Published in final edited form as:

Cell Calcium. 2013 October ; 54(4): . doi:10.1016/j.ceca.2013.08.001.

Synthesis and Properties of Asante Calcium Red –a Novel Family of Long Excitation Wavelength Calcium Indicators

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Abstract

Although many synthetic calcium indicators are available, a search for compounds with improved characteristics continues. Here, we describe the synthesis and properties of Asante Calcium Red-1 (ACR-1) and its low affinity derivative (ACR-1-LA) created by linking BAPTA to seminaphthofluorescein. The indicators combine a visible light (450–540 nm) excitation with deep-red fluorescence (640 nm). Upon Ca^{2+} binding, the indicators raise their fluorescence with longer excitation wavelengths producing higher responses. Although the changes occur without any spectral shifts, it is possible to ratio Ca^{2+} -dependent (640 nm) and quasi-independent (530 nm) emission when using visible (<490 nm) or multiphoton (~780 nm) excitation. Therefore, both probes can be used as single wavelength or, less dynamic, ratiometric indicators. Long indicator emission might allow easy $[\text{Ca}^{2+}]_i$ measurement in GFP expressing cells. The indicators bind Ca^{2+} with either high ($K_d=0.49\pm0.07\ \mu\text{M}$; ACR-1) or low affinity ($K_d=6.65\pm0.13\ \mu\text{M}$; ACR-1-LA). Chelating Zn^{2+} ($K_d=0.38\pm0.02\ \text{nM}$) or Mg^{2+} ($K_d\sim5\ \text{mM}$) slightly raises and binding Co^{2+} quenches dye fluorescence. New indicators are somewhat pH-sensitive ($\text{pK}_a=6.31\pm0.07$), but fairly resistant to bleaching. The probes are rather dim, which combined with low AM ester loading efficiency, might complicate *in situ* imaging. Despite potential drawbacks, ACR-1 and ACR-1-LA are promising new calcium indicators.

Keywords

confocal microscopy; dissociation constant; emission spectra; excitation spectra; fluorescence; intracellular free calcium concentration; magnesium ions; multiphoton microscopy; ratio imaging; neurons; zinc ions

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1. INTRODUCTION

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$) is typically performed with the use of fluorescent indicators [1–3]. Since the development of the first modern UV-excitable indicators, fura-2 and indo-1 [4] over a quarter century ago, a number of new indicators with different excitation/emission wavelengths and distinct affinities for calcium, have been synthesized [5–10]. While some of them including fura-2 [4] and fluo-3 [5], enjoy undying popularity, others like BTC [6] and Calcium Crimson [11] have attracted only a limited and short-lived interest. The analysis of their features and almost three decades of experience allows for the creation of a “perfect” fluorescent indicator profile. Such a probe should be compatible with modern confocal and multiphoton microscopy, preferably ratiometric with a large dynamic range, and long, visible light excitation to reduce phototoxicity [12–14] and autofluorescence [15, 16], while allowing the use of standard detectors and imaging setups. Furthermore, the indicator should be selective for Ca^{2+} ions and resistant to bleaching. Compatibility with fluorescent proteins such as GFP, commonly used as markers, would be an added benefit.

None of the currently available synthetic dyes seems to meet these criteria. The classic fura-2 [4] and its low affinity derivatives [7] are ratiometric, but require UV excitation. These probes cannot be effectively used with most laser scanning confocal and multiphoton microscopes (but see [17]). Furthermore, the effective use of fura-2 related indicators in the presence of GFP requires a post experimental fluorescence correction [18] or a physical modification of the imaging setup [19]. Such simple measures cannot eliminate GFP interference from the signal of spectrally related indicators [20] like the popular fluo-3 [5] and its analogues [21, 9]. Longer excitation wavelength indicators that are less prone to this problem [20], such as Calcium Orange and Calcium Crimson [11, 8], offer a very limited dynamic range [8] or, like X-Rhod-1 and Rhod-2 [5], are overly prone to accumulation in mitochondria and are often used to measure mitochondrial rather than cytosolic Ca^{2+} levels [22, 23]. Moreover, these visible light-excitable indicators lack the accuracy of their ratiometric counterparts. Attempts to solve this problem by the simultaneous imaging of fluo-3 and fura-red [24] have enjoyed limited success. This situation has prompted the development of new probes with improved characteristics such as ICPBC [25], calcium ruby [26, 27], CaSiR1 [28] and KFCA [29]. As they have become available only recently, very few examples of their applications have been described. It remains to be seen to what extent their limitations ranging from a small dynamic range (ICPBC; [25]) and mitochondrial accumulation (calcium ruby; [30]) to the lack of AM ester (KFCA) will affect their usage.

Here, we describe the synthesis of Asante Calcium Red-1 (ACR-1) and its low affinity derivative, Asante Calcium Red-1 Low Affinity (ACR-1-LA), red-shifted indicators developed recently [31] by chemists from Teflabs (Austin, TX). The new probes, which might feature some desired characteristics [32], have been first studied *in vitro* in calibration buffers to determine their basic properties. Afterwards, the indicators have been loaded into cells and briefly examined *in situ* to assess their usability for real-life calcium imaging. These experiments demonstrated that the new indicators, which offer several imaging modes and spectral compatibility with GFP, can be effectively, though not without certain challenges, used to measure intracellular free calcium concentrations $[Ca^{2+}]_i$.

2. MATERIAL AND METHODS

2.1. Synthesis of Asante Calcium-Red-1 (ACR-1) and Asante Calcium Red-1 Low Affinity (ACR-1- LA)

The synthesis was performed by first synthesizing 5-fluoro-7-chloro-1,6-dihydroxynaphthalene and then linking it to BAPTA aldehyde [4] (Supplemental Figures 1, 2) as described in Supplemental Data 1.

2.2. Calibration solutions

In vitro experiments were performed in solutions containing 100 mM KCl, 10 mM MOPS and desired concentrations of Ca^{2+} , Zn^{2+} and Mg^{2+} obtained by supplementing the basic buffer with different proportions of EGTA and metal ions using methods described previously [33]. The EGTA dissociation constants (K_d) were calculated using Chris Patton's MaxChelator program [34, 35] (<http://maxchelator.stanford.edu>). At our standard conditions ($T \sim 22^\circ\text{C}$, $\text{pH} = 7.2$, $I = 0.1 \text{ M}$), the K_d values were 149 nM for Ca^{2+} , 17.9 mM for Mg^{2+} and 2.89 nM for Zn^{2+} . The pH of calibration buffers was adjusted to the required level (7.20 ± 0.02 unless otherwise stated) using 0.1 M KOH or 0.1 M HCl. In the case of calcium calibration buffers, the desired $[\text{Ca}^{2+}]$ was obtained by cross-diluting 10 mM EGTA and 10 mM CaEGTA solutions, bought from Invitrogen (Carlsbad, CA, USA) or self-prepared and tested as described previously [36], using a protocol provided by Invitrogen. Buffers containing higher free $[\text{Ca}^{2+}]$ than that in 10 mM CaEGTA ($\sim 40 \mu\text{M} \text{Ca}^{2+}$) were prepared by adding aliquots of a 10 mM CaCl_2 stock solution to 10 mM CaEGTA. The concentration of free Ca^{2+} ions in these solutions was verified using a calcium-selective electrode (Model 97-20, Orion Research Inc., Beverly, MA, USA). Other calibration buffers contained 100 mM KCl, 10 mM MOPS, 1 mM EGTA, 0–100 mM Mg^{2+} or 0–10 μM free Zn^{2+} , concentrations obtained by adding aliquots of a 10 mM ZnCl_2 or a 5 M MgCl_2 stock solution to the basic buffer solution.

