
Substance P (SP), a neuropeptide, has been found in amacrine cells in a variety of vertebrate retinas. SP excited many of the ganglion cells we sampled in carp retina; many of these ganglion cells are directly excited by cholinergic agonists. It has been reported that SP modulates cholinergic synaptic transmission in some other neuronal systems by inhibiting or desensitizing cholinergic receptors. SP does not act in this way on the ganglion cell cholinergic receptors in isolated carp retina. Pulses of acetylcholine (ACh), applied to sensitive ganglion cells by microiontophoresis, were set to elicit consistent cellular responses. Application of SP to the retina through a nebulizing system (final concentration about $10^{-6}$M) did not reduce the excitation produced by the ACh pulse. In earlier work, we showed that the cholinergic receptors were nicotinic; in the present study, SP occasionally excited cells after the retina was treated with a nicotinic cholinergic antagonist, gallamine triethiodide. SP appears to excite cholinergic-sensitive ganglion cells at a postsynaptic site other than their nicotinic cholinergic receptor and via a pathway that does not require a presynaptic, SP-sensitive, ACh-releasing interneuron.

The neuropeptide substance P (SP) has been found in retinal tissue in various species, particularly in amacrine cells. SP excites ganglion cells in carp retina and mudpuppy retina. In the former it is predominantly the on-responding ganglion cells that are excited. Many of these neurons are also excited by acetylcholine (ACh) via postsynaptic cholinergic receptors. These receptors have been characterized as being excited by cholinergic agonists, blocked by the nicotinic antagonists d-tubocurarine and gallamine triethiodide, and unaffected by the muscarinic antagonist, atropine. Studies have shown that SP can block or desensitize the cholinergic receptor. In the cat, SP applied iontophoretically onto Ben-shaw cells blocks ACh-mediated excitation of these cells. SP desensitizes and inhibits nicotinic, cholinergic responses in cultured adrenal "para-neurons" and in a clonal line of cultured nerve cells.

In seeking evidence of an SP-ACh interaction at cholinergic receptors on carp retinal ganglion cells, we found that SP did not block the response of sensitive cells to repeated applications of ACh from a microiontophoretic pipette. Some ganglion cells had different responses to ACh and SP. In some cases SP could excite ganglion cells after the cholinergic receptors had been blocked with gallamine triethiodide. We therefore concluded that SP and ACh do not act at the same site on retinal ganglion cells.

**Materials and methods.** The experiments were performed on the isolated retina of the carp, Cyprinus carpio. After isolation from a fish, the retina was maintained receptor side up in an oxygenated atmosphere. Ganglion cell activity was recorded extracellularly with a metal-filled glass microelectrode. The ganglion cells were classified as on-center, off-center, or on/off center, sustained or transient types, by their response to spots and annuli of light flashed in their receptive fields. We assumed that when maximal action potential amplitude was obtained, the tip of the recording electrode was near the ganglion cell bodies. ACh and other pharmacologic agents were introduced into the retina through a cluster of three microiontophoretic pipettes whose tips were displaced approximately 30 µm from the tip of the recording electrode, toward the photoreceptor layer. Consequently, the 30 µm displacement probably located the microiontophoretic pipette tips in the inner plexiform layer, the locus of ganglion cell dendrites. This arrangement gave the best results in the iontophoretic studies.

ACh, applied to local groups of ganglion cells by microiontophoresis, excited some ganglion cells, particularly the on/off types. To control for current artifacts, iontophoretic current was passed through a NaCl-containing microiontophoretic pipette. SP was applied to the entire retina through a nebulizer. Nebulizers were activated by a stream of air at a regulated pressure of 5 to 7 psi (35 to 48 KiloPascals), which was pulsed on for precisely timed intervals by a solenoid-operated valve. The concentration of SP in the retina was estimated on the basis of the amount of material ejected from the nebulizer by each air pulse. Bovine serum albumin (0.1%) was added to the SP solutions to prevent the peptide from adhering to the glass walls of the nebulizer.

**Results.** Interaction between SP and ACh was tested by spraying SP onto the retina while repetitively exciting a ganglion cell with ion-
Fig. 1. Computer-generated raster display of the response of an on-center sustained ganglion cell to a 0.4 mm spot flashed in its receptive field and to ACh applied through an iontophoretic pipette placed near the cell. Each horizontal row of tick marks represents one episode of stimulation; each individual tick represents one action potential. Successive stimulus presentations are placed below one another in registration. Light stimuli were presented at 30 sec intervals; 20 sec of each stimulus episode are shown in each "sweep." **Top,** Response to the spot stimulus is shown at left; the response to a 1 sec pulse of ACh applied with 20 nAmp from the iontophoretic barrel is shown at right. A brief artifact at the make and break of the current pulse is seen. SP did not reduce the cell's response to exogenously applied ACh. **Bottom,** To control for pH and current artifacts, a 20 nAmp current was passed from an adjacent NaCl-filled barrel. Apart from the switching transient, the current had no effect on the activity of the cell.

