Cyclic AMP-dependent phosphorylation of a 16 kDa protein in a plasma membrane-enriched fraction of rat aortic myocytes

Chantal Boulanger-Saunier, David M. Kattenburg and Jean-Claude Stoclet

Laboratoire de Pharmacodynamie CNRS UA 600, Université Louis Pasteur 74, route du Rhin, 67400 Illkirch, France

Received 10 October 1985

Phosphorylation induced by cAMP-dependent protein kinase was examined in a plasma membrane-enriched fraction from control and β-adrenergic-stimulated rat aortic myocytes. Phosphorylation of a 16 kDa protein which copurified with the plasma membrane marker ($Na^+ + K^+$)-ATPase was most prominent. It was decreased by pretreatment of the myocytes with isoproterenol and the effect of isoproterenol was inhibited by propranolol. Both phosphorylation induced by cAMP-dependent protein kinase and its inhibition by isoproterenol pretreatment declined in preparations exposed to endogenous phosphatase. These results provide strong evidence that β-adrenergic stimulation of aortic myocytes induces in situ phosphorylation of a 16 kDa plasma membrane protein.

(Rat aorta) Myocyte β-Adrenergic stimulation cAMP-dependent phosphorylation Plasma membrane-enriched fraction

1. INTRODUCTION

cAMP has been implicated in smooth muscle relaxation induced by β-adrenergic agonists and other drugs [1,2]. It may also be involved in the regulation of other cell functions, such as cell division [3,4]. Target proteins for cAMP-dependent protein kinase have been studied in various tissues, especially cardiac muscle, but are not well-known in smooth muscle. cAMP-dependent phosphorylation of different substrate proteins has been reported in the cytosolic fraction of aortic smooth muscle [5,6]. One such substrate, myosin light chain kinase, may be implicated in smooth muscle relaxation [6,7]. It has been suggested that cAMP-dependent phosphorylation of plasma membrane [8,9] or endoplasmic reticulum [10] proteins could regulate membrane Ca^{2+} transport. However, the identification of such substrate proteins is still equivocal [6,10–14] and their subcellular localisation has not been clearly demonstrated.

To investigate the existence of membrane associated substrates for cAMP-dependent protein kinase in vascular smooth muscle cells, we have used membrane fractions derived from rat aortic myocytes in suspension or in culture. These membrane fractions appear to be less disrupted than comparable ones derived from whole tissues [15], and are not contaminated by membranes from non-smooth muscle cells.

Abbreviations: Iso, (−)-isoproterenol; Pro, dl-propranolol; PKA, cAMP-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

The aortic media was obtained from 10-week-old female Wistar rats after collagenase digestion [16,17]. Suspensions of aortic myocytes [18] were used directly to obtain membrane fractions or were cultured for 8 days in plastic petri dishes. Membrane fractions were then prepared from confluent cultures [16,17]. Microsomal fractions were ob-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

tained from aortic media and from myocytes in suspension or in culture as described by Kattenburg and Stoclet [15]. Plasma membrane-enriched fractions were obtained from the corresponding microsomal fractions by centrifugation on a discontinuous sucrose gradient [15]. $(Na^+ + K^+)$-ATPase and NADPH-cytochrome *c* reductase activities were used as enzyme markers for plasma membrane and endoplasmic reticulum, respectively [15]. These enzyme activities were measured according to Wuytack et al. [19] and Sottocasa et al. [20], respectively. The 2 lightest fractions F1 and F2 were enriched in plasma membrane marker activities and were free of mitochondrial contamination, but contained some endoplasmic reticulum. They were combined and used as the plasma membrane-enriched fraction. All protein assays were conducted according to Lowry et al. [21], with bovine serum albumin as standard.

Phosphorylation experiments were carried out in a total volume of 100 μl at 30°C, containing: 100 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EGTA (pH 7.0) and 15–20 μg membrane proteins, 0.2 mM [γ-^{32}P]ATP (Mg^{2+} salt) (2–5 μCi/assay) and 1 mM cAMP. Reactions were initiated by addition of the cAMP-dependent protein kinase purified according to Gilman [22,23] and stopped by adding 50 μl Laemmli solution [24] (182 mM Tris-HCl, pH 6.8, 7.5% SDS, 3.6% glycerol, 3.1% mercaptoethanol and 0.005% bromophenol blue) at 4°C. Boiling of the samples (5 min at 95°C) in the presence of 2.5% of SDS did not affect phosphate labelling.

