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Choline oxidase chemiluminèscent assay, after removal of eserine from medium, of acetylcholine released in vitro from brain slices *

M.A.M. Prado, T. Moraes-Santos, R.N. Freitas, M.A.R. Silva and M.V. Gomez

Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas UFMG, Belo Horizonte – Minas Gerais (Brazil)

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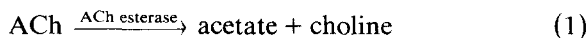
A chemiluminescent method has been used recently for the determination of acetylcholine with limitations such as the presence of a cholinesterase inhibitor in the incubation medium, which is indispensable for the study of acetylcholine release by various agents. A modified procedure is presented in which the cholinesterase inhibitor eserine (physostigmine) is extracted from the medium. The results showed complete recovery when labelled acetylcholine was used. This modified procedure was used to determine the release of acetylcholine evoked by tityustoxin and ouabain. The results were comparable to those obtained by bioassay using a strip of guinea pig ileum.

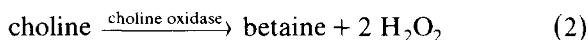
Introduction

Acetylcholine (ACh) was the first neurotransmitter to be documented. ACh can be measured by several methods, such as pyrolysis, gas chromatography-mass fragmentography (Jenden et al., 1973), biological methods using guinea pig ileum or leech muscle assay (MacIntosh and Perry, 1950), radiometric enzymatic determination (Goldberg and McCannan, 1973), and HPLC with electrochemical detection (Potter et al., 1983).

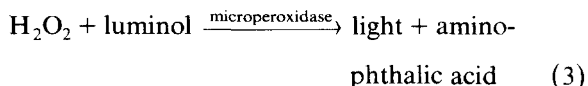
Mainly due to its specificity and sensitivity the chemiluminescent procedure for ACh assay (Israel and Lesbats, 1981a) has been used intensively for the determination of this neurotransmitter during continuous release. The method is based on the following 3 coupled enzyme reactions, whereas the choline assay involves only reactions (2) and (3):



$$\text{ACh} \xrightarrow{\text{ACh esterase}} \text{acetate} + \text{choline} \quad (1)$$



$$\text{choline} \xrightarrow{\text{choline oxidase}} \text{betaine} + 2\ H_2O_2 \quad (2)$$



$$H_2O_2 + \text{luminol} \xrightarrow{\text{microperoxidase}} \text{light} + \text{amino-phthalic acid} \quad (3)$$

If the concentrations of all the reactants except ACh are kept constant then the light emitted in reaction (3) is directly proportional to the concentration of ACh. This procedure permits the continuous monitoring of ACh release (Israel and Lesbats,1981b) and analysis of ACh from mammalian tissue extracts treated with oxidants (Israel and Lesbats, 1982). An adaptation of the method using periodate precipitation made it possible to assay endogenous ACh released from rat hemidiaphragm (Haggblad et al., 1983), but with a low recovery rate of ACh.

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Correspondence: Dr. Marcus Vinicius Gomez, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas-UFMG, Caixa Postal, 2486 Belo Horizonte – Minas Gerais, Brazil.

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A modification of the method that permits the assay of ACh in incubation fluids containing the cholinesterase inhibitor eserine (physostigmine) has been developed. The assay is based on the solubility of eserine free base in benzene. Eserine free base, being an apolar compound, is thus easily extracted by several washes with benzene, while ACh, which is a polar substance, remains in the washed extract.

The present paper describes this modification, which allows the determination of ACh in the presence of the cholinesterase inhibitor, eserine free base. Also, a typical application of the method for measurement of the ACh released from rat brain cortical slices stimulated by tityustoxin and ouabain is shown.

Material and methods

Acetylcholine chloride, eserine free base, eserine hemisulphate, ouabain, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), choline oxidase, acetylcholinesterase (*Electrophorus electricus*) and microperoxidase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $[^{14}C]$ACh ($[^{14}C]$methyl chloride; 54 mCi/mol) was from Amersham/Searle. All other chemicals were of analytical grade. Tityustoxin was purified according to a method previously described (Gomez et al., 1973).

