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ORIGINAL PAPER

Estrogen evokes a rapid effect on intracellular calcium in neurons characterized by calcium oscillations in the arcuate nucleus

Oliver Fricke · Lee-Ming Kow · Magda Bogun · Donald W. Pfaff

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Abstract Rapid estrogen effects became an interesting topic to explain estrogen effects not associated with the classical nuclear pathway. The rapid estrogen effect on intracellular calcium oscillations was characterized in neurons of the arcuate nucleus. Ratiometric calcium imaging (fura-2AM) was used to measure intracellular calcium in brain slices of female Swiss Webster mice (median of age 27 days p.n.). Calcium oscillations were dependent on intracellular calcium and also on calcium influx from the extracellular space. The perfusion of slices with calcium-free solution inhibited spontaneous calcium oscillations. The metabotropic glutamate receptor agonist t-ACPD (5 μM) and low concentrated ryanodine (100 nM) induced intracellular calcium release when slices were perfused with calcium-free solution. 17β-estradiol (10 nM) also induced intracellular calcium release in calcium-free ACSF. This effect was inhibited by the preceding administration of thapsigargin (2 μM) indicating the association of the rapid estrogen effect with intracellular calcium stores. The administration of the non-selective phospholipase C-inhibitor ET-18 (30 μM), but not U73122 (10 μM), and the inhibition of protein kinase A by H-89 (0.25 μM) suppressed the rapid estrogen effect. Analyses indicated a qualitative, but not quantitatively significant effect of 17β-estradiol on calcium oscillations.

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Introduction

The effect of estrogenic signals on brain development and behavior in mammals has been frequently discussed in the last few decades [1]. Primarily, research was focused on the classical nuclear pathway activated by estrogens [2]. But rapid estrogen effects using pathways localized in the cellular membrane and in the cytoplasmic area were suggested since the late 70s of the last century and were frequently discussed in the last few years [3–7]. Laboratory studies focused mainly on three important characteristics of rapid estrogen effects in the brain in the last 10 years: (1) rapid estrogen effects may enhance genomic estrogen effects [8, 9], (2) rapid estrogen effects might be associated to G proteins and protein kinases coupled pathways [10, 11] and, (3) rapid estrogen effects depend on cytoplasmic calcium and modify potassium and sodium channel activation [12, 13]. Thereby, physiological functions and behavior associated with hypothalamic structures stood in the center of research because estrogenic effects related to classical pathways were extensively investigated in the hypothalamus [14–17].

This area also became an important topic of neurobiological research since leptin was discovered because the hypothalamic network represents the primary feedback region for orexigenic and anorexigenic signals controlling feeding behavior [18, 19]. Interestingly, the appetite decreasing effect of estrogen has been well-known for many years and was partly explained by the effect of estrogen on neurons of the nucleus tractus solitarius which

is integrated into the cholecystokinine associated feedback of intestinal activity [20, 21]. Data on neuronal plasticity of connections between neurons of the arcuate nucleus (ARC) and the ventromedial hypothalamic area raise the question: do estrogens also decrease appetite by the modulation of the synaptic plasticity in the hypothalamic network? [22–24]. Data recently published gave evidence that estradiol mimics leptin's effect on rewiring of melanocortin cells in obese animals [25]. Thereby, this effect was mediated by the α-type estrogen receptor. Previous reports supported the hypothesis that the α-type estrogen receptor may be also expressed on the cell surface explaining rapid estrogen effects despite the classical nuclear estrogen signaling pathway [11]. Because studies on intracellular calcium oscillations in cortical and cerebellar neurons concluded that the presence of these oscillations is associated with gene expression and synaptic plasticity [26, 27], the question remains open if a putative rapid estrogen effect may be associated with the hypothalamic rewiring. Actually, limited knowledge is available on these calcium associated processes in the hypothalamic network. Therefore, the present study focuses on a suggested rapid effect of 17β-estradiol (E_2) on calcium oscillations in neurons of the ARC in female mice to make a first step to elucidate a possible role of a rapid estrogen effect on calcium mediated synaptic plasticity in hypothalamic neuronal networks.

Results

Perfusion with E_2 changes the pattern of intracellular calcium oscillations in neurons of the ARC

