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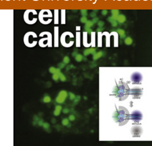

Fluoxetine suppresses calcium signaling in human T lymphocytes through depletion of intracellular calcium stores

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ABSTRACT

Selective serotonin reuptake inhibitors, such as fluoxetine, have recently been shown to exert anti-inflammatory and immunosuppressive effects. Although the effects on cytokine secretion, proliferation and viability of T lymphocytes have been extensively characterized, little is known about the mechanism behind these effects. It is well known that Ca^{2+} signaling is an important step in the signaling transduction pathway following T cell receptor activation. Therefore, we investigated if fluoxetine interferes with Ca^{2+} signaling in Jurkat T lymphocytes. Fluoxetine was found to suppress Ca^{2+} signaling in response to T cell receptor activation. Moreover, fluoxetine was found to deplete intracellular Ca^{2+} stores, thereby leaving less Ca^{2+} available for release upon IP_3- and ryanodine-receptor activation. The Ca^{2+}-modifying effects of fluoxetine are not related to its capability to block the serotonin transporter, as even a large excess of 5HT did not abolish the effects. In conclusion, these data show that fluoxetine decreases IP_3- and ryanodine-receptor mediated Ca^{2+} release in Jurkat T lymphocytes, an effect likely to be at the basis of the observed immunosuppression.

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1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) have been shown to exert anti-inflammatory and direct immunosuppressive effects such as suppression of T cell activation, cytokine secretion and proliferation and induction of apoptosis *in vitro* and *in vivo* [1–3]. Although it has been shown that these compounds have a high affinity for the serotonin transporter (SERT) in the central nervous system, it is not clear whether the immunological effects of SSRIs are mediated by inhibition of SERT-mediated serotonin (5HT) uptake in lymphocytes. On the contrary, several arguments oppose to the involvement of 5HT and SERT in the immunosuppressive effects of SSRIs, especially the discrepancy between the concentration needed for blockage of 5HT uptake on the one hand (nM range) and for *in vitro* immunosuppression on the other hand (μM range) [4,5]. The actual mechanism underlying the immunosuppressive effects of SSRIs has not been elucidated yet.

Elevation of the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is one of the key triggering signals for T cell activation. The $[Ca^{2+}]_i$ is regulated through an intimate interplay between Ca^{2+} in the extracellular space and intracellular storage sites such as the endoplasmic reticulum (ER). Ca^{2+} signaling mechanisms mostly rely on Ca^{2+} release from the ER through inositol 1,4,5-trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) following the activation of G-protein coupled receptors on the plasma membrane. Subsequent depletion of the ER triggers store-operated, capacitative Ca^{2+} entry to replenish the ER [6]. SSRIs have been shown to affect Ca^{2+} signaling in several cell types. Fluoxetine inhibited ATP-induced Ca^{2+} increases in PC12 cells through inhibition of both influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular stores [7]. Whereas fluoxetine has also been shown to suppress Ca^{2+} spikes in cultured rat hippocampal neurons, two other SSRIs, namely paroxetine and citalopram, did not [8]. Furthermore, chronic exposure of astrocytes to fluoxetine diminished RyR- and IP_3R-mediated Ca^{2+} release as well as the subsequent capacitative Ca^{2+} entry [9]. In microglia, pretreatment with paroxetine or sertraline reduced the amplitude of the Ca^{2+} increase induced by interferon-gamma (IFNγ) [10]. Oppositely, sertraline induced a Ca^{2+} rise in MG63 osteosarcoma cells [11]. Fluoxetine, paroxetine and citalopram induced a rise in $[Ca^{2+}]_i$ in Burkitt lymphoma

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cells [12]. In platelets, SSRIs (sertraline, paroxetine, fluoxetine) potentiated thrombin-mediated increases in intracellular Ca^{2+} [13]. Clearly, SSRIs are capable of interfering with Ca^{2+} signaling in a wide variety of cell types. Furthermore, it has been suggested that fluoxetine interferes with mitogen-induced Ca^{2+} influx in murine and human T lymphocytes as fluoxetine exerted similar effects as the Ca^{2+} ionophore A23187 on T cell proliferation, protein kinase C (PKC) degradation and cAMP levels [14,15].

Given the importance of Ca^{2+} signaling in T cell activation [6], we investigated whether interference with Ca^{2+} signaling might be at the basis of the immunosuppressive effects of fluoxetine in Jurkat T lymphocytes. In addition, we investigated whether the observed effects on Ca^{2+} signaling are related to the inhibition of 5HT uptake.

2. Experimental procedures

2.1. Cell culture

Jurkat T cells, clone E6-1, were cultured at 37 °C and 5% CO_2 in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin (100 U/ml penicillin G; 100 μg/ml streptomycin). All cell culture reagents were purchased from Life technologies (Carlsbad, CA, USA).

2.2. Buffers and chemicals

Krebs HEPES buffer contained 133.5 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.6 mM HEPES, 11.5 mM glucose and 1.5 mM $CaCl_2$, pH 7.4. In Ca^{2+}-free Krebs buffer, $CaCl_2$ was replaced by 4.5 mM EGTA (pH 7.4) preserving equimolarity with the Krebs HEPES buffer. No external 5HT was added to the buffer. 1,2-Bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM), D-*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester ("NPE-caged IP_3"), dextran Texas Red 10,000 MW (DTR), fluo3-AM and thapsigargin were purchased from Molecular Probes, Life technologies. Caffeine was from Sigma–Aldrich (St. Louis, MO, USA), ryanodine from Abcam (Cambridge, UK) and fluoxetine from ABC chemicals (Woutersbrakel, Belgium).

2.3. Visualization of intracellular Ca^{2+}

Dynamic changes in $[Ca^{2+}]_i$ were monitored using fluo3-AM. Cells in suspension were loaded with 5 μM fluo3-AM at 2×10^6/ml in Krebs buffer for 1 h at room temperature and subsequently washed 3× in Krebs buffer. Thereafter, cells ($0.5–1 \times 10^6$) were allowed to adhere on poly-L-lysine (0.1%) coated 18 mm diameter glass coverslips and left for 30 min at room temperature for de-esterification and settling on the dish. Cells were washed once in Krebs buffer to remove any unbound cells before imaging.

Intracellular Ca^{2+} imaging was performed in Krebs buffer at room temperature and was carried out using a Nikon Eclipse TE300 inverted epifluorescence microscope (Nikon Belux, Brussels, Belgium), equipped with a 40× oil-immersion objective (Plan Fluor, NA 1.30; Nikon) and an EM-CCD camera (QuantEM™ 512SC CCD camera, Photometrics, Tucson, AZ). We used a Lambda DG-4 filterswitch (Sutter Instrument Company, Novato, CA) to deliver excitation at 482 nm and captured emitted light *via* a 505 nm long-pass dichroic mirror and a 535 nm bandpass filter (35 nm bandwidth). Images (1/s) were generated with custom-generated QuantEMframes software written in Microsoft Visual C++ 6.0. Fluo3 fluorescence-intensity changes were analyzed with custom-developed FluoFrames software (generated by L.L., Ghent University, Belgium). Background fluorescence was subtracted from all images. Traces of individual cells were obtained by point analysis in FluoFrames software.

