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Neutral red as a hydrophobic probe for monitoring neuronal activity

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Abstract

Neutral red fluorescence has been used to monitor neuronal activity. Local changes in either pH or hydrophobicity are reported to increase neutral red fluorescence, but the mechanisms underlying the increases remain unclear. In this study, the pH-dependent fluorescence changes in the basic and acidic forms of neutral red preloaded in rat cerebellar slices were separately measured with two excitation wavelengths. Bath application of kainate, domoate or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) at 1 mM increased fluorescence of the acidic form and decreased that of the basic form irreversibly, consistent with a pH-dependent mechanism. In contrast, application of lower concentrations of AMPA, (*S*)-3,5-dihydroxyphenylglycine or KCl increased fluorescence of both forms transiently, suggesting that a pH-independent mechanism may also increase neutral red fluorescence. Electrical stimulation in the molecular layer also increased fluorescence of both forms. The response to weak electrical stimulation decayed in about 1 s, while response to intense stimulation lasted longer than 1 min. Neutral red binding to phospholipids was also detected as fluorescent spots on thin-layer chromatography, suggesting that interaction with phospholipids enhances neutral red fluorescence. Thus, the short-lasting signal of neutral red suggests its usage as a hydrophobic probe for neuronal activity.

Keywords: Neutral red; Fluorescence; Optical recording; Phospholipid; pH; Hydrophobic interaction

1. Introduction

Although rapid-responding voltage sensitive dyes are widely used for optical recording of neuronal activities, their response amplitudes are too small ($\sim 0.1\%$) for quantitative studies of synaptic transmission. Furthermore, staining procedures with highly lipophilic dyes often include treatment with detergents harmful to the tissue. Neutral red (NR) is a vital pH-indicator dye and was recently used as a probe for neuronal activity (Chen et al., 1996). NR stains neurons by simple incubation without any detergent and does not leak from living cells (Kado, 1993). Neuronal excitation increases NR fluorescence up to 10 times more than voltage sensitive dyes (Kado, 1993; Chen et al., 1998). Thus, NR seems to be a promising probe for monitoring neuronal activity. However, the origin of the fluorescence increase remains unclear. Chen et al. (1996, 1998) reported that NR fluorescence increased through a local decrease in pH, while other reports (Cohen et al., 1974; Lillie, 1977; Lulai and Morgan, 1992; Kado, 1993) have suggested that changes in environmental hydrophobicity may be the origin of the fluorescence increase.

The pink, acidic form of NR has a releasable proton (Lillie, 1977) and shows greater fluorescence than the yellow, basic form ($pK_a = 6.81$), (Lulai and Morgan, 1992). If acidification increases fluorescence through a fractional increase in the acidic form, it should be accompanied by a decrease in its basic counterpart. Therefore, according to pH theory, when fluorescence of the acidic form increases, that of the basic form should decrease. Based on this idea, each of the pH-dependent forms of NR was excited separately using two

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excitation filters in the present study. The results showed that intense stimulation with receptor agonists evoked the expected reciprocal changes in the acidic and basic forms of NR fluorescence. On the other hand, neuronal activation by weak electrical or pharmacological stimuli increased fluorescence of both acidic and basic forms of NR. This unexpected behavior of NR fluorescence is not consistent with a fractional change in the pH-dependent form of NR, and will be referred to as pH-independent change. Other experiments also showed that NR fluorescence increased on interaction with phospholipids, supporting the idea that changes in hydrophobic interactions also influence NR fluorescence.

2. Materials and methods

Male Wistar ST rats (7–10 weeks old) were decapitated under ether anesthesia and 250 μm thick parasagittal slices were prepared from the cerebellar vermis with a DTK2000 microslicer (Dosaka EM, Japan). After recovery (90 min), slices were incubated with 30 μM NR for 30 min at 32°C with continuous, gentle stirring. Extracellular NR was washed out for 30 min before making measurements in a submersion chamber maintained at 32°C. Slices were constantly perfused with artificial cerebrospinal fluid (1.5 ml/min), composed of (in mM), 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2.4 $CaCl_2$, 5 KH_2PO_4, 25 $NaHCO_3$ and 11 glucose, and constantly bubbled with 5% CO_2/95% O_2.

Images (12 bit, 512×512 pixels) were acquired with a cooled CCD camera (Photometrics, PXL37) and an upright fluorescence microscope (Olympus, BX50-WI) equipped with a dichroic mirror (600 nm), an emission filter (> 610 nm) and a $40 \times$ water-immersion objective lens. Two excitation filters, 460–490 nm (IB) and 540–580 nm (IY), were mounted in a filter changer (Olympus OSP-EXA) so that excitation light (150 W xenon lamp with a 25% ND filter) was alternately applied for 200 ms through each of the filters. Fluorescence images (50–100 ms exposure), one acidic and one basic, were taken with a 200 ms separation every min. For response time course measurements, images (2×2 binning, 256×256 pixels) were taken every 100 ms with 30 ms exposure using the IY filter, and the times from stimulation to the first image acquisition was varied under control by a Master-8 stimulator (AMPI, Israel).

