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Differential phosphorylation, desensitization and internalization of α1A adrenoceptors activated by norepinephrine and oxymetazoline.

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Running Title: α1A adrenoceptor regulation by oxymetazoline

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Abbreviations

AR: adrenoceptor; Bisindolylmaleimide I: 3-(N-[Dimethylamino]propyl-3-indolyl)-4-(3-indolyl)maleimide, 3-[1-[3-(Dimethylamino)propyl]1H-indol-3-yl]-4-(1H-indol-3-yl)1H-pyrrole-2,5dione; BMY-7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride); eGFP: enhanced green fluorescent protein; Gö6976: 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole; GRK: G protein receptor kinase; HEK: Human embryonic kidney cells; Hispidin: 6-(3,4-dihydroxystyrl)-4-hydroxy-2-pyrone; PKC: protein kinase C; Rottlerin: 1-[6-[(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one.

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ABSTRACT

Loss of response upon repetitive drug exposure, i.e. tachyphylaxis, is a particular problem for the vasoconstrictor effects of medications containing oxymetazoline (OXY), a α1-adrenoceptor (AR) agonist of the imidazoline class. One cause of tachyphylaxis is receptor desensitization, usually accompanied by phosphorylation, and internalization. It is well established that α1A-ARs are less phosphorylated, desensitized and internalized upon exposure to the phenethylamines norepinephrine (NE), epinephrine or phenylephrine (PE) than are the α1B and α1D subtypes. However, here we show in HEK-293 cells that the low efficacy agonist OXY induces G protein-coupled receptor kinase 2 (GRK2)-dependent α1A-AR phosphorylation, followed by rapid desensitization and internalization (~40% internalization after 5 min of stimulation), whereas phosphorylation of α1A-ARs exposed to NE depends to a large extent on PKC activity, is not followed by desensitization and the receptors undergo delayed internalization (~35% after 60 min of stimulation). Native α1A-ARs from rat tail artery and vas deferens are also desensitized by OXY, but not by NE or PE, indicating that this property of OXY is not limited to recombinant receptors expressed in cell systems. The results of the present study are clear indicative of agonist-directed α1A-AR regulation. OXY shows functional selectivity relative to NE and PE at α1A-ARs leading to significant receptor desensitization and internalization, which is important in view of the therapeutic vasoconstrictor effects of this drug and the varied biological process regulated by α1A-ARs.

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INTRODUCTION

The neurotransmitter in the central and peripheral (sympathetic) nervous system norepinephrine (NE) and the adrenal gland hormone epinephrine (EPI) regulate important biological processes through activation of α1 adrenoceptors (α1 ARs), including behavioral responses (Doze et al., 2011; Berridge et al., 2012), male reproductive performance (Sanbe et al., 2007; de Almeida Kiguti and Pupo, 2012), and contraction of vascular and non-vascular smooth muscles (Docherty, 2010). The products encoded by the three α1 AR genes are named α1A, α1B and α1D ARs. All three α1 ARs are 7 transmembrane domain receptors that activate Gq/11 heterotrimeric proteins leading to stimulation of phospholipase C, IP_3 and diacylglycerol production, and increases in cytosolic Ca^{2+} concentrations (Chen and Minneman, 2005).

The relatively large array of drugs that target α1 ARs either mimic (agonists) or inhibit (antagonists) the effects of the endogenous catecholamines NE and EPI, and are part of the therapeutic arsenal available for the relief of hypertension, prostatic hyperplasia and shock. In addition to reverting hypotension in shock, α1 AR agonists are vasoconstrictors when locally applied to the nasal mucous membrane or the eye, and are also components of over-the-counter oral medications for the common cold and flu.

Repeated exposure to an agonist may lead to diminished responses, a process known as tachyphylaxis (as defined in (Neubig et al., 2003). One cause of tachyphylaxis is receptor desensitization, usually accompanied by receptor phosphorylation and internalization. Tachyphylaxis is a particular problem for vasoconstrictors and nasal decongestants containing imidazoline α1 AR agonists such as oxymetazoline (OXY), naphazoline and xylomethazoline, and the rebound hyperemia and *rhinitis medicamentosa* observed with some of these drugs has been suggested to be linked to α1 AR desensitization (Vaidyanathan et al., 2010). α1 ARs are

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known to be subject to phosphorylation and internalization upon exposure to NE, EPI or phenylephrine (PE). However, there are substantial differences in the degree of phosphorylation, internalization and desensitization among the α1 AR subtypes. For instance, α1B ARs are phosphorylated to a greater extent in response to either NE or tetradecanoyl phorbol acetate (TPA, a protein kinase C activator) than α1A-ARs (Vazquez-Prado et al., 2000; Garcia-Sainz et al., 2004; Cabrera-Wrooman et al., 2010). Also, α1A ARs are less internalized upon exposure to NE, EPI or PE than are the α1B or α1D ARs (Chalothorn et al., 2002; Wang et al., 2007; Stanasila et al., 2008; Cabrera-Wrooman et al., 2010). When compared in the same cellular background, recombinant α1A ARs are much more resistant to desensitization induced by NE or TPA than α1B and α1D ARs (Vazquez-Prado and Garcia-Sainz, 1996; Vazquez-Prado et al., 2000; Cabrera-Wrooman et al., 2010). Therefore, as far as receptor phosphorylation, desensitization and internalization are concerned, the view that the α1A ARs are less tightly regulated than the other two subtypes is acceptable (reviewed in (Cotecchia, 2010). In this context, the tachyphylaxis observed for the vasoconstrictor effects of medications containing imidazoline agonists is intriguing, because these drugs are often selective ligands for α1A ARs (Minneman et al., 1994) given that this receptor subtype is much less susceptible to phosphorylation, desensitization and internalization than are the α1B and α1D ARs.

The present investigation extends early studies describing tachyphylaxis in responses of rat smooth muscle tissues to the α1A AR-selective and low efficacy agonist OXY (Ruffolo et al., 1977; Rice et al., 1991), and shows in HEK-293 cells that OXY induces rapid G protein-coupled receptor kinase 2 (GRK2)-dependent phosphorylation, desensitization and internalization of human recombinant α1A-ARs, whereas NE caused phosphorylation of receptors that is largely dependent on PKC and not followed by rapid desensitization and internalization. In addition to

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shedding light in the molecular mechanisms of the tachyphylaxis of the vasopressor and nasal decongestant effects of OXY and the resulting rebound hyperemia and *rhinitis medicamentosa*, these findings show clear evidence of distinct agonist-directed receptor phosphorylation, desensitization and internalization mechanisms. These findings are important in view of the varied biological functions regulated by these receptors.

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MATERIALS AND METHODS

Experiments in rat smooth muscle tissues

The experimental procedures were approved by the Ethics Committee for the Use of Experimental Animals from UNESP – Botucatu and are in accordance with the Guide for the Care and Use of Laboratory Animals (NIH). Male Wistar rats (16 to 20 weeks old, 260 to 380g) were killed by decapitation and selected tissues were excised and prepared for digital recording of isometric contractions as follows: the vas deferens (epididymal portion) and tail artery (distal segments) were cleaned of adherent tissues and mounted in organ baths under 9.8 mN (vas deferens) or 14.7 mN (tail artery) tension in a nutrient solution with the following composition (mM): NaCl 138; KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.36, $NaHCO_3$ 15, dextrose 5.5, (for vas deferens); NaCl 119, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, dextrose 5.5, (tail artery) prepared in glass-distilled, de-ionized water, maintained at 30 ^oC (vas deferens) or 37 ^oC (tail artery), pH 7.4, and continuously bubbled with 95%O_2/5%CO_2. All experiments were done in the presence of a cocktail of inhibitors containing cocaine (6 μM), corticosterone (10 μM), idazoxan (1 μM) and propranolol (0.1 μM) to block neuronal uptake, extraneuronal uptake, and α_2- and β-adrenergic receptors, respectively. After an equilibration period of 60 min with adjustments of basal tension and changes of physiological solution at each 20 min, the tissues were repeatedly challenged with KCl 80 mM at 20 min intervals until contractions of similar magnitude were obtained. Then, consecutive concentration response-curves to the agonists in the vas deferens (NE or OXY) and tail artery (PE or OXY) were obtained with 45 min intervals between each one. In some experiments, the vas deferens and tail artery were treated with 10 μM OXY or 10 μM NE for 5 minutes, extensively washed and 45 min later a concentration-response curve to PE or NE was constructed. PE was chosen as an agonist in the tail artery because there is

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a significant participation of α2 ARs in the contractions induced by NE in this vessel (Kamikihara et al., 2005). Curve fitting and pEC_{50} calculation was performed with the software package GraphPad Prism (version 4.00, San Diego, California, USA). All values are shown as means ± standard error of mean (SEM) of *n* experiments. Differences between mean values were tested for statistical significance ($P < 0.05$) using Student's paired or unpaired *t*-tests.

Cell Culture and transfections

HEK-293 cells were propagated in 100-mm dishes in Dulbecco's modified Eagle's Medium with sodium pyruvate, supplemented with 10% heat inactivated fetal bovine serum, 10 mg/ml streptomycin and 100 U/ml penicillin in a humidified atmosphere with 5% CO_2 at 37^{o}C. Confluent plates were subcultured at a ratio of 1:3. Cells were transfected with 15 µg of cDNA of the mammaliam expression plasmid pDT, containing N-terminal sequential hexahistidine and FLAG epitope-tagged human α1A (α1A-1 splice variant), α1B or N-terminal truncated mutant of α1D ARs (Δ^{1-79}α1D AR) by Lipofectamine® (Invitrogen), and when specified, stably transfected cells were selected with geneticin (400 µg/ml). The constructs were generously provided by Dr. K.P. Minneman and are described elsewhere (Vicentic et al., 2002; Pupo et al., 2003; Hague et al., 2004). In addition, HEK-293 cells were transfected with 20 µg of cDNA of the expression plasmid pcDNA1.1 encoding the sequence for a kinase-deficient mutant of bovine GRK2 in which lysine 220 was replaced by a methionine (C20-GRK2-K220M[3], described in (Ferguson et al., 1995), generously donated by Dr Maria de Fatima M. Lazari, from UNIFESP, and stably transfected cells were selected with geneticin (400 µg/ml) and designated DNGRK2 cells.

