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ORIGINAL PAPER

Short-term Effects of Endothelins on Tyrosine Hydroxylase Activity and Expression in the Olfactory Bulb of Normotensive Rats

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Abstract The olfactory system in rats is part of the limbic region with extensive afferent connections with brain areas involved in the regulation of behaviour and autonomic responses. The existence of the endothelin system and catecholaminergic neurons in the olfactory bulb suggests that endothelins may modulate noradrenergic transmission and diverse olfactory mediated processes. In the present work we studied the effect of endothelin-1 and -3 on neuronal norepinephrine release and the short-term regulation of tyrosine hydroxylase in the olfactory bulb. Results showed that both endothelins increased tyrosine hydroxylase activity through the activation of a non-conventional endothelin G-protein coupled receptor, coupled to the stimulation of protein kinase A and C, as well as Ca^{2+}/calmodulin-dependent protein kinase II. On the other hand, neither endothelin-1 nor endothelin-3 modified tyrosine hydroxylase total protein levels, but both peptides increased the phosphorylation of serine residues of the enzyme at sites 19 and 40. Furthermore, endothelins enhanced norepinephrine release in olfactory neurons suggesting that this event may contribute to increased tyrosine hydroxylase activity by reducing the feedback inhibition. Taken together present findings show a clear interaction between the endothelin system, and the catecholaminergic transmission in the olfactory bulb. Additional studies are required to evaluate the physiological functions regulated by endothelins at this brain level.

Keywords Endothelin-1 · Endothelin-3 · Norepinephrine release · Olfactory bulb · Tyrosine hydroxylase activity · Tyrosine hydroxylase expression

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Introduction

The olfactory bulb (OB) constitutes 4% of the total mass of the rat brain and communicates with areas strongly implicated in the central regulation of the cardiovascular function [1–4]. Diverse vasoactive peptides such as angiotensin II, natriuretic peptides and endothelins (ETs) are expressed in the OB [5–8]. ETs constitute a family of related isopeptides which includes ET-1, ET-2 and ET-3 [9, 10]. Their biological effects are mediated by two well characterized G-protein coupled receptors, ET_A and ET_B. The ET_A receptor exhibits higher affinity for ET-1 and ET-2 than for ET-3 whereas the ET_B receptor displays similar affinity for the three isoforms [9, 10]. Based on the observation of atypical responses in the presence of selective ET agonists and antagonists, several authors proposed the existence of additional ET receptors (termed atypical ET receptors) besides the conventional ET_A and ET_B receptors [11–15]. ETs are involved in the peripheral and central regulation of diverse biological functions including blood pressure, water and sodium balance, as well as neurotransmitter and hormone release [9, 10, 16]. Previous works support that ETs central effects on the cardiovascular function are mediated by changes in the sympathetic nervous system [10, 16]. ETs behave as

neurotransmitters and/or neuromodulators within the central nervous system. In this sense we reported that ETs modulate noradrenergic neurotransmission in the anterior and posterior hypothalamus of normotensive rats [11, 12, 15, 17, 18].

Tyrosine hydroxylase (TH) is the enzyme that catalyzes the rate-limiting step in the synthesis of catecholamines and its activity is tightly controlled by diverse mechanisms [19–21]. TH is expressed in the central nervous system (CNS), including the OB and it is a specific marker of catecholaminergic neurons [20, 22].

Recently, we observed that TH activity and expression as well as the neuronal NE transporter were modified in OB of DOCA-salt hypertensive rats (unpublished results). Based upon the existence of the ET system and catecholaminergic neurons in the OB and the various connections between this structure and regions of the CNS involved in the regulation of the cardiovascular function, we sought to establish the effect of ET-1 and ET-3 on the short-term regulation of TH activity and expression. We also assessed the receptors and intracellular mechanisms involved, as well as, the effects of both ETs on NE release.

Present findings show that in the OB, ET-1 and ET-3 increased TH activity presumably through the activation of an atypical receptor coupled to the adenylyl cyclase/protein kinase A (PKA), phospholipase C (PLC)/DAG/protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CamK-II) pathways. In addition, increased intracellular calcium resulting from its release by ryanodine sensitive stores and its influx from the extracellular compartment was also involved. Furthermore, ETs increased TH phosphorylation at Ser-19 and Ser-40 sites without affecting Ser-31 or total protein levels. Present findings suggest the ET-1 and ET-3 play an important role in the modulation of catecholaminergic transmission in the OB.

Materials and Methods

Animals and Chemicals

Male Sprague–Dawley rats weighing between 250 and 300 g (from the School of Pharmacy and Biochemistry, University of Buenos Aires) were used in all experiments. The rats were housed in steel cages and maintained at a temperature between 20°C and 23°C in a controlled room with a 12 h light–dark cycle. All animals had free access to water and commercial chow.

The following drugs, reagents and kits were used: ET-1, ET-3, (N,N-hexamethylene)carbamoyl-Leu-D-Trp(CHO)-D-Trp (BQ-610), and N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-l-methoxycarbonil-Trp-D-Nle (BQ-788); (Suc,Glu^{9},$Ala^{11,15}$)-ET-1, 8-21 (IRL-1620) and sarafotoxin S6b (SRTx-b) (American Peptide Company Inc., CA, USA). Catalase, desipramine hydrochloride, L-DOPA, bisindolylmaleimide I (GF-109203x), N-[2-((*p*-Bromocinnamyl) amino)ethyl]-5-isoquinolinesulfonamide 2HCl (H-89), hydrocortisone, 1-[N,O-bis-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), pargyline hydrochloride, suramin (SMN), 6-methyl-tetrahydrobiopterin and L-tyrosine (MP Biomedicals LLC, CA, USA). L-[3,5-^{3}H]tyrosine and DL-[7-^{3}H]NE (PerkinElmer Life and Analytical Sciences, MA, USA); PVDF membrane (GE Healthcare, Amersham Biosciences, UK); anti-actin polyclonal antibody (Ab) (Actin-Ab), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT), 8-bromoadenosine-3′,5′-cyclic monophosphate (8Br-cAMP), 8-bromoguanosine-3′,5′-cyclic monophosphate (8Br-cGMP), dantrolene sodium salt (DNT), forskolin (FSK), [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY-294002), Nω-nitro-l-arginine methyl ester (L-NAME), phorbol 12-myristate 13-acetate (PMA), protease inhibitor cocktail, streptavidin–alkaline phosphatase conjugate and 1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)-hexy]-1H-pyrrole-2,5-dione (U-73122) (Sigma, MO, USA). 2-Aminoethoxydiphenylborate (2-APB) (Calbiochem, CA, USA). Mouse anti-TH monoclonal Ab (TH-Ab) and rabbit anti-TH phospho-Ser-19, -31 and -40 (19 Ser-P, 31 Ser-P and 40 Ser-P, respectively), biotinylated anti-mouse Ab, and anti-rabbit Ab (Chemicon, CA, USA). Other reagents were of analytical or molecular biology quality and obtained from standard sources.