2.4. Electroporation loading with NPE-caged IP_3 and photoliberation

In order to study IP_3-mediated Ca^{2+} release from the ER, T cells were loaded with NPE-caged IP_3 through electroporation, as described elsewhere [16]. Briefly, cells seeded on coverslips were washed 3× with a low conductivity electroporation buffer (4.02 mM KH_2PO_4, 10.8 mM K_2HPO_4, 1.0 mM $MgCl_2$, 300 mM sorbitol, 2.0 mM HEPES, pH 7.4). The coverslips were then placed on the microscopic stage, 400 μm underneath a parallel wire Pt–Ir electrode and electroporated in the presence of 10 μl electroporation buffer containing 100 μM NPE-caged IP_3 and 100 μM DTR to visualize the electroporation zone. Electroporation was done with 50 kHz bipolar pulses applied as trains of 10 pulses of 2 ms duration each and repeated 15 times. The field strength was 100 V peak-to-peak applied over a 500 μm electrode separation distance. After electroporation, cells were thoroughly washed with Krebs buffer. Electroporation was performed after fluo3 loading and did not result in loss of fluo3 from the cells [17].

After loading with NPE-caged IP_3, coverslips were transferred to the microscope stage for Ca^{2+} imaging. Photoliberation of IP_3 was done by spot (20 μm diameter) illumination with 1-kHz pulsed UV light (349 nm UV laser Explorer, Spectra-Physics, Newport, Utrecht, The Netherlands) applied during 50 ms (50 pulses of 90 μJ energy measured at the entrance of the microscope epifluorescence tube).

2.5. Activation with anti-CD3/CD28 beads

T cell receptor activation was achieved by adding magnetic particles coated with antibodies against CD3 and CD28 (Dynabeads® Human T-Activator CD3/CD28, Life technologies) at a concentration of 25 μl per 10^6 cells (1:1 bead:cell ratio). Cells were visually inspected under the microscope at the end of each experiment to determine which cells were making contact with at least one magnetic bead.

2.6. Data analysis

The statistical analysis was conducted in 'R' [18]. Homoscedasticity and normality of residuals were visually checked using residuals *vs* fitted plots and QQ plots. If necessary, power transformations were applied, using a Box–Cox plot for guidance [19]. The datasets in Sections 3.1, 3.4 and 3.7 were analyzed using a one-way ANOVA or two-way ANOVA (to correct for the possible influence of time if the experiment was conducted for >1 day). The other datasets (Sections 3.2, 3.3, 3.5 and 3.6) were analyzed using the non-parametric Kruskal–Wallis test. Student's *T* tests (parametric) or Wilcoxon rank sum tests (non-parametric) were used for *post hoc* testing with a Holm–Bonferroni correction for multiple testing being applied. Data are presented as mean ± SD or median and range, for parametric and non-parametric data, respectively. Significance was set at $p < 0.05$, all tests were two-tailed.

Data are visually presented as boxplots showing median, first and third quartile. Whiskers represent lowest and highest data within 1.5 interquartile range (IQR). Data exceeding the 1.5 IQR were omitted from the graphs for clarity.

2.7. Digital droplet (dd)PCR

mRNA was isolated using the RNeasy® mini kit (QIAGEN, Hilden, Germany) and on column digestion of genomic DNA was performed using the RNase-free DNase set (QIAGEN). RNA concentrations were

determined with the Quant-it™ Ribogreen® RNA assay kit (Bio-Rad, Hercules, CA, USA). RNA quality assessment using microfluidic capillary electrophoresis (Experion RNA HighSens Chip, Bio-Rad) showed good quality RNA samples as determined by 18S/28S rRNA ratios (RNA quality index [RQI] 8.5 for positive control, 9.3–9.4 for representative T lymphocyte samples; a RQI > 7 indicates good quality). mRNA was transcribed to cDNA using the iScript™ advanced cDNA synthesis kit (Bio-Rad). cDNA concentrations were subsequently estimated using the Quant-it™ oligreen® ssDNA kit (Life technologies). All kits were used according to the manufacturer's instructions. A commercially available Taqman® assay (Hs00984349_m1; Life technologies) was used for amplification of serotonin transporter cDNA. 20 μl reactions were prepared containing 5 μl of cDNA (500 ng input material), 10 μl 2× ddPCR™ super mix for probes (Bio-Rad), 1 μl Taqman® assay and 4 μl water. ddPCR assays were performed as described previously [20]. Briefly, droplets were generated in 8-channel cartridges containing the 20 μl samples plus 50 μl droplet generating oil using the QX100™ droplet generator (Bio-Rad). Subsequently, droplet-in-oil suspensions were transferred to 96 well plates and placed into a T100™ Thermal Cycler (Bio-Rad). Cycling conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Subsequently, the droplets were automatically read by the QX100™ droplet reader (Bio-Rad) and the data were analyzed with the QuantaSoft™ analysis software 1.2.10.0 (Bio-Rad). All samples were tested in duplicate. No-template controls (NTCs) were included in every ddPCR run. HEK 293 cells stably transfected with hSERT (kind gift from Randy Blakely, Vanderbilt University) were used as a positive control.

2.8. Western blotting

10^7 cells were lysed in 1 ml radioimmunoprecipitation (RIPA) buffer containing 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 and supplemented with 5 mg/ml Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland), 10 μl/ml phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich) and 1 μl/ml benzonase® nuclease (Sigma–Aldrich). 50 μg of total protein was subjected to 10% sodium dodecyl sulphate polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane using Tris–glycine buffer (25 mM Tris base, 190 mM glycine, 0.05% SDS), as described elsewhere [21]. Serotonin transporter protein was detected with a 1:5000 dilution of ST51-1 (aa51-66) mouse monoclonal anti-human serotonin transporter antibody (Santa Cruz Biotechnology, CA, USA) in PBS + 0.3% Tween-20 + 10% nonfat dry milk overnight at room temperature. Incubation with secondary goat anti-mouse poly-HRP antibody (Thermo Fisher Scientific, Waltham, Massachusetts, USA) (1:1000 dilution) was performed during 1 h at room temperature in PBS + 0.3% Tween-20 + 5% nonfat dry milk. Protein bands were detected with enhanced chemiluminescence. HEK 293 cells, stably transfected with hSERT were used as a positive control. Specificity of the primary antibody was confirmed with a blocking peptide (PSPGAGDDTRHSIPAT; Thermo Fisher Scientific, Waltham, Massachusetts, USA). The ST51 antibody was incubated for 2 h at room temperature with the blocking peptide at 5-fold excess in PBS, as described elsewhere [21]. Then, the antibody-peptide mixture was further diluted in PBS containing 0.3% Tween-20 and 5% nonfat dry milk and added to the membrane.

2.9. Detection of T cell activation

In order to detect T cell activation, T cells were stimulated with anti-CD3/CD28 beads in a 1:1 bead:cell ratio for 5 h at 37 °C and 5% CO_2. Fluoxetine or BAPTA-AM were added 30 min before addition of the T cell stimulus and were maintained in the culture medium throughout the experiment. After 5 h incubation, cells were stained with anti-human CD69 PECy7 and anti-human CD3 PECy5 (eBioscience, San Diego, CA, USA) for 30 min in PBS + 1% bovine serum albumin and 0.1% NaN_3, washed once and analyzed on a FC500 (Beckman coulter, Fullerton, CA, USA).

