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CONDITIONAL INHIBITION OF SCREENING-PIGMENT AGGREGATION BY LIDOCAINE IN CRAYFISH PHOTORECEPTORS AND FROG RETINAL PIGMENT EPITHELIUM

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Summary

Lidocaine, at concentrations equal to or lower than those that inhibit fast axoplasmic transport, was found to interfere with the dark-adapting migration of the screening pigments along crayfish photoreceptors and within the cells of the frog retinal pigment epithelium (RPE). The effects of the anesthetic on pigment movements were studied in isolated eyes incubated under light or dark conditions in media of different ionic compositions. Treatment of crayfish eyes with 25 mmol l^{-1} lidocaine in normal Van Harreveld's saline arrested pigment migration to the dark-adapted position or caused migration towards the light-adapted position in the dark. Similar results were obtained with frog eyecups exposed to 5 mmol l^{-1} lidocaine in Ringer's solution. In each case, the inhibition of dark adaptation was reversible and dependent on the levels of Na^+ and Ca^{2+} in the incubation medium. A dark-adapted position of both pigments was compatible with lidocaine treatment provided that low-Na^+, or high-Ca^{2+} or Co^{2+}-containing solutions were used. These results indicate that light-adapted and dark-adapted pigment positions in both types of retinal cells can occur in the absence of local nervous input. Further, the data suggest a direct effect of lidocaine upon the photoreceptors or RPE cells. The inhibition of pigment aggregation is interpreted to be a consequence of an anesthetic-induced increase in the permeability of the plasma membrane, which in turn affects the intracellular ionic balance that controls pigment position.

Introduction

In addition to their effect on membrane excitability, local anesthetics are known to impair the transport of intracellular components along nerve cell processes. Procaine, lidocaine, tetracaine and other analogous compounds reversibly inhibit

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fast axoplasmic transport in mammalian and frog nerve at concentrations generally higher than those required to block the propagation of the electric impulse (Fink *et al.* 1972; Anderson and Edström, 1973; Aasheim *et al.* 1974; Fink and Kish, 1976; Lavoie, 1982*a*,*b*, 1983). These two actions of the local anesthetics are not necessarily correlated as regards their effective concentrations. Whereas dibucaine is slightly more potent than tetracaine on both excitability and axoplasmic transport, etidocaine is as potent as tetracaine on excitability but five times less effective in inhibiting transport (Lavoie, 1982*b*). Therefore, different actions of the drugs may be responsible for the effects observed on the two physiological processes.

In contrast to the vast literature concerned with the mechanisms of action of local anesthetics, comparatively few reports are available on the inhibitory action of these compounds on intracellular transport. Electron microscopy studies of axons treated with local anesthetics have shown reductions in the numbers of microtubules (Fink *et al.* 1972; Edström *et al.* 1973). Nevertheless, though such alteration of the cytoskeleton would be expected to interfere with its fundamental role in intra-axoplasmic motion, the decrease in microtubule count does not always correlate with inhibition of transport (Byers *et al.* 1973, 1979; Lavoie *et al.* 1989). The events leading to these morphological effects of the local anesthetics are far from understood, and it is conceivable that the drugs might have a concomitant, or even earlier, influence on other factors critical for intracellular transport. One of these, depletion of metabolic energy, has been ruled out as the cause of axoplasmic transport inhibition by lidocaine and has been shown to be, at most, only partly responsible for the inhibition observed with other local anesthetics (Lavoie *et al.* 1989).

This paper reports a first survey of the effects of lidocaine on two experimental models used for the study of intracellular transport: the migration of screening-pigment granules along the photoreceptors of the crayfish compound eye and within the cells of the frog retinal pigment epithelium (RPE). Like fast axoplasmic transport, screening-pigment migration in both types of retinal cells is dependent on microtubules (Frixione *et al.* 1979; Frixione, 1983*a*,*b*; Burnside *et al.* 1983) and on oxidative metabolism (Frixione *et al.* 1979; Mondragón and Frixione, 1989). Unlike fast axoplasmic transport, which is a rapid, continuous process, screening-pigment migration is a relatively slow response to changes in illumination and can be activated by light or darkness, even under *in vitro* conditions (Snyder and Zadunaisky, 1976; Frixione *et al.* 1979; Burnside and Basinger, 1983; Dearry and Burnside, 1984; Mondragón and Frixione, 1989). The movement of pigment in the RPE also differs from both fast axoplasmic transport and pigment migration in crustacean photoreceptors in that the pigment response to light – but not to darkness – is mediated by actin filaments in addition to microtubules (Burnside *et al.* 1983).

Pigment migration in the crayfish photoreceptor closely resembles fast axoplasmic transport in nerves in that the pigment granules move along the axon, advancing anterogradely – i.e. away from the nucleus and in the same direction

that the visual input is conducted – during dark-adaptation and retrogradely as they slip back into the cell body when returning to the light-adapted position. In contrast to typical axoplasmic transport, however, the pigment granules shuttle within a restricted area of the photoreceptor length, so that they never get down to the synaptic terminals. In the RPE cells, the pigment moves within several slender processes that stem from the cell body, invading these projections in the light and retreating towards the cell body in the dark. Although in both systems the pigment movements are bidirectional, the granules migrate in just one direction at any given time, the movement being in this regard analogous to that of chromatophores (Rodionov *et al.* 1991). As in chromatophores, movement in one direction aggregates the granules whereas the reverse motion causes dispersal, so the pigment redistributes according to the physiological state of the cells; in both the crayfish photoreceptors and the frog RPE the pigment aggregates as it moves to the dark-adapted position and is dispersed by the action of light. Dark-adaptation is in each case more dependent on metabolic energy than is light-adaptation (Frixione *et al.* 1979; Mondragón and Frixione, 1989).

Our experiments show that lidocaine blocks pigment migration to the dark-adapted position or promotes movement in the opposite direction in the dark in the two types of retinal cells at concentrations equivalent to, or lower than, those found to inhibit fast axoplasmic transport. Tests with ionic substitution in the incubation media indicate that the effect is dependent on the external levels of Na^+ and Ca^{2+}. The results suggest that lidocaine affects pigment migration by shifting the ionic balance of the cytoplasm in each case and argue against the necessary involvement of an efferent nervous influence in the control of pigment position.

Materials and methods

The procedures followed for dissection, incubation and fixation of the two types of eyes have been described in detail elsewhere (Frixione *et al.* 1979; Mondragón and Frixione, 1989). Briefly, eyestalks severed from adult crayfish of the species *Procambarus clarkii* (Girard) were dissected to remove the optic ganglia, leaving intact the lamina ganglionaris, which is intimately associated with the axons of the photoreceptors. Frog eyecups were prepared by enucleation of eyes excised from *Rana pipiens* (Schreber). In each case, three or four prepared eyes were then fastened to a rubber base at the bottom of a wide-mouthed 100 ml glass vial containing 6–10 ml of the desired incubation medium (see Tables 1 and 3) under an O_2-rich atmosphere. Lidocaine chlorhydrate (2 % stock solution from Astra Chemicals, S.A., México) was diluted in the test media to give the indicated concentrations. All solutions were buffered at pH 7.4 with 10 mmol l^{-1} imidazole, maintained at 17 °C, and changed under strictly light-proof conditions when so required. Incubation with frequent mild agitation was carried out for the specified periods in a dark-room or under approximately 500 lx of fluorescent illumination. Each group of eyes was then fixed (heat/formaldehyde method for crayfish eyes;

2.5 % glutaraldehyde in Ringer for frog retinas; see above references for details) at the appropriate time either in the light or in total darkness.

