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Essential role of TRPC1 channels in cardiomyoblasts hypertrophy mediated by 5-HT$_{2A}$ serotonin receptors

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ABSTRACT

Serotonin (5-HT) participates in the development of cardiac hypertrophy through 5-HT$_{2A}$ serotonin receptors. The hypertrophic growth of cardiomyoblasts induced by 5-HT$_{2A}$ receptors involves the activation of the Ca^{2+} responsive calcineurin/NFAT pathway. However, the mechanism whereby NFAT is activated by 5-HT$_{2A}$ receptors remains indeterminate. In this study, we examined whether transient receptor potential canonical (TRPC) channels participate in NFAT activation and hypertrophic response triggered by 5-HT. We demonstrate that TRPC1 expression is upregulated in 5-HT-treated rat cardiomyoblasts whereas TRPC6 is induced in a mouse model of heart hypertrophy. Moreover, TRPC1 knockdown by small interfering RNA inhibits NFAT activation and hypertrophic response mediated by 5-HT$_{2A}$ receptors.

These findings provide new insights about a mechanistic basis for the activation of the calcineurin/NFAT pathway by 5-HT$_{2A}$ receptors and highlight the critical role of TRPC1 in the development of cardiac hypertrophy.

Introduction

The adult heart responds to diverse stress signals such as hypertension, myocardial infarction, and pressure overload by hypertrophic growth. It is believed that hypertrophy is adaptative in the short term but becomes maladaptive in the long term and leads to heart failure. Therefore, there has been great interest in deciphering the intracellular pathways that govern maladaptive hypertrophy because of the potential therapeutic benefit of manipulating these mechanisms. The serotoninergic system undergoes significant alterations in human heart failure, suggesting its involvement in the development and progression of this disease [1,2]. In particular, serotoninergic 5-HT$_{2A}$ receptors are Gq-coupled receptors which are overexpressed in human heart failure, and whose importance in cardiac hypertrophy has been supported by *in vitro* and *in vivo* findings [3]. Indeed, 5-HT$_{2A}$ receptor blockade reverses hypertrophy during pressure overload in a mouse model of increased 5-HT levels, and reduces left ventricular hypertrophy associated to human hypertension [4,5]. In addition, 5-HT$_{2A}$ receptors mediate a hypertrophic response to 5-HT in cardiomyocytes [6].

The calcineurin/nuclear factor of activated T cells (NFAT) pathway was recently identified as a major determinant in 5-HT$_{2A}$-mediated cardiomyoblasts hypertrophy [6,7]. The phosphatase calcineurin, once activated by sustained elevation in intracellular Ca^{2+}, dephosphorylates a variety of cellular substrates including the NFAT transcription factors, which are sufficient to induce hypertrophy [8]. At present, the mechanism of action of 5-HT$_{2A}$ receptors on the calcineurin/NFAT pathway remains indeterminate. The stimulation of Gq-coupled receptors triggers the production of secondary messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 induces Ca^{2+} release from sarcoplasmic reticulum, producing a transient increase in intracellular Ca^{2+}, but the rise in Ca^{2+} is rapidly reversible and could not itself be solely responsible for calcineurin/NFAT activation. Recently, the possibility that hypertrophic agonists linked to G-protein coupled receptors elicit a sustained Ca^{2+} entry through transient receptor potential canonical (TRPC) channels has been raised [9]. The seven members of the TRPC family (TRPC1–7) are non-selective Ca^{2+}-permeable cation channels. The mode of activation of TRPC-mediated Ca^{2+} entry is still under debate, but TRPC channels respond to general stimuli following depletion of intracellular Ca^{2+} stores, receptor activation or membrane stretch [10]. To date, several groups have reported the involvement of TRPC channels in cardiac hypertrophy. Indeed, TRPC3 promotes cardiomyocyte hypertrophy through activation of calcineurin signaling and TRPC3 transgenic mice showed increased calcineurin/NFAT

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activation [11,12]. SiRNA-mediated knockdown of TRPC6 attenuates angiotensin II-induced NFAT activation and cardiomyocyte hypertrophy and TRPC6 transgenic mice exhibited increased NFAT activity and cardiomyopathy [13,14]. Ohba et al. [15] showed that overexpression of TRPC1 facilitated NFAT transcriptional activity and was involved in the development of cardiac hypertrophy induced by endothelin I.

