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STEROIDOGENIC EFFECT OF EXOGENOUS PHOSPHOLIPASE C ON BOVINE ADRENAL FASCICULATA CELLS

A.J. HADJIAN, M. CULTY and E.M. CHAMBAZ

Laboratoire de Biochimie Endocrinienne, INSERM U-244, CNRS ERA-942,
Université Scientifique et Médicale de Grenoble,
38402 Saint Martin d'Hères Cédex, France

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Phospholipase C (Bacillus cereus) added to the incubation medium stimulated the steroidogenic activity of bovine adrenal zona fasciculata cell suspensions to a level similar to that induced by optimal concentration of ACTH. This effect was not related to an increase of cyclic AMP ; it was calcium-dependent and was also induced by an other bacterial phospholipase C (from Clostridium perfringens) whereas phospholipases A2 and D were ineffective. Phospholipid metabolism was examined in these cells after radiolabeling with [^{14}C]-glycerol or [^{32}P]orthophosphate. Phospholipase C induced a very fast (5 seconds) increase in cellular [^{14}C]-1,2-diacylglycerol followed by [^{32}P] labeling of phosphatidic acid and phosphatidylinositol. These events preceded the stimulation of steroidogenesis which was detectable after 2 minutes of incubation. These observations suggest that activation of an endogenous phospholipase C activity may be considered as an early event in the response of bovine adrenocortical cells to steroidogenic effectors such as angiotensin II and acetylcholine.

Steroidogenesis of bovine adrenal fasciculata cells is stimulated by the adrenocorticotropic hormone (ACTH), the neurotransmitter acetylcholine (1) (through muscarinic receptors) and angiotensin II (2). The mechanism of action of ACTH is usually accepted to be mediated by cyclic AMP ; however, that of acetylcholine and angiotensin II is cyclic nucleotide independent (1,3). These two agents stimulate the incorporation of radiolabeled phosphate into phosphatidylinositol in several tissues, including bovine adrenal zona fasciculata (4). This effect precedes the increase of glucocorticoid synthesis and might thus be suggested as part of a causal cascade resulting in the steroidogenic response. The mechanism which underlies the increase of phosphatidylinositol labeling may be related to a de novo biosynthesis or/and to a stimulation of the "phosphatidylinositol cycle" with a resynthesis following an initial hydrolysis of this phospholipid. Considering this hypothesis, it was of interest to examine wether exogenous phospholipase C was able to mimic the steroidogenic cell response to acetylcholine and

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angiotensin II. To this end, we have studied both steroidogenesis and phosphatidylinositol metabolism after addition of a bacterial phospholipase C to bovine adrenal fasciculata cell suspensions. Our results show a pronounced effect on both parameters and provide new insights concerning the possible link between phospholipid metabolism and steroidogenesis activation in this system.

MATERIAL AND METHODS

- **Chemicals** : Acetylcholine was obtained from Serva (Heidelberg) and [Asp^1, Val^5] angiotensin II (Hypertensin) from Ciba-Geigy (Basel) ; [1,2,6,7 - ^3H] cortisol (100 Ci/mmol, [U-^{14}C] glycerol (150 mCi/mmol) and [^{32}P] H_3PO_4 were from the Commissariat à l'Energie Atomique (Saclay, France). Phospholipase C from Bacillus Cereus (ca 800 U/mg protein), phospholipase A2 from Crotalus durissus (ca 200 U/mg) and phospholipase D from cabbage (ca 0.3 U/mg) were purchased from Boehringer Mannheim (FRG). Phospholipase C from Clostridium perfringens (205 U/mg) was obtained from Sigma (Saint Louis, MO, USA) as well as all lipids and phospholipids model compounds.
- **Assay methods** : preparation and incubation of bovine zona fasciculata cell suspensions, cortisol and cyclic AMP radioimmunoassays were performed as reported in (1). Methods for cell phospholipid labeling with [^{32}P]Pi, extraction and analysis by thin layer chromatography have been described elsewhere (4). [^{14}C]glycerol-labeled 1,2-diacylglycerol was analyzed by thin layer chromatography according to Freeman and West (5). Cell viability was assessed using the trypan blue test which showed that in the presence of up to 10 µg/ml of phospholipase C (B. cereus) at least 75 % of the cells remained viable over a 60 minutes incubation period ; however, at higher enzyme concentration (30 µg/ml), the cell viability decreased to about 60 %.