After fixation, crayfish eyes were sagittally bisected and measurements of the pigment position over the longitudinal axis were taken with a micrometer installed in a stereomicroscope. The frog eyecups were divided into narrow strips, postfixed with 1 % OsO_4, dehydrated through ethanol, and embedded in Spurr's resin. The pigment position in the RPE was then micrometrically determined in semi-thin sections (150–200 nm) stained with Toluidine Blue and cut parallel to the photoreceptors located around the posterior pole of the eye. Micrographs of crayfish eyes were obtained with a 35 mm camera coupled to a stereomicroscope, and those of the frog RPE were produced with a photomicroscope. At least three eyes were included in each experiment with a given set of conditions, and all experiments were repeated at least twice, so the data shown in Fig. 2 and Tables 2 and 4 represent averages and standard deviations of determinations of pigment position in a minimum of six different eyes.

Results

Crayfish photoreceptors

Fig. 1A,B shows the sagittal plane of two crayfish eyes, fixed and bisected after being incubated in normal saline (solution A, Table 1, slightly modified from van

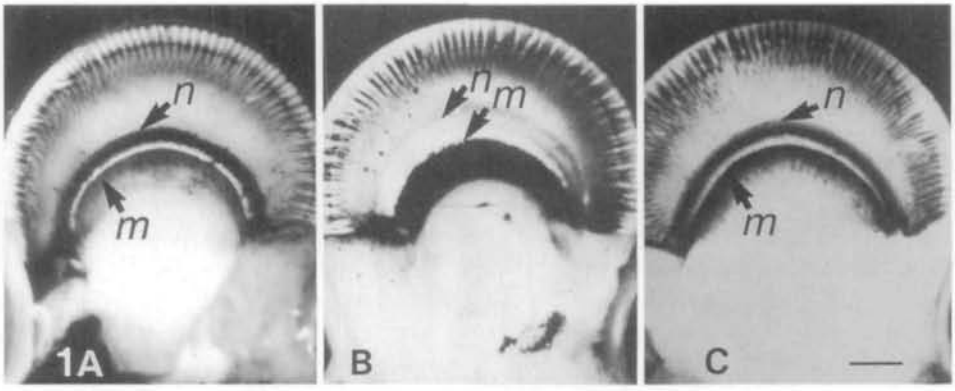

Fig. 1. Sagittal sections of crayfish eyes incubated in the light (A) or in darkness (B,C) in normal saline for freshwater crustaceans (A,B), or in saline containing 25 mmol l^{-1} lidocaine (C). Lidocaine antagonizes pigment aggregation to the dark-adapted position or promotes pigment dispersion in the absence of light. The basal membrane (*m*) and the nuclear ends (*n*) of the photoreceptors constitute the limits of the effective distance of migration and the landmarks of reference for determining the pigment index, as explained in the text. The incubation conditions correspond to those given in the footnotes of Table 2, as follows: A, condition *; B, condition †; C, condition ‖. Scale bar, 500 μm.

Table 1. *Composition of solutions used for incubation of crayfish eyes (in mmol l^{-1})*

	Solution	Na^+	K^+	Ca^{2+}	Mg^{2+}	Co^{2+}	Cl^-	Choline	Sucrose	Imidazole (pH 7.4)
A	Van Harreveld's (VH)	205	5.3	13.5	2.6	–	242.5	–	–	10
B	VH–choline	–	5.3	13.5	2.6	–	242.5	205	–	10
C	Sucrose	–	–	–	–	–	–	–	300	10
D	VH–high-Ca^{2+}	205	5.3	150	2.6	–	515.5	–	–	10
E	VH–Co^{2+}	205	5.3	13.5	2.6	15	272.5	–	–	10

Harreveld, 1936) for full adaptation to either light or darkness. In the light-adapted state (Fig. 1A), the photoreceptor layer of the retina appears to be filled with the screening pigment up to the distal or nuclear ends of the photoreceptors (see also scheme in Fig. 4; for histological details see Krebs, 1972; Nässel, 1976). In the dark-adapted condition (Fig. 1B), the pigment is found aggregated below the basal membrane of the retina, where it accumulates within the axons of the photoreceptors. When the pigment is so retracted, the photoreceptor cells become fully exposed, with a consequent increase in visual sensitivity. As the pigment recedes or expands between these extreme positions, its outer margin travels about 180 μm in an adult crayfish. Since the length of the cells themselves does not vary during these movements, a convenient pigment index (PI) can be obtained as the quotient of two measurements (Frixione and Aréchiga, 1981): (*a*) the distance from the basal membrane to the outer margin of the pigment mass, which varies with the level of adaptation; and (*b*) the distance from the basal membrane to the distal tip of the photoreceptors, which is constant for a given eye. Then $PI=a/b$ is a normalized figure which, under usual conditions, takes values ranging from 1.0 for maximal pigment dispersion in the light to 0.0 for extreme aggregation in the dark. The data shown in Fig. 2 and Table 2 are expressed in terms of this assessment convention.

Pre-incubation of crayfish eyes for 90 min in the light with saline containing 25 mmol l^{-1} lidocaine blocked pigment migration to the dark-adapted position (Table 2). A PI of nearly 1.0 was obtained in all treated eyes after subsequent incubation in the same solution for 120 min in darkness, when the controls were typically dark-adapted. The inhibitory effect of the anesthetic on pigment aggregation is largely reversible. Since, even under normal conditions, complete dark-adaptation occurs just once in isolated eyes, reversibility was tested with experiments that involved a single challenge in the dark. Eyes pre-incubated in the light for 90 min in the presence of 25 mmol l^{-1} lidocaine and then thoroughly washed with normal saline, aggregated the bulk of the pigment mass to the dark-adapted position when placed in darkness for 120 min. It should be noted, though, that the ability to recover from the treatment was not as homogeneous as might be inferred from the PI values in Table 2, because some of these eyes showed faint traces of pigment left in the proximal half of the effective migration zone. In a second protocol, the eyes were exposed to lidocaine for 30 min in the light and

Table 2. *Effect of 25 mmol l^{-1} lidocaine on screening-pigment position in crayfish photoreceptors*

Incubation	Pigment index
VH in the light (control light-adapted)*	1.00±0.03
VH in the light, then to dark (control dark-adapted)†	0.18±0.02
VH with lidocaine in the light, then to dark†	1.00±0.04
VH with lidocaine in the light, washed, then to dark‡	0.15±0.06
VH with lidocaine, in the light, then to dark, washed and kept in darkness§	0.23±0.08
VH with lidocaine in the dark‖	0.75±0.04
VH–choline with lidocaine in the dark‖	0.02±0.01
Sucrose solution with lidocaine in the dark‖	0.02±0.01
VH–high-Ca^{2+} with lidocaine in the dark‖	0.02±0.01
VH–Co^{2+} with lidocaine in the dark‖	0.02±0.01

Values are mean±S.D., $N \geq 6$.
See text for method of calculation of pigment index.
* Incubated for 90 min in the light.
† Pre-incubated for 90 min in the light and then kept in darkness for 120 min.
‡ Pre-incubated 90 min in the light, washed and then kept in darkness for 120 min.
§ Pre-incubated with lidocaine for 30 min in the light and 90 min in darkness, then washed and incubation in the dark continued for 120 min.
‖ Dark-adapted for 90 min in the corresponding solution, then lidocaine added and incubation continued for 180 min.

