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A WATER-SOLUBLE DERIVATIVE OF PRAZOSIN PRAZOSINAMINE HYDROCHLORIDE [1-(4′-AMINO-6′,7′-DIMETHOXYQUINAZOLIN-2′-YL)-4-(6″-AMINOHEXANOYL) PIPERAZINE HYDROCHLORIDE], REVERSIBLY INHIBITS THE CALCIUM-MOBILIZING ACTION OF α_1-ADRENERGIC AGONISTS IN THE PERFUSED RAT LIVER

WILFRED L. F. ARMAREGO,* JOSEPH G. ALTIN, RONALD C. WEIR and FYFE L. BYGRAVE†

Department of Biochemistry (Faculty of Science), * Department of Biochemistry (The John Curtin School of Medical Research), The Australian National University, Canberra, ACT 2601, Australia

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Abstract—A newly-synthesized derivative of prazosin, prazosinamine hydrochloride, was examined for its ability to antagonize the interaction of the α_1-adrenergic agonist phenylephrine with liver cells. Using a Ca^{2-}-selective electrode to measure changes in perfusate Ca^{2+} concentration, prazosinamine was found to be as effective as prazosin in inhibiting the phenylephrine-induced efflux of Ca^{2+} from the perfused liver. Maximal and half-maximal inhibition occurred at 150 nM and 25 nM prazosinamine, respectively. Prazosinamine appears to share the α_1-specificity of prazosin, but has other unique and desirable properties. Its solubility in aqueous media is about three orders of magnitude higher than that of prazosin. Also, its antagonistic effects are rapid in onset, and are reversed within seconds of terminating its infusion into the liver. These attributes seem to make this agent more useful than prazosin for adrenergic receptor studies in perfused tissues. The molecule can also be readily coupled to other ligands.

The study of many receptor-specific physiological responses that are induced by hormones has been furthered by a knowledge of the specificity of a particular hormone for a particular receptor. Synthetic receptor-specific antagonists such as dihydroergocryptine, prazosin and yohimbine, have been useful for characterizing the different adrenergic receptors in a variety of tissues including the nervous system, heart and liver [1–4]. In particular, the ability of these agents to distinguish between the different alpha-adrenergic binding sites has led to a subdivision of these receptors into the α_1-type, whose activation has been associated with the mobilization of cellular Ca^{2+} and the triggering of physiological responses [5–7]; and the α_2-type, whose activation elicits an inhibition of adenylate cyclase activity [7, 8].

To date most work on the characterization of alpha-adrenergic receptors has been conducted utilizing various radioligands, both agonists and antagonists, in competitive-binding type studies using plasma membrane preparations [9–11], or for relating the agonist–receptor binding with the physiological responses that are induced in isolated cells [5, 12]. These studies have been invaluable for determining the affinity of a ligand for a particular receptor, and for elucidating the responses that are associated with the activation of particular receptor types. However, the demonstration that the action of these agents is observable in the intact organ, such as the perfused liver [13, 14] is perhaps a clearer demonstration that the particular receptor–agonist interaction studied can be of both physiological and pharmacological importance.

In this paper we report that a newly-synthesized water-soluble derivative of prazosin (prazosinamine hydrochloride — see structural formula in Fig. 1) has α_1-adrenergic specificity, and is effective in inhibiting the mobilization of Ca^{2+}, and therefore other associated physiological responses such as respiration and glycogenolysis [13, 14], that are induced by the administration of the α_1-agonist phenylephrine to the perfused rat liver. Moreover, in contrast to prazosin, the antagonistic effects of prazosinamine are rapid in onset, and rapidly reversed, once the agent is removed. It seems that these attributes may render the use of this compound preferable to prazosin for receptor-related studies, especially in perfused tissues.

EXPERIMENTAL

Animals and perfusions. Male Wistar-strain albino rats weighing between 280 and 350 g and fed *ad libitum* were used in all experiments. The rats were anaesthetized with sodium pentobarbitone (50 mg/kg body wt), and the livers perfused with Krebs–Henseleit bicarbonate buffer [15] equilibrated with O_2/CO_2 (19:1) and containing 1.3 mM added $CaCl_2$. All perfusions were conducted in a non-recirculating

† Address for correspondence.