RESULTS

1. Steroidogenic effect of phospholipase C (B. cereus).

In a first set of experiments a single concentration of enzyme (10 µg/ml) was tested and cortisol concentration in the medium was determined as a function of time (Fig. 1). A progressive increase of net cortisol production was observed which reached a plateau, within 1 hour of incubation. Under these conditions, the activation of steroidogenesis was similar to that elicited by an optimal concentration of ACTH (1). No increase of cyclic AMP was detected at any time during this stimulation (data not shown). The steroidogenic activity of the cells was then examined as a function of phospholipase C concentration in the medium (Fig. 2) ; a gradual increase of activity was observed from 1 to 10 µg/ml of enzyme (final concentrations) with no further increase between 10 and 20 µg/ml. The specificity of the steroidogenic response was examined by

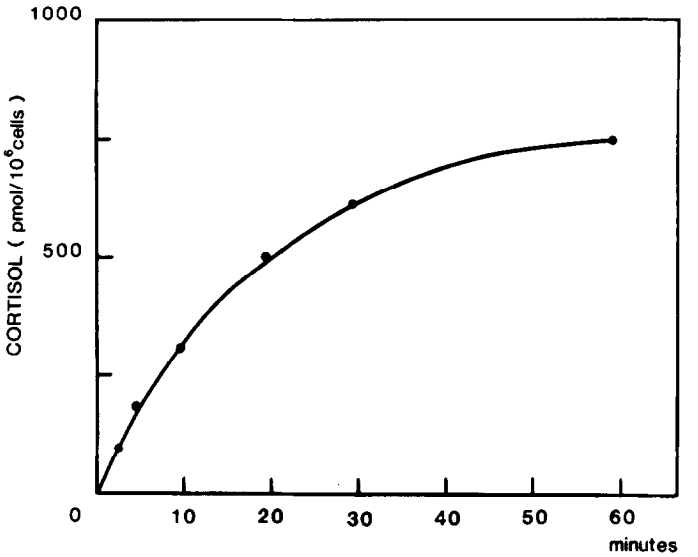

Figure 1. Time course of steroidogenesis induced in bovine adrenocortical cells by 10 µg/ml of phospholipase C. Results are expressed in pmol of cortisol per 10^6 cells (net cortisol production, after substraction of the corresponding control values). Results are the means for triplicate incubations in a single experiment, one of three that gave similar results.

using different phospholipases at concentrations ranging from 2 to 10 µg/ml : phospholipases A2 and D were unable to elicit any detectable increase of cortisol but phospholipase C from Clostridium perfringens was

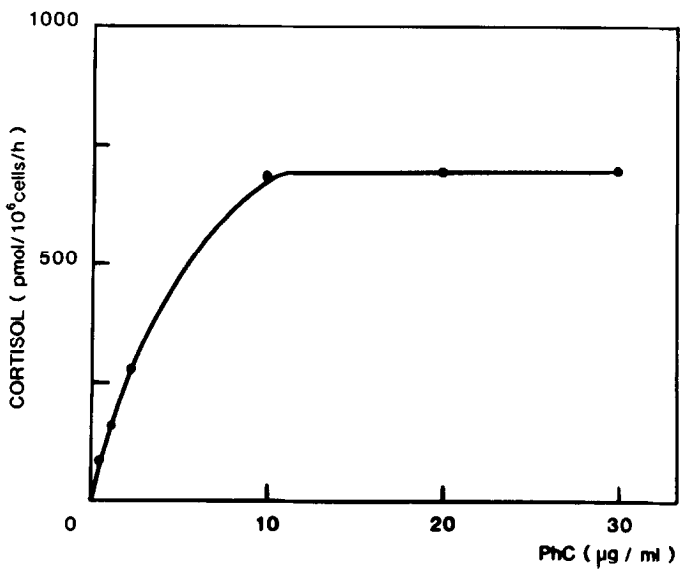

Figure 2. Dose-response of phospholipase C (Ph C) on cortisol production by bovine adrenocortical cells. The results plotted are means for triplicate incubations in one of three similar experiments.

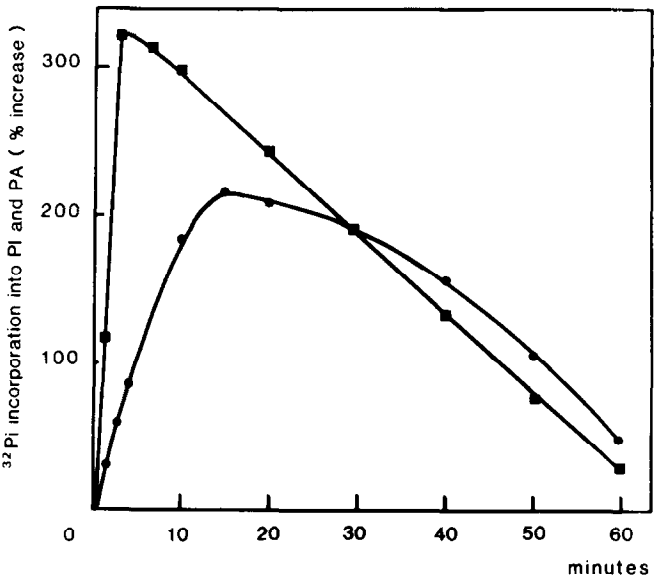

Figure 3. Effect of phospholipase C (10 µg/ml) on phosphatidic acid (PA) (■——■) and phosphatidylinositol (PI) (●——●) labeling ; [^{32}P]Pi incorporation is expressed as the net percentage increment per hour, after substraction of the corresponding control values (100 %). The values shown are means for duplicate samples in a representative experiment carried out two times.

