Increased sympathetic nerve activity with augmented release of catecholamines occurs in several forms of hypertension. The evidence for an endogenous substance with pressor and vascular sensitizing effects in hypertension is long standing, and a number of investigators have described circulating endogenous inhibitors of the sodium-potassium pump, also called digitalislike factors (DLFs) in animals and humans with hypertension. Whether there is a causal relation between endogenous DLFs and the enhanced sympathetic nerve activity in different forms of hypertension is unknown, but it is known that the cardiac glycosides inhibit norepinephrine uptake by adrenergic nerve terminals.

We designed experiments to investigate the role of a DLF in peripheral sympathetic neurovascular activity because such knowledge may help explain how DLFs may contribute to hypertension. In this research we used the steroid bufodienolide bufalin (Figure 1) as a model of an endogenous DLF since resibufogenin is not commercially available. Bufalin is a potent inhibitor of Na⁺,K⁺-ATPase and a stimulator of cardiovascular muscle contractile activity, is readily available commercially, and is almost identical in structure (differing only by one H⁺) (Figure 1) to the only chemically identified mammalian endogenous DLF, resibufogenin, a bufodienolide found in amphibians.

If the bufodienolides are to be considered candidates for the endogenous DLF, they should block K⁺-mediated vasodilation, potentiate norepinephrine vasoconstriction, increase vascular resistance, increase left ventricular dp/dt, raise arterial blood pressure, and produce natriuresis and diuresis, since all of these changes result from inhibition of Na⁺-K⁺ pump activity with the cardiac glycosides. Bufalin does, in fact, produce all of these changes in the anesthetized dog and rat. Using the isolated canine saphenous vein preparation, Brender demonstrated that cardiac glycosides increase basal tone and enhance vascular responsiveness to sympathetic nerve stimulation. α-Adrenergic receptor antagonists blocked the effects of cardiac glycosides suggesting that the neurotransmitter norepinephrine is involved in these effects. Lorenz et al demonstrated that digitalis derivatives such as acetylstrophanthidin contract vascular smooth muscle by displacing norepinephrine located in sympathetic nerve terminal vesicles, facilitating its release, reducing its metabolism, and inhibiting its reuptake. All these actions result in elevated concentrations of the norepinephrine in the neuromuscular junction and therefore potentiate vascular contractions.

This study was designed to investigate the vascular effects of bufalin, which is an endogenous digitalislike factor.
like factor. In particular, we were interested in whether bufalin altered release and uptake of the sympathetic neurotransmitter norepinephrine similar to the cardiac glycoside digitalis. We examined the effects of bufalin on endogenous norepinephrine release and overflow in basal and stimulated conditions and its effect on [3H]norepinephrine accumulation in isolated canine saphenous vein, a well characterized densely innervated blood vessel.15-20

Methods

All experiments were performed on lateral saphenous veins excised from mongrel dogs (15-25 kg) anesthetized with intravenously administered pentobarbital (30 mg/kg i.v.). The veins were cut into helical vein strips (4 mm wide, 10 cm long), suspended in a jacketed chamber, and superfused continuously (1.5 ml/min) with aerated Krebs-Ringer solution by a constant flow roller pump. The Krebs-Ringer solution had the following composition (mM): NaCl 118.3; KCl 4.7; MgSO4 1.2; CaCl2 2.5; NaHCO3 25; glucose 11.1, and disodium ethylenediaminetetraacetate (EDTA) 0.03. Sodium metabisulfite, 0.05 mM was added as an antioxidant. The solution was continuously aerated with a 95% O2-5% CO2 mixture.

Experiments in Stimulated Blood Vessels

Two platinum wires (26 gauge) were placed adjacent and parallel to the veins to stimulate norepinephrine release from the adrenergic nerve terminals in the blood vessel wall.15-17 Electrical impulses consisted of rectangular waves (9 V, 2 ms, 5 Hz) and were provided by a direct current power supply and a switching transistor triggered by a stimulator (S44, Grass Instrument Co., Quincy, Mass.). The preparations were connected to a force displacement transducer for continuous isometric tension recording, and basal tension was set at 2.0 g.

