The glucagon-induced activation of pyruvate dehydrogenase in hepatocytes is diminished by 4 β -phorbol 12-myristate 13-acetate

A role for cytoplasmic Ca²⁺ in dehydrogenase regulation

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1. Phenylephrine, vasopressin and glucagon each increased the amount of active (dephospho) pyruvate dehydrogenase (PDH_a) in isolated rat hepatocytes. Treatment with 4 β -phorbol 12-myristate 13-acetate (PMA) opposed the increase in PDH_a caused by both phenylephrine and glucagon, but had no effect on the response to vasopressin: PMA alone had no effect on PDH_a. As PMA is known to prevent the phenylephrine-induced increase in cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_c) and to diminish the increase in [Ca²⁺]_c caused by glucagon, while having no effect on the ability of vasopressin to increase [Ca²⁺]_c, these data are consistent with the notion that in intact cells an increase in [Ca²⁺]_c results in an increase in the mitochondrial free Ca²⁺ concentration, which in turn leads to the activation of PDH. 2. In the presence of 2.5 mM-Ca²⁺, glucagon caused an increase in NAD(P)H fluorescence in hepatocytes. This increase is taken to reflect an enhanced activity of mitochondrial dehydrogenases. PMA alone had no effect on NAD(P)H fluorescence; it did, however, compromise the increase produced by glucagon. 3. When the extracellular free [Ca²⁺] was decreased to 0.2 μ M, glucagon could still increase NAD(P)H fluorescence. Vasopressin also increased fluorescence under these conditions; however, if vasopressin was added after glucagon, no further increase in fluorescence was observed. Treatment of the cells with PMA resulted in a smaller increase in NAD(P)H fluorescence on addition of glucagon: the subsequent addition of vasopressin now caused a further increase in fluorescence. Changes in [Ca²⁺]_c corresponding to the changes in NAD(P)H fluorescence were observed, again supporting the idea that [Ca²⁺]_c indirectly regulates intramitochondrial dehydrogenase activity in intact cells. 4. PMA alone had no effect on pyruvate kinase activity, and the phorbol ester did not prevent the inactivation caused by glucagon. The latter emphasizes the different mechanisms by which the hormone influences mitochondrial and cytoplasmic metabolism.

INTRODUCTION

It is known that glucagon, vasopressin and phenylephrine each lead to an increase in the active, non-phosphorylated, form of the intramitochondrial enzyme pyruvate dehydrogenase (PDH_a) when added to suspensions of hepatocytes (Assimakopoulos-Jeannet *et al.*, 1983; Oviyasu & Whitton, 1984). Similarly, vasopressin has been shown to raise the PDH_a content of the perfused liver (Hems *et al.*, 1978; Sies *et al.*, 1983; Fisher *et al.*, 1985), and glucagon has been shown to be effective *in vivo* (McCormack, 1985b).

The interconversion of PDH is mediated by a specific kinase and phosphatase [for reviews see Reed (1981) and Wieland (1983)]. Ca²⁺ has been shown to activate the phosphatase (Denton *et al.*, 1972) and, for this reason, it has been proposed that the increase in PDH_a content occurring in response to glucagon, vasopressin and phenylephrine may be mediated by an increase in [Ca²⁺]_m, this being secondary to an increase in [Ca²⁺]_c (Denton & McCormack, 1980; and see Hansford, 1985). Consistent with this notion is the demonstrated increase in [Ca²⁺]_c in hepatocytes in response to these hormones (Charest *et al.*, 1983; Berthon *et al.*, 1984; Thomas *et al.*, 1984; Sistare *et al.*, 1985; Staddon & Hansford, 1986).

Further, the PDH_a content of isolated liver mitochondria can indeed be regulated by concentrations of extramitochondrial free [Ca²⁺] in the physiological range (McCormack, 1985a), in keeping with results obtained with mitochondria from other tissues (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981).

