7. Xue GP, Rivero BP, Morris RJ. The surface glycoprotein Thy-1 is excluded from growing axons during development: A study of the expression of Thy-1 during axogenesis in hippocampus and hindbrain. *Development.* 1991;112:161–176.
8. Ahmad I, Redmond LJ, Barnstable CJ. Developmental and tissue-specific expression of the rod photoreceptor cGMP-gated ion channel gene. *Biochem Biophys Res Commun.* 1990;173:463–470.
9. Mize RR. Quantitative image analysis for immunocytochemistry and in situ hybridization. *J Neurosci Methods.* 1994;54:219–237.
10. Perry VH, Morris RJ, Raisman G. Is Thy-1 expressed only by ganglion cells and their axons in retina and optic nerve? *J Neurocytol.* 1984;13:809–824.
11. Morris R, Raisman G. Estimation of Thy-1 in cryostat sections of nervous tissue. *J Neurochem.* 1983;40:637–644.

Carbachol Does Not Correct the Defect in the Phagocytosis of Outer Segments by Royal College of Surgeons Rat Retinal Pigment Epithelial Cells

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Purpose. To investigate the effect of carbachol on the phagocytosis of photoreceptor outer segments (OS) in cultures of normal Long–Evans and dystrophic Royal College of Surgeons (RCS) rat retinal pigment epithelial (RPE) cells.

Methods. Retinal pigment epithelial cells from normal and RCS rats were grown in tissue culture. On reaching confluence, they were presented with OS suspended in Krebs–Henseleit buffer in the presence or absence of carbachol and LiCl. The number of bound and ingested OS was quantitated using double immunofluorescence staining.

Results. LiCl inhibited the ingestion of OS by more than 90% but had no effect on the binding of OS by Long–Evans RPE cells. The addition of carbachol further reduced OS ingestion. Carbachol alone decreased OS ingestion by normal RPE cells by 30% but had no effect on OS binding. The effect of LiCl and carbachol on RCS RPE cells was similar to their effect on normal RPE cells.

Conclusions. Carbachol does not increase OS phagocytosis in normal or RCS rat RPE cells. The phagocytic defect in RCS rat RPE cannot be reversed or overcome by stimulation of the IP_3 pathway by carbachol. LiCl strongly inhibits the ingestion of OS by normal and by RCS RPE cells, and this effect is enhanced by carbachol. Invest Ophthalmol Vis Sci 1996;37:1473–1477.

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The phagocytosis of the shed tips of photoreceptor outer segments (OS) is one of the many important functions of the retinal pigment epithelium (RPE). Recent evidence suggests that this is a receptor-mediated process (for review see[1]), in which a receptor (phagocytosis receptor) on the surface of the RPE interacts with a ligand on the plasma membrane of the OS. As with most other receptor-mediated processes, it is likely that binding of an OS to the receptor triggers an intracellular signaling event, which causes the RPE to ingest the bound OS. The target of such a signal may well be the cytoskeletal elements that are abundant in both the RPE microvilli and in the cortical area underlying the plasma membrane.[2,3] Nothing is known about the signaling pathway that may operate in this phagocytic function. Previous studies from this laboratory have investigated the possible role of the protein kinase C pathway[4] as well as of the protein kinase A pathway[5,6] as mediators of OS ingestion. However, stimulation of both these pathways causes an inhibition of OS ingestion, without causing a parallel decrease in the binding of OS. This indicates that the effect of stimulating these pathways is intracellular, rather than extracellular (i.e. they do not affect the number or the affinity of OS-binding sites on the plasma membrane).

A well-studied model for investigating the process of OS phagocytosis is the Royal College of Surgeons (RCS) rat, which suffers from an inherited retinal degeneration.[7] The RPE of this animal displays limited phagocytosis of shed OS tips,[8,9] with a resultant slow degeneration of the OS. Studies using RPE cells grown in tissue culture have demonstrated clearly that the mutation affects the ingestion phase of OS phagocytosis because binding of OS occurs normally.[10] This suggests that either the phagocytosis receptor is defective and thus is unable to transmit a signal to the cell interior or that the intracellular signaling pathway to which the receptor is linked is defective.