When the number of oscillating neurons was counted in a time interval of 3 min, 39 of 120 measured regions of interest (ROI) (32.5%; 10 experiments in 5 animals) were characterized by oscillations of the intracellular calcium concentration. Slices were perfused with E_2 10 nM for 60 s (Fig. 1). Patterns of calcium oscillation were analyzed in 26 of 39 neurons before and after start of E_2 perfusion. About 13 ROI were excluded from the analysis because oscillations stopped before the start of E_2 perfusion. The time of observational intervals for analyses (mean 527 ± 116 s) was not different between before and after start of E_2 perfusion. The mean oscillation frequency was in the mean 0.023 ± 0.016 Hz before E_2 administration and 0.024 ± 0.018 Hz after starting E_2 perfusion. The mean difference of frequencies before and after starting E_2 administration was not significant ($P = 0.88$, paired t-test, two-tailed). The coefficient of variation (CV) was used to detect differences in oscillation patterns before and after start of E_2 perfusion. The CV was in the mean 0.240 ± 0.135 before and 0.241 ± 0.087 after start of E_2 10 nM administration. Thereby, the CVs were not significantly different before and after E_2 administration (post-hoc analysis with $P = 0.96$ for the mean difference of CVs; paired t-test, two-tailed). When the number of neurons with increasing CV after E_2 administration were counted, the significant majority of neurons (18 of 26 analyzed ROI) were characterized by an increase of CV after starting E_2 perfusion (post-hoc analysis, χ^2 test for distributions, $P = 0.049$ when the expected probability for an increase or decrease of CV was set to $P = 0.5$). The mean time interval of transients and the mean duration of transients were not significantly different before (mean time interval 74.2 ± 78.0 s; mean duration of transients 6.2 ± 1.5 s) and after (mean time interval 91.8 ± 94.4 s; mean duration of transients 5.7 ± 1.6 s) starting E_2 perfusion (post-hoc analyses, paired t-tests, two-tailed, $P = 0.38$ and a trend with $P = 0.17$ for the duration of transients).

Perfusion with calcium-free ACSF stops calcium oscillations

The perfusion with calcium-free ACSF ($CaCl_2 \cdot 2H_2O$ was replaced by $MgSO_4 \cdot 7H_2O$) stopped calcium oscillations in all measured cells ($n = 15$) after 3 min of calcium-free perfusion (one experiment in one animal; Fig. 2A). Controls were 39 neurons observed for 3 min under R-ACSF perfusion. About 13 of these 39 cells stopped calcium oscillations spontaneously within 3 min of observation. Post-hoc analysis showed that perfusion with calcium-free ACSF significantly stopped calcium oscillations 3 min after starting calcium-free perfusion (Fisher-test, $P = 0.01$).

Tetrodotoxin (TTX) inhibits intracellular calcium oscillations

TTX was used to inhibit sodium channels. Spontaneously oscillating cells were observed (13 cells from two slices of one animal) and TTX 1 μM was added for 300 s. About 10 of 13 cells stopped oscillations within this time interval (Fig. 2B). This result was controlled by the observation of seven oscillating cells in two slices of one animal. All seven cells continued oscillations over 480 s (post-hoc analysis with Fisher-test, $P = 0.04$).

Ryanodine evokes intracellular calcium release in neurons perfused with calcium-free ACSF

Ryanodine was applied in a low concentration to release calcium from intracellular storages. Slices were perfused with calcium-free ACSF (four slices from one animal). Ryanodine 100 nM was added. All measured neurons ($n = 6$) stopped calcium oscillations under perfusion with calcium-free ACSF and were characterized by a significant

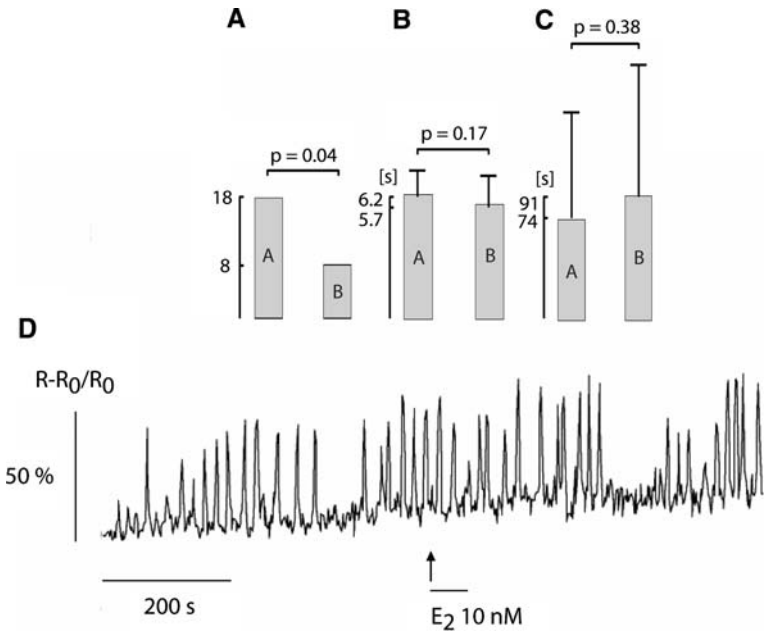

Fig. 1 (A) The significant majority of neurons was characterized by an increase (column A; $n = 18$) of the coefficient of variation (CV) after starting the administration of E_2 10 nM. The minority was characterized by a decrease of CV ($n = 8$). (B) Neurons ($n = 26$) reduced the mean duration of calcium transients after starting the administration of E_2 10 nM (statistical trend with $P = 0.17$ in post-hoc analysis). (C) Neurons ($n = 26$) increased the mean intervals of calcium transients due to administration of E_2 10 nM (not significant). (D) The figure displays a representative neuron. The neuron is characterized by spontaneous calcium oscillations. Spontaneous calcium oscillations were observed in 32.5% of recorded neurons (total $n = 120$ recordings). E_2 10 nM was applied for 60 s (arrow indicates the start of E_2 application). The illustrated cell increased CV from 0.287429 to 0.329018 after the start of E_2 administration. The vertical bar indicates 50% $R - R_0/R_0$. R is defined by the ratio of light intensities of 340–380 nm in the measured ROI. R_0 indicates the baseline ratio in the first minute of recording

