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Calcium-dependent mechanisms involved in the modulation of tyrosine hydroxylase by endothelins in the olfactory bulb of normotensive rats

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ABSTRACT

Endothelins (ETs) are widely expressed in the olfactory bulb (OB) and other brain areas where they function as neuropeptides. In a previous study we reported that in the OB ET-1 and ET-3 participate in the long-term regulation of tyrosine hydroxylase (TH), the key enzyme in catecholamine biosynthesis. ETs stimulate TH activity by increasing total and phosphorylated enzyme levels as well as its mRNA. ET-1 response is mediated by a super high affinity ET_A receptor coupled to adenylyl cyclase/protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) activation whereas that of ET-3 through an atypical receptor coupled not only to these signaling pathways but also to phospholipase C (PLC)/protein kinase C pathway. Given the participation of PLC and CaMKII in the regulation of TH by ETs in the OB we sought to establish the contribution of calcium to ETs response. Present findings show that calcium released from ryanodine-sensitive channels and extracellular calcium were necessary to stimulate TH by ETs through CaMK-II. On the other hand, intracellular calcium released by the endoplasmic reticulum partially mediated ETs-evoked increase in TH mRNA but calcium influx and CaMK-II inhibition abolished the response. However calcium mechanisms were not involved in ETs-evoked increase in TH protein content. Present findings support that different sources of calcium contribute to the long-term modulation of TH activity and expression mediated by ETs in the rat OB.

1. Introduction

Catecholamines (dopamine, norepinephrine and epinephrine) are important neurotransmitters involved in the regulation of numerous biological functions. Tyrosine hydroxylase (TH) is a specific marker of catecholaminergic neurons and the key enzyme in catecholamine biosythesis. It catalyzes the hydrolysis of L-tyrosine to L-Dopa and its regulation is a complex process involving short and long-term mechanisms (Kumer and Vrana, 1996; Dunkley et al., 2004; Daubner et al., 2011). TH is highly expressed in diverse areas and regions of the central nervous system (CNS) including the periglomerular layer of the olfactory bulb (OB) (Cigola et al., 1998). This brain area is an extension of the rostral telencephalon and plays a relevant role in the interaction between the animal and its environment (Cain, 1974). The OB connects to the hypothalamus and regions of the limbic system as the amygdala, septum, pyriform cortex and frontoorbito cortex (Kawasaki et al., 1980; Song and Leonard, 2005). Most of these brain regions and areas are involved in the regulation of the cardiovascular function and mood disorders. The loss of these connections by OB ablation causes behavioral, endocrine, immune and neural transmission changes similar to those observed in patients with depression (Kelly et al., 1997; Song and Leonard, 2005).

Endothelins (ETs) are a family of vasoactive related peptides comprised by three isoforms ET-1, ET-2 and ET-3 that are expressed in different areas of the mammalian CNS including the OB (Kurokawa et al., 1997; Wright and Harding, 1997; Schneider et al., 2007). ETs modulate neuronal activity acting as putative neurotransmitters or regulatory neuropeptides (Yamada and Kurokawa, 1998; Vatta et al., 2009). Neuropeptides act in the regulation of the synthesis, storage, release and reuptake of different neurotransmitters including catecholamines (Webber et al., 1998; Vatta et al., 2009). We previously reported that ETs modulate catecholamine synthesis in the OB by enhancing TH activity through an increase in the phosphorylated forms of the enzyme and its mRNA (Nabhen et al., 2011). ET-1 increases TH activity through adenylyl cyclase/PKA and CaMK-II pathways, whereas

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ET-3 not only through these pathways but also through the PLC/PKC pathway (Nabhen et al., 2011).

Given that calcium has been involved in the regulation of TH activity and expression (Kumer and Vrana, 1996; Dunkley et al., 2004) and that the physiological responses mediated by ETs can be calcium dependent or calcium independent (Tykocki and Watts, 2010), in the present study we sought to determine the relative contribution of calcium to the long-term regulation of TH by ETs in the OB of normotensive rats. In order to establish the participation of calcium the activity, total protein and mRNA levels of TH were evaluated in the presence of IP_3 receptors, ryanodine-sensitive channels, and CaMK-II inhibitors as well as in a low concentration calcium medium (LCM). Our results show that distinct sources of calcium contributed to the long-term regulation of TH activity and expression mediated by ETs in the OB of normotensive rats.

2. Materials and methods

2.1. Animals and chemicals

Male Sprague–Dawley rats weighing between 250 and 300 g (from the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires) were used in the experiments. Rats were housed in steel cages and maintained in a controlled room (temperature and humidity between 20–23 °C and 50–60%, respectively) and a 12 h light/dark cycle. All animals had free access to water and commercial chow.

The following drugs and reagents were used: ET-1, ET-3 (American Peptide Company Inc., CA, USA). Catalase, L-DOPA, 1-[N,O-bis-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), 6-methyl-tetrahydrobiopterin and L-tyrosine (MP Biomedicals LLC, CA, USA). L-[3,5–^{3}H]tyrosine and DL-[7-3H]NE (Perkin Elmer Life and Analytical Sciences, MA, USA); PVDF membrane (GE Healthcare, Amersham Biosciences, UK); anti-actin polyclonal antibody (Ab) (Actin-Ab), anti-TH monoclonal Ab (TH-Ab), protease inhibitor cocktail, dantrolene sodium salt (DNT) (Sigma, MO, USA). 2-Aminoethoxydiphenylborate (2-APB) (Calbiochem, CA, USA). Peroxidase conjugated anti-mouse Ab, and anti-rabbit Ab (Pierce, USA), Rabbit anti-TH phospho-Ser-40 (TH S40p Ab, from Invitrogen, CA, USA) and minimum essential medium MEM/EBSS (HyClone Lab. Inv., UT, USA). Other reagents were of analytical or molecular biology quality and obtained from standard sources.

2.2. Experimental protocol

Experiments were performed following the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication 1985, Revised 1996) and approved by the Institutional Animal Care and Use Committee of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. Animals were killed by decapitation between 9:00 and 12:00 h and the OB quickly removed according to Palkovits and Brownstein (1988). Tissues were pre-incubated for 30 min at 37 °C in Krebs bicarbonate solution supplemented with minimum essential medium MEM/EBSS (KBSS), pH 7.4, and bubbled with a gas mixture (95% O_2–5% CO_2) under continuous shaking. To determine the effect of OB long exposure to ET-1 or ET-3 on TH activity and expression, tissues were incubated for 240 min in the absence

