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Formation and Release of [^{3}H]Acetylcholine in the Rat Urinary Bladder Strip

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Abstract—The relationship between different frequencies of loading stimulation and [^{3}H]acetylcholine (ACh) formation and release from nerve terminals has been investigated in extratrigonal strips of the urinary bladder of the rat. An increase in frequency (0·2, 0·4 and 0·8 Hz) for the 30 min incubations with [^{3}H]choline produced an enhancement of storage of [^{3}H]ACh from 19·5 to 34% of total tritium content in the tissue. Higher frequencies (1·6 and 3·2 Hz) failed to increase storage further on. The [^{3}H]choline content did not vary significantly. Electrical field stimulation at 2 Hz (360 shocks) produced a release of tritium. The evoked outflow was higher when the strip was loaded at 0·8 Hz than at the other frequencies tested. Both [^{3}H]ACh and [^{3}H]choline were measured in the perfusate of strips preloaded at 0·8 Hz. Most of the induced outflow was found to be [^{3}H]ACh, as in previous experiments carried out using 0·2 Hz as a loading frequency. The findings suggest that in the rat urinary bladder strip loading at 0·8 Hz is suitable for increasing the formation and the resulting release of [^{3}H]ACh during electrical stimulation.

Studies in superior cervical ganglia (Collier & MacIntosch 1969), and in diaphragm muscles (Potter 1970), showed that the rate of incorporation of labelled acetylcholine (ACh) into ACh stores was greatly enhanced by stimulation of the cholinergic neurons. In the myenteric plexus also, although the rate of ACh formation is less affected by variations in neuronal activity than ACh turnover in other tissues (Szerb 1975), the content of [^{3}H]ACh increases with increasing loading frequency (Szerb 1976; Vizi et al 1984). It is assumed that in neuronal preparations the synthesis of ACh is regulated by the high affinity choline uptake system (Barker 1976; Weiler 1978). This system is enhanced in a frequency-dependent manner (Vaca & Pilar 1979, Antonelli et al 1981) and inhibited by hemicholinium-3 (Jope 1979). In the rat urinary bladder strip, [^{3}H] choline is taken up by a neuronal transport system, sensitive to hemicholinium-3, and converted into labelled ACh which can be released by electrical field stimulation and modulated by muscarinic agonists and antagonists (D'Agostino et al 1986). A considerable storage of labelled ACh is needed to study the potencies of these drugs at the presynaptic receptor level. We have investigated whether the [^{3}H]ACh formation in the rat urinary bladder depends upon the loading frequency, as in other tissues. Also, the relationship between [^{3}H]ACh storage and the release of [^{3}H]ACh upon delivery of the same number of shocks has been evaluated.

Methods

Preparation of the rat urinary bladder strip

Male albino rats, Wistar Morini strain, 190 to 210 g, were used. The whole urinary bladder was dissected and placed in Krebs-Henseleit solution of composition (mM): NaCl 118, KCl 5·6, $CaCl_2$ 2·5, $MgSO_4$ 1·19, $NaHCO_3$ 25, NaH_2PO_4 1·3, glucose 10. Four longitudinal muscle strips of the extratrigonal area (10 mm long, 1·5 mm wide, 9–12 mg) were isolated from the anterior portion of each bladder (Grana et al 1986).

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Labelling and release experiments

The procedures were described by D'Agostino et al (1986). Briefly, each preparation was positioned between two parallel platinum electrodes and suspended isometrically in a 2 mL perfusion organ bath filled with Krebs-Henseleit solution bubbled with a mixture of 95% O_2 and 5% CO_2 and containing 1 μM choline. During the first 15 min incubation tension was adjusted until it stabilized at 1 g.

The preparations were incubated for 30 min with 5 μ Ci [methyl-^{3}H]choline and stimulated continuously with supramaximal impulses of 1 ms duration at 0·2, 0·4, 0·8, 1·6 or 3·2 Hz. The stimulation parameters were monitored to ensure a constant stimulus strength. Superfusion was then stopped. At the end of the incubation with labelled choline, there was a 120 min washout period during which the strips were superfused at 37 °C and 2 mL min^{-1} (Gilson Minipulse 2 HP8 flow inducer). Starting at the 121st min, perfusion fluid was collected continuously in 3 min periods (6 mL samples). The strips were stimulated once during the collection of the 4th sample with square wave impulses (1 ms duration, supramaximal voltage) at a frequency of 2 Hz for 3 min (360 shocks) either in the absence or presence of 100 nM physostigmine. Physostigmine was added to the perfusion fluid 60 min before the stimulation. Aliquots (1 mL) of the superfusate were added to 7 mL of Picofluor 15 (Packard) and the tritium content measured by liquid scintillation spectrometry (Packard TRICARB 4530). Counting efficiency was determined by external standardization. Both resting and stimulation-evoked outflows of radioactivity were expressed in Becquerels per gram of tissue (Bq g^{-1}). The increase in release caused by stimulation was obtained from the difference between the total tritium outflow during 3 min stimulation plus that in the following 12 min (stimulation outflow period) and the calculated spontaneous outflow. Since preliminary trials had proved that the spontaneous outflow decreased exponentially during the experiment, the decline was calculated by fitting a linear regression line of the values expressed in Becquerels of all the samples except those of the stimulation outflow periods.