3. Results

3.1. Fluoxetine suppresses Ca^{2+} signaling in response to T cell receptor activation

In order to analyze the effect of fluoxetine on Ca^{2+} signaling when T cells are activated through the T cell receptor (TCR), we activated Jurkat T cells with magnetic particles coated with antibodies against CD3 and CD28 and analyzed the resulting changes in $[Ca^{2+}]_i$ through labeling with the fluorescent Ca^{2+} dye fluo3-AM. The fluorescent images obtained and the response of T cells to the anti-CD3/CD28 beads are illustrated in Fig. 1A. After each experiment, T cells making contact with at least one magnetic bead were visualized and selected for analysis. The changes in fluorescent signal over time (arbitrary units, A.U.), which relate to changes in $[Ca^{2+}]_i$, were plotted and result in a Ca^{2+} trace. The majority of cells in the control samples (83%) responded to contact with a bead with a short period of Ca^{2+} oscillations followed by a sustained increase in $[Ca^{2+}]_i$. A small percentage of cells showed an oscillatory pattern (11%) or transient response (6%) after contact with a bead (Fig. 1B). The same types of responses were found in T cells pre-incubated with 10 μM fluoxetine. A slight shift from sustained responses (75%) toward oscillatory (13%) and transient (12%) responses was observed, but no significant changes were detected as compared to control (Chi-square test, $p = 0.28$). However, fluoxetine did affect the magnitude of the response to TCR activation. T cells that were pre-incubated for 30 min with 10 μM fluoxetine (F10) responded with oscillations with smaller amplitude and a weaker sustained increase in $[Ca^{2+}]_i$. At 100 μM fluoxetine (F100), the response to TCR activation was almost completely absent (Fig. 1C). Viability of the cells was assessed by trypan blue staining at the end of the experiment and no increased cell death was observed in samples pre-incubated with fluoxetine compared to control samples (data not shown). The absence of a response in samples incubated with 100 μM fluoxetine was thus not due to loss of viability. In order to quantify the different responses, we calculated the difference between the maximum of the Ca^{2+} peak (maximum value of 0–720 s) and the baseline (mean of 0–120 s). Whereas the mean ± SD peak height of control T cells was 59.18 ± 30.38 (A.U.), the mean peak height of T cells pre-incubated with F10 was 50.97 ± 28.16 ($p = 0.041$) (Fig. 1D). Cells pre-incubated with F100 showed a dramatically reduced response to TCR activation, with a mean peak height of only 7.45 ± 7.05 ($p < 0.0001$). Similar results were obtained when the area under the $[Ca^{2+}]_i$ trace was analyzed instead of the peak $[Ca^{2+}]_i$ change (data not shown). These results show that fluoxetine inhibits the Ca^{2+} signaling pathway following TCR activation in a concentration-dependent manner in T lymphocytes.

3.2. Interference of fluoxetine with intracellular stores or capacitative Ca^{2+} entry

We next questioned whether the observed suppression of $[Ca^{2+}]_i$ responses by fluoxetine was due to either inhibition of capacitative Ca^{2+} entry or interference with the release of Ca^{2+} from intracellular stores. To this end we added thapsigargin (TG), a selective inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that prevents reuptake of Ca^{2+} into the ER, to the cells in Ca^{2+}-free

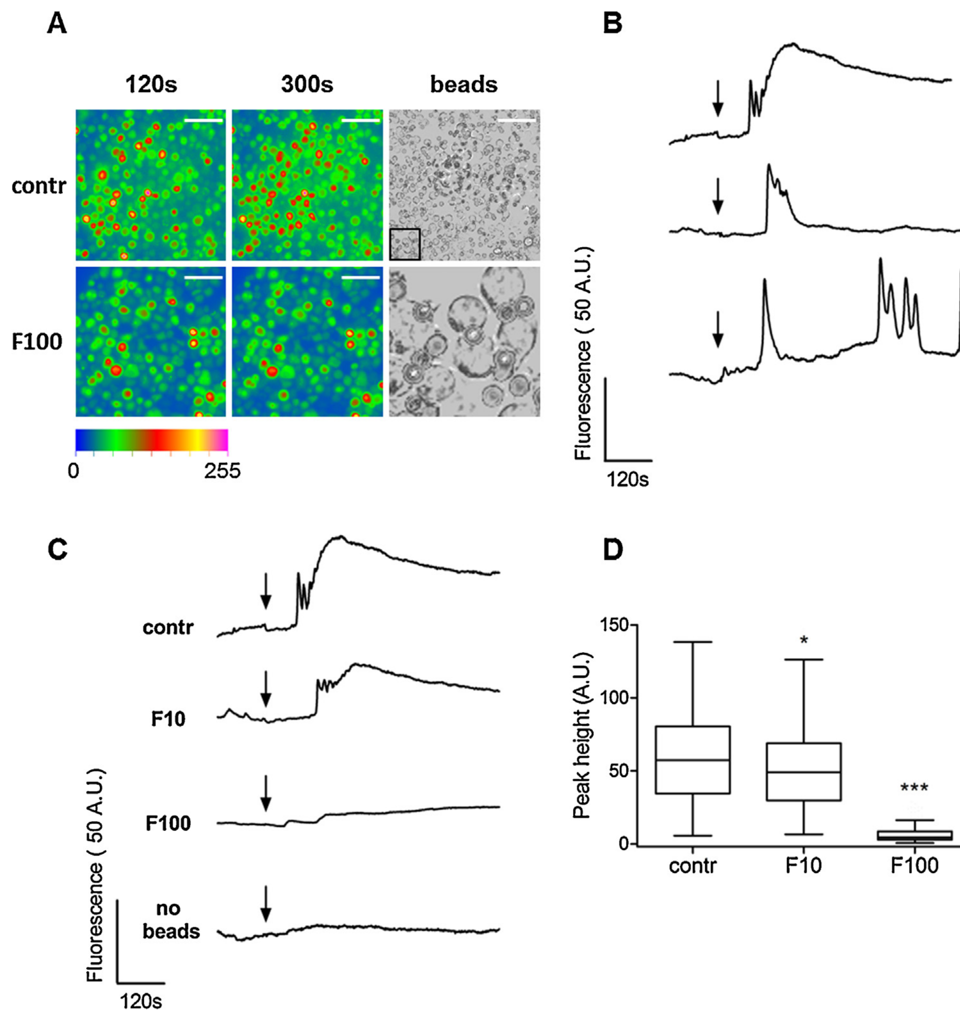

Fig. 1. Effect of fluoxetine on Ca^{2+} signaling in response to TCR activation. T cells were stimulated with magnetic beads coated with anti-CD3 and anti-CD28 antibodies. (A) It shows a series of images taken after addition of anti-CD3/CD28 beads (beads were added at 120 s). The upper right image is a bright field image taken at the end of the experiment, in which the location of the beads can be seen. The lower right image is a magnification of the marked area above. Scale bars are 50 μm. Color scale shows the pseudocolors in the RGB color mode assigned to the 256 shades of gray in an 8-bit image. (B) Representative Ca^{2+} responses induced by anti-CD3/CD28 beads in individual cells. Contact with a bead triggered sustained (top), transient (middle) or oscillatory (bottom) responses. The arrow indicates the addition of the beads. (C) Representative traces of T cells activated with anti-CD3/CD28 beads in Krebs buffer (contr), 10 μM fluoxetine (F10), 100 μM fluoxetine (F100) or without beads (negative control). For the negative control, the arrow indicates addition of an equal amount of buffer without beads. (D) Peak height of the Ca^{2+} response of T cells when stimulated with anti-CD3/CD28 beads in Krebs buffer (contr) or fluoxetine (10 μM, F10 and 100 μM, F100). Peak height was calculated as the difference between the maximum and the baseline. Each condition was repeated at least three times and data were pooled for analysis. In total, 89–146 bead-bound cells per condition were analyzed. * = $p < 0.05$. *** = $p < 0.0001$.

