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FKBP12.6 Regulates The Secretion of Adrenal Chromaffin Cell in Mice*

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Abstract FK506 binding protein 12.6 (FKBP12.6), a protein that binds to and regulates the ryanodine receptor type 2 (RyR2) Ca^{2+} release channels, may act as an important regulator of catecholamine secretion. In the present study, the role of FKBP12.6 in the control of chromaffin cell exocytosis has been investigated using FKBP12.6-null mice. The results showed that FKBP12.6 was expressed in mouse chromaffin cells; deletion of FKBP12.6 did not change the depolarization induced Ca^{2+} current and exocytosis. However, deletion of FKBP12.6 resulted in an enhanced caffeine-induced global Ca^{2+} transient and larger caffeine-induced exocytosis in chromaffin cells of mice. These results indicate that FKBP12.6 is involved in catecholamine secretion through regulation of Ca^{2+} release channel in mouse chromaffin cells.

Key words FKBP12.6, chromaffin cell, catecholamine, Ca^{2+}, exocytosis
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The "fight or flight" response is triggered by a surge of catecholamines epinephrine or norepinephrine that preparing the body to survive by combating an enemy or to flee from danger [1]. The physiological function of chromaffin cells consists in the exocytotic release of the catecholamines epinephrine and norepinephrine into the circulation in response to stress[2]. Since this release is a Ca^{2+}-dependent process[3], chromaffin cells have been widely used as models to study the correlation between Ca^{2+} and exocytosis [4]. However, studies about the Ca^{2+} and exocytosis response in chromaffin cells mainly focused on the Ca^{2+} influx through voltage gated Ca^{2+} channels in the plasma membrane [5-6], the relations between intracellular Ca^{2+} release and exocytosis are poorly understood.

FKBP12.6 is a member of FK506-binding protein family and widely expressed in many cell types. FKBP12.6 is generally considered to bind to and regulate RyR2 [7]. It has been shown that FKBP12.6 plays an important role in cardiac excitation-contraction coupling, learning and memory of the hippocampus cells, and insulin secretion [8-10]. The possibility that FKBP12.6 plays a role in catecholamine secretion is supported by studies in permeabilized bovine chromaffin cells, suggesting that cADPR causes elevation of cytosolic Ca^{2+} concentration requiring the FKBP12.6 and RyR [11]. However, the potential role of FKBP12.6 in catecholamine secretion remains unclear.

In this study, we have used FKBP12.6$^{-/-}$ mice to study the role of FKBP12.6 in regulating exocytosis in chromaffin cells. Our results show that FKBP12.6 is highly expressed in mouse chromaffin cells. Deletion of FKBP12.6 had no effect on depolarization-induced exocytosis but enhanced caffeine-induced global Ca^{2+} transients and increased exocytosis.

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1 Materials and methods

1.1 Single cell preparation

Mouse adrenal chromaffin cells isolation were performed according to a previously published protocol [12]. Briefly, wild-type or FKBP12.6$^{-/-}$ mice of either sex (8～12 weeks) were anesthetized by 5% chloral hydrate in accordance with the guidance of the Committee on Animal Care of Institute of Biophysics, Chinese Academy of Sciences, China. The adrenals were harvested, fat and cortex were removed in an oxygenated buffer containing (in mmol/L) 135 NaCl, 6 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 0.2 EDTA, 10 HEPES, and 10 glucose(pH 7.3). The medullae were then incubated in an enzyme solution containing 40 U/ml papain (Gibco, USA), 1 mmol/L DTT, and 0.5 g/L BSA at 37℃ for 30 min. Finally, the tissues were agitated firstly with a fire polished wide-bore 200 μl tip and then a 23 gauge syringe needle to release the single chromaffin cells in a H.DMEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/L streptomycin for 1～2 days at 37℃, 5% CO_2.

