The Effects of Acetylstrophanthidin and Ouabain on the Sympathetic Adrenergic Neuroeffector Junction in Canine Vascular Smooth Muscle

ROBERT R. LORENZ, DAVID A. POWIS, PAUL M. VANHOUTTE, AND JOHN T. SHEPHERD

SUMMARY We performed experiments to determine the effects of acetylstrophanthidin (ACS) and ouabain on the adrenergic neuroeffector junction in dog saphenous veins. In quiescent strips incubated with \(^{3}H\)-norepinephrine (\(^{3}H\)-NE), the drugs caused contraction and a progressive increase in overflow of \(^{3}H\)-NE and O-methylated metabolites; 3,4-dihydroxyphenylglycol (DOPEG) decreased. Tissue uptake of \(^{3}H\)-NE was partially inhibited. After surgical sympathectomy, both contraction and \(^{3}H\)-NE overflow were markedly attenuated. Following chemical sympathectomy with 6-hydroxydopamine, ouabain contractions were 11% of control, but the latter was Ca\(^{2+}\) dependent. Inhibition of the neuronal amine carrier by cocaine or desipramine and blockade of the neuronal \(\alpha\)-adrenoceptors with phentolamine or phenoxybenzamine attenuated the release of \(^{3}H\)-NE evoked by ACS and ouabain. During electrical stimulation, ACS augmented the overflow of \(^{3}H\)-NE. This was attenuated by cocaine, desipramine, and \(\alpha\)-adrenolytic drugs. ACS, like pargyline, augmented the overflow of \(^{3}H\)-NE evoked by tyramine and depressed that of DOPEG. These experiments suggest that acetylstrophanthidin and ouabain (1) cause contraction of vascular smooth muscle by displacement of norepinephrine from neuronal stores, (2) reduce neuronal monoamine oxidase activity, (3) facilitate and may trigger Ca\(^{2+}\)-dependent exocytotic release of norepinephrine, (4) partially inhibit the neuronal amine carrier mechanism but do not interfere with extraneuronal disposition of norepinephrine, and, finally (5) may have unexplained interactions with prejunctional \(\alpha\)-adrenoceptors.


EARLIER studies on the dog saphenous vein have shown that cardiac glycosides augment the contractile response to sympathetic nerve stimulation and exogenous vasoconstrictor agents (Brender et al., 1969). Prolonged incubation with the glycosides results in increases in basal tension (Brender et al., 1970). In other isolated blood vessels, the glycosides also cause contraction (e.g., Cow, 1911; Franklin, 1925; Matthews and Sutter, 1967; Hendricks and Casteels, 1974; Mikkelsen et al., 1979). The contraction can be attenuated by \(\alpha\)-adrenolytic drugs; thus it has been suggested that it is due in part to activation of postjunctional \(\alpha\)-adrenoceptors by norepinephrine liberated from sympathetic nerve endings in the blood vessel wall (Tsuru and Shigei, 1976; Karaki and Urakawa, 1977). The present investigation demonstrates that acetylstrophanthidin and ouabain (1) cause contraction of vascular smooth muscle by displacement of norepinephrine from neuronal stores, (2) reduce neuronal monoamine oxidase activity, (3) facilitate and may trigger Ca\(^{2+}\)-dependent exocytotic release of norepinephrine, (4) partially inhibit the neuronal amine carrier mechanism but do not interfere with extraneuronal disposition of norepinephrine, and, finally (5) may have unexplained interactions with prejunctional \(\alpha\)-adrenoceptors.

Methods

Experiments were performed on either helical strips or circular segments of lateral saphenous veins and on longitudinal strips of portal veins taken from dogs (15-30 kg) anesthetized with sodium pentobarbital (30 mg/kg, iv).