intracellular calcium release after administration of ryanodine (Fig. 2C). In the control group, none of the 15 observed cells was characterized of a significant calcium release after oscillations stopped under calcium-free perfusion. Post-hoc analysis showed that ryanodine administration significantly increased intracellular calcium after starting calcium-free perfusion (Fisher-test, $P < 0.001$).

t-ACPD evokes calcium transients in neurons perfused with calcium-free ACSF

t-ACPD was used to activate the metabotropic glutamate receptor inducing intracellular generation of inositol triphosphate (IP_3). Slices were perfused with calcium-free ACSF (four slices from one animal) and all cells stopped calcium oscillations. Subsequently, t-ACPD 5 μM was added. Calcium transients were visible in 9 of 10 measured cells in a time period of 120 s after start of t-ACPD perfusion (Fig. 2D). The intracellular calcium release was significantly related to the administration of t-ACPD ($P < 0.001$ in post-hoc analysis with Fisher-test; controls were 15 cells with calcium-free perfusion and without administration of t-ACPD. None of the controls was characterized by a significant calcium release.).

E_2 evokes calcium transients in neurons perfused with calcium-free ACSF

Slices were perfused with calcium-free ACSF (five slices from five different animals). E_2 10 nM was added to calcium-free ACSF for 60 s. About 13 of 28 measured cells (46%) were characterized by a significant increase of intracellular calcium within a time period of 120 s after start of E_2 perfusion (Fig. 3A). The present calcium release was significantly associated with the administration of E_2 ($P = 0.001$ in post-hoc analysis with Fisher-test; controls were 15 cells with calcium-free perfusion and without administration of E_2 or cyclodextrin. None of the controls was characterized by a significant increase of intracellular calcium.).

E_2 does not increase intracellular calcium in neurons pretreated with thapsigargin

Thapsigargin was used to inhibit the intracellular reuptake of released calcium. Slices were perfused with thapsigargin 2 μM in R-ACSF (two slices from two different animals). Calcium oscillations disappeared in all eight measured cells. Then, E_2 10 nM was added to R-ACSF for 60 s. None of the observed cells was characterized by an in-

