Biochem. J. (1990) 267, 245–247 (Printed in Great Britain)

Longer-term regulation of pyruvate dehydrogenase kinase in cultured rat cardiac myocytes

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The increased activity of pyruvate dehydrogenase (PDH) kinase induced in hearts of rats by starvation for 48 h was maintained following preparation of cardiac myocytes, and it was also maintained, though at a decreased level, after 25 h of culture in medium 199. This loss of PDH kinase activity was not prevented by n-octanoate, dibutyryl cyclic AMP or glucagon. The PDH kinase activity of myocytes from fed rats was increased to that of starved rats after 25 h of culture with n-octanoate, dibutyryl cyclic AMP or both agents together.

INTRODUCTION

Mammalian mitochondrial pyruvate dehydrogenase (PDH) complexes are regulated by reversible phosphorylation and this control mechanism regulates glucose oxidation in animals [1,2]. Phosphorylation catalysed by PDH kinase intrinsic to the complex is inactivating. In starved or diabetic animals PDH complex activity and glucose oxidation are decreased in most tissues as a result of activation of PDH kinase by products of lipid oxidation and by PDH kinase activator protein (KAP). The activity of KAP is subject to longer-term regulation, being increased by 2.5–3-fold over 24–48 h *in vivo* by starvation or alloxan-induced diabetes [2–4]. Longer-term regulation of KAP-mediated activation of PDH kinase has also been demonstrated in rat hepatocytes in tissue culture. In hepatocytes from fed rats, the activity of PDH kinase was increased during 25 h of culture by glucagon, n-octanoate, dibutyryl cyclic AMP or 8-bromo-cyclic AMP to levels comparable with those induced *in vivo* by starvation [5–7]. Measurement of PDH kinase activity in extracts measures indirectly the activity of KAP, because KAP is stable, whereas metabolite effectors of PDH kinase are lost when mitochondria are depleted of respiratory substrates by incubation with uncouplers such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [2–4,8]. The validity of this assumption has been verified by direct assay of KAP activity in relation to the effects of starvation [8] and of glucagon plus n-octanoate in cultured hepatocytes [5–7]. Muscle is of obvious quantitative importance to the regulation of glucose oxidation *in vivo*; we describe here the results of studies of the longer-term regulation of PDH kinase (by KAP) in cardiac myocytes in culture.

EXPERIMENTAL

Materials

Details of rats, sources of chemicals, biochemicals, tissue culture bottles, medium 199 and antibiotics are given in refs. [4,6,7].

Myocytes and culture

Calcium-tolerant cardiac myocytes were isolated by collagenase digestion of hearts from rats of 250–350 g by the method of Powell *et al.* [9]. The procedure adopted involved perfusion at 37 °C with bicarbonate-buffered saline [10] to wash out blood, followed by 4 min of perfusion with Ca^{2+}-free medium and then perfusion for 16.5 min/g of fresh heart with medium containing collagenase and 15 μM-Ca^{2+}. After dicing and further incubation (10–15 min), dispersion was completed by aspiration (plastic syringe), and myocytes were filtered through nylon mesh, allowed to settle under gravity, resuspended in saline medium [10] and again allowed to settle. After aspiration of the supernatant, the myocytes were resuspended in medium 199 containing 4% (v/v) foetal calf serum, other additions as given in Table 1 or in the text and antibiotics as in [5]. The yield of fresh cells was approx. 5×10^6/g of fresh heart; based on protein, citrate synthase or total PDH complex activity, this represented approx. 25–35% of total cells. Routinely, four Falcon bottles were plated. Because attachment of cardiac myocytes (unlike hepatocytes) is fragile, culture in air/CO_2 (19:1) at 37 °C was continued for 25 h without change of medium.

Viability was generally assessed by microscopic appearance, calcium-tolerant and viable cells being rod-shaped, whereas calcium-intolerant and non-viable cells were rounded [9], though in early studies Trypan Blue exclusion was also used. Approx. 85% of cells were adjudged viable at the beginning of culture, and based on citrate synthase activity, 87% of cells were recovered at the end of culture; i.e. the recovery of viable cells was apparently complete.

