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Rapid activation of glycogen phosphorylase by steroid hormones in cultured rat hepatocytes

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Testosterone (40–300 μM), oestradiol (20–500 μM), progesterone (20–500 μM), dexamethasone (10 nM–1 μM) and corticosterone (1–10 μM) activate glycogen phosphorylase rapidly when added directly to hepatocytes. The activation of phosphorylase was concentration-dependent and occurred after 10 min for dexamethasone, 30 min for testosterone and 60 min for oestradiol and progesterone. This rapid effect does not appear to be dependent on a stimulation of protein synthesis, it is independent of an increase in cyclic AMP, and it is not diminished by the presence of ornithine decarboxylase inhibitors. The stimulation of phosphorylase activity is diminished by depleting the incubation medium of Ca^{2+} in the presence of 0.5 mM-EGTA, and therefore it may involve changes in the distribution of Ca^{2+} in the hepatocytes. These results may explain some of the pharmacological effects of sex steroids, and also might contribute to the physiological actions of glucocorticoids.

INTRODUCTION

Mammalian liver is a major target organ for glucocorticoid actions. However, it was not usually considered to be a major site for the actions of sex steroids, since early studies were unable to demonstrate sex-steroid receptors in it. It is now recognized that the liver of mammals contains oestrogen and androgen receptors, although progesterone receptors have not been found yet [1].

Steroid hormones are thought to bind to cytoplasmic receptors and then translocate into the cell nucleus. This theory has been questioned by King [2,3], who has recently demonstrated [4] that the steroid receptors are mainly located within the nucleus. Nevertheless, the classical model of steroid actions involving changes in protein synthesis does not seem to be sufficient to account for all of their known effects. Steroid hormones have a stimulatory effect on hepatic glycogen phosphorylase activity *in vivo* which precedes and is independent of protein synthesis [5–10]. The stimulation does not involve an increase in cyclic AMP concentrations, since these are depleted at the same time or a little after phosphorylase activation. Studies from other laboratories have also shown steroid hormone effects which are independent of hormone interaction with the cell nucleus [11]. Interactions between steroid hormones and the plasma membrane have been suggested [12–16], and even the presence of steroid-hormone receptors located in the plasma membrane has been proposed [17–19].

The present work was performed to investigate whether the effects of steroid hormones on the glycogen phosphorylase activation could be observed in isolated hepatocytes. The aim of this study was to provide a controlled model for elucidating the mechanisms by which such effects are brought about. Hepatocytes were maintained in monolayer cultures for about 24 h before the experiments were started, in order to allow the cells to stabilize their metabolism.

MATERIALS AND METHODS

Animals and materials

The sources of the rats and most of the materials have been described previously [20–23]. Steroid hormones, adrenaline, vasopressin, phosphatidic acid, diltiazem, verapamil, actinomycin D and cycloheximide were from Sigma (London) Chemical Co. The cyclic AMP assay kit was from Amersham International, Amersham, Bucks., U.K. α-Difluoromethylornithine and 2*R*,5*R*-hept-6-yne-2,5-diamine ('RR-MAP') were generously given by the Merrell–Dow Research Institute, Cincinnati, OH, U.S.A.

Preparation and culture of hepatocytes

Hepatocytes were prepared and attached to collagen-coated tissue-culture dishes in a modified Leibovitz L-15 medium containing 10% (v/v) newborn-calf serum, 8.3 mM-glucose and 5 mM-galactose [22,23]. After 1–2 h 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 6–7 h, and the medium was then replaced by modified Leibovitz medium containing 0.2% (v/v) fatty-acid-poor bovine serum albumin, 8.3 mM-glucose and 5 mM-galactose, and the cells were incubated for a further 18–20 h. The medium was then replaced, and hormone additions were made so that the total volume of the medium changed by less than 2% [20]. At the times indicated, the cells were washed

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once with Leibovitz medium that did not contain serum or albumin. The cells were then scraped from the plates 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. They were then frozen in liquid N_2 and then stored at −20 °C until required [24].

