

EFFECT OF FLUOXETINE ON THE INHIBITION OF ADENYLATE CYCLASE ACTIVITY IN FOSKOLIN-STIMULATED MLTC-1 LEYDIG CELLS

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SUMMARY

Fluoxetine (FLX), a widely used antidepressant primarily acting as a selective serotonin reuptake inhibitor, has been shown to exhibit other mechanisms of action in various cell types. Cyclic adenosine monophosphate (cAMP) is a second messenger used for intracellular signal induction. Cyclic AMP is a nucleotide synthesized within the cell from adenosine triphosphate by the adenylyl cyclase enzyme, and is inactivated enzymatically to 5'AMP by hydroxylation with a group of enzymes called phosphodiesterase. The aim of this study was to determine the effects of FLX on MLTC-1 Leydig cells on intracellular cyclic AMP response to forskolin (FSK). MLTC-1 cells were incubated at 37°C in media supplemented with or without different doses of FLX (0, 0.156, 0.3125, 0.625, 1.25, 2.5, 5 and 10 µM). We then looked for how the concentration of FLX for a short-time (2 hours) and a long-time (24 hours) affects the concentration of intracellular cyclic AMP response to FSK and ATP levels on MLTC-1 cells. Our results show that FLX decreased the intracellular cAMP response to FSK depending on FLX concentration. FLX decreased significantly cAMP levels only at 10 µM after 2 hours of incubation but after 24 hours of incubation FLX caused an effect on cAMP levels at 5 µM and at 10 µM. Moreover, as expected, FLX also caused a decline of steroidogenesis, which is under the control of cAMP and ATP levels in the cells. Taken together, these findings demonstrate that the inhibition of cAMP synthesis by FLX is dose-dependent, and that FLX also inhibited hormone-induced steroidogenesis in MLTC-1 cells.

Keywords: AMPK, cyclic adenosine monophosphate, MLTC-1 cells, Fluoxetine, Forskolin

INTRODUCTION

Fluoxetine (FLX) is a phenylpropylamine that inhibits the neuronal reuptake of serotonin, which has a direct relationship on antidepressant activity. FLX has been shown to exhibit other mechanisms of action in various cell types and to have a role in the endocrine system of reproduction as well (Emslie *et al.*, 2012). FLX directly reduced the sexual hormones of depressed men and women (i.e. testosterone in men and estrogen and progesterone in women).

Forskolin (7beta-acetoxy-8, 13-epoxy-1a, 6β, 9a-trihydroxy-labd-14-en-11-one) is a diterpenoid isolated from plant *Coleus forskohlii* (Lamiaceae). It is a lipid-soluble compound that can penetrate cell membranes and stimulates the adenylyl cyclase

enzyme, and then increases intracellular cAMP levels. cAMP is a second messenger involved in intracellular signaling in response to many membrane-impermeable hormones (Rall, Sutherland, 1958). It plays an important part in many cellular processes like gene transcription, cell adhesion or ion channel gating (Bos, 2006; Tasken, Aandahl, 2004; Wong, Scott, 2004). Its levels are precisely regulated via both its rate of synthesis by adenylyl cyclase (AC) activity and its rate of degradation by an important group of cAMP-hydrolyzing phosphodiesterases (PDEs). For the most part, the effects of cAMP are dependent upon the activation of its downstream effectors protein kinase A (PKA) (Taylor *et al.*, 1990) and the exchange protein directly activated by cAMP (de Rooij *et al.*, 1998).

In Leydig cells, steroidogenesis is under the control of Luteinizing Hormone (LH) produced by the pituitary. Binding of LH to its receptor at the plasma membrane triggers the activation of adenylate cyclase, leading to an increase in the second messenger cyclic AMP (cAMP) intracellular level (Dufau *et al.*, 1980). At its turn, cAMP is the known mediator for steroidogenesis through its stimulation of StAR (Steroidogenic acute regulatory protein)-dependent cholesterol entry in the mitochondria (Stocco, 2001). Therefore, in order to understand more completely the mechanisms of FLX action in steroidogenesis, we explored whether FLX affected intracellular cAMP synthesis in MLTC-1 cell line, under forskolin (FSK) stimulation. Since FSK directly stimulates AC, it permits to determine more precisely the step(s) potentially affected by FLX. We then also analyzed the patterns of progesterone and ATP concentration in MLTC-1 Leydig 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 CellTiter-Blue Cell viability assay (G8080) were from Promega (France), XtremeGENE HP DNA transfection reagent was from Roche (France).

