Unsaturated fatty acids activate glycogen phosphorylase in cultured rat hepatocytes

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Oleate, linoleate, linolenate, arachidonate and eicosapentaenoate, but not myristate, palmitate and stearate, stimulated glycogen phosphorylase activity by 2–8-fold when added to cultured rat hepatocytes. Addition of BSA or Ca^{2+} to the incubation medium decreased the stimulating effects of the unsaturated fatty acids. The combination of oleate or linolenate, with corticosterone, testosterone or oestradiol produced synergistic stimulations of phosphorylase activity. The stimulation of glycogen phosphorylase activity by linolenate was inhibited by staurosporine or sphingosine. Staurosporine (80 nM) alone also decreased basal phosphorylase activities by about 60%. The results show that unsaturated fatty acids can be used as model agonists to stimulate phosphorylase activity by a mechanism that probably involves protein kinase C. On the basis of the fatty acid: BSA ratios used, this stimulation should only occur *in vivo* at high fatty acid concentrations when accompanied by hypoalbuminaemia.

INTRODUCTION

The breakdown of glycogen is controlled mainly by glycogen phosphorylase through mechanisms involving the phosphorylation and dephosphorylation of the enzyme. In the liver, two different signalling mechanisms for the phosphorylation and activation of glycogen phosphorylase have been described. The first involves an increase in the intracellular cyclic AMP concentrations, which can be achieved by hormones such as glucagon or β -agonists. The second involves a Ca^{2+} -dependent mechanism such as is seen with vasopressin, angiotensin II or α_1 -agonists [1–6].

The plasma membrane plays an essential role in regulating cell functions, and alterations in its chemical properties might have further implications on the signalling systems of the cell. In this respect, the concentration of fatty acids in the plasma might be of importance, since this may affect the composition and organization of lipids within the bilayer and also it might modify its fluidity properties. There is also considerable interest in the role of fatty acids in decreasing the effectiveness of insulin in tissues [7–10], and the mechanisms of its action are not fully understood. Several reports [1–17] have described a relationship between an increase in fatty acid concentrations and the mobilization of intracellular Ca^{2+} . This effect is mainly induced by arachidonate ($\text{C}_{20:4}$), but can also be caused by other fatty acids such as linoleate ($\text{C}_{18:2}$) and linolenate ($\text{C}_{18:3}$), though in a less effective manner [18]. Furthermore, fatty acids can stimulate protein kinase C [19–21].

We decided to use the activation of glycogen phosphorylase as a sensitive method of detecting changes in the metabolic balance of rat hepatocytes in order to study a possible effect of fatty acids. Glycogenolysis is normally accompanied by a mobilization of fatty acids from adipose tissue, since these two pathways are both activated by catecholamines and glucagon. It is therefore possible that the increased supply of fatty acids to the liver under these conditions might further modulate the activity of glycogen phosphorylase. The present work showed that unsaturated fatty acids could be used as model agonists to increase glycogen

phosphorylase activity. The fatty acids acted synergistically with the acute effects of pharmacological concentrations of oestradiol, progesterone and testosterone, or with physiological concentrations of corticosterone.

MATERIALS AND METHODS

Animals

Male Wistar rats (about 200 g) were obtained from Charles River (Québec, Canada). They were housed in a room that was lit from 08:00 to 20:00 h, and they were fed on Wayne Rodent Blox (Continental Grain Co., Chicago, IL, U.S.A.). The rats were used for the preparation of hepatocytes between 09:00 and 11:00 h. Hormones, sphingosine, fatty acids, 4 β -phorbol 12-myristate 13-acetate (PMA) and essentially fatty-acid-free BSA were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Staurosporine was from Kamiya Biomedical Co., Thousand Oaks, CA, U.S.A. The sources of other reagents are described by Gomez-Muñoz *et al.* [22].