Recent studies have suggested that the second messenger involved in OS phagocytosis may be inositol trisphosphate (IP_3). This is based on two lines of

evidence. First, the RCS RPE does not show the same pattern of protein phosphorylation when challenged with OS, as is observed in normal RPE.[11] Second, IP_3 levels are increased in normal RPE in response to challenge with OS, whereas RCS RPE does not show such an increase.[12] However, the cascade that produces IP_3 is intact in RCS RPE because it can be activated by carbachol.[12] These authors have suggested that the pathway for IP_3 production is intact in RCS RPE but is not activated by the binding of OS to the phagocytosis receptor. Based on these previous observations, it was reported recently that the phagocytic defect in RCS RPE can be overcome by stimulating the IP_3 cascade with carbachol during OS phagocytosis.[13] Because of the importance of this observation in providing a springboard for future investigation into the control of OS phagocytosis and for understanding the interactions of OS with the RPE, we have attempted to repeat this observation. To obtain results comparable to those published, we have duplicated the published procedures as closely as possible.

METHODS. Growth of Retinal Pigment Epithelial Cells. Retinal pigment epithelial cells are isolated from 9- to 12-day-old normal Long–Evans (LE) or RCS rats using a previously reported method.[14] They are grown either in RPMI-1640 (RPMI) or in Eagle's minimum essential medium (EMEM) containing 20% fetal bovine serum (FBS), plus 40 μg/ml kanamycin, 40 μg/ml gentamycin, and 2 mM glutamine in all cases. For phagocytosis studies, 30,000 RPE cells in 100 μl of one of the above media are seeded on 18-mm glass disks and used after reaching confluence in 6 to 7 days. Before use, disks are transferred to individual wells of a 12-well cluster plate.[10] The use and treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Quantitation of OS Phagocytosis. Outer segments are isolated from Long–Evans rats older than 30 days of age.[10] Before phagocytosis, OS are suspended in Krebs–Henseleit (KH) buffer containing 20% FBS.[13] LiCl is added at a final concentration of 50 mM if required. Phagocytosis is initiated by adding 1×10^7 OS to each well in 1 ml of KH buffer and incubating for 2 hours at 37°C. The OS-containing medium is removed and replaced with 1 ml of the same medium, with or without 1 mM carbachol, and incubation is continued for another 30 minutes.[13] Disks are rinsed in PBS to remove unbound OS and fixed in 3.5% formaldehyde. Bound OS are stained using an antiserum to bovine OS, followed by biotin-conjugated second antibody and finally with fluorescein–streptavidin. After permeabilizing the cells,[10] ingested OS are stained in the same manner with the exception that Texas Red conjugated streptavidin is used. Using this method, bound OS are stained green when viewed with a fluorescein filter set, whereas total OS (bound plus ingested) are stained red using a rhodamine filter set. Thus, by subtracting the number of bound OS from the total number of OS, the number of OS that are ingested can be calculated. Confluent areas of cells are selected randomly under transmitted light. Bound and ingested OS are then counted under epifluorescent illumination using a 1-cm^2 ocular grid at a final magnification of 375×. Each treatment within an experiment is carried out on duplicate disks, and at least five grid areas are quantitated on each disk.[10] Thus, $n = 10$ for each data point within an experiment. Each experiment was repeated at least twice. Means and standard deviations were calculated. Data were analyzed statistically using Student's t-test. P values < 0.05 were considered statistically significant.

RESULTS. In our first attempt to duplicate the published data, OS were fed to RPE cells in KH buffer containing 20% FBS and 50 mM LiCl according to Heth et al,[13] after growing the cells in EMEM containing 10% FBS. The ingestion of OS by LE RPE was essentially eliminated under these conditions (data not shown). The number of OS ingested by normal RPE cells in this buffer was identical to the number ingested by RCS RPE cells. Addition of carbachol further reduced OS ingestion by both cell types (data not shown).

