Ca²⁺-dependent activation of oxoglutarate dehydrogenase by vasopressin in isolated hepatocytes

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Vasopressin stimulated gluconeogenesis from proline in hepatocytes from starved rats; this was attributed to an activation of oxoglutarate dehydrogenase (EC 1.2.4.2) [Staddon & McGivan (1984) *Biochem. J.* **217**, 477–483]. The role of Ca²⁺ in the activation mechanism was investigated. (1) In the absence of extracellular Ca²⁺, vasopressin caused a stimulation of gluconeogenesis and a decrease in cell oxoglutarate content that were markedly transient when compared with the effects in the presence of Ca²⁺. (2) Ca²⁺ added to cells stimulated for 2 min by vasopressin in the absence of extracellular Ca²⁺ sustained the initial effects of vasopressin. Ca²⁺ added 15 min after vasopressin, a time at which both the rate of gluconeogenesis and the cell oxoglutarate content were close to the control values, caused a stimulation of gluconeogenesis and a decrease in cell oxoglutarate content. (3) Under conditions of cell-Ca²⁺ depletion, vasopressin had no effect on gluconeogenesis or cell oxoglutarate content. (4) Ionophore A23187 stimulated gluconeogenesis and caused a decrease in cell oxoglutarate content, but the phorbol ester 4 β -phorbol 12-myristate 13-acetate had no effects. (5) These data suggest that the initial activation of oxoglutarate dehydrogenase by vasopressin is dependent on an intracellular Ca²⁺ pool and independent of extracellular Ca²⁺. For activation of a greater duration, a requirement for extracellular Ca²⁺ occurs. The activation of oxoglutarate dehydrogenase by A23187 is consistent with a mechanism involving Ca²⁺, but the lack of effect of 4 β -phorbol 12-myristate 13-acetate indicates that protein kinase C is not involved in the mechanism of activation by vasopressin.

Vasopressin, in addition to its antidiuretic and pressor actions, has potent effects on hepatic intermediary metabolism (for review, see Williamson *et al.*, 1981), and the molecular mechanisms by which vasopressin causes these metabolic effects are being extensively studied. The stimulation of glycogenolysis by vasopressin is particularly well understood. Vasopressin in liver does not use cyclic AMP as a second messenger (Kirk & Hems, 1974), but the hormone does cause an increase in cytosolic [Ca²⁺], and this causes activation of phosphorylase *b* kinase and hence phosphorylation and activation of phosphorylase (see Charest *et al.*, 1983).

Other aspects of the mechanism of vasopressin action in liver are not so well understood as its stimulation of glycogenolysis. Gluconeogenesis

from proline in hepatocytes from starved rats was stimulated by vasopressin, and this stimulation is attributed in part to an activation of a mitochondrial enzyme, oxoglutarate dehydrogenase (Staddon & McGivan, 1984; see also Palmer *et al.*, 1983). The regulatory properties of purified oxoglutarate dehydrogenase have been studied *in vitro*. Activity can be increased by several mechanisms, i.e. by increases in [Ca²⁺], [NAD⁺]/[NADH], [ADP]/[ATP], [CoA]/[succinyl-CoA] and [H⁺] (see McCormack & Denton, 1979). The multitude of mechanisms by which enzyme activity can be increased *in vitro* obviously implies that there are several possible mechanisms by which vasopressin could activate oxoglutarate dehydrogenase in the intact cell.

The mitochondrial enzymes pyruvate dehydrogenase and NAD⁺-isocitrate dehydrogenase have some regulatory properties that are similar to those of oxoglutarate dehydrogenase. In particular, all

Abbreviation used: PMA, 4 β -phorbol 12-myristate 13-acetate.