buffer containing EGTA. Ca^{2+} exits the ER through a yet unidentified basal leak system, and blockage of SERCA consequently results in depletion of the ER. After a 5 min incubation period with TG that allowed the ER to be completely emptied (confirmed by subsequent addition of 50 μM cyclopiazonic acid or 100 μM thapsigargin, data not shown), Ca^{2+}-containing buffer (1.5 mM Ca^{2+}) was added, thus allowing the cells to refill their ER with Ca^{2+} through capacitative Ca^{2+} entry (Fig. 2A). The impact of fluoxetine on both steps was analyzed. Interestingly, 30 min pre-incubation of fluoxetine reduced the magnitude of the TG-induced Ca^{2+} release in a concentration-dependent manner (control median 7.90, range [−75.54–192.90]; F10 3.35, [−58.45–180.27], $p < 0.0001$; F100 1.12, [−22.14–99.43], $p < 0.0001$; Fig. 2B). No significant differences could be detected with respect to the magnitude of the peak after addition of Ca^{2+}-containing buffer (control median 38.00, range [7.0–217.3]; F10 44.55, [5.2–196.1]; F100 41.00, [3.3–208.7]; $p = 0.423$; Fig. 2C). Similar results were obtained when using anti-CD3/CD28 beads instead of TG to stimulate the cells. Whereas the initial response to the beads in Ca^{2+}-free buffer was suppressed by fluoxetine (control median 2.69, range [−6.2–85.2]; F10 1.84, range [−2.2–50.1], $p = 0.47$; F100 0.00, range [−6.7–46.1], $p < 0.0001$), the capacitative Ca^{2+} entry was not significantly affected by fluoxetine (control median 42.26, range [2.4–179.3]; F10 47.06, range [12.0–135.5], $p = 0.24$; F100 31.50, range [2.3–120.8], $p = 0.058$) (Fig. 2D–F). These data suggest that fluoxetine might interfere with Ca^{2+} release from intracellular stores. In contrast, fluoxetine does not appear to affect capacitative Ca^{2+} entry in T lymphocytes.

3.3. *Effect of fluoxetine on intracellular Ca^{2+} stores*

The observed inhibition of Ca^{2+} release from intracellular stores could be due to either depletion of the stores or direct inhibition of Ca^{2+} channels responsible for Ca^{2+} release from the ER. To differentiate between both options, a series of experiments was conducted studying the direct effect of fluoxetine on $[Ca^{2+}]_i$. Addition of fluoxetine to resting T cells induces a rise in $[Ca^{2+}]_i$ both in Ca^{2+}-containing and Ca^{2+}-free buffer (Fig. 3A and B). These data indicate that fluoxetine partially depletes the intracellular

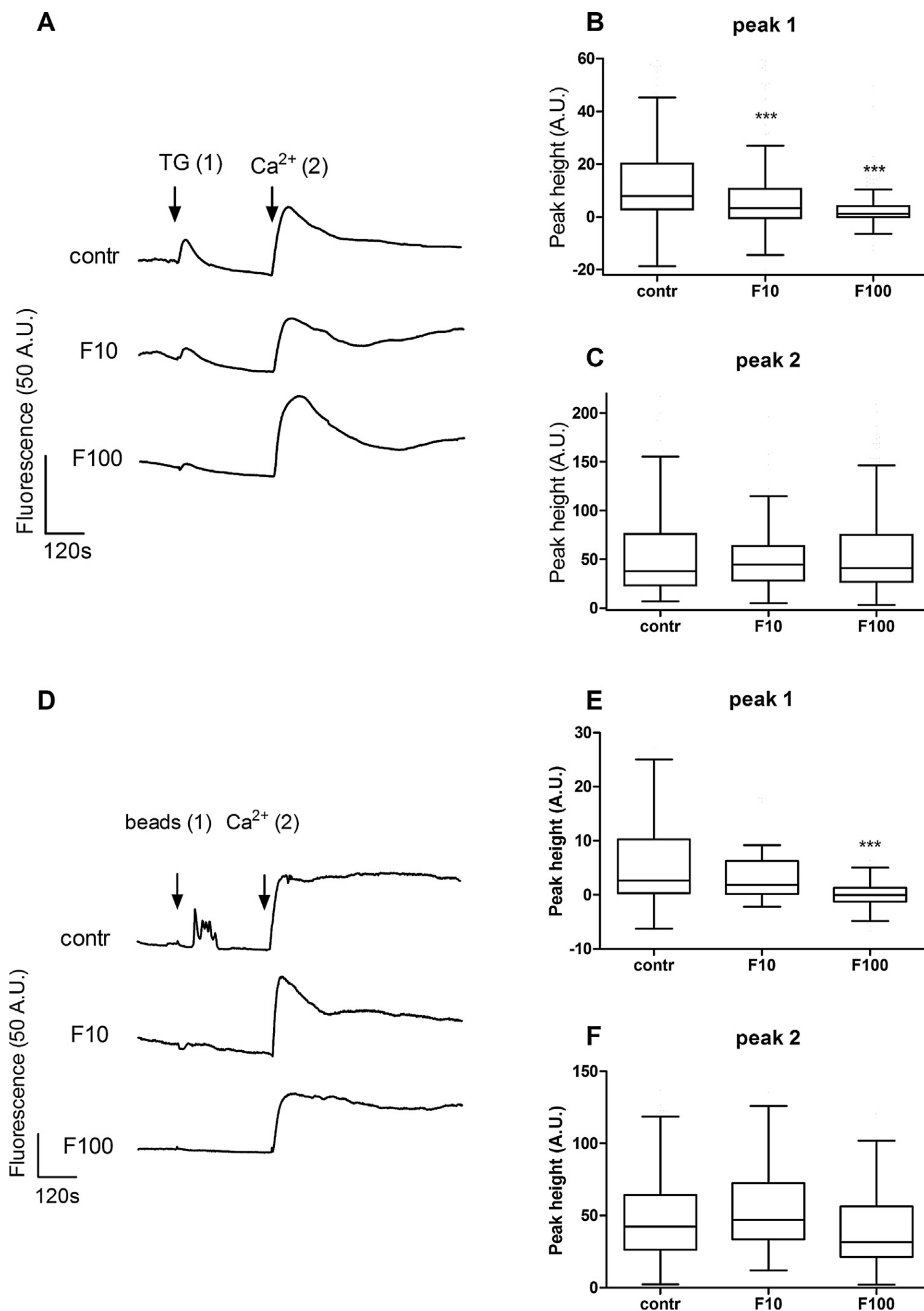

Fig. 2. Effect of fluoxetine on intracellular Ca^{2+} release and capacitative Ca^{2+} entry. Krebs buffer was replaced with Ca^{2+}-free buffer immediately before imaging and 10 μM TG (A–C) or 25 μl anti-CD3/CD28 beads (D–F) were added at 120 s. At 420 s, Ca^{2+}-free buffer was replaced with Ca^{2+}-containing buffer (1.5 mM Ca^{2+}). Cells were pre-incubated with fluoxetine (10 μM, F10 and 100 μM, F100) in Krebs buffer for 30 min, and fluoxetine was maintained in all added solutions. All conditions were repeated at least three times. Data were pooled for analysis. (A) Mean traces of cells in Ca^{2+}-free Krebs buffer (contr), 10 μM fluoxetine (F10) and 100 μM fluoxetine (F100). Arrows indicate addition of thapsigargin (TG, peak 1) and Ca^{2+}-containing buffer (Ca^{2+}, peak 2). (B) Peak height of the Ca^{2+} change induced by TG (peak 1). Peak height was calculated as the difference between the maximum and the baseline. (C) Peak height of Ca^{2+} change induced by re-introduction of Ca^{2+}-containing buffer (peak 2). (D) Representative traces of cells in Ca^{2+}-free Krebs buffer (contr), 10 μM fluoxetine (F10) and 100 μM fluoxetine (F100). Arrows indicate addition of anti-CD3/CD28 beads (beads, peak 1) and Ca^{2+}-containing buffer (Ca^{2+}, peak 2). (E) Peak height of the Ca^{2+} change induced by anti-CD3/CD28 beads (peak 1). Peak height was calculated as the difference between the maximum and the baseline. (F) Peak height of Ca^{2+} change induced by re-introduction of Ca^{2+}-containing buffer (peak 2). *** = $p < 0.0001$.

