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Role of Intracellular Divalent Cations on the Adenylate Cyclase Activation by Human LH in mLTC-1 Leydig Cells

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Abstract

Introduction: The Luteinizing Hormone (LH) regulates Leydig cell activities through LHR occupation, promoting Gs protein and/or β-arrestin activation. The activated $G s\alpha_{GTP}$ subunit stimulates Adenylyl Cyclase (ACs) and, therefore, intracellular cyclic Adenosine Monophosphate (cAMP) accumulation from the AC substrate Adenosine Triphosphate (ATP). The divalent cations, magnesium (Mg^{2+}) or manganese (Mn^{2+}) associate with ATP to form the real substrates of ACs. In addition, ACs are sensitive to calcium (Ca^{2+}) but in a different way than Mg^{2+} or Mn^{2+}. Indeed, LH increases the cytoplasmic calcium ion concentration ($[Ca^{2+}]_{cyt}$) but only when Ca^{2+} is present in the extracellular medium.

Materials and Methods: In the present study, the effects of intracytoplasmic Ca^{2+}, Mg^{2+}, and Mn^{2+} on the cyclic AMP response to human LH in mLTC-1 Leydig tumor cells were investigated. The mLTC-1 cells were incubated at 37 °C in media supplemented with and without 5 µM Ca^{2+}, 5 µM Mg^{2+}, or 5 µM Mn^{2+}. The intracellular cyclic AMP accumulation was then monitored under LH stimulation.

Results: Our findings revealed that only Mg^{2+} and Mn^{2+} in the extracellular medium potentiate the cAMP response to hLH, in contrast to Ca^{2+}. In addition, we also showed that HCO_3^- increased the stimulation of the adenylyl cyclase enzyme by Ca^{2+}, Mg^{2+} or Mn^{2+}.

Conclusions: In mLTC-1 cells, extracellular Mg^{2+} and Mn^{2+} might potentiate LH-stimulated ACs activity by favoring LH interaction with its receptor, whereas Ca^{2+} from internal stores might be mobilized towards the cytoplasm to increase ACs activity, possibly through the soluble isoform.

Keywords: Adenosine Monophosphate, Adenylyl Cyclase, mLTC-1, Luteinizing Hormone, Forskolin
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Introduction

In mammalian cells, the cAMP synthesis from ATP is catalyzed by ACs and its degradation into 5′-AMP is catalyzed by phosphodiesterases.[1] In response to the binding of extracellular ligands to specific G Protein-Coupled Receptors (GPCRs), and Gs protein activation, one or several ACs are activated. The cAMP released in the cytoplasm, acts as a second messenger by interacting with PKA or other targets (like EPAC) in independent cAMP signaling microdomain.[1,2] In mammalian cells, there are two distinct families of adenylyl cyclases; nine genes encode a family of transmembrane adenylyl cyclases (tmACs), and a single, alternatively spliced gene encodes a family of soluble Adenylyl Cyclase (sAC) isoforms. Also distinct from tmACs, the sAC activity is modulated by Ca^{2+},[3,4] and is sensitive to variations in intracellular ATP concentrations.[4,5] More than 25 years ago, when sAC activity was first discovered, it was predicted to be molecularly distinct from tmACs because its activity appeared to be dependent on the presence of the divalent cation, Mn^{2+}, and it was insensitive to forskolin and G protein regulation.[6,7]

The activity of adenylate cyclase is affected by many factors, and among these are divalent cations. The addition of either Mg^{2+} or Mn^{2+} to adenylate cyclase preparations is required to elicit enzyme activity, and it is generally accepted that the Mg^{2+} is the natural cofactor. The addition of Ca^{2+} in place of Mg^{2+} has no activating effect on the enzyme.[8]

It has been demonstrated, however, that the adenylate cyclase depends upon both Ca^{2+} and Mg^{2+} for maximal activity.[9] Maximal activities in the presence of Mg^{2+} depends upon trace amounts of Ca^{2+} which appear to be bound to the particulate matter containing the adenylate cyclase. The chelation of these ions by the relatively Ca^{2+}-specific chelator 1,2-bis-(2-dicarboxymethylaminoethoxy) ethane (EGTA) results in the inhibition of the enzyme which is reversed by the addition of Ca^{2+}.[10]