three can be activated by increases in $[Ca^{2+}]$ within the same concentration range (Denton *et al.*, 1972, 1978; McCormack & Denton, 1979). Denton & McCormack (1980, 1981) have proposed that these enzymes may be activated by hormones which increase cytosolic $[Ca^{2+}]$. Strong evidence in support of this has been obtained from studies on isolated rat heart mitochondria and intact perfused rat heart (Denton *et al.*, 1980; Hansford, 1981; McCormack & England, 1983; McCormack & Denton, 1984). In the liver hormones, such as phenylephrine and vasopressin, which increase cytosolic $[Ca^{2+}]$ (Charest *et al.*, 1983) have a number of effects in addition to the activation of oxoglutarate dehydrogenase which are compatible with an increase in intramitochondrial free Ca^{2+} . These include the activation of pyruvate dehydrogenase (Hems *et al.*, 1978; Assimacopoulos-Jeannet *et al.*, 1983) and increased $^{14}CO_2$ production from $[1-^{14}C]$ oleate (Sugden *et al.*, 1980a,b,c), $[1-^{14}C]$ butyrate and $[U-^{14}C]$ proline (Palmer *et al.*, 1983). These effects are much diminished in Ca^{2+} -depleted cells.

In the present paper the Ca^{2+} -dependence of the vasopressin-induced activation of oxoglutarate dehydrogenase in hepatocytes has been investigated in detail. Data are presented that indicate that the initial activation by vasopressin is independent of extracellular Ca^{2+} but dependent on an intracellular Ca^{2+} pool, but extracellular Ca^{2+} is required for full expression of the hormone-induced activation.

Experimental

Hepatocyte preparation, incubation and sampling

Hepatocytes were isolated from 200–300 g 24-h starved male Wistar rats by a modification (Krebs *et al.*, 1974) of the Berry & Friend (1969) method. The cells were incubated in bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing 2% (w/v) dialysed bovine serum albumin (fraction V); 2.5 mM- $CaCl_2$ was present or replaced with either 0.1 mM- or 0.5 mM-EGTA (Na^+ salt, pH 7.4) as indicated. All incubations were at 37°C in sealed plastic flasks containing an atmosphere of CO_2/O_2 (1:19). The cells (8–12 mg of cell protein/ml) were kept in suspension by rotatory shaking. For time-course experiments, 7 ml of cell suspension was preincubated with 5 mM-proline in a 50 ml flask for 25 min, then 0.6 ml of cell suspension was added to 0.06 ml of 35% (w/v) $HClO_4$. Additions were made as required to the remaining cells and subsequently samples were taken at the indicated times. The samples were vortex-mixed and then centrifuged (10000g for 2 min); the supernatant was removed and adjusted to approx. pH 7 by addition of 3M-KOH containing 0.25 M-Mops. After cooling on ice

and centrifugation (10000g for 2 min), the supernatant was used for metabolite assays.

Metabolite assays

Protein was determined by a biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard. Oxoglutarate was determined enzymically by the method of Bergmeyer & Bernt (1965). Glucose was determined essentially as described by Slein (1965), except that NAD^+ was used in conjunction with $NAD(P)^+$ -specific glucose-6-phosphate dehydrogenase.

Expression of results

Values for metabolites are expressed in units of nmol/mg of cell protein throughout. Where representative results are shown, similar results were obtained from at least three independent cell preparations. Other results are given as means $\pm$ S.E.M., and the significance of the results was assessed by a paired *t* test unless indicated otherwise. The numbers of independent cell preparations used to obtain the data are indicated.

Materials

Enzymes and coenzymes used in the metabolite assays were from Sigma Chemical Co., Poole, Dorset, U.K., or Boehringer Corp., Lewes, Sussex, U.K. Collagenase (type II) was from Worthington. $[Arg^8]$ Vasopressin (type V), A23187 and PMA were from Sigma.

Results

Vasopressin effects on oxoglutarate dehydrogenase activity in the presence and absence of extracellular Ca^{2+}

The rate of gluconeogenesis from proline in hepatocytes from starved rats may be used to measure flux through oxoglutarate dehydrogenase (Staddon & McGivan, 1984). The data of Hensgens *et al.* (1978) substantiate the assumption that oxoglutarate formed from proline is exclusively converted into glucose. In addition, the ratio of urea to glucose formed from proline under several hormonal conditions was close to the expected value of 1 (Staddon & McGivan, 1984). Gluconeogenesis from glutamine similarly involves flux through oxoglutarate dehydrogenase, and the stoichiometry of this conversion (Joseph & McGivan, 1978; Baverel & Lund, 1979) also corroborates the assumption that oxoglutarate is quantitatively metabolized to glucose. Increased flux through a non-equilibrium enzyme coincident with a decrease in its substrate concentration indicates activation of that enzyme. The measurement of the cell oxoglutarate content under conditions of increased oxoglutarate dehydrogenase flux may indi-

cate enzyme activation (see Staddon & McGivan, 1984).

