Effects of Three Sodium-Potassium Adenosine Triphosphatase Inhibitors

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Reports from several laboratories suggest the presence of an ouabainlike compound in plasma and various animal tissues, particularly during acute volume expansion and in low-renin hypertension. It has been hypothesized that this compound, through inhibition of the Na⁺-K⁺ pump, can constrict blood vessels, enhance vasoconstriction in response to agonists, increase cardiac contractility, raise blood pressure, and cause natriuresis/diuresis and therefore is implicated in the pathophysiology of the low-renin, volume-expanded type of hypertension. However, so far, only two steroid Na⁺-K⁺ pump inhibitors (namely, a bufodienolide derivative [resibufogenin], obtained from toad skin and plasma and a factor with the same carbon, oxygen, and hydrogen content as ouabain obtained from the plasma of volume-expanded humans) have been purified and structurally characterized. To determine whether such endogenous Na⁺-K⁺ pump inhibitors can in fact produce the above effects on the cardiovascular and renal systems, we infused commercially available bufalin (aglycone, identical to resibufogenin except for one H⁺), ouabain, and ouabagenin (aglycone) at equimolar doses in normotensive rats. Relative to ouabain, bufalin produced significantly greater dose-dependent increases in blood pressure, left ventricular rate of pressure change, heart rate, and excretion of urinary volume and sodium. Ouabagenin was without effect on any of these parameters. These data indicate that a Na⁺-K⁺ pump inhibitor can cause an increase in blood pressure despite potent diuretic and natriuretic effects and that, in rats, bufalin is much more potent in this respect than ouabain or ouabagenin. (Hypertension 1991;18:316-324)

It is now widely believed that a specific enzyme in the cell membrane of eukaryotic cells, sodium/potassium-dependent adenosine triphosphatase (Na⁺,K⁺-ATPase), is responsible for the active transport of Na⁺ and K⁺, causing uneven distribution of these ions across the cell membrane.1-3 It has been shown that inhibition of Na⁺,K⁺-ATPase in cardiovascular muscle cells by cardiac glycosides (ouabain, for example), resulting in inhibition of the active transport of Na⁺ and K⁺, causes increased cardiac contractility,3,4 vasoconstriction,5-7 increased responsiveness of blood vessels to vasoactive agents,8,9 and increased arterial pressure5-7,10 (particularly if diuresis does not occur).7 The vasoconstriction is thought to be mediated by electrogenic depolarization11-13 and/or an altered Na⁺/Ca²⁺ exchange mechanism.14

However, despite the importance of Na⁺,K⁺-ATPase, its physiological regulation is not clear. Cardiac glycosides such as ouabain and digoxin, derived from certain plants,15 have a specific inhibitory effect on Na⁺,K⁺-ATPase activity and sodium transport.16,17 The presence and significance of an endogenous Na⁺,K⁺-ATPase inhibitor has recently been the subject of intense debate. Such an endogenous inhibitor, if it were to exist, might play an important role in the regulation of blood pressure (BP). In animal tissues bufotoxin, a compound present in the venom of Bufo toads, is structurally analogous to the plant cardiac glycosides and has been shown to inhibit Na⁺,K⁺-ATPase.18 Several reports also suggest the presence of endogenous Na⁺,K⁺-ATPase inhibitors in mammalian tissues (e.g., plasma,19-23 cerebrospinal fluid,24 brain,25,26 urine,27 and adrenal gland28). Most of these inhibitors were purified to the point of homogeneity but have not been structurally characterized except in the case of lipids. Matthews et al recently reported that the Na⁺-K⁺ pump inhibitor isolated from human plasma has the same chemical composition as ouabain based on...
mass spectral analysis (the referenced abstract does not identify the compound; it was identified at the meeting). Furthermore, the Na⁺,K⁺-ATPase inhibitor present in toad skin that was first identified by Meyer and Linde⁶ has also been chemically characterized by Lichtstein et al.⁴⁰ as a steroidal dieniolide derivative, resibufogenin (monohydroxy-14,15-epoxy-20,22-dieniolide glycoside), which binds to glycoside (ouabain) receptors, inhibits Na⁺,K⁺-ATPase activity, and increases the force of contraction of cardiac tissue. The compound is also present in toad plasma. However, Brownlee et al.⁴¹ recently showed that the bufodienolide bufalin (aglycone), which differs from resibufogenin by only one H⁺, is equipotent to ouabain in inhibiting purified canine renal Na⁺,K⁺-ATPase but is only a weak natriuretic agent in rats. These investigators did not study the effect of bufalin on cardiovascular hemodynamics in rats.

