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The Induction of Cardiac Ornithine Decarboxylase by β_2-Adrenergic Agents Is Associated With Calcium Channels and Phosphorylation of ERK1/2

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ABSTRACT

The role that the induction of cardiac ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, by beta-adrenergic agents may have in heart hypertrophy is a controversial issue. Besides, the signaling pathways related to cardiac ODC regulation have not been fully elucidated. Here we show that in Balb C mice the stimulation of cardiac ODC activity by adrenergic agents was mainly mediated by β_2-adrenergic receptors, and that this induction was lower in the hypertrophic heart. Interestingly, this stimulation was abolished by the L-calcium channel antagonists verapamil and nifedipine. In addition, whereas the treatment with β_2-adrenergic agents was associated to both the increases in ODC, ODC-antizyme inhibitor 1 (AZIN1), c-fos and c-myc mRNA levels and the phosphorylation of CREB and MAP kinases ERK1 and ERK2 (ERK1/2), the co-treatment with L-calcium channel blockers differentially prevented most of these changes. These results suggest that the stimulation of cardiac ODC by β_2-adrenergic agents is associated with the activation of MAP kinases through the participation of L-calcium channels, and that by itself p-CREB does not appear to be sufficient for the transcriptional activation of ODC. In addition, post-translational mechanisms related with the induction of AZIN1 appear to be related to the increase of cardiac ODC activity. J. Cell. Biochem. 114: 1978–1986, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: ORNITHINE DECARBOXYLASE; POLYAMINES; BETA-ADRENERGIC AGENTS; L-CALCIUM CHANNELS; MAP KINASES; CREB

The polyamines putrescine, spermidine, and spermine are biological cations ubiquitously found in mammals and known to be essential for cell growth and differentiation [Cohen, 1998; Thomas and Thomas, 2001; Pegg, 2009]. In the heart, polyamine levels and the activities of the enzymes related to polyamine metabolism are associated to the action of drugs, hormones, and conditions that affect cardiac function and growth [Flamigni et al., 1986]. Although the precise role of polyamines in cardiac physiology has not yet been fully elucidated, several findings have indicated that these organic cations may function as intracellular mediators that affect cardiac contractility by binding to inward rectifier potassium channels [Ficker et al., 1994; Lopatin et al., 1994] and myofilament proteins [Harris et al., 2000], apart from regulating Ca^{2+} fluxes [Fan and Koening, 1998; Koenig et al., 1998]. In addition, ornithine decarboxylase (ODC), a rate-limiting enzyme in the polyamine biosynthetic pathway, can be induced in the heart by a variety of β-adrenergic agonists and other stimuli which cause cardiac hypertrophy [Warnica et al., 1975; Bartolome et al., 1980; Pegg, 1981; Copeland et al., 1982; Tipnis et al., 1989; Shimizu et al., 1991; Cubria et al., 1999; Schluter et al., 2000; Bordallo et al., 2001]. The possible role of the enhancement of ODC activity and polyamine levels in heart hypertrophy is far from being understood. Whereas the inhibition of cardiac ODC by α-difluoromethylornithine (DFMO) attenuated the heart hypertrophy elicited by isoproterenol, a β-adrenoreceptor agonist, the hypertrophy caused by thyroxine was not prevented by DFMO [Bartolome et al., 1980; Pegg, 1981]. In addition, DFMO attenuated the elevation of hypertrophic markers produced by isoproterenol in cultured cardiac myocytes, suggesting a

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central role of ODC in β-adrenoreceptor mediated hypertrophy [Schluter et al., 2000]. Interestingly, in the hearts of transgenic mice overexpressing the antizyme, a negative regulator of ODC, the increase in ODC activity and polyamine content produced by isoproterenol was prevented but the increase in cardiac growth was unaffected [Mackintosh et al., 2000]. On the other hand, mice with targeted overexpression of ODC in the heart did not show a hypertrophic phenotype in spite of having marked elevations in heart ODC activity and polyamine content, although in these transgenic mice β-adrenergic stimulation by isoproterenol resulted in a more severe heart hypertrophy (and even death) than in non-transgenic animals [Shantz et al., 2001]. More recent experiments have also shown that the overexpression of ODC decreases ventricular systolic function during induction of cardiac hypertrophy [Giordano et al., 2012].