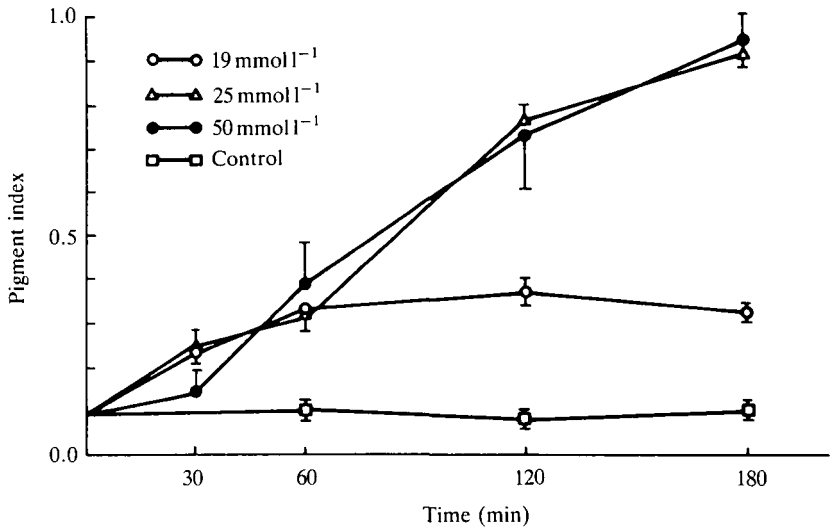

Fig. 2. Time course of pigment dispersion to the light-adapted position induced in the dark by exposure of dark-adapted crayfish eyes to various concentrations of lidocaine. Dark-adapted eyes transferred to fresh saline without the anesthetic maintain a stable pigment aggregation for several hours in the dark. Each point represents an average of at least four eyes, with standard deviation of the mean depicted by vertical lines.

90 min in the dark, before repeated washing and continued incubation in darkness for an additional 120 min. Pigment aggregation in this case was less complete (about 75 % of the maximum in average), but nevertheless characteristic of dark-adaptation and clearly different from the PI value of 0.87±0.07 (S.D., N=6) observed in the controls, which were not washed. Also, the region deserted by the pigment in the washed eyes was remarkably clean in comparison with the same region after treatment with the first protocol.

The anesthetic not only inhibits pigment aggregation, but also promotes pigment dispersion in the absence of light. When dark-adapted eyes were exposed to 25 mmol l^{-1} lidocaine in the dark the pigment dispersed to near the fully light-adapted position over 3 h, a period during which the photoreceptors can easily maintain a dark-adapted state (Figs 1C and 2). This light-adapting effect of lidocaine was considerably reduced when the concentration of anesthetic was lowered by just 25 % (i.e. down to 19 mmol l^{-1}) and it was virtually undetectable below this level. In contrast, doubling the concentration (to 50 mmol l^{-1}) produced pigment dispersion with the same time course as with 25 mmol l^{-1} lidocaine (Fig. 2).

Even though lidocaine produces pigment dispersion by itself, the natural response of the screening pigment to light could be detected in photoreceptors treated with the drug. Incubation of dark-adapted eyes with 25 mmol l^{-1} lidocaine for 2 h in the dark (i.e. when according to data in Fig. 2 the drug-induced migration has reached a PI value of about 0.75), followed by illumination for 60 min, resulted in a uniform PI of 1.0. This value is significantly higher (P<0.05 by the Student's t-test) than that produced by lidocaine during an equivalent total period in the dark (Fig. 2), indicating that the anesthetic does not interfere with the pigment movement elicited by light. This finding was not surprising because pigment dispersion is a relaxed state to which the cells revert when exposed to light and to many disruptive agents, including metabolic inhibitors (Frixione *et al.* 1979). Therefore, all the further tests of lidocaine on crayfish photoreceptors were designed to look at its effects on the ability of the cells to maintain the aggregation of pigment in the dark-adapted position, which is the active phase of adaptation. From the above results, a 2 h exposure of dark-adapted eyes to 25 mmol l^{-1} anesthetic was selected as the standard treatment.

Studies on the ionic dependence of the screening-pigment responses in arthropod photoreceptors have suggested that a light-elicited rise in intracellular levels of Ca^{2+} and/or Na^{+} is probably involved in the mechanism that drives the pigment to the light-adapted position (Kirschfeld and Vogt, 1980; Frixione and Aréchiga, 1981; Howard, 1984). Local anesthetics have recently been reported to alter the normal fluxes of Na^{+} and Ca^{2+} across plasma membranes in preparations of synaptosomes (García-Martín *et al.* 1990) and epithelial cells (Shibamoto *et al.* 1990), so one possible mechanism for the inhibitory effect of lidocaine on pigment aggregation is that it may increase Na^{+} and/or Ca^{2+} levels in the photoreceptor cells. To test this hypothesis, we tested the anesthetic in media lacking these ions (solutions B and C, Table 1). The eyes were first dark-adapted in the modified

solutions, which permit or even enhance pigment aggregation in the dark (Frixione and Aréchiga, 1981), and they were then transferred in complete darkness to the same medium containing lidocaine. Substitution of Na^+ with choline (solution B) eliminated the pigment dispersion induced by the anesthetic in the normal saline (Table 2). A similar protection was observed with an imidazole-buffered 300 mmol l^{-1} sucrose solution (solution C), that is in the absence of all the physiological ions.

It has been reported that abnormally high concentrations of Na^+ and Ca^{2+} in the external medium have opposite effects on pigment position (Frixione and Aréchiga, 1981). High Na^+ concentration inhibits, whereas high Ca^{2+} concentration facilitates, aggregation, and the addition of Co^{2+} to the normal saline also favors the dark-adapted state. Hence, it was of interest to find out whether the divalent cations could protect the photoreceptors against the dispersive action of lidocaine on the pigment. Eyes dark-adapted in high-Ca^{2+} or in Co^{2+}-containing media (solutions D and E, Table 1) were able to keep the pigment in the dark-adapted position despite the ensuing addition of the drug (Table 2). Most eyes incubated in the modified solutions presented the maximal pigment aggregation characteristically produced in such media in the absence of anesthetic.