Separation of radiolabelled compounds in the tissue and in the superfusate

Tritiated ACh and choline content was detected by the method of Barker et al (1972). When radiolabelled compounds were to be determined in the muscle strip, the tissue was homogenized in a micropotter in 0·5 mL of ice-cold 0·4 M perchloric acid. For the measurement of total tritium content, 100 μL of the 2 mL homogenate was added to 7 mL of Instagel (Packard) scintillator. Labelled choline and ACh were then extracted into 3 mL allylcyanide containing 15 mg of sodium-tetraphenylboron. The organic phase was evaporated to dryness and the residue was redissolved in 0·5 mL methanol applied to an anion exchange column (Dowex 2 × 8 Cl^-; 1 × 3·5 cm) and eluted with 9 mL methanol. The eluate was evaporated to dryness and the residue redissolved in 100 μL methanol containing unlabelled carrier choline and ACh (2 μmol each). 10 μL of this solution was spotted on cellulose thin layer chromatography sheets and developed in butan-1-ol–methanol–glacial acetic acid–H_2O (8:2:1:3 by vol). The choline and ACh bands were stained by iodine vapour and the chromatogram, in five sections, was scraped off into vials containing 1 mL of a mixture of methanol and 1 M HCl (19:1 by vol). The samples were counted in 9 mL of Instagel scintillator. Recoveries were determined by adding internal standards of [^{14}C]choline and [^{14}C]ACh (10 and 5 nCi, respectively) to the homogenate.

The recovery values were 61·2% ± 1·5 (n = 18) for choline and 66·8% ± 2 (n = 18) for ACh. All values for tritiated compounds were corrected accordingly.

By using this procedure, labelled choline and ACh could be separated quantitatively in the superfusate collected during 0–15, 15–30 and 30–45 min.

Statistics

The results are expressed as means ± s.e.m. Statistical analysis of the data was assessed by unpaired Student's *t*-test. *P* values of <0·01 were considered significant.

Materials

[methyl-^{3}H]Choline chloride (78 Ci $mmol^{-1}$; 2·89 TBq $mmol^{-1}$, [methyl-^{14}C]choline chloride (50 mCi $mmol^{-1}$; 1·85 GBq $mmol^{-1}$), [methyl-^{14}C]ACh chloride (58·3 mCi $mmol^{-1}$; 1·99 GBq $mmol^{-1}$) (all from Amersham, Bucks, UK). Choline chloride, ACh bromide, physostigmine sulphate were from Sigma Chemical Co., St Louis, USA.

Results

[^{3}H]ACh content

The homogenate of the tissue was assayed for [^{3}H]ACh, [^{3}H]choline and total tritium content.

The measurement in the strips preloaded at 0·2 Hz for tritiated ACh was 19·5 ± 2·6% of total ^{3}H content (228 793 ± 8894 Bq g^{-1}) (Fig. 1, Table 1).

When the labelling frequency was increased, the [^{3}H]ACh content was 23·3 ± 2·5% at 0·4 Hz, 34 ± 1·6% at 0·8 Hz, 28·1 ± 0·6 at 1·6 Hz and 25·9 ± 2 at 3·2 Hz of the total radioactivity (244 248 ± 9277 Bq g^{-1} and 248 538 ± 13 026 Bq g^{-1}, 227 213 ± 11 220 Bq g^{-1} and 214 373 ± 7838 Bq g^{-1} respectively) (Fig. 1, Table 1).

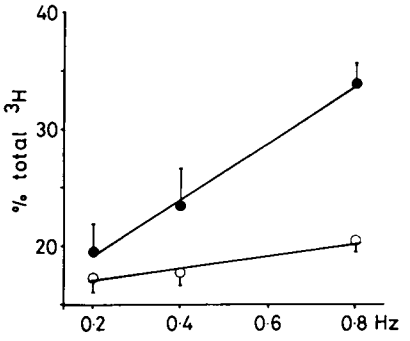

FIG. 1. [^{3}H]ACh (●) and [^{3}H]choline (○) amount in strips preloaded with [^{3}H]choline at different labelling frequencies. Percentage of total ^{3}H tissue content. Means ± s.e.m. from at least four experiments.