The molecular mechanisms by which β-adrenergic agents stimulate cardiac ODC are still under debate. Although initial pharmacological studies indicated that β_2-adrenoreceptors regulate the induction of myocardial ODC in mice [Copeland et al., 1982], more recent studies suggested that both β_1- and β_2-adrenoreceptors contribute to the elevation of ODC activity [Cubria et al., 1999]. Moreover, the responsiveness of the mouse heart to some β_2-adrenergic agonists such as clenbuterol appears to be dependent on the mouse strain [Shantz et al., 2001]. Distinct signaling pathways have been implicated in the action of β-adrenergic receptors in cardiomyocytes[Steinberg, 1999]. It is known that β_1-adrenoreceptors modulate cardiac contractility mainly through a cAMP-dependent mechanism, but the mechanisms implicated in the action of β_2-adrenoreceptors in the heart are less straight-forward. Gs, Gi, and Gαq signaling proteins, calcium ions, and the mitogen-activated protein kinase cascade appear to be involved in cardiomyocyte hypertrophy [Bogoyevitch et al., 1996; Adams et al., 1998; Zou et al., 1999; Xiang and Kobilka, 2003]. Regarding ODC induction, although in cell cultures of rat cardiomyocytes the action of isoproterenol was mimicked by butyryl-cAMP [Schluter et al., 2000], the possible mediation of cAMP and PKA in the in vivo induction of cardiac ODC by β-adrenergic receptor stimulation is not well defined. In addition, experiments using perfused rat hearts indicated that calcium ions were involved in cardiac ODC induction by isoproterenol [Guarnieri et al., 1983], but the underlying mechanisms were not explored. Here we report that both β_2-adrenoreceptor and L-type Ca^{2+} channels are implicated in the induction of cardiac ODC activity in mice and that this event is associated with the phosphorylation of ERK1/2 as well as with-increases in mRNA levels of ODC, ODC antizyme inhibitor 1 (AZIN1) and c-myc and c-fosprotooncogenes.

MATERIALS AND METHODS

MATERIALS

Isoproterenol (isoprenaline), clenbuterol, fenoterol, dobutamine, denopamine, verapamil, nifedipine, ±BayK8644, ICI-118,551, and Protease Inhibitor Cocktail (AEBSF, aprotinin, bestatin, E-64, leupeptin, pepstatin A) were obtained from Sigma–Aldrich. L-(1-^{14}C)ornithine (specific activity 56 mCi/mmol) was purchased from MoravekBiochemicals, Inc. (Brea, CA). The anti-pERK1/2 (pERK1/2) rabbit polyclonal IgG, the anti-ERK2 rabbit polyclonal IgG and the anti-p-CREB rabbit polyclonal IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Reagents for SDS–PAGE and Western blot were from Bio-Rad (Richmond, CA). Moloney murine leukemia virus (MMLV) reverse transcriptase, RNAlater®, RNAase free water and GenElute mammalian total RNA Miniprep kit were purchased from Sigma (St. Louis, MO). SYBR Green® PCR Master Mix was from Applied Biosystems (Warrington, UK). Primers were purchased from Sigma–Genosys (Suffolk, UK).

ANIMALS AND TREATMENTS

Animal procedures were carried out according to the institutional guidelines of the University of Murcia, in compliance with national (*RD 1201/2005*) and international laws and policies (European Union normative 86/009). Three-month-old male Balb/c adult mice were supplied by the Service of Laboratory Animals of the University of Murcia. Animals were fed with standard chow (UAR A03; Panlab, Barcelona, Spain) and water ad libitum, and maintained at 22°C and 55% relative humidity under a controlled 12:12 h light–dark cycle (light on from 0800 h). They were sacrificed by cervical dislocation after light anesthesia. Hearts were removed and frozen in liquid nitrogen until analysis. In the acute treatment, mice were randomly divided into several groups. For each group, mice were given intraperitoneal injections of β-adrenoreceptor agonists (isoproterenol, clenbuterol, fenoterol, or dobutamine) at a dose of 2 mg/kg (dissolved in 0.9% saline), and killed 5 h after injection. Denopamine was dissolved in dimethyl sulfoxide (DMSO). Control mice were injected with 0.9% saline or DMSO. In some groups, mice were injected with verapamil (30 mg/kg), nifedipine (20 mg/kg), ICI-118,551 (10 mg/kg), or BayK8644 (10 mg/kg) 30 min prior the β-adrenoreceptor agonist. To induce heart hypertrophy, mice were given daily intraperitoneal injections of either saline or 20 mg/kg isoproterenol for 10 days, and were killed 24 h after the tenth injection. The number of mice in each experimental group ranged from 4 to 6.

ORNITHINE DECARBOXYLASE ACTIVITY

ODC activity was determined as described elsewhere with some modifications [Bastida et al., 2005]. In brief, hearts were homogenized with the aid of a Polytronhomogeniser in buffer containing 25 mM Tris (pH 7.2), 2 mM dithiothreitol, 0.1 mM pyridoxal phosphate, 0.1 mM EDTA, and 0.25 M sucrose. The extracts were centrifuged at 20,000*g* for 20 min, and ODC activity was assayed in the supernatant by measuring $^{14}CO_2$ release from 30 µM L-[1-^{14}C] ornithine. The reaction was performed in glass tubes with tightly closed rubber stoppers, hanging from the stoppers two disks of filter paper wetted in 0.5 M benzethonium hydroxide dissolved in methanol. The samples were incubated at 37°C for 1 h, and the reaction was stopped by adding 0.5 ml of 2 M citric acid. $^{14}CO_2$ trapped in the paper disks was counted by liquid scintillation. Activity was expressed as pmol$^{14}CO_2$ produced per hour and per mg tissue wet weight.