Frog retinal pigment epithelium

The RPE cells are attached at their basal ends to Bruch's membrane of the retina and present long apical projections that interdigitate with the outer segments of the photoreceptors (Fig. 3 and scheme in Fig. 4; for a detailed morphological description, see Nguyen-Legros, 1978). Under bright illumination numerous pigment granules disperse along the narrow projections, forming a light-absorbing screen around the outer segment of each photoreceptor (Fig. 3A). In the dark the granules migrate towards the basal ends of the epithelial cells (Fig. 3B), so the full length of each photoreceptor becomes exposed and visual sensitivity is increased. As a result of these movements the distance from Bruch's membrane to the edge of the pigment mass changes by about 70 μm. Thus, as in the case of crayfish eyes, the pigment position can be expressed as a normalized index obtained from the quotient of two measurements (Burnside *et al.* 1983; Mondragón and Frixione, 1989): (*a*) the distance from Bruch's membrane to the edge of the pigment, which varies with adaptation; and (*b*) the distance from Bruch's membrane to the outer limiting membrane of the retina, which is fixed in each particular eye. Typical extreme values of $PI=a/b$ are 1.0 for full light adaptation and 0.3 for complete dark adaptation, both of which occur and are stable *in vitro* for up to 6 h when the eyecups are incubated in Ringer (solution A, Table 3) under the present conditions (Mondragón and Frixione, 1989).

Except for its effective concentration, lidocaine affected the RPE and the crayfish photoreceptors in a very similar way. Preliminary tests showed that 5 mmol l^{-1} anesthetic in the Ringer is a critical level, which has an effect on pigment position and can be tolerated by the tissue. Higher concentrations ($\geq$10 mmol l^{-1}) caused detachment of the retina from the RPE, particularly in

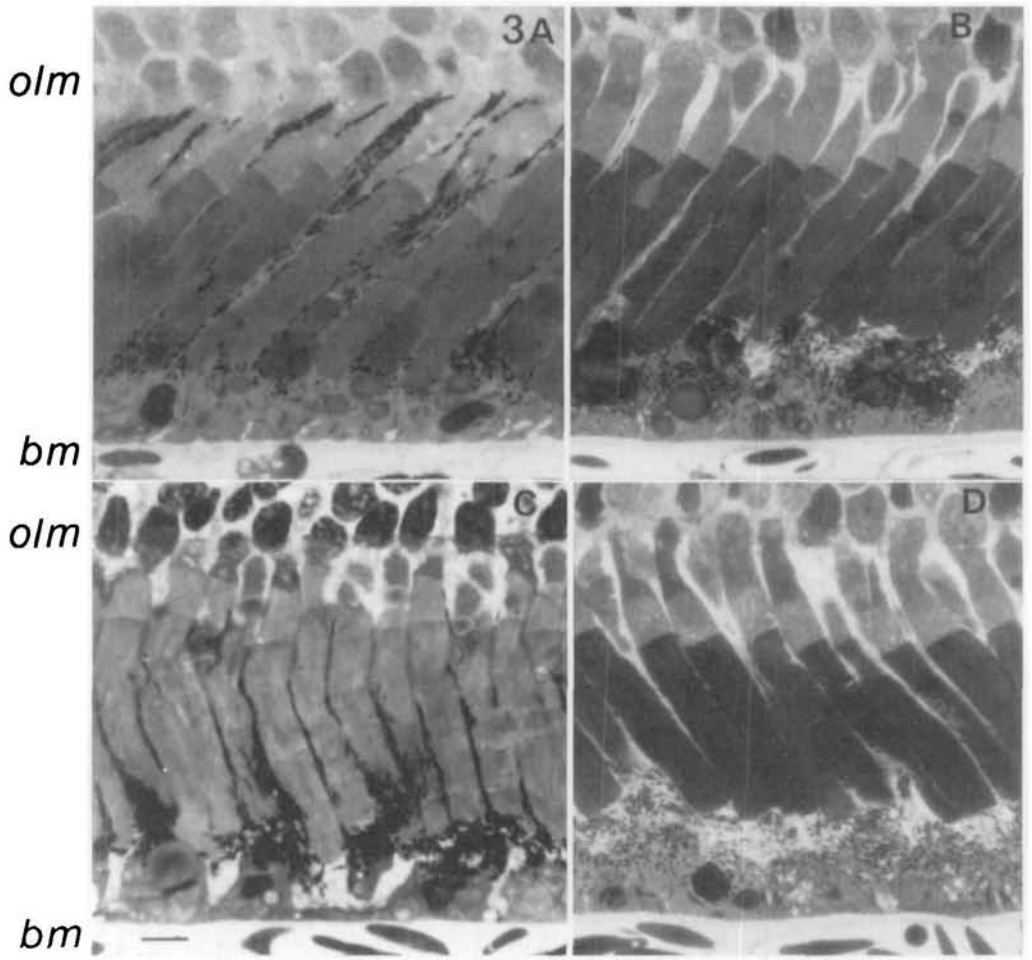

Fig. 3. Longitudinal sections of frog retinas incubated in the light (A) or in darkness (B–D) in normal Ringer (A,B), or in normal Ringer (C) or Na^+-free Ringer (D) containing 5 mmol l^{-1} lidocaine. Lidocaine antagonizes pigment aggregation to the dark-adapted position, i.e. it promotes pigment dispersion in the absence of light. Bruch's membrane (*bm*) and the outer limiting membrane (*olm*) of the retina constitute the limits of the effective distance of migration and the landmarks of reference for determining the pigment index (PI), as explained in the text. The incubation conditions correspond to those given in the footnotes of Table 4, as follows: A, condition *; B, condition †; C,D, condition §. Scale bar, 10 μm.

dark-adapted eyes, whereas lower levels (≤1 mmol l^{-1}) failed to produce clear and reproducible effects on pigment position. Therefore 5 mmol l^{-1} lidocaine was used for the following experiments.

Pigment aggregation to the dark-adapted position was arrested when the Ringer contained lidocaine (Table 4). All the eyecups gave a PI close to 1.0 after 3 h of dark incubation in the presence of the drug, in contrast with an average PI value of

Table 3. *Composition of solutions used for incubation of frog retinas (in mmol l^{-1})*

	Solution	Na^+	K^+	Ca^{2+}	Co^{2+}	Cl^-	Choline	Glucose	Imidazole (pH 7.4)
A	Ringer	110	2	1.8	–	115.6	–	20	10
B	Ringer–choline	–	2	1.8	–	115.6	110	20	10
C	Ringer–high-Ca^{2+}	110	2	9.0	–	130	–	20	10
D	Ringer–Co^{2+}	110	2	1.8	5	125.6	–	20	10

Table 4. *Effect of 5 mmol l^{-1} lidocaine on screening-pigment position in the frog retinal pigment epithelium*

Incubation	Pigment index
Ringer in the light (control light-adapted)*	1.00±0.02
Ringer in the light, then to dark (control dark-adapted)†	0.32±0.02
Ringer with lidocaine in the light, then to dark†	1.00±0.02
Ringer with lidocaine in the light, washed, then to dark‡	0.38±0.08
Ringer with lidocaine in the dark§	0.68±0.13
Ringer–choline with lidocaine in the dark§	0.33±0.02
Ringer–high-Ca^{2+} with lidocaine in the dark§	0.30±0.03
Ringer–Co^{2+} with lidocaine in the dark§	0.34±0.03

Values are mean±S.D., $N \geq 6$.
See text for method of calculation of pigment index.
* Incubated for 15 min in the light.
† Pre-incubated for 15 min in the light and then kept in darkness for 180 min.
‡ Pre-incubated for 15 min in the light, followed by 120 min in the dark with lidocaine, washed and kept in the dark for an additional 180 min.
§ Dark-adapted for 180 min in the corresponding solution, then lidocaine added and the incubation continued for 120 min.