Phosphorylase activities are expressed as means ±S.E.M. for three independent experiments, and the values for individual experiments were taken as means from triplicate dishes, unless otherwise indicated. Statistical significance was calculated by using a paired *t* test. Phosphorylase activities have been expressed relative to lactate dehydrogenase activities so as to compensate for small differences in the numbers of viable hepatocytes per culture dish [21,23].

Analytical procedures

Phosphorylase *a* and total phosphorylase activities were determined in homogenates [25]. A unit of phosphorylase is defined as the amount of enzyme that converts 1 μmol of substrate/min under the conditions of the assay. Lactate dehydrogenase was measured as described previously [21,23].

To determine cyclic AMP concentrations, cells were washed with ice-cold 0.16 M-NaCl and then 1 ml of 0.1 M-HCl was added. The cell suspensions were then transferred to small tubes and boiled for 5 min to deproteinize them. The concentration of cyclic AMP in the extract was determined by radioimmunoassay [26].

P_i and protein were determined by the methods of Fiske & Subbarow [27] and Lowry *et al.* [28] respectively.

RESULTS AND DISCUSSION

Testosterone (100 μM), oestradiol (100 μM) and progesterone (100 μM) activated ($P < 0.005$) glycogen phosphorylase after 10–60 min when added directly to hepatocytes (Figs. 1*a*–1*c*), and the effects were particularly pronounced with oestradiol and progesterone. These two hormones produced maximum increases of about 6-fold in phosphorylase activity (Fig. 2). The total phosphorylase activity in the hepatocytes was 204±21 munits/unit of lactate dehydrogenase (mean±S.E.M. for three independent experiments), which would have permitted a total stimulation of about 9-fold.

Dexamethasone, a synthetic glucocorticoid, at 100 nM produced a much smaller increase of about 20% ($P < 0.05$) in activity, but this effect occurred relatively quickly and reached a peak between 5 and 15 min (Figs. 1*d* and 3*a*). Similar stimulations were obtained with the natural glucocorticoid for the rat, corticosterone, at 1–10 μM (Fig. 3*b*). After 30 min the phosphorylase activity fell (Fig. 1*d* and Table 1), and a significant decrease ($P < 0.01$) was obtained after a 60 min incubation. This latter event is compatible with the results of Laloux *et al.* [29], who demonstrated a decreased phosphorylase activity in the livers of mice and rats treated 3 h previously with prednisolone.