WESTERN-BLOT ANALYSIS

The hearts were homogenized in PBS (137 mM NaCl; 2.7 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) containing 1% Igepal, 1 mM

EDTA, 0.1 mM PMSF an additional mixture of protease inhibitors (100 μM AEBSF, 0.2 μM aprotinin, 10 μM bestatin, 3.5 μM E-64, 5 μM leupeptin, 4 μM pepstatin A) and phosphatase inhibitors (2 mM imidazol; 1 mM NaF, 1.15 mM sodium molibdate, 1 mM sodium o-vanadate, and 4 mM sodium tartrate). The homogenates were centrifuged at 12,000*g* for 20 min, and equal amounts of protein from the supernatants, determined by using the Bradford reagent (Bio-Rad), were mixed with Laemmli sample buffer, heated at 95°C for 5 min and fractionated by electrophoresis in 10% polyacrylamide–SDS gels. The resolved proteins were electroblotted to PVDF membranes, and the resulting blots were incubated with 5% non-fat dry milk in PBS (0.01 M phosphate buffered saline pH 7.4) for 1 h. After washing in PBS + 0.1% Tween 20 (PBST) the blots were incubated at 4°C overnight with the primary antibody (anti-ERK2 rabbit antibody, anti-pERK1/2 rabbit antibody, or anti-pCREB rabbit antibody at a dilution 1:5,000). The blots were washed in PBST and incubated at room temperature for 1 h with a horseradish peroxidase-labeled goat secondary antibody (dilution 1:5,000). Immunoreactive bands were detected by using ECL+ detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and commercial developing reagents and films (Amersham). Comparable loading was ascertained by stripping and reprobing the membranes with an anti-ERK2 antibody. Stripping was performed by washing the membranes with PBS, followed by treatment with 0.5 M NaOH, 10 min at room temperature, and a final 10-min wash with PBS.

QUANTITATIVE REAL-TIME RT-PCR

Total RNA was extracted from hearts with GenElute mammalian total RNA Miniprep kit following the manufacturer's instructions. First-strand cDNA was obtained from total RNA using MMLV reverse transcriptase. One to 5 μg of total RNA was reverse-transcribed using 1 μl oligo (dT) as primer, 1 μl of 10 mM dNTP Mix, 1 μl of MMLV reverse transcriptase, 2 μl of buffer (containing 500 mM Tris–HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, and 5 mM DTT) and nuclease-free water up to 20 μl. RNA, oligo dT, and dNTPs were mixed and incubated at 70°C for 10 min. Mixture was put on ice for a few minutes and MMLV reverse transcriptase was added. After incubation at 37°C for 1 h, the transcriptase was denatured by heating at 90°C for 10 min. PCR amplification was carried out using a SYBR Green® PCR Master Mix (Applied Biosystems) and a 7500 Real Time instrument (Applied Biosystems). Different sets of primers and cDNA were used and the fluorescence data were collected and analyzed by means of 7500 SDS software (Applied Biosystems). The expression level of each gene was normalized against beta-actin. In some cases, L17 ribosomal protein or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also used for comparison [Ramos-Molina et al., 2012]. The values obtained for each gene were used to adjust the mRNA abundance. The following primers were used: mouse β-actin (forward, 5′-GATTACTGCTCTGGCTCCTAGCA-3′; reverse, 5′-GCTCAGGAGGAGCAATGATCTT-3′); Odc (forward, 5′-ATGGGTTCCAGAGGCCAAA-3′; reverse, 5′-CTGCTTCATGAGTTGCCACA-TT-3′); Azin1 (forward, 5′-CTTTCCACGAACCATCTGCT-3′; reverse, 5′-TTCCAGCATCTTGCATCTCA-3′); c-Myc (forward 5′-GCTGCATGAGGAGACACCGC-3′; reverse 5′-CAGACACCACATCAATTTC-3′); c-Fos (forward, 5′-ATGGTGAAGACCGTGTCAG-3′; reverse, 5′-AGCCTCAGGCAGACCTCCAG-3′); c-Jun (forward, 5′-GATCCAGCGCCCGCGGCTCCTG-3′: reverse, 5′-ATTTGCAAAAGTTCGCTC-3′);Gapdh (forward, 5′-CCTGCGACTTCAACAGC AAC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′); ribosomal protein L19 (forward, 5′-GGCTTGCCTCTAGTGTCCTC-3′; reverse, 5′-CTGATCTGCTGACGGGAGTT-3′).

STATISTICAL ANALYSIS

Data are expressed as mean ± SE. The significance of the differences observed was assessed by ANOVA, followed by the post hoc Newman–Keuls multiple range test, or by Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

β_2-ADRENERGIC RECEPTORS ARE IMPLICATED IN ODC INDUCTION IN THE MOUSE HEART

Figure 1 shows that both isoproterenol and clenbuterol at the dose of 2 mg/kg induced ODC activity (about sixfold over control levels, 5 h after administration) in the heart of Balb/c mice. This time length was selected because it corresponds with the peak of ODC activity. Similar results were obtained with CD1 mice (data not shown). Figure 1 also shows that fenoterol, a β_2-adrenergic agonist, elicited a response comparable to that of isoproterenol or clenbuterol, whereas the β_1-adrenergic agonists dobutamine and denopamine, did not significantly increase cardiac ODC activity under the same dose and conditions than the other agents. Even at a higher dose (20 mg/kg), they were unable to increase cardiac ODC activity (results not shown). It must be mentioned that fenoterol dissolved in DMSO produced the

