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α_2-Adrenoceptor blockade prevents cardiac glycoside-evoked neurotransmitter release from sympathetic nerves in dog saphenous vein

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1 The effect of α-adrenoceptor antagonists upon neurotransmitter release evoked by cardiac glycosides from sympathetic nerve terminals has been investigated in dog saphenous vein.

2 In rings of saphenous vein preloaded with [^{3}H]-noradrenaline, acetylstrophanthidin (ACS) caused a concentration-dependent efflux of ^{3}H (EC_{50} ca. 4.4 μM) that was attenuated by phentolamine and yohimbine but not by prazosin.

3 In helical strips of saphenous vein superfused with ACS at EC_{50} the efflux of ^{3}H-compounds in general, and of [^{3}H]-noradrenaline in particular, occurred after a short delay and increased with time to a maximum reached at 75 min. Phentolamine and phenoxybenzamine, but not prazosin reduced the efflux of [^{3}H]-noradrenaline and of total ^{3}H-compounds throughout the time-course of the ACS-evoked effect.

4 In helical strips of saphenous vein the glycoside ouabain also caused an increase in [^{3}H]-noradrenaline and in total ^{3}H-efflux that was attenuated by phentolamine.

5 By contrast with the above, in bovine adrenal medullary chromaffin cells, which appear to have no functional α-adrenoceptors, ACS caused a small, but significant increase in ^{3}H-efflux which was not prevented by phentolamine.

6 Phentolamine, at concentrations that attenuate markedly the ouabain- or ACS-evoked increase in ^{3}H-efflux from dog saphenous vein, did not cause significant inhibition of cocaine-sensitive [^{3}H]-noradrenaline uptake nor did it reduce the extent of the ^{3}H-efflux evoked either by tyramine or by reduced extracellular Na^+. These findings imply that phentolamine does not affect ACS-evoked neurotransmitter release by an action on the catecholamine uptake mechanism.

7 It is concluded that the cardiac glycoside-evoked increase in neurotransmitter release from noradrenergic nerve terminals of dog saphenous vein is modulated by a mechanism that involves an α_2-adrenoceptor.

Introduction

It is established that ouabain and other cardioactive steroids are able to evoke transmitter release from nerve terminals in a variety of tissues (see Powis, 1983). The mechanism, or mechanisms, by which this release occurs is still unresolved but it is probable that all pathways stem from the Na,K-ATPase inhibition that results from exposure to cardiac glycosides. The phenomenon of attenuation of glycoside-evoked release by α-adrenoceptor antagonists has been described briefly (Powis *et al.*, 1979; Lorenz *et al.*, 1980); the present paper describes a more detailed investigation and indicates that it is probable that the antagonists work specifically via α_2-adrenoceptors which, presumably, impinge at some point on the release pathway initiated by Na,K-ATPase inhibition. This paper does not dispute the possibility (see Powis, 1981) that Na,K-ATPase forms part of the pathway by which prejunctional α_2-adrenoceptors regulate neurotransmitter release from sympathetic nerve endings.

Methods

Rings (10–20 mg) or helical strips (ca. 150 mg) were cut from the lateral saphenous vein of dogs anaesthetized (30 mg kg^{-1}, i.v.) or killed (150 mg kg^{-1}, i.v.) with sodium pentobarbitone.

^{3}H-efflux experiments

In order to load the sympathetic nerve terminals with labelled neurotransmitter, the vein rings or strips were incubated for two 90 min periods at 37°C, each in a gassed (95% O_2:5% CO_2), modified Krebs solution (10 ml) containing (in mmol l^{-1}): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, (+)-glucose 11.1. This solution contained also (±)-[7-^{3}H]-noradrenaline (0.15 μM; specific activity 5–15 Ci $mmol^{-1}$, New England Nuclear or Amersham International) and ascorbic acid (0.1 μM). After loading with [^{3}H]-noradrenaline the preparations were rinsed three times with Krebs solution to remove extracellular radioactivity. Thereafter efflux of ^{3}H-compounds from rings or from strips in response to a particular experimental manoeuvre was measured as described by Powis & Madsen (1986a) and by Tan & Powis (1985). A chromatographic method based on adsorption and elution from alumina and Dowex-50 resin columns was used to separate [^{3}H]-noradrenaline from its major metabolites. This has been described elsewhere (Lorenz *et al.*, 1980). It may be noted here that under the conditions of these experiments, ^{3}H-efflux is a reasonable qualitative and quantitative index of [^{3}H]-noradrenaline efflux from dog saphenous vein (see Figures 4, 5 and 6, also Lorenz *et al.*, 1980; Tan & Powis, 1985). Accordingly it was considered unnecessary to separate total ^{3}H-efflux into its components in all experiments: neurotransmitter efflux in these cases was inferred from ^{3}H-efflux determinations (see Figures 1, 2 and 3).

