The Effects of Potassium Channel Openers and Blockers on the Specific Binding Sites for [³H]Glibenclamide in Rat Tissues

Toru Yamashita, Yukinori Masuda and Sakuya Tanaka

Shiraoka Research Station of Biological Science, Nissan Chemical Industries, Ltd., 1470 Shiraoka, Minamisaitama, Saitama 349-02, Japan

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ABSTRACT—The effects of K⁺ channel openers (PCOs), NIP-121, levcromakalim and nicorandil, and the blockers of the specific binding sites for [³H]glibenclamide, ATP-sensitive K⁺ channel blocker, were investigated in rat brain and cardiac ventricle membrane preparations. When the microsomes were incubated with [³H]glibenclamide, the specific glibenclamide binding was fully inhibited by unlabeled glibenclamide (1 μM) and apamin (100 μM). However, the specific glibenclamide binding was not influenced by excess NIP-121, levcromakalim and nicorandil, although glibenclamide antagonized the increase in the ⁸⁶Rb⁺ efflux by PCOs. On the other hand, the binding of [³H]glibenclamide after a long pre-incubation (60 min) at 37°C with NIP-121 and levcromakalim at pharmacological effective concentrations (10 nM to 1 μM) was significantly influenced. Both PCOs partially reduced both Kd and Bmax values of the specific [³H]-glibenclamide binding in a concentration-dependent manner that was not regulated by GTPγS. The dose-effect relationships for the Bmax’s of NIP-121 and levcromakalim seemed similar to those for vasorelaxation. These findings indicate that the pharmacological effect of PCO may be caused by the binding to its own specific sites but not to the specific sulfonylurea sites. The binding of PCOs may inhibit, in a negative allosteric manner the binding of sulfonylureas.

Keywords: [³H]Glibenclamide, K⁺ channel opener, NIP-121, Brain, Heart

Sulfonylurea derivatives such as glibenclamide and tolbutamide, which are clinically applied as orally active antidiabetic agents, are specific blockers for the ATP-sensitive potassium current (IₖATP). On the other hand, potassium channel openers (PCOs) specifically activate IₖATP, resulting in the increase of potassium ion permeability through the cell membrane (1, 2). Glibenclamide was reported to competitively antagonize the relaxation of the rat aorta (3–6), rat portal vein (4, 7) and guinea pig pulmonary artery (8) and the negative inotropic action of the canine atrial muscle (9) induced by PCOs such as NIP-121 (4–6), cromakalim (3–9), pinacidil (8, 9), minoxidil sulfate (7), RP 49356 (8) and nicorandil (9). In addition, the shortening of the action potential duration in the guinea pig ventricular cells with intracellular adenosine triphosphate (ATP) depletion, which is due to the activation of IₖATP, was reportedly inhibited by glibenclamide (10). In the canine atrial muscle, non-sulfonylureas tetaethylammonium (TEA), tetrabutylammonium (TBA) and BaCl₂ at low concentrations (<1 mM), but not TEA at a high concentration (3 mM), were reported to competitively antagonize the negative inotropic effect of cromakalim and pinacidil (11). On the other hand, Orito et al. reported that TEA and TBA non-competitively inhibited the levcromakalim-induced relaxation of the canine coronary artery (12). They suggested that TEA and TBA blocked the effect of PCOs by binding to a possible high-affinity site for tetraalkylammonium in the canine coronary artery (12). The slopes of the Schild plots provided proof for the above-mentioned competitive antagonisms. Therefore, the relationship between PCO and sulfonylurea derivatives is akin to that between agonists and antagonists, which is based upon classical pharmacological theory. However, in earlier receptor binding studies, PCOs reportedly did not displace the specific [³H]-glibenclamide binding in the competition assay protocol (13, 14). In addition, Bray and Quast (15) and Quast et al. (16) suggested that sulfonylurea derivatives inhibited in a negative allosteric manner the specific binding of PCO on the ATP-sensitive potassium channels.