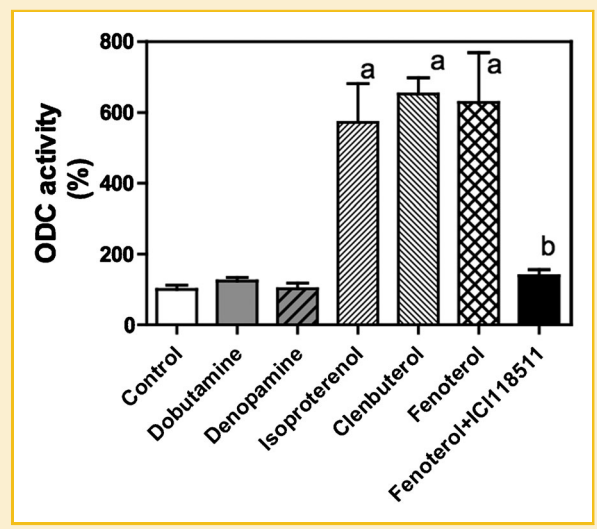

Fig. 1. Changes in cardiac ornithine decarboxylase (ODC) activity following the administration of β-adrenergic agonists and antagonists. Adult male mice were injected i.p. with the following compounds and doses: isoproterenol (2 mg/kg), clenbuterol (2 mg/kg), fenoterol (2 mg/kg), dobutamine (2 mg/kg), denopamine (2 mg/kg), fenoterol (2 mg/kg) + ICI-118,551 (10 mg/kg). Mice were killed 5 h after injection. Control mice received 0.9% NaCl. Results are expressed as % of control group and are presented as mean ± SEM (n = 5–6 animals per group). ODC activity in control group (100%): 1.63 ± 0.19 pmol $^{14}CO_2$/h mg tissue wet weight. Statistical significance: (a) $P < 0.001$ versus control group; (b) $P < 0.001$ versus fenoterol.

same effect when dissolved in saline, excluding the possibility that the lack of action of denopamine could be due to a solvent effect on ODC activity. Moreover, the pre-treatment of mice with ICI118511, a specific antagonist of β_2-adrenoreceptors, abrogated the induction of cardiac ODC in response to fenoterol. All these results indicate that in the mouse heart, the β_2-adrenoreceptors are the main mediators of ODC induction by β-adrenergic agents. In order to test whether the cardiac hypertrophy produced by beta adrenergic agents is associated to a permanent increase in cardiac ODC activity, mice were treated with isoproterenol for 10 days (daily injections of 20 mg/kg isoproterenol). Table I shows that this treatment produced a significant increase in heart weight (about 26%), but this process was not associated to a marked and permanent increase in cardiac ODC activity. This result indicates that the induction of ODC in cardiac and renal hypertrophies appears to be different since, in contrast to what was observed here, in the kidney hypertrophy produced by chronic treatment with testosterone, renal ODC remained markedly and permanently increased during the hypertrophic process [Tovar et al., 1995]. In addition, in the hypertrophic hearts of another group of mice exposed to the chronic treatment with isoproterenol, the induction of ODC 5 h after the last injection was about 28% of that achieved in mice with non-hypertrophic heart (results not shown), indicating that the induction of ODC by β-adrenergic agents is decreased by the hypertrophy. This could explain the poor induction of cardiac ODC by isoproterenol that we found in early observations with old mice (results not published), having higher values of the ratio of total heart weight/body weight than the control mice used in the present study.

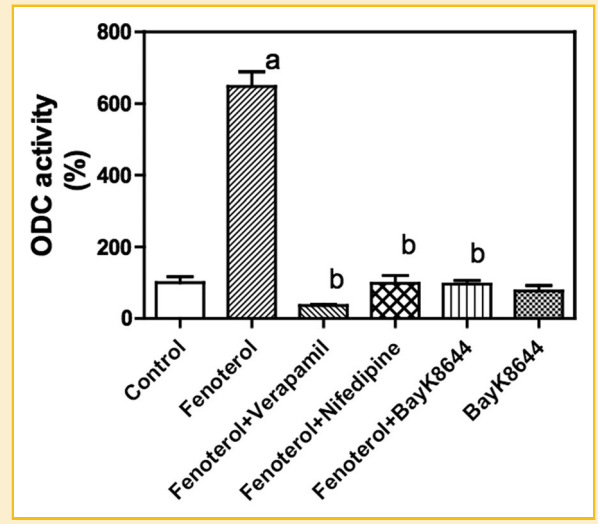

Fig. 2. Influence of L calcium channel antagonists on the stimulation of cardiac ornithine decarboxylase (ODC) activity by fenoterol. Adult male mice were injected i.p. with fenoterol (2 mg/kg) alone or in combination with the L calcium channel antagonist verapamil (30 mg/kg) or nifedipine (20 mg/kg) given 30 min prior fenoterol administration. Other groups were treated with BayK8644 (10 mg/kg), a calcium ion channel agonist, alone or in combination with fenoterol (2 mg/kg). Mice were killed 5 h after the last injection. Control mice received 0.9% NaCl. Results are expressed as % of control group and are presented as mean ± SEM (n = 5–6 animals per group). Statistical significance: (a) $P < 0.001$ versus control group; (b) $P < 0.001$ versus fenoterol.

