Regulation of pyruvate dehydrogenase by insulin and polyamines within electroporabilized fat-cells and isolated mitochondria

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1. Regulation of the mammalian pyruvate dehydrogenase (PDH) complex by insulin and polyamines has been examined by using electroporabilized rat epididymal fat-cells and isolated mitochondria. The complex could be regulated within the permeabilized cells not only by insulin, but also by certain low- M_r species, including Ca^{2+} and the polyamine spermidine. 2. Both spermine and spermidine increased the level of active dephosphorylated PDH (PDH_a) in isolated adipose-tissue mitochondria 2–3-fold, with half-maximal effects at 0.9 mM and 1.7 mM respectively. By contrast, PDH activity in rat heart mitochondria was essentially insensitive to the effects of these polyamines. 3. The effects on PDH activity of incubation of adipose-tissue mitochondria with spermine persisted through re-isolation and re-incubation of the mitochondria in the absence of the polyamine. 4. No evidence was found of any increase in the concentration of spermine associated with purified mitochondrial fractions prepared from insulin-treated tissue. 5. Overall, the data provide further evidence against a role for polyamines in the rapid stimulation of PDH by insulin, but suggest that polyamines may be important in mediating longer-term changes in the activity of the complex.

INTRODUCTION

The mammalian pyruvate dehydrogenase (PDH) system is located exclusively within the mitochondrial matrix, and exists in an inactive phosphorylated form (PDHP) and an active dephosphorylated form (PDH_a). Exposure of adipose tissue to insulin, which leads to the marked stimulation of fatty acid synthesis from glucose, also causes a rapid (5–15 min) increase in the level of PDH_a (Denton *et al.*, 1971). This apparently results from an increase in the activity of PDHP phosphatase, an enzyme specific for the complex (Hughes & Denton, 1976; Denton *et al.*, 1984; Thomas & Denton, 1986; Denton *et al.*, 1989). A striking feature of this activation is that it is still apparent after isolation and re-incubation of mitochondria with a respiratory substrate such as oxoglutarate (Denton *et al.*, 1984). Indeed, enhanced activity is still apparent even after permeabilization of the mitochondria, and is associated with a decrease in the $K_{0.5}$ value (concentration giving half-maximal effects) for Mg^{2+} (Thomas & Denton, 1986; Thomas *et al.*, 1986).

The mechanism by which insulin induces this effect on PDHP phosphatase remains obscure. Ca^{2+} has been shown to be a potent regulator of PDHP phosphatase *in vitro* (Denton *et al.*, 1971), and its effect within mitochondria is due largely to a decrease in K_m for Mg^{2+} (Thomas *et al.*, 1986; Midgley *et al.*, 1987). However, this second messenger is not apparently involved in the activation elicited by insulin (Denton *et al.*, 1984, 1989), but is important in the regulation of the PDH complex by other hormones (McCormack & England, 1983; Assimacopoulos-Jeannet *et al.*, 1986; McCormack *et al.*, 1990; Rutter & Denton, 1992).

Work by Damuni *et al.* (1984) and ourselves (Thomas *et al.*, 1986) has demonstrated that isolated PDHP phosphatase is also stimulated *in vitro* by the polyamines spermine and spermidine; again, the effect involves a diminution in the $K_{0.5}$ value for Mg^{2+} , raising the possibility that insulin might act on PDH by altering the concentration of polyamines within mitochondria. There

have been a number of suggestions that polyamines may be important in other actions of insulin (Lockwood & East, 1974; Tung *et al.*, 1985; Koenig *et al.*, 1989; Pedersen *et al.*, 1989; Richelsen *et al.*, 1989). However, we have been unable to detect any effects of either polyamine on the PDH_a level in mitochondria permeabilized to all low- M_r species with toluene (Thomas & Denton, 1986; Thomas *et al.*, 1986; G. A. Rutter, unpublished work).

We have recently described a preparation of electroporabilized rat epididymal fat-cells which retain full metabolic responses to insulin and other hormones, while allowing at the same time the manipulation of the concentrations of low- M_r compounds in the cytosol (Rutter & Denton, 1992). In the present paper we have used this preparation as well as isolated mitochondria to re-examine the regulation of fat-cell PDH by insulin and polyamines.

