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Interactions between nitrogen oxide-containing compounds and peripheral benzodiazepine receptors

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Nitrogen oxide-containing compounds displaced the peripheral benzodiazepine ligand [^{3}H]Ro5-4864 from guinea pig membrane preparations. Sodium nitroprusside (SNP) was the most potent ($IC_{50} = 5.61 \pm 1.72 \times 10^{-5}$ M). Moreover, its ability to bind to these receptors showed marked tissue variability (heart > kidney ≫ cerebral cortex). When tested on rat atrium, SNP by itself had no effect on basal inotropy or the increase in inotropy induced by (−)-S-BAY K 8644. In contrast, Ro5-4864 potentiated the marked increase in inotropy induced by (−)-S-Bay K 8644, and SNP completely abolished the potentiation of inotropy observed with Ro5-4864. Since peripheral benzodiazepine receptors are associated with calcium mobilization in the heart, these findings may indicate that some of the clinical effects of nitric oxide-generating drugs could be mediated by these receptors.

Benzodiazepine receptor, peripheral; Sodium nitroprusside; (Rat atrium; Guinea pig heart)

1. INTRODUCTION

Peripheral benzodiazepine receptors (PBR) exhibiting high affinity for [^{3}H]Ro5-4864 have been demonstrated in a wide variety of mammalian tissues [1–3]. The pharmacological profile and distribution of these receptors are distinct from those of central benzodiazepine receptors which mediate the effects of benzodiazepine agonists, e.g. diazepam, clonazepam [4]. Several lines of evidence indicate that PBR ligands affect calcium-dependent events in the guinea pig and rat heart [5–7,8] and mediate ventricular arrhythmias during myocardial ischemia in the dog [9]. Moreover, studies performed using cell cultures and synaptosomal preparations showed that PBR ligands may be associated with and modulate Ca^{2+} uptake via voltage-dependent calcium channels (VDCC) [10–12].

A large group of experimental and clinically useful vasodilators are thought to act by generating nitric oxide (NO) which in turn increases cyclic GMP (cGMP) production resulting in a modulation of intracellular calcium levels [13–15]. Recently, Karaki et al. [15] demonstrated that sodium nitroprusside (SNP), a potent vascular smooth muscle relaxing drug, inhibits the norepinephrine-induced $^{45}Ca^{2+}$ uptake in rat aortic strips. Most of the investigations studying the mechanism of action of nitrovasodilators have focused on the activation of guanylate cyclase [16], leaving other pathways poorly explored.

The present study describes an interaction between SNP and other nitrovasodilators with PBR in the guinea pig heart and the spontaneously beating rat atrium. We now report that these agents displace PBR ligands from guinea pig heart membrane preparations and that SNP inhibits the modulation of VDCC by Ro5-4864 in the rat atrium.

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2. MATERIALS AND METHODS

2.1. *Tissue preparation and radioligand binding*

Male Hartley guinea pigs (250–300 g, Charles River, Wilmington, MA) were killed by decapitation and the heart kidney and brain were rapidly excised and placed in ice-cold 10 mM Hepes/25% sucrose, pH 7.4 buffer. Following the removal of surrounding tissues from the heart and the dissection of the cerebral cortex, membranes were prepared as described by Sulakhe et al. [17] and frozen at −70°C until use. Thawed aliquots were resuspended in 250, 400 and 100 vols of 50 mM Tris-HCl, pH 7.4 buffer for heart, kidney and cerebral cortex, respectively, and 0.25 ml of membrane suspension added to each assay tube.

[^{3}H]Ro5-4864 (specific activity 79.7 Ci/mmol, New England Nuclear, Boston, MA) binding experiments were carried out in a total assay volume of 1 ml. In most studies, membrane preparations and drugs were incubated first at 30°C for 10 min, next transferred to an ice bath, radioligand added and the incubation continued at 0–4°C for 1 h. Binding was terminated by rapid filtration and two 5-ml washes with ice-cold buffer over Whatman GF/B glass fiber filters

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using a Brandel M-24R cell harvester (Brandel, Gaithersburg, MD). Non-specific binding was determined in the presence of 5×10^{-6} M PK 11195. Radioactivity was measured using a Packard scintillation spectrophotometer, model 2000 CA/LL. IC_{50} values and Hill coefficients were determined by the GRAFCAL IBM-PC computer program (Kaplan, D., Israel Institute for Biological Research, Israel).

