Domains of high Ca^{2+} beneath the plasma membrane of living A7r5 cells

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Theoretical models and indirect experimental observations predict that Ca^{2+} concentrations at the inner surface of the plasma membrane may reach, upon stimulation, values much higher than those of the bulk cytosol. In the past few years, we have shown that the Ca^{2+} -sensitive photoprotein aequorin can be intracellularly targeted and utilized for specifically monitoring the $[\text{Ca}^{2+}]$ of various organelles. In this work, we extend this approach to the study of the cytoplasmic rim beneath the plasma membrane. We have constructed a new aequorin chimera by fusing the photoprotein with SNAP-25, a neuronal protein which is recruited to the plasma membrane after the post-translational addition of a lipid anchor. The SNAP-25-aequorin chimera, expressed in the rat aortic smooth muscle cell line A7r5, appears correctly sorted as revealed by immunocytochemistry. Using this probe, we demonstrate that the mean $[\text{Ca}^{2+}]$ of this cytoplasmic region ($[\text{Ca}^{2+}]_{\text{pm}}$) can reach values >10-fold higher than those of the bulk cytosol ($[\text{Ca}^{2+}]_{\text{c}}$) upon activation of Ca^{2+} influx through plasma membrane channels. In unstimulated cells, the mean $[\text{Ca}^{2+}]_{\text{pm}}$ appears also to be higher than the bulk cytosol, presumably reflecting the existence of microdomains of high $[\text{Ca}^{2+}]$.

Keywords: calcium/plasma membrane/recombinant aequorin/targeting

Introduction

The rim of cytoplasm immediately beneath the plasma membrane is an important site of action of intracellular Ca^{2+} ions. In this restricted environment, a number of Ca^{2+} -dependent processes are activated, such as secretion, ion channel activity, production of second messengers, etc. Based, however, on the proximity to the Ca^{2+} channels, the Ca^{2+} changes under the plasma membrane are expected to be quite different from those measured in the rest of the cytoplasm. Pioneering work using the low Ca^{2+} affinity *n*-aequorin-J has shown that microdomains of $[\text{Ca}^{2+}]$ in the range 200–300 μM occur in the presynaptic terminal of the giant squid synapse during transmitter release (Llinás *et al.*, 1992, 1995; Silver *et al.*, 1994). This $[\text{Ca}^{2+}]$ appears to be necessary to trigger the exocytotic machinery efficiently (Heidelberger *et al.*, 1994). Apart from these models, however, direct evidence for a correlation between

local Ca^{2+} changes and effects on cell function has been very difficult to obtain. In fact, while the Ca^{2+} dependence of various processes occurring at the cell surface has been investigated thoroughly in a large number of cell models, the measurement of Ca^{2+} concentration in this restricted cytosolic space is a still unsolved experimental task.

We have recently described the targeting of the Ca^{2+} -sensitive photoprotein aequorin to the lumen of various cellular organelles, i.e. nucleus (Brini *et al.* 1993), mitochondria (Rizzuto *et al.*, 1992, 1993, 1994) and endoplasmic reticulum (Montero *et al.*, 1995), and the use of these targeted Ca^{2+} probes for a subcellular analysis of Ca^{2+} homeostasis. The same approach was extended here to the study of the subplasmalemmal space. Indeed, compared with lipid-bound fluorescent indicators (Etter *et al.*, 1994, 1996), a targeted photoprotein can be expected to exhibit a more selective localization and, by virtue of its low Ca^{2+} affinity (~10 and ~100 μM for wild-type aequorin and the Asp119→Ala aequorin mutant, respectively), a minor Ca^{2+} -buffering capacity.