The first samples of superfusate were collected 90 minutes after initial setup of the veins. The veins were superfused for a total of 210 minutes. Electrical stimulation (5 Hz) was applied for three 15-minute periods between 105 and 120 minutes (ES1), between 135 and 150 minutes (ES2), and between 195 and 240 minutes (ES3). In addition, the superfusate was collected continuously for 15 minutes before each stimulation period. This collection procedure allowed measurements of norepinephrine release under both basal and stimulated conditions. A total volume of 22 ml of superfusate was collected into cooled graduated cylinders containing sodium metabisulfite (100 μl, 0.5 M). The effect of bufalin on both basal and electrically stimulated release of norepinephrine was determined by supervising the vein with a Krebs-Ringer solution containing the drug for 15 minutes before and during the second period of electrical stimulation (ES2). Bufalin (Sigma Chemical Co., St. Louis, Mo.) was solubilized in a volume of 50% ethanol such that the final concentration of ethanol in the Krebs was less than 0.1%. This concentration of ethanol does not alter norepinephrine release.21

The response obtained with the drug was then compared with the response of the vein before and after exposure to the drug (ES1 and ES3).

In some experiments tetrodotoxin (TTX) (10^-6 M) was also added to the Krebs-Ringer solution 15 minutes before and during ES2. In these experiments, the control vessel was also exposed to TTX using this same protocol. One of the paired vessels was also treated with bufalin (10^-5 M) for 15 minutes before ES2 and during the stimulation. After ES2, both bufalin and TTX were discontinued, and the vessels were washed with drug-free Krebs-Ringer for 30 minutes before the final stimulation. In other experiments, the concentration of potassium ions in the Krebs-Ringer perfusing solution was increased from 5.9 to 100 mM by equimolar replacement of NaCl by KCl. After a basal collection, the 100 mM KCl solution was applied to the vessels and maximal tension developed within 15 minutes. Bufalin (10^-5
M) was then added to the superfusing Krebs and continued for 45 minutes. Superfusates were collected before the bufalin and at sequential 15-minute intervals for the remainder of the experiment. A paired vessel from the same animal was exposed to 100 mM KCl without bufalin and served as a control for tension and norepinephrine overflow.

The time dependence of the effects of bufalin was examined in experiments in which electrical stimulation was applied continuously for a period of 50 minutes with collection at 10-minute intervals. Bufalin (10^{-5} M) was added to the superfusing fluid after the initial collection of a pretreatment sample of superfusate.

**Experiments in Unstimulated Blood Vessels**

The blood vessels were prepared for superfusion as described above with electrical stimulation being omitted. Samples of superfusate were collected during 15-minute intervals for analysis of norepinephrine beginning 90 minutes after placement of the vessels in the superfusion chamber. After collection of a control sample, the superfusing Krebs-Ringer was switched to one containing 10^{-5} M bufalin.

At the end of each experiment, the vein was blotted dry and weighed. Norepinephrine was extracted in two aliquots of 5.0 ml of 1.0N acetic acid containing EDTA (0.03 mM) and ascorbic acid (1.1 mM). In previous experiments, it has been found that the efficiency and reproducibility of extracting norepinephrine by this method compares favorably with the technique of homogenizing vein tissue. Norepinephrine was isolated from tissue extracts by alumina adsorption and cation exchange chromatography. In addition a small section of the vein was extracted in the same manner before mounting it for superfusion so that the norepinephrine content could be compared before and after the experiment.

**Chromatographic Analysis of Norepinephrine**

Norepinephrine was isolated from superfusates using octadeclsilyl (C_{18}) separation cartridges according to the method of Hunter et al. Norepinephrine was eluted from the cartridges using 2 ml of 10% acetonitrile containing 0.15% trifluoroacetic acid. Eluates were lyophilized and reconstituted with 0.1N perchloric acid. The concentration of norepinephrine in 25 μl of this eluate was determined by liquid chromatography with electrochemical detection using a C_{18}Bondapak column and a mobile phase of 125 mM monochloroacetic acid, 1 mM sodium octane sulfonate, 0.75 mM disodium EDTA, and 1.5% methanol at a pH of 3.8.

The electrochemical cell used for quantitation of norepinephrine in the high-performance liquid chromatography column eluent was maintained at a potential of 0.65 V against a silver/silver chloride reference electrode. The lower limit of sensitivity for this method is 25 pg for norepinephrine, with a signal-to-noise ratio of 5.