Experimental Protocol

Experiments were performed following the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animal Guide (NIH Publication 1985, Revised 1996). Animals were decapitated between 9:00 and 12:00 h and the OB removed according to Palkovits and Brownstein [23]. Tissues were pre-incubated in a Dubnoff incubator for 30 min at 37°C in Krebs bicarbonate solution, pH 7.4, and bubbled with a gas mixture (95% O_2 and 5% CO_2) under continuous shaking. To determine the effect of ET-1 or ET-3 on TH activity and expression, tissues were incubated for 30 min in the absence (control) or in the presence of ETs. In other experiments tissues were pre-incubated for 15 min with the following antagonist or inhibitors prior to the addition of ETs: 100 nM BQ-610 (ET_A receptor antagonist), 100 nM BQ-788 (ET_B receptor antagonist), 500 nM SMN (G-protein inhibitor), 10 μM L-NAME (NO synthase (NOS) inhibitor), 500 nM H-89, 1 μM KN-62, 10 μM U-73122 and 100 nM GF-109203x, 5 μM LY-294002 (PKA, CaMK-II, PLC, PKC, inositol triphosphate (IP_3) kinase inhibitors, respectively) and 42 μM 2-APB (IP_3 receptor-selective antagonist), 50 μM DNT (ryanodine receptor

inhibitor). In other experiments, the OB was incubated for 30 min in the presence of 300 nM SRTx-b or 1 μM IRL-1620 (ET_A/ET_B and ET_B receptor agonist, respectively); 100 μM 8Br-cGMP or 100 μM 8Br-cAMP (Guanosone 3′,5′-cyclic monophosphate (cGMP) and adenosine 3′,5′-cyclic monophosphate (cAMP) analogue, respectively); and 1 μM PMA or 20 μM forskolin (PKC and adenylyl cyclase activator, respectively).

In other set of experiments, tissues were incubated with ETs for 30 min in a calcium free Krebs bicarbonate solution pH 7.4 in the presence of 0.5 mM EGTA.

Determination of TH Activity

TH activity was determined by the method of Reinhard et al. [24]. Following the incubation period OBs were homogenized in 500 μl buffer (5 mM KH_2PO_4 and 0.2% Triton X-100, pH 7.0). An aliquot was saved for protein determination and samples were centrifuged for 10 min at 10,000*g* at 4°C. An aliquot of the supernatant was incubated for 20 min at 37°C with 50 mM HEPES (pH 7.0), containing 15 nmol L-tyrosine containing 0.5 μCi ^{3}H-tyrosine, 420 mM *β*-mercaptoethanol, 1,000 U catalase, and 0.75 mM 6-methyl-tetrahydrobiopterin. The reaction was stopped by the addition of 1 ml of 7.5% activated charcoal suspension in 1 N HCl. The final mixture was vortexed and centrifuged at 500 g for 10 min followed by the supernatant separation where ^3H_2O was determined by conventional scintillation methods. Blank values were obtained by omitting 6-methyl-tetrahydrobiopterin from the mixture. Recovered ^3H_2O was determined as described by Reinhard et al. [24]. Results were expressed as percentage of control group ± SEM.

TH Western Blot Assay

Tissues 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). An aliquot of the supernatant was used for protein determination 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 gel at 100 Vts for 2.30 h. The gels were then transferred into PVDF membranes at 100 Vts for 75 min. The membranes were blocked overnight at 4°C in blocked solution (5% non fat powder milk in Tris-buffered saline containing 0.1% Tween 20 [TBS-T]) and the gels were stained overnight at 4°C with Coomasie Blue. After washing with TBS-T, the transfers were incubated with TH-Ab, 19 Ser-P, 31 Ser-P, 40 Ser-P (1/1000 overnight at 4°C); *β*-actin Ab (1/1500, 1 h at room temperature), biotinylated anti-mouse Ab or anti-rabbit Ab (1/50,000, 1 h at room temperature), streptavidin–alkaline phosphatase conjugate (1/3000, 1 h at room temperature) and BCIP-NBT. Bands were analyzed by densitometry and normalized to *β*-actin. Results were expressed as percentage of control group.

Determination of NE Release

Neuronal [^{3}H] NE release was measured according to Vatta et al. [25] with minor modifications. Briefly, OB were pre-incubated for 15 min at 37°C in Krebs bicarbonate solution, pH 7.4, and bubbled with a gas mixture (95% O_2 and 5% CO_2) under continuous shaking. Monoamine-oxidase (MAO) activity and extraneuronal NE uptake were inhibited by 50 μM pargiline and 100 μM hydrocortisone, respectively. NE stores were labelled with 2.5 uCi/ml [^{3}H] NE for 30 min followed by three consecutive washes of 10 min with Krebs buffer. Neuronal NE uptake was inhibited by the addition of 10 μM desipramine in the last wash. The tissues were then incubated for 35 min and seven consecutive samples of the incubation medium were collected every 5 min. The first sample corresponded to the basal release period whereas the remaining samples corresponded to the experimental period where tissues were incubated with 10 nM ET-1 or ET-3. [^{3}H]NE release was measured by conventional scintillation counting methods. Results were expressed as the area under the curve corresponding to the 30 min experimental period.

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.

Results

In order to assess the short-term effect of ET-1 and ET-3 on TH activity in the OB of normotensive rats, a concentration-response study was performed. Results showed that 10 nM ET-1, and 10 pM and 10 nM ET-3 increased TH activity by 32, 24 and 40%, respectively (Fig. 1). However, 10 fM ET-1 or ET-3 and 10 pM ET-3 did not modify the activity of the enzyme (Fig. 1).