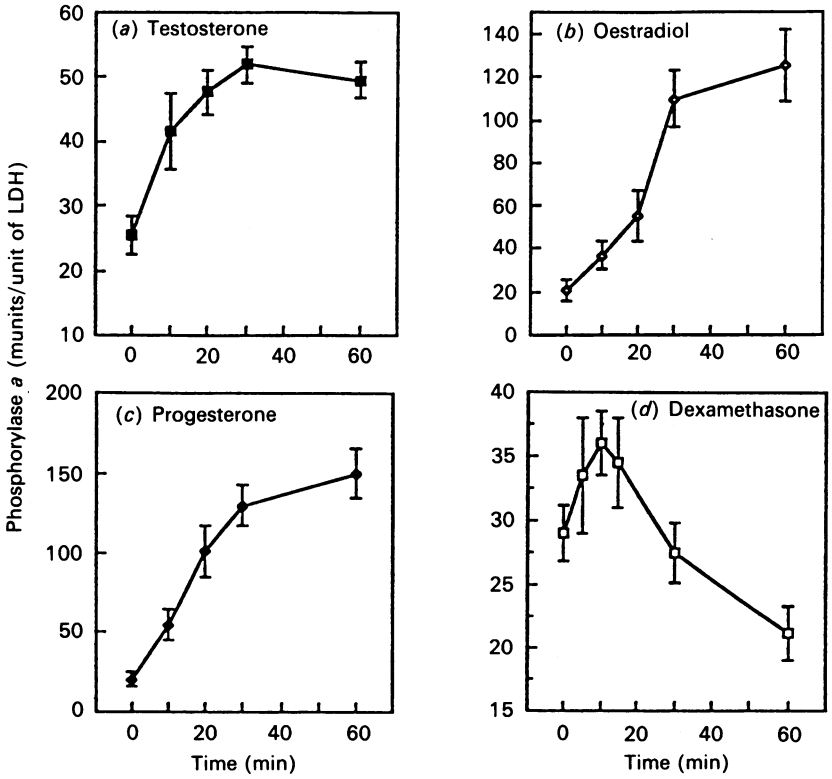

Fig. 1. Effects of testosterone, oestradiol, progesterone and dexamethasone on glycogen phosphorylase activity in rat hepatocytes

Monolayer cultures of rat hepatocytes were incubated at 37 °C with (*a*) 100 μM-testosterone, (*b*) 100 μM-oestradiol, (*c*) 200 μM-progesterone and (*d*) 100 nM-dexamethasone for the indicated times. Glycogen phosphorylase activity is expressed as munits of enzyme/unit of lactate dehydrogenase (LDH) present in the cells.

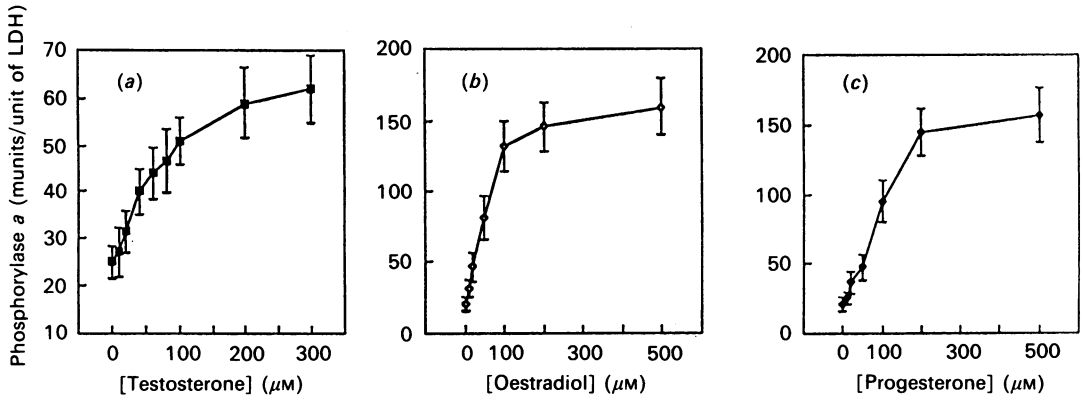

Fig. 2. Effects of increasing concentrations of testosterone, oestradiol and progesterone on glycogen phosphorylase activity

Monolayer cultures of rat hepatocytes were incubated at 37 °C for (*a*) 30 min with different concentrations of testosterone, (*b*) 60 min with different concentrations of oestradiol, or (*c*) 60 min with different concentrations of progesterone. Glycogen phosphorylase activity is expressed as munits of enzyme/unit of lactate dehydrogenase (LDH) present in the cells.

Many authors have reported that glucocorticoids exert a 'permissive' effect on the actions of several hormones and effectors such as catecholamines, prostaglandin E_1 and cyclic AMP [11,30,31]. The results in Table 1 indicate that dexamethasone potentiates the actions of other steroid hormones in activating glycogen phosphorylase, after incubation for 60 min. By this time the stimulating effect of dexamethasone itself had disappeared, and inhibitions were observed (Fig. 1*d*; Table 1).

The concentrations of testosterone, oestrogen and progesterone used in this work (Fig. 2) are far higher than those encountered physiologically. It has been reported [1] that the high doses required for steroid hormones to provoke effects in the liver appear to be predominantly caused by extensive metabolism of the steroids to inactive derivatives in the hepatocytes. Although androgen and oestrogen receptors have been demonstrated in mammalian liver [32], it is generally accepted that they are probably non-functional at endogenous steroid concentrations. A progesterone receptor in the liver of mammals still remains to be found [1,32].

