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The Release of Acetylated Choline Analogues by a Sympathetic Ganglion

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SUMMARY

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The present experiments tested the uptake, metabolism, and release of choline and two choline analogues, (2-hydroxyethyl)-*N*-ethyl-*N*,*N*-dimethylammonium (monoethylcholine) and *N*-hydroxyethyl-*N*-methylpyrrolidinium (pyrrolcholine), by the cat superior cervical ganglion. Both analogues were acetylated *in situ*, as was choline, and the acetylated derivatives, but not the parent compounds, were released upon subsequent preganglionic nerve stimulation. Like acetylcholine, the release of acetylmonoethylcholine and of acetylpyrrolcholine by nerve impulses required the presence of Ca^{++}. It is concluded that these choline analogues form cholinergic false transmitters. Acetylmonoethylcholine and acetylpyrrolcholine were substrates for acetylcholinesterase. Previous studies showed that acetylmonoethylcholine is less active than acetylcholine as a cholinergic agonist; the present study showed this also for acetylpyrrolcholine. Thus either false transmitter would reduce the safety factor for transmission at cholinergic synapses.

Studies with isolated choline acetyltransferase (EC 2.3.1.6) have demonstrated that certain analogues of choline can serve as substrates for the enzyme; however, in all cases choline is the preferred substrate (1-6). Two analogues of choline, monoethylcholine and *N*-hydroxyethylpyrrolidinium methiodide (pyrrolcholine), have been shown to be accumulated and acetylated by rat brain synaptosomes as efficiently as is choline (7). These findings suggested that monoethylcholine and pyrrolcholine, like triethylcholine (8), might form a cholinergic false transmitter, and the present experiments tested this by measuring the uptake, metabolism, and release of these analogues by the preganglionic nerve endings of the superior cervical ganglion.

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Cats were anesthetized with chloralose (80 mg/kg), and the right superior cervical ganglion was perfused (9, 10) with Krebs solution which contained eserine (NaCl, 120 mM; KCl, 4.6 mM; $CaCl_2$, 2.4 mM; $MgSO_4 \cdot 7H_2O$, 1.2 mM; $NaHCO_3$, 25 mM; glucose, 10 mM; eserine sulfate, 0.03 mM)

and which was equilibrated with 5% CO_2 in O_2 so that its pH at 37° was 7.3. In each experiment the ganglion was perfused (0.3–0.4 ml/min) with eserine-Krebs solution for 15 min, and then for 60 min with the same medium containing [^{3}H]choline, [^{3}H]pyrrolcholine, or [^{3}H]monoethylcholine (1 μM, 906 mCi/mmole); during exposure to choline or to the choline analogue, the preganglionic sympathetic nerve was stimulated (20 Hz, 0.3 msec, supramaximal voltage). Perfusion was then switched back to eserine-Krebs solution, nerve stimulation was stopped, and after 20 min the venous effluent from the ganglion was collected in 2-min samples. The release of accumulated radioactive materials was tested by stimulating the preganglionic nerve (20 Hz) for 2 min. In some experiments release was tested during perfusion with a Ca^{++}-free medium which contained no added $CaCl_2$ and contained ethylenebis(oxyethylenenitrilo)tetraacetic acid (100 μM). Radioactivity in the samples of ganglion effluent collected was determined by liquid scintillation spectrometry. In experiments with monoethylcholine or with pyrrolcholine, the identity of the radioactive material collected from the ganglion was determined by thin-layer chromatography on polyethyleneimine-impregnated cellulose plates, after the samples had been extracted by heptanone containing tetraphenylboron, and the bases recovered into HCl. In experiments with choline, acetylcholine was separated from unchanged choline by the method described before for distinguishing acetyltriethylcholine from triethylcholine (8): the samples were incubated with choline kinase to convert the choline, but not the acetylcholine, to phosphorylcholine, and the acetylcholine, but not the phosphorylcholine, was then extracted into heptanone containing tetraphenylboron. Ganglia were extracted with trichloracetic acid (10%, w/v), the trichloracetic acid was extracted with ether, and the aqueous extract was processed in the same way as the ganglion effluent; phosphorylated products – phosphorylcholine, phosphorylmonoethylcholine, or phosphorylpyrrolcholine – were not extracted by the heptanone containing tetraphenylboron. [^{3}H]Choline was bought from New England Nuclear, and [^{3}H]monoethylcholine and [^{3}H]pyrrolcholine were prepared as described before (7). Acetylated choline analogues were prepared by treating them with acetyl chloride, or by reaction with acetic anhydride; phosphorylated choline analogues were prepared by incubating the analogues with choline kinase (prepared from yeast). [^{14}C]Phosphorylcholine was obtained from New England Nuclear.