1.2 Adrenal slice preparation

We prepared adrenal medulla slices according to a previous study[13] with a minor modification. In brief, adrenal glands were removed from 8～12-week-old wild type (WT) or FKBP12.6-knockout (KO) mice and were immediately immersed in ice-cold, low Ca^{2+} bicarbonate-buffered saline (BBS) containing (in mmol/L) 125 NaCl, 2.5 KCl, 0.1 $CaCl_2$, 5 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose, pH 7.4, when gassed with 95% O_2/5% CO_2. After that, a single gland was glued with cyanoacrylate to the stage of a vibratome chamber and covered with the same cold, O_2-saturated BBS. Slices (100～200 μm) were cut parallel to the larger base of the gland (Vibratome 1000; Vibrotome). They were then incubated for 30 min at room temperature in normal BBS containing (in mmol/L) 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose gassed with 95% O_2/5% CO_2. Slices could be used for up to 8 h after cutting. For whole-cell recordings, slices were transferred to a recording chamber attached to the stage of an upright microscope equipped with an infrared-sensitive charge-coupled device camera (LUMPLanFI/IR 60×, BX51 WI; Olympus) and continuously superfused with normal BBS at room temperature.

1.3 Patch clamp recordings

Whole-cell recordings were performed using an EPC9/2 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) with Pulse software (HEKA Elektronik). The pre-recorded action potential was externally applied to the voltage-clamped cell. We used pipettes of 2～4 MΩ. For voltage-clamp experiments, all holding potentials were –70 mV. We analyzed data using Igor software (Wavemetrics, Lake Oswego, Oregon). The standard internal solution contained (in mmol/L) 145 CsCl, 8 NaCl, 1 $MgCl_2$, 10 HEPES and 2 ATP, 0.4 GTP (pH 7.2). All chemicals were from Sigma (St. Louis, Missouri).

1.4 C_m measurements

We measured the membrane capacitance using the software lock-in module of Pulse 8.30 together with an EPC-9/2 amplifier. A 1 kHz, 40 mV peak-to-peak sinusoid was applied around a DC holding potential of –70 mV. We analyzed the resulting current using the Lindau-Neher technique to give estimates of the C_m, membrane conductance and the series resistance.

1.5 Confocal Ca^{2+} imaging

Chromaffin cells were incubated with 10 μmol/L Fluo-4 AM (Invitrogen) in normal Tyrode solution containing (in mmol/L) 137 NaCl, 5.4 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 20 HEPES and 10 glucose for 20 min at 37℃ and transferred to a recording chamber. Cells were then perfused with normal Tyrode solution for 30 min for de-esterification. 10 mmol/L caffeine was rapidly added by a nearby pippete. Confocal line-scan imaging was performed by excitation with a 488 nm light from the argon laser of a Leica SP5 inverted confocal microscope (40×, 1.25 NA). Line-scan images were acquired at a sampling rate of 1.43 ms per line, along the longitudinal axis of the cell. Digital image processing was performed using MATLAB 7.1 (Math Works).

1.6 Immunostaining of RyR and FKBP12/12.6

Single cells isolated from adrenal medulla were fixed in 4% paraformaldehyde in PBS for 20 min. Fixed samples were washed three times, 5 min per time, in PBS and permeabilized in PBS containing 0.1% Triton-X 100 for 15 min before incubating in blocking buffer (2% BSA in PBS) for 2 h to block non-specific binding of the antibody. The cells were then incubated with anti-RyR antibody (1∶100, Pierce Antibody) and anti-FKBP12/12.6 antibody (1∶20, Abcam) overnight in 4℃. After another three 5 min

washes in PBS, secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 543-conjugated goat anti-rabbit IgG) (1∶1 000, molecular probes) was added to the blocking buffer and incubated with the cells for 1 h, and then washed out by three 5 min washes in PBS. Cells were then mounted on slides and examined using a laser scanning confocal microscope (Leica SP5, 40 × 1.25 NA oil immersion objective). Images were analyzed using ImageJ.

