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OPTICAL RECORDING OF ELECTRICAL ACTIVITY FROM PARALLEL FIBRES AND OTHER CELL TYPES IN SKATE CEREBELLAR SLICES *IN VITRO*

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SUMMARY

1. A reliable and simple fish brain slice preparation was obtained from the cerebellum of the skate, and its properties were described.

2. A potentiometric oxonol dye, RH-482, and multiple site optical recording of transmembrane voltage (MSORTV) were used to reveal the electrophysiological properties of the parallel fibre action potential and to measure its conduction velocity (0·13 m/s). The parallel fibre action potential was blocked in the presence of tetrodotoxin (TTX) and prolonged by tetraethylammonium (TEA), suggesting that the upstroke depends upon sodium entry and the repolarization upon potassium efflux. An after-hyperpolarization results from a calcium-dependent potassium conductance.

3. A second potentiometric dye, RH-155, differing only slightly from RH-482, exhibited a high affinity for glial cell membrane, and could be used to monitor changes in extracellular potassium concentration by detecting changes in glial membrane potential.

4. Calcium channel blockers such as cadmium ions blocked the optical signal that reflected the extracellular accumulation of potassium.

5. Interventions that modified the extracellular volume, and thereby affected the accumulation of potassium, produced large changes in the optical signal that monitored glial depolarization. Hypertonic and hypotonic bathing solutions resulted in decreases and increases, respectively, in the magnitude of the extrinsic absorption change that tracked potassium accumulation.

6. Blocking sodium–potassium pump activity by means of ouabain prolonged the time course of the optical signal that was related to potassium accumulation in the extracellular space.

7. Extracellular potassium accumulation was revealed to be critically dependent upon intracellular calcium ions.

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INTRODUCTION

The use of intracellular recording has provided important information about the electrophysiological properties of cerebellar Purkinje cells and their dendrites (Eccles, Llinás & Sasaki, 1966*a–d*; Martinez, Crill & Kennedy, 1971; Llinás & Hess, 1976). This knowledge has been extended largely through the implementation of the cerebellar *in vitro* slice preparation (Llinás & Sugimori, 1980*a,b*; Crepel, Dhanjal & Garthwaite, 1981). However, conventional intracellular recordings are limited to relatively large cell bodies or large dendrites, and therefore little is known directly about the ionic basis of the action potential and other electrical properties of the parallel fibres, a system of small non-myelinated axons. The parallel fibres are confined to a single layer of the cerebellum having a strict geometrical organization, the molecular layer; they have a large surface-to-volume ratio, and, collectively, they have a large membrane area. These properties suggest an alternative method for investigating the ionic basic of parallel fibre electrical activity, viz. optical recording using potential-sensitive molecular probes (for reviews see Cohen & Salzberg, 1978; Waggoner, 1979; Salzberg, 1983; Grinvald, 1985). Potentiometric probes allow one to monitor changes in membrane potential at a distance, using optical methods that exploit the linear changes in their absorption or fluorescence that follow voltage changes within microseconds (Cohen & Salzberg, 1978). These molecules have found a variety of applications because their use permits one to detect changes in voltage from membranes that are otherwise inaccessible, by measuring changes in light intensity in the image plane of an optical apparatus such as a microscope. Multiple photodetectors readily detect voltage changes in several cells (Salzberg, Grinvald, Cohen, Davila & Ross, 1977) and the technique of multiple site optical recording of transmembrane voltage (MSORTV) has already been applied to a variety of physiological preparations (Grinvald, Cohen, Lesher & Boyle, 1981; Kamino, Hirota & Fujii, 1981; Senseman, Shimizu, Horwitz & Salzberg, 1983; Salzberg, Obaid, Gainer & Senseman, 1983; Orbach & Cohen, 1983; Orbach, Cohen & Grinvald, 1985; Hirota, Kamino, Komuro, Sakai & Yada, 1985) including mammalian hippocampal slices (Grinvald, Manker & Segal, 1982).

In this paper we will describe some electrophysiological properties of parallel fibres, determined from optical measurements. We will also describe a slow optical signal, resulting from the stimulation of parallel fibres, and present evidence suggesting that it is of glial origin. The experiments were all carried out in a novel fish brain slice preparation that is extremely robust and reliable, and which has a molecular layer that is somewhat simpler than its mammalian counterpart (Nicholson, Llinás & Precht, 1969). In this system, optical recording of membrane potential from populations of small cells is possible with a signal-to-noise ratio approaching that obtained with microelectrodes. In addition, our studies have resulted in the unexpected finding that within a single preparation, different but closely related voltage-sensitive dyes bind selectively to different cell types. Some of these results have been communicated in preliminary form (Konnerth, Obaid & Salzberg, 1985; Konnerth, Obaid & Salzberg, 1986; Obaid, Konnerth & Salzberg, 1986; Salzberg, Obaid & Konnerth, 1986).

METHODS

Tissue preparation

Experiments were performed in adult specimens of the Atlantic skate, *Raja erinacea*, 22–30 cm across. The animals were anaesthetized by immersion in sea water containing tricaine methanesulphonate (Finquel, Ayerst Laboratories Inc., New York) for 10–15 min. The cranial cavity was opened from the dorsal side, and the brain exposed. Large blood vessels were removed from the surface of the brain, together with the adhering membrane. The brain was transected rostrally at the level of the olfactory bulb, and caudally at the posterior lateral line nerve, and transferred to the moist filter paper bottom of a dissecting dish. The cerebellum was dissected free, cleared of any remaining superficial blood vessels, and placed in a small beaker containing cold (4 °C) elasmobranch Ringer solution (see below) for approximately 2 min. The cerebellar mass was then removed to a slicing platform consisting of a plastic Petri dish to which a sheet of filter paper (Whatman No. 1) was attached with rubber cement. Coronal slices for most experiments were cut by hand using Gillette Super Stainless razor blades (Gillette, U.S.A.). The terminal 1–2 mm were cut away from each end of the cerebellum and discarded. Two 700 μm thick slices from each end of the cerebellum were cut using the razor blade and a simple jig for guidance. The jig was prepared by gluing two stacks of four No. 1 micro cover glasses (22 × 4 mm) (A. H. Thomas Company, Philadelphia, PA, U.S.A.) to the corners at one end of a microscope slide, parallel to its long axis. The jig was gently placed against one truncated end of the cerebellum, with the cover-glass stacks flanking the preparation, and a slice was cut by moving the blade back and forth along the cover-slip stacks with a gentle downward pressure. The cut slice was removed using a fine wetted camel-hair brush, and transferred to the recording chamber. We found that it was important that the filter paper on which the slices were cut be moist but not too wet, and that the blades be new and cleaned of grease with acetone before use. In our most recent experiments, a Vibroslicer (Campden Instruments, Ltd.) was used to cut coronal slices and these were at least the equal of the hand-cut slices.

Recording chamber and bathing solution

The recording chamber was constructed of black Delrin plastic and had a bottom consisting of a glass cover-slip to which a layer of Sylgard was attached. A brass pillar, fixed to the chamber, supported a small hydraulic micro-manipulator (MO-103, Narishige Scientific Instrument Laboratories, Tokyo), which held a bipolar stimulating electrode. The chamber was rigidly fixed to the stage of a large compound microscope (UEM, Carl Zeiss, Oberkochen). The chamber volume (4 ml) could be exchanged by passing 20 ml of solution, using gravity feed and suction removal, in less than 1 min. The cerebellar slice was held against the Sylgard bottom of the chamber by means of a parallel array of fine (13 μm) nylon monofilament threads glued at approximately 500 μm intervals to a U-shaped platinum wire (550 μm diameter) frame. This frame, with threads attached only to its top side, was able gently to immobilize 700 μm slices. Experiments were performed at room temperature (22–25 °C).