Three experiments tested the uptake and release of radioactivity by ganglia that had been perfused with [^{3}H]pyrrolcholine, and the result of a typical experiment is illustrated in Fig. 1a. Preganglionic nerve stimulation clearly released radioactive material, and this was shown in all experiments (total of eight tests). The radioactivity collected from ganglia at rest (preganglionic nerve not stimulated) was identified by thin-layer chromatography as unchanged pyrrolcholine; there was only a small amount of acetyl-

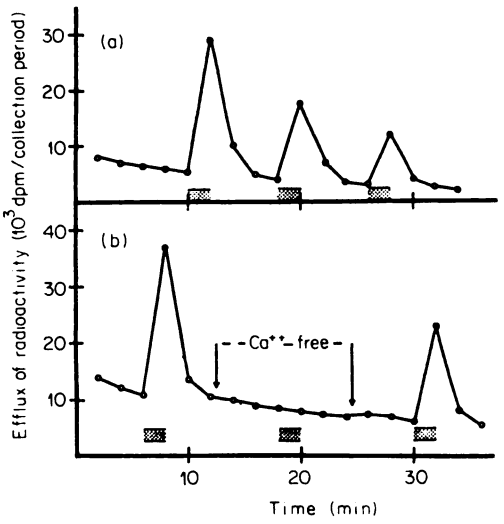

FIG. 1. *Release of radioactivity by preganglionic nerve stimulation from ganglia exposed to [^{3}H]pyrrolcholine*

Cat superior cervical ganglia were perfused with Krebs solution containing eserine and [^{3}H]pyrrolcholine. They were then perfused with eserine-Krebs solution, and during the times indicated by the stippled bars the preganglionic nerve was stimulated for 2 min at 20 Hz. In Fig. 1b perfusion was switched to Ca^{++}-free medium during the time indicated by the arrows.

pyrrolcholine in these samples (Fig. 2a). However, during preganglionic nerve stimulation, acetylpyrrolcholine was released; the ganglion effluent collected during nerve stimulation contained both unchanged pyrrolcholine and acetylpyrrolcholine (Fig. 2b). In five tests the amount of acetylpyrrolcholine identified in samples collected during nerve stimulation accounted for 99 ± 2% (mean ± SE) of the extra radioactivity released during nerve stimulation. Thus preganglionic nerve stimulation released acetylpyrrolcholine, but not unchanged pyrrolcholine. Two other experiments demonstrated that the release of acetylpyrrolcholine by nerve stimulation depended upon the presence of calcium ions; nerve stimulation released radioactivity before and after, but not during, perfusion with Ca^{++}-free medium (Fig. 1b), and in this respect the release of acetylpyrrolcholine resembles that of acetylcholine (11, 12).

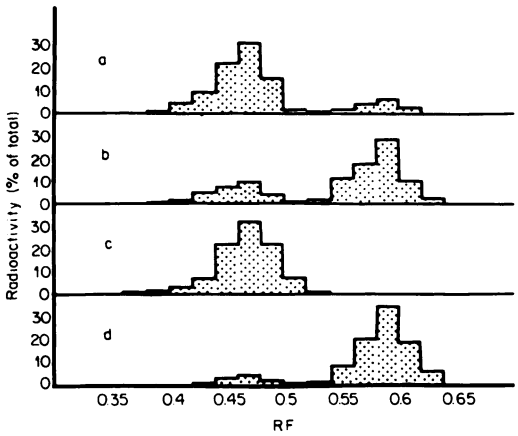

FIG. 2. *Thin-layer chromatography of pyrrolcholine (c), acetylpyrrolcholine (d), and ganglion effluent collected at rest (a) or during nerve stimulation (b)*

Samples (400 μl), in Krebs solution, were extracted by heptanone containing tetraphenylboron (10 mg/ml); the organic phase was shaken with HCl (1 N), and this acid phase was evaporated to dryness. The residue was dissolved in H_2O (15 μl), and 12 μl were applied to a polyethyleneimine plate (Macherey-Nagel) which was developed in a mixture of butanol, ethanol, acetic acid, and H_2O (8:2:1:3). The ganglion effluents were collected from a cat superior cervical ganglion treated in the same way as that in Fig. 1a.