A number of reports have provided evidence that Ca^{2+} may play a similar role in controlling the adenylate cyclase activity of other tissues as well.[11-13] The association of Ca^{2+} and Mg^{2+} has also been shown in some previous studies, but

the mechanism of their interaction is still limited. Ca^{2+} caused a competitive inhibition of Mg^{2+}-activated skeletal adenylate cyclase. Lowering the Mg^{2+} concentration increased the contribution of the high affinity Ca^{2+} binding site to the overall Ca^{2+} inhibition, and raising the Mg^{2+} concentration had an opposite effect.[14] Mahaffee et al. (1982) have shown that the sensitivity of adenylate cyclase of rat parathyroid membranes to Ca^{2+} inhibition was dependent upon the Mg^{2+} concentration.[15] Ca^{2+} may decrease the Parathyroid Hormone (PTH) secretion at least in part by a direct inhibition of adenylate cyclase. Mg^{2+} may promote PTH secretion either by enhancing the activation of adenylate cyclase by endogenous guanine nucleotides or by competing with Ca^{2+} for binding to a distinct regulatory site on the enzyme.[15] In addition, substitution of Mg^{2+} by Mn^{2+} abolished the inhibitory effect of Ca^{2+} on basal adenylate cyclase activity. Since Mg^{2+} and Ca^{2+} compete for a common allosteric site and Mn^{2+} abolished the effects of these cations, it would appear that Mn^{2+} also competes for the binding site of Mg^{2+} and Ca^{2+}.[16] In the present study, a kinetic analysis of the effects of Ca^{2+}, Mg^{2+}, and Mn^{2+} on the adenylate cyclase activity has been performed for the first time in mLTC-1 Leydig cells. It provides further evidence for the involvement of sAC in LH-stimulated cAMP synthesis in mLTC-1 cells. Our results are consistent with those from other reports about the catalytic role of divalent cations on LH-stimulated adenylate cyclase activation in cells.

Materials and Methods

Chemicals and Reagents

All chemicals used in this study were purchased from Sigma-Aldrich unless otherwise noted. Protease inhibitor cocktail was from Roche diagnostics (Mannheim, Germany). pGlosensor-22F cyclic AMP plasmid and Cell Titer-Blue Cell viability assay (G8080) were purchased from Promega (France), and the XtremeGENE HP DNA transfection reagent was from Roche (France).

mLTC-1 Cells

mLTC-1 cells[17] were obtained from the American Tissue and Cell Collection (ATCC) (LGC Standards, Molsheim, France). Cells were expanded in supplemented RPMI 1640 medium (Gibco, Invitrogen, 10% fetal bovine serum, 50 µg/ml gentamicin, 10 units of penicillin/ml and 10 µg/ml streptomycin), grown at 37 °C and 5% CO_2, which were used between their 6^{th} and 30^{th} passage.

Experiments

mLTC-1 cells (about 100.000 cells per well) on a 96-well Greiner white/clear bottom plate (Dutscher, Brumath France) were transfected with pGlosensor-22F cyclic AMP plasmid using XtremeGENE HP DNA transfection reagent as previously described for mLTC-1 cells.[18,19] Quantitations of intracellular cAMP were carried out as previously described for mLTC-1 cells.[18,19] The intracellular ATP levels were measured using the CellTiter-Glo 2.0 Assay (Promega, Madison, WI, USA) using the manufacturer's recommendations.[18,19] Cell viabilities were estimated using CellTiter-Blue Reagent (Promega, Madison, WI, USA) as recommended by the manufacturer.[18,19]

Area Under Curve (AUC) Calculations and Statistical Analyses

The GraphPad 5.00 package (GraphPad Software, San Diego CA) was used for the Area Under Curve (AUC) determinations of individual kinetics as well as for slope calculation by linear fitting of initial accumulation rate. Mean and SEM values for each triplicate AUCs were determined. One-way ANOVA with Dunnett's test was also performed using this package. The level of significance was at $p < 0.05$.

Results

Effect of Mg^{2+}, Mn^{2+} or Ca^{2+} on Intracellular Cyclic AMP Response to hLH in mLTC-1 Leydig Cells

In this study, we used mLTC-1 cells transiently expressing a chimeric cyclic AMP-responsive luciferase so that real-time variations of intracellular cyclic AMP concentration could be followed using oxiluciferin luminescence produced from catalyzed luciferin oxidation. The effects of Mg^{2+}, Mn^{2+} or Ca^{2+} were evaluated using the AUC of their kinetics over 60 min stimulation. To determine whether Mg^{2+}, Mn^{2+} or Ca^{2+} affects the expression of the cyclic AMP response to hLH, mLTC-1 cells were cultured for 90 min in the absence (control, Ctrl) or presence of 5 µM Mg^{2+}, 5 µM Mn^{2+} or 5 µM Ca^{2+}.

