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Evaluation of Voltage-Sensitive Dyes for Long-Term Recording of Neural Activity in the Hippocampus

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Abstract. We searched for an optimal voltage-sensitive dye for optical measurements of neural activity in the hippocampal slice by evaluating several merocyanine-rhodanine and oxonol dyes. The wavelength dependence (action spectra), pharmacological effects of staining, signal size, signal-to-noise ratio, and the utility of the dyes for long-term continuous recording were examined for four merocyanine-rhodanine dyes (NK2761, NK2776, NK3224 and NK3225), which had been reported to be optimal in embryonic nervous systems, and for two oxonol dyes (NK3630 (RH482) and NK3041 (RH155)), which have been among the most popular potentiometric probes for the hippocampal slice preparation. NK2761, NK3224 and NK3225 provided large signal-to-noise ratios, and proved to be useful for optical recordings lasting several hours. NK3630 was most suitable for long-term recording, although the signal-to-noise ratio was slightly inferior to that of the merocyanine-rhodanines. Using NK3630 (RH482) on the hippocampal slice preparation, we demonstrate here that long-term potentiation can be monitored stably for more than 8 hr.

Key words: Optical recording — Voltage-sensitive dye — Dye screening — Merocyanine-rhodanine — Hippocampal slice — Long-term potentiation

Introduction

Hippocampal slices constitute an organized laminar structure suitable for a physiological analysis of synaptic connections among neurons. The hippocampus is also used as an excellent model system for the analysis of long-term potentiation (LTP), which is considered to be a fundamental cellular mechanism responsible for the phenomena of learning and memory (Tsumoto, 1992; Bliss & Collingridge, 1993). For these investigations, conventional electrophysiological measurements have usually been applied, although they have some technical limitations: only a restricted number of electrodes can be placed in the preparation, and intracellular recording can be made only from large elements (e.g., cell bodies and large dendrites) and for relatively short durations.

Optical recording techniques with voltage-sensitive dyes have provided a powerful means for monitoring neural electrical activity offering two principal advantages over conventional electrophysiological techniques. One is that it is possible to monitor intracellular membrane potential changes directly and noninvasively. The other is that multiple sites of a preparation can be monitored simultaneously (for reviews *see* Cohen & Salzberg, 1978; Salzberg, 1983; Grinvald et al., 1988; Kamino, 1990, 1991). Since the first optical recording study in the hippocampal slice (Grinvald, Manker & Segal, 1982), many investigations have been devoted to this preparation, using absorption (Barish et al., 1996; Iijima et al., 1996; Nakagami, Saito & Matsuki, 1997; Sekino et al., 1997; Kojima et al., 1999), and fluorescent (Saggau, Galvan & Bruggencate, 1986; Albowitz & Kuhnt, 1991; Iijima et al., 1996) voltage-sensitive dyes (for a review *see* Ebner & Chen, 1995). Most of the recent works used oxonol dyes, such as RH155 and RH482, and they demonstrated that these absorption dyes provide usable signals (Barish et al., 1996; Iijima et al., 1996; Nakagami et al., 1997; Sekino et al., 1997; Kojima et al., 1999). On the other hand, we have previously reported that, in the embryonic nervous system, merocyanine-rhodanine dyes are better than the oxonols (Momose-Sato et al., 1995).

The choice of optimal dyes is an important consideration in optical recordings. Since transmission measurements are usually more advantageous than fluorescence in brain slice preparations (Grinvald et al., 1988; Wu & Cohen, 1993), we have compared the properties of

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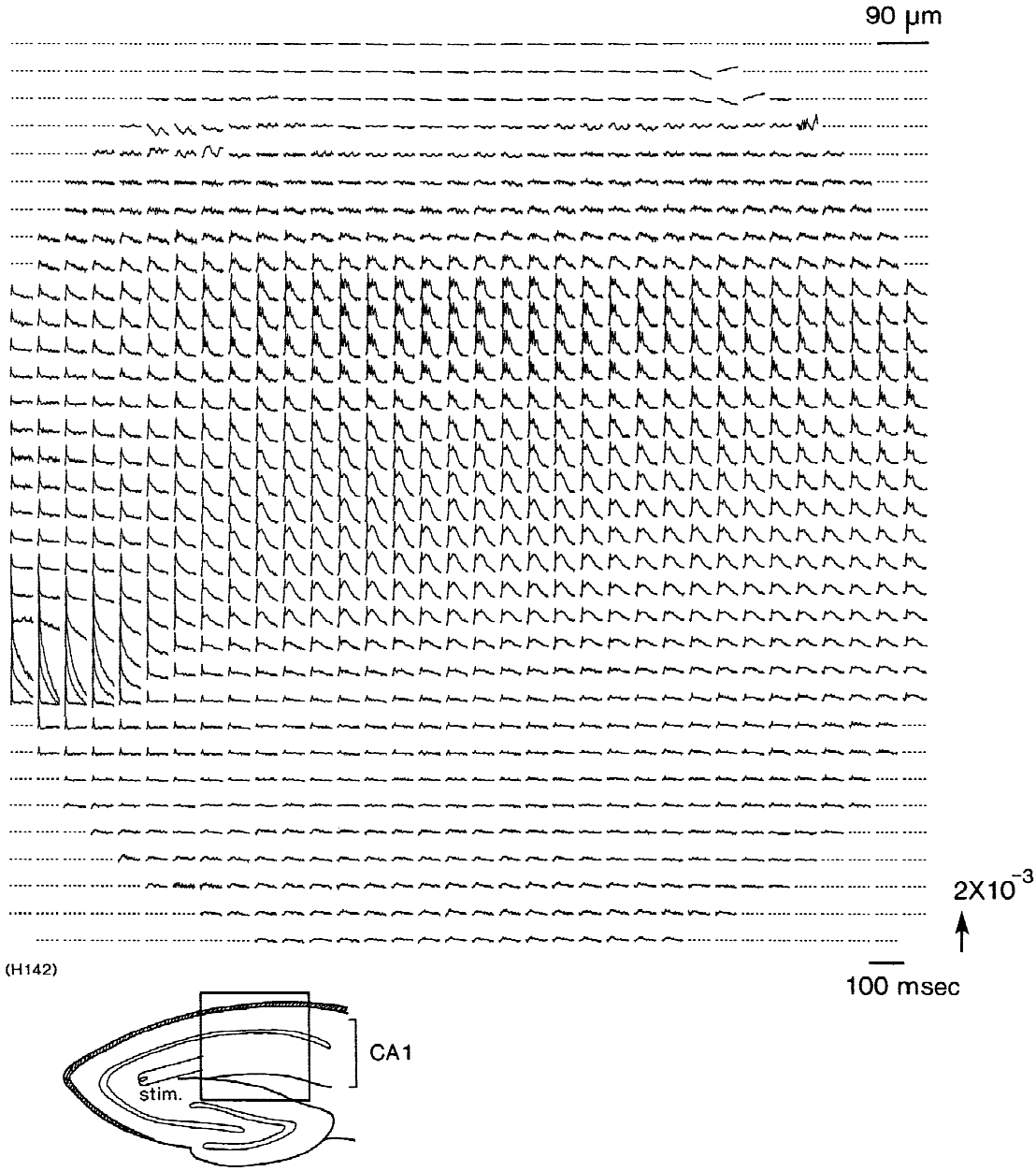

