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Subtype-Selective *Alpha*-1 Adrenoceptor Alkylation in the Rat Kidney and its Effect on the Vascular Pressor Response[1]

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ABSTRACT

Separate genes for *alpha*-1A and *alpha*-1B adrenoceptors have now been identified. Whereas *alpha*-1 adrenoceptors are known to mediate rat renal vasoconstriction, the relative importance of these *alpha*-1 adrenoceptor subtypes was unknown. We cannulated the right suprarenal artery of anesthetized male Sprague-Dawley rats to permit administration of the *alpha*-1A and *alpha*-1B alkylating antagonists, SZL-49 (SZL) and chloroethylclonidine (CEC), respectively, directly into the right kidney. Treated kidneys were homogenized to identify the doses of SZL and CEC that caused the maximum reductions in B_{max} for [^{3}H]prazosin, the relatively nonselective *alpha*-1 adrenoceptor antagonist. In other rats, a Doppler flow probe was placed around the right renal artery, and dose-peak response curves for boluses of the *alpha*-1 adrenoceptor agonist phenylephrine (PHE) were generated before and after supramaximal dosages of SZL or CEC. Renal vasoconstriction to PHE was nearly obliterated by SZL. In contrast, CEC caused only a modest rightward shift in the PHE DRC. SZL also abolished the renal vascular response to two other *alpha*-1 adrenoceptor agonists, cirazoline and methoxamine. Our data support the conclusion that the *alpha*-1 adrenoceptors at the level of the rat renal resistance vessels are predominantly *alpha*-1A adrenoceptors.

Renal vasoconstriction is mediated by *alpha* adrenergic receptors. Schmitz *et al.* (1981) were the first to demonstrate that selective *alpha*-1 adrenoceptor agonists caused vasoconstriction in the isolated perfused rat kidney. Although Wolff *et al.* (1989) have also recently demonstrated *alpha*-2 adrenoceptor-mediated renal vasoconstriction in conscious rats, several laboratories have verified the observation that *alpha*-1 adrenoceptor agonists were potent and efficacious renal vasoconstrictors, both *in vitro* and *in vivo* (*e.g.*, Cooper and Malik, 1985; DiBona and Sawin, 1987; Jeffries *et al.*, 1987; Wolff *et al.*, 1987).

The *alpha*-1 adrenoceptors mediating these responses are now known to include subpopulations of receptors. Data from several laboratories (see McGrath, 1982; Flavahan and Vanhoutte, 1986) had suggested the possibility of *alpha*-1 adrenoceptor heterogeneity. However, Morrow and Creese (1986) first clearly differentiated *alpha*-1A and *alpha*-1B adrenoceptor subtypes in rat cerebral cortex by their affinities for the competitive antagonists, WB4101, prazosin and phentolamine. More recently, Lefkowitz, Caron and colleagues have confirmed the presence of genes for *alpha*-1A (Lomasney *et al.*, 1991) and *alpha*-1B adrenoceptors (Cotecchia *et al.*, 1988). These investigators have also described a novel *alpha*-1C adrenoceptor (Schwinn *et al.*, 1990).

Several pharmacological agents with apparent selectivity for *alpha*-1A and *alpha*-1B adrenoceptors have been identified (Watson and Abbott, 1991). Minneman and colleagues (Han *et al.*, 1987; Minneman, 1988) demonstrated that an alkylating analog of clonidine, CEC, selectively inactivated *alpha*-1B adrenoceptors. In *in vitro* contractile studies, they demonstrated that CEC reduced the responsiveness of the spleen, but not that of the vas deferens to norepinephrine. Piascik, Kusiak and collaborators (Babich *et al.*, 1987; Kusiak *et al.*, 1989) identified an alkylating analog of prazosin, SZL, which selectively alkylated CEC-resistant *alpha*-1 adrenoceptors, presumably the *alpha*-1A adrenoceptor subtype.

The relative contribution of *alpha*-1 adrenoceptor subtypes to an *alpha*-1 adrenoceptor-mediated renal vascular response in the microvasculature of intact rat kidneys was unknown. We hypothesized that maximally efficacious doses of either SZL or CEC would obliterate renal vascular responses to *alpha*-1 adrenoceptor agonists if the responses were mediated relatively exclusively by *alpha*-1A or *alpha*-1B adrenoceptors, respectively.

