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ACETYLCHOLINE AS A POSSIBLE SENSORY MEDIATOR IN RABBIT CORNEAL EPITHELIUM*

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Abstract—Local denervation of rabbit corneas resulted in a decrease of 87–100 per cent of corneal epithelial acetylcholine thus suggesting a neuronal affiliation of this substance.

In other experiments, hemicholinium was injected either subconjunctivally or into the anterior chamber of the rabbit eye. When acetylcholine synthesis was inhibited by hemicholinium so that its level fell by at least 40 per cent, the cornea lost its touch sensitivity. This sensitivity returned concurrent with the return of acetylcholine to normal values. It is hypothesized that acetylcholine may have a role in pain perception in the cornea.

THE CORNEAL epithelium is considered to have the highest concentration of acetylcholine (ACh) of any mammalian tissue. Using an indirect procedure von Brücke[1] found 100–200 μg ACh/g of corneal epithelium in both rabbit and cow eyes. Subsequently, Williams and Cooper[2] observed that in cow eyes the ACh level was about 40 μg/g tissue. This unusually high concentration has stimulated some interest in determining the role of ACh in the epithelium.

Boros *et al.*[3] suggested a relationship between corneal transparency and ACh. They observed that the level of the ester was lower in cloudy as opposed to clear corneal transplants. However, Williams and Cooper[2] found no relationship between the degree of hydration of the cornea and the ACh content. Englehart[4] suggested a relationship between ACh content of the aqueous humor and light stimulation of the eye. He found that when rabbits were placed in the dark for several hours, no ACh could be found in the aqueous humor. However on subsequent exposure of one eye to light (the other being patched), ACh appeared in the aqueous humor of the light-treated eye only.

Two attempts to correlate ACh with the nerve terminals in the cornea have been made. In one, when the nerve supply was severed at the limbus, the cholinesterase activity of the epithelium was decreased 50 per cent at the end of 2 weeks.[5] In the other,[1] postganglionic section of the trigeminal nerve resulted in a decline in the ACh content of the epithelium of between 35 and 50 per cent.

In this paper, experiments are described which suggest that ACh may function in the corneal epithelium as a mediator of sensory impulse.

MATERIALS AND METHODS

Male Hartley guinea pigs weighing up to 325 g were used for the ACh bioassay. New Zealand white rabbits weighing 2·5–3 kg were used for all experiments. Extrac-

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tion and assay of ACh are described in detail in another paper.* Briefly, the procedure was as follows:

Corneal epithelium was scraped away from the stroma with a No. 1 Bard-Parker blade and transferred to a centrifuge tube containing cold 5% trichloracetic acid (TCA). After 1 hr, the tissue was homogenized, centrifuged, and the supernatant extracted with ether to remove the TCA. The extract was assayed for ACh using the guinea pig terminal ileum according to the method of Scott and Mautner.[6] The TCA precipitate from the epithelium was assayed for protein by the method of Lowry *et al.*[7]

Denervation experiments. Female rabbits were anesthetized with sodium pentobarbital. A 0·5% ophthalmic solution of tetracaine-HCl was dropped on the right eye which was then proptosed and immobilized by means of a curved hemostat. A 1:1000 solution of adrenalin was applied to the cornea near the limbus to minimize bleeding. Using a 10 mm corneal transplant trephine, a circle was incised in the center of the cornea about 0·3 mm deep. Then another circular incision was made outside the first, with a sharp razor blade held in a hemostat to regulate depth of cutting, 1 mm from the limbus and 0·3 mm deep, thereby forming two concentric circles 2 mm apart. The bridge of corneal tissue between the two circles was carefully removed with the aid of a fine corneal gill knife, taking care not to enter the anterior chamber. The completeness of the incision was ascertained by application of a 2% solution of fluorescein dye. The cornea was kept moist with saline during the operation. The contralateral, unoperated eye served as a control. To test for degeneration of nerves, a fine wisp of cotton was brushed across the cornea daily taking care to avoid touching the lashes. The corneal reflex in the operated eye disappeared completely by the tenth day. This was taken as a sign of complete nerve degeneration.[8] Corneal cells apparently retained their integrity since clouding was never observed. At this time the animals were sacrificed and the ACh content of the corneal epithelium of both control and operated eyes was determined as previously described.[9]