In the present paper, in an attempt to investigate whether changes in PDH_a content relate quantitatively to changes in [Ca²⁺]_c, we used glucagon, vasopressin and phenylephrine to elevate [Ca²⁺]_c in hepatocytes and PMA (an activator of protein kinase C; Castagna *et al.*, 1982) to antagonize the increases caused by both glucagon (Staddon & Hansford, 1986) and phenylephrine (Cooper *et al.*, 1985; Lynch *et al.*, 1985). Further, we examined the effects of these interventions on changes in cell NAD(P)H fluorescence, which are taken to be an indirect measure of changes in mitochondrial dehydrogenase activity. As such, this change in signal will reflect not only PDH activation but also that of the other intramitochondrial Ca²⁺-sensitive enzymes NAD⁺-isocitrate dehydrogenase (Denton *et al.*, 1978) and 2-oxoglutarate dehydrogenase (McCormack & Denton, 1979).

Abbreviations used: [Ca²⁺]_c, cytoplasmic free Ca²⁺ concentration; [Ca²⁺]_m, mitochondrial free Ca²⁺ concentration; PDH, pyruvate dehydrogenase; PDH_a, active (dephospho) pyruvate dehydrogenase; PMA, 4 β -phorbol 12-myristate 13-acetate.

MATERIALS AND METHODS

Isolation of hepatocytes

Hepatocytes were isolated from fed 150–300 g male Sprague–Dawley rats by a modification (Krebs *et al.*, 1974) of the Berry & Friend (1969) method.

Incubation of hepatocytes and sampling for enzyme activity

Hepatocytes were incubated at 37 °C in bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing 2.5 mM-CaCl₂ and 0.5% (w/v) dialysed bovine serum albumin (fraction V). The gas atmosphere was O₂/CO₂ (19:1) and the cells were maintained in suspension by rotary shaking. The cell concentration, as determined by a biuret method (Gornall *et al.*, 1949), was 3–7 mg of protein/ml. Portions of the cells were preincubated for 20 min, and then PMA was added (final concn. 500 nM) from a 1 mM solution in dimethyl sulphoxide. The control cells received the solvent only. After incubation for a further 3 min, portions (2 ml) of the cells were added to 400-fold concentrates of the required agonists. After 5 min, two 0.75 ml samples of the cell suspension from each incubation were centrifuged for 10 s in an Eppendorf 5414 Microfuge. The supernatant was aspirated and the cell pellet then frozen in liquid N₂.

Extraction and assay of PDH

The cell pellet was extracted essentially as described by Assimacopoulos-Jeannet *et al.* (1983). Specifically, ice-cold 0.1 M-potassium phosphate buffer (pH 7.8 at 20 °C) containing 2 mM-EDTA, 1 mM-dithiothreitol, 0.25% (v/v) Triton X-100 and 5% (v/v) rat serum was added to the cell pellet. The volume added was such that final cell protein concentration was about 15 mg/ml. The cell pellet was disrupted by five passages through a 23-gauge needle and then vortex-mixed for 30 s. The extract was centrifuged in an Eppendorf Microfuge for 30 s; the supernatant then was assayed for PDH activity.

PDH was assayed spectrophotometrically by coupling the production of acetyl-CoA to the acetylation of an azo dye (Coore *et al.*, 1971). The temperature was 30 °C. The supernatant (0.15 ml) was added to 0.85 ml of buffer (pH 7.8 at 20 °C) containing 0.12 M-Tris/HCl, 0.6 mM-EDTA, 1.2 mM-MgCl₂, 6 mM-pyruvate, 0.6 mM-NAD⁺, 0.12 mM-CoA, 1.2 mM-thiamin pyrophosphate, 6 mM-dithiothreitol, 60 μ M-*p*-(aminophenylazo)benzenesulphonic acid and 75 munits of arylamine acetyl transferase. The conditions of the assay permit the detection of changes in $V_{\max}$. The reaction rate was followed by measuring the decrease in A_{460} ; this was inversely proportional to the dilution of the enzyme extract.