MLTC-1 cells

MLTC-1 cells (Rebois, 1982) were obtained from the American Tissue and Cell Collection (ATCC) (LGC Standards, Molsheim, France). Cells were expanded in supplemented RPMI1640 medium (Gibco, Invitrogen, 10 % fetal bovine serum, 50 µg/ml gentamicin, 10 units of penicillin/ml and 10 µg/ml streptomycin) and used from passages P6 to P30.

Culture conditions

One day before each experiment, about 100,000 cells were seeded per well on a 96-well Greiner white/clear bottom plate (Dutscher, Brumath France) and incubated at 37°C under 5% CO₂ in 100 µL supplemented RPMI growth medium. Cells were then transfected with pGlosensor™-22F cyclic AMP plasmid using XtremeGENE HP DNA transfection reagent. This plasmid consists in firefly luciferase sequence fused to that of the protein kinase A cyclic AMP-binding domain in a way that allows control of

its enzymatic activity by cyclic AMP. Thirty minutes before transfection, DNA (100 ng plasmid per well) and XtremeGENE HP DNA transfection reagent (0.3 µL per well) were mixed together with serum-free RPMI medium (10 µL per well). Supernatants were then aspirated from each well and replaced by 100 µL of supplemented RPMI medium plus 10 µL of transfection mix. The plates were then incubated overnight at 37°C under 5% CO₂ before use of the cells in the assays.

Assays and measurements

Transfection supernatants were removed and replaced with medium deprived of fetal-calf serum (100 µL) and containing the luciferase substrate luciferin as well as 1 mM IBMX. The plates were then incubated during 1 hour before adding FLX at various concentrations in a 10 µL volume, and incubated a last time for 1 hour. Finally, individual stimulating hormones were added in a 10 µL volume in triplicate wells to reach 10 µM FSK (10 µM FSK together with the sub-stimulating concentration of 70 pM hLH) (Nguyen *et al.*, 2018). Cyclic AMP was measured using a Polarstar Optima (BMG Labtech Sarl, Champigny sur Marne, France).

MLTC-1 cells viability assessment

MLTC-1 cells were seeded in 96-well plates at 100,000 cells/well. Two days later, the medium was replaced with serum-free medium in the absence (control) or presence of FLX (0-10 µM) for 1 hour at 37°C before the addition of 20 µL of CellTiter-Blue® Reagent (Promega, Madison, WI, USA) to each well. After having incubated for 2 hours at 37°C, changes in fluorescence were recorded with a Spectra Gemini spectrofluorimeter (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The fluorescent signal from the CellTiter-Blue® Reagent is proportional to the number of viable cells.

Adenosine triphosphate (ATP) concentration measurement

After the incubation of cells with or without FLX, ATP concentration in cells was measured using the Cell-Titer-Glo Assay (Promega, Madison, WI, USA). Standards were prepared from ATP standard (Promega) using serial dilutions to obtain concentrations of 1×10^{-10} , 1×10^{-11} and 1×10^{-12} M. Briefly, the assay buffer and substrate were equilibrated to room temperature, and the buffer was transferred with the substrate. After 30 min, a 50 µL

sample of this solution was added to 100 μ L luciferin/luciferase reagent in 96-well white plates, the content was then mixed for 2 min and incubation was continued for 10 min at room temperature. The luminescence at integration time 1000 (ms) was read using an Ascent Luminoskan Luminometer (Thermo) with PBS as a blank for each experiment.