Fig. 1. Multiple-site optical recording of neural responses in a hippocampal slice preparation stained with an oxonol dye NK3630 (RH482) (0.5 mg/ml). The optical signals were evoked by applying a square current pulse (150 μA/250 μsec) to the Schaffer collateral pathway with a bipolar electrode. The evoked optical signals were detected using a 34 × 34 matrix photodiode array from the region indicated by a square in the lower inset. Four trials were averaged. The direction of the arrow on the right of the recording indicates a decrease in transmitted light (increase in dye absorption), and the length of the arrow represents the stated value of the fractional change (the change in the light intensity divided by DC-background intensity).

two major classes of absorption dyes, e.g., the merocyanine-rhodanines and the oxonols, in the present experiment. The second aim of this study is to evaluate the utility of the various voltage-sensitive dyes for long-term monitoring of hippocampal neural activity. Recently, a late phase of LTP, which lasts longer than 4 hr, has been distinguished from an early phase of LTP, and the importance of in vitro investigations with long-term recording has been emphasized (Abraham & Otani, 1991; Frey et al., 1988, 1996). Optical recording of intracellular membrane potential changes from multiple regions would be a useful tool for the study of LTP.

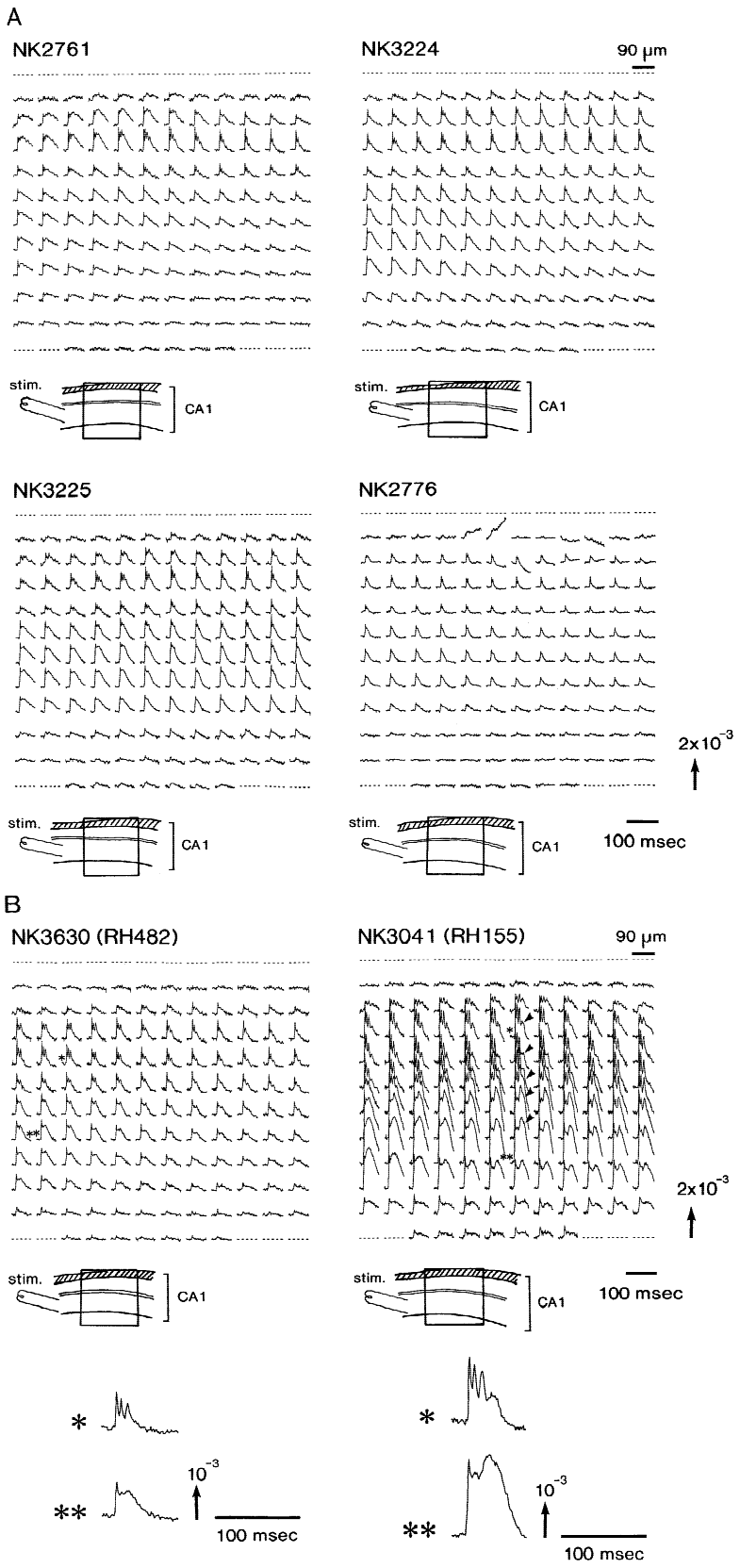

Fig. 2. Multiple-site optical recordings of neural responses in hippocampal slice preparations stained with four merocyanine-rhodanine dyes (*A*) NK2761, NK3224, NK3225 and NK2776, and two oxonol dyes (*B*) NK3630 (RH482) and NK3041 (RH155). In B, enlargements of the optical signals labeled with asterisks are presented on the bottom. The evoked optical signals were detected using a 12 × 12 matrix photodiode array from the CA1 region. In this and the following figures, two trials were averaged for the merocyanine-rhodanine dyes, and three trials were averaged for the oxonol dyes, except where noted.