1.7 Western blotting

Mouse adrenal medullae were harvested, quickly rinsed in PBS, immediately frozen in liquid nitrogen and stored at –80℃. Frozen tissues were homogenized in RIPA lysis buffer (Beyotime Biotchnology, China) containing a protease inhibitor cocktail (Roche). Tissue lysates were then centrifuged at 12 000 *g* for 5 min to remove insoluble debris. Protein concentrations were determined using the BCA assay. Adrenal medulla lysates were heated at 85℃ for 5 min in 1× sample loading buffer containing 5% β-mercaptoethanol before loading on gels. 20～30 micrograms of protein were size-fractionated on 15% SDS-polyacrylamide gels before transfer to PVDF membranes (Millipore) at 200 mA for 30 min. Membranes were probed with anti-GAPDH (1∶10 000, Sigma), anti-FKBP12/12.6 (1∶100, Abcam). Protein-signal densities were normalized to the corresponding GAPDH-signal densities.

1.8 RT-PCR

The cortex of adrenals was carefully removed and the medullae were then quickly frozen in liquid nitrogen. The total RNA of the medullae was isolated with the TRIzol (Invitrogen) method following the manufacturer's guidelines; 2 μg of RNA was then reverse transcribed to first-stand cDNA using random primers and M-MLV reverse tanscriptase (Promega) according to the manufacturer's protocol. The RT-PCR profile was as follows: 95℃ for 60 s, followed by 35 cycles of 95℃ for 20 s, 55℃ for 30 s, and 72℃ for 60 s. Primers used are as follows. RyR1: Forward-GCACACTGGTCAGGAGTCGTATG, Reverse- GGGTGTAGCACAGGATTTAT; RyR2: Forward- GAATTCATCATGGATACTCTACC, Reverse-GTCATGCACATTATCTTCTGCAT; RyR3: Forward-CCTGAGTTCACGACAAGCTACAG, Reverse-TAGCTG - CTTAAAGCTTTTCAAGC; FKBP12: Forward-CTCTCGGGACAGAAACAAGC, Reverse- AGAGTGGCATGTGGTGGGA; FKBP12.6: Forward-CCAGGAGACGGAAGGACAT, Reverse-CAAAGATGAGGGT - GGCATTG.

1.9 Statistical analysis

Statistical analysis was performed using SigmaPlot (Systat Software Inc., San Jose, CA, USA). Values given are $\bar{x} \pm s$. Data were tested for significance using the Student's t test. Only results with values of $P < 0.05$ were considered statistically significant.

2 Results

2.1 Both FKBP12 and FKBP12.6 are expressed in mouse adrenal chromaffin cells

Our RT-PCR results show that the mRNA of FKBP12 and FKBP12.6 are expressed in the mouse adrenal chromaffin cells (Figure 1a). The expressions of FKBP12 and FKBP12.6 messenger RNA are both abundant in wild-type adrenal medullae and a lack of FKBP12.6 mRNA in adrenal medullae of the FKBP12.6$^{-/-}$ mice. The FKBP12.6 protein was also present in chromaffin cells (Figure 1b). We also found that both RyR2 and RyR3, but not RyR1 are present in mouse adrenal chromaffin cells, which is in accordance with a previous report [14]. A ring-like pattern of fluorescence indicated RyR proteins are located mainly near the cell membrane (Figure 1d-*A*) and colocalized with FKBP12/FKBP12.6 (Figure 1d-*D*). It is reported that FKBP12.6 bind specifically to RyR2 [8], however, the role of FKBP12.6 in the regulation of exocytosis in chromaffin cells has not been examined yet in intact cells.

2.2 Deletion of FKBP12.6$^{-/-}$ in chromaffin cells results in unaltered response with depolarization induced exocytosis

Action potentials open plasma membrane Ca^{2+} channels of the chromffin cells and produce Ca^{2+} influx; the resulting cytosolic Ca^{2+} elevation triggers exocytosis [15–16]. The Ca^{2+} entry may cause Ca^{2+} release from nearby RyRs (Ca^{2+} induced Ca^{2+} release, CICR) located in the endoplasmic reticulum (ER) [17–18], which could amplify the Ca^{2+} and further enhance the exocytosis. To test whether FKBP12.6 also regulate voltage gated channels and CICR, whole-cell patch-clamped cells were stimulated by 5 s depolarization pulse train, membrane Ca^{2+} current and membrane capacitance change were simultaneously monitored (Figure 2). FKBP12.6-null chromaffin cells show similar voltage-gated calcium currents with WT cells ((291 ± 135) pA *vs* (353 ± 87) pA, $P = 0.33$, $n = 8$,