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[^{3}H]Prazosin binding in membrane preparations from HEK-293 cells

For radioligand binding measurements, confluent 100 mm plates were washed with phosphate buffered saline (PBS; 20 mM $NaPO_4$, 154 mM NaCl, pH 7.6) and cells were harvested by scraping. HEK-293 cells expressing recombinant human α_{1A}-, α_{1B}- or $\Delta^{1-79}\alpha_{1D}$-adrenoceptors were collected by centrifugation and homogenised with a Polytron; membranes were collected by centrifugation at 30,000 x g for 20 min and resuspended in 1X buffer A (25 mM HEPES, 150 mM NaCl, pH 7.4). Radioligand binding sites were measured by saturation analysis of specific binding of the α_1-adrenoceptor radioligand [^{3}H]prazosin (20 - 2000 pM). Nonspecific binding was defined as binding in the presence of 100 μM phentolamine. The pharmacological specificity of radioligand binding sites was determined by displacement of [^{3}H]prazosin (350 pM) by selected ligands, and data was analyzed by nonlinear regression analysis using the software package GraphPad Prism (version 4.00, San Diego, California, USA).

[^{3}H]Prazosin binding in intact HEK-293 cells

HEK-293 cells growing in 100 mm plates were transiently transfected with the expression plasmid pDT containing N-terminal sequential hexahistidine and FLAG epitope-tagged α_{1A} AR and 24 h later were transferred to poly-D-lysine treated 24-well microplates (100,000 cells/well). Approximately 48 h post transfection, the cells were incubated with 10 μM NE or 10 μM OXY for different times as indicated at 37 ^{o}C in DMEM, or with increasing concentrations of NE and OXY for 30 min and then washed twice with ice cold PBS (1.0 ml, pH 7.2). After washing, the cells were incubated with 1 nM [^{3}H]prazosin (80Ci/mmole, Amershan Biosciences) at 4^{o}C in PBS to reduce the partioning of the radioligand for 10 to 12 h (Leeb-Lundberg et al., 1987). Radioligand non-specific binding of was defined in presence of 100 μM phentolamine or 100 μM

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epinephrine. After the incubation with the radioligand, the cells were washed twice with 500 µl of ice cold PBS containing 0.1% BSA and then scrapped in 500 µl of MilliQ water. To each sample 4 ml of scintillation cocktail were added (OptiPhase "HiSafe" 3, PerkinElmer) and ^{3}H was counted in a Liquid Scintillation Analyzer (1900 TR – PACKARD, Canberra, Australia).

Intracellular Ca^{2+} Measurements

For intracellular Ca^{2+} (iCa^{2+}) mobilization assays, HEK-293 cells expressing recombinant human α1A ARs were cultivated overnight in black-walled clear bottom 96-well microplates covered with poly-D-lysine (30,000 cells/well). The next day, 100 µl of a modified Hanks' balanced saline solution (HBSS; 137 mM NaCl, 5.36 mM KCl, 1.18 mM, 1.26 mM $CaCl_2 \cdot 2H_2O$, 0.49 $MgCl_2 \cdot 6H_2O$, 0.41 $MgSO_4.7H_2O$, 0.44 KH_2PO_4, 4.17 $NaHCO_3$, 0.35 $NaHPO_4$, 5.5 mM D-glucose, 20 mM HEPES and 2.5 mM probenecid, pH 7.4) containing the fluorescent Ca^{2+} indicator FLUO-4 NW® (0.1% v/v, Invitrogen,) was added to each well according to manufacturer instructions for 1 h at 37°C. Before loading the fluorescent Ca^{2+} indicator, some wells were incubated with NE or OXY (both at 10 µM for 5 min), carefully washed at least 3 times with 200 µl of PBS (without Ca^{2+} and Mg^{2+}), and then incubated with 100 µl of modified HBSS containing the fluorescent indicator. After 1 h incubation with the fluorescent indicator, 90 µl of modified HBSS (without probenecid) was added to each well and basal fluorescence (excitation 485 nm, filter 485/20 nm, emission 525 nm, filter 516/20 nm) was measured in a microplate reader (Synergy 4, Biotek) every 2 s for 10 seconds. For the construction of concentration-response curves, 10 µl of assay buffer containing increasing concentrations of NE or OXY (final concentration in the well from 0.1 nM to 3 µM) were added through onboard dispensers at minimal speed (225 $\mu l.sec^{-1}$), and the effect on fluorescence was recorded every 2 s

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for 50 s. Data was collected online in a microcomputer, analyzed by the software Gen5 (Biotek), and the ratios between the peak minus basal fluorescence were taken and expressed as percentage of the maximal increase induced by NE 10 μM. The fittings for the concentration-response curves were calculated using the software package GraphPad Prism (version 4.00, San Diego, California, USA). In some experiments, cells were seeded in a 6-well plate (10^6 cells/well) and at least 12 h later incubated with Fura-2 AM (5 μM) for 1 h at 37ºC in a Krebs-Ringer HEPES buffer supplemented with 0.05% bovine serum albumin. After loading the calcium indicator, the cells were trypsinized, washed and resuspended in 2 ml of the above-mentioned buffer supplemented with 1.2 mM $CaCl_2$. Fluorescence readings were taken in an Aminco-Bowman Series 2 spectrometer (SLM Instruments Inc.,Urbana, IL, USA) with the excitation monochromator set at 340 and 380 nm and emission monochromator set at 510nm (Fura2-AM).

Confocal microscopy of HEK-293 cells

HEK-293 cells transiently transfected with the peGFP-N3 plasmid encoding recombinant human α1A ARs fused at the C-terminus with enhanced green fluorescent protein (α1A/eGFP, described in Hague et al., 2004 and kindly provided by Dr Chris Hague from University of Washington, Seattle, WA) were cultivated in quartz coverslips covered with poly-D-lysine for 24 to 36 h and imaged in a laser scanning confocal microscope (LSM510 META, Carl Zeiss, Jena, Germany) with Plan-Neofluar 40x oil immersion objective lens (Leica, Wetzlar, Germany), before and during the treatment with 10 μM NE or 10 μM OXY for 30 min at 37°C. In some experiments, the cells in the coverslips were treated with 30 nM prazosin 30 min prior the incubation of 10 μM OXY. eGFP fluorescence was excited using an argon laser at a wavelength of 488 nm and the emitted fluorescence was detected at 505 nm. Changes in fluorescence intensity were estimated

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using Image J 1.38x software (NIH, http://rsb.info.nih.gov/ij/) in areas just below the cell surface before (basal) and during the incubation with drugs. Data were normalized to a percentage of the fluorescence obtained before agonist treatment and the increases in fluorescence intensity above that observed before drug treatments were taken as measures of receptor internalization.

Phosphorylation of α1A ARs

HEK-293 cells transiently transfected with the peGFP-N3 plasmid encoding recombinant human α1A ARs fused at the C-terminus with enhanced green fluorescent protein eGFP (α1A/eGFP) were serum-starved for 24 h, maintained in phosphate-free Dulbecco's modified Eagle's medium for 1 h and then incubated in 1 ml of the same medium containing [^{32}P]Pi (150 μCi/ml) for 3–5 h at 37°C, as previously described (Vazquez-Prado et al., 2000). Labelled cells were stimulated as indicated and at the end of the incubation were washed with ice-cold phosphate-buffered saline and solubilized with 1.0 ml of ice-cold solubilization buffer containing the following: 100 mM NaCl, Tris 10 mM, Triton X-100 0.1%, Nonidet P40 0.1%, Sodium Dodecyl Sulfate 0.05%, 10 mM NaF, 1 mM Na3VO4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM p-serine, 1 mM p-threonine, and 1 mM p-tyrosine. The plates were maintained on ice for 1 h. The extracts were centrifuged at 12,700×g for 15 min at 4°C, and the supernatants were collected. Receptors were immunoprecipitated using rabbit antibodies generated in our laboratory and directed against eGFP, as described (Avendano-Vazquez et al., 2005; Cabrera-Wrooman et al., 2010). Receptor phosphorylation was detected with a Molecular Dynamics PhosphorImager and quantified with ImageQuant software (Amersham Biosciences Inc., Sunnyvale, CA, USA). Data fell within the apparatus' linear detection range and were plotted utilizing Prism 4.0 (GraphPad software, San Diego, CA, USA).

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Drugs

Drugs were obtained from the following sources: Bisindolylmaleimide I and rottlerin (Calbiochem, La Jolla CA, USA); corticosterone, hispidin, L-norepinephrine bitartrate salt, oxymetazoline HCl; phenylephrine HCl; oxymetazoline HCl; from Sigma Chemical Co, USA; cocaine (Cocainum Hydrochloricum puriss.) C.H. Boehringer, Germany; FURA2-AM and Fluo-4 NW (Invitrogen Life Technology, Carlsbad, CA, USA); BMY-7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride), 5-methylurapidil HCl, prazosin HCl, (±)-propranolol HCl, and idazoxan HCl from Research Biochemicals Inc. (RBI/SIGMA, U.S.A.). [^{3}H]prazosin (PerkinElmer Life and Analytical Sciences, Waltham, MA); Protein A_Agarose. (Upstate Biotechnology, Lake Placid, NY, USA) Nitrocellulose membranes (Bio-Rad, Hercules CA, USA); ^{32}P[Pi] (8500-9120 Ci/mmol; Elmer Life Sciences, Wellesley MA, USA); Chemiluminiscence kits, Pierce (Rockford IL, USA).

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RESULTS

Tachyphylaxis in contractions of the rat tail artery and vas deferens induced by OXY

The contractions of the rat vas deferens to NE (Pupo, 1998) and rat tail artery to PE (Kamikihara et al., 2005) under conditions of α2 and β ARs blockade are mediated by the activation of α1A ARs. At least three consecutive concentration-response curves to PE in the rat tail artery (Fig 1A) or NE in the vas deferens (Fig 1B) produced similar maximal effects (Emax) and *p*EC50 values (Table 1), showing no evidence for tachyphylaxis in the contractions induced by these agonists under the conditions investigated. OXY behaved as a partial agonist in the rat tail artery (Figure 1C) and vas deferens (Fig 1D) in relation to PE and NE, respectively. However, in contrast to the consecutive concentration-response curves for NE and PE, there was intense tachyphylaxis to contractions induced by OXY in the tail artery (Fig 1C) as reflected by a ~30-fold rightward shift and ~45% reduction in Emax (Table 1) and also in the vas deferens, where OXY failed to contract the tissue upon a second or third agonist exposure (Fig 1D).

