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The Action of Phloridzin and Sugars on (Na^+-K^+)-Activated ATPase

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Summary. The action of phloridzin and simple sugars on the (Na^+-K^+)-activated ATPase obtained from rabbit kidney has been studied. Phloridzin 10^{-4} to 10^{-3} M was found to inhibit the enzyme at Na^+:K^+ ratios less than optimal for enzyme activity, whereas stimulation was noted at Na^+:K^+ ratios greater than optimal for enzyme activity. Some sugars in concentrations of 0.1 to 0.5 M were found to inhibit the (Na^+-K^+)-activated ATPase. The sugars and related compounds could be ranked according to decreasing inhibitory potency as:

D-mannose > D-arabinose, D-xylose > L-xylose > D-glucose > fructose, L-arabinose > D-galactose, myo-inositol, mannitol = 0.

No stimulatory effect or interaction with K^+ was found with these compounds. The action of these substances on the (Na^+-K^+)-activated ATPase suggests an interaction of actively transported sugars and sodium-potassium transport at the level of the sodium pump that may be important in the biological coupling of the two systems.

A number of physiological studies have suggested a close connection between active sugar transport and sodium ion transport in biological systems. For example, in the intestine [3, 34] and in the kidney [23], active sugar transport requires the simultaneous presence of the sugar and Na^+. This relationship has been intensively studied in the intestine, where Na^+ enhances the affinity of actively transported sugars at some rate-limiting site [7, 13]. When both sugars and Na^+ are actively transported across an epithelial cell layer, the sugars and Na^+ appear to interact in active sugar transport at one surface while active Na^+ transport takes place at the opposite cell surface [9]. The observation that actively transported sugars stimulate Na^+ transport independently of associated modifications in cell metabolism [2, 35] might imply an interaction of sugars and Na^+ at the site of the sodium pump in addition to the interaction at the site of active

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sugar transport. However, Crane and his associates [6, 8], have pointed out that the low intracellular Na^+ maintained by active sodium pumping may provide the driving force for active sugar transport if sugars and Na^+ share a common carrier. Such a mechanism would account for the stimulatory effect of transportable sugars on Na^+ transport. (The sugars would aid Na^+ entry into the cell.) However, direct action of these sugars on the sodium pump itself has not been excluded. In an attempt to investigate the possibility of such a direct action, the effects of sugars on the (Na^+-K^+)-activated ATPase of rabbit kidney were examined. This ATPase has been intimately associated with the active sodium pump in a variety of tissues [36], and demonstration of direct action of actively transported sugars on that enzyme would support the possibility of direct action of sugars on the sodium pump.

Methods

(Na^+-K^+)-activated ATPase was obtained by a modification [4] of the method of Kinsolving, Post, and Beaver [20]. Assay of enzymatic activity was carried out by comparison of the rate of hydrolysis of ATP produced by the enzyme in the presence of Na^+ and K^+ to the rate in the presence of choline$^+$ [4]. Inorganic phosphate was assayed by the method of Taussky and Shorr [37] and protein by the biuret method of Gornall, Bardawill, and David [14].

Results

The relative affinities of the various sugars for the sugar transport system vary from tissue to tissue. In all tissues, however, phloridzin has a high affinity for the transport system compared to that of the simple hexoses and pentoses [1, 26, 30]. Consequently, the search for some direct action of sugars on the (Na^+-K^+)-activated ATPase of importance in sugar transport was begun with a survey of the effect of phloridzin on that enzyme.