^{3}H-uptake experiments

In some experiments uptake of [^{3}H]-noradrenaline into the nerve terminals was measured. Pairs of rings were equilibrated for a single 30 min period at 37°C in gassed, modified Krebs solution (see above) after which they were preincubated for 30 min with pargyline (30 μM; to prevent enzymic degradation of noradrenaline by monoamine oxidase) and corticosterone (30 μM; to inhibit extraneuronal accumulation of noradrenaline) plus phentolamine and/or acetylstrophanthidin (ACS) as required. In addition one ring of each pair was exposed to cocaine (30 μM) to block neuronal monoamine uptake. Subsequently the rings were incubated for 30 min with the same solutions which now also contained [^{3}H]-noradrenaline (0.15 μM).

Neuronal uptake of [^{3}H]-noradrenaline was calculated as the difference in tissue content of ^{3}H at the end of the incubation period measured in the presence and absence of cocaine. The vein rings were rinsed three times, each in 10 ml cold, modified Krebs solution, blotted dry and weighed. The rings were then solubilized (NCS, Amersham) at 50°C overnight, cooled, acidified with 0.1 ml glacial acetic acid and 10 ml scintillant was added. ^{3}H-content was measured by β-scintillation spectrometry.

This protocol to measure neuronal monoamine uptake has been validated for dog saphenous vein. In vein rings denervated acutely with 6-hydroxydopamine, the cocaine-sensitive component of [^{3}H]-noradrenaline or of [^{14}C]-tyramine uptake was abolished (D.A. Powis & G.M. Madsen, unpublished observations) indicating that this component indeed reflects monoamine uptake into the sympathetic nerves.

Bovine chromaffin cells

Chromaffin cells were prepared from bovine adrenal glands and maintained in primary tissue culture by the methods described by Powis & Baker (1986). The cells were dissociated by collagenase digestion of adrenal medullae and were plated at a density of 3–600,000 per well in 24-well cluster dishes and maintained at 37°C in an atmosphere containing 5% CO_2. They were used for experiments between days 3 and 21. In each experiment the effect of ACS upon catecholamine release was determined in the absence or presence of phentolamine using a single 24-well plate: 6 wells were used for control determinations and 6 wells each for the effects of phentolamine alone, for ACS alone and for ACS plus phentolamine. Phentolamine was added 15 min before ACS. Catecholamine secretion was measured at room temperature over a 45 min period. Cathecholamine secretion was estimated by measurement of ^{3}H-compounds released into the experimental medium after preloading the cells with [^{3}H]-noradrenaline (Powis & Baker, 1986). Some experiments were performed to confirm that ^{3}H-efflux under these conditions adequately mirrors the release of endogenous catecholamines from bovine adrenal medullary chromaffin cells maintained in tissue culture. It was shown within a single population of chromaffin cells that basal release of ^{3}H from cells preloaded with ^{3}H-noradrenaline was quantitatively similar to basal release of endogenous catecholamine (6.2 ± 0.37% of tissue content of ^{3}H released in 15 min compared with 5.2 ± 0.31% endogenous catecholamine over the same period; mean ± s.e., $n = 8$ comparisons). Furthermore 60 μM carbachol evoked the release of 25.3 ± 1.12% tissue ^{3}H content in 15 min compared with 21.0 ± 0.40% endogenous catecholamine ($n = 8$ paired determinations).

Analysis of data

All data are expressed as mean ± s.e. and the number of samples in a group is the number of experiments performed, each with tissue from a different dog. For each experiment with helical strips of saphenous vein,

two strips from the same dog were used, one exposed to the adrenoceptor antagonist, the other acting as its control. In experiments with rings, two or three rings from the same vein were exposed to the adrenoceptor antagonist, the same number acted as controls. The individual data obtained were combined to give a single value for inclusion in the group means. With adrenal chromaffin cells, each value contributing to the mean value shown in the results section was derived from a different preparation of cells. Statistical analysis was performed with Student's t test; P values less than 0.05 were considered to be significant.

