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The role of chloride ions in the regulation of steroidogenesis in rat Leydig cells and adrenal cells ☆

B.A. Cooke [a,*], L. Ashford [a], D.R.E. Abayasekara [b], M. Choi [a]

[a] *Department of Biochemistry and Molecular Biology, Royal Free Hospital School of Medicine, Rowland Hill Street, London, NW3 2PF, UK*
[b] *Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London, NW1 OTE, UK*

Abstract

The role of chloride ions in the regulation of steroidogenesis in rat Leydig cells and adrenal cells has been investigated. It was found that the chloride channel blocker 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) inhibited LH but not dibutyryl cAMP (dbcAMP)-stimulated steroidogenesis in the Leydig cells. This was found to be via an inhibition of cAMP production, because both LH- and forskolin-stimulated cAMP productions were inhibited by DIDS. The exclusion of chloride ions enhanced steroidogenesis during incubation of Leydig cells and adrenal cells with dbcAMP. The adrenal cells were found to be more sensitive to dbcAMP than Leydig cells and the enhancing effects of chloride removal were higher. In the presence of chloride ions, near maximum steroidogenesis was achieved with approximately 60 μM and 1 mM dbcAMP in the adrenal and Leydig cells, respectively. In the absence of chloride ions the concentrations required decreased approximately 50-fold and 10-fold, respectively. It is concluded that although LH may regulate DIDS sensitive chloride channels, the enhanced stimulation of cAMP-mediated steroidogenesis by chloride exclusion is not mediated via these channels. We propose a model based on the present and previous studies [1] with Leydig tumour (MA10) cells i.e. that intracellular chloride ion depletion enhances the action of cAMP on protein synthesis which results in increased synthesis of the Steroidogenic Acute Regulator (StAR) protein and consequently increased steroidogenesis.

Keywords: Leydig cell; Adrenal cell; Chloride; Steroidogenesis

1. Introduction

Chloride channels are important for the stimulation and regulation of many cellular processes [2]. Several types of chloride channels exist, the best known of which is regulated by cell volume changes. In addition, it is now established that volume-insensitive chloride channels, which can be linked to calcium channels (e.g. in cardiomyocytes) are regulated by protein kinase A (PKA) and protein kinase C (PKC) dependent phosphorylation [3]. Despite considerable biophysical information on the different types of chloride channels in plasma membranes and the mechanisms by which they are controlled, the intracellular effects of changes in chloride ion concentrations are not well understood. Recent data indicate that in addition to changes in membrane polarization, modulation of important metabolic pathways may occur.

There are several potential ways in which chloride channels could influence steroidogenesis. An efflux of chloride from the cell causes membrane depolarization. This can lead to an opening of calcium channels and result in an increase in intracellular calcium. Given the important role that calcium plays in steroidogenesis, especially in adrenal glomerulosa cells, this may be a mechanism for the modulation of calcium levels in steroidogenic cells. It is established that the rate-limiting step in steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial

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* Corresponding author. Tel.: +44-0171-830-2254; fax: +44-0171-794-5029.

E-mail address: bacooke@rfhsm.ac.uk (B.A. Cooke)

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membrane. Changes in mitochondrial membrane polarity by depolarization of the mitochondrial membranes induced by chloride efflux may also influence this process. With regard to potential control of other intracellular activities, it is also known that chloride ions are negative regulators of various enzymes and of especial significance with regard to steroidogenesis, of tyrosine kinase in T cell lymphocytes [4]. Thus in steroidogenic cells changes in cAMP-induced kinase activation enhanced by chloride may be important.

Electrophysiological studies have clearly established that chloride channels are present in Leydig cells [5] and can be modulated by LH and cAMP. Using patch clamping techniques, Joffre and co-workers have demonstrated that chloride and potassium channels are present in mature rat Leydig cells [5]. After stimulation of Leydig cells with human chorionic gonadotrophin (hCG), LH or cAMP, it was found that while there was little effect on potassium conductance, all of these ligands increased chloride conductance. These effects were found to be independent of extra-cellular calcium but were blocked by the chloride channel blocker SITS (4-acetamido-4′-isothiocyanostilbene-disulphonic acid). They concluded from further studies [6] that a hyper-polarization-activated chloride conductance in the plasma membrane of Leydig cells can be modulated by cAMP. This nucleotide acts by modifying the kinetics of the inward current and both the kinetics and amplitude of deactivating currents. Therefore, the depolarization of membranes which occurs when Leydig cells are exposed to LH/hCG can be explained by an increase of a cAMP-mediated chloride conductance allowing the chloride ions to exit the cell. Recently, Mattioli et al. have demonstrated that activation of PKA and PKC mediates the depolarizing effect of LH in ovine cumulus-corona cells [7].