Fig. 1 shows the effect of 2×10^{-4} M phloridzin on (Na^+-K^+)-activated ATPase activity plotted against [K^+] at constant ionic strength. A diphasic effect is observed. Phloridzin stimulates the (Na^+-K^+)-activated ATPase at low [K^+] and inhibits it at high [K^+]. This interaction of phloridzin and K^+ is depicted in greater detail in Fig. 2, where (Na^+-K^+)-activated ATPase activity is plotted against [phloridzin] at various Na:K ratios of constant ionic strength. With large Na^+:K^+ ratios, phloridzin stimulates (Na^+-K^+)-activated ATPase activity. This stimulation increases as [K^+] is decreased. If the relative concentration of K^+ is increased, the range of concentrations in which phloridzin stimulates becomes narrower until at K^+ concentrations equal to or greater than the optimum for enzyme activity only an inhibitory effect is found. In this inhibitory range, the

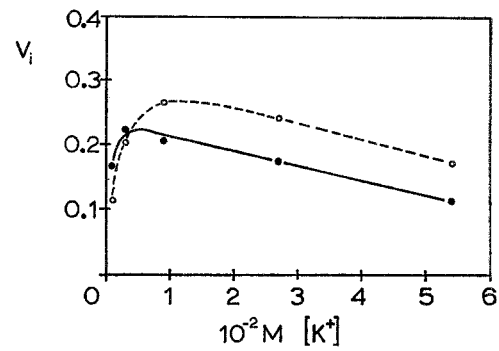

Fig. 1. Effect of phloridzin on the (Na^+-K^+)-activated (Na^+-K^+)-ATPase activity at various [K^+] with [$Na^+ + K^+$] constant at 0.096 M. V_i is in µmoles phosphate liberated per min per mg protein. The 1-ml reaction mixture contained MgATP (5 µmoles), Tris acetate buffer pH 6.7 (20 µmoles), enzyme (0.28 mg protein), and the indicated amounts of sodium and potassium acetate, either alone (o) or with 0.2 µmoles phloridzin (●). Incubation time was 15 min at 37 °C

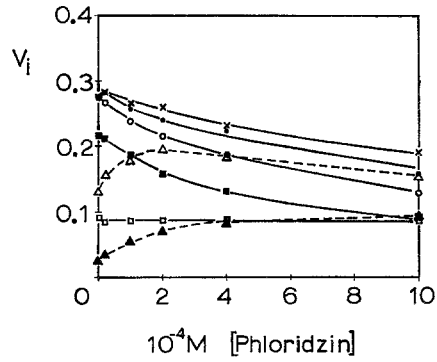

Fig. 2. Effect of phloridzin on (Na^+-K^+)-activated ATPase activity at various Na^+-K^+ ratios. V_i is in µmoles phosphate liberated per min per mg protein. The 1-ml reaction mixture contained MgATP (5 µmoles), enzyme (0.25 mg protein), phloridzin (as indicated), Tris acetate pH 6.7 (20 µmoles), $KC_2H_3O_2$ [0.3 µmoles (▲), 1 µmole (△), 3 µmoles (×), 9 µmoles (●), 27 µmoles (o), or 54 µmoles (■)], and $NaC_2H_3O_2$ so that the sum of sodium and potassium acetate was always 96 µmoles. For determination of Mg-dependent, (Na^+-K^+)-independent ATPase activity (□), choline acetate replaced sodium-potassium acetate. Incubation time was 15 min at 37 °C

inhibition produced by a given concentration of phloridzin increases with increasing [K^+].

If this action of phloridzin relates to sugar transport, then similar effects should be noted with simple sugars sharing the transport system. Because of the differences in relative affinities of the compounds for the transport system, it was anticipated that the concentrations of sugars required to demonstrate the effect would be large. With this end in mind,

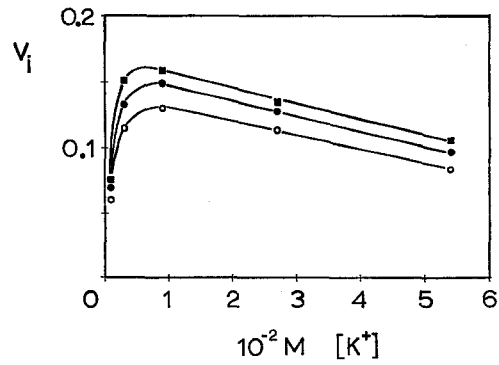

Fig. 3. Effect of D- and L-arabinose on (Na^+-K^+)-activated ATPase activity. V_i is in μmoles phosphate liberated per min per mg protein. The 1-ml reaction mixture contained Tris acetate pH 6.7 (20 μmoles), MgATP (5 μmoles), enzyme (0.304 mg protein), sodium and potassium acetate (0.096 μmoles in the ratio indicated) alone (■) or with L-(+)-arabinose [(300 μmoles) (●)] or D-(−)arabinose [(300 μmoles) (o)]. For determination of Mg-dependent, (Na^+-K^+)-independent ATPase activity, choline acetate (0.096 μmoles) replaced sodium-potassium acetate. Incubation time was 15 min at 37 °C