Superfusion Experiments

Helical strips were incubated for 2 hours in Krebs-Ringer solution (NaCl, 118.3 mm; KCl, 4.7 mm; CaCl\(_2\), 2.5 mm; KH\(_2\)PO\(_4\), 1.2 mm; MgSO\(_4\), 1.2 mm; NaHCO\(_3\), 25.0 mm; calcium disodium edetate, 0.026 mm; d-glucose, 5.5 mm) maintained at 37°C and aerated with a 95% O\(_2\)-5% CO\(_2\) mixture. The solution contained dl-(7-\(^{2}H\))norepinephrine (1.5 \(\times\) \(10^{-2}\) m; specific activity, 8.7 Ci/mmol, New England Nuclear) and ascorbic acid (0.5 mg/ml). The strips were transferred to a freshly labeled solution for an additional 2 hours, after which they were rinsed in Krebs-Ringer solution and mounted for superfusion (Vanhoutte et al., 1973). The strips were superfused with aerated Krebs-Ringer solution at 37°C by a constant flow roller pump (Holter, model 911) at a
rate of 3 ml/min. In some experiments, calcium-free solutions were used. For electrical stimulation, two platinum wire electrodes (10 cm long, 0.5 mm in diameter) were positioned parallel to and touching the vein strip so that both preparation and electrodes were superfused continuously. Rectangular stimuli of characteristics known to excite selectively the sympathetic nerves (9 V, 2 msec, 1-2 Hz) (Vanhouette et al., 1967) were applied to the electrodes by a DC power supply and switching transistor (RCA 2N 3034) triggered by a Grass stimulator (model SM6). The preparations were connected to a strain gauge for isometric tension recording. The initial tension was set at 3 g. After this initial stretch, the tension decreased and stabilized within 30 minutes. At this time, sampling of the superfusate was started.

The superfusate was collected at 2-minute intervals for direct estimation of total radioactivity. During selected 4-minute intervals, the superfusate was collected for subsequent chromatographic analysis.

**Tissue Uptake of ³H-Norepinephrine**

Strips (2 mm wide, 5 cm long) of saphenous veins were equilibrated for 30 minutes in Krebs-Ringer solution. They then were incubated for 15 minutes in solution containing ³H-norepinephrine (10⁻⁸ or 10⁻⁷ M). After the incubation, the strips were rinsed repeatedly with fresh, cold Krebs-Ringer solution, blotted dry, and weighed. The radioactivity was extracted (Levin, 1973) by placing each strip in 2.5 ml of ice-cooled 1 N acetic acid containing 0.03 mM Na₂EDTA and 5 mM ascorbic acid. After 30 minutes, the preparations were transferred to another set of test tubes containing the same extraction fluid. After another 30 minutes, the strips were removed, and the two 2.5-ml extraction portions were pooled for subsequent chromatographic analysis (Verbeuren et al., 1978).

**Column Chromatography**

To separate ³H-norepinephrine from its major metabolites [3,4-dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylglycol (DOPEG), 3-methoxy-4-hydroxyphenylglycol (MOPEG), 3-methoxy-4-hydroxymandelic acid (VMA), and normetanephrine (NMN)], a method was used that has been described elsewhere (Vanhouette et al., 1973; Verbeuren et al., 1977). Briefly, protective agents [0.2 ml of 3 N HCl and 20 mg of sodium metabisulphite and carriers (norepinephrine, DOMA, DOPEG, MOPEG, NMN, and VMA; 20 μg of each)] were added to the superfusion and extraction samples, which then were stored at -23°C until the column chromatography was performed. Catechol compounds (³H-norepinephrine, DOPEG, and DOMA) first were adsorbed at pH 8.4, on activated alumina; ³H-norepinephrine and DOPEG were eluted with 0.2 N acetic acid and DOMA with 1 N HCl.

³H-Norepinephrine was separated from DOPEG by adsorption on a Dowex 50 resin (200-400 mesh) previously cycled through the hydrogen and sodium forms. ³H-Norepinephrine was eluted from the Dowex 50 with 2 N HCl. In the effluent from the alumina column, NMN was separated from MOPEG and VMA by adsorption on Dowex 50 from which it was eluted with 6 N HCl:ethanol (1:1).