In order to check whether OXY induced tachyphylaxis in the contractions of the rat tail artery and vas deferens in response to PE and NE, the tissues were incubated with OXY (10 μM) for 5 min, extensively washed and after a 45 min recovery interval exposed to PE or NE. In rat tail artery (Figure 2A) and vas deferens (Figure 2B) pre-exposed to OXY (10 μM/5 min), PE and NE were 20-fold less potent than in time controls that had been treated with vehicle (Table 2). OXY (10 μM/5 min) was unable to induce tachyphylaxis to PE or NE if the treatment was performed in the presence of prazosin (30 nM), indicating that the effect depends on α-1 ARs activation (data not shown).

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Binding characteristics of α1A ARs expressed in HEK–293 cells

Saturation binding characteristics of [^{3}H]prazosin to membrane preparations from HEK-293 cells stably transfected with α1A ARs revealed a maximum binding capacity (Bmax) of 2042±158 fmol/mg protein and a K_D of 371±83 pM (mean±S.E.M. of four separate experiments). There was no specific binding of [^{3}H]prazosin to membrane preparations from non-transfected or mock transfected (empty pDT vector) HEK-293 cells (data not shown). Competition binding experiments were carried out using OXY (α1A-selective ligand), 5-methyl urapidil (α1A-selective) and BMY7378 (α1D-selective) in membrane preparations from HEK-293 cells expressing human α1A, α1B and a N-terminal truncated mutant of the human α1D ARs (Δ^{1-79}α1D AR, which traffics efficiently to the cell membrane (Pupo et al., 2003; Hague et al., 2004; Nojimoto et al., 2010)). All of the ligands competed for the binding of [^{3}H]prazosin, with the curves fitting a single site isotherm. As expected, 5-methyl urapidil (pK_i: α1A = 8.9±0.2; α1B =7.1±0.2 ; Δ^{1-79}α1D =7.7±0.2, n = 4) and OXY (pK_i: α1A = 6.8 ± 0.2; α1B = 5.2 ± 0.1; Δ^{1-79}α1D = 5.5 ± 0.1, n= 3 to 4) inhibited the binding of [^{3}H]prazosin with high relative affinities consistent with an interaction with α1A ARs, whereas BMY7378 (pK_i: α1A = 7.0±0.1 ; α1B = 6.8±0.2; Δ^{1-79}α1D =8.6±0.1) showed higher affinity in membrane preparations from cells expressing Δ^{1-79}α1D ARs. Note that OXY was ~20 to 40-fold more potent at α1A ARs than at the other two receptors.

Tachyphylaxis in HEK-293 cells expressing α1A ARs on responses to NE after pretreatment with OXY

OXY increased iCa^{2+} concentrations in HEK-293 cells stably transfected with human recombinant α1A ARs and was a partial agonist relative to NE (Figure 3A, Table 3). There was

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intense tachyphylaxis in the iCa^{2+} response to NE in HEK-293 cells expressing human α1A AR that were pre-treated with OXY (10 μM/5 min), but not in cells pre-treated with NE (10 μM/5 min) (Fig. 3B, Table 4). OXY did not induce tachyphylaxis in the iCa^{2+} increases induced by carbachol through activation of endogenous muscarinic receptors, suggesting that the desensitization is specific for α1A ARs (results not shown).

Rapid internalization of α1 ARs activated by OXY

The localization of α1A ARs in HEK-293 cells in the absence and presence of NE or OXY was assessed by confocal microscopy of cells expressing α1A ARs tagged with eGFP at the C-terminus (α1A/eGFP, Fig. 4). In non-stimulated cells, the α1A/eGFP were localized mainly at the plasma membrane (time 0` in Fig. 4). OXY (10 μM) induced a loss of fluorescence at the plasma membrane, which was already measurable after 10 min of exposure (Figs. 4A and 4B). In contrast, loss of fluorescence in the plasma membrane of cells treated with NE (10 μM) was modest and only observed after 60 min of exposure (data not shown). Treatment of HEK-293 cells expressing α1A/eGFP with prazosin (30 nM/30 min) inhibited the loss of fluorescence in the plasma membrane induced by OXY (10 μM), indicating that this effect depends on α1A/eGFP activation (Fig 4).

The efficacies of OXY and NE in internalizing α1A ARs (N-terminally FLAG tagged receptors) were further compared in HEK-293 cells transiently expressing the receptors and treated with these agonists for various times, washed twice with PBS and incubated with 1 nM [^{3}H]prazosin for 10 to 12h at 4^{o}C (to reduce the partitioning of the radioligand) to quantify receptors at the cell surface. Figure 5A shows the total and non-specific binding of [^{3}H]prazosin defined in presence of 100 μM phentolamine (lipophilic ligand) or 100 μM EPI (hydrophilic

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ligand). In these experiments phentolamine and EPI revealed similar amounts of non-specific binding, indicating that under these experimental conditions the radioligand labels cell surface receptors. The time-course of α1A AR internalization following NE (10 μM) or OXY (10 μM) addition is shown in Fig. 5B. OXY, but not NE, induced rapid loss of cell surface binding detectable within 5 min of stimulation and maximal after ~30 min. On the other hand, loss of cell surface binding following addition of NE was detected only after at least 45 min of stimulation. A full concentration response curve for the effect of NE and OXY on cell surface binding after 30 min of stimulation is shown in Fig. 5C; within 30 min of stimulation, NE up to 10 μM was unable to reduce cell surface α1A ARs (n = 3), whereas OXY internalized ~50% of the receptors with potency (pEC50) of 6.9 ± 0.2 (n = 3). To check if the reduction of the binding of [^{3}H]prazosin in OXY-treated cells was due to incomplete washing of the agonist, cells were treated with OXY (10 μM for 5 min), washed and then incubated with increasing concentrations of [^{3}H]prazosin at 4^{o}C for 10 h (Fig 5D); saturation analysis of [^{3}H]prazosin binding showed that OXY treatment (10 μM for 5 min) reduced the Bmax (248±25 *vs* 183±13 fmol/well, $P < 0.05$ in Student's *t* test; n =3) without changing affinity (K_D = 294±50 *vs* 360±69 pM, n = 3). As expected, treatment with NE (10 μM for 5 min) had no effect on [^{3}H]prazosin binding (Bmax = 255±14 fmol/well and K_D = 286±47 pM, n = 3; $P > 0.05$ compared to nonstimulated cells). The lack of reduction in affinity indicates that OXY was readily washed out and that the reduction of [^{3}H]prazosin binding was due to loss of binding sites (α1A ARs) and not related to "competition" due to incomplete removal of the agonist.

Phosphorylation of α1A AR following activation by NE and OXY

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The phosphorylation of α1A/eGFP activated by NE or OXY was determined in HEK-293 cells transiently transfected with the receptor and metabolically labeled with inorganic phosphate ([^{32}P]Pi); cells were treated for various times with the agonists and the α1A/eGFP-ARs were immunoprecipitated with an anti-serum against eGFP. Previous studies have shown that the phosphorylation pattern of α1A/eGFP in response to NE or TPA is similar to non-tagged α1A AR (Cabrera-Wrooman et al., 2010). Autoradiograms showing the time-courses of these phosphorylations are shown in Fig. 6. Increase in phosphorylation of α1A ARs was detected after 2 and 5 min of stimulation with NE and OXY, respectively, and was maximal after ~5 to 15 min. However, the receptors activated by NE displayed almost twice the levels of phosphorylation as those activated by OXY.

Effects of a dominant negative mutant of GRK2 and of PKC inhibitors on α1A AR phosphorylation, internalization and desensitization.

HEK-293 cells were transfected with a dominant negative mutant of GRK2 (in which lysine 220 was replaced by a methionine to disrupt the kinase activity of the enzyme, (Ferguson et al., 1995)) and a stable cell line was selected (DNGRK2 cells). NE (10 μM) caused an approximately doubling of phosphorylation of transiently transfected α1A/eGFP above basal in DNGRK2 cells (Fig. 7A), only slightly less to what was observed in HEK-293 cells. In addition, in DNGRK2 cells α1A ARs activated by NE also underwent modest and delayed internalization (Fig. 7B). However, in sharp contrast, phosphorylation of α1A/eGFP activated by OXY in DNGRK2 cells was greatly reduced (Fig. 7C). Internalization of α1A ARs by OXY in DNGRK2 cells was greatly reduced and occurred at a slower rate than that observed in HEK-293 cells (Fig. 7D). However, OXY did not induce tachyphylaxis for the iCa^{2+} response to NE in DNGRK2 cells (Fig.

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8), indicating an important role for GRK2 in the phosphorylation, desensitization and internalization of α1A ARs to OXY.

To check for the participation of protein kinase C isoforms, phosphorylation of α1A ARs activated by NE or OXY (both at 10 μM/15min) was determined in DNGRK2 cells treated with bisindolylmaleimide I (1 μM, non-subtype selective PKC inhibitor, (Toullec et al., 1991)); Gö6976 (0.1 μM, PKCα and β1 selective inhibitor, (Martiny-Baron et al., 1993)); hispidine (1 μM, PKCβ selective inhibitor, (Gonindard et al., 1997)); and Rottlerin (1 μM, PKCδ selective inhibitor, (Gschwendt et al., 1994)). The phosphorylation of α1A ARs in response to NE was greatly reduced by bisindolylmaleimide I and Gö6976, but not by hispidine or rottlerin, suggesting the involvement of PKCα (Fig. 9A). On the other hand, the small phosphorylation of α1A ARs in response to OXY in DNGRK2 was not affected by any of the protein kinase C inhibitors tested (Fig. 9B). The effect of the non-selective protein kinase inhibitor staurosporine (100 nM/30 min) on α1A AR internalization in HEK-293 cells was investigated. Staurosporine completely inhibited the delayed internalization of α1A ARs caused by NE (Fig 9C), but had little effect on internalization of receptors to OXY (Fig 9D).