**[^H]Norepinephrine Accumulation Experiments**

Canine saphenous veins were cut into longitudinal strips 2.0 cm x 4 mm and preincubated for 10 minutes (37°C) in 1.5 ml Krebs-Ringer solution or Krebs-Ringer solution containing bufalin (10^{-6} M or 10^{-5} M).

This preincubation was followed by incubation in a solution (1.5 ml) containing L-(7-[^H])-norepinephrine 2×10^{-7} M, (10–30 Ci/mmol), EDTA (0.05 mM) and L-ascorbic acid (0.3 mM) were added to the incubation solution to stabilize the amines. Incubation was for 2, 5, 10, or 15 minutes at 37°C in the presence of 2×10^{-7} M[^H]norepinephrine. After incubation, the veins were rapidly rinsed and placed in 2.5 ml of 1N acetic acid solution containing EDTA (0.03 mM) and ascorbic acid (1.1 mM). The tissues were extracted twice and the two extracts were combined. [^3^C]Orbitol was used to correct for carryover of extracellular[^H]norepinephrine by the tissue. Aliquots (0.5 ml) of the tissue extract were counted in a liquid scintillation counter, and the activity was expressed per milligram dry weight. In some experiments, corticosterone (2×10^{-4} M) was added to the Krebs-Ringer used in the[^H]norepinephrine accumulation studies to determine the effects of uptake_2 inhibition on bufalin-induced inhibition of accumulation.

**Statistical Methods**

The data are presented as mean±SEM. The data were analyzed using a blocked two-way analysis of variance. Means were compared either using the Student’s t test or, in the case of multiple comparisons, the Student-Newman-Keuls test.

**Results**

**Effects of Bufalin on Stimulated Veins**

**Electrical stimulation.** Transmural electrical stimulation (5 Hz) caused an increase in tension of 3.30±0.84 g and a norepinephrine overflow of 20±5 fmol/min/mg tissue from postganglionic sympathetic nerve terminals in canine saphenous veins during electrical stimulation. Norepinephrine measured in basal samples before ES, was always less than 5% of that during electrical stimulation, and the results on basal samples are not reported. Norepinephrine overflow, contractile tension, or tissue content of norepinephrine showed no significant changes with time during two additional 15-minute periods of electrical stimulation (ES_2 and ES_3) (Figure 2). In a separate series of veins, bufalin (10^{-5} M) applied 15 minutes before and continued during ES_2 significantly augmented norepinephrine overflow and tension (Figures 3A and 3B). Removal of bufalin caused a marked decrease in norepinephrine overflow but failed to influence tension during ES_3. Norepinephrine concentration of the tissue was unaltered bybufalin under these conditions (pretreatment concentration 2.40±0.25 ng/mg, posttreatment 2.18±0.16 ng/mg).

The increase in tension and norepinephrine overflow caused by bufalin during electrical stimulation...
Figure 2. Bar graph shows overflow of endogenous norepinephrine (NE) from canine saphenous veins exposed to three successive intervals of electrical stimulation (ES₁, ES₂, ES₃) (10 V, 5 Hz, 2 msec) of 15 minutes each with 30-minute intervals between stimulations (n=8 dogs). NE content of the tissue showed no change before (2.01 ±0.3 ng/mg) and after (1.96±0.3 ng/mg) stimulation.

was dose-related (Figures 4A and 4B). The lowest concentration that caused a significant increase in tension was 10⁻⁷ M and a plateau in the response was observed at about 10⁻⁶ M. In contrast, the effect of bufalin on norepinephrine overflow continued to increase up to concentrations of 10⁻⁵ M.

The time course of norepinephrine overflow when bufalin was applied to a vein already maximally stimulated electrically was also investigated. Veins treated with 10⁻⁵ M bufalin showed a significantly increased overflow after a 10-minute delay (Figure 5), which was not observed in untreated control vessels.

K⁺ stimulation (100 mM). Exposure of the vessels to Krebs-Ringer containing 100 mM KCl resulted in an increased norepinephrine overflow (Table 1) that showed a significant progressive decrease with time while tension remained constant. Paired vessels from the same animal treated with 10⁻⁵ M bufalin also showed an initial significant fall in norepinephrine overflow that was reversed by bufalin after an initial time delay (Table 1).