Images were stored in a personal computer (Power Macintosh 9500/200) and analyzed off line with IPLab software (Scanalytics, USA). To find regions of interest, a subtraction image (response peak minus pre-stimulus) was made and its pixel intensity histogram was calculated. The region of interest was selected among pixels showing changes larger than a half of the maximum change (either decrease or increase) in the histogram. Then, fluorescence changes in the selected region of interest over time were measured in the sequence of images. Finally, changes with maximum magnitude over 4SD of pre-stimulus control (usually 10–15 images) were taken as significant responses.

Drugs were bath applied for 1 min unless otherwise stated. Complete exchange of solution in the chamber took 1 min, judged from chloride potential shift measured by an Ag/AgCl electrode. A tungsten bipolar electrode (WPI, USA; tip resistance 2 MΩ) was positioned 150 μm beneath the slice surface in the molecular layer. Slices were stimulated 10 times at 0.5 Hz with 10 pairs of biphasic pulses (at 20 Hz), using a SEN7203 stimulator and a SS201J isolator (Nihon Kohden). The duration and intensity of stimulus pulses were 0.1 ms and 6 V, respectively, except where noted in the text.

Purity of NR (Molecular Probe, 'high purity') was more than 98% when checked by HPLC (Gilson) with a NovaPak ODS column (Waters) according to manufacturer's instructions. No fluorescent metabolite of NR was extracted with 20% methanol from the homogenate of the stained slices. Fluorescence spectra of NR in solution were obtained with a spectrofluorometer (Shimadzu RF5000) with a quartz cell. Phospholipids were extracted from rat cerebella using the method of Palmer (1971), and five authentic phospholipids (Sigma) were analyzed by high-performance TLC (Merck) with one of the following systems; $CHCl_3$: C_2H_5OH: 28% NH_4OH (65:35:5, volume ratio) or $CHCl_3$: $(CH_3)_2CO$: C_2H_5OH: CH_3COOH: H_2O (10:4:2:2:1). Two silica TLC plates were run simultaneously, one was soaked in 0.005% Rhodamine 6G (Sigma) for phospholipid detection and the other in 0.5 mM NR in methanol. Fluorescent spots on these plates were analyzed with a fluoroimager (excitation 532 nm, emission > 555 nm, Molecular Imager FX, BioRad). Kainic acid was purchased from Nakalai Tesque Co. Ltd. (Japan). Domoic acid, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), 6-cyano-7-nitroquinoxaline-2,3-dione sodium salt (Na–CNQX) and (*S*)-3,5-dihydroxyphenylglycine ((*S*)-DHPG) were purchased from Tocris–Cookson (UK).

3. Results

To find selective excitation filters for each form of NR, excitation spectra were measured in aqueous solutions at various pH. Fig. 1(A) shows fluorescence excitation spectra of aqueous NR solution at pH 5.5 and 10.5, representing nearly pure spectrum of each form, as NR is 99.98% basic at pH 10.5 and 95% acidic at pH 5.5, based on the reported value of pK_a 6.81, (Lulai and Morgan, 1992). Although these fluorescence spectra overlapped considerably, the peak at around 570 nm increased and that around 470 nm decreased as pH

decreased (Fig. 1(B)). Therefore, fluorescence intensity measured through filters passing these peak wavelengths should change in opposite directions with pH change. A calculated subtraction spectrum (pH 6.8 minus 7.4) showed that the excitation peaks of the acidic and basic forms and an isosbestic point were at 572, 467, and 514 nm, respectively (Fig. 1(C)). When the fluorescence of NR solutions was measured with a CCD camera through filters IY (540–580 nm) and IB (460–490 nm), NR fluorescence was increased and decreased, respectively, as pH decreased (Fig. 1(D)). Thus, IB and IY filters actually report fractional changes of each pH-dependent form of NR. The fluorescence measured through the IY and IB filters will be referred to as the acidic and basic form of NR fluorescence, respectively.

When cerebellar slices were incubated with NR (46 slices), entire Purkinje cells were prominently fluorescent as reported (Kado, 1993), while granule and other cells were stained weakly.