Preparation and culture of hepatocytes

Hepatocytes were prepared and attached to collagen-coated tissue-culture dishes in a modified Leibovitz L-15 medium containing 25 mM-Hepes, pH 7.4, 10% (v/v) newborn-calf serum, 8.3 mM-glucose and 5 mM-galactose [23,24]. After 45–60 min at 37 °C in humidified air, the unattached and non-viable cells were removed by replacing the medium. The incubation was continued for a further 4–5 h, and the medium was then replaced by modified Leibovitz medium containing 0.1 mM-essentially fatty-acid-free BSA and the cells were incubated for a further 18–20 h. The medium was then replaced and the BSA concentration was changed as indicated. Additions of fatty acids or hormones were made so that the total volume of the medium changed by less than 2%. Fatty acids were prepared in a 20% molar excess of KOH, dissolved by warming and then slowly pipetted with shaking into the BSA-containing medium [25]. At the times of incubation indicated, the cells were washed once

Abbreviations used: $\text{C}_{20:4}$, arachidonate; $\text{C}_{20:5}$, eicosapentaenoate; $\text{C}_{18:2}$, linoleate; $\text{C}_{18:3}$, linolenate; $\text{C}_{14:0}$, myristate; $\text{C}_{16:0}$, palmitate; $\text{C}_{18:1}$, oleate; $\text{C}_{18:0}$, stearate; PMA, 4 β -phorbol 12 β -myristate 13-acetate; LDH, lactate dehydrogenase.

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with ice-cold 0.16 M-NaCl. The cells were then scraped from the dishes in 1 ml of ice-cold 100 mM-glycylglycine buffer, adjusted to pH 7.4 with NaOH, and containing 100 mM-NaF, 20 mM-EDTA and 0.5 % glycogen [26,27]. They were frozen in liquid N₂ and then stored for up to 3 days at -70 °C until required for analysis.

Analytical procedures

Phosphorylase *a* and total phosphorylase activities were determined in homogenates [28]. A unit of phosphorylase is defined as the amount of enzyme that converts 1 μ mol of substrate/min under the conditions of the assay. Lactate dehydrogenase (LDH) was measured as described previously [24,29]. P_i was determined by the method of Fiske & Subbarow [30].

Expression of results

Phosphorylase activities are expressed as means $\pm$ S.E.M. unless stated to the contrary, and the values for individual experiments were taken as means from duplicate dishes each assayed in duplicate. Statistical significance was calculated by using a paired *t* test. Phosphorylase activities have been expressed relative to LDH activities so as to compensate for small differences in the numbers of viable hepatocytes per culture dish and the recovery from the dishes in the homogenates. Measurement of LDH activities also ensured for each condition that the fatty acids did not cause lysis of the hepatocytes [22–25], since loss of viability in the hepatocytes is accompanied by a leakage of LDH into the medium. Therefore any condition that produced lysis could be excluded. There was no significant loss of LDH activity from the hepatocytes in any of the conditions reported in this paper. The expression of the results relative to LDH is superior to presenting the results relative to protein or DNA, since non-viable and damaged cells can remain attached to the culture dishes. The content of LDH per dish of cells was 2.4 ± 0.1 μ mol of lactate oxidized/min (means $\pm$ S.E.M. from 15 independent preparations) and 1 μ mol/min is equivalent to 1 unit of activity at 22 °C. One dish of cells contained about 1.5 mg of protein, so that results can be readily calculated relative to protein if this is required for comparison with other work. However, the present incubations contained relatively high albumin concentrations and used collagen-coated dishes. Therefore the measurement of the relatively small concentration of hepatocyte protein might have been inaccurate for the routine determination of cell number. Total phosphorylase activity was 46.5 ± 2.5 nmol of P_i released/min (1 m-unit) per unit of LDH, and this was 8.4 ± 0.5 -fold higher than the basal phosphorylase *a* activity in 11 independent preparations. In four independent experiments, total phosphorylase activity was measured after incubation of the cells for 30 min with 1 mM-oleate (C_{18:1}) or 1 mM-C_{18:3} in the presence of 0.1 mM-BSA. This value was apparently 3.6 % lower in the presence of fatty acid, and the difference was not statistically significant when expressed as such or relative to LDH. Total and basal phosphorylase activities were consistently lower than those reported for previous experiments performed in England, where the sources of rats were different and the hepatocytes were also maintained in 0.03 mM- rather than 0.1 mM-BSA [22]. BSA can bind fatty acids and steroid hormones, and its effects in decreasing phosphorylase activity will be discussed below.

