

Imaging single-channel calcium microdomains by total internal reflection microscopy

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ABSTRACT

The microdomains of Ca^{2+} in the cytosol around the mouth of open Ca^{2+} channels are the basic 'building blocks' from which cellular Ca^{2+} signals are constructed. Moreover, the kinetics of local $[\text{Ca}^{2+}]$ closely reflect channel gating, so their measurement holds promise as an alternative to electrophysiological patch-clamp recording as a means to study single channel behavior. We have thus explored the development of optical techniques capable of imaging single-channel Ca^{2+} signals with good spatial and temporal resolution, and describe results obtained using total internal reflection fluorescence microscopy to monitor Ca^{2+} influx through single N-type channels expressed in *Xenopus* oocytes.

Key words: single-channel recording; calcium imaging; TIRFM; N-type Ca^{2+} channels

INTRODUCTION

Ion channels regulate the activity of virtually all cells, both electrically excitable and inexcitable. Our ability to study these channels was revolutionized following the invention of the patch-clamp technique by Erwin Neher and Bert Sakmann (Neher & Sakmann, 1976), which allows currents to be recorded through single channels with exquisite (sub-pA and sub-ms) resolution. Nevertheless, patch-clamping has some limitations. Among them, little or no information is obtained regarding the subcellular distribution of channels, it is not possible to record independently from more than one channel at a time, and trauma associated with giga-seal formation may disrupt the local cytoskeleton and thereby affect channel function.

For these reasons, there has been much interest in developing optical techniques that could allow us to non-invasively image the activity of numerous individual ion channels within a cell or even within cells in an intact tissue or organ. One approach involves the use of fluorescent probes that

sense the ions passing through a channel. This methodology provides a built-in amplification as thousands of ions per millisecond typically flow through an open channel; indeed, that is what makes patch-clamp recording possible. Moreover, optical recording offers further gain because a single fluorophore molecule can emit thousands of photons per millisecond. There is a limitation, however, as to which ions can be sensed, and Ca^{2+} ions are at present the only species for which detection at a single channel level is feasible. In part this is because of the availability of highly sensitive and selective fluorescent Ca^{2+} indicators, but more importantly because of the enormous changes in Ca^{2+} concentration that occur near the mouth of an open Ca^{2+} channel. Since the resting cytosolic $[\text{Ca}^{2+}]$ is maintained at a few tens of nM the local concentration can increase a thousand-fold when a Ca^{2+} -permeable channel opens, whereas corresponding changes for ions such as Na^+ or Cl^- are less than ten-fold.

The first unequivocal experiments imaging Ca^{2+} flux through single channels were made by Zou et al., (1999), who used

widefield fluorescence microscopy in conjunction with whole-cell electrophysiology to show fluorescence signals accompanying openings of individual caffeine-activated channels in the membrane of smooth muscle cells. The temporal and spatial resolution in their records was poor, however, and fluorescence signals rose and fell over a few hundred ms during and after channel openings and spread over tens of micrometers. This degradation arises simply from the diffusion of Ca^{2+} ions (and of Ca^{2+} -bound indicator molecules) away from the 'point source' of the channel. Improvements in spatial and kinetic resolution should thus be achieved by restricting fluorescence measurements to the close vicinity of the channel mouth, where changes in $[\text{Ca}^{2+}]$ are largest and rapidly track the opening and closing of the channel. Indeed, better results were subsequently obtained using confocal linescan microscopy to monitor fluorescence from sub femtoliter cytosolic volumes (Wang et al., 2001; Demuro and Parker, 2003). However, linescan microscopy has its own limitations in that spatial information is restricted to a single dimension, the scan line intersects only a few channels, and distorted signals arise from out-of-focus channels to either side of the scan.