Figure 1A shows that the typical kinetics of fluorescence increase upon stimulation of cyclic AMP-dependent luciferase transfected mLTC-1 cells by hLH in the presence of Mg^{2+}, Mn^{2+} or Ca^{2+} compared to the control. The relative activities are shown in Figure 1B using the kinetics AUCs. Mg^{2+} increases the cAMP response to hLH over a longer period of time than Mn^{2+} (Figure 1A), explaining its higher effect (Figure 1B). The data indicate that the presence of 5 µM Ca^{2+} in the extracellular medium has no effect on the cyclic AMP response to 0.7 nM hLH.

Effect of ATP with Mg^{2+} or Mn^{2+} on Intracellular Cyclic AMP Response to hLH in mLTC-1 Leydig Cells

The activity of the ACs is sensitive to variations in the relative concentrations of ATP and Mg^{2+} or Mn^{2+}. The interrelationship between ATP and Mg^{2+} or Mn^{2+} is illustrated by the experiments in Figure 2. The intracellular cAMP response to hLH significantly increased in mLTC-1 preparations treated with Mg^{2+} or Mn^{2+} in the presence of 25 µM ATP compared to those treated with Mg^{2+} or Mn^{2+} in the absence of 25 µM ATP.

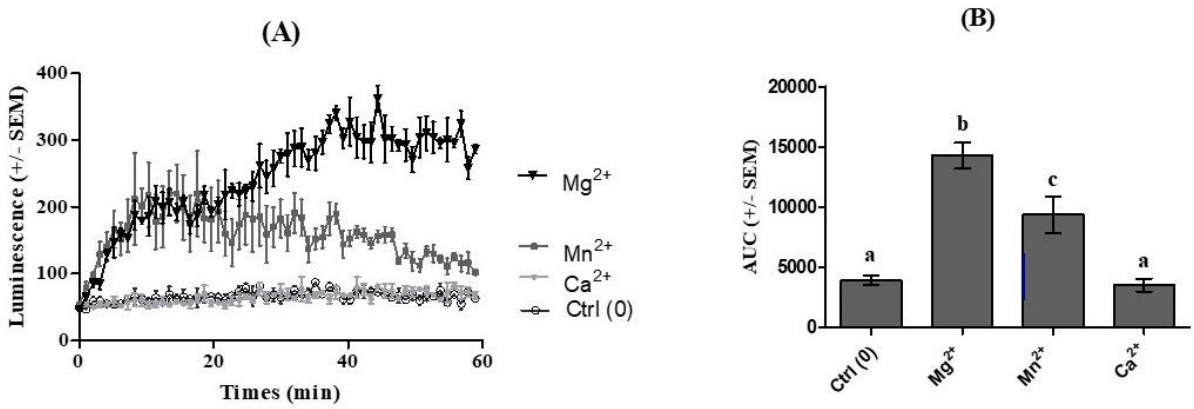

Figure 1. Effect of Mg^{2+}, Mn^{2+} or Ca^{2+} on Intracellular cAMP Response of mLTC-1 Cells to hLH. (A): Real-time recording of luminescence under stimulation of mLTC-1 cells by hLH in the presence of 5 µM Mg^{2+}, 5 µM Mn^{2+} or 5µM Ca^{2+}; (B): Dose-dependent effects of Mg^{2+}, Mn^{2+} or Ca^{2+} on hLH responses, respectively, determined by the Area Under Curve (AUC) of individual kinetics. Data represent mean ± SEM of 3 independent experiments (n=3). Results were analyzed by one-way ANOVA, followed by the Dunnett's test. Different letters indicate significant differences between control and treatment.

HCO_3^- Supports the Presumed Role of Extracellular Mg^{2+}, Mn^{2+} or Ca^{2+} in Intracellular Cyclic AMP Response to hLH in mLTC-1 Leydig Cells

In order to ascertain that the observed changes were actually due to Mg^{2+}, Mn^{2+} or Ca^{2+} -activation, we compared the effects of Mg^{2+}, Mn^{2+} and Ca^{2+} in the presence of bicarbonate (HCO_3). mLTC-1 cells were pre-treated with 60mM of HCO_3^- for 60 min, and then treated with 5 µM Mg^{2+}, 5 µM Mn^{2+} or 5 µM Ca^{2+} for 30 min. Our recent results have shown that HCO_3^- stimulates the intracellular cyclic AMP response to hLH in mLTC-1 Leydig cells after 90 min of incubation at 60 mM.[19] They also show that, when pre-incubating the cells for 60 min with HCO_3^- at 60 mM, extracellular Mg^{2+}, Mn^{2+} or Ca^{2+} increased the cAMP response to hLH (Figure 3).