Effects of Bufalin on Unstimulated Veins

In unstimulated saphenous veins 10⁻⁵ M bufalin caused delayed increase in tension that reached a maximum following about 30 minutes of drug exposure (Figure 6A). Superfusate collection revealed a coincident increase in norepinephrine overflow (Figure 6B). After termination of the bufalin, tension and norepinephrine overflow remained significantly elevated during the subsequent 30 minutes.

Effects of Tetrodotoxin on Bufalin Response in Stimulated Veins

In electrically stimulated preparations 10⁻⁶ M TTX significantly decreased the norepinephrine overflow during ES₂ (Figure 7). In the presence of TTX, 10⁻⁵ M bufalin did not increase the overflow. When both TTX and bufalin were discontinued and stimulation repeated (ES₃), the vessel that had previously been exposed to bufalin showed a marked augmentation of norepinephrine overflow.

Effects of Bufalin on [³H]Norepinephrine Accumulation

Control vessels incubated in [³H]norepinephrine showed an increasing norepinephrine accumulation with increasing incubation time (Figure 8). Pretreatment with bufalin (10⁻⁵ M) caused a significant inhibition of norepinephrine accumulation at 10 and 15 minutes but not following 2- and 5-minute incubations.

In another series of experiments, the uptake₂ blocker corticosterone (2×10⁻⁴ M) was added coincident with the bufalin. Blockade of uptake₂ did not alter the degree of inhibition observed with bufalin alone (n=5, data not shown).

Discussion

The experiments demonstrate that bufalin, structurally almost identical to a steroidal bufodienolide
TABLE 1. Effect of Bufalin on Norepinephrine Overflow From Isolated Canine Saphenous Veins Depolarized by 100 mM KCI

<table>
<thead>
<tr>
<th>Time</th>
<th>K⁺-Stimulated vein</th>
<th>K⁺-Stimulated +bufalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>00-15 min</td>
<td>14.5±4.3</td>
<td>12.0±2.6</td>
</tr>
<tr>
<td>15-30 min</td>
<td>10.1±3.1</td>
<td>8.8±1.8</td>
</tr>
<tr>
<td>30-45 min</td>
<td>7.1±1.6*</td>
<td>12.2±3.5†</td>
</tr>
<tr>
<td>45-60 min</td>
<td>6.7±1.5*</td>
<td>11.0±2.8†</td>
</tr>
<tr>
<td>90-105 min</td>
<td>4.9±1.7*</td>
<td>7.6±2.5†</td>
</tr>
</tbody>
</table>

Values shown as mean±SEM, expressed as femtomoles per minute per milligram. n=5 in each group. Each period of collection was 15 minutes. Bufalin (10⁻⁵ M) was applied to the treated vessel during the 15-60-minute interval. Before K⁺ stimulation, basal norepinephrine overflow was not significantly different between control and treated groups.

*Significantly different from the norepinephrine overflow during the 00-15-minute interval (by Student-Newman-Keuls test).
†Significantly different from the control vessel treated with KCl alone (paired t test).

endogenous to certain amphibians, affects sympathetic nerves innervating vascular smooth muscle in a number of ways. Bufalin markedly increases the norepinephrine overflow and contractile tension developed by canine saphenous veins in both unstimulated and stimulated preparations. It also significantly inhibits [³H]norepinephrine accumulation in saphenous veins. These effects are consistent with its known ability to inhibit Na⁺,K⁺-ATPase, and the effects of the cardiac glycosides on norepinephrine turnover in blood vessels. Here there is another property bufalin shares with the cardiac glycosides, one that could contribute to its ability to activate cardiac and vascular smooth muscle by increasing the concentration of norepinephrine in the neurovascular junction.

Postganglionic sympathetic nerve terminals located at the adventitial-medial border of the canine saphenous vein have a high content of norepinephrine that can be released by transmural electrical stimulation, K⁺, and other stimuli. In the present experiments, overflow of norepinephrine was measured; overflow is not, however, synonymous with release because with overflow both neuronal and extraneuronal uptake sites are relative to that function. These processes reduce the amount of norepinephrine in the sample actually released. In the

FIGURE 4. Graphs show dose-dependent effect of bufalin on tension (panel A) and norepinephrine (NE) overflow (panel B) in electrically stimulated canine saphenous veins (n=5–8). Figures show the increase of second period of electrical stimulation over the first. Results are presented as mean±SEM. *Difference from control is significant.