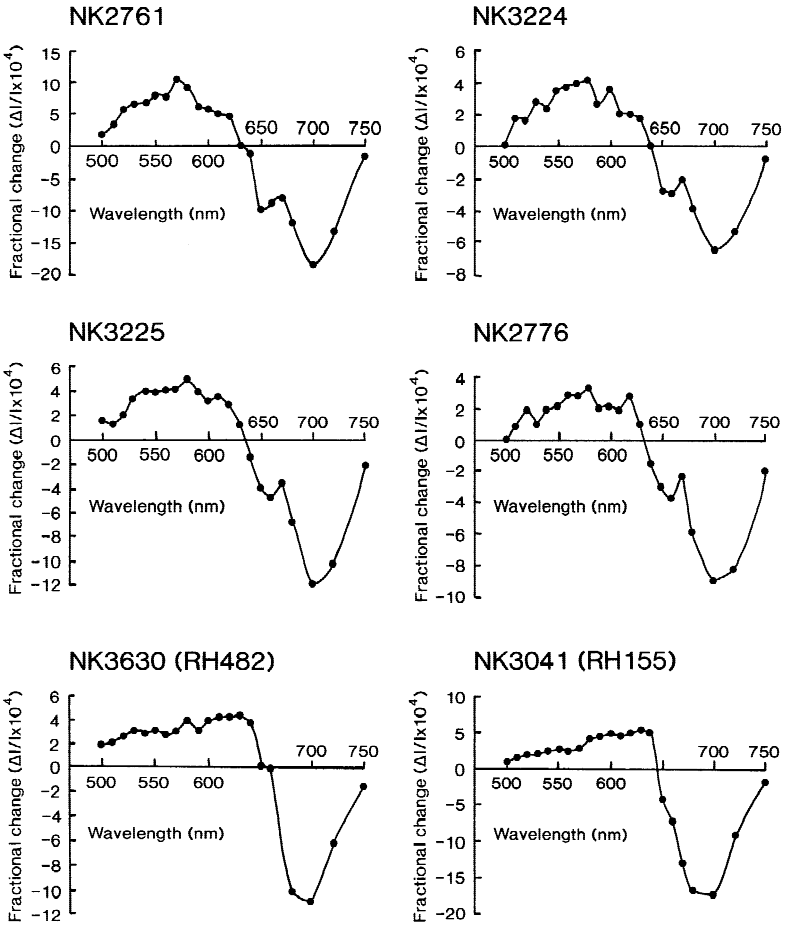

Fig. 3. Wavelength dependence of the optical signals. The amplitudes of the largest fast optical signal were plotted against the wavelength of the incident light.

Materials and Methods

Hippocampal Slice Preparations

Male Wistar rats (Saitama experimental animals supply, Saitama, Japan) 8–10 weeks of age were decapitated under ether anesthesia. Brains were quickly removed and cooled in iced artificial cerebrospinal fluid (ACSF). The solution contained (in mM): NaCl 124, KCl 5, $MgSO_4$ 1, $CaCl_2$ 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 22 and glucose 10, and was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). Transverse slices of hippocampus, 300 μm thick, were prepared using a rotorslicer (DTY-8700, Dosaka EM, Kyoto, Japan). The slices were maintained at room temperature (26–30°C) for at least 1 hr before use. The slice was transferred to a recording chamber and was continuously perfused with ACSF at a rate of 1–5 ml/min (usually 1 ml/min) at 30–32°C.

Electrical Stimulation

The Schaffer collateral pathway was stimulated using a bipolar tungsten electrode. A square current pulse (100–400 μA/250 μsec), which evoked nearly maximum responses in the CA1 region, was delivered at 0.05 Hz. In LTP experiments, the current intensity of test pulses was adjusted so as to elicit an excitatory postsynaptic potential (EPSP)-related slow optical signal of 30–50% of its maximal amplitude. LTP lasting longer than 5 hr was induced by tetanic stimulation using either three stimulus trains of 100 pulses (100 Hz/1 sec duration) with 10 min intertrain intervals (Frey et al., 1988, 1996), or 50 trains of 10 pulses (400 Hz/25 msec duration) presented as 10 bursts of 5 trains at 1 Hz (1 min between bursts) (Otani et al., 1989; Abraham et al., 1993).

Dye Staining

The slice was stained for 5 min in ACSF solution to which 0.2–0.5 mg/ml of the dye (usually 0.5 mg/ml) was freshly dissolved. After the staining, the preparation was washed with perfusion of normal ACSF, and was kept in the dark. The dyes used in the present experiment were as follows. Merocyanine-rhodanine: NK2761, NK2776, NK3224, NK3225. These dyes have been reported to be optimal for monitoring neural activity from early embryonic nervous systems (Momose-Sato et al., 1995). Oxonol: NK3041 (RH155), NK3630 (RH482). These dyes have been most frequently used in recent optical studies in hippocampal and other slice preparations (Konnerth, Obaid & Salzberg, 1987; Barish et al., 1996; Iijima et al., 1996; Nakagami et al., 1997; Sekino et al., 1997). The chemical structures of these dyes have been described previously (Konnerth et al., 1987; Momose-Sato et al., 1995); the dyes were purchased from Kankoh-Shikiso Kenkyusho (Okayama, Japan).

Optical Recording

The preparation chamber was mounted on the stage of an Olympus Vanox microscope (Type AHB-L-1). Bright-field illumination was

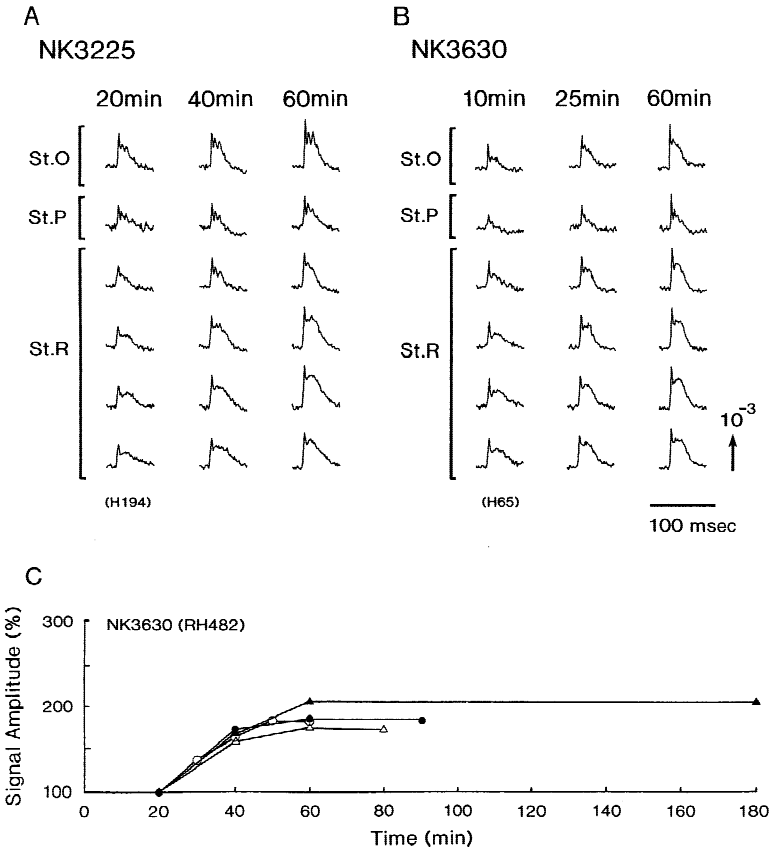

Fig. 4. Time-dependent change in the optical signals after staining. Enlargements of the optical signals obtained from the stratum oriens (St. O), stratum pyramidale (St. P) and stratum radiatum (St. R) are presented. Recordings were made 20, 40 and 60 min after the staining with NK3225 (*A*), and 10, 25 and 60 min after the staining with NK3630 (*B*). The dye concentration was 0.5 mg/ml. In *C*, normalized amplitudes of the slow optical signals (mean of eight signals) detected from the stratum radiatum are plotted against the time. Different symbols correspond to different preparations ($n = 4$).