Extraction and assay of pyruvate kinase

The cell pellet was extracted as described for PDH except that the buffer was ice-cold 0.1 M-Tris/HCl (pH 7.5 at 20 °C) containing 50 mM-NaF, 5 mM-EDTA and 0.1% Triton X-100. The final cell protein concentration was 5 mg/ml. Before assay the extract was centrifuged for 10 min in a Microfuge; the supernatant was then assayed for pyruvate kinase activity.

Pyruvate kinase was assayed spectrophotometrically by coupling the production of pyruvate to the oxidation of NADH. The temperature was 30 °C. The supernatant (50 μ l) was added to 1 ml of 0.1 M-Tris/HCl buffer (pH 7.5 at 20 °C) containing 1.5 mM-ADP, 25 mM-MgCl₂,

0.1 M-KCl, 0.15 mM-NADH and 2 units of lactate dehydrogenase. The endogenous rate of NADH oxidation was measured by following the decrease in A_{340} . Pyruvate kinase activity was then determined by measuring the increase in the rate of NADH oxidation after the addition of 0.5 mM-phosphoenolpyruvate; this sub-saturating concentration was chosen, as it permits the detection of changes in K_m (Engström, 1980; Garrison *et al.*, 1981). The rate of NADH oxidation was in inverse proportion to the dilution of the enzyme extract.

Measurement of NAD(P)H fluorescence

Isolated hepatocytes were suspended in Krebs & Henseleit (1932) bicarbonate-buffered saline, pH 7.4, containing 2% dialysed bovine serum albumin and no added CaCl₂. The cells were stored in 1 ml sealed batches on ice at a concentration of about 10 mg of cell protein/ml. For fluorescence measurements the supernatant was removed and the cell pellet was suspended in 2 ml of saline containing either 2.5 mM- or 0.5 mM-CaCl₂, as indicated. The albumin concentration was 0.5%. The cells were preincubated for 15 min in a rotary shaker and then transferred to the fluorimeter. The cells were continuously gassed with O₂/CO₂ (19:1).

Fluorescence was measured with a Farrand Ratio Fluorimeter-2 (Farrand Optical Co., New York, NY, U.S.A.). The excitation wavelength of 365 nm was selected by an interference filter (Farrand). The emitted light was measured at wavelengths greater than about 460 nm by using filter no. 3-73 from Farrand. The fluorimeter was fitted with a cuvette chamber thermostatically maintained at 37 °C. The cell suspension was stirred magnetically.

Measurement of the fluorescence of hepatocytes loaded with indo-1

Indo-1 (Gryniewicz *et al.*, 1985) was used as a sensitive indicator of changes in $[Ca^{2+}]_i$. Hepatocytes were loaded with indo-1 by using the membrane-permeant tetra-acetoxymethyl ester derivative, indo-1/AM (see Tsien *et al.*, 1982; Staddon & Hansford, 1986). The cells were suspended in saline (Krebs & Henseleit, 1932) containing 2.5 mM-CaCl₂ and 0.5% dialysed bovine serum albumin. The cell protein concentration was about 2.5 mg/ml. After 5 min incubation at 37 °C, indo-1/AM was added (final concn. 10 μ M) from a 5 mM solution in dimethyl sulphoxide. The incubation was continued for a further 30 min, after which time complete de-esterification of the dye was achieved (see Staddon & Hansford, 1986). The loading protocol resulted in an intracellular indo-1 concentration of approx. 0.1 mM. The cells were then washed and stored, and fluorescence measurements made as described by Staddon & Hansford (1986). The excitation and emission wavelengths of 365 nm and 400 nm (see Gryniewicz *et al.*, 1985) were selected by interference filters obtained from Farrand and Oriel respectively.

