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Acetylcholine release from the rabbit isolated superior cervical ganglion preparation

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Summary

1. The rabbit isolated superior cervical ganglion preparation has been used to measure the release of acetylcholine from the tissue at rest and during preganglionic nerve stimulation.

2. In the presence of physostigmine, the resting release of acetylcholine was $0{\cdot}13 \pm 0{\cdot}01$ (nmol/g)/min (10 experiments) and that during stimulation with 300 shocks at 10 Hz was $3{\cdot}1 \pm 0{\cdot}4$ (pmol/g)/volley in 4 experiments (means ± S.E.M.). The volley output was independent of the frequency of stimulation over the range 1 to 10 Hz but was higher at 0·3 Hz.

3. Tetrodotoxin, 0·8 μM, had no effect on the resting release of acetylcholine but reduced the stimulated release below detectable levels (2 pmol). Lowering the temperature of the bathing fluid to 5° C reduced to below detectable levels both the resting release and that produced by nerve stimulation.

4. The resting release of acetylcholine was increased by a potassium-rich (49·4 mM K^+) bathing solution and by replacing the sodium chloride in the solution with lithium chloride (113 mM Li^+).

5. (−)-Noradrenaline bitartrate, 3 μM, and (±)-adrenaline bitartrate, 1·5 μM, reduced by 70% the output of acetylcholine induced by stimulation at 0·3 Hz, but failed to reduce the resting release or that evoked by stimulation at 10 Hz. The inhibition was reversed by phentolamine.

6. It is concluded that the rabbit superior cervical ganglion *in vitro* is a suitable preparation for studying transmitter release and that the ganglion blocking effect of catecholamines is due to a reduction in transmitter release.

Introduction

The release of acetylcholine from preganglionic nerves has usually been studied *in situ* (Feldberg & Gaddum, 1934; Feldberg & Vartiainen, 1934; MacIntosh, 1938; Perry, 1953). In such experiments it is difficult to use very small perfusion volumes and often, when Locke solution is used, the tissue becomes oedematous. Moreover, collection of the perfusate is not easy and drugs under investigation may interfere with the fluid flow. To overcome some of these problems, we have tried to measure the release of acetylcholine from the rabbit isolated superior cervical ganglion and the effect upon it of noradrenaline and adrenaline under conditions not complicated by their vasoconstrictor effect.

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Methods

Ganglion preparation

Rabbits (1·5–2·5 kg) of either sex were anaesthetized with urethane (1·5 g/kg, i.v.) and the tracheae cannulated. Both superior cervical ganglia were removed, together with about 3 cm of the preganglionic sympathetic nerve and 1 cm of the postganglionic (internal carotid) nerve. The tissues were placed in a dish of Krebs solution, at room temperature, gassed with a mixture of 95% O_2, 5% CO_2, and the tissue sheath around the ganglion carefully teased away. One of the ganglia was then transferred to a small Perspex trough containing 1·5 ml of 15 μM physostigmine sulphate in Krebs solution, warmed to 37° C by means of a heating jacket and gassed with 95% O_2, 5% CO_2. The preganglionic nerve was laid across two platinum electrodes and suspended, during periods of stimulation, just above the surface of the solution. After a 30 min period of equilibration with the Krebs solution containing physostigmine sulphate in a concentration of 15 μM, the physostigmine sulphate concentration in the Krebs solution was reduced to 3 μM. At the end of the experiment, the ganglion (without nerve trunks) was weighed after removing excess moisture by pressing between filter papers.

The preganglionic nerve was stimulated by square wave pulses of 0·1 ms duration and 2 V strength, delivered *via* an isolation transformer. After a period of stimulation, the preganglionic nerve was resubmerged in the Krebs solution and an extra 7 min were allowed for the released acetylcholine to diffuse into the bathing solution. Aliquots (0·2–0·6 ml) of this solution were then assayed for acetylcholine content.

