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Interactions of K^+_{ATP} channel blockers with Na^+/K^+-ATPase

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Abstract Two K^+_{ATP} channel blockers, 5-hydroxydecanoate (5-HD) and glyburide, are often used to study cross-talk between Na^+/K^+-ATPase and these channels. The aim of this work was to characterize the effects of these blockers on purified Na^+/K^+-ATPase as an aid to appropriate use of these drugs in studies on this cross-talk. In contrast to known dual effects (activating and inhibitory) of other fatty acids on Na^+/K^+-ATPase, 5-HD only inhibited the enzyme at concentrations exceeding those that block mitochondrial K^+_{ATP} channels. 5-HD did not affect the ouabain sensitivity of Na^+/K^+-ATPase. Glyburide had both activating and inhibitory effects on Na^+/K^+-ATPase at concentrations used to block plasma membrane K^+_{ATP} channels. The findings justify the use of 5-HD as specific mitochondrial channel blocker in studies on the relation of this channel to Na^+/K^+-ATPase, but question the use of glyburide as a specific blocker of plasma membrane K^+_{ATP} channels, when the relation of this channel to Na^+/K^+-ATPase is being studied.

Keywords Glyburide · Glybenclamide · 5-Hydroxydecanoate · K^+ channel · Na^+/K^+-ATPase · Ouabain

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Introduction

Na^+/K^+-ATPase catalyzes the coupled active transport of Na^+ and K^+ across the plasma membranes of most mammalian cells, and this pumping function of the enzyme is specifically inhibited by ouabain and related digitalis drugs [1, 2]. In recent years, it has been recognized that Na^+/K^+-ATPase of intact cells may also function as a signal transducer; i.e., the ouabain-inhibited fraction of the enzyme interacts with neighboring membrane proteins such as Src and EGFR, causing the activations of multiple signal transduction pathways that link the initial cell membrane events to intracellular organelles and the nucleus [3, 4]. It is now well established that some of these ouabain-activated pathways lead to functional communication between Na^+/K^+-ATPase and mitochondria by mechanisms that involve the mitochondrial ATP-sensitive K^+ channel (K_{ATP} channel), but are not fully characterized [5–7]. In studies designed to explore the pathway that relays the ouabain message from Na^+/K^+-ATPase to mitochondria, 5-hydroxy-decanoate (5-HD) has been used as a well established blocker of mitochondrial K_{ATP} channel [5–7]. The tacit assumption in such studies is that 5-HD has no significant direct interaction with Na^+/K^+-ATPase. To our knowledge, however, there are no reported studies of 5-HD effects on Na^+/K^+-ATPase, in spite of the long history of the known effects of other long-chain fatty acids on Na^+/K^+-ATPase [8, 9]. The primary aim of this work, therefore, was to examine the effects of 5-HD on purified Na^+/K^+-ATPase. Because of the reported disagreements [10–12] on the interaction of Na^+/K^+-ATPase with another widely used K_{ATP} channel blocker, glyburide (glybenclamide), we have also examined the effects of this compound on the purified Na^+/K^+-ATPase.

Materials and methods

Materials

ATP, ouabain, glyburide, and the sodium salts of 5-HD, decanoic acid (capric acid), and dodecanoic acid (lauric acid) were obtained from Sigma (St. Louis, MO). Biomol Green was purchased from BIOMOL (Plymouth Meeting, PA). The common chemicals were of the highest purity available. Pig kidneys were purchased from a nearby slaughterhouse, and stored at −80°C until used for enzyme preparation. Male Sprague Dawley rats (300–350 g) were used according to the procedures and guidelines approved by the Institutional Animal Care and Use Committee.

Enzyme preparations

Pig kidneys were dissected to obtain outer medulla by procedures described before [13]. A microsomal fraction was then prepared from the outer medulla, treated with low concentrations of SDS to remove impurities without solubilizing the membranes, and the purified membrane-bound enzyme was collected after differential centrifugation by the angle rotor version of the procedure of Jorgensen [13]. The enzyme was suspended in a solution containing 0.25 M sucrose, 30 mM histidine, and 1 mM EDTA, and stored frozen until use. The specific activities of the ouabain-sensitive Na^+/K^+-ATPase of various kidney preparations, assayed at 5 mM ATP as described below, were in the range of 900–1,200 μmol/mg/h. The ouabain-sensitive activities of these preparations were greater than 95% of the total ATPase activity.

