The possible role of protein kinase C and phospholipids in the regulation of steroid production in rat Leydig cells

Axel P.N. Themmen, Jos W. Hoogerbrugge, Focko F.G. Rommerts and Henk J. van der Molen

Department of Biochemistry (Division of Chemical Endocrinology), Medical Faculty, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands

Received 17 May 1986

We have studied the possible involvement of the activation of calcium-dependent phospholipid-activated protein kinase (PK-C) in the stimulatory action of LHRH on Leydig cells, using 4β-phorbol-12-myristate-13-acetate (PMA) and phospholipase C (PL-C). LHRH agonist (LHRH-A) and PL-C had a large synergistic effect on LH-stimulated steroid production, whereas PMA inhibited the effect of LH. However, PMA always caused an increase in steroid production stimulated by various doses of dibutyryl cAMP. LH and PMA stimulated the phosphorylation of 17 and 33 kDa proteins, whereas LHRH-A and PL-C had no effect. Of all effectors used, LH had the most pronounced effect on the synthesis of 14, 27 and 30 kDa proteins. The present results suggest that the mechanisms of action of LHRH-A and PL-C on steroid production in Leydig cells may be similar and different from PMA, and may involve stimulation of a specific type of PK-C or hydrolysis of a specific pool of phospholipids.

(Leydig cell) *Phorbol ester* *Phospholipase C* *Steroidogenesis* *LH* *LHRH*

1. INTRODUCTION

It is generally accepted that cAMP plays an important role in the regulation of steroid production in testicular Leydig cells, since stimulation of the mitochondrial side chain cleavage enzyme by LH is accompanied by increased levels of cAMP, activation of protein kinase A and phosphorylation of at least six proteins [1–3]. Recently it was shown that LH action occurs concomitant with changes in the intracellular concentration of Ca^{2+} [4] and an increase in PI metabolism [5].

The pituitary decapeptide luteinizing hormone releasing hormone and its analogues (LHRH-A) have direct effects on Leydig cell steroid production [6,7], but the mechanism of action of LHRH or LHRH-A on Leydig cell steroidogenesis is not clear. Results from different investigators indicate that the action of LHRH is not mediated by cAMP [8,9]. It has been shown with isolated Leydig cells from mature rats that LHRH-A can cause an increase in the intracellular Ca^{2+} level [4] and a stimulation of incorporation of labelled phosphate into PI [10]. These results indicate that the action of both LH and LHRH involves changes in intracellular Ca^{2+} and PI turnover, although these hormones have different effects on cAMP and steroid production. Increased PI turnover may lead to production of diacylglycerol with subsequent stimulation of calcium-dependent phospholipid-activated protein kinase (PK-C) [11].

To investigate to which degree PK-C activation may play a role in the regulation of steroid production by LHRH, we have compared the steroidogenic effects of PMA and PL-C with those of LHRH-A. In addition, we have measured the effects of these agents on cellular protein phosphorylation and protein synthesis which may

Abbreviations: dbcAMP, N^6-2′-*O*-dibutyryl adenosine cyclic 3′,5′-monophosphate; PL-C, phospholipase C; PL-A_2, phospholipase A_2; PMA, 4β-phorbol-12-myristate-13-acetate; PA, 4β-phorbol-13-monoacetate; PI, phosphatidylinositol

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

reflect cellular activities after stimulation of specific membrane transducing systems.

2. MATERIALS AND METHODS

2.1. *Materials*

PMA, PA and PL-C (*Clostridium perfringens*, type XII) were obtained from Sigma (St. Louis, MO). Ovine LH (NIH-LH-S18, 1.03 IU/mg) was a gift from the Endocrinological Study Section of the National Institute of Health, Bethesda, MD. (D-Ser-t-bu^6,des-Gly^{10}-NH_2)-LHRH-ethylamide (HOE766, LHRH agonist) and Ac-(D)-pC1Phe-(D)pC1Phe-(D)Trp-Ser-Tyr-(D)Phe-Leu-Arg-Pro-(D)Ala-NH_2 (ORG30093, an LHRH antagonist) were kindly provided by Hoechst Pharma (Amsterdam, The Netherlands) and Organon (Oss, The Netherlands) respectively.

2.2. *Methods*

Leydig cells were isolated from 21–24-day-old rats and incubated as described [9,12]. Pregnenolone was measured by radioimmunoassay in the medium after incubation of the cells for 3 h with the indicated additions in the presence of inhibitors of pregnenolone metabolism cyanoketone (5 μM) and SU-10603 (19 μM) [13,14].