Acetylcholine assay procedure. The method of assay of released acetylcholine was that described by Paton & Vizi (1969). An 8 cm length of guinea-pig ileum was suspended in 3·5 ml Krebs solution at 37° C containing 6 nM physostigmine sulphate (to increase the sensitivity to acetylcholine) and 13 μM morphine sulphate (to reduce the release of endogenous acetylcholine). The minimal detectable amount of acetylcholine was 2 pmol. The oral end of the ileum was ligated and the the aboral end tied over an open-ended polythene tube which projected through the bottom of the organ bath, to allow the luminal contents to be extruded from the ileum without contaminating the bath. Samples for assay, or control acetylcholine solutions, were added to the assay bath at intervals of 1 min in volumes of 0·2 to 0·6 ml. The estimate of the acetylcholine content of samples was based on comparisons with concentration-response curves obtained with known concentrations of acetylcholine (containing the same concentration of any added drug as in the test samples) which were tested repeatedly throughout an experiment. The activity of assay samples could always be abolished by pretreatment of the assay organ with atropine sulphate, 0·3 μM, or by addition of alkali to the samples; moreover, mepyramine maleate, 10 μM, had no effect on the contractile responses.

The composition of the Krebs solution was (mM): NaCl 113, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4 \cdot 7H_2O$ 1·2, $NaHCO_3$ 25, KH_2PO_4 1·2, dextrose 11·5. When the potassium concentration was increased to 49·4 mM by adding KCl, the NaCl concentration was reduced appropriately. Exposure to the high potassium concentration was kept to a minimum and no increase in weight of the tissues was observed. When lithium was used, all the NaCl was replaced by LiCl to give 113 mM Li^+.

Statistical comparisons were made using Student's *t* test.

Drugs used

Acetylcholine bromide (B.D.H.), (±)-adrenaline bitartrate (B.D.H.), choline chloride (B.D.H.), lithium chloride (B.D.H.), (−)-noradrenaline bitartrate (B.D.H.), phentolamine methanesulphonate (Ciba), physostigmine sulphate (Burroughs Wellcome) and tetrodotoxin (Sankyo).

Results

Resting release of acetylcholine

The mean resting output of acetylcholine collected over periods of 20–30 min from 10 different ganglia was 0·13 ± 0·01 (nmol/g)/min, i.e. about 1·8 pmol/min for a preparation weighing 15 mg. This level of resting release was maintained for the duration of the experiments (2–3 h) and was not influenced by the addition of choline chloride (7 μM) to the bathing fluid.

Release of acetylcholine during preganglionic stimulation

The output of acetylcholine was increased by preganglionic nerve stimulation by amounts varying with the frequency and duration of stimulation. The increased output (corrected for resting release) is shown in Figure 1a. Addition of choline to the bathing fluid did not affect the acetylcholine output produced by stimulation at 1 Hz. Furthermore, the release of acetylcholine was quite consistent in a given tissue and did not alter significantly during successive periods of stimulation at 10 Hz for up to 2,000 shocks when applied at intervals of 7 min or more.

From Fig. 1a, it can be seen that the volley output decreased with increasing number of shocks at frequencies of 0·3 to 10 Hz.

For 200 or more shocks there was no statistically significant difference between the volley outputs at stimulation frequencies of 1 Hz, 3 Hz and 10 Hz (Figure 1b).

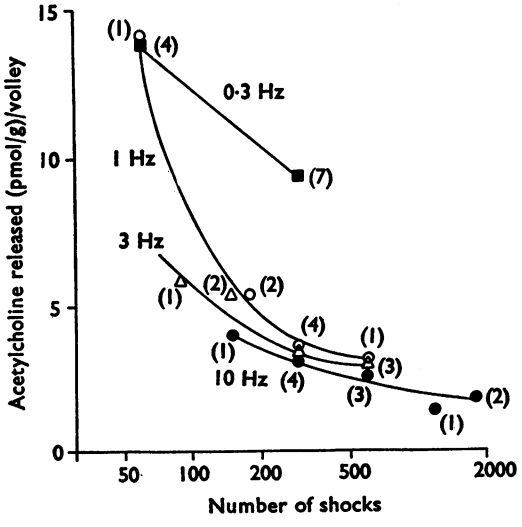

FIG. 1(a). Effect on output of acetylcholine from the rabbit isolated cervical ganglion obtained with different numbers of shocks at stimulation frequencies of 0·3 (■), 1(○), 3 (△) and 10 Hz (●). The figures in parentheses are the number of observations obtained on a different preparation; the abscissa is log scale.

