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Evidence for two independent pathways in the stimulation of steroidogenesis by luteinizing hormone involving chloride channels and cyclic AMP

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The possible role of chloride channels in luteinizing hormone (LH) action on steroidogenesis in rat Leydig cells had been investigated. A chloride channel blocker, SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid), inhibited LH-stimulated steroidogenesis at low (≤ 1 ng/ml), but not at high (100 ng/ml) LH concentrations. In addition, dibutyryl cyclic AMP- and forskolin-stimulated steroidogenesis was unaffected by SITS. The removal of extracellular chloride potentiated steroidogenesis stimulated by submaximal but not maximal doses of LH. These results suggest that at low levels of LH, steroidogenesis depends on chloride channels whereas with high levels, cyclic AMP is the mediator of LH action.

Luteinizing hormone; Cyclic AMP; Chloride channel; Calcium; Leydig cell; Steroidogenesis

1. INTRODUCTION

It is generally accepted that steroidogenesis in Leydig cells is regulated by luteinizing hormone (lutropin, LH) via the second messenger cyclic AMP. Cyclic AMP analogues and agents such as forskolin and cholera toxin in which increase cyclic AMP levels, will also stimulate steroidogenesis. However, it has been known for a long time that LH will stimulate near-maximal steroidogenesis before cyclic AMP production is detectable [1–3]. This suggests that other mechanisms in addition to cyclic AMP may be operating in the Leydig cell.

Our present work has focused on the involvement of chloride channels [4] in the regulation of steroidogenesis in rat Leydig cells. We have used a chloride channel inhibitor 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS) [5], and the removal of extracellular chloride by equimolar replacement with gluconate [6] in our investigations.

2. MATERIALS AND METHODS

Adult male Sprague-Dawley rats (230–250 g) were used in all experiments. Leydig cells were prepared according to an established protocol of collagenase dispersion, centrifugal elutriation and percoll density gradient centrifugation [7]. The Leydig cells (>95% pure as established by 3β-hydroxysteroid dehydrogenase cytochemistry [8]) were preincubated in DMEM (Gibco)-0.1% BSA (bovine serum albumin, Sigma), pH 7.4, in mini-culture wells (20000 cells/well) at 34°C for 2 h prior to the addition of agonists and antagonists.

The addition of SITS (Sigma), forskolin (Sigma) and the removal of extracellular chloride was achieved by the complete change of the preincubation medium to a medium containing additions or changes as indicated in the figure legends. LH (NIAMDD-oLH-22, 2.3 IU/mg, Bethesda, MD, USA) and dibutyryl cyclic AMP (Sigma) were dissolved in incubation medium and added as a 10 μl concentrate to give the required final concentration. Incubations were stopped with $HClO_4$ and stored frozen at −20°C. Samples were thawed and neutralized with K_3PO_4 prior to assay for testosterone and cyclic AMP by radioimmunoassay [9,10]. All data are expressed as the mean ± SD of triplicate incubations, normalized to 10^6 cells/incubation. Student's *t*-test was used for statistical analysis.

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3. RESULTS

Fig.1 shows the dissociation between testosterone and cyclic AMP production; from 0.01 to 0.1 ng/ml LH, testosterone production increased to half-maximal levels without there being any detectable cyclic AMP. From 0.5 to 1 ng/ml LH, there was an approximate doubling of the cyclic AMP production with no further change in testosterone synthesis. The large increase in levels of cyclic AMP with 10 and 100 ng/ml LH did not increase further testosterone production. SITS inhibited steroidogenesis at doses of LH $\leq$ 1 ng/ml, but had no effect at 100 ng/ml LH (fig.2). The basal testosterone production was decreased from 8.87 ± 0.50 to 6.08 ± 0.37 ng/10^6 cells per 2 h ($P < 0.05$) with 200 μM SITS. With 0.01 ng/ml LH, testosterone production decreased from 11.19 ± 0.67 to 7.20 ± 0.40 ng/10^6 cells per 2 h ($P < 0.01$) with 200 μM SITS. The ID_{50} for SITS with 1.0 ng/ml LH was 83 μM.