Drugs

Acetylstrophanthidin was synthesized from strophanthidin (Sigma S6626) by the method of Koechlin & Reichstein (1947); composition and purity of the preparation was confirmed by the Australian Microanalytical Service (Amdel, Port Melbourne, Victoria, Australia). Phentolamine mesylate was obtained from Ciba-Geigy, phenoxybenzamine hydrochloride injection from Smith, Kline & French, yohimbine hydrochloride from Sigma, prazosin hydrochloride from Pfizer, pargyline hydrochloride from Abbott Laboratories and cocaine hydrochloride (Merck) from the pharmacy of the Royal Newcastle Hospital (N.S.W., Australia). All of these drugs were dissolved in water and diluted with Krebs solution as required. Corticosterone (Sigma) was dissolved first in ethanol and diluted as required with Krebs solution. The proportion of ethanol in the final solution was never greater than 0.33% and was shown to be without effect upon ^{3}H-efflux.

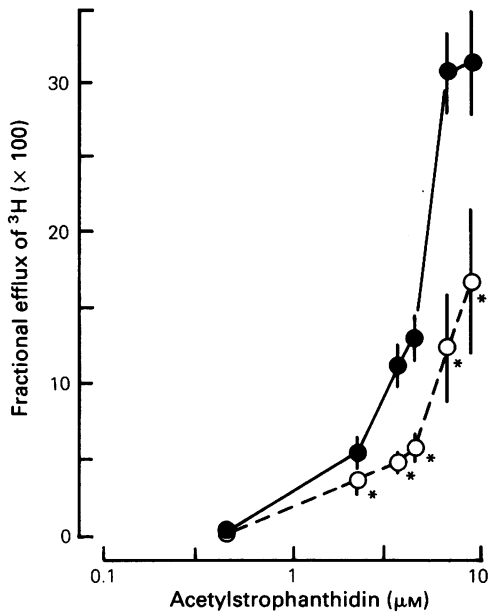

Figure 1 Effect of acetylstrophanthidin (ACS) upon ^{3}H-efflux from saphenous vein rings in the absence (●) and in the presence of phentolamine (○, 8.8 μM). In each experiment pairs of saphenous vein rings were used, one exposed to ACS alone, the other to ACS + phentolamine. Each point shown reflects the mean increase in fractional ^{3}H-efflux from basal over a period of 75 min. Each point shown is the mean of between 6–19 determinations; vertical lines show s.e.mean. * indicates a statistically significant difference between values measured in the presence and absence of phentolamine.

Results

Effects of acetylstrophanthidin upon neurotransmitter release from saphenous vein

Vein rings Spontaneous efflux of ^{3}H-compounds was measured over various time intervals to provide an estimate of the basal release of neurotransmitter from the nerve terminals in dog saphenous vein. Basal efflux was linear with time for at least 75 min ($r^2 = 0.93$, $n = 45$ determinations made at various times between 15–75 min); fractional efflux of ^{3}H-compounds was 0.125% of total tissue ^{3}H-content per minute. In 20 rings, fractional ^{3}H efflux over 75 min was 9.52 ± 0.43%.

ACS, in micromolar concentrations, caused an increase in efflux of ^{3}H-compounds from saphenous vein rings. Figure 1 shows the increase in fractional efflux from basal levels after 75 min exposure to the glycoside. Maximum efflux was evoked by ACS 9 μM at which concentration fractional ^{3}H-efflux was 41.1 ± 3.28% ($n = 6$) of total tissue content after 75 min. The EC_{50} for ACS was approximately 4.4 μM. It has been shown previously that the ^{3}H-compounds released by ACS come entirely from the sympathetic nerve terminals (6-hydroxydopamine experiments: Tan & Powis, 1985).

Effects of phentolamine The α-adrenoceptor antagonist phentolamine (< 8.8 μM) had no effect on the spontaneous efflux of ^{3}H-compounds from saphenous vein rings. In 20 rings spontaneous fractional efflux over 75 min in the presence of phentolamine (8.8 μM) was 9.05 ± 0.42% (cf. 9.52 ± 0.43% in untreated rings). Phentolamine (8.8 μM), however, did reduce the ^{3}H-efflux evoked by ACS: the ACS concentration vs. ^{3}H-efflux relationship was shifted to the right (Figure 1). In another series of experiments, a lower concentration (0.88 μM) of phentolamine was used. This attenuated ^{3}H-efflux evoked by ACS (< EC_{50}) but had little

effect upon that evoked by higher concentrations of ACS (Figure 2).

