Calmidazolium is a potent stimulator of steroidogenesis via mechanisms not involving cyclic AMP, calcium or protein synthesis

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This study reports an unexpected effect of calmidazolium on steroidogenesis. In contrast with previous work, which established that calmidazolium inhibits hormone-stimulated testosterone production in rat Leydig cells, the present study demonstrates that this compound is a potent stimulator of steroidogenesis when added by itself; this stimulation (approx. 10-fold in a 2 h incubation), was obtained over a narrow dose range (e.g. 1–10 μM) in mouse and rat Leydig cells and in rat adrenocortical cells. The same concentrations of calmidazolium decreased basal cyclic AMP to undetectable levels in rat Leydig cells. Also, cyclic AMP stimulated with luteinizing hormone (LH), cholera toxin and forskolin was inhibited by calmidazolium (ED_{50} 2 μM). In contrast with the actions of LH and cyclic AMP analogues on steroidogenesis, the effect of calmidazolium was not inhibited by removal of extracellular Ca^{2+} , or by the addition of La^{3+} (a Ca^{2+} -entry blocker), or the addition of cycloheximide (an inhibitor of protein translation). However, like dibutyl cyclic AMP, calmidazolium-stimulated steroidogenesis was inhibited by aminoglutethimide, an inhibitor of cholesterol side-chain cleavage. Another calmodulin inhibitor, trifluoperazine, did not stimulate steroidogenesis. It is concluded that calmidazolium has a similar effect on steroidogenesis to LH, but by-passes the requirements for cyclic AMP, Ca^{2+} , and protein synthesis. Calmidazolium is therefore a potentially important probe for elucidating the mechanisms of control of steroidogenesis.

INTRODUCTION

Calmodulin is a ubiquitous Ca^{2+} -binding protein that is involved in the regulation of a wide spectrum of cellular processes. It is the major sensor for the translation of intracellular Ca^{2+} changes into a biochemical signal essential to the regulation of many target enzymes (Cheung, 1980; Means *et al.*, 1982; Klee & Vanaman, 1982). In order to elucidate the mechanisms of calmodulin action, wide use of calmodulin antagonists has been made (Roufogalis, 1985). One, which has been frequently used and which binds with high affinity to calmodulin (Van Belle, 1981; Sobieszek, 1989), is calmidazolium {compound R 24571, 1-[bis-(*p*-chlorophenyl)methyl]-3-[2,4-dichloro- β -(2,4-dichlorobenzyloxy)phenethyl]imidazolium chloride}. We have previously shown that several calmodulin antagonists, including calmidazolium, inhibit luteinizing-hormone (LH)-stimulated cyclic AMP production and LH- and LH-releasing-hormone (LHRH)-stimulated testosterone production in rat Leydig cells (Sullivan & Cooke, 1985). These and other results indicate that Ca^{2+} and calmodulin have a positive role in the action of LH. Further experiments were carried out to investigate the effect of calmidazolium on steroidogenesis in rat Leydig cells, with the aim of continuing previous work on the role of calmodulin in steroidogenesis. However, in these investigations we unexpectedly found that calmidazolium, by itself, has a marked stimulatory effect on steroidogenesis. We have therefore investigated the mechanism of action of calmidazolium on steroidogenesis and compared it with LH and cyclic AMP analogues. The results obtained indicate that it may share a common site of action with LH, but by-passes the steps requiring cyclic AMP, Ca^{2+} , and protein synthesis. Furthermore, calmidazolium also stimulated steroid production in other steroidogenic cells. This suggests that

calmidazolium may stimulate processes involved in steroid production, which are common to all steroidogenic cells. A preliminary account of this work has been published in abstract form (Choi & Cooke, 1989).