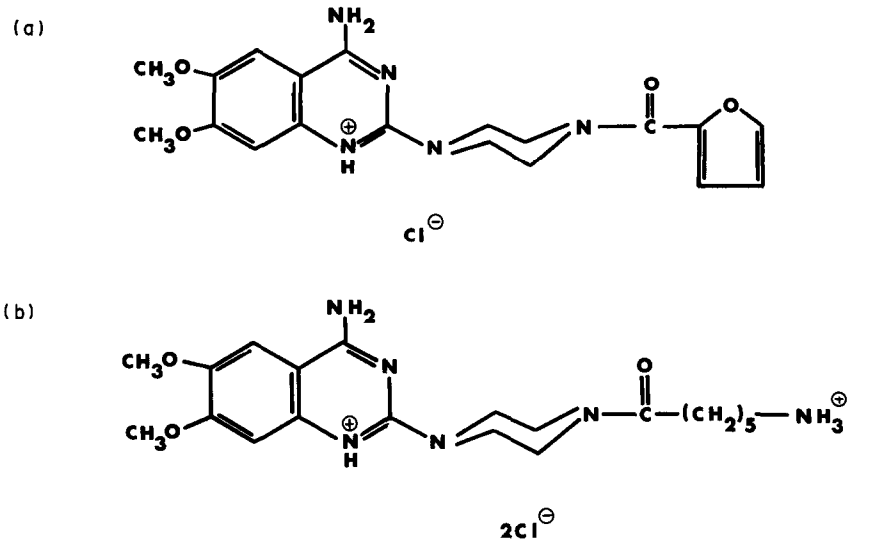

Fig. 1. Comparison of the structure of prazosin hydrochloride (a), with that of prazosinamine hydrochloride (b).

mode at a flow rate of 3.5 ml/min/g of wet liver. At the start of each experiment the liver was preperfused for 15–20 min before the infusion of any agonist or antagonist. Other details are exactly as described in [14].

Perfusate Ca^{2+} *measurements.* The perfusate Ca^{2+} concentration was monitored continuously with a Radiometer F2112 Ca^{2+}-selective electrode in a flow-through chamber placed on the outflow side of the liver [14]. The electrode was coupled to a Radiometer K801 reference electrode via an agarose–KCl salt-bridge, and the combined signals were fed via an Orion microprocessor ion-analyzer to a SP4100 computing integrator for recording and analysis. For other details see [16].

Chemicals and materials. Phenylephrine, [Arg^8] vasopressin, and [Val^5] angiotensin were obtained from the Sigma Chemical Co. St Louis, MO. Prazosin hydrochloride was obtained from Pfizer, Brooklyn, NY. Prazosinamine hydrochloride, a derivative of prazosin, was synthesized as detailed below. With the exception of prazosin which was dissolved in distilled water, all these agents were dissolved in Krebs–Henseleit buffer for infusion into the liver. Ca^{2+}-selective electrode membranes (F2112) and filling solutions S43316 were obtained from Radiometer, Copenhagen, Denmark. Other chemicals used were of analytical grade.