(Na^+-K^+)-activated ATPase activity was determined in the presence of 0.3 M concentrations of a number of hexoses and pentoses, and the resultant activities were compared. Inhibition varied from approximately 40% in the case of D-mannose to no detectable effect in the case of D-galactose. The degree of inhibition produced by a given sugar differed somewhat from one enzyme preparation to another. However, the ranking of sugars according to decreasing inhibitory potency was the same for all enzyme preparations. This ranking is: D-mannose > D-arabinose, D-xylose > L-xylose > D-glucose > fructose, L-arabinose > D-galactose = 0. The order is independent of $Na^+:K^+$ over the range 95:1 to 2.5:1, and was obtained by the type of direct comparison shown in Fig. 3. The consistent difference in inhibitory effect of D- and L-arabinose shown in Fig. 3 could be duplicated with D- and L-xylose. The observed difference in effect of the pairs of stereoisomers provides support for the concept of relatively specific interaction of sugars and (Na^+-K^+)-activated ATPase.

Because D-mannose appeared to be the most inhibitory of the sugars tested, it was selected for detailed study. Fig. 4 is a plot of (Na^+-K^+)-activated ATPase activity against D-mannose concentration at various $Na^+:K^+$ ratios at constant ionic strength. No stimulation of enzymatic activity was found. Inhibition occurred at D-mannose concentrations greater than 0.1 M regardless of the Na^+-K^+ ratio.

In the inhibitory action of D-mannose and the other sugars, the presence of pyranose or furanose ring structures may be important. This is suggested

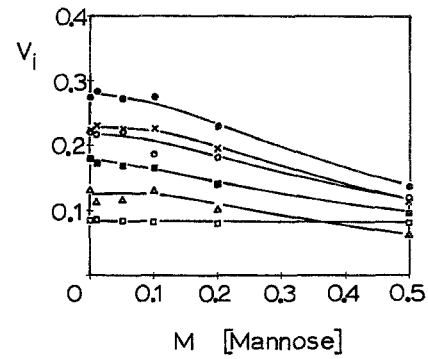

Fig. 4. Effect of D-mannose on (Na^+-K^+)-activated ATPase activity at various Na^+-K^+ ratios. V_i is in μmoles phosphate liberated per min per mg protein. The contents of the 1-ml reaction mixture and the conditions were the same as in Fig. 2 with the replacement of phloridzin by the indicated amounts of D-mannose. In particular, the content of $KC_2H_3O_2$ was 1 μmole (Δ), 3 μmoles (×), 9 μmoles (●), 27 μmoles (o), or 54 μmoles (■), with $NaC_2H_3O_2$ to a constant sum of sodium-potassium acetate of 96 μmoles

by the absence of any detectable inhibition of the (Na^+-K^+)-activated ATPase by 0.3 M mannitol. However, the ring structure cannot be the sole factor responsible for the observed inhibitory actions because of the variations observed among the various sugars. Moreover, 0.3 M myo-inositol, a compound known to be actively reabsorbed in the rat kidney by a mechanism requiring Na^+ and inhibited by phloridzin [15, 16, 17], had no detectable effect on (Na^+-K^+)-activated ATPase activity.