stores, leaving less Ca^{2+} available for release upon stimulation. If fluoxetine depletes ER stores, it could be expected that capacitative Ca^{2+} entry, which is activated upon emptying of the ER, might also be affected by fluoxetine. However, the peak height of the capacitative Ca^{2+} response after exposure of T cells to fluoxetine was not significantly different from the response elicited in control samples (control median 43.2, range [9.4–83.7]; F100 44.9, [23.8–161.1], $p = 0.19$, Fig. 3B), confirming previous observations that fluoxetine pre-incubation does not alter capacitative Ca^{2+} entry. Finally, it was confirmed that acute depletion of the ER stores with fluoxetine is followed by a lower Ca^{2+} peak in response to TG (Fig. 3C). From these data and those above, it can be concluded that fluoxetine depletes intracellular Ca^{2+} stores.

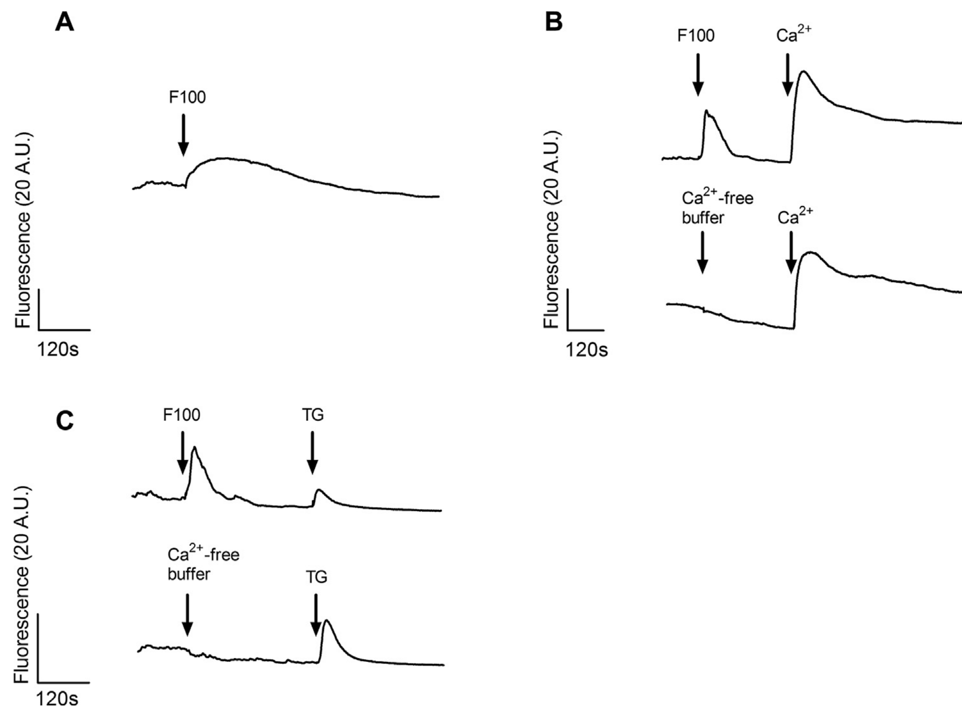

Fig. 3. Fluoxetine depletes intracellular calcium stores. (A) T cells were incubated in Ca^{2+}-containing Krebs buffer and 100 μM fluoxetine (F100) was added at 120 s. (B) Krebs buffer was replaced with Ca^{2+}-free buffer immediately before imaging and 100 μM fluoxetine (F100) or buffer (contr) was added at 120 s. At 420 s, Ca^{2+}-free buffer was replaced for Ca^{2+}-containing Krebs buffer (1.5 mM Ca^{2+}). (C) Krebs buffer was replaced with Ca^{2+}-free buffer immediately before imaging and 100 μM fluoxetine (F100) or Ca^{2+}-free buffer (contr) was added at 120 s. Thapsigargin was added at 420 s. The traces depict the mean response of at least 50 cells.

3.4. Interference of fluoxetine with IP_3-induced Ca^{2+} release

In order to study in more detail the functional effect of fluoxetine on the ER, T cells were loaded with NPE-caged IP_3 through electroporation, and IP_3 was released during imaging through flash photolysis. The height of the resulting $[Ca^{2+}]_i$ peak was measured. In accordance with the results of fluoxetine partially depleting intracellular Ca^{2+} stores, fluoxetine reduced the height of the Ca^{2+} peak after release of IP_3, although statistical significance was only reached at 100 μM fluoxetine (mean control 11.43 ± 11.81; F10 9.98 ± 12.94, $p = 0.11$, F100 2.13 ± 2.65, $p < 0.0001$) (Fig. 4). Thus, fluoxetine suppresses IP_3-mediated Ca^{2+} release from the ER.

3.5. Interference of fluoxetine with ryanodine receptor-mediated Ca^{2+} release

In addition to IP_3R, ryanodine receptors (RyR) are equally known to regulate Ca^{2+} release from intracellular stores. Ca^{2+} released by IP_3R may in turn activate RyR resulting in Ca^{2+}-induced Ca^{2+} release [22]. The functional effect of fluoxetine on RyR-mediated Ca^{2+} release was analyzed by addition of caffeine, which is known to activate RyR [23]. Preliminary experiments to select the most suitable concentration of caffeine showed a concentration-dependent increase in $[Ca^{2+}]_i$ upon exposure to caffeine in a range from 10 to 50 mM (data not shown). As 50 mM caffeine induced the strongest effect, we selected this concentration to study the impact of fluoxetine hereon (Fig. 5A). Responses of similar magnitude but shorter duration were obtained when the experiment was repeated in Ca^{2+}-free buffer, indicating that caffeine indeed released intracellular Ca^{2+} followed by capacitative Ca^{2+} entry (data not shown). In order to confirm that the rise in $[Ca^{2+}]_i$ induced by caffeine was due to RyR stimulation, cells were pre-incubated with an antagonistic concentration of ryanodine (200 μM). As shown in Fig. 5, ryanodine completely suppressed the rise in $[Ca^{2+}]_i$ induced by caffeine. Fluoxetine suppressed the rise in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 5A). In order to quantify the effect of fluoxetine on the RyR-mediated Ca^{2+} release, the difference between the maximal and minimal $[Ca^{2+}]_i$ was calculated. Control cells showed a median peak height of 17.5, range [2.8–74.9], F10 12.3, [0.4–50.8], $p < 0.0001$ and F100 4.1, [0.0–57.8], $p < 0.0001$ (Fig. 5B). Thus, fluoxetine inhibits RyR-induced Ca^{2+}-release in a concentration-dependent manner.

3.6. Involvement of 5HT and SERT in the fluoxetine-induced effects on Ca^{2+} signaling

As fluoxetine is known to inhibit serotonin uptake through the SERT, we next questioned whether the observed effects on Ca^{2+} signaling could be initiated by SERT inhibition. Therefore, we first analyzed whether Jurkat T lymphocytes express SERT. As shown in Fig. 6, T lymphocytes express SERT both at the mRNA level and the protein level. Whereas the positive control (hSERT transfected HEK293 cells) showed a protein band at ~80 and 60 kDa, only the 60 kDa band was found in T cells. As described previously, the ~80 kDa band presumably represents a highly glycosylated form of SERT, whereas the 60 kDa band is most likely the unmodified SERT protein [21]. Further, it should be noted that the large difference in expression levels between the positive control and the T cells is due to overexpression of SERT in the hSERT transfected cell line [21]. Specificity of the primary antibody was confirmed by incubation with a blocking peptide. The results of these experiments indicate that T lymphocytes do express SERT.

