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PRELIMINARY COMMUNICATIONS

CARDIAC GLYCOSIDES, CALCIUM AND THE RELEASE OF NEUROTRANSMITTER FROM PERIPHERAL NORADRENERGIC NERVES

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It is established that cardiac glycosides cause an increase in transmitter release from nerve endings in brain and from both somatic and autonomic nerves in a variety of other tissues(1). On the basis that on exposure of tissues to cardiac glycosides the only apparent biochemical consequence is Na, K-ATPase inhibition it is generally assumed that the mechanisms responsible for enhanced transmitter release all stem from inhibition of this enzyme on the neuronal cell membrane. Although some investigators have suggested that neurotransmitter release is a direct consequence of Na, K-ATPase inhibition(2,3,4) the favoured hypotheses are those that relate release to the increase in intracellular Na activity that results from such inhibition (See ref.1). From this point the opinions of different investigators are divergent. Some contend that the increase in intracellular Na promotes the influx of extracellular Ca which is the ultimate trigger for neurotransmitter release(5,6,7). Their conclusions rest on the finding that neurotransmitter release during exposure to cardiac glycosides is severely attenuated or abolished in the absence of extracellular Ca. However, others consider that the presence of extracellular Ca is irrelevant; they have reported that neurotransmitter release continues in zero-Ca containing media(3,8).

These experiments were undertaken to investigate further the role of extracellular calcium in the neurotransmitter release process initiated by exposure to cardiac glycosides.

Materials and Methods

Experiments were performed with rings (10-20 mg) cut from the lateral saphenous vein of dogs anaesthetized with sodium pentobarbital (30 mg.kg^{-1} i.v.). The rings 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): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.2 or 2.54, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, d-glucose 11.1. Each solution contained also dl(7-^{3}H) noradrenaline (1.5×10^{-7} mol/l; specific activity 5-15 Ci/mmol, New England Nuclear) and ascorbic acid (0.5 mg.ml^{-1}).

In some experiments in vitro chemical denervation of the saphenous vein rings was accomplished with 6-hydroxydopamine (6OHDA; 9) prior to incubation with radiolabelled noradrenaline. It was established that this treatment of the saphenous vein rings caused effective sympathetic denervation without destroying the functional integrity of the smooth muscle which contributes the bulk of the extraneuronal tissue: in an organ bath it was shown that field stimulation of treated rings caused no contraction but addition of exogenous noradrenaline produced a contractile response similar to untreated tissues (see also 10).

After loading the rings with noradrenaline they were rinsed for 45 min either with Krebs' solution or Ca-free Krebs' solution ($CaCl_2$ omitted; EGTA, 1 mmol/l added) to remove noradrenaline from the extracellular space. The vein rings were transferred to pre-incubation vessels containing gassed ($95\%O_2/5\%CO_2$) Krebs' solution or Ca-free Krebs' solution at 37°C and equilibrated for 15 min prior to transfer to incubation vessels containing the same basic solution ± ouabain (3×10^{-7} - 10^{-5} mol/l).

The vein rings were incubated for 75 min after which time aliquots (1 ml) of the incubation media were collected for estimation of total ^3H. The rings were then removed, rinsed with the appropriate Krebs' solution (4°C), blotted dry and weighed. To estimate the radioactivity remaining in the tissue after incubation, each vein ring was solubilised (NCS, Amersham) and aliquots were taken. Total ^3H in tissue and incubation media was determined by liquid scintillation spectrometry (Packard Model 3255). Corrections for quenching were made with an external standard; counting efficiency was 30-35%.

Analysis of data It has been found previously with the saphenous vein preparation that when cardiac glycosides cause an increase in total ^3H release this is paralleled quantitatively by release of ^3H-noradrenaline (10). Thus in these experiments no chromatographic separation of ^3H compounds was performed; changes in ^3H release were assumed to reflect adequately changes in ^3H-noradrenaline release. Since all incubations were performed over a 75 min period the release calculated during control and test conditions represents the accumulated release over this period. Release is expressed in dpm/mg. Changes in ^3H-release have been calculated in terms of the total ^3H potentially available for release and expressed as a percentage. Hence:

$$\text{control fractional release} = \frac{\text{basal } ^3H \text{ release into incubation medium}}{\text{tissue } ^3H \text{ content} + \text{basal } ^3H \text{ release}} \times 100\%$$

$$\text{increase in fractional release caused by cardiac cardiac glycosides} = \frac{\text{total } ^3H \text{ release} - \text{basal } ^3H \text{ release}}{\text{tissue content} + \text{total } ^3H \text{ release}} - \text{basal } ^3H \text{ release} \times 100\%$$

All data are expressed as mean ± s.e. and the number of samples is the number of vein rings used. Statistical analysis was performed with Student's "t" test; P values less than 0.05 were considered to be significant.

Results and Discussion

1. Spontaneous release of ^3H-compounds

Spontaneous release of ^3H-compounds from saphenous vein rings during the 75 min incubation period was determined in the presence of Ca (1.2 and 2.54 mmol/l) and in its absence. Under all three conditions release of ^3H was identical indicating that Ca plays no part in determining basal release (Table 1).