In order to identify the ET receptor involved, tissues were pretreated with 100 nM BQ-610 or 100 nM BQ-788 (ET_A and ET_B receptor selective antagonists, respectively) and then incubated for with 10 nM ET-1 and ET-3. Results showed that neither BQ-610 nor BQ-788 modified per se TH activity, but both antagonists abolished ET-1 and ET-3 response (Fig. 2a, b). To further evaluate the ET receptor

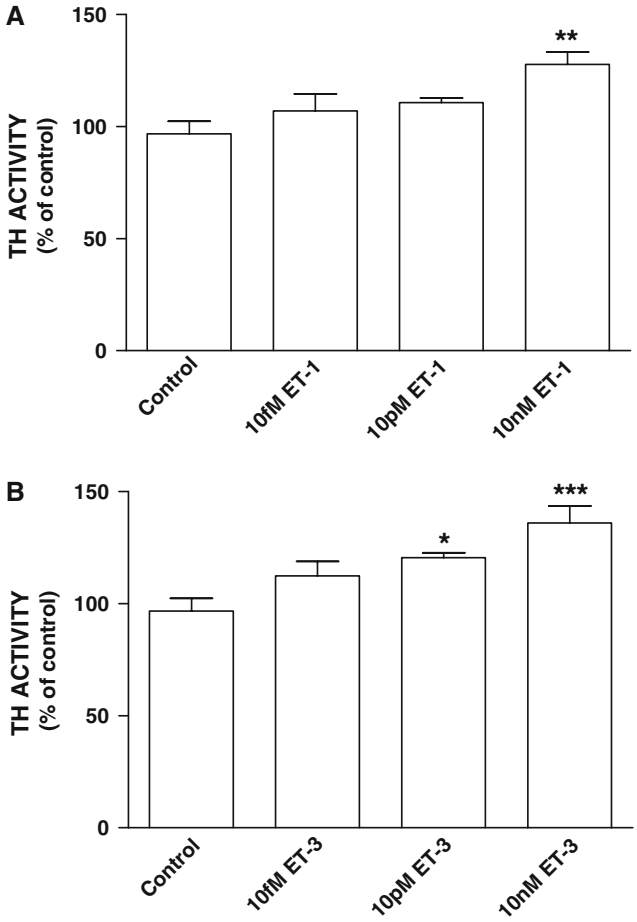

Fig. 1 Effect of endothelin-1 and -3 (ET-1 and ET-3) on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OB was incubated with increasing concentrations of ET-1 (**a**) and ET-3 (**b**). TH activity was determined as detailed in "Materials and Methods" and expressed as percentage of control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control. Number of experiments: 6–7

involved, tissues were incubated with SRTx-b (ET_A/ET_B receptor agonist) and IRL-1620 (selective ET_B agonists). Neither SRTx-b nor IRL-1620 modified TH activity (Fig. 2c). These findings support the idea that a non conventional or atypical ET receptor is involved. To determine whether this non-conventional ET receptor was a G-protein-coupled receptor, tissues were pre-treated with SMN (a G protein inhibitor at this concentration) [26]. Figure 3 shows that SMN did not modify TH activity, but it abolished the effect induced by ET-1 and ET-3.

ETs activate diverse protein kinases as PKA, protein kinase G, PKC and CaMK-II that are involved in TH regulation [19–21]. With the aim to identify the intracellular signalling pathways involved in the activation of TH by ETs, the activity of the enzyme was assessed in the presence of specific inhibitors or activators of the various intracellular pathways. The participation of the cAMP/PKA pathway was assessed by pre-treatment with H-89

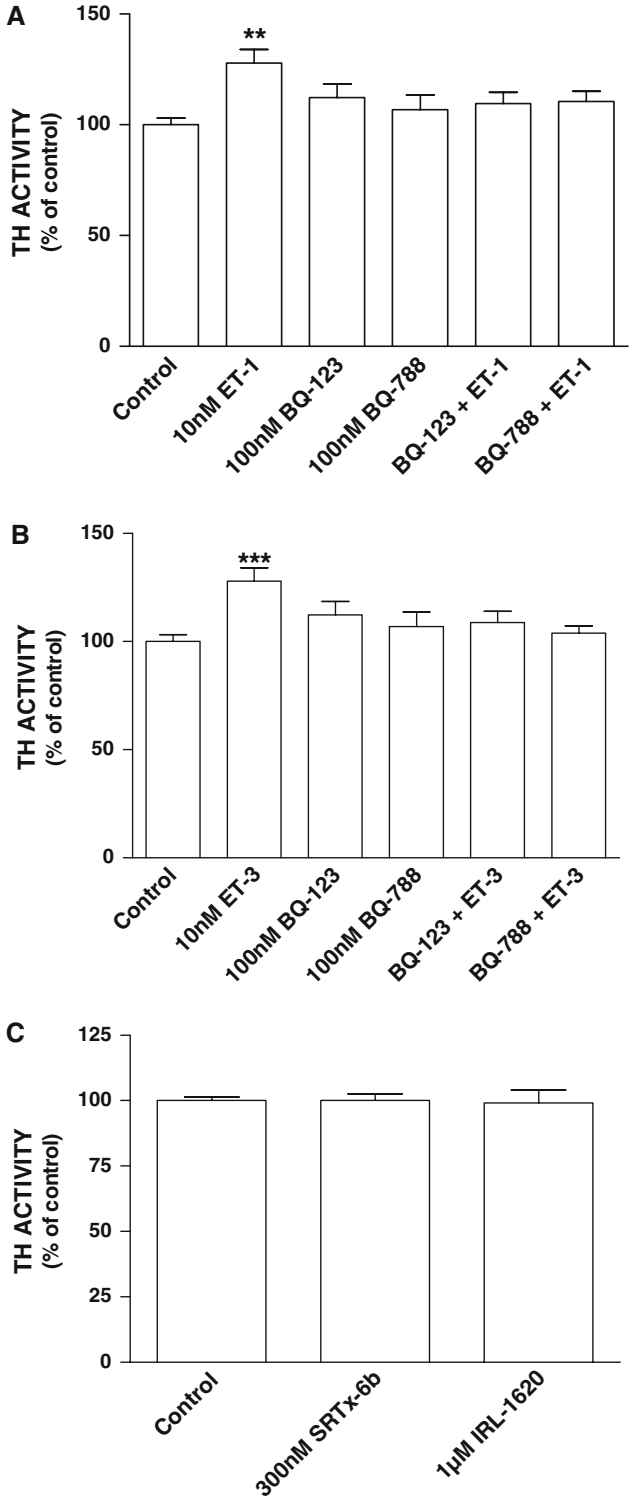

Fig. 2 Effect of endothelin (ET) receptor antagonists and agonists on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OB was pre-incubated with BQ-610 (ET_A antagonist) (**a**) and BQ-788 (ET_B antagonist) (**b**) before the addition of endothelins or incubated with sarafotoxin 6b (SRT-6b) (ET_A/ET_B agonist) and IRL-1620 (ET_B agonist) (**c**). TH activity was determined as detailed in "Materials and Methods" and expressed as percentage of control. ** $P < 0.01$ and *** $P < 0.001$ vs. control. Number of experiments: 6–8