MATERIALS AND METHODS

Materials

Mature male Sprague–Dawley rats (250 g) and mature BALBc mice were used in these investigations. Mini 96-well culture plates, Ham's F12 nutrient mixture and Dulbecco's modified Eagle's minimum essential medium, without NaHCO_3 (DMEM), were purchased from Gibco, Paisley, Scotland, U.K. HEPES, dibutyl cyclic AMP (dbcAMP), BSA (fraction V), Percoll, trypsin inhibitor (sterile filtered 1% solution), aminoglutethimide, calmidazolium and trifluoperazine were purchased from Sigma Chemical Co. EGTA (Pures grade) was obtained from Fluka Biochemicals. Ovine LH (oLH-22, 2.3 i.u./mg) was a gift from NIAMDD, Bethesda, MD, U.S.A. Type I collagenase was purchased from Worthington Biochemicals. Stocks of aminoglutethimide, calmidazolium and trifluoperazine were made in dimethyl sulphoxide and stored at -20°C .

Cell isolation

Rat Leydig cells were prepared by an established protocol of collagenase dispersion, centrifugal elutriation and Percoll-gradient (0–90%, v/v) centrifugation (Platts *et al.*, 1988) with the following modifications. The collagenase dispersion was carried out in DMEM/0.1% BSA, pH 7.4, containing 0.5 mg of collagenase and 20 μl of trypsin inhibitor/ml; each pair of decapsulated testes was placed in 7 ml of this solution in a 30 ml universal container.

Abbreviations used: calmidazolium, 1-[bis-(*p*-chlorophenyl)methyl]-3-[2,4-dichloro- β -(2,4-dichlorobenzyloxy)phenethyl]imidazolium chloride; LH, luteinizing hormone; LHRH, luteinizing hormone-releasing hormone; dbcAMP, dibutyl cyclic AMP (*N*⁶, *O*-dibutyladenosine 3':5'-cyclic monophosphate); DMEM, Dulbecco's modified Eagle's minimum essential medium.

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The universal container was then subjected to longitudinal shaking in a heated water bath. Previously, the testes were shaken at 80 strokes/min for 20 min at 37 °C. This has now been changed to 65 strokes/minute, and the incubations are continued until no interstitial connective tissue is visible. The digestion time for testes from 250 g rats is now approx. 42 min. This modified procedure has increased the yield of Leydig cells from 1×10^6 to over 4×10^6 cells per rat, without affecting the purity. After collagenase dispersion, the cells were filtered through nylon gauze of mesh size of 75 μm to remove fragments of seminiferous tubules. The cells from five animals were combined and subjected to centrifugal elutriation followed by Percoll-density-gradient centrifugation. Leydig-cell purity was routinely > 95% as established by 3 β -hydroxysteroid dehydrogenase cytochemistry (Cooke *et al.*, 1983).

Mouse Leydig cells were prepared as described previously (Hunter *et al.*, 1982). Briefly, the testes were decapsulated and dispersed by mechanical flushing, and the released cells were filtered through nylon gauze and then purified by Percoll-gradient (0–90%) centrifugation.

Rat adrenocortical cells were isolated by an established method with some minor modification (Haning *et al.*, 1970; Fattah *et al.*, 1977; Kramer, 1988). In brief, rat adrenal glands were added to ice-cold Ham's F12/15 mM-Hepes/NaOH, pH 7.4. Adhering fat was removed, and they were then ruptured by placing between two clean ice-cold glass plates. The medullae were carefully removed, and the remaining capsules and cortical cells were subjected to collagenase digestion with 1 mg of collagenase/ml of Ham's F12/2% BSA/15 mM-Hepes/NaOH, pH 7.4. The resulting cell suspension was filtered through nylon gauze to remove capsular material, and the suspended cells were pelleted in a bench centrifuge. The contaminating red cells were removed by Percoll-gradient centrifugation.