Bath application of 1 mM (*S*)-AMPA increased fluorescence of the acidic form, while fluorescence of the basic form was decreased ($n = 3$ out of 4, (Fig. 2(B–D)). In one case, (*S*)-AMPA application resulted in an increased fluorescence of both forms. The basic form fluorescence response to (*S*)-AMPA tended to show an 'overshoot' after an initial decrease ($n = 2$ out of 3). Chen et al. (1998) reported such a biphasic response as an indication of a pH-dependent mechanism. Other ionotropic glutamate receptor agonists, kainate ($n = 4$ out of 4, (Fig. 2(E)) or domoic acid ($n = 1$ out of 2), at 1 mM also increased fluorescence of the acidic form and decreased fluorescence of the basic form. These changes were consistent with a decrease in intracellular pH and were observed in the molecular layer and Purkinje cell soma, while the change in the granule cell layer was not significant (Fig. 2(B, C)). Although the onset of the fluorescence change fitted reasonably well with the time course of agonist bath application, the changes were irreversible with washout of the agonists for 15 min (Fig. 2(D, E)), suggesting that excitotoxic cell damage by high concentrations of glutamate receptor agonists involves cell acidification (Tang et al., 1998).

In contrast, application of 1–100 μM of (*S*)-AMPA ($n = 13$), an active AMPA receptor agonist, enhanced the fluorescence intensities for both acidic and basic forms of NR (Fig. 3(A, B, E)). Also, in contrast to 1 mM (*S*)-AMPA application, the biphasic response was not obtained after 1–100 μM (*S*)-AMPA application. Responses to 1 μM (*S*)-AMPA recovered to basal levels with washout of the agonist, whilst responses to higher concentrations lasted longer (Fig. 3(C, E)). Since the initial staining intensity differed among regions, dose-

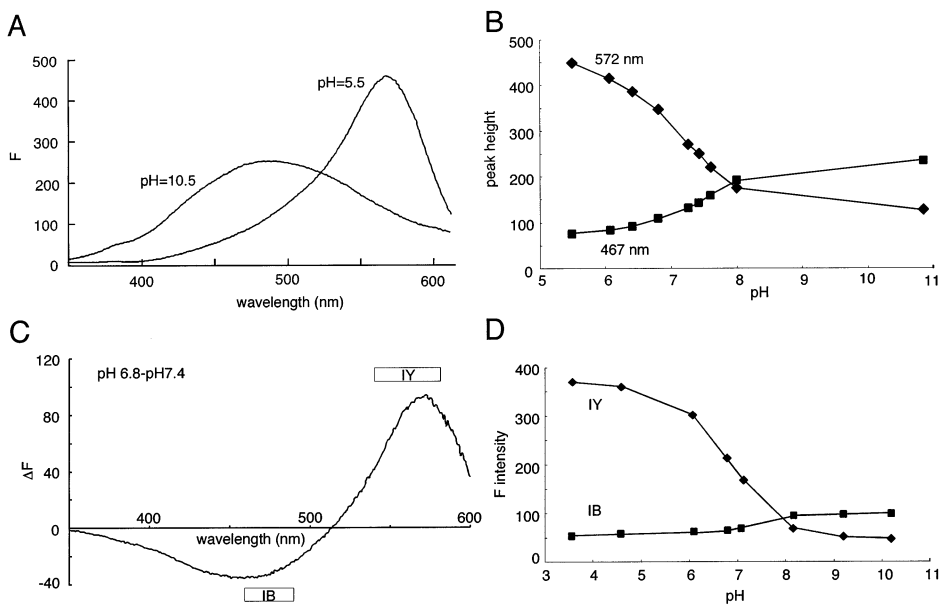

Fig. 1. Fluorescence excitation spectra of NR. (A) Fluorescence excitation spectra (emission at 634 nm) of NR (40 μM) dissolved in 100 mM triethanolamine-acetate buffer at pH 10.5 and 5.5. (B) Peak heights of fluorescence excitation spectra (emission at 634 nm) at various pH measured at 467 (squares) and 572 (diamonds) nm. (C) The subtraction spectrum (pH 6.8–7.4, emission at 634 nm). Horizontal bars (IY and IB) indicate the pass bands of each excitation filter used in the optical recording from slices. (D) The pH dependency of fluorescence intensity of NR solution measured through IB and IY filters. The pH of NR solution (40 μM in 10 mM sodium phosphate buffer) was adjusted by NaOH or acetic acid and measured with a pH meter (Beckman) after calibration. NR solution (800 μl) in a quartz cell was placed on the stage of an inverted fluorescence microscope (Zeiss, Axiovert S100) with the aid of a quartz cover glass. The fluorescence intensity was measured through 4 × objective lens, IY (diamonds) or IB (squares) filter, and other equipment was the same as in slice experiments. Images (10-ms exposure) were captured five times and averaged. Fluorescence intensity of buffer alone was subtracted.