To investigate if the effects of fluoxetine on Ca^{2+} signaling are mediated by SERT inhibition, we analyzed the influence of a large excess (1 mM) 5HT on the fluoxetine-induced suppression of RyR-mediated Ca^{2+} release (100 μM fluoxetine). If inhibition of SERT by fluoxetine causes the observed decrease in Ca^{2+} signaling, it can be expected that 5HT reverses this effect by competing with fluoxetine for binding to SERT. As shown in Fig. 6C, 5HT did not inhibit the suppression of RyR-mediated Ca^{2+} release by 10 or 100 μM fluoxetine

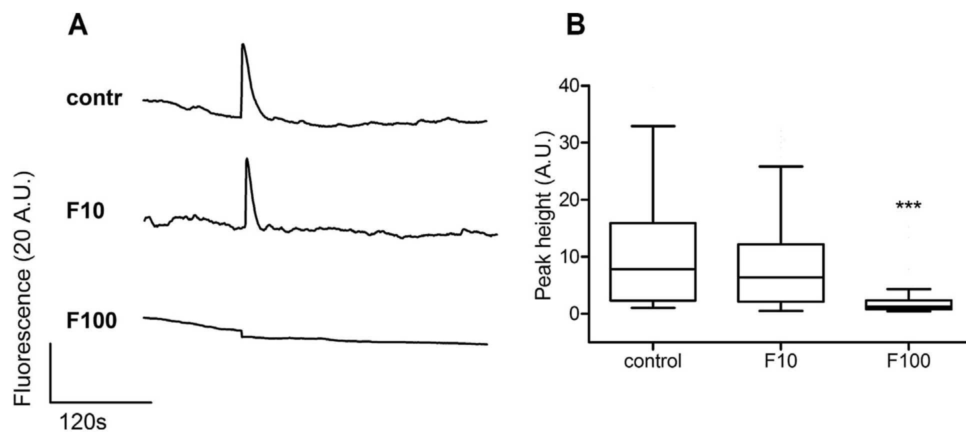

Fig. 4. Effect of fluoxetine on IP_3-mediated Ca^{2+} release. T cells were electroporated with NPE-caged IP_3 and IP_3 was released by flash photolysis after 120 s of imaging. Imaging was continued for 5 min. Fluoxetine (10 μM, F10 and 100 μM, F100) was added 30 min before the start of the experiment. The peak height of the Ca^{2+} change after photolytic release of IP_3 was calculated as the difference between the maximum and the baseline. A total of 61–111 cells per group were analyzed. (A) Mean traces of electroporated cells within the flash zone in Krebs buffer (contr), 10 μM fluoxetine (F10) and 100 μM fluoxetine (F100). (B) Calculated peak heights of the recorded Ca^{2+} changes after photolytic release of IP_3. *** = $p < 0.0001$.

(control median 21.70, [0.0–70.7] *vs* 5HT 20.70, [3.1–71.1], $p = 0.61$; F10 18.60, [0.9–60.3] *vs* F10 + 5HT 20.20, [−0.1–100], $p = 0.35$; F100 7.30, [−0.9–62.2] *vs* F100 + 5HT 5.25, [−0.6–93.6], $p = 0.18$). Thus, it is not likely that fluoxetine depletes Ca^{2+} from the ER through blockage of 5HT uptake by SERT. Notably, addition of 5HT (1 mM) did not induce Ca^{2+} changes on its own (data not shown).

3.7. The effect of fluoxetine on T cell activation is mimicked by buffering of intracellular Ca^{2+}

In order to investigate whether the observed effect of fluoxetine on intracellular Ca^{2+} stores is at the basis of its immunosuppressive effect, we analyzed the effect of fluoxetine on CD69 expression, an early activation marker. Incubation of T cells with anti-CD3/CD28 beads during 5 h in the absence of fluoxetine induced a strong upregulation of CD69 expression (Fig. 7). Non stimulated ('NS') cells showed a mean fluorescence intensity (MFI) of 10.29 ± 0.97, whereas stimulated cells ('S') had a MFI of 107.0 ± 3.0. Fluoxetine (100 μM) decreased the MFI to 9.77 ± 1.97 ($p < 0.0001$ compared to'S'). The same suppressive effect was found when cells were incubated with BAPTA-AM (50 μM), an intracellular Ca^{2+} chelator added to silence cytoplasmic Ca^{2+} changes, demonstrating that interference with intracellular Ca^{2+} signals after TCR stimulation indeed impairs T cell activation (MFI 18.27 ± 0.21, $p < 0.0001$ compared to'S'). Thus, the inhibitory effect of fluoxetine on CD69 expression can be mimicked by buffering the intracellular Ca^{2+} of the cells with BAPTA-AM. These data show that interference with Ca^{2+} signaling in T lymphocytes results in impaired T cell activation, as estimated from CD69 expression, and that the effect of fluoxetine is comparable in magnitude to the effect of buffering $[Ca^{2+}]_i$ with BAPTA-AM.

4. Discussion

In this report, we investigated the impact of fluoxetine on Ca^{2+} signaling in Jurkat T lymphocytes. Previous research has demonstrated that fluoxetine and other SSRIs exert anti-inflammatory and immunosuppressive effects on T lymphocytes [3,24]. Similar suppressive effects have been described in Jurkat T lympho-

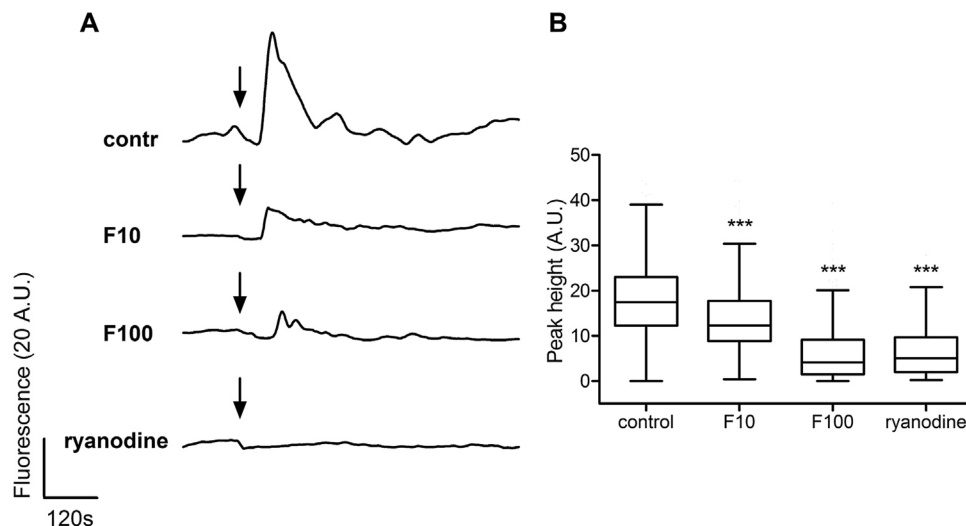

Fig. 5. Effect of fluoxetine on RyR-mediated Ca^{2+} release. T cells were stimulated with 50 mM caffeine in the presence of different concentrations of fluoxetine. The experiments were performed in calcium-containing Krebs solution. (A) Individual traces of cells in Krebs buffer (contr), 10 μM (F10), 100 μM (F100) fluoxetine or 200 μM ryanodine and stimulated with 50 mM caffeine at 120 s (arrow on the graph). Fluoxetine was added 30 minutes before the start of the experiment and maintained in all added solutions. (B) Calculated peak heights of the recorded Ca^{2+} changes. Per sample, 100 arbitrary cells were analyzed. Each condition was performed in duplicate and results were pooled for analysis. *** = $p < 0.0001$.