There is some evidence to suggest that sugars are surface-active [10, 31] and may in some cases bind specifically to cell membrane phospholipids [25, 32]. Since this type of surface interaction might be expected to depend on the ionic strength of the medium [10], the pattern of inhibition by D-mannose of the (Na^+-K^+)-activated ATPase was examined over a 64-fold range of [$Na^+ + K^+$] with the ratio of $Na^+ : K^+$ fixed at the optimal value of 0.86:0.09. The results, plotted in the form of Lineweaver and Burk [29] and given in Fig. 5, may be interpreted as indicating that mannose is a noncompetitive inhibitor of the Na^+-K^+-enzyme interaction. Analysis of the data of Fig. 5 by the method of least squares yields values of V_{max} (in μmoles phosphate liberated per min per mg protein) and of K_m (in M) for the uninhibited enzyme of $(7.9 \pm 0.3)\,10^{-2}$ and $(4.4 \pm 0.4)\,10^{-3}$, respectively. The corresponding values for the mannose-inhibited system are $(5.4 \pm 0.4)\,10^{-2}$ and $(3.6 \pm 0.8)\,10^{-3}$, respectively. Within the limits of error, the K_m values are identical, and no stimulatory effect of mannose would be expected at any concentration of [$Na^+ + K^+$] at the ratio of

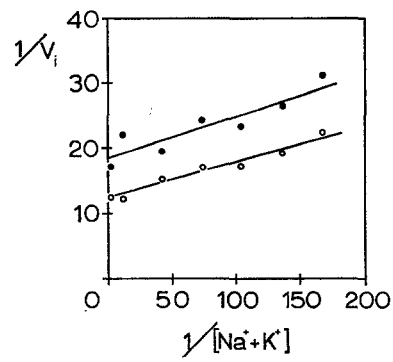

Fig. 5. Effect of 0.3 M mannose on (Na^+-K^+)-activated ATPase activity at various [$Na^+ + K^+$]. V_i is in μmoles phosphate liberated per min per mg protein; [$Na^+ + K^+$] in M. The 1-ml reaction mixture contained Tris acetate pH 6.7 (20 μmoles), MgATP (5 μmoles), enzyme (0.566 mg protein), sodium acetate-potassium acetate in the molar ratio 87:9 in the amounts indicated, alone (o) or with D-mannose (300 μmoles) (●). Incubation time was 15 min at 37 °C

0.86:0.09. The examination of the dependence of the action of mannose on the enzyme was continued at the extreme values of [Na^+-K^+], as the ratio of Na^+ to K^+ was varied widely on both sides of the optimum ratio. The nature of the inhibition did not change.

Neither the sugars nor phloridzin had any effect on the Mg-dependent, (Na^+-K^+)-independent ATPase present in the enzyme preparation.

Discussion

Phloridzin at concentrations of 10^{-4} to 10^{-6} M is a relatively specific inhibitor of active sugar transport in the kidney [30] and intestine [1], and of facilitated sugar transport in the red cell [26]. At higher concentrations of 10^{-4} to 10^{-3} M, equivalent to those used in the present study, phloridzin is known to inhibit aerobic oxidative metabolism and to bring about mitochondrial swelling [5, 19, 28] which is similar to the swelling produced by thyroxine, glutathione, and higher fatty acids. This swelling, which is reversed by the addition of high-energy phosphate compounds [19] and prevented by initial treatment of the mitochondria with uncoupling agents, has been related to an inability of mitochondria to maintain a selective ionic environment [28]. The demonstration in the present study of a complex interaction between Na^+, K^+, phloridzin, and (Na^+-K^+)-activated ATPase may serve to explain some aspects of the metabolic actions of phloridzin at high concentrations. The results imply that phloridzin would cause the

sodium pump, like the ion-activated ATPase, to undergo modification of activity and so would result in the loss of osmotic control and in the appearance of swelling. In mitochondria, the abnormal ionic environment and the increased permeability associated with the swelling would lead to changes in the reactions involving oxidative phosphorylation [33].