Protein phosphorylation was measured in intact cells. Cells (10^6) were incubated for 3 h in 0.5 ml Krebs-Ringer buffer without phosphate but containing 0.2% (w/v) glucose, 0.1% (w/v) BSA, in the presence of 100 μCi $^{32}PO_4$ (carrier free). The proteins were extracted and separated using polyacrylamide gradient (8–15%) gel electrophoresis in the presence of SDS as described by Bakker et al. [15]. For protein synthesis 10^6 cells were incubated in medium with 15–30 μCi [^{35}S]methionine. Cells were sonicated (2 times for 5 s, high setting) in 1 ml of 10 mM Tris-HCl (pH 7.4), 0.16 M NaCl, 3 mM $MgCl_2$, 5 mM KCl, 1 mM PMSF, and centrifuged (25 min, 10000 × *g*). Proteins were precipitated from the supernatant with 10% trichloroacetic acid, and separated on polyacrylamide gradient (10–20%) gels in the presence of SDS. The gels were dried and autoradiography was performed using Kodak SB-5 X-ray film. M_r was determined using an electrophoresis calibration kit from Pharmacia (Uppsala).

3. RESULTS

Isolated Leydig cells from immature rats were incubated with different amounts of LH in the absence or presence of 40 nM LHRH-A (fig.1A).

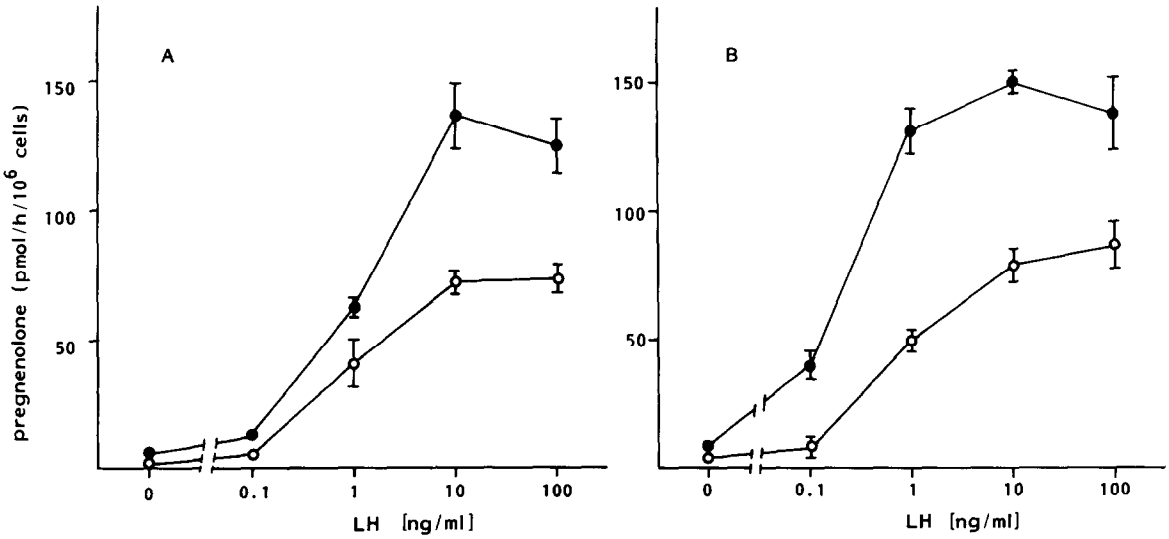

Fig.1. (A) Effect of LHRH-A on LH-stimulated pregnenolone production. Leydig cells were incubated with different amounts of LH for 3 h in the absence (○) or presence (●) of 40 nM LHRH-A. (B) Effect of PL-C on LH-stimulated pregnenolone production. Leydig cells were incubated with different amounts of LH for 3 h in the absence (○) or presence (●) of 1 U/ml PL-C. The values given are means ± SD (n = 4) of two different cell preparations. Steroid productions in the presence of LHRH-A or PL-C are significantly ($P < 0.01$) different from their respective controls (Student's t-test).

Pregnenolone production was stimulated up to 35-fold by LH alone, and could be further stimulated (60-fold) by the addition of 40 nM LHRH-A. LHRH-A alone also stimulated pregnenolone production in a dose-dependent manner with an ED_{50} of 0.1 nM LHRH-A and a 5-fold maximal stimulation (basal: 2.0 ± 0.1; 40 nM LHRH-A: 9.6 ± 0.2 pmol pregnenolone/h per 10^6 cells). All effects of LHRH-A on steroid production were completely abolished when the LHRH antagonist ORG30093D (400 nM) was included in the incubation medium (not shown).