2.3. Molar extinction coefficients (ϵ)

Molar extinction coefficients were determined by collecting absorption spectra (400–600 nm) of 1–20 μM indicator in calibration buffers using a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, CA). Absorbance maxima (540 nm) were then plotted against the indicator concentration and the ϵ values were determined by fitting the data to the Beer's equation (Sigma Plot, Systat, Chicago, IL).

2.4. Fluorescence spectra

The spectra of 5 μM ACR-1 and ACR-1-LA were recorded using a Fluorolog 3 (Horiba, Edison, NJ, USA) or QuantaMaster (PTI, Birmingham, NJ, USA) spectrofluorometer in calibration buffers. The spectra were excited over 400–550 nm, and emission was detected at 640 nm. Alternatively, we recorded emission spectra using 450, 480 or 540 nm excitation. All spectra were recorded at room temperature.

2.5. Dissociation constants (K_d)

Dissociation constants were determined as described previously [36] by relating an integrated indicator fluorescence (560–700 nm), (F) to free ion concentrations ($[\text{ion}]$) using the equation

$$[\text{ion}] = K_d(F - F_{\min}) / (F_{\max} - F) \quad (1)$$

where F_{min} and F_{max} are integrated intensities of free and ion bound indicators [4]. When plotted as $\log((F - F_{min}) / (F_{max} - F))$ against $\log[ion]$, the dye responses were linear and the x-intercept corresponded to K_d . Linear curve fits were performed using the graphing program SigmaPlot.

2.6. Acid dissociation constant (pK_a)

pK_a value was determined analogously to K_d as described before [37] using an integrated indicator fluorescence (560–700 nm) collected in calibration solutions, pH of which was adjusted to desired levels ($5.6 < \text{pH} < 7.8 \pm 0.02$) with 1 mM KOH and 1 M HCl.

2.7. Cultures of primary hippocampal neurons

Cell cultures were prepared as described previously [38]. Briefly, hippocampi removed from 0–3 postnatal day Sprague Dawley rat pups were treated with papain (1 mg/ml) and mechanically triturated. Cells were then plated in type I collagen coated (0.5 mg/ml) 35 mm glass-bottom dishes (Mattek, Ashland, MA, USA) at ~ 650 cells/mm² and cultured in Eagle's medium supplemented with serum, D-glucose, glutamine, and antibiotics at 37°C in a humidified incubator. Cytosine arabinoside (6.7 μM) was added 3–4 d after plating to inhibit cell division. Cells were imaged 10–14 days after plating. In some cases, 8–12 days after plating, cultures were transfected with GFP (0.5–2.5 μg) using DNA:Lipofectamine 2000 (Invitrogen) ratio of 1:1.5–2 according to the manufacturer's protocol and used 1–3 days later.

2.8. Other cell culture

All other cell types used in this study were gifts from colleagues from Washington University School of Medicine. The cells were plated on poly-D-lysine/laminin coated 35 mm glass-bottom dishes (Mattek) and cultured following standard procedures. Cells were imaged 2–3 days after plating when they became semi-confluent. In some cases, HEK293T cells were transfected with GFP using a standard CaPO₄ precipitation method [39] and used a day after transfection.

2.10. Imaging experiments

All imaging experiments were carried out at room temperature in a HEPES-buffered salt solution (HCSS) containing, in mM: 141 NaCl, 5.4 KCl, 1 NaH₂PO₄, 1.8 CaCl₂, 1 MgSO₄, 12 HEPES, and 5.5 D-glucose, pH of which was adjusted to 7.35 ± 0.05 with 0.1 M NaOH. Cells were loaded with ACR-1 or ACR-1-LA at room temperature by 60 min incubation with 10 μM AM ester in the presence of 0.1% Pluronic F-127 (Invitrogen), washed and incubated for another 30–60 min to allow for ester processing. Afterwards, cells were imaged using either a Zeiss LSM5 Pascal confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA) for single photon or an LSM510 META NLO imaging system (Zeiss) for multiphoton excitation experiments. The images were collected using a 40 $\times$ /1.2 lens (Zeiss). The indicator fluorescence was excited with 488 nm and 543 nm laser lines and the emission was collected through a long pass 560 nm and, for shorter excitation wavelengths, through a band pass 500–530 nm emission filters. The multiphoton excitation was provided by a tunable Chameleon Ultra I laser (Coherent, Santa Clara, CA, USA). When applicable, the long and short wavelength emissions were divided by one another to yield ratio values. The *in situ* indicator and GFP excitation spectra were determined by monitoring fluorescence intensity (<685 nm) while varying the excitation wavelength between 700 and 1000 nm at 10 nm intervals. The emission spectra were recorded using a Zeiss Meta-Head detector at 770 nm multiphoton excitation wavelength.

2.9. Indicators and chemicals

Acetoxymethyl (AM) esters and K^+ salts of ACR-1 and ACR-1-LA were purchased or received as samples from Teflabs (Austin, TX, USA). Other indicators including fura-2 and fluo-3 were bought from Teflabs, and X-Rhod-1 was purchased from Molecular Probes (Eugene, OR). Indicator stock solutions (1 mM) were prepared in distilled water and their AM esters were dissolved in dry DMSO. Ca^{2+} calibration kits were bought from Invitrogen. All other chemicals, including EGTA and MOPS, were bought from Sigma (St. Louis, MO, USA).

3. RESULTS

3.1. Design and structure of ACR-1 and ACR-1-LA

The design of ACR-1 and ACR-1-LA (Fig. 1) starts with optimal Ca^{2+} ion binding scaffold 1,2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid (BAPTA) [40]. To get a strong fluorescence enhancement upon binding Ca^{2+} , the fluorophore is linked directly to 5-methyl-BAPTA at the 5 position (para to the amino group). The semi-naphthofluorescein was chosen as a fluorophore to attain a red emission with a large Stokes shift. The pK_a of a closely related fluorophore had been previously reduced from 7.8 to 6.5 by monohalogenation of the naphtho moiety [41]. To lower it further, the naphtho moiety was dihalogenated, with a halogen at each of the two ortho positions, and the phenolic moiety monohalogenated, to give a physiologically useful pK_a of 5.5 for the fluorophore (measured at saturating $[Ca^{2+}]$). Thus, a one-pot reaction of 5-fluoro-7-chloro-dihydroxynaphthol and 4-fluoresoresorcinol with 5-formyl-5-methyl-BAPTA ethyl ester [4] gives the putative seminaphthofluorescein directly linked to BAPTA (ACR-1 and ACR-1-LA), as well as fluo-4 and putative dinaphthofluorescein by-products (Supplemental Figures 1 and 2).