EXPERIMENTAL

Materials

Chemicals and biochemicals were obtained from the sources given previously (Rutter & Denton, 1988, 1992; Thomas *et al.*, 1986); in addition, spermine, spermidine and putrescine (HCl form) were obtained from Sigma (Poole, Dorset, U.K.).

Isolation, permeabilization and incubation of fat-cells

Cells were isolated from the epididymal fat-pads of male Wistar rats (160–200 g) by collagenase digestion, and transferred to an 'intracellular medium', comprising 125 mM-KCl, 4.5 mM-NaCl, 12.5 mM-Hepes, pH 7.0, 0.5 mM-EGTA, 0.5 mM-N-(2-hydroxyethyl)ethylenediaminetriacetic acid ('HEDTA'), 5 mM- NaHCO_3 , 1 mM-glucose, 2 mM- MgCl_2 , 5 mM-2-oxoglutarate, 1 mM-malonnate, 2.4 mM- KH_2PO_4 and 1% (w/v) dialysed fatty-acid-free BSA, gassed with O_2/CO_2 (19:1), 37 °C (Rutter & Denton, 1992). Free Ca^{2+} concentrations in this medium were found to be < 5 nM, by using the Ca^{2+} indicator fura-2 (Rutter

Abbreviations used: PDH, PDH_a , PDHP, pyruvate dehydrogenase and its active (unphosphorylated) and inactive (phosphorylated) forms respectively. The concentrations of Ca^{2+} given in this paper refer to the free, unbound, form of the cation.

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& Denton, 1989), and were not affected by the addition of spermine or spermidine up to 10 mM. Cells were electroporated with six discharges from a 25 μ F capacitor (Gene Pulsar, Bio-Rad) at a field strength of 3 kV/cm (Rutter & Denton, 1992). Incubations were performed as given in the legend to Table 1, not more than 20 min after the electroporation procedure. At the end of the incubation period the infranatant was removed, ice-cold extraction buffer (0.1 M-KH₂PO₄, pH 7.2, 2 mM-EDTA, 1 mM-dithiothreitol, plus 50 μ l of rat serum/ml; 3 ml/g dry wt. of cells) was added, and the cells were briefly (1 s) vortex-mixed before freezing on liquid N₂. The cells were frozen and thawed twice more to ensure the complete disruption of mitochondria, and samples were centrifuged for 1 min at 10000 *g* before assay of PDH activity.

Isolation and incubation of mitochondria

Rat epididymal fat-pads were incubated with or without insulin (83 nM), and mitochondria were isolated in medium containing 250 mM-sucrose, 20 mM-Tris/HCl, pH 7.4, 2 mM-EGTA, 7.5 mM-GSH and 1% essentially fatty-acid-free BSA, at 0 °C, as described previously (Denton *et al.*, 1984). Rat heart mitochondria were isolated as described by Denton *et al.* (1980), except that the above isolation medium was used. Final mitochondria pellets were resuspended at 20–50 mg of protein/ml in isolation buffer and kept on ice until required.

Mitochondria were incubated at 37 °C in 'intracellular medium' (see above), and incubations terminated by sedimentation (25 s, 10000 *g*) and rapid freezing on liquid N₂. The pellets were extracted by freezing and thawing in P_i-based extraction buffer (see above and Denton *et al.*, 1980). For measurement of spermine content, mitochondria were further purified by centrifugation in a Percoll gradient (Belsham *et al.*, 1980).

Other methods

Assays of initial and total PDH activity in extracts and ATP contents of cells and mitochondria, were performed as described previously (McCormack & Denton, 1989; Rutter & Denton, 1992). Mitochondrial protein concentrations were estimated from measurements of total PDH activity, assuming levels of 80 m-units/mg of protein for adipose-tissue mitochondria prepared without Percoll fractionation (Denton *et al.*, 1984), where 1 unit catalyses the conversion of 1 μ mol of substrate/min at 30 °C. Free and bound concentration of metal ions and other species were calculated as in Rutter & Denton (1988), by using an iterative computer program. Spermine was determined by h.p.l.c. after derivative formation with benzoyl chloride as described by Verkoelen *et al.* (1988). Statistical significance was determined by Student's *t* test.