To investigate the effect of activation of PK-C on steroid production we incubated cells with increasing concentrations of LH in the presence of the phorbol ester PMA (100 ng/ml) (fig.2A). PMA alone stimulated steroid production 3-fold, but inhibited steroidogenesis at intermediate concentrations of LH, without affecting maximally stimulated cells. Results from incubation of cells with PMA in the presence of different concentrations of dbcAMP showed that the small steroidogenic effect of PMA was constant.

PL-C (1 U/ml) was added to the incubations to study the effects of phospholipid hydrolysis on Leydig cell steroid production (fig.1B). PL-C stimulated basal pregnenolone production 3-fold, and was able to stimulate LH-dependent steroid production further. These effects of PL-C were similar to those of LHRH-A.

To characterize further the effects of LH, LHRH-A, PL-C and PMA we have analyzed protein phosphorylation and protein synthesis by Leydig cells. During a 3 h incubation, LH as well as PMA stimulated the phosphorylation of a 17 and a 33 kDa protein, whereas addition of LHRH-A or PL-C had no discernable effects on protein phosphorylation. LH, LHRH-A, PL-C and PMA all stimulated the synthesis of 14, 27 and 30 kDa proteins after 5 h incubation, but the effects of LHRH-A, PL-C and PMA were much less pronounced than the effects of LH.

Addition of the phorbol ester PA (100 ng/ml) or $PL\text{-}A_2$ (5 U/ml) did not have any effects on steroid production, protein phosphorylation or protein synthesis.

4. DISCUSSION

The effects of two activators of PK-C on signal transduction pathways for regulation of steroid production in Leydig cells have been investigated: PMA which has been shown to activate PK-C [16] and PL-C which increases the concentration of

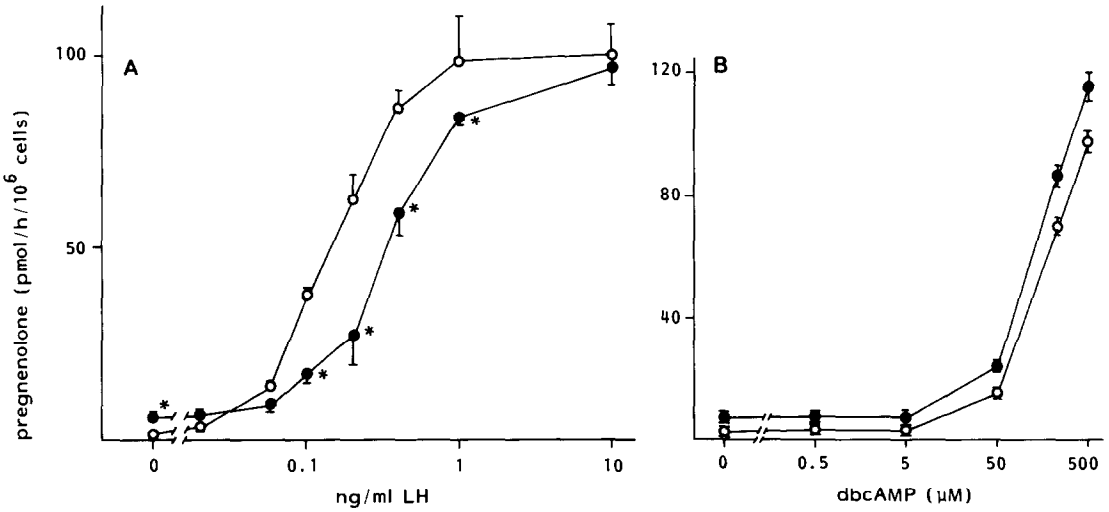

Fig.2. (A) Effect of PMA on LH-stimulated pregnenolone production. Leydig cells were incubated with different amounts of LH for 3 h in the absence (○) or presence (●) or 100 ng/ml PMA. (B) Effect of PMA on dbcAMP-stimulated pregnenolone production. Leydig cells were incubated with different amounts of dbcAMP in the absence (○) or presence (●) of 100 ng/ml PMA. The values given are means ± SD ($n = 4$) of two different cell preparations. Steroid productions in the presence of LHRH-A or PL-C are significantly ($P < 0.01$) different from their respective controls (Student's t-test).