For the preparation of the crude cardiac enzyme, ventricles of a fresh rat heart were minced, suspended in 15 ml of the above sucrose solution, homogenized by a Teflon-glass homogenizer, and further disrupted by a Polytron homogenizer. The cold homogenate was filtered through gauze, and centrifuged at 1,000*g* for 10 min at 4°C. The supernatant was then centrifuged for 1 h at 115,000*g* at 4°C. The sediment was suspended in the above sucrose solution, and used or stored frozen. The specific activities of the ouabain-sensitive Na^+/K^+-ATPase of these crude preparations, assayed at 5 mM ATP as described below, were in the range of 2–3 μmol/mg/h. The ouabain-sensitive activities of these preparations were 15–20% of total ATPase activity.

Na^+/K^+-ATPase assay

Enzyme activity was assayed at 37°C through the determination of the initial rate of release of Pi from ATP; i.e., enzyme concentration and reaction time were chosen to ensure that no more than 10–15% of the substrate was hydrolyzed. Reaction mixtures contained 100 mM NaCl, 12.5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM sodium azide (only in the case of crude cardiac enzyme), 20 mM Tris-HCl (pH 7.4), and either saturating ATP (5 mM) or suboptimal ATP (50 μM). That these ATP concentrations are optimal and suboptimal for the kidney and the cardiac enzymes is known [14, 15]. The assay under each specified condition was done in the presence of 1 mM ouabain and without ouabain in order to determine the ouabain-sensitive activity. Reactions were stopped by the addition of cold perchloric acid, cleared by centrifugation if necessary, and assayed for Pi.

For the study of the effects of glyburide on the enzyme activity, it was necessary to dissolve the drug in DMSO prior to addition to the reaction mixture. The final DMSO concentration did not exceed 0.02%. When appropriate, controls with DMSO alone were included.

Other assays

Pi was measured using the highly sensitive Malachite Green assay [16, 17], using the commercially available Biomol Green reagent. Protein was assayed using DC protein assay (Bio-Rad, Hercules, CA).

Data analysis

Data are means ± SE of the results of a minimum of three experiments. One-way analysis of variance (ANOVA) was performed and Bonferroni's correction for multiple comparisons was employed as appropriate. Statistical significance is reported at the $P < 0.05$. SPSS software was used for all analysis (SPSS Inc.; Chicago, IL).

Results

5-HD and other fatty acid effects on purified Na^+/K^+-ATPase

That long-chain fatty acids inhibit this enzyme was first noted by Ahmed and Thomas [8], and confirmed by others [18, 19]. Our subsequent studies [9, 15, 20–22] showed that fatty acids and related amphiphiles may exhibit different effects on Na^+/K^+-ATPase depending on substrate concentration: Inhibition at saturating ATP, and activation at suboptimal ATP. This is illustrated by the data of Fig. 1A, showing the effects of varying concentrations of lauric acid on the activities of the purified kidney enzyme at 5 mM and 50 μM ATP concentrations. Clearly, when ATP

