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Acetylcholine in bovine corneal epithelium

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AMONG the various mammalian tissues that have been studied for acetylcholine (ACh) content, the corneal epithelium contains the highest concentration. According to von Brucke,[1] bovine and rabbit corneal epithelium contain 100 to 200 μg ACh/g tissue. Although corneal epithelium is rich in nerve endings, the enormous concentration of ACh in these cells is not in accord with the level of ACh that is usually found in junctional tissue where it serves as a neurotransmitter. Thus, for example, in autonomic ganglia the concentration of ACh ranges from 6 to 44 μg/g of tissue.[2] For this reason it was thought that in corneal epithelium, ACh might be involved in another process, e.g. regulation of water and ion transport, than as a neurotransmitter.

This investigation was undertaken to explore this hypothesis; in addition to assaying ACh, the other components of the cholinergic triad, choline acetylase and cholinesterase, were also estimated in the cornea.

MATERIALS AND METHODS

Fresh bovine eyes, transported from the abattoir in crushed ice, were used in these experiments. Prior to incubation the whole eyes, excess muscle and connective tissue were removed. Eyes revealing corneal defects of any kind were discarded.

The corneal epithelium was removed by scraping the cornea with a no. 10 Bard-Parker blade. By means of a small glass rod the epithelium was rapidly transferred from the scalpel to a tared test tube containing 1·5 ml of 0·02 N HCl. The tissue was thoroughly minced with a glass rod, and the test tube containing the sample was then placed in a boiling water bath for 1 min. After cooling to room temperature, 0·5 ml of 20% TCA was added and the sample centrifuged. The supernatant solution was frozen at $-40°$ until assayed for ACh. The extensive dilution of the sample that was performed prior to bioassay obviated the removal of the TCA.

The extraction of ACh from corneal tissue, a modification of the method of Smallman and Fisher,[3] gave a quantitative recovery of ACh in preliminary experiments in which acetyl-^{3}H-choline was added to the minced epithelium.

After removing the epithelium, stroma was isolated by shaving off a thin layer of stromal tissue. The tissue was then treated in the manner described for epithelium. For determination of the cholinergic system in stroma and endothelium, after removal of epithelium, a sample of stroma and endothelium was taken and treated as described above for epithelium. No attempt was made to isolate endothelium alone.

Guinea pig ileum was used for ACh bioassay; on a few occasions ACh was also determined by the fluorometric procedure of Cooper.[4] Choline acetylase was assayed by the procedure of Bull *et al.*[5] Cholinesterase was determined by the colorimetric procedure of Ellman *et al.*[6] In this assay, corneal epithelium was homogenized with 0·1 M phosphate buffer, pH 8·0, and centrifuged at 900 *g* to remove large particles that interfered with the spectrophotometric assay. These particles were essentially devoid of cholinesterase activity.

The degree of corneal hydration was taken as an index of corneal permeability. After removal of the epithelium a full-thickness disk of 1-cm diameter was trephined from the centre of the cornea, blotted to remove excess water, and then weighed. The difference in weight between disks from control and experimental eyes was taken to represent a change in corneal hydration. Although relatively crude, this method was accurate within $\pm 7\%$ among replicate samples.

The medium used in all incubation experiments was Krebs-Ringer phosphate supplemented with 40 mM Tris buffer, pH 7·4, to increase the buffer strength. Unless otherwise indicated, during incubation the samples were continually gassed with O_2. In determining the effect of corneal hydration on the ACh content of the epithelium it should be emphasized that in all experiments outlined below, separate eyes were used in each step. At the end of the treatment the corneal epithelium was removed and a disk taken from the remainder of the cornea as outlined above. The following experimental procedures were employed.

I. *Hypothermia*

It has been well documented that the cornea will swell when cooled for a period of 20 to 40 hr and will subsequently deturgesce when incubated at 37° for 3 to 6 hr.[7–10] Eyes (3 to 5 per treatment) were subjected to: (a) incubation at 37° for 1 to 6 hr; (b) incubation at 4° for 20 to 40 hr; (c) step (b) followed by step (a).

II. *Anoxia*

A requirement of oxygen for the maintenance of corneal hydration has been demonstrated both *in vivo* and *in vitro*.[11–15] The following experiments were conducted to determine the effect of anoxia-induced corneal swelling and deturgescence on the ACh content of the epithelium: (a) incubation at 37° for 3 hr; (b) incubation at 37° for 3 to 6 hr with N_2 as the gas phase; (c) step (b) followed by step (a).

III. *Hypothermia and iodoacetate*

It was thought that a combination of hypothermia and a metabolic inhibitor might intensify the relationship, if any, between ACh content and corneal hydration. Iodoacetate was injected in a volume of 0·1 to 0·25 ml by means of a 25-gauge needle that was inserted through the sclera proximal to the limbus and angled forward to the anterior chamber. Mixing was accomplished by repeated withdrawal and ejection of the aqueous humor from the syringe. The concentration of the inhibitor was adjusted so that the dilution caused by the aqueous humor (estimated as 1·5 ml) would provide the desired final concentration. The following procedure was carried out: (a) injection of iodoacetate (6×10^{-3} M final concentration) and incubation at 37° for 3 hr; (b) injection of iodoacetate (6×10^{-3} M final concentration) and incubation at 4° for 20 hr; (c) step (b) followed by step (a).

