

## Full-length article

**Alteration of binding sites for [<sup>3</sup>H]P1075 and [<sup>3</sup>H]glibenclamide in reno-vascular hypertensive rat aorta<sup>1</sup>**Hua-mei HE<sup>2,3,5</sup>, Chao-liang LONG<sup>2,5</sup>, Le-zhi ZHANG<sup>3,5</sup>, Ai-ling FU<sup>3</sup>, Hai WANG<sup>4</sup><sup>2</sup>*Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing 100850, China;* <sup>3</sup>*Department of Pharmacology, Third Military Medical University, Chongqing 400038, China***Key words**

potassium channels; glibenclamide; P1075; pinacidil; hypertension; aorta; radioligand binding

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**Abstract**

**Aim:** The alterations of the binding sites for ATP-sensitive K<sup>+</sup> channel (K<sub>ATP</sub>) openers and blockers in aortic strips were investigated in hypertensive rats. **Methods:** Radioligand binding techniques were used to compare the specific binding properties of [<sup>3</sup>H]P1075 and [<sup>3</sup>H]glibenclamide (Gli) in normotensive (NWR) and reno-vascular hypertensive rat (RVHR) aortic strips. **Results:** The K<sub>D</sub> values of [<sup>3</sup>H]P1075 binding were increased by 1.5-fold, while the B<sub>max</sub> values were unchanged in RVHR. The IC<sub>50</sub> values of P1075 and pinacidil (Pin) for displacing the [<sup>3</sup>H]P1075 binding in RVHR were increased by 1.8- and 1.7-fold, respectively. The kinetic processes of association and dissociation of [<sup>3</sup>H]P1075 binding were slower in RVHR. Glibenclamide pretreatment slowed down the kinetic processes of the association and dissociation of [<sup>3</sup>H]P1075 binding in NWR, but failed to alter the kinetic processes of [<sup>3</sup>H]P1075 binding in RVHR. The IC<sub>50</sub> values of Gli for displacing the [<sup>3</sup>H]Gli binding at high-affinity sites were increased by 3-fold, while those at low-affinity sites remained to be unchanged in RVHR. The kinetic processes of association of [<sup>3</sup>H]Gli binding were decreased and those of the dissociation were accelerated in RVHR. The treatment with Pin slowed down the association kinetic processes but accelerated the process of the dissociation of [<sup>3</sup>H]Gli binding in NWR, but did not alter the kinetics of [<sup>3</sup>H]Gli binding in RVHR. **Conclusion:** The affinity of binding sites for [<sup>3</sup>H]P1075 and of high-affinity binding sites for [<sup>3</sup>H]Gli are decreased, and the negative allosteric interactions between the two binding sites are impaired in RVHR aorta.

**Introduction**

ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub>) have been identified in multiple cells<sup>[1,2]</sup>. In vascular smooth muscles, K<sub>ATP</sub> can regulate vascular tone<sup>[3]</sup>. The functions of vascular K<sub>ATP</sub> can be modulated by nucleotides, potassium channel blockers, sulfonylureas, such as glibenclamide (Gli), and K<sub>ATP</sub> openers, such as pinacidil (Pin)<sup>[4]</sup>. Recent molecular biological and electrophysiological studies on recombinant K<sub>ATP</sub> have identified that K<sub>ATP</sub> is a hetero-octameric complex composed of an inwardly rectifying K<sup>+</sup> channel (Kir<sub>6,x</sub>) serving as a pore-forming subunit and regulatory sulfonylurea receptors (SURs) belonging to the ATP-binding cassette protein superfamily<sup>[5]</sup>. The binding sites for nucleotides, sulfonylureas,

and potassium channel openers are localized in the SURs, which explains the complex sensitivity to these drugs<sup>[6]</sup>. Furthermore, it has been found that binding experiments have been carried out in vascular smooth muscles, using [<sup>3</sup>H]Gli and [<sup>3</sup>H]P1075, that the binding sites of K<sub>ATP</sub> openers are different from those of blockers, and negatively allosteric interaction exists between these two binding sites<sup>[7,8]</sup>. The functional alterations of K<sub>ATP</sub> in vascular smooth muscles from hypertensive animals have been investigated by using patch-clamp techniques; the dose-response curves of the K<sub>ATP</sub> openers pinacidil and aprikalim, for inducing relaxation of isolated aorta and basilar arteries, were shifted rightward<sup>[9]</sup>, and the effects of levromakalim on K<sub>ATP</sub> currents in mesen-

teric arteries were decreased in SHR<sup>[10]</sup>. But the mechanisms underlying the hypertensive changes of  $K_{ATP}$  remain to be investigated.

In the present study, the characteristics of [<sup>3</sup>H]P1075 and [<sup>3</sup>H]Gli binding in aortic strips, derived from normotensive and hypertensive rats, were compared for investigation of the hypertensive changes of  $K_{ATP}$  in vascular smooth muscles.