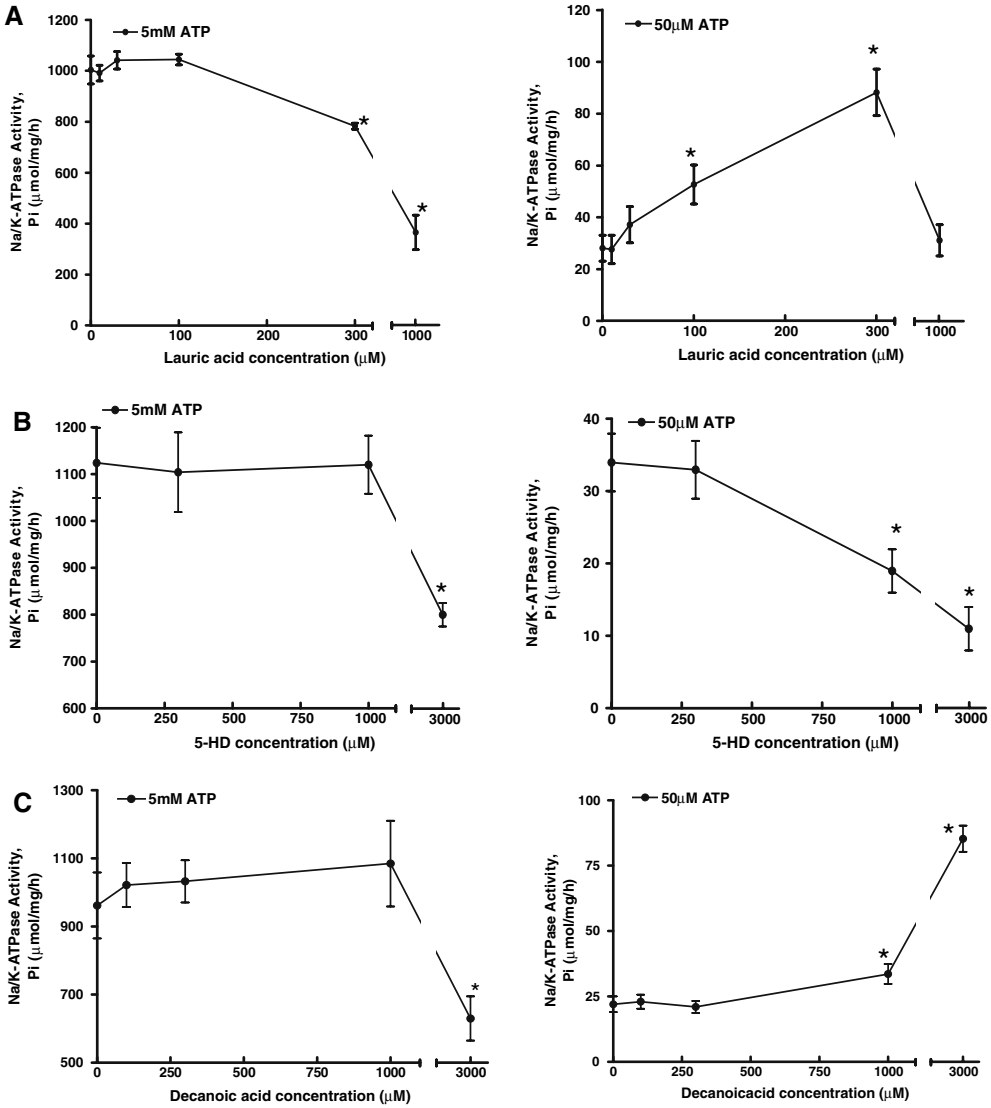

Fig. 1 Effects of varying concentrations of lauric acid, 5-hydroxydecanoic acid (5-HD), and decanoic acid on Na^+/K^+-ATPase activity of purified kidney enzyme at optimal and suboptimal ATP concentrations. Assays were done on the purified enzyme as described in Materials and methods, using 5 mM and 50 μM ATP. Several preparations of the purified enzyme with similar specific activities were used, as indicated in Materials and methods. $^*P < 0.05$

concentration was saturating (5 mM), increasing concentrations of lauric acid resulted in inhibition of the enzyme; whereas, at suboptimal ATP (50 μM), increasing lauric acid concentrations first activated the enzyme before causing inhibition (Fig. 1A). When experiments similar to those of Fig. 1A were conducted using varying concentrations of 5-HD, at both high and low ATP concentrations, only inhibitory effects of 5-HD were noted, though with different potencies (Fig. 1B). Decanoic acid, the parent compound of 5-HD, had inhibitory effects similar to that of 5-HD at 5 mM ATP (Fig. 1C); but at 50 μM ATP, decanoic acid activated the enzyme (Fig. 1C), unlike 5-HD (Fig. 1B). These findings are consistent with the proposition that activating and inhibitory effects of fatty acids on Na^+/K^+-ATPase are exerted at different sites, and that the introduction of the hydroxyl group to decanoic acid prevents its effect at the activating site. In our previous structure/activity studies on the effects of fatty acids and derivatives on Na^+/K^+-ATPase [9], we noted that some derivatives which did not activate the enzyme at suboptimal ATP (e.g., diacylglycerol) were capable of blocking the effect of an activator on Na^+/K^+-ATPase. 5-HD did not exhibit such blocking effects (data not shown).