provided by a 300 W tungsten-halogen lamp (Type JC-24V-300W, Kondo-Philips, Tokyo, Japan) driven by a stable dc-power supply. Incident light was made quasimonochromatic by an interference filter (703 ± 15 nm; Asahi Spectra, Tokyo, Japan) placed between the light source and the preparation. A microscope objective (×10, S plan Apo, 0.4 n.a.) and a photographic eyepiece (×1.67, ×2.5 or ×3.3) formed a magnified (×16.7, ×25 or ×33) real image of the preparation at the image plane. The transmitted light intensity at the image plane was detected using a multi-element silicon photodiode matrix array. In the present experiments, we used two optical recording systems, which were constructed in this laboratory. One is a 1020-site optical recording system with a 34 × 34-element silicon photodiode array (Hamamatsu Photonics, Hamamatsu, Japan) (for details *see* Hirota et al., 1995; Sato et al., 1998). The outputs from 1020 elements were fed into amplifiers via current-to-voltage converters and then passed to 32 sets of 32-channel analog multiplexers. Each output from the multiplexers was fed into a subranging type analog-to-digital (AD) converter system with a resolution of 18 bits and was sent to a computer. Another recording system is a 128-channel multiple-site optical recording system using a 12 × 12-element silicon photodiode array (MD-144-4PV, Centronic, Croydon, UK) (for details *see* Kamino, 1990, 1991; Momose-Sato et al., 1998). The output of each detector in the diode array was passed to an amplifier (AC coupling = 3 sec) via a current-to-voltage converter. The amplified outputs from 127 elements of the detector were recorded simultaneously on the videotape of a 128-channel data recording system (RP-890 series, NF Electronic Instruments, Yokohama, Japan) and were passed to a computer. The time resolution of these systems was ≈1 msec. In each recording, 2–4 trials were averaged, and no offline filtering was used.

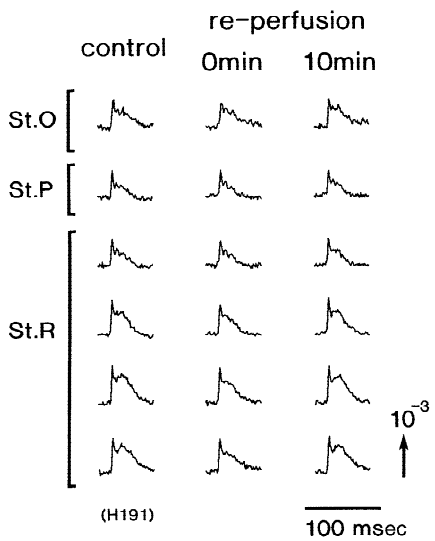

Fig. 5. Effects of stopping perfusion on the optical signals. Recordings were made before perfusion stop for 5 min (control), just after reperfusion (0 min) and 10 min after reperfusion (10 min). The preparation was stained with NK3630, and the control recording was made 3 hr after the staining.

Table 1. Rate of increase in signal size after staining

Dye	Preparation reference	Dye concentration (mg/ml)	Perfusion rate (ml/min)	Pre-incubation (hr)	90% recovery time (min)	
					Fast signal	Slow signal
NK2761	H181	0.5	1	3	61	60
	H182	0.2	1	5	59	60
	H184	0.2	5	4	52	59
	H192	0.2	5	4	36	50
	H190	0.2	5	7.5	56	50
NK2776	H195	0.5	1	1	97	93
	H197	0.5	1	1	112	120
NK3224	H196	0.5	1	1	86	96
	H201	0.5	1	1	77	60
NK3225	H194	0.5	1	1	104	108
	H199	0.5	1	1	80	94
NK3630 (RH482)	H107	0.5	1	1	42	48
	H132	0.5	1	2	30	41
	H113	0.5	1	6	40	38
	H65	0.5	6	1	35	42
	H131	0.5	6	1	45	42
NK3041 (RH155)	H198	0.5	1	1	<10	<10
	H193	0.5	1	1	<20	<20
	H141	0.5	1	5	<20	<20

The time required for attaining 90% of the maximum signal amplitude was evaluated for the fast and slow signals detected from the stratum radiatum. The values were measured from the plots shown in Fig. 4*C*.

Results

Optical Responses Evoked by Schaffer Collateral Stimulation

Figure 1 shows an example of optical recordings made in a hippocampal slice preparation stained with an oxonol dye, NK3630 (RH482). The signals were evoked by stimulation of the Schaffer collateral pathway, and the recording was made by averaging four trials using a 1020-element photodiode array. The magnification was ×33, and each pixel (element) of the array detected light transmitted by a square region (45 × 45 μm^2) of the preparation.

In this recording, the optical signals were evoked in a wide area of the CA1 region. The signals detected at the stratum radiatum consisted of two components, viz., fast spike-like and slow signals. It has been reported that, using the voltage-sensitive dyes (e.g., RH482), the fast and slow components represent action potentials and excitatory postsynaptic potentials (EPSPs), respectively, because the slow signal is eliminated in Ca^{2+}-free solution and the fast signal is blocked by tetrodotoxin (Grinvald et al., 1982; Nakagami et al., 1997). Similar results were obtained in the present experiment. The slow signal was also reduced by APV (DL-2-amino-5-phosphonovaleric acid; 190 μM) and CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione; 5 μM). At the strata pyramidale and oriens, multiple spike-like optical signals were triggered, which have been described as reflecting the action potential discharge (Grinvald et al., 1982). Because the optical signals near the stimulation electrode were contaminated with electrotonic potential-related signals, in the following experiments, we focused on the region which is 400–4500 μm from the tip of the electrode.

Figure 2 shows typical examples of original recordings made in a CA1 region stained with four merocyanine-rhodanine dyes (NK2761, NK3224, NK3225 and NK2776) (Fig. 2*A*), and two oxonol dyes (NK3630 (RH482) and NK3041 (RH155)) (Fig. 2*B*). For these recordings, a 12 × 12-element photodiode array with a magnification of ×16.7 was used. In this and the subsequent figures, two trials were averaged for the merocyanine-rhodanine dyes, and three trials were averaged for the oxonol dyes, except where noted. In the recordings shown in Fig. 2, the waveforms of the optical signals were almost identical for the various dyes, with the exception of NK3041. The signals provided by NK3041 appear to have another slow component with long duration (arrowheads in Fig. 2*B*). Konnerth et al. (1987) reported that RH155 exhibited a large slow wave in skate cerebellar slices, which is the result of an exceptionally high affinity of this dye for glial cell membrane, which monitors $[K^+]_o$. The large slow component observed in