RESULTS AND DISCUSSION

Effects of fatty acids on glycogen phosphorylase activity

Rat hepatocytes showed an increase in glycogen phosphorylase activity after incubation with 1 mM-C_{18:1} or 1 mM-C_{18:3} in the

presence of 0.1 mM essentially fatty-acid-free BSA. These effects were not observed after 2 min of incubation for C_{18:1}, but they were maximum after about 5 min for C_{18:1} and C_{18:3}, and the increase relative to the controls remained constant for the next 55 min (Fig. 1). The time courses for C_{18:3} were similar in the five experiments shown in Fig. 1, and the relatively large S.E.M. reflects differences in the extent of activation. In 15 independent experiments for C_{18:3} the stimulation was 4.1 ± 0.4 -fold ($P < 0.005$) at 30 min, and the equivalent value for 10 independent experiments for C_{18:1} was 2.0 ± 0.2 -fold ($P < 0.0005$). The effects of equivalent concentrations (1 mM) of six other fatty acids on the activation of glycogen phosphorylase after 30 min is shown in Table 1. The saturated fatty acids, myristate (C_{14:0}), palmitate (C_{16:0}) and stearate (C_{18:0}), had no significant effect on

Fig. 1. Effects of C_{18:1} and C_{18:3} on glycogen phosphorylase activity in rat hepatocytes

Monolayer cultures of rat hepatocytes were incubated at 37 °C with 0.1 mM essentially fatty-acid-free BSA and 1 mM-C_{18:1} (□) or 1 mM-C_{18:3} (■) for the time indicated. Glycogen phosphorylase activity in cells treated with fatty acids is expressed relative to that obtained at the same time in the absence of fatty acids and is given as means $\pm$ S.E.M. for four or five independent experiments for C_{18:3} and three to five experiments for C_{18:1}. The absolute value for the basal phosphorylase activity was 6.4 ± 1.1 m-units/unit of LDH at 5 min after changing the medium in eight independent experiments, and this rose in a linear manner to 9.2 m-units/unit of LDH at the 60 min point. Further values for the stimulations by C_{18:1} and C_{18:3} at 30 min are given in the text.

Table 1. Effects of fatty acid structure on the activation of glycogen phosphorylase in rat hepatocytes

Monolayer cultures of rat hepatocytes were incubated for 30 min at 37 °C with 0.1 mM essentially fatty-acid-free BSA and 1 mM concentrations of the fatty acids indicated. Results for the phosphorylase activity are means $\pm$ S.E.M. from three independent experiments.

Fatty acid added	Phosphorylase <i>a</i> (m-units/unit of LDH)	Significance of difference from control
None (control)	4.4 ± 0.7	
C _{14:0}	5.1 ± 0.2	
C _{16:0}	5.9 ± 0.2	
C _{18:0}	5.1 ± 0.2	
C _{18:1}	11.2 ± 1.3	$P < 0.025$
C _{18:2}	20.5 ± 1.3	$P < 0.01$
C _{18:3}	28.2 ± 2.7	$P < 0.005$
C _{20:4}	40.8 ± 4.4	$P < 0.01$
C _{20:5}	32.5 ± 4.5	$P < 0.01$

Fig. 2. Effects of the concentrations of BSA and C_{18:1} on the activity of glycogen phosphorylase in rat hepatocytes

Monolayer cultures of rat hepatocytes were incubated at 37 °C for 30 min with the concentration of C_{18:1} that is indicated and 0.03 mM (Δ), 0.09 mM (▲), 0.2 mM (○), 0.4 mM (□), 0.6 mM (●) and 1.0 mM (◇) essentially fatty-acid-free BSA. The results are from one experiment. They were confirmed in a second experiment and by the results shown in Fig. 4.

the glycogen phosphorylase activation. However, all of the unsaturated fatty acids caused enhancements which increased, for the C₁₈ series, with the degree of unsaturation.