## Materials and methods

**Drugs** [<sup>3</sup>H]Gli (specific activity 1850 GBq/mmol=50 Ci/mmol) was purchased from Dupont/ New England Nuclear (Boston, MA, USA). [<sup>3</sup>H]P1075 (specific activity 121 Ci/mmol) from Amersham International (Buckinghamshire, United Kingdom). The radiolabels were stored in ethanol at -20 °C. Gli, Pin, and Tris from Sigma Chemical Co (St Louis, MO, USA). HEPES from Boehringer Mannheim (Baden-Wuerttemberg, Germany). Dimethylsulphoxide ( $Me_2SO$ ) from Merck Company (West point, USA). P1075 was kindly donated by Leo Pharmaceuticals (Ballerup, Denmark). All other chemicals and reagents were of analytical grade. Both P1075 and Pin were dissolved in  $Me_2SO$  (25 g/L) and were diluted as appropriate so that the final concentration of  $Me_2SO$  was less than 0.1%.

**Animals** Wistar rats (NWR, male, weighing 235±21 g,  $n=106$ ), were provided by the Laboratory Animal Center of Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing, China.

**Reno-vascular hypertension** Reno-vascular hypertension was produced by coarctation of the abdominal aorta between the origin of the renal arteries, under ether anesthesia, as described by Liu *et al*<sup>[11]</sup>. The rats were used 30 d after the surgery. One day before the experiment, a polyethylene catheter was inserted into the carotid artery for blood pressure measurements with a two-channel physiological recorder (LMS-LB). Mean arterial pressure was 202±25 mmHg in RVHR and 140±11 mmHg in NWR. All animals were kept in a temperature-controlled room on a 14 h/10 h light/dark cycle and had free access to a standard rat chow diet and tap water.

**Aorta strips** All rats (NWR,  $n=55$ ; RVHR,  $n=51$ ) were decapitated and exsanguinated. Aorta strips were prepared as described in a previous study<sup>[12]</sup>. The preparations were then weighed and assigned into tissue holders and immersed in ice-cold HEPES-buffered physiological salt solution (PSS) containing (in mmol/L): NaCl 139, KCl 5,  $CaCl_2$  25,  $MgCl_2$  1.2, glucose 11, HEPES 5, gassed with 95%  $O_2$ /5%  $CO_2$  and titrated to pH 7.40 with NaOH at 37 °C.

### Equilibrium binding experiments

**Saturation assay** Binding assays of [<sup>3</sup>H]P1075 or [<sup>3</sup>H]Gli were routinely performed in duplicate in 0.50 mL PSS containing 3–5 mg wet weight of aorta strips at 25 °C or 37 °C. Nonspecific binding, determined in the presence of 50  $\mu$ mol/L unlabeled P1075 or 30  $\mu$ mol/L Gli, accounted for 48.6%±6.4% or 44%±6% of total bound ligand under the conditions employed. Specific binding was obtained by subtracting the nonspecific binding from the total binding.

For saturation experiments, aorta strips were incubated with different concentrations of label (0.25–30 nmol/L) for 90 min. They were then washed by adding 9 mL of ice-cold quench solution (NaCl 154 mmol/L, Tris 50 mmol/L, pH 7.40) for 1 min, blotted, and dissolved hermetically over 2 h in 50  $\mu$ L of 60% perchloric acid and 100  $\mu$ L of 30%  $H_2O_2$  at 80 °C. The samples were supplemented with 2.5 mL of ethylene glycol ethyl ether and 5 mL of dimethylbenzene containing 1% 2-(4-tert-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole for 8 h. Radioactivity was determined with liquid scintillation spectrometry (at an efficiency of 42%–55%). The equilibrium dissociation ( $K_D$ ) and the maximum number of binding sites ( $B_{max}$ ) of label were determined by Scatchard analysis, and Hill coefficient ( $n_H$ ) was calculated from Hill equation.

**Displacement assay** Aorta strips were incubated with 5 nmol/L [<sup>3</sup>H]P1075 or 3 nmol/L [<sup>3</sup>H]Gli and unlabeled inhibitors of interest, then washed and determined as described above. Specific binding of [<sup>3</sup>H]P1075 or [<sup>3</sup>H]Gli in the presence of unlabeled drugs was normalized to percentage of specific binding in the absence of these drugs. The concentration of drug inhibiting 50% of specific labeled-ligand binding ( $IC_{50}$ ) was analyzed using the weighted non-linear least-squares regression program. This program fits data assuming the presence of one or more binding sites and allows comparison of the relative goodness-of-fit using *F*-test. A two-site fit was accepted as superior to one-site fit only if significance exceeded  $P<0.05$ .