Light experiments. The corneas of rabbits of either sex were bathed in 0·1% diisopropyl fluorophate (DFP) in saline. One eye of each rabbit was patched with gauze-covered carbon paper. In some of the experiments the rabbits were then placed in a dark room for from 2·5 to 4 hr. They were removed and again treated with DFP. In other experiments the pretreatment in the dark was omitted. The animals were held in a standard rabbit box with their heads protruding and a tensor light was placed at a distance of 4 in. from the unpatched eye. The corneas were kept moist with 0·9% saline. After periods of exposure to light varying between 5 min and 5 hr the rabbits were placed under pentobarbital anesthesia. The corneal epithelium was removed and ACh was extracted and bioassayed as previously described.[9]

Hemicholinium experiments. A solution of 1% hemicholinium bromide (HC-3) was used in all experiments. Female rabbits weighing 2·5 kg were held in rabbit boxes and 2 drops of 0·5% xylocaine were applied to one eye. The eye was immediately proptosed to immobilize it and injection was made by means of a Hamilton microliter syringe fitted with a 30 gauge needle. In some experiments the HC-3 was delivered into the anterior chamber; in others injection was made subconjunctivally. It was considered that the animal had lost its corneal reflex if it no longer responded by blinking when the cornea was lightly touched with a small polished glass rod. Care was always taken to avoid pressure which might cause distortion of the globe thereby producing pain

* G. G. Fitzgerald and J. R. Cooper, unpublished observations.

in parts of the eye other than the cornea. When the corneal reflex disappeared, the epithelium was removed for determination of ACh. The contralateral eye of each rabbit served as a control with respect to the normal reflex as well as endogenous ACh concentration. The dose of HC-3 covered a range between 20 and 50 μl per eye because of the variation among animals in response to the drug. A sufficient quantity was given to cause loss of reflex without toxicity. At the end of the experiment, ACh was extracted from the corneal epithelium and bioassayed.

RESULTS

Since our experimental design involved a comparison of the ACh content of the control eye vs. the experimental eye, it was necessary in preliminary experiments to establish that the ACh content was identical in both eyes. Using 15 rabbits, the ratio of the ACh content of the left eye vs. the right eye was determined and found to be 1·03 with a standard deviation of 0·089. Although the variation between eyes of the same animal was minimal, the variation in the ACh content of the corneal epithelium in a population of rabbits was quite large. Thus, the left eye had a mean of 0·253 $\pm$ S.D. of 0·160 μg ACh/mg protein and the right eye, a mean of 0·263 $\pm$ S.D. of 0·180 μg ACh/mg protein.

Effect of light on ACh content of the corneal epithelium. In experiments conducted according to the method of Engelhart,[4] in which rabbits were pretreated in the dark for 2·5–14 hr before one eye was exposed to high intensity light, the results were scattered with no discernable trend. Similarly, when pretreatment in the dark was omitted and the rabbits were subjected to short lighting intervals varying from 1 to 15 min, inconclusive results were obtained. The results did not furnish sufficient evidence to warrant further consideration of a correlation between light stimulation of the cornea and ACh content of the epithelium.

Denervation experiments. Because von Brücke had reported that the ACh content of the cornea decreases following trigeminal section, we decided to investigate the possibility that ACh is affiliated with the fine nerve endings present. Trigeminal section is a rather difficult surgical procedure in rabbits so we chose to achieve denervation by local section of the nerves. Applying this technique, it was observed that the corneal reflex of the denervated eye began to disappear in 5–7 days after the incision was made, and was completely absent by the 10th day.* The reflex in the control eye remained brisk throughout. The results of four experiments appear in Fig. 1. The ACh content of the corneal epithelium in the denervated eyes had decreased by 87–100 per cent at a time when denervation was apparently complete in that cornea. These results furnish evidence for a relationship between ACh and intact nerves in this tissue.

Effect of HC-3 on ACh content and corneal reflex. Our local denervation studies implied the possibility of a neuronal affiliation for the ACh in corneal epithelium. We therefore sought to determine if ACh might be involved in the initiation of the sensory nerve impulse which results in pain perception in the cornea.