Table 2. Signal size and signal-to-noise ratio

Dye	Preparation reference	Stratum radiatum						Stratum pyramidale			Stratum oriens		
		Fast signal			Slow signal			Fast signal			Fast signal		
		ΔI/I Max ($\times 10^{-4}$)	I (arbitrary unit)	S/N	ΔI/I Max ($\times 10^{-4}$)	I (arbitrary unit)	S/N	ΔI/I Max ($\times 10^{-4}$)	I (arbitrary unit)	S/N	ΔI/I Max ($\times 10^{-4}$)	I (arbitrary unit)	S/N
NK2761	H153	14.0	0.86	13.5	16.0	0.84	11.5	8.0	0.75	7.7	10.9	0.67	7.9
(0.5 mg/ml)	H167	15.6	1.27	18.4	11.0	1.27	13.0	10.9	1.35	11.0	14.9	1.05	16.2
	H174	12.5	1.19	12.6	10.3	1.19	10.4	7.8	1.14	7.8	13.0	0.90	10.8
	H181	16.2	1.28	19.1	10.4	1.28	12.3	13.1	1.21	14.3	16.0	0.85	11.3
	H187	9.9	0.90	7.7	8.5	0.90	6.7	7.4	0.85	7.5	8.6	0.78	6.8
NK2761	H184	7.9	1.49	7.0	4.7	1.49	4.2	4.9	1.42	4.3	6.4	1.38	4.8
(0.2 mg/ml)	H189	3.9	1.42	3.0	1.5	1.42	1.2	2.6	1.43	3.1	3.0	1.11	2.4
	H190	3.9	1.28	2.8	2.7	1.28	1.9	3.0	1.15	1.8	4.0	0.94	2.3
	H192	4.0	1.52	3.5	3.1	1.52	2.7	3.5	1.36	3.5	2.6	1.28	2.3
NK2776	H195	8.5	1.21	10.0	5.4	1.21	6.4	4.7	1.07	6.0	5.7	1.10	5.8
(0.5 mg/ml)	H197	8.0	1.52	6.3	4.6	1.52	3.6	4.4	1.50	3.5	10.8	1.24	9.5
NK3224	H196	11.0	1.23	15.6	7.7	1.23	10.9	7.6	1.25	7.6	15.1	1.19	14.2
(0.5 mg/ml)	H201	15.6	1.04	12.3	13.0	1.04	10.2	7.0	1.11	8.2	12.1	0.84	14.3
NK3225	H194	15.7	1.00	13.9	14.7	1.06	11.5	10.5	1.00	7.1	15.5	0.94	12.2
(0.5 mg/ml)	H199	16.0	1.34	16.2	13.9	1.34	14.0	10.0	1.31	9.4	16.4	1.08	12.9
NK3630	H69	10.1	0.78	6.5	8.8	0.78	5.7	7.5	0.73	4.2	9.5	0.58	6.7
(0.5 mg/ml)	H105	10.9	0.60	9.0	8.2	0.60	6.8	7.4	0.70	6.1	9.5	0.49	5.5
(RH482)	H110	12.1	0.66	6.4	7.0	0.66	3.7	9.5	0.76	5.5	14.0	0.50	5.8
	H113	11.8	0.71	7.2	8.7	0.71	5.3	5.0	0.72	3.0	8.7	0.49	4.2
	H132	9.5	0.51	5.5	10.0	0.51	5.8	5.5	0.51	2.9	6.0	0.35	3.5
	H142	10.5	0.59	8.1	7.8	0.59	6.0	10.6	0.59	5.6	14.0	0.45	6.5
	H175	11.0	0.76	8.6	7.0	0.76	5.5	12.5	0.76	9.3	15.4	0.47	7.3
	H188	11.0	0.62	6.5	8.0	0.62	4.7	10.1	0.58	7.1	13.9	0.42	6.1
	H191	9.0	0.86	5.3	6.5	0.86	3.8	6.4	0.71	3.6	6.0	0.56	4.0
NK3041	H141	31.8	0.35	15.9	35.0	0.39	17.6	22.5	0.43	12.4	22.5	0.36	11.3
(0.5 mg/ml)	H193	18.4	0.74	14.5	18.0	0.81	12.7	13.0	0.74	9.2	11.0	0.62	8.6
(RH155)	H198	15.5	0.86	11.0	15.0	0.86	10.6	15.6	0.77	10.5	23.8	0.59	15.3

The signal size (ΔI/I: fractional change in transmitted light intensity), the background light intensity (I) and the signal-to-noise ratio (S/N) were evaluated for the best signals obtained from the stratum radiatum, stratum pyramidale and stratum oriens. The signal-to-noise ratio was measured in a single sweep recording.

the hippocampal slice preparation might also be related to such a glial depolarization. Among the merocyanine-rhodanine dyes, NK2776 usually exhibited relatively small optical signals and fewer multiple spike discharges. This dye often aggregated after the staining, and it is possible that the staining conditions were not as good as with the other merocyanine-rhodanine dyes.

Action Spectra

Voltage-sensitive dye absorption changes are well known to be dependent on the wavelength of the incident light (Waggoner & Grinvald, 1977; Cohen & Salzberg, 1978; Kamino, Hirota & Komuro, 1989). It is also known that the action spectrum of the potential-related optical signal differs from species to species (Ross & Reichardt, 1979; Senseman & Salzberg, 1980). Figure 3 shows the action spectra of the six dyes measured in the hippocampal slice preparation. The four merocyanine-rhodanine dyes exhibited the same action spectra: the transmitted light intensity changed in the positive direction in the range of 500–630 nm, and in the negative direction in the range of 640–750 nm, with the crossover occurring at 630–640 nm. The maximum absorption changes were obtained at 700 nm and 580 nm. These characteristics were the same as those observed in embryonic nervous systems (Momose-Sato et al., 1995), but slightly different from those obtained in adult and embryonic hearts (Hirota et al., 1985; Komuro et al., 1986). On the other hand, the shape of the action spectra of the oxonol dyes

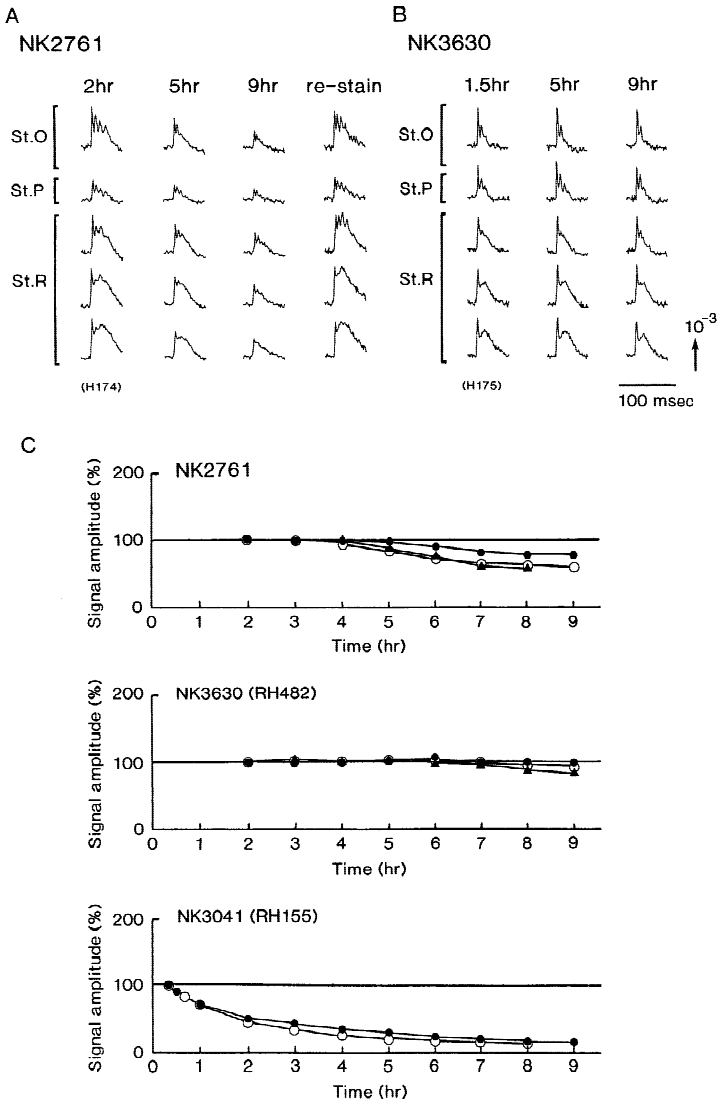

Fig. 6. Optical responses monitored for a long period. In *A*, recordings were made 2, 5 and 9 hr after the staining with NK2761, and in *B*, signals were obtained 1.5, 5 and 9 hr after the staining with NK3630. The dye concentration was 0.5 mg/ml. In *A*, the optical signals were restored after the preparation was restained with 0.5 mg/ml NK2761. (*C*) The amplitudes of the fast signals (mean of eight signals) detected from the stratum radiatum were plotted against the time from the staining (time 0). The optical signals were normalized with those at 2 hr for NK2761 (top) and NK3630 (middle), and at 20 min for NK3041 (bottom). The incident light was turned off except during the measuring period (about 5 sec per hr). Different symbols indicate different preparations ($n = 3$ for NK2761 and NK3630, $n = 2$ for NK3041).