RESULTS

Effects of insulin, Ca²⁺ and polyamines on PDH_a in electrically permeabilized fat-cells

In the absence of other additions, the level of PDH_a in the permeabilized fat-cells incubated in the presence of respiratory substrates (see the Experimental section) was increased 1.5–2.0-fold by incubation in the additional presence of 83 nM-insulin (Table 1). This stimulation is consistent with the activation of fatty acid synthesis by insulin under these conditions (Rutter & Denton, 1992).

The activity of PDH in the cells could also be readily manipulated by addition of CaCl₂ to give 1.8 μ M free Ca²⁺ (i.e. within the intracellular range of 0.1–5.0 μ M; see McCormack *et al.*, 1990). This activation is also observed in isolated mito-

Table 1. Effects of insulin, Ca²⁺ and polyamines on PDH_a in electrically permeabilized cells

Cells were incubated at 0.2 g dry wt. of cells/ml of 'intracellular medium' with the further additions shown for 15 min, and PDH activity was assayed in extracts, as described in the Experimental section. Data are means $\pm$ S.E.M. of four to six incubations using two to three separate preparations: † *P* < 0.05, †† *P* < 0.01, ††† *P* < 0.001 for effect of addition; * *P* < 0.05, for effect of insulin.

Addition	PDH _a (%)	
	Control	83 nM-insulin
None	11.9 $\pm$ 0.9	22.3 $\pm$ 3.3*
1.8 μ M-Ca ²⁺	31.4 $\pm$ 1.4††	39.9 $\pm$ 6.7†
None	19.3 $\pm$ 3.3	—
1 mM-spermidine	47.8 $\pm$ 2.5†††	—
None	15.2 $\pm$ 2.6	—
1 mM-spermine	18.7 $\pm$ 2.6	—

chondria from rat adipose and other tissues (Denton *et al.*, 1980, 1984; McCormack, 1985) and occurs as a result of uptake of Ca²⁺ into the mitochondrial matrix (Lukacs & Kapus, 1987; McCormack *et al.*, 1989) and the consequent stimulation of PDHP phosphatase activity.

The presence of spermidine (1 mM) also markedly increased PDH_a in the electroporated cells, but there was little effect of spermine (1 mM) (Table 1).

Effects of polyamines on PDH_a in isolated mitochondria

As observed previously (McCormack, 1989), spermine and spermidine at concentrations up to 10 mM had very little effect on PDH_a in intact, fully coupled heart mitochondria, incubated in the absence (< 10 nM) of Ca²⁺ (Fig. 1). By contrast, increasing concentrations of either spermidine or spermine increased the level of PDH_a in adipose-tissue mitochondria isolated and incubated under identical conditions (Fig. 1); putrescine was ineffective, however (results not shown). Treatment with spermine of mitochondria from control tissue increased PDH_a from 17.0 $\pm$ 2.5 to 46.4 $\pm$ 6.4% (see Fig. 1). The calculated value for the concentration giving half-maximal effects (*K*_{0.5} value) was 0.9 $\pm$ 0.4 mM (40 degrees of freedom). Spermidine was slightly less effective, increasing PDH_a to 39 $\pm$ 3.0% at saturating concentrations, with a *K*_{0.5} of 1.7 $\pm$ 0.4 mM (40 degrees of freedom). Fig. 2 illustrates that the effects of spermine and spermidine on PDH_a were rapid and essentially complete within 1–2 min.

As first reported by Denton *et al.* (1984), after incubation with a respiratory substrate, PDH_a in mitochondria isolated from insulin-treated tissue was consistently 1.5–2.0-fold higher than that in mitochondria from control tissue (Fig. 1). The activity was increased further (Fig. 1b) by incubation with spermidine (from 27.4 $\pm$ 1.1 to 49.2 $\pm$ 2.4% of total PDH activity; *K*_{0.5} 2.0 $\pm$ 0.3 mM; 40 degrees of freedom). Thus PDH_a was increased in mitochondria from insulin-treated tissue relative to those from control tissue, at all concentrations of spermidine. Spermine also increased the level of PDH_a in mitochondria from insulin-treated tissue (to 46.7 $\pm$ 4.0% of total PDH; *K*_{0.5} 0.7 $\pm$ 0.3 mM; 40 degrees of freedom). However, the difference in PDH_a content between mitochondria from control and insulin-treated tissue was less apparent when mitochondria were incubated with saturating levels of spermine (Fig. 1a). Neither polyamine caused any diminution in the mitochondrial content of ATP (Fig. 1).