FIGURE 5. Bar graph shows time course of norepinephrine overflow when bufalin (10⁻⁵ M) was added to saphenous vein preparations that were being supramaximally stimulated by electrical impulses. †Overflow was significantly different from a matched control vein and from pretreatment values.
present experiments, bufalin inhibited [3H]norepinephrine accumulation by 57%. The addition of the uptake2 blocker corticosterone did not affect these results, reflecting the fact that [3H]norepinephrine accumulation in this preparation was predominantly via neuronal uptake (uptake2) sites.Bufalin increased overflow of norepinephrine approximately seven times in unstimulated veins. The magnitude of this increase relative to the observed uptake inhibition suggests another mechanism occurs in addition to inhibition of norepinephrine uptake. We suggest bufalin directly augments release and accumulation of norepinephrine by membrane depolarization of the nerve terminals due to inhibition of the electrogenic Na+–K+ pump.

The increase in endogenous norepinephrine overflow seen with bufalin in the electrically stimulated preparations is probably due to exocytotic mechanisms since it is abolished by TTX. TTX prevents neuronal Na+ influx by blocking the fast Na+ channels. This conclusion is supported by the further evidence that vessels previously exposed to bufalin and TTX exhibit an increase in norepinephrine overflow and tension when the TTX is removed. This could be explained if the bufalin depolarization is more persistent than is the blockade of Na+ channels by TTX.

Addition of 100 mM K+ to superfusing solution increased the amounts of norepinephrine overflowing from vein preparations due to neuronal membrane depolarization in accordance with the Goldman equation. However, amounts of norepinephrine recovered progressively significantly decreased with time in the untreated preparations. The mechanism of this decrease is not clear. The fall-off in norepinephrine overflow was reversed when bufalin was added (Table 1), but overflow did not increase to the extent seen in preparations bathed in Krebs-Ringer solution (Figures 6 and 7).

**Figure 6.** Graphs show time dependence of tension (panel A) and response time dependence of norepinephrine (NE) overflow (panel B) in unstimulated veins during and subsequent to exposure to (10^{-5} M) bufalin. Data expressed as mean±SEM, n=5. *Significant difference relative to bufalin value.

**Figure 7.** Inhibition of bufalin-enhanced norepinephrine (NE) overflow by tetrodotoxin (TTX) (10^{-5} M) in canine saphenous veins exposed to three successive intervals of electrical stimulation (ES1, ES2, ES3) (10 V, 5 Hz, 2 msec) of 15 minutes each with 30-minute intervals between stimulations. Control veins received TTX during ES1, while the treated veins received TTX and bufalin (10^{-5}) during ES2.

**Figure 8.** Line graph shows time course for accumulation of [3H]norepinephrine in unstimulated saphenous veins (n=5). *Difference from paired control vein is time-control significant. NE, norepinephrine.
3B and 5). This suggests that bufalin influences overflow in part via a mechanism independent of membrane depolarization. Consideration was given to the possibility that bufalin might inhibit the mechanism of a proton pump, but it does not in a chromaffin vesicular preparation (N. Nelson, unpublished observation).

These studies show that bufalin, a Na⁺,K⁺-ATPase inhibitor and pressor agent structurally almost identical to a steroidal bufodienolide endogenous to the toad Bufo marinus, increases norepinephrine turnover in an adrenergic nerve rich vein. The increased turnover appears to result from increased norepinephrine release and decreased norepinephrine uptake. These changes are associated with increased tension. The results suggest that bufalin and, by inference, DLF increase the concentrations of norepinephrine in neu-rovascular clefts. Thus, in addition to direct muscle cell effects in heart and blood vessels, bufalin and DLF may also have indirect effects on these cells via the adrenergic nerve terminals, which would help to account for their effects on blood pressure.

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Key Words: digitalis • digitalis-like factors • glycoside • blood vessels • adrenergic receptors • norepinephrine • bufanolides
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