Synthesis of prazosinamine hydrochloride [1-(4′-*amino*-6′-7′-*dimethoxyquinazolin*-2′-*yl*)-4-(6″-*aminohexanoyl*)*piperazine hydrochloride*] (*Fig.* 1b). 6-Benzyloxycarbonylaminohexanoic acid [10.6 g, 1 mol, prepared in 90% yield as described for benzyloxycarbonylglycine [17], had m.p. 56–57° (Found: C, 63.1; H, 7.5; N, 5.3, $C_{14}H_{19}NO_4$ requires C, 63.4; H, 7.2; N, 5.3%), and the p.m.r. (200 MHz) spectrum in $CDCl_3$ (Me_4Si, δ O) has δ 1.34 (*m*, 2H, 4-CH_2), 1.46 (*m*, 2H, 3-CH_2), 1.61 (*m*, 2H, 5-CH_2), 2.29 (*t*, 2H, 2-CH_2), 3.12 (*m*, 2H, 6-CH_2), 5.08 (*s*, 2H, PhCH_2), 5.24 (*t*, 1H, N*H*), 7.33 (*s*, 5H, C_6H_5) and 10.4 (*br s*, 1H, CO_2H)ppm] in methylene chloride (160 ml) was treated with dicyclohexyl-carbodiimide (13.5 g, 1.6 mol) and stirred for 1 hr. The solid (dicyclohexylurea) that separated was filtered off, and dry pentachlorophenol (10.6 g, 1 mol), in methylene chloride (160 ml) was added to the filtrate and the mixture was stirred for 30 min. This solution was placed in a syringe and added slowly (30 min, through a SUBA seal) to a filtered solution of anhydrous piperazine (16.8 g, 5 mol.) in methylene chloride (400 ml) which had been standing over molecular sieves (44 g, Linde, type 4A) for 2 hr, and the mixture was stirred overnight. All operations up to this point were carried out with the exclusion of moisture. The large amount of white dicyclohexylurea was filtered off, the filtrate was evaporated to 70 ml and the further amount of urea that separated was filtered off. The filtrate was diluted with methylene chloride (200 ml) and the solution was washed with 0.1 M sodium hydroxide (3 × 100 ml, to remove free piperazine and neutral material, e.g. the 1,4-disubstituted piperazine) and extracted with 0.5 M hydrochloric acid (5 × 50 ml). The acidic extract was adjusted to pH 10.5–11.0 with 2 M sodium hydroxide, saturated with solid sodium chloride and the oil that separated was extracted with chloroform (5 × 100 ml). The dried (Na_2SO_4) extract was evaporated and the residual oil was kept under vacuum (0.1 mm Hg) overnight in the presence of P_2O_5 and NaOH. The i.r. spectrum of the oil, 1-(6′-benzyloxycarbonylaminohexanoyl)piperazine (4.12 g, 31%) had γ_{max} (film) 1635 (amide CO), 1715 (ester CO) and 3320 (NH) cm^{-1}, and p.m.r (200 MHz) spectrum in $CDCl_3$ [$(CH_3)_4Si$, δ Oppm] had δ 1.35 (*t*, 2H, 4′-CH_2), 1.52 (*m*, 2H, 3′-CH_2), 1.60 (*m*, 2H, 5′-CH_2), 2.30(*t*, 2H, CH_2CON), 2.47 (*br m*, 1H, piperazine-N*H*), 2.81 (*m* 4H, piperazine-3-CH_2), 3.15 (*m*, 2H, $CONHCH_2$), 3.40 (*t*, 2H, piperazine-2-CH_2), 3.59

(*t*, 2H, piperazine-2-CH_2), 5.10 (*s*, 2H, PhCH_2O), 5.52 (*t*, 1H, CON*H*CH_2) and 7.35 (*s*, 5H, C_6H_5)ppm indicated that it was better than 90% pure.

The oily benzyloxycarbonylhexanoylpiperazine (4.12 g, 1.1 mol) and 2-chloro-6,7-dimethoxyquinazolinyl-4-amine [2.64 g, 1mol, m.p.> 300° decomp, prepared in eight steps from veratraldehyde according to procedures similar to those used by Althuis and Hess [18] and Armarego and Reece [19] for preparing related 2-chloroquinazolinyl-4-amines (Found: C, 50.0; H, 4.1; N, 17.1. $C_{10}H_{10}ClN_3O_2$ requires C, 50.1; H, 4.2; N, 17.5%)] in isoamyl alcohol (55 ml) was boiled under reflux for 4 hr and set aside overnight. The mixture was cooled in ice and dry ether (80 ml) was added. The 1-(4′-amino-6′,7′ - dimethoxyquinazolin - 2′ - yl) - 4 - (6″ - benzyloxycarbonylaminohexanoyl)piperazine (5.48 g, 93%) that separated was collected, recrystallised from methanol and dried. It had m.p. 145–147° (effervescence) and its i.r. spectrum had γ_{max} (KBr) 1600 (C–N), 1640 (*br*, amide CO), 1700 (ester CO) and 3500 (NH) cm^{-1}, and the p.m.r. (90 MHz) spectrum in [$(CD_3)_2SO$; Me_4Si, δ O) at 55° had δ 1.38 (*br m*, 6H, 3″, 4″ and 5″ CH_2), 3.82 (*s* 3H, 6′ or 7′ OCH_3), 3.87 (*s*, 3H, 7′ or OCH_3), 7.25 (*s*, 5H, C_6H_5), 7.33 (*s*, 1H, quinazoline-5′-*H*), 7.65 (*s*, 1H, quinazoline-8′-*H*) and 8.60 (*s*, 1H, N*H*CO_2)ppm. (Found: C, 62.7; H, 7.4; N, 14.7. $C_{28}H_{36}N_6O_5$ requires C, 62.7; H, 6.8; N, 14.9%).

