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α_1-Adrenergic stimulation of Ca^{2+} mobilization without phosphorylase activation in hepatocytes from phosphorylase *b* kinase-deficient *gsd/gsd* rats

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Phenylephrine, vasopressin and the bivalent cation ionophore A23187 mobilized Ca^{2+} normally, but failed to activate phosphorylase, in hepatocytes from *gsd/gsd* rats with a deficiency of liver phosphorylase *b* kinase. These data provide strong evidence that phosphorylase *b* kinase is the site of action of the Ca^{2+} mobilized intracellularly during α_1-adrenergic activation of phosphorylase in liver cells.

Much evidence exists to show the involvement of Ca^{2+} in the α-adrenergic activation of hepatic phosphorylase (Assimacopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977; Blackmore *et al.*, 1978). It was originally postulated that Ca^{2+} acted by allosterically activating phosphorylase *b* kinase, thereby producing a conversion of phosphorylase *b* into phosphorylase *a*, which would serve to stimulate glycogen breakdown (Assimacopoulos-Jeannet *et al.*, 1977). The source of the Ca^{2+} for this activation has been shown by measurement of the Ca^{2+} content of liver organelles to be predominantly mitochondrial in origin (Blackmore *et al.*, 1979; Babcock *et al.*, 1979; Murphy *et al.*, 1980; Barritt *et al.*, 1981). Although some investigators using a different experimental approach have questioned this proposition (Poggioli *et al.*, 1980; Althaus-Salzmann *et al.*, 1980), the general consensus is that Ca^{2+} is mobilized from some internal store producing an increase in the free ionized Ca^{2+} concentration of the cytosol (Murphy *et al.*, 1980). Thereafter Ca^{2+} is expelled from the cell or is taken up into other subcellular organelles such as endoplasmic reticulum (Murphy *et al.*, 1980; Dehaye *et al.*, 1980). Finally, when the α-adrenergic stimulus ceases, it is postulated that there is a re-uptake of Ca^{2+} by the mitochondria, leading to a fall in cytosolic Ca^{2+}, which results in a cessation of glycogenolysis and re-uptake of Ca^{2+} by the cells (Exton, 1981).

Studies of the effects of α-adrenergic agonists, vasopressin and ionophore A23187 on the incorporation of $[^{32}P]P_i$ into hepatocyte cytoplasmic proteins, resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, have indicated that these agents increase the phosphorylation of phosphorylase (Garrison, 1978; Garrison *et al.*, 1979). However, these findings do not provide unequivocal proof that these agents act via phosphorylase *b* kinase, since the effect could be due to another Ca^{2+}-sensitive protein kinase or to a Ca^{2+}-inhibited phosphoprotein phosphatase.

Recently a strain of rats with a glycogen-storage disease (*gsd/gsd*) has been described that are deficient in hepatic phosphorylase *b* kinase (Malthus *et al.*, 1980; Watts & Malthus, 1980). Isolated hepatocytes from these rats have been used in the present study to test if phosphorylase *b* kinase is involved in the activation of phosphorylase by agents that induce Ca^{2+} mobilization in liver. In the present paper we show that activation of α_1-adrenergic receptors produces the same degree of Ca^{2+} mobilization as that seen in cells from unaffected rats; however, phosphorylase activation is not seen. Glucagon, vasopressin and the bivalent ionophore A23187 are also shown to mobilize intracellular Ca^{2+}, but not to cause activation of phosphorylase in hepatocytes from *gsd/gsd* rats. These results are consistent with phosphorylase *b* kinase being the target for Ca^{2+} mobilized during α-adrenergic activation of phosphorylase.

Experimental

Animals

Rats (140–160g) homozygous for glycogen-storage disease (*gsd/gsd*) were obtained from the Department of Clinical Biochemistry, Medical School, University of Otago, Dunedin, New Zealand. The primary defect in these animals is a deficiency in hepatic phosphorylase *b* kinase as described previously (Malthus *et al.*, 1980; Watts &

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Malthus, 1980). Sprague–Dawley rats (Harlan Industries, Indianapolis, IN, U.S.A.) were used as the source of control hepatocytes. All rats were maintained on Lab-blox chow (Allied Mills, Chicago, IL, U.S.A.) for at least 2 weeks before use.

Analytical methods

Methods for the preparation of hepatocytes, together with methods for the measurement of phosphorylase and Ca^{2+} efflux from hepatocytes have been described previously (Assimacopoulos-Jeannet *et al.*, 1977; Blackmore *et al.*, 1978). Units of phosphorylase are defined as μmol of [^{14}C]-glucose 1-phosphate incorporated into glycogen/min per g wet wt. of cells. In all the experiments reported, hepatocytes were washed and incubated in Krebs–Henseleit bicarbonate buffer containing 0.25mM-$CaCl_2$ to facilitate measurements of Ca^{2+} efflux. However, essentially identical results were obtained when the incubation medium contained the normal (2.5mM) concentration of Ca^{2+} (results not shown).