Incubation medium

For normal incubations of Leydig cells, DMEM containing 0.1% BSA, and 10 mM-Hepes/NaOH, pH 7.4, was used. In experiments involving the addition of La^{3+} and exclusion of extracellular Ca^{2+} , a simple salts medium was used consisting of (mM): 140 NaCl, 5 KCl, 1.8 CaCl_2 , 1 MgSO_4 , 10 glucose, 10 Hepes/NaOH, pH 7.4, plus 0.1% BSA. For the removal of Ca^{2+} , the CaCl_2 was replaced with 0.2 mM-EGTA to chelate any remaining Ca^{2+} . DMEM could not be used for experiments involving the addition of La^{3+} to the incubation medium, since La^{3+} forms insoluble precipitates with phosphates. Incubations of adrenocortical cells were performed in Ham's F12/0.2% BSA/15 mM-Hepes/NaOH, pH 7.4. In order to measure pregnenolone production, its further metabolism was blocked by addition of cyanoketone (5 μM) and SU10603 (20 μM) (inhibitors of 3 β -hydroxysteroid dehydrogenase and 17 α -hydroxylase respectively) to the incubation medium 30 min before stimulation of the cells.

Cell culture

Cells were plated at a density of 20000 cells/well in a final volume of 200 μl . Incubations were in an air incubator, at 34 °C for Leydig cells and 37 °C for adrenocortical cells. Leydig cells were preincubated for 2 h before addition of agonists and antagonists. This preincubation period has been shown to improve their responsiveness (Cooke *et al.*, 1977). The additions of calmidazolium, trifluoperazine and aminoglutethimide to Leydig cells were by the complete change of the incubation medium to a medium containing the appropriate concentrations as indicated in the Figures. Calmidazolium was added to adrenocortical cells in 100 μl of medium containing twice the final concentration of calmidazolium to 100 μl of medium and

cells within each well. The final concentration of dimethyl sulphoxide was constant for all the wells within each experiment and did not exceed 0.5% (v/v). LH and cyclic AMP analogues were dissolved in incubation medium and added as a 10 μl concentrate to give the required final concentration. Incubations were stopped with HClO_4 2 h after stimulation and stored at –20 °C. Samples were thawed and neutralized with K_3PO_4 before assay for testosterone, pregnenolone and cyclic AMP by radioimmunoassay (Steiner *et al.*, 1972; Verjans *et al.*, 1973; van der Vusse *et al.*, 1975; Harper & Brooker, 1975). All data are expressed as means $\pm$ s.d. of triplicate incubations, normalized to 10^6 cells/incubation. Student's *t* test was used for statistical analysis.

RESULTS

The present investigations have focused on the stimulatory effect of calmidazolium on steroidogenesis in rat Leydig cells. Table 1 shows the effect of calmidazolium (0–20 μM) on basal testosterone production. Calmidazolium caused a dose-dependent stimulation of testosterone production, which reached a maximum with approx. 5–10 μM and then declined to basal levels with 20 μM . When rat Leydig cells were stimulated with the same concentrations of calmidazolium, in the presence of LH (100 ng/ml), there was no additive stimulation of testosterone production, and the same decline to basal levels was obtained (results not shown). At these concentrations of calmidazolium there was no change in cell viability, as determined by a NADH-exclusion assay (Aldred & Cooke, 1983).

Fig. 1 shows the stimulation of steroidogenesis in rat Leydig cells. In all steroidogenic cells the initial site of regulation of steroidogenesis is the conversion of cholesterol into pregnenolone. The effect of calmidazolium on pregnenolone and testosterone production was compared with that produced with LH. Over the concentration range 1–10 μM , calmidazolium dose-dependently stimulated pregnenolone with a maximum of approx. 13-fold above basal (Fig. 1a). The same concentrations of calmidazolium stimulated basal testosterone by approx. 10-fold. In the same experiment stimulation with LH gave the expected dose-dependent increase in pregnenolone and testosterone production. With maximum stimulating concentrations of LH (1, 10 and 100 ng/ml), pregnenolone and testosterone production were increased by approx. 20-fold and 15-fold respectively (Fig. 1b).

These results show that the capacity of calmidazolium to stimulate steroidogenesis is similar to that of LH. This effect of calmidazolium was found not to be exclusive to rat Leydig cells, because this compound also stimulated steroidogenesis in mouse Leydig cells and rat adrenocortical cells (Tables 1 and 2); in both

Table 1. Stimulation of testosterone production in rat and mouse Leydig cells by calmidazolium

Incubations and assays for testosterone production were carried out as described in the Materials and methods section. The values for testosterone production are means $\pm$ s.d. for triplicate incubations.