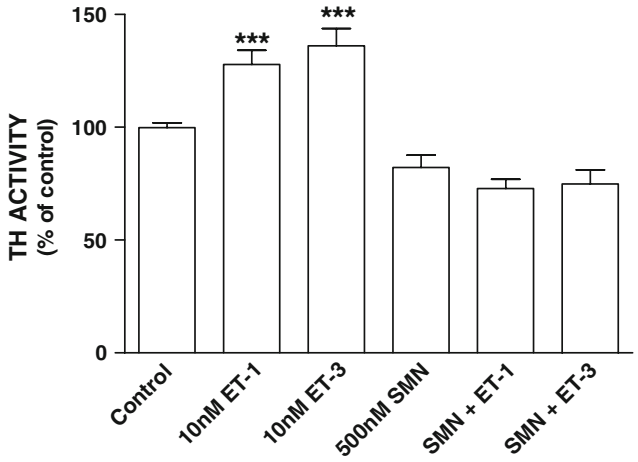

Fig. 3 Effect of a G-protein inhibitor on endothelin-1 and -3 (ET-1 and ET-3) response on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OB was pre-treated with SMN (G-protein inhibitor) and further incubated with ET-1 and ET-3. TH activity was determined as detailed in "Materials and Methods" and expressed as percentage of control. *** $P < 0.001$ vs. control. Number of experiments: 6–7

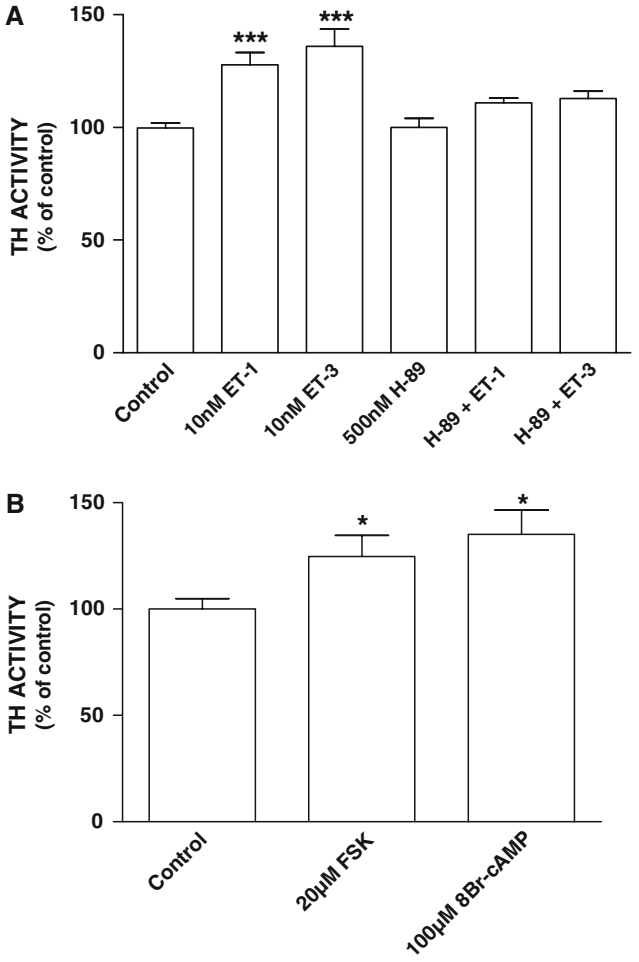

Fig. 4 Participation of the adenylyl cyclase pathway in endothelin-1 and -3 (ET-1 and ET-3) response on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OB was pretreated with H-89 (PKA inhibitor) and further incubated with ET-1 and ET-3 (**a**) or incubated with FSK (adenylyl cyclase activator) and 8Br-cAMP (cAMP analogue) (**b**). TH activity was determined as detailed in "Materials and Methods" and expressed as percentage of control. * $P < 0.05$ and *** $P < 0.001$ vs. control. Number of experiments: 6–8

(PKA inhibitor). PKA inhibition did not affect basal TH activity but it prevented the increase in TH activity induced by ET-1 and ET-3 (Fig. 4a). To further confirm the involvement of the cAMP/PKA pathway, experiments were carried out in the presence of a cAMP analogue (8Br-cAMP) and an adenylyl cyclase activator (FSK). Figure 4b shows that that both 8Br-cAMP and FSK increased TH activity by 35% and 25%, respectively.

To determine the role of the NO pathway in ETs response in the rat OB, experiments were performed in the presence of L-NAME (NOS inhibitor). Blockade of NOS by L-NAME failed to affect either basal or ETs-stimulated TH activity (Fig. 5a). Furthermore, incubation with a cGMP analogue (8Br-cGMP) did not modify TH activity (Fig. 5b).

The participation of the phosphoinositide (PLC and PKC) and CaMK-II pathways in ETs effect on TH activity, was assessed by pre-treating the OB with U-73122, GF-109203x and KN-62 (PLC, PKC and CaMK-II inhibitor, respectively). Results showed that none of the inhibitors modified basal TH activity (Fig. 6), but U-73122, GF-109203x and KN-62 inhibited the increase of TH activity induced by ETs (Fig. 6a, b, c). To confirm the involvement of PKC in ETs effect on TH activity, the OB was incubated with PMA (PKC activator). Activation of PKC by PMA led to an increase in TH activity (Fig. 6d).

Calcium has been shown to trigger TH activation in different tissues [27, 28]. In order to define the role of intracellular calcium in ETs response, the participation of IP_3 receptors, ryanodine-sensitive channels and extracellular calcium was assessed. Results showed that none of the inhibitors modified basal TH activity (Fig. 7). The response of ETs was not affected by 2-APB or LY-294002 (IP_3 receptor antagonist and IP_3 kinase inhibitor, respectively) (Fig. 7a, b). In addition, DNT (ryanodine-sensitive channel inhibitor) abolished ETs response on TH activity (Fig. 7c). The role of extracellular calcium was assessed by incubating the OB in a calcium free medium. The increase of TH activity induced by ETs was inhibited in a calcium free medium (Fig. 7d).

