We describe a simple and effective method for obtaining stable in vivo whole-cell recordings from cat visual cortical neurons. The core of the new approach is to prevent brain pulsation by retaining the dura mater. After being treated with an enzyme (collagenase), the dura became soft enough to allow easy penetration by a patch-clamp electrode with negligible damage to the tip. The procedure is as simple as those used for extracellular recordings, and all the intricate steps required for conventional techniques are no longer necessary. The reliability of this approach is demonstrated by stable and sustained intracellular recordings and high-quality intracellular staining. The method is especially effective for studying small neurons in the superficial layers immediately below the dura.

Introduction

The whole-cell patch-clamp recording technique was first used on isolated cells (Hamill et al., 1981; Sakmann and Neher, 1983) and later applied to brain slices (Konnerth et al., 1988; Blanton et al., 1989). Adapting the method for use with intact mammalian neocortex in vivo has previously been considered to be impractical, because cell search by the advancing electrode is blind. Furthermore, the extracellular matrix was thought to prevent the formation of a high-resistance seal. Two other general difficulties in intracellular recording from cortical areas have been: (i) instability of recordings caused by pulsation of vascular or respiratory origin, and (ii) the relatively small size of neurons in visual cortical areas.

Two groups have successfully applied the technique in recordings from neurons of visual cortex in vivo (Pei et al., 1991; Ferster and Jagadeesh, 1992). Essentially, their approach was to keep the patch-clamp electrode relatively clean of tissue debris by applying a continuous positive pressure during penetration. With the tip of the electrode closely resting on the cell membrane, the positive pressure is released and a small negative pressure is applied to the electrode. The shortcoming of their approach lies in the extensive procedures necessary to minimize respiratory-induced movements of the brain before recordings can be made. These procedures include mounting a hydraulic microelectrode holder on the skull, creating a bilateral pneumothorax, suspending the animal from the thoracic or cervical vertebrae, cementing a metal chamber over the craniotomy, and removing the dura above the cortex. These invasive procedures are not only time-consuming, but may incidentally result in a deterioration of the brain or indeed the general condition of the animal.

We describe a simple method for obtaining stable, in vivo whole-cell recordings in the cat visual cortex. The procedures used in the new method are as simple as those applied for making conventional extracellular recordings and all the additional steps described above, for providing stability of recording, become superfluous. The main element of the new approach is to prevent brain pulsation by retaining the dura. After being treated with an enzyme (collagenase), the dura is shown to be soft enough for the micropipette to penetrate with negligible damage to the tip. With this method, we demonstrate that high-quality intracellular recordings and staining can be obtained for neurons in all cortical layers, including the small cortical neurons in the superficial layers.

Materials and Methods

Animal Preparation

Experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health, USA (revised 1985). The animal preparation was similar to that used in our extracellular recording experiments (Li and Li, 1994; Li et al., 1999). Briefly, cats weighing 2–3 kg were anesthetized with Ketanest (30 mg/kg, i.m.) for initial surgical procedures. The trachea and the femoral vein were cannulated and a bilateral cervical sympathectomy was carried out. Lidocaine was applied to all wound margins and pressure points. To provide anesthesia, paralysis and physiological balance, intravenous infusion of gallamine triethiodide (10 mg/kg per h), urethane (20 mg/kg per h) and glucose (200 mg/kg per h) in Ringer’s solution (1.5 ml/kg per h) was administered during the experiment. The animal’s head was fixed in a stereotaxic frame. The animal was artificially respirated and vital signs (ECG, heart rate, end-tidal CO₂, rectal temperature) were continuously monitored. The nictitating membranes were retracted, the pupils dilated, the corneas protected by contact lenses, and refractive errors were corrected using spectacle lenses. Artificial pupils, 3 mm in diameter, were placed in front of the eyes.

Enzymatic Treatment of the Dura Using Collagenase

Our objective was to prevent brain pulsation by keeping the dura intact, and to allow the tip of a micropipette to penetrate the dura without being damaged and record intracellularly from the cortical cells. Collagenase was used to degrade collagen fibers, which make up most of the dura. A small hole (1.5 mm diameter) was made in the skull over the recording site in the striate cortex (area 17) and the exposed dura was treated with purified collagenase (Sigma Chemical Co., St Louis, MO). The enzymatic treatment was applied with a small piece of filter paper (the same size as the hole) saturated with the enzyme solution (50 mg/ml) on top of the dura for 20–30 min. The collagenase-containing filter paper was then removed, and the area was rinsed thoroughly with physiological saline. At this stage, thinning of the dura became apparent. Multiple or consecutive recording sites can then be studied. Holes can be filled with wax or agar upon completion of recording for those sites.