**Radioactivity Measurements**

Samples (1 ml) of superfusate and of the fractions obtained during the chromatographic procedure were added to 10 ml of Insta-Gel (Packard Instrument Corp.) and the radioactivity was measured in a liquid scintillation counter (Packard, model 3330). Corrections for quenching were made with the external standard method. The counting efficiency was about 32%. The samples were counted for 10 minutes.

**Denervation**

In two dogs, bilateral lumbar sympathectomy was performed 3 weeks before the saphenous veins were excised (Donald and Ferguson, 1970). Helical strips were placed for study in the superfusion apparatus. In other experiments, circular segments were placed in a 20-ml organ bath filled with physiological electrolyte solution, gassed with 5% CO₂ in O₂, maintained at 37°C, and allowed to equilibrate for 60 minutes. The segments were attached to a force transducer (Grass FT-03C) and changes in isometric tension recorded. Two rectangular electrodes were placed parallel to the segments for electrical stimulation (12 V, 2 msec). Some of these segments were denervated acutely with 6-hydroxydopamine according to the technique described by Aprigliano and Hermensmeyer (1976).

**Measurement of Norepinephrine Content**

The content of endogenous norepinephrine of the surgically denervated veins was assayed after extraction (Levin, 1973), using high-performance liquid chromatography with electrochemical detection (Muldoon et al., 1979).

**Drugs**

The following pharmacological agents were used: cocaine hydrochloride, desipramine, acetylstrophanthidin, norepinephrine, ouabain, pargyline hydrochloride, phentolamine mesylate, phenoxybenzamine hydrochloride, tetrodotoxin, 6-hydroxydopamine, and tyramine hydrochloride. All doses are expressed as molar concentrations in the incubation solution or in the superfusion fluid.

**Statistical Analysis**

For each group of preparations the number of strips reported is also the number of dogs used. All data are expressed as mean ± se. For statistical analysis of the data, Student's t-test for paired and unpaired observations was used; P values less than 0.05 were considered statistically significant. Unless it is stated otherwise, differences between control
Results

Overflow of \(^3\)H-Norepinephrine and Its Metabolites in Quiescent Saphenous Vein Strips

Six strips were superfused with acetylstrophanthidin (4.4 \(\times 10^{-6}\) M) for 80 minutes. After a latent period, there was a gradual rise in tension and a progressive increase in radioactivity of the superfusate. The overflow of \(^3\)H-norepinephrine and its metabolites increased with the exception of DOPEG, which decreased (Fig. 1).

Five strips were superfused with ouabain (3 \(\times 10^{-6}\) M) for 80 minutes. After a latent period, there was a gradual rise in tension and a progressive increase in radioactivity of the superfusate. The overflow of intact \(^3\)H-norepinephrine increased from a control value of 1,423 ± 212 dpm/min to 3,993 ± 1,039 dpm/min after exposure to ouabain for 34 minutes and to 75,338 ± 9,873 dpm/min after 74 minutes. The overflow of metabolites also increased with the exception of DOPEG which decreased.

Bilateral lumbar surgical sympathectomy was performed in two dogs 3 weeks before saphenous veins were taken for study. The content of endogenous norepinephrine in the denervated veins from the two dogs was 7.4 ng/g and 11.2 ng/g, respectively; innervated dogs’ saphenous veins contain more than 3,000 ng/g norepinephrine (Muldoon et al., 1979). In the denervated vein, with lowest norepinephrine content, acetylstrophanthidin applied for 74 minutes increased the overflow of \(^3\)H-norepinephrine from 206 to 249 dpm/min; tension increased from 2.2 to 3.6 g. In the other, acetylstrophanthidin increased the overflow from 207 to 1,157 dpm/min; tension increased from 1.9 to 5.5 g.

In five circular segments pretreated with 6-hydroxydopamine for 20 minutes, the response to electrical stimulation was abolished while that to exogenous norepinephrine was potentiated. The addition of ouabain caused only minimal increases in tension compared to the large contractions seen in control segments from the same vein. Subsequent addition of exogenous norepinephrine to the pretreated segments caused a marked increase in tension (Fig. 2).