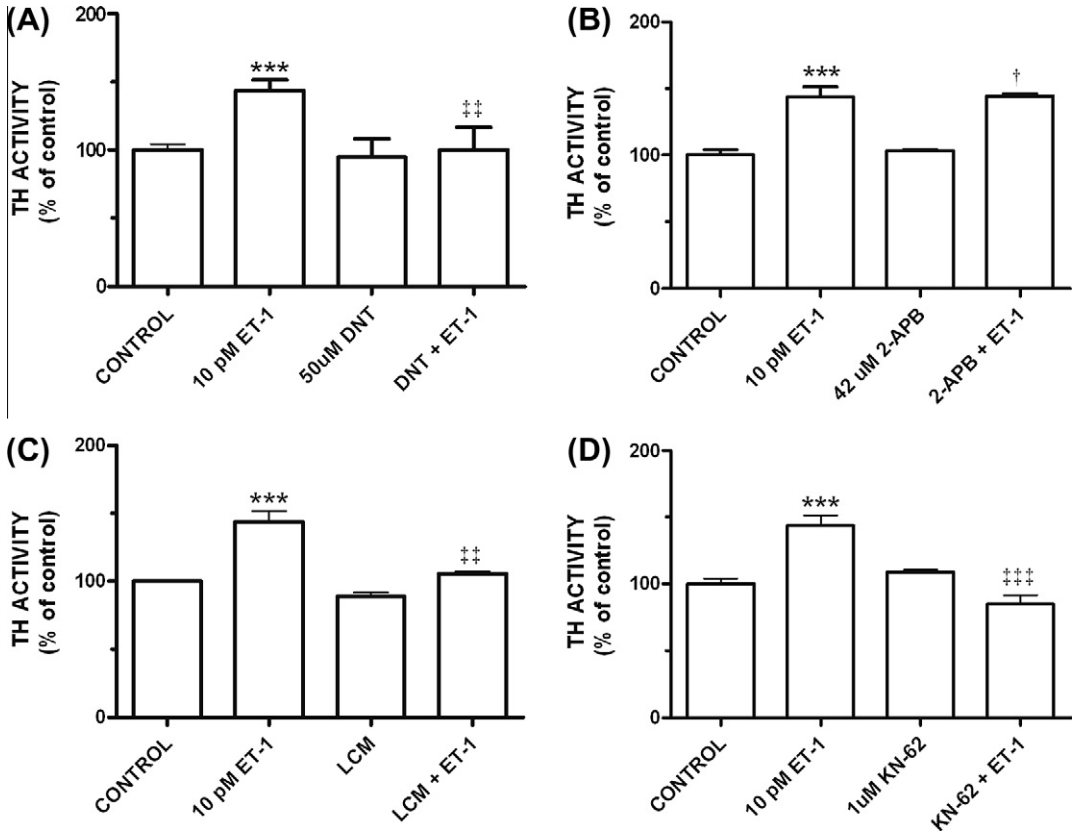

Fig. 1. Role of calcium in endothelin-1 (ET-1)-stimulated tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OB were incubated with ET-1 in the presence of DNT (ryanodine-sensitive channels inhibitor) (A), 2-APB (IP_3 receptor antagonist) (B), LCM (low calcium medium) (C) and KN-62 (CaMK-II inhibitor) (D). TH activity was expressed as percentage of control. $^{***}p < 0.001$ vs. control; $^{‡‡}p < 0.01$, $^{‡‡‡}p < 0.001$ vs. ET-1; $^{†}p < 0.05$ vs. 2-APB. Number of experiments: 5–7.

or presence of ETs (control and experimental groups, respectively). The following antagonists or inhibitors were added 15 min before and during the incubation period: 1 μM KN-62 (CaMKII inhibitor), 42 μM 2-APB(IP_3 receptor selective antagonist), and 50 μM DNT (ryanodine-sensitive channels inhibitor). In other experiments, tissues were incubated in a modified medium with low calcium concentration (LCM) (118 mM NaCl, 4.7 mM KCl, 200 μM $CaCl_2$, 12 mM $MgSO_4$ $7H_2O$, 1 mM NaH_2PO_4, 400 μM CdCl, 0.004 mM EDTA, 0.11 mM ascorbic acid, 26 mM NaH CO_3, 11.1 mM glucose).

2.2.1. Determination of TH activity

TH activity was assessed as reported by Reinhard et al. (1986). Briefly, following the incubation period, tissues were homogenized in 500 μl buffer (5 mM KH_2PO_4 and 0.2% Triton X-100, pH 7.0). After saving an aliquot for protein determination, samples were centrifuged for 10 min at 10,000*g* at 4 °C, and supernatants further incubated for 20 min at 37 °C with 50 mM HEPES (pH 7.0), containing 15 nmol L-tyrosine with 0.5 μCi ^{3}H-tyrosine, 420 mM β-mercaptoethanol, 1000 U catalase, and 0.75 mM 6-methyl-tetrahydrobiopterin. The reaction was stopped by the addition of 7.5% activated charcoal suspension (1 ml) in 1 N HCl. Samples were then vortexed and centrifuged at 500*g* for 10 min followed by ^3H_2O determination in supernatants by conventional scintillation methods. Blank values were obtained by omitting 6-methyl-tetrahydrobiopterin from the mixture. Recovered ^3H_2O was assessed as previously described (Reinhard et al., 1986). Results were expressed as percentage of control group ± SEM.

2.2.2. TH Western blot assay

The OB were homogenized in lysis buffer (20 mM Tris–Cl^- pH: 7.4, 1 mM PMSF, 5 mM EDTA, 25 mM NaF, 1% Triton X-100, 1% protease inhibitor cocktail) and then centrifuged for 20 min at 4 °C. A supernatant aliquot was saved to measure proteins and the remaining sample mixed with LAEMMLI buffer (62.5 mM Tris–Cl^- pH: 6.8, 2% SDS, 5% B-ME, 10% glycerol, 4% bromophenol blue), boiled for 5 min, and then subjected to SDS–PAGE at 100 V for 2.30 h. Proteins were then transferred to PVDF membranes at 100 V for 75 min and blocked overnight at 4 °C in 5% non-fat powder milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Membranes were then washed with TBS-T, and incubated with TH-Ab (1/3000), TH S40p Ab (1/2000) or β-actin Ab (1/3000), overnight at 4 °C; peroxidase conjugated anti-mouse Ab or anti-rabbit Ab (1/5000, 1 h at room temperature). Following TBS-T washes bands were detected with Super Signal West Femto kit (Pierce, IL, USA), analyzed by densitometry and normalized to β-actin. Results were expressed as percentage of control group ± SEM.

2.2.3. Quantitative real-time RT-PCR for TH mRNA

Total RNA from OB was isolated with TRI Reagent (Molecular Reasearch Center Inc., Cincinnati, OH, USA) according to the manufacturer's recommendations. Contaminating DNA was removed by incubation with RQ-1 RNase-Free DNase (Promega, WI, USA) at 37 °C for 40 min and RNA concentration measured by spectrophotometry.