The activation of glycogen phosphorylase by fatty acids was decreased as the concentration of BSA in the incubation medium increased (Fig. 2). This suggests that such an effect might be caused by the unbound or loosely bound fatty acids. Fig. 2 also shows the effect of BSA in the absence of exogenous fatty acids on glycogen phosphorylase activity. Increasing concentrations of BSA from 0.03 mM to 0.6 mM decreased ($P < 0.01$) the phosphorylase activity by $41 \pm 3\%$ in three independent experiments. The mechanism for this latter effect is not certain, although it

might be caused by the removal of fatty acids from the plasma membrane by the BSA, which in turn might cause a decrease in the concentration of the intracellular fatty acid pool.

We have previously reported [22] that physiological concentrations of corticosterone, or pharmacological concentrations of testosterone, oestradiol and progesterone, could rapidly activate glycogen phosphorylase in hepatocytes. For corticosterone, this stimulation reached a peak at 10 min and disappeared after 30 min (results not shown) in a similar manner to that described for dexamethasone [22]. The lack of an effect of corticosterone at 30 min is also seen in Tables 2 and 3. Addition of C_{18:1} together with corticosterone, testosterone, oestradiol or progesterone for 30 min produced a substantial synergism ($P < 0.05$) in the activation of glycogen phosphorylase activity (Table 2). A similar effect was found when C_{18:3} was added with corticosterone, testosterone or oestradiol. For progesterone and C_{18:3}, both of these reagents gave relatively high activation, and full synergism was not observed.

The mechanisms by which fatty acids stimulated phosphorylase activity were investigated further by using 0.1 mM-BSA and selected fatty acids. Glycogen phosphorylase can be activated through phosphorylation by phosphorylase *b* kinase, whose activity is increased by Ca²⁺ [31–33]. Furthermore, palmitate is able to enhance the stimulation of glycogen phosphorylase activity in contracting smooth muscle by a mechanism involving Ca²⁺ [34]. Experiments were therefore performed in the presence or in the absence of this cation in the incubation medium. The basal phosphorylase activities measured for hepatocytes incubated in the Ca²⁺-free medium appeared to be about 20% lower than in Ca²⁺-supplemented medium (Table 3), but with the three experiments shown this did not reach statistical significance. However, in previous work we demonstrated a significant time-dependent decrease in phosphorylase activity in Ca²⁺-depleted medium [22]. This effect is also compatible with data reported by Joseph *et al.* [35], who have demonstrated that the basal phosphorylase activity decreased by 30% in hepatocytes incubated in medium containing low Ca²⁺. By contrast, the

Table 2. Effects of steroid hormones, C_{18:1} and C_{18:3} on the activities of glycogen phosphorylase in rat hepatocytes

Monolayer cultures of rat hepatocytes were incubated for 30 min at 37 °C with 0.1 mM essentially fatty-acid-free BSA with 1 mM-C_{18:1}, 1 mM-C_{18:3} and steroid hormones as indicated. Results are means $\pm$ S.E.M. for two series of three independent experiments. The *P* values indicated as (a) show the significance of the difference between incubations that contained steroid hormones versus the appropriate incubation in the absence of hormone. The *P* values shown as (b) give the significance of the difference between appropriate incubations in the presence or absence of fatty acids.