To study the influence of β-adrenergic stimulation of aortic myocytes in culture on phosphorylation of a 16 kDa protein, confluent cells were incubated at 37°C under 5% CO_2/95% O_2 for various time in 3 ml physiological solution (composition in mM: 122 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.2 $MgCl_2$, 15.6 $NaHCO_3$, 11.5 glucose, 1 ascorbic acid). Iso (10^{-7} M) and Pro (5×10^{-7} M) were added as indicated. Unless otherwise indicated, membranes were prepared in the presence of 25 mM NaF, a phosphatase inhibitor. Cells were then homogenized and plasma membrane-enriched fractions were obtained as described [15]. Membrane fractions were phosphorylated by exogenous kinase, cAMP and labelled ATP as above, in the presence of 25 mM NaF and then submitted to electrophoresis. Phosphorylated samples were loaded on SDS-polyacrylamide slab gels consisting of a 5% stacking gel and a 12% separating gel according to Laemmli [24]. Phosphate incorporation was quantitated by cutting the radioactive band from the dried gels and subjecting it to liquid scintillation counting in 10 ml Aquassure.

Iso, Pro, Mg^{2+}-ATP and cAMP were purchased from Sigma. [γ-^{32}P]ATP and Aquassure were obtained from NEN, France. All other chemicals used were of reagent grade.

3. RESULTS

Phosphorylation of proteins of the plasma membrane-enriched fraction F1-2 isolated from cultured myocytes is illustrated in fig.1. A number of proteins were phosphorylated in the presence of labelled ATP and in the absence of exogenous protein kinase and cAMP (lane A). Among these was a protein of apparent 55 kDa which comigrates with the regulatory subunit of the exogenous type II cAMP-dependent protein kinase (lane E). Addi-

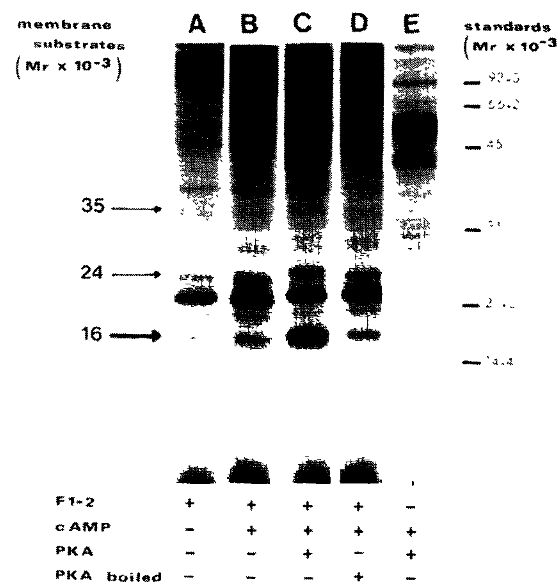


Fig.1. Representative autoradiogram of phosphorylated proteins of a plasma membrane-enriched fraction derived from rat aortic myocytes in culture, 8 days after plating. M_r markers: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysosyme (M_r 92 500, 66 200, 45 000, 31 000, 21 500 and 14 400, respectively).

tion of cAMP and exogenous protein kinase revealed substrate proteins indicated by arrows in fig.1 (16, 24 and 35 kDa). In other experiments (not shown), the same pattern of labelled proteins was obtained with plasma membrane-enriched fractions isolated from myocytes in suspension. In all cases, phosphorylation of the 16 kDa protein was the most prominent and was therefore studied in more detail.

Table 1 shows cAMP-dependent phosphorylation of the 16 kDa protein in cytosolic and microsomal fractions obtained from 3 different preparations: myocytes in culture (A), in suspension (B) and from the media layer (C). In microsomal fractions, the intensity of 16 kDa protein labelling was similar in A and B but lower in C. In cytosolic fractions, 16 kDa protein labelling was obvious in C but could not be detected in A and B. This indicates that homogenization of the media, a traumatic procedure, induces a loss of membrane 16 kDa protein content, whereas gentle homogenization of the myocytes does not.