The cDNA was reversed transcripted with M-MuLV Reverse Transcriptase (Fermentas Life Sciences, Burlington, ON, Canada), dNTPs and oligo (dT) primer. In each sample specific mRNA levels were measured in ROTOR GENE-Q termocycler (Qiagen, Germany) in a 20 μl final volume. Reactions were performed with 10 μl of 2X Real Mix containing Eva-green fluorocrome (Biodynamics, Argentina), 0.5 μl specific primers (10 μM) and 1 μl reverse transcriptase

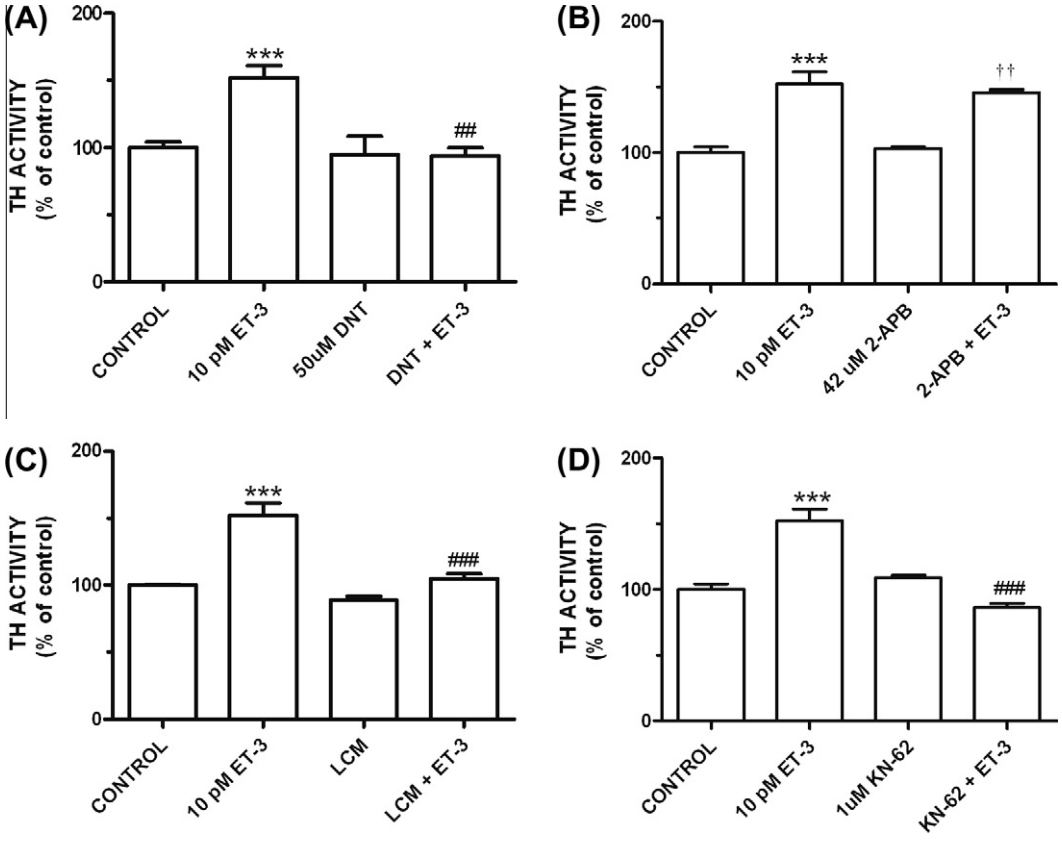

Fig. 2. Role of calcium in endothelin-3 (ET-3)-stimulated tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OB were incubated with ET-3 in the presence of DNT (ryanodine-sensitive channels inhibitor) (A), 2-APB (IP_3 receptor antagonist) (B), LCM (low calcium medium) (C) and KN-62 (CaMK-II inhibitor) (D). TH activity was expressed as percentage of control. $^{***}p < 0.001$ vs. control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. ET-3; $^{\dagger\dagger}p < 0.01$ vs. 2-APB. Number of experiments: 5–7.

product. Amplification consisted of 35 cycles of denaturation at 94 °C for 15 s, annealing at 54 °C for 45 s, and extension at 72 °C for 30 s. Fluorescence signals were monitored sequentially for each sample once per cycle at the end of extension. All samples were performed in duplicate.

An external standard RNA concentration curve for each primer pair was generated using pooled RNA samples and verified by agarose gel electrophoresis. The specificity of PCR products was confirmed by melting curves analysis showing the presence of a single sDNA product per primer pair, and by agarose gel electrophoresis revealing a single band of the predicted molecular weight for each product. To correct for minor variability among samples, TH expression level was normalized to the housekeeping gene β-actin. Relative expression levels quantification was performed with the standard curve method. The following primers were used:

–TH	forward	5'-AGGGCTGCTGTCTTCCTAC-3'
	reverse	5'-GCTGTGTCTGGGTCAAAGG-3'
–β-actin	forward	5'-TTCTGTGTGGATTGGT GGCTCTA-3'
	reverse	5'-CTGCTTGCTGATCCACATCTG-3'

2.3. Statistical analysis

Results were expressed as means ± SEM. The statistical analysis was performed by ANOVA followed by the Student–Newman–Keuls test. p values of 0.05 or less were considered statistically significant.

3. Results

In order to identify the calcium source involved in the long-term activation of TH by ET-1 and ET-3, the role of intracellular and extracellular calcium was assessed. To evaluate the participation of intracellular calcium stores, experiments were performed in the presence of selective inhibitors of IP_3 receptors (2-APB) and ryanodine-sensitive channels (DNT). None of the inhibitors affected basal TH activity (Figs. 1 and 2). The increase in TH activity induced by ETs was not modified by 2-APB (Figs. 1 and 2B) but it was abolished by DNT (Figs. 1 and 2A).

The role of extracellular calcium was assessed by exposing OB to ETs in a LCM. Figs. 1 and 2C show that enhanced TH activity