Progesterone production measurements

MLTC-1 cells were first seeded in 96-well plates (100,000 cells/well) for 3 days and then re-suspended in serum-free RPMI and pre-incubated with or without FLX (10 μ M) for either 2 or 24 hours. Afterwards, they were challenged for 3 hours with increasing doses of FSK/sub-hLH. Cell supernatants were collected and stored at -20°C until analysis. Progesterone production was measured with a previously described competitive ELISA assay. Briefly, a 96-wells plate was coated overnight at 4°C with a goat anti-mouse IgG antibody, 10 ng/well (UP462140, Interchim, Montluçon, France). After 3 washes with PBS 1X containing 0.1 % Tween 20, non-specific sites were saturated 1 hour with 200 μ L/well of PBS-Tween 20 supplemented with 0.2% BSA. Standard progesterone (Q2600, Steraloids, USA) in PBS-Tween 20-BSA or MLTC-1 cells supernatants (25 μ L per well of 1:50 or 1:100 dilution) were then plated on the empty plate. Progesterone-11-Hemisuccinate-HRP (Interchim) were added, together with 36 ng/well of mouse anti-P4 antibody (AbD Serotec, Biogenesis, Interchim). The plate was incubated for 4 hours at room temperature, washed, and 100 μ L/well of TMB ELISA substrate standard solution (Interchim) was added. The mixture was incubated for 20 min at room temperature in the dark. The reaction was stopped with 2 N H₂SO₄ and absorbance was measured at 450 nm using Sunrise™ reader (Tecan, France).

Area Under Curve (AUC) calculations and statistical analyses

The GraphPad 5.00 package (GraphPad Software, San Diego CA) was used for 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 FLX on intracellular cyclic AMP response to FSK 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 potency of different concentrations of FLX was evaluated using areas under the curves (AUC) of their kinetics over 2 and 24 hours stimulation. To determine whether FLX affects the expression of the cyclic AMP response to FSK, MLTC-1 cells were cultured for 2 and 24 hours in the absence (control, Ctrl) or presence of FLX (0, 0.156, 0.306, 0.625, 1.25, 2.5, 5 and 10 μ M).

Figure 1A and 1C show that the typical kinetics of fluorescence increase upon stimulation of cyclic AMP-dependent luciferase transfected MLTC-1 cells by FSK decrease in the presence of FLX. We observe the same for the areas under the curves (AUC) (Figures 1B and 1D). These decreases are highly significant as calculated by these kinetics AUCs (decrease by 12% at 10 μ M after 2 hours of incubation compared to control and by 15% at 5 μ M; by 29% at 10 μ M after 24 hours of incubation compared to control) (Figures 1B and 1D).

FLX reduces ATP content in MLTC-1 Leydig cells

To decipher the downstream events of the intracellular cyclic AMP response kinetics of Leydig cells to FSK activity after a FLX addition, we tested ATP concentration in cells after incubation with FLX. For that purpose, we measured ATP concentration using the Cell-Titer-Glo Assay. Our results showed that addition of FLX has resulted in significant decreases in ATP concentration: 40% with 10 μ M compared to control without FLX after 2 hours of incubation (Figure 2A) and 18% at 1.25 μ M; 31% at 2.5 μ M; 60% at 5 μ M; 72% at 10 μ M of FLX when compared to control after 24 hours of incubation (Figure 2B).

Effect of FLX on MLTC-1 Leydig cells viability

After 2 and 24 hours incubation in presence of FLX and a pre-incubation of 2 hours with CellTiter-Blue Cell viability assay, the percentage of cell viability was not affected after 2 hours of incubation when compared to control (Figure 3A), but it decreased at 5 and 10 μ M after 24 hours of incubation when compared to control (Figure 3B).