In the absence of extracellular Ca^{2+} , the stimulation of gluconeogenesis from proline by vasopressin as measured over a 30 min interval was considerably decreased when compared with the stimulation in the presence of Ca^{2+} (Staddon & McGivan, 1984). A time course of the effect of vasopressin on gluconeogenesis under these conditions shows that the stimulation of gluconeogenesis was transient (Fig. 1a). Glucose formed in the first 5 min after hormone addition was

6.3 ± 0.6 nmol/mg for control cells and 10.0 ± 1.2 (59% stimulation) in the presence of 15 nM-vasopressin ($n = 6$, $P \leq 0.01$). For the 5–30 min interval after hormone addition the glucose formed was 26.4 ± 1.4 nmol/mg for control cells, and this was not significantly different from that in the presence of 15 nM-vasopressin [24.8 ± 1.6 ($n = 4$)].

In the presence of 2.5 mM- Ca^{2+} , the glucose formed during the first 5 min after hormone addition was 8.3 ± 0.9 nmol/mg for control cells and 15.4 ± 1.4 (86% stimulation) in the presence of 15 nM-vasopressin ($n = 6$, $P \leq 0.001$). In the 5–30 min interval after vasopressin addition, the values for gluconeogenesis were 30.8 ± 3.4 nmol/mg for the control cells and 48.1 ± 4.2 (56% stimulation) in the presence of hormone ($n = 6$, $P \leq 0.01$). Even in the 20–30 min interval after vasopressin addition, a 36% stimulation of gluconeogenesis was still observed (results not shown). It is apparent that the initial (<5 min) stimulation by vasopressin of gluconeogenesis from proline is independent of extracellular Ca^{2+} , but for a stimulation of longer duration there is a requirement for extracellular Ca^{2+} .

Vasopressin, in the absence of extracellular Ca^{2+} , still caused a rapid decrease in the cell oxoglutarate content (Fig. 1b), which is similar to that observed in the presence of Ca^{2+} (see Staddon & McGivan, 1984). In the absence of extracellular Ca^{2+} , the cell oxoglutarate content 2 min after vasopressin addition was 3.93 ± 0.31 nmol/mg for control cells and 2.69 ± 0.30 in the presence of hormone ($n = 7$, $P \leq 0.001$). In the presence of Ca^{2+} the corresponding values for control and vasopressin-treated cells were 3.55 ± 0.52 and 1.59 ± 0.37 ($n = 4$, $P \leq 0.001$).

After 30 min in the presence of vasopressin, but in the absence of extracellular Ca^{2+} (Fig. 1b), the cell oxoglutarate content was 4.46 ± 0.25 nmol/mg, which was not significantly different from the control cell value of 4.48 ± 0.37 ($n = 7$). In the presence of extracellular Ca^{2+} the corresponding values were 3.64 ± 0.33 for control cells and 2.39 ± 0.27 in the presence of vasopressin ($n = 4$, $P \leq 0.01$). Sugden *et al.* (1980c) observed that vasopressin caused a Ca^{2+} -dependent decrease in cell oxoglutarate content in hepatocytes from fed rats, but no details of the times involved were given.

Consideration of the simultaneous effects of vasopressin in the absence of extracellular Ca^{2+} on the rate of gluconeogenesis from proline and the cell oxoglutarate content (Figs. 1a and 1b) indicates that a rapid activation of oxoglutarate dehydrogenase occurs that is similar in magnitude to that observed in the presence of extracellular Ca^{2+} . This activation was markedly transient in the absence of extracellular Ca^{2+} when compared with the activation in its presence.