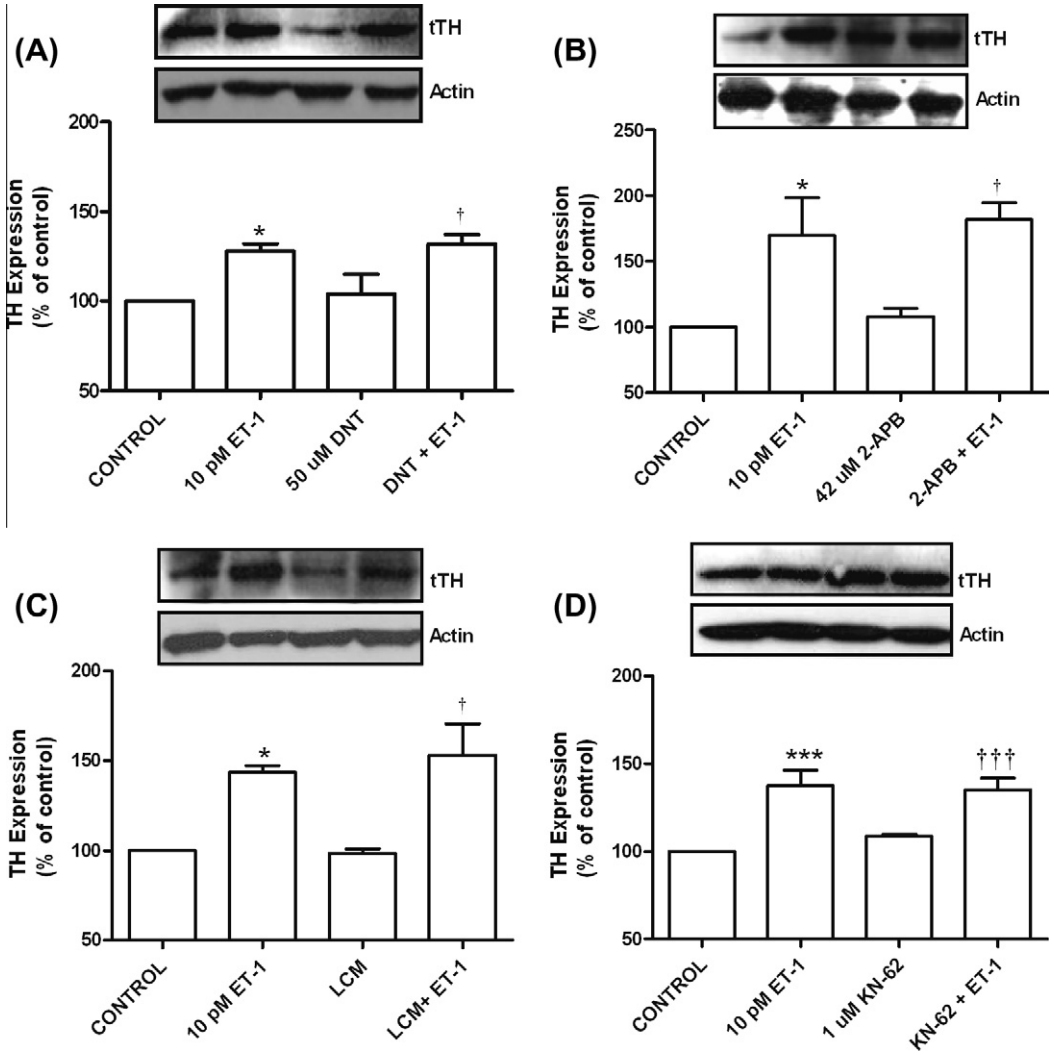

Fig. 3. Effect of calcium on endothelin-1 (ET-1) stimulation of total TH protein (tTH) level in the olfactory bulb (OB). The OB were incubated with ET-1 in the presence of DNT (ryanodine-sensitive channels inhibitor) (A), 2-APB (IP_3 receptor antagonist) (B), LCM (low calcium medium) (C) and KN-62 (CaMK-II inhibitor) (D). TH expression was determined by Western blot analysis as detailed in Material and methods. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control; $^{\dagger}p < 0.05$ vs. DNT, 2-APB or LCM; $^{\dagger\dagger\dagger}p < 0.001$ vs. KN-62. The Western blot assays shown are representative of at least three or four independent experiments.

evoked by ETs was inhibited by a LCM. As the increase in intracellular calcium stimulates CaMK-II which enhances TH activity by phosphorylation (Kumer and Vrana, 1996; Dunkley et al., 2004), we evaluated the participation of this kinase by pretreating tissues with KN-62 (CaMK-II inhibitor). Results showed that KN-62 did not modify basal TH activity, but it inhibited ET-1 and ET-3 response (Figs. 1 and 2D). Furthermore, similar results were obtained with another CaMK-II inhibitor. Pre-treatment of OB with 10 μM KN-93 also inhibited the stimulatory effect of ETs on TH activity (data not shown).

Western blot analysis showed that basal TH expression was changed neither by the inhibitors (DNT, 2-APB and KN-62) nor by a LCM (Figs. 3 and 4). Furthermore, ETs-evoked increase in TH expression was not affected by DNT (Figs. 3 and 4A), 2-APB (Figs. 3 and 4B), LCM (Fig. 3 and 4C) or KN-62 (Figs. 3 and 4D). TH activity can be regulated by protein phosphorylation at serine residues by a variety of protein kinases, being phsosphorylation at serine 40 the most important phosphorylation site implicated (Kumer and Vrana, 1996; Dunkley et al., 2004). Therefore serine 40 phosphorylation was evaluated in the presence of ETs either in a LCM or in OB pretreated with the inhibitors. Results showed that DNT, LCM and KN-62 did not affect basal TH phosphorylation at serine 40 site but they abolished the increase evoked by ET-1 and -3 (Figs. 5 and 6A,C,D). On the other hand, blockade of IP3 receptors did not change basal or ETs-stimulated phosphorylation at serine 40 site (Figs. 5 and 6B) further supporting the results observed on TH activity in the presence of ETs and the LCM and the inhibitors.

We next addressed whether ETs-induced calcium modifications were involved in TH mRNA changes. Real time PCR analysis revealed that DNT, 2-APB, KN-62 and LCM did not change basal TH mRNA expression (Figs. 7 and 8). However the increase in TH-mRNA levels induced by ETs was partially prevented by DNT and 2-APB (Figs. 7 and 8A, B) and completely abolished by KN-62 and LCM (Figs. 7 and 8C, D).

4. Discussion

The major finding of the present study is that in the rat OB calcium from distinct sources is involved in the modulation of TH activity and mRNA expression by ETs, supporting that the long-term modulation of TH by ETs is mediated by calcium dependent mechanisms.