If SP exerts its effect through the cholinergic receptor, then all cells sensitive to ACh should also be sensitive to SP. We tested 64 ganglion cells, including examples of all response types, with SP and cholinergic agonists. Only 14 cells (22%) were affected by both agents. In some cases, cobalt treatment, which blocks synaptic transmission, made the ganglion cell insensitive to one of the agents. This suggests that the SP and cholinergic agonists act at different sites. In the experiment illustrated in Fig. 2, Co++ blocked the cell's response to iontophoretically applied ACh. The unit shown in Fig. 1 gave an on-center sustained response to a 0.4 mm spot. ACh, ejected with 20 nAmp of iontophoretic current, strongly excited the cell. SP, at an estimated concentration of $2 \times 10^{-6}$M (10 times the threshold for SP excitatory actions), did not decrease the cell's response to ACh (Fig. 1, **top**). Small variations in background spontaneous firing rate or light response sometimes occurred but could not be distinguished from mechanical effects on retinal activity occasionally seen after application of pharmacologic agents through the nebulizer. To control for current effects, 20 nAmp of current was passed from a second, NaCl-filled barrel adjacent to the ACh barrel. Although a switching artifact was present at the make and break of the iontophoretic current (Fig. 1, **bottom**), it was clearly distinguishable from the ACh response of the cell (Fig. 1, **top**).
Fig. 2. Ratemeter record of an on/off ganglion cell (off response is more prominent than on response). Top trace, Upward deflections show the onset of the spot stimulus. Middle trace, Activity of the cell in impulses per second. Bottom trace, Application of pharmacologic agents to the cell. The upward deflection (marked "Co++") indicates when cobalt chloride solution was nebulized onto the retina, resulting in the loss of the light response and spontaneous activity. During the period of blockade, ACh was applied to the cell through an iontophoretic pipette using 80 nAmp of current (shown as downward deflections marked "ACh 0.80"). SP was applied with a nebulizer (time of application shown as an upward deflection marked "SP"). Note that SP was able to excite the cell during the cobalt blockade, and ACh was not. See text for further details.

Response both to light and to iontophoretically applied ACh, but SP applied through the nebulizer to an estimated retinal concentration of 10 μM evoked maintained firing from the cell. The lack of a light response during this period indicated that synaptic transmission was still blocked by Co++. At the right side of Fig. 2, the trace shows the light response recovering, during which time a pulse of ACh produced additional excitation in the cell.

Of the other ganglion cells, 19 (30%) responded only to cholinergic agonists, 17 (26%) responded only to SP, and 14 (22%) showed no response to either cholinergic agonists or SP. Considering the cells by their response type, we observed that unsustained ganglion cells frequently responded to SP only (8/19) and were the least likely to respond to both ACh and SP (3/19). Compared with cells of other response types, on/off and on-transient cells more frequently responded to both ACh and SP (4/15 and 3/8, respectively). However, 6/15 on/off cells responded to ACh only. No clear trends emerged in the other response classes.

These experiments required a cellular response to one of the agents and then the application of the other agent to a superthreshold level (typically 10 times the threshold). This concentration could be estimated when SP was applied through the nebulizer, but the concentration of ACh ejected from the iontophoretic pipette was difficult to determine. Moreover, the tip of the iontophoretic pipette might have been too far from the ACh receptor to activate the cell under study. In some experiments, however, CCh was applied to the retina through a nebulizer. Because the retinal concentration of this agent could be estimated after nebulizing, and because CCh resists hydrolysis by cholinesterases, the failure of SP-sensitive cells to respond to CCh in these experiments indicates non-interaction of SP and ACh.

On two occasions, SP excited ganglion cells after nicotinic, cholinergic receptors were blocked by application of gallamine triethiodide (Flaxedil; Davis and Geck) to the retina. In the experiment illustrated in Fig. 3, SP (5 × 10⁻⁷M) excited an on/off cell to an elevated rate of spontaneous discharge and an enhanced light response (Fig. 3, A and B). Flaxedil (8 × 10⁻⁶M) blocked the cell's light response (Fig. 3, C), but subsequent application of SP during the Flaxedil blockade again elicited increased, spontaneous firing (Fig. 3, D). During this period the absence of the light response indicated that the cholinergic receptors, which presumably mediate the light response, were still blocked by the antagonist.

Discussion. Our findings do not support the hypothesis that SP acts by directly modulating the cholinergic receptor. Our results, along with the determination that SP is effective at concentrations of 10⁻⁷M to 10⁻⁶M, strongly suggest that a specific receptor for SP is located on certain retinal neurons. We have observed that...
Fig. 3. Interval histograms show the response of an on/off cell treated with Flaxedil (a cholinergic antagonist) and SP. The vertical calibrations indicate five spikes and the interval (bin width) is 50 msec. Each series of five histograms shows the response of the cell after the application of the indicated agent. A, Unit gives an on/off response to 0.6 mm spot flashed in its receptive field. A 2 sec, 30 nAmp pulse of ACh (shown in middle of histograms) excited the cell. B, SP increased spontaneous activity and enhanced the cell’s light response. C, Flaxedil was applied 4 min after SP. Although there is little or no light response and no excitation by the iontophoretically applied ACh, spontaneous firing is markedly increased.

ganglion cells in the presence of synaptic blocking agents; thus the receptors are probably located on ganglion cell membranes, particularly on cells with an on-component in their light response. The present results, however, do not exclude the possibility of SP receptors on other retinal neurons.