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ABBREVIATIONS: B_{max}, maximum binding site density; CEC, chloroethylclonidine dihydrochloride; CIR, cirazoline hydrochloride; DRC, dose-response curve; METH, methoxamine hydrochloride; PHE, phenylephrine hydrochloride; RBF, renal blood flow; SZL, SZL-49 (Prazobind); MAP, mean arterial pressure.

Materials and Methods

Experiments were conducted in male Sprague-Dawley rats (Sasco, Inc., Omaha, NE) weighing 300 to 350 g. After the induction of pentobarbital anesthesia (50 mg/kg, i.p.), the rats were placed on a heated surgical table where body temperature was maintained at 37°C. The trachea was exposed and intubated with a short length of PE-240 tubing to provide a patent airway for spontaneous respiration, and a femoral vein was cannulated with PE-50 for 2.5-mg supplemental injections of pentobarbital when required. The abdominal cavity was opened through a ventral midline incision and the intestines were retracted to the left in gauze pads wetted with normal saline. The right suprarenal artery was located and its origination from the right renal artery was verified. The suprarenal artery was then carefully freed of connective tissue and was cannulated in the retrograde direction with tapered PE-10 tubing as described by Smits *et al.* (1983). The suprarenal artery catheter was connected to a Harvard syringe pump to facilitate the intrarenal arterial administration of drugs.

Radioligand binding assay methods. In order to determine the minimum dosages that produced the maximum *alpha*-1 adrenoceptor destruction, one of several doses of SZl or CEC dissolved in 3 ml of 0.45% NaCl was administered to individual kidneys *via* constant infusion over 1 hr. Forty-five min after stopping the infusion, the right kidney was removed and immediately frozen in liquid nitrogen. Kidneys were kept at −80°C for <1 week before radioligand binding studies were performed.

Renal membranes from vehicle and drug-treated kidneys were prepared according to the procedure of Williams *et al.* (1976) as modified by Schmitz *et al.* (1981). Each kidney was studied individually. After thawing the kidney and removing the capsule, it was weighed, minced and homogenized in 10 ml of ice-cold sucrose buffer (0.25 M sucrose, 1 mM $MgCl_2$, 5 mM Tris, pH 7.4). The remaining larger pieces were removed from the homogenate by filtering through two layers of gauze and centrifuging at 400 × *g* for 10 min at 4°C. The resulting supernatant was then centrifuged at 28,000 × *g* for 10 min at 4°C to pellet the renal membranes. The renal membranes were then washed by resuspending the pellet in ice-cold incubation buffer (10 mM $MgCl_2$, 50 mM Tris, pH 7.7) and were pelleted as before. This wash step was repeated, and the final pellet was suspended in sufficient incubation buffer to provide a protein concentration of 2 to 4 mg/ml.

[^{3}H]Prazosin, the radiolabeled *alpha*-1 adrenoceptor antagonist that binds with approximately equal affinity to *alpha*-1A and *alpha*-1B adrenoceptors (Morrow and Creese, 1986) was used as the radioligand. Six concentrations of radioligand (range = 0.3–6 nM) were used, and specific binding was defined as that which was inhibitable by 10 μM phentolamine, a nonselective *alpha* adrenergic antagonist.

Duplicate aliquots of the renal membranes were incubated with each concentration of radioligand ± 10 μM phentolamine for 30 min at 25°C in a final volume of 150 μl. At the end of the incubation period, the samples were diluted with 5 ml of ice-cold incubation buffer and were rapidly filtered through Whatman GF/C filters by a Brandel cell harvester. The filters were then washed with three additional 5-ml aliquots of ice-cold buffer, dried, punched into 7 ml scintillation vials and 5 ml of Scintiverse E (Fisher) were added. Radioactivity was counted with a Beckman 5000TD scintillation counter with a counting efficiency of approximately 49%.

The ligand was assumed to be bound at a single site, and the data were analyzed according to this model by the computer program, LIGAND, an iterative nonlinear regression program based on the law of mass action (Munson and Rodbard, 1980). The maximum number of binding sites (B_{max}) derived from these analyses were corrected for membrane protein concentration as determined by the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin standards.