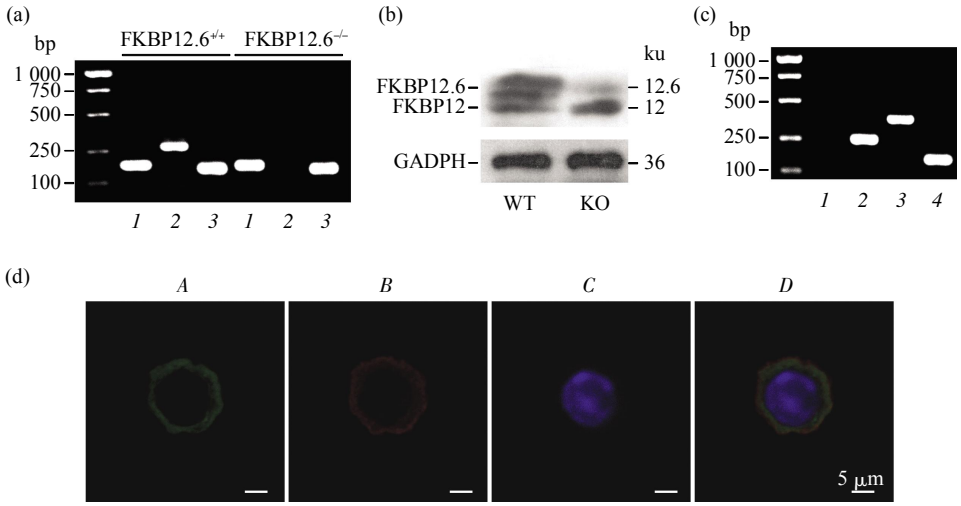

Fig. 1　Expression of FKBP12, FBP12 and RyRs in mouse adrenal chromaffin cells

(a) RT-PCR products of chromaffin cells, both FKBP12 and FKBP12.6 are present in the cells. *1*: FKBP12; *2*: FKBP12.6; *3*: GADPH. (b) Western blots analysis of the FKBP12.6 protein expression in mouse adrenal medullae. (c) RyR2 and RyR3, not RyR1, are present in mouse adrenal chromaffin cells. *1*: RyR1; *2*: RyR2; *3*: RyR2; *4*: GAPDH. (d) Immunostaining of RyR (*A*), FKBP12/ FKBP12.6 (*B*), and DAPI(*C*). The RyR colocalized with FKBP12.6 (*D*).