We chose HC-3 as the most specific means of achieving inhibition of ACh synthesis.

* It is unclear, both to us and to opthalmologists with whom we have consulted, why the corneal reflex takes 5–7 days to completely disappear. Part of the difficulty lies in the fact that very little is known about the innervation of the corneal epithelium.[10] It is possible that in the circumferential denervation technique, a few intact fibers remain and until they degenerate, these are sufficient to maintain the corneal reflex.

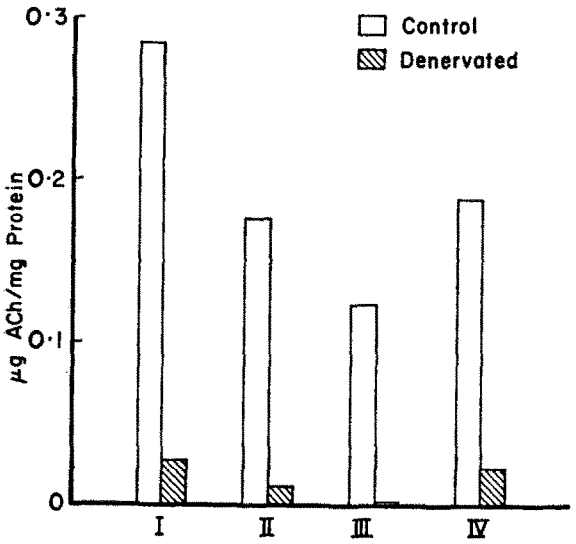

FIG. 1. ACh content of both operated and control eyes was determined 10 days after local denervation. Loss of corneal reflex was taken as the index of complete degeneration of epithelial nerves.

In the first approach, HC-3 was injected into one eye of a rabbit in sufficient quantity to result in loss of corneal reflex as tested by touching the cornea gently with the end of a polished glass rod. The contralateral eye served as a control. Table 1 shows the results of a time curve obtained from 6 animals so treated. The control eyes retained a normal, brisk reflex throughout. The reflex in the injected eye remained brisk 45 min after injection and this eye still contained 73 per cent as much ACh as the control. After 1 hr, the reflex in the HC-3 treated eye was absent, and remained so at 2 hr. The ACh levels in these eyes had fallen to 31 and 56 per cent of normal, respectively. At 4 and 6 hr, the reflex was normal again as was the ACh content.

TABLE 1. TIME COURSE OF EFFECT OF HC-3 ON CORNEAL REFLEX AND *IN VIVO* LEVELS OF RABBIT CORNEAL EPITHELIAL ACh*

Time after injection	Control eye (μg ACh/mg protein)	Control eye Corneal reflex	Treated eye (μg ACh/mg protein)	Treated eye Corneal reflex	ACh remaining in treated eye(%)
45 min	0·15	Brisk	0·11	Brisk	73
1 hr	0·19	Brisk	0·06	None	31
2 hr	0·16	Brisk	0·09	None	56
3 hr			0·23	Brisk	
4 hr	0·20	Brisk	0·22	Brisk	110
6 hr	0·07	Brisk	0·08	Brisk	113

* The anterior chamber of the "treated" eye was injected with 20 μl of a 1% solution of HC-3. Control eyes were not injected. Immediately upon loss of the corneal reflex in the treated eye, ACh was extracted into 5% TCA and bioassayed. The reflex in the control eye was checked at the same time and the tissue was similarly handled.

Data obtained from 13 animals (including those listed in Table 1) in which corneal reflex and ACh content were measured are summarized in Table 2. Each pair of figures represents one animal. It can be seen that in rabbits who lost the corneal reflex in the HC-3 treated eye, the ACh had fallen to 56 per cent or less of the control value. In one animal the reflex was just beginning to disappear and the level of ACh was 57 per cent of control. Animals that did not lose the reflex retained 73 per cent or more of the endogenous ACh. It is also clear that, in general, ACh levels of less than 0·1 μg/mg protein are correlated with loss of reflex. This is obviously not the case in rabbits with a low normal ACh content.