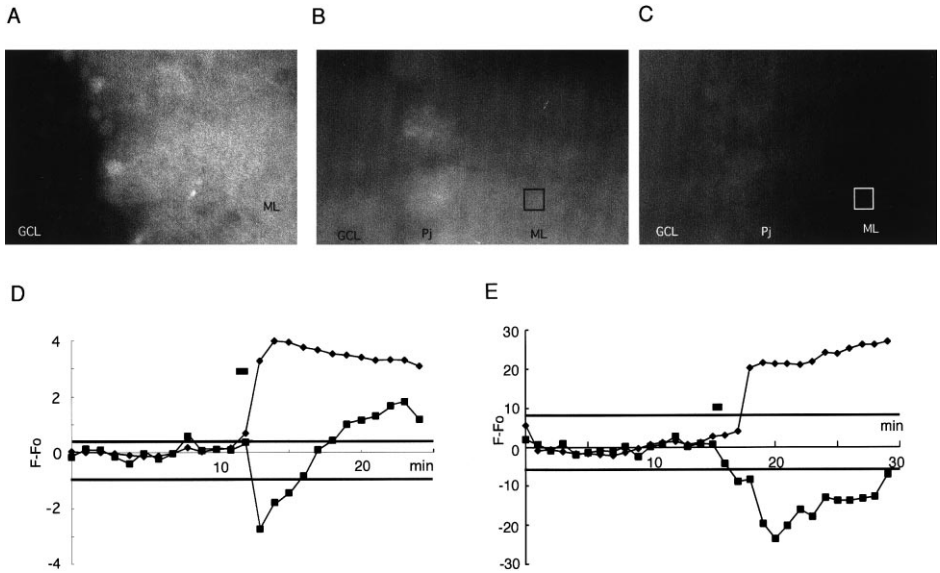

Fig. 2. The NR fluorescence increase by intense pharmacological stimuli. (A) Control fluorescence image excited through the IY filter (acidic form). Granule cells are weakly fluorescent, while molecular layer is intensely fluorescent. Soma and dendrite shafts of some Purkinje cells are seen. (B), (C) Subtraction images depicting changes in acidic (B) and basic (C) fluorescence with (*S*)-AMPA at 1 mM. Fluorescence increase and decrease are shown in white and black, respectively, and were significant in Purkinje cell soma (Pj) and molecular layer (ML) but not in granule cell layer (GCL). Squares show region of interest used in (D). (D) Time course of fluorescence changes evoked by 1 mM (*S*)-AMPA in the molecular layer. Fluorescence intensity in a region of interest (squares in B and C) was measured over time. Average of ten intensities measured before stimulus was subtracted. A representative of three independent experiments is shown. Acidic fluorescence (diamonds) increased and basic one (squares) decreased. Horizontal lines show 4SD levels of fluorescence prior to the stimulus (above for acidic, below for basic fluorescence). Application of 1 mM AMPA is indicated by a horizontal bar. (E) Time course of fluorescence changes evoked by 1 mM kainate in the molecular layer. A representative of four independent experiments is shown.

response relationships were analyzed after normalization by the initial fluorescence intensity. (*S*)-AMPA (1–10 μM) enhanced NR fluorescence in a dose-dependent manner of up to 20%, and regional differences in response magnitudes were not significant (Fig. 3(D)). (*R*)-AMPA, an inactive isomer, did not evoke any change in fluorescence ($n = 3$, (Fig. 3(E)). The selective AMPA receptor antagonist, Na–CNQX, inhibited the increase instated by (*S*)-AMPA ($n = 3$, (Fig. 3(G, H)), while Na–CNQX alone had no effect. These results showed that AMPA increased NR fluorescence through AMPA-receptor activation, and excluded a possible AMPA effect directly on NR fluorescence.

NR fluorescence in Purkinje cells was also increased transiently by 30 μM (*S*)-DHPG ($n = 5$, (Fig. 4(A)), the specific agonist of group 1 metabotropic glutamate receptors, which depolarizes Purkinje cells (Batchelor and Garthwaite, 1997). Furthermore, 15 mM KCl enhanced the NR fluorescence of both forms in granule cells as well as Purkinje cells ($n = 3$, (Fig. 4(B, C)). Thus, the NR fluorescence increase appeared to be related to depolarization of cell membrane.