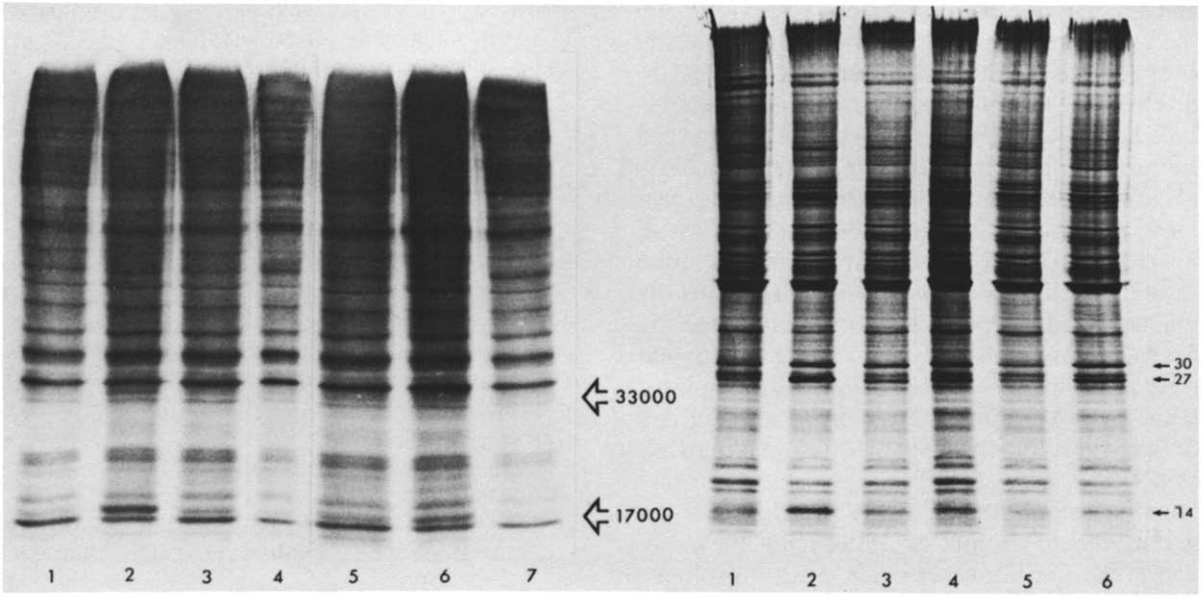

Fig.3. (Left) Protein phosphorylation patterns of Leydig cells. Cells were incubated for 3 h in the presence of $^{32}PO_4$. Proteins were extracted and separated on SDS-PAGE. The autoradiograph of the dried gel is shown. Lanes: 1, control; 2, 100 ng/ml LH; 3, 40 nM LHRH-A; 4, 1 U/ml PL-C; 5, 5 U/ml PL-A_2; 6, 100 ng/ml PMA; 7, 100 ng/ml PA. (Right) Protein synthesis pattern of Leydig cells. Cells were incubated for 5 h in the presence of [^{35}S]methionine. Proteins were extracted and separated on SDS-PAGE. The autoradiograph of the dried gel is shown. Lanes: 1, control; 2, 100 ng/ml LH; 3, 40 nM LHRH-A; 4, 100 ng/ml PMA; 5, 100 ng/ml PA; 6, 1 U/ml PL-C.

diacylglycerol in membranes [17,18] and elicits effects on protein phosphorylation in 3T3 cells which are supposed to be mediated by PK-C [19].

The synergistic effect of LHRH-A on LH-dependent steroid production was not observed with PMA. The phorbol ester stimulated basal steroid production and slightly inhibited the LH effects, but no inhibitory effects on dbcAMP- or maximally LH-stimulated steroidogenesis were found. These results indicate that PMA had no deleterious effects on the cells. Similar results have been obtained for mouse Leydig cells, and the inhibitory effect of PMA appeared to be located at the level of the regulatory GTP-binding protein of adenylate cyclase [20].

PL-C and LHRH-A stimulated basal steroid production as well as LH-dependent steroidogenesis, suggesting a common mechanism of action of PL-C and LHRH-A. LHRH-A and PL-C had also similar effects on protein phosphorylation and protein synthesis: i.e. the rate of protein phosphorylation was not affected, but the synthesis of proteins of 14, 27 and 30 kDa was slightly increased. These results suggest that LHRH and PL-C may share a common mechanism of action. In contrast, PMA stimulated protein phosphorylation to approximately the same extent as LH, but the effect of PMA on the newly synthesized proteins was much less pronounced than the effect of LH. These results indicate that patterns of protein synthesis are more sensitive to detecting actions of extracellular stimuli than protein phosphorylation patterns. Enzymatic amplification steps following protein phosphorylation could contribute to this.