The rapid enhancement of glycogen phosphorylase activity by steroid hormones suggests that it is unlikely to be explained by an increase in protein synthesis. Nevertheless, a series of experiments was designed to test this possibility, since glucocorticoids can inhibit glucose transport by rat thymus cells within 15 min by a mechanism that can be prevented by actinomycin D [11]. Incubations were performed in the absence or presence of 1 μg of actinomycin D/ml or 5 μg of cycloheximide/ml to inhibit protein synthesis [23,33]. These concentrations of antibiotics themselves stimulated glycogen phosphorylase activity, and these effects were additive to those of the steroid hormones (Table 2). This confirms that steroid hormones do not stimulate glycogen phosphorylase activity by promoting protein synthesis. Furthermore, Sistare & Haynes [34] have reported that a stimulation of gluconeogenesis in isolated rat hepatocytes by dexamethasone was seen after a lag averaging 9 min after the

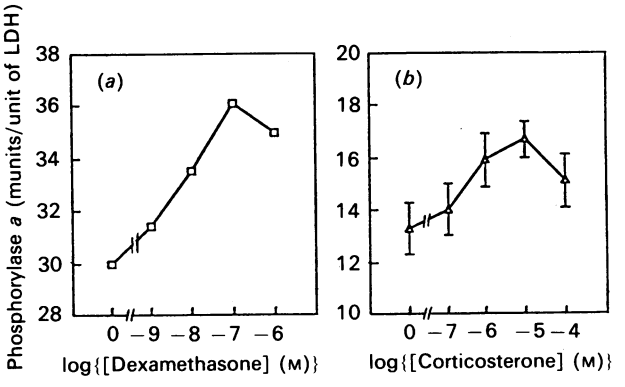

Fig. 3. Effects of Increasing concentrations of dexamethasone and corticosterone on glycogen phosphorylase activity

Monolayer cultures of rat hepatocytes were incubated at 37 °C for 10 min with different concentrations of (*a*) dexamethasone or (*b*) corticosterone. Glycogen phosphorylase activity is expressed as munits of enzyme/unit of lactate dehydrogenase (LDH) present in the cells.

Table 1. Effects of steroid hormones on the activity of glycogen phosphorylase in rat hepatocytes

Monolayer cultures of rat hepatocytes were incubated at 37 °C for 60 min as indicated. Glycogen phosphorylase activity is expressed as munits of enzyme/unit of lactate dehydrogenase (LDH) present in the cells. The significance of the difference between incubations containing sex steroids in the absence of dexamethasone is indicated by †$P < 0.005$ and ††$P < 0.001$. Differences between equivalent incubations in the presence or absence of dexamethasone are indicated by *$P < 0.05$ and **$P < 0.01$.

Sex steroid added	Phosphorylase *a* activity (munits/unit of LDH)	
	No dexamethasone	+ Dexamethasone (100 nM)
None (control)	22.5±2.5	16.4±2.0**
Testosterone (100 μM)	40.8±6.1††	50.6±6.3*
Oestradiol (100 μM)	135.4±19.0†	151.9±17.4*
Progesterone (200 μM)	158.1±24.6†	173.7±19.5*

Table 2. Effect of protein-synthesis-inhibiting antibiotics on steroid-hormone activation of phosphorylase in rat hepatocytes

Rat hepatocytes were incubated in monolayer cultures at the indicated times with 100 nM-dexamethasone, 100 μM-testosterone, 100 μM-oestradiol or 200 μM-progesterone in the absence or presence of 1 μg of actinomycin D/ml or 5 μg of cycloheximide/ml. Glycogen phosphorylase activity is expressed as munits of enzyme/unit of lactate dehydrogenase (LDH) present in the cells.