To understand more fully the cardiovascular and renal effects of these putative endogenous Na⁺,K⁺-ATPase inhibitors, we examined the effects of bufalin on BP, heart rate (HR), maximum rate of ventricular pressure change (dP/dt max), and renal excretion of urine volume (UV), sodium (UN,V), and potassium (Uk,V) and compared these with the effects of ouabain (another recently identified Na⁺-K⁺ pump inhibitor in human plasma) and ouabagenin (an aglycone of ouabain) in normotensive rats. We also examined the role of the autonomic nervous system and angiotensin II (Ang II) on these cardiovascular and renal effects of bufalin.

Methods

Animals

Male Wistar rats weighing 200–250 g were purchased from Charles River (Wilmington, Mass.). The rats were maintained on a normal rat chow diet (Bio-Serv Inc., Frenchtown, N.J.) containing 0.39% sodium and 0.9% potassium, housed in a constant-temperature room with a 12-hour light/dark cycle, and allowed free access to tap water. When the rats weighed 390–400 g, they were used in these studies. The rats were anesthetized with 120 mg/kg body wt i.p. inactin and placed on a heating pad (Gorman-Rupp Industries, Bellville, Ohio), with which rectal temperature could be maintained at 37°C. The trachea was cannulated with a PE-240 catheter (Clay-Adams, Parsippany, N.J.) to maintain free respiration. The right femoral artery was cannulated with PE-20 tubing to collect urine samples, and the hemodynamic parameters (arterial BP, HR, and dP/dt max) were recorded. The animals were studied for 4 hours after the initial 30 minutes of anesthesia and then received an intravenous infusion of 100 μg/ml bufalin at the rates of 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 μg/ml/min, each dose over 5 minutes.

In the first series of experiments, after a 30-minute postsurgical stabilization period, two 5-minute control urine samples were collected and arterial BP and HR were recorded (in these experiments dP/dt max was not recorded). The rats then received an intravenous infusion of bufalin in an isotonic solution of 10% ethanol, 90% saline at the rate of 0.005 ml/min for 20 minutes. The solution concentration was 20 μg/ml (0.1 μg/min), 40 μg/ml (0.2 μg/min), or 100 μg/ml (0.5 μg/min). Other animals received only vehicle at the same volume rate. Ten-minute urine samples were collected during and for 120 minutes after the infusion. Concentrations of sodium and potassium in the urine samples were measured with flame photometry (Klina Flame, Beckman Instruments, Palo Alto, Calif.), and 10-minute UV, UN,V, and Uk,V were calculated.

The results of these experiments indicated that only the highest dose of bufalin produced a BP response; the two lower doses were without effect. Therefore, in the second series of experiments, we examined the cumulative effects of increasing doses of bufalin infused intravenously at a concentration of 100 μg/ml. The rats were prepared as in the first series. After a 30-minute postsurgical stabilization period, two 5-minute control urine samples were collected and the hemodynamic parameters (arterial BP, HR, and dP/dt max) were recorded. The animals then received an intravenous infusion of 100 μg/ml bufalin at the rates of 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 μg/ml/min, each dose over 5 minutes. Five-minute urine samples were collected during and for 30 minutes after the infusion of each dose. Other animals were prepared and examined in the same manner except they received only vehicle instead of the solution containing bufalin. Volume infusion rates were the same. In other experiments we used the same protocol to study the cumulative effects of equimolar doses of ouabain dissolved in saline and equimolar doses of ouabagenin. We used ouabagenin to determine if any differences in the responses to bufalin and ouabain are related to the fact that ouabain is a glycoside.

In another series of experiments we examined the role of Ang II in the responses elicited by bufalin. We first blocked the action of Ang II by intravenously infusing the Ang II antagonist saralasin ([Sar⁶,Ala⁷]Ang II; Peninsula Laboratories, Belmont, Calif.) in saline, initially at the rate of 2 μg/min for 20 minutes and then at the rate of 1 μg/min for the remainder of the experiment. Bufalin was then infused cumulatively as described above. The Ang II blockade was confirmed
by test doses of Ang II at the beginning and following the completion of the experiment.