**Kinetic assay** To determine the association kinetics, the aorta strips were incubated with 3 nmol/L [<sup>3</sup>H]Gli or 5 nmol/L [<sup>3</sup>H]P1075 and aliquots were withdrawn at various time points for separation of bound and free ligand as described above. To measure the dissociation kinetics, the labels were equilibrated with aorta strips for 90 min. Dissociation was initiated by addition of a large excess of unlabeled ligands (Gli 30  $\mu$ mol/L, P1075 50  $\mu$ mol/L) and bound-label was determined at various time intervals ranging from 1 to 90 min. The constants of association and dissociation were calculated according to equations (1)  $LN[B_{eq} \cdot (B \cdot B)^{-1}] = K_{app} \cdot t$ , (2)  $K_{app} = K_1 \cdot [L] + K_2$ , and (3)  $LN(B \cdot B_{eq}^{-1}) = -K_2 \cdot t$  where *B* represents the amount of specific [<sup>3</sup>H]Gli or [<sup>3</sup>H]P1075 binding at dif-

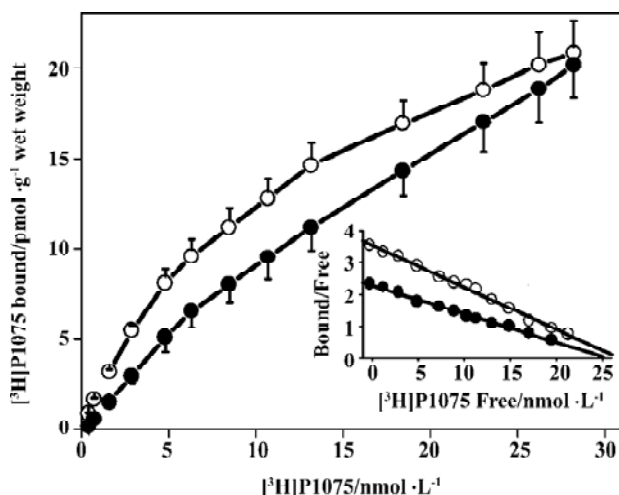
ferent time points,  $B_{eq}$  denotes the amount of the receptor-label complex at equilibrium and  $K_{app}$  the apparent rate constant of association, which depends on the rate constants of the association and dissociation ( $K_1, K_2$ ), and on the concentration of labeled ligand employed ( $[L]$ ).

**Statistical analysis** Results are expressed as mean±SD. Statistical significance of differences between groups of means was assessed by ANOVA followed by Dunnet's test using the Instat Programme (Graphpad SAS Software, San Diego, USA) and was accepted at the  $P<0.05$  level.

### Results

**[<sup>3</sup>H]P1075 bindings** [<sup>3</sup>H]P1075 bound in a saturated manner to aorta preparations in NWR and RVHR within the concentration range employed. Scatchard analysis of the data suggested that in NWR aorta strips [<sup>3</sup>H]P1075 bound to a single class of sites with a  $K_D$  value of  $7.7\pm 2.0$  nmol/L, and  $B_{max}$  value of  $26\pm 8$  pmol/g wet weight. In RVHR, [<sup>3</sup>H]P1075 also bound to a single class of sites. The  $K_D$  value, however, increased ~1.5-fold and the  $B_{max}$  value showed no change compared with those in NWR (Figure 1, Table 1).

$K_{ATP}$  opener P1075 and Pin, as well as blocker Gli, concentration-dependently inhibited specific [<sup>3</sup>H]P1075 binding. However, only  $K_{ATP}$  opener P1075 and Pin were able to inhibit the specific [<sup>3</sup>H]P1075 binding completely, whereas inhibition at saturation by Gli was incomplete only by up to 72% of maximal specific binding in NWR and RVHR



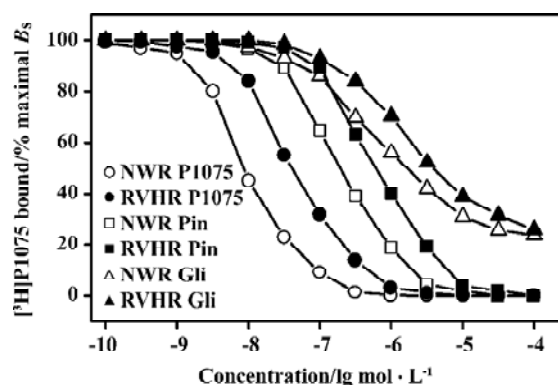
**Figure 1.** Equilibrium binding of [<sup>3</sup>H]P1075 to aorta strips in normotensive Wistar rats (NWR, open circle) and reno-vascular hypertensive rats (RVHR, solid circle). Strips were incubated with varying concentrations (0.01–25.00 nmol/L) of [<sup>3</sup>H]P1075 in a 0.50 mL assay volume at pH 7.40 and 37 °C. Inset: Scatchard analysis of the specific binding data. The data are summarized in Table 1.

**Table 1.** Scatchard analysis of [<sup>3</sup>H]P1075 binding in normotensive Wistar rats (NWR) and reno-vascular hypertensive rats (RVHR). Mean±SD. <sup>b</sup> $P<0.05$  vs NWR.