The preparation was bathed in an elasmobranch Ringer solution having the following composition (in mM): NaCl, 250; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 1; NaH_2PO_4, 0·2; glucose, 12; urea, 360; HEPES, 20; adjusted to pH 7·7 with NaOH. (The measured osmolarity was 879 mosM.) Oxygenation of the slices was maintained by blowing 100% O_2, previously saturated with water vapour at room temperature, at the surface of the bathing solution. Drugs used in the experiments, tetrodotoxin (TTX), tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), and ouabain, were obtained from Sigma Chemical Company (St Louis). TEA was recrystallized from ethanol before use. The dyes RH-482 and RH-155 are available from Dr Amiram Grinvald, The Weizmann Institute, Rehovot, Israel. RH-155 is also available, as NK-3041, from Nippon Kankoh-Shikiso Kenkyusho, Okayama, Japan.

Electrical stimulation and recording

The slices were stimulated by means of a bipolar electrode constructed from a pair of Teflon-coated 50 μm platinum wires, with their bared tips separated by 200–400 μm. The electrode was positioned in the molecular layer, most often oriented along a beam of parallel fibres. Brief square pulses (10–200 μs) were delivered by a stimulation isolation unit and field potentials were routinely

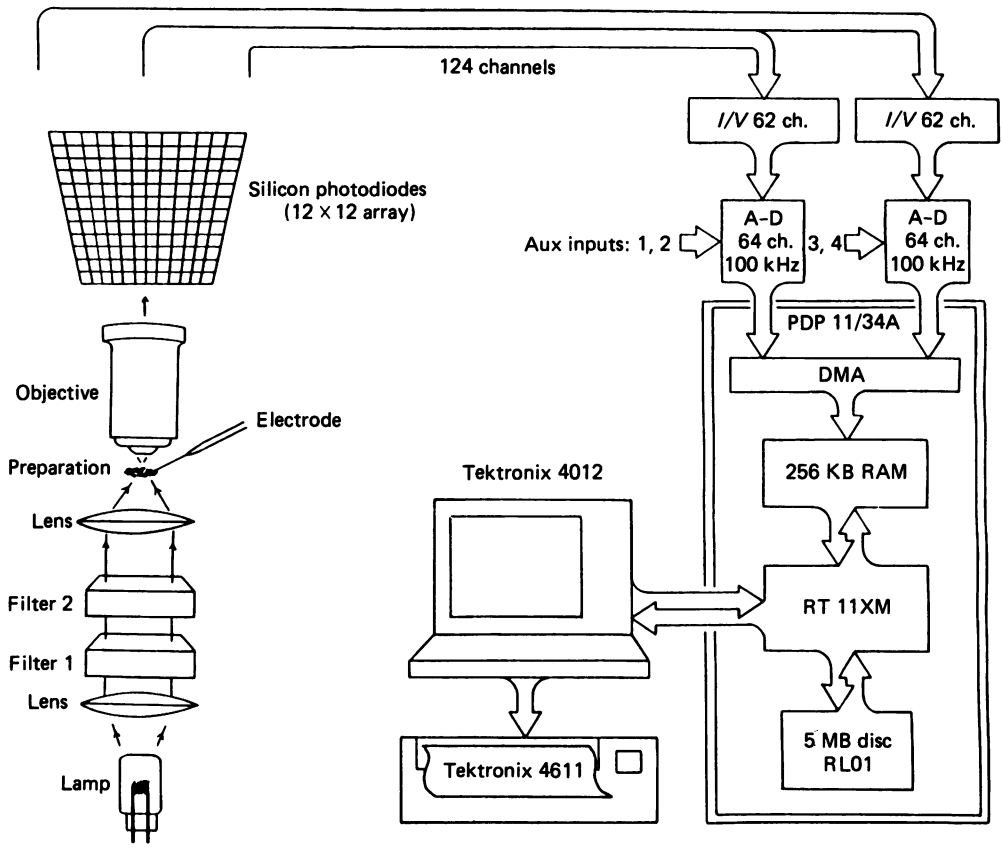

Fig. 1. Schematic diagram of the system for multiple site optical recording of transmembrane voltage (MSORTV). On the left is a compound microscope having a 12 x 12 silicon photodiode matrix array mounted on the trinocular tube in the image plane of the objective. A PDP 11/34A computer with two 64-channel multiplexers and two 12-bit analogue-to-digital (A–D) converters running at 100 kHz record a complete 124-pixel frame every 750 μs. Because the voltage outputs from each of the 124 current-to-voltage converters are a.c. coupled (typically with a 3 s time constant) to second-stage amplifiers, the effective resolution at the computer is approximately 18 bits.

recorded prior to optical measurements, using an NaCl (4 M)-filled micropipette (5–15 MΩ) and an M-701 electrometer (W.P. Instruments, Inc., New Haven, CT, U.S.A.).

Optical recording

The method that we have used for multiple site optical recording of transmembrane voltage (MSORTV) (Salzberg *et al.* 1977; Grinvald *et al.* 1981) is illustrated in Fig. 1. Light from a tungsten–halogen lamp was collimated, rendered quasi-monochromatic with a heat filter (KG-1, Schott Optical Company, Duryea, PA, U.S.A.) and one of several interference filters, and focused, by means of a bright-field condenser, on a region of the cerebellar slice centred on the molecular layer. Transmitted light was collected by a high numerical aperture (n.a.) objective (10 x ; 0·4 n.a. Fluotar; Wild Heerbrugg, Switzerland), converted to pseudo-water immersion by sealing the front element with epoxy, which formed a real image on a 12 x 12 element silicon photodiode matrix array (MS 144–0; Integrated Photomatrix Inc., Mountainside, NJ, U.S.A.), located in the image plane of the Zeiss UEM microscope. The photocurrents generated by the central 124

elements of the photodiode array were separately converted to voltages and amplified as described previously (Salzberg *et al.* 1977; Grinvald *et al.* 1981). All of the amplifier outputs were sent to a data-acquisition system based upon a PDP 11/34A computer (Digital Equipment Corp., Maynard, MA, U.S.A.) capable of acquiring a complete frame every 750 μs. This data-acquisition system is similar to that described previously by Grinvald *et al.* (1981) (see Cohen & Lesher, 1986), and employed in a variety of systems since (Grinvald *et al.* 1982; Orbach & Cohen, 1983; Senseman *et al.* 1983; Salzberg *et al.* 1983; Salzberg, Obaid & Gainer, 1985).

RESULTS

Optical recording of the parallel fibre action potential

Figure 2*A* shows the MSORTV display of the optical responses to a brief (50 μs) stimulus applied to the molecular layer of a coronal cerebellar slice after the preparation was stained for 60 min with a 100 μg/ml solution of the pyrazo-oxonol dye RH-482 (Grinvald, Hildesheim, Gupta & Cohen, 1980.) Each trace segment represents the changes in light intensity recorded as a function of time by a single element of the photodiode matrix array, each of which samples a region of the image plane corresponding to 100 μm on a side in the object plane. The short-latency optical signals, recorded in a single sweep, were localized to the molecular layer, and the evidence suggests that these spike-shaped signals indeed represent the parallel fibre action potential: (a) all of the evoked signals are confined strictly to the molecular layer and the maximal amplitudes are observed in the central portion of the beam of parallel fibres (Eccles *et al.* 1966*a*; Young, 1980*a*,*b*). (b) As expected, spread of the signals from the site of stimulation is observed, as demonstrated by the increasing latency with distance (Fig. 2*a*,*b*). (c) The optical signals are blocked in the presence of tetrodotoxin (TTX), in agreement with the behaviour of parallel fibres in the cerebelli of other animals (Gardner-Medwin & Nicholson, 1983). There was, in addition, a striking similarity between the shape of the field potentials recorded with extracellular microelectrodes from the molecular layer of our slice preparation *in vitro* and those recorded from the same animal *in vivo* (Young, 1980*a*). Also, we regularly observed that the field potentials recorded in the molecular layer of our slice preparation, shown to correspond primarily to the activation of parallel fibres (Eccles *et al.* 1966*a*; Young, 1980*a*), exhibited a spatial distribution within the slice that agreed closely with the distribution of the optical signals. For these reasons, we infer that the electrical activity, recorded optically by individual photodetectors, depicted in Fig. 2, represents the compound action potential originating in all of the stained, activated parallel fibres in the optical path. Since stimulation was always supramaximal, as determined from both field potential recordings and the magnitude of the optical signal, we assumed also that a constant number of fibres was activated in a given experiment. Because the width of the compound action potential recorded at different distances from the site of stimulation was constant, temporal dispersion resulting from different conduction velocities was not significant under our recording conditions, and therefore the time course of the compound action potential should reproduce the shape of the action potential in individual parallel fibres with good fidelity. (It is worth noting here that no absolute magnitude can, in general, be attached to voltage changes recorded optically (Cohen & Salzberg, 1978; Salzberg *et al.* 1983; Obaid, Orkand, Gainer & Salzberg, 1985); only the time course of the optical signal carries physiological significance. However, in the absence of significant