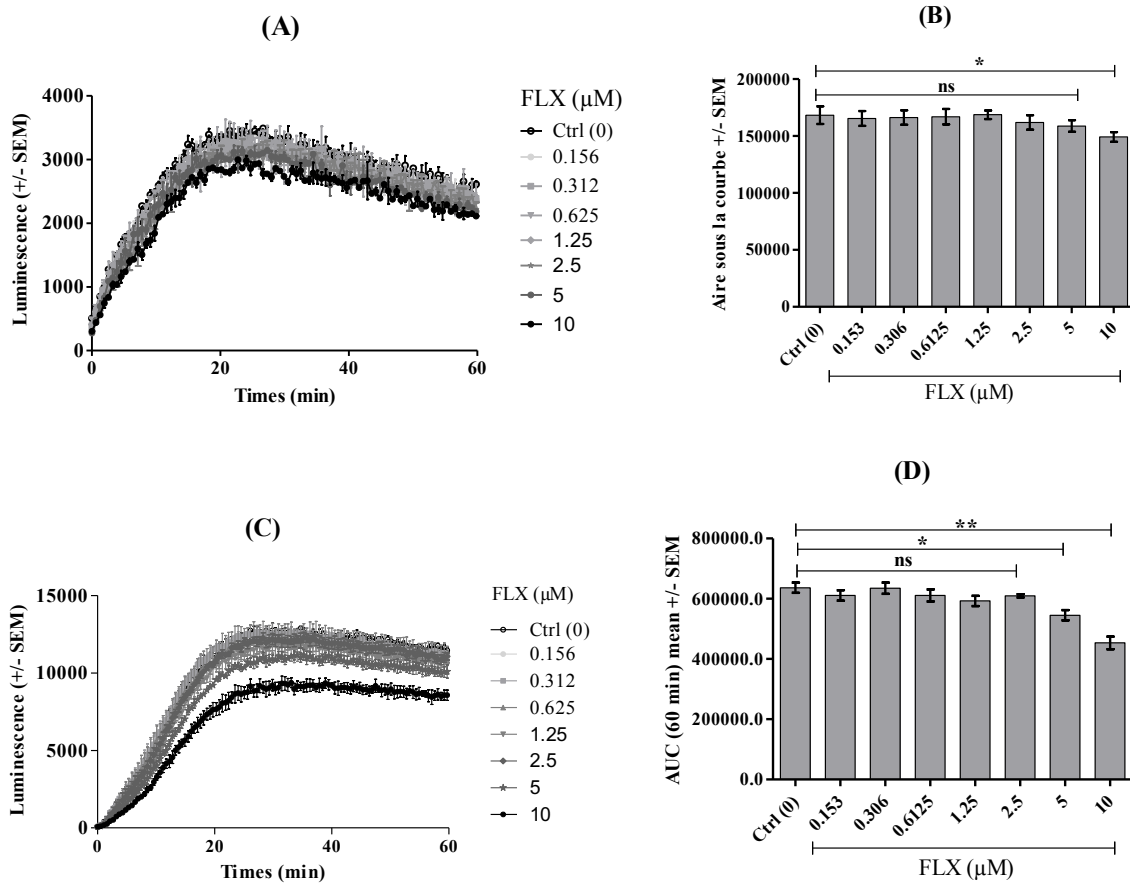


Figure 1. Effect of FLX on intracellular cAMP response of MLTC-1 cells to FSK. (A: after 2 hours), (C: after 24 hours): Real-time recording of luminescence under stimulation of MLTC-1 cells by FSK in the presence of 0 to 10 μM FLX; (B), (D): Dose-dependent effects of FLX on FSK responses respectively, determined by the Area Under Curve (AUC) of individual kinetics in figures 1A, 1C. Data represent mean ± SEM of 3 independent experiments (n=3). Results were analyzed by one-way ANOVA, followed by the Dunnett's test. *: significant difference (* p < 0.05; ** p < 0.01), ns: no significant difference.

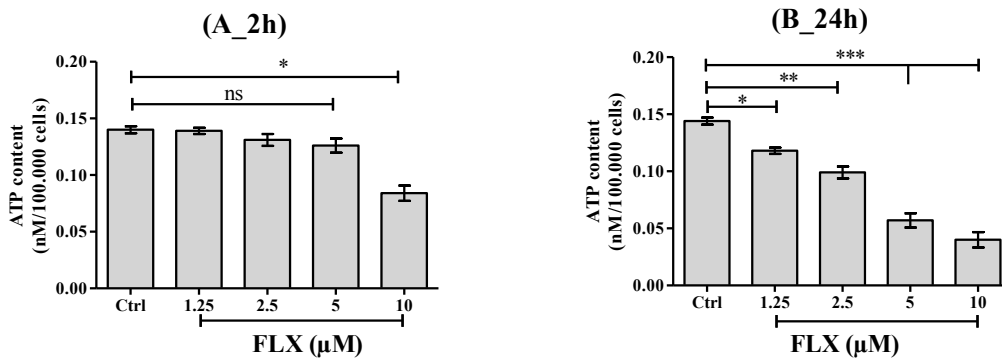


Figure 2. Effect of FLX on ATP concentration in MLTC-1 cells. ATP concentration in living cells was monitored using the Cell-Titer-Glo Assay. Cells were incubated at 37°C in the presence or absence of FLX after 2 hours of incubation (A) or after 24 hours of incubation (B). The experiments were repeated 6 times independently, values (%) are mean ± SEM (n=6). Results were analyzed by one-way ANOVA, followed by the Dunnett's test. *: significant difference (* P < 0.05; ** p < 0.01; *** p < 0.001), ns: no significant difference.