Fig. 1. Time courses of (a) the stimulation by vasopressin of gluconeogenesis from 5 mM-proline in the absence of extracellular Ca^{2+} (0.1 mM-EGTA added 5 min before hormone) and (b) the changes in cell oxoglutarate content. ●, Control; ■, +15 nM-vasopressin. For incubation details see the Experimental section. Glucose formation was measured from the end of the preincubation period. Results of a representative experiment are shown.

Effects of vasopressin on oxoglutarate dehydrogenase under conditions of intracellular Ca^{2+} depletion

Vasopressin had no effect on either the rate of gluconeogenesis from 5 mM-proline or the cell oxoglutarate content in hepatocytes preincubated for 25 min in saline containing 0.5 mM-EGTA and no added CaCl_2 (results not shown; experiments were carried out over the same time course as depicted in Fig. 1). The rate of gluconeogenesis under these conditions was approximately half that in the presence of 2.5 mM- CaCl_2 . These incubation conditions are expected to deplete intracellular Ca^{2+} stores, which suggests that the rapid initial activation of oxoglutarate dehydrogenase by vasopressin is dependent on such stores. This interpretation is equivocal, as the decreased rate of gluconeogenesis caused by 0.5 mM-EGTA indicates an impairment of cell metabolism. However, it was reported that hepatocytes responded to vasopressin under similar conditions in that increased inositol lipid turnover was still observed (Thomas *et al.*, 1983).

Effects of adding Ca^{2+} on oxoglutarate dehydrogenase in cells initially stimulated with vasopressin in the absence of extracellular Ca^{2+}

To investigate further the role of extracellular Ca^{2+} in the activation of oxoglutarate dehydrogenase by vasopressin, the effects of adding CaCl_2 to cells preincubated in the absence of extracellular CaCl_2 were studied. CaCl_2 added 2 min after vasopressin prevented both the stimulated rate of gluconeogenesis from returning to the control rate and the decreased cell oxoglutarate content from rapidly returning to control values (Figs. 2a and 2b). No readily discernible effects of CaCl_2 addition were observed on control cells. These observations indicate that the addition of extracellular Ca^{2+} 2 min after vasopressin prevents the subsequent return to control activity of oxoglutarate dehydrogenase after its initial activation.

When CaCl_2 was added 15 min after vasopressin in the absence of extracellular Ca^{2+} , a time at which both the cell oxoglutarate content and the rate of gluconeogenesis were similar to the control values, a rapid decrease in cell oxoglutarate content was observed and gluconeogenesis was stimulated (Figs. 3a and 3b). Glucose formed in the 15 min after the addition of CaCl_2 was 20.7 ± 2.5 nmol/mg in the control cells and 29.5 ± 2.8 (43% stimulation) in the presence of 15 nM-vasopressin ($n = 4$, $P \leq 0.01$). This is consistent with an activation of mitochondrial oxoglutarate dehydrogenase by the addition of extracellular Ca^{2+} . This apparent requirement by vasopressin for extracellular Ca^{2+} for oxoglutarate dehydrogenase activation must exist as early as 2 min, and last for at least 15 min after vasopressin addition.

Fig. 2. Effects of adding 2.5 mM- CaCl_2 2 min after vasopressin to hepatocytes initially incubated in the absence of extracellular CaCl_2 (see legend to Fig. 1) on (a) gluconeogenesis from 5 mM-proline and (b) cell oxoglutarate content. ●, Control; ■, + 15 nM-vasopressin. For incubation details see the Experimental section. Glucose formation was measured from the end of the preincubation period. Results of a representative experiment are shown.

Effects of A23187 and PMA on oxoglutarate dehydrogenase

Considerable evidence indicates that a bifurcated transducing mechanism is involved in the action of Ca^{2+} -mobilizing hormones (for review see Berridge, 1984). At least two messengers appear to be generated, namely increased cytosolic $[\text{Ca}^{2+}]$ and diacylglycerol, which activates protein kinase C (Takai *et al.*, 1979; Kishimoto *et al.*, 1980). Putative molecular dissectors of the two branches of the transducing mechanism are avail-