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DISCUSSION

The present study investigated the tachyphylaxis to the low efficacy partial agonist OXY in tissues and cells expressing rat native and human recombinant α1A ARs. The patterns of phosphorylation, desensitization and internalization of human recombinant α1A ARs exposed to OXY in HEK-293 cells sharply contrasted with the pattern observed for NE: α1A ARs activated by OXY are phosphorylated by GRK2, desensitized and undergo rapid internalization, whereas α1A ARs activated by NE are phosphorylated largely by PKC and internalize at a much slower rate.

Although OXY is a partial agonist relative to PE or NE in contractions of the rat tail artery and vas deferens (α1A AR-mediated effects) and in increases in iCa^{2+} in HEK-293 cells expressing human recombinant α1A ARs, it caused intense tachyphylaxis, whereas no tachyphylaxis was observed to the responses induced by the full agonists NE and PE. As partial agonists require higher receptor occupancies than full agonists to produce their effects, one could argue that tachyphylaxis may be an expected feature of responses to partial agonists, as they would be more affected by reduction in receptor density than full agonists. However, responses to NE and PE also showed tachyphylaxys in tissues or cells that had been previously exposed to OXY, but not to NE or PE, unveiling a distinctive property of this imidazoline. This ability of OXY to cause tachyphylaxis in contractions of the rat vas deferens to other adrenergic agonists had been previously described (Ruffolo et al., 1977; Rice et al., 1991) and it was concluded that it either resulted from slow dissociation of OXY from the receptor causing “pseudo-irreversible” antagonism or to an unique ability of this drug to desensitize/internalize α1 ARs (Rice et al., 1991). The present and previous studies do not support receptor inactivation due to “pseudo-irreversible” antagonism as an explanation. A full Schild analysis showed that OXY (0.1 to 30

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μM) behaves as a simple reversible competitive antagonist of contractions of the rat vas deferens and anococcygeous smooth muscle induced by NE (Kenakin, 1984; Campos et al., 2003). Saturation binding studies of [^{3}H]prazosin to intact HEK-293 cells expressing α1A ARs previously exposed to OXY (Fig. 5C) showed a reduction in the maximal binding without alteration in the pK_D and this effect of OXY is not seen in the binding of [^{3}H]prazosin to membrane preparations of HEK-293 cells expressing α1A ARs. In addition, the activation of α1A/eGFP in HEK-293 cells by OXY caused its translocation from the cell membrane to the intracellular compartment (Fig. 4).

Confocal microscopy and [^{3}H]prazosin binding experiments in intact cells showed that OXY caused rapid internalization of α1A ARs within ~5 min of addition, whereas internalization of α1A ARs by NE was much smaller and observed only after ~45 to 60 min of exposure. These findings can be compared with several studies using a variety of techniques to localize receptors that have reported only small and delayed internalization of α1A ARs, and all of these studies employed phenethylamine agonists to activate the receptor. For instance, confocal microscopy of HEK-293 cells expressing α1 AR subtypes tagged at the C-terminus with GFP showed that the α1A AR exposed to PE internalizes only after 50 minutes of stimulation (Chalothorn et al., 2002). Similarly, in HEK-293 cells expressing HA-tagged α1A ARs, EPI induced modest α1A AR internalization after 90 min, while precipitation of cell surface α1A ARs labeled with biotin does not reveal significant receptor internalization (Stanasila *et al.*, 2008). [^{3}H]Prazosin binding assays in intact rat-1 fibroblasts expressing human HA-tagged α1A AR (Price et al., 2002) and flow-cytometry of receptor localization (Morris et al., 2004) also confirm that NE induces HA-tagged α1A AR internalization after ~30 to 50 min. Thus, the present results with OXY show that the rate and extent of α1A ARs internalization depends on the nature of the agonist which is

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activating the receptor. This suggests biased agonism for OXY compared to NE towards the internalization pathway, and full concentration-response curves show that OXY was indeed more potent in internalizing α1A ARs than in increasing iCa^{2+} (Vanessa Lima, manuscript in preparation).

α1A ARs activated by NE were phosphorylated to a greater extent than those activated by OXY. However, the kinase involved in the phosphorylation of the α1A AR was agonist-dependent. Phosphorylation of α1A ARs exposed to OXY in cells expressing a dominant-negative mutant of GRK2 was drastically reduced, whereas the phosphorylation of receptors activated by NE was little affected. Conversely, the inhibition of PKC in DNGRK2 cells virtually abolished phosphorylation of α1A ARs exposed to NE, but had no clear effect on phosphorylation of receptors exposed to OXY. The involvement of PKC in the phosphorylation of α1A ARs by NE is well described (Vazquez-Prado and Garcia-Sainz, 1996; Vazquez-Prado et al., 2000; Price et al., 2002). In the present study, the phosphorylation of α1A ARs by NE was inhibited by bisindolylmaleimide I and Gö6976, but not by hispidine or rottlerin, suggesting that PKCα is the main isoform involved. However, it is important to mention that the selectivity of most protein kinase inhibitors has been recently questioned (Davies et al., 2000; Bain et al., 2007) and that caution should be taken to define the role of a particular kinase by the use of these compounds. There is indirect evidence for the involvement of GRK2 in the phosphorylation of α1A ARs in rat-1 fibroblasts, as the overexpression of GRK2, but not of GRK6, reduced maximal inositol phosphate accumulation in response to NE (Price et al., 2002). However, overexpression of GRK2 in HEK-293 cells had no effect on the internalization of α1A ARs activated by EPI (Stanasila et al., 2008). Interestingly, the modest and delayed internalization of α1A ARs after NE was inhibited by the protein kinase inhibitor staurosporine, whereas

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internalization of α1A ARs after OXY in HEK-293 cells expressing a dominant negative mutant of GRK2 was smaller and slower than in HEK-293 cells not expressing the dominant negative mutant. These results suggest that a specific protein kinase is recruited depending on the agonist activating the α1A AR, which might generate different patterns of receptor phosphorylation that in turn causes interactions with specific intracellular proteins leading to receptor desensitization/internalization. Similar agonist-dependent recruitment of kinases is well described for CCR7 and μ-opioid receptors; CCR7 receptors are phosphorylated by GRK3 and GRK6 upon exposure to the endogenous chemokine CCL19 and undergo internalization, whereas receptors exposed to the chemokine CCL21 are phosphorylated only by GRK6 and this is not followed by internalization (Zidar et al., 2009). Also, μ-opioid receptors activated by DAMGO are phosphorylated by GRK2 and undergo internalization, whereas PKC phosphorylates the receptors activated by morphine or fentanyl, which in turn are not internalized (Johnson et al., 2006; Hull et al., 2010). Such barcoding in receptor phosphorylation and the respective kinases involved have been recently elucidated for the μ-opioid receptor (Doll et al., 2011; Doll et al., 2012).

An important piece of information that remains to be clarified is the role of β-arrestins in the internalization of α1A ARs activated by OXY and NE, as biased agonism at β-arrestins is a well established concept (Reiter et al., 2012). Recent studies have shown that α1A ARs activated by EPI do not interact with β-arrestin 1 and 2 (Stanasila et al., 2008) and that the receptor activated by NE or labetalol recruit β-arrestin 2 only when heteromerizing with CXCR2 receptors (Mustafa et al., 2012). However, experiments employing confocal microscopy and co-immunoprecipitations of Flag-tagged α1A ARs and β-arrestin 2 failed to detect this recruitment

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in response to activation by NE and OXY (Pupo and Akinaga, unpublished observations), indicating that this will be a difficult task.

It has been recently shown that transgenic mice expressing a constitutively active mutant of the α1A, but not of the α1B AR, have an increased life span mainly as a result of cardiac and neuroprotection (Perez and Doze, 2011), broadening the potential therapeutic applications of drugs which selectively interfere with the signaling that results from activation of each of these α1 AR subtypes. To accomplish this, the understanding of the signaling profiles of α1 AR ligands in native and recombinant receptor systems is important. Functional selectivity for α1 AR ligands at α1A ARs expressed in CHO cells was recently described, and OXY was a full agonist in the extracellular acidification rate, a partial agonist for iCa^{2+} increase, but failed to stimulate cAMP production (Evans et al., 2011). The results of the present study indicate that manipulation of the functional selectivity/biased agonism of α1A AR ligands aiming for drugs that stabilize a receptor conformation less prone to desensitization would be advantageous for the maintenance of the vasoconstriction that underlies the therapeutic decongestant effects of these drugs.

In conclusion, our findings demonstrate the differential involvement of PKC and GRK2 in the phosphorylation of α1A ARs activated by NE and OXY, respectively, and the functional repercussion of this distinct recruitment on α1A AR desensitization and internalization. This is clear evidence of ligand-directed signaling for OXY at α1A AR towards the internalization pathway.

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Authorship Contributions

Participated in research design: Akinaga, Lima, Kiguti, García-Sáinz and Pupo

Conducted experiments: Akinaga, Lima, Hébeler-Barbosa, Alcántara-Hernández.

Performed data analysis: Akinaga, Lima, Alcántara-Hernández, García-Sáinz and Pupo.

Wrote or contributed to the writing of the manuscript: Akinaga, Kiguti, García-Sáinz and Pupo.

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Footnotes

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FIGURE LEGENDS

Fig. 1. Consecutive concentration-response curves for contraction of the rat tail artery to PE (A) and OXY (C) and vas deferens to NE (B) and OXY (D). The interval between the consecutive concentration-response curves was 45 min. Values are means±S.E.M. of four to six independent experiments performed with tissues from different rats.

Fig. 2. Concentration-response curves for the contraction of the rat tail artery (A) and vas deferens (B) in response to PE and NE, respectively, 45 min after treatment of the tissues with 10 μM OXY for 5 min or vehicle (matched control). Values are means±S.E.M. of four independent experiments performed with tissues from different rats.

Fig. 3. Concentration-response curves for iCa^{2+} increases induced by NE and OXY in HEK-293 cells stably expressing α1A ARs and loaded with the Ca^{2+} indicator Fluo-4 at 37 ^{o}C (A). In (B) are shown concentration-response curves for NE in cells that had been treated with 10 μM NE or 10 μM OXY for 5 min at 37 ^{o}C, washed twice and then loaded with Fluo-4. Real-time fluorescence were recorded every 2 s, with agonist additions after 10 s. Responses represent the difference between basal and peak fluorescence and are expressed as a percentage of the response to 10 μM NE. Values are means±S.E.M. of four independent experiments performed in duplicates.