In this study, the effects of PCOs, such as the pyranobenzoxadiazol derivative NIP-121 that is about 10 times more potent than the benzopyran derivative cromakalim (4–6, 17–20), levcromakalim ((−)-enan-
tiomer of cromakalim) and nicorandil, on the specific binding sites for \(^{[3]H}\)glibenclamide in rat brain and ventricle membrane preparations were thoroughly examined by not only the competition assay but also the saturation assay protocol. In addition, the properties of the specific binding sites for \(^{[3]H}\)glibenclamide were characterized by using the above-described potassium channel blockers and by using phenolamine, which is reported to be an antagonist for PCO (21–23).

**MATERIALS AND METHODS**

Male Wistar rats, each weighing 290 to 410 g, were purchased from Charles River Japan (Kanagawa). All animals were sacrificed by venesection under deep anesthesia induced by an intravenous injection of an excess of sodium pentobarbital.

**Membrane preparations of the rat aorta, heart and brain**

Crude membrane preparations of the rat aorta, heart and brain were prepared according to a modified procedure based on an earlier method (24), as follows: The tissues were dissected, and then adherent fat and connective tissue were removed from them in oxygenated Krebs-Henseleit solution (37°C) of the following composition: 118.4 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO\(_4\), 2.5 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 25.0 mM NaHCO\(_3\) and 11.7 mM glucose. The pooled tissues were finely minced with scissors and homogenized in 20 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) by ten 5-sec bursts on the Polytron (Kinematica, Lucern, Switzerland) at a setting of 7. The homogenate was centrifuged at 48,000 \(\times\) g for 60 min at 4°C. The pellet was resuspended in ice-cold Tris-HCl buffer by a 2-sec burst on the Polytron. This washed suspension was again sedimented by centrifugation at 48,000 \(\times\) g for 60 min at 4°C, and the final pellet was resuspended in 2 ml of fresh solution containing 1000 nM or the vehicle as the control for 60 min, in the presence or absence of 100 \(\mu\)M guanosine 5'-O-(3-thiotriphosphate) (GTP\(_\gamma\)S). The incubation mixture was diluted with 5 ml of ice-cold Tris-HCl buffer, vacuum-filtered through glass fiber filters (GF/C; Whatman, Maidstone, UK) and washed with 20 ml of ice-cold buffer at a flow rate of 1 ml/sec. The filters were then dried and placed in scintillation vials containing 5 ml scintillator (Econo-fluor; New England Nuclear, Boston, MA, USA) for at least 3 hr. The radioactivity was measured in a liquid scintillation counter (Tri-Carb 340CD; Packard, Zurich, Switzerland) at an efficacy of 45%. The specific binding of \(^{[3]H}\)glibenclamide was defined as the total binding minus the blanks determined in the presence of 10 \(\mu\)M unlabeled glibenclamide. The \(^{[3]H}\)glibenclamide dissociation constant (\(K_d\)) and the maximum number of specific binding sites (\(B_{max}\)) were computed by Scatchard analysis (26) for a single class of receptor sites or a non-linear least squares program for two or multiple classes of receptor sites.

To study the displacement of specific \(^{[3]H}\)glibenclamide receptor binding by other unlabeled compounds, the crude membrane preparations and \(^{[3]H}\)glibenclamide (1 nM for brain preparation and 0.5 or 2 nM for ventricle preparation) were incubated with increasing concentrations of NIP-121, levcromakalim, nicorandil, unlabeled glibenclamide, TEA and 4-aminopyridine (4-AP) for 20 min at 0, 25 or 37°C. Thereafter, the binding was studied as described above. The apparent \(K_d\) values were determined from the formula of Cheng and Prusoff (27), \(K_d = IC_{50}/(1 + L/K_d)\), where \(L\) = the radioligand concentration, and the apparent Hill coefficient or slope factor of the drug was determined from Hill or pseudo Hill plots, respectively (28).