When the molecular layer of the slice was electrically stimulated (seven slices), both acidic and basic forms showed fluorescence increases (Fig. 5(A–C)). Although the magnitude of the increase varied from slice to slice, it was dependent on the stimulus intensity within a slice (Fig. 5(D)). The fluorescence increase was observed in all three layers, but was more prominent in the molecular layer (Fig. 5(D)). Responses to electrical stimulation at 6 V had relatively fast time courses (Fig. 5(E)), while intense stimulation (≥ 16 V) evoked long lasting responses (Fig. 5(F)). The response was not biphasic, except when intense stimulation was repeatedly applied (data not shown). The recovery time courses were fitted well with single exponential curves, giving time constants of 0.80 ± 0.12 s (mean $\pm$ SD, $n = 4$) for responses to 6 V stimulation and 53.1 ± 18.8 s, ($n = 3$) for 16 V. The response to 6 V stimulation returned to the basal level (4SD of pre-stimulus intensity) within 1–3 s (1.9 ± 0.5 s, $n = 4$, (Fig. 5(E)), while that to 21 V stimulation did not return to the 4SD level within 1 min (Fig. 5(F)). NR fluorescence in the slice began to increase 60–100 ms after 6 V stimulation (Fig. 5(G)). Thus, the present results showed that brief molecular layer stimulation in the slice transiently enhanced NR fluorescence of both forms.

The pH-independent fluorescence increases may be explained by an increased quantum yield of NR, which is reported to occur in hydrophobic solvents (Singh et al., 1998). It was observed that addition of dimethylsulfoxide to aqueous solutions of NR dose-dependently enhanced the fluorescence (data not shown). NR is also known to bind with lipids (Lillie, 1977; Lulai and

Morgan, 1992). Therefore, it is possible that NR fluorescence is increased due to an enhanced interaction with membrane phospholipids which is somehow triggered by depolarization. When phospholipids extracted from rat cerebella were analyzed by high performance TLC, using either acidic or basic solvent system, several components that enhanced NR fluorescence were consistently observed (Fig. 6). Effects of phospholipids