Fig. 4. Localization of the α1A/eGFP monitored by confocal microscopy in HEK-293 cells in absence (time 0`) and presence of 10 μM NE (A), 10 μM OXY (B), or 30 nM prazosin plus 10 μM OXY (C) at 37^{o}C during 30 min. The images were taken at different times and are

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representative of three to four independent transfections. In (D) are shown the changes in intracellular fluorescence intensity in cells treated with NE or OXY and in cells treated with OXY in presence of prazosin. Data represent the mean ± S.E.M. of three to four independent transfections. Two-way ANOVA with Bonferroni post-test indicates differences from the effect of NE in the respective time (*$P < 0.05$, ** $P < 0.01$,*** $P < 0.001$) and differences from the effect of OXY plus 30 nM prazosin in the respective time (# $P < 0.05$, ## $P < 0.01$).

Fig. 5. [^{3}H]Prazosin binding in intact HEK-293 cells transiently transfected with α1A ARs to access cell surface receptors. In (A) are shown the total and non-specific binding of [^{3}H]prazosin displaced by 100 μM epinephrine or 100 μM phentolamine in intact HEK-293 cells to check for the labeling of cell surface receptors. (B) Cells were treated with either 10 μM NE or 10 μM OXY for various periods of time at 37^{o}C and the receptor populations at the cell surface were determined by [^{3}H]prazosin specific binding at 4 °C for 10 h and compared with the specific binding in cells not treated with the agonists (time 0`). (C) Concentration-response curves for NE and OXY for the loss of cell surface [^{3}H]prazosin specific binding after 30 min of stimulation with the agonists. In (D) are shown the saturation of specific binding of [^{3}H]prazosin (defined by 100 μM phentolamine) in intact HEK-293 cells previously treated with either 10 μM NE or 10 μM OXY for 5 min and washed twice at the end of the treatment. Data represent the mean ± S.E.M. of three to four independent experiments performed in duplicate from different transfections. In (A) Student's *t* test indicate that the non-specific binding of [^{3}H]prazosin in presence of epinephrine is not different from that in presence of phentolamine. Two-way ANOVA with Bonferroni post-test indicates differences from the effect of NE in the respective time (** $P < 0.01$,*** $P < 0.001$).

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Fig. 6. Time-courses of the effects of NE (A) and OXY (B) on phosphorylation of α1A/eGFP in HEK-293 cells. HEK-293 cells transiently transfected with α1A/eGFP were metabolically labeled with 150 μCi/ml [^{32}P]Pi and treated for the times indicated with 10 μM NE or 10 μM OXY. [^{32}P]-labeled α1A/eGFP were immunoprecipitated and visualized by SDS-PAGE and autoradiography. The phosphorylated receptors are expressed as percentage above basal values determined in nonstimulated cells (time 0`). Data represent the mean ± S.E.M. of three to four independent experiments performed with different transfections.

Fig. 7. Time-courses of the effects of NE and OXY on receptor phosphorylation and internalization in HEK-293 cells stably expressing a dominant-negative mutant of GRK-2 (DNGRK2 cells). In (A) and (C) are shown representative autoradiograms and the plot of phosphorylated α1A/eGFP receptors immunoprecipitated from DNGRK2 cells treated for the times indicated with 10 μM NE and 10 μM OXY, respectively. The specific binding of [^{3}H]prazosin to the surface of DNGRK2 cells transiently expressing α1A ARs treated for the times indicated with 10 μM NE and 10 μM OXY are shown in B and D, respectively. For comparison, the NE and OXY curves for receptor phosphorylation and internalization in HEK-293 cells treated with NE or OXY are shown as dashed lines in the respective graphs. Data represent the mean ± S.E.M. of three to four independent experiments performed with different transfections.

Fig. 8. Effects of the pre-treatment of HEK-293 and DNGRK2 cells with 10 μM OXY for 5 min in the iCa^{2+} increases induced by 10 μM NE. Cells transiently expressing α1A ARs were treated

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or not with 10 μM OXY for 5 min, washed, loaded with the fluorescent calcium indicator FURA-2 AM for 1h at 37^{o}C and then challenged with 10 μM NE. iCa^{2+} increases are expressed as percentage of the peak response to 10 μM thapsigargin in the respective cell line not pre-treated with OXY. Data represent the mean ± S.E.M. of three independent experiments performed with different transfections. $P < 0.05$ in unpaired Student's *t* test compared with the respective iCa^{2+} response in cells not pre-treated with OXY.

Figure 9. Effects of PKC inhibitors on receptor phosphorylation and internalization. In (A) and (B) are shown representative autoradiograms and the plot of phosphorylated α1A/eGFP immunoprecipatetd from DNGRK2 cells treated with 10 μM NE and 10 μM OXY for 15 min, respectively, in the absence (basal) and presence of the PKC inhibitors bisindolylmaleimide I (1 μM, non-selective); Gö6976 (0.1 μM, PKCα-selective); hispidine (1 μM, PKCβ-selective); and rottlerin (1 μM, PKCδ-selective) incubated 30 min before stimulation with agonists. The effects of the non-selective PKC inhibitor staurosporine (100 nM) in the specific binding of [^{3}H]prazosin to the surface of HEK-293 cells transiently expressing α1A ARs treated for the times indicated with 10 μM NE and 10 μM OXY are shown in B and D, respectively. For comparison, the NE and OXY curves for receptor phosphorylation and internalization in HEK-293 cells treated with NE or OXY in the absence of stauroporine are shown as dashed lines in the respective graphs. Data represent the mean ± S.E.M. of three to four independent experiments performed with different transfections. In (A) and (B) one-way ANOVA followed by Dunnett's test indicates differences from the effect of NE alone (* $P < 0.05$,** $P < 0.01$).

Table 1. Maximal contractions (g of tension) and pEC_{50} values of consecutive concentration-response curves for NE, PE and OXY in the rat tail artery and vas deferens.

			Consecutive concentration-response curve		
			First	Second	Third
Tail Artery	PE (n = 4)	Emax (g)	1.87 ± 0.05	2.19 ± 0.05**	2.31 ± 0.06***
		pEC_{50}	6.83 ± 0.08	6.65 ± 0.06	6.67 ± 0.07
	OXY (n = 5)	Emax (g)	1.54 ± 0.03	1.05 ± 0.06***	0.74 ± 0.04***
		pEC_{50}	7.15 ± 0.06	5.68 ± 0.13***	5.44 ± 0.11***
Vas Deferens	NE (n = 6)	Emax (g)	1.68 ± 0.04	1.81 ± 0.05	1.83 ± 0.08
		pEC_{50}	6.73 ± 0.07	7.00 ± 0.07*	7.00 ± 0.10*
	OXY (n = 5)	Emax (g)	0.62 ± 0.05	*no effect*	*no effect*
		pEC_{50}	6.70 ± 0.21	n.d.	n.d.

One-way ANOVA followed by Dunnett's test indicates differences from the respective value determined in the first concentration-response curve (* $P < 0.05$;** $P < 0.01$; ***$P < 0.001$). n.d., not determined.

Table 2. Maximal contractions (g of tension) and *p*EC50 values of concentration-response curves for PE and NE 45 min after treatment of tissues with 10 µM OXY for 5 min or with vehicle.

		Treatment	
		Vehicle (matched control)	10 µM OXY/5 min.
PE in tail artery (n = 4)	Emax (g)	1.89 ± 0.07	1.92 ± 0.04
	pEC_{50}	7.00 ± 0.10	5.72 ± 0.06***
NE in vas deferens (n = 4)	Emax (g)	1.86 ± 0.07	1.70 ± 0.05
	pEC_{50}	6.80 ± 0.09	5.54 ± 0.07***

*** Different from the respective value found in tissues treated with vehicle (matched control) ($P < 0.001$, unpaired Student's t test). n = number of experiments in tissues from different rats.

Table 3. Parameters of agonism (Emax and pEC_{50}) for NE and OXY in the increase of iCa^{2+} in HEK-293 cells expressing α1A ARs.

	Emax (% of 10 μM NE)	pEC_{50}
NE (*n* = 6)	102 ± 6	8.01 ± 0.06
OXY (*n* = 6)	52 ± 3	8.23 ± 0.07

n = number of experiments performed in duplicate.

Table 4. Parameters of agonism (Emax and pEC_{50}) for NE in the increase of iCa^{2+} in HEK-293 cells expressing α1A ARs treated with vehicle, 10 μM NE/5 min., or 10 μM OXY/5 min.

		Treatment		
		Vehicle	10 μM NE/5 min	10 μM OXY/5 min
NE (n = 6)	Emax (% of 10 μM NE)	104 ± 4	114 ± 6	35 ± 7***
	pEC_{50}	8.09 ± 0.11	8.11 ± 0.16	6.44 ± 0.17***

***One-way ANOVA followed by Dunnett's test indicates differences from the respective value determined in cells treated with vehicle ($P < 0.001$).