Similar results were obtained in experiments that used [^{3}H]monoethylcholine. Seven tests in three experiments (e.g., Fig. 3a) showed that preganglionic nerve stimulation released radioactivity from ganglia that had been perfused with [^{3}H]monoethylcholine. Thin-layer chromatography demonstrated that the spontaneous efflux of radioactivity was almost all due to unchanged monoethylcholine, and that nerve stimulation released acetylmonoethylcholine (Fig. 4); in five tests the amount of acetylmonoethylcholine released upon stimulation accounted for 100 ± 1% of the extra radioactivity collected during nerve stimulation. Two other experiments showed that the release of acetylmonoethylcholine was Ca^{++}-dependent (Fig. 3b).

At the end of all these experiments, the perfused ganglia were removed and extracted, and radioactive products in the extract were measured. The sum of the amount of the acetyl derivative released from and retained in ganglia that had been perfused with pyrrolcholine (50 ± 6 pmoles) was similar to that of ganglia that had been perfused with monoethylcholine (63 ± 12 pmoles). In similar experiments that measured the amount of acetylcholine synthesized from [^{3}H]choline, 75 ± 7 pmoles of [^{3}H]acetylcholine were formed. Thus, under the present experimental conditions, choline and the two choline analogues appear to be rather similar as substrates for choline acetyltransferase; the different acetylation of the substrates that can be shown *in vitro* using isolated choline acetyltransferase (7) is much less clear *in situ*.

In the present experiments acetylcholine and the two acetylated choline analogues also appeared to be equally available for release by nerve impulses: each 2-min period of stimulation released 19 ± 2% of the tissue content of acetylcholine, 21 ± 1% of the tissue content of acetylpyrrolcholine, and 20 ± 2% of the tissue content of acetylmonoethylcholine.

The present experiments confirm the previous observation (7) that pyrrolcholine and monoethylcholine can be acetylated by neuronal tissue, and they demonstrate

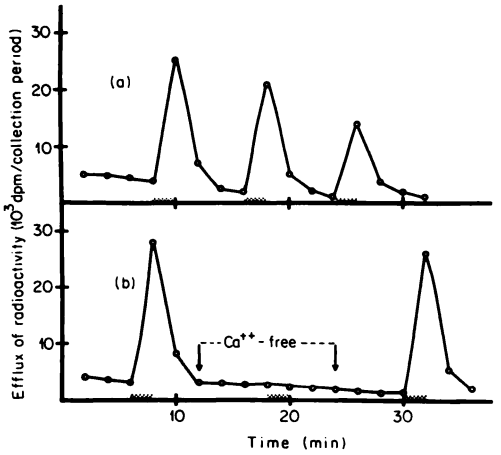

FIG. 3. *Release of radioactivity by preganglionic nerve stimulatiuon from ganglia exposed to [^{3}H]monoethylcholine*

Cat superior cervical ganglia were perfused with Krebs solution containing eserine and [^{3}H]monoethylcholine. They were then perfused with eserine-Krebs solution, and the preganglionic nerve was stimulated (20 Hz) for 2 min during the times indicated by the stippled bars. In Fig. 3b perfusion was switched to Ca^{++}-free medium during the time indicated by the arrows.

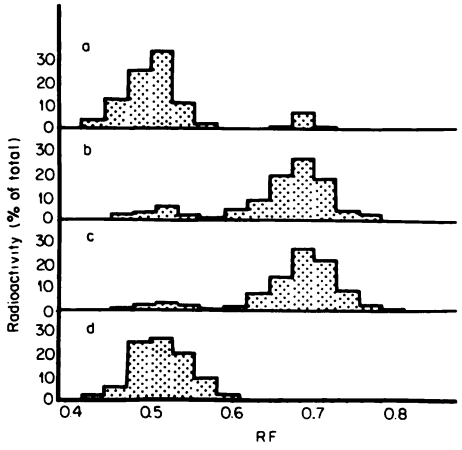

FIG. 4. *Thin-layer chromatography of monoethylcholine (c), acetylmonoethylcholine (d), and ganglion effluent collected at rest (a) or during nerve stimulation (b)*

Samples were treated in the same way as those used in Fig. 2. The ganglion effluents were collected from a cat superior cervical ganglion treated in the same way as that in Fig. 3a.

that these acetylated compounds are released from cholinergic nerve terminals by nerve impulses. Acetylpyrrolcholine and acetylmonoethylcholine are therefore cholinergic false transmitters, and, like acetylcholine (11, 12), they are released by a Ca^{++}-dependent process.