Since the minimal sequence requirements for the sorting to the plasma membrane are still not completely known, the targeting of aequorin to the subplasmalemmal space was based on the construction of a fusion protein including aequorin and SNAP-25, a protein which is synthesized on free ribosomes and recruited to the inner surface of the plasma membrane after the palmitoylation of specific cysteine residues (Hess *et al.*, 1992). Moreover, as the $[\text{Ca}^{2+}]$ of the subplasmalemmal region ($[\text{Ca}^{2+}]_{\text{pm}}$) could be expected to be, at least transiently, very high, we have constructed two SNAP-25-aequorin chimeras, differing only in the aequorin moiety: wild-type aequorin in the chimera designated pmAEQwt, the Asp119→Ala mutant, endowed with lower Ca^{2+} affinity, in the chimera named pmAEQmut, allowing measurements of $[\text{Ca}^{2+}]$ up to 150 μM . By expressing these chimeras in the A7r5 line, we were able to monitor selectively $[\text{Ca}^{2+}]_{\text{pm}}$ and demonstrate that, in unstimulated cells, the average $[\text{Ca}^{2+}]$ is apparently >10-fold higher than in the bulk cytosol and can reach, upon Ca^{2+} influx through plasma membrane channels, values as high as 100 μM .

Results

Construction and expression of the pmAEQ chimera

The start points for the construction were the wild-type SNAP-25 cDNA and two different HA1-tagged aequorin cDNAs: the wild-type aequorin (Brini *et al.*, 1985; Inouye *et al.*, 1995) and the Asp119→Ala mutant (Montero *et al.*, 1995), endowed with lower Ca^{2+} affinity. Since a *Clal* site is present at the 5' end of the aequorin cDNAs, through a PCR step a *Clal* site was inserted immediately upstream of the SNAP-25 stop codon; the PCR-modified

Fig. 1. Schematic map of the chimeric pmAEQ cDNA. The chimeric cDNA was constructed by fusing in-frame the cDNAs encoding SNAP-25 and HA1-tagged aequorin, as described in detail in Materials and methods. Coding and non-coding regions of the cDNA are indicated as boxes and lines, respectively. Within the coding region, the portions encoding SNAP-25, the HA1 epitope and aequorin are stippled, black and white, respectively. An asterisk indicates the position of the Asp119→Ala mutation of the aequorin cDNA (see text).

SNAP-25 cDNA was then fused to either the wild-type aequorin cDNA (to generate the chimera designated pmAEQwt) or the low-affinity mutant (generating the chimera designated pmAEQmut). Figure 1 shows a schematic map of the cDNA construct, while the details of the construction are discussed in Materials and methods. The chimeric cDNAs were subcloned in the expression vector pcDNA1 (Invitrogen), and used for transfecting the cell line A7r5 (Kimes and Brandt, 1976). The intracellular distribution of transiently expressed pmAEQ and, for comparison, cytosolic aequorin, cytAEQ (Brini *et al.*, 1995), was verified by immunostaining. Figure 2 shows confocal analysis of transfected cells. Three confocal sections of pmAEQ (Figure 2A–C) and cytAEQ (Figure 2E–G) are presented. In Figure 2A and B, a clear peripheral distribution of pmAEQ can be observed, with fine digitations appearing most strongly labelled; in Figure 2C, a diffuse labelling of the top surface of the cell can be appreciated. Conversely, the immunostaining of cytAEQ (Figure 2E–G) appears diffused to the whole cytoplasm, with partial nuclear exclusion. To further confirm the intracellular localization of the probes, confocal microscopy was performed on cells transiently expressing pmAEQ and cytAEQ after permeabilization with 100 μ M digitonin for 2 min prior to fixation. In Figure 2D, a clear staining of the plasma membrane is still evident in cells expressing pmAEQ while, under the same conditions (Figure 2H), no staining was observed in cells expressing cytAEQ.