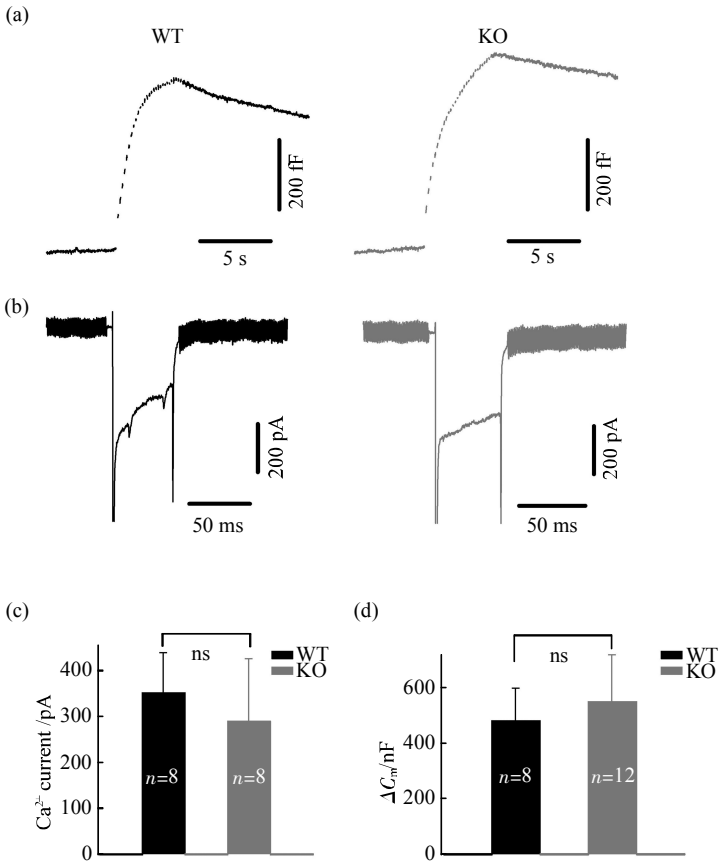

Fig. 2　FKBP12.6 deficient chromaffin cells display normal depolarization-induced exocytosis and calcium current

(a) Membrane capacitance (C_m) responses to step depolarization train in FKBP12.6 KO mice (right panel) versus WT mice (left panel). (b) Ca^{2+} current recording of single step depolarization in FKBP12.6 KO mice (right panel) versus WT mice (left panel). (c) Histograms show the amount of depolarization-induced Ca^{2+} current in two cell types. I_{Ca}= (353±87) pA (WT) and (291±135) pA (KO), P = 0.33. (d) Histograms show the amount of depolarization-induced exocytosis in two cell types. ΔC_m= (480±120) pF (WT) and (560±170) pF (KO), P = 0.29. Data are presented as the $\bar{x} \pm s$; n = 8～12 cells from 3 mice per group.

Figure 2c). Membrane depolarization induced secretion did not change (10%) in KO cells, compared to the WT cells ((48±120) nF to (560±170) nF, P = 0.29, Figure 2d). Taken together, these results indicate that deletion of FKBP12.6 has little effect in the depolarization induced Ca^{2+} current and exocytosis in mouse chromaffin cells.

2.3 Genetic deletion of FKBP12.6 enhanced caffeine-induced calcium transients and exocytosis

Deletion of FKBP12.6 causes Ca^{2+} leak in cardiomyocytes [19–20], which will influence the homeostasis of the intracellular Ca^{2+}. Here we sought to investigate whether FKBP12.6 has similar effect in mouse chromaffin cells. We firstly tried to measue the spontaneous Ca^{2+} release in mouse chromaffin cells, however, we did not detect spontaneous Ca^{2+} sparks in either WT or KO cells. Then 10 mmol/L caffeine was added to activate RyRs in the ER (Figure 3). High-dose caffeine induced a robust Ca^{2+} transients in both FKBP12.6$^{+/+}$ and FKBP12.6$^{-/-}$ cells and KO cells displayed a 47% higher Ca^{2+} transients amplitude than those of WT cells (from (3.41±0.41) to (5.01±0.44), P < 0.05, n = 10, Figure 3b and c). Thus, genetic deletion of FKBP12.6 enhanced caffeine-induced calcium transients.

In adrenal chromaffin cells, caffeine induces Ca^{2+} release from caffeine-sensitive (ryanodine) store. Removal of FKBP 12.6 from RyR2 leads to a larger caffeine-induced calcium transient. The released Ca^{2+} triggers large sense core vesicle exocytosis. Therefore, we took membrane capacitance method to monitor exocytosis. We first determined whether FKBP12.6 regulates the characteristics of caffeine-induced exocytosis. Local application of 10 mmol/L caffeine elicited a ΔC_m change in both FKBP12.6 deficient cells and WT cells (Figure 4). Two parameters of C_m trace were analyzed, peak C_m and delay time, which