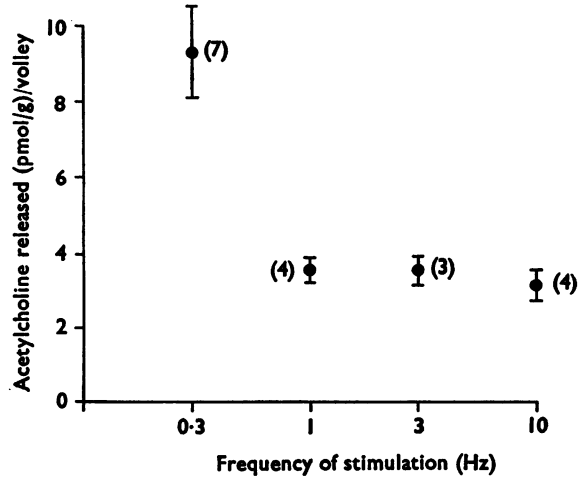

FIG. 1(b). Effect on output of acetylcholine from the rabbit isolated cervical ganglion obtained with 300 shocks at different frequencies of stimulation; means ±S.E.M. are shown. The figures in parentheses are the number of observations obtained on a different preparation; the abscissa is log scale.

However, for 300 shocks the output at 0·3 Hz was significantly higher than that at 1 Hz ($P<0·01$), 3 Hz ($P<0·05$) and 10 Hz ($P<0·005$). The absolute output per minute increased with frequency, rising to 4 (nmol/g)/min at a stimulation frequency of 30 Hz.

Effect of cooling

The effect of changing the bath temperature on release of acetylcholine is shown in Figure 2. On lowering the temperature of the preparation to 5° C, the resting

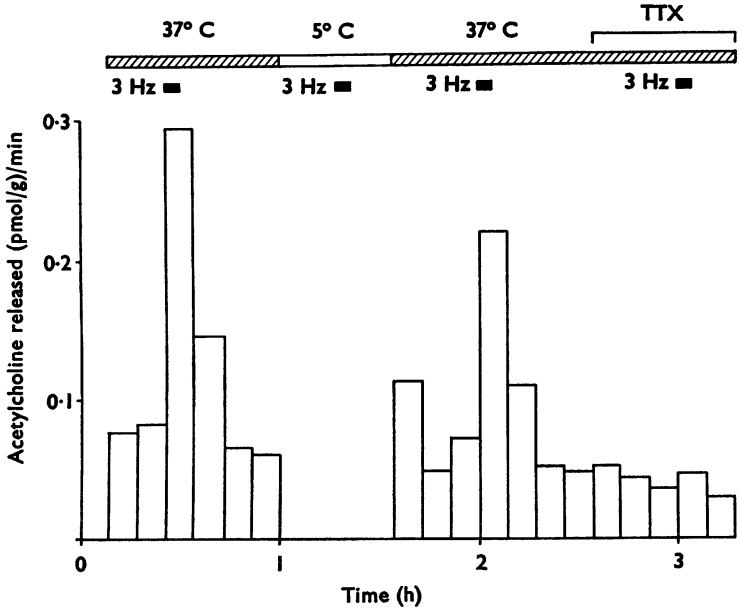

FIG. 2. The effect of lowering the temperature and 0·8 μM tetrodotoxin (TTX) on the resting and stimulated release of acetylcholine from the isolated superior cervical ganglion preparation. The columns represent the acetylcholine released during successive collection periods. The periods of stimulation at 3 Hz (300 shocks, 1 min 40 s) are indicated by the black bars.

release and that evoked in response to nerve stimulation at 3 Hz were reduced to below detectable levels (i.e. to <15·3 (pmol/g)/min or 1·5 (pmol/g)/volley). At a temperature of 15° C, the release, relative to that at 37° C, on stimulation at 10 Hz was reduced by 90%.

Effect of potassium

When the ganglion preparation was bathed for 7 min in a solution rich in potassium (49·4 mM K^+), the resting release of acetylcholine was increased from 0·079 ± 0·015 (nmol/g)/min to 0·25 ± 0·03 (nmol/g)/min (n=3).

Effect of tetrodotoxin

Tetrodotoxin (0·8 μM) had no effect on, or slightly reduced, the resting release of acetylcholine (Fig. 2), but abolished the release produced by nerve stimulation at 3 Hz (see Figure 2). Tetrodotoxin also did not significantly affect the increase in acetylcholine release evoked by potassium.

Effect of lithium

Replacement of sodium chloride by lithium chloride reduced the stimulated release at 10 Hz by about 71% but increased the resting release threefold (Figure 3).