0.32 for the controls. Treatment of dark-adapted eyes with the anesthetic in the dark dispersed the pigment up to a PI value of 0.68 within 2 h (Fig. 3C). Dark-adapted eyecups transferred in the dark to Ringer with anesthetic, and then incubated for 2 h in darkness before 60 min of illumination, gave a uniform PI of 1.0. Since the total incubation time in this case was 6 h, i.e. the maximum period during which the eyecups can maintain the dark-adapted state, full pigment dispersion under these conditions may reflect a decreased metabolic capacity of the cells in addition to the combined result of the treatment with the drug and the effect of illumination. However, as with crayfish eyes, it is clear that lidocaine blocks pigment aggregation but does not interfere with the movement to the full light-adapted position in RPE cells.

Lidocaine-treated eyecups showed morphological alterations of the retinal tissue. The photoreceptors appeared crumpled, and wide interstitial spaces separated the epithelial cells (Fig. 3C). These histological changes were not completely reversible, even though the pigment-transporting ability was recovered

after removal of the anesthetic. Eyecups incubated with lidocaine for 2 h in the dark, and then changed several times to fresh Ringer in darkness, had the pigment aggregated in the typical dark-adapted position after an additional 3 h of incubation.

The movement of pigment in the RPE has also been found to be sensitive to the ionic balance in the external and intracellular environments, particularly as regards Ca^{2+} and Na^{+} (Snyder and Zadunaisky, 1976; Dearry and Burnside, 1984; Mondragón and Frixione, 1989). Therefore, for the reasons already mentioned for crayfish eyes, we tested lidocaine in combination with manipulations of the incubation medium (solutions B–D, Table 3). Replacement of Na^{+} with choline in the Ringer (solution B) prevented the disruptive effects of the anesthetic on both dark-adaptation and retinal morphology (Table 4 and Fig. 3D). Equivalent results were obtained when lidocaine was applied in the presence of a fivefold increase in the normal concentration of Ca^{2+} (solution C) or with the addition of 5 mmol l^{-1} Co^{2+} to the Ringer (solution D).

Fig. 4 summarizes schematically these observations on the effects of lidocaine on screening-pigment behavior in the two types of retinal cells. In each case treatment of the isolated eyes with the anesthetic blocked pigment migration to the aggregated, energy-requiring state characteristic of dark-adaptation. The pigment was thus found in these eyes in a fully light-adapted position, equivalent to that shown by the drawings on the left. Moreover, lidocaine antagonized the aggregated state because it caused pigment reversion towards the dispersed, light-adapted position when administered to dark-adapted eyes subsequently maintained in the dark (drawings on the right). Nevertheless, the inhibitory action of lidocaine on dark-adaptation was found to depend on the composition of the incubation medium; low-Na^{+}, high-Ca^{2+} or Co^{2+}-containing solutions allowed dark-adapted eyes to hold the pigment in the fully aggregated state despite the presence of the drug. Therefore, the pigment position under these conditions was typical of dark-adaptation (illustrated by the drawings in the middle).

Discussion

Screening-pigment position is controlled through intrinsic and extrinsic mechanisms in arthropod photoreceptors. The cells are known to react to light and darkness as independent pigmentary effectors (Frixione *et al.* 1979; Kirschfeld and Vogt, 1980), but they are also subject to an efferent nervous input that influences the adaptation state according to a circadian rhythm (see Barlow *et al.* 1989). Recent evidence suggests that pigment aggregation in crayfish photoreceptors is facilitated through an efferent serotonergic pathway (Frixione and Hernández, 1989; Aréchiga *et al.* 1990). Likewise, dopamine, γ-aminobutyric acid and other neurotransmitters have been shown to modulate retinomotor movements in some vertebrate eyes (Dearry and Burnside, 1986*a*,*b*). Thus, although isolated eyes are disconnected from the central nervous system, it is possible that the local circuitry may still promote pigment aggregation in the dark, e.g. by spontaneous firing of

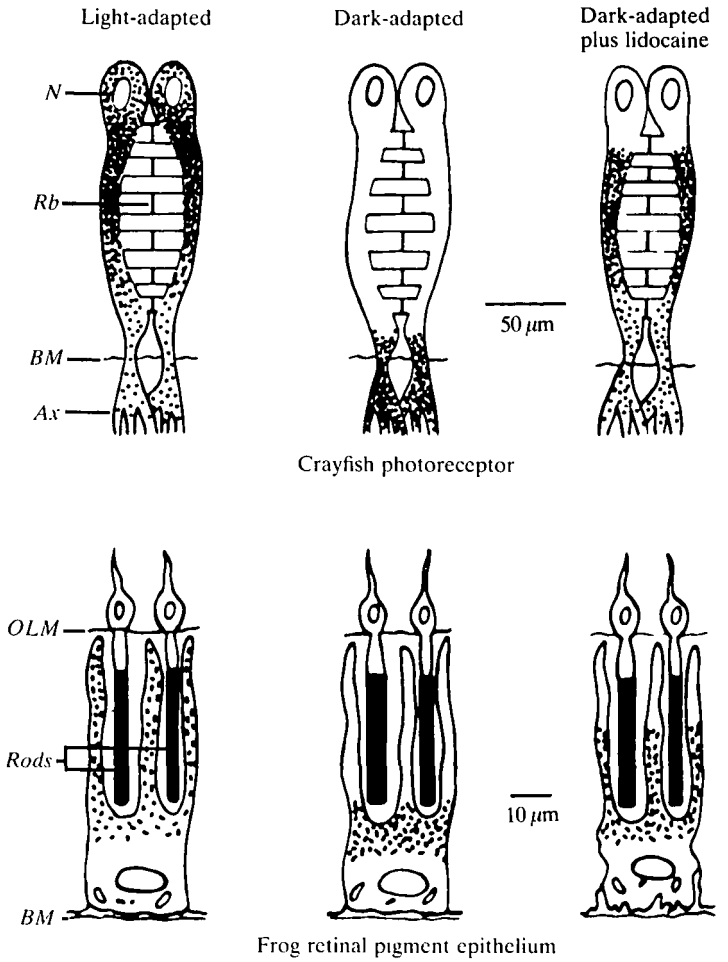

Fig. 4. Schematic illustration of the effects of lidocaine on the position of the screening pigments in crayfish photoreceptors (top) and frog RPE cells (bottom). Fully light-adapted eyes, or eyes placed in the dark while treated with lidocaine in the relevant saline, have the pigment dispersed along the corresponding cells (left). In contrast, eyes dark-adapted in the normal saline without anesthetic show cells with the pigment aggregated (middle). Dark-adapted eyes are able to maintain this state in the dark despite the addition of lidocaine if low-Na^+, high-Ca^{2+} or Co^{2+}-containing solutions are used for the incubation, but they revert towards the light-adapted condition in the dark if the drug is added to the normal saline (right). *N*, *Rb* and *Ax* indicate, respectively, the nucleus, the rhabdom or photosensitive structure, and the axon of crayfish photoreceptors. *OLM* and *Rods* indicate the outer limiting membrane and the photoreceptor cells in the frog retina. *BM* corresponds to the basal membrane of the crayfish eye and to Bruch's membrane in the frog retina.