3.2. Spectral properties and imaging modes of ACR-1 and ACR-1-LA

The newly synthesized indicators emit deep red fluorescence (F_{em} 600–700 nm) in response to visible light excitation (W_{exc} = 450–540 nm; Fig. 2A and B). Binding Ca^{2+} ions leads to a marked fluorescence increase, especially at long excitation wavelengths (540 nm) matching the indicator absorption maxima (Supplemental Figure 3). Although fluorescence of Ca^{2+} -free ACR-1 and ACR-1-LA (Fig. 2A–B) are very similar, ACR-1 responds more dynamically to Ca^{2+} binding (Fig. 2A) than its low affinity counterpart (Fig. 2B) at all tested excitation wavelengths. Membrane-permeable acetoxymethyl (AM) esters, commonly used to load indicators into cells [42], are weakly fluorescent with excitation profiles resembling those of a Ca^{2+} -free indicator (data not shown).

The molar extinction coefficients (ϵ) of free and Ca^{2+} -bound indicators ($\sim 50\text{--}56 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Supplemental Figure 3) are lower than those of most visible light excited Ca^{2+} probes ($\sim 75 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; [8]). Consequently, both indicators, especially when Ca^{2+} -free, are much dimmer than most of their analogues. It is also worth noting that Ca^{2+} binding causes a slight decrease in their absorbance (Supplemental Figure 3), which suggests that the emission rise results mostly, if not only, from the change in quantum yield.

The fluorescence intensity of both free and Ca^{2+} -bound indicators depends on excitation wavelengths. The longer the excitation, the brighter the fluorescence and the magnitude of fluorescence change upon Ca^{2+} binding (Fig. 2A, B). In particular, long excitation wavelengths ($\sim 540 \text{ nm}$; Fig. 2A–B, red lines) provide the brightest fluorescence (F_{540}^{630}) for ACR-1 that, upon Ca^{2+} binding, rises 35–45 times. If shorter excitation wavelengths are used, ACR-1 fluorescence rises 25–35 times (F_{480}^{630}) and 15–25 times (F_{450}^{630}) (Fig. 2A, C). The corresponding changes in ACR-1-LA fluorescence are somewhat lower (Supplemental

Figure 4). Therefore, both ACR-1 and ACR-1-LA can be used as dynamic, single wavelength Ca^{2+} indicators (Fig. 2C and Supplemental Figure 4).

An interesting alternative is suggested by the fact that Ca^{2+} binding results in a non-uniform fluorescence increase with most of the change occurring at longer emission wavelengths (560–700 nm; (F_{630}^{630}) whenever short excitation wavelengths are used (450–500 nm; Fig. 2A–B, green and blue lines). In particular, upon Ca^{2+} binding, the long wavelength fluorescence (F_{480}^{630}) rises 25–30 times (Fig. 2C; green symbols), while the shorter emission (500–550 nm; F_{480}^{525}) increases only 1.5–2.0 times (Fig. 1A; broken green line). Therefore, indicator fluorescence might be split into Ca^{2+} -dependent (F_{480}^{630}) and quasi-independent (F_{480}^{525}) components that, divided by each other, would yield an emission ratio ($F_{480}^{630}/F_{480}^{525}$). In this case, however, the difference between the Ca^{2+} sensitive and reference wavelengths is limited to the magnitude rather than direction of fluorescence change, as is usually the case [4]. Therefore, ratioing emission wavelengths leads to decreasing rather than increasing ACR-1 (Fig. 2D) and ACR-1-LA (Supplemental Figure 4) dynamic range. While the ratio changes are smaller than those attained with a 540 nm excitation (F_{540}^{630}) for the same $[\text{Ca}^{2+}]$ (Fig. 2C, D), recording data as ratios might provide advantages commonly associated with ratio imaging, such as increased signal stability and easy calibration [4].

It might also be interesting to note that both indicators are excited more effectively with longer (540 nm) than shorter (450–480 nm) wavelengths (Fig. 2A–B). In particular, the fluorescence intensity of Ca^{2+} -bound indicators (F_{630}^{630}) is 6–8 times higher when excited at long (540 nm) than short (450 nm) wavelengths. It might therefore be possible to create excitation ratios by dividing the Ca^{2+} -dependent fluorescence excited at long (540 nm; F_{540}^{630}) and short (F_{450}^{630} or F_{480}^{630}) wavelengths. Such ratios (e.g. $F_{540}^{630}/F_{480}^{630}$), however, offer very limited dynamic ranges for both ACR-1 (Fig. 2E) and ACR-1-LA (Supplemental Figure 4).

The reduction of dynamic range resulting from ratioing indicator emission (e.g. $F_{450}^{630}/F_{450}^{525}$) or excitation (e.g. $F_{540}^{630}/F_{450}^{630}$) might be bypassed by creating an excitation-emission ratio (Fig. 2F) in which the Ca^{2+} -dependent fluorescence (560–700 nm) excited at 540 nm (F_{540}^{630}) is divided by a reference emission (500–550 nm) excited at 450 or 480 nm (e.g. F_{450}^{525}). The dynamic range of such a ratio (e.g. $F_{540}^{630}/F_{450}^{525}$; Fig. 2F) approaches that of respective indicators excited at 540 nm (F_{540}^{630} , Fig. 2C, Supplemental Figure 4), but collecting the necessary images would require imaging systems that allow a quick change of both excitation and emission wavelengths like those described before [43, 44].

Like other Ca^{2+} indicators, ACR-1 and ACR-1-LA chelate not only Ca^{2+} , but also other divalent cations including Mg^{2+} and Zn^{2+} . Binding these ions increases the indicator fluorescence, but does not lead to spectral shifts (Fig. 3A–C). The magnitude of fluorescence rise is determined by both the type of ion bound and the excitation wavelength (Fig. 3). In any case, ACR-1 fluorescence changes induced by Mg^{2+} or Zn^{2+} binding are rather small and do not exceed 3 and 15 times (at 540 nm), respectively (Fig. 3D, Supplemental Figure 5).