Materials

Glucagon and ionophore A23187 were gifts from Eli Lilly Co., Indianapolis, IN, U.S.A. (–)-Adrenaline bis-tartrate, [arginine]vasopressin, (–)-phenylephrine hydrochloride, (–)-propranolol hydrochloride and yohimbine hydrochloride were from Sigma Chemical Co., St. Louis, MO, U.S.A. Phentolamine hydrochloride was a gift from Smith, Kline and French Labs., Philadelphia, PA, U.S.A. Prazosin hydrochloride was a gift from Pfizer Labs., New York, NY, U.S.A.

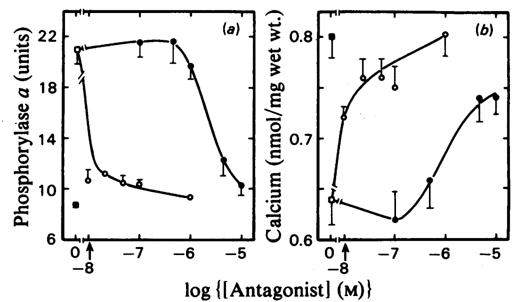

Fig. 1. *Dose response of prazosin and yohimbine on ability of adrenaline to stimulate Ca^{2+} efflux and phosphorylase activities in hepatocytes from unaffected animals*

Hepatocytes were incubated for 5min with 50nM-adrenaline (□) and various doses of the α_1-adrenergic antagonist prazosin (○) and the α_2-adrenergic antagonist yohimbine (●) after which time 0.5ml portions were removed for determination of calcium content and phosphorylase *a* activities. Prazosin was dissolved in dimethyl sulphoxide. Each value is the mean ± S.D. of duplicate assays performed on triplicate incubations. Results are representative of three separate experiments in which hepatocytes from two animals were pooled together. ■, Control value (saline).

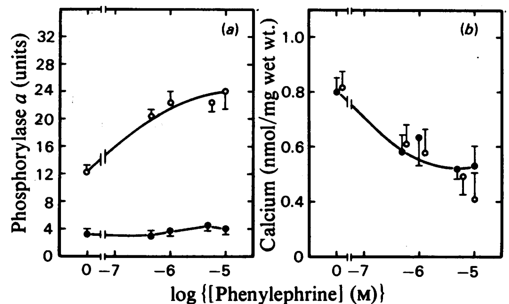

Fig. 2. *Dose response of the α-adrenergic agonist phenylephrine on phosphorylase activation and calcium content in hepatocytes from gsd/gsd (●) and unaffected (○) animals*

Hepatocytes were incubated for 5min with the various doses of phenylephrine. For other details see the legend to Fig. 1. Each value is the mean ± S.D. for duplicate assays performed on triplicate incubations. Results are representative of two separate experiments.

Results

To utilize the *gsd/gsd* rats as a means to confirm the hypothesis that Ca^{2+} fluxes are responsible for the activation of phosphorylase after α-adrenergic stimulation and that phosphorylase *b* kinase is the Ca^{2+}-sensitive enzyme in the cascade, it is essential to demonstrate that both Ca^{2+} fluxes and phosphorylase activation are mediated by the same cell-surface receptor. This is especially pertinent in the light of recent findings that more than one type of α-adrenergic receptor is found in hepatic plasma membranes (El-Refai & Exton, 1980*a*; Hoffman *et al.*, 1980*a*,*b*, 1981).

The results in Fig. 1 show that the effects of 50nM-adrenaline on phosphorylase activation and Ca^{2+} efflux are both potently inhibited by the α_1-antagonist prazosin, whereas the α_2-antagonist yohimbine is approximately two orders of magnitude less potent. These data support the view that Ca^{2+} mobilization and phosphorylase activation are mediated by the same α_1-adrenergic receptor, in hepatocytes from Sprague–Dawley rats.

Fig. 2(*a*) shows the effect of various doses of the specific α-adrenergic agonist phenylephrine on phosphorylase activation in hepatocytes from *gsd/gsd* and unaffected rats. It can be seen that with the cells from the mutant rats, there is minimal activation of phosphorylase by phenylephrine, and that the basal values are approx. 3.5 units compared with about 12

units in the normal hepatocytes. In the cells from unaffected animals, as expected (Blackmore *et al.*, 1978), phenylephrine potently activates phosphorylase by about 2-fold, with maximal effects being seen with 1 μM-phenylephrine. The Ca^{2+} content of hepatocytes from unaffected animals is approximately the same as that observed in those from *gsd/gsd* rats. The same dose response is seen for the effects of phenylephrine on Ca^{2+} efflux in both types of cell (Fig. 2*b*), and the response in both cells is antagonized by phentolamine, but not propranolol (results not shown). The close similarity of the Ca^{2+} effluxes induced by phenylephrine in the two situations suggests that the α_1-adrenergic receptors and the post-receptor mechanisms involved in Ca^{2+} mobilization are not modified in the hepatocytes of *gsd/gsd* rats.