Table 1

16 kDa protein phosphorylation in microsomal and cytosolic fractions from myocytes in culture, myocytes in suspension and from rat aorta media layer (pmol PO_4^{3-}/mg protein)

	Microsomal fraction	Cytosolic fraction
(A) Myocytes in culture	34.2 ± 0.9	n.d.
(B) Myocytes in suspension	32.6 ± 3.2	n.d.
(C) Media layer	7.5 ± 0.1	21.6 ± 2.1

20–25 μg protein was used in each assay. Results are given as mean SE ± mean (n = 3); n.d., not detectable

16 kDa protein phosphorylation was examined as a function of the plasma membrane enrichment of the fractions (table 2). The results show that the 16 kDa protein co-enriched with the plasma membrane marker ($Na^+ + K^+$)-ATPase (invariant ratio *a*) but did not co-enrich with the endoplasmic reticulum marker NADPH-cytochrome *c* reductase (variable ratio *b*). There is therefore strong evidence for plasma membrane localization of the 16 kDa protein.

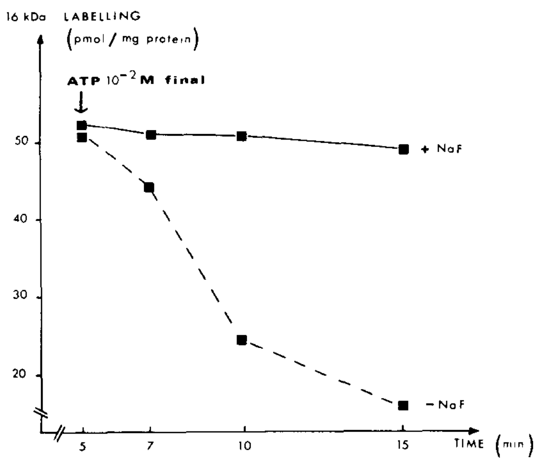
The influence of the phosphatase inhibitor NaF [25] on 16 kDa protein phosphorylation is described in fig.2. An excess of non-radioactive ATP (10^{-2} M final concentration) was added to maximally phosphorylated 16 kDa protein [26]. Under these conditions, the decrease of ^{32}P content of the protein during subsequent incubation was a

Table 2

Distribution of ($Na^+ + K^+$)-ATPase activity [19], NADPH-cytochrome *c* reductase activity [20] and phosphate incorporation into 16 kDa protein in F1, F2, F3 and F4 fractions [15] derived from the same myocyte culture, 8 days after plating (one representative experiment out of 7)

Membrane fractions	($Na^+ + K^+$)-ATPase		16 kDa protein labelling	NADPH-cytochrome *c* reductase	
	Spec. act.	*a*		Spec. act.	*b*
F1	108	1.9	56	3.6	0.06
F2	70	2.7	25.8	3.7	0.14
F3	42	2.6	16.2	4.7	0.29
F4	n.d.	–	n.d.	6.2	–

Units: ($Na^+ + K^+$)-ATPase, nmol P_i/mg per min; NADPH-cytochrome *c* reductase, μmol/mg per min; 16 kDa protein labelling, pmol/mg protein. *a* and *b* are the ratio of enzyme activity/16 kDa protein labelling for ($Na^+ + K^+$)-ATPase and NADPH-cytochrome *c* reductase, respectively; n.d., not detectable



← Fig.2. Representative experiment of 16 kDa protein dephosphorylation in the absence or presence of 10 mM NaF (n = 3) [26]. Plasma membrane-enriched fractions were phosphorylated as described in section 2. After 5 min incubation, 10^{-2} M non-labelled ATP was added and the decrease with time in PO_4^{3-} content of the 16 kDa protein was examined.

measure of phosphatase activity. In the absence of NaF, 16 kDa protein phosphorylation declined to control levels after 15 min incubation, but in the presence of NaF, phosphorylation was stable. In addition, 16 kDa protein phosphorylation was resistant to hydroxylamine treatment (92% radioactivity retained by the protein after 15 min

Table 3

Influence of β-adrenergic stimulation of aortic myocytes in culture on phosphorylation of the 16 kDa protein

Pre-treatment		NaF (25 mM)	n	16 kDa protein	
Drug	Time (min)			$^{32}PO_4^{3-}$ labelling (pmol/mg)	In situ phosphorylation[c] (pmol/mg)
Control	3	+	6	53.4 ± 0.9	–
Iso (10^{-7} M)	0.5	+	3	44.5 ± 0.8[d]	8.7 ± 0.5
	3	+	6	31.0 ± 1.0[d]	22.4 ± 1.1
	3	–[a]	3	32.7 ± 0.65[d]	20.8 ± 0.6
	3	–[b]	3	47.4 ± 1.3[d]	6.3 ± 1.4
Pro (5×10^{-7} M)	3	+	3	53.2 ± 0.4 N.S.	–
Iso (10^{-7} M) Pro (5×10^{-7} M)	3	+	3	49.8 ± 0.8[d]	3.2 ± 0.31