[Calmidazolium] (μM)	Testosterone production (ng/2 h per 10^6 cells)	
	Rat	Mouse
0	10.7 $\pm$ 0.8	24.5 $\pm$ 4.6
5.0	128.3 $\pm$ 1.9	86.2 $\pm$ 5.0
10.0	124.8 $\pm$ 1.0	239.1 $\pm$ 38.9
15.0	27.4 $\pm$ 2.8	–
20.0	13.3 $\pm$ 0.9	–

Fig. 1. Comparison between LH- and calmidazolium-stimulated pregnenolone and testosterone production in rat Leydig cells

(a) and (b) are dose-response curves to calmidazolium and LH respectively. Incubation and assays for testosterone (○) and pregnenolone (●) production were carried out as described in the Materials and methods section. All data points are means $\pm$ S.D. for triplicate incubations from the same experiment. Where S.D. values are not shown, these are smaller than the respective symbols.

Table 2. Stimulation of pregnenolone production in rat adrenocortical cells by calmidazolium

Incubations and assays for pregnenolone production were carried out as described in the Materials and methods section. The values for pregnenolone production are means $\pm$ S.D. for triplicate incubations.

[Calmidazolium] (μM)	Pregnenolone production (ng/2 h per 10 ⁶ cells)
0	34.1 $\pm$ 7.7
2.0	42.2 $\pm$ 5.7
4.0	91.8 $\pm$ 20.3
6.0	210.7 $\pm$ 17.3
10.0	340.9 $\pm$ 37.2

these cell types a maximum increase in steroidogenesis of 10-fold was obtained.

Fig. 2 shows the time course of pregnenolone and testosterone production in rat Leydig cells stimulated with calmidazolium (5 μM). There was a lag phase of approx. 10 or 20 min before an increase in pregnenolone or testosterone was observed, respectively. The earlier production of pregnenolone is consistent with the steroidogenic pathway, since it is a precursor of testosterone.

The conversion of cholesterol into pregnenolone can be prevented by inhibitors of the cholesterol side-chain-cleavage enzyme. Fig. 3 shows the effect of a commonly used inhibitor of this enzyme, aminoglutethimide, on testosterone production.

Fig. 2. Time course of pregnenolone and testosterone production in rat Leydig cells

Purified Leydig cells were stimulated with 5 μM-calmidazolium. Separate incubations were used for the determination of pregnenolone and testosterone. Incubations and assays for steroid production were carried out as described in the Materials and methods section. All data points are means $\pm$ S.D. for triplicate incubations from one of two similar experiments. Where S.D. values are not shown, these are smaller than the respective symbols.

Fig. 3. Effect of aminoglutethimide on basal, dbcAMP- and calmidazolium-stimulated testosterone production in rat Leydig cells

Purified Leydig cells were incubated with different concentrations of aminoglutethimide, an inhibitor of cholesterol side-chain cleavage. The cells were stimulated with dbcAMP or calmidazolium for 2 h. Incubations and assays for steroid production were carried out as described in the Materials and methods section. All data points are means $\pm$ S.D. for triplicate incubations from one of two similar experiments. Where S.D. values are not shown, these are smaller than the respective symbols. Symbols: ●, basal; △, +1 mM-dbcAMP; ▲, +5 μM-cadmidazolium.

Both dbcAMP (1 mM)- and calmidazolium (5 μM)-stimulated testosterone production were inhibited by aminoglutethimide, with an ED₅₀ of approx. 15 μM.

The effect of calmidazolium on cyclic AMP production was investigated. It inhibited cyclic AMP production with an ED₅₀ of approx. 2 μM, and similar results were obtained with LH, cholera toxin and forskolin stimulation (Table 3). At concentrations of calmidazolium which gave maximum stimulation of steroidogenesis, the basal cyclic AMP was undetectable.