METHODS

To circumvent the limitations of wide-field and confocal imaging, we explored the use of total internal reflection fluorescence microscopy (TIRFM) for rapid two-dimensional imaging of cytosolic Ca^{2+} signals arising very close to the cell membrane (Axelrod, 2003). TIRFM works by directing excitation light through a glass substrate toward an aqueous specimen at a sufficiently shallow angle that total internal reflection occurs due to the refractive index decrease at the glass/water interface. However, a very thin electromagnetic field (evanescent wave) is created in the liquid with the same wavelength as the incident light and decays exponentially with

distance from the interface (typically over one or a few hundred nm). Because this field is able to excite fluorophores near the interface while avoiding excitation further into the aqueous phase, it provides an 'optical sectioning' effect similar to but even narrower than that achieved by a confocal microscope. The utility of TIRFM for biological applications has expanded greatly in the last few years with the development of specialized oil-immersion objective lenses with very high numerical aperture (1.45 or greater). These allow the excitation light to be directed to the specimen at the necessarily shallow angle through the very edge of the lens while using a high-sensitivity c.c.d. camera to visualize fluorescence in the evanescent field through the same objective (Fig. 1). We tested the ability of TIRFM to resolve Ca^{2+} flux through individual N-type voltage-gated Ca^{2+} channels expressed in *Xenopus* oocytes loaded with the Ca^{2+} indicator fluo-4 dextran (Demuro & Parker, 2004). The oocyte was voltage-clamped by a two-electrode clamp at a holding potential of -80 mV, and depolarized to more positive voltages to induce openings of N-type channels.

RESULTS

After stripping away their surrounding vitelline envelope, oocytes adhered closely to a coverglass, so that the cell membrane and immediately adjacent cytoplasm lay within the evanescent field, with only a thin intervening film of extracellular solution. Depolarizing pulses then evoked numerous transient flashes of fluorescence ('sparklets': Fig. 2A). Several lines of evidence indicate that the sparklets reflect openings of single N-type channels. They were absent in control oocytes, their fluorescence magnitudes correspond to expected single-channel currents, their durations and inactivation properties are consistent with patch clamp data, and the voltage-dependence of their occurrence and magnitude match those expected for voltage-gated plasma membrane Ca^{2+} channels. Fluorescence measurements from

