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A simple method for measuring a picogram of acetylcholine using the clam *(Mya arenaria)* heart

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Summary

1. A simple and reliable method is described for assaying 1 pg or less of acetylcholine (ACh) using a strip of clam (*Mya arenaria*) heart.

2. Clam heart preparations generally are extremely selective for ACh. The preparation described is at least 10^5 times less sensitive to the following: acetyl coenzyme A, adrenaline, choline, dopamine, γ-aminobutyric acid, glutamic acid, histamine, 5-hydroxytryptamine and noradrenaline.

3. Because this preparation is one of the most sensitive known for detecting ACh, it should prove useful for assaying particularly low levels of this substance in biological extracts.

Introduction

Progress in biochemical and pharmacological research is often restricted by the sensitivity and specificity of methods available for detecting and measuring small amounts of different substances. During the course of studies designed to estimate very low levels of the enzyme choline acetyltransferase, it became necessary for us to have at our disposal a simple and reliable method for assaying picogram quantities of acetylcholine (ACh). It is known that some preparations of the leech dorsal muscle placed in a microbath (Szerb, 1962) respond to low levels of ACh, for example, 25–400 pg of AChCl (Szerb, 1962; Whittaker, Michaelson & Kirkland, 1964). However, in our hands, this preparation proved difficult to use because of the length of time required for reversal of effect, and relative lack of specificity. Furthermore, only very few of our preparations responded to amounts of ACh less than 100 pg (threshold of the best preparation was 60 pg AChCl).

Another particularly useful general preparation for ACh assay is the clam heart (Welsh & Twarog, 1960; Cottrell, Pentreath & Powell, 1968), but the entire heart preparations do not generally respond to doses less than about 1 ng of ACh. However, we have found that when a strip of *Mya arenaria* heart is placed in a microbath it will respond to very much lower levels of ACh and this report describes studies designed to evaluate the *Mya* heart strip as a preparation for detecting and measuring ACh. We also describe a cheap, simple and reliable method for recording the mechanical activity of such strips of muscle.

Methods

Specimens of the clam, *Mya arenaria*, were obtained from the estuary of the River Eden, Fife, and stored in circulating seawater tanks, but clams can also be

stored satisfactorily in bowls of seawater at 4° C. A method for preparing the entire *Mya* heart has been described (Cottrell *et al.*, 1968). In the present study, hearts were removed and pinned to cork at the base of a small dissecting dish filled with seawater. A strip of the ventricle muscle about $0{\cdot}6 \times 7$ mm in size was then cut away as shown in Fig. 1 and ligatured at each end with fine " Nylusta " nylon thread. The strip of muscle was then transferred to the tissue chamber of a micro-bath which contained 0·2 ml of liquid and immersed for 25 min in filtered seawater containing 5×10^{-5} g/ml Ergometrine maleate (B.D.H.) (Cottrell *et al.*, 1968) and then perfused with seawater alone. One end of the muscle strip was fastened with the thread to the base of the chamber and the other end to the tip of a 12·5 cm length of 0·5 mm diameter tungsten wire acting as a spring. A small piece of aluminium foil attached to the wire acted as a " flag " obstructing the passage of light from a 1·5 V " Pen-light " bulb to a 1GP 10 diode (Radiospares), converted to act as a photo-diode by scraping away the painted coating close to the germanium point contact. The tungsten wire and flag, and the bulb and photo-diode assembly, were carefully adjusted with respect to each other and to the vertically suspended strip of muscle, using Prior micromanipulators, so that any slight contraction of the muscle resulted in exposure of the germanium contact of the diode to light from the bulb. The diode was reverse-biassed by connecting its cathode to the positive terminal of a 45 V battery and the circuit completed via a 47 KΩ resistor. Changes in current flow through the circuit, resulting from changes in the amount of light reaching the photosensitive area of the diode, were measured as a voltage drop across the resistor and recorded on a Devices single channel pen recorder.