Table 1. In (d) and (e) the tritium release was measured in the presence of 100 nM physostigmine that was added to the perfusion fluid 60 min before stimulation. Means ± s.e.m. of (n) experiments are given.

Frequency		Tissue content (% of total ^{3}H)		^{3}H evoked by S (Bq g^{-1})
Labelling	Stimulation	[^{3}H]ACh	[^{3}H]choline	
(a) 0·2 Hz		19·5 ± 2·6 (6)	17·3 ± 1 (6)	
(b) 0·4 Hz		23·3 ± 2·5 (4)	17·8 ± 1 (4)	
(c) 0·8 Hz		34 ± 1·6 *(7)	20·6 ± 0·8 (7)	
(d) 0·2 Hz	2 Hz (360 shocks)	8·3 ± 0·6 *(4)	14·9 ± 1 (4)	4 477 ± 177 (4)
(e) 0·8 Hz	2 Hz (360 shocks)	17·6 ± 0·2**(4)	17·1 ± 1·5 (4)	6 368 ± 175*(4)
(f) 0·2 Hz	2 Hz (360 shocks)			7 616 ± 387 (4)
(g) 0·8 Hz	2 Hz (360 shocks)			11 502 ± 779*(15)

Significance of either increase or decrease vs 0·2 Hz (*) and 0·8 Hz (**) labelling experiments $P < 0·01$.

The [^{3}H]choline amounts, expressed as percentage of total ^{3}H content, were 17·3 ± 1, 17·8 ± 1, 20·6 ± 0·8, 21·4 ± 1·7 and 21·9 ± 2 at 0·2, 0·4, 0·8, 1·6 and 3·2 Hz respectively (Fig. 1, Table 1). These values are not statistically different.

[^{3}H]ACh release

Electrical field stimulation at 2 Hz (360 shocks) caused an increased outflow of radioactivity from the strip. The induced release was higher in the strips preloaded at 0·8 Hz than those at 0·2 Hz (Fig. 2, Table 1). The difference was statistically significant ($P < 0·01$) in the absence and presence of 100 nM physostigmine.

A marked decrease in [^{3}H]ACh but not in [^{3}H]choline content, compared with control experiments (Table 1) was shown when the tritium tissue content was assayed after the 2 Hz stimulation.

To identify labelled ACh and choline in the superfusate of the strips preloaded at 0·8 Hz, pooled samples were collected at 0 to 15, 15 to 30 and 30 to 45 min after washout.

In four experiments during the spontaneous outflow periods (0–15 and 30–45 min) the released radioactivity consisted prevailingly of [^{3}H]choline (82 ± 3·5% and 73 ± 5% of [^{3}H]ACh + choline, respectively) (Fig. 3). On the contrary, most of the evoked outflow (15–30 min) was [^{3}H]ACh (78·7 ± 2·5%) (Fig. 3). The sum of labelled choline plus ACh (15–30 min) accounted for 88·9 ± 8·5% of total released radioactivity.

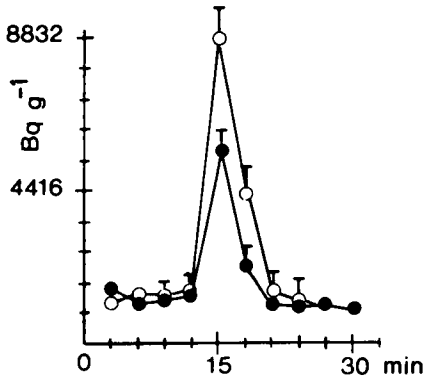

FIG. 2. ^3H release induced by stimulation (2 Hz, 360 shocks) in the absence of physostigmine in strips preloaded at 0·2 Hz (●) and at 0·8 Hz (○). Radioactivity (Bq) per gram of tissue. Means ± s.e.m. from at least four experiments.

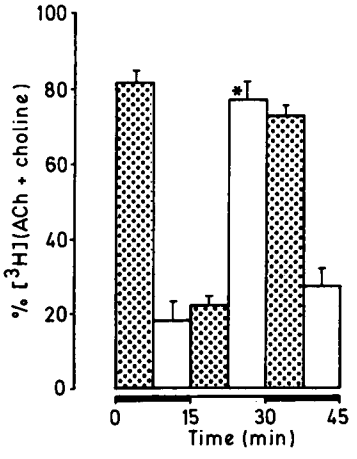

FIG. 3. Outflow of [^3H]ACh (open histogram) and of [^3H]choline (dotted histogram) in the superfusate of the rat urinary bladder during 0 to 15 min (spontaneous outflow), 15 to 30 min (stimulation-evoked outflow) and 30 to 45 min (spontaneous outflow) in the presence of 100 nM physostigmine. Percentage of [^3H]ACh plus choline. Means ± s.e.m. from four experiments. Significance of increase in evoked outflow of [^3H]ACh, $^*P < 0.01$.