**Effects on \(^{86}\)Rb\(^{+}\) efflux**

The thoracic aortas were dissected free of adherent fat and connective tissue and then cut into spiral strips, 3 mm in width and 15 mm in length, in modified Krebs solution (MKS) of the following composition: 121.9 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 15.5 mM NaHCO\(_3\) and 11.5 mM glucose, which was heated to 37°C and bubbled with 95% O\(_2\)/5% CO\(_2\). The endothelium of all preparations was removed using a cotton stick. The \(^{86}\)Rb\(^{+}\) efflux procedures were essentially as described by Hamilton et al. (29). After a 60-min equilibration period in the bathing solution maintained at 37°C and gassed with 5% CO\(_2\) in O\(_2\), tissues were loaded with \(^{86}\)Rb\(^{+}\) (74 kBq/ml) for 90 min. The \(^{86}\)Rb\(^{+}\) was then allowed to flow from the tissue into non-radioactive MKS. The tissues were transferred to 2 ml of fresh solution every 2 min. Thirty minutes after the start of efflux, the tissues were exposed to a solution containing NIP-121
(100 nM) or NIP-121 and glibenclamide (10-100 nM). At the end of the efflux study, the strips were placed in 0.5 ml Soluene-350 (Packard) at 50°C for 3 hr. The dissolved samples and 0.1 ml aliquots of the bathing solution were added to 5 ml of Hionic-Fluor (Packard) scintillation fluid, and the radioactive was measured.

Effects on vasorelaxant effect

In the tissue bath study, the isolated thoracic aorta was cut into a spiral strip, 3 x 15 mm, in Krebs-Henseleit solution at 37°C bubbled with 95% O₂/5% CO₂, and the endothelium was removed. Isometric tension was recorded under a resting tension of 1 g (TB-611T; Nihon Kohden, Tokyo). The aortic strips were contracted with a sub-maximal concentration (final concentration, 30 mM) of hypertonic KCl after the equilibration. NIP-121 or levcromakalim was cumulatively added to the preparation in half log unit increments after the contractile responses reached a plateau. At the end of the study, 100 μM papaverine was added to induce maximum relaxation. The degree of relaxation produced is expressed as a percentage of the maximum induced by papaverine.

**Drugs**

The following compounds were used: NIP-121, levcromakalim and nicorandil which were synthesized at the Central Research Laboratories of Nissan Chemical Industries, Ltd., Chiba; glibenclamide, TEA chloride, apamin, 4-AP, GTPγS and papaverine (Sigma Chemical Co.); phentolamine mesilate (Regitin inj.; Ciba Geigy Japan, Takarazuka). NIP-121 and levcromakalim (3 x 10⁻³ M) were dissolved in 50% dimethyl sulfoxide (DMSO) and serially diluted with distilled water. Nicorandil and phentolamine were dissolved in distilled water. Glibenclamide, TEA, apamin and 4-AP were dissolved in DMSO. The final concentrations of DMSO were from 1.7 x 10⁻⁸ to 0.17%. The vehicle used did not influence the binding of [³H]glibenclamide.

**Radioactive labeled ligands**

[Cyclohexyl-2,3-³H(N)]glibenclamide ([³H]glibenclamide), with a specific activity of 1883.3 GBq/µmol (radiochemical purity, 99%), and [⁸⁶RbCl], with a specific activity of 730 GBq/g (radiochemical purity, >99%), were purchased from DuPont-NEN (Boston, MA, USA). The [³H]glibenclamide, supplied in ethanol, was further diluted in distilled water. The final concentrations of the vehicle were from 0.003 to 0.1%. [⁸⁶RbCl] was supplied in a 0.5 M HCl solution and further diluted with distilled water.

**Statistical analyses**

In the saturation assay, the data were analyzed by means of two-way analysis of variance followed by Tukey’s multiple comparison test. In the [⁸⁶Rb] efflux experiment, the data were analyzed by means of Student’s paired t-test.