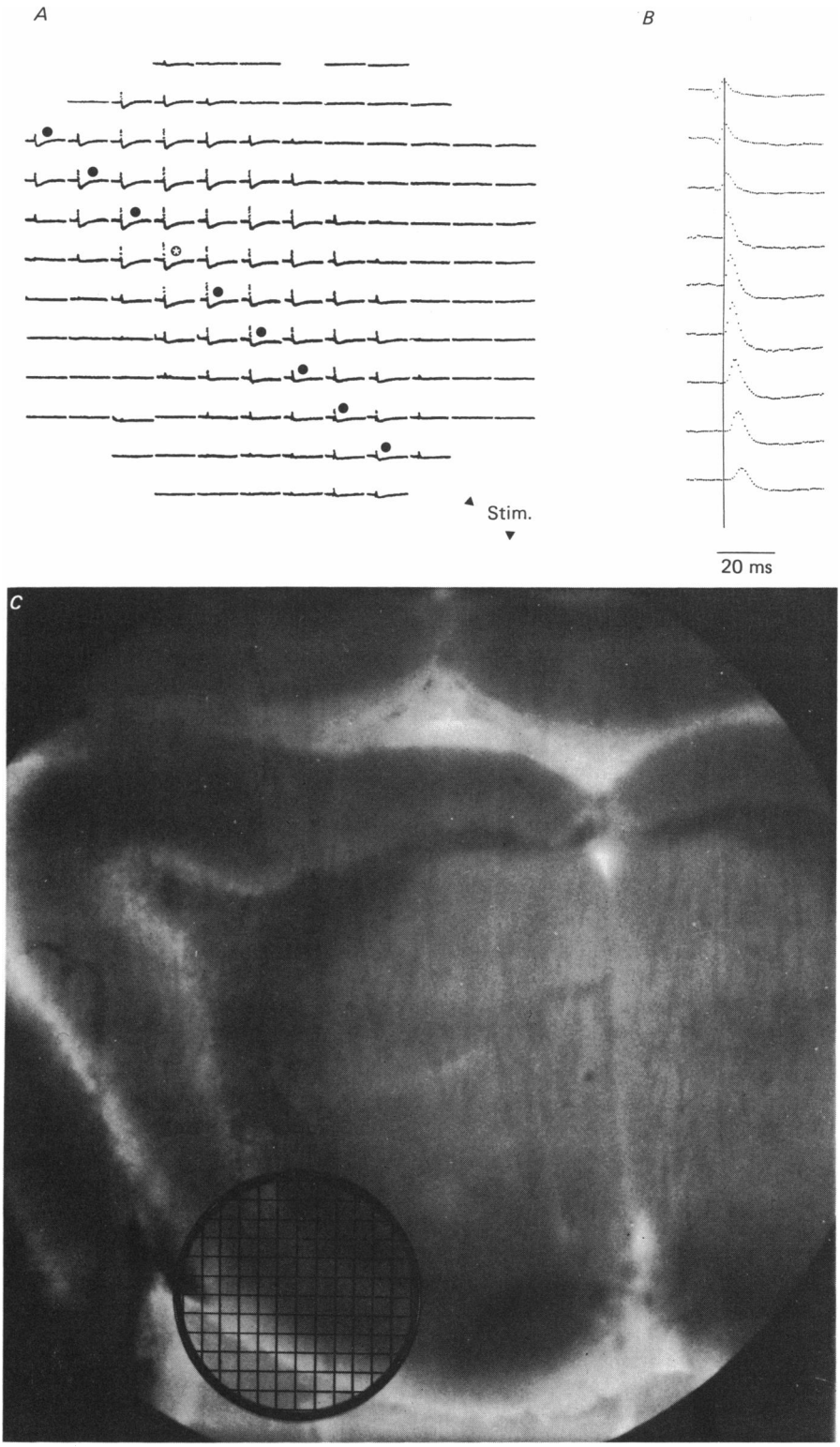

Fig. 2. For legend see opposite.

bleaching, successive optical signals from the same loci may be compared ratiometrically, and limited conclusions drawn.) Extrinsic optical signals that monitor changes in membrane potential frequently exhibit a complex dependence upon incident wavelength (Cohen, Salzberg, Davila, Ross, Landowne, Waggoner & Wang, 1974; Ross, Salzberg, Cohen, Grinvald, Davila, Waggoner & Wang, 1977; Morad & Salama, 1979), and this dependence may be used to distinguish a voltage-dependent fluorescence or absorption change from a light-scattering signal having a different origin (Salzberg, Davila & Cohen, 1973; Cohen *et al.* 1974). In addition, intrinsic optical signals such as changes in light scattering have been observed in mammalian brain slice preparations (e.g. Grinvald *et al.* 1983), and light-scattering changes following stimulation have been studied in preparations ranging from the squid axon (Cohen, Keynes & Landowne, 1972*a*,*b*) to the neurosecretory terminals of the mammalian neurohypophysis (Salzberg *et al.* 1985). Because these signals often represent physiological events other than changes in membrane potential (Salzberg *et al.* 1985), it is important to recognize their presence and to assess their contribution to the optical signals under consideration. The optical changes recorded from the molecular layer of the skate cerebellar slice, following staining with RH-482, reverse their sign between 630 and 690 nm, as expected for an extrinsic absorption change related to voltage. In addition, a small light-scattering signal is observed at all wavelengths examined, from 450 to 900 nm, and contributes to a slight degree to the shape of the parallel fibre action potential recorded optically. The presence of this small intrinsic optical signal thus introduces a slight distortion of the action potential's time course, but, qualitatively, the effect of the light-scattering component at wavelengths between 700 and 750 nm is to reduce slightly the

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Fig. 2. MSORTV display of the spatial distribution of optical responses to stimulation of parallel fibres and the propagation of the response from the site of stimulation. *A*, optical signals recorded by each of 124 elements of the photodiode array, following stimulation of the molecular layer of a coronal cerebellar slice with a single 50 μs pulse from a bipolar electrode located at the position indicated by the triangular symbols. The preparation had previously been stained for 1 h with 0·2 mg/ml of the pyrazo-oxonol dye RH-482 in elasmobranch Ringer solution. Each detector records an average signal from all of the activated cells in its light path. This display represents the membrane potential changes from many sites, each plotted against time. The optical signals represent compound action potentials from populations of parallel fibres. The filled circles mark the array elements whose outputs are shown in panel *B*, and the element indicated by a star would have been used for detailed analysis in this experiment. Single sweep. In this, and all succeeding Figures, the response time constant of the light-measuring system (10–90%) was 1·1 ms. The a.c. coupling time constant used here was 3 s. *B*, propagation of the parallel fibre action potential from the site of bipolar electrode stimulation. The optical signals marked by filled circles in Panel *A* are shown here with an expanded time base (calibration bar is 20 ms). The output of the photodiode closest to the position of the stimulating electrode is at the top, with the eight remaining traces representing the optical signals recorded from the eight locations along the diagonal beam of parallel fibres. The propagation velocity of the optical signal was 0·13 m/s. Temperature 23–25 °C. *C*, photograph of a representative coronal slice of skate cerebellum showing the placement of the photodiode array over the image of the molecular layer. Each square photodetector element monitors light passing through a region of the slice 100 μm on a side when a $\times 10$ microscope objective is used to form the image.

magnitude of the upstroke and to increase the apparent size of the after-hyperpolarization (see below and Fig. 3).