3.3. Metal ion binding properties

We determined the apparent dissociation constants (K_d) of the new indicators in EGTA-based calibration buffers (Fig. 4). We have found ACR-1 to have a very high affinity for zinc ($K_d = 0.48 \pm 0.02$ nM), low affinity for magnesium ($K_d \approx 5.0 \pm 2.3$ mM), and moderate ability to bind calcium ($K_d = 0.49 \pm 0.07$ μM). Mg^{2+} in near-physiological concentrations (1 mM; [45–50]) has practically no effect on ACR-1 affinity for calcium ($K_d = 0.53 \pm 0.07$ μM ;

Fig. 4). Therefore, Mg^{2+} ions are not expected to interfere with $[Ca^{2+}]$ measurements as, at physiological Mg^{2+} concentration, only a small amount of a weakly fluorescent Mg^{2+} -indicator complex would be formed. In contrast, both indicators bind Zn^{2+} ions with a high affinity, a process that might lead to a moderate increase in overall indicator fluorescence (Fig. 3). This effect however is limited by the availability of free Zn^{2+} ions in the cytosol of most cells ($[Zn^{2+}] \approx 1$ nM; e.g. [51–54], for review see [55]). The ACR-1-LA K_d for Ca^{2+} equals 6.65 ± 0.13 μM (Fig. 4), as expected of a low affinity indicator. Assuming an indicator can accurately report ion concentrations within 0.1–10 times its K_d , the new indicators cover a range of free Ca^{2+} concentrations (~ 0.05 – 70 μM) encompassing most of $[Ca^{2+}]_i$ reported to date.

Transition metal ions, Co^{2+} and, somewhat less effectively, Mn^{2+} , displace calcium bound to ACR-1 and reduce its fluorescence (Supplemental Figure 6). This feature might allow using previously developed quenching-based protocols to calibrate [56–58] and characterize ACR-1 and ACR-1-LA properties *in situ* [59–62].

3.4. pH sensitivity

To assess the impact of H^+ binding on ACR-1 fluorescence, we recorded indicator emission spectra in Ca^{2+} -free (10 mM EGTA) calibration buffers with pH ranging from 6.0 to 7.8 (Fig 5). In high pH buffers (pH ≈ 7.4), where ACR-1 was present mostly in its free (i.e. deprotonated) form, the indicator fluorescence was low. Raising the fraction of H^+ -bound ACR-1 by lowering the pH to 6.0 resulted in roughly a tenfold fluorescence rise without any spectral shifts (Fig. 5A). Lowering the pH further (5.0–pH 5.8), led to a fluorescence decline (data not shown), an effect described previously [5, 63] and attributed to overlapping multiple protonations [63].

Using the data corresponding to single proton binding (6.2–pH 7.8), we found ACR-1 pK_a to fall within a 6.2–6.4 range depending somewhat on the excitation wavelengths (Fig. 5C). Although this value is similar to that of fura-2 ($pK_a=6.35$ [37], ACR-1 protonation results in higher fluorescence rise (≈ 5 – 10) than that reported for fura-2 (~ 2.5 times [37]), indo-1 (3 times [63] or fluo-3 (<3 times [5]) over a comparable pH range. Even a moderate, physiologically relevant pH drop (to 6.6) might increase the ACR-1 fluorescence 2.5–4 times (Fig. 5C, circles, solid lines). In contrast, Ca^{2+} -bound ACR-1 fluorescence remained pH-independent (Fig. 5B, C; triangles, dashed lines).

In the absence of spectral shifts, a pH dependent fluorescence rise (Fig. 5A) cannot be distinguished from that induced by Ca^{2+} binding (Fig. 2A). The fluorescence would therefore be determined by both pH and free Ca^{2+} concentration, an effect most pronounced at short excitation wavelengths when $[Ca^{2+}] < K_d$, conditions under which most indicator molecules are Ca^{2+} -free and available for protonation (Fig. 5D). As a result, $[Ca^{2+}]$ change accompanied by environment acidification might be perceived as higher than the actual $[Ca^{2+}]$ rise.

3.5. ACR-1 and ACR-1-LA imaging in neurons

To assess usability of new indicators for *in situ* imaging, we loaded them into cultured neurons using a standard AM ester incubation protocol [64–66]. The rate of ACR-1 accumulation (~ 0.16 $\mu M/min$ or ~ 0.33 $\mu M/min$ at room temperature and $37^\circ C$, respectively) is lower than that of fluo-3 (~ 0.39 $\mu M/min$), fura-2 (0.8 $\mu M/min$), and X-rhod-1 (~ 3.1 $\mu M/min$) (Supplemental Figure 7). As a result, cytosolic ACR-1 concentrations achieved after 30–60 minutes of bath-loading does not exceed 10–20 μM , levels much lower than those typically achieved with similar procedures (50–150 μM ; [64–66]). Such a low loading efficiency might reflect a scanty diffusion, slow AM ester hydrolysis or poor indicator

retention in cells. As we have not seen a decline in neuron fluorescence within 1–3 hours after washing the ACR-1/AM off, the latter seems to be of secondary importance.

In most neurons, ACR-1 bath-loading, albeit weak, resulted in predominantly cytosolic staining (Fig. 6A, B). In some cells however, ACR-1 accumulated in discrete compartments, a process easy to spot when the indicator was excited at 488 nm (Fig. 6A; arrows), but not at 543 nm (Fig. 6B). A similar pattern has been observed in HEK293T cells (Fig. 6C, D). In contrast, there is very little, if any, evidence of indicator compartmentalization in immortalized mouse hippocampal cells HT22 (Fig. 6E, F), but the spotty indicator distribution is evident in N2A neuroblastoma cells (Fig. 6G, H). These data demonstrate that the level of ACR-1 compartmentalization varies widely among cell types ranging from very light, if present at all, to fairly substantial. Cultured hippocampal neurons and HEK293T cells seem to be moderately susceptible to this problem. The very high fluorescence of compartmentalized ACR-1 excited at 488 nm is somewhat surprising, as all factors analyzed in this study including protonation (Fig. 5A and C) and selected metal ion binding (Fig. 3) tend to rise the ACR-1 fluorescence excited at long (~543 nm) rather than short (~480 nm) wavelengths. This unexpected observation indicates that once confined in the intracellular organelles, ACR-1 responds to other factors such as other metal ions, free radicals, protein binding or dimerization. Though it would be interesting to find out what affects the signal of internalized ACR-1, it might cause well-known problems for routine calcium imaging [57, 1].