Fig. 3. Effects of adding 2.5 mM-CaCl₂ 15 min after vasopressin to hepatocytes initially incubated in the absence of extracellular CaCl₂ (see legend to Fig. 1) on (a) gluconeogenesis from 5 mM-proline and (b) cell oxoglutarate content ●, Control; ■, +15 nM-vasopressin. For incubation details see the Experimental section. Glucose formation was measured from the end of the preincubation period. Results of a representative experiment are shown.

able. Phorbol esters, such as PMA, can activate protein kinase C (Castagna *et al.*, 1982; Yamanishi *et al.*, 1983), but do not affect Ca²⁺ fluxes in hepatocytes (Garrison *et al.*, 1984). The Ca²⁺ ionophore A23187 may indicate cell responses dependent on changes in cell Ca²⁺ redistribution, for example the activation of phosphorylase (Blackmore *et al.*, 1978). A23187 and PMA had distinct effects on protein phosphorylation in hepatocytes; in combination the effects of vasopressin were mimicked, and it was surmised that Ca²⁺ and diacylglycerol may be the messengers responsible for the hormone effects (Garrison *et al.*, 1984).

A23187 caused a dose-dependent stimulation of gluconeogenesis from proline (net formation of

Table 1. A23187 titration of the stimulation of gluconeogenesis from 5 mM-proline

The incubation procedure was as follows. Hepatocyte suspension (7 ml) was preincubated in 50 ml plastic flasks for 25 min with 5 mM-proline, 0.5 ml was removed for assay, and then samples (1 ml) of the remaining cells were added to 2.5 µl of dimethyl sulphoxide or the required concentration of A23187 in dimethyl sulphoxide in 25 ml plastic flasks, and a sample taken for assay 30 min later. Glucose formation is expressed as the amount formed in the 30 min interval between sampling. Values given are means ± S.E.M. for the numbers of independent cell preparations indicated in parentheses. Statistical significance of the results with respect to the control was determined by Student's *t* test; **P* ≤ 0.01, ***P* ≤ 0.001.

A23187 (µM)	Glucose formed (nmol/mg in 30 min)
0 (control)	41.9 ± 2.9 (8)
1.0	42.9 ± 3.0 (4)
2.5	58.7 ± 5.0 (5)*
5.0	65.4 ± 4.4 (8)**
10.0	60.5 ± 3.7 (5)*

glucose in a 30 min period) in saline containing 2.5 mM-CaCl₂ (Table 1); 5 µM-A23187 was the optimal concentration tested. The ionophore solvent dimethyl sulphoxide, at concentrations (up to 0.25%, v/v) used had no effect on gluconeogenesis (results not shown). The time course of the stimulation of gluconeogenesis caused by 5 µM-A23187 is shown in Fig. 4(a). The stimulation was rapid and sustained for at least 30 min, and 5 µM-A23187 also caused a rapid and large sustained decrease in cell oxoglutarate content (Fig. 4b). These results indicate that A23187 caused an activation of oxoglutarate dehydrogenase and are consistent with a mechanism of activation involving Ca²⁺.

A23187 is an ionophore capable of bivalent-cation exchange, but can also exchange cations for protons, and hence it is capable of acting as a mitochondrial uncoupler (Reed & Lardy, 1972). The effects of A23187 on oxoglutarate dehydrogenase must be interpreted with care because of the complex actions of the ionophore. Mitochondrial uncouplers can stimulate gluconeogenesis from proline (Hensgens *et al.*, 1978) and hence the effects of A23187 may not be entirely due to its action as a Ca²⁺ ionophore. A23187 has been reported to cause a decrease in the ATP content of perfused liver (Friedmann *et al.*, 1979).

PMA used at 1 or 5 µg/ml had no effect either on gluconeogenesis from proline, measured over a 30 min interval, or on the cell content of oxoglutarate measured 30 min after PMA addition (results not shown). This negative result, assuming that under the conditions of the experiment protein

Fig. 4. Time courses of (a) the stimulation of gluconeogenesis from 5mM-proline by 5 μ M-A23187 and (b) the changes in cell oxoglutarate content

●, Control, ■, +5 μ M-A23187. For incubation details see the Experimental section. Glucose formation was measured from the end of the pre-incubation period. Results of a representative experiment are shown.

kinase C was activated, suggests that protein kinase C is not involved in the activation of oxoglutarate dehydrogenase by vasopressin. Garrison *et al.* (1984) in their studies on protein phosphorylation used a concentration of PMA similar to that used in our study.