Aequorin reconstitution

Native aequorin is composed of an apoprotein and a covalently bound co-enzyme, coelenterazine. The reconstitution of a functional photoprotein is accomplished by incubating the cell expressing recombinant aequorin with the membrane-permeant prosthetic group coelenterazine (Rizzuto *et al.*, 1995). The efficiency of this procedure depends on the relative rates of aequorin reconstitution and consumption. In cell compartments endowed, at rest, with low Ca^{2+} concentrations (such as the cytoplasm, the nucleus or the mitochondria), reconstitution of recombinant aequorin is highly effective (Rizzuto *et al.*, 1992, 1994; Brini *et al.*, 1993, 1995), while in an organelle with high $[\text{Ca}^{2+}]$, the endoplasmic reticulum, a good

Fig. 2. Confocal immunofluorescence of A7r5 cells transiently expressing pmAEQ (left panels) or cytAEQ (right panels). The pictures show 0.5 μ m (for pmAEQ, A, B and C) or 1 μ m (for cytAEQ, E, F and G) horizontal confocal sections of transiently transfected cells: in the case of pmAEQ, two sections (A and B), 1 μ m apart, near the base of the cell and one at the top (C) are shown; in the case of cytAEQ, the three sections (E, F and G) are each 4 μ m apart. The pmAEQ image refers to a cell expressing pmAEQmut; the same pattern was observed with cells expressing pmAEQwt. Given the relatively low level of expression of cytAEQ, thick confocal sections ($\sim 1 \mu$ m) were taken. Two relatively small, rounded cells are shown, in which the signal-to-noise ratio was particularly good. (D) (pmAEQ) and (H) (cytAEQ) refer to pictures of cells that have been permeabilized with 100 μ M digitonin for 2 min prior to fixation. Bar: 9 μ m (A–C and E–G), 14 μ m (D and H).

reconstitution can be obtained only upon drastic reduction of the luminal Ca^{2+} concentration (Montero *et al.*, 1995). Table I shows the efficiency of reconstitution of pmAEQ and cytAEQ in media with or without extracellular Ca^{2+} (expressed as a percentage of maximal). In contrast to cytAEQ, the reconstitution of the pmAEQ chimeras was reduced drastically in media containing physiological Ca^{2+} concentrations (1 mM). This effect was more pronounced with pmAEQwt ($11 \pm 1\%$) than with pmAEQmut ($21 \pm 4\%$), suggesting, albeit indirectly, that the $[\text{Ca}^{2+}]_{\text{pm}}$ is, in unstimulated cells, much higher than in the rest of the cytoplasm.

Ca^{2+} measurements with aequorin

Effect of Ca^{2+} readdition. In all experiments, measurements of $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{pm}$ were performed in monolayers of cells transiently transfected (Rizzuto *et al.*, 1995) with cytAEQ or pmAEQ, after a 45 min reconstitution with coelenterazine in Ca^{2+} -free Krebs–Ringer buffer (KRB). As shown in Figure 3A, in cells expressing cytAEQ, light emission, initially close to background values, underwent a minor increase upon addition of 1 mM CaCl_2 ; >95% of the total aequorin pool was discharged only by lysing the cells at the end of the experiment. The corresponding calibrated signal (Figure 3B) showed that $[\text{Ca}^{2+}]_c$ was 0.2 μM in Ca^{2+} -free medium and marginally increased upon Ca^{2+} readdition. Figure 3C shows that, with pmAEQwt, aequorin light

emission was also close to background values in Ca^{2+} -free medium, but underwent a very large transient rise when the perfusing buffer was supplemented with 1 mM CaCl_2 (>90% aequorin was consumed within ~2 min). The calibration of the aequorin signal into Ca^{2+} values (Figure 3D) showed a large transient increase (peak >8 μM), which rapidly declined towards basal. Figure 3E shows that also with pmAEQmut, a major fraction of aequorin (90% in 2 min) was consumed upon Ca^{2+} addition. Indeed, the calibration of the aequorin signal (Figure 3F) showed a transient $[\text{Ca}^{2+}]_{pm}$ increase which peaked at 120 μM . It is noteworthy that the rates of photon emission with pmAEQwt and pmAEQmut are quite similar, suggesting that, under these conditions, the increase in $[\text{Ca}^{2+}]$ of the subplasmalemmal region is so high as to saturate both probes. Accordingly, the values of $[\text{Ca}^{2+}]_{pm}$ would be largely artefactual. In order to get a more quantitative measurement of the changes in $[\text{Ca}^{2+}]_{pm}$, the cells were thus challenged with a much lower extracellular Ca^{2+} concentration.