Materials

Collagenase (type I) was from Cooper. Albumin, [Arg⁸]vasopressin, glucagon, phenylephrine and PMA were from Sigma Chemical Co. Ionomycin was from Calbiochem. Indo-1/AM and indo-1 were from Molecular Probes. All other reagents were of the highest grade commercially available. *p*-(*p*-Aminophenylazo)benzene-

Table 1. Activation of PDH by phenylephrine, vasopressin and glucagon in hepatocytes and the influence of PMA

Hepatocytes were preincubated for 20 min. PMA was then administered to the cells, and 3 min later the agonists were added. After 5 min in the presence of agonists the PDH_a content was measured as described in the Materials and methods section. PDH_a is expressed in terms of nmol/min per mg of cell protein. Values shown are means $\pm$ S.E.M., with the numbers of cell preparations indicated in parentheses. Statistical significance of the difference in values in each column with respect to the control is: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The following effects on PDH_a were without statistical significance: the effect of PMA alone; the effect of PMA on the vasopressin-induced activation; the effect of phenylephrine in the presence of PMA. The activation by glucagon in the control cells was significantly greater ($P < 0.001$) than that in the presence of PMA.

	Control	+ PMA (500 nM)
Control	1.01 $\pm$ 0.05 (8)	0.98 $\pm$ 0.06 (8)
Phenylephrine (25 μ M)	1.20 $\pm$ 0.09 (6)**	1.01 $\pm$ 0.08 (6)
Vasopressin (25 nM)	1.69 $\pm$ 0.13 (8)***	1.58 $\pm$ 0.11 (8)***
Glucagon (10 nM)	2.27 $\pm$ 0.22 (8)***	1.25 $\pm$ 0.13 (8)*

sulphonic acid was a gift from Dr. J. Lipsky (Johns Hopkins University, Baltimore, MD, U.S.A.).

Presentation of results

The fluorescence traces are representative of results obtained with several cell preparations. Where a statistical treatment of results has been adopted, the values are means $\pm$ S.E.M. for data obtained from the numbers of cell preparations shown in parentheses. Statistical significance of results was assessed by a paired *t* test.

RESULTS AND DISCUSSION

Hormonal effects on PDH_a and the influence of PMA

After a preincubation period to allow a steady state to be achieved, phenylephrine, vasopressin and glucagon each caused an increase in PDH_a in isolated hepatocytes (Table 1), essentially in confirmation of previous reports (Assimakopoulos-Jeannot *et al.*, 1983; Oviassu & Whitton, 1984). The total activity of PDH, as determined after a 10 min incubation in the presence of 30 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (a maximally effective concentration at the concentration of albumin used; and see Siess & Wieland, 1975) was 4.67 ± 0.28 (8) nmol/min per mg of cell protein. Thus resting PDH_a was 22% of total PDH activity. Phenylephrine, vasopressin and glucagon caused PDH_a to increase, respectively, to 26, 36 and 49% of total PDH.

PMA alone had no effect on PDH_a, at least after an 8 min exposure to the drug. However, PMA completely prevented the increase caused by phenylephrine, and significantly diminished the glucagon-induced activation. The activation by vasopressin was uninfluenced by PMA (Table 1). Expressed as a percentage of total PDH, PDH_a was 21% with PMA alone and 22%, 34% and 27% in combination with phenylephrine, vasopressin and glucagon respectively. The influence of PMA on the

ability of different agonists to increase PDH_a is thus entirely as expected, on the basis of its effects on agonist-induced increases in $[Ca^{2+}]_c$, if indeed $[Ca^{2+}]_c$ determines $[Ca^{2+}]_m$ and thereby PDH_a.

Hormonal effects on NAD(P)H fluorescence, and the influence of PMA

Further support for the proposal that the activity of the intramitochondrial Ca^{2+} -sensitive dehydrogenases is enhanced, indirectly, by an increase in $[Ca^{2+}]_c$ derives from NAD(P)H-fluorescence measurements. Glucagon in the presence of 2.5 mM- $CaCl_2$ caused an increase in NAD(P)H fluorescence in hepatocytes (Fig. 1a). A similar increase in fluorescence has been previously observed by others (Sugano *et al.*, 1980; Balaban & Blum, 1982; Charest *et al.*, 1983; Sistare *et al.*, 1985).