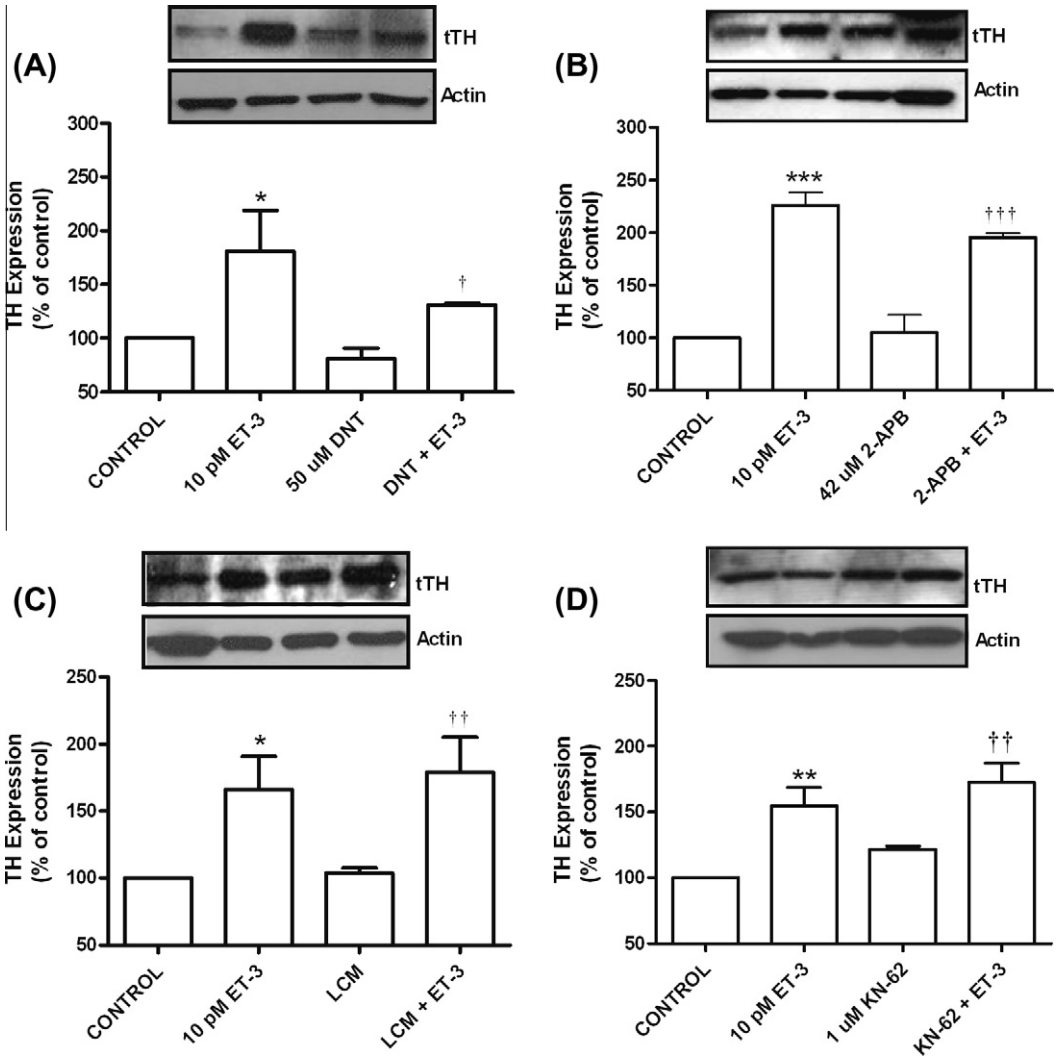

Fig. 4. Effect of calcium on endothelin-3 (ET-3) stimulation of total TH protein (tTH) level in the olfactory bulb (OB). The OB were incubated with ET-3 in the presence of DNT (ryanodine-sensitive channels inhibitor) (A), 2-APB (IP_3 receptor antagonist) (B), LCM (low calcium medium) (C) and KN-62 (CaMK-II inhibitor) (D). TH expression was assessed by Western blot analysis as detailed in Material and methods. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; $^{\dagger}p < 0.05$ vs. DNT; $^{\dagger\dagger}p < 0.01$ vs. LCM or KN-62; $^{\dagger\dagger\dagger}p < 0.001$ vs. 2-APB. The Western blot assays shown are representative of at least three or four independent experiments.

We previously reported that ETs enhance catecholaminergic transmission in the rat OB by increasing TH activity and expression (Nabhen et al., 2011). However, whether this modulation involved calcium dependent pathways is unknown so we designed the present study in an attempt to address this issue.

The OB is extensions of the rostral telencephalon and the integrity is necessary for organisms to elicit appropriate responses to environmental stimuli (Cain, 1974; Moffitt et al., 2002). The loss of connection with the forebrain and midbrain by OB removal (olfactory bulbectomy) results in neurochemical, behavioral and physiological changes (Moffitt et al., 2002; Song and Leonard, 2005). Although stressors influence mood and cardiovascular function, the specific central mechanisms underlying these changes remain elusive. The activation of diverse central regions to stress would result from a complex interaction of signals including classical neurotransmitters, neuropeptides and other neuroactive compounds as prostaglandins and nitric oxide (Mann et al., 2006). ETs and other neuropeptides like angiotensins and natriuretic peptides, expressed in different brain areas including the OB, modulate classical neurotransmission (Kurokawa et al., 1997; Wright and Harding, 1997; Schneider et al., 2007). ETs function as neuromodulators regulating catecholaminergic transmission in the CNS (Webber et al., 1998; Vatta et al., 2009). The physiological responses mediated by ETs may be calcium dependent or independent, although most responses involve calcium. ETs increase intracellular calcium by activating calcium influx or its release from intracellular stores depending on the tissue, the ET receptor involved and the response being measured (Tykocki and Watts, 2010). Furthermore in some cell types ETs induce transient increases in calcium whereas in others produce a slow and sustained calcium elevation (Tykocki and Watts, 2010). Evidence in the literature supports that calcium triggers the phosphorylation and subsequent activation of TH in different tissues (Salvatore et al., 2000; Takekoshi et al., 2001). In a previous study we reported that ETs participate in the long-term modulation of catecholaminergic transmission in the OB by increasing TH activity and expression as well as catecholamine release through diverse signaling pathways (Nabhen et al., 2011).

We first addressed the role of calcium released from intracellular stores in ETs-evoked TH activation. Intracellular calcium stores are located in the endoplasmic reticulum and calcium is released by activation of IP_3 receptors and ryanodine sensitive channels (Verma et al., 1992; Kawanabe et al., 2003; Tykocki and Watts, 2010). Pretreatment of OB with 2-APB or DNT showed that calcium