Effects of other α-adrenoceptor antagonists The effects of yohimbine and of prazosin upon ^{3}H-efflux evoked by ACS were measured. The relatively selective α_2-adrenoceptor antagonist yohimbine over the same concentration-range as phentolamine attenuated ACS-evoked ^{3}H-efflux (Figure 3). Prazosin, at concentrations which in dog saphenous vein are considered to block predominantly α_1-adrenoceptors (Sullivan & Drew, 1980; see also Guimaraes & Paiva, 1981) did not attenuate the ACS-evoked ^{3}H-efflux (Figure 3).

Effects of phentolamine upon the catecholamine uptake mechanism It has been suggested that cardiac glycosides might cause noradrenaline efflux from sympathetic nerve terminals by reversing the catecholamine uptake mechanism as a consequence of elevated Na_i caused by Na,K-ATPase inhibition (Powis, 1983; Tan & Powis, 1985). If this is so then it is possible that phentolamine prevents the noradrenaline releasing action of ACS by interfering with this mechanism. The following experiments were performed to evaluate this possibility:

(1) The effect of phentolamine upon cocaine-sensitive [^{3}H]-noradrenaline uptake was determined. In rings of dog saphenous vein, in the absence of phentolamine, the cocaine-sensitive uptake of [^{3}H]-noradrenaline was found to be linear at least to 75 min. In six determinations with pairs of rings (one of each pair pre-incubated with cocaine to inhibit neuronal catecholamine uptake) incubated in [^{3}H]-noradrenaline, cocaine-sensitive noradrenaline uptake was 11.1 ± 3.7 fmol mg^{-1} min^{-1}. Phentolamine (8 μM) had no significant effect upon [^{3}H]-noradrenaline uptake: in the presence of added phentolamine cocaine-sensitive noradrenaline uptake estimated from six other pairs of rings taken from the same veins was 9.5 ± 4.2 fmol mg^{-1} min^{-1}.

(2) Tyramine causes noradrenaline release from sympathetic nerve terminals as a consequence of its own entry into the nerve via the catecholamine uptake mechanism. The tyramine displaces stored transmitter which then may leave the cell on the reverse cycle of the pump (Bonisch, 1986). The effects of phentolamine on tyramine-evoked noradrenaline efflux were measured. In 6 pairs of rings of dog saphenous vein, in two experiments, tyramine at EC_{50} (30 μM, Powis & Madsen, 1986b) caused an increase in fractional ^{3}H-efflux from $2.87 \pm 0.33\%$ to $7.15 \pm 0.39\%$ total tissue content over a 15 min period (+ 149%). Phentolamine (8 μM) did not alter basal ^{3}H-efflux, but increased (rather than decreased) ^{3}H-efflux evoked by tyramine. In the presence of phentolamine, tyramine now caused

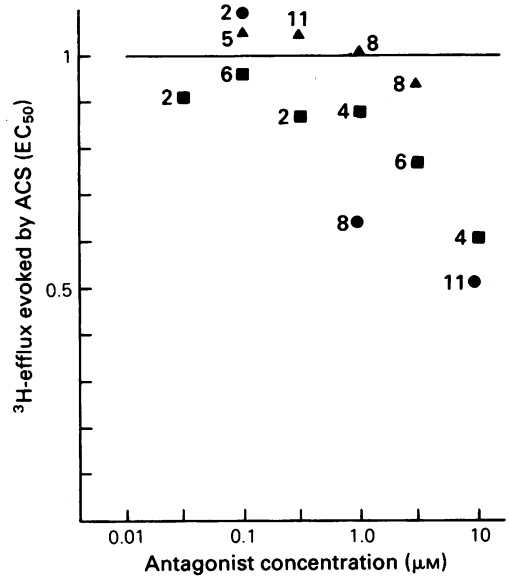

Figure 3 Effect of phentolamine (●), yohimbine (■) and prazosin (▲) upon ^{3}H-efflux evoked by acetylstrophanthidin (ACS; 4.4 μM) from rings of saphenous vein. The relative efflux evoked in a paired ring in the absence of antagonist was assigned a value of 1. The number of observations (i.e. sets of data from paired rings) is shown by each point.