In initial studies on the potential role of chloride ions in steroidogenesis, chloride in the buffer medium was replaced with equimolar concentrations of a membrane impermeant salt, gluconate. It was found that the exclusion of extracellular chloride markedly enhanced steroidogenesis in rat Leydig cells stimulated with sub-maximal but not maximal concentration of LH [8]. LH-stimulated steroidogenesis was found to be inhibited by the anion channel blocker SITS. Thus the biophysical and the biochemical results suggest that chloride efflux from Leydig cells may be regulated by LH/cAMP and that this may be an important regulatory mechanism in the control of steroidogenesis.

The aim of the present study was to extend our previous work on rat Leydig cells and determine the effects of another chloride channel blocker DIDS on cAMP and testosterone formation. We have also determined the effect of dbcAMP and chloride exclusion on steroidogenesis in rat Leydig and adrenal cells.

2. Materials and methods

2.1. Materials

Sodium chloride, potassium chloride, magnesium sulphate, glucose, calcium acetate, and HEPES were purchased from British Drug Houses Ltd. (Poole, Dorset, UK). Sodium gluconate, potassium gluconate, bovine serum albumin (fraction V), dibutyryl cyclic AMP (monophosphate sodium salt), pregnenolone, progesterone, gentamycin, Pregnenolone antibody was obtained from ICN Biomedicals (Thame, Oxon, UK). [7-^{3}H]Pregnenolone (19.9 Ci/mmol) and [1,2,6,7-^{3}H]testosterone (70 Ci/mmol) were obtained from Amersham International plc (Aylesbury, Bucks, UK). Other materials and methods were as previously described [9].

2.2. Incubation media

Chloride-replete buffer had the following composition: 140 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium sulphate, 1.8 mM calcium acetate, 10 mM glucose, 10 mM HEPES and 0.1% (*w*/*v*) BSA. Chloride-free buffer was prepared identically but the chloride salts of sodium and potassium were replaced with equimolar gluconate salts of sodium and potassium (or other substituting ions as indicated in the text). All the buffers were adjusted to pH 7.4 and had osmolarities in the range of 290–300 mOsm/l.

2.3. Isolation of cells

2.3.1. Leydig cells

Leydig cells were prepared from adult rat testes as previously described using collagenase dispersion, centrifugal elutriation and Pecoll gradient centrifugation [9].

2.3.2. Adrenal cells

Adrenal cells were prepared from adult rats as follows. The adrenal glands were added to ice-cold Ham's F12, 15 mM Hepes–NaOH pH 7.4. Adhering fat was removed and the adrenals were then ruptured by placing between two clean ice-cold glass plates. The adrenals were then subjected to collagenase digestion with 1 mg collagenase/ml Ham's F12, 2% BSA, 15 mM Hepes–NaOH pH 7.4. The resulting cell suspension was filtered through nylon gauze to remove undigested capsular material and the suspended cells were pelleted in a bench centrifuge.

2.4. Cell cultures

Cells were plated at a density of 20,000 cells/well in a final volume of 200 μl.

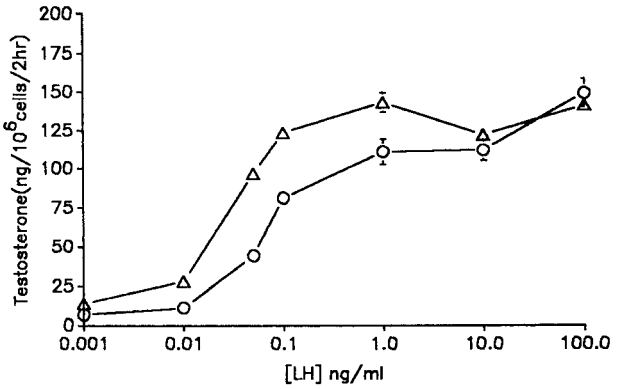

Fig. 1. Effect of removing extracellular chloride on LH-stimulated testosterone production. Leydig cells (2×10^4 cells/ well/200 μl) were incubated for 2 h with increasing concentrations of LH in the presence (○) and absence (△) of chloride ions. Each point represents the mean ± S.E. for triplicate incubations within one of at least three similar experiments.

