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REGULATION OF ATP-SENSITIVE K^+ CHANNELS BY CHRONIC GLYBURIDE AND PINACIDIL ADMINISTRATION

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Abstract—Treatment of rats with the $K^+_{(ATP)}$ channel antagonist sulfonylurea, glyburide (3 mg/kg/day, i.p., every 12 hr for 9 days), increased the B_{max} value of [^{3}H]glyburide binding to heart and whole brain total membranes by 30 and 24%, respectively. The ligand affinity was unaltered. Treatment with the K^+ channel activator, pinacidil (20 mg/kg/day, i.p., every 12 hr for 9 days), did not alter the B_{max} value for cardiac [^{3}H]glyburide binding sites, but decreased the B_{max} value in the brain by 21%. Chronic administration of hydralazine, which caused an acute reduction in systolic blood pressure equivalent to that of pinacidil, did not alter [^{3}H]glyburide binding in either heart or brain. Treatment with glyburide, pinacidil or hydralazine did not alter L-type calcium channels, assessed by [^{3}H]PN 200 110 binding, in cardiac and brain membranes or small size Ca^{2+}-activated K^+ channels in brain assessed by [^{125}I]apamin binding. These studies show that the ATP-sensitive class of K^+ channels can be regulated following chronic drug treatment in similar fashion to other receptor and channel systems.

ATP-sensitive K^+ channels ($K^+_{(ATP)}$) are present in a variety of tissues including heart, brain and pancreas [reviewed in Ref 1]. Channel closure depolarizes the cell and activates voltage-sensitive channels, and opening of these channels hyperpolarizes the cell to inhibit voltage-sensitive Ca^{2+} channel activation [2]. In cardiac and neuronal tissues, channel opening under conditions of reduced intracellular ATP levels as in ischemia and anoxia may limit Ca^{2+} influx and cellular damage [3, 4]. Additionally, neuronal $K^+_{(ATP)}$ channels may play roles in transmitter release [5]. The hypoglycemic sulfonylureas including glyburide and glypizide bind with high affinity to close the $K^+_{(ATP)}$ channels. The locus of action of the K^+ channel activator drugs, including pinacidil, cromakalim and diazoxide, is proposed to be a type of $K^+_{(ATP)}$ channel [6].

There is substantial evidence that ion channel proteins are subject to regulatory influences including chronic drug treatment and disease states [reviewed in Refs. 7 and 8]. Regulatory changes in expression or function of ion channel or receptor proteins underlie, in part, the phenomena of tolerance and withdrawal in a variety of systems. Tolerance to K^+ channel activators has been reported both *in vitro* and *in vivo* [9–12]. Chronic treatment with sulfonylurea antagonists has been reported to generate favorable cardiovascular effects under some circumstances [13, 14]. Additionally, a component of the long-term hypoglycemic effects of sulfonylureas is attributed, in part, to regulation of extrapancreatic insulin receptors [15]. We wished to examine whether $K^+_{(ATP)}$ channels are regulated by chronic ligand administration and whether this might contribute to any long-term effects of ligands active at these channels. Following chronic administration of an antagonist sulfonylurea, glyburide, and a putative activator, pinacidil, in rats, we examined the status of $K^+_{(ATP)}$ channels in heart and brain using [^{3}H]glyburide. L-type Ca^{2+} channels in both the heart and brain, and small size Ca^{2+}-activated K^+ channels in the brain were also assessed by [^{3}H]PN 200 110 and [^{125}I]apamin binding, respectively.

MATERIALS AND METHODS

Drugs and radioligands. [^{3}H]Glyburide (sp. act. 50.9 Ci/mmol), [^{3}H](+)PN 200 110 (sp. act. 74.1 Ci/mmol) and [^{125}I]apamin (sp. act. 2200 Ci/mmol) were purchased from Dupont-NEN (Boston, MA). Unlabeled drugs were obtained from the sources indicated: apamin (Sigma Chemical Co., St. Louis, MO), glyburide (Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ), PN 200 110 (Sandoz Inc., Basel, Switzerland) and pinacidil (Eli Lilly & Co., Indianapolis, IN). All other drugs and chemicals from the Sigma Chemical Co. or the Fisher Scientific Co. were of the highest purity routinely available. Stock solutions of pinacidil (20 mg/mL) and glyburide (2 mg/mL) were prepared in polyethylene glycol. Hydralazine (20 mg/mL) was prepared fresh daily in 0.9% saline.