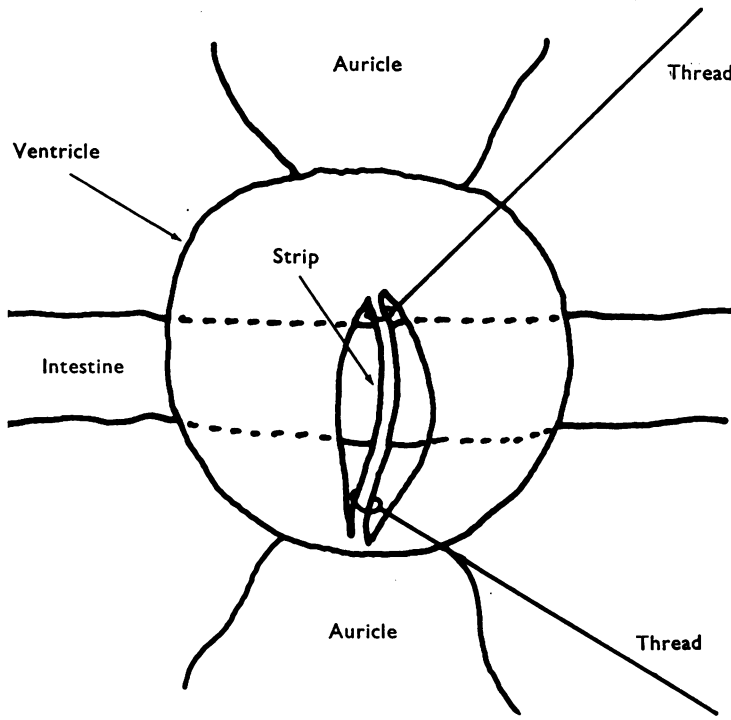

FIG. 1. Dorsal aspect of the *Mya arenaria* heart showing the position from which the strip of muscle was removed.

The experiments were completed in the spring and early summer.

The effects of the following drugs were tested on the activity of heart strips: acetylcholine chloride, choline chloride, dopamine hydrochloride, (−)-noradrenaline, adrenaline, 5-hydroxytryptamine creatinine sulphate, histamine acid phosphate, γ-aminobutyric acid, glutamic acid sodium salt, acetyl co-enzyme A sodium salt.

Drugs were dissolved in seawater and added to the preparation in 0·2 ml quantities. Dilutions of ACh solution were made using 10 μl Microcap pipettes which were discarded immediately after use.

Results

The majority (60–70%) of the strips of heart muscle, prepared as described above, began to beat in a rhythmical manner some 45 min after isolation. Those strips which did not beat were discarded.

ACh slowed, reduced the amplitude of movements, or completely stopped the rhythmical activity of heart strips in amounts as low as 0·1 pg, and generally with doses of 1 pg of AChCl. The activity of nearly all preparations was reduced when 3 pg of AChCl was added to the tissue chamber, but occasionally a preparation was obtained which did not respond to amounts of AChCl less than 200 pg. Often, with increased doses of AChCl over the range 0·2–5 pg, there was a graded response with respect to reduction in amplitude of beat, or frequency, or onset of response, or the rate of reversal of effect with washing (Fig. 2). However, sometimes the range of doses giving minimal to maximal responses at least with regard to the first three parameter above, was small, for example 2 to 4 times the threshold dose. The effect of ACh solutions on the muscle strips was rapidly reversed with washing so that generally doses of ACh could be added within 3 min intervals of each other.