Discussion

The results support the evidence that in the rat urinary bladder [^3H]ACh formation depends upon the loading frequency used (Fig. 1). Whereas the amount of [^3H]choline did not seem to be affected, increasing frequencies during the loading period produced an increased content of [^3H]ACh that was found to be enhanced by 70% at 0·8 Hz compared with 0·2 Hz. This is in agreement with results obtained in similar experimental conditions in the myenteric plexus preparation (Vizi et al 1984).

Higher frequencies (1·6 and 3·2 Hz) failed, not only to increase the [^3H]ACh storage, but rather they decreased it. Such a pattern parallels that reported in myenteric plexus (Gilbert et al 1973).

As far as [^3H]ACh content after 120 min washout is concerned, the difference between higher and lower frequencies of loading was some 35–40%. A similar result was obtained in myenteric plexus by Szerb (1976).

The data concerning the electrically induced outflow show that the higher the tissue content of [^3H]ACh, the higher was the amount of ^3H released when the same number of impulses was applied. Most of the released ^3H was [^3H]ACh. The separation of tritiated compounds released from strips preloaded with [^3H]choline at 0·8 Hz was 78·7 [^3H]ACh when ACh esterases were partially inhibited. The ACh/choline ratio was inverted during the resting outflow. A similar pattern for [^3H]ACh was found in the outflow of the strips loaded at 0·2 Hz (D'Agostino et al 1986). Evidence that the strip releases prevailingly ACh during stimulation is the fact that the tissue content assayed after stimulation was much decreased for ACh but not for choline (Table 1). Such a decrease, in relation to the higher induced outflow of [^3H]ACh, was more evident in tissue loaded at 0·8 Hz. The reduction of ^3H release in the presence of physostigmine is not surprising because the inhibitory feed-back mechanism via presynaptic muscarinic receptors is enhanced by drugs inhibiting ACh esterases (D'Agostino et al 1986).

To study the presynaptic potency of both muscarinic agonists and antagonists, [^3H]ACh outflow has to be evoked several times (Kilbinger & Wessler 1980; Kilbinger et al 1984) resulting in the depletion of [^3H]ACh storage. As both the decline of labelled stores during stimulation experiments and the presynaptic inhibitory action of drugs markedly affect the amount of the released neurotransmitter, the present research indicates a suitable method of increasing the formation and following the release of [^3H]ACh during electrical stimulation in the rat urinary bladder strip.

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References

Antonelli, L. A., Beani, L., Bianchi, C., Pedata, F., Pepeu, G. (1981) Br. J. Pharmacol. 72: 148–149P

Barker, L. A. (1976) Life Sci. 18: 725–732

Barker, L. A., Dowdall, M. J., Whittaker, V. P. (1972) Biochem. J. 130: 1063–1080

Collier, B., MacIntosh, F. C. (1969) Can. J. Physiol. Pharmacol. 47: 127–135

D'Agostino, G., Kilbinger, H., Chiari, M. C., Grana, E. (1986) J. Pharmacol. Exp. Ther. 239: 522–528

Gilbert, J. C., Hutchinson, M., Kosterlitz, H. W. (1973) Br. J. Pharmacol. 49: 166–167P

Grana, E., Lucchelli, A., Zonta, F., Santagostino Barbone, M. G., D'Agostino, G. (1986) Naunyn-Schmiedeberg's Arch. Pharmacol. 332: 213–218

Kilbinger, H., Wessler, I. (1980) Ibid. 314: 259–266

Kilbinger, H., Halim, S., Lambrecht, G., Weiler, W., Wessler, I. (1984) Eur. J. Pharmacol. 103: 313–320

Jope, R. S. (1979) Brain Res. Rev. 1: 313–344

Potter, L. T. (1970) J. Physiol. 206: 145–166

Szerb, J. C. (1975) Can. J. Physiol. Pharmacol. 53: 566–574

Szerb, J. C. (1976) Ibid. 54: 12–22

Vaca, K., Pilar, G. (1979) J. Gen. Physiol. 73: 605–628

Vizi, E. S., Ono, K., Adam-Vizi, V., Duncalf, D., Foldes, F. F. (1984) J. Pharmacol. Exp. Ther. 230: 493–499

Weiler, M. H., Jope, R. S., Jenden, D. J. (1978) J. Neurochem. 31: 789–796