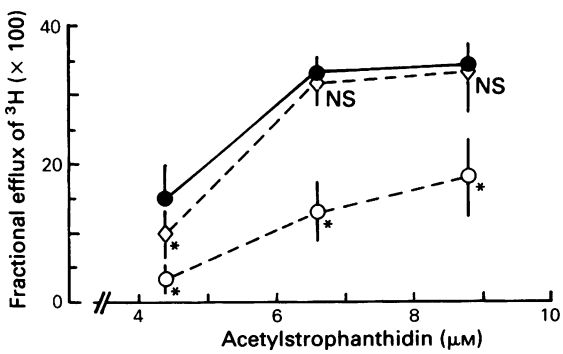

Figure 2 Effect of acetylstrophanthidin (ACS) upon ^{3}H-efflux from saphenous vein rings in the absence (●) and in the presence of phentolamine (0.88 μM, ◇; 8.8 μM ○). In each experiment three saphenous vein rings were used, one exposed to ACS alone, the others to ACS + phentolamine at each concentration. Each point shown reflects the mean increase in fractional ^{3}H-efflux from basal over a period of 75 min. Each point shown is the mean of 6 determinations; vertical lines show s.e.mean. * indicates a statistically significant difference between values measured in the presence and absence of phentolamine. NS, not significant.

an increase in fractional ^{3}H-efflux from 3.10 ± 0.24% to 9.88 ± 0.28% total tissue content in 15 min (+ 219%; $n = 6$ pairs of rings in two separate experiments).

(3) It is probable that low extracellular Na^+ causes increased noradrenaline efflux from sympathetic nerve terminals by reversal of the catecholamine uptake pump since inhibitors of this pump prevent the increase in efflux (Blaszkowksi & Bogdanski, 1972; Paton, 1973). The effects of phentolamine upon that efflux evoked by 25 mM Na^+ were measured. In 6 rings of dog saphenous vein, in three separate experiments, basal ^{3}H-efflux into normal Krebs solution over a 15 min period was 3.66 ± 0.27% of total tissue content. In 6 other rings in the same three experiments, ^{3}H-efflux into medium containing 25 mM Na^+ was 4.91 ± 0.37%. The increase (+ 34%) due to low Na was significant ($P < 0.0125$) and was preventable with cocaine (30 μM): the mean increase in ^{3}H-efflux caused by 25 mM Na^+ in the presence of cocaine was 3.76 ± 0.32% total tissue content over 15 min ($n = 6$ rings). Unlike cocaine, phentolamine (8 μM) did not prevent the increase in ^{3}H-efflux evoked by 25 mM Na^+: in normal Krebs solution containing phentolamine, basal efflux was 3.69 ± 0.33% ($n = 6$ rings in 3 experiments) and in low-Na^+ solution also containing phentolamine, ^{3}H-efflux was 4.99 ± 0.52% total tissue content in 15 min ($n = 6$ rings, three experiments).

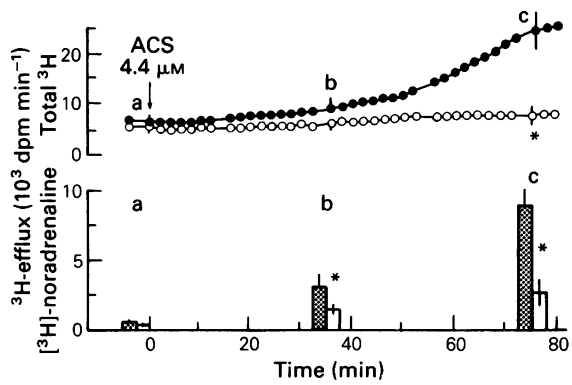

Figure 4 Effect of acetylstrophanthidin (ACS, 4.4 μM) on ^{3}H-efflux from superfused helical strips of dog saphenous vein in the absence and presence of phentolamine. The top panel shows the total ^{3}H-efflux appearing in the superfusate during successive 2 min periods, both before and after introduction of ACS (arrowed). At a, b and c samples were subjected to chromatographic separation and the [^{3}H]-noradrenaline in each is shown in the lower panel. Mean data from 6 strips in the absence of phentolamine (●, stippled columns) and from 5 strips in its presence (2.7 μM, ○, open columns) is shown; vertical lines show s.e.mean.
* indicates a statistically significant difference between observations in the presence and absence of phentolamine.