The sustained vasopressor effect of bufalin infusion was always associated with a sustained increase in HR; we wondered whether this was due to a decrease in parasympathetic tone or to an increase in adrenergic drive to the heart. To test this possibility, in some rats, cholinergic receptors or β-adrenergic receptors were blocked. The cholinergic receptor blockade was achieved with a bolus injection of 1 mg atropine in 0.05 ml saline and then a slow infusion of 10 μg/min atropine in 0.5 μl saline for the duration of the experiment. The β-adrenergic receptor blockade was achieved with a bolus injection of 2 mg propranolol in 0.05 ml saline and then a slow infusion of 20 μg/min propranolol in 0.5 μl saline for the duration of the experiment.32 The cholinergic or β-adrenergic receptor blockade was confirmed by a provocative bolus injection of 10–50 μg acetylcholine or 10–20 μg isoproterenol, respectively, before the beginning of bufalin infusion and following the end of the postinfusion period. Bufalin was infused cumulatively as described above.

In another series of experiments, we studied the effects of bufalin or ouabain on vascular smooth muscle cell membrane potential in tail arteries of normal rats in vitro using our standard technique.12,13 Briefly, transmembrane potentials were measured with glass microelectrodes filled with a 3 M KCl solution and having a tip resistance of 30–80 MΩ and tip potentials of <5 mV. Microelectrode impalements were made from the adventitial side of unopened caudal arteries, which were constantly suffused with physiological salt solution (NaCl 118.3, KCl 4.7, CaCl 2.5, KH2PO4 1.2, NaHCO3 1.2, and glucose 11 mmol/l) aerated with 95% O2/5% CO2. The pH was monitored with a small electrode placed in the bath and was held constant at 7.3–7.4. Following impalement and the recording of control transmembrane potentials, bufalin or ouabain was added to the bathing solution to achieve a concentration of 10⁻⁵ M and the effect on potassium potentials was recorded. This concentration was used because calculations based on several assumptions (e.g., very long half-lives of 12 hours or more, absence of degradation) indicated that the final plasma concentration of these agents achieved in our in vivo experiments was about 10⁻³ M.

Statistical Analysis of Data

All data are expressed as mean±SEM, and statistical significance was determined using a two-tailed t test for comparing the means of independent samples. Analysis of variance was used to detect possible significant differences among groups, and repeated-measures analysis of variance was used for comparisons within groups. Duncan’s multiple range test was used to determine significance of the F ratio; p<0.05 is considered significant.
Figure 2. Effects of intravenous infusion of 0.5 μg/min bufalin (●) or vehicle (○) for 20 minutes on excretion of urinary volume (UV), sodium (U\textsubscript{Na}V), and potassium (U\textsubscript{K}V) in rats. Same animals as in Figure 1. *p<0.05 different from before infusion; ‡p<0.05 different from vehicle.

Figure 3. Cumulative effects of intravenous infusion of bufalin (●), ouabain in saline (▲), or vehicle for bufalin (●) (n=8, 7, and 5, respectively) at six different rates for total of 30 minutes on blood pressure, heart rate, and rate of ventricular pressure change (dp/dt) in rats. *p<0.05 different from before infusion; ‡p<0.05 different from ouabain.

Figure 4. Ouabagenin in doses equimolar to those of bufalin had no effect on any parameter (Figures 5 and 6).

Exposure of vascular smooth muscle cells in the rat tail artery in vitro to 10\textsuperscript{-5} M bufalin produced a significant depolarization of the resting membrane potential, but the same concentration of ouabain was without effect (Figure 7).

Ang II blockade had no effect on the response to bufalin. Bufalin's effects on BP, HR, and dp/dt\textsubscript{max} did not differ in Ang II-blocked and normal rats (Figure 8). Additionally, UV, U\textsubscript{Na}V, and U\textsubscript{K}V following bufalin infusion did not differ in Ang II-blocked and normal rats (Figure 9). Similarly, cholinergic (n=4) or ß-adrenergic (n=4) receptor blockade had no effect on cardiovascular or renal responses to bufalin infusion.