2.2. *Intact tissue studies*

Male Sprague-Dawley rats weighing 175–200 g (Charles River, St. Constant, Quebec) were killed by decapitation. For the preparation of the atrium, hearts were rapidly removed and placed into a physiological salt solution (PSS), which was equilibrated with 95% O_2/5% CO_2, pH 7.2 at 37°C. The PSS had the following composition (in mM): 137 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 0.49 $MgCl_2$, 0.35 NaH_2PO_4, 5.6 dextrose and 11.9 $NaHCO_3$. The right and left atrium were dissected free from surrounding tissues and mounted with 0.5 g tension to a high compliance strain gauge transducer (Grass Model FT03C, Grass Instruments) in a 20 ml organ bath containing PSS at 37°C equilibrated with 95% O_2/5% CO_2. Atrial tissues were equilibrated for 60 min at 37°C with one change of bath medium every 15 min before any drug addition(s).

2.3. *Materials*

Specific drugs were obtained as follows: Ro5-4864, Hoffman-LaRoche, Nutley, NJ; PK 11195, Pharmuka Laboratories, Genevilliers, France; (−)-S-BAY K 8644, Miles Institute for Preclinical Pharmacology, West Haven, CT. All other chemicals were obtained from standard commercial sources.

3. RESULTS

3.1. *Radioligand binding studies*

SNP had only a marginal effect on the binding of [^{3}H]Ro5-4864 to guinea pig heart membranes incubated at 0–4°C for 1 h (less than 20% inhibition at a concentration of 10^{-3} M). Preincubation of SNP with heart tissue for 10 min at 30°C followed by the addition of radioligand and continuation of the reaction for 1 h at 0–4°C resulted in a concentration-dependent inhibition of [^{3}H]Ro5-4864 binding. The IC_{50} value for SNP in this preparation was $5.6 \pm 1.7 \times 10^{-5}$ M and the Hill coefficient 0.55 ± 0.06 ($n = 8$). The potency of sodium nitroprusside was the highest in the heart and was one or two orders of magnitude lower in kidney and cerebral cortex membranes, respectively (table 1). Other nitric oxide-generating agents, such as hydroxylamine (IC_{50} value of $5.12 \pm 1.86 \times 10^{-4}$ M), displaced [^{3}H]Ro5-4864 from its binding sites on cardiac membranes but demonstrated a significantly lower potency compared to SNP (table 2). The clinically useful drug isosorbide dinitrate (ISDN) showed a lower activity with an IC_{50} of $8.7 \pm 1.5 \times 10^{-4}$ M, which was somewhat higher than that of 4-nitroquinoline-*N*-oxide. Potassium ferricyanide, a substance known to inhibit guanylate cyclase activated by SNP, was about 200-fold less active than SNP in displacing [^{3}H]Ro5-4864 from guinea pig heart membranes. Two other nitrogen oxide-containing chemicals, $NaNO_2$ and $NaNO_3$, were only marginally active (table 2), while NaCN was completely inactive at a concentration of 10^{-2} M.

The Hill coefficients of all the substances tested were close to 0.5 and significantly different from 1.0 (tables 1,2; fig.1), suggesting multiple-site interaction(s) or negative cooperativity. In contrast, PK 11195, a potent antagonist at PBR, displaced [^{3}H]Ro5-4864 from heart membrane preparations with an IC_{50} value of $2.43 \pm 0.27 \times 10^{-9}$ M and a Hill coefficient of 1.06 ± 0.06 ($n = 5$). Additionally, SNP and hydroxylamine displaced [^{3}H]PK 11195 from guinea pig heart membrane

Table 1

Inhibition of [^{3}H]Ro5-4864 binding to PBR by SNP

	Heart	Kidney	Cerebral cortex
IC_{50} (M)	$5.61 \pm 1.72 \times 10^{-5}$	$3.0 \pm 0.2 \times 10^{-4}$	$3.7 \pm 0.3 \times 10^{-3}$
Hill coefficient	0.55 ± 0.06	0.67 ± 0.05	0.44 ± 0.14