The preceding benzyloxyquinazoline (2.5 g) was dissolved in boiling methanol (100 ml), diluted further with methanol (50 ml) and cooled. Palladium-on-charcoal (50%, 2.5 g) was added to the solution followed by three drops of 7 M methanolic hydrogen chloride (not more, otherwise a solid separates) and shaken with hydrogen at 720mm Hg and 25° for 18 hr. The p.m.r. spectrum of an aliquot indicated that hydrogenolysis was complete. i.e. the benzylic protons were absent. The catalyst was filtered off and the filtrate treated with 7 M methanolic hydrogen chloride (0.5 ml) and evaporated to a small volume. Dry ether was added and 1-(4′-amino-6′,7′-dimethoxyquinazolin - 2′ - yl) - 4 - (6″ - amino - hexanoyl)piperazine hydrochloride (1.94 g, 73%), m.p.> 210° (effervescence, slow heating) separated. It gave one spot on t.l.c. (R_f 0.45, on Merck Kieselgel $60F_{254}$ in butan-1-ol:acetic acid water: 10:3:7) (Found: C, 42.4; H, 6.8; N, 14.5; Cl, 15.6. $C_{20}H_{30}N_6O_3$. 2.5HCl.4.1H_2O requires C, 42.3; H, 7.2; N, 14.8; Cl, 15.6%). The i.r. spectrum had γ_{max} (KBr) 1600 (amide CO) and 3500 (*v br*, NH) cm^{-1}, and the p.m.r. (200 MHz) spectrum in D_2O (sodium 3-trimethylsilylpropanesulphonate, δ O) had δ 1.55 (*m*, 2H, 4″-CH_2), 1.80 (*m*, 4H, 3″ and 5″-CH_2), 2.62 (*t*, 2H, 2″-CH_2), 3.12 (*t*, 2H, 6″-CH_2), 3.80 (*s*, 3H, 6′ or 7′-OCH_3), 3.87 (7′ or 6′-$CO H_3$), 6.60 (*s*, 1H, quinazoline-5′-H), 6.84 (*s*, 1H, quinazoline-8′-H) ppm but the piperazine protons are under the broad base of the OCH_3 singlets.

I.r. spectra were measured on a Pye Unicam SP1050 spectrometer and p.m.r. spectra were measured on Jeol FX90Q (90MHz) and Varian XL200E (200MHz) n.m.r spectrometers at ambient temperatures unless otherwise stated.

Expression of data. All experiments were performed at least three times. Where indicated, data are expressed as means ± SEM for the number of independent experiments described.

RESULTS

Comparison of the effects of prazosin with those of prazosinamine in antagonizing the efflux of Ca^{2+} induced by the α_1-agonist phenylephrine