Discussion

Adenylyl cyclases require a divalent cation for catalytic activity. The structure of tmACs is characterized by two membrane-spanning domains and two cytosolic loops C1 and C2.[20] C1 and C2 form the catalytic core and are relatively conserved among the different AC isoforms. The catalytic core of AC also exhibits two regulatory sites for Mg^{2+}.[21] Mg^{2+} interacts with ATP, forming the biologically active chelate cation ATP complex and thus preparing the molecule for the nucleophilic attack by ACs.[21] Since Mn^{2+} is very similar to Mg^{2+} in terms of its chemical properties, Mn^{2+} is often used as a divalent cation in *in vitro* AC studies.[22] However, differences in catalytic and regulatory properties of ACs have been noted depending on whether Mg^{2+} or Mn^{2+} served as the metal cofactor.[23] Our data demonstrate

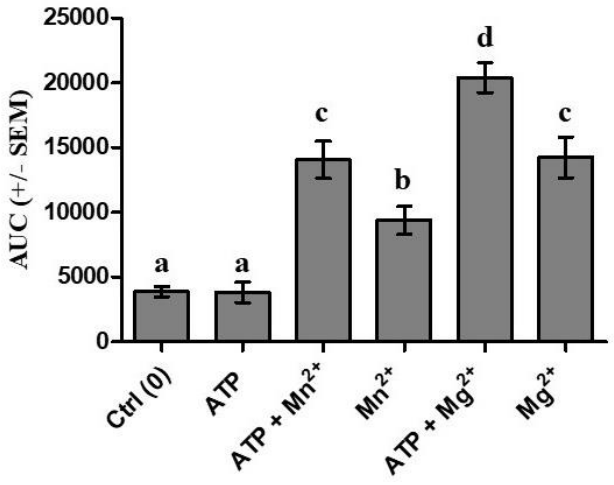

Figure 2. Effect of ATP and Mg^{2+} or Mn^{2+} on Intracellular Cyclic AMP Response to hLH in mLTC-1 Leydig Cells. Dose-dependent effects of 25 µM ATP and 5 µM Mg^{2+} or 5 µM Mn^{2+} on hLH responses, respectively, determined by the Area Under Curve (AUC) of individual kinetics. Data represent mean ± SEM of 3 independent experiments (n = 3). Results were analyzed by one-way ANOVA, followed by the Dunnett's test. Different letters indicate significant differences between control and treatment.

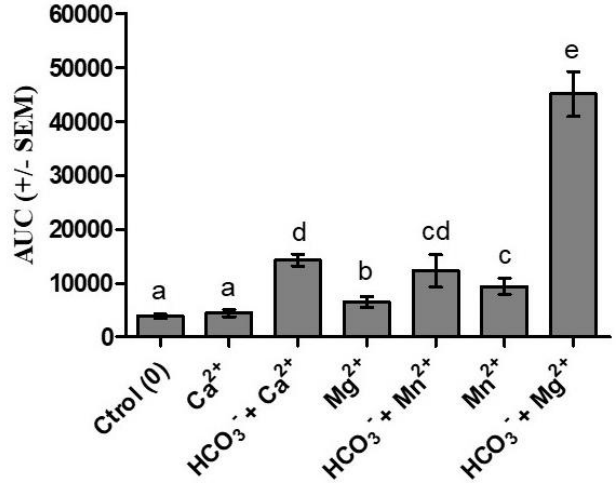

Figure 3. Effects of Extracellular Mg^{2+}, Mn^{2+} or Ca^{2+} in Presence of 60 mM HCO_3^- on the LH-Stimulated Intracellular cAMP Accumulation in mLTC-1. The cells were preincubated in HEPES buffer solution with 60 mM HCO_3^- for 60 min and then exposed to 5 µM Mg^{2+}, 5 µM Mn^{2+} or 5 µM Ca^{2+} extracellular for 30 min, before stimulation by 0.7 nM hLH. The values in all experiments are means ± SEM for 3 independent experiments (n = 3). p value of <0.05 was considered statistically significant using a one-way ANOVA followed by the Dunnett's test. Different letters indicate significant differences between control (0 µM) and each treatment at $p < 0.05$.

