58–72 phospholemman peptide subjected to thermolysin digestion and two-dimensional separation. Two major (a,b) and two minor (c,d) thermolytic phosphopeptides were seen following incubation with protein kinase C (Figure 4, panel B). In contrast, only one phosphopeptide, which co-migrated with peptide a, was obtained following incubation with the catalytic subunit of cAMP-dependent protein kinase (Figure 4, panel A). Amino acid sequence analysis of the thermolytic peptides derived from protein kinase C-catalysed phosphorylation was done after h.p.l.c. separation. Two major and two minor 32 P-labelled peptides (eluting at approximate retention times of 17 min, 32 min, 60 min and 66 min, respectively) were obtained (results not shown). The peptide eluting at 17 min co-migrated with thermolytic phosphopeptide a (Figure 4, panels C and D), and the peptide eluting at 32 min co-migrated with thermolytic phosphopeptide b (Figure 4, panels E and F). N-terminal amino acid sequencing of these peptides revealed the sequence LS*TRR (37-223*-107-118-51) for the peptide eluting at 17 min, and FRSS*IRR (60-45-51-183*-62-60-41) for the peptide eluting at 32 min (numbers in parentheses represent c.p.m. of samples taken from sequencing cycles and subjected to Cerenkov counting, * indicates selective increased 32 P-labelling). Hence, the peptide eluting at 17 min and corresponding to residues 67–71 in intact phospholemman contained 32 P-labelling in Ser-68, while the peptide eluting at 32 min and corresponding to residues 60–66 in intact phospholemman contained 32 P-labelling in Ser-63, in agreement with the chemical determination of phosphoserine described above.

When the 32 P-labelled h.p.l.c.-derived peptides which eluted at 60 and 66 min were subjected to two-dimensional separation on silica plates they co-migrated with the thermolytic phosphopeptides c and d, respectively (results not shown). These minor peptides were inconsistently found, and may derive from variable, incomplete thermolytic digestion. Therefore, they were not further analysed.

Figure 4 Autoradiogram showing thermolytic peptide mapping of the 32 P-labelled phospholemman peptide

Following incubation of the synthetic peptide with cAMP-dependent protein kinase or protein kinase C as described in the legend to Figure 1, the samples were purified by anion exchange chromatography, dried in a vacuum centrifuge and subjected to digestion with thermolysin (0.1 mg/ml) at 37 °C for 24 h. The resulting phosphopeptides, either singly or in combinations, were separated in two dimensions on silica thin layer plates as described in the text. Panel A, thermolytic phosphopeptide following phosphorylation with cAMP-dependent protein kinase; panel B, thermolytic phosphopeptides following phosphorylation with protein kinase C. Panels C–F, separate analyses of h.p.l.c.-purified thermolytic phosphopeptides following phosphorylation with protein kinase C. Panel C, h.p.l.c.-purified phosphopeptide eluting at 17 min; panel D, mixture of (B) and (C); panel E, h.p.l.c.-purified phosphopeptide eluting at 32 min; panel F, mixture of (B) and (E). (a)–(d) indicate labelling of those thermolytic phosphopeptides which are discussed in the text.

Figure 5 Autoradiogram showing regulation of ^{32}P -labelled phospholemman in intact rat diaphragm by adrenaline or insulin

(a) Rat diaphragms were prepared and prelabelled with ^{32}P , as described in the text, followed by incubation for 10 min in the absence or presence of adrenaline ($10\text{ }\mu\text{M}$). After homogenization, phospholemman was isolated from the 100 000 g pellet by chloroform/methanol/HCl extraction and acetone precipitation and solubilized in SDS-containing 'sample buffer', and equal amounts of protein were subjected to SDS/PAGE. Phosphorylated proteins were visualized by autoradiography. '15→' indicates the location of phospholemman. (b) Thermolytic peptide mapping of ^{32}P -labelled phospholemman in intact rat diaphragm incubated with adrenaline ($10\text{ }\mu\text{M}$) or insulin (100 nM) for 10 min. Phospholemman, isolated as described above, was located by autoradiography, cut out of the gel and subjected to digestion with thermolysin (0.1 mg/ml) at $37\text{ }^{\circ}\text{C}$ for 24 h. Aliquots of the resulting phosphopeptides, containing equal amounts of ^{32}P as ascertained by Cerenkov counting, were separated in two dimensions on silica thin layer plates as described above.