In studies addressing the relation of Na^+/K^+-ATPase to mitochondrial K_{ATP} channels, ouabain and 5-HD are often used together [5–7]. To explore the possibility of the interactive effects of the two inhibitors on Na^+/K^+-ATPase, the effect of a partially inhibitory concentration of 5-HD

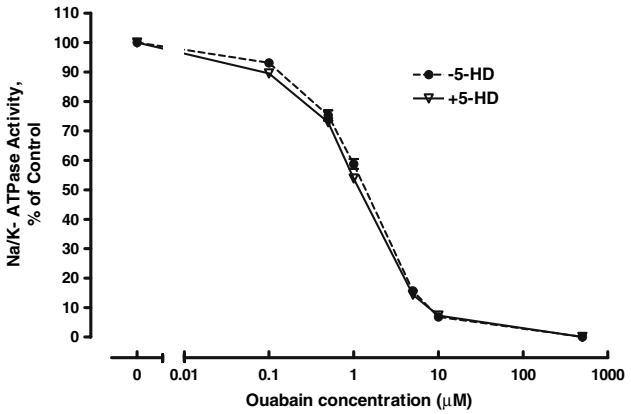

Fig. 2 Effects or varying concentrations of ouabain on Na^+/K^+-ATPase of the kidney enzyme in the absence and the presence of 5-hydroxydecanoate. Experiments were done as in Fig. 1, using 5 mM ATP and 3 mM 5-HD

was examined on the response of the enzyme to varying concentrations of ouabain. 5-HD did not have a significant effect on the dose–response curve for ouabain (Fig. 2).

Glyburide effects on purified Na^+/K^+-ATPase

Due to conflicting reports on the direct inhibitory effect of glyburide on Na^+/K^+-ATPase [10–12], we examined the effects of varying concentrations of this drug on the purified enzyme activity, using optimal and suboptimal substrate concentrations: 5 mM ATP and 50 μM ATP. This hydrophobic sulfonylurea, like most fatty acids and related amphiphiles, exhibited both activating and inhibitory effects depending on substrate concentration. Within the same concentration range of glyburide (100–750 μM), the drug was an inhibitor at 5 mM ATP, but an activator at 50 μM ATP (Fig. 3).

5-HD and glyburide effects on cardiac Na^+/K^+-ATPase

Limited experiments were done to compare the above-described effects of 5-HD and glyburide on the purified kidney enzyme with the drug effects on a crude preparation of cardiac Na^+/K^+-ATPase. Because of the low specific activity of the ouabain-sensitive Na^+/K^+-ATPase of this cardiac preparation (Materials and methods), we were unable to detect its activity at the suboptimal substrate concentration (50 μM ATP). Therefore, 5-HD and glyburide effects on the cardiac enzyme were only determined at saturating ATP (5 mM). Under this condition, both drugs exhibited only inhibitory effects (Fig. 4) as in the case of the kidney enzyme (Figs. 1 and 3). The sensitivities of the cardiac and the kidney enzymes to 5-HD were about the same or similar. However, glyburide clearly seemed to be more potent against the cardiac than against the kidney enzyme (compare Fig. 3 with Fig. 4). Though the cause of this difference is not known, it is of interest to note that the plasma membrane K_{ATP} channels also exhibit significantly different sensitivities to sulfonylureas, such as glyburide, in different cell types and tissues [23].

The ouabain-insensitive Mg^{2+}-ATPase activity of the crude cardiac preparation (Materials and methods) was also affected by glyburide and 5-HD. The highest concentrations of the two drugs used in Fig. 4, inhibited the Mg^{2+}-ATPase by about 15% (data not shown).

Discussion

In this study we have examined the effects of a fatty acid derivative (5-HD) and a sulfonylurea (glyburide) on Na^+/K^+-ATPase in order to clarify the appropriate use of these two K_{ATP} channel blocker drugs in studies dealing with the relations of K_{ATP} channels and Na^+/K^+-ATPase.