INFLUENCE OF L-CALCIUM CHANNELS ON CARDIAC ODC INDUCTION BY FENOTEROL

The induction of cardiac ODC activity obtained in response to fenoterol was prevented when mice were treated with the L-calcium channel antagonist verapamil, 30 min prior to fenoterol administration (Fig. 2). Similar results were obtained with nifedipine, another L-calcium channel antagonist. These results are in agreement with findings using the perfused rat heart as a model, in which manipulations of calcium levels affected ODC induction [Guarnieri et al., 1983]. Interestingly, BayK8644, an agonist of L-calcium channels, did not affect the basal level of cardiac ODC activity and also prevented the increase in ODC activity promoted by fenoterol (Fig. 2). These results suggest that Ca^{2+} fluxes through the L-calcium channels appears to be a critical factor for the induction of cardiac ODC by β_2-adrenergic agonists, and that both reduced and elevated cytosolic calcium concentrations may negatively affect this process. The negative effect of verapamil on ODC induction was also observed when isoproterenol was used as beta-adrenergic agonist (results not shown).

SEARCHING SIGNALING PROCESSES IMPLICATED IN CARDIAC ODC INDUCTION BY β_2-ADRENORECEPTORS

Since β-adrenergic agonists stimulate adenylate cyclase and the production of cAMP as second messenger, and this activates the cAMP-dependent protein kinase (PKA), we studied the phosphorylation of the transcription factor CREB (cAMP-response element binding protein) by PKA, an important step in the process of gene-activation mediated by cAMP [Sands and Palmer, 2008], and its possible role on cardiac ODC activation. On the other hand, since it is also known that adrenergic receptor stimulation activates the mitogen-activated protein kinase cascade [Bogoyevitch et al., 1996], we evaluated the role of the activation of extracellular signal-regulated kinases (ERKs) on the induction of cardiac ODC by

TABLE I. Influence of Repeated Isoproterenol Treatment on Heart Hypertrophy and ODC Activity

Treatment	Heart weight (mg)	Heart weight/body weight (mg/g)	ODC activity (pmol/h mg tissue wet weight)
Control (5)	168 ± 12	4.44 ± 0.47	1.23 ± 0.11
Isoproterenol (5)	212 ± 21[a]	5.59 ± 0.64[b]	1.54 ± 0.12

Mice were given daily intraperitoneal injections of either saline or 20 mg/kg isoproterenol for 10 days. Twenty-four hours after the tenth injection, mice were killed, and the hearts removed, weighed, and homogenized to measure ODC activity. Results are expressed as mean ± SEM.
[a] $P < 0.01$ versus control.
[b] $P < 0.05$ versus control.

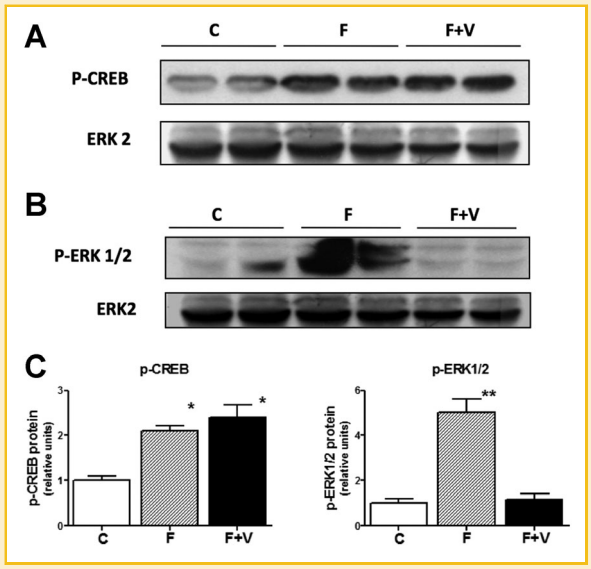

Fig. 3. Representative Western blots showing the effect of fenoterol (F, 2 mg/kg, 2 h) and fenoterol plus verapamil (F + V, fenoterol 2 mg/kg 2 h, verapamil 30/mg/kg 30 min before fenoterol) on cardiac p-CREB (A) and p-ERK1/2 (B). C: Quantification of p-CREB and pERK1/2 values in the different groups normalized to the values of ERK2 (n = 4); $^{*}P < 0.01$ versus C, $^{**}P < 0.001$ versus C and F + V.