We assumed that phagocytosis was reduced dramatically in the above experiment because of the use of KH buffer and, thus, repeated the experiment after suspending OS in normal growth medium or EMEM plus 20% FBS, as well as in KH buffer plus 20% FBS. It was apparent from that experiment that LiCl, which was included in the incubation medium, was responsible for the decrease in OS ingestion (data not shown). Thus, we performed the experiment shown in Figure 1. Cells were grown in EMEM or RPMI plus 20% FBS, and OS were fed in KH buffer plus 20% FBS. As shown in this figure, normal RPE cells ingest OS equally well whether they are grown in EMEM or RPMI, whereas RCS RPE cells do not phagocytose significant numbers of OS in either medium. When LiCl is included in the incubation medium, OS ingestion by LE RPE cells is inhibited by more than 90% whereas OS binding is not significantly affected. LiCl also significantly reduces the ingestion of OS by RCS RPE.

Figure 1 shows that LiCl alone exerts a significant inhibitory effect on OS ingestion. It seemed possible that a stimulatory effect of carbachol might be masked by the inhibitory effect of LiCl. We tested the effect of carbachol in both the presence and absence of LiCl.

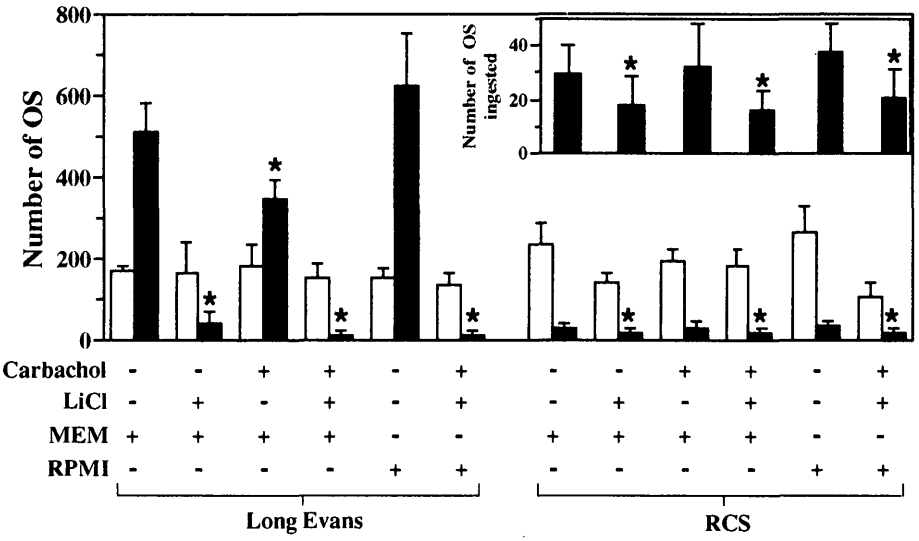

FIGURE 1. The effect of LiCl and carbachol on the phagocytosis of outer segments (OS) by Long–Evans and Royal College of Surgeons (RCS) rat retinal pigment epithelial (RPE) cells. Cells were grown in either RPMI or EMEM containing 20% fetal bovine serum (FBS). Outer segments were suspended in Krebs–Henseleit buffer plus 20% FBS and fed to RPE cells in this buffer for 2 hours, after which carbachol was added and incubation was continued for another 30 minutes. LiCl was present throughout the incubation. (*inset*) The number of OS ingested by RCS RPE is replotted on an expanded scale to show more clearly the differences between the various treatments. (□) = OS bound; (■) = OS ingested. *Significantly different from the respective control (EMEM or RPMI only) at $P < 0.05$. RPMI = RPMI-1640; EMEM = Eagle's minimum essential medium.

Carbachol alone reduces the ingestion of OS by normal RPE cells by 40% but did not reduce the ingestion of OS by RCS RPE cells. It is, however, obvious that carbachol alone does not increase OS ingestion by the mutant RPE cells. In fact, incubation with carbachol plus LiCl further reduces OS ingestion by normal and RCS RPE cells over that seen with LiCl alone.