The elevation of PDH_a activity elicited by spermine was apparently largely retained after sedimenting, washing in isolation medium and re-incubation (Table 2). A similar observation

Fig. 1. Effect of (a) spermine and (b) spermidine on PDH_a in mitochondria isolated from control (○) or insulin-treated adipose tissue (●) or heart (■)

Mitochondria were incubated at approx. 0.2 mg of protein/ml in KCl-based 'intracellular medium' (see the Experimental section) at 37 °C for 5 min before extraction and assay of initial and total PDH activity. PDH_a is given as % of total activity. Data are the means ± S.E.M. of four to eight separate observations on two to four entirely separate preparations of mitochondria. Data were fitted by a non-linear regression program (from Dr. P. J. England, Smith Kline Beecham, Welwyn, Herts., U.K.) to the following equation:

$$PDH_a = A/[1 + (B/X)^H] + C$$

where *A* is the increase in PDH_a elicited by the polyamine, *B* is the *K*_{0.5} value, *X* is the polyamine concentration, *H* is a variable coefficient and *C* is the level of PDH_a in the absence of added polyamine. Kinetic constants (for *n* = 44) are given in the text. ATP levels were measured in mitochondria from control (▽) or insulin-treated (▼) adipose tissue (see the Experimental section) in parallel experiments; data are the means of two observations which agreed to within 10%.

Fig. 2. Kinetics of the effects of (a) spermine and (b) spermidine on PDH_a in isolated adipose-tissue mitochondria from control (○, □, △) or insulin-treated (●, ■, ▲) mitochondria

Mitochondria were incubated as described in the legend to Fig. 1 for the times indicated, either in the absence (○, ●) of further additions or in the presence of 3.0 mM-spermine (□, ■) or 3.0 mM-spermidine (△, ▲). Data are the means ± range of duplicate incubations from a single preparation of mitochondria; closely similar results (not shown) were obtained with two further preparations of mitochondria.

Table 2. Retention of the effect of spermine on PDH_a in isolated adipose-tissue mitochondria

Mitochondria (approx. 0.1 mg of protein) from control or insulin-treated tissue were incubated (first incubation) in 0.5 ml of KCl-based 'intracellular' medium at 37 °C (see the Experimental section) for 2 min in the absence or presence of spermine. Mitochondria were then rapidly sedimented, washed in 1 ml of ice-cold isolation medium (see the Experimental section), resuspended in 50 μl of this medium and then re-incubated for a further 2 min (second incubation) in a further 0.5 ml of KCl-based medium. Data are means ± S.E.M. for four separate preparations of mitochondria: * effect of spermine *P* < 0.05 for paired observations; † effect of insulin *P* < 0.05.

Spermine concn. present during first incubation	PDH _a (%) after second incubation in mitochondria from:	
	Control tissue	Insulin-treated tissue
0 mM	36 ± 1.4	44 ± 1.1†
3 mM	45 ± 2.7*	55 ± 3.4*

was made for mitochondria from control adipose tissue, which were preincubated for 2 min in the absence or presence of 1 mM-spermine, and then diluted 10-fold with further incubation medium (minus spermine) before incubation for a further 2 min at the resulting sub-stimulatory spermine concentration. This gave levels of PDH_a at the end of the re-incubations of 30.0 ± 0.6% (initial incubation in the absence of spermine;

$n = 3$) and $47.3 \pm 5.6\%$ (initial incubation with 1 mM-spermine; $n = 3$, $P < 0.05$ for effect of preincubation with spermine).

The spermine content of mitochondria prepared from control and insulin-treated tissue was determined after further purification of the mitochondria by centrifugation in a gradient of Percoll. This was necessary to remove plasma membranes, which contain significant amounts of spermine and spermidine (T. A. Diggle, unpublished work). The effects of insulin on PDH was to increase its activity 2.4-fold above control values, but there was no evidence of any increase in spermine content (1.48 ± 0.17 nmol of spermine/mg of mitochondrial protein from insulin-treated tissue, compared with 1.98 ± 0.15 nmol/mg from control tissue; data are means $\pm$ S.E.M. for duplicate observations on three separate preparations). Amounts of spermidine were below the level of reliable assay.