Extracts and assays

At the end of culture CCCP was added to the culture medium to 10 μM and incubation was continued for 25 min to effect conversion of inactive PDH complex into active PDH complex. The myocytes were then dispersed into the culture medium with a rubber-coated spatula, the suspension was aspirated into a plastic centrifuge tube and the myocytes were spun down (2 min, 145 *g*, MSE Centaur 2 Centrifuge). The cells were resuspended in 0.5 ml of extraction buffer [8] containing 2.5% (v/v) Triton X-100. Extraction was completed by freezing (liquid N_2) and thawing three times. PDH complex was assayed in extracts by coupling to arylamine acetyltransferase [11]. Total PDH complex was assayed after conversion of inactive complex into active complex with PDH phosphatase as described in [12]. PDH kinase was assayed in extracts by the rate of ATP-dependent inactivation of PDH complex [5,8], and activity was calculated as the apparent first-order rate constant [3]. Citrate synthase was assayed as in [13], adenosine triphosphatase as in [8] and protein as in [14].

Abbreviations used: PDH, pyruvate dehydrogenase; KAP, PDH kinase activator protein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

RESULTS AND DISCUSSION

Cardiac myocytes: basic information

In earlier studies [5–7] PDH kinase was assayed in extracts of mitochondria isolated from hepatocytes. This was impracticable for myocytes, as heart weight is only 10% of that of liver, and PDH kinase was therefore assayed in extracts of myocytes. Recoveries of cardiac myocytes based on total PDH complex, citrate synthase and protein were 24, 27 and 35% respectively. By the same criteria, recoveries following culture were 62, 87 and 78% (medium 199) and 63, 87 and 80% (medium 199 + 50 μM-dibutyryl cyclic AMP). The proportion of PDH complex in the active form (PDH_a) was 32% for fresh heart, 72% in freshly prepared myocytes, 11% after culture in medium 199, and 5% after addition of 50 μM-dibutyryl cyclic AMP. The yield of myocytes was too low to allow routine assay of PDH_a as a percentage of total PDH. PDH complex activities in myocytes incubated with CCCP as a percentage of total activity measured with PDH phosphatase were 99% (fresh cells), 91% (medium 199) and 62% (dibutyryl cyclic AMP). These data are based on duplicate assays performed on a single batch of myocytes prepared from hearts of two fed rats.

The yields of active PDH complex in extracts prepared from myocytes incubated with CCCP sufficed to allow PDH kinase to be assayed at a concentration of PDH complex in the range 0.15–0.3 units/ml. Control experiments showed that the activity of PDH kinase was not influenced by the concentration of PDH complex over this range (results not shown). Control experiments showed also that the presence of up to 92% of the complex in the inactive (phosphorylated) form (the highest tested) had no effect on the measured PDH kinase activity when the concentration of PDH_a was kept constant (225 munits/ml; results not shown). The ATPase activity of myocyte extracts under the conditions of the kinase assay had an apparent first-order rate constant of 0.197 min^{-1} (18% hydrolysis in the first minute). We compute that this rate of ATP hydrolysis would decrease actual PDH kinase activity by 5% assuming a K_m for ATP of 20 μM and k_i for ADP of 100 μM (rat complex; [11]; D. R. Marchington, A. L. Kerbey & P. J. Randle, unpublished work).