Animals	<i>n</i>	$K_D$ / nmol·L <sup>-1</sup>	$B_{max}$ / pmol·g <sup>-1</sup> wet weight	$n_H$
NWR	5	7.7±2.0	26±8	1.2±0.4
RVHR	4	11.2±4.0 <sup>b</sup>	25±4	1.1±0.2

aorta preparations. Compared with those in NWR, inhibition curves of [<sup>3</sup>H]P1075 binding to aorta strips in RVHR by these modulators were shifted to the right along the concentration axis (Figure 2). Statistical analysis with the  $F$ -test showed that the fit of inhibition curves by P1075 and Pin to the one-site model was significantly superior to two-site model, which corresponded to their  $n_H$  being close to unity.  $IC_{50}$  for the inhibition of [<sup>3</sup>H]P1075 binding to RVHR aorta preparations by P1075 and Pin were increased by approximately 1.8- and 1.7-fold, respectively, compared with those in NWR. The change in  $IC_{50}$  value for inhibition of [<sup>3</sup>H]P1075 binding by P1075 in RVHR aorta strips was in fair agreement with that in the  $K_D$  value obtained in the saturation equilibrium experiments in Figure 1.

Inhibition of [<sup>3</sup>H]P1075 binding by Gli exhibited a biphasic plot (Figure 2) with  $n_H$  significantly different from unity (Table 3). In addition, statistical analysis with the  $F$ -test showed the fit of inhibition curve by Gli to the two-site

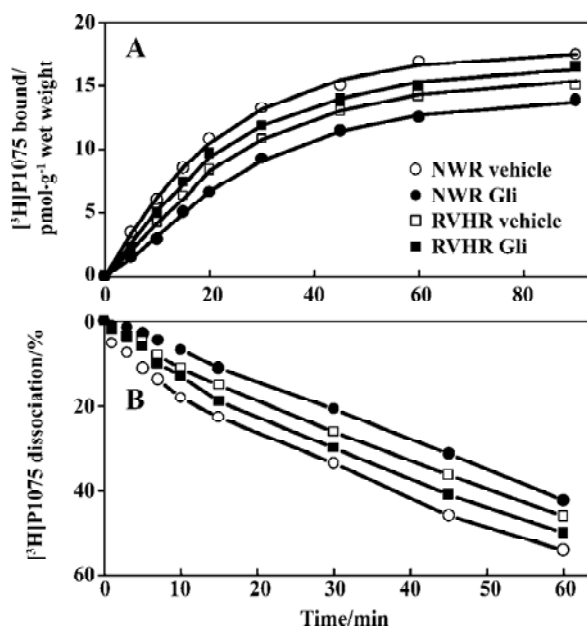


**Figure 2.** Inhibition of [<sup>3</sup>H]P1075 binding to aorta strips by P1075, Pin, or Gli in NWR and RVHR. Aorta strips were incubated with 5 nmol/L [<sup>3</sup>H]P1075 and increasing concentrations of these compounds for 90 min at 37 °C. Specific binding ( $B_s$ ) was  $10.5\pm 1.5$  fmol·mg<sup>-1</sup> wet weight, corresponding to  $51.4\pm 6.4\%$  of total binding. Data (as  $B_s$ ) were fitted with one-site and two-site model; the fitting parameters were listed in Table 2. Depicted were representative plots of 4–5 separate experiments performed in duplicate.

**Table 2.** Analysis of inhibition curves in Fig 2 of [<sup>3</sup>H]P1075 binding to aorta strips by  $K_{ATP}$  channel modulators. For experiment details see Figure 2.  $n=4-5$  experiments. Mean $\pm$ SD. <sup>c</sup> $P<0.01$  vs NWR.

Groups	Modulators	One-site fit		Two-site fit		<i>F</i> -test for hypothesis: one-site model is correct	
		IC <sub>50</sub> /nmol·L <sup>-1</sup>	$n_H$	IC <sub>50,H</sub> /nmol·L <sup>-1</sup>	IC <sub>50,L</sub> /μmol·L <sup>-1</sup>	<i>F</i> value	<i>P</i> <sup>1)</sup>
NWR	P1075	9.1 $\pm$ 1.3	1.14 $\pm$ 0.10	8.2 $\pm$ 0.9	0.03 $\pm$ 0.007	0.42	0.6651
	Pinacidil	200 $\pm$ 44	1.16 $\pm$ 0.14	181 $\pm$ 42	1.0 $\pm$ 0.2	0.76	0.4798
	Glibenclamide	589 $\pm$ 46	0.79 $\pm$ 0.08	204 $\pm$ 22	3.0 $\pm$ 0.5	164	<0.0001
RVHR	P1075	16.5 $\pm$ 0.8 <sup>c</sup>	1.13 $\pm$ 0.11	15.7 $\pm$ 1.1	2.1 $\pm$ 0.3	0.85	0.4429
	Pinacidil	337 $\pm$ 36 <sup>c</sup>	1.10 $\pm$ 0.13	258 $\pm$ 34	1.4 $\pm$ 0.2	1.65	0.2165
	Glibenclamide	1478 $\pm$ 81 <sup>c</sup>	0.74 $\pm$ 0.10	443 $\pm$ 109 <sup>c</sup>	3.4 $\pm$ 0.7	46.6	<0.0001

<sup>1)</sup> If  $P>0.05$ , the hypothesis will be accepted; If  $P<0.05$ , the hypothesis is rejected, and the fit of the two-site model is significantly superior, statistically, to the one-site model.

