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Oxytocin induces intracellular Ca^{2+} release in cardiac fibroblasts from neonatal rats

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ABSTRACT

Pituitary neuropeptide oxytocin is increasingly recognised as a cardiovascular hormone, in addition to its many regulatory roles in other organ systems. Studies in atrial and ventricular myocytes from the neonatal and adult rats have identified synthesis of oxytocin and the expression of oxytocin receptors in these cells. In cardiac fibroblasts, the most populous non-myocyte cell type in mammalian heart, the oxytocin receptors have not been described before. In the present study, we have investigated the direct effects of oxytocin on intracellular Ca^{2+} dynamics in ventricular myocytes and fibroblasts from new born rats. In myocytes, oxytocin increased the frequency of spontaneous Ca^{2+} transients and decreased their amplitude. Our data suggest that oxytocin receptors are also present and functional in the majority of cardiac fibroblasts. We used selective oxytocin receptor inhibitor L-371,257 and a number of intracellular Ca^{2+} release blockers to investigate the mechanism of oxytocin induced Ca^{2+} signalling in cardiac fibroblasts. Our findings suggest that oxytocin induces Ca^{2+} signals in cardiac fibroblasts by triggering endoplasmic reticulum Ca^{2+} release via inositol trisphosphate activated receptors. The functional significance of the oxytocin induced Ca^{2+} signalling in cardiac fibroblasts, especially for their activation into secretory active myofibroblasts, remains to be investigated.

1. Introduction

A nine amino acid neuropeptide oxytocin (OXT) is produced by the magnocellular cells of the hypothalamo-neurohypophysial system. OXT is released locally in many regions of the brain and systemically into the circulation. In the nervous system, OXT exerts a wide variety of effects ranging from its involvement in stress responses and modulation of nociception to the establishment of highly sophisticated social and reproductive behaviour [1–3]. Traditionally recognised for its role in uterine contractility and milk ejection, peripheral OXT is also involved in the regulation of many other organs and tissues [1]. In addition to male and female reproductive systems, the expression of OXT and oxytocin receptors (OXTR) has been documented in other tissues including heart [4], vasculature [5], endocrine pancreas [6], adipose tissue [7], retinal pigment epithelium [8] and the kidney [9].

In mammalian heart, the OXT-OXTR system has been initially associated with the right atrium where it is involved in the regulation of synthesis and release of atrial natriuretic peptide (ANP) in response to atrial distension [4,10]. Subsequent research has documented the expression of OXTR in all chambers of the neonatal and adult heart as well as in the aorta, coronary arteries and large veins prompting the recognition of OXT as an important regulator of cardiogenesis and cardiovascular function [5,11,12]. There is growing evidence for cardioprotective effects of oxytocin against acute ischemia/reperfusion injury, adverse cardiac remodelling after myocardial infarction, increased apoptosis of cardiomyocytes following heart transplantation and cardiac abnormalities associated with diabetes and obesity [11,13,14]. In contrast to these acute cardioprotective effects of OXT, more recent work using transgenic mice has found that cardiac-specific overexpression of OXTR leads to the development of cardiomyopathy accompanied by myocardial fibrosis culminating in cardiac failure [15]. This finding may suggest a potential involvement of cardiac fibroblasts in OXT-OXTR signalling although no direct evidence exists for such a phenomenon. There is a general consensus that, in addition to their classical role in the extracellular matrix synthesis and maintenance, cardiac fibroblasts contribute to the action potential propagation, cardiac mechano-sensitivity and many other normal and abnormal processes including hypertrophic growth of cardiomyocytes and their electrical remodelling [16–19]. Surprisingly, we found no data in the literature on the expression of OXTR in cardiac fibroblasts or the effects of OXT on intracellular Ca^{2+} $[Ca^{2+}]_i$ dynamics in this cell type even though a closely related neuropeptide, arginine vasopressin (AVP), has

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been implicated in the development of cardiac hypertrophy and fibrosis via stimulation of the cardiac fibroblast differentiation into secretory-active myofibroblasts [20–22]. An increase in $[Ca^{2+}]_i$ in response to different humoral factors has been shown to activate the transition of quiescent fibroblasts into highly active myofibroblasts [23]. The aim of this study was to investigate OXTR expression in neonatal cardiac fibroblasts and the effects of OXT on $[Ca^{2+}]_i$ in this cell type.