Vein strips The time course of ^{3}H-efflux evoked by ACS was investigated in superfused helical strips of dog saphenous vein. At ACS (EC_{50}) there was a delay of approx 6 min before increased efflux occurred; the rate of efflux then increased with time to reach a maximum at 75 min. The mean data from six strips is shown in Figure 4. In the presence of phentolamine (2.7 μM), ACS (EC_{50}) caused less efflux of ^{3}H-compounds, and, indeed, of unchanged [^{3}H]-noradrenaline (Figure 4, Table 1) than in the absence of phentolamine. Under similar conditions, phenoxybenzamine (3 μM) attenuated ACS-evoked ^{3}H-efflux but prazosin (0.1 μM, a concentration that blocks predominantly α_1-adrenoceptors in dog saphenous

Table 1 Effect of α- and β-adrenoceptor antagonists upon total ^{3}H and [^{3}H]-noradrenaline efflux evoked by acetylstrophanthidin (ACS, 4.4 μM) over 75 min from helical strips of dog saphenous vein

	No. of strips	*Spontaneous efflux at 75 min* (10^3 dpm per min)		*ACS-evoked efflux* (10^3 dpm per min)	
		Total ^{3}H	*[^{3}H]-NA*	*Total ^{3}H*	*[^{3}H]-NA*
Control	6	6.7 ± 0.89	0.54 ± 0.12	23.9 ± 3.45	8.9 ± 1.08
Phentolamine (2.7 μM)	5	5.8 ± 1.48	0.34 ± 0.06	8.0 ± 2.47*	2.6 ± 0.87*
Phenoxybenzamine (3.0 μM)	3	8.7 ± 1.47	1.17 ± 0.36	8.3 ± 1.20*	2.5 ± 0.69*
Prazosin 0.1 μM)	6	7.7 ± 1.17	—	21.1 ± 5.12	—
Propranolol (0.5 μM)	5	7.4 ± 1.23	0.73 ± 0.16	20.1 ± 2.94	6.7 ± 1.17

Mean values ± s.e.
*indicates that the value differs significantly from that in the absence of antagonist (i.e. from control).
Data taken from experiments of the type shown in Figure 4.

vein; Sullivan & Drew, 1980) and the non-selective β-adrenoceptor antagonist propranolol (0.5 μM) did not attenuate ACS-evoked ^{3}H-efflux (Table 1).

In five helical strips of saphenous vein superfused with ACS (EC_{50}), phentolamine (2.7 μM) was added to the superfusing fluid at 75 min. This caused an immediate downturn in total ^{3}H-efflux which continued to decline gradually (−19% after 10 min exposure to phentolamine). [^{3}H]-noradrenaline efflux declined by 23% ($P<0.01$) from a mean value of 8957 ± 1783(dpm)min^{-1} to 6878 ± 1542(dpm)min^{-1} after 10 min exposure to phentolamine. In five control strips superfused with ACS (4.4 μM = EC_{50}) from time = 0, but not then exposed to phentolamine at time = 75 min, there was no decline either in total ^{3}H-efflux or in [^{3}H]-noradrenaline efflux between time = 75 and 85 min (data not shown).

The glycoside ouabain (5 μM) caused an increase in ^{3}H-efflux from strips of saphenous vein after a delay of approx 20 min, and which reached a maximum within 40 min. Phentolamine (2.7 μM) attenuated the increase in total ^{3}H-efflux and in efflux of [^{3}H]-noradrenaline (Figure 5).

Effects of acetylstrophanthidin upon neurotransmitter release from bovine chromaffin cells

Chromaffin cells are functionally homologous to sympathetic neurones, and upon excitation with various secretagogues, including cardiac glycosides (Pocock, 1983) release catecholamines. However, unlike sympathetic neurones, bovine chromaffin cells appear to possess no functional α-adrenoceptors (Powis & Baker, 1986). Accordingly, it was of interest to determine the effects of phentolamine upon the release of adrenal medullary catecholamines evoked by ACS.