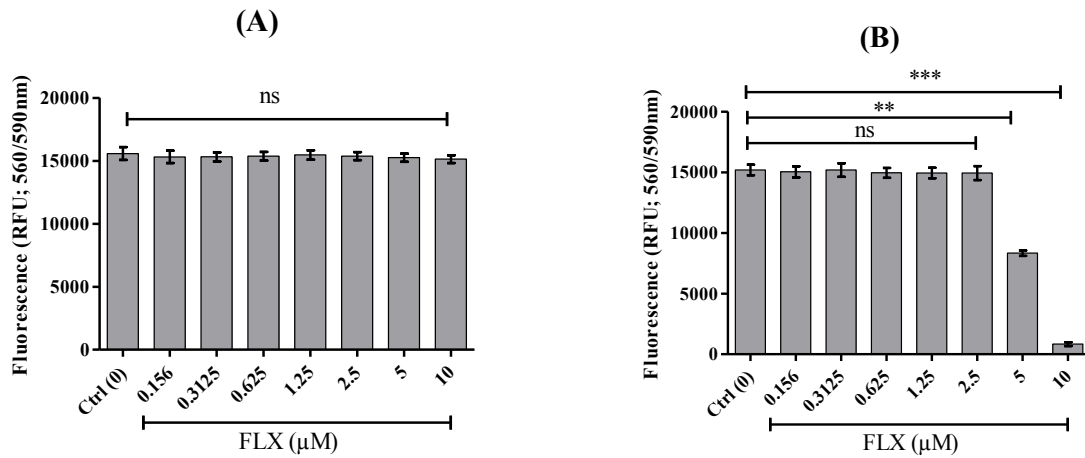


Figure 3. Effect of FLX on MLTC-1 cells viability. Cells were incubated at 37°C for 1 hour in the presence or absence of FLX after 2 hours of incubation **(A)** or after 24 hours of incubation **(B)**. The experiments were repeated 6 times, values (%) are mean \pm SEM (n=6). Results were analyzed by one-way ANOVA, followed by the Dunnett's test. *: significant difference (** p < 0.01; *** p < 0.001), ns: no significant difference.

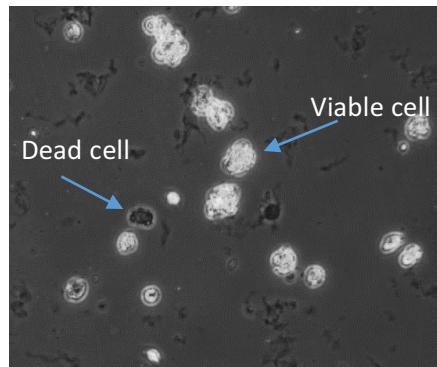


Figure 4. MLTC-1 cells after incubation for 2 hours with CellTiter-Blue Cell viability assay.

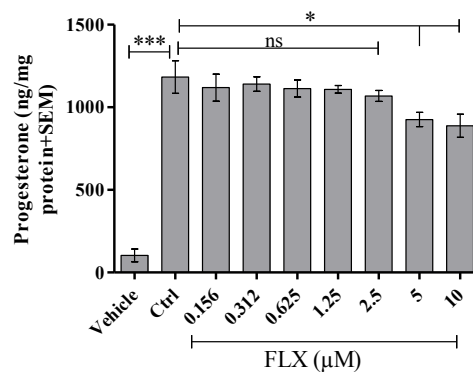


Figure 5. Effect of FLX in FSK/sub-hLH-promoted steroid production in MLTC-1. MLTC-1 cells were pre-incubated without (Ctrl) or with FLX for 24 hours and then the cells were stimulated for 3 hours with FSK/sub-hLH before progesterone productions were measured. Data are means \pm SEM of 3 independent experiments performed in duplicate (n=3). Results were analyzed by one-way ANOVA, followed by the Dunnett's test. *: significant difference (* p < 0.05; *** p < 0.001), ns: no significant difference.