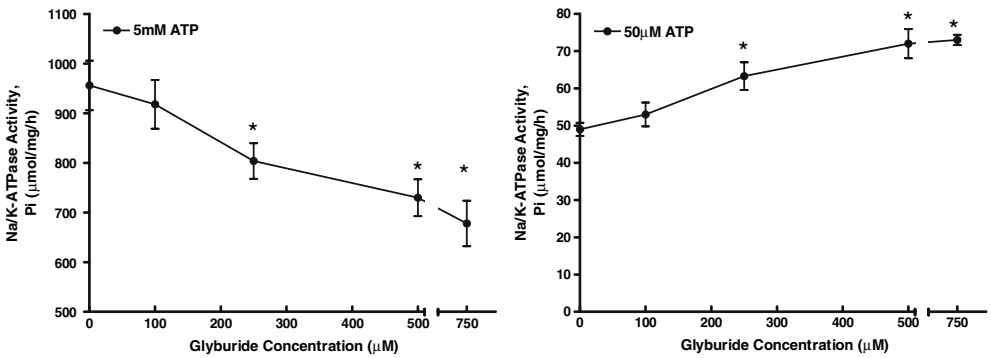

Fig. 3 Activating and inhibitory effects of glyburide on Na^+/K^+-ATPase of the kidney enzyme at suboptimal and optimal ATP concentrations. Experiments were done as described in Fig. 1 and Materials and methods. $*P < 0.05$

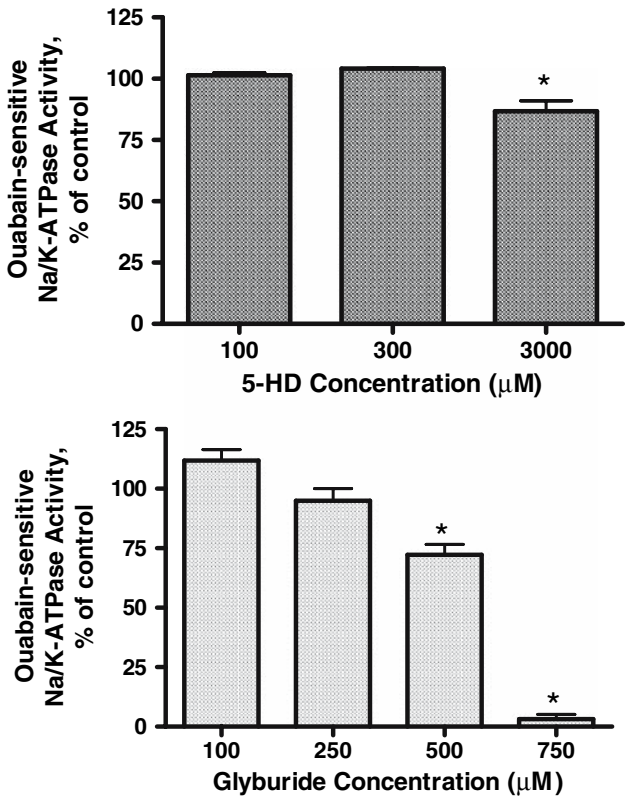

Fig. 4 Effects of 5-hydroxydecanoate (5-HD) and glyburide on Na^+/K^+-ATPase activity of a crude rat heart membrane preparation. Membranes were prepared and assays were done at 5 mM ATP as indicated in Materials and methods. $*P < 0.05$

Long-chain fatty acids have been known to affect Na^+/K^+-ATPase in two different ways, and perhaps at two different sites. The reversible inhibitory effect is non-competitive with ATP, not at the ouabain site, and not due to the denaturing detergent-like effects of fatty acids [8]. The reversible activating effect of a fatty acid is obtained at concentrations lower than or the same as inhibitory concentrations, and is evident when ATP is suboptimal, because the apparent Km for ATP is lowered in the presence of the fatty acid [9, 15]. Both activating and inhibitory effects are also demonstrable on the pumping function of Na^+/K^+-ATPase [22], and fatty acid potencies and efficacies in regard to both effects are influenced by chain-length, degree of unsaturation, and esterification [9].

The data presented here show that 5-HD, unlike the parent decanoic acid, exhibits only an inhibitory effect on Na^+/K^+-ATPase. Lack of activating effect by some fatty acid esters was noted before [9], but all previously tested fatty acids with chain lengths of eight or higher had activating effects [9]. Evidently, the introduction of the single hydroxyl group, such as that in 5-HD, is sufficient to prevent interaction, or reduce binding affinity, at the activating site. The inhibitory potency of 5-HD is similar to that of decanoic acid (Fig. 1), and in keeping with previous data on the relation of potency to chain length [8, 9].