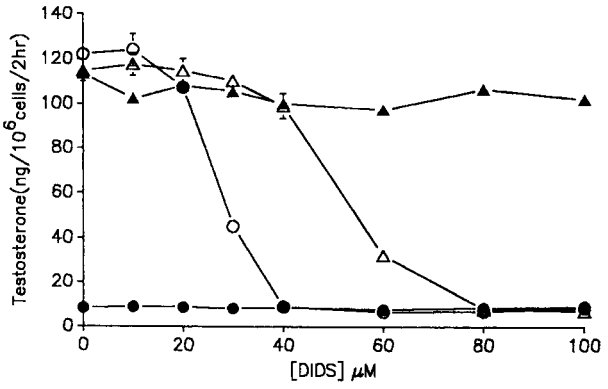

Fig. 3. Effect of DIDS on LH- and dbcAMP-stimulated testosterone production. Leydig cells (2×10^4 cells/well/200 μl) were incubated for 2 h with LH 1.0 (○) and 100 ng/ml (△) and dbcAMP (1 mM) (▲) in the absence and presence of increasing concentrations of DIDS. (●) represents basal values. Each point is the mean ± S.E. for triplicate incubations within one of two similar experiments.

Spent culture medium was removed from the cells and was replaced with modified salts buffer (containing 0.1% *w/v* BSA) and the cells were pre-incubated for 2 h. In experiments where the measurement of pregnenolone was required, the further metabolism of this steroid was blocked by the addition of cynoketone (5 μM) and SU10603 (20 μM) to the incubation medium. The medium was then replaced with identical, fresh buffer for a further 2 h in the presence and absence of LH, dbcAMP etc, after which the medium was acidified with 107 mM perchloric acid and the cells plus medium were frozen at −20°C until assayed for steroid or cAMP content. The latter was carried out by RIA [10] on the thawed medium after neutralization with 154 mM K_3PO_4 without extraction.

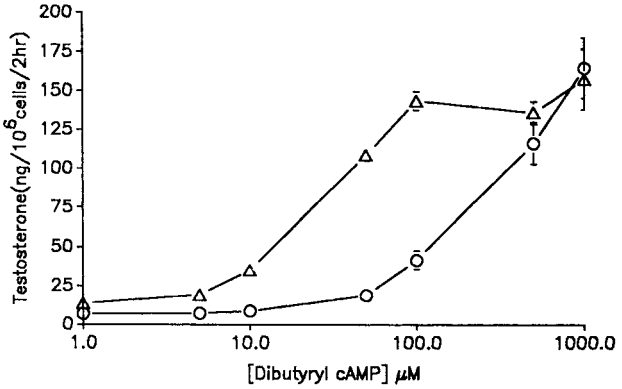

Fig. 2. Effect of removing extracellular chloride on dbcAMP-stimulated testosterone production. Leydig cells (2×10^4 cells/well/200 μl) were incubated for 2 h with increasing concentrations of dbcAMP in the presence (○) and absence (△) of chloride ions. Each point represents the mean ± S.E. for triplicate incubations within one of at least three similar experiments.

3. Results

The purified Leydig cells and adrenal cells were preincubated for 2 h, after which time the preincubation medium was removed and replaced by a simple salts medium containing glucose. For the removal of chloride, the chloride salts were replaced with equimolar concentrations of the appropriate gluconate salts (or other salts as indicated).