Total TH protein level was assessed by Western blotting. Results showed that neither ET-1 nor ET-3 modified total TH content (Fig. 8a). The phosphorylated forms of the enzyme (TH Ser 19p, TH Ser 31p and TH Ser 40p) were assessed to evaluate whether ETs-induced TH increase correlated with phosphorylation events. Results

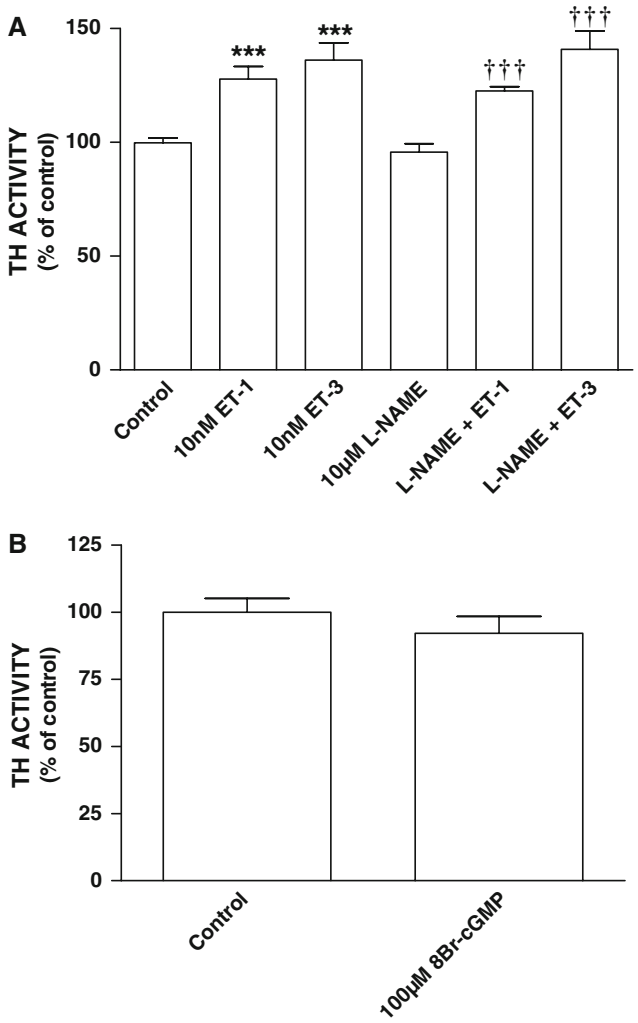

Fig. 5 Role of nitric oxide (NO) and cGMP in endothelin-1 and -3 (ET-1 and ET-3) effect on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OB was pre-treated with L-NAME (NO synthase inhibitor) before the addition of ET-1 and ET-3 (**a**) or incubated with 8Br-cGMP (cGMP analogue) (**b**). TH activity was determined as detailed in "Materials and Methods" and expressed as percentage of control. *** $P < 0.001$ vs. control; ††† $P < 0.001$ vs. L-NAME. Number of experiments: 6–8

showed that ET-1 and ET-3 enhanced TH Ser-19 and -40, whereas no changes were observed in TH Ser-31 level (Fig. 8b, c, d).

As the catecholamine biosynthetic pathway is tightly related to the neuronal amine secretion, the effect of ET-1 and ET-3 on neuronal NE release was determined. Both ETs increased neuronal NE release in the rat OB (Fig. 9).

Discussion

The major finding of the present study was that ET-1 and ET-3 were involved in the short-term regulation of TH in the OB.

The OB is an extension of the rostral telencephalon that plays a relevant role in the interaction between the animal and its environment [1]. This region has also numerous connections with different areas of the midbrain and forebrain [29]. The OB communicates with the dorsal medulla, specifically with the nucleus tractus solitarii which integrates the information of afferent carotid endings from sinus baroreceptor fibers. The OB is also related to regions of the límbic system like the amygdala, septum, pyriform cortex and frontoorbito cortex as well as the preoptical area and the ventromedial and posterior nucleus of hypothalamus [2–4, 30]. Many of these brain regions and areas are closely related to the control of the cardiovascular function.

The numerous connections of the OB with other regions and areas of the CNS, supports a wide spectrum of biological functions mediated by this structure. Thus, it is predictable that the removal of the OB has a major impact upon brain functions. Some of the changes observed in bulbectomized rats were endocrine alterations, body temperature regulation, increased exploratory behaviour, hyperactivity, sexual behaviour alteration as well as immune and cardiovascular changes [2, 29, 31, 32]. The impairment of these functional responses appears to be associated with the neurochemical alteration of neurotransmitters like NE, serotonin, acetylcholine, glutamate and GABA [29]. Pohorecky et al. [33] demonstrated that the ablation of the OB increased NE and monoamine oxidase activity in the brain stem. In addition, the integrity of the OB in rats is necessary for the brain to generate normal sympathoexcitatory responses to diverse physiological stimuli. In this regard, an attenuated response to hypotension, air jet stress, and smoke exposure, as well as elevated basal blood pressure, were observed in bulbectomized rats [31]. The exposure of conscious rats to smoke generates changes in blood pressure, breathing and sympathetic activity [34]. These findings support a close relationship between the OB and the control of the cardiovascular function.

Olfactory bulbectomy is also used as an experimental animal model of depression [29]. Various studies suggest that depression may influence the pathogenesis of cardiovascular diseases by inducing changes in the autonomic regulation [35, 36]. Patients with depression exhibit increased heart rate and sympathetic nervous system activity, as well as baroreflex alterations [37, 38]. It was suggested that olfactory bulbectomy exerts a strong influence not only on emotions but also on the central control of blood pressure, likely through the amygdala [2]. Recent results from our laboratory show that TH activity and expression were increased, whereas neuronal NE transporter was reduced in DOCA-salt hypertensive rats (unpublished data). However, little is known regarding the relationship between the OB and cardiovascular physiology.