[a] No preincubation
[b] 15 min preincubation at 30°C
[c] Induced by pretreatment
[d] $p < 0.01$, N.S., non-significant

Labelling of the protein was performed in acellular medium by measuring the incorporation of PO_4^{3-} after addition of labelled ATP, in the presence or absence of 25 mM NaF (to inhibit phosphatase activity). In situ phosphorylation induced by pretreatment was calculated in each experiment by subtracting 16 kDa labelling found on the membrane fraction obtained from pretreated cells from 16 kDa labelling in the membrane fraction obtained from control cells. Results are given as mean ± SE and were submitted to variance analysis. In each experiment control and pretreated cells came from the same pool of myocytes in the presence of 0.8 M NH_2OH at 30°C), as expected for a phosphoester linkage. The 16 kDa protein did not appear to be phospholamban-like because of its insensitivity to boiling (not shown).

Since Iso produces cAMP accumulation in rat aortic myocytes, it presumably induces activation of intracellular cAMP-dependent protein kinase and a subsequent increase in phosphate incorporation into target proteins. In vitro phosphorylation of these proteins should therefore be attenuated in membranes isolated from myocytes pretreated with Iso, provided that phosphoprotein phosphatase activity is inhibited during the isolation procedure. As shown in table 3, phosphorylation of the 16 kDa protein was reduced by 17 and 45% in membrane fractions obtained from aortic myocytes stimulated by 10^{-7} M Iso for 30 s and 3 min, respectively, 25 mM NaF being added to homogenisation and assay buffers. When NaF was omitted, the influence of Iso pretreatment disappeared with time: in vitro phosphorylation of the 16 kDa protein was attenuated by 38% when the assay was performed immediately after homogenization but only by 11% when the membrane fraction was incubated for 15 min at 30°C prior to assay. Thus the influence of Iso pretreatment of the cells decreased markedly under the influence of phosphoprotein phosphatase. In addition the effect of Iso pretreatment was inhibited by Pro which alone had no effect. Altogether these results show that β-adrenergic stimulation of the cells attenuated subsequent in vitro cAMP-dependent phosphorylation of the 16 kDa protein and that this phenomenon disappeared if membrane protein phosphatase was not inhibited.

4. DISCUSSION

Rapoport et al. [6] have recently studied phosphorylation of microsomal and soluble proteins in aorta stimulated with drugs which increase intracellular cAMP. They detected phosphorylation of cytosolic proteins only (21–49 kDa). Other studies have also concluded that cAMP did not increase smooth muscle microsomal phosphorylation [11–13]. However, cAMP-dependent phosphorylation of microsomal proteins (42 and 44 kDa) have been reported in rat aorta [10,14]. Three possible experimental differences may account for these different results and may also explain why phosphorylation of a 16 kDa membrane protein has not been previously detected. Firstly, homogenization of whole arterial tissue may induce membrane damage and, as shown here, loss of the 16 kDa protein which is not produced by gentle homogenization of cultured or isolated myocytes. Secondly, whole rat aorta as used in previous studies contains up to 50% of non-smooth muscle cells [27], whereas this study was performed on subcellular fractions of myocytes only. Lastly, phosphorylation experiments reported here were performed in an acellular medium, using a plasma membrane-enriched fraction. These conditions are much more sensitive for the detection of membrane substrates for protein kinase [28,29] than the incorporation of labelled phosphate into intact cells as used by Rapoport et al. [6].

Our results also provide strong evidence that β-adrenergic stimulation of aortic myocytes could induce in situ phosphorylation of a 16 kDa plasma membrane protein. Not only did Iso pretreatment of the myocytes decrease subsequent incorporation of labelled phosphate into the protein, but this effect could also be abolished by endogenous phosphatase activity. In addition, membranes from Iso-treated myocytes which had been dephosphorylated by endogenous phosphatase could then incorporate as much labelled phosphate as could control membranes from untreated myocytes.

The concentration of Iso used was close to the concentration producing half-maximal relaxation and half-maximal accumulation of cAMP in isolated rat aorta with a peak effect reached after 3 min [30]. At this concentration the effect of Iso on in situ phosphorylation of the 16 kDa protein could be detected after 30 s incubation, and after 3 min it reached 45% of the in vitro maximal cAMP-dependent phosphorylation of the protein. As expected, this effect was strongly depressed by 5×10^{-7} M Pro. These findings support the view that cAMP-dependent phosphorylation of a 16 kDa protein may play a role in the effect of β-adrenergic stimulation in aortic myocytes. While this role is still unknown, it might bear on plasma membrane function(s) since the 16 kDa protein copurified with a plasma membrane marker.