The exclusion of extracellular chloride enhanced testosterone production stimulated with submaximal, but not maximal levels of LH (Fig. 1). This effect, caused by removing extracellular chloride, was not exclusive to LH-stimulated testosterone production because it was found that dbcAMP-stimulated testosterone production was also similarly potentiated (Fig. 2). Maximum testosterone production is normally obtained with 1 mM dbcAMP, but in the absence of extracellular chloride, maximum steroidogenesis was obtained with 0.1 mM dbcAMP. The removal of extracellular chloride caused a greater potentiation of dbcAMP-stimulated, than LH-stimulated testosterone production.

The increased sensitivity of rat Leydig cells to stimulation by LH and dbcAMP in the absence of extracellular chloride, indicated the possible involvement of chloride channels in the regulation of steroidogenesis. This was assessed previously using SITS [8]. In further experiments using the chloride channel blocker, DIDS, it was found that testosterone production was unaffected by different concentrations of DIDS (0–100 μM) in the presence of maximum stimulating levels of dbcAMP (Fig. 3). However, testosterone production stimulated by 1 and 100 ng/ml LH were dose-dependently inhibited by DIDS with ID_{50}s of approximately 25 μM and 50 μM respectively (Fig. 3). The inhibition

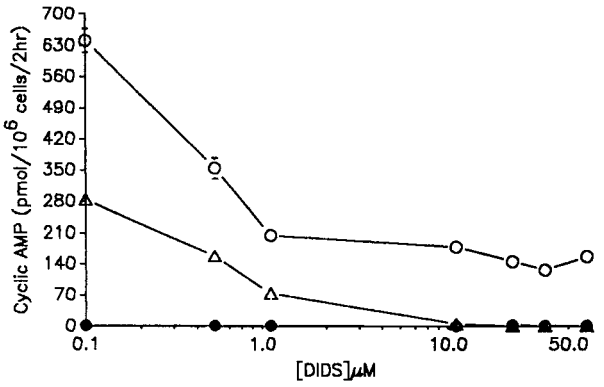

Fig. 4. Effect of DIDS on LH- and forskolin-stimulated cyclic AMP production. Leydig cells (2×10^4 cells/well/200 μl) were incubated for 2 h with LH (100 ng/ml (△) and forskolin 10 μM) (○) or buffer alone (●) in the absence and presence of increasing concentrations of DIDS. Each point represents the mean ± S.E. for triplicate incubations within one of two similar experiments.

of LH-stimulated testosterone production by DIDS (100 μM), could be overcome by the addition of dbcAMP to the incubation medium (results not shown), indicating that DIDS may be inhibiting LH-stimulated cyclic AMP production. The effect of DIDS on cyclic AMP production was therefore investigated in Leydig cells stimulated with LH (100 ng/ml) and forskolin (10 μM). Forskolin-stimulated cyclic AMP was decreased by two-thirds by 1 μM DIDS, and was not further inhibited by higher concentrations of DIDS (Fig. 4). However, LH-stimulated cyclic AMP was decreased to basal levels by increasing concentrations of DIDS (Fig. 4).

To investigate whether DIDS-sensitive chloride channels are involved in the potentiation of dbcAMP-stimulated steroidogenesis caused by the removal of extracellular chloride, DIDS (100 μM) was added to cells incubated in the absence and presence of extracellular chloride and stimulated by dbcAMP. No inhibition was found (Figs 5A and B).

To assess whether the potentiating effect of removing extracellular chloride ions on steroidogenesis is restricted to Leydig cells, investigations were carried out with rat adrenocortical cells. The replacement of chloride ions with gluconate, methanesulphonate or isethionate ions, markedly potentiated pregnenolone production in response to submaximum concentrations of dbcAMP (Fig. 6). The adrenal cells were found to be more sensitive to dbcAMP than Leydig cells and the enhancing effects of chloride removal were higher. In the presence of chloride ions, near maximum steroidogenesis was achieved with approximately 60 μM and 1 mM dbcAMP in the adrenal (Fig. 7) and Leydig cells (Fig. 1), respectively. In the absence of chloride ions the concentrations required decreased approximately 50-fold and 10-fold, respectively.