**RESULTS**

Identification of [³H]glibenclamide specific binding sites in the membranes from rat brain, heart and aorta

[³H]Glibenclamide bound reversibly and saturably to membranes from the rat brain and heart (<20 min) (data not shown). The saturation isotherms indicated specific and saturable binding sites for [³H]glibenclamide (Fig. 1), with non-specific binding representing less than 20% of

![Graph](image-url)
the total at 0.5 nM and about 30% at 2 nM. Scatchard analysis of the saturation isotherm resulted in a single plot for the rat brain membrane, indicating a single class of binding sites, and a biphasic plot for the rat ventricle membrane, indicating more than one type of binding site. The dissociation constant ($K_d$) was 0.245 nM for binding to the rat brain membrane and the maximum specific binding ($B_{\text{max}}$) was 115.6 fmoles/mg protein. The Hill coefficient was 1.12 ($r = 0.955, P < 0.001$), which also indicates a single class of binding sites for $[^3H]$glibenclamide. The apparent $K_d$ value in ventricular membranes corresponding to that of the high affinity binding site ($K_{dH}$ value) was 0.211 nM, whereas that corresponding to the low affinity binding site ($K_{dL}$ value) was 1.928 nM. The respective apparent $B_{\text{max}}$ concentrations ($B_{\text{maxH}}$ and $B_{\text{maxL}}$ values) were 53.6 and 146.6 fmoles/mg protein, respectively. The Hill coefficient was 0.495 ($r = 0.989, P < 0.001$), which was consistent with the results of nonlinear least squares analysis.

The effects of NIP-121, levromakalim and nicorandil on $[^3H]$glibenclamide specific binding (simultaneous incubation of all ligands)

When the microsome and $[^3H]$glibenclamide were simultaneously incubated with NIP-121, levromakalim or nicorandil, the PCOs did not displace $[^3H]$glibenclamide specific binding in the rat brain membrane at either 25°C or 0°C (Fig. 2) or that in rat ventricle membrane at concentrations of 0.5 or 2 nM $[^3H]$glibenclamide, respectively (Fig. 3).

![Fig. 2](image_url)

**Fig. 2.** Displacement of $[^3H]$glibenclamide by various potassium channel openers in rat brain membranes at 25°C (A) and 0°C (B). $[^3H]$Glibenclamide (1 nM) was incubated with 0.23 mg protein for 20 min in the presence of increasing concentrations of: □, NIP-121; △, levromakalim; ▽, nicorandil and ○, glibenclamide. Each value represents the mean of three separate experiments performed in triplicate.

![Fig. 3](image_url)

**Fig. 3.** Displacement of $[^3H]$glibenclamide by various potassium channel openers in rat ventricle membranes. 0.5 (A) or 2 nM (B) $[^3H]$glibenclamide was incubated with 0.35 mg protein for 20 min at 25°C in the presence of increasing concentrations of: □, NIP-121; △, levromakalim; ▽, nicorandil and ○, glibenclamide. Each value represents the mean of three separate experiments performed in triplicate.
The effects on \[^{3}H\]glibenclamide specific binding after the preincubation of PCOSs and the pharmacological effects

Under these conditions, \[^{3}H\]glibenclamide bound (0.0625 to 2 nM) to the specific sites in a reversible and saturable manner in the rat brain membrane. Scatchard analysis of the saturation isotherm elicited a monophasic plot, indicating the possible existence of a single class of binding sites. Table 1 shows the \(K_d\) value and \(B_{\text{max}}\) value of the specific \[^{3}H\]glibenclamide binding in the absence or presence of GTP\(_7\)S (100 \(\mu\)M) (control value). GTP\(_7\)S did not significantly change either the \(K_d\) or \(B_{\text{max}}\) value. All Hill coefficients from the saturation curves were close to 1, which also indicates a single class of binding sites for \[^{3}H\]glibenclamide. We confirmed in advance of this study that unlabeled glibenclamide fully inhibited, in a concentration-dependent manner, the \(B_{\text{max}}\) value (data not shown).

