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Acetylcholine output and foetal vascular resistance of human perfused placental cotyleda

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1 The foetal villous vessels of single cotyleda of human placentae have been perfused with a constant flow of Krebs solution, recording inflow pressure and passing the venous perfusate in cascade over guinea-pig ileum and rat stomach strip preparations *in vitro*.

2 Each cotyledon released for at least 4 h a substance that was probably acetylcholine. The perfusate caused contractions of both preparations which were inhibited by atropine or hyoscine and potentiated by physostigmine. Contractile activity was destroyed after incubation at 37°C of perfusate with acetylcholinesterase but not with acetylcholinesterase plus physostigmine.

3 When the perfusion temperature was lowered to 34°C or below, acetylcholine output was reduced, the extent depending on the fall in temperature.

4 No change in resistance of the villous vessels occurred during the changes in temperature or in the presence at 37°C of atropine, hyoscine, hexamethonium, (+)-tubocurarine, hemicholinium-3 or bretylium.

5 Submaximal vasoconstrictor responses of the villous vessels to the thromboxane A_2-mimetic U46619 were not affected by reduction of the perfusion temperature to 30°C, which lowered acetylcholine-like output by approximately 70%. Responses to U46619, at 37°C, were unchanged during the presence of atropine or hyoscine.

6 Acetylcholine is released into the foetal circulation of the human placenta but no evidence could be obtained that it affects villous vascular smooth muscle tone or vasoconstrictor responses.

Introduction

Acetylcholine (ACh) was first shown to be present in, and released from perfused human placental cotyleda by Chang & Gaddum (1933) and Chang (1936). Since then, although work has been done using biochemical techniques (Sastry *et al.*, 1976; Welsch & Wenger, 1980; Leventer *et al.*, 1982; reviewed by Sastry & Sadavongvivad, 1978) few studies have been carried out using the perfused cotyledon of the output of ACh and the mechanisms involved in its release. The human placenta contains large amounts of ACh and the enzyme responsible for its synthesis, choline acetyltransferase (Comline, 1946; Sastry *et al.*, 1976; Welsch & Wenger, 1980). This suggests that ACh plays an important localised role in placental function, since foetal blood contains cholinesterases (Jones & McCance, 1949) which would quickly hydrolyse the ACh being released into the vessels of the cotyledon. Whether this ACh is important in control of foetal placental vascular tone is not known. A constrictor effect of ACh in placental vessels was reported by Ciuchta & Gautieri (1964), whereas von Euler (1938) observed dilatation.

In the present studies, experiments were carried out which simultaneously measured placental foetal vascular tone and output of ACh. Efforts were made to throw more light on the mechanisms controlling ACh release and to determine whether it played any part in the control of placental foetal vascular resistance.

Methods

Human placentae were obtained after vaginal delivery or Caesarean section from women with uncomplicated pregnancies. Some, but not all, had received at least one of the following drugs during labour: oxytocin 2

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i.u. over 6–8 h, pethidine hydrochloride 100 mg i.m., promethazine hydrochloride 12.5–25.0 mg i.m., prochlorperazine maleate 12.5 mg i.m., metoclopramide hydrochloride 10–20 mg orally, and nitrous oxide and oxygen (70:30%). No differences between the responses of the tissues were noted which could be ascribed to these drugs.

The technique of perfusion of the placental cotyledon was a modification of that of Penfold *et al.* (1981) as described by Mak *et al.* (1984). As soon as possible after delivery (within 15 min) a suitable foetal vein and artery pair on the surface of the chorionic plate leading to a peripheral cotyledon were cannulated with plastic tubing (external diameter 1.34 mm for the artery and 2.2 mm for the vein). Each cotyledon was perfused by use of a Masterflex perfusion pump (model 7554.00 with pump head 7013) at a constant flow rate of 5 ml min^{-1}. The perfusion medium was an amniotic fluid-like Krebs solution containing (mM): NaCl 97.0, $NaHCO_3$ 30.0, KCl 3.0, KH_2PO_4 0.75, $CaCl_2.2H_2O$ 1.5, $MgSO_4.7H_2O$ 1.0, D-glucose 5.5; pH 7.3 when saturated with 95% O_2 and 5% CO_2. The whole placenta was immersed in the same solution. The maternal component of the cotyledon was also continually perfused (at approximately 5 ml min^{-1}) by inserting plastic tubing (1.34 mm external diameter) into two spiral arteries in the basal plate. Changes in vascular resistance on the foetal side were assessed from changes in inflow pressure, measured by means of a Statham P231D transducer and displayed on a Rikadenki (R22) chart recorder. Drugs were administered into the foetal artery by means of a Sage constant infusion pump (model no. 341A, Orion Research) after a stable villous perfusion pressure had been obtained.