In this context, we aimed to investigate whether TRPC channels contributed to 5-HT_{2A} receptor-mediated hypertrophic response.

Materials and methods

Materials. Culture medium, antibiotics and serum were purchased from Invitrogen (Cergy Pontoise, France). Chemicals (5-HT, DOI, EGTA) were purchased from Sigma–Aldrich (L'Isles d'Abeau Chesnes, France). M100907 [*R*-(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl-4-piperidinemethanol] was from Sanofi-Aventis (Paris, France). Following antibodies were used: polyclonal anti-TRPC1 and anti-TRPC6 (Alomone Labs, Jerusalem, Israel), polyclonal anti-ERK2 (Tebu-Bio, Le Perray en Yvelines, France), monoclonal anti-β-actin (Sigma–Aldrich). The ECL chemiluminescence kit was from Amersham Pharmacia Biotech (Orsay, France).

Cell culture. The H9C2 cardiomyocyte-like cell line (American Type Culture Collection, Molsheim, France) is a rat embryonic myoblast-derived cell line commonly used as an *in vitro* model of cardiomyocyte biology that shows similar hypertrophic and apoptotic responses as those seen in primary adult and neonatal cardiomyocytes [16,17]. Cells were maintained in MEM, supplemented with 10% fetal calf serum and 4 mM glutamine. Cells were grown at subconfluence in an atmosphere of 5% CO_2 and 37 °C and were serum starved in MEM supplemented with 1% dialyzed fetal bovine serum 24 h before experiments.

RNA extraction and RT-PCR. Cells were trypsinized and washed in PBS, then diluted in SV RNA lysis buffer (Promega, Charbonnieres, France). Total extract was purified with SV Total isolation system (Promega) and 1 μg of total RNA was reverse transcribed by using the high capacity cDNA reverse transcription kit (Applied Biosystems, Courtaboeuf, France). For PCR analysis, the expression of TRPC1, TRPC2, TRPC3, TRPC6 and β-actin, were determined by using the above cited primers. TRPC1: Forward 5′-TGCTGTTACT CAATCTCTACTC-3′, Reverse 5′-GACCAAATCATCCCAATAATCC-3′, TRPC2: Forward 5′-AGAAACAGTTTGTAGCACACC-3′, Reverse 5′-CA TGGTGAGGAAGATGAGGA-3′, TRPC3: Forward 5′-CATTCTCAATCAG CCAACAC-3′, Reverse 5′-TTTCAGTTCACCTTCGTTCAC-3′, TRPC6: Forward 5′-CTCCTCCCTAATGAAACCAG-3′, Reverse 5′-CCCATATCA TTCCTATTACCCA-3′ and β-actin: Forward 5′-TGTTACCAACTGGGA CGACA-3′, Reverse 5′-AAGGAAGGCTGGAAAAGAGC-3′.

Plasmid constructs and transfections. The luciferase reporter plasmid driven by four NFAT consensus binding sites (NFAT-Luc) was obtained from Stratagene (Agilent Technologies, Massy, France). Stable transfection of H9C2 cells was performed using Lipofectamine 2000 reagent (Invitrogen, Cergy Pontoise, France) as previously described [6].

siRNA knockdown of TRPC1. The selected siRNA specific to TRPC1 is 5′-AAG CUU UUC UUG CUG GCG UGC-3′ (Dharmacon, Surrey, United Kingdom). SiRNAs were transfected using the Dharmafect reagent (Dharmacon) according to the manufacturer's recommendations.

Luciferase detection assay. Expression of luciferase was monitored with the Luciferase Assay System (Promega France, Charbonnières) on a luminometer (Mithras LB940, Berthold Technologies, Thoiry, France).

[^{3}H]leucine measurements. H9C2 cells were plated in 24-well plates at subconfluence and treated with agonists for 24 h in the presence of 1 μCi/ml of [^{3}H]leucine (Amersham, Les Ullis, France). At the end of the incubation period, cells were washed with PBS, incubated in 5% trichloroacetic acid and neutralized with 0.25 M NaOH. Cell extracts were counted in a scintillation counter.