Although initially thought to be a blocker of the plasma membrane K_{ATP} channel [24], 5-HD has subsequently been shown to act as a highly selective inhibitor of the mitochondrial K_{ATP} channel [25–28]; and as such, 5-HD has been used to explore the relation of the ouabain-sensitive Na^+/K^+-ATPase to mitochondrial K_{ATP} channels [5–7]. From this point of view, the important findings of the present study are: (1) 5-HD concentrations that may have significant inhibitory effects on Na^+/K^+-ATPase (Figs. 1 and 4) far exceed those (30–300 μM) that effectively block the mitochondrial K_{ATP} channels [5–7, 26]. (2) There is no influence of 5-HD on the ouabain sensitivity of the purified Na^+/K^+-ATPase (Fig. 3). We may conclude, therefore, that in studies where communication between Na^+/K^+-ATPase and mitochondrial K^+_{ATP} channel is explored [5–7], it is reasonable to assume that the commonly used concentrations of this channel blocker (30–300 μM) have no direct effect on Na^+/K^+-ATPase.

It is appropriate to note that previous studies on the relation of mitochondrial K_{ATP} channels and Na^+/K^+-ATPase have been done mostly in the heart in relation to the possible cardioprotective effect of ouabain [5–7]. Because of this, we deemed it necessary to examine the effect of 5-HD not only on the purified kidney Na^+/K^+-ATPase, but also on a crude enzyme preparation from the heart (Fig. 4). Evidently, in both preparations significant inhibition of Na^+/K^+-ATPase requires 5-HD concentrations exceeding those that inhibit mitochondrial K_{ATP} channels, as indicated above. It is also of interest to note that the rat heart and the pig kidney Na^+/K^+-ATPases, with their known widely different sensitivities to ouabain [1, 2], both have similar or close sensitivities to 5-HD (Figs. 1 and 4). This provides further support for the independence of ouabain and fatty acid inhibitory sites of Na^+/K^+-ATPase, as already suggested [8].

Glyburide is the widely used prototypic sulfonylurea that blocks all K_{ATP} channels [26], the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels, and perhaps some calcium channels [12]. Since this drug has been used in a number of studies to explore the mechanism of suggested functional interaction between Na^+/K^+-ATPase and the plasma membrane K_{ATP} channels of renal epithelia and other cell types [29], the possibility of its direct interaction with Na^+/K^+-ATPase has been considered before [10–12]. However, when glyburide effects on Na^+/K^+-ATPase activities of crude isolated membranes or homogenates have been attempted, either inhibition [10] or lack of effect [11] have been reported. The present data on the purified kidney enzyme clearly show both activating and inhibitory effects of

glyburide (Fig. 3) similar to those of the fatty acids and their derivatives. At first sight, this similarity may seem strange. However, previous structure/activity studies on fatty acids and derivatives indicated the necessity of appropriate balance and spatial relation between the hydrophilic and hydrophobic moieties of these amphiphiles for their interaction with Na^+/K^+-ATPase [9]. Evidently, such balance also exists in the amphiphilic structure of glyburide, allowing its interaction with the postulated amphiphilic binding site on the enzyme [9]. Mechanistic issues aside, our present data indicate that depending on the prevailing ATP concentrations, Na^+/K^+-ATPase may indeed be either activated or inhibited by glyburide concentrations, up to 500 μM, that are often used for the presumed specific inhibition of the plasma membrane K_{ATP} channels in various cell types [10, 29]. This, and the possibility that plasma membrane K_{ATP} channels of different cells may have different sulfonylurea sensitivities [23], suggest the necessity of caution in the use of glyburide in studies where the proposed interaction between Na^+/K^+-ATPase and any K_{ATP} channel is examined.

In summary, the significance of this study is twofold: First, our findings justify the use of 5-HD, at concentrations of 300 μM or lower, as a highly specific inhibitor of the mitochondrial K^+_{ATP} channel in studies of cross-talk between these channels and Na^+/K^+-ATPase. Second, our data add significant weight to existing evidence questioning the use of glyburide and related sulfonylureas in studies of cross-talk between K_{ATP} channels and Na^+/K^+-ATPase.

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