It seems well established that the action of α_1-adrenergic agonists (e.g. phenylephrine) in liver is mediated by a mobilization of both intracellular and extracellular Ca^{2+} which leads to an increase in the cytosolic Ca^{2+} concentration, and consequently, a triggering of many Ca^{2+}-dependent physiological responses (reviewed in [20–23]). Like the action of other Ca^{2+}-mobilizing hormones, the binding of the α_1-adrenergic agonist phenylephrine to its specific receptor on the plasma membrane appears to be associated with the breakdown of phosphoinositides in the membrane that generate the second messenger inositol 1,4,5-trisphosphate, which appears to be involved in the release of Ca^{2+} from the endoplasmic reticulum [24–26]. The release of this intracellular hormone-sensitive pool of Ca^{2+} is accompanied by a concomitant net efflux of Ca^{2+} from the liver over a period of 3–4 min until the pool is depleted [14]. This pool is replenished from the extracellular Ca^{2+} pool once the agonist is removed. Although many workers (for example [5, 12, 27]) have correlated the degree of hormonal stimulation with the activation of glycogen phosphorylase, a Ca^{2+}-dependent enzyme involved in glycogenolysis, it seems that, at least in the early stages, the extent of hormone activation can be correlated with the amount of intracellular Ca^{2+} that is effluxed from the liver. Hence, the use of a Ca^{2+}-selective electrode to monitor the Ca^{2+} fluxes that are induced by the administration of an α_1-specific agonist or antagonist to the perfused rat liver, provides a convenient means to study the ability of agents like prazosinamine to antagonize agonist–receptor binding, and consequently, the associated Ca^{2+} flux response.

The Ca^{2+}-selective electrode trace in Fig. 2(a) shows the Ca^{2+} flux changes that are induced by the administration of a maximal dose of phenylephrine (2 μM) to the liver perfused with Krebs–Henseleit medium containing 1.3 mM Ca^{2+}. This results in a net efflux of approximately 140 nmol Ca^{2+}/g of liver (see also [14, 16]). The re-uptake of a similar amount of Ca^{2+} occurs when the agent is removed. A 5 min pre-infusion of prazosin (200 nM) results in a complete inhibition of the Ca^{2+} efflux response induced by a subsequent administration of phenylephrine (see Fig. 2b). Also, it can be seen that there is no significant reversal of this effect even after 30 min of terminating the infusion of prazosin. This suggests that the binding of prazosin to the site where it antagonizes the interaction of phenylephrine with the α_1-receptor is irreversible over this time period, thus making any further addition of the agonist or antagonist futile.

Comparison of Fig. 2(b) and 2(c) shows that the prazosin derivative prazosinamine (200 nM) is as effective as prazosin (200 nM) in antagonising the interaction of phenylephrine with the α_1-receptor. It is noteworthy that preliminary experiments had