The interpretation of the increase in fluorescence in Fig. 1(a) is complicated by the presence of NAD(P)H in both the mitochondria and cytoplasm. Small increases in NAD(P)H concentration are translated into large fluorescence increases owing to binding by dehydrogenases (see Sies, 1982, for review). Several lines of evidence may be adduced, however, in support of the conclusion that the increase in fluorescence is due to an increase in the rate of intramitochondrial NADH formation. (i) Inhibition of the respiratory chain is unlikely, as O_2 consumption was previously shown to be stimulated by glucagon in the perfused liver and isolated hepatocytes (Sugano *et al.*, 1980; Balaban & Blum, 1982). Phenylephrine and vasopressin also increased O_2 consumption in isolated hepatocytes (Balaban & Blum, 1982; Assimakopoulos-Jeannot *et al.*, 1983). (ii) Glucagon increased the ratio of β -D-hydroxybutyrate/acetoacetate in isolated hepatocytes, as shown by Siess & Wieland (1980) and confirmed here (results not shown). (iii) Prior treatment of the cells with 5 μ M-rotenone and 20 mM- β -DL-hydroxybutyrate, to reduce mitochondrial NAD^+ , greatly decreased the magnitude of the effect of glucagon. Conversely, pretreatment of the cells with 0.2 mM-amino-oxyacetate for 3 min, and then with 20 mM-ethanol for 90 s, did not prevent glucagon from causing the increase in fluorescence (results not shown). The latter conditions reduce cytoplasmic NAD^+ while blocking the transfer of redox equivalents into the mitochondria. (iv) Glucagon does indeed increase the PDH_a content (Assimakopoulos-Jeannot *et al.*, 1983; Oviassu & Whitton, 1984; Table 1) and oxoglutarate dehydrogenase activity in hepatocytes (see Staddon & McGivan, 1984).

PMA caused a small, non-reproducible, increase in nicotinamide nucleotide fluorescence, but consistently compromised the increase seen in response to glucagon (Fig. 1b). The increase in fluorescence in the presence of glucagon plus PMA was $54 \pm 5\%$ ($n = 11$; $P < 0.001$) of that with glucagon alone. Phenylephrine also caused an increase in NAD(P)H fluorescence (Fig. 1c; and see Sugano *et al.*, 1980; Charest *et al.*, 1983), which was greatly diminished to $9 \pm 2\%$ ($n = 3$; $P < 0.001$) by pretreatment with PMA (Fig. 1d). The effect of phenylephrine was also blocked by 50 μ M-phenolamine (results not shown), suggesting that the agonist was acting exclusively via α -receptors. The effect of vasopressin was uninfluenced by PMA (Fig. 1d): in the presence of PMA the increase in fluorescence was $106 \pm 6\%$ ($n = 5$) of that in its absence. When ionomycin was used to raise $[Ca^{2+}]_c$ (see Staddon & Hansford, 1986), an increase in NAD(P)H fluorescence was also observed (Fig. 1e). Thus

Fig. 1. Effects of glucagon and PMA on NAD(P)H fluorescence in hepatocytes incubated with 2.5 mM-CaCl₂.

Fluorescence was measured as described in the Materials and methods section. The final concentrations of added compounds were: 10 nM-glucagon; 500 nM-PMA; 25 μ M-phenylephrine; 25 nM-vasopressin; 10 μ M-ionomycin. The maximum increases in fluorescence caused by the agonists, expressed as a percentage of the increase in fluorescence obtained by adding 5 μ M-rotenone plus 20 mM-DL- β -hydroxybutyrate (results not shown), are as follows: (a) glucagon, 37%; (b) glucagon, 17%; (c) phenylephrine, 51%; (d) vasopressin, 44%; (e) ionomycin, 31%. Fluorescence is in arbitrary units.