DISCUSSION

Mechanism of the activation of PDH activity by polyamines within isolated adipose-tissue mitochondria

The increases in PDH_a observed in adipose-tissue mitochondria in response to spermine or spermidine are most simply explained by the entry of the polyamines into the mitochondrial matrix, leading to an increase in intramitochondrial polyamine concentration and a lowering of the $K_{0.5}$ for Mg^{2+} of PDHP phosphatase. Since rat heart PDHP phosphatase is known to be sensitive to polyamines (Thomas *et al.*, 1986), the much weaker effect on PDH_a in isolated heart mitochondria may be either because polyamines penetrate these mitochondria more poorly or because these mitochondria already contain sufficient concentrations of unbound polyamines to stimulate PDHP phosphatase maximally.

Polyamine transport by mitochondria has been relatively little studied. Only for liver mitochondria has uptake been demonstrated directly (Toninello *et al.*, 1985, 1988). The specificity of this uptake system was not extensively examined, but possibly involves co-transport of spermine (in the bivalent form) with phosphate. Less is known about efflux of polyamines from mitochondria.

The data shown in Fig. 2 suggest that the distribution of polyamines may rapidly achieve a steady-state distribution across the inner membrane of adipose-tissue mitochondria, determined by the kinetic properties of the uptake and efflux pathways. Such a model is analogous to the steady-state cycling of Ca^{2+} , which sets intramitochondrial free Ca^{2+} concentrations (Akerman & Nicholls, 1983; Crompton, 1985; McCormack *et al.*, 1990).

Since $K_{0.5}$ values for the effects of spermine and spermidine on PDH_a obtained with isolated mitochondria were similar to those for isolated PDHP phosphatase (Thomas *et al.*, 1986), it follows that the steady-state gradient of polyamines across the mitochondrial inner membrane is probably small and that the free concentration of both spermine and spermidine within isolated adipose-tissue mitochondria is considerably less than the total (about 1 mM; see above).

Use of electroporated fat-cells to study the mechanism of the activation of PDH by insulin

Electroporated fat-cells offer a novel system for investigating the mechanism of action of hormones, including insulin (Shibata *et al.*, 1991; Rutter & Denton, 1992). The present report extends previous studies to the action of insulin on PDH. Thus PDH activity was still sensitive to insulin, while also being sensitive to exogenously added low- M_r factors, including spermidine and Ca^{2+} . These molecules may enter the permeabilized cells both via the pores in the plasma membrane generated by the electroporation process as well as via any transport systems

normally present in the plasma membrane of these cells for the transfer of Ca^{2+} (Tsien & Tsien, 1990) and polyamines (Pegg & McCann, 1982). However, the former may be the more important, since (i) changes of Ca^{2+} in the millimolar range are necessary to elicit changes in PDH activity (Marshall *et al.*, 1984), rather than in the low-micromolar range found for the permeabilized cells; (ii) under the conditions used in the present study, addition of spermidine to intact cells did not significantly increase the activity of PDH (T. A. Diggle, unpublished work; see also below). In our previous report (Rutter & Denton, 1992) we argued that the stimulation of fatty acid synthesis by insulin did not involve any changes in intracellular Ca^{2+} concentration, since this was clamped at low-nanomolar levels by the presence of EGTA within the cells. The present data extend this observation to the control of PDH activity by insulin, consistent with our previous findings (Denton *et al.*, 1984, 1989).

Can changes in cellular polyamine content explain the mechanism of action of insulin on PDH activity?