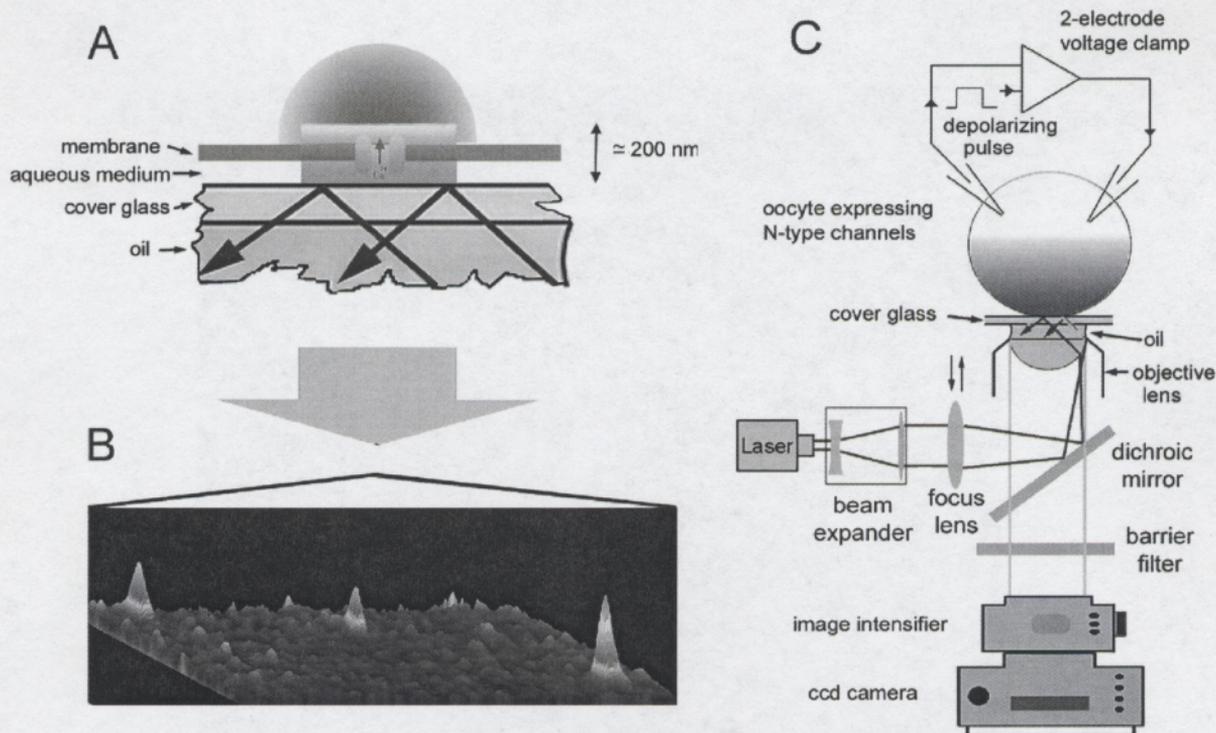


Figure 1. A: Principle of TIRFM imaging. Excitation light from a laser is directed at a shallow angle through a coverglass so that it undergoes total internal reflection at the interface with an aqueous solution (Ringer's solution). This creates an evanescent field extending ~ 100 nm from the interface, which can excite fluorescence in a dye-loaded cell in close proximity to the coverglass, allowing imaging of the microdomain of Ca^{2+} around the mouth of an open Ca^{2+} -permeable channel. B: Height-encoded representation showing 3 sparklets (single-channel openings) in a 60 μm square region of oocyte membrane. C: Schematic of the system used for TIRFM imaging and voltage-clamp activation of Ca^{2+} channels expressed in *Xenopus* oocytes (adapted from Demuro & Parker, 2004).

small regions of interest centered on sparklets showed stochastic pulsatile signals (Fig. 2B) that look remarkably like patch-clamp recordings, and although our temporal resolution was limited to about 30 ms, that was primarily due to the camera frame rate (30 fps) rather than any fundamental limitation.

Simultaneous records could be obtained independently from tens or hundreds of channels (Fig. 2B). Moreover, imaging gave information that electrophysiological measures could not. For example, the positions of sparklet sites remained stationary over several minutes, indicating that channels were anchored in the membrane (Fig. 2C); channel locations could be mapped with sub- μm precision (Fig. 2D), and simultaneous comparisons

between closely adjacent and nominally identical channels revealed surprising differences in gating kinetics (Fig. 2B).

DISCUSSION

We anticipate that optical imaging will evolve as a powerful adjunct to the patch-clamp technique as these methodologies have complementary advantages and limitations. Patch-clamping is unlikely to be surpassed for optimal kinetic resolution of channel kinetics and conductance, and it inherently provides control of membrane voltage. On the other hand, imaging is less invasive, can simultaneously monitor numerous channels, and provides spatial information. Moreover, TIRFM is relatively

simple and inexpensive to implement – for example, an existing fast Ca^{2+} imaging system could be adapted with the addition of little more than a laser and a specialized objective lens. Although our approach is restricted to imaging Ca^{2+} flux, it will be applicable to diverse channels, including those (such as nicotinic and NMDA receptors) that show appreciable Ca^{2+}

permeability even though they are not primarily considered as Ca^{2+} channels. In addition to applications in basic research, single-channel imaging holds promise for high-throughput screening of ion channel activity and may be more easily implemented than efforts to develop massively parallel arrays of patch-clamp electrodes.