Electrode Preparation and Penetration of the Dura

Using a programmable micropipette puller (APP-1, Stoelting Co.), electrodes were pulled to a tip size of 1–1.5 μm with a resistance of between 5 and 10 MΩ, using thin-walled borosilicate glass capillaries containing fibers (1.0 mm o.d. × 0.78 mm i.d., GC100TF-10, Clark Electromedical Instruments). It is recommended that the electrodes are prepared just before carrying out the experiment. They are prepared with a 10 s pre-pull in advance of the application of the pull force. During this period, the glass is heated to a stable equilibrium temperature, in order to achieve constant results. The electrode tip produced in this way, which proved to be most suitable for in vivo whole-cell patch recordings, is illustrated in Figure 1. Electrodes (with an inner filament) were back-filled with a solution buffered to pH 7.4 containing (in mM) KCl 90, NaCl...
When the membrane potential becomes stable, at a value between −30 and −60 mV, the negative pressure is released, in order to avoid sucking in the intracellular content. Sometimes, single positive current pulses (100 μA, 5–10 ms) were used to aid the penetration of the cell membrane. On average, intracellular recordings were usually maintained for more than 1 h, the longest recording time lasted for 3 h which was ended by intracellular injection. The experiment normally lasted 4–5 days, and three to five cells could be recorded, labeled and identified subsequent to the experiment, after the biocytin injection.

**Intracellular Injection of a Dye**

On completion of functional tests with intracellular recording, the cell was injected with biocytin (Horikawa and Armstrong, 1988). By passing negative current pulses, 0.5–1.0 nA in a 100 ms on/200 ms off cycle, for a period ranging from 10 to 30 min. During current injection, the normal responsiveness of the cell was continuously monitored using a visual stimulus. It is important to retain the normal responsiveness of the cell during the injection process. Then the electrode was withdrawn slowly from the cell interior, and the responsiveness of the cell was checked again extracellularly. For the next penetration, a new electrode should be used and the recording site should be placed at least 1 mm away from the previous penetration.

**Histological Procedures**

At the end of the experiment, the animal was deeply anesthetized and perfused through the heart; first with 0.9% saline and then with 2% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.3. Blocks of tissue containing the injected cells were removed and post-fixed overnight by submersion in cold (4°C) 4% paraformaldehyde in PBS. The tissue was sectioned on a vibratome at a thickness of 80 μm. The sections were thoroughly washed in PBS followed by Tris-buffered saline (TBS), and were pretreated for 6 h in a 0.5% solution of Triton X100 in PBS. Injected cells were identified by incubating the tissue overnight in the avidin-biotin-HRP complex (4°C) in dilution 1:2500 in PBS. The enzymatic reaction was revealed with diaminobenzidine (DAB) (0.06%) and H2O2 (0.003%) in 0.15 M Tris buffer (40°C) for 15 min. The sections were thoroughly rinsed and mounted onto gelatin-coated slides.

**Results**

The intact dura comprises several layers of dense connective tissue, which contain a matrix of collagen fibers running in different directions, parallel to the horizontal plane. This organization fulfills the mechanical requirements of the tissue. Figure 2 shows the effects of enzyme treatment with collagenase on the dura. Figure 2A illustrates a cross-section through the dura before treatment and Figure 2B after treatment. By comparing the two figures, the reduced fiber staining after collagenase treatment, as a result of a loss of collagen during the process of enzymatic digestion, is readily apparent. Soft tissue may develop on the outer surface of the dura (indicated by arrow) during the course of an experiment that lasted several days. These soft tissues can also be easily removed by topical application of collagenase.