To ensure a strong enough signal of weakly loaded cells, it might be necessary to use a higher than normal illumination intensity, an approach that might lead to indicator photobleaching and/or degradation. To assess these risks, we recorded the ACR-1 fluorescence in cultured neurons subjected to 50 mM K⁺ depolarization after collecting 10 or 75 images (Fig. 7A). As long as the excitation intensity was “low”, the indicator retained its brightness and, more importantly, its ability to respond to depolarization-induced [Ca²⁺]_i rise. Increasing the illumination intensity enhanced the bleaching rate and markedly reduced the ACR-1 response to [Ca²⁺]_i rise, a clear sign of indicator degradation [67, 68, 61]. To put these observations in perspective, we compared the fluorescence loss of ACR-1 and its well-established counterparts, fluo-3 and X-rhod-1. While acquiring sets of 100 images with 488 nm or 543 nm, we found ACR-1 to bleach at rates similar, if not lower, than those of fluo-3 and X-rhod-1, respectively (data not shown). Interestingly, the short (505–530 nm) ACR-1 emission declined slightly faster than its longer (>560 nm) fluorescence (Fig. 7B). Although probably trivial by themselves, the different bleaching rates might be important when attempting to derive an emission ratio, which tends to increase in resting neurons (Fig. 7B). Even though it seems to be an artifact, it is possible that the ratio change reflects a slow Ca²⁺ accumulation masked by indicator bleaching. To test this notion, we repeated the experiments with ACR-1-LA, which as a low affinity indicator would require a much higher [Ca²⁺]_i rise to change its ratio, but found a very similar pattern (data not shown). Therefore, in all likelihood, the ratio change in resting cells (Fig. 7B) resulted from an uneven fluorescence loss across indicator emission spectra, like that described before [69], rather than the Ca²⁺ influx induced by our imaging conditions. More intense illumination increased the rate of fluo-3 and ACR-1 bleaching to a similar extent, but led to an unexpected rise in X-Rhod-1 intensity (data not shown), a result suggesting either a change of indicator properties or light-induced indicator-mediated calcium accumulation.

Taken together, the data suggest that ACR-1 and ACR-1-LA are as resistant to bleaching and photodamage as fluo-3, and perform adequately as long as the illumination intensity remains near the minimum necessary for imaging (10% laser power in our case). The same excitation intensity might, however, render ACR-1 emission ratios unstable (Fig. 7B) and

make its interpretation difficult. During these experiments, we have not seen any signs of acute phototoxicity or cell damage.

To test these conclusions, we used ACR-1 in single wavelength ($F_{543}^{>560}$, Fig. 8A) and emission ratio ($F_{488}^{>560}/F_{488}^{>517}$; Fig. 8D) modes to monitor the depolarization-induced $[Ca^{2+}]_i$ rise in hippocampal neurons. This calcium influx led on average to a 3.5 fold increase of ACR-1 fluorescence ($F_{543}^{>560}$) excited at 543 nm (Fig. 8A; red line). Both the average and single cell responses recorded with the use of ACR-1 (Fig. 8A grey lines) were similar to those of its well-established counterparts, X-Rhod-1 (Fig. 8B) and fluo-3 (Fig. 8C) in terms of relative magnitude, kinetics and individual cell variability (Fig. 8A–C). A relatively quick fluorescence decline of ACR-1 (Fig. 8A), probably resulting from stronger illumination, was the only noticeable difference between ACR-1 and its peers.

To perform imaging experiments in emission ratio mode, we split the 488 nm excited indicator fluorescence into Ca^{2+} dependent ($F_{488}^{>560}$) and quasi-independent (F_{488}^{517}) components (Fig. 8D). While the former rose approximately 2.5 times (Fig. 8D; grey and red traces), the latter remained unchanged, though a bit shaky (Fig. 8D; black and green traces). As a result, the emission ratio, which fairly accurately traced the $[Ca^{2+}]_i$ change, was somewhat erratic (Fig. 8D, blue line). Attempts to improve the signal-to-noise ratio by increasing illumination intensity invariably led to a bleaching artifact and a slow ratio elevation similar to that described above (Fig. 7B). We have encountered similar problems when creating an emission ratio using 458 nm excitation (data not shown). It seems fair to say that the low intracellular indicator concentration achievable with the standard AM ester loading procedure (Supplemental Figure 7) might limit the usefulness of ACR-1 emission ratios in bath-loaded neurons. The use of more efficient loading methods, such as microinjection, might be needed to utilize the full potential of the ACR-1 emission ratios.

We then compared the ability of ACR-1 and ACR-1-LA to report $[Ca^{2+}]_i$ elevations induced by 50 mM K^+ depolarization and NMDA receptor stimulation (Fig. 8E, F). The high affinity ACR-1 ($K_d \sim 0.5 \mu M$; Fig. 4), responded similarly to both treatments (Fig. 8E), but its low affinity derivative, ACR-1-LA ($K_d \sim 6.6 \mu M$; Fig. 4), reported a much higher $[Ca^{2+}]_i$ in NMDA-treated than in depolarized neurons (Fig. 8F). It is well-known that NMDA receptor stimulation leads to a much higher Ca^{2+} influx and accumulation than K^+ induced depolarization (e.g. [70–72]), a difference often masked by excessive Ca^{2+} buffering by an indicator [71, 73, 66] and/or presence of optically unresolved Ca^{2+} domains [74]. In either case, the low affinity indicators are far more likely to report actual $[Ca^{2+}]_i$ than their high affinity counterparts [71, 73, 74]. The fact that ACR-1-LA, but not ACR-1, detects this difference shows that the indicators maintain their Ca^{2+} affinity status *in situ*, although their apparent dissociation constants (K_d) might vary from those determined *in vitro* (e.g. [75]).

3.6. The new indicators and multiphoton excitation

To see whether ACR-1 and its low-affinity derivative can be used with the multi-photon excitation, we acquired images of indicator-loaded neurons using excitation wavelengths ranging from 700 to 1000 nm. Both indicators were most effectively excited at 760–800 nm (Fig. 9A). The emission maxima remained unchanged around 640 nm and the emission spectra (Fig. 9B) closely resembled those recorded *in vitro* (Fig. 2A, B) and *in situ* with a single wavelength excitation (data not shown). Therefore, all considerations regarding possible imaging modes derived above (Fig. 2) and their limitations (Fig. 7) might be relevant to *in situ* experiments using multiphoton excitation.

It is worth noting that both ACR-1 and ACR-1-LA deep-red fluorescence is much longer than that of Green Fluorescent Proteins (Fig. 9B). While frequently used and very useful (for

review see [76, 77]), its expression in cells makes $[Ca^{2+}]_i$ measurement difficult, as its robust fluorescence characterized by broad emission spectra interferes with the signals of most commonly used indicators (e.g. [78, 18]). With its long emission wavelengths, ACR-1 might avoid this problem as long as its fluorescence is collected above 600 nm (Fig. 9B, dotted line). Such simple measures might also be effective for other “red” probes (e.g. X-Rhod-1), but are totally unsuitable for popular “green” dyes (e.g. fluo-3), whose spectra overlap to a lesser or greater extent with those of GFP and YFP (Supplemental Figure 8). As the Ca^{2+} -independent indicator emission (500–550 nm) overlaps with the dominant GFP fluorescence, it cannot be used as a reference in GFP expressing cells, which precludes ratioing ACR-1 emission wavelengths. In contrast, varying multiphoton excitation wavelengths is not likely to separate the indicator and GFP fluorescence, but might help to isolate GFP-positive cells.

