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Identification and characterization of platelet α_2-adrenoceptors and imidazoline receptors in rats, rabbits, cats, dogs, cattle, and horses

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ABSTRACT

This study aimed to pharmacologically identify and characterize α_2-adrenoceptors and imidazoline (I) receptors (I_1- and I_2-subtype) on canine, feline, bovine, equine, murine, and leporine platelet membranes. Saturation binding studies with both ^{3}H-yohimbine and ^{3}H-clonidine showed that α_2-adrenoceptors were expressed on canine, leporine, feline, and murine platelets but not on bovine and equine platelets. In competition studies, the rank order of affinity of 6 compounds for canine platelet α_2-adrenoceptors was similar to that of potency at α_{2A}-subtype reported in human platelets. Saturation binding studies in the presence of norepinephrine showed that canine, feline, bovine, and equine platelets had I_1-receptors defined by ^{3}H-clonidine binding, but neither murine nor leporine platelets had I_1-receptors; whereas, platelets of all species had I_2-receptors defined by ^{3}H-idazoxan binding. In competition studies, more potent compounds displayed biphasic competition curves with ^{3}H-clonidine. The rank orders of affinity of I_1 compounds for high-affinity components of I_1-receptors of canine, feline, bovine, and equine platelets and I_2-receptors of all species platelets were similar to those of compounds for high-affinity components reported in human I_1- and I_2-receptors, respectively. Guanine nucleotides inhibited the high-affinity component of naphazoline binding to canine I_1-receptors, but not to I_2-receptors. Furthermore, guanine nucleotides dose-dependently inhibited ^{3}H-clonidine binding to I_1-receptors; whereas, they did not interfere with ^{3}H-idazoxan binding to I_2-receptors, supporting the notion that I_1-receptors may belong to a G protein-coupled receptor superfamily in canine platelets. Interspecific variations of platelet α_2-adrenoceptor and imidazoline receptor expressions may explain different platelet responses to catecholamines and imidazoline α-adrenergic agents.

1. Introduction

Mammalian platelets vary widely in their responses to catecholamines and other α-adrenoceptor agonists (Kerry et al., 1984; Yu and Latour, 1977). It has been demonstrated in humans that epinephrine-induced platelet aggregation is mediated by α_2-adrenoceptors (Grant and Scrutton, 1979; Hsu et al., 1979; Lasch and Jakobs, 1979; Petrusewicz and Kaliszan, 1985). The α_2-adrenoceptors on human platelets have been found by radioligand binding studies (Lanza and Cazenave, 1985; Motulsky et al., 1980; Motulsky and Insel, 1982; Mustonen et al., 2000; Piletz and Sletten, 1993; Shattil et al., 1981). However, some imidazolines including clonidine exert complicated effects on platelets (Hsu et al., 1979; Lenox et al., 1985; Shattil et al., 1981; Stump and Macfarlane, 1983). From the effects of various imidazoline α-adrenoceptor agents on platelet aggregation, it was suggested that imidazoline compounds may interact with non-α_2-adrenoceptor binding sites on platelets (Ahn et al., 1990; Clare et al., 1984; Petrusewicz and Kaliszan, 1991; Pinthong et al., 2004). Two imidazoline compounds also inhibit platelet adenylate cyclase through non-α_2-adrenoceptor mechanisms (Ferry et al., 1986). Some studies have demonstrated nonadrenergic, imidazoline (I)-preferring binding sites (I_1- and I_2-receptors) in human platelets (Michel et al., 1990; Piletz et al., 1991; Piletz and Sletten, 1993; Zonnenschein et al., 1990).