Discussion

These data show that bufalin, a Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor, is a potent vasopressor agent in rats. Bufalin also produces a dose-dependent increase in HR and dp/dt\textsubscript{max}. These data, coupled with our previous findings that bufalin has a direct vasoconstrictor effect when infused into the dog forelimb vascular bed, strongly suggest that the vasopressor effect of bufalin results from an action on the heart as well as through its vasoconstrictor effect. The vasoconstrictor effect of bufalin probably results from depolarization of vascular smooth muscle cells since the concentration of bufalin that is pressor in vivo also depolarizes vascular smooth muscle cells in normal rat tail artery in vitro. The depolarization probably results from suppression of the electrogenic Na\textsuperscript{+}-K\textsuperscript{+} pump. It is also possible that the vasoconstriction induced by bufalin results in part from altered Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in vascular smooth muscle cells since, like other Na\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitors, bufalin inhibits active Na\textsuperscript{+} and K\textsuperscript{+} transport and thus alters the Na\textsuperscript{+} gradient across the cell membrane and therefore the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. In either case (altered voltage-dependent Ca\textsuperscript{2+} influx, as suggested by the depolarization observed in our experiments, or altered Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange), the end result will be an increase in the intracellular concentration of free...
Figure 4. Cumulative effects of intravenous infusion of bufalin (●), ouabain in saline (▲), or vehicle for bufalin (●) at six different rates for total of 30 minutes on excretion of urinary volume (UV), sodium (U NaV), and potassium (U KV) in rats. Same animals as in Figure 3. *p<0.05 different from before infusion; t* p<0.05 different from ouabain.

Ca²⁺ and therefore contraction of the vascular smooth muscle cells.

Our data also show that the increase in BP following the infusion of bufalin occurs in spite of a significant and dramatic increase in diuresis and natriuresis. The data further show that diuresis and natriuresis induced by bufalin is, at least in part, independent of BP because during the infusion of 0.5 μg/min bufalin for 20 minutes in our first series of experiments, diuresis and natriuresis occurred throughout the postinfusion period after BP had returned to the control level. This BP-independent natriuresis and diuresis is probably due to decreased renal tubular Na⁺ reabsorption subsequent to the inhibition of renal Na⁺,K⁺-ATPase. Admittedly, some of the natriuresis and diuresis that we observed during cumulative infusions of bufalin must have resulted from the large increase in BP. Perhaps the massive natriuresis and diuresis immediately following the end of bufalin infusion is due to additive factors such as 1) increased BP, 2) inhibited tubular Na⁺ and water reabsorption, and 3) decreased renal vascular resistance and increased renal blood flow due to cessation of bufalin infusion.

Thus bufalin, a Na⁺,K⁺-ATPase inhibitor, raises BP, possibly by inhibiting cardiovascular Na⁺-K⁺ pump activity. Furthermore, by inhibiting renal tubular cell Na⁺-K⁺ pump activity and by raising BP, bufalin also causes natriuresis and diuresis. However, these effects of bufalin do not explain the observed significant increase in U NaV. The kaluresis induced by infusion of bufalin is probably due to the massive increase in the excretion of Na⁺, which is then exchanged for K⁺ in the distal convoluted tubule. This is supported by the fact that infusion of ouabain, another Na⁺,K⁺-ATPase inhibitor, also causes natriuresis/diuresis and kaluresis.

Although infusion of equimolar doses of ouabain caused the same directional changes in all parameters except HR, the magnitudes of these changes were almost always less than those produced by bufalin (the exception was U NaV after infusion; Figures 3 and 4). These significantly lesser effects in response to equimolar doses of ouabain are probably...
due to the very low sensitivity of rat Na⁺,K⁺-ATPase to ouabain.³⁷

It must be recognized that we studied the acute effects of ouabain and bufalin. Since association rate constants for glycosides such as ouabain are often several-fold less than those of aglycones such as bufalin, the increased responses to bufalin that we observed may simply be due to the fact that bufalin binds more rapidly. Allen and Schwartz³⁷ have shown that the binding of cardiac glycosides to Na⁺,K⁺-ATPase from rat kidney is less stable than that to the enzyme from other species more sensitive to these glycosides. Therefore, effective doses of cardiac glycosides in rats initially are more likely to produce extrarenal electrolyte disturbances, leading to hyperkalemia and hyponatremia.³⁶ Despite the hyponatremia, high doses of cardiac glycoside cause increased U₄NaV due to inhibition of tubular Na⁺ reabsorption. The significantly increased U₄KV that we observed after ouabain infusion may be in part due to hyperkalemia.