^{32}P -labelling of phospholemman in intact muscle

Isolated rat diaphragm were prelabelled with ^{32}P , and phospholemman was isolated as previously described (Walaas et al., 1991). Under basal conditions phospholemman contained one thermolytic ^{32}P -labelled phosphopeptide (Figure 5b), and addition of insulin increased the ^{32}P -labelling of this phosphopeptide while another phosphopeptide also appeared (Figure 5b). This has also been observed in the presence of phorbol esters (Walaas et al., 1991). Addition of adrenaline ($10\text{ }\mu\text{M}$ for 10 min) also significantly increased (by 110% above control, $n = 3$) the ^{32}P -labelling of the phospholemman *in situ* (Figure 5a), an effect which is mimicked by isoproterenol ($10\text{ }\mu\text{M}$) (results not shown). These β -adrenergic agonists caused an increase in ^{32}P -labelling of that phosphopeptide which was present both under 'basal' conditions and following insulin stimulation (Figure 5b). This thermolytic peptide showed a migration identical to that of the phosphopeptide containing Ser-68, while the thermolytic peptides derived from insulin-treated preparations showed migrations

identical to the phosphopeptides containing Ser-63 and Ser-68 (Figure 4 and 5).

DISCUSSION

The results presented in this study demonstrate that protein kinase C and cAMP-dependent protein kinase can phosphorylate the membrane protein phospholemman at two sites in the carboxy terminal domain: Ser-63, which was phosphorylated by protein kinase C, and Ser-68, which was phosphorylated by both protein kinase C and cAMP-dependent protein kinase. Moreover, our results indicate that these sites are hormonally regulated, with insulin-induced phosphorylation of phospholemman taking place at Ser-63 and Ser-68, and adrenaline-induced phosphorylation taking place at Ser-68.

These conclusions are based on experiments performed with a synthetic peptide, taken from the intracellular carboxy terminal part of phospholemman, as predicted by the domain structure of the canine protein (Palmer et al., 1991). The evidence can be briefly summarized as follows: maximal phosphorylation *in vitro* of the synthetic phospholemman peptide with the catalytic subunit of cAMP-dependent protein kinase gave an approximate stoichiometry of 1 mol of phosphate/mol of peptide, while protein kinase C gave approx. 2 mol of phosphate/mol of peptide; additivity experiments indicated that one of these phosphorylation sites was shared by both kinases while the other was specific to protein kinase C. Furthermore, time course studies and kinetic data indicated that these phosphorylation sites might be of physiological importance. Amino acid sequencing showed that Ser-63 and Ser-68 represented the phosphorylation sites in the phospholemman peptide, with Ser-63 being phosphorylated by protein kinase C and Ser-68 being phosphorylated both by protein kinase C and cAMP-dependent protein kinase.

Based upon these results the phosphorylation sites in phospholemman in diaphragm muscle *in situ* have been analysed. Following insulin treatment, phospholemman contained two thermolytic phosphopeptides which show migrations identical to the peptides containing Ser-68 and Ser-63. Adrenaline treatment, in contrast, gives a thermolytic phosphopeptide which shows migration identical to that peptide containing Ser-68. Hence these results are consistent with the interpretation that, in intact muscle, both Ser-63 and Ser-68 of phospholemman are phosphorylated in response to insulin, with their phosphorylation being caused by the insulin-induced activation of protein kinase C (Cooper et al., 1987; Walaas et al., 1987; Egan et al., 1990; Farese et al., 1992). In contrast, only Ser-68 is phosphorylated in response to adrenaline, and this phosphorylation is presumably mediated by activation of cAMP-dependent protein kinase.

