WA11

Confocal imaging of identified neuronal populations using viral gene transfer

S. Kasparov

Physiology, Univeristy of Bristol, Bristol, UK

Confocal live cell imaging is a powerful tool for studying central neurones and well as glial and vascular cells abundant in brain. Expression of fluorescent proteins allows imaging of particular cellular phenotypes or intracellular elements which can be in this way distinguished from irrelevant background structures. Viral vectors are one of the best ways of introducing genes encoding for fluorescent constructs into differentiated postnatal brain neurones and glia. Using various promoter sequences it proves possible to selectively target at least certain cell types and to express a variety of fluorescent constructs. Several popular viral gene delivery systems are currently in use. These include vectors derived from adenovirus, lentivirus and adeno-associated virus genomes which all have their specific advantages for gene delivery in vivo and in vitro. Viral vectors can be introduced in vivo and fluorescent neurones imaged a few days later in either fixed or living tissue. In this way, certain neuronal groups can be transduced in the retrograde manner and cells which have certain axonal projections visualised specifically. However, the most favourable conditions for imaging which at the same time are compatible with viral gene delivery are provided by organotypic brain slice cultures. Such cultures can be transfected using one or more viral vectors and then remain viable and suitable for imaging experiments for several weeks. An adenoviral vector encoding for EGFP under the control of 3.7 kb of the GAD67 promoter has been used to fluorescently target GABAergic neurones in the nucleus tractus solitarii. These cells were then recorded in whole cell patch clamp mode using a red-shifted Ca2+ indicator Rhod-2 and the effects of nitric oxide on intracellular Ca2+ concentration in somata, dendrites and axons were studied in great detail. For imaging of noradrenergic neurones we use vectors based on an artificial PRSx8 promoter (Teschemacher et al., 2005). Both, EGFP and monomeric Red Fluorescent Protein (mRFP)-expressing vectors have been generated. mRFP distribution in many neurones is far from homogeneous and it frequently forms small aggregates which puts in question claims of its suitability as an intracellular tag. However as it leaves the green part of the spectrum open it is possible to patch mRFP-expressing neurones using fast Ca2+ dyes (such as Fluo-4) and study dynamics of the fast Ca2+ events in different cellular compartment including the putative release sites in axonal varicosities. These methods will be demonstrated in the course of the meeting.

Teschemacher, A. G., Wang, S., Lonergan, T., Duale, H., Waki, H., Paton, J. F. R., & Kasparov, S. (2005). Targeting specific neuronal populations in the brainstem using adeno- and lentiviral vectors: applications for imaging and studies of cell function. Experimental Physiology 90, 61-69.

British Heart Foundation and Wellcome Trust funded research

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

WA12

Imaging epithelial defense in the living gastrointestinal tract

M.H. Montrose

physiology, university of cincinnati, cincinnati, OH, USA

The epithelium lining the intestinal tract mediates the selective absorption of desirable substances from the gut lumen while acting as a remarkably effective barrier against undesirable materials (toxic substances, bacteria, etc). The basis for this barrier is a series of defense arranged serially with a set of pre-epithelial defenses (juxtamucosal pH control, mucus layer), epithelial defense (tight junctions, apical membrane impermeability), and subepithelial defenses (mucosal blood flow, neural regulation). Our laboratory has been developing and applying methods to explore the pre-epithelial and epithelial defenses used in the stomach and intestine. We have had some success using confocal and two-photon microscopy of these organs to explore tissue function with subcellular resolution in living (anesthetized) animals having their mucosal surfaces of the tissue surgically exposed to the exterior. In the first part of the presentation I will describe our work asking how the epithelial barrier is sustained during the rapid renewal of the small intestinal epithelium, where it is estimated that approximately 1 cell is lost per villus per minute. We have observed that the living epithelium appears discontinuous, with gaps in 3% of cell positions. These gaps do not take up vital stains that successfully mark neighbouring cell cytoplasm, nuclei, and apical membranes. When we track cell shedding in real time, we observe creation of gaps that are not rapidly filled by other cells after one cell departs. Importantly, when we add a fluorescent dye to the luminal fluid (Lucifer Yellow) it is not able to permeate into gaps. By this marker, and the presence of a substance in the gaps that reflects laser light, we conclude that an unknown substance plugs gaps. In the second half of the presentation, I will discuss our work in the stomach. Here we have created the first model in which rapid perturbation of the epithelium is restricted to microscopic regions, hopefully modeling the type of injuries that would serve as nucleating events in ulcer formation. We use two-photon absorpton to create micro-lesions that specifically kill 2-3 cells in the epithelium. In response to this insult, there is a spreading of damage to an annulus of innocent bystander cells (noted as increased permeability to a luminal dye, and loss of NAD(P)H fluorescence), and then all damaged cells are shed, apparently thru the force of the healthy epithelium migrating in to close off the damaged area. Coincident with the spreading damage is a large pulse of alkali from the tissue that provides a protective alkaline layer over the damaged tissue during subsequent events. The repair is regulated in part by COX-1, as COX-1 null mice have a smaller alkali pulse and a slower repair. In both the intestinal and gastric work, quantitative light microscopy provides windows into processes that are difficult if not impossible to detect by more classic methods.

funded by NIH (DK54940 & DK42457).