Figure 4A and C shows the increase in aequorin light emission induced by the addition of 100 μM CaCl_2 to cells transiently expressing pmAEQwt and pmAEQmut, maintained in Ca^{2+} -free medium. Figure 4A and C shows that the increase in light emission induced by Ca^{2+} readdition (which induces a negligible change of $[\text{Ca}^{2+}]_c$, not shown) was quite different with the two pmAEQ chimeras: 60–70% of total aequorin was consumed in

Table 1. Efficiency of reconstitution of the pmAEQ chimeras, and for comparison, of cytAEQ, with two different reconstitution protocols

	DMEM + 1% FCS	Ca^{2+} -free KRB
cytAEQ	88 ± 2	100
pmAEQwt	11 ± 1	100
pmAEQmut	21 ± 4	100

Coelenterazine (5 μM) was added to the indicated buffer; after 45 min, the total light output upon cell lysis was measured. For each chimera, the efficiency of reconstitution in DMEM + 1% FCS is expressed as percentage of that obtained in Ca^{2+} -free KRB.

Fig. 3. Effect of Ca^{2+} readdition (1 mM) to cells maintained in Ca^{2+} -free media. The upper traces (A, C and E) show aequorin light emission and the lower traces (B, D and F) show the corresponding calculated $[\text{Ca}^{2+}]$ values on coverslips of cells transiently expressing cytAEQ, pmAEQwt or pmAEQmut. After a 45 min reconstitution in Ca^{2+} -free media, the coverslip was transferred to the luminometer chamber and perfused with KRB containing 0.1 mM EGTA. Where indicated, KRB was supplemented with 1 mM CaCl_2 . At the end of the experiment, unconsumed aequorin was discharged ('cell lysis') by perfusing the cells with a hypotonic Ca^{2+} -rich solution (10 mM CaCl_2 in H_2O), in order to estimate the total photoprotein content. The inset in (A) shows the increase in the light emission of cytAEQ upon Ca^{2+} addition with an expanded scale.

Fig. 4. Effect of Ca^{2+} readdition ($100 \mu\text{M}$) to cells maintained in Ca^{2+} -free media. The upper traces (A and C) show aequorin light emission and the lower traces (B and D) show the corresponding calculated $[\text{Ca}^{2+}]_{\text{pm}}$ values, on coverslips of cells transiently expressing pmAEQwt or pmAEQmut. All conditions are as in Figure 3; where indicated, the perfusion buffer was supplemented with $100 \mu\text{M}$ CaCl_2 .

2 min with pmAEQwt (Figure 4A) compared with 10–20% with pmAEQmut (Figure 4C). The corresponding calibrated $[\text{Ca}^{2+}]_{\text{pm}}$ (Figure 4B and D) was similar with the two probes, in terms of both kinetics and peak amplitudes, i.e. $8 \pm 2 \mu\text{M}$ ($n = 5$) with pmAEQwt (Figure 4B) and $10 \pm 1.5 \mu\text{M}$ ($n = 5$; Figure 4D) with pmAEQmut. The steady-state $[\text{Ca}^{2+}]_{\text{pm}}$ ($2.0 \pm 0.5 \mu\text{M}$), as measured with pmAEQmut, was reached in 2 min after Ca^{2+} readdition. This value was significantly higher than basal $[\text{Ca}^{2+}]_{\text{c}}$ and lasted until Ca^{2+} was removed from the medium.