FLX inhibits FSK-induced steroidogenesis in MLTC-1 cells

Physiological significance of intracellular cAMP modulations by FLX was further studied by looking at the specific steroid secretion of MLTC-1 cells (progesterone). We observed that the increase of progesterone production by FSK stimulation in MLTC-1 cells was inhibited in the presence of FLX at 5 and 10 μM (Figures 5). However, we did not see the decrease on the progesterone production in MLTC-1 by FSK after 2 hours of incubation with FLX (not shown here).

DISCUSSION

In order to study the relative potency of molecules with LH activity, we set up a cellular model by transfecting MLTC-1 cell line with the GloSensor cyclic AMP responsive luciferase (Tranchant *et al.*, 2011) in our most recent study (Klett *et al.*, 2016). We transfected the MLTC-1 cells 24 hours before use and we found that they uniformly expressed the cyclic AMP-responsive luciferase as the luminescence responses to the same FSK challenge was identical in all wells.

In the present work, FLX at high concentration was found to exert a potentiating effect on the cyclic AMP response to FSK. This potentiation was observed after a 2 hours pre-incubation of MLTC-1 cells with FLX concentration at 10 μM , but no other concentrations. We also showed that the increase in cyclic AMP levels response to FSK was decreased strongly in MLTC-1 cells treated with 5 μM and 10 μM FLX after 24 hours of incubation. This suggests that the incubation time has a direct effect on cAMP production under the stimulating of FSK. Moreover, FLX also decreased progesterone production by stimulation of FSK in MLTC-1 cells. It is highly probable that this inhibition of steroidogenesis is due to inhibition of cyclic AMP accumulation, as shown in MLTC cells. What mechanism(s) cause(s) the observed quick decrease in cyclic AMP response to LH in the presence of FLX?

FLX is an antidepressant of the selective serotonin reuptake inhibitors (SSRI) class. It is a drug used to fight symptoms of conditions such as major depression, obsessive-compulsive disorder, bulimia nervosa and panic disorder, dysautonomia, postpartum depression, premature ejaculation, fibromyalgia or trichotillomania (Brambilla *et al.*, 2005; Guze, Gitlin, 1994). Moreover, it is now

assumed that adenylyl cyclase (AC)-cyclic AMP-PKA cascade is linked to pathogenesis and treatment of depression (Lin *et al.*, 2012). In Leydig cells, the binding of LH to its G-protein-coupled receptor (LHR) activates adenylate cyclase, which triggers an increase in cyclic AMP (cAMP) levels, which leads in turn to the activation of PKA (Dufau *et al.*, 1980). The mechanism of cyclic AMP formation catalyzed by the binding of LH to its G-protein-coupled receptor (LHR) on Leydig cells activates adenylate cyclase, triggering an increase in cyclic AMP (cyclic AMP) levels from the intracellular ATP (Dufau *et al.*, 1980). We have also examined the concentration of ATP in the cells after treatment with fluoxetine for 2 hours and our results clearly show that in fluoxetine-treated MLTC-1 cells, ATP levels are strongly decreased only at 10 μM while not affecting cell viability. Moreover, after 24 hours of incubation, we observed a decrease in the ATP levels at 1.25, 2.5, 5 and 10 μM FLX while cell survival rates only started to decline at 5 and 10 μM . This means that at concentrations of 5 and 10 μM , the decrease in cAMP may be partly due to cell death. However, at low concentrations it does not affect cell survival and the decrease in cAMP is due to a decrease in ATP concentration in cells by FLX. The effect of FLX was found to be dose-dependent and the results of our research are consistent with those of Charles E, *et al.* in 2016. These observations support the hypothesis that FLX partially decreases ATP synthesis by reducing respiratory chain activity (Charles *et al.*, 2016). FLX is known as selective serotonin reuptake inhibitor but it was found to possess a wide range of biological activities such as interaction with Na^+ and K^+ and Ca^{2+} channels (Pancrazio *et al.*, 1998; Deak *et al.*, 2000). In mitochondria, FLX indirectly affects electron transport and (F1Fo) ATPase activity, and thus inhibits oxidative phosphorylation (Curti *et al.*, 1999). These FLX effects on mitochondrial activities may also result from interaction with Voltage-dependent anion channels (VDAC) and conductance decreasing of a Ca^{2+} channel (Gincel *et al.*, 2001) adenine nucleotides (Rostovtseva, Colombini, 1997), other metabolites (Hodge, Colombini, 1997; Shoshan-Barmatz, Gincel, 2003), as well as preventing PTP opening by preventing the release of accumulated Ca^{2+} and by swelling of energized mitochondria and inhibiting release of cytochrome C from mitochondria (Nahon *et al.*, 2005). Recently our research has shown that the decrease in the cyclic AMP response to hLH in MLTC-1 cells incubated with FLX is due to indirect activation of AMPK (Nguyen *et al.*, 2019) at

