ACETYLCHOLINE KINETICS IN HEALTHY HUMAN PLACENTA AT TERM

by

P. Brahmayya Sastry
M. Satyanarayana
and
K. R. Rajeswari

Introduction

Acetylcholine (ACh) is a natural constituent of many parts of the nervous system. It is not entirely unique in function to the cholinergic neurones alone but is widely distributed in the animal and in plant kingdom also. During the past 50 years, several tissues, organs and organisms without innervation, or denervated chronically, have been shown to contain one or more components of the ACh system identified in the nervous system.

It is now well established that ACh exists in human placental syncytiotrophoblast (Barnes, 1968 and Tuchmann-Duplessis *et al* 1972) and it has been investigated more extensively for ACh system for the reason that, it is easily available for experimentation. ACh activity was reported first in extracts of the human placenta (Chang and Gaddum, 1933), later in incubated placenta (Chang, 1935) and in perfused placenta (Chang, 1936 and Berkovich, 1950).

Incubation of the tissue slices or mince was one of the classical methods used by various investigators for the study of ACh kinetics. Beznak (1934) for the first time showed that "pressed juice" of frog's heart produced ACh. Chang (1935), demonstrated the ACh production in incubated placental mince.

For "Invitro" study of ACh turnover, the placental mince technique was adopted from methods described by Quastel *et al* (1936) and Mann *et al* (1938, 1939a and 1939b). Chang *et al* (1940 and 1942) and Berkovich, 1950 demonstrated that placenta "Invitro" incubated at 37°C to 39°C, released ACh into the incubation medium. Raghavan and Brahmayya Sastry, 1970a also studied, on incubated placental mince in bicarbonate-buffered medium, the influence of certain physiological factors on released and bound ACh.

Over the past several years, sporadic reports have indicated that the human placenta at term contains fairly good amount of ACh but the results are inconsistant. This confusion is partly due to the inclusion, in many studies, of specimens from gravidas in whom there was either a superadded pre-eclamptic toxaemia or intrauterine-fetal death, complications which definetly affect the concentration of ACh in human placenta due to the pathological damage of its source, syncytiotrophoblast. In this study of placentas from healthy women, all complicating factors were specifically exclud-

Department of Physiology, Andhra Medical College, Visakhapatnam-530 001, A.P., India.
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ed and the procedure of ACh estimation was designed for "Invitro" study in phosphate-buffered medium on minced placental tissue derived from the healthy full-term women with live vaginal delivered babies to know the exact ACh kinetics.

Material and Methods

Preparation of placental mince: The placenta was collected within 2 minutes after vaginal delivery. It was thrown into hypothermic state (4°C) immediately in order to quickly produce an attenuation of placental cell metabolism and stop its oxygen-requirement. After 30 minutes of collecting, the placenta was spread on a rectangular ice-slab with the maternal surface up and the latter gently cleaned free from blood clots with cold Ringer-Locke's (RL). Random rectangular columns of placental tissue samples extending from the maternal to foetal surfaces were cut with fine knife from the healthy areas. They were further cut into fine mince with sharp fine scissors, washed clear of the blood traces with cold RL; mopped dry with wet and cold filter sheet; aliquots (600 to 800 mg) of the material weighed quickly on torsion balance and transferred to ice-cold phosphate-buffered eserinised (10^{-5} g/ml) RL (ERL) 10 ml each in four incubation tubes.

Incubation medium: Ringer-Locke's solution used for incubation has the composiontion used by Mann *et al* (1939a and 1939b) for brain slice incubation studied with slight modification and expressed in milli-moles per litre NaCl 154; KCl 5.6; $CaCl_2$ 1.7; NaH_2PO_4 0.25; Na_3HPO_4 2.1; Glucose 10; Eserine sulphate 10 μg/ml or 10^{-5} g/ml for final concentration and pH adjusted to 7.4 to 7.6. Instead of bicarbonate-buffered medium that required O_2 (95%) plus CO_2 (5%) mixtures, a phosphate-buffered medium was preferred as the latter needs only gasing by oxygen.