Cocaine and Desipramine

In six strips, acetylstrophanthidin in the presence of cocaine caused increases in tension and overflow of tritiated compounds which were less than those induced in control solution. Analysis of the superfusate at 34 and 74 minutes showed that the overflow of \(^3\)H-norepinephrine was less than that seen in the absence of cocaine. Qualitatively similar results were obtained in five strips treated with desipramine (Fig. 3).

Ca\(^{2+}\)-Free Solution

In five strips superfused with Krebs-Ringer solution containing no Ca\(^{2+}\), acetylstrophanthidin caused only a slight increase in tension. However, initially, the cardenolide augmented the overflow of radioactive compounds to a greater degree than that seen in Ca\(^{2+}\)-containing solutions (Fig. 4). In the absence of Ca\(^{2+}\), the acetylstrophanthidin-in-
duced overflow of $^3$H-norepinephrine reached a maximum at 34 minutes and at this time was no different from that obtained in the presence of Ca$^{2+}$. The overflow was maintained at this level for the remainder of the period of superfusion, whereas, in the presence of Ca$^{2+}$, it continued to increase. Addition of Ca$^{2+}$ (2.5 mM) to the Ca$^{2+}$-free superfusing fluid after the strips had been exposed to acetylstrophanthidin for 74 minutes caused a sudden and marked increase in tension to values that would have been anticipated had Ca$^{2+}$ been present throughout; the overflow of $^3$H-norepinephrine far exceeded control values (Fig. 4).

**Uptake of $^3$H-Norepinephrine**

Paired strips were incubated with $^3$H-norepinephrine. One served as a control; the other was exposed to acetylstrophanthidin or ouabain from 15 minutes before, to the end of the incubation period. The content of $^3$H-norepinephrine and its metabolites was determined both in the strips and the incubation fluid. With $2.2 \times 10^{-6}$ and $4.4 \times 10^{-6}$ M acetylstrophanthidin, the tissues contained less $^3$H-norepinephrine. Similar findings were obtained with $3 \times 10^{-6}$ M ouabain (Table 1). In six strips, preincubation for 15 minutes with $2.7 \times 10^{-6}$ M phentolamine had no significant effect on the uptake of $^3$H-norepinephrine. Incubation with $3 \times 10^{-5}$ M cocaine or $10^{-6}$ M desipramine reduced the tissue uptake of $^3$H-norepinephrine to $5.9 \pm 2.1$ and $4.3 \pm 0.4$% of control, respectively.

**Tetrodotoxin**

In five strips, ouabain in the presence of tetrodotoxin ($5 \times 10^{-6}$ M, a concentration sufficient to depress the response to 2 Hz electrical stimulation by $90 \pm 5$%), caused an increase in tension that was...
Table 1  Effect of Acetylstrophanthidin and Ouabain on \textsuperscript{3}H-Norepinephrine Uptake in Saphenous Vein Strips

<table>
<thead>
<tr>
<th>Condition</th>
<th>\textsuperscript{3}H-Norepinephrine uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dp m (\times 10^7) mg wet wt % of control</td>
</tr>
<tr>
<td>Control</td>
<td>10.63 ± 0.86</td>
</tr>
<tr>
<td>Acetylstrophanthidin (2.2 (\times 10^{-6}) M)</td>
<td>9.64 ± 0.96</td>
</tr>
<tr>
<td>Ouabain (3 (\times 10^{-6}) M)</td>
<td>12.87 ± 1.21</td>
</tr>
<tr>
<td>Ouabain (4.4 (\times 10^{-6}) M)</td>
<td>8.37 ± 0.91</td>
</tr>
<tr>
<td>Ouabain (3 (\times 10^{-6}) M)</td>
<td>12.41 ± 1.17</td>
</tr>
<tr>
<td>Control + Ouabain</td>
<td>10.08 ± 1.08</td>
</tr>
</tbody>
</table>

Experiments performed on paired strips of the same veins. Data shown as mean ± SE, \(n = 6\). Determined by column chromatography (see Methods).

greater than in its absence. After 34 minutes superfusion with ouabain, the overflow of \textsuperscript{3}H-norepinephrine was similar to that obtained in strips not pre-treated with tetrodotoxin, but at 74 minutes this overflow was depressed (Fig. 5).