Discussion

The results presented in this paper indicate that, for full expression of oxoglutarate dehydrogenase activation by vasopressin, there is a requirement for both intracellular and extracellular pools of Ca^{2+} . It is noteworthy that full activation of hepatic phosphorylase by α -adrenergic agonists

also requires both intracellular and extracellular Ca^{2+} pools (Reinhart *et al.*, 1984b). However, from the data presented it cannot be inferred whether the effect of Ca^{2+} on the enzyme is direct or indirect. It has been proposed (Denton & McCormack, 1980, 1981) that an increase in mitochondrial free $[\text{Ca}^{2+}]$ as a consequence of an increase in cytosolic $[\text{Ca}^{2+}]$, such as that caused by vasopressin in liver (Charest *et al.*, 1983), may be responsible for the hormonally induced activation of certain mitochondrial dehydrogenases. Reports of direct measurement of the effect of vasopressin on liver mitochondrial total Ca^{2+} are conflicting. Both an increase (Shears & Kirk, 1984) and a decrease have been reported (Exton, 1981). No doubt these discrepant results are due to the considerable methodological problems involved in such determinations (Reinhart *et al.*, 1984a).

It was shown by Berthon *et al.* (1984) that in the presence of extracellular Ca^{2+} vasopressin caused an increase in cytosolic $[\text{Ca}^{2+}]$ that was sustained for several minutes. Even in the presence of extracellular Ca^{2+} the activation of oxoglutarate dehydrogenase by vasopressin was not sustained to the extent that it was initially (Staddon & McGivan, 1984), and it would be of interest to establish if a decline in cytosolic $[\text{Ca}^{2+}]$ from its stimulated peak was responsible. Berthon *et al.* (1984) also demonstrated that in the absence of extracellular Ca^{2+} the increase in cytosolic $[\text{Ca}^{2+}]$ caused by vasopressin was transient. This transient effect may be responsible for the transient activation by vasopressin of oxoglutarate dehydrogenase, reported here, under similar conditions. The mechanism by which vasopressin causes an increase in cytosolic $[\text{Ca}^{2+}]$ appears to involve Ca^{2+} release from internal stores (Joseph *et al.*, 1984) and an increased plasma-membrane influx of Ca^{2+} (Berthon *et al.*, 1984). The increased plasma-membrane permeability to Ca^{2+} may explain the activation, reported here, of oxoglutarate dehydrogenase by the addition of extracellular Ca^{2+} to cells previously incubated with vasopressin but in the absence of extracellular Ca^{2+} .

This paper has concentrated on the role of Ca^{2+} in the mechanism by which vasopressin activates oxoglutarate dehydrogenase in hepatocytes. As discussed in the introduction, there are several mechanisms by which the enzyme may be activated, and hence an assessment of the role of effectors other than Ca^{2+} is required. The data of Siess *et al.* (1978) indicate that vasopressin did not cause a decrease in mitochondrial $[\text{ATP}]/[\text{ADP}]$ or $[\text{NADH}]/[\text{NAD}^+]$ ratios in isolated hepatocytes. It is therefore unlikely that changes in the concentrations of these effectors are responsible for the activation of oxoglutarate dehydrogenase by vasopressin.

In summary, results have been presented indicating that the activation of hepatic oxoglutarate dehydrogenase by vasopressin was dependent on intracellular and extracellular pools of Ca^{2+} . The enzyme was rapidly activated independently of the extracellular pool, but this activation was transient. The presence of extracellular Ca^{2+} sustained the initial activation.