Steroid added	Phosphorylase <i>a</i> (m-units/units of LDH)			
	Series 1		Series 2	
	No fatty acid	+C _{18:1} (1 mM)	No fatty acid	+C _{18:3} (1 mM)
None	4.4 $\pm$ 0.7	9.6 $\pm$ 1.2	6.7 $\pm$ 1.0	23.0 $\pm$ 5.2
Corticosterone (1 μ M)	4.3 $\pm$ 0.7	14.0 $\pm$ 1.8 <i>P</i> < 0.01 ^a <i>P</i> < 0.05 ^b	6.4 $\pm$ 0.6	30.5 $\pm$ 6.6 <i>P</i> < 0.01 ^a <i>P</i> < 0.05 ^b
Testosterone (100 μ M)	6.1 $\pm$ 0.6 <i>P</i> < 0.005 ^a	24.5 $\pm$ 3.6 <i>P</i> < 0.025 ^a <i>P</i> < 0.025 ^b	10.5 $\pm$ 1.2 <i>P</i> < 0.01 ^a	41.5 $\pm$ 7.7 <i>P</i> < 0.05 ^a <i>P</i> < 0.025 ^b
Oestradiol (100 μ M)	6.9 $\pm$ 0.4 <i>P</i> < 0.01 ^a	24.4 $\pm$ 4.5 <i>P</i> < 0.025 ^a <i>P</i> < 0.05 ^b	9.3 $\pm$ 1.6 <i>P</i> < 0.01 ^a	40.9 $\pm$ 12.7 <i>P</i> < 0.05 ^a <i>P</i> < 0.025 ^b
Progesterone (200 μ M)	21.0 $\pm$ 8.6 <i>P</i> < 0.05 ^a	34.2 $\pm$ 5.9 <i>P</i> < 0.025 ^a <i>P</i> < 0.05 ^b	26.8 $\pm$ 3.6 <i>P</i> < 0.01 ^a	39.4 $\pm$ 7.0 <i>P</i> < 0.05 ^a

Table 3. Effects of Ca^{2+} on the interactions of $\text{C}_{18:1}$ and $\text{C}_{18:2}$ with corticosterone on phosphorylase activity in rat hepatocytes

Monolayer cultures of rat hepatocytes were preincubated for 15 min at 37 °C with Leibovitz L 15 medium that contained no Ca^{2+} and which was supplemented with either 0.5 mM-EGTA or 1.3 mM- CaCl_2 . The media were then replaced by the equivalent media which contained 0.1 mM essentially fatty-acid-free BSA with $\text{C}_{18:1}$, $\text{C}_{18:2}$ and corticosterone as indicated. The hepatocytes were then incubated for 30 min at 37 °C. Phosphorylase activities are given as means $\pm$ S.E.M. for three independent experiments. The P values indicated as (a) show the significance of the difference in phosphorylase activity in the presence of the fatty acids compared with the appropriate control incubations in the absence. The P values shown as (b) give the differences between appropriate incubations in the presence or absence of corticosterone.

Fatty acid added	Phosphorylase <i>a</i> (m-units/units of LDH)			
	No Ca^{2+} + EGTA (0.5 mM)		+ Ca^{2+} (1.3 mM)	
	No corticosterone	+Corticosterone (1 μM)	No corticosterone	+Corticosterone (1 μM)
None	3.3 $\pm$ 0.49	3.0 $\pm$ 0.35	4.1 $\pm$ 0.91	4.4 $\pm$ 0.81
$\text{C}_{18:1}$ (1 mM)	14.0 $\pm$ 3.1 $P < 0.025^a$	14.6 $\pm$ 2.75 $P < 0.05^a$	6.4 $\pm$ 1.3 $P < 0.025^a$	8.0 $\pm$ 1.6 $P < 0.025^a$ $P < 0.05^b$
$\text{C}_{18:2}$ (1 mM)	21.1 $\pm$ 6.3 $P < 0.05^a$	21.8 $\pm$ 5.9 $P < 0.05^a$	9.9 $\pm$ 3.5 $P < 0.05^a$	13.4 $\pm$ 3.9 $P < 0.05^a$ $P < 0.05^b$

stimulating effects of $\text{C}_{18:1}$ and $\text{C}_{18:2}$ were enhanced ($P < 0.05$) in hepatocytes incubated in the absence of Ca^{2+} (Table 3). This is probably explained by the fact that Ca^{2+} forms insoluble soaps with fatty acids, making them less available to the hepatocytes. In fact, the Ca^{2+} -containing medium had a slightly more turbid appearance than the Ca^{2+} -depleted medium when $\text{C}_{18:1}$ was present.