PDH kinase activities in cardiac myocytes

The PDH kinase activities assayed in extracts of freshly prepared cardiac myocytes are shown for fed and 48 h-starved rats in line 1 of Table 1. The absolute activities were approx. twice those observed with mitochondrial extracts in [4]. The activities in total heart extracts were also greater than those in mitochondria prepared from the same heart (results not shown). The reason for this difference has yet to be established. The possibilities include loss of KAP or PDH kinase from mitochondria during their isolation or the presence of extramitochondrial KAP or PDH kinase. The effect of starvation in increasing PDH kinase persists in myocytes (Table 1, line 1), and, as measured in extracts of myocytes, it is comparable in magnitude to that observed in extracts of heart mitochondria [3,4]. Culture of myocytes from fed rats had no effect on PDH kinase activity compared with freshly prepared cells, but activity fell by 40% on culture of myocytes from starved rats (Table 1, line 2). This decline in activity in myocytes from starved rats was not prevented by n-octanoate, dibutyryl cyclic AMP, glucagon or glucagon plus octanoate (Table 1, column 2, lines 3, 4, 6 and 7). The activity of PDH kinase in myocytes from fed rats was increased by 1 mM-n-octanoate, by 50 μM-dibutyryl cyclic AMP and by octanoate plus dibutyryl cyclic AMP (Table 1, column 1, lines 3–5). The effects of these different agents were comparable (1.6–1.8-fold increase) and the level of activity achieved was close to that measured in myocytes from starved rats cultured under comparable conditions (Table 1). Glucagon (50 nM) had no effect on the PDH kinase activity of myocytes from fed rats (Table 1, line 6). There are a number of obvious possible explanations for the absence of a glucagon effect despite an increase in PDH kinase activity with dibutyryl cyclic AMP. One is a matter of experimental technique: because attachment of myocytes was fragile, the culture medium could not be changed and glucagon is sensitive to proteolysis. In liver, where change of culture medium was practicable, dibutyryl cyclic AMP was much more effective than glucagon [7].

Table 1. Effects of starvation of the rat and of 1 mM-n-octanoate, 50 mM-dibutyryl cyclic AMP and 50 nM-glucagon *in vitro* on the activity of PDH kinase in cardiac myocytes in culture

Myocytes prepared from rat hearts by collagenase digestion were cultured for 25 h in medium 199 containing 4% (v/v) foetal calf serum with additions as shown. Cells were then incubated with CCCP, and extracts were prepared and assayed for PDH kinase activity, expressed as the apparent first-order rate constant (means ± S.E.M.). The values in parentheses are the total numbers of assays, and there were two to eight assays per myocyte extract. The significance of differences was calculated both by the paired t test (parallel cultures) and by t tests on the total number of observations (analysis of variance showed that differences between myocyte preparations were not significantly greater than differences between PDH kinase assays). * $P < 0.01$ for effect of starvation; † $P < 0.01$ for effect of octanoate or dibutyryl cyclic AMP or both; for other differences, $P > 0.05$.

Culture	Rats...	PDH kinase activity (min^{-1}) Fed	Starved
None (fresh cells)		1.33 ± 0.08 (21)	3.01 ± 0.25* (6)
Control		1.20 ± 0.03 (77)	1.94 ± 0.12* (31)
+ n-Octanoate		1.94 ± 0.09† (38)	2.05 ± 0.10 (6)
+ Dibutyryl cyclic AMP		2.01 ± 0.09† (12)	2.04 ± 0.25 (12)
+ Octanoate + dibutyryl cyclic AMP		2.15 ± 0.14† (6)	–
+ Glucagon		1.07 ± 0.07 (38)	2.22 ± 0.10* (6)
+ Glucagon + octanoate		1.68 ± 0.09 (32)	2.49 ± 0.33* (6)

The effects of n-octanoate and dibutyryl cyclic AMP on the activity of PDH kinase in cardiac myocytes from fed rats and the effect of culture on this activity in myocytes from fed rats are comparable with those observed in hepatocytes [5–7]. In hepatocytes, glucagon induces a modest increase in PDH kinase activity and this is additive with effects of octanoate; these effects of glucagon were not seen in myocytes. The effect of culture in decreasing PDH kinase activity in hepatocytes from starved rats was prevented by n-octanoate or by glucagon plus n-octanoate [7], but these effects were not seen in cardiac myocytes. We conclude that fatty acids and cyclic AMP are longer-term regulators of PDH kinase activity through KAP in rat cardiac myocytes, as in rat hepatocytes.

We thank Dr. T. Powell of the Physiological Laboratory, University of Oxford, for helpful advice on the preparation of cardiac myocytes and Ms. A. E. Jones for skilled technical assistance. This work was supported by a grant from the British Heart Foundation.

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Received 24 October 1989/15 January 1990; accepted 26 January 1990