PL-C has been used also in other studies on regulation of steroidogenesis. PL-C had a strong synergistic effect on ACTH-stimulated cortisol production in isolated bovine adrenocortical cells [17], but inhibitory effects on Leydig cells have been reported also [21]. An explanation for these different effects of PL-C may be that these authors used a PL-C from another microbial source (*Bacillus cereus*). Since it has been shown in murine epidermal cells and in GH_4C_1 pituitary cells, that PL-C from *B. cereus* had very small effects compared to PL-C from *C. perfringens*, the

enzymes may have a different substrate specificity [18,22]. These results indicate that specific phospholipids in the cell membrane may be involved in the control of specific protein kinases. Similar specific phospholipids may be hydrolyzed after activation of the cells by LHRH-A or PL-C resulting in activation of a different type of protein kinase, which is not activated by PMA [23]. Alternatively, PL-C may cause changes in membrane fluidity, which have been shown to influence the number of LH receptors and their coupling to the adenylate cyclase system [24]. Arachidonic acid metabolites, which have been implicated to play a role in the regulation of steroid production [4], are not likely to be involved because phospholipase A_2 did not have any effect.

In conclusion, the present results show that the mechanisms of action of PMA and PL-C on Leydig cell steroidogenesis are clearly different. The similarities between the effects of LHRH-A and PL-C suggest that one primary effect of LHRH on Leydig cells may be the stimulation of hydrolysis of specific phospholipids. It appears therefore important to elucidate further the exact molecular mechanism of action of PL-C on these cells.

REFERENCES

[1] Bakker, G.H., Hoogerbrugge, J.W., Rommerts, F.F.G. and Van der Molen, H.J. (1983) Mol. Cell. Endocrinol. 33, 243–253.
[2] Cooke, B.A., Lindh, L.M. and Janszen, F.H.A. (1976) Biochem. J. 160, 439–446.
[3] Podesta, E., Dufau, M.L., Solano, A.R. and Catt, K.J. (1978) J. Biol. Chem. 253, 8994–9001.
[4] Cooke, B.A. and Sullivan, M.H.F. (1985) Med. Biol. 63, 259–263.
[5] Lowitt, S., Farese, R.F., Sabir, M.A. and Root, A.W. (1982) Endocrinology 111, 1415–1417.
[6] Hsueh, A.J.W. and Jones, P.C.B. (1982) Endocr. Rev. 2, 437–461.
[7] Cooke, B.A. and Sullivan, M.H.F. (1985) Mol. Cell. Endocrinol. 41, 115–122.
[8] Lin, T. (1984) J. Androl. 5, 193–200.
[9] Themmen, A.P.N., Hoogerbrugge, J.W., Rommerts, F.F.G. and Van der Molen, H.J. (1986) J. Endocrinol. 108, 431–440.
[10] Molcho, J., Zakut, H. and Naor, Z. (1984) Endocrinology 114, 1048–1050.
[11] Nishizuka, Y. (1984) Nature 308, 693–698.
[12] Rommerts, F.F.G., Molenaar, R. and Van der Molen, H.J. (1985) Methods Enzymol. 109, 275–288.
[13] Van der Vusse, G.J., Kalkman, M.L. and Van der Molen, H.J. (1974) Biochim. Biophys. Acta 348, 404–414.
[14] Van der Vusse, G.J., Kalkman, M.L. and Van der Molen, H.J. (1975) Biochim. Biophys. Acta 380, 473–485.
[15] Bakker, G.H., Hoogerbrugge, J.W., Rommerts, F.F.G. and Van der Molen, H.J. (1981) Biochem. J. 198, 339–346.
[16] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[17] Hadjian, A.J., Culty, M. and Chambaz, E.M. (1984) Biochem. Biophys. Res. Commun. 123, 33–40.
[18] Jaken, S. (1985) Endocrinology 117, 2301–2306.
[19] Rozengurt, E., Rodriguez-Pena, M. and Smith, K.A. (1983) Proc. Natl. Acad. Sci. USA 80, 7244–7248.
[20] Mukhopadhyay, A.K. and Schumacher, M. (1985) FEBS Lett. 187, 56–60.
[21] Papadopoulos, V., Carreau, S. and Drosdowsky, M.A. (1985) FEBS Lett. 188, 312–316.
[22] Fischer, S.M., Baldwin, J.K. and Adams, L.M. (1985) Biochem. Biophys. Res. Commun. 131, 1103–1108.
[23] Kiss, Z. and Luo, Y. (1986) FEBS Lett. 198, 203–207.
[24] Kolena, J., Blažíček, P., Horkovics-Kováts, Š., Ondriaš, K. and Šeböková, E. (1986) Mol. Cell. Endocrinol. 44, 69–76.