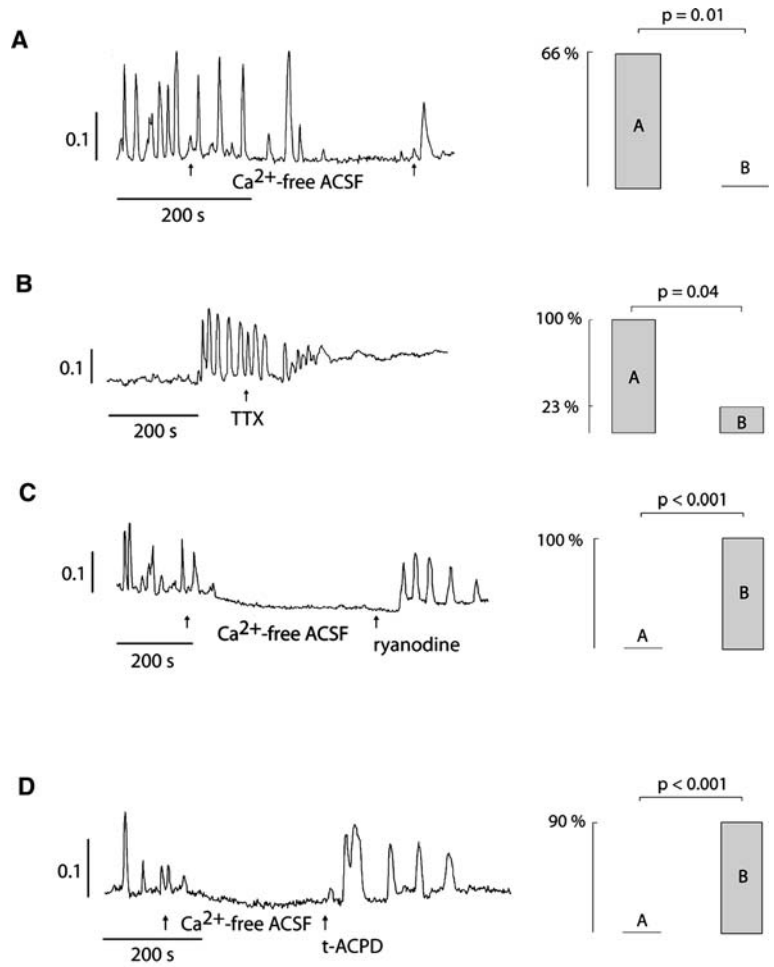

Fig. 2 (**A**) On the left a representative experiment is displayed. The substitution of calcium-free ACSF by R-ACSF could restitute calcium oscillations after the perfusion with calcium-free ACSF. The replacement of R-ACSF by calcium-free ACSF significantly stopped calcium oscillations in neurons of the ARC. Column A represents the control group with $n = 39$. Thereby, 33% of the cells stopped calcium oscillations spontaneously. Column B indicates the treatment group with $n = 15$. All cells of the treatment group stopped calcium oscillations. (**B**) The administration of 1 μM of the sodium channel inhibitor TTX significantly inhibited calcium oscillations, which is exemplarily displayed on the left. About 23% of the cells in the treatment group ($n = 13$) continued calcium oscillations (column B). In contrast, 100% of the cells in the control group ($n = 7$) sustained oscillations spontaneously (column A). (**C**, **D**) The administration of 100 nM ryanodine (right arrow, C; low concentrated ryanodine evokes intracellular calcium release) or 5 μM t-ACPD (right arrow, D; t-ACPD activates the metabotropic glutamate receptor to induce the production of IP_3) significantly reinstalled calcium transients that had been inhibited by perfusion of calcium-free ACSF (start is indicated by the left arrow). The control group is represented by column A ($n = 15$) and the treatment group by column B ($n = 6$ for the administration of ryanodine and $n = 10$ for the administration of t-ACPD). In all experiments, the vertical bar indicates 0.1 change of the measured intensity ratio for 340/380 nm

crease of intracellular calcium after administration of thapsigargin ($n = 8$). Controls were six cells (two slices from two animals) only perfused with thapsigargin without subsequent administration of E_2 or cyclodextrin. Calcium oscillations disappeared in all control cells when thapsigargin was administered.

The effect of ET-18 on E_2 induced intracellular calcium release in calcium-free ACSF

ET-18-OCH3 was applied as a non-selective inhibitor of phospholipase C (PLC). Slices were perfused with calcium-free ACSF (four slices from four animals). ET-18 30 μM was added after 3 min for 60 s. Subsequently, the slice was perfused with E_2 10 nM and ET-18 30 μM in calcium-free ACSF. About 6 of 23 measured cells (26%) were characterized by an intracellular calcium transient within 120 s after the administration of E_2 (Fig. 4A). This inhibition of the E2 induced calcium transient was a trend but not significant (post-hoc analysis with Fisher-test, $P = 0.16$; controls were 28 cells perfused with calcium-free ACSF and a subsequently with E_2 10 nM. About 13 of 28 controls were characterized by a significant calcium transient after E_2 administration).

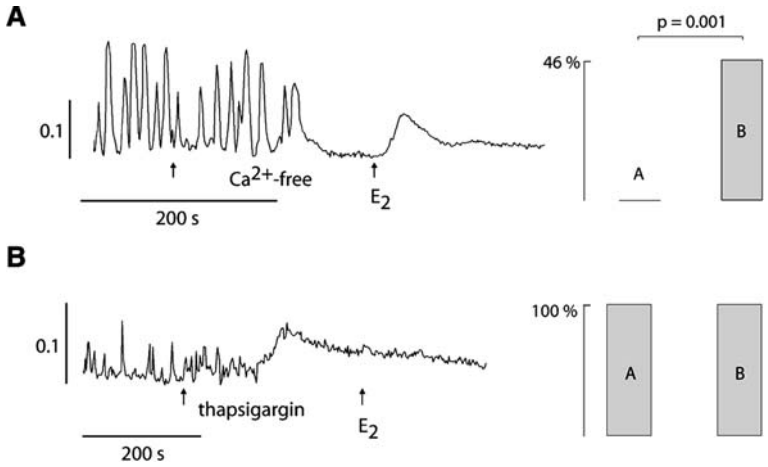

Fig. 3 (**A**) On the left, R-ACSF was replaced by calcium-free ACSF (left arrow) perfusing a spontaneously oscillating neuron. Calcium oscillation stopped under calcium-free ACSF. When E_2 10 nM was added for 60 s (right arrow) a calcium transient became visible in 13 of 28 (46% in column B on the right) recorded neurons. In contrast, none of the recorded neurons of the control group ($n = 15$) was characterized by a calcium transient. (**B**) The experiment exemplarily displays the administration of thapsigargin 2 μM (left arrow; thapsigargin inhibits the calcium reuptake into intracellular storages) effecting an increase of the intracellular calcium concentration. The later administration of E_2 10 nM for 60 s (right arrow) did not induce an elevation of the intracellular calcium concentration. This effect was significantly visible in the treatment group (column B with $n = 8$) in contrast to the control group (column A with $n = 6$). Taken together, E_2 10 nM induces a release of calcium from intracellular stores in seconds what was inhibited by the previous administration of thapsigargin. In all experiments, the vertical bar indicates 0.1 change of the measured intensity ratio for 340/380 nm