**Figure 3.** Modulation of association (A) and dissociation (B) kinetics of [<sup>3</sup>H]P1075 (5 nmol/L) with aorta strips in NWR and RVHR by vehicle or Gli 100 μmol/L preincubated for 10 min. Data fitted equation 1 and 3, respectively. The traces were representative of 4-7 experiments.

model was significantly superior to one-site model (Table 2). These results suggested that two binding sites for Gli were present. The IC<sub>50</sub> values for the high- and low-affinity binding sites of Gli were shown in Table 2. Their respective  $n_H$  values were found to be close to unity (not shown). IC<sub>50</sub> value for inhibition of [<sup>3</sup>H]P1075 binding in RVHR aorta strips by Gli at high affinity site increased by more than 2-fold compared with that in NWR aorta preparation, but there was no significant difference between IC<sub>50</sub> values at the low

affinity site in RVHR and in NWR (Table 2).

The kinetics of association and dissociation of [<sup>3</sup>H]P1075, measured at large excess of label over binding sites, are illustrated in Figure 3. In NWR, association kinetics of [<sup>3</sup>H]P1075 at 5 nmol/L followed a pseudo first-order kinetics, and the fit of this kinetics to equation 1 yielded a  $K_{app}$  value of 0.044 $\pm$ 0.004 min<sup>-1</sup>. After equilibrium was reached, dissociation of the receptor-label complex was initiated by addition of 50 μmol/L unlabeled P1075. The dissociation also followed a pseudo first-order kinetics with a  $K_2$  value of 0.029 $\pm$ 0.005 min<sup>-1</sup>. Using equation 2, these values of  $K_{app}$  and  $K_2$  allowed calculation of the second order rate constant of association,  $K_1$  to 0.009 $\pm$ 0.001 nmol·L<sup>-1</sup>·min<sup>-1</sup>. From these rate constants,  $K_D$  value of 3.3 $\pm$ 1.0 nmol/L, where the large error in this value is explained by the propagation of errors, was calculated.

After NWR aorta strips were pretreated with Gli 100 μmol/L for 10 min, association and dissociation kinetics of [<sup>3</sup>H]P1075 still followed the pseudo first-order kinetics. However, association and dissociation were slowed down with the reduced  $K_{app}$  and  $K_2$  value, but with increased  $K_D$  value compared to those in vehicle (Table 3).

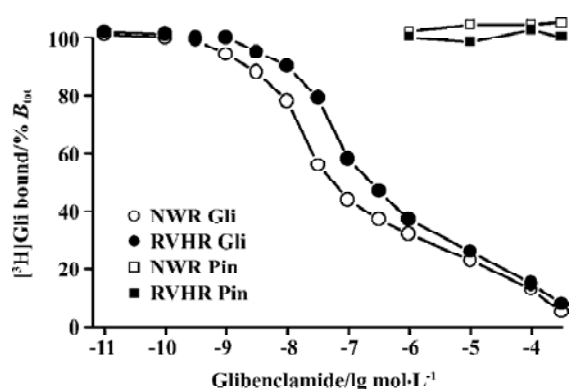
In RVHR, the association and dissociation of [<sup>3</sup>H]P1075 with aorta strips were also fitted the pseudo first-order kinetics, but slower association and dissociation kinetics were observed with increased  $K_D$  value compared to those in NWR. Gli at 100 μmol/L preincubated with aorta strips for 10 min, did not alter the association and dissociation of [<sup>3</sup>H]P1075 with RVHR aorta strips (Figure 3, Table 3).

**[<sup>3</sup>H]Gli binding experiments** The competitive inhibition curve of 3 nmol/L [<sup>3</sup>H]Gli binding to aorta strips in NWR and RVHR by unlabeled Gli exhibited biphasic plots with

**Table 3.** Modulation by vehicle or Gli 100 μmol/L, preincubated for 10 min, of the association and dissociation kinetics of [<sup>3</sup>H]P1075 with aorta strips in NWR and RVHR. *n*=4–7 separate determinations performed in duplicate. Mean±SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs NWR, <sup>e</sup>*P*<0.05, <sup>f</sup>*P*<0.01 vs vehicle. For experiment details see Figure 3.