A number of lines of evidence exist that have suggested that polyamines may play a role in the intracellular actions of insulin. Firstly, numerous studies have shown that polyamine levels may be increased in cells as a result of the induction of ornithine decarboxylase, a key controlling enzyme in polyamine biosynthesis (Blackshear *et al.*, 1991; Pegg, 1988). These changes, which are probably important in allowing enhanced rates of cell growth and differentiation, would seem too slow to explain the rapid activation of PDH which is observed in adipose tissue in response to insulin. However, there exists reports from one group (see Koenig *et al.*, 1989) of insulin-induced changes in these parameters in kidney cortex, liver and heart cells, which are apparently sufficiently rapid (1–2 min) to be compatible with being involved in rapid actions of insulin. Secondly, exogenously added polyamines can mimic a number of the effects of insulin on fat-cells. For example, spermine can increase lipogenesis (Lockwood & East, 1974) and exert a modest anti-lipolytic effect (Richelsen *et al.*, 1989). Further, spermine can also affect insulin binding to fat-cells (Pedersen *et al.*, 1989). However, it is likely that at least the effects on lipogenesis result from the formation of H_2O_2 via the action of polyamine oxidase present as a contaminant of some preparations of BSA (Livingston *et al.*, 1978). The purified preparations of BSA used in the present studies appeared to be free of this activity, as no detectable effects of spermine were found on fat-cell lipogenesis under conditions in which insulin was increasing rates by more than 10-fold (T. A. Diggle, unpublished work).

Turning to the observations made in the present study, the absence of any effect of polyamines on PDH_a in heart mitochondria over a range which increased this parameter 2–3-fold in adipose-tissue mitochondria (from control tissue) might be considered as weak evidence in support of a role of polyamines in insulin action, since rapid effects of insulin on PDH_a are not observed in heart. Further weak support is provided by the retention of the effect of spermine on PDH_a in mitochondria incubated first in the presence, and then in the absence, of the polyamine (Table 2). As mentioned in the Introduction, a key feature of the activation of PDH by insulin is that the effect persists through the preparation and subsequent incubation of mitochondria.

However, our other findings would appear to rule out changes in polyamine concentrations being largely responsible for the effect of insulin on PDH activity. These findings are the following: (i) the effects of spermidine on PDH_a levels were clearly additive to those of insulin (Fig. 1) (although this additivity was less apparent with spermine, it may be that this is in part due to the partial reversal of the stimulatory effects of spermine observed at

higher levels of the polyamine); (ii) there is no evidence of any increase in the amount of spermine associated with mitochondria purified from insulin-treated tissue; (iii) the effects of insulin on PDH are retained during incubation of electroporabilized cells (the present study) and also still persist in mitochondria made fully permeable to all small molecules by treatment with toluene (Thomas & Denton, 1986).

General conclusion: significance of the activation of PDH by polyamines

Although polyamines probably play no role in the rapid activation of fat-cell PDH by insulin, the findings of the present study do suggest that changes in polyamine concentrations may influence PDH activity in other situations where there is clearer evidence for increases in their concentration. Such changes occur after the induction of ornithine decarboxylase by growth factors and other hormones, and are often associated with enhanced cell growth and hence an increased requirement for acetyl-CoA formation and ATP synthesis. A recent report indicates that increases in polyamine levels in a pancreatic β -cell line may be linked to increases in oxidative metabolism (Sjöholm *et al.*, 1991).