2. Materials and methods

2.1. Cell isolation and culture

Cells were isolated from 1 day old rat pups using the Pierce Primary Cardiomyocyte Isolation Kit (Catalogue N 88281, Thermo Fisher Scientific, USA) according to the manufacturer recommended protocol. In brief, animals were euthanized in accordance with the Guidelines for the Care and Use of Laboratory Animals [24] and with approval from the United Arab Emirates University Animal Research Ethics Committee (Approval ERA_2017_5681). Ventricles from 5 pups were dissected and put into individual sterile 1.5 ml microcentrifuge tubes each containing 0.5 ml of ice-cold Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS, Thermo Scientific, USA). Using a pair of sterile scissors, the ventricles were cut into small pieces, washed three times with 0.5 ml ice-cold HBSS and incubated with a mixture of papain and thermolysin in HBSS for 30 min on a 37 °C heat block. At the end of incubation, the enzyme containing solution was carefully removed and the digested tissue was washed twice with ice-cold HBSS. After that, a 0.5 ml aliquot of Complete DMEM for Primary Cell Isolation culture medium (Thermo Scientific, USA) with 10% Fetal Bovine Serum (FBS) was added to each tube and the tissue was triturated with a wide-bore plastic pipette to release the cells. Once the tissue had dissociated into a single-cell suspension, a further 1 ml of the Complete DMEM for Primary Cell Isolation was added to each tube bringing the total volume to 1.5 ml. At this stage, the contents of all 5 microcentrifuge tubes were pooled, cells counted on a haemocytometer, diluted to a density of about 250 000 cells per cm^2 and plated into 35 mm glass-bottomed petri dishes (Bioptechs Inc., USA) for Ca^{2+} imaging experiments or onto 25 mm round glass coverslips placed into standard six-well plates for immunofluorescence experiments. The petri dishes and the six-well plates were incubated for 1 h at 37 °C in humidified 5% CO_2 incubator to facilitate fibroblast attachment. After that, the unattached cells were carefully removed and plated into new sets of petri dishes and onto coverslips. The attached cells in the first set of dishes and plates were supplemented with 2 ml of Complete DMEM for Primary Cell Isolation each and left undisturbed in the 5% CO_2 incubator for 24 h. This procedure yielded two sets of cell cultures: one enriched in cardiac fibroblasts and the other in cardiac myocytes. Cell culture medium was changed 24 h after plating and the 1000-fold diluted Cardiomyocyte Grows Supplement from the Pierce Primary Cardiomyocyte Isolation Kit added to the cardiomyocyte enriched cultures. Cardiac fibroblast cultures received Complete DMEM for Primary Cell Isolation without the Growth Supplement. The cells were used for experimentation 48 h later.