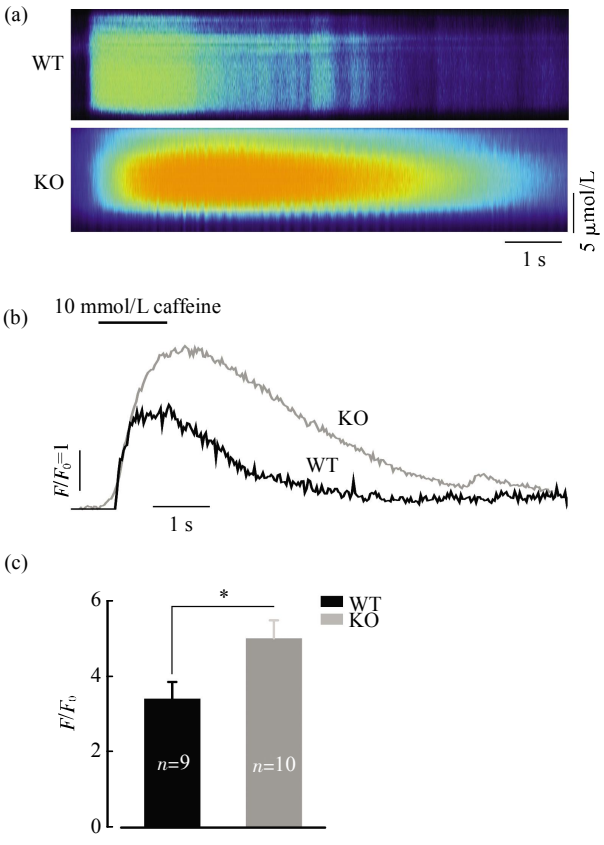

Fig. 3 Deletion of FKBP12.6 results in enhanced caffeine-induced calcium transient

(a) Representative confocal line-scan images of 10 mmol/L caffeine-induced Ca^{2+} transient in WT (upper) or KO (lower) mouse chromaffin cells. (b) Representative profile of 10 mmol/L caffeine-induced Ca^{2+} transient in wild-type (black) and knockout (gray) cells. (c) KO cells displayed a 47% higher Ca^{2+} transient amplitude than that of WT cells ((5.01±0.44) *vs* (3.41±0.41), P < 0.05). Data are presented as the $\bar{x} \pm s$; n = 10 cells from 3 mice per group; *P < 0.05 *vs* WT.

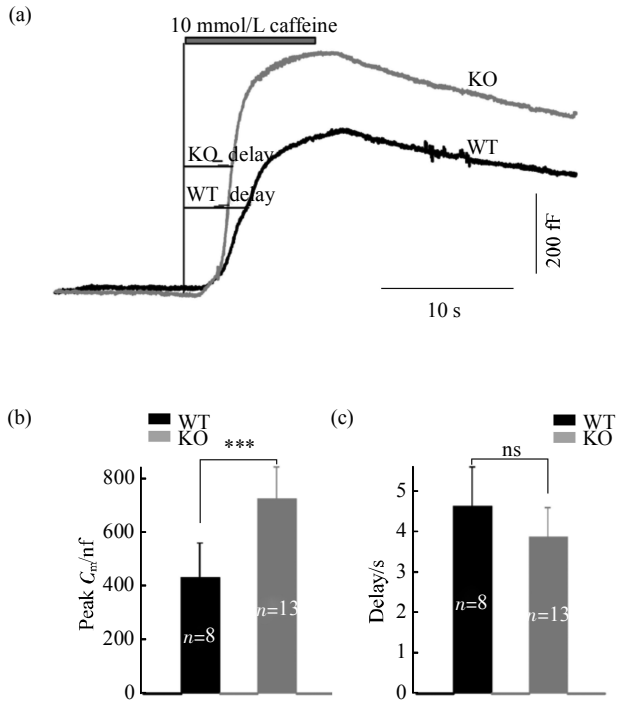

Fig. 4 FKBP12.6 deficiency displays increased caffeine-induced exocytosis in chromaffin cells

(a) Averaged membrane capacitance (C_m) traces in responses to a 10s-stimulus of 10 mmol/L caffeine show different C_m change in FKBP12.6 deficient cells versus WT cell. Two horizontal lines show delay time (from puff begin time to half-max C_m time) in two cell types. (b) Histograms show the amount of exocytosis in two cell types. ΔC_m=(430 ± 120) nF (WT) and (730 ± 0.12) nF (KO), P = 0.0002. (c) Histograms show the amount of delay time ((4.64±0.97) s (WT) *vs* (3.89 ± 0.74) s (KO)), P = 0.09. Data are presented as the $\bar{x} \pm s$; n = 8～13 cells from 3 mice per group; ***P <0.001 *vs* WT.

represent the amount of caffeine-induced exocytosis and its kinetics. Peak C_m was increased markedly in FKBP12.6 KO cells (WT, (430 ± 120) pF; KO, (730 ± 120) pF, $P < 0.001$, Figure 4b). However, no noticeable difference was detected in delay time in FKBP12.6 KO cells (WT, (4.64 ± 0.97) s; KO, (3.89 ± 0.74) s, $P > 0.05$, Figure 4c). These data suggest that FKBP12.6 play a role in caffeine-induced exocytosis in chromaffin cells.