The effect of U73122 on E_2 induced intracellular calcium release in calcium-free ACSF

U73122 was used to inhibit the hydrolysis of phosphoinositol phosphate (PPI) to inositol triphosphate (IP_3) by inhibition of the coupling of G protein for PLC activation. Slices were perfused with calcium-free ACSF (five slices from two animals). U73122 10 μM and E_2 10 nM were added simultaneously to ACSF. U73122 was not administered before E_2 to prevent the induction of a calcium

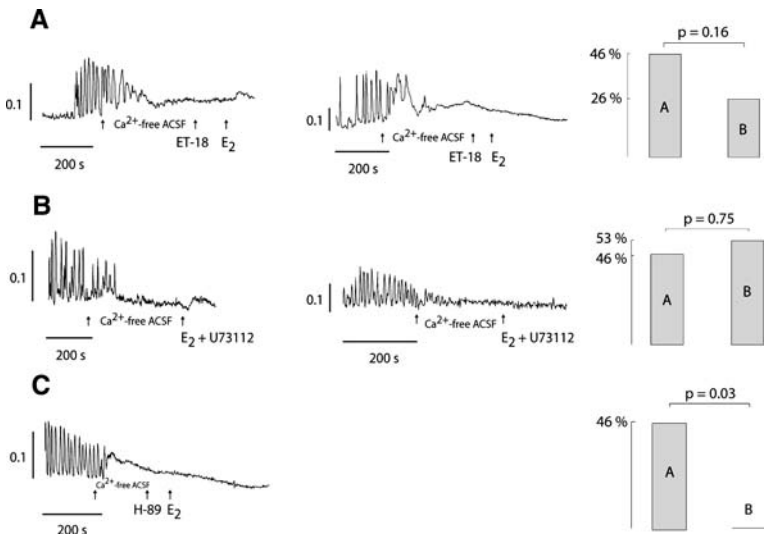

Fig. 4 **A** and **B** describe the effect of PLC inhibitors on REE. (**A**) The administration of ET-18 inhibited calcium transients induced by the application of E_2 10 nM in calcium-free ACSF (26% of the treated cells (total $n = 23$ in column B) were characterized by transients (left experiment) vs. 74% of cells without transients (right experiment) after E_2-administration in comparison to 46% of control cells ($n = 28$ in column A) with transients. (**B**) U73122 did not inhibit calcium transients induced by E_2 [53% of cells of the treatment group (total $n = 15$ in column B) were characterized by E_2 induced transients (left part of **B**) vs. 46% of controls (total $n = 28$ in column A)]. (**C**) The PKA-inhibitor H-89 significantly inhibited E_2 induced transients in the treatment group ($n = 7$ in column B), which is exemplarily displayed on the left. The control group ($n = 28$) was characterized by calcium transients in 46% of the cells. In all experiments, the vertical bar indicates 0.1 change of the measured intensity ratio for 340/380 nm

transient by the putative estrogenic effect of U73122. Subsequently, 8 of 15 cells were characterized by intracellular calcium release after the perfusion with E_2 10 nM (Fig. 4B). Controls were 28 cells, which were perfused with calcium-free ACSF and a subsequent administration of E_2 10 nM. About 13 of 28 controls were characterized by a significant calcium transient after E_2 administration. Post-hoc analysis (Fisher-test) showed that administration of U73122 did not significantly prevent E_2 induced intracellular calcium release ($P = 0.75$).

H-89 inhibits E_2 induced intracellular calcium release

H-89 was used for the inhibition of protein kinase A (PKA). Slices were perfused with calcium-free ACSF (two slices from one animal). H-89 0.25 μM was added. After 60 s $E_2$10 nM was added for further 60 s. None of the seven measured cells were characterized by intracellular calcium release after the addition of E_2 10 nM (Fig. 4C). The post-hoc analysis showed that H-89 significantly prevented the E_2 induced release of intracellular calcium ($P = 0.03$; Fisher-test; controls were 28 cells perfused with calcium-free ACSF and subsequently treated with E_2 10 nM. About 13 of 28 controls were characterized by a significant calcium transient after E_2 administration).

Discussion

The coefficient of variation (CV) was calculated to analyze the hypothesized rapid estrogen effect on the pattern of intracellular calcium oscillations in neurons of the ARC. Interestingly, the CV was relatively low (all calculated CVs were <0.68; mean of CVs = 0.24 ± 0.11) before and after E_2 administration indicating a highly regular pattern of oscillations. Thereby, the non-parametric test (post-hoc analysis with χ^2-test for distributions) indicated a significant rapid estrogen effect when CVs for each ROI were compared before and after start of E_2 administration. The majority of ROI was characterized by an increase of CV indicating a more irregular pattern of calcium oscillations after the start of E_2 administration. To quantify this effect, the putative difference of CVs before and after start of E_2 administration was analyzed by a parametric test in post-hoc analysis. The quantitative analysis (paired *t*-test) did not deliver a significant result for a hypothesized difference of CVs before and after E_2 administration. Therefore, the present rapid estrogen effect on the pattern of calcium oscillations was quantitatively too small to be significantly proven in the present post-hoc analysis.