Effect of Ca^{2+} store depletion. While Ca^{2+} readdition to cells incubated in Ca^{2+} -free medium causes minor changes in $[\text{Ca}^{2+}]_{\text{c}}$, much larger $[\text{Ca}^{2+}]_{\text{c}}$ increases can be elicited by the activation of store-operated Ca^{2+} influx (Byron and Taylor, 1995). We have thus investigated the effect of Ca^{2+} store depletion on the $[\text{Ca}^{2+}]_{\text{pm}}$. The corresponding $[\text{Ca}^{2+}]_{\text{c}}$ data are shown in Figure 5A. Transfected cells were first treated with the inositol 1,4,5 trisphosphate (IP_3)-generating agonist vasopressin, thus evoking a transient $[\text{Ca}^{2+}]_{\text{c}}$ rise (peak $1.4 \mu\text{M}$); Ca^{2+} influx was then triggered by adding $100 \mu\text{M}$ CaCl_2 to the perfusing medium. Ca^{2+} readdition caused a second increase of $[\text{Ca}^{2+}]_{\text{c}}$ at $0.8 \mu\text{M}$ and a sustained plateau at $\sim 0.4 \mu\text{M}$; finally, upon addition of EGTA, $[\text{Ca}^{2+}]_{\text{c}}$ returned to basal levels. With regard to the $[\text{Ca}^{2+}]_{\text{pm}}$, the release of stored Ca^{2+} caused a $[\text{Ca}^{2+}]_{\text{pm}}$ rise quite similar in amplitude and kinetics to that of the cytosol (demonstrating that the Ca^{2+} affinity of the pmAEQ is not affected by the fusion of the photoprotein

with SNAP-25 and/or by the local microenvironment). Similar results were obtained by releasing the internal stores with caffeine (data not shown). Conversely, a very large and rapid rise was observed upon readdition of Ca^{2+} , both with pmAEQwt (not shown) and with pmAEQmut (Figure 5B). The peak value, while close to the saturation of the probe with pmAEQwt, could be estimated to be $40 \pm 9 \mu\text{M}$ ($n = 5$) using pmAEQmut.

These conditions (readdition of Ca^{2+} to depleted cells) were aimed at magnifying store-operated Ca^{2+} influx. When Ca^{2+} influx was activated under more physiological conditions, i.e. the IP_3 -generating agonist was administered in the presence of external Ca^{2+} , the results were quite different. Cells transfected with either cytAEQ or pmAEQ were first exposed to $100 \mu\text{M}$ Ca^{2+} until a steady-state was reached and were then challenged with vasopressin. Figure 5C shows that, after a marginal increase upon Ca^{2+} readdition, stimulation with vasopressin induced a transient $[\text{Ca}^{2+}]_{\text{c}}$ increase, which peaked at $1.2 \mu\text{M}$. Figure 5D shows that $[\text{Ca}^{2+}]_{\text{pm}}$, monitored with pmAEQmut, after the large overshoot upon Ca^{2+} readdition (peak $\sim 12 \mu\text{M}$) underwent, upon stimulation with vasopressin, a rise from the plateau value of $2 \mu\text{M}$ to $\sim 5 \mu\text{M}$. This increase, larger than that of $[\text{Ca}^{2+}]_{\text{c}}$, was, however, >10 -fold smaller than that evoked by Ca^{2+} readdition to Ca^{2+} -depleted cells (Figure 3), probably reflecting the known inhibitory effect of Ca^{2+} on store-dependent Ca^{2+} current (Hoth and Penner, 1993; Zweifach and Lewis, 1995; Skutella and Rieg, 1996). Finally, the addition of EGTA caused the

Fig. 5. The effect of the IP_3 generating agonist vasopressin on $[\text{Ca}^{2+}]_{\text{pm}}$ and $[\text{Ca}^{2+}]_{\text{c}}$ measured with pmAEQmut and cytAEQ, respectively. All conditions are as in Figure 3. The calibrated $[\text{Ca}^{2+}]_{\text{c}}$ and $[\text{Ca}^{2+}]_{\text{pm}}$ traces are shown in the top and bottom part of the figure respectively. On the left, the cells, while perfused with Ca^{2+} -free KRB, were first challenged with 0.2 μM vasopressin (evoking a transient $[\text{Ca}^{2+}]_{\text{c}}$ and $[\text{Ca}^{2+}]_{\text{pm}}$ response); after the return to basal, the perfusion buffer was supplemented with 100 μM CaCl_2 ; after a few minutes (i.e. when a steady-state $[\text{Ca}^{2+}]_{\text{pm}}$ plateau was reached), the cells were challenged with 0.2 μM vasopressin, in the continuous presence of 100 μM CaCl_2 .

return of $[\text{Ca}^{2+}]_{\text{pm}}$ to a level indistinguishable from that of $[\text{Ca}^{2+}]_{\text{c}}$.

