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EFFECT OF COLLAGENASE PRETREATMENT ON CHOLINE AND ACETYLCHOLINE RELEASE FROM SLICES OF BOVINE SUPERIOR CERVICAL SYMPATHETIC GANGLIA

G. Sgaragli and J. R. Cooper

Department of Pharmacology

Yale University School of Medicine

New Haven, Connecticut 06510 U.S.A.

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A method has recently been reported for the preparation of relatively pure cholinergic synaptosomes from bovine superior cervical sympathetic ganglia (1, 2, 3). After disruption of the tissue by incubation with collagenase, homogenization and differential centrifugation yields a crude synaptosomal fraction (P_2) which can be further purified by centrifugation on density gradients. Electron microscopy indicated that the P_2 fraction so prepared was rich in synaptosomes and biochemical evidence indicated that the fraction retained most of the acetylcholine (ACh) and choline acetyl transferase (EC 2.3.1.6) activity of the homogenate. This preparation appeared to be suitable for performing biochemical and pharmacological studies. However, we observed that synaptosomes prepared by this procedure were unable to discharge ACh in the presence of a high extracellular potassium concentration. We briefly report here the evidence that the loss of this property by nerve endings is due to the preliminary treatment of tissues slices with collagenase.

Slices (total weight, 300 mg) of bovine superior cervical ganglia tissue were prepared as previously described (1) and incubated at 37°C for 1 hr in 5 ml of a modified Krebs-Ringer oxygenated buffer pH 7.0, of the following composition (mM): NaCl, 138; KCl, 5.6; $CaCl_2$, 0.22; $MgCl_2$, 1.0; CH_3COONa, 1.0; Na phosphate, 1.0; $NaHCO_3$, 12.0; glucose, 10.0. The incubation medium also contained 50 μM [^{14}-C-Me]-choline (5.66 μCi, Amersham-Searle), 5 mg of bovine serum albumin (Sigma Chem. Co., St. Louis, Mo.) with or without 15 mg of crude colla-

genase (EC 3.4.4.19, Type III, 190 U/mg protein, Worthington Biochem. Co., Freehold, N.J.) and 5 mg soybean trypsin inhibitor (Type I-S, Sigma Chem. Co.). During incubation the solution was gassed with 95% O_2-5% CO_2. Tissue was then removed, washed three times with 15 ml of fresh, prewarmed Krebs-Ringer buffer and finally resuspended in 5 ml of prewarmed incubation medium as above except that the medium now contained 2.2 mM Ca^{++} and 0.2 mM eserine sulfate. Either 0.4 ml of 700 mM Tris buffer pH 7.0 or 700 mM KCl were then added and incubation at 37° was initiated. After 20 or 40 min. incubation, 1 ml of the medium was removed and diluted with 3 ml 1N formic acid-acetone (1:3 = v:v). These extracts were then analyzed for [^{14}C] choline and [^{14}C] ACh by a slight modification of the paper high-voltage electrophoresis procedure as described by Hildebrand et al. (4). Aliquots of 50 to 100 µl of the formic acid-acetone extracts, together with 100 nanomoles of cold ACh and choline were applied to paper discs which were then placed on a sheet of Whatman 3 MM paper moistened with a buffer of 0.47 M formic acid-1.4 M acetic acid, pH 1.9. Flat plate electrophoresis was carried out at a constant voltage of 3,800 volts for 80 min, during which time the current increased from 90 to 105 amperes. The paper was then dried and exposed to I_2 vapor in order to reveal carrier choline and acetylcholine. The area containing the choline and ACh spots was cut in small strips and put in vials containing 1.8 ml water. Radioactivity was measured after addition of 20 ml Aquasol scintillation liquid.

As summarized in Table 1, in untreated slices in a "resting condition" (low potassium concentration), a leakage of radioactive choline and ACh occurred. In the presence of high potassium concentration, the release of both choline and ACh was stimulated, the former about 2 fold and the latter about 7 fold after a 40 min incubation.

However, in ganglion slices that were pretreated with collagenase a marked inhibition of K^+-induced release of both choline and ACh was observed. It is curious that with both choline and ACh, the inhibition by collagenase after 40 min incubation is slightly less than that at 20 min, suggesting that the effect

Table 1

Effect of collagenase pretreatment on [^{14}C]-choline and [^{14}C]-acetylcholine released into the medium from slices of superior cervical ganglia.