Sneyd et al. [28], and Shuttleworth and Thompson [29] reported that the calcium oscillation frequency is positively dependent on the calcium entry into the cell. In addition, the increase of the frequency of calcium oscillations also depends on an increase of cytplasmatic IP_3 in cells, which are characterized by a constant and not oscillating cytoplasmatic IP_3 concentration [30]. Thereby, both models of described oscillation generation (constant and oscillating IP_3 concentration) might be present in the same cell [30]. Our data give the suggestion that the frequency of calcium oscillations in neurons of the ARC mainly depended on the calcium entry in combination with an almost constant cytoplasmatic IP_3 concentration because the administration of E_2 induced intracellular calcium release due to the suggested IP_3 generation in calcium-free ACSF and was associated with a very small increase of variation of inter-transient intervals in R-ACSF perfused cells. Interestingly, the time duration of transients was shortened after administration of E_2 in a trend in the present post-hoc analysis ($P = 0.17$, two-tailed) which is in concordance to previously published data on the rapid estrogen effect in mouse oocytes [31]. Probably, longer time intervals of recording and a higher number of observed cells (lowering the variance in general) could have delivered a significant quantitative change for the time duration of transients due to E_2 administration also in the present study.

In the present study, the mean duration of transients was in the range of previously published data for developing and differentiating neurons in the cerebral cortex and the spinal cord [32, 33]. Previously published data also report that a subpopulation of adult gonadotropin releasing hormone neurons are characterized by fast type calcium transients with the mean of 13.7 ± 4.4 s ($n = 8$) which is slightly longer than transients reported in the present study (6.2 ± 1.5 s; $n = 26$) [34]. In contrast, astrocytes are typically characterized by calcium oscillations with a longer duration than the results of the present report and the most type of previously reported neurons [35]. The removal of extracellular calcium inhibited calcium transients in all measured cells within 3 min in the present study. Therefore, the autonomous generation of calcium transients was also dependent on extracellular calcium. Previously published data described that calcium oscillations are dependent on the influx of external calcium to replace internal calcium which has been lost into the extracellular space after the release from intracellular stores [28]. In cortical neurons, calcium transients are associated with the generation of action potentials [36]. In the present study, the inhibition of action potentials by the inhibition of sodium channels (administration of tetrodotoxin) stopped calcium oscillations indicating the possible induction of calcium transients by action potentials leading to voltage gated calcium currents (VGCC). The application of low concentrated ryanodine (activates the ryanodine receptor on intracellular calcium stores) or t-ACPD (induces the production of IP_3) activates the release of intracellular calcium

in neurons [37, 38] and could also induce calcium transients in the present study when extracellular calcium had been removed. Therefore, the sufficient source of intracellular increasing calcium was a necessary condition for the persistence of autonomous calcium oscillations in the present data. In conclusion, calcium transients could be evoked after removal of extracellular calcium, but spontaneous calcium oscillations were strictly dependent on the availability of also extracellular calcium in the present study.

After removal of extracellular calcium, E_2 induced an increase of the intracellular calcium concentration in observed neurons. The inhibition of the intracellular calcium release by thapsigargin inhibited the rapid estrogen effect. Therefore, E_2 likely induces the release of intracellular calcium in the present results. Qiu et al. came to similar conclusions when they investigated the effect of estrogenic agonists working on the cell membrane [39]. They described that estrogenic agents activate PLC in neurons expressing preopiomelanocortine (POMC) in the ARC [12, 39]. Thus, we investigated the dependence of estrogenically triggered intracellular calcium release on the activation of PLC. Interestingly, our results appeared different for two different antagonist of PLC. The non-specific PLC-inhibitor ET-18 may successfully inhibit the present estrogenic effect on intracellular calcium increase (trend with $P = 0.16$). In the previously reported study U73122 was applied to inhibit PLC [39], which was not significantly successful in the present study ($P = 0.75$). Interestingly, U73122 is characterized to be also an estrogenic agonist in the literature [40]. Because calcium transients are based on an all-or-none effect, inhibiting effects on calcium transients become only visible when the IP_3 concentration declines under a specific threshold. The inhibition of PLC by U73122 in combination with agonistic effects of U73122 on the estrogen receptor could be the reason that U73122 has not been characterized to be a significant inhibitor of rapid estrogen effect. In contrast to the present data, in the previously published report the inhibiting effect of U73122 on the rapid estrogen effect was based on the relative alteration of electrical currents which are not based on an all-or-none effect in contrast to transients [39].

The inhibition of PKA inhibited the increase of intracellular calcium due to E_2 administration. This result is concordant to previously published data in neuroblastoma cells published by Vasudevan [8]. PKA might support the induction of calcium by phosphorylation of ryanodine receptors of the endoplasmatic reticulum as previously shown for the effect of cannabinoids on ryanodine-sensitive storages [41].