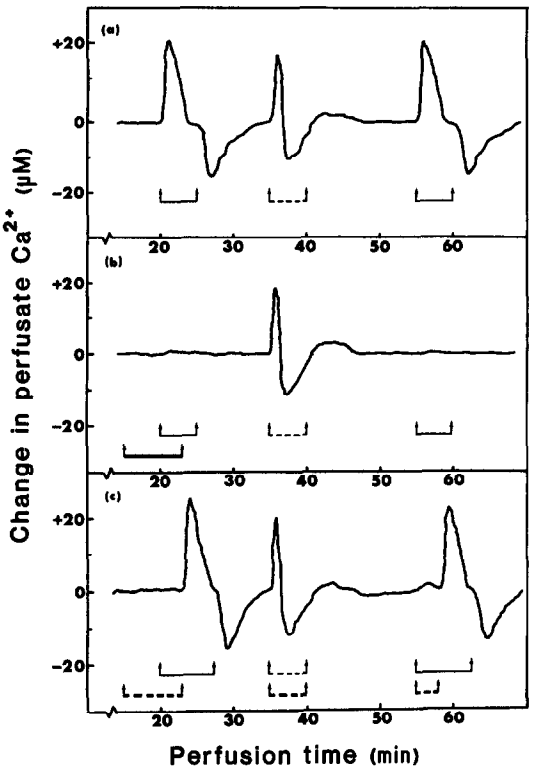

Fig. 2. Comparison of the antagonistic effects of prazosin (b), with those of prazosinamine (c), on the efflux of Ca^{2+} induced by the administration of phenylephrine to the perfused rat liver. Livers of fed rats were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM Ca^{2+}. After an equilibration period of 15–20 min agonists and antagonists were infused into the portal vein by infusion syringe for the times indicated by the arrows. The effluent perfusate was monitored continuously with a Ca^{2+}-selective electrode as described in the experimental section. Trace (a) shows the Ca^{2+}-response induced by separate infusions of maximal concentrations of phenylephrine (2 μM, thin line), and either vasopressin or angiotensin (each at 10^{-8} M, thin broken line), for the times indicated. The result for a similar experiment in which prazosin (0.2 μM, bold line) was infused prior to the first pulse of phenylephrine is shown in (b). Similarly, trace (c) shows the Ca^{2+} response obtained by infusing prazosinamine (0.2 μM, bold broken line) instead of prazosin. Each trace is a representative of between three and four experiments performed independently.

shown that the full effect of prazosinamine on the efflux of Ca^{2+} induced by the infusion of phenylephrine can be observed with only a 15 sec pre-administration of the agent (see Fig. 2b). This contrasts the use of prazosin where a 3–5 min pre-administration seems to be required to elicit complete inhibition of the Ca^{2+} efflux response (data not shown). From the Ca^{2+} flux changes that are observed (Fig. 2c) it seems clear that inhibition of agonist–receptor binding is maintained only for as long as the infusion of prazosinamine is continued. The termination of prazosinamine infusion results in a rapid onset of the Ca^{2+} efflux response that is normally induced by phenylephrine; the amount of Ca^{2+} effluxed is essentially the same as the control (Fig. 2a). Also, the time delay between the removal of the antagonist and the onset of the Ca^{2+} efflux response induced by the presence of phenylephrine (<10 sec), is not significantly different from that which occurs before the onset of Ca^{2+} efflux following the administration of phenylephrine alone. Hence, unlike the antagonism by prazosin which is essentially irreversible, the antagonistic effect of prazosinamine is rapidly reversed within seconds of terminating its infusion. As a consequence of this the Ca^{2+} response can be repeated many times, by subsequent prazosinamine and phenylephrine administrations, during the course of one experiment.

Concentration-dependence of the inhibition of phenylephrine-induced Ca^{2+} efflux by prazosinamine

The ability of different concentrations of prazosinamine to inhibit phenylephrine-induced Ca^{2+} efflux from the perfused liver is shown in Fig. 3. Each point on the curve represents the net amount of Ca^{2+} efflux induced by the infusion of phenylephrine 2 min after the infusion of prazosinamine at the specified concentration. It can be seen that the maximal and half-maximal effective doses of prazosinamine was approx. 150 nM, and 25 nM, respect-

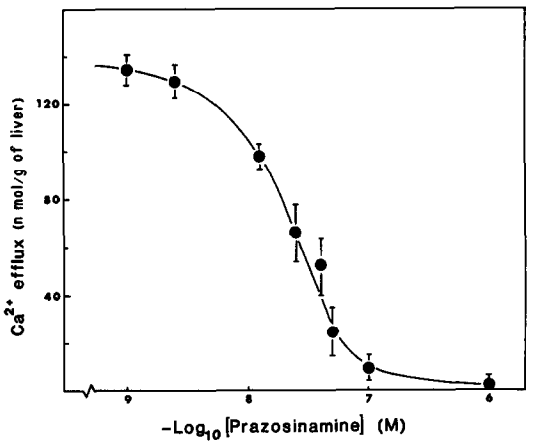

Fig. 3. Concentration-dependence of the antagonism of phenylephrine-induced Ca^{2+} efflux by prazosinamine. Liver perfusions were conducted as described in the legend to Fig. 2. After a pre-perfusion period of 15 min, repeated infusions of prazosinamine and phenylephrine were made. Each point on the curve represents the net amount of Ca^{2+} that is effluxed from the liver following the infusion of a maximal concentration of phenylephrine (2 μM), 2 min after the infusion of a specified concentration of prazosinamine. The infusions of both prazosinamine and phenylephrine was continued until no further Ca^{2+} was being effluxed. For the purposes of measuring the amount of Ca^{2+} efflux, a rest period of at least 10 min was allowed between the termination of prazosinamine and phenylephrine infusion, and any subsequent addition of these agents. Perfusion times were not permitted to run beyond 60 min; only the results of experiments for which the infusion of phenylephrine alone (after 60 min of perfusion) gave the usual amount of Ca^{2+} efflux, were used. Each point is the mean ± SEM of between four and six independent experiments performed at the specified concentrations of prazosinamine.