Animal groups	Pretreated factors	Association kinetics		Dissociation kinetics	
		<i>K</i> <sub>app</sub> /min <sup>-1</sup>	<i>K</i> <sub>1</sub> /nmol·L <sup>-1</sup> ·min <sup>-1</sup>	<i>K</i> <sub>2</sub> /min <sup>-1</sup>	<i>K</i> <sub>D</sub> /nmol·L <sup>-1</sup>
NWR	Vehicle	0.044±0.004	0.009±0.001	0.029±0.005	3.3±1.0
	Glibenclamide	0.031±0.002 <sup>f</sup>	0.002±0.001 <sup>f</sup>	0.020±0.005 <sup>e</sup>	8.3±2.1 <sup>f</sup>
RVHR	Vehicle	0.034±0.004 <sup>b</sup>	0.003±0.001 <sup>b</sup>	0.021±0.003 <sup>b</sup>	8.1±1.0 <sup>c</sup>
	Glibenclamide	0.035±0.004	0.003±0.001	0.022±0.004	8.5±1.2

*n*<sub>H</sub> significantly different from unity (Figure 4, Table 4). Inhibition curves were fitted with the one-site and two-site model, respectively. Statistical analysis with the *F*-test showed that the fit of curves of the two-site model was significantly superior to the one-site model (Table 4). Thus,



**Figure 4.** Inhibition of [<sup>3</sup>H]glibenclamide (Gli) binding to aorta strips in NWR and RVHR by unlabeled Gli and Pin. Aorta strips were incubated with [<sup>3</sup>H]Gli 3 nmol/L in the presence of various concentrations of unlabeled Gli or pinacidil for 90 min at 25 °C. Depicted are representative plots. *B*<sub>tot</sub> was 30.5±2.5 pmol·g<sup>-1</sup> wet weight. The Data are summarized in Table 4.

two distinct binding sites for [<sup>3</sup>H]Gli were present in NWR and RVHR aorta preparations. In NWR aorta strips, the IC<sub>50</sub> value for the inhibition of [<sup>3</sup>H]Gli binding at the high- and low-affinity sites by unlabeled Gli was 18±2 nmol/L and 21±4 μmol/L, respectively. The fit of one-site model gave *n*<sub>H</sub> equal to 0.80±0.09; a value in favor of heterogeneous class of binding sites with a negative cooperativity. In RVHR aorta strips, the IC<sub>50</sub> value for inhibition of [<sup>3</sup>H]Gli binding at low-affinity sites by unlabeled Gli showed no difference from that in NWR aorta preparations, but IC<sub>50</sub> at high-affinity sites increased approximately 4 fold. Pin, at concentrations up to 500 μmol/L neither inhibited the [<sup>3</sup>H]Gli binding to aorta strips in NWR nor in RVHR (Figure 4).

[<sup>3</sup>H]Gli binding to NWR aorta strips was rapid and approached equilibrium after a 40-50 min incubation period at 25 °C (data not shown). After equilibrium was reached, the dissociation of [<sup>3</sup>H]Gli-receptor complex was initiated by the addition of 30 μmol/L unlabeled Gli. Association and dissociation of [<sup>3</sup>H]Gli followed pseudo first-order kinetics. The fit of the association and dissociation to equation 1 and 3 yielded *K*<sub>app</sub> and *K*<sub>2</sub> of 0.046±0.004 and 0.007±0.001 min<sup>-1</sup>, respectively. After NWR aorta strips were pretreated with Pin (100 μmol/L) for 10 min, association and dissociation

**Table 4.** Displacement of [<sup>3</sup>H]Gli binding to aorta strips in NWR and RVHR by unlabeled Gli. For experiment details see Fig 4. Analysis with one-site model showed *n*<sub>H</sub> was significantly different from unity. *n*=4-5 experiments performed in duplicate. Mean±SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs NWR.

Group	One-site fit		Two-site fit		<i>F</i> -test for hypothesis: one-site model is correct	
	IC <sub>50</sub> /nmol·L <sup>-1</sup>	<i>n</i> <sub>H</sub>	IC <sub>50,H</sub> /nmol·L <sup>-1</sup>	IC <sub>50,L</sub> /μmol·L <sup>-1</sup>	<i>F</i> value	<i>P</i> <sup>1)</sup>
NWR	35±7	0.80±0.09	18±2	21±4	171	<0.0001
SHR	54±9 <sup>b</sup>	0.82±0.12	59±4 <sup>c</sup>	22±6	142	<0.0001

<sup>1)</sup> If *P*>0.05, the hypothesis will be accepted; If *P*<0.05, the hypothesis is rejected, and the fit of the two-site model is significantly superior, statistically, to the one-site model.

of [<sup>3</sup>H]Gli still followed pseudo first-order kinetics. However, when the association was retarded,  $K_{app}$  was reduced by 45.6%, but when the dissociation was accelerated,  $K_2$  was increased approximately 2 fold (Table 5).

In RVHR aorta strips, association and dissociation of [<sup>3</sup>H]Gli were also fitted pseudo first-order kinetics, but when association became slower,  $K_{app}$  was reduced by 39%, but when dissociation was faster,  $K_2$  was increased 1.5-fold. From these kinetics, the  $K_D$  value calculated was increased 3.3-fold compared with those in NWR aorta strips. After RVHR aorta strips were pretreated with Pin 100  $\mu$ mol/L for 10 min, the association and dissociation of [<sup>3</sup>H]Gli were not altered. From these kinetics, the  $K_D$  value calculated was also unchanged (Table 5).