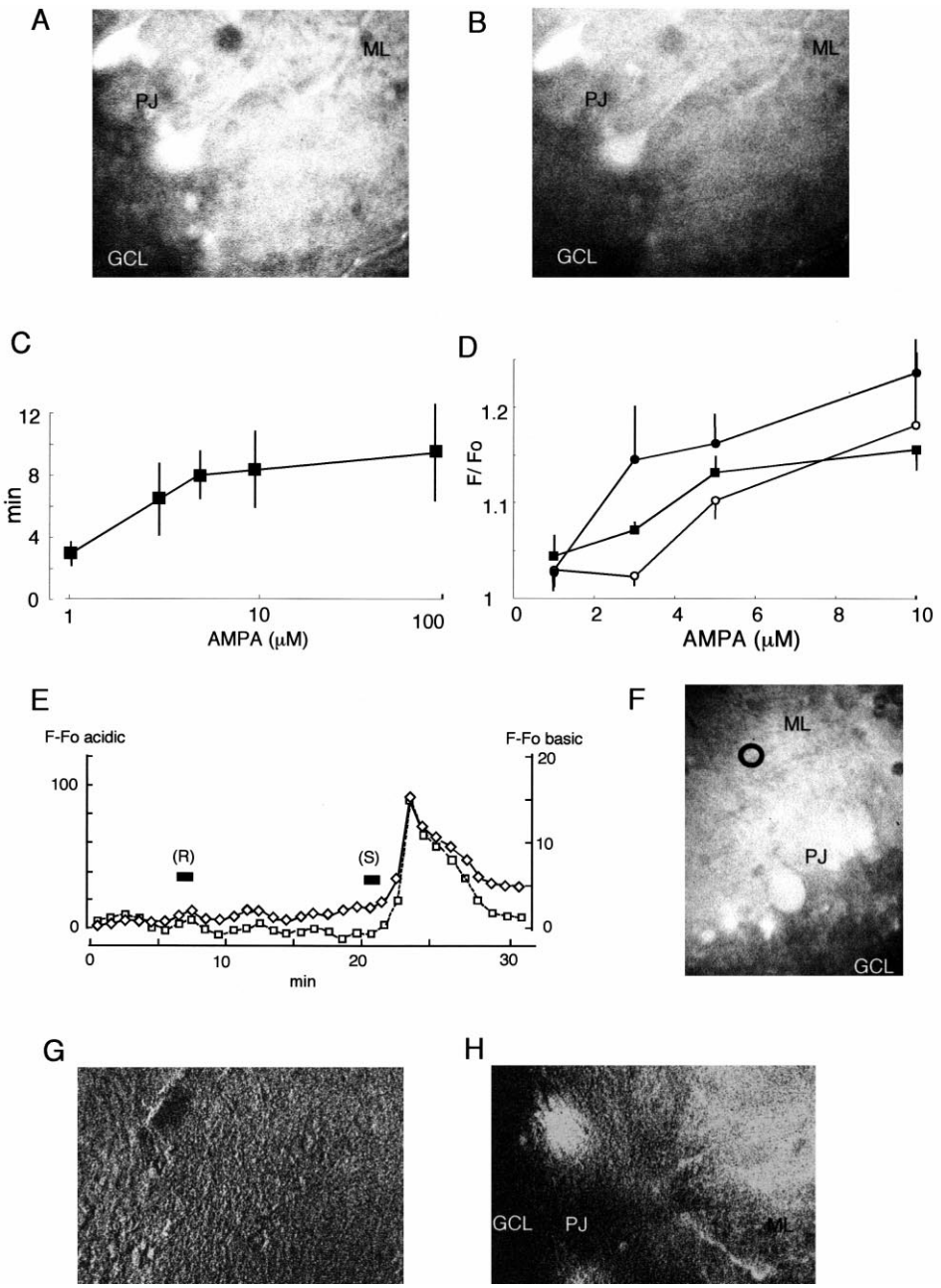

Fig. 3. NR fluorescence increase by AMPA application. (A), (B) Subtraction images (after-before application of 1 μM (*S*)-AMPA) of acidic (A) and basic (B) fluorescence. Fluorescence increase (white) is large in Purkinje cell soma (PJ) and molecular layer (ML), but small in granule layer (GCL). (C) Longer duration of response (acidic) was observed at higher (*S*)-AMPA concentrations. Duration of fluorescence increase in the molecular layer over 4 SD of the control was measured. Average of 3–5 experiments is shown with SEM (vertical bars). (D) Dependency on (*S*)-AMPA concentration. NR fluorescence increases (acidic) measured in Purkinje cell soma (closed circles), molecular layer (squares) and granule cell layer (open circles) were normalized to the pre-stimulus intensity. Averages of 2–4 experiments are shown with SEM (vertical bars). (E) Both forms of NR fluorescence were increased by (*S*)-AMPA at 3 μM, but not by (*R*)-AMPA. AMPA was bath applied during periods indicated by horizontal bars. Representative time course out of 3 experiments is shown. (F) Subtraction image (23–20 min) showing increase in acidic fluorescence. Time course is shown in (E). Fluorescence intensity was measured in the molecular layer as indicated by a circle. (G), (H) Subtraction images (after-before bath application of 1 μM (*S*)-AMPA) showing that the AMPA dependent fluorescence increase (acidic) was blocked by 30 μM CNQX (G). After washout of CNQX, a second application of (*S*)-AMPA caused fluorescence increase (H).