Renal vascular reactivity methods. Once optimal intrarenal arterial dosages of the irreversible subtype-selective *alpha*-1 adrenoceptor antagonists, SZL and CEC, were determined by radioligand binding assays, the renal vascular effects of these agents were assessed with minor modifications of our published methods (Wolff *et al.*, 1987). Rats were instrumented as above for the intrarenal arterial administration of drugs *via* a suprarenal artery catheter. In addition, a femoral artery was cannulated with PE-50 and was connected to a pressure transducer (Microswitch 156PC15GWL) and Grass tachograph (Model 7P4H) for continuous recording of blood pressure and heart rate on a Grass polygraph (Model 79E). A second cannula was placed in the femoral vein for infusion of 8% bovine serum albumin at a rate of 5 μl/min to maintain euvolemia (Maddox *et al.*, 1977), and the bladder was intubated. An ultrasonic flow probe (Titronics Medical Instruments) with a 1-mm lumen was placed around the renal artery between the suprarenal artery and aorta. The flow probe was connected to a directional pulsed Doppler flow meter (University of Iowa Bioengineering) and the Grass polygraph for continuous measurement of relative RBF.

A liquid chromatography sample injection valve (Valco Cheminert) with a 5-μl chamber was interspersed in the infusion line between the syringe pump and the suprarenal artery catheter. By injecting 5-μl boluses of *alpha*-1 adrenoceptor agonists in 150 μl of flush vehicle (0.45% NaCl) over 20 sec at approximately 3-min intervals, reproducible DRC were obtained. Various doses of the *alpha*-1 adrenoceptor-selective agonists, PHE, CIR or METH, were injected to obtain responses spanning the range of minimal to near maximal reductions in RBF. Responses typically lasted much less than 1 min, and were expressed as a percentage of the RBF immediately preceding the injection. Modest supramaximal dosages of SZL (10 μg/kg) or CEC (3 mg/kg) were then infused over 1 hr as for the radioligand binding studies to selectively remove subpopulations of the *alpha*-1 adrenoceptors. The renal vascular DRC were then repeated 30 min after stopping the infusion.

Materials. [^{3}H]Prazosin (24.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA). SZL and CEC were purchased from Research Biochemicals, Inc. (Natick, MA). PHE and METH were obtained from the Sigma Chemical Co. (St. Louis, MO). Phentolamine hydrochloride (Ciba Pharmaceutical Co., Summit, NJ) and CIR (Synthelabo, Paris, France) were gifts.

Statistics. Data are presented as mean ± S.E. Basal RBF and MAP values before and after SZL or CEC were statistically compared by paired *t* analysis, with $P < .05$ considered significant. A similarity in mean values for two to three dosages of SZL or CEC producing reductions in binding sites was taken as evidence of achieving a plateau of blockade. Our data concerning SZL and CEC were considered to be qualitative in nature, and were not subjected to a quantitative statistical analysis.

Results

Radioligand binding studies. Various dosages of SZL and/or CEC were administered directly into the right kidney of anesthetized rats to identify the minimal dosages that produced the maximal receptor alkylation. At least three rat kidneys were studied individually for each dose of the irreversible antagonists.

In control kidneys infused with saline ($n = 8$), Rosenthal plots of the specific equilibrium binding data for [^{3}H]prazosin yielded an *alpha*-1 adrenoceptor density of 91.1 ± 2.7 fmol/mg protein with a K_d of 0.71 ± 0.08 nM (fig. 1).

The infusion of SZL produced a dose-related decrease in *alpha*-1 adrenoceptor density. The B_{max} values after the intrarenal arterial infusion of 0.3 and 1 μg/kg of SZL over 1 hr were 33 ± 4 ($n = 3$) and 19 ± 3 fmol/mg protein ($n = 3$), respectively. The maximal effect, a reduction in the *alpha*-1 adrenoceptor density to 12.4 ± 1.9 fmol/mg protein ($n = 5$), was seen after the administration of 3 μg/kg of SZL (fig. 1A). Increasing the dose of SZL up to 10 μg/kg over 1 hr did not produce a further decrease in *alpha*-1 adrenoceptor density. When expressed as a percentage of the control B_{max}, these doses of SZL caused an 86% reduction in the number of renal *alpha*-1 adrenoceptors.