Preparations were apparently viable for at least 4–5 h. No significant changes occurred during this time, either in submaximal vasoconstrictor responses to the thromboxane A_2-mimetic U46619 or in ACh output into the foetal circulation (see below). Penfold *et al.* (1981) demonstrated that placentae perfused with Krebs solution containing 30g l^{-1} dextran (M_r approx. 70,000) maintained almost complete cellular viability for 1–2 h, as assessed by little change in the venous perfusate content of the intracellular substances potassium and lactic dehydrogenase and tissue levels of the respiratory nucleotides ATP and ADP. Although dextran was omitted from the Krebs solution in the majority of the present experiments, ACh output after 1 h of perfusion with Krebs solution only (0.114 ± 0.015 nmol min^{-1} g^{-1}, $n = 13$) was not significantly different from that measured when dextran was present in the above concentration (0.217 ± 0.067 nmol min^{-1} g^{-1}, $n = 4$). No difference was also found in ACh output 4 h after starting perfusion with either Krebs or Krebs plus dextran. The viability of our preparations, indicated by the reproducible responses to U46619 and little change in ACh output for up to 4 h prompted the use of Krebs only for perfusion, measuring resistance to flow and ACh output 1–2 h after starting perfusion, both in controls and in placentae subjected to the effects of drugs and temperature change.

ACh-like activity of the foetal perfusate from the placental cotyledon was assayed by the tissue cascade technique Vane (1969) with ACh used as a standard. The tissues employed were the rat fundus strip and guinea-pig ileum removed from animals after cervical dislocation and exsanguination. Stomachs were obtained from mature rats (Wistar, 200–250 g, of either sex) and the fundus strip prepared according to the method of Vane (1957). Segments of the distal ileum (approximately 6 cm in length) of guinea-pigs (400–500 g, of either sex) were also used. For the experiments which examined the effect of temperature on ACh output, a heating coil was interposed between the placenta and assay tissues to ensure that the cascade temperature of the perfusate was 37°C.

Assay tissues were suspended under a resting tension of 1g from Harvard isotonic force transducers (Model 380) and contractions recorded on the Rikadenki recorder. The tissues were superfused with Krebs solution (5 ml min^{-1}) which contained (μM) mepyramine 0.3, methysergide 0.4, propranolol 6.8, phenoxybenzamine 0.3, indomethacin 5.6 and physostigmine 2.4.

Assay tissue responses to submaximal concentrations of ACh infused into the superfusion fluid (usually 25–100 nM) were obtained and these were bracketed with responses to the placental effluent after a proportion of the latter was passed into the fluid so that it was diluted appropriately. At least four responses to a known concentration of ACh were bracketed with at least two concentrations of the perfusate. Assay errors were within 5–9% and 12–17% for the guinea-pig ileum and rat fundus strip, respectively. In those experiments which examined the effects of drugs on placental ACh output, each drug was continuously superfused over the assay tissues before passing it at the same concentration through the placenta.

Acetylcholine output was expressed in terms of the wet weight of the perfused cotyledon. The latter was readily identified at the end of each experiment due to the blood being washed out of the particular cotyledon. This was dissected out and weighed. (Mean weight = 27.7 ± 3.2 g, $n = 20$.)

Means and their standard errors (s.e.mean) were calculated. The significance of the differences between means were determined using Student's t test. The level of probability which was accepted as statistically significant was $P < 0.05$.