Slices from brain cortex of Wistar rats (2 months old, 200–250 g) were cut on a McIlwain tissue slicer, and incubated for 30 min in 3.0 ml incubation medium containing (in mmol/l): NaCl 136; KCl 2.7; $CaCl_2$ 1.35; NaH_2PO_4 0.36; $NaHCO_3$ 12; glucose 5.5; and eserine free base (physostigmine) 0.01, brought to pH 7.4 with 1 mol/l HCl. The presence of eserine in the medium protects acetylcholine against hydrolytic action of cholinesterase during the incubation. The slices were separated from the supernatant containing released ACh by centrifugation at $2500 \times g$ for 10 min. In order to eliminate protein interference, the supernatant was treated with 5% trichloroacetic acid and centrifuged at $10,000 \times g$ for 10 min. Trichloroacetic acid was removed by washing 4 times with diethyl ether. A 1.0-ml aliquot of washed extract, practically free of acid (pH 5.0–5.5), was then washed 6 times with 2 ml benzene. Traces of this solvent were removed by evaporation using an N-Evap model III apparatus (Organomation, Shrewsbury, MA). 100 μl of a 0.5% sodium metaperiodate solution was added to each sample to oxidize interfering substances present in the extracted solution (Israel and Lesbats, 1982). For the non-extracted sample, trichloroacetic acid was removed by ether washing, and ACh analyzed by bioassay.

Chemiluminescent assay for ACh

The assay was performed in a dark chamber with 960 μl of a mixture containing 1.5 U choline oxidase, 20 μg microperoxidase, 10 μmol/l luminol and 67 mmol/l glycine buffer, pH 8.6. Ten or 20 μl of each sample of the benzene-washed solution were added to the above medium. The first light reaction, due mainly to choline oxidation, was observed and recorded. When a steady-state line was obtained, 1.6 U acetylcholinesterase in a volume of 20 μl was added. The choline formed by ACh hydrolysis was then measured. ACh was quantified by measuring the height of the peaks using ACh standards submitted to the same treatment as the sample. In addition, an aliquot of $[^{14}C]$acetylcholine was used to determine the recovery rate. The labelled compound (145,003 dpm) was added for a parallel run and extracted with benzene as described above. All radioactivity (146,453 dpm) was found in the water extract and measured in a liquid scintillation spectrophotometer (LKB, Rack Beta, 'Primo').

ACh bioassay

The assay was performed according to the procedure previously described (Gomez et al., 1973) using a strip of guinea pig ileum suspended in Tyrode solution containing 10 mg morphine, 5 μg neostigmine, and 1.0 μg promethazine (phenergan) per liter, as described by Paton (1957), but following the precautions suggested by Feldberg (1945). The term ACh, as used in the bioassay, refers to material producing an acetylcholine-like effect on the guinea pig ileum. The active material was destroyed by boiling at pH 10 for 15 min, and the contraction of guinea pig ileum induced by

this material was completely abolished by atropine.

Results and discussion

Fig. 1 shows the results of light emission by the chemiluminescent assay of 20 pmol ACh standard analyzed under different experimental conditions. The presence of 0.01 mmol/l eserine free base (peak 2) or 0.01 mmol/l eserine hemisulphate (peak 3) in the solution of ACh standards reduced light emission by 22 and 30%, respectively (compare peaks 1 with 2 and 3). This inhibition means a corresponding reduction in the value of ACh, and is caused by the presence of the cholinesterase inhibitor. Washing the eserine free base-containing ACh standard with benzene gives a light emission identical (about 12 mV; peak 5) to that obtained for the control ACh standard without cholinesterase inhibitor (peak 1). This means that benzene washing removed the inhibitor, or eserine free base, in the chemiluminescent ACh assay. The ACh standard in the solution containing 0.01 mmol/l eserine hemisulfate washed with benzene showed a light emission similar (peak 6) to that observed without benzene washing (peak 3). Thus washing with benzene removed eserine free base from incubation fluids, but was ineffective in removing the cholinesterase inhibitor in its polar

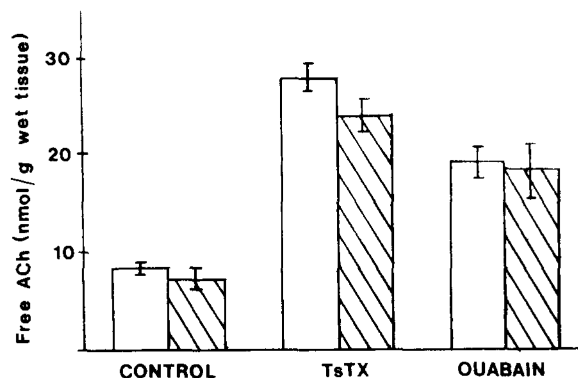

Fig. 2. Effects of 3 μmol/l tityustoxin and 10 μmol/l ouabain on the stimulated release of ACh from slices of rat cerebral cortex assayed by the modified chemiluminescent procedure (open bars) and bioassay method using guinea pig ileum (hatched bars). Incubation was carried out for 30 min at pH 7.4 in Tyrode solution containing 0.01 mmol/l eserine free base. The results of free ACh determinations are means ± SEM of 3 experiments. See text for other details.