In dogs and cats, epinephrine alone does not induce platelet aggregation but can potentiate platelet aggregation stimulated by other platelet agonists (Hart and Nolte, 1991; Hikasa et al., 1999; Kitzen et al., 1991; Meyers et al., 1983; Tschopp, 1970). Although epinephrine-potentiated platelet aggregation may be mediated by α_2-adrenoceptors on canine platelets, most imidazoline α_2-adrenergic agents inhibit epinephrine-potentiated platelet aggregation in dogs (Hikasa et al., 1998, 1999). It has been suggested that nonadrenergic imidazoline receptors similar to receptors in human platelets may also exist on canine platelets (Hikasa et al., 1999). Alternatively, bovine and equine platelets are nonresponsive to stimulation by epinephrine

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or norepinephrine (Bondy and Gentry, 1989; Meyers et al., 1979; Soloviev et al., 1999; Yokota et al., 2013b). In rats and rabbits, epinephrine alone does not induce platelet aggregation but potentiates platelet aggregation stimulated by other platelet agonists such as ADP and collagen (Jackson et al., 2003; Mori et al., 2010; Takano et al., 2001; Varga-Szabo et al., 2008; Yokota et al., 2013b; Yun-Chol et al., 2000). However, it has been reported that epinephrine does not potentiate ADP-induced aggregation of murine platelets, and that ^{3}H-yohimbine does not bind specifically to murine platelets (Glusa and Markwaldt, 1983). Although α_2-adrenoceptors have been found on murine, leporine, and human platelets (Kerry et al., 1984; Naselsky et al., 2001), discrepancies in the murine platelets may be responsible for the finding that α_2-adrenoceptor density on murine platelets was greatly lower compared with leporine and human platelets (Kerry et al., 1984). Therefore, comparative studies of platelet α_2-adrenoceptors and imidazoline receptors in various animals may be an important source of information on the mechanism of platelet activation. This study was conducted to pharmacologically identify and characterize α_2-adrenoceptors and imidazoline receptors on platelets of dogs, cats, cattle, horses, rats, and rabbits.

2. Materials and methods

2.1. Animals

Twenty-two healthy adult mixed-breed or beagle dogs of either sex (3–9 years old; 12 males and 10 females), 19 mixed-breed cats (2–8 years old; 15 males and 4 females), 7 cattle (2–5 years old; 5 castrated Japanese black beef cattle and 2 Holstein cows), 5 horses (9–14 years old; castrated Thoroughbred males), 50 Wistar-Imamichi rats (8–10 weeks old; males weighing from 250 to 300 g), and 6 Japanese white rabbits (6–8 months old; males weighing from 3.8 to 4.3 kg) were used throughout the study. Small animals were housed individually and fed commercial dry food and water *ad libitum*. Blood was repeatedly collected from each animal, except for rats. The experimental protocol was principally based on the Guide for the Care and Use of Laboratory Animals developed by the Institute of Laboratory Animal Research of the National Research Council of Japan and approved by the Animal Research Committee of Tottori University, Tottori, Japan.

2.2. Drugs and isotopes

The drugs and isotopes used are as follows: agmatine sulfate, clonidine hydrochloride, 5′-guanylylimidodiphosphate (Gpp(NH)p), guanosine 5′-O-(3-thiotriphosphate) (GTPγS), guanosine triphosphate (GTP), guanabenz, idazoxan hydrochloride, moxonidine hydrochloride, naphazoline hydrochloride, *l*-norepinephrine, oxymetazoline hydrochloride, phentolamine hydrochloride, prazosin hydrochloride, tolazoline hydrochloride, and yohimbine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); atipamezole hydrochloride, detomidine hydrochloride, and medetomidine hydrochloride (Farmos Group Ltd., Finland); and xylazine hydrochloride (Bayer, Federal Republic of Germany); ^{3}H-2-(2-benzofuranyl)-2-imidazoline (2-BFI) (specific radioactivity 68.0 Ci/mmol), ^{3}H-idazoxan (52.0 Ci/mmol), and ^{3}H-yohimbine (92.0 Ci/mmol) (Amersham, U.K.); and ^{3}H-clonidine (specific radioactivity 50–80 Ci/mmol, DuPont, Daiichi Chemical Co., Tokyo, Japan). Norepinephrine was dissolved in 0.04 mol/l HCl solution and then diluted with the incubation buffer. Prazosin was dissolved in sterile distilled water and then diluted with the incubation buffer. The other drugs were dissolved in the incubation buffer. Isotopes were stored at −20 °C in ethanol before reconstitution in the incubation buffer.