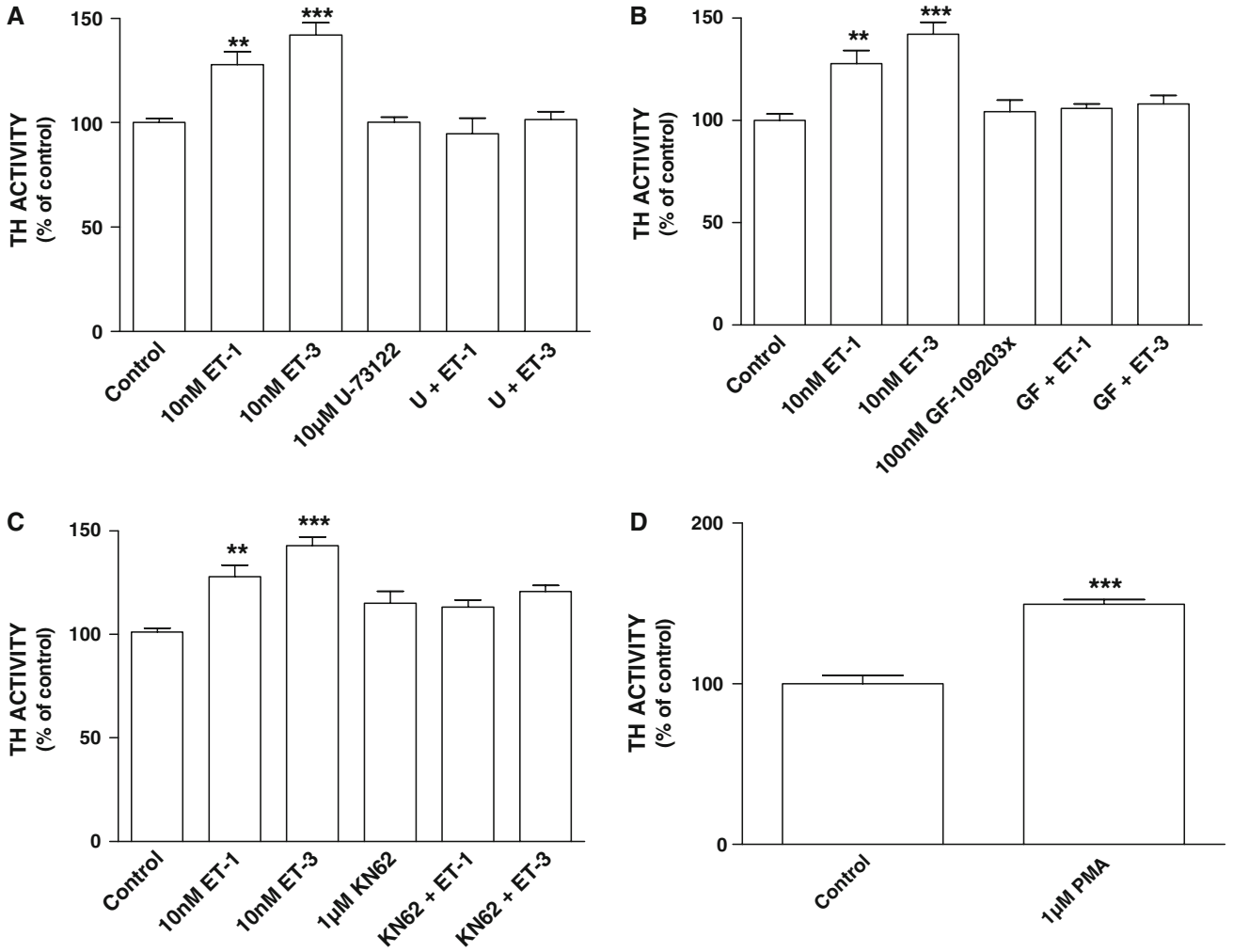

Fig. 6 Participation of PLC, PKC and CaMK-II in endothelin-1 and -3 (ET-1 and ET-3) effect on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). The OB was pre-treated with U-73122 (PLC inhibitor) (**a**), GF-109203x (PKC inhibitor) (**b**) or KN62 (CaMK-II inhibitor) (**c**) and further incubated with ET-1 and ET-3 or incubated with PMA (PKC activator) (**d**). TH activity was determined as detailed in "Materials and Methods" and expressed as percentage of control. ** $P < 0.01$ and *** $P < 0.001$ vs. control. Number of experiments: 6–8

The presence of ETs and their receptors in diverse regions and areas of the central nervous system including the OB is supported by different studies [16, 39]. In this regard, ET receptors binding sites were found in the glomerular layer of olfactory bulb and the olfactory ventricle of rats [5]. Moreover, the presence of ET_A receptors in the periglomerular area of this brain region has been reported [7].

Several works support that ETs function as neurotransmitters and/or regulatory neuropeptides within the CNS. ETs injection in the cerebral ventricles modifies blood pressure, heart rate and behaviour [16]. Most studies suggest that the cardiovascular effects of centrally applied ETs are mediated by changes in the sympathetic activity [16, 40].

In the present study ET-1 and ET-3 effects on TH activity and NE release in OB of normotensive rats were evaluated. We previously observed that 10 nM is the concentration of ETs that modifies hypothalamic catecholaminergic activity in vitro [11, 12, 15, 17, 18]. Endogenous ETs levels have been determined in cerebrospinal fluid and different regions of CNS, but not in the OB [16]. Present findings show that in the OB ET-1 and ET-3 increased TH activity through a G protein-coupled receptor, presumably an atypical or non-conventional ET receptor coupled to the activation of various intracellular signalling pathways. Selective ET_A and ET_B receptor antagonists (BQ-610 and BQ-788, respectively) did not affect TH basal activity but they both completely inhibited the increase induced by ETs. Furthermore, ET receptor agonists (SRTx-6b and IRL-620) failed to mimick ETs response. These findings in the presence of ET antagonists and agonists support that conventional ET receptors did not mediate the increase in TH activity evoked by ETs and