In none of our experiments did the stimulation of the molecular layer lead to the activation of the principal postsynaptic structures, the apical dendrites. This is suggested by the absence of an optical signal with the expected latency, and strongly

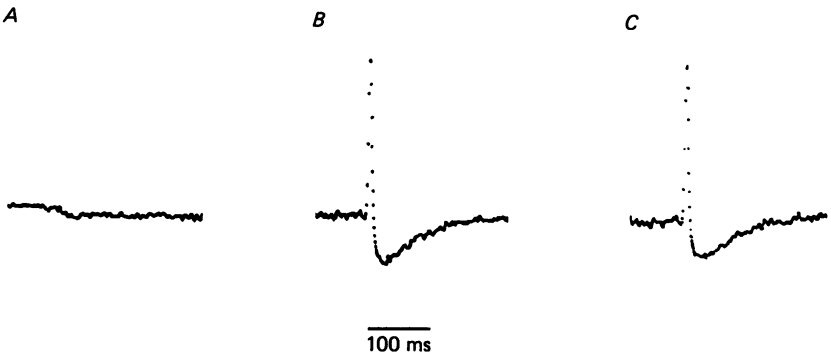

Fig. 3. The contribution of light scattering to the optical signal at 705 nm. *A*, optical signal recorded from a region of the skate cerebellar slice at 705 nm by three representative contiguous elements of the photodiode array before the preparation was stained. (The outputs of elements 36, 37 and 38 were summed.) *B*, optical signal recorded from the same region of the cerebellum following staining for 1 h in an elasmobranch Ringer solution containing 0·2 mg RH-482/ml. *C*, the optical signal in *B* corrected for the intrinsic signal in *A*. All of the records are single sweeps. The a.c. coupling time constant was 400 ms. Temperature 15 °C.

indicated by the absence of the long-lasting negativity (N2 wave) following the parallel fibre signal in the field potential recording (not shown) (Eccles *et al.* 1966*a*; Young, 1980*a*; Kocsis, Malenka & Waxman, 1983). This result is consistent with the observation in experiments *in vivo*, that a wide range of stimulus intensities applied to the skate molecular layer fail to activate the Purkinje cells (W. Young, personal communication; see also Fig. 4, Young, 1980). In addition, we performed an experiment in which we deliberately destroyed the Purkinje cells along the cell body layer within the optical field by means of a glass rod (*ca.* 150 μm tip diameter) mounted on a micro-manipulator. This treatment is expected to damage the apical dendrites of the Purkinje cells as well (R. Llinás, personal communication), but did not alter either the shape of the field potential or the time course of the action potential recorded optically. We conclude that nearly all of the electrical activity recorded optically from the molecular layer, following staining with RH-482, derives from the axons of the granule cells, the parallel fibres. Thus, the molecular layer of the present *in vitro* coronal slice preparation of the skate cerebellum is a most favourable system for monitoring in isolation the activity of a C.N.S. non-myelinated fibre system.

Electrical properties of the parallel fibre action potential

The shapes of the optical signals were highly uniform throughout the molecular layer (Fig. 2*A*) and propagation of the parallel fibre action potential within the molecular layer is apparent from the increase in the time to peak of the optical signal

with distance from the site of stimulation (Fig. 2*B*). Measurement of the propagation velocity from the nine sites indicated by filled circles in the MSORTV display yields a value of 0·13 m/s. Figure 2*B* illustrates more clearly the spread of excitation along the vector defined by these detector elements, which parallels the fibre beam.

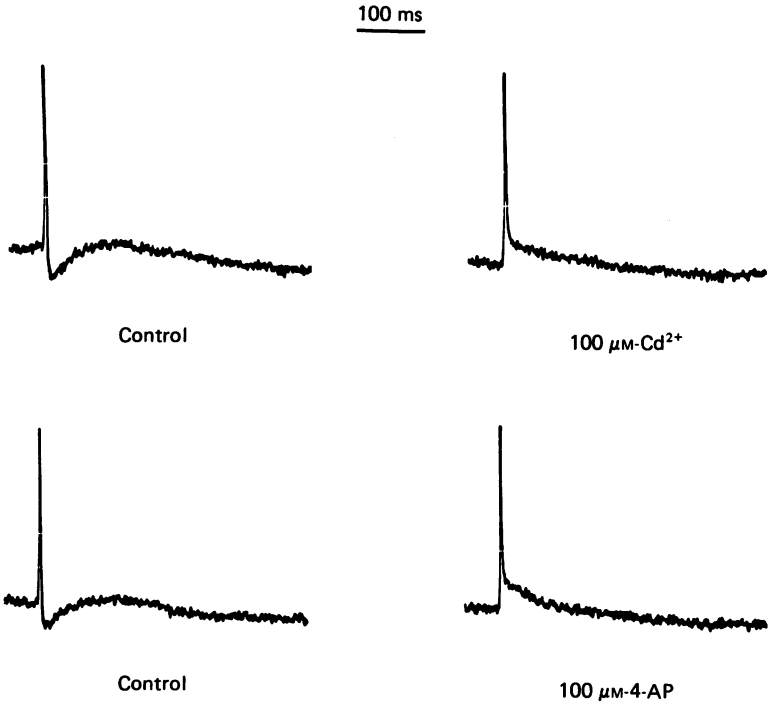

Fig. 4. The ionic nature of the parallel fibre action potential. The upper panel on the left shows a typical parallel fibre action potential, recorded by a single representative photodetector, selected at the beginning of the experiment, from a slice stained with the pyrazo-oxonol dye RH-482. This spike is blocked by TTX (not shown) and prolonged by TEA (see Fig. 9), suggesting that the upstroke depends upon sodium entry and that the repolarization depends upon potassium efflux. The prominent after-hyperpolarization resembles the after-hyperpolarization of other neurones, known to result from a calcium-activated potassium conductance ($g_{K,Ca}$). The upper panel on the right shows that Cd^{2+}, like other Ca^{2+} antagonists, reversibly blocks the after-hyperpolarization. The lower panel on the left shows the recovery after returning to normal Ringer solution. The lower panel on the right shows, in the same experiment, that 4-aminopyridine (4-AP) apparently has a similar effect on the *shape* of the action potential. Single sweeps. The a.c. coupling time constant was 400 ms. Temperature 23–25 °C.

In the results that follow, we display the analogue output of a single representative element of the photodiode matrix array, selected during the control experiment that always preceded any experimental intervention. The criteria for choosing a given array element included large signal amplitude to ensure good shape resolution, and long distance from the stimulating electrode in the object plane, to minimize contamination of the optical signal by stimulation artifacts. Once chosen, the same element was maintained throughout the experiment. For example, in the experiment illustrated in Fig. 2, the element indicated by a star was selected.