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REFERENCES

- Akerman, K. E. O. & Nicholls, D. G. (1983) *Rev. Physiol. Biochem. Pharmacol.* **95**, 149–201
- Assimakopoulos-Jeannet, F., McCormack, J. G. & Jeanrenaud, B. (1986) *J. Biol. Chem.* **261**, 8799–8804
- Belsham, G. J., Denton, R. M. & Tanner, M. J. A. (1980) *Biochem. J.* **192**, 457–467
- Blackshear, P. J., Nemenoff, R. A., Hovis, J. G., Halsey, D. L., Stumpo, D. J. & Huang, J.-K. (1991) *Mol. Endocrinol.* **1**, 44–52
- Crompton, M. (1985) *Curr. Top. Membr. Transp.* **25**, 231–276
- Damuni, Z., Humphreys, J. S. & Reed, L. J. (1984) *Biochem. Biophys. Res. Commun.* **124**, 95–99
- Denton, R. M., Coore, H. G., Martin, B. R. & Randle, P. J. (1971) *Nature (London)* **231**, 115–116
- Denton, R. M., McCormack, J. G. & Edgell, N. E. (1980) *Biochem. J.* **190**, 107–117
- Denton, R. M., McCormack, J. G. & Marshall, S. E. (1984) *Biochem. J.* **217**, 441–452
- Denton, R. M., Midgley, P. J. W., Rutter, G. A., Thomas, A. P. & McCormack, J. G. (1989) *Ann. N.Y. Acad. Sci.* **573**, 285–296
- Hughes, W. A. & Denton, R. M. (1976) *Nature (London)* **264**, 471–473
- Köenig, H., Goldstone, A., Lu, C. Y., Iqbal, Z., Fan, C. C. & Trout, J. J. (1989) in *The Physiology of Polyamines*, vol. 1 (Bachrach, V. & Heimer, Y. M., eds.), pp. 57–81, CRC Press, Boca Raton, FL
- Livingston, J. N., Amatruda, M. & Lockwood, D. H. (1978) *Am. J. Physiol.* **234**, E484–E488
- Lockwood, D. H. & East, L. E. (1974) *J. Biol. Chem.* **249**, 7717–7722
- Lukacs, G. L. & Kapus, A. (1987) *Biochem. J.* **248**, 609–613
- Marshall, S. E., McCormack, J. G. & Denton, R. M. (1984) *Biochem. J.* **218**, 249–260
- McCormack, J. G. (1985) *Biochem. J.* **231**, 581–595
- McCormack, J. G. (1989) *Biochem. J.* **264**, 167–174
- McCormack, J. G. & Denton, R. M. (1989) *Methods Enzymol.* **174**, 95–118
- McCormack, J. G. & England, P. J. (1983) *Biochem. J.* **214**, 581–585
- McCormack, J. G., Browne, H. M. & Dawes, N. J. (1989) *Biochim. Biophys. Acta* **973**, 420–427
- McCormack, J. G., Halestrap, A. P. & Denton, R. M. (1990) *Physiol. Rev.* **70**, 391–425
- Midgley, P. J. W., Rutter, G. A., Thomas, A. P. & Denton, R. M. (1987) *Biochem. J.* **241**, 371–377
- Pedersen, S. B., Hougaard, D. M. & Richelsen, B. (1989) *Mol. Cell. Endocrinol.* **62**, 161–166
- Pegg, A. E. (1988) *Cancer Res.* **48**, 459–774
- Pegg, A. E. & McCann, P. P. (1982) *Am. J. Physiol.* **243**, C212–C221
- Richelsen, B., Pedersen, S. B. & Hougaard, D. M. (1989) *Biochem. J.* **261**, 661–665
- Rutter, G. A. & Denton, R. M. (1988) *Biochem. J.* **252**, 181–189
- Rutter, G. A. & Denton, R. M. (1989) *Biochem. J.* **263**, 453–462
- Rutter, G. A. & Denton, R. M. (1992) *Biochem. J.* **281**, 431–435
- Shibata, H., Robinson, F. W., Benzing, C. F. & Kono, T. (1991) *Arch. Biochem. Biophys.* **285**, 97–104
- Sjöholm, A., Welsh, N., Hofteizer, V., Bankston, W. & Hellerstoröm, C. (1991) *Biochem. J.* **277**, 533–540
- Thomas, A. P. & Denton, R. M. (1986) *Biochem. J.* **238**, 93–101
- Thomas, A. P., Diggle, T. A. & Denton, R. M. (1986) *Biochem. J.* **238**, 83–91
- Toninello, A., DiLisa, F., Siliprandi, D. & Siliprandi, N. (1985) *Biochim. Biophys. Acta* **815**, 399–404
- Toninello, A., Miotto, G., Siliprandi, D., Siliprandi, N. & Garlid, K. D. (1988) *J. Biol. Chem.* **263**, 19407–19411
- Tsien, R. W. & Tsien, R. Y. (1990) *Annu. Rev. Cell Biol.* **6**, 715–760
- Tung, H. Y. L., Pelech, S., Fisher, M. J., Pogson, C. I. & Cohen, P. (1985) *Eur. J. Biochem.* **147**, 305–313
- Verkoelen, C. F., Romijn, J. C., Schroeder, F. H., Van Schalkwijk, W. P. & Splinter, T. A. W. (1988) *J. Chromatogr.* **426**, 41–54

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