3.7. ACR-1 in GFP expressing cells

To test this notion further, we compared ACR-1 fluorescence changes in HEK293T cells expressing or not expressing detectable quantities of GFP during stimulation of their native P2X/P2Y receptors using standard confocal microscopy (Fig. 10A). Application of receptor agonists, ATP and UTP, raised 543 nm excited ACR-1 fluorescence ($F_{543}^{>600}$) to virtually the same level in GFP positive and negative cells (Fig. 10B). We have, however, noticed a slight tendency of the ACR-1 peak response to decline as the GFP intensity increased (Fig. 10C). It seems therefore possible that, despite its broad emission separation (Fig. 9B), ACR-1 might underestimate $[Ca^{2+}]_i$ elevations in cells featuring very high GFP expression.

4. DISCUSSION

ACR-1 and ACR-1-LA are newly synthesized fluorescent calcium indicators with visible light excitation wavelengths. As such, they belong to a large family, whose development began with the synthesis of fluo-3 and rhod-2 [5]. Over the years, this indicator group has grown considerably in number and currently includes around 30 probes (e.g. [8]). While these indicators vary widely with respect to excitation wavelengths (430–590 nm), affinities for calcium (0.14–90 μ M) and dynamics of their response (2.5–100), they share, with a notable exception of fura-red [8], a small Stokes shift (25–30 nm) and absence of clear excitation or emission spectral shifts. These common features effectively curb their usage to measuring single fluorescence intensity (e.g. 500–550 nm) excited at a single wavelength (here 480–490 nm). This limit seems not to apply to ACR-1 and ACR-1-LA, which like fura-red, offer a large Stokes shift (100 nm) and a broad range of possible excitation wavelengths (Fig. 2). Therefore, in contrast to almost all of their counterparts ACR-1 and ACR-1-LA can be used either as highly dynamic single wavelength or, at the cost of somewhat smaller signal change, as ratiometric indicators (Fig. 2). Reduced as it might be, the dynamic range of ACR-1 and ACR-1-LA ratio, especially the most promising emission one, remains comparable to those of typical ratiometric indicators such as indo-1 or fura-2 (20–30; [4]) or, within its own league, higher than that of fura-red [79]. Apart from the higher dynamic range, the new indicators offer longer excitation wavelengths (Fig 2) and higher absorbance (Supplemental Figure 3) than fura-red [8]. Last, but not least, upon Ca^{2+} binding, the new indicators increase rather than decrease their fluorescence as is the case with fura-red. All these conclusions derived from *in vitro* spectral measurements, have been tested in a series of *in situ* experiments, which confirmed, albeit not without restrictions, their validity and demonstrated that the ACR-1 performance matches that of its well-known predecessors fluo-3 and X-Rhod-1 (fig.8). Taken together, these data suggest that ACR-1 and its low affinity derivative might be the most promising ratiometric indicators with visible light excitation synthesized to date.

This conclusion is further supported by our observations related to $[Ca^{2+}]_i$ imaging with the use of multiphoton excitation, which offers several advantages over one-photon excitation based confocal microscopy [12, 80]. Firstly, the new indicators combine long multiphoton excitation (760–800 nm, Fig. 9A), which holds a “sweet spot” between hemoglobin (<650 nm) and water (>900 nm) absorbance bands [81], with its 640 nm emission wavelength (Fig. 9B). This unique blend might allow collecting data from deeper layers of tissue due to a lower scattering of both wavelengths [82–84]. Secondly, the signal might be collected as an emission ratio due to the broad indicator emission spectra (Fig. 9B).

Furthermore, the long Ca^{2+} -dependent emission of the new indicators can be easily separated from that of GFP (Fig. 9B). Although in such situations, ACR-1 and ACR-1-LA can work only in single wavelength mode, they seem to be better suited for the job than most of their counterparts (Fig. 9, Supplemental Figure 8) and do, in fact, perform well in the presence of GFP (Fig. 10). Lastly, as fluorescence of ACR-1 and ACR-1-LA depends on quantum yield rather than absorbance changes (Supplemental Figure 3), they seem to meet the basic criteria [85] for fluorescence lifetime imaging (FLIM).

Another advantage of the new indicators is their relatively small response to binding Mg^{2+} and Zn^{2+} ions (Fig. 3). While the former seems to create problems only when Mg^{2+} indicators such as mag-fura-2 are used as low affinity Ca^{2+} indicators (e.g. [73, 36]), binding the latter often raises the indicator fluorescence more than Ca^{2+} ions [36]. This effect has been utilized to measure $[Zn^{2+}]_i$ with calcium indicators [86, 52, 87]. However, changes in $[Zn^{2+}]_i$, which often occur simultaneously or are induced by a $[Ca^{2+}]_i$ rise [88–90], have been reported to interfere with $[Ca^{2+}]_i$ measurements [91, 92]. ACR-1 and its low affinity analogue should be less prone to this problem.

On the other hand, ACR indicators are among the least bright members of the family, a result of their relatively low absorbance (Supplemental Figure 3). In addition, the indicator long emission, which seems advantageous in other contexts, might lie above the maximum sensitivity range of some standard detectors such as photomultiplier tubes and CCD cameras (450–550 nm). These features, combined with the low efficiency of AM ester based loading procedures (Supplemental Figure 7), may lead to the indicator intensity in cells being too low to be accurately measured, especially when the low intensity Ca^{2+} - independent fluorescence is needed for emission ratio imaging (Fig. 8D). Clearly, increasing the detection sensitivity would be the best way to solve this problem. Should this be impractical or impossible, a sufficient signal to noise ratio might be achieved by either increasing the indicator concentration or using a more intense illumination. While the former may lead to inaccurate $[Ca^{2+}]_i$ determination [66] and disturb Ca^{2+} signaling [93], the latter might cause indicator bleaching [94, 61], non-physiological responses and cell injury [95, 13].

As the ACR-1 affinity for calcium is relatively low ($K_d \sim 0.5 \mu M$), the indicator is less likely to cause Ca^{2+} -buffering related problems than its high affinity counterparts such as calcium-green-1 or Oregon-Green-BAPTA-1 ($K_d < 0.2 \mu M$; [8]). As a low affinity indicator, ACR-1-LA should be less prone to this problem. Therefore, both dyes can be present in cells at somewhat higher concentrations than other commonly used indicators. The question remains how this can be achieved, as bath-loading with currently available AM esters is rather inefficient (Supplemental Figure 7). As the free acid seems to be well retained in cells, it might be possible to reach a sufficient cytosolic dye concentration using other loading procedures (reviewed in [1]), of which microinjection seems an obvious, albeit tedious, option.