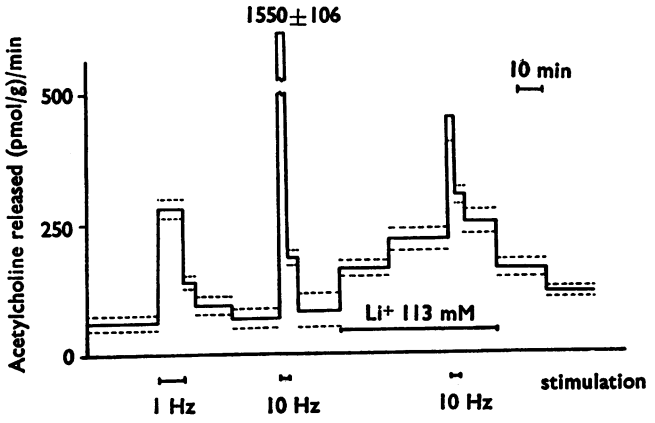

FIG. 3. The effect of replacing NaCl by LiCl in the fluid bathing the isolated ganglion preparation on the resting release of acetylcholine and on that evoked by preganglionic stimulation at 10 Hz. The dotted lines represent standard errors.

Effect of catecholamines

(±)-Adrenaline and (−)-noradrenaline, used as bitartrates, in a concentration of 3 μM, had no effect on the resting release of acetylcholine from the ganglion preparation. Thus, in the presence of noradrenaline, the mean resting release and standard error was 0·11 ± 0·012 (nmol/g)/min for 10 experiments compared with a control resting release of 0·13 ± 0·014 (nmol/g)/min for 10 experiments (P>0·1).

In contrast, the release of acetylcholine elicited by stimulation at 0·3 Hz was reduced by noradrenaline (3 μM) and by adrenaline (0·3 and 1·5 μM) added to

the organ bath 1 min before stimulation (Figure 4). No decrease in acetylcholine output at 10 Hz was observed. Phentolamine methanesulphonate (3 μM) prevented the inhibition by adrenaline of the release of acetylcholine. In 6 experiments, the control release produced by stimulation at 0·3 Hz was 17 ± 2 (pmol/g)/volley and in the presence of phentolamine alone (3 μM) was 18 ± 2 (pmol/g)/volley ($P>0{\cdot}5$; $n=3$). Adrenaline (1·5 μM) reduced the release to 3 ± 1 (pmol/g)/volley ($P<0{\cdot}001$; $n=3$), but in the presence of phentolamine (3 μM) and adrenaline (1·5 μM) the release was 17 ± 3 (pmol/g)/volley ($P>0{\cdot}5$; $n=3$).