NAD(P)H fluorescence changes in concert with $[Ca^{2+}]_c$.

The kinetics of the glucagon-induced increase in both NAD(P)H fluorescence and $[Ca^{2+}]_c$ are consistent with a causal relationship. The fluorescence of indo-1-loaded cells did not start to increase until about 6 s after glucagon addition and reached a peak at about 20 s (Fig. 2a). There was a lag of about 9 s before any increase in NAD(P)H fluorescence was observed, and this then reached a peak about 30 s after glucagon addition (Fig. 2b). Similar temporal relationships have been observed with phenylephrine and vasopressin (Charest *et al.*, 1983; Thomas *et al.*, 1984).

An estimate of values for $[Ca^{2+}]_c$ achieved in Fig. 2(a) was performed by using calibration protocols described previously (see Tsien *et al.*, 1982; Staddon & Hansford, 1986). Mn²⁺ (0.1 mM) was used to quench dye fluorescence. In the presence of 2.5 mM-CaCl₂ a correction was made for incomplete quenching, and then F_{min} was estimated from $F_{max} - AF$ (where AF is the fluorescence in the absence of dye), essentially as described by Gelfand *et al.* (1986). The K_d of indo-1 for Ca²⁺ was taken as 250 nM (Gryniewicz *et al.*, 1985). When ionomycin (20 μ M) was used to achieve F_{max} , $[Ca^{2+}]_c$ increased from 464 ± 23 to 1659 ± 163 nM (6) with glucagon; these values, for unknown reasons, are greater than those previously

estimated with quin2 (Staddon & Hansford, 1986), a problem similarly encountered by Gelfand *et al.* (1986). The use of Triton X-100 to achieve F_{max} gave lower values for $[Ca^{2+}]_c$, reminiscent of the quantification using quin2 (Staddon & Hansford, 1986): the increase was from 196 ± 11 to 353 ± 35 nM (6).

Glucagon caused an increase in NAD(P)H fluorescence even when the extracellular free $[Ca^{2+}]$ was decreased to about 0.2 μ M (Fig. 3a), a condition which allows mobilization of intracellular Ca²⁺ stores while minimizing Ca²⁺ entry into the cell. A transient increase in $[Ca^{2+}]_c$ occurred under these conditions (Fig. 3b; Staddon & Hansford, 1986): when ionomycin was used to estimate F_{max} , $[Ca^{2+}]_c$ increased from 346 ± 20 nM to peak of 1116 ± 158 nM; for F_{max} obtained by using Triton X-100, the corresponding values were 177 ± 21 nM and 348 ± 64 nM (4). Addition of vasopressin alone caused increases in NAD(P)H fluorescence and $[Ca^{2+}]_c$ (results not shown) similar to those observed with addition of glucagon. However, addition of vasopressin after glucagon caused no increase in NAD(P)H fluorescence (Fig. 3a) despite an increase in $[Ca^{2+}]_c$ (Fig. 3b) to a peak value of 755 ± 132 nM or 277 ± 42 nM (4), as estimated from F_{max} determined, respectively, by using ionomycin or Triton X-100. One explanation for the lack

Fig. 2. Early kinetics of the glucagon-induced increase in the fluorescence of indo-1-loaded hepatocytes (a) and the increase in NAD(P)H fluorescence (b)

Fluorescence was measured as described in the Materials and methods section. The final concentration of glucagon was 10 nM, and the extracellular $[CaCl_2]$ was 2.5 mM. Fluorescence is in arbitrary units.

of an increase in NAD(P)H fluorescence despite the increase in $[Ca^{2+}]_c$ is that glucagon may cause a time-dependent stimulation of NADH oxidation by the respiratory chain (see Halestrap *et al.*, 1983).