Data are presented as the mean ± SE of 3–8 experiments performed as described in section 2. The [^{3}H]Ro5-4864 final concentration was 1 nM in all tissues examined

Table 2

Effects of various compounds on the binding of [^{3}H]Ro5-4864 to guinea pig heart membranes

Agent	IC_{50} (M)	Hill coefficient
SNP	$5.61 \pm 1.72 \times 10^{-5}$	0.55 ± 0.06
Hydroxylamine	$5.12 \pm 1.68 \times 10^{-4}$	0.56 ± 0.05
ISDN[a]	$8.7 \pm 1.5 \times 10^{-4}$	0.65 ± 0.06
4-NQNO[b]	$2.0 \pm 0.2 \times 10^{-3}$	0.68 ± 0.08
$K_3Fe(CN)_6$	(52.9)[c]	
$NaNO_2$	(43.2)[c]	
$NaNO_3$	(25.4)[c]	

[a] Isosorbide dinitrate

[b] 4-Nitroquinoline-*N*-oxide

[c] Percent inhibition at 10^{-2} M

Data are presented as mean ± SE of 4–8 separate determinations. Binding assays were carried out as described in section 2 with a final ligand concentration of 1 nM

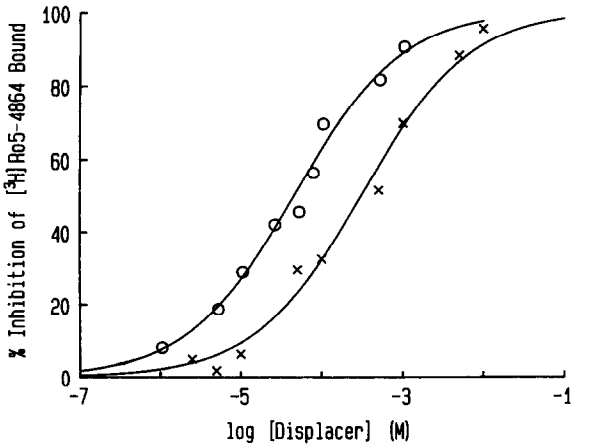

Fig.1. Inhibition of the binding of [^{3}H]Ro5-4864 to membranes of guinea pig heart by sodium nitroprusside (○) and hydroxylamine (×). Heart membranes were prepared as described in section 2.1. Shown are data from one experiment repeated 4–8 times with identical results. The final ligand concentration was 1 nM.

Table 3

Modulation of (−)-S-BAY K 8644-induced inotropy in the spontaneously beating rat atrium

Agent(s)	Chronotropy (beats/min)	Inotropy (% of basal response)
Control	240 ± 7	
(−)-S-BAY K 8644	300 ± 18[a]	135 ± 32[a]
+ SNP	298 ± 14[a]	128 ± 13[a]
+ Ro5-4864	291 ± 8[a]	237 ± 33[ab]
+ Ro5-4864 + SNP	270 ± 12[a]	141 ± 22[a]

[a] Significantly different from control, $P < 0.05$

[b] Significantly different from (−)-S-BAY K 8644, $P < 0.05$, Student's t-test

Results are presented as the mean ± SE of 3-s measurements (n = 7–10). SNP (100 μM) and Ro5-4864 (10 μM) were incubated for 10 min before the incubation of (−)-S-BAY K 8644 (1 μM) (5 min). Inotropy is presented as the percent increase compared to control force of contraction

preparations with IC_{50} values similar to those observed using [^{3}H]Ro5-4864 (results not shown).

In another set of experiments, it was observed that the binding of tritiated *N*-methylscopolamine ([^{3}H]NMS), a muscarinic antagonist, was not affected by concentrations of SNP above 10^{-3} M (results not shown).

3.2. *Intact tissue studies*

In order to further examine the interaction(s) between SNP and PBR in intact tissues, a spontaneously beating rat atrium was used. The atrium was incubated in an organ bath in the presence of the calcium channel activator (−)-S-BAY K 8644. A concentration of 1 μM (−)-S-BAY K 8644 induced a 135% increase in the force of contraction (i.e. inotropy) (table 3). SNP and Ro5-4864 (100 and 10 μM, respectively) had no effect on basal chronotropy or inotropy. A high concentration of SNP (100 μM) did not alter the chronotropy or inotropy induced by (−)-S-BAY K 8644, whereas Ro5-4864 (10 μM) significantly potentiated the inotropic response to (−)-S-BAY K 8644 by 2-fold. However, SNP completely inhibited the inotropic action of Ro5-4864 when added together (table 3). None of the drugs used had any effect on the positive chronotropic effect elicited by (−)-S-BAY K 8644.