On the other hand, preincubation with NIP-121 or levocromakalim for 60 min partially decreased, in a concentration-dependent manner, the \(K_d\) and \(B_{\text{max}}\) value of the specific \[^{3}H\]glibenclamide binding and maximally decreased both values at 100 or 300 nM, respectively (\(P<0.05\) vs control value) (Figs. 4 and 5A, and Table 1). The \(IC_{50}\) values of NIP-121 and levocromakalim on the \(B_{\text{max}}\) inhibition were 3.1±1.8 and 12.9±7.4 nM (\(P>0.05\)), respectively, and the maximum inhibition by NIP-121 and levocromakalim was 23.7±6.1 and 27.1±5.3%, respectively. On the other hand, the \(IC_{50}\) values of NIP-121 and levocromakalim on the vasorelaxant effect were 6.5±0.3 and 19.2±0.8 nM, respectively, and the maximum relaxation by NIP-121 and levocromakalim was 84.9±2.0 and 82.5±2.3%, respectively. The dose-effect relationships for the \(B_{\text{max}}\)'s of NIP-121 and levocromakalim seemed similar to those for vasorelaxation (Fig. 5).

### Table 1. Summary of the specific \[^{3}H\]glibenclamide binding in the absence and presence of GTP\(_7\)S (100 \(\mu\)M) and the inhibitory effects of NIP-121 (100 nM) and levocromakalim (300 nM) on the binding properties of \[^{3}H\]glibenclamide in rat brain membrane preparations

<table>
<thead>
<tr>
<th>Compound</th>
<th>(K_d) value (nM)</th>
<th>(B_{\text{max}}) value (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without GTP(_7)S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.216±0.018</td>
<td>126.4±18.4</td>
</tr>
<tr>
<td>NIP-121</td>
<td>0.068±0.009**</td>
<td>96.4±9.1*</td>
</tr>
<tr>
<td>Levocromakalim</td>
<td>0.084±0.011**</td>
<td>92.1±10.5*</td>
</tr>
<tr>
<td>With GTP(_7)S (100 (\mu)M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.194±0.020</td>
<td>139.0±15.3</td>
</tr>
<tr>
<td>NIP-121</td>
<td>0.052±0.007**</td>
<td>84.6±10.4*</td>
</tr>
<tr>
<td>Levocromakalim</td>
<td>0.062±0.008**</td>
<td>90.3±8.7*</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E.M. of four separate experiments performed in triplicate. *\(P<0.05\) and ** \(P<0.01\) vs each control group.

Displacement properties of various potassium channel blockers on \[^{3}H\]glibenclamide specific binding

Not only the unlabeled \(I_{K(ATP)}\) blocker glibenclamide, but also the small conductance calcium-activated potassium channel (ISK(Ca)) blocker apamin (30), fully displaced the specific binding of \[^{3}H\]glibenclamide in both membrane preparations in a concentration-dependent manner (Fig. 6). The voltage-dependent potassium channel blocker 4-AP and TEA in a pharmacologically effective concentration range (10 to 30 mM) partially inhibited the specific binding of \[^{3}H\]glibenclamide, especially in the ventricle membrane. The slope factors from the competition of unlabeled glibenclamide at all concentrations on brain and ventricle membranes were 0.502 (\(r=0.984, P<0.01\), means of three separate experiments performed in triplicate) and 0.400 (\(r=0.970, P<0.05\)), respectively, which indicate dual classes of binding sites for \[^{3}H\]glibenclamide. In the brain preparation, the result from the saturation assay was inconsistent with that from the competition assay. This discrepancy might be caused by the use of glibenclamide at different concentration ranges in the experimental protocols. If \[^{3}H\]glibenclamide of much higher concentrations (more than 10 nM) was used in a saturation as-
Fig. 5. Effects on the specific \([^3\text{H}]\text{glibenclamide binding after pre-}
\text{incubation with NIP-121 (O) and levermakalim (○) and the}
\text{vasorelaxant effects. A: Inhibition of the } B_{\text{max}} \text{ value of the specific}
[^3\text{H}]\text{glibenclamide binding in rat brain membrane preparations.}
\text{NIP-121 or levermakalim was pre-incubated with the membranes}
\text{for 60 min before each } [^3\text{H}]\text{glibenclamide saturation assay. Each}
\text{point represents the mean ± S.E.M. of four separate experiments per-
formed in triplicate. B: Vasorelaxant effect on 30 mM KCl-induced}
\text{contraction of the rat aorta. Each point represents the}
\text{mean±S.E.M. of five experiments.}