Incubation: Out of the four incubation tubes; three of them were transferred to a water-bath, motor-stirred, with temperature kept between 37°C to 38°C; for a period of 2 hours while the fourth tube remained as control in the refrigerator (4°C) for the same 2-hour period. The incubated tissue particles were constantly kept moving by fine oxygen bubbling into the bottom of the incubate.

In order to estimate the time-course of release of ACh during the 2-hour incubation, 2 ml of the clear supernatant fluid was drawn from each of the three incubation tubes every 30 minutes from the zero time and replaced by 2 ml of fresh ERL, each sample acidified to pH 4 to keep ACh stable. At the end of 2 hours the 3 incubation tubes were transferred to the refrigerator to arrest tissue activity and further synthesis of ACh. A 2 ml sample from the clear supernatant in the control tube also was collected and acidified to pH 4. The minced particles from all the 4 tubes (including the control tube) were separately collected by filtration, washed twice with fresh ERL, mopped dry with wet and cold filter sheet; 5 ml of fresh 10% trichloro acetic acid was added to each and incubated for 1½ hours at laboratory temperature for acid-extracted (MacIntosh and Perry, 1950), tissue-bound (B ACh); the extract separated by filtration; the acid removed by shaking with solvent ether cum water mixture to bring up the pH to 4 and the ether removed by aeration. Thus the supernatant samples containing released free ACh (F ACh) and the acid-extracted sample of tissue-bound ACh are kept in the refrigerator ready for estimation of ACh by bioassay.

Bioassay: The bioassay of free ACh and bound ACh was carried out on arterial blood pressure of young cat, anaesthetised by aetherchloralose sequence and eviscerated; and by means of bracketing the depressor responses to injections of suitable doses between similar responses to prepared standard ACh chloride solution and by matching similar responses to unknown ACh samples with known ACh standards. Many vasoactive substances in tissue extracts can produce a fall in the blood pressure. There was a necessity to make clear this fact that the depressor response was due to acetylcholine only. For this a few classical tests were done to identify the depressor substance as ACh in the acidified supernantant and the acid-extracted placental samples from the incubates (MacIntosh and Perry, 1950 and Brahmayya Sastry and Krishnamurthy, 1978).

Dry weight measurements: As there is likelihood of variation in water content of placenta, the dry weight of each placenta was separately determined by keeping the same in a hot air oven at 80°C to 90°C for 2-3 days till their successive weight readings were constant. All the results of ACh activity were expressed against dry weight, as ug/g of dry tissue.

Results

The ACh value expressed as (ug/30, 60, 90, 120 min/gm. dry weight) the parameters of evaluation of ACh dynamics worked out were: Basal or zero ACh values at 4°C were free Ia and bound IB: the experimental or activated ACh values at 37°C were IIa_1, IIa_2, IIa_3, IIa_4 free ACh and IIB bound ACh. The free ACh liberation values were plotted to show the rate of release by the slope of the curve; and the free, bound and total-synthesis values were calculated and shown in tables. Total-synthesis is a good index of the overall activity of the placental tissue. Free/Bound relation (ratio) as they stood at the time of termination of incubation were given as F/B ratio. The total-synthesis/initial bound relation (ratio) was also worked out and given. Every parameter has its own information to give but the best information is obtained by (1) total-synthesis, (2) total-synthesis/initial bound ratio, and (3) rate of release of ACh during the 2-hours incubation period (Vide: Figure) in order to understand ACh kinetics in human placenta in healthy states at term.