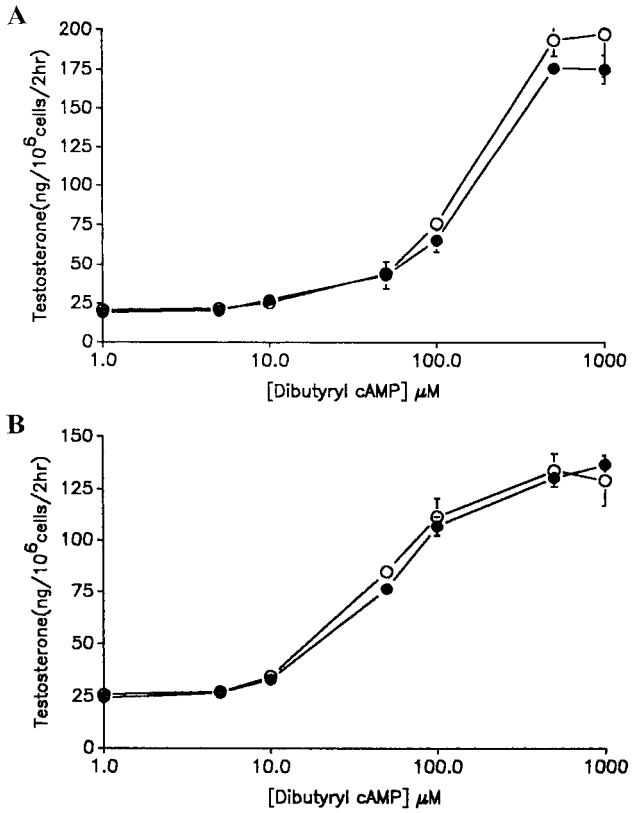

Fig. 5. Effect of DIDS on dbcAMP-stimulated testosterone production. Leydig cells (2×10^4 cells/well/200 μl) were incubated for 2 h with increasing concentrations of dbcAMP in the absence and presence of DIDS (100 μM) (●) and in the presence (fig 5A) absence (fig 5B) of extracellular chloride (○). Each point represents the mean ± S.E. for triplicate incubations within one of two similar experiments.

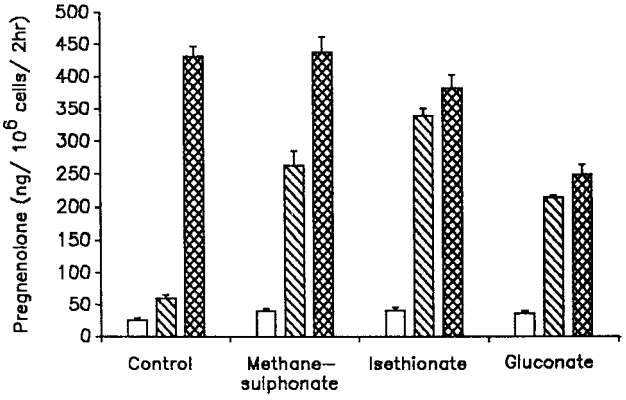

Fig. 6. Effect of removing extracellular chloride on dbcAMP-stimulated pregnenolone production in adrenocortical cells. Adrenocortical cells (2×10^4 cells/well/200 μl) were incubated for 2 h with different concentrations of dbcAMP. Each point represents the mean ± S.E. for triplicate incubations within one of two similar experiments.