Western blots analysis. Cells were lysed in solubilizing buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin) for 30 min on ice. Forty micrograms of protein cell extracts were resolved by SDS–polyacrylamide gel electrophoresis, transferred onto PVDF membranes (Millipore, Molsheim, France). Then membranes were probed with the indicated primary antibodies and revealed with the secondary antibodies coupled to horseradish peroxidase using the ECL chemoluminescence kit. Membranes were then stripped and reprobed with anti-β-actin antibody to control equal loading of proteins.

Measurements of intracellular Ca^{2+}. H9C2 cells were seeded onto Lab-Tek chambers (Nalgene Nunc, Rochester, NY) and loaded with 5 μm Fluo-3-AM (1 h, 37 °C) in Hank's balanced salt solution. Intracellular Ca^{2+} measurement studies were performed utilizing an LSM 510 confocal laser scanning microscope equipped with an Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany), using an 40× objective lens (Plan-APO, N.A. 1,2 Water) and an argon–krypton laser excitation source. For optimization of Fluo3-AM labeling the following settings in laser lines and filters on single-track mode were used: excitation at 488 nm with 3% transmission (argon laser line, output 25%) and emission using the BP 505–530 nm filter. Images were scanned at 1 s intervals and stored as a time series (approximately 4 min). Data are presented as the ration of fluorescence F/F_0 were F is the fluorescence at intermediate Ca^{2+} level and F_0 fluorescence at the resting level.

Ascendant aortic banding. C3H/HeOuJ mice (Harlan, France) were housed in a pathogen-free facility and handled in accordance with the procedures outlined in Council Directive 86/609/EEC. Ten-week-old male mice were anesthetized with isoflurane (4%), ventilated, and the left thorax was opened at the second intercostal space. Aortic constriction was performed by ligating the ascending aorta using a 7-0 Prolene suture under a dissecting microscope as previously described [5]. Age-matched animals underwent identical surgical procedure except for ligation of the aorta (sham-operated mice). Animals were analyzed for TRPC channels expression 6 weeks after aortic banding.

Statistical analysis. Results are expressed as means ± SEM. Statistical comparison of the data was performed using the *t*-test for comparison between two groups or one-way analysis of variance and the post hoc Tukey test for comparison of more than two groups. A value of $P < 0.05$ was considered significant.

Results

Increased expression of TRPC1 in 5-HT-treated cardiomyoblasts

In order to delineate whether TRPC channels might be involved in 5-HT mediated hypertrophy we first used RT-PCR to investigate the expression of TRPC in H9C2 cardiomyoblasts. We performed PCRs using custom synthesized primer pairs based on rat cloned TRPC sequences specific for TRPC1, TRPC2, TRPC3 and TRPC6. As shown in Fig. 1A, H9C2 cardiomyoblasts constitutively express TRPC1 and TRPC6 genes. To address whether 5-HT stimulation regulates the expression of TRPC1 and TRPC6, cardiomyoblasts were treated with 5-HT for increasing time periods. Western blots were performed from cardiomyoblasts after 0, 6, 12 and 24 h of 5-HT treatment. Our results show that TRPC1 protein level increased with a peak at 24 h corresponding to a fourfold stimulation compared to unstimulated cells (Fig. 1B). In contrast, the expression of TRPC6 was unchanged following 5-HT stimulation (Fig. 1B).

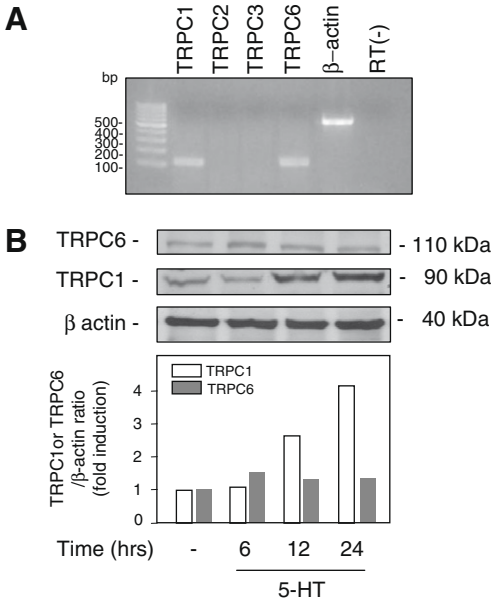

Fig. 1. TRPC channels expression in H9C2 cardiomyoblasts. (A) Analyse of TRPC1, TRPC2, TRPC3, TRPC6 and β-actin mRNA expression by RT-PCR as described under Materials and methods. Negative controls were performed without reverse transcriptase (RT −). (B) TRPC1 and TRPC6 protein expression following 5-HT stimulation were analyzed by immunoblotting experiments performed with TRPC1 and TRPC6 antibodies as described under Materials and methods. Cardiomyoblasts were stimulated with 5-HT (10 μM) for 6, 12 and 24 h. β-Actin expression was used as a loading control. The graph represents values of TRPC1 and TRPC6 band intensity after normalization for β-actin by densitometry. Results are representative of three independent experiments.