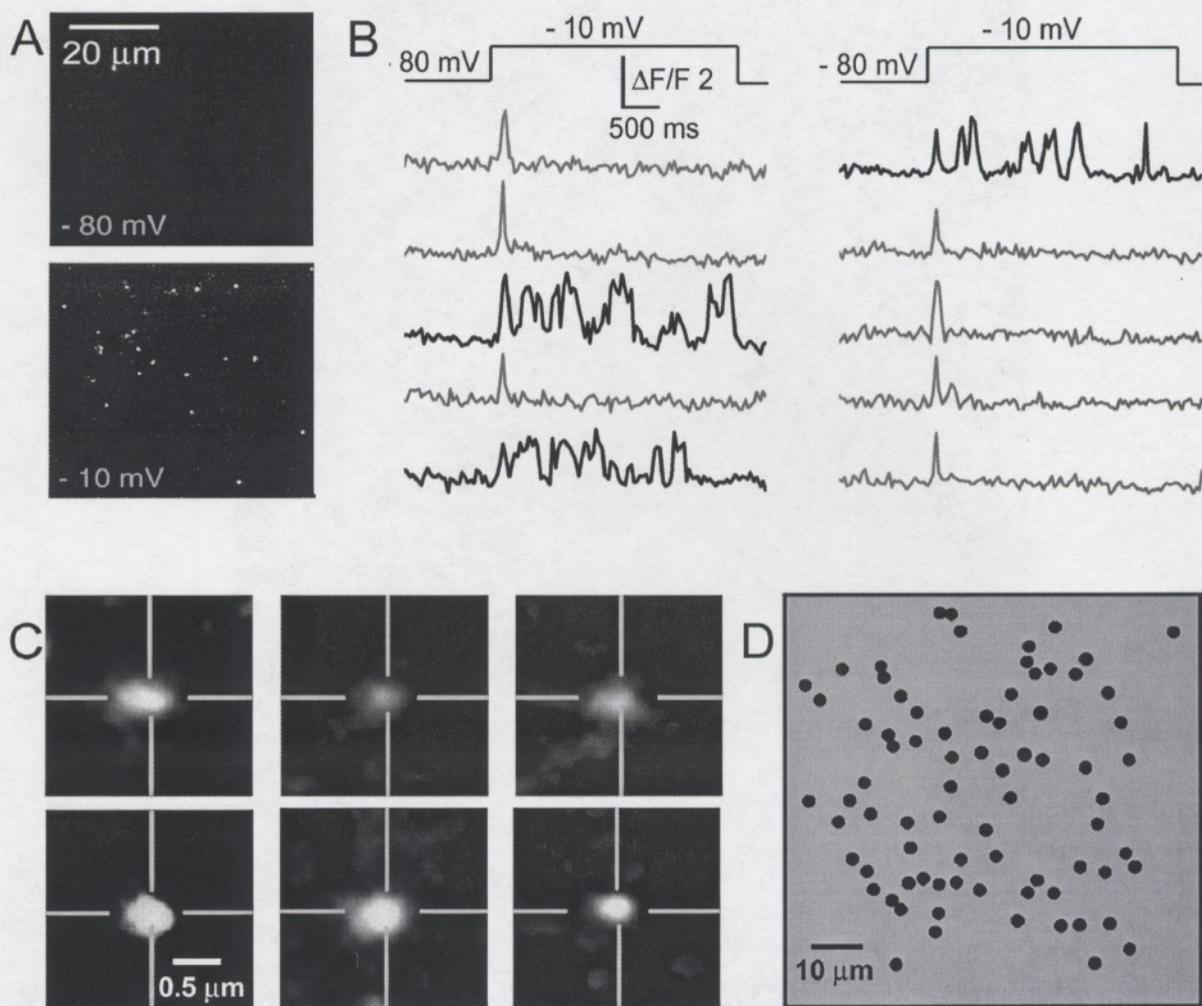


Figure 2. Imaging the activity and localization of single N-type channels expressed in the oocyte membrane. **A:** Single video-frames showing TIRFM fluorescence at rest, and after depolarizing to activate channels. Each spot (sparklet) in the lower panel arises from Ca^{2+} flux through an individual channel. **B:** Measurements of channel gating obtained by recording local fluorescence signals at sparklet sites. Examples are shown of simultaneous recordings from 10 channels. Note the variation in gating properties. Some channels (gray traces) showed only a single brief opening immediately on depolarization, whereas others (black) showed continued longer openings throughout the depolarizing pulse. **C:** N-type channels are immobile in the oocyte membrane. Panels show high-magnification views of sparklets at a given site during repeated depolarizing pulses over a period of about 5 min. Crosshairs are centered on the first event. **D:** Map shows the locations of sparklets within a region of membrane.

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