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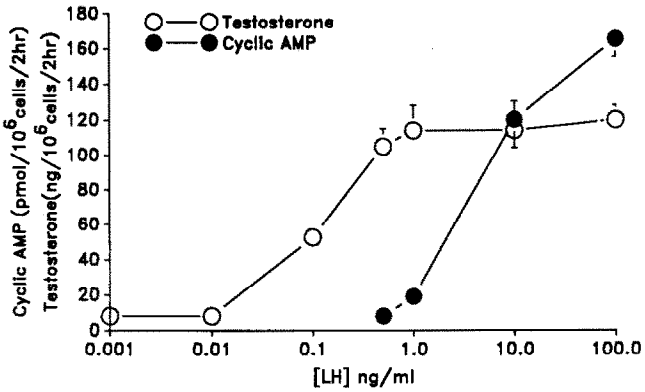

Fig.1. Dose–response curves for LH-stimulated testosterone and cyclic AMP production in rat Leydig cells. Leydig cells were incubated in DMEM-0.1% BSA, pH 7.4, at 34°C; LH was added for 2 h after a 2-h preincubation. Testosterone and cyclic AMP was determined as described in section 2. This result was typical of at least two similar experiments and the data points are the means ± SD for triplicate incubations. Where the SD values are not shown, these are smaller than the respective symbols.

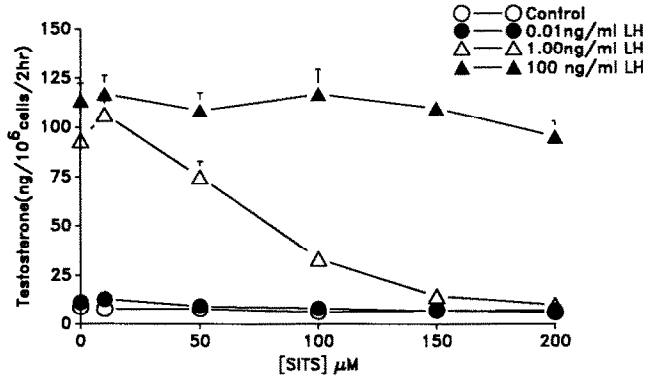

Fig.2. Effect of different concentrations of SITS on LH-stimulated testosterone production. Purified Leydig cells were preincubated for 2 h, after which the preincubation medium was removed and replaced with DMEM-0.1% BSA, pH 7.4, containing the appropriate concentration of SITS. LH was added for 2 h and testosterone was assayed as described in section 2. All data points are the means ± SD for triplicate incubations, and are representative of two similar experiments. For data points where the SD values are not shown, these are smaller than the respective symbols.

Table 1

The effect of SITS on testosterone production stimulated by dibutyryl cyclic AMP and forskolin

SITS (μM)	Testosterone production (ng/10⁶ cells per 2 h)			
	Dibutyryl-cAMP (1 mM)	Forskolin (1 μM)	Forskolin (5 μM)	Forskolin (10 μM)
0	113.1 ± 5.5	23.6 ± 3.0	70.1 ± 7.9	77.5 ± 9.8
1.0	114.4 ± 0.5	28.9 ± 6.3	75.5 ± 15.3	74.7 ± 5.5
10.0	114.3 ± 1.0	25.4 ± 3.7	87.2 ± 6.1	78.3 ± 1.4
50.0	115.8 ± 19.2	24.7 ± 2.9	74.2 ± 5.4	82.9 ± 9.0
100.0	119.3 ± 10.5	24.9 ± 1.3	72.9 ± 9.3	88.8 ± 2.3
150.0	122.7 ± 6.3	26.8 ± 6.4	86.2 ± 1.9	84.9 ± 4.2
200.0	127.7 ± 5.2	27.8 ± 6.8	76.8 ± 7.5	91.9 ± 8.5

SITS was added as described in fig.2. Dibutyryl cyclic AMP and forskolin were added for 2 h. Testosterone was determined according to section 2 and the values are means ± SD for triplicate incubations

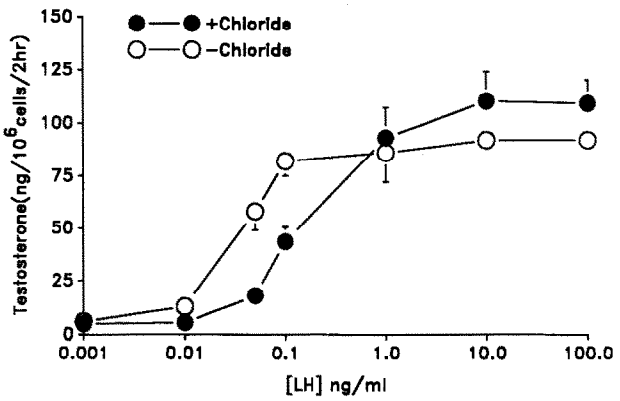

Fig.3. Effect of chloride removal on the dose–response curve for LH-stimulated testosterone production. Purified Leydig cells were preincubated for 2 h, after which the preincubation medium was removed and replaced by a simple salts medium containing glucose. The incubation solution consisted of (in mM): 140 NaCl, 5 KCl, 1.8 Ca-acetate, 1 $MgSO_4$, 10 Hepes-NaOH, 10 glucose, pH 7.4, 0.1% BSA. For the removal of chloride, the chloride salts were replaced with equimolar concentrations of the appropriate gluconate salts. LH was added for 2 h and testosterone was assayed as described in section 2. All data points are the means ± SD for triplicate incubations. Where SD values are not shown, these are smaller than the respective symbols.