say, dual classes of sites might be obtained in brain mem-
brane preparations. The apparent inhibition constants
(pK\(_i\)) of high and low affinity sites in the brain membrane
were 11.40 and 9.83, respectively, and those in the ventri-
cle membrane were 10.9 and 6.82, respectively. In the ven-
tricle membrane, the slope factors from the curves of
glibenclamide above about 10\(^{-7}\) M, apamin and 4-AP
were similar, being 1.18, 1.32 and 1.22, respectively. In ad-
dition, the slope factors from the curves of glibenclamide
at concentrations below about 10\(^{-7}\) M and TEA were also
computed to be about the same values, being 0.47 and
0.58, respectively. The apparent \(K_i\) of the potassium chan-
nel blockers were not calculated because the binding sites
for each blocker were not identified in this study.

The effect of phentolamine on \([^3\text{H}]\text{glibenclamide specific}
\text{binding}

Phentolamine, an \(\alpha_1\)-adrenoceptor antagonist that an-
tagonizes the effect of PCOs (4, 21–23), did not displace
[^3\text{H}]\text{glibenclamide specific binding in the rat brain or ven-
tricle membranes (Fig. 6).}

The antagonism of glibenclamide and apamin against the
\text{NIP-121-induced } ^{86}\text{Rb}\(^+\) efflux increment

NIP-121 (100 nM) significantly increased the rate of
^{86}\text{Rb}\(^+\) efflux above the basal level. Glibenclamide at 10 to
100 nM and apamin at the excess concentration of 10 \(\mu\text{M}

Fig. 6. Displacement of \([^3\text{H}]\text{glibenclamide by various potassium}
\text{channel blockers and phentolamine in rat brain (A) and ventricle}
\text{membranes (B). [^3\text{H}]Glibenclamide at 1 nM (brain) or 2 nM (ventri-
}
confirms that [H]glibenclamide also binds to a single class of binding sites in the brain membrane in the saturation assay examined at a wider concentration ranges (0.0625 to 10 nM) (data not shown).

In the present study, we failed to obtain specific binding sites for glibenclamide in the microsomal, cultured smooth muscle cell and tissue-strip preparations of rat, rabbit and canine aorta (unpublished observations). This may be due to the slow dissociation rate of glibenclamide from the specific sites, because in brain and ventricle preparations, PCOs could not displace the bound glibenclamide but inhibited the glibenclamide binding. Furthermore, antagonistic actions of glibenclamide on PCO-induced 86Rb⁺ efflux indicates the presence of specific sites of actions of glibenclamide in the rat aorta. Therefore, we conclude that glibenclamide is not a suitable ligand for studies on specific PCO binding sites in rat aorta. It may be necessary to use a radiolabeled ligand with higher affinity such as [H]P1075 (N-cyano-N(1,1-dimethyl[2,2,3,3-[H]propyl-N")-3-pyridinylguanidine) (15, 16) in binding studies not only for PCOs but also for potassium channel blockers, especially in blood vessels.