The present study is characterized by several methodic problems limiting the interpretation of the present data. The characterization of the observed neurons was only based on the visual characterization of ROI and the characterization of calcium transients. Because calcium imaging in perfused hypothalamic slices after non-specific bulkloading is characterized by limited observation periods due to methodic problems [34], the characterization of E_2 effects on the pattern of calcium oscillation was based on relatively short time periods of recording in the present study. Longer intervals of observation could have characterized the specificity of E_2-associated effects more in detail. Because the appearance of spontaneous calcium oscillations may be an intrinsic cellular process [30], the restitution of variables after the experimental intervention does not necessarily restitute calcium oscillations and limited the application of a longitudinal experimental control design in the present study.

In conclusion, present results support the hypothesis that E_2 induces the release of intracellular calcium in neurons of the ARC. Thereby, this rapid effect seemed to be depended on the availability of PLC and PKA. Limited conclusions can be drawn to the relevance of these present results on the synaptic plasticity in the hypothalamus and associated behavior. But present results are promising enough to open new doors in the understanding of hypothalamic physiology and related diseases.

Materials and methods

Reagents

Fura-2 acetoxymethyl ester 1 mM solution in dry 100% DMSO was purchased from Molecular Probes (USA) and diluted to 10 μM solution with R-ACSF (0.1% DMSO in the final solution for incubation).

Regular artificial cerebrospinal fluid (R-ACSF) consisted of glucose 10 mM, $NaHCO_3$ 26 mM, NaCl 124 mM, KCl 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4{\cdot}7H_2O$ 1.3 mM, $CaCl_2{\cdot}2H_2O$ 2.4 mM and distilled water. Calcium-free ACSF was used in two different versions. About 1.3 mM $CaCl_2{\cdot}2H_2O$ was replaced by $MgSO_4{\cdot}7H_2O$ in R-ACSF for the first version. Alternatively, 1 mM EGTA and 17.80 mM sucrose were added to ACSF without $MgSO_4{\cdot}7H_2O$ and $CaCl_2{\cdot}2H_2O$ for the second version. Chemicals were purchased from Fisher Scientific (Hampton, NH, USA).

17β-estradiol (E_2, water soluble and containing cyclodextrin) was purchased from Sigma (St. Louis, MO, USA) and diluted to 10 nM solution with R-ACSF.

U73122 (Sigma, St. Louis, MO, USA) was used to inhibit the hydrolysis of phosphoinositol phosphate (PPI) to inositol triphosphate (IP_3) by inhibition of the coupling of G protein for phospholipase C (PLC) activation. U73122

was dissolved in DMSO (1 mg/ml) and was diluted with R-ACSF to 10 μM solution.

H-89 (Sigma, St. Louis, MO, USA) was used to inhibit protein kinase A (PKA). H-89 was dissolved in DMSO (5 mg/ml) and diluted with R-ACSF to 0.25 μM solution.

Ryanodine (Sigma, St. Louis, MO, USA) was applied in a low concentration to release calcium from intracellular storages. Ryanodine was dissolved in DMSO (1 mg/ml) and diluted to 100 nM solution with R-ACSF.

t-ACPD (Sigma, St. Louis, MO, USA) was used to activate the metabotropic glutamate receptor inducing intracellular generation of IP_3. t-ACPD was dissolved in distilled water (2.5 mg/ml) and diluted to 5 μM solution with R-ACSF.

The non-selective PLC-inhibitor ET-18-OCH3 (Sigma, St. Louis, MO, USA) was administered to investigate the putative association of PLC to the rapid estrogen effect. ET-18 was dissolved in ethanol (15 mg/ml) and diluted to 30 μM solution with R-ACSF.

Thapsigargin (Sigma, St. Louis, MO, USA) was used to inhibit the intracellular reuptake of released calcium. Thapsigargin was dissolved in DMSO (5 mg/ml) and diluted to 2 μM solution with R-ACSF.

Tetrodotoxin (TTX, Fisher Scientific, Hampton, NH, USA) was administered to inhibit sodium channels. TTX was dissolved in R-ACSF and diluted to 1 μM solution with R-ACSF.

When DMSO was used for dissolving reagents, the applied solutions for perfusion contained ≤0.01% DMSO in their final concentration.

Animals

Female Swiss-Webster mice (n = 27) were purchased from Charles River Laboratories for the present experiments. Mice were housed in groups of 3–6 mice in plastic cages (30 × 20 × 15 cm) and were maintained on a 12:12 light/dark cycle (light on 10 p.m. through 10 a.m.) at constant room temperature (21–23°C). All animals had mouse chow and water ad libitum and were cared for in accordance with the National Institute of Health, The Rockefeller University Animal Care and Use Committee guidelines. Animals were not ovariectomized. The mean age of animals was 30.6 ± 11.0 days p.n. (median 27 days) when experiments were performed. The minimum age was 22 days p.n. Two animals were older than 35 days p.n. (77 and 41 days p.n.) and were used for experiments describing E_2 administration after perfusion with calcium-free ACSF. Therefore, the majority of mice was pre-pubertal (less than 38 days p.n.) according to Gehring [42] and Morrow [43] and should not have an ovarial synthesis of estrogens as suggested in pubertal mice.

