Measurements of local pH-transients in K^+-depolarized PC12 cells

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Recently we reported that rapid (~20 ms) acidification of the cloned "slow" rat $\alpha_3\beta_4$ nicotinic receptors enhances the agonist-activated current and accelerates its gating kinetics by increasing the affinity of the receptor to agonists [1]. This is a novel and distinctly different effect than the acidification-induced suppression of muscle nicotinic receptors and neuronal N-methyl-D-aspartate receptor and raises the possibility that that brief transient acidification or alkalization of the synaptic cleft may modulate postsynaptic receptors thereby providing plasticity to synaptic signaling. In support of synaptic acidification it has been found that: 1) postsynaptic potentials may be accompanied by brief (10 ms) acidic shifts [5,6]; 2) protons, stored in the vesicles [7] and co-released with transmitters, can modulate presynaptic Ca^{2+} channels [2,4]; and 3) buffering capacity of synaptic cleft is limited during brief synaptic events [10]. On the other hand, it has also been found that the initial acidification is followed by longer-lasting alkalization [6].

Here we describe experiments undertaken to directly measure local pH transients associated single exocytotic events. It is our goal to measure such pH transients within synaptic clefs, but as a first step in this direction, we decided to measure pH transients in the confined space between catecholamine-secreting PC12 cells (a line of rat pheochromacytoma cells) and the underlying glass substrate on which they are cultured. In this configuration we compared confocal and total internal reflectance fluorescence (TIRF) microscopy in conjunction with water-soluble (Snarf 4F, S23920 from Molecular Probes) and lipophilic (Fluorescein DHPE, F362 from Molecular Probes) fluorescent pH-indicator dyes. The dual objective was to improve the sensitivity of our pH-imaging techniques and to obtain preliminary recordings of local pH transients.

To improve signal-to-noise ratio (S/N), we have purchased a new CCD camera (iXon DV860 from Andor Technology, Northern Ireland), and added a green diode laser (λ_{ex} = 534 nm) to our Argon ion laser (λ_{ex} = 364, 457, 488, 514 nm). The new CCD camera (128 x 128 pixels) is exquisitely sensitive due to a novel CCD chip (E2V 60 from e2v Technologies, UK) with high quantum efficiency (~95%, back thinned design) and low readout noise (on-chip electron multiplication, Peltier cooling to –90 °C). With flexible binning, the frame rate can be increased from 450 per second for full frames (128 x 128 pixels), to well over 1 kHz.

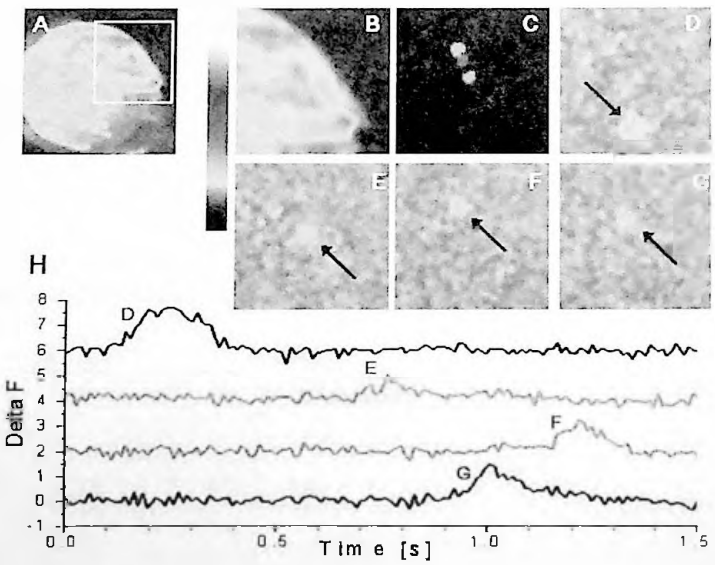

Figure 1. pH-sparks in a cultured PC-12 cell depolarized with K^+-rich solution. Panel A shows the average TIRF signal measured in 1000 frames over a period of 10.5 sec. The cell is ~20 μm in diameter. pH-sparks were observed within four regions (Panel, C: blue, green, orange and red) of the upper right hand quadrant (Panel B). The fluorescence intensity in these areas increased and declined again as indicated by the curves in panel H. Panels D to G show sample frames with background subtraction where each of the four pH-sparks appear red on a green background (Arrows). The used color scale is shown as an inset.