Our conclusion that SP and ACh act at different cellular sites is supported by similar findings in neurons of the interpeduncular nucleus and of the locus coeruleus. The retinal function of SP and the physiologic conditions mediating its release are unknown at present. The nature and duration of its effects, however, suggest it may have a role in resetting the long-term excitability of ganglion cells.


Key words: substance P, acetylcholine, retinal receptors, ganglion cells, carp

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$^{13}$C-Nuclear magnetic resonance studies of sugar cataractogenesis in the single intact rabbit lens. R. Gilberto Gonzalez, James Willis, James Aguayo, Patrick Campbell, Leo T. Chylack, Jr., and Thomas Schleich.

In this report we demonstrate that $^{13}$C-nuclear magnetic resonance spectroscopy may be used to monitor the sorbitol and glycolytic pathways in a single, intact, crystalline lens of the rabbit, thereby providing a new, noninvasive method for the study of sugar cataractogenesis. This approach is particularly useful for the measurement of intralenticular metabolic fluxes in single lenses under various conditions, at a time resolution previously unavailable in lens biochemistry. (Invest Ophthalmol Vis Sci 22:808-811, 1982.)

Exposure of the crystalline lens from several species to elevated glucose levels in vivo or in vitro leads to rapid opacification, the so-called "sugar" cataract. That the sorbitol pathway plays a fundamental role in sugar cataract formation in animals has been established by decades of careful, reproducible experimentation. The sorbitol pathway is also suspected to be an important factor in the approximately five-fold greater incidence of cataracts occurring in humans with diabetes mellitus. Recent publications, however, indicate that the susceptibility of the human lens to sorbitol accumulation differs from the animal lens in several significant aspects. In fact, one recent report suggests that the sorbitol pathway may actually protect the lens against osmotic stress derived from rapid fluctuations in blood-aqueous humor glucose concentrations. It is clear that the response of the human lens to high-glucose stress must be carefully quantitated, and not merely assumed to be similar to that of animal lenses. The scarcity of normal human lenses for experimentation and the atypical features of plentiful cataractous lenses make such study extremely difficult. This report describes a relatively new, noninvasive technique, $^{13}$C-nuclear magnetic resonance (NMR) spectroscopy, for the study of the sorbitol pathway in the intact crystalline lens. Because relatively few lenses are required to gain extensive metabolic information and because the time response of a single intact lens to high-glucose stress may be followed by this technique, it offers an improved means by which to study the role of the sorbitol pathway in human cataractogenesis.

This accessory pathway of glucose metabolism is comprised of two enzymes: aldose reductase, with nicotinamide-adenine dinucleotide phosphate as a cofactor, reduces glucose to sorbitol; polyol dehydrogenase, with reduced nicotinamide-adenine dinucleotide as cofactor, oxidizes sorbitol to fructose. When exposed to high glucose the lens accumulates sorbitol, and since this polyol is impermeable, its accumulation raises the cytoplasmic osmolarity. As water enters, the lens swells and ultimately opacifies.

Materials and methods. The lenses used for this study were obtained from New Zealand white rabbits weighing 0.7 kg. The rabbits were sacrificed by air embolus and the eyes were enucleated. The globe was entered posteriorly by way of a cruciate incision; the sclera was peeled back and cut, the vitreous was stripped off, and the zonules were cut. The lens was carefully removed with a plastic scoop and placed in a 10 mm NMR tube fitted with a Teflon stage to position the lens at the proper height. TC-199/bicarbonate incubation medium (290 ± 3 mOsm/L) containing supplemental glucose as previously described was used. The incubation medium contained 30% $^2$H$_2$O. The lens and the incubation medium were maintained under an atmosphere of 5% CO$_2$ and 95% air at 37°C and 100% humidity. The NMR tube also contained an eccentrically placed capillary tube filled with $^{13}$C-enriched methanol, which served as an external standard. In the experiments described here, one lens was subjected to 35.5 mM glucose 90% enriched with $^{13}$C at the C-1 position (obtained from Merck, Sharp & Dohme) for a total of 48 hr. The contralateral lens was subjected to a normal (5.5 mM) glucose load, which was similarly enriched with $^{13}$C, for 24 hr and was then subjected to a high-glucose load (35.5 mM) during the ensuing 24 hr. For spectral accumulation, the enriched medium was removed and the lens was washed with the corresponding unenriched medium and incubated in unenriched medium for the duration