In the experiments with low extracellular Ca^{2+} , the increase in NAD(P)H fluorescence observed with glucagon was diminished to $58 \pm 4\%$ ($n = 7$; $P < 0.001$) by pretreatment with PMA, and the subsequent addition of vasopressin now caused an increase in fluorescence (Fig. 3c); the increase in $[Ca^{2+}]_c$ caused by glucagon was

similarly attenuated (Staddon & Hansford, 1986; Fig. 3d) and that caused by subsequent addition of vasopressin enhanced (Fig. 3d). When indo-1 was used, $[Ca^{2+}]_c$ was estimated to increase from 335 ± 23 nM to peaks of 547 ± 108 nM with glucagon ($P < 0.05$ versus the increase in the absence of PMA) and 1348 ± 79 nM (4) with vasopressin, as determined by using ionomycin to estimate F_{max} ; with F_{max} obtained by the addition of Triton X-100 the corresponding values were 193 ± 27 , 276 ± 60 and 398 ± 69 nM.

In the presence of 2.5 mM- $CaCl_2$ (Fig. 3e), glucagon increased $[Ca^{2+}]_c$ from 442 ± 29 to 1564 ± 239 nM: PMA addition then decreased this to 602 ± 61 nM, and vasopressin increased it to 9575 ± 3373 nM (4). These values were derived by using ionomycin to estimate F_{max} ; with F_{max} obtained from Triton X-100, the corresponding values were 180 ± 6 , 323 ± 41 , 212 ± 7 and 466 ± 56 nM.

Hormonal effects on the activity of pyruvate kinase and the influence of PMA

The effects of the hormones and PMA on the activity of the cytoplasmic enzyme pyruvate kinase were investigated. Glucagon, via cyclic AMP, causes phosphorylation of the enzyme, with a concomitant increase in K_m for phosphoenolpyruvate (see Engström, 1980); phenylephrine and vasopressin similarly lead to an inactivation, albeit smaller, but act via Ca^{2+} and not cyclic AMP (see Garrison *et al.*, 1981). The effects of the hormones on pyruvate kinase are confirmed in Table 2. Quantitatively, the similarity of the effect of phenylephrine to that of vasopressin indicates that phenylephrine is acting only via α -receptors.

PMA alone had no effect on the activity of pyruvate kinase. However, PMA prevented the inactivation

Fig. 3. Effects of glucagon and PMA on NAD(P)H fluorescence in hepatocytes incubated with 0.2 μM free Ca^{2+} (except trace e, where the $CaCl_2$ concentration was 2.5 mM) and the corresponding fluorescence changes in hepatocytes loaded with indo-1

Fluorescence was measured as described in the Materials and methods section. The cells were preincubated with 0.5 mM- $CaCl_2$, except for those in (e), where the $CaCl_2$ was 2.5 mM. EGTA (0.75 mM), and/or PMA, was added (not shown) 3 min before addition of hormone in (a)–(d). The final concentrations of added compounds were: 10 nM-glucagon; 25 nM-vasopressin; 500 nM-PMA. Fluorescence is in arbitrary units. The maximum increases in NAD(P)H fluorescence caused by the agonists, expressed as a percentage of the increase in fluorescence obtained by adding 5 μM -rotenone plus 20 mM-DL- β -hydroxybutyrate (results not shown), are as follows: (a) glucagon, 35%; (c) glucagon, 19%.

Table 2. Inactivation of pyruvate kinase by phenylephrine, vasopressin and glucagon in hepatocytes and the influence of PMA

Hepatocytes were preincubated for 20 min. PMA was then added, and after a further 3 min the agonists were added. The activity of pyruvate kinase was determined after 5 min in the presence of agonists, as described in the Materials and methods section. Pyruvate kinase activity is expressed in terms of nmol/min per mg of cell protein. Values shown are means $\pm$ S.E.M., with the numbers of cell preparations indicated in parentheses. Statistical significance of the differences in values in each column with respect to the control is: * $P < 0.05$; *** $P < 0.001$. PMA alone had no significant effect on the activity of pyruvate kinase. Pyruvate kinase activity in the presence of glucagon was not significantly different from that in the presence of glucagon plus PMA.