Study design. Male Sprague–Dawley rats (Hilltop, Scottdale, PA), 300–340 g, were maintained under standard laboratory conditions and had free access to food and water during the course of the experiment. Glyburide (2.9 mg/kg/day) and pinacidil (20 mg/kg/day) were administered by intraperitoneal injection every 12 hr for 9 days. Similar doses of glyburide have been used in earlier chronic studies and shown to be effective in improving myocardial function associated with diabetic cardiomyopathy [14]. Since pinacidil administration was associated

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with hypotension and tachycardia, we also treated a group of rats with hydralazine (20 mg/kg/day, i.p., administered every 12 hr for 9 days), an antihypertensive with direct vasodilating properties, but with no known effects at $K^+_{(ATP)}$ channels. The systolic blood pressures and cardiac rates of rats were determined following initial administration of the vasodilator drugs. Blood pressure was measured by the tail cuff technique using a programmed electrosphygmomanometer PE-300 (Narco Bio-Systems, Houston, TX). Control animals received an equivalent volume of the polyethylene glycol (PEG) or 0.9% saline. Animals were killed 24 hr after the last injection of the drug or vehicle, and tissues were prepared for radioligand binding.

Membrane preparation. Hearts were rapidly excised and placed in ice-cold Tris–HCl buffer (50 mM; pH 7.2, 25°) and weighed. The ventricles were homogenized in 15 vol/g wet weight buffer using a Tekmar Polytron at a maximum setting for 7 sec followed by 10 passes with a glass–Teflon homogenizer using a Tri-R Stir-R at a setting of 7. The same procedure was adopted for the brain (minus brain stem) except that the initial homogenization step was omitted. Both homogenates were filtered through a double layer of cheesecloth and the filtrate was centrifuged at 45,000 *g* for 45 min. The pellet was resuspended in ice-cold Tris buffer and used in radioligand binding assays. Protein was determined by the method of Bradford [16] with bovine serum albumin as standard.

Radioligand binding. [^{3}H]Glyburide binding was performed as described earlier with minor modifications [17]. Briefly, ventricle (250–300 μg) and brain (150–200 μg) protein suspensions were incubated in a total 0.5-mL volume of buffer with [^{3}H]-glyburide (1.2×10^{-11} M to 1.5×10^{-9} M) for 60 min at 25°. Specific binding was defined as that displaced by 10^{-7} M unlabeled glyburide. [^{3}H]PN 200 110 binding was performed in a 5-mL assay volume containing protein suspensions and various concentrations of radioligand (9.1×10^{-12} M to 3.0×10^{-10} M) for 150 min and nonspecific binding was assessed in the presence of 10^{-7} M unlabeled PN 200 110 [18, 19]. Incubations were terminated by rapid filtration under vacuum with a Brandel cell harvester (model M-24R, Brandel Instruments, Gaithersburg, MD) over Whatmann GF/B filters, washed two times with 3- to 5-mL volumes of Tris buffer (5 mM). [^{125}I]Apamin binding was performed, with minor modifications as described earlier [20], by incubating brain membranes in 1 mL buffer (50 mM Tris–HCl containing 5.4 mM KCl and 0.1% bovine serum albumin) containing various concentrations of [^{125}I]apamin (4.9×10^{-12} M to 1.0×10^{-10} M) for 60 min at 0°. Unlabeled apamin, 1.0×10^{-6} M, was used to define specific binding. Reactions were terminated by rapid filtration under vacuum over GF/C filters presoaked in 0.3% polyethyleneimine and washed twice with 3 mL of buffer containing 5 mM Tris–HCl and 0.01% bovine serume albumin.

Data analysis. Radioligand binding data were analyzed using the non-linear least-squares analysis program LIGAND [21]. Statistical analysis was performed by Student's *t*-test or one way analysis of variance (ANOVA) using GraphPAD Instat (ISI software). All data are expressed as means ± SEM.