In five strips, tetrodotoxin was added after superfusion for 74 minutes with ouabain (3 \(\times 10^{-6}\) M). The tension decreased from 5.3 ± 0.6 to 4.8 ± 0.6 g; the overflow of tritiated compounds decreased from 46,849 ± 5,141 to 29,594 ± 2,676 dp m/ml collected, and the output of \textsuperscript{3}H-norepinephrine decreased from 75,339 ± 9,873 to 39,975 ± 3,881 dp m/min. Qualitatively similar effects of tetrodotoxin were seen in six strips superfused with 4.4 \(\times 10^{-6}\) M acetylstrophanthidin (mean decrease in efflux of tritiated compounds: from 6,251 ± 797 to 4,082 ± 472 dp m/ml collected).

Tyramine

Five strips were superfused with tyramine hydrochloride in control solution, in the presence of acetylstrophanthidin (2.2 \(\times 10^{-6}\) M, a concentration that does not alter basal \textsuperscript{3}H-norepinephrine overflow over the same time period), and again in control solution. Tyramine caused a sustained increase in tension and an increase in the overflow of \textsuperscript{3}H-norepinephrine and its metabolites with the exception of OMDA. In the presence of acetylstrophanthidin, the contractile response to tyramine was enhanced as was the overflow of \textsuperscript{3}H-norepinephrine and normetanephrine. The overflow of DOPEG decreased (Fig. 6, Table 2).

Adrenergic Blockade

In five strips, acetylstrophanthidin in the presence of phentolamine caused an increase in tension greater than that achieved by tyramine in the presence of acetylstrophanthidin in control solution (Table 2).

Figure 5  Effect of tetrodotoxin on changes in tension and efflux of \textsuperscript{3}H-norepinephrine and metabolites caused by ouabain. Data shown as mean ± SE (\(n = 5\)) and expressed as percent changes from basal values.

Figure 6  Effect of acetylstrophanthidin on the response to tyramine (Ty). Upper = tension; middle = total \textsuperscript{3}H-efflux (dp m/ml collected); lower = overflow of \textsuperscript{3}H-norepinephrine and its metabolites (dp m/min of collection). Data shown as mean ± SE (\(n = 5\)).
which was smaller than that induced in the absence of
the latter. The increase in radioactivity of the
superfusate normally evoked by acetylstrophan-
thidin was attenuated. Whereas the overflow of
3H-norepinephrine increased above control levels, the
increase was less than that obtained in tissues not
pretreated with phentolamine (Fig. 1). Qualitatively
similar results were obtained with ouabain.

Three strips were treated with phenoxybenzo-
amine (3.3 x 10^-6 M) before superfusion. This drug
also attenuated the increase in output of radioactive
compounds evoked by acetylstrophanthidin. After
superfusion for 74 minutes with acetylstrophan-
thidin (4.4 x 10^-6 M), the tension had increased from
0.7 ± 0.1 to 2.8 ± 1.1 g and the 3H-norepinephrine
overflow from 1,174 ± 364 dpm/min to only 2,544
± 688 dpm/min.

Electrical Stimulation

In seven strips, electrical stimulation at 2 Hz
caused a sustained increase in tension together with
an increased overflow of 3H-norepinephrine and its
metabolites. A second period of stimulation was
performed in the presence of acetylstrophanthidin.
The contractile response was enhanced and the
evoked overflow of 3H-norepinephrine and its me-
tabolites was augmented with the exception of DO-
PEG. In seven other unstimulated strips, the same
concentration of acetylstrophanthidin caused a mi-
nor increase in basal tension but did not alter the
overflow of radioactive compounds (Fig. 7).

In five strips electrical stimulation at 2 Hz caused
a sustained increase in tension and overflow of 3H
norepinephrine. The strips were subsequently su-
perfused with cocaine (3 x 10^-6 M) for 20 minutes.
A second period of stimulation was performed in
the presence of acetylstrophanthidin and cocaine.
The contractile response was slightly augmented;
however, the overflow of total radioactivity and 3H
norepinephrine did not increase (Fig. 8). Qualita-
tively similar results were obtained in five strips
treated with desipramine.