The rapid activation of glycogen phosphorylase by steroid hormones did not involve an increase in the intracellular cyclic AMP concentration, it was independent of the synthesis of proteins, and it was diminished by depleting the hepatocytes of Ca^{2+} [22]. $\text{C}_{18:1}$ and $\text{C}_{18:3}$ interacted synergistically ($P < 0.05$) with 1 μM -corticosterone in activating glycogen phosphorylase, provided that Ca^{2+} was present in the medium (Tables 2 and 3). However, in the absence of external Ca^{2+} , no synergistic effects of corticosterone on the activation of phosphorylase by $\text{C}_{18:1}$ and $\text{C}_{18:2}$ were observed (Table 3). It should also be noted that corticosterone alone produced no significant increase in glycogen phosphorylase activity after incubation of the hepatocytes for 30 min ([22], and Tables 2 and 3).

Previous work indicated that fatty acids could cause the activation of protein kinase C. We therefore decided to investigate whether acknowledged inhibitors of protein kinase C such as sphingosine or staurosporine [36] would block the activation of phosphorylase by $\text{C}_{18:3}$. Increasing the concentrations of sphingosine progressively decreased the stimulatory effects of $\text{C}_{18:3}$, and at 50 μM this inhibition ($P < 0.0005$) was $71 \pm 8\%$ in seven independent experiments. At 100 μM -sphingosine, there were no significant stimulatory effects of $\text{C}_{18:3}$ (Fig. 3a). However, incubation of the cells for 30 min with sphingosine significantly increased basal phosphorylase activity. The increase ($P < 0.025$) was $26 \pm 10\%$ for 50 μM -sphingosine in seven independent experiments, and reached $46 \pm 14\%$ ($P < 0.05$) when the cells were incubated in the presence of 100 μM -sphingosine in three independent experiments.

Staurosporine also antagonized the stimulatory effects of $\text{C}_{18:3}$ (Fig. 3b). Most of the decrease was obtained at 5 nM-staurosporine. At 80 nM, the staurosporine prevented $46 \pm 17\%$ of the stimulation specifically related to the addition of $\text{C}_{18:3}$ relative to the control ($P < 0.05$ for five independent experiments). However, unlike sphingosine, staurosporine decreased, rather than increased, the basal phosphorylase activity measured

Fig. 3. Effects of sphingosine and staurosporine on the activation of glycogen phosphorylase by $\text{C}_{18:3}$

Monolayer cultures of rat hepatocytes were incubated at 37 °C for 30 min with 0.1 mM essentially fatty-acid-free BSA in the presence (●, ■), or absence (○, □) of 1 mM- $\text{C}_{18:3}$ with the indicated concentrations of (a) sphingosine or (b) staurosporine. The results are means $\pm$ S.E.M. from four independent experiments for both inhibitors, and they are expressed relative to the results obtained in the absence of $\text{C}_{18:3}$, sphingosine and staurosporine. The absolute activity for this latter value was 8.0 ± 1.1 m-units/unit of LDH for five independent experiments.

in the absence of $\text{C}_{18:3}$ (Fig. 3b). This decrease ($P < 0.01$) was $61 \pm 14\%$ at 80 nM-staurosporine in five independent experiments. At present we do not know why staurosporine and sphingosine have different effects on phosphorylase activity when they are added alone. However, both of the reagents inhibited the stimulatory effects of $\text{C}_{18:3}$ on phosphorylase activity.

Fig. 4. Effects of corticosterone and a mixture of fatty acids on the activity of glycogen phosphorylase in rat hepatocytes

Monolayer cultures of rat hepatocytes were incubated for 30 min at 37 °C in medium containing either 0.14 mM- (□) or 0.6 mM- (■) essentially fatty-acid-free BSA. A mixture of fatty acids [consisting of: C_{14:0}, 2%; C_{16:0}, 28%; C_{18:0}, 15%; C_{18:1}, 34%; C_{18:2}, 14%; C_{18:3}, 1%; C_{20:4}, 6% (mol/100 mol)] was added at the concentrations indicated. The results are means $\pm$ S.E.M. for three or four independent experiments. The results are shown for incubations containing 1 μ M-corticosterone. The values were identical in the absence of corticosterone for most conditions, except with 0.14 mM-BSA and 1, 2 and 3 mM fatty acid, where the values were 21, 27 and 8% lower as expected from the synergism shown in Tables 2 and 3.