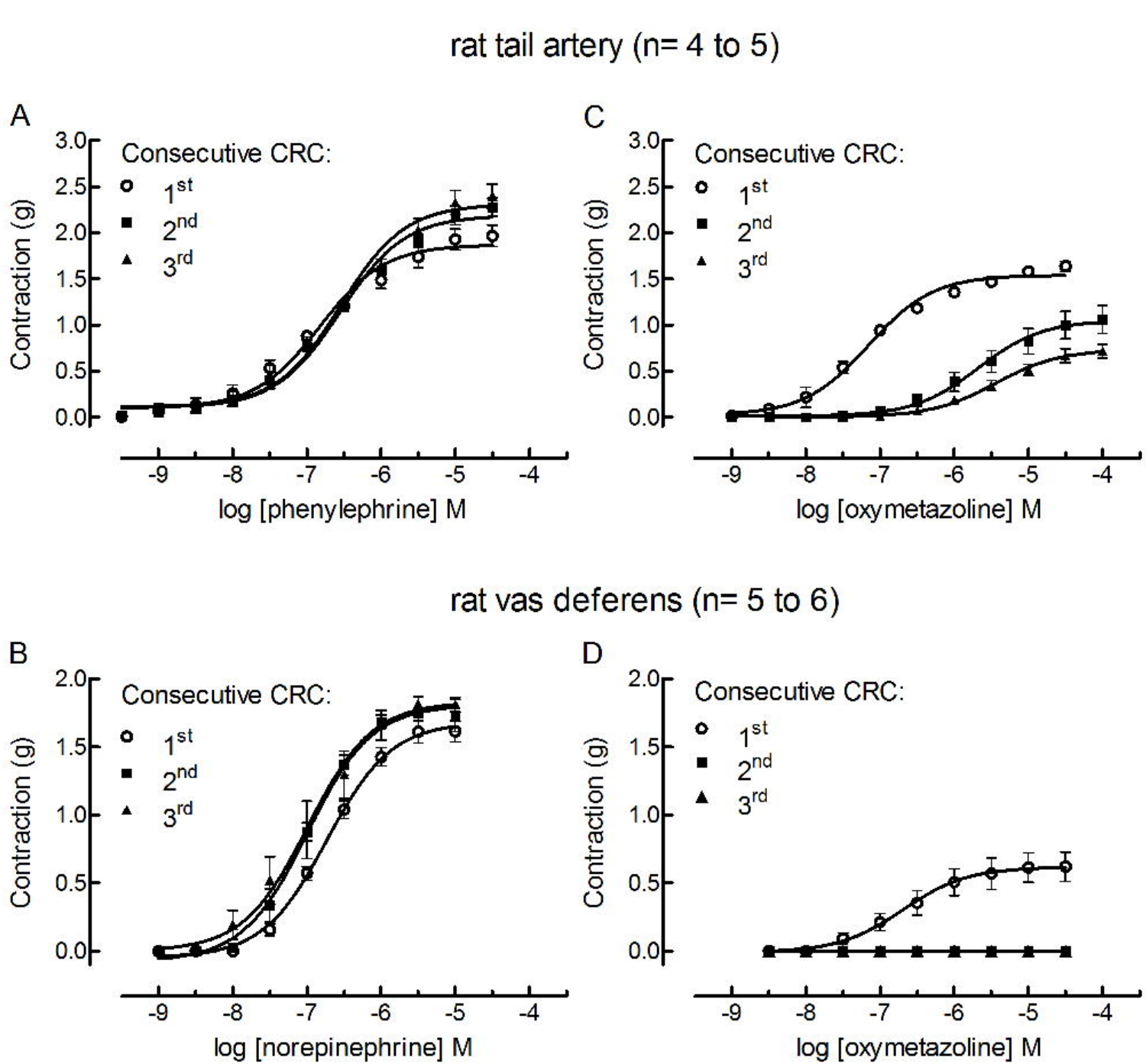
rat tail artery (n= 4 to 5)
A
Consecutive CRC:
1st
2nd
3rd
Contraction (g)
log [phenylephrine] M
C
Consecutive CRC:
1st
2nd
3rd
Contraction (g)
log [oxymetazoline] M
rat vas deferens (n= 5 to 6)
B
Consecutive CRC:
1st
2nd
3rd
Contraction (g)
log [norepinephrine] M
D
Consecutive CRC:
1st
2nd
3rd
Contraction (g)
log [oxymetazoline] M

figure 1

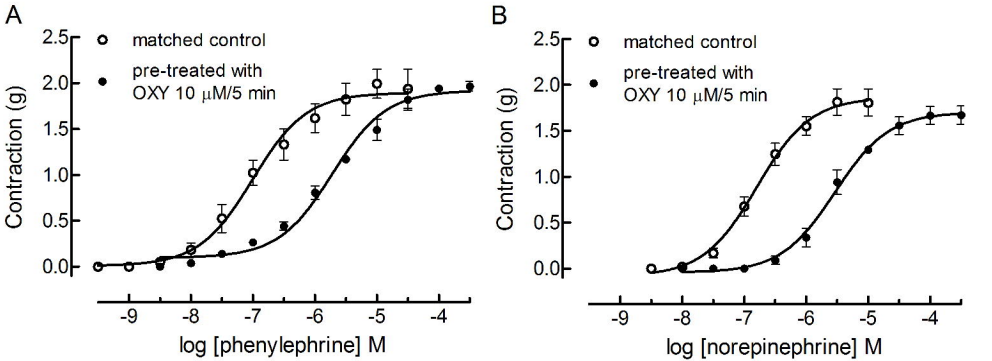
A
matched control
pre-treated with
OXY 10 μM/5 min
Contraction (g)
2.5
2.0
1.5
1.0
0.5
0.0
-9
-8
-7
-6
-5
-4
log [phenylephrine] M
B
matched control
pre-treated with
OXY 10 μM/5 min
Contraction (g)
2.5
2.0
1.5
1.0
0.5
0.0
-9
-8
-7
-6
-5
-4
log [norepinephrine] M

figure 2

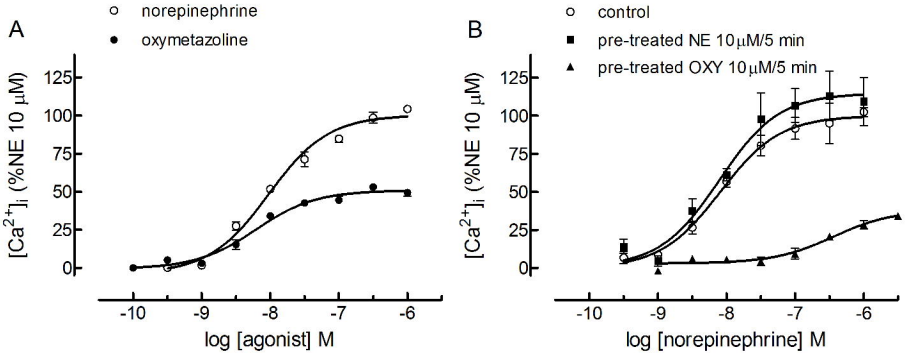
A
norepinephrine
oxymetazoline
[Ca2+]i (%NE 10 μM)
125
100
75
50
25
0
-10
-9
-8
-7
-6
log [agonist] M
B
control
pre-treated NE 10μM/5 min
pre-treated OXY 10μM/5 min
[Ca2+]i (%NE 10 μM)
125
100
75
50
25
0
-10
-9
-8
-7
-6
log [norepinephrine] M