**The effects of NIP-121, levcromakalim and nicorandil on [H]glibenclamide specific binding**

The effects of PCOs such as NIP-121, levcromakalim and nicorandil are competitively antagonized by sulfonylurea \( \text{K}_{\text{ATP}} \) blockers such as glibenclamide or tolbutamide (3–9, 13, 17, 36, 37), which yielded a slope of 1 on the Schild analysis. Therefore, if we assume that PCO and sulfonylurea are the competitive agonist and antagonist for the ATP-sensitive potassium channel, respectively, the binding sites for PCO and sulfonylurea should be the same. However, in this study, NIP-121, levcromakalim and nicorandil, which reportedly open ATP-sensitive potassium channels, did not displace the specific binding of [H]glibenclamide in rat brain and ventricle membranes when the membrane and [H]glibenclamide were simultaneously incubated with the PCOs. These findings are in agreement with those obtained earlier using PCOs such as cromakalim and diazoxide, but not RP 49356, at higher concentration ranges (13, 14). In this study, NIP-121 and levcromakalim also did not compete for either type of binding site in the rat ventricle membrane at 0°C. These findings suggest the following: The specific binding site for PCOs is completely different from that for sulfonylureas, because the same phenomenon was observed using different PCOs such as benzopyran or a nicorandil-like structure. Recently, the specific binding sites for sulfonylureas and ATP-sensitive potassium channels were reported to be separate molecular entities, since the cloned channels were not inhibited by a micromolar concentration of glibenclamide, although they were acti-

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**Identification of [H]glibenclamide specific binding sites in the membranes from rat brain, heart and aorta**

Specific binding sites for [H]glibenclamide were identified in membranes prepared from rat brain and heart. The sites of rat brain membrane determined from the saturation assay are consistent with the high affinity sites in the ventricle membrane, because their \( K_d \) values were similar. The computed \( K_d \) value of glibenclamide for the high affinity sites was the same as that previously reported, e.g., \( K_d \) values for intestinal smooth muscle (31), insulinaoma cells (32–34) and the cerebral cortex (14, 31, 32, 35). In both preparations, unlabeled glibenclamide displaced the specific binding of [H]glibenclamide in a concentration-dependent manner. However, the slope factor from the competition of unlabeled glibenclamide on brain membranes was less than 1, which indicates dual classes of binding sites for [H]glibenclamide, which is in agreement with an earlier report (14). The discrepancy might be caused by the use of glibenclamide at different concentration ranges in the two experimental protocols. We significantly inhibited this increase in the rate of 86Rb⁺ efflux in a concentration-dependent manner (Fig. 7). The maximum inhibition occurred in the presence of 50 nM glibenclamide. The \( pD_2 \) value of glibenclamide for the inhibition of 86Rb⁺ efflux increased by NIP-121 was 7.98 ± 0.75 (mean ± S.E.M. of five experiments).
vated by the PCO pinacidil (38, 39). In addition, Bray and Quast (15) and Quast et al. (16) found using the radiolabeled pinacidil-derivative [\textsuperscript{3}H]P1075 that sulfonylurea binding caused negative cooperative interaction of the PCO binding.

On the other hand, we found that pre-incubation with either NIP-121 or levromakalim for 60 min before [\textsuperscript{3}H]-glibenclamide application partially decreased in a concentration-dependent manner the K_d and B_{max} values of the specific [\textsuperscript{3}H]-glibenclamide binding. The partial inhibition of B_{max} may indicate that the binding of PCO may produce a negative regulation of [\textsuperscript{3}H]-glibenclamide binding rather than directly binding to the sites for sulfonylureas. We also confirmed that the effect of PCO is not controlled by GTP-binding protein. The decrease in B_{max} value by either PCO may be linked to the pharmacological effects, because the dose-effect relationships for the B_{max}'s of the two compounds seem similar to those for vasorelaxation. The discrepancy between the result in the competitive assay and that in the saturation assay might be caused by a difference in the time required to reach the steady state of PCO- (unknown at present) or glibenclamide-receptor complex formation (about 20 min in this study). If the time to reach the equilibrium of PCO-receptor complex formation is much longer than that of the glibenclamide-receptor complex formation, the effect of PCOs on the specific [\textsuperscript{3}H]-glibenclamide binding may be detected after the steady state of PCO-receptor complex formation. A change in state of the specific sulfonylurea binding site, which may be caused by a change in the membrane potential or in the enzyme activity such as phosphorylation by ATP (34, 40, 41), might be required to affect PCO binding, although the evidence for this was not obtained in this study. It may be necessary to use intact tissues or cells rather than membrane preparations when studying PCO binding using radiolabeled sulfonylurea.