3 Discussion

In the present study, FKBP12.6$^{-/-}$ mice have been employed to study the function of RyR2/FKBP12.6 in the regulation of chromaffin catecholamine secretion. Firstly, we demonstrated that both FKBP12 and FKBP12.6 are abundantly expressed in mouse chromaffin cell (Figure 1a, b), which is inconsistent with a previous study, suggesting that FKBP12 and FKBP12.6 protein are present in bovine adrenal medullary chromaffin cells[11]. RyR2 and RyR3, but not RyR1, are expressed in mouse chromaffin cell(Figure 1c). Immunofluorescence analysis indicated that RyRs are located perinuclear and near the plasma membrane, and colocalized with FKBP12/12.6 (Figure 1d), which is consistent with a previous report[14].

The adjacency of RyR2s to the plasma membrane let us wonder whether RyR2/FKBP12.6 can regulate the exocytosis through the CICR process. However, FKBP12.6-null chromaffin cells display normal depolarization-induced exocytosis (Figure 2d), which is consistent with a previous study that the CICR is small or nonexistent in mouse chromaffin cells[21]. The microdomain of Ca^{2+} influx from voltage-gated Ca^{2+} channel may distinct from the microdomain where RyR2 release Ca^{2+} in mouse chromaffin cells, which are supported by the studies that local intracelluar Ca^{2+} release happens at a site different from the microdomain where exocytosis occurs [14] and suppression of Ca^{2+} sparks increases spontaneous exocytosis in mouse adrenal chromaffin cells[22].

Then we investigate the role of FKBP12.6 in the global Ca^{2+} transients. A significant higher caffeine-induced Ca^{2+} transient was present in FKBP12.6$^{-/-}$ chromaffin cells (Figure 3). As FKBP12.6 binds to and stabilizes the Ca^{2+} release channel RyR2[23], removal of FKBP12.6 from RyR2 increases the open probability of the channel and causes an enhanced caffeine-induced Ca^{2+} transient. Concomitantly, FKBP12.6 deficient chromaffin cells display increased caffeine-induced exocytosis (Figure 4), which further confirmed that the Ca^{2+} dependent exocytosis is regulated by the RyR2/FKBP12.6 complex.

In summary, our study reveals that FKBP12.6 is involved in regulation of catecholamine secretion in mouse adrenal chromaffin cells.

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FK506 结合蛋白 12.6 调节小鼠嗜铬细胞分泌 *

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摘要 FK506 结合蛋白 12.6(FKBP12.6)能够结合并调控钙离子释放通道兰尼碱受体 2 型(RyR2)的开放，可能是儿茶酚胺分泌的重要调控器．利用 FKBP12.6 敲除小鼠模型，我们研究了 FKBP12.6 在肾上腺嗜铬细胞胞吐中的作用．结果表明，FKBP12.6 在小鼠肾上腺嗜铬细胞中表达，而敲除 FKBP12.6 小鼠的嗜铬细胞中有正常的去极化引起的钙电流和胞吐作用．然而，FKBP12.6 敲除会导致嗜铬细胞中出现增强的咖啡因引起的细胞整体钙瞬变和咖啡因引起的胞吐作用．结果提示，FKBP12.6 调控肾上腺嗜铬细胞儿茶酚胺的分泌，这种调控作用是通过调节钙离子的释放而实现的．FKBP12.6 是嗜铬细胞分泌的重要蛋白．

关键词 FK506 结合蛋白 12.6，肾上腺素嗜铬细胞，儿茶酚胺，钙离子，胞吐作用
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