Table I depicts the time-course of free (F) ACh (Mean ± SE) liberation in

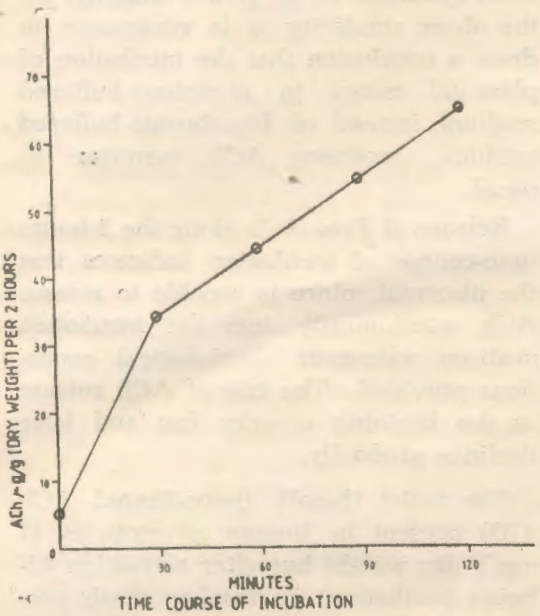
TABLE I

Time-course of Placental Free ACh Liberation

Temperature	Incubation Parameters Time-Course	*Placental ACh in μg/g. dry. Wt Tissue dry weight: 14.66%
		Mean ± SE
4°C	00-00 mts (Ia)	5 ± 0.09
37°C	00-30 mts (IIa_1)	34 ± 2.60
	30-60 mts (IIa_2)	44 ± 2.80
	60-90 mts (IIa_3)	53 ± 3.00
	30-120 mts (IIa_4)	63 ± 3.40

* 33 Placentas studied from healthy full-term mother with vaginal delivered live babies.

ug/g dry weight of tissue as 5 ± 0.9 per zero minutes (Ia), 34 ± 2.6 per 30 minutes (IIa_1), 44 ± 2.8 per 60 minutes (IIa_2), 53 ± 3 per 90 minutes (IIa_3) and 63 ± 3.4 per 120 minutes (IIa_4).



The above free ACh liberated values plotted to show the rate of release by the slope of the curve (Vide: Figure). The figure shows that the time-course of release of ACh in the 2-hour incubation period, revealed a linear relationship with rising 45° slope first and 40° slope later.

Table II lists the following different ACh Kinetics: Initial (Basal) free (Ia) and bound (IB) values at 4°C are 5 ± 0.9 (SE) and 47 ± 2.3 (SE) respectively. Experimental or activated free (F) and tissue bound (IIB) at the end of 2-hours incubation (37°C) are 63 ± 3.4 (SE) and 49 ± 2.8 (SE) respectively. The ratio of the Free-/Bound at the end of 2-hours incubation is 1.27 ± 0.05 (SE). The total-synthesis and totalsynthesis/ initial bound are calculated as 59 ± 3.2 (SE) and 1.26 ± 0.06 (SE) respectively.

Discussion

Most of our current knowledge about ACh kinetics originated from nervous tissues using either the cat superior cervical ganglion (Birks and MacIntosh, 1961) or the nerve-striated muscle preparations (Hubbard, 1973 and Quastel and HcKett, 1973). The human placenta is devoid of neuronal innervation, and is therefore a source of information about the process of ACh kinetics in non-nervous tissues.

TABLE II

Acetylcholine Kinetics in Healthy Term Placenta

Incubation Parameters (2-hours)		Placental ACh in μg/g. Dry Weight (33 Experiments) Tissue Dry Weight: 14.66%
		Mean ± SE
4°C	Free (Ia)	5 ± 0.09
	Bound (IB)	47 ± 2.30
37°C	Free (F)	63 ± 3.40
(Triplicates	Bound (IIB)	48 ± 2.80
	F/B (IIB)	1.27 ± 0.05 (ratio)
Total ACh synthesis		59 ± 3.20
Total-synthesis/Initial Bound (IB)		1.26 ± 0.06 (ratio)

In the present study, the incubation medium used (Vide: Material and Methods) was phosphate-buffered eserinised (10^{-5} g/ml) Ringer-Lock's with glucose 10 millimoles/L as recommended by Mann *et al,* 1939a; the same was used by Brahmayya Sastry and Krishnamurthy, 1978 for single cotyledon perfusion studies; and the medium was oxygenated. The solubility of oxygen at 37°C being 23.8 ml/L and the requirements worked out by Georke *et al,* 1961 (3.5 ml/kg/min) were adequately met; pH requirements have a wide margin of safety and that, used was narrow 7.4 to 7.6 and the glucose requirement was also satisfied Mann *et al,* (1939a and 1939b).