figure 3

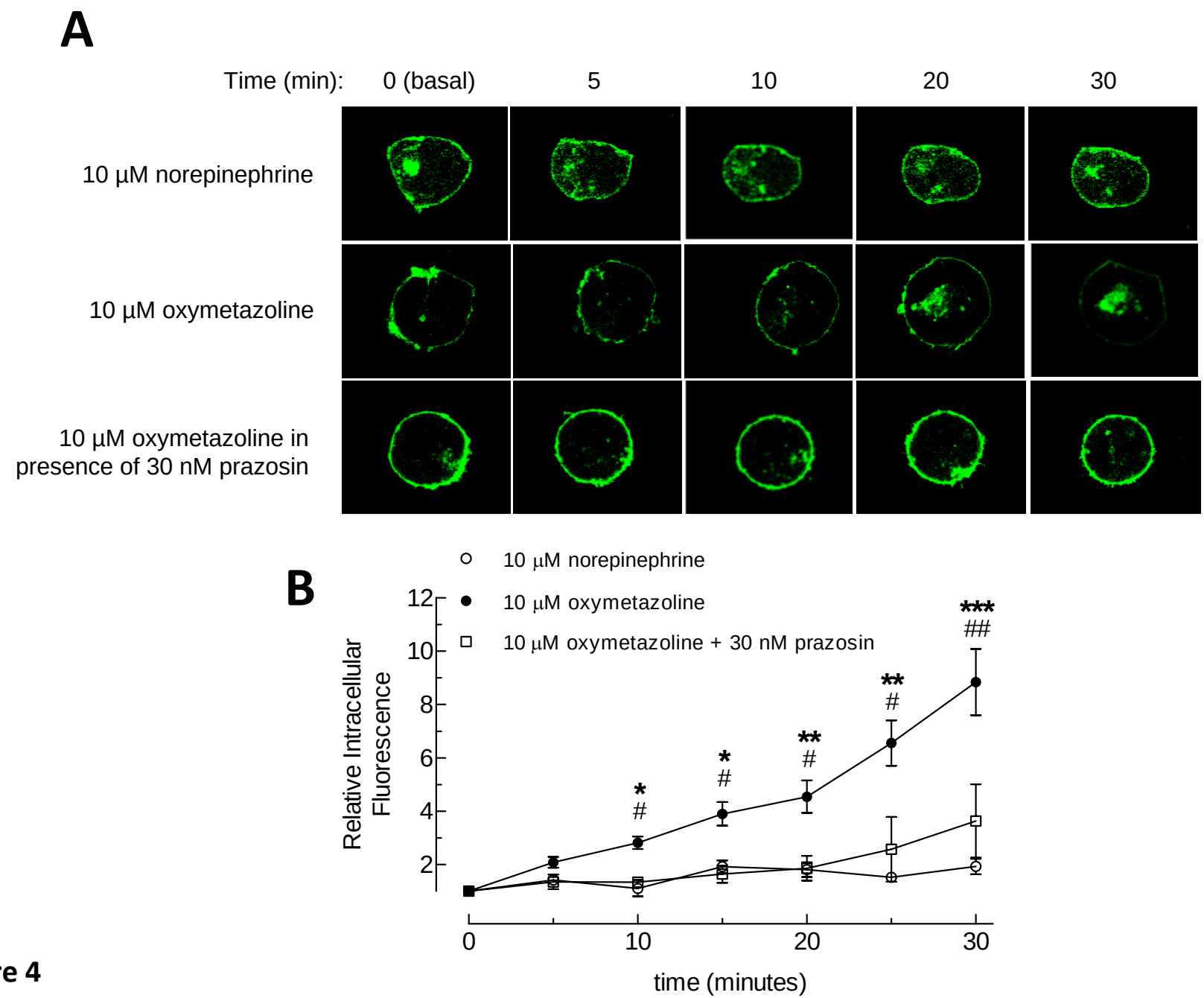

Figure 4

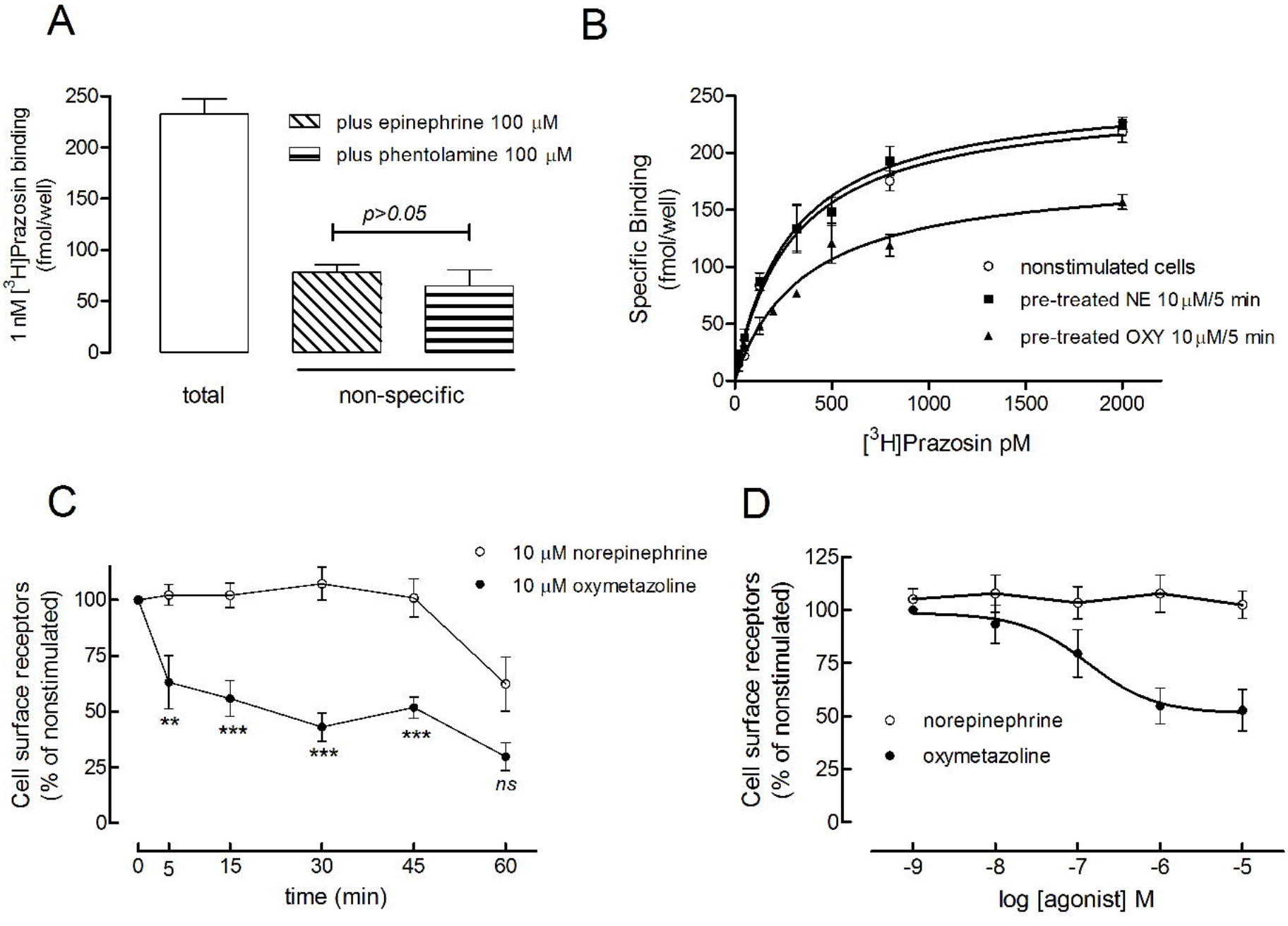
A
1 nM [3H]Prazosin binding (fmol/well)
250
200
150
100
50
0
plus epinephrine 100 μM
plus phentolamine 100 μM
p>0.05
total
non-specific
B
Specific Binding (fmol/well)
250
200
150
100
50
0
nonstimulated cells
pre-treated NE 10 μM/5 min
pre-treated OXY 10 μM/5 min
0
500
1000
1500
2000
[3H]Prazosin pM
C
Cell surface receptors (% of nonstimulated)
100
75
50
25
0
10 μM norepinephrine
10 μM oxymetazoline
**
***
***
***
ns
0
5
15
30
45
60
time (min)
D
Cell surface receptors (% of nonstimulated)
125
100
75
50
25
0
norepinephrine
oxymetazoline
-9
-8
-7
-6
-5
log [agonist] M