2.2. Immunofluorescence staining and cell imaging

Cell culture medium was removed from the six-well plates containing coverslips on which the cells have been cultured for 48 h; the cells were washed twice with phosphate buffered solution (PBS, Sigma-Aldrich, UK) and fixed in 10% neutral buffered formalin (10% NBF) for 10 min at room temperature followed by three 5 min washes with PBS. Coverslips were incubated overnight at room temperature with a mixture of two primary antibodies: rabbit polyclonal anti-OXTR (bs-1314R, BioSS, USA) and the mouse monoclonal anti-desmin (ab6322, Abcam, UK), which was used to identify the cardiac myocytes. For identification of cardiac fibroblasts expressing OXTR, the mixture of primary antibodies contained the same anti-OXTR antibodies and mouse monoclonal antibodies against a tyrosine kinase receptor discoidin domain receptor 2 (DDR2) (ab63337, Abcam, UK) [25]. Although markers uniquely specific to cardiac fibroblasts have not been unequivocally identified, the DDR2 is considered sufficiently discriminative marker for distinguishing cardiac fibroblast from the cardiac myocytes [26,27]. After the incubation with primary antibodies and thorough washing with PBS the coverslips were sequentially incubated with two secondary antibodies for 1 h each at room temperature in the dark. The secondary antibodies recognising the anti-OXTR were conjugated to the Alexa Fluor® 488 florescent dye while those recognising the anti-DDR2 or anti-desmin were conjugated to Alexa Fluor® 555. Cell nuclei were labelled with DAPI included in the mounting medium. Negative controls were prepared the same way but primary antibodies were omitted from the incubation medium. The cells were visualised using an upright Eclipse E400 (Nikon Instruments Inc., USA) fluorescence microscope equipped with the standard FITC, TRITC and DAPI filter sets and a low-light EMCCD camera (Andor Luca, Andor Technology, Belfast, UK) controlled by the Andor iQ 1.8 software. Images taken at the FITC, TRITC and DAPI spectral bands were saved to the hard drive in 16 bit TIFF format and subsequently imported into ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997–2018) for further processing. The imported images were background subtracted, auto-scaled and merged into 24 Bit RGB format. Merged images alongside with their corresponding grey scale component images were supplemented with 10 µm scale bars (see Fig. 1, Aa and Ba).

2.3. Ca^{2+} imaging and data analysis

For Ca^{2+} imaging experiments we choose to use the Ca^{2+}-sensitive dye Cal-520 due to its wide dynamic range and superior signal-to-noise ratio compared to other visible light Ca^{2+} indicators [28]. Cells were washed three times with physiological saline solution (PSS) and incubated with 10 µM of Cal-520/AM for 1 h at 30 °C. The loading solution contained 0.25% Pluronic F127 to facilitate loading of the dye into the cells. After loading, the cells were washed with PSS and incubated for 30 min at 30 °C to ensure complete de-esterification of the Cal-520/AM. The petri dish containing loaded cells was placed on the stage of an inverted microscope (Axiovert 405 M, Zeiss, Germany) and perfused with PSS preheated to 35 °C. Drug-containing solutions were applied locally to cells within the field of view of a 40x objective lens using a temperature controlled micro-perfusion device (MPRE8, Cell Micro-Controls, USA) connected to a computer controlled eight-channel pinch-valve delivery system (AutoMate Scientific, Inc., USA). The bath perfusion heater and the MPRE8 maintained the solution temperature at 35 ± 1 °C. Both heaters were powered by a modified MT-20 temperature controller (NPI electronic GmbH., Germany).

Spontaneous and OTX induced $[Ca^{2+}]_i$ transients were recorded at a rate of 40 frames per second using an Andor Luca EMCCD camera except for the experiments illustrated in Fig. 3C and D where Andor Zyla 4.2 sCMOS camera was used. Unless otherwise indicated, OXT was applied for 60 s after a 30 s long baseline recording. The recording continued for an additional 30 s following OXT washout. During this time interval, a brief pulse of 10 mM caffeine was applied to distinguish the cardiac myocytes from the fibroblasts as the latter cell type does not respond to caffeine [29]. Image acquisition and quantification were performed using the WinFluor software package (Dr. John Dempster, Strathclyde University). Mean fluorescence was measured from the ROIs placed over all cells in the field of view and presented as fluorescence self-ratio (F/F_0). The technical graphing and data analysis software package IgorPro 7.0 (Wavemetrics, USA) was used for curve fitting, statistical analyses and preparation of figures. The data were obtained from at least three different cultures. Statistical comparisons were made using paired *t*-test except where multiple comparisons were made in which case 1-way ANOVA followed by Dunnet's post-hoc test