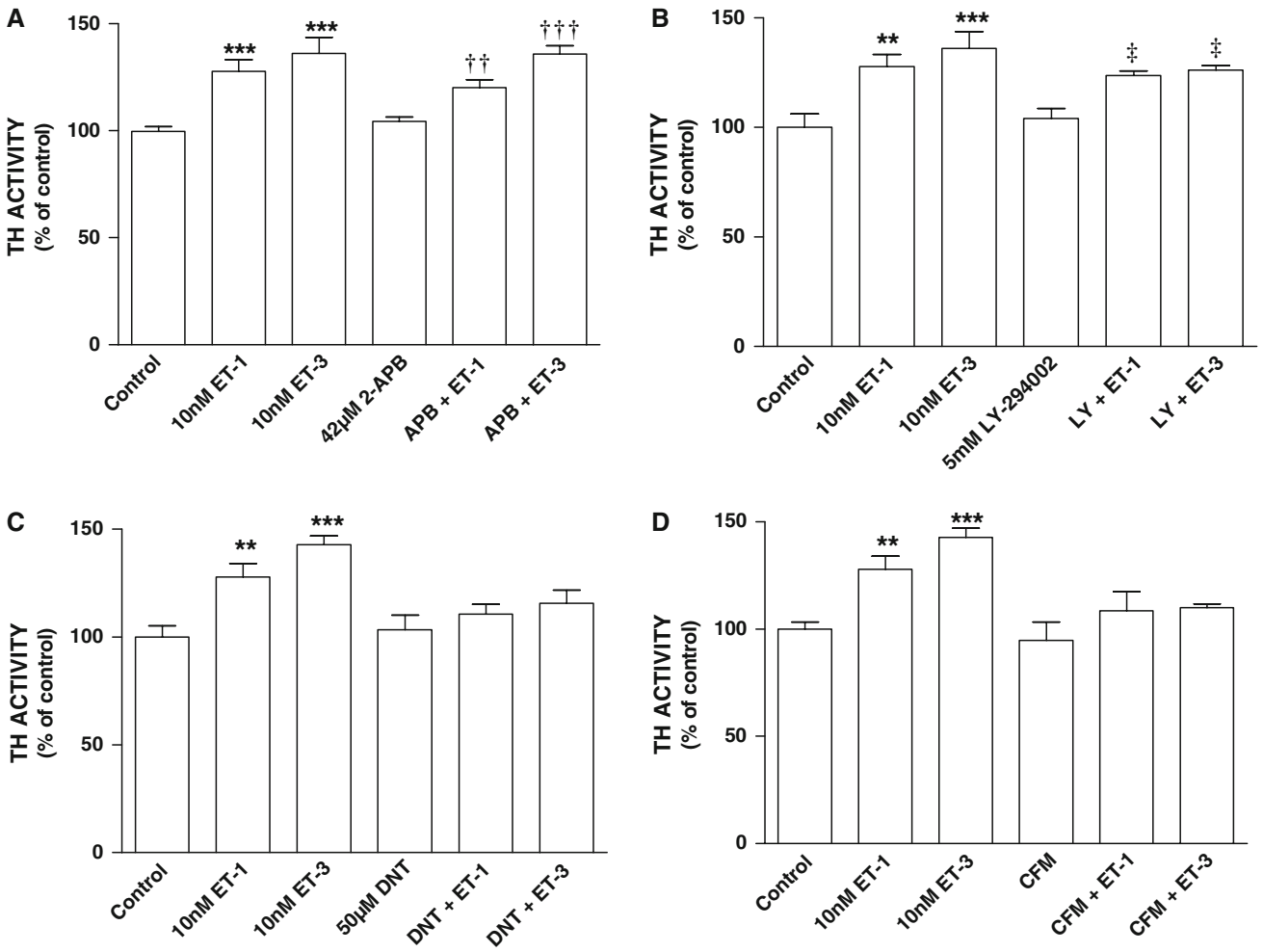

Fig. 7 Role of calcium in endothelin-1 and -3 (ET-1 and ET-3) effect on tyrosine hydroxylase (TH) activity in the olfactory bulb (OB). Before the addition of ETs the OB was pre-treated with 2-APB (IP_3 receptor antagonist) (**a**), LY-294002 (IP_3 kinase inhibitor) (**b**), DNT (ryanodyne receptor antagonist) (**c**) or incubated with ETs in a Ca^{2+} free medium (CFM) (**d**). TH activity was determined as detailed in "Materials and Methods" and expressed as percentage of control. ** $P < 0.01$ and *** $P < 0.001$ vs. control; †† $P < 0.01$ and ††† $P < 0.001$ vs. 2-APB; ‡ $P < 0.05$ vs. LY-294002. Number of experiments: 6–9

further suggest the participation of atypical ET receptors. Several studies reported the existence of atypical receptors termed ET_{AX} and ET_{BX} to differentiate them from the conventional ET receptors [11, 12, 15, 17]. In addition, functional and binding studies suggest the existence of a third receptor subtype, ET_C, that displays high affinity for ET-3 [11, 12, 41, 42]. ET_C amino acid sequence is 47 and 52% identical to ET_A and ET_B receptors, respectively. Since this receptor presents homology with both ET_A and ET_B receptors, each antagonist (BQ-610 and BQ-788), may bind to ET_C and abolish ETs response separately. However, the ET_C receptor has not been cloned in mammals yet. Recent reports suggest that the ETs atypical responses would be mediated by ET_A and ET_B receptor dimmerization. Experimental evidence shows that conventional ET receptors can form homo or hetero dimmers [43, 44]. Nevertheless, further studies are needed in order to support that the atypical response may result from the dimmerization of the conventional ET receptors or by atypical or other ET receptor subtypes not described yet. Present findings support that the non-conventional ET receptor that mediated ETs response on TH activity was a G-protein-coupled receptor since the response was abolished by a G-protein inhibitor. It is well known that ET receptors are able to stimulate multiple intracellular signalling pathways because they can activate distinct G-proteins [45].

TH catalyzes the rate-limiting and committed step in catecholamine biosynthesis [19–21]. The presence of TH mRNA and protein has been reported in rat OB periglomerular neurons [46]. TH activity is regulated by short-term mechanisms including feedback inhibition, allosteric regulation and phosphorylation [19–21]. Diverse

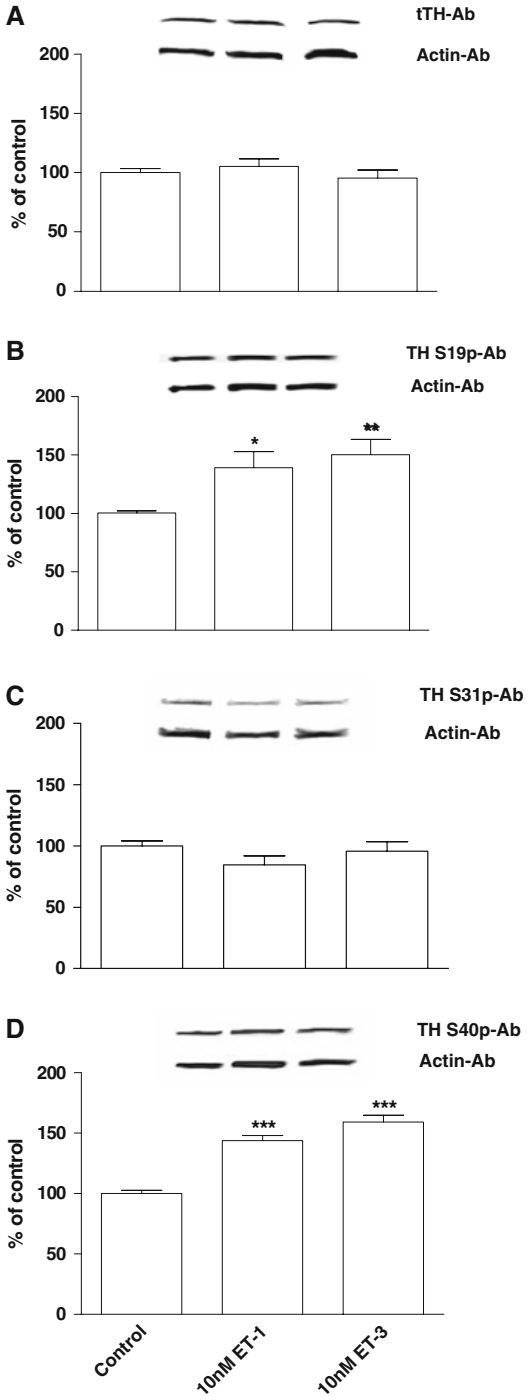

Fig. 8 Effect of endothelin-1 and -3 (ET-1 and ET-3) on the expression of total TH and the phosphorylated forms of the enzyme in the olfactory bulb. The expression of total TH level (tTH-Ab) (**a**) and the enzyme phosphorylated forms ar Ser 10 (**b**), 31 (**c**) and 40 (**d**) (THS19p-Ab, THS31p-Ab and THS40p-Ab, respectively) were determined by Western blot analysis and normalized to β-actin (Actin-Ab) as detailed in "Material and Methods". * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control. The Western blot assays shown are representative of at least three or four independent experiments