In five experiments with bovine chromaffin cells maintained in primary tissue culture, ACS (6.7 μM) caused a small (see also Pocock, 1983), but reproducible and statistically significant, increase in ^{3}H-efflux over a 45 min period from 2.67 ± 0.52% to 4.14 ± 0.45% of total cell ^{3}H content (+55%, $P<0.01$, $n = 28$ determinations in 5 experiments). In the same experiment, phentolamine (8.8 μM) itself had no significant effect on ^{3}H-efflux, nor did it attenuate ^{3}H-efflux evoked by ACS. ^{3}H-efflux in the presence of phentolamine alone was 3.32 ± 0.57% ($n = 29$ determinations in 5 experiments; cf. control values above) and efflux evoked by ACS in the presence of phentolamine was 5.22 ± 0.62% (+57%, $P<0.025$). Likewise in other experiments with bovine chromaffin cells in culture in which endogenous catecholamine release was measured by fluorimetry (Powis & Baker, 1986), the amount of catecholamine released by the glycoside ouabain was unchanged by phentolamine (data not shown).

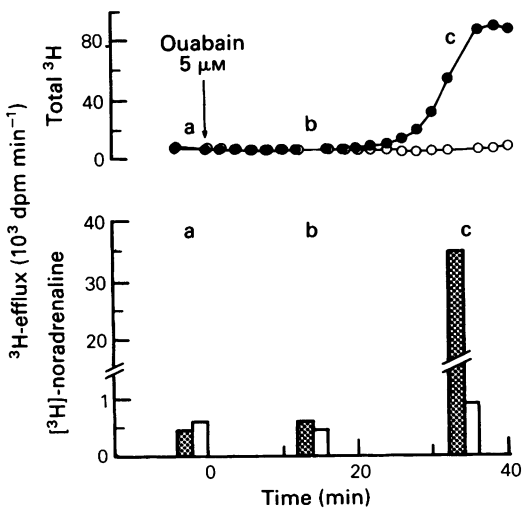

Figure 5 Effect of ouabain (5 μM) on ^{3}H-efflux from superfused helical strips of dog saphenous vein, one in the absence (●, stippled columns) and one in the presence (○, open columns) of phentolamine (2.7 μM). For other details refer to the legend for Figure 4.

Discussion

The experiments described here confirm that the cardioactive steroids, acetylstrophanthidin and ouabain, increase neurotransmitter efflux from the sympathetic nerve terminals of the canine saphenous vein, and from bovine adrenal medullary chromaffin cells. From the former tissue the efflux evoked by ACS and ouabain is substantial and its duration prolonged. From the latter the efflux is more modest (see also Pocock, 1983) but consistent and reproducible. Other experiments (P.K. Sullivan, D.A. Powis & R.R. Lorenz, unpublished but see Figure 6) have shown that ACS also evokes a substantial efflux of ^{3}H from the sympathetic nerves of dog atrial strips. With regard to the mechanism involved, cardiac glycoside-evoked neurotransmitter efflux is, in all probability, a consequence of Na,K-ATPase inhibition (see Powis, 1983; Tan & Powis, 1985).

The present experiments show clearly that the glycoside-evoked efflux of neurotransmitter from the sympathetic nerves of the saphenous vein and from atrial strips (Figure 6) is markedly attenuated by phentolamine over the time course of the effect (see also Powis *et al.*, 1979; Lorenz *et al.*, 1980). The experiments of Lorenz *et al.* (1980) showed in addition that ACS in concentrations insufficient to increase