The stimulation of testosterone production by LH and cyclic AMP analogues is acutely sensitive to the removal of extracellular Ca²⁺, and to the addition of protein-synthesis inhibitors to the incubation medium. The effect of these conditions on calmidazolium-stimulated testosterone production was investigated, with cells stimulated with LH and dbcAMP under the same conditions as for controls. As expected, the removal of extracellular Ca²⁺ caused a 50% decrease in LH (100 ng/ml)- and dbcAMP (1 mM)-stimulated testosterone pro-

Table 3. Effect of calmidazolium on basal, LH, cholera-toxin- and forskolin-stimulated cyclic AMP production in rat Leydig cells

Purified rat Leydig cells were incubated for 4 h in medium containing the above additions. The accumulated cyclic AMP was assayed by radioimmunoassay as described in the Materials and methods section. The values are means $\pm$ S.D. for triplicate incubations; N.D., not detectable.

[Calmidazolium] (μ M)...	Cyclic AMP (pmol/4 h per 10^6 cells)		
	0	2	10
Basal	13.8 $\pm$ 3.7	1.4 $\pm$ 0.2	N.D.
LH (100 ng/ml)	311.0 $\pm$ 60.0	145.9 $\pm$ 28.2	N.D.
Cholera toxin (2.5 μ g/ml)	79.8 $\pm$ 7.4	32.6 $\pm$ 0.2	8.0 $\pm$ 0.4
Forskolin (10 μ M)	230.1 $\pm$ 24.6	102.0 $\pm$ 12.1	6.5 $\pm$ 0.7

duction (Table 4). However, under the same conditions calmidazolium (5 μ M)-stimulated testosterone production was stimulated by approx. 35%. In further experiments the addition of La^{3+} to the incubation medium was investigated. La^{3+} was added because it has been reported to inhibit the entry of extracellular Ca^{2+} through the plasma membrane and would therefore be expected to have similar effects to the removal of Ca^{2+} (Weiss, 1974). Consistent with this, LH (100 ng/ml)-stimulated testosterone production was inhibited by La^{3+} (250 μ M). Calmidazolium-stimulated testosterone production was increased by addition of La^{3+} (250 μ M) to the incubation medium (Table 4).

Cycloheximide, an inhibitor of protein translation, was used to determine if the stimulatory action of calmidazolium required protein synthesis (Table 5). Testosterone production stimulated with LH (100 ng/ml) and dbcAMP (1 mM) was inhibited by cycloheximide (0.1 mM) to basal levels. In contrast, the stimulation of testosterone with calmidazolium (5 μ M) was unaffected by cycloheximide, even at 1 mM (results not shown).

In order to determine whether the observed effects of calmidazolium were a common property of calmodulin antagonists, the effect of trifluoperazine on basal and LH-stimulated testosterone production was investigated (Fig. 4). In agreement with previous findings (Sullivan & Cooke, 1985), it was found that trifluoperazine (1–20 μ M) dose-dependently inhibited LH-stimulated testosterone production, with an ED_{50} of approx. 6 μ M. However, no stimulation of basal testosterone production was observed.

Table 5. Effect of cycloheximide on LH-, dbcAMP- and calmidazolium-stimulated testosterone production in rat Leydig cells

Purified Leydig cells were preincubated with cycloheximide for 30 min and then stimulated with LH, dbcAMP and calmidazolium for a further 2 h. Assays for testosterone production were carried out as described in the Materials and methods section. All data are means $\pm$ S.D. for triplicate incubations for one of two similar experiments.

	Testosterone (ng/2 h per 10^6 cells)	
	Control	Cycloheximide (0.1 mM)
Basal	15.7 $\pm$ 5.4	12.1 $\pm$ 1.6
LH (100 ng/ml)	125.5 $\pm$ 16.1	14.4 $\pm$ 2.7
dbcAMP (1 mM)	118.7 $\pm$ 9.1	12.0 $\pm$ 2.6
Calmidazolium (5 μ M)	91.9 $\pm$ 2.5	87.3 $\pm$ 5.8

Fig. 4. Effect of trifluoperazine on basal and LH-stimulated testosterone production in rat Leydig cells

Purified Leydig cells were incubated with different concentrations of trifluoperazine and then stimulated with either medium (○, control) or LH (100 ng/ml; ●) for 2 h. Testosterone was assayed as described in the Materials and methods section. Each data point is the mean $\pm$ S.D. for triplicate incubations from one of two similar experiments. Where no S.D. values are shown, these are smaller than the respective symbols.