the severed efferent fibers or activation of certain interneurons. In the presence of an action-potential-blocking and neurotransmission-depressing agent such as lidocaine (Tabatabai and Booth, 1990) most, if not all, of these extrinsic

mechanisms may be expected to be inoperative. Therefore, the failure of the anesthetized eyes to aggregate their pigment or to keep it in that position could result from the lack of some facilitatory influence. This interpretation can be virtually excluded by the observation that in both preparations the effect of the drug was dependent upon the ionic composition of the incubation medium. The retinal cells were able to keep the screening pigment in the aggregated state in the presence of lidocaine provided that Na^+ was excluded from the medium; elevated Ca^{2+} concentration or the addition of Co^{2+} also prevented the inhibition. While a high Ca^{2+} concentration could perhaps promote the release of a facilitatory neurotransmitter and thus bypass the effect of lidocaine, a low Na^+ concentration and especially the presence of Co^{2+} should antagonize synaptic transmission (Dearry and Burnside, 1986*a*,*b*). Although an anesthetic-induced selective release of light-adapting neuromodulators cannot be rigorously ruled out, the present results seem to indicate that the light-adapted and dark-adapted positions of the screening-pigments can occur in both types of eye without the mediation of synaptic inputs. Moreover, the demonstrated ability of local anesthetics to inhibit fast axonal transport suggests that pigment migration may be impaired by a direct effect of lidocaine on the photoreceptors and RPE cells.

In the crayfish photoreceptor – a primary neuron – lidocaine affected the anterograde migration of the screening pigment to the dark-adapted position and its maintenance in the aggregated state at concentrations (19–25 $mmol\,l^{-1}$) equivalent to those reported to inhibit fast axoplasmic transport (Fink *et al.* 1972; Anderson and Edström, 1973; Byers *et al.* 1973; Lavoie, 1982*b*). However, the level of anesthetic in the interstitial spaces around the photoreceptors is probably lower than this, because even small ions appear to diffuse slowly into perfused nervous tissues of crustaceans (Abbott *et al.* 1975). Therefore, the effective concentration is likely to be closer to that found to inhibit pigment aggregation in the frog RPE (5 $mmol\,l^{-1}$), a much thinner and presumably more readily perfusable preparation. Such levels are within the range in which lidocaine abolishes excitability, but not axoplasmic transport, in nerve (Fink *et al.* 1972; Anderson and Edström, 1973; Lavoie, 1982*b*). Accordingly, pigment migration in the two types of retinal cells appears to be more sensitive to the anesthetic than is protein transport along axons. The accumulated evidence from these various systems suggests that lidocaine – and perhaps other local anesthetics – interferes with a basic mechanism common to different types of intracellular transport.

Pigment aggregation in the dark-adapted position is the active, energy-demanding phase of the adaptation cycle in both crayfish photoreceptors and RPE cells, whereas movement in the opposite direction seems to be the result of relaxation induced by light or limiting conditions (Snyder and Zadunaisky, 1976; Frixione *et al.* 1979; Mondragón and Frixione, 1989). Since oxidative metabolism in nervous tissue and other systems has been found to be sensitive to local anesthetics (Geddes and Quastel, 1956; Haschke and Fink, 1975; Tarba and Cracium, 1990), one reasonable assumption is that lidocaine might diminish the energy available in the retinal cells for aggregating the pigment and holding it in

that state. However, the occurrence of stable pigment aggregations in low-Na^+ media, despite the presence of lidocaine, is contrary to this view. The same observation argues against the inhibition being due to a specific effect of the anesthetic on microtubules (Fink *et al.* 1972; Edström *et al.* 1973), which are involved in pigment migration to the dark-adapted position in crayfish photoreceptors (Frixione *et al.* 1979; Frixione, 1983*a*,*b*) and RPE cells (Burnside *et al.* 1983). Hence, as has been concluded in the case of fast axoplasmic transport (Lavoie *et al.* 1989), the effect of lidocaine on the movement of the screening pigment is unlikely to be the result of an action of the drug on the structure or the fuelling of the translocation apparatus.

An alternative candidate to account for the vulnerability of pigment migration to lidocaine is the intracellular mechanism that controls pigment position in the retinal cells. A rise in cytoplasmic Ca^{2+} concentration has been associated with pigment migration to the light-adapted position in arthropod photoreceptors (Kirschfeld and Vogt, 1980; Frixione and Aréchiga, 1981; Howard, 1984) and RPE cells (Burnside and Basinger, 1983; Dearry and Burnside, 1984; Mondragón and Frixione, 1989). Since local anesthetics are known to inhibit the Ca^{2+}-storing ability of muscle sarcoplasmic reticulum (Bianchi, 1968; Johnson and Inesi, 1969), non-mitochondrial compartments in nerve terminals (Blaustein *et al.* 1978; Lavoie *et al.* 1986) and smooth endoplasmic reticulum of invertebrate photoreceptors (Walz, 1982), it is conceivable that lidocaine could hinder adequate buffering of internal Ca^{2+} and thus prevent the retinal cells from attaining the low levels required to keep the pigment in the dark-adapted position. This explanation is also unlikely, however, because the drug would be expected to affect the Ca^{2+}-sequestering organelles regardless of the ionic composition of the external medium, which is not the case with either preparation used in the present study. Moreover, the levels of lidocaine tested in our experiments are within the range found to stimulate rather than to inhibit Ca^{2+} uptake by lysed brain synaptosomes (Lavoie *et al.* 1986). Several local anesthetics may cause a net decrease in Ca^{2+} extrusion from nerve terminals as a result of inhibition of the Ca^{2+} pump at the plasma membrane, in spite of some stimulation of the Na^+/Ca^{2+} exchanger (García-Martín *et al.* 1990). Lidocaine could produce similar effects in the retinal cells and thus cause the pigment to move to the light-adapted position through Ca^{2+} accumulation in their cytosol. However, Na^+-deficient and Ca^{2+}-rich solutions would then be expected to enhance the light-adapting action of lidocaine, which is the reverse of the results of our experiments.

Conditions designed to increase internal Na^+ concentration promote pigment dispersion, or inhibit aggregation, in both crayfish photoreceptors and frog RPE cells (Mondragón and Frixione, 1989; Frixione and Pérez-Olvera, 1990). In both instances there is reason to suppose that elevation of Na^+ concentration might cause Ca^{2+} release from internal stores, which in turn could inactivate the energy-consuming mechanism that tends to aggregate the pigment in the dark (see also Frixione and Ruiz, 1988). The dependence of the lidocaine effect on external Na^+ concentration could, therefore, indicate that the anesthetic induces a higher than

normal Na^+ leakage into the cells, leading to internal Ca^{2+} release and inhibition of the pigment-aggregating mechanism. A similar mechanism has been considered to explain the Na^+-dependent inhibition of fast axoplasmic transport and saltatory organelle movement in nerve cells by batrachotoxin, a steroidal compound that opens the voltage-dependent Na^+ channels (Forman and Shain, 1981; Worth and Ochs, 1982). In this context it also becomes particularly significant that ouabain and veratridine, which have in common the ability to cause an abnormal increase in intracellular Na^+ concentration, are potent inhibitors of fast axoplasmic transport (Edström, 1977). Like lidocaine, ouabain inhibits pigment aggregation in crayfish photoreceptors (Frixione and Aréchiga, 1981; Frixione and Pérez-Olvera, 1990) and RPE cells (Mondragón and Frixione, 1989). A direct action of lidocaine on the Na^+/K^+-ATPase is unlikely because the anesthetic seems to be unable to alter the pumping rate of the enzyme, even at concentrations that were found to be effective in the present experiments (den Hertog and Ritchie, 1969).