Infusion of equimolar doses of the aglycone ouabagenin did not affect any hemodynamic or renal excretory parameter. This is not unexpected because the binding affinity of bufalin on most Na⁺ pump preparations is roughly 10−20-fold higher than that of ouabagenin. Thus, much higher doses of ouabagenin would probably be required to produce comparable renal effects. More importantly, however, our experiments show that the lesser responses to ouabain relative to equimolar doses of bufalin when administered acutely are not due to the fact that ouabain is a glycoside and bufalin is an aglycone.

In contrast to the results reported by Brownlee et al.,³⁸ in our experiments the natriuretic and diuretic effects of bufalin did not seem to be dependent on the action of Ang II. The magnitude and duration of the natriuretic/diuretic responses as well as the cardiovascular responses to bufalin infusion did not differ in normal rats and rats following Ang II blockade with saralasin, suggesting that Ang II is not involved in the observed responses. This discrepancy in our results and those reported by Brownlee et al.³⁸ may be due to a difference in the doses of bufalin used. Brownlee et al.³⁸ infused bufalin at one dose at the rate of 1 µg/min for 20 minutes, whereas we infused cumulative doses of bufalin ranging from 0.5 to 20 µg, each dose for 5 minutes, for a total duration of 30 minutes.

However, as in the study of Brownlee et al.,³¹ the diuretic and natriuretic response to bufalin was delayed; the maximal response was observed during the first 10−15 minutes after the infusion. We suspect that this massive diuresis and natriuresis resulted in part from the removal of renal vasoconstriction by cessation of the bufalin infusion. This is supported by our studies in dogs; infusion of bufalin into canine renal arteries caused increased renal vascular resistance and decreased blood flow. Following cessation
effects of angiotensin II blockade with saralasin (A) on blood pressure, heart rate, and rate of ventricular pressure change (dp/dt) in response to bufalin in rats (n=7). Bufalin data from Figure 3 (bullet) included for comparison. *p<0.05 different from before infusion.

of the infusion, renal vascular resistance returned to normal and was associated with the return of blood flow to normal and massive diuresis and natriuresis.39

Ouabain produced lesser effects even though it is equipotent to bufalin as an inhibitor of purified canine renal Na⁺,K⁺-ATPase.31 Perhaps this is not the case for rat Na⁺,K⁺-ATPase. Another possibility is that one or both agents have effects in addition to Na⁺,K⁺-ATPase inhibition. Weiland et al40 and Kim and Labella41 point out that certain steroid inhibitors of Na⁺,K⁺-ATPase, particularly the progesterone derivatives, are negatively inotropic when allowed to enter the cardiac cell but positively inotropic when restricted to the cell surface by attaching certain sugars to the molecule. Clearly, all Na⁺,K⁺-ATPase inhibitors do not produce the same hemodynamic and renal effects in rats.

The vasoconstrictor and natriuretic/diuretic effects of bufalin indicate that this agent has some of the properties required to be considered as a candidate for the circulating endogenous Na⁺,K⁺-ATPase inhibitor. A circulating Na⁺-K⁺ pump inhibitor has been reported to be present in several forms of low-renin hypertension20-23,42-44 and in the acutely volume-expanded state.23,44-46 However, in spite of several successes26-28,47,48 in purifying this endogenous inhibitor obtained from several tissues to homogeneity, the attempts to characterize it structurally have not been successful, except in the case of endogenous digitalislike activity in the skin and plasma of the toad Bufo marinus and more recently in human plasma. Using both receptor and immunoassay, Flier et al49 reported the presence of a circulating Na⁺-K⁺ pump inhibitor in B. marinus. Its presence in plasma of the toad suggests that this pump inhibitor may be involved in the physiological regulation of ion transport in various tissues. However, Flier et al49 also showed that Bufo spp. (as is the case with rats) are relatively resistant to the action of digitalislike compounds; for example, B. marinus requires 100 times more ouabain to inhibit Na⁺ transport across its bladder than Rana pipiens. Lichtstein et al30 have now purified and fully characterized the digitalislike compound in the plasma and skin of B. marinus and have shown it to be resibufogenin. Our data show that bufalin, which is closely related to

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resibufogenin, has a significantly greater effect on cardiovascular contractility and renal excretion of Na⁺ and water in rats than does ouabain, even though ouabain and bufalin have been shown to be equipotent inhibitors of canine renal Na⁺,K⁺-ATPase.³¹ These findings, therefore, suggest that the endogenous Na⁺,K⁺ pump inhibitor, at least in rats, may be more like bufalin in its physiological action than like ouabain.

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