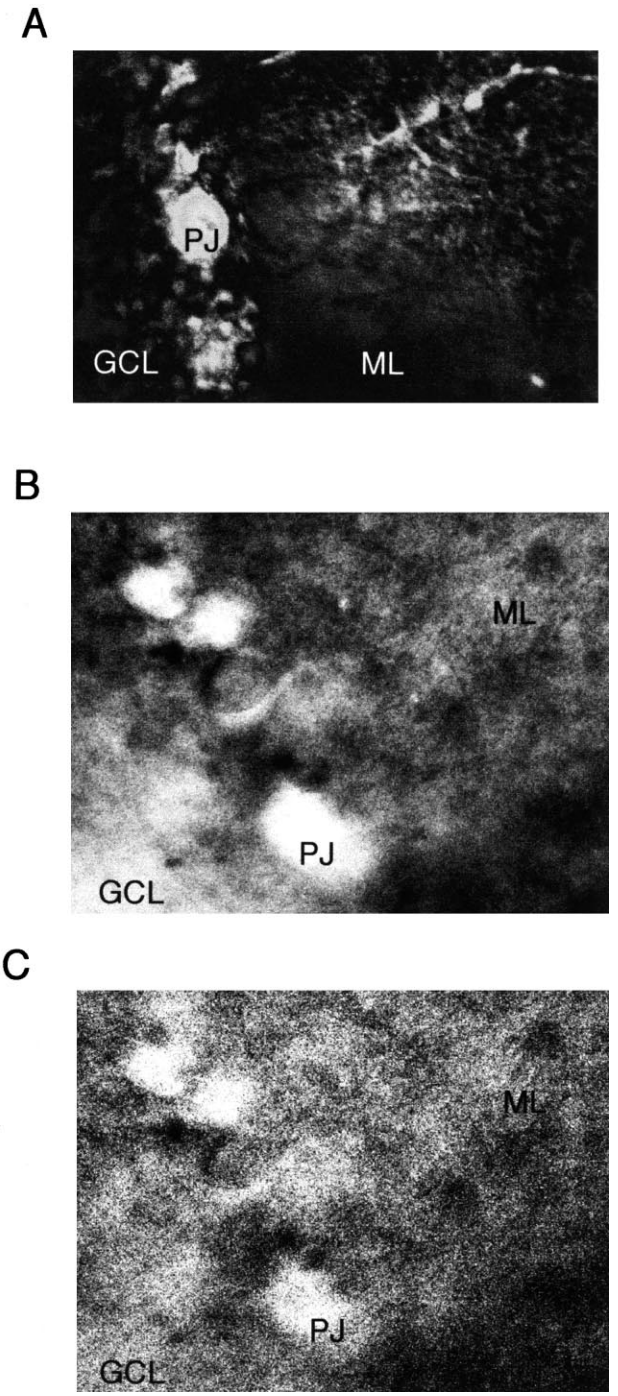

Fig. 4. Depolarizing reagents increased NR fluorescence. (A) A subtraction image (post stimulus–pre stimulus) showing that a 5-min application of 30 μM (*S*)-DHPG increased NR fluorescence in Purkinje cell dendrites and soma and in granule cells. (B), (C) Subtraction images (post stimulus–pre stimulus) showing KCl application increased fluorescence of acidic (B) and basic (C) forms in (mainly) Purkinje cell soma and granule cell layers.

were confirmed by using five authentic phospholipids with differently charged head groups. NR fluorescent spots appeared at the positions identified as phospholipids by rhodamine 6G (Fig. 6). These results indicated that phospholipids non-specifically bind to NR and increased fluorescence.

4. Discussion

In the present study, fractional changes in the pH-dependent forms of NR were distinguished using two excitation filters selected as described in (Fig. 1). Bath application of high concentrations of glutamate receptor agonists increased NR fluorescence in a pH-dependent manner (Fig. 2). In contrast, bath application of (*S*)-AMPA at low concentrations (Fig. 3), other depolarizing reagents (Fig. 4), or brief electrical stimulation (Fig. 5) increased the fluorescence measured through both filters, indicating a pH-independent increase in NR quantum yield. Thus, the present study showed that NR response has two components: one is long-lasting, pH-dependent and, evoked by intense stimuli, the other is transient, pH-independent and predominant when neurons are activated by weak stimuli.

The pH-independent NR responses are likely to be evoked by neuronal depolarization, because depolarizing reagents enhanced NR fluorescence (Figs. 3 and 4). Intracellular mechanisms that link depolarization to fluorescence enhancement are still unclear. Depolarization causes a variety of ionic and metabolic changes in neurons, and the present study suggested the contribution of mechanisms other than pH decreases. The present results showed that phospholipids with different charges (Fig. 6) and a spot likely to be free fatty acids (Fig. 6(B)) enhanced NR fluorescence, suggesting that NR binding to acyl moieties of phospholipids enhanced its fluorescence. Contribution of phospholipids in NR signals suggested that depolarization somehow altered accessibility of NR to membrane phospholipids. However, NR responses lasted for 1 s, which cannot be explained by a simple flip-flop mechanism driven by changes in membrane potentials. Since NR preferentially dissolves in hydrophobic solvents (eg. methanol), it is possible that dissociation from interacting lipids is retarded. At present, I hypothesize that redistribution of electrostatic field near/on the membrane accompanied with depolarization enhances access of NR to acyl moiety of phospholipids, causing fluorescence increase by hydrophobic interaction. After repolarization, NR may be slowly released from interacting lipids, which would be rate-limiting for the decay time course of NR response. Precise characteristics of the hydrophobic interaction and its relevance in NR fluorescence increase in neurons should be evaluated in the future.

The use of NR to monitor neuronal activity has advantages such as easy loading, reduced leakage, reduced toxicity and large magnitude of the signal. Chen et al. (1998) reported that cerebellar surface stimulation evoked pH-dependent increases in NR fluorescence that