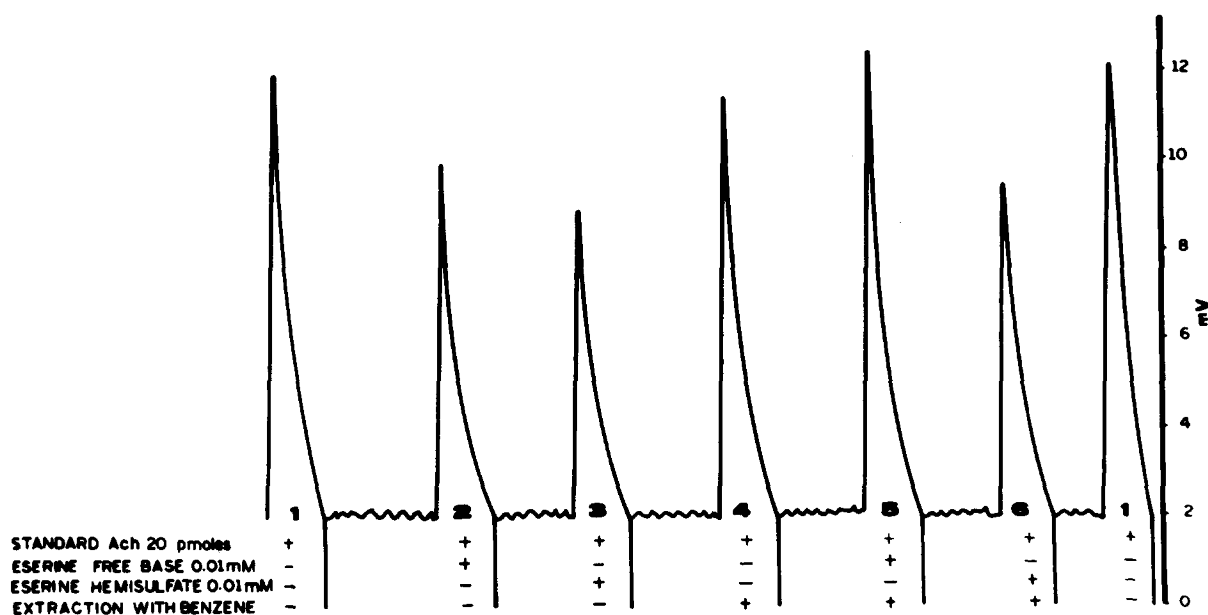

Fig. 1. Effects of eserine free base, eserine hemisulphate and extraction with benzene on light emission (mV) of 20 pmol ACh standard assayed by the chemiluminescent method. The height of the peaks represents the light emission at the experimental conditions shown.

form (eserine hemisulphate). Benzene-washing of the ACh standard solution without any of the cholinesterase inhibitors results in a light response similar (peak 4) to the control ACh standard (peak 1), i.e. about 12 mV. Thus, the benzene treatment does not interfere with the chemiluminescent ACh assay and is able to extract the eserine, which at the concentration used in the experiment, is soluble in the medium. In a parallel experiment, a sample of [*methyl*-^{14}C]acetylcholine submitted to the washing procedure with benzene showed that 100% of [^{14}C]ACh remains in the washed solution. In view of the fact that the inhibition of light emission caused by eserine free base totally disappeared after washing with benzene, no attempt was made to quantify the amount of cholinesterase inhibitor remaining in the washed extracts.

Fig. 2 shows the results of an experiment in which the method was applied to assay endogenous ACh released from rat brain cortical slices stimulated for 30 min with tityustoxin or ouabain (Gomez et al., 1973). In this experiment, ACh released into the incubating medium containing 0.01 mmol/l eserine free base was analyzed by the modified chemiluminescent method described here, as well as by a bioassay using guinea pig ileum. As can be seen, no statistical differences in the release of ACh were observed for either method. The present method is thus suitable for in vitro and in vivo determinations of ACh, even in the presence of the cholinesterase inhibitor, eserine free base. Besides a complete recovery of [^{14}C]ACh, the modified procedure showed results comparable to the biological assay. The method is, therefore, highly recommended for determining ACh, as opposed to the procedure which uses ACh precipitation by periodate which yields a very low recovery of around 30% (Haggblad et al., 1983).

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