Coldwell and Howlett (42) reported that unlabeled cromakalim did not compete with [\textsuperscript{3}H]-cromakalim binding to the rat heart, kidney, adrenal gland, spleen, lung, liver, aorta, platelets, red blood cells or brain in vivo and in vitro. Thus, they concluded that cromakalim did not have specific binding sites. On the other hand, Bray and Quast (15) and Quast et al. (16) reportedly identified a specific binding site for PCOs in the rat aorta using [\textsuperscript{3}H]-P1075, which was fully displaced by not only other types of PCOs but also by sulfonylurea derivatives such as glibenclamide, glipizide or AZ-DF 265. Why the [\textsuperscript{3}H]-cromakalim binding behavior was inconsistent with that of [\textsuperscript{3}H]P1075 has not been examined in detail. Furthermore, the reasons for the discrepancies among these studies and the present study, as well as between the binding properties and the pharmacological responses remain unclear, although Bray and Quast (15) and Quast et al. (16) suggested that the sulfonylurea binding causes negative cooperative regulation of the binding for PCOs.

**Displacement properties of various potassium channel blockers on [\textsuperscript{3}H]-glibenclamide specific binding**

This study showed that the I_{SK(Ca)} selective blocker apamin (30) also displaced the specific binding of [\textsuperscript{3}H]-glibenclamide in both membrane preparations in a concentration-dependent manner. However, apamin at the nanomolar range may not bind to the sulfonylurea binding sites or block I_{K(ATP)}, because the effective concentration range for the binding (\(> 10^{-6} \text{ M}\)) was about 100-times higher than that for pharmacological I_{SK(Ca)} channel blockade. In this study, apamin at 10 \text{nM} but not at 10 \text{nM} significantly inhibited the NIP-121 (100 nM)-induced \(86\text{Rb}^+\) efflux increment. In addition, Calder et al. reported that apamin at 1 \text{pM} did not influence the cromakalim-induced \(86\text{Rb}^+\) efflux increment in isolated guinea pig resistant arteries (43). This suggests that apamin at a high concentration (\(> 10^{-6} \text{ M}\)) may influence the function of I_{K(ATP)}. The full displacement and the slope of the competitive curve indicated that apamin directly binds to sulfonylurea sites, but does not cause negative allosteric regulation of specific sulfonylurea sites. However, it is not clear at present where the chemical homology lies, although sulfonylurea- (44, 45) and apamin- (46, 47) receptor proteins have been purified and identified. Some parts of the binding sites for these blockers might hold some analogous amino acid sequences or a similar electric charge relationship. In addition, we also found that the voltage-dependent potassium channel blocker 4-AP and TEA in the concentration range which produces a pharmacological response significantly displaced the specific [\textsuperscript{3}H]-glibenclamide binding, especially in rat heart.

**The effect of phentolamine on [\textsuperscript{3}H]-glibenclamide specific binding**

Phentolamine antagonizes the actions of cromakalim (4, 21–23). According to our earlier report (4), glibenclamide (10^{-7} \text{ M}) and phentolamine (3 \times 10^{-6}, 3 \times 10^{-5} \text{ M}) also antagonized the relaxation induced by NIP-121 and cromakalim. The present findings are the first to show that phentolamine did not displace [\textsuperscript{3}H]-glibenclamide from its specific binding sites in both the rat brain and heart. The pharmacological antagonism of phentolamine on the PCO-effect might be irrelevant to the binding for the specific sites of glibenclamide. The antagonism is reportedly caused by its chemical structure (imidazolidine derivative) and not by blockade of \(\alpha_1\)-adrenoceptors (4, 21–23). Phentolamine might bind to the other specific receptors related to the PCO-induced response.
Conclusion

PCOs, such as NIP-121 or levromakalim, showed pharmacological effects by binding to its own specific sites, but not to the specific glibenclamide sites. PCOs inhibit the specific binding of glibenclamide in a negative allosteric manner, although PCOs failed to displace the bound glibenclamide. The inhibition was not regulated by GTP, suggesting that the GTP-binding protein is not involved. In addition, a close relationship among the specific binding sites for various types of potassium channel blockers was suggested.

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