was different from that of the merocyanine-rhodanine dyes: the null wavelength was around at 650 nm, and the maximum absorption changes were observed at 700 nm and 630 nm. These values are similar to those reported by Konnerth et al. (1987) in skate cerebellum. According to these experimental results, we used an incident wavelength of 700 nm in the following experiments.

Time Dependent Change in the Optical Signals After Staining

For most dyes tested, the size of both the fast and slow optical signals was small just after the staining, and it gradually increased with time. Examples for NK3225 and NK3630 are presented in Fig. 4*A* and *B*. The size of the optical signals at 60 min was significantly larger than at 10–20 min. This behavior was observed in every layer of the CA1 region. Figure 4*C* shows the time course of the slow-signal amplitude detected from the stratum radiatum stained with NK3630. The abscissa is the time after the staining, and the ordinate is the amplitude of the slow signals (baseline-to-peak) normalized to the size of the signals at 20 min. The maximal signal size was attained 60 min after the staining.

In the present experiment, the perfusion was stopped for 5 min during the staining. We checked the effects of this procedure in a preparation stained with NK3630. In Fig. 5, optical signals detected before stopping perfusion for 5 min (control), just after reperfusion (0 min) and 10 min after reperfusion (10 min) were compared. The fast and slow signals were slightly reduced with the cessation of perfusion, but they fully recovered after 10 min. Therefore, the suppression of the optical signals observed initially after staining is not due to ischemia.

Table 3. Effective recording time with no discernible change in the optical signals

Dye	Preparation reference	10% Reduction time	
		Fast signal	Slow signal
NK2761	H167	6 hr 13 min	6 hr 3 min
	H169	5 hr<	4 hr 20 min
	H174	4 hr 20 min	4 hr 12 min
	H187	4 hr 33 min	5 hr 8 min
NK2776	H195	9 hr<	9 hr<
	H197	8 hr 40 min	9 hr<
NK3224	H196	5 hr	4 hr 56 min
	H201	5 hr 24 min	5 hr 27 min
NK3225	H194	7 hr	6 hr 43 min
	H199	5 hr 24 min	6 hr
NK3630	H67	7 hr 20 min	7 hr 9 min
(RH482)	H69	9 hr<	9 hr<
	H175	9 hr<	9 hr<
NK3041	H141	40 min	39 min
(RH155)	H193	31 min	43 min
	H198	32 min	34 min

The time required for 10% reduction of the optical signal amplitude was evaluated for the fast and slow signals detected from the stratum radiatum. The values were measured from the plots shown in Fig. 6*C*.

The time required for the maximum signal size depended on the dye. These results are summarized in Table 1. Of the dyes tested, NK3041 showed the fastest increase in signal size (90% < 10 min), followed by NK3630 > NK2761 > NK3224 > NK3225, and then NK2776 (90% ~1.5–2 hr). The rate of increase in signal was not changed by lowering the concentration of the dye, by increasing the perfusion rate, or by increasing the pre-incubation time (*see* NK2761 and NK3630 in Table 1).

Signal Size and Signal-to-Noise Ratio

The signal size and the signal-to-noise ratio provide a good indication of which dyes are likely to be useful for monitoring transmembrane potential (Cohen et al., 1974; Ross et al., 1977; Gupta et al., 1981; for a review *see* Cohen & Salzberg, 1978). Thus, we examined the fractional change in transmitted light ($\Delta I/I$) and signal-to-noise ratio (S/N) for the best signals obtained from the preparations stained with the different dyes. In Table 2, the maximum sizes of $\Delta I/I$ and S/N measured in single sweep recordings are compared for the fast and slow signals.

When the merocyanine-rhodanine dyes were applied to the hippocampal slice, NK2761, NK3224 and NK3225 usually gave large signals and good signal-to-noise ratios: $\Delta I/I$ was 7.0–16.4 × 10^{-4} and S/N was 6.7–19.1.

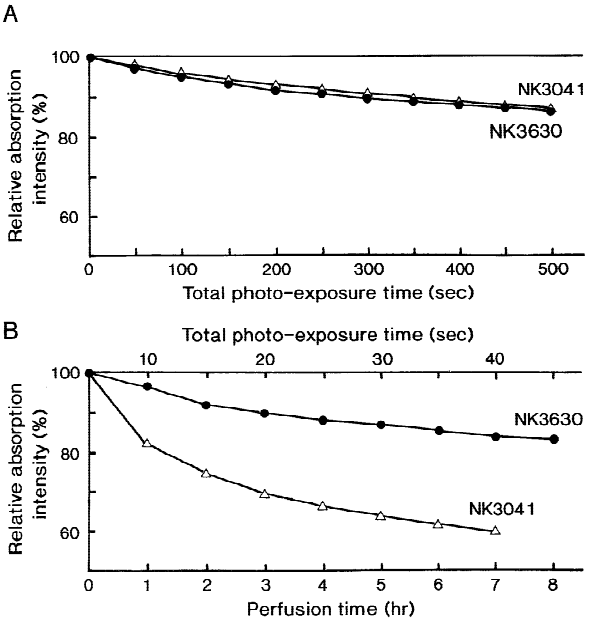

Fig. 7. (*A*) Effects of photo-illumination on the absorption intensity of the dye. The ordinate represents the relative absorption intensity (DC-background light intensity at time 0 divided by that at each time), and the abscissa is the time after the beginning of continuous illumination. (*B*) Effects of perfusion on the absorption intensity of the dye. The incident light was turned off except during the measuring period. The lower abscissa is the time after the staining (perfusion rate: 1 ml/min), and the upper abscissa is the total illumination time. Closed circles are for an experiment with NK3630 and open triangles are for an experiment with NK3041.

On the other hand, NK2776 provided smaller signals: $\Delta I/I$ was 4.4–10.8 × 10^{-4} and S/N was 3.5–10.0. When we used NK2761 with a concentration of 0.2 mg/ml, the signal size and the signal-to-noise ratio were markedly smaller, suggesting that this concentration is not optimal.