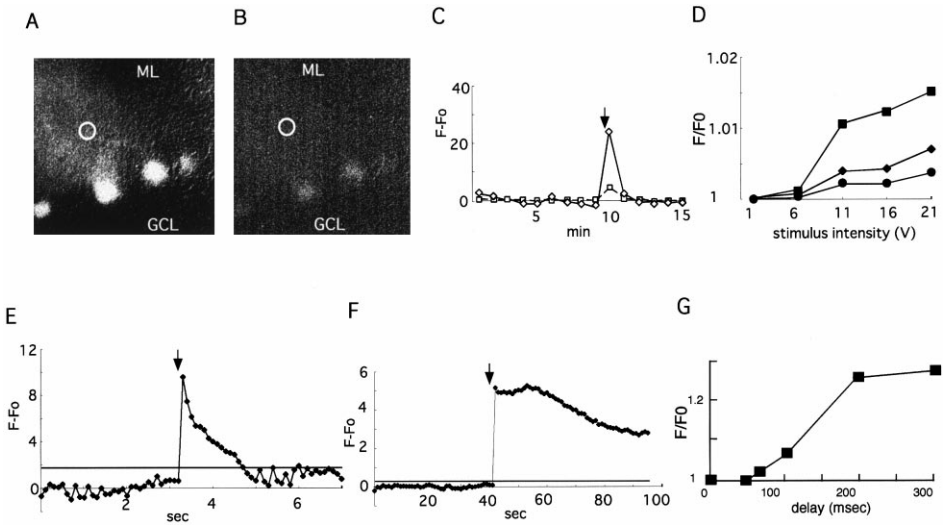

Fig. 5. NR fluorescence increases by electrical stimulation in the molecular layer. (A), (B) Subtraction images (post stimulus–pre stimulus) of the acidic (A) and basic (B) forms, showing fluorescence increase in Purkinje cells. Position of the stimulating electrode was near the upper-left corner. (C) Time course of fluorescence increase in acidic (diamonds) and basic (squares) forms, measured within the circles shown in (A) and (B). Arrow indicates timing of stimulation. (D) Relationship between stimulation intensity and acidic fluorescence increase in the molecular layer (squares), Purkinje cell soma (diamonds) and granule cell layer (circles) measured in a slice. Representative data of three experiments are shown. (E) Fluorescence intensities (acidic) measured every 100 ms in the molecular layer, showing recovery to the pre-stimulus (4SD) level (horizontal line) 1.4 s after 6 V stimulation (arrow). (F) Fluorescence intensity (acidic) measured every second in the molecular layer, showing stimulus at 21 V caused prolonged response. (G) The onset of fluorescence increases after stimulation indicated by the varied delay time between stimulation and image capture.

lasted for 1 min. Thus, NR method was considered as an indirect and slow measure of neuronal activity. The present study showed that the NR response to weak stimuli has more rapid time courses and suggested that changes in hydrophobic environments also affect NR fluorescence. Chen et al. (1998) obtained 2–4% of pH-dependent fluorescence change by their intense stimulation protocol (100–200 μA). In the present study, at least 10–50 times lower intensities were used, which may produce smaller pH-dependent changes. This stimulation condition, preferentially available to slices or isolated cells, reduces contribution of pH-dependent components, allowing pH-independent components to be observed.

NR seemed to stain preferentially Purkinje cells (Kado, 1993) and electrical stimulation which activates localized area in the molecular layer preferentially increased NR fluorescence in the molecular layer (Fig. 5(D)). Also activation of type I metabotropic glutamate receptor, whose concentration is high in Purkinje cells, highlighted NR fluorescence increases in Purkinje cells (Fig. 4(A)). These results suggested a usage of NR imaging for monitoring Purkinje cell activity. However, when entire slices were pharmacologically stimulated, regional differences in NR signals were not significant (Fig. 3(D)). Therefore, NR also stained other neurons and glial cells to a lesser extent, so that essentially NR signals can also be obtained from these components. It should therefore be noted that the duration of NR responses was largely dependent on the intensity of the electrical stimulation (Fig. 5). Intense electrical or pharmacological stimulation caused spreading depression mediated by glial depolarization, which lasts longer than neuronal depolarization. Thus, it is likely that brief stimulation transiently increases NR fluorescence in neurons, while the longer time course of the response may involve glial activities. However, cellular and subcellular components of NR responses cannot be identified under the spatial resolution available in the present study.

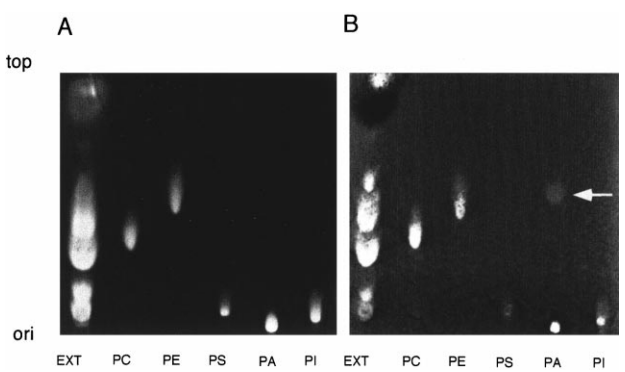

Fig. 6. TLC analysis of NR binding to phospholipids. Results of chloroform–methanol–ammonia system are shown. EXT: phospholipids extracted from rat cerebellum, PC, bovine brain phosphatidylcholine; PE, bovine brain phosphatidylethanolamine; PS, bovine brain phosphatidylserine; PA, synthetic phosphatidic acid; PI, bovine liver phosphatidylinositol. (A) Rhodamine-6G. (B) NR. Arrow indicates a fluorescent spot suggesting NR binding to free fatty acids.

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