DISCUSSION

The results obtained clearly demonstrate that calmidazolium exhibits a dose-dependent stimulatory and inhibitory effect on steroidogenesis. In previously published results (Sullivan &

Table 4. Effects of removal of extracellular Ca^{2+} and of added La^{3+} on testosterone production in rat Leydig cells

Purified rat Leydig cells were incubated in a simple salts medium. The Ca^{2+} -free medium contained 0.2 mM-EGTA and no added Ca^{2+} ; La^{3+} was added in the presence of normal Ca^{2+} concn. (1.8 mM). Assays for testosterone production were carried out as described in the Materials and methods section. All data are means $\pm$ S.D. for triplicate incubations from two separate experiments: * P < 0.01 and ** P < 0.001 for significant differences compared with the control.

	Testosterone (ng/2 h per 10^6 cells)			
	Control	Ca^{2+} -free	Control	250 μ M- La^{3+}
Basal	11.7 $\pm$ 1.0	10.7 $\pm$ 0.8	19.0 $\pm$ 0.8	24.6 $\pm$ 2.5
LH (100 ng/ml)	151.5 $\pm$ 5.2	73.8 $\pm$ 11.7**	168.2 $\pm$ 13.0	104.5 $\pm$ 8.5**
dbcAMP (1 mM)	123.9 $\pm$ 16.3	61.1 $\pm$ 6.8**	—	—
Calmidazolium (5 μ M)	79.7 $\pm$ 5.4	110.3 $\pm$ 8.7*	82.5 $\pm$ 7.5	111.3 $\pm$ 8.6*

Cooke, 1985), inhibition was obtained with nanomolar concentrations of calmidazolium. However, in the present study the inhibition of testosterone production was obtained with concentrations of calmidazolium $> 10 \mu\text{M}$. This micromolar concentration of calmidazolium is similar to that used for the inhibition of steroidogenesis in adrenal and granulosa cells (Tsang & Carnegie, 1983; Papadopoulos *et al.*, 1990). The inhibitory effects on LH-stimulated steroidogenesis support the conclusions of other workers (Hall *et al.*, 1981; Tsang & Carnegie, 1983; Sullivan & Cooke, 1985), i.e. that steroidogenesis is a calmodulin-dependent process. However, there have not been any previous reports showing a stimulatory effect of calmidazolium on steroid production.

Previous studies have shown that the omission of Ca^{2+} from rat Leydig-cell incubations resulted in marked decreases in testosterone production, but did not inhibit the LH-stimulated cyclic-AMP-dependent protein kinase activity (Janszen *et al.*, 1976). In support of this, the present study also shows that the exclusion of Ca^{2+} inhibited both the LH- and dbcAMP-stimulated testosterone production. This requirement for extracellular Ca^{2+} is further illustrated by the inhibition of testosterone production with LaCl_3 . La^{3+} inhibits the influx of Ca^{2+} into cells (Weiss, 1974), and has been shown to inhibit production of both cyclic AMP and steroids in adrenocortical cells and granulosa cells (Haksar *et al.*, 1976; Tsang & Carnegie, 1983). In contrast, testosterone production stimulated by calmidazolium was not inhibited by removal of extracellular Ca^{2+} or by the addition of La^{3+} to the incubation medium. This argues against a role for Ca^{2+} in the stimulatory action of calmidazolium. In fact, there was a significant enhancement of steroid production when extracellular Ca^{2+} was removed, or when La^{3+} was added. The reason for this stimulation is not known.