All chemicals for the Krebs solution were obtained from British Drug House Ltd, England, except for sodium choride and D-glucose which were from Ajax

Chemical Co., Sydney, Australia. Acetylcholine perchlorate, hemicholinium-3, indomethacin and acetylcholinesterase (from bovine erythrocytes) were obtained from Sigma Chemical Co., U.S.A.; (+)-tubocurarine chloride, hexamethonium bromide and atropine sulphate, Koch-Light Labs. Ltd., Colnbrook, England; U46619 ((15S)-hydroxy-11α, 9α-(epoxymethano)prosta-5z,13E-dienoic acid), Upjohn; bretylium tosylate, Wellcome Laboratories, England; hyoscine hydrochloride, Felton, Grimwade and Duerdins Pty. Ltd., Melbourne, Australia; propranolol hydrochloride, I.C.I. Australia Ltd.; mepyramine maleate, May and Baker Ltd., England; methysergide hydrogen maleate, Sandoz Australia Pty. Ltd.; phenoxybenzamine hydrochloride, Smith, Kline and French, Australia; physostigmine salicylate, David Bull Laboratories Pty. Ltd., Australia and dextran (M_r approx. 70,000) Fluka .G., Switzerland. All drugs were dissolved in 0.9% w/v saline prior to use, except bretylium which was dissolved in distilled water (50 mg ml^{-1}) prior to dilution with saline, and indomethacin which was dissolved in saline containing 0.07% (w/v) sodium carbonate.

Results

To confirm that the assay tissues were responding specifically to ACh in the placental perfusate, experiments were performed examining effects on responses to the perfusate of antagonists at muscarinic receptors and incubation with acetylcholinesterase. Both the rat stomach strip and the guinea-pig ileum exhibited sustained contractions when the perfusate from the placenta was diluted 10–100 fold by passage into the superfusion fluid. As shown in Figure 1, responses of the stomach strip were abolished by passing into the perfusate either hyoscine (0.2 μM) or atropine (1.4 μM). Removal of the antagonists from the perfusate was followed by a gradual return of the responses within 1 h. Analogous results were obtained with the guinea-pig ileum.

Three separate 10 ml aliquots of placental perfusate were incubated at 37°C for 30 min with 1 unit of acetylcholinesterase, or 1 unit of acetylcholinesterase plus 24 nmol physostigmine, or without either acetylcholinesterase or physostigmine. Responses of the assay tissues to perfusate were abolished only after it was incubated with acetylcholinesterase in the absence of physostigmine (Figure 2).

Output of ACh, as assayed every 30 min, did not change significantly from 1–4 h after the start of perfusion, varying from 0.108 ± 0.020 to 0.163 ± 0.025 nmol min^{-1} g^{-1} wet wt ($n = 4$). The perfusion pressure also did not alter significantly during this time.

Output of ACh from the placental lobule, after

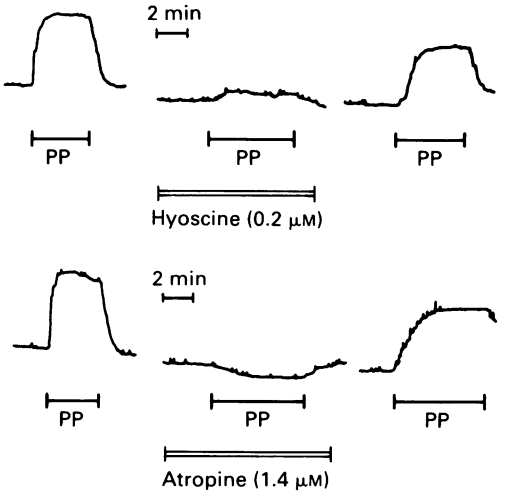

Figure 1 Effects of hyoscine and atropine on the response of the rat stomach strip to placental perfusate. Placental perfusate (PP) was passed over the tissue for the times indicated by the horizontal bars in either the absence or presence of atropine or hyoscine. The time interval between the initial responses and the responses in the presence of antagonists was 15 min, and initial responses were between 60 and 80% of the maximum response obtained. The traces on the right show responses 1 h after removal of the antagonists.

1 h of perfusion at 37°C was 0.114 ± 0.015 nmol min^{-1} g^{-1} wet wt ($n = 13$). As shown in Table 1, ACh output was significantly increased 2.6 fold when physostigmine (2.4 μM) was passed through the lobule. Table 1 also shows the ACh output and perfusion pressure during passage through the lobule of hexamethonium, (+)-tubocurarine, hemicholinium-3 or bretylium. In the concentrations used, none had a significant effect on ACh output or perfusion pressure. Neither atropine nor hyoscine affected perfusion pressure significantly, their presence in the perfusate precluding measurement of ACh by bioassay.

