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SHORT COMMUNICATION

Verification of rat brain acetylcholine antidepletion by morphine using gas chromatography

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UNTIL RECENTLY bioassays of various types have usually been used to measure acetylcholine (ACh). Such techniques, while very sensitive, have been criticized[1,2] as inherently nonspecific. Doubt has existed about the true identity of the substance measured. If drugs are used in the experiment, they are likely to appear in the extract and exert their own effect. Of the many recently developed chemical methods to measure tissue ACh, gas chromatography appears the most promising. It has been possible by this method and mass spectrometry[3,4] to demonstrate that the substance being measured in rat brain is indeed ACh.

We have recently reported[5] that morphine and related narcotic agonists have a dramatic ACh antidepleting action in doses that do not affect steady-state brain levels. This effect of morphine was observed in rats using doses of 1–10 mg/kg, i.p., after intraventricular (ivt.) hemicholinium-3 (HC-3) and acetylseco hemicholinium-3 (acetylseco HC-3) or i.p. scopolamine. This phenomenon was reversed by nalorphine and naloxone. In these experiments, the frog rectus abdominus muscle was employed as the means of bioassay to determine brain ACh after acid alcohol extraction.[6] It was most important to further validate our own data by a more specific chemical method. Morphine was used as a representative of the narcotic agonists to be studied.

Young male albino Holtzman rats from 20 to 30 days of age were used as described previously.[5] HC-3 was given ivt. in a dose of 20 μg under diethyl ether–air anesthesia. Narcotics were given i.p. immediately after HC-3 and termination of anesthesia. Animals were sacrificed by guillotine 30 min later. The brain minus the cerebellum was removed and assayed for ACh. In our previous work, as described above, the frog rectus assay of ACh was used. In the present study, the method of Schmidt *et al.*[7] was used to extract and determine ACh by gas chromatography. This method has the advantage of demethylation and chromatography occurring in one step. The whole brain minus the cerebellum was weighed and homogenized in acetonitrile containing 2% trichloroacetic acid (1·0 ml/0·1 g tissue) to which 25 nmoles of propinoylcholine iodide was added as internal standard. After centrifugation, an equal volume of water was added to the supernatant and extracted twice with equal volumes of diethyl ether. The ether layers were removed and residual ether was blown off by a stream of nitrogen. Two 2-ml samples were taken and diluted to 4 ml to which 5–6 μg of tetramethylammonium iodide was added as coprecipitant. The quaternary compounds were precipitated by addition of 0·15 ml iodide–iodine (1 g KI, 0·9 g I_2/5 ml). After centrifugation, the precipitate was redissolved in acetonitrile and placed in a Nuclear Chicago pyrolyzer. A Hewlett Packard 5750 gas chromatograph with 8 ft by $\frac{1}{4}$ in. stainless steel columns packed with 20% carbowax 6000 on 60/80 mesh Chromosorb W (HMDS) was used for the analysis. Gas flows were N_2, 75 ml/min, air, 1·5 l./min and H_2, 38 ml/min. Standard solutions of ACh and propionylcholine were assayed simultaneously with the tissues. Extracts were also treated with alkali as further evidence that the peaks measured were ACh and propionylcholine.

The results of gas chromatography and frog rectus bioassay are shown in Table 1.

TABLE 1. COMPARISON OF RESULTS BY BIOASSAY AND GAS CHROMATOGRAPHY

Treatment	Frog rectus bioassay		Gas chromatography assay	
	N*	Brain ACh (mean ± S.E. nmoles/g)	N	Brain ACh (mean ± S.E. nmoles/g)
Controls	8	18·1 ± 1·3	15	18·4 ± 0·7
HC-3 (20 μg)	9	10·1 ± 1·0†	10	9·3 ± 0·6†
Morphine (10 mg/kg)	8	19·1 ± 0·9	11	19·4 ± 0·8
Morphine (10 mg/kg) + HC-3 (20 μg)	12	17·2 ± 1·0	11	18·0 ± 0·6

* N = number of animals per group.

† $P < 0·01$ significantly different from control using a group comparison t test.

As can be noted, there is no significant difference in the results obtained by either method. Gas chromatography, however, further verifies the effect we have found with morphine. Our control data are in accord with that obtained by this method.[7] There is another method of gas chromatography of ACh recently reported which produces higher control values.[4] It remains for future research to determine why there is a discrepancy in the steady-state levels of brain ACh as measured by two rather similar gas chromatographic techniques. Nevertheless, this does not alter our present findings regarding the effect of morphine.

In conclusion, the ACh antidepletion action of morphine has been validated using two different methods of assay. The results obtained using the frog rectus bioassay and the gas chromatographic assay are remarkably similar.

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