In the presence of phenolamine, electrical stimu-
lation of six strips at 2 Hz resulted in small in-
creases in tension. The overflow of 3H-norepineph-
rine from these strips was three times greater than
in the absence of phenolamine; subsequent addi-
tion of acetylstrophanthidin did not enhance fur-
ther the overflow of 3H-norepinephrine but reduced
that of DOPEG (Table 3).

Experiments were done in four other strips using
a stimulation frequency of 1 Hz. In the presence of
phenolamine, the output of 3H-norepinephrine was
about half that observed at 2 Hz. Acetylstrophan-
thidin did not cause a significant increase in the
overflow of 3H-norepinephrine above that measured
during the first period of stimulation (Table 3).

Prejunctional α-Adrenoceptors

Electrical stimulation (1 Hz for 4 minutes) of four
portaI vein strips was performed in control solution,
in the presence of noradrenergine (1.2 x 10^-6 M),
Acetylstrophanthidin, 2.2 \times 10^{-6} M

**Figure 8** Effect of acetylstrophanthidin on response to electrical stimulation (ES) in presence of cocaine, 3 \times 10^{-5} M. Upper = tension; middle = total \(^{3}\)H-efflux (dpm/ml collected); lower = efflux of \(^{3}\)H-norepinephrine and its metabolites (dpm/min of collection). Data shown as mean ± SE (n = 5).

and again in control solution. This sequence was repeated in the presence of acetylstrophanthidin (2.2 \times 10^{-6} M); the frequency of electrical stimulation was reduced to 0.5 Hz to avoid excessive outpouring of tritiated compounds. Norepinephrine reduced the overflow of tritiated compounds evoked by electrical stimulation both in the presence and in the absence of acetylstrophanthidin (Table 4).

**Discussion**

**Effect of Acetylstrophanthidin and Ouabain on Tension and Release of Norepinephrine**

The present study extends earlier observations that, in canine saphenous veins, cardiac glycosides cause an increase in tension and an immediate potentiation of the response to sympathetic nerve stimulation (Brender et al., 1969, 1970). The experiments demonstrate that acetylstrophanthidin and ouabain markedly increase the overflow of labeled transmitter from the vein strips. This originates from the adrenergic terminals in the vein wall since the increase in \(^{3}\)H-norepinephrine overflow was virtually abolished after surgical sympathectomy. The contraction of the smooth muscle was due mainly to the release of norepinephrine. This was demonstrated by the fact that the contractile response to acetylstrophanthidin and ouabain was greatly attenuated by \(\alpha\)-adrenolytic drugs as is the case in other isolated blood vessels (Tsura and Shigei, 1976; Karaki and Urakawa, 1977) and that after pharmacological denervation the contractile response was only 11.4% of control.

The initial increases in \(^{3}\)H-norepinephrine overflow seen with acetylstrophanthidin and ouabain are unlikely to be due to exocytosis initiated by the generation or augmentation of spontaneous discharges of the neuronal membrane. This conclusion is based on the following observations: (1) tetrodotoxin, which prevents neuronal Na\(^{+}\) influx via fast Na\(^{+}\) channels, did not depress the initial pattern of release caused by ouabain; (2) the exocytotic release of norepinephrine is highly Ca\(^{2+}\)-dependent (see Vanhoutte, 1978). Ca\(^{2+}\)-free solution did not depress the initial increase in overflow evoked by acetylstrophanthidin; on the contrary, the total overflow of radioactive compounds was augmented. Omission of Ca\(^{2+}\) does not depress the release of acetylcholine evoked by ouabain from cholinergic nerve endings (Vizi, 1972).