The possibility that lidocaine might inhibit the pigment-aggregating mechanism by inducing an increase in Na^+ leakage into the retinal cells is supported by evidence that several anesthetics can enhance permeability to cations in artificial and natural membranes (Barchfeld and Deamer, 1985; Escudero and Gutiérrez-Merino, 1987). High levels of external Ca^{2+}, or the addition of Co^{2+}, which probably decrease Na^+ leakage into the cells (Blaustein and Goldman, 1968; Fulpius and Baumann, 1969), would be expected to protect the cells against this lidocaine effect. This is in accordance with our results. Competition between Ca^{2+} and local anesthetics has also been observed for active conductance changes in both frog and crustacean nerves (Aceves and Machne, 1963; Blaustein and Goldman, 1966). If our interpretation is correct, Ca^{2+} release from internal stores in response to a rise in Na^+ concentration in the cytoplasm should outweigh any stimulation of Ca^{2+} uptake that might be produced at the same time in these compartments by low doses of lidocaine (Lavoie *et al.* 1986). Direct measurements with ion-sensitive microelectrodes will be necessary to test this hypothesis for the action of lidocaine on pigment transport in the two types of retinal cells.

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References

Aasheim, G., Fink, B. R. and Middaugh, M. (1974). Inhibition of rapid axoplasmic transport by procaine hydrochloride. *Anesthesiology* **41**, 549–553.

Abbott, N. J., Moreton, R. B. and Pichon, Y. (1975). Electrophysiological analysis of potassium and sodium movements in crustacean nervous system. *J. exp. Biol.* **63**, 85–115.

ACEVES, J. AND MACHNE, X. (1963). The action of calcium and local anesthetics on nerve cells, and their interaction during excitation. *J. Pharmac. exp. Ther.* **140**, 138–148.

ANDERSON, K. AND EDSTRÖM, A. (1973). Effects of nerve blocking agents on fast axonal transport of proteins in frog sciatic nerves *in vitro*. *Brain Res.* **50**, 125–134.

ARÉCHIGA, H., BAÑUELOS, E., FRIXIONE, E., PICONES, A. AND RODRÍGUEZ-SOSA, L. (1990). Modulation of crayfish retinal sensitivity by 5-hydroxytryptamine. *J. exp. Biol.* **150**, 123–143.

BARCHFELD, G. L. AND DEAMER, D. W. (1985). The effect of general anesthetics on the proton and potassium permeabilities of liposomes. *Biochim. biophys. Acta* **819**, 161–169.

BARLOW, R. B., JR, CHAMBERLAIN, S. C. AND LEHMAN, H. K. (1989). Circadian rhythms in the invertebrate retina. In *Facets of Vision* (ed. D. G. Stavenga and R. C. Hardie), pp. 257–280. Berlin, Heidelberg: Springer-Verlag.

BIANCHI, C. P. (1968). Pharmacological actions on excitation–contraction coupling in striated muscle. *Fedn Proc. Fedn Am. Socs exp. Biol.* **27**, 126–131.

BLAUSTEIN, M. P. AND GOLDMAN, D. E. (1966). Competitive action of calcium and procaine on lobster axon. A study of the mechanism of action of certain local anesthetics. *J. gen. Physiol.* **49**, 1043–1063.

BLAUSTEIN, M. P. AND GOLDMAN, D. E. (1968). The action of certain polyvalent cations on the voltage-clamped lobster axon. *J. gen. Physiol* **51**, 279–291.

BLAUSTEIN, M. P., RATZLAFF, R. W., KENDRICK, N. C. AND SCHWEITZER, E. S. (1978). Calcium buffering in presynaptic nerve terminals. I. Evidence for involvement of a nonmitochondrial ATP-dependent sequestration mechanism. *J. gen. Physiol.* **72**, 15–41.

BURNSIDE, B., ADLER, R. AND O'CONNOR, P. (1983). Retinomotor pigment migration in the teleost retinal pigment epithelium. I. Roles for actin and microtubules in pigment granule transport and cone movement. *Invest. Ophth. visual Sci.* **24**, 1–15.

BURNSIDE, B. AND BASINGER, S. (1983). Retinomotor pigment migration in the teleost retinal pigment epithelium. II. Cyclic-3′,5′-adenosine monophosphate induction of dark-adaptive movement *in vitro*. *Invest. Ophth. visual Sci.* **24**, 16–23.

BYERS, M. R., FINK, B. R., KENNEDY, R. D., MIDDAUGH, M. E. AND HENDRICKSON, A. E. (1973). Effects of lidocaine on axonal morphology, microtubules and rapid transport in rabbit vagus nerve *in vitro*. *J. Neurobiol.* **4**, 125–143.

BYERS, M. R., O'NEILL, P. C. AND FINK, B. R. (1979). Lidocaine (without epinephrine) does not affect the fine structure or microtubules of the trigeminal nerve *in vivo*. *Anesthesiology* **51**, 55–57.

DEARRY, A. AND BURNSIDE, B. (1984). Effects of extracellular Ca^{++}, K^+, and Na^+ on cone and retinal pigment epithelium retinomotor movements in isolated teleost retinas. *J. gen. Physiol.* **83**, 589–611.

DEARRY, A. AND BURNSIDE, B. (1986*a*). Dopaminergic regulation of cone retinomotor movement in isolated teleost retinas. I. Induction of cone contraction is mediated by D2 receptors. *J. Neurochem.* **46**, 1006–1021.

DEARRY, A. AND BURNSIDE, B. (1986*b*). Dopaminergic regulation of cone retinomotor movement in isolated teleost retinas. II. Modulation by γ-aminobutyric acid and serotonin. *J. Neurochem.* **46**, 1022–1031.

DEN HERTOG, A. AND RITCHIE, J. M. (1969). The effect of some quaternary ammonium compounds and local anaesthetics on the electrogenic component of the sodium pump in mammalian non-myelinated nerve fibres. *Eur. J. Pharmac.* **6**, 138–142.

EDSTRÖM, A. (1977). Rapid axonal transport *in vitro*. Effects of derivatives of cyclic-AMP and other agents acting on the cyclic-AMP system. *J. Neurobiol.* **8**, 371–380.

EDSTRÖM, A., HANSSON, H. A. AND NORSTRÖM, A. (1973). Inhibition of axonal transport *in vitro* in frog sciatic nerves by chlorpromazine and lidocaine. *Z. Zellforsch. mikrosk. Anat.* **143**, 53–69.

ESCUDERO, B. AND GUTIÉRREZ-MERINO, C. (1987). Effects of local anesthetics on the passive permeability of sarcoplasmic reticulum vesicles to Ca^{2+} and Mg^{2+}. *Biochim. biophys. Acta* **902**, 374–384.