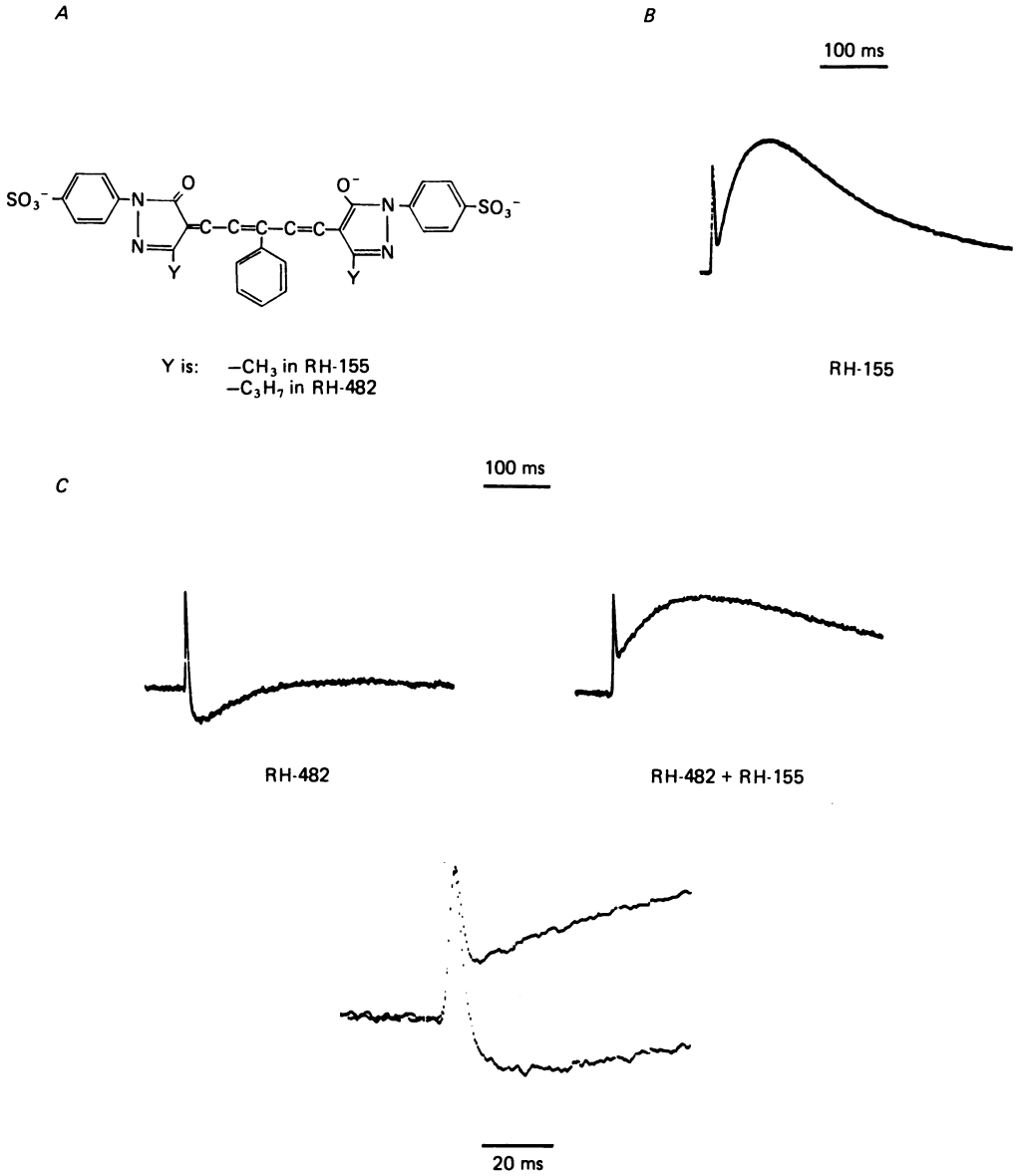

Fig. 5. RH-155 absorption signal reveals an additional component. *A*, structural formulae of two closely related pyrazo-oxonol dyes. RH-482 has symmetrically placed propyl groups which are shortened to methyl groups in RH-155. *B*, extrinsic absorption change obtained from a coronal slice that had been stained for 1 h with 0·2 mg RH-155/ml in elasmobranch Ringer solution, upon stimulation of the molecular layer with a bipolar electrode. This signal exhibits a large slow wave following the parallel fibre action potential. *C*, a double-staining experiment. The upper panel on the left shows the usual parallel fibre action potential obtained after staining the preparation with 0·2 mg RH-482/ml. The upper panel on the right shows the signal recorded from the same locus after re-staining with 0·2 mg RH-155/ml. The central panel at the bottom shows both signals, digitized and superimposed. Note the expanded timescale. All records are single sweeps, recorded by single, representative photodetectors from the photodiode array. The a.c. coupling time constant was 3 s in each of these records. Temperature 23–25 °C.

The normal parallel fibre action potential (Fig. 4, top left) is blocked in the presence of TTX, and prolonged (not shown) by TEA, suggesting that the upstroke depends upon sodium entry and the repolarization upon potassium efflux. The hyperpolarizing after-potential following the action potential recorded optically resembles the after-hyperpolarization in other neurones that results from a calcium-activated potassium conductance (Meech & Strumwasser, 1970; Llinás & Sugimori, 1980*a*,*b*; Salzberg *et al.* 1983). Indeed, the addition of the calcium channel blocking ion, Cd^{2+} (50–100 μM), to the normal Ringer solution containing 10 mM-Ca^{2+}, eliminated the after-hyperpolarization (Fig. 4, top right). When Cd^{2+} was added at low concentration (50–100 μM), and for short durations (5–10 min), the block was fully reversible (Fig. 4, top right and bottom left). Interestingly, 4-AP which blocks potassium channels in axons of the cockroach (Pelhate & Pichon, 1974), squid (Yeh, Oxford, Wu & Narahashi, 1976; Meves & Pichon, 1977), and frog (Ulbricht & Wagner, 1976) also blocks the after-hyperpolarization (Fig. 4, bottom right) without affecting the spike duration significantly. A similar effect is observed in guinea-pig hippocampal slices (Matsuda, Yoshida, Fujimura & Nakamura, 1986).

Staining with a different dye unmasks a slow signal

Cerebellar slices stained with the pyrazo-oxonol dye RH-482 yielded optical signals with satisfyingly large signal-to-noise ratios (Figs 2–4); consequently, no signal averaging was required and none was employed. Nevertheless, larger signals are always useful, as they may reveal smaller potential changes as well as potential changes in smaller structures. In an effort to improve the size of the signals, we have screened some additional dyes. Another pyrazo-oxonol dye originally synthesized by Rina Hildesheim, RH-155 (Grinvald *et al.* 1980), is a close analogue of RH-482, having its two alkyl side chains (Fig. 5*A*) shortened from propyl to methyl. Figure 5*B* shows the optical signal obtained from a coronal slice stained for 1 h with 0·2 mg/ml of the more hydrophilic dye, RH-155, in elasmobranch Ringer solution, when the molecular layer was stimulated with a bipolar electrode. This signal looks significantly different from that obtained with RH-482, in that it exhibits a large slow wave that follows the apparently unchanged parallel fibre action potential, and lasts for more than 500 ms. This record is once again the analogue output of a single representative photodetector, recorded in a single sweep.

Figure 5*C* shows a double-staining experiment in which the preparation was first stained with RH-482 and the record shown in the upper-left panel reproduces the familiar parallel fibre action potential. The upper panel on the right (in Fig. 5*C*) shows the signal recorded from the same locus after restaining for 1 h with RH-155. The doubly stained preparation exhibited an optical signal similar to that observed after staining with RH-155 alone (Fig. 5*B*). The central panel at the bottom shows both signals (in digitized form) superimposed, with the time-scale expanded. This experiment suggests that the two dyes have similar affinities for the parallel fibre membrane, but that RH-155 stains, in addition, one or more other types of cells. However, a possible explanation for the alteration in appearance of the optical signal is that RH-155 has some bizarre pharmacological effects. Compelling evidence for the former interpretation is presented in Fig. 6. Here, repetitive stimulation (10 Hz for 900 ms) leads to a dramatic decrease in the magnitude of the slow signals, while the

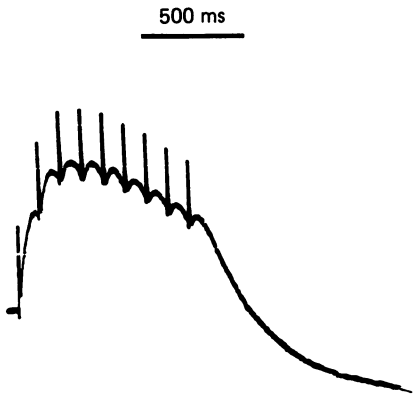

Fig. 6. The effect of repetitive stimulation at 10 Hz for 900 ms. A coronal slice was first stained in 0·2 mg RH-155/ml in elasmobranch Ringer solution. Analogue output of a single representative photodetector. Single sweep. The a.c. coupling time constant was 3 s. Temperature 23–25 °C.