It has been suggested that cardiac glycosides inhibit the neuronal amine carrier (Sharma and Banerjee, 1977). The present study demonstrates that in the dog saphenous vein, acetylstrophanthidin and ouabain partially inhibit the tissue uptake of tritiated norepinephrine. However, it is unlikely that this inhibitory effect of the cardenolides on the neuronal amine carrier mechanism could explain the increased overflow of \(^{3}\)H-norepinephrine the drugs cause in quiescent veins. In the same preparation, concentrations of cocaine and desipramine which completely block the amine carrier do not augment the basal overflow of tritiated norepinephrine and its metabolites (see Verbeuren et al., 1978; Dalemnas et al., 1978; Lorenz et al., 1979). The observation that, unlike cocaine (Clement et al., 1969, Verbeuren et al., 1978), acetylstrophanthidin augmented rather than reduced the response to the indirect sympathomimetic amine tyramine further indicates that the inhibitory effect of the cardenolide on neuronal uptake is functionally of little consequence (see Vanhoutte, 1978). An inhibitory effect of acetylstrophanthidin and ouabain on extraneuronal uptake and subsequent O-methylation is ruled out by the observations that the increases they cause in \(^{3}\)H-norepinephrine overflow are accompanied by increases in the overflow of O-methylated metabolites, in particular normetanephrine. An additional finding in this study is that acetylstrophanthidin caused changes in the overflow of \(^{3}\)H-norepinephrine and DOPEG similar to those seen with pargyline. Acetylstrophanthidin and ouabain affect the response to tyramine in a manner similar to monoamine oxidase inhibitors (Vanhoutte et al., 1977). These observations indicate that in the presence of acetylstrophanthidin the neuronal activity of monoamine oxidase is reduced. However, a decrease in monoamine oxidase activity is not sufficient to explain the total increase in \(^{3}\)H-norepinephrine overflow caused by acetylstrophanthidin, since in the presence of the drug the amount
of intact \(^3\text{H}\)-norepinephrine exceeds the total amount of \(^3\text{H}\)-norepinephrine and DOPEG present in the superfusate in basal conditions. In addition, besides \(^3\text{H}\)-norepinephrine, the total metabolite fraction also is augmented. When the action of monoamine oxidase was inhibited by pargyline, acetylstrophanthidin further augmented the release of \(^3\text{H}\)-norepinephrine caused by tyramine. Thus, it can be concluded that both depressed activity of monoamine oxidase and displacement of transmitter from neuronal stores combine to augment the overflow of \(^3\text{H}\)-norepinephrine.

**Cellular Mechanisms**

It seems logical to assume that the release of norepinephrine by acetylstrophanthidin and ouabain reported in the present experiments is initiated by binding of the drugs to the neuronal membrane, inhibition of neuronal Na\(^+\),K\(^+\)-ATPase, and consequent changes in the ionic composition of the neuromuscular junction. In particular, an increase in intracellular Na\(^+\) and Ca\(^2+\) (Bohr et al., 1969; Schwartz, 1977). In the dog saphenous vein, reduction of extracellular Na\(^+\) augments and incubation in \(K^+\)-free solution depresses the formation of deaminated metabolites (Vanhoutte et al., 1975; Beaty et al., 1978). Taken in conjunction with these findings, the present study suggests that either an increase in neuroplasmic Na\(^+\) or Ca\(^2+\) or a decrease in neuroplasmic K\(^+\) has an inhibitory effect on monoamine oxidase. Likewise, the possible trigger-
ing of the exocytotic process seen with prolonged exposure to acetylstrophanthidin and ouabain can be explained by Na\(^+\) loading of the adrenergic nerve endings permitting a high density Ca\(^{2+}\) influx into the adrenergic varicosities.