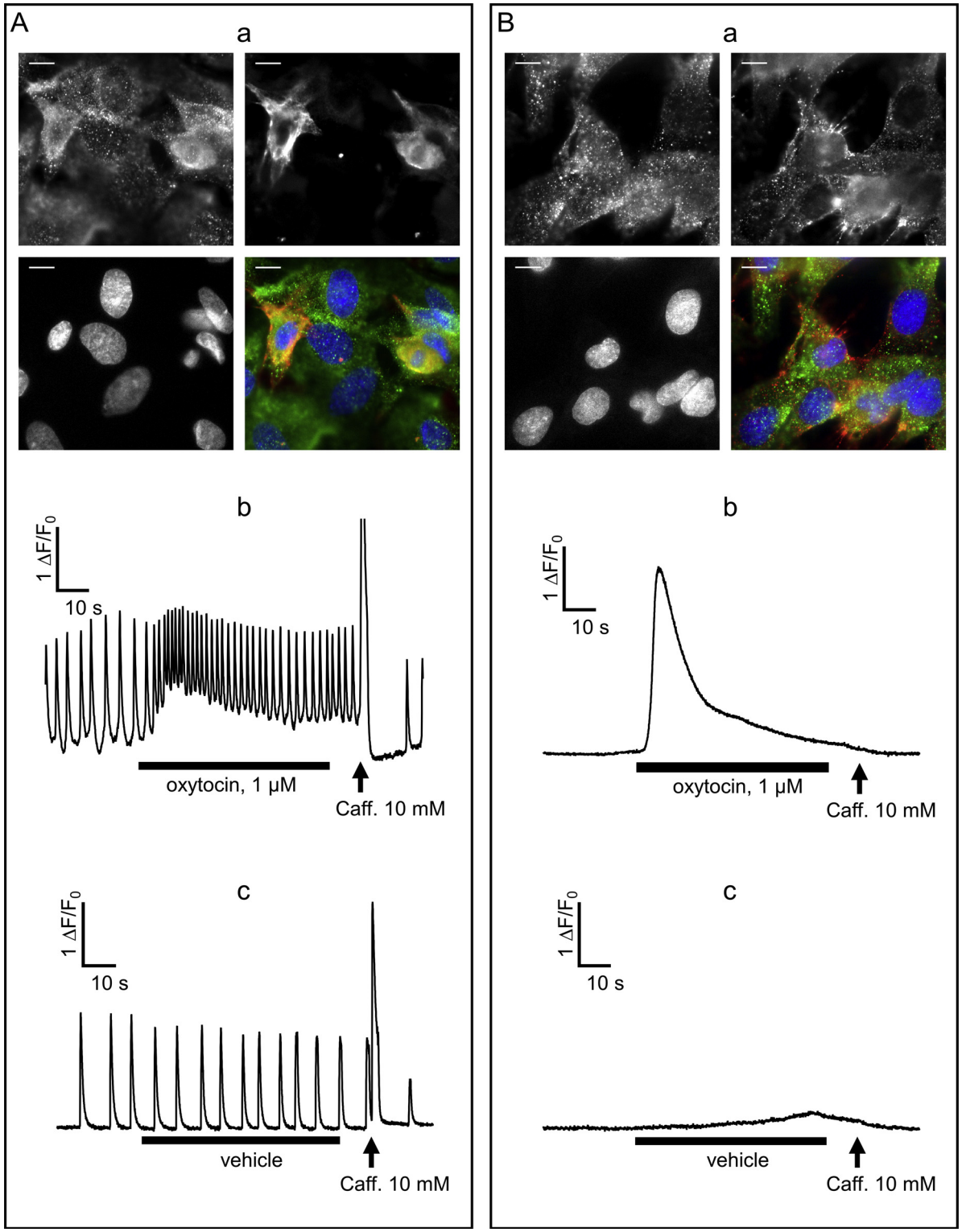

Fig. 1. Immunofluorescence images of cells and typical tracings of OXT- and caffeine-induced responses in ventricular myocytes (panel A) and fibroblasts (panel B) maintained in short term cell culture. Aa – Cells were labelled with antibodies recognising OXT receptors (top left image, green in the bottom right overlay) and cardiomyocyte marker desmin (top right image, red in the bottom right overlay). Nuclei were labelled with DAPI (bottom left image, blue in the bottom right overlay). Calibration bar corresponds to 10 μm. Ab – an application of OXT to cardiac myocytes had positive chronotropic effect accompanied by elevation of diastolic $[Ca^{2+}]_i$ and reduction in the amplitude of spontaneous $[Ca^{2+}]_i$ transients. Ac – an application of 0.003% acetic acid (vehicle) to cardiac myocytes had no effect on $[Ca^{2+}]_i$. Brief application of 10 mM caffeine (indicated by arrows) induced a release of Ca^{2+} from the SR resulting in high-amplitude $[Ca^{2+}]_i$ transients. Ba - Cells were labelled with antibodies against OXT receptors (top left image, green in the bottom right overlay) and cardiac fibroblast marker DDR2 (top right image, red in the bottom right overlay). Nuclei were labelled with DAPI (bottom left image, blue in the bottom right overlay). Calibration bar corresponds