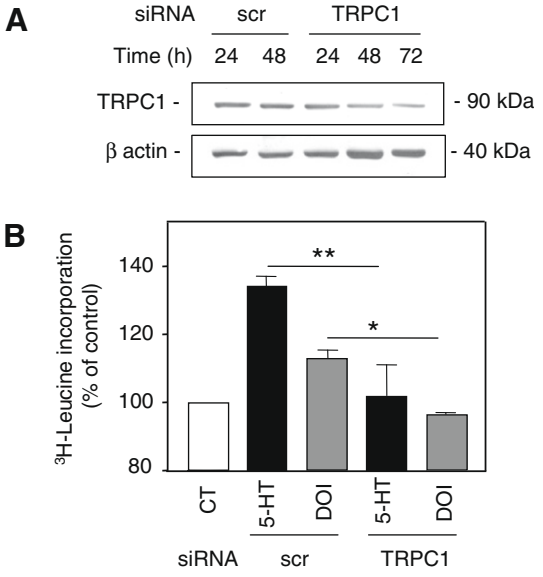

Fig. 2. TRPC1 knockdown decreases 5-HT-induced protein synthesis in H9C2 cardiomyoblasts. (A) TRPC1 expression in cardiomyoblasts at different time-points following transfection with 100 nM scramble (scr) or 100 nM TRPC1 siRNA. Blots are representative of three independent experiments. (B) Effect of TRPC1 knockdown on protein synthesis measured using [^{3}H]leucine incorporation. 48 h after scramble (scr) or TRPC1 siRNA transfection, cardiomyoblasts were treated 24 h with 5-HT (10 μM) or the 5-HT$_{2A}$ agonist DOI (1 μM). [^{3}H]leucine incorporation was measured as described under Materials and methods. Results are expressed as percentage of non-treated cells and are means ± SEM of four independent experiments ($^*P < 0.05$, $^{**}P < 0.01$ vs. indicated values).

These results suggest that 5-HT regulates TRPC1 expression in cardiomyoblasts.

Role of TRPC1 channel in the hypertrophic response induced by 5-HT$_{2A}$ receptors in cardiomyoblasts

We previously demonstrated that 5-HT promoted hypertrophic response in cardiomyoblasts and our current results show that 5-HT induces upregulation of TRPC1 expression. Since recent studies displayed strong evidences for a role of TRPC channels in development of cardiac hypertrophy [18] we hypothesized that TRPC1 gene knockdown should attenuate the hypertrophic response to 5-HT.

We used a siRNA approach to selectively inhibit TRPC1 expression in cardiomyoblasts. The inhibitory effect of TRPC1 siRNA on TRPC1 expression became obvious 48 and 72 h after transfection compared to scramble siRNA (Fig. 2A). To address whether TRPC1 knockdown attenuated the hypertrophic response, we examined the protein synthesis in cells treated with 5-HT (48 h post-transfection). We found that [^{3}H]leucine incorporation in response to 5-HT was significantly decreased in TRPC1 siRNA transfected cells compared to scramble siRNA transfected cells (Fig. 2B). Since we have recently showed that 5-HT$_{2A}$ receptors are responsible for the hypertrophic response induced by 5-HT in cardiomyoblast, we measured the protein synthesis in response to a 5-HT$_{2A}$ receptor agonist DOI. As shown in Fig. 2B, [^{3}H]leucine incorporation stimulated by 5-HT$_{2A}$ receptors is specifically inhibited by TRPC1 knockdown in cardiomyoblasts.