The ACh released from the human placenta is determined by bioassay method (MacIntosh *et al,* 1950). This method usually has high sensitivity upto 10^{-8} g/ml, was streamlined by assessment by bracketing method and tests confirming that the depressor substance in the incubate samples was ACh have been time-tested (Chang and Gaddum, 1933 and Emmelin and MacIntosh, 1956). This sensitivity is good enough for large quantities of ACh in placenta are available.

The vital materno-placental and placento-foetal links/barriers and fluids environment of the foetus and placenta: it was found proper to express ACh turnover as ug/time/g. dry weight of placental tissue, would offset the factor of varying water content in the placenta.

It is clearly evident from the results reported in this communication that placental mince releases ACh continuously in large quantities into the medium in which it is incubated.

In ug/2-hours/g (wet tissue weight) the mean ACh turnover in 33 experiments are F ACh 9.2, B ACh 7.2 and total-synthesis 8.7 (these wet weight values are equal to 63, 49 and 59 dry weight of tissue) are comparable to the similar values obtained by single cotyledon perfusion by Brahmayya Sastry and Krishnamurthi, 1978 (F ACh 9, B ACh 8.9 and Total-synthesis 10 ug/g. wet weight). By the above similarity it is reasonable to draw a conclusion that the incubation of placental mince in phosphate-buffered medium, instead of bicarbonate-buffered medium, produces ACh turnover as usual.

Release of Free ACh along the 2-hours time-course of incubation indicates that the placental mince is capable to release ACh continuously into the incubation medium whenever physiological conditions provided. The rate of ACh release in the begining is very fast and later declines gradually.

The initial (basal) tissue-Bound ACh (IB) present in human placenta is 47 mg/g dry weight but after activation (2-hours incubation) its Total-synthesis promoted to 63 ug/g dry weight. This ACh promoting action may be brought about by an increase in the cell permeability in placental mince during incubation and due to the plenty of choline deposites (145 ug/g) present in human placenta which is a chief substrate for ACh synthesis.

Conclusion

It is concluded that the healthy human full-term vaginal delivered placenta contain fairly high quantities of ACh and is continuously releasing into the incubation medium whenever activated by using choline deposits present in placenta. It is also proved that the rate of ACh released into the phosphate-buffered medium is same as that of the ACh released into the bicarbonate-buffered medium.

Summary

The present study reveals, the exact ACh kinetics in human placenta, purely at the healthy state of the mother-placenta-foetus. Placental mince in case of full-term vaginal deliveries of 33 healthy women was incubated *'In vitro'*, in triplicates, for 2 hous at 37°C in 10 ml of phosphate-buffered (pH 7.4) eserinised (5×10^{-5} g/ml) and oxygenated Ringer-Locke's fluid. Fluid incubate, collected every 30 minutes for free (F) ACh and acid-extracted tissue incubated at 2 hours for bound (B) ACh were assayed on recorded arterial blood pressure of chloralosed and eviscerated cat. Corresponding incubates, cold stored for 2 hours, served as controls. The placental ACh (Mean ± SE) in microgram/g (dry weight) was 63 ± 3.4 (F), 47 ± 3 (B) and 59 ± 3.2 (total-synthesis) in 2 hours of incubation. The time-course of ACh release was plotted. The results suggested that (1) The healthy vaginal delivered placenta at term contain fairly high quantities of ACh, (2) The rate of continuous release of ACh from the placental mince into the incubation medium along the 2-hours incubation time remained unchanged, revealed a linear relationship with rising 45° slope first and 40° slope later and (3) The incubation of placental mince in phosphate-buffered medium, instead of bicarbonate-medium, produced ACh turnover as usual.

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