Incubation time		Additions	Phosphorylase *a* activity (munits/unit of LDH)
10 min	A	None	23.4±3.2
	B	Dexamethasone	28.2±3.5
	C	Cycloheximide	31.4±4.6
	D	Actinomycin D	29.1±4.4
	E	Dexamethasone+cycloheximide	35.5±4.7
	F	Dexamethasone+actinomycin D	33.8±5.2
30 min	G	None	24.1±3.2
	H	Testosterone	47.4±6.4
	I	Cycloheximide	33.1±4.7
	J	Actinomycin D	30.3±3.7
	K	Testosterone+cycloheximide	58.4±6.0
	L	Testosterone+actinomycin D	56.6±7.3
60 min	M	None	25.0±4.1
	N	Oestradiol	150±16.3
	O	Progesterone	161±21.0
	P	Cycloheximide	32.1±5.3
	Q	Actinomycin D	32.3±6.4
	R	Oestradiol+cycloheximide	161±30.1
	S	Oestradiol+actinomycin D	158±26.7
	T	Progesterone+cycloheximide	180±24.8
	U	Progesterone+actinomycin D	178±29.6

time of steroid addition. This effect was also not dependent on protein synthesis, but the observed changes of control rates of gluconeogenesis by specific inhibitors of protein synthesis also demonstrate the difficulties of working with such inhibitors.

Further experiments were performed to establish whether the rapid effects of steroid hormones on glycogen phosphorylase activity were caused by increases in cyclic AMP. This was not the case (Table 3), in agreement with the results obtained *in vivo* with chickens [5,6,8–10], mice [7] and oocytes of *Xenopus laevis* [18,19,35]. In some of these reports cyclic AMP concentrations were in fact depleted [5,7–10,18].

Table 3. Effects of dexamethasone, testosterone, oestradiol and progesterone on the cyclic AMP concentration in cultured hepatocytes

Rat hepatocytes were incubated for various times with the hormones as indicated. The values are means for two independent experiments, which were derived from triplicate dishes. The means from the two experiments differed by less than 8% and errors are not given.

Addition		Time of incubation (min)	Cyclic AMP (pmol/mg of protein)
A	None	10	1.46
B	Dexamethasone (100 nM)	10	1.50
C	Testosterone (100 μM)	30	1.43
D	Oestradiol (100 μM)	60	1.38
E	Progesterone (200 μM)	60	1.27

It has been reported [36] that polyamines interact with muscle phosphorylase *b* and increase its affinity for AMP. Furthermore, it has been proposed that cortisol increases ornithine decarboxylase activity in the liver by a mechanism that is unknown [37]. This enzyme is responsible for polyamine synthesis, and might be rapidly activated by steroid hormones, thus causing an indirect phosphorylase activation. Studies in presence of ornithine decarboxylase inhibitors such as α-difluoromethylornithine and 'RR-MAP', an analogue of putrescine [38], demonstrated that phosphorylase *a* activation was not dependent on stimulation of polyamine synthesis (results not shown).

Glycogen phosphorylase can be activated by Ca^{2+} [30,31,39–43], and this occurs since phosphorylase *b* kinase is allosterically stimulated by Ca^{2+} [44]. Although it is known that intracellular Ca^{2+} mobilization is the main event for the activation of hepatic phosphorylase by vasopressin, catecholamines and angiotensin II, an influence of the extracellular Ca^{2+} concentration should not be ignored. The stimulation of phosphorylase activity by dexamethasone was abolished, and in fact a significant decrease was observed when the cells were incubated in a Ca^{2+}-free medium containing 0.5 mM-EGTA (Table 4, line B). The effect of testosterone was not entirely abolished, but the stimulation was decreased ($P < 0.01$) from 1.93- to 1.18-fold by Ca^{2+} depletion. The effects of oestradiol and progesterone were also decreased ($P < 0.05$) from 6.2- to 4.9-fold and 7.2- to 5.5-fold respectively by Ca^{2+} depletion. The preincubation in Ca^{2+}-depleted medium could cause a decrease of intracellular stores and attenuation of physiological responses [45,46]. In the present work the preincubation in Ca^{2+}-free medium plus EGTA caused a progressive decrease in