Of the dyes tested, the oxonol dye, NK3041, gave the largest signals: $\Delta I/I$ was 11.0–35.0 × 10^{-4}. NK3630 also provided large signals. However, the signal-to-noise ratio of these oxonol dyes, especially of NK3630, was not as good as expected. The S/N were 8.6–17.6 for NK3041 and 2.9–9.3 for NK3630. In general, signal-to-noise ratio is proportional to the square root of the transmitted background light intensity, if the dominant noise is shot-noise (Waggoner & Grinvald, 1977; Salzberg, 1983; Grinvald et al., 1988). In the present experiment, the background light intensity of the oxonol dyes was much smaller than that of the merocyanine-rhodanine dyes under equal staining conditions and with equal illumination intensity (Table 2:I). Thus, the low transmitted light intensity seems to be the cause of the poor signal-to-noise ratio of the oxonol dyes.

Effective Recording Time

To evaluate the utility of the dyes for long-term continuous recording, we examined how long measurements can

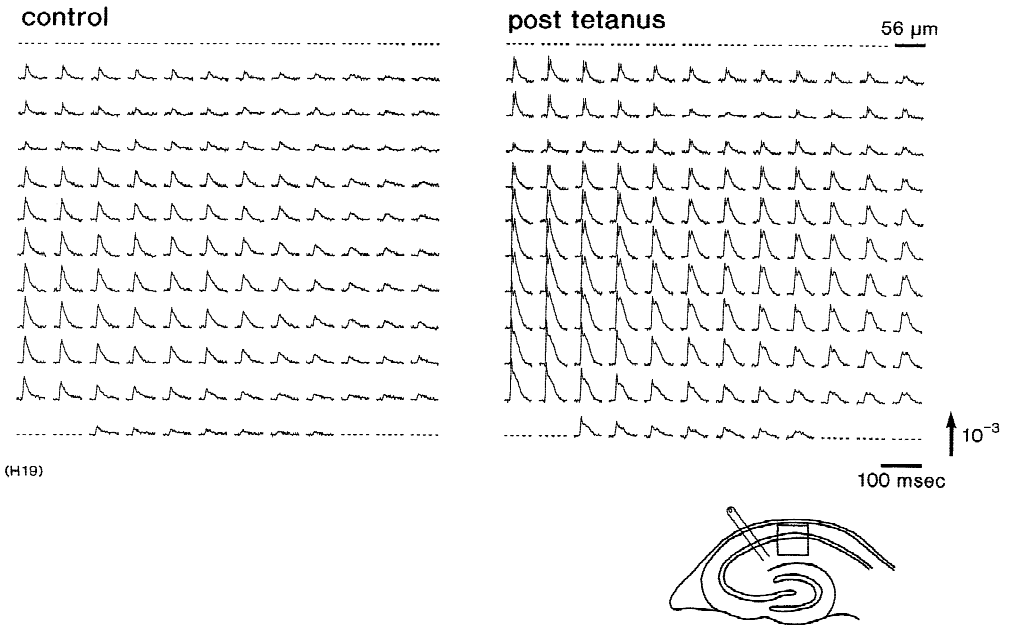

Fig. 8. Potentiation of the optical signals induced by tetanic stimulation (100 Hz/1 sec duration) delivered to the Schaffer collaterals. The preparation was stained with NK2761, and 8 trials were averaged.

be made with no discernible change in the optical responses. Figure 6*A* and *B* present optical signals monitored for 9 hr after the preparations were stained with NK2761 and NK3630. In this experiment, the incident light was turned off except during the measuring period (about 5 sec per hr). When we used NK2761 (Fig. 6*A*), the size of the fast and slow signals decreased gradually with time in every layer of the CA1 region. Both the signals were recovered in amplitude after the preparation was restained with NK2761, indicating that the deterioration of the optical signals is not due to decreased viability of the slice, but, rather, to lowered effectiveness of the dye. In the case of NK3630 (Fig. 6*B*), however, a significant reduction of the optical signals was not evident even after 9 hr.

In Fig. 6*C*, normalized signal amplitudes of the fast signals detected from the stratum radiatum are plotted against time, for several preparations stained with NK2761, NK3630 and NK3041. When we used NK2761 (Fig. 6*C*, top), the size of the optical signals was nearly constant for 4 hr, and then declined gradually. For NK3630 (Fig. 6*C*, middle), the optical signals were almost unchanged for 7 hr. On the other hand, when we applied NK3041 (Fig. 6*C*, bottom), the optical signal decreased rapidly, and the signal amplitude was reduced by 40% after 1 hr. Similar experiments were carried out using three other merocyanine-rhodanine dyes (NK3224, NK3225 and NK2776). The results are summarized in Table 3. Of the dyes tested, NK3041 exhibited the most rapid change in the optical signal size (10% reduction <40 min), followed by NK2761, NK3224, NK3225, and then NK2776/NK3630 (10% reduction >9 hr).

Two possible mechanisms of the time-dependent change in the optical signals can be considered. One is photobleaching, which is caused by an exposure of the stained preparation to the illumination light. Another is a reduction of the amount of dye bound to the cell membranes, which is caused by perfusion or other experimental procedures. To identify the mechanism(s), we examined the effects of illumination and perfusion. The results are shown in Fig. 7*A* and *B*, respectively. In Fig. 7*A*, the time course of photobleaching is compared for two oxonol dyes, NK3630 and NK3041. The abscissa is the illumination time, and the ordinate is the normalized background light intensity monitored without electrical stimulation under continuous illumination. Between NK3630 and NK3041, no significant difference was observed in the rate of increase in the transmitted light intensity (a decrease in dye absorption). In Fig. 7*B*, the effect of perfusion is compared for NK3630 and NK3041. In this experiment, the incident light was turned off except during the measuring period (about 5 sec per hr). The normalized transmitted light intensity is plotted against the time of perfusion (1 ml/min; lower abscissa), together with the time of total illumination (upper abscissa). In Fig. 7*B*, the rate of increase in the transmitted light intensity (a decrease in absorption) was more rapid in NK3041 than in NK3630. We suggest that this difference is due to a difference in a dissociation rate of the dye from the cell membrane, and this is the cause