Phloridzin, in its action on the (Na^+-K^+)-activated ATPase, appears either to act as a K^+ substitute or to shift the setting of the enzyme for optimal activity to a higher ratio of Na^+ to K^+. In this action, it appears similar to that of diphenylhydantoin, a compound known to produce relatively profound alterations in intracellular ionic homeostasis [12]. The shifts that phloridzin and diphenylhydantoin produce in the ratio of Na^+ to K^+ for optimal activity suggests that there is a relationship with the action of cardiac glycosides. The cardiac glycosides are efficient poisons of (Na^+-K^+)-stimulated ATPase and are known to compete with K^+ at low concentrations [11]. At the moment, the similarities of phloridzin, diphenylhydantoin, and cardiac glycosides are limited to structures containing multiple rings, inhibition of the (Na^+-K^+)-activated ATPase, and interaction with the K^+ site on that enzyme.

The action of simple sugars on the (Na^+-K^+)-activated ATPase appears to be less complicated than that of phloridzin. No stimulation was found and, in those cases where inhibition was discovered, the concentrations required to demonstrate the effect were about 10 times greater than the usual extracellular glucose concentration. Within the framework of the Crane hypothesis of sugar transport, inhibition of (Na^+-K^+)-activated ATPase and presumed inhibition of the Na^+ pump would be followed by decreased sugar transport. If sugars were equal in all respects except for their action on the (Na^+-K^+)-activated ATPase, the order of sugars ranked according to their rate of transport would be the reverse of the order of sugars ranked in decreasing inhibitory effect on the ion-activated ATPase. Unfortunately this comparison is difficult because the sugar transport system in the kidney is not well characterized. Studies of renal sugar transport involving comparisons of large numbers of sugars appear to be limited to the frog [18] in which the transport falls off according to the series: D-glucose > D-galactose > D-mannose > fructose, L-xylose > L-arabinose, and to the rabbit kidney [21, 22] where two phloridzin-sensitive sugar transport systems are indicated, one independent of and the other dependent on Na^+. The sugars transported by the Na^+-dependent system are D-galactose, D-glucose, α-methyl-D-glucoside, fructose, D-xylose, and 6-deoxy-D-glucose. Neither D- nor L-arabinose could be shown to belong to this group of sugars. Consequently, the available evidence suggests that

there is no simple relationship between the inhibitory effect of sugars on the renal (Na^+-K^+)-activated ATPase and the renal sugar transport system. The same conclusion emerges if comparison is made to the better-known red cell [24] and intestinal [38] sugar transport systems which admittedly are different from the renal transport system.

The lack of correspondence between the effects of sugars as inhibitors of the (Na^+-K^+)-activated ATPase and the known properties of the sugar transport system implies that inhibition of the ion-activated ATPase does not play a major role in determining sugar transport. However, it is possible that large concentrations of polyhydroxyl compounds might produce some change in the structure and activity of various enzymes, including the (Na^+-K^+)-activated ATPase, through modification of hydrogen bonding. Such modification might lead sugars to mimic the action of urea in the inhibition that compound produces on the (Na^+-K^+)-activated ATPase [36] and may account for the inhibition of dinitrophenol-stimulated ATPase by polyhydroxyl compounds [27]. (This latter enzyme was assayed in a Na^+-free medium, and a sequence of compounds of decreasing inhibitory potency was found which was markedly different from the corresponding sequence for the ion-activated ATPase; namely, inositol > mannitol > sucrose > glucose > fructose > xylose > glycerol = 0.)

The hydroxyl groups of sugars make the molecules more hydrophilic, and undoubtedly are the basis of their ability to concentrate at interfaces and to cause large changes in surface properties. For example, sucrose alters the properties of monolayers of several proteins [31], and several sugars have been shown to adsorb at a number of interfaces and to cause large changes in the charge density at the interface [10]. These studies indicate that the sugars not only adsorb at interfaces, but also displace ions in the process of adsorption. Although the data of Fig. 5 suggest that higher ionic strengths could not reverse any of the inhibition due to the sugars, the activation due to phloridzin at low [K^+] indicates that adsorption at the active site of the enzyme and displacement of the activating ions at higher ion concentrations are possible mechanisms of action of sugar-like compounds on the (Na^+-K^+)-activated ATPase. Such indirect effects produced by high intracellular concentrations of sugars will require attention in studies designed to assess the interrelationships of Na^+ and sugar transport.

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