The inhibition of basal and LH-, cholera-toxin- and forskolin-stimulated cyclic AMP production by calmidazolium suggests the involvement of calmodulin in the stimulation of adenylate cyclase in rat Leydig cells (Sullivan & Cooke, 1985). This has also been suggested for rat granulosa cells (Tsang & Carnegie, 1983), and demonstrated in adrenal (Papadopoulos *et al.*, 1990) and rat thymocyte plasma membranes (Segal, 1989). This also argues against the involvement of cyclic AMP in mediating the stimulatory action of calmidazolium on steroidogenesis.

In steroidogenic cells, the rate-limiting step for steroid production is the conversion of cholesterol into pregnenolone. This conversion is catalysed by the cytochrome *P*-450 cholesterol-side-chain-cleavage enzyme system, located in the mitochondrial inner membrane (Simpson, 1979). It is generally agreed that the acute regulation of steroidogenesis by the trophic hormones is localized in the mitochondria, but the precise mechanism of regulation is not known. It has been established for a long time that the stimulation of steroidogenesis by trophic hormones is acutely sensitive to the protein-synthesis inhibitor cycloheximide (Moyle *et al.*, 1971; Cooke *et al.*, 1975). It has also been shown that the transfer of cholesterol to the mitochondrial inner membrane can be inhibited by cycloheximide (Privalle *et al.*, 1983). Taken together, these observations suggest that trophic hormones stimulate the synthesis of protein(s) which allow the transfer of cholesterol to the mitochondrial inner membrane for side-chain cleavage. The inhibition by cycloheximide of LH- and dbcAMP-stimulated testosterone production is in agreement with previous findings (Cooke *et al.*, 1975). However, the insensitivity of calmidazolium-stimulated steroidogenesis to cycloheximide suggests that the mechanisms of this stimulation are different from that for LH or cyclic AMP. This suggests a direct action of calmidazolium on the mitochondria to stimulate the conversion of cholesterol into pregnenolone, and this suggestion is further supported by the inhibitory effect of amino-

glutethimide, an inhibitor of cholesterol side-chain cleavage. The time course of calmidazolium-stimulated steroidogenesis is similar to that for LH (Janszen *et al.*, 1976), which is suggestive of a common mechanism of action.

The stimulatory action of calmidazolium is probably not due to the inhibition of calmodulin, because another commonly used calmodulin inhibitor, trifluoperazine, did not stimulate testosterone production. However, it has recently been demonstrated, although not discussed in that paper, that low concentrations of trifluoperazine increased progesterone production stimulated by human chorionic gonadotropin and by cyclic AMP in mouse Leydig tumour cells (MA-10), and also by itself increased cholesterol synthesis *de novo* (Nagy & Freeman, 1990). These findings suggest that the use of calmidazolium and trifluoperazine to infer calmodulin involvement in the positive regulation of steroidogenesis should be made with caution.

Calmidazolium is chemically related to the imidazole fungicides isiconazole, miconazole and econazole. These compounds have been shown to inhibit testosterone production in Leydig cells. The inhibition was shown to occur over equivalent concentrations to those at which calmidazolium stimulated testosterone production, but no stimulation was reported (Schürmeyer & Nieschlag, 1984). Therefore it would appear that the stimulation of steroidogenesis is not a common property of these compounds.

It is concluded from the present study that calmidazolium, the commonly used calmodulin inhibitor, has a potent stimulatory effect on steroidogenesis. The levels of stimulation are similar to those obtained with LH and are not mediated by cyclic AMP. Unlike the stimulation of steroidogenesis with LH or cyclic AMP analogues, there are no requirements for extracellular Ca^{2+} and protein synthesis. Calmidazolium is therefore a potentially important probe for the further elucidation of the mechanisms which regulate steroidogenesis. Also, the use of calmidazolium as a specific calmodulin inhibitor in whole-cell studies, especially in relation to steroidogenesis, should be re-evaluated.

This work was supported by the Peter Samuel Royal Free Fund. M.S.K.C. is a Junior Research Fellow supported by the Royal Free Hospital School of Medicine.

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Received 23 May 1991/9 July 1991; accepted 12 August 1991