	Control	+PMA (500 nM)
Control	10.3 $\pm$ 0.7 (8)	10.2 $\pm$ 0.9 (8)
Phenylephrine (25 μ M)	8.9 $\pm$ 0.9 (8)*	10.2 $\pm$ 0.6 (7)
Vasopressin (25 nM)	8.3 $\pm$ 0.5 (8)***	7.8 $\pm$ 0.5 (8)***
Glucagon (10 nM)	3.1 $\pm$ 0.5 (8)***	3.6 $\pm$ 0.4 (8)***

induced by phenylephrine, but had no effect on that by vasopressin (Table 2), as expected from a role for Ca^{2+} in the inactivation. In marked contrast with its effect on the activation of PDH, PMA had no effect on the ability of glucagon to inactivate pyruvate kinase (Table 2). The increase in cyclic AMP content with 10 nM-glucagon may be greater than that required for activation of the cyclic AMP-dependent protein kinase: thus the partial inhibition by PMA of the increase in cyclic AMP (Heyworth *et al.*, 1984; García-Sáinz *et al.*, 1985) may be of no significance under these conditions.

To summarize, the activation of protein kinase C in hepatocytes leads to an attenuation of the ability of glucagon to increase $[\text{Ca}^{2+}]_c$ (Staddon & Hansford, 1986). The present work establishes a similar attenuation of both the glucagon-induced activation of pyruvate dehydrogenase and the increase in NAD(P)H content. The closely parallel behaviour of these three parameters strongly supports the proposal that changes in $[\text{Ca}^{2+}]_c$ caused by glucagon do exert meaningful effects on intramitochondrial Ca^{2+} -sensitive dehydrogenase activity via changes in $[\text{Ca}^{2+}]_m$. This contrasts with the response of the cytoplasmic enzyme pyruvate kinase, where PMA was without influence on the ability of glucagon to cause a decrease in activity. McCormack (1985b), with a different approach involving the isolation of liver mitochondria from rats injected with glucagon, also demonstrated that the increase in PDH_a seen in response to the hormone may be attributed to an increase in $[\text{Ca}^{2+}]_m$.

It is appreciated that the interconversion of PDH could, in theory, be achieved by mechanisms other than an increase in $[\text{Ca}^{2+}]_m$, as the kinase and phosphatase are subject to regulation by other effectors. In particular, the kinase activity is inhibited by ADP and pyruvate (Hucho *et al.*, 1972), and enhanced by increased $[\text{NADH}]/[\text{NAD}^+]$ and $[\text{acetyl-CoA}]/[\text{CoA}]$ ratios (Cooper *et al.*, 1975; Pettit *et al.*, 1975). It is clear from the results

presented here (Figs. 1–3) that a decrease in the mitochondrial $[\text{NADH}]/[\text{NAD}^+]$ ratio cannot be a factor in causing the increase in PDH_a content, and it is highly unlikely that a decreased mitochondrial $[\text{ATP}]/[\text{ADP}][\text{P}_i]$ ratio is involved. The latter conclusion follows from the near-equilibrium nature of oxidative phosphorylation (Holian *et al.*, 1977) and the hormonally induced increases in $[\text{NADH}]/[\text{NAD}^+]$ ratio. Published work on hormonally induced changes in these effectors is also inconsistent with their involvement in generating increases in PDH_a content (see, e.g., Siess *et al.*, 1977; Hems *et al.*, 1978; Assimacopoulos-Jeannet *et al.*, 1983).

The physiological importance of the increases in $[\text{Ca}^{2+}]_c$ and PDH_a produced by glucagon, and the antagonism by protein kinase C, however, remains to be elucidated.

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