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

WA13

Imaging dendritic calcium transients in pyramidal neurones

J. Waters

Physiology Department, Northwestern University, Chicago, IL, USA

2-photon microscopy has enabled real-time fluorescence imaging from the brains of living animals with cellular and sub-cellular resolution. By combining 2-photon microscopy with whole-cell patch-clamp techniques to load calcium indicator dyes, 2-photon microscopy can be used to monitor calcium transients in the dendrites of individual neocortical pyramidal neurones. Over the past several years I have been using these techniques to explore the roles of voltage-activated channels in dendritic function in anaesthetized and, more recently, awake animals.

Imaging in intact animals obviously presents a number of technical hurdles, such as movement of the preparation. I will highlight how some of these barriers have been overcome and present some of the results that I have obtained using these techniques.

Using these techniques, we have established that action potentials backpropagate into the dendritic trees of neocortical pyramidal neurones in urethane-anaesthetized rats, much as in slice preparations and that the effect of ongoing synaptic activity is minimal.

More recently, I have extended 2-photon imaging techniques to the awake, head-restrained rat. Here I observed spontaneously occurring calcium transients occurring every 5 seconds (on average) in the proximal apical dendrite (range: every 3-15 seconds, n = 5 neurons; imaging 80-100 mm from the soma). The amplitudes of these transients were variable. Comparison with measurements from anaesthetized animals, suggest that they result from single or bursts of backpropagating action potentials.

Backpropagation of single action potentials into more distal dendritic branches (150-200 mm from the soma) was also observed. However, the frequency of spontaneous transients was lower in distal than in proximal dendrites. This could be due to changes in the strength of backpropagation with different awake states or may simply result from a failure to detect some small calcium transients in distal dendrites.

Hence in awake rats dendritic channels support active propagation of action potentials, much as reported in slice preparations and in anaesthetized rats. Many possible roles of dendritic channels in synaptic integration have been identified in slice preparations. Which of these roles contribute to synaptic integration in awake animals is unclear, but 2-photon microscopy is likely to prove one of the most useful tools with which we can investigate this question.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

WA14

The use of 2-photon excitation of Fura dyes to measure intracellular Ca ion concentration

G.L. Smith[1], C.M. Loughrey[3] and D.L. Wokosin[2]

[1]Biomedical & Life Sciences, Glasgow University, Glasgow, UK, [2]Department of Physiology, Northwestern University, Chicago, IL, USA and [3]School of Veterinary Medicine, University of Glasgow, Glasgow, UK

With the increasing availability of reliable ultra-short pulsed near infrared (700nm to 1050nm) lasers, two-photon laser scanning microscopy is now possible on a commercial basis. Considerably lower photo-toxicity can accompany the use of longer wavelength illumination, allowing long-term imaging of biological tissue. Fluorescent dyes with absorption maxima < 400nm (e.g. Fura and Indo) can be excited by near-infrared laser light. Generally the Fura dyes are preferred over the equivalent Indo versions since the latter are less fluorescent and have problems with photo-isomerisation and photo-bleaching. Two-photon excitation (TPE) spectra of Fura-2, -4F, -6F, -FF and Furaptra were characterized using a tuneable (750nm to 850nm) ultra-short pulse laser. Two-photon fluorescence of these dyes was studied in free-solution and in the cytosol of isolated rabbit ventricular cardiomyocytes. The TPE spectra of the Ca2+-free and Ca2+-bound forms of the dyes were measured in free-solution and expressed in terms of the two-photon fluorescence cross-section. The Fura dyes displayed the same Ca^{2+}-free TPE spectrum in the intracellular volume of permeabilized and intact cardiomyocytes. Fluorescence measurements over a range of laser powers confirmed the TPE of both Ca^{2+}-free and Ca^{2+}-bound forms of the dyes. Single wavelength excitation at 810nm, was used to determine the effective dissociation constants (Keff) and dynamic ranges (Rf) of dyes: Fura-2, -4F, -6F, -FF and Furaptra. Single wavelength excitation of intracellular Fura-4F resolved diastolic and peak [Ca^{2+}] in isolated stimulated cardiomyocytes after calibration of the intracellular signal using reversible exposure to low (100μM) extracellular [Ca^{2+}]. Furthermore, TPE of Fura-4F allowed continuous, long-term (5-10min) Ca^{2+} imaging in ventricular cardiomyocytes using laser-scanning microscopy without significant cellular photo-damage or photo-bleaching of the dye.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.