The ability of the bivalent cation ionophore A23187 to activate phosphorylase and elicit Ca^{2+} mobilization was also examined in hepatocytes from *gsd/gsd* animals. The results in Fig. 3(*a*) show that in the hepatocytes from the mutant animals, ionophore A23187 causes no significant change in phosphorylase, whereas in the unaffected hepatocytes normal activation is observed. The Ca^{2+}-mobilizing ability of ionophore A23187 is identical in hepatocytes from both types of animal (Fig. 3*b*).

Table 1 also shows that vasopressin, another Ca^{2+}-dependent hormone, and glucagon are likewise ineffective at activating phosphorylase in the cells from *gsd/gsd* rats. However, they both elicit Ca^{2+} efflux, although the mobilization of Ca^{2+} is not involved in the action of glucagon on phosphorylase (Assimacopoulos-Jeannet *et al.*, 1977; Blackmore *et al.*, 1978).

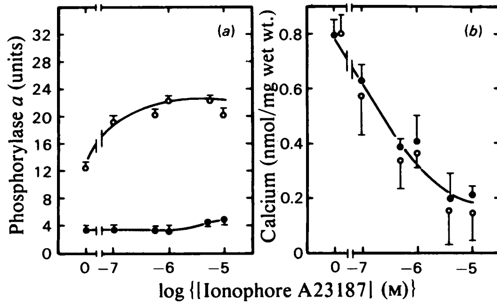

Fig. 3. *Dose response of the bivalent cation ionophore A23187 on phosphorylase activation and calcium content in hepatocytes from gsd/gsd* (●) *and unaffected* (○) *animals*

Hepatocytes were incubated for 5 min with the various doses of ionophore A23187, which were dissolved in dimethyl sulphoxide. Each value is the mean ± S.D. of duplicate assays performed on triplicate incubations. Results are representative of two separate experiments.

Discussion

Previous results from our laboratory have shown a close correlation between phosphorylase activation and Ca^{2+} efflux in isolated rat hepatocytes incubated with α-adrenergic agonists and vasopressin (Blackmore *et al.*, 1978). The receptor responsible for the adrenergic activation of phosphorylase has been shown by several groups to be α_1 in nature (Hoffman *et al.*, 1980*a*; El-Refai & Exton, 1980*b*). This is in agreement with our previous studies of 50 nM-[^{3}H]adrenaline binding to isolated rat liver plasma membranes (El-Refai *et al.*, 1979). More recent studies by Hoffman *et al.* (1981) have questioned this conclusion, and these workers have contended that adrenaline binding is predominantly to α_2-adrenergic receptors, whereas phosphorylase activation is α_1 in nature. However, in the studies reported by this group (Hoffman *et al.*, 1980*a*), the binding experiments were carried out with 10 nM-[^{3}H]adrenaline, whereas the hepatocyte studies employed 100 nM-adrenaline. This difference explains the basic discrepancy in their data, i.e. at low

Table 1. *Effect of vasopressin and glucagon on calcium content and phosphorylase a activity in hepatocytes from unaffected and gsd/gsd rats*

Hepatocytes were incubated for 5 min with either saline (control), vasopressin (10 nM) or glucagon (10 nM). For other details see the legend to Fig. 1. Values represent means of duplicate determinations from duplicate incubations. Results are representative of two separate experiments.

Animals	Addition	Phosphorylase *a* (units)	Calcium content (nmol/mg wet wt.)
gsd/gsd	Control	3.6	0.80
	Vasopressin	3.4	0.40
	Glucagon	4.1	0.40
Unaffected	Control	12.4	0.80
	Vasopressin	18.6	0.30
	Glucagon	29.4	0.54

adrenaline concentrations, binding is mainly to α_2-sites of unknown function, which have a K_D of about 10nM and a $B_{max.}$ (maximum binding) of 70fmol/mg of protein (Hoffman *et al.*, 1980*a*), whereas in the 50–100nM range, it binds mainly to the physiologically relevant α_1-sites, which have a K_D of approx. 50nM and a $B_{max.}$ of about 200fmol/mg of protein (El-Refai *et al.*, 1979).