figure 5

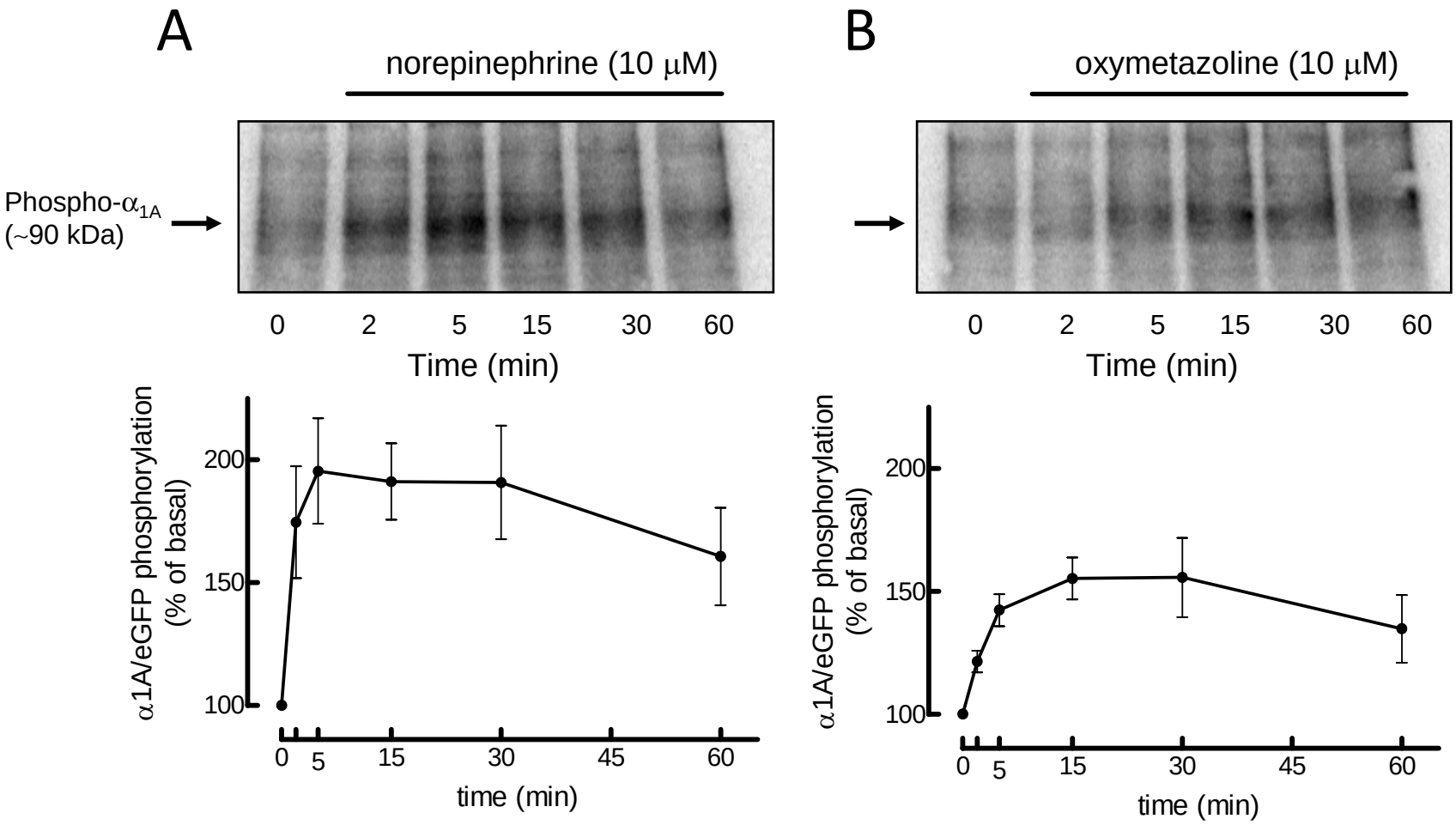

Figure 6

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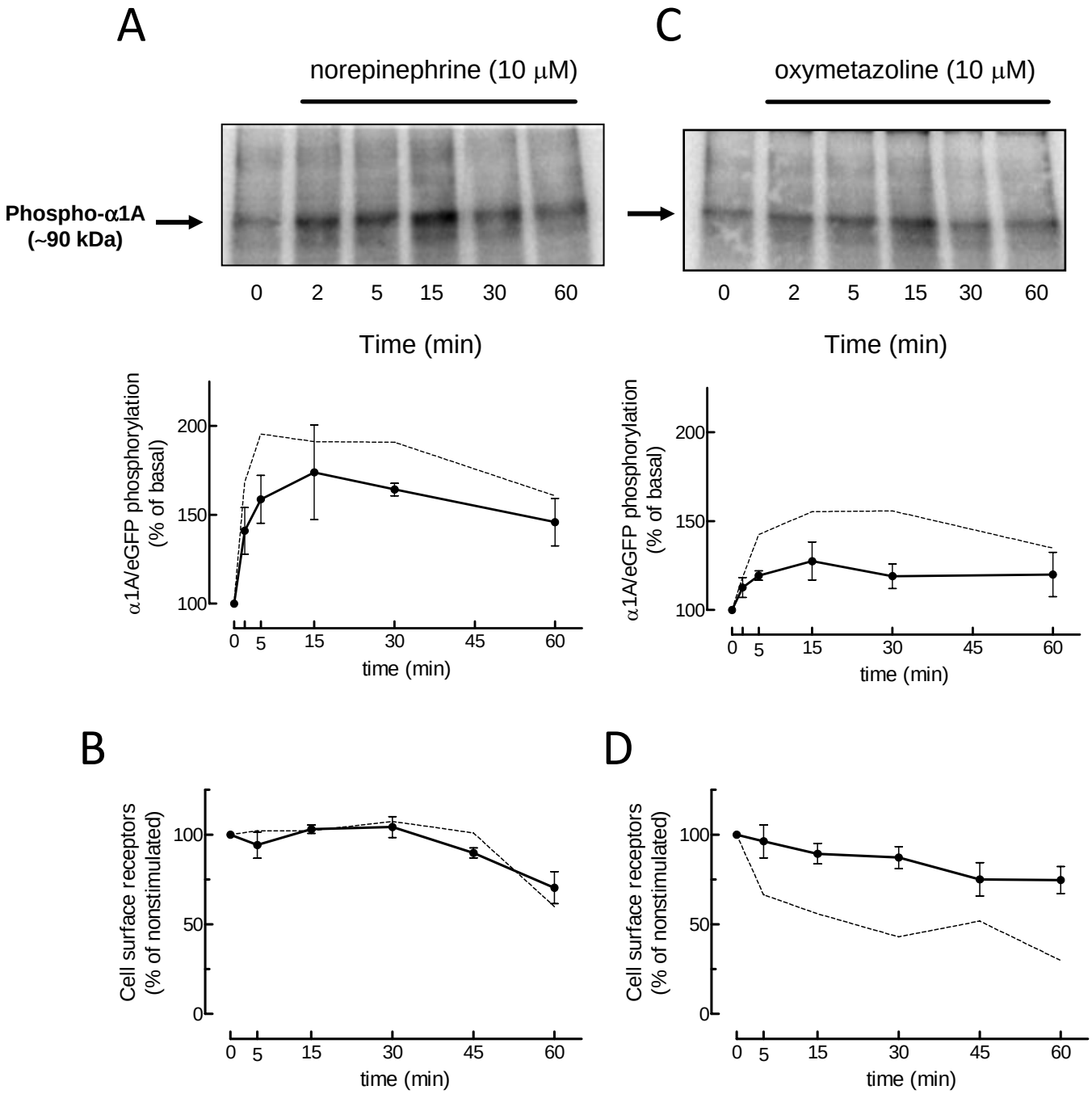

figure 7

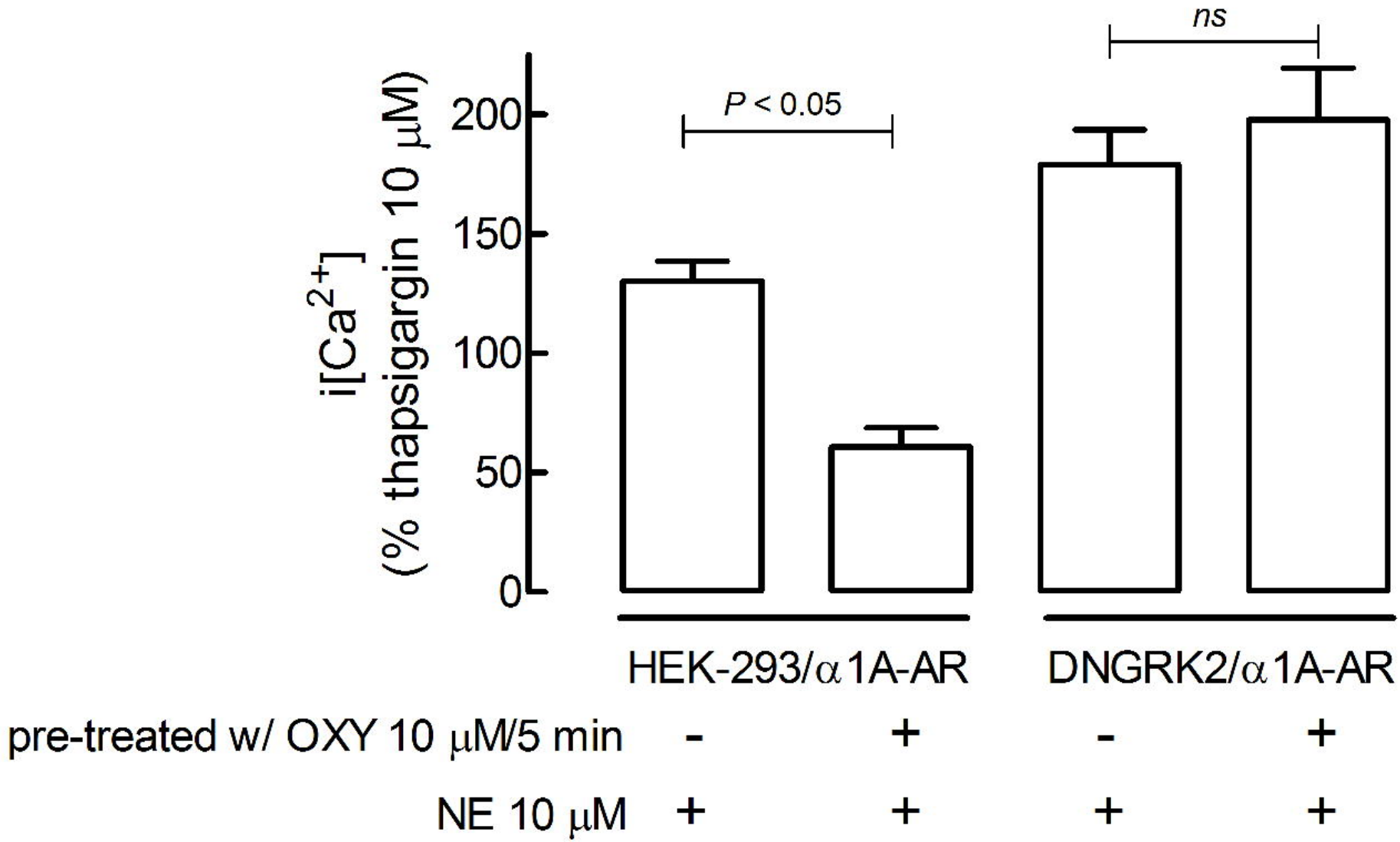
ns
P < 0.05
i[Ca2+]
(% thapsigargin 10 μM)
200
150
100
50
0
HEK-293/α1A-AR
DNGRK2/α1A-AR
pre-treated w/ OXY 10 μM/5 min - + - +
NE 10 μM + + + +

figure 8

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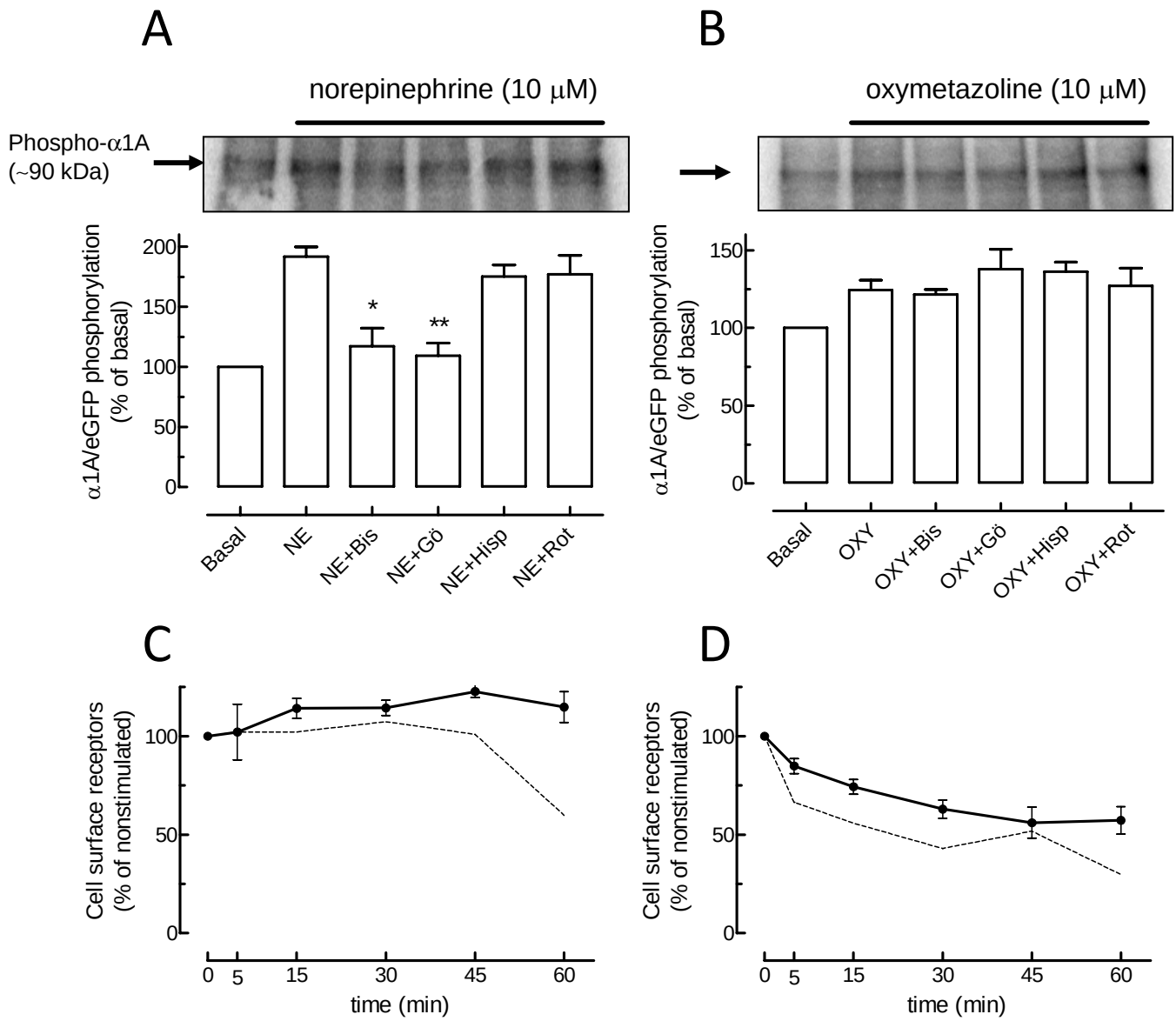

figure 9