DISCUSSION. We have attempted to repeat the observation of Heth et al[13] that stimulation of the IP_3 pathway by carbachol is able to correct the phagocytic defect in RCS RPE cells. Despite repeated experiments in which the experimental design was varied, we have been unable to confirm the reported ability of carbachol, and thus IP_3, to stimulate the ingestion of OS by RCS RPE. LiCl strongly inhibits the ingestion of OS by LE RPE and further reduces the already attenuated ingestion of OS seen in RCS rat RPE cells. Carbachol alone reduces the ingestion of OS by normal RPE but has no effect on the ingestion of OS by RCS RPE. Carbachol, in the presence of LiCl, increases the inhibition of OS ingestion over that caused by LiCl alone in both normal and RCS RPE.

If our observations are correct, it is important to define where an error in the analysis of data was made by Heth et al.[13] We have duplicated the incubation conditions reported in that article as closely as possible but see no difference between the medium used by Heth (RPMI) and that which we routinely use (EMEM). We think the problem lies with the inclusion of LiCl in the incubation medium for the phagocytosis studies and with the very small numbers of OS ingested by RCS RPE cells under any conditions. LiCl is used to inhibit the hydrolysis of IP_3. However, it inhibits OS ingestion almost completely and reduces the measurement of ingestion to the level of "noise" in our results and in the data of Heth et al.[13] Normal RPE cells ingest 400 to 600 OS per grid area in the absence of LiCl (Fig. 1). In the presence of LiCl, the number of OS ingested by normal RPE is reduced to less than 50 per grid area, and, in the presence of LiCl plus carbachol, the number is reduced to approximately 20 per grid area. This level of ingestion is probably nonspecific and is no different than the number of OS ingested by RCS RPE under the same conditions.

In the study reported by Heth et al,[13] the *total* number of OS present per grid area was reported to be 15.6 ± 3.9 (8.7% ingested = 1.4 OS) in the absence of carbachol and 9.5 ± 3.0 (33.7% ingested = 3.2 OS) in the presence of carbachol. In that study, carbachol (plus LiCl) decreased the total number of OS per grid (from 15.6 to 9.5). We observed an identical effect of carbachol plus LiCl in both normal and RCS rats, i.e., a decrease in the total number of cell-associated OS. If the difference reported by Heth et al[13] is real, the

observed increase of 1.8 (3.2 to 1.4) ingested OS per grid area in the presence of carbachol could be interpreted as a fourfold increase in the percent of OS ingested (8.7% versus 33.7%). However, with so few OS to count, these numbers are not distinguishable from noise and, as in our data, are caused by nonspecific ingestion. Additionally, this increase is less than the reported standard deviation of 3.

Heth et al[13] also state that treatment of RCS RPE with carbachol increases OS phagocytosis to "normal levels,"[10] that is, to the levels seen with normal rat RPE over the same period of incubation. Although it is true that approximately 35% of the cell-associated OS are ingested after a 30-minute incubation,[10] the actual number of OS ingested in 30 minutes in those experiments was 30 to 50 per grid area versus 3.2 OS per grid area as reported by Heth et al[13] (approximately 10 to 16 times more OS per grid). If carbachol corrected the defect in OS phagocytosis by RCS RPE, the number of ingested OS should be increased to the levels found in normal rat RPE. Obviously, this did not occur. It is misleading to state that phagocytosis in RCS RPE is increased to normal levels after carbachol treatment.

One difference between the methods reported here and those used in the previous study is the age of the rats from which RPE cells were isolated; Heth et al[13] used 5- to 6-day-old rats whereas we used 9- to 12-day-old rats. If OS phagocytosis is activated by IP_3 in the RPE cells of the younger rats, and not in the older rats, it is most likely that such activation is not specific to the phagocytosis of OS, because 5-day-old rats do not possess OS. It also seems unlikely that such an important signaling process would be turned off at an age when phagocytosis is about to occur.

We have not measured IP_3 levels on stimulation with carbachol. However, Osborne et al[15] and Heth et al[12,13] have shown a threefold to fourfold increase in IP_3 under the conditions we have used in the experiments reported here. Thus, we have no reason to doubt that such a stimulation of IP_3 production is occurring.