## Discussion

**Comparison of [<sup>3</sup>H]P1075 binding in NWR with that in RVHR** In the present study, saturation experiments in NWR showed that aorta strips [<sup>3</sup>H]P1075 bound to a single class of sites with high affinity ( $K_D=7.7\pm 2.0$  nmol/L) and relatively low density ( $B_{max}=26\pm 4$  nmol·g<sup>-1</sup> wet weight). These results are similar to those reported by Quast *et al*<sup>[13]</sup>. The  $K_D$  value determined here corresponded well to the potency of P1075 as a vasorelaxant agent<sup>[14]</sup>. The  $K_{ATP}$  opener P1075 and Pin, as well as blocker Gli, inhibited [<sup>3</sup>H]P1075 binding according to the Law of Mass Action. The  $IC_{50}$  value ( $9.1\pm 1.3$  nmol/L) for inhibition of [<sup>3</sup>H]P1075 binding by P1075 was in fair agreement with the  $K_D$  value obtained in the saturation experiments. The association and dissociation of [<sup>3</sup>H]P1075 with NWR aorta strips were time-dependent and monoexponential, these kinetic experiments allow a calculation of  $K_D$  value of  $3.3\pm 1.0$  nmol/L in reasonable agreement with the result of the saturation experiments (Table 1) and of competitive inhibition experiments (Table 2).

In the present study, [<sup>3</sup>H]P1075 binding in RVHR aorta strips was reported. The binding data showed that [<sup>3</sup>H]P1075 binding sites in RVHR aorta strips had similar qualities to those in NWR aorta preparations. However, some differences in [<sup>3</sup>H]P1075 binding in RVHR aorta strips were found: (1)  $K_D$  value of [<sup>3</sup>H]P1075 binding increased approximately 1.5-fold; (2)  $IC_{50}$  value for the inhibition of [<sup>3</sup>H]P1075 binding by P1075 and Pin increased by 1.8- and 1.7-fold, respectively; (3) association and dissociation became slower. These results suggest that the affinity of [<sup>3</sup>H]P1075 binding sites to  $K_{ATP}$  openers is reduced in RVHR aorta strips. Therefore, we suggest that chronic hypertension might reduce the affinity of binding sites for  $K_{ATP}$  openers in aorta. Furthermore, the  $B_{max}$  value of [<sup>3</sup>H]P1075 binding to aorta strips and the maximum inhibition of [<sup>3</sup>H]P1075 binding by P1075 and Pin showed no difference between RVHR and NWR. It is suggested that the number of [<sup>3</sup>H]P1075 binding sites is not altered in RVHR aorta strips. The present findings might explain why the dilatation of isolated aorta precontracted with norepinephrine or KCl in response to activation of  $K_{ATP}$  channels by Pin was impaired in SHR, why dilatation of the basilar artery by aprikalim was impaired in stroke-prone SHR *in vivo*<sup>[9]</sup>; and why the action of levromakalim on  $K_{ATP}$  channels in mesenteric artery muscle cells of SHR was decreased compared to normotensive rat cells<sup>[10]</sup>. The present findings also exclude the possibility that differences in response to Pin, aprikalim and levromakalim are related to genetic differences between normotensive rats and SHR that are unrelated to hypertension.

**Comparison of [<sup>3</sup>H]Gli binding in NWR with RVHR** In the present study, the binding of [<sup>3</sup>H]Gli in NWR aorta strips was specific and reversible. Using a wide range of concentrations for Gli, we obtained evidence for the presence of both high and low affinity Gli-binding sites in aorta preparations: the former ranging from  $1\times 10^{-11}$  to  $1\times 10^{-7}$  mol/L

**Table 5.** Modulation by Pin(100  $\mu$ mol/L) preincubated for 10 min, of the association and dissociation kinetics of [<sup>3</sup>H]Gli binding to aorta strips in NWR and RVHR.  $n=4-7$  separate determinations performed in duplicate. Mean $\pm$ SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs NWR; <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs Vehicle. For experiment details see Figure 3.

Animal groups	Pretreated factors	Association kinetics		Dissociation kinetics	
		$K_{app}/\text{min}^{-1}$	$K_1/\text{nmol}\cdot\text{L}^{-1}\cdot\text{min}^{-1}$	$K_2/\text{min}^{-1}$	$K_D/\text{nmol}\cdot\text{L}^{-1}$
NWR	Vehicle	0.046 $\pm$ 0.004	0.013 $\pm$ 0.001	0.007 $\pm$ 0.001	0.55 $\pm$ 0.14
	Pinacidil	0.025 $\pm$ 0.007 <sup>c</sup>	0.004 $\pm$ 0.002 <sup>f</sup>	0.014 $\pm$ 0.003 <sup>e</sup>	3.50 $\pm$ 0.87 <sup>f</sup>
SHR	Vehicle	0.028 $\pm$ 0.009 <sup>c</sup>	0.006 $\pm$ 0.003 <sup>c</sup>	0.011 $\pm$ 0.002 <sup>b</sup>	1.83 $\pm$ 0.67 <sup>c</sup>
	Pinacidil	0.026 $\pm$ 0.005	0.005 $\pm$ 0.002 <sup>f</sup>	0.012 $\pm$ 0.004	2.00 $\pm$ 0.42