Cocaine, in a concentration that inhibits neuronal uptake of norepinephrine and the response to tyramine (Clement et al., 1969; Verbeuren et al., 1978), greatly attenuated the increase in \(^3\)H-norepinephrine overflow seen with acetylstrophanthidin. Similar results were obtained with desipramine in concentrations that inhibit the tissue uptake of \(^3\)H-norepinephrine, demonstrating that the inhibitory effect of cocaine cannot be attributed to the local anesthetic properties of the compound. From these experiments and the finding that acetylstrophanthidin augments rather than depresses the release of \(^3\)H-norepinephrine by tyramine (whose action depends on a functional neuronal carrier), it can be concluded that a functional neuronal carrier for norepinephrine is necessary for the effect of acetylstrophanthidin and ouabain on the adrenergic nerve endings. A similar conclusion has been reached in rabbit atria (Paton, 1973). An increase in the neuronal concentration of Na\(^+\) or Ca\(^{2+}\) could cause reversal of the neuronal amine carrier or increase the affinity of its internal aspect for norepinephrine; this would explain the attenuation of \(^3\)H-norepinephrine efflux evoked by acetylstrophanthidin and ouabain by drugs that block this mechanism. Such an action, in particular if partial, would also explain why short exposure to acetylstrophanthidin and ouabain moderately depresses the neuronal uptake of \(^3\)H-norepinephrine, but does not prevent the indirect sympathomimetic effect of tyramine which has a greater affinity for the neuronal carrier. An alternative explanation is that the neuronal amine carrier is linked to the Na\(^+,K^+\)-ATPase enzyme (Berti and Shore, 1967), so that inhibition of the carrier renders the enzyme non-functioning and thus acetylstrophanthidin and ouabain ineffective.

An unexpected finding in the present study was that the overflow of \(^3\)H-norepinephrine evoked by acetylstrophanthidin and ouabain was greatly attenuated by \(\alpha\)-adrenoceptor antagonists. This occurred both in quiescent strips and in those stimulated electrically. In the latter situation prior to administration of acetylstrophanthidin, the overflow of norepinephrine already was enhanced due to removal of the inhibition of the prejunctional \(\alpha\)-adrenoceptors by phentolamine (Lorenz et al., 1979; Starke, 1977). This raised the possibility that no further increase in norepinephrine release might be possible with acetylstrophanthidin. However, when the overflow of norepinephrine was reduced in the presence of phentolamine by lowering the frequency of stimulation from 2 Hz to 1 Hz, acetylstrophanthidin still did not cause any major increase in overflow. Evidence that acetylstrophanthidin was not acting as an \(\alpha\)-adrenoceptor antagonist and thereby causing increased \(^3\)H-norepinephrine release by removal of \(\alpha\)-adrenoceptor-mediated inhibition comes from the observation that exogenous norepinephrine, in the presence of acetylstrophanthidin, was able to inhibit the overflow of \(^3\)H-norepinephrine evoked by electrical stimulation. The portal vein was used because, in this tissue, prejunctional \(\alpha\)-adrenoceptors can be demonstrated without blockade of the neuronal uptake mechanism (Lorenz et al., 1979). Since \(\alpha\)-adrenoceptor blocking agents attenuated the release of norepinephrine evoked by acetylstrophanthidin and ouabain, it is conceivable that neuronal \(\alpha\)-adrenoceptors are situated close to or at the sites where acetylstrophanthidin and ouabain bind to the membrane bound Na\(^+,K^+\)-ATPase. If this is so, then phentolamine or phenoxybenzamine could act by: (1) binding to the ATPase in such a way as to cause an allosteric change in the enzyme molecule which precludes acetylstrophanthidin or ouabain binding; (2) physically hindering the action of acetylstrophanthidin or ouabain by steric interference with the binding sites; or (3) altering a surface charge mechanism which is also necessary for the effect of acetylstrophanthidin and ouabain. Support for the suggestion of an interplay between \(\alpha\)-adrenoceptors and neural Na\(^+,K^+\)-ATPase comes from experiments that have shown that norepinephrine stimulates this enzyme prepared from rat brain; the stimulation is prevented by phentolamine (Gilbert et al., 1975). Furthermore, ouabain and norepinephrine exert opposing actions on rat brain homogenate Na\(^+,K^+\)-ATPase activity (Yoshimura, 1973). The postulated association between \(\alpha\)-adrenoceptors and the Na\(^+,K^+\)-ATPase may highlight a possible first step in the chain of events in which prejunctional \(\alpha\)-adrenoceptors influence neurotransmitter release caused by the nerve impulse.

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