2.3. Platelet preparation

Platelets were prepared by modifying the methods described previously (Hikasa et al., 1998). Blood was collected from the jugular vein of dogs, cats, cattle, horses, and rabbits using a 21- to 18-gauge needle into 0.1 volume of 3.2% sodium citrate as the anticoagulant. Blood was collected by cardiocentesis from rats using a 22-gauge needle under isoflurane anesthesia into the same anticoagulant solution. To obtain platelet-rich plasma, the blood was centrifuged for 15–20 min at 110–140 × *g*, and the supernatant was further centrifuged for 3–5 min at 200 × *g*. Platelet-rich plasma was then centrifuged for 10 min at 2000 × *g* to obtain a platelet pellet. The platelet pellet was stored at −70 °C until further use. Platelet yields were over 98%. After thawing, platelet lysates were resuspended in ice-cold washing buffer (5 mM Tris HCl, 5 mM EDTA, pH 7.4) and homogenized using a Polytron homogenizer (setting 7 for 10 s, 3 times) at 4 °C. The homogenate was centrifuged for 20 min at 50,000 × *g* and 4 °C. The resulting membrane pellet was washed once more with the same buffer and centrifugation procedure. The final crude membranes were resuspended in the incubation buffer (50 mM Tris HCl, 0.5 mM EDTA, 0.5 mM $MgCl_2$, pH 7.4) at a protein concentration of 1–2 mg/ml.

2.4. Radioligand binding assays

2.4.1. Saturation binding study

Saturation binding experiments to platelet membranes were performed by slightly modifying a previously published method (Hikasa et al., 1998). ^{3}H-Yohimbine and ^{3}H-clonidine were used for the bindings to α_2-adrenoceptor sites in each animal platelet. Aliquots of platelet membranes suspended in incubation buffer were incubated in duplicate or triplicate with 6–8 concentrations of ^{3}H-yohimbine (0.1–8 nM) and ^{3}H-clonidine (0.25–32 nM). Nonspecific binding of ^{3}H-yohimbine or ^{3}H-clonidine was defined as binding in the presence of 10 μM norepinephrine. Specific binding was defined as the difference between the total binding and the nonspecific binding.

Radioligand binding assay to platelet nonadrenergic I_1 and I_2 sites was performed using ^{3}H-clonidine and ^{3}H-idazoxan, respectively, as previously reported (Piletz and Sletten, 1993; Regunathan and Reis, 1996). Specific binding of ^{3}H-clonidine to the I_1 site was determined in the presence of 10 μM norepinephrine to mask α_2-adrenoceptors and with further addition of 100 μM clonidine to define nonspecific binding. Incubations were performed in duplicate or triplicate with 6–10 concentrations (0.5–64 nM) of ^{3}H-clonidine. The specific binding of ^{3}H-idazoxan to the I_2 site was determined in the presence of 10 μM norepinephrine with further addition of 100 μM idazoxan to define nonspecific binding. Incubations were performed in duplicate or triplicate with 6–10 concentrations (0.5–64 nM) of ^{3}H-idazoxan. As an additional experiment on feline platelets, the specific binding of ^{3}H-2-BFI to the I_2 site was determined in the presence of 10 μM norepinephrine with further addition of 100 μM idazoxan to define nonspecific binding. Incubations were performed in duplicate or triplicate with 6 concentrations (1–16 nM) of ^{3}H-idazoxan.

2.4.2. Competition binding study

Competition binding studies were performed with ^{3}H-yohimbine (for α_2-site), ^{3}H-clonidine (for I_1-site), and ^{3}H-idazoxan (for I_2-site) at the equilibrium dissociation constant (K_d) concentration from saturation experiment to each animal platelet, in the absence or presence of various concentrations of competing drugs (10^{-12}–10^{-3} M, 15–17 concentrations). Incubations were performed in duplicate or triplicate at each concentration of the competing agent. An additional competition binding experiment with ^{3}H-2-BFI (for I_2-site) at the K_d concentration in feline platelets was also performed