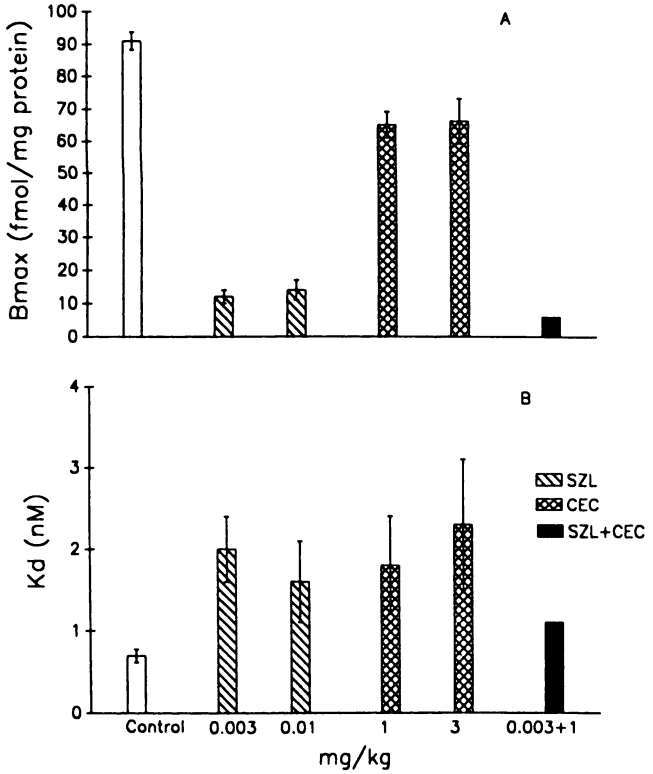

Fig. 1. B_{max} (panel A) and K_d (panel B) for [^{3}H]prazosin binding to membranes from kidneys infused for 1 hr with vehicle or the subtype-selective *alpha*-1 adrenoceptor alkylating agents, SZL and CEC ($n \geq 3$ for each dose). Data are shown as mean ± S.E.

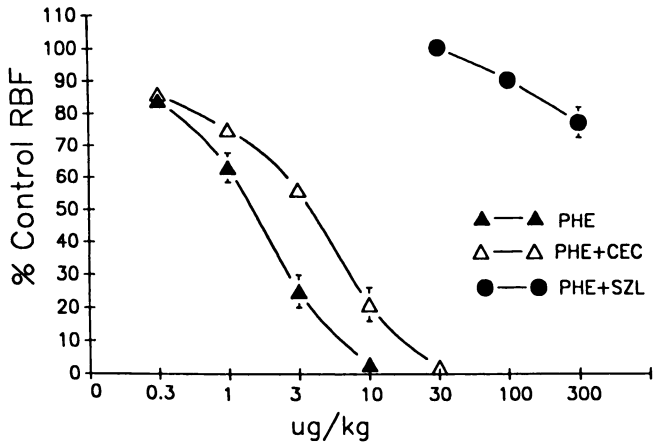

Fig. 2. Renal vascular reactivity to PHE before and after SZL ($n = 6$) or CEC ($n = 6$). Responses are shown as mean ± S.E.

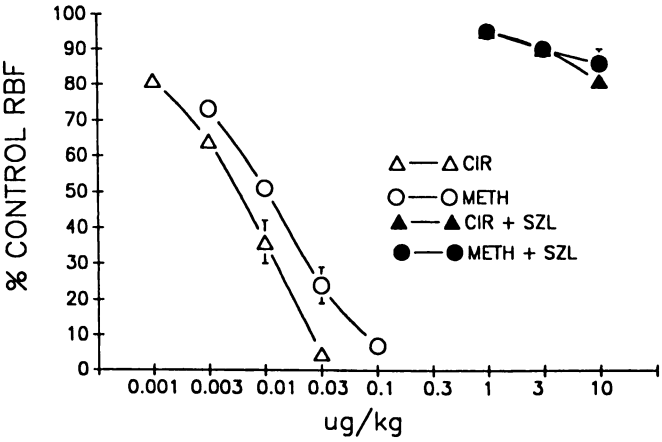

Fig. 3. Renal vascular reactivity to CIR and METH before and after alkylation of *alpha*-1A adrenoceptors by SZL ($n = 3$). Responses are shown as mean ± S.E.

The population of *alpha*-1 adrenoceptors remaining after SZL alkylation displayed a small variable decrease in affinity, as shown in figure 1B.