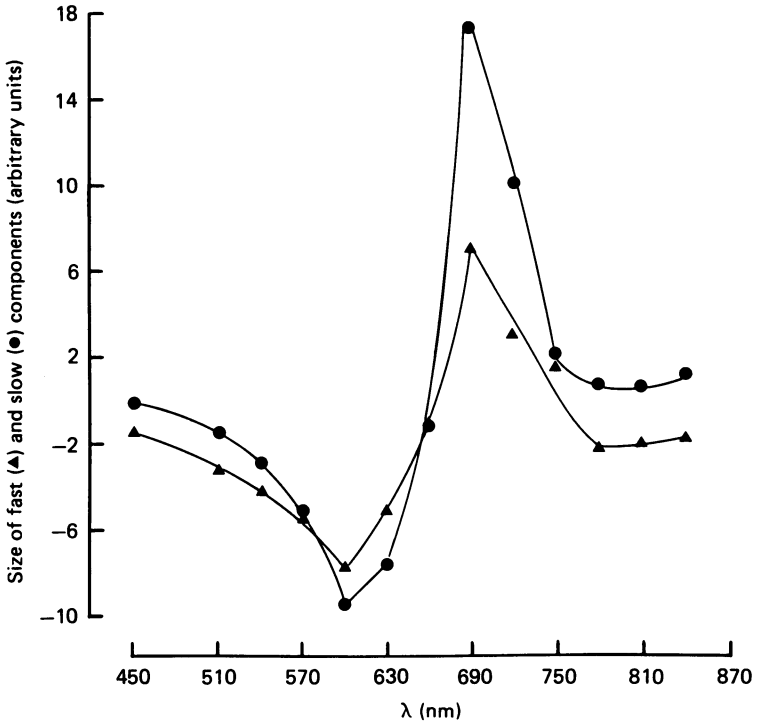

Fig. 7. Wavelength dependence of the fast (▲) and slow (●) components of the absorption signal obtained with RH-155. A coronal slice was stained in 0·2 mg RH-155/ml in elasmobranch Ringer solution, and the preparation stimulated once every 5 min. The corresponding changes in absorption were recorded at different wavelengths ranging from 840 to 510 nm. At 450 nm, the small size of the optical signal required the averaging of ten sweeps, recorded at 3 s intervals. The values plotted here represent the magnitudes of the optical signals normalized to the resting transmitted light intensity at each wavelength. The 'fast' and 'slow' components, at each wavelength, were defined, for this purpose, as the magnitudes of the optical signals at the times corresponding to the early and late peak values at 705 nm. Ordinate values in arbitrary units. Temperature 23–25 °C.

spikes decrease only slightly. If the composite optical signal represented the voltage change in a homogeneous cell population, we should be forced to the unlikely conclusion that the later spikes in the train are initiated from a membrane potential more positive than the peak of the first action potential. A far more probable interpretation of this effect is that the parallel fibre action potentials are 'riding' on

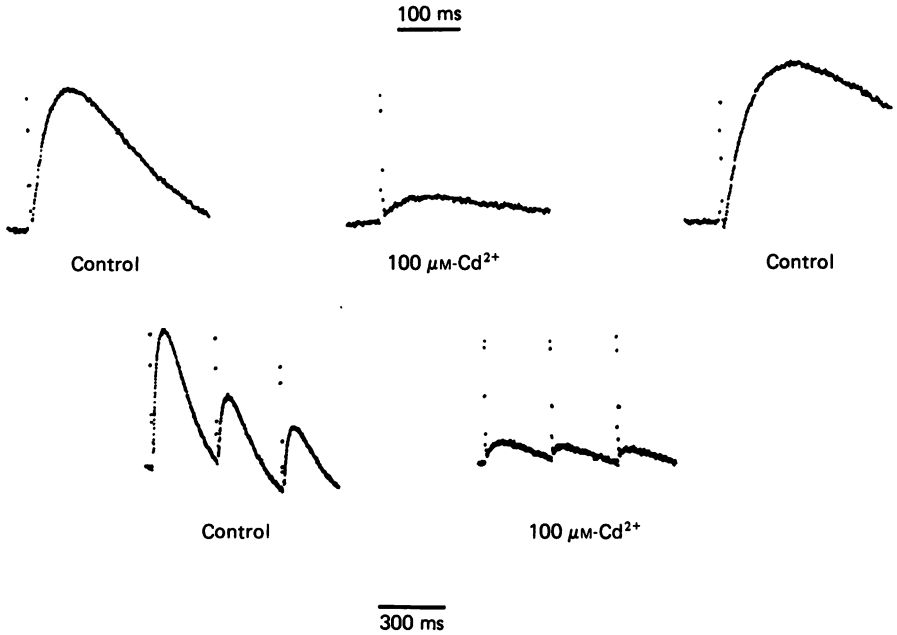

Fig. 8. The effect of Cd^{2+} on the composite optical signal obtained following staining with RH-155. The upper panel on the left illustrates the composite optical signal, comprised of the parallel fibre spike and the slow wave representing membrane potential changes in another cell type, recorded upon stimulation of the molecular layer of a cerebellar slice with a bipolar electrode in an elasmobranch Ringer solution identical to control except that KCl was reduced to 6 mM. The upper central panel shows the selective elimination of the slow wave in the presence of 100 μM-Cd^{2+}. The upper panel on the right illustrates the reversibility of the Cd^{2+} effect. The second row of traces shows the blocking effect of Cd^{2+} on the slow wave during a train of stimuli delivered at 3·3 Hz for 1 s. All of the records in this experiment are the digitized outputs of a single representative photodetector. Single sweeps. The a.c. coupling time constant was 400 ms. Temperature 23–25 °C.

a saturable signal of different origin. Possible candidates for the source of the slow wave optical change are the Purkinje cell dendrites and the glial cells. Preliminary evidence for the glial origin of the slow signal is provided by its time course during repetitive stimulation: the amplitude of the signal saturates when a maximal ('ceiling') level of the signal is reached. This is similar to the behaviour of glial membranes during repetitive stimulation (Orkand, Nicholls & Kuffler, 1966), and consistent with the observation (Heineman & Lux, 1977) that the extracellular accumulation of potassium ions also reaches a 'ceiling' concentration during supramaximal repetitive stimulation (see also Nicholson *et al.* 1969).