Role of TRPC1 channel in NFAT activation induced by 5-HT$_{2A}$ receptors in cardiomyoblasts

Because it has been shown that Ca^{2+} influx through TRPC channels is essential for NFAT-mediated hypertrophy, we examined the potential role of TRPC1 in the activation of NFAT by 5-HT$_{2A}$ receptors.

We first verified that in cardiomyoblasts, 5-HT$_{2A}$ receptors triggered a rapid increase in intracellular Ca^{2+} which might be the initiatory event in subsequent TRPC activation. Changes in intracellular Ca^{2+} of individual cells are recorded using the fluorescent ratio of Fluo3-AM probe. As displayed in Fig. 3A, 5-HT produced a rapid increase in Ca^{2+} which was abolished by the 5-HT$_{2A}$ receptor antagonist M100907.

The activation of NFAT was measured using cardiomyoblasts that stably express a reporter construct harboring four NFAT consensus binding sequences in front of the luciferase gene. As shown in Fig. 3B, NFAT activation induced by 5-HT is inhibited by the antagonist M100907 and by the Ca^{2+} chelator agent EGTA, confirming that 5-HT-triggered NFAT activation is dependent on 5-HT$_{2A}$ receptors and Ca^{2+} signaling. The potential role of TRPC1 in mediating NFAT activation was evaluated using TRPC1 knockdown. NFAT activation induced by 5-HT and DOI was significantly decreased in TRPC1 siRNA transfected cells compared to scramble siRNA transfected cells (Fig. 3C). Altogether, our results demonstrate that TRPC1 channels participate in the stimulation of the calcineurin/NFAT pathway by 5-HT$_{2A}$ receptors in cardiomyoblasts.

Expression of 5-HT$_{2A}$ receptors and TRPC channels in a model of heart hypertrophy

In order to further investigate the involvement of 5-HT$_{2A}$ receptors and TRPC channels during the development of cardiac hypertrophy *in vivo*, we analyzed their expression in aortic-banded mice, a typical model of pressure-overload induced hypertrophy. Western blot and densitometric analysis revealed a significant increase in 5-HT$_{2A}$ receptors and TRPC6 channels in aortic-banded hearts compared to sham hearts (Fig. 4). Unexpectedly, the expression of TRPC1 protein was not modified in this model of cardiac