By contrast with the effects with C_{18:3}, 80 nM-staurosporine and 50 μ M-sphingosine had no significant effect on the stimulation of phosphorylase activity (results not shown) that can be produced by 100 μ M-testosterone, 100 μ M-oestradiol or 200 μ M-progesterone, as described in Table 2.

Experiments were performed to try to simulate the concentrations of fatty acids relative to albumin that might be encountered *in vivo*. Concentrations of 0.14 mM- and 0.6 mM-BSA were used, since these are representative of serum albumin concentrations in severe nephrotic syndrome [37] and in normal conditions [38–40] respectively. Fatty acids were added as a mixture (see legend to Fig. 4) that resembled the normal composition of saturated and mono-, di-, tri-, tetra- (and higher) unsaturated fatty acids that are unesterified in human blood [40]. Corticosterone was added at 1 μ M, which is about twice the diurnal peak concentration [41]. In stress conditions the corticosterone concentrations in the blood of rats can rise to 3 μ M [42–44]. Addition of the fatty acid mixture at 2 mM and 3 mM increased ($P < 0.025$ and 0.005 respectively) phosphorylase activities at 0.14 mM-BSA by 2.1- and 4.5-fold respectively (Fig. 4). At 4 mM of the fatty acid mixture, the hepatocytes began to lyse and to leak LDH, and so the results were discarded. When the concentration of BSA was raised to 0.6 mM, the fatty acid mixture at up to 4 mM failed to stimulate phosphorylase activity.

Most of the stimulations of glycogen phosphorylase activity that were observed in the present work were obtained at a fatty acid:albumin molar ratio of 10:1. This ratio is much higher than occurs under normal physiological conditions, where the serum concentration of albumin is normally about 0.6 mM [38–40], and fatty acid concentrations can rise to about 1.2 mM [45]. However, fatty acid concentrations in severe stress, trauma, hypoxia, toxic conditions, myocardial infarction and diabetic ketoacidosis can rise to 2 mM or higher [46,47]. The peak concentrations of fatty acids after myocardial infarctions in treated and untreated diabetics can be as high as 2–3.3 mM. Similar high concentrations of fatty acids occur after by-pass surgery in non-diabetic patients (G. D. Lopaschuk, personal communication). These high fatty

acid concentrations would be unlikely to stimulate phosphorylase activity at normal physiological concentrations (0.6 mM) of albumin, assuming that the hepatocyte model and the conditions used provide a reasonable simulation of the situations that occur *in vivo* (Fig. 4). However, what is clearly more important in this respect is the concentration of albumin, which can decrease as a result of poor nutrition, in aging, liver disease and particularly in severe nephrotic syndrome, where concentrations of 0.14 mM can occur [37]. Stimulations of 2.1-fold in phosphorylase activity were observed at 2 mM of a physiological mixture [40] of fatty acids (Fig. 4). Our results (Figs. 2 and 4) therefore indicated that fatty acid:albumin ratios of 10:1 could stimulate glycogen phosphorylase activity. Such ratios could occur in severe stress conditions with an albumin concentration of less than about 0.3 mM ([46,47]; G. D. Lopaschuk, personal communication). The importance of albumin is also emphasized in Fig. 2, where BSA decreased the basal phosphorylase activity which was obtained in the hepatocytes in the absence of exogenous fatty acids.

The fatty acid:albumin ratio of 10:1 that we employed is widely used to study fatty acid metabolism. It is therefore likely that in such experiments fatty acids could be causing cell activation as well as providing substrates for lipid metabolism. The possible effects of fatty acids in causing cell lysis were excluded by measuring LDH activities and discarding any condition where there was leakage from the cells. The reversal of the effects of C_{18:3} by relatively low concentrations of sphingosine and staurosporine also point to a specific rather than a non-specific effect of the unsaturated fatty acids. These experiments were used to investigate some of the mechanisms by which fatty acids might activate phosphorylase. In most cases, we chose not to use C_{20:4}, so as to avoid the additional possibilities of cell activation through the synthesis of eicosanoids. It has been well established that fatty acids are implicated in the mobilization of Ca²⁺ [11–18], in alteration of mitochondrial Ca²⁺ transport [48] and in the stimulation of protein kinase C [19–21]. These effects appear to be relatively specific for polyunsaturated fatty acids rather than saturated acids, which was also the case for the activation of glycogen phosphorylase (Table 1). The effects of unsaturated fatty acids on Ca²⁺ metabolism and protein kinase C activity were observed in other work in the micromolar range. However, it should also be stressed that either no albumin [16–21,48,49] or low albumin concentrations [34] were present in the incubations used by the other authors.