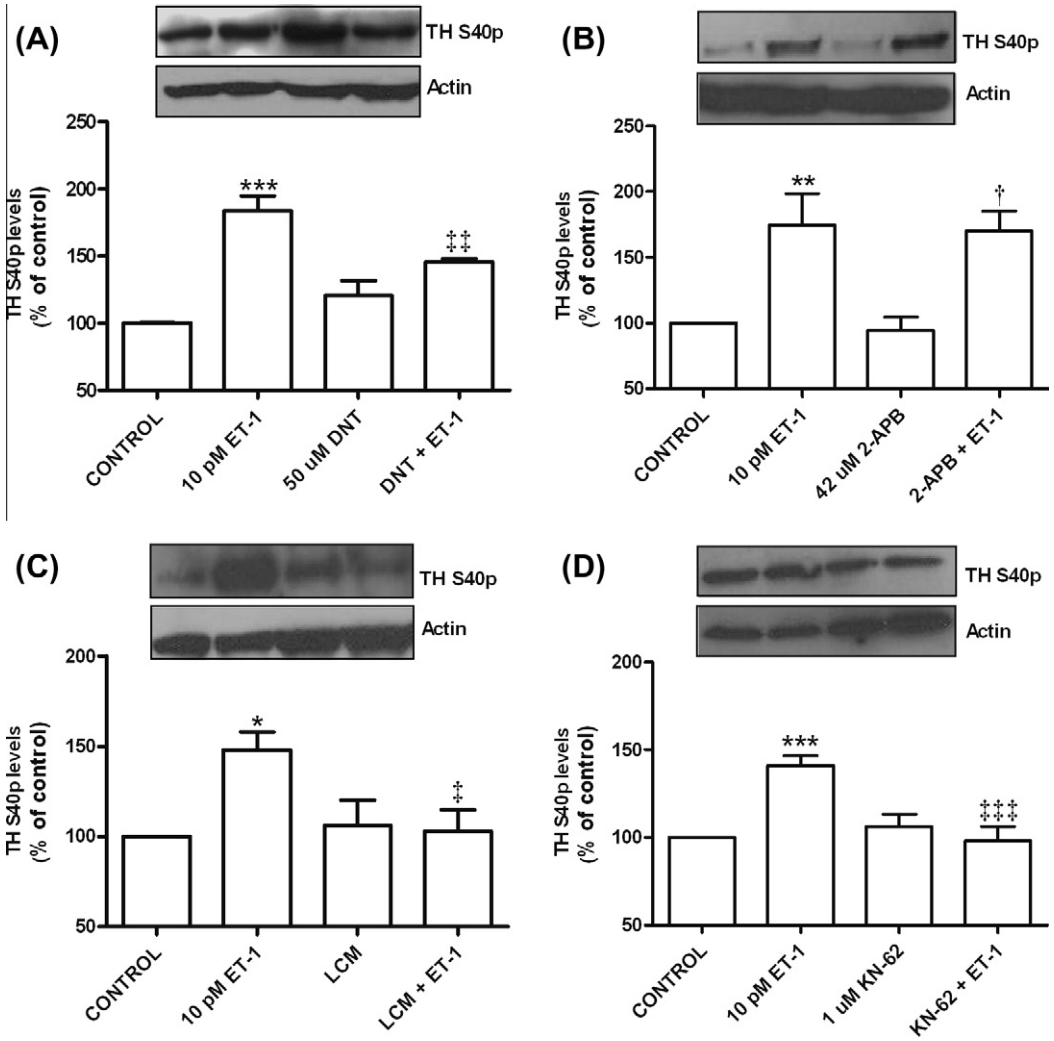

Fig. 5. Effect of calcium on endothelin-1 (ET-1) stimulation of TH phospho-Ser-40 (TH S40p) level in the olfactory bulb (OB). The OB were incubated with ET-1 in the presence of DNT (ryanodine-sensitive channels inhibitor) (A), 2-APB (IP_3 receptor antagonist) (B), LCM (low calcium medium) (C) and KN-62 (CaMK-II inhibitor) (D). TH S40p was determined by Western blot analysis as detailed in Material and methods. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; $^{‡}p < 0.05$, $^{‡‡}p < 0.01$ and $^{‡‡‡}p < 0.001$ vs. ET-1; $^{†}p < 0.05$ vs. 2-APB. The Western blot assays shown are representative of at least three or four independent experiments.

from ryanodine-sensitive channels was involved in the long-term modulation of TH by ETs. Early activation of the enzyme by ETs (5 min) depends on calcium released from IP_3-sensitive stores (data not shown) but activation at longer periods involves calcium from ryanodine-sensitive channels. Calcium released by intracellular stores in response to ETs is followed by sustained calcium influx (Neylon, 1999; Tykocki and Watts, 2010). The specific voltage-dependent calcium channels (VGCCs) activated by ETs differ due to the wide spectrum of responses mediated by these peptides. ETs through ET_A or ET_B receptors regulate VGCCs acting as calcium channel openers or indirectly by altering voltage-gating properties (Tykocki and Watts, 2010). However in some excitable cells, VGCCs are not activated by ET-1 (Pollock, 2004). It has been shown that inhibition of VGCCs fails to abolish the inward calcium currents caused by ET-1, suggesting that ET-1 remaining calcium influx is mediated by any of the several voltage-independent calcium entry pathways. The present study shows that extracellular calcium contributes to ETs-evoked TH activation suggesting that VGCCs channels are activated in the OB by ETs. However from the present study it is not possible to identify the specific VGCC implicated in the response.

Regardless of the source, intracellular calcium binds to calmodulin and modulates diverse intracellular pathways as CaMK-II activation, a kinase directly implicated in TH calcium-dependent phosphorylation (Kumer and Vrana, 1996; Clerk et al., 2002). We found that TH activation by ETs was mediated by CaMK-II in OB. Furthermore ETs-induced phosphorylation at serine 40 site was abolished by CaMK-II inhibition. Although phosphorylation is a rapid process, it was reported that long-term regulation of TH activity (hours to days) involves changes not only in total enzyme content but also in the phosphorylated forms supporting that phosphorylation is a short and long-term mechanism implicated in TH activation (Arbogast and Hyde, 2000; Gozal et al., 2005). Furthermore, studies from our laboratory also show that increased TH activity following prolonged exposure to ET-1 and ET-3 involves phosphorylation events (Perfume et al., 2008; Nabhen et al., 2011). The present study suggests that CaMK-II activation by ETs mediates the phosphorylation of TH at serine 40 in the regulatory domain resulting in increased activity of the enzyme (Kumer and Vrana, 1996; Dunkley et al., 2004; Nabhen et al., 2011). However phosphorylation of TH at Ser-40 by CaMK-II is still a matter of debate (Dunkley et al., 2004). Whether Ser-40 phosphorylation in-