Table 4. Effects of Ca^{2+} depletion on the effects of steroids on glycogen phosphorylase

Hepatocytes were preincubated for 15 min in Ca^{2+}-free Leibovitz medium containing 0.5 mM-EGTA and then for the time indicated in the same medium with steroid hormone. Phosphorylase activity of the control was 24.6±3.6, 24.5±4.7 and 21.2±3.0 munits/unit of lactate dehydrogenase present in the cells incubated for 10, 30 and 60 min respectively. The significance of the difference between groups incubated in the presence or absence of steroids is indicated by $*P < 0.05$, $**P < 0.005$, $***P < 0.0025$, compared with the equivalent medium.

	Additions	Incubation time (min)	Relative phosphorylase *a* activity (%) Normal medium	Relative phosphorylase *a* activity (%) Ca^{2+}-free medium + 0.5 mM-EGTA
A	None	10	100	83.7±11.8
B	Dexamethasone (100 nM)	10	121±19.8*	54.0±5.0*
C	None	30	100	70.3±10.2
D	Testosterone (100 μM)	30	193±27.8***	83.3±19.1
E	Dexamethasone (100 nM)	30	89.8±11.4	47.1±8.1***
F	Testosterone (100 μM) + dexamethasone (100 nM)	30	223±28.2***	67.1±11.8
G	None	60	100	63.5±15.8
H	Oestradiol (100 μM)	60	620±96.4***	312±57.7**
I	Progesterone (200 μM)	60	723±110***	373±80.5**

phosphorylase activity measured at 10 min ($P < 0.05$), 30 min ($P < 0.01$) and 60 min ($P < 0.01$) in the absence of steroid hormones. Furthermore, the absence of Ca^{2+} from the medium decreased phosphorylase activities for all of the incubation conditions (Table 4).

Thus, although Ca^{2+} influx may not be a prerequisite for the initial increase in the intracellular Ca^{2+} concentrations, it might be required for prolongation of the response. A series of experiments was also performed with the Ca^{2+}-channel blockers diltiazem and verapamil, but these substances activated phosphorylase by 1.7- and 1.5-fold respectively. Studies from other laboratories have shown that phosphatidic acid [47] and vasopressin [43,48] each interact synergistically with glucagon to stimulate Ca^{2+} influx in the perfused rat liver and in isolated rat hepatocytes, respectively. Our results (Table 5) suggest that similar effects might be caused by sex steroids, since synergistic effects with either vasopressin or potassium phosphatidate in activating phosphorylase were demonstrated with testosterone and oestradiol. By contrast, this was not the case for dexamethasone, since the activity obtained with dexamethasone and vasopressin together was lower ($P < 0.05$) than that with vasopressin alone. We cannot explain this discrepancy. However, we have also found that dexamethasone and testosterone each interact synergistically with adrenaline to activate phosphorylase (Table 6). Therefore, steroid hormones might potentiate the Ca^{2+} influx and accelerate the release of Ca^{2+} from the intracellular stores.

It has been proposed that plasma membranes bind more Ca^{2+} in the presence of cortisol [49]. Also, some steroids such as progesterone, medroxyprogesterone, 17α-hydroxyprogesterone, cyproterone and prednisolone 3,20-bisguanylhydrazone cause partial inhibition of the Na^+/K^+-ATPase, which can be accompanied by a specific conformational change in the enzyme. According to this hypothesis, the change in protein conformation alters the affinity of Ca^{2+} for lipids associated with Na^+/K^+-ATPase and increases Ca^{2+} release into the cell [50]. Thus partial inhibition of the Na^+ pump may significantly change