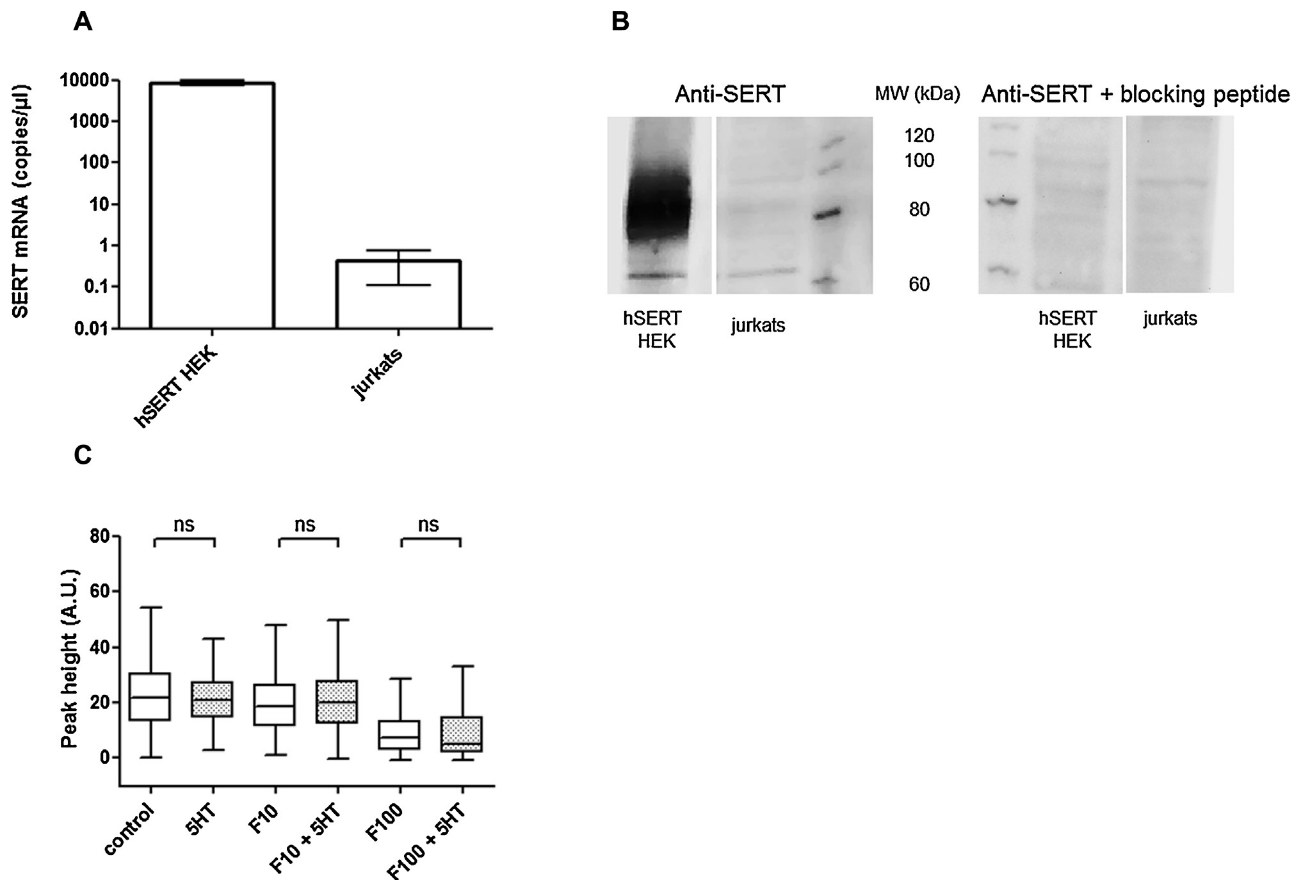

Fig. 6. Analysis of SERT expression in Jurkat T lymphocytes and involvement of 5HT in the fluoxetine-induced effects. (A) SERT mRNA expression was detected with ddPCR. Samples were analyzed in duplo and compared to a positive control (hSERT transfected HEK293 cells); (B) SERT protein expression in hSERT HEK cells (positive control) and T cells. Left: detection with anti-SERT; Right: detection with anti-SERT and a blocking peptide; (C) Results of competition experiments with 5HT (1 mM) on fluoxetine (10 and 100 µM) inhibition of caffeine-induced Ca^{2+} release. Cells were incubated with fluoxetine and/or 5HT 30 min before the start of the experiment. All conditions were analyzed in triplicate and data were pooled for analysis. Peak height expresses the caffeine-induced change in Ca^{2+}. ns = not significant.

cytes [25]. Although several hypotheses on the mechanism behind the observed effects were investigated (reviewed in [2]), the exact mechanism by which fluoxetine suppresses T cell activation and proliferation was not clarified. SSRIs have been shown to affect Ca^{2+} signaling in several cell types including neurons [8], astrocytes [9], microglia [10], osteosarcoma cells [11], platelets [13] and adrenal medulla PC12 cells [7,26]. Since elevation of intracellular Ca^{2+} plays a major role in the pathway leading to T cell activation in response to antigens [6], we investigated if SSRIs, in particular fluoxetine, interfere with this signaling pathway in T cells.

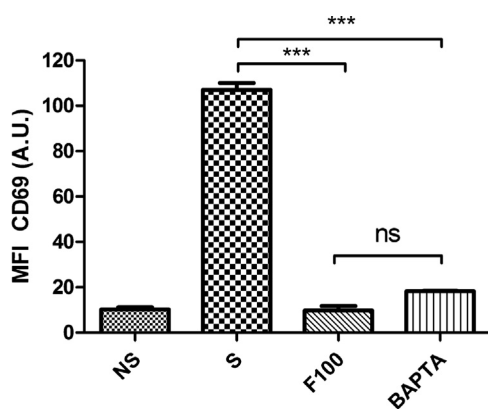

Fig. 7. Effect of fluoxetine and BAPTA-AM on CD69 expression in activated T cells. T cells were activated with anti-CD3/CD28 beads. BAPTA-AM (50 µM) or fluoxetine (100 µM, F100) were added 30 min before addition of the beads and the cells were incubated for 5 h at 37 °C and 5% CO_2. Cells were stained with anti-human CD3 PECy5 and CD69 PECy7 and analyzed by flow cytometry. NS = non-stimulated cells. S = cells stimulated with anti-CD3/CD28 beads. Mean ± SD of mean fluorescent intensities (MFI) are shown. Each condition was analyzed in triplicate. *** = $p < 0.0001$. ns = not significant.