Since the two closely related pyrazo-oxonol dyes, RH-482 and RH-155 appear to

have different relative affinities for two different membranes in the same slice, it would seem that these two membranes must differ in some significant way. Thus, it seemed possible to us that the extrinsic absorption changes arising from the two membranes stained with RH-155 might exhibit differences in their wavelength dependence. That any such differences are small is shown in Fig. 7. Since the precise waveforms of the two signals are not known, and the pure light-scattering

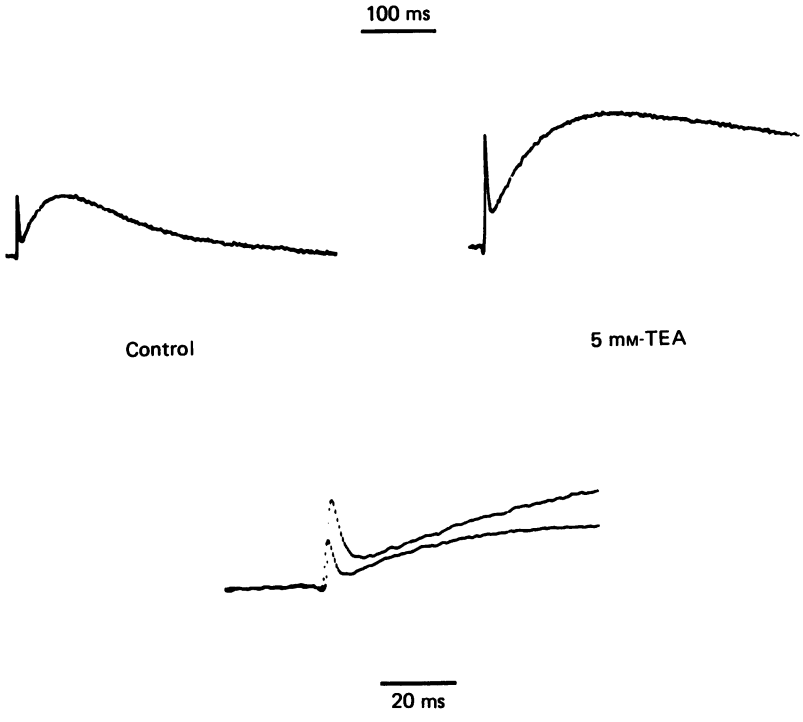

Fig. 9. Effect of tetraethylammonium (TEA) on the composite optical signal. The upper panel on the left shows the optical signal obtained in control elasmobranch Ringer solution after staining for 1 h in 0·2 mg RH-155/ml. The upper panel on the right shows that 5 mM-TEA increases the magnitude and duration of both the parallel fibre action potential and the slow wave. These records are the analogue outputs of a single representative photodetector. Single sweeps. The a.c. coupling time constant was 3 s. The bottom panel shows the superposition of the control and TEA optical signals, in digitized form, on an expanded timescale. Temperature 23–25 °C.

contribution, in the presence of dye, is also undetermined, we can only conclude that the wavelength dependence of the extrinsic absorption signals from the two (or more) cell types are approximately the same. The contribution of light scattering to the optical signal at 705 nm can be estimated from the records shown in Fig. 3. It is clear that this relatively small signal contributes negligibly to the slow component of the RH-155 signal (at 705 nm).

Effects of cadmium on the composite optical signal

In an effort to identify the origin of the slow-wave optical signal, we were led to re-examine the effect of cadmium on the absorption change recorded from the coronal

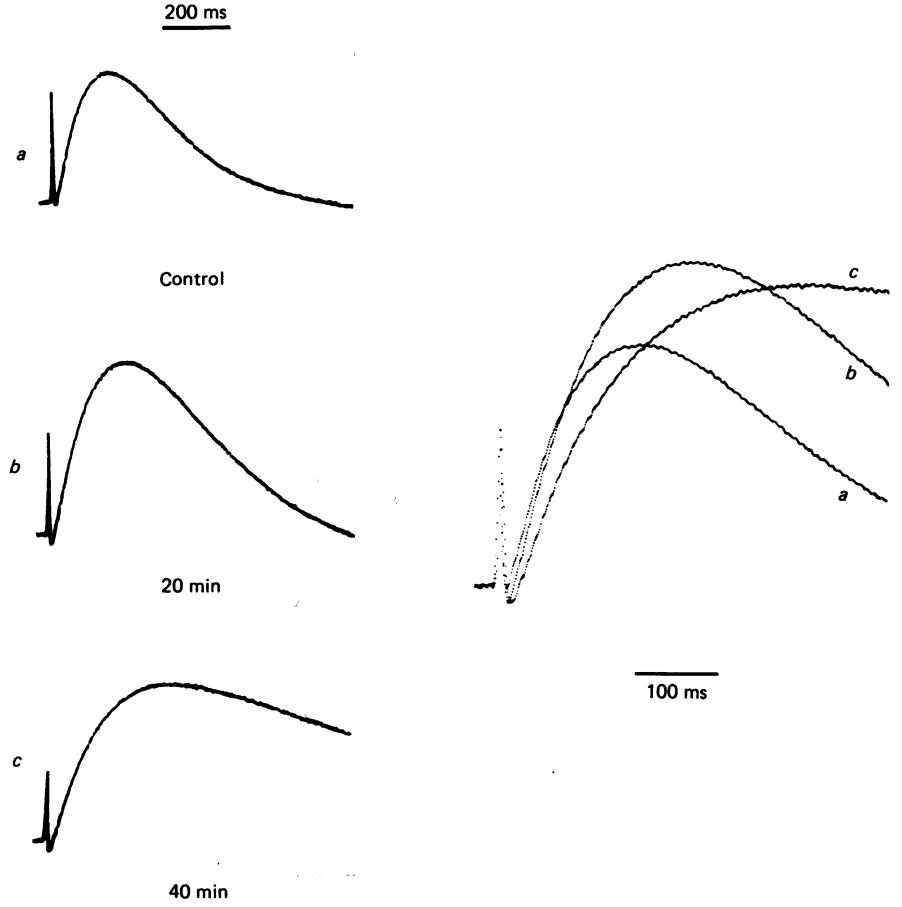

Fig. 10. Effect of ouabain on the slow component of the extrinsic absorption signal obtained from the molecular layer following staining with RH-155. *a*, optical signal recorded from a cerebellar slice in control elasmobranch Ringer solution after staining for 1 h in 0·2 mg RH-155/ml. *b*, compound optical signal recorded 20 min following the addition of 10^{-5} M-ouabain to the bath. *c*, compound optical signal recorded 40 min following the addition of 10^{-5} M-ouabain to the bath. In the panel on the right, the three traces are superimposed, and displayed on an expanded timescale. Each of the records was recorded in a single sweep and represents the summed outputs of five representative and contiguous photodetectors in the array. The a.c. coupling time constant was 3 s. Temperature 13 °C.

slice preparation. Figure 8 illustrates the selective nature of cadmium ion's effect on the composite optical signal observed from the cerebellar slice after staining with RH-155. The upper panel on the left shows that when the molecular layer is stained with RH-155, the optical signal is comprised of the parallel fibre spike and the slow wave that we conclude represents membrane potential changes in another cell type. The remaining panels in the upper row of Fig. 8 show that the slow wave is selectively and reversibly eliminated in the presence of 100 μM-Cd^{2+}. The second row of Fig. 8 shows the blocking effect of Cd^{2+} on the slow wave, during a train of stimuli at 3·3 Hz. We observed that during the train, the slow waves rapidly saturate, or reach a ceiling level (see also Fig. 6).