TABLE 2. EFFECT OF HC-3 ON CORNEAL REFLEX AND LEVELS *IN VIVO* OF RABBIT CORNEAL EPITHELIAL ACh*

Animal no.	Control eye (μg ACh/mg protein)	Control reflex	Treated eye (μg ACh/mg protein)	Corneal reflex	ACh remaining in treated eye(%)
1	0·19	Brisk	0·06	None	31
2	0·16	Brisk	0·09	None	56
3	0·23	Brisk	0·10	None	43
4	0·14	Brisk	0·05	None	36
5	0·07	Brisk	0·04	Slow	57
6	0·15	Brisk	0·11	Brisk	73
7	0·20	Brisk	0·22	Brisk	110
8	0·07	Brisk	0·08	Brisk	113
9			0·03	None	
10			0·03	None	
11			0·01	None	
12			0·10	Brisk	
13			0·23	Brisk	

* From 20 to 50 μl of 1% HC-3 was administered by subconjunctival injection or injection into the anterior chamber of the "treated" eye. As soon as the corneal reflex had disappeared, the epithelium from both treated and control eyes was scraped into 5% TCA and ACh was measured as before. Each pair of determinations was obtained from one animal. The control levels for animals 9–13 were not determined.

In order to assess the possibility that the HC-3 which was entering the general circulation might be exerting an effect on ACh synthesis in the control eye without depleting it sufficiently to interfere with corneal reflex in that eye, a second approach was undertaken. Table 3 summarizes results obtained when ACh in the control eye was assayed prior to injecting the experimental eye with HC-3 to produce a loss of reflex. There was no difference in ACh content from injected animals providing further substance to the observation that loss of reflex is associated with a decline in ACh to approximately 60 per cent or less of its original concentration.

In a final aspect of the study (Table 4), the preceding approach was reversed. After determining the ACh content of the experimental eye as soon as the reflex disappeared, the animals were allowed to recover for 1 week. At the end of this time, the control eye was injected with the same amount of HC-3 and the reflex was lost in that eye. After 3 days the reflex in the control eye had returned and ACh was measured. In these animals the absolute values for ACh were very low in control eyes, possibly due to a

TABLE 3. EFFECT OF HC-3 ON CORNEAL REFLEX AND LEVELS *IN VIVO* OF RABBIT CORNEAL EPITHELIAL ACh*

Control eye (μg ACh/mg protein)	Corneal reflex	Treated eye (μg ACh/mg protein)	Corneal reflex	ACh remaining in treated eye (%)
0·29	Brisk	0·06	None	21
0·15	Brisk	0·04	None	27
0·20	Brisk	0·12	None	60

* The ACh level in the control eyes was determined prior to injecting the anterior chamber of the "treated" eyes with sufficient 1% HC-3 to cause loss of corneal reflex. ACh was determined as soon as the reflex disappeared. Each pair of determinations was obtained from one animal.

TABLE 4. EFFECT OF HC-3 ON CORNEAL REFLEX AND LEVELS *IN VIVO* OF RABBIT CORNEAL EPITHELIAL ACh*

Control eye (μg ACh/mg protein)	Corneal reflex	Treated eye (μg ACh/mg protein)	Corneal reflex	ACh remaining in treated eye (%)
0·06	Brisk	0·03	None	50
0·09	Brisk	0·06	None	67
0·11	Brisk	0·07	None	64

* Sufficient 1% HC-3 was injected into the anterior chamber of the "treated" eyes to cause loss of corneal reflex and the epithelial ACh was extracted and assayed. After 1 week, the contralateral control eyes of the same animals were injected with the same quantity of 1% HC-3 and the reflex disappeared. Seventy-two hr later the reflex had returned in the control eyes and the ACh levels were then determined in the same manner. Each pair of determinations was obtained from one animal.

residual HC-3 effect. However, the relative amounts of ACh in control vs. experimental eyes were again in a range similar to that observed in the preceding experiments.