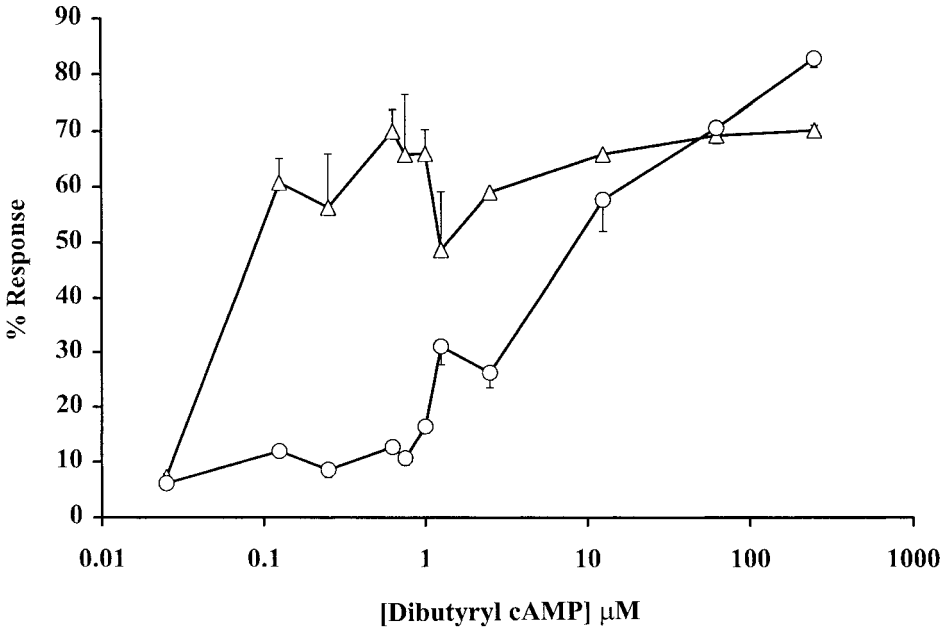

Fig. 7. Effect of removing extracellular chloride ions on dbcAMP-stimulated pregnenolone production in adrenal cells. Adrenal cells (2×10^4 cells/well/200 μl) were incubated for 2 h with increasing concentrations of dbcAMP in the presence (○) and absence (△) of chloride ions. Each point represents the mean ± S.E. for triplicate incubations from three experiments. Within each experiment the results were calculated as a percentage of the maximum steroidogenesis.

4. Discussion

The results obtained in the present study demonstrate that chloride removal enhances dbcAMP stimulated steroidogenesis both in rat Leydig cells and adrenal cells. These, together with similar results using MA10 cells [1], strongly indicate that this is a general phenomenon in the control of steroidogenesis. The present results also demonstrate that the chloride channel blockers inhibit LH but not dbcAMP stimulated steroidogenesis; this was found to be via an inhibition of LH-stimulated cAMP production.

The well-established dissociation between testosterone and cAMP production in Leydig cells stimulated with low concentrations of LH, has given rise to the suggestion that additional transducing mechanisms in addition to those involving cyclic AMP, could mediate testosterone production (see review [11]). Our initial results demonstrated that LH-stimulated steroidogenesis was inhibited by the chloride channel antagonist SITS [8]. This inhibition was obtained only with LH concentrations ≤ 1 ng/ml and not at 100 ng/ml. Therefore, these initial observations suggested that at low levels of LH, steroidogenesis was dependent on chloride channels whereas with high levels of LH, cyclic AMP was the mediation of LH action. However, the results of the present study with the chloride channels inhibitor, DIDS, showed that testosterone production stimulated with all concentrations of LH was inhibited by DIDS, although progressively higher concentrations were required for inhibition as the stimulating concentration of LH was increased. This argues against the involvement of chloride channels in steroidogenesis only when stimulated with low levels of LH. The different observations with SITS and DIDS could be explained by differences in their potencies for the inhibition of chloride channels.

The lack of effect of SITS and DIDS on dbcAMP-stimulated testosterone production suggest that these compounds may be acting through the inhibition of cyclic AMP production at a site prior to the activation of adenylate cyclase. When cyclic AMP production was measured, this was shown to be the case. The inhibition of both LH- and forskolin-stimulated cyclic AMP production suggested multiple sites of action of DIDS. The effect of DIDS on the action of forskolin suggests one of these sites to be the adenylate cyclase enzyme, but the inhibition was incomplete. The other site of action appears to be at the LH-receptor. However, the possibility exists that SITS and DIDS may also be inhibiting a chloride-dependent pathway involved in the stimulation of testosterone production by LH, which is independent of cyclic AMP production. The potentiation of both sub-maximal LH- and dbcAMP- stimulated testosterone production by the removal of extra-cellular chloride, suggest that this effect is via processes distal to the production of cAMP. The effect of removing extra-cellular chloride does not appear to be via SITS- or DIDS-sensitive chloride channels. This is supported by the inability of 100 μM DIDS to inhibit the potentiation of steroidogenesis caused by the removal of extra-cellular chlor-