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References

- Assimakopoulos-Jeannet, F. D., McCormack, J. G. & Jeanrenaud, B. (1983) *FEBS Lett.* **159**, 83–88
- Baverel, G. & Lund, P. (1979) *Biochem. J.* **184**, 599–606
- Bergmeyer, H. U. & Bernt, E. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 324–327, Academic Press, New York and London
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Berthon, B., Binet, A., Mauger, J.-P. & Claret, M. (1984) *FEBS Lett.* **167**, 19–24
- Blackmore, P. F., Brumley, F. T., Marks, J. L. & Exton, J. H. (1978) *J. Biol. Chem.* **253**, 4851–4858
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773
- Denton, R. M. & McCormack, J. G. (1980) *FEBS Lett.* **119**, 1–8
- Denton, R. M. & McCormack, J. G. (1981) *Clin. Sci.* **61**, 135–140
- Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163
- Denton, R. M., Richards, D. A. & Chin, J. G. (1978) *Biochem. J.* **176**, 899–906
- Denton, R. M., McCormack, J. G. & Edgell, N. J. (1980) *Biochem. J.* **190**, 107–117
- Exton, J. H. (1981) *Mol. Cell. Endocrinol.* **23**, 233–264
- Friedmann, N., Divakaran, P., Kirkland, K., Kimura, S. & Wood, J. (1979) *J. Pharmacol. Exp. Ther.* **211**, 127–132
- Garrison, J. C., Johnsen, D. E. & Campanile, C. P. (1984) *J. Biol. Chem.* **259**, 3283–3292
- Gornall, H. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–756
- Hansford, R. G. (1981) *Biochem. J.* **194**, 721–732
- Hems, D. A., McCormack, J. G. & Denton, R. M. (1978) *Biochem. J.* **176**, 627–629
- Hensgens, H. E. S. J., Meijer, A. J., Williamson, J. R., Gimpel, J. A. & Tager, J. M. (1978) *Biochem. J.* **170**, 699–707
- Joseph, S. K. & McGivan, J. D. (1978) *Biochim. Biophys. Acta* **543**, 128–131
- Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 3077–3081
- Kirk, C. J. & Hems, D. A. (1974) *FEBS Lett.* **47**, 128–131
- Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 2273–2276
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Krebs, H. A., Cornell, N. W., Lund, P. W. & Hems, D. A. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F. & Tygstrup, N., eds.), pp. 726–750, Munksgaard, Copenhagen, and Academic Press, New York
- McCormack, J. G. & Denton, R. M. (1979) *Biochem. J.* **180**, 533–544
- McCormack, J. G. & Denton, R. M. (1984) *Biochem. J.* **218**, 235–247
- McCormack, J. G. & England, P. J. (1983) *Biochem. J.* **214**, 581–585
- Palmer, T. N., Watts, D. I. & Sugden, M. C. (1983) *Biochem. Int.* **6**, 433–441
- Reed, P. W. & Lardy, H. A. (1972) *J. Biol. Chem.* **247**, 6970–6977
- Reinhart, P. H., Van de Pol, E., Taylor, W. M. & Bygrave, F. (1984a) *Biochem. J.* **218**, 415–420
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1984b) *Biochem. J.* **220**, 35–42
- Shears, S. B. & Kirk, C. J. (1984) *Biochem. J.* **220**, 417–421
- Siess, E. A., Brocks, D. G. & Wieland, O. H. (1978) *Biochem. Soc. Trans.* **6**, 1139–1144
- Slein, M. W. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 117–123, Academic Press, New York and London
- Staddon, J. M. & McGivan, J. D. (1984) *Biochem. J.* **217**, 477–483
- Sugden, M. C., Ball, A. J., Illic, V. & Williamson, D. H. (1980a) *FEBS Lett.* **116**, 37–40
- Sugden, M. C., Tordoff, A. F. C., Illic, V. & Williamson, D. H. (1980b) *FEBS Lett.* **120**, 80–84
- Sugden, M. C., Ball, A. J. & Williamson, D. H. (1980c) *Biochem. Soc. Trans.* **8**, 591–592
- Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979) *J. Biol. Chem.* **254**, 3692–3695
- Thomas, A. P., Marks, J. S., Coll, K. E. & Williamson, J. R. (1983) *J. Biol. Chem.* **258**, 5716–5725
- Williamson, J. R., Cooper, R. H. & Hoek, J. B. (1981) *Biochim. Biophys. Acta* **639**, 243–295
- Yamanishi, J., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1983) *Biochem. Biophys. Res. Commun.* **112**, 778–786