To determine whether collagenase treatment of the dura has any adverse effect, the integrity of cellular morphology and the functional properties of the underlying cortical neurons were examined. Figure 3A is a histological cross-section of the cortical tissue just underneath the enzyme-treated dura; a cell located in layer II was labeled from which visual responses were recorded and intracellular recordings were made (see Fig. 4), prior to being injected with intracellular dye. The axons and dendrites of the cell can be seen projecting upward into layer I, reaching the surface of the cortex just below the dura. A high-power magnification of the dendrite terminals with dendritic spines is illustrated in Figure 3B. Figure 3C is a camera lucida drawing of the cell. The integrity of the axon (thin lines) and dendrite collaterals (thick lines) of the neuron is evident. The axon collaterals can be seen extending over several millimeters in

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**Whole-cell Recordings In Vivo**

While maintaining a positive pressure (3–5 kPa), the electrode pipette was advanced slowly into the cortex to search for visually responsive cells. When the large action potentials changed from being bipolar to unipolar, the positive pressure was reduced to 1–2 kPa, and the resistance was continuously monitored with current pulses (0.1 nA, 10 ms, 1 pulse/s). Close contact with a neuron is recognized by an increase in the resistance of the electrode and/or a sudden increase in the spontaneous discharge rate. At this point, the positive pressure is released and a small negative pressure (0.5–1.0 kPa) is applied. This often results in a gradual entry into the cell interior, as is indicated by a slow increase in membrane potential. When the membrane potential becomes stable, at a value

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**Figure 1.** Photograph of the tip of the recording electrode. The shape of the tip was shown to be most suitable for in vivo whole-cell patch recordings in our experiment. Calibration bar, 10 μm.

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10, potassium EGTA 5 and HEPES buffer 10. For subsequent intracellular staining of a neuron, the solution also contained 2–4% biocytin.

Before penetrating the dura, the hole in the skull was filled with 4% agar dissolved in saline, to dampen cortical pulsations. A hydraulic, pulse-motor driving unit (PF5-1, Narishige) was used to advance or retract the electrode. Successful penetration of the electrode through the dura was confirmed by monitoring the change of tip resistance during an applied pulse of electrical current (100 μs, 0.1 nA, interval 500 ms), as compared with the original resistance tested in agar. To prevent the electrode tip from becoming blocked, a positive pressure of 20 kPa was applied while advancing the electrode. When the electrode tip touched the surface of the dura, both the tip resistance and the baseline noise would increase 5–10 times compared with the reference value. This increase in the tip resistance persisted until the electrode pierced the dura. This process may occur only once, or repeat itself a few times, presumably reflecting going through different layers depending on the degree of enzymatic digestion of the dura. A final decline in the tip resistance (to the reference value tested in agar) and the baseline noise level, coupled with the appearance of neuronal discharges, indicates the tip’s successful entry into the cortical tissue. At this point, the positive pressure applied to the electrode should be decreased to 3–5 kPa.

To avoid damage to the fine tip, it is important that insertion of the electrode should be perpendicular to the surface of the cortex, and the agar should be placed in position before the electrode touches the dura. When the dura was sufficiently digested, the electrode could pierce it without difficulty. In our experiment, only one or two electrodes might be damaged at the beginning of the first penetration. Repeated damage of the electrode tip indicates insufficient digestion of the dura. We checked the tip of the recording electrode under microscope before and after each penetration. As a rule, neither debris build-up nor damage of the tip was seen after the successful recordings.

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Figure 2. Photomicrographs of cross-sections of the dura of cat showing the morphological effect of collagenase in vivo. (A) The normal dura with densely packed collagen fibers. Calibration bar, 50 µm. (B) The dura after treatment with collagenase. The fibers become looser and show less staining for collagen. The arrow indicates the new-developed soft tissue on the outer surface of the dura during the course of the experiment. Calibration bar, 50 µm. The sections were stained with Anilin blue and acid fuchsin.