The functional role of phospholemman remains unknown. Expression of the protein in *Xenopus* oocytes leads to the occurrence of a chloride current with several unusual characteristics (Moorman et al., 1992). Other workers have suggested that the protein may be involved in ion channel regulation (Atali et al., 1993). The latter proposal appears to be supported by the similarities between the intracellular domain of phospholemman and the regulatory domain of phospholamban (Palmer et al., 1991), a sarcoplasmic reticulum protein involved in the regulation of $\text{Ca}^{2+}/\text{ATPase}$ in this organelle (James et al., 1989). Although insulin and adrenaline have generally opposite effects on cellular function in rat diaphragm (e.g. Walaas, 1955a,b), both hormones induced phosphorylation of the carboxy terminal domain of phospholemman, albeit with partial overlap between the labelled sites. Such partially overlapping phosphorylation induced by antagonistic hormones, which also has been found in other

proteins (e.g. Diggle and Denton, 1992), might indicate that the functional effects following phosphorylation of Ser-68 alone may be distinct from those following simultaneous phosphorylation at both Ser-68 and Ser-63. The identification of the hormone-regulated phosphorylation sites in phospholemman presented in this study should contribute to defining the functional importance of this protein.

Following completion of this work, a study by Lu et al. (1994) has shown that the Ser-63 residue of a phospholemman peptide consisting of residues 42–72 represents a preferred substrate for the NIMA kinase derived from the *nimaA* gene from *Aspergillus*. This peptide is also phosphorylated *in vitro* by protein kinase C and cAMP-dependent protein kinase, and the authors have predicted, based on peptide mapping and comparison with preferred kinase consensus sequences, that cAMP-dependent protein kinase phosphorylates Ser-68, while protein kinase C phosphorylates Ser-63, as shown to occur in our work. The authors also propose that protein kinase C would phosphorylate a second site on Ser-69. However, this is not supported in their work since the peptide substrate employed contains Thr-69, which is not phosphorylated.

This study was supported by grants from The Juvenile Diabetes Foundation International (New York), The Norwegian Diabetes Society (Oslo), Nordic Insulin Foundation (Gentofte, Denmark) and California Metabolic Research Foundation (La Jolla, CA, USA). Identification of S-ethylcysteinyl derivatives, performed by protein sequencing analysis, was provided by The Rockefeller University Protein Sequencing Facility, which is supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment. Identification of ³²P-labelled amino acids was performed at the Protein Sequencing Facility, Forskningsparken, University of Oslo. We thank Ms. Siri Mathiesen and Ms. Iren Sefland for assistance.