4. DISCUSSION

SNP is a potent vasodilator acting as a vascular smooth muscle relaxing drug [12,15,16,21]. This drug has been shown to potently increase cGMP levels in various cell systems [16]. In addition, other agents sharing a similar pharmacological profile, e.g. ISDN, hydroxylamine, have also been shown to elicit cGMP accumulation [18]. Recent studies have demonstrated that the activity of nitrovasodilators [16] on muscle cells is associated with a decrease in the level of intracellular calcium [15], apparently as a consequence of cGMP accumulation [13]. The processes involved in this pathway, culminating in the relaxation of the smooth muscle, may be a direct (or indirect) result of the interaction between nitrogen oxide(s) and sulfhydryl groups [13,14,16].

The guinea pig heart was selected for the study since it has been shown to possess a high density of PBR [7] that are functionally linked to voltage-dependent calcium channels [5–7]. Data presented in table 1, demonstrate that SNP exhibited a marked tissue specificity as evidenced by the differential binding affinities to membranes prepared from heart, kidney and cerebral cortex. The ability of SNP to displace [^{3}H]Ro5-4864 from PBR in heart membrane preparations was 19- and 151-fold higher than in renal and cerebral cortex membranes, respectively. Furthermore, the IC_{50} values in heart of SNP and other substances investigated in this study are similar to the concentrations and doses required to elicit cGMP formation [19,20] and reduce blood pressure, respectively [21].

The effects of SNP on peripheral benzodiazepine receptors in guinea pig heart membranes appeared to be specific, as concentrations of this drug higher than its IC_{50} for [^{3}H]Ro5-4864 binding did not affect the binding of [^{3}H]NMS. An additional aspect of the specificity of SNP [$Na_2Fe(NO)(CN)_6$] at PBR is evident from the observations regarding the lack or marginal activity of compounds such as NaCN, $NaNO_3$ and $Na_3Fe(CN)_6$ (table 2). Although these substances share some of the same chemical functions, they differ dramatically from SNP in their potency to displace [^{3}H]Ro5-4864 from cardiac tissue.

A functional relationship between PBR and SNP is indicated by observations reported in table 3. PBR ligands have been shown to interact with voltage-dependent calcium channels in their activated state both in vitro [6,7,22] and in vivo [8,9,22]. In the spontaneously beating rat atrium, a tissue which responds to Ro5-4864 and (−)-S-BAY K 8644 in an identical manner to guinea pig atrium (Bolger, G.T., unpublished results), SNP did not alter either basal or (−)-S-BAY K 8644-activated states. However, the potentiation of the positive inotropic effect of (−)-S-BAY K 8644 by Ro5-4864 was completely abolished by SNP (table 3).

It should be noted that the effects of SNP and all other compounds examined with regard to [^{3}H]Ro5-4864 binding were detected following preincubation at 30°C. Under such conditions nitric oxide or other reactive nitrogen oxide intermediates may be released and react with thiol groups on membranous protein(s), forming labile *S*-nitrosothiols [13,14]. The generation of such compounds can elicit alterations in PBR binding characteristics. Benavides et al. [23] and Lueddens et al. [24] reported on the effects of the

histidine-modifying reagent diethylpyrocarboxylate and of AHN 086, a specific alkylating agent of PBR, respectively. In these studies the interaction of a reactive chemical with rat kidney PBR produced dramatic alterations in the binding of the PBR ligand PK 11195. Our results demonstrate that thiol groups localized on or in the vicinity of the PBR may affect significantly the binding of [^{3}H]Ro5-4864, as do histidine moieties.

Thus, several lines of evidence suggest that SNP, a potent vasodilator, as well as other nitrovasodilators, interact with PBR on cardiac tissue. Since PBR are associated with calcium mobilization, our findings may indicate that the clinical effects (vasodilatation) of nitrogen oxide-containing drugs could be mediated by an interaction with PBR and an inhibition of calcium mobilization.

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