TIME* (min)		PRETREATMENTS: NONE				COLLAGENASE				COLLAGENASE + SBTI			
		NK		HK		NK		HK	% inhibition‡	NK		HK	% inhibition‡
20	Choline	9,450 ±	1,944	21,705 ±	705	3,024 ±	2,268	9,429 ±	519	47.7	2,053 ±	1,673	11,394 ± 2,916	23.8
40		15,147 ±	2,025	32,830 ±	7,562	11,016 ±	4,104	22,767 ±	2,612	33.5	10,110 ±	3,198	24,300 ± 162	19.8
20	ACh	860 ±	760	12,900 ±	590	432 ±	270	1,377 ±	351	92.1	1,242 ±	378	3,483 ± 27	89.7
40		4,347 ±	27	30,997 ±	1,513	729 ±	567	9,909 ±	1,701	63.1	2,619 ±	837	9,153 ± 3,591	75.5

Slices were preincubated at 37°C for 1 hr in oxygenated Krebs-Ringer buffer containing 50 μM [^{14}C]-choline (5.66 μCi with or without 2850 units of collagenase and 5 mg of soybean trypsin inhibitor (SBTI). After this preincubation, slices were washed and incubated at 37°C in oxygenated Krebs-Ringer buffer containing 0.2 mM eserine sulfate and 2.2 mM Ca^{++}. The K^{+} concentration was either 5.6 mM (NK) or 57.45 mM (HK). Aliquots of the medium were removed at 20 and 40 min and assayed as described in the text. Values (average ± S E M) were derived from 3 or 4 experiments and represent the total CPM of the medium in the form of either choline or ACh.

‡ Percent inhibition is calculated as: $\frac{\Delta^{o}(HK-NK) - \Delta^{1}(HK-NK)}{\Delta^{o}(HK-NK)} \times 100$ where Δ^{o} refers to no treatment and Δ^{1} refers to the treatment with either collagenase or collagenase + SBTI.

* Incubation time after pretreatment.

Table 2

Effect of collagenase pretreatment on [^{14}C]-acetylcholine content in slices of superior cervical ganglia.

TIME* (min)	PRETREATMENTS: NONE NK	NONE HK	COLLAGENASE NK	COLLAGENASE HK
0	42,380 ± 2,840	43,180 ± 3,140	40,850 ± 1,700	41,670 ± 2,600
40	35,650 ± 1,720	11,050 ± 2,320	39,500 ± 1,350	30,250 ± 2,450

Experimental conditions are the same as reported in Table 1. Slices were homogenized with formic acid-acetone solution, and after centrifugation an aliquot of the supernatant solution was spotted on paper and subjected to high voltage electrophoresis as described in the text. Values (average ± S E M) were derived from 3 or 4 experiments and represent the total radioactive ACh (CPM) in the formic acid-acetone extract.

* Incubation time after pretreatment.

of collagenase may be diminished with increased incubation time. Since crude collagenase contains trypsin-like activity, soybean trypsin inhibitor was added in one set of experiments with similar results to that obtained with crude collagenase. This indication that the inhibition of release of ACh is due to collagenase itself is further supported by some preliminary experiments in which purified collagenase was employed. Again the results were identical with previous experiments which utilized the crude preparation. Also of interest are other preliminary findings in our laboratory that synaptosomes prepared from collagenase-treated slices also fail to exhibit a K^+-induced release of ACh compared to synaptosomes prepared from untreated slices. Finally, it should be noted that this effect of collagenase appears to be on the release mechanism only since the total radioactivity of acetylcholine in the slices after the preincubation period was the same, regardless of the presence or absence of collagenase in the incubation medium (Table 2).

Betz and Sakmann (5) recently observed that the application of collagenase to a frog neuromuscular junction produced a loss of acetylcholinesterase, a partial depletion of "synaptic cleft material" and electrophysiological effects typically seen with acetylcholinesterase inhibitors. Our experiments indicate an effect of collagenase on the release of ACh. This effect appears to correlate with an effect on neurostenin, (Berl, personal communication), an actomyosin-like protein found in nervous tissue that is hypothesized to be implicated in transmitter release (6).

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