high concentration of FLX (50 and 100 μM) and that FLX acts at low concentrations through another pathway, independent of AMPK. FLX at low concentrations could directly inhibit adenylate cyclase activity through depletion of its substrate ATP (Nguyen *et al.*, 2019).

CONCLUSION

The results of our study showed that the antidepressant FLX at μM concentrations exerted a strong inhibition on FSK-stimulated cyclic AMP synthesis and progesterone secretion in MLTC-1 cells. Furthermore, FLX also reduced ATP levels and viability of MLTC-1 cells. We think that it should provide novel lines of investigation concerning long-term mechanisms by which steroidogenesis might be impaired in depressed patients treated with FLX.

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ỨC CHẾ HOẠT ĐỘNG ADENYLATE CYCLASE BỞI FLUOXETINE TRONG TẾ BÀO LEYDIG MLTC-1 ĐƯỢC KÍCH THÍCH BẰNG FORSKOLIN

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TÓM TẮT

Fluoxetine (FLX), một loại thuốc chống trầm cảm được sử dụng rộng rãi, chủ yếu hoạt động như một chất ức chế tái hấp thu serotonin có chọn lọc, đã được chứng minh là có nhiều cơ chế hoạt động khác nhau trong các loại tế bào. Adenosine monophosphate vòng (AMPC) là chất truyền tin thứ hai được sử dụng cho cảm ứng tín hiệu nội bào. AMPC được tổng hợp trong tế bào từ adenosine triphosphate (ATP) bởi adenylyl cyclase, và bị bất hoạt thành 5'AMP bằng cách hydroxyl hóa bởi enzyme phosphodiesterase. Mục đích của nghiên cứu này là xác định ảnh hưởng của FLX trong tế bào Leydig MLTC-1 đáp ứng AMPC theo chu kỳ nội bào được kích thích bởi forskolin (FSK). Các tế bào MLTC-1 được ủ ở 37°C trong môi trường có bổ sung hoặc không bổ sung FLX ở các nồng độ khác nhau (0, 0,156, 0,3125, 0,625, 1,25, 2,5, 5 và 10 µM). Sau đó, ảnh hưởng của FLX đến AMPC nội bào và ATP trong tế bào MLTC-1 trong thời gian ủ ngắn (2 giờ) và thời gian ủ dài (24 giờ) được xác định. Kết quả của chúng tôi cho thấy FLX đã làm giảm AMPC nội bào đáp ứng với FSK tùy thuộc vào liều. FLX giảm đáng kể AMPC tại 10 µM sau 2 giờ ủ và tại 5 và 10 µM sau 24 giờ ủ. Hơn nữa, FLX cũng gây ra sự suy giảm steroidogenesis, nằm dưới sự kiểm soát của mức AMPC và ATP trong các tế bào. Kết hợp lại với nhau, những phát hiện này chứng minh rằng sự ức chế tổng hợp AMPC của FLX phụ thuộc vào liều và FLX cũng ức chế sự sản sinh steroid hormone trong tế bào MLTC-1.

Keywords: Adenosine monophosphate, AMPK, MLTC-1, Fluoxetine, Forskolin