Some of the ^3H would have been released by the nerves of the saphenous vein rings; some would have come from the extraneuronal tissue. An idea of the magnitude of each component was obtained from those experiments in which chemical sympathectomy had been performed. In six vein rings that had been treated with 6OHDA total tissue ^3H content was 3954 ± 359 dpm/mg

compared with 25117 ± 2580 dpm/mg in 23 normal rings (16%). The first value is assumed to reflect predominantly the capacity of the extraneuronal compartment. It was found that a larger proportion of ^3H was released from this compartment than from the neuronal compartment during a subsequent 75 min incubation. In the presence of Ca 1084 dpm/mg of a total store of 3243 dpm/mg were released from three 6OHDA treated rings compared with 1934 dpm/mg of a total store of 25117 dpm/mg in the 23 untreated rings. These data indicate that roughly half of the basal release during the 75 min incubation derives from extraneuronal sources.

The situation is precisely the same in the absence of Ca; the presence or absence of Ca is therefore irrelevant with respect to mobilization of intra- and extraneuronal stores of ^3H under resting conditions.

Table 1. Effect of external Ca on spontaneous release of ^3H-compounds from saphenous vein rings

Ca (mmol/l)	^3H-release (fractional release x 100)
0	7.70 ± 0.36 (27)
1.20	7.71 ± 0.48 (12)
2.54	7.74 ± 0.42 (25)

mean ± s.e; number of determinations in parentheses.

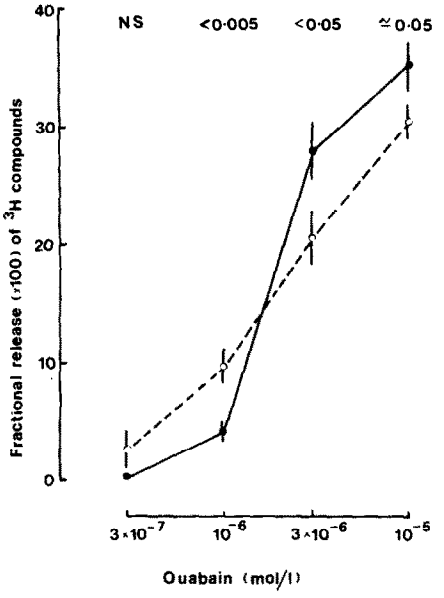

Fig.1: Effect of ouabain in the presence of Ca (2.54 mmol/l; ●—●) and in its absence (o--o) upon fractional release (× 100) of ^3H compounds. Each point shown is the mean ± s.e. of at least 6 determinations. The statistical significance of the difference between the results obtained in the presence or absence of Ca at each concentration of ouabain is shown.

2. Release of ^3H by cardenolides

a) In the presence of Ca. Increasing concentrations of ouabain (3×10^{-7} - 10^{-5}mol/l) caused increased release of ^3H from saphenous vein rings in the presence of Ca (2.54 mmol/l; Figure 1). At Ca (1.2 mmol/l) the concentration:release curve of ouabain was similar.

b) In the absence of Ca. Figure 1 shows also that in the absence of Ca there is still a concentration dependent release of ^3H evoked by ouabain from saphenous vein rings. However, there is a difference between the curves obtained in the presence and absence of Ca. At low concentrations of ouabain (<10^{-6} mol/l) the ^3H release in the absence of Ca is greater than in its presence whereas at higher concentrations (> 3×10^{-6} mol/l) the release in the absence of Ca is less than in its presence.

A precisely similar observation has been made with saphenous vein rings exposed instead to the cardenolide acetylstrophanthidin (ACS): at lower concentrations of ACS (2.2×10^{-6} mol/l) the release of ^3H in the absence of Ca was 8.9 ± 1.95% compared with 5.6% ± 2.65% in its presence. At a higher concentration of ACS (4.5×10^{-6} mol/l) the release of ^3H in the absence of Ca was 9.7 ± 2.65% while in its

presence it was 13.2 ± 1.54%. This indicates that the phenomenon is not a peculiarity unique to ouabain but may be a property of the cardenolides in general.

To evaluate the possibility that the differential effect of different concentrations of cardenolides is not the result of low concentrations mobilizing preferentially the extraneuronal sources of ^{3}H, the effect of ACS in the presence or absence of Ca was tested in 6OHDA treated tissues. It was found that neither in the presence or absence of Ca did ACS (4.5×10^{-7} - 6.7×10^{-6} mol/l) cause an increase in ^{3}H release above basal levels.

It appears, therefore, that the curious differential effects of low and high concentrations of cardiac glycosides evident in the presence and absence of Ca is due to a differential effect on neuronal stores. This phenomenon is of considerable interest as it might give further insight into the mechanisms of action of cardiac glycosides on neurotransmitter release.

The present experiments show that in the canine, innervated saphenous vein preparation Ca is not required for cardiac glycoside evoked release of ^{3}H-noradrenaline from nerve endings; however, its presence does modify release obtained. Further work is required to determine the role played by Ca in the release process.

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