We have tested the effects of several other compounds on the activity of heart strips. These substances and the amounts of them required to arrest or slow the rhythmic activity of heart strips are listed in Table 1. Unlike ACh, noradrenaline

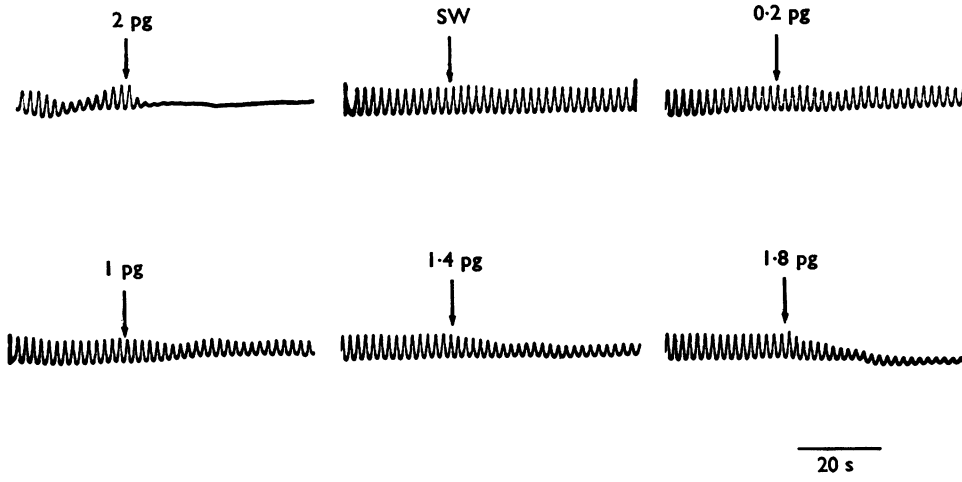

FIG. 2. Responses of a strip of muscle to small quantities of ACh.

and dopamine caused the muscle to cease rhythmic activity in a contracted state. Lower doses of noradrenaline and dopamine (about 0·5 to 5 μg) increased the amplitude (and sometimes the frequency) of beating.

Discussion

The results obtained in this report show that a suitably prepared strip of the *Mya arenaria* heart makes one of the most sensitive preparations known for detecting ACh and that the effect of ACh can be rapidly reversed by washing for about 1 min, after a threshold dose causing complete arrest of the muscle. Furthermore ACh was shown to be at least 10^5 times more active than any of the other pharmacological agents tested on the strips. These data suggest that *Mya* heart strips will prove to be extremely valuable for assaying very small amounts of ACh in biological extracts.

As with other microbath preparations care must be taken to ensure that the composition of the bathing medium is not altered when preparing extracts, otherwise spurious results may be obtained. Furthermore care must be taken to ensure that all glassware used is thoroughly clean.

When attempting to detect ACh in individual snail neurones, we discovered that interfering substances diffused from the walls of small soda glass tubes which were made in this laboratory. It is therefore preferable to prepare all solutions in Pyrex or other non-soda glass containers to avoid this source of contamination.

One point of pharmacological interest made during this study is that whereas 5-hydroxytryptamine excites the intact isolated heart causing an increase in amplitude and/or frequency, it inhibits the activity of muscle strips after perfusion with ergometrine. Further experiments on entire hearts show that the difference in response is due to the ergometrine (Powell & Cottrell, unpublished). Thus ergometrine has the ability to alter completely the response of the *Mya* muscle to 5-hydroxytryptamine, the presumed transmitter released from the clam cardioaccelerating nerves (Cottrell & Laverack, 1968). The mechanism underlying this change in activity is unknown.

Specimens of *Mya arenaria* can easily be stored for months under appropriate conditions (Cottrell *et al.*, 1968).

TABLE 1. *Amounts required of some different substances to inhibit rhythmic activity of the muscle strip*

	pmol
ACh	0·006
Choline	8×10^4
Dopamine	1×10^4
Noradrenaline	4×10^4
Adrenaline	3×10^4
5-Hydroxytryptamine	1×10^3
Histamine	4×10^4
GABA	3×10^5
Glutamic acid	2×10^4
Acetyl coenzyme A	1×10^4

Results are based on five experiments in which each substance was tested on at least three different preparations.

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