Alternatively, until the launch of more effective AM esters, cell brightness might be increased with stronger illumination intensity. Although the least desirable option, it might still be used within limits, without causing excessive indicator damage (Fig. 7) and cell injury. One of the first adverse effects of more intense excitation is the loss of stability and hence, usability of the emission ratio, which tends to rise in the absence of $[Ca^{2+}]_i$ changes (Fig. 7B). Stronger illumination damages dyes rendering them insensitive to $[Ca^{2+}]_i$ changes even in single wavelength mode (Fig. 7A).

Another factor that might affect the reliability of ACR-1 under some experimental conditions is its pH sensitivity. As indicator protonation leads to a marked fluorescence increase of its Ca^{2+} -free form (Fig. 5), cytosolic acidification, which often accompanies $[Ca^{2+}]_i$ changes [96–100], might contribute to the overall fluorescence. A parallel experiment might therefore be required to verify that the pH_i remains constant or to make an allowance for the effect of pH_i on the reported $[Ca^{2+}]_i$. If this is ignored, the data may not reflect the actual $[Ca^{2+}]_i$ changes in either absolute or relative terms, a matter further complicated by the pH effect on indicator affinity [63, 37]. Using proper pH specific calibration data might alleviate the problem, but a full correction for large and dynamic pH shifts might be difficult.

Taken together, the presented data show that the newly synthesized indicators, ACR-1 and ACR-1-LA, offer a number of distinct features that set them apart from other visible light excitable dyes. A unique combination of excitation and emission wavelengths that allows flexible imaging in different modes, including an emission ratio option, seems to be their main advantage. Other benefits of the new indicators include spectral compatibility with GFP, weak response to Mg^{2+} and Zn^{2+} binding, and a potential suitability for FLIM. The indicator usability might be limited by relatively weak fluorescence, inefficient loading with currently available AM esters, and, under some conditions, pH sensitivity. Despite these potential drawbacks, the performance of the new indicators in our model systems matched that of other commonly used probes. It is up to further experiments to find out how the new indicators would fare in real life applications and determine areas in which they would be most useful.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Drs. S. Achilefu, M. Berezin and C. Frieden of Washington University School of Medicine (WUSM) and members of their laboratories for their help with spectrofluorometry. We are very grateful to Dr. S. Mennerick and members of his laboratory at WUSM, Ms. A. Benz and Dr. D. Crawford, for providing the primary cultures of hippocampal neurons and transfecting them with GFP, respectively. We appreciate our WUSM colleagues for their generous gifts of GFP transfected HEK293T (Ms. N. Husic), HT22 (Dr. B. Wice) and N2A neuroblastoma (Dr. Q. Xiao) cells. We thank Mr. Gary London for his help in preparing the manuscript. This work was supported by NIH Neuroscience Blueprint Center Core Grant P30 NS057105 to Washington University, NIH grants P01 NS032636 and R01 NS36265 (to MPG), NIH R01 MH078823 (to Dr. S. Mennerick), and funds from the Center for Investigation of Membrane Excitability Diseases (CIMED) at Washington University.

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ACR-1 (AM):	$R^1 = \text{CH}_2\text{OCOCH}_3$,	$R^2 = \text{Me}$,	$R^3 = \text{H}$
ACR-1 (K ⁺ Salt):	$R^1 = \text{K}^+$,	$R^2 = \text{Me}$,	$R^3 = \text{H}$
ACR-1-LA (AM):	$R^1 = \text{CH}_2\text{OCOCH}_3$,	$R^2 = \text{F}$,	$R^3 = \text{F}$
ACR-1-LA (K ⁺ Salt):	$R^1 = \text{K}^+$,	$R^2 = \text{F}$,	$R^3 = \text{F}$

FIGURE 1.

Chemical structure of K⁺ salts and acetoxymethyl (AM) esters of Asante Calcium Red (ACR-1) and Asante Calcium Red-1-Low Affinity ACR-1-LA) fluorescent Ca²⁺ indicators. The nature and properties of the compounds are determined by substitutions R¹, R², and R³ in the common base structure. The synthetic pathways are shown in Supplemental Figures 1 and 2 and described in detail in Supplemental Data 1.

FIGURE 2.

A–B. Representative emission spectra of Ca^{2+} -free (dashed lines) and Ca^{2+} -bound (solid lines) ACR-1 (**A**) and ACR-1-LA (**B**) in calibration buffers containing either 10 mM EGTA or 1 mM free calcium collected at 450 (blue), 480 (green) or 540 nm (red) excitation. The spectra were normalized to the maximum emission of Ca^{2+} -bound ACR-1 excited at 540 nm. **C.** The relationship between $[\text{Ca}^{2+}]$ and integrated ACR-1 fluorescence intensity (560–700 nm; F_{Wi}^{630}) excited at 450 (blue), 480 (green) or 540 nm (red). **D.** ACR-1 emission ratios of Ca^{2+} -dependent (560–700 nm; F_{Wi}^{630}) and quasi-independent (500–550 nm; F_{Wi}^{525}) fluorescence excited at 450 (blue) or 480 nm (green) plotted as a function of $[\text{Ca}^{2+}]$. **E.**

ACR-1 excitation ratios of Ca^{2+} -dependent fluorescence excited at 540 nm (F_{540}^{630}) and 450 (F_{450}^{630} , blue) or 480 nm (F_{480}^{630} , green) as a function of $[\text{Ca}^{2+}]$. **F.** Mixed excitation/emission ratios were calculated by dividing Ca^{2+} -dependent fluorescence excited at 540 nm (F_{540}^{630}) by Ca^{2+} -independent emission excited at 450 (blue) or 480 nm green), F_{450}^{525} or F_{480}^{525} respectively, and plotted against $[\text{Ca}^{2+}]$. The data (mean $\pm$ SEM) were derived from 3–4 sets of spectra recorded in calibration buffers and normalized to the minimum values in Ca^{2+} -free buffers.

FIGURE 3.

A–C. Representative emission spectra of ACR-1 in the absence (black) and presence of saturating concentrations of Ca^{2+} (1 mM; red), Mg^{2+} (100 mM; blue) and Zn^{2+} (1 μM ; green) recorded in EGTA-based calibration buffers using 450 nm, 480 nm and 540 nm excitation wavelengths. All spectra were normalized to the maximum intensity of Ca^{2+} -bound indicator. **D.** The relative change of ACR-1 fluorescence excited at 450, 480 and 540 nm upon Ca^{2+} (red), Mg^{2+} (blue) and Zn^{2+} (green) ion binding. The data (mean $\pm$ SEM) were derived from 2–5 experiments by dividing the fluorescence intensity of ion-bound and free (10 mM EGTA) ACR-1 at corresponding excitation wavelengths.

FIGURE 4.