Discussion

The possibility of measuring the $[\text{Ca}^{2+}]$ in the subplasma-membral space, a cytosolic region which plays a key role in the control of Ca^{2+} homeostasis and in a number of physiological processes (secretion, ion and metabolite transport, etc.), is a major goal in the study of cell physiology. However, the methodologies now available to achieve this goal are hampered by technical and theoretical limitations. Indeed, given the spatial resolution of optical microscopes (under ideal conditions that of incident light, i.e. 300–500 nm), at best a cytosolic fluorescent Ca^{2+} indicator, such as fura-2 (Grynkiewicz *et al.*, 1985), will only measure the average of a very steep gradient. In an attempt to overcome this limitation, fluorescent Ca^{2+} indicators have been anchored to the plasma membrane via the addition of a hydrophobic fatty acid tail (Etter *et al.*, 1994, 1996). The kinetics of $[\text{Ca}^{2+}]$ increase measured by lipid-bound Ca^{2+} dyes upon opening of plasma membrane channels were found to be much faster than those recorded with the same indicator loaded in the cytosol, though, surprisingly, the peak increases were not very different. Here we present the development and the use of a specifically targeted aequorin chimera. Moreover,

through the use of aequorin moieties with different Ca^{2+} affinities, a wide range of $[\text{Ca}^{2+}]$ can be measured accurately.

With this selective probe, the study of the $[\text{Ca}^{2+}]$ dynamics in the rim of cytoplasm immediately beneath the plasma membrane revealed an interesting, and in part unexpected, scenario. In particular, the mean $[\text{Ca}^{2+}]_{\text{pm}}$ appears much higher than that of the bulk cytosol both when Ca^{2+} influx is activated and in unstimulated cells. This latter conclusion is supported by both the reduced reconstitution of the pmAEQ chimeras in Ca^{2+} -containing media (Table I) and by the direct monitoring of the steady-state $[\text{Ca}^{2+}]_{\text{pm}}$ (see Figure 4). However, the maintenance of a gradient between $[\text{Ca}^{2+}]_{\text{pm}}$ and bulk $[\text{Ca}^{2+}]_{\text{c}}$ would imply a continuous flux of Ca^{2+} into the cytosol, whereas in resting cells there is no net flux of Ca^{2+} across the plasma membrane. Two non-mutually exclusive possibilities could thus account for the high $[\text{Ca}^{2+}]_{\text{pm}}$ values. $[\text{Ca}^{2+}]_{\text{pm}}$ could be heterogeneous also under resting conditions. In particular, hotspots could be generated transiently in the proximity of Ca^{2+} channels during spontaneous activity. The local $[\text{Ca}^{2+}]$ would lead to activation of Ca^{2+} extrusion, and, on average, the net flux would be zero. The pmAEQ signal would average the $[\text{Ca}^{2+}]_{\text{pm}}$ values but, since the rate of photon emission of aequorin is proportional to the 2nd–3rd power of $[\text{Ca}^{2+}]$, the mean signal would be dominated by the regions at high Ca^{2+} . Alternat-

ively, the negative surface potential of the inner leaflet of the plasma membrane, which was estimated in other cell systems to be ~ 20 – 30 mV (Hille *et al.*, 1975), could account for a steady-state difference in the resting $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{pm}$ levels. However, this interpretation appears unlikely to us for at least two reasons: (i) while transient microdomains might have escaped the spatial resolution of previous studies using lipid-bound fura-2, a homogeneous $[\text{Ca}^{2+}]_{pm}$ of 1–2 μM should have been readily revealed as a rim of high $[\text{Ca}^{2+}]$ at the cell periphery; and (ii) the electrophysiological characterization of the basal activity of Ca^{2+} -dependent channels argues against this model. For instance, Ca^{2+} -dependent K^+ channels present in A7r5 cells are activated by a $[\text{Ca}^{2+}]$ ranging from 0.1 to 1 μM (Van Renterghem and Lazdunski, 1992) and, therefore, would be fully activated by a mean $[\text{Ca}^{2+}]$ of 1–2 μM underneath the plasma membrane. Such an activation is not found in resting cells.