RESULTS

Hemodynamic and general characteristics. The general characteristics of rats following chronic treatments are summarized in Table 1. A small, but significant, cardiac hypertrophy was observed 9 days following chronic administration of pinacidil and hydralazine, but not after glyburide. In control rats, acutely administered pinacidil (10 mg/kg body weight) elicited a sustained decrease in systolic blood pressure (vehicle, 124.1 ± 3.8 mm Hg vs pinacidil, 52.5 ± 4.3 mm Hg) and an increase in cardiac rate (vehicle, 470 ± 10 beats/min vs pinacidil, 600 beats/min; N = 5–6) within 15–20 min. A significant hypotensive effect was still observed 5 hr post-administration. Initial acute administration of hydralazine (10 mg/kg body weight) elicited changes in systolic blood pressure and cardiac rate equivalent to those observed following the administration of pinacidil, in agreement with earlier studies [22].

Radioligand binding to cardiac and brain membranes. [^{3}H]Glyburide binds specifically and with high affinity to cardiac and neuronal $K^+_{(ATP)}$ channels [17, 23]. In the radioligand concentration range (0.01 to 1.4 nM) examined, monophasic Scatchard analysis and linear Hill plots of unit slopes were found in both tissues consistent with [^{3}H]-

Table 1. General characteristics of rats following chronic drug treatment

Treatment	Body wt (g)	Heart wt (g)	Heart:Body wt (g:kg × 10^{-3})	Brain wt (g)
Control (6)	363 ± 7	1.42 ± 0.05	3.92 ± 0.12	2.03 ± 0.12
Vehicle (9)	333 ± 5	1.35 ± 0.04	4.05 ± 0.10	1.97 ± 0.04
Pinacidil (9)	342 ± 6	1.54 ± 0.05*	4.53 ± 0.13*	2.11 ± 0.04
Hydralazine (8)	338 ± 5	1.59 ± 0.05†	4.59 ± 0.13†	2.14 ± 0.07
Glyburide (9)	348 ± 7	1.42 ± 0.05	4.19 ± 0.11	2.14 ± 0.07

Values are means ± SEM; the number of animals per group is shown in parentheses. See text for details of drug treatments.

* Significantly ($P < 0.05$) different with respect to vehicle group.

† Significantly ($P < 0.05$) different with respect to general group.

Table 2. [^{3}H]Glyburide binding to rat cardiac and brain membranes following chronic drug treatments

	[^{3}H]Glybiride				[^{3}H]PN 200 110				[^{125}I]Apamin	
	Heart		Brain		Heart		Brain		Brain	
	B_{max} (fmol/mg protein)	K_D [10^{-11} M]	B_{max} (fmol/mg protein)	K_D [10^{-11} M]	B_{max} (fmol/mg protein)	K_D [10^{-11} M]	B_{max} (fmol/mg protein)	K_D [10^{-11} M]	B_{max} (fmol/mg protein)	K_D [10^{-12} M]
Control	42.3 ± 1.0	12.8 ± 1.9	223.5 ± 4.9	9.5 ± 0.8	225.8 ± 3.8	3.7 ± 0.4	359.8 ± 18.5	3.5 ± 0.4	ND*	ND
Vehicle	47.5 ± 1.5	11.2 ± 0.7	239.4 ± 7.3	8.4 ± 0.2	250.3 ± 11.1	4.5 ± 0.5	335.1 ± 10.1	3.2 ± 0.4	27.6 ± 3.4	28.2 ± 3.9
Glyburide	61.8 ± 3.4†	12.6 ± 0.9	297.1 ± 14.8‡	8.4 ± 0.4	254.4 ± 12.6	5.3 ± 0.8	363.6 ± 16.6	3.1 ± 0.5	26.3 ± 2.8	23.7 ± 2.1
Pinacidil	46.5 ± 1.8	9.4 ± 0.7	188.7 ± 8.0§	8.2 ± 0.3	246.1 ± 10.1	3.9 ± 0.3	330.3 ± 20.8	3.0 ± 0.5	20.3 ± 1.9	25.8 ± 1.6
Hydralazine	46.6 ± 1.7	9.2 ± 0.7	231.2 ± 8.6	9.4 ± 0.7	242.7 ± 21.5	3.3 ± 0.3	371.9 ± 8.7	3.9 ± 0.5	ND	ND

Values are means ± SEM; number of animals in each group: N = 6–9 except for apamin binding where N = 4. See text for details of drug treatments.
* Not determined.
† P = 0.001 with respect to vehicle group.
‡ P = 0.002 with respect to vehicle group.
§ P = 0.01 with respect to vehicle group.