Basal perfusion pressures were found not to be significantly different from the mean control value at 37°C (45.8 ± 6.3 mmHg, $n = 13$) when the temperature of the lobule was altered in 3.5–5°C increments between 20 and 40°C (data not shown). However, ACh output was significantly reduced at temperatures of 33.5°C and below (Figure 3). At 20°C, mean ACh output was 5.9 ± 3.7% ($n = 4$) of that at 37°C. Figure 3 also shows mean vasoconstrictor responses to a concentration of the thromboxane A_2-mimetic U46619 (28.6 nM) which caused a submaximal effect at 37°C, an increase in perfusion pressure to 86.6 ± 13.3 mmHg ($n = 7$). The maximum increase

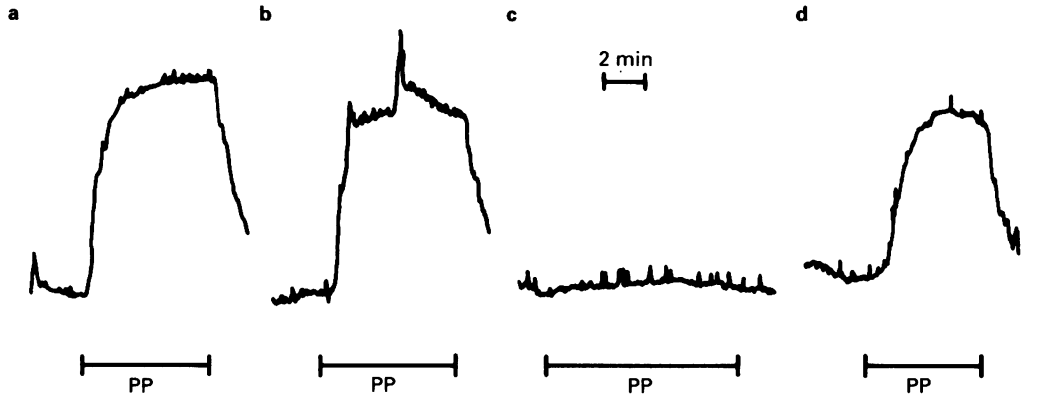

Figure 2 Effect of incubation of the placental perfusate (PP) with acetylcholinesterase. Response of the rat stomach strip to (a) placental perfusate, (b) perfusate incubated with saline, (c) perfusate incubated with acetylcholinesterase (0.1 unit ml^{-1}), (d) perfusate incubated with acetylcholinesterase (0.1 unit ml^{-1}) plus physostigmine (2.4 μM). Incubation was for 30 min at 37°C. Solutions were superfused over the assay tissue during the periods indicated by the horizontal bars and the interval between each measurement was approximately 10 min. The response shown at (a) was 60% of the maximum response obtained.

obtained to U46619 was 194 ± 14 mmHg ($n = 8$). As shown in Figure 3 lowering of temperature to 20–25°C caused reduced responses to U46619, the magnitude of the response being significantly less at 20°C than at 37°C ($P < 0.05$).

The rise in perfusion pressure in response to U46619 (28.6 nM) at 37°C was not affected by the presence of either atropine or hysocine. The mean initial rise in perfusion pressure to U46619 was compared to subsequent responses to U46619 in the same preparation

Table 1 Mean acetylcholine (ACh) output in foetal perfusate and foetal cotyledon vessel perfusion pressure