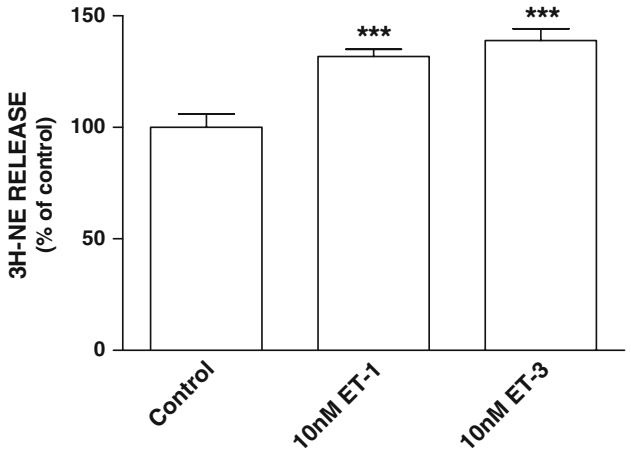

Fig. 9 Effects of endothelin-1 and -3 on neuronal norepinephrine (NE) release in the olfactory bulb. The OB was incubated with 10 nM ET-1 or ET-3 neuronal NE release was determined as detailed in "Materials and Methods" and expressed as percentage of control. *** $P < 0.001$ vs. control. Number of experiments: 5

intracellular signalling pathways underlie TH complex regulatory mechanisms. Phosphorylation is considered the major mechanism of TH activation, being serine residues (Ser) 8, 19, 31 and 40 phosphorylated by diverse protein kinases [19, 21]. However, phosphorylation of each serine site does not affect TH activity equally. The Ser 8 residue is phosphorylated by cdc/ciclina A, but there is no experimental evidence supporting that this event increases TH activity. On the other hand, Ser 19 is a target for MAP kinases 1 and 2 (ERK1 and ERK2) as well as for CaMK-II and its phosphorylation results in a slightly increase in TH activity. The protein kinases that induce TH phosphorylation at Ser 31 are ERK1 and ERK2 which induce a modest increase in the enzyme activity. The Ser 40 residue is the most promiscuous site since it can be phosphorylated by PKA, PKC, CaM-PKll and PKG. Present finding show that ET-1 and ET-3 enhanced TH activity through the cAMP/PKA and PLC/DAG/PKC pathways. In addition, it is well known that neuronal mechanisms controlling cytoplasmatic calcium concentrations are important for diverse cellular functions. Thus, several reports show that calcium triggers TH phosphorylation and subsequent activation in different tissues [27, 28]. The increase of intracellular calcium involved in TH modulation may result from trans-membrane calcium fluxes as well as from calcium released from IP_3-dependent stores and/or ryanodine sensitive channels. Present findings showed that a calcium free medium and DNT but not 2-APB, abolished the effect of ETs on TH activity, supporting that increased intracellular calcium resulted from calcium released by ryanodine sensitive channels and calcium incorporated from the extracellular compartment. It has been reported that calcium released

from IP_3 sensitive stores turns off IP_3 release and initiates the activation of ryanodine sensitive channels [47]. Therefore it is likely that IP_3 receptors were involved in the initial intracellular calcium peak in response to ETs, but at 30 min the ryanodine sensitive channels would be the predominant intracellular calcium source. In addition ryanodine receptors are also activated by calcium entry through voltage gated calcium channels [48]. Thus, TH activation would result likely from calcium released from ryanodine sensitive channels in response to a rise in intracellular calcium generated by the activation of voltage-gated calcium channels. Calcium binds to calmoduline and the calcium–calmodulin complex activates CaMK-II which in turn induces TH phosphorylation. Present findings support that CaMK-II was involved in ETs stimulation of TH activity. Neither ET-1 nor ET-3 modified total TH protein levels, but they increased the phosphorylation of serine residues at 19, and 40 sites. Phosphorylation of TH at these sites supports the involvement of PKC, PKA and CaMK-II in the increase of the enzyme activity induced by ET-1 and ET-3.

Growing evidence supports that the stimulation of a kinase system may activate other kinases by "cross-talk" mechanisms. Although, our results showed the implication of diverse kinase pathways in TH activation it is not possible to determine whether the activation of each pathway was induced separately or it resulted from cross-talk mechanisms.

It is widely accepted that TH acts as a sensor of the local concentration of catecholamine products [19–21]. Pterin is the first substrate that binds to TH, and catecholamines also bind to the same site of the enzyme therefore modulating TH activity by feedback inhibition [19, 20]. Present findings show that ETs also augment neuronal NE release from the OB of normotensive rats. Several works show that ETs increase neuronal NE release from the cerebral cortex and posterior hypothalamus [11, 39]. The increase in NE release leads to a reduction of the TH–NE complex facilitating the TH–pterin complex formation, thus resulting in enhanced enzyme activity.

Taken together, present findings suggest that the short-term increase of TH activity in response to ET-1 and ET-3 may result from increased neuronal NE release and TH phosphorylation, but not from changes in de novo synthesis of the enzyme.

In conclusion, our findings show that in the rat OB, both ETs increased TH activity through a non-conventional ET receptor coupled to a G-protein that stimulates the cAMP/PKA, PLC/PKC, and CaMKII pathways. Furthermore, TH regulation by ETs involved the increase of intracellular calcium mediated by ryanodine receptor activation and calcium influx. The rise in intracellular calcium likely led to CaMKII activation which contributed to increased TH activity. To our knowledge this is the first report to show a clear interaction between ETs and the catecholaminergic transmission in the OB. However, further studies are necessary in order to elucidate the role of this interaction in the OB and its relationship with the different biological functions regulated at this level.

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