TIRF experiments with cultured catecholamine-secreting PC-12 cells stained with F-362, demonstrate the typical patters of adhesion (Fig.1A). With optimal staining the camera gives excellent S/N at frame/rates as high as 1200 Hz. Most intriguingly, when activated by depolarizing K^+-rich solution pulses, the PC12 cells produced focal pH-transients lasting ~200 ms (Fig. 1H). Surprisingly, our findings suggest that the "spark-like" increases in fluorescence reflect transient alkalization. The confocal measurements in Fig. 2A, demonstrate the F-362 dye responds as expected to imposed pH-changes both at the cell surface and in the "cleft-space" between the cells. In particular, they verify that alkaline solution increases fluorescence intensity as observed for the pH-transients in Fig. 1. Attempts to measure paracellular pH with a water-soluble pH-indicator (SNARF 4F) were not encouraging (Fig. 2B) since fluorescence from dye within the narrow spaces between (and under) cells was swamped by the fluorescence from the bulk of the solution.

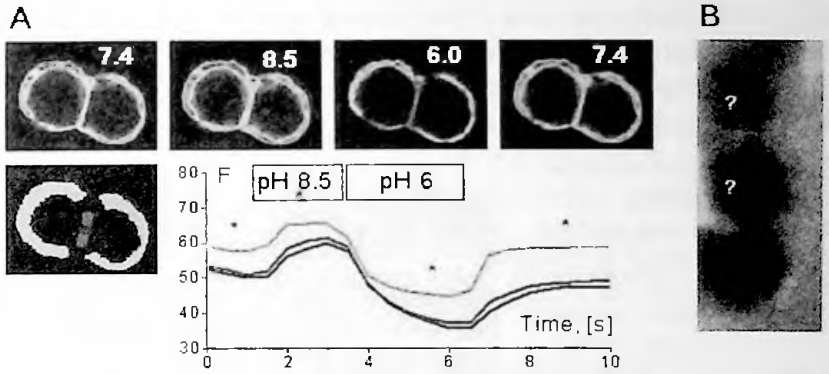

Figure 2. Comparison of confocal measurements with lipophilic (Panel A. F-362) and water-soluble (Panel B. SNARF 4F) dyes. Panel A: Measurement of interstitial pH measured between 2 PC12 cells stained with F362 and exposed to test solutions with pH 7.4 (control), 8.5, 6, and 7.4 (sample frames at top corresponding to *s in graph). The curves and regions of interest correspond the exposed membrane surface (green) and membranes at different depths (red and blue) within the cleft between the cells. Notice, that on return to control solution (at time ~7s), the pH-response in the cleft lags ~1s after that at the surface. Panel B: Fluorescence from SNARF 4F distributed around, and possibly between (?) a string of adhering PC-12 cells .

Application of K^+-rich depolarizing solution to PC-12 cells stained by F-362 produced clear TIRF images where transient focal fluorescence signals (Fig. 1) were superimposed on the steady state image (Fig. 1 AB). The focal signals were seen roughly along a line, were stronger in areas of adhesion than in between (Fig. 1 trace/panel D *vs.* E) and lasted ~200 ms, so that they were clearly seen in multiple frames when recording at 95 frames per second (Fig. 3). These focal fluorescence signals may correspond to pH-transients produced by exocytosis of single secretory vesicles. Detailed analysis of pH-sparks in consecutive frames, was performed with a computerized algorithm that determined a Gaussian approximation and quantified the amplitude, center and full-width at half amplitude (FWHA) as well as the "size" of the pH-spark, *i.e.* its overall fluorescence (proportional to amplitude times $FWHA^2$, [3]). From the first appearance of the pH-spark, the size increased over a period of ~50 ms, and then declined slowly in the following period of ~ 200 ms. FWHA was remarkably constant at ~ 2μm compared the slowly spreading Ca-sparks observed *e.g.* in atrial cells [11]. This may reflect that the membrane-bound pH-dye, unlike the dyes generally used for measurements of Ca-sparks, are not freely diffusible in paracellular spaces.