Table 5. Phosphorylase activation by the co-administration of dexamethasone, testosterone or oestradiol with phosphatidate or vasopressin

Hepatocytes were incubated in monolayer cultures for 10 min with the combinations indicated. The activities of phosphorylase are expressed relative to that obtained in control incubations, which was 24.1±2.9 munits/unit of lactate dehydrogenase present in the cells. The significance of the difference between the control and incubations in the absence of vasopressin and phosphatidate is indicated by $*P < 0.05$ and $**P < 0.01$. The significance of the difference between incubations to which phosphatidate or vasopressin was added, compared with equivalent incubations in the absence of these compounds, is indicated by $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$. The phosphorylase activity obtained after adding phosphatidate plus vasopressin was 193±19%, which did not differ significantly from that with vasopressin alone.

Additions	Relative phosphorylase *a* activity (%) None	+ Phosphatidate (100 μM)	+ Vasopressin (10 nM)
None (control)	100	90.5±11.6	181±18.7††
Dexamethasone (100 nM)	123±16.2*	166±18.3†	163±13.0†
Testosterone (100 μM)	161±19.9**	225±26.6†	255±30.0†
Oestradiol (100 μM)	213±20.7**	247±15.4†	333±23.2††

Table 6. Phosphorylase activation by the co-administration of dexamethasone or testosterone with adrenaline

Hepatocytes were incubated in monolayer cultures with the combinations of hormones indicated. The activities of phosphorylase are expressed relative to that obtained in control incubations, which were 28.1 ± 4.0 and 30.5 ± 3.5 munits/unit of lactate dehydrogenase for 10 and 30 min respectively. The significance of the difference between groups is indicated by $*P < 0.005$.

	Additions	Time of incubation (min)	Relative phosphorylase *a* activity (%)
A	None (control)	10	100
B	Adrenaline (2 μM)	10	529 ± 25.4
C	Dexamethasone (100 nM)	10	120 ± 11.6
D	Adrenaline (2 μM) + dexamethasone (100 nM)	10	642 ± 34.7 B versus D*
E	None (control)	30	100
F	Adrenaline (2 μM)	30	361 ± 26.7
G	Testosterone (100 μM)	30	194 ± 19.2
H	Adrenaline (2 μM) + testosterone (100 μM)	30	626 ± 46.3 F versus H*

Ca^{2+} movements [51]. Furthermore, it has been reported that dexamethasone affects polyphosphoinositide metabolism in different tissues [16,52,53], and this could also modify the distribution of intracellular Ca^{2+}. Finally, we have found indications of specific binding of steroids to liver plasma membranes [54].

In conclusion, this paper shows for the first time a rapid activation of glycogen phosphorylase by steroid hormones in rat hepatocytes in monolayer cultures. This effect is independent of protein synthesis, it does not involve an increase in intracellular cyclic AMP concentration and it is not diminished by inhibitors of ornithine decarboxylase.

Phosphorylase activation by the steroid hormones is dependent on Ca^{2+}, although the exact mechanism for the involvement of this cation is not yet known. These findings are in agreement with an interaction between steroid hormones and the plasma membranes. The nature of such an interaction remains unknown, but does not appear to correspond to the classical mode of action of these hormones. It may be significant that micromolar concentrations of bile acids, which are structurally related to the steroid hormones used in the present work, are able to mobilize intracellular Ca^{2+} in hepatocytes [55,56].

The results establish that part of the effect of pharmacological doses (micromolar concentrations) of sex steroids in activating glycogen phosphorylase can result from their direct action on the liver. Furthermore, glucocorticoids also have significant rapid effects in this respect (Fig. 3) at concentrations that are encountered *in vivo* [57,58]. These effects might therefore have physiological relevance for the action of glucocorticoids alone, or in terms of facilitating the actions of catecholamines (Table 6).

We believe that further investigation performed on cultured hepatocytes and purified membrane fractions will contribute to elucidating the relevance of these extragenomic effects of steroid hormones.

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