The experiments reported here do not support the suggestion that the generation of IP_3 can correct the phagocytic defect in the RCS RPE. Our results conclusively show that this does not occur. We suggest reasons for the interpretation of data that led Heth et al[13] to the conclusion that IP_3 production is deficient in OS phagocytosis by RCS RPE.

Unfortunately, these results leave us without a viable explanation of the phagocytic defect in the RCS RPE or of a confirmed second messenger involved in the initiation of OS ingestion. The three sites proposed by Heth et al[13] are still candidates for the expression of the mutation in the RCS rat. These are: the cytoplasmic tail of the receptor (it is clear that OS binding occurs normally in this animal[10,16]—thus, the external environment of the phagocytosis receptor seems to be normal); an abnormality in the linkage (G protein?) between the receptor and the signaling pathway; and a defect in a component of the signaling pathway itself. Because OS phagocytosis is a unique and highly specialized function of the RPE, it is possible that this cell expresses unique components of the known signaling pathways or has developed a pathway specialized to this one function.

Key Words

carbachol, outer segments, phagocytosis, retinal pigment epithelium, Royal College of Surgeons rat

References

1. McLaughlin BJ, Cooper NGF, Shepherd VL. How good is the evidence to suggest that phagocytosis of ROS by RPE is receptor mediated? In: Osborne NN, Chader GJ, eds. *Progress in Retinal and Eye Research.* Oxford: Pergamon; 1994;13:147–164.
2. Lutz DA, Chen XM, McLaughlin BJ. Characterization of phagosome-associated cytoskeleton isolated from retinal pigment epithelial cells. ARVO Abstracts. *Invest Ophthalmol Vis Sci.* 1992;33:910.
3. Burnside B, Laties AM. Actin filiments in apical projections of the primate pigmented epithelial cell. *Invest Ophthalmol Vis Sci.* 1976;15:570–575.
4. Hall MO, Abrams TA, Mittag TW. ROS ingestion by RPE cells is turned off by increased protein kinase C activity and by increased calcium. *Exp Eye Res.* 1992;52:591–598.
5. Hall MO, Abrams TA, Mittag TW. The phagocytosis of rod outer segments is inhibited by drugs linked to cyclic adenosine monophosphate production. *Invest Ophthalmol Vis Sci.* 1993;34:2392–2401.
6. Kuriyama S, Hall MO, Abrams TA, Mittag TW. Isoproterenol inhibits rod outer segment phagocytosis by both cAMP-dependent and independent pathways. *Invest Ophthalmol Vis Sci.* 1995;36:730–736.
7. Dowling JE, Sidman RL. Inherited retinal dystrophy in the rat. *J Cell Biol.* 1962;14:73–109.
8. Bok D, Hall MO. The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. *J Cell Biol.* 1971;49:664–682.
9. Herron WL, Riegel BW, Meyers DE, Rubin ML. Retinal dystrophy in the rat-a pigment epithelial disease. *Invest Ophthalmol Vis Sci.* 1969;8:595–604.
10. Chaitin MH, Hall MO. Defective ingestion of rod outer segments by cultured dystrophic rat pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 1983;24:812–820.
11. Heth C, Schmidt SY. Protein phosphorylation in retinal pigment epithelium of Long–Evans and Royal College of Surgeons rats. *Invest Ophthalmol Vis Sci.* 1992;33:2839–2847.

12. Heth C, Marescalchi P. Inositol triphosphate generation in cultured rat retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 1994;35:409–416.
13. Heth C, Marescalchi P, Ye L. IP_3 generation increases rod outer segment phagocytosis by cultured Royal College of Surgeons retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 1995;36:984–989.
14. Mayerson PL, Hall MO, Clark VM, Abrams TA. An improved method for isolation and culture of rat retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 1985;26:1599–1609.
15. Osborne NN, FitzGibbon F, Schwartz G. Muscarinic acetylcholine receptor-mediated phosphoinositide turnover in cultured human retinal pigment epithelium cells. *Vision Res.* 1991;31:1119–1127.
16. Hall MO, Abrams TA. Kinetic studies of rod outer segment binding and ingestion by cultured rat RPE cells. *Exp Eye Res.* 1987;45:907–922.