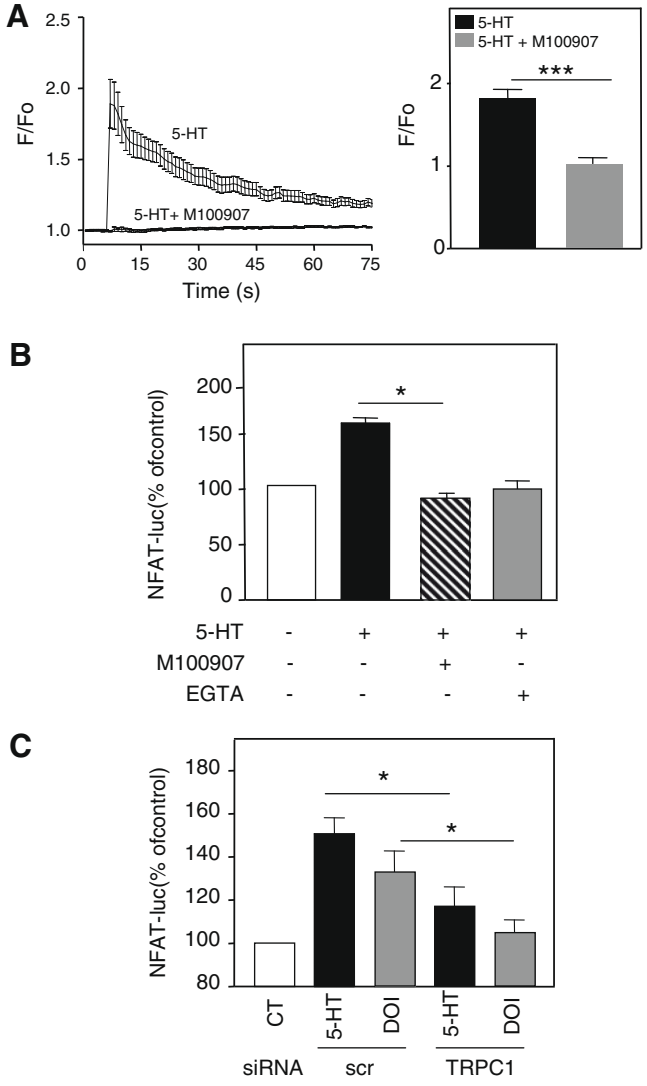

Fig. 3. Role of TRPC1 in NFAT activation induced by 5-HT_{2A} in H9C2 cardiomyoblasts. (A) Time-course of cytosolic Ca^{2+} in cardiomyoblasts stimulated with 5-HT in the presence or absence of the 5-HT_{2A} antagonist M100907. The variation of intracellular Ca^{2+} was detected by fluorescence intensity using Fluo3-AM as described under Materials and methods. Left panel, representative records of the variation of intracellular Ca^{2+} in cardiomyoblasts stimulated with 5-HT (10 μM) in the presence or absence of the 5-HT_{2A} antagonist M100907 (1 μM). Right panel, statistical data for the variation of intracellular Ca^{2+} are presented as the means ± SEM for 13 5-HT treated cells and 15 M100907 + 5-HT treated cells (***$P < 0.001$ vs. indicated values). (B) NFAT-luc reporter stimulation by 5-HT (10 μM) for 24 h in the presence of the 5-HT_{2A} antagonist M100907 (1 μM) or EGTA (1 mM). Results are expressed as percentage of non-treated cells and are means ± SEM of three independent experiments (*$P < 0.05$ vs. indicated values). (C) NFAT-luc reporter stimulation by 5-HT (10 μM) and DOI (1 μM) for 24 h in cardiomyoblasts transfected with 100 nM scramble (Scr) or 100 nM TRPC1 (TRPC1) siRNA. Results are expressed as percentage of non-treated cells and are means ± SEM of three independent experiments (*$P < 0.05$ vs. indicated values).

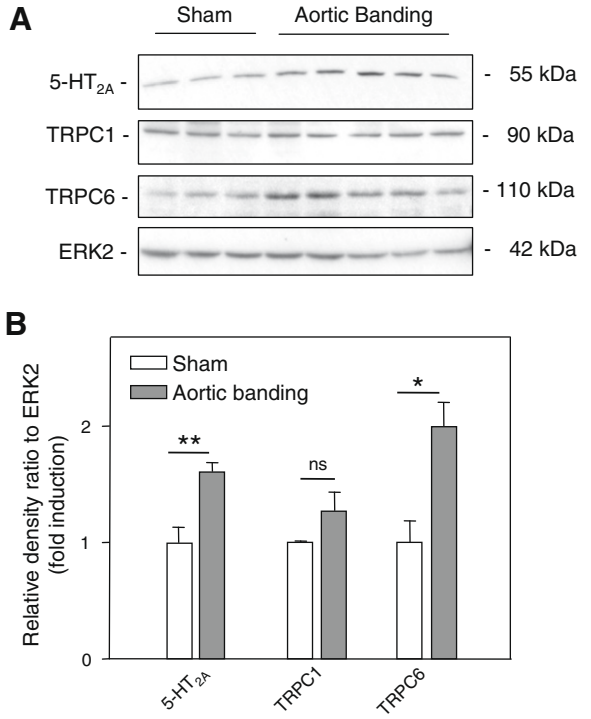

Fig. 4. Regulation of TRPC1 and TRPC6 expression in a mouse model of cardiac hypertrophy induced by aortic banding. Ten-week-old mice were subjected to ascendant aortic banding or sham operation to trigger pressure-overload hypertrophy of the heart. Six weeks after aortic banding, animals were sacrified and TRPC levels in cardiac protein lysates were analyzed by immunoblotting. (A) Immunoblots were performed on cardiac samples from sham and aortic-banded mice with anti-5-HT_{2A} receptor, anti-TRPC1 and anti-TRPC6 antibodies. ERK2 expression serves as a loading control. (B) Graph represents densitometric analysis of 5-HT_{2A} receptor, TRPC1 and TRPC6 protein levels in sham or aortic-banding mice. (*$P < 0.05$, **$P < 0.01$ between sham and banded mice).

hypertrophy. Finally, these results demonstrate that TRPC channels expression and 5-HT_{2A} receptors are upregulated during pressure overload in the heart which supports their critical function in the development of cardiac hypertrophy.