Even more evident is the difference between $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{pm}$ upon activation of Ca^{2+} influx. The simple manoeuvre of shifting the cells from Ca^{2+} -free media to media containing 1 mM Ca^{2+} induces such a dramatic change in $[\text{Ca}^{2+}]_{pm}$ that, due to the rapid consumption of the aequorin pool, only a lower limit (~ 120 μM) of the real peak value can be obtained. Only when a 10-fold lower $[\text{Ca}^{2+}]$ is added to the cells can the amplitude (~ 10 μM) and the kinetic behaviour of the $[\text{Ca}^{2+}]_{pm}$ transient be measured accurately. Under these latter conditions, it is also apparent that the $[\text{Ca}^{2+}]_{pm}$ rise caused by Ca^{2+} readdition is ~ 5 -fold increased if the Ca^{2+} influx pathway dependent on depletion of IP_3 -sensitive stores (Hoth and Penner, 1992; Fasolato *et al.*, 1994; Clapham, 1995) is activated previously. Interestingly, despite the low $[\text{Ca}^{2+}]$ in the extracellular medium, the peak values of the $[\text{Ca}^{2+}]_{pm}$ increases appear much higher than the predicted values at the mouth of store-dependent Ca^{2+} channels (Zweifach and Lewis, 1995) and approach those estimated for the active zones of presynaptic terminals (Heidelberger *et al.*, 1994). In conclusion, the present data indicate that the activity of Ca^{2+} channels induces very high $[\text{Ca}^{2+}]$ increases (> 100 μM) not only in specialized domains but also in wide regions under the plasma membrane. The described experimental approach, which may in the future also allow the targeting of aequorin chimeras to the mouth of a Ca^{2+} channel, now offers the possibility of quantitatively investigating the role of these $[\text{Ca}^{2+}]$ changes in modulating the biological events occurring at the inner surface of the plasma membrane.

Materials and methods

Construction of the SNAP-25–aequorin chimeras

The chimeric cDNAs were constructed as follows. A PCR run was performed using the SNAP-25 cDNA as a template and the following primers: forward, GAATTCACCATGGCCGAGGACGCAGACATGCGTAAT; reverse, ATCGATACCACTTCCAGCATCTTTCT. The forward primer specifies an *EcoRI* site immediately in front of nucleotide -4 of the SNAP-25 cDNA, whereas at the $5'$ end of the reverse primer a *Clal* site substitutes the native stop codon of the cDNA. After 30 PCR cycles (95°C 1 min, 62°C 2 min, 72°C 1 min), a single PCR product was identified, isolated and subcloned according to standard procedures into the *SmaI* site of PBS+ (Stratagene). The PCR-modified SNAP-25 cDNA (pSNAP-25) was then excised from the vector by a *Clal*–*KpnI* digestion and inserted into appropriately cut pBSK⁺ plasmids, containing the aequorin cDNAs. For the construction of pmAEQwt,

pSNAP-25 was subcloned upstream of a *HindIII*–*EcoRI* fragment encoding HA1-tagged aequorin (Brini *et al.*, 1995). For the construction of pmAEQmut, the pSNAP-25 was subcloned upstream of a *HindIII*–*EcoRI* fragment encoding the Asp119→Ala aequorin mutant (Montero *et al.*, 1995). In both cases, fusion occurred in-frame, as verified by DNA sequencing. The entire inserts (pmAEQwt and pmAEQmut) were then excised via *EcoRI* digestion and inserted into the expression vector pcDNA1 (Stratagene). The recombinant plasmids (pmAEQwt/pcDNA1 and pmAEQmut/pcDNA1) were utilized for the transfections as previously described (Rizzuto *et al.*, 1995).