DISCUSSION

That ACh acts as the chemical mediator in the transmission of nerve impulses at certain types of synapses in the peripheral nervous system has been well documented. To date, no substance has been identified as a neuro-transmitter in sensory fibers. However, ACh has been implicated as the initiator of impulses in carotid body chemoreceptors,[11–15] thermal receptors,[16] taste fiber endings,[17] cutaneous mechanoreceptors[18,19] and in the auditory system.[20] In Loewi-type experiments, electrical stimulation of a "donor" carotid body induced an increase in the sensory discharge of the downstream preparation.[14] This effect was potentiated by eserine, depressed by hexamethonium and blocked by acetylcholinesterase. Also, HC-3 depressed the response of chemoreceptors to natural stimulation.[13–15] Many reports have been based primarily on studies in which applied or injected ACh caused an afferent discharge by the sensory fibers; for example, Diamond[18] found that the close arterial injection of ACh into regenerating sural nerves of rabbits resulted in a burst of impulses. Under the same conditions, no such response was seen with regenerating motor

nerve fibers nor with normal motor nerves. In other experiments, ACh synthesis in the cat cochlear nucleus was inhibited by an injection of HC-3 and depletion of residual ACh by olivary stimulation. Under these conditions, the normal physiological effects of stimulating the superior olive were blocked.[20]

There have also been reports in the literature which suggest a relationship between ACh and pain. The injection of a high concentration of ACh into the brachial artery in man[19] was observed to produce, in addition to transient paresis of motor power, severe pain in the extremity. In a study on the nature of the fluid in the common nettle hair (Urtica Urens),[21] histamine and ACh were identified. The ACh content of a single hair was reported as 0·01–0·4 μg. In an attempt to reproduce the burning pain caused by the nettle hair fluid, solutions of comparable concentrations of ACh alone, histamine alone, and ACh in combination with histamine were pricked into the skin. ACh alone produced no sensation and histamine alone was responsible for the triple response and itching. When both were present, however, the painful sensation was achieved. It seems reasonable that histamine may have acted in this case to remove permeability barriers so that ACh achieved access to receptors. Other investigators[22] found that ACh (10^{-5} g/ml) applied to base of an exposed cantharidin blister produced pain which began as soon as the solution touched the blister area, and disappeared in 15–45 sec. The area was then refractory to further applications for 5 min. In the presence of atropine, the touch response of the rabbit cornea was abolished, also suggesting a role for ACh in the mediation of sensory impulses.[23]

The results which we obtained following local corneal denervation in rabbits, in which there was a decrease of 87–100 per cent in epithelial ACh at the time of complete nerve degeneration, may be construed as supportive evidence for a neuronal location of ACh in this tissue. It is further substantiated by the correlation between loss of corneal reflex in response to touch and the decrease in ACh in the epithelium to 60 per cent or less of its original level following HC-3 inhibition of its synthesis. That this loss of corneal sensitivity with HC-3 is not due merely to a local anesthetic effect of the drug is demonstrated by the fact that the reflex disappeared only after 45 min. The observed effect was not caused by the neuromuscular blocking action of HC-3 since the animals retained full mobility and were able to retract their heads from a painful stimulus. Furthermore, the blinking reflex could be demonstrated if the eye was approached slowly from an angle at which the animal could readily observe. The fact that touch sensitivity in the rabbit cornea disappears when roughly 40 per cent of the ACh stores are depleted suggests that a certain critical level of ACh is requisite to pain perception. It is also possible that two pools of the neurohumor are present in this tissue, one which is important in pain and one which is not. If this is the case, it may be that greater than a 40 per cent decrease actually exists in the pool which is significant in pain perception at the time when corneal sensitivity disappears.

Arguments against the consideration of a role for ACh in sensory perception are based on the fact that the effect of exogenously administered ACh can be blocked by large doses of nicotine or ACh plus eserine, as well as compounds such as hexamethonium or D-tubocurarine, while the response to physiological stimuli remains intact.[24–28] It is possible that these drugs may be more readily accessible to the receptors than ACh, or that free nerve endings in the cornea are different from other sensory endings. Zander and Weddell[29, 30] observed two types of fibers in the cornea, plain and beaded, with the latter exhibiting axonal swelling of various degrees. Prince[31]

assumes that each bead may be a sensory element which serves to amplify the effect of the ending. The evidence that ACh is not only affiliated with nerves but must be present in a certain minimum concentration for the sensation of pain to be experienced suggests that a role for ACh as a sensory mediator must be further considered.

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