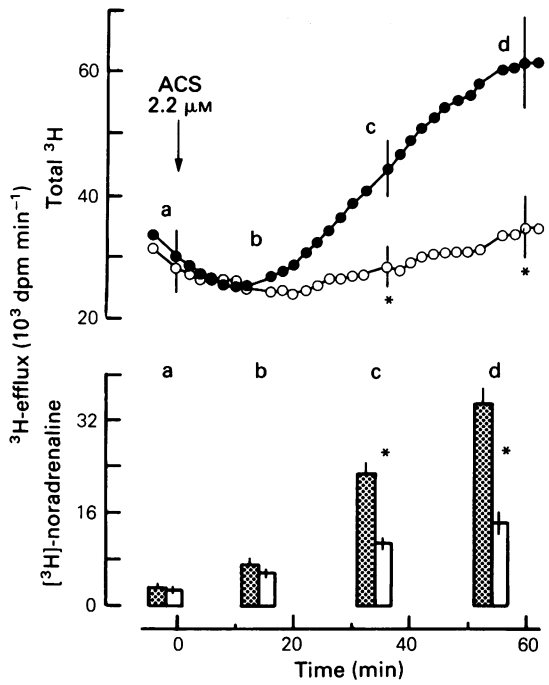

Figure 6 Effect of acetylstrophanthidin (ACS, 2.2 μM) on ^{3}H-efflux from superfused strips of dog atrial appendage (wet weight 160–350 mg, previously loaded with [^{3}H]-noradrenaline) in the absence (●, stippled columns) and in the presence (○, open columns) of phentolamine (2.7 μM). Mean data from 6 strips in the absence and 6 strips in the presence of phentolamine; vertical lines show s.e.mean. For other details refer to the legend for Figure 4.
(P.K. Sullivan, D.A. Powis & R.R. Lorenz, unpublished experiment).

spontaneous ^{3}H-efflux, markedly augmented ^{3}H-efflux evoked by depolarizing stimuli and, further, that phentolamine prevented the augmentation due to ACS.

There are many possible explanations for the attenuating effects of phentolamine upon cardiac glycoside-evoked neurotransmitter efflux: (1) that the attenuation is the result merely of a non-specific chemical interaction between phentolamine and glycoside which prevents the effects of the latter; (2) that phentolamine acts directly on Na,K-ATPase preventing inhibition of the enzyme by cardiac glycosides; (3) that phentolamine acts at the neurotransmitter release site preventing the glycoside-evoked efflux. In this regard it has been suggested that cardiac glycosides, by inhibiting Na,K-ATPase cause elevated internal [Na^+] which when sufficiently pronounced, causes reversal of the catecholamine uptake system (Paton, 1973; see also Powis, 1983) which thereby increases neurotransmitter efflux; (4) that phentolamine acts at an α-adrenoceptor, occupation of which by an antagonist interferes with the glycoside-evoked release.

The fact that ACS-evoked efflux of neurotransmitter can be blocked equally well with phenoxybenzamine and with yohimbine shows that the effect is not a peculiarity of the pharmacology of phentolamine. The likelihood of a non-specific chemical reaction between phentolamine and ACS (a substituted aglycone) providing the explanation is reduced by the finding that the chemically dissimilar compounds phenoxybenzamine and yohimbine are also effective, and further, that phentolamine also blocks the effects of ouabain (a glycoside).

It is unlikely that phentolamine blocks at the site of neurotransmitter efflux, at least if reversal of uptake is the mechanism by which this occurs in the presence of cardiac glycosides. The present experiments show that phentolamine, even at the highest concentration used throughout the series, has no effect upon cocaine-sensitive [^{3}H]-noradrenaline uptake in dog saphenous vein, nor does it reduce the effects of either tyramine (these experiments and see also Starke & Montel, 1974) or low extracellular Na^+ (these experiments) on [^{3}H]-noradrenaline efflux, both of which require a functional catecholamine transporter mechanism (Bonisch & Rodrigues-Pereira, 1983; Bonisch, 1986).

The experimental results obtained with the chromaffin cells of the bovine adrenal medulla are crucial for discriminating between the possibilities listed. In bovine chromaffin cells, which appear to have no α-adrenoceptors (Powis & Baker, 1986), phentolamine fails to attenuate ACS-evoked efflux of neurotransmitter. This observation detracts considerably from the first three possibilities listed above and, incidentally, adds substantial weight to the fourth possibility, that phentolamine blocks cardiac glycoside-evoked efflux via its interaction with an α-adrenoceptor.

More specifically the saphenous vein experiments show that the α-adrenoceptor involved is an α_2-adrenoceptor in as far as yohimbine and phentolamine attenuate the ACS-evoked ^{3}H-efflux but prazosin at concentrations which block α_1-adrenoceptors in dog saphenous vein (Sullivan & Drew, 1980) is without effect.

From these findings it may reasonably be concluded that interference by phentolamine and yohimbine with cardiac glycoside-evoked neurotransmitter release from sympathetic nerve terminals in the dog saphenous vein is a consequence of blockade of an α_2-adrenoceptor which impinges on the pathway initiated by Na,K-ATPase inhibition.

On a speculative note, α_2-adrenoceptors on sympathetic nerve terminals have often been described and are considered to regulate neurotransmitter release evoked by depolarizing stimuli (Starke, 1977) but their

mechanism of action has never been satisfactorily explained. The present data suggest, once again, the possibility that the mechanism might involve the participation of Na,K-ATPase (see Powis, 1981).

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