	Perfusion pressure (% control)	*ACh output* (% control)
Physostigminine salicylate		
2.4 μM, 1 h	97.3 ± 3.7 (6)	264.0 ± 27.2 (14)*
24.2 μM, 1 h	127.7 ± 10.8 (4)	234.0 ± 48.1 (4)*
Hexamethonium bromide		
27.6 μM, 1 h	91.8 ± 4.8 (4)	109.5 ± 16.3 (4)
276.1 μM, 1 h	101.7 ± 4.1 (4)	94.3 ± 9.7 (4)
(+)-Tubocurarine chloride		
1.3 μM, 1 h	105.7 ± 6.1 (5)	153.3 ± 25.3 (4)
12.7 μM, 1 h	117.8 ± 13.5 (4)	91.3 ± 20.7 (4)
Hemicholinium-3 100 μM, 2 h	114.8 ± 7.6 (5)	101.1 ± 14.7 (4)
Bretylium tosylate 24.1 μM, 2 h	105.2 ± 7.8 (4)	100.7 ± 10.8 (4)
Atropine sulphate 575.7 μM, 1 h	92.6 ± 18.8 (4)	—
Hyoscine hydrochloride 22.8 μM, 1 h	108.1 ± 7.0 (4)	—

Placental lobules were perfused for 1 h. Each drug was added to the foetal perfusate only, ACh output being assayed and perfusion pressure measured after perfusion for the time indicated. Figures show means ± s.e.mean (n). Control perfusion pressure was 45.8 ± 6.3 mm Hg, and control ACh output was 0.114 ± 0.015 nmol min^{-1} g^{-1} wet wt ($n = 13$).
*Significantly different from control, $P < 0.05$.

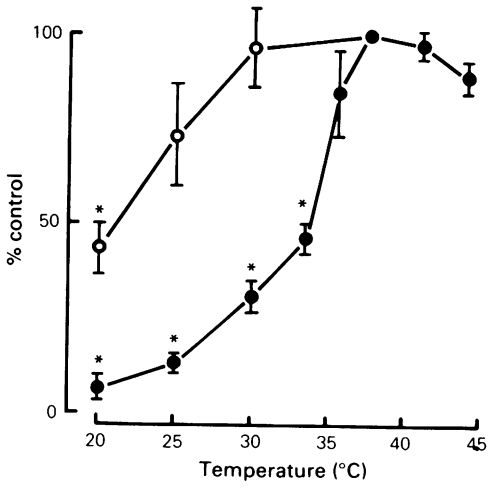

Figure 3 Effect of temperature on placental release of acetylcholine (ACh) and responses to U46619. Measurements were made of increases in perfusion pressure in response to U46619 (2.86 nM) (○) and of ACh output from placental lobules 1 h after the start of perfusion (●). Vertical bars show s.e.mean ($n = 4$). *Significantly different from mean control value, $P < 0.05$.

after addition to the perfusion fluid of saline, atropine (575.7 μM) or hyoscine (22.8 μM). After saline the mean response to U46619 was 96.2 ± 5.7% of the initial response, in the presence of atropine 111.8 ± 4.2%, and in the presence of hyoscine 81.9 ± 7.6% ($n = 4$). These differences were not significant.

Discussion

In the present study, acetylcholine was continuously released from the perfused human placental cotyledon for at least 4 h. Output, in the presence of physostigmine (2.4 μM) was found to be 0.301 ± 0.031 nmol-min^{-1} g^{-1} wet wt. This value is similar to that reported by Sastry & Krishnamurty (1978). Since the placenta is devoid of innervation, the ACh found in the perfusate must therefore originate from non-nervous tissue, possibly from the syncytiotrophoblast layer (Olubadewo & Sastry, 1978). The present experiments revealed that release was temperature-dependent. Output was markedly reduced by cooling the placenta, the magnitude of the decrease being dependent on the extent of the temperature reduction.

The results of these studies provided no evidence to support the hypothesis that the ACh appearing in the lumen of the villous vessels affected placental foetal vascular resistance. Neither muscarinic nor nicotinic antagonists affected basal vascular tone, as indicated by no significant change in perfusion pressure. Nevertheless it could be postulated that the released ACh might affect vascular tone via a mechanism that is not susceptible to the drugs used. This does not appear to be the case. Lowering the temperature of the placenta to 30°C caused a fall in ACh output of approximately 70% but caused no significant change in either basal perfusion pressure or the sensitivity of the preparation to the vasoconstrictor effects of U46619.

The results of this study therefore confirm earlier findings that ACh is continually released into the human placental villous vessels but the endogenous release does not appear to have a role in the control of basal vascular resistance or to modify vasoconstrictor responses in the villi. It has been suggested that the ACh may be important in control of amino acid transport across the placenta (Bull *et al.*, 1961; Rowell & Sastry, 1981).

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