Acetylmonoethylcholine is about 2–8 times less active than acetylcholine on muscarinic receptors (13, 14) and about 5 times less active on nicotinic receptors (13). In the present study acetylcholine and acetylpyrrolcholine were compared as agonists on isolated guinea pig ileum and on frog rectus abdominis preparations. Cumulative dose-response curves were generated (Fig. 5), and the ED_{50} values were estimated graphically. The potency of acetylpyrrolcholine relative to acetylcholine was estimated from the concentrations required to produce a response that was 50% of the maximal response obtained with acetylcholine. Acetylpyrrolcholine was 29 ± 5.2 times less active than acetylcholine on the ileum preparation, and similar results have been reported by others (15). In the present experiments, unlike those reported by Cho *et al.* (15), the maximal response produced by acetylpyrrolcholine was less (83 ± 7%) than that produced by

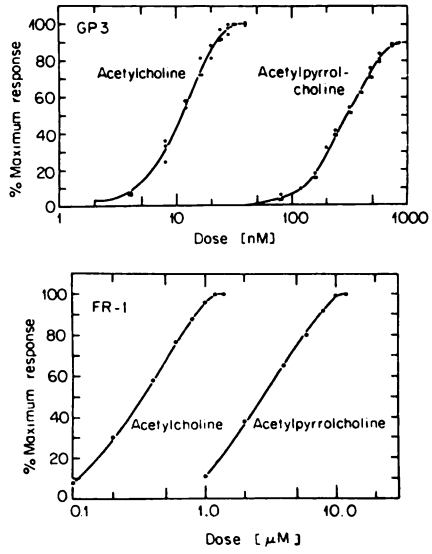

FIG. 5. *Dose-respnse curves of guinea pig ileum (above) or frog rectus abdominis preparations (below) to acetylcholine and to acetylpyrrolcholine*

The rectus abdominis was used in the presence of eserine (10 μM), and the ileum was used without an anticholinesterase agent.

acetylcholine. The study on the nicotinic receptors of the frog rectus abdominis preparation showed that acetylcholine was 11.6 ± 0.6 times more active than acetylpyrrolcholine; the maximal response of this tissue was similar to either compound.

Acetylcholine, acetylmonoethylcholine, and acetylpyrrolcholine (1–2 μM) were also studied as substrates for rat brain cholinesterases by the radiometric procedure described previously (16, 17); this measures the amount of acetate generated by the hydrolysis. The substrates were prepared by allowing the appropriate tertiary amino alcohol to react with [^{3}H]acetic anhydride (500 mCi/mmole, Amersham/Searle) in acetone, followed by treatment with excess methyl iodide; the esters were purified by ion-exchange chromatography. Both acetylmonoethylcholine and acetylpyrrolcholine were substrates for rat brain cholinesterases: acetylmonoethylcholine was found to be hydrolyzed as rapidly as acetylcholine (see also ref. 13), and acetylpyrrolcholine was hydrolyzed 70% as rapidly as acetylcholine.

Thus the present experiments show that monoethylcholine and pyrrolcholine are substrates for choline acetyltransferase at preganglionic nerve endings, and that the acetylated derivatives are released as false transmitters upon nerve stimulation. Both false transmitters are pharmacologically less active than acetylcholine. The safety factor for transmission at cholinergic synapses varies; it was shown to be between 4 and 12 for skeletal muscle (18) and was estimated to be about 4 at a sympathetic ganglion (19). Since acetylmonoethylcholine is 5 times and acetylpyrrolcholine is 12 times less active than acetylcholine on nicotinic receptors, and since both false transmitters are hydrolyzed by cholinesterases, complete replacement of transmitter by acetylmonoethylcholine or by acetylpyrrolcholine would reduce or abolish this safety factor.

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