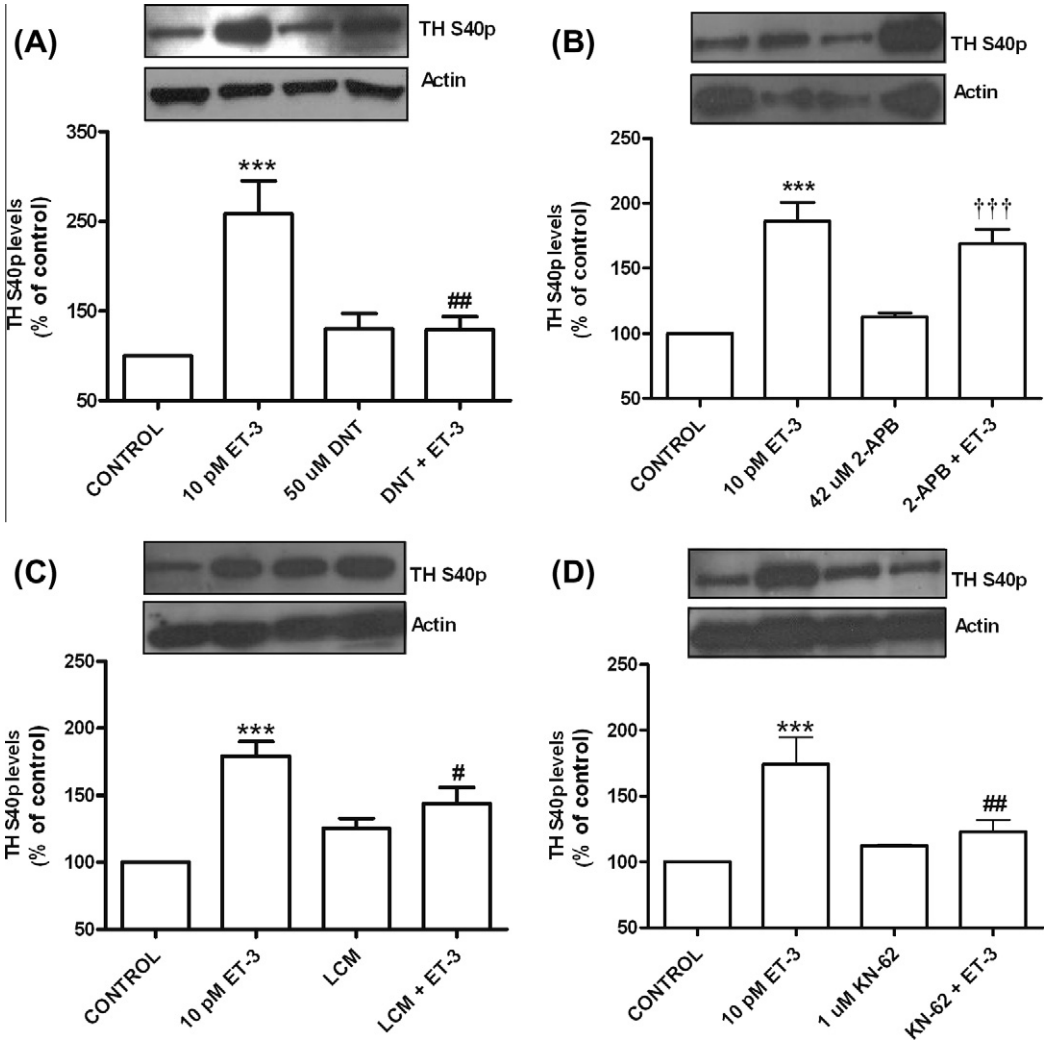

Fig. 6. Effect of calcium on endothelin-3 (ET-3) stimulation of total TH protein level in the olfactory bulb (OB). The OB were incubated with ET-3 in the presence of DNT (ryanodine-sensitive channels inhibitor) (A), 2-APB (IP_3 receptor antagonist) (B), LCM (low calcium medium) (C) and KN-62 (CaMK-II inhibitor) (D). TH S40p was assessed by Western blot analysis and normalized to β-actin as detailed in Material and methods. $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. ET-3; $^{\dagger\dagger\dagger}p<0.001$ vs. 2-APB. The Western blot assays shown are representative of at least three or four independent experiments.

duced by ETs is directly and/or indirectly mediated by CaMK-II is not possible to assess. However the present study permit us suggest that CaMK-II may be involved in the long-term regulation of TH activity mediated ET-1 and ET-3.

We previously reported that ETs also increase total TH mRNA and protein levels in the rat OB (Nabhen et al., 2011). When the contribution of calcium was investigated, we found that blockade of IP_3 receptors and ryanodine-sensitive channels partially reduced ETs-induced increase in TH mRNA, whereas, incubation in a LCM suppressed ETs response. It was reported that in PC12 cells bradykinin or thapsigargin induce calcium release from intracellular stores leading to enhanced TH mRNA levels (Menezes et al., 1996). However the increase in TH mRNA by nicotine requires extracellular calcium (Sabban, 1997). Present results suggest that TH gene expression activation by ETs involves both intracellular and extracellular calcium. ETs-induced early rise in calcium from intracellular stores seems sufficient to trigger initial events involved in TH gene expression. However extracellular calcium appears to be essential for ETs long-term regulation of TH mRNA levels. Calcium contribution was further confirmed by the observation that CaMK-II inhibition prevented the elevation of TH mRNA induced by ETs.

The increase in TH protein content induced by ET-1 and ET-3 was not affected by a LCM or inhibition of IP_3 receptors, ryanodine-sensitive channels or CaMK-II. These findings support that neither intracellular nor extracellular calcium mediate ETs-induced changes in TH protein content. Several works show that stimuli known to modulate mRNA levels like hypoxia and stress do not necessarily induce the expected changes in protein content supporting the participation of post transcriptional mechanisms (Czyzyk-Krzeska et al., 1997; Xu et al., 2007). In this regard, it was reported that during long-term hypoxia increased TH mRNA level was due to its enhanced stability (Czyzyk-Krzeska et al., 1997). In PC12 cells a cytoplasmic protein which binds to a pyrimidine rich sequence in the 3′ untranslated region of the TH mRNA mediates the increase in TH mRNA stability (Sabban, 1997). Therefore, the maintenance of protein levels may result from an increase in the transduction rate of previous existing mRNA-TH or in its stability (Kumer and Vrana, 1996; Xu et al., 2007). Nevertheless it is also possible that no changes are evident due to TH long half-life (over 4 h). Present results suggest that the increase in TH protein content induced by ETs is not mediated by calcium dependent mechanisms. Whether ETs increase the protein transduction rate or TH-mRNA stability remains to be established.

Present findings support that calcium dependent mechanisms are implicated in ET-1 and ET-3 regulation of catecholaminergic neurotransmission in the rat OB (Nabhen et al., 2009; Nabhen et al., 2011). However, it is well known that ETs and its receptors are also present in the glia of regions like OB and can affect its function (Rogers et al., 2003; Vincent et al., 2003). Furthermore, astrocytes activation may induce excitatory and inhibitory neuronal effects depending on the receptor involved and the signaling pathway activated by ETs (Filosa et al., 2012). ET-1 induces the mobilization of intracellular calcium through ET_B receptors in hypothalamic astrocytes and calcium changes activate glutamate and nitric oxide signaling pathways which in turn has excitatory and inhibitory neuronal effects, respectively (Filosa et al., 2012). The fact that astrocytes undergo changes in neuron activity