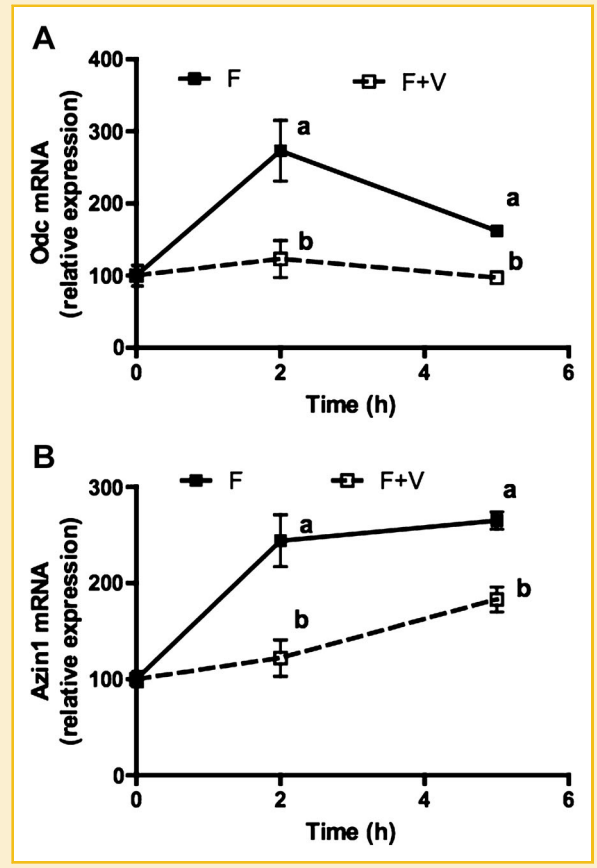

Fig. 4. Influence of fenoterol (F, 2 mg/kg) and fenoterol + verapamil (F + V, 2 and 30 mg/kg, respectively) treatments on ODC (A) and AZIN1 (B) mRNA levels in mouse heart. mRNA contents were analyzed by quantitative real-time RT-PCR analysis, and the values were normalized to β-actin. Each point is the mean ± SEM of four animals. Statistical significance: (a) $P < 0.01$ versus time 0; (b) $P < 0.05$ versus F.

fenoterol. Figure 3A shows that fenoterol stimulated the phosphorylation of CREB in the mouse heart, and that the pretreatment of mice with verapamil did not apparently affect the activation of CREB, although ODC induction was prevented. This result suggests that either CREB does not participate in the induction of cardiac ODC or that CREB activation is not by itself sufficient to increase ODC activity and hence, other mediators regulated by calcium fluxes could be essential for the induction of cardiac ODC by CREB. From Figure 3B it can be seen that fenoterol stimulated the phosphorylation of the MAP kinases ERK1 and ERK2 (ERK1/2), and that verapamil prevented the phosphorylation of these enzymes. The parallelism between this modification of ERKs and the induction of ODC strongly suggests that the activation of ERKs may have a certain role in the process of cardiac induction by β_2-adrenergic agonists.

INFLUENCE OF β_2-ADRENERGIC AGENTS AND L-CALCIUM CHANNELS ON CARDIAC TRANSCRIPT LEVELS

We next examined how the different treatments affected the levels of ODC mRNA in the mouse heart, as well as those other genes that have been related to the action of β-adrenergic agonists in cardiac hypertrophy, such as c-myc or c-fos [Gan et al., 2005; Robbins and Swain, 1992]. Figure 4A shows that fenoterol produced a significant increase (higher than 100% after 2 h) in ODC mRNA in the heart that was almost totally inhibited by verapamil. A similar pattern was observed for AZIN1 mRNA, although in this case mRNA levels were still elevated after 5 h of treatment (Fig. 4B). No changes were detected for antizyme 1 mRNA levels (results not shown). The β_2-adrenoreceptor agonist also produced marked increases in c-myc and c-fos mRNA levels after 2 h of treatment, decreasing thereafter (Fig. 5A,B). These effects were also prevented by verapamil (Fig. 5A, B). The effect of fenoterol on c-jun mRNA content was not statistically significant and was not affected by the L-calcium channel antagonist (Fig. 5C). These results suggest that both β_2-adrenergic agents and L-calcium channels are required for the transcriptional activation of c-myc and c-fos. In addition, the increase of AZIN1 mRNA, encoding the AZIN1 protein, a positive regulator of ODC [Fujita et al., 1982; Mangold, 2006; López-Contreras et al., 2010], could also explain why the enhancement of ODC activity is higher than that of its mRNA, suggesting then that the β_2-adrenergic agents affect ODC expression at both transcriptional and post-translational levels.