In the case of T lymphocytes, Ca^{2+} is stored in the ER and release from the ER is mediated predominantly by binding of IP_3 to IP_3R, and is further regulated by RyR [27]. The majority of research conducted on the effect of antidepressants, including SSRIs, on Ca^{2+} signaling in other cell types suggests interference with intracellular Ca^{2+} stores [7,9,11,13]. In accordance with these data, we demonstrated that fluoxetine interferes with the ER Ca^{2+} stores in T lymphocytes. As opposed to tricyclic antidepressants, we found that fluoxetine inhibits IP_3-induced Ca^{2+} release [28]. More specifically, we demonstrated that fluoxetine suppresses the rise in $[Ca^{2+}]_i$ in response to TCR activation. Additionally, we showed that the decreased Ca^{2+} signaling is due to the inhibition of Ca^{2+} release from ER stores, rather than the blockage of capacitative Ca^{2+} entry. There are two possible explanations for the inhibition of the Ca^{2+} release from intracellular stores: either fluoxetine causes a depletion of stored Ca^{2+} thus leaving less Ca^{2+} available for release after IP_3R or RyR activation, or fluoxetine directly interferes with the Ca^{2+} channels blocking the Ca^{2+} release in response to IP_3R or RyR activation. In accordance to Serafeim et al., who found that fluoxetine and other SSRIs induced a rise in $[Ca^{2+}]_i$ in malignant B cells [12], the addition of fluoxetine to resting T cells resulted in an increase of the cytoplasmic Ca^{2+} concentration. Subsequent addition of TG

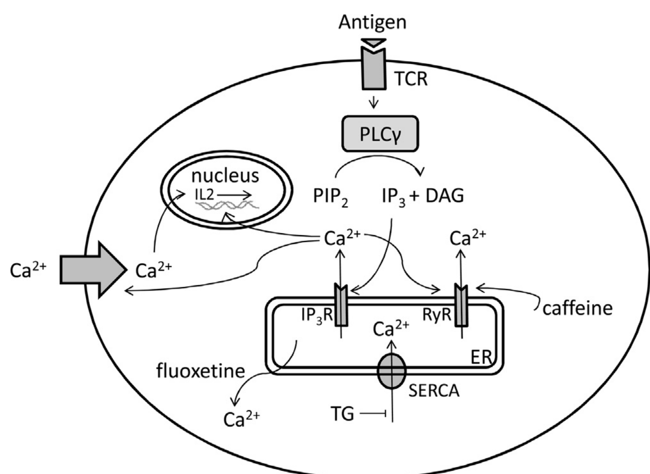

Fig. 8. Schematic representation of fluoxetine-induced effects on Ca^{2+} signaling in T lymphocytes. In T cell activation, binding of an antigen to the TCR results in activation of PLCγ, which converts PIP_2 to IP_3 and DAG. IP_3 induces Ca^{2+} release from the ER through activation of IP_3R. Ca^{2+} may in turn activate RyR thereby further stimulating Ca^{2+} release. RyR agonists such as caffeine also induce Ca^{2+} release by RyR. Secondary to the release of Ca^{2+} from the ER, influx of Ca^{2+} through the plasma membrane is induced. The rise in cytoplasmic Ca^{2+} eventually leads to transcription of a pleotropic set of genes, including IL2, resulting in T cell activation and proliferation. Fluoxetine depletes intracellular Ca^{2+} stores, thereby leaving less Ca^{2+} available for release upon IP_3R or RyR activation. As Ca^{2+} release from intracellular stores is an indispensable step in the pathway leading to T cell activation, inhibition of Ca^{2+} signaling by fluoxetine results in impaired T cell activation and proliferation. Intermediate steps in the signaling transduction pathway were omitted for clarity. TCR = T cell receptor, PLCγ = phospholipase Cγ; PIP_2 = phosphatidylinositol 4,5-bisphosphate; IP_3 = phosphatidylinositol 3,4,5-trisphosphate; DAG = diacylglycerol; ER = endoplasmic reticulum; TG = thapsigargin.

resulted in a significantly lower amount of Ca^{2+} being released from the ER. Therefore, these data suggest that fluoxetine depletes the ER stores, thereby leaving less Ca^{2+} available for release after IP_3R or RyR activation (Fig. 8).

Jurkat and primary T lymphocytes have been shown to express several types of 5HT receptors (5HT1A, 5HT1B, 5HT2A, 5HT3 and 5HT7), as well as tryptophan hydroxylase indicating that these cells are capable of synthesizing and responding to 5HT [29,30]. Furthermore, T cells are capable of releasing 5HT into the extracellular space in response to stimulation [30]. Although the precise role of 5HT in T lymphocyte function has not been elucidated, 5HT has been identified as an important factor in T cell activation and proliferation [31]. Fluoxetine was designed to selectively inhibit the serotonin transporter, which is responsible for uptake of 5HT into the cell. Although no external 5HT was added to the incubation buffer in our experiments, T cells have been demonstrated to secrete 5HT themselves and therefore it is possible that 5HT was present in the microenvironment during the experiments. Given the presumed importance of 5HT in T cell activation and proliferation, it could be expected that the anti-proliferative effects of fluoxetine might be related to its capability to inhibit 5HT uptake in T cells. Here we show that fluoxetine depletes Ca^{2+} from intracellular stores, thereby disturbing the main signaling transduction pathway leading to T cell activation. Nevertheless, we demonstrated that the depletion of ER stores is not mediated through blockage of 5HT transport by SERT since addition of even a large excess of 5HT did not abrogate the effect of fluoxetine on Ca^{2+} signaling. Instead, it has been proposed that fluoxetine, being a highly lipophilic molecule, interacts with the membrane lipid bilayer and thereby influences the ion channel structure and function [7]. Future research will be needed to elucidate how fluoxetine interacts with Ca^{2+} channels at the molecular level.

Finally, we demonstrated that the immunosuppressive effects of fluoxetine – under the form of decreased CD69 expression in response to TCR activation – can be mimicked by buffering of intracellular Ca^{2+} with BAPTA-AM. Others have shown that inhibition of IP_3- or RyR-mediated Ca^{2+} release downregulates Jurkat T cell proliferation and IL2 production [27]. In primary human T cells, inhibition of RyR equally inhibited T cell proliferation [32]. These data suggest that inhibition of IP_3- and RyR-mediated Ca^{2+} release from ER stores plays an important role in the immunosuppressive effects of fluoxetine, although it cannot be excluded that other mechanisms contribute to the immunosuppressive outcome.

It should be noted that the concentrations of fluoxetine used in this report are considerably higher than the plasma concentrations found in depressive patients. Whereas plasma concentrations of fluoxetine are usually below 1 μM, we applied concentrations of 10–100 μM to study the effects of fluoxetine on Ca^{2+} signaling. The applied concentrations are based on previous reports on *in vitro* T cell immunosuppression by SSRIs [3]. However, since SSRIs are lipophilic compounds that accumulate in tissues, significantly higher concentrations in organs than in plasma can occur. In that respect, it has been demonstrated that SSRIs can reach 10-fold higher concentrations in spleen than in plasma [33]. As the meeting of a naïve T cell and its antigen occurs in lymphoid tissue such as the spleen or lymph nodes, it can be expected that T lymphocytes going through the activation process in lymphoid tissue are actually exposed to fluoxetine concentrations up to 10 μM, a concentration which we have demonstrated to exert acute inhibitory effects on Ca^{2+} signaling *in vitro*. Furthermore, it has been shown that the effects of fluoxetine on IP_3R and RyR are time and concentration dependent [9]. The EC_{50} for the chronic effects of fluoxetine in astrocytes was almost 10 times lower than for the acute effects, suggesting that the potency of fluoxetine to interfere with Ca^{2+} signaling increases with longer exposure time. Therefore, chronic exposure of T lymphocytes to fluoxetine might result in immunosuppression at lower concentrations (0.5–1 μM) which are within the same range as plasma concentrations found in depressive patients.

Finally, we selected fluoxetine to study the effects on Ca^{2+} signaling in T lymphocytes. As other SSRIs also induce immunosuppressive effects in T lymphocytes [3], it would be interesting to investigate whether these compounds also affect Ca^{2+} signaling in T lymphocytes.

In conclusion, these data show that fluoxetine suppresses intracellular Ca^{2+} signaling in Jurkat T lymphocytes through depletion of Ca^{2+} from intracellular stores, an effect likely to be at the basis of the observed immunosuppression.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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