steroidogenic with a relative potency equivalent to one third of Bacillus cereus phospholipase C activity (not shown). In addition, the effect of this latter enzyme was not changed after heating the preparation at 60°C for 20 min., conditions which should inactivate potential contaminants such as the theta toxin (6, 7).

2. Effect of phospholipase C (B. cereus) on phosphatidylinositol turnover.

Labeling experiments with [^{32}P]Pi were conducted to test the effect of phospholipase C on phosphatidylinositol metabolism. As shown in figure 3, an optimally steroidogenic concentration of enzyme (10 µ g/ml) induced a sharp peak of phosphatidic acid labeling at 2 minutes of incubation, followed by a linear decrease toward basal levels until 60 minutes. A delayed increase in phosphatidylinositol labeling occured between 10 to 20 minutes and was followed by a gradual decrease until the end of the experiment. Phosphatidylinositol labeling was then examined as a function of enzyme concentration as shown on figure 4 : after a sharp increase observed for enzyme concentrations of 0.5 to 2 µg/ml, a plateau was

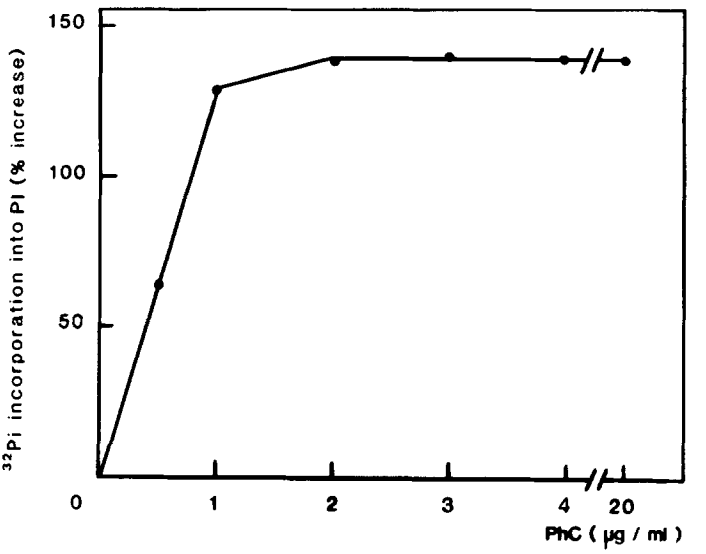

Figure 4. Dose-response of phospholipase C on [^{32}P]Pi incorporation into phosphatidylinositol (PI) at 15 minutes of incubation. Results are the means for duplicate incubations in one of two similar experiments.

obtained until 20 µg/ml. To gain further insight in the initial step of phospholipid turnover, cells were prelabeled with [^{14}C]-glycerol and thereafter stimulated in the presence of 10 µg/ml of phospholipase C. As might have been expected, a rapid release of [^{14}C]- labeled 1,2-diacylglycerol was observed (Fig.5) which was detectable after 5 seconds of incubation. This release thus appeared to be the earliest detectable biological response of the cells following addition of the enzyme.

3. **Influence of extracellular calcium on phospholipase C stimulation of steroidogenesis and phospholipid metabolism.**

In a calcium-free medium (absence of calcium and presence of 1 mM EGTA), the phospholipase effect was markedly decreased : by 46 % for steroidogenesis and by 71 % and 84 % for phosphatidic acid and phosphatidylinositol labeling respectively (table I). These observations are in accordance with the known calcium-dependence of phospholipase C catalytic activity (8).