The logarithm of relative fluorescence change of ACR-1 (circles) and ACR-1-LA (triangles) plotted against the log of free concentrations of a - Zn²⁺ (green symbols), b and d - Ca²⁺ alone (red symbols), c - Ca²⁺ in the presence of 1 mM free Mg²⁺ (pink symbols), and e - Mg²⁺ (blue symbols) ions. The data (mean $\pm$ SEM) were pooled from 3–5 experiments. The solid lines represent fits to equation 1, the horizontal dashed line is an x intercept and the vertical dotted lines mark estimated K_d values. For comparative purposes, we marked the approximate free cytosolic concentrations of the tested ions.

FIGURE 5.

A–B. Representative emission spectra of ACR-1 excited at 450 nm in calibration buffers of different pH containing 0 (10 mM EGTA) or 1 mM free Ca^{2+} . **C.** The relationship between the fluorescence intensity of Ca^{2+} -free (circles) and Ca^{2+} -bound (triangles) ACR-1 excited at 450 (blue), 480 (green) and 540 nm (red) and the buffer pH. All data (mean $\pm$ SEM) were pooled from 3–4 experiments and normalized to Ca^{2+} intensity of Ca^{2+} -free ACR-1 in a standard calibration buffer (pH=7.20) at appropriate excitation wavelengths. The lines represent fits to a four parameter dose-response sigmoid curve. **D.** Estimated fluorescence of partly $\text{Ca}^{2+}/\text{H}^{+}$ -bound ACR-1 over a range of buffer pH and $[\text{Ca}^{2+}]$ excited at 480 nm. Total fluorescence intensity was calculated as a linear superposition of free, H^{+} - and Ca^{2+} -bound

ACR-1 intensities under given conditions and normalized to the maximum intensity of a fully Ca^{2+} -bound indicator.

FIGURE 6.

A–H. Confocal images of cultured hippocampal neurons (A, B), HEK293T cells (C, D), immortalized mouse hippocampal cells HT22 (E, F), and N2A neuroblastoma cells (G, H) loaded with ACR-1 (10 μ M, 60 minutes at room temperature) collected through a long pass 560 nm emission filter using 488 nm ($F_{488}^{>560}$; left hand panel) or 543 nm ($F_{543}^{>560}$; right hand panel) excitation. Please note the uniform, apparently cytosolic indicator distribution in most neurons (A, B) and HEK293T cells (C, D) and instances of compartmentalization (arrows) well visible in 488 nm (A, C), but not 543 nm (B, D), excited images. Widespread compartmentalization is evident in N2A cells (G, H), but virtually absent in HT22 cells (E, F). The scale bar corresponds to 20 μ m.

FIGURE 7.

A. ACR-1 fluorescence (>560 nm) excited at 543 nm in hippocampal neurons subjected to 50 mM K⁺ depolarization after 10 (green circles) or 75 (red circles) “low” (10% laser power; circles) or “high” (75% laser power; triangles) intensity illumination cycles. **B.** Changes of Ca²⁺-dependent (>560 nm; green circles), Ca²⁺-independent (505–530 nm; red circles) fluorescence intensity and their ratio (triangles) of ACR-1 in resting cultured hippocampal neurons excited at 488 nm (10% laser power) during 100 image acquisition cycles. The datasets were described using linear regression (solid lines); the dotted line corresponds to the starting value of 1. In both A and B, the images (512×512 pixels, 4x averaging) of bath-loaded neurons (10 μ M ACR-1/AM, 60 min) were acquired at 5 sec intervals. The fluorescence intensity was first normalized for individual neurons to that of the mean intensity of the first five images, and then the normalized values were averaged to yield the presented data (mean $\pm$ SEM). The data were pooled from 3–4 experiments, each containing 5–10 neurons. For clarity only every fifth point is shown.

FIGURE 8.

A–C. Single cell (gray lines) and mean fluorescence intensity (red and green lines) of Ca^{2+} dependent fluorescence of ACR-1 (A), X-Rhod-1 (B) and fluo-3 (C) in cultured hippocampal neurons subjected to 50 mM K^+ depolarization. The data (F_{exc}^{em}) were collected at 5 second intervals using indicated excitation and emission wavelengths and presented as normalized values. **D.** Calcium dependent ($F_{488}^{>560}$) and quasi-independent (505–530 nm; F_{488}^{517}) fluorescence of ACR-1 in hippocampal neurons exposed to 50 mM K^+ . The single cell traces are shown as gray and black lines, respectively; the mean values are shown as red and green lines. The blue line represents the mean emission ratio ($F_{488}^{>560}/F_{488}^{517}$) calculated by averaging ratios of individual cells. Graphs A–D show complete results of single experiments selected out of 2–5 experiments performed. The horizontal dashed lines represent the normalized fluorescence value at the onset of an experiment ($F/F_0=1$). **E–F.** The mean fluorescence ($F_{543}^{>560}$) of ACR-1 (E) and its low affinity derivative, ACCR-1-LA (F), excited at 543 nm in cultured hippocampal neurons depolarized with 50 mM K^+ (green circles) or exposed to 100 μM NMDA (red circles). The presented data (mean $\pm$ SEM) were pooled from 2–3 experiments, each containing 5–10 neurons. For clarity every third point is shown.

FIGURE 9.

A. Multiphoton excitation spectra of ACR-1 (red), ACR-1-LA (blue) and GFP (green) in cultured hippocampal neurons were determined by measuring the average fluorescence intensity (<685 nm) over a range of excitation wavelengths (700–1000nm). B. The emission spectra of ACR-1(red), ACR-1-LA (blue) and GFP (green) excited at 770 nm multiphoton excitation wavelength were recorded in cultured hippocampal neurons using Zeiss LSM510 Meta head. The dashed line shows a possible separation between GFP and ACR-1 fluorescence. In A and B, data collected in 3–5 experiments were first normalized to the maximum fluorescence for individual cells, averaged and plotted as mean $\pm$ SEM.

FIGURE 10.

A. A sample confocal image of HEK293T cells transfected with GFP and loaded with ACR-1 in which GFP was visualized through a 505–530 nm band-pass filter using 488nm excitation (F_{488}^{517} ; green) and the Ca^{2+} indicator fluorescence ($F_{543}^{>600}$; red) as excited at 543 nm. **B.** The ACR-1 fluorescence change in cells expressing (green symbols) or not expressing GFP (red symbols) during combined application of ATP and UTP, agonists of p2X/P2Y receptors. The data (mean $\pm$ SEM) were pooled from 3 experiments each including 40–60 cells, of which 30–40% contained detectable amounts of GFP. **C.** The relationship between the peak ACR-1 response and the GFP fluorescence intensity. The data derived from experiments shown in panel B. represent single cells with (green circles) or without (red circles) detectable GFP expression. Please note that as the GFP intensity increases, the expected peak ACR-1 response of GFP positive cells (solid green regression line) becomes slightly smaller than the average peak indicator response recorded in GFP negative cells (dotted red line).