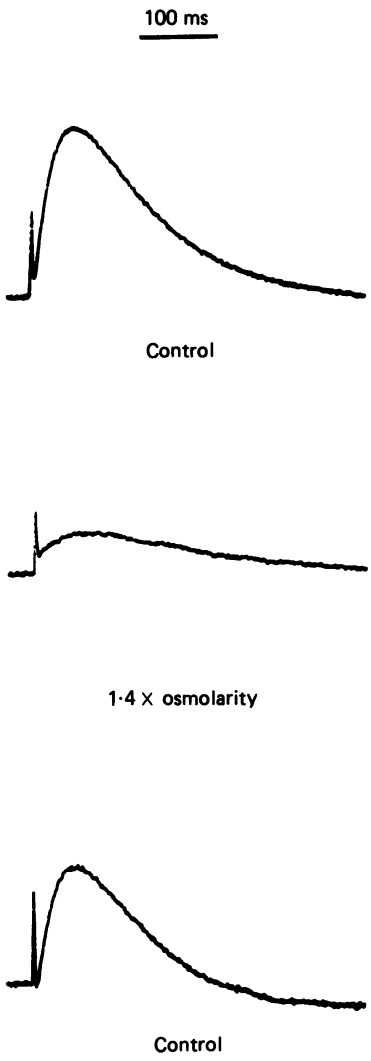

Fig. 11. The effect of hyperosmotic Ringer solution on the composite optical signal recorded from cerebellar slices stained with the dye RH-155. The upper panel shows the composite optical signal recorded from the molecular layer in a control Ringer solution following staining for 1 h in 0·2 mg RH-155/ml. The centre panel illustrates the effect of enlarging the extracellular space by means of a Ringer solution made 1·4 times hypertonic by the addition of sucrose. The lower panel shows the partial recovery upon return to control Ringer solution. The traces represent the outputs of a single representative photodetector during a single sweep. The a.c. coupling time constant was 3 s. Temperature 23–25 °C.

The effect of tetraethylammonium on the composite optical signal

Figure 9 illustrates the effect of tetraethylammonium (TEA) on the compound optical signal. The upper panel on the left shows the control record, and that on the right shows that 5 mM-TEA increases the magnitude and the duration of both the parallel fibre action potential and the slow wave. The record below shows the superposition of the control and TEA signals on an expanded time base. The slow

wave increases in size some 40% more than does the spike. We suppose that the increase in magnitude of the spike represents an increase in the number of parallel fibres activated in the presence of TEA, and that TEA actually increases the extracellular accumulation of potassium from each fibre, as a result of its prolongation of the spike.

Modification of the magnitude and duration of the extracellular accumulation of potassium

The rise time of the slow signal and its long duration suggest that this component of the extrinsic absorption change recorded from the skate cerebellar slice reflects potassium accumulation in the extracellular space, and is probably of glial origin. Voltage-sensitive dyes have previously been used to record from a variety of glial cell types (Salzberg, Obaid, Shimizu, Orkand & Senseman, 1982; Astion, Obaid, Orkand & Salzberg, 1986; Konnerth & Orkand, 1986; Lev-Ram & Grinvald, 1986) and there is no reason to suppose that glial potential changes in the molecular layer of the elasmobranch cerebellum could not be monitored optically. One would then expect that a sodium–potassium pump, either neuronal or glial or both, would contribute to the clearing of the accumulated potassium, and that pharmacological block of the pump would perturb the time course of the slow wave. Tang, Cohen & Orkand (1980) showed that in the optic nerve the axonal pump is most important for the removal of excess extracellular K^+, but in the skate cerebellum, the relative contributions of the two pumps are not known. Figure 10 shows that ouabain, at 10 μM, significantly prolonged the time course of the slow-wave recovery, but the effect was not reversible. The panels on the left show the output of a single photodetector in analogue form, with the slice bathed in control Ringer solution, and after exposure to 10 μM-ouabain for 20 and 40 min, respectively. On the right are shown, superimposed, the summed outputs of four contiguous photodetectors, in digitized form, obtained simultaneously with the analogue records shown on the left. It is clear that when the sodium–potassium pump is inhibited by ouabain, the slow component of the compound optical signal becomes bigger, tends to rise more slowly, and decays with a much longer time course. (It should be noted that there is a distortion of the shape of the signal, recorded a.c., which becomes more important as the real time course approaches the a.c. coupling time constant. The a.c. coupling time constant in our recording system is 3 s, and our slow signal peaks, under control conditions, at 150–200 ms. As the kinetics of this signal slow down, the rate of its decay is increasingly distorted, but under the experimental conditions illustrated here, the effects are so dramatic, that despite the a.c. distortion, there can be no doubt about the result.) We suppose that by inhibiting the sodium–potassium pump, and thereby diminishing the rate of clearance of potassium from the narrow cleft, we are increasing the size of the signal that reflects depolarization of the glia, and we are greatly prolonging its recovery time.

If the slow wave optical signal does indeed represent the glial membrane potential response to extracellular potassium accumulation, one would expect that modifying the extracellular volume would alter the magnitude of this effect. In particular, we would expect that an increase in the size of the extracellular space would reduce the effective increase in extracellular potassium concentration upon parallel fibre

stimulation. This condition can be achieved, at least transiently, by making the Ringer solution 40% hypertonic with sucrose, and this is illustrated in the middle panel of Fig. 11. Here, it is clear that the slow wave is selectively reduced (30% reduction in spike amplitude, 75% reduction in the size of the slow wave) by this procedure, and that the effect is largely reversible. Figure 12 illustrates the

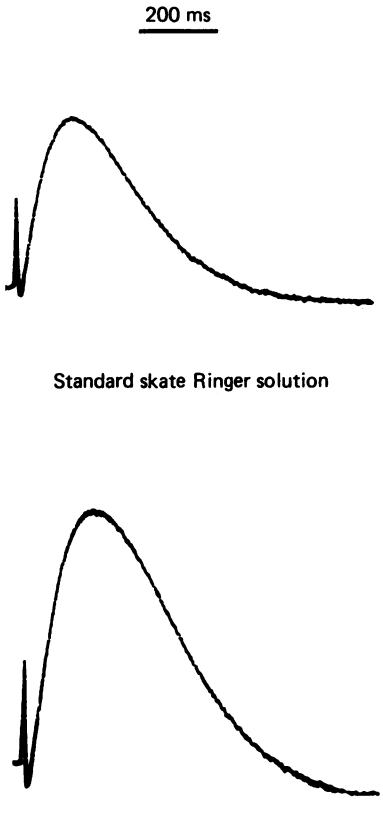

Fig. 12. The effect of hypo-osmotic Ringer solution on the composite optical signal recorded from cerebellar slices stained with the dye RH-155. The upper panel shows the composite optical signal recorded from the molecular layer in a control Ringer solution following staining for 1 h in 0·2 mg RH-155/ml. The lower panel shows the effect of diluting the elasmobranch Ringer solution to 80% of normal tonicity by the addition of distilled water. This is expected to reduce the extracellular volume in this preparation. The traces were recorded in single sweeps by single representative photodetectors. The a.c. coupling time constant was 3 s. Temperature 14 °C.

complementary experiment. Here, we observe an increase in the size of the slow component of the optical signal upon a decrease in the tonicity of the extracellular medium. Although the preparation remained responsive for hours after this treatment, the effect could not be reversed by washing with control Ringer solution.

Both of these experiments are consistent with the idea that the slow component of the optical signal results from potassium accumulation in the extracellular space

upon stimulation of the parallel fibres, and reflects the depolarization of the glial cells that behave as potassium electrodes.

DISCUSSION

The results presented here suggest several conclusions that we believe are significant. First, we have demonstrated a reliable and very simple fish brain slice preparation. This preparation has the advantage of simplicity, but that simplicity has a price, viz. the apparent absence of viable, or, at least, easily excitable postsynaptic structures. This situation may be ameliorable by cutting thicker slices, so that the Purkinje dendritic arborization is spared, but it is not certain that an increase in slice thickness sufficient to preserve postsynaptic structures will permit adequate oxygenation of the preparation.

Secondly, we have been able to characterize, at least in a preliminary fashion, the action potential in a small unmyelinated C.N.S. fibre system, the parallel fibres, whose individual elements are far too small to impale with microelectrodes.

Thirdly, we have shown that there exist closely related dyes having different relative affinities for different membranes in the same preparation. We expect that this finding may prove quite useful for the study of brain structures containing different cell types.

Finally, the extraordinary sensitivity of the slow component of the optical signal to calcium and calcium channel blockers suggests that much of the potassium that accumulates in the cerebellum of the skate passes through calcium-dependent potassium channels, and that, at least in this preparation, intracellular calcium is critically involved in potassium homeostasis.

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