DISCUSSION

The molecular mechanisms implicated in the action of β-adrenergic agents on cardiac ODC induction and heart hypertrophy are not yet completely understood. The present results indicate that in the mouse heart, the β_2-adrenoreceptors play a major role in the process of ODC induction by adrenergic drugs and that Ca^{2+} current through the L-type Ca^{2+} channels appears to be a critical requirement for the

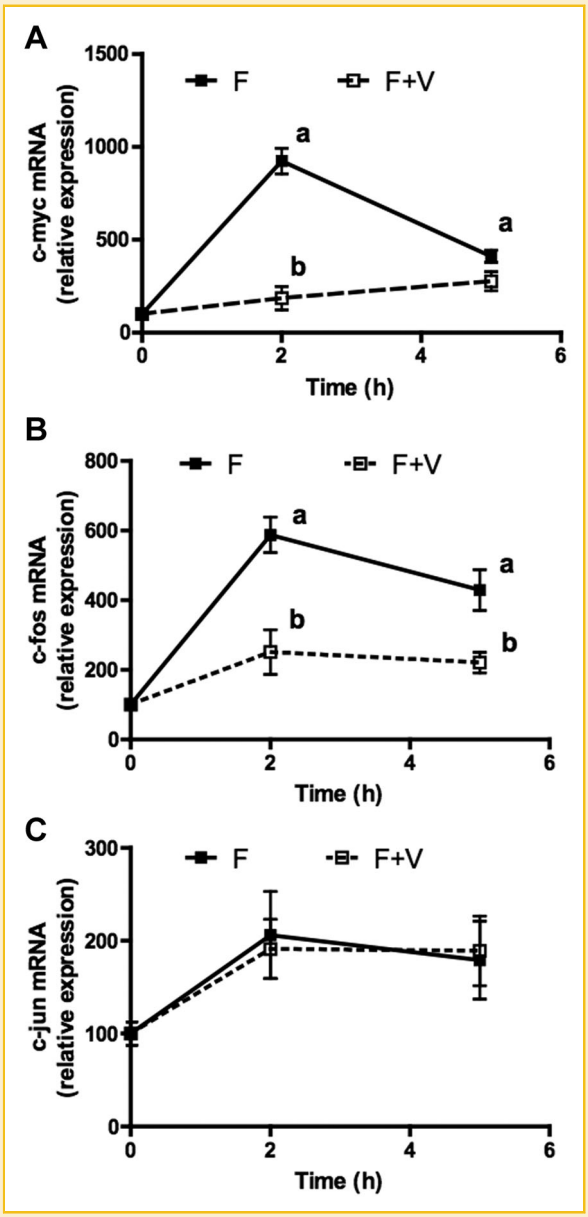

Fig. 5. Influence of fenoterol (F, 2 mg/kg) and fenoterol + verapamil (F + V, 2 and 30 mg/kg, respectively) treatments on c-myc (A), c-fos (B), and c-jun (C) mRNA levels in mouse heart. mRNA contents were analyzed by quantitative real-time RT-PCR analysis, and the values were normalized to β-actin. Each point is the mean ± SEM of four animals. Statistical significance: (a) $P < 0.001$ versus time 0; (b) $P < 0.01$ versus F.

induction of cardiac ODC elicited by the β_2-adrenergic agents. These results are in agreement with previous results obtained in experimental models different from that of adult mice [Copeland et al., 1982; Guarnieri et al., 1983; Schluter et al., 2000]. These findings may be relevant in order to understand the molecular mechanisms of cardiac ODC induction and its role in cardiac physiology. Since ODC and polyamines are required for cell growth [Cohen, 1998; Thomas and Thomas, 2001], it was believed that the induction of cardiac ODC by β-adrenoreceptor agonists, which stimulated the growth of cardiac myocytes, could be an obligatory step in heart hypertrophy induced by catecholamines (Bartolome et al., 1980, 1982). However, experiments using transgenic mice over-expressing ODC in the heart demonstrated that the robust expression of ODC is not sufficient by itself to promote heart hypertrophy but indicated that ODC may cooperate with factors inducing cardiac growth [Shantz et al., 2001]. In this regard, it has been suggested that putrescine may function as a low affinity agonist on β-adrenoreceptors and modulate acute responses mediated by β-adrenoreceptors [Bordallo et al., 2008]. Our results suggest that the stimulation of ODC by the beta adrenergic agonists is a characteristic of normal heart mice, which is markedly diminished once that the heart becomes hypertrophic.