As shown in figure 1A, the maximally efficacious intrarenal arterial dose of CEC (1 mg/kg over 1 hr) reduced the *alpha*-1 adrenoceptor density to 64.7 ± 4.5 fmol/mg protein ($n = 6$). Lower doses of CEC [B_{max} after 0.1 mg/kg = 78 ± 2.6 fmol/mg protein ($n = 3$), B_{max} after 0.3 mg/kg = 73 ± 2.0 fmol/mg protein ($n = 3$)] did not alkylate all CEC-sensitive sites, and increasing the dosage of CEC to 3 mg/kg over 1 hr did not cause additional renal *alpha*-1 adrenoceptor alkylation. The maximum decrease in [^{3}H]prazosin binding sites represented a 29% reduction relative to control kidneys. The *alpha*-1 adrenoceptor population remaining after CEC alkylation also exhibited a variable decrease in affinity for [^{3}H]prazosin, as shown in figure 1B.

When SZL (3 μg/kg) and CEC (1 mg/kg) were administered simultaneously over 1 hr, [^{3}H]prazosin binding sites were reduced 93% to 6.8 ± 0.7 fmol/mg protein (fig. 1).

In vivo renal vascular responses. Intrarenal arterial bolus injections of *alpha*-1 adrenoceptor agonists produced transient dose-dependent reductions in RBF. As shown in figure 2, the peak vasoconstrictor response seen after a 10 μg/kg bolus of PHE reduced RBF to approximately zero during the control period.

After the administration of a supramaximal dose of SZL (10 μg/kg over 1 hr), neither RBF (4.5 ± 0.2 to 4.3 ± 0.3 kHz shift) nor MAP (105 ± 5 to 103 ± 4 mm Hg) were changed ($n = 6$). However, there was a large rightward shift (≥100-fold) in the PHE DRC. After the SZL infusion, 300 μg/kg boluses of PHE only produced 23 ± 4.7% reductions in RBF. We did not administer larger doses of PHE because the PHE that recirculated after this dose caused large increases in systemic pressure.

RBF (4.8 ± 1.1 to 4.4 ± 1.1 kHz shift) and MAP (105 ± 3 to 105 ± 3 mm Hg) were also unchanged ($n = 6$) when CEC was administered in a supramaximal dose (3 mg/kg over 1 hr). In contrast to the near obliteration of renal vascular responsiveness to PHE after the SZL infusion, the infusion of CEC produced a comparatively modest parallel rightward shift (approximately 3-fold) in the PHE DRC.

As shown in figure 3, DRC were obtained for two additional *alpha*-1 adrenoceptor-selective agonists, CIR and METH. The responses to these two agonists were also shifted markedly to the right by the SZL infusion ($n = 3$), similar to the PHE DRC shown in figure 2.

Discussion

Morrow and Creese (1986) used pharmacological methods to divide *alpha*-1 adrenoceptors into *alpha*-1A and *alpha*-1B adrenoceptor subtypes. The validity of this division has been recently confirmed by the demonstration of distinct genes for these receptors (Cotecchia *et al.*, 1988; Lomasney *et al.*, 1991). Piascik and colleagues (Piascik *et al.*, 1989; Kusiak *et al.*, 1989) and Minneman and colleagues (Han *et al.*, 1987; Minneman, 1988) have extended our pharmacological capabilities by dem-

onstrating, respectively, that SZL alkylated *alpha*-1A adrenoceptors and CEC alkylated *alpha*-1B adrenoceptors. However, these two subtype-selective irreversible antagonists had not been utilized as tools to assess the relative contribution of *alpha*-1A and *alpha*-1B adrenoceptors to the *alpha*-1 adrenoceptor-mediated vascular response of rat renal resistance vessels.

Earlier *in vitro* studies by Han *et al.* (1987) had established that rat renal membranes contained both CEC-sensitive and CEC-resistant subpopulations of *alpha*-1 adrenoceptors. Because of our intention to investigate the function of these receptors *in vivo*, our initial objective was to identify minimal intrarenal arterial dosages of SZL and CEC that produced the maximal amount of renal *alpha*-1 adrenoceptor inactivation. To ascertain the appropriate amounts of SZL or CEC, radioligand binding studies were performed on treated kidneys. The values for [^{3}H]prazosin binding that were obtained from vehicle-treated kidneys are in agreement with previous results from this laboratory (Schmitz *et al.*, 1981; Smyth *et al.*, 1984). In addition, our *in vivo* demonstration of a 29% reduction in the number of renal *alpha*-1 adrenoceptors is consistent with *in vitro* data from the Minneman group (Han *et al.*, 1987).