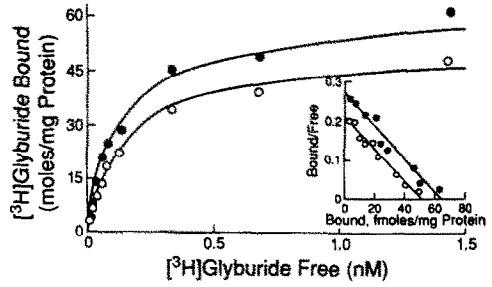

Fig. 1. Saturation analysis of the binding of [^{3}H]glyburide to heart membranes from chronic glyburide (●) and vehicle (○) treated groups. Inset: Scatchard transformation of the data. Shown is a plot representative of 7–9 experiments of chronic glyburide-and vehicle-treated animals.

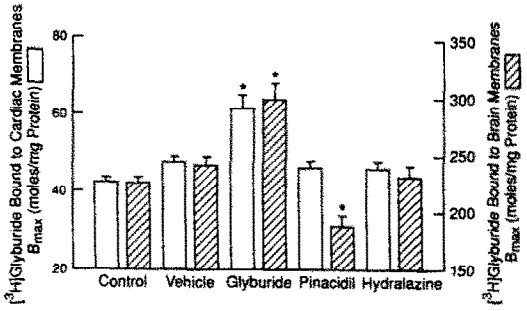

Fig. 2. Influence of chronic glyburide, pinacidil and hydralazine on [^{3}H]glyburide binding to the heart (left Y-axis) and brain (right Y-axis) membranes. Values are means ± SEM, N = 6. Key: (*) represents values differing significantly ($P < 0.05$) from these corresponding values from the vehicle group.

glyburide binding specifically to a single class of sites. The apparent maximum number (B_{max}) of specific [^{3}H]glyburide binding sites in cardiac membranes was 47.5 ± 1.5 fmol/mg protein, and the binding affinity of [^{3}H]glyburide was $11.2 \pm 0.7 \times 10^{-11}$ M. In the brain, the B_{max} value for [^{3}H]glyburide was 239.4 ± 7.3 fmol/mg protein and the K_D value, $8.4 \pm 0.2 \times 10^{-11}$ M (Table 2).

Effects of chronic drug administration in vivo. Chronic administration of glyburide, 3 mg/kg/day for 9 days, increased the high-affinity [^{3}H]glyburide binding site density in cardiac total membranes by 30% (Fig. 1) and in the brain membranes by 24% without change in affinities. Chronic pinacidil administration, 20 mg/kg/day for 9 days, did not change the B_{max} value for [^{3}H]glyburide binding in the heart, but did decrease binding site density in the brain by 21%. Vehicle treatment alone did not affect [^{3}H]glyburide binding site densities or affinities in the heart or brain compared to control animals (Fig. 2; Table 2).

Administration of pinacidil or glyburide did not alter significantly L-type Ca^{2+} channels in the heart or in the brain as revealed by [^{3}H]PN 200 110

binding, suggesting that the changes observed may be specific to the $K^+_{(ATP)}$ channel. Chronic administration of hydralazine, at a dose which lowered systolic blood pressure like pinacidil, failed to affect [^{3}H]glyburide binding or [^{3}H]PN 200 110 binding to cardiac and brain membranes compared to the control values.

[^{125}I]Apamin binding to brain membranes revealed a single saturable binding site with a B_{max} value of 28 ± 3 fmol/mg protein and a K_D value of $2.8 \pm 0.4 \times 10^{-11}$ M. Densities and affinities were not altered significantly in brain membranes from animals treated with glyburide compared to the control. Specific [^{125}I]apamin binding sites could not be detected in cardiac membranes. In rats treated with pinacidil, [^{125}I]apamin binding to brain regions showed a small but statistically insignificant reduction in B_{max} values; K_D values did not differ significantly.