The results in Fig. 1 show that the Ca^{2+} fluxes induced in hepatocytes by 50nM-adrenaline are mediated by α_1-adrenergic receptors as shown previously for phosphorylase activation. Both effects are inhibited more potently by the α_1-antagonist prazosin than by the α_2-antagonist yohimbine, indicating that they are mediated by the same α_1-adrenergic receptor as that previously identified in hepatic plasma membranes by us (El-Refai & Exton, 1980*b*).

Since α_1-adrenergic stimulation of cells from *gsd/gsd* rats produces the same degree of Ca^{2+} mobilization (dose response and maximum effect) as in cells from normal rats, the lack of phosphorylase activation can be attributed to the very low phosphorylase *b* kinase activity present in the livers of these animals. [It is assumed that, as in two strains of Sprague–Dawley rats (the present paper; Hoffman *et al.*, 1980*a*) and in Wistar rats (Aggerbeck *et al.*, 1980), α_1-adrenergic receptors also mediate the effects of adrenaline on hepatic phosphorylase in *gsd*/+-heterozygotes and +/+ rats in the New Zealand colony from which the *gsd/gsd* rats arose.] The lack of correlation between Ca^{2+} mobilization and phosphorylase activation in hepatocytes from *gsd/gsd* rats is illustrated in Fig. 4. In normal cells there is a good correlation between the two parameters up to a Ca^{2+} mobilization equivalent to 0.15nmol/mg of cells.

There have been two recent reports of mammalian protein kinases that are stimulated by Ca^{2+}, but are separate from phosphorylase *b* kinase. One of these phosphorylates glycogen synthase and requires the Ca^{2+}-dependent regulatory protein calmodulin (Payne & Soderling, 1980). The other is usually assayed with histone H1, but can act on glycogen synthase (Kishimoto *et al.*, 1978). Its sensitivity to Ca^{2+} is greatly increased by the addition of a phospholipid such as phosphatidylcholine and also of an unsaturated diacylglycerol such as dioleoylglycerol (Kishimoto *et al.*, 1980). The present results showing that phenylephrine, vasopressin and ionophore A23187 produced no significant activation of hepatic phosphorylase in *gsd/gsd* rats indicate that neither of these protein kinases is capable of mediating the effects of Ca^{2+} on phosphorylase in this tissue. In addition, these results exclude the possibility that Ca^{2+} exerts its effects on the activation state of the enzyme through inhibition of phosphorylase phosphatase.

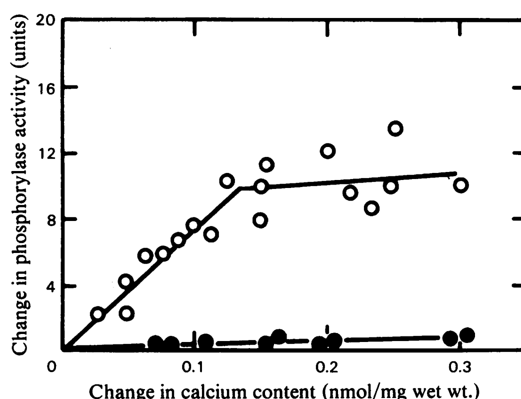

Fig. 4. *Correlation between the change in calcium content and change in phosphorylase a activities in hepatocytes from gsd/gsd* (●) *and unaffected* (○) *animals elicited by various doses of phenylephrine*

The *gsd/gsd* values are taken from the data in Fig. 2, whereas the values for unaffected animals are taken from the data in Fig. 2 plus data from Blackmore *et al.* (1978).

Two possibilities could influence the results presented here. The first relates to a possible decrease in the total phosphorylase activity in the hepatocytes from *gsd/gsd* rats. However, these cells have been shown to possess near normal activities of total phosphorylase when the activity is measured after the conversion of phosphorylase *b* into phosphorylase *a* by muscle phosphorylase *b* kinase added *in vitro* (Malthus *et al.*, 1980). We have confirmed these data (T. D. Chrisman & J. H. Exton, unpublished work). We have also assayed total phosphorylase activity in the presence of AMP and 1,2-dimethoxyethane (Uhing *et al.*, 1979). Phosphorylase *a* and total phosphorylase ($a+b$) activities in hepatocytes from *gsd/gsd* animals were 3.6 and 28.5 respectively and in unaffected animals were 12.4 and 29.4 respectively. The second possibility is that the Ca^{2+} sensitivity of the liver phosphorylase *b* kinase of the mutant rats might be markedly decreased, which may be related to a deficiency of calmodulin. However, this possibility would not explain the near absence of assayed enzyme activity in liver homogenates (Malthus *et al.*, 1980) or the failure of glucagon to increase its activity in hepatocytes.

In conclusion, the experiments described in the present paper demonstrate again the utility of genetic mutants lacking a key regulatory enzyme in establishing or refuting hypotheses regarding mechanisms involved in the hormonal regulation of metabolism.

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