After having established maximally efficacious doses of SZL and CEC, renal vascular reactivity to PHE, METH or CIR was assessed *in vivo* before and after the administration of SZL or CEC. Once alkylated with SZL or CEC, the receptors are inactive. Until spare receptors are eliminated, receptor alkylation simply causes a parallel rightward shift in DRC. With additional receptor alkylation, there is both a rightward shift in the DRC and a diminution in the maximum response. Ultimately, all of the receptors are inactivated and the response approaches zero. An extension of this logic predicts that if two receptor subtypes were contributing to the renal vascular response to *alpha*-1 adrenoceptor agonists, then selective inactivation of one receptor subtype should not completely eliminate the response.

For our *in vivo* renal vascular reactivity studies, we administered doses of SZL and CEC that were proven by our biochemical studies to have reached a plateau or maximal effect. However, the pharmacological selectivity of these drugs has been challenged. Schwinn *et al.* (1990) have recently shown that CEC also inactivates *alpha*-1C adrenoceptors. This recently described *alpha*-1 adrenoceptor subtype does not, however, appear to have a widespread distribution. In addition, investigators studying the smooth muscle of various organs *in vitro* have questioned the selectivity of SZL (P. Abel, personal communication). Our binding data are consistent with the notion that SZL has some affinity for CEC-sensitive receptors because slightly overlapping populations of renal *alpha*-1 adrenoceptors were apparently inactivated (*i.e.*, 86% + 29% > 100%). Finally, although comparatively low, [^{3}H]prazosin does have an affinity for rat renal *alpha*-2B adrenoceptors (Bylund, 1988). This may explain the small amount of residual displaceable [^{3}H]prazosin that was still present after the combined administration of SZL and CEC.

Nonetheless, renal vascular responses to the *alpha*-1 adrenoceptor agonist, PHE, were essentially obliterated by SZL, whereas CEC caused only a modest parallel rightward shift, as would be expected for competitive antagonism. SZL also blocked the renal vascular responsiveness to two other *alpha*-1 adrenoceptor agonists, CIR and METH. Our data, therefore, suggest that vascular responses to *alpha*-1 adrenoceptor agonists in rat renal resistance vessels were mediated by *alpha*-1A adrenoceptors.

Our data are consistent with those of Piascik *et al.* (1989), which demonstrated that SZL caused rightward shifts in the pressor responses to i.v. PHE in anesthetized rats without affecting the base-line blood pressure. Neither SZL nor CEC caused changes in RBF in agreement with observations that the competitive *alpha*-1 adrenoceptor antagonist prazosin does not dilate the renal vasculature of anesthetized rats (DiBona and Sawin, 1987). Han *et al.* (1990) recently reported that *alpha*-1A adrenoceptor-mediated vasoconstriction predominated in rat renal artery rings. Our data extend these observations down to the rat renal resistance vessels. Morrow and Creese (1986), in fact, predicted our results based on their analysis of the study by Schmitz *et al.* (1981). However, our data do not rule out the possibility of *alpha*-1B adrenoceptor localization to renal vascular elements, such as the glomerular mesangial cells and renal veins, which have minimal contribution to overall renal vascular resistance.

Rat renal membranes do contain both *alpha*-1A and *alpha*-1B adrenoceptors. Our renal vascular data do not exclude a tubular role for either *alpha*-1A or *alpha*-1B adrenoceptors. By isolating proximal tubules and medullary thick ascending limbs from dispersed nephron segments, we have recently found both *alpha*-1A and *alpha*-1B adrenoceptors in proximal tubules, and predominantly *alpha*-1B adrenoceptors in medullary thick ascending limbs (Feng *et al.*, 1991). Additional exploitation of subtype-selective *alpha*-1 adrenoceptor antagonists as pharmacological probes of renal physiology and pathophysiology is, therefore, suggested.

In summary, maximally efficacious intrarenal arterial dosages of the *alpha*-1A and *alpha*-1B adrenoceptor alkylating drugs SZL and CEC, respectively, were identified by radioligand binding studies of treated rat kidneys. Renal vascular reactivity to *alpha*-1 adrenoceptor agonists was then assessed *in vivo* before and after the administration of modest supramaximal dosages of SZL or CEC. The vasoconstrictor responses to *alpha*-1 adrenoceptor agonists were essentially obliterated by SZL, whereas CEC only caused a small rightward shift in the DRC. We conclude that the *alpha*-1 adrenoceptor vasoconstrictor response of rat renal resistance vessels is mediated predominantly by the *alpha*-1A adrenoceptor subtype in the pentobarbital-anesthetized rat.

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