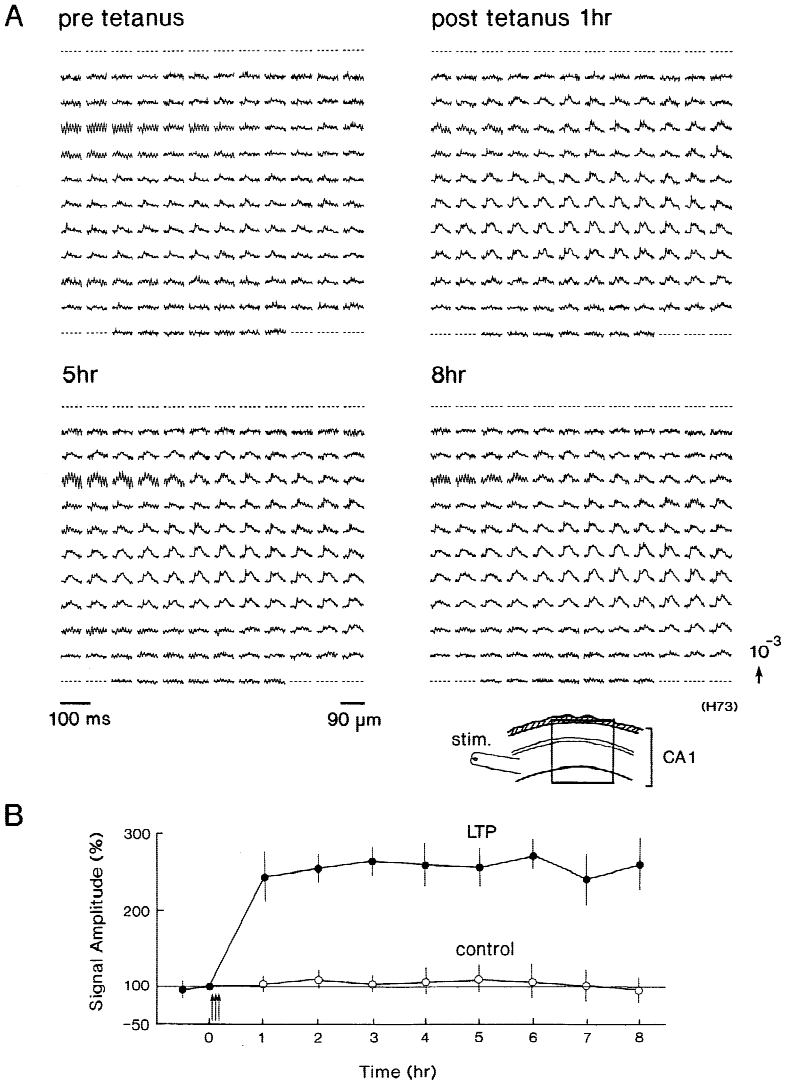

Fig. 9. (*A*) Long-term recording of the potentiation of the optical signals induced by tetanic stimulation (400 Hz/25 msec duration ×5 trains ×10 bursts: *see* Methods). The preparation was stained with NK3630, and two trials were averaged. (*B*) Normalized signal amplitudes of the EPSP-related slow optical signals detected from the stratum radiatum. Mean ± SD ($n = 8$ positions) of the signal amplitude is plotted against the time. Closed and open circles are the data obtained with and without tetanization, respectively. Arrows indicate tetanization.

of the difference in the time course of the optical signal change observed in Fig. 6 and Table 3.

Application to Monitoring LTP

Based on the experimental results represented above, we applied the multiple-site optical recording method to the hippocampal slice to monitor long-term potentiation. Figure 8 shows a typical example of the potentiation of the optical signals induced by tetanic stimulation. The preparation was stained with NK2761 and the tetanus was delivered to the Schaffer collateral pathways. Tetanization increased the amplitude of the EPSP-related slow optical signals. In addition, the initial spike portion seems enhanced, and in some regions a second spike appeared. The change was most evident in the region of the stratum radiatum. Thus, in the following experiment, we focused on the EPSP-related signals evoked in this region.

Figure 9 shows a long-term recording of optical signals made for 8 hr after tetanization. In this experiment, we used NK3630, and the recordings were made only twice every hour (average of two trials in each recording) to minimize the effects of photobleaching. Although the signal-to-noise ratio of the original recording was not large, it is clearly demonstrated that the slow signal amplitude was increased significantly by the tetanus, and that this potentiation lasted for 8 hr. In Fig. 9*B*, the time course of the slow signal amplitude, normalized at time 0 (just before tetanization), is presented. From these data, it is demonstrated that the optical recording tech-

nique with a voltage-sensitive dye can be used effectively for the study of LTP in in vitro slice preparations.

Discussion

In the present experiments, we screened several voltage-sensitive dyes with an emphasis on absorption in the hippocampal slice preparation. Screening of dyes in a new preparation seems to be crucial for a successful application of the optical technique, because it has been shown that the sensitivity (the signal size and the signal-to-noise ratio), wavelength dependence, and other characteristics of the dyes differ from species to species and from preparation to preparation (Ross & Reichardt, 1979; Senseman & Salzberg, 1980; Grinvald et al., 1988). Indeed, an oxonol dye, NK3041 (RH155), provided large optical signals in the hippocampal slice, although it gave very small signals in the embryonic nervous systems (Momose-Sato et al., 1995).

The ideal voltage-sensitive dye is sensitive to changes in transmembrane potential and has little or no pharmacological and/or phototoxic actions. In addition, it is required that bleaching of the dye is small. The present results demonstrate that useful absorption probes of membrane potential are available from among the merocyanine-rhodanines and the oxonols. In the present experiment, large signal-to-noise ratios were obtained for NK2761, NK3224, NK3225 and NK3041 (RH155). Although NK3041 showed the largest signal and the fastest increase in the signal size after staining, this dye seems not ideal for monitoring neural responses in the hippocampal slice. First, NK3041 often exhibited a second slow component with long duration, which was not observed with other dyes. Second, the reduction of the optical signals due to dye washout was so rapid that a stable recording could not be performed. The shape and the size of the optical signals using NK2761, NK3224 and NK3225 were nearly constant for 4 to 6 hr, so these dyes appear to be useful for a short-term recording in the hippocampal preparations. The signal-to-noise ratio of NK3630 (RH482) was slightly smaller than that of the merocyanine-rhodanine dyes. However, this dye permitted optical measurements with no discernible change in the signal size over periods of 8 hr or longer. Thus, for long-term continuous recording, NK3630 seems best in the present experiment. The decrease in the optical signals is likely to be due to a dissociation of the dye molecule from the cell membrane caused by perfusion. Therefore, if these dyes are applied to a preparation that does not require perfusion (e.g., cultured slices), even longer recordings might be possible.

When we applied the merocyanine-rhodanine dyes and the oxonol dye NK3630, the size of the fast and slow signals was usually small just after the staining, and it gradually increased with time. This phenomenon has not been observed either in embryonic nervous systems or in cardiac tissues (Kamino, 1990, 1991; Momose-Sato et al., 1995), suggesting that the interaction between the dye and the cell membrane is a complex one in a variety of preparations.

Long-Term Potentiation

The optical recording technique has been applied to the hippocampal slice preparation for short-term recording of LTP (Saggau et al., 1986) and epileptiform potentials (Albowitz & Kuhnt, 1991). In the present experiment, we have succeeded in monitoring LTP for at least 8 hr. As is the case with behavioral memory, LTP in the hippocampal CA1 region and in the dentate gyrus consists of different stages: late LTP, lasting longer than 4 hr, can be distinguished from early LTP, lasting minutes or several hours, using inhibitors of protein synthesis (Frey et al., 1988; Otani et al., 1989; Frey & Morris, 1997). LTP is also classified into three phases, viz., LTP1, LTP2 and LTP3, according to the time constants of their decay (Abraham & Otani, 1991; Abraham et al., 1993). It has been suggested that the late phase of LTP is dependent on *de novo* synthesis of mRNA. However, the experimental effects of an RNA synthesis blocker, actinomycin D, are still confusing (Otani et al., 1989; Nguyen et al., 1994; Nguyen & Kandel, 1996; Frey et al., 1996). We are now investigating the effects of some inhibitors of protein synthesis on LTP, using the optical recording technique, and the voltage-sensitive dyes that have proven to be useful in the present experiment.

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