Other proteins of similar apparent molecular mass have recently been found in heart and liver plasma membranes. The heart protein (15 kDa) is phosphorylated following Iso stimulation, is different from phospholamban and is also a substrate for protein kinase C [28]. The liver protein (16 kDa) is phosphorylated by diacylglycerol-activated protein kinase C [31]. The role of these proteins is still unknown. However, it appears that the plasma membrane of several tissues, including rat aortic myocytes, contains low molecular mass proteins which are substrates for functionally important protein kinases and may play a part in hormonal regulation of cell functions.

ACKNOWLEDGEMENTS

The authors wish to thank Dr J. Zwiller for his advice. D.M.K. was supported by a postdoctoral fellowship from the Canadian Heart Foundation.

REFERENCES

[1] Namm, D.H. and Leader, J.P. (1976) Blood Vessels 13, 24–47.
[2] Bar, H.P. (1974) Adv. Cyclic Nucleotide Res. 4, 194–237.
[3] Chatelain, R.E., Dardik, B.N. and Robson, R.D. (1985) Eur. J. Pharmacol. 112, 271–275.
[4] Pastan, I.H., Johnson, G.S. and Anderson, W.B. (1975) Annu. Rev. Biochem. 44, 491–498.
[5] Adelstein, R.S., Conti, M.A., Hathaway, D.R. and Klee, C.B. (1978) J. Biol. Chem. 252, 8347–8350.
[6] Rapoport, R.M., Draznin, M.B. and Murad, F. (1982) Proc. Natl. Acad. Sci. USA 79, 6470–6474.
[7] Miller, J.R., Silver, P.J. and Stull, J.T. (1983) Mol. Pharmacol. 24, 235–242.
[8] Scheid, C.R., Honeyman, T.W. and Fay, F.S. (1979) Nature 277, 32–36.
[9] Meisheri, K.D. and Van Breeman, C. (1982) J. Physiol. 331, 429–441.
[10] Bhalla, R.C., Webb, R.C., Singh, D. and Brock, T. (1978) Am. J. Physiol. 234, H508–H514.
[11] Krall, J.F. and Korenman, S.G. (1982) Proc. Soc. Exp. Biol. Med. 170, 245–250.
[12] Hogaboom, G.K., Emler, C.A., Butcher, F.R. and Fedan, J.S. (1982) FEBS Lett. 139, 309–312.
[13] Hirata, M. and Kuriyama, H. (1980) J. Physiol. 307, 143–161.
[14] Chiesi, M. and Carafoli, E. (1984) Biochem. Biophys. Res. Commun. 124, 797–806.
[15] Kattenburg, D.M. and Stoclet, J.C. (1984) Biochim. Biophys. Acta 777, 221–231.
[16] Chamley-Campbell, J., Campbell, G.R. and Ross, R. (1979) Physiol. Rev. 59, 1–61.
[17] Travo, P., Barrett, G. and Burnstock, G. (1980) Blood Vessels 17, 110–116.
[18] Bucher, B., Travo, P., Laurent, P., Stoclet, J.C. (1982) Cell Biol. Int. Rep. 6, 113–123.
[19] Wuytack, F., DeSchutter, G. and Casteels, R. (1981) Biochem. J. 198, 265–271.
[20] Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438.
[21] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[22] Gilman, A.G. (1970) Proc. Natl. Acad. Sci. USA 67, 305–312.
[23] Myamoto, E., Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 6395–6402.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Pato, D.M. and Adelstein, R.S. (1983) J. Biol. Chem. 258, 7047–7054.
[26] Rudolph, S.A. and Krueger, B.K. (1979) Adv. Cyclic Nucleotide Res. 10, 107–133.
[27] Warshaw, D.M., Mulvany, M.J. and Halpern, W. (1979) Circ. Res. 45, 250–259.
[28] Presti, C.F., Jones, L.R. and Lindeman, J.P. (1985) J. Biol. Chem. 260, 3860–3867.
[29] Forn, J. and Greengard, P. (1978) Proc. Natl. Acad. Sci. USA 75, 5195–5199.
[30] Schoeffter, P. and Stoclet, J.C. (1982) Eur. J. Pharmacol. 77, 183–186.
[31] Cooper, R.H., Kobayashi, K. and Williamson, J.R. (1984) FEBS Lett. 166, 125–130.