that extracellular Mg^{2+} or Mn^{2+} activated adenylyl cyclase in mLTC-1, but Ca^{2+} did not do so. Both cations are necessary for maximal enzyme activity. The addition of these divalent cations has increased the activity of the ACs enzyme through increased intracellular cAMP concentration. The activation of adenylyl cyclases by Mg^{2+} is undoubtedly due to its effect on enzyme affinity for its substrate which in fact is ATP-Mg.[12] The sensitivity of adenylyl cyclases increases in the presence of Mg^{2+} and ATP, which was also observed in adipocytes and myocardial cells.[12,24,25] The authors have proposed that the substrate for the enzyme is the Mg-ATP chelate.[12] Free ATP competes with Mg-ATP chelate for catalytic sites on enzymes. The increased activity of Mg^{2+} was also observed with adenylyl cyclase in fat cells[12] and cardiac tissue.[24] ACs enzyme is also known to be more active in the presence of Mn^{2+}, a distinctive feature that led to its discovery in mammals.[6] Mn^{2+} cations are potent stimulators of human sperm motility through the stimulation of the ACs enzyme activity.[26] *In vivo*, Mg^{2+}-ACs activity increases the affinity for ATP of the mammalian ACs enzyme.[4] In the structure- and kinetics-based model, ATP, with Ca^{2+} binds to its γ-phosphate in the ACs catalytic center. This results in an open ACs state. Then, Mg^{2+} ion binds to the α-phosphate of ATP, leading to a distinct set of catalytic residue interactions referred to as the closed state. This change, from an open state to a closed state, induces the esterification of the α-phosphate with the ribose in adenosine and the concomitant release of the β- and γ-phosphates.[27]

cAMP and Ca^{2+} are arguably the prototypical second messengers that control cellular homeostasis. The mammalian ACs, which catalyze cAMP synthesis are potentially regulated either directly by Ca^{2+} and/or calmodulin (CaM), or indirectly either by CaM kinase (CaMK), protein kinase C (PKC), or calcineurin (CaN), all of which are potentially activated when $[Ca^{2+}]_i$ is increased.[28-30] The nine plasma membranes AC isoforms are classically grouped according to the ability of Ca^{2+} to regulate their activity. It has been accepted that AC1, AC3, and AC8 are activated by Ca^{2+} via CaM, whereas Ca^{2+} alone inhibits AC5 and AC6, but has no effect on AC2, AC4, AC7, or AC9. In our study, the incubation of extracellular calcium at 5 µM with mLTC-1 cells did not affect intracellular cAMP concentrations. However, the incubation of extracellular calcium in the presence of HCO_3^- causes an increase in the cAMP response to hLH. A similar result is also observed for Mn^{2+} and Mg^{2+}. We know that cAMP is produced by adenylyl cyclase enzymes (including soluble adenylyl cyclase (sAC) and transmembrane adenylyl cyclase (tmAC)) that use ATP as a substrate. sAC activity is directly stimulated by HCO_3^-, and sAC has been confirmed to be a HCO_3^- sensor in a variety of mammalian cell types.[31] Calcium directly stimulates sAC activity while functioning independently from calmodulin to increase the affinity of sAC for its substrate ATP-Mg^{2+}.[4] A micro-domain consisting of sAC and cyclic nucleotide gated calcium channels could explain the observed cAMP/calcium oscillations in cells.[32] Because of the synergy between calcium and bicarbonate, even small intracellular changes in calcium or subtle changes in intracellular pH and/or carbon dioxide, which will be in equilibrium with bicarbonate, will result in significant changes of cellular cAMP. Thus, our results show that divalent cations are required for the activation of adenylyl cyclases, but the mechanisms by which they produce activation are quite dissimilar.

Conclusion

Collectively, the results show that the AC response to hLH in mLTC-1 requires extracellular Mg^{2+} or Mn^{2+}, but not Ca^{2+}. Adding HCO_3^- with Ca^{2+}, Mg^{2+} or Mn^{2+} leads to an increase of the intracellular cAMP response to hLH. Our data favor the conclusion that the catalytic component of adenylyl cyclase contains a divalent cation allosteric site in many different cellular models. We also showed that HCO_3^- increases the stimulating role of Ca^{2+}, Mg^{2+} or Mn^{2+} in ACs activity.

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Authors' Contributions

TMDN conceived and supervised the project. TMDN designed the experiments. TMDN, VGT, TCHN, and BNN carried out the laboratory work. TMDN wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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