FINK, B. R., KENNEDY, R. D., HENDRICKSON, A. E. AND MIDDAUGH, M. E. (1972). Lidocaine inhibition of rapid axonal transport. *Anesthesiology* **36**, 422–432.

FINK, B. R. AND KISH, S. J. (1976). Reversible inhibition of rapid axonal transport *in vivo* by lidocaine hydrochloride. *Anesthesiology* **44**, 139–146.

FORMAN, D. S. AND SHAIN, W. G., JR (1981). Batrachotoxin blocks saltatory organelle movement in electrically excitable neuroblastoma cells. *Brain Res.* **211**, 242–247.

FRIXIONE, E. (1983*a*). The microtubular system of the crayfish retinula cells and its changes in relation to screening pigment migration. *Cell Tissue Res.* **232**, 335–348.

FRIXIONE, E. (1983*b*). Firm structural associations of migratory pigment granules with microtubules in crayfish retinula cells. *J. Cell Biol.* **96**, 1258–1265.

FRIXIONE, E. AND ARÉCHIGA, H. (1981). Ionic dependence of screening pigment migrations in crayfish retinal photoreceptors. *J. comp. Physiol.* **144** A, 35–43.

FRIXIONE, E., ARÉCHIGA, H. AND TSUTSUMI, V. (1979). Photomechanical migrations of pigment granules along the retinula cells of the crayfish. *J. Neurobiol.* **10**, 573–590.

FRIXIONE, E. AND HERNÁNDEZ, J. (1989). Modulation of screening-pigment position in crayfish photoreceptors by serotonin: possible involvement of Na^+/K^+-ATPase activity. *J. exp. Biol.* **143**, 459–473.

FRIXIONE, E. AND PÉREZ-OLVERA, O. (1990). Light-adapting migration of the screening-pigment in crayfish photoreceptors is a two-stage movement comprising an all-or-nothing initial phase. *J. Neurobiol.* **22**, 238–248.

FRIXIONE, E. AND RUIZ, L. (1988). Calcium uptake by smooth endoplasmic reticulum of peeled retinal photoreceptors of the crayfish. *J. comp. Physiol.* **162** A, 91–100.

FULPIUS, B. AND BAUMANN, F. (1969). Effects of sodium, potassium, and calcium ions on slow and spike potentials in single photoreceptor cells. *J. gen. Physiol.* **53**, 541–561.

GARCÍA-MARTÍN, E., GONZÁLEZ-CABANILLAS, S. AND GUTIÉRREZ-MERINO, C. (1990). Modulation of calcium fluxes across synaptosomal plasma membrane by local anesthetics. *J. Neurochem.* **55**, 370–378.

GEDDES, I. C. AND QUASTEL, J. H. (1956). Effects of local anesthetics on respiration of rat brain cortex *in vitro*. *Anesthesiology* **17**, 666–671.

HASCHKE, R. H. AND FINK, B. R. (1975). Lidocaine effects on brain mitochondrial metabolism *in vitro*. *Anesthesiology* **42**, 737–740.

HOWARD, J. (1984). Calcium enables photoreceptor pigment migration in a mutant fly. *J. exp. Biol.* **113**, 471–475.

JOHNSON, P. N. AND INESI, G. (1969). The effect of methylxanthines and local anesthetics on fragmented sarcoplasmic reticulum. *J. Pharmac. exp. Ther.* **169**, 308–314.

KIRSCHFELD, K. AND VOGT, K. (1980). Calcium ions and pigment migration in fly photoreceptors. *Naturwissenschaften* **67**, 516–517.

KREBS, W. (1972). The fine structure of the retinula of the compound eye of *Astacus fluviatilis*. *Z. Zellforsch. mikrosk. Anat.* **133**, 399–414.

LAVOIE, P.-A. (1982*a*). Inhibition of fast axonal transport *in vivo* by tetrocaine: an increase in potency at alkaline pH, and no change in potency in calcium-depleted nerves. *Can. J. Physiol. Pharmac.* **60**, 1715–1720.

LAVOIE, P.-A. (1982*b*). Block of fast axonal-transport *in vitro* by the local-anesthetics dibucaine and etidocaine. *J. Pharmac. exp. Ther.* **223**, 251–256.

LAVOIE, P.-A. (1983). Inhibition of fast axonal-transport *in vitro* by the local-anesthetics prilocaine, mepivacaine and bupivacaine. *Can. J. Physiol. Pharmac.* **61**, 1478–1482.

LAVOIE, P.-A., KHAZEN, T. AND FILION, P. R. (1989). Mechanisms of the inhibition of fast axonal transport by local anesthetics. *Neuropharmacology* **28**, 175–181.

LAVOIE, P.-A., MEKHAIL-ISHAK, K. AND SHARKAWI, M. (1986). Biphasic effect of local anesthetics on the adenosine triphosphate-dependent calcium uptake by lysed brain synaptosomes. *Can. J. Physiol. Pharmac.* **64**, 218–221.

MONDRAGÓN, R. AND FRIXIONE, E. (1989). Retinomotor movements in the frog retinal epithelium: dependence of pigment migration on Na^+ and Ca^{2+}. *Expl Eye Res.* **48**, 589–603.

NÄSSEL, D. R. (1976). The retina and retinal projections on the lamina ganglionaris of the crayfish *Pacifastacus leniusculus* (Dana). *J. comp. Neurol.* **167**, 341–360.

NGUYEN-LEGROS, J. (1978). Fine structure of the pigment epithelium in the vertebrate retina. *Int. Rev. Cytol. (Suppl.)* **7**, 287–328.

RODIONOV, V. I., GYOEVA, F. K. AND GELFAND, V. I. (1991). Kinesin is responsible for centrifugal movement of pigment granules in melanophores. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4956–4960.

SHIBAMOTO, S., HORI, T., HAYAKAWA, M., NANGO, M., CRAGOE, E. J., OKU, N. AND ITO, F.

(1990). Inhibitory effect of local anesthetics on Na^+/H^+ antiporter in brush border membrane-reconstituted vesicles. *Life Sci.* **47**, 1129–1133.

SNYDER, W. Z. AND ZADUNAISKY, J. A. (1976). A role for calcium in the migration of retinal screening pigment in the frog. *Expl Eye Res.* **22**, 377–388.

TABATABAI, M. AND BOOTH, A. M. (1990). Mechanism of action of local anesthetics on synaptic transmission in the rat. *Anesth. Analg.* **71**, 149–157.

TARBA, C. AND CRACIUM, C. (1990). A comparative study of the effects of procaine, lidocaine, tetracaine and dibucaine on the function and ultrastructure of isolated rat liver mitochondria. *Biochim. biophys, Acta* **1019**, 19–28.

VAN HARREVELD, A. (1936). A physiological solution for freshwater crustaceans. *Proc. Soc. exp. Biol. Med.* **34**, 428–432.

WALZ, B. (1982). Ca^{2+}-sequestering smooth endoplasmic reticulum in an invertebrate photoreceptor. II. Its properties as revealed by microphotometric measurements. *J. Cell Biol.* **93**, 849–859.

WORTH, R. M. AND OCHS, S. (1982). Dependence of batrachotoxin block of axoplasmic-transport on sodium. *J. Neurobiol.* **13**, 537–549.