ively. These concentrations are similar to those required for inhibition of phenylephrine-induced Ca^{2+} efflux by prazosin (data not shown). It should be noted, however, that the time required for the association of prazosin (0.2 nM) with its binding site to reach equilibrium in isolated plasma membranes appears to be around 15–20 min [12]. Since inhibition of Ca^{2+} efflux by prazosinamine seems to occur more rapidly than when prazosin is used (data not shown) it is clear that a direct comparison of the effective concentrations must also take into account these differences in kinetics. Using the equation of Cheng and Prusoff [28], the apparent dissociation constant for the inhibition of phenylephrine-induced Ca^{2+} efflux by prazosinamine was calculated to be approximately 1.2 nM.

DISCUSSION

The major point revealed in this study is that prazosinamine, a newly-synthesized water-soluble derivative of the α_1-specific antagonist prazosin, has antagonistic properties similar to prazosin as judged by its ability to inhibit the phenylephrine-induced efflux of Ca^{2+} in the perfused rat liver. The inhibition appears to be specific for the α_1-receptor since the Ca^{2+} response induced by other Ca^{2+}-mobilizing hormones such as vasopressin and angiotensin seems unaffected by a pre-administration of even high doses (1 μM) of prazosinamine (see also Fig. 2b, c). Although the effective concentrations of prazosinamine for maximal and half-maximal inhibition of the Ca^{2+}-efflux response induced by phenylephrine, are similar to the concentrations of prazosin required for a corresponding inhibition of Ca^{2+}-efflux (Fig. 2b, c) or glucose output [13], our results show that prazosinamine has other desirable properties which are not shared with prazosin.

Firstly, the solubility of prazosinamine is much higher than that of prazosin. The solubility of prazosin was determined empirically for these experiments and was found to be approx. 350 μM; however, the solubility of prazosinamine was found to be in excess of 1 M. The higher solubility of prazosinamine is attributed to the additional highly basic aliphatic amino group ($pK_a ca.10.5$) attached to the piperazine moiety of the prazosin molecule by five methylene groups and an amide function, which are expected to make the molecule charged and polar, and hence more hydrophilic. At physiological pH, i.e. *ca.*7, prazosinamine (estimated $pK_a > 7.5$) must contain a high proportion (>76%) of the diprotonated species (*cf* Fig. 2b; pK_a of 2,4-diaminopyrimidine is 7.23 and a fused benzene ring, and alkyl groups on the 2-amino group are all base strengthening by at least 0.3 pK_a units [29]) and the rest exist as the species protonated on the aliphatic group. Prazosinamine behaves like a detergent in aqueous solution because it froths on shaking.

Another characteristic property of prazosinamine is its ability to rapidly reverse its inhibition of phenylephrine-induced Ca^{2+}-efflux, once its infusion is terminated. It thus appears that the prazosinamine molecule is able to dissociate itself from the α_1-specific binding site much more rapidly than prazosin. The removal of Ca^{2+}-efflux inhibition was apparent only seconds after terminating prazosinamine infusion, though no significant reduction of inhibition by prazosin was apparent 30 min after its removal. The reason for this is not clearly understood, but its seems likely that it could be related to the greater solubility of prazosinamine in the aqueous perfusate.

Finally, we suggest that the attributes of high solubility, and reversible nature of the α_1-specific antagonism exhibited by prazosinamine, may make this the compound of choice in pharmacological studies on adrenergic receptors. This is especially so in studies involving perfused tissues where it is expected that many separate administrations of agonist and antagonist are desired during the one experiment. Furthermore, preliminary experiments have shown that prazosinamine can be readily coupled to immunogenic molecules like hemocyanin, and to a Sepharose-4B matrix. This suggests that the compound can also be used to raise anti-idiotypic antibodies against the α_1-receptor, and to produce an affinity column for the purification of these receptors.

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