The activation of glycogen phosphorylase by C_{18:3} may have involved the stimulation of protein kinase C, since it was prevented by sphingosine and partially reversed by staurosporine (Fig. 3). Diacylglycerols and phorbol esters also stimulate phosphorylase activity [2,50]. However, these latter stimulations were small by comparison with those produced by C_{18:3}, and in the present work amounted to an increase ($P < 0.01$) at 25 μ M-PMA of only 23% (results not shown). The effects of phorbol esters and diacylglycerols can be synergistically increased by the Ca²⁺ ionophore A23187 [2], and it is known that unsaturated fatty acids can also stimulate the mobilization of intracellular Ca²⁺ [11–18]. Depletion of extracellular Ca²⁺ might not be sufficient to decrease the initial rise in the intracellular Ca²⁺ pool and to prevent the activation of phosphorylase by fatty acids [51]. In fact, lowering extracellular Ca²⁺ could increase the availability of fatty acids by decreasing the formation of Ca²⁺ soaps (Table 3). Thus the stimulation of glycogen phosphorylase by unsaturated fatty acids could require the simultaneous transmission of at least two signals, e.g. increased intracellular Ca²⁺ and diacylglycerol. The effect of diacylglycerols or phorbol esters alone on kinase C is insufficient to express the full activation, but nevertheless staurosporine or sphingosine is

able to prevent partially the activation by fatty acids. It has also been suggested that activation of kinase C has a feedback-inhibitory effect on the stimulation of phosphorylase by α_1 -agonists [50].

Glucocorticoid concentrations increase with those of fatty acids in stress conditions. The synergism between fatty acids and corticosterone is probably explained by the different mechanisms of action of these agents. The stimulating effects of fatty acids are increased by depleting the medium of Ca^{2+} , whereas that of corticosterone is decreased ([22]; Table 3). Cortisol can increase the binding of Ca^{2+} to plasma membranes [52], and other steroids may cause increased release of Ca^{2+} into cells through effects on Na^+/K^+ -ATPase [53,54], or, in the case of dexamethasone, by modifying polyphosphoinositide metabolism [55–57]. Bile acids, which are structurally related to steroid hormones, are also able to mobilize intracellular Ca^{2+} in hepatocytes [58,59]. Thus the action of steroid hormones in mobilizing Ca^{2+} may explain why they synergize the effects of fatty acids in stimulating glycogen phosphorylase (Tables 2 and 3). The release of cortisol in severe stress, trauma and diabetes might be a factor in a rapid enhancement of glycogenolysis. Glucocorticoids are also often administered clinically after myocardial infarction and surgery. It may also be significant that both fatty acids [7–10] and glucocorticoids [60,61] decrease the sensitivity of tissues to the actions of insulin. This could promote the release of glucose from the liver and the fatty acids could spare the hepatic oxidation of glucose [7]. However, in the long term, glucocorticoids can decrease glycogen breakdown and stimulate glycogen synthesis [22,62].

Our results demonstrate that unsaturated fatty acids can be used as model ligands for studying the control of phosphorylase activity, possibly through mechanisms involving the stimulation of protein kinase C. The net effect on phosphorylase activity probably depends on a complex interaction with Ca^{2+} mobilization and increases in cyclic AMP concentrations. However, any involvement of fatty acids in stimulating glycogen breakdown *in vivo* is likely to depend on either the generation of locally high concentrations of polyunsaturated fatty acids or a very low concentration of circulating albumin.

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