to 10 μm. Bb – an application of OXT to cardiac fibroblasts caused a bi-phasic $[Ca^{2+}]_i$ response: large transient followed by a smaller sustained elevation in $[Ca^{2+}]_i$. Bc – vehicle application caused a slow developing elevation in $[Ca^{2+}]_i$ in some cells. Caffeine application to cardiac fibroblasts failed to induce any response in $[Ca^{2+}]_i$.

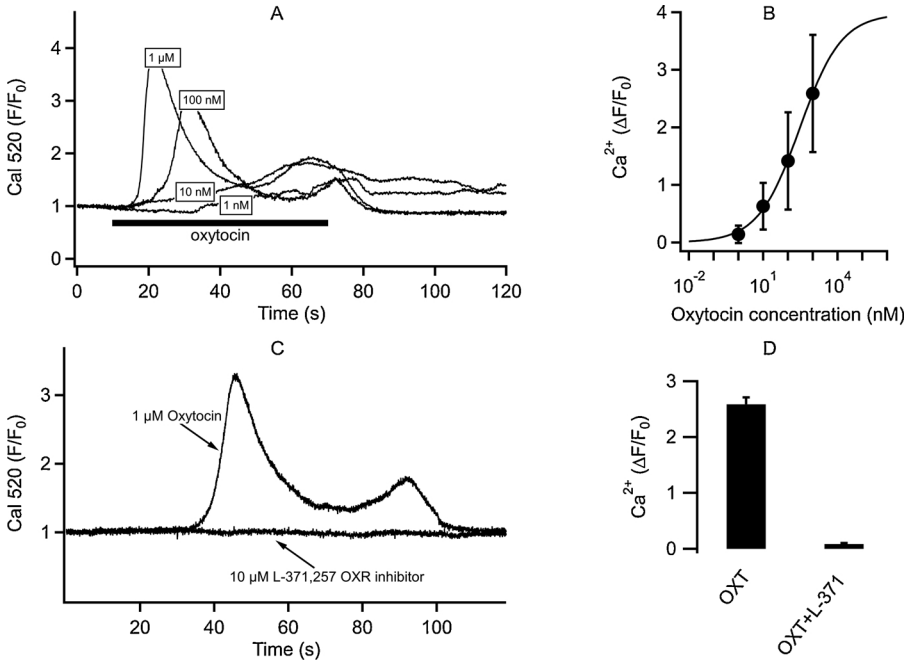

Fig. 2. A – representative traces of $[Ca^{2+}]_i$ responses to different concentrations of OXT (indicated by a solid bar). The concentrations used are shown on top of each trace. B – Dose-effect relationship of $[Ca^{2+}]_i$ responses to OXT. Traces were obtained from different cells each receiving only one application of OXT to alleviate possible desensitisation. Data points represent mean ± standard deviation of 24 to 77 measurements. Solid line represents best fit of data to the Hill equation. C - OXT induced $[Ca^{2+}]_i$ transients are eliminated by the inhibition of OXT receptors: $[Ca^{2+}]_i$ responses to OXT alone or in the presence of selective OXTR inhibitor L-371,257. D - Averaged values of $[Ca^{2+}]_i$ responses to OXT in control and in the presence of OXTR inhibitor. Data presented as mean ± standard error of mean were recorded from 77 cells in control and 33 cells pre-treated with 10 μM L-371,257 ($p < 0.01$).