Figure 3. A biocytin-injected cortical neuron in layer II. (A) Photomicrograph of the cell. The fine processes of the cell are well preserved following treatment with collagenase. Calibration bar, 50 µm. (B) High-power magnification of the dendritic terminals in layer I (indicated by the square in A) of the same cell. Note that the dendritic processes immediately below the dura are well preserved with intact dendritic spines. Calibration bar, 10 µm. (C) Camera lucida drawing of the cell showing the processes of the apical dendrites (thick lines) and the thin horizontally directed axon collaterals (thin lines) within layers I and II. Calibration bar, 200 µm.

layers II and I. Figure 4 illustrates the functional properties and intracellular response of the cell shown in Figure 3. The cell has a 6° receptive field (Fig. 4A) and a facilitatory surround, exhibiting sharp orientation selectivity at 90°, with complete suppression of response to stimulus movement in the opposite direction (270°) at the optimal stimulus orientation (Fig. 4B), and narrow-band spatial frequency tuning (Fig. 4C). Intracellular recordings from the cell (Fig. 4D) show that the membrane potential was depolarized or hyperpolarized by a sinusoidal grating stimulus set at the preferred orientation, and that distinct EPSPs and IPSPs could be recorded.

Figure 5 illustrates two cells located in layer II. The densely labeled cell at the top is a biocytin-injected cell, and the lower one a dye-coupled cell. Figure 5B shows a high-power magnification of the dendrite terminals in layer I. The details provided by the magnification confirm that the tiny dendritic spines in the most superficial layer are well preserved.

Our recordings were not confined to the superficial layers. With positive pressure applied to the micropipette during penetration to keep the electrode tip opening clear of tissue debris while advancing the electrode through the cortical tissue, whole-cell recordings and intracellular injection of dyes could be obtained for cells in the deeper layers of the cortex. The penetration illustrated in Figure 6A shows the course of an electrode (arrow) as it was advanced tangentially down the medial bank of the lateral gyrus. The tip of the electrode was shown to traverse a distance of 1950 µm as indicated by the histological reconstructions. A cell located at the end of the electrode track was recorded and filled with dyes. Figure 6B is the high-power magnification of the cell. This cell was located in layer VI, with its basal dendrites extending in the same layer and apical dendrite reaching upwards to layer I.

In total, intracellular recordings were made from 107 cells using this method. Thirty-three of these cells were injected with biocytin and subsequently identified under the microscope. Of the sample, 11 cells were located in layers II/III, and the dendritic terminals of all these superficial layer neurons were visible up to the surface of the cortex. The remaining recording sites were located in the deep layers below layer III. Physiological
tests for the population of cells yielded normal tuning characteristics and intracellular responses. The membrane potential for most of the cells ranged from –30 to –60 mV, depending on the quality of the membrane seal and presumably the size of the soma. Stability of the recordings was not affected by applying hyperpolarizing and depolarizing currents during the recording.

Clearly, collagenase digestion appeared to have little effect on the parenchyma of the cortex. As shown, the morphology of cortical neurons appeared to be normal. Moreover, at the site of entry of the electrode, there was also no evidence of damage to neural tissue by the dimpling effect on the entry of the electrode.

Discussion
The dura in the intact animal acts as a protective barrier for the brain. To neurophysiologists interested in making intracellular recordings from the cortex, the dura represents a hindrance for micropipettes to penetrate. In earlier in vivo experimental approaches, the dura above the recording area had to be

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**Figure 4.** Functional tuning properties and intracellular responses of a layer II cell after the dura was treated with collagenase. The morphology of the cell is depicted in Figure 3. The visual stimulus was a sine-wave grating presented on a CRT screen. (A) An occlusion test (Li and Li, 1994) was used to determine the size of the receptive field. In this test, a circular homogeneous field (luminance 8.3 cd/m²) of increasing dimensions was presented at the center of the receptive field while a background grating of optimal stimulus parameters drifted continuously over the whole stimulus screen. Response amplitude decreased gradually with increasing extent of occlusion. The receptive field size was judged by the point at which the response curve meets the baseline of spontaneous activity of the cell. (B) Orientation tuning curve with a maximum at 90°. (C) Spatial frequency tuning curve. (D) Intracellular responses of the cell to a sinusoidally modulated grating (3 Hz) drifted at the preferred orientation. The EPSPs and IPSPs are shown by the fluctuations above and below the resting membrane potential. Calibration bars, 10 mV and 200 ms.

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**Figure 5.** Cell morphology of superficial cortical neurons after the dura had been treated with collagenase. (A) A pair of dye-coupled cells in layer II; the densely labeled cell on the top is the original injected cell. Calibration bar, 50 µm. (B) High-power magnification of the dendritic terminals in layer I of the same cell (indicated by the square in A). Note that the dendritic processes immediately below the dura are well preserved with intact dendritic spines. Calibration bar, 10 µm.