The main finding of these experiments is that TIRF microscopy used in conjunction with a lipohilic fluorescent pH-indicator dye is capable of measuring highly localized fluorescence transients that may represent alkalization associated with exocytosis of single secretory vesicles. The alkalization is surprising considering the acidic contents of secretory vesicles. To substantiate the present findings, it would be useful to perform more extensive experiments with a ratiometric lipophilic pH-dye in order to ascertain that the action spectrum of the local pH-transients display the same wavelength-dependence as observed when the cell are exposed to test solutions with different pH. Similarly, higher frame rates should be used to test if the observed alkalization is preceded by brief acidification

(*Cf.* [6]). It should also be noticed that the local pH-transients produced by exocytosis may be expected to depend not only on the excess of protons within the secretory vesicles, but also on their contents of pH-buffers and proton transporters. In fact at pH 5, it may be calculated that a secretory vesiscle contains only about one free proton, but has ~10,000 protons and transmitter molecules that are possibly bound to proteoglycans, the ion-exchange properties of which, remain to be fully explored [8]. Thus it is possible that both the magnitude and polarity of local pH-transients may depend on proton fluxes generated by a number of proton-binding and –transporting molecules. Furthermore, it has been proposed that the exocytotic process does not always run rapidly to completion, but that the fusion pore only briefly opens and then closes again ("kiss-and-run" model[9]). Considering the sensitivity of the TIRF technique, as demonstrated in the present results, it is likely that this technique may help to elucidate these processes and their role in controlling pH within synaptic clefts. Supported by NIH RO1 HL 16152 and R21 EB003473.

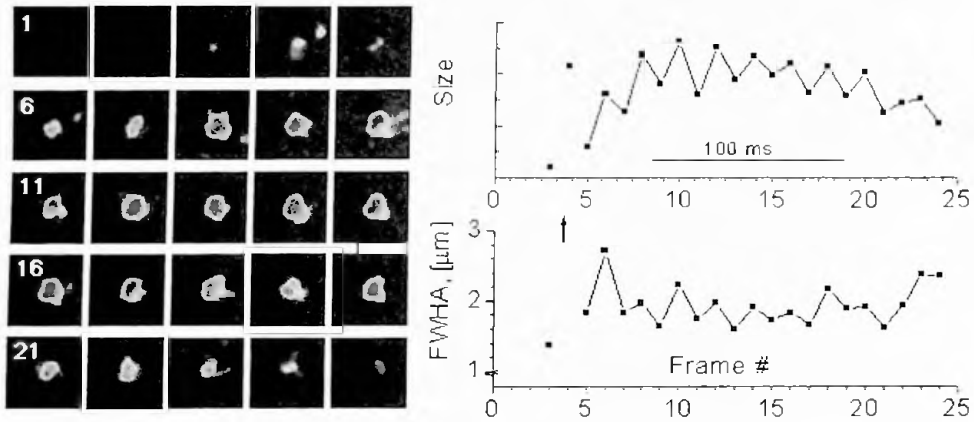

Figure 3. Consecutive frames showing the development and decline of one of the pH-sparks (Spark D, blue in Fig. 1.). The graph shows the time course of the size (overall fluorescence intensity) full-width at half maximum (FWHA) determined with same computer program used routinely for analysis of Ca^{2+}-sparks [3].

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