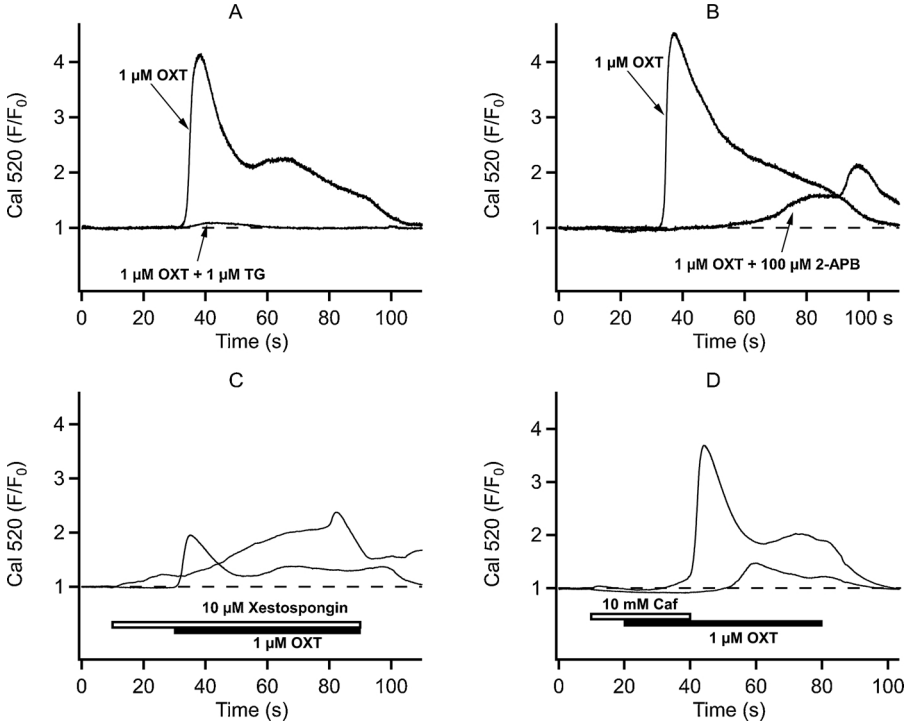

Fig. 3. Effects of a specific SERCA pump inhibitor thapsigargin and IP_3 receptor antagonists on the OXT induced $[Ca^{2+}]_i$ transients. A – representative traces of $[Ca^{2+}]_i$ responses to 1 μM OXT alone and 1 μM OXT in the presence of thapsigargin. B – Representative traces of $[Ca^{2+}]_i$ responses to 1 μM OXT alone and 1 μM OXT in the presence of 100 μM 2-APB. The onset and duration of applications were the same as Fig. 1Bb. C – Two types of responses to 1 μM OXT (solid bar) observed in the presence of 10 μM Xestospongin C (open bar): a reduction in the peak amplitude with preserved shape of the $[Ca^{2+}]_i$ transient and no visible effect of Xestospongin C itself on the resting $[Ca^{2+}]_i$ or a slow rise in resting $[Ca^{2+}]_i$ upon Xestospongin C application with complete abolition of the OTX induced $[Ca^{2+}]_i$ transient peak. D – $[Ca^{2}]_i$ responses to OXT are inhibited in the presence of 10 mM caffeine. Application of caffeine to cardiac fibroblasts (open bar) had no discernible effect on resting $[Ca^{2+}]_i$ but completely prevented OXT induced $[Ca^{2+}]_i$ transients. Upon washout of caffeine in continuous presence of OXT, $[Ca^{2+}]_i$ transients reappeared ranging from almost identical to control to much smaller and slower rises in $[Ca^{2+}]_i$. None of the cells responded to caffeine with a rise in $[Ca^{2+}]_i$, confirming them as fibroblasts.

was used.

2.4. Solutions and reagents

Unless indicated otherwise, all the reagents and fluorescent dyes used in this study were purchased from Thermo Fisher Scientific (USA). PSS was composed of (in mmol/l): NaCl 133, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, HEPES 5, Glucose 10. The pH of this solution was adjusted with 1 M NaOH to 7.4 at 35°C.