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**Figure 6.** Histological reconstruction of a biocytin-injected cell in layer VI. (A) The electrode track (arrow) is seen above the cell body, which is parallel to the medial bank of the lateral gyrus. Calibration bar, 500 µm. (B) High-power magnification of the injected cell. Calibration bar, 50 µm.
removed or incised in order to insert the micropipette for either sharp-electrode or whole-cell patch intracellular recordings (Pei et al., 1991; Ferster and Jagadeesh, 1992; Matsumura et al., 1996). The brain tissue consequently becomes exposed, making it vulnerable to dehydration and bacterial infection. In addition, freed from its retaining boundary, the brain is subject to pulsation of both vascular and respiratory origin. The pulsation often makes a stable intracellular recording impossible.

By applying collagenase locally over the dura at the recording site, we have shown that the dura can be transformed from an impermeable barrier into a permeable tissue for the inserted micropipette. The effect is caused by the enzymatic digestion of the dura resulting in a change in the tissue laxity shown as tissue expansion at the site of application in the histological sections. The method, therefore, effectively eliminates not only the need for opening the dura, but also surgical procedures that were either associated with the open-dura approach or used to reduce the tissue pulsations. The retention of the dura resulted in a significantly improved stability, and the rate of successes, in obtaining high-quality whole-cell recordings while still allowing the dura to act as a physical barrier and to keep the integrity of the cerebral–spinal fluids and microenvironment surrounding the brain tissue.

Collagenase is known to be a proteinase with a very high degree of specificity for its substrates which comprise only collagen and gelatin (Nagai, 1961). This enzyme has been widely used with in vitro studies for primary disaggregation of adrenal and brain tissue. Cells can withstand prolonged exposure to this enzyme apparently without causing significant damage (Lasfargues and Moore, 1971; Freshney, 1987). Despite the fact that collagenase is known to be generally non-cytotoxic, there are still concerns that the highly concentrated enzyme may cause damage to the brain tissue. With regard to cell morphology, earlier studies have demonstrated that isolated neurons from rat cerebral cortex retained their synaptic boutons on the plasma membrane in the presence of collagenase in the perfusion medium (Huttner et al., 1979). In addition, presynaptic terminals with mitochondria, vesicles and synaptic cleft could also be observed. These isolated neurons of the mammalian central nervous system have maintained well-preserved morphology and electrochemical membrane properties of the voltage-dependent channels after the enzymatic treatment (Kaneda et al., 1988).

We believe that in our study the adverse effect by the applied enzyme on the cortical tissue, if there is any, can be considered insignificant. This is supported by the fact that the cells that have been recorded and stained from the most superficial layers, which are supposed to be most vulnerable to the enzymatic action, appeared to be normal both morphologically and functionally. As illustrated in Figures 3, 4 and 5, they exhibited long dendrites with numerous processes and spines, and extensively projected horizontal axons that could be traced for several millimeters within the superficial layer. In addition, normal responsiveness and stimulus selectivity can also be shown from these cells. Similar to what has been shown in the previous studies, our method is also effective for recording of cells in the deeper layers, by applying a continuous positive pressure to the micropipette during penetration.

With some of the surgical procedures such as bilateral pneumothorax being avoided and the dura kept intact, the physiological condition of the cortical tissue can be maintained relatively stable over a longer period of time to allow continuous intracellular recordings to be carried out on the animal for up to 5 days. The benefit of being able to sustain a stable condition over a long period of time for intracellular recording of cortical neurons in an in vivo preparation is especially significant when intracellular injection of dyes is among the study objectives. Adequate time can be taken to allow the dyes to diffuse up to the smallest axon collaterals. Our results show that cells could be labeled and well identified, with a success rate of 35% on average, 1–4 days after intracellular injection of biocytin. In addition, cells sampled over a period of 5 days showed apparently normal tuning characteristics.

To summarize, our method greatly simplifies preparatory procedures, while at the same time improving the general condition of the cortex, as a result of the retention of the protective dural membrane. While the method was developed for the specific purpose of making intracellular recordings from the visual cortex, it can be applied more generally to other cortical structures. The method therefore has a broad potential for application in neurophysiological research.

Notes
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References