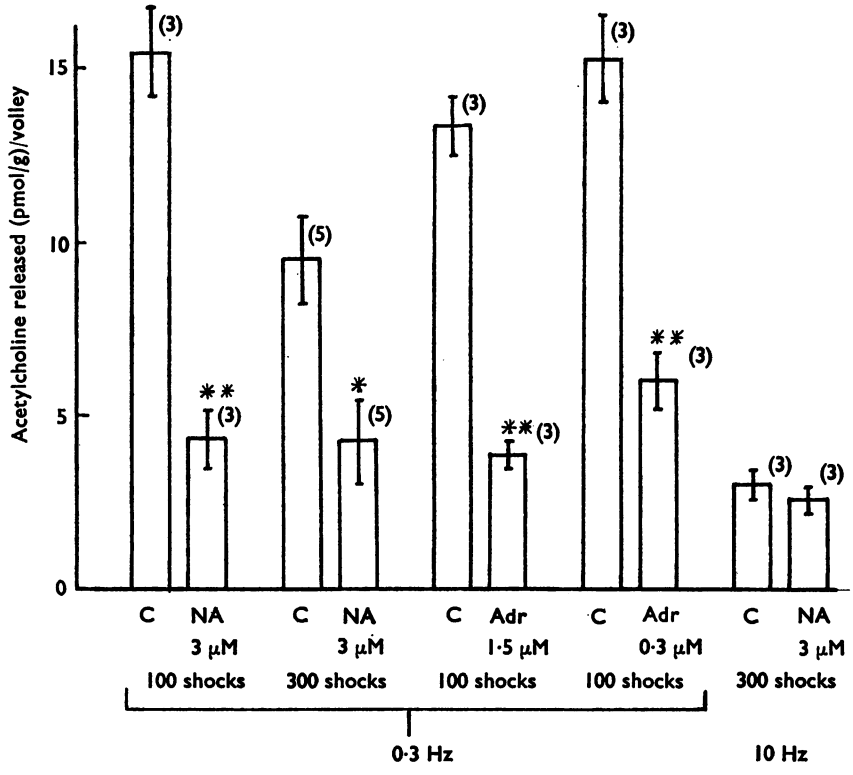

FIG. 4. The effect of noradrenaline (NA, 3 μM) and adrenaline (Adr, 0·3 and 1·5 μM) on the release of acetylcholine from the rabbit isolated superior cervical ganglion stimulated preganglionically. The control release is represented by the columns marked C, the bars indicate S.E.M. and the figures in parentheses are the number of experiments. Two asterisks indicate $P<0{\cdot}001$ and one asterisk $P<0{\cdot}01$. At 10 Hz, $P>0{\cdot}5$. The same preparations were used for the control and the observation in the presence of a catecholamine.

Discussion

The rabbit isolated superior cervical ganglion appears to be a suitable preparation in which to measure acetylcholine release. The amounts of acetylcholine released at rest and during repetitive preganglionic nerve stimulation were quite similar to those previously reported for perfused cat ganglia (Perry, 1953 ; Birks & MacIntosh, 1961). The effects of stimulus frequency upon acetylcholine release are of interest since we have been able to cover a wider frequency range than hitherto. In agreement with previous observations, we have found the volley output to be fairly constant for frequencies from 1 to 10 Hz, but to be appreciably greater

at 0·3 Hz. The release at high frequencies might be limited by the rate of acetylcholine synthesis (cf. Perry, 1953). In the perfused cat superior cervical ganglion, the presence of choline in the perfusion fluid is required for sustained release at 10 Hz (Matthews, 1963). In the rabbit isolated superior cervical ganglion, choline did not augment acetylcholine release at 1 Hz and so its absence could not account for the fall in volley output on increasing the stimulus frequency from 0·3 to 1 Hz. The effect of choline at higher frequencies is uncertain.

Since tetrodotoxin had no effect on the resting release of acetylcholine, propagated nervous activity is not involved in this process. A similar result was obtained in studies of the neuromuscular synapse (Elmqvist & Feldman, 1965 ; Katz & Miledi, 1967). Reduction of the bath temperature abolished the resting release, as well as that evoked by nerve stimulation, indicating an energy requirement for release. The effect of lithium ions in increasing the resting release of acetylcholine is possibly related to the reduction in extracellular sodium concentration which, as shown in squid axon, increases the influx of calcium (Baker, Blaustein, Hodgkin & Steinhardt, 1969). Intracellular accumulation of lithium, which is pumped from cells less efficiently than is sodium (Keynes & Swan, 1959), may then contribute to a block of synthesis of acetylcholine which thereby precludes release following nerve stimulation, as has been proposed in rat cortex slices (Vizi, Illes, Ronai & Knoll, 1972).

Using the longitudinal muscle strip of guinea-pig ileum, Paton & Vizi (1969) observed that both adrenaline and noradrenaline reduced the resting release of acetylcholine. In the isolated superior cervical ganglion preparation, however, no effect on the resting release was produced by (±)-adrenaline or (−)-noradrenaline in concentrations of 3 μM. In contrast, noradrenaline and adrenaline reduced the evoked release at a low frequency of stimulation (0·3 Hz), although no such effect was seen at a frequency of 10 Hz. The greater effectiveness of catecholamines at lower frequencies is in good agreement with the findings of Knoll & Vizi (1971). Paton & Thompson (1953) observed that adrenaline reduced acetylcholine release in the perfused superior cervical ganglion of the cat. Furthermore, catecholamines have also been shown to depress ganglionic transmission (Marazzi, 1939 ; Lundberg, 1952 ; Matthews, 1956 ; Eccles & Libet, 1961 ; McIsaac, 1966 ; de Groat & Volle, 1966 ; Christ & Nishi, 1969 ; Kayaalp & McIsaac, 1970), especially at low frequencies of stimulation, and Christ & Nishi (1971) have recently shown electrophysiologically that the reduction in transmission *in vitro* is a presynaptic effect. Our result, that noradrenaline and adrenaline are capable of reducing acetylcholine release from the preganglionic nerve terminals of the isolated superior cervical ganglion, provides evidence that the reduction in transmission frequently observed *in vitro* with noradrenaline can be explained by depression of acetylcholine release. Since sympathetic ganglia have been shown to contain and to release adrenaline on preganglionic stimulation (Lissak, 1939 ; Bülbring, 1944), the possibility is provided for a modulatory role of catecholamines on acetylcholine release.

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