The significance of the results of all experiments was determined by the Student's '*t*' test. All values are given with the standard error of the mean.

RESULTS AND DISCUSSION

The distribution and concentration of ACh, choline acetylase, and cholinesterase are shown in Table 1. ACh is localized essentially in the corneal epithelium; the trace amount that appears to be

TABLE 1. ACH, CHOLINE ACETYLASE, AND CHOLINESTERASE IN THE BOVINE CORNEA

	ACh (μg/g)	Choline acetylase (μg ACh synthesized/g/hr)	Cholinesterase (μg AcSCh hydrolyzed/g/hr)
Epithelium	38 ± 2·2 (39)*	4,722 ± 179 (17)	4,640 ± 184 (12)
Stroma		113 ± 11 (4)	2,947 ± 225 (5)
Stroma and endothelium	0·15 ± 0·06 (5)		2,660 ± 151 (12)

* Figures in parentheses refer to the number of samples.

present in stroma and endothelium was of a slow-reacting nature as determined by the bioassay procedure and probably is not acetylcholine. Although the localization of the neurohumor in the epithelium agrees with the finding of von Brucke, the concentration of ACh is considerably higher in his experiments. Von Brucke reported a value of ACh of 100 to 200 μg/g in both rabbit and bovine corneal epithelium, whereas we find values of 38 μg/g. This discrepancy is difficult to explain, although it should be pointed out that von Brucke estimated the concentration of ACh indirectly and, in addition, gave data only on two rabbits.

The values reported in this paper for choline acetylase activity, 4,722 μg ACh synthesized/g epithelium/hr, are considerably higher than those obtained by van Alphen,[16] who reported a value of 1,000 μg ACh synthesized/g epithelium/hr. This difference may be explained either by species difference (Van Alphen used rabbit corneal epithelium) or by experimental procedure, since we used the disrupted whole cell procedure of Bull *et al.*, whereas Van Alphen used an acetone powder preparation. As shown in Table 1, more than 97% of the choline acetylase activity in the cornea is localized in the epithelium.

In contrast, cholinesterase is present in epithelium and stroma, although the epithelium contains almost twice as much activity as the stroma. It is of interest to note that the endothelium contains no cholinesterase. The value of cholinesterase activity in corneal epithelium, 4,640 μg ACh hydrolyzed/g/hr, compares with that obtained by Hebb[17] for ventral spinal roots, 2,500 to 5,000 μg/g/hr. Whether the cholinesterase that occurs in cornea is of the true or pseudo type remains to be determined.

Table 2 depicts the effect of a variety of experimental conditions on the relationship between epithelial ACh levels and corneal permeability as measured by corneal hydration. It is apparent that

TABLE 2. RELATIONSHIP BETWEEN ACH LEVELS AND CORNEAL HYDRATION

Condition	ACh concentration (μg/g)	Significance (P value)	Corneal wt. (mg)	Significance (P value)
Control	38 ± 2·2 (39)		66·0 ± 2 (20)	
Incubation at 37°	44 ± 7·4 (23)	N.S.*	63·7 ± 1·2 (17)	N.S.
Incubation at 4°	24 ± 2·0 (24)	<0·001	87·6 ± 1·6 (15)	<0·001
Incubation at 4° followed by incubation at 37°	40·7 ± 1·0 (40)	N.S.	77·0 ± 1·6 (23)	<0·001
Incubation at 37° with nitrogen	42 ± 6·0 (10)	N.S.	67·0 ± 1·0 (3)	N.S.
Incubation at 37° with nitrogen followed by oxygen	57 ± 9·2 (10)	<0·01	77·6 ± 6·3 (3)	N.S.
Incubation at 37° with iodoacetate	39·6 ± 4·4 (10)	N.S.	78·9 ± 1·9 (10)	<0·001
Incubation at 4° with iodoacetate	12·3 ± 2·6 (7)	<0·001	86·3 ± 1·6 (7)	<0·001
Incubation at 4° with iodoacetate followed by incubation at 37°	84 ± 1·5 (10)	<0·001	86·2 ± 2·0 (10)	<0·001

* N.S. = not significant ($P < 0·05$).

if a relationship exists, it is not a simple one. Thus, under hypothermic conditions, the ACh concentration drops, although the cornea increases in weight during the turgescent phase, and this same situation obtains in the condition of hypothermia plus iodoacetate. However, in the deturgescent phase, ACh levels return to a normal value with hypothermia alone, whereas corneal weight, although significantly decreased, does not reach control values. In the procedure of hypothermia and iodoacetate, both ACh content and corneal hydration remain significantly elevated after attempted deturgescence. In other words, the level of ACh appears to vary independently of the state of hydration of the cornea. Similarly, in the experiment involving anoxia, the ACh concentration in the cornea is elevated in the subsequent oxygenation phase of the experiment, but no significant change in corneal weight is observed. Experiments are in progress on the metabolism of ACh under these experimental conditions in order to gain some insight into the role of the neurohumor in the corneal epithelium.