DISCUSSION

Ion channels, like membrane receptors, are subject to a variety of regulatory influences including chronic treatments and diseases [reviewed in Refs. 7 and 8]. In general, chronic administration of antagonist or agonist ligands results in up-regulation or down-regulation, respectively, of channel density and function. This has been demonstrated for Na^+ channels and for L-type Ca^{2+} channels. Chronic activation of Na^+ channels in cultured muscle cells by batrachotoxin results in down-regulation of both channel function and number, and blockade of electrical activity produces a corresponding up-regulation of these channels [24]. Similarly, chronic treatment of PC12 cells with the 1,4-dihydropyridine Ca^{2+} channel antagonists nifedipine, and the activator S-Bay K 8644, results in up- and down-regulation, respectively, of channel density and function [25]. The observed up-regulation of high affinity cardiac and neuronal sulfonylurea binding sites, following *in vivo* glyburide administration, is consistent with antagonist-induced regulation of other ion channel and receptor systems.

Chronic *in vivo* pinacidil administration failed to alter high-affinity glyburide binding site density in cardiac membranes. This suggests that activators do not regulate cardiac ATP-sensitive K^+ channels or, that if such regulation exists, it is probably not mediated by direct interaction at the sulfonylurea binding sites. Although sulfonylureas including glyburide are competitive inhibitors of K^+ channel activator-induced smooth muscle relaxation and Rb^+ efflux, the activators have been shown to be ineffective as inhibitors of high-affinity sulfonylurea binding [17]. In the brain, a 20% down-regulation in [^{3}H]glyburide binding site density was observed, however, in the pinacidil-treated group. It is possible that the relatively high dose of pinacidil employed here might have neuronal effects, although lower doses of pinacidil have been reported to have a poor central nervous system distribution profile [26]. The observed changes, however, are not likely to be due to the hypotensive effects of pinacidil since hydralazine was without similar effect.

Our observations provide evidence that the $K^+_{(ATP)}$ channel proteins are in a tonic state and are subject to regulatory influences. Alterations in ATP-sensitive K^+ channels have already been reported under conditions of cardiac hypertrophy [27], experimental cardiac failure [28] and diabetes [29; Gopalakrishnam M and Triggle DJ, unpublished observations]. Mozaffari *et al.* [14] have shown that chronic glyburide therapy prevents the reduction in cardiac function observed in rat diabetic cardiomyopathy. However, these effects were attributed, in part, to altered carbohydrate metabolism and Ca^{2+} transport processes. Viewed in the context of our present studies, regulation of the $K^+_{(ATP)}$ channels cannot be ruled out.

The mechanism(s) underlying $K^+_{(ATP)}$ channel regulation by sulfonylureas is not understood. Our preliminary studies have shown that chronic treatment of cardiac myocytes with glyburide reduces high-affinity sulfonylurea binding site density in contrast to the up-regulation observed *in vivo*. This difference suggests the absence of regulatory processes or circulating factors *in vitro* that contribute to the *in vivo* processes. However, cells chronically depolarized by elevated K^+ did show up-regulation of [^{3}H]glyburide binding [Gopalakrishnan M and Triggle DJ, unpublished observations]. Alterations in membrane electrical activity have been reported to alter a number of plasmalemmal proteins including muscarinic acetylcholine receptors, opiate receptors, α-bungarotoxin receptors, and voltage-dependent Na^+ and Ca^{2+} channels [24, 30, 31]. Earlier studies from this laboratory have shown that depolarization-induced events, probably via increasing intracellular Ca^{2+} levels, regulate voltage-sensitive Ca^{2+} channels in chick neural retina cells [32]. Alternatively, it is possible that up-regulation of binding sites following glyburide may be a compensatory phenomenon subsequent to antagonist occupation of the channel, similar to that observed with other membrane receptors. Endogenous regulatory peptides capable of displacing [^{3}H]glyburide binding have been identified [33]. The levels of these peptides could dictate channel regulation *in vivo*. Additionally, *in vivo* sulfonylurea treatment could exert metabolic changes [14, 34]. Currently, little is known about tolerance or desensitization, and its mechanism(s), to sulfonylureas and K^+ channel activators. Tolerance to cromakalim and its cross-desensitization with nicorandil has been reported in vascular and uterine smooth muscle [11, 12, 25]. Changes in expression and/or function of K^+ channels could, in principle, underlie these phenomena. Our study provides evidence that the ATP-sensitive class of K^+ channels is subject to drug-induced regulatory influences. Further investigations will undoubtedly be necessary to examine the functional consequences of these changes and the mechanism(s) involved. These studies are ongoing.

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