Discussion

5-HT has recently been described as a hypertrophic factor in cardiac cells, and we previously demonstrated that part of this effect was mediated through 5-HT_{2A} receptors [6,19]. 5-HT_{2A} receptor-mediated hypertrophy involves the activation of calcineurin/NFAT which is a prominent hypertrophic signaling pathway in the heart. The calcineurin phosphatase is activated by a sustained increase in calcium. However the upstream source of calcium responsible for the activation of the calcineurin/NFAT in response to 5-HT_{2A} receptors is not defined. In the current study, we provide evidence for the first time that TRPC1 channels are necessary for the stimulation of NFAT and subsequent hypertrophic response to 5-HT_{2A} receptors. Indeed, TRPC1 knockdown inhibited NFAT activation and protein synthesis induced by 5-HT stimulation in cardiomyoblasts. Our findings are consistent with a recent study demonstrating that TRPC1−/− mice were protected against maladaptative cardiac hypertrophy during pressure overload [20]. Therefore, TRPC1 channels may represent novel pharmacological targets for modulating pathologic calcineurin/NFAT signaling in the heart.

Some additional support for the involvement of TRPC channels in the development of cardiac hypertrophy has been provided by several independent studies demonstrating upregulation of TRPC1, TRPC3 and TRPC6 in some models of hypertrophy [9]. We reported here that 5-HT stimulation strongly upregulated the expression of TRPC1 by an unknown mechanism. Interestingly, among the potential signaling intermediates that regulate TRPC gene expression are the NFAT transcription factors themselves. TRPC1, TRPC3 and TRPC6 have conserved NFAT consensus sites in their promoter and recent study indicated that calcineurin inhibition was able to block the agonist-dependent induction of TRPC3 mRNA in rat cardiac myocytes [11]. Altogether, these data and our current study strongly support the fact that, once activated by TRPC channels,

NFAT transcription factors might stimulate TRPC expression through a positive feedback loop that further contributes to pathological remodeling.

The mechanism by which 5-HT_{2A} receptors activate TRPC channels is presently unknown. 5-HT_{2A} receptors are Gq-coupled receptors that trigger the production of IP3 and DAG through phospholipase C activation and subsequent Ca^{2+} release from ER stores. Indeed, our results show that 5-HT stimulation induces a rapid increase in intracellular Ca^{2+} which is abolished by the 5-HT_{2A} receptor antagonist M100907. Therefore, we hypothesize that, in our model, TRPC1 channel is activated by the Ca^{2+} depletion of ER stores induced by 5-HT_{2A} receptor stimulation. However, we cannot exclude that PLC-mediated production of IP3 and DAG induced by 5-HT_{2A} receptor could directly activate TRPC1, as previously demonstrated with other Gq-coupled receptors [13].

In vivo experiments indicate that, in cardiac ventricles from aortic-banded mice, 5-HT_{2A} receptors are upregulated which is consistent with their role in the development of cardiac hypertrophy. Unexpectedly, in this model of pressure-overload hypertrophy we found that TRPC6 expression was upregulated instead of TRPC1. One possible explanation for such discrepancy would be a crucial species difference. A large number of animal models of cardiac hypertrophy have been described in the literature. While the majority of studies perform in mice demonstrated upregulation of TRPC6 but not TRPC1 in ventricular hypertrophy induced by aortic banding, those performed in the rat identified changes in TRPC1 but not TRPC6 expression [14,15,21]. In addition, other subtypes like TRPC3 and TRPC5 have been found to be upregulated in different models of cardiac hypertrophy [18]. Therefore, a controversial issue would be to determine which TRPC isoform is most important in cardiac hypertrophy. Indeed, while demonstrating the importance of TRPC1 channel in 5-HT-mediated hypertrophy, our findings do not preclude a role for others TRPC channels since TRP proteins are thought to form heteromeric assemblies. Multimer formation of distinct TRPC proteins (TRPC1/TRPC4/TRPC5) has been observed by intermolecular resonance energy transfer measurements in heterologous expression systems [22]. Therefore, it is possible that functionality of TRPC channel in cardiac hypertrophy is dependant on the formation of specific complexes.

Although there is still discrepancy as to which subtype of the TRPC channels is the most important in cardiac hypertrophy, our data demonstrate an essential role for TRPC1 in NFAT activation and hypertrophic stimulation through 5-HT_{2A} receptors.

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