Cell culture and transfection

Rat aortic smooth muscle cells of the A7r5 cell line (Kimes and Brandt, 1976) (passages 15–25) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (200 U/ml) and streptomycin (0.2 mg/ml) in 25 cm² Falcon flasks. For transient expression experiments, the cells were seeded onto 13 mm coverslips and allowed to grow to 75% confluence. At this stage, transfection with the recombinant plasmids cytAEQ, pmAEQwt and pmAEQmut (4 μg DNA/coverslip) was carried out as previously described (Rizzuto *et al.*, 1995).

Ca^{2+} measurements

For aequorin reconstitution, as indicated in the text, the cells were transferred to modified KRB (125 mM NaCl, 5 mM KCl, 1 mM Na₃PO₄, 1 mM MgSO₄, 5.5 mM glucose, 20 mM HEPES, pH 7.4, 37°C), supplemented with 0.1 mM EGTA (in some experiments EGTA was omitted, with no significant difference in the count yield) and 5 μM coelenterazine. After 45 min, the coverslip was transferred to the luminometer chamber, and perfused with KRB containing 0.1 mM EGTA. All experiments were performed in KRB medium, supplemented, where indicated, with 0.1 mM EGTA, 100 μM or 1 mM CaCl₂. The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+} -rich solution (10 mM CaCl₂ in H₂O), thus discharging the remaining aequorin pool. The light signal was collected and calibrated into $[\text{Ca}^{2+}]$ values as previously described (Brini *et al.*, 1995; Rizzuto *et al.*, 1995). In brief, the transfected cells were placed in a perfused, thermostatted chamber placed in the close proximity of a low-noise photomultiplier, with built-in amplifier–discriminator. The output of the discriminator was captured by a Thorn-EMI photon counting board, and stored in an IBM-compatible computer for further analyses. The aequorin luminescence data were calibrated off-line into $[\text{Ca}^{2+}]$ values, using a computer algorithm based on the Ca^{2+} response curve of wild-type and mutant aequorins, as previously described (Brini *et al.*, 1995; Montero *et al.*, 1995).

Immunolocalization of the HA1-tagged recombinant aequorin

At 48 h after transfection, A7r5 cells were processed for immunofluorescence as follows. The cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min, washed three times with PBS and then incubated for 30 min in PBS supplemented with 50 mM NH₄Cl. Permeabilization was achieved with a 5 min incubation with 0.1% Triton X-100 in PBS, followed by a 1 h wash with 1% gelatin (type IV from calf skin) in PBS. The cells were then incubated for 1 h at 37°C in a wet chamber with a 1:100 dilution (in 1% gelatin in PBS) of the monoclonal antibody 12CA5 (initially a kind gift from Dr Jacques Pouyssegur, Nice, France; then obtained from BAbCo, Berkeley, CA), which recognizes the HA1 tag (Field *et al.*, 1988). The cells were then incubated for 30 min in a wet chamber with a 1:50 dilution (in 1% gelatin in PBS) of the tetramethylrhodamine (TRITC)-labelled anti-mouse IgG secondary antibody. After each antibody incubation, the cells were washed four times with PBS. Fluorescence was then analysed with a 100 $\times$ objective on a RCM8000 real-time Nikon confocal microscope using a krypton–argon ion laser.

Acknowledgements

We thank G. Ronconi and M. Santato for technical assistance, A. Negro for kindly providing the SNAP-25 cDNA and P. Pacaud and C. Frelin for the gift of the A7r5 cell line. This work was supported by grants from 'Telethon', from the EU programs 'Biomed2', 'Human Capital and Mobility' and 'Copernicus', from the 'Human Frontier Science Program', from the Italian Research Council (CNR) Special Project 'Oncology', from the Italian University Ministry and from the British Research

Council to T.P. and R.R. R.M. was supported by a EU fellowship 'Human Capital and Mobility'.

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Received on August 8, 1996; revised on November 15, 1996