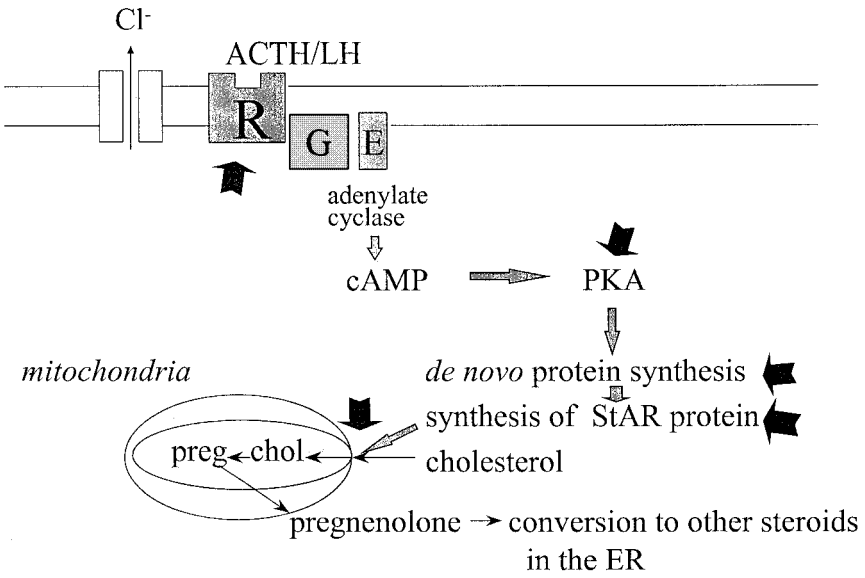

Fig. 8. The negative regulation of steriodogenesis by chloride ions in Leydig and adrenal cells. The possible sites of negative regulations by chloride ions are indicated by the filled arrows ([syAb]); these include PKA, protein synthesis, synthesis of the StAR protein and the mitochondrial membranes.

ide. The potentiating effect of removing extra-cellular chloride on steroidogenesis was also demonstrated in rat adrenal cells using different replacement ions. These cells were found to be more sensitive than Leydig cells to dbcAMP in the absence of chloride.

In our previous studies using the mouse Leydig (MA10) tumour cells [1], the mechanisms of the effect of chloride omission on dbcAMP-stimulated steroidogenesis was investigated. It was found that chloride omission enhanced progesterone production 2- and 4-fold in the absence and presence, respectively, of submaximally stimulating levels of dbcAMP (0.1 mM) during incubation for 2 h. This enhancement in stimulation increased continuously with time since after 6 h dbcAMP-stimulated progesterone production was 15-fold higher in the absence of chloride. Omission of chloride ions still enhanced progesterone production in the absence of absence of calcium, indicating that calcium ions are not involved in the potentiating effects on steroidogenesis observed in chloride-free buffer. Total protein synthesis was found to be 4-fold higher in cells incubated in chloride-free compared with chloride-replete media in the presence of 0.1 mM dbcAMP. Omission of chloride was also found to increase (4-fold) the level of the Steroidogenic Acute Regulatory (StAR) protein in the cells incubated with dbcAMP (0.1 mM). There was no increase in either the levels or activities of cytochrome P450 cholesterol side chain cleavage enzyme (cytP450$_{scc}$) or 3β hydroxysteroid dehydrogenase (3βHSD). These results were consistent with a cAMP-dependent regulatory role of chloride ion efflux in the control of steroidogenesis that requires protein synthesis but not calcium ions. It was proposed that this occurs by increases in StAR protein synthesis via a general increase in cAMP-dependent protein synthesis and/or by the enhancement of the steroidogenic effects of StAR.

The possible sites for the negative control of steroidogenesis by chloride ions are illustrated in Fig. 8. Based on the electrophysiological studies of Joffre and our biochemical studies, LH via its receptor, may directly open chloride channels via SITS/DIDS sensitive chloride channels. However, the enhanced stimulation of cAMP-mediated steroidogenesis by chloride exclusion is not mediated by SITS/DIDS sensitive chloride channels. The chloride-mediated action of cAMP may involve enhanced activation, via PKA, of protein synthesis that results in increased synthesis of the StAR protein. Lastly, the efflux of chloride ions may depolarize the outer mitochondrial membranes and thus enhance the transport of cholesterol into the inner mitochondrial membrane.

Acknowledgements

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