REFERENCES

- Atali, B., Guillemare, E., Lesage, F., Honoré, E., Romey, G., Lazdunski, M. and Barhanin, J. (1993) *Nature* (London) **365**, 850–852.
- Boulanger-Saunier, C. and Stoclet, J.-C. (1987) *Biochem. Biophys. Res. Commun.* **143**, 517–524.
- Boulanger-Saunier, C., Kattenberg, D. M. and Stoclet, J.-C. (1985) *FEBS Lett.* **193**, 283–288.
- Cooper, D. R., Konda, T. S., Standaert, M. L., Davis, J. S., Pollet, R. J. and Farese, R. V. (1987) *J. Biol. Chem.* **262**, 3633–3639.
- Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P. and Lewis, R. E. (1988) *J. Biol. Chem.* **265**, 947–954.
- Dent, P., Lavoie, A., Nakielnny, S., Caudwell, F. B., Watt, P. and Cohen, P. (1990) *Nature* (London) **348**, 302–308.
- Denton, R. M. (1986) *Adv. Cyclic Nucleotide Res.* **20**, 293–341.
- Diggle, T. A. and Denton, R. M. (1992) *Biochem. J.* **282**, 729–736.
- Egan, J. J., Saltis, J., Wek, S. A., Simpson, I. A. and Londos, C. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1052–1056.
- Farese, R. V., Standaert, M. L., Arnold, T., Yu, B., Ishizuka, T., Hoffman, J., Vila, M. and Cooper, D. R. (1992) *Cell. Signalling* **4**, 133–143.
- Holmes, C. F. B. (1987) *FEBS Lett.* **215**, 21–24.
- James, P., Inui, M., Tada, M., Chiesi, M. and Carafoli, E. (1989) *Nature* (London) **342**, 90–92.
- Jones, L. R., Besch, H. R., Jr., Fleming, J. W., McConaughy, M. M. and Watanabe, A. M. (1979) *J. Biol. Chem.* **254**, 530–539.
- Jones, L. R., Maddock, S. W. and Besch, H. R., Jr. (1980) *J. Biol. Chem.* **255**, 9971–9980.
- Jones, L. R., Maddock, S. W. and Hathaway, D. R. (1981) *Biochim. Biophys. Acta* **641**, 242–253.
- Kemp, B. E. and Pearson, R. B. (1990) *Trends Biochem. Sci.* **15**, 342–346.
- Kemp, B. E., Benjamini, E. and Krebs, E. G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1038–1042.
- Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685.
- Lu, K. P., Kemp, B. E. and Means, A. R. (1994) *J. Biol. Chem.* **269**, 6603–6607.
- Manalan, A. S. and Jones, L. R. (1982) *J. Biol. Chem.* **257**, 10052–10062.
- Moorman, J. R., Palmer, C. J., John, J. E., Durieux, M. E. and Jones, L. R. (1992) *J. Biol. Chem.* **267**, 14551–14554.
- Nairn, A. C. and Greengard, P. (1987) *J. Biol. Chem.* **262**, 7273–7281.
- Palmer, C. J., Scott, B. T. and Jones, L. R. (1991) *J. Biol. Chem.* **266**, 11126–11130.
- Presti, C. F., Jones, L. R. and Lindemann, J. P. (1985) *J. Biol. Chem.* **260**, 3860–3867.
- Smith, P. K., Krohn, R. I., Hermansen, G. Y., Mallia, A. G., Gartner, F. H., Provanzone, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85.
- Walaas, E. (1955a) *Acta Physiol. Scand.* **35**, 109–125.
- Walaas, E. (1955b) *Acta Physiol. Scand.* **35**, 126–137.
- Walaas, O., Walaas, E., Lystad, E., Alertsen, Aa. R., Horn, R. S. and Fossum, S. (1977) *FEBS Lett.* **80**, 417–422.
- Walaas, O., Walaas, E., Lystad, E., Alertsen, Aa. R. and Horn, R. S. (1979) *Mol. Cell. Endocrinol.* **16**, 45–55.
- Walaas, O., Horn, R. S. and Walaas, S. I. (1991) *Biochim. Biophys. Acta* **1094**, 92–102.
- Walaas, S. I., Horn, R. S., Adler, A., Albert, K. A. and Walaas, O. (1987) *FEBS Lett.* **220**, 311–318.
- Walaas, S. I., Horn, R. S., Nairn, A. C., Walaas, O. and Adler, A. (1988a) *Arch. Biochem. Biophys.* **262**, 145–158.
- Walaas, S. I., Horn, R. S., Albert, K. A., Adler, A. and Walaas, O. (1988b) *Biochim. Biophys. Acta* **968**, 127–137.
- White, M. F. and Kahn, C. R. (1994) *J. Biol. Chem.* **269**, 1–4.
- Witt, J. J. and Roskoski, R., Jr. (1975) *Anal. Biochem.* **66**, 253–258.
- Woodgett, J. R. and Hunter, T. (1987) *J. Biol. Chem.* **262**, 4836–4843.