3. Results

Our initial experiments compared the expression of OXTR and the $[Ca^{2+}]_i$ responses to application of OXT in cardiac myocytes (Fig. 1, panel A) and in cardiac fibroblasts (Fig. 1, panel B). We found that OXTR immuno-reactivity is present in many cells positive for cardiac myocyte marker desmin (Fig. 1Aa) and for cardiac fibroblast DDR2 (Fig. 1 Ba). The majority of cardiac myocytes loaded with Ca^{2+} sensitive indicator generated spontaneous $[Ca^{2+}]_i$ transients. The most consistent effect of OXT on spontaneously active myocytes was an increase in the frequency of $[Ca^{2+}]_i$ transients associated with a reduction in their amplitude (illustrated in Fig. 1 Ab). Less consistent was the OXT effect on diastolic $[Ca^{2+}]_i$ ranging from no effect at all to a quite substantial transitory rise. As expected, application of caffeine to cardiac myocytes induced large $[Ca^{2+}]_i$ responses due to Ca^{2+} release from the SR [30]. Application of vehicle had no effect on spontaneous

$[Ca^{2+}]_i$ transients in cardiac myocytes (Fig. 1 Ac).

Application of 1μM OXT to cardiac fibroblasts induced large $[Ca^{2+}]_i$ transients comprised of a peak followed by a sustained elevation above the resting level (Fig. 1 Bb). Removal of the extracellular Ca^{2+} eliminated the sustained component but did not affect the peak (not illustrated). Vehicle application induced a slow rising small increase in $[Ca^{2+}]_i$ (Fig. 1 Bc). The same $[Ca^{2+}]_i$ responses were observed when PSS was applied from the fast application pipette instead of the vehicle suggesting that the slow rise in $[Ca^{2+}]_i$ was due to the shear stretch activation of mechano-sensitive channels in the fibroblasts [31]. Application of caffeine was without effect in cardiac fibroblasts. We used this unresponsiveness of fibroblasts to caffeine together with the lack of contractions and absence of spontaneous Ca^{2+} transients in our subsequent experiments to confirm that the cell under study was indeed a fibroblast not a quiescent myocyte.

Since OXT induced $[Ca^{2+}]_i$ signalling in cardiac fibroblasts has not been characterised before we focused our attention primarily on this cell type. As illustrated in Fig. 2A, the effect of OXT on $[Ca^{2+}]_i$ in cardiac fibroblasts was dose-dependent. In our hands, OXT concentrations below 1 nM failed to induce measurable changes in $[Ca^{2+}]_i$. Increasing the OXT concentration to 1 nM or above induced the $[Ca^{2+}]_i$ responses with progressively increasing peak amplitude (Fig. 2A). To exclude possible desensitisation of OXTR, especially by higher doses of OXT, we tested only one concentration of OXT per culture dish. Averaged results from 24 to 77 measurements per dose plotted against the OXT concentrations are shown in Fig. 2B. Fitting the dose-effect curve to the Hill equation yielded an apparent EC50 value of about 300 nM. As this value is much higher than that found in e.g. myometrial cells and OXT is a partial agonist of AVPR, we used selective OXTR inhibitor L-371,257 (Tocris Bioscience, UK) with 800 times higher affinity to the OXTR over AVPR to test whether the effects observed in our experiments were indeed mediated by the OXTR. The results of our experiments with L-371,257 revealed complete inhibition of OXT induced $[Ca^{2+}]_i$ transients in the presence this OXTR inhibitor at 10 μM concentration (Fig. 2C and D).