$K_{app}$  denote apparent rate constant of association,  $K_1$ : rate constant of association,  $K_2$ : rate constant of dissociation.

with nanomolar affinity constant, and the latter from  $1 \times 10^{-7}$  to  $1 \times 10^{-3.5}$  mol/L with micromolar affinity constant. Our results are consistent with those reported previously in retarding vascular smooth muscle<sup>[12]</sup>.

[<sup>3</sup>H]Gli binding in these preparations had similar qualities to those in NWR. However, [<sup>3</sup>H]Gli exhibited slower association and faster dissociation with an increased  $K_D$  value in RVHR than in NWR (Table 5). The  $IC_{50}$  value for the inhibition of [<sup>3</sup>H]Gli binding by Gli at the high affinity component was increased, but was unchanged at the low affinity component. This indicates that the affinity of high affinity sulfonylurea sites is decreased in RVHR aorta strips.

**Interaction of binding sites for  $K_{ATP}$  openers and blockers in NWR** In the present study, [<sup>3</sup>H]P1075 bound to a single site in NWR aorta strips, whereas [<sup>3</sup>H]Gli bound to both high and low affinity sites in NWR aorta strips. [<sup>3</sup>H]P1075 binding can be inhibited by opener P1075 and Pin, but not completely by blocker Gli, whereas [<sup>3</sup>H]Gli binding could be inhibited by Gli, but not by Pin. Furthermore, [<sup>3</sup>H]P1075 and [<sup>3</sup>H]Gli binding present a similar association rate (0.044 and 0.046 nmol·L<sup>-1</sup>·min<sup>-1</sup>) but different dissociation rate (0.029 and 0.007 nmol·L<sup>-1</sup>·min<sup>-1</sup>, respectively). The most plausible explanation for these results is that  $K_{ATP}$  openers and blockers bind to different binding sites. In addition, Pin, preincubated with NWR aorta strips, slowed down the association of [<sup>3</sup>H]Gli and sped up the dissociation of [<sup>3</sup>H]Gli-receptor complex. It is suggested that the binding sites for  $K_{ATP}$  channel opener Pin negatively allosterically couple to the binding sites for  $K_{ATP}$  channel blocker Gli. The plausible mechanism is that the binding of Pin to its sites in SURs brings about configuration alteration in the Gli-sites, which hinders the binding of [<sup>3</sup>H]Gli to its sites and facilitates faster release of the radiolabel from receptor-label complex. Likewise, Gli, preincubated with NWR aorta strips, also slowed down the association of [<sup>3</sup>H]P1075 and sped up the dissociation of [<sup>3</sup>H]P1075-receptor complex. It was indicated that the sites for  $K_{ATP}$  channel blocker Gli also negatively allosterically coupled to the sites for  $K_{ATP}$  channel opener P1075. Alternatively, Gli down-regulates the affinity of the P1075 binding sites through inducing its configuration alteration. The evidence for a negative allosteric coupling between the sites for P1075 and Gli has been previously obtained for the rat aorta<sup>[8]</sup>. In the experiments, however, rat aorta preparations were only labeled with [<sup>3</sup>H]P1075, and the dissociation of [<sup>3</sup>H]P1075-receptor complex was initiated by addition of an excess of Gli rather than P1075 after [<sup>3</sup>H]P1075 binding reached equilibrium. In the present study, aorta strips were pretreated with Gli, and dissociation was initiated by addition of an excess of P1075

rather than Gli. A negative allosterism between the sites for openers and blockers was identified in a different experimental protocol in this study.

**Interaction of binding sites for  $K_{ATP}$  openers and blockers in RVHR** In the present paper, we studied the interaction between the binding sites for  $K_{ATP}$  openers and blockers in RVHR aorta strips. The evidence that  $K_{ATP}$  openers and blockers bound to different binding sites was also obtained in RVHR. In contrast to NWR, pretreatment with Gli failed to alter the association and dissociation kinetics of [<sup>3</sup>H]P1075 with aorta strips in RVHR. It is suggested that the negative allosteric action of sulfonylurea Gli on the binding sites for the opener P1075 was damaged in RVHR aorta strips. Similarly, pretreatment of Pin did not alter the association and dissociation kinetics of [<sup>3</sup>H]Gli with RVHR aorta strips. It is indicated that the negative allosteric action of opener Pin on the binding sites for sulfonylurea Gli is also damaged in SHR aorta strips.

In conclusion, the affinity of P1075 binding sites and high-affinity sulfonylurea binding sites are reduced, and the negative allosteric interactions between the two binding sites are impaired in RVHR aorta strips.

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