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REFERENCES

1. H. VON BRUCKE, H. F. HELLAUER and K. UMRATH, *Ophthalmologica (Basel)* **117**, 19 (1949).
2. F. C. MACINTOSH, *J. Physiol. (Lond.)* **99**, 436 (1941).
3. B. N. SMALLMAN and R. W. FISHER, *Canad. J. Biochem.* **36**, 575 (1958).

4. J. R. COOPER, *Biochem. Pharmac.* **13**, 795 (1964).
5. C. BULL, C. HEBB and D. RATKOVIC, *Biochim. biophys. Acta* **67**, 138 (1963).
6. G. L. ELLMAN, K. D. COURTNEY, V. ANDRES and R. M. FEATHERSTONE, *Biochem. Pharmac.* **7**, 88 (1961).
7. A. DAVSON, *J. Physiol. (Lond.)* **125**, 15P (1954).
8. B. SCHWARTZ, B. DANES and P. J. LEINFELDER, *Am. J. Ophthal.* **38**, 182 (1954).
9. F. J. PHILPOT, *J. Physiol. (Lond.)* **128**, 504 (1955).
10. D. M. MAURICE, *The Transparency of the Cornea, A Symposium*, p. 67. Blackwell, London (1960).
11. G. K. SMELSER and V. OZANICS, *Science* **115**, 140 (1952).
12. G. K. SMELSER and V. OZANICS, *Arch. Ophthal.* **49**, 335 (1953).
13. M. E. LANGHAM and I. S. TAYLOR, *Brit. J. Ophthal.* **40**, 321 (1956).
14. M. E. LANGHAM, *The Transparency of the Cornea, op. cit.*, p. 87.
15. J. E. HARRIS, *ibid.*, p. 73.
16. G. W. H. M. VAN ALPHEN, *Arch. Ophthal.* **58**, 449 (1957).
17. C. HEBB, *Handbuch der experimentellen Pharmakologie*, vol. 15, p. 55. Springer-Verlag, Berlin (1963).

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Effects of norepinephrine, acetylcholine and calcium on the oxidation of glucose in the submaxillary gland of the rat

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IN A prior communication[1] which pertained primarily to the oxidation of glucose labeled in C-1, it was stated that norepinephrine or acetylcholine with eserine *in vitro* stimulated the production of $^{14}CO_2$ from radioactive glucose by submaxillary gland slices of rat, but only in the presence of calcium. The present report extends the comparison of the oxidation to carbon dioxide of glucose labeled in C-1 and C-6, under conditions in which some of the pathways of normal metabolism were blocked. Submaxillary (submandibular) glands from mature male Sprague-Dawley rats were used, and the procedures for tissue preparation, incubation and measurement of $^{14}CO_2$ production were the same as described previously.[1]

RESULTS

The results of eight separate experiments are shown in Fig. 1. Each grouping shows the effects of either norepinephrine (295 μM) or acetylcholine (342 μM, with 360 μM eserine), of iodoacetic acid alone (10 mM), and of the combination of iodoacetic acid with norepinephrine or acetylcholine (plus eserine). Each control value (when nothing extra was added) is presented as 100%, so each response greater than that represents a stimulation, whereas anything below 100% shows an inhibitory effect.

It may be noted, as previously reported,[1] that when calcium was omitted from the incubation medium, neither norepinephrine nor acetylcholine had any effect on $^{14}CO_2$ production when glucose-1-^{14}C (G-1-^{14}C) was the substrate (Fig. 1-A). With glucose-6-^{14}C (G-6-^{14}C) as the substrate under the same conditions, norepinephrine and acetylcholine both had inhibitory effects of approximately 20% (Fig. 1-B).

With calcium included in the incubation medium in the normal amount for Krebs-Ringer bicarbonate (2·54 mM), the stimulatory effect of norepinephrine was considerable, whereas that of acetylcholine was less, but still definite, both with G-1-^{14}C as the substrate (Fig. 1-C and Ref. 1) and with G-6-^{14}C (Fig. 1-D). Norepinephrine did not always have as great a stimulation on the $^{14}CO_2$ production as in these particular experiments (90% or more), but its effect was usually greater than that of acetylcholine plus eserine.

Iodoacetic acid alone at a concentration of 10 mM caused considerable inhibition of $^{14}CO_2$ production with either substrate, and with or without added calcium (Fig. 1). The inhibition was greater with G-6-^{14}C (88–95%) than with G-1-^{14}C (67–86%).