Subsequent experiments were designed to test the involvement of the endoplasmic reticulum (ER) and inositol trisphosphate (IP_3) receptors in the OXT induced $[Ca^{2+}]_i$ in cardiac fibroblasts. Fig. 3A illustrates that the inhibition of the Sarco-(Endo-)plasmic Reticulum Ca^{2+} (SERCA) pump with thapsigargin eliminated the OXT induced Ca^{2+} transients indicating that the endoplasmic reticulum was the sole source of Ca^{2+} release in response to OXT in cardiac fibroblasts. As the lack of response to caffeine in cardiac fibroblasts suggests that ryanodine receptors are unlikely to be responsible for the OXT induced Ca^{2+} transients, we have tested the involvement of the IP_3 receptors using three known inhibitors, namely 2-Aminoethoxydiphenyl borate (2-APB), Xestospongin C and caffeine. Application of 2-APB had little effect on its own but drastically reduced the OXT induced Ca^{2+} transients (Fig. 3B). Interestingly, upon washout of the OXT and 2-APB mixture, the $[Ca^{2+}]_i$ transiently increased before falling to its resting level. Application of 10 μM Xestospongin C induced either no change or a slow rise of $[Ca^{2+}]_i$ (Fig. 3C). The responses to OXT in the presence of Xestospongin C were reduced but not eliminated completely. However, after 10 min incubation with 10 μM of Xestospongin C the fibroblasts responses to OXT were abolished (not illustrated). Application of 10 mM caffeine to cardiac fibroblasts had no discernible effect on its own but completely supressed the OXT induced $[Ca^{2+}]_i$ transients. Upon washout of caffeine in continuous presence of OXT, the $[Ca^{2+}]_i$ transiently increased ranging from a transient almost indistinguishable from control to much smaller and slower rises in $[Ca^{2+}]_i$ (Fig. 3D). These results are compatible with the notion of IP_3 receptors being the main route for the OXT induced Ca^{2+} release in cardiac fibroblast.

4. Discussion

Previous studies have firmly established OXT as a cardiovascular hormone (see [11] for detailed review). However, no data is available on the expression and function of OXTR in cardiac fibroblasts. To address this, we have applied immunofluorescence and Ca^{2+} imaging techniques to primary cultures of cells from the newborn rat ventricles to test whether the OXTR are expressed in cardiac fibroblasts and whether OXT can induce $[Ca^{2+}]_i$ signalling in this cell type. Our findings suggest that, in addition to cardiac myocytes, cardiac fibroblasts express OXTR and respond to OXT with a dose-dependent elevation of $[Ca^{2+}]_i$. The results show that the affinity of the receptors activated by OXT in cardiac fibroblasts is lower than that in the myometrium or in mammary glands. This raises the possibility that the observed effect was due to a cross-activation of the AVPR which are known to have lower sensitivity to OXT compared to their natural ligand AVP [1,32]. In our experiments, the responses to OXT were abolished by the selective OXTR inhibitor. This leads us to the conclusion that the responses were mediated by OXTR and not AVPR although the latter type of receptors is also present in cardiac fibroblasts [21] and can contribute to the growth of cardiac fibroblasts and development of cardiac hypertrophy [22,33,34].

Although none of the inhibitors used in our experiments is specific to the IP_3 receptors and all have been shown to exert multiple off-target effects [35–38], the combined results of our experiments with three different inhibitors show consistent inhibition of the OXT induced $[Ca^{2+}]_i$ transients suggesting that these were mediated by the activation of the IP_3 receptors on the ER of cardiac fibroblasts. The sustained component of the OXT-induced $[Ca^{2+}]_i$ elevation is likely due to the activation of store-operated Ca^{2+} entry (SOCE). The identity of ion channels that underlie SOCE remains to be uncovered although several candidate channels have been described in cardiac fibroblast and implicated in the process of fibroblast activation into pro-fibrotic secretory active myofibroblasts [29,39–42].

In conclusion, the results of our study show that OXT triggers Ca^{2+} signalling in cardiac fibroblasts via OTXTR expressed on the surface membrane of these cells. The relationship between the OXT effects on cardiac myocytes and cardiac fibroblasts requires further experiments.

Overall, our findings are compatible with the idea that the oxytocin induced Ca^{2+} signalling in cardiac fibroblasts is mediated by mechanisms similar to that operating in the myometrium and in mammary myoepithelial cells, namely, G-protein induced activation of phospholipase C leading to the production of IP_3 and diacylglycerol that trigger Ca^{2+} release from the ER followed by store-and receptor-operated Ca^{2+} entry [1,43]. Importantly, the same mechanisms mediate Ca^{2+} signalling induced by vasopressin, a hormone involved in cardiac hypertrophy and fibrosis [20–22]. This similarity of mechanisms implies that, while having cardioprotective effects in some situations, oxytocin may also promote cardiac fibrosis potentially leading to heart failure.

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