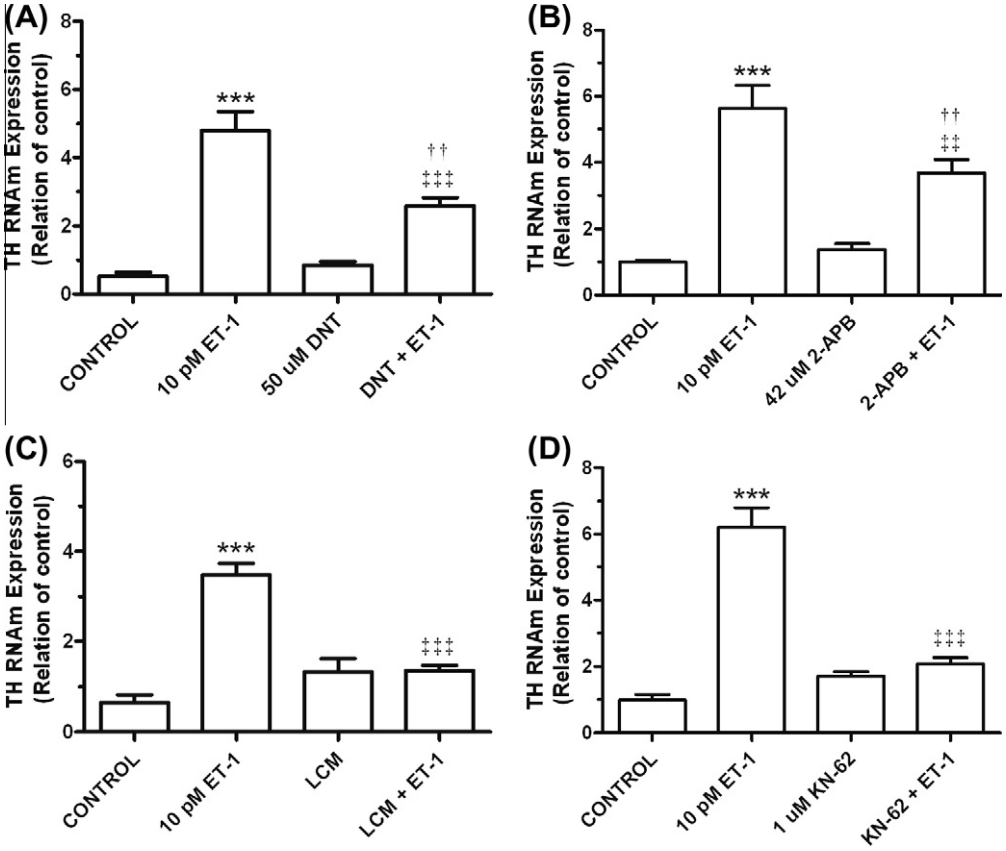

Fig. 7. Effect of calcium on endothelin-1 (ET-1) on the expression of TH mRNA in the olfactory bulb. The OB were incubated with 10 pM ET-1 in the presence of DNT (ryanodine-sensitive channels inhibitor) (A), 2-APB (IP_3 receptor antagonist) (B), LCM (low calcium medium) (C) and KN-62 (CaMK-II inhibitor) (D). The expression of TH mRNA was determined as detailed in Materials and methods and expressed as relation of control. $^{***}p<0.001$ vs. control; $^{‡‡}p<0.01$, $^{‡‡‡}p<0.01$ vs. ET-1; $^{††}p<0.01$ vs. DNT or 2-APB. Number of experiments: 4. Graphs illustrate the results obtained in the exponential phase of the real time PCR assay.

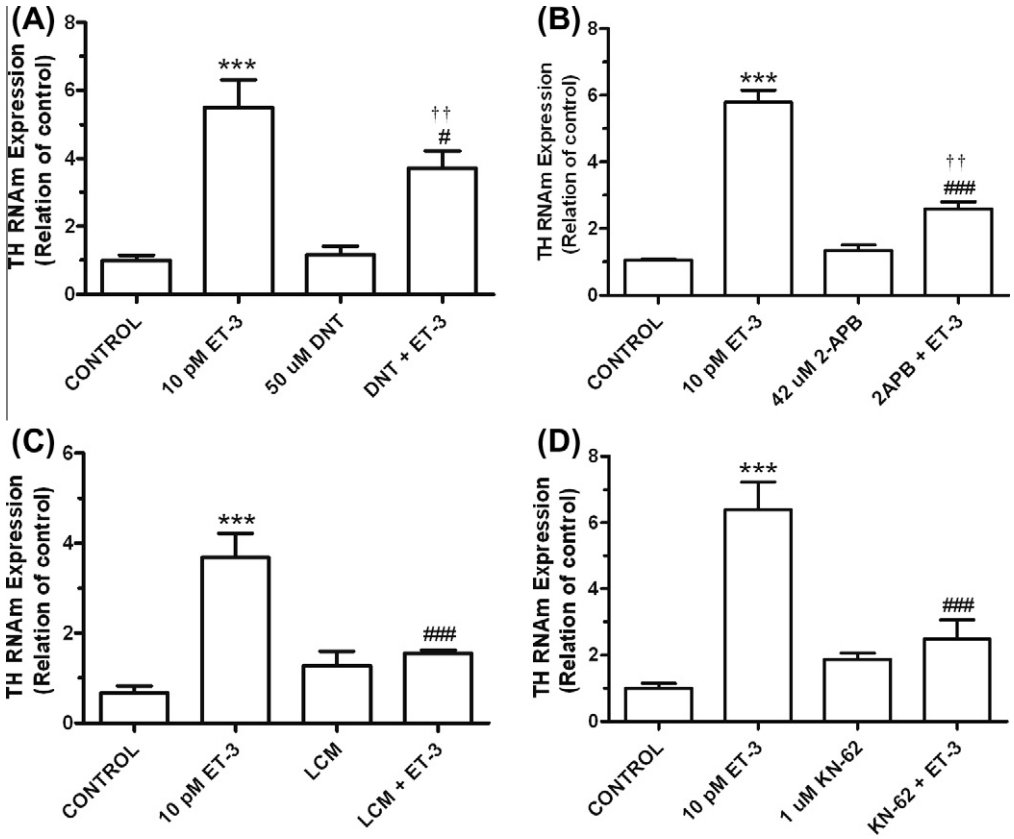

Fig. 8. Effect of calcium on endothelin-3 (ET-3) on the expression of TH mRNA in the olfactory bulb. The OB were incubated with 10 pM ET-3 in the presence of DNT (ryanodine-sensitive channels inhibitor) (A), 2-APB (IP_3 receptor antagonist) (B), LCM (low calcium medium) (C) and KN-62 (CaMK-II inhibitor) (D). The expression of TH mRNA was determined as detailed in Materials and methods and expressed as relation of control. $^{***}p<0.001$ vs. control; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. ET-3; $^{\dagger\dagger}p<0.01$ vs. DNT or 2-APB. Number of experiments: 4. Graphs illustrate the results obtained in the exponential phase of the real time PCR assay.

suggests that these cells could play a significant role in the modulation of central neurotransmission. Thus, whether the effect of ETs on TH regulation within the OB is direct and/or indirect, still remains to be elucidated.

5. Conclusions

Brain catecholamines play a relevant role in controlling different biological functions centrally regulated. Diverse factors including neuropeptides like ETs modulate catecholaminergic transmission. Although the ET system and ETs are known to be expressed in the OB, little is known about this brain region and catecholamine transmission. Previous and present studies from our laboratory show that ET-1 and ET-3 act as neuromodulators in this brain area by increasing TH activity. The present study provides novel evidence showing that intracellular and extracellular calcium participate in the regulation of TH activity by ET-1 and ET-3. The role of the ETs-catecholamines–OB interaction and the underlying regulatory mechanisms are not presently well defined. The OB is connected with central regions intimately involved in the regulation of the cardiovascular function and mood. This region is essential for responses evoked following environmental stimuli like stressors (Song and Leonard, 2005; Grippo and Johnson, 2009). Exposure of rats to stressors increases body temperature, heart rate and blood pressure diminishes exploration and escape performance supporting a link between stressors and depressive syndromes (Van den Buuse et al., 2001; Harkin et al., 2002). In addition, they are associated with signs and symptoms of cardiovascular impairment (Grippo and Johnson, 2009). Present findings contribute to the understanding of the regulation of catecholaminergic transmission by ETs in the rat OB. Although preliminary studies from our laboratory support impaired regulation of catecholamine metabolism by ETs in the rat OB of hypertensive rats, further studies are necessary to clearly establish the physiological and pathophysiological role of catecholamines and ETs in the OB and its connection with the cardiovascular function.

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