On the other hand, at present, it is not clear whether β-adrenergic stimulation of cardiac ODC is mediated by the classic Gs–adenylate cyclase–cAMP–PKA signaling pathway, nor if the changes in ODC activity correlate with transcriptional activation or with any other post-transcriptional events. Although both β_1- and β_2-adrenoreceptors are present in the mouse heart, and both of them stimulate the cAMP/PKA pathway [Kaumann, 1997; Steinberg, 1999; Rockman et al., 2002; Xiang and Kobilka, 2003] our results indicate that only activation of β_2-adrenoreceptors appears to be effective for the induction of ODC. Available evidence suggests that whereas β_1-adrenoreceptors only interact with Gs proteins, β_2-adrenoreceptors can be coupled to both Gs and Gi proteins, activating bifurcated signaling pathways [Steinberg, 1999; Xiao et al., 1999; Zheng et al., 2004]. Thus, the release of βγ subunits after activation of Gi may stimulate the extracellular signal-regulated kinase (ERK) cascade [Faure et al., 1994; Daaka et al., 1997]. Our results showing that cardiac ODC induction by fenoterol, a β_2-adrenoreceptor agonist, was associated to the phosphorylation of ERK1/2 and CREB, and that the blockade of ODC induction by L-calcium channel antagonists also prevented the phosphorylation of ERKs, strongly suggest that the activation of the mitogen-activated protein kinase cascade by adrenergic receptor stimulation appears to be important for the induction of cardiac ODC by catecholamines, but requires critical levels of free intracellular calcium. In this regard, it was reported that isoproterenol induces ERK activation and cardiomyocyte hypertrophy [Zou et al., 1999], and that the elevation of intracellular Ca^{2+} is a critical factor for MAPK activation in rat cardiomyocytes [Bogoyevitch et al., 1996]. Although a role for Ca^{2+} rather than cAMP has been established in the β-adrenergic regulation of myocardial gene expression [Bishopric et al., 1992], our results showing that the agonist of L-calcium channels BayK8644, in the absence of exogenous adrenergic agents, did not stimulate the induction of cardiac ODC suggest that increases in both cAMP and Ca^{2+} signals appear to be critical for the stimulation of ODC by the β_2-adrenoreceptor agonists. In addition, the inhibition of the fenoterol-mediated stimulation of ODC by BayK8644 suggests that prolonged activation of L-calcium channels or altered Ca^{2+} fluxes may alter the signaling processes implicated in ODC induction. The fact that only the β_2-adrenoreceptor agonists stimulated cardiac ODC induction, in spite that β_1-adrenoreceptors predominate in the mouse heart, when the stimulation of both β-adrenoreceptors subtypes increases L-type Ca^{2+} currents [Steinberg, 1999; Xiao et al., 1999], could be explained by the postulated specific and functional

compartmentalization of β-adrenoreceptors with L-calcium channels and other signaling intermediates in cardiomyocytes [Gao et al., 1997; Kaumann, 1997; Zhou et al., 1997; Xiao et al., 1999; Xiang and Kobilka, 2003; Fischmeister et al., 2006]. This could be critical to trigger the signaling pathway leading to ODC induction. The failure to induce ODC in cultured mouse cardiomyocytes in response to isoproterenol precludes the use of direct ERK1/2 inhibitors to confirm that the activation ERKs is essential for ODC induction.

The present results also demonstrate that fenoterol markedly increased the levels of different mRNAs in the mouse heart, including those coding for ODC, ODC antizyme inhibitor 1 (AZIN1), c-myc, c-fos, and c-jun. More interestingly, verapamil prevented the rise in the levels of all these mRNAs except that of c-jun. In mammals, there is ample evidence that ODC is regulated at transcriptional, translational and post-translational levels [Pegg, 2006]. In rats and mice, post-translational control mechanisms have been proposed to explain the increase in cardiac ODC activity after β-adrenergic agonists [Lau and Slotkin, 1982; Cubria et al., 1999]. Our results suggest that both transcriptional and post-translational mechanisms are likely to be implicated in cardiac ODC induction. The increases in ODC and c-myc mRNA levels, associated to the fact that ODC is a known transcriptional target of c-myc [Bello-Fernandez et al., 1993; Packham and Cleveland, 1997] support the hypothesis that c-myc could participate in the transcriptional activation of cardiac ODC. In particular, the increase in abundance of c-myc mRNA induced by fenoterol seen here is in agreement with reported findings showing such an increase in the heart of mice treated with isoproterenol, precedes the increase in cardiac mass [Robbins and Swain, 1992]. Increased c-fos expression in ventricular cardiomyocytes has also been found associated with the activation of PKA and ERK1/2 [He et al., 2010]. Besides, the rise in AZIN1 mRNA, which encodes a protein that negates the stimulatory effect of antizymes on ODC degradation [Coffino, 2001; Kahana, 2009], also support the possibility of a post-translational control of ODC by AZIN1, which would be consistent with a previous report showing increased ODC half-life by clenbuterol in the mouse heart [Copeland et al., 1982].

One of the best-known transcription factors that participates in the specific changes in gene expression associated to the cAMP/PKA signaling pathway is CREB. After phosphorylation of CREB by PKA, it can bind to consensus cAMP-response element (CRE) in the DNA modulating gene transcription [Sands and Palmer, 2008]. Although CRE-like elements have been found in mouse, rat and human ODC genes [Palvimo et al., 1991; Abrahamsen et al., 1992], their role in ODC induction has not been clearly established. In our study, the fact that fenoterol stimulated the phosphorylation of this transcription factor, and that verapamil, although preventing cardiac ODC induction, did not inhibit the phosphorylation of CREB suggests that CREB phosphorylation by itself does not appear to be sufficient for the transcriptional activation of ODC. One possibility is that other factors activated in the mouse heart through ERK signaling, such as c-myc or c-fos, could be necessary for the induction of cardiac ODC by CREB. In fact, cross-talk between extracellular signal-regulated kinase and cAMP signaling has been reported [Houslay and Kolch, 2000], as well as CREB with fos/jun on a single cis-element in steroidogenic cells [Manna and Stocco, 2007]. In conclusion, our results indicate that the induction of cardiac ODC by β_2-adrenergic agents is partially mediated by transcriptional mechanisms, and that it requires the participation of L-calcium channels, associated to the activation of the ERK signaling pathway.

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