Preparation and incubation of brain slices

Mice were anaesthesized by the intraperitoneal injection of urethane. Anaesthesized mice were sacrificed by use of a gilloutine. The complete brain was removed and secured in ice-cooled R-ACSF gazed with 95% O_2 and 5% CO_2 (temperature around 5°C). A tissue block containing the hypothalamus was cut under the binoculars. The tissue block was fixed on a plate and set into a microtome chamber circulated by gazed R-ACSF at a temperature of 5°C. The microtome (Leica VT 1000 S, Leica, Nussloch, Germany) was used to cut brain slices containing the hypothalamus in a frontal orientation with a thickness of 200–300 μm. Slices were stored on a grid in R-ACSF gazed with 95% O_2 and 5% CO_2 at room temperature (21–23°C) for 1 h minimum. Then, a single slice was incubated in a chamber (volume 3 ml) in R-ACSF containing 10 μM fura-2AM for 1 h under protection from light. Thereby, slices were gazed with 95% O_2 and 5% CO_2 at room temperature. Finally, the slice was transferred into the recording chamber and washed by oxygenated (95% O_2) and buffered R-ACSF (5% CO_2) for 30 min in the darkness. Normal light microscopy was used at 10× and 40× to detect the ARC and neurons in the slice before recording was started.

Measurement of changes of the intracellular [Ca^{2+}]

ACSF, E_2 and reagents were administered with a perfusion at room temperature (21–23°C). The dead time was 60 s (flow 1.8 ml/min) and 30 s (flow 3.6 ml/min) between the vials of treatment solution and the chamber (volume 1 ml) where the brain slice was perfused. All displayed results were corrected by the dead time in figures when the application of reagents is indicated by an arrow in the timeline. Neurons were identified microscopically by their typical shape and morphology in the slice and by calcium oscillations with spontaneous transients of less than 10 s duration.

The measurements were performed on a electrophysiological recording unit equipped with upright microscope (Olympus, Tokyo, Japan), xenon lamp, CCD camera (Media Cybernetics, Silver Spring, MD, USA) and shutter-system with a filter exchanger (Lambda 10-2, Sutter Instrument Company, Novato, CA, USA) with filters for 340 and 380 nm controlled by a PC using the MetaFluor software (Universal Imaging, Dowingtown, PA, USA). Signals were collected from regions of interest (ROI) corresponding to a single cell also using the MetaFluor program. Background measurements were made from an area without cells equally sized to the observed ROI. Signals were obtained in dual excitation mode (ratio 340/380 nm) at 510 nm filtered. The time lapse of signal

acquisition was set to 1 s in all experiments. Binning was set to 2 and gain was adjusted at 2500. The time of light exposition was adjusted in every experiment individually. Therefore, measured intensities were not comparable between different experiments. After background subtraction, the obtained signal was transformed in percentage of ratio change in relation to the lowest ratio (R_0) obtained in the first minute of data acquisition by the formula $R\% = 100 (R - R_0)/R_0$ in all experiments describing the spontaneous and estradiol induced calcium oscillation pattern. When calcium transients of oscillations were analyzed, a transient was identified after subtraction of the background signal and transformation to $R\%$ by the use of a self-written program based on EXCEL 2003 (Microsoft, Redmond, WA, USA). To detect the start of a transient the criteria was an increase of $\Delta R\% \geq 25\%$. The end of a transient was indicated by $\Delta R\%$ between –25 and +25% when the previous $\Delta R\%$ had been negative. The program was used to count the number of transients, to determine the duration of transients and to calculate the frequency of calcium oscillations. Three consecutive increases of the ratio were set to indicate a significant increase of intracellular calcium in experiments focused on intracellular calcium release due to administered reagents. In this way, the present study used different criteria to detect an increase of intracellular calcium and to indicate an autonomous generated calcium transient to distinguish calcium transients in oscillations from isolated transients due to reagent stimulated intracellular calcium release. These different criteria were used because calcium transients of calcium oscillations were often characterized by a higher slope and shorter duration than the intracellular calcium release due to administered reagents.

Statistics

The coefficient of variation (CV = standard deviation/mean) was used to describe a variation of the calcium oscillation pattern (variation of the interval between transients before and after start of E_2 administration) [31]. The number of ROI with increasing CV after E_2 administration was compared to the estimated number of the H_0 (50% chance of an increase or decrease of CV after starting the administration of E_2) by χ^2 test for distributions in post-hoc analysis. Quantitative differences of CVs before and after start of the E_2 administration were calculated. The mean of differences was analyzed for significance by a paired t-test, (two-tailed). The number of ROI with events (increase of intracellular calcium or calcium transient) was compared to controls by post-hoc Fisher-test in reagent treated slices. A P value of less than 0.05 was considered as significant for all statistical tests. The software package NCSS (Kaysville, UT, USA) was used for statistical analyses.

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