DISCUSSION

The present data demonstrate that the addition of a bacterial phospholipase C stimulates both phosphatidylinositol metabolism and steroido-

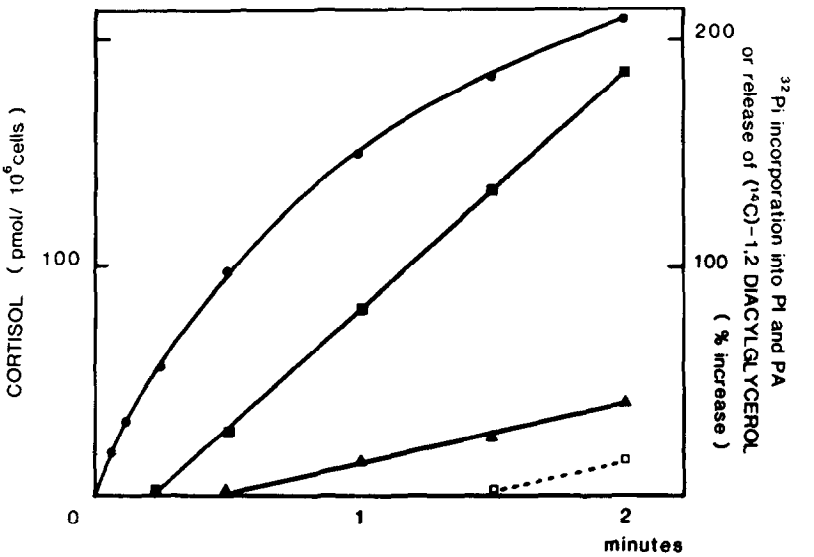

Figure 5. Release of [^{14}C]-1,2-diacylglycerol (●——●) from prelabeled adrenocortical cell phospholipids following addition of phospholipase C (10 µg/ml). Phosphatidic acid (PA) (■——■)and phosphatidylinositol (PI) (▲——▲) labeling are shown on the same graph to illustrate the sequence of events preceding the increase of cortisol production (□------□). The results plotted are means for duplicate incubations in one of three similar experiments.

TABLE I

Influence of extracellular calcium on the activation by phospholipase C (10 µg/ml) of steroidogenesis and [^{32}P]Pi incorporation into phosphatidic acid and phosphatidylinositol

	Incubation medium	
	containing 2.5 mM Ca^{2+}	calcium free
Cortisol production (pmol/10^6 cells/hour)	988 ± 490	529 ± 324 [a]
Phosphatidic acid labeling (after 2 min. of incubation)	244 ± 56	70 ± 50 [a]
Phosphatidylinositol labeling (after 15 min. of incubation)	211 ± 112	34 ± 15 [a]

Steroidogenesis was expressed as net cortisol production (see legend of figure 1) and [^{32}Pi] incorporation into phospholipids as net percentage increment (see legend of figure 3).
a : statistically significant ($P < 0.001$) from results obtained in presence of calcium (Student t test) (mean ± S.E.M. ; $n \approx 6$).

genesis in bovine adrenal zona fasciculata cell suspensions whereas phospholipase A2 and D were ineffective. This first observation of a steroidogenic effect of phospholipase C on adrenocortical cell is reminiscent of previous reports concerning several cell types on which the enzyme has been shown to mimic the biological response to specific stimuli, e.g. platelets (9), neuroblastoma cells (10), pancreatic Langerhans islets (11), hepatocytes (12) and adipocytes (13).

In the present study the steroidogenic response to phospholipase C would bring into focus the possible involvement of a phosphoinositide metabolism in the triggering of steroidogenesis in response to agents such as acetylcholine and angiotensin II. Indeed, these effectors have been shown to stimulate phosphatidylinositol turnover in adrenocortical cells before any increase in steroid production could be detected (1). In response to angiotensin II, the first detectable event is a very fast (15 seconds) decrease of prelabeled polyphosphoinositides ; furthermore, acetylcholine and angiotensin II induce an increase of intracellular inositol phosphates (submitted for publication) which strongly suggest that stimulation of an endogenous phospholipase C activity is involved. As a result, two kinds of potential intracellular messengers may be generated i.e. inositol triphosphate, which has recently been proposed as an intracellular mediator of calcium mobilization (14) and diacylglycerol which is a potent activator of the calcium and phospholipid dependent protein-kinase C (15). The C kinase has recently been characterized in bovine adrenocortical cells (16). The synergistic effect of calcium and a diacylglycerol analog to elicit a full physiological secretory response in platelets has been used to suggest that protein kinase C was involved in the process (17). A similar approach has been used with our model in which the combination of a calcium ionophore and a synthetic diacylglycerol (1-oleoyl-2-acetyl-glycerol) or a potent phorbol ester such as 12-O-tetradecanoyl-phorbol-13-acetate mimics the steroidogenic effect of antiotensin II (18). Undoubtedly, in the present model, the phospholipase C-dependent phosphoinositide metabolism remains to be more thoroughly examined

as a possible link between the receptor occupancy at the membrane level and the intracellular events leading to steroidogenic response. This pathway would be of special interest in the response to cyclic nucleotide-independent adrenocortical effectors such as angiotensin II and acetylcholine.

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