Dibutyryl cyclic AMP- (1 mM) and forskolin- (1, 5 and 10 μM) stimulated testosterone production were not inhibited by SITS (table 1).

In order to investigate further the role of chloride in Leydig cell steroidogenesis, the extracellular chloride was replaced with equimolar gluconate. Fig.3 shows that testosterone production stimulated by low concentrations of LH (0.01, 0.05 and 0.1 ng/ml) was potentiated by approximately 2–3-fold by chloride removal, but maximum testosterone production was unaffected.

4. DISCUSSION

The original observations on the marked dissociation of the testosterone and cyclic AMP dose–response curves to LH [1–3] were made with relatively insensitive methodology. Since that time, the sensitivity of the cyclic AMP assay has increased by several orders of magnitude, and highly hormone-sensitive cell preparations have been developed. Using the latter, in the present study virtually the same results were obtained, indicating that a cyclic AMP-independent pathway is operating at low levels of LH. Our results using a chloride channel blocker and by excluding chloride, strongly indicate that this other pathway involves chloride channels.

From patch clamp experiments, it has been reported that an increase in the intracellular Ca^{2+} (from 10^{-7} to 10^{-6} M) activates an outward chloride current in rat Leydig cells [4]. Our results demonstrate that the inhibition of chloride channels using SITS inhibited steroidogenesis in rat Leydig cells. However, this in-

hibition was seen only at LH concentrations ≤1 ng/ml and not at 100 ng/ml. More importantly, this inhibition was not seen when cyclic AMP was the stimulating agent, such as with 1 mM dibutyryl cyclic AMP and forskolin (1, 5 and 10 μM). Even at concentrations of forskolin where steroidogenesis was submaximal, SITS had no effect. These results suggest that chloride currents are important for the stimulation of steroidogenesis, at concentrations of LH that stimulate little or no cyclic AMP synthesis. Furthermore, this effect of LH is independent of cyclic AMP, because in the presence of dibutyryl cyclic AMP and forskolin and at a high concentration of LH (100 ng/ml) where there is a large increase in cyclic AMP synthesis, no SITS effect was found.

The potentiation of submaximal LH-stimulated testosterone production by chloride exclusion, would argue for a stimulatory role for an outward chloride current. Submaximal levels of LH may stimulate an outward chloride current via an increase in the intracellular Ca^{2+} [11]. However, it is not known how this plays a role in the regulation of steroidogenesis. One possibility is that the chloride channels are coupled to an enzyme at the plasma membrane, which, when activated/inactivated will stimulate steroidogenesis.

In conclusion, these results provide strong evidence for two pathways in the regulation of steroidogenesis in rat Leydig cells. The primary pathway which may operate at physiological levels of LH (≤1 ng/ml) and be independent of cyclic AMP, involves chloride channels. Another pathway which is cyclic AMP-dependent becomes fully operational at concentrations of LH where the stimulated cyclic AMP levels are sufficiently high, e.g. with 100 ng/ml.

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REFERENCES

[1] Catt, K.J. and Dufau, M.L. (1973) Nature New Biology 244, 219–221.
[2] Moyle, W.R. and Ramachandran, J. (1973) Endocrinology 93, 127–134.
[3] Rommerts, F.F.G., Cooke, B.A., Van der Kemp, J.C.M. and Van der Molen, H.J. (1973) FEBS Lett. 33, 114–118.
[4] Duchatelle, P. and Joffre, M. (1987) FEBS Lett. 217, 11–15.
[5] Bader, C.R., Bertrand, D. and Schlichter, R. (1987) J. Physiol. 394, 125–148.
[6] Tolkovsky, A.M. and Richards, C.D. (1987) Neuroscience 22, 1093–1102.
[7] Platts, E.A., Schulster, D. and Cooke, B.A. (1988) Biochem. J. 253, 895–899.
[8] Cooke, B.A., Aldred, L.F., Hunter, M.G., Sullivan, M.H.F. and Dix, C.J. (1983) J. Steroid Biochem. 19, 359–366.
[9] Verjans, M.L., Cooke, B.A., De Jong, F.H., De Jong, C.M.M. and Van der Molen, H.J. (1973) J. Steroid Biochem. 4, 665–676.
[10] Harper, T.F. and Brooker, G. (1975) J. Cycl. Nucleotide Res. 1, 207–218.
[11] Sullivan, M.H.F. and Cooke, B.A. (1986) Biochem. J. 236, 45–51.